Caterpillars on a phytochemical landscape: the case of alfalfa and the Melissa blue butterfly

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Abstract

Modern metabolomic approaches that generate more comprehensive phytochemical profiles than were previously available are providing new opportunities for understanding plant-animal interactions. Specifically, we can characterize the phytochemical landscape by asking how a larger number of individual compounds affect herbivores and how compounds covary among plants. Here we use the recent colonization of alfalfa (*Medicago sativa*) by the Melissa blue butterfly (*Lycaeides melissa*) to quantify plant metabolites and the performance of caterpillars as affected by both individual compounds and suites of covarying phytochemicals. We find that survival, development time and adult weight are all associated with variation in nutrition and toxicity, including biomolecules associated with plant cell function as well as putative anti-herbivore action. The plant-insect interface is complex, with clusters of covarying compounds in many cases encompassing divergent effects on different aspects of caterpillar performance. Individual compounds with the strongest associations are largely specialized metabolites, including alkaloids, phenolic glycosides and saponins. The saponins are represented in our data by more than 25 individual compounds with beneficial and detrimental effects on *L. melissa* caterpillars, which highlights the value of metabolomic data as opposed to approaches that rely on total concentrations within broad defensive classes.

Keywords: *Lycaeides melissa, Medicago sativa*, metabolomics, plant defense, specialization

1 | INTRODUCTION

One of the conceptual pillars of trophic ecology is the idea that herbivores must overcome the barrier of plant defensive chemistry before extracting the nutrients necessary for growth and reproduction (Feeny, Rosenthal, & Berenbaum, 1992). The success of this idea is reflected in areas of research that include coevolution (Agrawal, Petschenka, Bingham, Weber, & Rasmann, 2012), ecological specialization (Dyer, 1995), and nutrient flow in ecosystems (Hättenschwiler & Vitousek, 2000). In most cases, progress has been made by chemical ecologists focusing on small subsets of the specialized metabolites produced by plants and consumed by herbivores. The focus on a few charismatic molecules or classes of compounds, such as furanocoumarins (Berenbaum, 1983) or cardiac glycosides (Zalucki, Brower, & Alonso-M, 2001), was at least in part necessitated by early methods in natural products chemistry that were targeted and not easily optimized for the discovery of large suites of co-occurring metabolites (Dyer et al., 2018; Maag, Erb, & Glauser, 2015). As technological limitations have dissipated, the opportunity now exists for a more comprehensive understanding of the challenges faced by herbivores, with the possibility of discovering, among other things, novel compounds and synergistic interactions among compounds (Prince & Pohnert, 2010; Richards, Dyer, Smilanich, & Dodson, 2010; Sardans, Penuelas, & Rivas-Ubach, 2011). More generally, an important task is to quantify the phytochemical complexity of the antagonistic interaction between plants and herbivores, with an eye towards understanding constraints on the evolution of both players (Fordyce & Nice, 2008; Macel, van Dam, & Keurentjes, 2010) and predicting the formation of new plant-herbivore interactions (Erbilgin, 2018). Here we use the example of a specialized herbivore and a recently colonized host plant to investigate the phytochemical landscape from the perspective of developing caterpillars. By the "phytochemical landscape" we mean metabolomic variation

among individual plants and associated toxic and nutritional effects on, in our case, a focal herbivore (Glassmire et al., 2019; Hunter, 2016; Wu, Wilson, Chang, & Tian, 2019).

The Melissa blue butterfly, *Lycaeides melissa*, is specialized on larval host plants in the pea family (Fabaceae), primarily in the genera *Astragalus* and *Lupinus*. Within the last 200 years, *L. melissa* has colonized introduced alfalfa, *Medicago sativa* (Fabaceae), at least twice and probably multiple times (Chaturvedi et al., 2018), forming a heterogeneous patchwork of association throughout the range of the butterfly in western North America, often with naturalized or weedy patches of *M. sativa*. In general, *M. sativa* is a suboptimal host plant for *L. melissa*: individuals that feed on the plant have reduced survival and performance relative to individuals feeding on native hosts (Forister, Nice, Fordyce, & Gompert, 2009). *M. sativa*-associated populations do, however, show evidence for a slight increase in the ability to develop on the novel resource relative to populations that remain on native plants (Gompert et al., 2015). Additional evolutionary change in populations associated with the novel host is evidenced by reduced female oviposition preference for native hosts (Forister et al., 2012) and reduced caterpillar performance on native hosts (relative to populations that have not shifted to the exotic) (Gompert et al., 2015).

The genetic architecture of host use in this system is known to be polygenic and characterized by loci with conditionally neutral (host-specific) effects and ongoing local adaptation (Gompert et al., 2015). What is needed next is an understanding of which plant traits most affect *L. melissa* fitness. Previous work has suggested that phytochemical variation among host populations is biologically significant for caterpillars eating *M. sativa* (Harrison et al., 2016), but the magnitude of these effects and the salient compounds are unclear. Moreover, caterpillars do not encounter compounds in isolation, but in combinations of covarying

89 molecules, and it is unknown how variation among hosts in phytochemical mixtures affects
90 herbivore evolution. For example, will the trajectory of further local adaptation by *L. melissa* to
91 *M. sativa* be a matter of evolving the ability to detoxify one or a large number of compounds? A
92 better understanding of how key compounds covary among individual plants could also shed
93 light on the potential for evolutionary response of the plant to herbivores in its introduced North
94 American range. Here we use a common garden approach and caterpillars individually reared in
95 a controlled environment to address these questions while describing the effects of metabolomic
96 variation in *M. sativa* on *L. melissa*.
97

98 **2 | METHODS**

99 **2.1 | Plants and caterpillars**

100 Plants used in this project were grown at the University of Nevada, Reno, Main Station
101 experimental farm. The common garden was planted in 2016 with seeds collected the previous
102 year from 45 plants (previously studied by [Harrison et al., 2018]) growing in a fallow field in
103 north-western Nevada on the western edge of the Great Basin Desert. The focal butterfly, *L.*
104 *melissa*, was present in the source field but has not colonized the university farm where
105 experimental plants were grown. The 45 maternal plants each contributed 15 offspring to a
106 randomized grid design in the common garden, irrigated with broadcast sprayers in 2016 and
107 drip in 2017, without supplemental fertilization. A single plant was randomly selected from each
108 maternal family for use in the rearing experiment reported here as a way to capture as much
109 genetic and phenotypic variation as possible.

110 On 17 and 18 July 2017, a total of 45 *L. melissa* females were collected from an alfalfa-
111 associated population near Verdi, NV, and confined to oviposition arenas (three females per

112 arena, 500 mL plastic cups) with host plant leaves and mesh lids sprayed with Gatorade®, a
113 sports drink with sugar, water, carbohydrates, salt and other ingredients that has been used
114 elsewhere for captive butterflies (Mattila & Otis, 2003). After three days, eggs were removed
115 from leaves, pooled, and kept at room temperature until hatching, at which time caterpillars were
116 placed individually in Petri dishes (100 x 25 mm) with leaves of a particular *M. sativa* individual
117 (which became the only plant from which they were fed throughout the experiment). Ten
118 caterpillars were assigned to each of the 45 experimental *M. sativa* plants (for a total of 450
119 independently-reared caterpillars) and kept in a growth chamber set to 25ºC and a 12 hour light /
120 12 hour dark cycle. Caterpillars were given new, undamaged leaves as needed, approximately
121 every 2-3 days. From each caterpillar we recorded survival to adult, sex, date of eclosion (if
122 successful) and adult weight to the nearest 0.01 mg on a Mettler Toledo XP26 microbalance.
123 Adult weight is taken as a proxy for fitness in *L. melissa* (Forister et al., 2009).
124

125 **2.2 | Phytochemistry and plant traits**

126 Metabolomic variation among individual plants was characterized with liquid chromatography–
127 mass spectrometry (LC-MS) (Jorge, Mata, & António, 2016) using leaves collected on a single
128 day at the start of the rearing experiment (as described above, one plant was randomly selected
129 from each of 45 maternal lines in a common garden). Leaves were taken haphazardly from four
130 different stems, avoiding the youngest and oldest leaves, and combined in a single paper
131 collection envelope; we also avoided damaged leaves, although plants were exposed to constant,
132 low levels of natural herbivory from insect and small mammal herbivores before and during the
133 experiment (thus the present study does not address plasticity of defense in response to
134 herbivore attack). Vacuum-dried, ground leaves (10 mg) were extracted in 2 mL of 70%

aqueous ethanol and injected into an Agilent 1200 analytical high performance liquid chromatograph paired with an Agilent 6230 Time-of-Flight mass spectrometer via an electrospray ionization source. Resulting chromatograms were analyzed using MassHunter Quantitative Analysis (v.B.06.00, Agilent, Santa Clara, CA), and major classes of compounds were identified using characteristic relative mass defects (Ekanayaka, Celiz, & Jones, 2015), as described further in the appendix. Leaf protein content was quantified with three replicates (~2 mg each) per plant using the Bicinchoninic acid assay (Pierce Biotechnology, Waltham, MA). Before grinding, five dried leaflets from each sample were weighed to the nearest 0.1 mg, scanned, and area was measured using ImageJ (v.1.52a); specific leaf area (SLA) was calculated as leaf area divided by dry mass. Finally, leaf toughness was measured on fresh material in the common garden, at the start of the experiment (mid-July, when leaves were also sampled for chemistry and protein) and at the end of the experiment (mid-August), from three leaves per plant at each date, with a penetrometer (Chatillon 516 Series) through the center of the middle leaflet, as in (Harrison et al., 2018); the three leaves were selected haphazardly, avoiding the oldest and youngest leaves. Leaf toughness (averaged across the three leaves per plant at each collection) was correlated between early and late in the season ($r = 0.36$), but we focus on the measurements taken at the first time point in subsequent analyses for consistency with samples taken at that time for metabolomics.

2.3 | Overview of analyses of plant traits and caterpillar performance

Our analytical strategy to understand the association between phytochemical variation and caterpillar performance followed two complementary paths, one focusing on reducing the number of variables (through dimension reduction and feature selection) to produce relatively

simple models, and the other on the estimation of effects of all individual compounds on caterpillars (without reducing the number of predictor variables). For the first path, involving dimension reduction, we utilized an approach developed for gene transcription studies that identifies groups or modules of correlated variables with hierarchical clustering (Langfelder & Horvath, 2008); after clustering, we reduced the number of independent variables by selecting among modules and other plant traits (specific leaf area, protein and leaf toughness) using lasso regression (Ogutu, Schulz-Streeck, & Piepho, 2012). Lasso regression shrinks coefficients for less important variables to zero, and is thus useful for model selection, in contrast to ridge regression which constrains coefficients (providing stable estimates) while not excluding variables. Modules (and other plant traits) selected in the lasso regression step were subsequently analyzed in Bayesian linear models that are useful in this context because they allowed us to quantify our confidence in the sign of effects (positive or negative) as continuous probabilities (as opposed to relying on arbitrary significance cutoffs). For the second analytical path, we utilized ridge regression (Ogutu et al., 2012) to estimate effects for all compounds simultaneously, which allowed us to investigate the distribution of effects among compounds and classes of compounds. Both analytical paths incorporated cross-validation during the lasso and ridge regressions (further details below in section 2.4.2), and as a means of evaluating the predictive success of the Bayesian models. We also used randomization tests to compare the performance of modules and individual compounds with randomly-chosen suites of compounds.

2.4 | Dimension reduction and feature selection

2.4.1 | Clustering of phytochemical variables

180 We chose an approach (the first set of analyses mentioned above) that reduces the number of
181 independent variables while allowing us to learn about the correlational structure of the data,
182 specifically unsupervised hierarchical clustering as implemented in the blockwiseModules
183 function of the WGCNA package (Langfelder & Horvath, 2008) in R (R Core Development
184 Team, 2016). Among the options in the pipeline, we used positive correlations among variables
185 ("signed" network type), merge cut height at 0.25, and correlations raised to the power of five
186 (which is where the scale free topology index reached a plateau). Through experimentation, we
187 found that our results with LC-MS data were robust to variation in these choices, including the
188 choice of signed or unsigned networks. After an initial round of clustering, we took a remaining
189 19 unassigned compounds and put them through a second round of clustering (although the
190 majority of consequential compounds were identified in the first round). One output of the
191 WGCNA procedure is the first eigenvector from each cluster of compounds, which reduced our
192 number of predictor variables by a factor of ten.
193

194 **2.4.2 | Lasso regression and Bayesian models**

195 The resulting eigenvectors plus protein, SLA (specific leaf area) and leaf toughness were then
196 put through the feature reduction step of lasso regression (Ogutu et al., 2012), a penalized
197 regression that allows beta coefficients to be constrained to zero (thus excluding variables). We
198 used the cv.glmnet function of the glmnet package (Friedman, Hastie, Simon, & Tibshirani,
199 2016) with cross-validation during error reduction set to leave out one plant (and associated
200 caterpillars) at each iteration. The variables selected by the lasso were then put into a Bayesian
201 linear model to estimate coefficients and associated credible intervals using JAGS (version 3.2.0)
202 run in R with the rjags package (Plummer & others, 2003). Two Markov chains were run for

10,000 steps for each analysis (no burn in was required) and chain performance was assessed by plotting chain histories, and calculating the Gelman and Rubin convergence diagnostic and effective sample sizes (Brooks & Gelman, 1998; Gelman, Rubin, & others, 1992). For all models, minimally-influential priors for the regression coefficients were modeled as a normal distribution with a mean of zero and variance of 100 (variance = 1/precision). We quantified our confidence in the sign of coefficients (positive or negative) as the fraction of the posterior samples that were less than zero (for coefficients with a median negative value) or greater than zero (for coefficients with a median positive value).

All analyses were done using the R statistical language (R Core Development Team, 2016) on scaled (z-transformed) predictor variables, and both lasso and Bayesian models used binomial (for survival), Poisson (for development time) and Gaussian (for adult weight) errors. The latter two analyses (development time and adult weight) included sex as a factor. The analysis of development time also included adult weight as a covariate; while (reciprocally) the analysis of adult weight included development time as a predictor. These variables are negatively correlated (r = -0.52), and they function as useful covariates of each other, allowing us to investigate the possibility of unique plant effects on weight gain and development time, which could not be discovered if, for example, these variables were combined into a single performance index.

2.4.3 | Cross-validation and resampling to judge model performance

The success of models developed with the dimension reduction and feature selection pipeline was judged in two ways. We used a cross-validation procedure in which we left out one plant (and associated caterpillars) in each iteration of the Bayesian model and then used the

226 estimated coefficients (for phytochemical variables and other plant traits) to predict the
227 performance of the unobserved caterpillars. After 45 iterations (one for each plant), we
228 calculated a simple correlation coefficient between the observed and predicted performance of
229 caterpillars across plants. In addition, we repeatedly resampled the original LC-MS data to
230 match the structure of the reduced set of predictor variables to ask to what extent randomly
231 assembled modules could outperform the empirically-derived modules (in other words, if a
232 model contained two modules with 15 and 20 compounds, simulated predictors would include
233 modules based on 15 and 20 randomly-selected compounds).
234

235 **2.5 | Individual compound effects**

236 The second path of our two-part analytical strategy involved simultaneous estimation of the
237 effects of all individual chemical compounds on caterpillar survival, development time and adult
238 weight. For this approach, we again used penalized regression (in the glmnet package [Friedman
239 et al., 2016]), but this time with ridge regression (instead of lasso) which constrains beta
240 coefficients to avoid variance inflation but does not eliminate variables. As with the analyses
241 above, ridge regression was done using error structures appropriate to the specific response
242 variables, and included additional covariates where possible (in models of development time and
243 adult weight). The resulting coefficients associated with all individual compounds were
244 examined as a second perspective on the modules examined in the first set of analyses, and were
245 used to ask to what extent individual compound effects could be predicted by the degree to
246 which they vary among individual plants as quantified with the simple coefficient of variation.
247 To assess confidence in the results of ridge regressions, we used a bootstrap approach, repeatedly
248 resampling the data and estimating coefficients 1000 times, noting the compounds whose

249 bootstrap confidence intervals did or did not overlap zero (Delaney & Chatterjee, 1986). We
250 also allowed for the discovery of interactions among compounds using penalized regression on
251 all individual compounds and all pairwise interactions between compounds. For ease of
252 interpretation, this final analysis of potential interactions used lasso (not ridge) regression, letting
253 the coefficients for many of the individual compounds and pairwise interactions go to zero.
254

255 **3 | Results**

256 Of the 450 caterpillars that started the experiment, 261 were reared to eclosion as adults (a
257 mortality rate similar to previous work with this system [Gompert et al., 2015]) on leaves from
258 45 individual alfalfa plants that were characterized for protein, leaf toughness, specific leaf area
259 and 163 individual metabolomic features (see Figure 1 for variation among plants in caterpillar
260 performance and a subset of plant traits, and appendix Table A1 for a list of compounds).
261 Hierarchical clustering identified 14 subsets (or modules) of compounds with generally low
262 correlations among modules and high correlations within modules (see appendix Figures A1 and
263 A2 for correlations within and among modules, and Figure A3 for module variation among
264 plants). The correlational structure of the phytochemical data is illustrated as an adjacency
265 network in Figure 2 (and in Figure A4 colored by compound class instead of module), where it
266 can be seen that some modules (e.g., modules 1 and 2) contain a great diversity of compound
267 types, while other modules are made up of more narrow classes (e.g. modules 7 and 8 are mostly
268 saponins, a class of defensive metabolites [Levin, 1976]). From the 14 eigenvectors
269 summarizing variation in the modules, as well as the other plant traits, lasso regression (Ogutu et
270 al., 2012) produced a reduced set of potential predictors which were then used in Bayesian
271 multiple regression models that included between six and seven independent variables (Table 1).

The models had reasonably high performance in leave-one-out cross-validation: correlations between observed and predicted values were between 0.50 and 0.59 (Table 1), and thus model predictions explained between 25 and 35% of the observed variation in caterpillar performance. Resampling analyses were similarly successful (Supporting Information Figure A5), with only a small fraction (never more than 3%) of randomly-generated models exceeding the variance explained by the models reported in Table 1.

Variation among plants in the suites of covarying compounds had large effects on caterpillar performance: for example, the beta coefficient of -0.40 (on the log-odds scale) associated with module 3 corresponds to a 0.10 reduction in the probability of survival (relative to average) associated with a one unit change in that phytochemical module (Table 1; note that in Table 1 and elsewhere negative coefficients for development time are associated with fewer days, and thus can be thought of as potentially beneficial effects, in contrast to negative coefficients for survival and weight that are detrimental to caterpillars). The phytochemical predictor variables are eigenvectors from clustering analysis, and thus are not entirely straightforward to interpret, especially when the clustering analysis was itself based on z-transformed data. It is important to note that our LC-MS data (used in clustering analysis) consists of peak areas divided by the peak of an internal standard, and again divided by the dry weight of the sample (thus, in total, referred to as "relative abundance per dry weight"; see appendix for additional details including choice of standard). Variation in these numbers reflects variation in concentrations within compounds (among plants), but care should be used in comparing among compounds because of different ionization responses relative to the standard (thus the use of z-transformation for among-compound analyses). Nevertheless, the effects reported in Table 1 reflect real variation in suites of compounds, as can be seen in correlations

295 between eigenvectors and individual compounds in Supporting Information Figure A2, and in
296 variation among plants in average z-scores in Figure A3.

297 In some cases, modules included in multiple regression models had common effects
298 across response variables (e.g., the positive association of module 10 with both survival and
299 adult weight or the negative association of module 3 also with survival and weight), while other
300 modules had more specific effects on a single response (e.g., modules 11 and 13 on survival).
301 Specific leaf area had a negative association with survival and adult weight, with the coefficients
302 for specific leaf area (-0.32 for survival and -0.35 for weight) being of similar magnitude to some
303 of the phytochemical effects. Neither leaf toughness nor protein had sufficiently strong
304 associations with any of our caterpillar response variables to pass the initial filter of the cross-
305 validated lasso regressions.

306 Module-based analyses (as in Table 1) focused on feature reduction with lasso regression;
307 as a complementary analytical approach, we also used ridge regression (Ogutu et al., 2012) on all
308 of the individual compounds (ridge regression estimates effects of compounds without excluding
309 variables as in lasso regression). Analyses of individual compounds by ridge regression (Figure
310 3) were broadly consistent with the strongest module-specific effects, as can be seen, for
311 example, with module 10 having positive effects on survival and adult weight in module
312 analyses (Table 1) and in compound-specific analyses (Figure 3). Similarly, the individual
313 compounds in module 3 had negative compound-specific effects on survival (Figure 3), and that
314 module had the strongest negative effect on survival in the eigenvector-based analyses in Table
315 1. Not surprisingly, the larger modules (with a greater number of covarying compounds,
316 including many primary metabolites) tended to have a more complex mix of positive and
317 negative effects (for example, modules 1 and 2, Figure 3). For ease of interpretation, the

318 coefficients from compound-specific regressions of survival and development time (in Figures 3
319 and 4) have been back-transformed to be on the scales of probability and days (respectively), and
320 displayed as changes relative to intercepts. For example, a compound with a relatively large
321 effect on survival in Figure 3 could be associated with a 0.005 reduction in the probability of
322 survival relative to average survival and while holding other compounds constant.

323 We saw some variation among classes of compounds in their effects on caterpillars
324 (Figure 4). All classes included positive and negative effects, with saponins, alkaloids and
325 phenolic glycosides including some of the stronger negative effects of individual compounds,
326 while lipids and sterols tended towards positive associations with survival and development
327 (Figure 4). We also considered potential pairwise interactions among individual compounds, and
328 found few interactions that passed the filter of the penalized regression (Supporting Information
329 Table A2), at least relative to the large number of potential interactions. Saponins and alkaloids
330 tended to be overrepresented in the interactions that were detected, and phenolic glycosides were
331 involved in stronger negative interactions relative to other compounds (Figure A6). We did not
332 find evidence that more or less variable compounds (among individual plants) had differential
333 effects on caterpillars (Figure A7).

334

335 **4 | Discussion**

336 The results reported here represent a dissection of the phytochemical landscape facing a
337 specialized insect herbivore attacking a recently-colonized host plant (Hunter, 2016). The
338 phytochemical landscape is both physical, referring to variation in compounds among individual
339 plants in a common garden (Figure A3), and hypothetical to the extent that effects of individual
340 compounds on caterpillars are estimated, although compounds are of course not encountered in

341 isolation. Our exploration of the phytochemical landscape facing *L. melissa* on *M. sativa* is
342 necessarily a first draft based on a single point in time. Despite the snapshot nature of our study,
343 models including suites of covarying metabolites and other plant traits had predictive success for
344 caterpillar performance and suggested different natural products affecting survival, development
345 time and adult weight. Previous work with *M. sativa* and other insect herbivores has focused on
346 sapogenic glycosides (Levin, 1976), and a simple outcome from our study could have been that
347 one or a small number of saponins have anti-herbivore properties that reduce fitness of our focal
348 insect. Instead, we find large numbers of compounds with potentially consequential effects on
349 caterpillars (Figure 3), and which were in some cases of similar magnitude or greater than the
350 effects of morphological features, including leaf toughness and specific leaf area (Carmona,
351 Lajeunesse, & Johnson, 2011).

352 We find that prominent classes of specialized metabolites in our focal plants, such as
353 saponins and peptides, include compounds with both positive and negative effects on survival
354 and development of caterpillars. Positive effects of these compounds are potentially associated
355 with feeding stimulation, as has been observed (along with other positive effects) for other
356 specialist herbivores and plant toxins (Seigler & Price, 1976; Smilanich, Fincher, & Dyer, 2016).
357 Negative effects of saponins on insects potentially include disruption of hormone production
358 (Chaieb, 2010), although exact modes of action on *L. melissa* will await further study. Although
359 many of the compounds with strong effects are specialized metabolites (including alkaloids and
360 phenolic glycosides, as well as saponins and peptides), we also find both positive and negative
361 effects on caterpillar performance associated with primary metabolites (Figure 4), especially
362 phospholipids (Figure 2). These could be direct effects if a compound is suboptimal for
363 development, or they could be associated with nutritional imbalance (Behmer, 2009), such that

too much of one nutrient makes it difficult for caterpillars to consume a balanced diet. It has been suggested that the presentation of unbalanced nutrition can be a kind of anti-herbivore strategy (Berenbaum, 1995), although this has not been studied in the *L. melissa*-*M. sativa* interaction.

The finding that our specialist herbivore is affected by a wide range of metabolites that vary greatly even within a single host population has implications for our understanding of heterogeneity in the system, and also for local adaptation of the herbivore to the novel host. *Lycaeides melissa* typically colonizes weedy or feral patches of *M. sativa* on roadsides or integrated into natural communities, and previous work has documented dramatic variation among individual alfalfa locations (often in close proximity) in the extent to which they can support caterpillar development (Harrison et al., 2016). Previous phytochemical data with a lower resolution was less successful in explaining that variation (Harrison et al., 2016), but the results reported here suggest that among-patch variation could be explained by future studies using detailed metabolomic data. The within-population complexity described in the current study also raises the possibility that the novel host presents a multi-faceted and potentially ever-shifting target from the perspective of evolving butterfly populations (Chaturvedi et al., 2018; Gompert et al., 2015; Harrison et al., 2016). In particular, it is possible that *M. sativa* defense against a specialist herbivore might be realized through different combinations (within and among populations) of individually-acting compounds, thus making it less likely that butterflies in any one population possess an effective suite of alleles that improve fitness on *M. sativa*.

The correlational structure of the phytochemical variation that we observed has implications for the evolution of plant defense and the accumulation of insect herbivores on *M. sativa*. Specifically, correlations among modules (which are themselves composed of a diversity

387 of compound types) should make it possible to hypothesize directions of least resistance for
388 defense evolution. Module 3, including an alkaloid with a prominent effect on caterpillars but
389 also phospholipids and saponins, had a negative effect on survival (Table 1, Figure 3). Module 3
390 negatively covaried with module 2, which was itself positively associated with caterpillar
391 survival (including a peptide of large effect but many other compound types as well). Thus an
392 increase in module 3 and an associated decrease in 2 would be beneficial for the plant, at least
393 with respect to herbivory by our focal herbivore. Predicting evolutionary response by *M. sativa*
394 would of course depend on a genetic understanding of the relevant plant traits, which the present
395 study does not include. However, a recent study of *M. melissa* performance on a related plant,
396 *Medicago truncatula*, found that genetic variation in the plant explained a substantial proportion
397 of phenotypic variation (between 8 and 57%) in phytochemical and structural traits but also in
398 caterpillar performance (Gompert et al., 2019). Of course, most plants do not have the luxury of
399 optimizing defense against a single herbivore, and it is easy to imagine that improvements in
400 defense against one enemy could lead to increased attraction to another (Salazar et al., 2018),
401 especially given the diversity of effects even within major classes studied here, including
402 saponins and phenolic glycosides. Compounds in the latter class (phenolics) were found to have
403 strong positive and negative effects on assemblages of arthropods associated with the maternal
404 plants from which seeds were collected to start the common garden used in the present study
405 (Harrison et al., 2018).

406 The results reported here raise a number of avenues for future exploration, including the
407 apparent overrepresentation of both saponins and alkaloids in interactions with other compounds
408 (Figure A6). Relative mass defect (RMD) is a useful tool for the categorization of compounds
409 (Table A1), but it has limitations in complex mixtures; we are developing methods that use other

410 data from high resolution mass spectrometry to further refine categorization of *Medicago*
411 metabolites (Philbin & Forister, *in prep*). Also, in the present study, we have not attempted to
412 separate constitutive and induced defenses (Jansen et al., 2009) as the plants in the common
413 garden were exposed to natural and continuous levels of herbivory. We also acknowledge that
414 feeding under laboratory conditions is of course not natural, although we found in a previous
415 study that genetic variants (in caterpillars) associated with success in laboratory feeding trials
416 were at least partially predictive of genetic variation associated with alfalfa use by *L. melissa* in
417 the wild (Chaturvedi et al., 2018). Thus it is clear that metabolomic data, such as those analyzed
418 here, have the potential to both open up new avenues of conceptual development in plant-insect
419 interactions and to link micro-evolutionary trajectories across hosts and herbivores.
420

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427

428 **CONFLICT OF INTEREST**

429 The authors declare no competing interests.
430

431 **AUTHOR CONTRIBUTIONS**

MLF: designed experiment, conducted analyses, wrote first draft. SY: conducted experiment and contributed to experimental design. CSP, CDD, BH: generated and interpreted phytochemistry and protein data. MLF, JGH, OS: developed and maintained common garden. JAF, ZHM, CCN, LAR: contributed to analyses and experimental design. CAB, JAF, ZG, CCN: contributed to experimental design. All authors: contributed to writing.

DATA AVAILABILITY STATEMENT

Data will be made available from the Dryad Digital Repository: *link to be determined*.

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574 **Figure legends** (also reproduced below individual figures)

575

576 **FIGURE 1** Variation among plants in caterpillar survival (a), development time (b) adult
577 weight (c), three individual compounds (d-e) and two external plant traits, specific leaf area (g)
578 and leaf toughness (h). The three example compounds shown here (out of the 163 assayed) were
579 among the top five most influential compounds for survival, development time and adult weight:
580 cpd. 9 is an alkaloid with a negative association with survival, cpd. 94 (a peptide) has a negative
581 association with development time, and cpd. 160 is a phospholipid with a negative association
582 with adult weight. Individual plants in all panels are organized from left to right by decreasing
583 caterpillar survival in the top panel (a). Standard errors are shown for panels b, c, g and h. The
584 units for d-e are compound relative abundance per dry weight of sample; the units for specific
585 leaf area are cm^2/mg, and grams/newton for leaf toughness.

586

587 **FIGURE 2** Illustration of correlational structure among compounds: each node in the network is
588 a compound, and compounds are linked by a line if they are correlated among individual plants
589 at 0.5 or above (links among compounds in modules 12-14 represent weaker correlations, greater
590 than 0.1; see main text for details). Nodes are clustered in space for ease of visualization, but
591 relative distances among nodes (and the relative lengths of lines) convey no additional
592 information. Two letter codes within nodes indicate compound classes, as explained in the
593 legend. Colors of nodes correspond to membership in modules as determined by hierarchical
594 cluster analysis; the color key to the 14 modules is shown in the lower left (also see Figure A4
595 where nodes are colored by compound class). Not shown are a small number of compounds with

596 weak connections to all other compounds, including two compounds that were not included in
597 any module (shown as module zero in Figure 3).
598
599 **FIGURE 3** Effects of individual compounds on survival, development time and adult weight, as
600 estimated by ridge regression (using binomial, Poisson and Gaussian models, respectively). The
601 strength of effect for each compound is indicated by the horizontal extent of each bar, and
602 compounds are grouped by modules (m1, m2, etc.); the order of compounds along the vertical
603 axis is arbitrary within modules and fixed across columns. Orange colors indicate negative
604 effects on survival, development and weight, while blue colors are positive effects (note that
605 negative effects for development time correspond to fewer days, or more rapid development).
606 The darker shades of orange and blue mark coefficients whose 95% confidence intervals did not
607 overlap zero in 1,000 bootstrap samples. Values for survival and development time have been
608 back-transformed from units on the log-odds and log scales to units of probability and days to
609 pupation, and are shown as changes from the mean or intercept values. For example, a negative
610 (orange) survival coefficient of 0.005 means a reduction of that amount from the average
611 probability of survival associated with variation in a particular compound. The fifteen
612 compounds with the largest coefficients (by absolute value) and bootstrap intervals not
613 overlapping zero are labelled by compound classes (see Figure 2 for abbreviations) in each
614 panel. Structural annotations are shown to the right for six compounds based on matches from
615 the METLIN metabolomics database, as follows by compound number: 154 (unidentified sterol);
616 9 (unidentified alkaloid); 60 (soyasaponin A3); 40 (unidentified saponin); 46 (medicagenic acid
617 3-O-triglucoside); 45 (medinoside E). Those same compounds are identified in parentheses in
618 the main panels next to the bars corresponding to their individual effects.

619

620 **FIGURE 4** Violin plots of compound-specific effects (coefficients from ridge regressions)
621 summarized by chemical classes. Sample sizes for each category are shown above the top panel
622 ("Other" includes 1 sugar, 2 pigments and 2 halogenated compounds). Categories are arranged
623 from left to right based on the gradient of median positive to negative effects on survival.
624 Coefficients for survival (a) and development time (b) have been back-transformed from the
625 units of log-odds and log to probability and days to pupation, respectively, and shown as
626 deviations from the mean or intercept value (as in Figure 3). Note that negative effects for
627 development time correspond to fewer days (more rapid development). Violin plots show
628 medians (black dots) and interquartile ranges (boxes); vertical lines are upper and lower fences
629 (the third quartile + 1.5 * the interquartile range, and the lower quartile - 1.5 * the interquartile
630 range, respectively) surrounded by kernel density envelopes.

631

TABLE 1 Results from Bayesian regressions of module eigenvectors and covariates predicting caterpillar survival, development time and adult weight (as binomial, Poisson, and Gaussian regressions, respectively, with corresponding units in log-odds, log number of days, and milligrams).

	Survival		Development time		Weight	
	coefficient (CI; prob.)		coefficient (CI, prob.)		coefficient (CI, prob.)	
m2	0.14	(-2.06, 0.48; 0.80)	-0.01	(-0.04, 0.02; 0.77)		
m3	-0.40	(-0.67, -0.14; >0.99)			-0.44	(-0.84, -0.03; 0.98)
m4					0.29	(-0.14, 0.70; 0.91)
m6			-0.01	(-0.03, 0.01; 0.80)		
m9	-0.30	(-0.63, 0.03; 0.96)	0.01	(-0.02, 0.04; 0.79)		
m10	0.35	(0.08, 0.62; >0.99)			0.40	(-0.01, 0.82; 0.98)
m11	0.36	(0.14, 0.58; >0.99)				
m13	-0.18	(-0.43, 0.06; 0.93)				
m14			0.01	(-0.02, 0.03; 0.71)		
SLA	-0.32	(-0.56, -0.08; >0.99)			-0.35	(-0.72, 0.012; 0.97)
Sex		/	0.06	(0.02, 0.10; >0.99)	1.12	(0.40, 1.84; >0.99)
mg		/	-0.03	(-0.05, -0.01; >0.99)		/
Days		/		/	-1.41	(-1.76, -1.05; >0.99)
Intercept	0.34	(0.14, 0.54; >0.99)	3.48	(3.45, 3.52; >0.99)	10.36	(9.81, 10.91; >0.99)
Validation	0.59		0.59		0.50	

Note: For each regression coefficient, numbers in parentheses are 95% credible intervals (CI, the first two numbers) and the probability that the coefficient has the estimated sign (e.g., 0.80 for the m2 survival coefficient of 0.14 indicates a 80% probability that the m2 module has a positive effect on survival). Note that negative coefficients for development time indicate faster caterpillar development (fewer days) associated with variation in a particular module. Modules (listed in the left column) are only shown if they were included in one of the three regressions following feature selection using lasso regression (see main text for additional details). Empty spaces in the Table appear if a particular module was selected through lasso regression for one or two analyses but not all three (m3, for example, was not selected by lasso regression for development time). Slash marks (/) indicate variables not considered for a particular analysis (e.g., sex, adult weight [mg] and development time [days] were not possible for the survival analysis because they are not observed on dead individuals). Values for "validation" shown in the last row are the correlation between observed and predicted values in cross-validation (Supporting Information Figure S4).

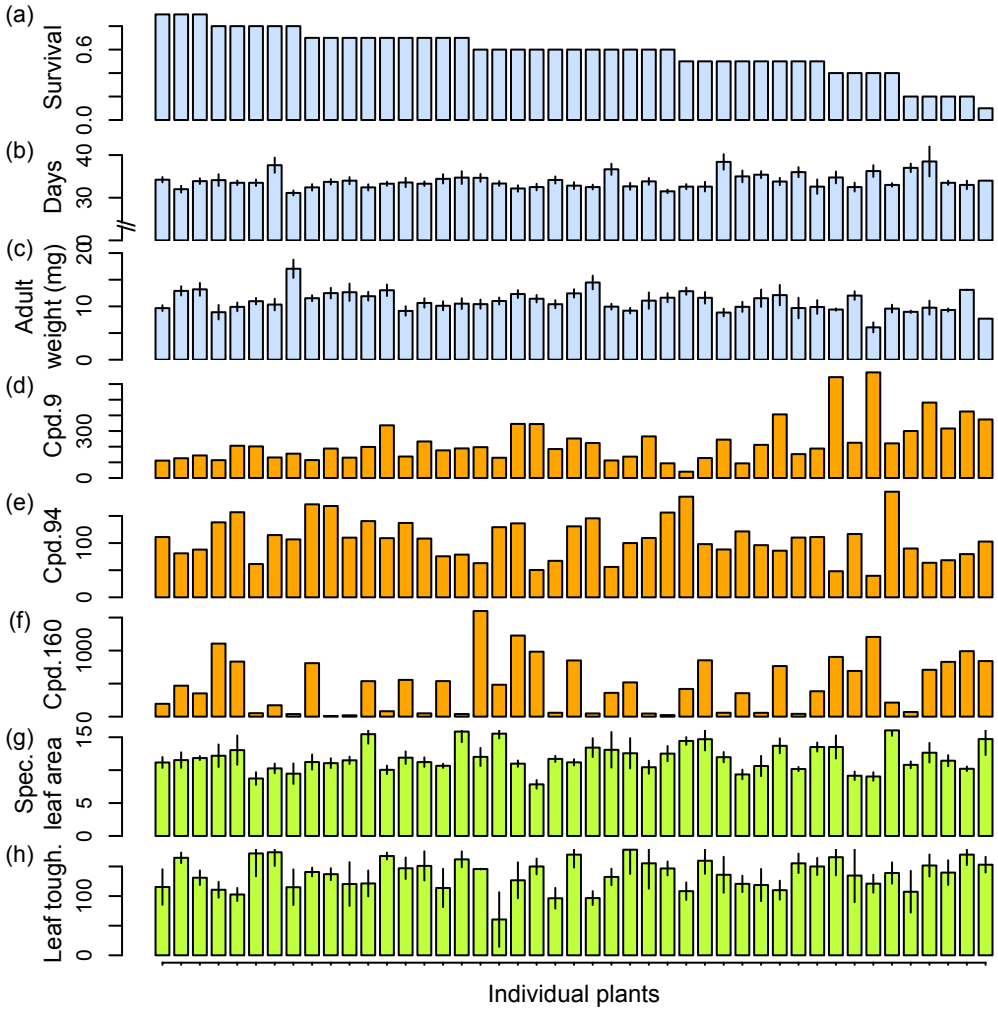
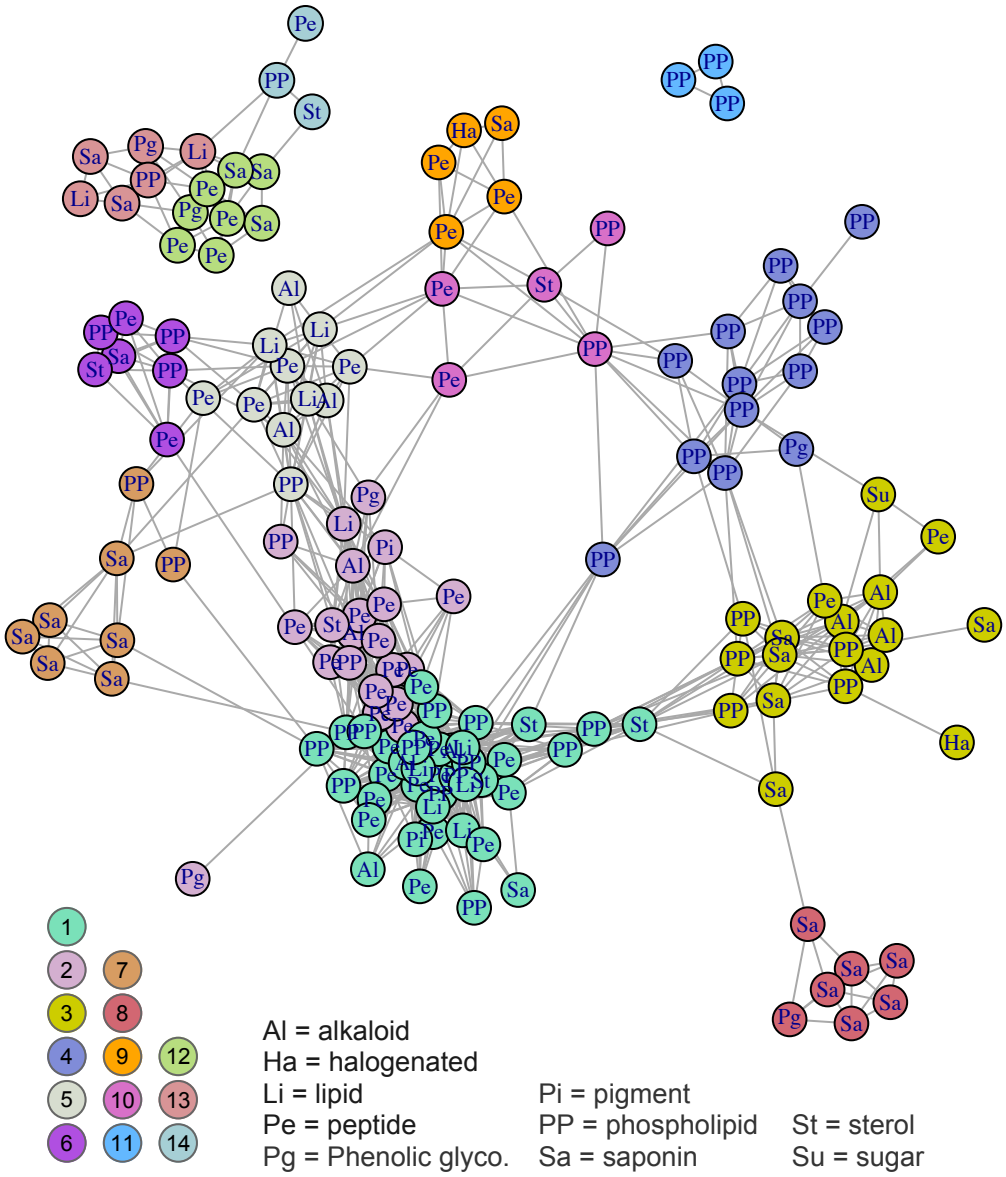


FIGURE 1 Variation among plants in caterpillar survival (a), development time (b) adult weight (c), three individual compounds (d-e) and two external plant traits, specific leaf area (g) and leaf toughness (h). The three example compounds shown here (out of the 163 assayed) were among the top five most influential compounds for survival, development time and adult weight: cpd. 9 is an alkaloid with a negative association with survival, cpd. 94 (a peptide) has a negative association with development time, and cpd. 160 is a phospholipid with a negative association with adult weight. Individual plants in all panels are organized from left to right by decreasing caterpillar survival in the top panel (a). Standard errors are shown for panels b, c, g and h. The units for d-e are compound relative abundance per dry weight of sample; the units for specific leaf area are cm^2/mg, and grams/newton for leaf toughness.



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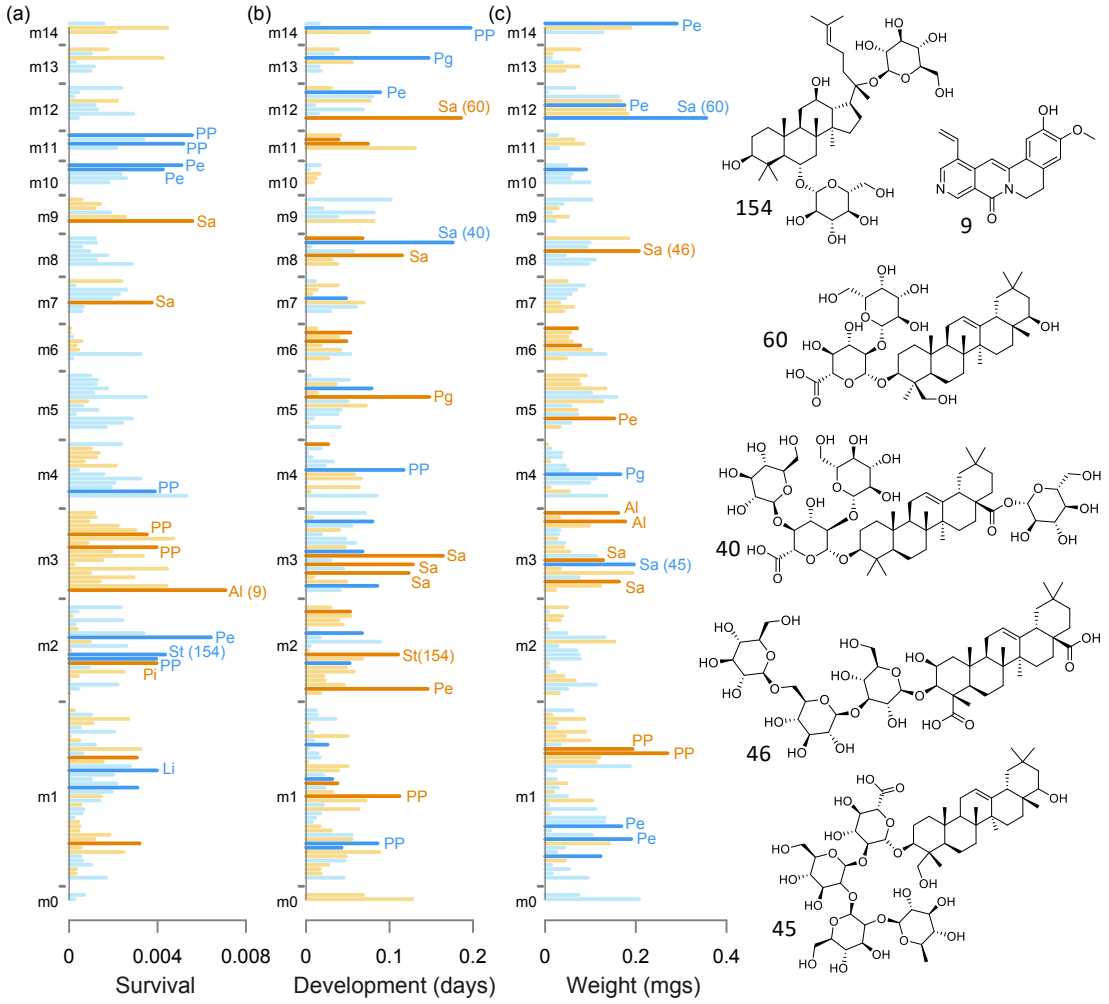

FIGURE 3 Effects of individual compounds on survival, development time and adult weight, as estimated by ridge regression (using binomial, Poisson and Gaussian models, respectively). The strength of effect for each compound is indicated by the horizontal extent of each bar, and compounds are grouped by modules (m1, m2, etc.); the order of compounds along the vertical axis is arbitrary within modules and fixed across columns. Orange colors indicate negative effects on survival, development and weight, while blue colors are positive effects (note that negative effects for development time correspond to fewer days, or more rapid development). The darker shades of orange and blue mark coefficients whose 95% confidence intervals did not overlap zero in 1,000 bootstrap samples. Values for survival and development time have been back-transformed from units on the log-odds and log scales to units of probability and days to pupation, and are shown as changes from the mean or intercept values. For example, a negative (orange) survival coefficient of 0.005 means a reduction of that amount from the average probability of survival associated with variation in a particular compound. The fifteen compounds with the largest coefficients (by absolute value) and bootstrap intervals not overlapping zero are labelled by compound classes (see Figure 2 for abbreviations) in each panel. Structural annotations are shown to the right for six compounds based on matches from the METLIN metabolomics database, as follows by compound number: 154 (unidentified sterol); 9 (unidentified alkaloid); 60 (soyasaponin A3); 40 (unidentified saponin); 46 (medicagenic acid 3-O-triglucoside); 45 (medinoside E). Those same compounds are identified in parentheses in the main panels next to the bars corresponding to their individual effects.

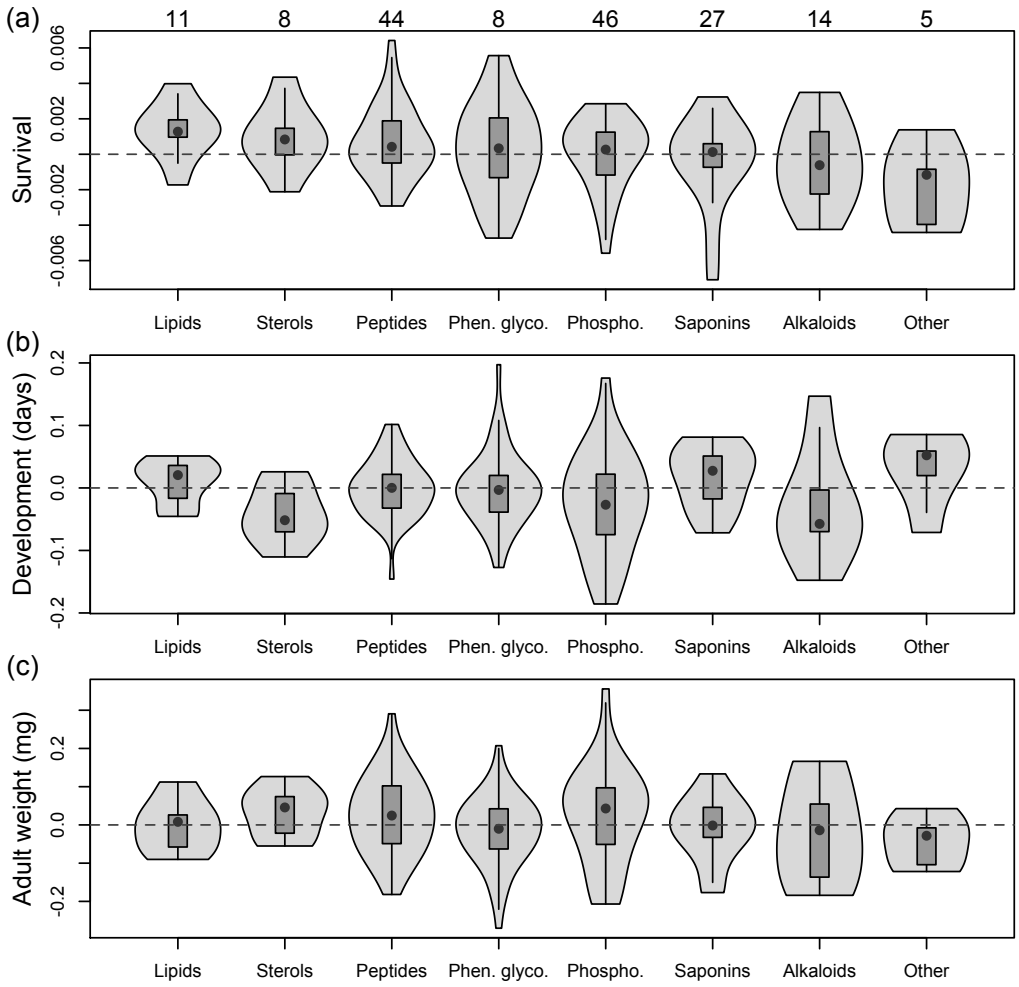

FIGURE 4 Violin plots of compound-specific effects (coefficients from ridge regressions) summarized by chemical classes. Sample sizes for each category are shown above the top panel ("Other" includes 1 sugar, 2 pigments and 2 halogenated compounds). Categories are arranged from left to right based on the gradient of median positive to negative effects on survival. Coefficients for survival (a) and development time (b) have been back-transformed from the units of log-odds and log to probability and days to pupation, respectively, and shown as deviations from the mean or intercept value (as in Figure 3). Note that negative effects for development time correspond to fewer days (more rapid development). Violin plots show medians (black dots) and interquartile ranges (boxes); vertical lines are upper and lower fences (the third quartile + 1.5 * the interquartile range, and the lower quartile - 1.5 * the interquartile range, respectively) surrounded by kernel density envelopes.

APPENDIX

Additional phytochemical methods 1: LC-TOF analysis of foliar plant tissue

Foliar tissue was dried *in vacuo* and individual leaves were selected haphazardly from individual plants and finely ground (TissueLyser II, Quiagen; Hilden, Germany). Approximately 10 mg of ground foliar tissue was weighed and extracted in 2.00 ml of 70% aqueous ethanol, and briefly vortexed before 15 minutes of sonication. This suspension was centrifuged (500 rpm) for 10 min, then 1 ml aliquots of the supernatant were filtered through a 96-well filter (AcroPrep, 1 mL, 1 μm glass fiber) into glass vial inserts and capped with a silicone mat before analysis. Chromatography was performed on an Agilent 1200 analytical HPLC equipped with a binary pump, autosampler, column compartment and diode array UV detector, coupled to an Agilent 6230 Time-of-Flight mass spectrometer via an electrospray ionization source (ESI-TOF; gas temperature: 325 °C, flow: 10 L/m; nebulizer pressure: 35 psig; VCap: 3500 V; fragmentor: 165 V; skimmer: 65 V; octopole: 750 V). Extracts (1.00 μL) were co-injected with 0.50 μL of digitoxin internal standard (0.200 mM, Sigma-Aldrich) and eluted at 0.500 mL/min through a Kinetex EVO C18 column (Phenomenex, 2.1 x 100 mm, 2.6 μ, 100 Å) at 40 °C. The linear binary gradient was comprised of buffers A (water containing 0.1 % formic acid) and B (acetonitrile containing 0.1 % formic acid) changing over 30 minutes accordingly: 0-1 min 5% B, ramp to 50% B at 4 min, ramp to 100% B at 21 min, 21-25 min 100% B ramping to 1.00 mL/min, before re-equilibrating the column from 25-30 min at 5% B, 0.5 mL/min.

Individual compounds were quantified relative to the digitoxin internal standard using Agilent MassHunter Quantitative Analysis. Digitoxin is a commercially available cardenolide which has previously been used as an internal standard in saponin analysis (Balsevich, Bishop, & Deibert, 2009). Its structural similarity to, and lack of coelution with, saponins and its absence in *Medicago* extracts make it an ideal internal standard for quantitation of saponins, the focal phytochemical class in this study. While digitoxin allows for the quantitation of saponins as "digitoxin equivalents", this does not extend to other phytochemical classes due to differences in ionization efficiency inherent in structural differences for other classes. In these cases, the digitoxin internal standard still serves to partially correct for between run variation in instrument response. We do not make quantitative assertions between phytochemical classes, only assertions based on their within-class variation for this reason.

Putative phenolics (200-400 ppm) and saponins (400-650 ppm) were annotated using the relative mass defect (RMD) characteristic of each phytochemical (see next appendix section). Compounds with RMD greater than 650 were presumed to be lipids or sterols. These assignments were revised by identifying presumed peptides based on even *m/z* features. Mass spectra of presumed phenolics, saponins and lipids were cross-referenced against the METLIN database (Smith et al., 2005) to further categorize annotations into phospholipids, vitamins (vitamin D derivatives), carotenoids, sterols, amino acids, alkaloids and sugars. One compound displayed an isotope distribution characteristic of a chlorinated structure and was designated as being halogenated. Due to the lack of structural information in ESI-TOF mass spectra, annotation beyond this classification was not possible.

Additional phytochemical methods 2: relative mass defect (RMD)

Relative mass defect is a recently-developed method for inferring structural information from high resolution mass spectrometry data (Ekanayaka et al., 2015) which we have used to aid in the classification of metabolites in *M. sativa* and to a lesser extent propose putative structures. Here we describe the theoretical background for the calculation and use of relative mass defect. Mass defect is the deviation of atomic mass (see definitions below) from its mass number (e.g. hydrogen: $A_H = 1$, $m_H = 1.00784$ Da, $d_H = 0.00784$). Relative mass defect of an atom (RMD_a) in ppm is calculated as:

$$RMD_a = \frac{m_a - (A_a)}{m_a} * 10^6$$

Where m_a is the atomic mass and A_a is the mass number of that atom. Although H has a positive mass defect ($RMD_H = 7740$ ppm), mass loss due to the strong nuclear force (Einstein, 1905) leads to increasingly negative mass defect as A increases. Atoms commonly found in natural products ($RMD_N = 221$, $RMD_C = 0$, $RMD_O = -319$, $RMD_P = -846$, $RMD_S = -873$ ppm) have a relative mass defect which is a full order of magnitude lower than that of hydrogen. As a result, the relative mass defect (RMD_M) of a molecule estimates the number of hydrogen atoms relative to other atoms in a natural product: a high RMD molecule has a higher %H than a low RMD molecule. When chemical formula is known, computing the theoretical RMD_M of a molecule M is facile:

$$RMD_M = \sum_{a=1}^{N} n_a RMD_a * 10^6$$

Where n_a is the count of the ath element in the set of elements composing a natural product, and N is the number of elements in that natural product. As a chemical formula this would appear in the form $C_{nC}H_{nH}N_{nN}O_{nO}P_{nP}S_{nS}$. When the chemical formula of a natural product is unknown, the RMD of a molecule M can be calculated from HRMS data:

$$RMD_M = \frac{m/z_M - m/z_{Mt}}{m/z_M} * 10^6$$

Where m/z_M and m/z_{Mt} are the mass to charge ratio and nominal mass-to-charge ratio of molecular ion M, respectively.

The RMD of a molecular ion then serves as an experimental estimate of degree of unsaturation; or more generally, how many H are present per unit of molecular mass. This metric can be very useful for discriminating natural products which may have the same nominal mass but differ in exact mass, obtained via HRMS data which is accurate at a sub-ppm level. In Figure A1 (left), three structures are listed which have the same nominal mass (270 Da) but have increasing RMD as the contribution of H to the exact mass increases. Pinostrobin, the most unsaturated and oxidized molecule has the lowest %H and lowest RMD (330 ppm) and as the %H increases to estrone and then methyl palmitate, the RMD of each molecule increases (600 ppm and 948 ppm respectively). This approach is useful when trying to discriminate flavonoid glycosides, such as the apigenin glycoside (Figure A8, right, RMD = 255 ppm) from saponins such as medicagenic acid glycoside (Figure A8, right, RMD = 494) in *M. sativa* extracts. However, other phytochemicals in these extracts may also have similar RMD, and this metric should not be solely relied upon for annotation. Daughter ions may also differ in RMD from their parent ions due to fragmentation or loss of H to form cationic species. Other information, such as relative retention time, molecular mass and odd molecular masses indicating nitrogenous compounds can also inform classification and annotation.

Definitions:

Atomic mass (*m*): exact mass of an atom measured in daltons (Da).

Mass number (*A*): the total number of nucleons (protons + neutrons) in an atom.

Mass to charge ratio (*m/z*): ion mass divided by charge. The measured unit of mass in a mass spectrometer. When $z = 1$, m/z equals ion mass.

Nominal mass to charge raio (m/z_n): mass to charge ratio truncated to zero decimals (or floor function). For example, the nominal mass of $m/z = 270.2559$ is $m/z_n = 270$.

High Resolution Mass Spectrometry (HRMS): Mass spectrometric techniques which yield masses accurate to four decimal places which allows for prediction of putative chemical formulae.

Isobaric: molecules having the same mass

Molecular ion: Ionic species representing an intact molecule

Parent ion: Molecular ion which becomes fragmented into daughter ions

Daughter ions: Resultant ions from the fragmentation of parent ions

TABLE A1 Details on individual compounds (numbered along the left column), including module numbers (see Figure 1), chemical class assignments, ridge regression beta coefficients (for caterpillar survival, development time and adult weight as in Figure 3), and mass spectra results: *m/z*, retention time (RT ave.) and relative mass defect (RMD). Compounds are organized by module number followed by compound number (an arbitrary designation).

Cpd.	Module	Class	Surv.	Dev.	Weight	*m/z*	RT ave.	RMD
65	m. 0	Phospholipid (PP)	0.00026	-0.1273	0.2072	299.2719	6.451733	909
180	m. 0	Sterol (St)	0.00067	-0.0684	0.0740	485.3591	27.309711	740
23	m. 1	Peptide (Pe)	0.00065	-0.0629	0.1109	492.2455	4.985533	499
30	m. 1	Alkaloid (Al)	0.00059	0.0172	-0.0105	366.1097	5.301200	300
35	m. 1	Saponin (Sa)	0.00020	0.0112	0.1319	459.2233	5.533244	486
37	m. 1	Peptide (Pe)	-0.00041	0.0073	0.1304	1564.7065	5.623800	452
50	m. 1	Peptide (Pe)	-0.00048	-0.0165	0.1683	1238.5847	5.966289	472
53	m. 1	Peptide (Pe)	-0.00042	-0.0301	0.0121	1106.5421	6.010844	490
57	m. 1	Peptide (Pe)	-0.00183	0.0547	0.1034	256.2642	6.141222	1031
58	m. 1	Peptide (Pe)	-0.00114	-0.0546	0.1899	1192.5417	6.211267	454
63	m. 1	Phospholipid (PP)	-0.00320	0.0857	-0.1410	518.3253	6.374000	628
64	m. 1	Peptide (Pe)	-0.00053	0.0427	0.0425	562.322	6.399089	573
67	m. 1	Phospholipid (PP)	-0.00246	-0.0880	0.0248	284.2959	6.617089	1041
70	m. 1	Peptide (Pe)	0.00052	-0.0476	0.1226	1168.6682	7.022178	572
78	m. 1	Peptide (Pe)	0.00060	0.0464	-0.0439	694.402	7.380578	579
84	m. 1	Phospholipid (PP)	0.00100	-0.0265	0.0137	293.2112	8.701933	720
89	m. 1	Phospholipid (PP)	-0.00032	-0.0165	0.0532	546.3438	10.332689	629
93	m. 1	Alkaloid (Al)	-0.00029	-0.0176	0.0149	358.2468	10.897978	689
95	m. 1	Lipid (Li)	0.00168	0.0453	0.0941	425.2903	11.479511	683
111	m. 1	Alkaloid (Al)	-0.00022	0.0119	0.0602	592.5035	15.707178	850
113	m. 1	Sterol (St)	0.00100	0.0130	-0.0143	661.3032	15.991800	458
128	m. 1	Peptide (Pe)	-0.00269	0.0354	-0.0869	584.4235	17.597156	725
129	m. 1	Peptide (Pe)	-0.00106	-0.0033	-0.0254	567.4209	17.818844	742
136	m. 1	Peptide (Pe)	0.00051	0.0017	0.0215	932.6342	18.698356	680
139	m. 1	Phospholipid (PP)	0.00205	0.0077	-0.0888	792.5648	18.853422	713
146	m. 1	Sterol (St)	-0.00004	-0.0499	-0.0437	451.3596	20.131378	797
149	m. 1	Phospholipid (PP)	-0.00047	0.0087	-0.0978	784.5599	20.769978	714
157	m. 1	Sterol (St)	0.00117	0.0258	0.0326	429.3741	21.812222	871
160	m. 1	Phospholipid (PP)	-0.00322	0.0023	-0.1929	747.6095	21.920444	815
161	m. 1	Phospholipid (PP)	0.00061	0.0140	-0.2701	552.4296	22.245667	778
162	m. 1	Phospholipid (PP)	-0.00307	0.0165	-0.1205	907.5367	22.382756	591
163	m. 1	Peptide (Pe)	-0.00153	-0.0016	-0.1119	886.5581	22.381711	630
164	m. 1	Peptide (Pe)	0.00277	-0.0499	0.1871	830.676	22.432867	814
165	m. 1	Lipid (Li)	0.00398	-0.0385	0.0233	537.5365	22.754333	998
167	m. 1	Phospholipid (PP)	0.00199	0.0207	0.0001	708.5134	23.041267	725
169	m. 1	Lipid (Li)	0.00100	0.0316	0.0242	565.5681	23.807289	1004
170	m. 1	Phospholipid (PP)	0.00214	-0.0376	-0.0473	885.5552	24.037489	627
171	m. 1	Lipid (Li)	0.00310	0.0220	0.0281	536.4387	24.087178	818
172	m. 1	Phospholipid (PP)	0.00194	-0.0314	-0.0176	869.559	24.216778	643
173	m. 1	Phospholipid (PP)	-0.00145	-0.1120	0.0485	787.427	24.262511	542
175	m. 1	Pigment (Pi)	0.00138	-0.0712	-0.1041	871.5766	24.897822	662
177	m. 1	Lipid (Li)	-0.00052	0.0206	0.0080	208.1347	27.036422	647
18	m. 2	Phen. glyco. (Pg)	-0.00246	-0.0574	0.0201	323.0738	4.163533	228
33	m. 2	Phen. glyco. (Pg)	-0.00040	-0.0210	-0.0409	493.1329	5.430044	505
69	m. 2	Peptide (Pe)	0.00001	-0.0226	-0.0657	1018.537	6.846578	527
92	m. 2	Lipid (Li)	0.00220	-0.0455	0.1122	375.2549	10.640356	679
94	m. 2	Peptide (Pe)	0.00041	-0.1458	0.0496	618.4291	11.238667	694
99	m. 2	Alkaloid (Al)	-0.00001	-0.0174	-0.0306	618.4291	12.470689	694
100	m. 2	Peptide (Pe)	0.00235	-0.0290	-0.0485	600.4193	13.376289	698
101	m. 2	Alkaloid (Al)	0.00040	-0.0528	0.0074	618.4291	13.049600	694
103	m. 2	Peptide (Pe)	-0.00012	-0.0534	-0.0377	603.4311	13.358222	714

104	m. 2	Peptide (Pe)	0.00242	-0.0386	-0.0329	600.4193	13.376289	698
109	m. 2	Peptide (Pe)	0.00025	-0.0441	0.0031	600.4193	15.398533	698
112	m. 2	Peptide (Pe)	-0.00036	0.0008	-0.0056	600.4193	15.860356	698
114	m. 2	Peptide (Pe)	0.00337	0.0671	0.0466	584.4235	16.288622	725
122	m. 2	Peptide (Pe)	0.00642	0.0166	0.1315	584.4235	16.758622	725
133	m. 2	Phospholipid (PP)	-0.00094	0.0888	-0.1533	716.5243	18.284733	732
134	m. 2	Peptide (Pe)	0.00260	0.0043	0.0275	568.4294	18.417311	755
135	m. 2	Peptide (Pe)	-0.00007	-0.0027	0.0696	937.5896	18.675222	629
154	m. 2	Sterol (St)	0.00435	-0.1104	0.0743	693.425	21.485978	613
155	m. 2	Phospholipid (PP)	0.00395	-0.0670	0.0774	635.3816	21.488511	601
166	m. 2	Pigment (Pi)	-0.00396	0.0522	-0.0076	871.5766	22.912311	662
168	m. 2	Peptide (Pe)	0.00091	-0.0479	0.0062	1057.7337	23.685733	694
2	m. 3	Halogen (Ha)	-0.00085	0.0590	0.0426	199.0946	0.546622	475
5	m. 3	Alkaloid (Al)	-0.00443	0.0446	-0.0332	217.0692	0.628311	485
6	m. 3	Peptide (Pe)	-0.00292	-0.0089	0.0744	309.1795	0.638667	581
7	m. 3	Sugar (Su)	-0.00441	0.0855	-0.1219	365.1066	0.653400	292
9	m. 3	Alkaloid (Al)	-0.00708	0.0404	-0.0229	321.1156	0.696467	360
12	m. 3	Alkaloid (Al)	-0.00114	0.0704	-0.1614	186.0765	0.831267	411
13	m. 3	Alkaloid (Al)	-0.00088	0.0796	-0.1770	140.071	0.843044	1008
14	m. 3	Peptide (Pe)	-0.00221	0.0548	-0.0971	225.0855	1.036511	380
34	m. 3	Phospholipid (PP)	-0.00396	-0.0466	-0.0396	547.3472	5.507867	634
38	m. 3	Saponin (Sa)	-0.00193	0.0677	-0.0541	633.348	5.635956	549
41	m. 3	Saponin (Sa)	-0.00335	-0.1640	0.1125	471.3488	5.634111	740
42	m. 3	Saponin (Sa)	-0.00151	0.0292	-0.1282	631.3333	5.725444	816
45	m. 3	Saponin (Sa)	-0.00020	-0.1281	0.1960	1105.5828	5.781400	527
55	m. 3	Saponin (Sa)	-0.00096	-0.1231	-0.1934	617.3527	6.043333	678
62	m. 3	Saponin (Sa)	-0.00139	-0.0482	-0.1629	825.4299	6.314022	521
120	m. 3	Phospholipid (PP)	-0.00122	-0.0073	-0.0314	732.5644	16.529933	770
141	m. 3	Phospholipid (PP)	-0.00302	-0.0398	0.0321	963.6051	18.994089	628
143	m. 3	Phospholipid (PP)	-0.00352	0.0180	0.0291	951.6048	19.629644	636
174	m. 3	Phospholipid (PP)	-0.00473	0.0474	-0.0259	805.6846	23.370044	759
27	m. 4	Phen. glyco. (Pg)	0.00157	-0.0575	0.1663	441.2984	5.217978	676
80	m. 4	Phospholipid (PP)	0.00323	-0.0657	0.1113	518.3253	7.756911	628
83	m. 4	Phospholipid (PP)	0.00205	0.0001	0.0979	520.3405	8.404400	654
85	m. 4	Phospholipid (PP)	0.00189	-0.0632	-0.0102	454.2938	8.763000	647
86	m. 4	Phospholipid (PP)	0.00389	-0.0043	-0.0528	496.3412	8.889378	687
91	m. 4	Phospholipid (PP)	0.00532	0.0847	0.1361	524.3727	10.499178	711
140	m. 4	Phospholipid (PP)	0.00237	-0.0266	-0.0049	797.5204	18.879244	653
142	m. 4	Phospholipid (PP)	-0.00099	0.0177	0.0110	714.5088	19.293533	711
144	m. 4	Phospholipid (PP)	-0.00135	0.0014	0.0366	756.5562	19.707467	735
148	m. 4	Phospholipid (PP)	-0.00123	0.0070	0.0349	716.525	20.684533	732
151	m. 4	Phospholipid (PP)	-0.00066	0.0324	-0.0100	801.5511	20.976333	688
153	m. 4	Phospholipid (PP)	-0.00212	0.0224	0.0440	758.5721	21.488156	754
159	m. 4	Phospholipid (PP)	0.00041	0.1169	0.0507	803.5978	22.069778	744
4	m. 5	Alkaloid (Al)	0.00060	-0.0718	0.0563	104.1077	0.595533	1035
8	m. 5	Alkaloid (Al)	0.00026	0.0377	0.0717	144.1024	0.663689	711
10	m. 5	Lipid (Li)	0.00096	0.0048	-0.0902	301.2133	0.700111	708
11	m. 5	Alkaloid (Al)	0.00122	-0.0359	-0.0768	158.1181	0.705733	747
15	m. 5	Peptide (Pe)	0.00110	-0.0136	0.1019	188.0714	2.198778	380
22	m. 5	Phen. glyco. (Pg)	0.00349	-0.1477	0.1581	623.1272	5.055600	506
32	m. 5	Phen. glyco. (Pg)	-0.00083	0.0498	-0.1267	507.1149	5.435600	638
54	m. 5	Peptide (Pe)	0.00129	0.0416	-0.0698	351.2153	6.034822	613
87	m. 5	Peptide (Pe)	0.00284	0.0082	-0.1526	1455.421	9.179689	57
96	m. 5	Phospholipid (PP)	0.00240	-0.0021	0.0564	377.2674	11.743289	691
97	m. 5	Lipid (Li)	0.00167	0.0404	-0.0329	279.2328	11.961267	834
102	m. 5	Lipid (Li)	0.00128	0.0511	-0.0726	281.2485	13.161200	884
124	m. 5	Peptide (Pe)	0.00173	0.0786	-0.1331	1099.6413	16.987733	583
49	m. 6	Alkaloid (Al)	0.00323	0.0532	0.1335	445.369	5.779267	829
98	m. 6	Saponin (Sa)	0.00015	-0.0269	-0.0464	607.2933	12.283867	483
107	m. 6	Phospholipid (PP)	-0.00005	-0.0122	-0.0705	593.2777	14.143022	468
108	m. 6	Sterol (St)	-0.00004	-0.0533	-0.0551	621.3084	14.628844	496
110	m. 6	Peptide (Pe)	0.00014	-0.0388	-0.0504	635.3816	15.485111	601

121	m. 6	Phospholipid (PP)	-0.00057	-0.0485	-0.0593	607.2933	16.585889	483
126	m. 6	Phospholipid (PP)	-0.00031	-0.0179	-0.0777	621.3084	17.422600	496
132	m. 6	Peptide (Pe)	-0.00042	-0.0410	-0.1018	635.3816	18.238778	601
66	m. 7	Saponin (Sa)	-0.00238	0.0110	-0.0479	1087.5707	6.218867	525
68	m. 7	Saponin (Sa)	0.00025	-0.0381	0.0862	943.529	6.721333	561
74	m. 7	Saponin (Sa)	0.00259	-0.0129	0.0691	585.4277	7.205911	731
75	m. 7	Saponin (Sa)	0.00227	-0.0067	0.0576	907.5367	7.203356	591
76	m. 7	Saponin (Sa)	0.00190	0.0480	0.0452	1069.5625	7.218822	526
79	m. 7	Saponin (Sa)	-0.00375	-0.0688	-0.0314	923.5027	7.572822	544
81	m. 7	Phospholipid (PP)	0.00063	0.0602	-0.0630	277.2167	8.279133	782
82	m. 7	Phospholipid (PP)	0.00058	0.0288	-0.0403	317.209	8.321022	659
31	m. 8	Phen. glyco. (Pg)	0.00117	-0.0678	-0.1842	684.2517	5.347200	368
40	m. 8	Saponin (Sa)	0.00123	0.1758	0.0983	1119.5627	5.806133	503
43	m. 8	Saponin (Sa)	0.00055	0.0050	0.0909	973.504	5.631911	518
46	m. 8	Saponin (Sa)	0.00093	0.0569	-0.2069	989.4993	5.818644	505
47	m. 8	Saponin (Sa)	0.00173	-0.1150	0.0430	843.4409	5.873844	523
52	m. 8	Saponin (Sa)	0.00121	-0.0305	0.1098	973.504	6.013467	518
56	m. 8	Saponin (Sa)	0.00285	-0.0381	0.0958	827.4455	6.058733	538
16	m. 9	Peptide (Pe)	-0.00141	-0.0005	0.0386	141.0881	3.049089	624
19	m. 9	Alkaloid (Al)	0.00186	0.0813	0.0131	422.1973	4.658444	467
72	m. 9	Peptide (Pe)	-0.00253	0.0377	-0.0505	1170.1459	7.037956	125
73	m. 9	Saponin (Sa)	-0.00558	-0.0807	0.0211	402.1992	7.065644	495
125	m. 9	Peptide (Pe)	-0.00057	0.1016	0.1027	584.4235	16.991733	725
181	m. 9	Halogen (Ha)	-0.00116	0.0197	-0.0283	127.1233	27.473089	970
117	m. 10	Peptide (Pe)	0.00509	0.0172	0.0481	595.2837	16.515511	477
118	m. 10	Peptide (Pe)	0.00425	0.0036	0.0906	613.4849	16.515178	790
137	m. 10	Sterol (St)	0.00234	-0.0163	0.0588	335.2595	18.765067	774
138	m. 10	Phospholipid (PP)	0.00259	-0.0120	0.0542	613.4849	18.837933	790
150	m. 10	Phospholipid (PP)	0.00178	-0.0083	0.0982	775.535	20.840600	690
39	m. 11	Saponin (Sa)	0.00214	-0.1306	0.0287	527.2482	5.672422	471
176	m. 11	Phospholipid (PP)	0.00557	-0.0410	0.0270	861.4798	26.956867	557
178	m. 11	Phospholipid (PP)	0.00338	-0.0390	-0.0627	835.5451	27.192933	652
179	m. 11	Phospholipid (PP)	0.00517	-0.0743	-0.0850	834.5424	27.162333	650
20	m. 12	Saponin (Sa)	0.00021	0.0792	0.1608	409.1823	4.755400	313
21	m. 12	Phen. glyco. (Pg)	-0.00217	-0.0762	-0.1660	481.2056	4.897222	427
24	m. 12	Peptide (Pe)	0.00115	0.0097	0.1753	374.1461	5.014222	390
25	m. 12	Saponin (Sa)	0.00127	0.0686	-0.1764	475.2191	5.100889	461
48	m. 12	Peptide (Pe)	0.00290	0.0146	-0.1820	728.3151	5.887111	433
60	m. 12	Saponin (Sa)	0.00040	-0.1856	0.3557	959.5744	6.105156	599
116	m. 12	Peptide (Pe)	0.00237	-0.0299	0.0653	959.5744	16.460289	599
147	m. 12	Peptide (Pe)	0.00043	0.0889	0.0042	965.6208	20.478333	643
26	m. 13	Phen. glyco. (Pg)	-0.00424	0.1468	0.0130	683.1501	5.126178	215
51	m. 13	Saponin (Sa)	0.00026	-0.0548	0.0378	915.4514	5.947444	659
61	m. 13	Saponin (Sa)	0.00113	0.0149	-0.0740	351.2153	6.255356	613
88	m. 13	Lipid (Li)	0.00097	0.0176	-0.0429	383.2564	9.921756	669
105	m. 13	Lipid (Li)	-0.00173	-0.0380	-0.0770	613.4839	13.432956	790
158	m. 13	Phospholipid (PP)	0.00100	0.0318	-0.0148	816.6582	21.926822	806
145	m. 14	Peptide (Pe)	0.00156	0.0153	0.2906	939.5958	19.884289	634
152	m. 14	Phospholipid (PP)	-0.00445	0.1972	-0.1885	941.6201	21.136822	659
156	m. 14	Sterol (St)	-0.00212	-0.0755	0.1265	427.3947	21.699578	924

TABLE A2 Compounds and coefficients from lasso regression allowing for possible effects of all individual compounds (as in Figure 2) as well as all pairwise interactions among compounds. Listed here are interactions selected by lasso regression, using binomial and Gaussian regressions for survival and adult weight, respectively (with units on those scales, as in Table A1). Results for development time are not shown here as none of the potential interactions for development time were selected by lasso regression.

Survival regression		Weight regression			
Compounds	Coefficient	Compounds	Coefficient	Compounds	Coefficient
cpd.9	-0.080	cpd.25	-0.085	cpd.43 x cpd.47	-0.030
cpd.122	0.067	cpd.49	0.0081	cpd.49 x cpd.138	0.051
cpd.154	0.0078	cpd.5 x cpd.7	-0.10	cpd.55 x cpd.79	-0.025
cpd.5 x cpd.6	-0.042	cpd.5 x cpd.9	-0.059	cpd.60 x cpd.65	0.016
cpd.5 x cpd.7	-0.0095	cpd.8 x cpd.11	-0.018	cpd.60 x cpd.79	-0.048
cpd.6 x cpd.34	-0.024	cpd.12 x cpd.152	0.019	cpd.60 x cpd.132	-0.080
cpd.6 x cpd.79	-0.11	cpd.14 x cpd.91	0.11	cpd.65 x cpd.133	-0.22
cpd.7 x cpd.133	0.078	cpd.18 x cpd.73	-0.18	cpd.85 x cpd.161	0.16
cpd.19 x cpd.54	-0.0063	cpd.18 x cpd.79	0.0048	cpd.86 x cpd.141	0.052
cpd.21 x cpd.60	-0.038	cpd.19 x cpd.85	-0.024	cpd.88 x cpd.118	0.063
cpd.45 x cpd.54	0.00024	cpd.19 x cpd.124	0.013	cpd.91 x cpd.122	-0.062
cpd.48 x cpd.142	0.019	cpd.23 x cpd.24	-0.011	cpd.95 x cpd.175	-0.011
cpd.61 x cpd.82	-0.034	cpd.25 x cpd.60	0.086	cpd.105 x cpd.125	0.024
cpd.62 x cpd.79	-0.010	cpd.25 x cpd.117	-0.019	cpd.105 x cpd.152	-0.090
cpd.83 x cpd.164	-0.017	cpd.25 x cpd.145	-0.078	cpd.117 x cpd.149	-0.10
cpd.99 x cpd.109	-0.039	cpd.32 x cpd.66	-0.37	cpd.117 x cpd.156	0.047
cpd.152 x cpd.174	-0.18	cpd.34 x cpd.94	0.00025	cpd.121 x cpd.140	0.25
		cpd.35 x cpd.49	-0.062	cpd.125 x cpd.148	0.0035
		cpd.41 x cpd.179	0.144	cpd.124 x cpd.176	0.13

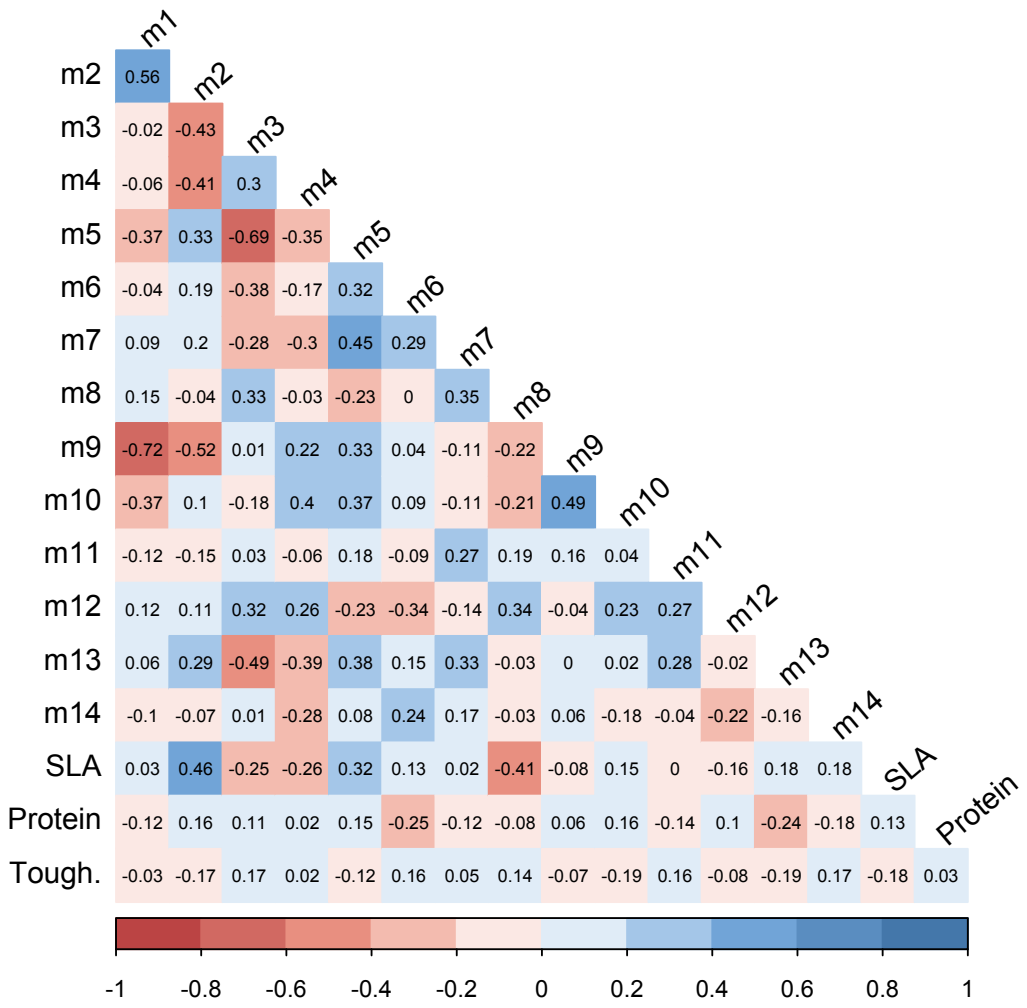

FIGURE A1 Pairwise correlations between phytochemical modules (represented by eigenvectors m1, m2, etc.) and plant traits specific leaf area (SLA), protein and leaf toughness. Values shown are Pearson product-moment correlation coefficients.

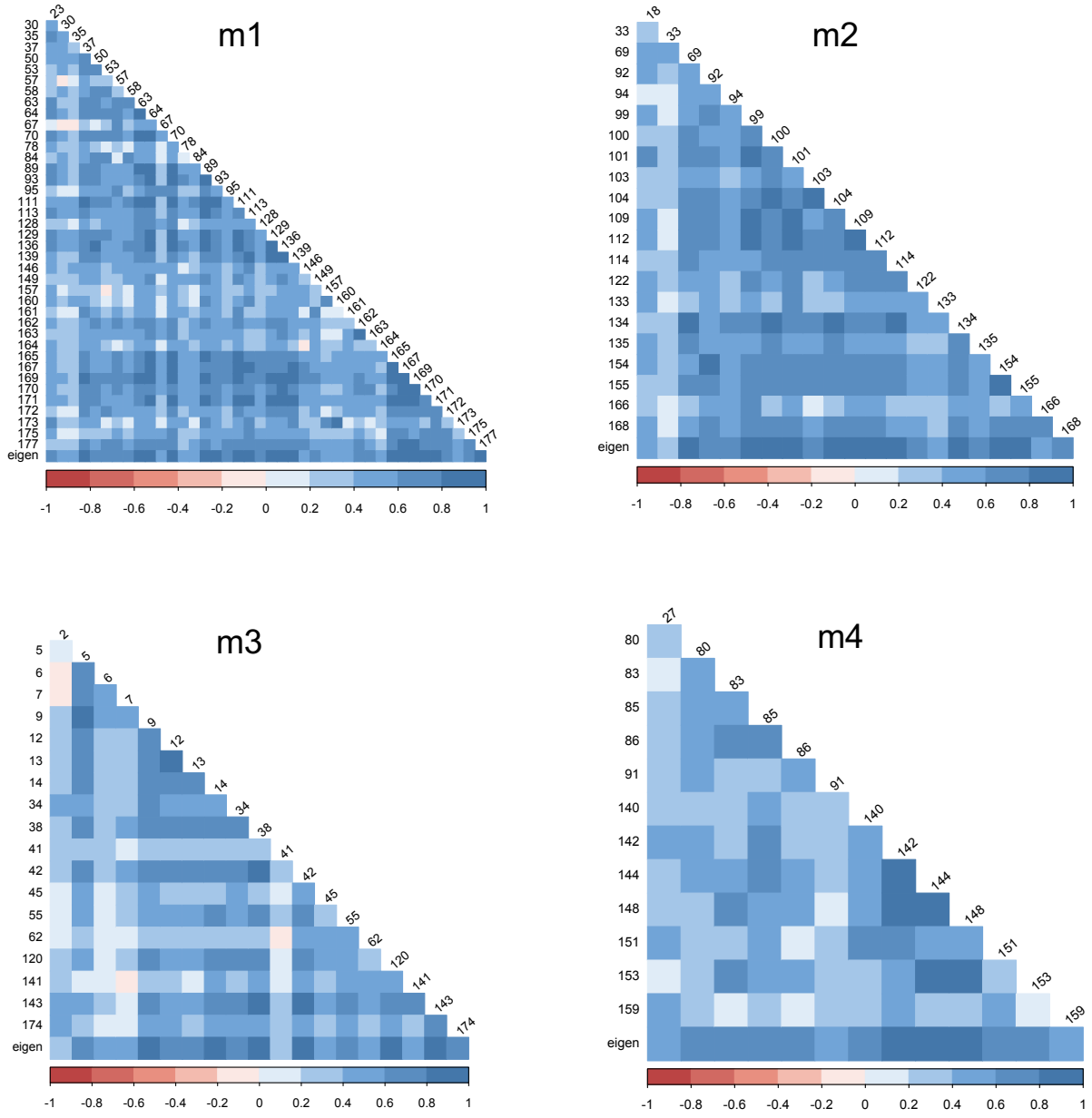

FIGURE A2a Pairwise correlations (as in Figure A1), but among compounds within modules m1 to m4. The bottom row in each graph shows correlations among individual compounds and the eigenvector used in other analyses for the given module.

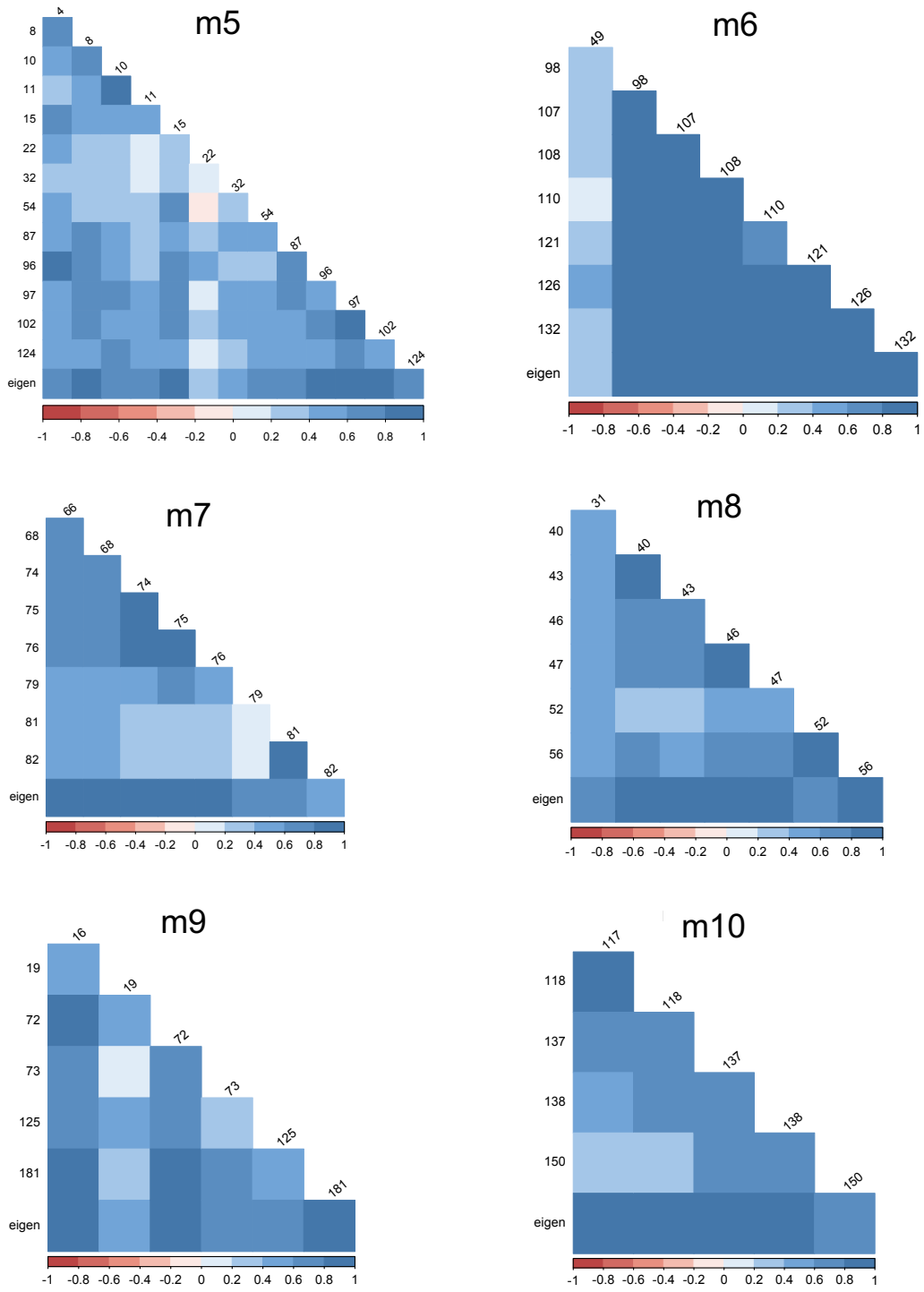

FIGURE A2b Pairwise correlations (as in Figure A1), but among compounds within modules m5 to m10. The bottom row in each graph shows correlations among individual compounds and the eigenvector used in other analyses for the given module.

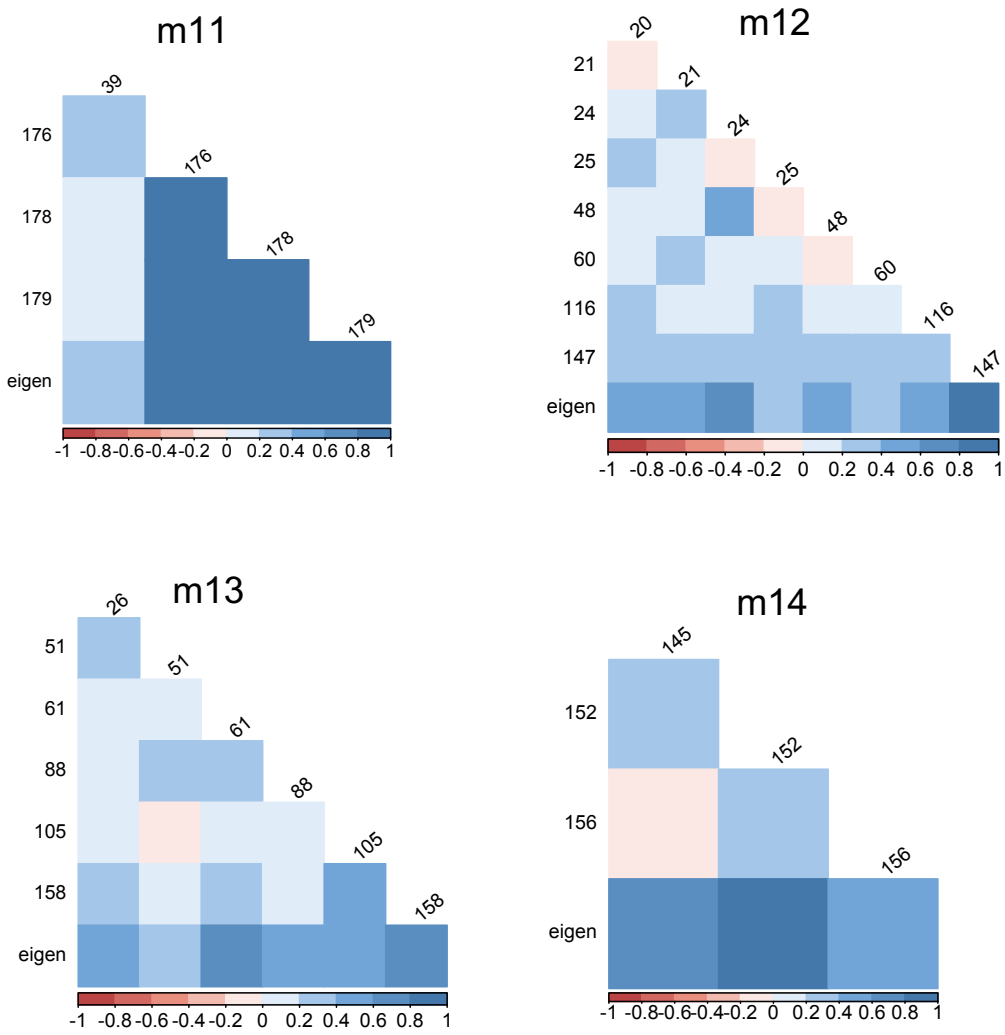

FIGURE A2c Pairwise correlations (as in Figure A1), but among compounds within modules m11 to m14. The bottom row in each graph shows correlations among individual compounds and the eigenvector used in other analyses for the given module.

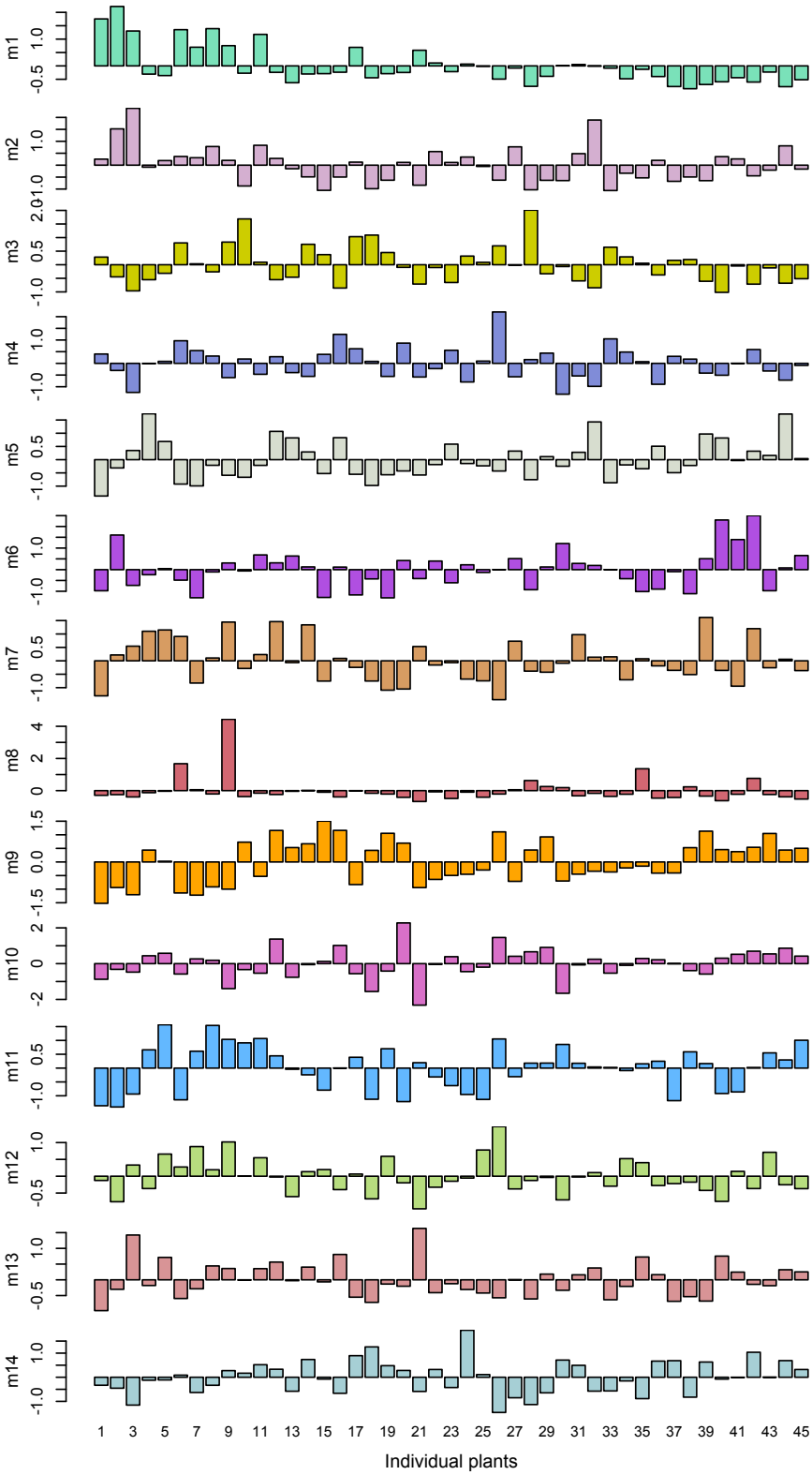

FIGURE A3 Visualization of variation among plants (bars) in phytochemical modules. Each bar is the average of z-scores for compounds comprising a given module. Colors correspond to modules as in Figure 1. The order of plants (along the x-axis) is arbitrary.

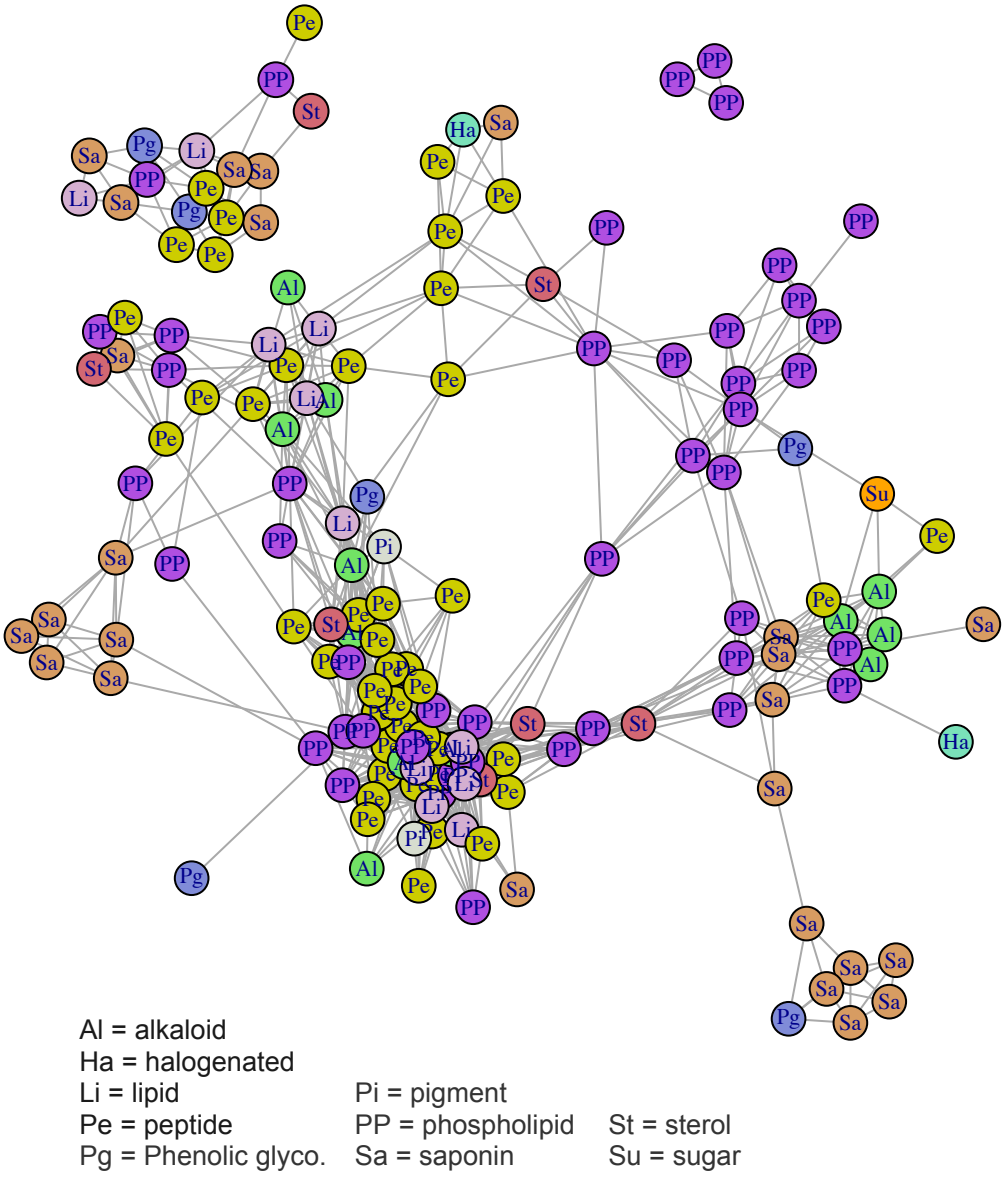

FIGURE A4 Illustration of correlational structure among compounds, as in Figure 2, but here color coded by compound class identities instead of by module assignment.

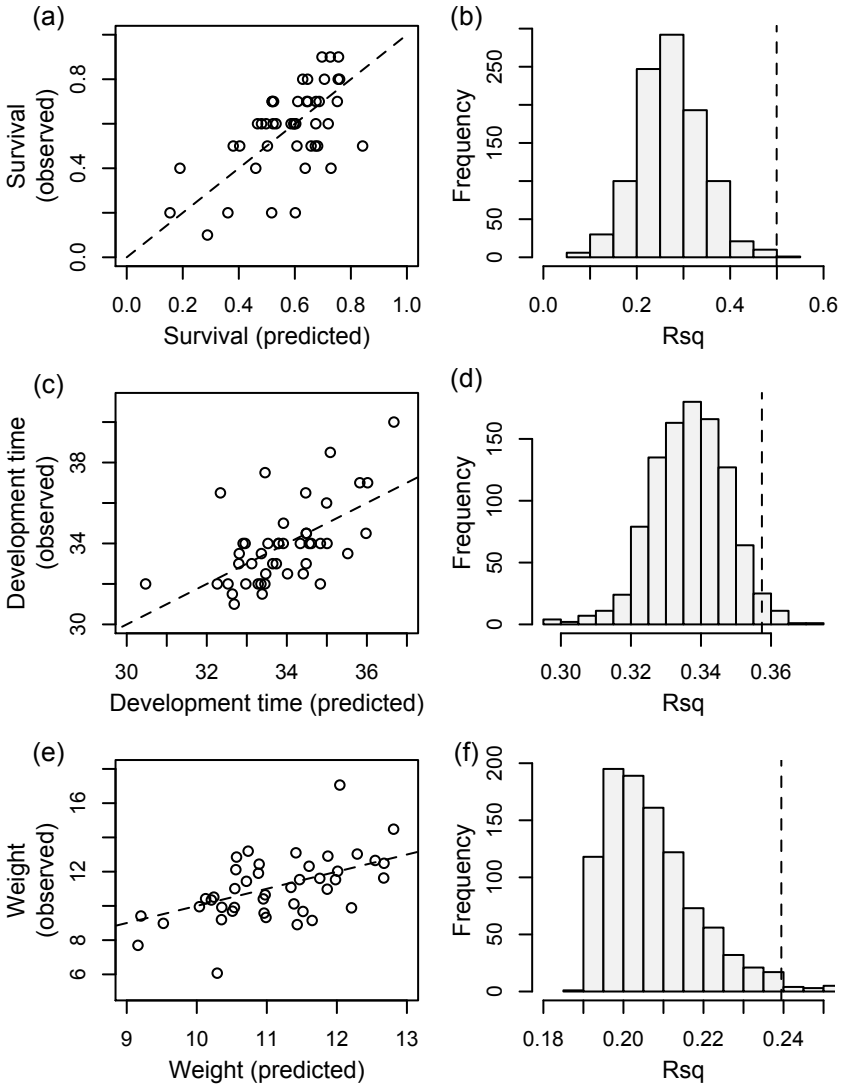

FIGURE A5 Results from cross-validation (a, c, e) and resampling analysis (b, d, f) of Bayesian regressions reported in Table 1. In the observed vs predicted plots (a, c, e), each dot represents the observed and predicted performance of caterpillars associated with individual plants, which was the level at which cross-validation was conducted (i.e., one plant was left out of each iteration). In the resampling analyses (b, d, f), "modules" were constructed based on randomly assembled collections of compounds selected to match the structure of the initial analyses. For example, the survival regressions included modules 2, 3, 9, 10, and 11 (Table 1). Because m2 includes 21 compounds, for one iteration of the resampling analysis 21 compounds were randomly selected, from which the first eigenvector was taken for use in the analysis (and same for the other modules). From each of 1000 randomly-created sets of modules, R^2 values were retained and summarized in plots (b, d, f), along with dashed lines representing the R^2 values of empirical collections of compounds and associated eigenvectors. In all cases, the empirical models outperformed all but a tiny fraction of simulated models. The fraction of the simulated models with R^2 values greater than the empirical models are as follows: 0.007 (b), 0.027 (d), and 0.023 (f).

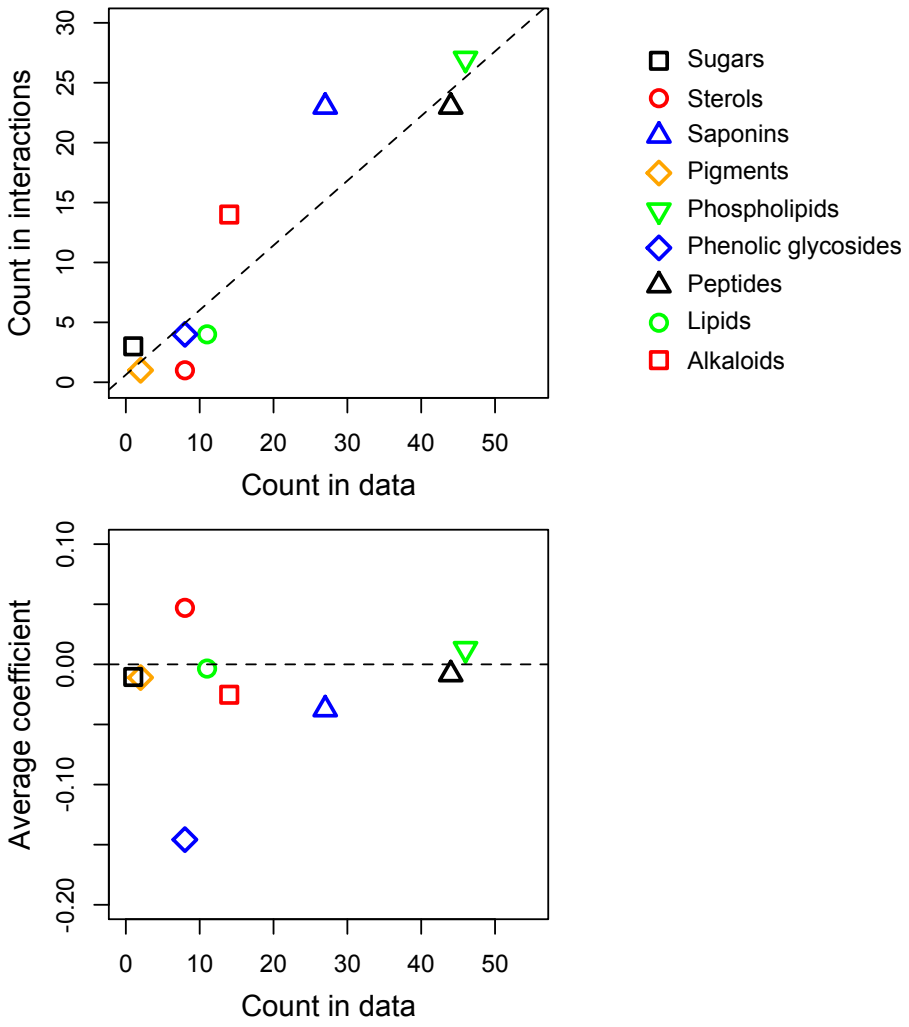

FIGURE A6 Additional details on interactions by compound class. Top panel shows the number of times that compounds of a certain class appear in pairwise interactions (as in appendix Table A2) as a function of the total numbers of those compounds in different classes ("Count in data"). Classes that are represented by a greater number of distinct compounds in the total dataset are, in general, more likely to be observed in pairwise interactions: the dotted line shows the expected frequency of observation given random sampling from the total pool of compounds. Saponins and alkaloids are overrepresented in interactions, appearing in 44 and 41% (respectively) more pairwise interactions than expected by chance. Bottom panel shows average interaction coefficients for all interactions involving compounds of particular classes: most pairwise interactions are relatively small in magnitude, with the exception of interactions involving phenolic glycosides, that tend to be strongly negative. For both panels, interactions are considered from analyses of caterpillar survival and weight; no interactions were detected for development time (Supporting Information Table A2).

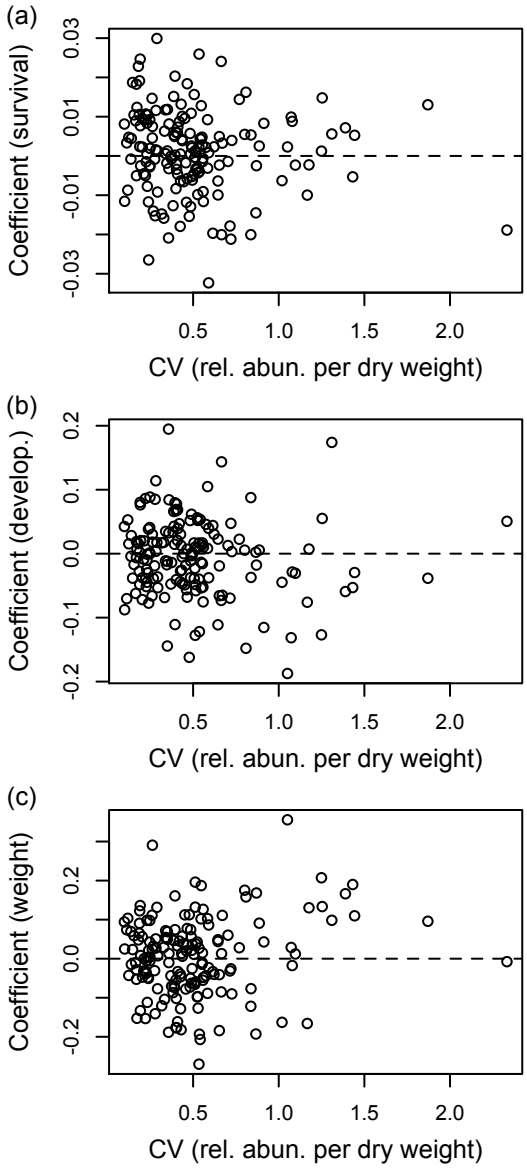

FIGURE A7 Compound specific effects vs coefficients of variation for individual compounds. The compound specific effects based on ridge regression are the same as those listed in Table A1.

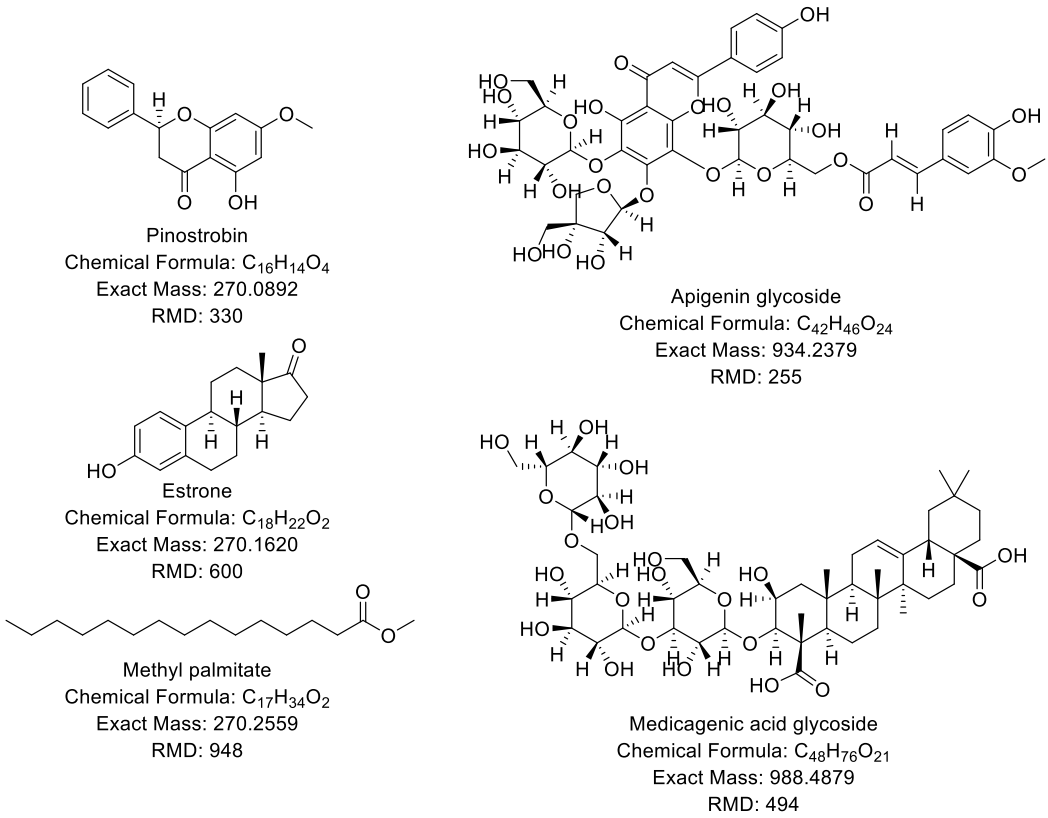

Figure A8. Relative mass defect of natural products. **Left:** A series of isobaric molecules (by nominal mass) which have increasing RMD as %H increases. **Right:** Characteristic metabolites from *M. sativa* which can be classified by RMD.