Floral volatiles in *Mimulus*: chemical ecology, insect olfaction, genetics, and reproductive isolation

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Abstract

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Floral diversity is immense, with more than 250,000 species of angiosperms known. The number of phylogenetically diverse floral species that share common characteristics implies a background pattern of selection acting on floral traits. Unique combinations of these floral traits, or "pollination syndromes," are hypothesized to reflect selective pressure imposed by certain classes of pollinators. One flower character in particular, scent, has been hypothesized to operate as an unseen signal to attract certain pollinators, particularly when combined with other signals such as color and shape. However, the contribution of scent in pollinator-mediated selection between sister taxa has nearly always been inferred and rarely directly tested, and pollinator sensory mechanisms that drive attraction often remain unclear. The genus *Mimulus* (Phrymaceae) forms a developing model system for studying floral diversity and pollinator-driven speciation using a combination of genetic tools and field ecology. Two sister species of *Mimulus*, *M. cardinalis* and *M. lewisii*, are pollinated by hummingbirds and

bumblebees respectively, and present a unique system in which to examine the sensory mechanisms and signals that might mediate pollinator-driven speciation. Using a combination of scent collection, gas chromatograph-coupled multi-unit recording, and behavioral experimentation, we investigated the role of multiple *Mimulus* volatiles on bumblebees (*Bombus vosnesenskii*). Three key compounds are found at different concentrations in the two species, with notable effects on electrophysiology and behavioral responses by bumblebees. Using linkage mapping and *in vitro* assays, we found that species-specific differences in abundance of these three compounds are due to two terpene synthase genes, which are expressed and functional in the bumblebee-pollinated species but are nonfunctional or not expressed in the hummingbird-pollinated species. RNAi knockdowns of these two terpene synthases in the bumblebee-pollinated species indicated the functional role that these two genes play in species-specific differences. When the RNAi knockdown plants were introduced to free-flying *Bombus impatiens* in a greenhouse experiment, one knockdown (*LIMONENE-MYRCENE SYNTHASE*) had no effect on bumblebee visitation, but the other (*OCIMENE SYNTHASE*) had a significant effect, decreasing bumblebee visitation compared with the wild-type bumblebee-pollinated flowers. By integrating chemistry, electrophysiology, behavior, and genetics, we have identified a significant phenotypic trait (*E*-β-ocimene levels), identified its genetic underpinnings (*OCIMENE SYNTHASE*), and have shown that it has a significant effect on pollination. Together, these results suggest that scent alone may be a sufficient force to drive differential pollinator attraction to sister species, providing a mechanism for speciation and maintenance of reproductive isolation in angiosperm taxa.

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DEDICATION

To my mother, Karolen Irene Paularena (1957-2001), who taught me by word and example what it means to practice science not just as a way of knowing but as the logical next step from curiosity.

Let us roll all our strength and all
Our sweetness up into one ball,
And tear our pleasures with rough strife
Thorough the iron gates of life:
Thus, though we cannot make our sun
Stand still, yet we will make him run.
- Andrew Marvell, "To His Coy Mistress"

Her willingness to address difficult issues was one of her strengths. If something seemed morally correct, she did it. – John Richardson

INTRODUCTION

Angiosperm diversity, insect pollination, and the origin of species

In 1879, Charles Darwin wrote a letter to Joseph Hooker, puzzling over the rapid diversification of the flowering plants. He commented that

"*The rapid development as far as we can judge of all the higher plants within recent geological times is an abominable mystery... Saporta believes that there was an astonishingly rapid development of the high plants, as soon* [as] *flower-frequenting insects were developed and favoured intercrossing. I sh*^{d} [sic] *like to see this whole problem solved."* (Darwin 1879)

Darwin's interest in pollination went beyond this letter, including an entire book on the subject (Darwin 1862). The puzzle of this rapid diversification has inspired generations of research, particularly focused on the idea of Gaston de Saporta that the angiosperm radiation was driven primarily by plant-insect interactions (Friedman 2009). The idea that coevolution between pollinating insects and flowering plants has been the primary driver of this diversification is now fairly well established (Grant 1949, Stebbins 1970, Feinsinger 1983), although there is still some disagreement on how strong the effect on species diversity truly is (Gorelick 2001). Although the various orders of pollinating insects arose by the Jurassic, well before the origin of the angiosperms in the Cretaceous, the arrival of angiosperms heralded a massive radiation within these orders in pollinating insect families. In particular, the hymenopteran suborder Aculeata (bees and wasps) first appears in the fossil record in the Cretaceous, and the earliest true bee, *Trigona* (Apidae) shows up in the late Cretaceous, well after the arrival of angiosperms. The late Cretaceous also serves as the backdrop for the overall massive radiation of bee-associated angiosperms (Crepet *et al.* 1991, Grimaldi 1999, Dötterl & Vereecken 2010); the origin of the crown bees is simultaneous with the origin of the core eudicots (Cardinal & Danforth 2013). Despite this understanding of the role that insect pollinators play in the evolution of angiosperm diversity, the underlying genetic mechanisms for this process are still largely unknown (Fenster *et al.* 2004).

A concrete definition of species boundaries is a critical starting point for any study of speciation. In1942, Ernst Mayr defined species as "groups of actually or potentially interbreeding natural populations that are reproductively isolated from other such groups" (Mayr 1942); today, this is termed the Biological Species Concept. Working from Mayr's Biological Species Concept, one of the principal means of studying the origins of diversity among species is to investigate the origin of reproductive isolation - namely, which factors lead to isolation between sister taxa?

Factors leading to reproductive isolation are typically divided up into pre-zygotic (*e.g.* mate choice, reproductive timing, physical compatibility, gamete compatibility) and post-zygotic (*e.g.* hybrid inviability, hybrid sterility) factors. Due to their relative position in time, pre-zygotic factors can have a very large effect on reproductive isolation (Ramsey *et al.* 2003, Coyne & Orr 2004, Rieseberg & Willis 2007); post-zygotic factors are responsible for the remainder of reproductive isolation (Coyne & Orr 1989; Ramsey *et al.* 2003). Historically work on "speciation

genes" has focused on post-zygotic incompatibility factors, which are genetically more tractable (Coyne & Orr 2004). In flowering plants, the most critical factor in pre-zygotic reproductive isolation is the attraction of animal pollinators. Coyne and Orr (2004, p. 441) write that:

"Our major conclusion is that two sets of key factors - traits increasing sexual selection in animals, and traits promoting animal pollination in plants - appear to increase the rate of speciation."

Understanding the factors leading to the attraction and behavioral response of animal pollinators is thus key to understanding the massive and rapid radiation of angiosperms over which Darwin and Hooker were puzzling. Evidence from numerous studies suggests that differences in attractive factors between sister taxa can both create and maintain reproductive isolation (reviewed in Fenster *et al.* 2004, Lowry *et al.* 2008a, Harder & Johnson 2009, Kay & Sargent 2009, Van der Niet *et al.* 2014).

Pollinator shifts: mechanisms and evidence

"Pollination syndromes" are often defined as suites of floral traits that suggest convergence onto a single pollinator guild's preferences and morphology (Faegri & van der Pijl 1979, Fenster *et al.* 2004) – therefore, one speaks of "hummingbird flowers," "bee flowers," etc. By attracting different guilds of pollinators, species that are otherwise interfertile may maintain prezygotic reproductive isolation and thus prevent population admixture. The "Most Effective Pollinator Principle" (MEPP) was defined by G. L. Stebbins in 1970, pointing out that plants should evolve to match the morphology and attractive preferences of their most effective pollinators, with some attention to the effects of secondary pollinators (Stebbins 1970). The existence of pollination syndromes has been debated for the last several decades (Herrera 1996, Ollerton 1996, Waser *et al.* 1996); however, as Stebbins pointed out, the role of secondary pollinators is often ignored in this discussion, as is the difference between extreme obligate mutualists and less extreme specialists. A recent literature analysis has shown that the MEPP holds true across 417 plant species, with strong correlations between predicted pollination syndrome and pollinator effectiveness (Rosas-Guerrero *et al.* 2014). Several common trends of pollination syndrome transitions have been demonstrated in a variety of taxa in this analysis, most commonly transitions from bee to moth pollination, bee to bird pollination, and bird to moth pollination (Van der Niet & Johnson 2012, Rosas-Guerrero *et al.* 2014).

Transitions from bee to bird pollination are one of the most well-documented pollinator shifts (Van der Niet & Johnson 2012), and are common in the western North American flora (Grant 1994). In these shifts, two phenomena are at work: the shift towards the novel bird pollinator and the shift away from the ancestral bee pollinator (Thomson & Wilson 2008). Changes in phenotype can thus serve to attract birds and facilitate their pollination activity (termed "pro-bird" adaptations, Castellanos *et al.* 2004); alternately, they may serve to discourage bee visitation ("anti-bee" adaptations). *Via* some ecological process, perhaps shifts in weather or colonization of novel habitat, transition to bird pollination is favored; the phenotypic changes in the flower necessary to conform to bird pollination result in exclusion of bees, which then act as parasites by wasting pollen and nectar (Thomson & Wilson 2008). At the end of this process,

the bird-pollinated derived species may be almost completely reproductively isolated from its bee-pollinated ancestor by pollinator preference alone, though very few studies have quantified reproductive isolation (Ramsey *et al.* 2003). In most systems, this transition appears to be unidirectional towards hummingbird pollination; reversals seem to only occur when other non-bee pollinators also play a role (Beardsley *et al.* 2003, Kay *et al.* 2005, Wilson *et al.* 2006, Whittall & Hodges 2007, Van der Niet & Johnson 2012).

Reproductive isolation *via* pollination can occur in one of two major ways: differential pollinator attraction (ethological isolation, which this thesis focuses on) and differential pollen deposition and placement (mechanical isolation, not discussed hereafter) (Grant 1949). In the former case, many different factors may influence pollinator choice, including floral color (Bradshaw & Schemske 2003, Zufall & Rausher 2004, Streisfeld & Kohn 2005, Hoballah *et al.* 2007, Streisfeld & Kohn 2007, Dell'Olivo *et al.* 2013), shape (Hodges 1997, Fulton & Hodges 1999, Alexandersson & Johnson 2002, Strakosh & Ferguson 2005, Whittall & Hodges 2007, Owen & Bradshaw 2011), display size (Thomson 1989, Conner & Rush 1996, Grindeland *et al.* 2005), pattern (Goyret 2010, Owen & Bradshaw 2011, Shang *et al.* 2011, Yuan *et al.* 2013b), orientation (Fulton & Hodges 1999, Hodges *et al.* 2002), texture (Kevan & Lane 1985, Goyret & Raguso 2006, Whitney *et al.* 2009), and scent (Dodson *et al.* 1969, Galen & Kevan 1983, Williams & Whitten 1983, Raguso & Pichersky 1995, Kessler *et al.* 2008, Raguso 2008a,b, Riffell *et al.* 2008, Peakall *et al.* 2010, Klahre *et al.* 2011,Schiestl *et al.* 2011, Schlüter *et al.* 2011, Xu *et al.* 2011, Morse *et al.* 2012, Friberg *et al.* 2013). Multimodal learning by pollinators is a serious consideration, so combinations of these traits may be perceived independently of their individual values (Kulaci *et al.* 2008, Leonard *et al.* 2011). Each of these factors may be under the control of diverse metabolic and developmental processes, driven by suites of genes ranging in identity from transcription factors to biosynthetic enzymes; genes controlling attractive phenotypes are thus a logical target for the study of reproductive isolation *via* pollinator choice.

A body of work discusses the genetics of floral traits relevant to differential pollinator attraction, but most of the examples are incomplete. In some cases, a locus has been identified that is important for the trait of interest and has known ecological significance, but it has not been mapped to a single gene, or the mapping is based on a candidate gene approach rather than an unbiased mapping approach and thus the true underlying gene may be misidentified. In other cases, a trait is suggested to be important for pollinator choice and the genetic basis is known, but experiments with pollinators have not been conducted. Overall, very few examples exist which link trait description, genetics, and behavioral experiments in the field or greenhouse when describing traits relevant for pollinator attraction and choice (Yuan 2013a).

The role of floral scent in animal pollination

The fact that flowers use scent in attracting insects has been known for millennia. In his *Inquiries on Animals* (350 BCE), Aristotle wrote:

"…insects have all the senses, for they can see, smell, and taste. Insects, whether they have wings or are apterous, can smell from a great distance, as the bee and the snipes do scent

honey, for they perceive it from a long distance, as if they discovered it by the scent." (tr. Richard Cresswell, 1878)

Floral scent is a major attracting factor (Raguso 2008a,b), but despite this early recognition of its role in pollination, it has been less well-studied than visual cues, although exceptions exist in systems under agricultural cultivation, such as roses (Goulson 2010). Scent has the potential to attract insects from a very large distance - certainly much further than visual displays. Olfactory lures attract euglossine bees from as far as 1 kilometer (Dötterl & Vereecken 2010) and moths from tens to hundreds of meters (even in the absence of immediately visible targets, Raguso & Willis 2003). This is particularly true for species that are pollinated by nocturnal pollinators such as bats and hawkmoths (Pellmyr 2009). On the other hand, floral scent may also play a role in close-range landing decisions once a pollinator has been attracted by visual signals from a greater distance (Butler 1951, Galen & Kevan 1980, Galen & Kevan 1983, Lunau 1992, Dobson *et al.* 1999, Majetic *et al.* 2009, Pellmyr 2009, Dötterl & Vereecken 2010); this is especially true in weakly scented flowers (Dötterl & Vereecken 2010, Parachnowitsch *et al.* 2012). Conversely, hummingbirds have little sense of smell and display minimal scent learning (Goldsmith & Goldsmith 1982, Ioalé & Papi 1989), and scent does not appear to play a large role in hummingbird pollination (Knudsen *et al.* 2004).

Floral scent is learned differently by different pollinators, and floral scent learning is strongly affected by the level of pollinator specialization and pollinator social structure. Bee species that specialize on particular groups of flowering plants appear to depend on innate olfactory search images, which can then be modified through learning; after learning occurs, these bees may rely less on olfactory cues and more on landmarks, color, shape, and location (Dötterl & Vereecken 2010). Similarly, hawkmoths have innate preferences for certain floral odors, but are able to adapt to feed on alternate food sources when preferred food sources are scarce (Riffell *et al.* 2008).

By contrast, generalist, social species such as bumblebees learn scent in the hive before emerging to forage for the first time (Dornhaus & Chittka 2004), and thus are rarely truly naïve (Lunau 1992, Dötterl & Vereecken 2010). Most individual bumblebees learn to specialize on one or two flower species, which improves foraging performance (Heinrich 1976). Social communication within the hive also plays a role in foraging; in bumblebees, workers that have just foraged recruit other workers, who begin to probe the nectar pots into which the forager just unloaded her nectar, and some proportion of these bees immediately begin foraging (Dornhaus & Chittka 2001). The strongest display of learning occurs when a successful forager brings in nectar containing the scent and combines it with foraging behavior. Contributions to learning within the hive have been estimated at 23% from the floral scent in the air, 48% from the floral scent in the nectar pot, and 29% from the behavior of the recruiting worker (Molet *et al.* 2009). Bumblebees do not transmit direct location information (Dornhaus & Chittka 2004). While honeybees may have evolved the dance language to cope with patchy resources in dense forests, bumblebees largely evolved in more well-distributed habitats, with potentially less need for lengthy and error-prone navigational instruction (Dornhaus & Chittka 1999).

Research on bumblebees indicates that they retain floral resource information best when learning a combination of visual and olfactory signals. The presence of olfactory signals can sharpen color discrimination in artificial flower trials (Kunze & Gumbert 2001). Combining visual and olfactory stimuli can make bumblebees choose rewarding flowers correctly more often: although combining visual and olfactory stimuli does not make bumblebees choose the correct flowers any faster, it does lead to a higher success rate (Kulachi *et al.* 2008). Other pollinators, such as some hawkmoths, require a synergy of visual and olfactory cues to elicit a feeding response (Raguso & Willis 2003). Olfactory signals should thus be considered within a multimodal framework of pollinator attraction, allowing synergy with visual and tactile cues such as color, shape, display size, orientation, and texture.

Floral volatiles can even serve as "olfactory nectar guides" (by analogy to visual nectar guides), with emission concentrated in specific areas of the flower that serve as positional cues for insect attraction (Lunau 1992, Kolosova *et al.* 2001b, Dötterl & Jürgens 2005); in some species, these olfactory nectar guides may coincide with visual and tactile guides (Lunau 1992, Kolosova *et al.* 2001b), leading to optimal positioning of the pollinator for pollen transfer. Floral volatiles can also transmit information in other ways; for example, production and emission of floral volatiles can be rhythmic (Loughrin *et al.* 1990, Kolosova *et al.* 2001a, Raguso & Willis 2003, Verdonk *et al.* 2003, Picone *et al.* 2004, Oyama-Okubo *et al.* 2005, Majetic *et al.* 2007), corresponding to the primary time of day of pollinator activity (Loughrin *et al.* 1990, Kolosova *et al.* 2001a, Raguso & Willis 2003, Verdonk *et al.* 2003, Majetic *et al.* 2009). Many plants emit modified floral volatiles after pollination (Tollsten & Bergström 1989, Tollsten & Bergström 1993, Schiestl *et al.* 1997, Schiestl & Ayasse 2001, Negre *et al.* 2003), perhaps as a way to avoid attracting florivores , parasites, and seed predators (Dötterl & Jürgens 2005, Muhlemann *et al.* 2006) or to direct pollinators to other flowers on the same individual (Schiestl & Ayasse 2001).

Although plant volatiles are perhaps best known for their impact on pollinator visitation, they may play multiple roles in the plant's interaction with its environment (Raguso 2008b), including both direct (Levin 1976, Heil 2004) and indirect (Levin 1976, Paré & Tumlinson 1999, Kessler & Baldwin 2001,) defenses against herbivory, defense against microbial spoilage of nectar resources (Huang *et al.* 2012) and mediation of below-ground interactions with beneficial microbes (Wenke *et al.* 2010). They can also function in ways that are detrimental to plant fitness, such as attracting florivores (Theis *et al.* 2007) or pollinator predators (Heiling *et al.* 2004). As a result, selective pressures on plant volatiles may vary, particularly in cases where a single target of volatile signaling acts in multiple roles (*e.g.*, pollinating insects that oviposit on the plant's leaves, leading to larval herbivory). The conflict between selective factors can lead to varied patterns of volatile emissions that attract alternative pollinators (Kessler *et al.* 2010) or to altered emission of volatiles in some tissues (Effmert *et al.* 2008).

Synthesis of floral volatiles

Floral volatiles are secondary compounds, and many are thought to have originally played roles in herbivory defense (Schiestl 2010). They can be divided into up to seven categories: aliphatics, aromatics (benzenoids), terpenes, C5-branched chain compounds, nitrogenous and sulfur-containing compounds, and miscellaneous cyclic compounds (Knudsen *et al.* 2006). Of

these, the aromatics and terpenes are perhaps the best-studied classes of floral compounds. Floral volatile production goes through a two-step process from an upstream precursor: first, the basic skeleton of the volatile is produced by a synthase gene, and second, any modifications (such as oxidization) occur (Dudareva *et al.* 2004). Aromatic compounds are largely produced by the shikimate pathway (Maeda & Dudareva 2012), thus sharing a common pathway with anthocyanin pigments (Zuker *et al.* 2002, Maeda & Dudareva 2012), while terpenes are synthesized from polymers of isoprene (Dudareva *et al.* 2004) and share a common pathway with the carotenoid pigments (Simkin *et al.* 2004). Genes involved in the biosynthesis of floral scent have been shown to be under positive selection in some lineages (Barkman 2003, Schlüter *et al.* 2011).

Terpene floral volatiles come in three primary forms: monoterpenes (C10 compounds, synthesized from geranyl pyrophosphate in the plastid), sesquiterpenes (C15 compounds, synthesized from farnesyl pyrophosphate in the cytosol), and diterpenes (C20 compounds, synthesized from geranylgeranyl pyrophosphate in the plastid); longer terpenes exist but are not volatile (Chen *et al.* 2011). Terpene synthase enzymes are thought to have evolved from an ancestral bifunctional copalyl diphosphate synthase/kaurene synthase (CPS/KS, involved in gibberellin biosynthesis); in angiosperms this CPS/KS gene duplicated and subfunctionalized once to form separate CPS and KS genes. From this duplication of CPS and KS, a series of seven terpene synthase gene subfamilies have evolved, with separate evolutionary events leading to monoterpene synthesis in gymnosperms and angiosperms. Within angiosperms, the subfamilies TPS-a, b, and c are generally responsible for sesquiterpene, monoterpene, and diterpene production, respectively (Chen *et al.* 2011).

Nearly half of all known terpene synthases are multifunctional (Degenhardt *et al.* 2009), perhaps best exemplified by the particularly outstanding diversity of monoterpenes produced by *Nicotiana suaveolens* from a single enzyme, CIN (1,8-cineole synthase). When provided with geranyl pyrophosphate, the common precursor of monoterpenes, CIN produces seven monoterpenes *in vitro* in a bacterial overexpression system: 1,8-cineole, β-myrcene, limonene, sabinene, *E*-β-ocimene, α-terpineol, and α-pinene (Roeder *et al.* 2007). This production occurs from a single active site *via* the production of a highly reactive carbocation intermediate, which then can be stabilized into one of a number of monoterpene products (Bohlmann *et al.* 1998, Davis & Croteau 2000, Degenhardt *et al.* 2009).

Floral scent and reproductive isolation

Current progress in the field of floral volatiles and pollinator choice has focused on two main areas: description of floral scent profiles across a variety of taxonomic groups and characterization of the biosynthesis of floral volatiles (Whitehead & Peakall 2009). Floral scent can be a valuable cue in differentiating cryptic species that appear visually or even genetically indistinguishable but that display different volatile mixtures (Mant *et al.* 2005, Whitehead & Peakall 2009, Peakall & Whitehead 2014).

Independently from work on floral volatiles, research on pollinators has often focused on preference through electrophysiological and behavioral assays, but this is rarely integrated into

an overall picture of the evolution of pollination syndromes and pollinator shifts. A few exceptions exist, including some research linking floral scent to direct measures of fitness (Miyake & Yafuso 2003, Majetic *et al.* 2009, Parachnowitsch *et al.* 2012), but none thus far have integrated these areas to test the potential role of specific floral volatiles in pollinator shifts, with the exception of work in *Petunia*.

Some of the earliest work on floral volatiles and pollination comes from studies on the alpine herb *Polemonium viscosum*, which has both sweet and skunky morphotypes. At higher altitudes, the sweet morph of *P. viscosum* dominates, where it is preferentially visited by bumblebees; the lower-altitude skunky morph is visited less often by bumblebees and more often by muscid flies (Galen & Kevan 1980). It appears that sweet floral scent serves as a bumblebee attractant in this system, while skunky scent serves primarily to deter florivory and nectar robbing by ants (Galen 1983, Galen & Kevan 1983). Seed set of the two morphs in reciprocal transplants behaved in accordance with their altitudinal patterns: sweet morphs set more seeds at higher altitude sites, while skunky morphs set more seeds at lower altitude sites; as both were pollen-limited, this suggests that insect preference for floral scent may have a fitness effect (Galen 1985), but the exact mechanism remains unclear.

Following upon the work in *Polemonium*, floral scent research focused on two areas: the role of floral scent in the extremely specialized sexually deceptive orchid genera *Ophrys* and *Chiloglottis* and the biochemical and genetic mechanisms behind floral scent production, initially in *Clarkia breweri*. In both *Ophrys* and *Chiloglottis* the volatiles known to be important for pollinator attraction are known (Schiestl & Ayasse 2000, Ayasse *et al.* 2003, Schiestl *et al.* 2003, Peakall *et al.* 2010). Recent work suggests that stearoyl-acyl carrier protein desaturases may be responsible for species-specific production of alkenes in *Ophrys* (Schlüter *et al.* 2011), but the genetic basis of differential production of chiloglottones in *Chiloglottis* remains elusive. In *Clarkia breweri*, the *Lis* gene produces S-linalool synthase at high levels in floral tissue, while it is expressed only minimally in the unscented *C. concinna* (Dudareva *et al.* 1996); earlier work had shown that linalool elicited electroantennogram responses in *Hyles lineata*, a hawkmoth that pollinates *C. breweri* (Raguso *et al.* 1996). However, studies manipulating *Lis* in *C. breweri* and testing the effect on hawkmoth pollinators have not been done to date. Following the work in *Clarkia*, many terpene synthases (and, to a lesser extent, other volatile synthesis genes) have been identified in other plant systems (Gang 2005, Chen *et al.* 2011), but they have rarely been tied to pollination (Whitehead & Peakall 2009).

The best-studied link between floral scent, genetics, and pollinator preference is in the sister species *Petunia axillaris* and *P. exserta*. White-flowered *P. axillaris* is pollinated primarily by nocturnal hawkmoths and emits large amounts of aromatic compounds (particularly benzaldehyde, benzyl alcohol, and methyl benzoate), while the closely related red-flowered, hummingbird-pollinated *P. exserta* emits no measurable scent (Galliot *et al.* 2006, Klahre *et al.* 2011). Crosses between the two species were used in a quantitative trait locus (QTL) mapping approach (Klahre *et al.* 2011), revealing the presence of two major loci explaining differences in floral volatile emission. One of the two major loci on chromosome II was absolutely required for floral volatile emission, but the authors were unable to identify a gene underlying the

effects on volatile emission. The locus on chromosome VII contained a candidate gene, *ODORANT1* (*ODO1*), previously shown to regulate benzenoid emission in *P. hybrida* (Verdonk *et al.* 2005). The authors then created four near-isogenic lines (NILs): white, scented flowers (heterozygous for the chromosome II locus); white, unscented flowers (homozygous *exserta* at the chromosome II locus); red, scented flowers (heterozygous at both chromosome II and VII loci); and red, unscented flowers (heterozygous at the chromosome VII loci and homozygous *exserta* at the chromosome II locus). Testing with hawkmoths in a wind tunnel revealed that moths preferentially visited the scented flowers within a given color; when presented with conflicting stimuli (a white, unscented flower versus a red, scented flower), the moths initially chose both types equally. Unfortunately, *ODO1* has not been directly verified as the causative gene underlying the locus on chromosome VII, and the critical gene underlying the locus on chromosome II has not been identified. Regardless, this is the most complete study to date that links volatile genetics with direct tests of pollinator choice.

Given what is currently known about the role of floral scent in reproductive isolation via pollinator choice, several open questions are evident. For example, how do species-specific differences in floral volatiles arise - are the differences qualitative (number and identity of volatiles), or are they quantitative (proportion and absolute amount of shared volatiles)? When working in systems with different pollinators, do changes in floral volatile emission attract one pollinator, deter the other, or both? Are the genes responsible for species-specific differences regulatory genes such as transcription factors, or structural genes such as terpene synthases? How much change in volatile emission is required to effect a pollinator shift, and to what extent do other changes in floral phenotype affect this?

The genus *Mimulus*: a novel study system for floral scent

The potential of the genus *Mimulus* (monkeyflowers; Phrymaceae) as a study system for pollinator-mediated pre-zygotic reproductive isolation was first published by the Plant Biology group at the Carnegie Institute of Washington in 1971, although the group had been working with the system since 1929 (Hiesey *et al.* 1971). Within *Mimulus*, section *Erythranthe* has experienced two independent transitions from ancestral bee pollination to derived hummingbird pollination (Beardsley *et al.* 2003). An extant species pair, bumblebee-pollinated *M. lewisii* Pursh and hummingbird-pollinated *M. cardinalis* Douglas ex Benth, illustrates this transition beautifully; within the zone of sympatry of the two species, 97.6% of reproductive isolation is due to differential pollinator preference (Ramsey *et al.* 2003). Prior work has implicated several loci of large effect in this differential pollinator attraction (Schemske & Bradshaw 1999, Bradshaw & Schemske 2003). The genus *Mimulus*, and specifically the sister species *M. lewisii* and *M. cardinalis*, is ideal for the study of pollinator-mediated reproductive isolation for a number of reasons:

(1) *Mimulus* displays a wide variety of floral phenotypes in terms of color, shape, size, and visual pattern, as well as a diversity of pollinators; specifically, *M. lewisii* and *M. cardinalis* vary widely in color, shape, size, nectar content, and reproductive organ placement, and have different pollinators.

(2) *M. lewisii* and *M. cardinalis* are sister taxa (Beardsley *et al.* 2003), and readily produce vigorous hybrids with hand pollination. Cuttings can readily be propagated to clone plants to replicate exact genotypes. Both parents and their hybrids have a fairly short generation time of 12 weeks and flower in 8 weeks after seeds are sown. Each pollination produces approximately 1000 seeds which have high germinability, no dormancy, and a long storage life.

(3) Genetic resources for the two species exist and are robust; in particular, the related species *Mimulus guttatus* has been sequenced, though not yet published. Marker maps and draft genome assemblies for *M. lewisii* and *M. cardinalis* exist as well. The two genomes are approximately 500Mb in size, making them tractable. Since the ratio of physical distance to genetic distance is fairly low (between 20-250 kb/cM), mapping does not require unreasonably large mapping populations. The recent development of stable transgenesis in *M. lewisii* (Yuan *et al.* 2013c) allows rapid verification of genetic function to a high standard of evidence, as well as tests of transgenics with pollinators in the greenhouse.

(4) Much prior work exists on *Mimulus* as a developing model system for studying plant-pollinator interactions and floral traits (Pollock *et al.* 1967, Hiesey *et al.* 1971, Vickery 1990, Sutherland & Vickery 1992, Vickery 1992, Vickery & Sutherland 1994, Bradshaw *et al.* 1995, Vickery 1995, Wilbert *et al.* 1997, Bradshaw *et al.* 1998, Schemske & Bradshaw 1999, Beardsley *et al.* 2003, Bradshaw & Schemske 2003, Ramsey *et al.* 2003, Streisfeld & Kohn 2005, Streisfeld & Kohn 2007, Angert *et al.* 2008, Lowry *et al.* 2008b, Wu *et al.* 2008, Pince 2009, Streisfeld & Rausher 2009, Bodbyl Roels & Kelly 2011, Owen & Bradshaw 2011, Fishman *et al.* 2013, Streisfeld *et al.* 2013, Wu *et al.* 2013, Yuan *et al.* 2013a,b,c).

Discovery of floral scent in *Mimulus lewisii* and *M. cardinalis*

A recurring pattern of shifts from hummingbird to hawkmoth pollination has characterized speciation in many western North American plant taxa (Grant 1993, Whittall & Hodges 2007), but in the genus *Mimulus* (monkeyflowers) section *Erythranthe* the evolution of hawkmoth pollination from hummingbird-pollinated ancestors has not occurred. "Hawkmoth flowers" share several characteristics with "hummingbird flowers," including a large volume of dilute nectar and a long tubular corolla. But most hummingbird flowers are red, hence not easily visible to hawkmoths, whose visual sensitivity does not extend into the longer wavelengths (Cutler *et al.* 1995). Hawkmoth flowers are usually white (or pale) and highly reflective (Grant 1993), adapted for detection by crepuscular and nocturnal hawkmoths.

The initial goal of my graduate research was to design and synthesize a new *Mimulus* species, pollinated by hawkmoths and reproductively isolated from its red-flowered, hummingbird-pollinated ancestor, *M. cardinalis*. The red color of *M. cardinalis* flowers is produced by the combination of high concentrations of anthocyanin (pink) and carotenoid (yellow) pigments. To selectively eliminate either (or both) floral pigments, we crossed *M. cardinalis* to a white-flowered (bumblebee-pollinated) *M. lewisii* homozygous for a recessive allele (*boo1*) unable to produce anthocyanins, and homozygous for a dominant suppressor of carotenoid pigmentation (*YUP*, Bradshaw & Schemske 2003). *M. cardinalis* is homozygous for the alternative alleles (*BOO1 yup*). We self-pollinated the F_1 (*BOO1/boo1 yup/YUP*) to produce a segregating F_2

population (*N* = 500), from which we recovered the "ancestral" red phenotype (*BOO1 yup*), and the "derived" yellow (*boo1 yup*), pink (*BOO1 YUP*), and white (*boo1 YUP*) phenotypes (Figure 1). Segregants of each color (*N* = 3 per color) were matched as closely as possible for corolla size, shape, and nectar volume.

Figure 1: Red, yellow, pink, and white *Mimulus* flowers from a single F2 population.

Using 28 naïve captive-bred hawkmoths (*Manduca sexta*) in a dimly-lit flight chamber (Figure 2) with one flower of each color in each visitation trial, we counted the total number of pollinator visits as well as recording the initial visit of each hawkmoth. There were significant differences in visits among the flower color phenotypes (Figure 3; $N = 447$, $X^2 = 134$, df=3, $p = 10^{-28}$). Hawkmoths also showed significant differences in initial, first-choice floral preference (overall $X^2 = 20$, $p = 0.00017$, df = 3; red vs. yellow: $X^2 = 4$, $p = 0.0455$, df=1; red vs. dark pink: $X^2 = 8$, $p = 0.0047$, df = 1; red vs. white: $X^2 = 16$, $p = 6.33 \times 10^{-5}$, df = 1; yellow vs. dark pink: $X^2 = 1.3333$, $p = 0.2542$, df = 1; yellow vs white: $X^2 = 7.2$, $p = 0.00729$, df = 1; dark pink vs. white $X^2 = 2.6667$, $p = 0.1025$). There were no initial visits to red flowers; white was preferred to yellow ($p = 0.0073$) but not to dark pink ($p = 0.1025$). During further observation, moths appeared to ignore the red flowers, consistent with their retinal receptor profile (Cutler *et al.* 1995). These results indicate that hawkmoths are innately and repeatedly attracted to flowers at least one allele substitution step (yellow or pink) away from the red flower color characteristic of the ancestral hummingbird-pollinated *M. cardinalis*, and have the strongest innate preference for the two-allele substitution (white).

Figure 2: Carolina hawkmoth (*Manduca sexta*) feeding from white *Mimulus* segregant in flight chamber.

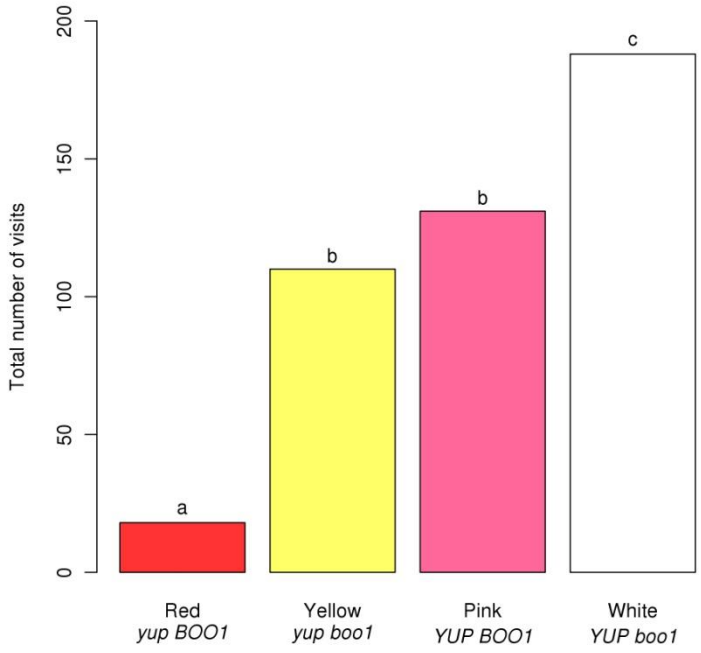

Figure 3: Total number of visits by Manduca sexta to Mimulus F2 flowers. Letters (a,b,c) indicate significant differences in floral preference red vs. yellow: $X^2 = 66.125$, $p = 10^{-16}$, df=1; red vs. pink: $X^2 = 85.698$, $p = 10^{-20}$, df = 1; red vs. white: $X^2 = 140.2913$, $p = 10^{-32}$, df = 1; yellow vs. pink: $X^2 = 1.8299$, $p = 0.1760$, df = 1; yellow vs. white: $X^2 = 20.4161$, $p = 6.23 \times 10^{-6}$, df = 1; pink vs. white $X^2 = 10.185$, $p = 0.001416$).

Floral scent is critical to hawkmoth visitation, with the exception of some diurnal species such as *Macroglossum stellatarum* that are purely visual feeders that do not distinguish between odors (Kelber & Pfaff 1997, Raguso & Willis 2003, Balkenius & Kelber 2006). Olfactory displays attract hawkmoths from tens to hundreds of meters (even in the absence of immediately visible targets) and increase visitation parameters such as visit time and number of visits (Raguso & Willis 2003). Hawkmoths can detect most floral volatiles, and chemical compositions can vary widely (terpenoids, esters, nitrogenous volatiles, *etc.*) (Raguso & Willis 2003). Based upon this crucial aspect of hawkmoth behavior, I hypothesized that *Mimulus lewisii* and/or *M. cardinalis* must be producing some floral scent, given that *Manduca sexta* was willing to forage on it in the flight chamber. Prior work (Schemske & Bradshaw 1999) had dismissed any strong role of floral scent in pollinator attraction in *Mimulus*:

"Neither species has an odor detectable by humans, and our observations suggest that pollinator visitation is influenced primarily by flower color, size, shape, and nectar reward."

but it seemed that floral scent might indeed play a role in pollinator attraction. With two exceptions (both unpublished theses: *M. guttatus*, Martin 1988; *M. aurantiacus*, Büsser 2004), scent in *Mimulus* has never been formally investigated, and its potential role in the isolation of these two species was unclear.

Main objectives and research questions

Given the potential existence of floral scent in *Mimulus lewisii* and/or *M. cardinalis*, the course of study was clear: identify the volatiles present in the two species, determine which were important for the attraction of bumblebee pollinators, and discover their genetic basis; further, if tools were available, synthesize a scent-free *M. lewisii* to determine the effect of scent loss on bumblebee pollination. As the bumblebee pollinators of *M. lewisii* are a generalist species, this work had the potential to add to our limited knowledge of the role of floral volatiles in mediating interactions between generalist pollinators and their floral resources. Additionally, due to the role of *Mimulus* as a model system for pollination research, my hope was to synthesize floral volatile research into the overall question of pollinator attraction in the genus, and to perhaps serve as a model for integrating floral volatiles into other model plant-pollinator systems.

<u>The central research questions of my thesis were:</u>

(I) Which floral volatiles are emitted by *Mimulus lewisii* and *M. cardinalis*, and in what amounts? (Chapter 2)

(II) Of these, which floral volatiles are important for bumblebee attraction? (Chapter 2)

(III) What is the genetic basis of species-specific differences in these important volatiles? (Chapter 3)

(IV) What is the response of bumblebees to *M. lewisii* plants with reductions in emission of specific floral scents? (Chapter 3)

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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

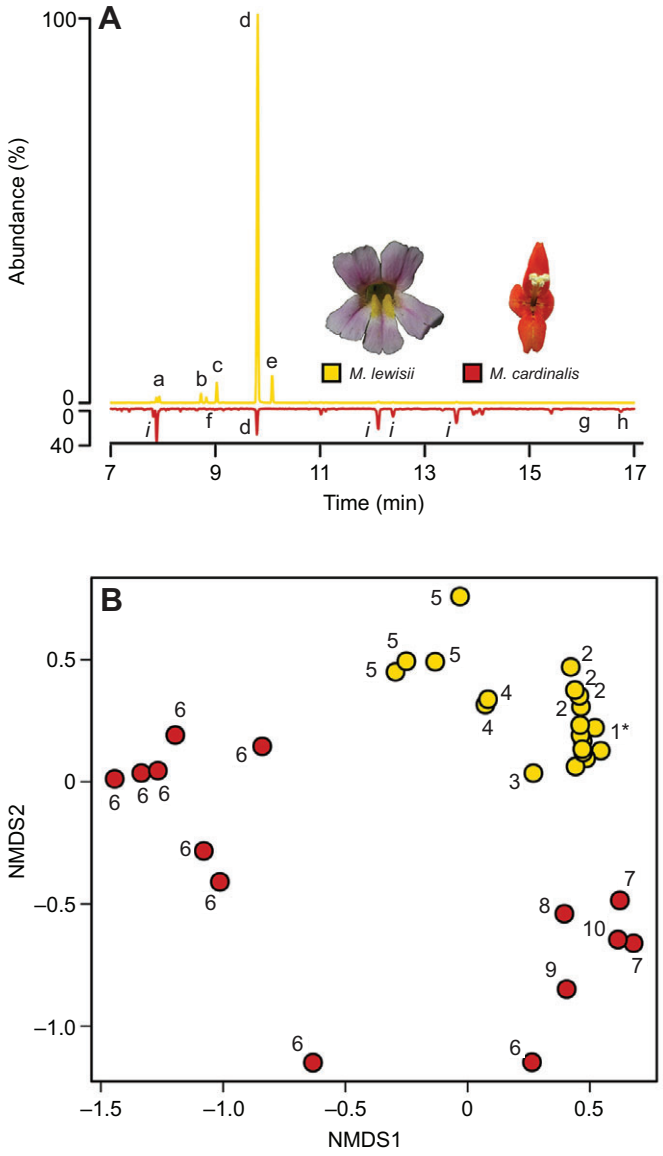

Fig. 1. Floral volatiles emitted from *Mimulus lewisii* and *M. cardinalis*. (A) Gas chromatography mass spectrometry (GCMS) analysis of floral volatiles from bumblebee-pollinated *Mimulus lewisii* (top, yellow) and hummingbird-pollinated *M. cardinalis* (bottom, red). Labels specify individual volatiles: 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

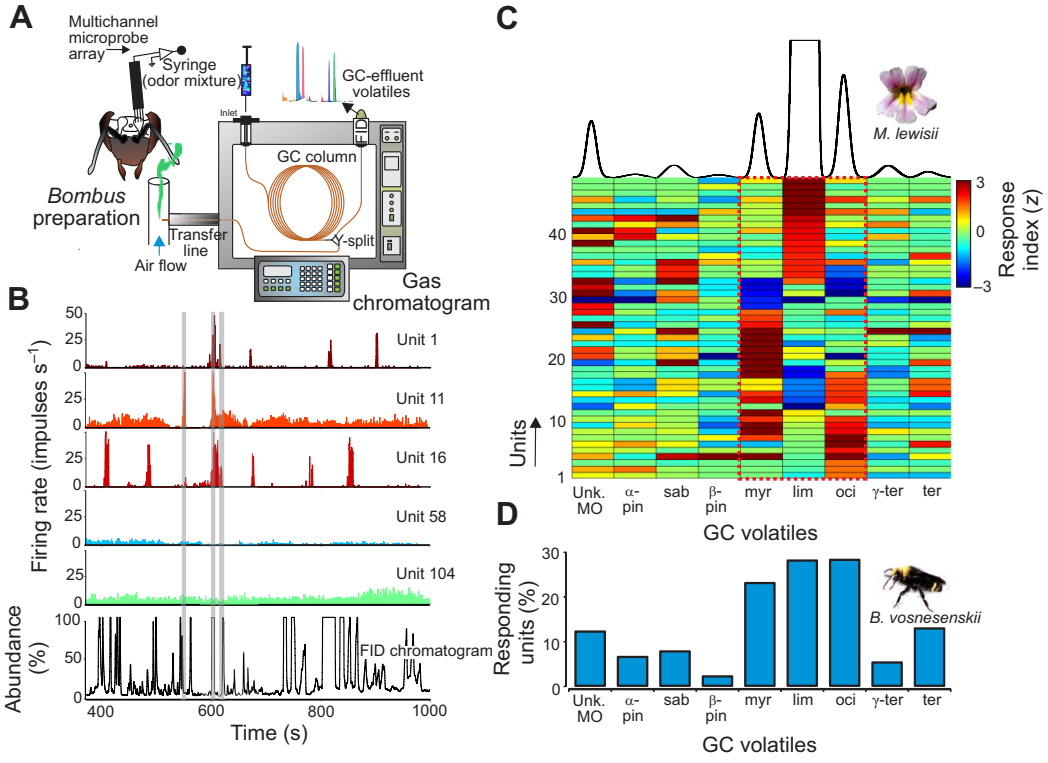

Fig. 2. Responses of *Bombus vosnesenskii* antennal lobe neurons to gas chromatography-fractionated scent from *M. lewisii* flowers. (A) Depiction of gas chromatography multichannel recording (GCMR). Effluent from the GC is split such that half enters the GC's detector (flame ionization detector, FID) 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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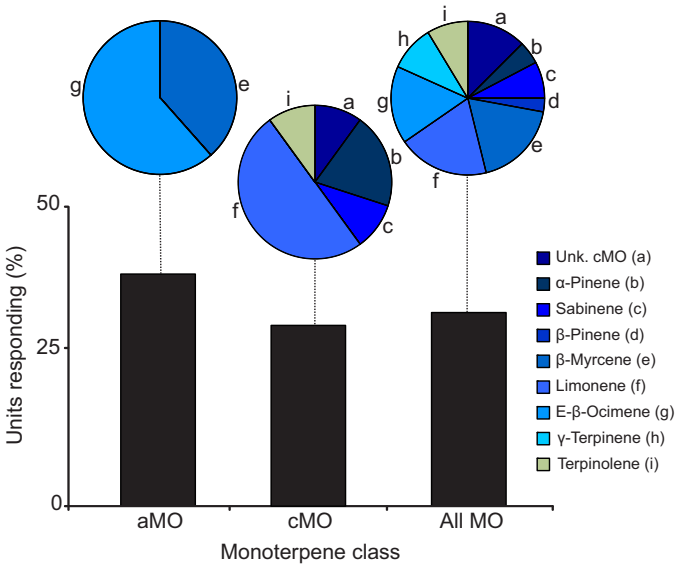

Fig. 3. Identification of bioactive volatiles and their chemical class using GCMR. Analysis of the population-level neural activity in response to the different volatiles revealed that units were responsive to acyclic monoterpenes (aMO) and cyclic monoterpenes (cMO), but units were also broadly responsive 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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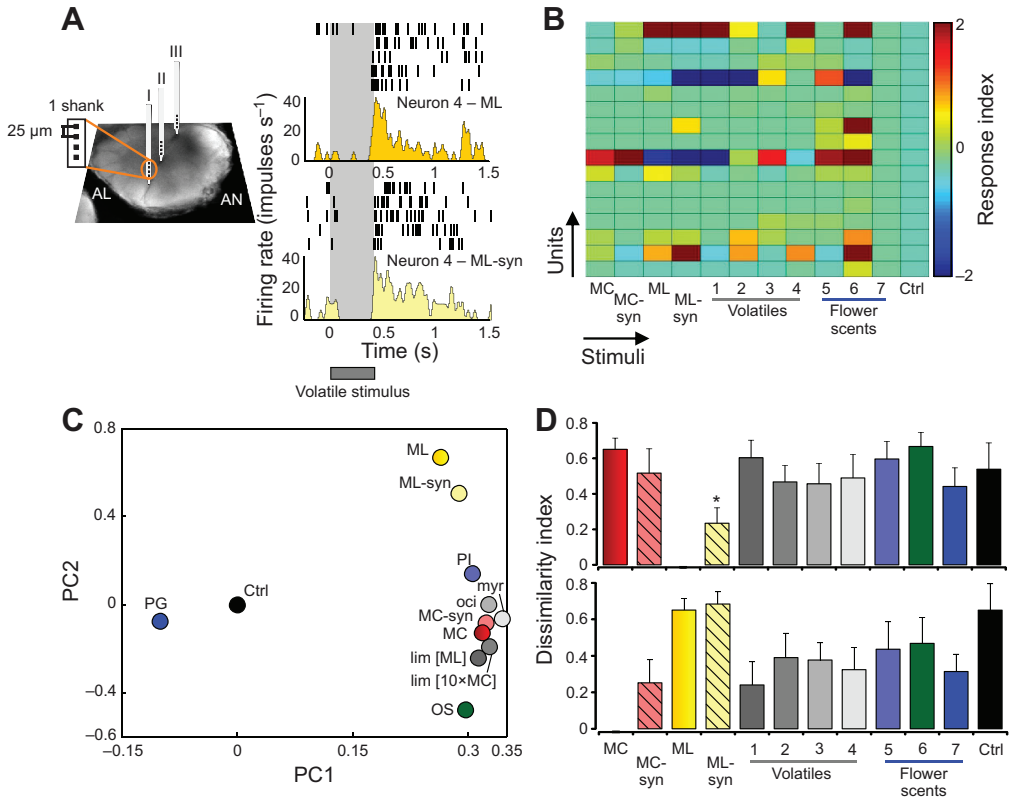

Fig. 4. AL neural responses to floral extracts, artificial mixtures and single volatiles. (A) Multichannel recording in the bee's AL allowed examination of the bee's ability to discriminate between volatile stimuli. On the right are peristimulus time histograms (PSTHs) and raster plots of a unit that 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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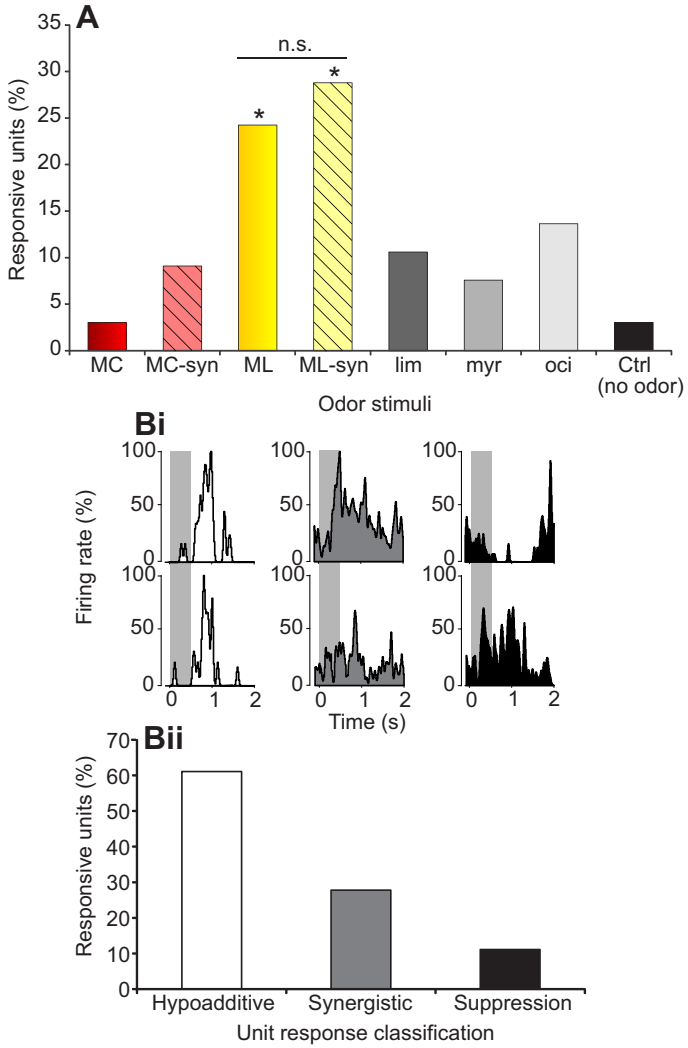

Fig. 5. Unit responses to mixtures and single volatiles. (A) The percentage of responsive units relative to the flower scents, synthetic mixtures and single volatiles. The *M. lewisii* scent (ML) and synthetic *M. lewisii* (ML-syn) evoked a significantly greater proportion of the neural units relative 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.

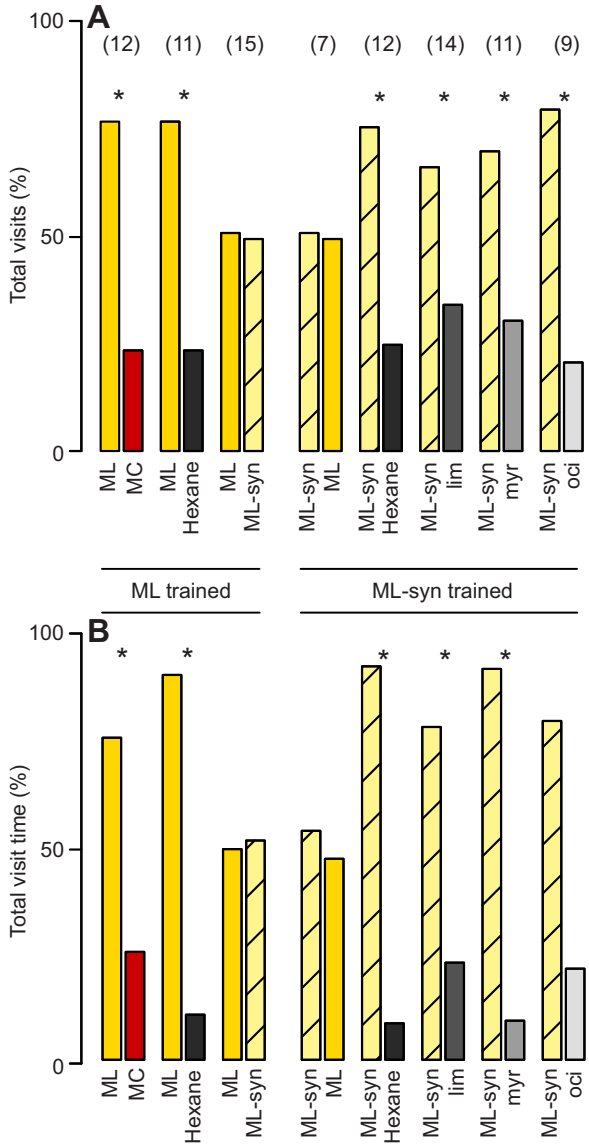

Fig. 6. Behavioral responses of *B. vosnesenskii* to floral bouquets, synthetic mixtures (hatched bars) and individual floral volatiles. (A) Total flower choices by experienced *B. vosnesenskii* workers trained to either natural ML or the synthetic mixture. Bars are the mean percentage of animals responding 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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Floral volatile alleles can contribute to pollinator-mediated reproductive isolation in monkeyflowers (*Mimulus*)

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Accession numbers for sequences:
KF857264, *M. lewisii LIMONENE-MYRCENE SYNTHASE* mRNA, complete cds
AHI50308, *M. lewisii* LIMONENE-MYRCENE SYNTHASE protein product
KF857262, *M. lewisii OCIMENE SYNTHASE* mRNA, complete cds
AHI50306, *M. lewisii* OCIMENE SYNTHASE protein product
KF857263, *M. cardinalis OCIMENE SYNTHASE* mRNA, complete cds
AHI50307, *M. cardinalis* OCIMENE SYNTHASE protein product

Summary

Pollinator-mediated reproductive isolation is a major factor in driving the diversification of flowering plants. Studies of floral traits involved in reproductive isolation have focused nearly exclusively on visual signals, such as flower color. The role of less obvious signals, such as floral scent, has been studied only recently. In particular, the genetics of floral volatiles involved in mediating differential pollinator visitation remains unknown. The bumblebee-pollinated *Mimulus lewisii* and hummingbird-pollinated *M. cardinalis* are a model system for studying reproductive isolation *via* pollinator preference. We have shown that these two species differ in three floral terpenoid volatiles - D-limonene, β-myrcene, and *E*-β-ocimene - that are attractive to bumblebee pollinators. By genetic mapping and *in vitro* enzyme activity analysis we demonstrate that these interspecific differences are consistent with allelic variation at two loci – *LIMONENE-MYRCENE SYNTHASE* (*LMS*) and *OCIMENE SYNTHASE* (*OS*). *M. lewisii LMS* (*MlLMS*) and *OS* (*MlOS*) are expressed most strongly in floral tissue in the last stages of floral development. *M. cardinalis LMS* (*McLMS*) has become a pseudogene and is not expressed. *M. cardinalis OS* (*McOS*) is expressed, but the encoded enzyme produces no *E*-β-ocimene. Reducing the expression of *MlLMS* by RNAi in transgenic *M. lewisii* produces no behavioral difference in pollinating bumblebees; however, reducing *MlOS* expression produces a 6% decrease in visitation. Allelic variation at the *OCIMENE SYNTHASE* locus likely contributes to differential pollinator visitation, and thus promotes reproductive isolation between *M. lewisii* and *M. cardinalis*. *OCIMENE SYNTHASE* joins a growing list of "speciation genes" ("barrier genes") in flowering plants.

Introduction

The rapid diversification of the world's estimated 275,000 species of flowering plants has often been attributed to their specialized association with different animal pollinators (Grant, 1949; Stebbins, 1970). Flowering plants use a variety of signals to advertise the presence (or illusion) of a reward to their associated pollinators; the association between pollinator type and suites of signals gives rise to the concept of pollination syndromes (Fenster *et al.,* 2004). Perhaps the most well-known and easily studied signal is floral color, which has been investigated in a variety of pollination syndromes (Rausher, 2008). Other visual signals, such as texture, pattern, orientation, anthesis time, size, and shape have been investigated to some extent in a variety of systems (Harder and Johnson, 2009; Kay and Sargent, 2009; Yuan *et al.*, 2013a).

Floral scent – the amount, relative ratios, and identities of volatile compounds emitted by the flower – is a generally understudied signal, despite the long understanding that it may play a strong role in attracting pollinators (Raguso, 2008a). The recent development of techniques for studying floral scent, including chemical analysis of floral scent, analysis of pollinator neural activity at both the receptor and higher-order processing levels, and genetic and genomic tools, has allowed some progress in this area. However, although floral scent is frequently characterized, and genes responsible for the production of floral volatiles are occasionally identified, a synthesis of floral scent biochemistry, neurobiology, genetics, ecology, and evolution has been lacking. Those systems with well-characterized volatiles that affect pollination are separate from those with well-characterized genetics (Raguso, 2008a; Raguso, 2008b; Whitehead and Peakall, 2009; Parachnowitsch *et al.*, 2012).

Much of the work discussing speciation involving floral volatiles has been done in extremely specialized systems where scent is crucial to plant-pollinator interactions (Raguso, 2008b), most notably the

sexually deceptive orchids in the genera *Chiloglottis* (Schiestl and Peakall, 2005; Peakall *et al.*, 2010) and *Ophrys* (Schiestl and Ayasse, 2002; Mant *et al.*, 2005; Vereecken *et al.*, 2010; Xu *et al.*, 2012), as well as the non-deceptive genus *Gymnadenia* (Huber *et al.*, 2005). Recent work has begun expanding this to non-orchid systems such as *Silene* (Waelti *et al.*, 2008), *Linanthus* (Chess *et al.*, 2008), and *Petunia* (Klahre *et al.*, 2011). While there are a growing number of studies demonstrating the importance of floral volatiles in mediating these largely specialized plant-pollinator interactions, the genetic pathways controlling volatile production in these systems remain unknown.

In contrast, the genes underlying volatile production are known in a diverse range of angiosperm systems (Gang, 2005), including *Clarkia* (Pichersky *et al.*, 1995; Dudareva *et al.*, 1996; Dudareva *et al.*, 1998; Wang and Pichersky, 1998), *Antirrhinum* (Dudareva *et al.*, 2000; Dudareva *et al.*, 2003), *Petunia* (Koeduka *et al.*, 2006; Orlova *et al.*, 2006; Dexter *et al.*, 2007), *Silene* (Gupta *et al.*, 2012), *Arabidopsis* (Bohlmann *et al.*, 2000; Chen *et al.*, 2003), and many species of agricultural importance. Although our knowledge of the genetic underpinnings of volatile production and emission has grown as a result of these systems, there is a paucity of research linking floral volatiles and plant speciation with the genetic and molecular basis for those effects.

Petunia is the only well-developed model for the role that a specific volatile plays in differential attraction of pollinators between sister species (Klahre *et al.*, 2011). The sister species *P. axillaris* and *P. exserta* differ in their production of methyl benzoate, a volatile attractive to the hawkmoth pollinators of *P. axillaris*. *ODO1*, one of the genes hypothesized to underlie this difference, encodes a MYB transcription factor that appears to be differentially expressed in the two species. The hawkmoth *Manduca sexta* is attracted more strongly to near isogenic lines with high levels of methyl benzoate production, suggesting that a change in volatile production mediated by a single gene can lead to

differential pollinator attraction; however, the underlying genetic mechanisms mediating species-specific volatile emission in this system remain unknown.

The sister species *M. lewisii* and *M. cardinalis* have served as a model system for studying pollinator-mediated reproductive isolation for several decades (Hiesey *et al.*, 1971), and the combination of ecological and genetic resources has led to the discovery of multiple loci impacting differential pollinator attraction between the two species (Bradshaw and Schemske, 2003; Yuan *et al.*, 2013b; Yuan *et al.*, 2013c). *Mimulus lewisii* is a bumblebee-pollinated alpine species, while its sister, *M. cardinalis*, is a lower-elevation hummingbird-pollinated species (Hiesey *et al.*, 1971; Schemske and Bradshaw, 1999). Within areas of sympatry, pollinator fidelity is responsible for 98% of reproductive isolation between *M. lewisii* and *M. cardinalis* (Ramsey *et al.*, 2003). Previous work has shown that three floral volatiles produced by *M. lewisii* – D-limonene, β-myrcene, and *E*-β-ocimene – are important for the attraction of bumblebee pollinators, including *Bombus vosnesenskii*, the native pollinator of *M. lewisii* in the central Sierra Nevada mountains of California. Of the three volatiles, *M. cardinalis* produces only D-limonene, released at just 0.9% the rate of *M. lewisii* (Byers *et al.*, 2014).

Therefore, we ask some global questions, which we begin to address in this manuscript: What are the genetic underpinnings of the differential emission of floral volatiles between *M. lewisii* and *M. cardinalis*? How many genes are responsible, and how do the species differ in gene expression and protein function? What role, if any, do these scent differences play in differential pollinator visitation, and through this, reproductive isolation? In keeping with other discussions of "speciation genes" in plants (Rieseberg and Blackman, 2010), are these genes of large effect or small effect, coding or regulatory genes? *Mimulus*, with its known attractive volatiles, genetic and genomic tools, and well-studied ecology, is an obvious choice for filling in this missing piece of the floral scent-speciation link.

Results

Genetic mapping of species-specific differences in floral volatiles

Construction of an F_1 cross between *M. lewisii* inbred line LF10 and *M. cardinalis* inbred line CE10 revealed patterns of inheritance of loci controlling the emission of D-limonene, β-myrcene, and *E*-β-ocimene. The emission rate of D-limonene for the F_1 (47.2 ng/flower/hr) was similar to that of the *M. lewisii* parental inbred line (55.1 ng/flower/hr, p = 0.599, t = 0.570, df = 4) and much higher than that of the *M. cardinalis* parental inbred line (0.5 ng/flower/hr, p = 0.028, t = -4.033, df = 2), suggesting that high levels of D-limonene emission are inherited from the *M. lewisii* parent in a dominant manner. The pattern was similar for β-myrcene (F_1: 2.6 ng/flower/hr, *M. lewisii*: 3.3 ng/flower/hr, *M. cardinalis*: 0.0 ng/flower/hr; F1 vs. *M. lewisii* p = 0.555, t = 0.663, df = 3; F1 vs. *M. cardinalis* p = 0.058, t = 2.677, df = 2). For *E*-β-ocimene, the *M. lewisii* allele appears to be semidominant (F_1: 2.8 ng/flower/hr; *M. lewisii*: 7.6 ng/flower/hr; *M. cardinalis*: 0.0 ng/flower/hr; F1 vs. *M. lewisii* p = 0.027, t = 2.897, df = 6; F1 vs *M. cardinalis* p = 0.070, t = -2.381, df = 2). Complete or partial dominance of the *M. lewisii* alleles for these floral volatiles is consistent with other traits that differ between the species (Bradshaw *et al.*, 1998).

When a backcross (F_1 x *M. cardinalis*) population of 100 plants was scored for the presence or absence of emission of β-myrcene and *E*-β-ocimene, it segregated approximately 1:1 for both volatiles (0.52:0.48 β-myrcene present:absent; 0.38:0.62 *E*-β-ocimene present:absent), suggesting that alleles at Mendelian loci might control the difference in emission of these monoterpenes between *M. lewisii* and *M. cardinalis*. D-limonene and β-myrcene emission rates were very highly correlated (r = 0.975), but neither was particularly highly correlated with *E*-β-ocimene emission rate (r = 0.474 versus D-limonene, r = 0.574 versus β-myrcene). Therefore, we considered a two-locus model for the difference in these three compounds between the two species – one locus controlling the production of D-limonene and β-

myrcene, and another, unlinked, locus controlling *E*-β-ocimene. A larger backcross population (N = 768) was constructed to map the two loci with greater precision.

Identification and characterization of a bifunctional LIMONENE-MYRCENE SYNTHASE (LMS) in *M. lewisii* flowers

The locus associated with D-limonene and β-myrcene emission was mapped to a 15 cM interval between markers M02_510K and M02_1500K, *ca*. 5.3 cM from M02_1500K. Using the assembled and annotated *M. guttatus* genome v1.1 as a reference (http://www.phytozome.net/cgi-bin/gbrowse/mimulus/), the ortholog of M02_1500K maps to *M. guttatus* scaffold 89 at position 201 kbp. On scaffold 89 between positions 206 kbp and 226 kbp there is a cluster of three terpene synthases/cyclases – excellent candidates for controlling D-limonene and β-myrcene emission.

Indel markers developed for two of the *M. lewisii/cardinalis* candidate genes in the terpene synthase cluster revealed no recombinations (in 768 backcross plants) between themselves or the putative *LIMONENE-MYRCENE SYNTHASE* (see Methods). The very tight linkage among the candidate terpene synthases within the cluster made it impractical to resolve the identity of the D-limonene and β-myrcene synthases by recombination. RT-PCR showed that, of the three candidates, only the *M. lewisii* ortholog (KF857265) of the *M. guttatus* terpene synthase on scaffold 89 at position 321 kbp (mgv1a003660m) is transcribed in *M. lewisii* flowers. The marker genotype at M02_1500 accounted for 92% of the difference between *M. lewisii* and *M. cardinalis* emissions of D-limonene and 98% of the difference in β-myrcene emissions, consistent with a single-locus model for D-limonene and β-myrcene production. No transgressive segregation was observed in the backcross population. The predicted AHI50308gene product contains the conserved DDxx(D/E) and (N,D)Dxx(S,T,G)xxxE (NSE/DTE) motifs

required for Mg^{2+} binding during the terpene synthesis process (Nieuwenhuizen *et al.*, 2013), as well as the RRx_8W motif required for cyclic terpene formation (Dudareva *et al.*, 2003).

The *M. lewisii* cDNA (KF857264) orthologous to mgv1a003660m, designated *TS321K*, was overexpressed in *E. coli* (as in Bohlmann *et al.*, 2000). A crude lysate from the *E. coli* culture was supplied with geranyl pyrophosphate (GPP) as a substrate, yielding D-limonene and β-myrcene in the same proportions as observed in the authentic headspace collection from *M. lewisii* flowers (Table S1, Fig. 2*A*). This suggests that the high correlation between D-limonene and β-myrcene emission in the backcross mapping population is due to the pleiotropic effect of a bifunctional LIMONENE-MYRCENE SYNTHASE (LMS) encoded by a single *LMS* gene in *M. lewisii* (*MlLMS*). This is consistent with the frequent occurrence of multi-product terpene synthases (Dudareva *et al.*, 2004). Of note, this is not a strict demonstration that *MlLMS* is the gene underlying this locus; other factors may be responsible for the species-specific differences in D-limonene and β-myrcene emission, and further experiments including transgenic rescue of the *M. cardinalis* allele would be necessary to be completely certain.

Identification and characterization of OCIMENE SYNTHASE (OS) in *M. lewisii* flowers

The locus associated with *E*-β-ocimene emission was mapped to a 7.5 cM interval midway between markers sc4_2325K and M13_2620. The marker genotype at sc4_2325 accounted for 98% of the difference between *M. lewisii* and *M. cardinalis* emissions of *E*-β-ocimene, consistent with a single-locus model for *E*-β-ocimene production. No transgressive segregation was observed in the backcross population. The orthologous region of the *M. guttatus* genome lies in a 484 kbp interval (2325 kbp – 2809 kbp) on scaffold 4. There is a cluster of five terpene biosynthesis loci on scaffold 4 at position 2538 kbp – 2577 kbp. The gene at position 2538 kbp (mgv1a020487m) is annotated as a terpene synthase, while the other four loci are annotated as sesquiterpene cyclases (Fig. 1). An indel marker developed for

the *M. lewisii/cardinalis* ortholog of mgv1a003660m, designated *TS2538*, revealed no recombinations (in 768 backcross plants) with the putative *OCIMENE SYNTHASE* (see Methods).

When overexpressed in *E. coli* and supplied with GPP as a substrate, the *M. lewisii TS2538* cDNA (KF857262) encodes a functional OCIMENE SYNTHASE (MlOS, AHI50306) (Fig. 2*A*, Table S1). However, under the same conditions the *M. cardinalis TS2538* cDNA (KF857263) does not encode an enzyme (McOS, AHI50307) capable of synthesizing any monoterpene that we could detect. McOS differs from MlOS at 19 amino acid residues, including insertion of a leucine residue at position 238 in McOS and deletion of an arginine residue at position 308 in McOS (Fig. S1). Both sequences contain the same DDxx(D/E) and NSE/DTE Mg^{2+} binding motifs, as well as the RRx_8W cyclase motif, which are unaltered by the 19 nonsynonymous amino acid substitutions (Fig. S1). Of note, this is not a strict demonstration that *MlOS* is the gene underlying this locus; other factors may be responsible for the species-specific differences in *E*-β-ocimene emission, and further experiments including transgenic rescue of the *M. cardinalis* allele would be necessary to be completely certain.

LMS* and *OS* expression *in vivo

Using RT-PCR with six different stages of flowering tissue from early bud (8d prior to anthesis, 5mm) to open flower (see Yuan *et al.*, 2013c), we found that both *MlLMS* and *MlOS* were expressed in the last three days prior to anthesis (15mm and 20mm) of floral development, as well as in the open flower (Fig. 2*B*). By contrast, *McLMS* was not expressed in any flowering stage. *McOS* is expressed in a similar temporal pattern to *MlOS*. It appears that the lack of *E*-β-ocimene production in *M. cardinalis* is due to coding rather than regulatory sequence changes. Whether *McLMS* was initially inactivated *via* regulatory or coding sequence mutation is unclear, but it was followed by further degeneration into a non-expressed pseudogene.

Construction of RNAi knockdowns of *MlLMS* and *MlOS* in stably transformed *M. lewisii*

Using RNA interference *via Agrobacterium*-mediated *in planta* transformation of hairpin RNAi constructs into *M. lewisii* (Yuan *et al.*, 2013c), we were able to knock down the expression of both *MlLMS* and *MlOS* to produce much lower floral volatile levels, comparable to those produced in plants homozygous for the *M. cardinalis* alleles at *LMS* and *OS*. This allowed us both to verify *LMS* and *OS* gene function *in vivo* and to determine the effect of decreased emission of specific floral volatiles on pollinating bumblebees.

We recovered 24 *M. lewisii* (inbred line LF10) T_1 plants carrying the *MlLMS*-RNAi transgene, and assayed each transgenic plant in triplicate for floral volatile production. All T_1 plants had lower emission rates of D-limonene and β-myrcene relative to the wild-type *M. lewisii* LF10 (D-limonene: range = 1.2%-56.1%, mean = 10.2%; β-myrcene: range = 4.1-50.0%, mean = 12.8%). Interestingly, most T_1 plants showed a decrease in emission of terpinolene (range = 0.0%-132.6%, mean = 18.0%), indicating that MlLMS may be responsible for synthesizing an additional minor compound in *M. lewisii*. One of these T_1 transgenics (LMS321K-8) was selfed as the parent of T_2 plants used for pollinator studies (for data on three other T_2 lines from independent T_1 transgenics, see Table S3). The original T_1 LMS321K-8 had very low emission rates of D-limonene and β-myrcene, with a mean of 2.8% D-limonene production and 9.1% β-myrcene production relative to the *M. lewisii* LF10 T_0 parent. Notably, LMS321K-8 had an increase in *E*-β-ocimene of 452.8% compared with the *M. lewisii* T_0 parent (Table 1, Fig. 3). All other T_1 plants had a similar increase in *E*-β-ocimene production relative to the wild-type parent (range = 190.4-493.9%, mean = 383.4%).

A total of 71 T_2 plants from the self-pollinated progeny of T_1 LMS321K-8 were assayed using headspace collection of floral volatiles to select the greenhouse population for the bumblebee pollinator behavioral

experiment. The 24 individuals selected for the experiment produced much less D-limonene and β-myrcene compared to the wild-type *M. lewisii* ancestor (D-limonene: range = 0.1-2.4%, mean = 1.9%; β-myrcene: range = 0.0-4.8%, mean = 0.6%), and more *E*-β-ocimene than the *M. lewisii* wild-type ancestor (range = 93.0-510.5%, mean = 247.2%). The D-limonene and β-myrcene levels were similar to those found in *M. cardinalis* (D-limonene: range = 0.03-2.8%, mean = 0.9% of wild-type *M. lewisii*; β-myrcene is absent from *M. cardinalis*).

Only two T_1 plants carrying the *MlOS*-RNAi transgene were recovered, but both had the desired *E*-β-ocimene knockdown phenotype relative to *M. lewisii* LF10 (*E*-β-ocimene: range = 0.8%-2.9%, mean = 1.8%; D-limonene: range = 39.6%-58.9%, mean = 49.2%; β-myrcene: range = 28.3%-41.2%, mean = 34.8%). T_1 plant OS2538-1, which was self-pollinated to create a T_2 population for pollinator studies, had a much lower emission rate of *E*-β-ocimene (0.8%) relative to *M. lewisii* LF10, as well as lower emission rates of D-limonene (39.6%) and β-myrcene (28.3%)(Fig. 3). T_1 plant OS2538-2 flowered substantially later than OS2538-1, and so a T_2 line was not created from this plant. A total of 80 T_2 plants were produced from OS2538-1, and these produced similar amounts of D-limonene and β-myrcene as the *M. lewisii* LF10 ancestor (D-limonene: range = 61.3-127.4%, mean = 83.6%; β-myrcene: range = 57.3-144.5%, mean = 88.7%), but much less *E*-β-ocimene (range = 0.9-3.9%, mean = 1.9%).

Effects of *MlLMS* and *MlOS* knockdowns on bumblebee pollinator behavior

Two experiments, one for the *MlLMS*-RNAi transgenics and one for the *MlOS*-RNAi transgenics, were performed to assay the impact of reduced monoterpene production on bumblebee (*Bombus impatiens*) visitation in a captive greenhouse setting. During each experiment, both preference (expressed as the proportion of total visits to each flower type) and constancy (expressed as the tendency of an individual bumblebee to deviate from random choices, exclusive of preference, see Waser, 1986) were measured.

A total of 1682 visits were observed to flowers in the *MlLMS*-RNAi knockdown experiment. Visits were defined as observable contact with the sexual organs of the flower – *i.e.,* the bumblebee entered the flower fully, as required to effect pollination. Of 1682 visits, 833 (49.52%) were to the wild-type *M. lewisii* and 849 (50.48%) were to the *M. lewisii MlLMS*-RNAi transgenic plants, showing no significant difference ($X^2 = 0.15$, $p = 0.70$, Fig. 4). Bumblebees appeared to show no overall qualitative behavioral difference towards either flower type.

A total of 39 bumblebee foraging bouts were assayed for constancy, with an average Bateman's index of -0.0114 (-1 indicates complete inconstancy – regular switching between types; 0 indicates random visitation patterns; +1 indicates complete constancy, always within types). To determine whether this constancy was significantly different from random visitation, the same bumblebee foraging bouts were used with 100,000 simulated runs of randomly permuted plant locations, resulting in an average Bateman's index of -0.1141. A total of 96,648 simulations had more divergent Bateman's index values than the actual data, showing that bumblebees demonstrated no constancy when presented with these flowers ($p = 0.97$).

For the *MlOS*-RNAi knockdown experiment, a total of 2202 visits were observed. Of these visits, 1166 (52.95%) were to wild-type *M. lewisii* and 1036 (47.05%) were to the *M. lewisii MlOS*-RNAi plants, showing a significant preference for the wild-type *M. lewisii* flowers ($X^2 = 7.67$, $p = 0.0056$, Fig. 4). Bumblebees approaching the *MlOS*-RNAi flowers were noted to frequently wave their antennae and contact the flower with their antennae prior to aborting a potential visit, suggesting that *E*-β-ocimene may operate as a near-field olfactory cue, but this behavior was not noted for the wild-type flowers in this experiment or either flower type in the *MlLMS*-RNAi experiment. Constancy was also absent in the

MlOS-RNAi experiment, with a total of 46 bumblebee foraging bouts showing an average Bateman's index of 0.0149; the simulation described above was repeated using these foraging bouts, with an average Bateman's index of -0.1142 (p = 0.95).

Discussion

Mimulus lewisii produces three floral volatiles with significant neurophysiological and behavioral effects on bumblebees – D-limonene, β-myrcene, and *E*-β-ocimene, while *M. cardinalis* produces only D-limonene at much lower levels (0.9% of *M. lewisii*) (Byers *et al.*, 2014). These differences are due to changes in gene expression or coding sequence in two genes: *LIMONENE-MYRCENE SYNTHASE* (*MlLMS*, *McLMS*) and *OCIMENE SYNTHASE* (*MlOS*, *McOS*). In quantitative genetic terms, allelic variation at LMS and OS accounts for 92-98% of the phenotypic difference between *M. lewisii* and *M. cardinalis* in floral emission of D-limonene, β-myrcene, and *E*-β-ocimene. The very low level of volatile emission from *M. cardinalis* flowers can be explained at the molecular genetic level; *McLMS* has become a pseudogene that is not expressed, while *McOS* has multiple coding sequence differences that eliminate its ability to produce *E*-β-ocimene, though it is still expressed. Functional copies of both genes are necessary to produce D-limonene, β-myrcene, and *E*-β-ocimene *in vivo*. Although we lack strict evidence that these are the genes underlying these loci, we present strong circumstantial evidence (the lack of product from McOS activity *in vitro* and the lack of transcription of *McLMS*) that is consistent with this, and RNAi knockouts show that the loss-of-function *LMS* and *OS* alleles can recapitulate the *M. cardinalis* volatile emission phenotypes necessary to test for differential pollinator visitation. Formal demonstration would require transforming *M. cardinalis* with the functional *M. lewisii* allele, but this was not done owing to the difficulty of *in planta* transformation of *M. cardinalis*.

Surprisingly, despite the high level of production of D-limonene and β-myrcene in *M. lewisii* flowers, substantially knocking down emission of these two compounds produces no significant effect on bumblebee visitation in the greenhouse. In contrast, knocking down emission of *E*-β-ocimene results in a modest (6%) but significant decrease in bumblebee visitation, suggesting that alternative alleles of *OCIMENE SYNTHASE* contribute to reproductive isolation between the bumblebee-pollinated *M. lewisii* and the hummingbird-pollinated *M. cardinalis*. Although 6% is a modest effect size in quantitative genetic terms, in evolutionary genetic terms a selection coefficient of 0.06 (130 greater visits to the wild-type plant / 2202 total visits) would sweep the beneficial allele to fixation very quickly in natural populations (Hartl and Clark, 1997), so we designed our pollinator visitation experiments to detect a difference in visitation as small as 5%. Assuming an infinite population size, the probability of fixation of the allele is 2s, or 12%; an effective population size greater than five individuals would allow selection to exceed drift as an evolutionary force at this locus ($N_e = 1/(4s) = 4.17$).

Why does the loss of D-limonene and β-myrcene have no effect on bumblebee visitation? First, the T_2 plants used in the greenhouse experiment had surprisingly high levels of *E*-β-ocimene, perhaps due to rerouting of a common pool of the shared precursor geranyl pyrophosphate (GPP). Terpene synthesis is a flexible but complex process, and buildups of precursors can be utilized by alternate metabolic pathways (Gang, 2005). Given the much higher emission of *E*-β-ocimene in the *MlLMS*-RNAi transgenic plants, *M. lewisii* may be prone to this effect. As the RNAi technique used here is an analogous (but weaker) representation of the phenotypes resulting from a loss-of-function mutation in a wild population, fluctuations in volatile production as found here are reflective of the system's physiology and the effects that might occur in a natural setting.

It is also possible that the high production of D-limonene and β- myrcene in *M. lewisii* serves another function within the plant, such as defense against herbivores, nectar robbers, or disease (Kessler *et al.*,

2013), as these volatiles are known anti-herbivory compounds (Levin, 1976). Although the three volatiles have similar physical properties, D-limonene and β-myrcene may serve to mediate long-distance attraction at the patch level rather than at the level of the individual flower; long-distance attraction has been shown to be important for honeybee (*Apis mellifera*) navigation (Bogdany and Taber, 1979). The high production of D-limonene and β-myrcene may be a remnant of some previous pollination syndrome, environmental context, or merely the byproduct of some other metabolic process within the plant. Similarly, although a significant effect on bumblebee visitation was seen with the loss of *E*-β-ocimene, it is possible that the main role of this volatile may lie elsewhere (Kessler *et al.*, 2013), for example in herbivory defense (Arimura *et al.*, 2004) with a secondary role in the attraction of bumblebee pollinators. Data on herbivory, florivory, or pathogen infestation in wild populations of *M. lewisii* and *M. cardinalis* is currently lacking, limiting our ability to speculate on these possibilities. Future field experiments will increase our understanding of the multiple roles these volatiles may be playing in *M. lewisii* and *M. cardinalis*.

Finally, it is possible that these effects differ from those that would be found with wild *Bombus vosnesenskii*. However, both species are generalist floral visitors, and the *M. lewisii* scent elicits similar olfactory responses in both bee species. Moreover, *B. impatiens* has been used as a model for bumblebee-flower interactions in other systems, including those involving *B. vosnesenskii* (Bodbyl Roels and Kelly, 2011), thus we feel that *B. impatiens* is an excellent model for these experiments (see SI Materials and Methods for a full explanation). Although these results differ in detail from those we found in previous behavioral experiments with artificial and extracted floral scents, in which all three monoterpenes were required for maximum bumblebee response (Byers *et al.*, 2014), the greenhouse experiments offer a more realistic assay for the effect of scent on pollinators by allowing them to integrate multiple floral cues.

What role does scent play in pollinator interaction within this system? Many studies have shown that scent plays a strong role in landing decisions by diurnal pollinators such as bumblebees and honeybees (Butler, 1951; Galen and Kevan, 1980; Galen and Kevan, 1983; Lunau, 1992; Majetic *et al.*, 2009; Dötterl and Vereecken, 2010) – the initial approach may be guided by patch-level visual signals, followed by a visually-guided approach to an individual flower. At that point, the final landing decision may be influenced by floral scent, especially in relatively weakly scented flowers such as *M. lewisii* (Dötterl and Vereecken, 2010; Parachnowitsch *et al.*, 2012). Therefore, even in the densely-flowered greenhouse experiments, signals such as the presence or absence of *E*-β-ocimene may play a significant role in final landing decisions. Additionally, densities in the greenhouse experiments were similar to those found in wild populations of *M. lewisii*, which grows along montane streambeds in large clusters, so the dense greenhouse conditions are a better indicator of the potential effect of a single change in scent in a wild population.

How might a loss-of-function allele of *OS* promote a pollinator switch from bumblebees to hummingbirds? Hummingbirds have a very limited sense of smell (Ioalé and Papi, 1989), and retain scent information very poorly (Goldsmith and Goldsmith, 1982), so the loss of scent in a hummingbird-pollinated flower such as *M. cardinalis* (an "anti-bee" but not "pro-bird" shift, to use the language in Castellanos *et al.*, 2004) would likely have no fitness cost, and might even increase fitness by discouraging bumblebee visitors from transferring heterospecific pollen to the stigma and carrying away nectar or pollen. In combination with the difference in visual signals and mechanical access found between *M. lewisii* and *M. cardinalis*, such a loss of *E*-β-ocimene might serve to reinforce visitation behavior. Whether these changes in floral volatiles evolved in allopatry or as reinforcement during secondary contact is unclear; investigating the volatile profiles and orthologous terpene synthase genes of other species in *Mimulus* section *Erythranthe* may provide some insight into this question. The fact that the *OS* polymorphism between *M. lewisii* and *M. cardinalis* is in a structural gene contradicts the

current thinking that genes involved in prezygotic reproductive isolation – often referred to as "speciation genes" (Coyne, 1992) or "barrier genes" (Noor and Feder, 2006) – are nearly always regulatory genes (Rieseberg and Blackman, 2010). However, the limited number of genes with known effects in prezygotic reproductive isolation should preclude any general conclusions from being drawn about this process. We would suggest, however, that the potential for structural genes to contribute to this process should not be ignored.

Although many systems used in the study of floral volatiles have relatively strong scents that are detectable by the human nose, scent can also be a factor in reproductive isolation in systems where it is easily missed, as in *Mimulus*. The role of strong emissions of floral volatiles in attracting nighttime pollinators from a distance is well documented (Raguso and Willis, 2003). The potential role of changes in floral scent in pollinator-mediated reproductive isolation involving generalist, daytime pollinators such as bumblebees is largely unknown, and no examples integrating floral scent genetics and pollinator reproductive isolation in sister species with generalist pollinators have been reported. Some authors have commented that the role of floral scent in reproductive isolation is questionable in generalist cases, as floral scents thus serve less as "private channels" and pollinators are attracted to multiple floral scent profiles (Schiestl and Ayasse, 2002).

Floral scent should be considered as an attractive factor even in generalist systems, along with more easily-measured visual signals such as floral color and pattern. Here, the sister species *M. lewisii* and *M. cardinalis* can be used as a model for the study of reproductive isolation involving floral volatiles – one can begin by looking at species-specific differences, identifying critical volatiles within a complex mixture *via* electrophysiological and behavioral assays (Riffell *et al.*, 2013; Byers *et al.*, 2014). Then, studies can proceed by determining the genetic basis of these phenotypic differences, creating high-resolution

genetic materials (near-isogenic lines, transgenics), and, finally, assaying of the results of these genetic changes in ecologically relevant greenhouse or field settings. Nearly all previous studies of the role of floral volatiles in speciation have only answered a subset of these questions, but our work with *Mimulus*, an emerging model system, shows that a comprehensive, integrative study is possible.

Experimental Procedures

QTL and fine mapping

Volatiles were first assayed in triplicate in an F1 cross of *M. lewisii* inbred line LF10 and *M. cardinalis* inbred line CE10 (LF10 x CE10) and compared with previous results for the parent lines (9 samples each; see Byers *et al.*, 2014) using Welch's t-test for unequal sample sizes and unequal variances. A coarse mapping population consisting of 100 individuals of a cross between LF10 and CE10, backcrossed to CE10 [(LF10 x CE10) x CE10], was then constructed. Headspace volatiles were collected in the manner described in Byers *et al.*, 2014 (see also SI Materials and Methods and methods below) and assayed for emission rates of D-limonene, β-myrcene, and *E*-β-ocimene. Pearson correlation coefficients were calculated pairwise for the three scents to investigate potential linkage or pleiotropy. A subset of 24 backcross plants with the two most divergent phenotypes (high D-limonene/β-myrcene and low *E*-β-ocimene; low D-limonene/β-myrcene and high *E*-β-ocimene) were screened at 34 indel markers evenly spaced across the genome (Table S4) with the intent of creating a low-resolution quantitative trait locus (QTL) map. However, it was clear from inspection of the genotypic and phenotypic data that the emission of D-limonene/β-myrcene and *E*-β-ocimene were, to a first approximation, segregating as Mendelian traits.

A larger backcross population (N = 768) was constructed and screened with markers flanking the putative *LIMONENE-MYRCENE SYNTHASE* (M02_510 and M02_1500), and flanking the putative *OCIMENE SYNTHASE* (sc4_2325K and M13_2620) (Table S4). Markers used in the mapping process were

developed from *M. lewisii* and *M. cardinalis* genome sequences, and amplify codominant markers in the backcross. To reduce the effort required to score flowers for volatile production, only those backcross plants with informative recombinations between markers flanking *LMS* (N = 107) or *OS* (N = 52) were phenotyped for scent, using a direct extraction assay from flowers rather than the more labor- and time-intensive headspace collection method. For further details, see Supplemental Information.

The *Mimulus guttatus* genomic region corresponding to the *M. lewisii* region containing *LMS* or *OS* was examined, and candidate genes were identified based upon their map position relative to the flanking molecular markers and the *M. guttatus* annotation. For the *LMS* locus controlling D-limonene and β-myrcene emission, primers were designed to amplify indel polymorphisms in two of the terpene synthases/cyclases on *M. guttatus* scaffold 89. The candidate genes were designated LC250K and TS306K (see Table S5 for all primers). No recombination events were observed among the two candidate genes and the putative *LMS*, defining a candidate region of less than 0.1 cM. For the *OS* locus controlling *E*-β-ocimene emission, primers were designed to amplify an indel polymorphism in a terpene synthase designated TS2538 on *M. guttatus* scaffold 4. No recombination events were observed between TS2538 and *OS*, defining a candidate region of less than 0.1 cM.

In vitro assay for terpene synthase activity

For details, see Supplemental Information and (Fäldt *et al.*, 2003).

Terpene synthase expression _in vivo_

Total RNA was extracted from various plant tissues using the Spectrum Plant Total RNA Kit (Sigma-Aldrich) and quantified. For tissue-specific expression, RNA was collected from leaf, corolla tube, petal lobe, and nectar guide. Flower buds were collected at 5, 8, 10, 15, and 20mm stages in *M. lewisii* and equivalent stages in *M. cardinalis*. cDNA was prepared from total RNA extracts using the SuperScript III

First-Strand Synthesis System (Invitrogen). RT-PCR for both loci for both species was performed with these cDNA, using *MlUBC* as a control for background expression levels as described in Yuan *et al.*, 2013c. Primers used were the following: *MlLMS* RNAi forward/reverse for *MlLMS* and *McLMS*; *MlOS* RNAi sense forward/reverse for *MlOS* and *McOS*; and *MlUBC*_foward/reverse for *MlUBC*.

Construction of *MlLMS* and *MlOS* RNAi transgenic *M. lewisii*

Transgenesis was done in the *M. lewisii* background, as insect pollination is inferred to be the ancestral state in this clade (Beardsley *et al.*, 2003) and construction of transgenic *M. cardinalis* is infeasible. Hairpin RNA interference (RNAi) transgenes targeted to knock down the expression of *MlLMS* or *MlOS* were constructed in pFGC5941 (Kerschen *et al.*, 2004; Arabidopsis Biological Resource Center, CD3-447) as described in (Yuan *et al.*, 2013c). In each case, target specificity of the RNAi fragment was assured by BLAST search against the *M. lewisii* LF10 genome sequence. For *MlLMS*, a 106 bp fragment of *M. lewisii* nectar guide cDNA was amplified and directionally cloned into the pFGC5941 *Nco*I/*Asc*I (sense) and *Bam*HI/*Xba*I (antisense) sites. For *MlOS*, a 289 bp sense fragment was amplified and directionally cloned into the *Nco*I/*Asc*I site of pFGC5941. A 180 bp antisense fragment (entirely within the 289 bp *Nco*I/*Asc*I amplicon) was amplified and directionally cloned into the *Bam*HI/*Xba*I site. Constructs were verified by sequencing, then electroporated separately into *Agrobacterium tumefaciens* strain GV3101 and used for *in planta* transformation of *M. lewisii* LF10 following (Yuan *et al.*, 2013c).

Greenhouse experiments

For details of experimental design, see Supplemental Information.

Observations of bumblebee behavior were recorded for the first six hours of the first three days by two observers using voice recorders, each following one or two bumblebees at a time. At the start of each day prior to the first bumblebee activity, old flowers were removed and newly opened flowers were

counted and equalized between the two types of plants (wild-type and RNAi transgenic) to ensure that bumblebees had an equal chance of encountering a given flower of each type on each day.

Data were transcribed and analyzed for preference (proportion of total visits) and constancy. For constancy, visits were "collapsed" to the plant level – *i.e.*, multiple visits to one plant in sequence were reduced to a single visit, since flower numbers were unequal between plants and flowers were often tightly clustered. Bumblebees were only used for constancy analysis if they visited ten or more plants in a foraging bout. Constancy was calculated using Bateman's method (described in Waser, 1986), which is independent of preference; equalizing flowers at the start of the day gave pollinators equal access to each type, as required by this metric. To determine if observed constancy was different from the null expectation, the same foraging data were used in a permutation test with shuffled plant identities, repeated 100,000 times; the fraction of the simulations with a greater than observed deviation from zero (complete randomness) was used to estimate the *p*-value.

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Legends for Supporting Information

Supporting experimental procedures.

Table S1: Products of the *in vitro* terpene synthase assays.

Table S2: Volatile production in the 2-4 best T1 plants recovered from RNAi experiments.

Table S3: Volatile production in T2 plants from four separate T1 parents recovered from RNAi knockdown of *MlLMS.*

Table S4: Molecular markers used during QTL and fine mapping.

Table S5: Sequences of oligonucleotide primers used.

Figure S1: Protein sequences of MlOS and McOS with differences highlighted.

Figure S2: Schematic of the greenhouse experimental setup.

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Tables:

Table 1: Volatile production in transgenic (T_1 parent plants of greenhouse experiment lines) and wild-type *M. lewisii* and *M. cardinalis*. Values are an average of three independent headspace samples. See Table S2 for complete data on all volatiles produced by *M. lewisii*.

Volatile	LF10 (ng/hr)	CE10 (ng/hr)	*MlLMS*-RNAi (ng/hr)	*MlOS*-RNAi (ng/hr)	*MlLMS*-RNAi (% LF10)	*MlLMS*-RNAi (% CE10)	*MlOS*-RNAi (% LF10)	*MlOS*-RNAi (% CE10
β-myrcene	3.313	absent	0.257	1.116	7.75%	n/a	33.68%	n/a
D-limonene	55.113	0.516	1.216	23.820	2.21%	235.61%	43.22%	4616.27%
***E*-β-ocimene**	7.624	absent	25.125	0.054	329.55%	n/a	0.71%	n/a

Figures:

Figure 1: *Mimulus lewisii* and *M. cardinalis* and their terpene synthases. (A) *Mimulus lewisii* and *M. cardinalis*. (B) Genetic maps of *MlLMS* and *MlOS* and the homologous regions in *M. guttatus*. Positions on the lower half of each are from the *M. guttatus* genome scaffolds; annotations are from queries of the *M. guttatus* transcripts with BLASTx. Putative terpene synthases are highlighted. *M. lewisii* limonene-myrcene synthase is homologous to a terpene synthase at 319,982 bp on *M. guttatus* scaffold 89, and *M. lewisii* ocimene synthase is homologous to a terpene synthase at 2,538,727 bp on scaffold 4.

Figure 2: Terpene synthase activity *in vitro* and *in vivo*. (A) Products of terpene synthases using a bacterial overexpression system and *in vitro* enzyme assay. Using geranyl pyrophosphate (GPP, the common monoterpene precursor), the MlLMS enzyme produces D-limonene (orange) and β-myrcene (green) in the same relative proportion as in the floral volatile emission. *M. lewisii* MlOS produces *E*-β-

ocimene (blue), but *M. cardinalis* McOS does not. (B) Temporal expression of terpene synthases *in vivo.* *MlLMS* is expressed just prior to flowering and in open flowers, but *McLMS* is not expressed at any stage of floral development. *MlOS* shows a similar expression pattern to *MlLMS*, and *McOS* is expressed at the same stages, despite producing no terpenoid volatile that we could detect.

Figure 3: Stable RNAi knockdowns of *MlLMS* and *MlOS* in *M. lewisii* produce plants with low emission levels of D-limonene (orange)/β-myrcene (green) and *E*-β-ocimene (blue), respectively.

Figure 4: Greenhouse experiments with *Bombus impatiens* and *M. lewisii* wild-type and transgenic lines. (A) Image of a typical bumblebee visit. (B) Response of bumblebees to *MlLMS* RNAi knockdowns, *MlOS* RNAi knockdowns, and the wild-type parent. Bumblebees show the same visitation response to *MlLMS* knockdown transgenics as to wild-type *M. lewisii*. Bumblebees preferentially visit wild-type *M. lewisii* over *MlOS* knockdown transgenics.

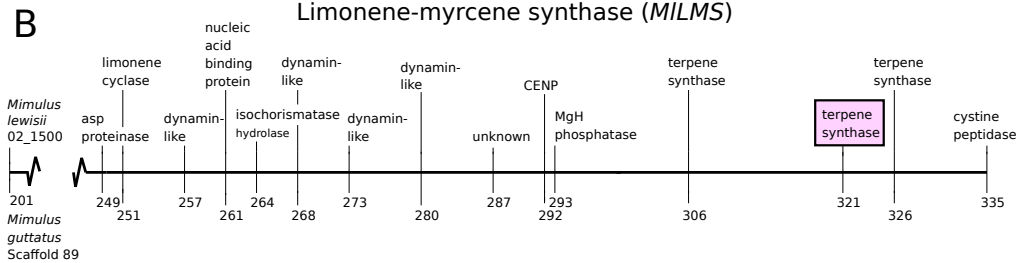
A

Mimulus lewisii

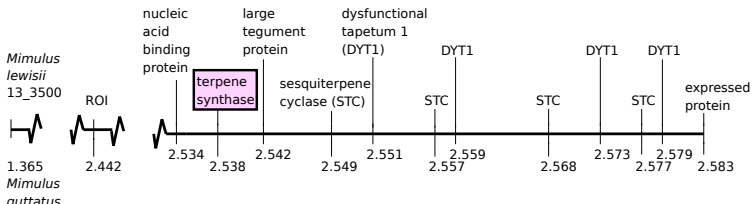
Mimulus cardinalis

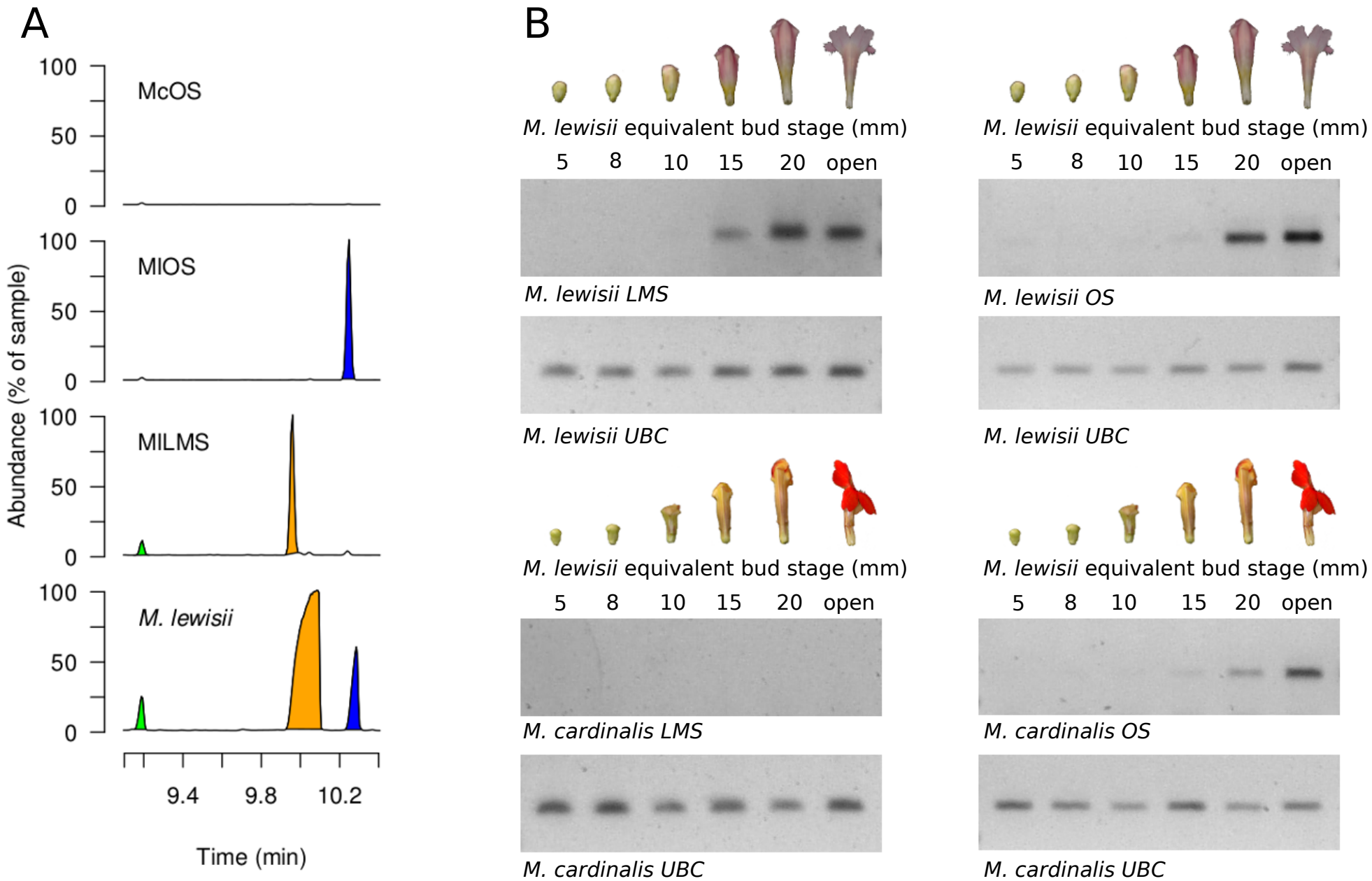
B

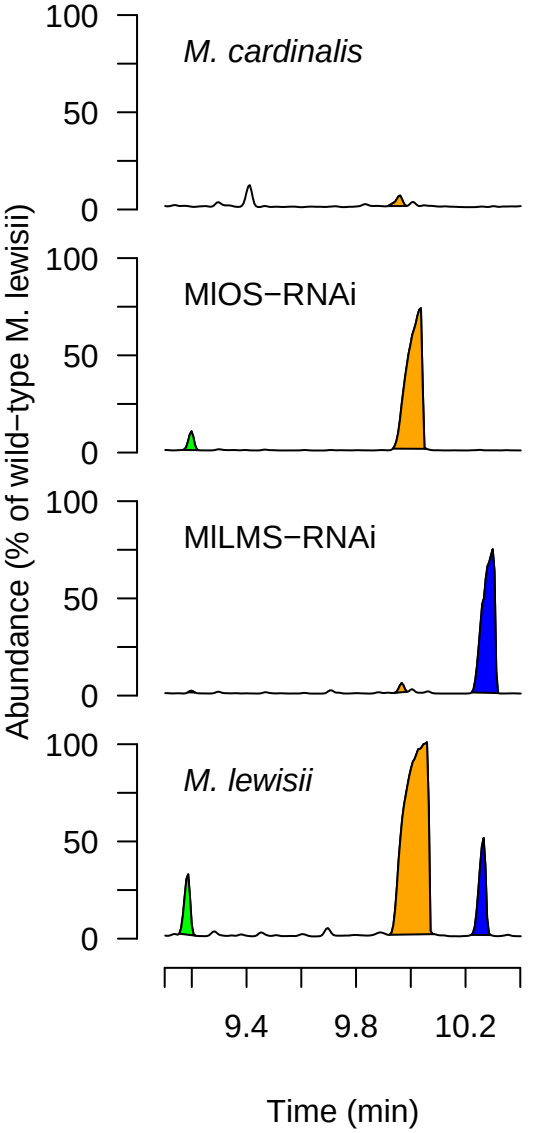
Limonene-myrcene synthase (*MlLMS*)



Ocimene synthase (*MlOS*)

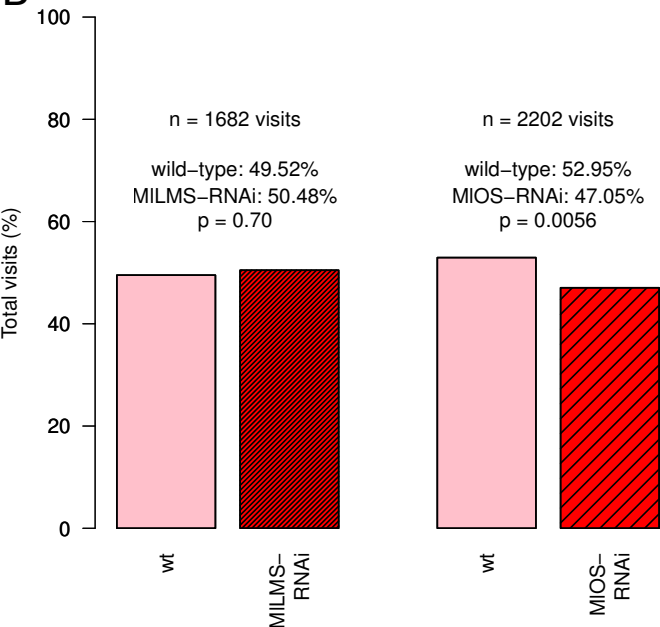



A
100
50
0
McOS
MIOS
MILMS
M. lewisii
Abundance (% of sample)
9.4
9.8
10.2
Time (min)
B
M. lewisii equivalent bud stage (mm)
5
8
10
15
20
open
M. lewisii LMS
M. lewisii OS
M. lewisii UBC
M. cardinalis LMS
M. cardinalis OS
M. cardinalis UBC


100
50
0
M. cardinalis
MlOS−RNAi
MlLMS−RNAi
M. lewisii
Abundance (% of wild−type M. lewisii)
9.4
9.8
10.2
Time (min)

A

B


Total visits (%)
100
80
60
40
20
0
n = 1682 visits
wild−type: 49.52%
MILMS−RNAi: 50.48%
p = 0.70
n = 2202 visits
wild−type: 52.95%
MIOS−RNAi: 47.05%
p = 0.0056
wt
MILMS−RNAi
wt
MIOS−RNAi

Supplemental Materials and Methods

Volatile headspace collection and analysis

Volatile headspace samples were collected from pairs of flowers with intact pedicels detached from the parent plant, which were placed in a 3L plastic oven bag (Reynolds, Richmond, VA, USA) for a 24-hour period. Samples were collected on volatile traps (constructed by placing 100mg of Porapak Q adsorbent (mesh size 80-100, Waters Corp., Milford, MA USA) in between layers of silanized glass wool in a 7mm borosilicate glass tube. A push-pull system (Raguso and Pellmyr, 1998; Riffell *et al.*, 2008) was used to pull headspace air through the bag using a diaphragm pump (400-1901, Barnant Co., Barrington, IL, USA) at a flow rate of 1L/min. All samples were collected for a period of 24 hours, as shorter collection periods do not capture the weak volatile emission of *M. cardinalis* adequately (Byers *et al.*, 2014).

Volatiles were eluted from traps using 600μL of hexane and stored in a 2 mL borosilicate glass vial with a Teflon-lined cap at -80°C. 150μL of hexane was removed from the vial and concentrated down to 15μL under nitrogen gas. A 3μL aliquot of each sample was run on 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; 30m, 0.25mm, 0.25μm) was used, and helium was used as carrier gas at constant flow of 1 cm^3/min. The initial oven temperature was 45°C for 4min, followed by a heating gradient of 10°C/min to 230°C, which was then held isothermally for 4min. Compound identities were determined by comparison with retention times of published volatiles from *Mimulus lewisii* (Byers *et al.*, 2014).

Volatile extraction collection and scoring

Two flowers from each plant (a single pair on a node) were removed from the plant. The corollas were separated from the calyx and pistil, crushed with hand pressure, and soaked in 500 μL of HPLC-quality

hexane for 2 hours in a scintillation vial. Extracts were concentrated and run on the GCMS using the same methods as the headspace samples, except the GC temperature method used the following protocol: initial oven temperature was 45°C for 3.5min, followed by a heating gradient of 20°C/min to 70°C, 10°C/min to 130°C, and 30°C/min to 285°C, which was then held isothermally for 5min.

The area under the GC peaks for β-myrcene and *E*-β-ocimene was used to assign *LMS* and *OS* genotypes to each recombinant plant. Plants with β-myrcene or *E*-β-ocimene peaks >$1x10^5$ counts*milliseconds were scored as heterozygotes at *LMS* or *OS*, respectively; all other recombinant plants lacked β-myrcene or *E*-β-ocimene and were scored as *M. cardinalis* homozygotes at the relevant locus. Recombination fraction between the flanking molecular markers and the *LMS* or *OS* genotype was used to estimate the map position of the loci controlling the volatile emission phenotypes.

***In vitro* assay for terpene synthase activity**

To test the biochemical function of the candidate terpene synthases, the candidate cDNAs were overexpressed in *E. coli* and the terpene synthase activity assayed *in vitro* as described in (Fäldt *et al.*, 2003). The coding region (including stop codon) of *MlLMS* and *MlOS* were amplified from *M. lewisii* inbred line LF10 corolla cDNA (see Table S5 for all primers) and TOPO cloned separately into pET100 (Life Technologies, Grand Island, NY). *McOS* was amplified from *M. cardinalis* inbred line CE10 open corolla cDNA and TOPO cloned into pET100. Construct integrity was verified by sequencing.

Induction of *LMS* or *OS* overexpression, crude protein extraction, and *in vitro* terpene synthase activity assay were carried out as described in Fäldt et al. (2003), with geranyl pyrophosphate (Sigma-Aldrich, St. Louis, MO) as the substrate. Enzyme reactions were terminated by extraction with 500 µL of HPLC-grade hexane. Hexane-soluble reaction products were assayed using the GCMS system described above, with an initial oven temperature of 45°C for 4min, followed by a heating gradient of 10°C/min to 230°C, which was then held isothermally for 4min. Product identities were confirmed by comparison of

retention times and mass spectra with *M. lewisii* headspace collections; the identity and retention time of volatiles in these headspace collections was previously verified using authentic standards (Byers *et al.*, 2014).

Greenhouse experiments

Single colonies of bumblebees (*Bombus impatiens*, Biobest, Belgium) were used in these experiments. *Mimulus lewisii* is pollinated in the wild by several species of bumblebees, dominated by *Bombus vosnesenskii* (Schemske and Bradshaw, 1999), but also including *B. balteatus*, *B. centralis*, and *B. flavifrons* (Hiesey *et al.*, 1971). *B. impatiens* was chosen in place of *B. vosnesenskii* because it is commercially available year-round and has been used in previous experiments with *Mimulus* (Bodbyl Roels and Kelly, 2011). Additionally, *B. impatiens* is very closely related to *B. vosnesenskii* (Cameron, 2007), and is nearly identical in size (*B. impatiens* workers: length 9-15mm, abdominal width 4.5-8mm; *B. vosnesenskii* workers: length 9-14mm, abdominal width 5-8mm) (Franklin, 1912). Both species are generalists (Harder and Barrett, 1993; Alarcón *et al.*, 2008).

Bumblebees were first trained on a uniform array of wild-type *M. lewisii* LF10 plants for a total of 7-10 days. Bumblebees are known to demonstrate in-hive learning of floral volatiles (Dornhaus and Chittka, 2005; Molet *et al.*, 2009). This training reproduces the expected situation in the wild, where foragers would become familiar with a particular "major" flower (Heinrich, 1976) and learn to exploit it. Bees were given unrestricted access to the plants at all times during the training and testing phases of the experiment, and were kept at temperatures between 17-24°C and a 15:9 light:dark cycle in the greenhouse *via* artificial lighting with high pressure sodium vapor lamps. No supplemental nectar or pollen was provided to the bees during the training or testing phases.

After completion of the 7-10 day training phase, the training plants were removed and replaced with a randomized hexagonal array consisting of 24 wild-type *M. lewisii* LF10 plants and 24 RNAi transgenic *M.*

lewisii plants, each separated by a distance of 35cm from its neighbors (Figure S2). Randomization was generated using the rand() command in Perl 5.14.2, which calls drand48 to generate pseudo-random numbers utilizing a linear congruential algorithm. *MlLMS* and *MlOS* RNAi transgenic plants were tested in separate experiments. Although 35cm may seem quite dense, this arrangement most closely replicates the density of *M. lewisii* patches in the wild.

Supplemental References

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Supplemental Figures and Tables

Figure S1: Protein sequences of MlOS and McOS with differences highlighted. The three boxed sets of residues correspond to the conserved RRx_8W motif required for cyclic terpene formation and the DDxx(D/E) and (N,D)Dxx(S,T,G)xxxE (NSE/DTE) motifs required for Mg^{2+} binding during the terpene synthesis process.

Figure S2: Schematic of the greenhouse experimental setup.

Table S1: Products of the *in vitro* terpene synthase assays. RT, retention time. No other terpene products were found in these assays.

MlOS		**McOS**		**MlLMS**	
RT (min)	**Product**	**RT (min)**	**Product**	**RT (min)**	**Product**
-	-	-	-	9.265	β-myrcene
-	-	-	-	10.038	D-limonene
10.331	*E*-β-ocimene	-	-	-	-
13.255	*cis*-geraniol	13.256	*cis*-geraniol	13.252	*cis*-geraniol
13.626	*trans*-geraniol	13.625	*trans*-geraniol	13.626	*trans*-geraniol

Table S2: Volatile production in the 2-4 best T1 plants recovered from RNAi experiments relative to wild-type *M. lewisii* and *M. cardinalis*. Values shown are averages across three independent headspace samples of each of 4 plants (MlLMS-RNAi T1s) and 2 plants (MlOS-RNAi T1s); values from wild-type plants are taken from three independent headspace samples taken concurrently with the transgenics. Numbers in parentheses correspond to the 10% and 90% values for the given volatile. Unk. MT: unknown monoterpene.

Volatile	**LF10 (ng/hr)**	**CE10 (ng/hr)**	**MlLMS-RNAi T1 (ng/hr)**	**MlOS-RNAi T1 (ng/hr)**	**MlLMS-RNAi T1 (%LF10)**	**MlLMS-RNAi T1 (%CE10)**	**MlOS-RNAi T1 (%LF10)**	**MlOS-RNAi T1 (%CE10)**
Unk. MT	0.30 (0.26, 0.34)	Absent	0.34 (0.23, 0.50)	0.20 (0.18, 0.22)	114% (75.1%, 166%)	n/a	67.1% (60.4%, 71.7%)	n/a
α-pinene	1.17 (1.10, 1.25)	0.11 (0.04, 0.19)	2.44 (1.51, 3.57)	1.30 (0.68, 1.97)	209% (129%, 306%)	2305% (1430%, 3379%)	112% (58.4%, 169%)	1235% (646%, 1863%)
Sabinene	0.89 (0.83, 0.98)	Absent	3.33 (1.99, 5.04)	1.51 (0.96, 1.98)	374% (223%, 566%)	n/a	169% (108%, 223%)	n/a
(-)-β-pinene	1.01 (0.92, 1.10)	Absent	1.63 (1.06, 2.40)	0.78 (0.47, 1.04)	162% (105%, 238%)	n/a	77.7% (46.2%, 103%)	n/a
β-myrcene	2.83 (2.05, 3.79)	Absent	0.21 (0.15, 0.26)	1.14 (0.65, 1.66)	7.40% (5.43%, 9.31%)	n/a	40.4% (22.9%, 58.7%)	n/a
D-limonene	43.21	1.02	1.07	24.63	2.47%	105%	57.0%	2412%

	(35.53, 50.70)	(0.65, 1.42)	(0.59, 1.92)	(15.70, 34.22)	(1.36%, 4.45%)	(57.4%, 188%)	(36.3%, 79.2%)	(1537%, 3351%)
E-β-ocimene	5.55 (4.31, 7.04)	Absent	22.80 (17.06, 29.08)	0.11 (0.04, 0.22)	411% (308%, 524%)	n/a	1.96% (0.67%, 3.99%)	n/a
γ-terpinene	0.06 (0.04, 0.08)	Absent	0.05 (0.02, 0.08)	0.01 (0.006, 0.02)	90.0% (37.2%, 141%)	n/a	22.3% (10.8%, 30.0%)	n/a
Terpinolene	0.18 (0.14, 0.21)	Absent	0.03 (<0.01, 0.03)	0.07 (0.03, 0.12)	17.9% (1.60%, 16.6%)	n/a	39.3% (15.2%, 66.6%)	n/a

Table S3: Volatile production in T2 plants from four separate T1 parents recovered from RNAi knockdown of *MlLMS*, relative to wild-type *M. lewisii*. Samples were collected via direct extraction (see Supporting Experimental Procedures). Values are averages across all samples, presented in counts*milliseconds. Where given, numbers in parentheses correspond to the 10% and 90% values for the given volatile. Sample sizes for the lines were 99 T2 plants (Line 8), 29 T2 plants (Line 14), 38 T2 plants (Line 21), and 8 T2 plants (Line 24). Of note, the genotypes of these T2 plants is not known, and as a result these values may be higher than homozygous T2 plants would be. Line 8 was chosen for the greenhouse experiments with the *MlLMS* RNAi knockdown plants.

Volatile	***M. lewisii* LF10**	***M. cardinalis* CE10**	**MlLMS-RNAi-8 T2 plants**	**MlLMS-RNAi-14 T2 plants**	**MlLMS-RNAi-21 T2 plants**	**MlLMS-RNAi-24 T2 plants**
β-myrcene	$1.84x10^5$	Absent	$1.57x10^3$ ($0.00x10^0$, $0.00x10^0$)	$6.47x10^3$ ($0.00x10^0$, $2.23x10^4$)	$3.33x10^2$ ($0.00x10^0$, $0.00x10^0$)	$4.95x10^3$ ($0.00x10^0$, $1.74x10^4$)
D-limonene	$4.60x10^6$	$1.63x10^4$	$4.98x10^4$ ($0.00x10^0$, $3.40x10^4$)	$1.69x10^5$ ($1.01x10^3$, $6.04x10^5$)	$6.10x10^4$ ($5.51x10^3$, $1.50x10^5$)	$8.37x10^4$ ($1.72x10^3$, $2.88x10^5$)
E-β-ocimene	$2.17x10^5$	Absent	$3.47x10^5$ ($7.97x10^4$, $7.48x10^5$)	$1.48x10^5$ ($3.47x10^4$, $2.69x10^5$)	$2.53x10^5$ ($9.63x10^4$, $4.95x10^5$)	$1.06x10^5$ ($3.15x10^4$, $1.66x10^5$)

Table S4: Molecular markers used during QTL and fine mapping.

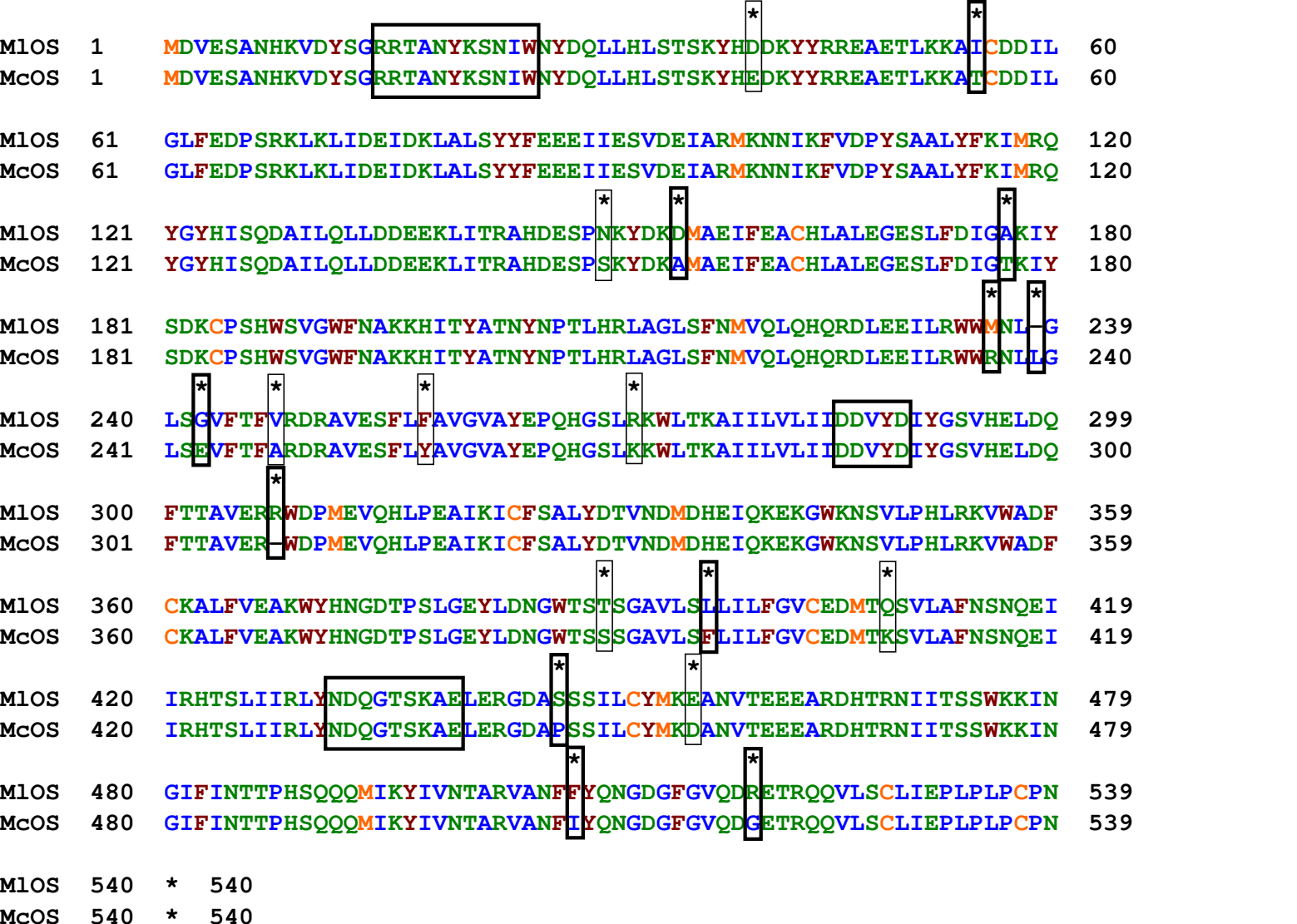
Marker Name	**Forward primer**	**Reverse primer**	**Length (M. lewisii LF10)**	**Length (M. cardinalis CE10)**
01_500	CCATACTGGTTCTGAAATTGCC	AATCTCGCCAAGAAGTAGGAAC	130	180
01_1460	CATCTCTTGTGGCCAGCTTGTA	CAACAACCAAGTGTCCAATGCTA	210	265
01_2490	ACGATGAACGGATTTATGGCTC	CTCAAACCACATTATACAGCTGC	110	150
02_510	CGACATTTCTACGAGCGACGA	AATCTACGTACGCCTAGAGTGA	207	235
02_1500	CATAAACGTTCTCTAGTGGATC	TCACTTGTGCATAAATTGGCTG	260	330

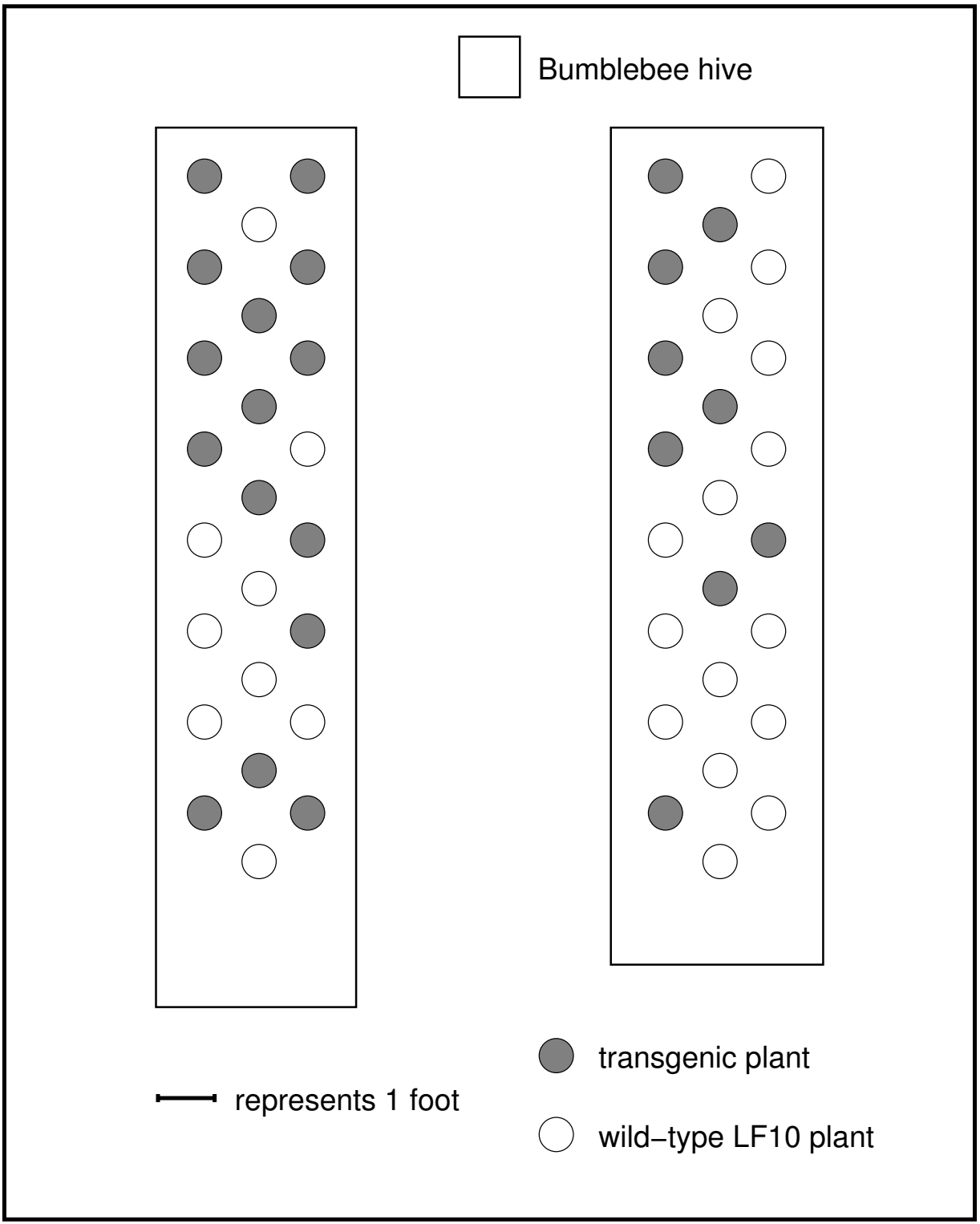
02_2900	TTTCTGGTTCACCCGAACTTAA	TAAAATCCCCTCCAAGCACTC	196	166
02_3500	ATGATAAGCAACCCTTAGTGTC	TACCCTTCACCAGTATAAGTTC	175	135
03_520	GGACCTTGATTACTGCAAGGCTA	TCTGCCACTGTCTTCGAAGCAC	200	150
04_2500	TGTAAGGGATGTCCAGGAAGC	GAGCCCAAGTCACATAGAATCA	500	470
06_470	ACGTAGCGTTTAATGAACCGAA	TATCGGGTGAGGAATATGTGTA	480	435
06_2500	GGAATTTGGCTCATCTTGGCAC	GGAGAAACTGATGTAAGACCCA	414	380
06_4500	TCGTTTCGATTGGCGGAAACCA	GGGATGATGATCAAGGTCCTGA	160	100
07_500	CAGTTGGTTGACGATCACAGA	TCCATGATCACTGATGCTGCA	200	230
07_1400	AATGATGGAGAACATCCATGTCC	CAGTAAAACGTTCCTGCTCGAGT	236	266
07_2100	CAGGGAATGCATTTCTAGTGCA	TCCGCTTAAAGGCACATGACAC	220	250
08_1000	TGACATCTGATAGCAGAGGGCA	GAACCATGGAGAGGTAGAGTGT	160	190
08_3200	TCCAAGCAAGTTTAGTGGGATC	TGACAAGGAGGCTGAGCAGAA	160	190
08_5100	CTCGATCAGTTTAGAGCTGCGA	TGTCTGCACTGTACATTCGAGC	170	200
09_600	GAGTCCGCTGATAGTACTGTGA	CCTCTCAGCATACTCCTCTGGA	195	170
10_2800	TGAGGATTTAAGCAACCACTCC	TGTGACCACTCTTACTCTTGAG	225	188
11_2600	TTTGAGCTATACGCCGTTCAC	CAGCTCGATTTTACAGAGTCC	150	207
12_2530	CGGTTCGTGTTCTTGTATGGA	GCTCAACTGTCAAAGCTGCATA	380	420
13_440	TTCACCCGAAGCGTGTACGCATA	GCACCACGATTCTGTTCCTCCAA	385	350
13_1600	CAGTGTTTCTTCGAGTGTGTAC	TGACTGCACCAGCATTACGCA	390	430
sc4_2325K	TGATCCCTTCGTCTCAAACTTTCCATA	TCATACCTCGGATACTTTTGCTTTTCA	140	170
13_2620	CTCCAGTAAGCCTGATGCCTCA	TCTTACGTGCTTCCTGCATGGTC	480	530
13_3500	AGCCCATTCACTTCATCCTGTGA	TGTGTTCCAGGGTCTTCATCGA	310	255
13_4480	CGCCGTTACCCACTTACCTCAA	ATGTCAATGACTCGCCACAAGGA	360	395
14_600	GCTCTGAAGCCAGAGTGTAGC	GYCTAATCGTTCGCACTTCGC	450	400
14_1520	GACAGAAGCTATGAAACCATCC	ACTGTCCAACACTCTTTCGTGC	420	390
14_2580	AAGCTCACATTGACTGACAAGGC	TCTCGATCTCCTCTCAAGCCAAC	460	500
14_4480	GCGTTTTGCCTTGTGTCAAGAC	AGCGTGTTTGCTTCCACTTCTC	250	290
14_6540	CAAGTGTATGTGACACGACGAC	CGCTCACGTCTCAAAGCTGCTA	445	500
14_7500	TGGCAGTTACCTCTATTGTGGA	CAGGTTTAATTTGCCGTCGTAC	260	220
14_8420	CACAGAGTTTGACATTCCAGCAC	GATAGCTCATTCACTCTGCTGC	465	500

Table S5: Sequences of oligonucleotide primers used. Where relevant, the TOPO linker (CACC) or linkers with restriction sites for directional cloning for RNAi constructs are bolded and underlined.

Primer Name	**Primer Sequence (5'-3')**
LC250K forward	CTAGCCTTTATAGCAAGATGCTGC
LC250K reverse	CATCTCAGACGTCTCCTTTGGTTC
TS306K forward	CCACTGCGATATTCGATGATCCTC
TS306K reverse	CCATCTGCAAAAGAAAAGGGCAGC
TS2538 forward	AATTAAGTTCAGGGGCGCATCTAC
TS2538 reverse	GGACGAAACTTGCAACTCTTCCGC

MlLMS in vitro forward	**CACC**ATGTCTGCTACCCTTATAATGCA
MlLMS in vitro reverse	TCAAATGTCAGTGTGGACTCCCCATT
MlOS/McOS in vitro forward	**CACC**ATGAAGAATAACATAAAATTCGTCGATCCA
MlOS/McOS in vitro reverse	TTAATTAGGGCAAGGTAGAGGAAGA
MlLMS RNAi forward	**GTTCTAGACCATGG**CAATCAAATCTCTTTCTTCGAAAAAATCA
MlLMS RNAi reverse	**GTGGATCCGGCGCGCC**GCATTGCATTGTATTGATGCACAAGGCTC
MlOS RNAi sense forward	**AAACCATGG**CGCGTCTTCATCGATCCTATGTTAC
MlOS RNAi sense reverse	**ATTGGCGCGCC**TTAATTAGGGCAAGGTAGAGGAAGA
MlOS RNAi antisense forward	**TAATCTAGA**AATACTACTCCTCATTCTCAGCAAC
MlOS RNAi antisense reverse	**ATTGGATCC**TTAATTAGGGCAAGGTAGAGGAAGA
MlUBC forward	GGCTTGGACTCTGCAGTCTGT
MlUBC reverse	TCTTCGGCATGGCAGCAAGTC


MlOS 1 MDVESANHKVDYSGRRTANYKSNIWNYDQLLHLSTSKYHDDKYYRREAETLKKAICDDIL 60
McOS 1 MDVESANHKVDYSGRRTANYKSNIWNYDQLLHLSTSKYHEDKYYRREAETLKKATCDDIL 60
MlOS 61 GLFEDPSRKLKLIDEIDKLALSYYFEEEIIESVDEIARMKNNIKFVDPYSAALYFKIMRQ 120
McOS 61 GLFEDPSRKLKLIDEIDKLALSYYFEEEIIESVDEIARMKNNIKFVDPYSAALYFKIMRQ 120
MlOS 121 YGYHISQDAILQLLDDEEKLITRAHDESPNKYDKDMAEIFEACHLALEGESLFDIGAKIY 180
McOS 121 YGYHISQDAILQLLDDEEKLITRAHDESPSKYDKAMAEIFEACHLALEGESLFDIGTKIY 180
MlOS 181 SDKCPSHWSVGWFNAKKHITYATNYNPTLHRLAGLSFNMVQLQHQRDLEEILRWWMNL-G 239
McOS 181 SDKCPSHWSVGWFNAKKHITYATNYNPTLHRLAGLSFNMVQLQHQRDLEEILRWWRNLLG 240
MlOS 240 LSGVFTFVRDRAVESFLFAVGVAYEPQHGSLRKWLTKAIILVLIIDDVYDIYGSVHELDQ 299
McOS 241 LSEVFTFARDRAVESFLYAVGVAYEPQHGSLKKWLTKAIILVLIIDDVYDIYGSVHELDQ 300
MlOS 300 FTTAVERRWDPMEVQHLPEAIKICFSALYDTVNDMDHEIQKEKGWKNSVLPHLRKVWADF 359
McOS 301 FTTAVER-WDPMEVQHLPEAIKICFSALYDTVNDMDHEIQKEKGWKNSVLPHLRKVWADF 359
MlOS 360 CKALFVEAKWYHNGDTPSLGEYLDNGWTSTSGAVLSLLILFGVCEDMTQSVLAFNSNQEI 419
McOS 360 CKALFVEAKWYHNGDTPSLGEYLDNGWTSSSGAVLSFLILFGVCEDMTKSVLAFNSNQEI 419
MlOS 420 IRHTSLIIRLYNDQGTSKAELERGDASSSILCYMKEANVTEEEARDHTRNIITSSWKKIN 479
McOS 420 IRHTSLIIRLYNDQGTSKAELERGDAPSSILCYMKDANVTEEEARDHTRNIITSSWKKIN 479
MlOS 480 GIFINTTPHSQQQMIKYIVNTARVANFFYQNGDGFGVQDRETRQQVLSCLIEPLPLPCPN 539
McOS 480 GIFINTTPHSQQQMIKYIVNTARVANFIYQNGDGFGVQDGETRQQVLSCLIEPLPLPCPN 539
MlOS 540 * 540
McOS 540 * 540


Bumblebee hive
transgenic plant
represents 1 foot
wild–type LF10 plant

DISCUSSION

The sister species *Mimulus lewisii* Pursh and *M. cardinalis* Douglas ex Benth. (Phrymaceae) exist in sympatry along the western slopes of the Sierra Nevada mountains in California. Within this zone of sympatry, differential pollinator attraction is responsible for 97.6% of the reproductive isolation between the two species (Ramsey *et al.* 2003). Previous work in this system had indicated the role of color in mediating pollinator preference (Schemske & Bradshaw 1999, Bradshaw & Schemske 2003). However, like many weakly scented species (Raguso 2008; Whitehead & Peakall 2009), the role of scent in pollinator attraction was assumed to be negligible, as the two species are scentless to the human nose (Schemske & Bradshaw 1999). Experiments with captive hawkmoths indicated that floral scent was likely present in the system in large enough amounts to encourage flower visitation, so I set out to determine what role scent could be playing in pollinator isolation within this system.

Research contributions of this thesis

In Chapter 2 (published in Byers *et al.* 2014), I described the first steps towards answering this question: the identification of the floral volatiles present in both species and the discovery of which volatiles were most electrophysiologically and behaviorally attractive to pollinating bumblebees, *Bombus vosnesenskii*. Using headspace collection and gas chromatography-mass spectroscopy, the volatiles emitted by both species were identified. *Mimulus lewisii* flowers produce nine monoterpene volatiles. *Mimulus cardinalis* flowers produce two monoterpene volatiles, two sesquiterpenes, and one alkene alcohol. The volatile profile of *M. lewisii* is dominated by three monoterpenes: D-limonene, β-myrcene, and *E*-β-ocimene, which together make up 93% of the total volatiles emitted. Of these three, *M. cardinalis* only produces D-limonene, but at 0.9% the rate emitted by *M. lewisii*; overall, the floral scent emitted by *M. lewisii* is approximately 65 times stronger than that emitted by *M. cardinalis*.

As *Bombus vosnesenskii* is the main pollinator of *M. lewisii* in the Sierra Nevada (Schemske & Bradshaw 1999), we collected this species in the Seattle area for electrophysiological and behavioral experiments. Electrophysiological preparations were made from live bumblebees, and extracellular recordings were done using a multi-channel electrode inserted into the antennal lobe (Byers *et al*. 2013). The results revealed that, while all nine monoterpenes emitted by *M. lewisii* excited a response in the antennal lobe, three monoterpenes – D-limonene, β-myrcene, and *E*-β-ocimene – elicited a much stronger response. When the preparation was stimulated with *M. lewisii* and *M. cardinalis* headspace extracts, it responded more strongly to *M. lewisii*. An artificial mixture of the three volatiles that elicited the largest response was made; bumblebee preparations responded to this mixture more strongly than to its individual components, and responded equally strongly to the natural *M. lewisii* headspace extract. Both the *M. cardinalis* headspace extract and an equivalent concentration of limonene were far less excitatory.

Electrophysiological results do not always predict behavioral results - as an example, a mouse will exhibit electrophysiological stimulation to cat urine, but it will not move towards the cat urine in a behavioral arena. However, in this case, the three volatiles (D-limonene, β-myrcene,

and *E*-β-ocimene) that elicited the strongest antennal lobe responses had the same effects in behavioral trials with *B. vosnesenskii*. Bumblebees preferred the natural scent of *M. lewisii* to that of *M. cardinalis*, but treated *M. lewisii* and its three-component artificial mimic equivalently. As in the electrophysiological experiments, the three components of the artificial mimic when tested individually against the mimic were not able to recapitulate the complete response.

As a result of the electrophysiological and behavioral results, we demonstrated the importance of these three monoterpenes in bumblebee preference for the scent of *M. lewisii*. However, the role of these volatiles in the whole-flower context – in other words, what happens when they are altered in a real flower – remained unclear. Although bumblebees rely on floral scent for a variety of foraging decisions (Dötterl & Vereecken 2010), they will forage on flowers that produce little to no scent (Keasar *et al.* 1997, Chittka *et al.* 2001), so it was unclear whether differences in floral scent in this system might actually contribute to pollinator-mediated reproductive isolation. As a first step towards elucidating their role in reproductive isolation, it was necessary to determine the genetic basis of these species-specific differences in volatile emission. Once the genetic basis was known, experimental genetic manipulation of floral scent emission could be trialed with free-flying bumblebees. These experiments are discussed in Chapter 3.

We used an unbiased quantitative trait locus (QTL) mapping approach to determine the genetic basis of differences in these volatiles. A small backcross mapping population (seeds from an F_1 plant of *M. cardinalis* x *M. lewisii* which was backcrossed to *M. cardinalis*) was created and scanned at a small number of marker loci distributed evenly across the genome, which were then correlated with levels of emission of D-limonene, β-myrcene, and *E*-β-ocimene. Emission of D-limonene and β-myrcene was found to be highly correlated ($r = 0.975$), suggesting a single locus, or tightly linked loci, for these two volatiles, but neither was correlated with *E*-β-ocimene emission. Moreover, in this small population, emission of D-limonene/β-myrcene and *E*-β-ocimene appeared to behave as Mendelian traits, with D-limonene/β-myrcene production behaving in a dominant fashion (*M. lewisii* allele dominant over the *M. cardinalis* allele) and *E*-β-ocimene production behaving in a semidominant fashion, again with the *M. lewisii* allele partially dominant over the *M. cardinalis* allele.

Using a larger mapping population, a more dense set of markers in the regions containing the presumptive loci for D-limonene/β-myrcene and *E*-β-ocimene, and transcriptome data from *M. lewisii* flowers, we were able to identify two candidate genes, both monoterpene synthases. Upon verification with *in vitro* terpene synthesis assays (Fäldt *et al.* 2003), the two candidate genes were named *LIMONENE-MYRCENE SYNTHASE* (*LMS*) and *OCIMENE SYNTHASE* (*OS*). *LIMONENE-MYCENE SYNTHASE* in *M. lewisii* (hereafter *MlLMS*) is expressed in the final stages of floral development and produces D-limonene and β-myrcene *in vitro* (and also produces terpinolene in small amounts *in vivo*); the *M. cardinalis* allele, *McLMS*, has degenerated into a pseudogene and is not expressed in floral tissue. *OCIMENE SYNTHASE* in *M. lewisii* (*MlOS*) is expressed in the same floral development stages as *MlLMS* and produces only *E*-β-ocimene *in vitro*; while the copy in *M. cardinalis* (*McOS*) is also expressed (albeit at slightly lower levels), it

does not produce any product *in vitro*, which is presumably due to one or several of the 19 amino acid residue differences between MlOS and McOS. Using *Agrobacterium*-mediated RNAi knockdown stable transgenesis (Yuan *et al.* 2013c), we were able to dramatically decrease expression of *MlLMS* and *MlOS* in the *M. lewisii* background, suggesting that allelic variation at *LMS* and *OS* is responsible for the species-specific differences in volatile emissions of these three monoterpenes.

However, in order to demonstrate that the differences in floral volatile emission play a role in reproductive isolation *via* pollinator choice, it was necessary to conduct greenhouse experiments using these transgenic plants and free-flying bumblebees (Yuan *et al.* 2013a). We set up two experiments: one to test the impact of reduction of D-limonene and β-myrcene *via* knockdown of *MlLMS* in *M. lewisii* and another to test the impact of reduction of *E*-β-ocimene *via* a knockdown of *MlOS*. In each case, bumblebees were trained on wild-type *M. lewisii*, then trialed for three days with an equal number of plants (and equal number of flowers) of the wild-type and transgenic knockdowns. Visitation counts (defined as a visit that could effect pollination) and individual bumblebee constancy were assessed (Waser 1986). In neither case did bees demonstrate constancy. Despite the demonstrated importance of D-limonene and β-myrcene in bumblebee electrophysiology and behavior, bumblebees did not respond significantly to a decrease in D-limonene and β-myrcene in the first experiment (49.5% visitation to the wild-type, 50.5% visitation to the knockdown plants). By contrast, knocking down emission of *E*-β-ocimene resulted in a modest but significant decrease in visitation (53% visitation to the wild-type, 47% visitation to the knockdown plants). Although a 6% decrease in visitation seems modest, this is a sufficiently large difference in fitness (knockdown plants are only 94% as fit as wild-type plants) to potentially lead to fixation of the functional *OS* allele in a bumblebee-pollinated species.

D-limonene, β-myrcene, and *E*-β-ocimene are three of the four most common floral volatiles among angiosperms (Knudsen *et al.* 2006, Schiestl 2010), and their existence is assumed to be ancestral across the seed plants (Schiestl 2010). The results discussed in this thesis demonstrate that even common, widely used volatiles (here, *E*-β-ocimene) can play a role in reproductive isolation.

Why are D-limonene and β-myrcene emitted at such high levels from *M. lewisii* despite playing no role in bumblebee visitation (contrary to the electrophysiological and behavioral response)? D-limonene, β-myrcene, and *E*-β-ocimene are also common secondary metabolites involved in plant defense (Levin 1976, Arimura *et al.* 2004). It is possible that D-limonene and β-myrcene are playing a role in herbivory defense – specifically defense against florivory – in *M. lewisii*. As *M. lewisii* grows in more alpine environments, resources for florivores may be scarcer than at lower altitudes, leading to a requirement for direct defense against florivores. However, without further study of florivory in both species and the role of these volatiles in defense in *Mimulus*, this is purely speculation. Finally, it is possible that D-limonene and β-myrcene are functioning at a different stage in bumblebee attraction to *M. lewisii*; unlike the demonstrated role of *E*-β-ocimene in close-range landing decisions, they may be functioning at the patch level to attract pollinators from a longer distance.

The exact role of loss of *E*-β-ocimene in the evolution of *M. cardinalis* remains unclear, and cannot be fully elucidated by studies of this type. Loss of *E*-β-ocimene emission in *M. cardinalis* appears to be due to a loss of function in the protein due to amino acid residue changes, rather than a shift to production of other monoterpenes or a loss of expression. Although not discussed in this thesis, the common ancestor of section *Erythranthe* appears to emit *E*-β-ocimene, as does *M. parishii* and the yellow morphotype of *M. verbenaceous*. Loss of *E*-β-ocimene production could have occurred while *M. lewisii* and *M. cardinalis* evolved in allopatry; alternatively, it could have evolved once secondary contact was established in order to discourage bumblebee visitation to *M. cardinalis*. In the words of Castellanos *et al.* 2004, this acts as an anti-bee trait rather than a pro-bird trait. Finally, in contrast to suggestions in the literature (Rieseberg & Blackman 2010), *MlOS* is a structural rather than a regulatory gene, and its loss of function is *via* coding rather than regulatory shifts. Opinions differ on the importance of structural and regulatory genes in pollinator shifts, with a recent review suggesting that

"…variation in structural genes, as well as changes in their expression levels, are likely mechanisms allowing rapid evolutionary responses to fluctuating pollinator communities." (Clare *et al.* 2013)

Future research directions

With this thesis, I have addressed the role that three floral volatiles play in floral isolation in *Mimulus lewisii* and *M. cardinalis*. However, additional work remains to be done within the scope of this question. First, testing the transgenic plants, induced mutants, or high-resolution near-isogenic lines, in the field in a region of sympatry between *M. lewisii* and *M. cardinalis* would be ideal. The development of novel genetic tools such as CRISPR (Belhaj *et al.* 2013) will simplify this substantially. Additionally, understanding the phylogenetic patterns of floral volatiles across section *Erythranthe* (as well as how they relate to each species' pollinator, habitat, sympatry with other species in the section, *etc*.) may help explain some of the evolutionary history of floral volatiles in *M. lewisii* and *M. cardinalis* – *e.g*., whether the presence of farnesene isomers in *M. cardinalis* represents a novel gain of function or whether they were lost in *M. lewisii*. Similarly, understanding the molecular evolution and expression of terpene synthesis genes in *M. lewisii*, *M. cardinalis*, and the rest of section *Erythranthe* may help shed light on the selective pressures underlying changes in terpene production. As mentioned above, investigating alternate roles for terpenes in *M. lewisii* and *M. cardinalis* – long-distance attraction, herbivory defense (either direct or indirect), defense against pathogens, mediation of below-ground interactions, *etc.* – may help explain the presence of volatiles that seem to be less critical for bumblebee pollination.

Although the discovery of the role that floral scent plays in reproductive isolation in this system is novel, much is known about the genetic basis of other traits mediating pollinator attraction in *Mimulus lewisii* and *M. cardinalis*. In particular, floral color has been very well studied. We know that the *YELLOW UPPER* (*YUP*) locus, which controls carotenoid deposition, has a very strong role in pollinator attraction (Bradshaw & Schemske 2003); however, the *YUP* near-isogenic line contains normal levels of carotenoids (not discussed in this thesis), so *M. lewisii* is

not converting unused carotenoids into terpene volatiles as might be expected (Simkin 2004). The gene underlying *ROSE INTENSITY* (*ROI*) , another locus controlling anthocyanin intensity, has recently been identified (Yuan *et al.* 2013c). Prior work had shown that anthocyanin intensity showed a tradeoff in pollinator attraction (Schemske & Bradshaw 1999); however, *ROI1* is tightly linked with *OCIMENE SYNTHASE*, and the F_2 plants in this study with high levels of anthocyanin would very likely have also emitted almost no *E*-β-ocimene whatsoever, leading to suppression of bumblebee attraction, and more recent work with high-resolution near-isogenic lines with a recombination between *ROI1* and *OS* has failed to replicate this effect (Bradshaw, personal communication). The region of the genome containing *ROI1* and *OS* also contains major quantitative trait loci (QTL) responsible for corolla length and pollen viability, as well as being under heterozygote-excess transmission distortion (Fishman *et al.* 2013), so additional factors may be co-segregating with *OCIMENE SYNTHASE* that play a role in reproductive isolation.

We also know that nectar guides play a role in bumblebee attraction and entry orientation (Owen & Bradshaw 2011) and are under control of the *GUIDELESS* gene (Yuan *et al.* 2013b). Their loss in the *M. lewisii guideless* mutant does not decrease scent production (not discussed in this thesis), so the dual nectar guide cues of carotenoid pigmentation/trichomes and *E*-β-ocimene emission must both be required for correct orientation and floral visitation by bumblebees. In this way, these two traits and the two genes (*GUIDELESS* and *OCIMENE SYNTHASE*) must be working synergistically to mediate bumblebee pollination, and may be co-regulated as a result.

Schemske and Bradshaw (1999) also discussed nectar volume and projected area as attractive traits affecting pollination; a more thorough project with a similar F_2 population measuring more floral traits may uncover additional cases where floral scent is working synergistically with other attractive traits. Finally, modeling bumblebee and hummingbird visitation decisions as a behavioral chain of events (Lynn 2003, Chittka & Raine 2006) may help clarify which factors are influencing pollinator choice at each decision point.

Moving beyond floral volatiles in *Mimulus*

Globally, many further areas of work remain open in the general field of scent-mediated plant-pollinator interactions. A variety of work has shown that scent can play a role at long distance for some specialist pollinators (up to 1 kilometer for euglossine bees, Dötterl & Vereecken 2010; tens to hundreds of meters for hawkmoths, Raguso & Willis 2003). However, little is known about the role of scent in attracting generalist pollinators such as bumblebees, short-tongued bees, syrphid and other flies, and non-hawkmoth lepidopterans at a distance. Some information is known about close-range landing decisions (e.g. Butler 1951, Galen & Kevan 1980, Galen & Kevan 1983, Lunau 1992, Dobson *et al*. 1999, Majetic *et al.* 2009, Dötterl & Vereecken 2010), but very little is known about how scent might function in generalist pollinator attraction at a distance. Overall, most examples of scent-mediated plant-pollinator interactions that have been extensively studied have involved relatively rare, specialized systems such as sexual, oviposition, and food deception (e.g. *Ophrys*, *Chiloglottis*, *Arum*, and *Disa*; Schiestl & Johnson 2013). Where scent has been investigated in rewarding systems with

generalist pollinators, its effect on pollinators has been tested empirically only infrequently (*e.g*. through electrophysiological and behavioral assays in the lab or field setting).

Some recent studies have focused on empirical tests in rewarding flowers that are pollinated by more generalist pollinators (e.g., Pellmyr & Patt 1986, *Lysichiton* and staphylinid beetles; Raguso *et al.* 1996 and Raguso & Light 1998, *Clarkia* and hawkmoths; Odell *et al.* 1999, *Antirrhinum* and bumblebees; Miyake & Yafuso 2003, *Alocasia* and drosophilid flies; Ashman *et al.* 2005, *Fragaria* and small bees; Huber *et al.* 2005, *Gymnadenia* orchids and multiple lepidopteran species; Waelti *et al.* 2007, *Silene* and multiple insect pollinators; Kessler *et al.* 2008, *Nicotiana* and hummingbirds and hawkmoths; Riffell *et al.* 2008, *Datura* and *Agave* and hawkmoths; Schlumperger & Raguso 2008, *Echinopsis* and hawkmoths; Majetic *et al.* 2009, *Hesperis* and bumblebees and syrphid flies; and Klahre *et al.* 2011, *Petunia* and hawkmoths). Unfortunately, most of these systems have not been investigated further (and fewer than half have measured fitness as a function of floral volatiles), and little work overall has been done on the role of scent in attracting pollinators other than hawkmoths and social bees. Moreover, nearly all work studying the role of scent in attracting pollinators has been done in an artificial laboratory environment that often lacks other floral signals. More work is clearly needed in the areas of sensory processing in other generalist pollinators such as short-tongued bees, flies, and non-hawkmoth lepidopterans; additionally, more work on learning and memory in heavily learning-focused pollinators such as bumblebees in real-world settings would be beneficial, as this work has nearly exclusively been done in a laboratory setting with artificial flowers and scent blends. Similarly, as recent work has shown that many pollinators use multimodal cues to navigate flowers (Dötterl & Vereecken 2010), additional studies investigating multimodal attraction in both generalist and specialist pollinators remain to be done.

The work discussed in this thesis points to the need to investigate floral scent in systems previously assumed to rely only on visual cues for pollinator choice. Floral scent can be a subtle cue, but - as I have shown here – it has the potential to play a role in reproductive isolation, even in weakly scented species. Human researchers are innately focused on visual cues as a result of our biased sensory systems; although we may be able to distinguish one trillion different volatile blends (Bushdid *et al.* 2014), the majority of the work done on the genetics of traits affecting pollinator choice has been visual (Raguso 2008, Yuan *et al.* 2013a). Scent is difficult to study for a variety of reasons, including the amount of effort required to collect samples, the expensive equipment necessary for basic analysis, and the lack of knowledge of the role of scent in reproductive isolation outside of specialist cases (Raguso 2008). In many research groups, studying floral scent requires either collaboration with chemists (who may not be knowledgeable about the specific chemistry of floral volatiles) or with the limited number of floral scent researchers with an interest in evolution. These barriers combine to make scent a difficult target of mapping approaches (as phenotyping scent is far less trivial than phenotyping other traits such as floral color or symmetry). As a result, some promising studies of floral scent genetics and pollinator isolation have stopped at large regions, choosing to follow up candidate genes or to drop the region entirely (as in Klahre *et al.* 2011), resulting in a biased approach towards floral scent genetics.

From the evolutionary and ecological side, the majority of knowledge around floral scent is restricted to either descriptive studies of floral volatiles in particular species or to the genetics of floral scent pathways (particularly terpene and aromatic volatiles) (Whitehead & Peakall 2009). Little is known about the effect of floral scent on plant fitness or pollinator choice. Eliciting the role of floral scent in pollinator transitions is of interest in understanding pollinator-mediated reproductive isolation and speciation. As an example, a common pattern in western North American flora is the transition from bee to hummingbird to hawkmoth pollination (Grant 1993, Whittall & Hodges 2007, Rosas-Guerrero *et al.* 2014); however, hummingbird-pollinated species are usually not strongly scented (Knudsen *et al.* 2004). How does the transition to hawkmoth pollination (which requires a scent cue, Raguso & Willis 2003) occur in the absence of floral scent in its predecessor, or are hummingbird-pollinated intermediates scented? Other common transitions often include the emission of novel compounds (e.g. sulfur compounds involved in bat pollination, Knudsen & Tollsten 1995); understanding the genetic and biochemical mechanisms allowing emission of novel volatile classes will be a key part of explaining these pollinator shifts.

Still less is known about population-level variation in floral scent and whether it varies with abiotic factors such as temperature and soil nutrients, negative biotic factors such as herbivory and competition, or positive biotic factors such as pollinator distribution. Floral scent is largely absent in research addressing the "geographic mosaic of coevolution" (Thomson 1999, 2005). Overall, manipulative studies of floral scent in field settings are rare, limiting our ability to discover general patterns of the effect of floral scent on fitness and reproductive isolation. More integrative studies that – as here - combine floral scent characterization, electrophysiological and behavioral studies, genetic mapping of floral volatile loci, and greenhouse or field experiments with molecularly-defined alternative alleles will benefit our understanding of the role of floral scent in species diversification.

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monkeyflowers (*Mimulus*). *Proc Natl Acad Sci USA* 96(21):11910-11915.

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Research interests

I am interested in investigating the genetic basis for speciation events in natural populations, with a particular focus on the role that floral scent plays in pollinator-mediated speciation in wildflowers. Angiosperms (flowering plants) total more than 275,000 named species and are one of the most diverse groups on the planet. Animal pollination occurs in over 80% of angiosperm species, and is a major driver of angiosperm speciation and floral trait diversity. The massive insect-driven radiation of angiosperms during the Cretaceous indicates the strong role pollination syndromes and pollinator-mediated selection have on speciation and global biodiversity.

Education

University of Washington, Seattle, WA
2008-2014 (Thesis defense: 5 June 2014; graduation: 14 June 2014)
Ph.D. candidate, Department of Biology
Advised by Dr. H. D. "Toby" Bradshaw, Jr. and Dr. Jeffrey A. Riffell

Massachusetts Institute of Technology (MIT), Cambridge, MA
2003-2007
S.B., Biology

Minuteman Regional High School, Lexington, MA
1999-2003
Biotechnology Academy Program
(600 hours/year of biology-specific lectures, labs, and research in a vocational setting)

Research experience

Melinda Denton Writing Fellow, University of Washington (Winter Quarter 2014)
Continuing work on the role of floral scent in pollinator-mediated reproductive isolation in *Mimulus*; analyzing scent in multiple species of *Mimulus*; manuscript preparation.

Plant Biology Fellow, Bradshaw Laboratory, University of Washington
(Fall Quarter 2013)
Continuing work on the role of floral scent in pollinator-mediated reproductive isolation in *Mimulus*.

National Science Foundation Graduate Research Fellow, Bradshaw and Riffell Laboratories, University of Washington (Fall Quarter 2010 - Summer Quarter 2013)
Studying the role of floral scent in the evolution of ornithophily using *Mimulus* wildflowers and various pollinators, particularly *Bombus* bees, including scent profiling, electrophysiology,

behavioral experimentation, mapping of loci responsible for floral scent, and greenhouse experiments with transgenic plants.

Plant Biology Fellow, Bradshaw Laboratory, University of Washington
(Summer Quarter 2009 - Summer Quarter 2010)

Studied the role of floral color and scent in the evolution of sphingophily from ornithophily using *Mimulus* wildflowers and *Manduca* moths.

Graduate Opportunity Fellow, Peichel Laboratory, Fred Hutchinson Cancer Research Center, University of Washington (Spring Quarter 2009)

Studied the expression of pigment patterning genes in *Gasterosteus aculeatus*, the threespine stickleback, using *in situ* hybridization of whole larval fish.

Graduate Opportunity Fellow, Bradshaw Laboratory, University of Washington
(Winter Quarter 2009)

Studied the role of floral color in a possible sphingophilic pollinator shift in *Mimulus*, the monkeyflowers, using the Carolina Sphinx (*Manduca sexta*) as a model pollinator.

Graduate Opportunity Fellow, Di Stilio Laboratory, University of Washington
(Autumn Quarter 2008)

Studied the sequences and expression of five floral transcription in *Thalictrum delavayi*, a basal eudicot, using consensus sequencing and *in situ* hybridization probe preparation.

Technical Research Assistant, Bulyk Laboratory, Harvard Medical School (2007 - 2008)

Determined the DNA binding specificity of over 100 transcription factors in yeast using protein-binding microarrays and *in vitro* transcription/translation, developing a streamlined high-throughput process for hybridization and downstream data analysis.

Undergraduate Researcher, Polz Laboratory, MIT (2005)

Assembled and closed genomes from two environmentally-isolated species of *Vibrio*, developing a streamlined methodology for efficient contig-to-genome reconstruction.

Undergraduate Researcher, Kaiser Laboratory, MIT (2004)

Investigated flavin membrane transporters in the endoplasmic reticulum of yeast using a novel yeast strain overexpressing *RIB4*, a riboflaving biosynthesis pathway gene, and searching for candidate genes upregulated during the unfolded protein response (UPR).

Bioinformatics Research Intern, Human Genome Sequencing Center, Baylor College of Medicine (2002)

Developed annotation methodology for the assembled human genome and annotated regions of Chromosome 9 using a consensus of various bioinformatics tools and available sequence.

Awards and fellowships

2013	Melinda Denton Writing Fellowship, Department of Biology
2013	Best graduate student talk, Department of Biology annual retreat
2009	NSF Graduate Research Fellowship
2008	Achievement Rewards for College Scientists (ARCS) Foundation Fellowship
2008	GenOM Project Graduate Fellowship
2008	Plant Biology Fellowship
2008	Graduate Opportunity Program Research Assistantship

Research funding

2012-2014	NSF Doctoral Dissertation Improvement Grant ($14,562) DISSERTATION RESEARCH: Contribution of specific floral odorants to

differential attraction and reproductive isolation in monkeyflowers (*Mimulus*). Note: this grant was selected as an NSF "Highlight" by the IOS program director, titled "The science behind a flower's sweet scent"; please see http://go.usa.gov/8tPY for details.

Teaching experience and educational outreach

Teaching experience

Spring Quarter 2014: Biology 428 (Sensory Physiology and Ecology), teaching assistant; two discussion sections and guest lecture
Summer Quarter 2013: Biology 317 (Plant Classification and Identification), guest lecturer
Winter Quarter 2012: Biology 457 (Chemical Communication), guest lecturer
Winter Quarter 2010: Biology 180 (Introductory Biology), teaching assistant; three lab sections and interactive lectures
Autumn Quarter 2009: Biology 481 (Experimental Evolutionary Ecology), teaching assistant; lab section and course organization (including all grade records and electronic assignments)

Mentoring and supervisory experience

2013	Field team, Yosemite National Park (Bradshaw Lab: training, experimental design, data analysis, surveys)
2012-2013	Mary Sargent (technician, Bradshaw Lab)
2011-2013	Elischa Sanders (undergraduate, Riffell Lab)
2010-2013	Riane Young (undergraduate, Bradshaw Lab)
2010	Stefanie Sternagel and Beth Dingman, undergraduate Advanced Experimental Evolutionary Ecology students (Bradshaw Lab)
2009-2010	Kali Brandt (undergraduate, Bradshaw Lab)
2008	Sean McMaster (undergraduate, Bulyk Lab)
2007-2008	Mita Shah (undergraduate, Bulyk Lab)
2012-2014	Melissa Lacey (new graduate student)
2011-2014	Henry Hunter (new graduate student)
2010-2014	William Hardin (new graduate student)

Educational outreach

2014	Docent enrichment lecture, Washington Park Arboretum
2013	Panelist for a graduate experience discussion for undergraduates, Univeristy of Washington
2013	Lab tour for NSF REU students working at Friday Harbor Labs
2013	Lab tour for Seattle community members
2012	Undergraduate Diversity mentor, Evolution 2012, Ottawa, Canada
2011-present	Contributor, Calphotos and BugGuide
2011	Panelist for a graduate experience discussion for undergraduates, University of Puget Sound
2011	News media appearances and docenting related to *Amorphophallus titanum* bloom, University of Washington Botany Greenhouse
2010-2014	Docent, Woodland Park Zoo, Seattle, WA
2009-2014	Ambassador, Woodland Park Zoo, Seattle, WA
2008-2012	Docent, University of Washington Botany Greenhouse and Medicinal Herb Garden

Publications

K.J.R.P. Byers, J.P. Vela, J.A. Riffell, & H.D. Bradshaw, Jr. Floral volatile alleles contribute to pollinator-mediated reproductive isolation in monkeyflowers (*Mimulus*). *The Plant Journal*, in revision.

K.J.R.P. Byers, H.D. Bradshaw, Jr., & J.A. Riffell. 2014. Three floral volatiles contribute to differential pollinator attraction in monkeyflowers (*Mimulus*). *Journal of Experimental Biology* **217**(4): 614-623.

Yuan Y., **K.J.R.P. Byers**, & H.D. Bradshaw, Jr. 2013. The genetic control of flower-pollinator specificity. *Current Opinion in Plant Biology* **16**(4): 422-428.

K.J.R.P. Byers, E. Sanders, & J.A. Riffell. 2013. Identification of olfactory volatiles using gas-chromatography-multi-unit recordings (GCMR) in the insect antennal lobe. *Journal of Visualized Experiments*, **72**, e4381, doi:10.3791/4381. http://www.jove.com/video/4381/.

C. Zhu*, **K.J.R.P. Byers***, R.P. McCord*, *et al.* 2009. High-resolution DNA binding specificity analysis of yeast transcription factors. *Genome Research* **19**: 556-566. (* these authors contributed equally to this work)

Invited presentations and seminars

Byers, Kelsey J.R.P. 2014. The role of three floral volatiles in pollinator-mediated reproductive isolation in monkeyflowers (*Mimulus*)." Gordon Research Conference: Plant Volatiles 2014 (Ventura, CA).
Byers, Kelsey J.R.P. 2014. Making sense of floral scents: floral scent differences between sister species of monkeyflowers - chemical ecology, neurobiology, genetics, and evolution. Gordon Research Seminar: Plant Volatiles 2014 (Ventura, CA).
Kelsey Byers. 2010. Rational design of a novel plant-pollinator interaction in a developing model system. Seattle Area Model Plant Labs Spring Seminar.

Presentations and seminars

Byers, Kelsey J.R.P., J.A. Riffell, & H.D. Bradshaw, Jr. 2013. The role of three floral volatiles in pollinator-mediated reproductive isolation in monkeyflowers (*Mimulus*). Evolution 2013 (Snowbird, UT).
Byers, Kelsey J.R.P., J.A. Riffell, & H.D. Bradshaw, Jr. 2012. Three floral odorants contribute to differential pollinator attraction between two species of monkeyflowers (*Mimulus*). Evolution 2012 (Ottawa, Canada).
Byers, Kelsey J.R.P., H.D. Bradshaw, Jr., & J.A. Riffell. 2012. Specific floral odorants contribute to differential pollinator attraction in monkeyflowers (*Mimulus*). SICB 2012 (Charleston, SC).
Byers, Kelsey J.R.P., H.D. Bradshaw, Jr., & J.A. Riffell. 2011. Specific floral odorants contribute to differential pollinator attraction in monkeyflowers (*Mimulus*). Evolution 2011 (Norman, OK).
Byers, Kelsey J.R.P., J.A. Riffell, & H.D. Bradshaw, Jr. 2011. Scents and sensibility: *Mimulus*

and its multimodal attraction system. *Mimulus* Meeting 2011 (Chapel Hill, NC).
Byers, Kelsey J.R.P. & H.D. Bradshaw, Jr. 2010. Rational design of a novel pollinator interaction. Evolution 2010 (Portland, OR).

Posters

Byers, Kelsey J.R.P., Jeffrey A. Riffell, & H.D. Bradshaw, Jr. 2014. "The role of three floral volatiles in pollinator-mediated reproductive isolation in monkeyflowers (*Mimulus*)." Gordon Research Conference: Plant Volatiles, 2014.
Byers, Kelsey J.R.P., H.D. Bradshaw, Jr., & J.A. Riffell. 2012. Specific floral odorants contribute to differential pollinator attraction in monkeyflowers. Gordon Research Conference: Plant Volatiles, 2012.
Byers, Kelsey, L. D'Souza, K. Martin, K. Worley. 2002. Developing methodology for human genome annotation. Keck Center for Computational Biology Undergraduate Research Training Program Mini-Symposium (Houston, TX).
Byers, Kelsey. 2000. Effects of various sage extracts on tobacco mosaic virus. BIO2000 Biotechnology Industry Organization Conference (Boston, MA).

Professional service

2014-present	Reviewer, *Evolution*.
2011-2013	Member, Faculty Appointments Committee, Department of Biology
2010-2011	Member, UW Biology Graduate Student Coordinating Committee
2009-2014	Member, University of Washington Institutional Review Board B
2009-2010	Member, Graduate Program Committee, Department of Biology
2005-2007	Member, MIT Committee on the Use of Humans as Experimental Subjects (IRB)

Society memberships

Society for the Study of Evolution