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REVIEWS

Concerted Changes in Floral Colour and Scent, and the Importance of Spatio-Temporal Variation in Floral Volatiles

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Abstract | Many floral phenotypes are naturally dynamic, changing shape, colour or reward availability as flowers age. Here we explore the dynamic nature of floral volatiles, with special reference to the potential for concerted changes in floral colour and scent and their functional integration as multimodal floral signals. We review the evidence for concerted changes in floral colour and scent in the literature and survey the most appropriate content- and efficacy-based hypotheses for multimodal floral function in such cases. Finally, we analyze unpublished data on concerted changes in floral colour and scent collected from the colour-changing flowers of the pan-tropical invasive weeds *Lantana camara, L. montevidensis* and *Heliotropium amplexicaule*. In each species, older, colour-changed flowers emitted reduced amounts of linalool and its oxides, along with common aromatic esters and alcohols, whereas abundant sesquiterpene volatiles emitted by floral calyces and the plants' leaves and stems did not change. Although these compounds were not the most abundant floral volatiles, they are known to show behavioral and electrophysiological–olfactory activity for several species of butterflies. We conclude with a discussion of the conceptual and methodological reasons why dynamic patterns of floral scent emission often are overlooked, and identify opportunities for further progress in these areas.

Keywords: *deception, multimodal communication, mutualism, pigment, pollination, volatiles*

1 Introduction

Floral colour change is a widespread phenomenon usually associated with post-reproductive or unrewarding flowers that are retained on the plant for several days before abscission.[1] Floral retention may arise as a physiological constraint of fertilization or pollen tube growth,[2,3] but the resulting increase in floral display size is often correlated with increased pollinator attraction from a distance.[4–7] Within an inflorescence, colour differences between pre- and post-reproductive flowers facilitate pollinator discrimination against the latter, which may enhance foraging and/or pollination efficiency and reduce geitonogamy or pollen discounting.[8–10] A wide range of pollinators, including butterflies,[11] moths,[12] bees[13,14] and flies[15] discriminate between pre- and post-change flowers. The repeated evolution of floral colour change, which involves diverse pigment pathways, floral organs and phylogenetic lineages (over 77 angiosperm families), suggests that it is an important general feature of plant–pollinator communication (reviewed by Weiss).[16]

Do flowers that change colour show concerted changes in scent emission rates or composition? During the earliest studies of floral colour change,[17,18] the analytical approaches necessary to answer this question were neither sensitive enough nor generally accessible to botanists or floral ecologists.[19] On the other hand, few of the researchers who have studied floral colour change have explicitly considered the possibility that

Geitonogamy: a kind of self-pollination in which pollen is transferred (by wind or pollinators) between different flowers on the same plant, as contrasted with autonomous self-pollination, in which an individual flower is pollinated by its own pollen.

Pollen discounting: in addition to inbreeding depression, another potential fitness cost of self-pollination is the lost opportunity to export pollen to another plant, which is essentially a siring cost (reduced male fitness).

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floral scent might change in concert with colour, nor what it would mean for pollinator behaviour if it did. Studies of pollinator attraction and foraging behaviour have long focused on single sensory modalities.[20] Until recently, major concepts in pollination ecology and floral signal evolution, such as flower constancy, pre-zygotic reproductive isolation and reinforcement, and reproductive character displacement, have remained either anosmic or colour-blind.[21,22] Nevertheless, recent developments in chemical analysis and conceptual advances in multimodal communication, including that between plants and pollinators, have made it possible to address this question from proximate and ultimate standpoints.[23–25] In this paper, we explore the potential for concerted changes in floral signals, both by reviewing the relevant literature and by examining previously unpublished data from *Lantana* and *Heliotropium* species. We then consider the larger subject of temporal variation in the emission and chemical composition of floral volatiles, within which concerted floral changes are embedded, through a discussion of the analytical methods most suitable for tracking rhythmic or ontogenic changes in floral scent, as well as a review of the different physiological, ecological and evolutionary contexts in which such variation should be important.

Multimodal communication: when organisms communicate utilizing multiple sensory channels, such as the exaggerated visual courtship display put on by a male lyrebird while it is singing a repertoire of learnt songs and sounds to prospective mates.

Flower constancy: the tendency for a pollinator to over-visit the same kind of flower during a foraging bout, resulting in more effective pollen transfer among conspecific flowers. Constancy can result from several causes, including innate sensory bias, associative learning and constraints on working memory.

Anthocyanin: highly substituted phenolic compounds that constitute a major class of floral pigments, derived from aromatic amino acids. Anthocyanin molecules are bound to sugars, are sensitive to pH and tend to be stored within the vacuoles of petal cells.

Gas chromatogram: the graphical output of an analytical process by which blends of volatile compounds are separated due to differences in their boiling temperature and relative affinity for the stationary phase within a capillary glass column. The output takes the form of distinctive peaks (see Figs. 2–4), which can be quantified algebraically by comparing their areas to those of authentic standards using external standard (dose-response) curves.

There are proximate and ultimate reasons why concerted changes in floral colour and volatile emissions should be expected. The proximate reasons concern the physiology of floral development and senescence. Changes in pigmentation are known to result directly from fertilization or pollen tube growth or as a consequence of flower age, through a variety of mechanisms including shifts in vacuolar pH[26] and differential expression of anthocyanin biosynthetic genes.[27] Similarly, post-pollination scent reduction has been documented in many plants[28] and generally accompanies floral senescence.[29] Both pigment and volatile changes in aging flowers are regulated at some level by ethylene, through the key biosynthetic enzymes and their structural genes or through flux in their substrates.[30,31] Furthermore, floral pigments and scent compounds often are products of the same biosynthetic pathways,[32] and pleiotropic interactions between pigments and related volatiles have been observed in flowers (anthocyanins and benzenoid volatiles in carnation)[33] and in fruits (carotenoids and norisoprenoid volatiles in tomato and melon).[34] Although not all colour–scent correlations are pleiotropic,[35,36] the experimental ablation of volatile benzyl acetone emissions from *Nicotiana attenuata* flowers through gene silencing of chalcone synthase, a key enzyme in anthocyanin biosynthesis,[37] reinforces the idea that floral colour and scent can be tightly co-regulated.

The ultimate reasons to expect concerted changes in floral colour and scent concern the behavioral responses of animal pollinators to floral advertisements,[38] and their consequences for plant reproductive success. Most flowers present pollinators with combinations of visual and olfactory signals,[39] which elicit feeding behavior in some animals through synergistic interactions.[40] Visual cues provide contextual information when paired with olfactory stimuli during classical conditioning of honeybees,[41] and the presence of olfactory cues allows bumblebees to discriminate between highly similar flower models.[42,43] Bumblebees exhibit higher flower constancy when flowers differ in both colour and scent than when flowers differ in colour alone.[44] However, their foraging rates at complex flowers decrease, due to an increase in time spent moving between flowers. Kulahci *et al.* independently demonstrated that bumblebees learn multimodal floral traits (shape and scent) more quickly and accurately than single modality traits and make more accurate choices when they have trained on multimodal artificial flowers.[45] Such complex floral signals also may be temporally dynamic, in that the various constituent components may change over floral ontogeny, either individually or in concert.

Subtle age-specific changes in floral scent combined with colour change could allow pollinators to more accurately discriminate between rewarding and empty flowers, just as bumble bees utilize scent marks to avoid depleted or recently visited flowers.[46–48] At present, the evidence for concerted changes in floral scent and colour is limited. Lex[49] used honeybee conditioning and panels of human observers to show that colour changes in the nectar guides (banner petals) of *Aesculus hippocastanum* (Sapindaceae) are associated with perceptible changes in odour quality. Casper and LaPine[18] were the first to apply chemical analysis to this question in flowers of *Cryptantha humilis* (Boraginaceae). Solvent extracts of 400 pre- and post-colour change *Cryptantha* flowers were analyzed using GC-FID, resulting in age-specific (but chemically unidentified) gas chromatogram peaks some of which increased in size while others decreased in conjunction with flower age-associated colour

change. Although the logistical ease, temporal resolution and sensitivity of such analyses have improved dramatically (see later) in the 30 years since this study, few chemically-informed analyses have been brought to bear on this subject.

The available data, while meager, suggest at least two patterns. First, while flower colour changes, scent composition remains qualitatively similar, but may increase in magnitude, creating a sequential signal in which an enhanced odour plume attracts a pollinator from a distance, with visually-guided landing or probing at close range. Such a pattern was described for hawkmoth-pollinated *Quisqualis indica* (Combretaceae), in which newly opened white flowers are upright but become red and pendant by the following evening, while continuing to emit a strong, sweet fragrance.[50] Chemical data collected for another hawkmoth-pollinated plant, *Lonicera japonica* (Caprifoliaceae), support this pattern, as newly opened white flowers and day-old yellow flowers have strong, linalool-dominated emissions.[51,52] Second, both colour and odour change in concert as a multi-modal signal making a pollinator less likely to visit the post-change flowers. Such a pattern is consistent with available data on *Tibouchina* (Melastomataceae) species in Brazil. With age and/or pollination, the flowers of *T. pulchra* and *T. sellowiana* turn from white to pink, and buzz-pollinating bees strongly discriminate against the older, pink flowers,[53] which also differ in scent composition, with decreased emissions of heptanal and nonanal and increased emissions of limonene, chavicol and indole.[54] Manipulative field assays indicate that changes in both colour and scent impact bee visitation.[53] Another Brazilian species, *Brunfelsia australis* (Solanaceae), shows similar concerted changes in colour and scent[55] although it is butterfly-pollinated, with nectar as the primary floral reward.[56] In Box 1, we take a closer look at patterns of concerted colour–scent change in three butterfly-pollinated plants.

2 A Case Study with Butterfly-Pollinated Taxa

Our previous observations of colour-changing flowers suggest that concerted changes in floral scent and colour are potentially widespread and are worthy of systematic study. Here we examine two *Lantana* (Verbenaceae) species in which floral colour change and its repercussions for insect foraging behavior have been well studied.[57] *Lantana camara* L. and *L. montevidensis* (Spreng.) Briq. are invasive Neotropical weedy shrubs,[58] whose well-documented attractiveness to diverse flower-feeding Lepidoptera[59–61] has contributed to their widespread cultivation in butterfly gardens and commercial butterfly houses throughout the world.[62,63] Each species produces capita (heads) of tubular, zygomorphic flowers that mature in centrifugal fashion. Flowers of *L. camara* are yellow upon opening, turning deep orange, scarlet or pink with age, depending upon the cultivar[61] (Fig. 1). Flowers of *L. montevidensis* initially are lavender with concentric white and yellow rings at the mouth of the corolla tube; these rings are lost in older, uniformly lavender flowers (Fig. 1). Bagged or greenhouse-grown *Lantana* flowers change colour as they age, but may continue to hold nectar if not visited.[11,57] However, in field settings with regular insect visitation, nectar typically is absent in flowers that have changed colour, as it is not replenished after removal from older flowers[16] Thus, colour change is physiologically independent of nectar removal in *Lantana* flowers, but functions as a *de facto* honest signal for nectar availability under typical levels of flower visitation. In addition, we studied *Heliotropium amplexicaule* Vahl (Boraginaceae), another widespread, invasive weed of Neotropical origin. This heliotrope produces cymes of small, funnel shaped lavender-purple flowers that are remarkably similar to those of *L. montevidensis*, including the loss of a yellow pigmented "target" in the throat of older flowers (Fig. 1). Although the attraction of these plants for butterflies has largely been studied in the context of acquisition of alkaloidal toxins from cut foliage by adult male danaine nymphalid butterflies,[64] the flowers themselves are highly attractive to butterflies and bees at our study sites in Columbia, South Carolina (USA), Berkeley, California (USA) and likely elsewhere, given their similarity to those of *L. montevidensis*. As with *Lantana* species, insect visitors show a marked avoidance of older, colour-changed flowers of *H. amplexicaule*. For example, on 4 and 7 Sept. 2002, we observed 11 bouts by honey bees (*Apis mellifera* L.) averaging 16 flower visits per bout, and only 11 of 175 observed visits were to older purple flowers (whose frequency varied from 15–40% in a given cyme). Similarly, of 6 observed bouts by the fiery skipper butterfly (*Hylephila phyleus* (Drury)), averaging 7.3 flowers visited per bout, only 1 of 44 observed visits was made to an older purple flower. Thus, the colour-changed flowers of *H. amplexicaule*, like those of *Lantana*, presumably are retained in dense inflorescences to contribute to distance attraction,

Zygomorphy: a term used to describe flowers with bilateral symmetry (e.g. typical of the Bignoniaceae), which tend to be presented laterally from an upright stem, and thus accommodate foraging pollinators with a landing platform and vestibular space.

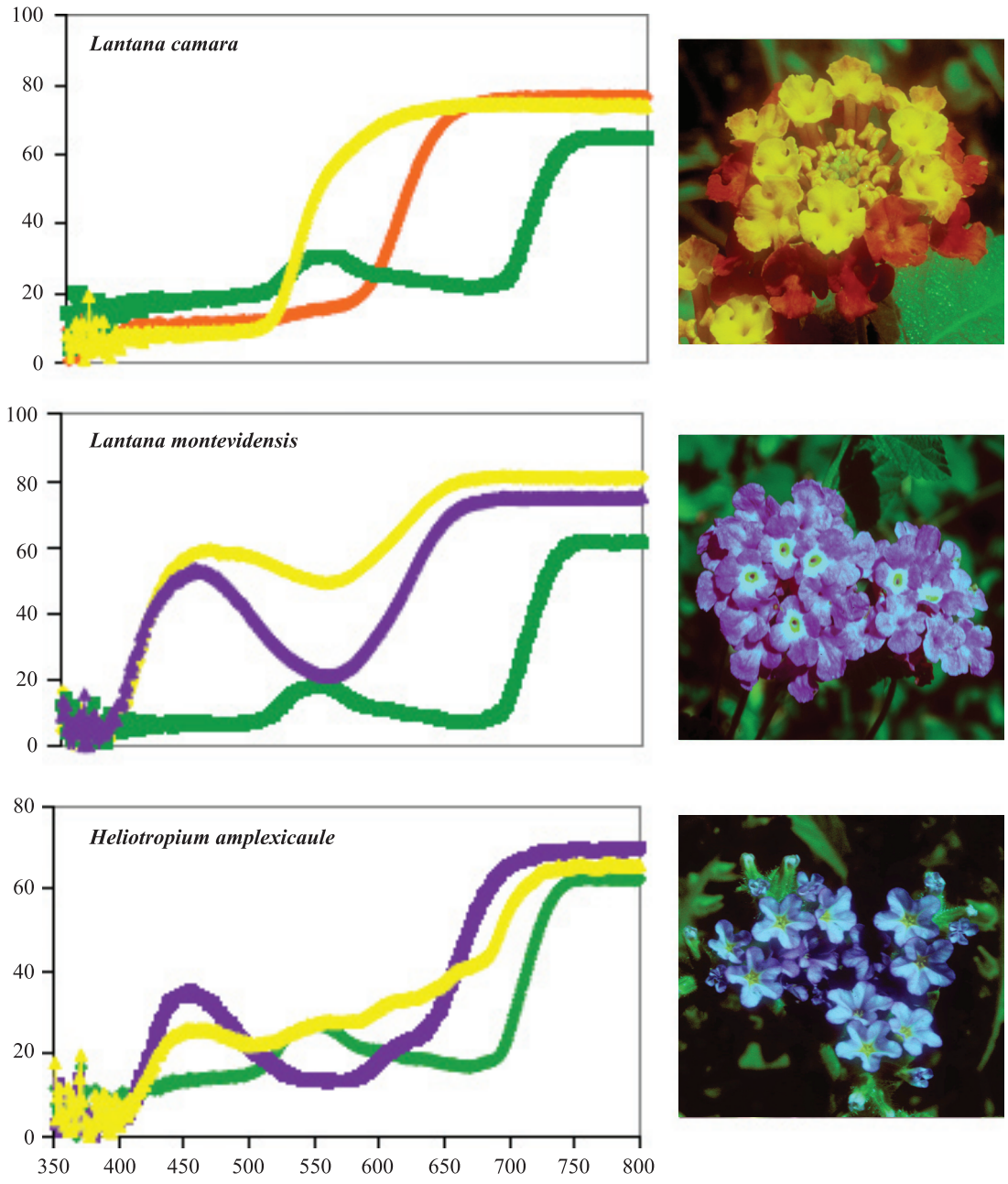

Figure 1: Reflectance spectra of floral colour change in focal species of this study. Vertical axis is reflectance as a % of a white standard, whereas horizontal axis is wavelength of reflected light (nm). In each panel, the upper (yellow) trace is a young, open flower with nectar, the middle (orange or purple) trace is an older, nectarless flower whose colour has changed, and the lower (green) trace is background vegetation. Recordings were made using a Spectral Instruments, Inc. fiber optic spectrophotometer with a tungsten bulb for illumination and an integration sphere.

but are largely ignored by flower visitors once they have landed.

We designed experiments using cultivated *L. camara* and *L. montevidensis* plants, as well as wild-growing plants of *H. amplexicaule* to address the following questions:

1. Does floral scent emission change qualitatively or quantitatively in association with floral colour change in these species?
2. Are changes in scent emission uniform, or do certain compounds or compound classes change more than others?

3. To what extent do changes in scent emission reflect the availability of nectar?

These experiments should reveal whether floral olfactory signals contrast with or reinforce patterns of visual display, and should generate testable hypotheses for how butterflies and other insects learn to utilize the multiple sensory signals proffered by flowers of *Lantana* and similar plants.

2.1 *Plant materials*

Plants of *Lantana camara* (var. "Patriot") and *L. montevidensis* were purchased from Mesquite Valley Growers, Inc. in Tucson, Arizona (USA) and grown in a greenhouse under ambient conditions at the University of South Carolina in 2001 and 2002. Three cuttings from each plant were rooted with 0.1% indole-3-butyric acid, propagated in 60% Baccto, Inc. potting soil: 40% sand, fertilized monthly with Miracle Gro™ (15%K: 30%P: 15%N) fertilizer, and transplanted into 4 L pots. This "clonal" approach was expressly adopted in order to reduce as many sources of error as possible (see[19]), including genetic variation. The fact that our resulting trial replicates (see below) are pseudo-replicates is irrelevant to our primary question, which concerns the proof-of-concept on whether subtle changes in floral scent can be detected reproducibly above background noise using current analytical methods. For *Heliotropium amplexicaule* (Boraginaceae), we studied living, naturalized plants in weedy populations growing near athletic fields on the University of South Carolina campus during summer June–August of 2002 and 2003.

2.2 *Spectral reflectance*

The spectral properties of *Lantana* and *Heliotropium* flowers were measured using a Spectral Instruments, Inc. (Tucson, AZ) SI-440 CCD array UV-VIS Spectrophotometer. This instrument was connected by a 400 μm fiber optic probe to a Labsphere, Inc. (North Sutton, NH) 9 cm ID integration sphere. Using a 10 W tungsten light source, we collected reflectance data from 350 nm (UV) to 800 nm (IR) wavelengths. The integration sphere, fitted with a 5 mm diameter sampling port, was placed over 3–4 grouped flowers with a black felt cloth as background. This method allowed us to capture transmitted light scattered by the textured surfaces typical of flower petals. Data were collected as percent transmission in comparison to a white pigment (Duraflect™) standard. This standard reflected evenly in all non-UV wavelengths, with a 5% drop-off from 400 to 350 nm (Labsphere, Inc. product literature). The reflectance of the black cloth background was negligible (0.5–1% of standard) for all wavelengths tested.

2.3 *Volatile collection:*

The following methods of scent collection were performed for all species:

1. Individual flowers of different age (and colour) were carefully excised and immediately sealed, 30 at a time, within square headspace bags. Bags were prepared by cutting and re-sealing clear nylon resin (Reynolds, Inc.) oven bags to 9 × 9 cm dimensions, using an impulse heat sealer (American International Electric, Inc.). After 30 min equilibration time, we exposed a solid phase micro-extraction (SPME) fiber coated with polydimethylsiloxane (PDMS, 100 μm film thickness) within this headspace for an additional 30 minutes. Pilot experiments showed that additional equilibration time and exposure time did not result in novel compounds or alter peak ratios (data not shown). We sampled nectar scent in *Lantana* species by removing nectar from 20 newly opened, intact flowers using wedge-shaped wicks cut from Whatman #2 filter paper, then sealing the wet filter paper wicks into a headspace bag and repeating SPME exposure as described by Raguso.[65] Control analyses were performed using dry filter paper wicks and empty headspace bags.
2. SPME analyses were repeated on single, intact inflorescences from which pre- or post-colour change flowers were selectively removed, to test whether patterns observed using excised flowers were artifacts of wounding. Inflorescences were enclosed within 12 × 9 cm headspace bags cinched at 6 cm (halfway) with scentless plastic ties. Equilibration and SPME exposure time were as described above. Control collections were performed on foliage and inflorescence bracts in order to distinguish between floral and non-floral volatiles.
3. Floral scent was collected from intact, bagged inflorescences using dynamic headspace methods.[19,66] Plants were placed within a Precision, Inc. growth chamber at 24°C at 10:00 hrs and watered. One hr later, 9 × 12 cm headspace bags were placed over single, intact inflorescences and cinched halfway. Two slits were cut into the bags, one to admit ambient air, the other to hold a cut pasteur pipette filled

Headspace: a term borrowed from the field of Food Science, in which the air above a beverage saturates with the volatile fraction of flavor and fragrance compounds in equilibrium with the liquid phase below. In the context of floral volatiles, headspace can refer either to the naturally emitted volatiles emanating from living flowers, or the suite of volatiles collected from such flowers using the static or dynamic analytical methods described in this paper.

with 100 mg SuperQ adsorbent (80/100 mesh size, divinylbenzene/ethylvinylbenzene polymer sandwiched between plugs of silanized quartz wool; see Levin et al.[67] for details). Volatile-laden air was drawn through this cartridge using a Supelco PAS-500 vacuum pump, powered by a 9V battery, at a flow rate of 200 ml air/min, over 4 hrs. Trapped scent compounds were eluted with 3 ml hexane, concentrated to 75 μL with a flow of N_2 gas, and supplemented with 16 ng of toluene (5 μL of 0.03% solution in hexane; see Svensson *et al.*[68]) as an internal standard. Samples were stored in Teflon-capped 4 mL borosilicate glass vials at –20°C for 3–5 days, then were analyzed using gas chromatography-mass spectrometry (GC-MS).

2.4 *GC-MS analysis*

Sample aliquots of 1 μl were injected into a Shimadzu GC-17 A with a Shimadzu QP5000 electron impact, quadrupole mass spectrometer (MS) as a detector. Analyses were made using splitless injections at 240°C on a polar GC column [0.25 mm ID, length 30 m, film thickness 0.25 μm (EC WAX); Alltech Associates, Inc.]. Additional replicates were performed on a non-polar GC column (EC5) to confirm compound identity with authentic standards (see Raguso *et al.*[69]). Ultra high purity helium was used as a carrier gas, with a flow rate of 1 ml/min and a split ratio of 12:1. Oven temp was held constant at 60°C for 3 min, then increased at 10°C per min until 260°C and held for 7 min. Details of the pressure program were provided by Levin *et al.*[67] Putative compound identification via comparison with mass spectral libraries [Wiley and NIST libraries (>120,000 mass spectra)] was verified whenever possible through co-injection of known standards or essential oils for which constituent analyses have been published.[69] GC peak areas were integrated using Shimadzu's Class-5000 and GC-MS Solutions software and quantified by comparison with the internal standard, then were expressed as ng scent (toluene equivalents) per flower per hr or ng scent per g flower (fresh mass) per hr (see Svensson *et al.*[68]).

2.5 *Floral manipulations and statistical analyses*

We tested whether changes in the floral scent of *Lantana camara* and *L. montevidensis* are correlated with colour-change and/or nectar removal using two different approaches. First, for 18–20 inflorescences of each species, we selectively removed pre- or post-colour-change flowers, resulting in uniformly coloured treatments. Nectar was removed from half of these treatments by inserting filter paper wicks into the corolla tubes for 10 min and removing them prior to headspace collection. Previous studies indicate that nectar refill in yellow flowers of *L. camara* requires 6 hrs to reach the original volume, while orange/red flowers do not replenish nectar.[70] We used dynamic headspace methods to collect floral scent from these inflorescences over 4–4.5 hrs. For all scent compounds shown to change with flower colour in SPME-GC-MS analyses, we combined standardized volatile emission data to create an aggregate dependent variable. Variation in scent emission among these compounds was analyzed using ANCOVA, with flower colour, nectar presence/absence and colour × nectar interaction as factors and individual plant as a covariate (SPSS 10.0). When plant individual was found not to contribute to variance in emissions, it was dropped from subsequent analyses. For *L. montevidensis*, we also partitioned these compounds into two aggregate dependent variables; linalool-derived and benzoic acid-derived compounds. In this way, we could test the hypothesis that odour changes occur through coordinated scent biosynthesis and pathway flux.

Flowers of *Heliotropium amplexicaule* had not been studied previously by Weiss,[16,57] so an additional experiment was performed to test whether ontogenic changes in floral colour (and scent) are triggered by nectar removal or pollen deposition. We prepared four groups of young inflorescences (n = 5 for each treatment) for experiments by gently removing older flowers with forceps, leaving 14 newly opened, yellow-centered flowers. We collected headspace volatiles from these using SPME-GC-MS as described above for four treatments: 1) day of anthesis control, 2) 24 hrs later (sham control, touching filter paper to corolla), 3) 24 hrs after removing nectar with filter paper, and 4) 24 hrs after hand-pollinating flowers with a fine insect pin (as a surrogate proboscis). Mean standardized peak areas (counts) were compared using one-way ANOVA with treatment as a factor and the expectation that one or more treatments would produce significantly lower mean volatile emissions than the control(s).

Floral and vegetative tissues of *Lantana camara, L. montevidensis* and *Heliotropium amplexicaule* emit highly complex volatile blends, with at least 77 compounds identified from isolated flowers of these species (Table 1). For *L. camara*, results were largely consistent with previously published data using steam distillation-essential oil-GC-MS[71] and dynamic headspace GC-MS.[72] For each species, vegetative volatiles presented a contrast with floral

scent in both chemical composition and number of compounds present, and were dominated in number and relative amount by over 100 different monoterpene and sesquiterpene hydrocarbons (data not shown), some of which also were emitted by floral tissues. In each case, SPME analyses of dissected flowers (Table 1), as compared with SPME analyses from intact inflorescences (data not shown), revealed numerically simpler blends of largely or exclusively floral volatiles, comprised of aromatic esters and alcohols, apocarotenoids (e.g. 4-oxoisophorone), ocimene- and linalool-derived oxygenated monoterpenoids (Table 1).

For *Lantana camara*, trans-β-ocimene, along with trans-β-caryophyllene, α-humulene and germacrene-D were the most abundant floral volatiles, and did not change appreciably with flower colour change (Table 1, Fig. 2). These compounds were abundant in involucres, leaves and stems as well as in floral tissues. In contrast, the emissions of linalool-derived compounds and benzaldehyde were reduced by at least half in old, orange-red flowers (Fig. 2). SPME analyses of filter paper wicks soaked with nectar samples drawn from *L. camara* flowers revealed trace amounts of the most abundant sesquiterpenes and the pyranoid linalool oxides, likely due to passive absorption into the nectar from corolla tissues. However, when the combined emissions of benzaldehyde and linalool-derived compounds over 4 hrs were analyzed using ANOVA, only flower colour was significantly associated with scent emission per flower and per g (fresh mass) flower (Table 2). Neither the presence of nectar nor the interaction between nectar and flower colour contributed significantly to quantitative variation in flower-specific volatiles.

Similar patterns were observed for flowers of *Lantana montevidensis* (Tables 1, 2). The colour change from yellow- and white-ringed new lavender flowers to older, pure lavender flowers was accompanied by a pronounced reduction in the emission of linalool, its various oxides and several aromatic compounds, including methyl benzoate, methyl salicylate, benzaldehyde, phenylacetaldehyde, 2-phenylethyl acetate and 2-phenyl ethanol (Fig. 3). Again, significant variation in the pooled emissions of these compounds was associated only with colour change, whether standardized per flower or per g fresh floral mass (ANOVA, Table 2). When ANOVA was repeated using linalool-related compounds and aromatic compounds as separate dependent variables, we found that this effect was primarily due to aromatic compounds (Table 2), especially phenylacetaldehyde and 2-phenylethanol, which are dramatically reduced in older flowers (Fig. 3). In contrast, linalool derivatives did not show as consistent reductions with floral age (Fig. 3). Unlike in *L. camara*, no scent compounds were detected in the SPME analyses of nectar from *L. montevidensis* soaked into filter paper wicks.

Results for *Heliotropium amplexicaule* were even more straightforward (Table 1). Flower-specific volatiles such as trans-β-ocimene, linalool and its oxides, methyl benzoate and 1,4-dimethoxybenzene were largely reduced or absent in older, lavender-coloured flowers (Fig. 4), obviating the use of ANOVA to explore quantitative changes. Manipulative experiments revealed that neither the removal of nectar nor the pollination of first-day flowers is sufficient to spur colour change or scent changes (Fig. 4) within 24 hrs, as there were no significant treatment effects (ANOVA; $F = 1.26$, $P = 0.32$) associated with variation in the mean aggregate GC peak area counts for summed flower-specific compounds. Thus, ontogenic changes in floral colour and scent appear to co-occur with flower age, rather than to reflect rapid physiological responses to nectar removal or pollination.

Apocarotenoids: a class of secondary metabolites consisting of compounds that are catabolized from β-carotene and similarly large, non-volatile compounds and thus have isoprenoid origins, but are not synthesized through the same mechanisms as monoterpenes or sesquiterpenes.

2.6 *Concerted changes in* Lantana *and* Heliotropium *flowers*

The experiments outlined above provided clear answers to three questions. In *Lantana camara* and *L. montevidensis*, floral colour change was correlated with largely quantitative changes in floral scent chemistry (Figs. 2, 3), and similar changes in scent were observed in *Heliotropium amplexicaule* (Fig. 4). However, these changes did not occur uniformly in all floral volatiles or in the most abundant compounds, and were not tightly correlated with the presence or removal of nectar (Table 2, Figs. 2–4). Rather, they were limited to two separate biosynthetic groups of volatile compounds: linalool and its furanoid and pyranoid oxides (monoterpenoids), and a suite of common aromatic esters, alcohols and aldehydes (benzenoids). It is unlikely that direct pleiotropy with carotenoid pigment degradation or anthocyanin deposition would explain the specific reduction of these volatiles in aging flowers.[35] Although these compounds comprise small peaks in the floral chromatograms of each species (Figs. 2–4), they, along with 4-oxoisophorone (Fig. 3) were identified as a core group of shared compounds in a comparative study of 22 species of butterfly-pollinated plants from 13 angiosperm families.[72] Moreover, Andersson[73] and Andersson and Dobson[74] found these compounds to be among the most potent antennal stimulants

Pleiotropy: a term from genetics in which a single mutation or allelic substitution can have multiple phenotypic effects, usually due to the gene product's participation in a biosynthetic or developmental pathway. **Epistasis** describes the converse phenomenon, in which a single trait requires the expression of multiple genes.

Table 1: Headspace volatiles identified from *Lantana* and *Heliotropium* flowers using SPME-GC-MS. (Mean ± SEM % of total ion current GC-MS peak areas from SPME analyses of standardized numbers of flowers)

			Lantana camara					*Lantana montevidensis*					*Heliotropium amplexicaule*			
			new, yellow flws (n=3)		old, red flws (n=3)			new, yellow eye (n=3)		old, purple (n=3)			new, yellow eye (n=3)		old, purple (n=3)	
Volatile compound	Ret time	ID	mean	sem	mean	sem	V?	mean	sem	mean	sem	V?	mean	sem	detectable	V?
alpha-pinene	2.24	1	0.0479	0.0011	0.0438	0.0076	V	0.0390	0.0289	0.0298	0.0000	V				
camphene	2.62	1						0.1752	0.1301	0.2208	0.0058	V				
beta-pinene	3.34	1	0.1219	0.0091	0.1024	0.0408	V	0.0691	0.0405	0.0819	0.0261	V				
sabinene	3.65	1	0.0907	0.0128	0.0942	0.0323	V	1.4201	1.0080	1.6763	0.2365	V				
myrcene	4.59	1	1.1194	0.2300	0.8200	0.1789	V	0.3402	0.0571	0.2582	0.1306	V				
limonene	5.29	1	0.0558	0.0163	0.0569	0.0156	V	0.3776	0.2486	0.6389	0.1059	V				
1,8 cineole	5.45	1						0.3528	0.2313	0.6880	0.1029	V				
cis-beta-ocimene	6.04	1	3.7349	0.9561	2.8251	0.7396		1.3052	0.2560	0.9763	0.1102		0.38	0.10		
gamma-terpinene	6.17	1						0.0201	0.0134	0.0758	0.0326	V	66.94	6.92	x	
trans-beta-ocimene	6.38	1	50.5111	12.4727	48.8260	10.1362		60.3204	9.7057	58.3547	5.5562					
alpha-terpinolene	6.90	1						0.1095	0.0623	0.1804	0.0236	V				
trans-4,8-dimethylnona-1,3,7-triene	7.35	1	0.7082	0.1910	0.5211	0.1349	V	0.1714	0.0404	0.3573	0.1046					
anisole	7.89	1	0.0218	0.0110	0.0332	0.0147										
ocimene epoxide	8.17	2	0.1224	0.0212	0.0636	0.0272							0.52	0.28		
41(100), 69(54), 84(32), 91(24), 55(15), 53(14), 79(12), 67(11), 68(10), 119(8)	8.36	3	0.0526	0.0118	0.0232	0.0108	V									
allo-ocimene	8.42	1	0.0920	0.0271	0.0669	0.0240	V									
ocimene epoxide	8.77	2	1.2453	0.2756	0.5239	0.2189										
nr. 3,3 dimethyl hexene (41(100), 69(86), 55(79), 53(14), 97(13), 70(8), 83(5), 56(5), 43(5), 42(4))	9.32	3	0.0817	0.0199	0.0427	0.0255	V									
cis-furanoid linalool oxide	**9.44**	**1**	**0.4125**	**0.1043**	**0.0305**	**0.0148**		**0.3956**	**0.1025**	**0.3649**	**0.0623**		0.31	0.05		
nr. trans-2,6-dimethyl-1,3,5,7-octatetraene (unsaturated trans-beta-ocimene)	9.54	3	0.1024	0.0385	0.0714	0.0346	V	0.0447	0.0068	0.0641	0.0321					
trans-furanoid linalool oxide	**9.85**	**1**	**0.3566**	**0.0431**	**0.0408**	**0.0154**		**0.3563**	**0.0734**	**0.2062**	**0.1041**		7.58	3.03		
pyranoid linalool oxide ketone	**9.90**	**1**	**0.1726**	**0.0649**	**0.0000**	**0.0000**		**0.4927**	**0.0027**	**1.6288**	**0.1001**		2.16	0.40	x	
delta-elemene	9.92	**1**	0.2315	0.0305	0.3320	0.0319	V									
trans-myroxide (ocimene oxirane)	10.09	**2**	0.2665	0.0778	0.1836	0.0648										

gamma-terpineol	10.09	**2**						0.2369	0.0375	0.6297	0.0721	V				
alpha-copaene	10.24	**1**	0.3810	0.0497	0.3508	0.0545	V	0.1741	0.0728	0.4927	0.0883	V				
trace sesquiterpene (m/z 93, 105, 119…)	10.33	**3**	0.1318	0.0680	0.0952	0.0059	V					V				
camphor	10.53	**1**						0.1106	0.0649	0.3364	0.0704	V				
benzaldehyde	**10.58**	1	**0.1578**	**0.0199**	**0.1323**	**0.0532**		**0.6161**	**0.1811**	**0.0000**	**0.0000**		0.46	0.36		
beta-bourbonene	10.72	2											0.12	0.12		V
C15H24: 150(100), 41(63), 161(60), 55(58), 91(55), 119(54), 133(53), 81(41), 79(29), 107(29), 43(26), 59(23)…	10.75	**3**	0.0343	0.0082	0.0303	0.0099	V									
linalool	**10.78**	1	**0.0453**	**0.0076**	**0.0309**	**0.0188**		**17.1503**	**4.4872**	**3.9461**	**1.0643**	V	1.00	0.12		
sesquithujene	10.82	2	0.0491	0.0091	0.0581	0.0140	V									
beta-cubebene	10.85	1	0.5870	0.0752	0.4498	0.0764	V									
C15H24: 119(100), 41(58), 105(55), 93(51), 161(46), 91(43), 121(43), 55(33), 79(31), 69(28), 77(28), 120(24), 43(21)…	10.93	3	0.0689	0.0086	0.0772	0.0160	V									
alpha-zingiberene isomer	11.09	2	0.3770	0.0462	0.3901	0.0300	V									
alpha-cedrene	11.27	1	0.3872	0.0578	0.3747	0.0633	V									
beta-ylangene	11.33	2	0.0289	0.0024	0.0497	0.0079	V	0.0530	0.0174	0.1044	0.0313	V				
nr. beta-elemene	11.37	3	0.1813	0.0283	0.2029	0.0334	V	0.0712	0.0268	0.1899	0.0651	V				
trans-alpha-bergamotene	11.45	2	0.0227	0.0051	0.0253	0.0016	V	0.0110	0.0018	0.2650	0.1117	V				
beta-elemene	11.55	2	1.5675	0.2536	1.9339	0.2915	V	1.2719	0.3832	3.9780	0.8859	V				V
beta-caryophyllene	11.65	1	14.3065	1.7226	15.6895	2.2010	V	1.2060	0.5256	2.9271	0.5828	V	0.40	0.38		V
nr. cadina-1(2,4)-diene	11.76	3	0.4999	0.0645	0.5362	0.0720	V					V				
methyl benzoate	**11.87**	**1**	**0.1505**	**0.0416**	**0.0910**	**0.0120**		**0.1846**	**0.0292**	**0.0000**	**0.0000**		11.41	4.47	x	
trace sesquiterpene (m/z 79, 91, 105, 119…)	11.96	3	0.0161	0.0053	0.0344	0.0114	V									
gamma-elemene	12.11	2	0.1255	0.0287	0.1786	0.0332	V									
phenylacetaldehyde	**12.15**	1						**1.4490**	**0.4360**	**0.0628**	**0.0375**					
cis-beta-farnesene	12.22	2	0.2023	0.0376	0.2328	0.0228	V									
allo-aromadendrene	12.27	1	0.4599	0.0347	0.4347	0.0678	V	0.3543	0.0309	0.4034	0.0651	V				V
C15H24: 119(100), 121(83), 93(71), 105(64), 41(60), 79(57), 81(49), 77(49), 91(46), 67(38), 69(38, 133(34), 189(34)…	12.39	3	0.0910	0.0213	0.0881	0.0434	V									
trans-beta-farnesene	12.44	2	0.2423	0.1446	0.8059	0.4154	V									

(*Continued*)

Table 1: (Continued)

			Lantana camara					*Lantana montevidensis*					*Heliotropium amplexicaule*			
			new, yellow flws (n=3)		old, red flws (n=3)			new, yellow eye (n=3)		old, purple (n=3)			new, yellow eye (n=3)		old, purple (n=3)	
Volatile compound	ret time	ID	mean	sem	mean	sem	V?	mean	sem	mean	sem	V?	mean	sem	detectable	V?
alpha-humulene	12.56	1	7.7087	0.9126	9.6534	1.1581	V	0.1294	0.0411	0.3321	0.0981	V	0.16	0.16		V
C15H24: 41(100), 69(97), 56(60), 44(60), 93(58), 79(33), 109(30), 162(29), 91(26), 55(24), 119(23), 133(20), 106(20)…	12.70	3	0.1051	0.0273	0.1332	0.0364	V									
4-oxoisophorone	12.78	2						0.3639	0.0550	0.1678	0.0472					
gamma-curcumene	12.78	2	2.8010	0.4426	2.7987	0.2549	V									
C15H24: 161(100), 41(92), 91(76), 105(54), 120(52), 69(46), 133(38), 93(36), 56(33), 121(33), 65(31), 79(28), 119(28)…	12.83	3	0.1192	0.0265	0.1637	0.0527	V									
germacrene D	13.04	1	3.6545	0.3587	3.8249	0.5053	V	6.3898	2.6338	13.9526	2.0791	V	2.04	0.80	x	V
alpha-zingiberene isomer	13.11	1	2.8009	0.2593	3.2244	0.2465	V									
C15H24: 41(100), 105(85), 93(60), 69(51), 91(41), 94(40), 79(36), 55(31), 67(31), 81(30), 119(28), 161(26)…	13.18	3	0.1455	0.0063	0.0325	0.0173	V	0.1474	0.0464	0.5966	0.1907	V				
1,4 dimethoxybenzene	13.22	2											3.48	2.11	x	
cis-pyranoid linalool oxide	**13.24**	**1**	**0.7000**	**0.1792**	**0.0325**	**0.0173**		**0.2387**	**0.0802**	**0.1750**	**0.0322**		1.53	0.50	x	
bicyclogermacrene	13.31	2	0.5046	0.0826	0.8781	0.1489	V	2.0451	0.8015	4.3666	0.7214	V	1.15	0.60	x	V
beta-curcumene	13.35	2	0.6298	0.1040	0.6350	0.0418	V	0.1176	0.0599	0.0000	0.0000	V				
trans,trans-alpha-farnesene	13.41	1	0.1545	0.0346	0.1640	0.0316	V									

trans-pyranoid linalool oxide	**13.45**	**1**	**0.1224**	**0.0330**	**0.0000**	**0.0000**		**0.3157**	**0.0983**	**0.1850**	**0.0542**			
delta-cadinene	13.53	2	0.0825	0.0179	0.1060	0.0537	V	0.0451	0.0087	0.0694	0.0381	V		
gamma-cadinene	13.59	2	0.0788	0.0212	0.1201	0.0149	V							
beta-sesquiphellandrene	13.70	2	0.0707	0.0140	0.1037	0.0151	V	0.0912	0.0232	0.3640	0.0553	V		V
alpha-curcumene	13.73	2	0.0926	0.0242	0.1688	0.0572	V							
methyl salicylate	**13.74**	1	0.0000	0.0000	0.0397	0.0366	V	**0.3514**	**0.0559**	**0.0573**	**0.0574**			
trans,trans-4,8,12-trimethyl-1,3,7, 11-tridecatetraene	14.09	2	0.0384	0.0221	0.1255	0.0474	V	0.0950	0.0526	0.0982	0.0343			V
2-phenylethyl acetate	14.17	1						0.1116	0.0306	0.0780	0.0408			
C15H24: 41(100), 91(100), 69(68), 79(63), 105(58), 53(50), 77(50), 81(47), 117(45), 43(45), 131(36), 67(35), 93(33), 119(29)…	14.20	3	0.0673	0.0172	0.0924	0.0219	V							
germacrene B	14.40	2	0.2498	0.0376	0.4078	0.0596	V	0.0994	0.0267	0.2365	0.0649	V		
2-phenyl ethanol	**15.14**	1						**0.5330**	**0.2279**	**0.1832**	**0.0288**			
81(100), 41(86), 43(43), 79(40), 93(38), 53(35), 55(34), 68(33), 67(33), 69(31), 95(30), 107(21), 77(19), 109(18)…	16.62	3	0.0341	0.0109	0.0353	0.0115	V							
81(100), 41(86), 43(43), 79(40), 93(38), 53(35), 55(34), 68(33), 67(33), 69(31), 95(30), 107(21), 77(19), 109(18)…	16.75	3	0.0950	0.0217	0.1754	0.0324	V							

Retention times (RT) are given in minutes, from EC-Wax GC column (30 m, 0.25 mm ID, 0.25 um film thickness)—see Sections 2.3 and 2.4.
1 = co-retention and MS match with authentic standard.
2 = verified by comparison with essential oil or natural product for which published data are available (esp. Ngassoum et al.[71] and Andersson et al.[72]).
3 = unidentified. If MS library match is >90% and no negative evidence is available, a provisional name is given.
Otherwise, at least 10 most abundant ms ion fragments are listed in descending order of m/z.
V = also present in vegetation. nr. = near
Compounds in **bold font** were used in ANOVA analyses due to their loss in colour-changed flowers.

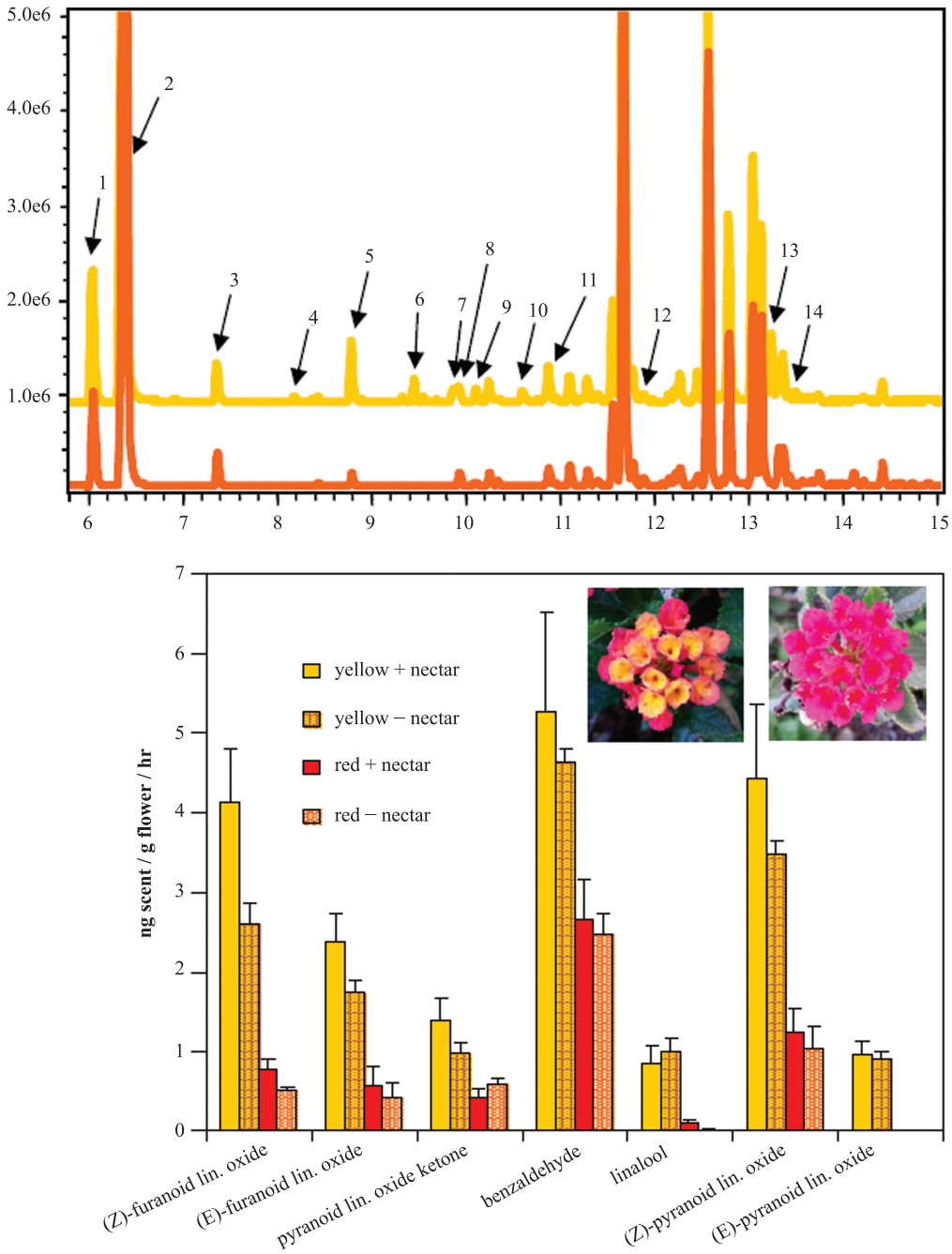

Figure 2: Upper panel: comparison of SPME-GC-MS total ion current traces from 30 young, nectar-rewarding yellow flowers (upper trace) vs. 30 older, nectarless orange-red flowers (lower trace) of *Lantana camara*, on EC-Wax polar GC column. Numbered compounds are (1) cis-β-ocimene, (2) trans-β-ocimene, (3,4,5) ocimene epoxides, (6,7) cis and trans-furanoid linalool oxides, (8) pyranoid linalool oxide ketone, (9) ocimene epoxide, (10) benzaldehyde, (11) linalool, (12) methyl benzoate, (13,14) cis and trans pyranoid linalool oxides. Unlabeled peaks are sesquiterpene hydrocarbons present in the floral calyces and vegetation. X axis is GC column retention time, min.; Y axis is TIC peak area counts. Lower panel: mean + SEM emissions (ng/g fresh mass/hr) of volatiles that track colour change in *L. camara*, with or without nectar removed (mean ± SEM (N) = 15.5 ± 0.9 flowers/treatment).

for four distantly related butterfly species. Considering these patterns and the attractiveness of *Lantana* flowers to diverse butterflies worldwide, it is likely that these compounds represent a convergent signal to attract butterflies, and may have evolved independently in plants that track pre-existing biases in the sensory capabilities of butterflies as a specific group of pollinators.[75]

Table 2: ANOVA of changes in floral scent in *Lantana* species.

Dep. Variable	Source	MS	df	F	P
L. camara ng/flower/hr	colour (age)	0.147	1	14.95	**0.002**
	nectar	0.008	1	0.85	0.37
	colour * nectar	0.003	1	0.28	0.61
	plant	0.007	1	0.73	0.41
	error	0.010	15		
L. camara ng/g flower/hr	colour (age)	729.60	1	27.64	**<0.001**
	nectar	22.96	1	0.87	0.37
	colour * nectar	10.85	1	0.41	0.53
	plant	19.04	1	0.72	0.41
	error	26.40	15		
L. montevidense ng/flower/hr	colour (age)	0.689	1	7.72	**0.015**
	nectar	0.190	1	2.13	0.17
	colour * nectar	0.013	1	0.15	0.71
	error	0.089	14		
L. montevidense ng/g flower/hr	colour (age)	12605.64	1	7.90	**<0.001**
	nectar	3122.17	1	1.96	0.18
	colour * nectar	83.26	1	0.05	0.82
	error	1595.14	14		
linalool-derived compounds, pooled ng/g flower/hr	colour (age)	37.32	1	0.26	0.62
	nectar	147.34	1	1.03	0.33
	colour * nectar	96.77	1	0.68	0.42
	error	142.98	14		
aromatic compounds, pooled ng/g flower/hr	colour (age)	13390.61	1	10.75	**0.005**
	nectar	1971.50	1	1.58	0.23
	colour * nectar	360.27	1	0.29	0.60
	error	1245.38	14		

However, these ubiquitous compounds, especially 2-phenylethanol and phenylacetaldehyde, also are emitted by many flowers with generalized pollination systems and are attractive to several families of moths, flies and bees as well as butterflies (reviewed by [76,77]).

Previous studies have measured the behavioral responses of butterflies to *L. camara* flowers, initially with respect to inflorescence size and colour change. Weiss demonstrated that from a distance *Agraulis vanillae* (Nymphalidae) butterflies prefer to visit larger inflorescences irrespective of colour.[57] At close range, although both *A. vanillae* and *Junonia coenia* (Nymphalidae) butterflies innately prefer yellow (young) flowers over red (old) flowers, they rapidly learn to associate yellow flowers with the presence of nectar, and continued experience results in greater discrimination in favour of the rewarding colour. *Battus philenor* (Papilionidae) butterflies similarly show a rapid learned association between floral colour and nectar, and individuals readily shift their foraging efforts to a newly rewarding colour if the location of the nectar is changed.[78] Further experiments by Weiss suggest that learned colour bias in *A. vanillae* likely overshadows other floral traits, because the butterflies visit older, red flowers painted yellow with acrylic paint, and conversely, they ignore young, yellow flowers painted red.[11]

Andersson and Dobson experimentally decoupled colour and scent in *L. camara*, using a related butterfly (*Heliconius melpomene* (Nymphalidae)) as a model pollinator.[79] Naïve *H. melpomene* were more likely to probe at yellow artificial flowers when combined with the scent of concealed *L. camara* flowers than with the scent of concealed *L. camara* leaves. However, once the butterflies have been conditioned on yellow artificial flowers, they show a hierarchical preference for yellow regardless of scent composition. When yellow colour and floral scent were decoupled, yellow artificial flowers with vegetative scent elicited stronger feeding responses than did green artificial flowers with floral scent, suggesting that although innate responses to scent trigger nectar foraging and facilitate flower learning in naïve *H. melpomene*, they are overridden by learned colour preferences

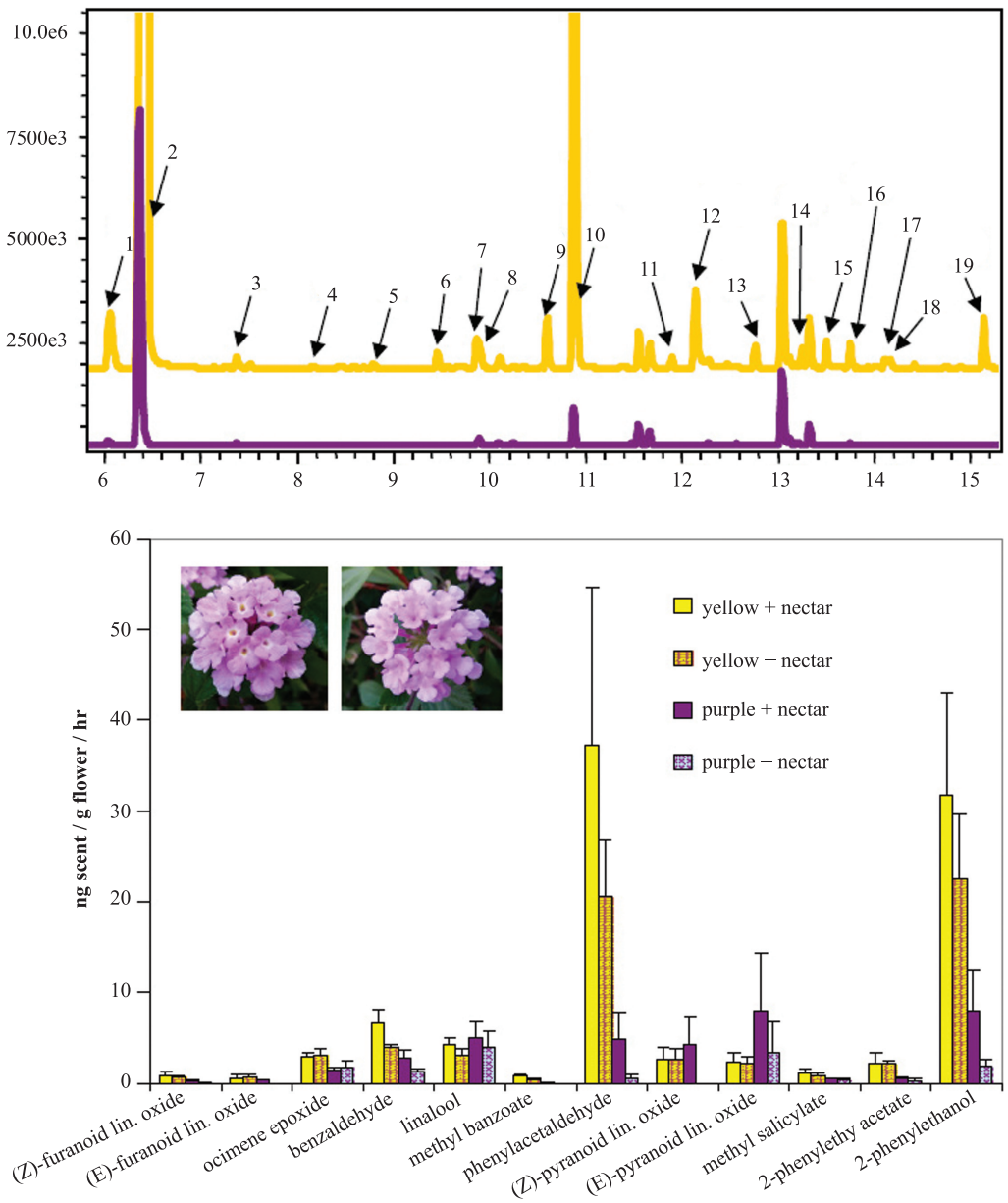

Figure 3: Upper panel: comparison of SPME-GC-MS total ion current traces from 30 young, nectar-rewarding yellow-centered flowers (upper trace) vs. 30 older, nectarless purple flowers (lower trace), of *Lantana montevidensis* on EC-Wax polar GC column. Numbered compounds are (1) cis-β-ocimene, (2) trans-β-ocimene, (3) trans-4,8-dimethyl-1,3,7-nonatriene, (4,5) ocimene epoxides, (6,7) cis and trans-furanoid linalool oxides, (8) pyranoid linalool oxide ketone, (9) benzaldehyde, (10) linalool, (11) methyl benzoate, (12) phenylacetaldehyde, (13) keto-isophorone, (14,15) cis and trans pyranoid linalool oxides, (16) methyl salicylate, (17) trans, trans-4,8,12-trimethyl-1,3,7,11-tridecatetraene, (18) 2-phenylethylacetate, (19) 2-phenylethanol. Unlabeled peaks are sesquiterpene hydrocarbons present in the floral calyces and vegetation. X axis is GC column retention time, min.; Y axis is TIC peak area counts. Lower panel: mean + SEM emissions (ng/g fresh mass/hr) of volatiles that track colour change in *L. montevidensis*, with or without nectar removed (mean ± SEM (N) = 15 ± 0.7 flowers/treatment).

in experienced butterflies. Similar conclusions were drawn by, Lewis and Lipani[80] and Ômura and Honda[81] in behavioral studies with *Pieris rapae* and *Vanessa indica* butterflies, respectively, for which floral scent enhances attraction to non-preferred colours but is overshadowed in the presence of preferred colours. However, flowers are visited by diverse lineages of butterflies that utilize visual and olfactory floral information in complex ways.[82,83] What the experiments described

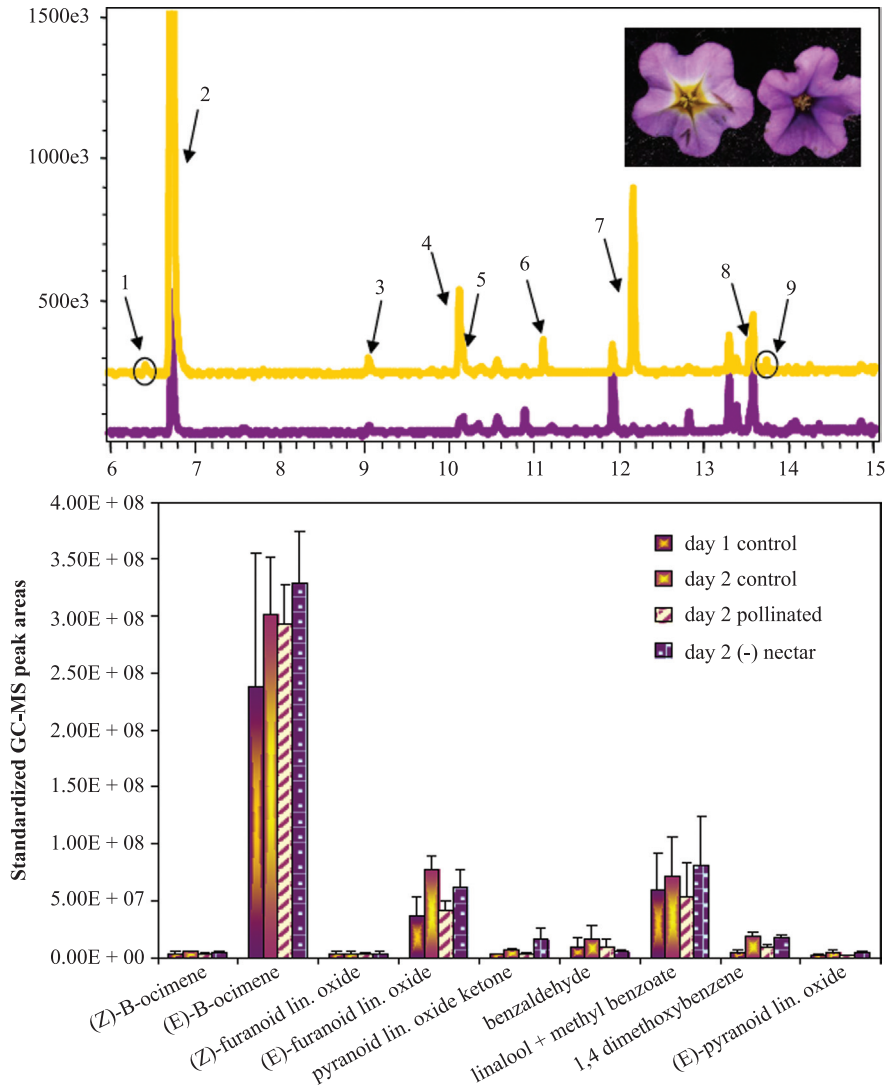

Figure 4: Upper panel: Comparison of SPME-GC-MS total ion current traces from 25 young flowers of *Heliotropium amplexicaule* with yellow centers (upper trace) vs. 25 older flowers lacking a yellow center (lower trace), on EC-Wax polar GC column. Numbered compounds are (1) cis-β-ocimene, (2) trans-β-ocimene, (3) ocimene epoxide, (4) trans-furanoid linalool oxide, (5) pyranoid linalool oxide ketone, (6) linalool, (7) methyl benzoate, (8) 1,4-dimethoxybenzene, (9) pyranoid linalool oxide. Unlabeled peaks are sesquiterpene hydrocarbons present in the floral calyces and vegetation. X axis is GC column retention time, min.; Y axis is TIC peak area counts. Lower panel: mean + SEM (SPME-GC-MS) peak areas of volatiles that track colour change in 14 flowers of *H. amplexicaule*, at anthesis vs. 24 hrs later with no treatment, nectar removed or hand-pollinated.

above did not address is the potential impact of concerted changes in colour and scent on flower choice by pollinators, either in the *Lantana* system or more generally. Below we consider alternative hypotheses for the function of dynamic, multimodal floral signals.

3 Concerted Floral Changes as Multimodal Floral Signals

Recently, hypotheses developed to explain the evolution of multimodal signals in intraspecific animal communication[23] have been applied to interspecific signaling between plants and pollinators.[25,84] These non-mutually exclusive sets of hypotheses have been partitioned into *content-based hypotheses* (addressing what kinds of messages are conveyed) and *efficacy-based hypotheses* (addressing how the messages are conveyed), with the potential for inter-signal interactions (e.g. functions of attention or context) in each category[20] (see Table 3). Content-based hypotheses include the potential for multiple messages encoded by multimodal signals (e.g. species identity enhancing constancy, flower

Table 3: Rationalizing multimodal signal hypotheses (after Leonard et al. [20,84]) with factorial combinations of floral colour and scent changes.

	Likely to be relevant
NO	Not supported by evidence
???	Insufficient evidence
Lantana	Example provided in text

	Content-based hypotheses						Efficacy-based hypotheses					Inter-signal interaction	
	Multiple messages					Redundant signals	Signal transmission		Signal detection	Signal processing		Attention altering	Context
	Species ID (constancy)	Flw. Type (handling)	Location in: (habitat)	(patch)	Flw. Guide (nectar/ pollen)	Increased accuracy	Efficacy backup	Efficacy tradeoff	Increased speed or success	Parallel processing	Perceptual variability	Sequential signals	Synergistic signals
I. Static Floral Signals													
1. Colour and scent do not change					NO, deception								
II. Dynamic Floral Signals													
1. Colour changes, scent does not change	???					NO	NO	NO	NO				
2. Colour changes, scent also changes													
a. Quantitative changes in floral scent													
i. Increase in overall emissions	NO	???	*Quisqualis indica, Lonicera japonica*			???		???	*Bombus*		*Lonicera*	*Quisqualis and Lonicera*	
ii. Decrease in overall emissions	NO	NO	NO	NO	*Pueraria?*	*Pueraria?*	NO	NO	NO	NO		NO	NO
b. Qualitative changes in floral scent													
i. Specific compounds diminish with change	???	*Tibouchina*	*Lantana and Heliotropium?*			*Lantana?*	NO	???	*Bombus*	*Bombus*	*Heliotropium*	NO	NO
ii. Novel compounds are emitted with change	???	*Tibouchina*	*NO*	*Brunfelsia?*		*Tibouchina?*	NO	???	*flower choice*	*flower choice*	???		
3. Colour does not change, scent changes													
a. Advertisement													
i. Quantitative changes in scent (see 2a)	NO	???	NO	NO	NO	NO	NO	NO	*Bombus*				
ii. Qualitative changes in scent (see 2b)	NO	???	NO	NO	NO	NO	NO	NO	*flower choice*				
b. Reward													
i. Nectar is scented, removal explains change	NO	???	NO	NO		NO	NO	NO		NO	NO		NO
ii. Pollen is scented, removal explains change	NO	???	NO	NO		NO	NO	NO		NO	NO		NO

type indicating handling efficiency and resource location at habitat, patch and intrafloral scales, including the use of nectar guides) and redundant signals that improve pollinator accuracy (e.g. the trait variability hypothesis of Gegear and Laverty[39]). Efficacy-based hypotheses include aspects of signal transmission and detection in variable or noisy environments, signal processing by pollinator sensory and nervous systems, and the potential for multiple signal channels to communicate with a broader spectrum of floral visitors or functional groups thereof (e.g. in generalized pollination systems, as discussed above for honey bees and skipper butterflies likely pollinating *Heliotropium*).

The full spectrum of multimodal hypotheses outlined by Leonard et al.[20] was developed largely to explain flowers with static signal displays, and it is apparent from Table 3 that changes in colour, scent or both traits places limitations on which multimodal hypotheses are likely to impact flower–pollinator communication. The differences between static floral display, single modality floral change and concerted floral change also highlight the fact that floral communication is not always honest or cooperative.[38,65] The redundant signal hypothesis posits that multiple signal components (colour and scent together) improve the accuracy of the information conveyed, but the condition of redundancy requires that both floral colour and scent change, or that neither change (Table 3). For example, visitors might more effectively learn to avoid older lavender flowers of *L. montevidensis* that also lack phenylacetaldehyde and related aromatic volatiles (Fig. 3). We suspect that this phenomenon is common in nature, whether the concerted change in scent is quantitative, such as the reduction of the only two floral volatiles (linalool and methyl anthranilate) in older flowers of kudzu (*Pueraria montana*; Fabaceae; data not shown) whose purple banner petals have lost their yellow spot, or qualitative, such as the emission of novel compounds (indole and chavicol) in older flowers of *Tibouchina pulchra* (Melastomataceae) that have turned pink and lost their pollen.[54] The relevance of the multiple messages hypothesis to different plant–pollinator systems would depend upon the scale at which floral signals evoke pollinator behavior. These effects would range from the sequential dynamics of patch choice, landing and probing described above for *Lantana* and *Heliotropium*, to cases in which the floral reward itself (nectar, pollen, oil, resin) is the source of scent, and its removal by pollinators abolishes the function of intrafloral olfactory guide,[65] affects floral constancy[22] or flower type recognition with respect to handling efficiency.[39] Our assignments of hypotheses in Table 3 are not exhaustive, but are meant to indicate how little we currently know about concerted changes in floral colour and scent and how they impact pollinator behavior.

Concerted changes in visual and olfactory floral signals also could function in efficacy-based signal processing, potentially through parallel processing, in which multimodal signals are processed more quickly or effectively by pollinators through parallel neural pathways. Earlier, we reviewed several examples in which bumblebees acquire better or more accurate information about visual signals in the presence of floral scent. Further studies have shown that bees make more decisive colour choices when scent is present, even if they have not learned the flower as a complex signal.[43] If the experimental design used by Kudo *et al.*[14] to decouple flower retention, nectar absence and colour change were extended to include specific colour–scent pairings, that would provide an appropriate test of the parallel processing hypothesis for the speed and accuracy of bumblebee floral choice, as well as a test of the multiple-messages hypothesis guiding decisions of bees to leave an inflorescence (and the dynamics of pollen flow). Finally, two kinds of inter-signal interactions are likely to impact concerted changes in floral colour and scent. Attention-altering interactions occur when one signal focuses a pollinator's attention on a second signal, such as colour-guided floral approaches or probing triggered by the presence of an odour plume.[76] A subtle alternative occurs when inter-signal interactions are contextual, such that two signals synergize a feeding response that is not observed in response to either signal alone: the former interactions are sequential, whereas the latter are synchronized.[83] These mechanisms may be appropriate for the hawkmoth-pollinated plants (*Lonicera japonica* and *Quisqualis indica*) described above, in which floral scent is emitted on a nocturnal rhythm and is maintained in older, colour-changed flowers, enhancing olfactory display and either calling attention to or providing the context for visitation to white, newly opened flowers.[50,52] Depending upon the species of visiting moth, this could also represent multiple messages (Table 3). Given the scant attention paid to concerted changes in floral colour and scent to this point (and the corresponding lack of explicit examples in Table 3), there is a clear opportunity to explore the multimodal hypotheses discussed above in field and laboratory experiments that involve other kinds of pollinators (as well as floral

larcenists) with diverse behavioral responses to floral signals (see [76,82]).

4 Physiological and Ethological Aspects of Dynamic Floral Volatile Emissions

In this review, we have focused on concerted changes in floral colour and scent, but there are other biologically interesting cases in which floral volatile emissions change during the lifetime of a flower, blossom or otherwise integrated inflorescence that are worthy of consideration here. Central to such a discussion is the issue of costs.[85] When does the production and emission of floral volatiles bear tangible metabolic costs,[86] and when do the ecological costs of apparency to enemies become limiting?[77,87] When either category of costs is realized, we predict that floral scent, like any sensory component of display in animal courtship, should be withheld or minimized when it is not required, as is observed when autogamous, self-pollinating plants are derived from an outcrossing ancestor.[88,89] This expectation is most commonly met in the post-pollination loss of scent in senescing flowers,[90] in which pollen tube fertilization of embryos triggers a pulse of ethylene that down-regulates floral volatile biosynthesis.[30] The entrainment of scent emission to diurnal or nocturnal rhythms in plants with long-lived flowers that are pollinated during those specific activity periods[91] is thought to result from similar selective forces.[28] Finally, recent attempts to reconcile studies of floral biology and plant defence in a whole-plant context reveal that herbivore-induced changes in plant systemic volatile emission also affect the chemical signals of subsequent flowers produced by the induced plant. Kessler and Halitschke[92] demonstrated that herbivory by tobacco hornworm caterpillars (*Manduca sexta*) on a wild tomato species (*Solanum peruvianum*) induced plant-wide changes in phenolic content (including pollen and floral tissues) as well as distinctive changes in floral volatile emissions, both of which were correlated with reduced visitation and pollination by bees. The flowers of *Acacia (Vachellia) seyal-fistula* provide another example where floral volatiles are shaped also by plant defence. The ants that otherwise defend this tree species appear to be repelled by the primary pollen volatile, (*E,E*)-α-farnesene, and avoid the flowers until bees have removed the pollen.[93] Depending upon life history strategies and mating system, chemical cross-talk between plant defence and floral ecology should be common, providing another dimension of dynamism in floral scent.

Finally, floral volatiles might be expected to vary temporally in the case of dichogamy—the temporal segregation of male and female floral functions. Perfect flowers of the common pawpaw (*Asimina triloba*; Annonaceae) have sequential female and male phases staggered by at least one day, with marked changes in volatile emissions from acetoin-dominated female flowers to butanediol dominated male flowers.[94] A similar example is that of the dead horse arum (*Helicodiceros muscivorus*; Araceae), in which volatile sulfides attract carrion flies to the female-phase inflorescence (in which they are trapped) on day 1, but are no longer emitted on the following day, when the male florets have matured and the flies leave the floral chamber covered with pollen.[95] Protogyny is common in kettle flowers that engage in deceptive pollination,[96] in which visual, tactile, morphological and chemical features combine to function as an integrated pollinator trap. In such cases, we expect spatial and temporal variation in floral volatiles to reflect a chemical division of labor, in which pollinators are attracted and manipulated to optimize both male and female floral functions,[97] and antagonistic or ineffective visitors are filtered out through chemical and/or morphological filters.[21,98] The extraordinary spatial and temporal variation in volatile chemistry demonstrated for the voodoo lily (*Sauromatum guttatum*; Araceae), a trap inflorescence that engages in brood-site deception,[99] speaks to the great potential for such division of labor in complex flowers that trap their pollinators.[100]

Protogyny: one of two outcomes of dichogamy, the temporal separation of sex expression in hermaphroditic flowers. In protogyny, the stigma is receptive before pollen matures and is dehisced from anthers; thus the flower shows female expression before male expression. Trap or kettle flowers often are protogynous, such that pollen from trapped insects is acquired on the stigma before the flower deposits its own pollen on the trapped insect, which is released afterwards.

5 Methodological Considerations for Studying Floral Volatiles

We conclude with a discussion of emerging analytical methods that should allow investigators to track dynamic floral emissions with finer temporal resolution and detector sensitivity. The earliest studies of floral scent expressly recognized the rhythmic nature of volatile emissions.[101,102] However, the low sensitivity of chromatographic methods available during the first 25 years of research on floral volatiles required protracted sampling, often from large numbers of flowers, resulting in low spatial and temporal resolution.[19] Most of the data presented in this paper as a case study were collected using two complementary approaches that facilitate the analysis of volatiles from living or recently excised flowers (as opposed to vacuum steam distillation or essential oil preparation). Dynamic headspace approaches involve sampling scent-laden air from floral headspace with replacement, using pumps to push or pull filtered air through a glass or inert plastic headspace chamber onto a pre-column adsorbent cartridge, from which the trapped volatiles are eluted with solvent or directly with

heat.[66,103] A clear benefit of dynamic headspace methods is the measurement of emission rates standardized per floral number or mass, which can be used to experimentally manipulate floral traits.[104,105] Improvements in the performance and sensitivity of capillary GC-MS, combined with the capacity to reduce the volume of pre-column headspace sorbent traps and elution solvents, allowed researchers to sample repeatedly at 1–3 hr intervals from the same, living flower(s), and thus to track diel and ontogenic changes in scent composition and emission rates with finer temporal resolution.[106,107]

In contrast, static headspace methods, in which floral volatiles equilibrate within the headspace chamber and are adsorbed onto a solid matrix, provide cleaner (solvent-free), more abundant volatile samples for chemical identification but do not provide quantitative data on emission rates. Solid phase micro-extraction (SPME) fibers, constructed of materials used as stationary phases in GC or LC columns (e.g. polydimethylsiloxane (PDMS) or divinylbenzene (DVB)), are used as adsorptive surfaces to trap headspace volatiles, which then are inserted (like a syringe) into a GC injection port with a modified capillary glass liner.[108,109] This method allows the sensitive analysis of volatiles from individual flowers or their dissected tissues, equilibrated in small vials or cuvettes, rapidly enough to avoid the artifacts of senescence.[94,110] Variations on this theme involve SPME-like devices embedded within syringe needle traps,[111] those with expanded surface area (stir-bar SPME or Twister™ probes),[112] sample-enrichment probes fitted with silicone rubber[113,114] or incubation of floral samples with lengths of the silicone tubing itself, combined with thermal desorption and cryo-focusing before GC-MS analysis.[115]

A hybrid approach combining dynamic sampling and direct thermal desorption-GC-MS is the micro-solid phase extraction (mSPE) method developed by Amirav and Dagan.[116] In this method, the adsorbent cartridges used in dynamic headspace approaches are miniaturized to quartz tubes with 1–5 mg of a thermally stable sorbent (e.g Tenax TA), attached to vacuum pumps using reduction tubing, and used to collect volatiles on the scale of minutes, rather than hours. This approach also requires a modified injection port, in which the cartridges are placed directly into a modified liner for thermal desorption. Dötterl and Jürgens,[117] in particular, have applied mSPE-GC-MS to rapidly and reproducibly sample volatiles from freshly dissected flower parts, including anthers and pollen,[118] and in comparative studies of plants with highly volatile compounds often masked by elution solvents.[119] If the investigator is careful to reduce variation between cartridges and protect against sample break-through, mSPE-GC-MS is appropriate for high temporal resolution volatile sampling.

The methodological approaches described above are well suited to exploratory or comparative analyses in which there is a need to identify or track tens to hundreds of unknown volatiles. When the compounds of interest already have been identified, two additional methods may be used to more sensitively track spatial and temporal dynamics of volatile emissions from living plants. The first is a device called the zNose™, which combines a fast, portable GC (1 m column) with a surface acoustic wave (SAW) detector, providing temporal resolution as low as 3 min.[120]. The potential for continuous, unmonitored sampling makes the zNose ideal for tracking diel or ontogenic changes in floral volatiles as well as herbivore- or pathogen-induced changes in vegetative emissions. The second is the marginally portable but extremely sensitive proton transfer reaction mass spectrometer (PTR-MS), which allows real time tracking of known volatile components on the scale of seconds (or less), through the combination of a proton transfer drift tube with a quadrupole mass spectrometer.[121] The most recent application of this method to floral volatiles was by Riffell et al.,[122] who used PTR-MS to track the temporal and spatial dynamics of behaviorally active components of the floral scent blend of *Datura wrightii* (e.g. benzaldehyde and linalool) with increasing distance from living flowers. Several reviews published within the last decade compare the suitability, portability and cost of these methods from academic vs. commercial standpoints.[66,103,123]

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Robert Raguso grew to appreciate chemically mediated insect–plant interactions during his undergraduate research on butterflies at Yale University. He studied the genetics, physiology and ecology of floral volatiles for his doctoral research at the University of Michigan and insect olfaction and behavior for his postdoctoral work in the Center for Insect Science at the University of Arizona, where he met his co-author, Martha Weiss. He currently serves as chair of the Department of Neurobiology and Behavior at Cornell University. His research interests include multi-modal communication and signal evolution, the role of "private channels" in obligate mutualism and the impact of geographic mosaics of coevolution on variation in chemical phenotypes.