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RESEARCH ARTICLE

Three floral volatiles contribute to differential pollinator attraction in monkeyflowers (*Mimulus*)

Kelsey J. R. P. Byers, H. D. Bradshaw, Jr and Jeffrey A. Riffell*

ABSTRACT

Flowering plants employ a wide variety of signals, including scent, to attract the attention of pollinators. In this study we investigated the role of floral scent in mediating differential attraction between two species of monkeyflowers (*Mimulus*) reproductively isolated by pollinator preference. The emission rate and chemical identity of floral volatiles differ between the bumblebee-pollinated *Mimulus lewisii* and the hummingbird-pollinated *M. cardinalis*. *Mimulus lewisii* flowers produce an array of volatiles dominated by D-limonene, β-myrcene and *E*-β-ocimene. Of these three monoterpenes, *M. cardinalis* flowers produce only D-limonene, released at just 0.9% the rate of *M. lewisii* flowers. Using the *Bombus vosnesenskii* bumblebee, an important pollinator of *M. lewisii*, we conducted simultaneous gas chromatography with extracellular recordings in the bumblebee antennal lobe. Results from these experiments revealed that these three monoterpenes evoke significant neural responses, and that a synthetic mixture of the three volatiles evokes the same responses as the natural scent. Furthermore, the neural population shows enhanced responses to the *M. lewisii* scent over the scent of *M. cardinalis*. This neural response is reflected in behavior; in two-choice assays, bumblebees investigate artificial flowers scented with *M. lewisii* more frequently than ones scented with *M. cardinalis*, and in synthetic mixtures the three monoterpenes are necessary and sufficient to recapitulate responses to the natural scent of *M. lewisii*. In this system, floral scent alone is sufficient to elicit differential visitation by bumblebees, implying a strong role of scent in the maintenance of reproductive isolation between *M. lewisii* and *M. cardinalis*.

KEY WORDS: Floral scent, Insect behavior, Antennal lobe, Olfaction, Terpene, Speciation

INTRODUCTION

Flowering plants and their pollinators are classical examples of mutualistic associations, where many plants produce flowers exhibiting traits that operate as 'advertisements' to attract specific pollinators into contact with the plant's reproductive structures. In turn, the pollinators must perceive the floral advertisements in order to receive the reward (e.g. nectar, pollen) (Kevan and Baker, 1983; Schemske and Bradshaw, 1999; Fenster et al., 2004; Raguso and Willis, 2005; Schäffler et al., 2012). One of these floral traits – scent – is particularly important in driving pollinator behavior and mediating reproduction in flowering plants (Galen and Newport, 1988; Weiss, 2001; Jürgens et al., 2003; Dobson, 2006; Raguso, 2008; Vereecken et al., 2010; Klahre et al., 2011). The contribution of scent can be very specialized; examples include the sexually deceptive orchid *Chiloglottis trapeziformis* (Peakall, 1990; Schiestl et al., 1999; Ayasse et al., 2000; Schiestl et al., 2003), where the flower releases the scent mimic of the sex pheromone produced by female *Neozeleboria cryptoides* wasps in order to attract male wasps as pollinators (Schiestl et al., 2003). Scent can also mediate differential attraction of pollinators between two closely related flower species; for example, *Petunia axillaris* emits a scent profile attractive to crepuscular moths, whereas bee- and hummingbird-pollinated *Petunia* (*P. integrifolia* and *P. exserta*, respectively) exhibit visual and olfactory characteristics that are attractive to their cognate pollinators (Hoballah et al., 2005; Klahre et al., 2011). Floral scent has also been shown to operate synergistically with the visual display of the flower – an excellent example being the combined effects of the visual and odor display of the *Ophrys heldreichii* orchid in attracting male *Tetralonia berlandi* bees (Spaethe et al., 2007). Nonetheless, for both the orchid and *Petunia* systems, scent is critical for pollinator-mediated reproduction, but for the vast majority of plant–pollinator associations the link between floral scent and pollinator attraction remains unexplored.

There are three important gaps in our understanding of the role of floral scent in mediating pollinator attraction: (1) the identity of the behaviorally effective floral volatiles; (2) the manner in which volatiles are processed by the pollinator sensory systems to drive the plant–pollinator association; and (3) the genetic basis of floral volatile production and pollinator perception to provide insight into the evolution of the mutualism. The relationship between plants and pollinators – including co-evolution, pollinator sensory bias and associative learning (Schiestl and Johnson, 2013) – is particularly important for closely related floral species whose reproductive isolation is mediated by differential pollinator preference (Fulton and Hodges, 1999; Schemske and Bradshaw, 1999; Ramsey et al., 2003; Hodges et al., 2004; Aldridge and Campbell, 2007; Klahre et al., 2011). In many such cases the composition and class of volatiles in the scents overlap (Jürgens, 2004; Svensson et al., 2006; Waelti et al., 2008; Steiner et al., 2011). How do pollinators discriminate between the different floral species, and which subset of volatiles in the floral bouquet is necessary and sufficient for mediating the differential pollinator visitation? For insects, mixtures of volatiles emitted from flowers are especially critical for eliciting behavior (Miyake and Yafuso, 2003; Riffell et al., 2009a; Riffell et al., 2009b), with specific volatile identities and ratios necessary for perception of the scent (Wright et al., 2005; Piñero et al., 2008; Najar-Rodriguez et al., 2010). Moreover, the individual chemical constituents of the floral bouquet rarely show the same potency as the complete bouquet or a synthetic mixture of a key subset of floral volatiles (Riffell et al., 2009a; Riffell et al., 2009b; Stökl et al., 2010). Modification of a few key volatiles in a flower's bouquet could potentially have strong effects on pollinator visitation and reproductive isolation in nature, but these effects are largely unknown (Parachnowitsch et al., 2012), the main exception being methyl benzoate in *Petunia* (Klahre et al., 2011).

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List of abbreviations

AL	antennal lobe
CE10	*Mimulus cardinalis* inbred line
GCMR	gas chromatography multichannel recording
GCMS	gas chromatography mass spectrometry
LF10	*Mimulus lewisii* inbred line
lim	Limonene
MC-natural	*Mimulus cardinalis* natural scent
MC-synthetic	*Mimulus cardinalis* synthetic scent
ML-natural	*Mimulus lewisii* natural scent
ML-synthetic	*Mimulus lewisii* synthetic scent
myr	β-myrcene
NMDS	non-metric multidimensional scaling
oci	β-ocimene
PSTH	peristimulus time histogram
RI	response index

To gain insight into the role of scent in mediating differential pollinator attraction in closely related flower species, we investigated two *Mimulus* (Phrymaceae) species that are models of reproductive isolation and speciation (Hiesey et al., 1971; Schemske and Bradshaw, 1999; Ramsey et al., 2003; Bradshaw and Schemske, 2003). The sister species (Beardsley et al., 2003) *Mimulus lewisii* Pursh and *M. cardinalis* Douglas ex. Benth (Fig. 1A) have overlapping ranges at middle elevation in the Sierra Nevada mountains of California, but are reproductively isolated by pollinator choice (Schemske and Bradshaw, 1999; Ramsey et al., 2003; Bradshaw and Schemske, 2003) – *M. lewisii* is pollinated by bumblebees (*Bombus* sp., largely *Bombus vosnesenskii* Radoszkowski 1862), while *M. cardinalis* is pollinated by hummingbirds. Differential pollinator attraction is responsible for 98% of the reproductive isolation between the two *Mimulus* species in sympatry (Ramsey et al., 2003). Although phenotypic traits such as visual characteristics (flower color, flower size) and reward (nectar content) have been shown to be important for differential pollinator visitation (Schemske and Bradshaw, 1999; Bradshaw and Schemske, 2003), the role of scent has never been examined. This system thus offers an opportunity to explore the sensory basis of plant–pollinator interactions by determining the minimal subset of floral volatiles necessary and sufficient to drive the olfactory and behavioral preferences of bumblebees for *M. lewisii* flowers. Ultimately, the availability of sophisticated genomic tools in *Mimulus* (Wu et al., 2008; Owen and Bradshaw, 2011; Yuan et al., 2013a; Yuan et al., 2013b) will permit elucidation of the genetic basis of reproductive isolation between *M. lewisii* and *M. cardinalis*.

In this study, we examined the olfactory mechanisms controlling the preference of bumblebees for *M. lewisii* over *M. cardinalis*. Using an integrative combination of chemical analytical, electrophysiological and behavioral methodologies, we demonstrate that three floral monoterpenes – D-limonene, β-myrcene and *E*-β-ocimene – are processed in the bumblebee's olfactory system to mediate preference for *M. lewisii* flowers, and that these three volatiles alone are necessary and sufficient to drive differential bumblebee visitation between *M. lewisii* and *M. cardinalis*.

RESULTS

Characterization of floral scent

Mimulus lewisii (inbred line LF10) and *M. cardinalis* (inbred line CE10) differ both qualitatively and quantitatively in their scent profiles (Fig. 1A). *Mimulus lewisii* produces nine volatile compounds that are exclusively monoterpenes (chiefly D-limonene, β-myrcene and *E*-β-ocimene, which together make up 93% of the total emission). By contrast, *M. cardinalis* produces five volatile compounds, chiefly monoterpenes (53% of the total emission), with the remainder comprising sesquiterpenes (31%) and 1-octen-3-ol (16%). *Mimulus lewisii* produces approximately 65 times as much

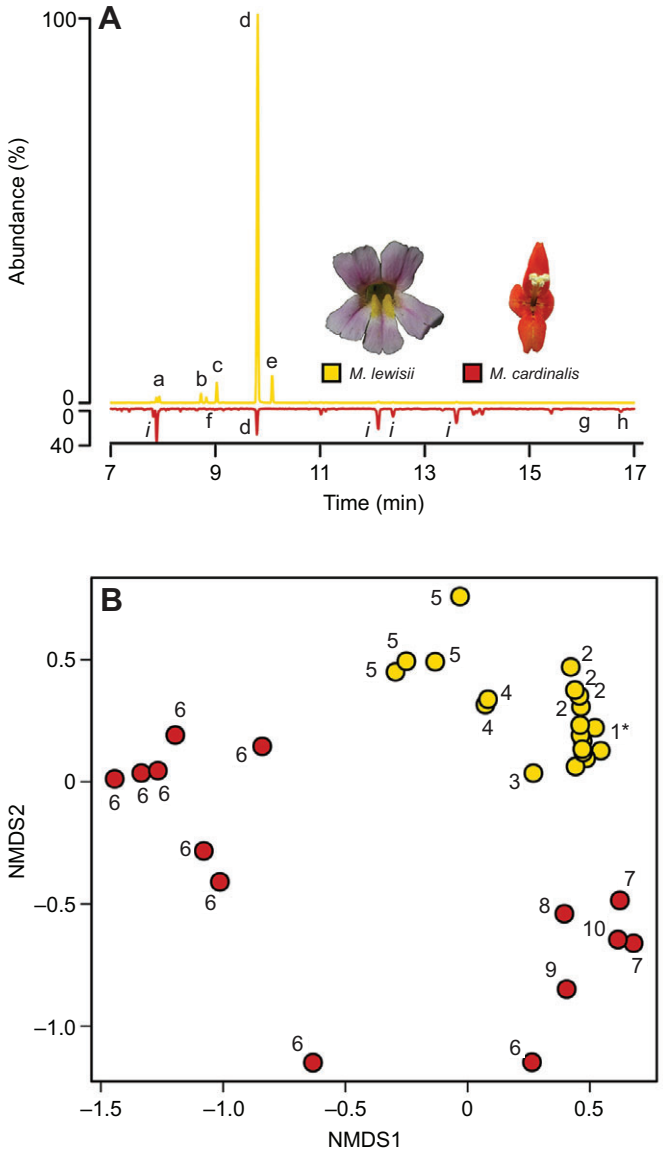

Fig. 1. Floral volatiles emitted from *Mimulus lewisii* and *M. cardinalis*. (A) Gas chromatography mass spectrometry (GCMS) analysis of floral volatiles from bumblebee-pollinated *Mimulus lewisii* (top, yellow) and hummingbird-pollinated *M. cardinalis* (bottom, red). Labels specify individual volatiles: a, α-pinene; b, sabinene and β-pinene (left and right smaller peaks, respectively); c, β-myrcene; d, D-limonene (note visible presence in both *M. lewisii* and *M. cardinalis*); e, *E*-β-ocimene. The unknown monoterpene at 7.92, γ-terpinene and terpinolene are not labeled due to low abundance. *Mimulus cardinalis*-specific volatiles include 1-octen-3-ol (f); and farnesene isomers (g and h). Notable contaminants are indicated with *i*. (B) Non-metric multidimensional scaling (NMDS) plot of the 12 volatiles present in *M. lewisii* and *M. cardinalis* (see supplementary material Table S1 for details); stress=0.088. Individual points represent single headspace collections of populations of each species: populations 1–5 represent *M. lewisii* and 6–10 represent *M. cardinalis*, with 1 and 6 representing the inbred lines used to determine scent composition for each species. The cluster (1*) on the right side of the plot indicates the close clustering of the *M. lewisii* inbred line, with nine samples in the cluster. For a list of individual populations in this figure, see supplementary material Table S1.

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total floral scent as *M. cardinalis* (mean 71±29 versus 1.1±0.9 ng flower^{-1} h^{-1}; P<0.0001, t=−7.28, d.f.=8).

Seven of the monoterpenes produced by *M. lewisii* are absent in *M. cardinalis*, including two of the three most abundant compounds (β-myrcene and *E*-β-ocimene). D-Limonene, the most abundant compound in both species, is emitted at a 107-fold higher rate in the floral bouquet of *M. lewisii* compared with *M. cardinalis* (mean 55.1±23.2 versus 0.52±0.56 ng flower^{-1} h^{-1}, P=0.00013, t=6.88, d.f.=8). The other shared monoterpene, α-pinene, is also far more abundant in *M. lewisii* (25-fold higher, P=0.0008, t=–5.28, d.f.=8). With the exception of these two monoterpenes, all other compounds are exclusive to one species or the other.

To determine whether the inbred lines are representative of their species, we collected and compared the floral bouquets among five populations of *M. cardinalis* (including inbred line CE10) and five populations of *M. lewisii* (including inbred line LF10), all originally collected from the Sierra Nevada mountains (supplementary material Table S1). All were generally consistent with the original inbred lines in both qualitative and quantitative measures (Fig. 1B; ANOSIM: *M. lewisii* versus *M. cardinalis*, R=0.7672, P=0.001; *M. lewisii* inbred line LF10 versus *M. cardinalis* inbred line CE10, R=0.9674, P=0.001; *M. lewisii* wild lines versus *M. cardinalis* wild lines, R=0.8889, P<0.01). The spread of the *M. cardinalis* inbred line CE10 in the non-metric multidimensional scaling (NMDS) plot is likely due to the occasional presence of an '*M. lewisii*' monoterpene such as sabinene at the absolute limit of detection, and does not represent an overall high variance in this inbred line. Vegetative samples from several of these populations show a much reduced emission of monoterpenes in both species, indicating that vegetation is not serving as a proxy scent source in lieu of floral volatiles (Raguso and Willis, 2003).

Antennal lobe responses to *Mimulus* floral extracts and synthetic mixtures

To identify the volatiles in the *M. lewisii* scent that elicit robust olfactory responses and thus may drive pollinator behavior, we used the floral extracts as stimuli in simultaneous gas chromatography with multichannel recording (GCMR) experiments in the bumblebee's (*B. vosnesenskii*) antennal lobe (AL). The GCMR technique allows identification of bioactive volatiles in a complex bouquet (Riffell et al., 2009a).

Using *M. lewisii* scent and the bumblebees as the detectors, we found that volatiles eluting from the GC evoked significant responses in ~40% of the recorded units (49 out of 119 total units, N=7 preparations used for GCMR experiments), with the remaining units showing no significant change in activity (Fig. 2B). We next examined the percentage of units in an ensemble that significantly responded to the nine volatiles eluting from the GC. Analysis of the

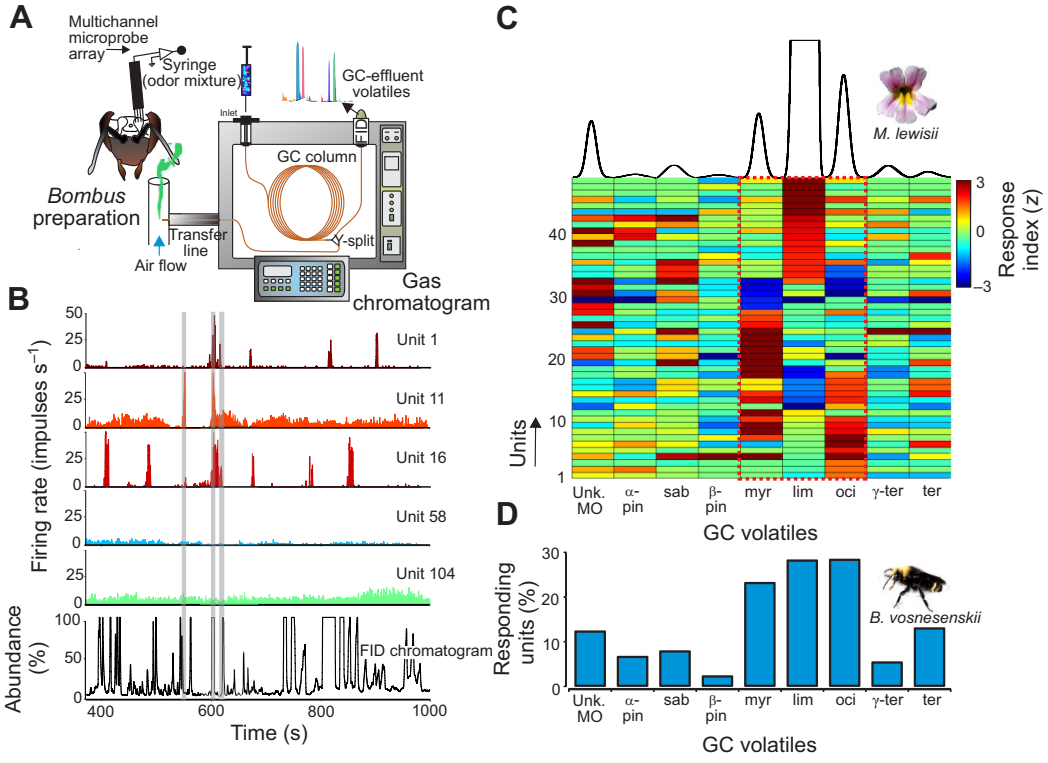

Fig. 2. Responses of *Bombus vosnesenskii* antennal lobe neurons to gas chromatography-fractionated scent from *M. lewisii* flowers. (A) Depiction of gas chromatography multichannel recording (GCMR). Effluent from the GC is split such that half enters the GC's detector (flame ionization detector, FID) while the other half arrives simultaneously at the bee's antenna. (B) Rate histograms (bin=200 ms) of neural unit responses to the eluting compounds from the *M. lewisii* headspace extract (3 µl injection). Certain volatiles evoked significant unit responses [e.g. myrcene (myr), limonene (lim) and ocimene (oci); gray bars]. However, not all units were responsive to the eluting volatiles (e.g. units 58 and 104). (C) Unit responses for each volatile eluted from the GC. The top plot shows the chromatogram with each peak corresponding to a volatile. Only those units that demonstrated significant responses (response index, RI>2.0, RI<–2.0 s.d.) are shown (color scale, bottom plot). Note that the population responses clustered around a group of three volatiles (myr, lim and oci; outlined by a red box) within the floral headspace. Volatiles are ordered corresponding to the retention time, except for those volatiles that gave robust responses (volatiles myr, lim and oci), which were rearranged for clarity. (D) The percentage of responsive units in each ensemble was determined for each volatile in the floral headspace and plotted for each preparation. A threshold of 2 s.d. of the entire data set for each species was used to identify the volatiles that evoked the greatest activity: D-limonene, *E*-β-ocimene and β-myrcene. Volatiles that evoked significant unit responses are α-pinene (α-pin), sabinene (sab), β-pinene (β-pin), terpinolene (ter), γ-terpinene (γ-ter) and an unknown monoterpene (Unk. MO).

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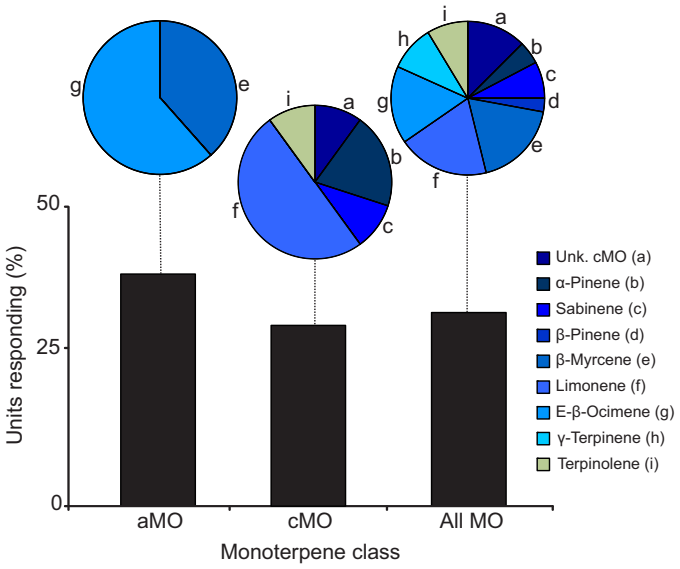

Fig. 3. Identification of bioactive volatiles and their chemical class using GCMR. Analysis of the population-level neural activity in response to the different volatiles revealed that units were responsive to acyclic monoterpenes (aMO) and cyclic monoterpenes (cMO), but units were also broadly responsive to both monoterpene types (e.g. D-limonene, ocimene and myrcene) (All MO). Pie charts at the top are the percentage of units responding to the individual volatiles; letters next to the pie charts denote the individual volatiles.

neural population showed that many units were broadly responsive to different monoterpenes, but, in particular, three monoterpenes elicited significant responses: D-limonene, β-myrcene and *E*-β-ocimene, which elicited responses in 21–27% of the total units. Further analysis of the neural population showed that these three volatiles from the floral bouquet elicited the strongest inhibitory and excitatory responses by AL units (Fig. 2C; Kruskal–Wallis test: χ^2=77.2, P<0.0001). Moreover, volatiles showed significant differences in their activation potency in the AL (Kruskal–Wallis test with multiple comparisons: P<0.05), with the three volatiles above activating significantly higher percentages of units than the other floral volatiles (Fig. 2D).

To examine whether AL units differentially responded to the different classes of monoterpenes (acyclic, cyclic), we analyzed unit responses to the volatiles eluting from the GC. The results from this analysis showed that the majority of responsive units (68%) were specifically tuned to one of the two classes of monoterpenes, whereas the remainder (~31%) were more broadly responsive across the two classes (Fig. 3; χ^2: P<0.001). However, both selective and broadly tuned units were strongly responsive to the volatiles D-limonene, β-myrcene and *E*-β-ocimene (Fig. 3).

Are the volatiles that we have identified through GCMR analysis, either singly or as a mixture, as effective as the complex natural floral bouquet? Synthetic mixtures of these three compounds at their natural concentrations and ratios in the authentic bouquet of *M. lewisii* were prepared. First, the *M. lewisii* natural floral scent (ML-natural) and synthetic mixture (ML-synthetic) evoked significant responses in individual units (Fig. 4A). Analysis of all responsive units showed that the ML-synthetic had the same percentage of responding units as the ML-natural (Fig. 5A). In addition, the ML-natural and ML-synthetic scents elicited a higher percentage of responsive units compared with both the single volatiles and the *M. cardinalis* (MC) volatiles, together suggesting that the ML scents are processed in a non-additive manner in the AL (Fig. 5A). Comparison of single unit responses support this hypothesis – more than 38% of the units showed either response suppression or synergy to the ML-synthetic relative to the single most effective volatile constituent (Fig.5B). Thus, unique responses by units may underlie the singular percept of the ML-synthetic bouquet.

To examine further the neural representation that permits the discrimination of the floral scents, the scent-evoked responses at the level of the neural ensemble were analyzed. The three-component mixture (ML-synthetic) elicited an overlapping pattern of ensemble activity to that of the ML-natural; however, both the natural *M. lewisii* and its synthetic mimic elicited different patterns of ensemble activity when compared with natural *M. cardinalis* (Fig. 4B). To investigate the relationship between the single volatiles and the floral bouquets, we examined the population responses in multivariate space (principal components analysis). For a single preparation, this analysis revealed that the ensemble responses distinctly separated the ML scents (natural and synthetic mixture), the single volatiles and the MC scent (Fig. 4C). However, responses to the ML scents may be due to the higher intensity of the stimuli or, alternatively, the ability of the neural ensemble to effectively process different mixtures. To address this, we stimulated the bumblebee with limonene at the same intensities as in MC and ML scents, and at a 10-fold higher intensity than in the MC scent. Furthermore, three different flower scents, all at the same intensity as *M. lewisii*, were tested: *Petunia integrifolia*, a bee-visited flower; *Peniocereus greggii*, a moth-visited flower; and *Oenothera speciosa*, a moth- and bee-visited flower (Riffell et al., 2013). The results showed that the AL ensemble effectively separated mixture stimuli (Fig. 4C). Examining the normalized Euclidean distances (dissimilarity indices) between the ML-natural and the other stimuli for all preparations revealed a similar trend, with the ML-natural scent being dissimilar from the single volatiles and other flower extracts (Fig. 4D; Kruskal–Wallis test: χ^2=31.1, P<0.01), but not dissimilar to the ML-synthetic (multiple comparisons: P>0.05). Similarly, the MC-natural and the MC-synthetic (containing only limonene) were not significantly different from one another in their dissimilarity indices, but were different from the ML scents (Fig. 4D; multiple comparisons: P>0.05). Together, these results suggest that bumblebees can differentially perceive the two flower species and that the neural response to the complex scent of *M. lewisii* can be recapitulated with a mixture of just three volatile monoterpenes.

Behavioral responses of bumblebees to *Mimulus* scents and synthetic mixtures

We exposed experienced *B. vosnesenskii* workers to complete natural floral bouquets from *M. lewisii* and *M. cardinalis* (Fig. 6). Bumblebees were trained to *M. lewisii* scent (ML-natural), and then exposed to a two-choice array consisting of artificial paper disk flowers moistened with either *M. lewisii* or *M. cardinalis* (MC-natural) headspace samples. When exposed to the authentic complete bouquets, bumblebees chose to land on the artificial flower bearing the *M. lewisii* scent more often than on the *M. cardinalis* scent (χ^2: P<0.001). Both the total number of choices for each bumblebee and the total time spent investigating the artificial flowers showed a clear preference for the *M. lewisii* odor (Fig. 6; χ^2: P<0.001 for total choices; P=0.02, t=2.53 for total time; N=12 bumblebees). Similar effects were seen with *M. lewisii*-trained bumblebees when exposed to *M. lewisii* versus a control solvent odor (χ^2: P<0.001for total choices; P<0.001, t=4.89 for total time; N=11 bumblebees).

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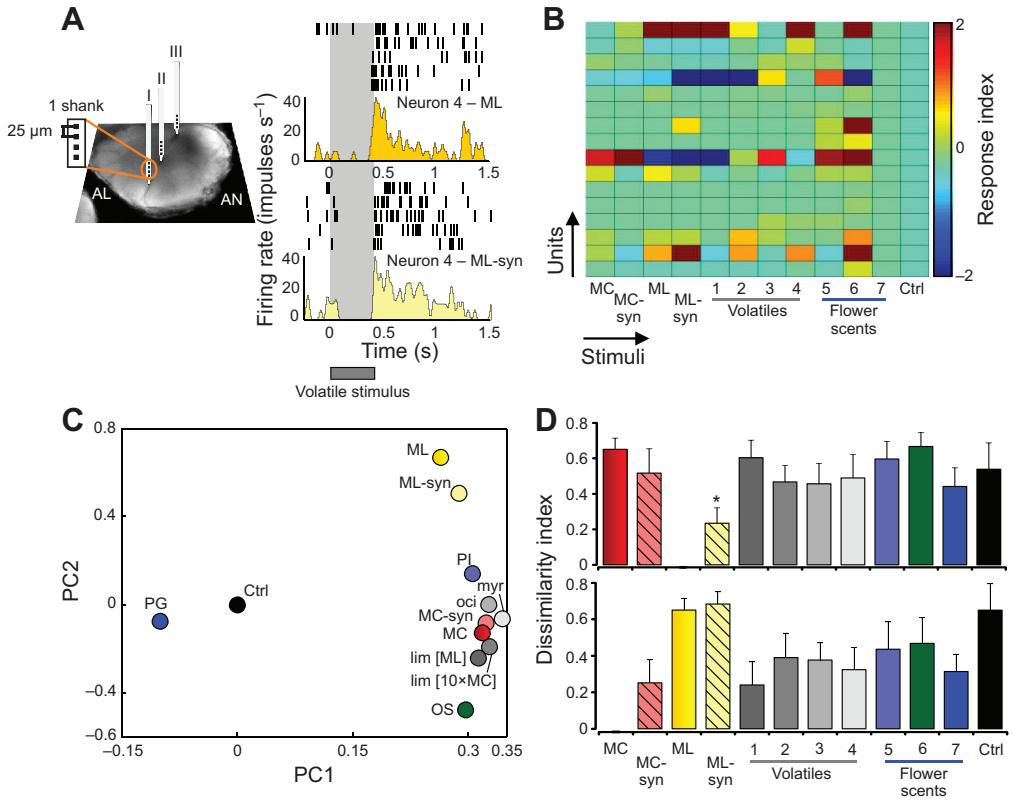

Fig. 4. AL neural responses to floral extracts, artificial mixtures and single volatiles. (A) Multichannel recording in the bee's AL allowed examination of the bee's ability to discriminate between volatile stimuli. On the right are peristimulus time histograms (PSTHs) and raster plots of a unit that showed significant responses (based on CUMSUM test) to both floral headspace and artificial mixture. (B) Response of one 16-unit ensemble to the different volatile stimuli shown, plotted as color-coded response matrices across all units (rows 1–16) and volatile stimuli (columns 1–12): *M. cardinalis* (MC), *M. cardinalis* synthetic stimulus (MC-syn; contains only MC-intensity limonene), *M. lewisii* (ML), *M. lewisii* synthetic mixture (ML-syn), ML-intensity limonene (volatile 1), 10×MC-intensity limonene (volatile 2), ML-intensity myrcene (volatile 3), ML-intensity ocimene (volatile 4), *Petunia integrifolia* (PI, scent 5), *Oenothera speciosa* (OS, scent 6) and *Peniocereus gregii* (PG, scent 7). In addition, hexane was tested as a negative control (Ctrl). (C) Principal components analysis of ensemble responses. Yellow circles correspond to the natural ML and synthetic ML, red circles to the natural MC and synthetic MC, and gray circles to the single volatiles. Note the clustering of the natural and synthetic ML relative to the single volatiles and other mixtures. (D) Dissimilarity indices in the ensemble firing rates in response to volatile stimuli (N=8 preparations, from as many bees). Dissimilarity indices are shown with the ML as the origin (top) or MC as the origin (bottom). Hatched bars designate the synthetic flower scents (ML-synthetic, MC-synthetic). Bars are the mean ± s.e.m.; asterisks denote a significant difference between treatments and the control (P<0.05).

To determine whether bumblebees will respond equivalently to *M. lewisii* scent and to the simplified '*M. lewisii*' synthetic bouquet (ML-synthetic), composed only of D-limonene, β-myrcene and β-ocimene (mixture of isomers), aliquots of ML-natural or ML-synthetic were loaded onto artificial flowers, and experienced *B. vosnesenskii* workers were tested as described above. The bumblebees found the two ML scents indistinguishable based on visitation behavior (bumblebees trained on ML-natural: χ^2: P=0.86 for total choices; P=0.72, t=–0.354 for total time investigating each flower, N=15 bumblebees; bumblebees trained on ML-synthetic: χ^2: P=0.90 for total choices; P=0.491, t=–0.734 for total time investigating each flower, N=7 bumblebees).

To examine whether the individual volatiles of the artificial bouquet were capable of recapitulating the effects of the overall bouquet, we tested bumblebees trained to the three-component *M. lewisii* synthetic mixture against its individual constituent volatiles. The total number of choices was significantly higher to ML-synthetic than to any of the individual volatiles (χ^2: P<0.01 for D-limonene; χ^2: P=0.02 for β-myrcene; χ^2: P<0.001 for β-ocimene) and, with the exception of β-ocimene, bumblebees spent significantly more time investigating ML-synthetic than its individual components (P<0.001, t=4.67 for D-limonene; P<0.01, t=4.24 for β-myrcene; P=0.11, t=1.80 for β-ocimene isomer mixture). The three-component mixture of D-limonene, β-myrcene and β-ocimene is capable of eliciting the same behavioral response as the native scent of *M. lewisii* itself, but each individual component fails to recapitulate the overall bouquet.

DISCUSSION

Although absolute abundance of a given volatile does not necessarily correlate with its perception by the pollinator or its behavioral importance, the three dominant monoterpenes in *M. lewisii* are the most important volatiles driving bumblebee behavior in this system. GCMR analysis of headspace samples from *M. lewisii* shows that these three compounds disproportionately affect antennal lobe activity in *B. vosnesenskii*, the native pollinator of *M. lewisii*. Additionally, when considered as an entire floral bouquet, bumblebees show significantly higher AL activity when exposed to the authentic headspace bouquet of *M. lewisii* in comparison to the authentic headspace bouquet of *M. cardinalis*, and this effect is not due to the simple difference in total volatile emission between the two species. Consistent with these results, behavioral assays with bumblebees show that those trained to *M. lewisii* odor paired with a sucrose reward (as workers would experience in the field and hive)

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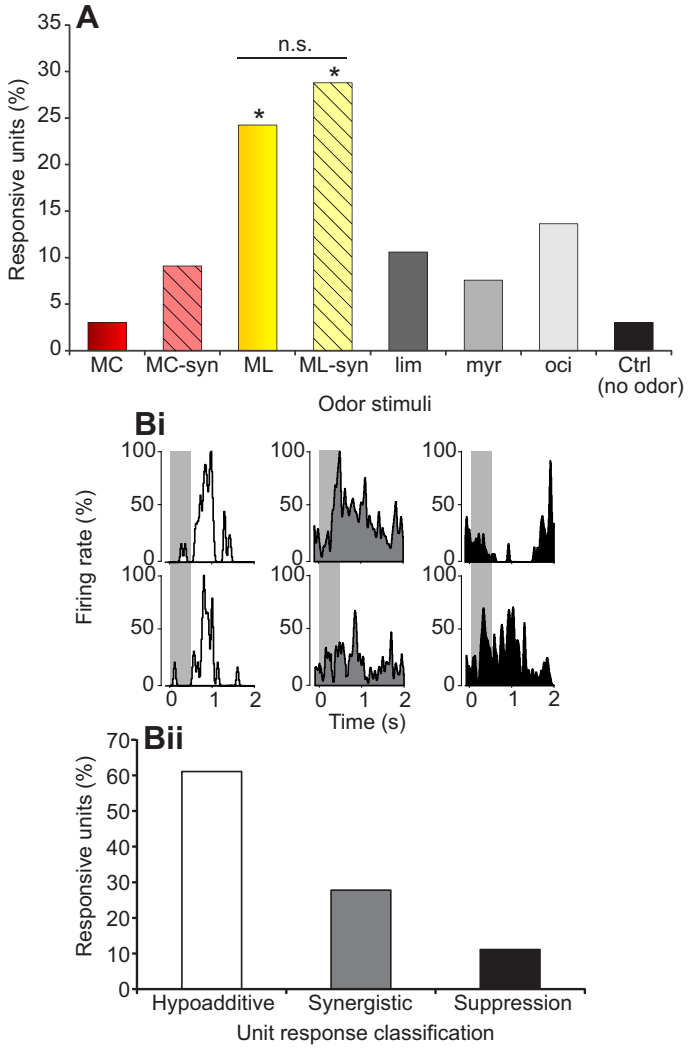

Fig. 5. Unit responses to mixtures and single volatiles. (A) The percentage of responsive units relative to the flower scents, synthetic mixtures and single volatiles. The *M. lewisii* scent (ML) and synthetic *M. lewisii* (ML-syn) evoked a significantly greater proportion of the neural units relative to the mineral oil (no odor) control or the *M. cardinalis* scent (two-by-two χ^2: **P*<0.001). n.s., not significantly different. (Bi) Units that showed similar ('hypoadditive'; white bars), synergistic (gray bars) or suppressive (black bars) responses to the mixture (top) relative to the single volatiles (bottom) that evoked the greatest responses. For these three units (each from a different preparation), limonene elicited the greatest response. Gray bars denote the stimulus duration (500 ms). (Bii) Percentage of responsive units that showed hypoadditive, synergistic or suppressive responses.

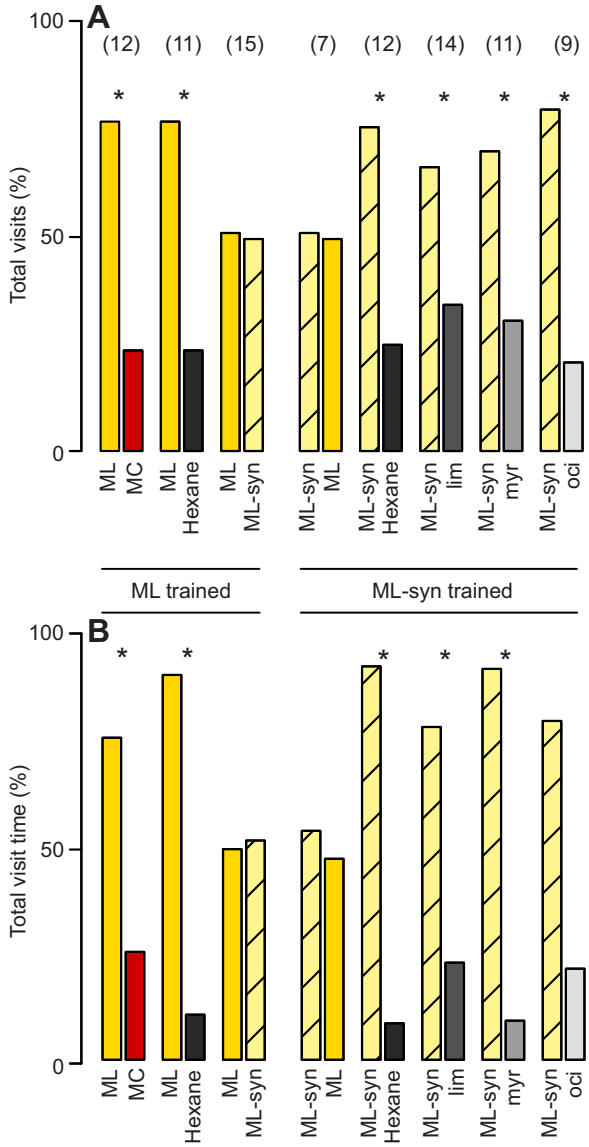

Fig. 6. Behavioral responses of *B. vosnesenskii* to floral bouquets, synthetic mixtures (hatched bars) and individual floral volatiles. (A) Total flower choices by experienced *B. vosnesenskii* workers trained to either natural ML or the synthetic mixture. Bars are the mean percentage of animals responding to the two-choice treatments. (B) The time the bees spent attempting to feed from the two-choice treatments. Bars are the mean percentage of total time for each treatment. Asterisks denote a significant difference between the two-choice treatments (*t*-test or χ^2-test: *P*<0.05). Numbers in parentheses indicate the number of bees used in each two-choice treatment. ML-syn, synthetic mixture of three compounds.

prefer *M. lewisii* authentic headspace samples to those of *M. cardinalis*.

It is possible to reduce the complexity of the *M. lewisii* authentic bouquet to a synthetic mixture of just these three monoterpenes while still capturing the same AL responses. When considered at the level of both single neural units and the neural ensemble, this synthetic mixture is perceived equivalently to the authentic natural bouquet of *M. lewisii*, and both are perceived differently from authentic *M. cardinalis* and a synthetic mixture of *M. cardinalis* consisting of D-limonene only. The individual volatiles in this synthetic mixture are less effective than the mixture as a whole, showing that the AL processes the bouquet of *M. lewisii* in a non-additive fashion. Moreover, bumblebees show no behavioral difference in their response between the synthetic artificial mixture of D-limonene, β-myrcene and β-ocimene and the authentic *M. lewisii* headspace sample, but prefer the synthetic artificial mixture to each of its components.

Reducing the nine volatiles emitted by *M. lewisii* to a smaller set of just three key volatiles in this fashion – and showing that these three volatiles are critical for AL and behavioral processing of the *M. lewisii* bouquet – increases the probability that a species-specific change in one or more of these volatiles may be a powerful driver of pollinator-based reproductive isolation between *M. lewisii* and *M. cardinalis*. Subsets of key volatiles have been shown to be important

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in a variety of plant–pollinator interactions, most impressively those involving sexually deceptive orchids; however, they also play a role in less specialized systems. In *Silene latifolia*, for example, the pollinating moth *Hadena bicruris* responds most strongly to lilac aldehydes (Dötterl et al., 2006), despite the presence of more than 40 volatiles in the total bouquet (Jürgens et al., 2002); these lilac aldehydes alone were able to replicate the behavioral effects of the full floral bouquet where other bouquet components were not. In work with *S. latifolia* and the closely related *S. dioica*, manipulating the emission of one key volatile, phenylacetaldehyde, had significant effects on pollen transfer; when the two species had similar levels of phenylacetaldehyde, interspecific transmission of pollen increased (Waelti et al., 2008). In *Petunia axillaris*, genetic manipulation of the production of methyl benzoate influenced both floral attraction and visit order by pollinating hawkmoths (Klahre et al., 2011), despite the presence of multiple other compounds in the floral bouquet, including an equal emission amount of benzaldehyde (Hoballah et al., 2005). Methyl benzoate and other oxygenated aromatic volatiles, like phenylacetaldehyde and benzyl alcohol, strongly activate moth antennal receptor neurons and AL projection neurons (Shields and Hildebrand, 2001; Riffell et al., 2013), thus providing a direct link between the composition of the floral bouquet and sensory processing and behavior.

As a mediator of pollinator attraction, floral scent can play a key role in the origin and maintenance of reproductive isolation between sister taxa of flowering plants, which are often separated primarily (or solely) by pollinator-based prezygotic reproductive isolation (Grant, 1949; Coyne and Orr, 2004). Hummingbird pollination is the derived character state in section *Erythranthe* of *Mimulus*, with bumblebee pollination inferred to be ancestral (Beardsley et al., 2003). The evolution of hummingbird pollination from bee-pollinated ancestors is a recurring theme in the flora of western North America (Grant, 1949). *Mimulus cardinalis* is known to harbor recessive (i.e. loss-of-function) alleles at several loci controlling traits that contribute to pollinator discrimination (Bradshaw et al., 1995; Bradshaw et al., 1998). It seems likely that genes responsible for species-specific differences in floral scent between *M. lewisii* and *M. cardinalis* – particularly genes influencing the emission of D-limonene, β-myrcene and *E*-β-ocimene – might follow this pattern, and thus may play a role in the evolution of hummingbird pollination in this system. This suggests that further investigation of floral scent as a driver of pollinator-based speciation may be tractable, particularly given the forward and reverse genetics tools available in *Mimulus*, including the ease of creating stable transgenics in *M. lewisii* (Yuan et al., 2013a). In the present study, we provide strong impetus to identify the genetic mechanisms for the evolution of derived hummingbird pollination from ancestral bumblebee pollination.

The approach shown here – characterizing volatile production in sister taxa and identifying volatiles that are behaviorally significant to their pollinators – can be expanded to other systems. Prior work done on the production of benzenoid volatiles in *P. axillaris* and the resulting effects on pollinator choice (Klahre et al., 2011), differential expression of *S*-linalool synthase in scented *Clarkia brewerii* and scentless *C. concinna* (Dudareva et al., 1996), and work on the importance and synthesis of a single volatile in *Silene* (Kaminaga et al., 2006; Waelti et al., 2008) suggest that the genetic basis of production of key floral volatiles may be relatively simple, increasing the tractability of investigating scent as a key factor in pollinator-based reproductive isolation in animal-pollinated angiosperms. Indeed, an integrative synthesis of volatile chemistry, pollination ecology and genetics is needed to answer broader questions about reproductive isolation (Whitehead and Peakall, 2009). Investigation into sensory mechanisms of pollinators in conjunction with their floral resources may also provide broader insights into the evolution of plant–pollinator interactions, particularly in tightly linked mutualistic or exploitative pollination relationships. These same techniques are also applicable to applied problems in modern agriculture such as managing pollinator decline and containment of transgenic pollen by promoting pollinator switches driven by volatile emissions of insect-pollinated agricultural crops.

MATERIALS AND METHODS

Floral specimens

Mimulus lewisii and *M. cardinalis* inbred lines (LF10 and CE10, respectively), derived by >10 generations of single seed descent from wild plants originally collected in their zone of sympatry in the central Sierra Nevada mountains (CA, USA), were used for initial floral volatile analysis. Additional populations of each species (N=3 populations for *M. lewisii*; N=3 populations for *M. cardinalis*), and a separately derived inbred line from each, were obtained from nearby areas (see supplementary material Table S1) to rule out potential geographic and inbreeding differences between the two species. All plants used for this study were grown in the same controlled greenhouse conditions to minimize any effect of abiotic factors on scent production.

Scent collection and analysis

Scent was collected from greenhouse-grown flowers using a push–pull system (Raguso and Pellmyr, 1998; Riffell et al., 2008). Two flowers cut from the parent plant with pedicels attached were placed in a plastic oven bag (Reynolds, Richmond, VA, USA) ~3 l in volume. Diaphragm pumps (400-1901, Barnant Co., Barrington, IL, USA) were used to pull fragrant headspace air through sorbent cartridge traps at a flow rate of 1 l min^{-1}. Traps were constructed by packing 100 mg of Porapak Q adsorbent (mesh size 80–100, Waters Corp., Milford, MA, USA) in borosilicate glass tubes (7 mm) plugged with silanized glass wool. Purified air enters the top of each bag (1 l min^{-1}). Collections began during the day and continued overnight for 24 h to control for the effects of any potential circadian scent emission on floral volatile abundance. Shorter collection periods were inadequate to capture the volatiles present in *M. cardinalis*, so 24 h collections were used for both species. Nine replicates of inbred lines LF10 and CE10 (supplementary material Table S1) were collected, along with smaller numbers of replicates of additional populations. An NMDS plot was prepared from these data using Wisconsin double standardization and square-root transformed emission rates and the Bray–Curtis dissimilarity index using the vegan package in R (Oksanen et al., 2013).

Trapped volatiles were eluted from sorbent cartridges using 600 µl of HPLC-grade hexane. Each sample was stored in a 2 ml borosilicate glass vial with a Teflon-lined cap at –80°C until concentration and analysis. An aliquot of the sample was concentrated 10-fold under a stream of nitrogen gas. A 3 µl aliquot of this concentrated volatile sample was analyzed using an Agilent 7890A GC (gas chromatograph) and a 5975C Network Mass Selective Detector (Agilent Technologies, Palo Alto, CA, USA). A DB-5 GC column (J&W Scientific, Folsom, CA, USA; 30 m, 0.25 mm, 0.25 µm) was used, and helium was used as the carrier gas at a constant flow of 1 cc min^{-1}. The initial oven temperature was 45°C for 4 min, followed by a heating gradient of 10°C min^{-1} to 230°C, which was then held isothermally for 4 min. Chromatogram peaks were identified tentatively with the aid of the NIST mass spectral library (ca. 120,000 spectra) and verified by chromatography with available authentic standards and published Kovats indices. Peak areas for each compound were integrated using ChemStation software (Agilent Technologies) and are presented in terms of nanograms per flower per hour in Table 1.

Electrophysiology

Experimental preparation

Wild-caught *B. vosnesenskii* worker bumblebees, a native pollinator of *M. lewisii* (Schemske and Bradshaw, 1999), were used in multi-unit recording

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Table 1. Mean volatile emission by *Mimulus lewisii* and *Mimulus cardinalis*

Volatile	RT (min)	Emission rate (ng h^{-1})	
		M. lewisii	*M. cardinalis*
Unk. MO [*mz*=53,77,91,105,121,**136**]	7.92	0.34 (0.21, 0.51)	Absent
α-Pinene	8.08	1.80 (0.98, 2.77)	0.07 (<0.01, 0.23)
Sabinene	8.88	1.49 (0.73, 2.72)	Absent
(-)-β-Pinene	8.98	1.23 (0.77, 1.59)	Absent
1-Octen-3-ol	8.99	Absent	0.17 (0, 0.43)
β-Myrcene	9.18	3.31 (1.79, 4.60)	Absent
D-Limonene	9.94	55.11 (36.32, 81.86)	0.52 (0.04, 1.19)
E-β-Ocimene	10.25	7.62 (3.96, 11.96)	Absent
γ-Terpinene	10.47	0.07 (0.03, 0.11)	Absent
Terpinolene	10.95	0.26 (0.11, 0.48)	Absent
β-Farnesene	16.33	Absent	0.19 (0, 0.37)
α-Farnesene	16.98	Absent	0.14 (0, 0.30)

Numbers in parentheses correspond to the 10% and 90% values for the given volatile, respectively. Volatiles listed were identified to retention time (RT) with synthetic standards and Kovats indices. *E*-β-Ocimene was further verified using the retention time of a *Datura wrightii* headspace sample. Unk. MO, unknown monoterpene.

experiments from the AL. Although regional differences are a potential confounding factor in work with this widely distributed species (Herrera et al., 2006; Skorupski et al., 2007; Ings et al., 2009), *B. vosnesenskii* is a broadly generalist species (Alarcón et al., 2008), and the volatiles in question, all commonly found across many plant taxa, may be provoking a pre-existing sensory bias rather than a region-specific response. Additionally, *M. lewisii* has been observed being visited by other *Bombus* species in the field (*B. balteatus*, *B. centralis* and *B. flavifrons*), as well as by honeybees (*Apis mellifera*) (Hiesey et al., 1971), so the species is not solely attractive to California populations of *B. vosnesenskii*.

Ten *B. vosnesenskii* workers – typical replicate numbers for these types of experiments (Fernandez et al., 2009; Riffell et al., 2009b; Brill et al., 2013) – were used in this study, with the spiking activity from a total of 159 isolated neurons (hereafter termed 'units'). Multi-unit recording experiments permit stable, long-duration (>4 h) recordings of AL neural ensemble responses. In preparation for recording, the bumblebee was placed in a 1 ml Gilson pipette tip and secured with dental wax, leaving the head and antennae exposed. The head was opened to expose the brain, and the pipette tip was fixed to a recording platform attached to a vibration-isolation table. The sheath overlaying one AL was carefully removed with a pair of fine forceps and the brain was superfused with physiological saline solution [in mmol l^{-1}: 150 NaCl, 3 $CaCl_2$, 3 KCl, 25 sucrose, 10 *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer, pH 6.9]. After the experiment was completed, the brain was excised and immersed in 1–2% glutaraldehyde in 0.1 mol l^{-1} phosphate buffer to facilitate locating probe tracks in order to examine the consistency of the recording electrode placement in the AL. Brains were fixed for 6–12 h, then dehydrated with a graded ethanol series, cleared in methyl salicylate, and finally imaged as whole mounts with a laser-scanning confocal microscope (Zeiss 510 Meta equipped with a 457 nm argon laser). While identification of participating glomeruli in the encoding of the flower mixture is important for assigning functional significance to how the olfactory information is processed in the AL, this is beyond the scope of our study.

Olfactory stimulation

Olfactory stimuli were delivered two different ways. First, stimuli were delivered to the antenna by pulses of air from a constant air stream diverted through a glass syringe containing a piece of filter paper bearing collected floral scent or single or mixed volatile compounds. Synthetic single volatiles used in electrophysiological and behavioral experiments were β-myrcene, β-ocimene (mixture of isomers) and D-limonene (Sigma-Aldrich, St Louis, MO, USA; purity >90% for myrcene and ocimene; >97% for D-limonene). Aliquots (10 µl) of volatiles or mixtures were added to the filter paper such that the final amount loaded was 6 µg of D-limonene, 165 ng of β-myrcene and/or 800 ng of the β-ocimene mixture. In addition, to examine the AL neural ensemble responses to complex flower scents, three flower extracts were also tested: *O. speciosa* (bee- and butterfly-visited), *P. integrifolia* (bee-visited) and *P. gregii* (moth-visited) (Riffell et al., 2013). These concentrations of volatile and mixture stimuli were scaled to the natural emissions of the *M. lewisii* flower (and verified by gas chromatography mass spectrometry, GCMS), except for limonene, which was tested at three different intensities: equal to ML, 10×MC and equal to MC ('synthetic MC', as this was the only volatile in the MC scent that elicited consistent AL responses; data not shown). The stimulus was pulsed by means of a solenoid-activated valve controlled by Tucker-Davis acquisition software (OpenEx Suite, Tucker-Davis Technologies, Alachua, FL, USA). The outlet of the stimulus syringe was positioned 2 cm from and orthogonal to the center of the antennal flagellum ipsilateral to the AL of interest. Stimulus duration was 500 ms, and each train of five pulses was separated by a 5 s interval. The control solvent for the floral headspace extracts was hexane.

In the second method to deliver olfactory stimuli, we used GC coupled with multi-channel recording (GCMR) to identify compounds in the floral scent that can be detected by the bumblebees (Riffell et al., 2009a; Byers et al., 2013). The effluent from the GC served to stimulate the preparation and allowed identification of compounds in the flower scent that elicit significant neural activity in the AL owing to the high degree of convergence of olfactory receptor neurons into AL neurons (Riffell et al., 2009a). A 3 µl sample of collected headspace volatiles was injected (splitless, 30 s) into an Agilent 7820A GC (Agilent Technologies) equipped with a flame ionization detector (FID) and a DB-5 column (J&W Scientific, Folsom, CA, USA). Effluent was split 1:1 between the FID of the GC and the bumblebee antenna using a universal glass '**Y**' connector (J&W Scientific). Effluent to the antenna passed through a heated transfer line (Syntech, Hilversum, The Netherlands) set at 250°C into a glass odor-delivery tube and mixed with a stream of charcoal-filtered, humidified air flowing through the delivery tube to the side of the antenna at a rate of 70 ml min^{-1}.

Ensemble recording and data analysis

For recording the neural activity in the AL in response to the odor stimuli, we used a 16-channel silicon multielectrode recording array (a 4×4–3 mm-50-177; NeuroNexus Technologies, Ann Arbor, MI, USA) inserted into the bumblebee AL. Extracellular activity was acquired with a RZ2 base station (Tucker-Davis Technologies) and a RP2.1 real-time processor (Tucker-Davis Technologies), and extracellular activity in the form of action potentials, or spikes, was extracted from the recorded signals and digitized at 25 kHz using Tucker-Davis Technologies data-acquisition software (Byers et al., 2013; Riffell et al., 2013). Threshold and gain settings were adjusted independently for each channel, and spikes were captured in the 4-channel, or 'tetrode', recording configuration: any spike that passed threshold on one channel triggered the capture of spikes recorded on the other three channels on the same shank. Offline Sorter v.3 (Plexon Neurotechnology Research Systems, Dallas, TX, USA) was used to sort extracellular spikes based on their waveform shape (Gray et al., 1995), and spikes were assigned timestamps to create raster plots and

calculate peristimulus time histograms (PSTHs). The recorded neural ensembles likely consist of mixed populations of local interneurons and projection neurons, the identities of which are not currently identifiable for this species (but see Lei et al., 2011), but the dimensions and spacing of the recording array make it possible to record stimulus-evoked neural activity from multiple sites across the AL.

A unit was considered to be responsive if its control-subtracted PSTH was above (excitatory) or below (inhibitory) the 95% confidence limits derived from the CUMSUM test. We quantified the control corrected response for every unit by calculating a response index (RI). RI values reflect the deviation from the mean response of all units across all odors in one ensemble, as RI=(R_{odor}–R_m)/s.d., where R_{odor} is the number of spikes evoked by the test odor minus the number evoked by the control stimulus, R_m is the mean response and s.d. is the standard deviation across the data matrix. The RI values for the non-responsive units fell between −2.0 and +2.0, based on the CUMSUM test. To determine how unit responses to individual volatile compounds may differ from natural floral scent- or synthetic mixture-evoked responses, we compared mixture responses with those of the most effective volatile compound. For each volatile compound and mixture tested, we placed each unit into one of three different categories depending on mixture responses: equal to (*Z*-score within ±2.0 of the response), lower ('suppression'; *Z*-score ≤2.0 of the response) or higher ('synergy'; *Z*-score ≥2.0 of the response) than the individual volatile that produces the greatest response. Finally, representation of the single volatile and mixtures was examined at the level of the neural population through multivariate analysis and calculation of the Euclidian distances between olfactory stimuli (Riffell et al., 2009b; Riffell et al., 2013).

Behavioral experiments

Worker individuals of *B. vosnesenskii* (wild-caught in Seattle, WA, USA) were trained to scents for a period of 18 h, rested for 6 h without stimulus, and then were tested in a free flight arena in a two-choice bioassay. Training consisted of exposing individual bumblebees to natural or synthetic floral odor that was loaded on to a filter paper, while providing a constant source of 30% sucrose on a cotton swab. Testing consisted of providing individual bumblebees with a choice between two side-mounted artificial flowers dosed with 10 µl of concentrated headspace collection, synthetic mixture, single volatile or hexane alone. Bumblebees were allowed to acclimate to the testing chamber for 1 min and were then observed for 3 min. In total, 79 bumblebees were tested; each individual bumblebee was trained to only one odor and then subsequently tested before being discarded. Because of the number of treatments and the limited time this wild-caught species is available during the summer months, 7–15 individual workers were used in each treatment. However, this number is often typical of behavioral studies using commercially available bumblebees (Kulahci et al., 2008; Kaczorowski et al., 2012).

Several treatments were performed: *M. lewisii* versus *M. cardinalis* (to ensure that species-specific behavioral differences exist), *M. lewisii* versus a synthetic mixture consisting of D-limonene, β-myrcene and a mixture of isomers of β-ocimene (to investigate the necessity and sufficiency of these compounds to mimic the complete bouquet of *M. lewisii*), the synthetic mixture versus each of its components, and each of the synthetic mixture and *M. lewisii* versus a control solvent (hexane) odor. In all cases of the synthetic mixtures and single odorants, emission rates and ratios were scaled to simulate those emitted by the natural flowers (as determined by GCMS). An equal mixture of the sample and mineral oil was pipetted onto the artificial paper disk flower to provide a medium for continued emission of volatiles over a longer period. The number of visitations (both initial choice and total choices) between treatments was compared using a chi-square goodness-of-fit test, while time differences were assessed using a paired *t*-test.

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Competing interests

The authors declare no competing financial interests.

Author contributions

K.J.R.P.B., H.D.B. and J.A.R. conceived the study; K.J.R.P.B. and J.A.R. conducted and analyzed the data; and K.J.R.P.B., H.D.B. and J.A.R. prepared the manuscript.

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Supplementary material

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Table S1. Populations of *M. lewisii* and *M. cardinalis* used in the NMDS analysis in Figure 1B

Species	Population name	Numbering in Figure 1B	Replicate collections	Source	County	Nearest road	Latitude	Longitude	Elevation
M. lewisii	LF10	1	9	Inbred	Tuolumne	Evergreen Rd.	37.817	-119.867	1350
M. lewisii	SL9	2	4	Inbred	Tuolumne	Evergreen Rd.	37.817	-119.867	1350
M. lewisii	ML52	3	1	Wild	Mariposa	Tioga Pass Rd.	37.83	-119.48	2690
M. lewisii	SL2	4	2	Wild	Tuolumne	Evergreen Rd.	37.817	-119.867	1350
M. lewisii	WL3	5	4	Wild	Mariposa	Wawona Rd.	37.53	-119.65	1208
M. cardinalis	CE10	6	9	Inbred	Tuolumne	Evergreen Rd.	37.817	-119.867	1350
M. cardinalis	SC12	7	2	Inbred	Tuolumne	Evergreen Rd.	37.817	-119.867	1350
M. cardinalis	FC2	8	1	Wild	Mariposa	Old Coulterville Rd.	37.703	-119.753	1316
M. cardinalis	SC62	9	1	Wild	Tuolumne	Evergreen Rd.	37.82	-119.87	1320
M. cardinalis	WC32	10	1	Wild	Mariposa	Wawona Rd.	37.53	-119.65	1208

Latitude, longitude, and elevation reflect the location of the original collection of the population; inbred lines have been inbred for over ten generations. All populations are drawn from the central Sierra Nevada mountain range in California USA.