Phytochemistry 100 (2014) 51–59

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Floral scent emitted by white and coloured morphs in orchids

L. Dormont [a,*], R. Delle-Vedove [a,c], J.-M. Bessière [b], B. Schatz [a]

[a] Centre d'Ecologie Fonctionnelle et Evolutive (CEFE), UMR 5175 CNRS, 1919 Route de Mende, 34293 Montpellier, France
[b] Ecole Nationale Supérieure de Chimie de Montpellier, Laboratoire de Chimie Appliquée, 8 rue de l'Ecole Normale, 34296 Montpellier, France
[c] Laboratoire de Génétique et Evolution des Populations Végétales (GEPV), Université des Sciences et Technologies de Lille 1, 59655 Villeneuve d'Ascq, France

ARTICLE INFO

Article history:
Received 15 July 2013
Received in revised form 15 January 2014
Available online 10 February 2014

Keywords:
Floral polymorphism
Colour
Odour
Orchids
Insect pollination
Chemical ecology

ABSTRACT

Polymorphism of floral signals, such as colour and odour, is widespread in flowering plants and often considered to be adaptive, reflecting various pollinator preferences for particular floral traits. Several authors have recently hypothesized that particular associations exist between floral colour and scent, which would result from shared biochemistry between these two floral traits. In this study, we compared the chemical composition of floral volatiles emitted by white- and purple-flowered morphs of three different orchid species, including two food-deceptive species (*Orchis mascula* and *Orchis simia*) and a food-rewarding species (*Anacamptis coriophora fragrans*). We found clear interspecific differences in floral odours. As expected from their pollination strategy, the two deceptive orchids showed high inter-individual variation of floral volatiles, whereas the food-rewarding *A. c. fragrans* showed low variation of floral scent. Floral volatiles did not differ overall between white- and coloured-flowered morphs in *O. mascula* and *A. c. fragrans*, while *O. simia* exhibited different volatile profiles between the two colour morphs. However, a detailed analysis restricted to benzenoid compounds (which are associated with the production of floral anthocyanin pigments) showed that white inflorescences emitted more volatiles of the shikimic pathway than coloured ones, both for *O. mascula* and *O. simia*. These results are consistent with the current hypothesis that shared biochemistry creates pleiotropic links between floral colour and scent. Whether intraspecific variation of floral signals actually affects pollinator attraction and influences the reproductive success of these orchids remains to be determined.

Introduction

Many flowering plants show substantial variation in floral traits at individual or population levels (Galen, 1999; Warren and MacKenzie, 2001; Weiss, 1995). Ecologists have focused primarily on interpreting colour variation in flowers, asking why different colour morphs have evolved and how they are maintained in populations. In many flowering plant species, pollinator insects have been shown to play a key role. Pollinator-mediated selection is frequently proposed to explain floral colour polymorphism, with different colour morphs likely reflecting selection driven by different pollinators with different colour preferences (Brown and Clegg 1984; Eckhart et al., 2006; Jones and Reithel, 2001; Juillet et al., 2010; Malerba and Naterro, 2012; Suchet et al., 2011). For example, the two main pollinators of *Mimulus aurantiacus* have been shown to exhibit strong preferences for either the red-flowered morph (hummingbirds) or for the yellow-flowered morph (hawkmoths) (Streisfeld and Kohn, 2007), and floral divergence has been experimentally demonstrated to be governed by insect preference. In the deceptive orchid *Disa ferruginea*, the flower colour shift has been shown to be driven by geographical variation of colour preference of the same pollinator: allopatric red and orange floral morphs of this orchid mimic the flowers of sympatric nectar-producing species, and the butterfly *Aeropetes tulbaghia* prefers red or orange floral signals in different parts of its range (Newman et al., 2012). More recently, pleiotropic effects of selection exerted by agents other than pollinators have been reported to explain flower colour variation in some cases (Coberly and Rausher, 2008). Colour polymorphism might reflect multiple selection pressures, involving not only pollinators but also herbivore-protection strategies, local abiotic conditions or indirect selection (Majetic et al., 2009; Schemske and Bierzychudek, 2007; Strauss and Whittall, 2006).

Together with floral colour, odour emitted by flowers represents a key floral signal used by insects to detect and select rewarding flower species (Chittka and Raine; 2006; Delle-Vedove et al., 2011; Suchet et al., 2011; Tremblay et al., 2005; Raguso, 2008a; Wang et al., 2013). The importance of floral scent has long been neglected in studies of pollination biology (Adler and Irwin, 2012; Raguso, 2008b). Most flower-visitor species orient their

* Corresponding author. Tel.: +33 (0)4 67 41 33 00; fax: +33 (0)4 67 61 33 36.
E-mail address: laurent.dormont@cefe.cnrs.fr (L. Dormont).

http://dx.doi.org/10.1016/j.phytochem.2014.01.009
0031-9422/

flight behaviour by exploiting simultaneously volatile and visual cues provided by flowers (Burger et al., 2010; Dötterl et al., 2011; Milet-Pinheiro et al., 2012). Recent studies have shown experimentally that colour and scent can be equally important for pollinator choices (Glover, 2011; Klahre et al., 2011; Kunze and Gumbert, 2001). The chemical composition of floral volatiles has been investigated and detailed in many plant species (reviewed by Knudsen et al., 2006; Schiestl, 2010), but contrary to colour variation, the variability of floral scent within a plant species and its effect on pollination rate have received limited attention (Delle-Vedove et al., 2011; Dormont et al., 2010a; Soler et al., 2012).

Some authors have compared the composition of floral volatiles in plants showing intraspecific variation of floral colour, and found that colour morphs of the same species consistently differ in scent chemistry (Flamini et al., 2002; Olesen and Knudsen, 1994; Salzmann and Schiestl, 2007; Zuker et al., 2002). It has been suggested that specific floral scent-colour combinations may result from related biochemical processes, because volatile compounds and colour pigments often share common biosynthetic pathways (Knudsen et al., 2006; Majetic et al., 2007; Zuker et al., 2002). If precursor molecules are channelled to production of one product, less of the other product may be formed. For example, volatile benzenoid compounds and anthocyanin-derived pigments responsible for blue, red or purple flower colouration are both produced by the phenylpropanoid biosynthetic pathway. This biosynthetic connection might explain specific floral colour-scent associations, such as white flower morphs that emit more benzenoid volatiles than coloured morphs in some plant species (Majetic et al., 2007; Zuker et al., 2002).

In this study, we investigated the composition of floral scent emitted by different colour morphs in orchids, which represent numerous very interesting cases of floral polymorphism. The great diversity of floral colour characters in this family is generally associated with animal (mainly insect) pollination (Claessens and Kleynen, 2011; Van der Cingel, 1995). Approximately one-third of all orchid species achieve pollination through food deception, i.e., flowers contain no nectar or other rewards but resemble or mimic floral signals of rewarding plants to attract pollinators (Jersakova et al., 2006). Consequently, intraspecific variation in floral traits is expected to be high in food-deceptive orchids, because flowers must delay the avoidance learning of pollinators (Jersakova et al., 2006; Schiestl, 2005). After visiting flowers that did not offer a nectar reward, insects have been observed to fly greater distances, or to switch to flowers with different form or colour characters (Smithson and MacNair, 1997). Because frequent floral morphs are more quickly recognized and avoided by pollinators, rare morphs could gain a selective advantage by being more frequently visited and pollinated. This rare-morph advantage through negative frequency-dependent selection has been hypothesized to explain the maintenance of floral polymorphism in rewardless orchids, at least for colour traits (Gigord et al., 2001; Smithson and MacNair, 1997). For example, in *Dactylorhiza sambucina*, frequencies of the yellow- and red-coloured morphs have been shown to reflect pollinator preference for the rare colour morph (Gigord et al., 2001). Among the wide range of colour variants in orchid flowers, the occurrence of rare hypochromatic inflorescences (very pale morphs and even entirely white flowers) remains intriguing. Many orchid species occasionally show a few white-flowered individuals within natural populations of the common coloured morph (Bournérias and Prat, 2005; Weiss, 1995). Until recently, it was unknown whether such white orchid flowers differ from coloured morphs in their production of olfactory signals. If they do, this may affect pollinator behaviour and thereby also reproductive success of the white morphs (Ackerman and Carromero, 2005; Koivisto et al., 2002; Schatz et al., 2013). In other plant families in which similar floral colour polymorphisms have been described, the white-coloured flower morphs have been shown to clearly differ in scent chemistry from pigmented morphs (Li et al., 2006; Majetic et al., 2007; Zuker et al., 2002). In deceptive orchids, it could be expected that different colour morphs also exhibit different scent profiles, so that pollinators just deceived by one colour morph can be attracted by a flower showing simultaneously distinct colour and odour (Kunze and Gumbert, 2001). In a preliminary study, we showed for the food-deceptive orchid *Orchis mascula* that purple-flowered morphs did not clearly differ in their floral scent from the white-flowered morphs. Overall, floral volatiles were highly variable in both morphs (Dormont et al., 2010a, 2010b).

Here, we ask whether this situation also characterizes two other orchid species with different reproductive strategies. Like *Orchis mascula*, *O. simia* and *Anacamptis coriophora fragrans* typically exhibit common coloured flowers (purple, pink, and red/brown, respectively), but in each of these, rare white-flowered individuals can also be observed, always at low frequency, within populations dominated by coloured-flowered individuals (Fig. 1). We analyzed and compared the floral scent emitted by white and coloured inflorescences in these three orchid species. They display three different strategies of pollinator attraction: both *Orchis* species are food-deceptive species, in which pollinator attraction is based on pollinator naiveté in *O. mascula* and on visual mimicry in *O. simia*, while *A. c. fragrans* is nectar-rewarding. More specifically, we aimed at answering two questions: (1) Does floral scent differ between white- and coloured-flowered morphs in these three orchid species? We focused particularly on benzenoid compounds in floral volatiles of the two morphs, as these compounds are hypothesized to be linked to the production of anthocyanin pigments. (2) Does scent difference between colour morphs depend on reproductive strategy, i.e. between non-rewarding (food-deceptive) species and food-rewarding species? As explained above, it can be expected that intraspecific variation of floral volatiles will be higher in food-deceptive orchids than in food-rewarding species, in order to minimize the avoidance learning ability of pollinators.

Results and discussion

Chemical composition of floral volatiles from both colour morphs

A total of 47 volatile compounds were identified from emissions of *O. mascula* inflorescences (Table 1). The volatile profile was largely dominated by terpene products: 38 of the 47 compounds (81%) originated from the terpenoid biosynthetic pathway. The major components (each with >10% of the profile) were (E)-ocimene, limonene, (Z)-3-hexenyl acetate, and linalool. No difference was observed between the two populations studied.

In *O. simia*, 41 volatile compounds were isolated and identified from emissions of flowers. As in *O. mascula*, terpene products were predominant in the floral scent of *O. simia*: α-pinene, myrcene, β-phellandrene. However, several lipids were found to dominate the volatile profile in a few individuals: (Z)-3-hexenyl acetate (mean 17% of the profile in the white morph, but up to 35% in some individuals of the white morph), nonanal and decanal.

A total of 27 volatile compounds were found in emissions of inflorescences of *A. c. fragrans*, including 17 compounds from the shikimic pathway. The chemical composition of the floral scent was largely dominated by two benzenoid compounds: p-anisaldehyde and p-dimethoxybenzene, which accounted for 38% and 31% of all floral volatiles, respectively. These two major compounds were followed by two other benzenoid components: methyl anisate (9%) and 1,2,4-trimethoxybenzene (7%). These four major compounds accounted for 85% of the total volatile profile in the coloured morph (82% in the white morph), whilst the overall terpene products represented less than 1% of the profile.

Fig. 1. White- and coloured-flowered morphs of *Orchis mascula*, *Orchis simia*, and *Anacamptis coriophora fragrans*. Pictures taken in the study sites by the authors. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The three orchid species studied here have already been examined for flower scent using a different sampling technique. Purple *O. mascula* flowers have been sampled with dynamic headspace adsorption (onto the porous polymer Porapak Q) by Nilsson (1983) and Salzmann et al. (2007a), who detected 30 and 33 floral volatile compounds, respectively. We found roughly similar profiles for *O. mascula* and the same dominant compounds as in these previous studies, but collection with SPME fibers allowed us to isolate 14 more compounds from floral emissions. Schatz et al. (2010) also used the dynamic headspace adsorption method to collect *O. simia* volatiles, and found fewer compounds (23 compounds) than with SPME in our study (41 compounds). Salzmann et al. (2007a) also used dynamic headspace on Porapak Q to trap flower volatiles from *A. coriophora*, and isolated 54 floral volatile compounds, of which 15 were identified. In our study, we found fewer compounds (27), but we found the two same dominant compounds (p-anisaldehyde and p-dimethoxybenzene). However, we do not know if these authors sampled *A. c. fragrans* or the closely related subspecies *A. c. coriophora*.

Interspecific variation of floral volatiles and influence of reproductive strategies

Profiles of the floral odours of the three different orchid species were clearly distinct: the NMDS (stress = 0.10) conducted on the relative proportions of the most abundant compounds (compounds occurring at >1% level in the profile) showed a clear separation among the volatiles of the three species (Fig. 2), and especially between those of *A. c. fragrans* and the two other species. The PERMANOVA performed on the global dataset confirmed these differences (Table 2). The compounds that accounted for these differences were those that dominated the volatile profile and were different among the three orchid species (see above). The volatile profile of *A. c. fragrans* inflorescences was particularly characterized by benzenoid compounds, which accounted for 82-85% of the total compounds, while volatiles from *O. mascula* and *O. simia* mainly consisted of terpene products (81% and 78%, respectively).

Regarding the reproductive strategies of the three orchids, the two deceptive orchids, *O. mascula* and *O. simia*, also differed from the rewarding *A. c. fragrans* by two other characteristics. First, the total number of volatile compounds in the floral scent was much lower in *A. c. fragrans* (27 compounds) than in the two deceptive species *O. mascula* (47 compounds) and *O. simia* (41 compounds). Second, as mentioned above, the two deceptive orchids exhibited high inter-individual variation of floral volatiles, whereas the food-rewarding *A. c. fragrans* showed low variation of floral scent. The mean SE (mean SE of the relative proportions of compounds calculated for all individuals in each species) was higher for *O. mascula* (0.655) and *O. simia* (0.545) than for *A. c. fragrans* (0.398). A multivariate Levens's test followed by Tukey HSD comparisons showed that the variances for the three orchid species were significantly heterogeneous ($F_{(2;70)} = 52.15$, $P < 0.0001$).

The differences we observed in the volatile profiles across species are consistent with the predictions of high among-individual variation of floral traits in deceptive orchids. The frequent occurrence of such intraspecific variability is commonly considered to be a strategy to minimize avoidance-learning by pollinators: because insects face varying floral traits, it will take a longer time for them to recognize inflorescences without reward (Jersakova et al., 2006; Juillet et al., 2011; Salzmann et al., 2007b; Schiestl, 2005). Salzmann et al. (2007b) also reported lower variation of floral scent in the food-rewarding *A. c. fragrans* when compared with the closely related food-deceptive orchid *Anacamptis morio*. High intraspecific variation of floral scent has also been observed in other food-deceptive orchids, e.g. in *Dactylorhiza romana* (Salzmann and Schiestl, 2007), *Orchis pauciflora* (Salzmann et al., 2007a), and *Epidendrum ciliare* (Moya and Ackerman, 1993).

Intraspecific variation of floral volatiles and differences between colour morphs

Variation between white and purple morphs

Considering the three orchid species, and considering the whole set of compounds, the NMDS analysis showed no clear separation between white and purple morphs, but the PERMANOVA revealed a significant colour effect (Table 2). As the interaction term between colour and species effects also proved significant (Table 2), we tested the colour effect independently for each species. These analyses showed that the colour effect revealed by the PERMANOVA (Table 2) was mainly attributable to the significant variation between white and purple morphs of *O. simia* (Fig. 2; $F_{(1,16)} = 11.38$, $P < 0.001$), whereas for *O. mascula* and for *A. c. fragrans* inflorescences the volatile profile showed no significant variation between the two colour morphs (Fig. 2; *O. mascula*: $F_{(1,35)} = 1.10$, $P = 0.33$; *A. c. fragrans*: $F_{(1,19)} = 0.63$, $P = 0.73$). A few qualitative differences were observed between the two colour morphs of *O. mascula*. Eleven compounds were found only in the volatile emissions from *O. mascula* purple inflorescences, while 9 other compounds present in volatiles from white inflorescences were not detected in any of the purple samples. In *A. c. fragrans*, 4 and 5 compounds were found only in coloured and only in white morphs, respectively. All these "colour-specific" components were found only as traces (<1%), and were found only in a few individuals (fewer than half of individuals for each colour morph). In *O. simia*, consistent differences were found between the volatile profiles of white- and coloured-flowered morphs. Several compounds showed large differences in their relative proportions between the two colour morphs. The variation was particularly

Table 1
Mean composition of floral volatiles emitted by white- and coloured-flowered morphs of *O. mascula*, *O. simia* and *A. c. fragrans*. Values are expressed as a percentage relative to total volatile compounds. RI: retention index; SE: standard error; O: occurrence.

Compound	RI	*Orchis mascula*				*Orchis simia*				*Anacamptis coriophora fragrans*			
		Purple (n = 24)		White (n = 24)		Purple (n = 9)		White (n = 8)		Purple (n = 10)		White (n = 11)	
		Mean ± SE	O	Mean ± SE	O	Mean ± SE	O	Mean ± SE	O	Mean ± SE	O	Mean ± SE	O
Fatty acid derivatives													
(Z)-3-Hexenol	850	0.65 ± 0.63	6	1.26 ± 0.42	15	–		1.87 ± 0.63	4	–		–	
Decane	1000	–		0.88 ± 0.88	4	0.44 ± 0.44	2	–		–		–	
(Z)-3-Hexenyl acetate	1006	6.46 ± 2.44	19	12.20 ± 3.89	20	3.21 ± 2.44	7	17.17 ± 2.44	7	0.05 ± 2.44	1	–	
Nonanal	1008	–		–		5.47 ± 1.28	9	6.76 ± 0.28	7	–		0.90 ± 0.28	8
Methyl octanoate	1123	0.53 ± 0.39	4	–		–		–		–		–	
2-Decanone	1190	–		0.22 ± 0.22	2	–		–		–		–	
(Z)-3-Hexenyl butyrate	1184	1.44 ± 0.59	12	0.29 ± 0.14	12	–		–		–		–	
Dodecane	1200	–		–		–		–		–		0.02 ± 0.16	1
Decanal	1208	–		–		2.15 ± 0.34	9	7.49 ± 0.34	6	0.89 ± 0.34	10	1.67 ± 0.34	9
Methyl decanoate	1325	0.93 ± 0.31	6	–		–		–		–		–	
Tridecane	1400	–		–		0.04 ± 0.01	1	–		0.03 ± 0.01	1	–	
Methyl dodecanoate	1524	1.69 ± 1.27	8	–		–		–		–		–	
Hexadecene-1	1586	0.83 ± 0.83	9	0.36 ± 0.36	8	–		–		–		–	
Benzenoids													
p-methylanisole	1015	–		–		0.96 ± 0.23	1	0.99 ± 0.15	2	0.14 ± 0.13	1	0.65 ± 0.19	2
Methyl benzoate	1089	–		–		–		–		0.36 ± 0.23	2	0.11 ± 0.09	1
p-Cresol	1071	–		–		–		0.55 ± 0.55	1	–		–	
Phenylethanal	1121	0.64 ± 0.49	13	2.68 ± 1.33	18	–		–		–		–	
1,4-Dimethoxybenzene	1171	–		–		–		–		31.45 ± 7.29	10	30.55 ± 14.25	10
2-Phenylethanol	1106	0.93 ± 0.64	15	1.86 ± 1.59	13	–		–		–		0.08 ± 0.03	1
2-(p-Methoxyphenyl)ethanol	1249	–		–		–		–		–		0.08 ± 0.05	1
p-anisaldehyde	1265	–		–		–		–		38.48 ± 10.79	10	38.39 ± 18.50	10
Cinnamaldéhyde (E)	1267	–		0.87 ± 0.35	7	–		3.87 ± 1.35	3	–		2.34 ± 1.05	2
p-Anisic acid	1273	–		–		–		–		0.08 ± 0.05	2	0.07 ± 0.03	3
p-Anisyl alcohol	1293	–		–		–		–		0.77 ± 0.29	7	0.91 ± 0.20	6
Methyl cinnamate (Z)	1299	–		0.89 ± 0.61	6	–		–		2.48 ± 1.31	10	2.91 ± 2.10	10
p-Methoxyphenylacetic acid	1341	–		–		–		–		0.19 ± 0.15	2	0.12 ± 0.05	3
1,2,4-Trimethoxybenzene	1378	–		–		–		–		7.04 ± 4.35	10	6.10 ± 1.85	10
Methyl p-anisate	1384	–		–		–		–		9.13 ± 3.95	10	8.58 ± 3.83	10
Methyl cinnamate (E)	1395	–		0.15 ± 0.11	7	–		–		–		–	
Veratraldehyde	1489	–		–		–		–		0.08 ± 0.04	2	0.08 ± 0.03	1
4-Phenylbutanone	1521	–		0.36 ± 0.17	9	–		–		–		–	
1,2,4,5-Tetramethoxybenzene	1537	–		–		–		–		3.11 ± 0.81	10	3.07 ± 1 .01	10
2,4,6-Trimethoxybenzaldehyde	1605	–		–		–		–		0.33 ± 0.25	3	–	
Coumarine	1621	–		–	–	–		0.38 ± 1.31	1	–		–	
Buthyl p-methoxy benzoate	1685	–		–	–	–		–		1.85 ± 0.95	10	1.48 ± 1.10	9
Terpenoids													
α-Pinene	935	2.52 ± 0.64	21	4.12 ± 0.78	19	11.49 ± 2.86	9	15.67 ± 3.96	7	–		0.19 ± 0.09	3
Sabinene	969	2.89 ± 0.67	20	2.41 ± 0.65	19	6.12 ± 1.65	9	3.98 ± 2.11	5	–		–	
β-Pinene	979	2.15 ± 0.76	18	3.84 ± 1.91	13	4.58 ± 2.78	9	1.32 ± 0.88	5	–		–	
6-Methylhepten-2-one	981	2.49 ± 1.93	11	4.16 ± 1.20	19	–		3.97 ± 1.93	7	–		–	
Myrcene	988	3.88 ± 1.22	19	5.23 ± 2.00	18	7.12 ± 1.66	9	6.82 ± 1.61	6	0.01 ± 0.01	1	0.25 ± 0.20	3
α-Terpinene	1014	–		–		0.84 ± 0.62	7	0.54 ± 0.28	3	–		–	
p-Cymene	1027	–		–		0.88 ± 0.21	5	0.84 ± 0.66	4	0.08 ± 0.04	1	–	
Limonene	1031	10.67 ± 4.26	20	12.88 ± 2.74	21	4.26 ± 0.95	8	3.56 ± 0.98	7	0.67 ± 0.34	1	0.83 ± 0.56	4
β-phellandrene	1034	–		–		9.03 ± 1.29	9	4.19 ± 1.60	6	–		–	
1.8-Cineole	1035	3.95 ± 2.86	15	2.52 ± 0.75	18	0.95 ± 0.38	5	0.77 ± 0.18	7	–		–	
Ocimene-(Z)	1039	2.35 ± 0.50	22	1.87 ± 0.52	20	–		–		–		–	
Ocimene-(E)	1047	22.68 ± 7.20	19	16.30 ± 3.60	21	0.88 ± 0.41	6	0.29 ± 0.23	2	–		–	
γ-Terpinene	1057	–		–		3.67 ± 0.31		4.44 ± 0.83	5	0.12 ± 0.14	1	0.05 ± 0.04	2
Sabinene hydrate trans	1072	0.12 ± 0.83	8	0.29 ± 0.29	10	3.58 ± 0.44	9	0.27 ± 0.27	1	–		–	
Terpinolene	1088	–		–		2.46 ± 0.35		–		–		–	
Linalool oxide trans	1090	4.75 ± 1.27	21	8.30 ± 2.20		–		0.28 ± 0.28	1	–		–	
Camphenol-6 al	1091	–		–	–	4.95 ± 0.44	9	1.40 ± 0.74	3	–		–	
N.I.[a] (m/z 39 67 96 109)	1102	–		–	–	10.40 ± 1.07	9	2.42 ± 1.70	2	–		–	
Linalool	1103	13.15 ± 5.89	21	3.46 ± 0.78	21	–		2.72 ± 1.09	4	–		–	
Allo-ocimene-(Z)	1128	2.52 ± 0.86	14	1.74 ± 0.71	15	–		–		–		–	
α-Campholenal	1129	–		–		1.49 ± 0.19	9	0.51 ± 0.34	2	–		–	
2,6-Dimethyloctatetraene	1128	–		0.73 ± 0.58	5	–		–		–		–	
Allo-ocimene-(E)	1144	0.53 ± 0.22	15	0.62 ± 0.23	8	–		–		–		–	
Pinocarveol	1147	–		–		1.05 ± 0.45	4	0.39 ± 0.39	1	–		–	
4-Oxoisophorone	1151	0.65 ± 0.47	5	0.35 ± 0.27	8	–		–		–		–	
β-Terpineol trans	1157	0.73 ± 0.43	8	0.30 ± 0.16	5	–		–		–		–	
Pinocarvone	1160	0.61 ± 0.49	8	0.48 ± 0.33	5	1.84 ± 0.21	9	0.59 ± 0.40	2	–		–	
Hydroxy-1,8-cineole	1171	0.55 ± 0.55	5	–		–		–		–		–	
Terpinene-4-ol	1183	0.82 ± 0.69	5	–		4.88 ± 1.04	9	2.68 ± 0.80	6	–		–	
Pinanone	1185	–		–		0.86 ± 0.16	7	–		–		–	
p-Cymen-8-ol	1190	0.12 ± 0.12	4	–		0.24 ± 0.15	3	–		–		–	

Table 1 (*continued*)

Compound	RI	*Orchis mascula*				*Orchis simia*				*Anacamptis coriophora fragrans*			
		Purple (n = 24)		White (n = 24)		Purple (n = 9)		White (n = 8)		Purple (n = 10)		White (n = 11)	
		Mean ± SE	O	Mean ± SE	O	Mean ± SE	O	Mean ± SE	O	Mean ± SE	O	Mean ± SE	O
α-Terpineol	1199	1.19 ± 0.52	18	1.39 ± 0.48	15	1.71 ± 0.19	9	0.07 ± 0.37	4	–		–	
Carveol trans	1215	–		–		0.80 ± 0.15	8	–		–		–	
Cinerone	1231	0.75 ± 0.75	4	0.38 ± 0.38	6	–		–		–		–	
Cuminaldehyde	1238	–		–		0.72 ± 0.12	8	–		–		–	
Carvone	1247	0.36 ± 0.36	8	–		0.72 ± 0.14	8	0.07 ± 0.07	1	–		–	
Pinocarveol trans	1249	0.18 ± 0.18	11	0.78 ± 0.53	7	–		–		–		–	
2,3-Pinanediol	1258	–		–		0.29 ± 0.14	4	–		–		–	
8-Hydroxylinalool	1310	–		0.79 ± 0.79	5	–		–		–		–	
α-Terpinyl acetate	1346	–		–		–		0.36 ± 0.36	1	–		–	
α-Copaene	1381	0.22 ± 0.22	5	–		–		–		–		–	
β-Bourbonene	1387	0.44 ± 0.38	11	0.13 ± 0.72	6	–		–		–		–	
β-Caryophyllene	1417	0.23 ± 0.19	5	0.26 ± 0.28	5	1.89 ± 0.73	6	0.80 ± 0.65	2	–		–	
α-Bergamotene trans	1432	0.15 ± 0.13	7	1.12 ± 0.71	2	–		–		–		–	
β-Farnesene	1454	1.38 ± 0.79	4	–		–		–		–		–	
Geranylacetone	1484	–		0.89 ± 0.44	5	–		0.15 ± 0.15	1	–		–	
Germacrene D	1485	0.84 ± 0.84	5	–		–		–		–		–	
α-Curcumene	1487	0.72 ± 0.50	9	0.50 ± 0.42	5	–		–		–		–	
α-Muurolene	1498	0.69 ± 0.69	2	0.73 ± 0.73	6	–		–		–		–	
Germacrene A	1509	0.12 ± 0.83	12	0.84 ± 0.58	5								
		–		–		–		–					
δ-Cadinene	1518	0.47 ± 0.47	8	0.55 ± 0.47	7	–		–		–		–	
(E.E)-α-Farnesene	1522	0.23 ± 0.18	7	–		–		–		–		–	

[a] N.I. = Not identified (α-pinene oxide + another co-eluted compound).

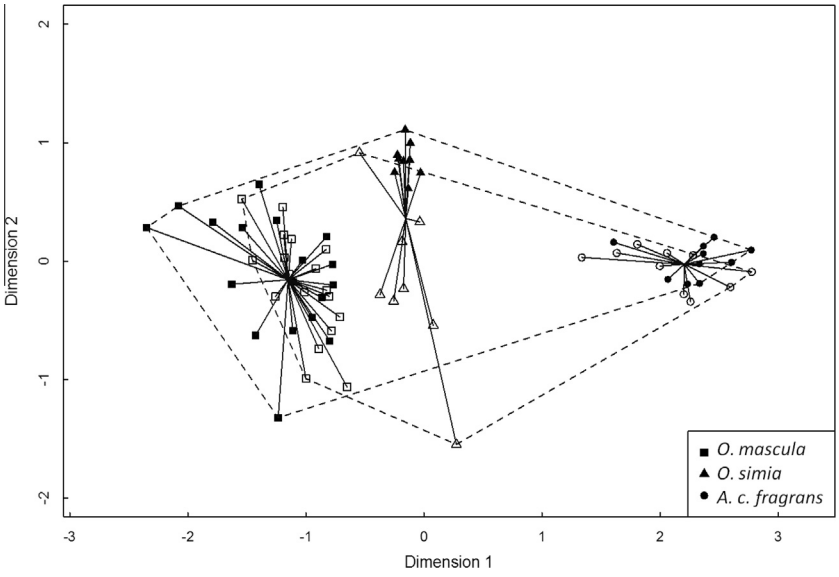

Fig. 2. Non-metric multi-dimensional scaling of the relative proportions of floral volatiles emitted by white- (open plots) and coloured-flowered (closed plots) morphs of *O. mascula*, *O. simia* and *A. c. fragrans* based on the Bray-Curtis dissimilarity index (stress = 0.10). The analysis included the relative proportions of the major volatile compounds (>1% of the total volatiles). The centroid of samples from the same species is indicated. The dashed line groups samples of the same colour.

Table 2
PERMANOVA of the relative proportions of floral volatiles emitted by individuals, testing for the effects of species, of colour and the interaction between these two factors.

Sources of variation	Df	MS	F	P
Species	2	6.5	3.69	<0.001
Colour	1	0.47	51.55	0.0082
Species*Colour	2	0.44	3.47	0.0022
Residuals	67	0.13		

clear for (Z)-3-hexenyl acetate (17% in the white morph, 3% in the coloured morph) and for NI (not identified, 2% in the white morph, 10% in the coloured morph). The NMDS analysis conducted on the most abundant compounds (>1%) showed a separation of the *O. simia* white and coloured morphs (Fig 2).

When focusing more specifically on the group of benzenoid compounds, which is thought to be associated with the production of anthocyanin-derived pigments, significant differences were found between coloured and white morphs for two species, *O. mascula* and *O. simia* (Table 3). In both species, white inflorescences exhibited significantly more benzenoid compounds than coloured ones, and the relative proportion of most of these benzenoids increased in the white inflorescences when compared to coloured ones (Table 1). Interestingly, one shikimic compound, cinnamaldehyde, was isolated in many white inflorescences of the three orchids, but has never been detected in any of the purple-coloured inflorescences. Methyl cinnamate (Z and E) were also detected only

Table 3
Comparison (PERMANOVA) of the relative proportions of benzenoid floral volatiles emitted by white- and coloured-flowered morphs, for *O. mascula*, *O. simia* and *A. c. fragrans*.

	Mean (±SE)			
	White	Purple	F	P
O. mascula	6.09 (±7.81)	0.73 (±1.97)	7.511	0.002
O. simia	10.05 (±11.95)	1.09 (±3.08)	4.169	0.038
A. c. fragrans	96.07 (±5.42)	97.55 (±3.22)	0.493	0.534

in the white form of *O. mascula*, and occurred in both forms of *A. c. fragrans*, but in greater proportions in the white form.

The first important result of this study is thus that floral volatiles emitted by these orchids did not clearly differ between white- and purple-flowered morphs, when all the volatile compound classes are considered. Only *O. simia* exhibited few differences between white and coloured morphs, white flowers being observed to emit higher amounts of lipid products (e.g. (Z)-3-hexenol, 6-methylhepten-2-one, (Z)-3-hexenyl acetate, decanal) than purple flowers. In other studies, the chemical composition of floral volatiles has been shown to vary consistently between distinct colour morphs (Flamini et al., 2002; Li et al., 2006; Majetic et al., 2007; Odell et al., 1999; Salzmann and Schiestl, 2007). However, other authors did not find clear differences in floral odours between colour morphs of a same species (Majetic et al., 2008; Olesen and Knudsen, 1994; Wang et al., 2013). Interestingly, our second analysis restricted to the volatile benzenoid compounds produced by these orchids showed larger differences between white and coloured morphs, both for *O. mascula* and *O. simia*. It would be also interesting in a further study to sample nocturnal volatile emissions of orchid flowers, because white flowered morphs have been observed to emit more benzenoid compounds during night in some cases (Raguso et al., 2003; Majetic et al., 2007). Recent studies have suggested that particular associations may exist between floral colour and scent, which would result from shared biochemistry between these two floral traits (Majetic et al., 2007, 2010; Zuker et al., 2002). Such processes have been proposed to explain the differences in floral scent in polymorphic plants showing co-occurring white- and coloured-flowered morphs in natural populations. In these cases, white flowers have been shown to emit more benzenoid products than purple flowers (Majetic et al., 2007; Zuker et al., 2002). These authors hypothesized that the flavonoid precursors of floral pigments (anthocyanins responsible for purple colouration) in blocked biosynthetic pathways may be converted into volatile benzenoid compounds, leading to a direct biosynthetic connection between such colour and odour characters. Higher amount of benzenoids in white morphs has also been hypothesized to be an adaptation maximizing attraction of night-flying moth pollinators (Raguso et al., 2003). Other plant species, however, did not show a similar link between colour and scent: for example, the white morph of *Syringa oblata* flowers has been shown to emit lower amounts of benzenoid compounds, e.g. benzaldehyde, than the purple morph (Li et al., 2006). In our case, the significantly higher number of benzenoid compounds in *Orchis* white inflorescences are consistent with the hypothesis that shared biochemistry creates pleiotropic links between colour and scent, in which the absence of purple pigmentation (shikimate pathway) would result in an increase of volatile benzenoid compounds. For example, compounds such as cinnamaldehyde and methyl cinnamate, which were observed to be absent or occurred in much lower proportions in purple inflorescences than in white inflorescences (Table 1), are known to represent key metabolites leading to flavonoid pigments responsible for floral coloration (Holton and Cornish, 1995; Dewick, 2002; Dudareva et al., 2013), e.g. anthocyanins in purple flowers. Some anthocyanin pigments (e.g. cyaniding-3,5-diglucoside, orchicyanins I and II) have already been identified in *O. mascula* and *O. simia* flower parts (Arditti, 1992).

In another polymorphic deceptive orchid species, *Calanthe sylvatica*, which exhibits three colour morphs, with white, lilac and purple flowers, floral scent emitted by white flowers does not differ from those emitted by coloured flowers (Delle-Vedove et al., 2011). In fact, two floral scent profiles were identified in the white-flowered morph, either similar to floral scent of lilac flowers, or similar to floral scent of purple flowers. No more benzenoid compounds were found in the white-flowered individuals than in other morphs. However, the underlying biochemical and/or ecological processes responsible for the white floral colour in *C. sylvatica* may differ from those for the three orchids studied here: white morphs are not rare in *C. sylvatica*, and floral colour polymorphism is characterized by three floral colour morphs (lilac, white, and purple), equally abundant, and usually separated in distinct populations and adapted to ecologically different situations (Juillet et al., 2010).

Several different mutations in both structural and regulatory genes of the shikimate pathway may affect anthocyanin production and cause flower colour change, e.g. a lack of pigment production in white-flowered morphs (Levin and Brack, 1995; Majetic et al, 2008; Nakatsuka et al., 2005; Rausher, 2008). More precisely, it has been shown that distinct mutations acting on different genes of the anthocyanin pathway can cause effective different changes in floral scent emission, even within a single plant species (Zuker et al., 2002; Zvi et al., 2008). A mutation located upstream of the biosynthesis of both volatiles and pigments would lead to a positive correlation, whereas a mutation located downstream from volatile synthesis but upstream of pigment synthesis would lead to a negative correlation (Majetic et al., 2008; Wang et al., 2013). In our case, whether the white-flowered variants observed in the three orchid species result from similar mutations or from different/independent mutations affecting separate branches of the anthocyanin biosynthetic pathway is unknown.

Variation between individuals

In *O. mascula* and *O. simia*, there was considerable variation among individuals for most of the compounds of the volatile profiles. For example, in *O. mascula*, the level of (E)-ocimene varied from 0% to 76.8% of the profile between individuals. (Z)-3-hexenyl acetate, limonene, α-pinene, linalool, linalool oxide, and 6-methylhepten-2-one also showed very large differences in their relative proportions among individuals, regardless of the colour morph considered. In *O. simia*, the major compounds also showed great variation in their relative proportion among individuals, some compounds ranging from 0% to more than 20% in many cases. In *A. c. fragrans*, the overall variation of floral volatiles among individuals was much lower than that observed in the two *Orchis* species.

Conclusion

We showed that floral volatile signals emitted by two orchids did not clearly differ between white- and purple-flowered morphs. Only *O. simia* exhibited different volatile profiles between the two colour morphs. Interestingly, white inflorescences were found to emit more benzenoid compounds than coloured inflorescences in two orchid species, aligning with the shared biochemistry hypothesis. But for *O. simia*, coloured and white morphs also differed by the level of some lipid products. Other authors found higher levels of terpenoids in a population of the white form of *Hesperis matronalis* (Majetic et al., 2007). Finally, white morphs can thus be associated with increased volatile amounts of benzenoids, terpenoids, or fatty acid derivatives. As expected from their pollination strategy, the two deceptive orchids showed high inter-individual varia-

tion of floral volatiles, whereas the food-rewarding *A. c. fragrans* showed low variation of floral scent.

Variation of floral scent in purple and white orchid morphs might simply be explained by floral colour change, but may also be influenced by multiple and/or conflicting selection pressures acting on pleiotropic effects of flower traits (Coberly and Rausher, 2008; Rausher, 2008; Sánchez-Lafuente, 2002). Whether pollinator-imposed selection may have a direct impact on floral scent emissions, independent of biochemical constraints due to floral colour, remains unanswered. In *O. mascula*, odour signals from flowers have been hypothesized to play a minor role for pollinator orientation towards purple or white morphs (Dormont et al., 2010a). In the two other orchids, *O. simia* and *A. coriophora*, the effects of floral colour and scent on pollinator attraction have never been investigated. It would be interesting to measure and compare the reproductive success of white and coloured morph in these orchids. Further olfactory tests, e.g., GC-EAD experiments that examine pollinator responses to the minor compounds specific to each colour morph, are also needed to further explore whether there is any variation in the olfactory cues for pollinators between purple and white morphs. In some other plant-insect systems, host location and recognition by insects has been proved to be partly governed by particular minor volatile compounds emanating from the host-plant (Balao et al., 2011; Bernays and Chapman, 1994; Bruce and Pickett, 2011; Schatz et al., 2009; Soler et al., 2012). Other tests, such as olfactometer bioassays, will also help to evaluate whether floral odours in these orchids may help pollinators to distinguish between purple and white morphs.

Experimental

Organisms and study sites

Orchis mascula L. is a perennial non-rewarding orchid species, widely distributed in Europe, western Asia, and northern Africa. Its elongated inflorescences consist generally of 5 to 20 purple flowers, which bloomed in mid-April in the study area. *O. mascula* flowers are visited and pollinated by numerous species present early in the spring such as bumblebees (*Bombus* spp.), honeybees (*Apis mellifera*), cuckoo bumblebees (*Psithyrus* spp.), solitary bee species of several genera (*Eucera*, *Nomada*, *Andrena*, *Apis*), and several coleopteran and dipteran species (Bournérias and Prat, 2005; Claessens and Kleynen, 2011; Cozzolino et al., 2005; Nilsson, 1983). We also sampled *O. mascula* floral volatiles in a preliminary study (Dormont et al., 2010a), and we here extended the sampling to more individuals and additional populations.

Orchis simia Lamarck is a non-rewarding orchid species, and is also widely distributed from Europe to northern Africa and Iran. Its spherical inflorescences typically display 10 to 40 pink flowers which bloomed at the beginning of May in the study area. The only confirmed pollinators of *O. simia* are the beetle *Cidnopus pillosus* and the moth *Hemaris fuciformis* and some hymenopterans such as honeybees (Bournérias and Prat, 2005; Claessens and Kleynen, 2011; Schatz, 2006).

Anacamptis coriophora subsp. *fragrans* (L.) Bateman, Pridgeon and Chase, is a food-rewarding species, producing nectar, whose range is restricted to the Mediterranean area. Its elongated inflorescences have 5 to 25 flowers showing red-brown colouration. Honeybees, several bumblebees and some moths of the genus *Zygaena* are known to be the most frequent pollinators of *A. c. fragrans* flowers (Claessens and Kleynen, 2011; Dafni and Ivri, 1979; Van der Cingel, 1995).

Locally, white-coloured individuals are regularly observed within natural populations (Fig. 1). Their frequency within the populations of coloured individuals is less than 1% for *O. mascula* (n >20,000 observed individuals) and for *O. simia* ($n > 10{,}000$) and less than 3% for *A. c. fragrans* ($n > 3000$) (B. Schatz, pers. obs.). All the experiments were carried out in the "Causse du Larzac", an extensive limestone plateau located roughly 70 km north of Montpellier, in southern France.

Field sampling of floral volatiles

Floral volatiles were monitored using solid-phase microextraction (SPME), a non-destructive, solvent-free sampling technique ideally adapted to the sampling of volatiles on living plant individuals *in situ* (Flamini et al., 2002; Musteata and Pawliszyn, 2007). A total of 48 *O. mascula* individuals (24 white morphs and 24 purple morphs), 17 *O. simia* individuals (8 white and 9 purple), and 21 *A. c. fragrans* (11 white and 10 purple), were randomly selected and sampled for scent collection between 11:00 and 16:00. The period of floral volatile sampling *in situ* was defined with respect to both the flowering period and the period of maximum activity of insects during the day, i.e., between 11:00 and 16:00.

Sampling by SPME was performed using 65 μm polydimethylsiloxane/divinylbenzene (PDMS-DVB) fibres (Supelco®). The whole inflorescence was enclosed in a bag made from polyethylene terephtalate (Nalophan®; Kalle Nalo GmbH, Wursthüllen, Germany), a nonreactive plastic. After the equilibration time (15 min), the fibre was introduced with a manual holder into the Nalophan® bag containing the inflorescence. The fibre was exposed in close proximity (2 cm) to flowers for 45 min, which was found in preliminary tests to be the best duration time for collecting volatiles. For each sampled population, a control bag was also sampled: an SPME fibre was inserted into an empty Nalophan® bag, in order to monitor volatiles from the air surrounding the plant.

Gas chromatography-mass spectrometry of floral volatiles

GC-MS analyses of the SPME extracts were performed using electronic impact ionization mode on a Varian Saturn 2000 ion trap spectrometer, interfaced with a Varian GC CP-3800 apparatus. The Varian CP-3800 was equipped with a 1079 split-splitless injector (260 °C) and a 30 m × 0.25 mm × 0.25 μm film thickness ID WCOT CPSil-8CB fused silica capillary column (Chrompack®, Bergen op Zoom, The Netherlands), with helium as carrier gas (1 ml min^{-1}), and programmed 2 min isothermal at 50 °C, then 50 °C to 220 °C at 4 °C min^{-1}. Mass spectra were recorded in electronic impact (EI) at 70eV, and identified by matching the mass spectra with data of three MS libraries (NIST, Adams 2007, and Wiley), as well as with data from the MS bank of the CEFE laboratory. For all compounds, identification was also performed by comparison of calculated retention index (RI) with data from libraries and published data (Adams 2007). Peaks were quantified using Star Chromatography Software®. The relative importance of each compound was expressed with respect to total volatiles in order to compare the volatile profiles of the samples.

Data analysis

The chemical composition of floral volatiles from coloured- and white-flowered individuals in the three orchid species was analysed in R 2.14.1 (R Development Core Team, 2011) using multivariate analysis incorporated in the Vegan package (Oksanen et al., 2012). For each species and for each colour morph, the relative proportion of each compound that accounted for more than 1% of total volatiles was included in the analyses. We also conducted additional analyses specifically for benzenoids, considering all the compounds (i.e. including compounds that accounted for less than 1% of total volatiles). Following Soler et al. (2012), the data were transformed into a data matrix of pairwise Bray-Curtis dissimilarity

indices between samples, and a non-metric multi-dimensional scaling (NMDS) was first used to visualize similarities among samples. Secondly, a Permutational Multivariate Analysis of Variance (PERMANOVA) on the distance matrices based on 4999 permutations (Anderson, 2001) was performed to test for differences in the floral scent among the three orchid species, among the two colour morphs and also among these different factors combined. The interaction between colour and species effects was shown to be significant, so that scent variation between purple and white morphs was then analysed separately within each species using PERMANOVA. For groups that were significantly different, a similarity percentage (SIMPER) was used to identify the compounds responsible for the variations. Finally, the difference of variance in total scent composition within an orchid species was tested using a multivariate analogue of Levene's test for homogeneity of variance.

Acknowledgments

We thank B. Buatois and N. Barthes, from the CEFE's platform of chemical analyses in ecology (PACE), for technical assistance in chemical analyses. We are grateful to D. McKey, Université Montpellier II and CEFE-CNRS, for comments on the manuscript and for useful discussions.

References

Ackerman, J.D., Carromero, W., 2005. Is reproductive success related to colour polymorphism in a deception pollinated tropical terrestrial orchid? Caribbean J. Sci. 41, 234–242.
Adler, L.S., Irwin, R.E., 2012. What you smell is more important than what you see? Natural selection on floral scent. New Phytol. 195, 510–511.
Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecol. 26, 32–46.
Arditti, J., 1992. Fundamentals of orchid biology. John Wiley, New York.
Balao, F., Herrera, J., Talavera, S., Dötterl, S., 2011. Spatial and temporal patterns of floral scent emission in *Dianthus inoxianus* and electroantennographic responses of its hawkmoth pollinator. Phytochemistry 72, 601–609.
Bernays, E.A., Chapman, R.F., 1994. Host-plant selection by phytophagous insects. Chapman & Hall, New York.
Bournérias, M., Prat, D., 2005. Les orchidées de France, Belgique, et Luxembourg. Coll. Parthénope. Biotope, Mèze.
Brown, B.A., Clegg, M.T., 1984. Influence of flower color polymorphism on genetic transmission in a natural population of the common morning glory, *Ipomoea purpurea*. Evolution 38, 769–803.
Bruce, T.J.A., Pickett, J.A., 2011. Perception of plant volatile blends by herbivorous insects - finding the right mix. Phytochemistry 72, 1605–1611.
Burger, H., Dötterl, S., Ayasse, M., 2010. Host-plant finding and recognition by visual and olfactory floral cues in an oligolectic bee. Funct. Ecol. 24, 1234–1240.
Chittka, L., Raine, N.E., 2006. Recognition of flowers by pollinators. Curr. Opin. Plant Biol. 9, 428–435.
Claessens, J., Kleynen, J., 2011. The flower of the European orchid – form and function. Jean Claessens and Jacques Kleynen, Geuelle.
Coberly, L.C., Rausher, M.D., 2008. Pleiotropic effects of an allele producing white flowers. Evolution 62, 1076–1085.
Cozzolino, S., Schiestl, F., Muller, A., De Castro, O., Nardella, A.M., Widmer, A., 2005. Evidence for pollinator sharing in Mediterranean nectar-mimic orchids: absence of premating barriers? Proc. Roy. Soc. London B 272, 1271–1278.
Dafni, A., Ivri, Y., 1979. Pollination ecology of, and hybridization between *Orchis coriophora* L. and *O. collina*. Sol. ex. Russ (Orchidaceae) in Israel. New Phytol. 83, 181–187.
Delle-Vedove, R., Juillet, N., Bessière, J.-M., Dormont, L., Pailler, T., Schatz, B., 2011. Colour-scent associations in a tropical orchid: three colours but two odours. Phytochemistry 72, 735–742.
Dewick, P.M., 2002. The shikimate pathway: aromatic amino acids and phenylpropanoids. In: Medicinal Natural Products: a Biosynthetic Approach, second ed. John Wiley & Sons Ltd, Chichester, pp. 121–166.
Dormont, L., Delle-Vedove, R., Bessière, J.-M., Hossaert-McKey, M., Schatz, B., 2010a. Rare white-flowered morphs increase the reproductive success of common purple morphs in a food-deceptive orchid. New Phytol. 185, 300–310.
Dormont, L., Delle-Vedove, R., Bessière, J.-M., Hossaert-McKey, M., Schatz, B., 2010b. Helping in food-deceptive orchids? A possible new mechanism maintaining polymorphism of floral signals. Plant Signal. Behav. 5, 1–2.
Dötterl, S., Milchreit, K., Schäffler, I., 2011. Behavioural plasticity and sex differences in host finding of a specialized bee species. J. Comp. Physiol. A 197, 1119–1126.
Dudareva, N., Klempien, A., Joëlle, K., Muhlemann, J.K., Kaplan, I., 2013. Biosynthesis, function and metabolic engineering of plant volatile organic compounds. Tansley reviews. New Phytol. 198, 16–32.
Eckhart, V.M., Rushing, N.S., Hart, G.M., Hansen, J.D., 2006. Frequency-dependent pollinator foraging in polymorphic *Clarkia xantiana* ssp. *xantiana* populations: implications for flower colour evolution and pollinator interactions. Oikos 112, 412–421.
Flamini, G., Cioni, P.L., Morelli, I., 2002. Analysis of the essential oil of the aerial parts of *Viola etrusca* from Monte Labbro (South Tuscany, Italy) and *in vivo* analysis of flower volatiles using SPME. Flavour Frag. J. 17, 147–149.
Galen, C., 1999. Why do flowers vary? The functional ecology of variation in flower size and form within natural plant populations. Bioscience 49, 631–640.
Gigord, L.D.B., Macnair, M.R., Smithson, A., 2001. Negative frequency-dependent selection maintains a dramatic flower color polymorphism in the rewardless orchid *Dactylorhiza sambucina* (L.) Soò. Proc. Natl. Acad. Sci. USA 98, 6253–6255.
Glover, B.J., 2011. Pollinator attraction: the importance of looking good and smelling nice. Curr. Biol. 21, 307–309.
Holton, T.A., Cornish, E.C., 1995. Genetics and biochemistry of anthocyanin biosynthesis. Plant Cell 7, 1071–1083.
Jersakova, J., Johnson, S.D., Kindlmann, P., 2006. Mechanisms and evolution of deceptive pollination in orchids. Biol. Rev. 81, 219–235.
Jones, K.N., Reithel, J.S., 2001. Pollinator-mediated selection on a flower color polymorphism in experimental populations of *Antirrhinum* (Scrophulariaceae). Am. J. Bot. 88, 447–454.
Juillet, N., Delle-Vedove, R., Dormont, L., Schatz, B., Pailler, T., 2010. Differentiation in a tropical deceptive orchid: colour polymorphism and beyond. Plant Syst. Evol. 289, 213–221.
Juillet, N., Salzmann, C.C., Scopece, G., 2011. Does facilitating pollinator learning impede deceptive orchid attractiveness? A multi-approach test of avoidance learning. Plant Biol. 13, 570–575.
Klahre, U., Gurba, A., Hermann, K., Saxenhofer, M., Bossolini, E., Guerin, P.M., Kuhlemeier, C., 2011. Pollinator choice in Petunia depends on two major genetic Loci for floral scent production. Curr. Biol. 21, 730–739.
Knudsen, J.T., Eriksson, R., Gershenzon, J., Stahl, B., 2006. Diversity and distribution of floral scent. Bot. Rev. 72, 1–120.
Koivisto, A.-M., Vallius, E., Salonen, V., 2002. Pollination and reproductive success of two colour variants of a deceptive orchid, *Dactylorhiza maculata* (Orchidaceae). Nordic J. Bot. 22, 53–58.
Kunze, J., Gumbert, A., 2001. The combined effect of color and odor on flower choice behavior of bumble bees in flower mimicry systems. Behav. Ecol. 12, 447–456.
Levin, D.A., Brack, E., 1995. Natural selection against white petals in *Phlox*. Evolution 49, 1017–1022.
Li, Z.G., Lee, M.R., Shen, D.L., 2006. Analysis of volatile compounds emitted from fresh *Syringa oblate* flowers in different florescence by headspace solid-phase microextraction-gas chromatography-mass spectrometry. Anal. Chim. Acta 276, 43–49.
Majetic, C.J., Raguso, R.A., Tonsor, S.J., Ashman, T.-L., 2007. Flower color-flower scent associations in polymorphic *Hesperis matronalis* (Brassicaceae). Phytochemistry 68, 865–874.
Majetic, C.J., Raguso, R.A., Ashman, T.-L., 2008. The impact of biochemistry vs. population membership on floral scent profiles in colour polymorphic *Hesperis matronalis*. Ann. Bot. 102, 911–922.
Majetic, C.J., Raguso, R.A., Ashman, T.-L., 2009. Smell of success: floral scent affects pollinator attraction and seed fitness in *Hesperis matronalis* (Brassicaceae). Funct. Ecol. 23, 480–487.
Majetic, C.J., Rausher, M.D., Raguso, R.A., 2010. The pigment-scent connection: do mutations in regulatory vs. structural anthocyanin genes differentially alter floral scent production in *Ipomoea purpurea*? S. Afr. J. Bot. 76, 632–642.
Malerba, R., Naterro, J., 2012. Pollinator response to flower color polymorphism and floral display in a plant with a single-locus floral color polymorphism: consequences for plant reproduction. Ecol. Res. 27, 377–385.
Milet-Pinheiro, P., Ayasse, M., Schlindwein, C., Dobson, H.E.M., Dötterl, S., 2012. Host location by visual and olfactory floral cues in an oligolectic bee: innate and learned behaviour. Behav. Ecol. 23, 531–538.
Musteata, F.M., Pawliszyn, J., 2007. In vivo sampling with solid phase microextraction. J. Biochem. Biophys. Meth. 70, 181–193.
Nakatsuka, T., Nishihara, M., Mishiba, K., Yamamura, S., 2005. Two different mutations are involved in the formation of white-flowered gentian plants. Plant Sci. 169, 949–958.
Newman, E., Anderson, B., Johnson, S.D., 2012. Flower colour adaptation in a mimetic orchid. Proc. R. Soc. B. 279, 2309–2313.
Nilsson, L.A., 1983. Anthecology of *Orchis mascula* (Orchidaceae). Nordic J. Bot. 3, 157–179.
Oksanen J., Blanchet G.F., Kindt R., Legendre P., Minchin P.R., O'Hara B. et al., 2012. Vegan: Community Ecology Package. R version 2.0-3. Available at: http://vegan.r-forge.r-project.org/.
Olesen, J.M., Knudsen, J.T., 1994. Scent profiles of colour morphs of *Corydalis cava* (Fumariaceae) in relation to foraging behaviour of bumblebee queens (*Bombus terrestris*). Biochem. Syst. Ecol. 22, 231–237.
Moya, S.J.D., Ackerman, J.D., 1993. Variation in the floral fragrance of *Epidendrum ciliare* (Orchidaceae). Nordic J. Bot. 13, 41–47.
Odell, E., Raguso, R.A., Jones, K.N., 1999. Bumblebee foraging responses to variation in floral scent and color in snapdragons (*Antirrhinum*: Scrophulariaceae). Am. Midl. Nat. 142, 257–265.
R Development Core Team, 2011. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.
Raguso, R.A., 2008a. Wake up and smell the roses: the ecology and evolution of floral scent. Annu. Rev. Ecol. Evol. Syst. 39, 549–569.

Raguso, R.A., 2008b. Start making scents: the challenge of integrating chemistry into pollination ecology. Entomol. Exp. Appl. 128, 196–207.
Raguso, R.A., Levin, R.A., Fooze, S.E., Holmberg, M.W., McDade, L.A., 2003. Fragrance chemistry, nocturnal rhythms and pollination "syndromes"' in *Nicotiana*. Phytochemistry 63, 265–284.
Rausher, M.D., 2008. Evolutionary transitions in floral color. Int. J. Plant Sci. 169, 7–21.
Salzmann, C., Cozzolino, S., Schiestl, F., 2007a. Floral scent in food-deceptive orchids: species specificity and sources of variability. Plant Biol. 9, 720–729.
Salzmann, C., Nardella, A.M., Cozzolino, S., Schiestl, F.P., 2007b. Variability in floral scent in rewarding and deceptive orchids: the signature of pollinator-imposed selection? Ann. Bot. 100, 757–765.
Salzmann, C.C., Schiestl, F.P., 2007. Odour and colour polymorphism in the food-deceptive orchid *Dactylorhiza romana*. Plant Syst. Evol. 267, 37–45.
Sánchez-Lafuente, A.M., 2002. Floral variation in the generalist perennial herb *Paeonia broteroi* (Paeoniaceae): differences between regions with different pollinators and herbivores. Am. J. Bot. 89, 1260–1269.
Schatz, B., 2006. Fine scale distribution of pollinator explains the occurrence of the natural orchid hybrid x*Orchis bergonii*. Ecoscience 13, 111–118.
Schatz, B., Djieto-Lordon, C., Dormont, L., Bessière, J.-M., McKey, D., Blatrix, R., 2009. A simple, non-specific chemical signal mediates defence behaviour in a specialised ant-plant mutualism. Curr. Biol. 19, 361–362.
Schatz, B., Geoffroy, A., Dainat, B., Bessiere, J.-M., Buatois, B., Hossaert-McKey, M., Selosse, M.A., 2010. A case study of modified interactions with symbionts in a hybrid Mediterranean orchid. Am. J. Bot. 97, 1278–1288.
Schatz, B., Delle-Vedove, R., Dormont, L., 2013. Presence, distribution and effect of white, pink and purple morphs on pollination in the orchid *Orchis mascula*. Eur. J. Environ. Sci. 3, 119–128.
Schiestl, F., 2005. On the success of a swindle: pollination by deception in orchids. Naturwissenschaften 92, 255–264.
Schiestl, F., 2010. The evolution of floral scent and insect chemical communication. Ecol. Lett. 13, 643–656.
Schemske, D.W., Bierzychudek, P., 2007. Spatial differentiation for flower color in the desert annual *Linanthus parryae*: was Wright right? Evolution 61, 2528–2543.
Smithson, A., MacNair, M.R., 1997. Negative frequency-dependent selection by pollinators on artificial flowers without rewards. Evolution 51, 715–723.
Soler, C., Proffit, M., Bessiere, J.-M., Hossaert-McKey, M., Schatz, B., 2012. Evidence for intersexual chemical mimicry in a dioecious plant. Ecol. Lett. 9, 978–985.
Strauss, S.Y., Whittall, J.B., 2006. Non-pollinator agents of selection on floral traits. In: Harder, L.D., Barrett, S.C.H. (Eds.), Ecology and Evolution of Flowers. Oxford University Press, Oxford, UK, pp. 120–138.
Streisfeld, M.A., Kohn, J.R., 2007. Environment and pollinator-mediated selection on parapatric floral races of *Mimulus aurantiacus*. J. Evol. Biol. 20, 122–132.
Suchet, C., Dormont, L., Schatz, B., Giurfa, M., Simon, V., Raynaud, C., Chave, J., 2011. Floral scent variation in two *Antirrhinum majus* subspecies influences the choice of naïve bumblebees. Behav. Ecol. Sociobiol. 65, 1015–1027.
Tremblay, R.L., Ackermann, J.D., Zimmerman, J.K., Calvo, R.N., 2005. Variation in sexual reproduction in orchids and its evolutionary consequences: a spasmodic journey to diversification. Biol. J. Linn. Soc. 84, 1–54.
Van der Cingel, N.A., 1995. An Atlas of Orchid Pollination - European Orchids. Balkema, Rotterdam.
Wang, H., Conchou, L., Bessiere, J.-M., Cazals, G., Imbert, E., Schatz, B., 2013. Flower color polymorphic *Iris lutescens* (Iridaceae): biochemical analyses in light of plant-insect interactions. Phytochemistry 94, 123–134.
Warren, J., Mackenzie, S., 2001. Why are all colour combinations not equally represented as flower-colour polymorphisms? New Phytol. 151, 237–241.
Weiss, M.R., 1995. Floral color-change - a widespread functional convergence. Am. J. Bot. 82, 167–185.
Zuker, A., Tzfira, T., Ben-Meir, H., Ovadis, M., Shklarman, E., Itzhaki, H., Forkmann, G., Martens, S., Neta-Sharir, I., Weiss, D., Vainstein, A., 2002. Modification of flower colour and fragrance by antisense suppression of the flavonone 3-hydroxylase gene. Mol. Breed. 9, 33–41.
Zvi, M.M.B., Negre-Zakharov, F., Masci, T., Ovadis, M., Shklarman, E., Ben-Meir, H., Tzfira, T., Dudareva, N., Vainstein, A., 2008. Interlinking showy traits: co-engineering of scent and colour biosynthesis in flowers. Plant Biotechnol. J. 6, 403–415.