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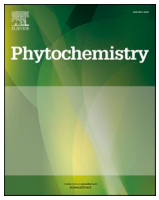

Organ-specific volatiles from Sonoran desert *Krameria* flowers as potential signals for oil-collecting bees

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ABSTRACT

The evolution of flowers that offer oils as rewards and are pollinated by specialized bees represents a distinctive theme in plant-pollinator co-diversification. Some plants that offer acetylated glycerols as floral oils emit diacetin, a volatile by-product of oil metabolism, which is utilized by oil-collecting bees as an index signal for the presence of floral oil. However, floral oils in the genus *Krameria* (Krameriaceae) contain *β*-acetoxy-substituted fatty acids instead of acetylated glycerols, making them unlikely to emit diacetin as an oil-bee attractant. We analyzed floral headspace composition from *K. bicolor* and *K. erecta*, native to the Sonoran Desert of southwestern North America, in search of alternative candidates for volatile index signals. Using solid-phase microextraction, combined with gas chromatography-mass spectrometry, we identified 26 and 45 floral volatiles, respectively, from whole flowers and dissected flower parts of these two *Krameria* species. As expected, diacetin was not detected. Instead, *β*-ionone emerged as a strong candidate for an index signal, as it was uniquely present in dissected oil-producing floral tissues (elaiophores) of *K. bicolor*, as well as the larval cells and provisions from its oil-bee pollinator, *Centris cockerelli*. This finding suggests that the floral oil of *K. bicolor* is perfused with *β*-ionone in its tissue of origin and retains the distinctive raspberry-like scent of this volatile after being harvested by *C. cockerelli* bees. In contrast, the elaiophores of *K. erecta*, which are not thought to be pollinated by *C. cockerelli*, produced a blend of anise-related oxygenated aromatics not found in the elaiophores of *K. bicolor*. Our findings suggest that *β*-ionone has the potential to impact oil-foraging by *C. cockerelli* bees through several potential mechanisms, including larval imprinting on scented provisions or innate or learned preferences by foraging adults.

1. Introduction

The chemistry of floral display – the pigments and volatile compounds by which flowers advertise the presence of nectar, pollen, or other nutritious rewards – represents a major axis of phytochemical diversification (Pichersky and Raguso, 2018; Nadot and Carrive, 2021). Floral colors and scents often attract nectar- or pollen-seeking animals by exploiting pre-existing sensory biases or preferences learned during foraging (Raine and Chittka, 2007; Leonard and Papaj, 2011; Schiestl and Johnson, 2013; Russell et al., 2018).

There are many categories of honest signals. Conventional signals (*sensu* Guilford and Dawkins, 1995), so named for their arbitrary, statistical associations with floral rewards, are deemed to be honest when they reliably predict the presence of a reward (Wright and Schiestl, 2009). For example, phenylacetaldehyde, a floral volatile common to at least 29 angiosperm families (Knudsen et al., 2006), was shown to have the strongest statistical association with nectar and pollen in *Brassica rapa* flowers and, accordingly, was shown to be acquired as a learned preference when *Bombus terrestris* bees foraged *ad libitum* from *B. rapa* flowers (Knauer and Schiestl, 2015). The arbitrary association of conventional floral signals with rewards in some plants also allows them to be employed deceptively by other plants whose flowers lack nectar or

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unconcealed pollen (Renner, 2006; Salzmann et al., 2007). Such food-deceptive strategies can enhance plant fitness through siring success, often at the expense of pollinator foraging efficiency (Jersakova and Johnson, 2006; Castillo et al., 2012).

Floral signals may also be considered honest if they emanate directly from the floral rewards, as has been described for scented pollen (Dobson and Bergström, 2000), scented nectar (Raguso, 2004) and colored nectar (Hansen et al., 2007; Roy et al., 2022). Such cases may be considered floral examples of index signals, which are far less likely to be deceptive (Maynard-Smith and Harper, 1995), if they are inexorably linked to floral rewards through genetic, developmental, or metabolic relationships, as opposed to being maintained by selection as honest signals of quality due to the physiological costs of signal production (Weaver et al., 2017). Floral index signals are thought to be adaptive for the plant when pollinator services are limiting, for example in the colored floral nectar of some plant species endemic to oceanic islands, where pollinators may be rare or endangered (Hansen et al., 2006).

In chemical ecology, index signals are best known from insect communication systems in which volatile pheromones indicate male quality to choosy females (when derived from larval-sequestered pyrrolizidine alkaloids in tiger moths; Iyengar et al., 2001) or signal female reproductive state to male burying beetles (via shared biosynthetic pathways with juvenile hormone; Engel et al., 2016). From a pollinator's perspective, a signal's reliability is paramount when floral resources are scarce or expensive to acquire, allowing foragers to accurately evaluate the presence of floral rewards without expending additional time and energy while visiting the flower (Howell and Alarcón, 2007). In such circumstances, sensory cues created by nectar itself (e.g. UV nectar fluorescence [Thorp et al., 1975] or humidity gradients produced by nectar [von Arx et al., 2012]) might provide more reliable (i.e. less "cheatable") indicators of nectar presence than would conventional signals such as floral colors or scents, which can be more easily decoupled from the presence of nectar (Ackerman et al., 2011).

Nutritious floral rewards are not limited to nectar and pollen (rev. by Simpson and Neff, 1981). Vogel (1969, 1974) described a highly specialized plant-pollinator mutualism between plants that secrete non-volatile floral oils (acetylated glycerols or free fatty acids of moderate [C16-18] chain length) and bee pollinators that collect floral oils as rewards (rev. by Buchmann, 1987; Machado, 2004; Renner and Schaefer, 2010; Neff and Simpson, 2017). Although less common than the collection of nectar rewards, floral oil collection by bees has evolved at least seven times, and, in plants, oil as the floral reward has arisen independently at least 28 times (Schäffler et al., 2015). Globally, the mutualism between oil flowers and oil bees involves ~1700 species in 10 angiosperm families (Schäffler et al., 2015; Neff and Simpson, 2017), and 370 species in two families of oil-harvesting bees (Apidae, Melittidae), representing 2.2% of described bee species (Danforth et al., 2019).

Diffuse coevolution is thought to have contributed to diversification in oil plant lineages (e.g. over 300 species of *Calceolaria* [Calceolariaceae]; Cosacov et al., 2009) and oil bee lineages (with more than 250 described species of *Centris* bees [Anthophorinae; Apidae]; Vivallo, 2020; Martins et al., 2015; Martins and Melo, 2015) in the Americas. For instance, oil flowers such as those present in the family Malpighiaceae present oils in glands termed "elaiophores" (Vogel, 1969), a trait that is expressed in nearly half of the 1300+ described species in this family (Vogel, 1974; Anderson, 1979). Similar structures furnishing floral oils have evolved independently in many plant lineages. Floral oils are harvested by female *Centris* and *Epicharis* bees to be used in nest construction or combined with nectar and pollen as provisions for larval brood cells (Buchmann, 1987; Neff and Simpson, 2017; Sabino et al., 2020). Parallel co-diversification has resulted in the evolution of ~140 spp. of oil plants in Southern Africa, including ~70 spp. in the genus *Diascia* (Scrophulariaceae) (Vogel, 1974; Steiner and Whitehead,1991) and 54 spp. of orchids in the Coryciinae (Pauw, 2006; Waterman et al., 2009), along with a smaller radiation (~25 spp) of specialized oil bees in the genus *Rediviva* (Melittidae) (Vogel and Michener, 1985; Steiner and Whitehead, 1991; Kahnt et al., 2017).

The non-volatile chemistry and often concealed location of floral oils raise the question of whether oil bees routinely use conventional signals to find and utilize oil flowers, or whether the non-volatile oils might reveal themselves to oil-foraging bees through volatile index signals related to their biosynthetic pathways. Dötterl et al. (2011) provided the first insights by experimentally decoupling the traits of an oil flower species (*Lysimachia punctata* L.; Primulaceae) in behavioral assays with its oil bee pollinator (*Macropis fulvipes*; Melittidae). Flower-naïve females respond more strongly to the unusual scent of *L. punctata* flowers than to their yellow coloration, but experienced females respond more flexibly to scent or color, while retaining a preference for the combined traits over scent or color presented singly (Dötterl et al., 2011). Male *M. fulvipes* bees, which do not collect oils, show a stronger visual orientation to *L. punctata* flowers, independent of experience. A subsequent study by Schäffler et al. (2015) revealed that the floral scent of *L. punctata* contains the volatile fatty acid derivative diacetin (a combination of glycerol 1,3-diacetate and glycerol 1,2-diacetate), which was shown to be attractive to *M. fulvipes* bees in behavioral assays. These authors suggested a biosynthetic link between diacetin and the acetylated glycerols (e.g. 1-[(3*R*)-acetoxystearoyl]-2/3-acetylglycerol) that are dominant constituents of floral oils in *L. punctata*. Diacetin occurs in over 80% of oil plant species surveyed globally thus far. In electrophysiological assays (electroantennograms or EAGs), the antennae of *M. fulvipes* and South African *Rediviva neliana* oil bees respond to diacetin, whereas those of related bees that do not collect oils (*Melitta haemorroidalis* and *Apis mellifera*, respectively) do not (Schäffler et al., 2015). Thus, diacetin satisfies the criteria for an index signal that reliably indicates the presence of floral oils and may constitute a private communication channel between oil plants and their bee pollinators (Schäffler et al., 2015; Castañeda-Zárate et al., 2021).

Not all floral oils share the same chemical composition. Diacetin is unlikely to serve as an index signal in oil plant lineages whose floral oils lack acetylated glycerols and thus would not inexorably produce diacetin as a volatile by-product of oil biosynthesis (Neff and Simpson, 2017). One genus in such a lineage is *Krameria* (Krameriaceae), with 18–20 spp. of root-parasitic plants distributed in subtropical deserts and other habitats across the Americas (Simpson, 1989, 2007; Simpson et al., 2004). *Krameria* floral oils lack acetylated glycerols and are uniquely characterized by β-acetoxy substitutions to free fatty acids (e.g. 3-acetoxyhexadecanoic acid, 3-acetoxyoctadecanoic acid, and 3-acetoxyeicosanoic acid) with both even- and odd-numbered carbon chain lengths varying from C13 to C22 (Seigler et al., 1978; Simpson et al., 1979; Seipold, 2004).

Given that diacetin is unlikely to be produced by *Krameria*, it is unclear whether *Centris* bees utilize conventional or index signals to find or gather oil from *Krameria* flowers. Index signals (or a close approximation) remain an option if distinctive, lipophilic volatiles are absorbed within and emitted exclusively from *Krameria* floral oils, as has been demonstrated for some floral nectars (Raguso, 2004; Howell and Alarcón, 2007). As a first step towards addressing this question, we characterized the floral volatile chemistry of *Krameria* plants in the Sonoran Desert of southeastern Arizona, USA, where *K. bicolor* (formerly *grayi;* see Simpson, 2013) S. Watson and *K. erecta* Schult are widespread and often locally abundant. The flowers of *K. bicolor* are visited by *Centris cockerelli* Fox, and sometimes *C. caesalpiniae* Cockerell and *C. rhodopus* Cockerell, the former a common Sonoran Desert bee species that visits these flowers to collect oils but not pollen or nectar. Palynological analyses of brood cell larval provisions indicate that *C. cockerelli* females gather pollen only from two species of desert leguminous trees (*Parkinsonia* spp.) and from creosote bush (*Larrea tridentata* [DC.] Coville), but do not harvest *K. bicolor* pollen as larval food (S.L. Buchmann and W. Sabino, unpublished results). Currently, little is known about the pollination biology of *K. erecta*, which we have included here because it can co-occur in the same habitats in the Sonoran Desert as *K. bicolor*, despite its only partially overlapping flowering phenology. We have

recently observed floral visitation by *Centris rhodopus* and *Centris atripes* Mocsáry (D.R. Papaj, unpublished observations), whereas we have not observed *C. cockerelli* bees visiting flowers of *K. erecta*.

Krameria flowers bear specialized elaiophores (Vogel, 1969, 1974), formed as blisters of thin-walled epidermal tissues filled with oil (Buchmann, 1987), the presence of which is fundamental for the fitness of both oil-collecting bees (larval provision) and the plant (seed set) (Carneiro et al., 2019). As in the Malpighiaceae, *Krameria* plants are pollinated by oil-specialized bees in the genus *Centris* (Apidae). Female *Centris* bees use modified front leg setal combs to rupture elaiophores, collect the viscous oil and transfer it to their hindlegs for transport back to their nests, where it is mixed with pollen and nectar to form the larval brood cell provisions. Inside their nests, female *Centris* oviposit directly onto a surface layer of floral oil covering their pollen provisions at the bottom of each brood cell. Evidence suggests that in some *Centris* species the oil may be enzymatically transformed (possibly with salivary gland secretions) into hardened waxy secretions to form the brood cell's lining (S.L. Buchmann and W. Ludger, unpublished results). It is hypothesized that these waxy cell linings derived from floral lipids serve as water-proofing and structural support for the cells (Neff and Simpson, 2017).

During field studies, we noticed that the flowers of *K. bicolor* produce a distinctive fragrance akin to ripe red raspberry fruit (*Rubus idaeus* L; Rosaceae), which is apparent to the human nose from at least 5m downwind of blooming plants. In contrast, flowers of the sympatric congener *K. erecta,* which bloom during the Sonoran Desert summer, release a qualitatively distinct – but less distinctive - sweet scent. The intense, raspberry-like fragrance of *K. bicolor* is noteworthy because the magenta flowers are relatively small (~33 mg fresh mass) and are borne mostly singly in leaf axils, rather than in congested inflorescences. Interestingly, female bees (*C. cockerelli, C. rhodopus, C. caesalpiniae*) netted at blooming *K. bicolor* plants had the same raspberry-like aroma as the flowers of *K. bicolor*, and the provisioned brood cells of *C. cockerelli* and *C. caesalpiniae* smell strongly of raspberry when cut open, even when they are one year old (e.g. from emerged brood cells the following spring; S.L. Buchmann, personal observation).

We aimed to characterize the volatile organic compounds (VOCs) that oil collecting *C. cockerelli* bees could potentially use to locate patches of blooming *Krameria* plants and their individual flowers. We collected headspace from intact flowers of *K. bicolor* and, for comparison, the co-occurring *K. erecta,* and used gas chromatography-mass spectrometry (GC-MS) to separate and identify the components of volatile blends. In addition, we characterized volatiles emitted by dissected flower parts, including the specialized elaiophores known to secrete floral oils in *Krameria*. Finally, we evaluated volatile profiles associated with brood cells and larval provisions (a mix of pollen, nectar and oil) of *C. cockerelli* bees, to determine whether floral volatiles found in *K. bicolor* also occurred in those brood cells, presumably absorbed within the collected oils.

2. Results and discussion

2.1. Volatile organic compounds identified from Krameria floral headspace

We identified 26 volatile organic compounds (VOCs) from the floral headspace of *Krameria bicolor* and 45 VOCs from flowers of *K. erecta* (Fig. 1; Table S1). These compounds include a rich variety of aliphatic alcohols, ketones, aldehydes and esters as well as terpenoids and aromatic compounds (Table S1). Only two of the 30 aliphatics (n-heptanol, pentyl acetate), five of the 17 benzenoids (benzyl alcohol, 2-phenyethanol, benzaldehyde, methyl benzoate, ethyl benzoate) and two of the 25 terpenoids ((*E*)-*β*-ocimene, 6-methyl-5-hepten-2-one) identified were shared between *K. bicolor* and *K. erecta,* suggesting gross qualitative scent differences between species, as visualized in a heat map (Fig. 1). Importantly, as we expected, diacetin was not detected in any of our samples.

2.2. Species-specific and organ-specific differences in Krameria floral scent

Exploratory ordination of a Bray-Curtis index of dissimilarity revealed significant quantitative differences between the volatile headspace of *K. bicolor* and *K. erecta* (two-way ANOSIM, species: $R = 1.0$, $p = 0.0016$; flower parts: $R = 0.67$, $p = 0.0001$), visualized as distinct clusters using non-metric multidimensional scaling (NMDS; Fig. 2). SIMPER analysis revealed that the VOCs with significant contributions to the quantitative differences between species were two aromatics (1,4-dimethoxybenzene, methyl benzoate) and several sesquiterpenes (*α*-copaene, *α*-cubebene, *δ*-cadinene, germacrene D, (*E*)-*β*-caryophyllene, and gleenol; Table S2a). All these compounds are present in *K. erecta* but not in *K. bicolor,* except for methyl benzoate, which was found in both species but with a low contribution for *K. bicolor* (Fig. 1, Table S2a).

Flowers of both *Krameria* species were systematically dissected into functionally distinct flower parts, including the showy (pink) petaloid sepals, the visually contrasting zygomorphic banner petals, the sponge-like oil-producing glands (elaiophores) and the remaining sexual organs (androecium) (Fig. 3). There were significant differences among floral parts for each species (ANOSIM, *K. bicolor*: $R = 0.72$, $p = 0.0065$; *K. erecta*: $R = 0.57$, $p < 0.0001$). For *K. bicolor*, the SIMPER analysis showed that the primary differences among floral parts were due to terpenoids, including (*E*)- and (*Z*)-*β*-ocimene, linalool, (*E,E*)-*α*-farnesene, *β*-ionone and 6-methyl-5-hepten-2-one; and to the aromatics benzyl alcohol and hexyl benzoate (Table S2b). In *K. erecta*, SIMPER analysis also showed that aromatics (1,4-dimethoxybenzene, *p*-methylanisol) and terpenoids (germacrene D, *α*-cubebene, *α*-copaene, among others) contributed most to the differences observed among the dissected flower parts (Table S2c). Aliphatic compounds were present in all dissected parts of *K. bicolor*, but only in the androecium and elaiophores of *K. erecta* (Fig. 1, S1). Aromatic volatiles were not detected in *K. bicolor* banners, whereas terpenoids were found in all floral tissues of both *Krameria* species (Fig. 1, S1).

The oil-producing elaiophores of *K. bicolor* were the sole floral source for a number of volatiles, including 2,2-dimethyl-1-pentanol, the aromatics benzyl alcohol and 2-amino benzaldehyde, the sesquiterpene (*E, E*)-*α*-farnesene, and the irregular terpenoid *β*-ionone, which lends the distinctive, raspberry-like scent to the flowers of this species (Fig. 1). In contrast, the elaiophore-specific volatiles of *K. erecta* were characterized by n-heptanol, pentyl acetate, nonadecane, aliphatic ketones (1-methyl-3-methylene-2-pentanone, 2-tridecanone, 2-pentadecanone) and several oxygenated aromatics (e.g., *p*-methylanisol, methyl-*p*-anisate, benzyl acetate, *p*-anisyl alcohol, *p*-anisaldehyde), most of which were absent in the same floral organs of *K. bicolor* (Fig. 1, S1). Finally, four unidentified compounds with unusual mass spectra (Fig. S2) were associated with the elaiophores of the *Krameria* species, two in *K. bicolor* (unknowns 1 and 4) and three in *K. erecta* (unknowns 4, 5, and 6) (Fig. 1; Table S1).

2.3. Oil-rich brood cell pollen provision of Centris bees

We identified 30 volatile compounds from the headspace of opened *Centris cockerelli* brood cells and their contents (Fig. 1; Table S1). These VOCs do not show a specific pattern of differences between cell wall linings and provisions, except for 3-hydroxy-2-butanone, butyrolactone and 2-methylbutanoic acid, which were absent in the cell wall samples. These three compounds appear to be specific to bee larval food provisions (Fig. 1), whereas aromatics and terpenoids were scarce (Fig. 1). Independent studies of the brood cells of oil-harvesting centridine bees (including this population of *C. cockerelli*) have revealed the presence of a diverse brood cell microbiome community. Unpublished data (S.L. Buchmann, unpublished results) indicate the presence of *Apilactobacillus* species, along with other bacterial genera, but not yeasts. Thus, some of the brood cell VOCs identified here may be metabolic byproducts from these actively fermentative bacteria. Additionally, aromatics were

Fig. 1. Volatile organic compounds found in *Centris cockerelli* brood cells (left panel), *Krameria bicolor* flowers (middle panel), and *Krameria erecta* flowers (right panel). Compounds are grouped by classes (aliphatics, aromatics, terpenoids, miscellaneous -mis- and unknowns -unk-). Blue shading is based on total ion current GC peak area log scale (see Table S1 for more details about compound identification, retention times and Retention Indices). Abbreviations:cell + prov: cells containing food provisions; cell walls: cell walls alone; provisions: food provisions alone. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

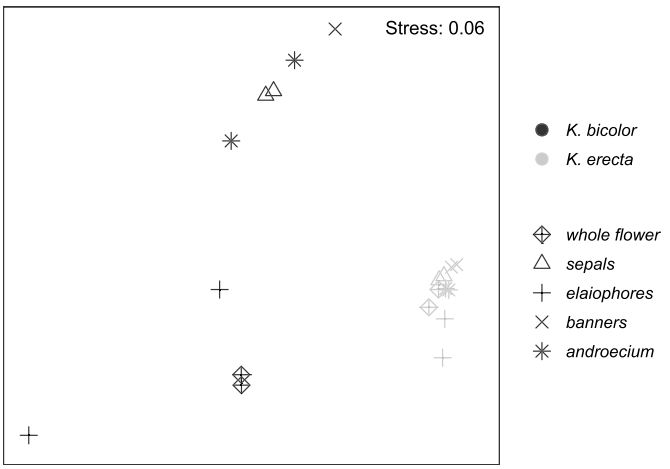

Fig. 2. Volatile organic compounds emitted by *Krameria*. Non-metric multidimensional scaling (NMDS) plot showing the relationship of the floral volatile composition between *Krameria bicolor* (black) and *Krameria erecta* (grey), and their dissected floral parts (represented by symbols).

represented by 2-phenylethanol and terpenoids by 6-methyl-5-hepten-2-one and β-ionone (Fig. 1). The latter compound provides a connection between the strong raspberry scent of the provision samples and the elaiophore-specific emissions of β-ionone by flowers of *K. bicolor*, whereas no other elaiophore-specific compounds (e.g. (*E,E*)-α-farnesene) were present in provision (Figs. 1 and 4). Although few comparative data are available, in an independent study of a nocturnal bee *Ptiloglossa latecalcarata* (Colletidae), which utilizes bat-pollinated flowers of *Caryocar brasiliense* (Caryocaraceae) as a principal source of nectar and pollen for its larvae, de Araujo et al. (2020) showed that the VOCs of the brood cells of *Ptiloglossa* differ significantly from the floral volatiles of *C. brasiliense* but, as we found, they present compounds that may be products of larval food fermentation.

2.4. Candidates for index signals in Krameria floral oils

Volatile β-ionone was uniquely attributed to the dissected elaiophores of *K. bicolor* flowers, was found in the larval provisions of the oil bee pollinator, *C. cockerelli*, and was observed to persist in subterranean nests at least one year after oil harvesting by female bees. These observations suggest that the floral oil of *K. bicolor* is perfused by β-ionone and that foraging *C. cockerelli* bees could utilize this VOC both as a distance attractant and as an intrafloral guide to locate and collect oil from elaiophores. If behavioral assays were to confirm these predictions, subsequent experiments should address whether behavioral attraction is innate or learned. Exposure to scented nest provisions during larval development creates a situation in which larval imprinting on β-ionone and subsequent scent-mediated attraction of oil-foraging adult bees to flowers of *K. bicolor* is possible (see Dobson et al., 2012). However, careful rearing experiments using provision samples with vs. without β-ionone would be needed to distinguish between the competing hypotheses of volatile imprinting of larvae vs. innate or learned preferences by adults in floral host location by *C. cockerelli* bees (see Praz et al., 2008).

The patterns described above for *K. bicolor* suggest that β-ionone (or other elaiophore-specific compounds absorbed into floral oils) could be candidates for index signals in oil flower-pollinator mutualisms lacking diacetin. There is some question as to whether volatile compounds that

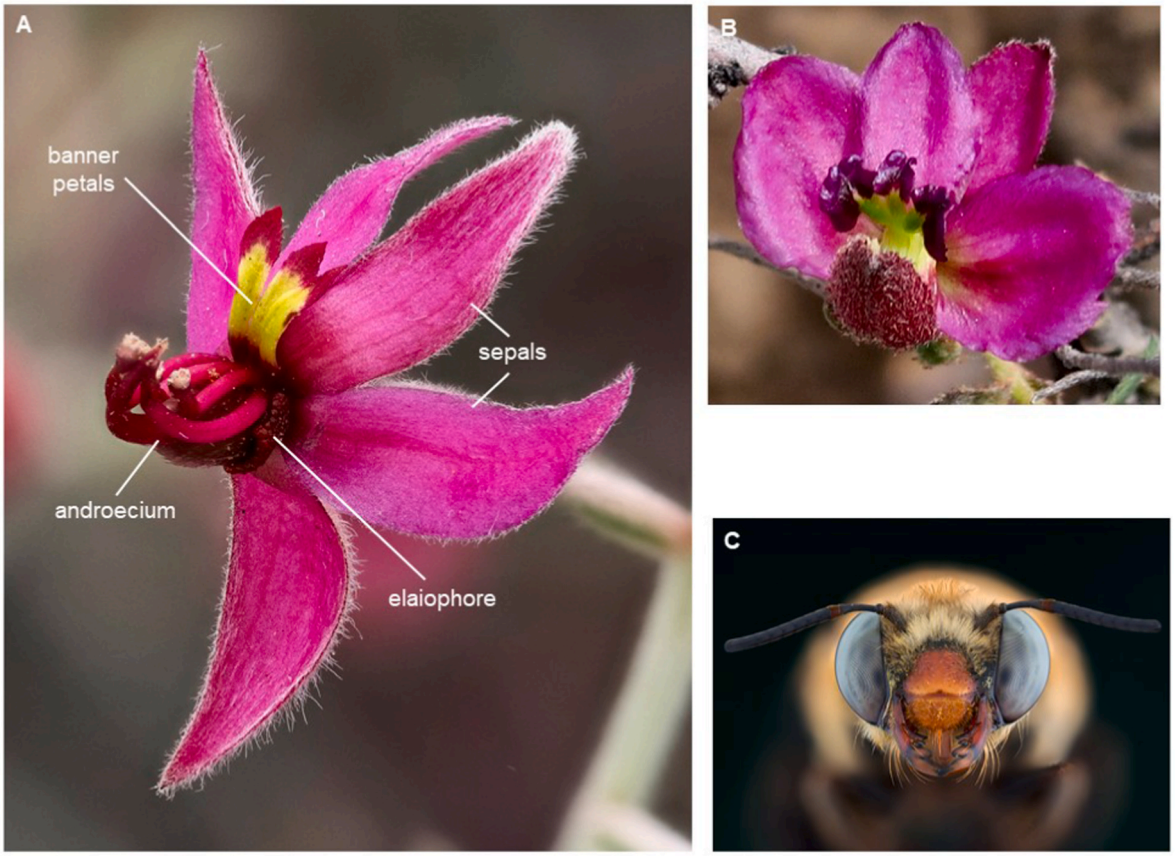

Fig. 3. *Krameria* species studied along with their main bee visitors. (A) *Krameria bicolor* flower with porose anthers extruding whitish oily pollen above and modified petals (elaiophore glands) below. (B) *Krameria erecta* flower with broad flag-like banner petals. (C) Frontal view of a female oil-collecting bee *Centris cockerelli* Fox. Figures A and C courtesy of Bruce Taubert. Figure B from Wikimedia Commons, by Daniel R. Papaj.

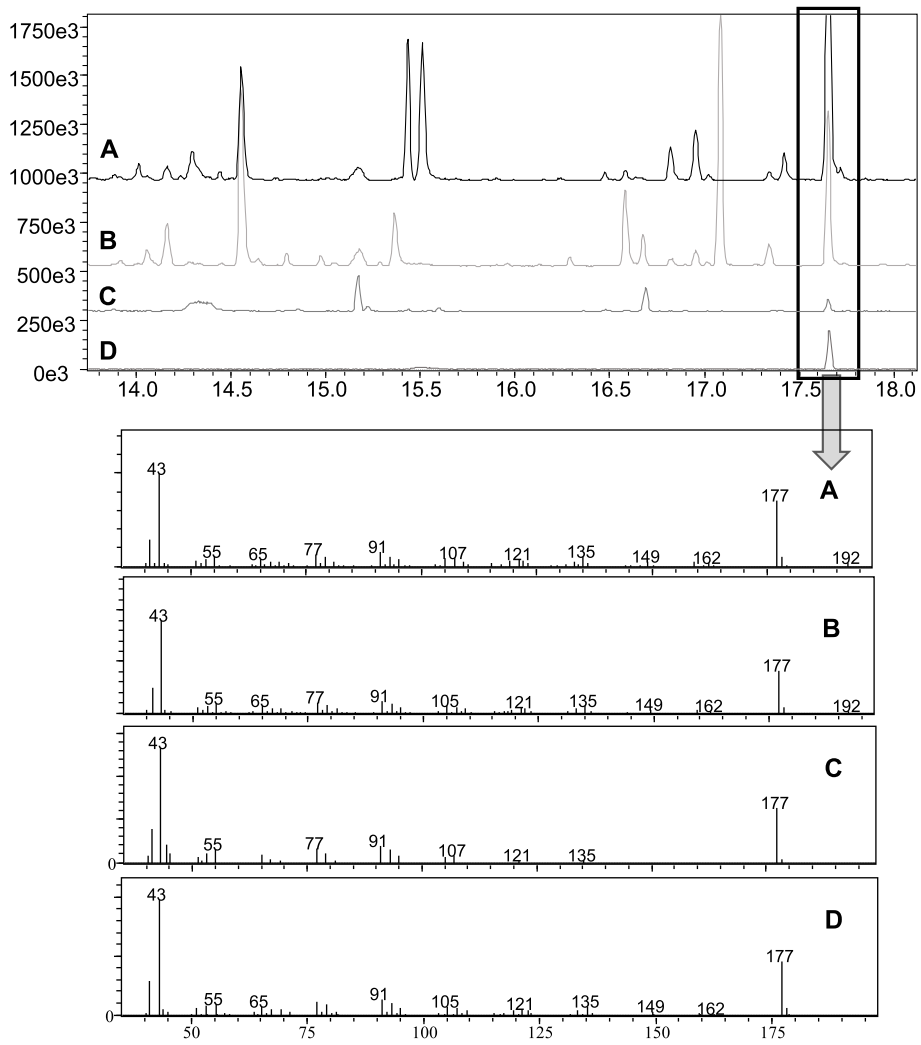

Fig. 4. Gas chromatographic (GC) and mass spectral (MS) data for β-ionone. Upper panel - total ion chromatogram (TIC) traces from *Krameria bicolor* (100 cut flowers; A), nest cell contents (oils, pollen, nectar) provisioned by *Centris cockerelli* bees (B), fresh raspberry fruit (C) and *β*-ionone authentic standard (D). Lower panel – 70 eV quadrupole EI mass spectra representing TIC peaks at retention time 17.665 min for each sample in the upper panel (A–D); data are consistent with an identification of *β*-ionone.

dissolve into liquid floral rewards (nectar or oils) are less reliable signals than VOCs biosynthetically derived from the reward, such as diacetin in flowers with acetylated glycerols. For example, (*S*)-(+)-linalool is produced exclusively in the nectar spurs of the flowers of *Penstemon digitalis* (Plantaginaceae), is dissolved in the nectar (Burdon et al., 2015) and was demonstrated to be under positive natural selection (Parachnowitsch et al., 2012). However, linalool emission was decoupled from nectar rewards when it continued to be emitted by flowers of *P. digitalis* after nectar had been experimentally removed (Burdon et al., 2020), thereby failing to satisfy the first criterion – the "unfakeability" - of index signal definition (Weaver et al., 2017). Additional assays would be required to determine whether flowers of *K. bicolor* naturally or experimentally depleted of floral oils continue to emit *β*-ionone. The second criterion, that a putative index signal such as *β*-ionone is not maintained by costs associated with condition or quality, is more difficult to test without considering the full suite of selective agents, including pollinators, herbivores, and pathogens.

Our analyses of floral scent in *K. erecta* reveal a more complex blend of elaiophore-specific volatiles, including some widespread oxygenated aromatics (Fig. 1 and Table S1). The most intriguing constituents include two para-methoxy-substituted aromatics - *p*-anisaldehyde and *p*-methylanisole – volatile compounds known to display a range of functions in plant-pollinator interactions. For example, *p*-anisaldehyde is highly attractive to honey bees (*Apis mellifera*) in the fragrance of wild thistles and other flowers (Theis, 2006), and was demonstrated to function as a conventional signal for bumble bees (*Bombus terrestris*) when experimentally associated with less variable sugar rewards (Knauer and Schiestl, 2015). In contrast, *p*-methylanisole was described as a species-specific host signal and potential "private channel" of communication between an Asian fig species (*Ficus semicordata*) and its highly specific fig wasp pollinator (*Ceratosolen gravelyi*; Chen et al., 2009), and between the oligolectic bee *Protodiscelis palpis* (Colletidae) and its aquatic host plant *Hydrocleys martii* (Alismataceae) (Torres Carvalho et al., 2014). Also, this compound attracts the florivorous scarab beetle *Cyclocephala forsteri* (Melolonthidae) to the female flowers of the macauba palm (*Acrocomia aculeata*, Arecaceae) (Maia et al., 2020). Interestingly, the elaiophores of *K. erecta* also emit 2-tridecanone, a compound that is almost as widespread as diacetin in oil flower species, and which triggers antennal responses in oil-collecting bees (Schäffler et al., 2015). Moreover, Castañeda-Zárate et al. (2021) have suggested that 2-tridecanone could be responsible of the pollinator shift, from moths to oil-collecting bees, in the African orchid *Satyrium longicauda* (Orchidaceae). As such, 2-tridecanone is a potential index signal for floral oil in *K. erecta*. Additional studies will be needed to determine which additional *Centris* bee species utilize *K. erecta* as an oil source and which floral traits they use to find and handle those flowers.

Although we lack direct behavioral studies of the pollinators of *K. bicolor* and *K. erecta* in Arizona, USA, a recent study of *Krameria grandiflora* A. St.-Hil. in Xique-Xique, Brazil provides insight into how floral traits might guide orientation and flower handling by *Centris* bees (Carneiro et al., 2019). In this study, the authors systematically excised petaloid sepals, banner petals, the combination of these floral parts, or elaiophores, then measured bee visitation, oil gathering behavior and subsequent seed set in flowers of *K. grandiflora*. Only petaloid sepal removal significantly reduced flower visitation by *Centris byrsonimae* Mahlmann & Oliveira and *C. xanthomelaena* Moure & Castro bees, whereas the removal of elaiophores eliminated oil gathering behavior and, conversely, their presence alone (when both sepals and banner petals had been excised) was sufficient to elicit oil gathering (Carneiro et al., 2019). Either the removal of sepals + petals or the removal of elaiophores resulted in loss of seed formation, suggesting that both visual display and integrated floral rewards are required for functional pollination in *K. grandiflora*. It remains to be determined how floral scent, especially the elaiophore-specific compounds, contribute to floral attraction and handling by *Centris* bees in the Sonoran Desert.

2.5. Distribution and Function(s) of β-ionone in floral scents

Apart from the elaiophores of *Krameria bicolor*, β-ionone has been reported as a floral headspace component from species in at least 14 angiosperm families (Knudsen et al., 2006). Some of these include *Narcissus* (Amaryllidaceae; Dobson et al., 1997), orchids (Orchidaceae; Gerlach and Schill, 1991; Bergström et al., 1992; Kaiser, 1993), roses (Rosaceae; Brunke et al., 1992; Flament et al., 1993), and cacti (Cactaceae; Kaiser and Tollsten, 1995). In the context of bee pollination, a recent study by Rabeschini et al. (2021) explored whether flowers pollinated by large carpenter bees (*Xylocopa*, Apidae) show any specific, shared floral scent components. Multivariate analyses revealed that two VOCs, β-ionone and (*E*)-methyl cinnamate, can be considered reliable statistical predictors of pollination by these large bees.

In addition, field assays in tropical Brazil showed that baits containing β-ionone were attractive to wild carpenter bees (Rabeschini et al., 2021). It is noteworthy that β-ionone is present as a floral scent component of the highly fragrant *Gelsemium sempervirens* (L.) (Johnson et al., 2019), which is both pollinated and nectar-robbed by *Xylocopa virginica* bees in southeastern USA (Adler and Irwin, 2006).

3. Conclusions

Our study of floral volatiles in two species of *Krameria* confirms that diacetin does not serve as a signal of the presence of floral oils in all oil plant lineages. We propose β-ionone, a volatile compound produced by floral elaiophores and present in bee provision, as an alternative index signal in the *Krameria bicolor-Centris* system. If correct, a different volatile (e.g., *p*-methylanisole, 2-tridecanone) or blend thereof might be used as a pollinator-oriented signal by the closely related *Krameria erecta*, which lacks β-ionone in its floral bouquet. The presence of key *K. bicolor* volatiles in the brood cell linings of *Centris cockerelli* along with the absence of key *K. erecta* volatiles, is consistent with field behavioral observations and phenology records suggesting that *C. cockerelli* collects floral oils from *K. bicolor* but not from the co-occurring *K. erecta*. Future studies should include electro-physiological (EAGs) and behavioral tests of these predictions, using both flower-naïve and experienced *C. cockerelli* bees and controlling for possible multi-modal interactions with floral visual display, which seem likely based on studies of *K. grandiflora* and its pollinators in Brazil (Carneiro et al., 2019).

4. Experimental

General Experimental Procedures. The headspace volatile collections were done using solid-phase microextraction fiber (SPME, Supelco) and air sampler vacuum pumps (PAS-500, Spectrex) connected to a cartridge containing Super-Q adsorbent powder. Chemical analyses were done by GC-MS using a Shimadzu GC17A gas chromatograph.

Plant materials and volatile collections. Materials from wild plants and bee nests were collected from upper Sonoran Desert thorn scrub habitats in southeastern Arizona (AZ), USA, during spring and summer, 2018. *Krameria bicolor* flowers were obtained from a population located at the Pima Community College west campus, Tucson, Pima Co. AZ (32°13'34.5"N, 111°01'07.3"W) on April 30. *Centris cockerelli* sealed brood cells were excavated from flower beds on the grounds of St. Mary's Hospital in Tucson on May 2 (32°13'36.3"N, 111°00'04.1"W). Flowers of *Krameria erecta* were gathered on August 2, in Montosa Canyon, Santa Cruz Co., AZ (31°40'32.0"N, 110°55'31.0"W). Samples were placed in plastic bags and packed into coolers with Blue Ice and mailed via courier to Cornell University (Ithaca, NY, USA), where they were refrigerated (4 °C) until chemical analysis were performed. Due to unforeseen delays in transit, analyses took place 10 days after flowers were harvested. The problem of old samples (and early degradation products) was rectified during spring, 2023 when we collected a new set of *K. bicolor* of intact, whole flower headspace volatiles in the morning of the same day that the VOC traps were purged with hexane solvent.

To evaluate spatial variation of floral volatiles, flowers from both *Krameria* species were dissected into the following component organs (per flower): 5 reflexed sepals, 2 oil-bearing elaiophores and 3 banner petals along with the remaining male and female sexual organs (androecium plus pistil), attached to the receptacle. Floral dissections coupled with chemical analysis provide insights to pollinator behavior when flowers show tissue-specific production of attractants or rewards (Dobson et al., 1999; Jürgens and Dötterl 2004; Martin et al., 2017). Dissected parts from 12 to 18 flowers of *K. bicolor*, and 18–36 flowers of *K. erecta* were pooled into 1.5 ml glass vials capped with a nylon resin oven bag gasket (Reynolds Consumer Products) for 30 min to allow equilibration. After this time, a solid-phase microextraction fiber (SPME; 65 μm, polydimethylsiloxane/divinylbenzene (PDMS/DVB); Supelco, Bellefonte, PA, USA) was exposed to the equilibrated headspace for an additional 30 min, followed by immediate gas chromatography-mass spectrometry (GC-MS) analysis. Volatiles were also collected from whole (undissected), excised flowers (28 *K. bicolor*, 30 *K. erecta*, and 100 from both species). The methodology used was the same as described above, except that for *K. bicolor*, the equilibration time was 120 min, as determined using pilot assays with different equilibration times. For all samples, ambient controls were collected to differentiate floral volatiles from background contaminants. Volatiles from flowers attached to stems, stems without flowers, and cut leaves of *K. bicolor* also were analyzed to distinguish vegetative compounds present in our floral samples.

To identify possible compounds as artifacts of floral tissue storage during transport (fermentation-related) and damage during dissection, we performed a 4 h dynamic headspace collection of flowers attached to the plants (see Table S4). The volatile collections were made in August 2018 for *K. erecta* (Montosa Canyon, AZ) and in May 2023 for *K. bicolor* (Pima Community College west campus, Tucson, AZ). Stems with leaves and flowers (15 new open flowers for *K. erecta*, and 30–66 for *K. bicolor*) were enclosed in a Reynolds (nylon resin) oven bag (16 × 13 cm) affixed with plastic ties. Volatiles were collected in a cartridge containing 10 mg of Super-Q (Alltech Associates) adsorbent powder packed with glass wool into a Pasteur pipette. Air from the headspace was pulled through the cartridge using an air sampler vacuum pump (PAS-500, Spectrex) at a flow rate of 200 ml/min. Ambient and vegetative (stems without flowers attached) control samples were collected in parallel. Trapped VOCs were eluted with 300 μl GC-MS purity hexane. Samples were packed into coolers and mailed next day via courier to Cornell University where they were concentrated to 50 μl using a stream of gaseous nitrogen and stored at −20C until chromatographic analyses were performed.

Centris *nest provisions.* To study the presence of *Krameria* related volatiles in five *C. cockerelli* nests, we sampled the headspace of brood

cells containing larval provisions (N = 5). Additionally, volatiles from isolated fragments of cell walls (N = 5) and larval provisions (N = 4) from different brood cells were collected separately. All samples were placed into 4 ml glass vials, and the procedure for headspace collection via SPME fibers was the same as described above for dissected floral parts.

GC-MS analysis. Both SPME and dynamic headspace samples were analyzed by GC-MS, using a Shimadzu GC17A gas chromatograph equipped with an EC WAX polar GC column (30 m long, 0.25 mm internal diam, 0.25 μm film thickness; Grace, Deerfield, IL, USA), operated with a constant carrier flow of 1 ml/min (ultra-high purity He), and coupled to a Shimadzu QP-5000 quadrupole mass spectrometer (electronic ionization, 70 eV) as a detector. The GC oven temperature was programmed from 40 °C (3 min), increasing by 10 °C/min, to 240 °C (5 min). The injection port temperature was 240 °C and the interface temperature was 260 °C. Peaks present in the chromatograms were integrated manually using the Shimadzu GCMS Solutions 4.45 software.

Volatile compound identifications were initially aided through the use of mass spectral libraries (NIST, Wiley) and confirmed whenever possible by matching retention times and mass spectra with those of authentic standards. Retention Index (RI) values were calculated for each compound using retention times from an n-alkane blend (C7 – C30) and compared to values derived from comparable analytical conditions, as published in the NIST webbook online database (https://webbook.nist.gov/). Compounds that could not be identified using either of these criteria were classified as "unknowns", for which the ten most abundant ion fragments from their mass spectra are provided (Table S1).

Multivariate statistical analysis. Multidimensional scaling (MDS) was used to visualize the variation in scent composition between *Krameria* species, using data generated by SPME-GC-MS. To perform the MDS, the peak area (abundance) of each compound was square root transformed to de-emphasize the contributions of the largest peak areas, and then was used to generate a Bray-Curtis similarity index (Clarke, 1993). Then, differences in scent composition among species and floral parts were compared by a two-way crossed ANOSIM (Analysis of Similarity), using 9999 random permutations to obtain the R-values (an R-value close to 1 indicates dissimilarity between groups). To assess dissimilarities of the floral parts of *K. bicolor* and *K. erecta* we performed a one-way ANOSIM for each species using the same criteria as above. When ANOSIM indicated significant differences, it was followed by a similarity percentage test (SIMPER) to evaluate the average contribution of specific compounds to the differences (see Arguello et al., 2013). All analyses were performed using the *vegan* package (Oksanen et al., 2019) of the software R 4.0.0 (R Development Core Team, 2020), except for the two-way ANOSIM done with PAST 4.07 (Hammer et al., 2001).

CRediT authorship contribution statement

Maria Sol Balbuena: Data curation, Formal analysis, Writing - original draft, Writing - review & editing. **Stephen L. Buchmann:** Conceptualization, Resources, Visualization, Writing - review & editing. **Daniel R. Papaj:** Conceptualization, Resources, Supervision, Writing - review & editing. **Robert A. Raguso:** Formal analysis, Methodology, Project administration, Resources, Supervision, Validation, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2023.113937.

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