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ARTICLE

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Plant defense synergies and antagonisms affect performance of specialist herbivores of common milkweed

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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 effective when co-expressed with others compared to their independent efficacy. However, this hypothesis has rarely been tested outside of phytochemical mixtures, 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 chewing herbivores on plants in natural populations. We employed regression and a novel application of random forests to identify synergies and antagonisms between defense traits. We found the first direct empirical evidence for two previously hypothesized defense synergies in milkweed (latex by secondary metabolites, latex by trichomes) and identified numerous other potential synergies and antagonisms. Our strongest evidence for a defense synergy was between leaf mass per area and low nitrogen content; given that these “leaf economic” traits typically covary in milkweed, a defense synergy could reinforce their co-expression. We report that each of the plant defense traits showed context-dependent effects on herbivores, and increased trait expression could well be beneficial to herbivores for some ranges of observed expression. The novel methods and findings presented here complement more mechanistic approaches to the study of plant defense diversity and provide some of the best evidence to date that multiple classes of plant defense synergize in their impact on insects. Plant defense synergies against highly specialized herbivores, as shown here, are consistent with ongoing reciprocal evolution between these antagonists.

KEYWORDS

cardenolide, chemical ecology, common milkweed (*Asclepias syriaca*), defense hypothesis, herbivory, latex, plant defense, plant–insect interactions, random forest, swamp milkweed beetle (*Labidomera clivicollis*), 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 (Coley et al., 2018; Kant et al., 2015; Kursar et al., 2009). Consequently, ecologists have spent decades developing

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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 (Agrawal & Fishbein, 2006; Duffey & Stout, 1996; Salazar et al., 2018). Explaining why plants invest in multiple defenses is an open question, especially since there is evidence that selection has favored specific combinations of defense traits ("defense syndromes"; Agrawal & Fishbein, 2006; da Silva & Batalha, 2011; Sanczuk et al., 2020; cf. Moles et al., 2013). Several nonexclusive hypotheses have been proposed to explain both the prevalence of multitrait defense strategies and the specific combinations of defenses that are observed (Agrawal, 2011).

Jones and Firn (1991) articulated the problem of multiple phytochemical defenses clearly: Individual plants often express many different types of secondary metabolites, and it is difficult to explain why. One of the leading explanations for multiple secondary metabolites is synergistic interactions between different compounds (Duffey & Stout, 1996; Richards et al., 2016; Wetzel & Whitehead, 2020). For example, the furanocoumarin xanthotoxin is more toxic when mixed with other furanocoumarins than expected from its effects alone (Berenbaum et al., 1991); similarly, amides in *Piper* plants are more effective in combination than separately (Dyer et al., 2003; Richards et al., 2010; Scott et al., 2002; Whitehead & Bowers, 2014). Synergies have also been detected in phytochemical defenses against microbes (e.g., Muroi & Kubo, 1993; Stermitz et al., 2000) and fungi (Cipollini & Stiles, 1992). Conversely, ecologists have also detected antagonistic relationships, where compounds reduce the efficacy of each other, including among furanocoumarins (Diawara et al., 1993) and amides (Whitehead & Bowers, 2014) and between pyrrolizidine alkaloids and chlorogenic acid (Liu et al., 2017). Tests for synergies and antagonisms between secondary metabolites are still relatively rare (Richards et al., 2016), but 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 (Agrawal, 2011; Rasmann & Agrawal, 2009; Richards et al., 2016, but see Green et al., 2001; Steppuhn & Baldwin, 2007).

Studies of synergies between phytochemicals generally measure the responses of herbivores to artificial diets with manipulated concentrations of phytochemicals in an ANOVA design (e.g., Berenbaum et al., 1991; Dyer et al., 2003; Scott et al., 2002; Stamp & Yang, 1996). 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 Hay, 1996; Nelson & Kursar, 1999; Pennings, 1996). For experimental designs with continuously varying defense traits, the logical extension of this approach is regression of herbivore performance (e.g., growth, survival) on levels of defense traits (e.g., concentrations of different phytochemicals) with a term for the interaction between two traits that captures potential synergies or antagonisms (Richards et al., 2016) (compare Figure 1a,b with Figure 1c,d). However, implicit in this regression modeling approach is the assumption of a *bilinear* interaction: that the synergistic benefit (or antagonism) of one additional unit of defense trait A for each unit of defense trait B is the same regardless of the initial levels of A or B (e.g., Figure 1c,d). However, other relationships are biologically reasonable. The synergistic benefit of one trait might be a threshold effect (e.g., Figure 1e,f). This could occur if sufficient quantities of a defense causes a switch in herbivore behavior (e.g., *Trichoplusia ni* exhibit trenching behavior on latex-bearing plant species and not on others) and the effect of a second defense is impacted by herbivore behavior (Dussourd & Denno, 1994). Alternatively, a synergy might have diminishing marginal returns (e.g., Figure 1e,f), with the benefit of the synergy most prominent when increasing from low to moderate levels of trait expression.

To identify complex relationships like these in experimental data, in this paper we introduce tests for synergies and antagonisms using random forest regression. Random forests are a popular form of machine learning that fit data using an ensemble (effectively, model averaging) of many decision trees (James et al., 2021). Because of this structure, random forest models are very flexible and can capture nonlinear trends in data. Recent innovations have provided a framework for harnessing the flexible nature of random forests to make inferences about interactions, as we do here (Hooker, 2007; Hooker & Mentch, 2019). To our knowledge, this approach for identifying interactions with random forests has not been used in ecology, so we include thoroughly annotated code on Figshare (Edwards et al., 2022).

Our experiments involved herbivores feeding on common milkweed, *Asclepias syriaca*, a well-characterized system for studying plant defenses. Milkweed produces toxic phytochemicals—cardenolides—that are potent defenses and severely limit which species of herbivores that can feed on it, leading to a simplified herbivore community (Agrawal, 2017). In addition to cardenolides, *A. syriaca* also employs 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 shut (Agrawal & Konno, 2009), and leaf toughness and low nutrient content may reduce insect preference or performance (Agrawal & Fishbein, 2006). Furthermore, expression of these defensive traits varies

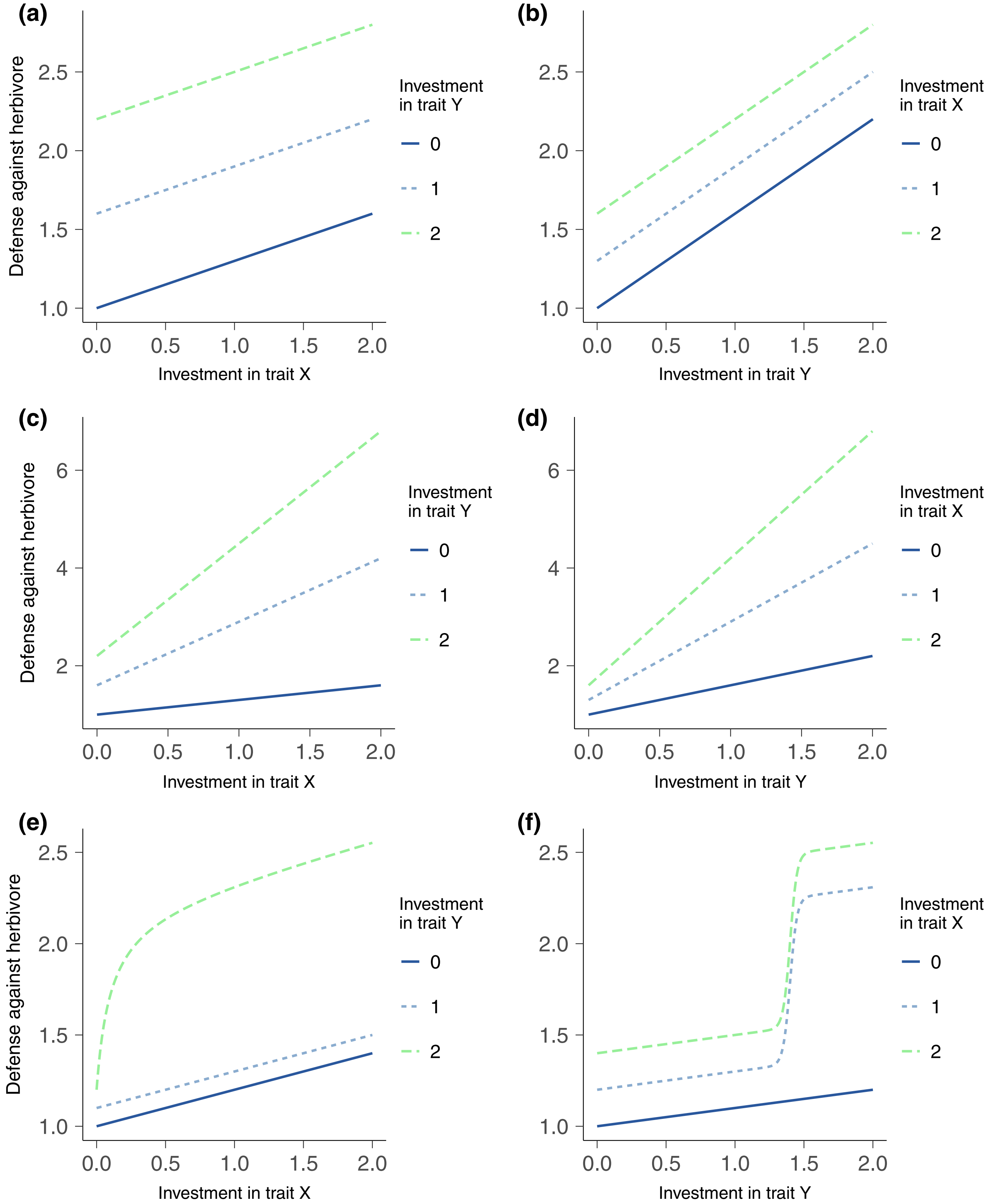


FIGURE 1 Legend on next page.

substantially in natural conditions within species, allowing experiments to capture broad ranges of defense traits in a field setting.

Two putative defense synergies have been proposed for milkweed plants (Agrawal, 2011): latex with trichomes and latex with cardenolides. First, freshly hatched monarch butterfly caterpillars were observed to first carefully chew off trichomes in a small feeding region and then to bite into the leaf tissue (Zalucki et al., 2002). At this point, latex exudes, and the caterpillar must struggle free before it can consume the leaf tissue. Many caterpillars fail to survive this first exposure to latex (Zalucki et al., 2001). Trichomes and latex may thus act synergistically, with higher densities of trichomes leaving neonate caterpillars more exhausted when they encounter the first-bite latex, and thus less able to struggle free. There is indirect evidence for a synergy between these traits: across *Asclepias* species, these two traits also show correlated evolution (Agrawal & Fishbein, 2006). Second, *A. syriaca* latex contains cardenolides that mirror the concentrations found in leaves (Agrawal et al., 2014; Züst et al., 2019), so latex may be a vehicle to deliver toxins directly into the mouth of feeding herbivores in addition to physically gumming up mouthparts. In this way, latex acts as both a chemical and a physical barrier when cardenolide expression is high, suggesting a synergy between latex and cardenolide expression. 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). Although latex by trichome and latex by cardenolide synergies in milkweeds have been proposed over the last two decades, no direct empirical evidence for either has been produced. Both synergies include latex, which is most prominently a defense against chewing herbivores; the latex by trichome synergy is specifically hypothesized from observations of monarch caterpillar behavior and may not be relevant for other herbivores with different feeding behaviors.

No specific antagonisms have been hypothesized for *A. syriaca* or even widely discussed in the broader literature outside of antagonisms between individual secondary metabolites (e.g., Calcagno et al., 2002; Diawara et al., 1993; Liu et al., 2017; Whitehead & Bowers, 2014). For specialist insects that are able to sequester plant toxins, we might expect an antagonistic relationship between secondary metabolites and traits that increase predation—indirect defenses that attract natural predators. Indeed, any plant traits that are somewhat effective against herbivores but also have negative effects on natural enemies of herbivores could be antagonistic with indirect defenses.

In this study we evaluated plant defense synergies and antagonisms using herbivore performance on common milkweed *A. syriaca* under field conditions, employing both well-established regression approaches and a novel application of random forests. Although the majority of existing studies of synergies have used artificial diets, we measure herbivore performance under field conditions, relying on natural variation in defense traits in wild plants. The use of observational field studies meant sacrificing controlled conditions, but it provided a realistic context for plant defenses and allowed us to study defenses that are not easily manipulated in the lab. Using two specialist chewing herbivores (the swamp milkweed beetle *Labidomera clivicollis* and the monarch butterfly *Danaus plexippus*), we tested the defense synergy hypothesis for two predicted synergies: latex by cardenolide and latex by trichome. We also explored other potential synergies and antagonisms between cardenolides, trichome density, latex, leaf toughness, carbon, nitrogen, and leaf mass per area. Where synergies and antagonisms have not been previously hypothesized, we sought evidence for trait interactions that are worthy of more targeted experiments. Our methods provide a road map for future tests of the synergy hypothesis

FIGURE 1 Conceptual diagram of types of trait synergies. (a and b) Without a synergy between defense traits X and Y, the per-unit benefit to the plant of investing in one trait does not depend on the investment level of the other. This leads to linear relationships between investment in a trait and the resulting benefit to the plant, with differences in investment in the second trait changing the intercept but not the slope. These relationships can be captured with a regression model that does not include an interaction term. (c and d) With a bilinear synergy, the per-unit benefit of investing in one trait varies linearly with investment in the other. This leads to linear relationships between investment in a trait and the resulting benefit to the plant, with both the slope and the intercept depending on the investment level in the other trait. These relationships can be captured by an interaction term in a regression model; they are also shown in Figure 3a. (e and f) Non-bilinear synergies produce more complicated relationships between investment in traits and benefit, which are not well represented by interaction terms in regression models. We present an approach to identifying the presence of these synergies (but not their functional form) using random forests. Here we show a synergy in which the benefit depends in part on the product of a saturating function of investment in trait X and a threshold function of investment in trait Y. (a) and (b) benefit $= a + b \times$ (investment in X) $+ c \times$ (investment in Y); (c) and (d) benefit $= a + b \times$ (investment in X) $+ c \times$ (investment in Y) $+ d \times$ (investment in X) $\times$ (investment in Y); (e) and (f) benefit $= a + b \times$ (investment in X) $+ c \times$ (investment in Y) $+ d \times f$ (investment in X) $\times g$ (investment in Y); $f(\mathrm{X}) = \mathrm{X}/(\mathrm{X} + \mathrm{h})$; $g(\mathrm{Y}) = 1/(1 + \exp(m \times (\mathrm{Y} + q)))$. In all cases, $a = 1$, $b = 0.3$, $c = 0.6$. When present, $d = 1$, $h = 0.1$, $m = -20$, $q = -1$.

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 found in recently disturbed habitats including roadsides and fields. Larvae of *D. plexippus* and *L. clivicollis* are both specialist herbivores that commonly feed on the leaf tissues of *A. syriaca* in our study area. Both of these herbivores have modifications to their Na,K-ATPase (sodium pumps) that increase their tolerance of cardenolides (Dobler et al., 2012) but do not provide complete immunity (Agrawal, 2005; Jones et al., 2019; Tao et al., 2016; Zalucki et al., 2001).

Field experiments

In September 2015, we conducted an experiment to assay the growth and survival of neonate monarch caterpillars, *D. plexippus*, on 117 *A. syriaca* plants near Ithaca, NY (42.39 N, 76.39 W). Caterpillars were obtained from a laboratory colony (Jones & Agrawal, 2019). We selected plants from a natural population 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 and collecting a leaf disc for measuring trichome density and leaf mass per area (LMA), the inverse of specific leaf area. We then removed that paired leaf to be dried for measurement of cardenolides, carbon, and nitrogen.

Latex, leaf toughness, LMA, and cardenolides were quantified following standardized methods (for a detailed description, see 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; LMA (kg dry mass/m^2) was measured by drying and weighing a 31.67 mm^2 leaf disc; cardenolides were separated and measured from dried and ground leaf tissue using high-performance liquid chromatography (HPLC) following the methods described in Züst et al. (2019). Observed cardenolide compounds did not match currently identified compounds, so we report their identity based on the retention time (e.g., during HPLC analysis, cardenolide 10.7 eluted at 10.7 min). Trichome density was measured by first imaging the underside of leaf discs with the ZEISS SteREO Discovery V20, then using ImageJ to draw vertical and horizontal transects through the middle of the disc and counting all hairs that crossed the transects (Schindelin et al., 2012). These transect counts were highly correlated with densities obtained by counting all hairs in a quadrant of the leaf disc but were faster and more robust to overlapping hairs (Collin B. Edwards, 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 were then removed and weighed. In a few cases, field conditions prevented collection on Day 6, and caterpillars were instead collected on Day 7; we account for this discrepancy by including a random effect of plant in our regression models. Missing caterpillars were presumed dead. In several cases, plants died during the experiment, necessitating their removal from the study. In total, we obtained 165 weighed larvae and 183 measures of survivorship for 92 plants.

In September 2016, we conducted an experiment to assay the growth and survival of neonate swamp milkweed beetles, *L. clivicollis*, on 129 *A. syriaca* plants using the same methods as described previously, with the following exceptions. First, we used a separate field site ~300 m away, at Dunlop Meadow (a Cornell University Natural Area). Second, because *L. clivicollis* aggregate in their early life stages, we placed five larvae in each enclosure rather than two. In a few cases, loss of larvae during placement left us with only four larvae in an enclosure. Third, owing to unusual weather patterns, many of the plants experienced accelerated senescence during the experiment; we did not include these samples. Fourth, owing to contamination of tissue samples, we were unable to measure LMA or carbon and nitrogen concentrations. In total, we obtained 133 weighed larvae and 464 records of survivorship for 95 plants.

Analysis

We took two approaches to analyzing our data. First, we looked for bilinear synergies and antagonisms using a traditional linear regression approach, where synergies and antagonisms were represented with a statistical interaction term. Second, we implemented a novel (for ecology) extension of random forests to identify synergies and antagonisms that were not bilinear. In all cases, our metric of herbivore growth was log final mass. For regression analyses, we rescaled plant traits so that their mean was zero and their SD was 1; for random forest analyses we rescaled plant traits so that their maximum was 1.

In total we had two 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” and “Beetles.” These two data sets have some overlap in the defense traits measured, but the cardenolide compounds expressed in each year were different, and, owing to sample contamination, we were unable to measure LMA, carbon, or nitrogen in the Beetles data. To facilitate interpretation, we recontextualize percent nitrogen as “non-nitrogen” (100 minus percent nitrogen), so that all analyzed traits are expected *a priori* to have a negative effect on herbivores. Although carbon and nitrogen concentrations are sometimes combined into the carbon-to-nitrogen ratio (C:N) as a proxy for nutritional quality, here we treat carbon and (non-)nitrogen as independent, as we and others have done elsewhere (e.g., Agrawal, 2004a,b). This decision was driven by three factors. First, the purpose of our study was to quantify trait interactions, and precombining two traits in a fixed fashion as in the C:N ratio instead presupposed a specific trait interaction. Second, herbivores sometimes respond to carbon and nitrogen separately; analyzing them as separate traits allowed us to detect this. Third, we found that variation in C:N was driven almost exclusively by variation in (non-)nitrogen (correlation coefficient of 0.95, $p < 0.001$) and was uncorrelated with carbon (correlation coefficient of -0.077, $p = 0.3$) (Appendix S1: Figure S1).

Trait correlations

We computed pairwise across-plant trait (phenotypic) correlations for each of our data sets using Pearson correlation coefficients. Each of our data sets contained several cardenolide compounds that were highly correlated (Monarchs: cardenolides 17.4, 17.7, 18.4; Beetles: cardenolides 10.7, 18.1). For each of these suites of covarying cardenolides, we used principal component analysis (PCA) to identify a single measure—the first principal component (PC)—that represented 95% (Monarchs) and 98% (Beetles) of the variation in those cardenolides. All further analysis used these first PCs in place of the individual cardenolides in question, labeled “cardenolide suite 2015” and “cardenolide suite 2016.” For the remaining cardenolides, which were not highly correlated (Monarchs: cardenolides 10.5, 18.6; Beetles: cardenolides 8.4, 10.2), we treated each compound as a separate defense trait.

Linear regression

For each trait pair (e.g., latex by trichomes, latex by cardenolide for each of the independent compounds [10.5, 18.6, 10.2, 8.4] and the two cardenolide suites), we fit a regression model that included main effects for the two focal traits and an interaction between those two traits. We also include a random effect of plant identity to account for structure in the data; this term captured the shared experience of multiple herbivores on the same plant as well as variation associated with the day herbivores were put on plants. We then used marginal hypothesis testing to identify the significance of the interaction term. Because this analysis involved many comparisons, we discourage viewing *p*-values as part of a null hypothesis significance test (NHST) to “definitively” prove (or find lack of proof for) specific synergies or antagonisms. Instead, we emphasize the value of *p*-values as evidence (or a lack of evidence) for synergies or antagonisms (Muff et al., 2022); synergies and antagonisms with the smallest *p*-values are those that the data most strongly support. Following Muff et al. (2022), we designate $p < 0.01$ as strong evidence, $0.01 < p < 0.05$ as moderate evidence, and $0.05 < p < 0.1$ as weak evidence. We note that focusing on *p*-values as evidence rather than a binary NHST is more consistent with the actual definition and statistical best practices for *p*-values and is a good idea even without multiple comparisons.

In what follows, we develop a technical basis for interpreting the regression interaction coefficient, then describe the intuition this provides. For a pair of scaled traits x_1 and x_2 with main effect coefficients β_1 and β_2 (these represent the direct effect on the herbivore of variation in either trait when the other is at its average) and interaction coefficient β_{12}, we can rewrite our expectation for the expected herbivore performance y from the standard regression form

$$E[y] = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2 \tag{1}$$

to

$$E[y] = \beta_0 + (\beta_1 + \beta_{12} x_2) x_1 + \beta_2 x_2. \tag{2}$$

From Equation (2) we see that the expected effect of varying the level of Trait 1 (x_1) depends on both β_1 (the direct effect of Trait 1) and the product of β_{12} (the interaction coefficient) and x_2. Because we are working with scaled trait values, x_1 and x_2 represent deviations from the mean trait values across our experiment. When β_{12} is negative, if Trait 2 is above the mean for that trait (x_2 is positive), higher-than-average values of Trait 1 (positive values of x_1) are associated with reduced herbivore performance relative to when Trait 2 is at its mean.

Because Equation (1) can just as easily be rewritten to focus on variation in Trait 2, in the form

$$E(y) = \beta_0 + (\beta_2 + \beta_{12}x_1)x_2 + \beta_1 x_1 \tag{3}$$

we see the interpretation is symmetrical, and β_{12} is modifying the effect of each trait in the context of the other. When herbivore survival is the response, y is the logit transformation of survival, but this interpretation still applies to survival because the logit and its inverse are monotonic.

To identify an interaction as a synergy or an antagonism, we look to the sign of the interaction coefficient, β_{12}. Because the response is insect performance, a negative coefficient for the interaction term represents a potential synergy between traits; herbivore performance is lower when the traits are both above average expression levels compared to what we would expect from the separate effect of each of the trait's expression levels. Similarly, a positive interaction coefficient represents an antagonism between traits. Because each trait is rescaled to have mean 0 and SD 1, the interaction term can be interpreted as modifying the effect of one trait based on how far the other trait is from the mean. Because the classification of a trait as a defense against herbivores could be context-dependent (see following discussion), we took the inclusive approach of identifying synergies and antagonisms based solely on the sign of the interaction coefficient.

An underlying assumption of many plant–insect ecologists (including ourselves) is that for a given pair of plant and herbivore species, a plant trait generally is or is not a defense. However, a key insight from Equation (2) is that, depending on the strength of trait interactions, a focal trait can be harmful or helpful to herbivores depending on the expression levels of other traits. From Equation (2) we find that the net effect on herbivores of increasing Trait 1 changes sign (e.g., switches from being defensive to beneficial to herbivores or vice versa) when $\beta_1 = -\beta_{12}x_2$. This switching point depends on the coefficients of the main effect (β_1) and interaction (β_{12}), as well as how far Trait 2 is from its mean (x_2). For a linear model with a nonzero interaction term, a switching point will always exist, but it may be associated with extreme, biologically unreasonable trait values. We calculated the switching point for each trait pairing, treating a switching point as biologically reasonable if it occurred when the nonfocal trait value was within one SD of its mean. We designated a trait as "context-dependent" if, when paired with each other trait, at least one of the switching points was biologically reasonable. If no switching points were biologically reasonable, we designated the trait as "defensive" or "helpful" based on whether it was harmful or helpful to herbivore performance when other traits were at their mean.

Because the interaction 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 (logit-link) and growth (identity-link) 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, we developed two new metrics, which we introduce here. The first, the "predictable response range" (PRR), captures the variation in response values that can be attributed to a model. The second, the percentage of predicted response variation explained by the interaction (%PRV), captures the relative role of nonadditivity (e.g., synergy or antagonism) in explaining model predictions.

Predictable response range is a metric for any model, and describes the range of model predictions for the data (Figure 2). Much like the coefficient of determination, R^2, PRR is a tool to understand the variation in the dependent variable that can be explained by the model. Unlike R^2, however, PRR focuses on explaining how the dependent variable is expected to change (e.g., from modeled effects, after excluding the variation from random noise and unmodeled variables) if predictor values were changed from the least favorable to the most favorable observation in the data. For regression models, PRR was simply the range of $\hat{y}$, the model predictions of the data (Figure 2). For the random forest analysis, calculation was slightly more complex (Appendix S1) but produced an analogous range. When applied to our models of two defense traits and their interaction, PRR captured how much the plant with the highest predicted herbivore performance could expect to benefit if it were able to modify its two modeled traits to those of the plant with the lowest predicted herbivore performance. PRR thus represented all explained effects of the trait combination on herbivores in our data: the combination of direct effects of the traits, bilinear synergies or antagonisms, and, in the case of random forests, any other nonadditive relationships.

%PRV is a metric measuring how much of the variation explained by a model can be attributed to nonadditive terms. We calculated this by first using the fitted model in question to predict response values (survival or log final mass) across a grid of the two focal trait values, then fitting an additive model to those predictions, with the trait values treated as factors. This new additive model should perfectly fit any variation in the response that can be attributed to additive effects (e.g., will exactly fit data predicted from a linear model without an interaction term); any residuals of this new additive model are therefore due to nonadditivity. We calculate %PRV from a Type III ANOVA of the additive model: %PRV is the ratio of the residual sum of squares to

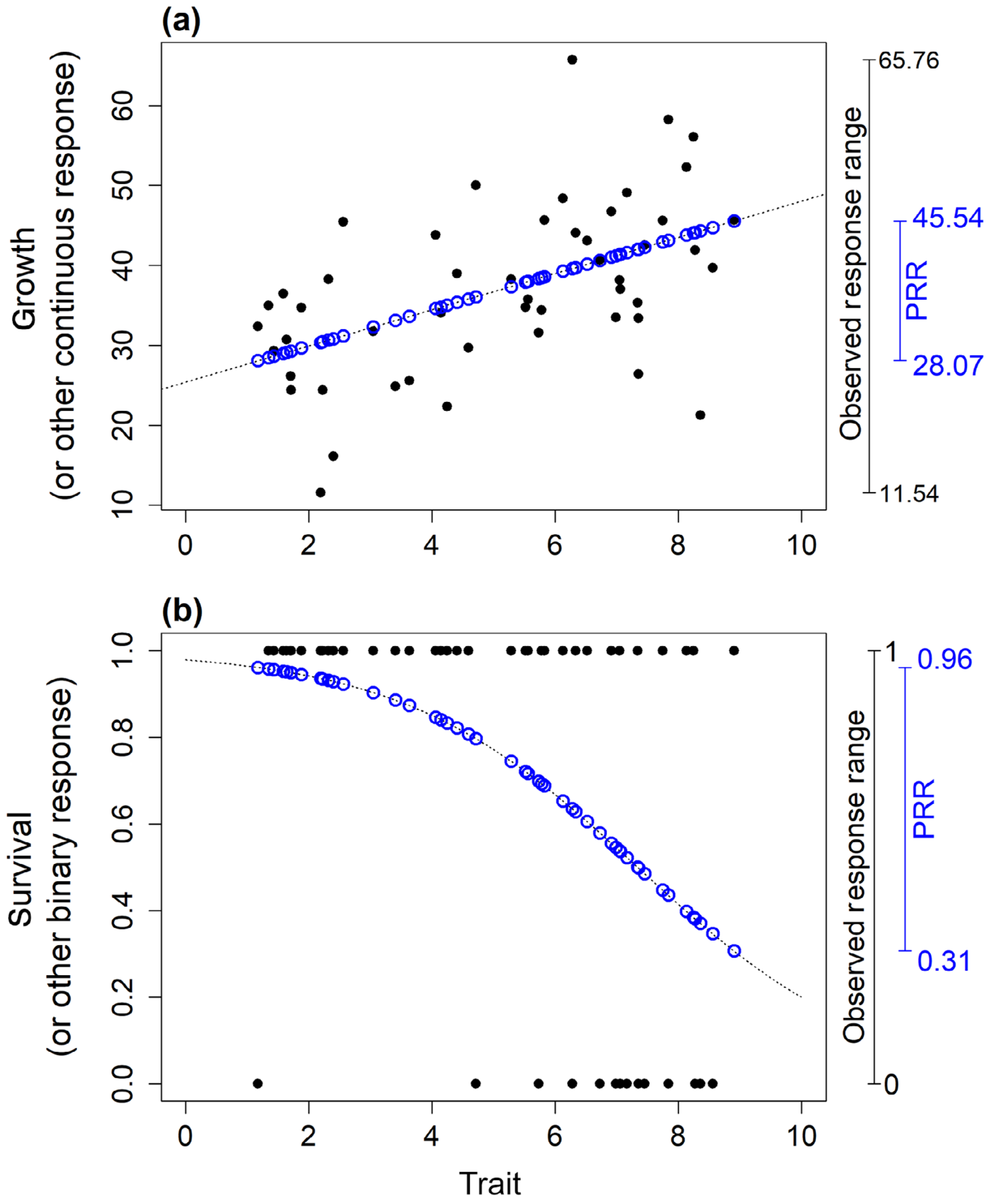


FIGURE 2 Example of predictable response range (PRR) for simplified simulated data. Black points represent simulated data; blue circles show predictions for those points from a fitted model. PRR captures the range of response values (e.g., growth or survival) predicted for data points. Here 30 trait values were drawn from a uniform distribution ranging from 1 to 9, and (a) growth and (b) survival responses to traits were simulated. The dashed line shows models fit to the data of the form "response ~ trait" using (a) linear regression or (b) logistic regression.

the total sum of squares, multiplied by 100 to convert to percentages. This makes %PRV somewhat analogous to R^2 converted to percentages: A value of 50% means that half of the variation in model predictions is due to nonadditive model components. To avoid %PRV depending on regions of extrapolation, we used a weighted ANOVA with weights corresponding to nearness to actual data points, calculated using multivariate kernel density estimation (see Edwards et al., 2022 for implementation details).

Predictable response range and %PRV have natural units that are comparable between models: range in the response (log weight or survival) and percentages, respectively. Because random forests are more flexible than regression models, we expect the noninteractive components of our random forest decomposition (see following discussion) to explain a larger fraction of the variation in the data than would our regression models, resulting in a lower %PRV for random forest analyses than regression models.

Random forest

Like many machine learning algorithms, random forests can be very effective at prediction (e.g., "How fast do caterpillars grow on a plant with these specific defense traits?") but do not directly provide information (analogous to regression coefficients) about the basis for those predictions (e.g., "How much does each plant trait affect caterpillar growth rate?"). Hooker (2007) provided a 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. 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 across a grid of trait values. The predicted herbivore performance was thus the result of any linear and nonlinear patterns in the real data that the random forest model captured. We carried out simulated experiments for each pair of defense traits and analyzed the resulting simulated data to determine whether the response was additive (no interactions) or nonadditive (synergy, antagonism, or other relationship). For a detailed walkthrough of simulated experiments and their analysis, see Appendix S1 (annotated code for implementing this technique is available on Figshare; Edwards et al., 2022).

Simulated synergies and antagonisms

Because random forest models are equation-free, the method presented above for using random forest models can capture non-bilinear synergies (or antagonisms), but the fitted model does not provide information for the functional form of that relationship, nor does PRR or %PRV. To determine how to identify when fitted random forest with nonadditive relationships represented synergies or antagonisms, we simulated data as if it had been generated by the random forest analysis procedure described earlier, but with known functional forms of synergies or antagonisms (see Appendix S1 for details). As in the previously described methodology, we fit this simulated data with an additive linear model. Synergies and antagonisms represent nonadditive processes and might be identifiable from the residuals of this model; we plotted those residuals (the third column in Figure 3a–f). We explored many potential trait synergies and antagonisms but here present results for three synergies (linear, threshold, diminishing marginal returns [DMR]) and three antagonisms (linear, threshold, decaying).

Classifying random forest results

Based on our simulations, we found that synergies and antagonisms had residuals that could be separated into four quadrants based on the sign of the residuals (Figure 3). For simplicity, we label the four quadrants Q1, Q2, Q3, and Q4 starting in the bottom left (nearest the origin) and moving clockwise. We found that synergies were represented by herbivores underperforming the additive model (negative residuals) in Q1 and Q3 (the quadrants on the diagonal) and overperforming the additive model (positive residuals) in Q2 and Q4 (the quadrants on the off-diagonal). The reverse was true for antagonisms. To quantify how much a given trait pair matched one of these qualitative patterns, we defined a new test statistic, θ. θ was calculated as the sum of the signs of residuals in Q1 and Q3 minus the sum of the signs of residuals in Q2 and Q4 divided by the total number of predictions summed. Residuals of 0 were given a sign of 0. For any pair of breakpoints separating the trait space into four quadrants, θ represented the average tendency toward synergy (negative values) or antagonism (positive values), with $\theta = -1$ if each prediction followed the described pattern for synergies, $\theta = 1$ if each prediction followed the described pattern for antagonisms, and 0 if residuals were evenly split between those patterns. For each fitted random forest model, we chose the breakpoints that maximized the absolute value of θ, i.e., the breakpoints that best represented a synergy or antagonism. To classify an interaction as a synergy or antagonism, we simulated 999 permutations of the random forest predictions, in which the predictions of each grid point were randomly reassigned. For each of these simulated data sets, we then identified the new optimal breakpoints and calculated the new θ, generating the null distribution of θ for that trait pair. We designated an interaction "unclear" if the observed θ was between the 0.025 and 0.975 quantiles of the null distribution and "synergy" or "antagonism" if it was below or above those thresholds, respectively.

Software and packages

All analyses and simulations were carried out in the R programming language (R Core Team, 2021). We used the following key packages: mgcv (Wood, 2011) to fit mixed-effects models; randomForest (Liaw & Wiener, 2002) to fit random forest models; car (Fox & Weisberg, 2019) for marginal hypothesis testing and calculating sum of squares; the tidyverse package suite (Wickham et al., 2019) for data cleaning and manipulation; and ggplot2 (Wickham, 2016), ggpubr (Kassambara, 2020), and cowplot (Wilke, 2020) for creating figures. The scripts used to carry out all our analyses are available on Figshare (Edwards et al., 2022).

RESULTS

Trait correlations

Several patterns of trait co-expression emerged in our field studies (Appendix S1: Figures S1 and S2). In 2015, several of the physical traits showed positive phenotypic correlations; notably, leaf toughness was correlated with both latex exudation from leaves ($r = 0.17$, $p < 0.021$) and leaf trichome density ($r = 0.49$, $p < 0.001$). Percentage leaf

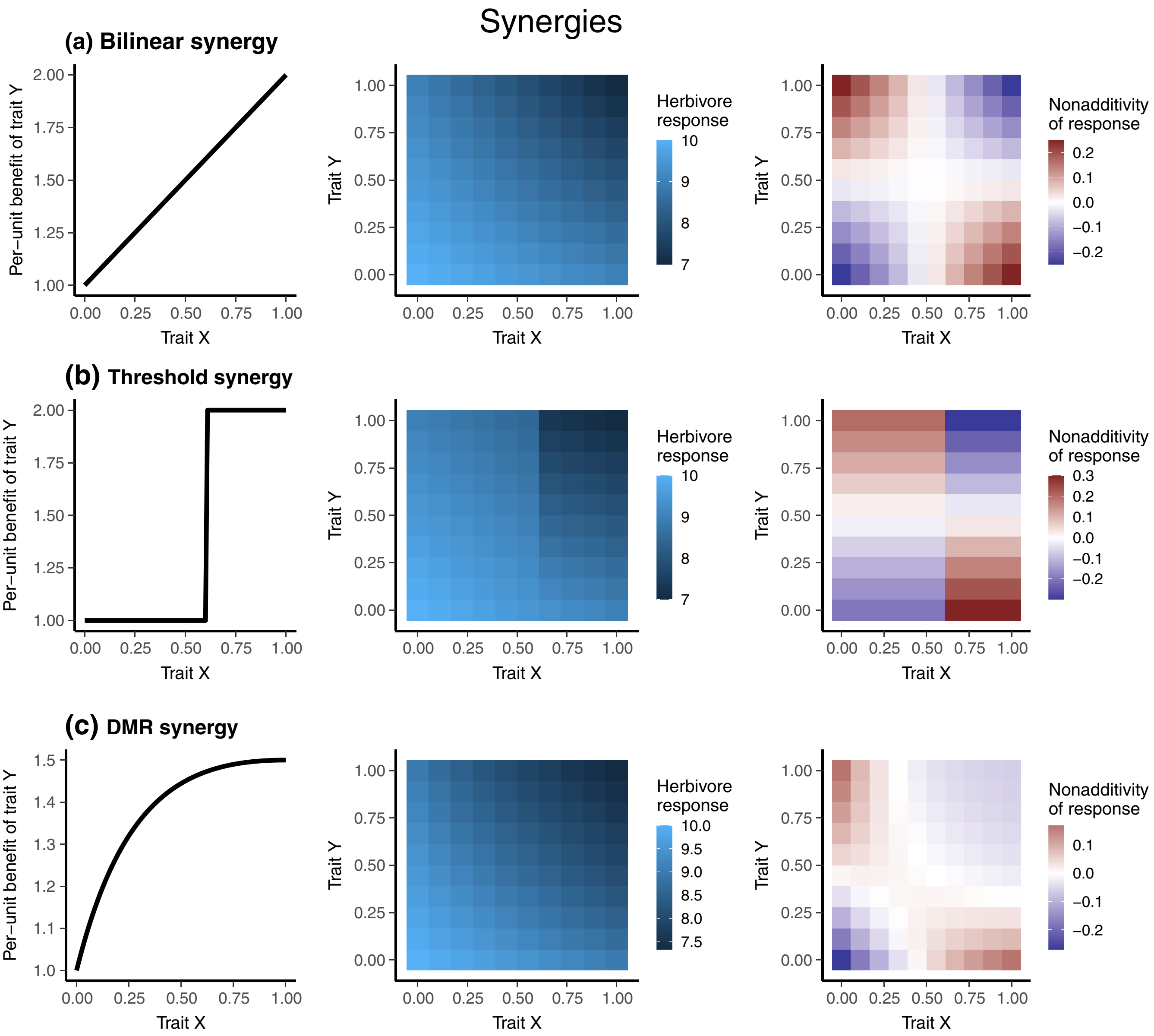
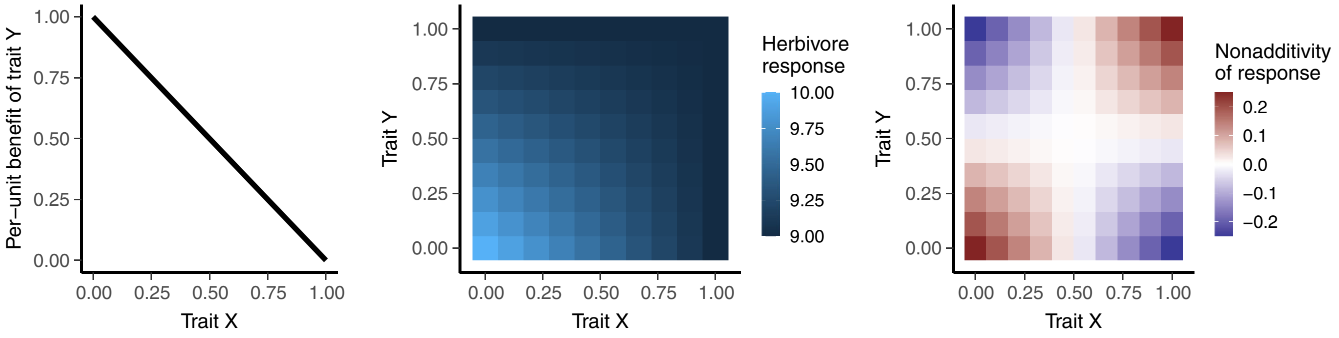


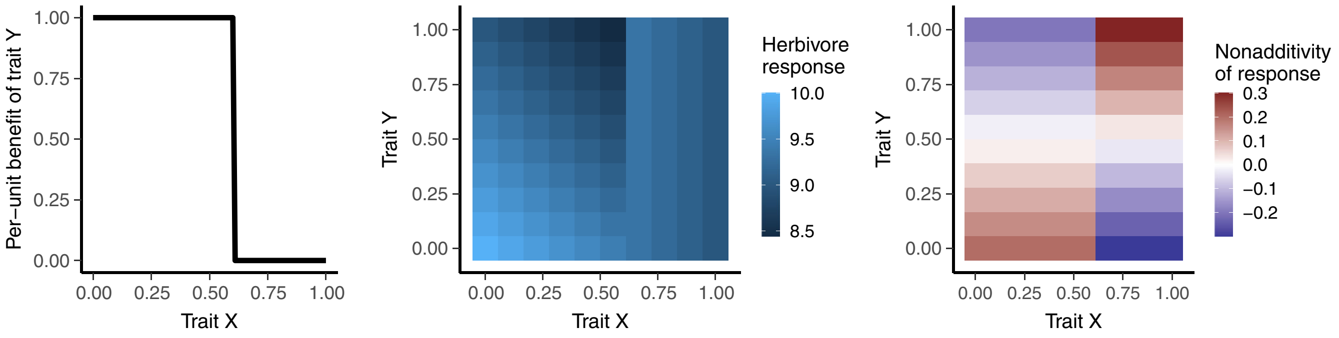
FIGURE 3 Data simulated from known synergies or antagonisms. For each relationship, we first show the per-unit benefit of investment in trait Y (when Y is at 0.5) as trait X is varied (left), then herbivore performance, which could represent growth or survival depending on units and rescaling (middle), then the residuals of herbivore performance when fit with an additive model (right). In the residual plots, positive values show herbivores overperforming 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 overperform when one trait is high and the other is low. (d–f) For antagonistic relationships, herbivores overperform the additive model when both traits are high or both are low and underperform when one trait is high and the other is low.

Antagonisms

(d) Bilinear antagonism



(e) Threshold antagonism



(f) Decaying antagonism

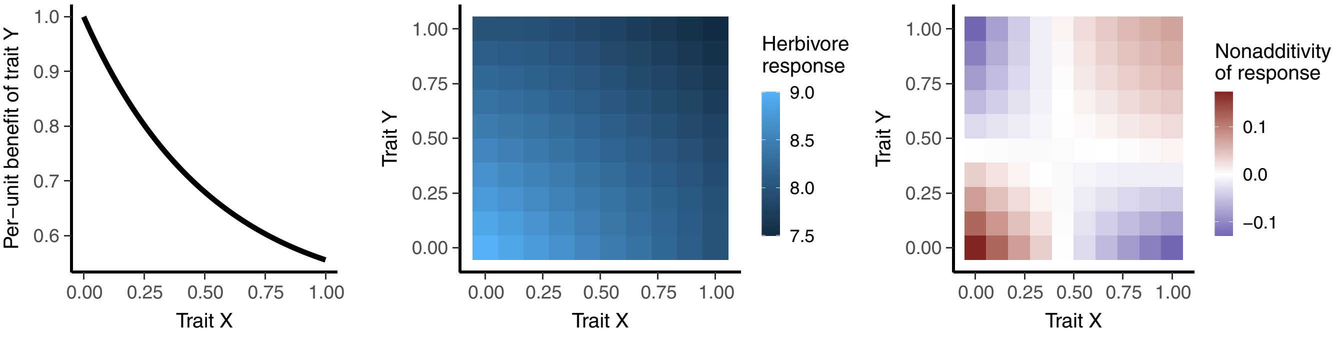


FIGURE 3 (Continued)

carbon was positively correlated with the two traits that clearly incorporated carbon, trichome density ($r = 0.49$, $p < 0.001$) and leaf toughness ($r = 0.21$, $p < 0.01$). In 2016, physical defense traits (trichomes, toughness, and latex) all had significant positive correlation with each other (latex and toughness, $r = 0.38$, $p < 0.01$; latex and trichomes, $r = 0.13$, $p < 0.04$; toughness and trichomes, $r = 0.30$, $p < 0.01$) and were generally negatively correlated or essentially uncorrelated with cardenolides.

Linear regression

For supressing monarch growth, we found strong evidence for a bilinear synergy between non-nitrogen and LMA, moderate evidence for a bilinear synergy between leaf toughness and carbon and between non-nitrogen and trichome density, and weak evidence for a bilinear synergy between leaf toughness and trichome density and between leaf toughness and non-nitrogen (Table 1). For suppressing monarch growth, we also found moderate evidence for a bilinear antagonism between cardenolide 18.6 and non-nitrogen, between cardenolide 18.6 and leaf toughness, and between cardenolide 18.6 and LMA. For reducing monarch survival, we found weak evidence for a synergy between cardenolide 18.6 and latex. For suppressing *L. clivicollis* growth, we found weak evidence for a synergy between latex and cardenolide 10.2 and between latex and leaf toughness. We also found weak evidence for an antagonism between

TABLE 1 Summary of regression analysis for bilinear interactions, showing all trait pairs with $p < 0.1$; lower p-values represent synergies or antagonisms with stronger support.

Data set	Trait 1	Trait 2	Relationship	F or χ^2	p	%PRV	PRR
Beetle growth	Latex	Cardenolide 10.2	Synergy	3.9439	0.051	27	−6.71 to −5.92
Beetle growth	Latex	Leaf toughness	Synergy	3.836	0.054	96	−7 to −5.7
Beetle growth	Cardenolide suite 2016	Cardenolide 8.4	Antagonism	2.8265	0.096	60	−6.69 to −5.67
Beetle survival	Latex	Cardenolide 10.2	Synergy	2.6981	0.1	20	0 to 0.84
Monarch growth	Non-nitrogen	LMA	Synergy	10.3651	0.002	53	−5.77 to −4.51
Monarch growth	Non-nitrogen	Cardenolide 18.6	Antagonism	5.7922	0.018	84	−5.62 to −4.58
Monarch growth	Leaf toughness	Carbon	Synergy	5.2086	0.025	61	−5.84 to −4.57
Monarch growth	Leaf toughness	Cardenolide 18.6	Antagonism	5.187	0.025	98	−5.39 to −4.58
Monarch growth	Cardenolide 18.6	LMA	Antagonism	4.9287	0.029	75	−5.52 to −4.58
Monarch growth	Trichome density	Non-nitrogen	Synergy	4.2549	0.042	34	−5.37 to −4.38
Monarch growth	Leaf toughness	Trichome density	Synergy	3.5435	0.063	34	−5.46 to −4.5
Monarch growth	Leaf toughness	Non-nitrogen	Synergy	3.0646	0.083	61	−5.32 to −4.7
Monarch survival	Latex	Cardenolide 18.6	Synergy	3.1335	0.077	82	0.66 to 1

Note: LMA is leaf mass per area, the inverse of specific leaf area. F-statistic and chi-square statistic are for a single model term with 1 df; relationship is identified by the sign of the interaction coefficient (negative = synergy). %PRV measures the importance of nonadditivity in explaining model fit (see *Methods*); PRR ("predictable response range") measures the range of response values predicted by the model (see *Methods*). For growth, PRR is in units of log (grams final weight), and for survival, PRR is the expected probabilities of herbivore survival.
Abbreviation: PRV, predicted response variation.

cardenolide 8.4 and the covarying cardenolide suite 2016. For reducing *L. clivollis* survival, we found weak evidence for a synergy between latex and cardenolide 10.2.

All of our presumed defense traits showed context-dependent effects. Of the 204 interaction pairs, 93 (46%) had switching points that were within one SD of the mean of the second trait (e.g., Figure 4) (see "context-dependence-all.csv" in Edwards et al., 2022). Only 75 of the pairs (37%) had switching points more than two SDs from the mean. Moreover, of all the traits measured, each of them were context-dependent rather than consistently defensive (or consistently beneficial to herbivores) in at least one trait pairing for either growth or survival (Table 2).

Simulations to classify random forests

Data simulated from synergies always produced residual plots in which the bottom left and top right regions showed herbivores underperforming compared to predictions from an additive model (an ANOVA model with no interaction terms, fitted to the simulated data), and the top left and bottom right showed herbivores overperforming compared to predictions from an additive model (e.g., Figure 3a–c). The reverse was true for antagonisms: Herbivores always overperformed compared to predictions from an additive model in the bottom left and top right and underperformed compared to predictions from an additive model in the top left and bottom right regions (e.g., Figure 3d–f). Depending on the functional form of the synergy or antagonism, patterns of residuals did not always separate perfectly into four quadrants (e.g., Figure 3c, right panel), but the general pattern held true for all synergies and antagonisms we evaluated (see code in Edwards et al. [2022] to explore additional synergies and antagonisms).

Random forests

Random forests identified many additional synergies and antagonisms that were not detected using regression approaches (recall, differences in results are expected, because the regression analysis specifically tests for bilinear departures from linearity, whereas the random forests analysis tests for arbitrary deviations from additivity). Because our metrics for evaluating the nonadditivity of the modeled relationship are new, there are no clear guidelines as to what constitutes strong or weak evidence. We chose to emphasize synergies and antagonisms with a %PRV of 20 or higher (i.e., 20% or more of the sum of squares of the random forest model predictions cannot be explained by additive models) for which the relationship was clear (i.e., θ outside the 95% range of the null distribution). We chose this reporting threshold because we judged that it identified a reasonable but not overwhelming number of highest-impact

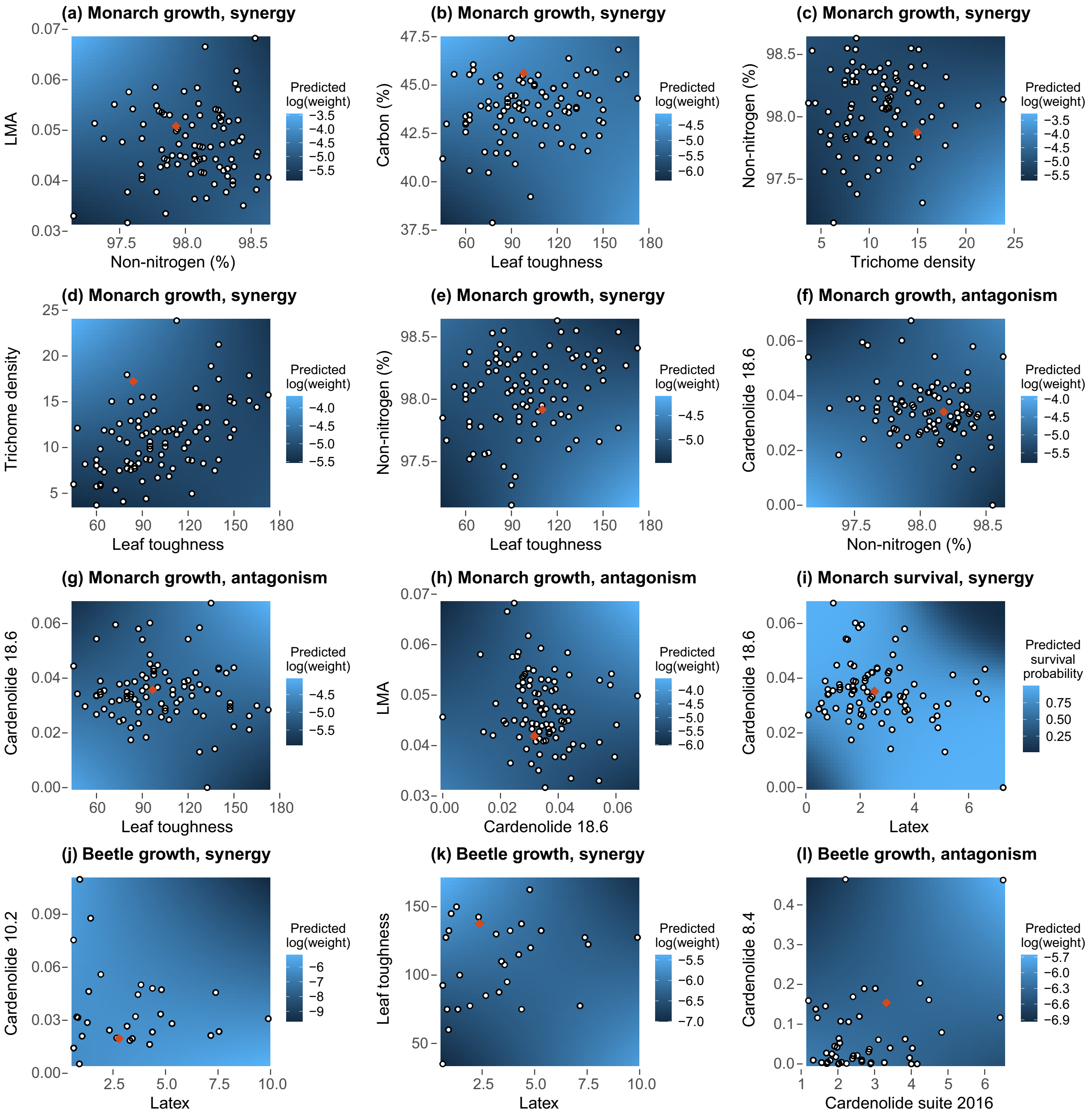


FIGURE 4 Bilinear synergies fit to observed data using regression models, for all interaction terms with $p < 0.1$ (Table 1). Heat maps show predicted survival or growth from fitted models (analogous to central column of Figure 3); points correspond to trait values of observed plants. Orange diamonds show the "switching points" where the effect on herbivores of increasing a plant trait changes from being helpful to harmful or vice versa. Note that the values of the surface in regions without data are unlikely to represent meaningful patterns (i.e., extrapolation away from the data should not be trusted). Units: Leaf mass per area is shown in grams per 10 cm^2 dry mass, carbon and non-nitrogen in percentage of dry mass, leaf toughness in grams of force, trichomes in counts per millimeter of transect, cardenolides in milligrams per gram of dry mass, latex in milligrams of fresh mass, and cardenolide suites are unitless.

interactions on which to focus. We provide readers with all results with clear relationships and a %PRV larger than 5 in Table 3 (and all results are available in "results-correspondence.xlsx" in Edwards et al., 2022). All 10 synergies and antagonisms with a %PRV of 20 or greater were detected for monarch survival. The relationship with the highest %PRV (33%) was a synergy between latex and trichome density, with an expected probability

TABLE 2 Context dependence of traits; traits were identified as context-dependent if their effect on herbivores switched sign (positive to negative) in response to another trait changing values within one SD of its mean.

Trait	Response	Context-dependent on
Carbon	Monarch growth	...
Carbon	Monarch survival	Cardenolide 18.6, leaf toughness, cardenolide suite 2015
Non-nitrogen	Monarch growth	Leaf mass per area (LMA), cardenolide 10.5, cardenolide 18.6, carbon, trichome density, leaf toughness, cardenolide suite 2015
Non-nitrogen	Monarch survival	LMA, cardenolide 18.6
Cardenolide 10.2	Beetle growth	Cardenolide 8.4, leaf toughness, trichome density, latex
Cardenolide 10.2	Beetle survival	...
Cardenolide 10.5	Monarch growth	LMA, cardenolide 18.6, non-nitrogen, carbon, latex, leaf toughness, cardenolide suite 2015
Cardenolide 10.5	Monarch survival	Cardenolide suite 2015
Cardenolide 18.6	Monarch growth	LMA, cardenolide 10.5, non-nitrogen, carbon, latex, leaf toughness, cardenolide suite 2015
Cardenolide 18.6	Monarch survival	LMA, cardenolide 10.5, non-nitrogen, carbon, latex, trichome density, cardenolide suite 2015
Cardenolide 8.4	Beetle growth	Cardenolide 10.2, leaf toughness, trichome density
Cardenolide 8.4	Beetle survival	...
Cardenolide suite 2015	Monarch growth	Non-nitrogen, leaf toughness
Cardenolide suite 2015	Monarch survival	Cardenolide 10.5, cardenolide 18.6, carbon, latex, trichome density
Cardenolide suite 2015	Beetle growth	Cardenolide 8.4, cardenolide 10.2, trichome density
Cardenolide suite 2015	Beetle survival	Cardenolide 10.2
Latex	Monarch growth	...
Latex	Monarch survival	LMA, cardenolide 10.5, cardenolide 18.6, carbon, trichome density, leaf toughness, cardenolide suite 2015
Latex	Beetle growth	Cardenolide 10.2, leaf toughness, trichome density
Latex	Beetle survival	Trichome density
LMA	Monarch growth	Cardenolide 18.6, non-nitrogen, trichome density, cardenolide suite 2015
LMA	Monarch survival	Cardenolide 10.5, cardenolide 18.6, non-nitrogen, carbon, latex
Leaf toughness	Monarch growth	Cardenolide 10.5, cardenolide 18.6, non-nitrogen, carbon, latex, trichome density, cardenolide suite 2015
Leaf toughness	Monarch survival	Carbon
Leaf toughness	Beetle growth	Latex
Leaf toughness	Beetle survival	Cardenolide 10.2, cardenolide suite 2016, trichome density
Trichome density	Monarch growth	...
Trichome density	Monarch survival	Cardenolide suite 2015
Trichome density	Beetle growth	Cardenolide 8.4, cardenolide 10.2, cardenolide suite 2015, latex
Trichome density	Beetle survival	Cardenolide 10.2, cardenolide suite 2016, leaf toughness, latex

of survival ranging between 0.68 and 0.93 across observed trait values (PRR). Of the 10 interactions with a %PRV of 20 or higher, physical traits of leaves—trichome density, leaf toughness, and LMA—were present in all but one, half of the interactions involved one of the two nutritional quality traits (carbon or non-nitrogen), and three of the five synergies involved one cardenolide compound, cardenolide 10.5.

Correspondence between regression and random forest results

Eight of the 13 bilinear synergies and antagonisms identified using regression were similarly classified as synergy or antagonism using random forests (for joint summaries of regression and random forest analyses, see "results-correspondence.xlsx" in Edwards et al., 2022).

TABLE 3 Summary of random forest analysis showing all trait pairs with %PRV of five or higher (after rounding) with clear synergies or antagonisms (θ outside the 95% limits of the null distribution from permutation testing).

Data set	Trait 1	Trait 2	Relationship	%PRV	PRR
Monarch survival	Latex	Trichome density	Synergy	33	0.68 to 0.93
Monarch survival	LMA	Cardenolide 10.5	Synergy	26	0.78 to 0.94
Monarch survival	Leaf toughness	Trichome density	Antagonism	26	0.7 to 0.93
Monarch survival	Cardenolide 10.5	Carbon	Synergy	25	0.8 to 0.93
Monarch survival	Trichome density	Cardenolide 10.5	Synergy	24	0.66 to 0.93
Monarch survival	Trichome density	Carbon	Antagonism	23	0.7 to 0.92
Monarch survival	Latex	Leaf toughness	Antagonism	22	0.69 to 0.94
Monarch survival	LMA	Carbon	Antagonism	21	0.79 to 0.92
Monarch survival	LMA	Non-nitrogen	Antagonism	20	0.7 to 0.93
Monarch survival	Trichome density	Non-nitrogen	Synergy	20	0.66 to 0.93
Monarch survival	Leaf toughness	Carbon	Antagonism	19	0.76 to 0.93
Monarch survival	Leaf toughness	Cardenolide 10.5	Synergy	18	0.78 to 0.94
Monarch growth	Leaf toughness	Carbon	Synergy	18	−5.37 to −4.9
Monarch survival	Non-nitrogen	Carbon	Synergy	17	0.74 to 0.93
Monarch survival	LMA	Leaf toughness	Antagonism	16	0.73 to 0.93
Monarch survival	Latex	Cardenolide suite 2015	Synergy	16	0.69 to 0.93
Monarch survival	Latex	Cardenolide 18.6	Synergy	16	0.7 to 0.93
Monarch survival	Cardenolide 10.5	Cardenolide suite 2016	Antagonism	15	0.77 to 0.93
Monarch survival	Cardenolide 18.6	Carbon	Antagonism	13	0.8 to 0.92
Monarch growth	Cardenolide suite 2015	Carbon	Antagonism	11	−5.33 to −4.9
Monarch survival	Cardenolide 18.6	Non-nitrogen	Synergy	9	0.71 to 0.92
Monarch growth	Trichome density	Carbon	Antagonism	9	−5.39 to −4.88
Monarch growth	Non-nitrogen	Carbon	Synergy	8	−5.3 to −4.9
Monarch growth	LMA	Non-nitrogen	Synergy	8	−5.31 to −4.95
Beetle growth	Leaf toughness	Cardenolide 10.2	Synergy	8	−6.51 to −5.92
Monarch growth	LMA	Carbon	Antagonism	7	−5.35 to −4.88
Beetle growth	Leaf toughness	Cardenolide suite 2016	Synergy	7	−6.57 to −6.03
Beetle growth	Cardenolide 10.2	Cardenolide suite 2016	Synergy	6	−6.59 to −6.06

Note: %PRV and PRR are the same metrics as in Table 1.

Abbreviations: LMA, leaf mass per area; PRR, predictable response range; PRV, predicted response variation.

In four cases, regression identified bilinear interactions that our random forest method did not clearly identify as either synergy or antagonism. For the interaction between cardenolide 8.4 and the cardenolide 2016 suite on *L. clivicollis* growth, the "unclear" designation derives from poor statistical power (only four of the 100 grid cells in trait space were usable) (Appendix S2: Figure S1). The other three interactions (latex by leaf toughness on *L. clivicollis* growth, cardenolide 18.6 by leaf toughness on monarch growth, non-nitrogen by leaf toughness on monarch growth) likely reflect complicated functional forms that did not fall clearly into synergies or antagonisms using random forests but were identifiably synergies or antagonisms when simplified to bilinear interactions. Of the bilinear synergies identified using linear regression, only the leaf toughness by trichome density interaction on monarch growth conflicted with random forest results (regression: weak evidence for a synergy; random forest: antagonism with %PRV = 3). Of the synergies identified using random forests, none of the 10 interactions with %PRV 20 or higher had any evidence of bilinear synergy ($p > 0.1$ in all cases). In fact, of the 37 interactions random forest analyzed with a %PRV of 5 or greater, only four interactions (synergies) had p-values < 0.1. In general, %PRV was much higher for regression models than for random forests. This was true for all bilinear interactions with $p < 0.1$, 65% of interactions with random forest %PRV greater than or equal to 5, and 70% of all interactions.

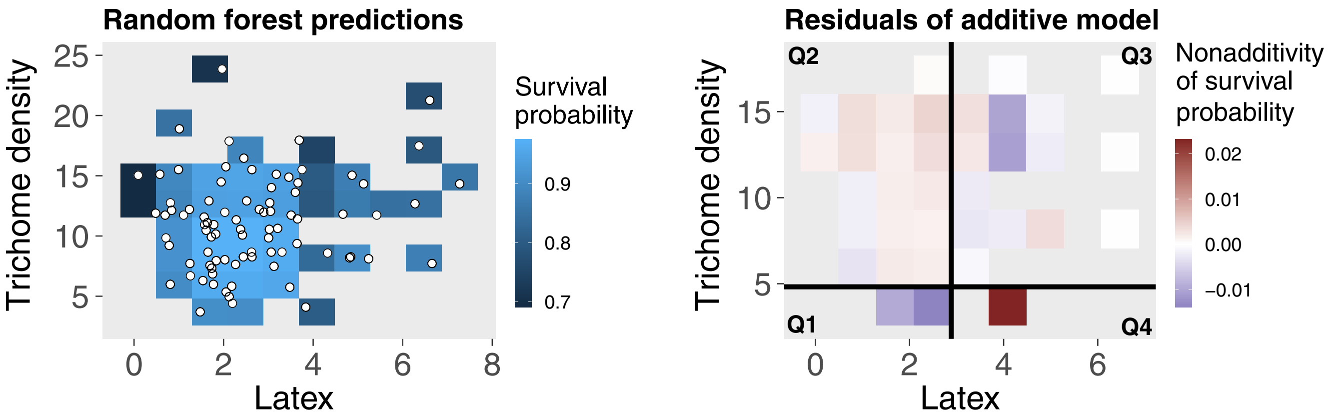
DISCUSSION

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. We found the first direct empirical support for previously hypothesized synergies between latex and cardenolides (Figure 4i,j) and between latex and trichomes (Figure 5a) on monarch survival. We also found evidence for new synergies and antagonisms. The strongest evidence was for a bilinear synergy between non-nitrogen and LMA on monarch growth (Figure 4a): monarch growth was worse on thick, low-nitrogen leaves than the leaf thickness or low nitrogen alone would imply. For low values of LMA (thin leaves), increased non-nitrogen led to *increased* herbivore growth; similarly, for low values of non-nitrogen, increased LMA increased herbivore growth. However, at higher levels of either trait—past the switching point—increasing the level of the other trait led to decreased herbivore performance. Lack of nitrogen and high LMA may thus jointly contribute to low nutritional value for insect herbivores (Wright & Cannon, 2001). Indeed, comparisons across milkweed species previously suggested low nutritional quality as a convergent defense strategy employed in this genus (Agrawal & Fishbein, 2006). In addition to potentially shaping defense strategies on its own, this synergy between non-nitrogen and LMA reinforces a pattern of covariation between LMA and non-nitrogen associated with the leaf economics spectrum (Fajardo & Siefert, 2018; Wright et al., 2004); these trait correlations have been explained by physiological costs and benefits associated with resource acquisition and leaf lifespan (Shipley et al., 2006). Previous work in *A. syriaca* showed this relationship among wild plants in the field and among genotypes grown in a common environment (Agrawal, 2020). Our findings suggest that the co-expression of LMA and low leaf nitrogen, identified as part of the leaf economic spectrum, coincides with a defense synergy: Nitrogen-poor plants suppress herbivore growth even more with increasing LMA than do plants with higher nitrogen content. This inference must be balanced, however, by our finding that there was generally an antagonism between LMA and non-nitrogen on monarch survival; a detailed model would be needed to integrate the combined effects of leaf economics, herbivore growth, and herbivore survival.

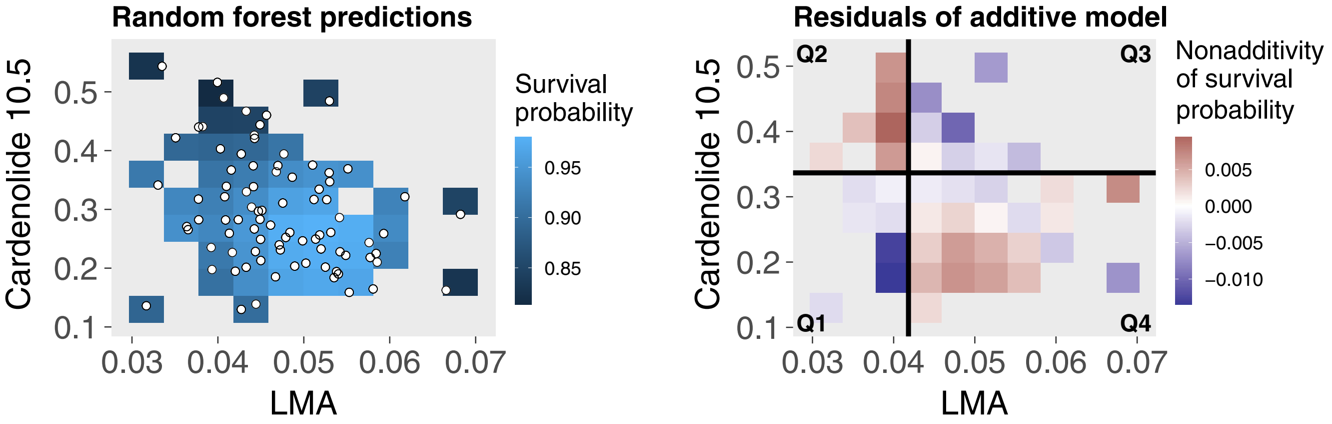
Regression and random forest analyses produced both overlapping and unique results, suggesting these tools are complementary rather than redundant. When regression analysis identified a significant bilinear synergy or antagonism, random forest analysis usually agreed with the type of interaction. However, the strongest synergies and antagonisms identified by the random forest analysis did not show evidence for bilinear interactions. Using only regression analysis, we would have identified a single synergy for monarch survival (latex by cardenolide 18.5). However, by applying our random forest methods, we identified an additional five notable synergies affecting monarch survival (%PRV ≥ 20), including the latex by trichome density synergy previously hypothesized based on the behavior of *D. plexippus* caterpillars (Zalucki et al., 2002). In contrast, many bilinear relationships identified using regression did not have clear patterns or had small %PRV when examined using random forests. This reflects the different strengths of these two methods; the relative simplicity of regressions provides more power when relationships are largely bilinear, allowing us to detect such relationships more easily. In contrast, random forests are more flexible, which allows them to detect relationships that are not bilinear but makes them more data-hungry.

We were surprised to find that our analysis identified nearly every "defensive" trait as context-dependent (Table 2, Figure 4). Our regression models predicted that the effect of increasing any given trait could be helpful or harmful to herbivores depending on the expressed levels of other traits (for example, the switching point in the LMA by non-nitrogen synergy described above). This context dependence is consistent with the adaptive redundancy hypothesis (Rasmann & Agrawal, 2009) and may help to explain why studies often find inconsistent benefits of defensive traits (Agrawal, 2011). For example, although cardenolides and latex are generally correlated with resistance to caterpillars, sometimes they have no apparent effect (Agrawal, 2005; Agrawal & Fishbein, 2006). Although experiments might find different or even conflicting results for many reasons, our study suggests that the effect of a focal "defense" trait could differ or even reverse if another key (potentially unmeasured) trait differed sufficiently in expression between two experiments. Such variation in interacting traits is likely, as studies have found that expression levels of individual defense traits can vary substantially based on environmental conditions. For example, latex can differ in expression based on water availability and plant ontogeny (Agrawal et al., 2014; Barton, 2014), and prickle and trichome density can vary based on light and water availability (Agrawal et al., 2012; Barton, 2014; Ehleringer, 1982). Taken together, this suggests plants face a puzzle of interacting traits whose benefits all depend on one another, with environmental conditions impacting or constraining some of those traits.

(a) Monarch survival, synergy



(b) Monarch survival, synergy



(c) Monarch survival, antagonism

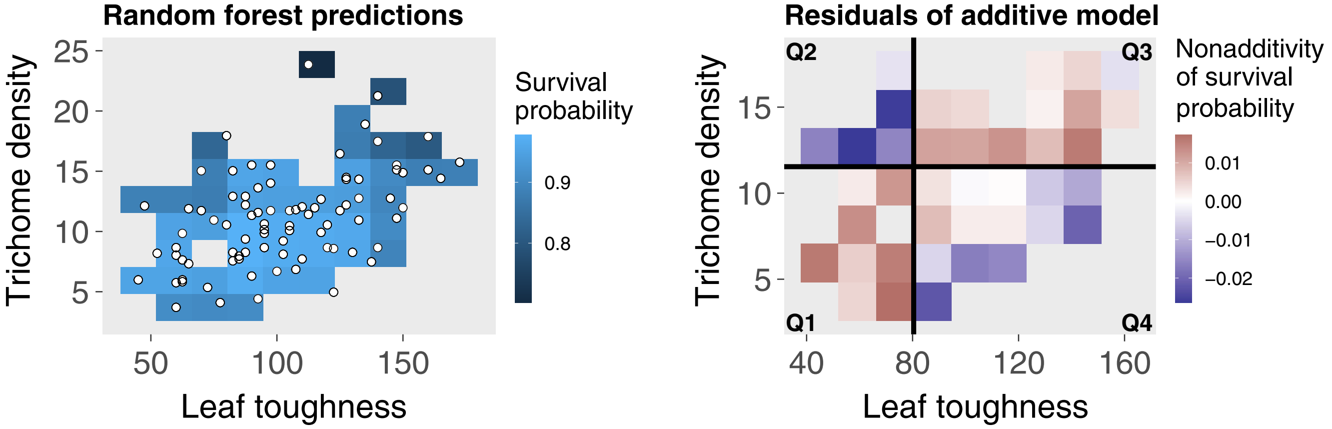


FIGURE 5 Plots of the three synergies and antagonisms identified from random forest analyses that showed the highest percentage of predicted response variation (%PRV); all happen to be from the "Monarch" survival data. On the left, predictions of the random forest for pairs of trait values calculated as described in the methods (analogous to the second column of Figure 3). White points represent actual data; only grid cells containing actual data are plotted. On the right, residuals from fitting an additive model to the predictions on the left (analogous to the third column of Figure 3). Black lines represent the quadrant separation that maximizes the difference in residual between Q1 + Q3 (bottom left and top right quadrants) versus Q2 and Q4 (top left and bottom right quadrants) (i.e., the separation that maximizes the absolute value of θ). Note that because additive models will perfectly fit grid cells that are alone in their row or column (necessarily leading to residuals of 0), we removed these entries from the plots. Comparing this to Figure 3, we can see that the residuals of (a) and (b) are consistent with synergies, and the residuals of (c) are consistent with antagonisms (permutation testing confirms this). Similar plots for every trait pair of each data set appear in Appendix S2: Figure S1. (a) $\theta = -0.56$, %PRV = 33, PRR = 0.683–0.934, (b) $\theta = -0.67$, %PRV = 26, PRR = 0.782–0.937, (c) $\theta = 0.70$, %PRV = 26, PRR = 0.696–0.929. Units are as described in Figure 3. LMA, leaf mass per area; PRR, predictable response range; PRV, predicted response variation.

The herbivores species used in our experiments were both *Asclepias* specialists and have adaptations that partially mitigate many of the defenses of milkweed plants (e.g., Agrawal, 2005; Dobler et al., 2012; Jones et al., 2019; Tao et al., 2016; Zalucki et al., 2001). As such, low nutrient availability is expected to play a large role in determining herbivore performance (Feeny, 1976) and has been proposed as a strategy for *Asclepias* specifically (Agrawal & Fishbein, 2006). Three of the bilinear synergies identified by regression analysis and two of the notable interactions identified by random forest analysis (one synergy, one antagonism) involve non-nitrogen. These synergies and antagonisms involving non-nitrogen may reflect the expected importance of low nutrition as a defensive trait. Equivalently, these interactions between non-nitrogen and other defense traits suggest that the effect of those other traits—trichome density, LMA, cardenolides, leaf toughness—vary based on the resources available to the herbivore. Just as the effects of environmental stresses on plants can vary substantially based on resources available to those plants (e.g., Eneji et al., 2008; Zahoor et al., 2017), we expect the effects of plant defense traits to be different if the herbivore is limited by nitrogen or has it in relative excess.

Several of the identified synergies involved latex: latex by various cardenolides, latex by leaf toughness, latex by trichome density, and latex by LMA. Latex is present in nearly 10% of angiosperm species and has no known function in plants other than defense (Agrawal & Konno, 2009). Latex can be quite harmful to herbivores on its own (e.g., Van Zandt & Agrawal, 2004; Zalucki & Brower, 1992), but its commonness across plant families is all the more understandable if it synergizes with other defenses. Here our measure of latex was the wet mass exuded upon leaf damage, which in *Asclepias* typically correlates negatively with insect performance. Particularly notable were the latex by cardenolide synergies identified in this study, which had previously been proposed but not tested (Agrawal, 2011). Interpreting latex by cardenolide synergies is potentially complicated by the fact that variation in wet latex mass could represent dilution of the latex, in which case higher cardenolide content in the plant tissues might be needed simply to counterbalance dilution. However, because each of our analyses only compared latex measured at a single site in one season, we did not expect high variation in water availability. Instead, measured variation in latex likely reflects differences in the physical defense provided by latex. 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 monarchs or *L. clivicollis* feeding on *A. syriaca*. The latex of most plants contains secondary metabolites, often at elevated levels compared to other plant tissues (Agrawal & Konno, 2009). Further, a recent study of the common dandelion (*Taraxacum officinale*) found that herbivore damage increased when sesquiterpene levels in latex were reduced; whereas this study did not explicitly address synergies, their findings imply a latex by sesquiterpene synergy (Huber et al., 2016). To our knowledge, our study is the first to find explicit evidence for latex by secondary metabolite synergies, but we speculate that such synergies may be common across plant taxa.

Secondary metabolites are often emphasized in studies of plant defenses because of their generally unambiguous function in resistance to herbivores (Ehrlich & Raven, 1964). We found numerous synergies and some antagonisms between cardenolides and other defensive traits in *A. syriaca*; this likely reflects the importance of cardenolides in resisting milkweed herbivores (Agrawal et al., 2021; Tao et al., 2016; Zalucki et al., 2001). However, a meta-analysis of plant traits and herbivore resistance found that physical resistance traits were more reliably correlated with herbivore resistance, particularly when the focus is on specialist herbivores (Carmona et al., 2013). The three purely physical resistance traits represented in our study (LMA, leaf toughness, trichome density) were present in 60% of the identified bilinear interactions and 90% of the identified random forest interactions. The importance of physical resistance traits in plant defenses across species and the prevalence of synergies and antagonisms between physical traits and other defenses in *A. Syriaca* suggest that the suites of traits employed by individual plants may be structured by the costs and indirect benefits of physical traits.

We found PRR to be a useful tool for examining the effects of trait variation on response, measured in meaningful units. We caution that this metric should only be used for data in which variation in traits comes from natural sources (e.g., observational data). In a controlled experiment in which we could manipulate the expression levels of traits, PRR could generally be inflated by increasing the range of trait values outside any biologically meaningful region. %PRV is a valuable tool for interpreting the role of nonadditivity in model fits (and is a necessary step for our use of random forests), but model predictions should be weighed by their proximity to actual data points to avoid %PRV values being driven by extrapolation.

Multivariate plant defense strategies have been explored through observations of trait co-expression. This has been done at the level of between-species comparisons (e.g., Agrawal & Fishbein, 2006; da Silva & Batalha, 2011; Moles et al., 2013; Sanczuk et al., 2020), between-population comparisons (e.g., Barton, 2014), or between individuals within a population (e.g., Agrawal et al., 2014). Trait co-expression can provide indirect support for synergies, but these same signals might represent other forms of advantage or disadvantage for specific combinations. Among *Asclepias* species, comparative work has found interspecific patterns of co-expression between latex and trichomes and between LMA and non-nitrogen, but not between latex and cardenolide expression (Agrawal & Fishbein, 2006). The statistical framework we provide here helps explicitly identify traits that are associated with synergistic (or antagonistic) impacts on herbivores.

Tests of synergies between classes of defenses (rather than between secondary metabolites within a class) are rare, but ours is not the first. A few studies in aquatic systems found 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 artificial diets, suggesting that plant digestion inhibitors might synergize with defense 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 trypsin proteinase inhibitors (TPI, a form of digestion inhibitor) synergized with toxic nicotine to defend against generalist *Spodoptera exigua* (Steppuhn & Baldwin, 2007). Follow-up work in the same system also 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). Like Steppuhn and Baldwin (2007), Green et al. (2001) found phytic acid (an antinutritive defense) reduced insect metabolism of defensive xanthotoxins, implying a potential synergy with phytic acid interfering with specialist herbivore counterdefenses. However, this relationship was not extended to measure the ultimate effect of the phytic acid by xanthotoxin interaction 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 studies to date have used artificial diets (e.g., Whitehead et al., 2021), and nonmetabolite defenses like latex, leaf toughness, and trichomes do not lend themselves to such controlled experiments. Similarly, the most elegant current approaches to testing phytochemical synergies identify an interaction index based on the dose necessary to cause 50% mortality (ED_{50}) (Nelson & Kursar, 1999; Richards et al., 2016; Tallarida, 2000). These tools are well suited to artificial diet experiments in which an ED_{50} is likely 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. The synergies and antagonisms between classes of traits identified here and in the few other studies described earlier suggest that such trait interactions are common, even if the study of them is not. We hope the analytical tools provided here will support a broader exploration of synergies in the study of plant defenses. The benefits of being able to conduct such rigorous tests under natural conditions make this approach an essential complement to more controlled and mechanistic studies.

AUTHOR CONTRIBUTIONS

Collin B. Edwards and Anurag A. Agrawal conceptualized and designed the experiments, and Collin B. Edwards performed the field experiment, lab work, and data analysis. Collin B. Edwards and Stephen P. Ellner developed analytical methods and simulations. Collin B. Edwards wrote the paper with substantial input from Stephen P. Ellner and Anurag A. Agrawal.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data and code (Edwards et al., 2022) are available on Figshare at https://doi.org/10.6084/m9.figshare.20421633.

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SUPPORTING INFORMATION

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

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