

# The missing stink: sulphur compounds can mediate a shift between fly and wasp pollination systems

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The radiation of the angiosperms is often attributed to repeated evolutionary shifts between different pollinators, as this process drives diversification of floral forms and can lead to reproductive isolation. Floral scent is an important functional trait in many pollination systems but has seldom been implicated as a key mechanism in pollinator transitions. In this study, we suggest a role for sulphur compounds in mediating a shift between specialized carrion-fly and pompilid-wasp pollination systems in *Eucomis* (Hyacinthaceae). Flowers of closely related *Eucomis* species pollinated by carrion flies or pompilid wasps have very similar greenish-white flowers, but differ markedly in floral scent chemistry (determined by GC–MS analysis of headspace extracts). Comparison of the floral colours of the four *Eucomis* species in the visual systems of flies and wasps suggests that colour plays little role in pollinator discrimination. Nectar properties and morphology also do not differ strongly between fly- and wasp-pollinated flowers. By comparing floral scent bouquets and experimentally manipulating the scent of plants in the field, we demonstrate that shifts between wasp and fly pollination in these four congeners can depend on the production or suppression of sulphur compounds (dimethyl disulphide and dimethyl trisulphide) in the fragrance bouquet. This suggests that mutations affecting the production of particular scent compounds could precipitate shifts between pollinators, independently of floral morphology, colour or nectar properties.

**Keywords:** pollinator transition; floral evolution; pollination syndrome; myophily; oligosulphide

## 1. INTRODUCTION

Pollinator-mediated selection on floral traits typically arises from the morphology, behaviour and sensory modalities of particular pollinator types (Harder & Johnson 2009). Evolutionary shifts between pollinators can therefore diversify floral forms and may result in reproductive isolation (Grant 1949; Stebbins 1970; Johnson 2006). Understanding the mechanisms responsible for precipitating a shift from one pollen vector to another is thus of particular interest, as it may often represent the initial stage of plant speciation. To this end, a number of studies have investigated the functional significance of morphology or floral colour for pollinator attraction in closely related species (Bradshaw & Schemske 2003; Irwin & Strauss 2005; Hoballah *et al.* 2007; Cooley *et al.* 2008; Thomson & Wilson 2008). However, the role of floral scent in these transitions has generally been ignored.

Floral scent functions as a pollinator attractant in many pollination systems (Raguso 2001; Dudareva & Pichersky 2006) and often forms the basis for highly specialized plant–pollinator interactions (Schiestl *et al.* 1999, 2003; Brodmann *et al.* 2008; Shuttleworth & Johnson 2009a,b,c). The adaptive significance of floral scent has also been suggested by studies that demonstrate associations between scent composition and various pollinator types (e.g. Knudsen & Tollsten 1993, 1995;

Raguso & Pichersky 1995; Jürgens *et al.* 2002, 2003; Knudsen *et al.* 2004; Terry *et al.* 2004). The functional role of floral scent in many plant–pollinator interactions (e.g. Raguso & Pichersky 1995; Schiestl *et al.* 1999, 2003; Raguso 2001; Brodmann *et al.* 2008; Chen *et al.* 2009; Shuttleworth & Johnson 2009a,b,c) suggests that changes in the production of particular volatiles by flowers may, in some systems, alter their attractiveness and initiate shifts between different pollinator types (Kessler *et al.* 2008).

Groups of closely related species that show divergence in pollinators and floral scent, rather than morphology or colour, are ideal for exploring the role of fragrance evolution in pollinator transitions. The African genus *Eucomis* L'Hér. (Hyacinthaceae) represents a promising study system, as the 10 species have morphologically unspecialized flowers with exposed nectar and yet apparently have highly specialized and divergent pollination systems (Shuttleworth & Johnson 2009a; this study). In a previous study, we showed that pollinator attraction in the specialized pompilid-wasp pollination systems of *Eucomis autumnalis* and *E. comosa* is based primarily on floral fragrance (Shuttleworth & Johnson 2009a). Preliminary observations of several closely related *Eucomis* species (*E. bicolor*, *E. humilis*, *E. schijffii*, *E. montana* and *E. vandermerwei*) indicated that they differ dramatically from the wasp-pollinated species in scent chemistry and are pollinated by carrion flies. We thus hypothesized that the divergent pollination systems in *Eucomis* are mediated by differences in floral scent chemistry rather than morphology, floral colour or nectar properties.

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The aims of this study were (i) to establish whether some *Eucomis* species depend on carrion flies for pollination, (ii) to compare the floral fragrance, colour, morphology and nectar properties of these fly-pollinated species with wasp-pollinated *Eucomis* species to establish which traits are associated with divergent evolution, and (iii) to determine whether a pollinator shift could be induced by experimental manipulation of floral scent.

## 2. MATERIAL AND METHODS

### (a) *Pollination systems and dependence on pollinators*

This study focused on the putatively fly-pollinated taxa *E. bicolor* Baker and *E. humilis* Baker, and was conducted in the montane grasslands of Royal Natal National Park (RNNP) in the South African Drakensberg mountains (28°42'54" S, 28°53'43" E, altitude 2400 m). Additional visitor observations for *E. bicolor* were obtained in a small population on Gilboa Estate in the Karkloof mountains of KwaZulu-Natal (29°15'01.6" S, 30°15'21.6" E, altitude 1532 m). Floral visitors to both species were recorded between 1999 and 2009 (electronic supplementary material, table S1). Representative insects were collected for quantification of pollen loads in the laboratory.

The dependence of *E. bicolor* and *E. humilis* on pollinator visits was established by comparison of seed set from naturally pollinated and bagged (for autonomous seed set) flowers at RNNP in the 2007/2008 flowering season. Plants were bagged at the bud stage and left for *ca* six weeks to allow fruit development (*E. bicolor*:  $n = 793$  flowers on nine plants; *E. humilis*:  $n = 610$  flowers on 10 plants). Natural seed set was measured from plants adjacent to the bagged plants and at the same stage of development (*E. bicolor*:  $n = 689$  flowers on nine plants; *E. humilis*:  $n = 537$  flowers on eight plants). Means were calculated per plant and used as replicates for obtaining a grand mean for each treatment group.

### (b) *Morphometrics and spectral reflectance analysis of flowers*

Morphometrics were measured from specimens housed in the Bews Herbarium (University of KwaZulu-Natal, Pietermaritzburg). Lengths and widths of the inflorescence (cm) and of individual tepals (mm) were measured for *E. autumnalis* subsp. *clavata* ( $n = 22$ ), *E. bicolor* ( $n = 18$ ), *E. comosa* ( $n = 17$ ) and *E. humilis* ( $n = 20$ ). Plant means for tepal dimensions were calculated from measurements of three individual flowers. Morphometric measurements were plotted in two dimensions using non-metric multi-dimensional scaling (NMDS) and analysed as described for the fragrance analysis (see below).

*Eucomis bicolor* flowers are dull greenish-white with purple along the edges, whereas *E. humilis* flowers are more variably coloured, ranging from plain yellow-green to entirely purple (figure 1). Spectral reflectance (%) across the 300–700 nm range was measured using methods described in Shuttleworth & Johnson (2009a). The tepals (each from a separate plant) of four *E. bicolor* and eight *E. humilis* flowers (four green and four purple, to test whether the plants' pollinators perceived them differently) from RNNP were measured in December 2007 and December 2006, respectively. Spectra for *E. autumnalis* and *E. comosa* are presented in Shuttleworth & Johnson (2009a).

The similarity of these flowers as perceived by their pollinators was determined by plotting the reflectance spectra of

the four *Eucomis* species as loci in the bee colour hexagon (Chittka 1992) and the blowfly colour vision model (Troje 1993). Although the visual abilities of pompilid wasps are not known, the colour hexagon is a suitable model for most higher Hymenoptera (Chittka *et al.* 1992). Pompilid wasps probably have a similar trichromatic visual system to the honeybee (*Apis mellifera*), although the peak sensitivities ( $\lambda_{\max}$ ) of the wasp photoreceptors may differ slightly. Colour distance in the hexagon was calculated as the Euclidean distance between loci. The blowfly model is based on behavioural experiments with *Lucilia* sp. calliphorids and should thus be well suited to modelling the visual capabilities of the carrion- and blowfly pollinators of *E. bicolor* and *E. humilis* (Troje 1993). According to this model, flies exhibit categorical colour vision, such that spectral stimuli are discriminated only when they fall within separate categories with boundaries at 400 and 515 nm (Troje 1993; Arnold *et al.* 2009). The opponent system is based on the relative excitations of the two p-type and two y-type receptors, so the perceived colour depends on the receptor of each pair that is stimulated most strongly. Thus, the four possible colour categories (p+y+, p+y-, p-y+ and p-y-) correspond to the difference in excitation between the receptors of each type (Troje 1993; Arnold *et al.* 2009). All stimuli within each category are considered indistinguishable to flies (Troje 1993).

### (c) *Nectar properties*

Nectar production during 24 h was measured for *E. bicolor* and *E. humilis* in December 2007. Plants were cut and kept in vases overnight. Nectar present at the beginning of the period was removed. Nectar volume ( $\mu$ l) and concentration (percentage sucrose equivalents by mass) were measured using 5  $\mu$ l capillary tubes and a Bellingham & Stanley 0–50% or 45–80% sugar concentration hand-held refractometer. Means were calculated per plant and these used to calculate a grand mean for the population. Differences in nectar properties between pollination systems were compared using an ANOVA with species nested within pollination system. Mean nectar volumes were log transformed to achieve homogeneity of variance.

Nectar sugar composition was determined by high-performance liquid chromatography using methods described in Brown *et al.* (2009). Nectar was collected from plants at RNNP in December 2007 (*E. bicolor* and *E. humilis*), Midmar Nature Reserve in November 2007 (*E. autumnalis* subsp. *clavata*) and Gilboa Estate in January 2008 (*E. comosa*; see Shuttleworth & Johnson (2009a) for details of the latter two study sites).

### (d) *Fragrance analysis and behavioural assays*

Floral scent for *E. bicolor* and *E. humilis* was collected using dynamic headspace sorption methods (described in Shuttleworth & Johnson 2009a) and analysed by coupled gas chromatography–mass spectrometry (GC–MS). Solvent samples (*E. bicolor* S1 and *E. humilis* S1, see electronic supplementary material, table S2, for sampling details) were analysed by Dr Roman Kaiser (Givaudan, Switzerland) using methods described in Kaiser & Tollsten (1995). Thermal desorption samples (five for each species; see electronic supplementary material, table S2, for sampling details) were analysed on a Varian CP-3800 GC (Varian, Palo Alto, CA, USA) coupled to a Varian 1200 quadrupole mass spectrometer with a Varian 1079 injector equipped with a 'ChromatoProbe' thermal desorption device using methods

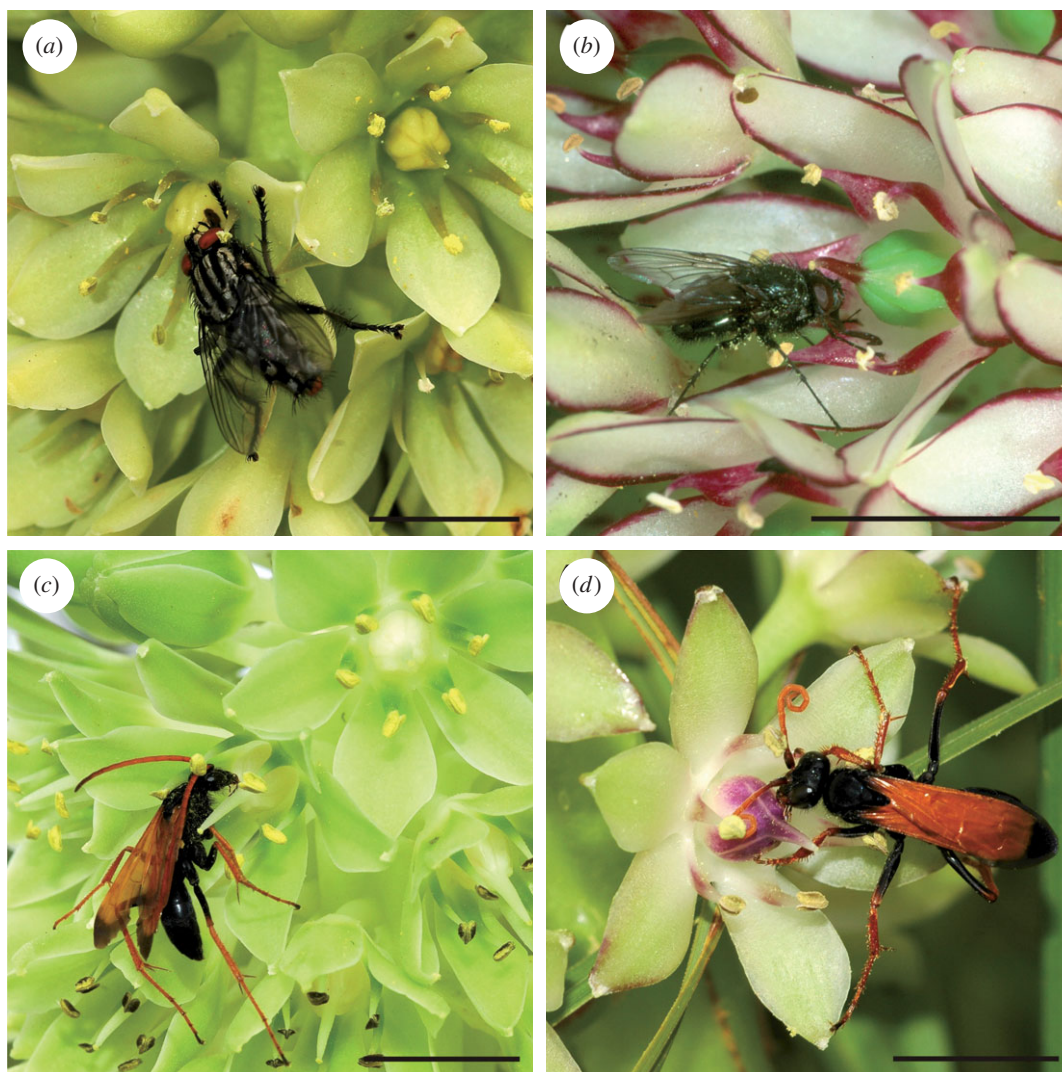


Figure 1. Flowers of the four *Eucomis* species with their pollinators. (a) *E. humilis* being visited by Sarcophaginae sp. 1, Witsieshoek Resort. (b) *E. bicolor* being visited by *Phaonia* sp. 1, Sentinel Peak. (c) *E. autumnalis* being visited by *Hemipepsis capensis*, Vernon Crookes Nature Reserve. (d) *E. comosa* being visited by *H. capensis*, Gilboa Estate. Scale bars, 10 mm.

described in Shuttleworth & Johnson (2009a). Quantification was based on 68 synthetic standards injected and thermally desorbed under identical conditions to the samples (Shuttleworth & Johnson 2009a).

To determine whether differences in the floral scents are related to the pollination system, we plotted fragrance data for the four *Eucomis* species (data for the wasp-pollinated species are presented in Shuttleworth & Johnson (2009a)) in two dimensions with NMDS using Primer 6.1.6 (2006) (Clarke & Warwick 2001; Clarke & Gorley 2006). NMDS was based on Bray–Curtis similarity and data were square-root transformed prior to analysis. Differences in the fragrance profiles between species and pollination systems were tested using ANOSIM (Clarke & Gorley 2006), a non-parametric permutation procedure based on the similarity matrix underlying the ordination (Clarke & Warwick 2001). The test statistic  $R$  compares average rank similarities within and among groups.  $R$  close to unity indicates complete separation of groups, whereas  $R$  close to zero indicates minimal separation among groups. Observed  $R$ s were compared with the distribution of  $R$  generated by up to 10 000 random permutations of the sample labels to assess statistical significance, which depends on replicate

sample size and must be interpreted accordingly (Clarke & Warwick 2001). Volatiles characterizing the fragrance of each species and each pollination system were explored using the similarity percentages (SIMPER) function in Primer (Clarke & Gorley 2006). SIMPER identifies compounds that contribute most to the average similarity within a particular group (a species or pollination system).

To establish the effects of sulphur-compound production by non fly-pollinated *Eucomis* flowers on pollinator preference, we added a 1:1 blend of dimethyl disulphide (DMDS) and dimethyl trisulphide (DMTS) to inflorescences of the wasp-pollinated *Eucomis* species and then recorded floral visitor behaviour. Two similar-sized inflorescences were cut and placed in vases. A small (10 ml) vial with a cotton dispenser wick and containing either 100  $\mu$ l DMDS, 100  $\mu$ l DMTS and 800  $\mu$ l white mineral oil (to slow evaporation) or 1000  $\mu$ l of white mineral oil only (for the control) was then attached to the base of each inflorescence and concealed from view with leaves. Emission rates of oligosulphides (quantified by injection and thermal desorption of synthetic standards under identical conditions to the flower samples) from vials identical to those used for the experimental inflorescences, quantified at 30 min

intervals during 3.5 h, ranged from 31.5 to 8.8  $\mu\text{g min}^{-1}$  for DMDS and from 35.9 to 19.0  $\mu\text{g min}^{-1}$  for DMTS. Experimental inflorescences were placed *ca* 1 m apart and the insects visiting flowers on each inflorescence were noted. The absence of sulphur compounds from the natural scents of the two inflorescences were confirmed by GC–MS after the experiments were completed (sufficient time was allowed for any residual sulphur compounds to evaporate prior to analysis). Flowers on both experimental and control inflorescences were emasculated to avoid contaminating gene pools.

Bioassays with *E. autumnalis* subsp. *clavata* were conducted near a patch of *E. humilis* at RNNP during December 2008. The inflorescences were moved to new positions three times during the 3.5 h of this experiment, but control and scent treatments were not switched between plants. Bioassays with *E. comosa* were conducted at Gilboa Estate (see Shuttleworth and Johnson (2009a) for details of the study site) during January 2009. The sulphur and the control vials were switched between inflorescences once and the inflorescences were moved to new positions once during the 2 h of this experiment.

### 3. RESULTS

*Eucomis bicolor* and *E. humilis* are both visited almost exclusively by flies in the families Calliphoridae, Muscidae and Sarcophagidae (electronic supplementary material, table S1; figure 1a,b). Pollen is deposited haphazardly on the flies' bodies as they lap nectar, which is secreted by the sepal nectaries and gathers at the ovary base. Pollen transfer occurs as flies move around on flowers and brush against the erect stigmas (figure 1). Naturally pollinated flowers of both species produced significantly more seeds than bagged flowers (*E. bicolor*: bagged =  $0.003 \pm 0.002$ , naturally pollinated =  $1.7 \pm 0.28$ ,  $t = 5.87$ ,  $p < 0.001$ ; *E. humilis*: bagged =  $0.1 \pm 0.04$ , naturally pollinated =  $2.1 \pm 0.36$ ,  $t = 5.30$ ,  $p = 0.001$ ; means  $\pm$  s.e. seeds per flower per plant).

Floral morphologies of fly- and wasp-pollinated *Eucomis* species are not strongly divergent (figure 1 and electronic supplementary material, figure S1). Tepals and individual flowers of the four *Eucomis* species have similar dimensions, although the inflorescences of *E. comosa* are larger than those of the other species (figure 1 and electronic supplementary material, figure S1). Morphometric data did not separate into discrete clusters in the NMDS analysis, although some species differed significantly (global  $R = 0.43$ ,  $p < 0.001$ ; figure 2b) because of the differences between *E. comosa* and the other species (figure 2b). Floral morphology differed significantly between pollination systems ( $R = 0.168$ ,  $p < 0.001$ ), although  $R$  was much smaller than for scent differences.

Colours of fly- and wasp-pollinated *Eucomis* flowers are also similar (electronic supplementary material, figure S2; Shuttleworth & Johnson 2009a). *Eucomis bicolor* flowers have a dirty white spectrum with low overall reflectance (electronic supplementary material, figure S2). *Eucomis humilis* flowers have low overall reflectance that peaks between 500 and 600 nm (green; electronic supplementary material, figure S2) with the purple flowers exhibiting an additional peak between 600 and 700 nm (red; electronic supplementary material, figure S2). Neither species reflects ultraviolet light (electronic

supplementary material, figure S2). These reflectance spectra (especially for *E. humilis* and the two wasp-pollinated species) are similar to the spectra of background vegetation (Shuttleworth & Johnson 2009a).

Floral-colour loci for the four *Eucomis* species all fall in the blue–green to green region of the colour hexagon (figure 3a) and in the green quadrant of the blowfly colour vision model (with the exception of *E. bicolor*, which is marginally in the blue quadrant; figure 3b). These floral colours are also similar to the colour of green foliage background (represented by the centre of the hexagon; figure 3a). Loci for green and purple *E. humilis* flowers are close together in the colour hexagon and fall within the same category in the fly model (as a result of the limited sensitivity of Hymenoptera and fly photoreceptors in the red end of the spectrum (Troje 1993; Chittka & Waser 1997; figure 3). Using 0.1 hexagon units as a practical threshold for colour discrimination (Chittka *et al.* 1997, but see Dyer & Chittka 2004a,b; Dyer *et al.* 2008), wasps would not distinguish *E. comosa* (wasp-pollinated) from *E. humilis* (fly-pollinated), but would be able to discriminate between the other species (figure 3a). Flies would be unable to distinguish *E. humilis* from the two wasp-pollinated species (figure 3b).

Nectar properties were not clearly associated with pollination systems in the four *Eucomis* species (electronic supplementary material, table S3). Nectar volume was not significantly different between pollination systems ( $F_{1,14} = 0.697$ ,  $p = 0.418$ ). Nectar concentration varied considerably among species (electronic supplementary material, table S3) and differed significantly between pollination systems ( $F_{1,14} = 76.4$ ,  $p < 0.001$ ; see electronic supplementary material, table S3, for species comparisons). All four *Eucomis* species exhibit hexose-dominant nectar (electronic supplementary material, table S3).

All four *Eucomis* species produce many floral volatiles encompassing various compound classes (electronic supplementary material, table S2; Shuttleworth & Johnson 2009a). Compounds characterizing the fragrance of each species (accounting for 50% of the similarity between conspecific samples) were similar for all four species, aside from the sulphur-containing compounds, especially DMDS and DMTS, which were only produced by the fly-pollinated species (table 1 and electronic supplementary material, table S2; Shuttleworth & Johnson 2009a). Linalool contributed a large percentage to conspecific similarity for all species (table 1). When species were grouped by the pollination system, linalool, 3,5-dimethoxytoluene and hotrienol contributed to the similarity of the scents of both fly- and wasp-pollinated species. However, (*E*)-ocimene contributed only to the wasp-pollinated species while DMDS, DMTS and (*E*)-linalool oxide (furanoid) contributed only to the fly-pollinated species (table 1).

Fragrance data for the four species separate into discrete clusters in the NMDS analysis (global  $R = 0.816$ ,  $p < 0.001$ ; figure 2a). The relatively large  $R$  values for pairwise comparisons (greater than 0.6) indicate distinct separation between the fragrance profiles of all species (figure 2a). The greatest separation was between the fly-pollinated *E. bicolor* and both wasp-pollinated species (*E. comosa* and *E. autumnalis*), as well as between the fly-pollinated *E. humilis* and the wasp-pollinated

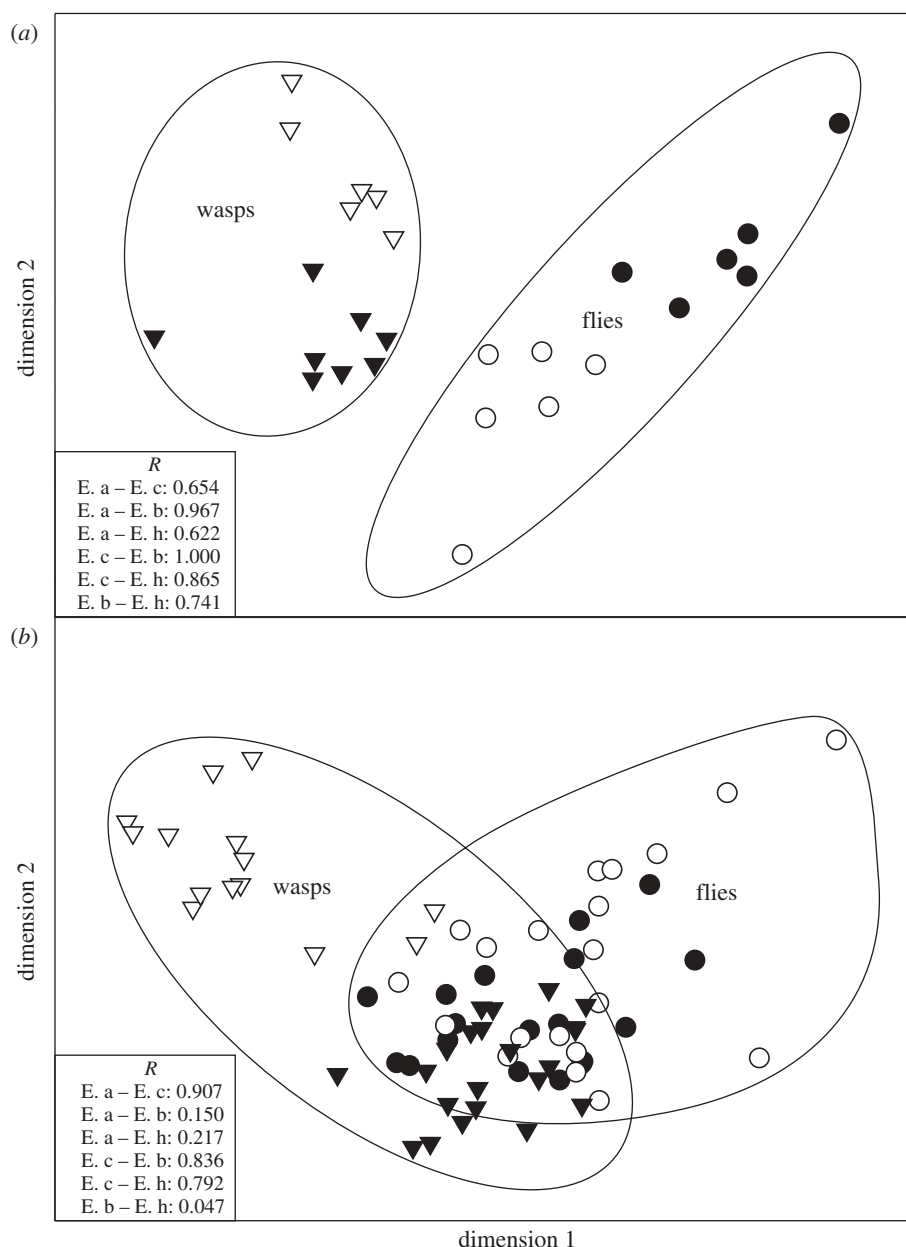


Figure 2. (a) Floral scent profiles (stress = 0.12) and (b) morphometric measurements (stress = 0.05) of fly- and wasp-pollinated *Eucomis* species plotted in two dimensions based on non-metric multi-dimensional scaling (NMDS). E. a, *E. autumnalis*; E. b, *E. bicolor*; E. c, *E. comosa*; E. h, *E. humilis*. Filled circles, *E. bicolor*; open circles, *E. humilis*; filled triangles, *E. autumnalis*; open triangles, *E. comosa*.

*E. comosa* (figure 2a). Fragrance profiles differed significantly between pollination systems (global  $R = 0.678$ ,  $p < 0.001$ ).

In the bioassays, inflorescences of the normally wasp-pollinated species with sulphur compounds experimentally added attracted large numbers of flies (mostly calliphorids and sarcophagids) that would not typically visit these flowers (Binomial test comparing fly visits to experimental and control inflorescences,  $p < 0.001$  for both *Eucomis* species; figure 4 and electronic supplementary material, table S4). Addition of sulphur compounds did not deter pompilid wasps (binomial test,  $p = n.s.$  for both species). Flies landing on experimental flowers exhibited differing behaviours: some perched on the inflorescences and then periodically explored flowers and drank nectar, whereas others (especially sarcophagids) immediately lapped nectar after arriving at flowers.

Visiting flies frequently contacted stigmas. Very few flies crawled down to the base of the inflorescence to explore the vials from which sulphides were emitted.

#### 4. DISCUSSION

Sulphur compounds in floral scent primarily differentiate fly and wasp pollination systems in *Eucomis*. Differences in floral colour (figure 3), nectar properties (electronic supplementary material, table S3) and morphologies (figures 1 and 2b; electronic supplementary material, figure S1) among the four species were minor and not clearly associated with the two pollination systems. Indeed, inflorescences of the fly-pollinated *E. humilis* are often difficult to distinguish from those of the wasp-pollinated *E. autumnalis* in the field (figure 1; Shuttleworth & Johnson 2009a). In contrast, floral scent chemistry

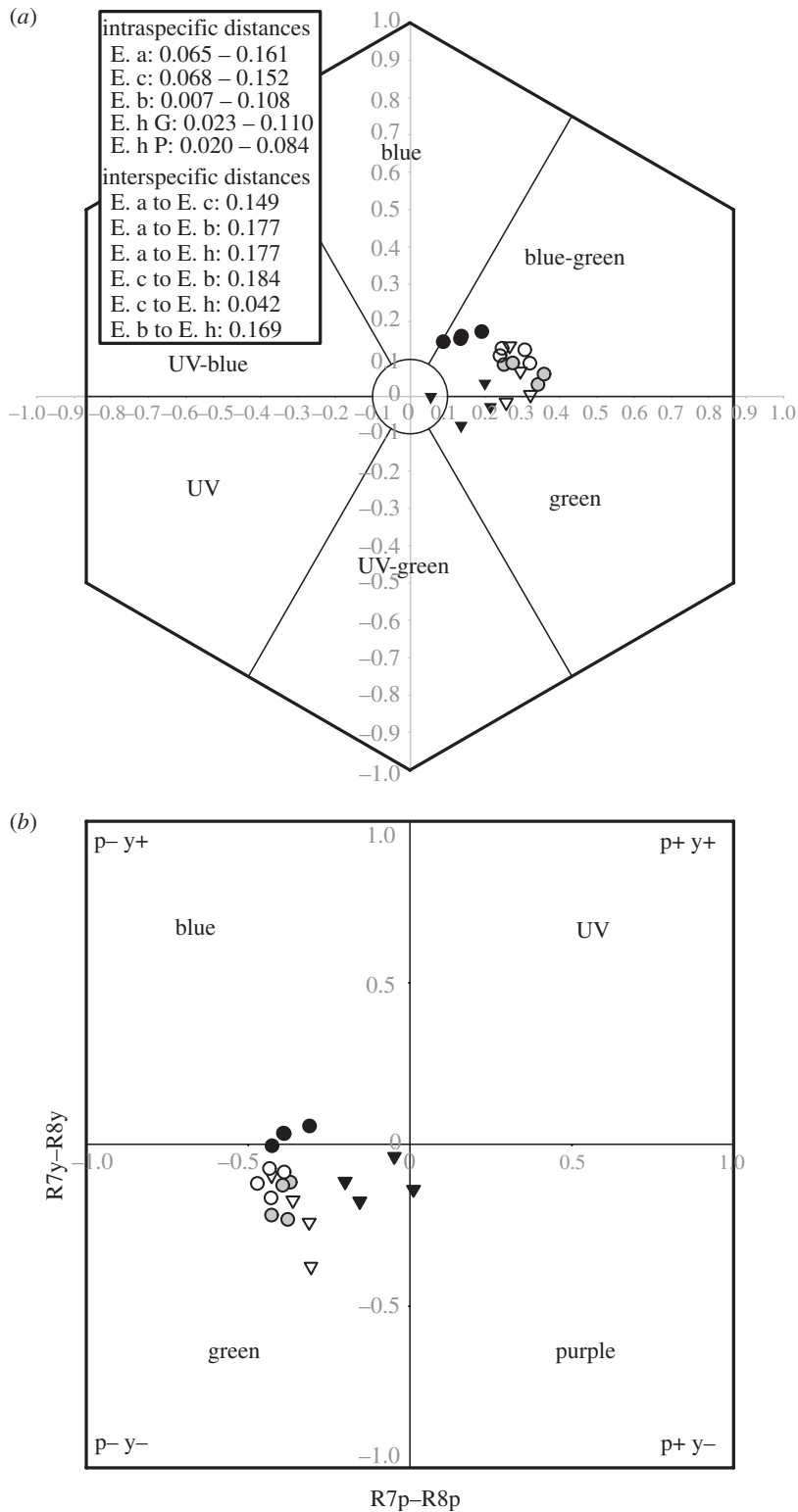


Figure 3. Floral colours of the four *Eucomis* species in bee and fly colour space. (a) Loci in the bee colour hexagon (in this case used as a model of wasp vision; Chittka 1992). The six segments represent the six categories of bee colour vision. Loci are calculated from the relative stimulation of the three receptor types (UV, blue and green) by the spectral reflectance of the flowers. Distance between loci in the hexagon correlates to discriminability of the colours by bees (or in this case wasps). Intraspecific distances (hexagon units) represent the range of distances between conspecific samples. Interspecific distances (hexagon units) represent the distances between the mean loci for each species (human green and human purple *E. humilis* pooled). E. a, *E. autumnalis*; E. b, *E. bicolor*; E. c, *E. comosa*; E. h, *E. humilis* (green and purple flowers indicated by G and P, respectively). (b) Loci in the blowfly colour vision model suggested by Troje (1993). Flies exhibit a categorical colour vision system such that spectral stimuli with loci in the same quadrant would not be discriminated (Troje 1993; Arnold *et al.* 2009). Filled triangles, *E. autumnalis*; open triangles, *E. comosa*; filled black circles, *E. bicolor*; filled grey circles, *E. humilis* human green; open circles, *E. humilis* human purple.

Table 1. Percentage contributions of particular scent compounds to the average similarity (based on Bray–Curtis similarity coefficient) between intraspecific fragrance samples for each *Eucomis* species (compounds listed here account for the first 50% of overall intraspecific similarity).

| compound                               | wasp-pollinated      |                  | fly-pollinated    |                   | fly  | wasp |
|--|----------------------|------------------|-------------------|-------------------|------|------|
|  | <i>E. autumnalis</i> | <i>E. comosa</i> | <i>E. bicolor</i> | <i>E. humilis</i> |      |      |
| linalool                               | 26.4                 | 15.1             | 16.3              | 17.7              | 21.7 | 24.8 |
| 3,5-dimethoxytoluene                   | —                    | 19.3             | 7.1               | 3.2               | 5.8  | 9.5  |
| hotrienol                              | 11.9                 | 8.1              | —                 | 10.3              | 4.3  | 12.1 |
| ( <i>E</i> )-linalool oxide (furanoid) | 5.0                  | 4.0              | —                 | 7.3               | 4.4  | —    |
| ( <i>E</i> )-ocimene                   | 3.9                  | 6.7              | —                 | —                 | —    | 5.7  |
| 1-octen-3-ol                           | 4.0                  | —                | —                 | —                 | —    | —    |
| dimethyl disulphide                    | —                    | —                | 9.9               | 4.9               | 9.1  | —    |
| dimethyl trisulphide                   | —                    | —                | 6.4               | —                 | 5.2  | —    |
| ( <i>Z</i> )-linalool oxide (furanoid) | —                    | —                | 6.4               | —                 | —    | —    |
| indole                                 | —                    | —                | 5.7               | —                 | —    | —    |
| 4-methylpentan-2-one                   | —                    | —                | —                 | 5.0               | —    | —    |
| 4-methylpent-3-en-2-one                | —                    | —                | —                 | 3.4               | —    | —    |
| total                                  | 51.2                 | 53.1             | 51.8              | 51.9              | 50.5 | 52.1 |
| average similarity                     | 61.3                 | 68.3             | 64.5              | 61.5              | 49.7 | 53.9 |

differed qualitatively between wasp- and fly-pollinated species, with the latter producing large amounts of sulphur-containing compounds that are absent from the scents of the two wasp-pollinated species (table 1 and electronic supplementary material, table S2; Shuttleworth & Johnson 2009a). Experimental addition of sulphur compounds to wasp-pollinated flowers induced a clear ecological shift in that experimental flowers immediately attracted flies, thus confirming the functional significance of sulphur compounds for fly-pollinated *Eucomis* plants (figure 4 and electronic supplementary material, table S4).

The overlap in the colours of wasp- and fly-pollinated *Eucomis* species in the colour hexagon and the blowfly model suggests that colour is not used by pollinators to discriminate these species (figure 3; Chittka 1992; Troje 1993). Floral colour in *Eucomis* species probably evolved as a form of crypsis to minimize visits by non-pollinating insects (Shuttleworth & Johnson 2009a) rather than as a pollinator attractant. The open, morphologically unspecialized, flowers of *Eucomis* species (figure 1) allow for pollen removal by a diverse spectrum of insects, and the costs of pollen loss to unfaithful floral visitors (Hargreaves *et al.* 2009) may be the basis for the evolution of this cryptic colouring.

Unlike other floral traits, odours clearly differ between pollination systems in these four *Eucomis* species (table 1 and figure 2a). Specialization in the wasp-pollinated *Eucomis* species is based on floral scent (Shuttleworth & Johnson 2009a), and this also appears to be the case in the fly-pollinated species. The sulphur compounds that characterize the scents of the two fly-pollinated species (table 1, figure 2a and electronic supplementary material, table S2) are commonly produced during protein decomposition and are known blowfly attractants, presumably guiding flies to feeding and oviposition sites (Stensmyr *et al.* 2002; Jürgens *et al.* 2006). Oligosulphides are also important floral volatiles of sapromyophilous staphylinids that mimic carrion odours (Jürgens *et al.* 2006). Our results suggest that sulphur compounds are a key functional trait that determines whether carrion- and blowflies visit (and potentially pollinate) *Eucomis* flowers

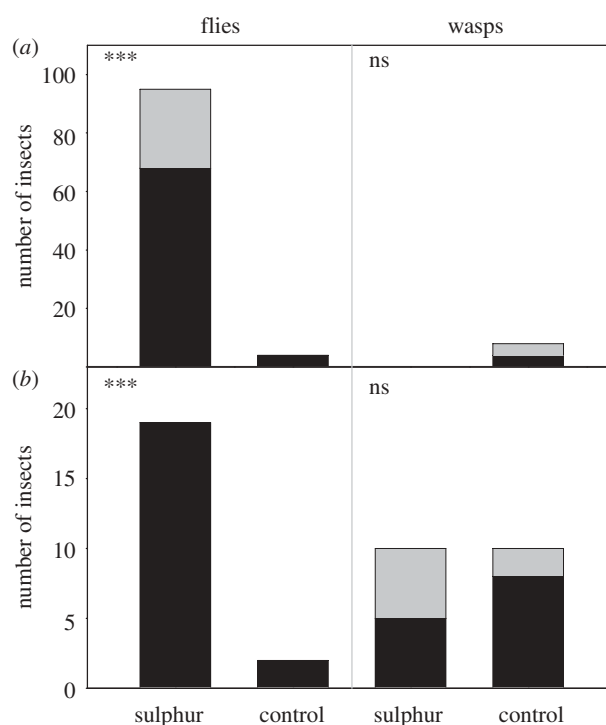


Figure 4. Attraction of flies and wasps to flowers of the wasp-pollinated (a) *E. autumnalis* subsp. *clavata* and (b) *E. comosa* with a 1:1 blend of DMDS and DMTS experimentally added to the inflorescences. The number of individual visits (excluding approaches) by different pollinator types to the inflorescences was compared with binomial tests. Approaches represent instances in which an insect flew up to the inflorescence, but did not actually land on the flowers. Grey bars, approaches; black bars, visits. \*\*\*,  $p < 0.001$ ; ns,  $p$  not significant.

(figure 4 and electronic supplementary material, table S4). However, flies seldom sought the actual oligosulphide-emitting vial in these experiments which suggests that visual cues within a scent context may play a role at short distances.

Attraction of insects by scent cues alone has traditionally been associated with sexually deceptive systems (Schiestl *et al.* 1999, 2003), brood site mimicry (Stensmyr *et al.* 2002) and the highly coevolved obligate mutualisms of figs and fig wasps (Chen *et al.* 2009) and yuccas and yucca moths (Svensson *et al.* 2005, 2006). However, our results suggest that this phenomenon also occurs in typical food-rewarding plants. Although showy to the human observer, *Eucomis* flowers are cryptically coloured and likely do not stand out from the background vegetation in the eyes of their pollinators (figure 3; Shuttleworth & Johnson 2009a). Furthermore, *E. autumnalis* flowers attract their wasp pollinators even when completely concealed from view, suggesting that floral scent is the primary attractant (Shuttleworth & Johnson 2009a). This study of *Eucomis* shows that in such systems, simple changes in the production of particular compounds could dramatically alter the assemblage of visitors attracted, which could, in turn, lead to shifts between different pollinators.

Most *Eucomis* species appear to be either wasp- or fly-pollinated, suggesting one or more shifts between these pollination systems. One scenario is that a mutant wasp-pollinated *Eucomis* began producing sulphur compounds, thereby attracting two different pollinator types (*sensu* Stebbins' (1970) 'intermediate stage of double function principle'). Differences in effectiveness of the two pollinator types could then drive specialization to one or the other, ultimately resulting in a complete transition between pollinators (Stebbins 1970). In such a situation, differences in effectiveness may depend on the abundance of the different pollinator types in particular regions or habitats. Both fly-pollinated *Eucomis* species are more common in high-altitude grasslands than are the wasp-pollinated species, possibly correlating with higher fly abundance and lower wasp abundance in this habitat (Arnold *et al.* 2009).

Pollinator transitions have generated considerable interest, as they may often represent the beginning of plant speciation (Grant 1949; Stebbins 1970; Johnson 2006). We have shown that changes in the production of sulphur compounds have the potential to precipitate a shift from wasp to fly pollination in *Eucomis* without associated changes in the morphology, colour or nectar properties of flowers. This study is one of the first to show convincingly that simple changes in the floral scents of plants can profoundly influence the assemblage of floral visitors that they attract. Although the sulphur compounds involved in the *Eucomis* systems are attractive to carrion flies, the principle applies broadly to any shifts involving pollinators that are attracted exclusively by particular floral scents, and so deserves more focused study.

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