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Chemistry of floral rewards: intra- and interspecific variability of nectar and pollen secondary metabolites across taxa

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Abstract. Floral chemistry mediates plant interactions with pollinators, pathogens, and herbivores, with major consequences for fitness of both plants and flower visitors. The outcome of such interactions often depends on compound dose and chemical context. However, chemical diversity and intraspecific variation of nectar and pollen secondary chemistry are known for very few species, precluding general statements about their composition. We analyzed methanol extracts of flowers, nectar, and pollen from 31 cultivated and wild plant species, including multiple sites and cultivars, by liquid-chromatography–mass-spectrometry. To depict the chemical niche of each tissue type, we analyzed differences in nectar and pollen chemical richness, absolute and proportional concentrations, and intraspecific variability. We hypothesized that pollen would have higher concentrations and more compounds than nectar, consistent with Optimal Defense Theory and pollen's importance as a male gamete. To investigate chemical correlations across and within tissues, which could reflect physiological constraints, we quantified chemical overlap between conspecific nectar and pollen, and phenotypic integration of individual compounds within tissue types. Nectar and pollen were chemically differentiated both across and within species. Of 102 compounds identified, most occurred in only one species. Machine-learning algorithms assigned samples to the correct species and tissue type with 98.6% accuracy. Consistent with our hypothesis, pollen had 23.8- to 235-fold higher secondary chemical concentrations and 63% higher chemical richness than nectar. The most common secondary compound classes were flavonoids, alkaloids, terpenoids, and phenolics (primarily phenylpropanoids including chlorogenic acid). The most common specific compound types were quercetin and kaempferol glycosides, known to mediate biotic and abiotic effects. Pollens were distinguished from nectar by high concentrations of hydroxycinnamoyl-spermidine conjugates, which affect plant development, abiotic stress tolerance, and herbivore resistance. Although chemistry was qualitatively consistent within species and tissue types, concentrations varied across cultivars and sites, which could influence pollination, herbivory, and disease in wild and agricultural plants. Analyses of multivariate trait space showed greater overlap across sites and cultivars in nectar than pollen chemistry; this overlap reflected greater within-site and within-cultivar variability of nectar. Our analyses suggest different ecological roles of nectar and pollen mediated by chemical concentration, composition, and variability.

Key words: cultivar variation; dynamic range boxes; floral chemistry; floral rewards; intraspecific variation; n-dimensional hypervolume; phenotypic integration; plant secondary metabolites; plant–microbe interactions; plant–pollinator interactions; site variation.

Introduction

Floral reward chemistry is central to ecology, mediating interactions with pollinators, flower-visiting antagonists, and microbes (Strauss and Whittall 2006, Irwin et al. 2010, Huang et al. 2012, Good et al. 2014, McArt et al. 2014) that influence plant reproductive success. Alkaloids, phenolics, terpenoids, and proteins have been found in nectar (Baker 1977, Adler 2000, Nicolson and Thornburg 2007, Heil 2011, Stevenson et al. 2017). Numerous secondary metabolites, including phenolic compounds (De-Melo and de Almeida-Muradian 2017), alkaloids (Wink 1993, Dübecke et al. 2011), and terpenoids (Flamini et al. 2003) occur in pollen. Nectar chemicals can deter nectar robbers (Barlow et al. 2017), preserve nectar from spoilage (Herrera et al. 2010), or act as floral filters that conserve food rewards for effective pollinators (Tiedeken et al. 2016), but could also occur as a pleiotropic consequence of plant defense against foliar herbivory (Adler 2000, Heil 2011). Pollen secondary chemistry is also central to plant reproduction, mediating interactions with pollinators, microbes, and the abiotic environment (Dobson and Bergstrom 2000, Murphy 2000, Pacini and Hesse 2005, Arnold et al. 2014).

Floral chemistry can have effects that depend on organism, dose, and context. First, many floral compounds attract pollinators, but repel ants and other non-pollinating insects (Stephenson 1982, Junker and Blüthgen 2010, Galen et al. 2011, Junker et al. 2011a) and inhibit microbes (Dobson

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and Bergstrom 2000, Huang et al. 2012, Junker and Tholl 2013). In some cases, however, nectar chemicals can deter consumption by pollinators (Hagler et al. 1990, Hagler and Buchmann 1993, Kessler et al. 2008, Barlow et al. 2017), with negative as well as positive effects on plant reproduction in different systems (Adler and Irwin 2005, 2012, Kessler et al. 2008, Thomson et al. 2015). Second, the same compound can have different consequences at different doses. For example, low concentrations of caffeine in nectar improved pollinator memory and increased pollination services to artificial flowers (Wright et al. 2013, Thomson et al. 2015), but high concentrations of caffeine and other compounds deterred pollinators (Singaravelan et al. 2005, Wright et al. 2013). Third, compounds may have different effects in the context of chemical mixtures. For example, individual floral volatiles may be attractive only as components of a blend (Hebets and Papaj 2005).

Despite the importance of chemical concentration and context in floral ecology, challenges associated with chemical analysis of nectar and pollen have limited the number of species for which secondary chemistry has been fully and quantitatively described. Although qualitative assays of particular compound classes date back many decades (Baker 1977, Dobson 1988), quantitative assessments are still limited to a handful of plant species, and often target particular compounds. Within species, chemical composition of floral rewards can vary at the scale of individual plants, patches, and populations (Kessler et al. 2012, Egan et al. 2016), and this variation can influence plant–pollinator interactions (Kessler et al. 2012, Thomson et al. 2015, Barlow et al. 2017). However, even in well-studied species, little is known about the extent of, or contributors to, intraspecific variation in nectar and pollen chemistry.

The relative costs and benefits of attraction and defense may be different for pollen than for nectar. Chemical defense of pollen makes intuitive sense because pollen is the male gamete and therefore requires chemicals for development (Grienenberger et al. 2009) and for protection from insects, microbes, and abiotic stressors such as desiccation and UV light (Pacini and Hesse 2005), whereas the sole purpose of nectar is to reward mutualists. Optimal defense theory predicts that defensive chemicals are preferentially allocated to a plant's most valuable tissues (Zangerl and Rutledge 1996). Therefore, we might expect pollen to have higher concentrations of defensive compounds than nectar has (Cook et al. 2013). Indeed, in two *Delphinium* species, anther alkaloid concentrations were 150- to 3,000-fold higher than nectar concentrations, and comparable to levels in leaves, flowers, and fruits (Cook et al. 2013). However, in *Chelone glabra*, iridoid glycoside concentrations were similar in nectar and pollen (Richardson et al. 2016), and in *Brugmansia aurea*, alkaloid concentrations were higher in nectar than pollen (Detzel and Wink 1993). These examples emphasize the need to compare differences in chemical concentrations of pollen and nectar in a wider range of plant species to make general statements about relative amounts in nectar vs. pollen.

Within a single species, the chemistry of nectar and pollen may be interdependent. Studies on other plant parts reported chemical correlations between leaves and fruits (Wink 1988, Agrawal et al. 2002), leaves and flowers (Kessler and Halitschke 2009, Kessler et al. 2011), leaves and nectar (Adler et al. 2012), and flowers and nectar (Barlow et al. 2017). These correlations suggest the hypothesis that secondary chemical concentrations in floral rewards may reflect pleiotropic consequences of natural selection for greater defense of leaves or flowers against herbivores (Adler 2000), or of artificial selection for lower secondary compound concentrations in the edible parts of cultivated plants (Wink 1988). On the other hand, many compounds are exclusive to either nectar, pollen, or leaves (Kessler and Baldwin 2007, Manson et al. 2012, Marlin et al. 2014, Stevenson et al. 2017), which suggests that plants can selectively allocate secondary compounds both quantitively and qualitatively. This selectivity could enable plants to transcend ecological costs through maintenance of tissue-specific chemical composition and consequent ecological function. For example, in *Nicotiana africana*, multiple insect-deterrent alkaloids occur in leaves, but these compounds are absent from nectar; this selective distribution may facilitate defense against herbivores without repellence of pollinators (Marlin et al. 2014). A survey that assesses overlap between nectar and pollen chemical composition across a range of species would help to elucidate the extent of interdependence between nectar and pollen chemistry, and the degree to which chemistry of these two plant parts can evolve independently.

Covariation among nectar and pollen compounds, termed "phenotypic integration" (Pigliucci 2003), may mediate attractiveness to and repellency of specific chemical combinations (Junker et al. 2018). In other words, covariation among compounds may modulate the effects of individual chemicals and concentrations. For example, in many host-seeking herbivore/pollinators, individual volatiles from host plants are less attractive than multi-compound blends (Bruce and Pickett 2011). In pollinators, multiple integrated signals can help floral visitors learn to associate food, or toxicity, with specific visual, olfactory, and gustatory stimuli (Dobson 1988, Cook et al. 2005, Junker and Parachnowitsch 2015). This learning of reward-associated signal patterns, which is facilitated by within-species consistency of multiple floral traits, promotes efficient resource collection by pollinators and effective pollination of plants (Heinrich 1975). In pollen specifically, integrated synthesis and degradation of different metabolites may be critical to development and maturation of the pollen grain and surrounding pollenkitt (Pacini and Hesse 2005, Blackmore et al. 2007), and therefore essential for plant fecundity. However, to our knowledge, phenotypic integration of nectar and pollen has not been investigated in any species (Dobson 1988, Cook et al. 2005, Junker and Parachnowitsch 2015).

Thorough characterizations of floral reward secondary chemistry in a diverse array of species are needed to test ecological hypotheses related to tissue-specific differences in composition, constraints between nectar and pollen chemistry of the same species, and the extent of intraspecific variation across genotypes and environments. Therefore, we conducted a comprehensive LC-MS-based characterization of nectar and pollen secondary chemistry from 31 cultivated and wild plant species in 21 angiosperm families to address the following questions: (1) What are the common classes of secondary compounds in nectar and pollen? (2) How diverse are secondary metabolites in nectar and pollen across

species? (3) How do conspecific nectar and pollen differ quantitatively and qualitatively? (4) Within species, how does chemistry vary across cultivars and across sites? (5) Within a species and tissue type, what is the level of phenotypic integration, and is integration of nectar correlated with integration of pollen?

Materials and Methods

Study sites and sampling design

Nectar, pollen, and flower samples (hereafter referred to as "tissue types") were collected from 31 phylogenetically diverse species of flowering plants from 21 families in Massachusetts, Vermont, and California, United States, in 2013 and 2014 (Appendix S1: Table S1). To characterize intraspecific variation in cultivated species, we collected up to 10 samples from each of three cultivars; for wild species, we collected up to 10 samples from each of three sites (see Appendix S1: Table S1; Data S1: Species_metadata.txt, Sites.txt, and Cultivars.txt for all species names, sample sizes, site locations, and cultivar codes). Samples were obtained from local farms, natural areas, or along roadsides (after obtaining permission where necessary), and in some cases plants were purchased from nurseries (*Antirrhinum majus*, two cultivars of *Dicentra eximia*, *Digitalis purpurea*, *Eupatorium perfoliatum*, *Lobelia siphilitica*, and *Penstemon digitalis*). We chose a mix of native and introduced species, with an emphasis on common species that are bee pollinated or for which we had prior knowledge of floral secondary chemistry to facilitate analyses. For crop plants, we focused on species whose yield is improved by pollination (Delaplane et al. 2000).

Sample collection

Nectar was collected with microcapillary tubes from flowers bagged in mesh for 24 h to allow nectar to accumulate. For most species, nectar was pooled across individual flowers and, when necessary, across plants to obtain a sufficient volume for analysis. Care was taken to avoid contamination of samples with pollen. Depending on the plant species, we collected nectar either from the top or bottom of the corolla after removing the flower from the plant. Each nectar sample contained at least 5 μL but typically 20 μL nectar, added to 80 μL EtOH to prevent spoilage. Samples were kept on ice in the field, then stored at −20°C until lyophilization. Alcohol from *Thymus vulgaris* nectar samples was evaporated at room temperature. For *Antirrhinum majus* and *Rhododendron prinophyllum*, nectar was initially too viscous to collect with microcapillary tubes. To facilitate collection, we added 20 μL deionized water to each flower's nectary, and collected the resulting liquid several hours later. Concentrations and composition of these species' nectar should therefore be interpreted with caution.

Pollen was collected from plants with mature undehisced or newly dehiscing anthers. For 17 species, we could only obtain sufficient quantities of pollen by collecting anthers, and, for *Solidago canadensis*, whole flower tops. Anther samples consisted of pollen, the pollen sac, and a small amount of filament. For simplicity, we refer to both anther and pollen samples as "pollen." We aimed to collect at least 5 mg/sample. In most species, pollen was pooled across flowers within plants, but not across plants. Samples were lyophilized and stored at −20°C until extraction. Flowers were also collected. These were mainly used to confirm identification of compounds found in nectar and pollen, but full chemical profiles were analyzed for nine species. The flower sample consisted of the entire flower for five species, the flower without anthers for two species, the flower without carpel for one species, and the flower without calyx for one species (see Appendix S1: Table S1).

Sample processing and chemical analyses

Lyophilized nectar was redissolved in 50 μL methanol. Pollen samples were extracted in methanol as previously described (Arnold et al. 2014, Palmer-Young et al. 2016). Dried, unground pollen or flowers (5–50 mg) were sonicated for 10 min with 1 mL methanol in a 2 mL microcentrifuge tube, then incubated without shaking for 24 h at room temperature. Samples were centrifuged for 5 min at 11,000 *g*, and the supernatant transferred to a glass vial.

Extracts were analyzed by liquid chromatography (LC)-Electrospray Ionization Mass Spectroscopy (ESIMS) and UV spectroscopy using a Micromass ZQ LC-MS (Waters, Elstree, Herts, UK). Aliquots of nectar or pollen extract (10 μL) were injected directly onto a Phenomenex (Macclesfield, Cheshire, UK) Luna C18(2) column (150 × 3.0 mm inner diameter, 5 μm particle size). Samples were eluted with solvents A, MeOH; B, H_2O; C, 1% HCO_2H in MeCN with the following program: A = 0%, B = 90% at t = 0 min; A = 90%, B = 0% at t = 20 min; A = 90%, B = 0% at t = 30 min; A = 0%, B = 90% at t = 31 min; solvent C was a constant 10% throughout the run. Column temperature was 30°C and flow rate 0.5 mL/min. To facilitate compound identification, High Resolution ESIMS data were recorded on a subset of samples using a Thermo LTQ-Orbitrap XL mass spectrometer (Waltham, Massachusetts, USA) coupled to a Thermo Accela LC system performing chromatographic separation of 5 μL injections on a Phenomenex Luna C18(2) column (150 mm × 3.0 mm inner diameter, 3 μm particle size). The Orbitrap used the same mobile phase gradient, column temperature, and flow rate as described for the ZQ-LCMS. Spectra were recorded in positive and negative modes at high resolution (30,000 FWHM [full width at half maximum]).

Compounds were identified by comparison with mass spectra in the National Institute for Standards and Technology (USA) spectral database version 2.0 (Kramida et al. 2013) and, when possible, spectral comparisons with authentic standards in the library at Royal Botanic Gardens, Kew, UK. Compound quantities were calculated from external standard curves based on mass spectra or UV absorbance of the same compound; if the compound was not available, a standard curve for a compound with the same chromophore was used instead. All concentrations are given in micromolar (μmol/L original volume for nectar, μmol/kg dry mass for flower and pollen). Nectar samples were typically too small to obtain accurate dry masses, which obligated the use of fresh volume-based concentrations, and pollen is generally partially dehydrated at maturity (Heslop-Harrison 1979, Pacini et al. 2006), suggesting that dry and fresh mass-based concentrations are

reasonably similar for pollen. Most amino acids eluted in the solvent front and could not be quantified; therefore, we quantified only phenylalanine and tryptophan. "Alkaloids" as defined in the figures include all nitrogen-containing compounds except amino acids, including spermidine derivatives, and we note here that the boundaries of the alkaloid chemical class are not universally agreed upon (Hesse 2002). "Chlorogenic acids" refer to all phenylpropenoid derivatives of quinic acid.

Statistical analyses

All analyses were conducted in R version 3.3 for Windows (R Core Team 2014).

Species accumulation curves.—To visualize chemical diversity across species, chemical species accumulation curves were computed with the vegan package v2.5, function specaccum (Oksanen et al. 2017), and graphed with ggplot2 v2.2 (Wickham 2009), cowplot v0.9 (Wilke 2016), and ggdendro v0.1 (de Vries and Ripley 2016). Color palettes used in figures were recommended by P. Tol (Tol 2012). Within- and cross-species accumulation curves were computed separately. We assessed accumulation of new compounds as more samples of a given species were analyzed within species, and as additional species were analyzed across species.

Random forest.—Distinctiveness of species and tissue types was assessed by random forest machine-learning algorithm (Breiman 2001). This technique determined whether samples could be reliably assigned to their correct species and tissue type based on proportional composition. It has been used previously to distinguish between bacterial communities (Junker and Keller 2015), and different blends of floral volatiles (Junker et al. 2011*b*). To convert absolute concentrations to proportions, the absolute concentration of each compound (in μmol/L) within each sample was divided by the sample's total concentration of quantifiable compounds. The analysis was implemented in R package randomForest v4.6 (Liaw and Wiener 2002) with 10,000 iterations and 10 randomly sampled compounds used for each split in the tree (mtry = 10). The out-of-basket rate indicated the proportion of incorrectly assigned samples.

Nonmetric multidimensional scaling.—Clustering of sample chemical compositions by species and tissue type was visualized with non-metric multi-dimensional scaling (NMDS) based on Bray-Curtis distances between each sample's proportional concentrations with function vegdist (Oksanen et al. 2017). NMDS of the distance matrix was performed with function isoMDS (Venables and Ripley 2002). Within-species ordinations were produced with function metaMDS, which applies a Wisconsin double standardization and square-root transformation to the original data matrix, then computes an ordination based on Bray-Curtis distances between samples (Oksanen et al. 2017). The metaMDS ordination method was not used for the full cross-species data set because it resulted in convergence errors, but was used for visualization of within-species variation because it allows creation of convex hulls for each within-species group.

Differences in chemical composition across tissue types, cultivars, and sites.—Statistical differences between tissue types, sites, and cultivars were assessed with permutational MANOVA function adonis in R package vegan (Oksanen et al. 2017). This function conducts an analysis of variance based on distance matrices using a permutation test to compute F statistics and R^2 values. Model R^2 values are calculated as the sum of squares for each factor divided by the total sum of squares for the model; they indicate the proportion of variance explained by each factor in the model (Oksanen et al. 2017), and are henceforth referred to as "percentage of variance explained." Permutational MANOVA models were run separately from the NMDS ordinations, which were used for visualization. When comparing across tissue types, we used proportional chemical concentrations because nectar, pollen, and flower concentrations were measured on different scales (by fresh volume for nectar, but by dry mass for flower and pollen). However, we used absolute concentrations when comparing within a species and tissue type. We elected to use absolute concentrations because we felt that they were a more direct reflection of the collected data, possibly more ecologically meaningful for interactions with mutualists and antagonists (Tiedeken et al. 2016, Barlow et al. 2017), and more relevant to future bioassays that test activity of specific compounds. In addition, they are statistically more appropriate for many analyses (Morton et al. 2017), and robust to different levels of ability to quantify co-occurring compounds.

Comparisons of absolute concentrations and chemical species richness by tissue type.—We used general linear mixed models, fit with the lme4 package v1.1 (Bates et al. 2015), to compare absolute chemical concentrations of each chemical class in nectar and pollen. Within each sample, we calculated total concentration of each compound class by summation of the micromolar concentrations of each constituent compound. Median species-level concentration was then computed for each chemical class and tissue type. To conform to distributional assumptions of the model, only non-zero (i.e., positive) values for median concentration were used. Although this approach obscures within-species variation in concentrations, which were pursued in detail in subsequent analyses, our aim in this analysis was to compare in general terms the concentrations found in nectar and pollen. Models used a Gaussian error distribution with species-level median ln(concentration + 1) (measured as μmol/L) within each chemical class as the response variable, and tissue type (nectar or pollen) as the predictor variable. Plant species was used as a random effect to account for possible non-independence of nectar and pollen concentrations in samples from the same species. To compare chemical concentrations for species where we collected anthers rather than pure pollen, a t test was used to compare species-level median log-transformed concentrations for chemical classes that were represented in at least six species of each pollen type (alkaloids, amino acids, and flavonoids). To test for differences in chemical species richness between nectar and pollen, we used a generalized linear mixed model with a Poisson error distribution. Chemical richness (i.e., number of compounds found) was the response variable, tissue type the predictor

variable, and plant species the random effect. For this and subsequent lme4 models, homogeneity of variance and distribution of residuals were inspected with quantile-quantile and residuals vs. fitted-value plots to check for conformation to model assumptions (Bolker et al. 2009).

Trait space overlap between nectar and pollen and across cultivars and sites.—We used the dynamic range boxes package v0.10 (Junker et al. 2016) to assess differences in volume and overlap of multivariate chemical trait spaces (niche hypervolumes) across tissue types, and across cultivars (for cultivated species) or sites (for wild species) within individual species. Independent analyses were performed for each species (for comparisons across tissue types) or for each species and tissue type (for comparisons across cultivars or sites). The "dynamic range box" is a multivariate measure of the chemical trait space occupied by a tissue type, with each compound considered as a separate dimension of the *n*-dimensional trait space. The size of the range box in each dimension corresponds to the variability in concentration of each compound. Hence, a voluminous range box indicates a high variability in chemical concentration of the compounds. For comparisons of trait space volume between nectar and pollen, proportional (rather than absolute) concentrations were used to compute the sizes of range boxes. We used proportional concentrations because nectar and pollen concentrations were measured on different scales (fresh volume vs. dry mass), and because large differences in absolute concentrations were already obvious based on visual inspection of the data. By using proportional data, the composition of tissues with differences in absolute concentration can be compared. Differences in trait space volume between tissue types were tested with Gaussian family linear mixed-effects models using size of the *n*-dimensional hypervolume as the response variable, tissue type as the predictor variable, and plant species as a random effect.

Proportional overlap between groups of samples was measured as the arithmetic mean of overlap in chemical concentrations for each compound, i.e., in each dimension of trait space (dynamic range boxes aggregation method "mean"). Proportional overlaps are, by construction, asymmetric. This is because each group of samples occupies a different total volume of trait space (Junker et al. 2016). Therefore, any shared trait space may represent a relatively small proportion of total trait space for a group that occupies a large trait space, but a relatively large proportion of total trait space for a group with that occupies a smaller trait space. In the case of chemical trait space, asymmetric overlap indicates that one type of sample encompasses a larger fraction of the number of compounds found in the other group, and/or spans a larger spectrum of concentrations for compounds shared between the two groups. For example, if nectar contains one compound, and pollen contains the same compound, at the same concentrations, but also three additional compounds, then pollen will occupy a larger proportion of nectar trait space than nectar does of pollen. As a result, we can expect pollen to perform many of the chemically mediated functions performed by nectar in terms of, e.g., the number of microbe, herbivore, or pollinator species that are attracted or repelled. Further examples can be found elsewhere (Kuppler et al. 2017, Junker and Larue-Kontić 2018). Asymmetry in trait space overlap was tested in Gaussian family general linear mixed models that used the proportional trait space overlap (i.e., shared trait space divided by total trait space) as the response variable, tissue type as the predictor variable, and plant species as a random effect.

Coefficients of variation (CV) were calculated as the ratio of standard deviation to mean concentration for each compound within each species and tissue type. The coefficient of variation was calculated at two levels of resolution: the "species level" (i.e., a CV calculated for each compound within each species and tissue type, without consideration of sites and cultivars) and the "within-species" level (i.e., a CV calculated for each compound within each combination of species, tissue type, and site or cultivar). A Gaussian family linear mixed model was fit with coefficient of variation as the response variable; tissue type, level of resolution, and their interaction as predictors; and species as a random effect. Post-hoc pairwise comparisons with Tukey adjustment for multiple tests were made using R package lsmeans v2.27 (Lenth 2016). We also tested for differences in CV for compounds from different chemical classes within each tissue type. Square root-transformed CV was the response variable, chemical class, and tissue type were the predictor variables, and plant species was the random effect to account for non-independence of CV for different compounds within the same species.

Phenotypic integration.—We assessed the extent of covariation among different compounds within each species or tissue by calculating phenotypic integration (Pigliucci 2003). High phenotypic integration indicates that compounds have consistent relative concentrations; low phenotypic integration indicates variability in relative concentrations. Phenotypic integration was determined for each species and tissue type with at least 8 samples following previously described approaches for plant volatiles (Junker et al. 2018). Pearson's correlation coefficient *r* was computed for all concentrations (in μmol/L) of all pairs of compounds. Eigenvalues were calculated for the resulting correlation matrix. Raw phenotypic integration index was measured as the variance of the eigenvalues with a correction for sample size (Wagner 1984, Herrera et al. 2002, Junker et al. 2018). This index can be compared across species and tissue types with different numbers of compounds and samples.

In addition to calculating the integration index using complete chemical profiles, we also calculated within-module phenotypic integration (Junker et al. 2018). "Modules" are groups of well-correlated compounds, defined by hierarchical cluster analysis of a dissimilarity matrix of chemical concentrations (R function hclust). The optimal number of modules was determined with the silhouette function (Maechler et al. 2005). The mixture was divided into the optimal number of modules with the cutree function, and phenotypic integration was computed separately for each module.

Differences in phenotypic integration between nectar and pollen were assessed with a linear mixed-effects model that used integration index as the response variable (Gaussian distribution), tissue type (flower, nectar, or pollen) as the predictor variable, and species as a random effect. Post-hoc pairwise comparisons with Tukey adjustment for multiple tests were made using R package lsmeans (Lenth 2016).Correlation

between phenotypic integration of nectar and pollen was assessed with a Pearson correlation for all species with at least eight samples each for both nectar and pollen.

To assess the effects of shared biosynthetic pathways on correlation between concentrations of compound pairs, we computed all pairwise correlation coefficients for species and tissue types represented by at least eight samples. Correlations were grouped as within class (i.e., both compounds belonged to the same chemical class) or between class (i.e., the two compounds belonged to different classes). We compared correlation strength (Pearson's r) for within- vs. between-class correlations in a general linear mixed model. The model used Pearson's r as the response variable (Gaussian distribution); tissue type, relationship between compounds (within vs. between class), and their interaction as predictor variables; and plant species as the random effect. Pairwise contrasts were computed with Tukey correction for differences between tissue types. Additional comparisons were made for the effect of chemical relationship within each tissue type. Whereas the phenotypic integration analysis treated each species and tissue type as one observation, this analysis used each pair of compounds within a species and tissue type as one observation. As a result, it had greater power to distinguish effects of tissue type and shared biosynthetic pathway on covariation among compounds.

Phylogenetic signal.—We tested for phylogenetic signal in total concentrations of flavonoids, alkaloids and spermidines, and terpenoids in nectar and pollen, and phenotypic integration index of nectar and pollen. We used function congeneric.merge in the pez package v1.1 (Pearse et al. 2015) to obtain a time-scaled, rooted tree by extraction of our species from an unparalleled molecular phylogeny of flower plants (Zanne et al. 2014). Phylogenetic signal was assessed with the function phylosig in R package phytools v0.6 (Revell 2012), which uses a permutation test (10,000 iterations) to compute Bloomberg's K (Blomberg et al. 2003).

Results

Patterns of composition and diversity

Our survey identified 102 compounds across samples of flowers (9 species), nectar (26 species), and pollen (28 species). The most common secondary compound classes were flavonoids, alkaloids including spermidine derivatives, terpenoids, and chlorogenic acids (Fig. 1). Phenylpropanoids other than chlorogenic acids consisted of acylated sugars (feruloyl glucose in *Fragaria* pollen and *Silene* nectar), rosmarinic acid (*Monarda* pollen and *Thymus* nectar), and a lignin glycoside (*Penstemon* pollen). Also ubiquitous were the free amino acids phenylalanine and tryptophan, which were recorded in 92% of nectars and 100% of pollens. The most frequently recorded compounds were the flavonoids quercetin and kaempferol glycosides, which were among the five most common compounds for all three tissue types (Table 1). Many pollens (71% of species) contained hydroxycinnamoyl-spermidines, mainly triscoumaroyl and trisferuloyl spermidines.

Aside from these common compounds, cross-species diversity of flower, nectar, and pollen samples was high. Most compounds were found in only a single species (Fig. 2a), and new compounds were discovered with each additional species sampled (Fig. 2b). Within species, however, the qualitative composition of compounds was consistent (Fig. 2c). Because lyophilization likely resulted in loss of the most volatile sample components, and we could not simultaneously optimize our chromatographic methods for all possible compounds, the true diversity of compounds in the samples is even greater than what is depicted here. We would therefore encourage the analysis of fresh samples and the use of alternative methods of separation and detection, such as GC-MS, to identify additional chemical components.

Differentiation across species and tissue types

Each species and tissue type exhibited characteristically unique phytochemistry, visible using NMDS multivariate

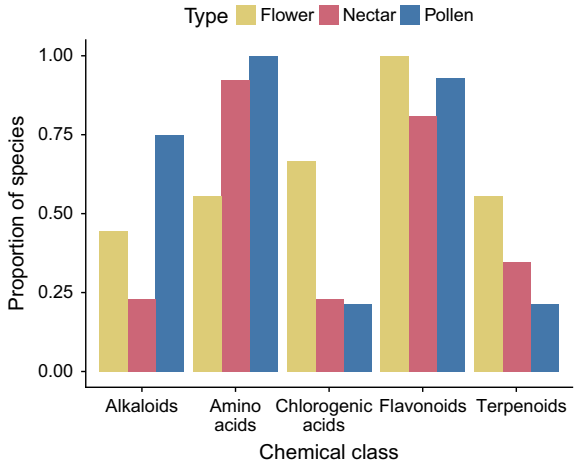

Fig. 1. Prevalence of major compound classes in flowers (9 species), nectar (26 species), and pollen (28 species). Alkaloids include all nitrogen-containing compounds except the amino acids, including spermidine derivatives. Chlorogenic acids refer to all phenylpropenoid derivatives of quinic acid.

Table 1. Most common compounds by tissue type.

Type and compound	Presences	Prevalence (%)
A) Flower (9 species)		
Quercetin-*O*-glycoside	8	88.9
Chlorogenic acid	6	66.7
Kaempferol-*O*-glycoside	6	66.7
Tryptophan	5	55.6
Acylated sugar	4	44.4
B) Nectar (26 species)		
Phenylalanine	24	92.3
Tryptophan	17	65.4
Quercetin-*O*-glycoside	9	34.6
Chlorogenic acid	6	23.1
Kaempferol-*O*-glycoside	5	19.2
C) Pollen (28 species)		
Phenylalanine	27	100
Tryptophan	25	92.3
Kaempferol-*O*-glycoside	19	67.9
Quercetin-*O*-glycoside	14	50.0
Triscoumaroyl spermidine	11	39.3

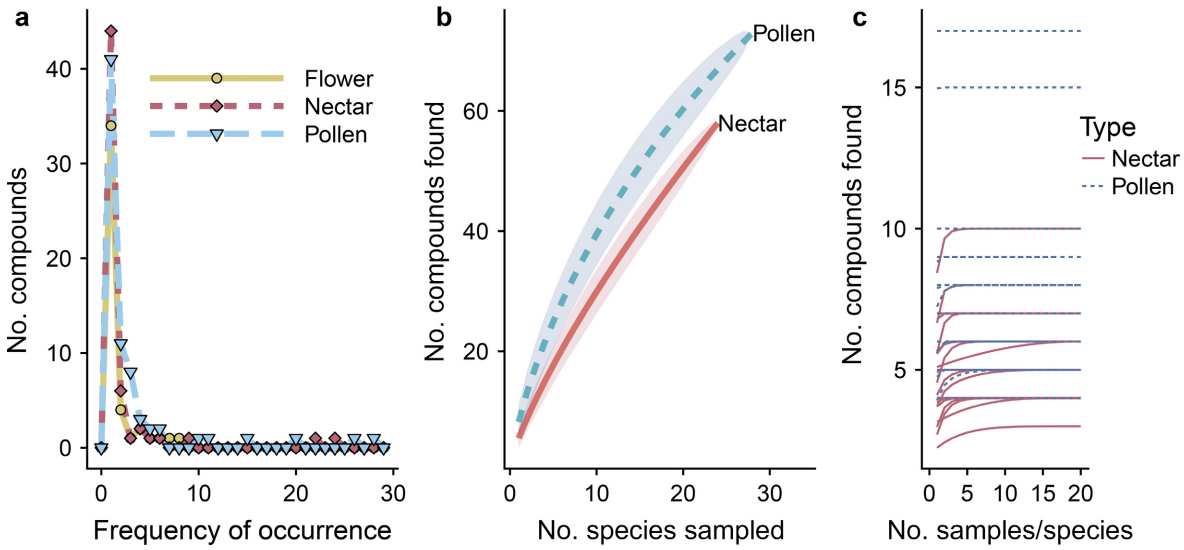

FIG. 2. Chemical diversity in nectar, pollen, and floral samples. (a) Most compounds were found in only a single species. Flower samples, solid yellow line; nectar samples, dotted red line; pollen samples, dashed blue line. (b) Chemical species accumulation curves indicated that new compounds were found for each additional species sampled. Neither nectar nor pollen accumulation curves approached saturation. Lines and shaded bands show mean ± SD. (c) Within-species chemical species accumulation curves. All compounds within each species were found after analysis of the first few samples for both nectar (solid red lines) and pollen (dashed blue lines).

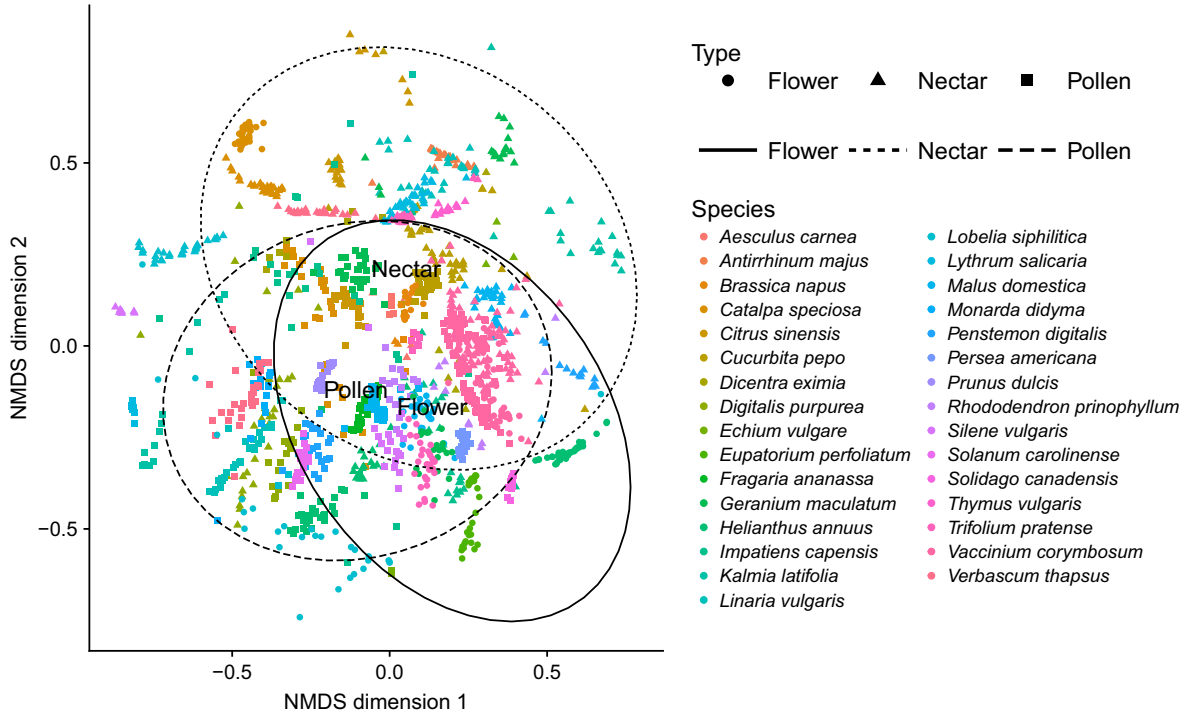

FIG. 3. Nonmetric multidimensional scaling-based ordination of Bray-Curtis distances between flower (circles), nectar (triangles), and pollen (squares) samples. Samples clustered strongly by species and tissue type, with significant differences between tissue types ($F_{2,1,482} = 65.9$, $P = 0.001$). Random forest discriminant analysis showed that 98.6% of samples could be assigned to the correct species-tissue type combination. Ellipses show 95% confidence bands for flower (solid line), nectar (dotted line), and pollen (dashed line). Colors indicate different species. Ordination is based on proportional chemical composition.

ordination based on proportional composition (Fig. 3). Species and tissue type explained $R^2 = 86.6\%$ of the variation among samples. A random forest analysis assigned compounds to the correct plant species and tissue type with 98.6% accuracy.

On an absolute scale, pollen had much higher concentrations of secondary metabolites than did nectar. Non-zero median pollen concentrations were 23.8- (terpenoids) to 235-fold (flavonoids) higher than those in nectar (Fig. 4; pairwise comparisons; alkaloids, $t = 6.76$, $P < 0.001$; amino acids, $t = 9.27$,

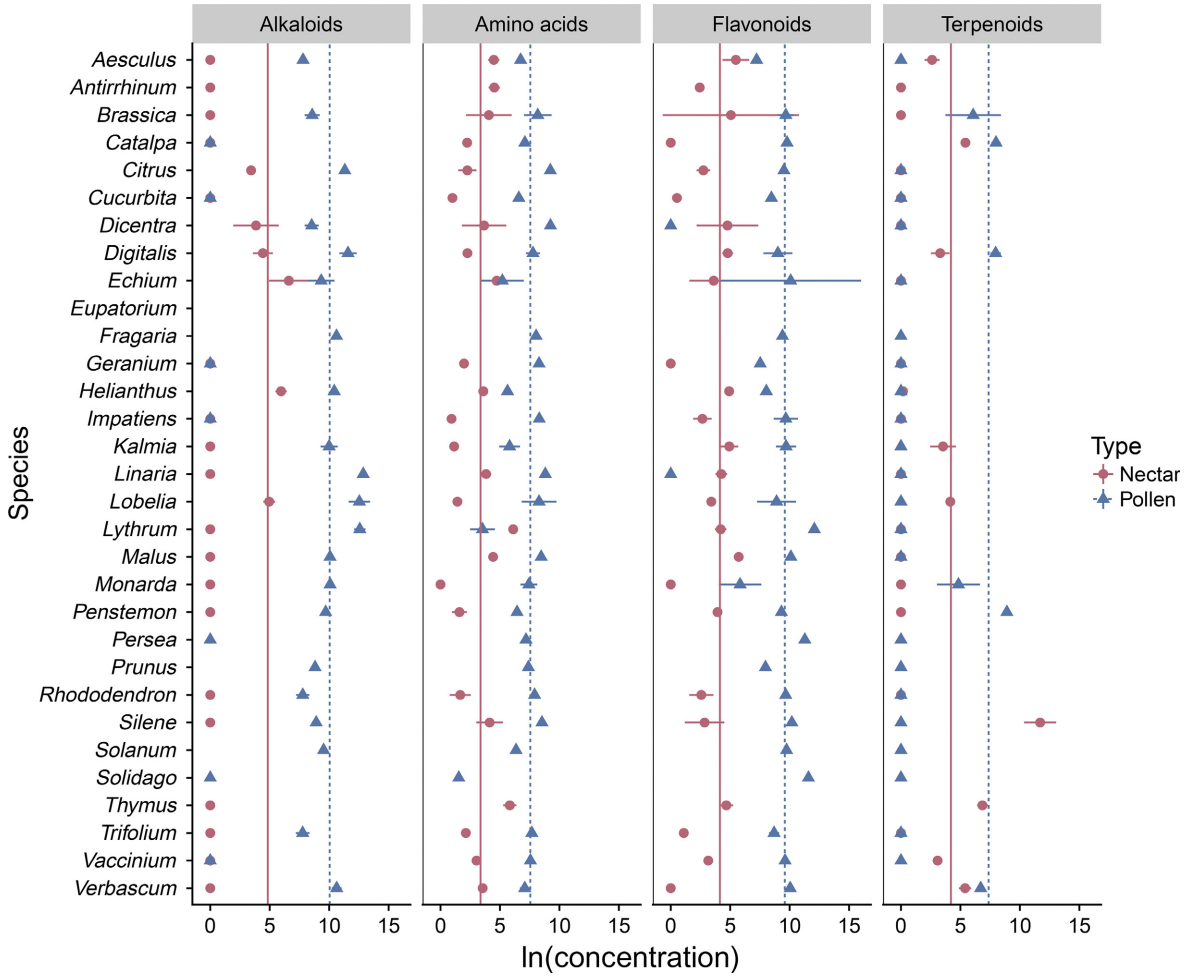

FIG. 4. Absolute ln(concentration + 1) (measured as μmol/L) of all compound classes were 23.8- to 235-fold lower in nectar (red circles) than in pollen (blue triangles). Vertical lines show median non-zero concentrations in nectar (solid red line) and pollen (dashed blue line). Points and error bars show means and 95% confidence intervals. Where no error bars are visible, either all concentrations are zero or error bars are smaller than symbols for points. Concentrations are in μmol/L for nectar and μmol/kg dry mass for pollen. Alkaloids include all nitrogen-containing compounds except the amino acids, including spermidine derivatives.

$P < 0.001$; flavonoids, $t = 12.06$, $P < 0.001$; terpenoids, $t = 2.27$, $P = 0.025$). Pollen concentrations did not differ between species where we collected anthers rather than pollen (t test $P > 0.20$ for alkaloids, amino acids, and flavonoids).

Flowers, nectar, and pollen also had distinct proportional composition at the level of both individual compounds (permanova: $F_{2,\ 1482} = 65.9$, $P = 0.001$, $R^2 = 0.081$, Fig. 3) and compound classes ($F_{2,58} = 4.18$, $P = 0.001$, $R^2 = 0.125$). Flowers had the highest proportion of flavonoids (53% of documented chemical composition) and the lowest proportion of alkaloids (9%) and free amino acids (4%, Fig. 5), nectar had the highest proportion of free amino acids (23%) and terpenoids (19%, Fig. 5), and pollen had the highest proportion of alkaloids and spermidines (42%) and the lowest proportion of terpenoids (1%, Fig. 5). Most samples not covered by these chemical classes were dominated by chlorogenic acids, which comprised 85% of composition of *Helianthus* flowers, 33% of *Dicentra* nectar, 62% of *Penstemon* nectar, and 60% of *Rhododendron* nectar. Both nectar and pollen of *Geranium* were dominated by tannins.

Of the nectars with a high (>15% documented chemistry) proportion of alkaloids and spermidines, *Citrus* contained only caffeine (42% of total concentration); *Dicentra* contained aporphine-, aconitine-, and isoquinoloid-type alkaloids (total 17%); *Digitalis* (41%) and *Helianthus* (71%) contained acylated spermidines; *Echium* contained several pyrrolizidine alkaloids as echimidine derivatives (total 81%); and *Lobelia* contained two piperidyl and one pyridyl alkaloid (total 51%).

Pollen also differed qualitatively and quantitatively from nectar (Fig. 6). Across all species, nectar and pollen shared on average only 34% of compounds. Much of this overlap was due to phenylalanine and tryptophan, which were common in both nectar and pollen (Fig. 1). When amino acids were excluded, the qualitative contrast was even more stark (22% nectar only, 57% pollen only, 22% shared). Pollen contained, on average, 63% more compounds than did nectar (9.3 ± 0.67 compounds in pollen vs. 5.7 ± 0.51 compounds per species in nectar [mean ± SE], $Z = 4.41$, $P < 0.001$).

Chemical trait space overlap between conspecific nectar and pollen

We used dynamic range boxes to obtain quantitative estimates of trait space overlap between nectar and pollen of

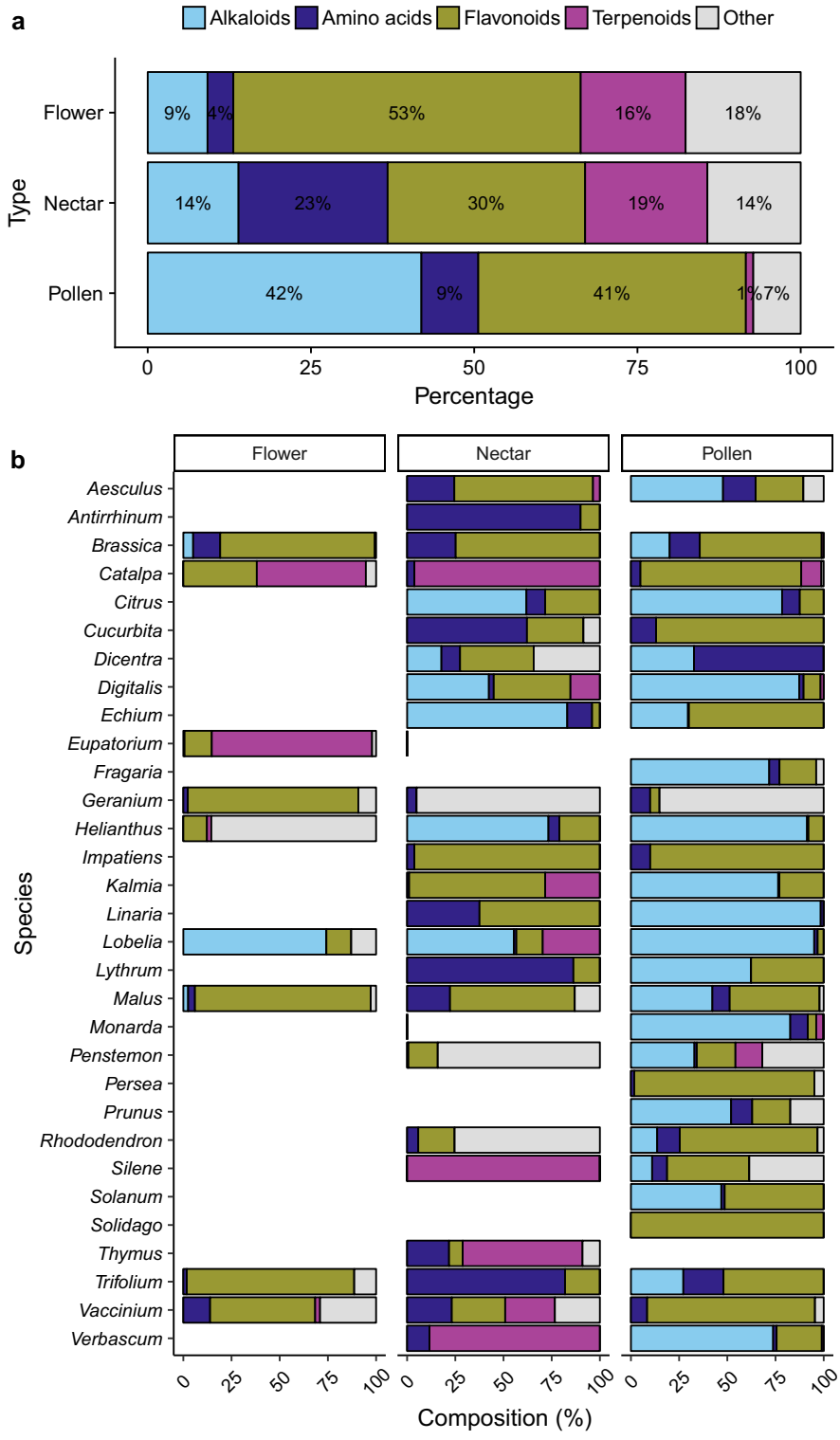

FIG. 5. Median proportional compositions of flower, nectar, and pollen samples by chemical class. (a) Bar chart showing median proportions across (b) all species. Tissue types differed significantly in class-wise proportional composition (permutational MANOVA on median proportional composition for each species and tissue type, $F_{2,58} = 4.18$, $P = 0.001$). Tissue type explained 12.5% of variance in proportional composition across species. Alkaloids include all nitrogen-containing compounds except the amino acids, including spermidine derivatives.

the same species. Despite the higher number of compounds in pollen, which allowed for variation in more chemical dimensions, nectar and pollen occupied similar amounts of chemical trait space based on proportional composition (nectar and pollen hypervolumes both had size 0.71 ± 0.03). There was, accordingly, little asymmetry in trait space overlap between the two tissue types, with median trait space overlap of 0.14 (Fig. 7). This low overlap, which reflects

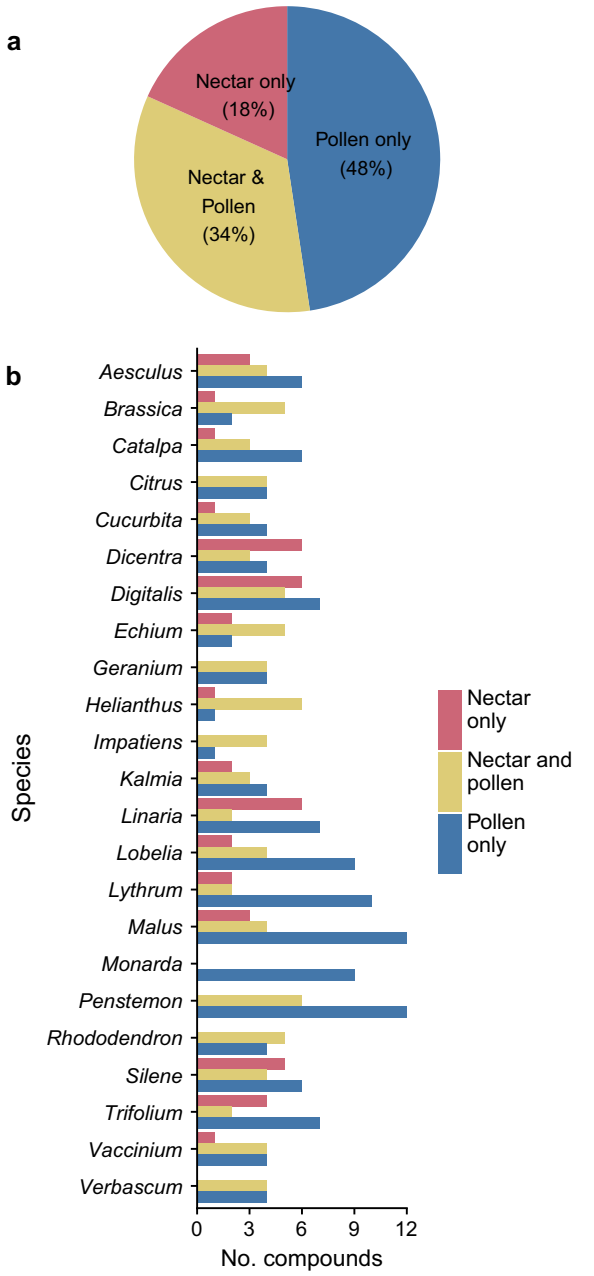

FIG. 6. Number of quantifiable compounds detected in nectar, pollen, and both nectar and pollen. (a) Pie chart indicates totals aggregated across all species. (b) Individual species. Pollen contained on average 63% more compounds than did nectar (9.3 ± 0.67 vs. 5.7 ± 0.51 compounds/species [mean ± SE], $\chi^2 = 19.5$, df = 1, $P < 0.001$).

both the proportion of shared compounds (Fig. 7) and their relative concentrations (Fig. 5), adds further evidence of phytochemical differentiation between nectar and pollen within a single species. When the same analysis was run on absolute concentrations rather than proportional composition, trait space overlap was near zero (Appendix S1: Fig. S2), reflecting higher absolute concentrations found in pollen (Fig. 4). On the absolute scale (Appendix S1: Fig. S2), trait space overlap between nectar and pollen was greatest in species that lacked unique compounds in nectar (*Impatiens*, *Rhododendron*, and *Verbascum*; Fig. 6). In these cases, pollen trait space overlapped more than one-half of nectar trait space (Appendix S1: Fig. S2).

Intraspecific differences across cultivars and sites

Across cultivars of the same species, permutational MANOVA showed significant variation in chemical concentrations for 11 of 15 comparisons (2/2 species for flowers, 4/5 for nectar, 5/8 for pollen). These comparisons were chosen a priori to reflect species with high levels of replication. Cultivar explained 32.5% of intraspecific variation across samples on average (Table 2A). Across sites for wild species, we found significant variation in chemical concentrations for 8 of 14 comparisons (0/1 for flower, 3/5 for nectar, 5/7 for pollen), and site explained $R^2 = 21.1\%$ of intraspecific variation across samples on average (Table 2B).

We analyzed intraspecific trait space overlap across cultivars and sites with dynamic range boxes (Fig. 8). Linear mixed model post-hoc comparisons indicated that, for both cultivar- and site-level comparisons, nectar trait spaces had significantly greater overlap across within-species groups than did pollen trait spaces (cultivars, $t = 2.1$, $P = 0.039$; sites, $t = 3.74$, $P < 0.001$).

The greater overlap in nectar than pollen likely reflected higher intraspecific coefficients of variation (CV) in nectar chemical concentrations than in pollen or flowers (Fig. 9). Nectar concentrations had on average 90% higher CV than pollen; this difference was consistent whether CV was calculated based on variation in concentrations at the species level ($t = 10.50$, $P < 0.001$) or the within-species level (i.e., variation within sites and cultivars, $t = 12.77$, $P < 0.001$). Accounting for sites and cultivars significantly reduced CV by 14% relative to when variation was calculated at the species level (species-level CV = 0.82 ± 0.04; within-species CV = 0.70 ± 0.04, $t = -4.17$, $P < 0.001$). No significant effect of chemical class on CV was found for flowers, nectar, or pollen (class effect, $F_{4, 310} = 1.77$, $P = 0.13$; $P > 0.20$ for all Tukey-corrected pairwise contrasts between classes within tissue types).

Domesticated apple (*Malus domestica*) exemplified chemical separation across tissue types and cultivars within a single species (Fig. 10). Flowers, nectar, and pollen were completely distinguished from one another, and tissue type explained $R^2 = 81\%$ of variation across samples (MANOVA $F_{2,84} = 207.4$, $P = 0.001$, Fig. 10a). Within nectar and within pollen, cultivars exhibited almost complete separation in chemical trait space (nectar, $F_{2,29} = 8.58$, $P = 0.001$, $R^2 = 0.39$; pollen, $F_{2,29} = 13.93$, $P = 0.001$, $R^2 = 0.51$, Fig. 10b, c).

Phenotypic integration

Chemical mixtures were generally less integrated in flowers (least squares mean 9.91 ± 4.59) than in nectar (21.30 ± 2.96) and pollen (21.53 ± 3.17), but these differences were not statistically significant ($F_{2,39.6} = 2.37$, $P = 0.10$, Fig. 11a). However, integration of chemical modules varied significantly across tissue types ($F_{2,36.4} = 4.31$, $P = 0.021$). Within-module integration was significantly higher in nectar (46.1 ± 4.30) than in flowers (26.2 ± 6.26,

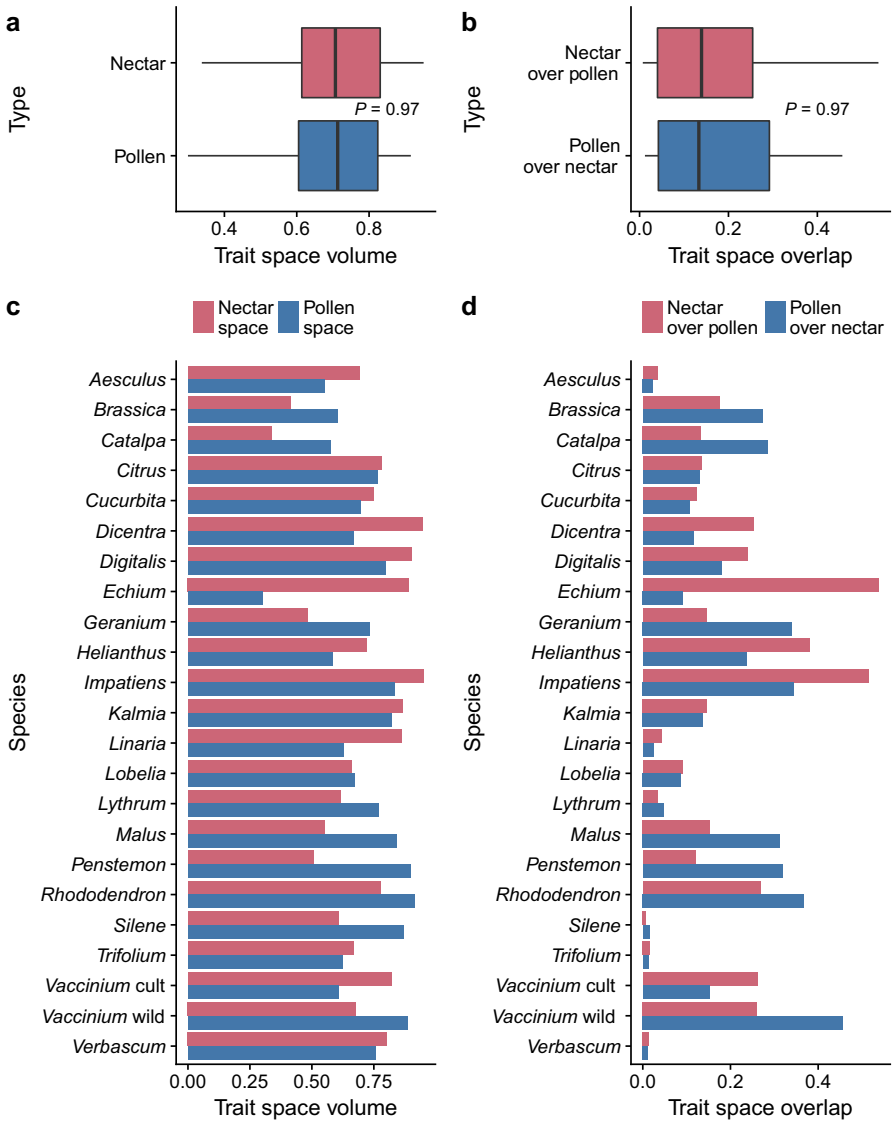

FIG. 7. Nectar and pollen exhibited similar levels of variability in proportional composition, with no significant asymmetry in trait space overlap of one tissue type by the other. Graphs show dynamic-range-boxes-based trait space volume of nectar (red bars) and pollen (blue bars) and overlap between the two types. (see *Materials and Methods: Statistical analyses: Trait space overlap between nectar and pollen and across cultivars and sites.* (a) Median hypervolume size and (b) proportional hypervolume overlap, aggregated across species. (c) Hypervolume size and (d) proportional overlap for each individual species. The hypervolume size indicates the variability of proportional concentrations. Trait space overlap indicates how much the nectar trait space covers the pollen trait space ("nectar over pollen") and vice versa. Calculations are based on proportional composition. *Vaccinium corymbosum* samples are separated into samples from cultivated (cult) and wild taxa. *P* values in panels a and b are for generalized linear mixed model pairwise comparisons between (a) nectar and pollen volume size and (b) asymmetry in overlap between nectar and pollen in. See Appendix S1: Fig. S2 for trait space volumes and proportional overlap based on absolute concentrations. In boxplots, dark line represents median, box boundaries represent first and third quartiles, and whiskers extend to the most extreme data point that is less than 1.5 times the interquartile range from the box. Points represent observations that lie outside the range of the whiskers.

$t = 2.76$, $P = 0.024$, Fig. 11b). Within-module integration of pollen was intermediate (35.33 ± 4.01) and not significantly different from either nectar ($t = -1.98$, $P = 0.13$) or flowers ($t = 1.26$, $P = 0.42$, Fig. 11b). Integration of nectar and pollen were not significantly correlated ($t = -0.538$, $P = 0.60$, Fig. 11c).

Consideration of individual species showed that compounds tended to cluster by biosynthetic relatedness. For example, in *Malus domestica* nectar (Appendix S1: Fig. S3), there were seven pairwise correlations with *r* values above 0.80. All were between pairs of flavonoids or a flavonoid and chlorogenic acid (Appendix S1: Fig. S3). Chlorogenic acid is an ester of quinic and caffeic acids. Caffeic acid, like other flavonoids, is synthesized via the phenylpropanoid pathway (Rice-Evans et al. 1996). These shared metabolic precursors may explain correlations between concentrations of chlorogenic acid and flavonoids. Likewise, in *Digitalis purpurea* pollen, 9 of the 10 strongest correlations (highest *r* values) were between chemically similar spermidine derivatives (Appendix S1: Figs. S4, S5).

Analysis of all pairwise correlations between compounds indicated stronger positive correlations for within-class (i.e.,

TABLE 2. Results of permutational MANOVA tests for intraspecific variation in chemistry across cultivars and sites.

Species	Type	*N*	Cultivars	*F*	Df	*P*	R^2
a) Cultivars							
Helianthus annuus	**flower**	**40**	**4**	**2.44**	**3,36**	**0.023**	**0.17**
Malus domestica	**flower**	**29**	**3**	**11.29**	**2,26**	**0.001**	**0.46**
Citrus sinensis	**nectar**	**23**	**2**	**13.09**	**1,21**	**0.001**	**0.38**
Cucurbita pepo	nectar	45	3	1.77	2,42	0.062	0.08
Digitalis purpurea	**nectar**	**30**	**3**	**1.96**	**2,27**	**0.02**	**0.13**
Helianthus annuus	**nectar**	**20**	**4**	**5.99**	**3,16**	**0.001**	**0.53**
Malus domestica	**nectar**	**30**	**3**	**8.58**	**2,27**	**0.001**	**0.39**
Citrus sinensis	**pollen**	**23**	**2**	**19.84**	**1,21**	**0.001**	**0.49**
Cucurbita pepo	pollen	32	3	1.77	2,29	0.138	0.11
Digitalis purpurea	pollen	17	3	0.57	2,14	0.913	0.08
Fragaria ananassa	**pollen**	**30**	**3**	**7.78**	**2,27**	**0.001**	**0.37**
Helianthus annuus	pollen	30	3	0.91	2,27	0.406	0.06
Malus domestica	**pollen**	**30**	**3**	**13.93**	**2,27**	**0.001**	**0.51**
Persea americana	**pollen**	**30**	**3**	**86.00**	**2,27**	**0.001**	**0.86**
Prunus dulcis	**pollen**	**30**	**3**	**4.88**	**2,27**	**0.007**	**0.27**
B) Sites							
Geranium maculatum	flower	21	3	2.03	2,18	0.1	0.18
Geranium maculatum	nectar	19	2	0.72	1,17	0.508	0.04
Impatiens capensis	**nectar**	**31**	**3**	**2.55**	**2,28**	**0.036**	**0.15**
Kalmia latifolia	**nectar**	**20**	**3**	**4.16**	**2,17**	**0.004**	**0.33**
Linaria vulgaris	**nectar**	**31**	**4**	**1.85**	**3,27**	**0.031**	**0.17**
Lythrum salicaria	nectar	33	3	0.96	2,30	0.444	0.06
Verbascum thapsus	nectar	27	2	2.14	1,25	0.101	0.08
Geranium maculatum	**pollen**	**30**	**4**	**4.70**	**3,26**	**0.001**	**0.35**
Impatiens capensis	**pollen**	**24**	**3**	**12.14**	**2,21**	**0.001**	**0.54**
Kalmia latifolia	**pollen**	**15**	**3**	**2.97**	**2,12**	**0.033**	**0.33**
Linaria vulgaris	**pollen**	**32**	**5**	**2.24**	**4,27**	**0.046**	**0.25**
Solanum carolinense	pollen	28	3	2.18	2,25	0.07	0.15
Solidago canadensis	**pollen**	**25**	**3**	**3.41**	**2,22**	**0.014**	**0.24**
Verbascum thapsus	pollen	29	2	2.70	1,27	0.091	0.09

Notes: Boldface type indicates $P < 0.05$. *N*, number of samples. Numerator degrees of freedom (df) are listed first, followed by denominator degrees of freedom.

both compounds belonged to the same chemical class) than between-class compound pairs ($F_{2, 1238} = 12.35$, $P < 0.001$). Within each tissue type, the effect of chemical relatedness was significant for both nectar ($t = 4.26$, $P < 0.001$) and for pollen ($t = 4.59$, $P < 0.001$). The effect of chemical relatedness did not vary significantly across tissue types (relationship × type interaction, $F_{2, 1280} = 2.28$, $P = 0.10$), although the estimate for the effect of chemical relatedness tended to be higher for nectar (0.21 ± 0.043) than for pollen (0.13 ± 0.028 SE, Appendix S1: Fig. S6). Across all compound pairs, correlation coefficients were higher in nectar than in pollen (estimate of differences, 0.11 ± 0.030, $t = 3.82$, $P < 0.001$), and marginally higher in pollen than in flowers (estimate 0.075 ± 0.032, $t = 2.36$, $P = 0.048$, Appendix S1: Fig. S6).

Phylogenetic signal

No significant phylogenetic signal was found for median total concentrations of alkaloids, amino acids, flavonoids, or terpenoids in nectar or pollen (Bloomberg's *K* randomization test, $K = 1.09$, $P = 0.07$ for nectar terpenoids, $P > 0.25$ for all others), nor for number of compounds or phenotypic integration of nectar or pollen (Bloomberg's *K* randomization test, $P > 0.45$ for all).

DISCUSSION

In the most comprehensive qualitative and quantitative cross-taxon description of nectar and pollen chemistry to date, we found marked differentiation of nectar and pollen across species, clear quantitative and qualitative distinction between nectar and pollen of the same species, and intraspecific variation in both nectar and pollen chemistry across cultivars and sites. Pollen had higher concentrations and more compounds than did nectar, consistent with Optimal Defense Theory. These data provide a new level of insight into the secondary chemistry of nectar and pollen, and provide a framework for future research on the heritability, ontogeny, and ecological consequences of chemical variation in floral rewards.

Common compounds and potential functions

Most secondary chemicals were from a few common classes —flavonoids, alkaloids, chlorogenic acids, and terpenoids. Flavonoids are widespread among plants and tissue types (Taylor and Grotewold 2005). Flavonoids in our samples, mainly quercetin and kaempferol glycosides, were among the most frequently recorded compounds in flowers, nectar, and pollen, where they may mediate both biotic and abiotic interactions. First, flavonoids can serve primary functions as plant

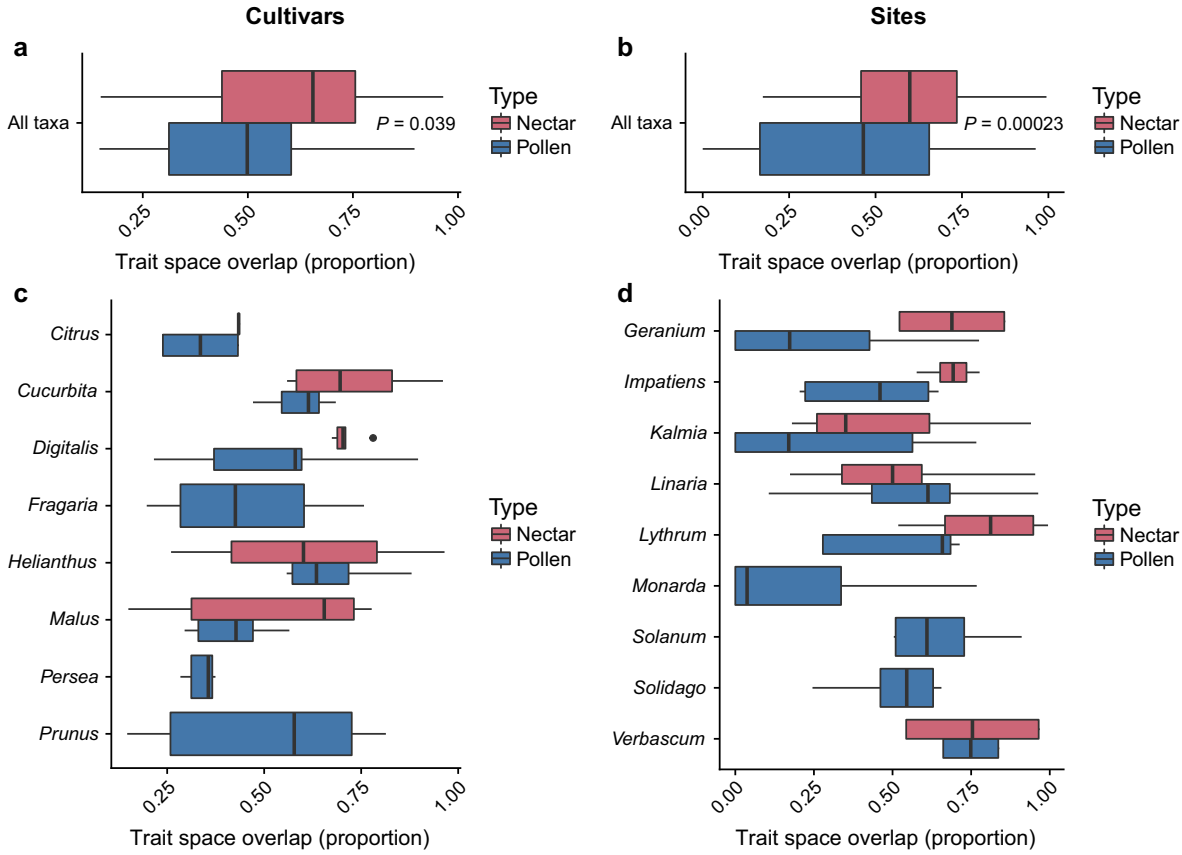

FIG. 8. Intraspecific variation in nectar and pollen composition across cultivars (cultivated species; a, c) and sites (wild species; b, d). Horizontal axis shows median proportional overlap of trait space (*n*-dimensional hypervolume) for all pairs of sites and cultivars, as quantified by dynamic range boxes. Median proportional hypervolume overlap in panels a and b are pooled across species. The trait space overlap indicates how much trait space is shared between a typical pair of cultivars or sites. Analyses are based on proportional composition. *P* values in panels a and b are for generalized linear mixed model pairwise comparisons between nectar and pollen site- or cultivar-wise overlap. Nectar chemistry overlapped more across both sites and cultivars than did pollen chemistry (cultivars, $t = -2.1$, $P = 0.039$; sites, $t = -3.74$, $P = 0.0002$). In boxplots, dark line represents median, box boundaries represent first and third quartiles, and whiskers extend to the most extreme data point that is less than 1.5 times the interquartile range from the box. Points represent observations that lie outside the range of the whiskers.

growth regulators (Taylor and Grotewold 2005). For example, flavonoids can govern pollen fertility (Mo et al. 1992). These growth-regulating properties could also contribute to the allelopathic activity of flavonoids against microbes and insects (Taylor and Grotewold 2005), and inhibit germination of competing, heterospecific pollen (Murphy 2000). Second, flavonoids can act as antioxidants, which could improve tolerance of pollen grains to abiotic stressors that may reduce viability (Schoper et al. 1986). While hydroxycinnamic acids have superior absorption of UVB irradiation, flavonoids also absorb wavelengths in the UV spectrum, and accumulation is stimulated by both visible and UV light exposure, as well as by other abiotic stressors that generate reactive oxygen species (Agati and Tattini 2010). The high flavonoid concentrations in our pollen samples (median non-zero concentrations >14,000 μmol/L) were similar to those reported for leaves grown in full sunlight (Agati and Tattini 2010), which suggests that pollen has comparable abilities to withstand potentially damaging radiation. Third, flavonoids can regulate biotic interactions with mutualists and antagonists. Flavonoids generally reduce herbivory and infection (Karpinski et al. 2003, Cushnie and Lamb 2005). In multiple plant species, high constitutive and inducible leaf flavonoid content has been correlated with insect and pathogen resistance (Treutter 2005). Protection of nectar and pollen from microbial and insect antagonists may help to preserve these resources for plant reproduction. Flavonoids may also be an honest signal for insects with vision in the UV spectra; nectar with flavonoids fluoresces under UV light (Thorp et al. 1975) and could visually guide pollinators to rewarding flowers.

Alkaloids and spermidines in our samples were dominated by the spermidine conjugates in pollen. Spermidines were generally esterified to one or more cinnamic acids, e.g., triscoumaroyl and trisferuloyl spermidines. These compounds likely play both developmental and ecological roles. Found in all plants, hydroxycinnamoyl-spermidines are thought to have phytohormone-like roles in plant development and abiotic stress tolerance; synthesis is induced by exposure to heat, UV, salinity, and dessication (Gill and Tuteja 2010) as well as by herbivory (Bassard et al. 2010). In *N. attenuata*, foliar concentrations of 520 μmol/L reduced herbivore growth rates by 50%; the median nonzero alkaloid concentration in our pollen samples (23,000 μmol/L) was 44-fold higher (Kaur et al. 2010).

Both developmental and ecological functions of spermidines are likely important for pollen, which must endure abiotic

stresses that can reduce viability (Schoper et al. 1986) before it germinates to fertilize ovules. In *Arabidopsis*, deficiency of spermidine conjugates caused pollen grains to become deformed, indicating the developmental role of these compounds (Grienenberger et al. 2009). Prior to germination, pollen may be exposed to insects and pathogens, which can be inhibited by spermidines (Walters et al. 2001), and UV irradiation, which can be absorbed by spermidines (Gill and Tuteja 2010). In *Arabidopsis* pollen, hydroxycinnamoyl-spermidines are concentrated in the pollen coat, an ideal location to function in UV absorption and inhibition of insects and pathogens (Grienenberger et al. 2009). Despite their multifunctionality and developmental importance, nearly one-third of our tested pollens lacked spermidines, suggesting that these compounds are dispensable for some species.

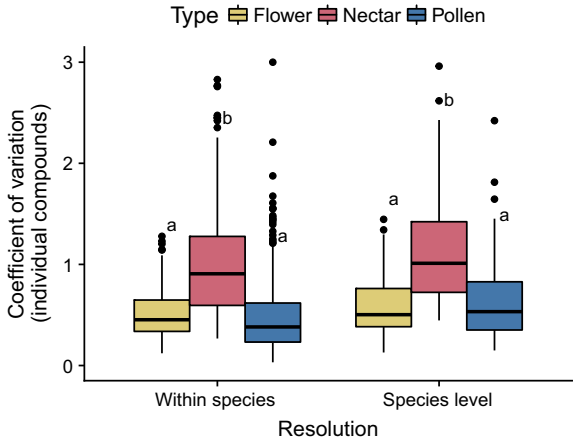

FIG. 9. Nectar chemical concentrations were relatively more variable than either flower or pollen concentrations, whether variation was calculated at the level of species (left) or the level of cultivars (for cultivated species) and sites (for wild species; right). Coefficients of variation were calculated as the ratio of the standard deviation to the mean for each compound within each species and tissue type (species level), or for each compound within species, tissue type, and site or cultivar (within species). Different lowercase letters indicate significant differences ($P < 0.05$) between tissue types within each level of resolution in linear mixed model post-hoc comparisons. In boxplots, dark line represents median, box boundaries represent first and third quartiles, and whiskers extend to the most extreme data point that is less than 1.5 times the interquartile range from the box. Points represent observations that lie outside the range of the whiskers.

We recorded spermidine conjugates in nectar of *Helianthus annuus* and *Digitalis purpurea*. Spermidines have not been previously reported in nectar, although they have been found in xylem and phloem, and the enzymes that catalyze their synthesis have been found in nectar (Friedman et al. 1986, Shah et al. 2016). In *H. annuus* and *D. purpurea*, nectar and pollen contained the same spermidine conjugates, suggesting that spermidines in nectar could be a result of contact with pollen. Regardless of their origin, the occurrence of spermidines in nectar may still be ecologically relevant to organisms that interact with these species.

Overall, alkaloids comprised >15% of recorded metabolite concentrations in the nectar of 6 of 26 species. Nectar alkaloids included caffeine in *Citrus*; aconitine and isoquinoline alkaloids in *Dicentra*, pyrrolizidine alkaloids in *Echium*, and piperidine and pyridyl alkaloids in *Lobelia*. Alkaloids have antimicrobial and insect-deterrent properties (Wink 1993), which may defend nectar against bacteria and non-pollinating insects that can deplete floral rewards (Good et al. 2014, Barlow et al. 2017). Whether nectar alkaloids are beneficial for pollination per se remains a matter of debate. Effects may depend on ecological context. For example, alkaloids reduced plant reproduction in *Gelsemium sempervirens* through deterrence of pollinators (Adler and Irwin 2005), but increased outcrossing in *Nicotiana attenuata* by enforcement of modest drinking behavior (Kessler et al. 2008), and had dose-dependent benefits for pollination of artificial flowers (Thomson et al. 2015). Nectar alkaloids could benefit pollination when they are preferred over alkaloid-free

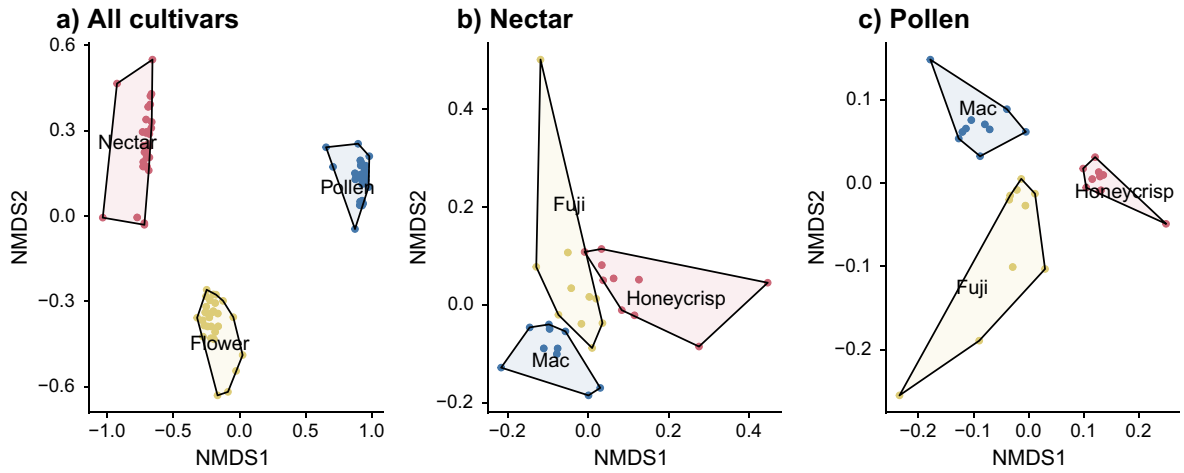

FIG. 10. Example of (a) distinct chemical compositions of flower, nectar, and pollen and (b, c) intraspecific variation in nectar and pollen composition across cultivars in *Malus domestica*. Graphs show ordinations based on Bray-Curtis distances after Wisconsin double standardization of concentrations measured as μmol/L. Permutational MANOVA showed that tissue type ($F_{2,84} = 207$, $P = 0.001$) explained $R^2 = 81\%$ of variation across samples in panel a. Differences between cultivars were significant for both nectar ($F_{2,27} = 8.58$, $P = 0.001$, panel b) and pollen ($F_{2,27} = 13.93$, $P = 0.001$, panel c). Cultivar abbreviations: Fuji, Fuji-Autumn Red; Mac, Macintosh. See Table 2 for full results of cultivar-wise permutational MANOVA.

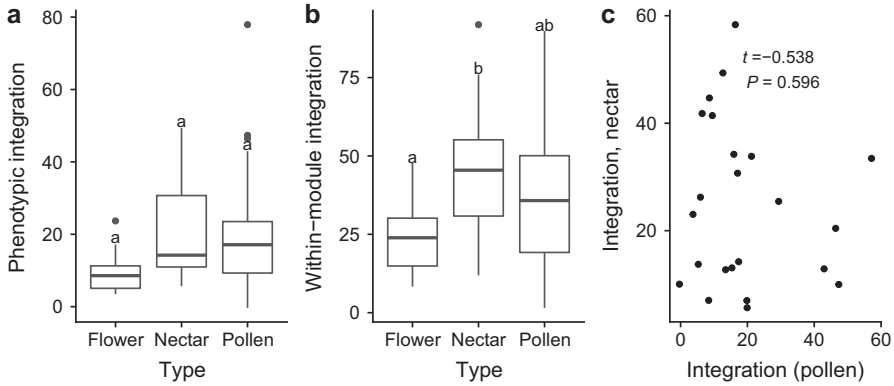

FIG. 11. Median species-wise phenotypic integration of flower, nectar, and pollen samples. (a) Integration of the full chemical mixture was generally higher in nectar and pollen, but did not differ significantly across tissue types (linear mixed model $F_{2,2.42} = 39.6$, $P = 0.11$). (b) Integration within modules of compounds within each mixture (defined by hierarchical clustering) indicated significant differences across tissue types ($F_{2,36.4} = 4.31$, $P = 0.021$). Nectar had higher within-module integration than did flowers ($t = 2.76$, $P = 0.024$). (c) No significant correlation was found between species-level nectar integration and pollen integration. In boxplots, dark line represents median, box boundaries represent first and third quartiles, and whiskers extend to the most extreme data point that is less than 1.5 times the interquartile range from the box. Points represent observations that lie outside the range of the whiskers.

solutions by honey and bumble bees (Singaravelan et al. 2005, Thomson et al. 2015); enhance pollinator memory and associative learning (Wright et al. 2013, Baracchi et al. 2017); or deter nectar robbers, which preserves rewards for pollinators (Barlow et al. 2017). For example, 10 μmol/L caffeine in nectar of artificial flowers resulted in more pollination from bumble bees than 100 μmol/L or no caffeine (Thomson et al. 2015), and 129 μmol/L caffeine at artificial feeders increased recruitment of honey bees (Couvillon et al. 2015). The caffeine concentrations in our *Citrus* nectar samples (median 25.6 μmol/L, interquartile range 14.7–50.4 μmol/L) are within the concentration range that may benefit pollination by several of these mechanisms.

Differentiation across species

Across the species surveyed, each species and tissue type was chemically unique. Most compounds were recorded only once, and new compounds were recorded with each additional species sampled (Fig. 2). This is likely due, at least in part, to our phylogenetically diverse set of species, which came from 21 plant families. Despite quantitative variation within species, random forest (machine-learning) algorithms assigned samples to their correct taxon and tissue type with over 98% accuracy. Each tissue type within a species was characterized by a unique combination of chemicals not found in any other species, or even in other floral tissues of the same plant. Nectar and pollen of the same species were chemically distinct in proportional composition, absolute concentrations, and chemical identity, all of which suggest chemical regulation to accomplish specific ecological functions. These results, which are consistent with prior surveys that revealed high floral phytochemical diversity (Junker et al. 2011a, Courtois et al. 2016), suggest that nectar and pollen chemistry of the same plant can take independent evolutionary trajectories. Prior studies of floral volatiles and nectar have shown lower levels of insect-repellent compounds in species that benefit from animal pollination, which is thought to reflect the high costs of pollinator deterrence for obligate outcrossers (Abel et al. 2009, Adler et al. 2012). Future studies should test whether pollen exhibits the same chemical trends as these other tissue types, with reduced levels of defensive chemicals in pollinator-dependent species.

Pollen and nectar of the same species had distinct phytochemistry

Differences between nectar and pollen are exemplified by alkaloids and spermidines, where concentrations in nectar were orders of magnitude lower than those in pollen, consistent with the lower concentrations of alkaloids in *Nicotiana* spp. nectar relative to leaves and flowers (Adler et al. 2012). In our samples, caffeine concentrations in *Citrus* nectar were 2,900-fold lower than those in pollen. In a variety of *Coffea* and *Citrus* spp., nectar caffeine concentrations were always below the taste thresholds of honey bees, but were sufficient to enhance honey bee memory for floral cues associated with a reward (Wright et al. 2013). Many alkaloids and spermidines present in pollen were absent from nectar, which indicates that the presence of alkaloids in nectar is not necessarily constrained by their presence in other tissues, at least in pollen. This finding is consistent with previously documented lack of nectar alkaloids in *Nicotiana africana* (Marlin et al. 2014), and nectar limonoids in *Citrus sinensis* (Stevenson et al. 2017). Generally, our results suggest selection for lower alkaloid levels in nectar to minimize pollination-related costs (Adler et al. 2012), and are consistent with the disposability of nectar, a dedicated floral reward, relative to the male gametes in pollen (Hargreaves et al. 2009).

We still have much to learn about mechanisms of nectar production, and the degree to which nectar chemistry reflects secondary metabolism in other parts of the plant (Heil 2011, Stevenson et al. 2017). Whereas pollen development, including the production of pollenkitt, have been described in detail (Heslop-Harrison 1979, Pacini and Hesse 2005, Blackmore et al. 2007), including at the molecular level (Grienenberger et al. 2009, Yonekura-Sakakibara et al.

2014), the molecular basis of sugar transport in nectar was only elucidated recently (Lin et al. 2014). Greater knowledge of nectar production would help to clarify physiological constraints on chemical composition. Correlations between nectar and corolla chemistry (Cook et al. 2013, Richardson et al. 2016, Barlow et al. 2017) may relate to the mode of nectar secretion. For example, in Ranunculaceae, some species secrete nectar through cuticular microchannels, whereas others release nectar by rupture of epidermal cells that line the nectary (Antoń and Kamińska 2015). The latter mechanism releases the entire cytoplasmic contents into the nectary, which could be a less selective process than secretion through microchannels (Antoń and Kamińska 2015). Constraints between nectar and phloem chemistry may reflect sites of secondary compound synthesis. For example, locally synthesized or adsorbed nectar chemicals (Raguso 2004) might be less constrained by phloem chemistry relative to compounds that are synthesized remotely and transported via xylem or phloem. For remotely synthesized compounds, pleiotropic costs of foliar defenses could impose a lower limit on nectar concentrations (Adler et al. 2012), whereas autotoxicity could impose an upper limit (Baldwin and Callahan 1993). We also do not know to what extent nectar composition is environmentally vs. genetically determined (Mitchell 2004). Future study on regulation of nectar synthesis and provisioning with phytochemicals in diverse species will indicate which phytochemicals are constrained by, vs. independent from, chemistry of other plant parts. Overall, our data suggest strong independence of nectar and pollen secondary chemistry. They indicate that nectar chemistry can evolve separately from that of pollen, both in terms of composition and concentration.

Intraspecific variation across cultivars and sites

Across cultivars and sites, within-species nectar and pollen phytochemistry was qualitatively conserved but quantitatively heterogeneous. Intraspecific differences were not only statistically significant, but also of large magnitude. A median pair of cultivars or sites shared less than two-thirds of chemical trait space for nectar and less than half for pollen, with possible implications for disease resistance, herbivore resistance, and pollinator behavior, as discussed in the following three paragraphs.

We found the clearest differentiation in chemistry across cultivars. This likely reflects the consequences of strong artificial selection, as well as the homogeneous age and genetic background of cultivated plants relative to those in the wild, although we cannot exclude some effects of environmental factors or maternal environment. In other work, nectar traits such as volume and sugar composition had high heritability, but were generally measured in greenhouse rather than field settings (Mitchell 2004). Genetic control over non-sugar nectar constituents has not been explicitly addressed except with transformed plant lines (Kessler and Baldwin 2007), and no other study to our knowledge has examined intraspecific variation in pollen composition. Inter-cultivar variation in chemistry suggests a need for future study on how cultivars vary in attractiveness to managed and wild pollinator communities, particularly in species where yields are pollen limited (Garibaldi et al. 2013). In addition, cultivar differences illustrate how pleiotropic effects of selection on non-floral traits can alter nectar and pollen chemistry, which may complicate theories of floral phytochemical evolution in wild species.

We found less consistent, but still statistically significant, variation across sites in chemistry of wild species. These differences may reflect genetic or environmental effects, or their interactions. Genetic differences across populations likely explain some differences (Mitchell 2004). For example, deterministic effects of genetics on floral traits are demonstrated by the within-species consistency of floral morphology (Heinrich 1975), the low inducibility of floral chemical defenses relative to those of other tissues (Zangerl and Rutledge 1996), and the qualitative consistency of conspecific nectar amino acid samples from widely separated sites (Baker and Baker 1977). However, the environment can also have profound effects on floral traits. These include scent emission (Dötterl et al. 2009, Kessler et al. 2011), floral color morph (Baker and Baker 1977), diurnal rhythm of flowering (Kessler et al. 2010), and pollinator attraction (Kessler et al. 2011). Nectar traits can also be influenced by the environment. For example, nectar grayanotoxin concentrations were correlated with heat load across *Rhododendron* populations (Egan et al. 2016), and nectar alkaloid levels were experimentally modified by herbivory and nutrient addition (Adler et al. 2006). Each of these studies demonstrates ways in which the environment can influence floral chemistry. Finally, genotype by environment interactions have been found for nectar production rates (Boose 1997) and could exist for nectar and pollen chemistry as well. Future experiments using plant genotypes grown under different conditions could clarify the relative importance of genetics and environment to nectar and pollen chemistry. Additional experiments could test the inducibility of secondary chemical concentrations in response to environmental cues including fertilization, herbivory, and pathogen challenge.

Chemical differences between sites have implications for both pollinator behavior and plant evolution. Site-specific chemistry could alter pollinator foraging preferences, potentially shaping inter- and intraspecific resource competition, nest site selection, and population dynamics. Individual bumble bees, in particular, have a broad foraging range but consistent site- and plant-specific preferences that are retained over multiple weeks (Heinrich 1976, Ohashi and Thomson 2009). For plants, optimal chemistry of floral rewards may differ in response to abiotic conditions; pollinator availability, effectiveness, and chemical sensitivity (Tiedeken et al. 2014); and presence of non-pollinating insects and pathogens. Local selective pressures that act on preexisting variation could create chemical divergence across populations, as found in *Rhododendron ponticum* (Egan et al. 2016), which could in turn shape flower–insect interaction networks (Tiedeken et al. 2016). A related question is the scale at which pollinators make foraging decisions. Nectar phytochemical concentrations can influence local interactions (Adler and Irwin 2005, Kessler and Baldwin 2007), but can also vary by orders of magnitude among flowers of a single inflorescence (Kessler et al. 2012). It is unknown whether pollinators can detect inter-site differences against this background of within- and between-individual variation. If they can, differences in

chemical concentrations could be one driver of preferences for plant species and foraging sites.

Phenotypic integration

Our results indicate that nectar (mean integration index = 21.5) and pollen (mean 21.3) have levels of integration that are similar to those of leaf volatiles (mean 22.0), which were more integrated than flower volatiles (mean 10.8; Junker et al. 2018) and flower methanolic extracts (mean 9.9; this study). The generally low levels of integration in flowers may reflect several factors. First, flowers are physiologically complex, including petals, corolla, stigma, and anthers that differ in chemical composition (Flamini et al. 2002). This heterogeneity may reduce the chemical integration of the pooled floral tissue. Second, flowers undergo rapid chemical changes during maturation, bloom, and senescence that result in different chemical ratios in samples that differ slightly in developmental stage (Schiestl et al. 1997). Third, flowers may accomplish ecological functions with single compounds, which may lessen the need for integration of the whole flower. For example, variation in the floral volatile 2-phenylethanol was sufficient to alter both pollinator attraction and ant repellence in *Polemonium viscosum* (Galen et al. 2011). Likewise, a single compound, the monoterpenoid linalool, was sufficient to alter growth of some bacteria from *P. digitalis* flowers (Burdon et al. 2018).

In our study, correlations between different compounds were partly explained by biosynthetic similarity. Overall, concentrations of compound pairs that belonged to the same chemical class were more strongly correlated than were pairs that belonged to different chemical classes (Appendix S1: Fig. S6). For example, in *Malus domestica* nectar, the seven strongest correlations were all between pairs of flavonoids or a flavonoid and chlorogenic acid (Appendix S1: Fig. S3). All of these compounds are synthesized via the phenylpropanoid pathway (Rice-Evans et al. 1996). Similarly, in *Digitalis purpurea* pollen, 9 of the 10 strongest correlations were between spermidine derivatives (Appendix S1: Figs. S4, S5). These findings are consistent with prior analyses of phenotypic integration in scent bouquets, where biosynthetic similarity between compounds was correlated with strength of covariation (Junker et al. 2018).

On the other hand, both *Malus* and *Digitalis* (Appendix S1: Figs. S3–S5), as well as the entire data set (Appendix S1: Fig. S6), showed numerous strong correlations between compounds from different classes. These correlations could reflect similar solubilities or transport (in nectar), or selection for specific chemical ratios or combinations that function in pollinator attraction, defense, or development. Multimodal signals that combine scents with color can attract and condition pollinators to rewards (Junker and Parachnowitsch 2015). For example, carbon dioxide, floral volatiles, and leaf volatiles all functioned in concert with visual cues to attract adult *Manduca sexta* to artificial flowers; in females, carbon dioxide was only attractive against a background of host-plant leaf volatiles (Goyret et al. 2008). In nectar, which exhibited the highest within-module integration (Fig. 11) and strongest average correlation between compound pairs (Appendix S1: Fig. S6), consistent secondary chemical ratios could promote pollinator constancy by allowing pollinators to associate species-specific flavors with food rewards. This hypothesis has also been suggested to explain the consistency of amino acid composition of conspecific nectars (Baker and Baker 1977) and the morphological similarity of conspecific flowers (Heinrich 1975). Further research is needed to determine the primary and secondary significance of correlations between secondary compounds in nectar and pollen, and how covariation is differentially regulated in the two tissue types. Manipulative studies are necessary to determine whether damage by herbivores reduces the level of integration in nectar and pollen, as found for leaf volatiles (Junker et al. 2018).

There was no significant correlation between the integration of a species' nectar and the integration of its pollen. This is an important result, because it indicates that forces acting on phenotypic integration of nectar may be different from those acting on phenotypic integration of pollen, and that integration of these two tissues may be independently regulated. For example, *Malus domestica* had the second highest integration of all species for nectar (PI = 49.4), but the ninth lowest integration for pollen (PI = 12.7). Likewise, *Catalpa speciosa* had second highest integration for pollen (47.3), but below average integration for nectar (10.0). Together with the low levels of chemical overlap between nectar and pollen, this finding emphasizes that secondary chemistry of conspecific nectar and pollen can chemically diverge from one another. This divergence may reflect the unique selective pressures exerted on their different ecological roles.

This description of nectar and pollen secondary chemistry complements an expanding knowledge of scent- and morphology-mediated interactions between flowers, insects, and microbes (Junker and Blüthgen 2010, Junker et al. 2011*a*, Junker and Parachnowitsch 2015). Nectar and pollen secondary chemistry mediates interaction with pollinators, floral antagonists, and pathogens, and thereby influences the ecology and evolution of many plant communities. Our analyses summarize the variety of chemical strategies used in floral food rewards of diverse plant taxa.

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