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The role of volatile organic compounds, morphology and pigments of globeflowers in the attraction of their specific pollinating flies

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Summary

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Key words: *Chiastocheta*, floral morphology, insect attraction, nursery pollination mutualism, pigments, *Trollius europaeus*, volatile organic compounds.

- Floral scents and visual cues of the globeflower *Trollius europaeus* may play a key role in the attraction of *Chiastocheta* flies, involved in a highly specific nursery pollination mutualism.
- Here, headspace collection and GC-MS were used to identify and quantify the volatile organic compounds emitted by the globeflower.
- Scents are produced in three different floral parts by four structures: secretory glands and flat epidermis cells in the abaxial sepal epidermis, conical cells in the adaxial sepal epidermis, and pollen. The blend is made up of 16 compounds commonly found in floral scents. Geographical variation among populations is low compared with variation amongst individuals within populations. Electroantennographic analyses revealed that six compounds emitted by both anthers and sepals are detected by *Chiastocheta* flies. Removing the anthers hidden inside the globe from flowers in the field decreased the number of fly visits to globeflowers.
- A multivariate analysis of the effect of several floral traits on pollinator visitation rate conducted in the field showed that both floral scents and visual flower cues play a role in pollinator attraction. However, their relative roles and the intensity of the selective pressures exerted on floral traits by pollinators appear to vary in time and space.

Introduction

The main cues involved in pollinator attraction are visual and olfactory. Both the genetics and the ecology of flower colour and shape have been thoroughly studied (Clegg & Durbin, 2003). Flower scents have been studied less, but recent technical developments in the collection and analysis of volatile compounds (Tholl & Röse, 2006) have facilitated research on plant volatiles and led to an increase in scent-related studies. Until now, very few generalizations could be made about flower scent functions: each compound can have no (e.g. waste product or by-product of the synthesis of other molecules), one or several functions (e.g. pollinator

attractant, insect repellent or antibacterial activity; Dudareva & Pichersky, 2006). Nursery pollination mutualisms are highly specific interactions in which the specific pollinator's only egg-laying site is located on its host plant's reproductive structures (Dufay & Anstett, 2003). Amongst the nursery pollination mutualisms reported so far, odours produced by the host plants were studied for figs (Gibernau *et al.*, 1997, 1998; Grison-Pigé *et al.*, 2002), dwarf palms (Dufay *et al.*, 2003; Caissard *et al.*, 2004), *Macrozamia* cycads (Terry *et al.*, 2004), *Silene* species (Jürgens *et al.*, 2002; Dötterl & Jürgens, 2005), yuccas (Svensson *et al.*, 2005) and *Glochidion* trees (Okamoto *et al.*, 2007). Fig wasps were stimulated by the odour of their associated fig species but, in

most cases, not by the odour of another species (Grison-Pigé *et al.*, 2002; Proffit *et al.*, 2007). Synergistic effects between volatile compounds emitted by *Ficus hispida* appeared to be involved in both the host-finding and floral-stage discrimination of the pollinator (Chen & Song, 2008), whereas in *Ficus semicordata* one single unusual compound in *Ficus* ensured specific pollinator attraction (Chen *et al.*, 2009). *Epicephala* moths are able to discriminate their *Glochidion* host from nonhost plants by means of floral scents (Okamoto *et al.*, 2007). The nursery pollinator of *Silene latifolia*, that is *Hadena bicurris* (Lepidoptera, Noctuidae), mainly reacts to lilac aldehydes (Dötterl *et al.*, 2006). *Cycadotrrips chadwicki* thrips are attracted by ocimene isomers and β -myrcene produced by their cycad host plant (Terry *et al.*, 2007). No behavioural assays were conducted in the *Yucca*–*Tegeticula* pollination system; however, the pollinator of *Yucca filamentosa*, *Tegeticula cassandra* (Lepidoptera, Prodoxidae), responded strongly in electroantennographic analyses to an uncommon and so far undescribed dioxygenated compound (C₁₁H₁₈O₂), which might be responsible for attracting the moths (Svensson *et al.*, 2005). Plants involved in nursery pollination may produce either specific molecules to attract the nursery pollinator, as has been suggested in *Yucca*, or rely on a more or less specific combination of common compounds, such as 2-phenylethyl alcohol and 2-phenylaceto-nitrile in the *Breynia vitis-idaea*–*Epicephala* system (Svensson *et al.*, 2010).

The pollination of the globeflower *Trollius europaeus* L. (Ranunculaceae) depends entirely on specific Anthomyiidae flies of the *Chiastocheta* Pokorny genus. Six *Chiastocheta* species were described based on male genitalia morphology: *C. rotundiventris* Hennig, *C. dentifera* Hennig, *C. inermella* Zetterstedt, *C. macropyga* Hennig, *C. setifera* Hennig and *C. trollii* Zetterstedt (Collin, 1954). The adults spend most of their lifetime inside the globes where they mate and feed on nectar, and where the females lay eggs. They are almost never found on other flowers (Ibanez *et al.*, 2009a). The flies' ability to detect globeflowers is therefore critical from the moment the adult emerges. The *Trollius*–*Chiastocheta* mutualism involves large, colourful flowers and diurnal pollinators, which suggest that visual cues may be important in pollinator attraction and host finding. Several studies have indeed revealed the importance of floral shape and colour in this association. Pellmyr (1989) used simple yellow bowls as traps and showed that the colour alone can attract large numbers of flies. Ibanez *et al.* (2009a) showed that artificially opened flowers received fewer fly visits than the naturally occurring globose phenotype. However, *Chiastocheta* prefer to visit the artificially opened *Trollius* flowers over other yellow bowl-shaped flowers in the genus *Ranunculus* (SI, pers. obs.), which suggests that olfactory stimuli might also be at play in the specific attraction.

Here, we characterize the floral scent emitted by different globeflower populations, and investigate the role of these

volatile organic compounds (VOCs) in attracting their specific pollinating and seed-eating flies. We also compare the importance of floral scents in comparison to other floral traits in attracting the flies to the flowers. Specifically, we address the following questions: which compounds are emitted by globeflowers; which of these compounds can be detected by the flies; which floral structures produce the odours; and how important are the VOCs in attracting flies in comparison to other floral traits, such as globe morphology and pigment concentration?

Materials and Methods

The *Trollius europaeus*–*Chiastocheta* spp. system

The European globeflower is an arctic–alpine perennial species, growing in moist meadows. In the Alps, natural populations range from 700 to 2500 m altitude (Jaeger & Després, 1998; Després *et al.*, 2007). Flowering is synchronized within populations and typically lasts 2–3 wk. The hermaphroditic flowers are composed of yellow sepals fully enclosing the sexual parts of the flower (stamen and gynoecium, Fig. 1a) and are passively pollinated by six species of *Chiastocheta* flies (Després & Cherif, 2004; Pompanon *et al.*, 2006). Both male and female flies visit and pollinate the globe-shaped flower in which they eat nectar and pollen, and mate (Després, 2003). Females deposit eggs onto or between the carpels: each larva eats several seeds and falls on to the soil to pupate and overwinter. *Chiastocheta* larvae feed only on *T. europaeus* seeds; they are obligate associates of globeflowers (Després & Jaeger, 1999).

Headspace volatile collection and scent analyses

Four study sites were chosen in two mountain massifs in the French Alps: Banchet (1206 m asl, 5°47'E 45°18'N); Col de Porte (1326 m asl, 5°47'E 45°17'N); Lautaret (2100 m asl, 6°24'E 45°03'N); and Pré Gelé on the north side of the Col du Galibier (2300 m asl 6°24'E 45°04'N). Pairwise distances between sites ranged from 1.5 to 100 km. We analysed the blend of nine to 13 first-day flowers randomly chosen at each of the four sites in 2003 (see Table 1 for the sample size for each population). In total, 47 flowers were analysed to determine the scent compounds emitted by *T. europaeus* flowers, and to study scent variations within and across populations. Floral odours were collected using dynamic headspace sampling. First-day flowers are easily recognized as their outer stamens are just starting to dehisce. Each flower was enclosed in a Nalophan® bag (Kalle GmbH, Wiesbaden, Germany), and air cleaned through a charcoal filter was blown into the bags at 400 ml min⁻¹ and extracted at 300 ml min⁻¹ through an adsorption tube containing 30 mg of Alltech SuperQ (ARS Inc., Gainesville, FL, USA). Negative controls were yielded by

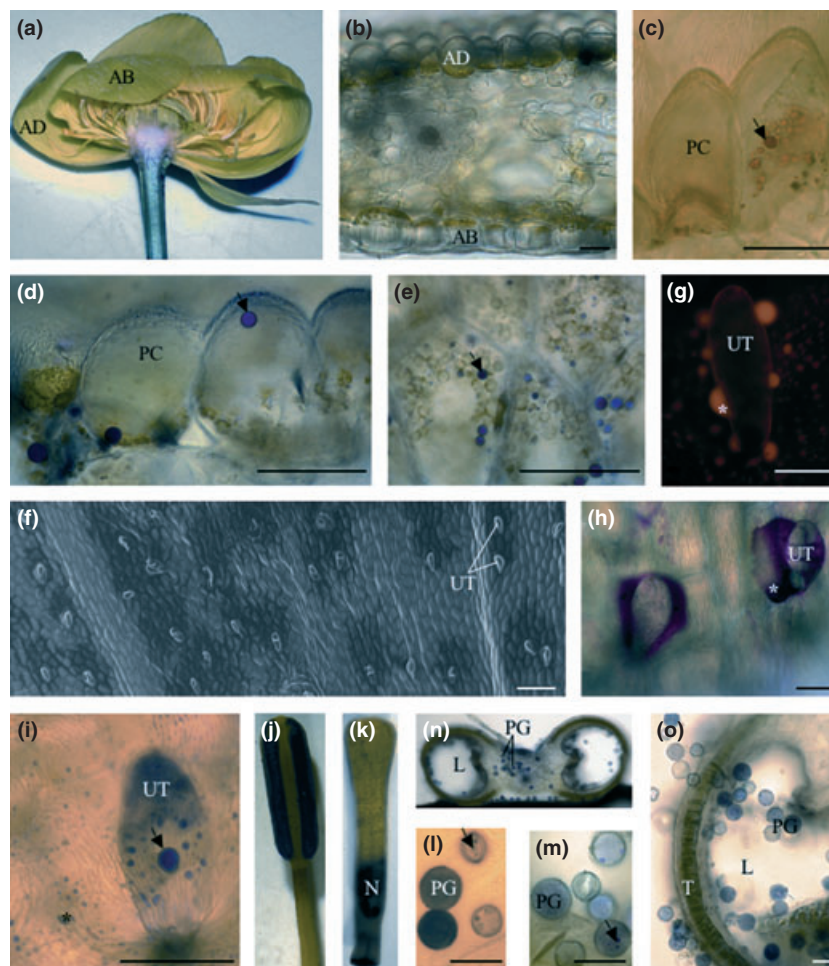


Fig. 1 Globeflower (*Trollius europaeus*) anatomy and the histochemical localization of lipids and terpenes. (a) Half-cut flower showing globe-shaped sepals enclosing sexual parts. (b) Unstained section of a sepal. (c, d) Sepal adaxial epidermis stained with Sudan red IV (c) or NaDi reagent (d). (e) Paradermal optical section of the sepal abaxial epidermis stained with NaDi reagent. (f) Environmental scanning electron microscopy (ESEM) top view of the sepal abaxial epidermis showing excretory glands. (g–i) Trichome of the sepal abaxial epidermis stained with Nile red (g), Nile blue (h) or NaDi reagent (i). (j, k) Stamen (j) and staminode (k) stained with Sudan black. (l, m) Pollen grain stained with Sudan black (l) or NaDi reagent (m). (n, o) Section of an anther stained with NaDi reagent (n) and detail showing tapetum (o). AB, abaxial epidermis; AD, adaxial epidermis; L, locellus; N, nectary; PC, papillate cell; PG, pollen grain; T, Tapetum; UT, unicellular trichome; *, extracellular droplets; arrows, intracellular droplets. Bars, 20 μm , except for (f), which is 100 μm .

the same process applied to flower-less bags. Each odour collection lasted for 2 h (increasing the duration of volatile collection did not lead to the detection of additional compounds). The adsorption tubes were eluted with 150 μl CH_2Cl_2 . To each sample we added 4 μg each of two internal standards (nonane and dodecane) for an estimation of compound quantities. The samples were analysed qualitatively using a Varian CP3800 gas chromatograph coupled with a Varian Saturn 2000 mass spectrometer (Varian Inc., Palo Alto, CA, USA). We injected 1 μl into a 1079 injector (200°C, splitless mode) and used a CP sil 8 CB column (30 m, 0.25 mm inner diameter, 0.25 μm film thickness) with helium as the carrier gas (constant flow rate: 1 ml min^{-1}). The oven temperature was kept at 50°C for the first 3 min, then programmed to increase 3°C min^{-1} to 100°C, 2.7°C min^{-1} to 140°C, 2.4°C min^{-1} to 180°C, and then 6°C min^{-1} to 250°C. To identify the compounds, we compared the mass spectra of the samples with those of Adams (1995), MassFinder, Wiley 6 and NIST98 libraries and with the spectra of authentic compounds. We also compared the retention indices with those for known authentic compounds. For quantification, extracts

were injected into a CP3800 gas chromatograph (FID detector, column EC-1, length 30 m, internal diameter 0.25 mm, film thickness 0.25 μm , carrier gas helium, oven temperature as for GC–MS analysis). The compound quantities were estimated using the two internal standards nonane and dodecane.

Floral scent was additionally collected in the laboratory to get a sample for electroantennographic measurements. Forty flowers of *T. europaeus* were randomly chosen on 11 June 2008 at Col de Porte, cut including the floral stem, and sent via express mail to Bayreuth (Germany) where the scent was collected on 12 June 2008 for 6.5 h in the afternoon. The flowers were enclosed in an oven bag (Topitts, Cofresco Frischhalteprodukte, Minden, Germany) and emitted volatiles were trapped in an adsorbent tube filled with 10 mg of Tenax-TA 60–80 and 10 mg of Carbotrap 20–40. The air was sucked from the bag over the adsorbents (150 ml min^{-1}) by a membrane pump (G12/01 EB; Rietschle Thomas, Puchheim, Germany). Volatiles were eluted from the adsorbents with 80 μl of acetone (SupraSolv; Merck KGaA, Darmstadt, Germany) in order to obtain a sample for the electrophysiological analyses.

Table 1 Occurrence (in brackets the number of samples collected in each population), median and range (relative amount in %) of the scents emitted by first-day flowers (headspace collection) of four different *Trollius europaeus* populations (Banchet, Col de Porte, Lautaret and Pré Gelé, French Alps)

	(1) Banchet			(2) Col de Porte			(3) Lautaret			(4) Pré Gelé		
	Occurrence (13)	Median	Range	Occurrence (12)	Median	Range	Occurrence (9)	Median	Range	Occurrence (13)	Median	Range
Total amount (ng per 2 h per flower)		342	16–2290		222	72–603		548	119–2420		370	23–1536
Fatty acid derivatives												
Z-3-hexen-1-ol ¹	10	4	0–99	11	5	0–38	9	4	01–38	13	5	01–49
Nonanal ¹	3	0	0–10	7	5	0–25	8	3	0–25	13	4	01–51
Z-jasmone ¹	9	tr ²	0–3	6	tr	0–3	5	tr	0–3	4	0	0–tr
Benzenoids												
Methyl benzoate ¹	4	0	0–32	4	0	0–30	8	4	0–30	7	2	0–10
Methyl salicylate ¹	12	1	0–5	9	tr	0–1	9	tr	tr–1	11	tr	0–1
Monoterpenoids												
E-β-ocimene ¹	7	4	0–32	4	0	0–21	7	5	0–35	9	7	0–31
Linalool ¹	9	9	0–30	9	15	0–32	9	26	15–41	12	22	0–47
Perillene	0	0	0–0	5	0	0–32	5	1	0–32	4	0	0–7
Sesquiterpenoids												
β-bourbonene	1	0	0–6	0	0	0–0	3	0	0–3	1	0	0–9
β-caryophyllene ¹	12	49	0–86	9	41	0–65	9	27	14–65	11	24	0–48
β-copaene	1	0	0–1	0	0	0–0	1	0	0–0	1	0	0–2
α-humulene ¹	1	0	0–1	2	0	0–9	1	0	0–9	0	0	0–0
Germacrene D ¹	3	0	0–38	3	0	0–12	4	0	0–16	6	0	0–48
Z,E-α-farnesene ¹	1	0	0–2	0	0	0–0	2	0	0–4	1	0	0–2
E,E-α-farnesene ¹	9	8	0–23	6	4	0–19	6	2	0–29	10	8	0–35
Caryophyllene oxide ¹	7	1	0–12	3	0	0–7	9	6	01–18	7	2	0–6

¹Identification is based on the comparison of the retention time and mass spectrum with an authentic standard.
²tr, relative amount was < 0.5%.

Gas chromatography coupled to electroantennographic detection (GC-EAD)

The electrophysiological analyses of the floral scent sample were carried out using a GC-EAD system as described earlier (Dötterl *et al.*, 2005a): Vega 6000 Series 2 gas chromatograph equipped with a Phenomenex ZB-5 column; GRAPHACK 3D/2 splitter provided by Gerstel; heated transfer line and two-channel USB acquisition controller provided by Syntech. Five *Chiastocheta* females and nine males were tested. Among the males were three *C. macropyga*, two *C. rotundiventris*, two *C. intermella*, and two *C. trollii* individuals. The females could not be identified to species level because the genitalia on which determination is based were too dry to be dissected following GC-EAD. The flies were caught on 11 June 2008 at Col de Porte, where they were found inside the collected flowers and also sent via express mail to Bayreuth. For the measurements, the head was excised from the thorax and the postoccipital region was subsequently placed in a glass capillary electrode containing insect ringer (8.0 g l⁻¹ NaCl, 0.4 g l⁻¹ KCl, 0.4 g l⁻¹ CaCl₂). The tip of one antenna was placed in another glass capillary electrode (recording electrode) also containing insect ringer. The electrodes were connected to silver wires. A quantity of the odour sample (0.1–1 µl) was injected splitless (injector temperature 250°C) at 60°C oven temperature; the split vent was then opened after 1 min and the oven heated at a rate of 10°C min⁻¹ to 200°C. The end temperature was held for 5 min.

To identify the EAD-active compounds, 0.1 µl of the scent sample was analysed on a Varian Saturn 2000 mass spectrometer and a Varian 3800 gas chromatograph fitted with a 1079 injector. Component identification was carried out using the NIST 02 mass spectral database, or MassFinder 3, and confirmed by comparison with retention times and mass spectra of authentic standards.

Microscopic study and histochemistry

In order to locate the floral structures emitting odours, we used light microscopy with various stains and environmental scanning electron microscopy (ESEM) on different parts of the flowers of five potted plants.

For the ESEM observations, the sepals were pasted directly on to a stage in the low-pressure chamber (110 Pa, 15 kV) of an S-3000N Hitachi microscope (Tokyo, Japan) and then cooled using the Pelletier effect. For histochemistry, freehand sections of the sepals and anthers were observed using a Leitz DMRB microscope.

To reveal lipids, we used five different stains: Sudan red IV, Sudan black B, Nile blue, Nile red and NaDi. Sudan red IV was used to stain fats, oils and waxes orange, and Sudan black B to stain phospholipids and free fatty acids black. The samples were rinsed in 50% ethanol, stained

for 5–20 min with Sudan red IV or Sudan black B in 70% ethanol, rinsed again in 50% ethanol and observed in water. Nile blue was used to stain phospholipids and free fatty acids blue and neutral lipids red. Samples were stained for 5 min with 1% Nile blue at 60°C then rinsed in water and in 1% acetic acid and observed in water. Nile red was used to make neutral lipids fluorescent. Samples were directly stained for 5 min with 10⁻⁴ to 10⁻⁵% Nile red in phosphate-buffered saline before observation in the same buffer (excitation filter 450–490 nm and barrier filter 515 nm). The NaDi reaction was used to reveal terpenes: NaDi stains lipids blue and terpenes purple. Samples were placed for 15–45 min in a freshly prepared mixture of 10⁻³% 1-naphthol, 10⁻³% N,N-dimethyl-*p*-phenylenediamine dihydrochloride and 0.4% ethanol in 100 mM sodium cacodylate-HCl buffer (pH 7.2) and then observed in the same buffer.

Behavioural test: the attraction of flies to anther odours

In order to test whether VOCs play a role in fly attraction, we first tried unsuccessfully to attract *Chiastocheta* to globe-flower scent in a Y-tube design. We also tested attraction in the field to artificial and to natural flowers with or without added synthetic compounds (all single EAD-active compounds and mixtures thereof which resembled the scent emitted by the flowers). However, no significant differences in fly attraction were found, which may show that odours are not involved in fly attraction or that our experimental design was unsuitable. We finally decided to manipulate floral scent using a more natural method, by testing the effect of removing the anthers, as anthers were shown to contain VOCs (Jürgens & Dötterl, 2004). Ten patches of 1 m², each containing 10 immature flower buds, were randomly selected in Lautaret (population 3) in June 2007. All the buds were emasculated. One week later, fresh open anthers were collected from neighbouring flowers outside each patch; these were placed inside the globes of five randomly chosen emasculated flowers in each patch. Five observers were then randomly successively assigned to four patches for 30 min. In total, each patch was observed twice (2 × 30 min) by two different observers. The number of *Chiastocheta* flies that landed on and entered the flowers was recorded. The total number of flowers observed was 94; six emasculated buds did not develop properly.

Observation of flies visiting natural globeflowers and measurement of flower traits

To test whether fly visits can be explained by the scent of visited flowers or by other floral traits, the number of fly visits to specific flowers was correlated with their VOC content, their morphology and their pigment concentration.

Fly observations In two patches A and B (distance 200 m), located at Pré Gelé (population 4), we bagged 50 immature flowers each in order to prevent visits from flies and pollination before the experiment. On the 20 and 28 June 2007, in sunny, windless conditions, we removed the bags, and five observers recorded the number of visits of *Chiastocheta* flies entering these flowers; that is to say, if a single *Chiastocheta* entered a flower, went out and entered again, it was counted twice. When the flies move rapidly, it can be difficult to differentiate *Chiastocheta* from other flies, but *Chiastocheta* are the only flies which move freely between the sepals and enter the globe-flowers. Each globe-flower was observed three times for 30 min; the observers and observation periods were randomized.

Morphological traits We measured *in situ* the globe's distance from the ground (flower height), the globe's outer diameter (defined as the diameter of the last circle of sepals), the globe's inner diameter (defined as the diameter of the first circle of sepals), the globe's height and the number of sepals.

Pigments We chose to investigate carotenoids, as they are often involved in yellow colours in plants (Grotewold, 2006), and adonivernith, a phenolic pigment close to luteolin which is particularly abundant in the sepals (4 mg g⁻¹; Gallet *et al.*, 2007). Following the observations, we cut the flowers and took them to the laboratory where we immediately dissected the sepals and anthers. A quarter of the sepals were kept frozen at -20°C and the adonivernith concentration was later determined (see Gallet *et al.*, 2007 for a detailed description of the protocol). Another quarter were weighed and immersed in a 70% acetone : 30% H₂O solution for 15 min. The solution was then filtered and the absorbance at 470 nm was measured, from which the concentration of carotenoids was determined (Lichtenhalter, 1987).

VOCs It was not possible to collect the floral scent from all the flowers observed using the time-consuming dynamic headspace technique described earlier. We therefore extracted the VOCs in a solvent in order to determine the VOC content of the flowers observed. All flowers were in a similar developmental stage and age (unpollinated first-day flowers at the start of observation period), and we assumed that a flower which contains a higher total content of VOCs than another flower is likely to emit more scent.

The remaining half of the sepals and all the anthers of the flowers were weighed and immersed in twice their mass of hexane containing 40 mg l⁻¹ camphor as an internal standard, for 24 h in separate tubes (two tubes per flower, one including the sepals and one the anthers), and then stored at -20°C until analysed. GC-FID (flame ionization detector) analyses were carried out on a GC (Agilent 6850; Agilent

Technologies, Santa Clara, CA, USA) equipped with a FID. Nitrogen was used as carrier gas at a flow rate of 1 ml min⁻¹. A glass HP-Innowax 1909N-133E capillary column (30 m × 25 mm inner diameter, 0.25 µm film thickness) was used in the following conditions: 3 min at 40°C, then 2°C min⁻¹ up to 160°C and 12°C min⁻¹ to 240°C with 2 min hold time. An aliquot (1 µl) of the extract was injected in split mode (10 : 1 ratio). Quantitative peak estimation was achieved by comparison with the internal standard and a molar response of one was assumed for all components (Picone *et al.*, 2004). The quantity of each VOC contained in a flower was determined using the following formula: 2 × quantity in the sepals + quantity in the anthers.

The solvent extracts contained long-chain alkanes and alkenes, which are known to be important in other pollination systems (e.g. sexual deceptive orchids; see Schiestl *et al.*, 1999). *Chiastocheta* flies did not, however, respond to these compounds in preliminary electroantennographic measurements, but did respond to other compounds found in the headspace samples. We therefore only included compounds with a Kovats retention index below 2000 for chemical and other further analyses of these solvent extracts.

Data analysis

To test for differences in scent composition (headspace volatile collections) among the different populations, we carried out a permutational multivariate analysis of variance using distance matrices (PERMANOVA) based on Bray-Curtis indices. This method is available in the 'vegan' package of R 2.7.2 software (R Development Core Team, 2009) under the function name 'adonis'. Adonis is a multivariate procedure directly analogous to MANOVA (Anderson, 2001; McArdle & Anderson, 2001), and commonly used in community ecology. Instead of using the quantitative data, we used the percentage amount (relative amount) of compounds as we found wide variations in the total amount of scent even within populations (see the Results section). In order to determine the compounds responsible for the differences in the percentage amount of scent emitted between populations, we used a SIMPER analysis in Primer (Clarke & Gorley, 2006). Following statistical analyses were done using the software R 2.7.2.

The dataset for visits to flowers manipulated for anther odour was analysed using a generalized mixed model ('glmmPQL' function in package 'MASS') with the treatment as fixed effect and the patch as random effect. The total number of visits received by each flower was obtained by pooling the number of visits received during the two observational trials.

The morphological and biochemical traits of unmanipulated observed flowers were highly correlated (of the 91 pairwise correlation coefficients between traits, 35 were

significantly different from zero in patch A and 27 in patch B), so we did not use classical multiple regression. Instead, we modelled *Chiastocheta* visits to natural flowers against the traits measured using generalized partial least-squares (gPLS) regression. The number of *Chiastocheta* that entered the globes was modelled using 'Poisson' family (function 'glm', package 'stats' in R). Following Bastien (2005), we built an algorithm which estimated the coefficient of each trait in the regression and a 95% confidence interval (1000 bootstraps) using the R package 'boot', as well as the coefficient of determination (R^2) for the model. For each model, six PLS components were included in order to compare R^2 among models. All the traits measured were included in each gPLS component in order to avoid arbitrary trait selection based on P -values.

Results

Floral scent of *T. europaeus* and how it varies between populations

A single flower emitted a median of 342 ng of scent per 2 h, and there were no significant differences between the populations (KW-ANOVA: $H(3, n = 47) = 4.60, P = 0.20$).

Sixteen floral scent compounds were detected in the 47 samples collected by dynamic headspace (Table 1; all, except *Z*-3-hexen-1-ol and perillene, are also found in EAD samples from cut flowers). These 16 compounds included eight sesquiterpenoids, three monoterpenoids, three fatty acid derivatives and two benzenoids. The scent was highly variable and the compounds occurred, on average, in only $49 \pm 8\%$ (mean \pm SE) of samples. No compound was detected in all of the samples, and only four compounds occurred in $> 80\%$ of the samples: *Z*-3-hexen-1-ol (43 samples), methyl salicylate (41), β -caryophyllene (41) and linalool (39). Most of the compounds were emitted in small amounts, and only three compounds contributed on average $> 5\%$ to the total scent blend (median: β -caryophyllene 34%, linalool 18%, *E,E*- α -farnesene 7%).

Eleven of the 16 compounds occurred in all of the four populations analysed, among them *E*- β -ocimene, linalool, β -caryophyllene, *E,E*- α -farnesene and β -caryophyllene oxide, which in at least one population contributed $> 5\%$ (median) to the total amount of scent emitted. The five other compounds occurred in three of the populations each, and these compounds were, when present, mostly emitted in relatively small amounts.

In two populations, the scent samples were dominated by β -caryophyllene (median 49, 41%), while in the others, similarly high amounts of linalool (median 26, 22%) and β -caryophyllene (median 27, 24%) were emitted (the 14 other compounds contributed on average $< 10\%$ (median) to the scent of the different populations). This difference in the relative amount of linalool and β -caryophyllene (as

indicated by a SIMPER analysis) is mainly responsible for the differences in scent pattern found among the populations in a multivariate approach (PERMANOVA: $R^2 = 0.08, P = 0.0039$). However, although the differences are significant, the low R^2 value indicates that there was a big overlap in scent samples between the populations. One explanation for this finding is that variations in scent were also high within the populations, and this is true for both the main (β -caryophyllene, linalool) and the minor compounds (Table 1).

Histology

Trollius flowers are composed of yellow sepals fully enclosing the sexual parts of the flower (stamen and gynoecium, Fig. 1a). An unstained section of a petal (Fig. 1b) shows that the adaxial epidermis (inside the flower) is composed of conical cells while the abaxial epidermis cells are flat. Staining of the conical cells of the adaxial epidermis revealed lipidic droplets (Sudan red VI, Fig. 1c) containing terpenes (NaDi reagent, Fig. 1d). Terpenic droplets are also present in the abaxial epidermis (Fig. 1e). The abaxial epidermis also presents unicellular glands (Fig. 1f). These glands, or trichomes, exude lipids (Fig. 1g) that became neutral once exuded (red coloration of Nile blue, Fig. 1h). Lipids are often esterified spontaneously once secreted, which is perhaps the case here. Esterified lipids, such as, for example, terpene-fatty acid polymers, could be more glutinous and eventually hinder insects' movement on the globe-flower. All these lipids, acid inside the cells and neutral when secreted, contain terpenes (Fig. 1i). In the rest of the flower, only the anthers (Fig. 1j), the nectar-producing region at the base of the staminodia (Fig. 1k) and the pollen (Fig. 1l) produce lipids. The staining of the pollen with NaDi shows terpene content (Fig. 1m), but the tapetum does not contain terpenes (Fig. 1o). In summary, terpenes are found in four different parts of the flower (adaxial epidermis of the sepals, abaxial epidermis of the sepals, trichomes and pollen) and in four different kinds of cells: conical cells, flat epidermis cells, unicellular glands (i.e. trichomes) and pollen, respectively.

Electrophysiologically active compounds

The sample used for the electroantennographic measurements was collected from 40 cut flowers and contained all the compounds found in the samples collected *in situ* and listed in Table 1, except for *Z*-3-hexen-1-ol and perillene (Supporting Information, Table S1). During EAD measurements, the baseline was quite unstable; however, distinguishable antennal responses were elicited in four females and six males (see Fig. 2, where two measurements are given as examples). Flies of both sexes responded consistently (at least in three of the four females, and five of the six males)

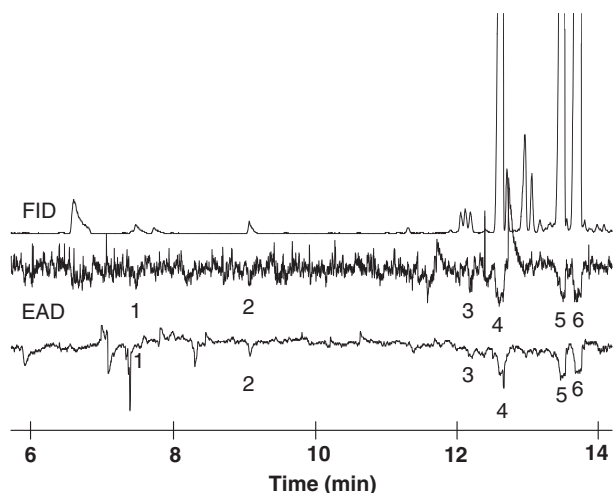


Fig. 2 Coupled gas chromatographic and electroantennographic detection (GC-EAD) of a *Trollius europaeus* flower scent sample tested on two *Chiastocheta* males (*C. macropyga* and *C. trollii*). 1, linalool; 2, methyl salicylate; 3, Z-jasmone; 4, β -caryophyllene; 5, germacrene D; 6, E,E- α -farnesene. FID, flame ionization detector.

to the sesquiterpenes β -caryophyllene, germacrene D and E,E- α -farnesene, which dominated the scent sample used for these measurements. The flies' antennae also responded consistently to methyl salicylate, Z-jasmone and linalool, which occurred only in small amounts in this scent sample. In males there seems to be some variation in the antennal responses of different species (e.g. Z-jasmone may elicit stronger responses in *C. rotundiventris* and *C. trollii* compared with the other species). However, the noisy baseline and the small number of individuals measured per species mean that a definitive conclusion about differences among species cannot be drawn.

Observation of flies visiting natural globe flowers

Thirty-three and 41 flowers were observed in patches A and B, respectively. We recorded 502 and 317 events of flies entering the flowers in patches A and B, respectively, which represents 15 ± 0.27 and 7.7 ± 0.14 (mean \pm SE) visits per flower.

In the solvent extracts for these flowers (Table 2) we found most of the compounds (among them, all EAD-active ones), which were also detected in the headspace analyses of the corresponding population (Pré Gelé), but not perillene, β -copaene, Z,E- α -farnesene and caryophyllene oxide, which occurred only in some headspace samples and mostly in small amounts. In comparison to the headspace samples, the solvent extract samples were dominated by green leaf volatiles, such as hexanal and E-2-hexenal, which were not found in the headspace samples (Table S1). The relative proportion of VOCs differed significantly between flower parts, with anther scent being dominated by

E,E- α -farnesene and sepal scent by β -caryophyllene and germacrene D (Fig. 3).

Two EAD-active compounds were only present in a fraction of the solvent extract samples: germacrene D and methyl salicylate. Their presence was associated with a higher visitation rate in both patches (Table 3; in patch A the effect of germacrene D was only marginally significant), which was generally not true for the inactive compounds present in a fraction of the flowers. The presence of EAD-inactive compounds was generally not associated with a higher visitation rate in either patch (except β -bourbonene in patch B). The presence of Z-3-hexenyl acetate influenced the visitation rate negatively in patch A.

The variation in the visits could be partly described by the variation in the total amount of EAD-active VOCs in patch B (Table 4, $R^2 = 0.46$) but not in patch A ($R^2 = 0.01$). This must be compared with the variation in the visits as a result of morphological and pigment traits ($R^2 = 0.28$ in B; $R^2 = 0.10$ in A). The R^2 of the full model was 0.13 in patch A and 0.56 in B. In patch B, where the variation in the traits measured explained most of the variation in the visits, increases in the globe diameter, the number of sepals and the amount of germacrene D were linked to an increase in the number of visits; and an increase in the concentration of carotenoids in the sepals was linked to a decrease in the number of visits (Fig. 4).

The attraction of flies to manipulated flowers

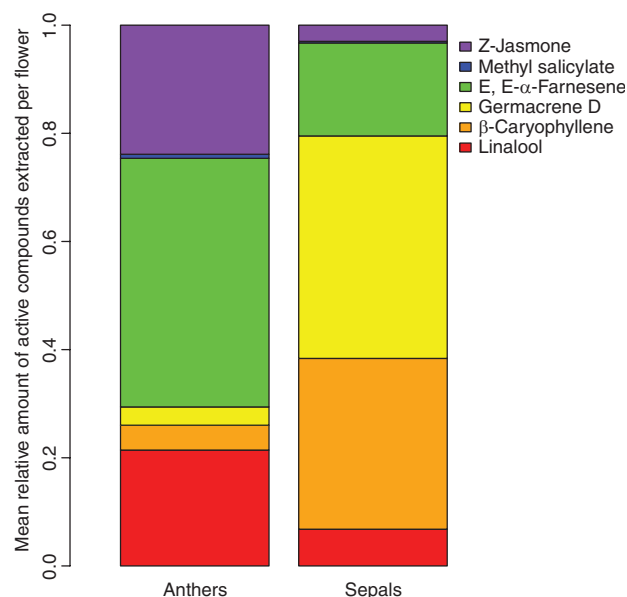
The presence of anthers inside the flowers did not have any effect on the number of flies that landed on the globe (7.5 ± 4.6 and 7.9 ± 5.6 with and without anthers, respectively; $t_{1,83} = 0.502$, $P = 0.617$), but had a significant positive effect on the number of flies that entered the globe (4.2 ± 3.0 and 2.6 ± 2.3 , respectively; $t_{1,83} = 3.32$, $P = 0.001$).

Discussion

Although *T. europaeus* is involved in a highly specialized nursery pollination mutualism, the flowers did not emit uncommon compounds, but rather widespread fatty acid derivatives, benzenoids and terpenoids, which make up the floral scent of many other plants (Knudsen *et al.*, 2006). The two most abundant compounds in the headspace samples, linalool and β -caryophyllene, are among the most common of all floral scents. The emission of common floral scent compounds by a plant involved in a specialized pollination system is not exceptional and has also been found in other plants interacting with nursery pollinators. Several compounds identified in the headspace samples for the present study, such as linalool and E,E- α -farnesene, were also found in a previous study of *Trollius* anther scent (Jürgens & Dötterl, 2004). β -Caryophyllene, the main compound

Table 2 Median and range of the traits measured in two patches at Pré Gelé (French Alps) and, for volatile organic compounds, (VOCs) the number of flowers containing each individual compound (total number of flowers sampled per patch in brackets)

	Patch A			Patch B		
	Occurrence (33)	Median	Range	Occurrence (41)	Median	Range
VOCs (mg per flower)						
<i>Physiologically active compounds</i>						
Linalool	33	0.793	0.16–2.73	41	1.06	0.21–7.22
β -caryophyllene	33	3.474	0.10–21.61	41	1.337	0.23–4.77
Germacrene D	28	1.863	0–15.52	31	2.836	0–15.51
E,E- α -farnesene	33	2.019	0.13–10.54	41	2.318	0.48–6.57
Methyl salicylate	13	0	0–0.53	5	0	0–0.42
Z-jasmone	33	0.824	0.45–1.94	41	1.003	0.18–2.42
<i>Physiologically inactive compounds</i>						
Hexanal	33	11.52	0.42–35.21	41	3.979	0.34–17.09
E-2-hexenal	33	15.96	4.17–68.77	41	11.75	1.73–31.7
Z- β -ocimene	31	0.108	0–0.40	41	0.217	0.01–0.83
Z-3-hexenyl acetate	26	0.09	0–0.38	39	0.221	0–1.57
1-hexanol	33	1.291	0.09–4.11	41	1.391	0.20–5.74
Z-3-hexenol	33	4.868	1.50–14	41	4.692	1.51–14.76
Nonanal	31	0.236	0–0.83	41	0.952	0.04–3
β -bourbonene	10	0	0–0.38	23	0.122	0–0.64
Methyl benzoate	15	0	0–0.10	24	0.013	0–0.35
Hexanoic acid	15	0	0–0.40	40	0.272	0–1.39
Nerolidol	26	0.631	0–7.61	34	0.168	0–4.19
Globe morphology (cm)						
Outer diameter		2.8	2.1–3.1		3.3	2.7–3.8
Inner diameter		2.3	1.8–2.7		2.6	2–3.1
Distance to ground		22.5	16–27.5		27	21–34
Globe height		1.5	1.2–1.9		1.6	1.3–1.8
Number of sepals		12	10–19		12	10–16
Sepal pigments (mg g ⁻¹ fresh mass)						
Adonivernith		8.47	3.2–16.72		6.32	2.47–16
Carotenoids		0.21	0.11–0.33		0.2	0.08–0.46

**Fig. 3** Relative amounts of the EAD-active compounds extracted (hexane) from the anthers and the sepals of *Trollius europaeus* flowers.

found in this study, was not listed in Jürgens & Dötterl (2004)'s study. This study shows that the relative amount of VOCs emitted by sepals and anthers is different, with β -caryophyllene dominant in the sepals and E,E- α -farnesene in the anthers.

We found wide variations not only in the relative amounts of compounds emitted within and between populations, but also in the scent quality. None of the compounds present in the headspace samples was found in all the samples analysed, and most of the compounds did not even occur in all of the replicate samples within populations. Such high degrees of variation are unusual for a plant involved in a highly specific nursery pollination system. In *Yucca* species, for example, scent variability is much lower, and a strong conservatism even between species was found (Svensson *et al.*, 2005, 2006). In *Silene latifolia*, which is the host of the nursery pollinator *Hadena bicruris*, variation in scent within and between populations was also high, but lilac aldehyde, which dominated most of the samples (Dötterl *et al.*, 2005b), and which is most attractive to *Hadena* (Dötterl *et al.*, 2006), was found in all the samples analysed.

Table 3 Generalized linear models of the number of visits to flowers in two patches in Pré Gelé, depending on the presence of single compounds (only compounds which were absent in at least three flowers in any patch were included in the analysis)

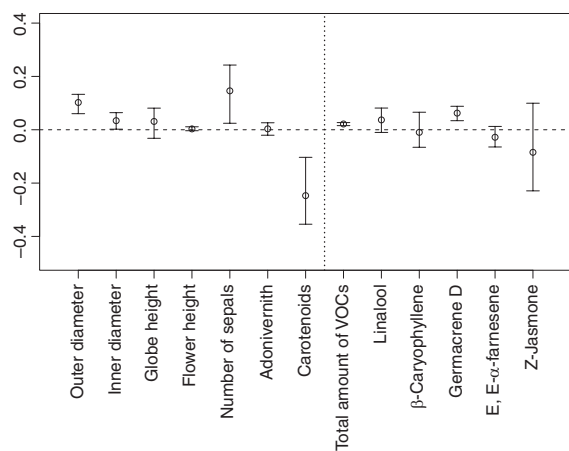
	Patch A				Patch B			
	Estimate	SE	Z	P	Estimate	SE	Z	P
Germacrene D	0.185	0.104	1.786	0.074	0.220	0.098	2.255	0.024
Methyl salicylate	0.183	0.070	2.615	0.009	0.224	0.111	2.011	0.044
Z-3-hexenyl acetate	-0.220	0.080	-2.746	0.006	Na	Na	Na	Na
β -bourbonene	-0.011	0.076	-0.144	0.885	0.185	0.081	2.291	0.022
Methyl benzoate	0.008	0.070	0.114	0.909	0.072	0.081	0.897	0.370
Hexanoic acid	-0.002	0.070	-0.025	0.980	Na	Na	Na	Na
Nerolidol	-0.045	0.084	-0.541	0.589	0.158	0.111	1.426	0.154

Na, the compound was absent in only one or two flowers, in which case the test is not robust. The first two compounds (germacrene D and methyl salicylate) are electrophysiologically active and the remaining compounds are inactive.

Table 4 Coefficient of determination of each generalized partial least-squares (gPLS) model explaining visitation rates to the flowers in each patch, according to either all the traits measured or the volatile organic compounds (VOCs) only, or flower morphology and sepal pigmentation only

	All traits	Only VOCs	All except VOCs	Visits
Patch A	0.129	0.014	0.104	15.21 \pm 9.08
Patch B	0.561	0.460	0.286	7.73 \pm 5.69

Each model includes three PLS components. Mean number of visits per flower per h \pm SD.

**Fig. 4** Estimated coefficients and 95% bootstrap confidence intervals of the partial least-squares (PLS) regression for each floral trait explaining globeflower (*Trollius europaeus*) fly visits in patch B. The results for patch A are not shown because the PLS regression has a very poor explicative power in this patch (see Table 4). The confidence interval and coefficient for methyl salicylate are not shown because this compound is present in only 12% of the individuals of patch B, which makes the confidence interval very high.

Most of the compounds found in the headspace samples from population 4 were also found in the solvent extract samples of the flowers which were observed for fly visits in

the field (patches A and B in population 4) before extraction (Table S1). However, there are two main differences between the headspace and the solvent extract samples. First, all the solvent extracts contained several C6 compounds (e.g. E-2-hexenal, hexanal), which are known as typical green leaf volatiles (GLVs), and which were not found in any of the headspace samples. Such GLVs are known to be most commonly produced and released after injury to the plant tissue (Matsui, 2006), and in our case it is likely that they were produced after cutting the anthers and the sepals before they were extracted in hexane. Second, three of the EAD-active compounds (linalool, β -caryophyllene, E,E- α -farnesene) were found in all the solvent extracts, but only in a few (one to three) of the headspace samples. Notably, we did not find these compounds in very weak headspace samples, and it may be that these compounds were emitted as minor compounds by these flowers in quantities too small to be detected.

The histo-location of lipids and terpenes reveals four distinct odour production and/or secretion structures: the conical cells of the adaxial sepal epidermis; the flat epidermis cells of the abaxial sepal epidermis; the unicellular glands (trichomes) on the abaxial epidermis of the sepals; and the pollen in the anthers. The biosynthesis of volatiles usually occurs in epidermal flat or conical cells in flowers, whereas secretory glands are usually found on vegetative organs such as leaves (Pichersky *et al.*, 2006). These different locations reflect the different functions of the volatiles emitted: the volatiles emitted by conical cells of the flower typically attract pollinators, and the volatiles from vegetative organs, which are usually released upon disruption, typically repel herbivores. Globeflower sepals appear to combine petal (conical cells) and leaf (secretory glands) characteristics, in addition to their role in the very particular floral morphology of the globeflower, unique in its genus, which is central to pollination specialization as it forms a totally closed corolla excluding nonspecific pollinators. As a result, the abaxial epidermis of the sepals represents the only flower

part directly exposed to nonspecialized visitors. The secretory glands observed could therefore be involved in herbivore or nonspecific pollinator repulsion. By contrast, the emission of volatiles inside the globeflower by both the pollen and the conical cells of the adaxial epidermis may be involved in specific pollinator attraction inside the globe at a short distance, once the fly has alighted on the globe, attracted by other cues (floral colour and shape). Anther scent appears to be central in fly behaviour once flies have landed on the globeflower. Indeed, removing anthers had no effect on long-distance attraction but had a negative effect on the probability of a fly entering the flower. Therefore, floral scent perhaps plays a more important role in attracting and guiding flies which have already landed than in long-distance attraction.

Our electrophysiological tests revealed that flies have antennal receptors for six compounds emitted (methyl salicylate, *Z*-jasmone, β -caryophyllene, germacrene D, E, *E*- α -farnesene, linalool). We also found a positive correlation between the amount of EAD-active compounds in the solvent extracts of flowers and the visitation rate (patch B, Fig. 3). Flowers with a high total amount of these compounds were visited more often by *Chiastocheta* than flowers with a low amount of these compounds. If these six active compounds are therefore treated as signals, and the others as noise according to Raguso (2003), the variability of the signalling compounds is lower than the variability of the other compounds in the headspace samples (*t*-test: $t_{df=14} = -2.11$; $P = 0.052$). The active compounds occurred in $68 \pm 9\%$ of collected samples, and the inactive in only $38 \pm 9\%$, indicating that natural selection stabilizes the production of signalling compounds (see also Ayasse *et al.*, 2000). When testing separately for differences in the relative amounts of EAD-active and -inactive compounds between populations, we still find differences in both cases (PERMANOVA: $P < 0.05$ each) and once again high variability within populations. Flowers containing germacrene D had more fly visits in the field than flowers that did not contain this compound in patch B, and we also found a positive correlation between the amount of this compound in the solvent extract of flowers and the visitation rate of flies in that patch. These results suggest that this compound plays a role in attracting flies and that it may work in synergy with other compounds. Similarly, the presence of one compound in the solvent extracts, methyl salicylate, found in a large number of headspace samples, but only in a few solvent extract samples, resulted in an increase in the *Chiastocheta* visitation rate. The six identified EAD-active compounds are not only detected by *Chiastocheta*, but are also known to be perceived by, or to attract, other insects, including dipterans (Bengtsson *et al.*, 2001; Zhu *et al.*, 2003; James, 2005; Siderhurst & Jang, 2006; Jhumur *et al.*, 2007).

The wide variation in scent composition both within and between plant populations indicates that pollinator-driven

selection on floral scent in this mutualism is weak in comparison to other similar systems analysed, for example, yucca plants, where a strong conservation of the floral scent signal has been documented both within populations of the same species (Svensson *et al.*, 2005) and between closely related species (Svensson *et al.*, 2006).

In addition to floral scent, visual floral cues play a role in attracting *Chiastocheta* to *Trollius* flowers. As shown in Fig. 4, the three traits with greatest impact on fly visitation rate are all visual. Both globe diameter and the number of sepals (which are correlated traits; Pearson's $r = 0.25$, $t_{1,72} = 2.27$, $P = 0.02$) were positively associated with fly visits, whereas carotenoid concentration was negatively associated with fly visits. Therefore, flies are attracted to larger flowers, but are also sensitive to colour differences in flowers. We were, for example, able to attract flies to scentless fake flowers, whose shape and, in particular, yellow colour may have been responsible for attracting flies (indeed, blue, red or green fake flowers do not attract *Chiastocheta* flies, and attract very few other flies). Many fly species are known to respond to yellow colours (Lunau & Maier, 1995), and this is also true for *Chiastocheta*. Pellmyr (1992) managed to attract *Chiastocheta* flies to yellow scentless traps and also found that small changes in the colour of the traps strongly influenced the number of *Chiastocheta* flies trapped. We found a wide variation in the concentration of carotenoid pigments, and if we assume that carotenoids influence the colour of the flowers, our results indicate that flies respond to the colour differences which naturally occur within *Trollius* populations. Adonivernith is close to the yellow pigment luteolin (it has been identified as luteolin 8- β -D-glucopyranosyl-2''-O-D-xylopyranoside; Gallet *et al.*, 2007), but there was no correlation between its concentration in the sepals and the fly visitation rate. When located in the carpel walls, adonivernith is also involved in the plant's reaction to the presence of *Chiastocheta* larvae (Gallet *et al.*, 2007; Ibanez *et al.*, 2009b), so it is unclear how flies should respond to it. Recently, an ongoing strong directional selection pressure stabilizing the closed phenotype (Ibanez *et al.*, 2009a) has been demonstrated, driven by *Chiastocheta* behaviour, and the coevolutionary trajectories of flower morphology and fly behaviour have been modelled (Ibanez & Després, 2009).

Our results revealed the compounds emitted by *Trollius* flowers and indicate that both floral scents and visual flower cues play a role in the *Chiastocheta*–*Trollius* interaction. The relative importance of the visual and the olfactory cues may, however, varies and depends on several factors. On the day we observed the flies, VOC variability explained a negligible fraction of the variability in visits in patch A, but almost half the variability in patch B. The other traits measured also played a role, higher than for the VOCs in patch A, but lower in patch B. In patch B, *Chiastocheta* flies seemed to prefer larger flowers emitting high total amounts

of EAD-active VOCs, especially germacrene D, and with low concentrations of carotenoids. As flowers that experience high visitation rates are likely to be better pollinated and, more importantly, will export more pollen (Ibanez *et al.*, 2009a) and have a higher male fitness, we expect the traits involved in *Chiastocheta* attraction to be under selection pressure. The selective pressures on floral traits 'at work' over a whole flowering period cannot be predicted from our single-day observations, which suggest that they are likely to be variable in time and space. However, there seems to be directional selection pressure on the presence of the EAD-active compounds in comparison to the inactive compounds. Some other traits, such as the carotenoid content of the sepals, may also be under *Chiastocheta* selection pressure. Interestingly, in this study the variability of the visits could be explained by the measured flowers' traits when the visitation rate was low but not when it was high. It would have been possible to draw a more solid conclusion if the number of observed patches had been higher than two, but more intense selection in harsh ecological conditions has been observed elsewhere (Wilson *et al.*, 2006). Indeed, when *Chiastocheta* flies are more abundant and when the flowers are crowded, the flies are likely to be less selective. When fly activity is low, the plants enter into competition to attract pollinators, and the selective pressures on flower scent may only be active during these situations of competition between plants. Generally speaking, selection on globeflower floral traits might be more intense in populations with a low density of *Chiastocheta* flies and in years when climatic conditions reduce fly activity leading to pollinator limitation.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Compounds found with the three different methods used to measure variation among populations, to identify electroantennographic detection (EAD)-active compounds and to correlate fly visits with volatile organic compounds (VOCs).

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