Do color polymorphisms reflect pollinator shifts?: Integrating floral traits as signals to a novel pollinator to understand species divergence

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ABSTRACT

Pollinators are important in the evolution of flowering plants, but questions remain about how early stages of pollinator-mediated speciation arise. Studying intraspecific variation in floral traits can shed light on early stages of floral trait divergence and identify how traits evolve as signals to novel pollinators, either acting separately or in concert. Among two hummingbird-pollinated species of monkeyflowers (*Mimulus* section Erythranthe), *M. cardinalis* and *M. verbenaceus*, yellow floral morphs have arisen from more common, red-flowered morphs and have become well-established in several populations. To identify 1) whether these floral transitions involve additional floral traits beyond color and 2) if they represent incipient pollinator shifts, we characterized yellow-flowered morphs of *M. verbenaceus* and *M. cardinalis* and their more common red-flowered forms across numerous floral traits (reflectance, pigmentation, volatile organic compounds, morphology, and nectar) and assessed pollinator perception and preference of floral signals by a novel bumblebee pollinator (*Bombus terrestris ssp. audax*). The two yellow morphs appeared to follow independent evolutionary paths, resulting in distinct hues of yellow that varied in reflectance, pigmentation, and perception by bees. For floral scent, both yellow forms differed from their red conspecific forms: in one case increasing total emissions of a similar scent profile (*M. verbenaceus*), and in the other, emitting a distinct scent profile at similarly low levels (*M. cardinalis*). In both species, yellow morphs had wider, less restrictive corolla openings and greater herkogamy than red flowers but produced similar nectar rewards. Collectively, these floral trait differences resulted in a clear preference for yellow floral morphs in both species, with naïve bumblebees choosing yellow flowers twice as often as red forms. Despite the increased attractiveness of yellow morphs, bumblebees had difficulty handling flowers to access rewards, suggesting they may be ineffective pollinators to current floral phenotypes. This study reveals that parallel transitions in floral color in two monkeyflower species involve a suite of changes to other floral traits which directly impacts interactions with potential pollinators. This work highlights the value of integrative studies of intraspecific floral phenotypes and behavioral responses of pollinators to understand incipient pollinator shifts and the potential for future pollinator-mediated floral evolution.

INTRODUCTION

Pollinators are important for the evolution and diversification of flowering plants (van der Niet et al., 2014), but questions remain about how pollinator-mediated speciation arises at early stages (Kay and Sargent, 2009), particularly its likelihood of arising among sympatric groups (Waser and Campbell, 2004). Studying intraspecific variation in floral traits can shed light on early stages of floral trait divergence mediated by pollinators (Herrera et al., 2006; Wenzell et al., 2023) and how this might generate reproductive isolation among distinct floral morphs, which could potentially lead to speciation (Johnson, 2006; Kay and Sargent, 2009). Previous work suggests that genes of large effect can alter floral traits and therefore patterns of pollinator visitation in ways that can facilitate reproductive isolation, i.e., via pollinator shifts (Bradshaw and Schemske, 2003). Such studies have often highlighted examples involving floral color transitions, as changes in color can result from a small number of gene changes and result in strong effects on pollinator behavior (Hopkins and Rausher, 2012; Stankowski et al., 2017; Liang et al., 2023). Nonetheless, questions remain regarding if color transitions are accompanied by changes in other floral traits, and if so, how different traits might impact pollinator behavior (Sutherland and Vickery, 1993; Schiestl and Johnson, 2013). By exploring variation in additional floral traits in intraspecific floral color transitions, hypothesized to represent possible pollinator shifts (Vickery, 1995), we can gain insight on the nature of early stages of pollinator-mediated floral divergence, by better understanding the order and function of different floral traits' transitions in an incipient pollinator shift.

Within the genus *Mimulus* (monkeyflowers, Phrymaceae, (Lowry et al., 2019)), section Erythranthe offers ideal case studies to investigate the evolution of floral traits via pollinator-mediated selection (Bradshaw and Schemske, 2003; Byers et al., 2014) and has become an emerging model system in evolutionary ecology, evo devo, and functional genetics of floral traits (Wu et al., 2008; Yuan et al., 2013; Yuan, 2019). Section Erythranthe is believed to include two independent shifts to hummingbird pollination from insect-pollinated ancestors (Beardsley et al., 2003). These shifts to hummingbird pollination correspond to transitions to red floral pigmentation, which results from high concentrations of anthocyanin and carotenoid pigmentation (Yuan et al., 2014; LaFountain et al., 2015), in addition to changes in floral morphology, i.e., to more tubular corollas with more reflexed petal lobes and more abundant, sucrose-rich nectar (Beardsley et al., 2003). More recent work on one of these transitions to bird pollination, *M. cardinalis*, from its bee-pollinated sister species *M. lewisii*, also found that floral scent plays a role in this pollinator shift, as key scent components were linked to increased visitation by bumblebees to *M. lewisii* (Byers et al., 2014), demonstrating that floral scent—a key but often overlooked component of pollinator attraction—can influence pollinator preference and reproductive isolation.

Among the widespread hummingbird-pollinated members of sect. Erythranthe, *M. cardinalis* and *M. verbenaceus*, naturally-occurring yellow-flowered morphs have arisen from more common red forms and have become well-established in several populations at range edges (Vickery, 1995). Previous work on these yellow variants found evidence that they were preferred by bumblebee pollinators compared to red-flowered forms in both species (Vickery and Vickery,

1992), as well as evidence of assortative mating within the uncommon yellow morph due to pollinator fidelity (Vickery, 1995). Yellow floral color in both species is believed to result from homozygosity at a single recessive locus (Vickery, 1995), which in *M. cardinalis* has been demonstrated to involve the locus PELAN, which is responsible for the expression of anthocyanins in petal lobes, and which is not expressed in either of two independent yellow forms (Yuan et al., 2014). Despite previous work on these intriguing color polymorphisms, including both their molecular underpinnings and their influence on pollinator behavior, it remains unknown whether these uncommon floral variants also differ in other floral traits important for pollination, such as floral scent, morphology, and reward quality.

In order to identify whether this floral color transition, which may represent an incipient pollinator shift, also involves additional floral traits, we characterize these yellow-flowered morphs of *M. verbenaceus* and *M. cardinalis* and their more common, red-flowered conspecific forms across numerous floral traits. First, we quantify floral color reflectance and analyze floral pigments responsible for these color differences, before characterizing floral scent, morphology, and nectar properties. We then investigate how these phenotypic differences may impact pollinator behavior in a novel bumblebee (*Bombus terrestris* ssp. *audax*) pollinator, by examining its perception of color and scent signals and testing whether it exhibits a preference for one floral morph over another. In doing so, this study asks the following research questions: 1) Do yellow floral morphs vary from red morphs in multiple floral traits (scent, morphology, nectar) or only in color?; 2) Are differences in floral traits of yellow morphs consistent among species (e.g., is there evidence for convergent in floral transitions)? 3) Is there evidence that yellow morphs represent an adaptation to bee pollination, via increased perception and/or preference for yellow morphs? By investigating whether a suite of floral traits varies among floral morphs within and across species, this study aims to identify whether color alone may drive potential pollinator shifts, or if—and to what degree—other traits may be involved. By expanding our understanding of the direction, order, and integration of the evolution of various floral traits in response to a potential pollinator shift, this work sheds light on how early stages of pollinator-mediated divergence may arise.

METHODS

***Plant material*—**The yellow-flowered color morph of *M. verbenaceus* (known as MVYL) was originally collected from a population located at Vassey's Paradise in the Grand Canyon, Arizona, USA, near the northwestern extent of the species range (Vickery and Vickery, 1992), about 100 miles north of the source population of the red-flowered *M. verbenaceus* (MVBL). MVBL was collected along the West Fork Trail in Sedona, AZ, USA (34.9883°N, 111.7485°W). Yellow-flowered morphs of *M. cardinalis* have been characterized at two populations: one at the northern extent of the species range in the Siskiyou Mountains of Jackson County, Oregon, USA (known as SM), and one from the southern range extent in Cedros Island, Baja California. For this study, we characterize the yellow morph from the northern population (SM), though we note that previous work with pollinators include the southern Cedros Island

population (Vickery and Vickery, 1992; Vickery, 1995). The red-flowered reference line of *M. cardinalis* (CE-10) was collected near South Fork Tuolumne River, Tuolumne County, California, USA (37.817°N, 119.867°W). Plants used in experiments represent reference lines inbred for at least ten generations and were grown from seed at John Innes Centre, Norwich, UK.

Prior to germinating, seeds were sterilized in dilute (1.2%) sodium hypochlorite solution for 5-10 minutes, then rinsed three times with sterilized distilled water. Seeds were then sown on MS growth media in Petri dishes and placed in a controlled environment room (CER) under 14-hour day lengths at 20°C day/19°C night. Following germination, seedlings were transferred to potting soil to establish in CERs before being moved to the glasshouses at John Innes Centre.

Floral color: reflectance and pigments**—**Floral color was quantified by measuring reflectance of fresh corolla tissues (collected immediately prior from plants growing in a glasshouse) using a reflectance spectrometer (FLAME-S-UV-VIS-ES Assembly, 200-850nm, Ocean Insight), with light source from a pulsed xenon lamp (220 Hz, 220-750 nm, Ocean Insight) on four areas of the corolla: the upper petal lobe, the central (bottom) petal lobe, the lower side petal lobe, and the throat of the corolla tube/nectar guide. The reflectance spectrometer collected readings every 5ms and averaged 25 scans per reading (Ocean View spectroscopy software, Ocean Insight). Readings were standardized with an absolute white color reference (Certified Reflectance Standard, Labsphere) and electric dark was used as the black standard. Three readings were taken from each tissue of each flower measured, and three flowers were measured per individual plant, with at least 5-15 plants characterized per line. Reflectance curves at wavelengths from 300-700nm were visualized for all four tissues. All three petal lobe tissues showed similar reflectance curves (Supplemental Materials, Figure S1), so we present and analyze only the central petal lobe tissue hereafter, as this petal lobe is forward facing (not strongly reflexed) to pollinators approaching the front of the flowers.

Floral color (reflectance) of the central petal lobe was compared among lines using a principal component analysis (PCA) using R package psych ((Revelle, 2023) to allow for the varimax rotation, which prioritizes loading each trait (i.e., wavelength) onto only one PC axis), based on the intensity of reflectance at a given wavelength (based on intervals of every ten nanometers from 300-700nm, which captures wavelengths relevant to the vision of most pollinators). First, a PCA was run based on intensity values at all 41 wavelengths; then, only factors with an eigenvalue > 1 were used in the PCA, which resulted in a final PCA of 3 factors. To visualize whether these flowers reflect in the ultraviolet (UV) range, which is important for bee vision, we photographed flowers of 1-3 individuals of all four color morphs under UV light using a UV-sensitive camera (Nikon D610, converted to a Full Spectrum camera by removing the Kolari Vision UV bandpass filter, with a Micro-Nikkor 105mm lens) in a dark room illuminated by a UV black light.

Characterizing floral pigments**—** To characterize the biochemical basis of floral color differences, we analyzed the anthocyanin and carotenoid pigments present in corolla tissues of five individuals (1 flower per individual plant for pigment group) for all four lines. Pigments were extracted from corollas collected on their first day of opening and immediately frozen in

liquid Nitrogen and stored at -80°C until use. Anthocyanins were extracted following methods modified from Butelli et al. (2008), and carotenoids using a modified protocol from Sérino et al. (2009). Total anthocyanins were quantified by measuring absorbance at 525 nm and total carotenoids at 450 nm, using a spectrophotometer (DS-11 FX UV-vis-spectrophotometer, Cambridge Bioscience, UK). When initial absorbance readings were above the saturation point of the spectrophotometer, samples were diluted with the addition of extraction buffer (1:1 of 1% solution of hydrochloric acid in methanol for anthocyanins and 4:1 ethyl acetate for carotenoids). Because the size of corollas varied among floral morphs (Supplemental Materials), and thus likely contributed different amounts of tissue, we standardized the absorbance values relative to corolla size by dividing absorbance by the average mass (g) of a fresh corolla for each line (based on an average mass from one corolla from five individual plants), and used these values for analysis (though we note that overall patterns were similar to those using un-standardized absorbance values). Differences among lines (nested in species) in total anthocyanins and total carotenoids were assessed by performing separate ANOVAs (aov() function in R) for each pigment type, followed by post doc Tukey tests (TukeyHSD() function with 95% CI).

Floral scent— We characterized floral volatile organic compounds (VOCs) for 3 repetitions each of at least 5-10 plants per line, from plants growing in the glasshouse at John Innes Centre. Floral VOCs were collected using dynamic headspace collection. Pairs of freshly cut flowers were placed in 50 mL glass beakers containing approximately 40 mL of sterile H_2O, which were contained in oven roasting bags (Sainbury's, UK), sealed and connected to scent traps with aluminum twist ties. Scent traps were composed of modified glass Pasteur pipettes filled with 100mg Porapak Q (Merck/Sigma), contained within pea-sized amounts of glass wool on either side, and were connected to plastic tubing of Spectrex PAS-500 volatile pumps (Merck/Sigma), which were run at a flow rate of 100 mL/min for 24 hours. Floral VOCs were then eluted from scent traps using 600 uL of extraction solvent (10% acetone in HPLC-grade hexane) into 2mL screw-top amber glass vials, which were stored at -20°C in a dedicated scent freezer before concentration and GCMS analysis.

Compound identification, quantification, and analysis— Sample aliquots of 150ul were first concentrated to 50ul prior to injection of 3ul into an Agilent GC-MS system (7890B GC with 5977A/B MS) using a Gerstel MPS autosampler system with a splitless inlet held at 250C. The oven temperature was held at 50C for 4 minutes, then raised at 5C/minute to 230C, where it was held for 4 minutes. The column was a Phenomenex 7HG-6015-02-GGA (35m x 250um x 0.1um) and the carrier gas was helium at a flow rate of 1.0603 ml/minute. The MS was run in scan mode for ion masses between 50 and 600, with the MS source held at 230C.

Data files were first analyzed using Agilent Unknowns software (v.10.1) to integrate peaks and give the top three tentative NIST library identifications. We first excluded peaks with an area under the curve of $1x10^5$ due to the extremely sensitive nature of the Unknowns integrator, as well as excluding peaks that eluted after 30 minutes which are less likely to be volatile pollinator attractants. Peaks with the same tentative library identification as a peak within 0.1 minutes in the blank sample and with less than a 5-fold higher area in the sample of

interest were excluded, as were peaks where all three library hits contained silica or phthalate contaminants. Following this, remaining uncertain peaks were compared to the nearest blank peaks (within 0.1 minute) by visual comparison of spectra and retained or discarded as above. To further verify the identity of retained peaks, an alkane ladder (Merck, 49452-U) was run at the same time as the samples and the Kovats Retention Index of each peak was calculated and compared with published Kovats indices available via NIST. Compounds with an available authentic reference standard available for purchase were then verified by injection on our GCMS system and integrated areas converted to ng/flower/hour using a concentration curve run with each standard. Compounds without a standard available, but with matching Kovats Retention Indices to the literature, had the areas converted using a similarly structured reference compound. Compounds with no matching standard or Kovats Retention Index are listed in Table 1 as "Unknowns" and quantified using a similarly structured reference compound to the "best guess" of the NIST library. Scent data were visualized in R v.4.2.2 (R Core Team 2022) using the vegan package v.2.6-4 (Oksanen et al., 2022) for NMDS plotting and Welch's t-tests for comparisons between morphs within species for both individual volatile and total volatile emissions.

***Floral morphology and nectar*—**Approximately three flowers each of 5-15 individuals of each line were measured using 150mm digital calipers (Linear Tools, RS Components) for each morphological floral trait. Measured traits included corolla tube length (measured from base of corolla to mouth opening), floral display height (vertical spread of petal lobes as viewed from the front), display width (horizontal spread of petal lobes), tube opening height (vertical space within mouth of corolla tube), and opening width (horizontal space within mouth); these traits play important roles in pollinator attraction/visual signals and access to nectar rewards. Herkogamy, the distance between the anthers and stigma of an individual flower, was assessed by measuring the length of the stamens (from the receptacle to the anthers) and the length of the pistil (from the receptacle to the stigma) of three flowers from five individual plants of each line. Herkogamy was calculated as the difference between the length of the pistil and the length of the anthers (allowing for a possible negative herkogamy value if stamens were longer than the pistil).

For nectar measurement, at least three flowers per plant (5-15 plants per line) were sampled on the first day of opening, to ensure consistent time for nectar production across lines. Flowers were collected from plants growing in the glasshouse and immediately sampled for nectar volume and sucrose concentration. Nectar was collected from the base of corollas into microcapillary tubes (Cat. No. 2930210, 1.55 mm external diameter, Paul Marienfeld GmbH & Co. KG, Germany), and the height of nectar in tubes was measured in mm using digital calipers, which was then converted into uL (based on capillary tube dimensions (1.15mm internal diameter and 100mm length, via manufacturer's specifications) and the geometric formula for volume of a cylinder [height of nectar in mm/100*103.87=h/100*(π*0.5752)]. Concentration of sucrose was then measured using a nectar refractometer (0 to 50% (BRIX) sugar, Bellingham and Stanley UK Ltd.).

Multivariate differences in morphological traits (corolla length, display height and width, and opening height and width) were assessed among lines using a nonmetric multidimensional scaling (NMDS) ordination using Bray distances in R package vegan (Oksanen et al 2022), followed by a MANOVA of these traits by species and line nested within species. Differences among lines for each floral trait were assessed using a linear mixed model (the lmer() function in R package lme4 (Bates et al., 2015)) with line nested within species and individual plant included as a random effect, followed by Type II Wald chi-square tests using the Anova() function in package car (Fox et al., 2020).

***Pollinator perception of color and scent*—**To characterize how floral colors may be perceived by bee pollinators, we plotted the reflectance data of lower central petal lobes on trichromatic models of bee (*Apis mellifera*) visual systems using R package pavo (Maia et al., 2019), which estimates level of contrast of a color signal against a vegetative (green) background.

To test whether the floral scents of different color morphs elicit different electrophysiological responses in bumblebees, we performed electroantennography (EAG). We exposed antennae from nine naïve *Bombus terrestris* individuals (see below; one antenna per individual) to floral scent extractions (concentrated down to half volume) of both yellow-flowered morphs (MVYL and SM) and their conspecific red-flowered reference lines (MVBL and CE10 respectively) and measured the electrophysiological response to these extracts, in addition to a positive control floral scent VOC (0.5 ng/ul phenylacetaldehyde (PAA), a compound known to elicit a response to *Bombus terrestris* (Knauer and Schiestl, 2015)), a negative control (a puff of air through a scent cartridge with no VOCs) and the extraction solution in which floral VOC blends were suspended (10% acetone in hexane).

Using micro-dissection scissors, single antennae were excised from live bees, which were first chilled for 20 minutes at 4C, and the tip (last 1.5 flagellomeres) of the antenna was removed using a micro-dissection scalpel. The antenna was then placed onto an antennal fork using electrode gel and placed into an MP-15 micromanipulator-electrode holder mounted to a magnetic base plate. The antenna was allowed to equilibrate for 10 minutes prior to recording commencing. Recording signals were sent to an IDAC-2 and from there to a computer, where they were recorded using GcEad/2014 software. Stimuli were delivered via a CS-55 stimulus controller. Continuous air flowed over the antenna, with pulses of the same air flow strength controlled via a foot pedal. All EAG equipment was supplied by Ockenfels SYNTECH GmbH, Buchenback, Germany.

The order of scent stimulants was randomized (1 round of 6 exposures each, comprised of 3 pulses 0.5 seconds apart, delivered every thirty seconds), apart from the positive control (PAA) which was delivered in three rounds of 4 exposures and was always the first, fourth, and final stimulant delivered to each antenna sample. This was done because antennae decrease in responsiveness over time, so the positive control signal was used to normalize antennal responses to other stimuli throughout the course of the EAG assay, and the final corrected amplitude value was then scaled to the initial PAA response (following Byers et al., 2020). Additionally, the first

response to a novel stimulus (scent) often results in a disproportionately large peak, so the first response to each stimulus was removed as an outlier for each trial. This resulted in a total of 1,230 recorded responses to stimuli (across nine trials using one antenna from each of nine bees), which were measured and recorded using the software Gc-Ead 2014 v 1.2.5 (2014-05-03; SYNTECH, https://www.ockenfels-syntech.com/).

We tested whether the scaled and corrected amplitude of the antennal response varied among stimuli using a linear mixed model (lmer()) with trial (individual antenna) included as a random effect, along with a random effect term of stimuli nested within trial to account for the repeated exposures of each stimuli to a single individual antenna. Pairwise comparisons among stimuli were assessed using the function lsmeans() with Sattherwaite degrees of freedom and lmer test limit set to 6000 in R package lmerTest (Kuznetsova et al., 2017).

To investigate whether other functional groups of novel pollinators may respond to floral signals in these species, identical electroantennography experiments were also conducted with nine virgin female *Manduca sexta* hawkmoths, which were reared on a standard artificial diet (Frontiers Scientific Services, Newark, DE, USA). Phenylacetlaldehyde was again used as a control, as it is known to be detectable by *Manduca sexta* (Fraser et al., 2003). Details of antennal preparation (although no chilling was used prior to antenna removal), stimulus delivery, and stimulus identity and statistical analysis were identical to the *Bombus terrestris audax* experiments.

***Bumblebee choice experiment*—**To assess whether bumblebees exhibited a preference for visiting one floral color morph over another, we performed a pairwise choice experiment using naïve *Bombus terrestris* workers. Hives of *Bombus terrestris* ssp. *audax* were sourced from Agralan, Ltd. (Wiltshire, UK) and were maintained in a controlled environment room (CER) in the Entomology Department at the John Innes Centre, Norwich, UK, which was kept at 22C during the day and 20C at night with 14 hour day lengths. Hives were kept in a polycarbonate cage and were given access to commercial bee pollen (organic raw bee pollen, Sevenhills Wholefoods, Wakefield, UK) and 25% sucrose (Fischer Scientific) solution. The day (ca. 16:00-17:00) preceding each experimental trial, sucrose solution was removed from the cage, in order to motivate workers to forage during the trial the following day. For use in trials, workers were collected from outside the hive and were kept in individual ventilated 50 mL Falcon tubes, kept in darkness until use.

Open, receptive flowers of yellow- and red-flowered reference lines were cut from plants maintained in the glasshouse and were immediately placed into damp floral foam (Cappstan UK) in 15 mL Falcon tubes. For each within-species pairwise comparison, four flowers of the yellow morph and 4 flowers of the red morph were randomly arranged in one of two 2x2 grid arrays on the walls of a 65 x 80 x 78 mm polycarbonate flight cage. Comparisons were made only within-species, so each trial consisted of a pairwise choice of either the red or yellow morph of *M. verbenaceus* (MVBL versus MVYL) or of *M. cardinalis* (CE10 versus SM) presented in a 50:50 ratio. The background behind the floral arrays was covered in green poster roll paper (Meadow

Green, House of Card & Paper, UK) to simulate a vegetative background. The flight cage was placed inside a CER kept at 20C during experiments.

For each trial, one naïve worker bee was placed in the flight cage with the randomized array of flowers and their foraging behavior was recorded. A choice (floral visit) was recorded any time the bee intentionally contacted the corolla of any flower; if the bee entered the throat of the corolla tube (i.e., to attempt to access the nectar at the base of the corolla tube), this visit was also recorded as a probe. The color morph of each choice was recorded, as well as the handling time (how many seconds the bee handled the flower during each visit/choice). Each trial lasted 30 minutes, after which time, the bee was removed from the flight chamber and euthanized. If a bee failed to forage after 20 minutes in the chamber, it was removed and euthanized. Flowers that came in contact with bees during a trial were replaced before the next trials and the arrangement of floral morphs in each array was re-randomized for each trial. The flight chamber was cleaned with 70% EtOH between trials of different species.

Trials were performed until each species comparison had ten successful trials (i.e., ten individual bees had made a choice). Preference of bees was assessed by comparing the observed total number of flower choices made for each color morph to the number of choices expected under the null hypothesis of no color preference (=50%) using a Chi-square goodness of fit test (chisq.test() function in R), with a separate test run for each species, with choices pooled across trials. We also tested for difference among color morphs of the first choice made by each bee (i.e., the color of the flower each bee contacted first) in the same manner. In addition, because preference could vary among individual worker bees, we also assessed preference in a second way using generalized linear mixed models (GLMM) using the glmer() function in package lme4, which included a random effect for individual bee. These GLMMs used one of the choice metrics (number of visits, number of probes, and total handling time) as the response variable with floral color morph as the predictor, with a Poisson error distribution useful for count data, and separate models were performed for each species comparison.

RESULTS

Floral color: reflectance and pigments**—**Reflectance curves of petal lobes showed different patterns for red and yellow flowers (Figure 1B). Red flowers of both species showed little to no reflectance at wavelengths below ca. 560nm but showed high levels of reflectance from wavelengths 600nm and above. The two yellow-flowered forms showed some overall similarity in reflectance patterns, with sharp increases in reflectance at wavelengths of 500nm and above. However, the two yellow morphs varied somewhat at lower wavelengths: *M. verbenaceus* MVYL shows low levels of reflectance from 400-500nm and *M. cardinalis* SM shows a small reflectance peak below 400nm, in the UV range. To assess whether this small peak may translate to increased visibility under UV wavelengths for SM flowers, we photographed all four floral morphs under UV light (Supplemental Figure S3). These photographs revealed that while the petal lobes of red morphs of both species reflect little to no light in the UV range, the yellow *M. verbenaceus* MVYL and in particular the yellow *M.*

cardinalis SM are comparatively brighter under UV light, suggesting they may be more visible to bees, whose eyes have receptors sensitive to UV wavelengths (Briscoe and Chittka, 2001). While reflectance curves of different petal lobe tissues were largely similar, reflectance patterns of the throat of corollas varied slightly for SM, showing a small peak around 550nm (Supplemental Figure S1) that reflects the red spots of the nectar guide, which is absent in the *M. verbenaceus* yellow morph MVYL (Figure 1A).

We next analyzed reflectance data of petal lobes using a PCA and found that axes PC1 and PC2 resulted in clear separation of both yellow lines, MVYL and SM, both from each other and from the red-flowered lines (*M. verbenaceus* MVBL and *M. cardinalis* CE10), which overlapped with each other substantially (Supplemental Figure S2). Similar to the patterns seen in plots of reflectance curves, this suggests that both *M. verbenaceus* MVBL and *M. cardinalis* CE-10 red-flowered lines are characterized by a similar color of red, while the two yellow-flowered morphs vary in their particular hue of human-vision yellow.

Total anthocyanins (absorbance measured at 525nm) did not vary significantly between species ($F_{1, 16}$= 0.89, p =0.36; Figure 1C) but did vary among lines within species ($F_{2,16}$= 207.8, $p < 0.0001$). Both yellow-flowered lines had significantly lower absorbance than their red counterparts (Supplemental Table S1), indicating very low levels of anthocyanins in tissues of yellow flowers. For total carotenoids, absorbance at 450nm varied significantly among species ($F_{1, 16}$= 15.2, p = 0.001; Figure 1D) and lines within species ($F_{2,16}$ = 14.04, p = 0.0003). Notably, both yellow-flowered morphs had significantly higher absorbance at wavelengths associated with carotenoids than did the red-flowered conspecific lines. This suggests that not only are red flowers different from yellow due to the presence of increased anthocyanins, but that yellow forms may also produce greater amounts of carotenoids compared to their more common red-flowered forms.

Floral scent— Overall, *M. verbenaceus* emitted floral scent at a considerable rate, while *M. cardinalis* produced very little scent overall (Figure 2A). Scent composition was strongly different between morphs within species (Figure 2B, 2C). We identified 39 distinct compounds in the floral scent of *M. verbenaceus* (across both floral morphs: three aromatics, two fatty acid-derived compounds, 26 terpenoids, and eight unknowns), compared to 10 compounds (across both floral morphs: one aromatic, two fatty acid-derived compounds, one lactone, three terpenoids, and three unknowns) in *M. cardinalis* (Table 1). Notably, the composition of floral VOCs of *M. verbenaceus* contained a high diversity of terpenoids (26 of 39 total compounds) and was overall similar across color morphs (Figure 2D), though a subset of compounds specific to only one color morph were identified (in particular, terpenoid alcohols were mostly absent from red *M. verbenaceus*), with more unique compounds found in the yellow form than the red (26 terpenoids in at least ⅔ of yellow samples vs. 10 in red, Table 1). Additionally, the yellow MVYL had significantly higher emission rates compared to the red-flowered MVBL, with approximately 2.32-fold greater total scent emission than the red form (Welch's t-test, t = 8.064, df = 22.214, $p < 0.0001$). In contrast, the yellow and red forms of *M. cardinalis* produced markedly different bouquets of floral VOCs (Figure 2E) which they emitted overall at different,

relatively low levels of total volatiles, with yellow form emissions approximately 1.68-fold higher than red form emissions (Welch's t-test, $t = 3.738$, $df = 20.904$, $p = 0.002$). None of the compounds were found in common between the two species, meaning that visualization of all samples via NMDS was not possible.

***Floral morphology and nectar rewards*—**Floral morphological traits varied significantly among species (MANOVA: approximate $F_{1,97} = 81.628$, $p < 0.0001$) and between lines nested within species (MANOVA: approximate $F_{2,97} = 18.613$, $p < 0.0001$). Our ordination based on an NMDS of these morphological traits reveals that while the two species are largely separated, patterns of morphological variation among red and yellow floral morphs vary by species (Figure 3A). Whereas the red MVBL and the yellow MVYL lines of *M. verbenaceus* overlap considerably and appear similar in floral morphology, the yellow SM line of *M. cardinalis* grouped distinctly from the red-flowered CE-10.

For individual morphological traits, corolla length varied significantly among species ($\chi^2_{1, 117} = 10.2$, $p = 0.001$) and among lines nested within species ($\chi^2_{2, 117} = 53.99$, $p < 0.0001$; Figure 3B). For pairwise comparisons among lines within species, the yellow *M. verbenaceus* line MVYL had significantly longer corolla tubes than the red MVBL, while the yellow *M. cardinalis* SM had significantly shorter corollas than the red CE-10 (statistics for pairwise comparisons located in Supplemental Table S2). Floral display height varied significantly among species ($\chi^2_{1, 99} = 49.42$, $p < 0.0001$) and lines ($\chi^2_{2, 99} = 58.16$, $p < 0.0001$; Supplemental Figure S4). Within species, MVYL had a significantly taller floral display than MVBL, and the yellow *M. cardinalis* SM did not differ from the red-flowered CE-10. Display width varied significantly among species ($\chi^2_{1, 99} = 25.08$, $p < 0.0001$) and lines nested within species ($\chi^2_{2, 99} = 11.29$, $p = 0.004$), however, no pairwise comparisons of lines within species was significantly different. Both opening height and width varied significantly between species (height: $\chi^2_{1, 98} = 33.88$, $p < 0.0001$; width: $\chi^2_{1, 98} = 53.51$, $p < 0.0001$) and among lines (height: $\chi^2_{2, 98} = 65.635$, $p < 0.0001$; width: $\chi^2_{2, 98} = 57.03$, $p < 0.0001$). For both species, red-flowered lines (MVBL and CE10) had significantly narrower openings than their conspecific yellow-flowered lines (MVYL and SM, respectively), with the same patterns observed for both opening height and width. Herkogamy (distance between anthers and stigma) did not vary between species ($\chi^2_{1, 63} = 2.68$, $p = 0.1$) but did vary significantly among lines nested within species ($\chi^2_{2, 63} = 116.34$, $p < 0.0001$), with both yellow flowered lines (MVYL and SM) displaying greater herkogamy than their red-flowered conspecific lines.

For nectar characteristics, volume of nectar produced per flower varied between species ($\chi^2_{1, 93} = 48.94$, $p < 0.0001$) but not between lines within species ($\chi^2_{2, 93} = 2.28$, $p = 0.32$; Figure 3B). Percent of sucrose concentration varied both between species ($\chi^2_{1, 93} = 74.25$, $p < 0.0001$) and among lines ($\chi^2_{2, 93} = 17.38$, $p = 0.0002$), but within-species differences were only significant for *M. verbenaceus*, where the yellow MVYL had significantly higher sucrose concentration than the red-flowered MVBL (Supplemental Materials).

Pollinator perception and preference

***Perception of color and scent*—**We approximated how bees would perceive the colors of floral morphs by plotting central petal lobe reflectance in bee visual space (Figure 4A), which revealed that both red-flowered lines overlapped with each other and showed low contrast from a green background (represented at the center of the hexagonal plot), while the yellow-flowered lines of *M. verbenaceus*(MVYL) and *M. cardinalis* (SM) appear distinct both from each other and from the red flowers, and fall further out on the axes, indicating greater contrast with a green background compared to the red-flowered morphs.

With regard to scent, bumblebees' electrophysiological responses varied significantly among scent stimuli ($\chi^2 = 78.28$, df = 6, $p < 0.0001$), and pairwise comparison tests showed that all four floral scent extracts elicited a significantly greater response than did the air-only negative control (Table S3), suggesting these floral scents can be perceived by potential bumblebee pollinators. However, antennal responses to floral scent extracts were only significantly greater than those in response to extraction solvent alone for two floral stimuli, the two *M. cardinalis* morphs (CE-10 $p = 0.027$; SM $p = 0.0001$), and were marginally significantly greater for the yellow *M. verbenaceus* MVYL ($p = 0.0565$), and was not significantly different for the red *M. verbenaceus* MVBL ($p= 0.63$). Additionally, only one pairwise comparison among the different floral scents was significantly different (SM response was significantly higher than MVBL, $p = 0.019$), suggesting that while *Bombus terrestris* workers could perceive the floral scents of all focal floral morphs, we did not find evidence that conspecific floral morphs were likely to vary in attractiveness to bees based on their floral scent profiles alone. Additionally, responses to PAA were significantly lower than responses to all floral scents ($p < 0.05$; Table S3) and did not significantly vary to responses to air and solvent ($p > 0.05$), possibly due to the concentration used (0.5 ng/uL). EAG experiments with *Manduca sexta* hawkmoths revealed similar patterns to those of *Bombus terrestris*, with moths displaying significantly higher antennal responses to scents of all floral morphs compared to controls, but no significant variation in response among floral scents (Supplemental Figure S5, Table S4).

***Pollinator preference: bumblebee choice experiment*—** When naïve worker bees were offered equal numbers of red and yellow flowers of *M. verbenaceus* in an experimental array, they made a total of 89 floral visits over ten trials (ten individual bees; Supplemental Figure S6). Fifty-nine (66.3%) of these visits were to the yellow morph (MVYL) and 30 visits (33.7%) were to red flowers (MVBL), suggesting a significant preference for the yellow-flowered morph over the red ($\chi^2_1= 9.45$, $p = 0.002$). For *M. cardinalis*, bees made a total of 150 visits over ten trials, visiting the yellow morph (SM) significantly more frequently, with 99 visits (66%) to SM and only 51 visits (34%) to the red CE10 ($x^2_1= 15.36$, $p < 0.0001$). Despite this apparent preference for yellow for total number of choices made by all bees, we did not find evidence that bees were significantly more likely to choose a yellow flower for the first flower they visited (6 bees' first choice was to yellow and 4 to red, a pattern that was repeated out of ten trials for both species; $\chi^2_1= 0.4$, $p = 0.53$).

When analyzed using GLMMs, we found evidence for a significant preference for yellow flowers of *M. verbenaceus* over red, in terms of total number of visits ($\chi^2_1= 9.44$, $p = 0.002$) and

handling time (χ^2_1= 11.74, p = 0.0006), but only marginal evidence for a difference in number of flowers probed (χ^2_1= 3.65, p = 0.056). For *M. cardinalis*, the yellow flowers were significantly preferred to red in terms of number of visits (χ^2_1= 15.125, p = 0.0001), number of flowers probed (χ^2_1= 10.07, p = 0.002), and handling time (χ^2_1= 74.07, p < 0.0001). Despite an apparent preference of bees for yellow flowers in both species, worker bees were often observed to have difficulty handling flowers and attempting to access floral rewards, particularly in the longer-tubed MVYL. Bees were commonly observed crawling around the back of the corolla in an attempt to nectar rob (or check for existing robbing holes), and when bees made persistent efforts to enter corollas to reach nectar at the base of floral tubes, this could result in considerable damage to flowers, including bruising and ripping of corolla tissues (Figure 4D).

DISCUSSION

We characterized two yellow floral morphs, established in otherwise red-flowered hummingbird-pollinated species, *M. verbenaceus* and *M. cardinalis*, across a suite of floral traits hypothesized to be important to pollinator attraction and effectiveness. In doing so, we found evidence that, in addition to the previously proposed loss of anthocyanins in petal lobes resulting in a shift from red to yellow flowers (Yuan et al., 2014), numerous other changes in floral phenotype differentiated these uncommon floral morphs. For floral color, we found that the two yellow morphs displayed distinct hues of yellow, which differed in reflectance at both UV and human-visible wavelengths. Furthermore, not only did yellow morphs contain little to no anthocyanins, but both contained increased carotenoid pigments relative to red forms. With regard to floral scent, both yellow forms differed from their red conspecific forms, though in contrasting ways: in *M. verbenaceus*, the yellow MVYL produced an overall similar scent profile as the red MVBL (with some unique VOCs), but emitted them at greater rates, while in *M. cardinalis*, both color morphs produced little scent overall but with contrasting VOC profiles. Floral morphology further distinguished yellow forms from red: in both species, yellow morphs had wider, less restrictive corolla openings and greater herkogamy, but while the yellow *M. verbenaceus* MVYL displayed longer corolla tubes than red MVBL, the yellow *M. cardinalis* SM had significantly shorter corolla tubes than red CE-10. Despite these differences, nectar rewards were largely similar across color morphs within species.

Subsequently, we found evidence to suggest that bees were more likely to visually perceive the yellow flowers of both species more readily than the typical red flowers, but that floral scent of yellow morphs was not apparently more perceptible to bumblebees, though all floral scents appeared to elicit a response in potential bee pollinators. Collectively, these floral trait differences resulted in a clear preference of naïve bumblebees for yellow morphs in both species: bumblebees chose yellow flowers twice as often as red flowers in both species. Overall, this study reveals that parallel transitions in floral color within two closely related hummingbird-pollinated species may follow independent evolutionary paths, accompanied by changes across a suite of floral traits, which strongly impact responses of novel pollinators. Thus, even intraspecific floral variation can influence pollinator behavior, with the potential to facilitate a

pollinator shift, which has implications for reproductive isolation and pollinator-mediated floral evolution.

Variation in floral traits: color, scent, morphology, and nectar— In this study, we expand on previous work in these intriguing, naturally occurring color polymorphisms in two hummingbird-pollinated species (Vickery and Vickery, 1992; Vickery, 1995), by quantifying differences among conspecific floral morphs in color (reflectance and pigments), in addition to scent, morphology, and nectar. By quantifying color differences within and among species, we found that shades of the two yellow morphs are quantitatively different, and result not only from a near lack of anthocyanins as expected, but also from an increase in quantity of carotenoid pigments present in corolla tissues. This could suggest that while a single gene may result in a shift from red to yellow flowers by "turning off" expression of anthocyanins in petal lobes (Vickery and Vickery, 1992; Yuan et al., 2014), it appears that in these yellow morphs, this may also be accompanied by an increase in the expression of carotenoid pigments. Furthermore, particularly in *M. verbenaceus*, floral scent is characterized by a high diversity of terpenoid compounds, which, similar to carotenoid pigments, are produced at much higher rates in the yellow MVYL form than in the red MVBL. Because precursors of terpenoid VOCs and carotenoid compounds are produced by a shared biochemical pathway (Mostafa et al., 2022), apparent upregulation of both of these classes of compounds in the yellow-flowered MVYL could point to an overall upregulation of the pathway. While preliminary, this represents an intriguing finding and should be explored further in future research.

Interestingly, while both forms of *M. verbenaceus* emitted much greater amounts of floral VOCs than either of the *M. cardinalis* lines, this higher emission did not translate to a detectable increase in electrophysiological response in naïve bumblebees, as measured by antennal responses. Nor did we find statistically significant evidence that the scent of either yellow morph elicited a greater response from bees than their red conspecific form, despite greater scent emission rates and a differing scent profile (in *M. cardinalis*). It is possible that our EAG analysis was impacted by the relatively small sample size of individual workers (N = 9) and may have been complicated by our selected concentration of the positive control stimulus. Phenylacetaldehyde (PAA) has been previously reported to elicit an antennal response in *Bombus terrestris audax*, but in our case, resulted in responses that did not differ significantly from air-only negative control or extraction solvent stimuli, possibly due to an inadvertently low concentration used in trials. Because all four of our floral scent stimuli elicited a significantly greater response than the air negative control, we feel confident that workers of *Bombus terrestris audax* are capable of detecting the floral scent of these floral morphs, though we interpret differences among individual stimuli with caution. To better tease apart the contributions to pollinator attraction of individual signals (e.g., color vs scent), future work should use Y-tubes experiments or artificial flowers with different combinations of color and scent in behavioral assays. Additionally, we note that the scent profile of the *M. cardinalis* reference line CE-10 characterized here differs markedly from that described in Byers et al.

(2014), which is likely a result of more strict filtering of compounds found in blank samples and a more sensitive GCMS system used in this study.

Finally, with regard to floral morphology and nectar, we found that differences between conspecific yellow and red floral morphs were concentrated in traits responsible for mechanical fit of pollinators and reward access but not necessarily reward quality. Floral display varied among morphs only for one dimension (height) for one species (*M. verbenaceus*, Figure 1A), while corolla length and opening width and height (all of which determine the ability of pollinators to access nectar rewards) varied among floral color morphs. In both yellow morphs, corolla openings were taller, wider, and therefore less restrictive, which likely function to allow access to pollinators other than narrow-billed hummingbirds (e.g., bumblebees). Interestingly, for corolla length, yellow forms of *M. verbenaceus* and *M. cardinalis* varied from their red conspecific forms in contrasting ways: yellow *M. verbenaceus* MVYL had longer corolla tubes, while yellow *M. cardinalis* SM had shorter tubes. While this difference aligns with expectations of bumblebee-pollination for SM, the longer corollas of MVYL in *M. verbenaceus* are surprising, and their implication for potential bumblebee pollination are discussed further below. Nectar rewards did not vary among color morphs in *M. cardinalis*, either in terms of quantity of nectar or sucrose concentration, while in *M. verbenaceus*, yellow flowers had higher sucrose content but no difference in nectar volume. These findings are noteworthy as reward quality is an important trait in determining pollinator preference (Kaczorowski et al., 2005; Cronk and Ojeda, 2008). Nonetheless, even if the quantity of nectar remains unaltered, it is possible that bee pollinators had greater access to nectar given shorter corollas (in yellow *M. cardinalis*) and wider corolla openings (in both yellow morphs), which could also function to increase rewards available to bumblebees, thus potentially influencing their behavior.

***Bumblebee preference for yellow flowers*—**We found strong evidence for a preference among bumblebees for yellow flowers in both species based on several metrics: total number of visits, handling time, and number of flowers probed (in *M. cardinalis*). It is noteworthy that yellow color morphs of both species resulted in a consistent 2:1 ratio of visits to yellow versus red flowers (Supplementary Figure S6). Despite this, we did not find significant preference based on first flower choice. While this metric was likely hindered by its smaller sample size (each bee can only make one first choice, thus N=10 for each species), it is somewhat surprising because pollinator preference in first choice of flowers is often used as a metric for pollinator attraction (Huang et al., 2006; Byers and Bradshaw, 2021), which we hypothesized to be the primary mechanism by which bumblebees would choose yellow flowers over red (i.e., via color and possibly scent). This is because previous research and models of bee vision suggest that red flowers provide poor visual contrast to bees (particularly against a green vegetative background (Briscoe and Chittka, 2001; Schiestl and Johnson, 2013), which was supported by our plot of flower color in bee visual space (Figure 4A). Despite this expectation that color played a large role in bee preference due to increased attractiveness, our finding that bees did not significantly choose yellow flowers first suggests that other floral traits may play a role in bee foraging behavior.

With this in mind, this suggests that floral traits involved in pollinator attraction, such as color and scent, may not fully explain bumblebees' observed 2:1 preference for yellow flowers, suggesting that other traits play important roles in determining pollinator foraging decisions. As discussed above, nectar volume did not vary among color morphs, though other morphological differences may have nonetheless allowed *B. terrestris* workers to better access nectar rewards. Indeed, corolla opening width and height were significantly larger in both yellow-flowered morphs compared to their red-flowered conspecifics (Figure 3B), which could have provided bees better access to nectar rewards in both yellow floral morphs (particularly for SM, which had shorter average corollas than its red counterpart). Nonetheless, during the experiment, bees were observed to have difficulty handling flowers across color morphs and species. In particular, bumblebees seemed to enter the corolla in many different orientations, often poorly contacting plant sexual organs (Figure 4D), which would hinder efficiency of pollen transfer, and bees often seemed to struggle to fit far enough inside corolla tubes to access nectar rewards. Many bees were observed to probe flowers and then exit flowers, crawl around the back of the petal lobes and attempt to rob or look for previous nectar robbing holes in order to access nectar in this manner. On several occasions, bees were observed to tear open corolla tubes in their persistence to crawl deeper into corolls, which severely damaged the corollas of these flowers (Figure 4D), which was observed to be most severe in *M. verbenaceus* (possibly due to MVYL's longer corolla tubes).

Bees' observed difficulty handling MVYL flowers could reflect, not only its long corollas, but also its lack of nectar guides (Figure 1Ai), in contrast to the yellow SM of *M. cardinalis*, which lacks anthocyanins only in petal lobes but maintains red spots at the corolla throat as nectar guides (Figure 1Aiii). This difference in nectar guides, present in SM but lacking in MVYL, could contribute to the difference in probing behavior observed between species, wherein bees probed yellow *M. cardinalis* flowers significantly more often than red but this same trend was not significant in *M. verbenaceus*, where bees were observed to spend time crawling over and around flowers before entering corollas (possibly due to a lack of orienting signals from a nectar guide). Taken together, observations of bumblebees interacting with flowers in controlled experimental arrays showed strong preference for yellow morphs but poor fit overall, suggesting that current phenotypes of tested *M. verbenaceus*, and to a lesser extent *M. cardinalis*, may be ill-suited to pollination by workers of *Bombus terrestris*. We acknowledge that *B. terrestris* ssp. *audax* is not native to western North America, where these plant species occur, which could influence our analyses. Nonetheless, our results align with previous work which found significant bumblebee preference for both species in and near their natural distributions, which reported *B. appositus*, and *B. huntii* as common visitors (Vickery and Vickery, 1992; Vickery, 1995). Thus, while we acknowledge that other species of *Bombus* may be better adapted to pollination of these species, the remarkably long and relatively narrow corollas of *M. verbenaceus* likely still pose a hurdle to pollination by bumblebees.

Given this observation, we hypothesize that the yellow morph of *M. verbenaceus* MVYL could potentially reflect an incipient pollinator shift not necessarily to bee pollination but to

hawkmoth pollination, given its pale coloration, long corolla tube, increased floral scent emission, and lack of nectar guides, compared to both its red conspecific form and both morphs of *M. cardinalis*. While speculative, the potential for hawkmoth pollination in this system is consistent with our EAG results for the hawkmoth *Manduca sexta*, which were similar to patterns shown by *Bombus*, indicating hawkmoths are capable of detecting scent profiles of all floral morphs (Supplemental Figure S4). Additionally, *Manduca sexta* preferred yellow flowers over red in controlled experiments with crosses of *M. cardinalis* and *M. lewisii*, lending support for this possibility (Byers and Bradshaw, 2021). Additionally, shifts from hummingbird to moth pollination have occurred repeatedly in other well characterized systems such as *Aquilegia* (Whittall and Hodges, 2007), and may reflect that many hawkmoths are generalist visitors (Wenzell et al., 2023, others), which are often underreported when pollinator observations take place only during daylight hours, as in previous observations of visitors to these floral morphs (Vickery 1992). Alternatively, observed floral trait variation could reflect selection from other biotic or abiotic drivers (Strauss and Whittall, 2006) or drift, which is expected to be stronger at range edges (Nadeau and Urban, 2019) where both yellow morphs occur. While other explanations of floral trait variation warrant further study, our reported 2:1 preference among bumblebees for yellow morphs in both species provide convincing evidence that these floral traits are likely to be subject to selection mediated by pollinators. Future research into the native pollinators and other environmental conditions of yellow floral morphs of both species *in situ*, along with nearby red-flowered populations, is needed to more fully investigate the eco-evolutionary conditions which may favor a pollinator shift within these taxa.

CONCLUSIONS

In this study, we document that two intraspecific floral color polymorphisms are accompanied by variation in a suite of additional floral traits, with implications for the attraction, mechanical fit, and reward access of a novel pollinator group. The yellow floral morphs in both otherwise red-flowered, hummingbird-pollinated species were strongly preferred by naïve bumblebees in experimental arrays, and our results suggest this preference reflects multiple floral traits acting in concert to influence pollinator foraging decisions. We found that the two yellow variants have likely followed independent evolutionary paths and vary in numerous floral traits beyond color in contrasting ways. Furthermore, these contrasting patterns of intraspecific trait variation have implications for the preference and effectiveness of novel pollinators. This suggests that while increased preference by bumblebees for yellow morphs may provide the potential for a shift in pollinators, which could influence reproductive isolation, any potential shift appears to be incomplete, as even preferred phenotypes appear ill-suited to these pollinators. This could suggest that current phenotypes may occupy a fitness valley or may instead be adapted for a different pollinator still. Ultimately, this study reveals that suites of floral traits act in concert to shape the attraction, fit, and preference of pollinators, and while changes in attraction traits may initiate the process of a pollinator shift, additional changes to fit traits must follow to allow reward access and ensure efficient pollen transfer. Thus, this study

sheds light on the order in which floral traits may evolve in an incipient pollinator shift and their impact on the behavior of a novel pollinator, with implications for early stages of potential pollinator-mediated divergence and speciation.

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FIGURES

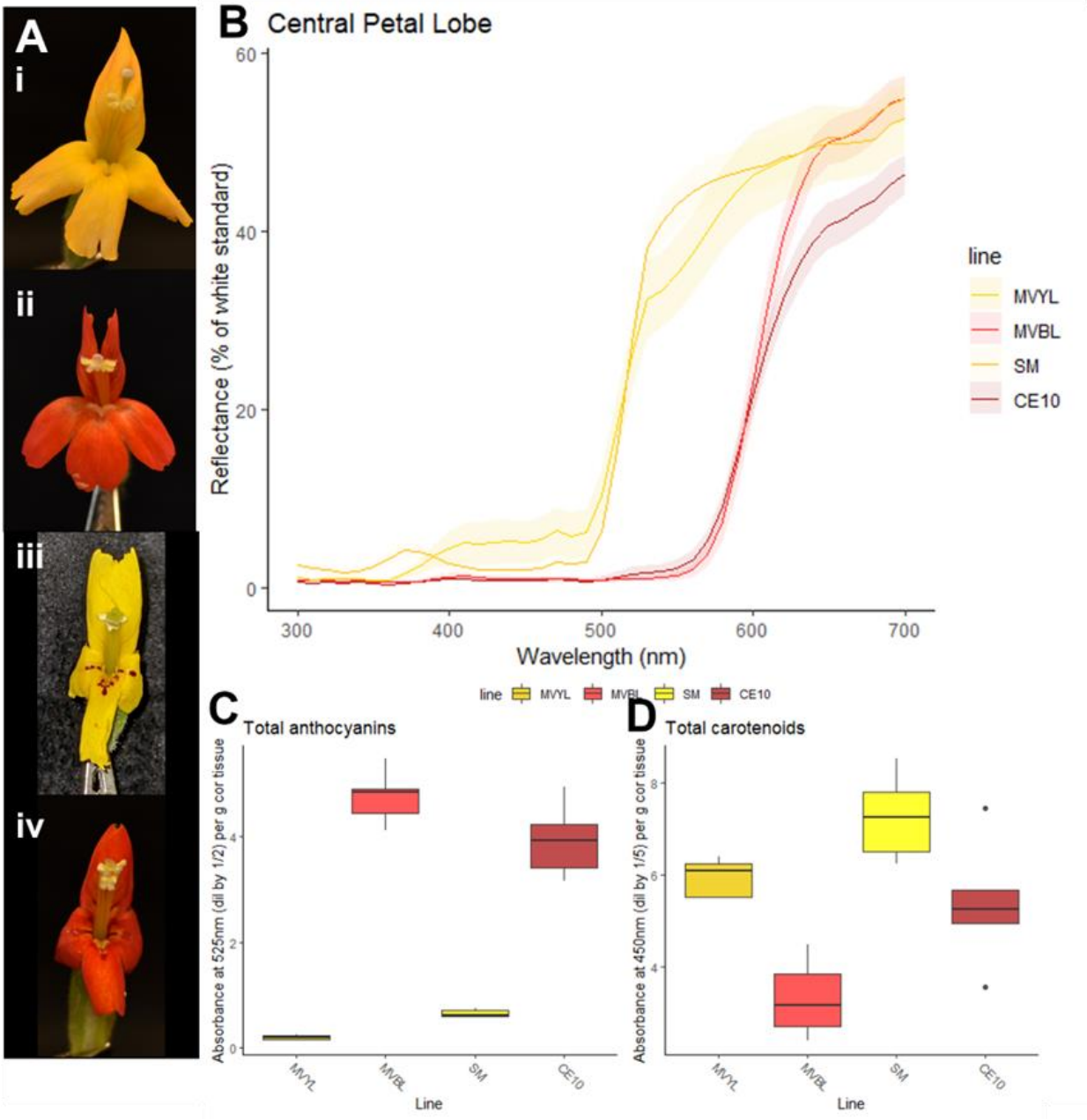

Figure 1. (A) Focal floral morphs: *M. verbenaceus*, yellow form, MvYL (i) and red form, MvBL (ii); *M. cardinalis*, yellow form, SM (iii) and red form, CE-10 (iv). (B) Reflectance curves of lower central petal lobes of each floral morph, shown as reflectance (as a percent of reflectance of a white standard) at wavelengths from 300-700nm. (C) Total anthocyanins extracted from corolla tissues of each floral morph, measured by absorbance at 525nm and (D) total carotenoids, measured as absorbance at 450nm, relative to average mass (g) of corolla per line.

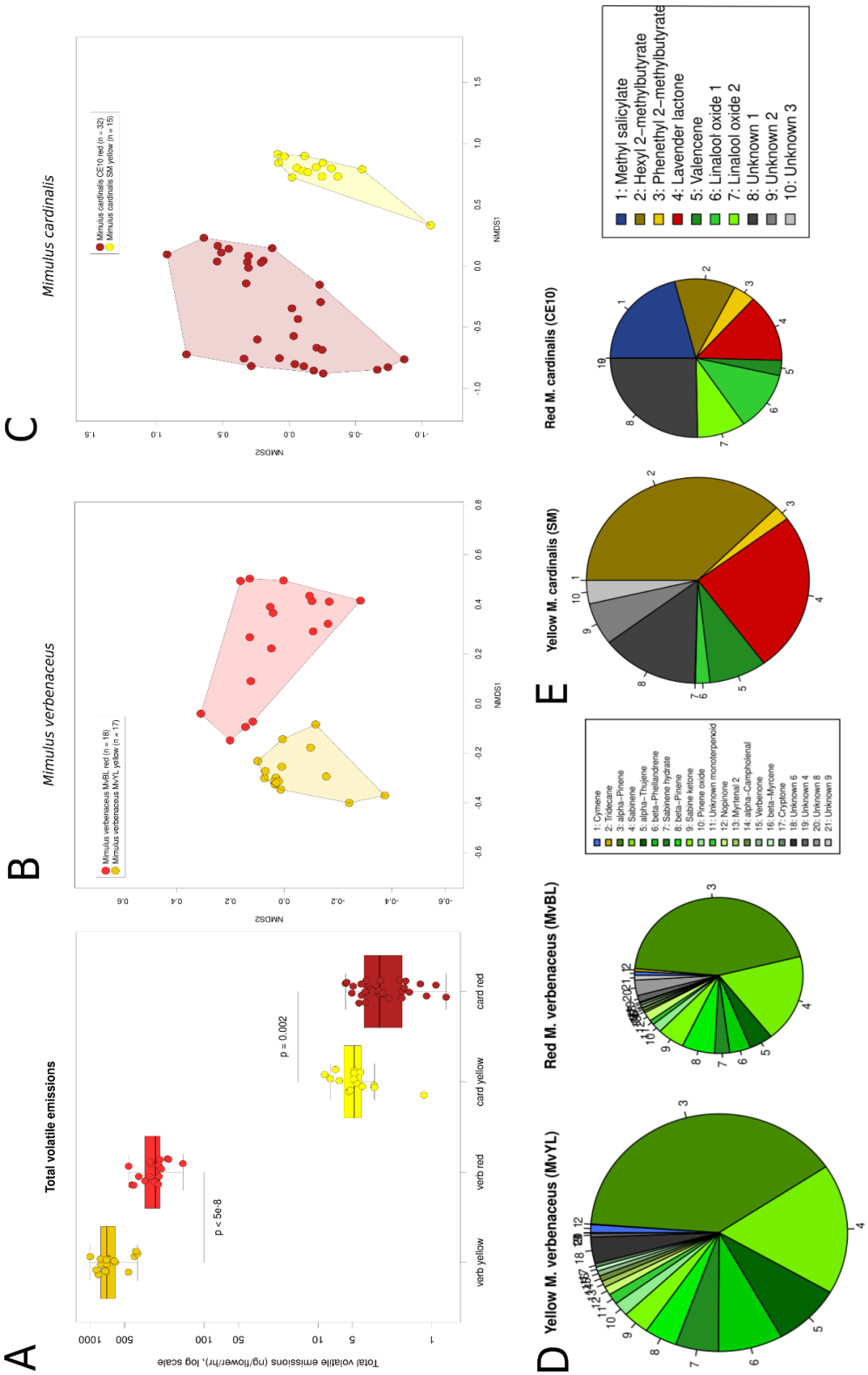

Figure 2. Floral scent emissions vary in total volatiles (both species) and global composition of VOCs (*M. cardinalis*) between red and yellow conspecific floral morphs. A: Comparison of total volatile emissions of the four species-color morph lines, with the y-axis on a log scale. B: NMDS

plot of floral scent samples in *M. verbenaceus*. C: NMDS plot of floral scent samples in *M. cardinalis*. D: Floral scent composition in color morphs of *M. verbenaceus*. E: Floral scent composition in color morphs of *M. cardinalis*. Note that no unified NMDS plot of *M. verbenaceus* and *M. cardinalis* is possible due to a lack of volatile sharing between species.

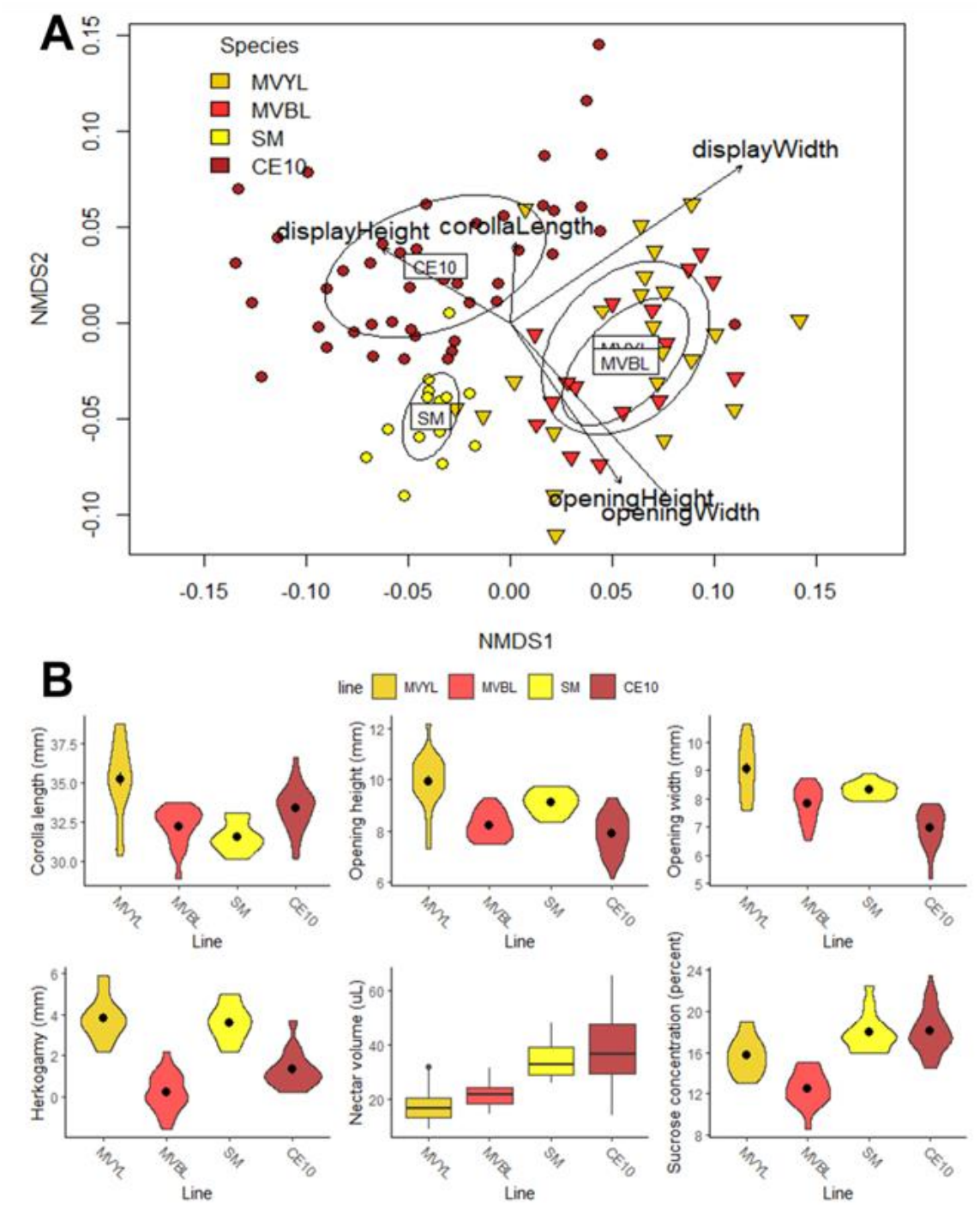

Figure 3. (A) NMDS ordination of floral morphological traits by line. Line centroid is labeled, with circles showing standard deviation. Individual trait loadings with $p < 0.05$ (envfit()) are shown. (B) Violin plots of selected morphology and nectar traits by line, with mean +/- standard error (except for Nectar volume: boxplot with median (central bar), first and third quartiles (box)).

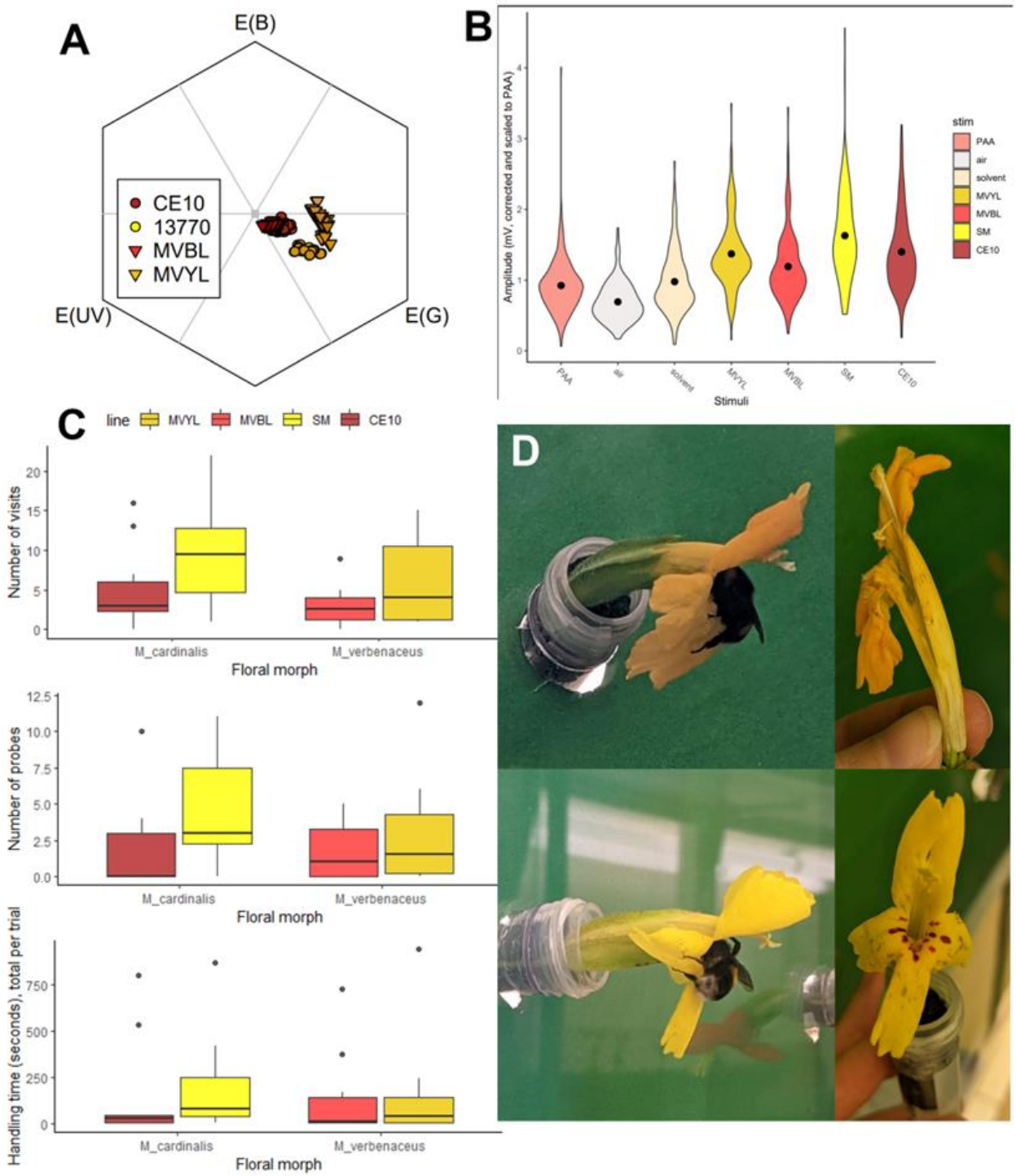

Figure 4. (A) Floral color perception by bees. Central petal lobe reflectance data for each floral morph plotted on a model of bee visual space, where the center point represents low visual contrast to a green vegetative background. (B) EAG responses to scent stimuli, including phenylacetaldehyde (PAA, a floral VOC known to be detectable by *Bombus terrestris*), air (negative control), extraction solvent alone, and floral scent extractions of MVYL, MVBL, SM,

and CE10. (C) Bee preference metrics in pairwise choice experiments between conspecific pairs of floral color morphs (N= 10 trials per species comparison). (D) Bumblebees probing yellow morphs, often with poor contact to anthers and stigma (left), and resulting damage to flowers (right) of *M. verbenaceus* (top) and *M. cardinalis* (bottom). Note corolla tube split open from bee entering corolla in *M. verbenaceus* (top photos).

TABLES

Table 1. Volatile emissions (ng/flower/hour ± standard error of the mean) of each volatile identified from the four lines. Numbers in parentheses after each emission value indicate the number of samples a compound was found in from that line (total sample numbers for each line are in the table header). Under the Compound header, superscript letters: A: compound identity validated using authentic reference standards; B: compound identity validated using published Kovats Retention Indices, our calculated Kovats Retention Indices, and NIST Library spectrum matching; C: compound identity could not be validated and compound is listed as an unknown (and, where its type could be determined, as an unknown of that type). Values in bold text differed significantly between red and yellow forms.

Compound Name	**Type**	**Kovats Retention Index**	**Yellow *M. verbenaceus*MvYL (n = 17)**	**Red *M. verbenaceus*MvBL (n = 18)**	**Yellow *M. cardinalis* SM (n = 15)**	**Red *M. cardinalis* CE10 (n = 32)**
Total emissions (summed across all compounds)	-	-	**675.830 ± 43.538**	**291.169 ± 19.487**	**5.036 ± 0.493**	**2.985 ± 0.240**
Cymene[A] (m/o/p structure could not be determined with standards)	aromatic	1021	**7.096±0.860 (16)**	**2.374±0.361 (17)**	Absent (0)	Absent (0)
Cuminaldehyde[A]	aromatic	1236	**1.784±0.280 (15)**	**0.407±0.125 (10)**	Absent (0)	Absent (0)
Cuminol[A]	aromatic	1290	**1.037±0.211 (13)**	**0.071±0.042 (3)**	Absent (0)	Absent (0)

1-Octen-3-ol[A]	FAD	979	**3.342±0.397 (16)**	**0.299±0.180 (3)**	Absent (0)	Absent (0)
Tridecane[A]	FAD	1300	**0.496±0.144 (10)**	**1.699±0.413 (12)**	Absent (0)	Absent (0)
α-Thujene[B]	terpenoid	923	**55.323±5.055 (17)**	**12.770±2.420 (18)**	Absent (0)	Absent (0)
α-Pinene[A]	terpenoid	929	**254.941±18.331 (17)**	**126.776±8.250 (18)**	Absent (0)	Absent (0)
Camphene[A]	terpenoid	942	**2.132±0.288 (16)**	**0.580±0.044 (18)**	Absent (0)	Absent (0)
Sabinene[A]	terpenoid	971	**114.602±9.888 (17)**	**51.895±4.510 (18)**	Absent (0)	Absent (0)
β-Pinene[A]	terpenoid	972	**26.593±3.216 (17)**	**17.861±0.767 (18)**	Absent (0)	Absent (0)
β-Myrcene[A]	terpenoid	990	**3.630±0.267 (17)**	**1.365±0.176 (15)**	Absent (0)	Absent (0)
α-Terpinene[A]	terpenoid	1013	**1.670±0.278 (13)**	**0.395±0.114 (11)**	Absent (0)	Absent (0)
β-Phellandrene[A]	terpenoid	1025	**52.585±3.663 (17)**	**11.589±1.592 (18)**	Absent (0)	Absent (0)
Eucalyptol[A]	terpenoid	1026	**2.797±0.304 (17)**	**0.750±0.089 (18)**	Absent (0)	Absent (0)
γ-Terpinene[A]	terpenoid	1055	**2.050±0.405 (12)**	**0.125±0.071 (3)**	Absent (0)	Absent (0)
Sabinene hydrate[A]	terpenoid	1063	**34.796±5.026 (15)**	**8.154±1.800 (11)**	Absent (0)	Absent (0)
Terpinolene[A]	terpenoid	1085	**0.587±0.133 (13)**	**0.068±0.032 (4)**	Absent (0)	Absent (0)
Pinene oxide[A]	terpenoid	1092	**12.466±1.461 (17)**	**5.428±0.545 (17)**	Absent (0)	Absent (0)
Unknown monoterpenoid[C]	terpenoid	1095	**9.281±1.130 (17)**	**2.636±0.337 (17)**	Absent (0)	Absent (0)

β-Thujone[A]	terpenoid	1113	**1.146±0.190 (15)**	**0.311±0.067 (14)**	Absent (0)	Absent (0)
α-Campholenal[B]	terpenoid	1123	**5.080±0.513 (17)**	**1.864±0.267 (18)**	Absent (0)	Absent (0)
Nopinone[A]	terpenoid	1133	7.468±0.526 (17)	5.563±1.046 (18)	Absent (0)	Absent (0)
Sabinol[B]	terpenoid	1137	**2.111±0.397 (15)**	**Absent (0)**	Absent (0)	Absent (0)
(*E*)-Verbenol[A]	terpenoid	1142	**2.493±0.221 (17)**	**0.175±0.101 (3)**	Absent (0)	Absent (0)
Sabine ketone[B]	terpenoid	1153	21.484±2.918 (17)	14.803±2.303 (18)	Absent (0)	Absent (0)
Pinocarvone[B]	terpenoid	1157	**2.041±0.178 (17)**	**0.555±0.128 (12)**	Absent (0)	Absent (0)
Myrtenal isomer 1[A]	terpenoid	1181	**1.105±0.178 (14)**	**0.188±0.109 (3)**	Absent (0)	Absent (0)
Cryptone[B]	terpenoid	1182	**3.186±0.381 (17)**	**1.597±0.179 (18)**	Absent (0)	Absent (0)
α-Terpineol[A]	terpenoid	1188	**0.070±0.010 (16)**	**Absent (0)**	Absent (0)	Absent (0)
Myrtenal isomer 2[A]	terpenoid	1191	**5.490±0.436 (17)**	**1.115±0.402 (6)**	Absent (0)	Absent (0)
Verbenone[A]	terpenoid	1205	**4.426±0.784 (12)**	**0.268±0.184 (2)**	Absent (0)	Absent (0)
*M. verbenaceus*Unknown 1[C]	unknown	894	**1.047±0.219 (14)**	**0.139±0.045 (7)**	Absent (0)	Absent (0)
*M. verbenaceus*Unknown 2[C]	unknown	1176	**1.130±0.168 (15)**	**0.438±0.126 (9)**	Absent (0)	Absent (0)
M.	unknown	1273	3.348±0.44	3.922±0.98	Absent (0)	Absent (0)

*verbenaceus*Unknown 3[C]			8 (16)	3 (15)		
*M. verbenaceus*Unknown 4[C]	unknown	1312	1.710±0.228 (15)	1.375±0.173 (18)	Absent (0)	Absent (0)
*M. verbenaceus*Unknown 5[C]	unknown	1392	**23.187±3.656 (16)**	**0.521±0.357 (2)**	Absent (0)	Absent (0)
*M. verbenaceus*Unknown 6[C]	unknown	1396	1.517±0.460 (11)	1.256±0.212 (14)	Absent (0)	Absent (0)
*M. verbenaceus*Unknown 7[C]	unknown	1942	**0.537±0.207 (8)**	**9.071±1.868 (17)**	Absent (0)	Absent (0)
*M. verbenaceus*Unknown 8[C]	unknown	2028	**0.044±0.030 (2)**	**2.766±0.607 (16)**	Absent (0)	Absent (0)
Methyl salicylate[A]	aromatic	1191	Absent (0)	Absent (0)	**Absent (0)**	**0.626±0.047 (29)**
Hexyl 2-methylbutyrate[A]	FAD	1238	Absent (0)	Absent (0)	**1.888±0.132 (15)**	**0.343±0.032 (29)**
Phenethyl 2-methylbutyrate[A]	FAD	1485	Absent (0)	Absent (0)	0.115±0.028 (10)	0.124±0.015 (25)
Lavender lactone[B]	lactone	1041	Absent (0)	Absent (0)	**1.265±0.160 (15)**	**0.412±0.069 (22)**
Linalool oxide isomer 1[A]	terpenoid	1070	Absent (0)	Absent (0)	**0.105±0.023 (10)**	**0.360±0.036 (30)**

Linalool oxide isomer 2[A]	terpenoid	1086	Absent (0)	Absent (0)	**0.005 (1)**	**0.272±0.045 (27)**
Valencene[A]	terpenoid	1482	Absent (0)	Absent (0)	**0.421±0.093 (13)**	**0.093±0.043 (7)**
M. cardinalis unknown 1[C]	unknown	950	Absent (0)	Absent (0)	0.717±0.093 (13)	0.755±0.181 (13)
M. cardinalis unknown 2[C]	unknown	1052	Absent (0)	Absent (0)	**0.334±0.074 (10)**	**Absent (0)**
M. cardinalis unknown 3[C]	unknown	1687	Absent (0)	Absent (0)	**0.185±0.018 (14)**	**Absent (0)**