Standing Armies and Crystal Swords: Defense Evolution Across Wild Grape

by

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Dedication

I dedicate this dissertation to my family. To my parents, who always made sure I had access to anything I wanted to read and indulged my many hobbies. To my grandmother, who is the toughest and bravest person I know. To my sister, Nora, who I talked (and complained) to almost every day during this process. To my husband, Jacob, who is my anchor. And especially to my cats, who may have at times made this process more stressful, but also cuddled me through it.

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Abstract

The diversity of adaptations that plants demonstrate to defend themselves from herbivory has fascinated biologists for decades. To explain this wealth of defense traits, researchers have developed a body of theory that uses patterns of ecological pressures in the environment to predict investment in defense. However, the importance of biological and geographic scale in determining which selective pressures dominate is only beginning to be tested. In this dissertation, I explore the patterns and processes of defense trait evolution in plants, examining whether defense investment across scales matches up with plant defense theory. Using the wild grape genus *Vitis* as my primary study system, I synthesize comparative trait and occurrence data with mechanistic investigations of the processes that shape trait investment. My first data chapter addresses a long-standing hypothesis about correlated defense evolution: that there is a trade-off between direct and indirect defense traits. In this chapter, I use a phylogenetic framework to investigate the relationship between direct defense and mite domatia, an indirect defense trait that is often neglected in studies of plant defenses, discovering a negative interspecific correlation between these defense traits that is consistent with the hypothesized trade-off. My second data chapter expands on the previous chapter's hypothesized patterns of correlated trait evolution by comparing and contrasting how broad trait investment patterns shift from the intraspecific to the interspecific scale. I found that trait-trait correlations are rare across *Vitis* species compared to trait-environment correlations, but that both types of interactions are scale-dependent. These results suggest that defenses in grape largely evolve independent of each other, but that features of the environment are relatively better predictors of defense trait evolution. My third and fourth data chapters center on calcium oxalate crystals in plant leaves, an understudied trait that I use as a model to understand the mechanisms of plant defense evolution. Researchers have long hypothesized that calcium oxalate crystals are defensive against herbivory, but recent literature has challenged this assumption. In one chapter, I address the evolution and defense ecology of these crystals, characterizing how herbivores interact with different types of calcium oxalate crystals and whether the crystals act synergistically with plant chemistry, as well as what environmental conditions lead to calcium oxalate biomineralization. I found that needle-shaped raphide crystals derived from *Vitis riparia* do not impact herbivores, and that soil calcium addition, but not herbivory, induce their production. These results challenge the long-standing hypothesis that raphides operate as a defense against chewing arthropods, and provide evidence that calcium oxalate serve as a dynamic storage system in grape. In my final data chapter, I describe the phylogenetic and geographic distribution of known occurrences of calcium oxalate crystals across the plant kingdom, reporting over 2,000 species that biomineralize calcium oxalate from ~400 peer reviewed publications. I find that crystal morphologies correlate differentially with geographic parameters, suggesting that this morphological variation evolved in response to different ecological pressures. By linking phylogenetic comparative and experimental approaches in tests of theory, my dissertation provides a broader understanding of plant defense evolution in wild grape and beyond.

Chapter 1 : Testing Predictions From Plant Defense Theory

1.1 INTRODUCTION

Species interactions, including mutualism, competition, predation, herbivory, and parasitism, are ubiquitous in nature and are of fundamental importance to evolutionary biology and the study of the generation and maintenance of biodiversity (Hembry and Weber 2020). For example, few evolutionary ecologists would dispute the importance of mutualism in the case of animal-facilitated pollination of plants, and that these interactions have driven divergence in flower color and morphology (Kay and Sargent 2009), and predation is similarly recognized for its strength in driving evolution through adaptations such as crypsis, aposematism, and mimicry (Vamosi 2005; Przeczek et al. 2008). Despite the importance of species interactions for evolutionary research, evidence of species interactions past is hard to come by in the fossil record. Instead, our best clues to the ancient interactions come from observing modern-day patterns in traits and their impacts on interactors. Comparisons across extant relatives in a clade then allow us to then make and test hypotheses about how these traits evolved. Here, I focus on adaptations that allow plants to reduce damage (hereafter defense traits), which result from the selective pressure imposed by herbivory (Futuyma and Agrawal 2009).

Plants and the animals that eat them make up an estimated 80% of the total of biomass on the planet, demonstrating the widespread nature of their interactions (Bar-On et al. 2018). Correspondingly, this multitude of interactions has lead to the evolution of a broad array of plant defensive traits that act in varied ways to discourage herbivory. Plant defense theory predicts myriad patterns of defensive trait evolution based on which strategies are favorable in specific ecological contexts. For example, several broad-scale theoretical frameworks predict how plants balance the threat of herbivory with abiotic pressures, such as the competitive/stress-tolerant/ruderal triangle (CSR triangle; Grime, 1977), and the resource availability hypothesis (Coley et al. 1985). Other hypotheses focus on the costs or benefits of defensive traits in the context of other plant phenotypes, including apparency theory (Feeny 1976), defense syndrome theory (Agrawal and Fishbein 2006), and growth-defense trade-offs (e.g., the growth-differentiation balance framework, Herms & Mattson, 1992). Though these frameworks can help us understand how plants invest in defense, many oversimplify the ways in which defense traits interact with each other and with the broader environment. Additionally, few tests of plant defense theory recognize the importance of scale in maintaining or breaking-down associations between traits. Thus, biologists acknowledge that plant defense theory is rife with conflicting and incomplete predictions that are likely being applied to inappropriate levels of biological organization (Endara and Coley 2011; Hahn and Maron 2016; Agrawal 2020). Experimental and phylogenetic work is thus needed to make sense of the many hypothesized interactions between traits and between traits and the environment (Weber and Agrawal 2012; Agrawal 2020).

Many of our hypotheses about plant defense evolution derive from observations of variation in the world around us, including how plants and insects behave, or how interactions are arranged in the environment. For example, a repeated association between slow growth/high investment in defense plant phenotypes and resource-limited environments may lead authors to propose that there is a causality driving this association. Thus, trait-trait and trait-environment associations can allow us to test hypotheses about the adaptive value of traits. The availability of large-scale datasets, combined with innovations in statistical methods for testing for correlations across taxa now means that many of our hypotheses about plant defense evolution can be tested by looking for repeated associations between traits or traits and environments. Thus, I study plants' adaptation to herbivory by combining experimental manipulations of herbivores with trait quantifications across clades, using contemporary trait and interaction data to infer the processes that have shaped defense expression.

1.1.1 Trade-offs, synergisms, and trait-trait interactions

Trade-offs and synergisms are the foundation for many predicted patterns of trait expression across phylogenies, though trait-trait correlations must be interpreted with care, as many mechanisms can result in correlated evolution. Trade-off patterns (i.e. negative correlations between traits) can occur when investment in one defense strategy precludes investment in another, which might be the case when multiple traits that are selected for cannot be co-expressed because of a limiting resource, meaning that fitness cannot be maximized (Agrawal et al. 2010; Agrawal 2020). As a result, trade-offs between traits rely on the assumption that defense traits are costly, and that the resources that a plant can invest in traits are limited (Rehr et al. 1973; Herms and Mattson 1992; Mole 1994). Negative trait correlations may also result from adaptation (Agrawal 2020). Adaptive negative correlations between traits may be predicted when defenses operate against the same herbivores, and thus are redundant, or when the two defense traits are ecologically incompatible. For example, in situations where a plant has both direct defenses (i.e., defenses that directly impact herbivores, like toxic metabolites or trichomes) and indirect defenses (i.e., defenses that recruit mutualist defenders, such as extrafloral nectaries or ant domatia), ant mutualists could be poisoned by secondary metabolites leaking into extrafloral nectar, or larger predatory mite mutualists may be impeded by dense trichomes on leaf surfaces (e.g. Adler, 2000; Krips et al., 1999; Strauss et al., 2002). Negative correlations between defense traits thus have a strong theoretical basis, but evidence supporting their presence and the mechanisms behind them is limited (see meta-analyses by Koricheva et al., 2004; Moles et al., 2013).

If two traits enhance rather than interfere with each other's function, we can expect to see positive correlations between them through evolutionary time (i.e. across multiple tips of a phylogenetic tree) because selection has favored their co-occurrence (Berenbaum and Neal 1985; Rasmann and Agrawal 2009). Synergisms between defense traits are predicted to result in a reduction in herbivory that is greater than the impacts of either of the defensive traits on their own. For example, Konno et al. (2014) found that a mechanical defense and a chemical defense worked in concert to reduce the growth and increase the mortality of a lepidopteran herbivore. Positive associations between traits may also be predicted in ecological or environmental contexts where multiple types of herbivore pressure are present, such as in geographic regions where herbivore pressure is particularly high and diverse (e.g. the tropics, Schemske et al., 2009). Positive evolutionary correlations have been experimentally demonstrated between a variety of paired defense (Berenbaum and Neal 1985; Clauss et al. 2006; Weber et al. 2012, 2016; Konno et al. 2014; Richards et al. 2016).

Plant defense theory has also sought to blend observations of correlated traits into the concept of syndromes, which are typically defined as suites of co-occurring resistance or tolerance traits across species that result from shared ecological interactions (Agrawal and Fishbein 2006). Evidence for syndromes has been observed for select species, or even within genera or families, but less so across the plant kingdom. Indeed, despite decades of concentrated research into when and where suites of defense traits should be adaptive, few patterns observed within a particular plant clade are broadly applicable across all plants or environments (Moles et al. 2013). These inconsistent patterns highlight the need for more phylogenetically-informed empirical studies that can examine variation in defense trait investment across the plant kingdom. Ultimately, investigations of correlations between traits will allow the scientific community to identify potential drivers of defense trait evolution (Agrawal 2020).

1.1.2 The role of biotic and abiotic factors in the evolution of plant defense

Plant defense theories must incorporate an understanding that defense is but one energetic expenditure for plants that needs to be balanced with growth and reproduction. Thus, the expression of multiple defense traits should only be adaptive in certain abiotic or biotic conditions. For example, the study of resistance and tolerance to herbivory in plants is frequently evaluated in the context of resource availability (the resource availability hypothesis, RAH; Coley et al. 1985; Endara and Coley 2011). It has been broadly observed that highly defended species frequently originate from resource-stressed environments (Hahn and Maron 2016), an example of which is the high spinescence demonstrated by plants in desert environments. We might also expect the seasonality of an environment to indirectly impact the defense investment of plants by determining the period of time that a plant species is exposed to herbivory (Karban 2007; Pearse and Hipp 2012). It is important to note that patterns in any given resource or environmental variable do not necessarily correspond to patterns in other resources. For example, plants in the desert have seemingly unlimited sunlight access but low water and nutrient access, while plants in tropical environments may have abundant water, but low nutrients and high competition for sun.

Biotic and abiotic interactions are inexorably linked, where the effects of environment on defense evolution can be indirectly mediated by the impacts of environment on herbivory pressure. This interconnectedness of abiotic habitat variables and biotic herbivory pressure makes it difficult to tease apart the impacts of climate and herbivores on plant defense investment. Indeed, biotic pressure is predicted to vary along the same gradients as climate in many cases, with higher abundances of more diverse arthropod herbivores in the tropics (Mittelbach et al. 2007; Schemske et al. 2009; Baskett and Schemske 2018), and more resource-rich habitats (Hahn and Maron 2016). The extent to which these patterns of herbivore abundance, rates of herbivory, and plant defenses themselves are generalizable is a subject of debate in the plant defense community (Moles et al. 2013; Anstett et al. 2016). Further, relationships between defensive traits and environmental variables can be additionally complicated if phenotypes have multiple functions. For example, some physical traits that influence herbivory, such as specific leaf area, leaf toughness, and trichomes, are also implicated in water retention and UV protection, and thus their expression may be correlated with water availability or sun exposure in addition to or instead of herbivory (Onoda et al. 2011; Karabourniotis et al. 2020b). Overall, and the relative importance of environmental interactions to plant defense evolution is not well understood, and additional studies are needed that explore the ecological context of defense investment.

1.2 OVERVIEW OF CHAPTERS

In this dissertation, I explore the patterns, processes, and scale-dependency of defense trait evolution to evaluate hypotheses for how defenses evolve relative to each other and the environment. I do this by pairing common garden experiments, trait quantifications, occurrence data, and literature surveys (chapters 2, 3, and 5) with mechanistic investigations (chapter 4). In three of my dissertation chapters, I use the agriculturally and economically important *Vitis* genus of wild grapes as my study system due to its wide range of defensive phenotypes and well-characterized and accessible germplasm resources (Ma et al. 2018; Singh and Acevedo 2024). *Vitis* consists of approximately 60 to 70 accepted species in two subgenera, *Muscadinia* (four species) and *Vitis* (around 60 species), and there are two major hotspots of grape diversity: North America and east Asia (Moore 1991; Péros et al. 2023). *Vitis vinifera ssp. sylvestris*, the wine grape, is the only grape species native to Europe, and has been cultivated profusely. *Vitis* vines are woody, deciduous perennials that are frequently grown from hardwood cuttings, and suffer damage from a range of arthropod herbivores and fungal pathogens (Gerrath et al. 2015; Singh and Acevedo 2024).

In chapter two, I test a classic hypothesis about the patterns of plant defense evolution: a trade-off between direct and indirect defense. In 1966, Janzen observed that plants that did not have ant bodyguards were more bitter to the palate than congeners with ants, and hypothesized that a trade-off in defense strategies was responsible for this pattern. This simple observation spurred decades of research trying to retrieve this pattern across and within a variety of taxa, finding inconsistent evidence of a negative correlation between chemical defense and indirect defense traits such as extrafloral nectaries, ant-domatia, and volatiles (Rehr et al. 1973; Steward and Keeler 1988; Heil et al. 2000, 2002; Dyer et al. 2001; Eck et al. 2001; Ballhorn et al. 2008). Very few of these studies investigated these patterns across species, and those that did largely failed to account for phylogeny in their analyses (but see Rudgers et al., 2004). My work is the most comprehensive analysis of interspecific patterns between direct and indirect traits within a clade, capitalizing on advances in phylogenetic comparative methods to test for negative correlations between direct defense in the form of secondary metabolism and trichomes and indirect defense in the form of mite domatia across 33 *Vitis* species. I did detect a negative evolutionary correlation between domatia and secondary metabolite diversity in *Vitis* leaf tissue consistent with a trade-off between these defense strategies, which implies an as-of-yet untested trait-trait ecological conflict or physiological constraint.

In chapter three, I identify whether patterns of trait evolution in *Vitis* persist or break down across biological scales. Evolutionary trait correlations have the potential to shift dramatically across scales (e.g., within vs across species), and can reveal the underlying causes and consequences of trait interactions because the types of forces that are likely to dominate in shaping defense evolution and expression may vary. For example, evolutionary correlations between traits may be masked at the intraspecific level due to plasticity in those traits, a phenomenon that is unlikely to play as large a role in trait variation at the species level (Agrawal 2020). Conversely, resource allocation trade-offs that result in negative correlations between defense strategies within a species may be ameliorated by variation in resource acquisition across species (van Noordwijk and de Jong 1986; Agrawal and Hastings 2019). Despite this compelling theory, little work has examined how predicted relationships from plant defense theory translate across biological and environmental scales. In this chapter, I conducted a multi-scale study of trait correlations between plant defenses by quantifying a large variety of defense-related traits both across and within *Vitis* species. I found that strong correlations between defense traits, and between defenses and environmental variables, are rare, and, when present, do shift across scales, challenging our assumptions about the appropriateness of the application of plant defense theory to all evolutionary timescales. In general, correlations that are likely related to specialization in defensive strategies, such as physical vs chemical defense were observed at the interspecific scale, while physiological constraint dictates trait-trait correlations within species. At both scales, variation in environmental variables is a relatively better determinant of defense-trait investment for *Vitis* vines, implying that defense traits have evolved independently of each other in this clade.

In chapter four, I narrow my scope to an experimental investigation of the impacts of a putatively defensive trait on herbivores, and the environmental stimuli that induce the formation of said trait. Leaves of *Vitis riparia* vines have both bundles of needle-like calcium oxalate crystals, termed raphides, and smaller more organically shaped crystals, termed druses. Proposed functions of these crystals include from storage of excess calcium, storage of CO_2 in cases of water stress, and defense against chewing herbivores (Franceschi and Nakata 2005; Karabourniotis et al. 2020a; Khan et al. 2023). However, experimental evidence of the function of calcium oxalate crystals in *Vitis* specifically is absent in the literature, and the defensive function of calcium oxalate crystals in general has recently come under fire (Paiva 2021). Ultimately, the significant debate about the role of calcium oxalate in plants can only be addressed through carefully designed experimental manipulations of the ecological pressures imposed by and on these crystals. In a laboratory study manipulating herbivore diet as well as a greenhouse study manipulating soil calcium and simulated herbivory, I found no support for the hypothesis that *Vitis riparia* calcium oxalate crystals function in plant defense, but support for the hypothesis that their production is induced by soil calcium levels. This work adds to the growing body of knowledge about the ecology of calcium oxalate crystals in plants, challenges their roll in mediating plant-insect interactions, and suggests that calcium oxalate crystals are formed passively in response to abiotic environmental factors in this taxon.

In chapter five, I expand beyond *Vitis* to gain insight into the ecology and evolution of calcium oxalate crystals by describing their phylogenetic and geographic extent in plants. Crystalline forms of calcium oxalate within leaves are widespread trait across the plant kingdom and diverse in form (McNair 1932; Franceschi and Horner 1980; Lawrie et al. 2023). Despite their wide distribution and the potential for this trait to mediate ecological interactions, the evolution of calcium oxalate crystals has been understudied. In order to study their evolution, we need to know when and where they occur in plants, both geographically and phylogenetically. In this chapter, I systematically survey the current literature on the presence and morphology of calcium oxalate crystals in taxa across the plant kingdom, and use modern advances in phylogenetic comparative methods to reveal patterns in the distribution of these structures across phylogenetic and geographic scales. I found that calcium oxalate crystals are found in taxa from at least half of all recognized plant families, and that different crystal morphologies are correlated with different geographic gradients. In particular, this work suggests that calcium oxalate crystal morphologies have evolved in response to different selection pressures, including herbivory and drought stress.

Finally, in my concluding remarks, I enumerate some of the remaining gaps in our understanding of plant defense across scales and the evolution and ecology of calcium oxalate crystals, with recommendations for specific work that should be undertaken. I observe that trait-trait correlations seem to be the exception and not the rule for the evolution of defense in *Vitis* and that latitude is a relatively better determinant of investment in specific defense traits, especially at the interspecific level. In my later chapters I conclude that calcium oxalate likely plays different roles in different plant taxa, and that these roles may be determined by crystal morphology. My dissertation synthesizes theory from the last several decades to understand the evolution of often-overlooked traits that mediate plant-insect interactions, and both tests and generates hypotheses about the drivers of defense diversity.

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Chapter 2 : Evolutionary Signatures of a Trade-Off in Direct and Indirect Defenses Across the Wild Grape Genus *Vitis*

2.1 ABSTRACT

Evolutionary correlations between chemical defense and protection by mutualist bodyguards have been long predicted, but tests of these pattern 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 correlate 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.

2.2 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 (Rhoads and Cates 1976; Agrawal et al. 2010; Agrawal 2020). 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 (Felsenstein 1985; Agrawal 2006). While many evolutionary trade-offs and synergisms between traits are hypothesized in evolutionary ecology, many trait combinations have yet to be tested at the 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 and 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 an 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 defend a plant (Riddick and 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 (Campbell and Duffey 1979; Agrawal et al. 2002). 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 and Fishbein 2006; Webber and 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 and 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 and 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 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 and 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 (Schemske et al. 2009; Baskett and Schemske 2018).

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 (Letourneau and Barbosa 1999; Heil et al. 2000, 2002; Dyer et al. 2001; English-Loeb and Norton 2006; Ballhorn et al. 2008). Though this work 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 work, 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 which 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 (1) 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*? And, (2) do abiotic conditions predict evolutionary investment in certain types of defensive strategies?

2.3 METHODS

2.3.1 Study design

We sampled defensive phenotypes of 33 *Vitis* species grown in pre-established vineyards outside of Davis, California (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.

2.3.2 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 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 (Grostal and O’Dowd 1994; Karban et al. 1995; English-Loeb et al. 2002). We measured domatia size and hair density on dried, pressed *Vitis* leaves (185 plants x 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 locations of the domatia on the leaf, Figure 2.1A). Domatia hair density scores were assigned on a scale of 0 to 9, with 0 representing no hairs present in the vein axil and 9 being a densely packed, full domatium (Figure 2.2). 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).

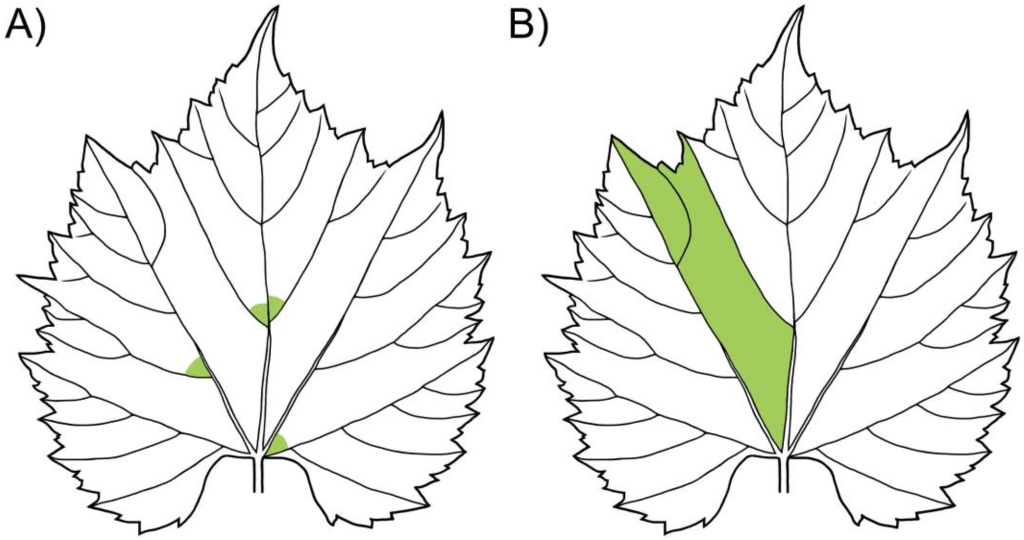

Figure 2.1 Physical defense phenotype sampling method for *Vitis* leaves, demonstrating A.) the locations of domatia measured on each leaf and B.) the region of the lower leaf surface scanned for trichome density. Methods were adapted from (Loughner et al. 2008).

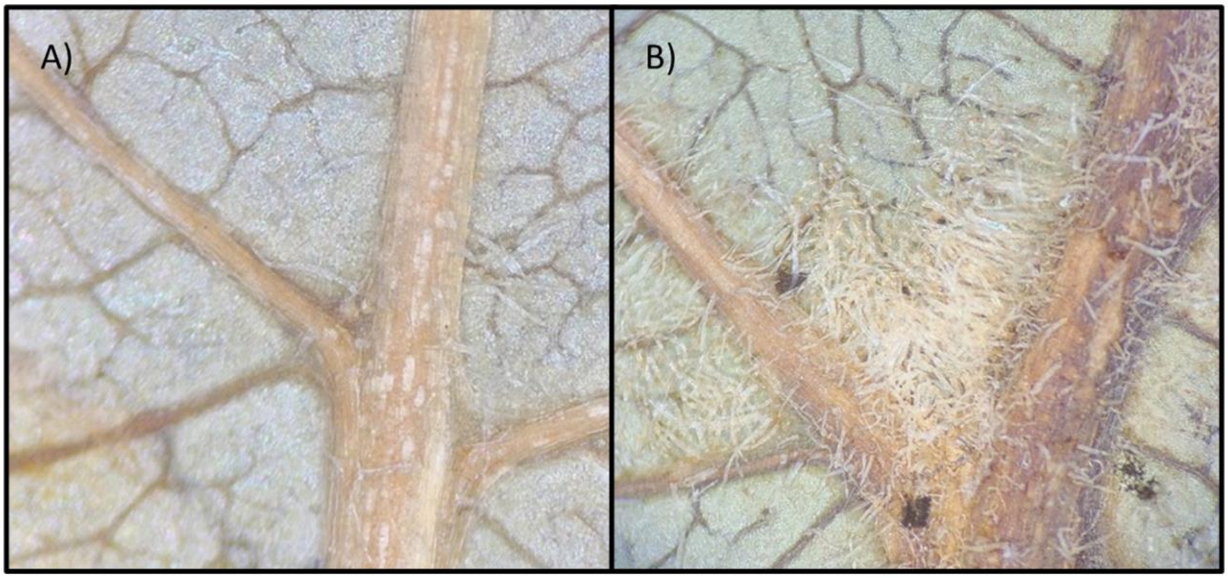

Figure 2.2 Range of domatia densities in the common garden *Vitis* leaf samples. A) Domatium assigned a density of 1 (almost no hairs, not overlapping) from *V. arizonica*. B) Domatium assigned a density of 9 (completely full domatium) from *V. vulpina*.

2.3.3 Direct defense

For direct defense, we quantified chemical diversity and trichome density. While a large body of evidence has linked trichomes (Levin 1973; Carmona et al. 2011; Bar and Shtein 2019) and secondary chemistry (Rhoads and Cates 1976; Hartmann 2007) to herbivore deterrence in a variety of systems, both traits play multiple roles in plant physiology and adaptation (Karabourniotis et al. 2020c). 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 dissection microscope using the O-084/U-33 nine-point scale, remaining consistent with the region sampled for each leaf (Figure 2.1B, 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 have 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. 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 leaf the 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 hours. 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.

LCMS 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 x 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 start of 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 an full MS/all-ion fragmentation (AIF) method with a scan range of m/z 100-1500 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 minutes 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 as well as 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 (Wetzel and Whitehead 2020; Salgado et al. 2023). We calculated species averages by taking the mean richness, total abundance, and Shannon diversity values per species.

To filter our LCMS dataset of unidentified peaks to putatively defensive secondary metabolites, we used relative mass defect (RMD) filtering. RMD filtering is a method for categorizing compound peaks in large LCMS datasets 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 ppm values. Before performing any analyses on our chemical data, we dropped all compounds with RMD values above 1200 so as 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 3rd instar larva for a period of seven 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 hour photoperiod for the duration of the palatability trials. We scanned remaining leaf tissue at the end of the seven 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 seven 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, Rasband 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.

2.3.4 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, 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 and 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.

2.3.5 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 6th, 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 and 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. Non-overlapping 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 100bp. We ensured sequences were in the correct orientation using the Adjust_Direction.py script. Multiple sequence alignment was conducted using MAFFT (Katoh and 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 (AIC) through the R package *phanghorn* (Schliep 2011). Resulting best model fits are reported in Appendix Table A.1.

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 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 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 post-burnin 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 2.3 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).

2.3.6 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 controlling for non-independence due to phylogenetic relatedness (Revell and 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).

2.4 RESULTS

2.4.1 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 max of 12.2 for *V. vulpina*, which had large, full domatia (Appendix Figure A.1). *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, 1,969 of which 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 respectively. The Shannon diversity of metabolites was highest in *V. labrusca* and lowest in *V. riparia* (Appendix Figure A.2).

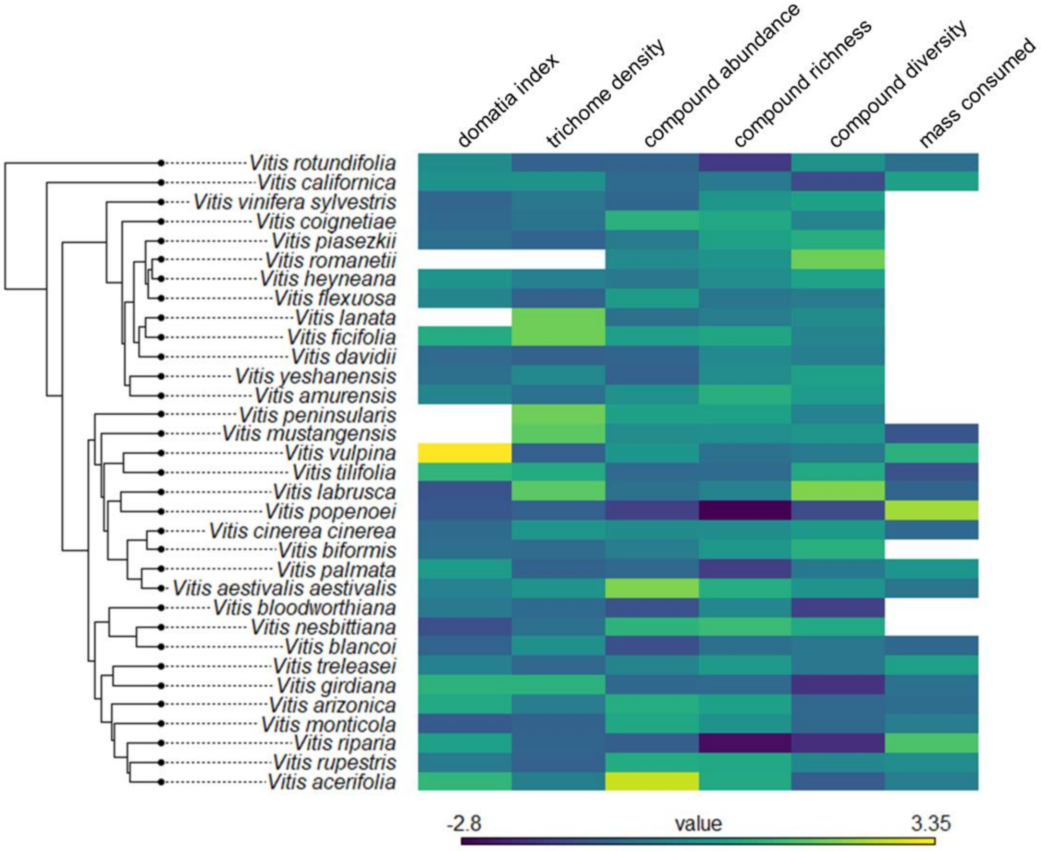

Figure 2.3 Maximum clade credibility tree of *Vitis* species investigated in this study, accompanied by standardized mean trait values for six defense related metrics across *Vitis* species. White space represents traits for which data could not be collected for a given *Vitis* species.

2.4.2 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 2.4B,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 2.4A). The results of these analyses are summarized in Table 2.1.

Additionally, there was a strongly negative correlation between trichome density on the lower leaf surface and the mass of grape leaf tissue consumed (p=0.00014, Appendix Figure A.3b).

Table 2.1 Results of phylogenetic 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	-	0.216
Palatability ~ trichome density	-0.00297 ± 0.00064	<0.001***
Palatability ~ chemical abundance	-	0.111
Palatability ~ chemical richness	$-4.7\times10^{-5} \pm 1.3\times10^{-5}$	0.002**
Palatability ~ shannon diversity	-0.0485 ± 0.0124	0.001**

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 metabolites in all three RMD classes correlated significantly with the mass of leaf tissue consumed by the herbivores (Appendix Tables A.2, A.3, and A.4). Interestingly, for peaks in the 100-250ppm RMD range typical of flavonoids, this correlation was positive, while the other two RMD ranges, the correlation was negative (Supplementary Table A.3). Peak diversity in the terpenoid and phenolic RMD ranges were also negatively correlated with mass of leaf tissue consumed (Supplementary Table A.2 and A.4).

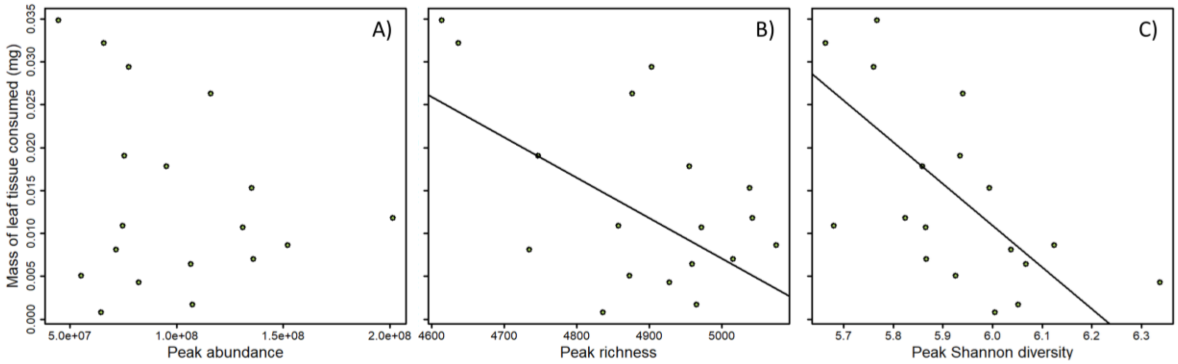

Figure 2.4 Relationships between our LCMS chemistry results and the results of the herbivory assays using *S. exigua* in our common gardens. The average mass of leaf tissue consumed in grams per *Vitis* species plotted against (A) the average abundance of metabolite peaks in tissue extracts, (B) the average richness of peaks, and (C) the average Shannon diversity of peaks. Significant trendlines from PGLS models are shown (alpha = 0.05).

2.4.3 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=0.0237, Figure 2.5), 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 nor richness of compounds using PGLS models.

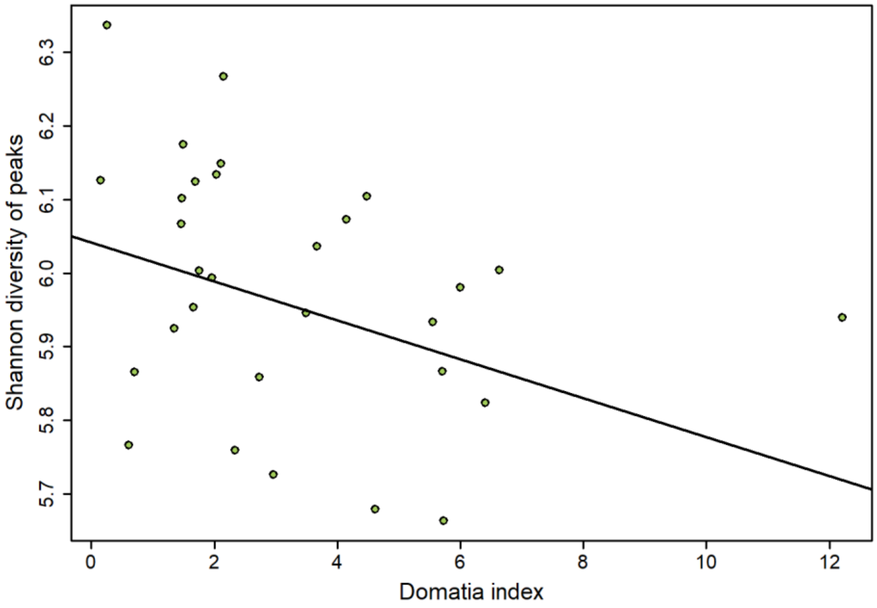

Figure 2.5 Negative correlation between the domatia index, a measure of indirect defense, and the Shannon diversity of peaks, a measure of direct defense, in grape leaves. Each point represents the mean for a *Vitis* species. Significant trendline from the PGLS model is shown (p=0.0237).

Because plant defense theory predicts the existence of suites of coexpressed defense traits that act together to reduce herbivory (Agrawal and 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=0.0115), 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 evolve 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 datasets filtered down to specific metabolites classes. RMD filtering narrowed our chemical peaks down to 394 compounds that fit into the 100-250ppm RMD range typical of flavonoids, 977 in the 200-400ppm 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=0.0212; Appendix Table A.2), 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=0.0437; Appendix Table A.3), 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 correlated negatively with trichome density (p=0.00597; Appendix Table A.3), while compounds in the range of neither terpenoids nor phenolics showed any significant relationship with trichomes.

2.4.4 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 least variability in temperature throughout the year. The most northern species in the dataset 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* which 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 *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 dataset (Appendix Table A.5). The only significant evolutionary correlations between abiotic variables and defense traits were between overall latitude and chemical abundance (p=0.0446) and temperature seasonality and chemical abundance (p=0.0332; Appendix Table A.5). Both of these correlations were positive, with the average abundance of peaks in the *Vitis* species increasing with latitude and temperature seasonality.

2.5 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. 1) 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, 2) 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, 3) 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.

2.5.1 Evolutionary trade-off among direct and indirect defense

This paper 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 paper 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 non-comparative methods and found mixed results (e.g. Rehr et al. 1973; Letourneau and Barbosa 1999; Heil et al. 2000, 2002; Dyer et al. 2001; Eck et al. 2001). 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*. Further, 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 (Steward and Keeler 1988, Romeo et al. 1996). 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). Further, 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 and 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 (Vásquez et al. 2008; Ma et al. 2014), 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.

2.5.2 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 chemical defense, domatia, nor trichomes 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 (Moles et al. 2011; Anstett et al. 2016). 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 and Coley 2011a; Pearse and 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 do 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.

2.5.3 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. Richards et al. 2015; Glassmire et al. 2019; 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 and 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 and 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; Jones and Firn 1991; Carmona et al. 2011; Wetzel and 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 and 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 and 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 and 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 3rd 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 (Grostal and O’Dowd 1994; Agrawal and Karban 1997; 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 decreases with increasing trichome density, albeit not in grape (Krips et al. 1999). However, the absence of a negative correlation between domatia and trichomes on grapes does not support this hypothesized no-mutualist defense syndrome.

2.6 CONCLUSIONS

Since first proposed in the 60s, the direct-indirect trade-off 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, nor 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.

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Chapter 3 : Evolutionary Trajectories of Multiple Defense Traits Across Phylogenetic and Geographic Scales in *Vitis*

3.1 ABSTRACT

The processes driving defense trait correlations may vary within and between species based on ecological or environmental contexts. However, most studies of plant defense theory fail to address this potential for shifts in trait correlations across scales. In this work, we tested for correlations between multiple defensive traits (secondary chemistry, carbon to nitrogen ratio, domatia, leaf toughness, trichomes, and pearl bodies) across a common garden of twenty-one *Vitis* species and eighteen genotypes of the species *Vitis riparia* to identify when and where patterns of defense trait evolution persist or break down across biological scales. Additionally, we asked whether *Vitis* defense trait investment correlates with environmental variables as predicted by plant defense theory, using environmental metrics for each *Vitis* species and *V. riparia* genotype from the GBIF and WorldClim databases. We tested for correlations between defense trait investment, herbivore palatability, and environmental variables using phylogenetically informed models. Beyond a few likely physiological exceptions, we observed a lack of correlations between defense traits at both intra- and interspecific scales, indicating that these traits evolve independently of each other in *Vitis* rather than forming predictable defense syndromes. We did find that investment in carbon:nitrogen (at both scales) and pearl bodies increases with proximity to the equator, demonstrating support for plant defense theory's prediction of higher investment in defenses at more equatorial environments for some, but not all, defense traits. Overall, our results challenge commonly held hypotheses about plant defense

evolution, namely the concept of syndromes, by demonstrating that strong correlations between defense traits are not the prevailing pattern both across and within *Vitis* species. Our work also provides the first comprehensive evaluation of the evolutionary divergence in approaches that *Vitis*, a genus with significant agricultural value, have evolved to defend themselves against herbivores.

3.2 INTRODUCTION

Plants display an impressive array of traits to reduce or deter herbivory, producing sophisticated defense arsenals that make up for their lack of mobility to escape damage. There are many hypothesized drivers of this stunning diversity of defense traits, but plant defense theory generally neglects to predict what drivers of diversity dominate at different levels of biological scale (i.e. within versus across plant species). Recent theory has proposed that the processes that shape trait correlations are likely to shift across environmental, spatial, and temporal sample scales, thereby influencing what patterns we detect and ultimately the conclusions we come to about the drivers of trait divergence when we look only within *or* across species (Laughlin et al. 2017; Agrawal 2020; Zhou et al. 2022). For example, a given resource may be limiting at the within species scale, manifesting as a negative correlation between traits, while that same resource may be more available at the between species scale, thereby attenuating the negative trait correlation (Hahn and Maron 2016; Zhou et al. 2022). Thus, we may see different evolutionary correlations between the same defense traits depending on whether we are comparing individuals within a species versus between species.

Theory about the drivers of defense variation often focuses on aspects of the plants' biology that are predicted to impact selection on defenses. For example, plants are hypothesized to specialize in certain defense strategies or traits at the expense of others, either as the result of resource-based trade-offs, which prevent the simultaneous expression of two traits with the same metabolic precursor(s) (Agrawal et al. 2010; Agrawal 2020) or due to an ecological conflict between the traits (Strauss et al. 2002). For example, it might be disadvantageous to provide extrafloral nectar to mutualist arthropods if the toxic metabolites within a plant leak into that nectar and potentially poison the defenders. There are also many reasons to expect there to be positive correlations between defenses, oftentimes termed “syndromes” (Kursar and Coley 2003; Agrawal and Fishbein 2006). Syndromes might occur when traits interact synergistically to provide defense (Berenbaum and Neal 1985; Rasmann and Agrawal 2009), are genetically or developmentally linked, or in situations where plants are subject to damage from a range of generalist and specialist herbivores (e.g. in the tropics, Schemske et al. 2009) resulting in multiple seemingly-redundant defense traits being advantageous.

Geographic patterns in environmental variables are also hypothesized drivers of defense trait evolution, although these patterns are also rarely examined across scales. For example, one prominent theory, sometimes referred to as the latitudinal herbivory defense hypothesis (LHDH), predicts that plants invest more in defenses closer to the equator because the tropics are diversity hotspots with high levels of herbivory pressure (Coley and Aide 1991; Coley and Barone 1996; Anstett et al. 2016). Equatorial climes also have longer growing seasons, lengthening the amount of time that plants are threatened by arthropod herbivores. Resource availability may also influence the evolution of defense traits in plants. Plants are often observed to be highly defended against herbivory in resource-poor environments, a pattern hypothesized to result from selection for plants to protect hard-won resources in their relatively expensive-to-produce photosynthetic tissue (the resource availability hypothesis (RAH); Coley et al. 1985; Endara and

Coley 2011). While mean environmental temperature and growing season length typically vary closely with latitude, resource availability may not. Thus, the LHDH and the RAH may produce conflicting patterns of defense investment, such as in arid environments far from the equator where the growing seasons are short (suggesting that defense investment should be low) but nutrients are limited (suggesting that defense investment should be high; Coley et al. 1985).

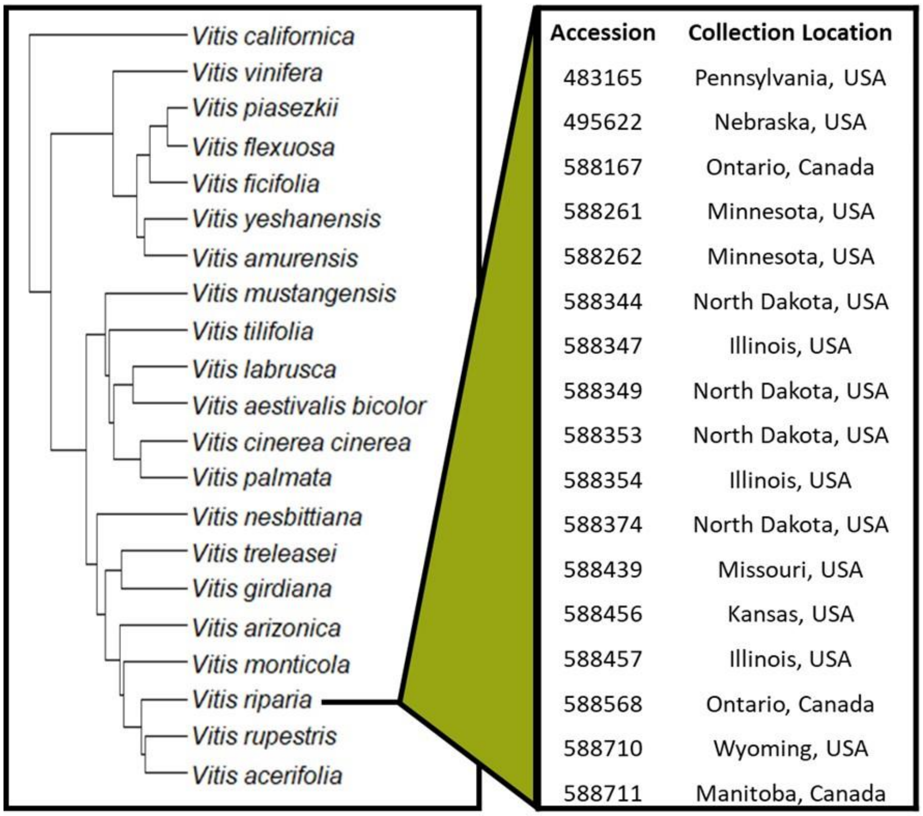
Although rare, several studies have tested whether the theorized correlations between traits, or between traits and environmental variables, persist or break-down across levels of biological organization. In a 2012 meta-analysis of papers measuring correlations between direct and indirect defenses in plants that participate in defensive mutualisms using ants, Koricheva and Romero observed a negative correlation between the defense strategies that persisted both within and across species. In contrast, broad support has been found for the aforementioned resource availability at the interspecific level, but less so within species (Hahn and Maron 2016). Finally, Agrawal and Hastings (2019) found that a trade-off between constitutive and induced chemical defenses is apparent within individual *Asclepias* species, but breaks down when sampling across species. These observations emphasize that the scale at which we choose to evaluate a pattern can give us different ideas of what factors shape the evolution of defense traits. Studies that address the scale-dependency of trait-trait and trait-environment correlations require thorough phenotyping of multiple traits across many species and individuals to comprehensively quantify defensive phenotypes in a plant group. As a result, few tests of plant defense theory test scale as an influence on the drivers that maintain or break-down associations between traits.

In this study, we aim to evaluate trait-trait and trait-environment correlations between an array of defense traits and environmental variables at both the inter- and intraspecific scale to explicitly test the scale-dependency of plant-defense syndromes. We do this using wild grape genus *Vitis* as our study system. *Vitis* is a charismatic, agriculturally important clade of about sixty species of woody vines, native to North America, Europe, and Asia (Moore 1991; Zhiduan et al. 2007; Aradhya et al. 2013; Gerrath et al. 2015; Péros et al. 2023). *Vitis* vines have a wide range of traits thought to be directly and indirectly defensive against herbivores, making them an excellent system in which to investigate correlated defense trait evolution (Appendix Figure B.1). We specifically ask at both the interspecific and intraspecific level (1) whether traits are correlated (positively or negatively) with one another, and (2) whether features of the abiotic environment predict defense trait investment. We do this by synthesizing defense trait quantifications, species occurrence data, climate data, and herbivore bioassays to achieve a holistic picture of defense in *Vitis* species, and ultimately gain insight into the evolutionary forces that are driving the evolution of *Vitis* defense traits at different levels of biological organization.

3.3 METHODS

3.3.1 Experimental plants

We collected defense trait data on grapevines grown in a greenhouse common-garden. A common garden holds the environment constant, meaning that phenotypic variation between plants grown in that common environment can be assumed to be produced by genotypic variation between the plants. We attempted to grow all *Vitis* species available from the USDA-ARS germplasm network, which we rooted as cuttings in the spring of 2021 (across genus vines) and 2022 (*V. riparia* vines). Two of the species in our data (*V. vinifera* and *V. rupestris*) are noted in the germplasm database as having been donated as cultivated material, while all others are either specified as collected as wild material or do not have this information available. The breadth of species of *Vitis* used in this study represent those we were able to obtain and root successfully (approximately one third of the ~60 species in the *Vitis* genus), and are thus biased towards species native to North America. However, the species in our dataset are representative of much of the latitudinal range of *Vitis*, and are native to the hotspots of *Vitis* diversity in North America and East Asia (Moore 1991; Zhiduan et al. 2007). We quantified defense metrics across twenty-one species of *Vitis* (1-6 individuals per species, mean = 4.38; all of the same genotype) and eighteen *V. riparia* genotypes (4-5 individuals per *V. riparia* genotype, mean = 4.94; Fig 3.1). All traits were collected on mature leaves, and, unless otherwise noted, we collected the trait data on a consistent area on the underside of each leaf adjacent to the midvein.



Accession	**Collection Location**
483165	Pennsylvania, USA
495622	Nebraska, USA
588167	Ontario, Canada
588261	Minnesota, USA
588262	Minnesota, USA
588344	North Dakota, USA
588347	Illinois, USA
588349	North Dakota, USA
588353	North Dakota, USA
588354	Illinois, USA
588374	North Dakota, USA
588439	Missouri, USA
588456	Kansas, USA
588457	Illinois, USA
588568	Ontario, Canada
588710	Wyoming, USA
588711	Manitoba, Canada

Figure 3.1 *Vitis* species (left) and *V. riparia* genotypes (right) that we sampled to characterize multi-trait defense arsenals

3.3.2 Metrics of plant defense

Chemistry - Numerous studies have demonstrated the negative impacts of specialized metabolites on herbivores by poisoning them, slowing their growth, or encouraging them to eat elsewhere (Levin 1976; Mithöfer and Boland 2012). We quantified grape secondary metabolism using liquid chromatography mass spectrometry (LCMS) of leaf extracts. We collected leaf punches from five mature leaves per experimental plant and pooled them so that we had one sample per plant that would capture a broader picture of the plant's metabolism. We immediately submerged the leaf punches in an extraction solution made up of isopropanol:acetonitrile:water 3:3:2 containing 0.1% formic acid and 104 nM telmisartan as an internal standard. We soaked the leaf material overnight, and then separated the extracts from the leaf tissue and stored the extracts at -20˚C until analysis. We dried the remaining leaf tissue and measured its dry mass so we could standardize the quantity of metabolites per mass of leaf tissue.

We analyzed the extracts (diluted 1:1 in ultrapure water) at the Michigan State University Mass Spectrometry and Metabolomics Core Facility using the facility's Thermo Q-Exactive mass spectrometer and Thermo Vanquish Flex UHPLC system with a Waters BEH-C18 reverse phase column. We used the same instrumentation and analysis parameters as reported in (Graham et al. 2023). The result of this analysis was raw peak data for each analyzed sample. We standardized all peak areas by subtracting out the peak areas of the averaged blanks, normalizing to the telmisartan peak, and dividing by the mass of the leaf tissue extracted. Additionally, we used the relative mass defects of the detected compounds to remove compounds with defects of over 1,200, which are typically inorganic ions not of biological origin (Ekanayaka et al. 2015; Graham et al. 2023). Finally, we summarized the metabolic profiles of the samples in two ways: metabolite abundance (sum of the peak areas of all compounds in the sample), and metabolite richness (count of unique peaks in the samples).

Carbon-to-nitrogen ratio - We use C:N as a proxy for leaf nutritional content, following plant defense literature which finds that leaves that are higher in nitrogen (and thus have a lower C:N ratio) are more nutritious and thus more palatable to herbivores (Mattson 1980; Agrawal and Fishbein 2006). We collected and dried one leaf per experimental plant in a drying oven for three days at 45°C. We then ground the dried leaf material into a smooth powder using a HG-600 Geno/Grinder® 2010 (Cole-Palmer, Metuchen, NJ, USA) and packaged approximately 2 to 5 mg of the samples in tin foil capsules. We used an ECS 4010 - CHNS-O Elemental Combustion system (Costech Analytical Technologies, Inc., Valenica, CA, USA) to determine the carbon and nitrogen content per mass of sample for each species and *V. riparia* genotype. We used atropine as a standard with a known carbon and nitrogen content. The carbon to nitrogen ratio within the samples was calculated using the percent carbon and nitrogen contents supplied by the chromatograms, and the mass of the sample combusted.

Domatia **-** Domatia are plant-formed structures that shelter mutualistic mites, who in turn patrol the leaf surface eating fungus and microarthropods, thus serving as an indirect defense (Agrawal and Karban 1997; Norton et al. 2000, 2001; Romero and Benson 2004, 2005). In *Vitis,* these structures are formed out of leaf hairs that cluster in vein junctions creating a “canopy” mites can nestle into and lay their eggs within. We scored size and hair density of four domatia on three dried, pressed leaves per experimental plant under a dissecting microscope. For size, we measured the radius of each domatium using a micrometer and scored the density of the hairs within the domatium on a nine point scale adapted from the OIV code O-085/U-33 scale used by plant breeders to assess leaf hair density (IPGRI et al. 1997). On our scale, a domatium density of zero represents a junction between veins that is devoid of hairs, while a score of nine represents a dense, full domatium with no leaf surface visible underneath. For leaf undersides that were fully pubescent, we were unable to assign a domatium radius measurement, as there is no discernable difference between domatium and laminar trichome cover. For other species, we multiplied the radius and the density score together to get a metric we term “domatia index” (for figures showing the measurement strategy and more detail on the ranking system used, see Graham et al., 2023). Previous literature has established that domatia index is a strong predictor of mutualistic mite abundance, and thus the strength of the mutualistic defense, on grape leaf surfaces (Loughner et al. 2008).

Toughness - Leaf toughness is frequently cited in the literature as a direct defense against herbivory (e.g. Coley, 1983; Malishev & Sanson, 2015; Salgado-Luarte et al., 2023). To quantify the toughness of the leaves, we punctured three leaves per plant with a force gauge penetrometer (Wagner, Greenwich, CT, USA). Because there was high noise in this toughness data we collected five measurements per leaf.

Trichomes – Trichomes are hair-like projections on the surface of plant tissue, which are hypothesized to serve a variety of purposes for plants, one of the most prominent of which is as a direct defense against chewing herbivores (Fig 2 Figure S1c; Levin 1973; Karabourniotis et al. 2020). For three dried, pressed leaves per plant, we characterized the density of trichomes on the abaxial leaf surfaces using the same nine point hair density scale used to measure domatium density.

Pearl bodies - Pearl bodies are microscopic orbs made of leaf epidermal cells that largely contain polysaccharides, proteins, and lipids (Paiva et al. 2009; Gerrath et al. 2015). These structures are primarily found on leaf undersides or on stems and have been hypothesized as a food source for plant mutualists such as mites and ants (Ozawa and Yano 2009; Paiva et al. 2009). We quantified the number of pearl bodies per leaf underside on fresh leaves using a dissecting microscope. We then standardized by leaf area to obtain the density of pearl bodies per centimeter squared of leaf surface.

Palatability - We characterized the palatability of grape leaf tissue to a generalist herbivore via a no-choice bioassay as a metric of the overall direct defense capacity of a plant. We used third instar *Spodoptera exigua* larvae, a generalist lepidopteran pest known to feed on grape (Merkx-Jacques et al. 2008). We placed a single larva on a grape leaf (mean = 3.3 leaves per *Vitis* species or genotype) in a closed cup and allowed it to feed for six days. At the end of this time period, we removed the leaf, assessed the survival of the herbivore, and scanned the leaf. We quantified the area of leaf tissue removed from the leaf using ImageJ (Rasband 2012), and converted this to mass removed using the average leaf mass per area metric calculated from the dry leaves used in the domatia/trichome/pearl body measurements.

3.3.3 Environmental variables

We downloaded occurrence data for each *Vitis* species from the Global Biodiversity Information Facility (GBIF) database using the R package rgbif (Chamberlain et al. 2012; GBIF.org 2023). We were unable to obtain locality data for two *Vitis* species (*V. treleasei* and *V. yenshanensis*) because they did not exist in the GBIF database. As a result, the climate analyses are conducted with nineteen *Vitis* species rather than the full twenty-one listed in Fig 3.1. We cleaned our dataset of occurrences in two ways. First, we filtered down the occurrence records to only those marked as being from research-grade preserved specimens. Then, we used the R package CoordinateCleaner to remove occurrences around country capitals, the GBIF headquarters, and institutions of biodiversity research, as well as duplicate occurrences, occurrences with equal latitude and longitude coordinates, coordinates at country centroids, zeros, outliers, and coordinates within bodies of water (Zizka et al. 2020). This resulted in a total of 16,641 occurrences for the species in this study.

We did not have precise locality of origin information available in the USDA-ARS germplasm database; for most accessions, the most precise location available was at the state or province level. For accessions that did have location data available, we manually verified that the collection location fell within the range of species occurrences obtained from GBIF. To obtain occurrence points for *V. riparia* genotypes, we binned the 1,748 research-grade occurrences for *V. riparia* by state/province of origin and randomly sampled an occurrence from each state that our accessions originated from. Rather than using the state centroid, this approach produced an in-state occurrence point from habitats with confirmed *V. riparia* presence (Appendix Figure B.2). Using this cleaned set of occurrences, we then extracted climate data for each latitude/longitude coordinate from the WorldClim database using the R package raster (Fick and Hijmans 2017; Hijmans 2022). We calculated mean location coordinates and environmental variable values for each of the 21 *Vitis* species used in this study (Appendix Figure B.2).

To test for patterns of defense trait expression consistent with plant defense theory, we narrowed down the available GBIF and WorldClim data to four metrics that we expect to correlate with defense traits according to the LHDH and RAH: absolute value of latitude (a measure of distance from the equator), temperature seasonality (bio4), annual precipitation (bio12), and precipitation of the driest month (bio14). In our analyses, tests of the latitudinal herbivory defense hypothesis (LHDH) are made by comparing defense traits and latitude or seasonality. We include either latitude or seasonality in this hypothesis testing because, while correlated, we expect that temperature seasonality is representative of the relative growing season length that plants are exposed to herbivory, and thus may be expected to correlate with defense investment in ways that are distinct from latitude alone.Tests of the resource availability hypothesis (RAH) are made by comparing defenses and metrics of precipitation because aridity is an environmental stressor predicted (though not always found) to impact plant defense (Coley et al. 1985; Koricheva et al. 1998; Pearse and Hipp 2012).

3.3.4 Statistical analyses

All analyses were performed using the R programming language, version 4.4.1 (R Core Team 2021). We computed species/genotype means for each trait and environmental variable investigated. To test for intraspecific correlations between traits via the Pearson method, we used the "cor" and "cor.test" functions in base R on trait pairs, and constructed correlograms using the function "corrplot" in the R package corrplot, version 0.94. To test whether features of the abiotic environment predict the defense trait investment of *V. riparia* genotypes, we ran separate linear models for each environmental variable with that variable as the predictor and defense trait values as response variables. We chose not to combine the environmental predictors into one model for two reasons: the predictor variables are correlated with each other, and thus it would be statistically inappropriate to include them together in the same model, and because we were interested in teasing apart the distinct relationships between different facets of the abiotic environment and defense evolution rather than combine them into a less interpretable metric.

To test for correlations between defense traits across *Vitis* species, we corrected for non-independence using phylogenetic independent contrasts (PICs). PICs were computed using the "pic" function in the package "phytools." This required a *Vitis* phylogenic tree pruned to the species in this study, which we sourced from (Graham et al. 2023). We then used the "cor.test" function to test for evolutionary correlations between the phylogenetically corrected trait values.

To test whether there is evidence for the LHDH or the RAH across *Vitis* species, we used the phylogenetic generalized least squares (PGLS) method implemented through the function “gls” in the package nlme version 3.1-166 using a Brownian motion correlation structure with environmental variables as predictor variables and defense traits as response variables. We ran these models separately for each environmental predictor the same reasons described in the above paragraph. Because each test represents a distinct hypothesis, we did not correct for multiple comparisons (Streiner 2015; Rubin 2021). Specifically, we treat each a priori hypothesis as distinct, predicting that traits would assort into defense syndromes, and that variation in defense traits would be tied to proximity to the tropics and resource availability. While correcting for multiple comparisons can reduce the likelihood of falsely rejecting a null hypothesis, it can also obscure biologically meaningful trait relationships. As a result, we follow the statistical advice of Streiner (2015) and Rubin (2021) and present uncorrected p-values for each hypothesis with an associated linear relationship in Appendix Tables B.1 and B.2.

In order to assess whether defense traits measurements from *Vitis* vines cluster into groups of plants with similar trait values, which would be indicative of plant-defense syndromes, we additionally performed a principal components analysis (PCA) using plant-level defense trait means at both the intraspecific and interspecific levels. This was implemented via the prcomp() function in base R.

3.4 RESULTS

3.4.1 Intraspecific trait-trait correlations

At the intraspecific level, we observed only two significant correlations between defense traits (Fig 3.2). Tougher leaves had a lower carbon to nitrogen ratio, meaning that nitrogen makes a greater contribution to the mass of the leaf in tougher *Vitis* leaves (p=0.0115). Additionally, the richness of metabolites in leaf samples was negatively correlated with the abundance of those metabolites (p= 0.0300). According to this result, leaves with a higher number of metabolites have less metabolite investment overall, perhaps demonstrating a trade-off between chemical defense strategies. No *V. riparia* genotypes exhibited trichomes on their leaf surfaces outside of domatia, so we dropped this trait from the intraspecific analyses. Principal component analysis revealed a lack of clustering of defense trait values at the intraspecific level, providing no evidence of coherent defense syndromes (Appendix Figure B.3).

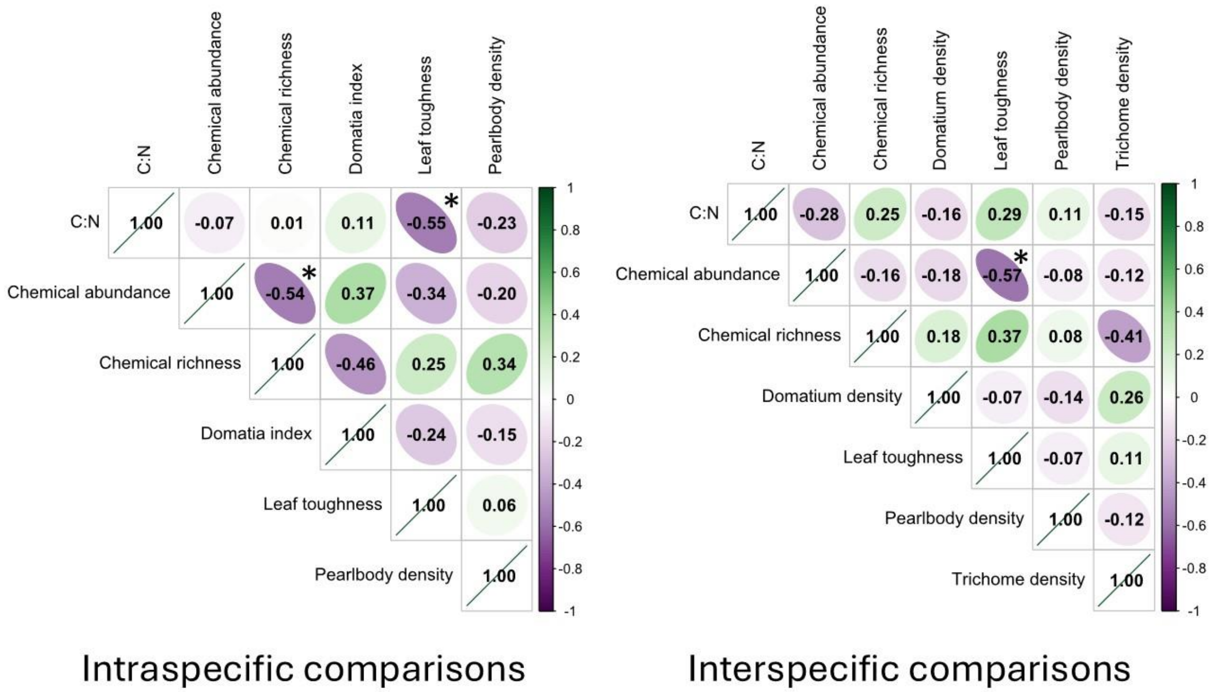


Figure 3.2 Intraspecific (left) and interspecific (right) correlations between putatively defensive traits in *Vitis*. Greener colors represent more positive correlations, whereas more purple colors represent stronger negative correlations. Numbers inside the ellipses are Pearson’s correlations coefficients calculated for trait pairs. Variables that are significantly correlated are indicated with asterisks. Correlations were computed using PICs rather than raw trait values in the case of the interspecific comparisons to account for phylogenetic relatedness.

3.4.2 Intraspecific trait-environment correlations

In our test of the latitudinal herbivory-defense hypothesis, we detected intraspecific trait-environment relationships between leaf toughness and latitude (p=0.0118, Fig 3.3a, Appendix Table B.1) as well as between leaf C:N ratio and latitude (p=0.000147, Fig 3.3b, Appendix Table B.1). The direction of these effects reveals that *Vitis riparia* genotypes further from the equator have more tough, but less nitrogen-rich leaves. Leaf C:N content also correlated with temperature seasonality, where environments with more seasonal temperature variation have lower carbon-to-nitrogen ratios (p=0.0136, Appendix Figure B.4). This means that *V. riparia* genotypes in more variable regions have a greater proportion of nitrogen in their leaves than those native to regions with longer growing seasons, which corresponds with the expectations of the latitudinal herbivory-defense hypothesis. However, we did not detect a significant correlation between leaf toughness and seasonality (p=0.577). We present all effect sizes, degrees of freedom, and p-values for intraspecific linear relationships between traits and latitude in Appendix Table B.1. We found no support for the hypothesis that resource availability in the form of precipitation predicts investment in defense traits across *V. riparia* genotypes.

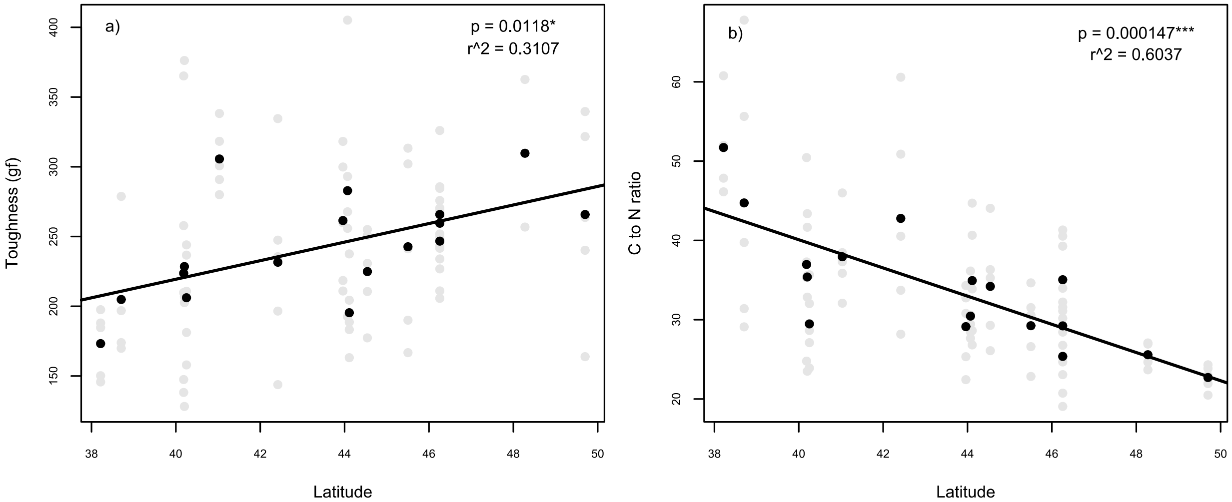


Figure 3.3 Contrasting correlations between defense traits and latitude within *Vitis riparia*. Black points represent genotype means, while grey points depict plant level means. Trendlines represent linear model estimates for the relationships between defense traits and latitude.

3.4.3 Interspecific trait-trait correlations

At the across-species (interspecific) scale, we also saw few trait-trait correlations (Fig 3.2). However, we did detect a significant negative correlation between secondary metabolite abundance and leaf toughness (p=0.00841), which is in line with a trade-off between physical and chemical defense across species. For a depiction of how our measured traits vary across the *Vitis* phylogeny, see Appendix Figure B.5. Again, we saw no evidence of clustering of defense trait values across species via PCA that would be indicative of defense syndromes; instead PC values are relatively evenly distributed across trait space (Appendix Figure B.3B). Plant-level PCA scores for traits did cluster with other points from plants of the same species (see colored hulls in figure).

3.4.4 Interspecific trait-environment correlations

We found mixed support for the LHDH, and no support for the RAH, across *Vitis* species. Pearl bodies (p=0.0273, Fig 3.4a, Appendix Table B.2) and C:N ratio (p=0.0299, Fig 3.4b, Appendix Table B.2, note that while significant, the model where latitude predicts the investment in C:N does not have strong predictive power) correlate negatively with latitude, while chemical abundance correlates with latitude positively (p=0.0224, Fig 3.4c, Appendix Table B.2). These first two observations are in line with our prediction that more equatorial environments have more defended, less palatable plant species. However, secondary metabolite abundance shows the opposite pattern with latitude, where more northern environments have more abundant metabolites. Carbon-to-nitrogen ratio was also negatively correlated with latitude at the intraspecific level, suggesting that this relationship persists across scale. Only metabolite abundance correlated with seasonality, again positively (p=0.0162, Appendix Figure B.6).

Additionally, we detected a significant relationship between herbivory, a metric of overall plant defense, and the latitude of the home environments of *Vitis* species (p=0.0083, Fig 3.4d, Appendix Table B.2), whereby the generalist herbivore in our bioassay ate less leaf material from plants that originate closer to the equator. We present all effect sizes, degrees of freedom, and p-values for linear interspecific relationships between traits and latitude in Appendix Table B.2. As seen at the intraspecific scale, no defense traits correlated with annual precipitation or precipitation of the driest month across *Vitis* species.

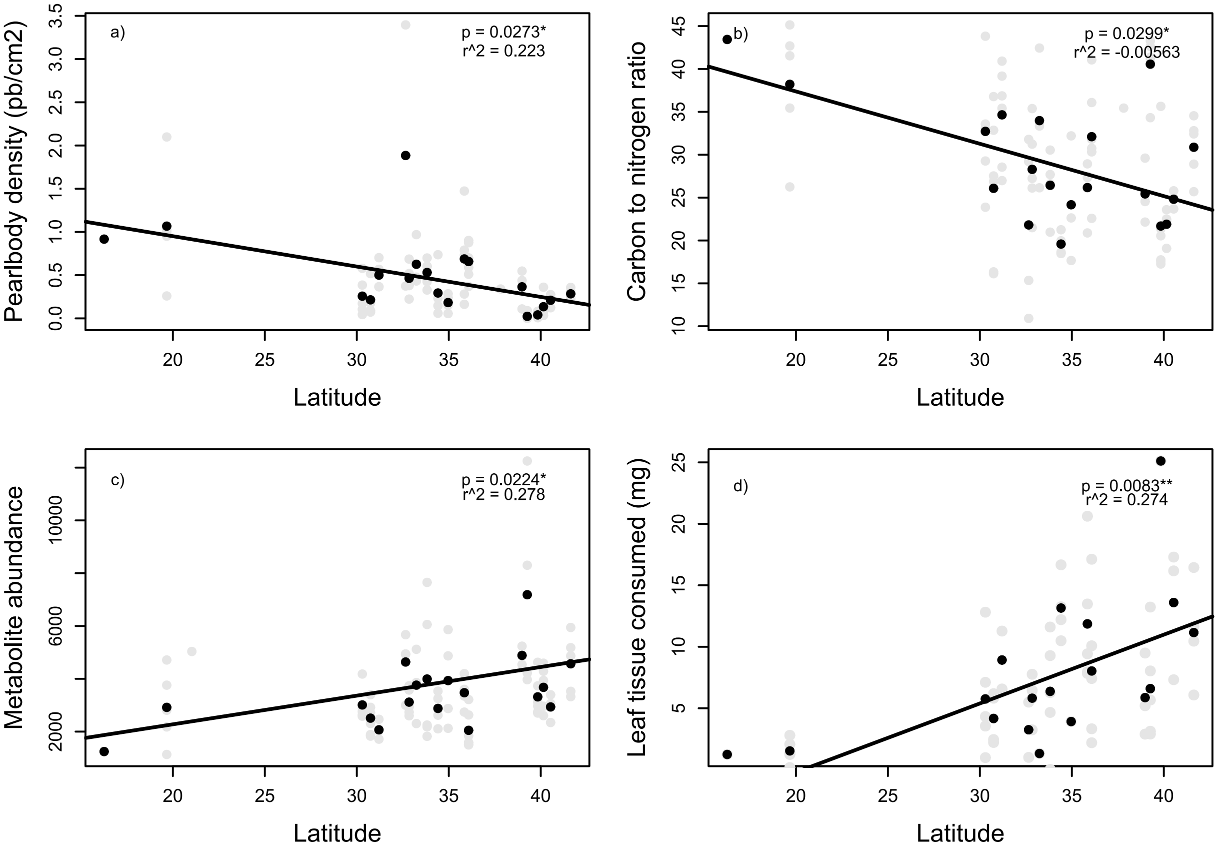


Figure 3.4 Interspecific relationships between defense metrics and species latitude. All relationships are significant at the α = 0.05 level. Black points represent species means. Grey points are plant-level averages for the trait.

3.5 DISCUSSION

We used a phylogenetic comparative framework and a powerful common garden experimental design to test the scale-dependance of plant defense syndromes and trait-environment correlations in the wild grape genus *Vitis*. Because the common garden method controls for trait plasticity, any significant relationships detected implies that evolution has shaped present-day variation in these traits. Our results demonstrate that strong correlations between *Vitis* defense traits, especially those indicative of synergism, are rare both across and within species, and we thus conclude that these traits largely evolve independently of each other. Indeed, instead of sorting into predictable defense syndromes, we saw stronger relationships between traits and the abiotic environment, specifically latitude of origin and seasonality.

3.5.1 Trait-trait correlations across scales

The general lack of correlations between traits at either level of biological organization within this study stands in contrast to plant-defense theory that predicts that plants should exhibit complex arrays of positively or negatively covarying defense traits. Syndromes have long fascinated plant biologists and garner some support in experimental tests of defense trait expression (e.g. Agrawal and Fishbein 2006; Johnson et al. 2014; Farias et al. 2020; Zhao et al. 2020). However, we detected no support for trait-trait synergisms at either scale within this study, instead observing solely negative correlations between traits. This echoes the findings of Moles and colleagues (2013), where only five out of forty-five pairwise correlations between physical and chemical defense traits collected across a broad array of plant taxa were significant, and with three of the five pairs exhibiting trade-off patterns. Similarly, while Johnson et al. (2014) found that twenty-six species of evening primroses exhibited covariation between traits primarily indicative of trade-offs. This inconsistency of the syndromes across taxa underscores the importance of incorporating multiple scales into investigations. Indeed, all of the investigations of plant defense syndromes that we are aware of to date are tested at the interspecific scale, meaning that interesting within-species trait-trait interactions are likely being missed.

Recent theory suggests that correlations that are due to genetic or physiological constraint are more likely to be detectable at the intraspecific scale but break down at the interspecific scale, while correlations between traits that result from niche specialization (e.g., competitive/stress-tolerant/ruderal strategies, plant apparency) may be more likely to be observed across species (Agrawal and Hastings 2019; Agrawal 2020; Zhou et al. 2022). One reason for this is that life-history strategies such as growth form or fast growth are likely to be fixed at the species level, producing little variation within species (Futuyma 2010). Zhou and colleagues (2022) suggest that evaluating intraspecific correlations between traits is a more direct assessment of physiological or evolutionary constraint than assessing correlations across species, which may be complicated by environmental drivers of trait investment. Our results are in support of this theory, where the correlation between leaf toughness and leaf carbon:nitrogen content, likely a biproduct of plant physiology, is strong within but not across species. Additionally, our results suggest that investing in chemical defense may be at the expense of one form of physical defense (i.e. a trade-off pattern), as we observed a negative relationship between leaf toughness and metabolite abundance across but not within species. We did not find evidence of the oft hypothesized trade-off between direct and indirect defense traits across nor within species, a pattern which has received mixed support in previous investigations of defense investment in *Vitis* (English-Loeb and Norton 2006; Graham et al. 2023).

Why might trajectories of defense trait evolution shift across spatial or biological scales? One reason may be that the geographic ranges of populations within a single species are likely to be much smaller than the ranges of multiple species within a genus. Thus, the area occupied by a single species may not encompass enough of a gradient in resources or herbivore communities to impose constraints leading to a trade-off between traits in a single species (Hahn and Maron 2016). Indeed, our intraspecific comparisons were conducted in *V. riparia*, which, while having a range that is much larger than that of many other wild grape species, still has a much more limited geographic range than the genus as a whole. As our common garden design should eliminate variation in trait expression due to plasticity, we expect that a lack of variation in defense strategies between populations produced the broad lack of trait correlations at the intraspecific scale observed in this study.

Conversely, as observed by Agrawal and Hastings (2019), resource allocation trade-offs that are present within species may be masked by variation in resource acquisition across species (see also van Noordwijk and de Jong 1986). A theoretical example used to explain this phenomenon is the “car-house paradox,” which proposes that within any one household (i.e. within species), financial constraint means that owners must choose between spending more of their money on either a house or a car, but across households (i.e. across species), there is enough variation in financial acquisition that there is generally a trend that homeowners with more expensive houses tend to own more expensive cars (van Noordwijk and de Jong 1986; Arnold 1992). For this reason, we may be more likely to observe trade-offs within species, but positive correlations across species. While we did observe trade-offs between traits at the intraspecific level, we also observed a different negative correlation between chemical abundance and leaf toughness at the interspecific level, and no positive correlations between traits. If the intraspecific correlations between chemical abundance and richness and between leaf toughness and carbon-nitrogen content are the result of resource limitation, the lack of these trade-off patterns at the interspecific level might suggest that variation in resource acquisition across species might be producing this pattern.

While we did not find evidence of plant defense syndromes in wild grape, there are likely aspects of defense against herbivory in the grape genus that we may be missing by virtue of only measuring select traits. For example, we did not intentionally induce defense traits in this study, meaning that we did not address the often predicted trade-off pattern between constitutive and induced defenses (Karban and Myers 1989; Brody and Karban 1992; Koricheva et al. 2004; Agrawal and Hastings 2019). Because we sampled defenses one at a time with week-long intervals between collection, we do not think that our sampling caused measurable induction of defensive chemicals or physical traits. However, it is possible that some induction of defenses was caused by removal of plant tissue. Other defense-related traits to investigate in *Vitis* in the future might include calcium oxalate phytoliths or volatile chemicals that attract natural enemies, though the function of the former trait has not been well established in *Vitis* (Arnott and Webb 2000; Schwab and Wüst 2015).

3.5.2 Trait-environment correlations across scales

The LHDH, an offshoot of the more all-encompassing latitudinal biotic interactions hypothesis, has captivated and puzzled evolutionary ecologists for decades (e.g. Anstett et al. 2016; Baskett and Schemske 2018; Baskett et al. 2020). To our knowledge it has not been tested at multiple levels of biological organization simultaneously. Our findings demonstrate mixed support for its prediction that plants at more equatorial latitudes will be more defended than plants in temperate climes. We found that one form of indirect defense (pearl bodies) was more prevalent at lower latitudes, and that plants in these regions also have less nutrient-dense leaves (i.e. have a higher C:N). The former finding compliments a growing body of accounts of higher investment in indirect defense that provide food to bodyguard mutualists at more equatorial latitudes (Keeler 1980; Oliveira and Leitao-Filho 1987; Fiala and Linsenmair 1995). For example, latitude, present-day annual temperature, and precipitation were found to be strong predictors of the diversity of EFN- and ant domatia-bearing flowering plants (Luo et al. 2023). We also detected this negative correlation between latitude and C:N across *V. riparia* genotypes, suggesting that this evolutionary trajectory is repeated across biological scales. In contrast, *Vitis* species in more temperate climes had greater investment in secondary metabolite abundance. It is interesting to note that when we use temperature seasonality as the predictor in our trait-environment models in the place of latitude, only two correlations are significant (one at the intraspecific scale and one at the interspecific scale; Fig S4 and S6). Though latitude and temperature seasonality are highly correlated, seasonality does not vary 1:1 with latitude. Thus, we might predict that some other unmeasured feature of the environment that is influenced by latitude (e.g. herbivore pressure) is more important in shaping defense investment in and across *Vitis* species. Indeed, the observation that different traits correlate with latitude in opposite directions within *V. riparia* may suggest a niche-specialization trade-off, whereby different herbivore communities in lower versus higher latitude environments have selected for different defense strategies in grape species. Evolutionary biologists often expect that specialization in diet increases towards the equator, but investigations of patterns in the specialization of insects on host plants do not always report consistent correlations with latitude (Salazar and Marquis 2012; Anstett et al. 2014). Though latitudinal characterizations of herbivore community on *Vitis* species are rare, one study did find that more northern species actually had a higher incidence of attack by the specialist grape phylloxera *Daktulosphaira vitifoliae*, a pattern largely driven by the susceptibility of *V. riparia* to the herbivore.

We also observed that *Vitis* species seem to be better defended overall closer to the equator, as the generalist herbivore *S. exigua* consumed less leaf tissue from grape species native to Central America than from species native to more northerly regions of America. Because our grape leaves were not defended by mutualist bodyguards during our bioassay experiment, it is unlikely that pearl bodies contributed to this trend. Plant nutritive value may be implicated in this pattern, but we did not see a direct effect of C:N ratio on herbivore performance. Thus, we may interpret our results to mean that no one defense strategy is the magic bullet to defend against *S. exigua* in this controlled setting, but instead some un-tested combination of defenses reduces their feeding. Additional tests factorially combining distinct defense traits would then be helpful to evaluate which combinations of traits impact herbivory in *Vitis* species.

A recent meta-analysis of papers exploring biogeographical patterns of ant-plant mutualism found that such mutualisms are more effective at improving plant performance in arid environments, presenting evidence of an advantage to investing in indirect defense in low precipitation environments (Leal and Peixoto 2017). Anecdotally, *V. arizonica*, a species of grape native to the Southwestern United States, experiences some of the lowest annual rainfalls in our dataset, and also produces huge quantities of pearl bodies (Fig S5). However, our overall results suggest that precipitation is not a significant driver of defense evolution within nor across *Vitis* species. It is important to note that the *Vitis* vines used in this experiment were not grown in nutrient scarce environments that would mimic what they encounter in their home ranges. Instead, the plants were grown in a common garden environment and thus all plants were subjected to the same environmental conditions. It is possible, therefore, that the vines would demonstrate plasticity in their defense phenotypes if sampled in their natural ranges, and correlations between traits and environmental variables might thus be observed. Though we did not find evidence that environmental stress due to precipitation is a determinant of *Vitis* defense investment, water is but one resource that plants need for growth. Other tests of the impacts of resource availability have considered essential nutrients such as nitrogen and even light availability and have found that different resources may or may not correlated with particular plant defenses (Koricheva et al. 1998; Endara and Coley 2011b).

3.6 CONCLUSIONS

Using a powerful pairing of common garden trait quantifications and a comparative phylogenetic framework we demonstrated a lack of hypothesized correlations between defense traits both across and within *Vitis* species. Instead, latitude was a strong predictor of plant defense investment at both levels of biological organization, although trait-latitude correlations shifted across scales and traits. This work thus challenges the oft-hypothesized trade-off and synergism framework for defense trait evolution in plants, and demonstrates the scale dependency of hypotheses that related this defense evolution to the abiotic environment. Future work on plant defense evolution should carefully consider the relevancy of plant defense hypotheses at different geographic and biological scales.

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Chapter 4 : Characterizing the Causes and Consequences of Calcium Oxalate Crystal Presence in *Vitis riparia*

4.1 ABSTRACT

Understanding the physiological and ecological functions of phenotypes is one of the foremost goals of biologists. Calcium oxalate biomineralization is one phenotype in plants that is phylogenetically widespread and morphologically diverse, but for which the function is still an area of active debate. While there are several hypotheses for the function of these inorganic crystals, such as defense against chewing herbivores and storage of excess calcium, no single function is reliably demonstrated across taxa. Indeed, the variety of environmental conditions that produce the crystals, as well as their failure to provide consistent antiherbivore defense across plant and herbivore species, suggests that different crystal morphologies might have different functions. In this study we experimentally investigated the environmental influence on the formation of calcium oxalate druse and raphide needle crystals in *Vitis riparia,* the riverbank grape, as well as their putative defensive function against a generalist lepidoptera herbivore. To test the inducibility of crystals, we manipulated the growing conditions of *V. riparia* to alter the presence of excess calcium and simulated herbivory. To test the defensive properties of extracted *Vitis* crystals, we performed a no-choice herbivore bioassay using the generalist herbivore *Spodoptera exigua*. We manipulated herbivore diet for the presence of *V. riparia* and amorphous calcium oxalate crystals and *V. riparia* secondary metabolites to test for impacts of crystal shape on herbivore growth, both alone and with plant chemistry. We found that the addition of calcium to *V. riparia* soil increased the density of both raphide and druse crystals in leaves. Contrary to

expectations, the herbivory treatment decreased the density of raphide crystals in leaves, and *V. riparia*-derived crystals did not impact the weight gain, time to pupation, nor the survival of moth larvae. However, amorphous crystals negatively impacted herbivore growth and survival. We also detected no interactive effect between crystals and leaf chemistry on herbivores. Our results suggest that *Vitis* calcium oxalate crystals serve a function other than defense against chewing herbivores in *Vitis riparia*, and that crystal abundance is determined at least in part by soil calcium levels. Our multifaceted test of the formation and function of calcium oxalate crystals in *V. riparia* demonstrates that abiotic factors are a relatively stronger determinant of crystal production, and, contrary to hundreds of years of speculation on their function, these crystals do not seem to mediate plant-insect interactions in this taxon.

4.2 INTRODUCTION

Since calcium oxalate (CaOx) crystals were first discovered via microscope by Antonie van Leeuwenhoek in the 1600s, plant biologists have hypothesized about their formation and functions (van Leeuwenhoek 1675; Webb 1999; Franceschi and Nakata 2005). Nearly 350 years later, the ecological role of role of CaOx crystals is still an area of active debate (Paiva 2019, 2021; Khan et al. 2023; Lawrie et al. 2023). Plants in at least 200 families have CaOx crystals within their leaves, stems, roots, flowers, and/or fruits (Franceschi & Nakata, 2005; McNair, 1932), taking on a multitude of forms, from sharp needles to prisms to conglomerations (Webb 1999; Raman et al. 2014). Many hypotheses have been proposed for the potential selective pressures that produce and maintain this variation in their morphologies, but we lack manipulative experiments that simultaneously test the causes and consequences of CaOx presence in plant organs.

The CaOx crystal expression is often hypothesized and demonstrated to hinge on the presence of excess calcium in a plant's environment. Calcium oxalate crystals have thus been proposed to be a dynamic storage for excess calcium (Paiva 2019), but evidence for increased calcium oxalate crystal production in high calcium soil conditions is inconsistent, and may be species specific. For instance, soil calcium concentration did not impact calcium oxalate production in *Pancratium sickenbergeri*, indicating that the crystals are not inducible in this plant species (Ruiz et al. 2002). Paradoxically, Molano-Flores (2001) showed that *Sida rhombifolia* seedlings that were subjected to calcium-scarce conditions had the highest quantities of druse crystals in their plant parts. Thus, the degree to which soil calcium plays a role in crystal presence and density is difficult to generalize across taxa.

One of the most compelling additional hypotheses for the presence of calcium oxalate crystals in plants is that they act as a defense against chewing herbivores. Herbivory is a strong selective pressure for plants due to their inability to move to escape predation. As a result, plants have evolved a diversity of traits that deter potential antagonists. Calcium oxalate crystals are proposed to be a physical deterrent to herbivory by causing internal irritation upon ingestion. Indeed, the sharp, abrasive nature of these crystals has long been implicated in dental wear and external and internal membrane irritation in mammals, including humans (Perera et al. 1990; Gardner 1994; Danielson and Reinhard 1998; Salinas et al. 2001). In the case of arthropod herbivores, a major group of plant antagonists, the picture is less clear. Some researchers have found clear fitness impacts on arthropods that are fed with calcium oxalate crystals (e.g. Korth et al., 2006; Park et al., 2009), while others have not (e.g. Nagaoka et al., 2010). There is some evidence that herbivory can induce the formation of crystals, further suggesting that CaOx does play a role in defense in some plant taxa (Molano-Flores 2001).

Calcium oxalate crystals in isolation may not be an effective deterrent to herbivores, but instead rely on a synergism with other defense traits, complicating investigations of the impacts of crystals on herbivores. In a 2014 paper, Konno et al. found that kiwifruit raphides and cysteine proteases, a class of defense metabolites, worked together at biologically relevant levels to decrease the growth and increase the mortality of Eri-silkmoth (*Samia ricini*) larvae to a degree that was greater than the sum of the impacts of the two defenses on their own, a phenomenon they dubbed the "needle effect". Thus, the negligible effect of CaOx crystals on some herbivores may be because raphides are only an effective defense in the presence of secondary metabolism. The work by Konno also demonstrated that it was specifically the needle shape of the crystals that facilitated this synergism, as crystal grains did not show evidence of enhancing the rate of mortality amongst the larvae.

Work by Volk and colleagues proposed that different crystal morphologies may play different functions in plants, and thus, the aforementioned hypotheses may not be mutually exclusive (Volk et al. 2002). For instance, the works highlighted above found defensive properties of raphide needle crystals and prismatic crystals (Korth et al. 2006; Konno et al. 2014), while druses or crystal sand morphotypes are not found to decrease herbivore growth (Nagaoka et al. 2010). Based on this literature, we might expect that the formation of druse and crystal sand morphologies would be related to storage, and would be induced by calcium levels in soil and act as a form of calcium storage, while raphide or prismatic morphologies additionally or alternatively provide defense for plants. Determining the degree to which CaOx is constitutively versus facultatively expressed via environmental induction, either by herbivory or coil calcium, can help elucidate the functions of these widespread crystals, especially when paired with experimental manipulations of crystal presence in an herbivore's diet.

Riverbank grape (*Vitis riparia*) presents a valuable system in which to test the ecology and inducibility of CaOx crystals because their organs contain at least two distinct crystal morphologies: raphide bundles and druses (Jáuregui-Zúñiga et al., 2003; Webb et al., 1995; Fig 4.1a). Raphides and druses can be found in predictable localities within grape leaves; raphides are sporadically distributed throughout the mesophyll tissue in idioblasts, while druses are exclusively found in bundle sheath cells clustered in rows along veins in grape leaves (Arnott & Webb, 2000; Kolyva et al., 2023; Webb et al., 1995; Fig 4.1a). This differential locality may hint at differential roles; suggesting raphides are distributed throughout the leaf to maximize their defensive capacity, while druses form near vascular tissue where calcium is deposited. Previous studies of calcium oxalate crystals in *Vitis* have not tested these roles.

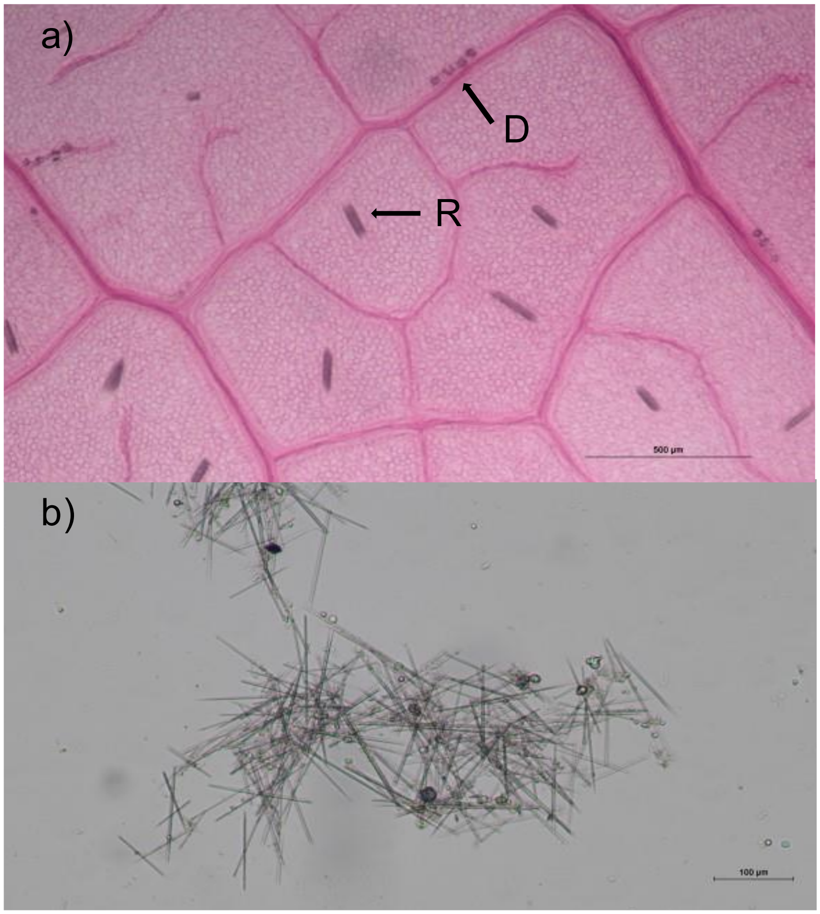

Figure 4.1 *Vitis riparia* calcium oxalate crystals a) within stained leaf tissue and b) isolated from ground leaf tissue. Structures labeled R are raphide bundles, while those labeled D are druse crystals.

Here, we provide experimental tests of the inducibility and function of crystal types in riverbank grape. First, we aim to explore the causes of crystal formation, testing whether environmental stimuli influence their formation. We performed a greenhouse-based experimental manipulation to study if different calcium oxalate crystal morphologies (raphides and druses) are differentially inducible through calcium soil addition or simulated herbivory in *Vitis*. We predict that raphides are formed in response to herbivory, while druses are induced through calcium addition. Second, we conducted a series of bioassays using generalist herbivores with a tightly controlled factorial design to test whether crystals provide antiherbivore defense. We specifically asked: is herbivorous arthropod growth impacted by the presence of *Vitis* raphides in their diet, does crystal shape determine the impact of CaOx on herbivore growth, and if raphides operate synergistically with grape metabolites to the detriment of herbivores. We predict that *Vitis*-derived CaOx crystals will be a more effective defense in the presence of grape chemical defenses than they are on their own. In comparison to previous work on the trophic ecology of CaOx crystals, this bioassay strategy lets us examine the long term impacts of different crystal morphologies on herbivores to more closely replicate the realities of sustained crystal diets in the wild.

4.3 METHODS

4.3.1 Inducibility of calcium oxalate crystals in Vitis riparia

We rooted genetically identical *Vitis riparia* cuttings collected in Michigan in a common environment in the spring of 2024. Once the vines had begun to leaf out, we separated the cuttings into three experimental treatments: calcium addition, simulated herbivory, and control. Calcium addition plants were watered with a solution of 15 mM calcium chloride twice a week, achieving a soil calcium concentration of ~1700 ppm (as opposed to ~100 ppm in the control treatment). Herbivory treatment plants received the same amount of water without calcium but were sprayed with 100mg of jasmonic acid dissolved in 250 mL of 0.1% acetone twice during the 6 week growing period. We also simulated herbivory on plants in the herbivory treatment by removing 1/3 of the area of each mature leaf twice in the growing period, at the same time points as the leaf tissue removal. All plants from all treatments were handled when the simulated herbivory treatment was applied. Control treatment plants again received the same amount of water and were sprayed with a control spray made up of 0.1% acetone in water.

After a six week growing period, we removed one mature, undamaged leaf from each vine, and dried it in a drying oven at 60C until fully dehydrated. We cleared, stained, and mounted leaves using a procedure adapted from (Machesky et al. 2025). In brief, we soaked the leaves in sodium hydroxide until they became transparent, washed them in distilled water, and soaked them in bleach until decolorized. We then dehydrated leaves in increasing concentrations of ethanol, up to pure ethanol, and soaked them in 0.5% Safranin O dye in ethanol to stain the leaves. After washing off excess dye in ethanol, we fixed the leaves in cedar oil and examined them under a compound microscope at 4x magnification. We took pictures of the samples with the microscope, and then counted the number of raphide bundles and druses visible within the resulting standardized image size.

Statistical Analyses: To test whether calcium oxalate crystals are inducible, we constructed generalized linear models where the predictor is treatment (control, Ca addition, or simulated herbivory) and response variables are either raphide or druse counts (per a standardized area). Because the data, especially in the case of the druse counts, were overdispersed and zero-inflated, we implemented models with a negative binomial correlation structure with a zero-inflation extension using the r function glmmTMB in the package of the same name (Brooks et al. 2017).

4.3.2 Effects of grape leaf calcium oxalate crystals on herbivores

To test the defensive function of *V. riparia*-derived secondary chemistry and CaOx crystals, we used a factorial no-choice bioassay varying the presence of grape leaf CaOx crystals, amorphous CaOx crystals, and grape leaf chemistry.

Acquiring chemical and crystal diet additives: To control for the impact of other defense traits present in *Vitis* leaves on herbivores, crystals and leaf metabolites were extracted from wild grape leaf material and introduced into artificial diet. To extract leaf metabolites, we collected mature, undamaged leaves from wild *Vitis riparia* vines near Ann Arbor, Michigan in the summer of 2023 and dried them at room temperature using silica gel beads. Once the leaves were fully dry, we ground them into a fine powder using a mortar and pestle. We then submerged the powdered leaf material in 100% methanol for 10 minutes to extract leaf metabolites. We centrifuged these extracts so that solid leaf material could be separated from methanol supernatant. We stored the supernatant extract at -20C until just prior to the bioassay. In the days leading up to the bioassay, we once again centrifuged the extract to remove any remaining solid particles. We then let the methanol evaporate off in a fume hood at room temperature for about 48 hours, leaving behind a leaf metabolite residue. Finally, we resuspended this residue in 1% sunflower seed oil in DI water, with the goal of including both polar and non-polar compounds in this chemical stock solution. This resulted in a grape leaf metabolite extract of approximately 47.44g/L (w/v).

To extract leaf raphides, we isolated calcium oxalate crystals from the leaf pulp pellet that resulted from the chemical extraction and centrifugation of ground leaf material described above. To do this, we used a modified version of the CaOx extraction procedure described in Konno et al 2014. In brief, we separated unwanted leaf material from calcium oxalate by using a solution of 6.35M cesium chloride and 0.4M calcium chloride, with a density of approximately 1.8g/mL. This solution had a density intermediate between the densities of the unwanted leaf material and the CaOx crystals, and when centrifuged, produced a pellet of CaOx crystals and a surface layer of leaf pulp. We repeated this dilution and centrifugation procedure three times, each time discarding the unwanted leaf material layer. We then washed the pellet an additional three times with distilled water to obtain uncontaminated calcium oxalate crystals. We then allowed the pellet to dry so we could accurately weigh the crystals into artificial diet. Examination of extracted crystals under light microscopy indicated that the initial drying and grinding of the grape leaf material did not damage the crystals. However, the raphide crystals were loosened from their bundles (Fig 4.1b). The vast majority of crystals extracted in this manner were raphide needles, with some druses dispersed throughout. Amorphous CaOx crystals were purchased as a purified reagent (Thermo Scientific Chemicals, Pittsburgh Pennsylvania, USA). These crystals are most similar in form to crystal sand crystals, and do not exhibit a needle-shape.

Bioassay Design: To text the individual and combined effects of calcium oxalate crystals and grape leaf chemistry on herbivore fitness, we performed a no-choice bioassay with the generalist herbivore, *Spodoptera exigua* (Frontier Scientific Services Agriculture, Newark Delaware, USA). The herbivores were enclosed in individual plastic cups containing one of six possible artificial diet treatments (Fig 4.2). Each diet cup contained 5mL of general lepidopteran artificial diet (Frontier Scientific Services Agriculture, Newark Delaware, USA) prepared in one large batch according to the instructions provided and dispensed into the cup. While the diet in each cup was still liquid, we thoroughly mixed in 1mL of treatment stock solution. We then allowed the diet to set and stored the cups at 4C until the following day, when we set up the bioassay.

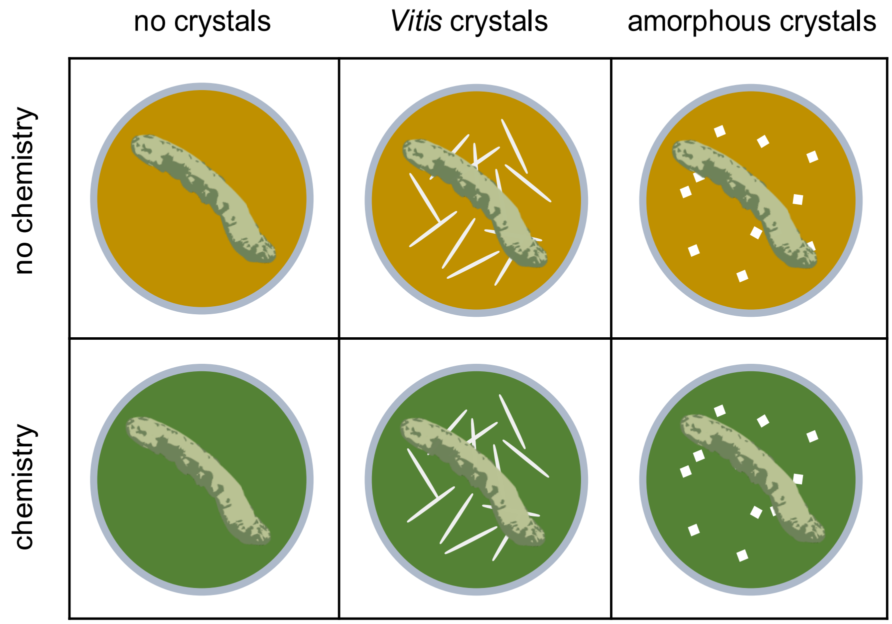

Figure 4.2 Factorial design of the bioassay, allowing us to test the impacts of crystal shape and presences as well as grape chemical presence on *S. exigua* growth.

The treatment stock solutions were designed so that dispensing 1mL of solution into the diet cups would introduce 2.85mg of CaOx crystals (either *Vitis*-derived or amorphous) and/or 47.44mg of grape metabolites. All treatment solutions had a base of 1% sunflower seed oil in DI water. After allowing the diet cups to warm to room temperature, we introduced one ~3rd instar *S. exigua* larva (9-15 mg in mass, $\bar{x}$ = 11.7 mg) to each diet cup. We allowed them to feed until pupation, which took between 11 and 21 days depending on the treatment. The cups were stored within trays (each holding 30 cups) in the incubator, which was set to 21°C with 9 hours of light. Treatment cups were randomized within trays at the outset of the experiment, but the trays were consistently kept in the same incubator locations. We included more than enough diet to the caterpillars to get them to pupation; no caterpillars ate all provided diet.

Every day during the bioassay we weighed each larva and surveyed them for survival and/or pupation. Pupae were weighed in the same order each day to maintain roughly consistent feeding intervals across days. After ten days, we sacrificed five larvae per treatment for further analysis by immediately freezing them at -20C. Finally, we collected all pupae and weighed them to get a final wet mass measurement.

Statistical Analyses: To capture a holistic picture of the impacts of our treatments on the herbivores, we analyzed multiple metrics of success/fitness, including the maximum mass they achieved, the number of days it took for them to reach this maximum mass, whether they survived to pupation (binary 0/1), and the mass of the pupa if they did pupate. We converted the raw larval mass data to percent growth to account for any differences in the initial masses of the larvae.

$$percent\ growth = \frac{m_{final} - m_{initial}}{m_{initial}} \times 100$$

We also dropped larvae from the analyses that perished without any evidence of having eaten the artificial diet (14 larvae across all treatments).

We used analysis of variance to compare max larval and pupal masses between treatments, and we used binomial logistic regressions to assess survival. All models included terms for individual effects of CaOx treatment (raphides or amorphous crystals) and chemistry, as well as CaOx-chemistry interaction terms. Additionally, in preliminary analyses we included tray as a random effect in mixed effects models using the package lme4, but discovered no qualitative impact on the results, so we excluded this random effect from the final models. All analyses were performed in R version 4.2.1 (R Core Team 2021).

4.4 RESULTS

4.4.1 Are different calcium oxalate crystal morphologies differentially inducible through calcium soil addition or simulated herbivory in **V. riparia?**

The calcium addition treatment had a significant positive impact on both the density of raphide bundles and druses in *V. riparia* leaves (p<<<0.05 for both, Fig 4.3). Contrary to our expectations, vines in the herbivory treatment had a lower density of raphide bundles as compared to the control treatment (p=0.00138). There was no effect of simulated herbivory on druses, and very few leaves from the control or herbivory treatments even had druses.

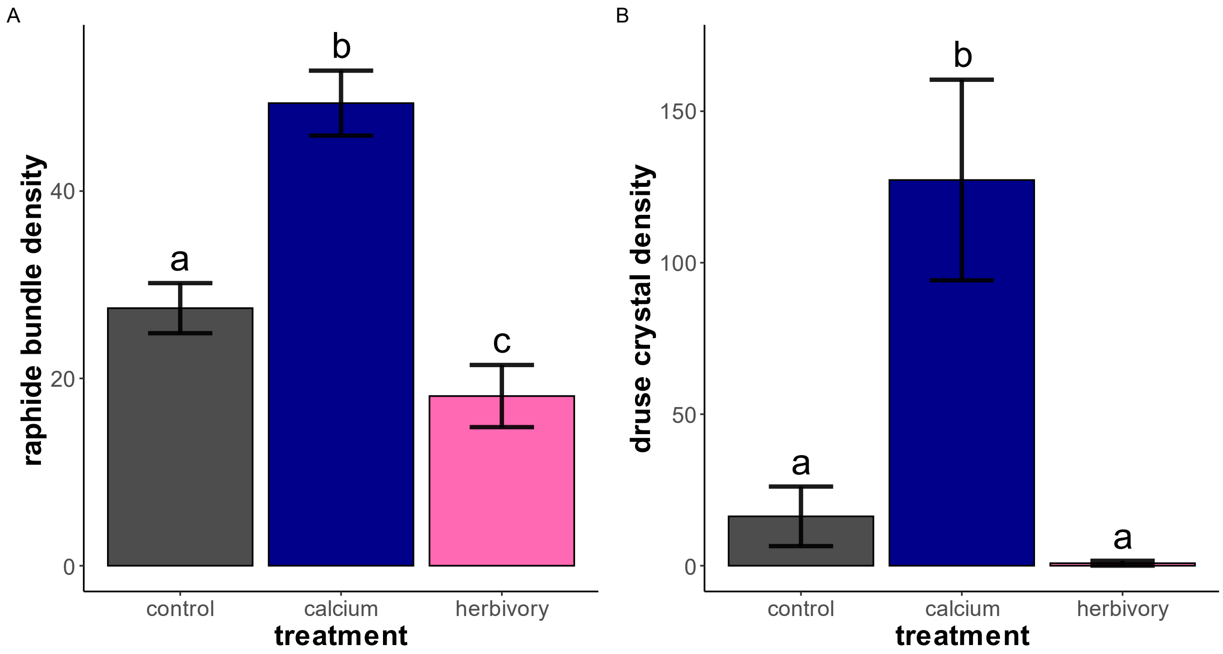

Figure 4.3 Average leaf A) raphide bundle and B) druse crystal densities from vines subjected to the three induction treatments. Error bars represent standard error in measurements calculated for the raw density data. Sample size was 34 control leaves, 28 calcium leaves, and 35 herbivory leaves. Bars labelled with different letters have model estimates significantly different from each other at the p=0.05 level.

4.4.2 Do **Vitis riparia***-derived calcium oxalate crystals impede herbivore growth?*

Contrary to our predictions, we found no significant impact of the *V. riparia* CaOx crystals on any of our metrics of herbivore success. Larvae in this treatment reached a similar average maximum percent growth (control = 2371±85% growth, *Vitis* CaOx = 2269±104% growth; Fig 4.5) and pupal mass (control = 123±5mg, *Vitis* CaOx = 110±6mg) compared to the control treatment and were not significantly more likely to pupate (Table 4.1). Because the results for the maximum percent growth and wet pupal mass were qualitatively identical, we have elected to only graph the percent growth results. Additionally, the larvae fed *V. riparia* crystals reached their maximum mass at the same time as the control larvae, meaning that the calcium oxalate crystals did not slow their growth (Fig 4.4).

Table 4.1 Effects of treatment on the odds of larval survival to pupation. Note that the total and deceased larvae counts do not include the five larvae per treatment that were sacrificed. Significance comparisons are made to the control in the case of the additive effects, while the interaction effects are treated as interactions in the models.

Treatment	Total larvae in treatment (n)	Count of larvae that died	Log odds of survival	Standard Error	p-value
Control	22	3	0.463	0.621	-
Vitis crystals	22	5	1.22	0.803	0.438
Leaf chemistry	24	14	-0.595	0.747	0.00347**
Amorphous crystals	22	14	-0.560	0.763	0.00162**
Vitis crystals + chemistry	15	14	-1.872	0.981	0.439
Amorphous crystals + chemistry	21	15	-0.9163	0.787	0.0661

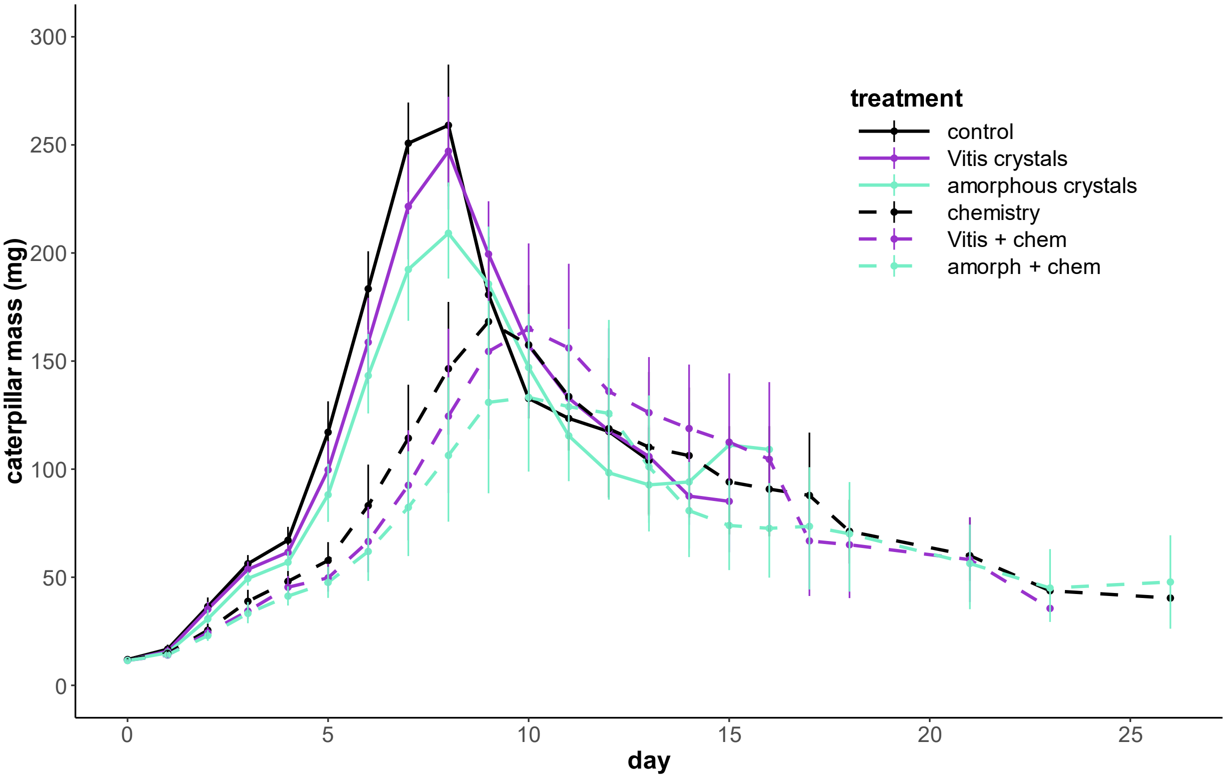

Figure 4.4 Average growth curves of larvae by treatment. Error bars represent confidence limits for the caterpillar mass means per treatment at each time point.

4.4.3 Does crystal shape determine the impact of calcium oxalate crystals on herbivore growth?

Contrary to our prediction, we found that crystal grains had a significant negative impact on herbivore growth in comparison to the control treatment. This was true for both the average maximum percent growth of the larvae during the bioassay (p=0.0103, Fig 4.5), as well as for the average pupal masses (p=0.00656).

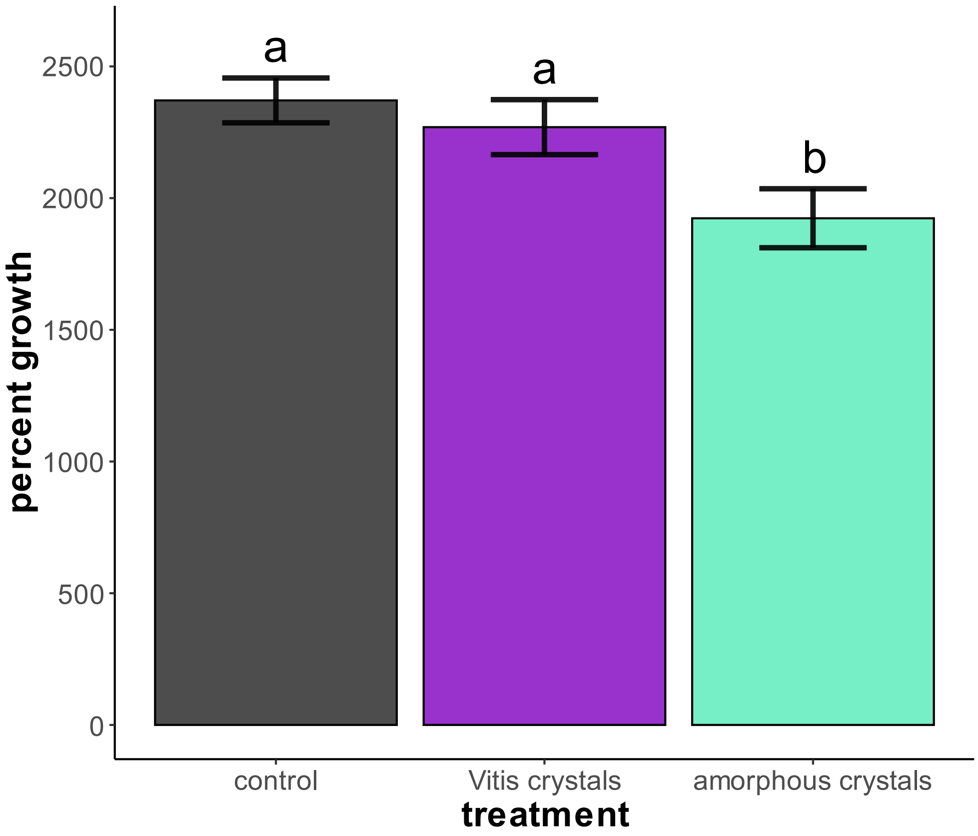

Figure 4.5 Effect of crystal shape on the percent growth of caterpillars. Error bars represent standard error in measurements calculated for the percent growth data. Bars labelled with different letters have model estimates significantly different from each other at the p=0.05 level.

4.4.4 Do calcium oxalate crystals synergize with grape metabolites to the detriment of herbivores?

We did not detect a significant interaction between *Vitis* CaOx crystals and leaf chemistry on herbivore survival, nor on our other metrics of herbivore success. Leaf chemistry did decrease the maximum percent growth the larvae achieved compared to the control ($p<0.00001$, Fig 4.6). Larvae in the leaf chemistry treatment also had a lower odds of surviving to pupation ($p=0.00347$, Table 4.1). Of the few larvae that did make it to pupation under the chemistry treatment, the pupae subjected to the chemistry treatment were also significantly smaller than the control pupae ($p<0.0001$). We found no evidence for a synergism between chemistry and amorphous CaOx crystals.

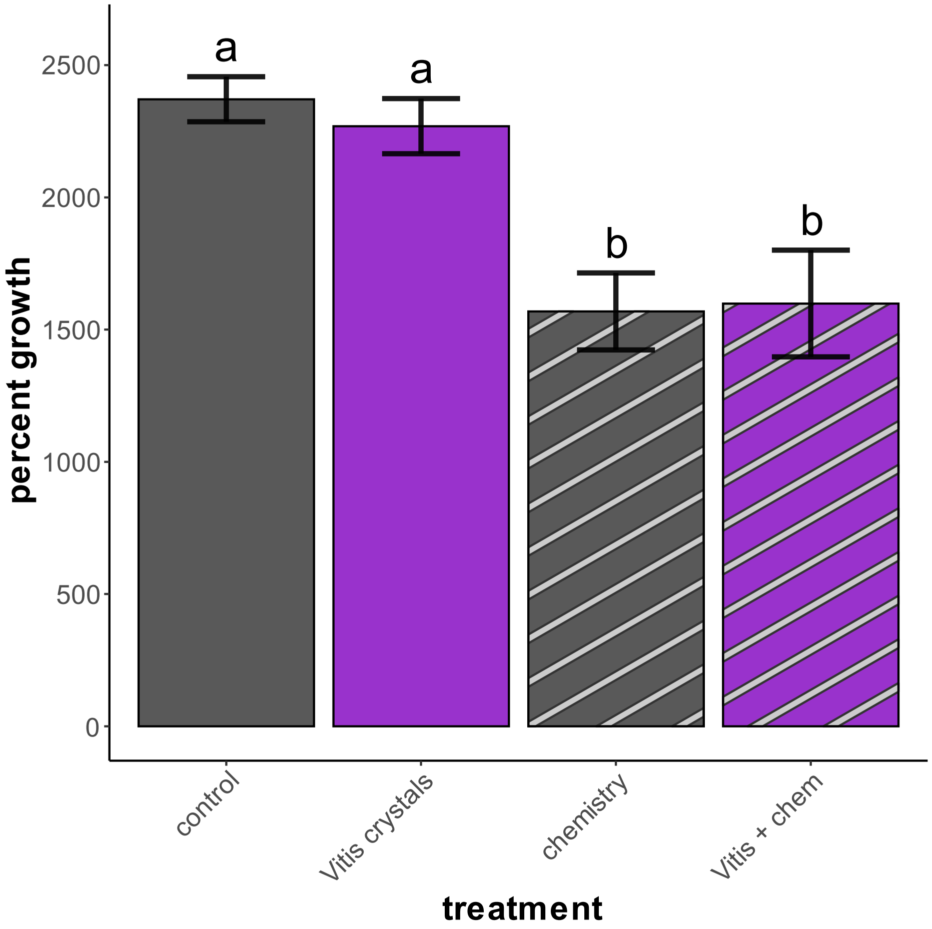

Figure 4.6 Lack of a synergistic effect of raphide crystals and grape leaf chemistry on caterpillar percent growth. Error bars represent standard error in measurements calculated for the percent growth data. Bars labelled with different letters have model estimates significantly different from each other at the $p=0.05$ level.

Larvae in the chemistry treatment also reached their maximum masses more slowly than those in the control treatment ($p=0.00175$). It took an average of about nine days for the control, raphide, and amorphous treatment larvae to reach their maximum mass, while larvae in the chemistry treatment took an average of almost two days longer (Fig 4.4).

4.5 DISCUSSION

The causes and consequences of calcium oxalate crystal presence in plants has sparked significant debate, which has recently been renewed by several review articles (Paiva 2019, 2021). Here, we demonstrate that CaOx crystal density is inducible via Ca soil addition in *V. riparia*, but not through simulated herbivory. Additionally, we show that *V. riparia*-derived calcium oxalate crystals do not impact the feeding of a generalist herbivore, and do not seem to enhance the defensive function of *V. riparia* secondary chemistry. The results of this study challenge the assumption that raphide calcium oxalate crystals function as a growth reducer for chewing herbivores, and fail to support the hypothesized “needle effect” synergism of needle-shaped crystals and chemical defense (Konno et al. 2014).

This work joins the growing body of research suggesting that foliar calcium oxalate crystals are over-emphasized as a defense against insect herbivores (Paiva 2021). However, we acknowledge that there is compelling evidence for a post-ingestive impact of CaOx crystals, especially in the case of the needle-shaped morphologies, in other plant taxa. Why do *Vitis* crystals, which primarily take the form of bundles of sharp raphides, fail to impact the growth of this generalist herbivore? One thought is that the herbivores in our bioassay experiment were able to avoid the crystals. Some evidence suggests that mature caterpillars are able to sense CaOx crystals and avoid them (Doege 2003), an advantage that small arthropod herbivores might have over mammal herbivores. We hypothesize that the caterpillars in our study were able to avoid the larger raphide needles and druses, but not the smaller amorphous crystals used for the crystal shape control, consistent with the result that amorphous crystals *did* have a negative impact on herbivore growth. Since no herbivores ate all the provided diet, it is possible that the larvae were not forced to eat the *Vitis*-derived crystals due to food scarcity. In the first five days of eating, the growth curves of caterpillars on the control, raphides, and amorphous crystals treatments closely follow each other, implying that the impact of the amorphous CaOx in the diet was not immediate. Gradually, the amorphous crystal growth curve begins to separate from the other two treatment curves, which might indicate that the impact of the crystals is cumulative. It is important to note that while mandible wear may be a component of this gradual effect, *S. exigua* caterpillars shed their mandibles upon transition between instars, meaning that any growth impact of worn mandibles on the larvae is likely only temporary.

In addition, *Vitis*-derived CaOx crystals did not modify the effect of leaf chemistry on herbivores in our bioassays. This is in contrast with Konno et al., (2014), who found that kiwi-derived raphides had a negative impact on growth in the short term, but no change in mortality. There are several reasons why our results may contrast with those of Konno (2014). One key factor is that the caterpillars used in the 2014 study were neonates while our larvae were around 3rd instar when placed on the diet. It is thus possible that raphides are only a detriment to young lepidopteran larvae, and that at larger sizes, the larvae can tolerate the presence of crystals. Additionally, the raphides in the Konno bioassay were applied to the surface of castor oil leaves, rather than mixed into artificial diet, leaving the possibility that the crystals produced a physical pre-ingestive effect on the neonate larvae that was absent when the raphides were mixed into our diet cups. Ultimately, our results do not support a pre- or post-digestion defense function for calcium oxalate crystals in grape.

In general, we saw that the presence and abundance of druse crystals in *V. riparia* leaves across all treatments were more variable than those of raphides, suggesting that druses are more plastic and perhaps more ephemeral than raphide bundles. We predicted that druse crystals would be sensitive to calcium levels in the soil, while raphides would not be, and our results that the densities of both crystal types increase in calcium-rich soil support the former, but not the latter prediction. Calcium oxalate crystals have long been hypothesized to be a dynamic storage system for calcium in plants (Volk et al. 2002; Franceschi and Nakata 2005; Paiva 2019). Mounting evidence suggests that there are both environmental and genetic components to the phenotypic expression of CaOx crystals (Webb 1991). Calcium oxalate crystal formation can be genetically engineered in plant species that do not naturally form these crystals (Nakata 2012a), and evidence suggests that different crystal types are formed via different biosynthetic pathways (Nakata and McConn 2007). In the case of raphides, bundles of needle-crystals which are formed in specialized vacuoles called idioblasts, cell-direction is almost definitely implicated (Franceschi & Nakata, 2005). However, our understanding of the genetic regulation of crystal morphology and abundance is still nascent. Another investigation of differential inducibility of CaOx crystal morphologies found that moving *Pistia stratiotes* plants to calcium scarce conditions from control concentrations caused a decrease in druses but no change in raphides, demonstrating that druses seem to be more responsive to soil calcium than raphide bundles in that system (Volk et al 2002). In that study, both types of crystals were more prevalent and larger in high calcium conditions. Similarly to *V. riparia*, druse crystals of *P. stratiotes* form near veins, while raphides are in the aerenchyma (Volk et al. 2002).

A growing body of work has implicated calcium oxalate crystals in “alarm photosynthesis,” whereby carbon is collected and stored as CaOx crystals (Tooulakou et al. 2016a,b). In drought conditions when stomata are closed, the crystals are hypothesized to be degraded by oxalate oxidase to provide CO_2 for photosynthesis, with H_2O_2 and Ca^{2+} ions as biproducts (Tooulakou et al. 2016a). In studies of pigweed, levels of CaOx crystals fluctuate throughout the day, and were shown to decrease when plants encountered artificial CO_2 stress conditions (Tooulakou et al. 2016a, 2019). The location of *V. riparia* druses in bundle sheath cells near leaf veins supports their role in this biosynthetic pathway. Indeed, work on *V. vinifera*, a cultivated congener of *V. riparia*, found that druse crystal, but not raphide crystal densities, decreased slightly during the day, and more dramatically after application of a water stress treatment (Kolyva et al. 2023). Water availability was not variable across our treatments, and we expect that variation in crystal abundance in our plants was minimally influenced by water availability, and instead that we primarily measured variation due to calcium availability and leaf damage. Regardless of this untested additional component to CaOx expression in *V. riparia*, our results support the hypothesis that CaOx abundance is tied to calcium availability.

4.6 CONCLUSIONS

Here we add to the growing body of scholarship elucidating the causes and consequences of calcium oxalate crystal formation in plants, explicitly testing the inducibility and putative defensive function of these mysterious structures. We provide evidence that calcium oxalate production can be induced by excess calcium, and that *Vitis*-derived crystals do not impair the growth and survival of a generalist chewing herbivore. This work sheds light on the causes and consequences of CaOx production in *Vitis riparia*, and generates new questions about the context dependency of CaOx ecology.

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Chapter 5 : Characterizing the Phylogenetic and Geographic Distribution of Calcium Oxalate in Vascular Plants

5.1 ABSTRACT

Despite being discovered over 300 years ago, there is still active debate about the ecological functions and evolutionary origins of calcium oxalate crystals in vascular plants. Here, we describe a systematic literature survey of the presence, morphology (raphide, prism, druse, styloid, crystal sand, or other), and tissue location of calcium oxalate crystals in vascular plants. Using this incidence data, we additionally tested for geographic trends in calcium oxalate presence, aimed at shedding light on the processes that have generated and maintained variation in this trait. Our survey revealed calcium oxalate crystals in over 2000 plant species from 219 vascular plant families, and that particular crystal morphologies are not restricted to specific parts of the plant phylogeny nor specific plant organs. Phylogenetic regressions of crystal morphotype presence and geographic variables revealed positive associations between the presence of needle-like raphide crystals and equatorial, humid environments, while prismatic crystals were associated with arid, more poleward environments. We hypothesize that these patterns may result from raphides playing a primary role in plant defense against herbivory, since herbivory is higher at more equatorial latitudes, while prismatic crystals may play a role in plant tolerance to drought. Our results indicate that calcium oxalate biomineralization is an ancient trait common to angiosperms, gymnosperms, and ferns, and that the evolution of different crystal morphologies may have been influenced by different selective forces.

5.2 INTRODUCTION

Calcium oxalate (CaOx) biomineralization occurs in many forms across all five kingdoms of living organisms (Milton and Axelrod 1951; Franceschi and Horner 1980; Gadd et al. 2014; He et al. 2014; Pueschel 2019). Reports of CaOx crystals in plant tissues date back to the invention of microscopy (van Leeuwenhoek 1675), and for just as long plant biologists have been hypothesizing about CaOx crystal function. Many early descriptions of CaOx crystals appear in plant family descriptions. A list of these early reports was tabulated by McNair (1932), who found that 215 plant families contained CaOx crystals, which together represented diverse morphologies. Since McNair's work in 1932, the distribution and form of CaOx crystals has been documented in a variety of new plant taxa, and has been reviewed thoroughly within a small number of plant clades. The order Piperales (Horner et al. 2015), families Araceae (Keating 2004), Cactaceae (Hartl et al. 2007), and Oleaceae (Lersten and Horner 2009), tribe Cardueae (Kartal 2016), and genus *Prunus* (Lersten and Horner 2000) all have particularly strong documentation of the occurrence and diversity of these crystals within their plant parts. In 2023, Lawrie and colleagues published a systematic review of the incidence of raphide needle morphology of CaOx crystals in angiosperms, finding that these needle crystals are present in 46 plant families and almost 800 species. The morphology and presence/absence of CaOx crystals varies considerably across plant species, but the phylogenetic distribution and evolutionary origin of this variation is unexplored.

Of the different types of CaOx crystals documented thus far in plants, the needle-like raphide form is the most well-studied (Lawrie et al. 2023). However, other common CaOx morphologies include prisms, rod-shaped styloids, spheroids called druses, and small grains called crystal sand (Franceschi and Horner 1980; Prychid and Rudall 1999; Webb 1999; Franceschi and Nakata 2005). Calcium oxalate crystals are naturally produced through a combination of calcium uptake and oxalic acid biosynthesis (Khan et al. 2023). The morphology of CaOx crystals is hypothesized to be determined by the size, orientation, and intravacuolar matrix composition of the specialized vacuoles within idioblast cells that the crystals form within (Webb 1999; Nakata 2012b; Cuéllar-Cruz et al. 2020; Khan et al. 2023). There is also evidence that alternate genes and biosynthetic pathways play a role in crystal morphology differentiation (Nakata 2003; Nakata and McConn 2007). However, despite this variation, CaOx crystals are consistent enough in form and location within a species that they can be used as diagnostic characters (e.g. Gerrath et al., 2015; North & Willis, 1971).

Several articles review possible functions of plant CaOx crystals, frequently citing storage, detoxification of heavy metals, and defense against herbivory as the leading hypotheses (Franceschi and Nakata 2005; Tooulakou et al. 2016b; Paiva 2019, 2021; Cuéllar-Cruz et al. 2020; Karabourniotis et al. 2020a; Khan et al. 2023; Lawrie et al. 2023). There has been conjecture that CaOx crystals of different morphologies have different physiological or ecological functions in plants; for example, needle-like raphides might be more likely to play a role in defense, while druses could primarily be a dynamic storage system for calcium and/or CO_2 (Volk et al., 2002). For example, needle CaOx crystals are implicated in the irritating mouthfeel of kiwifruit for humans (Perera et al. 1990), and decrease the weight gain of chewing arthropod herbivores, especially when in conjunction with toxic phytochemicals (Konno et al. 2014). Other work on CaOx crystal formation and function has implicated the crystals in storage of calcium, as well as of CO_2 for plants in drought stressed conditions. This CO_2 storage pathway, termed “alarm photosynthesis” proposes that CaOx is a dynamic storage system for CO_2 that plants can access when they have closed their stomata to stem water loss (Tooulakou et al. 2016b,a, 2019).

These different hypothesized functions for CaOx crystals in plants generate different macroevolutionary and macroecological predictions, which are largely untested. If CaOx crystals act as defense in plants, we might expect their incidence to correlate negatively with distance from the equator. Latitudinal gradients in plant defense have long been hypothesized to result from greater herbivory pressure at equatorial latitudes (Schemske et al. 2009; Salazar and Marquis 2012). Plants in more tropical climates also have longer-lived leaves and are subject to less seasonality. Taken together, these observations lead to the prediction that tropical plants should evolve higher levels of defense (the latitudinal herbivory defense hypothesis; Anstett et al., 2016; Coley & Aide, 1991; Coley & Barone, 1996). In McNair's (1932) systematic investigations of CaOx presence across plants, he observed that the majority of CaOx bearing plant families are native to the tropics, providing preliminary evidence that herbivory pressure could be a driver of CaOx crystal evolution. Alternatively, under the calcium storage hypothesis, we might expect a correlation between CaOx crystal evolution and aridity. Several papers have observed that plants native to arid environments have more crystals in their tissues (Ci et al. 2010; Karabourniotis et al. 2020b). Indeed, members of the Cactaceae, which are known for their adaptations to living in water-stressed conditions, are rife with CaOx crystals that are diverse in form (Garvie 2006; Hartl et al. 2007). Thus, testing these compelling hypotheses about CaOx crystal macroevolution would advance our understanding of their ecological and physiological functions.

Despite the significant advancements in phylogenetic comparative methods that have occurred since the last systematic investigation of CaOx crystals of all types in plants, we lack a modern, comprehensive synthesis of the phylogenetic and geographic distribution of this trait, limiting our ability to hypothesize about and test CaOx crystal evolution. Here, we sought to aggregate and synthesize studies of CaOx occurrence in plant structures to characterize the phylogenetic distribution of this trait and analyze the presence and morphology data using phylogenetic comparative methodology to visualize and assess the patterns of this trait's evolution. Specifically, the aims of this study were to 1) describe the phylogenetic distribution of CaOx crystal types, and 2) test for associations between CaOx crystal presence and environmental predictors such as aridity and latitude consistent with the hypotheses that the crystals play a role in drought tolerance and defense.

5.3 METHODS

5.3.1 Calcium oxalate crystal records

We gathered all papers available on Web of Science that matched the terms "calcium oxalate" AND "plant" AND "crystal." We then manually confirmed that papers were primary sources on CaOx presence and contained species-level information on the crystal morphology (raphide, prism, druse, styloid, crystal sand, or other), as well as the location of the crystals in the plant (flower, fruit, leaf, root, seed, or stem). Because of the prevalence of other phytoliths in plants, we verified that each paper referred to crystals formed specifically from CaOx and not other compounds such as silica. For the purposes of thoroughly describing the phylogenetic distribution of CaOx morphologies, we included additional non-primary source records from two prior systematic reviews (McNair 1932; Lawrie et al. 2023). Note that in converting the crystal data from McNair 1932, we classified "typical crystals" as druses for our purposes based on process of elimination. This family-level data is included in the family-level tree (Fig 1), but not in the species-level analyses because McNair only contained family-level crystal occurrences, and did not specify tissue type.

We applied taxonomic corrections to the dataset using the function “TNRS” in the R package TNRS (Boyle et al. 2013). We then manually assigned taxonomic corrections to families that TNRS was unable to correctly update using World Flora Online and the World Checklist of Vascular Plants species list (Govaerts et al. 2021; Elliott et al. 2024). Species that were unable to be matched to accepted species names were excluded from downstream analyses.

5.3.2 Geographic data collection and cleaning

We downloaded occurrence data from the Global Biodiversity Information Facility (GBIF) database using the R package rgbif (Chamberlain et al. 2012; GBIF.org 2025). We limited our search to occurrences that accompanied preserved specimens so that the records were of research grade. We subjected the occurrences to further cleaning using the function “clean_coordinates()” from the R package CoordinateCleaner (Zizka et al. 2019). We specifically used the "capitals", "centroids", "duplicates", "equal", "gbif", "institutions", "zeros", “outliers” and “seas” tests to account for typical biases and errors present in GBIF data. Additionally, approximately 1.5% of the taxa in the dataset are classified as commonly cultivated according to the (World Programme for the Census of Agriculture 2010 2005). For the purposes of our geographic analyses, we excluded these taxa to reduce the risk of agricultural crops distorting natural species ranges. We downloaded aridity index data from the Global Aridity Index and Potential Evapotranspiration Database (Zomer et al. 2022) for all occurrences in our dataset using the R package raster (Hijmans 2022). We then calculated mean absolute value of latitude and aridity index values for each of the species used in this study.

5.3.3 Statistical analyses

To visualize the family-level phylogenetic distribution of CaOx crystal morphologies, we used the GBOTB extended mega tree of vascular plants (Zanne et al. 2014; Smith and Brown 2018). We plotted the tree using the function “plotFanTree.wTraits” in the R package phytools (Revell 2012). We generated a species-level phylogenetic tree of the 1615 species in our dataset using “phylo.maker” in the R package V.PhyloMaker using the scenario three parameter (Jin and Qian 2019). This function also uses the GBOTB mega tree.

To eliminate the influence of duplicate crystal observations for any given species, we only counted a species as having a crystal morphology in a particular organ once. We assumed that other crystal morphologies were absent in a given organ in species with another type of morphology reported. For example, a species with raphides and druses in their leaves was assumed to not have prisms, styloids, or crystal sand morphologies in its leaves. However, within our data we did not assume that just because crystals have not been found in a species’ organ they are not there. For example, a species with a record of CaOx in its leaves was not assumed to lack crystals in its flowers, fruit, seeds, stems, or roots.

We used phylogenetic binomial logistic regressions implemented via the “glmmadmb()” function with a brownian motion correlation structure using the species-level tree generated above (Skaug et al. 2010). In these models, we analyzed the relationship between crystal type presence/absence within a tissue and aridity or the absolute value of latitude. Available data for all tissue types were included in these analyses.

5.4 RESULTS

Starting with a list of 900 papers, we collected data on 2596 taxa from 394 scientific papers on CaOx occurrence. Our systematic survey of the literature revealed that 2325 species and 219 families of vascular plants have CaOx crystals in at least one of their organs (Fig. 5.1). Our work thus expands the list of vascular plant families known to have CaOx biomineralization by about 30 families, bringing the total to almost half of all currently recognized vascular plant families (Elliott et al. 2024). Raphides are the most commonly reported CaOx crystal type in the literature, with prisms and druses the second and third most reported morphologies respectively (Appendix Table C.1; Fig. 5.1). Most crystal observations are made from plant leaves, with flowers and stems also having many crystal occurrences. Compared with counts of raphides in flowers (194 species) and stems (189 species), prisms are relatively more commonly found in these plant parts (238 species for flowers, 300 for stems; Appendix Table C.1).

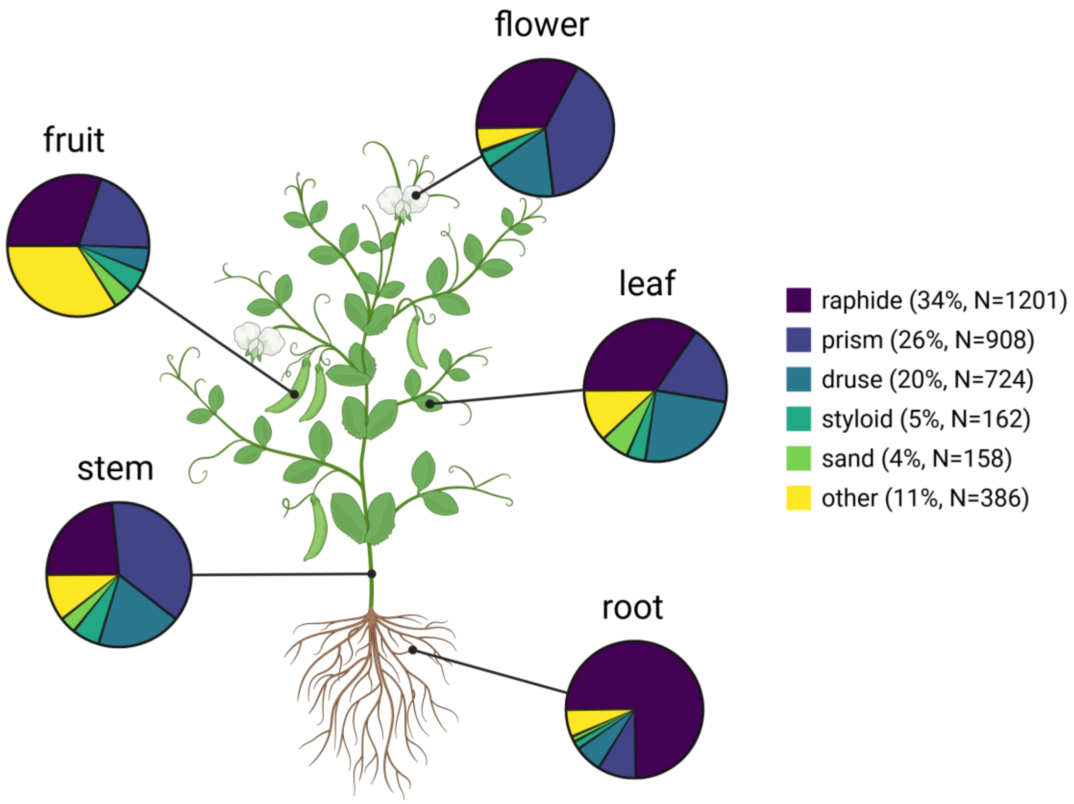

Figure 5.1 Distribution of calcium oxalate crystal morphologies across plant organs, incorporating records of 2,596 vascular plant species. Created in BioRender. Graham, C. (2025) https://BioRender.com/qeg4zji

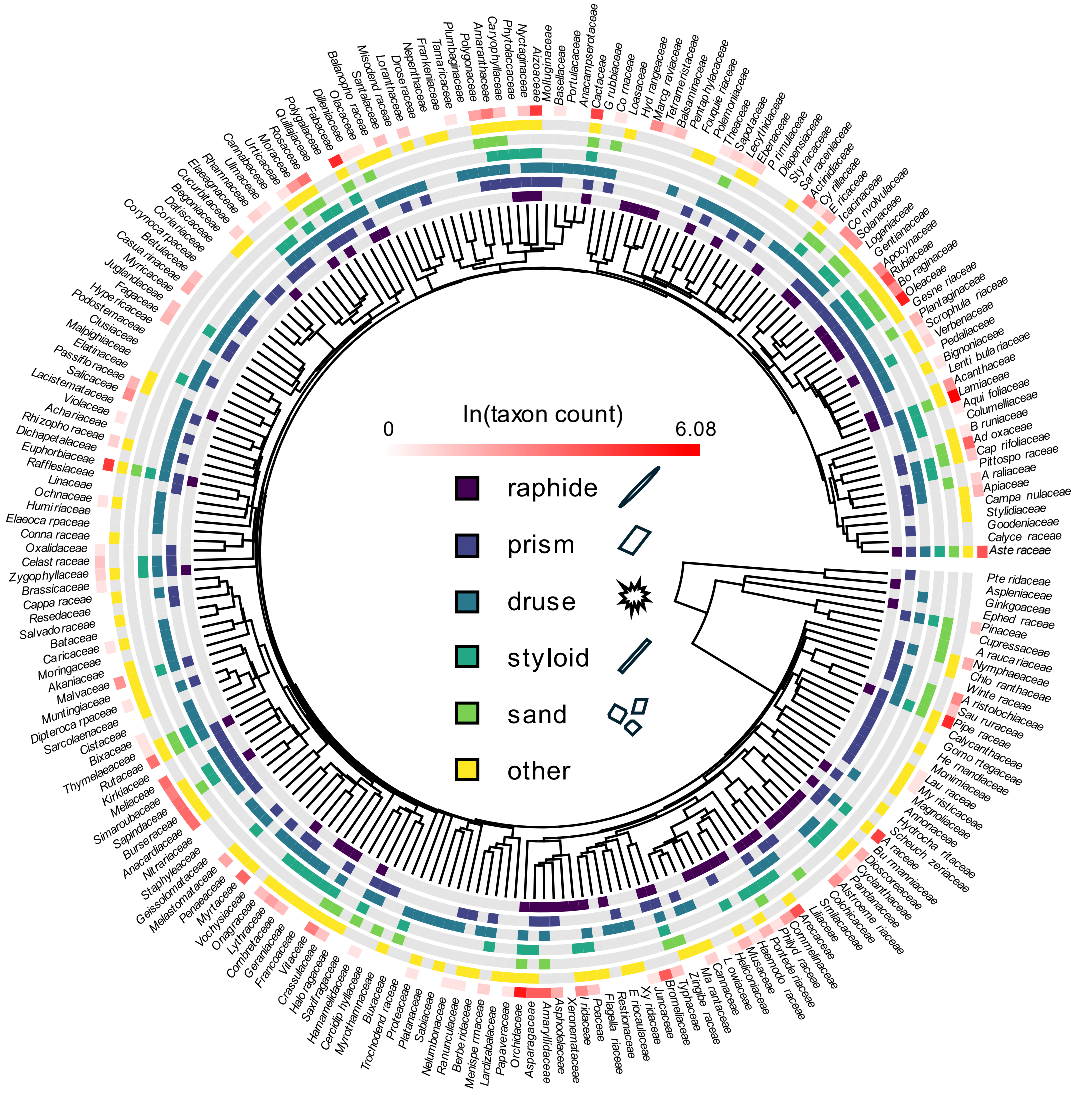

Figure 5.2 Phylogenetic distribution of calcium oxalate crystals across vascular plants. Crystal presence squares are filled in for a family if at least one taxon in that family has a particular crystal morphology in any tissue type. The red squares indicate the number of taxa sampled per family, plotted on a log scale to facilitate interpretability.

We found that associations between crystal presence and geographic parameters were crystal-morphology specific (Table 5.1). Across plant species, raphide presence within sampled tissues was more likely at equatorial latitudes (test of latitude), and within more humid environments (test of aridity). These trends are consistent with the hypothesis that raphides are a form of defense and thus can be expected to be more common in tropical environments according to the latitudinal herbivory defense hypothesis. Prismatic crystals on the other hand showed the opposite trend, where crystals were more likely to be present at more poleward latitudes, and were also more likely to be present in more arid environments. The geographic pattern evidence for prismatic crystals is thus more supportive of a drought-stress function for this crystal morphology. We detected no significant relationship between the druse, styloid, or crystal sand morphologies and either geographic predictor.

Table 5.1 Estimated effect sizes of latitude and aridity on crystal presence across species. Effect sizes are given as the increase in the log odds of having a crystal type present in a sampled tissue for every 1° in latitude or 1 unit increase in aridity score (n = 2835 tissue observations from 2319 species).

	Effect of latitude	p-value	Effect of aridity	p-value
raphide	**-0.041 ± 0.0159**	**0.010***	**1.314 ± 0.378**	**0.001****
prism	**0.013 ± 0.004**	**0.002****	**-0.618 ± 0.109**	**1.6E-08*****
druse	0.007 ± 0.018	0.698	-0.423 ± 0.458	0.356
styloid	0.037 ± 0.030	0.219	-0.339 ± 0.780	0.664
crystal sand	-0.0001± 0.038	0.997	0.116 ± 0.886	0.896

5.5 DISCUSSION

Here, we demonstrate the wide breadth of vascular plant clades that biomineralize CaOx. The morphologies of calcium oxalate crystals are widely distributed across plants. Since the work of McNair (1932) many families that were once distinct have been combined. Thus, our updated list adds about 30 families to the ~190 families of vascular plants that contain CaOx biomineralization identified by McNair. This means that an astounding 45% of recognized plant families contain taxa with CaOx biomineralization. This ubiquity suggests that CaOx crystals may have evolved early in the evolution of vascular plants, especially given that the ferns *Acrostichum aureum* and *Hymenasplenium cardiophyllum,* and gymnosperms such as *Pinus flexilis, Juniperus osteosperma,* and *Ginkgo biloba* biomineralize CaOx (Anthoons 2017; de

Arruda et al. 2021; Malekhosseini et al. 2022; Lawrie et al. 2023; Pearce and Pearce 2023). However, not all plants produce CaOx crystals, and when present, crystals can take a variety of shapes, leading to the alternative hypothesis that CaOx crystals originated independently many times in different lineages (Franceschi and Nakata 2005). Calcium oxalate phytoliths do not preserve well in plant fossils, limiting our ability to gain insight to their evolution from the fossil record (Malekhosseini et al. 2022). Thus, systematic investigations describing their phylogenetic distribution like this work are some of our best tools for learning about CaOx crystal evolution.

The geographic patterns in crystal morphology presence we observed support the hypothesis that different CaOx crystal morphologies have different functions in plants. For example, we found that raphide crystals are more likely to be present in plant tissues of species native to more equatorial environments, a pattern that substantiates the observation McNair (1932) made almost 100 years ago. This pattern of raphide presence is consistent with the hypothesis that raphides play a role in plant defense, because plants at more equatorial latitudes are predicted to invest more in traits that provide defense against the high levels of herbivore diversity present in those regions (Salazar and Marquis 2012). Raphide crystals are commonly implicated in defense against herbivory, especially in the case of chewing herbivores. For mammal herbivores, raphides reduce grazing, and ingesting the crystals causes irritation to the mouth and digestive system (Perera et al. 1990; Wood et al. 1997; Bradbury and Nixon 1998; Ruiz et al. 2002). For chewing arthropod herbivores, the efficacy of CaOx as a defense has been demonstrated to be enhanced by plant secondary metabolism (Konno et al. 2014). Thus, future work should examine whether there are evolutionary correlations between raphides and certain classes of secondary metabolites, such as the cysteine proteases found to cause acridity kiwifruit or the allergenic proteins associated with taro raphides (Paull et al. 1999, 2022; Konno et al.

2014). The family Araceae may be particularly promising for this work due to the high densities and acridities of raphides in these taxa (Gardner 1994; Coté and Gibernau 2012; Valenzona 2023).

In contrast to raphide crystals, we found that across species, prismatic crystals were more likely to be observed in organs of plants from more arid environments, suggesting that they may play a role in drought stress. Prismatic crystals have been ascribed a variety of functions in plants, including heavy metal detoxification and defense against herbivory (Mazen 2004). Indeed, prisms in *Medicago truncatula* have been observed to decrease the growth and increase the mortality of chewing insect herbivores (Korth et al. 2006; Park et al. 2009); thus, we do not suggest that prisms only function for drought stress tolerance. Prisms are considered one of the most simplistic crystal shapes, and are commonly observed in cell walls and cells surrounding leaf vasculature (Nakata and McConn 2000, 2007; Franceschi and Nakata 2005; Nakata 2012b). In our characterization of patterns in CaOx, prisms are the second most commonly observed morphology, and are widely found in plant leaves, stems, and flowers across vascular plants. One mechanism by which CaOx crystals may function in drought stress is through the alarm photosynthesis pathway, by which plants accumulate CO_2 and store it as oxalate for photosynthetic use when their stomata are closed. Thus far, only druse CaOx crystal density has been found to fluctuate with photosynthetic rates, particularly in *Amaranthus hybridus* and *Vitis vinifera*, though the size of both druses and raphides also seems to be negatively impacted by CO_2 starvation (Tooulakou et al. 2016b,a, 2019; Kolyva et al. 2023). Prior work on CaOx temporal plasticity has indicated that druses may be more readily broken down than raphides (Volk et al. 2002). Karabourniotis et al (Karabourniotis et al. 2020a) found that across clades native to regions with lower mean annual precipitation had greater proportions of species with

CaOx crystals, though we believe this analysis was agnostic to the crystal morphology. We suggest that future work should examine the potential for prismatic crystals to participate in alarm photosynthesis similarly to the druse crystal type.

The lack of geographic patterns in the incidence of druse, styloid, and sand crystal morphotypes might indicate that these crystal types are more ephemeral in plant tissues, or that their function is not tied to ecological interactions that tend to correlate with environment. Indeed, the aforementioned work on druses in *A. hybridus* and *V. vinifera* has found that these crystals vary substantially in number and size during the day, meaning that standardized sampling times must be implemented in order to detect their presence across plant samples (Tooulakou et al. 2016a, 2019; Kolyva et al. 2023). Other hypothesized function for calcium oxalate in plants is as a storage system for calcium and heavy metal detoxification, functions that may yield more complex geographic patterns in CaOx incidence(Nakata 2003; Franceschi and Nakata 2005; He et al. 2014). More detailed examination of calcium oxalate crystal type presence across plant organs may provide further clues to their functions. For example, Karabourniotis et al (2020) speculate that root CaOx may function in storage and metal detoxification and leaf CaOx in alarm photosynthesis, with CaOx in all structures functioning in defense. We should also note that is also possible that geographic associations for other crystal morphologies do exist, but are of small enough effect that they were not able to be detected in these analyses. Thus, more sampling must be conducted to obtain a more complete picture of the ecological parameters associated with CaOx production.

Our literature search indicates that most plant clades have not been thoroughly surveyed for CaOx presence. Qualitatively, plant families with high sampling have a higher diversity of crystal morphologies in their plant tissues. The families Asteraceae, Piperaceae, Araceae, Aracaceae, Orchidaceae, Euphorbiaceae, Fabaceae, and Oleaceae all have high numbers of sampled taxa in this dataset, and this high crystal diversity. However, these families also have large numbers of accepted taxa (especially Asteraceae), and thus only a small percentage of taxa in each of these families have actually been sampled. Unlike some plant traits, CaOx presence is not easy to score without destructive, often lab-based sampling. Common sampling techniques including clearing and staining leaves to look at them under microscopy or burning plant material to isolate and measure the non-combustible crystals left behind (Ensikat et al. 2023). Confirming that crystalline structures in plants are indeed made of CaOx (and not for example silica) also requires sophisticated techniques such as acid solubility comparisons, IR spectroscopy, or scanning electron microscopy combined with electron dispersive x-ray spectroscopy (Molano-Flores 2001; Pueschel 2001; Baran et al. 2010; Sokol et al. 2024). These challenges mean that records of CaOx *absence* are also hard to come by, limiting our ability to perform ancestral state reconstructive methods to estimate gains and losses of CaOx morphologies across the plant phylogeny.

5.6 CONCLUSIONS

Here, we confirm that CaOx biomineralization is phylogenetically and geographically widespread in vascular plants. Additionally, we provide evidence that differential ecological interactions have shaped the evolution of different CaOx crystal morphologies. This database is valuable for both testing and generating hypotheses about the ecology and evolution of these mysterious crystals. Yet, more sampling is needed to make better predictions about when and how many times CaOx biomineralization evolved in plants. Future directions include testing for associations between crystal types and plant functional traits, such as leaf longevity, photosynthetic activity, and woodiness.

5.7 CITATIONS

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Chapter 6 : Concluding Remarks

Evolutionary biologists hypothesize that herbivory pressure has led to the evolution of the observed broad array of plant defensive traits with varied modes of action. My work indicates that biotic and abiotic ecological pressures have interacted to produce patterns of plant trait expression that vary across levels of biological organization and environmental gradients. Together, chapters two and three of this dissertation reveal that correlations related to specialization in defensive strategies, such as direct vs indirect defense and physical vs chemical defense may be more likely to be observed at the interspecific scale, while correlations in defenses that likely result from physiological constraint are more prevalent within species. I thus suggest that future comparative work on correlated defense evolution involve hypothesis testing at multiple biological scales in order to gain more insight into the evolutionary mechanisms that have produced patterns in trait variation. Also, as the mechanism behind the trade-off pattern observed in chapter two is unclear, additional work should be conducted to test whether plants do risk poisoning their bodyguards by investing in both attracting bodyguards and producing toxic metabolites. To my knowledge, no studies have tested this potential ecological interference between direct and indirect defenses; instead, prior work on this trade-off pattern primarily cites avoiding redundancy as the cause for such a pattern (e.g Ballhorn et al., 2008; Dyer et al., 2001; Rehr et al., 1973).

Taken together, my final two chapters reveal that tests of mechanisms can produce results that are at odds with broad scale patterns in trait expression. Specifically, within *Vitis riparia,* calcium oxalate crystals do not seem to contribute to defense against herbivory for a generalist lepidopteran herbivore. However, patterns in the presence of calcium oxalate raphide crystals across taxa correlate with proximity to the equator, a pattern we would expect for traits that provide plants with defense against herbivores. This dissertation therefore provides valuable clues to the evolution and function of calcium oxalate crystals in plants. My work, along with recent reviews of the field of calcium oxalate research, indicates that these crystals may have multiple functions in plants, and that it is likely that different crystal morphologies contribute to these different functions (Volk et al. 2002; Konno et al. 2014; Khan et al. 2023; Lawrie et al. 2023). Selection on a given trait is the result of a variety of pressures. Any given trait can have a multitude of functions that facilitate biotic and abiotic ecological interactions. Indeed, few defense-related traits are believed to have a single function (an important exception of which is domatia, which are currently only demonstrated to function in attracting beneficial mites to a leaf's surface). I thus propose that the pairing of experimental and comparative approaches for studying evolution can paint a more complete picture of the selective forces that act on multi-functional traits, such as calcium oxalate crystals.

To conclude what I have learned about calcium oxalate evolution and ecology, I argue that, based on the results of chapters four and five, calcium oxalate crystal presence and density is influenced by environmental calcium levels, and may have primarily evolved to serve as a dynamic storage system, whether that be storage of calcium or carbon dioxide. However, I propose that, despite the results of my fourth chapter, raphides are a calcium oxalate crystal morphology that have evolved to provide defense in some plant taxa. The efficacy of this morphology as a defense may be related to the size or density of the crystal bundles, or the pairing of the crystals with irritating secondary metabolism (Konno et al. 2014; Paull et al.

2022). Thus, future comparative work should be conducted to characterize the size of raphides across plant taxa, and to identify whether there is evidence of a repeated evolutionary association between this crystal morphology and classes of metabolites or metabolite diversity. Additionally, isolating raphides of different sizes from plants and performing bioassays similar to those described in chapter 4 could illuminate the roll of crystal size in defense. Finally, I suggest that future bioassays test the functions of raphides as a defense against a variety of herbivores to determine whether, as suggested by Paiva (2021), chewing arthropod herbivores are insulated from the impact of these crystals due to their anatomy.

In sum, my work reveals that the discordance between theory and observed patterns in defenses may be the result of conflicting ecological and physiological pressures at different levels of biological organization. This discordance can only be alleviated through repeated testing of theory through both comparative and experimental means, as I have done here (Weber and Agrawal 2012; Agrawal 2020). My work is powerful because I approach patterns of plant defense evolution from multiple biological scales, such as from across the plant kingdom to within individual species. Additionally, I examine defensive traits that are often overlooked in studies of plant defense, such as mite domatia, food bodies, and calcium oxalate crystals. Thus, my dissertation provides a more holistic picture of plant defense expression in an attempt to address the conflicting patterns predicted by evolutionary theory.

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Appendices

Appendix A: Supplementary Material for Chapter 2

Appendix Table A.1 List of genetic markers with corresponding best nucleotide substitution model fits.

Marker Name	Supercrunch Search Term 1	Supercrunch Search Term 2	Nuclear or plastid	Model
At103	At103	Mg-protoporphyrin monomethyl ester cyclase	nuclear	HKY+I
atpBrbcL	atpB-rbcL;rbcL-atpB	atpB-rbcL intergenic spacer;complete genome	chloroplast	HKY+I
atpHatpF	atpH-atpF	atpH-atpF intergenic spacer;complete genome	chloroplast	HKY+I
GAI1	GAI1	GAI-like protein 1	nuclear	HKY+I
GM1165	1165	gene marker 1165 genomic sequence	nuclear	HKY+I
GM1313	1313	gene marker 1313 genomic sequence	nuclear	GTR+I
GM1314	1314	gene marker 1314 genomic sequence	nuclear	HKY
GM1973	1973	gene marker 1973 genomic sequence	nuclear	HKY+I
GM2129	2129	gene marker 2129 genomic sequence	nuclear	HKY+I
GM241	241	gene marker 241 genomic sequence	nuclear	HKY+I
GM2415	2415	gene marker 2415 genomic sequence	nuclear	HKY+I
GM3221	3221	gene marker 3221 genomic sequence	nuclear	HKY+I
GM3389	3389	gene marker 3389 genomic sequence	nuclear	HKY
GM5069	5069	gene marker 5069 genomic sequence	nuclear	HKY
GM5693	5693	gene marker 5693 genomic sequence	nuclear	JC+I
GM590	590	gene marker 590 genomic sequence	nuclear	HKY+I
GM6054	6054	gene marker 6054 genomic sequence	nuclear	HKY
GM6670	6670	gene marker 6670 genomic sequence	nuclear	HKY+I
GM689	689	gene marker 689 genomic sequence	nuclear	HKY+I
GM7022	7022	gene marker 7022 genomic sequence	nuclear	HKY
GM7029	7029	gene marker 7029 genomic sequence	nuclear	HKY+I
GM7230	7230	gene marker 7230 genomic sequence	nuclear	GTR+I
GM7312	7312	gene marker 7312 genomic sequence	nuclear	HKY+I
GM7362	7362	gene marker 7362 genomic sequence	nuclear	HKY+G
GM7386	7386	gene marker 7386 genomic sequence	nuclear	HKY+I
GM7413	7413	gene marker 7413 genomic sequence	nuclear	GTR+I
GM7434	7434	gene marker 7434 genomic sequence	nuclear	GTR+I
GM7447	7447	gene marker 7447 genomic sequence	nuclear	HKY+G
GM765	765	gene marker 765 genomic sequence	nuclear	GTR+G
matK	matK	maturase K;complete genome	chloroplast	HKY+I
PHYA	PHYA	phytochrome A1	nuclear	HKY+I
psbA_trnK	psbA-trnK;trnK-psbA	psbA-trnK intergenic spacer;(psbA) gene;complete genome	chloroplast	HKY+I
psbAtrnH	psbA-trnH;trnH-psbA	psbA-trnH intergenic spacer;complete genome	chloroplast	HKY+I
RPB2	RPB2	RNA polymerase II second largest subunit	nuclear	HKY+I
rpl32trnL	rpl32-trnL;trnL-rpl32	rpl32-trnL intergenic spacer;complete genome	chloroplast	HKY+I
rpoBtrnC	rpoB-trnC;rpoB;trnC	rpoB-trnC intergenic spacer;complete genome	chloroplast	HKY+I

rps16	rps16	ribisomal protein S16;complete genome	chloroplast	GTR+I
SQD1	SQD1	SQD1 gene	nuclear	HKY+I
trnCpetN	trnC-petN;petN-trnC	trnC-petN intergenic spacer;complete genome	chloroplast	HKY+I
trnFndhJ	trnF-ndhJ;ndhJ-trnF	trnF-ndhJ intergenic spacer;complete genome	chloroplast	GTR+I
trnK	trnK	tRNA-Lys;complete genome	chloroplast	GTR+I
trnKrps16	trnK-rps16;rps16-trnK	trnK-rps16 intergenic spacer;complete genome	chloroplast	HKY+I
trnL	trnL	tRNA-Leu;complete genome	chloroplast	HKY+I
trnStrnG	trnS-trnG;trnG-trnS	trnS-trnG intergenic spacer;complete genome	chloroplast	HKY+I
trnTtrnL	trnT-trnL;trnL-trnT	trnT-trnL intergenic spacer;complete genome	chloroplast	HKY+I
ycf1	ycf1	ycf1 gene; putative RF1 ;complete genome	chloroplast	GTR+I

Appendix Table A.2 Correlations between LCMS peaks in the 440-640 ppm RMD range (typical of terpenoids) and our other *Vitis* leaf traits.

	Domatia index		Trichome score		Mass consumed	
	Effect size	p-value	Effect size	p-value	Effect size	p-value
Abundance	-	0.572	-	0.585	-	0.0945
Richness	-	0.168	-	0.213	-8.06E-5	0.000612***
Diversity	-6.34	0.0212*	-	0.140	-5.15E-2	0.000202***

Appendix Table A.3 Correlations between LCMS peaks in the 100-250 ppm RMD range (typical of flavonoid compounds) and our other *Vitis* leaf traits.

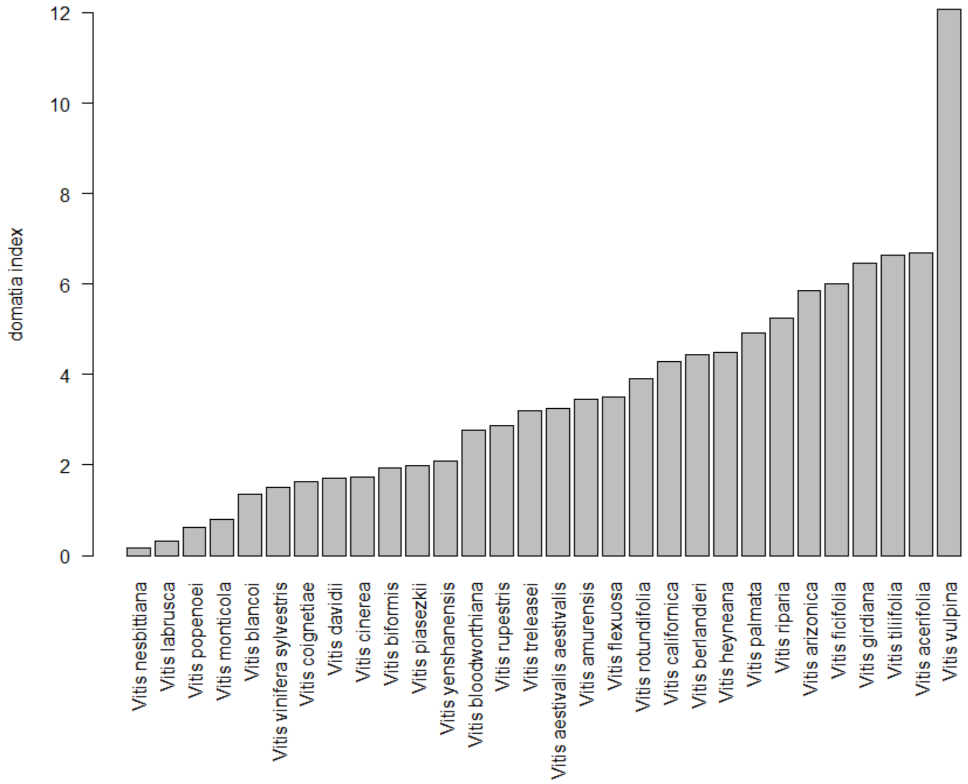
	Domatia index		Trichome score		Mass consumed	
	Effect size	p-value	Effect size	p-value	Effect size	p-value
Abundance	-	0.344	-3.61E-7	0.00597**	-	0.194
Richness	-	0.196	-	0.0521	2.32E-4	0.0230*
Diversity	2.38	0.0437*	-	0.287	1.12E-2	0.0391

Appendix Table A.4 Correlations between LCMS peaks in the 200-400ppm RMD range (typical of phenolic compounds) and our other *Vitis* leaf traits.

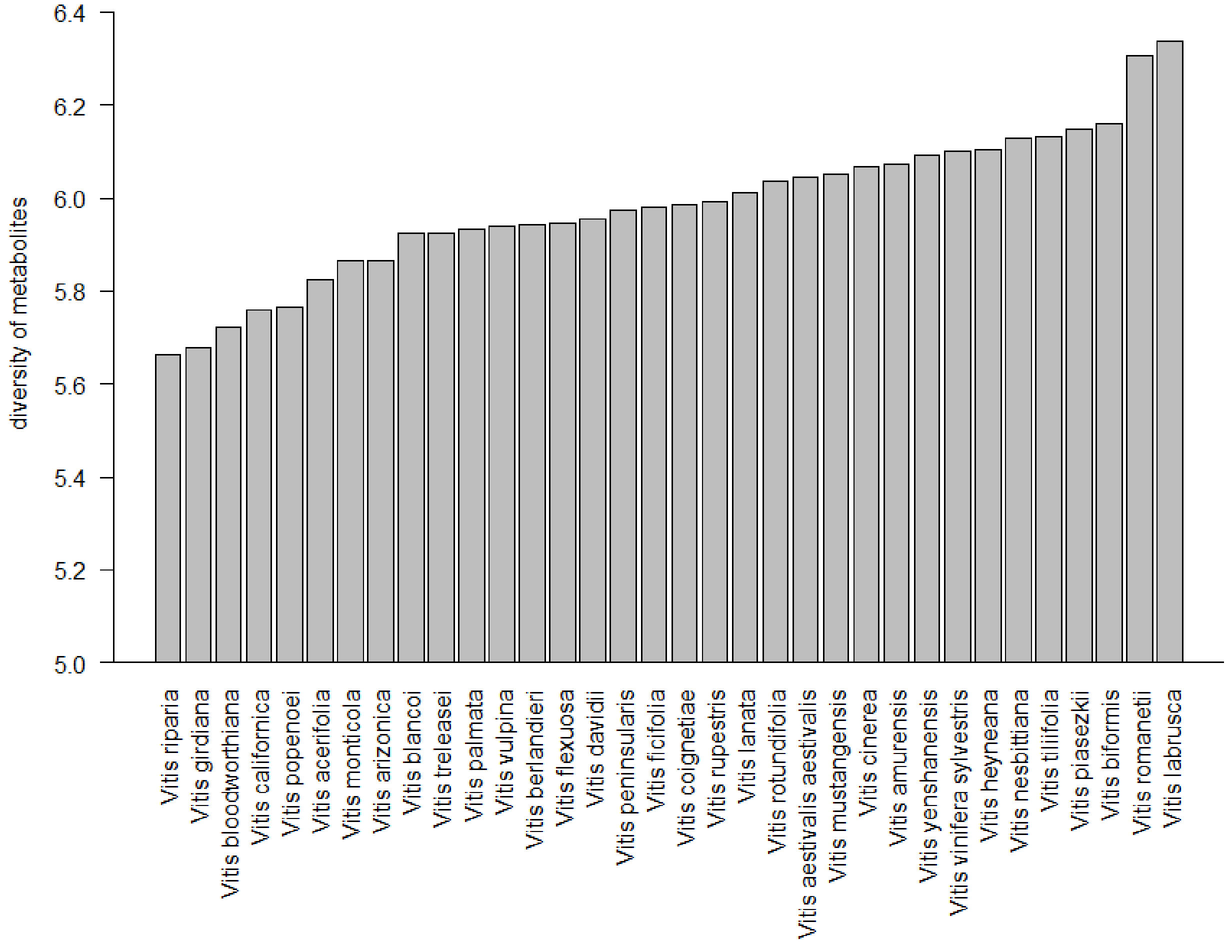
	Domatia index		Trichome score		Mass consumed	
	Effect size	p-value	Effect size	p-value	Effect size	p-value
Abundance	-	0.620	-	0.613	-	0.474
Richness	-	0.727	-	0.488	-2.38E-4	0.0187*
Diversity	-	0.783	-	0.0759	-1.19E-02	0.0258*

Appendix Table A.5 Correlations between climate variables and *Vitis* defense metrics. Results are from PGLS models with climate variables are predictors and defense metrics as response variables. Standardized effect sizes and p-values are shown

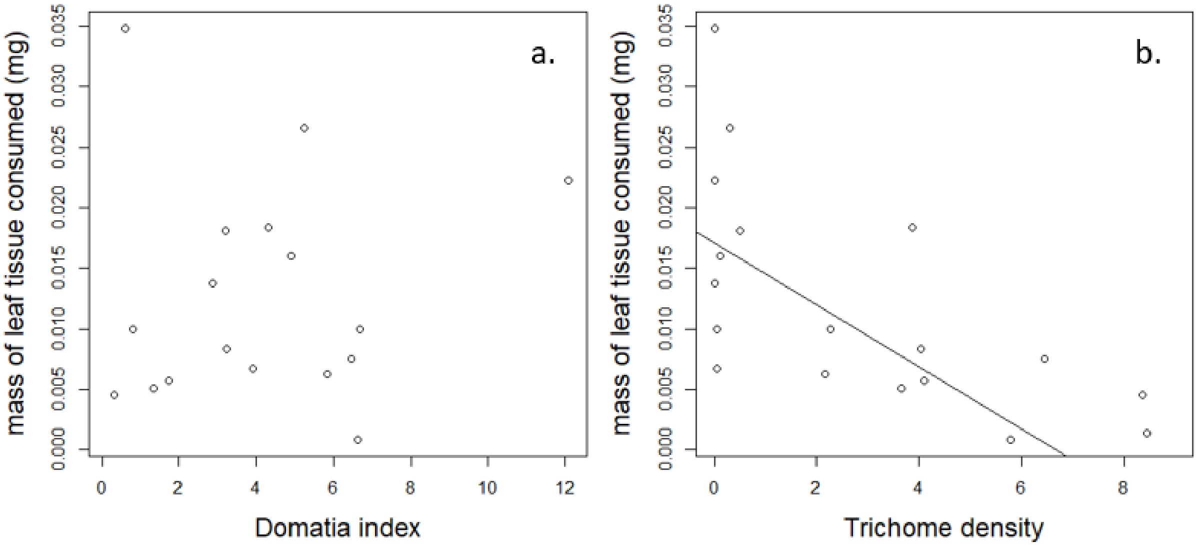
	Domatia index	Trichome density	Chemical abundance	Chemical richness	Chemical diversity
|Latitude|	0.191, p=0.356	0.170, p=0.474	0.409, p=0.0446*	0.305, p=0.168	0.123, p=0.497
Temperature Seasonality	0.214, p=0.300	0.135, p=0.563	0.430, p=0.0332*	0.259, p=0.240	0.151, p=0.402
Precip. of driest month	0.0111, p=0.955	-0.276, p=0.196	-0.0507, p=0.804	-0.348, p=0.103	-0.229, p=0.190



Appendix Figure A.1 Distribution of average domatia index scores for the *Vitis* species measured in this study.



Appendix Figure A.2 Distribution of average Shannon diversity of metabolites scores for the *Vitis* species. Note that the y-axis does not begin at 0.



Appendix Figure A.3 Relationship between a.) average domatia index, and b.) average trichome density on the palatability of leaf tissue to *S. exigua* larvae using leaves from the greenhouse common garden. Significant trendlines from PGLS models are shown (alpha = 0.05).

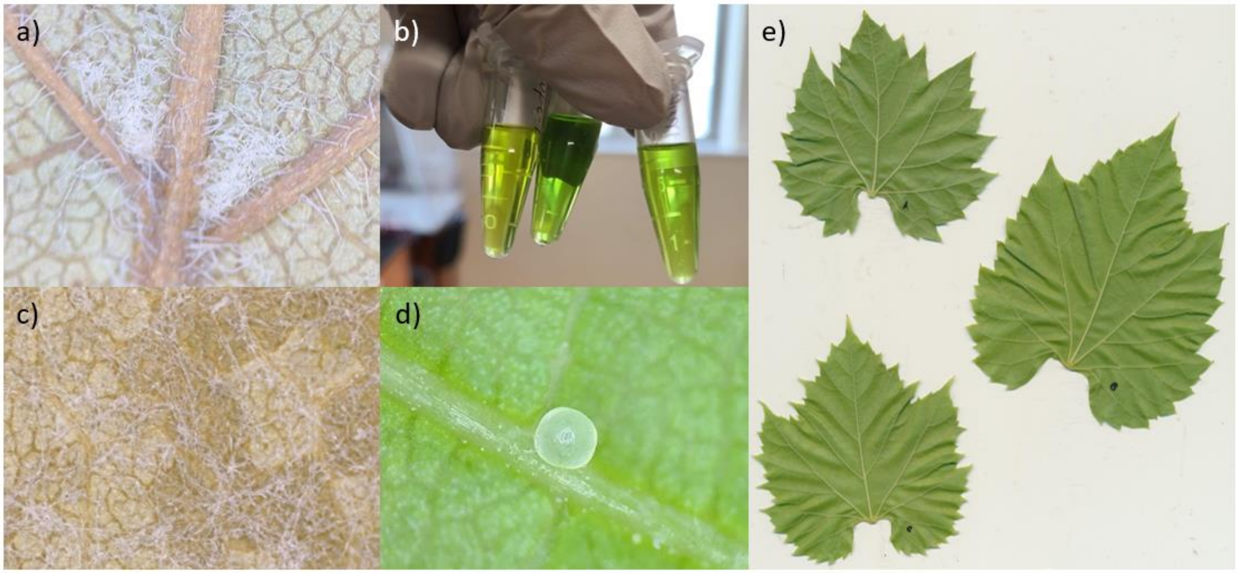
Appendix B: Supplementary Material for Chapter 3

Appendix Table B.1 Results of linear models of defensive traits in response to |latitude| for *Vitis riparia* (intraspecific level). D.f. = degrees of freedom, p = the p-value, with significance denoted relative to α = 0.05.

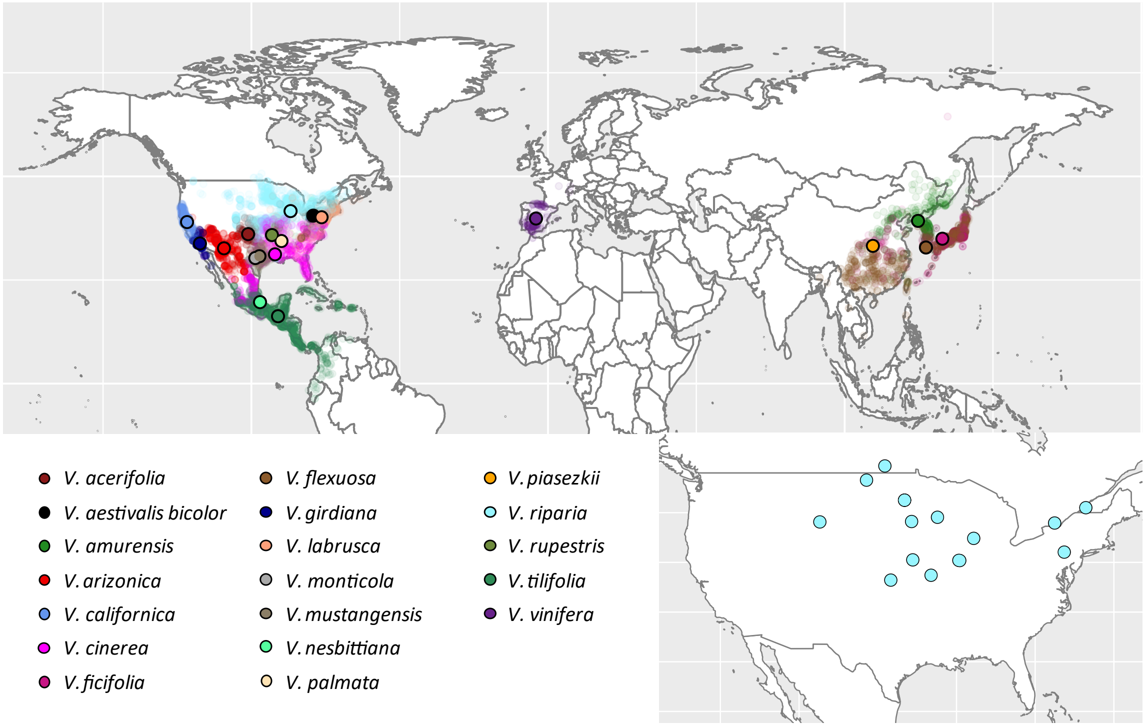
Defense metric	Effect size	d.f.	p-value
C:N	-1.778 ± 0.353	15	0.000147**
Chemical abundance	-20.18 ± 140.05	14	0.887
Chemical richness	0.874 ± 5.572	14	0.878
Domatia index	0.0336 ± 0.160	15	0.837
Leaf toughness	6.656 ± 0.0118	15	0.0118*
Pearlbody density	0.00140 ± 0.00853	15	0.872
Mass consumed	-0.273 ± 0.217	13	0.229

Appendix Table B.2 Results of linear models of defensive traits in response to |latitude| across *Vitis* species (interspecific level). D.f. = degrees of freedom, p = the p-value, with significance denoted relative to α = 0.05.

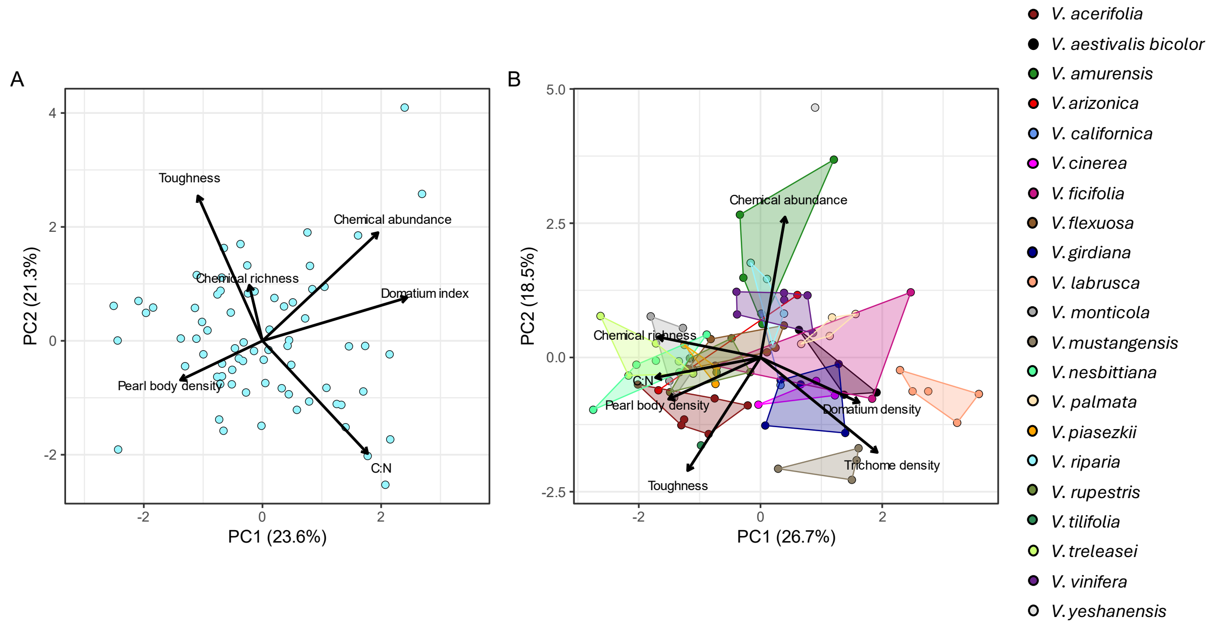
Defense metric	Effect size	d.f.	p-value
C:N	-0.610 ± 0.257	19	0.0299*
Chemical abundance	108.570 ± 43.221	19	0.0224*
Chemical richness	-1.863 ± 3.028	19	0.547
Domatia index	0.202 ± 0.103	16	0.0697
Leaf toughness	-3.792 ± 2.520	19	0.151
Pearlbody density	-0.0351 ± 0.0145	19	0.0273*
Trichome density	-0.00267 ± 0.112	19	0.981
Mass consumed	0.560 ± 0.186	18	0.0083**



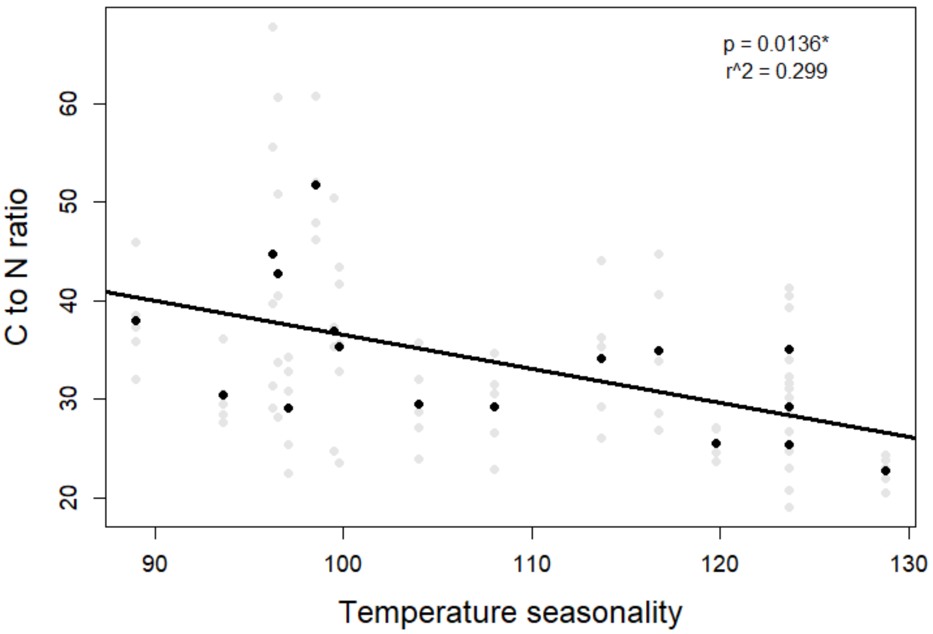
Appendix Figure B.1 Examples of *Vitis* defense traits measured in this study, including a) mite domatia, b) secondary chemistry extracts, c) trichomes, and d) a pearl body. Also included is e) a scan of V. riparia leaves.



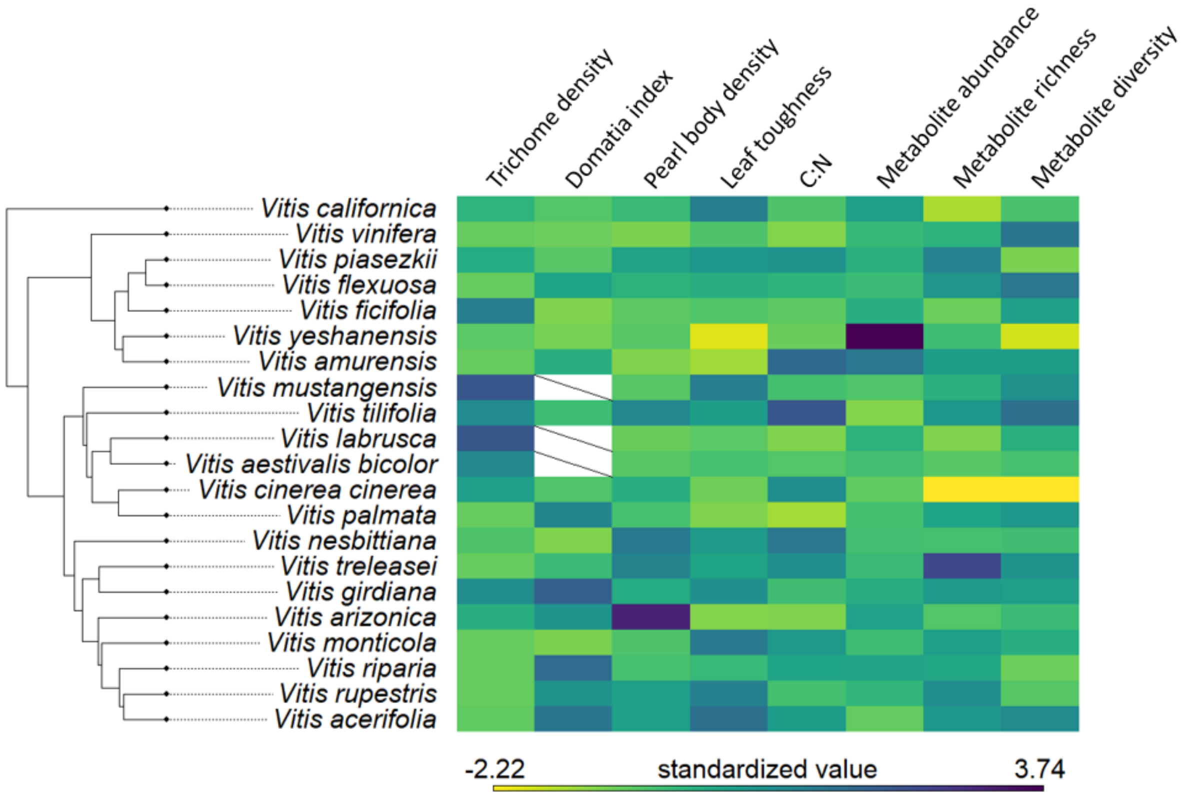
Appendix Figure B.2 Geographic distribution of *Vitis* species assessed in this study. Bolded points represent average occurrence location of each species, while the transparent points represent raw occurrence locations. The inset shows *V. riparia* accession locations from which we extracted climate variables.



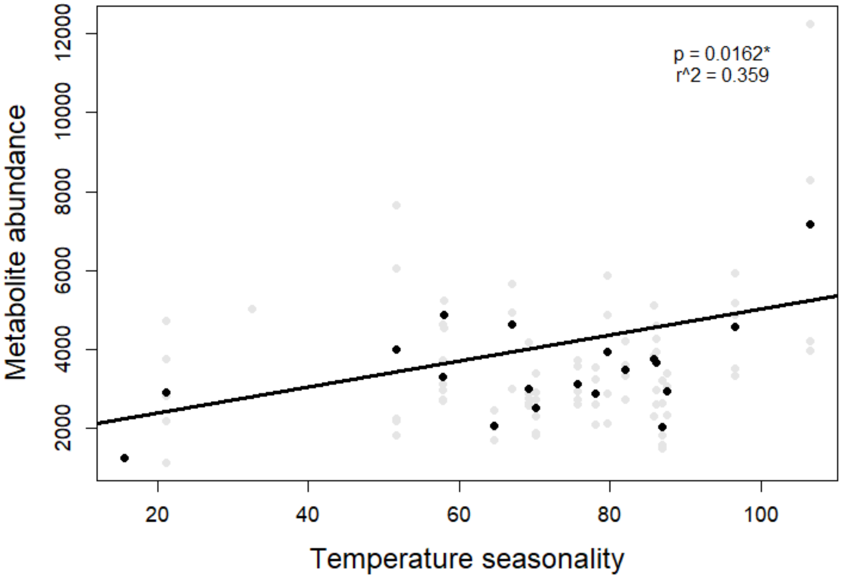
Appendix Figure B.3 Principal component analysis of defense-related traits in A) *Vitis riparia* and B) across *Vitis* species. Hulls in the second panel connect PC values calculated for plants from the same species.



Appendix Figure B.4 Relationship between C:N and temperature seasonality across *Vitis riparia* genotypes (intraspecific level). Temperature seasonality is measured as standard deviation from the mean yearly temperature, representing the annual range of temperature fluctuation. The linear relationship is significant at the α = 0.05 level. Black points represent genotype means. Grey points are plant-level averages for the trait.



Appendix Figure B.5 Interspecific variation in defense traits of *Vitis* species. Trait values have been standardized so that the same scale, in standard deviation units, can be used for all traits.



Appendix Figure B.6 Relationship between metabolite abundance and temperature seasonality across *Vitis* species (interspecific level). Temperature seasonality is measured as standard deviation from the mean yearly temperature, representing the annual range of temperature fluctuation. The linear relationship is significant at the α = 0.05 level. Black points represent species means. Grey points are plant-level averages for the traits

Appendix C: Supplementary Material for Chapter 5

Appendix Table C.1 Number of species in the dataset that contain each crystal type, broken down by the organ in which the crystals were recorded. The total number of species included in this analysis was 2596.

location	raphide	prism	druse	styloid	crystal sand	other
flower	194	238	101	24	2	32
fruit	33	22	6	6	5	37
leaf	634	329	451	79	118	219
root	115	14	10	3	2	10
seed	36	5	2	0	1	3
stem	189	300	154	50	30	85
total	**1201**	**908**	**724**	**162**	**158**	**386**