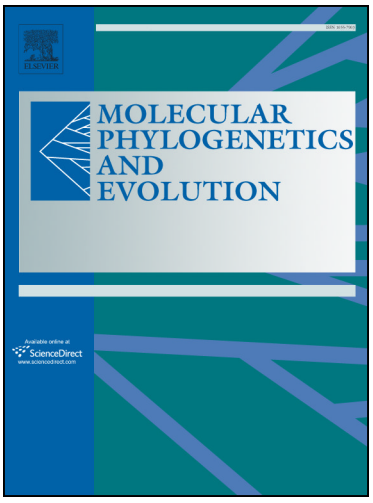

Evolutionary and Ecological Drivers of Plant Flavonoids Across A Large Latitudinal Gradient

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Evolutionary and Ecological Drivers of Plant Flavonoids Across A Large Latitudinal Gradient

Running title: Flavonoids in *Ruellia*

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ABSTRACT

Flavonoids are important secondary metabolites that play an integral role in protecting plants against UV radiation and other forms of environmental stress. Given widespread impacts of environmental effects associated with latitude on a multitude of biological systems and a well-documented increase in solar radiation towards the equator, plant flavonoid production is expected to increase as a response to factors associated with decreasing latitude. Using data from a Neotropical genus (*Ruellia*) that spans an exceptionally broad latitudinal gradient, we tested a hypothesis of a positive latitudinal gradient in flavonoid concentration and assessed other factors that influence flavonoid production including habitat type (xeric vs. wet), altitude, phylogenetic relatedness, and pleiotropic effects. Two flavones with peak absorbance in ultraviolet wavelengths, apigenin and luteolin, were detected across all species. Transcriptome data confirm high expression of the gene required for flavone biosynthesis, *flavone synthase* (*FNS*). Contrary to our prediction, data revealed a positive correlation between flavone concentration and higher latitudes. Further, we recovered strong impacts of xeric habitat, pleiotropy, and phylogenetic relatedness on flavone concentrations. This study documents a complex interplay of ecological, historical, phylogenetic relatedness, and pleiotropic factors driving plant flavonoid production.

KEYWORDS

Flavone, flavonol, latitude, pleiotropy, *Ruellia*, xeric

1. INTRODUCTION

Across latitudes, there exists a steep, positive gradient in UV radiation, with greater intensities of UV-B wavelengths towards lower latitudes (Caldwell *et al.,* 1980; Caldwell 2013). UV radiation is a significant source of environmental stress on plants because of its disruptive impacts on the photosynthetic apparatus, on biomass production, and its mutagenic effects (Robberect *et al.,* 1980; Epel *et al.,* 1999; Flenly 2011; Shaukat *et al.,* 2013; Comont *et al.,* 2012).

One of the most common plant responses to increases in UV radiation is the manufacturing of increased amounts of flavonoids. Flavonoids are phenolic compounds widely regarded as one of the most important classes of plant secondary metabolites (Grotewold 2006). These compounds are central to a variety of functional responses to environmental stimuli including protection against UV radiation, heat, drought, salinity, pathogens, and herbivores (Sisson & Caldwell 1977; Harborne & Williams, 1998; Williams & Grayer 2004; Jaakola & Hohtola 2010; Pollastri 2011). Most flowering plants produce anthocyanins in addition to one or more classes of non-anthocyanin flavonoids (Winkel-Shirley 1996; Fig. 1; Table S1). Studies on plant UV sunscreening have, in particular, highlighted the role of two classes of flavonoids invisible to the human eye–flavones and flavonols–because these absorb ultraviolet wavelengths between ~290–320 nm (UV-B, shortwave) and ~320–400 nm (UV-A, longwave; Koostra 1994; Sasaki & Takahasi 2002; Casati & Walbot 2005; Agati & Tattini 2010; Shaukat *et al.,* 2013). Rather than act as UV attenuators themselves, unlike other phenylpropanoids, flavonoids have been proposed to act as essential antioxidants in photoprotection (Agati *et al.* 2012; Agati *et al.* 2013). These two classes of compounds are widespread across the phylogeny of flowering plants, with available data indicating that 88% of all surveyed species produce one or both types of compounds (33%, both flavonols and flavones; 45%, only flavonols; 10%, only flavones; 12%, neither; Table S1).

Large-scale latitudinal gradients in UV radiation have been explored in a limited number of studies that have tested the effects of latitude on flavonoid presence and/or concentration, and these studies have yielded mixed results. For example, Koski & Ashman (2015) demonstrated a

higher concentration of UV-absorbing floral compounds towards lower latitudes in Silverweed Cinquefoil (*Argentina anserina*) and del Valle *et al.* (2015) found that decreasing latitude helps to explain increased levels of flavonoids in leaves and calyces of Shore Campion (*Silene littorea*). In contrast, negative associations between lower latitudes and flavonoid content have been documented in Common Juniper leaves (*Juniperus communis*; Martz *et al.,* 2009) and in Bilberry fruits (*Vaccinium myrtillus*; Lätti *et al.,* 2008). A negative association between latitude and leaf flavone content was also found in White Birch (*Betula pubescens*) but a positive gradient was documented for leaf flavonols sampled from the same studied individuals (Stark *et al.,* 2008); in these studies, negative associations were explained by other environmental variables, including day length or light quality.

These contrasting patterns of flavonoid production across latitudinal gradients are likely attributable to a greater complexity of factors impacting plant secondary metabolites beyond latitude (Millet *et al.,* 2018). In particular, other studies have shown significant co-variation among altitude, UV-B radiation, and flavonoid content (Caldwell *et al.,* 1980; Casati & Walbot 2005; Bakhshi & Arakawa 2006; Pereira *et al.,* 2006; Berardi *et al.,* 2016a). Similarly, associations have been found between drought stress or arid conditions and flavonoid content (Tattini *et al.,* 2004; Treutter 2006; Agati *et al.,* 2013; Hughes *et al.,* 2013). Phylogenetic history also influences plant secondary metabolites, although within the flavonoid pathway, most research to date has emphasized anthocyanins (Campanella *et al.,* 2014; Ng & Smith 2016) rather than non-anthocyanin flavonoids. Finally, pleiotropic and correlated effects among biochemical pathways that share similar enzymes may help to explain patterns of flavonoid production (Berardi *et al.,* 2016b, Sheehan *et al.,* 2016). Taken together, disentangling meaningful signatures of selection on plant flavonoids across large-scale environmental gradients is likely to be most informative when considering numerous different interacting factors.

To estimate the relative contributions of multiple factors on flavonoid production, we assembled a large comparative matrix of non-anthocyanin flavonoid occurrence and quantity

across a species-rich genus that spans one of the largest latitudinal gradients of any lineage of flowering plants: ca. 80 degrees (i.e., ranging from ~43˚N near Milwaukee, Wisconsin to ~37˚S in central Argentina). The genus *Ruellia* (Wild Petunias: Acanthaceae; Fig. 2) contains upwards of 400 species, most of which are Neotropical. Species in this genus are markedly ecologically diverse and range from wet rainforest understories to myriad xeric to seasonally dry ecosystems (e.g., caatinga, campos rupestre, savanna, dry tropical forests; Tripp 2007; Tripp & Tsai 2017). In addition to considerable diversity in habitat, latitude, and pollination systems (cf. Tripp & Manos 2008), species of *Ruellia* inhabit a broad range of altitudes, ranging from sea level to ~2,800 m.

In this study, our first objective was to assess latitudinal, altitudinal, and habitat impacts on plant flavonoids. Specifically, we test the hypothesis that lower latitudes drive higher plant flavones. Second, we explore the degree to which flavonoid production reflects evolutionary history. Finally, because enzymes responsible for flavones also contribute to anthocyanin production in corollas and in leaves, we examine potential pleiotropic interactions among flavone content, tissue type, and flower color.

2. MATERIALS AND METHODS

2.1 Plant Tissues

We focused our sampling on plants growing at tropical to sub-tropical latitudes (i.e., ~23˚N to 23˚S; Fig. S1) because this region experiences one of the steepest gradients in UV radiation worldwide (NOAA Report 2008). Tissues of species of *Ruellia* used in this experiment (Fig. 2) derived primarily from wild field collections throughout the Neotropics, which represents the center of diversity for *Ruellia* (Table S2). Voucher specimens representing these collections are housed at The University of Colorado Herbarium or The Missouri Botanical Garden Herbarium (Table S2). For 16 accessions, flavone data were generated from herbarium rather than field material (Table S2; we note that we tested whether flavone concentrations varied by tissue collection in the field or herbarium with an ANOVA and found no difference ($F_{(1,23)} = 0.113$, $p = 0.740$). Taxon names, geographical origins, voucher specimens, month sampled, floral color,

tissue type, and latitude are provided in Table 1. Because our goal was to focus on broad-scale geographical and macroevolutionary questions, we sampled tissue from many species across a wide range. To account for species with disparate ranges and also some within-species variation, we attempted to sample second accessions when possible, for a total of 72 accessions of *Ruellia* representing 55 species. These accessions span a broad diversity of variables (e.g., latitude, habitat, altitude, flower color, phylogenetic divergence) that we specifically aimed to explore in this study. Of these, flavonoid data from 54 leaf samples and 49 corolla samples were generated to explore variation in concentration and composition of compounds.

2.2 Extraction and HPLC Analysis of Flavones

Because this was the first study to broadly examine the presence of non-anthocyanin flavonoids across *Ruellia*, we initially screened for the presence of flavonols (kaempferol, quercetin, myricetin) as well as flavones (apigenin, luteolin, tricetin) using analytical standards (flavonols from Sigma Aldrich, St. Louis, Missouri; apigenin and luteolin from Cayman Chemical, Michigan; tricetin from Extrasynthese, Genay, France). No flavonols were detected in pilot runs consisting of 20 samples and thus subsequent screening focused only on flavones,

Plant tissues were dried prior to extraction by removal of nectar and patting to dryness, then placement in a paper mesh bag surrounded by silica gel beads, as in Berardi *et al.,* (2016b). A total of 25 mg of dried corolla tissue or 50 mg of dried leaf tissue was utilized in each extraction. All extracts were subject to acid hydrolysis to remove *O*-glycosides following Harborne (1998) and Markham (1982), using modifications detailed in Berardi *et al.,* (2016b). Briefly, corolla and leaf tissues were hydrolyzed in 2M HCl for 90 min at 103°C. This extract was cooled, washed twice with ethyl acetate, and the resulting supernatant containing flavones was dried and stored at 4°C in a dark container until further analysis. Acid hydrolysis removes glycosides and other moieties attached to the flavonoid via an oxygen molecule (i.e., *O*-glycosylated flavonoids) thus converting them into their flavonoid aglycone forms (e.g., apigenin, luteolin). Flavonoids resistant to acid hydrolysis are typically *C*-glycosides wherein glycosides are directly attached to a carbon molecule on the core flavonoid structure (Harborne

1998; Markham 1982). Thus, we assumed that any flavonoid peaks present in HPLC chromatograms other than those corresponding to aglycone standards and with an appropriate spectral reflectance banding pattern reflected a *C*-glycosylated flavonoid glycoside present in tissues (Harborne 1998). It should be noted that any additional moieties, such as hydroxycinnamic acids connected to a flavonoid glycoside by an ester linkage (also known as acyl groups) would also be removed in the acid hydrolysis procedure.

Dried extracts were re-suspended in 200ul of MeOH:HCl (99:1, v/v). Injections (10 ul) were analyzed using an Agilent Infinity 1260 Series High Performance Liquid Chromatography system with a 100x4.6 mm Chromolith Performance RP-18 endcapped column (Merck Millipore, Darmstadt, Germany). Flavones were separated by gradient elution at a flow rate of 2.0 mL/min at 25°C using solvents A (water with 0.7% TFA), B (acetonitrile), and C (methanol with 0.5% TFA). Starting conditions were 75% A, 0% B, 25% C, and the method proceeded as follows: 0-4 min 57.5% A, 7.5% B, 35% C, 4-8 min hold at 57.5% A, 7.5% B, 35% C, 8.4-9.5 min 50% A, 10% B, 40% C, 9.5-12 min hold at 50% A, 10% B, 40% C, 12-15 min 40% A, 0% B, 60% C, 15-17 min 75% A, 0% B, 25% C. Peaks were detected at 340 and 365 nm (for flavones and pilot flavonol runs, respectively) using photodiode array detector (DAD) scanning from 200 to 600 nm at a step of 2 nm. Chromatograms were visualized using Agilent ChemStation for LC 3D Systems (Rev. B0403, Waldbronn, Germany). Quantification of each compound was conducted by creating a dilution series of a standard compound of each flavonoid aglycone.

Flavonoid aglycone peaks were identified by comparison to retention times and UV spectra of chemical standards. Because many flavonoid *C*-glycoside standards are unavailable, we identified these compounds through similarity in UV spectrum to flavonoid aglycone standards (in this case, either luteolin or apigenin; tricetin was not present in any samples; Fig. S2). Flavone glycosides have very similar and sometimes nearly identical UV spectra, e.g., their maxima, minima, shoulders, and general shape. Thus, *C*-glycoside compounds were quantified as either luteolin or apigenin equivalents. For statistical analyses, we considered four classes of compounds for downstream analyses: *A*-apigenins (any aglycone or *O*-glycosidic apigenins that were hydrolyzed and detected as apigenin aglycone), *A*-luteolins (any aglycone or *O*-glycosidic luteolins that were hydrolyzed and detected as luteolin aglycone), *C*-glycosidic apigenins

(hereafter *C*-apigenins), and *C*-glycosidic luteolins (hereafter *C*-luteolins). We further binned *A*- and *C*-type compounds into total apigenin vs. total luteolin for additional statistical comparisons.

2.3 Gene Expression Analysis of *FLS* and *FNS*

Production of flavones requires expression of the gene *FNS* (for apigenin biosynthesis) or expression of *F3'H* followed by *FNS* (for luteolin), as shown in Fig. 1. To validate our HPLC results, we examined expression levels of *FNS* for samples for which we have available transcriptome data. *F3'H* functions broadly in the production of anthocyanins in most of our targeted species (Zhuang & Tripp 2017a, 2017b) and thus was excluded from further expression study conducted here. We also assessed expression levels of *FLS*, which directs flux towards flavonol rather than flavone production. To identify *FNS* (*Arabidopsis*: XP_020877933) and *FLS* (*Arabidopsis*: NP_196481, NP_201163.1, NP_201164.1, NP_680463.1, NP_201165.1 and NP_680388.1) homologues present in *Ruellia*, we *de novo* assembled tissue specific transcriptomes from leaves and/or corollas of 12 species of *Ruellia*: four red-flowered species (*R. brevifolia*, *R. chartacea*, *R. elegans*, *R. fulgida*), five purple-flowered species including one experimental hybrid (*R. breedlovei*, *R. elegans x speciosa*, *R. hirsuto-glandulosa*, *R. longepetiolata*, *R. simplex*), and three yellow-flowered species (*R. bourgaei*, *R. lutea*, *R. speciosa*; six of these species were also included in our flavone concentration study [Table S2]). *De novo* assembly and homology assessments were conducted as described in Zhuang & Tripp (2017b).

Two classes of flavone synthase have been characterized in dicots, with *FNSII* being much more phylogenetically widespread than *FNSI* (which is restricted to the Apiaceae, Gebhardt *et al.,* 2007), *FLS* largely exists as a single-copy gene in the Asterids (Harborne 1994). Thus, amino acid sequences of six *FLS* genes from *Arabidopisis* (GI:1063730777, GI:1063743028, GI:1063743031, GI:30697866, GI:79332034, GI:22327555) plus three *FNSII* genes from *Glycine max* (GI:318054536, GI:318054538) and *Arabidopsis* (GI: 297806938) were retrieved from NCBI and employed as references for homolog searches using Proteinortho (Lechner *et al.,* 2001).

To measure expression levels of *FLS* and *FNS* homologs identified in *Ruellia*, trimmed reads from previous tissue-specific RNA-seq libraries (SRA BioProject ID: PRJNA323650) were

mapped back to predicted gene sequences using BWA (Li & Durbin 2009) and default parameters. A counts table was generated using eXpress v1.5.1 (Roberts *et al.,* 2013). Data were visualized using the heatmap.2 program available within the gplots package in R (Warnes *et al.,* 2009).

2.4 Environmental and Phenotypic Predictor Variables

The following variables were analyzed to assess potential impacts on flavonoid production: latitude, habitat type, altitude, flower color, tissue type (corolla, leaf), and phylogenetic history. Geographical visualization of samples used in this study (Table 1) was facilitated by the RgoogleMaps package (Loecher & Ropkins 2015). Latitudinal and altitudinal data were taken directly from field collection information or secondarily inferred using Google Earth for previously non-georeferenced herbarium specimens. Species sampled in the present study ranged specifically from ~22.3˚N to ~21.2˚S. and from sea level to ~1700 m elevation. Habitat was scored as a binary character: wet or xeric. Here, wet habitats were taken to be those that remain everwet year-round and lack a pronounced dry season; they are primarily forested ecosystems. Xeric habitats were taken to be those that are marked by a strong dry season and, if forested, experience a deciduous or subdeciduous event annually. Xeric habitats encompass a broad variety of ecosystems ranging from forested environments to savannas to landscapes in between (e.g., “selva baja caducifolia” and “selva mediana subcaducifolia / subperennifolia” in Mexico and adjacent Central America; cerrado, campos rupestre, chiquitano, chaco, and pampas in South America). In this study, 36 species were scored as having affinity to wet habitats and 36 were scored as belong to xeric habitats. No species sampled in the present study spanned both categories, and this condition is unknown in *Ruellia*.

Flower color was scored in one of two ways, first as a binary character (presence of anthocyanins [red, pink, purple flowers] vs. absence of anthocyanins [yellow, white flowers]) and second as an unordered, multistate character (red, pink, purple, yellow, or white flowers). In both cases, color categories were for the most part delimited by spectrophotometer data measured in the field using an Ocean Optics JAZ-COMBO with 600 lines blazed at 300 nm coupled with a ILX511b detector, UV2/OFL-V-4 filter, and an L2 lens with a 50 uM slit. Three

measurements were taken per corolla/species. Following Altshuler (2003), the median wavelength for each sample was used to bin into flower color as follows: ~600–650nm = red; ~420–460 *and* 600–650nm = pink; ~420–460nm = purple; ~500–550nm = yellow; ~420–700nm = white (Fig. S3; File S1). All red, pink, and purple-flowered species of *Ruellia* manufacture anthocyanins whereas all yellow and white-flowered species examined to date do not (Bloom 1976; E. Tripp *et al.,* ms in prep.). In 16 accessions in which flavone data were generated from herbarium rather than fresh field material, color was assigned based on specimen label data or information provided in the species protologue.

2.5 Phylogenetic signal in *Ruellia*

To estimate impacts of shared evolutionary history on resulting flavone data, we constructed a phylogenetic hypothesis using newly generated data from ddRAD loci. All wet lab procedures including library preparation and sequencing are described in Tripp et al. (2017) and summarized here. Briefly, DNA was extracted from leaf tissue using CTAB (Doyle & Doyle 1987), and two restriction enzymes, *EcoR1* and *Mse1*, were used to digest DNA. Custom-designed oligos with an in-line unique barcode affixed to the 5' end were annealed to reads. Following pooling to achieve multiplexing of 96 samples per lane, the 250-500 bp region was size selected though gel excision to reduce the genome sequenced. RAD library preps were conducted in-house (E. Tripp's molecular lab, University of Colorado). Libraries were submitted to the University of Colorado's BioFrontiers Sequencing Facility for QC, column cleanup, and sequencing on an Illumina NextSeq to yield 1x75 bp reads. Resulting reads were demultiplexed using ea-utils (Aronesty 2011) then trimmed of adapters and filtered for quality using cutadapt (Martin 2011). Read quality was checked before and after trimming using FastQC (Andrews 2010). To assemble loci and generate phylip files for downstream phylogenetic analysis, data were processed using pyRAD (Mindepth:5, Wclust:0.85, MinCov:20; Eaton 2013). A best estimate maximum likelihood tree was generated with 100 bootstrap replicates under the GTRGAMMA model using RAxML (Stamatakis 2014). All data were processed using the University of Colorado's SUMMIT High Performance Cluster (Anderson et al. 2017).

Because we additionally generated flavone data for some accessions not represented in our phylogeny, we grafted terminals for these additional taxa (n=3 for leaf tissue and n=5 for corolla tissue) onto the phylogeny using the R package phytools (Revell 2013). For multiple accessions of a species already represented in the phylogeny, these were placed in a sister group relationship with the already represented species. For species not yet represented in the phylogeny, taxa were grafted as an unresolved polytomy nearest to the most recent common ancestor of the clade to which its membership was predicted based on knowledge of morphological synapomorphies (see Tripp 2007). Newly added branches were assigned an arbitrary length of 0.001. To test for phylogenetic signal in our flavone data, we used Moran's I test for autocorrelation and then assessed significance via the function "lipaMoran" implemented in the R package phylosignal (Keck *et al.,* 2016) using both grafted and un-grafted phylogenetic trees. Data were visualized using R package phytools (Revell 2013). Data from a total of 54 leaf samples plus 48 corolla samples were used in these analyses.

2.6 Statistical Analyses

To test for differences in flavone concentration among tissues and species, and for correlations between and among flavone concentration and explanatory variables (latitude, elevation, habitat [xeric or wet], and flower color [binary or multistate]), flavone concentration was first square root transformed. Normality of transformed data was evaluated via Shapiro tests, and homoscedasticity was assessed using the Fligner–Killeen statistic (Crawley 2007). We tested for general differences in flavone concentrations with a two-way ANOVA with specimen type (herbarium or field collected) as a statistical block using the aov function in the base R package "stats" (R Core Team 2014). We conducted tests for correlations using three approaches: (1) Pairwise Pearson's product moment correlations between variables (assuming an FDR adjusted p-value threshold of < 0.05) using the corr.test function in the R package "psych" (Revelle 2017), with p-values adjusted using the Holm-Bonferroni method; (2) phylogenetic generalized least squares (PGLS) tests for each explanatory variable using simple linear regression model $Y \sim X$; and (3) PGLS tests using a multiple linear regression model $Y \sim X_1 + X_2 .. X_n$. All statistical analyses

were conducted using package Phytools (Revell 2013) within the R environment (R Core Team 2014).

3. RESULTS

3.1 Flavone Classes and Distributions

Flavone data from 54 leaf samples and 48 corolla samples were generated to explore variation in concentration and composition of compounds. Total flavones and total apigenins, but not luteolins, varied significantly among species (total flavones $F_{(23,53)} = 7.98$, $p < 0.0001$; total apigenins $F_{(23,53)} = 8.37$, $p < 0.0001$) and tissues (total flavones $F_{(1,23)} = 104.53$, $p < 0.0001$; total apigenins $F_{(1,23)} = 103.75$, $p < 0.0001$), with significant species*tissue interactions (total flavones $F_{(1,23)} = 3.95$, $p < 0.001$; total apigenins $F_{(1,23)} = 4.27$, $p < 0.001$). Corollas produced higher apigenin concentrations than leaf apigenins (Table 2). Across all species, apigenins and especially *C*-apigenins were the most widespread and abundant compounds (Table 2). Across *Ruellia* leaf tissues, *C*-apigenin concentrations were ~10x higher than were *A*-apigenins, but species that contained both *C*- and *A*-apigenins had similar proportions of each type in both leaves and corollas (Table 2 parenthetical values). Whereas the relative amount of *C*-apigenins was not tissue specific, *A*-apigenins were more abundant in corollas than leaves (Table 2). Luteolins were less frequently detected across samples and were consistently low in concentration compared to the apigenins; *A*-luteolins were the most frequent luteolin present in 20 (37.04%) leaf samples (Table 2). The corollas that contained *A*-luteolins produced moderate amounts (0.20 mg per g dried tissue), ~6x higher than the *A*-luteolins produced in leaf samples. Overall levels of intraspecific variation in flavone concentrations (i.e., between two different accessions of a single species) were lower than interspecific variation for all compound comparisons (Table S3).

3.2 Expression analysis of *FLS* and *FNS* gene

Through blast-based homolog searches, we recovered one copy of *FLS* (*flavonol synthase*) and one copy of *FNS* (*flavone synthase*), the latter homologous to the conserved angiosperm *FNS II* gene (Table S4). Expression of *FLS* was extremely low in both leaf and corolla samples (Fig. 3). In

contrast, *FNSII* was highly expressed in both leaf and corolla tissues in most of our samples (low to moderate expression in the corollas of *Ruellia hirsuto-glandulosa* and *R. longepetiolata* and low expression in the leaves of *R. elegans* and *R. speciosa* (Fig. 3).

3.3 Relationship Between Vegetative and Floral Flavones

We recovered a significant correlation between total apigenins present in the leaves and corollas of sampled species (p=0.024; Fig. 4a; correlation coefficients therein). Additionally, total flavone concentration was also positively correlated in leaves and corollas (p=0.013; Fig. 4b; correlation coefficients therein). No significant relationship was found between total luteolins and either leaf or corolla tissues (Fig. 5).

3.4 Geographical, Habitat, Tissue Type, and Floral Color Influences on Flavones

Impacts of several variables on flavone concentration varied depending on statistical approach, specifically whether or not phylogenetic correction was employed (see results of simple correlation analyses vs. the two analyses conducted using PGLS). Because of evidence for phylogenetic signal detected in our dataset (see below), results from tests using PGLS (latter two columns in Table 3) are emphasized. In contrast to our prediction, we recovered a significant negative association between total flavone concentration and lower latitudes in both leaf and corolla tissues, using all three statistical approaches (Figs. 5 & 6, Table 3). Instead, a significant positive association between total flavone concentration and xeric habitats was recovered in our leaf dataset, with the strongest association deriving from our *C*-apigenin dataset (Figs. 5 & 7). We additionally detected a significant correlation between total floral flavone concentration and flowers containing anthocyanins vs. those lacking anthocyanins (*p=0.029*; Fig. 8, Table 3), but this relationship was not significant when flower color was scored as a multistate character (Fig. S4). Between the two classes of flavones produced, there was no relationship between total apigenin and total luteolin produced (Fig. 5). Finally, we failed to recover a relationship between elevation and flavone concentration.

3.5 Phylogenetic Impacts on Flavone Composition

Using our phylogenetic tree that included grafted taxa, we detected evidence of significant positive autocorrelation in the leaf dataset ($p<0.05$) between total flavone concentration and phylogenetic relatedness in three clades (Fig. 9a: the *Ruellia inundata* + *galeottii* clade, within the *Ruellia amoena* lineage, and within the *Ruellia novogaliciana* lineage). All three of these clades contain species that inhabit xeric or seasonally dry environments. Summed across all clades, phylogenetic correlograms, which express changes in a given trait value as a function of relative phylogenetic patristic distance, indicate that the phylogenetic signal present in our flavone dataset is strongest among close relatives and decays quickly at intermediate phylogenetic distances (Fig. 9b). No significant correlation between flavone concentration and phylogenetic relatedness was recovered in our corolla dataset (Figs. 9c & 9d). Similar results were observed for data analyzed with un-grafted phylogenetic trees (Fig. S5).

4. DISCUSSION

Given widespread impacts of latitude on a multitude of biological systems, we expected to recover a strong pattern of increased flavone production with decreasing latitude (sensu Koski & Ashman 2015; del Valle *et al.,* 2015). In contrast, our study of flavone content across 72 accessions of *Ruellia* spanning ca. 40˚ in latitude yielded support for the opposite pattern, which is driven in part by occurrence in xeric or seasonally dry environments. This association can be further explained by phylogenetic history and niche conservatism among xeric environment-inhabiting plant lineages. Flavone concentrations were furthermore positively correlated in leaves and corollas, and concentrations of these metabolites were higher in plants that manufacture anthocyanins in flower tissues suggesting pleiotropic impacts of gene function in different tissues and in connected biosynthetic pathways.

4.1 A Reverse Latitudinal Gradient in Flavone Content and Composition

A gradient in UV radiation and exposure exists from the Earth's Northern and Southern Hemispheres towards the equator that is strongly correlated with latitude, with few regional exceptions (exceptions on a local scale such as variation in cloud cover to be expected). In plants, the mechanism by which flavones and flavonols protect against UV exposure relates to

their antioxidant capacities and their affinity for absorbing solar energy wavelengths (Einbond *et al.,* 2004). Flavones and flavonols exist in stable, hydroxylated states, and hydroxyl groups reduce damaging effects of reactive oxygen species (ROS; e.g., free-radicals) through hydrogen donation. In particular, functional groups attached to the core flavonoid B-ring of these molecules are especially important for such functions (reviewed in Kumar & Pandey 2013). Prior to the present investigation, numerous studies have demonstrated a correlation between flavonoid concentration and UV radiation, including: positive associations between UV and quercetin concentration in white birch (Stark 2008), quercetin and luteolin in *Ligustrum vulgare* leaves (Tattini *et al.,* 2004), flavones and flavonols in several arctic and alpine plants (Nybakken *et al.,* 2004), and non-anthocyanin flavonoids in maize and *Arabidopsis* leaves (reviewed in Winkel 1996, Treutter 2006). Thus, there can be little doubt that flavonoids respond broadly to increased UV radiation and oxidative stress in numerous plant groups across the globe. In contrast to the above studies, our results indicate that a weak, albeit significant "reverse" latitudinal gradient characterizes flavone content in *Ruellia* (Figs. 5 & 6) in which higher concentrations of these metabolites occur towards higher latitudes. This result was driven primarily by apigenin (rather than luteolin) and was recovered in both our vegetative and floral tissue datasets (Fig. 6). One possible explanation for the discordance between results presented herein and prior studies is that ours emphasized among-species comparisons—species of which have widely different habitats—whereas many prior works emphasized within-species study systems wherein different populations of a given species likely span similar (rather than divergent) habitat types.

Another difference between our results and prior studies is that flavonoid accumulation under UV, drought, and other oxidative stress is most often associated with the accumulation of dihydroxylated flavonoids (e.g. luteolin, quercetin, cyanidin; Fig. 1), which are preferentially produced due to superior antioxidant abilities compared to monohydroxylated flavonoids, like apigenin (Agati *et al.,* 2012). If flavones act as efficient antioxidants in *Ruellia* tissues, then why not produce more luteolin than apigenin? There are several suitable and not mutually exclusive explanations: (1) luteolin production may be highly inducible (especially in leaves, where

protection of photosystem II is essential), and was simply not captured at the time of sampling; (2) the apigenin glycosides produced in *Ruellia* may contain other moieties such as acyl groups that increase UV attenuation and antioxidant capacity; (3) distinct apigenin and luteolin compounds may play different roles in leaves and flowers; (4) flavonoid pathway pleiotropy and enzymatic interactions (discussed below) may simply prevent luteolin production in large quantities.

4.2 Effects of Habitat, Phylogeny, Flower Color, and Altitude on *Ruellia* Flavones

We recovered a significant positive association between flavone content and xeric rather than wet habitats in our leaf dataset. Similarly, flavonoid induction via xeric and/or drought-stress conditions has been documented in many other plant species, thought to be in response to water-deficit induced oxidative stress. In *Silene littorea*, total flavonoid content was negatively associated with increased precipitation (Del Valle *et al.,* 2015). In *Ginkgo biloba*, low soil moisture content helps promote flavonoid biosynthesis and accumulation in leaf tissues (Wang *et al.,* 2015). In *Matricaria chamomilla,* apigenin compounds consistently increased in concentration under drought treatments (Hojati *et al.,* 2011). Given this reasoning, xeric conditions could be a major influence on the accumulation of flavones in *Ruellia*.

In this study, equal numbers of species derived from xeric vs. wet habitats (*n=36* vs. *n=36*) such that biased sampling is unlikely to contribute to the above results. However, our finding of higher flavone content in samples of *Ruellia* deriving from xeric habitats is partially confounded by an association between xeric habitats and higher latitudes (Figs. 5 & 7). Xeric habitats such as shrublands, grasslands, and seasonally dry tropical forests, which originated and became widespread following the onset of drying across the Neotropics ca. 15 MY before present (Burnham & Johnson 2004), are especially well-represented in portions of Mexico, Brazil, Paraguay, and Bolivia. These habitats lay at comparatively higher latitudes (N or S of the Equator) than wetter forests that dominate equatorial regions of Costa Rica, Panama, Colombia, and Venezuela.

Although the mechanism(s) that link flavonoid production with xeric conditions are not well understood, we here speculate that xeric habitats marked by a short to long periods of

reduced canopy cover (cf. Mexican dry forests, caatinga) or no canopy cover whatsoever year-round (cf. portions of the cerrado) may in effect 'mimic' those of lower latitudes or higher altitudes that are characterized by high UV and light exposure. Oxidative stress resulting from increased exposure (as a function of reduced canopy cover) and associated xeric conditions typical of these environments are well-recognized factors that induce flavonoid production (Close & McArthur 2002; Tattini *et al.,* 2004; Treutter 2006). Alternatively, higher flavone contents found in lineages occupying xeric or seasonally dry environments may reflect greater investment in anti-herbivory strategies given high costs of manufacturing tissues lost due to damage, and numerous studies have shown that flavonoids help protect against herbivore stress through a variety of mechanisms (reviewed in War *et al.,* 2012; Mierziak *et al.,* 2014). Additional studies in other lineages that span wet to xeric environments are needed to further refine or refute these working hypotheses.

That phylogenetic niche conservatism is likely the rule rather than the exception in *Ruellia* (Tripp 2007; Simon *et al.,* 2009; Pennington & Dick 2010) accentuates the potential impact that phylogeny may have in explaining patterns of plant secondary metabolites across geography. Our study detected significant positive correlation between leaf flavone content and phylogenetic relatedness in three clades, all of which inhabit xeric environments (Figs. 9a & 9b): the *Ruellia inundata* + *galeottii* clade, within *Ruellia amoena*, and within *Ruellia novogaliciana*. These three groups inhabit seasonally dry forests in the Sierra Madre del Sur in Mexico, a region with exceptional representation of intact, dry habitats. However, the southern mountains of Mexico, especially portions of Chiapas, are additionally home to extensive tracts of wet forests. In this study, we sampled several species of *Ruellia* that inhabit these wetter environments in Mexico including *Ruellia jussieuoides*, *R. maya*, *R. matudae*, *R. matagalpae*, *R. megasphaera*, *R. oaxacana*, and *R. stemonacanthoides*, and none showed evidence of significant phylogenetic signal in flavone content. These data yield strong evidence that flavone content is highest among xeric habitat lineages but that xeric lineages also tend to be marked by phylogenetic niche conservatism. We aim to disentangle these two interacting variables in a future, expanded investigation of flavonoid content across broader geographical and taxon sampling of *Ruellia* (E. Tripp *et al.*, in prep.).

Whereas other studies have documented significant associations among altitude, UV radiation, and flavonoid content (Caldwell *et al.,* 1980; Barnes *et al.,* 1987; Murai *et al.,* 2015; Berardi *et al.,* 2016a), we failed to detect a link between flavones and altitude in our study. One potential explanation for this lack of relationship may relate to the overall low altitudes inhabited by species of *Ruellia*. Although our total elevational range of *Ruellia* tissue collections exceeded 1700 m (sea level to 1706m), this range spans relatively low elevations compared to known altitudinal impacts on UV ratios, especially common in high alpine environments much above 2000 m elevation (Caldwell *et al.,* 1980; Liu *et al.,* 2016; Nybakken *et al.,* 2004). However, we caution that our study lacks point data on precise levels of UV radiation and other environmental variables that impact flavonoids (e.g., precipitation, temperature, soil moisture, light, and annual/inter-annual fluctuation in these variables; Graglia *et al.,* 2001; Manetas 2006; Nerg *et al.,* 1994; Pereira *et al.,* 2006; Anderson *et al.,* 2013; Hectors *et al.,* 2014).

4.3 Pleiotropy as a Driver of Flavonoid Composition Across *Ruellia*

The utility of flavonoids in a broad variety of plant functions, manifested in different organs, in different biosynthetic pathways, and at different stages of maturation, yields a number of hypotheses that can be put forward regarding pleiotropic impacts on flavonoid production. *First*, expression of flavonoids in one tissue may be correlated to expression of these same compounds in other tissues through direct or indirect selection. For example, the presence of a specific flavone or flavonol present in flowers may help explain the presence of this same compound in leaf tissues (Armbruster 2002; Berardi *et al.,* 2016b). In contrast, if flavonoid production is instead more responsive to environmental cues, we may expect higher production in leaves than in corollas owing to protection of the photosynthetic apparatus or for antioxidant capacity (Agati *et al.,* 2013). *Second*, because enzyme sharing is common in the flavonoid pathway, production of one class of flavonoids may be correlated to production of a different class of flavonoids that uses one or more same enzymes. For example, expression of the enzyme F3'H can and does affect anthocyanin production as well as flavone and flavonol production (Fig. 1; Berardi *et al.,* 2016b, Larter *et al.* in press). Thus, high expression in one pathway that utilizes a given enzyme such as F3'H may be correlated to high expression of

another pathway that utilizes the same enzyme; in other words, when pathways are 'on', they are 'really on'. An example of the resulting phenotype would be that of “co-pigmentation”, where both anthocyanins and non-anthocyanin flavonoids present in plant tissues result in a shifted visible color (Asen *et al.* 1970). Alternatively, and *third*, because of enzyme sharing, production of compounds in one pathway may instead compete with production in another. For example, the core enzymes CHS, CHI, and F3'H are required for cyanidin (a type of anthocyanin) production, but CHS, CHI, and F3'H are also required to manufacture luteolin (Fig. 1; Kitada *et al.,* 2001; Ueyama *et al.,* 2002). As such, one prediction is that flavone and/or flavonol production may be higher in white- or yellow-flowered species that lack anthocyanin production, where flux would be shunted away from the anthocyanin-specific pathway by DFR and re-directed towards flavone or flavonol pathways, such as in *Petunia* (Sheehan *et al.* 2016).

Our survey of flavone production in leaf and corolla tissues across species of *Ruellia* with quantifiable floral colors permits assessment of the above hypotheses. Specifically, we found (1) a moderate correlation between flavone concentration in leaves and corollas; (2) generally higher concentrations of flavones in corolla compared to leaf tissues, rejecting a hypothesis that photosynthetic leaf tissues should be more protected (by flavones) than floral tissues; and (3) a strong association between red, pink, or purple flowers (i.e., those manufacturing the anthocyanins) and concentration of floral flavones, thus rejecting a hypothesis of competitive interactions between flavone- and anthocyanin-specific pathways utilizing shared enzymes. However, (4) enzymatic competitive interactions may explain a particular within-pathway dynamic in *Ruellia* resulting in more apigenin than luteolin in vegetative and corolla tissues. Even though dihydroxylated flavonoids have greater antioxidant and UV attenuation capacity than monohydroxylated flavonoids and may be more selectively advantageous (Tattini *et al.,* 2004; Agati *et al.,* 2013), the *Ruellia* F3’H enzyme could have greater activity towards dihydrokaempferol than naringenin, leaving FNS to produce mostly apigenin and only very small quantities of luteolin.

Taken together, an association between highly pigmented flowers and flavone concentration indicates the presence of pleiotropic impacts on expression of enzymes involved in multiple biosynthetic pathways. Additionally, correlated high expression patterns across

different tissue suggests either pleiotropic impacts across these different tissues or direct selection on all tissues as a response to environmental stimuli. Finally, that flavone concentration was higher in corollas than in leaves suggests a more complex set of processes, stimuli, and/or genetic interactions that may be controlling flavone production in these tissues in *Ruellia.* Namely, it may be that flavone production is better explained by whether or not the anthocyanin pathway is being expressed than whether corolla tissue vs. photosynthetic tissue is being protected. We are currently in the process of generating data to assess this possibility.

4.4 Flavone and Flavonol Biosynthesis

Outside of the anthocyanins, flavones and flavonols are two of the most widespread and abundant classes of flavonoids. Because of their similarity in chemical structure, it is plausible that plants that produce only one of the two classes would not suffer serious consequences; both absorb in the UV range (albeit minimally in the UV-B range) and have antioxidant capacity (Agati *et al.,* 2012). However, when both flavones and flavonols are produced, functional role diversification can occur (Zhang *et al.* 2009). These compounds are produced through the branching activity of one of two key enzymes: FNS and FLS, respectively. Flavonol production is initiated with the precursor compound dihydrokaempferol, and sequential action by the enzyme *FLS* or action of *F3'H/F3'5'H* followed by *FLS* yields kaempferol, quercetin, and myricetin, respectively (Fig. 1). In contrast, direct action by the enzyme *FNS* or action of *F3'H* followed by *FNS* yields apigenins and luteolins, respectively (Fig. 1), with subsequent activity by glycosyltransferases to yield either flavone *O*- or *C*-glycosides (Jiang *et al.,* 2016). It is not yet clear whether flavone *O*- or *C*-glycosides differ in function or selective advantage in plants; most flavone roles have been associated with both groups (e.g. sunscreening, antioxidants, antiherbivory, allelopathy; Jiang *et al.*, 2016). Additionally, their dedicated biosynthesis is not well described outside of model and horticultural species, and gene homologs have not yet been detected in *Ruellia*. Thus, it is difficult at this time to conjecture as to why *Ruellia* produce more flavone *C*-glycosides than *O*-glycosides; this could be pursued through future research.

While both flavone and flavonol biosynthesis use hydroxylating enzymes, their presence is controlled by either *FNS* or *FLS* activity. In the present study, we found homologs of both *FLS*

and *FNSII* in our *Ruellia* transcriptome data. HPLC profiling documented the presence of only flavones, not flavonols, across all species surveyed in *Ruellia,* and thus we predicted that *FNSII* would be expressed to a greater degree than *FLS*. Our transcriptome expression data support these HPLC results: *FLS* was expressed at very low levels in both leaf and corolla tissues (Fig. 3). In contrast, *FNSII* was expressed at moderate to high levels in all sampled species and tissues except the leaves of *R. longepetiolata* and the corollas of *R. elegans* and *R. speciosa* (Fig. 3). Although we lack flavone data from these latter two samples, it is likely that HPLC profiling will reveal production of flavones in both.

5. Conclusions

The latitudinal gradient is one of the single, most powerful phenomena impacting a multitude of biological systems and biochemical pathways. For example, higher rates of molecular evolution towards the equator driven by increased UV exposure remains a leading hypothesis to explain widespread higher biodiversity at lower latitudes (Dowle *et al.,* 2013). Data presented in this study support prior findings demonstrating that plant flavonoid content is likely to be driven primarily by other interacting factors besides environmental impacts of latitude, as originally hypothesized. Among these factors are substantial impacts of habitat type, phylogenetic history, and pleiotropy on flavonoid biosynthesis. Future studies that fail to incorporate a large complement of potentially impactful factors may yield incomplete understanding of what drives or constrains plant flavonoids.

AUTHORS' CONTRIBUTIONS

ET designed the experiment. ET collected field data. MS & HS conducted flavone extractions; MS & AB generated and quantified HPLC data. YB, AB, MS, and ET conducted data analyses. ET wrote the manuscript. YB and AB contributed to writing the manuscript. MS and HS contributed text to the methods and results. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors have no competing interests to declare.

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Table 1. Materials used in this study. Numbers following Latin name refer to molecular ID accessions. Sample source refers to whether tissue derived from wild field collections or herbaria. Voucher refers to herbarium collection number and herbarium that specimen is housed in.

Latin Name	Tissue	Floral color	Source	Altitude	Voucher	Latitude (˚)
Ruellia amoena6	Corolla	Purple	Field	459m	Tripp & Ruple 5727 (COLO)	19.4607778
Ruellia amoena6	Vegetative	Purple	Field	459m	Tripp & Ruple 5727 (COLO)	19.4607778
Ruellia amoena7	Corolla	Purple	Field	7m	Luján 535 (COLO)	21.165639
Ruellia amoena7	Vegetative	Purple	Field	7m	Luján 535 (COLO)	21.165639
Ruellia bourgaei7	Vegetative	Yellow	Field	1554m	Tripp 428 (DUKE)	20.2938611
Ruellia breedlovei4	Corolla	Purple	Field	1009m	Tripp *et al.,* 5753 (COLO)	16.8031167
Ruellia breedlovei4	Vegetative	Purple	Field	1009m	Tripp *et al.,* 5753 (COLO)	16.8031167
Ruellia ciliatiflora6	Vegetative	Purple	Field	280m	Tripp *et al.,* 5170 (COLO)	9.6249
Ruellia ciliatiflora8	Vegetative	Purple	Field	12m	Tripp *et al.,* 5168 (COLO)	10.6504333
Ruellia cuatrecasasii	Vegetative	White	Field	68m	Tripp *et al.,* 5186 (COLO)	7.56935
Ruellia donnellsmithii4	Corolla	Purple	Field	74m	Tripp *et al.,* 5765 (COLO)	15.5283
Ruellia donnellsmithii4	Vegetative	Purple	Field	74m	Tripp *et al.,* 5765 (COLO)	15.5283
Ruellia eumorphantha4	Corolla	Pink	Field	1092m	Luján 554 (COLO)	-20.49311
Ruellia eumorphantha4	Vegetative	Pink	Field	1092m	Luján 554 (COLO)	17.38125
Ruellia foetida5	Corolla	White	Field	82m	Tripp & Ruple 5732 (COLO)	19.4956667
Ruellia foetida5	Vegetative	White	Field	82m	Tripp & Ruple 5732 (COLO)	19.4956667
Ruellia foetida6	Vegetative	White	Field	22m	Tripp & Ruple 5735 (COLO)	19.6509444
Ruellia foetida7	Corolla	White	Field	12m	Luján 550 (COLO)	17.788444
Ruellia foetida7	Vegetative	White	Field	12m	Luján 550 (COLO)	17.788444
Ruellia fulgida5	Corolla	Red	Herbarium	150m	Tripp & Luján 494 (RSA)	10.4418056
Ruellia fulgida5	Vegetative	Red	Herbarium	150m	Tripp & Luján 494 (RSA)	10.4418056

Ruellia galeottii3	Corolla	Purple	Field	330m	Tripp & Ruple 5726 (COLO)	19.6038889
Ruellia galeottii3	Vegetative	Purple	Field	330m	Tripp & Ruple 5726 (COLO)	19.6038889
Ruellia galeottii4	Corolla	Purple	Field	22m	Tripp & Ruple 5733 (COLO)	19.6509444
Ruellia galeottii4	Vegetative	Purple	Field	22m	Tripp & Ruple 5733 (COLO)	19.6509444
Ruellia grisea	Vegetative	Red	Field	636m	Tripp *et al.,* 5241 (COLO)	2.5360167
Ruellia hirsutoglandulosa2	Corolla	Purple	Herbarium		Ramirez-Amezcua *et al.,* 1098 (RSA)	21.0653889
Ruellia hirsutoglandulosa2	Vegetative	Purple	Herbarium		Ramirez-Amezcua *et al.,* 1098 (RSA)	21.0653889
Ruellia hookeriana5	Corolla	Purple	Field	968m	Tripp *et al.,* 5750 (COLO)	16.7093889
Ruellia hookeriana5	Vegetative	Purple	Field	968m	Tripp *et al.,* 5750 (COLO)	16.7093889
Ruellia humboldtiana5	Vegetative	Red	Field	315m	Tripp *et al.,* 5233 (COLO)	1.4187222
Ruellia humboldtiana6	Vegetative	Red	Field	315m	Tripp *et al.,* 5233 (COLO)	6.2648611
Ruellia inundata6	Corolla	Purple	Field	1090m	Tripp *et al.,* 5752 (COLO)	16.7078889
Ruellia inundata6	Vegetative	Purple	Field	1090m	Tripp *et al.,* 5752 (COLO)	16.7078889
Ruellia jaliscana3	Corolla	Yellow	Field	1380m	Tripp & Deregibus 199 (DUKE)	20.6339167
Ruellia jaliscana3	Vegetative	Yellow	Field	1380m	Tripp & Deregibus 199 (DUKE)	20.6339167
Ruellia jaliscana5	Corolla	Yellow	Herbarium	1500m	Guerrero & Chazaro 275 (MO)	20.8573889
Ruellia jaliscana6	Corolla	Yellow	Field	1730m	Luján 536 (COLO)	20.702806
Ruellia jaliscana6	Vegetative	Yellow	Field	1730m	Luján 536 (COLO)	20.702806
Ruellia jussieuoides6	Vegetative	Purple	Field	34m	Tripp *et al.,* 5185 (COLO)	7.6705
Ruellia jussieuoides8	Corolla	Purple	Field	250m	Tripp *et al.,* 5748 (COLO)	17.2556111
Ruellia jussieuoides8	Vegetative	Purple	Field	250m	Tripp *et al.,* 5748 (COLO)	17.2556111
Ruellia longifilamentosa3	Vegetative	Yellow	Field	1453m	Tripp *et al.,* 5262 (COLO)	4.9355556
Ruellia macarenensis	Corolla	White	Field	373m	Tripp *et al.,* 5248 (COLO)	3.2786389
Ruellia macarenensis	Vegetative	White	Field	373m	Tripp *et al.,* 5248 (COLO)	3.2786389
Ruellia macrophylla12 var. lutea	Vegetative	Yellow	Field	185m	Tripp *et al.,* 5148 (COLO)	11.2959444
Ruellia macrophylla13	Vegetative	Red	Field	260m	Tripp *et al.,* 5174 (COLO)	9.4891333
Ruellia matagalpae5	Corolla	Purple	Field	1100m	Tripp *et al.,* 5749 (COLO)	16.9690556
Ruellia matagalpae5	Vegetative	Purple	Field	1100m	Tripp *et al.,* 5749 (COLO)	16.9690556

Ruellia matagalpae6	Corolla	Purple	Field	966m	Tripp *et al.,* 5760 (COLO)	15.6019722
Ruellia matagalpae6	Vegetative	Purple	Field	966m	Tripp *et al.,* 5760 (COLO)	15.6019722
Ruellia matudae4	Corolla	Pink	Field	1020m	Tripp *et al.,* 5754 (COLO)	15.7508056
Ruellia matudae4	Vegetative	Pink	Field	1020m	Tripp *et al.,* 5754 (COLO)	15.7508056
Ruellia maya3	Corolla	Purple	Field	250m	Tripp *et al.,* 5747 (COLO)	17.2556111
Ruellia maya3	Vegetative	Purple	Field	250m	Tripp *et al.,* 5747 (COLO)	17.2556111
Ruellia mcvaughii3	Corolla	Red	Field	1487m	Luján 537 (COLO)	20.226444
Ruellia mcvaughii3	Vegetative	Red	Field	1487m	Luján 537 (COLO)	20.226444
Ruellia megasphaera3	Corolla	Pink	Field	1090m	Tripp *et al.,* 5756 (COLO)	15.7524167
Ruellia megasphaera3	Vegetative	Pink	Field	1090m	Tripp *et al.,* 5756 (COLO)	15.7524167
Ruellia novogaliciana2	Corolla	Red	Field	1087m	Luján 540 (COLO)	19.346833
Ruellia novogaliciana2	Vegetative	Red	Field	1087m	Luján 540 (COLO)	19.346833
Ruellia novogaliciana3	Corolla	Red	Field	1087m	Luján 541 (COLO)	19.346833
Ruellia novogaliciana3	Vegetative	Red	Field	1087m	Luján 541 (COLO)	19.346833
Ruellia nudiflora8	Corolla	Purple	Field	10m	Tripp *et al.,* 5737 (COLO)	19.1721389
Ruellia nudiflora8	Vegetative	Purple	Field	10m	Tripp *et al.,* 5737 (COLO)	19.1721389
Ruellia oaxacana3	Vegetative	White	Field	558m	Tripp *et al.,* 5771 (COLO)	15.8806944
Ruellia paniculata5	Vegetative	Purple	Field	12m	Tripp *et al.,* 5169 (COLO)	10.6504444
Ruellia paniculata6	Vegetative	Purple	Field	12m	Tripp *et al.,* 5167 (COLO)	10.6504444
Ruellia paniculata7	Vegetative	Purple	Field	280m	Tripp *et al.,* 5171 (COLO)	9.6248889
Ruellia pereducta5	Corolla	Pink	Field	240m	Davidse *et al.,* 20523 (MO)	16.9822222
Ruellia pereducta6	Corolla	Pink	Field	215m	Tripp *et al.,* 5744 (COLO)	16.9822222
Ruellia pereducta6	Vegetative	Pink	Field	215m	Tripp *et al.,* 5744 (COLO)	16.9822222
Ruellia pereducta7	Vegetative	Pink	Field	342m	Tripp *et al.,* 5746 (COLO)	17.1569167
Ruellia petiolaris6	Corolla	Yellow	Herbarium	1000m	Croat 45880 (MO)	17.04
Ruellia petiolaris7	Corolla	Yellow	Field	320m	Tripp *et al.,* 5767 (COLO)	15.8565833
Ruellia petiolaris7	Vegetative	Yellow	Field	320m	Tripp *et al.,* 5767 (COLO)	15.8565833
Ruellia phyllocalyx	Corolla		Herbarium	-	Rimachi 3773 (MO)	-2.0644722

Ruellia pittieri5	Corolla	Purple	Herbarium	100m	Croat & Grayum 59899 (MO)	8.6916667
Ruellia potamophila2	Vegetative	White	Field	702m	Tripp *et al.,* 5220 (COLO)	3.5838056
Ruellia potamophila3	Vegetative	White	Field	829m	Tripp *et al.,* 5219 (COLO)	3.5775556
Ruellia praeclara2	Corolla	White	Herbarium	200m	Rodríguez 1928 (MO)	9.73
Ruellia prostrata3	Corolla	White	Herbarium	-	Prévost 4998 (MO)	4.9480556
Ruellia proxima3	Corolla	White	Herbarium	-	Daly *et al.,* 9034 (MO)	-7.5
Ruellia pulverulenta4	Corolla	White	Herbarium	800-1200m	Liesner & González 9950 (MO)	10.4333333
Ruellia radicans4	Corolla	Red	Herbarium	-	Skog *et al.,* 7145 (MO)	3.1333333
Ruellia rufipila	Corolla	White	Herbarium	-	-	-14.0625
Ruellia ruiziana3	Corolla	Red	Herbarium	1487m	Villarroel *et al.,* 24 (MO, USZ)	-18.1963889
Ruellia saccata	Corolla	Red	Herbarium	250m	Schmidt-Lebuhn 60 (GOET, LPB, US)	-14.45
Ruellia saccata	Vegetative	Red	Herbarium	250m	Schmidt-Lebuhn 60 (GOET, LPB, US)	-14.45
Ruellia sanguinea4	Corolla	Red	Herbarium	1021m	Terán *et al.,* 2728 (BOLV, MO)	-17.1219444
Ruellia sarukhaniana5	Corolla	Yellow	Field	1016m	Luján 546 (COLO, RSA)	18.659806
Ruellia sarukhaniana5	Vegetative	Yellow	Field	1016m	Luján 546 (COLO, RSA)	18.659806
Ruellia simplex6	Corolla	Purple	Herbarium	200m	Zardini & Vera 53576 (AS, MO)	-22.1291667
Ruellia spnov2	Vegetative	White	Field	246m	Tripp *et al.,* 5228 (COLO)	1.450362
Ruellia sp26	Vegetative	White	Field	953m	Tripp *et al.,* 5264 (COLO)	5.2530278
Ruellia sp27	Vegetative	White	Field	333m	Tripp *et al.,* 5266 (COLO)	5.8929444
Ruellia speciosa5	Vegetative	Yellow	Field	1700m	Tripp & Acosta 175 (DUKE)	17.0405556
Ruellia sprucei3	Corolla		Herbarium	120m	Liesner 7202 (MO)	1.9166667
Ruellia standleyi4	Corolla		Herbarium	800-1100m	Stevens *et al.,* 21381 (MO)	13.2333333
Ruellia stemonacanthoides5	Corolla	Purple	Field	423m	Tripp *et al.,* 5762 (COLO)	15.3788333
Ruellia stemonacanthoides5	Vegetative	Purple	Field	423m	Tripp *et al.,* 5762 (COLO)	15.3788333
Ruellia tubiflora	Corolla	White	Herbarium	1050m	McDade & Stein 933 (MO)	4.0833333
Ruellia yurimaguensis3	Corolla	Purple	Herbarium	207.5m	Reynel et al., 5331 (MO)	207.5 Reynel *et al.,* 5331 (MO)

Table 2. Flavone composition and concentrations across accessions of *Ruellia* sampled. Percentages reflect the number of either leaf or corolla samples containing a given class of compounds compared to the total number of leaves or corollas sampled. Mean concentration units are mg of flavone per gram of dry tissue. Values in parentheses reflect adjusted means and standard deviations, where species that did not produce a given class of flavones were removed from the dataset.

	A-Apigenin		C-Apigenin		A-Luteolin		C-Luteolin		Total Apigenin		Total Luteolin	
	Leaf	Corolla	Leaf	Corolla	Leaf	Corolla	Leaf	Corolla	Leaf	Corolla	Leaf	Corolla
Percent (%)	7.41	79.59	98.15	91.84	37.04	22.45	31.48	14.29	98.15	91.84	44.44	30.61
Mean Conc.	0.03 (0.36)	0.42 (0.53)	0.37 (0.37)	0.69 (0.75)	0.01 (0.03)	0.04 (0.20)	0.03 (0.09)	0.01 (0.09)	0.39	1.11	0.04	0.06
SD	0.12 (0.33)	0.48 (0.48)	0.30 (0.30)	0.62 (0.61)	0.02 (0.02)	0.12 (0.19)	0.06 (0.08)	0.04 (0.06)	0.32	0.86	0.08	0.15

Table 3. P-value comparisons of correlation analyses using three different statistical approaches, the latter two correcting for phylogenetic relatedness (see methods). Significance is denoted at the 0.05 and 0.01 thresholds. Direction of relationships (significantly positively correlated or negatively correlated) is denoted with (+) or (-) following the p-value. The flower color variable is based on multistate scoring of this trait.

	Simple corr.test		PGLS (single variable)		PGLS (multiple variables)	
	Leaf	**Corolla**	**Leaf**	**Corolla**	**Leaf**	**Corolla**
Flower.color	0.044* (+)	0.057	0.135	0.047* (+)	0.105	0.035* (+)
Elevation	0.713	0.417	0.530	0.384	0.924	0.071
Habitat	0.000** (+)	0.113	0.002** (+)	0.134	0.004** (+)	0.380
Latitude	0.018* (-)	0.038* (-)	0.047* (-)	0.025* (-)	0.017* (-)	0.041* (-)

*P<0.05
**P<0.01

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Figure legends

Fig. 1 Simplified flavonoid pathway showing three of seven major groups of compounds comprising the flavonoid pathway: flavones, flavonols, and anthocyanins. Enzymes are denoted via abbreviations (capital letters) along arrows; substrates are denoted between the arrows. Apigenin and luteolin are the core flavones produced by *Ruellia* and are highlighted in a gray box. To date, the trihydroxylated flavone tricetin has been detected primarily in non-flowering plants such as liverworts (Campos *et al.,* 2002).

Fig. 2 Floral phenotypic diversity of select species of *Ruellia* included in this study. (a) *Ruellia maya* [*E. Tripp et al. 5747*]. (b) *Ruellia novogaliciana* [*M. Luján et al. 540*]. (c) *Ruellia longifilamentosa* [*E. Tripp et al. 5262*]. (d) *Ruellia donnell-smithii* [*E. Tripp et al. 5765*]. (e) *Ruellia petiolaris* [*E. Tripp et al. 5767*]. (f) *Ruellia matudae* [*E. Tripp et al. 5754*]. (g) *Ruellia mcvaughii* [*M. Luján et al. 537*]. (h) *Ruellia proxima* [*E. Tripp et al. 6001*]. (i) *Ruellia matagalpae* [*E. Tripp et al. 5760*]. (j) *Ruellia megasphaera* [*E. Tripp et al. 5756*]. (k) *Ruellia humboldtiana* [*E. Tripp & Luján 495*]. (l) *Ruellia speciosa* [*E. Tripp & S. Acosta 175*]. (m) *Ruellia ochroleuca.* (n) *Ruellia ruiziana* [*E. Tripp et al. 6028*]. (o) *Ruellia paniculata* [*E. Tripp et al. 5905*]. Herbarium vouchers of all collections deposited at the University of Colorado Herbarium (COLO).

Fig. 3 Heat map showing expression levels of homologs of *FLS* and *FNSII* recovered in *Ruellia* (a) corolla and (b) leaf tissues. High expression intensities represented by red; low expression intensities represented by yellow to yellowish-brown.

Fig. 4 Pearson correlations of (a) apigenin and (b) total flavone content between leaf and corolla tissues. Empirical data points shown as black dots, red line shows linear regression, gray area indicates 95% confident interval.

Fig. 5 Pairwise Pearson correlations of flavone concentrations and environmental factors for (a) corolla and (b) leaf tissues. Pearson's associations visualized at an adjusted *p=0.05* threshold. Significant correlations among variables shown via presence of a dot. Size of dot indicates strength of the correlation. Color of dot indicates direction of correlation (negative, red; positive, blue). For habitat, red indicates association with xeric environments whereas blue indicates association with wet environments. Flower.color.M (a) indicates results from coding flower color as a multistate character (red, pink, purple, yellow, white). Flower.color.B (b) shows results from coding flower color as a binary character ('pigmented' or with anthocyanins [pelargonidin, cyanidin, and/or delphinidin], vs. 'unpigmented' or without anthocyanins), and positive association is between flavones and presence of anthocyanins (see text).

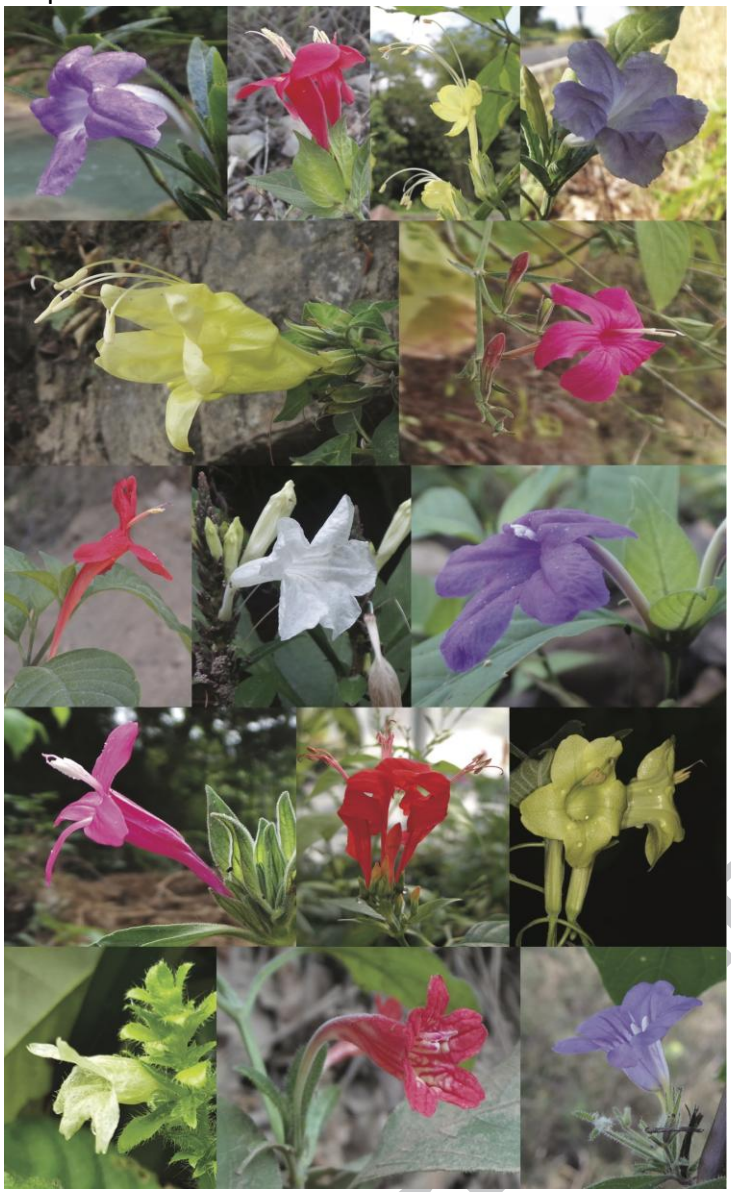
Fig. 6 Relationship between (a) latitude and apigenin concentration and (b) latitude and total flavone concentration in leaf tissues; relationship between (c) latitude and apigenin concentration and (d) latitude and total flavone concentration in corolla tissues. Black line shows linear regression.

Fig. 7 Boxplots (median, quartiles, and range) showing relationship between habitat and (a) apigenin and (b) total flavone concentrations in leaves; relationship between habitat and (c) apigenin and (d) total flavone concentrations in corollas.

Fig. 8 Boxplots (median, quartiles and range) showing a lack of correlation between 'unpigmented' (i.e., yellow or white) flowers and flavone concentration but a positive correlation between 'pigmented' (i.e., red, purple, pink) flowers and flavone concentration.

Fig. 9 Phylogenetic distribution of total flavone concentrations (= the trait value, on the scale) in leaf (a) and corolla (c) tissues. Samples with significant phylogenetic autocorrelation marked by red star and samples manually grafted onto phylogeny marked via a black asterisk. Phylogenetic correlograms showing autocorrelation of total flavone concentration at different phylogenetic lagtimes in the leaf (b) and corolla (d) datasets. Horizontal black line indicates the expected value of Moran's I under a null hypothesis of no phylogenetic autocorrelation. Solid black curved line represents the Moran's I index of autocorrelation. Dashed black lines represent the lower and upper bounds of the confidence envelope (95%). The colored red bar in (b) shows where positive autocorrelation was detected.

Graphical abstract



Highlights

- Flavones play an integral role in protecting plants against UV radiation and other forms of environmental stress.
- Flavone data from a species-rich radiation of Neotropical plants were employed to test hypotheses of extrinsic and intrinsic drivers of biochemical evolution.
- Xeric conditions, but not latitude, drive high flavone content in plant leaves and flowers.
- Pleiotropy additionally drives higher accumulation of flavones in plants that manufacture floral anthocyanins than plants that do not.
- Both ecological and evolutionary factors drive plant flavone content.