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Among- and within-population variation in morphology, rewards, and scent in a hawkmoth-pollinated plant

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Abstract

Premise: Floral scent is a complex trait that mediates many plant–insect interactions, but our understanding of how floral scent variation evolves, either independently or in concert with other traits, remains limited. Assessing variation in floral scent at multiple levels of biological organization and comparing patterns of variation in scent to variation in other floral traits can contribute to our understanding of how scent variation evolves in nature.

Methods: We used a greenhouse common garden experiment to investigate variation in floral scent at three scales—within plants, among plants, and among populations—and to determine whether scent, alone or in combination with morphology and rewards, contributes to population differentiation in *Oenothera cespitosa* subsp. *marginata*. Its range spans most of the biomes in the western United States, such that variation in both the abiotic and biotic environment could contribute to trait variation.

Results: Multiple analytical approaches demonstrated substantial variation among and within populations in compound-specific and total floral scent measures. Overall, populations were differentiated in morphology and reward traits and in scent. Across populations, coupled patterns of variation in linalool, leucine-derived compounds, and hypanthium length are consistent with a long-tongued moth pollination syndrome.

Conclusions: The considerable variation in floral scent detected within populations suggests that, similar to other floral traits, variation in floral scent may have a heritable genetic component. Differences in patterns of population differentiation in floral scent and in morphology and rewards indicate that these traits may be shaped by different selective pressures.

KEYWORDS

floral traits, floral volatiles, genetic variation, intraspecific variation, nectar, Onagraceae, plant–pollinator interactions

Floral scent can mediate interactions with mutualists and antagonists, both of which may act as agents of selection (e.g., Galen et al., 2011; Kessler et al., 2013). Flowering plants often emit a large number of volatile compounds that are products of related biosynthetic pathway branches (e.g., Barkman, 2001; Cai et al., 2016; Pichersky and Raguso, 2018). As such, floral scent is a quantitative trait that is both functionally and analytically complex. Recent studies have increased our understanding of complex scent phenotypes by demonstrating that floral scent mediates interactions in generalized systems (e.g., Johnson and Hobbhahn, 2010; Schiestl et al., 2018; Kantsa et al., 2019), and that floral scent blends often comprise a core set of common components (reviewed by Knudsen and Gershenzon, 2006). However, most studies of floral scent variation to date have been descriptive, such that our understanding of the evolutionary drivers of variation in floral scent remains limited, outside of a small number of systems (Kessler and Halitschke, 2009; Parachnowitsch et al., 2012; Gervasi and Schiestl, 2017).

One prerequisite for determining how scent variation evolves is to determine whether scent is variable at different levels of biological organization. In particular, for scent to evolve via natural selection, scent must vary among individuals in a population, and this variation must have a heritable component. Standing genetic variation may form

the basis for adaptation to novel environments (Barrett and Schluter, 2008), and intraspecific trait variation can alter the outcome of ecological interactions (Bolnick et al., 2011). Across a number of study systems, there is considerable variation in scent at different scales. At the macroevolutionary scale, scent varies among species adapted to different pollinators (Raguso et al., 2003; Waelti et al., 2008; Byers et al., 2014; Bischoff et al., 2015) and is similar among species that have converged on the same pollinator (Dobson, 2006; Jürgens et al., 2013; Schiestl and Johnson, 2013). On the microevolutionary scale, scent varies among populations that span species' ranges where plants may interact with different mutualists or antagonists (e.g., Suinyuy et al., 2012; Souto-Vilarós et al., 2018; Friberg et al., 2019). Relatedly, pollinator shifts and mating system variation have been associated with gains and losses of floral scent (Raguso et al., 2007; Doubleday et al., 2013; Sas et al., 2016; Petrén et al., 2021) and shifts in structural genes (Amrad et al., 2016).

Despite this evidence for variation in floral scent at different scales, variation within populations has not been extensively characterized. Of the 151 records of intraspecific variation in scent compiled in a recent review (Delle-Vedove et al., 2017), 62 records of intraspecific variation in scent did not contain any measures of variation within populations, and an additional 43 records contained only basic descriptions of intrapopulation variation, e.g., coefficients of variation. Although the genetic underpinnings of floral scent production are increasingly well investigated, most such studies address the structural genes and enzymes responsible for scent production (e.g., Pichersky and Gang, 2000; Tholl et al., 2005; Chen et al., 2011), biosynthetic pathway dynamics among such entities (e.g., Gershenzon, 1994; Pasquet et al., 2017; Jantzen et al., 2019) or inbred lines prepared to test pollinator responses (Klahre et al., 2011; Byers et al., 2014). Despite this growing literature, only one study thus far has addressed the heritability of measurable variation in floral scent (Zu et al., 2016). As such, our understanding of the potential for scent to evolve via natural selection is currently limited.

Although floral scent has been historically studied in isolation from other floral traits, there are several reasons why variation in floral scent at different levels should be investigated in concert with variation in morphology and rewards (Raguso, 2008; Junker and Parachnowitsch, 2015). First, most flowers have traits that engage multiple sensory modalities used by foraging pollinators, such as olfactory, visual, and tactile stimuli. Multi-modal floral communication may serve to increase the overall information content or efficacy of signals that attract pollinators to plants (Leonard et al., 2011a, b). As a result, floral scent may serve as a pollinator attractant independent of, or in concert with, other types of floral traits (reviewed by Junker and Parachnowitsch, 2015). Second, variation in floral scent could also be a product of variation in another floral trait, for instance, if scent production is a function of flower size (Ashman, 2009; Galen et al., 2011; Parachnowitsch et al., 2012; Eisen et al., 2022). One way to begin to investigate whether floral scent may be under selection independent of or together with other floral traits is to determine whether populations are differentiated in both scent and other floral traits and whether patterns of variation in scent parallel patterns of variation in other floral traits across populations.

In this study, we investigated variation in floral scent at three scales—within plants, among plants, and among populations—and determined whether population differentiation occurs in scent traits, morphology and reward traits, or both types of traits in *Oenothera cespitosa* subsp. *marginata* (Nutt. ex Hook. & Arn.) Munz (Onagraceae), hereafter *O. c. marginata*. A self-incompatible, perennial herb with large, white, night-blooming, and sweet-smelling flowers, *O. c. marginata* represents an excellent model system for investigating sources of variation in quantitative floral traits because there is considerable variation in the abiotic and biotic environment across its range, which spans most biomes in the western United States (Wagner et al., 1985; Patsis et al., 2021; see Study system section below). In addition, other species in the genus exhibit considerable variation in quantitative floral traits in greenhouse common garden studies of the allometry of floral morphology (Summers et al., 2015) and floral advertisement and reward traits (Raguso et al., 2007; Gallego-Fernández and García-Franco, 2021). We conducted a greenhouse common garden study using seeds sourced from five populations that were distributed across the natural range of *O. c. marginata*. By eliminating variable environmental effects on trait values, the common garden enabled us to assess whether phenotypic variation has a genetic basis. These data were used to address two explicit questions about the nature of variation in scent and morphology and reward traits across and within populations: (1) What is the repeatability of floral scent as a measurable phenotype? (2) Are populations differentiated in morphology and rewards, floral scent, or both types of traits?

MATERIALS AND METHODS

Study system

Oenothera cespitosa is a perennial herb with a rosette growth form, a deep tap root, and large, white, night-blooming flowers that produce a strong, sweet fragrance (Wagner et al., 1985). We note that the newer spelling of the species epithet has precedence over the older spelling of *O. caespitosa* (Wagner, 2005). The long-tubed, nectar-rich flowers of *O. cespitosa* are self-incompatible and are pollinated by a guild of long-tongued hawkmoths and, occasionally, by large bees (Stockhouse, 1975; Hodges, 1987; Artz et al., 2010). Five described subspecies of *O. cespitosa* are distributed across western USA, with edaphic affinities for alkali clays (*O. cespitosa* subsp. *cespitosa*) or sandstone (*O. cespitosa* subsp. *navahoensis*) at low elevation, and for

loamy, neutral soils (*O. cespitosa* subsp. *macroglottis*), limestone or dolomite-derived soils (*O. cespitosa* subsp. *crinita*) at high elevation (Wagner et al., 1985; Artz et al., 2010). The fifth subspecies, *O. c. marginata*, is the most widespread and ecologically generalized subspecies of *O. cespitosa*, ranging from the Sonoran Desert along the Arizona–Mexico border to lava fields and talus slopes of the northern Great Basin in Idaho and eastern Oregon (Wagner et al., 1985; Patsis et al., 2021), and a recent phylogenetic study suggests that *O. c. marginata* is monophyletic (Patsis et al., 2021). The expansive distribution of *O. c. marginata*, spanning most noncoastal biomes in western North America, suggests the possibility of latitudinal variation in floral traits, which we explore in the present study.

Greenhouse common garden methods

Plants were grown from field-collected seeds from five populations that span the range of *O. c. marginata*: Pinal County, Arizona; Inyo County, California; near Zion National Park in Washington County, Utah; Logan Canyon in Cache County, Utah; Oneida County, Idaho (see Appendix S1, Table S1). For convenience, we refer to these populations as follows: Arizona, Inyo, Zion, Logan, and Idaho. Seeds were collected opportunistically by harvesting mature, semidehiscent capsules from up to 10 distinct individual plants per population, when possible (see Appendix S1, Table S1 for sample sizes), and were stored in paper coin envelopes with silica gel at −20°C until germination. Seeds from different maternal plants were combined for each population, surface-sterilized for 1 min in 1% v/v aqueous bleach solution and air-dried. Because the seeds used for this study were pooled from different maternal plants, the sampled plants likely vary in their contribution to the samples collected for each population in the greenhouse. This limitation of the experimental design was due to variation in seed production and germination rates and could bias estimates of genetic diversity.

To break dormancy, we soaked approximately 50 seeds per population overnight on moist filter paper, and then the seed coats were cut with a sharp razor under a dissecting microscope. The embryos were removed and placed on damp towels in Petri dishes sealed with Parafilm. The dishes were placed under a light source for 12 h/day until cotyledons became green and the radicle extended. After 2 weeks, seedlings were transplanted into compartmentalized seed trays and filled with Metro-Mix 360 (Sun-Gro Horticulture Canada, Montreal, Quebec, Canada) enriched with perlite (3:1 MetroMix–perlite). All the trays were bottom-watered and protected with a clear plastic cover. After 2–3 weeks, 30 plants from each population (150 total) were transplanted into larger terra cotta pots (20 cm diameter; 4 L volume) and were assigned randomized positions within the greenhouse. Plants were watered once a day, fertilized weekly with a 21-5-20 fertilizer (nitrogen-phosphate-potash), and kept under natural sunlight.

Measurements of morphology and reward traits

Seven morphological traits were measured for each plant using digital calipers (see Appendix S2, Figure S1 for diagram) to generate five morphological trait values: corolla diameter, corolla flare, hypanthium length, pedicel length, and herkogamy. Average corolla diameter per flower was calculated based on two measurements per flower. Corolla flare was measured as the longest chord across the face or opening of the hypanthium (=nectar tube). Hypanthium length was measured from the distal end of the ovaries to the base of the corolla, where sepals attach to the base of the petals, and this measure of floral size may be independent of some components of vegetative size, as it was correlated with the length of the axillary leaf in only one of three populations for which leaf length data were collected (Appendix S2, Figure S2). The length of the pedicel was measured from the proximal end of the ovaries to the axis of the subtending leaf. Because subspecies *marginata* is the only subspecies of *O. cespitosa* that consistently produces a pedicel, it is a diagnostic field character for this taxon (Wagner et al., 1985). Pedicel length was measured as a potential internal standard for pollinator-independent floral variation—it has no obvious function in pollinator attraction or reward, as it is concealed within the vegetative rosette. While we expect that variation in the pedicel is not related to variation in pollinators, pedicel length could be under selection by another agent of selection, for instance, if pedicel length affects the apparency of flowers to herbivores and/or variation in pedicel length could be the product of drift. The lengths of the style and longest stamen for each flower were measured from the opening of the nectar tube to the attachment of the anther to the filament (for stamens) and to the base of the four stigma lobes (for the style). Herkogamy (distance between stamens and the stigma) was calculated by subtracting the longest stamen length from style length, which resulted in small negative values in some cases. The flowers are neither protandrous nor protogynous, because the stamens are dehiscent and stigma lobes are receptive at anthesis and the distance between them does not change until the morning after anthesis.

In addition to the five morphological traits, two nectar traits were measured: nectar volume and nectar sugar concentration. Nectar volume was estimated by extracting nectar into a 50 µL microcapillary tube and measuring the height of the nectar in the tube (mm), which was then converted to a volume (µL) using an equation ($y = 0.5518x + 0.0506$) generated by measuring known volumes (10–50 µL) of a solution that matched the mean measured nectar sugar concentration (23% w/v sucrose). Nectar sugar concentration was measured by analyzing 5 µL from each sample using a handheld refractometer, within a range of 0 to 50% sucrose equivalents (brix) by mass.

Floral volatiles collection methods

Floral volatiles were collected using dynamic headspace methods (see Galen et al., 2011) from 21 May to 21 July

2009 in natural lighting in a well-aerated corridor adjacent to the common greenhouse. Samples were collected opportunistically from 1–8 flowers per plant and 9–27 plants per population (see Appendix S1, Table S1 for exact sample sizes).

Single, newly opening flowers were inserted within nylon resin oven bags (Reynolds, Lake Forest, IL, USA) standardized to c. 500 mL volume for headspace sampling, with a fresh bag used for each sample. Headspace samples were collected using PAS-500 Micro Air Sampler pumps (Spectrex, Redwood City, CA, USA) connected to Pasteur pipette-derived glass traps that contained 0.0100 g of Super Q 80/100 adsorbent sandwiched between plugs of silanized quartz wool (Alltech Associates [W.H. Grace], Deerfield, IL, USA). Pump flow rates were set to 200 mL/min. Scent was collected during the first hour of anthesis, the period of greatest pollinator activity in natural populations (Hodges, 1987; Artz et al., 2010). Flowers are borne individually within the axil of the subtending leaf and are not organized in inflorescences. However, all flowers that open on the same day do so synchronously. Floral scent and color do not change until the morning after anthesis, and the dimensions of floral sex organs do not change until floral senescence. Nevertheless, we collected data during the first hour of anthesis to best reflect the traits that would be encountered by foraging hawkmoths at dusk. Each evening of sampling, one ambient control sample was collected from an empty oven bag placed elsewhere in the corridor.

Following headspace collection, traps were eluted with 300 µL of GC-MS-quality hexane (Burdick & Jackson GC2; Honeywell Life Science, Muskegon, MI, USA). Sampled flowers were removed from the plant and weighed (to 0.01 g), then dried for 24 h at 50°C and weighed again. Although removing flowers from plants to measure floral mass could generate wounding volatiles, none were detectable in the chemical profiles of subsequent samples from the same plant because a minimum of 22 h elapsed between the removal of a flower and the collection of a subsequent sample. Eluted volatile samples were concentrated to 50 µL with a flow of gaseous N_2 and spiked with 23 ng of toluene (5 µL of a 0.03% v/v solution in hexane) as an internal standard in preparation for analysis with gas chromatography–mass spectrometry (GC-MS). Samples were stored at −20°C until analysis.

Scent analysis via GC-MS

Volatile samples were analyzed using a GC17A gas chromatograph coupled with a QP5000 quadrupole mass spectrometer (Shimadzu Scientific Instruments, Kyoto, Japan). One microliter of the solvent eluted samples was injected (splitless mode) at 240°C onto a polar GC column (EconoCap EC Wax, W.M. Grace, Columbia, MD, USA; 30 m length, 0.25 µm film thickness, 0.25 mm inner diameter). The GC oven programs increased from 40°C to 260°C at either 15°C/min or 10°C/min, with a 2- or 4-min hold at the maximum temperature, respectively. The use of a faster GC program reduced the total time of analysis but did not affect peak resolution to baseline. Electrical ionization mass spectra were generated at 70 eV (scanning range 40–350 m/z), and resulting mass spectra were compared with those of MS libraries (Wiley, NIST [National Institute of Standards and Technology], Adams) using Shimadzu GCMSolutions software. Volatile organic compounds (VOCs) were identified via direct comparison of retention time and mass spectra with those of authentic, co-injected standards. When standards were not available, we calculated Kovats retention indices (KRI) by comparison with the retention times of n-alkanes on the same GC column using the 10°C/min GC program (see Schlumpberger and Raguso, 2008), then comparing KRI values with online, searchable data on the NIST webbook (https://webbook.nist.gov/) and Pherobase (https://www.pherobase.com; El-Sayed, 2021).

Scent data extraction and processing

Peak areas were integrated manually using Shimadzu GCMSolutions software, resulting in a data set of 40 VOCs that occurred in one or more floral sample(s). To exclude ambient contaminants, we compared chromatograms of floral samples to the ambient control sample collected on the same day. None of the floral compounds retained in our data set were present in substantial amounts in the ambient control samples.

Emission rates were normalized by dividing total ion current (TIC) peak areas by that of the internal standard (Svensson et al., 2005), then were calculated algebraically using response factors generated using external standard dose-response curves generated from log-dilutions of six floral volatiles identified in these analyses [(*E,E*)-α-farnesol, geraniol, β-caryophyllene, linalool, methyl benzoate, and toluene; see Table S2]. Emission rates are presented as micrograms scent per gram fresh floral mass per hour.

Statistical analyses

All R code and data are available on GitHub (github.com/kate-eisen/Oenothera) and Zenodo (doi:10.5281/zenodo.6572548). All analyses were performed in R studio version 1.2.5033 using R version 3.6.3 (R Core Team, 2020).

Before the analyses to address our two questions, we reduced the number of floral scent variables for analysis because we identified 40 volatile compounds in our floral scent sampling (Appendix S1, Table S2). To this end, we examined patterns of correlation among these compounds across all populations and within each population. Specifically, we explored the potential for correlation among biosynthetically related VOCs (e.g., compounds structurally related to geraniol), given that products of related biosynthetic pathway branches often co-occur in floral

headspace (Barkman, 2001). By determining which of these pathway clusters were significantly correlated (in the entire data set and at the population level) after correcting for multiple tests, we identified 12 scent variables for subsequent analysis (Table 1). Four of these variables are single volatile compounds that were not strongly correlated with other compounds, and the remainder are groups of three to six compounds produced via the same or similar biosynthetic pathways.

Question 1 (Q1)

To investigate the repeatability of floral scent across flowers, plants, and populations, we estimated the variance partitioned among flowers, plants, and populations for floral scent summary metrics (total emission rate, compound diversity, compound group diversity) and the 12 composite floral scent variables (see above). For the univariate floral scent summary metrics, we estimated repeatability, which represents an upper limit on the narrow-sense heritability of the trait (Falconer and Mackay, 1996), using the rptR R package (Stoffel et al., 2017). We used the rptGaussian function to estimate the repeatability of total emission rate and the rptPoisson function to estimate the repeatability of compound diversity and compound group diversity. We note that our estimates of repeatability should be interpreted with caution because they could be influenced by maternal effects and maternal sibships. While repeatability generally places an upper limit on the narrow-sense heritability of the trait (Falconer and Mackay, 1996), our estimates of repeatability provide a starting point for understanding the heritability of floral scent in this system and could be additionally verified in a future study that more explicitly tracks scent variation in progeny of different maternal lines (see Future Directions below).

To determine how variance is partitioned among populations, plants, and flowers for each scent variable, we performed a hierarchical modeling of species communities (Hmsc) analysis using the Hmsc R package (Tikhonov et al., 2020). This approach is a framework for joint species distribution modeling, where species occurrences or abundances (here, emission rates of scent variables) are related to environmental covariates, species traits, or phylogenetic relationships (here, the flowers, plants, and source populations sampled). As such, this approach accounts for the multivariate nature of floral scent data, where emissions of specific compounds or groups of compounds may not be

TABLE 1 Abbreviations and corresponding volatile compounds for the 12 scent variables analyzed. For IUPAC names and retention times, see Appendix S1, Table S2.

Abbreviation	Pathway description and compounds
Monoterpenes	
GER	Geraniol-related: citronellol, neral, geranial, nerol, geraniol
ALT	α-Terpineol
LIN	Linalool
LID	Linalool-derived: (*Z*)-furanoid linalool oxide, (*E*)-furanoid linalool oxide, pyranoid-linalool oxide-ketone, (*Z*)-pyranoid linalool oxide, (*E*)-pyranoid linalool oxide
OCI	Ocimene-related: β-myrcene, (*Z*)-β-ocimene, (*E*)-β-ocimene
Sesquiterpenes	
CAR	Caryophyllene-related: β-caryophyllene, α-humulene, caryophyllene oxide, farnesol
FAR	Farnesene-related: β-farnesene, (*Z*,*E*)-α-farnesene, (*E*,*E*)-α-farnesene, farnesene epoxide
NER	(*E*)-Nerolidol
ISO	Isophytol
Aromatics and amino acid-derived volatile organic compounds	
PHE	Phenylalanine-derived: 2-phenylethanol, phenylacetonitrile, nitro-2-phenylethane, phenylacetaldoxime
LEU	Leucine- and valine-derived: 3-methylbutyronitrile, nitro-3-methyl-butane, (*Z*)−3-methylbutyraldoxime, (*E*)−3-methylbutyraldoxime, (*Z*)-isobutyraldoxime, (*E*)-isobutyraldoxime
ILE	Isoleucine-derived: 2-methylbuyronitrile, nitro-2-methyl butane, (Z)−2-methylbutyraldoxime, (*E*)−2-methylbutyraldoxime, 2-methylbutylbenzoate

independent. We ran this model on the mean-standardized scent data. Our model included a fixed effect of population and random effects of plant, flower, and sampling date, to account for any effects of variation in conditions across sampling days. Because we were specifically interested in detecting variation among plants, we adjusted the shrinkage in our Hmsc model for the plant-level random effect because the default priors shrink the variances of random effects close to zero, such that a very strong signal is required to overcome the shrinkage (Ovaskainen and Abrego, 2020). Thus, we tested different values of a2 using the setPriors function and found that a2 = 5 maximized the amount of variance explained by the model and also generated a covariance matrix on an equivalent scale to the raw data (results not shown).

Question 2 (Q2)

To determine whether populations are differentiated in morphology and reward traits, scent traits, or both types of traits, we began by conducting three parallel sets of multivariate analyses on the morphology and rewards, scent, and combined data sets. Because a low number of unique plants were sampled from the Idaho population, it was excluded from these analyses. To determine the amount of variance explained by population in the morphology and rewards, scent, and combined data sets, respectively, we performed a permutational multivariate analysis of variance (PERMANOVA) using a Bray–Curtis distance matrix and the adonis function from the vegan package (Oksanen et al., 2018). Our opportunistic sampling of flowers and plants in the greenhouse common garden resulted in different numbers of flowers sampled per plant. Given the challenges associated with performing PERMANOVA with unbalanced design, these analyses were performed using only the measurements of the first flower sampled per plant.

To determine which specific scent and morphological and rewards variables contribute to population differentiation, we performed a canonical analysis of principal (CAP) coordinates with a Bray–Curtis distance dissimilarity index using the capscale function from the vegan package (Oksanen et al., 2018). Similar to the PERMANOVAs that addressed overall population differentiation (described above), we performed three CAP analyses, using the floral scent data, the morphology and rewards data, and the combined data; each analysis used only the measurements of the first flower sampled per plant.

To examine patterns of variation in specific traits across populations, we then performed univariate analyses using general linear mixed-effects models, which were implemented using the lme4 package (Bates et al., 2015). Models were visually assessed to ensure normally distributed residuals with homogenous variance. These models all contained source population as a fixed effect and plant nested within source population as a random effect, which enabled us to use data from multiple flowers per plant where available. The significance of the fixed effect of source population was assessed using the ANOVA function in the lmerTest package ver. 2.0-29 (Kuznetsova et al., 2017) to perform type III *F*-tests using the Kenward–Roger approximation for the denominator degrees of freedom. When ANOVAs returned significant *F*-values, we used Tukey's honest significant difference tests to determine which group means were significantly different using the emmeans function with the pairwise option in the emmeans package (Lenth, 2019).

These univariate analyses were performed on two types of variables. First, we analyzed two floral scent summary metrics: total scent emission rates and the number of compounds detected in a sample. Second, we analyzed scent and morphological and rewards variables that were correlated with one or both of the first two CAP axes. These variables had significant Pearson correlation coefficients with one or both axes at $P < 0.01$ after applying a false discovery rate correction for the number of variables in that version of the data set ($N = 12$ for scent only, $N = 8$ for morphology and rewards only, $N = 20$ for the combined data set); in all significant correlations, $|r| > 0.35$. For the variables that were correlated with one or both of the CAP axes, we analyzed all measurements from a plant using general linear mixed-effects models with plant nested within population included as a random effect (see above). We applied a false discovery rate correction for the number of variables significantly correlated with the CAP axes for each data set ($N = 6$ for scent only, $N = 6$ for morphology and rewards only, $N = 5$ for the combined data set; see Results) on the *P*-values associated with *F*-tests for a significant effect of source population. We then tested for differences between source populations using the emmeans function as described above.

RESULTS

Overview of floral volatile diversity

GC-MS analyses of headspace from 170 flowers, representing 76 individual plants in five geographically distinct populations led to the identification of 40 volatile organic compounds (VOCs). These compounds included 15 monoterpenoids, nine sesquiterpenoids, one diterpene (isophytol), 11 compounds whose C skeletons were wholly or partially derived from the amino acids LEU, VAL or ILE, and four aromatic compounds with or without N atoms, derived from PHE (Table S2). Correlation analysis revealed that subsets of these compounds, many of which are biosynthetically related, tended to occur as "blocks" of correlated traits, identified by Spearmen's rho correlation coefficients and visualized using heat maps (Appendix S2, Figure S3).

The frequency of individual VOCs across all samples ranged from linalool and the stereoisomers of 2-methylbutyl- and 3-methylbutylaldoxime (present in at least 157 of 170 total

samples) to a block of aromatic compounds found in 10 or fewer samples, only in the Logan, UT population (Appendix S1, Tables S2 and S3). Not surprisingly, the most frequent compounds also were among those emitted in the highest amounts per flower; linalool (up to 60.5%) and the LEU and ILE-derived aldoximes (up to 49%) dominated total scent emissions across the five populations (Appendix S1, Table S3). At the other end of the spectrum, we detected scarce compounds that were only found in certain populations or when specific compounds had high emission rates. In addition to the four PHE-derived aromatics, (*E*)-farnesene epoxide and pyranoid linalool oxide ketone (6-ethenyl-2,2,6-trimethyloxan-3-one) were found only in the Logan, UT population (Appendix S1, Table S3). Citronellol, neral, nerol, and geranial combined represented less than 0.025% of total emissions in the Arizona population (Appendix S1, Table S3), and the mean emission rate of geraniol, a structurally related and more abundant compound, was 9.32 μg/g fresh mass/h when these compounds were not detected and 670.07 μg/g fresh mass/h when at least one compound was detected (two sample *t*-test with unequal variances: $t = -5.8836$, df = 22.024, $P = 6.37\text{e-}06$). Similarly, samples that contained (*Z*)-β-ocimene and/or β-myrcene had high amounts of (*E*)-β-ocimene (mean emission rate: 947.92) relative to samples that only contained (*E*)-β-ocimene (mean emission rate: 1.52) ($t = -5.2032$, df = 106, $P = 9.637 \times 10^{-7}$). (*Z*,*E*)-α-farnesene occurred in samples with high amounts of (*E*,*E*)-α-farnesene (mean emission rate: 3260.43), relative to samples that only contained (*E*,*E*)-α-farnesene (mean emission rate: 10.80) ($t = -6.848$, df = 17.005, $P = 2.827 \times 10^{-6}$). Lastly, caryophyllene oxide and/or α-humulene were detected in samples with high amounts of (*E*)-β-caryophyllene (mean emission rate (47.12), relative to samples that only contained (*E*)-β-caryophyllene (mean emission rate 3.97) ($t = -4.571$, df = 46.624, $P = 3.573 \times 10^{-5}$).

Interestingly, some compounds would have been omitted from analysis if only the first flower of each plant had been analyzed, which reflects a degree of within-plant variation in floral scent (see also Q1 below). These compounds included neral and geranial in the Inyo, CA population and (*E*,*E*)-α-farnesene in the Arizona population (Appendix S1, Table S3). The latter compound is especially noteworthy because it is inconsistent among flowers of the same plant and, when present, can be emitted in very large amounts, ranging from <1% (Arizona) to 80% (Logan) of total emissions by peak area (Appendix S2, Figure S4).

Q1. What is the repeatability of floral scent?

The repeatability of total scent emission was 0.36, while the repeatability of compound diversity (number of compounds) was 0.32, and the repeatability of compound groups (number of groups of compounds) was 0.

An Hmsc model revealed substantial variation in floral scent at multiple levels. "Population" explained 14% of variation on average and explained 25% or more of the variation in four scent variables: linalool (25%), LEU compounds (leucine- and valine-derived compounds) (27%), isophytol (25%), and α-terpineol (26%) (Figure 1; Appendix S2, Table S5). "Plant" explained 47% of variation on average and ranged from explaining less than 10% of variation (LID [linalool-derived] compounds) to explaining greater than 75% of variation (FAR [farnesene-related], GER [geraniol-related], and OCI [ocimene-related] compounds). "Flower" explained between 0 and 3% of the variation in most scent variables but explained 10% of variation in isophytol. "Sampling date" generally explained between 0 and 2% of the variation overall but explained a larger (7–10%) amount of variation in three scent variables (ILE [isoleucine-derived] and LEU compounds, α-terpineol). On average, about one third of the variance in scent variables was unexplained by population, plant, flower, or sampling date but the amount of unexplained variance ranged widely across scent variables (minimum: 3%, maximum: 84%).

Q2. Are populations differentiated in morphology and rewards, floral scent, or both types of traits?

The PERMANOVAs of the floral scent data, the morphology and rewards data, and the combined data all revealed main effects of source population. Specifically, source population explained 23.6% of the variation in floral scent ($P < 0.001$), 35.1% of the variation in morphology and rewards traits ($P < 0.001$), and 27.0% of the variation in the combined data set ($P < 0.001$).

Total scent emission varied among source populations ($F_{3,68.005} = 6.51$, $P = 0.0006$) (Figure 2A). Emission rates (scent per gram fresh floral mass per hour) of plants from Arizona and Inyo were about 2.2 times higher than emission rates of plants from Logan, even when differences in floral mass were standardized (Appendix S2, Table S6). Compound diversity per floral scent sample also varied across the source populations ($F_{3,67.73} = 4.37$, $P = 0.0071$) (Figure 2B). More compounds were detected in samples from Arizona (estimated marginal mean ± 1 SE: 16.2 ± 0.74 compounds) and Inyo (17.6 ± 1.06 compounds) relative to Logan (13.0 ± 0.88 compounds) (Appendix S2, Table S6).

In the CAP analysis of the floral scent data, source population explained 22% of the total variation in the data (Appendix S2, Figure S5A). CAP axis 1 explained 19% of the total variation in the data, while CAP axis 2 explained 2% of the total variation in the data. Four scent variables were positively correlated with CAP axis 1 (ILE compounds, LEU compounds, linalool, and isophytol). Four scent variables were positively correlated with CAP axis 2 (LEU compounds, linalool, OCI compounds, and FAR compounds) (Appendix S2, Table S7). Emission rates of the LEU compounds, linalool, and isophytol all varied among the source populations (Appendix S2, Table S8), with higher emission rates of LEU compounds and linalool in Inyo and

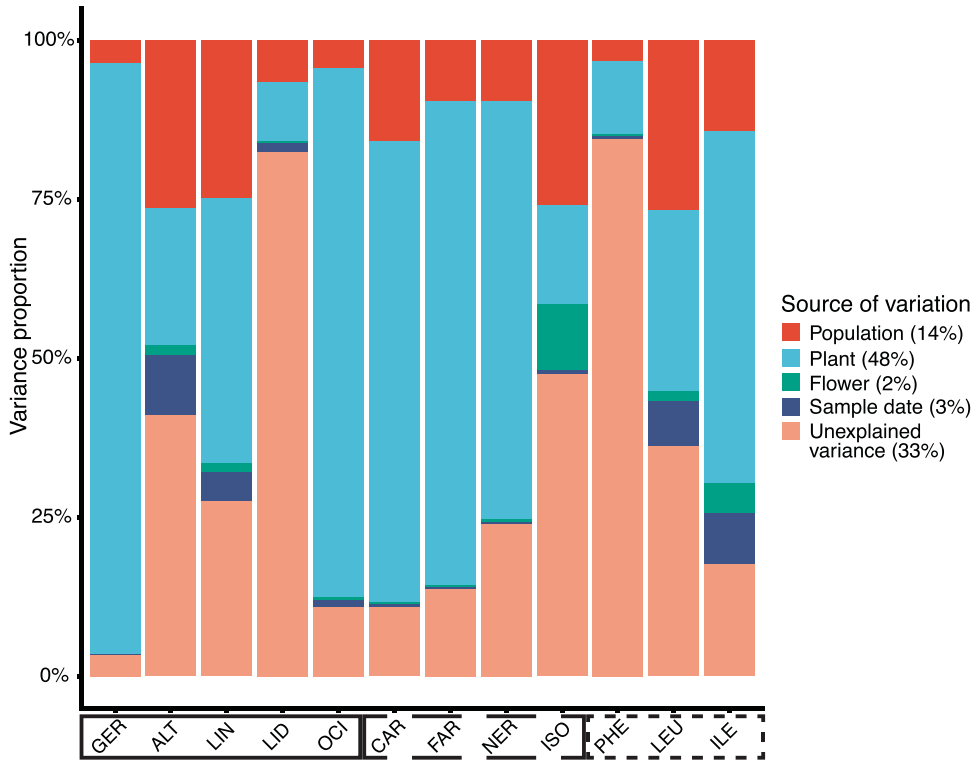

FIGURE 1 Visualization of the proportion of variance in each scent variable explained by source population (red), plant (light blue), flower (green), sampling date (dark blue), and variance unexplained by these factors (salmon). Scent variables are grouped along the *x*-axis by broad-scale compound type: monoterpenes (solid box), sesquiterpenes and diterpenes (dashed box), and aromatics and amino acid-derived volatile organic compounds (dotted box). The percentages in parentheses in the legend are the average percentage of variance explained by the given factor across all of the scent variables.

Arizona relative to Logan (Figure 2C and D; Appendix S2, Table S9) and higher emission rates of LEU compounds in Inyo relative to Zion (Figure 2C; Appendix S2, Table S8). Emission rates of isophytol were higher in Arizona relative to all other source populations (Figure 2E; Appendix S2, Table S9). Emission rates of ILE, OCI, and FAR compounds did not vary among source populations (Appendix S2, Table S8).

In the CAP analysis of the morphology and rewards data, source population explained 29% of the total variation in the data (Appendix S2: Figure S5B). CAP axis 1 explained 24% of the total variation in the data, while CAP axis 2 explained 4% of the total variation in the data. Five morphological and rewards traits (for means ± SE by population, see Appendix S2: Table S4) were positively correlated with CAP axis 1 (mean corolla width, tube flare, hypanthium length, nectar volume, percent nectar sugar), while four traits were positively correlated with CAP axis 2 (mean corolla width, tube flare, nectar volume, pedicel length) (Appendix S2, Table S10). Pedicel length was negatively correlated with CAP axis 1. All of these traits varied across the source populations with the exception of percentage nectar sugar (Appendix S2, Table S11). Mean corolla width and tube flare were generally highest for plants from Zion, and intermediate for plants from Inyo and Arizona, relative to plants from Logan (Figure 3A, B, 4A, B; Appendix S2, Table S12). Hypanthium length was shorter for plants from Logan relative to all other populations (Figure 4C; Appendix S2, Table S12). Pedicel length was longer for plants from Logan and Zion relative to plants from Arizona (Figure 4D; Appendix S2, Table S12). Nectar standing crop volume was greater for plants from Arizona and Inyo relative to plants from Logan (Figure 4E; Appendix S2, Table S12).

In the CAP coordinates of the combined floral scent and morphology and rewards data, source population explained 28% of the total variation in the data (Figure 3C). CAP axis 1 explained 20% of the total variation in the data, while CAP axis 2 explained 6% of the total variation in the data. One morphological trait—hypanthium length—and three scent variables—ILE compounds, LEU compounds, and linalool—were positively correlated with CAP axis 1 (Appendix S2, Table S13), and linalool was also positively correlated with CAP axis 2. These traits were also identified in the scent-only and morphology and rewards-only analyses, and patterns of variation across populations are described above.

DISCUSSION

Because the evolution of floral scent via natural selection necessitates variation in floral scent across levels of biological organization, we investigated the potential for variation in floral scent at three scales: within plants, among

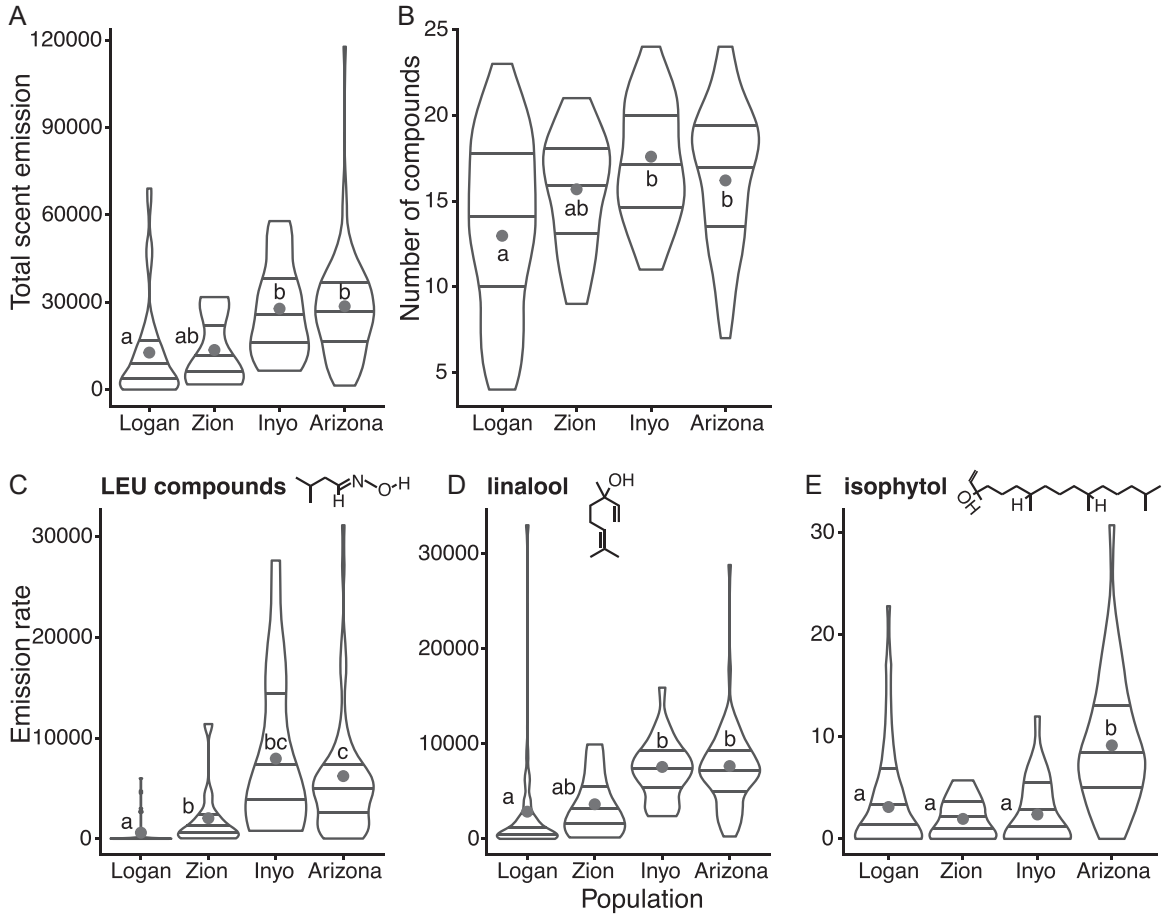

FIGURE 2 Variation in total scent emission rates (A), number of compounds detected in a sample (B), emission rates of leucine-derived compounds (chemical structure is shown for (*E*)−3-methylbutyraldoxime, which was the most abundant of the LEU compounds) (C), linalool (D), and isophtyol (E) across populations. Violin plots represent raw scent data and are scaled to have the same maximum width across populations and horizontal lines on the violins indicate the quartiles for each population. Different lowercase letters indicate differences in the estimated marginal means (dark gray points) between populations.

plants, and among populations of a hawkmoth-pollinated species with a large distribution in the western US. We found considerable variation especially among plants and also among populations in multiple floral scent measures, including total scent emission rates, VOC diversity, and emission rates of several, biosynthetically informed scent variables. In addition, because floral scent may evolve in concert with other floral traits due to selection on multimodal floral signals or trait correlations, we determined whether populations are differentiated in floral scent, morphology and rewards, or both types of traits. Overall, populations were differentiated in morphology and rewards and floral scent, with population explaining about 10% more of the variation in the morphological and rewards traits compared to the scent data. Across populations, we detected patterns of variation in scent variables and morphological and rewards traits that are consistent with pollinator syndromes. Here we discuss the implications of these findings in terms of our growing understanding of the factors that contribute to the evolution of variation in floral scent.

Floral scent is generally repeatable

Determining whether trait variation has a heritable component is a critical precondition for determining whether a trait can evolve via natural selection. Using a greenhouse common garden experiment to minimize plasticity, we estimated the repeatability, which places an upper limit on narrow-sense heritability, for three univariate measures of floral scent and used hierarchical modeling of species communities (Hmsc) to determine how variance is partitioned among populations, plants, and flowers for each of our 12 scent variables. For total scent emission rate, the number of floral scent compounds present in a sample, and the majority of our scent variables, our estimates of repeatability and variance at the plant level were generally in line with the mean heritabilities reported for different types of floral traits, such as corolla traits (46%), flower number (34%), and reward traits (21%) (Ashman and Majetic, 2006). Similarly, the one study that estimated narrow-sense heritability for floral volatiles found an average heritability of 18% across 13 floral volatiles in

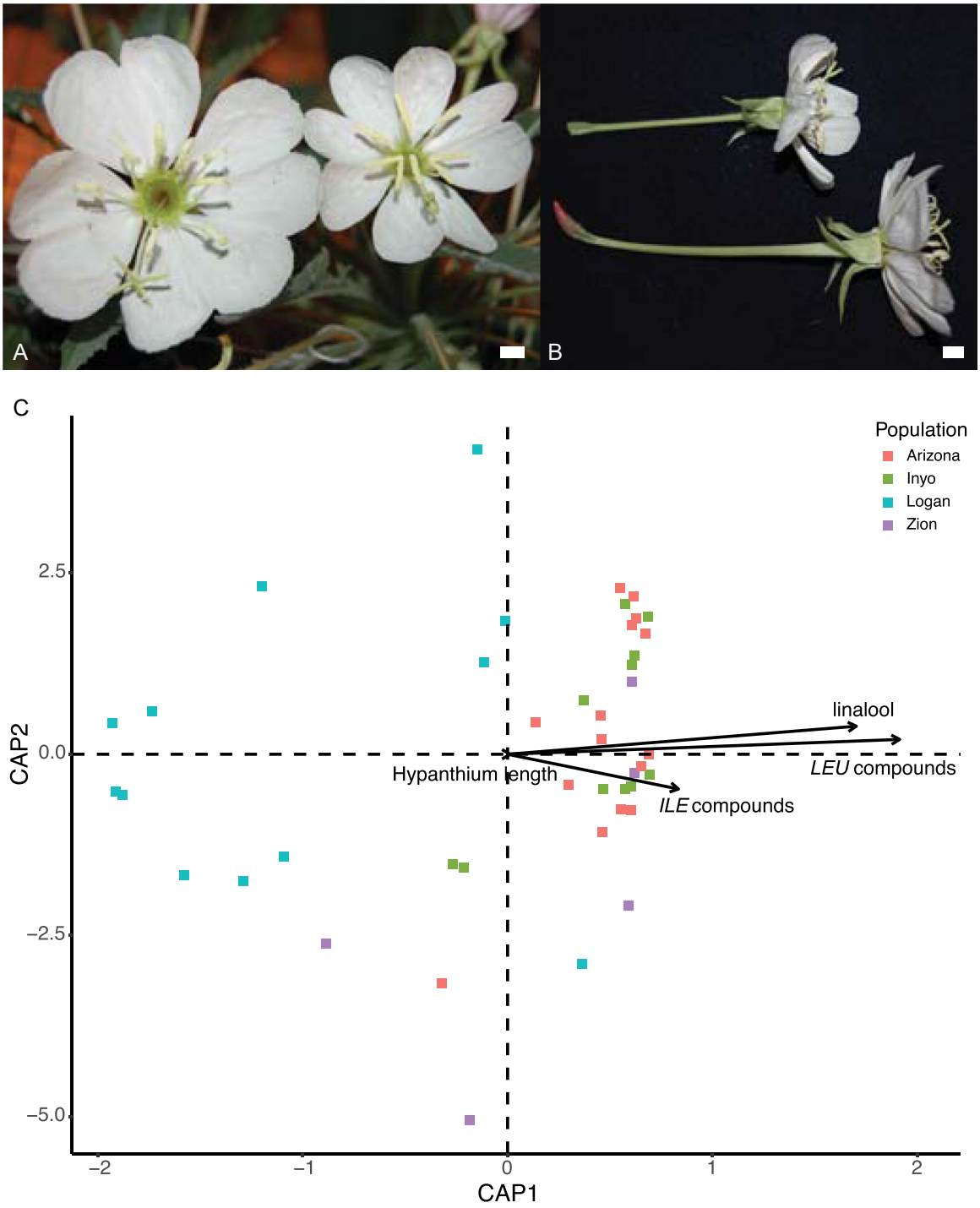

FIGURE 3 Variation in corolla width (A; scale bar = 1 cm) and hypanthium length (B; scale bar = 1 cm; see Appendix S2, Figure S1 for a diagram of specific floral parts; flowers in this image were cut at the ovary and do not include the pedicel), with larger flowers from Inyo, CA and smaller flowers from Logan, UT. Hypanthium length was the only morphological trait, along with three floral scent variables commonly associated with long-tongued hawkmoth pollination, correlated with one or both of the axes of a canonical analysis of principal coordinates (C) separating the populations using the combined scent and morphology data set.

Brassica rapa (Zu et al., 2016), although our results should be interpreted with some caution, given that seeds were pooled from multiple plants and that a greenhouse generation was not used to control for maternal effects. While further work is required to develop a general understanding of the extent of heritable genetic variation in floral scent, our results support the idea that floral scent could evolve in response to natural selection.

For the number of compound groups (the 12 scent variables analyzed in this study) present in a sample, our estimate of repeatability was zero, which likely results from the low amount of variance in this metric of scent diversity

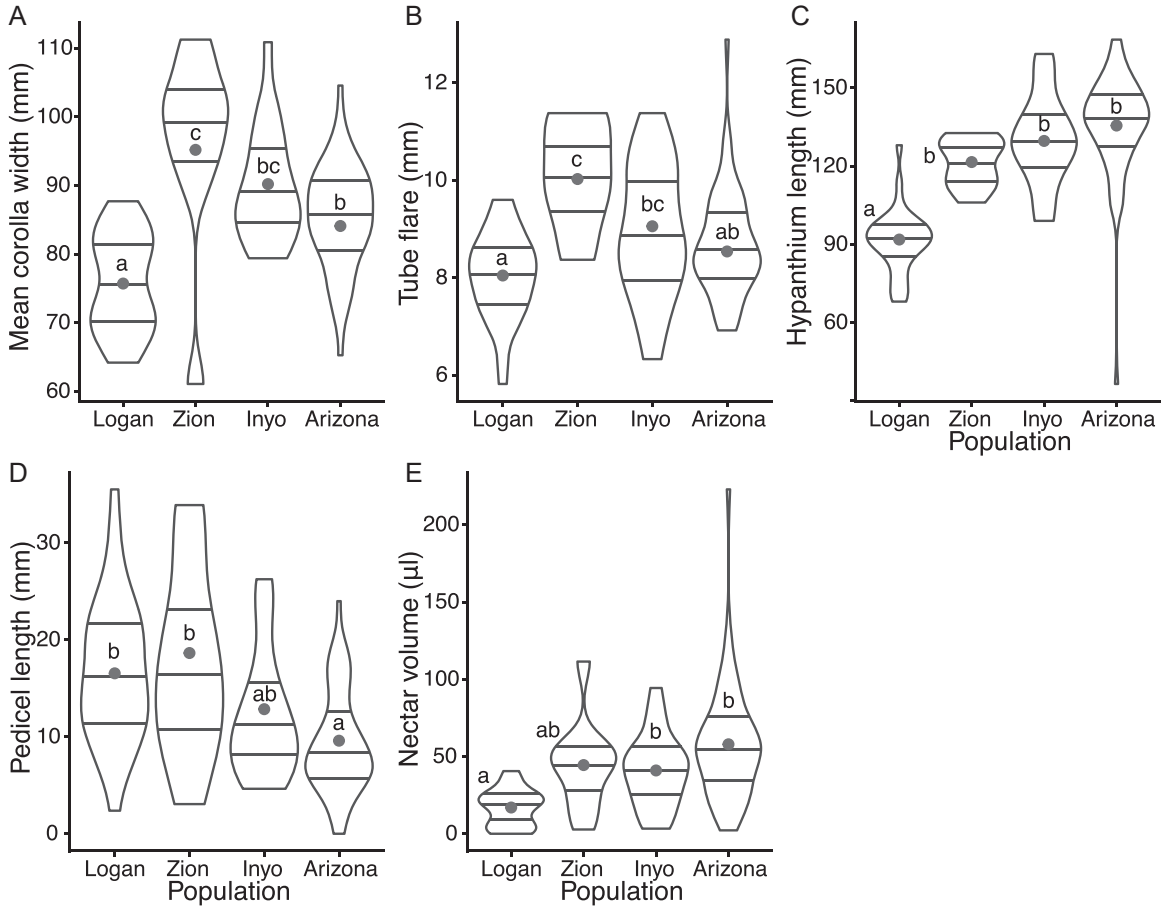

FIGURE 4 Variation in five morphological traits across populations: (A) mean corolla width, (B) tube flare, (C) hypanthium length, (D) pedicel length, and (E) nectar volume. Violin plots represent raw trait data and are scaled to have the same maximum width across populations and horizontal lines on the violins indicate the quartiles for each population. Different lowercase letters indicate differences in the estimated marginal means (dark gray points) between populations.

(mean ± SD: 7.20 ± 1.64 compound groups/sample). However, converting our 40 compounds into 12 scent variables allowed us to analyze the data in a biosynthetically informed way, and reduced the potential for drawing conclusions based on metabolic noise, such as variation in the production of minor products that are an artifact of the abundance of the major product of a given pathway (see Fähnrich et al., 2011; Schiestl et al., 2011). Together, these findings demonstrate the challenges associated with representing scent variation in a way that is analytically tractable, controls for biosynthetic artifacts among major and minor products, and preserves the potentially biologically relevant complexity of multivariate scent phenotypes. These challenges are particularly prevalent in systems where upward of 50 VOCs are produced from a small number of biosynthetic pathways (Chapurlat et al., 2019; Friberg et al., 2019; Gfrerer et al., 2021; Szenteczki et al., 2021), as these scent phenotypes are highly dimensional, but each dimension may not be an independently produced trait (Barkman, 2001; Junker, 2018). Moving forward, approaches that rely on knowledge of biosynthetic pathways (Levin et al., 2003; Pasquet et al., 2017) or use ordinations to find linear combinations of scent variables that explain the greatest amount of variation among groups (Bischoff et al., 2014; Campbell et al., 2019; Eisen et al., 2022) to reduce dimensionality and facilitate analysis will continue to increase our understanding of the functional consequences of multivariate variation in floral scent.

Population differentiation in morphology and rewards and scent

We found that population explained a somewhat larger amount of variation in our data set of morphological and rewards measurements, relative to the data sets of floral scent variables and both types of traits. Our greenhouse common garden reduced environmental variation, including drought, soil differences, natural enemies, and competition that could induce plasticity in floral trait values, including floral scent (Majetic et al., 2009; Burkle and Runyon, 2016; Friberg et al., 2017; Campbell et al., 2019). As such, our results suggest that there may be more genetically based variation in floral scent relative to morphology and rewards traits within populations. There are multiple possible explanations for this pattern. First, by reporting

and analyzing all floral VOC emissions, our approach captures more of the phenotypic space, relative to studies that restrict their analyses to either (1) GC-EAG active compounds that may be particularly likely to be under pollinator-mediated selection (e.g., Knauer and Schiestl, 2015; Chapurlat et al., 2019) or (2) the most abundant compounds (e.g., Schiestl et al., 2011; Cozzolino et al., 2015; Majetic et al., 2015; Zu et al., 2016), which could increase variation in scent profiles. However, analyzing floral scent as composite scent variables based on correlations and knowledge of biosynthetic pathways, rather than single compounds, should subsume some variation that may result from the production of multiple compounds from a single biosynthetic pathway (e.g., major and minor products; Barkman, 2001; Junker, 2018). Second, floral scent and morphology and rewards may be subject to different past or contemporary selective pressures that could result in different amounts of variation within populations. For instance, if floral scent is subject to conflicting or oscillating selective pressure from pollinators and antagonists, as detected in other systems (Kessler et al., 2013; Knauer et al., 2018), these dynamics could maintain more variation in scent relative to morphology or rewards. Given that flower-bud galling moths (*Mompha* species) are frequent visitors to *Oenothera* plants (Jogesh et al., 2017), including other *O. cespitosa* subspecies (Artz et al., 2010), the potential for conflicting selection from multiple agents could be investigated in future studies of this system (see Future directions below).

Variation in scent, morphology, and rewards is consistent with pollination syndromes

Across our four well-sampled populations, we found patterns of trait variation that are consistent with different pollination syndromes. Specifically, plants from the Arizona and Inyo populations tended to have greater emissions of linalool and LEU-derived compounds (Figure 2), and longer hypanthia (Figure 4C), which together are associated with pollination by long-tongued moths (Knudsen and Tollsten, 1993; Anderson et al., 2010). Previous studies have identified that long-tongued (8–13 cm) *Sphinx* and *Manduca* hawkmoth species are key pollinators in southern populations of *O. c. marginata* (Gregory, 1964; Stockhouse, 1976; Hodges, 1987), while Great Basin and prairie populations produce smaller flowers pollinated by short-tongued (4–6 cm) *Sphinx* and *Hyles* hawkmoth species (Wagner et al., 1985; Artz et al., 2010). Our morphological and rewards data likely reflect this shift in pollinators because the Logan population (our well-sampled northern population) had smaller, narrower flowers with less nectar relative to the other populations (Figures 3, 4A). Given that the Logan population also had the largest pedicels, variation in floral size does not seem to be associated with variation in some plant size traits, although overall plant size was not measured as part of this study. While more work is needed to definitively associate this trait variation with variation in the pollinator community and to demonstrate that it results from pollinator-mediated selection (see Future directions below), our data point to the potential for geographic variation in pollinators to shape variation in the floral traits of *O. cespitosa*, which has been observed in some systems with multiple pollinators (Herrera et al., 2006; Gómez et al., 2009; Suinyuy et al., 2015; reviewed by Phillips et al., 2020, but see Svensson et al., 2005).

Implications of trait variation for floral evolution

The patterns of trait variation at different levels of biological organization identified in this study provide insight into the evolutionary dynamics that may be at play in this system. For our floral scent variables, more variance was explained at the plant level (47% on average), relative to the population level (14% on average). This result suggests that there may be a relatively large amount of standing genetic and phenotypic variation for floral scent profiles in this system, which could be adaptive if plants are employing a generalist approach to attracting a diverse array of pollinators in highly variable environments (Burkle and Runyon, 2019). Future studies that more explicitly track scent variation in progeny of different maternal lines are particularly needed to increase our understanding of the heritability of floral scent because these types of studies have been rare to date (e.g., Zu et al., 2016; Friberg et al., 2017). In addition, the relatively smaller amount of variation across populations also suggests that there is a core set of common, shared compounds, despite the potential for geographic variation in selective agents and pressure (see above). This variation in selective pressure may be shaping patterns of variation in the small number of scent variables that were more differentiated across populations: linalool, LEU-derived compounds, and isophytol (Figures 2, 4B). Finally, the presence of the PHE-derived compounds that were only detected in the Logan population could reflect dynamics that are unique to the local environment, such as potential introgression with a nearby population of an edaphically distinct subspecies, *O. c. cespitosa*, as is common in this section of the genus (Wagner et al., 1985).

Future directions

Our study raises multiple avenues for future research that would provide further insight into the dynamics of this system and increase our knowledge of the mechanisms driving variation in floral scent, morphology, and rewards at different geographic scales and levels of biological organization. One potential extension would be finer scale sampling of populations across the subspecies' range, which covers a large portion of the western US. To date, studies that measure intraspecific variation in floral scent in a large

number of populations (e.g., upwards of 10) have been relatively rare (but see Tholl et al., 2005; Sanaa et al., 2012; Friberg et al., 2019). This type of intensive sampling at the population-level would help to disentangle the relative importance of abiotic and biotic environmental variation to observed patterns of floral scent variation, especially when transplants or common gardens are used to supplement data from in situ populations (Majetic et al., 2009; Doubleday et al., 2013). Pairing sampling of floral phenotypes at this level with genetic or genomic data to calculate F_{ST} (fixation index) and Q_{ST} (genetic differentiation of a quantitative trait among populations) on scent, morphological, and rewards traits would also increase our understanding of the extent to which drift, population isolation, and founders' effects contribute to observed patterns of variation. However, previous studies have documented that hawkmoth foraging patterns result in gene flow among metapopulations and populations in closely related taxa (Stockhouse 1976; Skogen et al., 2019), which could mitigate the potential for strong drift. In addition, another experiment that would further our understanding would involve estimating selection on scent in natural populations, which has been done in a handful of systems including *Penstemon* (Parachnowitsch et al., 2012), *Gymnadenia* (Gross et al., 2016; Chapurlat et al., 2019), and *Brassica rapa* (Gervasi and Schiestl, 2017; Knauer and Schiestl, 2017). A pollen supplementation and herbivore manipulation experimental framework (e.g., Sletvold et al., 2015) would provide estimates of selection mediated by pollinators, herbivores, and other agents and conducting this type of experiment in combination with pollinator and herbivore observations would help to determine whether geographic variation in pollinator communities generates variation in patterns of contemporary selection on floral scent.

Finally, studies that take multiple measures of floral scent at the whole-flower level within a plant remain rare (Euler and Baldwin, 1996; Baldwin et al., 1997). Across nine plants for which four flowers were measured from the Arizona population, we observed considerable variation in floral scent profiles of flowers from the same plant (Appendix S2, Figure S6)—for some plants, all flowers formed a tight clump in nonmetric multidimensional scaling (NMDS) space, while the flowers of other plants occupied the full range of observed variation across NMDS axis 1 (see pink and purple ellipses in Appendix S2, Figure S6). However, these data came from a relatively small number of plants from a single population, and more systematic, extensive repeated measurement of scent at this level are needed to test hypotheses about the plasticity of scent in response to the pollination environment. For example, relative to subsequent flowers, the first flower on a plant may produce more scent, to facilitate pollinator attraction, or less scent, if there are steep costs associated with investing in scent in a potentially unfavorable pollination environment; these relationships could also vary with life history attributes including mating system, self-compatibility, and perennialism (Evans et al., 2005; Theiss et al., 2010). It is also as of yet unknown if removing flowers from a plant (as was done here, to enable calculating scent emission rates per floral fresh mass) could alter resource allocation or physiology and thus the size, scent, or nectar of subsequent flowers, or allocation to plant defense. In addition to variation in total emissions, flowers may exhibit qualitative variation in floral scent composition, which could reflect variation in the plastic responses of different biosynthetic pathways to environmental variation. We found that pyranoid linalool oxides and (*E,E*)-α-farnesene were particularly variable among flowers within a plant, and emissions of (*E,E*)-α-farnesene were shown to increase with the severity of experimental drought treatments in a separate study of *Ipomopsis aggregata* (Campbell et al., 2019). Relatedly, it is unknown if scent emissions (along with flower size and nectar standing crop) in new flowers vary with the pollination status of previously open flowers.

CONCLUSIONS

Using a perennial species with a widespread distribution that spans multiple biomes in the western United States, we provide evidence for quantitative and qualitative variation in floral scent at multiple levels of biological organization. The results of our greenhouse common garden suggest that variation in floral scent likely has a heritable component, which would enable it to evolve via natural selection. We found that morphology and rewards contributed more than floral scent or a combination of both types of traits to population differentiation, potentially due to greater within-plant variation in floral scent or differences in selective pressures. Patterns of trait differentiation across populations were consistent with pollination syndromes for the key pollinators that vary across the species' range. Future studies of fine-scale variation in scent across and within plants and populations, in addition to estimating selection in wild populations, would increase our understanding of the evolution of floral scent, a complex trait that mediates many plant–insect interactions.

AUTHOR CONTRIBUTIONS

R.M. and R.A.R. designed the study. R.M. collected the data, with guidance and chemical analysis provided by R.A.R. K.E.E. analyzed the data, with input from R.A.R. K.E.E. led the manuscript writing. All others contributed to editing the manuscript.

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DATA AVAILABILITY STATEMENT

All data and code are available on GitHub (github.com/kate-eisen/Oenothera) and Zenodo (doi:10.5281/zenodo.6572548).

OPEN RESEARCH BADGES

This article has been awarded Open Materials and Open Data badges. All materials and data are publicly accessible via the Open Science Framework at https://doi.org/10.5281/zenodo.5882370. Learn more about the Open Practices badges from the Center for Open Science: https://osf.io/tvyxz/wiki

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1.

Table S1. Identifying information for the five source populations used in the greenhouse common garden.

Table S2. Volatile organic compounds (VOCs) as assigned to scent variables for multivariate statistical analysis.

Table S3. Summary statistics of volatile organic compounds (VOCs) identified from flowers of *Oenothera cespitosa* subsp. *marginata*.

Table S4. Measurements of traits related to floral size, display, rewards, and function from flowers of *Oenothera cespitosa* subsp. *marginata*.

Appendix S2.

Figure S1. Lateral view of a simplified *O. cespitosa* subsp. *marginata* flower, illustrating morphological measurements.

Figure S2. Correlations of length of axillary leaf subtending each flower with hypanthium length in three populations.

Figure S3. Example of the correlation heatmaps used to condense the 40 observed volatile organic compounds into 12 floral scent variables for analysis.

Figure S4. Sample total ion chromatograms showing iterative similarity (upper) or difference (lower) between replicate flowers sampled from the same individual plants of *Oenothera cespitosa* subsp. *marginata*.

Table S5. Variance in scent variables explained by an Hmsc model.

Table S6. Estimated marginal means contrasts for models of total scent emission and number of compounds detected.
Figure S5. Visualizations of three canonical analysis of principal coordinates using floral scent, morphology, and the combined data set.
Table S7. Correlations of floral scent variables with CAP axes 1 and 2.
Table S8. ANOVA outputs of the effect of source population on the six scent variables that were correlated with CAP axis 1 or 2.
Table S9. Estimated marginal means contrasts for models of the three scent variables that varied across populations.
Table S10. Correlations of morphological traits with CAP axes 1 and 2.
Table S11. ANOVA outputs of the effect of source population on the six morphological traits that were correlated with CAP axis 1 or 2.
Table S12. Estimated marginal means contrasts for models of the five morphological traits that varied across populations.
Table S13. Correlations of scent and morphological traits with CAP axes 1 and 2.
Figure S6. Nonmetric multidimensional scaling (NMDS) plot of plants from Arizona where four unique flowers per plant were measured.

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