

Phenotypic selection to increase floral scent emission, but not flower size or colour in bee-pollinated *Penstemon digitalis*

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Summary

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- Fragrance is a putatively important character in the evolution of flowering plants, but natural selection on scent is rarely studied and thus poorly understood. We characterized floral scent composition and emission in a common garden of *Penstemon digitalis* from three nearby source populations.
- We measured phenotypic selection on scent as well as floral traits more frequently examined, such as floral phenology, display size, corolla pigment, and inflorescence height.
- Scent differed among populations in a common garden, underscoring the potential for scent to be shaped by differential selection pressures. Phenotypic selection on flower number and display size was strong. However, selection favoured scent rather than flower size or colour, suggesting that smelling stronger benefits reproductive success in *P. digitalis*. Linalool was a direct target of selection and its high frequency in floral-scent bouquets suggests that further studies of both pollinator- and antagonist-mediated selection on this compound would further our understanding of scent evolution.
- Our results indicate that chemical dimensions of floral display are just as likely as other components to experience selective pressure in a nonspecialized flowering herb. Therefore, studies that integrate visual and chemical floral traits should better reflect the true nature of floral evolutionary ecology.

Introduction

Floral scent is an important but frequently ignored component of the floral phenotype (e.g. Raguso, 2008a). Although volatile organic compound (VOC) emission has been catalogued for many species (e.g. Knudsen *et al.*, 2006) and its ecological roles have been elucidated in some plant–animal interactions (e.g. Dobson, 2006; Raguso, 2008b; Junker *et al.*, 2010; Becklin *et al.*, 2011), basic information on phenotypic variation and natural selection on scent is generally lacking. Scents often are predicted to be shaped by natural selection (Raguso, 2001), but the environment can play a large part in phenotypic differences and may skew the relationship among traits and fitness (Rausher, 1992). Volatile compounds can be particularly sensitive to micro-habitat and climate variables, making it challenging to assess the magnitude and causes of observed differences (Majetic *et al.*, 2009a; Becklin *et al.*, 2011), and their evolutionary consequences (Whitehead & Peakall, 2009). However, integrating scent into the broader framework of floral evolutionary ecology is crucial if we are to reach a more balanced understanding of selection on complex floral phenotypes. For example, several lines of evidence suggest that pollinators can perceive scent in concert with floral colour or form (Spaethe *et al.*, 2007; Leonard *et al.*, 2011). Therefore, studies that measure selection on floral visual display

alone may overlook stronger complementary, redundant or synergistic effects of correlated variation in volatile emissions (Majetic *et al.*, 2009b). However, the scarcity of estimates of natural selection on scents has hindered our understanding of the evolution of VOC function as signals and cues (Allison & Hare, 2009; Galen *et al.*, 2011; Kessler & Heil, 2011; Schiestl *et al.*, 2011), and provides the primary motivation for this study.

We measured phenotypic selection on floral phenotypic variation in *Penstemon digitalis*, using an experimental design that measured chemical and visual aspects of floral display and minimized environmental variation through the use of a common garden. The genus *Penstemon* is diverse, with multiple transitions from bee to hummingbird pollination (Wolfe *et al.*, 2006). The roles of flower colour and morphology (but not scent) have been studied extensively in *Penstemon*, both within species and across pollination syndromes (e.g. Castellanos *et al.*, 2004; Wilson *et al.*, 2004; Thomson & Wilson, 2008). In particular, our previous work provides evidence for pollinator-mediated selection to increase flower size and display in *P. digitalis* (Parachnowitsch & Kessler, 2010). To our noses, *P. digitalis* does not have a strong or distinctive smell; therefore we had no *a priori* expectations of its potential role in this system. However, even scents that are barely perceptible to humans (e.g. pollen odours) can be important in pollinator attraction (e.g. Ashman *et al.*, 2005; Cook

et al., 2005). Therefore, we measured scent, floral morphology, corolla colour and life history traits in plants from three populations of bee-pollinated *P. digitalis*. Specifically, we asked:

- (1) What is the phenotypic variation of volatiles?
- (2) Is there phenotypic selection on scent?
- (3) How does selection on scent compare with more commonly measured components of the floral phenotype such as morphology, colour, phenology and display?

Materials and Methods

Study system

Penstemon digitalis Nutt. ex Sims (Plantaginaceae) is a native North American wild flower found in meadows and prairies (synonym: *Penstemon laevigatus* subsp. *digitalis* (Nutt. ex Sims) Benn.). *Penstemon digitalis* corollas are white or have purple striping within the throat of the corolla tube. The purple colour is probably attributable to delphinidin-based anthocyanin (Scogin & Freeman, 1987) and appears black under UV light, suggesting that it may act as a nectar guide for pollinators (Silberglied, 1979). Glandular trichomes cover the flowers and flowering stems (but not leaves); however, they may do little to protect plants from seed predation (Thomas, 2003). Flowers are protandrous and self-compatible (Zorn-Arnold & Howe, 2007); however, bagged flowers fail to reproduce, suggesting that pollinators are necessary for seed set. Bees, ranging from small to large-bodied, are the main pollinators/visitors of *P. digitalis* flowers (Clinebell & Bernhardt, 1998; Mitchell & Ankeny, 2001; Dieringer & Cabrera, 2002). In our study area (central NY State), *Bombus* spp. appear to be the primary floral visitors (> 50%; A. L. Parachnowitsch, pers. obs.). Both micro-lepidopterans and dipterans are known pre-dispersal seed predators of this species (Mitchell & Ankeny, 2001; Thomas, 2003), but at our field sites, fruits are attacked primarily by an unidentified micro-lepidopteran (Parachnowitsch & Kessler, 2010).

Field experiment

We planted a common-garden population just before flowering (3 June) in 2007 to increase our power to detect selection on all traits by potentially extending phenotypic trait variation (discussed in Agrawal, 2011). The common garden also facilitated scent sampling by placing physically dispersed phenotypes in one location (see Supporting Information Notes S1 for further details) and increased our ability to detect phenotypic variation by reducing the confounding impact of micro-environmental factors such as local temperature and humidity on variation in scent. Plants were chosen from three source populations (NR, WF, TH; see Table 1 for details) known to differ in morphological floral traits (Parachnowitsch, 2011) that were located in Tompkins County, NY, USA in old fields (maximal distance between populations is < 10 km). The garden was located in a managed field ($42^{\circ}29'249''\text{N}$ $77^{\circ}25'671''\text{W}$) where above-ground vegetation had been removed by mowing and herbicide application. It was separated from the nearest *P. digitalis*

Table 1 *Penstemon digitalis* source population locations, the predictor volatiles that distinguish each population when grown in a common garden from the other two and their frequency in the Random Forest models

Population	Location	Predictor volatiles	Model frequency
NR (Neimi Rd)	$42^{\circ}30'092''\text{N}$	α -farnesene	0.840
	$-76^{\circ}26'204''\text{W}$	β -cedrene	0.810
		Unknown-4	0.870
TH (Turkey Hill Rd)	$42^{\circ}26'428''\text{N}$	<i>trans</i> -2-octenal	1.000
	$-76^{\circ}25'743''\text{W}$	<i>cis</i> - β -ocimene	0.690
WF (Whipple Farm)	$42^{\circ}26'436''\text{N}$	<i>trans</i> -2-octenal	0.775
	$-76^{\circ}25'892''\text{W}$	α -farnesene	0.555

population (WF) by abandoned agricultural ‘old’ fields dominated by *Solidago* spp. and a c. 25-m-wide tree line. Although *P. digitalis* plants are perennial, observations from these populations suggest that survival after flowering is rare and individuals may more commonly be semelparous. Plants were randomly chosen for transplanting from across each population just before flowering. The bolting plants were growing at least 1 m apart to ensure that they represented separate genets. Thus, the common garden controls for environmental effects on traits post flower initiation but does not eliminate differences accumulated before flowering. In the garden, plants were arranged in a randomized complete block design with 35 blocks consisting of one plant from each of the three populations. Plants were arranged in seven rows (15 plants per row) with spacing of 0.5 m between plants and 1 m between rows. Plants flowered for c. 7 wk and were visited by naturally recruited bumblebees (*Bombus* spp., generally *Bombus impatiens*) and other small-bodied bees, as well as the occasional hummingbird (*Archilochus colubris*). Plant mortality, failure to flower and missing data led to a final sample size of 88 plants (NR = 30, TH = 26 and WF = 32).

Plant volatile collections, quantification and identification We collected VOCs from the entire inflorescence using an open-flow dynamic headspace trapping design described in Kessler & Baldwin (2001). Collections were taken over 4 d (21–24 June) and on the final day, we re-sampled six plants (two from each previous collection date) to assess the repeatability of volatile emission. For the six plants with two VOC collections, mean volatile production was used in the analyses. Further details of collections, quantification and the identification of volatiles can be found in Notes S1.

Morphological, visual and life history traits For at least three flowers per plant, six morphological dimensions of the tubular flower were measured (the width and length of the centre lower petal, the width and length of the bell of the corolla tube, the full length of the tube, and the width of the constriction floral tube around the ovary) as in Parachnowitsch & Kessler (2010), and reduced to a single floral size measure by a geometric mean. To quantify corolla pigment, a single observer counted the number of lines and scored their intensity on a four-point scale (0, no colour; 1, light; 2, medium; 3, dark purple) on flowers measured

for size. The corolla pigment presented is the mean of line number \times intensity.

We checked plants every 2–3 d to assess first flower date, mean daily display size and days flowering. When the fruits had developed (mid-August), we measured plant height, counted total flower number, fruit number and pre-dispersal seed predation. We measured the fruit diameter (mm) of all fruits and scored them as damaged or not. Female fitness was estimated as the diameter of undamaged fruits plus one half the diameter of damaged fruits. Previous work demonstrated that fruit diameter is correlated with seed number and, on average, pre-dispersal seed predators consume half of the seeds of undamaged fruits (Parachnowitsch & Kessler, 2010). Although damage varied among plants, it was not generally related to the traits we measured (data not shown). Therefore, for simplicity, we did not include damage in our analyses.

Scent variation in wild populations

To assess whether scent variation was found among plants growing in the naturally occurring populations, we sampled 14 plants per population during peak flowering in 2010. Plants were haphazardly chosen within the confines of the sampling apparatus tubing at least 1 m apart. Each population was sampled once on a sunny day (NR and WF on 21 June; TH on 23 June), making variance attributable to population differences confounded with variance attributable to day. In addition to volatile collections, the numbers of open flowers/plant were counted. Volatile samples were collected and processed as described in Notes S1.

Statistical analyses

Population variation To determine population variation in our phenotypic traits (flower size, corolla pigment, flowering phenology, display size, plant height and scent) or fitness correlates (damage, aborted fruits, seed production, and fruit number and size) we used MANOVAs followed by ANOVAs with block included as a random factor. For these models, scent was reduced to a single measure (total scent) by summing standardized peak areas of all compounds for each individual. Here (and elsewhere), we used Wilk's lambda to assign significance to MANOVA models and the Ryan, Elinot, Gabriel, Welsh's post hoc test to determine population differences for each trait.

To specifically assess whether scent production was influenced by population origin in the common garden, we used two further approaches. First, because important variation may be lost in a composite trait such as total scent (Mant *et al.*, 2005), we used a MANOVA including all 23 compounds followed by ANOVAs of individual scents. We used both total emission from the inflorescence and a per-flower estimate of scent production (divided each scent compound by the number of open flowers). Models included population, block and measurement date as independent variables (random factors). Secondly, we visualized population differences using the Random Forests classification algorithm (RF) described in Ranganathan & Borges (2010), because these kinds of algorithm are especially suited for

examining differences in complex traits such as volatile blends. RF provides a minimum set of predictor volatiles necessary to distinguish each population from the others and a ranking of the relative importance of each volatile for population differentiation. We ran three separate analyses with two categories: (1) population of interest, and (2) the two remaining populations classified as 'others'. We used 200 bootstrap iterations for each analysis (Ranganathan & Borges, 2010) with the package *VarSelRF* in the statistical software program R (R Development Core Team, 2009). The mean decrease in accuracy for removal from the models was calculated for each VOC and the probability of a plant belonging to a group was calculated using the average 'out of the bag' probability of membership (Ranganathan & Borges, 2010).

To examine differences in floral VOC emission among the three wild population collections we used the same methodology (MANOVA followed by ANOVAs and RF). However, unlike the common-garden comparisons, we were unable to block by a spatial or temporal factor in these models because all plants within a population were measured on the same day.

Correlations To determine correlations among phenotypic traits, we calculated Pearson correlations followed by the Dunn–Šidák correction for multiple comparisons for the eight traits (flower size, corolla pigment, day of first flower, days flowering, daily display, height, flower number and total scent). Because floral pigments and other chemical compounds can be related through shared pathways (Rausher, 2008) and visual and volatile cues can be perceived by pollinators in an integrated fashion (e.g. Raguso and Willis, 2005), we also calculated correlations between pigment and the 23 scents. However, none of the correlations was significant after controlling for multiple comparisons and pigment was also not correlated with total scent ($r = 0.110$; $P = 0.31$). Therefore, we only present correlations between pigments and the scents under selection (see the Results section).

Phenotypic selection To measure phenotypic selection on *P. digitalis* phenotypic traits we used univariate generalized linear models (version 9.2; SAS Institute Inc., Cary, NC, USA) for all traits where selection differentials (S) are estimated as the covariance between relative fitness and the traits. S measured the strength and direction of total phenotypic selection on plant volatiles ($N = 23$ volatiles), flower size, corolla pigment, day of first flower, days flowering, daily display size, final height, and total flower number. For these analyses, relative female fitness was relativized (individual fitness/population mean) and each trait standardized to Z-scores (mean of 0, and variance of 1). However, S includes direct selection on a trait as well as selection via correlated characters. Thus, to measure direct selection (targets), multivariate models were used to control for trait correlations (Lande & Arnold, 1983). Because of the number of traits (30), we did not have the power to detect direct selection (selection gradients, β) using a single multivariate model. Therefore, we reduced scent variables in two ways: (1) using total scent, and (2) including only those traits with

significant S (see the Results section). We included *trans*- β -ocimene in these models because its differential was nearly significant ($P = 0.051$). For all scent variables we used emission from the inflorescence rather than a per-flower measure because it is likely that the entire bouquet was perceived by visiting insects, and because selection on per-flower emission was not significant (not shown). All phenotypic selection models included population identity and block as factors; however, values for selection on these factors are not shown.

Mean-standardized selection gradients have been suggested as a useful alternative to quantify selection (Hereford *et al.*, 2004). However, our results show a similar pattern whether we use mean or variance-standardized traits (not shown). Because the majority of selection coefficients reported are variance-standardized (e.g. Harder & Johnson, 2009; Siepielski *et al.*, 2009), we present these data to compare selection on scents with more commonly measured traits.

Results

Population variation

In the common garden, we found that *P. digitalis* populations differed when considering all traits (MANOVA: $F_{16,54} = 4.87$; $P < 0.0001$) or fitness correlates (MANOVA: $F_{10,60} = 3.99$;

$P = 0.0003$). Specifically, flower size, corolla pigment, first flower date, days flowering and seed predator damage differed among populations (Table S1). Conversely, there were no population differences in total scent, daily display, total flower number, plant height, fruit number or size, and female fitness (Table S1).

Despite lack of population differentiation in total scent (sum of peak areas), when we used the individual compounds, we found overall population differences in scent emission (MANOVA: $F_{46,24} = 3.03$; $P = 0.0023$). These differences remained when we standardized emissions by flower number ($F_{46,24} = 2.31$; $P = 0.015$). Moreover, populations did not differ in open flower number on sampling days ($F_{2,87} = 1.99$; $P = 0.15$) or total flower number over the season (Table S1; $P = 0.35$), suggesting that the differences in scent were not simply driven by variation in flower production. Over half (16/23) of the volatiles differed significantly among populations (Table 2); however, the minimum VOCs needed to distinguish the populations ranged from two to three in the RF analyses (Table 1). Increased production of α -farnesene, β -cedrene and an unidentified compound (unknown-4) distinguished population NR, whereas population WF was separated by reduced scent of two volatiles (*trans*-2-octenal and α -farnesene). Conversely, population TH differed from the others by producing more *trans*-2-octenal but less *cis*- β -ocimene. The mean decrease in accuracy for removing compounds from the analyses is presented in Table S2. Based on 'out

Table 2 Volatile compounds, retention times (RTs), mean peak areas and percentage of the headspace (%) from *Penstemon digitalis* quantified with GC-MS

Compound class	Name	RT	Peak area \pm SE	%	Population variation	$F_{2,87}$
Monoterpenes	<i>cis</i>-β-ocimene	10.25	0.081 \pm 0.010	1.25	NR = WF > TH	4.75*
	<i>trans</i>-β-ocimene	10.58	0.355 \pm 0.063	5.46		2.62
	Linalool	15.26	0.434 \pm 0.068	6.68		2.16
	β-citronellol	18.41	0.130 \pm 0.020	2.01		2.50
Sesquiterpenes	<i>cis</i>-jasmone	21.80	0.012 \pm 0.002	0.18	NR > TH = WF	3.48*
	α-copaene	14.53	0.081 \pm 0.010	1.25		6.24**
	α-cedrene	15.58	0.094 \pm 0.017	1.45		4.67*
	Bergamotene	15.83	0.381 \pm 0.061	5.87		0.64
	β-cedrene	16.03	0.137 \pm 0.032	2.11		14.16****
	Caryophyllene¹	16.15	0.164 \pm 0.039	2.54		12.39****
	Germacrene D¹	17.76	0.239 \pm 0.044	3.68		2.84*
	α-farnesene	18.03	0.094 \pm 0.018	1.44		18.16****
	Thujol¹	19.62	0.090 \pm 0.012	1.39		5.82**
Aromatics	Nerolidol	23.39	0.039 \pm 0.008	0.60	NR > WF = TH	13.12****
	Methyl benzoate	16.50	0.132 \pm 0.045	2.03		4.55*
	Methyl salicylate	19.04	0.221 \pm 0.067	3.41		7.72**
	Methyl cinnamate	24.10	0.175 \pm 0.036	2.70		2.53
Fatty acid derivatives	<i>trans</i>-2-octenal	13.58	0.597 \pm 0.086	9.19	TH > WF = NR	13.15****
	<i>cis</i>-6-nonenal	13.87	0.510 \pm 0.101	7.85		0.98
Unidentified	Unknown-1	14.45	1.543 \pm 0.779	23.74	NR = TH = WF	1.08
	Unknown-2	11.61	0.804 \pm 0.133	12.36		3.43*
	Unknown-3	22.65	0.100 \pm 0.019	1.54		3.61*
	Unknown-4	24.26	0.083 \pm 0.015	1.27		1.24*

Floral-specific volatiles are in italics and compounds under selection are in bold (see Fig. 2). Corolla and/or other reproductive tissue probably produce the floral-specific volatiles and unknown-1, *cis*-6-nonenal and *trans*-2-octenal are emitted from the sticky trichome exudates based on SPME (solid-phase microextraction). Population variation was determined with ANOVAs followed by Ryan, Elinot, Gabriel, Welsh's post hoc tests comparing plants from three populations flowering in a common garden. Compound quantities are expressed as standardized peak areas (relative to tetralin as internal standard).

$N = 88$.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

¹Identified by library search rather than direct comparison.

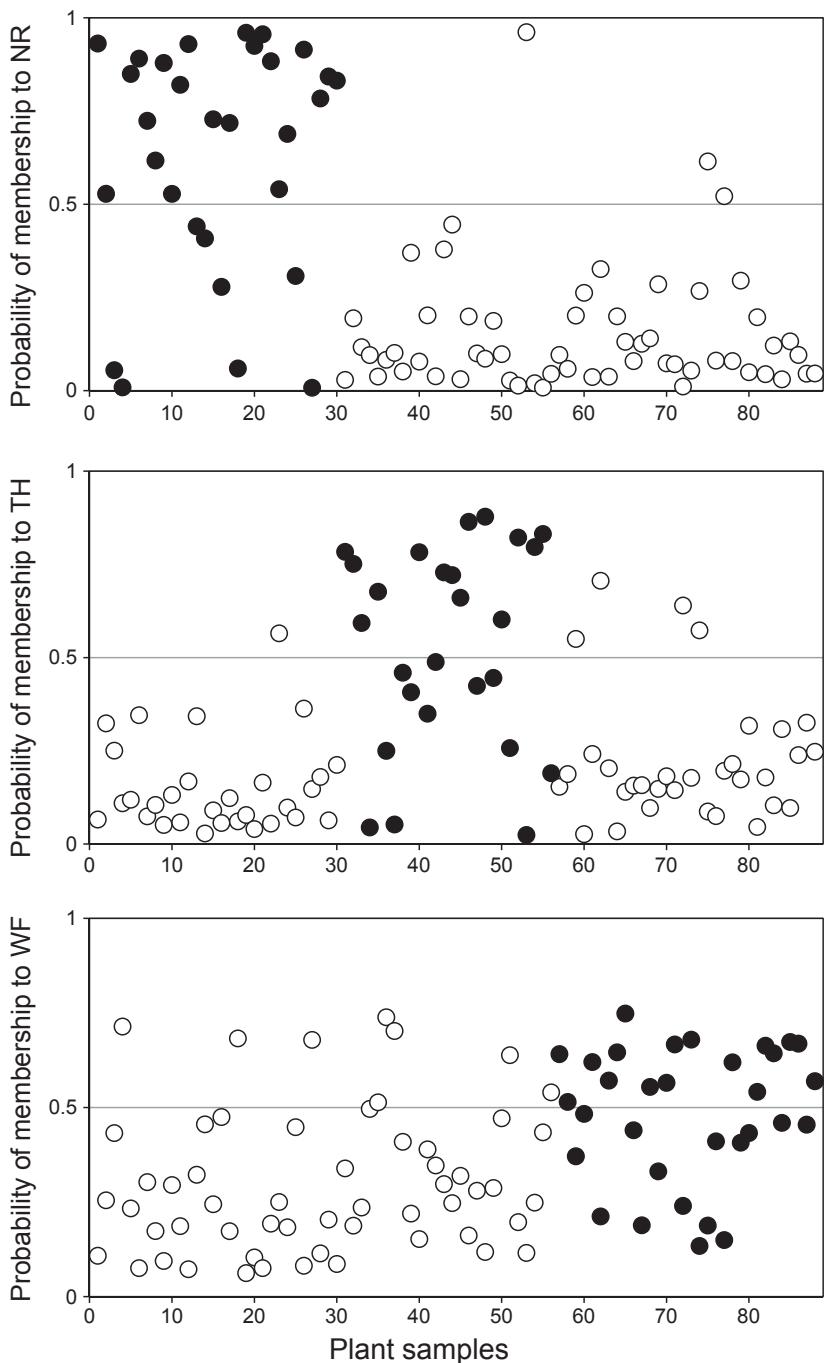


Fig. 1 Comparisons of volatile emissions from three populations of *Penstemon digitalis* grown in a common garden using the 'out of the bag' probability of membership. Each population was compared with the other two (classified as the same) in a separate analysis: (a) NR, (b) TH and (c) WF (see Table 1 for locations). Each point represents a single plant sample; complete separation of populations would have all samples from each population falling on opposite sides of the 0.5 line. Closed circles are the population being compared to the 'others' (open circles). Each individual plant occupies the same position on all three graphs.

of the bag' probabilities, not all individuals from a population grouped together, suggesting an expected overlap in scent profiles in the three populations (Fig. 1). However, NR and TH appear to be more distinct, whereas WF was not strongly separated from the other two.

In situ volatile sampling of the three populations showed a similar trend of population differentiation to the common garden. Populations differed based on MANOVA ($F_{40,40} = 1.95$; $P = 0.019$) and RF separated the populations (Table S3). However, no individual comparison of compounds revealed significant differences (data not shown) and two compounds found in

the common garden (methyl benzoate and β -cedrene) were not emitted in detectable amounts in these plants.

Phenotypic selection

We found phenotypic selection on floral display, scent and phenology in *P. digitalis*. There were significant differentials (S) for an earlier flowering date, with larger daily displays. There was also significant selection on size; taller plants and those with greater total flower number had higher relative fitness (Table 3). Additionally, total scent was under significant positive selection

Table 3 Phenotypic selection on phenotypic traits of *Penstemon digitalis*

Trait	$S \pm SE$	$\beta \pm SE$
Flower size	0.11 ± 0.11	0.01 ± 0.04
Corolla pigment	-0.02 ± 0.09	-0.02 ± 0.03
First flower date	-0.27 ± 0.08	-0.05 ± 0.03
Daily display	0.48 ± 0.05	0.26 ± 0.05
Days flowering	0.10 ± 0.08	-0.002 ± 0.04
Plant height	0.22 ± 0.08	0.008 ± 0.03
Flower number	0.52 ± 0.05	0.28 ± 0.05
Total scent	0.35 ± 0.06	0.10 ± 0.03

Selection differentials (S) and gradients (β , targets of selection) are presented for eight traits; values in bold are statistically significant ($P < 0.05$). Coefficients are from models that include both population identity and block as factors ($N = 88$). Detailed descriptions of the traits can be found in the Materials and Methods section.

(Table 3) and specifically there were significant differentials to increased emissions of eight floral volatiles: linalool (absolute configuration unknown), both *cis*- and *trans*- β -ocimene (marginally significant; $P = 0.051$), β -citronellol, *cis*-6-nonenal, α -copaene, *trans*-2-octenal and the unknown-1 (Fig. 2). Conversely, we found no direct selection on flower size or corolla pigment ($P_s > 0.30$; Table 3) and this was also true when individual flower size measures or colour estimates were used (not shown). When we controlled for correlations among traits in a multivariate regression model to measure the direct targets of selection (gradients, β), we found similar targets whether we used total scent or the volatiles with significant differentials in the model. Floral display (total flower number and daily display; Tables 3,S4) and scent (linalool; Fig. 2) were direct targets, suggesting that total selection on the other traits was driven by correlations with these three traits (Tables S5,S6).

Discussion

Three geographically close populations of *P. digitalis* differed consistently in floral scent composition, either when flowering in a common garden (Fig. 1) or when sampled in their source populations. Furthermore, because some scent compounds showed different patterns among the three populations (Table 2), this affected both overall scent emissions and also the ratios

among blend components. Considering the body of published work on honeybee olfactory discrimination, these plants have the potential to be perceived as smelling differently by visiting insects (Smith *et al.*, 2006; Wright & Schiestl, 2009). Other studies have documented spatial variation in scents (Knudsen, 2002; Dötterl & Jürgens, 2005; Salzmann *et al.*, 2007; Chess *et al.*, 2008; Majetic *et al.*, 2008; Schlumpberger & Raguso, 2008; Soler *et al.*, 2011) but this is generally measured over much larger geographic ranges than the *P. digitalis* populations that we sampled (but see Salzmann *et al.*, 2007). Furthermore, these *in situ* sampling designs are limited in their ability to control for microclimate differences between populations. Until recently, floral scent was assumed not to vary substantially within or between populations (Raguso, 2008b), or to be selectively neutral when it does, as shown in an early study of fitness on *Tolumnia* orchids (Ackerman *et al.*, 1997). For *P. digitalis*, the pollinators and seed predators present in the three wild populations are the same (A. L. Parachnowitsch, pers. obs.). Therefore, gross differences in insect community are unlikely to explain the population variation we found (as in Ellis & Johnson, 2009).

We found significant phenotypic selection to increase floral scent in a common garden of *P. digitalis* (Fig. 2). Given the potential importance of scents to pollinators and other floral visitors (Raguso, 2009), significant selection on these traits is perhaps not surprising. Scent variation can be related to fitness in other systems (see Miyake & Yafuso, 2003), suggesting that selection could be acting on this trait. Additionally, experimentally increasing a pollinator-deterring scent resulted in intensified selection on other floral characters in *Polemonium viscosum* (Polemoniaceae; Galen *et al.*, 2011), indicating that selection on scent could act in an integrated fashion with other floral traits. The single other study to measure Lande–Arnold selection gradients on floral scents showed that selection on compounds detected by antennae of the pollinators of *Gymnadenia odoratissima* (Orchidaceae) was positive whereas selection acted to reduce undetected scents (Schiestl *et al.*, 2011). In our study, the volatiles under selection are common floral scents (Knudsen *et al.*, 2006) and many bees perceive them (Dötterl & Vereeken, 2010), suggesting that selection to increase detected volatiles may be common. Signal detection is a necessary condition for direct pollinator-mediated selection; however, pollinator-mediated selection on volatiles has yet to be tested for any plant species.

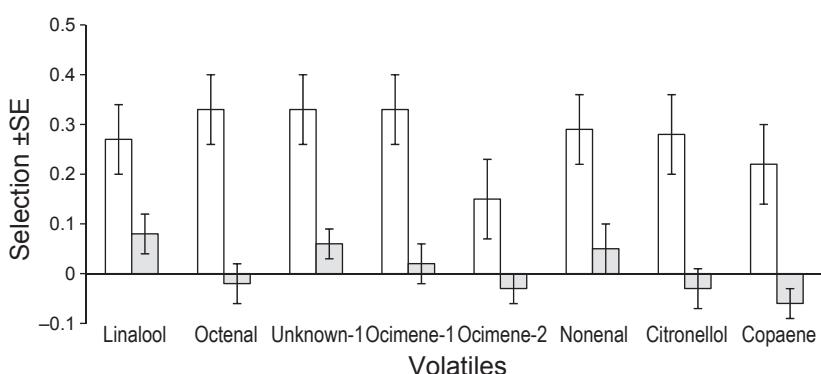


Fig. 2 Phenotypic selection on floral volatiles of *Penstemon digitalis*. Only those volatiles with statistically significant differentials ($P < 0.05$) are shown ($P = 0.051$) with the inclusion of *trans*- β -ocimene ($P = 0.051$). Selection gradients (grey bars) are from a model that included all traits with significant differentials (these eight VOCs plus first flower date, daily display, flower number and plant height). Ocimene-1, *cis*- β -ocimene; ocimene-2, *trans*- β -ocimene. Only linalool had a gradient significantly different from zero. Coefficients are from models that include both population identity and block as factors ($N = 88$).

Linalool was a direct target of selection in *P. digitalis* (Fig. 2). Furthermore, correlations with linalool probably drive total selection on the five other compounds with significant differentials (Table S5), suggesting that direct selection on linalool will lead to an overall increase in scent production. Linalool (in either or both of its enantiomeric forms) is one of the more common components of floral volatile blends and is present in c. 70% of characterized angiosperm families (Knudsen *et al.*, 2006). Thus, it is likely to have diverse context-dependent roles, such as pollinator attraction (Raguso & Pichersky, 1999) and antagonist repellence (e.g. nectar-robbing ants Junker & Blüthgen, 2008). However, we do not know the effects of linalool on pollinator behaviour for *P. digitalis* and found no relationship between seed predation and linalool emissions (not shown). It is important to note that the chirality of linalool may affect both pollinator and herbivore behaviour (e.g. Reisenman *et al.*, 2010) and we have not yet determined the absolute configuration of the linalool volatiles emitted by *P. digitalis* flowers.

Phenotypic selection on floral scents could contribute to or constrain population variation in *P. digitalis*. For example, compounds tied to functions such as pollinator attraction or herbivore repellence may not vary among populations with similar insect communities, while other compounds may be free to vary either by differential selection or by random processes such as genetic drift (Azuma *et al.*, 2001; Raguso, 2008b). In *P. digitalis*, generally floral volatiles under phenotypic selection did not vary among populations (Tables 1,2,S3), suggesting a possible functional role, especially for the target of selection, linalool. However, experiments measuring pollinator- or seed predator-mediated selection in different populations are necessary to elucidate the role of plant–insect interactions in shaping floral scent and the processes that are probably responsible for population differentiation.

Because floral colour and shape are important for pollinator transitions in the genus *Penstemon* (Wilson *et al.*, 2004) and we previously found pollinator-mediated selection on floral size and display in this species (Parachnowitsch & Kessler, 2010), one might hypothesize that selection on these traits would be common. Yet, unlike our earlier findings (Parachnowitsch & Kessler, 2010), there was no selection to increase flower size in the common garden. Moreover, we found stronger selection on floral scents than on more commonly measured visual aspects of *Penstemon* flowers, such as corolla size and colour. The selection gradients on either total scent or on linalool were also stronger than selection on flower size detected previously in a wild population (TH; Parachnowitsch & Kessler, 2010). Selection on scent was not as strong as that on plant-level reproductive traits such as daily display size and total flower number (Table 2). However, significant selection on flower number and display size is frequent in nature (Harder & Johnson, 2009) and thus strong selection on these traits in *P. digitalis* was not surprising. If selection on scent is commonly stronger than that on other corolla characters, then these data highlight the potential importance of volatiles in floral evolutionary ecology.

Although progress in chemical analytical methodology has allowed for greater ease of VOC characterization (Tholl *et al.*,

2006), relevant biological and statistical interpretation is lagging. The difficulty arises from the number of compounds that can comprise a volatile blend, not all of which may have biological relevance. Statistical techniques have been applied to reduce scent variables and account for inter-correlations (van Dam & Poppy, 2008); however, these ordination/informatic techniques do not provide a simple solution for studying phenotypic selection. For example, phenotypic selection on *P. digitalis* principal components (as in Schiestl *et al.*, 2011) generally supported our conclusions but did not allow determination of the targets of selection because multiple traits loaded on a single component (Notes S2). Furthermore, these statistics have the disadvantage of reducing the comparability among studies; the majority of selection coefficients on floral characters are differentials and gradients of individual traits (reviewed in Harder & Johnson, 2009). Therefore, we suggest first measuring selection differentials on all compounds to estimate total selection and then using these differentials and correlations among traits to inform further analyses. Differentials can be dissatisfying because one cannot determine the targets of selection (Lande & Arnold, 1983; Brodie *et al.*, 1995) and the chances of falsely detecting significance increase with complex volatile blends. Measuring selection gradients will circumvent this problem to some extent. However, for most volatile blends, some form of data reduction will be necessary to have power to run these multivariate models. Thus, depending on the complexity of the blend, the nature of total phenotypic selection and the correlation matrix, the most appropriate data reduction should be applied. Models that include a subset of the traits may be useful (as in our study) or some form of summary statistics such as total scent, although the ‘signal’ and ‘noise’ components of scent can be lost in a composite trait (see Mant *et al.*, 2005). Alternatively, when authors have a biologically relevant hypothesis (Scheiner *et al.*, 2000), path analyses maybe a useful tool to determine selection (such as between detected and nondetected VOCs; Schiestl *et al.*, 2011). Finally, to improve our ability to assess selection on floral scents in an integrated framework, we suggest studies on the evolutionary ecology of floral scents also include at least one other measure of the floral phenotype such as flower/display size, colour and reward.

Our study of *P. digitalis* shows that floral scents can be highly variable and an important trait in the microevolution of floral characters, even in a relatively weakly scented species. The next steps are to determine the agents of selection on scent and how phenotypic selection might vary in natural populations. In particular, the influence of pollinators is hypothesized to be important in scent evolution and, despite the lack of evidence of selection by seed predators in this study, antagonists may also play a role in *P. digitalis* scent evolution. In general, more examples of natural selection on scents are necessary to evaluate the relative role of scents in a natural-selection framework.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Population difference in floral, plant and reproductive traits of three populations of *Penstemon digitalis* flowering in a common garden

Table S2 The mean decrease in accuracy produced by removing a particular compound from the three Random Forest models comparing each population to a pooled category of the other two populations

Table S3 Predictor volatiles that distinguish each population of *Penstemon digitalis* from the other two based on local collections

Table S4 Complete set of phenotypic selection coefficients of *Penstemon digitalis* floral traits

Table S5 Pearson correlation coefficients among the phenotypic traits of *Penstemon digitalis*

Table S6 Pearson correlation coefficients among the seven volatiles under phenotypic selection in *Penstemon digitalis* as well as floral pigmentation and total scent

Notes S1 Detailed methodology of volatile collection, analyses and variation.

Notes S2 Phenotypic selection analyses using principal components.

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