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Research

Floral scent and pollinator visitation in relation to floral colour morph in the mixed-mating annual herb *Collinsia heterophylla*

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Even though floral scent is of major importance for pollinator attraction, it is less investigated than other floral traits. Previous studies suggest the importance of joint exploration of olfactory and visual floral cues to understand plant–pollinator interactions. We investigated flower scents in *Collinsia heterophylla*, a bee-pollinated, annual herb a with mixed-mating system combining self- and outcross-pollination. In *Collinsia*, floral size and development variation is related to mating system, ranging from large-flowered mixed-mating species to small-flowered self-pollinated species. However, to our knowledge, flower scent has not been described in any species in the genus. We also studied whether flower-emitted volatiles were coupled to presence versus absence of a coloured band on the upper lip within a population in *C. heterophylla*, and if these colour morphs affected pollinator visitation. We performed headspace collections of volatiles in the greenhouse from potted flowering plants, and compared these to controls in the bud stage. Flower-specific volatiles were highly dominated by terpenoid compounds typical of bee-pollinated plants, such as β-myrcene, (*Z*)- and (*E*)-ocimene and sesquiterpenes (*E*)-α-bergamotene and β-sesquiphellandrene. The aliphatic ester methyl hexanoate was also prominent, together with additional esters, whereas methyl cinnamate constituted the only aromatic compound. Floral colour morphs showed no qualitative difference in volatiles, but the coloured morph produced significantly higher quantities for seven of the 26 individual flower compounds. A field experiment performed within a natural population, using behavioural observations and florescent dyes dusted on the flowers, could not detect any differences in pollinator visitation between colour morphs. We conclude that *C. heterophylla* flowers emit volatile compounds commonly associated with attraction of their most important pollinators. It would be highly interesting to explore the function of floral scent for pollinator attraction and relate floral scent to mating system variation across *Collinsia* for a better understanding of pollinator influence on floral evolution.

Keywords: *Collinsia heterophylla*, floral scent, flower colour polymorphism, mixed mating, pollinator attraction

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Introduction

Floral trait variability in the angiosperms is known to be extremely large, and is often believed to be linked to selection exerted by pollinating agents (Van Der Niet et al. 2014, Farré-Armengol et al. 2015, Gervasi and Schiestl 2017) or to other factors (floral antagonists and abiotic factors, Strauss and Whittall 2006, Caruso et al. 2018, Friberg et al 2019). Floral traits of significance for plant–pollinator interactions include both visual and olfactory cues, such as large and colourful flowers or inflorescences with scents and rewards, e.g. nectar and pollen (Harder and Johnsson 2009, Parachnowitsch and Manson 2015). Of the various traits involved in pollinator attraction, it appears that floral scent/fragrance is less studied than other traits (Delle-Vedove et al. 2017). Interestingly, Dellinger (2020) showed that predicting pollinator syndromes (convergent floral adaptations to specific functional pollinator groups, Fenster et al. 2004), is most reliable for reward (nectar), and that scent and floral shape are more reliable than flower colour. It is also possible that colour appears more unreliable because of the difference in how humans (that performed the study) and pollinating insects perceive colour variation (Paine et al. 2019). Similarly, olfactory cues can be perceived very differently by humans and insects, potentially leading to floral scents being overlooked in a species. However, floral scents are known to be of importance for pollinator attraction also in species that appear scentless or weakly scented to the human nose (Ashman et al. 2005, Parachnowitsch et al. 2012).

Insect-pollinated flowers are typically characterized by a rich bouquet of volatile organic compounds, where functional pollinator groups appear to be connected to differences in particular compounds rather than to the composition of the bouquet (Farré-Armengol et al. 2020). For example, bee-pollinated flowers often emit terpenoid floral volatiles (Dobson 2006, Farré-Armengol et al. 2020). Bee species are also known to be attracted to particular floral colours, based on ancient, well-conserved visual systems of ultraviolet-, blue- and green-sensitive photoreceptors (Dyer et al. 2012, Kantsa et al. 2017). Previous studies have shown that floral scents and colours can be used in combination by foraging bees for more reliable detection of rewards (Srinivasan et al. 1998, Burger et al 2010, Kantsa et al. 2017). The same is true for visual floral patterns linked with similar scent patterns (Lawson et al. 2018). There is also evidence for a biochemical link between anthocyanin pigments (blue, red or purple pigments) and aromatic floral scents (Dudareva et al. 2004, Schuurink et al. 2006). However, in *Gymnadenia rhellicani* floral colour morphs, linked to differences in an anthocyanidin synthase gene and pollinator choice, were independent of floral scent differences (Kellenberger et al 2019). These studies suggest the benefit of investigating both floral colour and scent variation for understanding floral evolution.

Variation in mating system (e.g. outcrossing versus self-fertilization, or their combination in mixed mating) can also explain variability in floral traits (Karron et al. 2012, Barrett 2013). For example, outcrossing species are expected to invest more in floral attraction and rewards compared to selfing species (Smith-Huerta and Huerta 2015, Tedder et al. 2015, review by Barrett and Harder 2017). Floral scents have been shown to be lost or severely reduced in selfing compared to outcrossing species or populations (Doubleday et al. 2013, Sas et al. 2016) or only marginally affected (Majetic et al. 2019), potentially suggesting some pollination-dependence or other benefits of producing the floral scents in selfers, e.g. defensive functions. More studies investigating floral scents in mixed mating species would be of interest for gaining knowledge about the link between floral scents and mating system.

Collinsia heterophylla is a mixed-mating herb that belongs to a genus with extensive variation in mating system, from self-pollinating to mixed-mating species (Armbruster et al. 2002, Kalisz et al. 2012). Floral traits, including flower size, delayed selfing, and the separation of male and female reproductive functions in time, are linked with outcrossing rates in *Collinsia* (Elle et al. 2010, Kalisz et al. 2012). Previous studies of floral trait variation in *Collinsia* (ca 23 species), while extensive, have not included descriptions of floral scent, to the best of our knowledge. Moreover, we have never noted any apparent scent from *C. heterophylla* flowers in the field or the greenhouse. Self-compatible *C. heterophylla* is outcrossing to a mean of ca 50% (range 0.29–0.84, Kalisz et al. 2012, Strandh et al. 2017) and pollinated by long-tongued, nectar-feeding bees, which may also collect pollen (primarily species of *Osmia*, *Bombus*, *Anthophora* and *Habropoda*) (Armbruster et al. 2002, Hersh et al. 2015). Floral traits of importance for pollinator attraction in *C. heterophylla* could include inflorescence size, flower size and shape, and flower colour (Lankinen et al. 2017, Strandh et al. 2017). *Collinsia heterophylla* flowers vary in colour, determined by the anthocyanidins delphinidin, cyanidin and peonidin or pelargonidin (Garber 1958). The white and purple five-lobed corolla with one upper and one lower lip vary in the intensity of purple between populations (from very light pink to dark purple), and some populations are polymorphic for presence/absence of dark pigment/band on the upper lip (Weil and Allard 1964, Lankinen 2009) (Fig. 1). Studies of pollinator visitation patterns would be useful for determining the importance of attraction of these floral traits.

In the current study we aimed to investigate the presence of floral-specific volatiles in *C. heterophylla* grown under controlled conditions in the greenhouse, as this knowledge could be highly important for future studies on the association between mating system and floral variation of both visual and olfactory cues. The absence of scent from bee-pollinated flowers such as *Collinsia* spp. would be unexpected, unless investment in pollinator attraction is reduced because of the intermediate selfing rate. A second aim was to explore a possible link between qualitative and quantitative differences in floral volatiles and the flower colour polymorphism on the upper lip. A final aim was to investigate pollinator visitation rate in a natural population, using florescent dyes dusted on the flowers, to study if pollinators preferred one of the two floral colour morphs and also if they preferred plants with a

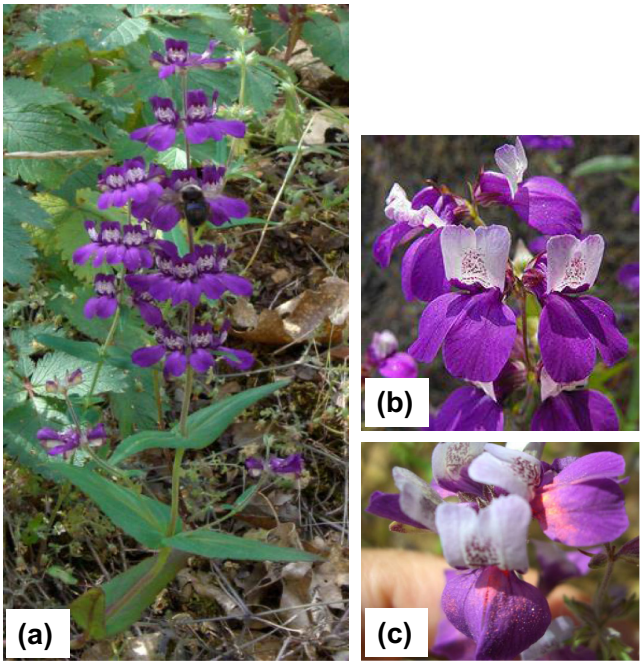

Figure 1. Bee-pollinated *Collinsia heterophylla* in a population in Sierra Nevada, California that is polymorphic for (a) presence versus (b) absence of a dark band on the upper lip petal (the white morph is an example taken from a different population). (c) In this population we studied pollination visitation to the two morphs using florescent dyes dusted on the petals. Photographs: (a, c) J. A. Madjidian, (b) Stickpen (licence in the public domain).

greater number of open flowers (as an indication of a preference for large inflorescence size).

Material and methods

Plant material and study populations

Collinsia heterophylla Buist (Plantaginaceae) is a hermaphroditic winter-annual herb native to California (Newson 1929, Neese 1993). Plants are found at sites below 1000 m a.s.l., typically growing on dry slopes in meadow-like environments shaded by trees. Flowering starts between March and June depending on latitude, elevation and site conditions. Plants commonly grow together with several co-flowering species (e.g. *Allium*, *Artemisia*, *Castilleja*, *Clarkia*, *Delphinium*, *Helianthus*, *Lupinus*, *Mimulus*, *Silene*, Lankinen et al. 2017, in 23 populations a mean of 4.3 ± 2.6 (SD) coflowering species were found, Lankinen and Madjidian unpubl.). These species may compete for the same pollinators.

The zygomorphic flowers are arranged in whorls on long spikes. The lower lip of the flower is folded to form a keel-like structure, which encloses the fertile parts, similar to pea flowers. For successful pollination, visiting insects need to depress the corolla keel, allowing the underside of their thorax to contact the fertile parts of the flower. *Collinsia heterophylla* might be described as generalized because it is pollinated by some 14 species of animals, yet it is more cogently viewed as specialized because the pollinators are all large-bodied, long-tongued bees in a community containing potential pollinators of much larger functional diversity (W. S. Armbruster, unpubl. in Fenster et al. 2004). Reward traits include pollen and nectar. When the flowers first open, the style is short, the stigma is unreceptive and the anthers are undehisced. The anthers dehisce sequentially ca one per day during 3–4 days, whereas the style elongates and becomes receptive to incoming pollen (Armbruster et al. 2002). Pollen can thus be collected by pollinators from about one day after flower opening. Pollinators visit flowers at the different floral developmental stages, i.e. with 1–4 dehisced anthers (Hersh et al. 2015). Nectar production is on average about 10 µl per day (Hersh et al. 2015). No difference in nectar production was found between the floral developmental stages (1–4 dehisced anthers).

Pollinator visitation studies were conducted in a natural population in Mariposa County, California (37°50'20.434''N, 119°56'55.103''W, outcrossing rate = 0.41 (Strandh et al. 2017), proportion flowers of the white morph = 0.12 (Lankinen et al. 2017)). Plants used for volatile collections originated from another natural population in Mariposa County, California (37°30'07.056''N, 120°07'24.959''W, outcrossing rate = 0.45 (Strandh et al. 2017), proportion flowers of the colour morph without pigment on the upper lip = 0.05 (Lankinen et al. 2017)). Plants were collected as seeds from ca 50 open-pollinated plants and grown in the greenhouse for two generations to establish an outcrossed base population. Seeds were cold-stratified and plants were grown under pollinator-proof conditions in a semi-automated greenhouse. Plants grew in unfertilized potting compost (peat with 10% clay and 2% calcium) mixed with sand (4:1) without additional fertilizer in pots of volume 565 000 mm^3). We watered plants as needed and rotated all plants among positions on benches several times during their lifetime.

Collection of volatiles in the greenhouse

For collection of volatiles, potted plants were moved into another greenhouse chamber to avoid contamination from other plants. The above-ground parts of whole plants were enclosed in 5 l polyacetate oven bags tied together at the base of the plant stem and supported by two flower sticks placed into the soil. Polyvinylchloride (PVC) tubing (I.D. 4 mm) connected the bag to a single 12V diaphragm air pump, that sucked air out from the bag at one of the upper edges at 300 ml min^{-1} through an adsorption filter tube, and unfiltered ambient air was let in through a small opening on the other side of the bag. Filter tubes were made from teflon (TFE) tubing (inner diameter 3 mm, length 50 mm) containing the adsorbent polymer Porapak Q (25 mg; 50–80 mesh). The Porapak adsorbent was held in place with rolled balls of polypropylene wool, secured by short pieces of smaller teflon tubing (inner diameter 1.5 mm, length 2 mm) inserted into the main tube on both sides of the adsorbent material.

Simultaneous collections were made with five triplets of *C. heterophylla* plants comprising one individual in the bud stage (before onset of flowering) (total n = 5) and two individuals in the flowering stage, one if which was of the colour morph with pigment on the upper lip (total n = 5) and one of the colour morph without pigment on the upper lip (total n = 5). The colour morphs are hereafter referred to as 'banded' and 'white', respectively. The number of open flowers were also counted on each flowering plant at the time of odour collection. With each collection round of one or more triplets of plants, collections were also made from an empty oven bag arranged in the same configuration as the plants, with flower sticks placed in a flower pot with soil only, in order to distinguish plant-produced odours from those of any other parts of the system or the ambient air in the collection chamber. Collection of volatiles lasted for 5 h during the middle of the day, between 10:00 and 15:00 h.

Immediately after collections were completed, adsorption filter tubes were eluted with 250 µl of hexane added in two aliquots and gently pushed through with a constant nitrogen gas flow after each aliquot. Anethole was added as an internal standard with 200 ng per sample. Eluted hexane samples were stored in 1.5 ml screw neck glass vials with butyl/PTFE seal screw caps at −18°C until analysis. Adsorption filter tubes were rinsed with 3 × 300 µl of hexane followed by 3 × 300 µl of acetone before reuse.

Analysis of volatiles

From each sample, 2 µl were injected in splitless mode with an autoinjector into a gas chromatograph equipped with a DB-Wax capillary column (polyethylene glycol, 30 m × 0.25 mm ID × 0.25 µm film thickness), interfaced to a 5975 mass selective detector. Helium was used as carrier gas (constant flow rate of 35 cm s^{-1}), injector temperature 225°C, and a temperature program of 30°C for 5 min, then 10°C min^{-1} up to 225°C with a 10-min hold. The mass spectrometer was set with a 5-min solvent delay and spectra were taken in electron impact ionization (EI) mode at 70 eV, with a scanning range of 30–350 m/z. Before the series of samples a Kováts index blend with aliphatic n-hydrocarbons C8–C20 was also injected.

The chromatograms from different samples were compared visually with Agilent ChemStation software (ver. E.02.02.1431) using the overlay function, and the presence or absence of compounds in individual collections determined by comparisons of mass spectra and retention times across the full range of samples. Amounts of compounds per sample were determined by comparison with the internal standard. Compounds considered flower volatiles in the present study were selected according to either of two different criteria: 1) They were collected at significantly higher mean amounts per plant from flowering plants (n = 10) than control plants in the bud stage (n = 5) according to a two-sided T-test with unequal variances. 2) Their presence could be detected in a significantly higher proportion of samples from flowering plants than control plants, according to Fisher's exact test. Quantitative differences in floral volatiles between flower morphs were analysed based on amounts per sample (= individual plants) and controlling for the number of flowers per plant. We tested for statistical differences between floral colour morphs in 1) the total amount of floral volatiles with a general linear model and 2) the amounts of individual floral volatiles with a multivariate general linear model, including the factor morph and the number of flowers (standardized by subtracting the mean and dividing with SD) as covariate using SPSS (ver. 26, SPSS 2019). Additionally, we performed a principle component analysis (PCA) to explore potential clustering between the banded and white colour morph across the amounts of floral volatiles using SPSS.

Compounds of interest were identified by matching their mass spectra to reference spectra in the Wiley (10th edition) and NIST (NIST 14) commercial mass spectral databases. In many cases their identity was confirmed by comparing mass spectra, retention times or Kováts indices to those of commercially obtained standards.

Investigating pollinator visitation in a population polymorphic for floral colour

We investigated if pollinators discriminated between the two floral colour morphs (banded and white), and if number of open flowers in a plant was important for pollinator attraction, in a natural population that was polymorphic for colour on the upper lip. First, we followed 13 individual bees or bumblebees and recorded the colour morph of each flower visited (17 ± 5.2 (SD) flowers per insect). Second, we dusted petals with fluorescent dyes to estimate the potential transfer of pollen between flowers (Fig. 1c). We dusted the petals of all open flowers on plants growing ca 1 m apart, using different colours for the two colour morphs. For practical reasons, we dusted more flowers of the banded morph than the white morph, as the proportion of the banded morph was higher (0.88) than the white morph (0.12). In one plot, we dusted 15 plants of the banded morph (five with blue dye and ten with yellow dye) and five plants of the white morph (red dye). In another plot, we dusted ten plants of the banded morph (blue dye) and five plants of the white morph (red dye). The two plots were separated by 10 m and each plot covered a 5 × 10 m area. We used two dye colours for the banded morph in one plot to investigate the occurrence of visits between plots and to detect whether the dye colour affected visitation rate within the same morph. We found no evidence that any of the dye colours influenced pollination rate, as there was no difference in how often the unique colours were found.

After 24 h, we collected 15 plants per plot (ten of the banded morph and five of the white morph), growing in the vicinity of the dusted flowers. We collected more plants with the banded morph because of the higher frequency of this morph. We avoided the closest neighbours because it is possible that we dropped a small amount of dye on closely adjacent plants while marking flowers. We scored all flowers on the collected plants for presence or absence of dye under an ultraviolet lamp. We used a χ^2 test to test for statistical differences

between the flower morphs in proportion visually observed pollinator visits or retrieval of unique dye proportions.

To investigate the relation between number of open flowers in a plant and the proportion of dye in these flowers, as an indication of pollinator visits, we used Kendall partial rank-order correlation coefficient.

Results

Floral volatiles

Chromatograms of volatile collections from *Collinsia heterophylla* plants contained a great number of individual peaks. Comparisons between collections from plants in flowering and bud stages revealed that many compounds were present mainly in collections from flowering plants (Fig. 2). The Supporting information presents all compounds considered in the chemical analysis, based on their presence in more than an occasional sample from flowering plants. Twenty-six compounds were considered floral odours, and used in subsequent analyses, based on significantly higher release rates and/or their presence almost exclusively in samples from flowering plants. Total amount of these 26 floral volatiles collected per hour was approximately 1600 ng per inflorescence or 13 ng per flower. Five compounds were noted as potential candidates released at near significant rates or present in a limited number of samples from flowering plants, but not used in subsequent analyses. Ten compounds were found in similar amounts in both flowering plants and control plants in the bud stage, and assumed to be general plant odours primarily released from green tissues of *C. heterophylla*. Some additional peaks were present only in occasional samples or found in considerable amounts in empty control samples, and are not presented here.

Five compounds were released at rates above 100 ng per plant per hour, including four flower volatiles: the terpenoids β-myrcene, (*Z*)-β-ocimene, (*E*)-β-ocimene, and the aliphatic ester methyl hexanoate, but also the green leaf volatile (*Z*)-3-hexenyl acetate (Fig. 3). Most compounds detected at lower rates were also found primarily in collections from flowering plants (Fig. 4). Among these were predominantly the sesquiterpenes (*E*)-α-bergamotene, and β-sesquiphellandrene, several other mono- and sesquiterpenes, a few aliphatic esters and alcohols, and the benzenoic compound methyl cinnamate. Among aliphatic alcohols and aldehydes, 1-hexanol, 1-octanol and 1-nonanol were significantly or nearly significantly associated with flowering plants, whereas the corresponding aldehydes were found in similar quantities in both the flowering and the bud stage. Apart from these

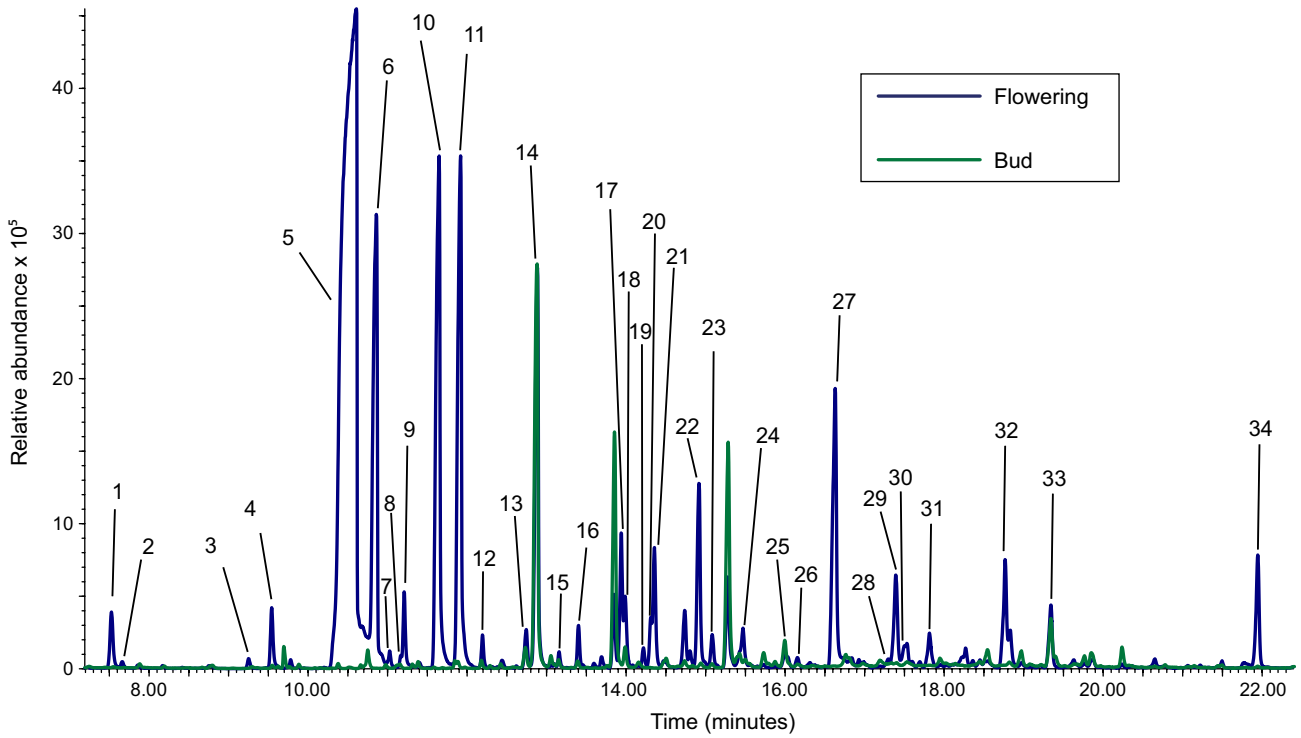

Figure 2. An overlay of chromatograms from collections from *C. heterophylla* plants in the flowering (blue) and bud stage (green), respectively. Numbers refer to individual compounds: 1) α-pinene, 2) α-thujene, 3) β-pinene, 4) sabinene, 5) β-myrcene, 6) methyl hexanoate, 7) limonene, 8) β-phellandrene, 9) 1,8-cineole, 10) (*Z*)-β-ocimene, 11) (*E*)-β-ocimene, 12) hexyl acetate, 13) (*E*)-4,8-dimetyl-1,3,7-nonatriene (DMNT), 14) (*Z*)-3-hexenyl acetate, 15) 6-methyl-5-hepten-2-one (sulcatone), 16) 1-hexanol, 17) methyl octanoate, 18) nonanal, 19) unknown 1, 20) hexyl butanoate, 21) unknown 2, 22) unknown 3 (ester), 23) (*Z*)-3-hexenyl-α-metylbutanoate, 24) unknown 4, 25) linalool, 26) 1-octanol, 27) (*E*)-α-bergamotene, 28) unknown 5 (farnesene-like), 29) (*Z*)-3-hexenyl hexanoate, 30) (*E*)-β-farnesene, 31) unknown 6 (farnesene-like), 32) β-sesquiphellandrene, 33) anethole (internal standard), 34) methyl cinnamate

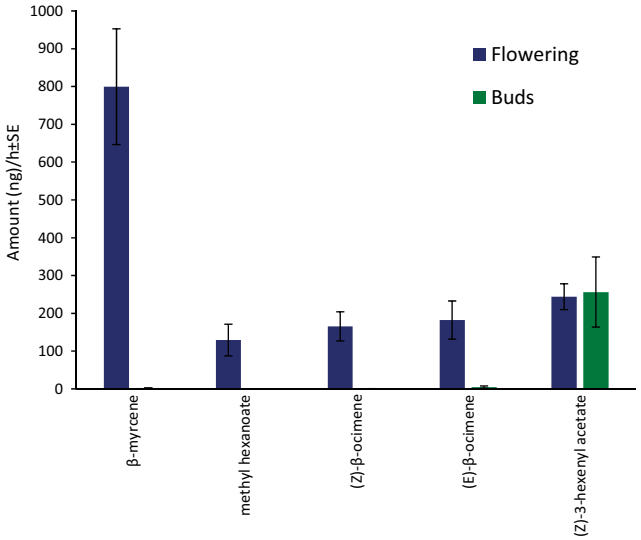

Figure 3. Amounts of five major compounds collected at > 100 ng h^{-1} from *C. heterophylla* plants in the flowering (n = 10) and bud stages (n = 5), respectively. Compounds in the graph are presented in order of retention time and include four major floral volatiles and (*Z*)-3-hexenyl acetate, which is an example of a green leaf volatile collected at similar amounts in both the flowering and bud stages.

aldehydes, a few more compounds like hexyl acetate, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), 6-methyl-5-hepten-2-one (sulcatone), linalool, acetophenone and methyl salicylate appeared to be produced in similar quantities in both groups of plants. Seven compounds, most of which associated primarily with flowering plants, were not completely identified and were listed as unknown. Of these, we could not provide any candidates for numbers 1, 2, 4 and 7. Number 3 was an ester with MS very similar to (*Z*)-3-hexenyl isobutyrate, but the retention index (14.56) does not correspond to the synthetic compound (13.83). Numbers 5 and 6 are sesquiterpenes with MS similar to farnesene isomers, but we did not compare retention indices with synthetic standards. (Fig. 4).

When comparing floral scent of banded colour morphs to those with white colour morphs, there were no qualitative differences between the two groups of plants (Supporting information). Thus, no compounds appeared to be unique to either banded or white morphs. The number of flowers per plant varied greatly between individual plants (range 50–300), but there were no significant differences between the mean numbers of flowers on plants with banded (mean ± SE = 118 ± 27 flowers/plant) and white (141 ± 38 flowers/plant) morphs, respectively (t-test; $p = 0.67$). Quantitative estimates showed that the summed total amount of the 26 flower-related compounds found in each sample was positively correlated with the number of open flowers during the collection (Pearson correlation; $r^2 = 0.66$, ANOVA; $F_{1,7} = 8.19$, $p = 0.024$). There was also a non-significant trend that the banded flower morph produced more volatiles than the white morph ($F_{1,7} = 5.52$, $p = 0.051$) (Fig. 5, 6). A multivariate analysis controlling for number of flowers per plant revealed no significant effect of flower morph on the total amount of the 26 flower specific compounds captured ($F_{1,7} = 2.04$, $p = 0.49$). Among the individual compounds, amounts of seven compounds were significantly higher in the banded flower morph compared to in the white morph (Fig. 5, 6, Supporting information). Four of these compounds, as well as three additional compounds, were also significantly affected by the number of flowers (Supporting informations).

PCA based on the respective amounts of the 26 floral volatiles showed that five principal components (eigenvalue > 1) explained 96% of the variation. No apparent clustering was seen between the banded and white flower morphs for any of the principal components (Supporting information).

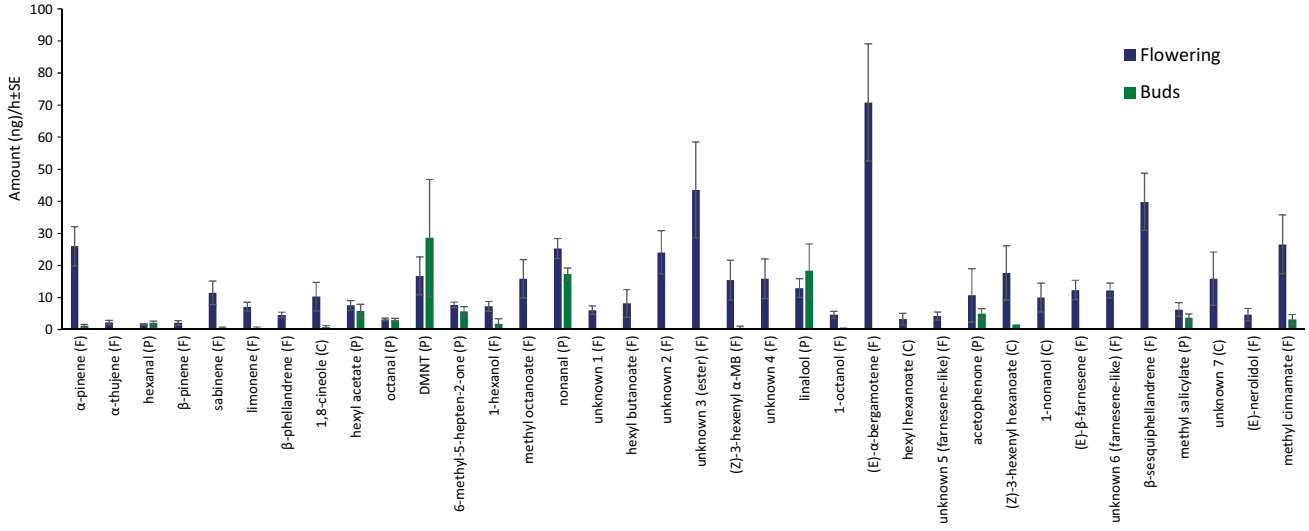

Figure 4. Amounts of 36 different compounds collected at up to 80 ng h^{-1} from flowering *C. heterophylla* plants (n = 10), and the corresponding amounts collected from control plants in the bud stage (n = 5). (F) = compounds designated as flower odours. (C) = compounds suggested as potential candidates. (P) = compounds designated as general plant-derived odours.

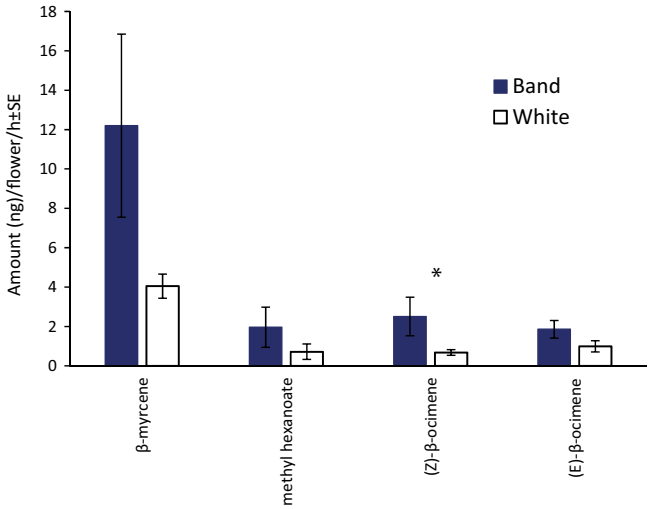

Figure 5. Amounts of the four major flower volatiles collected per flower per hour from *C. heterophylla* plants with and without dark bands on the upper lip, respectively. n = 5 for each group. Among these individual compounds, the amount of (*Z*)-β-ocimene (asterisk) was significantly higher in banded flowers in a multivariate analysis taking into account both the number of flowers and flower morphs.

Pollinator visitation and floral colour variation

Analyses of visitation sequences in a wild population revealed no floral morph-specific difference in visitation rate, either for ten bumblebees (193 flowers visited, $\chi^2 = 0.0005$, df = 1, $p > 0.1$) or for three bees (29 flowers visited, $\chi^2 = 0.653$, df = 1, $p > 0.1$), compared to that expected from morph frequencies in the population (12% with white upper lip).

Our colour dye experiment showed that pollinator activity can be quite high in wild populations. The proportion of individual plants that received at least one visit during about 24 h was as high as 0.87 ± 0.092 (mean ± SD, n = 30). Furthermore, of all investigated flowers (n = 261), the proportion visited was at least 0.21 ± 0.006. Seven flowers had more than one colour, i.e. were visited more than once. No visits could be detected between the plots, as the colour only used in one of the plots was not found in the other plot. There was no difference between colour morphs in the proportion of individuals (0.90 white versus 0.85 banded, $\chi^2 = 0.14$, df = 1, $p > 0.1$) or the proportion of flowers (0.17 white versus 0.22 banded, $\chi^2 = 1.49$, df = 1, $p > 0.1$) that had received dye on the petals. In flowers that had received dye, the origin of the dye (either from the white or dark morph) could be traced because the morphs were connected to unique colours. The unique colours were retrieved equally often on either morph (white: 5 red (= white origin) and 14 blue or yellow (= banded origin); banded: 15 red and 40 blue or yellow, $\chi^2 = 0.007$, df = 1, $p > 0.1$), indicating that pollinators fly between morphs as often as they fly within morphs. We found no evidence that one of the dye colours was found more often than the others (plot 1 (three colours): $\chi^2 = 3.96$, df = 2, $p > 0.1$; plot 2 (two colours): $\chi^2 = 1.93$, df = 1, $p > 0.1$), suggesting that pollination rate was equal between manipulated flowers with different dye colours.

Plants with a higher number of open flowers had a higher number of dyed flowers in an analysis controlling for floral morph (Kendall partial rank-order correlation coefficient;

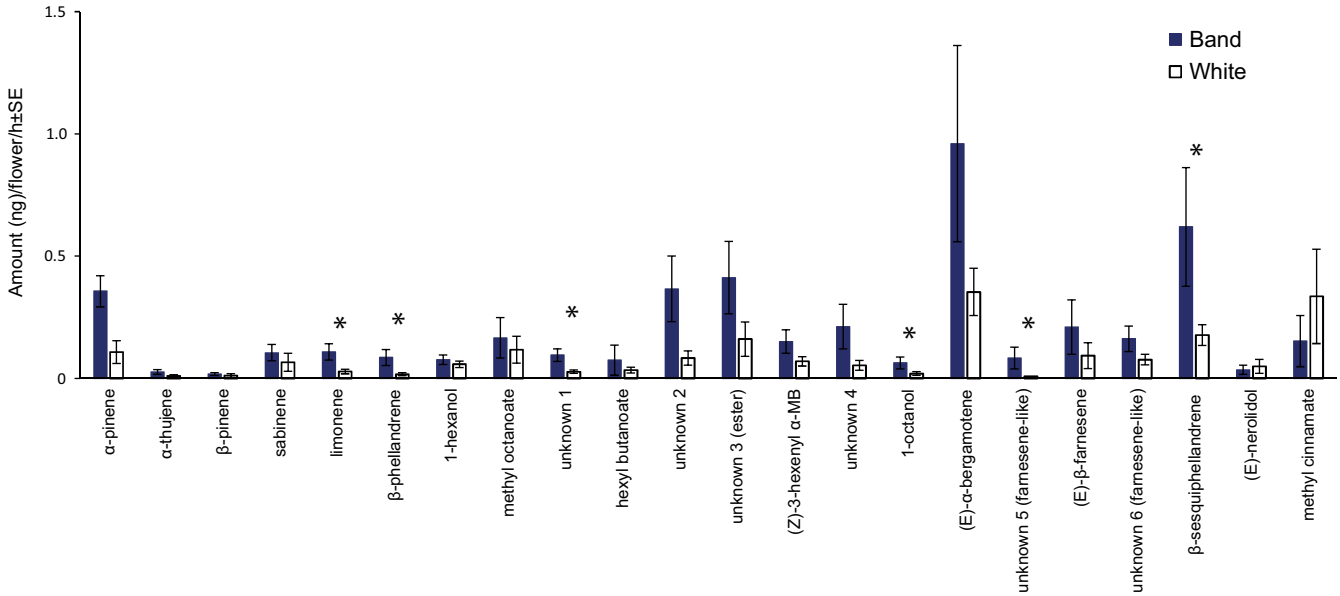

Figure 6. Amounts of minor flower volatiles collected per flower from *C. heterophylla* plants with and without dark bands on the upper lip, respectively (total amounts collected per plant, divided by the number of flowers on each plant). n = 5 for each group. There are non-significant trends for higher amounts of compounds collected from plants with banded flowers, with a few exceptions like nerolidol and methyl cinnamate, but only six compounds marked by asterisks were significantly affected by flower morph in a multivariate analysis taking into account both the number of flowers and flower morphs.

T = 0.323, $p < 0.01$, n = 30). This indicates that plants with larger inflorescences received more pollinator visits independent of floral morph. Floral colour morphs did not differ in number of open flowers (white morph = 7.6 ± 4.38 (mean ± SD), n = 10, dark morph = 9.3 ± 4.40, n = 20; t-test; $p > 0.1$).

Discussion

This study demonstrates that *Collinsia heterophylla* releases a rich bouquet of defined volatile organic compounds from its flowers. Our comparisons of volatile release in the bud and flowering stages, respectively, constitutes strong evidence that the compounds under study are associated specifically with the floral display, although our volatile collections are not limited only to the flowers themselves or individual parts thereof (compare Parachnowitsch et al. 2012, Burdon et al. 2015). In contrast to our own sensory impressions, we found substantial amounts of floral compounds collected from *C. heterophylla* flowers (1600 ng per inflorescence or 13 ng per flower), which is within the lower range of release rates from many plant species reported in a review by Farré-Armengol et al. (2020). Comparable to rates reported for e.g. *Lysimachia punctata* with 516 ng per inflorescence (Dötterl and Schäffler 2007) or several *Nicotiana* species with ca 1–8 ng per flower (Raguso et al. 2003).

The volatile bouquet of *C. heterophylla* was highly dominated by several terpenoid compounds including the monoterpenes β-myrcene, (*Z*)-β-ocimene and (*E*)-β-ocimene, and the sesquiterpenes (*E*)-α-bergamotene and β-sesquiphellandrene. A similar general dominance of mono- and sesquiterpenes, including some individual shared compounds such as ocimenes and bergamotene, is found in *Penstemon digitalis* (Parachnowitsch et al. 2012, Burdon et al. 2015), which belongs to a genus closely related to *Collinsia* within the family Plantaginaceae (Albach et al. 2005). In *P. digitalis*, several floral volatiles, including primarily terpenoids, were under positive phenotypic selection from pollinators. Interestingly, Parachnowitsch et al. (2012) also point out the absence of any distinctive scent from *P. digitalis*. Both *C. heterophylla* and *P. digitalis* are generally bee pollinated, with pollinator communities primarily composed of solitary bees and bumblebees (Armbruster et al. 2002, Dieringer and Cabrera 2002, Hersh et al. 2015). The connection between bee-pollinated flowers and terpenoid floral volatiles is not limited to a local phylogenetic context, but represents a general functional convergence across a great number of unrelated plant families (Dobson 2006, Farré-Armengol et al. 2020).

The biological signalling functions of terpenoid compounds commonly associated with floral volatiles appear to be very complex, with many compounds emitted to a greater or lesser degree from both flowers and green plant tissues in different plant species. β-ocimenes are among the most ubiquitous floral volatiles across different angiosperms, but are also commonly associated with induced compounds released from green plant tissues in response to herbivory (Farré-Armengol et al. 2017). In *C. heterophylla*, β-myrcene was the dominant component among the floral volatiles, collected at 5–4 times higher amounts than either of the β-ocimenes. Myrcene and ocimenes appear to be biosynthetically related, often produced in the same enzymatic reactions, and co-occuring in volatile collections (Farré-Armengol et al. 2017). Depending on the context, both volatiles could apparently constitute an indicator of floral signals or larval host plants for herbivorous pollinators like moths. In the moth *Spodoptera littoralis*, unmated females were attracted for feeding to flower odour from lilac, dominated by (*E*)-β-ocimene, whereas mated females switched their preference to the larval host plant cotton, whose odour bouquet was more characterized by β-myrcene (among many other compounds) (Saveer et al. 2012, Binyameen et al. 2014). Olfactory receptor neurons tuned to ocimenes and/or myrcene have been characterized in several moth species (Røstelien et al. 2000, Binyameen et al. 2014).

The functional diversity of plant terpenoids in general is further emphasized by the presence of several terpenoids found in similar quantities from both flowering and control plants in this study, suggesting that they are primary released from green plant tissues in *C. heterophylla*. This includes linalool and 6-methyl-5-hepten-2-one, which are both among the most common floral volatiles across many angiosperm families, but also frequently produced by green plant tissues (Knudsen et al. 2006). In contrast, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) is most commonly characterized as a signalling cue for herbivory and elicitor associated with induced defences in plants (Meents et al. 2019). However, it is also found as a component of flower odours, including a dominant role in the floral scent of yuccas and some specialized orchids (Svensson et al. 2005, Wiemer et al. 2009). Different saturated and (*Z*)-unsaturated aliphatic esters constituted the second most prevalent group of floral volatiles in *C. heterophylla*, and many of these were more or less exclusively detected in the flowering stage. Among other aliphatic compounds produced at lower amounts and associated with the flowering stage, but not exclusively, were the unsaturated alcohols, whereas corresponding aldehydes showed no indication of being specifically associated with the floral stage. All of these types of compounds are otherwise fairly common among floral odours and associated with insect pollination (Knudsen et al. 2006, Farré-Armengol et al. 2020). The floral-associated volatiles in *C. heterophylla* displayed a conspicuous lack of benzenoic compounds, apart from the single aromatic ester methyl cinnamate, which was among the ten most prevalent floral volatiles. Although individual compounds may vary, the overall composition of compounds and compound classes in the floral odour of *C. heterophylla* is reminiscent of the overall composition of floral volatiles found in different flower parts of *P. digitalis*, including the presence of methyl cinnamate as a conspicuous aromatic component (Burdon et al. 2015).

Previous studies have shown that at least some volatile compounds can be under phenotypic selection by pollinators (Majetic et al. 2009, Schiestl et al. 2011, Parachnowitsch et al.

2012, Joffard et al. 2020). In future studies of floral evolution in *C. heterophylla* it would be of interest to investigate how pollinators respond to the identified floral scents, and if any compounds are under selection. The more commonly investigated floral traits display size or number of flowers, are frequently increased by pollinator-mediated selection (Harder and Johnsson 2009, Caruso et al. 2018). Our field study indicated a pollinator preference for plants with greater numbers of open flowers, i.e. larger inflorescences. It would be interesting to know if inflorescence size is positively linked with amounts of volatile compounds in the field, as appeared to be the case in the greenhouse study, and if these traits are indicative of higher rewards (honest signals, Wright and Schiestl 2009). Alternatively, the higher visitation rate could follow from more flowers being available (e.g. leading to more efficient foraging, Tsujimoto and Ishii 2017). Because the difference in number of open flowers was substantial between the greenhouse and the field, it is also uncertain if differences in floral scent produced per plant individual would be detectable by pollinators in the small plants in natural populations.

Flower colour polymorphism within populations can be selectively neutral or be maintained over time by conflicting selection pressures, mediated by local pollinators, herbivores or pleiotropic relationships with other plant traits (Strauss and Whittall 2006, Rausher 2008). Floral colour morphs are also known to sometimes vary in floral scent (Salzmann and Schiestl 2007). In the present study, we did not find any qualitative differences in floral scent between the two investigated floral morphs. However, our results suggest that the morph with pigment on the upper lip produced higher amounts of some volatiles, based on their significant association with floral morph. However, these results should be interpreted with caution, given the limited number of individual plants used in our study. There could be a biochemical relationship between floral odours and floral pigment, providing an explanation by the fact that the banded morph has more of the dark pigment than the white morph (on both lower and upper lip). However, no link was found between flower morph and amounts of the aromatic compound methyl cinnamate, which would have been expected, given that aromatic compounds have similar biochemical pathways as the anthocyanidins providing the purple colour of the flowers in *C. heterophylla* (Dudareva et al. 2004, Schuurink et al. 2006). In *Hesperis matronalis* colour polymorphism and floral scents were linked in some populations but not in others, suggesting a more complex relation than a shared biochemical association (Majetic et al. 2008).

In our field study, we could not find any evidence that pollinators preferred one of the morphs over the other. This is in line with a previous study on *C. heterophylla* (Weil and Allard 1964), potentially ruling out pollinators as the main reason for the persistence of this polymorphism. Previous greenhouse studies have not been able to find significant fitness differences between floral morphs (Lankinen and Madjidian 2011). Thus, the higher amounts of some of the floral scents in the banded morph is a first indication that the colour polymorphism may not be selectively neutral. However, it is important to note that this is a small study conducted only in one population. Potential benefits of the different floral morphs and associated scents need to be studied further to confirm differences and to understand why this polymorphism exists. Because the dark band on the upper lip is more common in some geographical regions than in others (Lankinen et al. 2017), it could be hypothesised that the banded morph and higher amounts of some floral scent compounds could be more beneficial in some populations than in others, and e.g. linked to presence of co-flowering species (Norton et al. 2015), herbivores (Ramos and Schiestl 2019) or temperature (Harrap et al. 2020).

In conclusion, this study identified floral-specific scents in *C. heterophylla*. Because these compounds are typical for bee-pollinated plant species, it is highly probable that they are involved in attracting pollinators in mixed-mating *C. heterophylla*. Importantly, this knowledge could have implications for future studies on the function of floral scents for pollinator attraction in this species and its relation with other floral traits. *Collinsia heterophylla* populations as well as *Collinsia* species vary substantially in mating system and a set of floral morphological and developmental traits (Kalisz et al. 2012, Strandh et al. 2017), which are proposed to at least partly be linked to pollination predictability (Armbruster et al. 2002). Adding information about floral scent variation across populations and species would be highly interesting for our understanding of how floral scents and mating system covary and evolve, and potentially lead to speciation (Baldwin et al. 2011).

Data availability statement

Data are available from the Dryad Digital Repository: <http://doi.org/10.5061/dryad.866t1g1qb> (Larsson et al. 2021).

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Author contributions

Mattias C. Larsson: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Project administration (equal); Resources (equal); Validation (lead); Visualization (equal); Writing – original draft (equal); Writing – review and editing (equal). **Josefin A. Madjidian**: Conceptualization (supporting); Data curation (supporting); Funding acquisition (supporting); Investigation (supporting); Methodology (supporting); Project administration (supporting); Writing – review and editing (supporting). **Åsa Lankinen**: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Funding acquisition (lead); Investigation

(equal); Methodology (equal); Project administration (equal); Resources (equal); Supervision (lead); Visualization (equal); Writing – original draft (equal); Writing – review and editing (equal).

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