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Original Article

Evolutionary signatures of a trade-off in direct and indirect defenses across the wild grape genus, *Vitis*

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Abstract

Evolutionary correlations between chemical defense and protection by mutualist bodyguards have been long predicted, but tests of these patterns remain rare. We use a phylogenetic framework to test for evolutionary correlations indicative of trade-offs or synergisms between direct defense in the form of plant secondary metabolism and indirect defense in the form of leaf domatia, across 33 species in the wild grape genus, *Vitis*. We also performed a bioassay with a generalist herbivore to associate our chemical phenotypes with herbivore palatability. Finally, we tested whether defensive traits correlated with the average abiotic characteristics of each species' contemporary range and whether these correlations were consistent with plant defense theory. We found a negative evolutionary correlation between domatia size and the diversity of secondary metabolites in *Vitis* leaf tissue across the genus, and also that leaves with a higher diversity and richness of secondary metabolites were less palatable to a generalist herbivore, consistent with a trade-off in chemical and mutualistic defense investment. Predictions from plant defense theory were not supported by associations between investment in defense phenotypes and abiotic variables. Our work demonstrates an evolutionary pattern indicative of a trade-off between indirect and direct defense strategies across the *Vitis* genus.

Keywords: *Vitis*, indirect defense, direct defense, chemical defense, domatia

Introduction

Trade-offs and synergisms are central to our understanding of the factors that promote and maintain trait diversity. These interactions between traits contribute to the adaptive advantage a trait or suite of traits confers to an organism, and thus are predicted to produce complex patterns of trait investment across organisms. Negative evolutionary correlations between traits, sometimes referred to as trade-offs, are produced by several mechanisms, including redundancy, limiting resources, or ecological conflict (Agrawal, 2020; Agrawal et al., 2010; Rhoads & Cates, 1976). Contrastingly, trait synergisms occur when multiple traits act in concert to provide a selective advantage greater than each trait on its own. At the macroevolutionary scale, synergisms may manifest as positive evolutionary correlations between traits. Testing for evolutionary patterns consistent with trade-offs and synergisms requires the use of phylogenetic comparative methods to disentangle whether correlations between traits across species are due to repeated evolution or simply similarity due to common descent, ideally using common gardens (Agrawal, 2006; Felsenstein, 1985). While many evolutionary trade-offs and synergisms between traits are hypothesized in evolutionary ecology, many trait combinations have yet to be tested at a broad evolutionary scale.

The study of plant defense against herbivory is rich in long-theorized evolutionary trade-offs and synergisms between the incredibly diverse traits plants express to combat enemies (Agrawal & Fishbein, 2006; Johnson et al., 2014). Within this body of theory, one of the most compelling yet elusive hypotheses is that of a trade-off between direct and indirect plant defenses. Direct plant defenses act by physically impairing or poisoning herbivores (e.g., thorns, trichomes, secondary metabolism), while indirect defenses operate by attracting or retaining mutualistic predatory arthropods that reduce herbivory by consuming or discouraging herbivores (e.g., extrafloral nectaries, domatia). A negative correlation between direct and indirect defenses was first proposed by Dan Janzen in 1966, when he observed that *Acacia* plants occupied by bodyguard ants had foliage that tasted less bitter than those without defensive ants. We may expect this negative correlation between direct and indirect defenses to occur for several reasons. First, plants have limited resources that they can allocate to defense, and thus may experience selection against redundancy in their defenses. As a result, having both a direct defense that discourages a herbivore group and an indirect defense that targets the same group may not be an efficient use of an organism's resources. Direct and indirect defenses may also interfere with each other in ways that limit their selective benefit. For example, having hairy or sticky leaves as a direct defense against chewing herbivores may make it challenging for mutualists to inhabit and adequately

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defend a plant (Riddick & Simmons, 2014). Similarly, plants with microfauna bodyguards may be less likely to have toxic metabolites if those compounds inhibit the growth of or kill their mutualists (Agrawal et al., 2002; Campbell & Duffey, 1979). Thus, there are both physiological and ecological reasons why having direct and indirect defenses may be maladaptive.

Alternatively, rather than negative macroevolutionary correlations, direct and indirect defenses may also be predicted to be positively correlated across species. If species have been subjected to selection pressures that favor multiple types of active defenses at any given time, such as attack from a range of herbivores with diverse feeding modes, having both direct and indirect defensive traits may be adaptive (Agrawal & Fishbein, 2006; Webber & McKey, 2009). As previously mentioned, defense traits may also be positively evolutionarily correlated if they act synergistically, meaning that the presence of two or more traits at once provides greater defense against herbivores than any of the traits on their own. Positive relationships between defense traits have been experimentally demonstrated in several contexts, particularly in the case of chemical defenses (Berenbaum & Neal, 1985; Richards et al., 2016), pairings between chemical and physical defenses (Clauss et al., 2006; Konno et al., 2014), and between different types of traits that attract mutualist defenders (Weber et al., 2012, 2016).

Plant defense theory additionally emphasizes the importance of the biotic and abiotic environment in patterns of defense trait evolution. This school of thought suggests that macroevolutionary trajectories of plant defense traits are largely driven by adaptation to local environmental conditions rather than conflicts or synergisms between traits. Under this framework, plant defensive traits are predicted to display patterns of evolutionary convergence, whereby traits are correlated with abiotic or biotic environmental factors hypothesized to have driven their adaptive change (Agrawal, 2006). For example, plant defense theory predicts that plants from water-stressed environments exhibit more pronounced defenses against herbivory (Hahn & Maron, 2016), and the dramatic defense traits expressed by cacti in arid regions are evidence for this hypothesis. Similarly, Pearse and Hipp (2012) found that physical and chemical defenses across oaks increased with a decrease in minimum precipitation in their home range, indicating that plants evolving in environments with a higher risk of drought invest more in their defenses. Seasonality, another abiotic ecological factor, is also predicted to impact plant defense investment by shortening the period of time that plant species are exposed to herbivory. Temperature seasonality, in particular, has been implicated as a predictor of defense investment because it limits a plant's growing season and thus the time it is vulnerable to herbivores (Karban, 2007; Pearse & Hipp, 2012). Features of the abiotic environment can also indirectly influence levels of defense in plants through biotic interactions. For instance, the higher levels of herbivory and more diverse herbivore communities characteristic of tropical regions may exert stronger selection for multiple defense traits in tropical climates, resulting in a pattern of higher expression of multiple defensive traits at more equatorial latitudes (Baskett & Schemske, 2018; Schemske et al., 2009).

Despite these compelling and sometimes conflicting hypotheses, few studies have tested for correlations in direct and indirect defense across species using a common garden and comparative phylogenetic approach (but see Rudgers et al., 2004). What work has been done has largely investigated patterns in direct and indirect trait investment within a limited range of plant species and has found mixed results (Ballhorn et al., 2008; Dyer et al., 2001; English-Loeb & Norton, 2006; Heil et al., 2000, 2002; Letourneau & Barbosa, 1999). Though this study contributes to our overall understanding of plant defense evolution, patterns of direct and indirect defensive strategies across species remain a subject of debate in evolutionary biology, and studies that use a phylogenetically informed approach are needed to disentangle patterns of investment at the macroevolutionary scale.

In this study, we use phylogenetic comparative methods to test for patterns of defense evolution consistent with trade-off, synergism, or geographic hypotheses across the wild grape genus, *Vitis* (Vitaceae). The genus *Vitis* is composed of about 80 species that are distributed across the temperate zones of North America, Europe, and Asia. *Vitis* vines are woody, deciduous perennials and demonstrate a variety of both direct and indirect defensive phenotypes that are purported to reduce herbivory. Using common garden studies, we quantified evolutionary patterns in both direct and indirect defensive traits across 33 species of *Vitis*. We combined this trait data with contemporaneous climatic data for each species to test whether patterns of defense evolution are consistent with a priori plant defense hypotheses. Specifically, we asked (a) is there evidence for positive or negative evolutionary correlations between direct defense (trichomes, secondary metabolism, and palatability) and indirect defense traits (domatia size and density) across *Vitis*? (b) Do abiotic conditions predict evolutionary investment in certain types of defensive strategies?

Methods

Study design

We sampled defensive phenotypes of 33 *Vitis* species grown in pre-established vineyards outside of Davis, CA (Wolfskill vineyard at 38.5033°N, −121.9803°W; UC Davis Viticulture and Enology Collections at 38.5382°N, 121.7617°W). This design allows us to isolate evolutionary differences in plant defenses via a shared habitat, reducing the confounding effect of plasticity inherent in collecting trait data from herbarium sheets or living plants collected from very different native habitats. We measured defensive traits on three genotypes per *Vitis* species (with the exception of *V. peninsularis*, which only had two genotypes available). Fresh leaves were packaged on dry ice and mailed to Michigan State University overnight for processing. All assays and traits were measured on mature, fully expanded leaves collected in late summer 2019.

Indirect defense

For indirect defense, we quantified investment in mite domatia, which are small tufts of trichomes found in junctions between veins on the undersides of the leaves that facilitate a mutualistic relationship with predacious and fungivorous mites. Mite domatia provide mites with shelter from unfavorable microclimate features or predation (Lundström, 1887; Walter, 1996), and in turn, mites provide plant hosts with protection from phytophagous microarthropods and deleterious fungi. A large body of research has demonstrated that domatia are an effective indirect defense in *Vitis*. Domatia size and density are correlated with the population size of predatory and fungivorous mites, which in turn correlates with increased defense against herbivores and pathogens (English-Loeb et al., 2002; Grostal & O'Dowd, 1994; Karban et al., 1995). We measured domatia size and hair density on dried, pressed *Vitis* leaves (185 plants × 3–6 leaves per plant = 621 leaves). Under a dissection microscope, we measured the hair density and radius of four domatia per leaf (remaining consistent in the locations of the domatia on the leaf, Figure 1A). Domatia hair density scores were assigned on a scale of 0–9, with 0 representing no hairs present in the vein axil and 9 being a densely packed, full domatium (Figure 2). This was in accordance with the OIV code O-085/U-33 scale, a standard trichome density ranking system used by plant breeders (IPGRI et al., 1997). We marked leaves that were fully pubescent, where no distinction between domatium and foliar trichomes could be made, as having domatia hair density scores of 0. We also measured the radius of each domatium using an ocular micrometer. We combined domatia size and density into a single domatia index score by multiplying the hair density score by the radius for each domatium. This choice is consistent with previous literature that combined these scores because domatia radius and density are strongly correlated and because domatia index is a strong predictor of predatory mite abundance (Loughner et al., 2008).

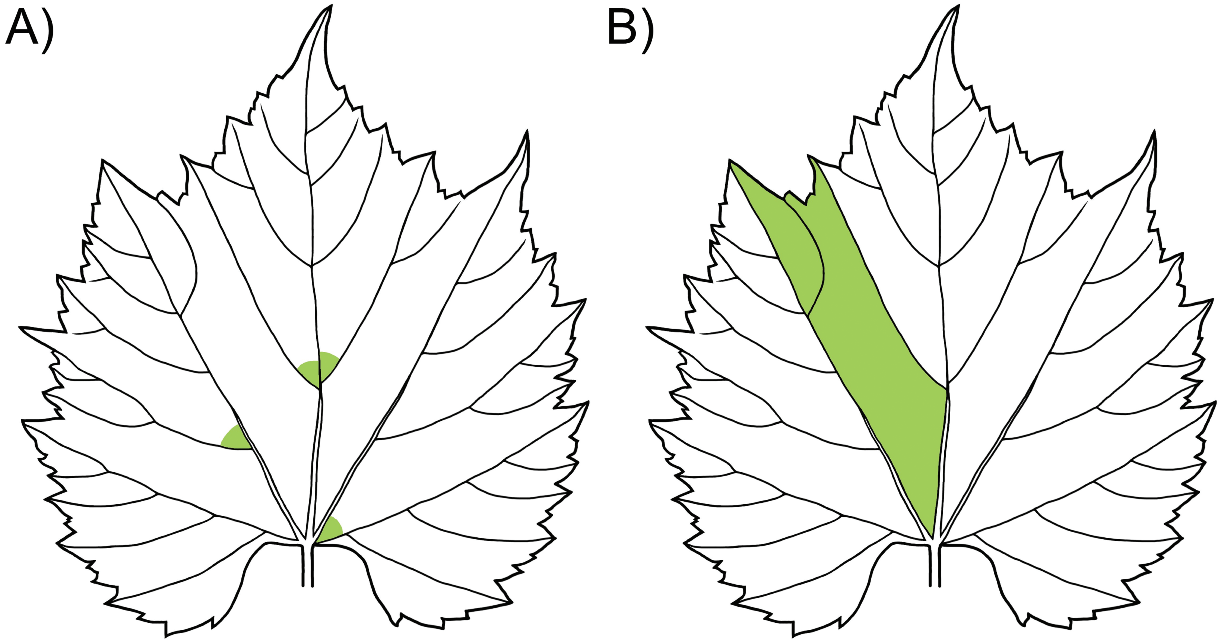

Figure 1. Physical defense phenotype sampling method for *Vitis* leaves, demonstrating (A) the locations of domatia measured on each leaf and (B) the region of the lower leaf surface scanned for trichome density. Methods were adapted from Loughner et al. (2008).

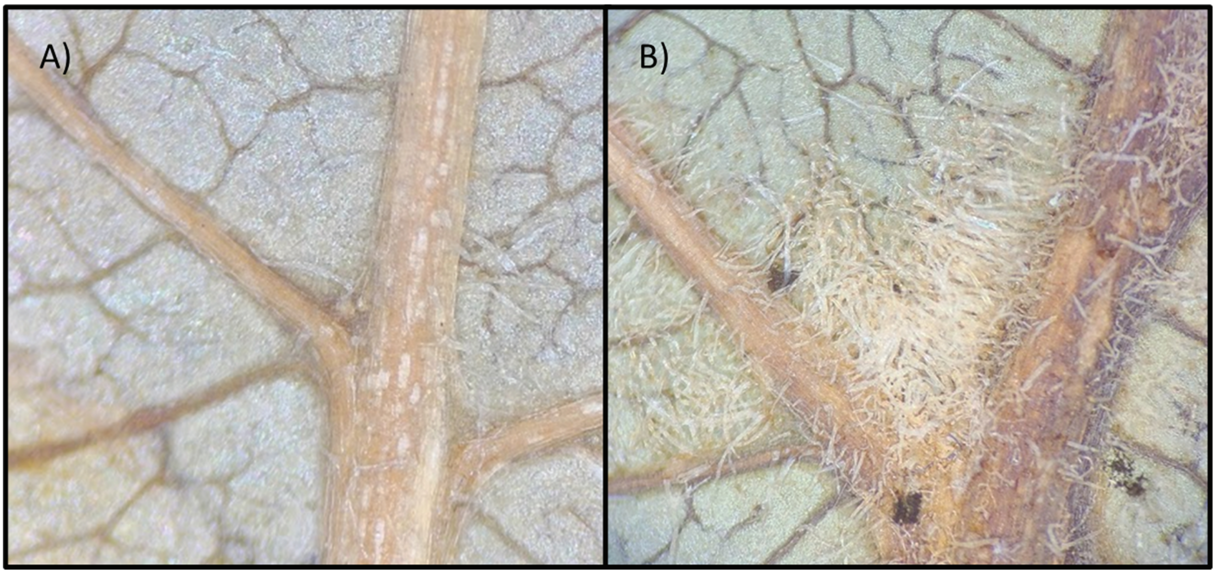

Figure 2. Range of domatia densities in the common garden *Vitis* leaf samples. A) Domatium assigned a density of 1 (almost no hairs, not overlapping) from *V. arizonica*. (B) Domatium assigned a density of 9 (completely full domatium) from *V. vulpina*.

Direct defense

For direct defense, we quantified chemical diversity and trichome density. While a large body of evidence has linked trichomes (Bar & Shtein, 2019; Carmona et al., 2011; Levin, 1973) and secondary chemistry (Hartmann, 2007; Rhoads & Cates, 1976) to herbivore deterrence in a variety of systems, both traits play multiple roles in plant physiology and adaptation (Karabourniotis et al., 2020). To confirm the validity of these traits as measures of direct defense in *Vitis*, and to quantify a more holistic metric of direct defense across species, we also quantified leaf palatability using a generalist herbivore bioassay.

Leaf trichomes

We scored leaf trichomes on the lower leaf surface under a dissection microscope using the O-084/U-33 nine-point scale, remaining consistent with the region sampled for each leaf (Figure 1B, IPGRI et al., 1997).

Leaf chemical diversity

Grape leaves contain a variety of compound classes that may act in defensive capacities, including flavonols, anthocyanins, and other phenolic compounds, as well as terpenes (Fernandes et al., 2013; Kedrina-Okutan et al., 2018; Pintać et al., 2019). Analysis of the metabolite profiles of grape accessions has primarily focused on the economically important *V. vinifera*, and thus, variation in metabolism across *Vitis* species is not well characterized. We quantified secondary metabolite abundance, richness, and diversity of *Vitis* species using liquid chromatography–mass spectrometry (LCMS). This method allowed us to measure *Vitis* chemical diversity without necessitating compound identification. We sampled three leaves per plant from three plants per *Vitis* species (118 total plants). We collected a single 2.53 cm^2 semicircle punch from each sampled leaf (n = 354) and immediately flash-froze the leaf punches in liquid nitrogen. Leaf punches were transported to the lab on dry ice and stored at −80 °C until extraction. We then incubated the frozen leaf tissue in 1 ml of extraction solvent (isopropanol:acetonitrile:water 3:3:2 containing 0.1% formic acid and 104 nM telmisartan as an internal standard) at room temperature for 16 hr. Extracts were stored at −20 °C. Before chemical analysis was performed, we centrifuged the extracts to remove precipitates and diluted the extracts 1:1 in MilliQ water. We dried and weighed the remaining leaf punch samples to standardize chemical composition per mass of tissue.

Liquid chromatography-mass spectrometry was performed at the Michigan State University Mass Spectrometry and Metabolomics Core Facility using a Thermo Q-Exactive mass spectrometer interfaced with a Thermo Vanquish Flex UHPLC system. Samples were injected onto a Waters BEH-C18 reverse phase UPLC column (2.1 × 100 mm) and separated using the following gradient: initial conditions 98% A (water + 10 mM ammonium formate, pH 3.0) and 2% B (acetonitrile + 0.1% formic acid), hold for 1 min at initial conditions, ramp to 99% B at 7 min, hold for 1 min at 99% B, return to initial conditions at 8.1 min, and hold at 2% B for 2 min before the start of the next injection. Column flow rate was 0.3 ml/min and column temperature was 40 °C. Data were acquired in positive ion electrospray mode using a full MS/all-ion fragmentation (AIF) method with a scan range of m/z 100–1,500 and HCD cell collision energy steps of 10, 30, and 60 V for the AIF scans. Capillary voltage was set to 3.5 kV, sheath gas was 47.5, aux gas was set to 11.25, probe heater was set to 412.5 °C, and the S-lens RF level was set at 50. Raw LCMS spectra were processed with Progenesis QI (Waters, Non-Linear Dynamics), and peak areas were normalized to the telmisartan peak and dry sample mass.

For analysis, we excluded peaks that eluted after 8.00 min to avoid the inclusion of membrane lipids. We also filtered out compounds that displayed maximum abundance values in the blanks and compounds with maximum abundances below 10,000. We subtracted average compound abundances in the blanks from each sample and divided abundances by the dry mass of the sample's respective leaf disk to standardize per mass of tissue. We calculated overall chemical abundances by summing the individual compound abundances for each sample and chemical richness as the number of unique chemicals in each sample. We additionally calculated the Shannon diversity of each sample, which takes into account both the diversity of peaks in each sample and the abundance of each peak. We chose Shannon diversity because a body of research suggests that chemical richness and evenness together constitute important elements for defense against herbivory (Salgado et al., 2023; Wetzel & Whitehead, 2020). We calculated species averages by taking the mean richness, total abundance, and Shannon diversity values per species.

To filter our LCMS data set of unidentified peaks for putatively defensive secondary metabolites, we used relative mass defect (RMD) filtering. RMD filtering is a method for categorizing compound peaks in large LCMS data sets into structurally similar classes of compounds (Ekanayaka et al., 2015). RMD scores were calculated for each peak by dividing the mass defect (values following the decimal point of the mass-to-charge ratio, m/z) by the measured monoisotopic mass and multiplying this result by 10^6 to receive parts per million (ppm) values. Before performing any analyses on our chemical data, we dropped all compounds with RMD values above 1,200 to remove compounds with a negative mass defect, which are typically inorganic ions not of biological origin. We used three RMD bins to categorize our compounds: 100–250 ppm for flavonoid compounds, 200–400 ppm for phenolics (Harrison et al., 2018), and 440–640 ppm for terpenoids (Ekanayaka et al., 2015).

Palatability assay

To assess the palatability of leaf tissue from *Vitis* species to a generalist herbivore, and to test whether measures of defense correlate with palatability, we conducted no-choice assays using larvae of the beet army worm, *Spodoptera exigua* (Frontier Scientific Services Agriculture). We used performance of a generalist caterpillar as an integrative measure of plant quality likely to be affecting other generalists such as herbivorous mites targeted by domatia-dwelling predators. *Spodoptera exigua* is a commonly used generalist herbivore in palatability bioassays and was selected for this assay because their methodological ease of use (available to order and measure) and because individuals consumed more grape leaf tissue than *Heliothis virescens* larvae in preliminary bioassays. Palatability assays were performed on plants in the University of California–Davis greenhouses grown from dormant cuttings of outdoor vineyard plants, to ensure that no history of pesticide treatment impacted herbivore performance. We subjected up to three whole leaves from each *Vitis* plant (18 species, 73 plants, n = 217 leaves) to herbivory from a single third-instar larva for a period of 7 days. Freshly picked leaves from California were packed into individual plastic ziplock bags with a moist paper towel and shipped overnight to Michigan on dry ice for use in these trials. Feeding larvae were contained in 3.25 oz plastic sauce cups (Fabri-Kal, USA) and were incubated at 22 °C with a 16-hr photoperiod for the duration of the palatability trials. We scanned remaining leaf tissue at the end of the 7-day period and used imageJ to calculate the standardized pixel area of missing leaf tissue. No leaves were entirely consumed by the larvae in the 7-day period. In order to associate leaf area removed by the herbivore with the mass of tissue consumed, we calculated the average leaf mass per area (LMA, g/pixel) for each *Vitis* species using dried leaves from the domatia/trichome quantifications. We divided the dry mass of each leaf by the leaf's area while fresh (as measured from leaf scans with imageJ, Schneider et al., 2012) and averaged the results for each *Vitis* species. Because leaves vary in thickness, leaf area removed may not be fully representative of the tissue lost by the plant. We thus averaged the area consumed per leaf across each *Vitis* species and multiplied these values by the LMA of each species to obtain the average mass of leaf tissue lost in milligrams.

Abiotic environment

To look for patterns between the environmental characteristics of the geographic origins of our *Vitis* species and their defense phenotypes, we used data from the Global Biodiversity Information Facility (GBIF) database. We downloaded latitude and longitude coordinates for recorded occurrences of our investigated *Vitis* species from this database (GBIF.org, 2022). We then cleaned the coordinate data by limiting the reported occurrences to preserved specimens from herbaria to increase our confidence in the plant ID. We additionally used the clean_coordinates function in the CoordinateCleaner package in R 3.6.1 to remove points around capitals, country centroids, biodiversity institutions, and the GBIF headquarters, as well as those with equal latitude and longitude coordinates and those with coordinates that place them in the ocean, zeros, duplicates, and outliers (Zizka et al., 2020). This process produced 13,561 valid occurrences for 35 *Vitis* species. For all cleaned *Vitis* occurrence points, we downloaded data for abiotic environmental variables from the WorldClim database (Fick & Hijmans, 2017). To capture the variation in abiotic factors that are associated with latitudinal or tropical/temperate variation, we pulled data on annual mean temperature, annual precipitation, and temperature seasonality at our occurrence points. Because we were interested in testing the theory surrounding the relationship between plant defenses and temperateness/tropicality divide, we calculated mean latitude and temperature seasonality (BIO4) values for each *Vitis* species. To test for the impacts of drought stress on the evolution of *Vitis* defenses, we additionally calculated the mean precipitation of the driest month (BIO14) for each species.

Phylogenetic estimation

We reconstructed a relative time-calibrated phylogeny for the *Vitis* species in our study. We identified phylogenetically informative markers in *Vitis* using the phylotaR package (Bennett et al., 2018), which identifies orthologous clusters of sequences in GenBank through BLAST alignment. We searched the GenBank nucleotide database (genomic DNA/RNA) on January 6, 2021, excluding clusters that contained fewer than six representative *Vitis* species as well as clusters explicitly involved in sex determination, fruit color, or stress tolerance (e.g., FRIGIDA, TFL1, C-repeat binding factors, MybA1, MTB1-4, DHN1a) to avoid patterns of convergence misleading phylogenetic inference. This search resulted in 47 target clusters for downstream phylogenetic analyses. We created a species-level supermatrix for our *Vitis* species and subspecies for our identified markers using the superCRUNCH pipeline (Portik & Wiens, 2020). We removed all sequences with the name "hybrid" in the description, as well as accessions determined to be hybrids or misidentified based on Zecca et al. (2020). Sequence search terms and taxonomy matching terms are available in the Dryad submission for this study. Matching sequences were filtered using the reference mc-megablast extract method using representative fasta files from our phylota search as reference sequences. Nonoverlapping blast coordinates were merged using the -span option with a 100 bp bridge. We further filtered sequences down to one representative sequence per species for each locus with the -oneseq option. Representative sequences for each species were chosen by length, with a minimum sequence length threshold of 100 bp. We ensured sequences were in the correct orientation using the Adjust_Direction.py script. Multiple sequence alignment was conducted using MAFFT (Katoh & Standley, 2013), and aligned sequences were relabeled using the Fasta_Relabel_Seqs.py script. Sequences were trimmed by removing columns with data for two or fewer species using trimAL implemented with the Trim_Alignments_Trimal.py superCRUNCH function. All included markers had over 15% taxon representation. We used jModeltest to determine appropriate substitution models for each partition based on Akaike's information criterion through the R package *phanghorn* (Schliep, 2011). The resulting best model fits are reported in Supplementary Table S1.

We estimated the joint posterior distribution of topologies and relative node divergence times using BEAST 2.6.3 (Bouckaert et al., 2019). Each marker was partitioned with its own unlinked previously estimated substitution model. We utilized one uncorrelated exponential relaxed clock model to estimate node heights for all of the partitions, applying a birth–death tree model. We conducted three Markov chain Monte Carlo (MCMC) searches, each run for 2,000,000,000 generations sampled every 10,000 generations using three random starting trees. Trees were rooted using *V. rotundifolia* Michaux as an outgroup following convention based on previous findings (as cited by Ma et al., 2018). Convergence of each Bayesian run was assessed by plotting the log-likelihood of sampled trees and parameters using Tracer (Rambaut et al., 2018). The first 25% of sampled trees were removed from each run as a burnin, and a maximum clade credibility (MCC) tree was identified from the combined postburnin output of the three MCMC runs using the maxCladeCred function in the R package phangorn. For full tree and associated GenBank accession numbers, see Figure 3 and the data deposited on Dryad. All analyses were run on the MCC tree, with consensus branch lengths calculated using the least squares method via the "consensus.edges" function in the package "phytools" (Revell, 2012).

Statistical analyses

We tested for evolutionary correlations between traits and between traits and environmental variables, using phylogenetic generalized least squares (PGLS) analyses. PGLS models test for evolutionary correlations between traits while

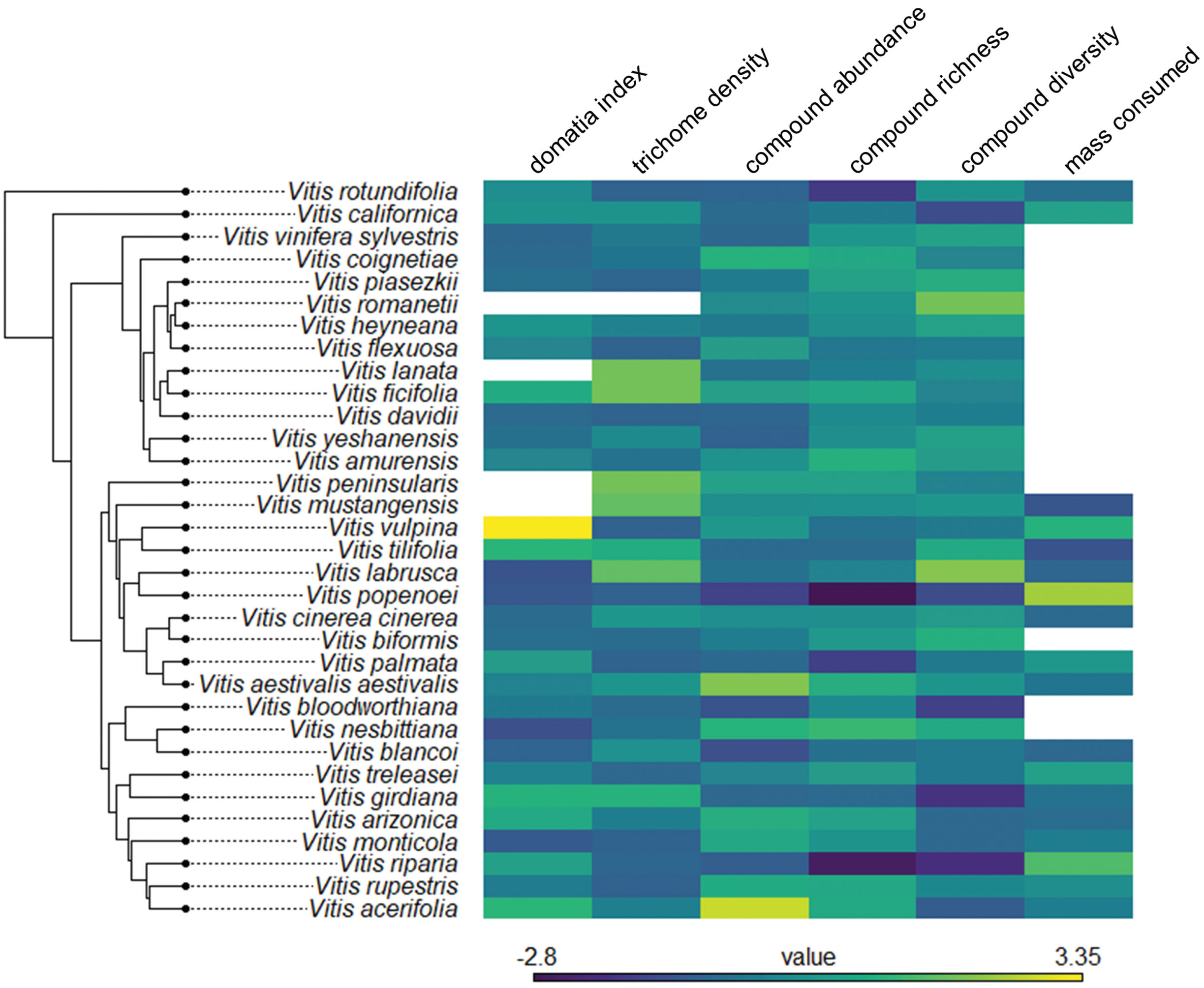

Figure 3. Maximum clade credibility tree of *Vitis* species investigated in this study, accompanied by standardized mean trait values for six defense-related metrics across *Vitis* species. White space represents traits for which data could not be collected for a given *Vitis* species.

controlling for nonindependence due to phylogenetic relatedness (Revell & Harmon, 2022). We used the "gls" function in the R package "nmle," incorporating a Brownian motion correlation structure. All analyses were conducted using R version 4.2.1 (R Core Team, 2021), and all scripts and data will be provided upon publication.

Results

Defense phenotypes

There was high variability in defensive phenotypes across the *Vitis* genus. Species of *Vitis* had mean domatia indices that ranged from 0.15 for *V. nesbittiana*, indicating that domatia were largely absent in this species, to a maximum of 12.2 for *V. vulpina*, which had large, full domatia (Supplementary Figure S1). *Vitis vulpina*, *V. rupestris*, and *V. popenoei* had mean trichome density scores of 0, indicating that they were fully glabrous, while *V. ficifolia*, *V. lanata*, and *V. romanetti* had fully pubescent leaf undersides with mean trichome densities of 9.

Chemical phenotypes were also highly variable. LCMS methods produced spectra for 5,549 unique compounds, of which 1,969 were present in all leaf extracts. *Vitis acerifolia* leaves had the highest average abundance of compounds in their tissue, while *V. aestivalis* had the highest chemical richness. *Vitis popenoei* had the minimum values for both chemical peak abundance and richness. The Shannon diversity of metabolites was highest in *V. labrusca* and lowest in *V. riparia* (Supplementary Figure S2).

Palatability assay

To test whether our direct defense phenotypes reflect palatability of leaves to generalist herbivores, we conducted no-choice assays using larvae of *S. exigua*. We found significant negative correlations between the mass of the leaf tissue consumed by the *S. exigua* larvae and the richness and Shannon diversity of metabolites in the tissue of our grape species (Figure 4B and C), consistent with chemical Shannon diversity and richness serving as defenses in *Vitis*. The leaf mass consumed was not correlated with the abundance of metabolites per unit leaf tissue in the leaves (Figure 4A). The results of these analyses are summarized in Table 1. Additionally, there was a strongly negative correlation between trichome density on the lower leaf surface and the mass of grape leaf tissue consumed (p = .00014, Supplementary Figure S3b).

To establish whether generalist herbivore performance responds to specific classes of metabolites, we additionally tested whether the abundance, richness, and Shannon diversity of peaks in the three RMD chemical classes explained palatability in the bioassay. We found that the richness of

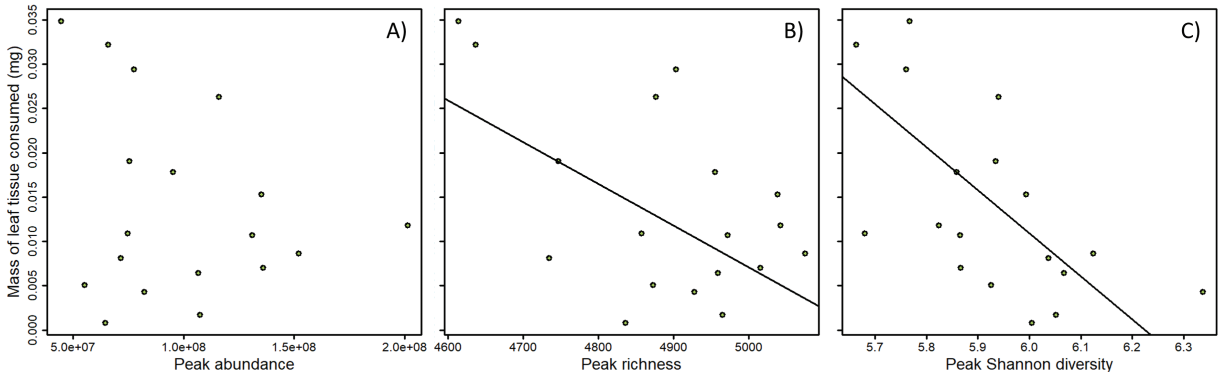

Figure 4. Relationships between our LCMS chemistry results and the results of the herbivory assays using *S. exigua* in our common gardens. The average mass of leaf tissue consumed in grams per *Vitis* species plotted against (A) the average abundance of metabolite peaks in tissue extracts, (B) the average richness of peaks, and (C) the average Shannon diversity of peaks. Significant trendlines from phylogenetic generalized least squares models are shown (α = .05).

Table 1. Results of phylogenetic generalized least squares models comparing leaf palatability (average leaf mass consumed in grams) to a generalist herbivore and our defensive phenotypes. All comparisons were made between species means.

Comparison	Effect size (mg of tissue consumed)	*p*-value
Palatability ~ domatia index	—	.216
Palatability ~ trichome density	−0.00297 ± 0.00064	<.001***
Palatability ~ chemical abundance	—	.111
Palatability ~ chemical richness	$-4.7 \times 10^{-5} \pm 1.3 \times 10^{-5}$	.002**
Palatability ~ Shannon diversity	−0.0485 ± 0.0124	.001**

Significance: **P < 0.01, ***P < 0.001.

metabolites in all three RMD classes correlated significantly with the mass of leaf tissue consumed by the herbivores (Supplementary Tables S2–S4). Interestingly, for peaks in the 100–250 ppm RMD range typical of flavonoids, this correlation was positive, while for the other two RMD ranges, the correlation was negative (Supplementary Table S3). Peak diversity in the terpenoid and phenolic RMD ranges was also negatively correlated with the mass of leaf tissue consumed (Supplementary Tables S2 and S4).

Direct–indirect defense trait correlations

In accordance with the prediction that direct chemical defense and indirect defense may exhibit a negative relationship with each other due to ecological conflicts or resource limitation, we found that Shannon diversity of metabolites and domatia index were significantly negatively correlated across *Vitis* species ($p = .0237$, Figure 5), where there was a 0.0264 ± 0.0111 decrease in Shannon diversity for every 1 unit of increase in average domatia index. Contrary to our initial expectation, there was no significant relationship between domatia index and the total abundance or richness of compounds using PGLS models.

Because plant defense theory predicts the existence of suites of coexpressed defense traits that act together to reduce herbivory (Agrawal & Fishbein, 2006), we additionally tested for a positive correlation between direct defense traits. Indeed, there was a significant positive relationship between trichome density and the Shannon diversity of metabolites across species ($p = .0115$), whereby plants with more diverse chemistry also had more leaf trichomes. Domatia index and trichome density were not correlated in these species, indicating that these traits evolved independently.

Because we were interested in which classes of metabolites drive the observed negative relationship between direct and indirect defense, we additionally performed the same PGLS analyses with chemistry data sets filtered down to specific metabolite classes. RMD filtering narrowed our chemical peaks down to 394 compounds that fit into the 100–250 ppm RMD range typical of flavonoids, 977 in the 200–400 ppm range typical of phenolics, and 2,774 in the 440–640 range typical of terpenoids. We found that the Shannon diversity of peaks in the range of terpenoids was negatively correlated with the domatia index of *Vitis* species ($p = .0212$; Supplementary Table S2), pointing to terpenoids as the major compound class shaping the negative correlations between chemical defense and indirect defense. Indeed, about 50% of the peaks detected in our grape samples were in the 440–640 ppm RMD range, hinting that the larger patterns associated with metabolite diversity could be driven by compounds within this RMD range in the extracts. Conversely, the diversity of peaks in the flavonoid range was positively correlated with domatia index across species ($p = .0437$; Supplementary Table S3), revealing that not all chemical compound classes follow the more general trade-off pattern.

To test for predicted positive correlations among different direct defense traits, we looked for correlations between specific chemical classes and leaf trichomes. We did not find support for positive correlations. The abundance of peaks in the flavonoid range was instead negatively correlated with trichome density ($p = .00597$; Supplementary Table S3), while compounds in the range of neither terpenoids nor phenolics showed any significant relationship with trichomes.

Abiotic environment

There was considerable variation in habitat variables across the home ranges of *Vitis* species in the study. The most southerly ranged species was *V. tiliifolia*, with occurrences that averaged at 16° 20′ latitude. This species also experiences the

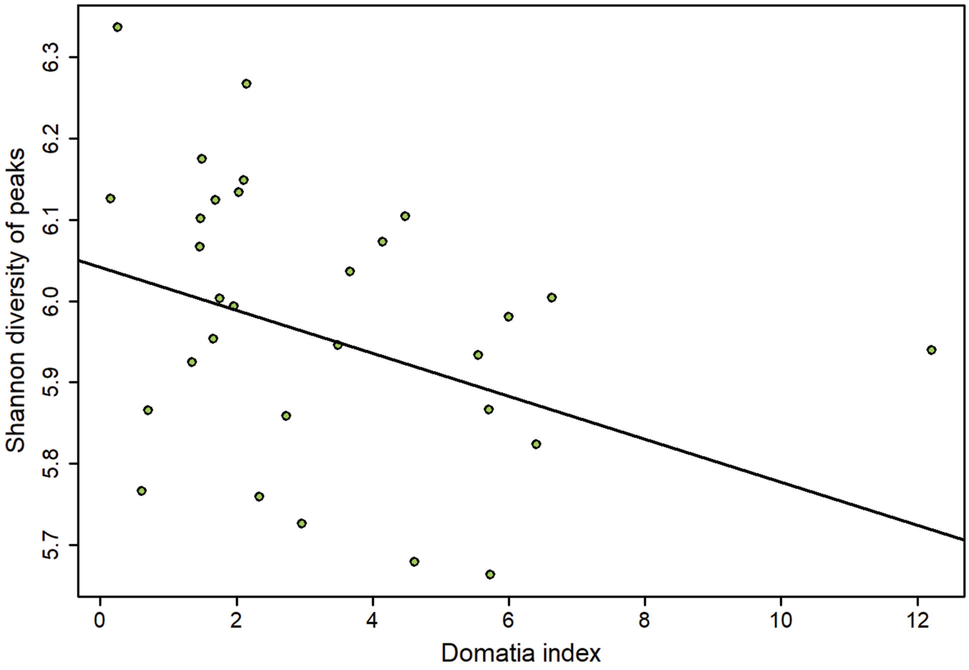

Figure 5. Negative correlation between the domatia index, a measure of indirect defense, and the Shannon diversity of peaks, a measure of direct defense, in grape leaves. Each point represents the mean for a *Vitis* species. Significant trendline from the phylogenetic generalized least squares model is shown (p = .0237).

least variability in temperature throughout the year. The most northern species in the data set was *V. vinifera*, averaging at 41° 56′ latitude, though the east Asian native *V. amurensis* experiences the greatest variability in climate of the species in this study. Grapes such as *V. girdiana*, *V. arizonica*, and *V. peninsularis* that are native to the southwest United States and northern Mexico experience the least annual precipitation and driest months, respectively, while vines of the Central America natives such as *V. popenoei* and *V. tiliifolia* experience much wetter climates. Despite the range of climates that *Vitis* species are exposed to, we discovered few evolutionary correlations between latitude, temperature seasonality, and precipitation of the driest month and the defensive traits measured for *Vitis* species in our data set (Supplementary Table S5). The only significant evolutionary correlations between abiotic variables and defense traits were between overall latitude and chemical abundance (p = .0446) and temperature seasonality and chemical abundance (p = .0332; Supplementary Table S5). Both of these correlations were positive, with the average abundance of peaks in the *Vitis* species increasing with latitude and temperature seasonality.

Discussion

Plants have evolved a diverse suite of defensive phenotypes, which have captivated biologists for centuries. Plant defense theory posits that evolutionary variation in plant defense is driven by processes including trade-offs and synergisms among different defensive traits, as well as selection from environmental conditions that shape divergence across space and time. Here, we use a phylogenetic comparative and common garden approach to test whether patterns of defense trait evolution are consistent with several classic predictions of plant defense theory in the grape genus, *Vitis*. Our analyses revealed three main findings. (a) Phylogenetic analyses uncovered a negative correlation between investment in mite domatia and plant chemical diversity across *Vitis*, consistent with a hypothesized trade-off between direct and indirect defense investment over evolutionary time. In contrast, (b) there was a stark absence of support for predicted correlations between defense investment and climate variables hypothesized by plant defense theory to impact trait evolution. Finally, (c) we found evidence that palatability to a generalist herbivore was correlated with plant chemical diversity across species of *Vitis*, consistent with recent work in plant chemical ecology, positing that chemical richness and evenness contribute together to defense. Below, we discuss each of these results in turn, including related literature, caveats, and future directions.

Evolutionary trade-off among direct and indirect defense

This article is the first comparative phylogenetic study to find evidence for the existence of the long-hypothesized trade-off between direct and indirect defense across plant species. Only one other study to our knowledge has investigated direct and indirect defense trait correlations across species using modern phylogenetic comparative methods (Rudgers et al., 2004). That article did not detect a correlation between extrafloral nectaries, which provide food to mutualist ant defenders, and direct defense in the form of trichomes and gossypol glands in the cotton clade Gossypieae. Other studies have investigated evidence of a direct–indirect defense trade-off using noncomparative methods and found mixed results (e.g., Dyer et al., 2001; Eck et al., 2001; Heil et al., 2000, 2002; Letourneau & Barbosa, 1999; Rehr et al., 1973). For example, a 2006 study by English-Loeb and Norton tested for but did not detect intraspecific correlations between mite domatia and direct defense against pathogenic fungi in *Vitis riparia*. Furthermore, most of the investigations of direct–indirect defense trade-offs thus far have focused on ant–plant mutualisms, which is perhaps not surprising given that the initial hypothesis was based on observations in ant systems by Janzen, who observed a trend of higher leaf bitterness in plants without mutualists. By setting our findings in the context of the broader literature, it is clear that whether or not a trade-off between direct and indirect defense exists (or should even be expected) is likely a function of the specific system and scale. It is additionally clear that work in alternative indirect defense systems, including mite and volatile-based systems, is needed to determine the parameters under which a direct–indirect trade-offs is expected more broadly.

While our study revealed evolutionary patterns consistent with a trade-off in direct and indirect defense in *Vitis*, the specific mechanism driving this pattern is unknown. The observed negative evolutionary correlation could be produced through several mechanisms. For example, resource limitation, such as through a metabolic precursor, could constrain the expression of both domatia and metabolite diversity (Agrawal et al., 2010). However, a specific shared precursor for domatia and chemical traits is unknown in this system. Selection could also act against the coexpression of multiple defenses if they are costly to the plant and redundant with each other or if they conflict with one another's ecological function (Romeo et al., 1996; Steward & Keeler, 1988). The specific cost and redundancy of defensive traits in *Vitis* are unknown. However, chemical defenses have been shown to be costly more generally, particularly in Arabidopsis (Neilson et al., 2013). Furthermore, the production of more diverse compound mixtures may be more costly than producing more metabolites overall because more biosynthetic machinery is needed to generate more unique metabolites (Gershenzon, 1994), which could explain why we saw a negative correlation between domatia and compound diversity, rather than compound abundance. To our knowledge, no work to date has evaluated the metabolic cost of producing domatia, but trichome production, which is key to the production of domatia, is costly (Agren & Schemske, 1994; Hare et al., 2003; Sletvold et al., 2010). In terms of redundancy, it is unclear whether the targets of domatia and chemical defense overlap in *Vitis*. However, it is not unreasonable to expect that the generalist herbivores targeted by domatia-dwelling predators would be affected by the same chemical traits impacting the generalist herbivore in our bioassay. Secondary metabolites have been demonstrated to reduce fitness of herbivorous mites in several systems, including grape (Ma et al., 2014; Vásquez et al., 2008), and there is evidence that the same resistance traits induced by spider mites affect the *Spodoptera exigua* caterpillars used in this study (Karban, 1988). Finally, rather than being simply redundant and costly, domatia and chemical defense could actively conflict with one another leading to an ecological cost of having both traits. If chemical defensive traits poison domatia-dwelling plant bodyguards, either through direct contact (e.g., through waxes, pollen, nectar, or pearl bodies) or through consuming prey that have fed upon defensive plant compounds, selection would be expected to reduce the coexpression of these defenses over time. Agrawal et al. (2002) found that predatory mites were less fecund when fed with spider mites that had been grown on a bitter cucumber variety compared to a sweet variety, indicating a potential mechanism by which mutualists are being poisoned by metabolites in leaf foliage. In other mutualistic plant–insect interactions, mutualists that feed on nectar are disrupted by host-plant toxicity (Adler, 2000), suggesting an ecological cost of having both direct and indirect defenses could exist across systems. For future work, *Vitis* presents an exciting system for experiments testing between putative mechanisms behind direct–indirect defense evolutionary trade-offs.

Lack of predicted correlations between defense investment and climate variables

Our results suggest that evolutionary investment in grape defenses is largely not explained by the native climatic environment of *Vitis* species as predicted by plant defense theory. We tested two hypotheses of environmental influences on plant defense evolution: that defense expression correlates with latitude and seasonality and that defense correlates with water limitation, with the expectations that plants from less seasonal, tropical environments and those from water-stressed environments will be more defended against herbivory. We found no significant negative correlations between defense trait investment and the latitude of origin of the *Vitis* species, nor with the seasonality of the temperatures those species experience. Contrary to our expectations, we did observe a positive correlation between peak abundance and both latitude and temperature seasonality across the genus, a pattern that is the opposite of what is predicted by the latitudinal herbivory defense hypothesis. Support for a latitudinal gradient in plant defenses in the literature is mixed, with sporadic support in particular taxa but a lack of consensus across plants more broadly (Anstett et al., 2016; Moles et al., 2011). In the case of this study, the species we sampled may not span enough of a range of latitudes to produce a detectable pattern in defense investment. Indeed, grapes are most commonly found in temperate and subtropical regions rather than the tropics themselves, and thus as a genus may not experience as much variation in biotic pressure from herbivores as other groups more likely to display a latitudinal gradient in defense.

Finally, we found no correlation between defense investment and mean annual precipitation (a proxy of the drought stress that the species experiences in their native range). Again, this contrasts with plant defense theory, which predicts that defense investment should be higher in more resource-stressed environments where photosynthetic tissue is more expensive to produce (Coley et al., 1985; Endara & Coley, 2011; Pearse & Hipp, 2012). Since latitudinal biotic interactions and resource stress are not as directly related to each other as other bioclimatic variables (e.g., latitude and seasonality), variation in defense investment produced by these geographic patterns may complicate the straightforward patterns predicted by defense theory. Additionally, the lack of evolutionary correlations seen in our common garden study does not rule out the existence of a latitudinal pattern in the field, as phenotypic plasticity could result in an observed relationship between climate variables and grape defense investment in plants growing in their native environments. Trends in defense investment due to plasticity may be more detectable in plants sampled directly from the environment of origin.

Chemical diversity as a predictor of palatability to a generalist herbivore

Our research adds to a growing body of literature supporting the role of chemical diversity in resistance to herbivory (e.g., Glassmire et al., 2019; Richards et al., 2015; Sedio et al., 2020; Whitehead et al., 2021). Our bioassay experiment indicated that *Vitis* species with more rich and diverse metabolites in their leaves sustain less damage from a generalist herbivore than those with less diverse metabolic profiles, suggesting that secondary metabolite composition is an important defense against enemies for grape species. Because the structural diversity of compounds, which we measure here, is associated with the functional diversity of compounds (Wetzel & Whitehead, 2020; Whitehead et al., 2021), leaves with higher compound diversity may impact herbivores more strongly simply by virtue of delivering a diversity of toxicity mechanisms at once. However, metabolite diversity has also been proposed to be detrimental to herbivores for reasons distinct from the overall number of defensive metabolites in the leaves. One hypothesis posits that diverse cocktails of metabolites work together synergistically to the detriment of herbivores (Berenbaum & Neal, 1985; Richards et al., 2016). In this hypothesis, a metabolite is proposed to be more effective against an herbivore in the presence of another metabolite or metabolites. Another hypothesis suggests that the more unique metabolites in a mixture, the more likely there will be a metabolite or group of metabolites that will be effective against any given herbivore, thereby enhancing plant fitness (the screening hypothesis; Carmona et al., 2011; Jones & Firn, 1991; Wetzel & Whitehead, 2020). Shannon diversity takes into account the evenness of the compounds in the mixture, where more even mixtures have a higher Shannon diversity than those with the same number of unique metabolites but in more uneven abundances. Our finding that leaf palatability is more strongly correlated with Shannon diversity of metabolites than metabolite richness alone therefore suggests that metabolite evenness is an important component of the plant's defense. Observations of plants with more even mixtures of compounds might suggest a situation where most of the plant's compounds contribute to its fitness, as would be the case in situations where the metabolites act synergistically to provide defense for the plant (Wetzel & Whitehead, 2020). Interestingly, most studies that have shown a negative impact of metabolite diversity on herbivores had the expectation that richness was the key factor at play, not evenness (Richards et al., 2016; Salgado et al., 2023). Indeed, few if any studies have attempted to disentangle the impact of metabolite evenness versus richness on insect herbivores (Wetzel & Whitehead, 2020), making this study an important contribution to our understanding of the impacts of chemical diversity on herbivores.

Though herbivory was negatively related to the richness and diversity of metabolites across *Vitis* species, domatia size had no direct relationship with leaf palatability. This result is unsurprising for two reasons. First, domatia on their own are not anticipated to be a defense in the absence of plant mutualists. The process of collecting and shipping the leaves likely eliminated any mites that were present upon leaf sampling. Second, as previously mentioned, mite mutualists are unlikely to be a deterrent to "large" chewing herbivores such as caterpillars of the size used in our study (although predatory mites may consume small immature insects and insect eggs, e.g., Knapp et al., 2013; Patel & Zhang, 2017). Most predatory mites are several hundred micrometers in size (da Silva et al., 2016; Vangansbeke et al., 2020), negligibly small compared to the several centimeter-long third-instar *S. exigua* larvae that we used in our bioassays. Instead, mutualistic mites have been shown to reduce numbers of herbivorous mites, such as spider mites, as well as fungal hyphae on leaf surfaces (Agrawal & Karban, 1997; Grostal & O'Dowd, 1994; Norton et al., 2000).

Our results also support existing evidence that trichomes are a physical defense against herbivory. Dense trichomes impair the movement of herbivores and make it more difficult to reach the leaf surface, thus serving as a direct defense against many antagonistic arthropods (Levin, 1973). The positive correlation between trichome density and chemical diversity across these *Vitis* species could indicate the existence of a grape defensive strategy of investing in both chemical and physical defenses against herbivores. Such an association could be akin to a no-mutualist-mite strategy, where the presence of mutualist bodyguards is not favored by plant traits, namely dense trichomes and high chemical diversity. Indeed, while having trichomes on a leaf surface may protect mutualistic mites from predators as well as microclimate stressors, high trichome densities may impede the ability of mites to navigate the leaf surface and reach their prey. There is evidence that the speed and predation rate of a predatory mite decrease with increasing trichome density, albeit not in grapes (Krips et al., 1999). However, the absence of a negative correlation between domatia and trichomes in grapes does not support this hypothesized no-mutualist defense syndrome.

Conclusions

Since first proposed in the 1960s, the direct–indirect tradeoff hypothesis has been a staple of plant defense theory. In this study, we present evidence that secondary metabolite diversity, a direct defense against herbivores, trades off with mite domatia, an indirect defense, across 33 species in the genus *Vitis*. While there was a positive association between trichomes and secondary metabolite diversity, we did not find support for the existence of other synergisms between traits or for trait–environment correlations consistent with plant defense theory. Additionally, our results suggest that secondary metabolite diversity is an important metric of defense for *Vitis* vines. Though we demonstrated that these traits are negatively correlated across species, future work must be done to identify the mechanism behind this pattern.

Supplementary material

Supplementary material is available online at *Evolution* (https://academic.oup.com/evolut)

Data availability

The data and scripts for this manuscript are available on Dryad at https://doi.org/10.5061/dryad.vdncjsz15.

Author contributions

C.D.K.G. and M.G.W. conceptualized and designed the study. E.J.F. provided plant material. E.J.F. and A.T.Z. collected plant samples. A.L.S. assisted with the design and collection of chemistry data. C.D.K.G. collected trait data, performed the herbivory experiment, and analyzed data. M.G.W. built the phylogenetic tree. C.D.K.G. and M.G.W. wrote and revised the manuscript.

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Conflict of interest: The authors declare no conflict of interest.

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