The Complicated Relationship Between Floral Volatile Production and Olfactory Signaling Within the Genus *Mimulus*

Jemima Elsherbini

Cardiff, Wales, United Kingdom & Marion, Illinois

B.S in Integrative Biology, University of Illinois, 2020

A Thesis presented to the Graduate Faculty of the University of Virginia in Candidacy for the Degree of Master of Science

December 2023

Department of Environmental Sciences

Committee:

David E. Carr (Advisor)

Matthew Reidenbach (Out of Area Member)

T'ai Roulston

Table of Contents

Dedication

A vast amount of people were (and still are) vital to the completion of this project, and I have an unbelievable amount of gratitude to express towards everyone who helped me along the way. First, I would like to thank my advisor, David E. Carr, who took a chance on me as a graduate student and allowed me, fresh out of college, an opportunity to be here and pursue this dream. Dave was supportive from the beginning, despite numerous confusions and hardships and the letter "u" in behavioural. He challenged me to become a more independent, resourceful, and creative scientist. I learned so much through this process of how to design a research project, grant writing, creating a poster, and presenting a poster – just numerous new skills that I could keep listing. Thank you for giving me an opportunity to grow and learn as a scientist.

I want to thank those who have worked with me tirelessly throughout this whole thesis: Judy Masi and Maggie McCartney at Blandy Experimental Farm, for patiently explaining reimbursements and funding too many times to me, as well as ordering project equipment and managing expenses; Austin Eichhorn and Stephanie Petrovick for the million spur-of-the-moment edits I'd ask for - you two are the real MVPs of this thesis and my heart; and last but certainly not least thank you, Kelsey Schoenemann and Emily Spindler, for helping me adjust to my first field season and UVA – Blandy Gals!

Thank you to my family for all your love and support throughout graduate school. I would not have made it here without all of you, so thank you for supporting me and listening to my complaining when the bees attacked me. Thank you to all my friends who supported me, gave me advice, and comfort – I appreciate all of you from the bottom of my heart. Thank you to everyone who helped me through this thesis and helped push me to be the best scientist I could be, no matter how hard it got.

Chapter 1:

Bumble Bee Olfactory Preference and VOC Emissions Dependent on Pollination Status of *Mimulus* Flowers

Abstract

Floral odors in the form of volatile organic compounds (VOCs) play a vital role in attracting insect pollinators to flowers. Pollinators use these floral odors to aid in locating floral rewards - nectar and pollen - for sustenance. Floral rewards may change in quantity or quality post-visitation from a pollinator. After a pollinator visits a flower, this can trigger an odor change that may increase foraging efficiency for pollinators. In this study, I examined behavioral preference with *Bombus impatiens* to evaluate the ability of bees to discriminate between pollinated and unpollinated floral volatiles in *M. guttatus* and *M. lewisii*. I also used a GC-MS to investigate the differences in floral volatile profiles between the unpollinated and pollinated flowers to link changes in pollinator behavior with changes in olfactory signaling. My results found that *M. guttatus* had a shift in volatile composition between unpollinated and pollinated flowers but no significant difference in bumble bee preference. In *M. lewisii*, I found a significant behavioral preference for unpollinated flowers but no difference in volatile organic compound production. My VOC analysis did not detect an innately attractive compound (β-trans-bergamotene) previously reported in *M. guttatus* that could have accounted for the non-significant preference for unpollinated flowers. However, experiments in both species provide evidence of changes in odor composition after pollination, and future studies with larger sample sizes are recommended to determine the ecological importance of these changes.

Introduction

Pollinators use a complex mix of visual and olfactory cues emitted from flowers to make foraging decisions when searching for sustenance. Sustenance comes in the form of two floral rewards: nectar (carbohydrates) and pollen (lipids and proteins) (Nicolson et al., 2018). Pollinators learn to associate visual and olfactory cues with their preferred rewards and base their foraging decisions accordingly (Schiestl & Johnson, 2013). Floral rewards are not static throughout the flower's life. Pollinator visitation decreases the quantity of nectar and pollen, making subsequent pollinator visits less rewarding (Michener, 2007). Variations in the quantity and quality of the rewards caused by removal could impede the pollinators' learned associations between signals and rewards (Schiestl & Johnson, 2013). A change in visual or olfactory cues that reflect changes in reward status would mitigate any signal mismatch when rewards are removed. This change in cues would allow an increase in foraging efficiency for the pollinator and increase pollinator fidelity to the plant (Raguso, 2008).

Floral visual cues guide pollinators to rewards and can include any visible part of the flower, such as the corollas or the sepals (Leonard & Papaj, 2011; Faegri & Pijl, 2013; Reverté et al., 2016). In some plants, there is a color change after pollinators visit. In response, pollinators will notice the color change and decrease their visitation to the plant since there is less reward to be gained, allowing them to be more efficient in foraging (Delph & Lively, 1989; Farzad et al., 2002; Hansen et al., 2006; Makino & Ohashi, 2017; Schaal & Leverich, 1980). For example, in *Viola cornuta,* a color change is triggered by pollination, either self-pollination or cross-pollination (Farzad et al., 2002). However, insect pollinators were not used in this study, so we do not know the pollinator's response to this color change. Another study observing *Lupinus*

texensis found that the plant's base petal changes color after a pollinator's visitation, causing pollinator visitation to significantly decrease (Schaal & Leverich, 1980).

Olfactory cues are emitted from flowers in the form of volatile organic compounds (VOCs). As with visual cues, they entice pollinators to visit and gain rewards. Insect-pollinated plants have higher VOC emission and chemical richness than plants pollinated by wind or other forms of pollination (Farré-Armengol et al., 2015), suggesting that insects select plants to pollinate based on these signals. Floral VOCs can be released from any floral tissue, such as the corollas, stamen, or pistil, or even directly from pollen or nectar (Baldwin, 2010).

Bees are able to track and learn odors for foraging by being attracted or repulsed by specific volatile compounds, but not all compounds emitted by a flower elicit a response. For example, of the 108 VOCs found in the European Pear, only 17 of them elicit a neurological response in honeybees (Lukas et al., 2019). In bees, the specificity of the olfactory detection process begins in the antenna. The antenna bears all of the olfactory organs and uses olfactory receptors (ORs) to detect and track odors through olfactory sensory neurons (OSNs) (Molet et al., 2009). Airborne chemicals can then bind to specific ORs inside the hairs, called sensilla, on the antennae (Mertes et al., 2021; Paoli & Galizia, 2021). Once a chemical is detected, signals are transmitted along OSN axons from the antennae to the antennal lobe (AL) (Gomez Ramirez et al., 2023; Mertes et al., 2021). The AL is the first olfactory center in the brain and is made of glomeruli, which are associated with olfactory receptors from particular ORNs (Mertes et al., 2021). Different odors evoke a different glomerular activity that is dependent on the molecules' chemical structure (Mertes et al., 2021).

Riveros et al. (2009) used Pavlovian conditioning with sugar water and scent to train *Bombus occidentalis* to exhibit a proboscis extension reflex (PER) in response to scent alone,

discovering that they are able to retain learned olfactory cues for up to five hours. If bees were allowed to forage prior to training, this quickened the learning recognition, but Riveros et al. also discovered that even naïve day-old bees were able to learn this association. Molet et al. (2009) found that within the nest, learning of floral odors is facilitated by floral odors released directly into the hive, and the learning performance is increased when produced into nectar in honeypots. This olfactory specificity and sophisticated learning capability can enable bees to associate specific chemical signals with the highest rewarding flowers.

VOCs that show a positive correlation between the floral reward amount and the strength of the volatile signal could be termed "honest signals" (Stanton & Preston, 1988; Knauer & Schiestl, 2015; Ito et al., 2021). Pollinators can develop a preference for the high signal strength of VOCs associated with a high reward quantity (Dobson et al., 1999; Ashman, 2005; Howell & Alarcón, 2007; Wright & Schiestl, 2009; Knauer & Schiestl, 2015; Haber et al., 2019; Burdon et al., 2020). This association reinforces the correlation between the cue and reward and strengthens the learned behavior.

Honest signaling can reflect a change in rewards post-pollination to maximize pollinator foraging efficiency by allowing them to avoid flowers with fewer rewards, but few studies have tested whether olfactory signals change after pollination or a change in reward status (Rodriguez-Saona et al., 2011; Lucas-Barbosa et al., 2016; Barragán-Fonseca et al., 2020). Lucas-Barbosa et al. (2016) devised an experiment that tested if butterflies had a preference for unpollinated or pollinated *Brassica nigra*. They discovered pollination status influenced the behavior of the butterflies and observed that the butterflies landed and lingered on unpollinated flowers, whereas they did not spend a long time, or even landed, on pollinated flowers. The researchers also found a VOC change between pollinated and unpollinated *B. nigra*. Rodriguez-Saona et al. (2011)

found that unpollinated blueberry flowers had greater visitation and greater VOC emissions than pollinated blueberry flowers. Lastly, the butterfly *Pieris brassicae* uses a mix of visual and olfactory cues when searching for floral rewards on *B. nigra* and *Raphanus sativus,* and *B. nigra* floral VOC composition was altered by pollination but not *R. sativus* (Barragán-Fonseca et al., 2020). The researchers tested whether hand-pollination or emasculation would affect the volatile composition and pollinator preferences relative to unpollinated controls. These studies found that pollinator visitation and VOC emission in *B. nigra* were influenced by pollination status but not *R. sativus* flowers.

Olfactory signals can be honest with pollinators, but this may not always be the case. Dishonest signals (or sensory traps) exploit a sensory bias in the pollinator. Plants could be dishonest for various reasons, such as cutting metabolic energy costs, ensuring higher reproductive benefits for both female and male aspects of the plant, or drawing in pollinators despite reduced reward offerings. The best-known examples of dishonest signaling have been found in rewardless orchid volatiles that mimic sex pheromones to attract their pollinators (Johnson, 2000; Ayasse et al., 2003; Ellis & Johnson, 2010; Steiner et al., 2011). For example, flowers in the genus *Orphys,* the bee orchids, visually resemble a bee perched on the flower and emit VOCs that replicate bee mating pheromones, thus saving metabolic energy by not providing any rewards but still attracting pollinators (Borg-Karlson, 1990; Zito et al., 2018). The production of cues that exploit a sensory bias in the pollinator may be a mechanism for attracting pollinators independent of the flower's reward status.

It may not always be beneficial for a plant to signal a change in rewards to pollinators. For example, in *Mimulus ringens*, multiple visits from pollinators help ensure a high reproductive output for both male and female reproduction (Karron et al., 2006; Christopher et

al., 2019). Color or olfactory changes that signal a change in reward status can result in a cost to a plant's reproductive fitness if they discourage subsequent visitation. These signals can result in favoring plants that can create a deceptive signal that will draw pollinators despite a reduced ability to offer rewards.

The genus *Mimulus* (Phyrmaceae) contains extensive phenotypic, ecological, and genomic diversity, rendering it a near-ideal system to explore the evolution and function of VOCs due to the high variety of pollination syndromes that appear within its evolutionary history (Wu et al., 2008). The genus is mostly herbaceous and native to open, wet habitats, with life histories varying from annual to perennial. However, very few species from the genus have had their VOCs recorded. Nothing is known about how pollination status affects VOCs in the genus *Mimulus*. However, Haber et al. (2019; 2021) discovered a strong pollinator sensory bias for the volatile β-trans-bergamotene in *M. guttatus*. The pollinator's preference for β-trans-bergamotene can override learned preferences for VOC compounds that are positively correlated with the amount of pollen in a flower (Haber et al., 2021). This type of deception may be common among insect-pollinated plants, but it has not received much attention.

This research focuses on two species, *Mimulus lewisii* and *M. guttatus*. Both species are pollinated by bumble bees, produce pollen, have zygomorphic flowers, and are the only species within the genus to have their volatiles analyzed. They share three of the same VOCs that influence bumble bee attraction (D-limonene, β-myrcene, and E-β-ocimene) (Byers et al., 2014; Haber et al., 2019). The two species differ in floral color, size, reward amount, and life cycle. *Mimulus lewisii* produces nectar, has pink petals, and has a perennial life history. *Mimulus guttatus*, on the other hand, has yellow petals and can be annual or perennial, depending on the population. Among the 17 floral VOCs detected in *M. guttatus* is β-trans-bergamotene, and

pollinators' innate preference for it can override other learned sensory preferences that may be more advantageous for the bumble bees (Haber et al., 2021). This makes β-trans-bergamotene a potentially dishonest signal that exploits a sensory bias. The three VOCs that both species emit can influence bee floral attraction, and in *M. guttatus*, they are potential honest signals as they provide cues to pollinators that correlate with their reward value (Haber et al. 2019). However, it is unknown if the relationship between these cues and rewards is present in *M. lewisii*.

In this study, I proposed to analyze the behavioral responses of experienced bees to pollinated and unpollinated *Mimulus guttatus* and *M. lewisii.* I will analyze the VOCs from *M. guttatus* and *M. lewisii* before and after pollination. I will then test pollinator preferences in pair-wise choice tests for VOCs from pollinated and unpollinated flowers of each species. I expect to see both preference and VOC differences between pollination status in *M. lewisii* but not in *M. guttatus*. I expect that pollinators will not show a strong preference for unpollinated *M. guttatus* because of its reliance on a dishonest signal, β-trans-bergamotene (Haber et al., 2021).

Methodology

Study System: Mimulus and Bombus impatiens

The *M. guttatus* used in this study are descendants of seeds collected from over 500 random maternal families in an annual population in Napa County, CA (Snell Valley: 38° 42' 20.0'' N, 122° 24' 29.0" W). The *M. lewisii* are descendants of seeds collected from a perennial population in Skamania County, WA (Mount St. Helens National Volcanic Monument: 46° 14' 43" N, 122° 11' 04" W). The plants used in the current study were grown in a greenhouse at Blandy Experimental Farm in Boyce, VA, USA (photoperiod:18 D:6 N). Approximately 20 seeds from each population were sown in a 3-inch square pot filled with Promix BK25 soilless

potting mix. There were 20 pots per tray with bottom watering. Once I saw germination in each of the original pots, I randomly transplanted four plants from each maternal family into their own pots to be used in pairs during the trials.

The bumble bee*, Bombus impatiens*, was used for the behavioral preference studies. It is a generalist pollinator, and *Bombus* spp. are among the most important pollinators for most *M. guttatus* and *M. lewisii populations* (Kiang, 1972; Bradshaw & Schemske, 2003; Vickery, 1999). Although its range does not overlap with either *Mimulus* species, *Bombus impatiens* was used in this study due to its commercial availability. I used two Koppert Natupol Hives – one for each *Mimulus* species. Bees were provided with sugar water *ad libitum* and occasionally supplemented with additional "nectar" that was supplied with the hive by Koppert. Pollen was supplied within the hive through fresh flowers (*M. guttatus* or *M. lewisii,* depending on the trial being run) placed within an arena surrounding the hive.

Bumble Bee Choice Training

Prior to running the preference tests, the bees were exposed to the outdoors for a weekend to entice foraging behavior. Afterward, they were left in a grey training arena (80x60x62 cm) to be trained on a single flowering species. The training arena was covered with a sheet of plexiglass for viewing accessibility and for easily replacing old plants with fresh ones within the arena. For example, before the *M. lewisii* tests, the bumble bees were exposed only to *M. lewisii* plants in the arena. This allowed the bumble bees to learn the volatiles of that specific species.

I placed nine live plants and five faux plants with sugar water in the training arena. The faux flowers were made from yellow (*M. guttatus*) or pink (*M. lewisii)* construction paper (Y-HUE and V-LP, respectively, from Color Aid Corporation, Hudson Falls, NY) and pressed into a 6-petal flower using a 'Cuttlebug Scribble Flower™' and a Sizzix® press (Lake Forest, CA). The real plants were exchanged every 24 hours for fresh plants, and the sugar water (1:1 ratio of sugar:water) was replaced twice daily during the morning and night. The faux and real plants were mixed within the training arena to allow the bees to create an association between them.

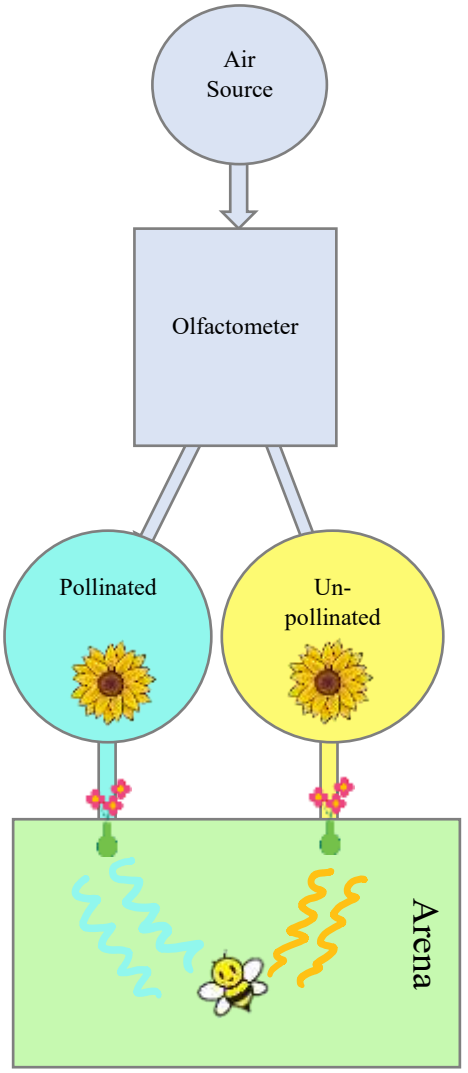

Figure 1. This depicts the baseline experiment for all paired choice tests. The air within the chamber will cause the bee to smell the pollinated and unpollinated flowers. If the bee has a preference for that scent, it should visit one of the flowers and attempt to pollinate.

Pollination

I used pollination to induce potential changes in volatile production. Pollination, by itself, has been observed to produce changes in VOC emissions in *Brassica nigra* (Barragán-Fonseca et al., 2020; Lucas-Barbosa et al., 2016). In nature, reward removal would usually be accompanied by pollination.

The flowers used within all trials were pairs of plants from the same maternal family to control for potential genetic differences between plants. Prior to behavioral trials, I hand-pollinated all open flowers on one plant and left the flowers on another unpollinated. I would match the open flowers for pollinated to unpollinated. Within the Blandy Greenhouse headhouse, I used a tuning fork to vibrate the pollen grains from the anthers of a pollen-donor plant, and using a small paintbrush, I would "paint" the pollen grains from the donor plant onto

the trial plant. For *M. guttatus*, I would choose a pair of sibling plants with at least three or more open flowers. In the case of *M. lewisii*, sibling plants with one or more open flowers were used. Hand-pollinations were done 12 hours before trials for *M. guttatus* and an hour before for *M. lewisii*. *Mimulus lewisii* was pollinated an hour before due to the fast nature of corolla abscission soon after pollination (personal observation). I observed in *M. lewisii* fast corolla abscission, as originally, pollination took place at 12 hours similar to *M. guttatus.* However, all petals fell off when observed for 12 hours for trials. Afterward, I tried at six hours, which also had corolla abscission, and finally at one hour, which observed no abscission. Thus altering my protocol to pollinating an hour before trials.

Behavioral Trials

The choice trials were conducted in a closed arena (separate from the training arena) (Fig.1). This arena was 80 cm l x 60 cm w x 62 cm h, with four sides and the bottom of the arena made of plywood and painted gray. In one wall, two ports spaced 13 cm apart allowed air and volatiles to be streamed into the arena. Compressed air was split into two equally pressurized streams through a 2-Channel Air Delivery System (Analytical Research Systems, Gainsville, FL). The streams flowed at 2 L/min through two Teflon tubes into a pair of 5400 ml glass chambers. The 2-piece chambers allowed the isolation of flowers in the upper section (900 ml) from the lower section with a partition of aluminum foil. This prevented volatiles from the rest of the plant (leaves, stems, roots) from being mixed with the floral volatiles. The chambers were connected by tubing to the faux flowers mounted into one arena wall. To make the “corolla” of the faux flowers, I removed the cap and bottom of a clear 1.5 ml microcentrifuge tube and inserted it into the center of the faux flower petal. Each volatile airstream entered the arena

through a tube running through the faux flower. Due to the chambers being made of glass, they lacked any odor, and similarly, all tubes were Teflon. To ensure fresh volatiles, each pair of plants (one pollinated, one unpollinated) was changed after five trials, and each pair came from a different full-sibling family.

A single bee was released into the arena with the faux flowers for 15 minutes. During the trial, the bee was monitored, and a choice was determined by counting the first faux flower the bee crawled or landed on. Bees that did not reach a decision within the allotted time were omitted from the dataset. Bees observed to be motionless for 30 seconds were poked by the observer to entice an action.

Behavioral Preference Study Analysis

To test for preferences of experienced bees for pollinated versus unpollinated flowers, I used a Chi-Squared goodness-of-fit test to evaluate the null hypothesis that the choice was random (P[pollinated] = P[unpollinated] = 0.5). Separate tests were run for the *M. guttatus* and *M. lewisii* trials.

Volatile Collection

I collected floral volatiles in Fall of 2022 and processed them through GC-MS in June and July of 2023. I sampled VOCs from 10 *M. guttatus* and 10 *M. lewisii.* For each species, five of the plants were unpollinated, and five were pollinated. I always simultaneously collected the VOCs in pairs of unpollinated and pollinated plants from the same family.

Floral volatiles were collected using a pull-push collection system (Sigma Scientific LLC, Micanopy, FL). This system pumps air into a chamber containing the flowers and vacuums

air through a filter exiting the chamber (Fig. 2). The filters were VCT 3.5" with 30mg ± 5 Porapak™ Type Q (Sigma Scientific LLC, Micanopy FL). The flow rate for air into the chamber is 1.5 LPM, and the flow rate for air out is 2 LPM. Plants were housed in glass chambers (Sigma Scientific LLC, Micanopy, FL) for an environment without external volatiles. The same glass chambers (Sigma Scientific LLC, Micanopy, FL) used in the behavioral assays experiment were reused for these experiments, as well as a taller set of glass chambers (6400 mL) for taller plants. All floral volatiles were collected for eight hours at minimum to ensure volatile collection into the filter. Between each experiment, the glass chambers were wiped down with 70% ethanol, and to minimize the spread of contamination, nitrile FisherBrand© gloves were worn while handling filters and glass chambers. Once collected, the filters were removed from the tubing, wrapped in aluminum foil, and stored at -80 °C.

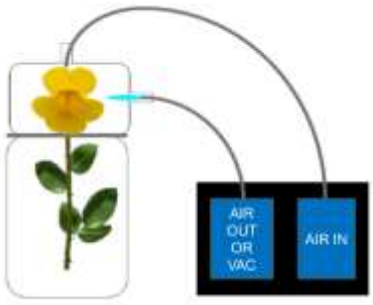

Figure 2. Push-Push collection system for extracting volatiles from Mimulus. Teal shape represents the collection filter, while the grey bar represents aluminum foil to separate the stem from the floral volatiles.

Volatile Elution and Identification

I eluted the trapped compounds with 150 µl dichloromethane (CH_2Cl_2) and added 5 µL of a mix (dissolved in CH_2Cl_2) containing the internal standards n-octane (40 ng⁄µL) and nonyl acetate (80 ng⁄µL). Samples were injected into 1-µl aliquots using a splitless injector into a Rxi-5Sil MS column (0.25 mm internal diameter, 0.25 µm film thickness, 30.0 m length) in a Shimadzu GCMS-QP2010S and then separated and detected using a Shimadzu GC-2010. The column started at 50°C with no hold time and then increased at 8°C/min to 240°C. Pure helium was the carrier gas. Injection began at 250.0°C. Each sample was run for 23.75 minutes. This

process allowed us to establish the presence of a compound, but the GCMS could not provide a quantitative estimate.

Each sample's volatiles were identified by choosing chemical peaks on each chromatogram with distinct curved or pointed peaks. These chemical peaks represented the retention times of different chemical compositions within each sample. After identifying a chemical peak, I used its mass spectrum to identify the most likely VOC. The mass spectrometer bombards the sample with energetic electrons, causing the sample (the analyte) to lose an electron due to electron repulsion, and further electrons cause the sample's ions to fragment. After fragmentation, these ions are passed into a mass analyzer and sorted by their mass-to-charge ratio. NIST 2014 software compares the analyte's mass spectrum to a database of mass spectra, generating a likelihood for the five best matches in order of most to least likely. Once the software identified the volatiles, they were summarized in Excel with alternative names and their likelihoods. These likelihoods were placed out of 100 and determined how likely the structure was to other existing chemicals in the database. The closer to 100, the more accurate the machine was of the match. Once the chemicals were identified, all non-plant-based chemicals or chemicals with likelihoods below 60 and all chemicals with only one occurrence across all samples within a species were removed. I then identified the chemical class using PubChem, Classyfire (Djoumbou Feunang et al., 2016), and the National Institute of Science and Technology website. All chemicals not identified as sesquiterpenes, monoterpenes, monoterpenoids, esters, or benzenoids were removed from the dataset. This resulted in a total of 22 volatiles within *Mimulus guttatus* and 27 volatiles for *Mimulus lewisii*.

Volatile Analysis

I tested for VOC differences between unpollinated and pollinated samples within each individual species using a mixed model ANOVA with the SAS "Mixed" procedure. The first dependent variable was the total number of VOC compounds. The independent variables included a fixed treatment effect and a plant maternal family as a random effect. I created a second dependent variable by calculating principal component 1 (PC1) from a principal component analysis (PCA) using the covariance/variance matrix instead of the correlation matrix in the SAS "Princomp" procedure. For each species, singleton compounds were removed from the PCA analysis. I based the PCA on a covariance-based matrix to accommodate the binary data. The PCA for *M. guttatus* used 22 non-zero VOCs. The PCA for *M. lewisii* used 27 non-zero VOCs.

Results

Behavioral Trials Results

In *Mimulus guttatus* trials, experienced bumble bees showed a slight preference for unpollinated flowers, but this preference was not significant (60% vs. 40%, respectively; $\chi^2 = 1.2$, $p = 0.273$, $n = 30$) (Fig. 3a). I ran a total of 114 trials, in 84 there was no choice made by the bee. In the *M. lewisii* trials, I found that bumble bees had a significant preference for unpollinated flowers (69% vs. 31%; $\chi^2 = 4.17$, $p = 0.041$, $n = 29$) (Fig. 3b). I ran a total of 88 trials, in 59 trials the bumble bees did not make a choice.

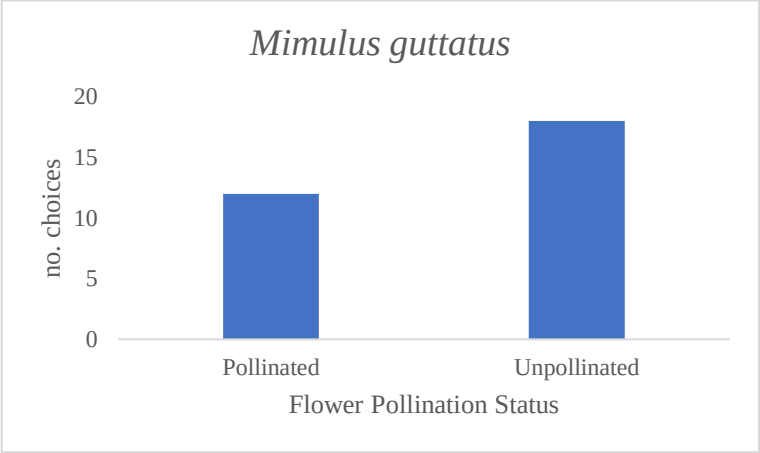

Figure 3a. M. guttatus trials, bumble bee workers did not demonstrate a significant preference for either unpollinated or pollinated flowers (60% vs. 40%, respectively; $\chi 2 = 1.2$, $p = 0.273$.). Bars represent the number of choices the bees made.

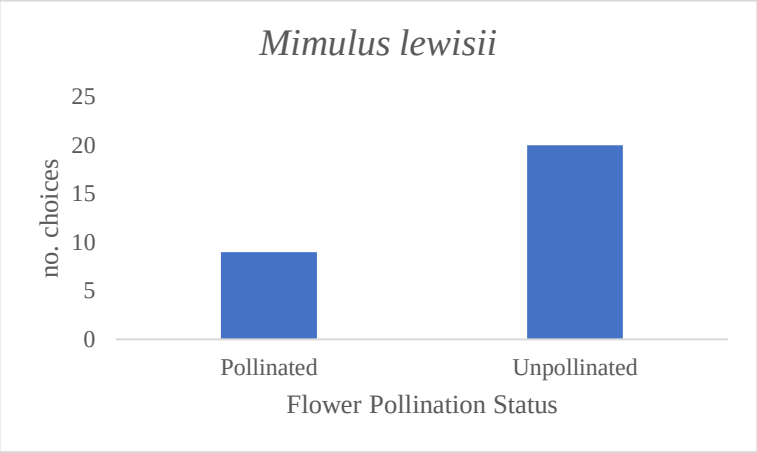

Figure 3b. M. lewisii trials, bumble bees had a significant preference for unpollinated M. lewisii (69% vs. 31%; $\chi 2 = 4.17$, $p = 0.041$). Bars represent the number of choices the bees made.

Volatile Identification Results

Across both species, there was a total of 37 unique floral volatiles (Table 1). In *Mimulus guttatus*, I identified a total of 18 volatiles in pollinated and 15 volatiles in unpollinated flowers (Supplementary Table 1). Within the pollinated flowers, there were three monoterpenes, one monoterpenoid, six esters, six benzenoids, and two sesquiterpenes. In the unpollinated flowers, there was one monoterpene, four monoterpenoids, four esters, ten benzenoids, and one sesquiterpene. There were 11 volatiles shared between pollinated and unpollinated flowers. There were seven unique to pollinated flowers and four unique to unpollinated.

In *Mimulus lewisii*, I identified a total of 26 volatiles in pollinated and 26 volatiles in unpollinated flowers (Supplementary Table 1). Within the pollinated flowers, there were three monoterpenes, seven monoterpenoids, six esters, seven benzenoids, and eight sesquiterpenes. In the unpollinated flowers, there were three monoterpenes, five monoterpenoids, five esters, eight benzenoids, and six sesquiterpenes. There were 25 volatiles shared between pollinated and

unpollinated florals. There was one volatile unique to pollinated flowers and one unique to unpollinated.

Volatile Data Analysis

I ran two analyses within the dataset. The first was a mixed model ANOVA comparing the mean number of total VOC compounds produced by pollinated versus unpollinated plants. Separate analyses were run for each species. Within *M. guttatus,* the mixed model ANOVA, which included family as a random effect, found that there was consistent variation (variance component = 0) among individual plant families in VOC*,* and the fixed treatment effect (pollinated vs unpollinated) showed no difference in the mean total VOC production ($F_{1,4} = 0.91$, $p = 0.3931$).

In *M. guttatus*, PC1 accounted for 39.9% of the variance in the VOC dataset. PC1 is a measure of the VOC blend based on the presence or absence of the volatiles within each sample. In PC1, the five volatiles with the highest weights included: 5,9-Undecadien-2-one, 6,10-dimethyl (0.3684); Phenol, 4-(1,1-dimethylpropyl) (0.3684); p-Toulic acid, 2-ethylhexyl ester (0.3684); Acetic acid, 2-ethylhexyl ester (0.3418); and linalool (-0.3169). A mixed model ANOVA with family as a random effect compared the means of PC1 for pollinated and unpollinated *M. guttatus*. There was some consistent variation among the families, with 15.5% of the total random effect variation attributable to families. This means some families differed in VOC blend regardless of the pollination treatment. The pollination treatment fixed effect showed a significant difference in mean PC1 ($F_{1,4} = 30.11$, $p = 0.0054$) between pollinated and unpollinated *M. guttatus*, suggesting a VOC shift between the two treatments (Fig 4a).

In *M. lewisii,* the mixed model ANOVA found no consistent variation among families in total VOC production (variance component = 5.75). This means that some families produced more VOCs regardless of whether they were pollinated or unpollinated, with 55.5% of the variance of the total random effect attributed to family differences. The fixed effect showed no significant effect of pollination status on mean total VOC production ($F_{1,4} = 2.63$, $p = 0.1802$) (Fig. 4b).

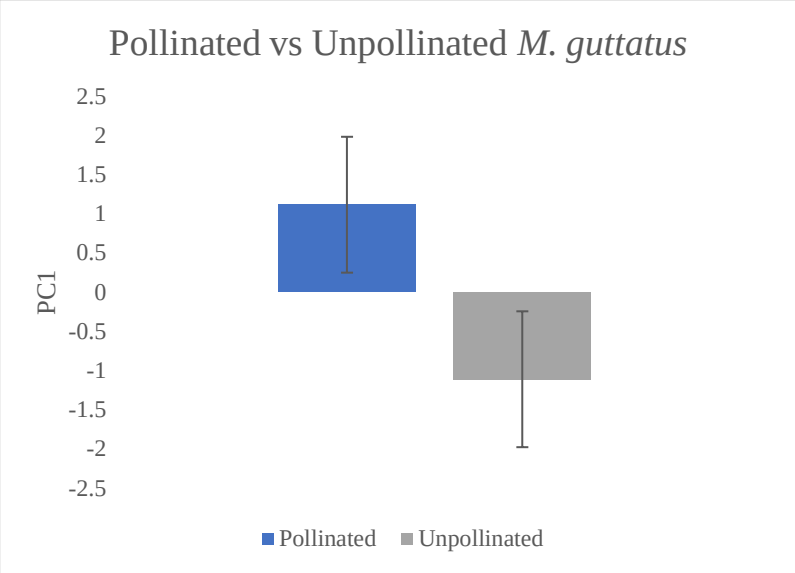

Figure 4a. Mixed Model ANOVA with pollination treatment as a fixed effect for PC1 on M. guttatus. The mean PC1 for pollinated M. guttatus differed from the mean PC1 for unpollinated plants ($F_{1,4} = 30.11$, $p = 0.0054$). Error bars indicate 95% confidence intervals.

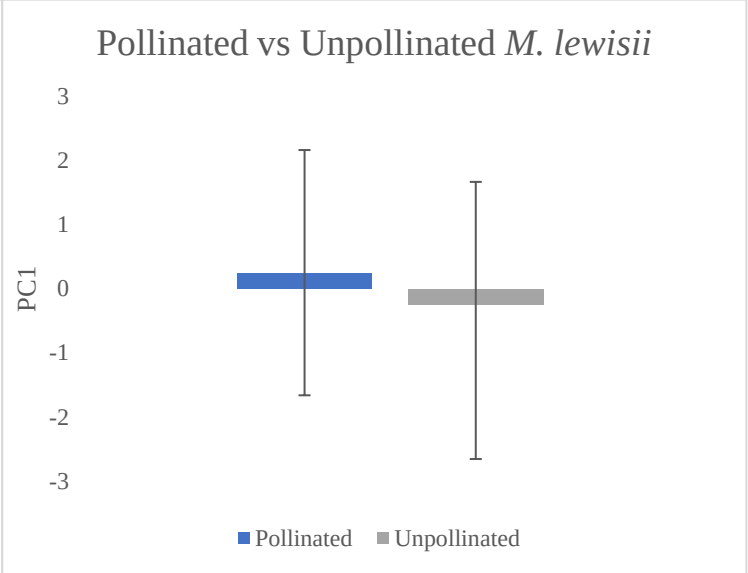

Figure 4b. Mixed Model ANOVA with pollination treatment as a fixed effect for PC1 on M. lewisii. The mean PC1 for pollinated M. lewisii did not differ from the mean PC1 for unpollinated plants ($F_{1,4} = 2.00$, $p = 0.0228$). Error bars indicate 95% confidence intervals.

PC1 accounted for 35.07% of the variation in the VOC dataset for *M. lewisii*. Within PC1, the six volatiles that had the highest weights over the dataset included: 5,9-Undecadien-2-one, 6,10-dimethyl (-0.341); Biphenyl (0.3410); Caryophyllene oxide (0.3095); 2,6-Dimethyl-8-(tetrahydropyran-2-yloxy)-octa-2,6-dien-1-ol (-0.3069); Caryophyllene (0.2573); 10s,11s-Himachala-3(12),4-diene (0.2573). The mixed model ANOVA for *M. lewisii* showed no

variation among families in the mean of PC1. Mean PC1 did not differ significantly between the pollination treatments ($F_{1,4} = 0.26$, $p = 0.6370$).

Discussion

Through my analysis of VOC emissions, I found that *Mimulus guttatus* volatiles have a chemical shift, demonstrating that post-pollination VOCs have a different volatile cocktail mixture than pre-pollination. Despite this difference in VOC composition, my behavioral trials found that bumble bees had no significant preference for the volatiles emitted by pre- or post-pollination flowers in *M. guttatus.* For *M. lewisii*, I found that the chemicals released have no consistent shift in VOC production after pollination. However, my behavioral experiment demonstrated that bumble bees significantly preferred non-pollinated floral volatiles of *M. lewisii*.

My findings for both species were paradoxical. In *M. guttatus*, I found a significant difference between unpollinated and pollinated volatiles, yet pollinators did not respond to this change in signaling. This suggests that Haber et al. (2019; 2021) may be correct about the presence of a dishonest volatile presence masking honest signals (e.g., like limonene) in *M. guttatus*. Haber et al. (2019) found a strong pollinator sensory bias for β-trans-bergamotene, and Haber et al. (2021) found that β-trans-bergamotene is capable of altering the response of pollinators to honest signals. If pollinated flowers produce β-trans-bergamotene, it could interfere with pollinators' learning or response capability to the differences in VOC emissions from pollinated and unpollinated flowers. However, my results did not pick up the dishonest chemical β-trans-bergamotene. It also did not detect 12 other volatiles that Haber et al. (2019) reported, so I cannot fully support a dishonest volatile as an explanation for the failure of bees to

make a strong distinction between pollinated and unpollinated flowers. Compared with Haber et al. (2019), my study found four of the same compounds (α-pinene, limonene, trans-limonene, and linalool) in *M. guttatus.* I was unable to identify any other compound Haber et al. (2019) identified in *M. guttatus*, especially β-trans-bergamotene. This could suggest issues with our data collection or seasonal timing affecting the collection of the volatiles.

Unstable plant-pollinator mutualisms can cause conflict between selection pressures from the pollinators for honest signals and the selection pressures from plants to reduce costs associated with reward production. These conflicting selective pressures could result in a relationship where the plant still displays the true or honest relationship between rewards and the strength of a signal, but the honest signal is masked by a chemical or physical change that overrides an innate bias. This can allow a reduced metabolic cost by reducing the amount of biological work a plant has to do to entice pollinators, such as creating dual rewards, they can produce a single reward. In plants like *M. guttatus* that have a reduced attractiveness due to lacking nectar, it could be that volatiles can compensate by releasing a dishonest masking signal. From prior studies, I can suggest that deceptive strategies or sensory biases can allow plants with low rewards to compete more highly for pollinators against dual-reward flowering species (Christopher et al., 2019; Howell & Alarcón, 2007; Karron et al., 2006; Knauer & Schiestl, 2015). This has been witnessed in *M. guttatus,* which has a VOC emittance masking any potential honest signals from pollinators (Haber et al., 2019, 2021). In addition, several studies have suggested that receiver or pollinator biases can be selected for the signals transmitted from the plants (Steiner et al., 2011; Zu et al., 2016; Joffard et al., 2020). However, my study did not find β-trans-bergamotene, previously demonstrated as producing a biased response of pollinators

(Haber et al. 2019, 2021), and did not uncover any other candidate "dishonest" signals in *M. guttatus*.

The ability of pollinators to show a preference for unpollinated flowers based on olfactory cues alone suggested a VOC shift, but this was not supported by my chemical analysis of the volatiles in *M. lewisii*. Chemical ecology and volatile collection are variable, leading me to believe that there could be a shift detected by the bumble bees that the GC-MS or my statistical analyses are unable to detect. The small sample size in each pollination class ($n = 5$) may have also been an issue. If I had increased the sample size, we would have had a higher statistical power, which may have lent itself to more pronounced trends. For example, I had a compound that appeared in four out of five unpollinated plants but only two out of the five pollinated. These could have been possible trends in my data, but they could not be fully realized due to the small sample size. Lastly, I was unable to estimate the volume or rates of VOC emission, which could have found differences in my data that would be undetectable from only the comparison of presence and absence data.

Of the three main compounds (D-limonene, β-myrcene, and E-β-ocimene) that Byers et al. (2014) found in *M. lewisii,* which accounted for 93% of the VOCs by volume, I found only D-limonene. This could suggest issues with my VOC collection and processing, or the timing of seasonal collection was influencing the volatiles. The collection of floral volatiles is fraught with uncontrolled variables that may have influenced my results.

Unlike olfactory signals, visual signals that change after pollination are plentiful within the literature. For example, some species show a change in floral colors in response to a change in reward status (Schaal & Leverich, 1980; Delph & Lively, 1989; Gori, 1989; Weiss, 1991; van Doorn, 1997; Weiss & Lamont, 1997; Farzad et al., 2002; Hansen et al., 2006; Makino &

Ohashi, 2017). A review by Weiss and Lamont (1977) included 450 species from 75 families that have documented floral color change, most often in response to a change in floral reward. *Lupinus texensis*, for example, has a base petal change color after visitation by a pollinator, which signals to pollinators that floral rewards have been removed (Schaal & Leverich, 1980). In comparison, there are very few studies that have tested for changes in olfactory cues. In blueberries and *B. nigra*, volatiles decrease post-floral pollination (Barragán-Fonseca et al., 2020; Lucas-Barbosa et al., 2016; Rodriguez-Saona et al., 2011). In another study, male *Glochidion rubrum* flowers showed a decrease in two major volatiles after pollinator visits, and all volatiles were reduced in female flowers (Okamoto et al., 2022). One significant difference between my study and previous studies is that I isolated the olfactory cues and did not allow the bumble bees any visual cues. My study is therefore the first to test how instrumental olfactory cues are without the effect of visual cues.

In conclusion, there was a significant chemical composition change in *M. guttatus* VOC emissions between unpollinated and pollinated flowers, but pollinators did not have a significant preference to either treatment. The presence of an innately attractive VOC (β-trans-bergamotene) discovered in earlier studies of *M. guttatus* (Haber et al. 2019; 2021) could explain the paradox, but I did not detect this compound in either pollinated or unpollinated plants. On the other hand, my conclusion that there was no significant preference for unpollinated flowers could be a type II error. If my behavioral dataset had increased to 100 trials, selecting unpollinated plants 60% of the time would have suggested a significant preference. In *M. lewisii*, pollinators detected a VOC shift that could not be verified by chemical analysis. An increase in statistical power through increased sample size in these analyses may have aided in uncovering trends in the chemical analysis. Despite the shortcomings of this experiment, data from both *M. guttatus* and *M. lewisii*

provide some evidence of a signal change after pollination. This is only the fifth study to provide evidence of such a change and the first to completely isolate the role of olfactory cues on pollinator response. In future studies, I suggest more stringent protocols such as keeping a sterile space to take volatiles, eliminating potential contaminants from being brought in (i.e. laundry chemicals or hand soaps), and less time between collection and analysis of species. In addition, I would also suggest an increase in VOC sampling of *M. lewisii* and *M. guttatus* volatiles pre- and post-pollination.

Acknowledgments

Rupesh Kariyat provided volatile collection equipment and filters. Jill Venton provided access to her GC-MS, and Earl Ashcraft provided operational help for the GC-MS. Judy Masi and Elizabeth Jackson provided administrative and logistical assistance. Austin Eichhorn and Stephanie Petrovick provided edits to earlier additions of this chapter. David E. Carr, for his continuous guidance and support throughout this chapter. I'd also like to thank my committee members, T'ai H. Roulston and Matthew A. Reidenbach, for their thesis comments and their development in the methodology for the project. Funds for this project were provided by the University of Virginia's Department of Environmental Science Exploratory Award and the Blandy Experimental Farm Fellowship.

Literature Cited

Ashman, T.-L. (2005). The limits on sexual dimorphism in vegetative traits in a gynodioecious plant. *The American Naturalist*, *166 Suppl 4*, S5-16. https://doi.org/10.1086/444598

Ayasse, M., Schiestl, F. P., Paulus, H. F., Ibarra, F., & Francke, W. (2003). Pollinator attraction in a sexually deceptive orchid by means of unconventional chemicals. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *270*(1514), 517–522. https://doi.org/10.1098/rspb.2002.2271

Baldwin, I. T. (2010). Plant volatiles. *Current Biology*, *20*(9), R392–R397. https://doi.org/10.1016/j.cub.2010.02.052

Barragán-Fonseca, K. Y., van Loon, J. J. A., Dicke, M., & Lucas-Barbosa, D. (2020). Use of visual and olfactory cues of flowers of two brassicaceous species by insect pollinators. *Ecological Entomology*, *45*(1), 45–55. https://doi.org/10.1111/een.12775

Borg-Karlson, A.-K. (1990). Chemical and ethological studies of pollination in the genus *Ophrys* (orchidaceae). *Phytochemistry*, *29*(5), 1359–1387. https://doi.org/10.1016/0031-9422(90)80086-V

Bradshaw, H. D., & Schemske, D. W. (2003). Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. *Nature*, *426*(6963), Article 6963. https://doi.org/10.1038/nature02106

Burdon, R. C. F., Raguso, R. A., Gegear, R. J., Pierce, E. C., Kessler, A., & Parachnowitsch, A. L. (2020). Scented nectar and the challenge of measuring honest signals in pollination. *Journal of Ecology*, *108*(5), 2132–2144. https://doi.org/10.1111/1365-2745.13432

Büschges, A., & DiCaprio, R. A. (2008). Somatosensation in Invertebrates. In *The Senses: A Comprehensive Reference* (pp. 355–362). Elsevier. https://doi.org/10.1016/B978-012370880-9.00362-5

Byers, K. J. R. P., Bradshaw, H. D., Jr, & Riffell, J. A. (2014). Three floral volatiles contribute to differential pollinator attraction in monkeyflowers (*Mimulus*). *Journal of Experimental Biology*, *217*(4), 614–623. https://doi.org/10.1242/jeb.092213

Christopher, D. A., Mitchell, R. J., Trapnell, D. W., Smallwood, P. A., Semski, W. R., & Karron, J. D. (2019). Hermaphroditism promotes mate diversity in flowering plants. *American Journal of Botany*, *106*(8), 1131–1136. https://doi.org/10.1002/ajb2.1336

Delph, L. F., & Lively, C. M. (1989). The Evolution of Floral Color Change: Pollinator Attraction Versus Physiological Constraints in *Fuchsia Excorticata*. *Evolution*, *43*(6), 1252–1262. https://doi.org/10.1111/j.1558-5646.1989.tb02572.x

Djoumbou Feunang, Y., Eisner, R., Knox, C., Chepelev, L., Hastings, J., Owen, G., Fahy, E., Steinbeck, C., Subramanian, S., Bolton, E., Greiner, R., & Wishart, D. S. (2016). ClassyFire: Automated chemical classification with a comprehensive, computable taxonomy. *Journal of Cheminformatics*, *8*(1), 61. https://doi.org/10.1186/s13321-016-0174-y

Dobson, H. E. M., Danielson, E. M., & Wesep, I. D. V. (1999). Pollen odor chemicals as modulators of bumble bee foraging on *Rosa rugosa Thunb*. (*Rosaceae*). *Plant Species Biology*, *14*(2), 153–166. https://doi.org/10.1046/j.1442-1984.1999.00020.x

Ellis, A. G., & Johnson, S. D. (2010). Floral Mimicry Enhances Pollen Export: The Evolution of Pollination by Sexual Deceit Outside of the *Orchidaceae*. *The American Naturalist*, *176*(5), E143–E151. https://doi.org/10.1086/656487

Faegri, K., & Pijl, L. V. D. (2013). *Principles of Pollination Ecology*. Elsevier.

Farré-Armengol, G., Filella, I., Llusià, J., & Peñuelas, J. (2015). Pollination mode determines floral scent. *Biochemical Systematics and Ecology*, *61*, 44–53. https://doi.org/10.1016/j.bse.2015.05.007

Farzad, M., Griesbach, R., & Weiss, M. R. (2002). Floral color change in *Viola cornuta L*. (*Violaceae*): A model system to study regulation of anthocyanin production. *Plant Science*, *162*(2), 225–231. https://doi.org/10.1016/S0168-9452(01)00557-X

Gori, D. F. (1989). Floral Color Change in *Lupinus Argenteus* (*Fabaceae*): Why Should Plants Advertise the Location of Unrewarding Flowers to Pollinators? *Evolution*, *43*(4), 870–881. https://doi.org/10.1111/j.1558-5646.1989.tb05184.x

Gomez Ramirez, W. C., Thomas, N. K., Muktar, I. J., & Riabinina, O. (2023). The neuroecology of olfaction in bees. *Current Opinion in Insect Science*, 56, 101018. https://doi.org/10.1016/j.cois.2023.101018

Haber, A. I., Sims, J. W., Mescher, M. C., Moraes, C. M. D., & Carr, D. E. (2019). A key floral scent component (β-trans-bergamotene) drives pollinator preferences independently of pollen rewards in seep monkeyflower. *Functional Ecology*, *33*(2), 218–228. https://doi.org/10.1111/1365-2435.13246

Haber, A. I., Sims, J. W., Mescher, M. C., Moraes, C. M. D., & Carr, D. E. (2021). A sensory bias overrides learned preferences of bumblebees for honest signals in *Mimulus guttatus*. *The Royal Society Proceedings B*, *288*, 8. https://doi.org/288

Hansen, D. M., Beer, K., & Müller, C. B. (2006). Mauritian coloured nectar no longer a mystery: A visual signal for lizard pollinators. *Biology Letters*, *2*(2), 165–168. https://doi.org/10.1098/rsbl.2006.0458

Howell, A. D., & Alarcón, R. (2007). Osmia bees (Hymenoptera: Megachilidae) can detect nectar-rewarding flowers using olfactory cues. *Animal Behaviour*, *74*(2), 199–205. https://doi.org/10.1016/j.anbehav.2006.11.012

Ito, K., Suzuki, M. F., & Mochizuki, K. (2021). Evolution of honest reward signal in flowers. *Proceedings of the Royal Society B: Biological Sciences*, *288*(1943), 20202848. https://doi.org/10.1098/rspb.2020.2848

Joffard, N., Arnal, V., Buatois, B., Schatz, B., & Montgelard, C. (2020). Floral scent evolution in the section *Pseudophrys*: Pollinator-mediated selection or phylogenetic constraints? *Plant Biology*, *22*(5), 881–889. https://doi.org/10.1111/plb.13104

Johnson, S. (2000). Batesian mimicry in the non-rewarding orchid *Disa pulchra*, and its consequences for pollinator behaviour. *Biological Journal of the Linnean Society*, *71*(1), 119–132. https://doi.org/10.1006/bijl.1999.0430

Karron, J. D., Mitchell, R. J., & Bell, J. M. (2006). Multiple pollinator visits to *Mimulus ringens* (*Phrymaceae*) flowers increase mate number and seed set within fruits. *American Journal of Botany*, *93*(9), 1306–1312. https://doi.org/10.3732/ajb.93.9.1306

Kiang, Y. T. (1972). Pollination Study in a Natural Population of Mimulus guttatus. *Evolution*, *26*(2), 308–310. https://doi.org/10.2307/2407041

Knauer, A. C., & Schiestl, F. P. (2015). Bees use honest floral signals as indicators of reward when visiting flowers. *Ecology Letters*, *18*(2), 135–143. https://doi.org/10.1111/ele.12386

Leonard, A. S., & Papaj, D. R. (2011). 'X' marks the spot: The possible benefits of nectar guides to bees and plants. *Functional Ecology*, *25*(6), 1293–1301. https://doi.org/10.1111/j.1365-2435.2011.01885.x

Lucas-Barbosa, D., Sun, P., Hakman, A., Beek, T. A. van, Loon, J. J. A. van, & Dicke, M. (2016). Visual and odour cues: Plant responses to pollination and herbivory affect the behaviour of flower visitors. *Functional Ecology*, *30*(3), 431–441. https://doi.org/10.1111/1365-2435.12509

Mertes, M., Carcaud, J., & Sandoz, J.-C. (2021). Olfactory coding in the antennal lobe of the bumble bee *Bombus terrestris*. *Scientific Reports*, *11*(1), Article 1. https://doi.org/10.1038/s41598-021-90400-6

Makino, T. T., & Ohashi, K. (2017). Honest signals to maintain a long-lasting relationship: Floral colour change prevents plant-level avoidance by experienced pollinators. *Functional Ecology*, *31*(4), 831–837. https://doi.org/10.1111/1365-2435.12802

Michener, C. D. (2007). *The Bees of the World* (2nd ed). Johns Hopkins University Press.

Molet, M., Chittka, L., & Raine, N. E. (2009). How floral odours are learned inside the bumblebee (*Bombus terrestris*) nest. *Naturwissenschaften*, *96*(2), 213–219. https://doi.org/10.1007/s00114-008-0465-x

Nicolson, S. W., Da Silva Das Neves, S., Human, H., & Pirk, C. W. W. (2018). Digestibility and nutritional value of fresh and stored pollen for honey bees (*Apis mellifera scutellata*). *Journal of Insect Physiology*, *107*, 302–308. https://doi.org/10.1016/j.jinsphys.2017.12.008

Ochieng, S. A., Hallberg, E., & Hansson, B. S. (1998). Fine structure and distribution of antennal sensilla of the desert locust, *Schistocerca gregaria* (Orthoptera: Acrididae). *Cell and Tissue Research*, *291*(3), 525–536. https://doi.org/10.1007/s004410051022

Paoli, M., & Galizia, G. C. (2021). Olfactory coding in honeybees. *Cell and Tissue Research*, *383*(1), 35–58. https://doi.org/10.1007/s00441-020-03385-5

Raguso, R. A. (2008). Wake Up and Smell the Roses: The Ecology and Evolution of Floral Scent. *Annual Review of Ecology, Evolution, and Systematics*, *39*(1), 549–569. https://doi.org/10.1146/annurev.ecolsys.38.091206.095601

Reverté, S., Retana, J., Gómez, J. M., & Bosch, J. (2016). Pollinators show flower colour preferences but flowers with similar colours do not attract similar pollinators. *Annals of Botany*, *118*(2), 249–257. https://doi.org/10.1093/aob/mcw103

Riveros, A. J., & Gronenberg, W. (2009). Olfactory learning and memory in the bumblebee *Bombus occidentalis*. *Naturwissenschaften*, *96*(7), 851–856. https://doi.org/10.1007/s00114-009-0532-y

Rodriguez-Saona, C., Parra, L., Quiroz, A., & Isaacs, R. (2011). Variation in highbush blueberry floral volatile profiles as a function of pollination status, cultivar, time of day and flower part: Implications for flower visitation by bees. *Annals of Botany*, *107*(8), 1377–1390. https://doi.org/10.1093/aob/mcr077

Schaal, B. A., & Leverich, W. J. (1980). Pollination and Banner Markings in *Lupinus texensis* (*Leguminosae*). *The Southwestern Naturalist*, *25*(2), 280–282. https://doi.org/10.2307/3671260

Schiestl, F. P., & Johnson, S. D. (2013). Pollinator-mediated evolution of floral signals. *Trends in Ecology & Evolution*, *28*(5), 307–315. https://doi.org/10.1016/j.tree.2013.01.019

Stanley-Stahr, C. A., & Pitts-Singer, T. L. (2023). Odor Discrimination after Olfactory Conditioning of Managed Solitary Bees, *Osmia Lignaria* and *Megachile Rotundata*. *Journal of Insect Behavior*, *36*(3), 180–194. https://doi.org/10.1007/s10905-023-09825-8

Stanton, M. L., & Preston, R. E. (1988). Ecological Consequences and Phenotypic Correlates of Petal Size Variation in Wild Radish, *Raphanus Sativus* (*brassicaceae*). *American Journal of Botany*, *75*(4), 528–539. https://doi.org/10.1002/j.1537-2197.1988.tb13471.x

Steiner, K. E., Kaiser, R., & Dötterl, S. (2011). Strong phylogenetic effects on floral scent variation of oil-secreting orchids in South Africa. *American Journal of Botany*, *98*(10), 1663–1679. https://doi.org/10.3732/ajb.1100141

van Doorn, W. G. (1997). Effects of pollination on floral attraction and longevity. *Journal of Experimental Botany*, *48*(314), 1615–1622.

Vickery, R. K. (1999). Remarkable Waxing, Waning, And Wandering Of Populations Of *Mimulus Guttatus*: An Unexpected Example Of Global Warming. *The Great Basin Naturalist*, *59*(2), 112–126.

Weiss, M. R. (1991). Floral colour changes as cues for pollinators. *Nature*, *354*(6350), Article 6350. https://doi.org/10.1038/354227a0

Weiss, M. R., & Lamont, B. B. (1997). Floral Color Change and Insect Pollination: A Dynamic Relationship. *Israel Journal of Plant Sciences*, *45*(2–3), 185–199. https://doi.org/10.1080/07929978.1997.10676683

Wright, G. A., & Schiestl, F. P. (2009). The evolution of floral scent: The influence of olfactory learning by insect pollinators on the honest signaling of floral rewards. *Functional Ecology*, *23*(5), 841–851. https://doi.org/10.1111/j.1365-2435.2009.01627.x

Wu, C. A., Lowry, D. B., Cooley, A. M., Wright, K. M., Lee, Y. W., & Willis, J. H. (2008). *Mimulus* is an emerging model system for the integration of ecological and genomic studies. *Heredity*, *100*(2), Article 2. https://doi.org/10.1038/sj.hdy.6801018

Zito, P., Rosselli, S., Bruno, M., Maggio, A., & Sajeva, M. (2018). Floral scent in a sexually deceptive *Ophrys* orchid: From headspace collections to solvent extractions. *Plant Signaling & Behavior*, *14*(1), 1552056. https://doi.org/10.1080/15592324.2018.1552056

Zu, P., Blanckenhorn, W. U., & Schiestl, F. P. (2016). Heritability of floral volatiles and pleiotropic responses to artificial selection in *Brassica rapa*. *New Phytologist*, *209*(3), 1208–1219. https://doi.org/10.1111/nph.13652

Supplementary Table

Supplementary Table 1. Floral volatile presence in pollinated and unpollinated M. lewisii and M. guttatus. Volatiles were identified by a GC-MS and then narrowed down by classifications. Numbers in parenthesis refer to how many times the volatiles occurred within the dataset, "x" denotes the volatiles presence , and a "" displays which singleton volatiles were removed from the PCA analysis.*

		Presence			
		M. lewisii		*M. guttatus*	
	Volatile	**Unpollinated (n)**	**Pollinated (n)**	**Unpollinated (n)**	**Pollinated (n)**
Sesquiterpenes					
	1,1,7,7a-Tetramethyl-1a,2,6,7,7a,7b-hexahydro-1H-cyclopropa[a]naphthalene		x (1)*		
	10s,11s-Himachala-3(12),4-diene	x (1)	x (2)		
	2-Pentadecanone, 6,10,14-trimethyl	x (3)	x (3)	x (5)	x (3)
	beta-Bisabolene	x (1)	x (1)		
	Dodecane, 2,6,10-trimethyl				x (3)
	Caryophyllene	x (1)	x (2)		
	Caryophyllene oxide	x (1)	x (3)		
	Isocalamendiol		x (1)*		
	geranyl-.alpha.-terpinene	x (1)	x (1)		
Esters					
	2-Hexanol, acetate	x (2)	x (2)	x (1)	x (1)
	Acetic acid, 2-ethylhexyl ester	x (4)	x (2)		x (5)
	Acetic acid, decyl ester	x (1)	x (2)	x (1)	x (1)
	Acetic acid, heptyl ester	x (1)	x (2)	x (1)	x (3)
	Dihydrocitronellyl acetate		x (1)*		x (2)
	Lauryl acetate	x (3)	x (5)	x (4)	x (4)
Benzenoids					
	1-Butanol, 3-methyl, benzoate			x (2)	
	1H-Indene, 2,3-dihydro-1,1,3-trimethyl-3-phenyl	x (3)	x (3)	x (3)	x (4)
	2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-			x (2)	
	4-Trifluoromethylbenzoic acid, 4-hexadecyl ester	x (1)*		x (1)*	
	Benzenepropenamide, N-(phenylmethyl)	x (2)		x (1)	x (1)
	Benzoic acid, 2-ethylhexyl ester	x (5)	x (4)	x (5)	x (5)
	Biphenyl	x (2)	x (3)	x (1)	x (1)
	Homosalate	x (1)	x (1)	x (3)	x (3)
	Phenol, 4-(1,1-dimethylpropyl)				x (4)
	p-Toulic acid, 2-ethylhexyl ester	x (2)	x (1)	x (4)	
	Mandelic acid, 2TBDMS derivative		x (2)		
	m-Toulic acid, 2-ethylhexyl ester	x (1)	x (1)	x (1)*	
Monoterpenes					
	1-Hexanol, 5-methyl-2-(1-methylethyl)	x (2)	x (3)	x (5)	x (2)
	5,9-Undecadien-2-one, 6,10-dimethyl	x (3)	x (2)		x (4)
	Menthyl acetate	x (2)	x (2)		x (2)
Monoterpenoids					
	2,6-Dimethyl-8-(tetrahydropyran-2-yloxy)-octa-2,6-dien-1-ol	x (2)	x (2)		
	Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl)-,(1.alpha.,2.alpha.,5.alpha.)		x (1)*		
	Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)	x (3)	x (2)	x (1)*	
	D-Limonene	x (2)	x (2)	x (1)*	
	Limonene-1,2-diol	x (1)	x (2)	x (1)	x (1)
	Linalool	x (1)	x (1)	x (4)	
	α-Pinene		x (1)*		

Chapter 2:

How Interspecific and Intraspecific Variation Shape Floral Volatile Composition in the Genus *Mimulus*

Abstract

Interspecific (within-species) and intraspecific (between populations) variations in floral volatile organic compounds (VOCs) play an important role in plant evolution. The relationship between the two is tantamount to understanding how lineages break and form in the past and the future. Many forces, such as pollinators, phylogenetics, or abiotic environmental factors may shape volatile production and signaling throughout a species' range or within a genus. In this study, I examined floral volatile profiles within five species of the genus *Mimulus* and three populations of the species *M. guttatus*. I used a GC-MS to investigate how volatile composition varied throughout the genus and across several populations, and how it affected the genus's pollination biology trajectory. I found that no overarching selection force or constraint by phylogenetics, reward systems, or pollinators across species within the genus. I found differences among M. guttatus populations that could be due to abiotic ecosystem factors. These results suggest that phylogenetic constraints do not drive interspecific variation, but interspecific variation is more likely influenced by individual species' adaptive solutions to entice pollinators or is influenced by genetic drift.

Introduction

Plants evolved many lures and cues to entice pollinators to visit their flowers and exchange pollen. Flowers entice pollinators to visit by releasing attractive scents made of complex metabolites called volatile organic compounds (VOCs). Floral VOCs can be released from any floral tissue, such as the corolla, stamen, or pistil (Baldwin, 2010; Schiestl & Johnson, 2013). Floral VOCs are divided into four organic chemical groups: aromatics, monoterpenes, sesquiterpenes, and fatty acid derivates (Knudsen et al., 2006; Schiestl, 2010). The most common angiosperm VOCs are limonene, (E)-β-ocimene, myrcene, linalool, α- and β-pinene (Knudsen et al., 2006). However, most floral VOCs are understudied. These VOCs vary across angiosperms; there are constraints or forces that affect the patterns of their production and evolution (Raguso, 2008). I examined patterns of interspecific and intraspecific variation in VOC production in the genus *Mimulus*.

Pollinator-mediated selection is one of the factors that affect plant volatile production. Shifts in pollinators lead to divergent floral VOC compositions. This shift between pollinators has a cascading effect upon the VOCs emitted by the plants as changes in pollinators occur. For example, in fig plants, fig wasps typically pollinate a single *Ficus* species and distinguish between sympatric *Ficus* species based on their specific floral scents (Okamoto & Su, 2021). In moth-pollinated and other nocturnal-pollinated floral species, floral volatiles have an increased output at nighttime compared to during the daytime, likely a result of the plant evolving to attract a nocturnal pollinator (Borges, 2018; Borges et al., 2016; Dobson, 2006; Dötterl et al., 2012; Jürgens et al., 2002; Knudsen & Tollsten, 1993, 1995; Krug et al., 2018; Pereira et al., 2014; Pettersson et al., 2003; Raguso et al., 2003; Siqueira et al., 2018). The moth genus *Greya,* a pollinating-parasitic moth, was found to drive the evolution of floral volatiles in the entire plant

genus of *Lithophramga* on a species and population level (Friberg et al., 2019). In the genus *Mimulus,* hummingbird-pollinated species (*M. cardinalis*) emitted fewer volatiles than bumble bee-pollinated species (*M. lewisii*) (Byers, Bradshaw, et al., 2014; Byers, Vela, et al., 2014). When petals are colored red, hummingbirds have exclusive access to floral rewards (Lunau et al., 2011), largely due to the inability of bees to see red wavelengths, resulting in a lack of petal detection. Most Ecuadorian hummingbird-pollinated floral species do not produce volatiles (Knudsen et al., 2004. It has been suggested that this is yet another evolutionary adaptation to avoid bee detection (Coimbra et al., 2020; Knudsen et al., 2004). A similar effect has been seen in other flowering species pollinated by sunbirds in the Old World (Chen et al., 2020; Rodríguez-Gironés & Santamaría, 2004). Bird-pollinated species of the South African genus *Protea* were found to have significantly fewer floral volatiles than those that are beetle-pollinated (Steenhuisen et al., 2012). Despite hummingbirds and sunbirds evolving nectar feeding independently, these two distantly related families of birds have shaped floral evolution by driving the loss of scent in red flowers.

Phylogenetic relatedness may also shape similarities and differences in VOC emissions. Volatile emissions can potentially be carriers of phylogenetic information, and this information can be used to reveal patterns of non-pollinator-mediated compounds' production history throughout a species (Barkman, 2001; Gögler et al., 2009; Raguso et al., 2003, 2006). In the genus *Ophrys*, shifts in pollination account for changes in alkene and alkadiene emission within the sexually deceptive orchids, but phylogenetic relatedness explains volatile patterns of fatty acids and esters (Joffard et al., 2020). In the oil-secreting orchid tribe Diseae, a phylogeny was discovered to be more vital than pollinators in explaining trends of VOC production and emission throughout the genus, *Coryciinae* and ecotypes (Steiner et al., 2011). Outside orchids,

volatiles emitted from nine species of tree peony of the genus *Paeonia* fell into four phylogenetic clusters that were consistent with their geographic locations (Luo et al., 2020).

It has long been suspected that intraspecific variation in floral volatiles represents an evolutionary step toward interspecific variation, but very few studies have been published that specifically investigated intraspecific variation (Ackerman et al., 1997; Azuma et al., 2001; Delle-Vedove et al., 2017; Knudsen, 2002; Schlumpberger & Raguso, 2008). Variation among populations in floral odors might evolve through pollinator-mediated selection, genetic drift, introgression of floral traits, and pleiotropic effects within molecular pathways, or populations might differ due to environmental phenotypic plasticity (Burkle et al., 2020; Raguso, 2008).

Intraspecific variation, when observed, is most likely a result of pollinator-mediated selection. Evidence of this has been reported in both rewarding and deceptive orchids (Ackerman et al., 1997; Moya & Ackerman, 1993). One subspecies of *Linanthus dichotomus* attracted more noctuid moths than another, which attracted more general pollinators. This was due to the subspecies attracting noctuid moths having a higher volatile emission rate of lilac aldehydes, while the other subspecies had a higher emission rate of phenylacetaldehyde (Chess et al., 2008). In the cycad, *Encephalarto ghellinckii,* two pollination ecotypes differ in their dominant volatiles, and these compounds attract different species of pollinating beetles (Suinyuy & Johnson, 2021). In the species *Conopodium majus*, three populations were studied, each with a distinct volatile composition leading to the conclusion that specific pollinators or accidental differences influence each population's volatiles due to urbanization (Tollsten & Øvstedal, 1994).

Abiotic environmental factors could play a role in intraspecific volatile production. A study by de Manincor er al. (2022) investigated the differences between four perennial plants in wild and controlled populations and found that in one species (*Anthyllis vulneraria*), geographic

differences influenced volatile compositions. However, another species (*Ranunculus bulbosus*) showed VOC geographic variation between the controlled and wild population, suggesting phenotypic plasticity. Soler et al. (2011) observed differences in floral volatiles between East Asian and Indian populations of two species of *Ficus*. They hypothesized that geographic barriers between the two regions were disrupting gene exchange. The pattern underlies the importance of intraspecific variation to understanding the lineage differences or the branching of species over time. The between-population (intraspecific) differences observed today are the beginnings of future between-species (interspecific) differences.

Unstable plant-pollinator mutualisms influence differences in VOC production (Edwards & Yu, 2007). The most likely force of the destabilization of plant-pollinator mutualism is energic cost reduction towards pollinator attraction. In plants, the interaction cost would be floral rewards or nectar/pollen production. Plants could reduce this cost by decreasing pollen amount or size or decreasing nectar production. If a plant lowers nectar or pollen production without compromising pollinator visitation, this would be advantageous as it reduces the energetic cost of creating more rewards. A reduction or elimination of rewards could lead to a divergence in VOC signaling to signals that are innately attractive to a pollinator ("a sensory trap"). If the VOC signaling is strongly attractive, it would allow the plant to gather pollinators without expending the energic cost of creating and refreshing floral rewards such as pollen or nectar, creating a dishonest signal. The best-studied examples of this shift in VOC emissions are the rewardless orchids, which emit scents that mimic pollinator pheromones (Ayasse et al., 2003; Ellis & Johnson, 2010; Johnson, 2000; Steiner et al., 2011).

The genus *Mimulus* (Phrymaceae) contains extensive phenotypic, ecological, and genomic diversity, rendering it a near-ideal system to explore the evolution and function of

VOCs due to the high variety of pollination syndromes that appear within its evolutionary history (Wu et al., 2008). The genus is mostly herbaceous and native to open, wet habitats, with life histories varying from annual to perennial. Very few species from the genus have had their VOCs recorded, but there is some research about the discovery of a strong pollinator sensory bias for the volatile β-trans-bergamotene in *M. guttatus* (Haber et al., 2021). Byers et al. (2014) studied volatile production by *M. cardinalis* and *M. lewisii*, two sister taxa within the genus, discovering that *M. cardinalis* (hummingbird pollinated) has very weak expression of floral volatiles as compared to *M. lewisii* (bumble bee pollinated). My study examined five different species of *Mimulus*, and three different *M. guttatus* populations.

The *M. guttatus* species complex is one of the most studied sections of the genus. *Mimulus guttatus* is a mixed-mating perennial herb with yellow petals and zygomorphic flowers that are commonly pollinated by bumblebees (Kiang, 1972; Pennel, 1951) but are also capable of self-pollination (mean outcrossing rate ~70%, (Ritland & Ritland, 1989)). It produces little or no nectar, rewarding pollinators with only pollen. It is the most wide-ranging member of the genus in North America, occupying wet habitats in the west, from the Mexican border north to Alaska (Kiang, 1972). Most populations are annual, but populations in areas that are wet year-round have evolved a perennial life history (Kiang, 1972; Pennel, 1951). Annual and perennial populations differ in the UV reflectance of their corollas (DeMarche et al., 2015), and it is therefore possible that there is a difference in volatile production. *Mimulus glaucescens* is a member of the *M. guttatus* complex. It is an annual herb native to a small, restricted range in California, pollinated by bumblebees, and, like *M. guttatus*, does not produce nectar.

M. lewisii and *M. cardinalis* are sister species. *M. lewisii* is a perennial herb with bright pink petals native to northwestern North America. It is pollinated by bumblebees and produces

both pollen and nectar (Bradshaw & Schemske, 2003). *M. cardinalis* is a perennial herb native to western United States from Southern California to Washington. It produces pollen and nectar and is pollinated primarily by hummingbirds. Nectar production is much greater than *M. lewisii*, and its red petals are typical of bird-pollinated flowers. *M. lewisii* and *M. guttatus* share three VOCs that influence bumble bee attraction; D-limonene, β-myrcene, and E-β-ocimene (Byers, Vela, et al., 2014; Haber et al., 2019).

Mimulus ringens is one of only two members of the genus native to the eastern United States and occupies open habitats (Windler et al., 1976). It is a perennial herb with light purple petals and pollinated by bumble bees. It produces both pollen and nectar. Unlike most monkeyflower species, its flowers only last a single day.

In this study, I propose to analyze interspecific and some intraspecific differences in floral volatiles among five species in the genus *Mimulus*. I propose two questions: 1) what are the patterns of floral volatile production in a group of related species in the genus *Mimulus* that differ in their pollination ecology, and 2) what is the degree of intraspecific variation in volatile production in *M. guttatus*? I expect to see similar volatiles in nectar-producing, bee-pollinated species (*M. ringens* and *M. lewisii).* In bumble bee-pollinated *M. ringens*, I expect to see the three main volatiles (D-limonene, β-myrcene, and E-β-ocimene) as in the nectar-producing *M. lewisii*. In *M. cardinalis*, due to its hummingbird pollinators, I expect the three main volatiles to be absent or extremely reduced. In *M. glaucescens*, I expect to see a similar volatile composition as *M. guttatus* and possible evidence of β-trans-bergamotene. Within the intraspecific *Mimulus* populations, I expect to see some variation in volatiles between the populations, especially between perennial and annual populations.

Methods

Study System: Mimulus

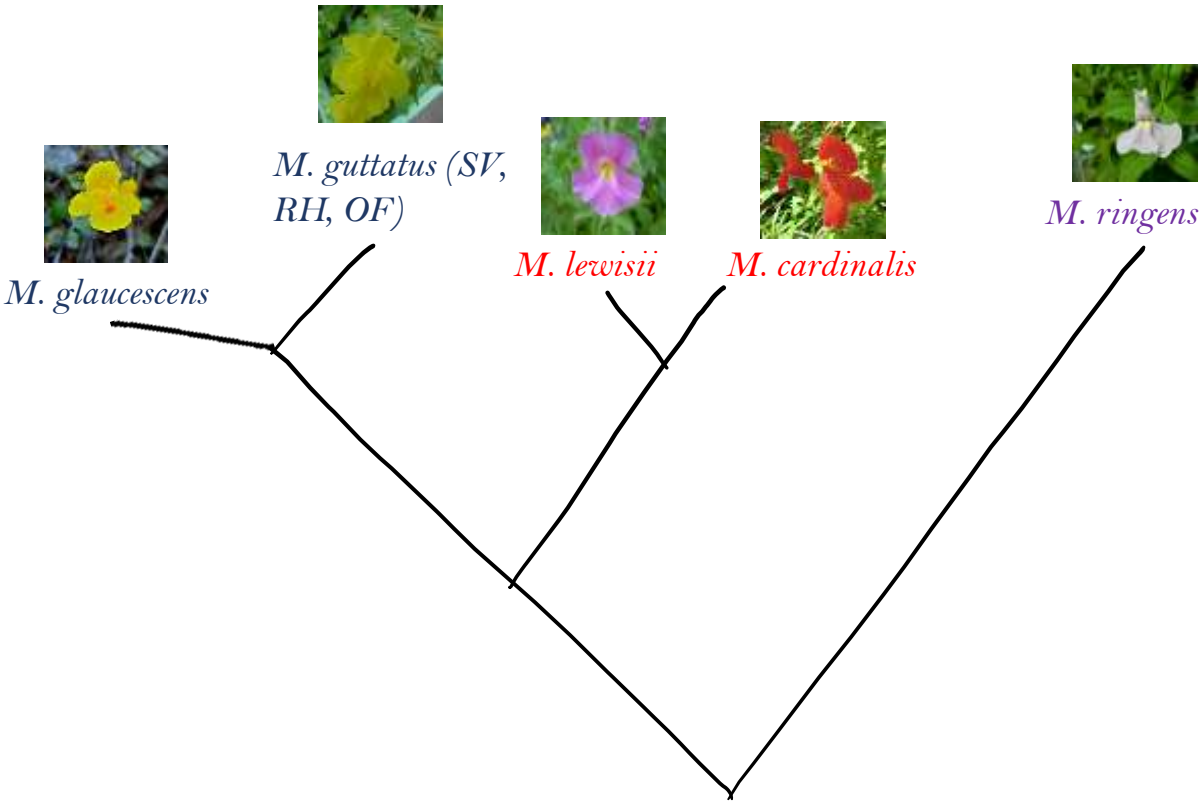

Figure 1. Phylogenetic tree displaying the relationships of focal species in the genera Mimulus. Barker et al. 2012

All three *M. guttatus* populations that were used in this study are descendants of seeds collected from random maternal families across three counties (Napa, Marin, and Tuolumne) in California (Table 1, Fig. 2). The SV and RH populations are annual, but the OF (Marin County) population is perennial. The *M. lewisii* are descendants of seeds collected from a population in Skamania County, WA (Mount St. Helens National Volcanic Monument) (Table 1). The *M. ringens* are descendants of seeds collected from a population at Blandy Experiment Farm in Clarke County, VA (Table 1). *M. glaucescens* are descendants of seeds collected from Butte

County in Northern California. The plants used in the current study were grown in a greenhouse at Blandy Experimental Farm in Boyce, VA, USA (photoperiod:18 D:6 N). Approximately 20 seeds from each population were sown in a 3-inch square pot filled with Promix BK25 soilless potting mix. There were 20 pots per tray with bottom watering. Once germination was witnessed in each of the "original pots," we randomly transplanted four plants from each maternal family into their own pots. Due to a whitefly outbreak, *M. ringens* had to be sprayed with neem oil.

Figure 2. California locations of each M. guttatus population that was used in this study.

Table 1. All Mimulus species and M. guttatus populations below are used within this study. All populations were grown and observed in a greenhouse at Blandy Experimental Farm in Clarke County, VA

Species	Region	County	Site	Code	N Latitude	W Longitude
M. guttatus (OF)	Coast	Marin	Johnson's Oyster Co.	OF	38° 05.05'	122° 55.90'
M. guttatus (SV)	Inner Coast Range	Napa	Snell Valley	SV	38° 42.06'	122° 24.5'
M. guttatus (RH)	Sierra Foothills	Tuolumne	Red Hills	RH	37° 50.31'	120° 28.14'
M. lewisii	Pacific Northwest	Skamania	Mount St. Helens	MSH	46° 14' 43"	122° 11' 04"
M. ringens	Atlantic Northeast	Clarke	Blandy Experimental Farm	MR	39° 3' 40"	38° 3' 47"
M. glaucescens	Northern California	Butte	--	SH	39° 47' 00"	121° 44' 39"
M. cardinalis	Pacific Northwest	Skamania	Mount St. Helens	BC	--	--

Volatile Collection

I collected floral volatiles in May, June, and July of 2023 when the species started flowering. The SV population of *M. guttatus* and *M. lewisii* had their volatiles collected in the fall of 2022 and analyzed through a GC-MS in June and July of 2023. The rest of the floral volatiles were analyzed in September and October of 2023. The order of collection was determined simply by the order in which plants began to flower. I attempted to sample volatiles from five unpollinated plants of each species, but due to low levels of flowering, I was able to collect from only a single *M. glaucescens* and a single plant from the RH population of *M. guttatus.* Immediately, post-volatile collection we collected pollen from each plant and recorded the corolla width of each species.

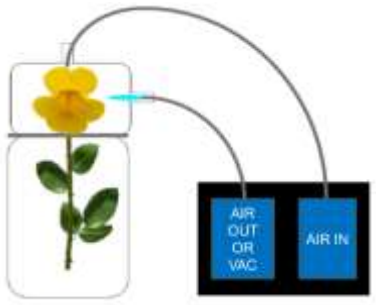

Figure 4. Push-Push collection system for extracting volatiles from Mimulus. Teal shape represents the collection filter, while the grey bar represents aluminum foil to separate the stem from the floral volatiles.

Floral volatiles were collected using a pull-push collection system (Sigma Scientific LLC, Micanopy, FL). This system pumps air into a chamber containing the flowers and vacuum air through a filter exiting the chamber (Fig. 4). The filters were VCT 3.5" with 30mg ± 5 Porapak™ Type Q (Sigma Scientific LLC, Micanopy FL). The flow rate for air into the chamber is 1.5 LPM, and the flow rate for air out is 2 LPM. Plants were housed in the glass chambers (Sigma Scientific LLC, Micanopy, FL) to allow for an environment without external volatiles. Glass chambers (5400 mL) were used to collect volatiles, as well as a taller set of glass chambers that are 6400 mL (Sigma Scientific LLC, Micanopy, FL). All floral volatiles were collected for eight hours at minimum to ensure volatile collection into the filter. Between each experiment, the glass chambers were wiped down with 70% ethanol, and to minimize the spread of contamination, nitrile FisherBrand© gloves were worn while handling filters and glass chambers. Once

collected, the filters were removed from the tubing, wrapped in aluminum foil, and stored at -80 °C.

Two species required a different volatile collection set-up due to their height (*M. ringens,* and *M. cardinalis*). We created a "guillotine" for these species (Fig. 5a). The "guillotine" had a plywood base and a PVC frame (total: 110 cm h, 22 cm w, 20 cm d). I mounted an adjustable plastic mesh platform on the PVC frame that held the plant pot. A wood block on top of the PVC frame served as the base for the headspace chamber. I drilled two holes, one for the plant stem and the other for the incoming Teflon airline (Fig. 5b). The block was cut in half around the hole used to insert the plant stem. To keep the halves airtight, a screwing hinge passed through the block. I covered the top of the block with a sheet of Teflon so that the wood was not exposed to the collection chamber and potentially picked up by the filter collection. A glass dome (400 mL) with an exit port was placed on top of the Teflon sheet, and the vacuum line removed air from the chamber into the port. A silicon lining kept the glass dome airtight against the Teflon floor of the chamber, as well as two rubber bands over the top to apply more force for an airtight seal.

Figure 5ab. Floral Headspace Guillotine Device. The image on the left (5a) shows the full design of the guillotine with a plant inside to demonstrate the collection. The image on the right (5b) shows the headspace where the florals are filtered out.

Volatile Elution, Identification, and Analysis

I eluted the trapped compounds with 150 µl dichloromethane (CH_2Cl_2) and added 5 µL of a mix (dissolved in CH_2Cl_2) containing the internal standards n-octane (40 ng/µL) and nonyl acetate (80 ng/µL). Samples were injected into 1-µl aliquots using a splitless injector into a Rxi-5Sil MS column (0.25 mm internal diameter, 0.25 µm film thickness, 30.0 m length) in a Shimadzu GCMS-QP2010S and then separated and detected using a Shimadzu GC-2010. The column started at 50°C with no hold time and then increased at 8°C/min to 240°C. Pure helium was the carrier gas. Injection began at 250.0°C. Each sample was run for 23.75 minutes. This data only allowed us to establish the presence of a compound, not a quantitative estimate.

Volatiles from each sample were identified by chemical peaks on each chromatogram. These chemical peaks represented the retention times of different chemical compositions within each sample. I used the mass spectrum from each peak to identify the most likely VOC. The

mass spectrometer bombards the sample with energetic electrons, causing the sample (the analyte) to lose an electron due to electron repulsion, and further electron bombardment causes the sample's ions to fragment. After fragmentation, these ions are passed into a mass analyzer and sorted by their mass-to-charge ratio. NIST 2014 software compares the analyte's mass spectrum to a database of mass spectra, generating a likelihood for the five best matches in order of most to least likely. Once the software identified the volatiles, they were summarized in Excel with alternative names and their likelihoods. All non-plant-based chemicals with likelihoods below 60 and those with only one occurrence across all samples were removed. I then identified the chemical class of each compound using PubChem, Classyfire (Djoumbou Feunang et al., 2016), and the National Institute of Science and Technology website. All chemicals not identified as sesquiterpenes, monoterpenes, monoterpenoids, esters, or benzenoids were removed from the dataset.

Volatile Analysis

I characterized floral blends with a principal component analysis (PCA) with the PRINCOMP procedure in SAS. Due to only having presence/absence data for each compound, I calculated the PCA based on the covariance/variance matrix of the data. I created a dendrogram of all the populations based on the first three principal components using an unweighted pair group method with arithmetic mean (UPGMA) algorithm using the CLUSTER procedure in SAS. I compared the mean total VOCs across all populations with a 1-way ANOVA followed by a Tukey's Test for pairwise comparisons among the populations with the SAS GLM procedure.

Results

The general breakdown of all the volatiles seemed to trend heavily in favor of esters and benzenoids (Table 2, Supplementary Table 1). All species had esters, and all but *M. glaucescens* had benzenoids. In the *M. guttatus* SV population, there are 19 total volatile compounds (including VOCs recorded in multiple samples) with four esters and nine benzenoids. In the OF population, there are nine total volatiles with seven esters and one benzenoid. In the RH population, there are two total volatiles, and they were both esters. In *M. glaucescens*, there are five total volatiles with four esters and one monoterpenoid. In *M. lewisii*, there are 27 total volatiles, which are eight esters, two benzenoids, and six monoterpenoids. In *M. cardinalis*, there are 17 total volatiles with eight esters, seven monoterpenoids, and two benzenoids. In *M. ringens*, there are 16 total volatiles with eight esters, two benzenoids, and six monoterpenoids. The least frequently occurring are monoterpenes and sesquiterpenes, appearing in the SV population and *M. lewisii*. Across the entire dataset, the two most frequently occurring VOCs are benzoic acid, 2-ethylhexyl ester, which occurred 17 times, and acetic acid, 2-ethylhexyl ester, which occurred 12 times across the dataset.

Table 2. The number of volatile compounds by chemical classes within each species and the total number of individual volatile compounds within species.

	Esters	Benzenoids	Monoterpenoids	Monoterpenes	Sesquiterpenes	Total Individual Volatiles
M. guttatus (SV)	4	9	4	1	1	19
M. guttatus (RH)	2	0	0	0	0	2
M. guttatus (OF)	7	1	1	0	0	9
M. glaucescens	4	0	1	0	0	5
M. lewisii	5	8	6	2	6	27
M. cardinalis	8	2	7	0	0	17
M. ringens	8	2	6	0	0	16

I ran a PCA for all species and populations within our dataset (Fig. 6). Each principal component (PC) characterized the floral scent based on the presence and absence of the volatiles. Loadings within each eigenvector represent the strength of the relationship between each individual VOC and the PC. The first three PCs accounted for 47.46% of the total variation in the dataset. PC1 accounted for 24.93% of the total variation in the data. In PC1, the eight volatiles with the highest loadings included: 2-Pentadecanone, 6,10,14-trimethyl (0.36055), 1-Hexanol, 5-methyl-2-(1-methylethyl) (0.3411), Linalool (0.28239), Tetrahydrolavandulyl acetate (-0.2754), and 1H-Indene, 2,3-dihydro-1,1,3-trimethyl-3-phenyl (0.26925). PC2 accounted for 13.61% of the total variation. In PC2, the five volatiles with the highest loadings included: Acetic acid, 2-ethylhexyl ester (0.4277), Benzoic acid, 2-ethylhexyl ester (0.42334), p-Toluic acid, 2-ethylhexyl ester (0.35876), 2-Heptenoic acid, isobutyl ester (0.34536), and Tetrahydrolavandulyl acetate (0.26996). PC3 accounted for 9.74% of the variation in the dataset. In PC3, the five volatiles that had the highest loadings included: Limonene-1,2-diol (0.44245), 2-Hexanol, acetate (-0.4017), 4-Methyl-2-pentyl acetate (0.29307), Menthyl acetate (-0.2567), and 5,9-Undecadien-2-one, 6,10-dimethyl (-0.2496).

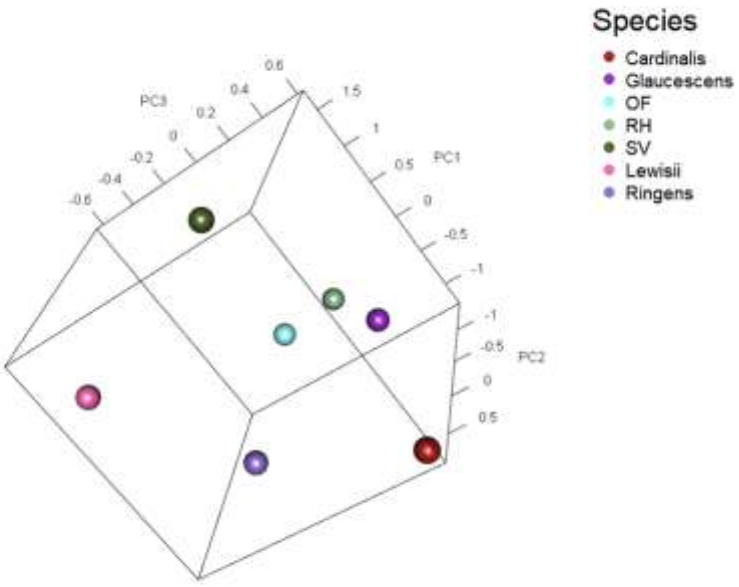

Figure 6. PCA for all species and populations within the dataset. This image depicts where the means for each species and population are located within PC1, PC2, and PC3. Each principal component has characterized the floral scent based on the presence or absence of the volatiles. The first three PC's accounted for 47.46% of the total variation in the dataset.

The plot of PC 1-3 did not show strong phylogenetic clusters (Fig. 6). There was a cluster of two of the *M. guttatus* populations (OF, RH). There was an additional cluster of *M. glaucescens* and *M. ringens*. *M. cardinalis* and *M. lewisii* were far away from each other and in between the *M. ringens/glaucescens* cluster. In a different analysis of the clusters by UPGMA algorithms (Fig. 7), I found three differing clusters: 1) *M. ringens* and *M. cardinalis*, 2) two other *Mimulus* populations (RH and OF) with *M. glaucescens,* and 3) *M. guttatus* (SV) and *M. lewisii.* There does not seem to be strong phylogenetic constraints against floral scent.

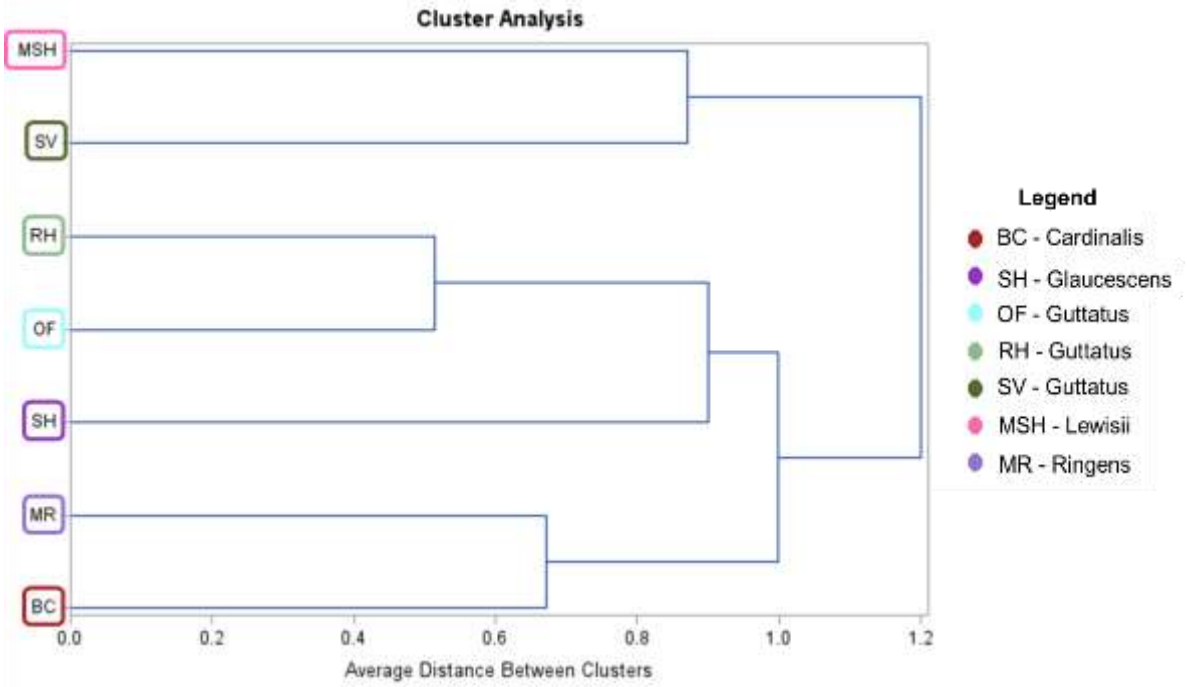

Figure 7. UPGMA cluster analysis for all populations. There are three clusters, the first is for M. lewisii and M. guttatus SV population. The second clustering is made up of the rest of the M. guttatus complex (M. glaucescens, RH, OF). The third cluster is made up of M. ringens and M. cardinalis.

I ran an ANOVA comparing the mean number of total VOC compounds across all populations (Fig. 8). Populations varied significantly in the mean total number of VOCs emitted ($F_{6,20} = 4.26$, $p = 0.0064$). A Tukey Test on the pairwise comparisons for among the populations found only two significant differences. The mean total VOC produced for *M. lewisii* is significantly higher ($t = 4.17$, $p = 0.0072$) than the mean total VOC production by *M. guttatus* OF population. I also found that the SV population had a higher mean total VOC production than the OF population ($t = -3.15$, $p = 0.0300$). No other populations were significantly different from each other.

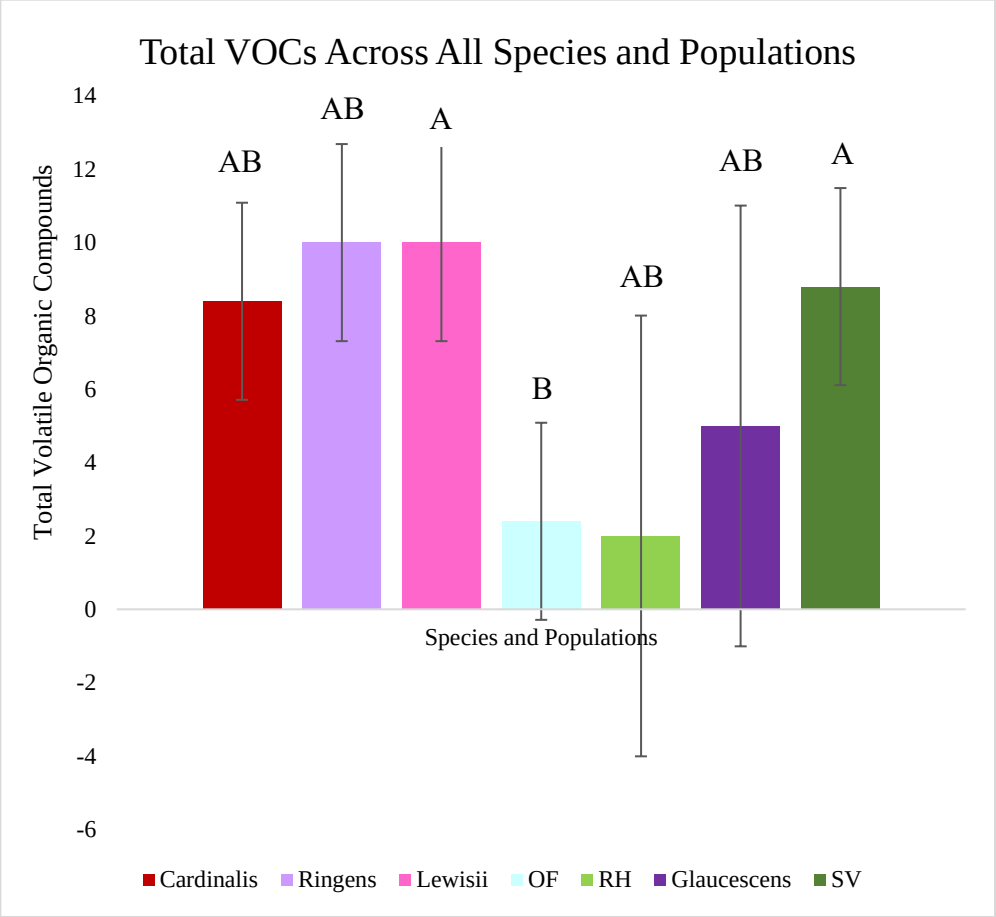

Figure 8. Mixed Model ANOVA on all species and M. guttatus populations. Each bar represents the mean number of volatile compounds produced by each species. The overall ANOVA demonstrated significant differences among species/populations ($F_{6,20} = 4.26$, $p = 0.0064$). Error bars represent the lower and upper confidence intervals in the mixed model ANOVA. Means that share a letter are not significantly different based on a Tukey HSD test.

Discussion

My findings indicate no strong phylogenetic pattern from floral scent in the genus *Mimulus*. Clusters based on VOC emission included: 1) *M. guttatus* (SV) and *M. lewisii*, 2) *M. guttatus* (RH and OF) and *M. glaucescens,* and 3) *M. ringens* and *M. cardinalis*. My results found no pollinator-mediated pattern, as clustering species did not organize into pollinator

groups. Additionally, I found no clustering pattern towards dual floral rewards (nectar and pollen) and singular rewards (only pollen) within the genus.

A clustering split that I observed may be coming from when the volatiles were collected and analyzed. I collected the *M. guttatus* (SV) and *M. lewisii* samples in the fall of 2022 and processed them in June 2023. I collected the floral volatiles from the rest of the populations in the summer of 2023 and analyzed them throughout the Fall of 2023. The chromatographs also looked different between both sides of this split. Due to this split, I believe the clustering within my dataset was highly influenced by the collection period and longer-term storage, with potential seasonal differences.

When compared to the literature, my findings were not similar. For *M. guttatus* population SV, I detected 19 volatiles. In comparison, Haber et al. (2019) detected 17 volatiles from this same population, and my study found only four of the same compounds (α-pinene, limonene, trans-limonene, and linalool). I was unable to detect any other compound Haber et al. (2019) identified, including β-trans-bergamotene, a chemical that overrode learned preferences in bumblebees (Haber et al. 2021).

In *M. lewisii*, Byers et al. (2014) found that three main compounds accounted for 93% of total VOC emissions (D-limonene, β-myrcene, and E-β-ocimene), whereas I observed only D-limonene among one sample that I identified. Byers et al. (2014) found only five volatiles in *M. cardinalis*. I detected 17 volatiles in *M. cardinalis*, and only α-pinene and limonene were in common with those reported by Byers et al. (2014). I attribute most of these differences to *M. guttatus, M. lewisii,* and *M. cardinalis* as issues with data collection or the timing of processing/analysis of the data.

The most interesting comparison between the literature and my species was *M. cardinalis*, as the literature states that hummingbird-pollinated flowers should have a decreased amount of volatiles compared to bumblebee-pollinated flowers (Byers et al., 2014; Knudsen et al., 2004). However, within my data, *M. cardinalis* had the same, if not more, volatiles as the bumblebee-pollinated flowers. The increase in VOCs in comparison to Byers could be evidence that hummingbirds use their olfactory receptors more often than commonly believed. Ioale & Papi (1988) also found that the White-vented Violetear was able to discriminate against multiple different scents. Another study found that hummingbirds utilize olfaction in nectar foraging to avoid defensive chemicals emitted by wood ants (*Formica francoeuri*) and aggregation pheromones of Argentine ants (*Linepithema humile*) (Kim et al., 2021). However, I believe the most likely cause of the increase in *M. cardinalis* VOCs is sample collection or sample processing errors**.** (Friberg et al., 2019; Ioalé & Papi, 1989; Joffard et al., 2020; Knudsen et al., 2004; Steiner et al., 2011)

Many forces, such as pollinator-mediated selection or phylogenetic patterns, can shape interspecific variation in VOCs. Friberg et al. (2019) found that pollinating-parasitic *Greya* moths drove floral scent variation within the genus Lithophragma at the species and population levels. In Joffard et al. (2020), pollinators seemed to be the cause of differences in alkene and alkadiene emission, and phylogenetic relatedness explained trends in fatty acids and esters in sexually deceptive orchids in the genus *Ophrys*. Steiner et al. (2011) found that phylogeny made a bigger impact on explaining trends of VOC emissions in oil-secreting orchid species and ecotypes within the tribe *Diseae*. The patterns within my data do not show a strong phylogenetic pattern, or a strong pattern of pollinator-mediated selection, or a strong pattern of reward-based selection. It is, therefore, difficult to attribute VOC variation among species to selection based on

my results. However, many adaptive solutions could be potentially occurring like differences in bumble bee species collection of pollen and pollination. Another possibility is that the variation is random due to genetic drift. Additionally, there could be problems with our data collection that are influencing my results.

The *M. guttatus* populations displayed intraspecific variation. For these populations, intraspecific variation may be caused by environmental differences and potentially local pollinator communities. A study by DeMarche et al. (2015) found that annual and perennial populations in *M. guttatus* had different nectar guides under UV lighting across 37 populations in northern California, directly showing intraspecific variation. Based on those findings, I expected M. guttatus populations to cluster by life history, but my data did not cluster by annual versus perennial. Populations RH and OF clustered together despite the fact that RH is an annual and OF is a perennial. Azuma et al. (2001), reported that most of the variation in *Magnolia kobus* across 32 populations in Japan was found among individuals, not populations. They concluded that it is unlikely that pollinator communities were structuring the variation. Therefore, they proposed that selection on scent might be weak within the range of the *Magnolia kobus* species distribution. My study had a similar outcome in finding lots of variation among individuals rather than consistent variation among populations. It could be possible that variation in scent among pollinations is swamped by within-population variation.

Low statistical power limited this study. For some populations (*M. glaucescens* and *M. guttatus* RH population), I was able to get only a single sample. If I had increased the sample size, there would have been a higher statistical power, and I may have detected more pronounced trends. I was unable to estimate the volume or rates of VOC emission, which could have revealed differences that would be undetectable from only the comparison of presence and absence data.

The biggest limitation of this study seemed to be the unexpected inconsistencies in VOC collection, especially the differences observed between the early and later collections. Many of the clustering patterns seem to follow that dichotomy.

In conclusion, our data suggests that intraspecific variation might happen in the *M. guttatus* complex. It does not suggest any pollinator-mediated selection or selection based on reward type for the phenotypic clustering with interspecific variation. Our data also suggests that hummingbird-pollinated species may release more volatiles than expected, as they released the same or even more volatiles than my recorded bumblebee species. For future studies, I would suggest an increased sampling of all species and populations and more stringent protocols when collecting floral volatiles.

Acknowledgements

Rupesh Kariyat provided volatile collection equipment and filters. Avehi Singh and Ariela Haber for their volatile procedures. Kylie Bill for building the "guillotine." Chris Ivey for the *M. glaucescens* seeds. Jill Venton provided access to her GC-MS, and Earl Ashcraft provided operational help for the GC-MS. Judy Masi and Elizabeth Jackson provided administrative and logistical assistance. Maggie McCartney for aiding in lab safety and coordinating lab use. Austin Eichhorn and Stephanie Petrovick provided edits to earlier additions to this chapter. David E. Carr, for his continuous guidance and support throughout this chapter. I'd also like to thank my committee members, T'ai H. Roulston, and Matthew A. Reidenbach, for their thesis comments and their development of the methodology for the project. Funds for this project were provided by the University of Virginia's Department of Environmental Science Exploratory Award and the Blandy Experimental Farm Fellowship.

Literature Cited

Ackerman, J. D., Meléndez-Ackerman, E. J., & Salguero-Faria, J. (1997). Variation in pollinator abundance and selection on fragrance phenotypes in an epiphytic orchid. *American Journal of Botany*, *84*(10), 1383–1390. https://doi.org/10.2307/2446136

Ayasse, M., Schiestl, F. P., Paulus, H. F., Ibarra, F., & Francke, W. (2003). Pollinator attraction in a sexually deceptive orchid by means of unconventional chemicals. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *270*(1514), 517–522. https://doi.org/10.1098/rspb.2002.2271

Azuma, H., Toyota, M., & Asakawa, Y. (2001). Intraspecific Variation of Floral Scent Chemistry in Magnolia kobus DC. (Magnoliaceae). *Journal of Plant Research*, *114*(4), 411–422. https://doi.org/10.1007/PL00014006

Baldwin, I. T. (2010). Plant volatiles. *Current Biology*, *20*(9), R392–R397. https://doi.org/10.1016/j.cub.2010.02.052

Barkman, T. J. (2001). Character coding of secondary chemical variation for use in phylogenetic analyses. *Biochemical Systematics and Ecology*, *29*(1), 1–20. https://doi.org/10.1016/S0305-1978(00)00031-4

Borges, R. M. (2018). Dark Matters: Challenges of Nocturnal Communication Between Plants and Animals in Delivery of Pollination Services. *The Yale Journal of Biology and Medicine*, *91*(1), 33–42.

Borges, R. M., Somanathan, H., & Kelber, A. (2016). Patterns and Processes in Nocturnal and Crepuscular Pollination Services. *The Quarterly Review of Biology*, *91*(4), 389–418. https://doi.org/10.1086/689481

Bradshaw, H. D., & Schemske, D. W. (2003). Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. *Nature*, *426*(6963), Article 6963. https://doi.org/10.1038/nature02106

Burkle, L. A., Glenny, W. R., & Runyon, J. B. (2020). Intraspecific and interspecific variation in floral volatiles over time. *Plant Ecology*, *221*(7), 529–544. https://doi.org/10.1007/s11258-020-01032-1

Byers, K. J. R. P., Bradshaw, H. D., Jr, & Riffell, J. A. (2014). Three floral volatiles contribute to differential pollinator attraction in monkeyflowers (Mimulus). *Journal of Experimental Biology*, *217*(4), 614–623. https://doi.org/10.1242/jeb.092213

Byers, K. J. R. P., Vela, J. P., Peng, F., Riffell, J. A., & Bradshaw, H. D. (2014). Floral volatile alleles can contribute to pollinator-mediated reproductive isolation in monkeyflowers (*Mimulus*). *The Plant Journal*, *80*(6), 1031–1042. https://doi.org/10.1111/tpj.12702

Chen, Z., Niu, Y., Liu, C.-Q., & Sun, H. (2020). Red flowers differ in shades between pollination systems and across continents. *Annals of Botany*, *126*(5), 837–848. https://doi.org/10.1093/aob/mcaa103

Chess, S. K. R., Raguso, R. A., & Lebuhn, G. (2008). Geographic divergence in floral morphology and scent in Linanthus dichotomus (Polemoniaceae). *American Journal of Botany*, *95*(12), 1652–1659. https://doi.org/10.3732/ajb.0800118

Coimbra, G., Araujo, C., Bergamo, P. J., Freitas, L., & Rodríguez-Gironés, M. A. (2020). Flower Conspicuousness to Bees Across Pollination Systems: A Generalized Test of the Bee-Avoidance Hypothesis. *Frontiers in Plant Science*, *11*. https://www.frontiersin.org/articles/10.3389/fpls.2020.558684

Delle-Vedove, R., Schatz, B., & Dufay, M. (2017). Understanding intraspecific variation of floral scent in light of evolutionary ecology. *Annals of Botany*, *120*(1), 1–20. https://doi.org/10.1093/aob/mcx055

DeMarche, M. L., Miller, T. J., & Kay, K. M. (2015). An ultraviolet floral polymorphism associated with life history drives pollinator discrimination in *Mimulus guttatus*. *American Journal of Botany*, *102*(3), 396–406. https://doi.org/10.3732/ajb.1400415

Djoumbou Feunang, Y., Eisner, R., Knox, C., Chepelev, L., Hastings, J., Owen, G., Fahy, E., Steinbeck, C., Subramanian, S., Bolton, E., Greiner, R., & Wishart, D. S. (2016). ClassyFire: Automated chemical classification with a comprehensive, computable taxonomy. *Journal of Cheminformatics*, *8*(1), 61. https://doi.org/10.1186/s13321-016-0174-y

Dobson, H. (2006). Relationship between Floral Fragrance Composition and Type of Pollinator. In E. Pichersky & N. Dudareva (Eds.), *Biology of Floral Scent* (pp. 147–198). CRC Press. https://doi.org/10.1201/9781420004007.sec4

Dötterl, S., David, A., Boland, W., Silberbauer-Gottsberger, I., & Gottsberger, G. (2012). Evidence for Behavioral Attractiveness of Methoxylated Aromatics in a Dynastid Scarab Beetle-Pollinated Araceae. *Journal of Chemical Ecology*, *38*(12), 1539–1543. https://doi.org/10.1007/s10886-012-0210-y

Edwards, D. P., & Yu, D. W. (2007). The roles of sensory traps in the origin, maintenance, and breakdown of mutualism. *Behavioral Ecology and Sociobiology*, *61*(9), 1321–1327. https://doi.org/10.1007/s00265-007-0369-3

Ellis, A. G., & Johnson, S. D. (2010). Floral Mimicry Enhances Pollen Export: The Evolution of Pollination by Sexual Deceit Outside of the Orchidaceae. *The American Naturalist*, *176*(5), E143–E151. https://doi.org/10.1086/656487

Friberg, M., Schwind, C., Guimaraes Jr., P. R., Raguso, Robert. A., & Thompson, John. N. (2019). Extreme diversification of floral volatiles within and among species of Lithophragma (Saxifragaceae). *PNAS*, *116*(10), 4406–4415. https://doi.org/10.1073

Gögler, J., Stökl, J., Sramkova, A., Twele, R., Francke, W., Cozzolino, S., Cortis, P., Scrugli, A., & Ayasse, M. (2009). Ménage À Trois—Two Endemic Species of Deceptive Orchids and One Pollinator Species. *Evolution*, *63*(9), 2222–2234. https://doi.org/10.1111/j.1558-5646.2009.00712.x

Haber, A. I., Sims, J. W., Mescher, M. C., Moraes, C. M. D., & Carr, D. E. (2019). A key floral scent component (β-trans-bergamotene) drives pollinator preferences independently of pollen rewards in seep monkeyflower. *Functional Ecology*, *33*(2), 218–228. https://doi.org/10.1111/1365-2435.13246

Haber, A. I., Sims, J. W., Mescher, M. C., Moraes, C. M. D., & Carr, D. E. (2021). A sensory bias overrides learned preferences of bumblebees for honest signals in Mimulus guttatus. *The Royal Society Proceedings B*, *288*, 8. https://doi.org/288

Ioalé, P., & Papi, F. (1989). Olfactory bulb size, odor discrimination and magnetic insensitivity in hummingbirds. *Physiology & Behavior*, *45*(5), 995–999. https://doi.org/10.1016/0031-9384(89)90227-8

Joffard, N., Arnal, V., Buatois, B., Schatz, B., & Montgelard, C. (2020). Floral scent evolution in the section Pseudophrys: Pollinator-mediated selection or phylogenetic constraints? *Plant Biology*, *22*(5), 881–889. https://doi.org/10.1111/plb.13104

Johnson, S. (2000). Batesian mimicry in the non-rewarding orchid Disa pulchra, and its consequences for pollinator behaviour. *Biological Journal of the Linnean Society*, *71*(1), 119–132. https://doi.org/10.1006/bijl.1999.0430

Jürgens, A., Witt, T., & Gottsberger, G. (2002). Flower scent composition in night-flowering Silene species (Caryophyllaceae). *Biochemical Systematics and Ecology*, *30*(5), 383–397. https://doi.org/10.1016/S0305-1978(01)00106-5

Kiang, Y. T. (1972). Pollination Study in a Natural Population of Mimulus guttatus. *Evolution*, *26*(2), 308–310. https://doi.org/10.2307/2407041

Kim, A. Y., Rankin, D. T., & Rankin, E. E. W. (2021). What is that smell? Hummingbirds avoid foraging on resources with defensive insect compounds. *Behavioral Ecology and Sociobiology*, *75*(9), 132. https://doi.org/10.1007/s00265-021-03067-4

Knudsen, J. T. (2002). Variation in floral scent composition within and between populations of Geonoma macrostachys (Arecaceae) in the western Amazon. *American Journal of Botany*, *89*(11), 1772–1778. https://doi.org/10.3732/ajb.89.11.1772

Knudsen, J. T., Eriksson, R., Gershenzon, J., & Ståhl, B. (2006). Diversity and distribution of floral scent. *The Botanical Review*, *72*(1), 1. https://doi.org/10.1663/0006-8101(2006)72[1:DADOFS]2.0.CO;2

Knudsen, J. T., & Tollsten, L. (1993). Trends in floral scent chemistry in pollination syndromes: Floral scent composition in moth-pollinated taxa. *Botanical Journal of the Linnean Society*, *113*(3), 263–284. https://doi.org/10.1111/j.1095-8339.1993.tb00340.x

Knudsen, J. T., & Tollsten, L. (1995). Floral scent in bat-pollinated plants: A case of convergent evolution. *Botanical Journal of the Linnean Society*, *119*(1), 45–57. https://doi.org/10.1111/j.1095-8339.1995.tb00728.x

Knudsen, J. T., Tollsten, L., Groth, I., Bergström, G., & Raguso, R. A. (2004). Trends in floral scent chemistry in pollination syndromes: Floral scent composition in hummingbird-pollinated taxa. *Botanical Journal of the Linnean Society*, 9.

Krug, C., Cordeiro, G. D., Schäffler, I., Silva, C. I., Oliveira, R., Schlindwein, C., Dötterl, S., & Alves-dos-Santos, I. (2018). Nocturnal Bee Pollinators Are Attracted to Guarana Flowers by Their Scents. *Frontiers in Plant Science*, *9*. https://www.frontiersin.org/articles/10.3389/fpls.2018.01072

Lunau, K., Papiorek, S., Eltz, T., & Sazima, M. (2011). Avoidance of achromatic colours by bees provides a private niche for hummingbirds. *Journal of Experimental Biology*, *214*(9), 1607–1612. https://doi.org/10.1242/jeb.052688

Luo, X., Yuan, M., Li, B., Li, C., Zhang, Y., & Shi, Q. (2020). Variation of floral volatiles and fragrance reveals the phylogenetic relationship among nine wild tree peony species. *Flavour and Fragrance Journal*, *35*(2), 227–241. https://doi.org/10.1002/ffj.3558

Molet, M., Chittka, L., & Raine, N. E. (2009). How floral odours are learned inside the bumblebee (Bombus terrestris) nest. *Naturwissenschaften*, *96*(2), 213–219. https://doi.org/10.1007/s00114-008-0465-x

Moya, S., & Ackerman, J. D. (1993). Variation in the floral fragrance of Epidendrum ciliare (Orchidaceae). *Nordic Journal of Botany*, *13*(1), 41–47. https://doi.org/10.1111/j.1756-1051.1993.tb00009.x

Okamoto, T., & Su, Z.-H. (2021). Chemical analysis of floral scents in sympatric Ficus species: Highlighting different compositions of floral scents in morphologically and phylogenetically close species. *Plant Systematics and Evolution*, *307*(4), 45. https://doi.org/10.1007/s00606-021-01767-y

Pennel, F. W. (1951). Mimulus. In L. R. Abrams & R. S. Ferris, *An illustrated flora of the Pacific States: Washington, Oregon, and California* (Vol. 3, pp. 688–731). Stanford University Press. https://www.biodiversitylibrary.org/item/28124

Pereira, J., Schlindwein, C., Antonini, Y., Maia, A. C. D., Dötterl, S., Martins, C., Navarro, D. M. do A. F., & Oliveira, R. (2014). Philodendron adamantinum (Araceae) lures its single cyclocephaline scarab pollinator with specific dominant floral scent volatiles. *Biological Journal of the Linnean Society*, *111*(3), 679–691. https://doi.org/10.1111/bij.12232

Pettersson, S., Ervik, F., & Knudsen, J. T. (2003). Floral scent of bat-pollinated species: West Africa vs. the New World. *Biological Journal of the Linnean Society*, *82*(2), 161–168. https://doi.org/10.1111/j.1095-8312.2004.00317.x

Raguso, R. A. (2008). Wake Up and Smell the Roses: The Ecology and Evolution of Floral Scent. *Annual Review of Ecology, Evolution, and Systematics*, *39*(1), 549–569. https://doi.org/10.1146/annurev.ecolsys.38.091206.095601

Raguso, R. A., Levin, R. A., Foose, S. E., Holmberg, M. W., & McDade, L. A. (2003). Fragrance chemistry, nocturnal rhythms and pollination "syndromes" in Nicotiana. *Phytochemistry*, *63*(3), 265–284. https://doi.org/10.1016/S0031-9422(03)00113-4

Raguso, R. A., Schlumpberger, B. O., Kaczorowski, R. L., & Holtsford, T. P. (2006). Phylogenetic fragrance patterns in Nicotiana sections Alatae and Suaveolentes. *Phytochemistry*, *67*(17), 1931–1942. https://doi.org/10.1016/j.phytochem.2006.05.038

Ritland, C., & Ritland, K. (1989). Variation of Sex Allocation Among Eight Taxa of the Mimulus Guttatus Species Complex (scrophulariaceae). *American Journal of Botany*, *76*(12), 1731–1739. https://doi.org/10.1002/j.1537-2197.1989.tb15163.x

Rodríguez-Gironés, M. A., & Santamaría, L. (2004). Why Are So Many Bird Flowers Red? *PLOS Biology*, *2*(10), e350. https://doi.org/10.1371/journal.pbio.0020350

Schiestl, F. P. (2010). The evolution of floral scent and insect chemical communication. *Ecology Letters*, *13*(5), 643–656. https://doi.org/10.1111/j.1461-0248.2010.01451.x

Schiestl, F. P., & Johnson, S. D. (2013). Pollinator-mediated evolution of floral signals. *Trends in Ecology & Evolution*, *28*(5), 307–315. https://doi.org/10.1016/j.tree.2013.01.019

Schlumpberger, B. O., & Raguso, R. A. (2008). Geographic variation in floral scent of Echinopsis ancistrophora (Cactaceae); evidence for constraints on hawkmoth attraction. *Oikos*, *117*(6), 801–814. https://doi.org/10.1111/j.0030-1299.2008.16211.x

Siqueira, E., Oliveira, R., Dötterl, S., Cordeiro, G. D., Alves-dos-Santos, I., Mota, T., & Schlindwein, C. (2018). Pollination of Machaerium opacum (Fabaceae) by nocturnal and diurnal bees. *Arthropod-Plant Interactions*, *12*(5), 633–645. https://doi.org/10.1007/s11829-018-9623-z

Steenhuisen, S.-L., Raguso, R. A., & Johnson, S. D. (2012). Floral scent in bird- and beetle-pollinated Protea species (Proteaceae): Chemistry, emission rates and function. *Phytochemistry*, *84*, 78–87. https://doi.org/10.1016/j.phytochem.2012.08.012

Steiner, K. E., Kaiser, R., & Dötterl, S. (2011). Strong phylogenetic effects on floral scent variation of oil-secreting orchids in South Africa. *American Journal of Botany*, *98*(10), 1663–1679. https://doi.org/10.3732/ajb.1100141

Suinyuy, T. N., & Johnson, S. D. (2021). Evidence for pollination ecotypes in the African cycad Encephalartos ghellinckii (Zamiaceae). *Botanical Journal of the Linnean Society*, *195*(2), 233–248. https://doi.org/10.1093/botlinnean/boaa079

Tollsten, L., & Øvstedal, D. O. (1994). Differentiation in floral scent chemistry among populations of Conopodium majus (Apiaceae). *Nordic Journal of Botany*, *14*(4), 361–368. https://doi.org/10.1111/j.1756-1051.1994.tb00619.x

Windler, D. R., Wofford, B. E., & Bierner, M. W. (1976). Evidence Of Natural Hybridization Between Mimulus Ringens And Mimulus Alatus (Scrophulariaceae). *New England Botanical Club, Inc*, *78*(816), 641–649.

Wu, C. A., Lowry, D. B., Cooley, A. M., Wright, K. M., Lee, Y. W., & Willis, J. H. (2008). Mimulus is an emerging model system for the integration of ecological and genomic studies. *Heredity*, *100*(2), Article 2. https://doi.org/10.1038/sj.hdy.6801018

Supplementary Table

Supplementary Table 2. Floral volatile presence in unpollinated M. lewisii, M. ringens, M. guttatus (SV), M. guttatus (OF), M. guttatus (RH), M. cardinalis, and M. glaucescens. Volatiles were identified by a GC-MS and then narrowed down by classifications. Numbers in parenthes refer to how many times the volatile compounds occurred within the dataset, "x" denotes the volatile compound presence, and a "" displays which singleton volatiles were removed from the PCA analysis.*

		Presence						
	Volatile	*M. lewisii*	*M. ringens*	*M. gutattus (SV)*	*M. gutattus (OF)*	*M. gutattus (RH)*	*M. cardinalis*	*M. glaucesens*
Sesquiterpenes								
	1,1,7,7a-Tetramethyl-1a,2,6,7,7a,7b-hexahydro-1H-cyclopropa[a]naphthalene							
	10s,11s-Himachala-3(12),4-diene	x (1)						
	2-Pentadecanone, 6,10,14-trimethyl	x (3)		x (5)				
	beta-Bisabolene	x (1)						
	Dodecane, 2,6,10-trimethyl							
	Caryophyllene	x (1)						
	Caryophyllene oxide	x (1)						
	Isocalamendiol							
	geranyl-.alpha.-terpinene	x (1)						
Esters								
	2-Hexanol, acetate	x (2)	x(1)	x (1)	x(2)			x(1)
	Acetic acid, 2-ethylhexyl ester	x (4)	x(3)				x(4)	
	Acetic acid, decyl ester	x (1)		x (1)				
	Acetic acid, heptyl ester	x (1)		x (1)				
	Dihydrocitronellyl acetate		x(3)				x (1)	
	Lauryl acetate	x (3)	x (1)	x (4)			x (1)	
	2-Heptenoic acid, isobutyl ester		x(3)				x(4)	
	3-Hydroxypropionic acid cyclic butaneboronate		x(1)		x(1)		x(1)	
	4-Methyl-2-pentyl acetate		x (2)		x(3)	x(1)	x(2)	x(1)
	E-8-Methyl-7-dodecen-1-ol acetate							
	Nonanoic acid, nonyl ester		x (1)				x (4)	
	Octyl thioglycolate					x(1)	x(2)	
	2-Octanol, acetate				x (1)			x (1)
	4-Hydroxy-4-methylhex-5-enoic acid, tert.butyl ester				x(1)	x(1)		
	Cyclopropanetetradecanoic acid, 2-octyl, methyl ester				x(1)			x(1)
Benzenoids								
	1-Butanol, 3-methyl, benzoate			x (2)				
	1H-Indene, 2,3-dihydro-1,1,3-trimethyl-3-phenyl	x (3)		x (3)				
	2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-			x (2)				
	4-Trifluoromethylbenzoic acid, 4-hexadecyl ester	x (1)*		x (1)*				
	Benzenepropenamide, N-(phenylmethyl)	x (2)		x (1)				
	Benzoic acid, 2-ethylhexyl ester	x (5)	x(3)	x (5)	x(1)		x(3)	
	Biphenyl	x (2)		x (1)				
	Homosalate	x (1)		x (3)				
	Phenol, 4-(1,1-dimethylpropyl)							
	p-Toulic acid, 2-ethylhexyl ester	x (2)	x (1)	x (4)			x(3)	
	Mandelic acid, 2TBDMS derivative							
	m-Toulic acid, 2-ethylhexyl ester	x (1)		x (1)*				
Monoterpenes								
	1-Hexanol, 5-methyl-2-(1-methylethyl)	x (2)		x (5)				
	5,9-Undecadien-2-one, 6,10-dimethyl	x (3)						
	Menthyl acetate	x (2)	x (2)				x (1)	
Monoterpenoids								
	2,6-Dimethyl-8-(tetrahydropyran-2-yloxy)-octa-2,6-dien-1-ol	x (2)						
	Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl)-,(1.alpha.,2.alpha.,5.alpha.)							
	Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)	x (3)		x (1)*				
	D-Limonene	x (2)	x (1)	x (1)*			x(3)	
	Limonene-1,2-diol	x (1)	x (1)	x (1)	x(1)		x (4)	x(1)
	Linalool	x (1)		x (4)				
	α-Pinene						x(2)	
	Tetrahydrolavandulyl acetate		x (4)				x (5)	
	trans-Geranylacetone		x (1)				x (1)	
	α-Citral		x (1)				x (1)	