

Floral volatiles from *Clarkia breweri* and *C. concinna* (*Onagraceae*): recent evolution of floral scent and moth pollination

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Abstract: *Clarkia breweri* (*Onagraceae*) is the only species known in its genus to produce strong floral fragrance and to be pollinated by moths. We used gas chromatography-mass spectrometry (GC-MS) to identify 12 abundant compounds in the floral headspace from two inbred lines of *C. breweri*. These volatiles are derived from two biochemical pathways, one producing acyclic monoterpenes and their oxides, the other leading from phenylalanine to benzoate and its derivatives. Linalool and linalool oxide (pyran form) were the most abundant monoterpenoids, while linalool oxide (furan form) was present at lower concentrations. Of the aromatic compounds detected, benzyl acetate was most abundant, whereas benzyl benzoate, eugenol, methyl salicylate, and vanillin were present as minor constituents in all floral samples. The two inbred *C. breweri* lines differed for the presence of the additional benzenoid compounds isoeugenol, methyleugenol, methylisoeugenol, and veratraldehyde. We also analyzed floral headspace from *C. concinna*, the likely progenitor of *C. breweri*, whose flowers are odorless to the human nose. Ten volatiles (mostly terpenoids) were detected at low concentrations, but only when headspace was collected from 20 or more flowers at a time. Trans- β -ocimene was the most abundant floral compound identified from this species. Our data are consistent with the hypothesized recent evolution of floral scent production and moth pollination in *C. breweri*.

A sweet, penetrating floral fragrance characterizes many moth-pollinated flowers (BAKER 1961, GRANT 1983). Volatile compounds from such flowers attract moths of several families (CANTELO & JACOBSON 1979 a, b; GABEL & al. 1992; HEATH & al. 1992), releasing visual search behaviors in sphingid moths and functioning as nectar guides and landing stimuli for noctuid moths (BRANTJES 1973, 1978).

Moth pollination has evolved independently at least four times within the angiosperm family *Onagraceae* and may be the ancestral mode of outcrossing in the tribe *Onagreae* (MACSWAIN & al. 1973, RAVEN 1979). Indeed, 53% of all outcrossing species in the *Onagreae*, including over 40 species of *Oenothera*, are moth-pollinated (RAVEN 1979). Floral scent may have played an important role in the evolution of these species. GREGORY (1963, 1964) and WAGNER (1981, 1986) have qualitatively described the floral fragrances of some North American *Oenothera* species, but to

our knowledge only the scent compounds of *Oenothera odorata* JACQU. (*Onagraceae*) and *O. erythrosepala* BORBAS. have been chemically analyzed (ZHENG & al. 1989, LIU & al. 1991).

Floral scent production is generally absent in *Clarkia*, a genus of 44 species of annuals native to western North America (LEWIS & LEWIS 1955). Although self-compatible, most diploid *Clarkia* species are outcrossers, pollinated by oligolectic bees (MACSWAIN & al. 1973). The only *Clarkia* species pollinated by moths is *C. breweri* (GRAY) GREENE (*Onagraceae*) (MACSWAIN & al. 1973, RAVEN 1979). Its pale pink, funnel-shaped flowers produce both copious nectar and a strong, sweet-spicy fragrance that is unique in the genus (MACSWAIN & al. 1973). The flowers of *C. concinna* (FISCHER & MEYER) GREENE, a species closely related to *C. breweri*, are smaller and darker pink than those of *C. breweri* and are pollinated by bees, flies, and butterflies rather than by moths (MACSWAIN & al. 1973). The flowers of *C. concinna* are scentless to the human nose, as are those of all other *Clarkia* species with the exception of *C. breweri* (LEWIS & LEWIS 1955).

The phylogenetic placement of the scented *C. breweri* within the generally non-scented genus *Clarkia* suggests that scent production in *C. breweri* is recently evolved. Based on morphological and genetic data, both *C. breweri* and *C. concinna* are placed in the derived section *Eucharidium* (LEWIS & LEWIS 1955, GOTTLIEB & WEEDEN 1979). *Eucharidium* is delimited by the presence of several traits considered apomorphic in *Clarkia*, including four stamens (all other species have eight) and a long nectar tube (LEWIS & LEWIS 1955). *Clarkia breweri* and *C. concinna* have the same haploid chromosome number ($n = 7$) and, unlike any other pair of *Clarkia* species, they can be experimentally crossed to yield partially fertile F_1 hybrids in the laboratory (RAGUSO & PICHERSKY, unpubl.). *Clarkia concinna* is found in mesic oak/pine woodlands throughout northern California, while *C. breweri* populations are restricted to a few xeric canyons at the southern edge of *C. concinna*'s range. Thus, *C. concinna* and *C. breweri* fit the well established progenitor-derivative species relationship in the genus *Clarkia* (LEWIS 1962, MACSWAIN & al. 1973).

Despite numerous surveys and discussions of floral scent production among closely related plant species (DODSON & al. 1969, THIEN & al. 1975, GREGG 1983, PELLMYR 1986, GROTH & al. 1987, LOUGHRIN & al. 1990), the biological mechanisms of floral scent evolution remain obscure. For example, little is known about the biochemical pathways, enzymes and genes that are responsible for the production of floral scent components (PICHERSKY & al. 1994), and few investigations have dealt with the action of natural selection on this character (HILLS & al. 1972, GALEN & KEVAN 1983). Since it appears likely that the floral scent of *C. breweri* evolved after *C. breweri* split from *C. concinna*, this relatively recently evolved scent may not be as complex as some other well-characterized floral scents, some of which may have in excess of 50 components (KNUDSEN & TOLLSTEN 1993). We have thus chosen *C. breweri* as a model for genetical, biochemical, and ecological studies of floral scent production. Here we report the chemical composition of its floral fragrance.

Material and methods

Plant material. *Clarkia breweri* plants were grown from seed stocks originally collected from two field locations in the Central Coast Range Mountains of California, USA. Plants

from line 1 (Fig. 1) were derived from seeds collected in May 1990 from a population on Mt Hamilton, located 5 km east of the summit (at the 3.14 mile marker) on San Antonio Valley Rd, Santa Clara Co., CA. Seeds used to generate line 2 (Fig. 1) were collected in May 1991 in Del Puerto Canyon, 20 km W of Patterson on Del Puerto Cyn. Rd, Stanislaus Co., CA. To minimize quantitative variation, inbred lines were prepared by self-pollinating plants from each accession for 4 generations. *Clarkia concinna* plants were grown from seeds collected by L. GOTTLIEB and V. FORD (accession G-8740) at the junction of Highway 128 and Lower Chiles Valley Rd, Napa Co., CA. Seeds were stored at 8 °C in coin envelopes surrounded by silica gel desiccant (Sigma Corp.).

Culture. Seeds were germinated in vermiculite under a 12L/12D photoperiod at 17 °C in growth chambers. After 10 days, seedlings were transferred to 60% sand : 40% Baccto

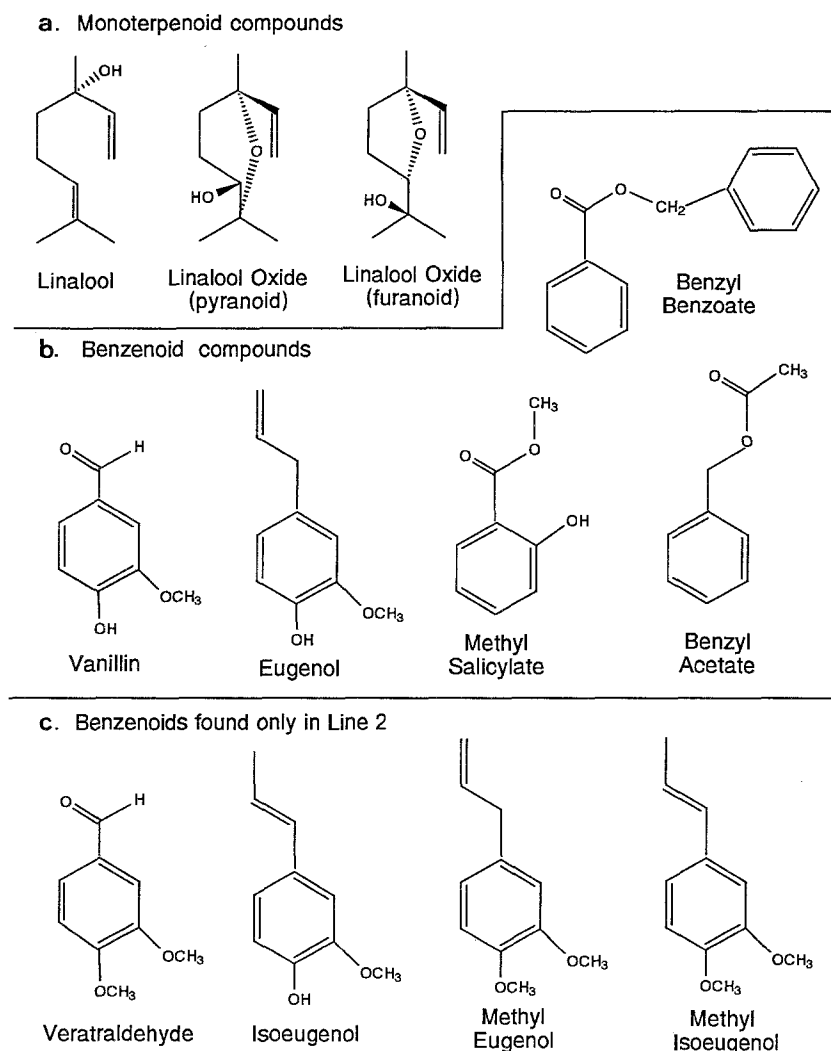


Fig. 1. Reconstructed ion chromatograms (GC-MS) of *Clarkia* floral volatiles isolated by headspaces adsorption on tenax/charcoal cartridges. Chromatograms are representative of floral volatiles from: *a* *C. breweri* line 1, *b* *C. breweri* line 2, and *c* *C. concinna*. Numbered peaks are identified in Tables 1 and 2. Peak labelled "G" represents geraniol internal standards. Unlabelled peaks represent contaminants also found in ambient controls

potting soil in 3 × 3 in. cylindrical plastic pots and moved to the Univ. of Michigan's Matthaei Botanical Gardens. After the third week the plants were placed beneath an array of high pressure 1000 Watt sodium lamps under a 14L/10D regime. The plants flowered 6–8 weeks after germination. Plants were exposed to greenhouse temperatures that approximated the natural range of temperatures in their native habitat (15°C–25°C).

Volatile collection. Flowers of *C. breweri* typically open in the morning and remain open for 4 to 6 days. Pollen deshisces during the first evening after anthesis and stigma lobes recurve and become receptive during the second evening. Floral scent production begins immediately at anthesis, reaches a peak during the second day and declines markedly within 24–36 h after pollination (PICHERSKY & al. 1994).

Floral scent components were concentrated by enclosing a single (unless otherwise indicated) living, two-day-old hermaphroditic flowers within 0.5 L cylindrical plexiglas vessels supported by ring stands. Air spaces surrounding petioles were sealed with aluminium foil gaskets. Incoming air was drawn over filters of activated charcoal into the plexiglas chambers, over the flowers and through glass adsorbent columns by an LKB (2016 Vacugene) electrical vacuum pump at a flow rate of 1 L air/min. This flow rate exceeded rates cited in other floral headspace studies but was found to maximize sample concentration of volatiles.

Collection of floral scent compounds began at dusk and proceeded for 24 h under greenhouse conditions. Volatile collections were repeated for 23 *C. breweri* plants and for 9 plants of *C. concinna*. Glass columns (7 mm int. D) were packed with 200 mg of activated SK-4 carbon and 100 mg of Tenax TA adsorbent (both 60/80 mesh, Alltech, Inc.) and plugged with silanized glass wool. Adsorbents were pre-conditioned with numerous washes of hexane and ethyl ether, then dried at ambient temperature. Adsorbent columns were covered with aluminum foil and connected to ducts in plexiglas concentration vessels by Tygon tubing, oriented so that volatile compounds passed first through Tenax, then through activated charcoal (*sensu* WILLIAMS & WHITTEN 1983). Glass Y-tubes were used to facilitate simultaneous collections of volatiles from vegetative and ambient sources, which were later subtracted from floral GC-MS profiles.

GC-MS analyses. After floral scent collection, adsorbent columns were eluted with 2.4 ml of HPLC-grade hexane and the eluant stored in teflon-capped glass vials at – 20 °C. Samples were prepared for GC-MS by concentrating to 75 µl with a stream of purified air at ambient temp. An internal standard of 0.2% v/v geraniol was added to all samples prior to injection in order to correct for sample preparation artefacts. Samples (4 µl) were analyzed using a Finnigan 9610 gas chromatograph (splitless injector at 200 °C, temp. programmed at 50 °C for two min increasing to 275 °C at 15 °C/min) equipped with a DB 5 column (30 m; 0.32 mm int. D, 1 µm film thickness). A Finnigan 4021 mass spectrometer (1 sec/scan, m/z 35–500, 2 kV) was used as a detector.

Volatiles were tentatively identified through an on-line MS library, then confirmed by comparison of retention times and mass spectra with those of known standards (Aldrich, Inc.). Relative abundances of volatiles were determined by comparison of RIC (reconstituted ion chromatogram) values with calibrations based on data from serial dilutions of standard concentrations.

Data analysis. In order to statistically compare differences in volatile production between the two inbred lines of *C. breweri*, we calculated µg compound per g fresh weight of flower for each volatile detected. Means were computed and found not to conform to a normal distribution (data not shown), one of the requirements for performing a T-test (ROTHMAN & ERICSON 1987). The original volatile data from each inbred line of *C. breweri* were resampled with replacement for 1000 iterations using a “bootstrapping” algorithm written in Pascal, in which mean volatile production values were calculated for each iteration and a grand mean was calculated for the entire stimulation (EFRON 1982). The standard deviations of grand means for each bootstrapped data set were used to calculate the test

statistic Z (as modified from ROTHMAN & ERICSON 1987) for each volatile in both inbred lines, such that:

$$Z = \frac{\bar{X}_1 - \bar{X}_2}{(\text{SE}_1^2 + \text{SE}_2^2)^{1/2}}$$

where \bar{X}_1 = mean $\mu\text{g/g}$ fr. wt. volatile a in line 1, \bar{X}_2 = mean $\mu\text{g/fr. wt.}$ volatile a in line 2, SE_1 = standard error for volatile a in line 1, SE_2 = standard error for volatile a in line 2.

In this case, bootstrapped standard deviations were substituted for standard errors and median values of the original volatile data were substituted for means in order to calculate Z . The latter substitution is preferable because medians are less strongly affected by outlier data points than means are. The significance of calculated Z -values was determined by consulting a table of areas beneath the normal curve (ROTHMAN & ERICSON 1987: 732).

Results

We detected 12 compounds in the floral headspace collections from *Clarkia breweri* (Fig. 1, Table 1). The monoterpene alcohol linalool and its trans-pyranoid oxide constituted major fractions of floral volatile output in both lines of *C. breweri*

Table 1. Floral headspace volatiles identified from *Clarkia breweri*

Compound	Ret. ¹ time (sec)	Peak # on Fig. 1	Relative amounts (%) ² <i>C. breweri</i> ³	
			I	II
1. Monoterpenoids				
Linalool	490	2	38.65 (9.16)	27.63 (5.57)
Trans-linalool oxide (pyranoid)	546	4	20.35 (4.19)	10.72 (1.51)
Cis-linalool oxide (furanoid)	483	1	1.67 (0.39)	0.77 (0.14)
2. Aromatics				
Benzyl acetate	535	3	22.32 (3.28)	42.15 (7.65)
Benzyl benzoate	895	12	6.80 (1.54)	3.93 (1.22)
Eugenol	666	6	5.10 (1.80)	1.32 (0.43)
Methyl salicylate	563	5	4.38 (0.63)	3.32 (0.68)
Vertraldehyde	734	10	—	5.64 (1.54)
Methyleugenol	688	7	—	1.91 (0.59)
Methylisoeugenol	744	11	—	1.44 (0.66)
Vanillin	693	8	0.72 (0.17)	0.99 (0.42)
Isoeugenol	720	9	—	0.19 (0.16)

¹ GC-MS; 50° to 275°C at an increase of 15°C/min temp. program.

² Percentages of total volatiles produced are given as means of areas beneath GC peaks followed by standard errors in parentheses. Compounds identified from one plant line but not observed in the other line are indicated by —.

³ Inbred plant lines; I = *C. breweri* line 1 (n = 13 plants), II = *C. breweri* line 2 (n = 10 plants).

examined, as did the aromatic ester benzyl acetate. Linalool (38.65%) was the most abundant scent compound detected from flowers of line 1 ($n = 13$ plants), while benzyl acetate (42.15%) dominated the headspace volatiles collected from flowers of line 2 ($n = 10$ plants). Minor constituents in both lines included linalool oxide (cis-furanoid), benzyl benzoate, eugenol, methyl salicylate and vanillin (Table 1). Four additional compounds – isoeugenol, methyleugenol, methylisoeugenol, and veratraldehyde – were detected in all flowers from line 2 but were absent in plants from line 1 (Table 1). Variation in amounts of individual scent compounds collected over 24 hours was generally high, especially for linalool and benzyl acetate in line 1 and eugenol in both lines (see Standard Errors, Table 1).

In these experiments, we collected abundant headspace compounds from single, living flowers of *C. breweri*. We did not detect any volatile compounds from similar amounts of floral tissues of the closely related species, *C. concinna* ($n = 4$ trials). Headspace analyses with 20–30 living *C. concinna* flowers (3 plants combined \times 3 trials) revealed 10 volatile compounds present at low concentrations (Fig. 1, Table 2). Three compounds – linalool, its trans-pyranoid, and cis-furanoid oxides – were shared with *C. breweri*. The remaining constituents of *C. concinna* included the aliphatic cis-3-hexenyl acetate, the cyclic monoterpenes α -pinene and limonene, the acyclic monoterpenes myrcene and trans- β -ocimene and two unidentified sesquiterpenes ($C_{15}H_{24}$, mw 204). The dominant volatile, trans- β -ocimene, constituted over 50% of total fragrance production. We did not detect any benzenoid compounds in *C. concinna* floral headspace collections. Results from recent

Table 2. Floral headspace volatiles identified from *Clarkia concinna*

Compound	Ret. ¹ time (sec)	Peak # on Fig. 1	Relative amounts (%) ² <i>C. concinna</i>
1. Monoterpenoids			
Trans β -ocimene	448	17	55.92 (10.29)
Trans-linalool oxide (pyranoid)	545	4	10.79 (1.83)
Limonene	440	16	10.53 (0.13)
Linalool	486	2	8.09 (1.04)
α -pinene	349	13	0.51 (0.06)
Myrcene	401	14	0.36 (0.07)
Cis-linalool oxide (furanoid)	582	1	0.34 (0.18)
2. Sesquiterpenes			
Sesquiterpene 2	719	19	3.82 (0.83)
Sesquiterpene 1	665	18	1.46 (0.18)
3. Aliphatic acids			
Cis-3-hexenyl acetate	410	15	8.18 (3.33)

¹ GC-MS; 50° to 275 °C at an increase of 15 °C/min temp. program.

² Percentages of total volatiles produced are given as means of areas beneath GC peaks followed by standard errors in parentheses ($n = 3$ trials, each with 3 plants, combined).

headspace analyses (data not shown) suggest that limonene, myrcene, and trans- β -ocimene also may be present at very low concentrations in the floral scent of *C. breweri*.

Relative amounts of floral scent production in two lines of *C. breweri* plants and in *C. concinna* were calculated in units of micrograms of scent compound per gram fresh weight of flower, over 24 hours (Table 3). Flowers from line 2 of *C. breweri* emitted nearly twice the amount of total floral fragrance (mean = 437.34 $\mu\text{g/g}$) as did those of line 1 (mean = 254.34 $\mu\text{g/g}$). Among compounds common to both lines, the strongest disparity was observed for benzyl acetate, which was detected in 3-fold greater quantities in flowers of line 2 than in those of line 1. Benzyl acetate was the only compound for which differences between inbred lines were found to be significantly different at the $p < 0.05$ level (Table 3). Not surprisingly, the quantity of total floral volatiles emitted by *C. breweri* plants was roughly 8–15 times greater than that emitted by *C. concinna* (mean = 30.42 $\mu\text{g/g}$).

Table 3. Volatile compound production (μg per g fresh weight) of *Clarkia* flowers

Compound	Volatile production ¹		
	<i>C. breweri</i> I	<i>C. breweri</i> II	<i>C. concinna</i>
1. Monoterpenoids			
Linalool	88.45 (21.09)	103.05 (20.78)	0.38 (0.04)
Trans-linalool oxide (pyranoid)	48.80 (10.33)	45.48 (7.60)	0.52 (0.02)
Cis-linalool oxide (furanoid)	4.06 (0.98)	3.04 (0.57)	0.02 (0.01)
Trans- β -ocimene	–	–	27.40 (1.17)
2. Aromatics			
Benzyl acetate ²	65.19 (8.22)	191.02 (35.23)	–
Benzyl benzoate	17.66 (4.06)	16.91 (5.36)	–
Methyl salicylate	13.62 (2.19)	17.28 (3.57)	–
Veratraldehyde	–	33.52 (9.01)	–
Eugenol	13.69 (4.75)	5.70 (1.86)	–
Vanillin	2.87 (0.66)	6.40 (2.69)	–
Methyleugenol	–	8.59 (2.64)	–
Methylisoeugenol	–	5.66 (2.44)	–
Isoeugenol	–	0.81 (0.70)	–
Other <i>C. concinna</i> volatiles, combined	–	–	2.07 (0.39)
Totals:	254.34 (52.28)	437.46 (92.45)	30.42 (1.63)

¹ Given as μg compound per g fresh weight flowers over 24 h, with standard errors in parentheses. Sample sizes are the same as those listed in Tables 1 and 2.

² Difference in amount of benzyl acetate production between *C. breweri* lines is significant at the $p < 0.005$ level. All other compounds shared by both lines were not signif. different at $p < 0.05$ level; see text.

Discussion

The floral scents of *C. breweri* and *C. concinna* are qualitatively and quantitatively distinct. Most of the floral volatiles of *C. breweri* are common components of a wide array of scented angiosperm flowers (KNUDSEN & al. 1993). They have been found in flowers pollinated by bees (WILLIAMS & WHITTEN 1983), beetles (THIEN & al. 1975) and butterflies (PELLMYR 1986, GROTH & al. 1987), but conform most strongly to the "white floral olfactory image" of compounds common to many night blooming and/or moth pollinated flowers such as *Jasminum sambac* (L.) AITON (*Oleaceae*; KAISER 1991), *Platanthera bifolia* (L.) RICH. (*Orchidaceae*; NILSSON 1983, TOLLSTEN & BERGSTRÖM 1989), *Plumeria rubra* f. *acutifolia* (POIR.) WOODSON (*Apocynaceae*; OMATA & al. 1991) and *Selenicereus hamatus* BRITTON & ROSE (*Cactaceae*; KAISER 1991). The strong, sweet floral scent of *Clarkia breweri* is rich in linalool and aromatic esters, but lacks the N-bearing indole and oximes found in many moth-pollinated flowers (KNUDSEN & TOLLSTEN 1993), including *Gaura drummondii* (SPACH.) TORR. & GRAY within the *Onagreae* (TERANISHI & al. 1991).

Aromatic esters such as methyl salicylate and benzyl acetate are ubiquitous floral scent components in moth-pollinated flowers (KNUDSEN & TOLLSTEN 1993). Similar compounds, such as amyl salicylate and amyl benzoate, have been shown to function as attractants to the hawkmoth species *Hyles lineata* (FABR.) (*Sphingidae*: *Lepidoptera*) and *Manduca sexta* (L.) (*Sphingidae*) in field bioassays (MORGAN & LYON 1928). Preliminary electrophysiological data suggest that the antennae of the hawkmoth pollinators of *C. breweri* (*Hyles lineata* and *Sphinx parelegans*, HENRY EDWARDS) (*Sphingidae*) are more sensitive to linalool, methyl salicylate and benzyl acetate than to the other 9 scent compounds (RAGUSO, LIGHT & PICHERSKY, unpubl.).

Although the flowers of *C. breweri* are strongly scented, unlike other moth-pollinated relatives within the *Onagraceae* (*Oenothera*, *Gaura* spp.) they do not close or wilt during the day following anthesis, nor do they exhibit any pronounced circadian fluctuations in volatile emission (PICHERSKY & al. 1994). Fragrance periodicity and diurnal floral closure appear to evolve generally as secondary modifications of moth-pollinated flowers, enhancing floral attractiveness to moths while withholding nectar and pollen from some diurnal floral visitors (STEBBINS 1970). At present, the flowers of *C. breweri* can attract and reward foraging moths but cannot exclude diurnal bees, flies, butterflies, and hummingbirds from visiting its flowers. Nevertheless, all diurnal visitors combined account for less than 20% of pollen transfer in populations of *C. breweri* while the remaining 80% is accomplished by crepuscular and nocturnal moths (RAGUSO & PICHERSKY, unpubl.).

The faint floral fragrance of *C. concinna* was discovered only when more than 20 flowers were used for headspace collections. It is more similar in chemical composition and intensity to the floral fragrance of bee-pollinated alfalfa (*Medicago sativa* L., *Fabaceae*, HENNING & TEUBER 1992) than it is to that of *C. breweri*. In addition to having a different scent profile, the total volatile output of *C. concinna* flowers was much smaller than that of *C. breweri* flowers. The lower volatile output of *C. concinna* was largely attributable to the abundant benzenoid compounds unique to the floral scent of *C. breweri*. It should be noted that volatile detection is dependent both on the collection methods used vis-à-vis the specific volatiles

and the sensitivity of the instruments. Thus, it is quite possible that both *C. breweri* and *C. concinna* emit additional volatiles not yet detected in our experiments.

However, a large disparity was also noted when only compounds common to both species (linalool and its oxides) were compared. One potential explanation for the greater levels of linalool/linalool oxide production in *C. breweri* is that its flowers (mean 0.22–0.23 g fr. wt./flower) are more than four times as large as those of *C. concinna* (0.05 g fr. wt./flower). An allometric increase in surface area and number of secretory cells in petals and styles could result in a proportional increase of linalool/linalool oxide emission without corresponding changes in enzymatic regulation. However, when scent emission is tabulated on a per fresh weight basis (Table 3), the differences between species remain profound. Thus, flower size alone does not explain the still greater than hundred-fold disparity in production of common volatiles between the two species.

Moreover, we did not encounter any specialized osmophoric structures (e.g., glandular trichomes, papillate cells, see STERN & al. 1986, 1987) associated with fragrance volatilization in either species, despite examination of floral tissues with scanning electron microscopy and histological methods. Therefore, differences in floral scent production in *C. breweri* and *C. concinna* cannot be attributed simply to floral allometry or to the presence of novel morphological structures, as might be expected in an older taxon, but must have arisen via changes in enzymatic regulation (PICHERSKY & al. 1994). These data are consistent with the hypothesized recent origin of floral scent production in *C. breweri*.

The floral fragrances of the two inbred *C. breweri* lines also differed from each other both qualitatively and quantitatively. We observed intra-specific variation for a modification reaction in the flowers: namely, the methylation of a para-hydroxy group on a benzene ring. Individuals from line 2 (Fig. 1) of *C. breweri* produced the para-methoxy aromatic compounds methyleugenol, methylisoeugenol, and veratraldehyde. In contrast, only the para-hydroxy compounds eugenol and vanillin were detected from plants of line 1 (Fig. 1). YAZAKI & al. (1991) described substrate-specific enzymatic conversion of para-coumaric acid to para-hydroxybenzaldehyde and para-hydroxybenzoate in *Lithospermum* plants (*Boraginaceae*). Similarly, para-specific methyl transferases might be responsible for the synthesis of methyleugenol, methylisoeugenol, and veratraldehyde from eugenol and vanillin precursors in flowers of *C. breweri* plants from line 2. We also detected isoeugenol in flowers from line 2, but not in those from line 1. Intraspecific variation for production of similar compounds (isoeugenol and 1-methoxy-4-[1-propenyl]benzene) has been reported from flowers of *Cimicifuga simplex* WORMSK. (*Ranunculaceae*), in which divergent scent chemotypes are strongly correlated with the retention of different pollinators on flowers (PELLMYR 1986). We have not yet addressed this possibility in *Clarkia breweri*. Quantitative differences in emission were also observed between the two lines, as significantly greater levels of benzyl acetate were produced in flowers of line 2 (Table 3). Increased production of benzyl acetate and the presence of four novel scent compounds in flowers of line 2 adequately explain the two-fold disparity in fragrance production among flowers of the two inbred *C. breweri* lines.

Examination of the chemical structure of the 12 compounds found in the floral headspace of *C. breweri* reveals that they fall into two groups (Fig. 2). In the first

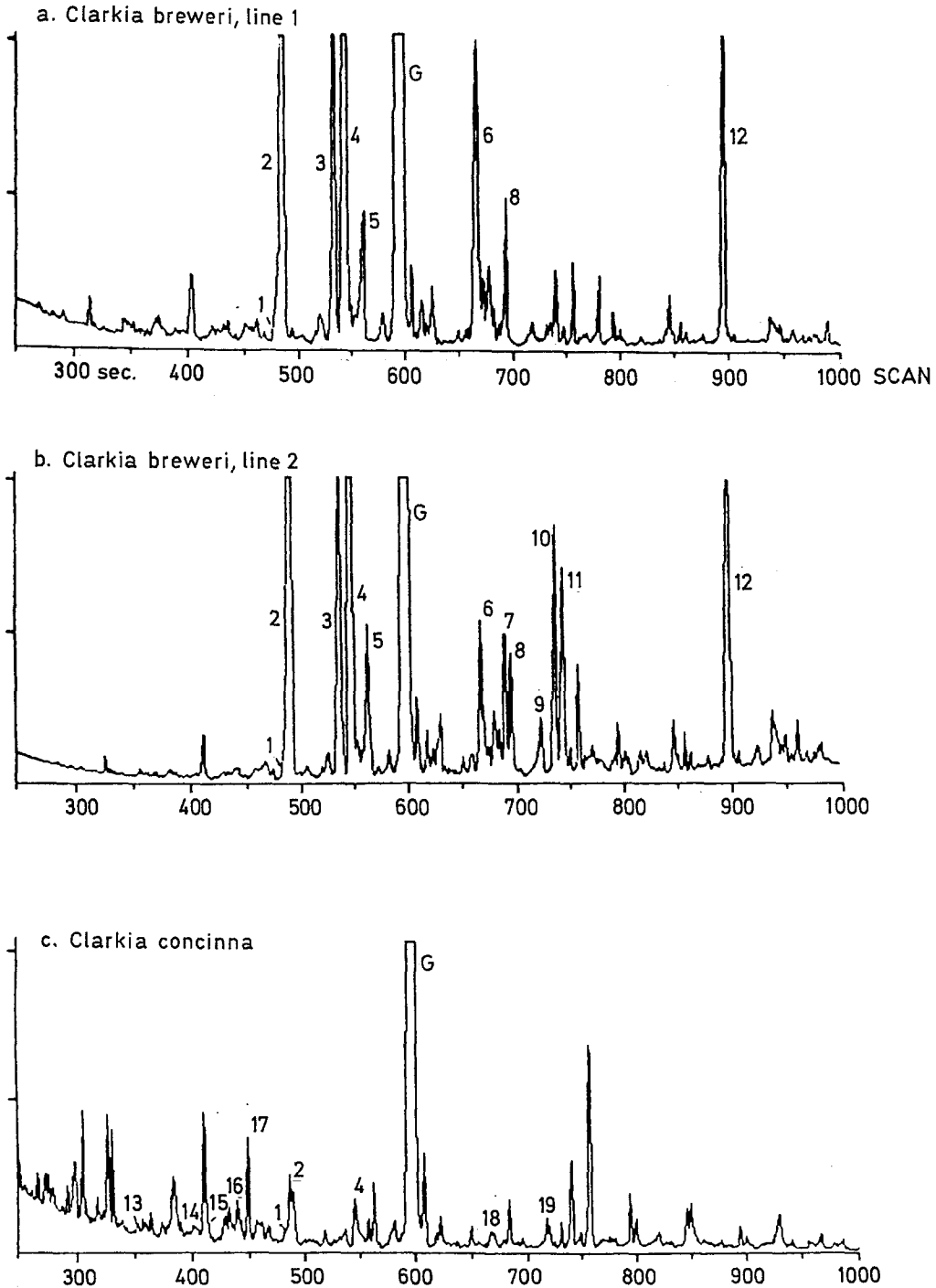


Fig. 2. Chemical structures of floral scent compounds from *Clarkia breweri*: *a* monoterpene compounds, *b* benzenoid compounds, and *c* benzenoids found only in line 2

group are the monoterpenoids; linalool and the two linalool oxides. Linalool is produced in plants from the universal monoterpene precursor, geranyl pyrophos-

phate (GPP), by a single enzymatic reaction (PICHESKY & al. 1994). Both linalool oxides presumably could be produced from linalool via a 6,7 peroxide intermediate. These three compounds are also produced at much lower concentrations in flowers of *C. concinna*, along with several other cyclic and acyclic monoterpenes and two sesquiterpenes. The most abundant of these, ocimene, is an acyclic monoterpene that may be derived from linalool via linalyl pyrophosphate (CORI & al. 1986).

The remainder of the *C. breweri* floral volatiles are aromatic compounds with a common benzene skeleton. While there is no general agreement on the enzymatic steps that lead from phenylalanine to benzoate, the major intermediates in the pathway are known (YAZAKI & al. 1991, SCHNITZLER & al. 1992). The nine aromatic compounds found in the floral scent of *C. breweri* appear to be modified forms of one or another of these intermediates. Three are aromatic esters (benzyl benzoate, benzyl acetate, and methyl salicylate) and the other six are methoxy-containing compounds (eugenol, isoeugenol, methyleugenol, methylisoeugenol, vanillin, and veratraldehyde).

Thus, it appears that only two biosynthetic pathways are responsible for floral scent production in *C. breweri*. Since both are general pathways in plants, the evolutionary changes leading to strong scent production in *C. breweri* are most likely to have involved enzymatic up-regulation within existing pathways or blockage of such pathways at critical positions. By either mechanism, intermediate metabolites could accumulate and undergo additional modification, such as the putative methylation of para-hydroxy aromatic compounds described above. Further research will be required in order to elucidate the genetical and biochemical details of these mechanisms.

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