

# Natural selection on floral volatile production in *Penstemon digitalis*

## Highlighting the role of linalool

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**Keywords:** corolla color, floral scent, flower size, linalool, phenotypic selection, *Penstemon digitalis*, pollination

Natural selection is thought to have shaped the evolution of floral scent; however, unlike other floral characters, we have a rudimentary knowledge of how phenotypic selection acts on scent. We found that floral scent was under stronger selection than corolla traits such as flower size and flower color in weakly scented *Penstemon digitalis*. Our results suggest that to understand evolution in floral phenotypes, including scent in floral selection, studies are crucial. For *P. digitalis*, linalool was the direct target of selection in the scent bouquet. Therefore, we determined the enantiomeric configuration of linalool because interacting insects may perceive the enantiomers differentially. We found that *P. digitalis* produces only (S)-(+)-linalool and, more interestingly, it is also taken up into the nectar. Because the nectar is scented and flavored with (S)-(+)-linalool, it may be an important cue for pollinators visiting *P. digitalis* flowers.

### Phenotypic Selection on Floral Scent

In our recent paper “Phenotypic selection to increase floral scent emission, but not flower size or color in bee-pollinated *Penstemon digitalis*” in *New Phytologist*,<sup>1</sup> we found scent was under stronger selection than more commonly measured visual components of a floral display (Fig. 1). Scents are an important signal in plant-insect communication and therefore are expected to be under pollinator and antagonist-mediated selection.<sup>2,3</sup> Animal-mediated selection on scent is predicted to be balancing due to conflicting selection pressures, if scents attract both mutualists and antagonists<sup>4</sup> but directional if a scent simultaneously attracts pollinators and repels enemies.<sup>5</sup> Of course, scent blends are generally composed of many volatiles and, thus, it is possible that different components of the blend will be under different forms of selection.<sup>6</sup> However, due to the difficulty of conducting large-scale scent collections in the field, as well as the necessity of specialized equipment and expertise, our knowledge of the evolutionary ecology of scents is elementary. Therefore, incorporating floral chemistry into studies that include floral color, size and density of display should provide unbiased measurements of the relative importance of scent, particularly in systems in which it is not a conspicuous trait. Accordingly, we chose a weakly scented North American species of *Penstemon* (Plantaginaceae), a well-studied genus in pollination research, for which we had no strong expectations for scent to be a dominant floral character. We measured phenotypic selection on scents as well as other floral traits

such as flower size, corolla color, display size and phenology in plants from three local populations of *P. digitalis* in New York. Although natural selection has been estimated for floral traits in many systems, the bulk of the research has been conducted on visual cues, mechanical fit traits and floral phenology.<sup>7</sup> Thus, it is not only interesting and informative to measure selection on scents but it is also important to embed this knowledge within the framework of what we already know about floral evolutionary ecology.

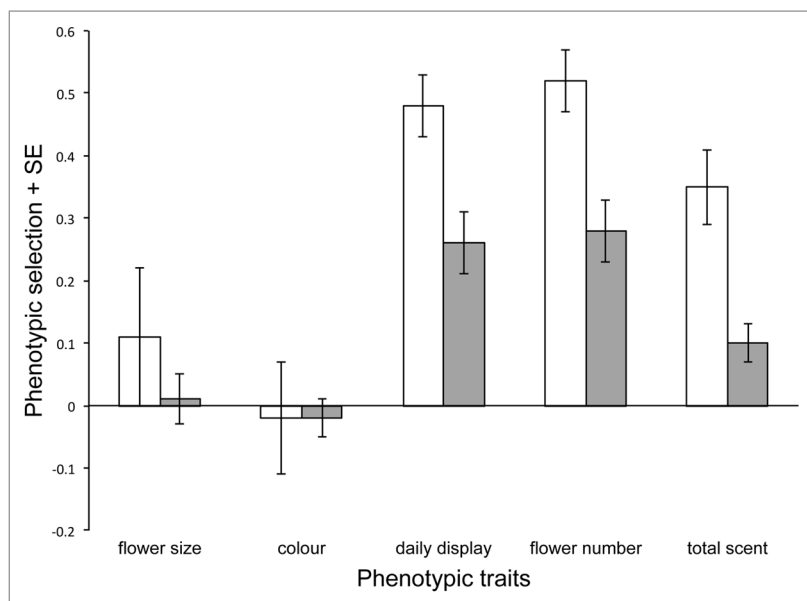
For our study of *P. digitalis*, we took advantage of a common garden experiment for its relative ease of collecting floral scents from many individuals over a short time. We used the methods outlined by Lande and Arnold,<sup>8</sup> which require phenotypic measurements of traits and a fitness estimate for each plant to determine which traits confer higher relative fitness. We measured eight traits [scent (n = 23 volatiles), flower size, corolla pigment, day of first flower, days flowering, daily display size, final height and total flower number] and estimated female fitness (seed set) to determine and compare phenotypic selection on scent, visual and phenological traits. Specifically, we used selection gradients obtained from multivariate regression analyses that control for correlations among traits to determine the direct targets of selection. That phenotypic selection on scent was stronger than for other corolla characters, such as floral color and size in *P. digitalis*, which was particularly interesting for two reasons. First, because pollinators frequently show consistent color preferences, floral color is generally hypothesized to be adaptive and a likely

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Submitted: 10/16/12; Revised: 10/29/12; Accepted: 10/29/12

<http://dx.doi.org/10.4161/psb.22704>

Citation: Parachnowitsch A, Burdon R, A. Raguso R, Kessler A. Natural selection on floral volatile production in *Penstemon digitalis*: Highlighting the role of linalool. *Plant Signal Behav* 2013; 8:e22704; PMID: 23221753; <http://dx.doi.org/10.4161/psb.22704>.



**Figure 1.** Phenotypic selection on floral and inflorescence traits of *Penstemon digitalis*. White bars represent total selection (via direct selection and selection on correlated traits) while gray bars represent the targets of selection (direct selection) obtained from multivariate models that control for correlations among traits. Total and direct selection were significant for daily display size, total flower number and total scent. Flower size is the geometric mean of six floral measurements, floral color was estimated as the number  $\times$  intensity of purple lines, daily display is the mean of display size, flower number is the total number of flowers produced and total scent is the sum of all relativized peak areas of 23 compounds emitted from the inflorescence. Traits not shown: first flower date, days flowering, plant height. Modified from table 3 (Parachnowitsch et al. 2012).<sup>1</sup>

target of selection.<sup>9</sup> Moreover, when corolla pigmentation varies, such as in *P. digitalis* [for example coefficients of variance (CV):  $CV_{\text{pigment}} = 1.67$ ,  $CV_{\text{total scent}} = 0.69$ ,  $CV_{\text{linalool}} = 0.75$ ], this color variation may be maintained by conflicting or fluctuating selection, as suggested in other systems.<sup>10–12</sup> Thus, although our work suggests that floral color variation in *P. digitalis* is selectively neutral,<sup>11,13,14</sup> this was not the initial expectation. Second, our previous study found direct selection by pollinators on flower size in a nearby population,<sup>13</sup> suggesting that it is an important trait for pollinator attraction in this system.

Of the entire blend of 23 scent compounds we found in *P. digitalis*, linalool was the target of selection.<sup>1</sup> To determine which compounds were targets, we again used multivariate regression models but here we included individual compounds instead of total scent as a single-character state. Linalool is a common component of floral scents,<sup>15</sup> suggesting that it plays important and diverse roles in floral communication.<sup>16</sup> Our study demonstrates that this compound can also be under natural selection, and at least in *P. digitalis*, it is also the single target of selection from the scent bouquet. Our investigations suggested that linalool is a floral-specific volatile and therefore could be used as an odor cue by pollinators. However, linalool is a chiral compound with two enantiomeric forms that could influence insect behavior differentially and we had not determined which of the two enantiomers (or proportion of each) the flowers emitted. Therefore we returned in 2012 to assess the exact configuration of linalool emitted from *P. digitalis* flowers.

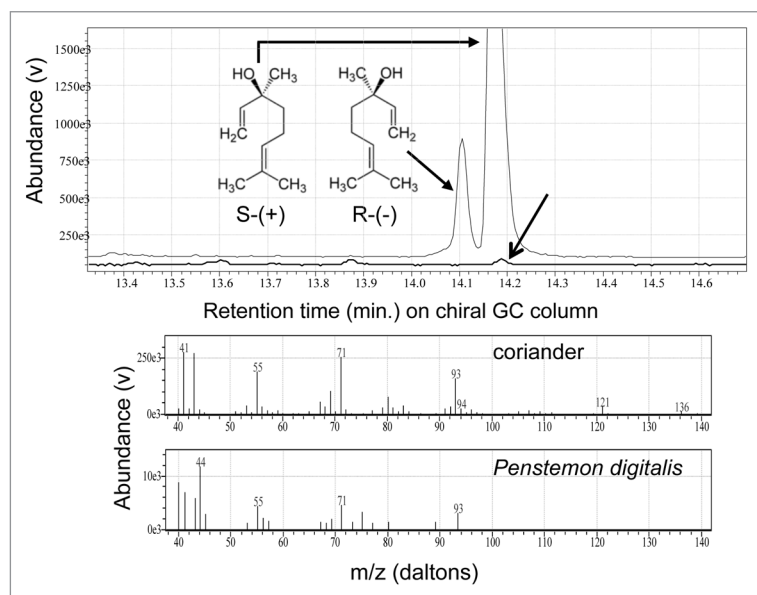
### Linalool in *Penstemon digitalis* Flowers

Both insect mutualists and antagonists can potentially respond differently to the two optical isomers of linalool. Studies of the model insect, *Manduca sexta*, demonstrated two distinct olfactory processing channels for linalool, such that enantiomer-specific antennal receptor neurons project to different glomeruli in the antennal lobes of the moth's brain.<sup>17</sup> A differential perception by

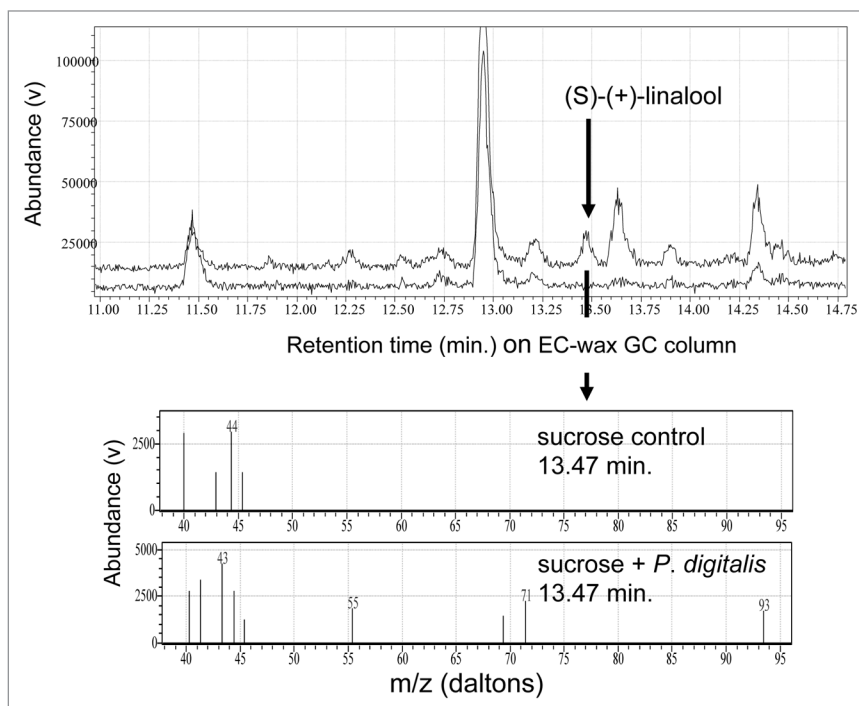
the insect potentially allows for alternate ecological functions of the enantiomers<sup>18</sup> and, thus, differential natural selection on the two compounds. Therefore, to better understand how linalool might function in *P. digitalis*, we used a chiral column (Cyclosil-B,

Agilent-J&W GC columns) to analyze its optical characteristics. We compared headspace from freshly cut *P. digitalis* flowers using SPME (solid-phase microextraction) with racemic linalool (Aldrich L260-2; 97% pure), (*R*)-(-)-linalool (Aldrich 62139, > 95% pure) and the odor of powdered coriander seed (*Coriandrum sativum*), which is dominated by (*S*)-(+)-linalool.<sup>19</sup> Methods generally followed those we used previously (Note S1);<sup>1</sup> collecting odors on 100  $\mu\text{m}$  polydimethylsiloxane (PDMS) fibers from headspace chambers constructed using oven-baked glass scintillation vials with a gasket of nylon resin oven bag material (Reynolds, Inc.). For flower emissions, a 10 mL vial was packed with six cut flowers (from three populations we previously studied), to maximize the intensity of the linalool peak which can be weak for some plants.<sup>1</sup> We used a 1.5 mL vial with approximately 0.5 g of coriander for comparison and collected scents from the two vials for 30 min. Using direct comparison of the GC-MS (gas chromatograph-mass spectrometer) output, we were able to determine that *P. digitalis* produces (*S*)-(+)-linalool only (Fig. 2). This likely has implications for plant-insect interactions in the system and most importantly will allow for manipulations of the compounds produced by *P. digitalis* in future experiments. For example, pollinators may use linalool as a cue after smelling it in nectar-rewarding flowers. Alternatively, linalool, like other monoterpenoids, can have repellent functions for antagonists in floral bouquets.<sup>20</sup> Although we did not detect a relationship between linalool and predispersal seed predation in our common garden experiment,<sup>1</sup> it is possible that the benefit of increased linalool is from its defensive rather than attractive function. Thus, further experimental tests with (*S*)-(+)-linalool in *P. digitalis* are necessary to address its functional role in the system.

Because linalool is a polar compound, it could be absorbed into the nectar of *P. digitalis* if it is emitted from corolla tissue near the nectary.<sup>21</sup> Thus, linalool not only would signal the presence of flowers, but also could indicate the nectar reward sought by pollinators. We tested this hypothesis by first trying to detect linalool in naturally occurring nectar samples. Unfortunately, *P. digitalis* does not produce large quantities of nectar and



**Figure 2.** Enantiomeric configuration of linalool emitted by flowers of *Penstemon digitalis*. Upper panel: comparison of linalool emitted from coriander (upper GC trace) vs. cut *P. digitalis* flowers (lower trace) on a chiral GC column. Coriander shows a racemic mix dominated by (S)-(+)-linalool (right peak), whereas *P. digitalis* emits only that enantiomer (arrow). GC trace for (R)-(-)-linalool not shown (see text). Lower panel: EI-quadrupole mass spectra for (S)-(+)-linalool from coriander (above) and for linalool emitted by flowers of *P. digitalis* (below).



**Figure 3.** Evidence that *Penstemon digitalis* floral nectar is scented with (S)-(+)-linalool. Upper panel shows GC traces from SPME trapping of volatiles emitted by 35% sucrose solution on filter paper, either after 1 h incubation in the nectar tube of *P. digitalis* (upper trace) or from control filter papers (lower trace). The arrow indicates (S)-(+)-linalool, verified by MS and retention index on polar GC column using an authentic standard. Lower panel compares EI-quadrupole mass spectra from sucrose control (upper box) vs. flower-incubated sucrose (lower box), at the retention time (13.47 min) of (S)-(+)-linalool. The incubation of sucrose within the flower effectively reproduces the process of passive but selective absorption of polar volatiles by nectar in the corolla tubes of *P. digitalis* flowers.

even with pooling from many individuals we were unable to detect scents from field-collected nectar samples. Thus, we used 3  $\mu\text{L}$  of ~35% sucrose solution (approximately similar to the sanding crop of naturally produced nectar) inserted into > 40 flowers on eight fresh-cut stems from one population to demonstrate whether linalool would be passively taken up by nectar within the flower as in ref. 21. After 1 h, nectar was collected from the flowers using filter paper and scent was collected as previously but from 4 mL vials. We compared scent from this surrogate nectar with scent emitted from sucrose solution-soaked filter paper. Indeed, we found that the surrogate nectar was scented with linalool, suggesting that *P. digitalis* nectar is likely flavored as well as scented (Fig. 3). Thus, pollinators could learn to use linalool as an honest cue for the presence of reward in *P. digitalis* flowers.<sup>22</sup>

The ecology and evolution of nectar in general and scented nectar in particular is poorly understood.<sup>21,23,24</sup> Moreover, scented nectar may serve different functional roles in attraction for mutualists and repellence of antagonists.<sup>25,26</sup> Therefore, as with linalool itself, direct tests of the function of linalool-scented nectar in *P. digitalis* are necessary to determine its role. However, the potential for linalool to act as an honest indicator of nectar-reward suggests that selection to increase linalool may be pollinator-mediated through their preference for higher rewarding plants in this system.

## Conclusions

Our results suggest that by continuing to ignore floral scents, our overall perspective on natural selection on floral characters will remain skewed toward assuming the importance of visual characters. Moreover, as scent sampling methods, chemical analyses and data handling of the large data sets produced by measuring scent bouquets advance,<sup>27-29</sup> large-scale field-based ecological experiments are becoming more attainable.<sup>6,30-32</sup> Thus, future floral evolutionary research will likely increasingly incorporate scents to understand how integrated floral phenotypes evolve.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Acknowledgments

We thank all those involved with the original publication of the selection data, as well as Frantisek Baluska for the opportunity to expand our thoughts and results of *Penstemon digitalis* floral scent. GC-MS analyses of linalool were supported by NSF grant DEB-0746106 to R.A.R. and the 2012 field season was supported by Kungliga Vetenskapsakademien grant FOA11H-317 to A.L.P.

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