RESEARCH PAPERS

JOURNAL OF Evolutionary Biology ₀ₑₛₑᵦ WILEY

# Evolutionary history and ecology shape the diversity and abundance of phytochemical arsenals across monkeyflowers

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**Funding information**
US National Science Foundation, Grant/Award Number: IOS-1558035; University of Louisiana start-up; Northern Arizona University start-up

## Abstract

We examine the extent to which phylogenetic effects and ecology are associated with macroevolutionary patterns of phytochemical defence production across the *Mimulus* phylogeny. We grew plants from 21 species representing the five major sections of the *Mimulus* phylogeny in a common garden to assess how the arsenals (NMDS groupings) and abundances (concentrations) of a phytochemical defence, phenylpropanoid glycosides (PPGs), vary across the phylogeny. Very few PPGs are widespread across the genus, but many are common to multiple sections of the genus. Phytochemical arsenals cluster among sections in an NMDS and are not associated with total concentration of PPGs. There is a strong phylogenetic signal for phytochemical arsenal composition across the *Mimulus* genus, whereas ecological variables such as growing season length, latitude, and elevation do not significantly influence arsenal. In contrast, there is little phylogenetic signal for total PPG concentration, and this trait is significantly influenced by several ecological factors. Phytochemical arsenals and abundances are influenced by plant life history form. Both phylogenetic effects and ecology are related to phytochemical patterns across species, albeit in different ways. The independence of phytochemical defence concentrations from arsenal compositions indicates that these aspects of defence may continue to evolve independently of one another.

**KEYWORDS**
defence, macroevolution, *Mimulus*, monkeyflower, phylogenetic comparative analysis, phytochemical

## 1 | INTRODUCTION

As sessile organisms, plants have evolved to produce a great diversity of defences to resist herbivory (Erb et al., 2012; Hanley et al., 2007; Rasmann & Agrawal, 2009). Plant secondary compounds are key among these defences and are present in all higher plants (Fraenkel, 1959; Wink, 2003). Divergence in phytochemical defences can occur through the evolution of novel compounds and/or the evolution of novel combinations of compounds (multivariate arsenals). For example, Berenbaum (1978, 1983) used the wild parsnip system to elegantly demonstrate that plants can escape from adapted herbivores by producing ecologically novel compounds, often from the same chemical precursor. Alternatively, in the tropical plant genus *Inga*, closely related species produce different combinations (and

presence/absence patterns) of commonly produced compounds (Coley et al., 2018). Ultimately, the degree of divergence among phytochemical arsenals reflects both constraints that promote conservation and covariance of individual phytochemical defences across phylogeny and ecological factors that diversify defences—a process that may even lead to speciation (Coley & Kursar, 2014; Endara et al., 2015; Maron et al., 2019; Marquis et al., 2016).

Comparative phylogenetic approaches can reveal broad patterns of trait evolution across taxa and may lead to further investigation of the processes underlying the patterns (Futuyma & Agrawal, 2009; Kariñho-Betancourt, 2018; Weber & Agrawal, 2012). Common patterns of defence production and/or common trait correlations that occur across species (i.e. phylogenetic signal; Ehrlich & Raven, 1964; Losos, 2008; Vermeij, 1994) might reflect constraints. For phytochemical traits, there may be physiological or genetic limitations in the biosynthetic origins of phytochemicals that constrain evolutionary diversification. Alternatively, closely related species may have similar phytochemical defence if they have experienced similar selective pressures (Agrawal et al., 2009).

Although there is often consistency in broad patterns of classes of compounds across closely related taxa (Liscombe et al., 2005; Pearse & Hipp, 2009; Wink, 2003; Wink & Mohamed, 2003), phylogenetic signal is not always strong. Disparate patterns of defence production and/or trait correlations across species are reflected in a weak or nonexistent phylogenetic signal (Agrawal, Fishbein, et al., 2009; Agrawal et al., 2008; Becerra, 1997; Johnson et al., 2014; Kursar et al., 2009). A number of evolutionary processes can influence the strength of phylogenetic signal (Revell et al., 2008). These traits may be less developmentally or physiologically constrained and are able to evolve independently across species, possibly in response to differing selection pressures (Ackerly, 2009).

The degree of divergence in defence production across species can be influenced by trade-offs either between defence traits or between defences and other plant traits. One type of evolutionary trade-off, between life history strategy and defence production, has been phenotypically characterized both within and across plant species, although the genetic basis of these trade-offs is less firmly elucidated (Kooyers et al., 2020; Lowry et al., 2019; Prasad et al., 2009). Multiple hypotheses exist to predict variation in levels of plant defence among and within species, including the Resource Availability Hypothesis (RAH) and optimal defence theory (ODT). The RAH was formulated based on inter-species differences in growth rate and defence investment, where species in low resource environments tend to be composed of slower growing, better defended species, whereas species in high resource environments tend to contain faster growing, more poorly defended species (Coley et al., 1985; Endara & Coley, 2011). Optimal defence theory predicts that plants will evolve levels of defence that are positively related to levels of herbivory and negatively related to allocational or ecological costs (Herms & Mattson, 1992; Koricheva, 2002; Rhoades, 1979). Intraspecific patterns are often not consistent with RAH, with positive relationships between the length of the growing season along an environmental gradient and the level of defence (Hahn & Maron, 2016; Kooyers

et al., 2017). These patterns are more consistent with ODT and could be driven by selection for resource allocation to rapid reproduction in short growing seasons, lower herbivore pressure in habitats with shorter growing seasons and/or less apparency of plants with shorter growing seasons (Feeny, 1976; Smilanich et al., 2016).

Multiple species in the monkeyflower genus, including *Mimulus guttatus* and the *M. lewisii* species complex are model systems for the study of evolution (e.g. Lowry et al., 2019; Wu et al., 2008; Yuan, 2019). Work in *M. guttatus* has increased understanding of the evolutionary ecology and underlying genetics of both physical and phytochemical plant defences (Holeski, 2007; Holeski et al., 2010, 2013; Kooyers et al., 2017). However, with the exception of the resin that gives the 'sticky monkeyflower' *M. aurantiacus* its name (Hare, 2002; Sobel et al., 2019), there is nearly no information on the diversity of defences across the genus. The ~ 120 species in the genus have a worldwide distribution with over 75% of the species occurring across diverse habitats in western North America (Beardsley & Olmstead, 2002). Phenylpropanoid glycosides (PPGs) are bioactive secondary compounds implicated in plant defence against herbivores in multiple plant species (Cooper et al., 1980; Mølgaard, 1986, 1992; Rotter et al., 2018). PPGs are the dominant phytochemical defences in at least one species in the *Mimulus* genus in terms of concentration (*M. guttatus*; Holeski et al., 2013; Keefover-Ring et al., 2014; Rotter et al., 2018), whereas the phytochemistry of other species in the genus is largely unknown.

Here, we investigate macroevolutionary patterns of phytochemical defence trait production across the *Mimulus* phylogeny. Using plants grown from 21 species representing the five major sections of the *Mimulus* phylogeny, we address the following questions:

1. How does phytochemical defence (PPG) abundance and multivariate composition (arsenal) vary across the *Mimulus* phylogeny?
2. How strong are phylogenetic effects for total PPG levels and arsenals?
3. What is the role of ecology as a predictor of interspecific patterns of defence abundances and arsenals?

## 2 | METHODS

### 2.1 | Germplasm and common garden experiment

We used seed collections from 21 different species or varieties that had been previously collected from natural populations between 2004 and 2007 (Table S1). Latitude, longitude and elevation of each population was documented at time of collection. For each site, climate normals derived from 1960–1990 and 1981–2010 were downloaded from ClimateWNA (Wang et al., 2012). The selected taxa span the diversity of the genus *Mimulus*, representing species from five of the major taxonomic sections described by Grant (1924): Diplacus, Oenoe, Eunanus, Simiolus and Erythranthe. Species boundaries in section Diplacus have been the subject of recent debate (Chase et al., 2017), and we have elected to refer to

some taxa as separate species here that may be grouped as a single species elsewhere (*M. aridus*, *M. aurantiacus*, *M. australis*, *M. parviflorus* and *M. puniceus* are all considered subspecies of *M. aurantiacus* by Thompson, 2005). Further, recent taxonomic work has split the genus *Mimulus* into separate genera (Barker et al., 2012). However, in order to ease comparisons with previously published work, we have elected to continue using the name *Mimulus* to refer to this genus (see Lowry et al., 2019; Nesom et al., 2019 for discussion).

Individuals from 1–6 maternal lines (average 3.6) were grown from a single population of each species in the University of Oregon greenhouses. Plants were grown in a blend of potting mix, crushed granite and perlite in Stuewe Cone-tainers, with standardized watering and fertilizing with macro- and micro-nutrients. Plants were grown to an early adult stage (as flowering was just initiating), and leaf samples were collected from the youngest 3–4 nodes on multiple growing stems per plant. Samples were flash frozen on liquid nitrogen and stored in a −80°C freezer before being shipped on dry ice to Northern Arizona University for analysis.

## 2.2 | Phytochemical analysis

All tissue was lyophilized, ground to a fine powder, and a portion weighed in preparation for phytochemical analysis. We used methanol to extract PPGs from the dried, ground samples with 10 min of sonication and allowed extracts to sit overnight in the dark. After centrifugation, a 100 μl aliquot of each methanol extract was vacuum dried and then redissolved with vortex mixing in 100 μl of an aqueous catechol (Sigma-Aldrich, St. Louis, MO, USA) internal standard solution (Holeski et al., 2013). We analysed the solutions using ultra-high-performance liquid chromatography (UHPLC) with photodiode array detection (PDA) and negative electrospray ionization single quadrupole mass spectrometry (MS; Waters Acquity I-Class UPLC and 3100 SQ MS, Milford, MA, USA) using the same UHPLC conditions as Falk et al. (2018). We used a standard curve of methanol solutions of conandroside purified from *M. guttatus* (Keefover-Ring et al., 2014), also containing catechol as an internal standard, and calculated the concentrations (mg compound/ g dry weight) of all PPGs as conandroside equivalents. We also used small amounts of calceolarioside A, calceolarioside B, mimuloside, verbascoside and several unknown PPGs purified from *M. guttatus* (Keefover-Ring et al., 2014) as retention time standards for peak identification of these compounds in the various *Mimulus* species. Total PPGs was calculated as the sum of all PPGs for each individual.

## 2.3 | Statistical analysis

We calculated summary statistics including means, standard deviations and standard errors of concentrations for each PPG and total PPGs for each species using the *plyr* package v1.8.4 (Wickham, 2011) in R v3.5.3 (R Foundation for Statistical Computing, Vienna, Austria). We assessed correlations between PPGs using the rcorr() function in

the *Hmisc* package v4.2-0 (Harrell, 2015); mean values for each PPG for each species were used in this analysis. Because levels of many PPGs were highly correlated and because of the number of PPGs that were absent in multiple species, we conducted a nonmetric multidimensional scaling (NMDS) analysis using the *vegan* library v2.5-6 in R (Oksanen et al., 2019). We input a bray dissimilarity matrix using all individuals in the experiment and restricted our analysis to two dimensions. We averaged both NMDS axis 1 and axis 2 values for each species and used these values as a proxy for phytochemical arsenal in the comparative analyses below. We assessed whether there was significant clustering of NMDS scores by phylogenetic section using a permutational MANOVA implemented using the adonis function in the *vegan* library v2.5-6 (Oksanen et al., 2019). All individuals in the experiment were included in this analysis. Distances were calculated using euclidean distances and statistical significance assessed with 1,000 permutations. Correlations between each NMDS axis and each individual PPG as well as total PPGs were calculated as done with the individual PPGs above.

## 2.3.1 | Phylogeny construction and comparative analyses

We downloaded sequences from three regions (ETS, ITS, trnL) from all species in the analysis from Genbank using the read.Genbank function in the *ape* library v5.3 (Paradis et al., 2004). Specific accessions used are listed in Table S2. These sequences were originally generated for the most recent *Mimulus* phylogenetic reconstruction (Beardsley *et al.*, 2004). We used Clustlx to align each of the three loci individually. We then used Supermatrix function in *evobir* library to concatenate sequences resulting in a dataset with 2028 total base pair across the three loci. We evaluated which mutation model best fit our data using the *phangorn* library v2.5.5 (Schliep, 2011).

We inferred phylogenetic trees using the Bayesian framework implemented in BEAST v1.10.4 (Drummond & Rambaut, 2007). An input files was created with BEAUti v1.10.4 (Drummond et al., 2012). Model parameters include a GTR + Γ+I substitution model, a strict molecular clock, and a speciation:Yule process option for a tree prior. We conducted three independent BEAST MCMC runs with the same parameters; each run included $1 \times 10^8$ generations sampling every 1,000 generations with a burnin of 10%. We viewed the results of each run separately to assess variation in tree topology before combining the results of all three runs using LogCombiner v1.10.4. Mixing and convergence of runs was assessed in Tracer v1.7.1 (Suchard et al. 2018) and all estimated sample sizes were >200. We summarized our posterior distribution of trees by calculating the maximum clade credibility (MCC) tree using TreeAnnotator v1.10.4 (https://beast.community/treeannotator). Posterior probabilities of clades were visualized using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). Since topology did not vary among runs, we used the MCC tree deviated from the combined runs within all downstream phylogenetic comparative analyses (Figure S1). This tree also has the same topology as in the most recent phylogenetic

reconstruction of the *Mimulus* genus (Beardsley *et al.* 2004) and all nodes are well resolved.

We conducted ancestral character reconstruction by using the fastAnc function to estimate character states and 95% confidence intervals at interior nodes in the *phytools* library v0.6-99 (Revell, 2012). We reconstructed character states for total level of PPGs as well as phytochemical arsenal using NMDS axis 1 and NMDS axis 2 as proxies. We assessed the strength and significance of phylogenetic signal for each PPG, NMDS axis and total levels of PPGs using Pagel's $\lambda$ and Blomberg's $K$ (Blomberg et al., 2003; Pagel, 1999) implemented in *phylosignal* library v1.3 (Keck et al., 2016). Both statistics compare observed trait values to trait evolution that follows a Brownian motion model that tests a null model of no phylogenetic signal. Pagel's $\lambda$ is a maximum likelihood approach that examines the transformation necessary for the observed values to fit a Brownian motion model of trait evolution, whereas Blomberg's $K$ is the standardized deviation of observed trait data in tips of a phylogeny to a phylogenetically corrected model under the assumption of Brownian motion trait evolution. For either statistic, values of zero indicate phylogenetic independence of traits, whereas values of one (Pagel's $\lambda$) or one or greater (Blomberg's $K$) indicate stronger trait similarity than expected for closely related species (i.e. strong phylogenetic signal). We assessed both statistics as they do not always give similar conclusions (Münkemüller et al., 2012). Species that did not produce a particular individual PPG were included in both ancestral character reconstruction and phylogenetic signal analyses as zeros.

### 2.3.2 | Phylogenetic general least squared (PGLS) models

To examine associations between phytochemical arsenals, environmental and life history strategy while accounting for evolutionary history, we employed phylogenetic general least squared regression via a maximum likelihood model implemented in the *ape* library v5.3 (Paradis et al., 2004). Our MCC tree from above was used within this model to account for phylogenetic structure between traits and predictor variables assuming a Brownian motion error structure. Response variables for these models included species means for NMDS axis 1, NMDS axis 2 and total PPGs. To test hypotheses about regarding how plant life history is associated with defences, we examined whether the transition between annual and perennial plants is associated with differences in defence amount or arsenal. To assess the relationship between abiotic environmental factors and defences, we examined the association between total PPGs and latitude. We also ran this model with elevation as a covariate and annual mean temperature instead of latitude as predictor variables. To assess the relationship between growing season length and defences, we conducted PGLS regressions examining associations between total PPGs and number of frost-free days in the growing season, the per cent of precipitation as snow, or the beginning of frost-free growing season. To examine whether differences in defence arsenals are associated with temperature, latitude or the

growth season start date or duration, we substituted NMDS axis 1 or axis 2 for total PPGs in the above model.

Finally, to examine whether defence abundance and defence arsenals evolve in concert across the phylogeny, we conduct separate univariate PGLS regressions with total PPGs as the response variable and each NMDS axis as a predictor variable. As with the PGLS models above, we account for phylogenetic history assuming a Brownian motion error structure. For all of the above tests, we chose not to apply any correction for multiple tests to our PGLS results as a number of the independent variables are strongly correlated (García, 2004). However, for reference, a strict Bonferroni correction for six independent tests per response variable would result in a significant threshold of $p < .0083$.

## 3 | RESULTS

### 3.1 | Patterns of PPG abundances and arsenals

Multivariate PPG composition varied substantially among species and included significant clustering among sections of the phylogeny (pseudo-$F_{5,16} = 7.6$, $p = .007$; Figure 1). Our phytochemical analyses detected nine different PPGs previously reported from *M. guttatus* studies (conandroside, mimuloside, verbascoside, calceolarioside A, calceolarioside B and unknown PPGs 1, 4, 6, and 7; Holeski et al., 2013; Keefover-Ring et al., 2014). Unknown PPGs 1, 4, 6 and 7 found in this study correspond to unknown PPGs 10, 8, 15 and 16, respectively, from Keefover-Ring et al. (2014). We also detected three novel putative PPGs (unknown PPGs 2, 3 and 5, identified as
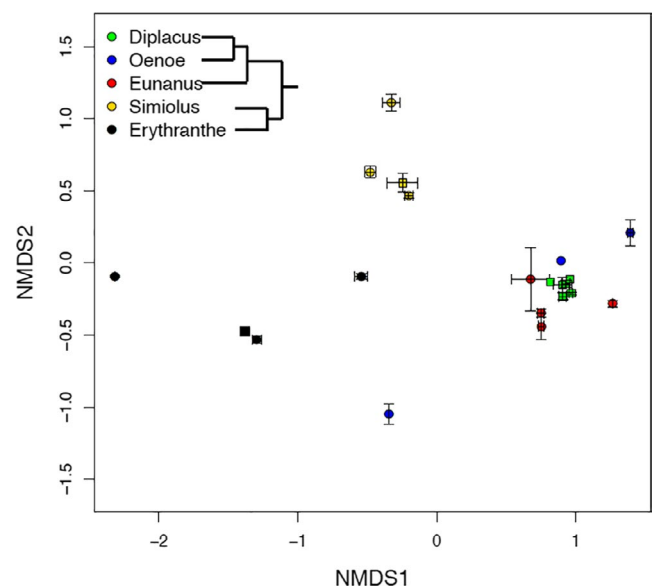


**FIGURE 1** NMDS of PPG arsenals across five sections of monkeyflowers. Section Paradanthus is divided into Erythranthe and Simiolus-related sections as it is paraphyletic in our phylogeny. Circles and squares represent species with annual and perennial life histories, respectively. Error bars are standard deviations. Stress value is 0.057.

PPGs by their UV profiles and molecular weights; Figures S2, S3 and S4; Table S3) across the 21 species assessed. No individual PPG was present within every species, although verbascoside was found in 18/21 species surveyed (Table S1).

Mean total PPG concentrations ranged from 0.28 mg/g plant dry weight in *M. palmeri* to 41.85 mg/g plant dry weight in *M. norrisii* (Table S1). Plants in sections Diplacus, Oenoe and Eunanus had relatively high levels of verbascoside and lower diversity of other PPG compounds. Diplacus and Oenoe also have relatively high levels of unknown PPG 3. Plants in the Simiolus and Erythranthe sections of the phylogeny have a larger diversity of compounds with lower levels of verbascoside, compared to Diplacus, Oenoe and Eunanus. Although some PPGs such as verbascoside and unknown PPG 7 are distributed across the phylogeny, other more rarely occurring compounds occur in only 1–4 species and tend to be in closely related species. For example, unknown PPG 1 occurs only in sister species *M. norrisii* and *M. floribundus*, whereas unknown PPG 2 occurs only in the closely related species *M. cardinalis*, *M. filicaulis*, *M. bicolor* and *M. palmeri* (Table S1).

Although the degree of correlation between individual PPGs is variable, all significant correlations between individual PPGs are positive relationships (Table 1). To examine how phytochemical arsenals vary across the phylogeny, we conducted an NMDS analysis. NMDS axis 1 is influenced strongly by verbascoside with smaller contributions from unknown PPGs 2 and 3, whereas NMDS axis 2 is influenced by calceolarioside A and B, conandroside, mimuloside and others (Table 2). NMDS axes largely reflect divergence among the different sections of the phylogeny. The species in the Diplacus, Oenoe and Eunanus section have higher values than species in Paradanthus and Erythranthe sections on NMDS axis 1 and species in Simiolus have higher values than other clades on NMDS axis 2. There were no significant correlations between either NMDS axis 1 or 2 and total PPG concentrations, indicating that total levels of PPGs can evolve independently from individual arsenal components (Table 2).

## 3.2 | The role of evolutionary history in phytochemical production

A phylogenetic signal exists for some aspects of phytochemical defence within the genus (Figure 2). There was a phylogenetic signal in the arsenals of PPGs, that is in both NMDS axes (Figure 2a,b; NMDS I: Pagel's $\lambda = 1.02$, $p = .001$, Blomberg's $K = 1.67$, $p = .001$; NMDS II: Pagel's $\lambda = 0.97$, $p = .001$, Blomberg's $K = 0.71$, $p = .001$). For individual PPGs, verbascoside showed the strongest phylogenetic signal (Pagel's $\lambda = 0.92$, $p = .001$, Blomberg's $K = 0.99$, $p = .001$), with several other individual PPGs also showing moderate and significant phylogenetic signal (Table S4). There was not a significant phylogenetic signal in total levels of PPGs (Figure 2c; Pagel's $\lambda = 0.63$, $p = .175$, Blomberg's $K = 0.28$, $p = .081$), suggesting that concentration of total PPGs may evolve independently from specific PPG arsenal, and that different evolutionary

**TABLE 1** Correlations between individual PPGs and total PPGs

| | Calc. A | Calc. B | Verbascoside | Conandroside | Mimuloside | Unkn. PPG 1 | Unkn. PPG 2 | Unkn. PPG 3 | Unkn. PPG 4 | Unkn. PPG 5 | Unkn. PPG 6 | Unkn. PPG 7 | Total PPGs |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Calc. A | | .583 | .103 | .365 | **.006** | .804 | .749 | .312 | .611 | .833 | .203 | .262 | .639 |
| Calc. B | −0.12 | | .145 | .616 | .512 | .691 | .097 | .335 | **.001** | .712 | .569 | .588 | .622 |
| Verbascoside | −0.36 | −0.32 | | .293 | .155 | .328 | .109 | .251 | .539 | .353 | .223 | .843 | **.014** |
| Conandroside | 0.20 | −0.11 | −0.23 | | **<.001** | **<.001** | .634 | .559 | .772 | **<.001** | **<.001** | .303 | **.034** |
| Mimuloside | **0.57** | −0.09 | −0.31 | **0.82** | | **<.001** | .486 | .407 | .673 | **<.001** | **<.001** | .186 | .106 |
| Unkn. PPG 1 | 0.06 | −0.09 | −0.22 | **0.98** | **0.71** | | .712 | .620 | .822 | **<.001** | **<.001** | .354 | .054 |
| Unkn. PPG 2 | 0.07 | 0.36 | −0.35 | −0.11 | −0.16 | −0.08 | | .414 | .730 | .730 | .590 | .122 | .907 |
| Unkn. PPG 3 | −0.23 | −0.22 | 0.26 | −0.13 | −0.19 | −0.11 | −0.18 | | .621 | .642 | .503 | .389 | .595 |
| Unkn. PPG 4 | −0.11 | **0.65** | −0.14 | −0.07 | −0.10 | −0.05 | −0.08 | −0.11 | | .833 | .743 | .388 | .563 |
| Unkn. PPG 5 | 0.05 | −0.08 | −0.21 | **0.97** | **0.70** | **1.00** | −0.08 | −0.10 | −0.05 | | **<.001** | .381 | **.047** |
| Unkn. PPG 6 | 0.28 | −0.13 | −0.27 | **0.99** | **0.88** | **0.95** | −0.12 | −0.15 | −0.07 | **0.95** | | .249 | **.047** |
| Unkn. PPG 7 | 0.25 | −0.12 | −0.04 | 0.23 | 0.29 | 0.21 | −0.34 | −0.19 | −0.19 | 0.20 | 0.26 | | .840 |
| Total PPGs | 0.11 | 0.11 | **0.52** | **0.45** | 0.35 | 0.42 | −0.03 | 0.12 | 0.13 | **0.43** | **0.43** | 0.05 | |

*Note:* Pearson correlation coefficients are below the diagonal, with associated *P*-values above the diagonal. Significant values at *p* <.05 are in bold.

Abbreviations: Calc., calceolarioside; Unkn., unknown.

**TABLE 2** NMDS axis composition

| PPG | NMDS axis 1 | | NMDS axis 2 | |
|---|---|---|---|---|
| | r | p | r | p |
| Calceolarioside A | −.35 | .107 | **.49** | **.021** |
| Calceolarioside B | −.39 | .074 | **−.55** | **.008** |
| Verbascoside | **.63** | **.002** | −.13 | .555 |
| Conandroside | −.15 | .494 | **.69** | **<.001** |
| Mimuloside | −.23 | .306 | **.81** | **<.001** |
| Unknown PPG 1 | −.12 | .586 | **.60** | **.003** |
| Unknown PPG 2 | **−.54** | **.010** | −.29 | .187 |
| Unknown PPG 3 | **.47** | **.028** | .13 | .569 |
| Unknown PPG 4 | −.12 | .594 | **−.47** | **.026** |
| Unknown PPG 5 | −.12 | .606 | **.58** | **.004** |
| Unknown PPG 6 | −.18 | .425 | **.75** | **<.001** |
| Unknown PPG 7 | .14 | .531 | .32 | .144 |
| Total PPGs | .16 | .463 | .22 | .335 |
| NMDS axis 2 | −.02 | .929 | | |

*Note:* Higher values for NMDS axis 1 correspond to higher levels of verbascoside and unknown PPG 3, and lower levels of unknown PPG 2. Higher values for NMDS axis 2 correspond with higher levels of calceolarioside A, conandroside, mimuloside and unknown PPGs 1, 5 and 6, and lower levels of calceolarioside B and unknown PPG 4. Significant values are in bold.

processes may underlie current patterns in PPG arsenals versus concentrations.

## 3.3 | The role of ecology in phytochemical evolution

We observed phylogenetically independent associations between PPGs and other traits that can at least partly be explained by differences in ecology. Use of climate normals from 1960–1990 versus 1981–2010 did not alter our results (Table 3 versus Table S5) with the exception of the relationship between Total PPGs and bFFP (beginning of the frost-free period). This relationship was marginally significant when 1960–1990 climate data were used (Table 3), and significant when 1981–2010 climate data were used (Table S5). Perennial plants have higher values than annuals on NMDS axis 1 suggesting that they produce a defence arsenal including more verbascoside than annual plants, whereas there is no significant difference between life history strategies for NMDS axis 2 (Figure 3a,b; Tables 3 and S5). There is a strong association between total concentration of PPGs produced and life history strategy in the raw data, whereby perennial species generally produce higher concentrations of PPGs than annual species (Figure 3c), although this relationship becomes only marginally significant when phylogenetic relatedness is taken into account (Tables 3 and S5).

Neither NMDS 1 nor NMDS 2 was significantly associated with latitude, elevation, length of growing season (number of frost-free days), beginning of growing season, precipitation as snow, or mean annual temperature (Table 3). In contrast, total PPG concentrations were

strongly associated with multiple abiotic factors. Plants from areas with a longer growing season (higher values for NFFD) tend to produce higher concentrations of PPGs than species from areas with shorter growing seasons (Figure 4c, Table 3). Patterns of total PPG concentrations across taxa were also significantly affected by elevation and amount of precipitation as snow, with decreasing PPG concentrations as elevation and amount of precipitation as snow increase (Figure 4a,d; Table 3), and mean annual temperature, with PPG concentrations increasing as temperature increases (Figure 4b, Table 3).

## 4 | DISCUSSION

We investigated macroevolutionary patterns of phytochemical defence trait production across the *Mimulus* phylogeny, using 21 species representing the five major sections of the genus. In assessing how phenylpropanoid glycoside (PPG) abundance and multivariate arsenal composition vary across the phylogeny, we found that closely related *Mimulus* species cluster into distinct phenylpropanoid glycoside defence arsenals, and that these vary independently of phytochemical concentrations. Within these arsenals, all of the significant relationships between individual compounds are positive, and there are many nonsignificant relationships between individual compounds. The latter pattern is possibly due in part to low power with use of species means, or could indicate that individual PPGs are evolving independently, with little genetic or biosynthetic constraints. We found a strong phylogenetic signal for phytochemical defence arsenals, but no significant phylogenetic signal for total PPG concentrations. Finally, we assessed how ecology may influence interspecific patterns of defence abundances and arsenals. We found some evidence for phylogenetically independent trade-offs between life history strategy and defence. The abiotic environment affects phytochemical concentrations, but there is little evidence for influence of abiotic environmental factors on phytochemical arsenal across species.

## 4.1 | Patterns of PPG abundances and arsenals

We found some individual PPGs to be widely distributed across the phylogeny, whereas others are more narrowly distributed and tend to occur in closely related species. The former pattern is consistent with inheritance through a common ancestor, whereas the most parsimonious explanation for the latter pattern is through more recent evolution of novel PPGs. We note that our study assessed PPGs only; although these are the main bioactive secondary compounds in *M. guttatus*, other species may utilize other classes of compounds as well.

The phytochemical patterns that we found occurring across species are, in part, similar to those found within the species *Mimulus guttatus*. Previous work in *M. guttatus* examined chemistry from common garden experiments in plants derived from many annual and perennial populations (Rotter et al., 2019), or only annual populations within the species (Kooyers et al., 2017). Verbascoside does
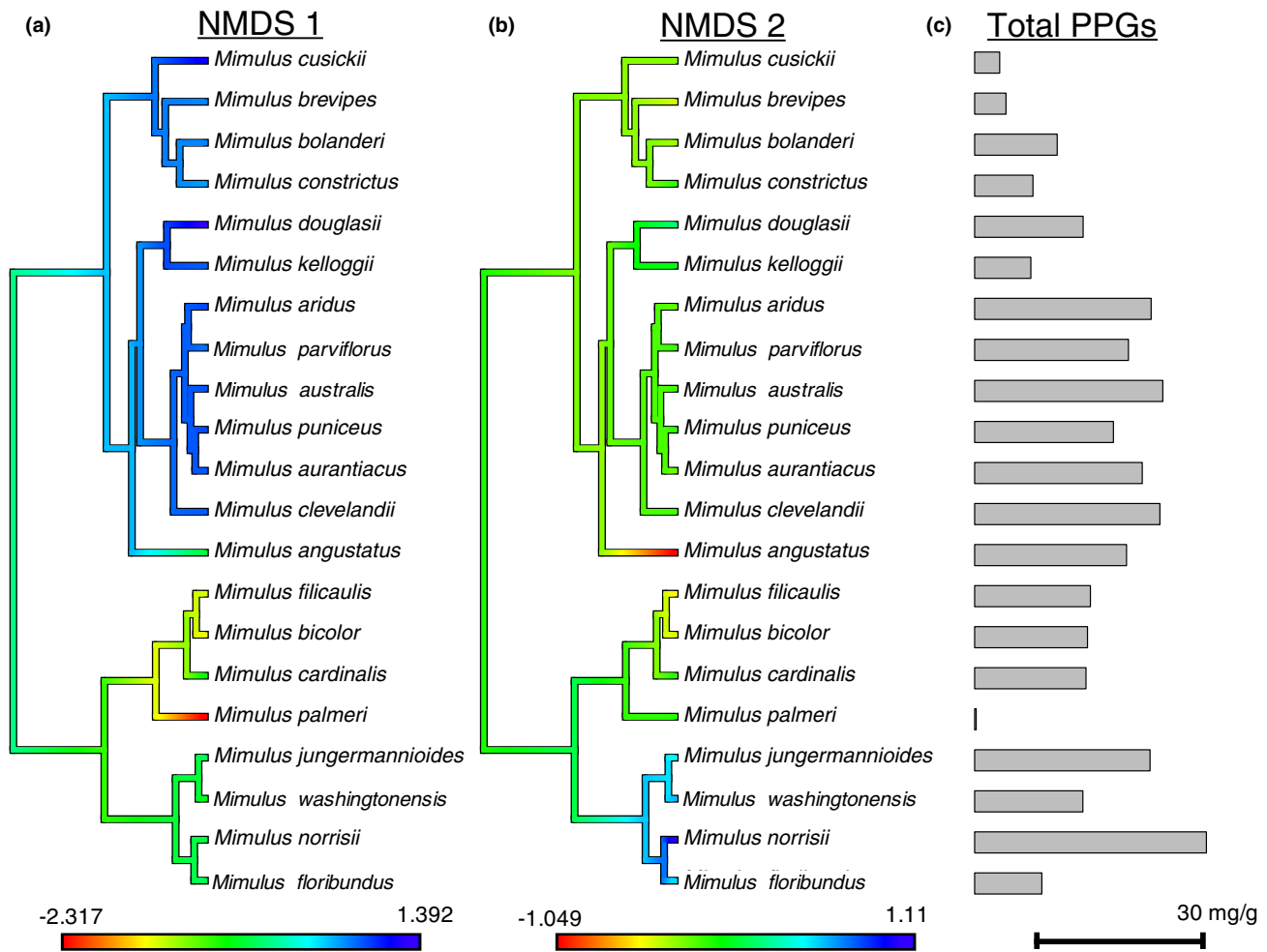
**FIGURE 2** Ancestral character reconstruction for (a) NMDS axis 1, (b) NMDS axis 2 and (c) total PPGs (concentration). Colder values reflect higher values on NMDS axes.

not cluster with other PPGs within *M. guttatus* in principle component analyses (PCA) in previous work, and in the current study verbascoside is associated with only two putative PPGs that have not been found in *M. guttatus*. NMDS axis 2 overlaps in composition with the first PCA axis (chemPC1) in both of the *M. guttatus* studies, with all containing calceolarioside B, conandroside and mimuloside. However, one difference between chemPC1 and NMDS axis 2 is for the PPG calceolarioside B, which is highly positively correlated with conandroside and mimuloside within *M. guttatus*, but is negatively correlated here, across species.

Our result that individual PPGs covary to different extents across species is consistent with results from other plant genera. Previous work has shown the evolution of plant defence syndromes, suites of positively correlated traits that typically include phytochemical defences, physical defences and traits influencing nutritional quality, across phylogenies (Agrawal & Fishbein, 2006; Johnson et al., 2014; Kursar & Coley, 2003). Positively covarying traits are assumed to act synergistically in some way (e.g. Calcagno et al., 2002), or may act only partially redundantly against different herbivores. For example, phytochemical defences can be less

effective against specialist herbivores than generalists, whereas physical defences may also deter specialists (Mauricio, 1998; Rotter et al., 2018). In contrast, if defences are entirely redundant, one might expect to find negative correlations. Such negative correlations between traits have been often looked for, particularly between phytochemical and physical defences, or classes of phytochemical compounds, but with mixed support (Agrawal, 2007; Koricheva et al., 2004; Rudgers et al., 2004; Mason et al., 2016). Notably, trade-offs between phytochemical and physical traits varied across ontogeny in *Datura* (Kariñho-Betancourt et al., 2015), illustrating the importance of sampling at the same developmental stage, as we have done in this study.

Most prior work has assessed relationships between classes of phytochemical compounds. Examination of phylogenetic signal and trait relationships for individual compounds within the same phytochemical class, as examined in our study, is relatively rare. We found relatively strong phylogenetic signal for phytochemical arsenals, and moderate to low phylogenetic signal for some individual compounds. Results from other studies have found low to moderate phylogenetic signal and a mixture of positive (Johnson et al., 2014),

| Response phenotype | Predictor | Covariate | Value | Std. Error | t-value | p-value |
|---|---|---|---|---|---|---|
| Total PPGs | bFFP | – | −0.09 | 0.05 | −1.94 | .067 |
| Total PPGs | life history | – | 9.35 | 4.88 | 1.92 | .071 |
| Total PPGs | NFFD | – | 0.12 | 0.03 | 3.73 | **.001** |
| Total PPGs | PAS | – | −0.23 | 0.04 | −5.27 | **<.001** |
| Total PPGs | MAT | – | 2.64 | 0.56 | 4.73 | **<.001** |
| Total PPGs | Latitude | – | 0.21 | 0.84 | 0.26 | .801 |
| Total PPGs | Elevation | – | −0.01 | 0.00 | −3.30 | **.004** |
| NMDS1 | bFFP | – | 0.00 | 0.00 | −0.15 | .883 |
| NMDS1 | life history | – | 0.42 | 0.17 | 2.47 | **.023** |
| NMDS1 | NFFD | – | 0.00 | 0.00 | 0.29 | .776 |
| NMDS1 | PAS | – | 0.00 | 0.00 | 0.20 | .844 |
| NMDS1 | MAT | – | 0.01 | 0.03 | 0.27 | .792 |
| NMDS1 | Latitude | – | 0.04 | 0.03 | 1.39 | .182 |
| NMDS1 | Elevation | – | 0.00 | 0.00 | −1.61 | .124 |
| NMDS2 | bFFP | – | 0.00 | 0.00 | −1.38 | .184 |
| NMDS2 | life history | – | −0.01 | 0.13 | −0.11 | .913 |
| NMDS2 | NFFD | – | 0.00 | 0.00 | 1.24 | .230 |
| NMDS2 | PAS | – | 0.00 | 0.00 | −1.67 | .111 |
| NMDS2 | MAT | – | 0.03 | 0.02 | 1.73 | .100 |
| NMDS2 | Latitude | – | −0.01 | 0.02 | −0.28 | .783 |
| NMDS2 | Elevation | – | 0.00 | 0.00 | −1.29 | .212 |
| Total PPGs | Latitude | Elevation | −0.62 | 0.72 | −0.86 | .402 |
| NMDS1 | Latitude | Elevation | 0.03 | 0.03 | 0.91 | .376 |
| NMDS2 | Latitude | Elevation | −0.02 | 0.02 | −0.77 | .450 |
| Total PPGS | NMDS1 | – | 4.61 | 6.14 | 0.75 | .462 |
| Total PPGS | NMDS2 | – | 12.18 | 8.67 | 1.40 | .176 |

**TABLE 3** Phylogenetic generalized least squared (PGLS) model results

*Note:* bFFP is beginning of the frost-free period (Julian days), NFFP is the number of frost-free days, PAS is precipitation as snow (August-July, mm), and MAT is mean annual temperature. P-values in bold are those significant at α = 0.05. P-values in italics are marginally significant. A conservative Bonferroni correction for six independent tests would result in a significance threshold of $p > .0083$.

negative (Kariñho-Betancourt et al., 2015) or no significant relationships between individual compounds (Cacho et al., 2015). Our results suggest that although biosynthetic constraints may exist for some phytochemical compounds, response to natural selection or other evolutionary processes is also possible. The lack of consistent negative relationships among individual compounds in our study as well as in previous studies reinforces the hypothesis that individual compounds are not necessarily redundant with one another but may act independently or synergistically against herbivores (e.g. Agrawal, 2007).

Across a phylogeny, the most parsimonious explanation for congruence in production of particular classes of compounds is that the biosynthetic pathway to produce particular types of compounds has evolved once or only a few times and is highly conserved (Brock et al., 2006; Wink, 2003). In *Inga*, for example, different patterns of presence/absence in particular compounds between closely related species are hypothesized to be due to differences in gene regulation

across species, rather than independent evolution of the ability to synthesize these compounds that are common across the phylogeny (Coley et al., 2018). The production of novel compound classes within a family is rarer but does occur, perhaps most notably in Brassicaceae (e.g. Brock et al., 2006; Züst et al., 2020). A recent study found a stronger phylogenetic signal for concentrations and diversity of cardenolides (evolutionarily novel in the genus) than for glucosinolates (ancestral to the genus) across species in the genus *Erysimum* (Brassicaceae). No significant relationships were found between the classes of compounds for either diversity or concentration, indicating that they are evolving independently of one another (Züst et al., 2020).

We did not find evidence for a phylogenetic signal in total PPG concentration in our study. Weak phylogenetic signal can be associated with a number of different evolutionary processes and scenarios (Revell et al., 2008), so we are not able to pinpoint specific processes that may underlie this result. However, our result is compatible with evidence in the literature for evolution of concentration
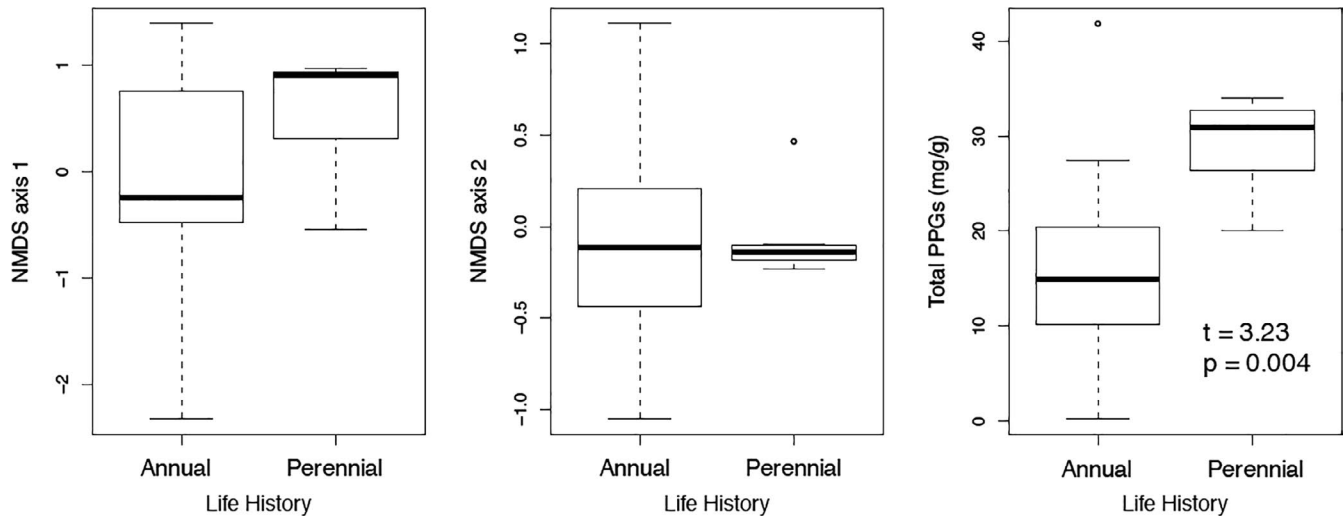
**FIGURE 3** Comparisons of (a) NMDS axis 1, (b) NMDS axis 2 and (c) total PPG concentration in annual and perennial species. Regression lines and statistics reflect linear models without corrections for phylogenetic correlation. Statistics for PGLS models can be found in Table 3. In each boxplot, the bottom and top of the box represent first and third quartiles and the centre line is the median. Whiskers represent the less extreme value of either the minimum/ maximum value or 1.5 times the interquartile range.

of defensive compounds. Among the few studies in the literature that have assessed phylogenetic signal for phytochemical concentrations, there is either no phylogenetic signal for phytochemical concentration, or a significant phylogenetic signal with evidence for reduction in concentrations towards the tips of the phylogeny (Agrawal et al., 2012; Becerra, 2015; Cacho et al., 2015). One caveat with our result is that we did have a limited sample size within species. Concentrations of PPGs are known to vary substantially across populations within at least one *Mimulus* species (*Mimulus guttatus*; Holeski et al., 2013; Kooyers et al., 2017; Rotter et al., 2019); thus, additional sampling might provide more insight into the evolution of phytochemical concentrations across the *Mimulus* phylogeny.

## 4.2 | Defences and life history strategy

Phylogenetically independent trade-offs exist between life history strategy and at least one aspect of defence arsenals; perennial plants produce higher values for NMDS1 than do annual plants. Our raw data show that perennials also produce higher concentrations of total PPGs than do annuals, although this result becomes only marginally significant when phylogenetic corrections are made, as many of the perennial plants in our study are from the same section (Diplacus).

We did not measure growth rate or resource availability for the species in our study, and so cannot directly assess the predictions of the inter-species hypothesis RAH, that shorter life cycles are synonymous with rapid growth rate and lower levels of defence as the negative impact of losing leaf area is low in these species (Coley et al., 1985; Endara & Coley, 2011). However, some annual *Mimulus* do not have higher vegetative growth rates than perennials (Dole, 1992; van Kleunen, 2007). Annual *Mimulus* species tend to have rapid development to reproduction and relatively little

investment in vegetative tissue, although the latter is plastic and can vary from year to year with precipitation (J. Sobel, personal observation). The RAH was developed based upon work in woody plants; it is possible that growth rate/life history relationships differ in some herbaceous systems. In previous studies across phylogenies in herbaceous plants, RAH has had mixed support. In a study of 38 species of milkweed, variation in growth rate did not explain variation in three resistance traits, trichomes, cardenolides and latex (Agrawal & Fishbein, 2008). Across 30 species of *Streptanthus*, some support for the RAH was found; glucosinolate richness increased with habitat bareness, although no relationship was found between glucosinolate production and habitat or soil nutrient variables (Cacho et al., 2015).

Our results showing PPG abundance at the broader scale reflect similar resource allocation trade-offs that we have observed within *M. guttatus* that follow the predictions of ODT. Our results show that species with longer growing seasons and perennials tend to have more investment into constitutive defence than rapid cycling annual plants. Within annual *M. guttatus*, there is a genetic-based trade-off between rapid development to flowering and investment in resistance (Kooyers et al., 2017; Kooyers et al., 2020), compatible with the predictions of ODT. We do not measure time to flowering in this experiment, but it is possible that rapid development time is similarly important across other annual species in the genus and that similar allocational trade-offs underlie our observed patterns.

In addition to the higher values displayed for perennial versus annual species for PPG arsenal and concentration, there was greater variance among annual species for both arsenal composition and PPG concentration than for perennial species. There are multiple potential explanations for the variance pattern. The perennial species represent either mostly perennial sections or are recently derived perennial species nested within groups of annuals. It is possible that the recently derived perennials are evolving differences in defence
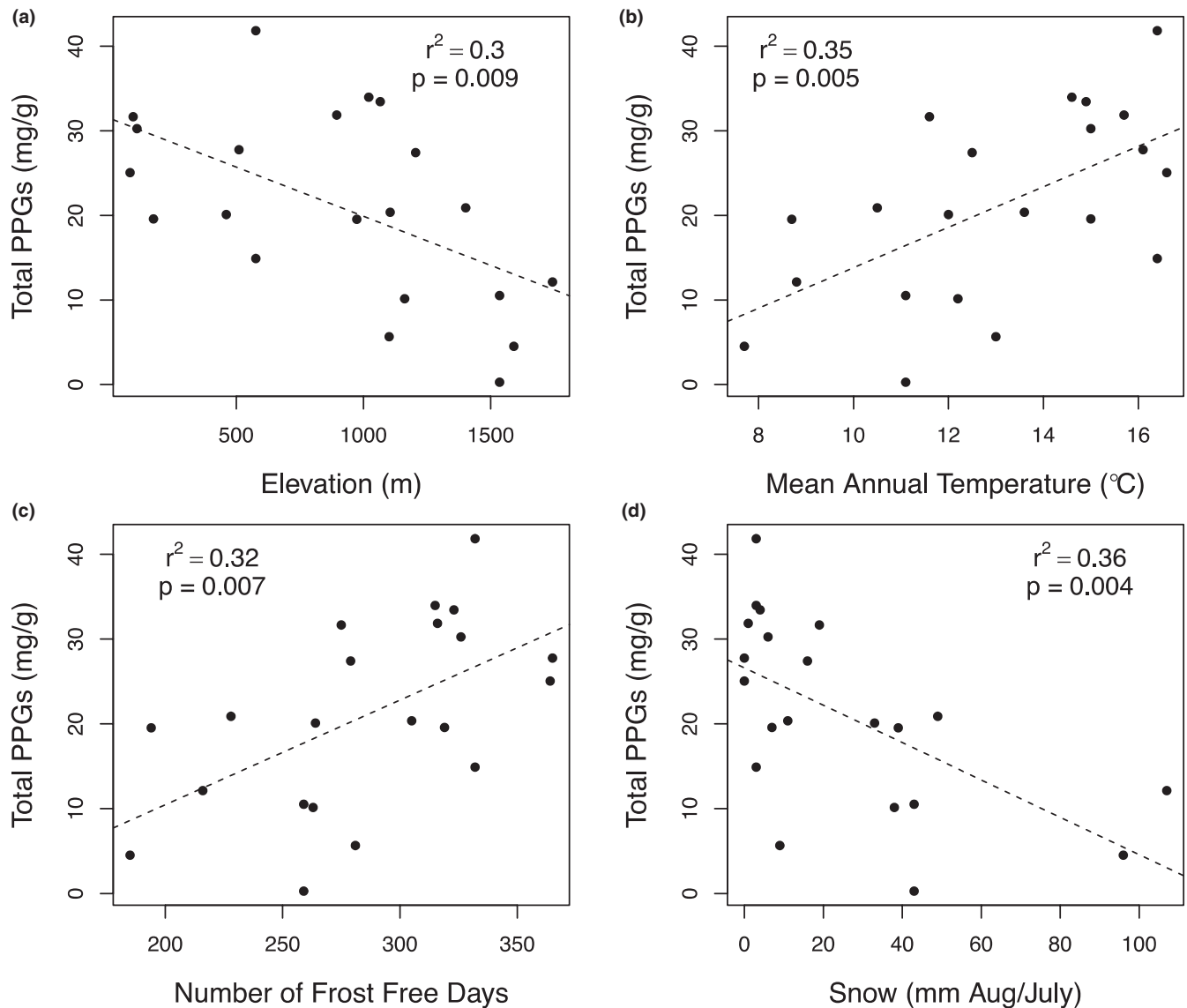
**FIGURE 4** Relationship between total PPG concentration and a) elevation, b) mean annual temperature, c) number of frost-free days and d) amount of precipitation as snow. Regression lines and statistics reflect linear models conduced use the lm() function in R without correction for phylogenetic structure. Statistics for PGLS models can be found in Table 3

from the annuals due to the shift in life history, but the evolution is constrained by developmental canalization and/or genetic variation within the clade. It is also possible that annual species invest primarily in diverse arsenals of constitutive PPGs rather than high levels of PPGs, whereas perennial species invest in arsenals that are less diverse from one another but consist of higher concentrations. A larger sampling of species across the genus and inclusion of assessment of induced PPGs would help to clarify this pattern and would possibly provide more information on the underlying mechanism.

## 4.3 | Defences and the abiotic environment

Variation in the abiotic environment is strongly associated with phytochemical concentrations, although there is little evidence for direct influence of abiotic environmental factors on phytochemical

arsenal across species. Species from areas with a longer growing season (measured as number of frost-free days) tend to produce higher concentrations of PPGs than species from areas with shorter growing seasons. Elevation, amount of precipitation as snow and mean annual temperature also affected PPG concentrations, and are also likely tied to length of the plant growing season. Correlations between these environmental variables and PPG concentrations could be due to abiotic and/or biotic drivers that we are not able to disentangle within the framework of this study. Fewer frosts and warmer mean annual temperatures can maintain increased herbivore pressure, driving the evolution of increased levels of defence (Coley & Barone, 1996; Pennings et al., 2009; Schemske et al., 2009), as well as leading to longer growing seasons. The latter can affect defence levels as well, with evidence for plants with limited growing seasons investing less in defence and more in rapid growth and reproduction (Kooyers et al., 2017). However, growing season length is difficult to

indirectly measure across multiple species (Kooyers et al., 2015). For example, a desert annual species might have a very short growing season but may live in a habitat with a very large number of frost-free days. Several species within our phylogeny may fall into this category, where number of frost-free days is not a reliable indicator of growing season length. We did not find a significant relationship between latitude and phytochemical concentrations or arsenals, contrary to the predictions of the latitudinal herbivory defence hypothesis (Schemske et al., 2009). Future evolutionary ecology studies within multiple species would be necessary to parse apart potential connections between abiotic environment, life history and associated functional traits, and variation in phytochemical arsenals.

## 4.4 | Conclusions

We examined the diversity and abundances of individual phytochemical compounds within a single class across a widespread and diverse plant genus. We found strong phylogenetic signal for phytochemical arsenals. Compound abundances appear to be able to evolve independently from arsenals and show little phylogenetic signal. Compound abundances are significantly influenced by ecology, here assessed as life history and the abiotic environment. Future work might include increased representation of species across the phylogeny, investigation of biotic factors and additional abiotic factors (e.g. drought stress) involved in species ecology, and investigation of both constitutive and induced phytochemistry. In addition, further work might investigate phytochemistry of floral tissue, as a complement to the study of foliar phytochemistry done here (e.g. De La Pascua et al., 2020). Combined, future work may elucidate how intraspecific trade-offs, environmental factors and evolutionary processes contribute to phytochemical variation across species.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.