

Drought and leaf herbivory influence floral volatiles and pollinator attraction

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Abstract

The effects of climate change on species interactions are poorly understood. Investigating the mechanisms by which species interactions may shift under altered environmental conditions will help form a more predictive understanding of such shifts. In particular, components of climate change have the potential to strongly influence floral volatile organic compounds (VOCs) and, in turn, plant–pollinator interactions. In this study, we experimentally manipulated drought and herbivory for four forb species to determine effects of these treatments and their interactions on (1) visual plant traits traditionally associated with pollinator attraction, (2) floral VOCs, and (3) the visitation rates and community composition of pollinators. For all forbs tested, experimental drought universally reduced flower size and floral display, but there were species-specific effects of drought on volatile emissions per flower, the composition of compounds produced, and subsequent pollinator visitation rates. Moreover, the community of pollinating visitors was influenced by drought across forb species (i.e. some pollinator species were deterred by drought while others were attracted). Together, these results indicate that VOCs may provide more nuanced information to potential floral visitors and may be relatively more important than visual traits for pollinator attraction, particularly under shifting environmental conditions.

Keywords: *Campanula rotundifolia*, climate change, floral display, floral scent, *Heterotheca villosa*, *Phacelia hastata*, plant–pollinator interactions, pollinator community, *Potentilla recta*, volatile organic compounds

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Introduction

One of the main challenges facing ecologists today is to gain a better understanding of how climate change is affecting ecological interactions among species so that we may maintain and restore the essential ecosystem services they provide, like pollination. To better understand the mechanisms by which shifts in species interactions could occur, ecologists quantify traits potentially involved in mediating these interactions. Volatile organic compounds (VOCs) are traits that can be influenced by climate change and strongly mediate a wide array of interactions between plants and insects, either in an attractive or repellent manner. VOCs have been most extensively studied in the context of tri-trophic (plant–herbivore–natural enemy) interactions where they play important roles in plant defense (e.g. De Moraes *et al.*, 2001; Kessler & Baldwin, 2001; Xiao *et al.*, 2012). Although less studied, VOCs emitted from flowers can mediate mutualistic interactions between plants and their pollinators (Dudareva *et al.*, 2006). For example, pollinators use these airborne chemical signals while searching for nectar and pollen (Schiestl,

2015). To date, pollination biology has focused largely on the role of visual cues for pollinator attraction (Raguso, 2008a), which contribute to community-level patterns in plant–pollinator interaction networks (Vazquez *et al.*, 2009). The importance of floral odors for pollination is increasingly appreciated (e.g. Kessler *et al.*, 2008; Raguso, 2008a,b; Morse *et al.*, 2012; Riffell *et al.*, 2013), yet how they structure plant–pollinator interactions, particularly in the context of climate change, is relatively poorly studied. Given their crucial roles in directing other plant–insect interactions (e.g. in the context of herbivory), plant VOCs likely exert important influences on plant–pollinator interactions and community dynamics.

Components of climate change, including increasing temperatures and CO₂ levels and altered precipitation patterns, can affect plant traits important for pollinator attraction (e.g. Jablonski *et al.*, 2002; Scaven & Rafferty, 2013). For instance, elevated temperatures can influence flower production, flower size, floral phenology, plant height, and the production and composition of nectar and pollen (e.g. Delph *et al.*, 1997; Wahid *et al.*, 2007; Liu *et al.*, 2012). Importantly, studying the effects of climate change on these traits of plants has done little to advance our understanding of how plant–pollinator interactions and community dynamics may shift

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because (1) plant species often respond similarly to the same environmental cue (i.e. community-wide reductions in flower production due to drought, or advancement of flowering phenology due to warmer spring temperatures); (2) when species-specific responses occur, it has proved difficult to leverage this information in a predictive way, and (3) a slow recognition that pollinator foraging behavior is not static (i.e. interactions within a community are flexible consequences of context-dependent pollinator choices) hinders our understanding of how cascades of shifts in foraging patterns may occur in communities altered by climate change. Thus, studying floral VOCs – information-rich signals that are highly responsive to the environment – and plant–pollinator interactions in the context of climate change will likely enhance our ability to form a more predictive understanding of how plant–pollinator interactions will change. In fact, increasing temperatures have been found to increase the quantity of floral VOCs and alter the quality (i.e. shift ratios of compounds in floral bouquets) with potentially important, but unknown, effects on pollinators (Farré-Armengol *et al.*, 2014).

Reduced precipitation resulting in drought is occurring as a result of anthropogenic climate change, and drought is predicted to become more severe in many places across the globe (e.g. Pederson *et al.*, 2010; Dai, 2013; Cook *et al.*, 2015). Water availability can have strong effects on plant growth as well as plant traits important for pollination and other species interactions. For example, drought stress reduced flower size and nectar volume in fireweed (*Epilobium angustifolium*) (Carroll *et al.*, 2001) and the wild tobacco species *Nicotiana quadrivalvis* (Halpern *et al.*, 2010). Given shifts in plant quality under drought conditions, the amount and consequences of herbivory are also likely to be altered (Huberty & Denno, 2004). In fact, drought may have the strongest effects on herbivory compared to other components of climate change (Scherber *et al.*, 2013). Moreover, herbivory and pollination do not occur in isolation (i.e. plants are simultaneously exposed to herbivores and pollinators). Plant herbivory is ubiquitous, predicted to increase as the climate warms (Currano *et al.*, 2010), and herbivore feeding has recently been shown to affect flower volatile emissions and pollinators (Theis *et al.*, 2009; Kessler *et al.*, 2011; Pareja *et al.*, 2012; Bruinsma *et al.*, 2014). If the ability of pollinators to locate appropriate flowers within the landscape becomes compromised due to climate change, plant communities and agriculture will likely suffer serious reproductive consequences (Farré-Armengol *et al.*, 2013). Additionally, the *interactive* effects of climate change factors on plant secondary chemistry and VOCs are poorly understood

(Bidart-Bouzat & Imeh-Nathaniel, 2008). Thus, it will be important to not only investigate the effects of multiple climate change factors and their interactions (e.g. Schweiger *et al.*, 2010; Hoover *et al.*, 2012), but also multiple types of species interactions (Tylianakis *et al.*, 2008; Fontaine *et al.*, 2011).

Environmental conditions that induce stress in plants (e.g. drought and herbivory) have the potential to change floral VOCs and affect pollination attraction, but this remains poorly understood. Here, we experimentally manipulated drought and herbivory for four species of forbs in a fully factorial design to investigate effects on (1) traditional measures of pollinator attraction (i.e. plant size, floral display, and flower size), (2) floral VOC quantity (i.e. total volatile production per flower) and quality (i.e. shifts in VOC composition and dispersion of compounds), and (3) subsequent rates and composition of pollinator visitors. Our results suggest that floral VOCs represent a strong, consistent link between plant physiological responses to drought and information-rich signals by which pollinators make foraging choices. We also show that herbivory can modify the effects of drought on plant traits including floral VOCs, at least in some species.

Materials and methods

Plant material and growth conditions

We used three species of widespread native forbs: *Campanula rotundifolia* L., harebell; *Heterotheca villosa* (Pursh) Shinners, hairy false goldenaster; and *Phacelia hastata* Douglas ex Lehm., silverleaf phacelia as well as one invasive forb: *Potentilla recta* L., sulphur cinquefoil; native to Eurasia. Sixty individuals of each species were grown from seed in a greenhouse with a day/night temperature regime of 26 °C/15 °C and a 16 h photoperiod with supplemental lighting provided by metal halide lamps. Seeds were collected locally at the Mt. Ellis field site (see below). Plants were grown in 6.5 cm wide by 25 cm tall plastic cone-tainers using Sunshine Mix #1 soil (Sun Gro Horticulture, Agawam, MA, USA) with added fertilizer (1 teaspoon or ca. 5 mL of Osmocote per pot; The Scotts Company, Marysville, OH, USA). Seeds were planted in the fall (October), allowed to grow and establish for 6 weeks, and then vernalized in a climate-controlled chamber at approximately 4 °C with a 12 h photoperiod for 100–130 days. Plants removed from the cold room and returned to the greenhouse began flowering within 4–6 weeks.

Drought and herbivore treatments

Once plant species were flowering, we then experimentally manipulated drought and herbivory in a completely randomized, fully crossed design to create the following treatments: control, herbivore, drought, and herbivore + drought ($n = 8$ individual plants of each forb species per treatment). For the

drought treatment, water was withheld until the first signs of wilting were observed and then plants were watered until water drained freely through the holes in the bottom; this cycle was repeated for 2 weeks (14 days) prior to measuring plant traits and field observations (see below). The time it took each individual to wilt varied with species, plant size, drought tolerance, etc.: *C. rotundifolia* and *H. villosa* wilted after about 3 days, *P. hastata* in about 2 days, and *P. recta* wilted about 1 day after water was withheld. These pulsed drought treatments are thought to mimic natural conditions better than continuous drought (Huberty & Denno, 2004), and plants subjected to continuous drought did not produce flowers (L.A. Burkle and J.B. Runyon, unpublished data). All plants were well-watered on the morning of the experiments. Plants in the control and herbivore only treatments were watered thoroughly every day and never water-stressed.

For the herbivore treatment, we used the lepidopteran *Trichoplusia ni* (Hübner) (cabbage looper), a species that has been used extensively as a model generalist herbivore in plant-herbivore interaction studies. Eggs of *T. ni* were obtained from Benzon Research (Carlisle, PA, USA), and developing larvae were reared on an artificial diet until use. Two second-instar larvae were caged on one fully expanded leaf of each plant using small clip cages (36.5 × 25.4 × 9.5 mm; Bioquip Products, Rancho Dominguez, CA, USA); larvae were starved for 24 h before use (e.g. Simpson *et al.*, 1999; Nielsen *et al.*, 2013) and caterpillars were moved to a new leaf every day. Larvae occasionally died and were replaced. Larvae were allowed to feed for 4 days prior to experiments, so that each plant had four leaves that had been fed upon at time of volatile collection and field observations. We used low levels of herbivory to mimic realistic levels of insect damage for these forb species (e.g. Agrawal & Kotanen, 2003) and to minimize the visual signs of damage. Cages (and caterpillars within them) were removed to minimize potential visual or olfactory cues from caterpillars themselves, before taking plants to the field for pollinator observations.

Measuring visual plant traits

We assessed several plant traits that are ostensibly important in pollinator attraction: plant size, floral display, and flower size. All traits were measured on the same day as field observations of pollinators (see *Assessing pollinator visitation* below). The height of each plant was measured from soil surface using a meter stick. We measured plant area (length × width) for *P. hastata* rather than height since area is a better measure of plant size for this species due to its prostrate, sprawling growth form. The number of open flowers (i.e. floral display) was counted on each plant except we measured the number of capitula (flower heads) for *H. villosa* and estimated the number of *P. hastata* flowers by determining the mean number of flowers per cyme and counting the number of cymes on each plant. Mean flower width for each forb species in each treatment was determined using digital calipers by randomly subsampling three to four flowers from at least eight different plants of each treatment (although some plants in the drought treatment

had fewer flowers); for *H. villosa*, we measured the width of capitula rather than individual flowers.

Measuring floral volatiles

We also measured floral scent on the same day as field observations of pollinators. Volatiles were collected by enclosing flowers in 950 mL clear polyethylene cups with clear dome lids (Dart Container Corporation, Mason, MI, USA) and pulling air (0.5 L min⁻¹) through volatile traps containing 30 mg of the adsorbent HayeSep-Q (Restek, Bellefonte, PA, USA) using portable volatile collection systems (Volatile Assay Systems, Rensselaer, NY, USA); filtered air was not pushed into the cup. Volatiles were collected for 1 h from six replicates of each species/treatment combination; three replicates were collected before field observations (0900–1100) and three after field observations (1300–1500). Due to the available number and size of flowers as well as plant architecture, the number of flowers from which volatiles were sampled in a given collection varied for *C. rotundifolia* (1–15), *H. villosa* (1–6 capitula), *P. hastata* (13–48), and *P. recta* (1–3), but all amounts of volatile emissions are reported on a per flower basis (ng flower⁻¹ h⁻¹). Volatiles were eluted from traps with 200 µL of dichloromethane, and 500 ng of *n*-nonyl-acetate was added as an internal standard. Samples were analyzed using an Agilent 7890A gas chromatograph (GC) coupled with a 5975C mass spectrometer and separated on a HP-1 ms column (30 m × 0.25 mm inside diameter, 0.25 µm film thickness); helium was used as the carrier gas. The GC oven was maintained at 35 °C for 3 min and then increased by 5 °C min⁻¹ to 125 °C, then 25 °C min⁻¹ to 250 °C. Quantifications were made relative to the internal standard using CHEMSTATION software (Agilent Technologies, Wilmington, DE, USA), and identifications of compounds were made using NIST 08 Mass Spectral Search Program (National Institute of Standards and Technology, Gaithersburg, MD, USA) and confirmed by comparing retention times and mass spectra with commercial standards, when available.

Assessing pollinator visitation

To quantify the effects of drought and herbivory on pollinator visitation, we transported the plants to a diverse Northern Rocky Mountain native plant community ca. 5 miles southeast of Bozeman, Montana, USA (N 45°37.39', W 110° 57.70'). This plant community is a large meadow located at 1600–1700 m elevation at the base of Mt. Ellis, and through our work in this system over the last several years, we have identified 50 species of forbs and at least 70 bee species that visit them (unpublished data). Typical to this region, plants begin blooming after snow melt (late April–May) and continue through August. Our four focal species are found in this meadow or surrounding areas, and the timing of our field observations was near peak-bloom (July) in the field system on calm, sunny days. On observation days, we created an assemblage of each forb species containing two or four individuals of each treatment for observation in the field. Individuals in each treatment were placed in a group (ca. 1 m² area), and each

treatment group was separated by at least 2 m. For each replicate assemblage, we observed each forb species for 1 h (30 min in the morning and 30 min in the afternoon to more thoroughly assess the pollinator community) and captured any floral visitors contacting the reproductive parts of flowers using insect nets. Each floral visitor that we captured was placed in a labeled vial for later identification, and we recorded the identity and frequency of these plant–pollinator interactions. We created these assemblages and performed observations twice for each forb species, in early- and mid-July, using different plant individuals each time. Observation period was included as a covariate in analyses (below) and removed if not significant ($P > 0.05$). Pollinators (88% bees) were identified to species or lowest taxonomic level possible in the lab. We calculated per-plant and per-flower pollinator visitation rates because both are known to be important for individual plants and the communities in which they occur (e.g. Burkle & Irwin, 2010).

Statistical analyses

We investigated treatment effects and their interactions on traditional measures of plant traits important for pollinator attraction (plant size, flower size, and floral display) as well as pollinator visitation rates (per-plant and per-flower) using separate two-way MANOVAS for each forb species. Floral display data were square-root transformed for normality for *C. rotundifolia*, *P. hastata*, and *P. recta*. *Phacelia hastata* plant area data were square-root transformed as well. Significant MANOVAS ($P < 0.05$ in all cases) were followed by two-way ANOVAS, and we focused on reporting these ANOVA results. We investigated treatment effects and their interactions on volatile emission using separate two-way ANOVAS for each forb species. Significant ANOVAS were followed by Tukey's HSD tests to further determine differences among treatments. Occasionally, the Tukey's tests detected a significant effect between treatments where an overall treatment effect was not found by the main ANOVA model. In these cases, we report the more conservative ANOVA results. We tested for treatment effects on the composition of volatile compounds and variance in composition (i.e. estimate of dispersion) using multivariate analysis of variance ('adonis' in vegan package in R, Oksanen *et al.* (2012) based on Bray-Curtis dissimilarities) and 'betadisper' in vegan package in R respectively. We visualized any treatment effects using nonmetric multidimensional scaling. To determine which compounds, if any, contributed most strongly to the composition differences in volatiles among treatments, we used similarity percentage analyses ('siper' in vegan package in R). Lastly, we tested for treatment effects on the composition and dispersion of pollinator visitors to each forb species using 'adonis' and 'betadisper' respectively.

Results

Visual plant traits

Campanula rotundifolia. Although drought did not influence *C. rotundifolia* plant height, drought

significantly reduced flower width by 32% and floral display by 30%, and herbivory mediated the effects of drought on flower width (Table 1; Fig. 1a, e).

Heterotheca villosa. Although drought did not influence *H. villosa* height, drought significantly reduced flower width by 18% and floral display 38-fold (Table 1; Fig. 1b, f).

Phacelia hastata. Drought significantly reduced plant area by over half, flower width by 10%, flower depth by 5% ($F_{1,26} = 42.81$, $P < 0.0001$), and floral display by over eightfold (Table 1; Fig. 1c, g). Herbivory mediated the effects of drought on flower depth ($F_{1,26} = 18.25$, $P < 0.0001$) and width, resulting in particularly small flowers in the herbivore + drought treatment (Table 1, Fig. 1c).

Potentilla recta. Drought reduced height by 13%, flower width by 18%, and floral display by almost sevenfold (Table 1; Fig. 1d, h).

Floral volatiles

Campanula rotundifolia. Flowers of *C. rotundifolia* emitted the same 26 volatile compounds in all treatments (Table S1). Drought more than double the total volatiles released per flower (Table 2 and S1, Fig. 1i), influenced the composition (i.e. relative abundances) of compounds (Fig. 2a; $F_{1,20} = 2.85$, $P = 0.022$), and increased the dispersion (i.e. variation among plants) of compounds produced per flower ($F_{1,22} = 4.86$, $P = 0.038$). The compounds that contributed most strongly to the difference in composition between the drought and watered control treatments were the irregular terpene 6-methyl-5-hepten-2-one, the benzenoid benzaldehyde, and the monoterpene α -pinene. Collectively, these three compounds explained 45% of the variation in composition of VOCs between the drought and watered control treatments. 6-methyl-5-hepten-2-one, benzaldehyde, and α -pinene are common VOCs emitted by flowers and are predicted to play roles in attracting pollinators (Knudsen *et al.*, 2006). The levels of these compounds were over twice as high in the drought treatment compared to the watered controls.

Heterotheca villosa. Flowers of *H. villosa* emitted the same 40 volatile compounds in all treatments (Table S2). Flowers of plants in the drought treatment tended to produce less total volatiles than those in the watered controls, but these effects were not statistically significant (Tables 2 and S2, Fig. 1j). Herbivory

Table 1 Two-way ANOVA results testing for the main and interactive effects of drought and leaf herbivory treatments on plant traits (plant size, flower width, and floral display) for each of the four focal species. Whole model MANOVAS were significant ($P < 0.0014$) for each species. P values in boldface are significant at $\alpha = 0.05$

Source	Plant size*			Flower width			Floral display†		
	df	F	P	df	F	P	df	F	P
<i>Campanula rotundifolia</i>									
Whole model	4,26	0.27	0.89	4,26	30.67	<0.0001	4,26	5.47	0.0025
Drought	1,26	0.89	0.35	1,26	45.98	<0.0001	1,26	9.20	0.0054
Herbivory	1,26	0.06	0.81	1,26	1.96	0.16	1,26	0.011	0.92
Water × herbivory	1,26	0.09	0.77	1,26	6.10	0.015	1,26	1.67	0.21
<i>Heterotheca villosa</i>									
Whole model	4,20	2.25	0.10	4,20	15.88	<0.0001	4,20	5.79	0.0029
Drought	1,20	1.21	0.28	1,20	24.74	<0.0001	1,20	22.48	0.0001
Herbivory	1,20	0.54	0.47	1,20	0.12	0.73	1,20	0.16	0.69
Water × herbivory	1,20	0.47	0.50	1,20	2.08	0.15	1,20	0.19	0.67
<i>Phacelia hastata</i>									
Whole model	4,26	13.87	<0.0001	4,26	14.75	<0.0001	4,26	18.38	<0.0001
Drought	1,26	16.03	0.0005	1,26	45.21	<0.0001	1,26	47.84	<0.0001
Herbivory	1,26	1.43	0.24	1,26	2.42	0.12	1,26	1.60	0.22
Water × herbivory	1,26	2.12	0.16	1,26	5.76	0.018	1,26	2.09	0.16
<i>Potentilla recta</i>									
Whole model	4,31	22.30	<0.0001	4,31	20.15	<0.0001	4,31	129.4	<0.0001
Drought	1,31	14.43	0.0006	1,31	56.55	<0.0001	1,31	368.0	<0.0001
Herbivory	1,31	0.005	0.94	1,31	0.25	0.62	1,31	0.83	0.37
Water × herbivory	1,31	0.41	0.53	1,31	4.70	0.032	1,31	7.79	0.009

*Height for all forbs except *P. hastata*, where plant area (square-root transformed) was used.

†Square-root transformed for *Campanula rotundifolia*, *P. hastata*, and *P. recta*.

($F_{1,20} = 2.08$, $P = 0.049$), but not drought ($F_{1,20} = 0.80$, $P = 0.60$), influenced the composition of compounds per flower (Fig. 2b). The compounds that contributed most strongly to the difference between herbivore treatments were the benzenoid benzaldehyde, the irregular terpene 6-methyl-5-hepten-2-one, the monoterpene eucalyptol, and two unidentified monoterpenes (MT1 and MT2). Collectively, these five compounds explained 44% of the variation in composition of VOCs between herbivory treatments. The levels of benzaldehyde and eucalyptol were 50% higher in the herbivore treatment, while MT1 and MT2 were over four times higher in the control treatment. Neither herbivory ($F_{1,22} = 0.42$, $P = 0.52$) or drought ($F_{1,22} = 2.31$, $P = 0.14$) significantly influenced the dispersion of compounds.

Phacelia hastata. Flowers of *P. hastata* emitted the same 40 volatile compounds in all treatments (Table S3). Drought influenced the composition of chemical compounds present in flowers of *P. hastata* (Fig. 2c; $F_{1,20} = 2.18$, $P = 0.038$). The compounds that contributed most strongly to the difference between the drought and watered control treatments

were the monoterpenes β -myrcene, limonene, and eucalyptol, the irregular terpene 6-methyl-5-hepten-2-one, and the green leaf volatile z -3-hexenyl acetate. Collectively, these five compounds explained 48% of the variation in composition of VOCs between drought and watered control treatments. The levels of β -myrcene, limonene, and eucalyptol were up to twice as high (per flower) in the watered controls compared to the drought treatment, while z -3-hexenyl acetate and 6-methyl-5-hepten-2-one were 75% and 64%, respectively, higher in the drought treatment. Neither herbivory ($F_{1,22} = 0.044$, $P = 0.84$) or drought ($F_{1,22} = 1.94$, $P = 0.18$) significantly influenced the dispersion of compounds.

Potentilla recta. Flowers of *P. recta* released the same 29 volatile compounds regardless of treatment (Table S4). Drought increased the emission of total volatiles per flower by 67% (Table 2 and S4, Fig. 11). Drought also influenced the composition of chemical compounds present (Fig. 2d; $F_{1,20} = 4.84$, $P = 0.002$), but not the dispersion of compounds ($F_{1,22} = 0.42$, $P = 0.52$), in flowers of *P. recta* compared to watered control plants. The

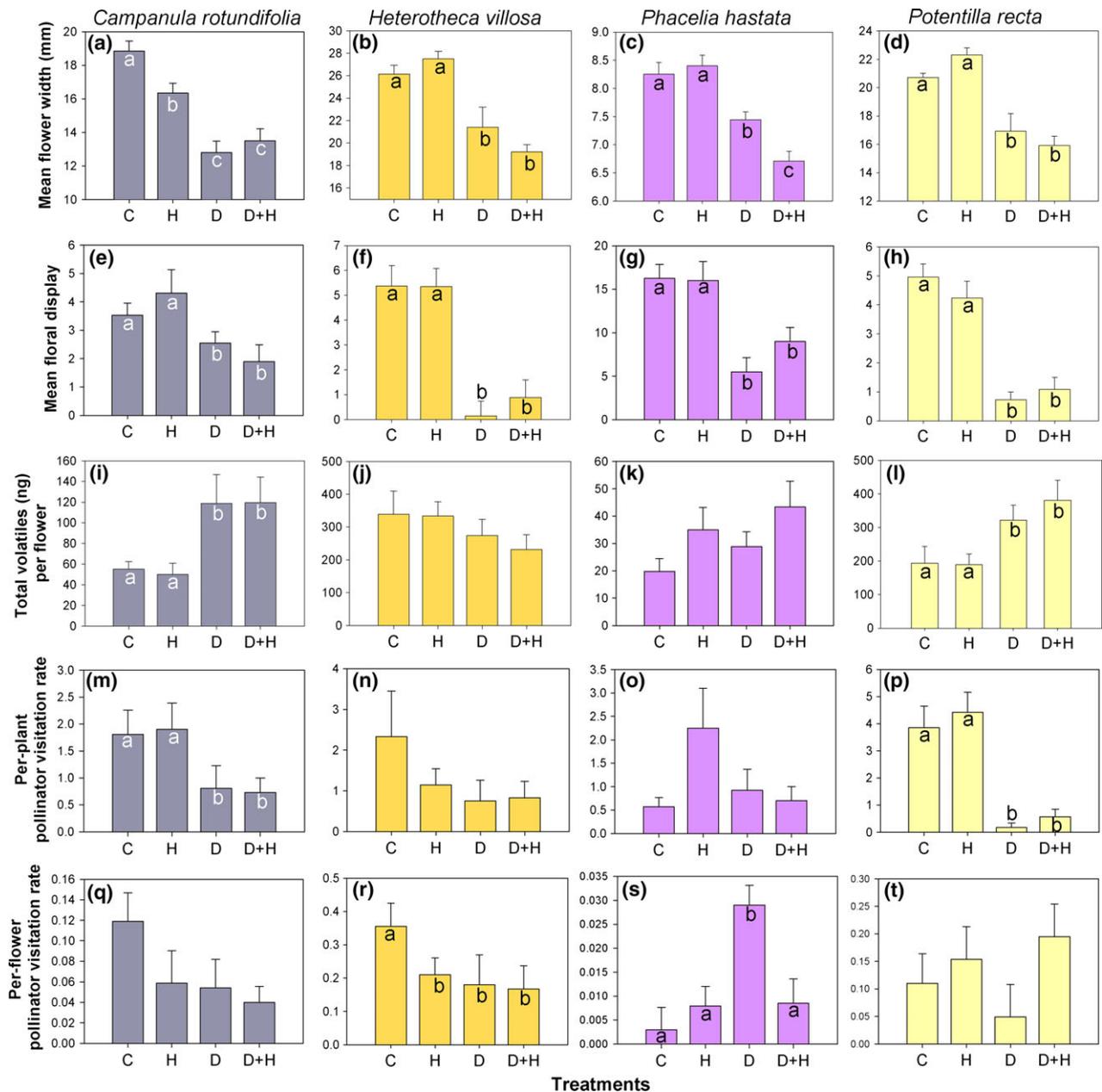


Fig. 1 Treatment effects on plant traits (panels a–h), total volatiles (ng) per flower (panels i–l), and pollinator visitation rates (panels m–t) for *Campanula rotundifolia* (purple), *Heterotheca villosa* (mustard yellow), *Phacelia hastata* (pink), and *Potentilla recta* (lemon yellow). Bars are means \pm 1 SE. Different letters indicate significant differences between treatments within species ($P < 0.05$) from *post-hoc* Tukey HSD tests. Treatments are: C = control, H = herbivore, D = drought, D + H = herbivore and drought.

compounds that contributed most strongly to the difference between drought and watered controls were the green leaf volatiles *z*-3-hexenyl acetate and *z*-3-hexenol. These two compounds together explained 54% of the variation in composition of VOCs between drought and watered control treatments. The levels of these compounds (per flower) were three times higher in

flowers of plants in the drought compared to control treatment.

Pollinator visitation

Campanula rotundifolia. Drought reduced per-plant (Table 2, Fig. 1m) and per-flower (Table 2, Fig. 1q)

Table 2 Two-way ANOVA results testing for the main and interactive effects of drought and leaf herbivory treatments on total volatiles (ng) produced per flower and on per-plant and per-flower pollinator visitation rates for each of the four focal forb species. *P* values in boldface are significant at $\alpha = 0.05$

Source	Total volatiles (ng)			Per-plant visitation rate			Per-flower visitation rate		
	df	<i>F</i>	<i>P</i>	df	<i>F</i>	<i>P</i>	df	<i>F</i>	<i>P</i>
<i>Campanula rotundifolia</i>									
Whole model	3,20	3.74	0.028	3,20	8.98	0.0003	3,20	3.67	0.024
Drought	1,20	11.19	0.0032	1,20	9.79	0.0055	1,20	2.20	0.16
Herbivory	1,20	0.012	0.92	1,20	0.0	1.00	1,20	1.74	0.20
Water × herbivory	1,20	0.022	0.88	1,20	0.058	0.81	1,20	0.67	0.43
<i>Heterotheca villosa</i>									
Whole model	3,20	0.91	0.45	3,20	1.19	0.34	3,20	5.04	0.041
Drought	1,20	2.84	0.11	1,20	2.06	0.17	1,20	2.06	0.17
Herbivory	1,20	0.19	0.66	1,20	0.70	0.41	1,20	6.63	0.023
Water × herbivory	1,20	0.12	0.74	1,20	0.93	0.35	1,20	2.78	0.12
<i>Phacelia hastata</i>									
Whole model	3,20	1.91	0.16	3,20	2.30	0.11	3,20	4.95	0.0086
Drought	1,20	1.46	0.24	1,20	1.36	0.26	1,20	9.15	0.0080
Herbivory	1,20	4.25	0.052	1,20	3.36	0.082	1,20	3.13	0.096
Water × herbivory	1,20	0.0017	0.97	1,20	2.00	0.17	1,20	8.25	0.011
<i>Potentilla recta</i>									
Whole model	3,20	2.73	0.050	3,20	14.3	<0.0001	3,20	0.95	0.46
Drought	1,20	5.66	0.027	1,20	42.84	<0.0001	1,20	0.031	0.86
Herbivory	1,20	0.16	0.69	1,20	0.70	0.41	1,20	2.64	0.12
Water × herbivory	1,20	0.22	0.64	1,20	0.018	0.89	1,20	0.75	0.40

pollinator visitation rate to *C. rotundifolia* by half, though this latter effect was not significant.

Heterotheca villosa. Treatments did not affect per-plant pollinator visitation rate (Table 2, Fig. 1n), but herbivory reduced per-flower pollinator visitation rate by 41% compared to watered controls (Table 2, Fig. 1r).

Phacelia hastata. Although treatments did not significantly affect per-plant pollinator visitation rates (Table 2, Fig. 1o), drought increased per-flower visitation 10-fold, and herbivory negated this effect (Table 2, Fig. 1s).

Potentilla recta. Drought drastically reduced per-plant pollinator visitation 22.5-fold (Table 2, Fig. 1p), but these effects did not translate to reductions in per-flower pollinator visitation (Table 2, Fig. 1t).

Pollinator community composition. More than 25 species of flower-visiting insects were observed in this study, 88% of which were bees (Hymenoptera, Apoidea); flies (Diptera); and butterflies (Lepidoptera) comprised the remaining 12% of visitors (Table S5). Plant species ($F_{3,14} = 1.75$, $P = 0.045$) and drought ($F_{1,14} = 3.21$, $P = 0.007$) influenced the community composition of visiting pollinators (Fig. 3). Herbivory ($F_{1,14} = 0.84$,

$P = 0.57$) and the interaction between drought and herbivory ($F_{1,14} = 1.51$, $P = 0.16$) had no effect on the pollinator community for any plant species. Three species of bees in the genus *Lasioglossum* (*Dialictus*) and one skipper butterfly species (Hesperiidae) contributed most strongly (i.e. explained 50% of the variation) to the differences in the community of pollinator visitors between drought and watered controls across forb species. There was no difference in the dispersion of the pollinator community between drought and watered controls ($F_{1,13} = 0.35$, $P = 0.56$) (Fig. 4).

Discussion

All four forb species were affected by experimental drought and/or herbivory, but the effects were species-specific. Drought, but not leaf herbivory, influenced visual traits important for pollinator attraction, and, for all forb species, the values of these traits were reduced by drought (i.e. smaller flowers and floral display). Drought also increased total VOCs released per flower in two of the forb species tested, despite having smaller flowers. Moreover, drought influenced the composition of volatiles emitted by flowers in three of the four forb species. Additionally, for one plant species (*C. rotundifolia*), drought increased the dispersion of volatile compounds, indicating that drought broadly altered a

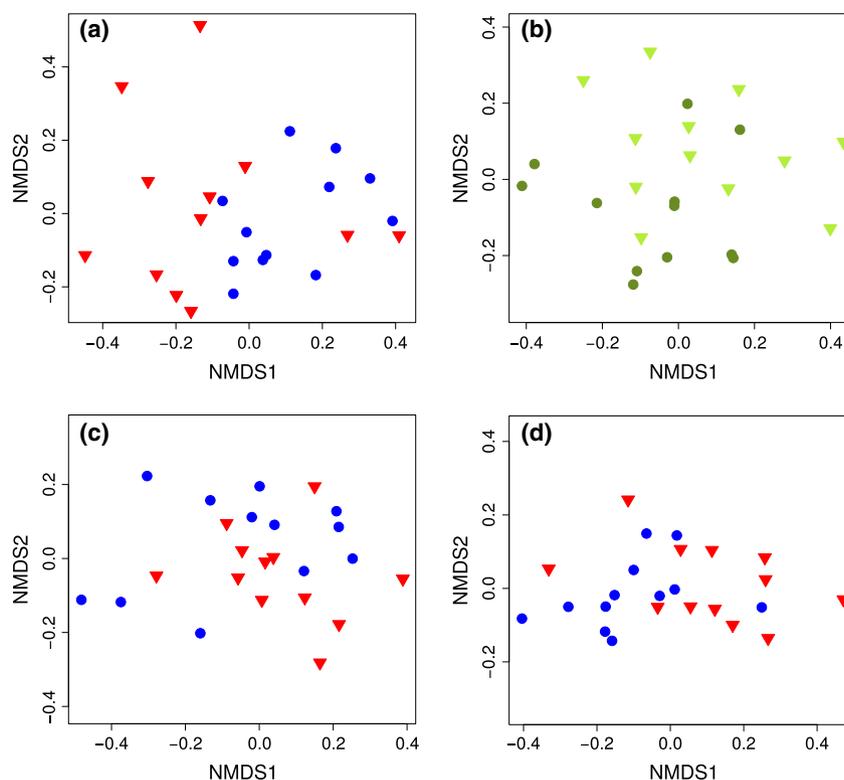


Fig. 2 Treatment effects on floral volatile composition. Drought (red triangles) influenced the composition of chemical compounds present in flowers of *Campanula rotundifolia* (a), *Phacelia hastata* (c) and *Potentilla recta* (d) compared to watered controls (blue circles), suggesting a mechanism by which pollinators can differentiate plants experiencing water stress. (b) Leaf herbivory (dark green circles) influenced the composition of VOCs in flowers of *Heterotheca villosa* compared to no-herbivory controls (light green triangles).

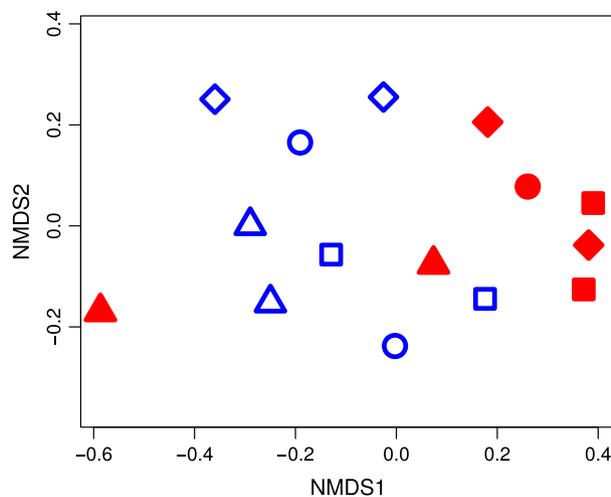


Fig. 3 Treatment effects on pollinator composition. Drought (red filled symbols) influenced the composition of pollinator visitors across all forb species: *Campanula rotundifolia* (circles), *Heterotheca villosa* (triangles), *Phacelia hastata* (squares), and *Potentilla recta* (diamonds). Watered controls are blue open symbols.

diversity of VOCs and VOC signaling pathways. These effects of drought on visual traits and floral VOCs were accompanied by effects on pollinator visitation, sug-

gesting that plant fitness may also be influenced if plant reproduction is pollen limited. Because both visual and volatile traits were influenced by drought for most of the species tested, it is difficult to infer which traits most strongly influenced pollinator attraction. However, while drought reduced flower size and floral display similarly for all forb species, the effects of drought on VOC emission per flower and the composition of those VOCs were species-specific, indicating that shifts in VOCs as a result of environmental changes, like drought, and subsequent effects on pollinator foraging choices, may provide a more predictive understanding of shifts in species interactions under climate change. In particular, investigating both visual and volatile responses of forbs to environmental changes was the most informative when trying to explain pollinator responses.

Overall, drought had strong effects on both visual and olfactory plant traits, though often in opposing ways. For example, while drought reduced the visual apparency of the plants (by reducing plant size, flower size, and/or floral display), drought tended to increase the odor apparency of the plants by increasing VOC emission by flowers. Interestingly, higher temperatures have been shown to increase VOC emission by flowers

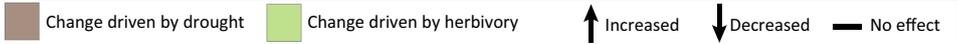
	Plant traits			Floral VOCs			Pollinator attraction	
	Plant height	Flower width	Floral display	Total VOCs per flower	VOC composition	VOC dispersion	Pollinator visits per flower	Pollinator visits per plant
 <i>Campanula rotundifolia</i>	—	↓	↓	↑	✓	↑	↓	↓
 <i>Heterotheca villosa</i>	—	↓	↓	—	✓	—	↓	—
 <i>Phacelia hastata</i>	↓	↓ width depth	↓	—	✓	—	↑	—
 <i>Potentilla recta</i>	↓	↓	↓	↑	✓	—	—	↓
								

Fig. 4 Summary of treatment effects on plant traits, floral VOCs, and pollinator attraction for *Campanula rotundifolia*, *Heterotheca villosa*, *Phacelia hastata*, and *Potentilla recta*. The ✓ symbol indicates statistically significant effects of drought (brown) or herbivory (green) on VOC composition.

as well (Farré-Armengol *et al.*, 2014), suggesting that there will be general enhancement of these pollinator cues in a warmer and drier world. However, the magnitude of reduction in floral display by drought was greater than the magnitude of increase in VOC emissions, indicating that the total volatiles emitted by all flowers of a plant in the drought treatment were likely lower than that produced by control plants. Despite these patterns, it is not clear how visual and olfactory plant traits mechanistically combine (and possibly interact) to determine pollinator attraction under drought conditions.

Leaf herbivory affected pollinator visitation to one species, *H. villosa*. In this case, the effects of herbivory on pollinator visitation, driven at least in part by shifts in VOCs, were stronger than the effects of drought on pollinator visitation through reduced floral display. Herbivory also mediated the effects of drought on flower size in *C. rotundifolia* and *P. hastata* and on pollinator visitation rate to *P. hastata*, indicating the importance of considering interactions between multiple components of climate change (e.g. Rosenblatt & Schmitz, 2014). Changes in floral volatile emissions induced by herbivores feeding on leaves has been

shown thus far to occur in a handful of other plant species (Effmert *et al.*, 2008; Kessler *et al.*, 2011; Pareja *et al.*, 2012; Bruinsma *et al.*, 2014). Such herbivore induced changes can decrease or increase pollinator visitation (Lucas-Barbosa *et al.*, 2011; Bruinsma *et al.*, 2014). However, the degree to which folivory affects floral VOCs and pollinator attraction is likely dependent on the herbivore and plant species involved (i.e. these plant species may lack or have low induced responses in flowers from leaf damage) and the magnitude of feeding damage. It is important to note that in this study we selected a generalist caterpillar species that is not found naturally in this system and restricted its feeding time on these plants to maintain the level of leaf damage at a low level that is typically observed for these species. Thus, the minimal effects of herbivory in this study may be due in part to low levels of damage. Future studies may consider a wider range of feeding levels, different herbivore species, and direct florivory, especially given positive relationships observed between damage levels and VOCs (e.g. Gouinguéné *et al.*, 2003; Copolovici *et al.*, 2011).

Are changes in visual or olfactory cues driving changes in pollinator visitation? Pollinator visitation to

C. rotundifolia and *P. recta* appears to be driven by both reductions in floral visibility and shifts in the composition of floral VOCs. By contrast, pollinator visitation to *H. villosa* and *P. hastata* appears to be influenced primarily by shifts in the composition of VOCs induced by leaf herbivory and drought respectively. For *H. villosa*, although drought reduced floral visibility, only leaf herbivory reduced pollinator visitation per flower (Table 2), suggesting that herbivore-induced changes in VOC composition are affecting pollinator attraction. For *P. hastata*, drought reduced floral visibility but increased pollinator visitation per flower, again possibly acting through changes in VOC composition. Some volatile compounds that were affected by drought and/or herbivory are known to influence pollinator behavior including limonene and β -myrcene (Byers *et al.*, 2014), benzaldehyde (Theis, 2006), eucalyptol and α -pinene (Schiestl & Roubik, 2003), further supporting changes in VOCs as a mechanism underlying pollinator choice in this study. Plant VOCs are highly responsive to the environment and represent information-rich signals that can provide details about the status of the emitting plant to foraging insects (e.g. Ponzio *et al.*, 2014). Therefore, it is perhaps not surprising that VOCs might provide a more predictive understanding of changes in species interactions caused by environmental change.

In summary, for all forbs tested (four species), experimental drought universally reduced flower size and floral display, but there were *species-specific* effects of drought on volatile emission per flower, the composition of compounds produced, and subsequent pollinator visitation rates. Moreover, the community of pollinating visitors was influenced by drought across plant species (i.e. some pollinator species were deterred by drought while others were attracted). Given that specific ratios of each VOC can convey information to pollinators (Raguso, 2008c), it is not surprising that environmentally mediated shifts in VOC composition have strong effects on the identity and frequency of pollinating visitors, and changes in floral VOCs may be a mechanism that pollinators use to differentiate plants undergoing water stress. If water stress impedes the ability of pollinators to locate floral resources in their environment or if floral resource quality and quantity is lower in floral species they chose to visit based on VOCs under water stress, pollinators may be negatively affected as the frequency and severity of droughts increase due to climate change. Together, these results indicate that VOCs may provide more nuanced information to potential floral visitors and may be relatively more important than visual traits for pollinator attraction, particularly under shifting environmental conditions.

Plant–pollinator interactions are essential to the health of natural ecosystems, and much of the human diet depends on animal pollination (Winfrey *et al.*, 2011). Because of this importance, the worldwide decline in pollinators (e.g. Potts *et al.*, 2010; Burkle *et al.*, 2013; Kerr *et al.*, 2015), and additional threats posed by climate change, a more complete understanding of the mechanisms shaping plant–pollinator communities is needed to protect and restore ecosystems and their services (Menz *et al.*, 2011). We illuminated such mechanisms by combining experimental and observational approaches to examining floral VOCs and pollinator attraction. This novel perspective and our focus on floral VOCs as cues for pollinators hold considerable potential to advance our understanding of the structure and function of plant–pollinator interactions under climate change. Additional research is needed to explore the roles that stress-affected VOCs play in pollinator behavior as well as investigating the degree to which these patterns hold for other plant species. Looking forward, such approaches will be important for providing information essential to address far-reaching questions about how the interactive effects of climate change impact entire communities through interlinked networks, including pollinators and herbivores.

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References

- Agrawal AA, Kotanen PM (2003) Herbivores and the success of exotic plants: a phylogenetically controlled experiment. *Ecology Letters*, **6**, 712–715.
- Bidart-Bouzat MG, Imeh-Nathaniel A (2008) Global change effects on plant chemical defenses against insect herbivores. *Journal of Integrative Plant Biology*, **50**, 1339–1354.
- Bruinsma M, Lucas-Barbosa D, ten Broeke CJM, van Dam NM, van Beek TA, Dicke M, van Loon JJA (2014) Folivory affects composition of nectar, floral odor and modifies pollinator behavior. *Journal of Chemical Ecology*, **40**, 39–49.
- Burkle LA, Irwin RE (2010) Beyond biomass: measuring the effects of community-level nitrogen enrichment on floral traits, pollinator visitation and plant reproduction. *Journal of Ecology*, **98**, 705–717.
- Burkle LA, Marlin JC, Knight TM (2013) Plant–pollinator interactions over 120 years: loss of species, co-occurrence, and function. *Science*, **339**, 1611–1615.
- Byers KJRP, Bradshaw HD, Riffell JA (2014) Three floral volatiles contribute to differential pollinator attraction in monkeyflowers (*Mimulus*). *The Journal of Experimental Biology*, **217**, 614–623.
- Carroll AB, Pallardy SG, Galen C (2001) Drought stress, plant water status, and floral trait expression in fireweed (*Epilobium angustifolium*) (Onagraceae). *American Journal of Botany*, **88**, 438–446.
- Cook BI, Ault TR, Smerdon JE (2015) Unprecedented 21st century drought risk in the American Southwest and Central Plains. *Science Advances*, **1**, e1400082.
- Coplovic L, Kännaste A, Rimmel T, Vislap V, Niinemets U (2011) Volatile emissions from *Alnus glutinosa* induced by herbivory are quantitatively related to the extent of damage. *Journal of Chemical Ecology*, **37**, 18–28.

- Curran ED, Labandeira CC, Wilf P (2010) Fossil insect folivory tracks paleotemperature for six million years. *Ecological Monographs*, **80**, 547–567.
- Dai A (2013) Increasing drought under global warming in observations and models. *Nature Climate Change*, **3**, 52–58.
- De Moraes CM, Mescher MC, Tumlinson JH (2001) Caterpillar-induced nocturnal plant volatiles repel conspecific females. *Nature*, **410**, 577–580.
- Delph LF, Johannsson MH, Stephenson AG (1997) How environmental factors affect pollen performance: ecological and evolutionary perspectives. *Ecology*, **78**, 1632–1639.
- Dudareva N, Negre F, Nagegowda DA, Orlova I (2006) Plant volatiles: recent advances and future perspectives. *Critical Reviews in Plant Sciences*, **25**, 417–440.
- Effmert U, Dinse C, Piechulla B (2008) Influence of green leaf herbivory by *Manduca sexta* on floral volatile emission by *Nicotiana suaveolens*. *Plant Physiology*, **146**, 1996–2007.
- Farré-Armengol G, Filella I, Llusia J, Peñuelas J (2013) Floral volatile organic compounds: between attraction and deterrence of visitors under global change. *Perspectives in Plant Ecology, Evolution and Systematics*, **15**, 56–67.
- Farré-Armengol G, Filella I, Llusia J, Niinemets Ü, Peñuelas J (2014) Changes in floral bouquets from compound-specific responses to increasing temperatures. *Global Change Biology*, **20**, 3660–3669.
- Fontaine C, Guimarães PR Jr, Kéfi S *et al.* (2011) The ecological and evolutionary implications of merging different types of networks. *Ecology Letters*, **14**, 1170–1181.
- Gouinguéné S, Alborn H, Turling TCJ (2003) Induction of volatile emissions in maize by different larval instars of *Spodoptera littoralis*. *Journal of Chemical Ecology*, **29**, 145–162.
- Halpern SL, Adler LS, Wink M (2010) Leaf herbivory and drought stress affect floral attractive and defensive traits in *Nicotiana quadrivalvis*. *Oecologia*, **163**, 961–971.
- Hoover SER, Ladley JJ, Shchepetkina AA, Tisch M, Gieseg SP, Tylianakis JM (2012) Warming, CO₂, and nitrogen deposition interactively affect a plant–pollinator mutualism. *Ecology Letters*, **15**, 227–234.
- Huberty AF, Denno RF (2004) Plant water stress and its consequences for herbivorous insects: a new synthesis. *Ecology*, **85**, 1383–1398.
- Jablonski LM, Wang X, Curtis PS (2002) Plant reproduction under elevated CO₂ conditions: a meta-analysis of reports on 79 crop and wild species. *New Phytologist*, **156**, 9–26.
- Kerr JT, Pindar A, Galpern P *et al.* (2015) Climate change impacts on bumblebees converge across continents. *Science*, **349**, 177–180.
- Kessler A, Baldwin IT (2001) Defensive function of herbivore-induced plant volatile emissions in nature. *Science*, **291**, 2141–2144.
- Kessler D, Gase K, Baldwin IT (2008) Field experiments with transformed plants reveal the sense of floral scents. *Science*, **321**, 1200–1202.
- Kessler A, Halitschke R, Poveda K (2011) Herbivory-mediated pollinator limitation: negative impacts of induced volatiles on plant–pollinator interactions. *Ecology*, **92**, 1769–1780.
- Knuudsen JT, Eriksson R, Gershenzon J, Stahl B (2006) Diversity and distribution of floral scent. *The Botanical Review*, **72**, 1–120.
- Liu Y, Mu J, Niklas KJ, Li G, Sun S (2012) Global warming reduces plant reproductive output for temperate multi-inflorescence species on the Tibetan plateau. *New Phytologist*, **195**, 427–436.
- Lucas-Barbosa D, van Loon JJA, Dicke M (2011) The effects of herbivore-induced plant volatiles on interactions between plants and flower-visiting insects. *Phytochemistry*, **72**, 1647–1654.
- Menz MHM, Phillips RD, Winfree R, Kremen C, Aizen MA, Johnson SD, Dixon KW (2011) Reconnecting plants and pollinators: challenges in the restoration of pollination mutualisms. *Trends in Plant Science*, **16**, 4–12.
- Morse A, Kevan P, Shipp L, Khosla S, McGarvey B (2012) The impact of greenhouse tomato (Solanaceae: Solanaceae) floral volatiles on bumble bee (Hymenoptera: Apidae) pollination. *Environmental Entomology*, **41**, 855–864.
- Nielsen JK, Kjaer C, Schoonhoven LM (2013) *Proceedings of the 11th International Symposium on Insect-Plant Relationships*, 257 pp. Springer Science & Business Media.
- Oksanen J, Blanchet FG, Kindt R *et al.* (2012) *vegan*: Community Ecology Package. R package version 2.0-4. Available at: <http://CRAN.R-project.org/package=vegan> (accessed 21 November 2015).
- Pareja M, Qvarfordt E, Webster B, Mayon P, Pickett J, Birkett M, Glinwood R (2012) Herbivory by a phloem-feeding insect inhibits floral volatile production. *PLoS ONE*, **7**, e31971.
- Pederson GT, Graumlich LJ, Fagre DB, Kipfer T, Muhlfeld CC (2010) A century of climate and ecosystem change in Western Montana: what do temperature trends portend? *Climatic Change*, **98**, 133–154.
- Ponzio C, Gols R, Weldegergis BT, Dicke M (2014) Caterpillar-induced plant volatiles remain a reliable signal for foraging wasps during dual attack with a plant pathogen or non-host insect herbivore. *Plant, Cell and Environment*, **37**, 1924–1935.
- Potts SG, Biesmeijer JC, Kremen C, Neumann P, Schweiger O, Kunin WE (2010) Global pollinator declines: trends, impacts and drivers. *Trends in Ecology & Evolution*, **25**, 345–353.
- Raguso RA (2008a) Start making scents: the challenge of integrating chemistry into pollination ecology. *Entomologia Experimentalis et Applicata*, **128**, 196–207.
- Raguso RA (2008b) The “invisible hand” of floral chemistry. *Science*, **321**, 1163–1164.
- Raguso RA (2008c) Wake up and smell the roses: the ecology and evolution of floral scent. *Annual Review of Ecology, Evolution, and Systematics*, **39**, 549–569.
- Riffell JA, Lei H, Abrell L, Hildebrand JG (2013) Neural basis of a pollinator's buffet: olfactory specialization and learning in *Manduca sexta*. *Science (New York, NY)*, **339**, 200–204.
- Rosenblatt AE, Schmitz OJ (2014) Interactive effects of multiple climate change variables on trophic interactions: a meta-analysis. *Climate Change Responses*, **1**, 8.
- Scaven VL, Rafferty NE (2013) Physiological effects of climate warming on flowering plants and insect pollinators and potential consequences for their interactions. *Current Zoology*, **59**, 418–426.
- Scherber C, Gladbach DJ, Stevnbak K *et al.* (2013) Multi-factor climate change effects on insect herbivore performance. *Ecology and Evolution*, **3**, 1449–1460.
- Schiestl FP (2015) Ecology and evolution of floral volatile-mediated information transfer in plants. *The New Phytologist*, **206**, 571–577.
- Schiestl FP, Roubik DW (2003) Odor compound detection in male euglossine bees. *Journal of Chemical Ecology*, **29**, 253–257.
- Schweiger O, Biesmeijer JC, Bommarco R *et al.* (2010) Multiple stressors on biotic interactions: how climate change and alien species interact to affect pollination. *Biological Reviews*, **85**, 777–795.
- Simpson SJ, Mordue AJ, Hardie J (1999) *Proceedings of the 10th International Symposium on Insect-Plant Relationships*, 304 pp. Springer Science & Business Media.
- This N (2006) Fragrance of Canada thistle (*Cirsium arvense*) attracts both floral herbivores and pollinators. *Journal of Chemical Ecology*, **32**, 917–927.
- This N, Kesler K, Adler LS (2009) Leaf herbivory increases floral fragrance in male but not female *Cucurbita pepo* subsp. *texana* (Cucurbitaceae) flowers. *American Journal of Botany*, **96**, 897–903.
- Tylianakis JM, Didham RK, Bascompte J, Wardle DA (2008) Global change and species interactions in terrestrial ecosystems. *Ecology Letters*, **11**, 1351–1363.
- Vazquez DP, Bluthgen N, Cagnolo L, Chacoff NP (2009) Uniting pattern and process in plant-animal mutualistic networks: a review. *Annals of Botany*, **103**, 1445–1457.
- Wahid A, Gelani S, Ashraf M, Foolad MR (2007) Heat tolerance in plants: an overview. *Environmental and Experimental Botany*, **61**, 199–223.
- Winfree R, Bartomeus I, Cariveau DP (2011) Native pollinators in anthropogenic habitats. *Annual Review of Ecology, Evolution, and Systematics*, **42**, 1.
- Xiao Y, Wang Q, Erb M *et al.* (2012) Specific herbivore-induced volatiles defend plants and determine insect community composition in the field. *Ecology Letters*, **15**, 1130–1139.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Volatile compounds released by *Campanula rotundifolia* flowers (mean ± SE) subjected to drought, leaf herbivory, and drought + leaf herbivory.

Table S2. Volatile compounds released by *Heterotheca villosa* flowers (mean ± SE) subjected to drought, leaf herbivory, and drought + leaf herbivory.

Table S3. Volatile compounds released by *Phacelia hastata* flowers (mean ± SE) subjected to drought, leaf herbivory, and drought + leaf herbivory.

Table S4. Volatile compounds released by *Potentilla recta* flowers (mean ± SE) subjected to drought, leaf herbivory, and drought + leaf herbivory.

Table S5. Summary of pollinators observed visiting each of the treatment plant species. C is watered control, D is drought, H is herbivory, and N is no herbivore control.