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Rare white-flowered morphs increase the reproductive success of common purple morphs in a food-deceptive orchid

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Summary

• How floral colour polymorphism can be maintained in evolutionary time is still debated. In rewardless orchids, it is unknown whether rare white-flowered morphs differ in scent chemistry from pigmented morphs, and whether such intraspecific variation in floral signals may have an impact on reproductive success.

• We compared the chemical composition of floral volatiles emitted by white- and purple-flowered morphs of *Orchis mascula*, and recorded the fruit set of both colour morphs. We also used white ping-pong balls to mimic white-flowered morphs in field bioassays.

• We found that colour polymorphism was not associated with floral odour polymorphism. Surprisingly, when populations of purple-flowered plants included a few white-flowered individuals, the fruit set of the purple morph increased significantly (from 6 to 27%), while that of the white morph remained low. We obtained the same fourfold increase in fruit set when using ping-pong balls as visual lures, demonstrating the association between colour variation and fruit set, and the key role of visual signals in pollinator attraction.

• Our results are incompatible with negative frequency-dependent selection, a hypothesis invoked to explain colour polymorphism in other rewardless orchids. We propose several hypotheses to explain the maintenance of white morphs in *O. mascula*.

Introduction

Colour polymorphisms are widespread in both animals and plants (Weiss, 1995; Bond, 2007). Why different colour morphs have evolved, and how they are maintained in populations have long intrigued ecologists. In particular, many flowering plants show substantial intraspecific variation in floral colour (Weiss, 1995; Galen, 1999; Warren & Mackenzie, 2001). Current explanations for polymorphism in floral signals most frequently rely on the key role of insects through pollinator-mediated selection: insects use diverse floral signals (flower colour, odour, size and shape) to detect and select the flower species they visit in search of rewards (Chittka & Raine, 2006). For example, colour polymorphism (Brown & Clegg, 1984; Jones & Reithel, 2001), flower height (Dickson & Petit, 2006) and variation in floral scent (Knudsen, 2002; Raguso *et al.*, 2003; Majetic *et al.*, 2009) have been reported to be associated with various pollinator preferences. More recently, it has been proposed that intraspecific variation of floral traits may also reflect multiple and conflicting selection pressures, involving not only pollinators but also herbivore-protection strategies, local abiotic conditions or indirect selection via pleiotropic effects (Warren & Mackenzie, 2001; Schemske & Bierzychudek, 2007; Coberly & Rausher, 2008). Whether pollinators are the primary selective agents influencing floral polymorphism, or whether such polymorphisms are driven mainly by nonpollinator agents of selection, remains under debate (Strauss & Whittall, 2006; Rausher, 2008).

Orchids present a great diversity of floral characters associated with animal pollination. Approximately one-third of

all orchid species achieve pollination through food deception; that is, flowers contain no nectar or other rewards but resemble or mimic floral signals of rewarding plants to attract pollinators (Jersakova *et al.*, 2006a). Consequently, variation in floral traits is expected to be high in food-deceptive orchids, because pollinators will learn to avoid common unrewarding floral phenotypes (Schiestl, 2005; Jersakova *et al.*, 2006a). 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 & 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 (NFDS) has been hypothesized to explain the maintenance of floral polymorphism in rewardless orchids, at least for colour traits (Smithson & MacNair, 1997; Gigord *et al.*, 2001). 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). However, colour polymorphism in this species has also been recently explained by other hypotheses (Jersakova *et al.*, 2006b; Smithson *et al.*, 2007). Regarding another important floral signal, olfactory cues, variation in floral scent composition at individual or population levels has also been reported in some orchid species (Moya & Ackerman, 1993; Schiestl *et al.*, 1997; Salzmann & Schiestl, 2007). However, to what extent colour and odour polymorphism may influence reproductive success remains poorly understood.

Among the wide range of colour variants in orchid flowers, the occurrence of rare hypochromic inflorescences (pale morphs and even entirely white flowers) has long intrigued ecologists. Many orchid species occasionally show a few white-flowered individuals within natural populations of the common-coloured morph (Weiss, 1995; Bournérias & Prat, 2005). In other plant families, where white-coloured flower morphs have been observed, pollinators have been shown to discriminate among colour morphs, and insect behaviour can thus explain, at least partly, differential reproductive success of the colour morphs (Waser & Price, 1981; Brown & Clegg, 1984; Stanton *et al.*, 1989; Odell *et al.*, 1999; Jones & Reithel, 2001; Raguso *et al.*, 2003). However, it remains unclear whether the presence of white flowers is simply the result of repeated spontaneous mutations or inbreeding, or whether pollinator-mediated selection may have contributed to maintenance of hypochromic morphs. In orchids, the behavioural responses of pollinators to white inflorescences within populations of a coloured morph, and the possible consequences for plant reproductive success, have been poorly investigated (Koivisto *et al.*, 2002; Ackerman & Carromero, 2005). It is also unknown whether such white orchid flowers differ from coloured morphs in their production of olfactory signals. Recent studies examining the floral scent composition of different colour morphs in polymorphic plant species have reported consistent flower colour–flower scent associations (Flamini *et al.*, 2002; Majetic *et al.*, 2007; Salzmann & Schiestl, 2007). In particular, in diverse plant families, white flower morphs have been shown to clearly differ in scent chemistry from coloured morphs (Olesen & Knudsen, 1994; Zucker *et al.*, 2002; Raguso *et al.*, 2003; Li *et al.*, 2006; Majetic *et al.*, 2007). For example, light- or white-coloured flowers have been reported to emit more benzenoid compounds than other colour morphs, and this has been hypothesized to be an adaptation maximizing attraction of night-flying moth pollinators (Raguso *et al.*, 2003). Because volatile aromatic compounds and anthocyanin-derived pigments responsible for flower coloration both originate from the same phenylpropanoid biosynthetic pathway, Zucker *et al.* (2002) and Majetic *et al.* (2007) hypothesized that colour–scent associations in flowers may result from particular biochemical processes, by which flavonoid precursors of flower pigmentation in blocked biosynthetic pathways may be converted into volatile aromatic compounds.

For these reasons, effects of floral colour and odour signals have to be considered together in studies of pollinator attraction and plant reproductive success (Majetic *et al.*, 2009). In recent reviews, Raguso (2008a,b) emphasized that food-deceptive plants primarily use visual cues to elicit pollinator behaviour, not because odour is unimportant, but rather because of how odour–colour combinations are learned. He also stressed the importance of taking into account behavioural interplay between visual and olfactory components of floral phenotype, in order to develop a more integrated understanding of how pollinators experience flowers and shape their evolution (Raguso, 2008a).

The 'early purple orchid', *Orchis mascula* L., is a food-deceptive orchid distributed throughout Europe which typically exhibits red-purple flowers. However, rare white-flowered individuals can be regularly observed within populations of purple-flowered ones. According to the NFDS hypothesis proposed for rewardless orchids (Gigord *et al.*, 2001), we would expect differences in the reproductive success between the two *O. mascula* colour morphs: avoidance of the common purple morph by deceived pollinators would lead to more frequent visitation and pollination of the rare morph, conferring a selective advantage to the white morph. In this study, we addressed the following questions: first, is there a flower scent–flower colour association for the two *O. mascula* morphs; secondly, does reproductive success differ between the two colour morphs; and thirdly, if there is a difference in reproductive success, how do floral signals influence this parameter? Is the colour difference alone important? Based on our results, we offer new arguments to explain tentatively the maintenance of rare white-flowered plants in orchids.

Materials and Methods

Study sites and organism

The experiments were carried out in two different sites in a mountainous area of south-central France. The first site was located *c.* 70 km north of Montpellier, on the Causse du Larzac, an extensive limestone plateau (43°53′N, 3°15′E, 740 m altitude). The second site was situated on the Causse du Blandas, another calcareous plateau 10 km from the first site (43°55′N, 3°29′E, 710 m altitude). In each site, we surveyed several patches of *O. mascula* individuals, each of which consisted of *c.* 20–100 individuals and was separated by at least 50 m (usually 300 m or more) from the nearest neighbouring patch. In this study, and because most patches were very distant from each other, each patch of 20–100 individuals was considered as a 'population'.

Orchis mascula L. is a perennial nonrewarding orchid species, widely distributed in Europe, western Asia and northern Africa. Inflorescences generally consist of five to 20 purple flowers. In some populations, a few white-flowered individuals occur mixed with the purple-flowered individuals. *O. mascula* flowers are pollinator-dependent (Nilsson, 1983; B. Schatz, unpublished) and are visited and pollinated by bumblebees (*Bombus* spp.), cuckoo bumblebees (*Psithyrus* spp.), solitary bee species of several genera (*Eucera*, *Nomada*, *Andrena*, *Apis*) and a chafer beetle (*Cetonia aurata*) (Nilsson, 1983; Bournérias & Prat, 2005; Cozzolino *et al.*, 2005). Flowering occurs early in spring, and *O. mascula* is known to exploit newly emerged insect pollinators, suggesting that pollination in this species is effected mainly by visits of naïve, inexperienced insects (Nilsson, 1983; Van der Cingel, 1995). Increased pollinator abundance in the vicinity of nectariferous coflowering species (Van der Cingel, 1995; Johnson *et al.*, 2003) is unlikely to be an important factor in the reproductive success of *O. mascula*, as in our study sites very few other plants were observed to grow and flower during the early period of flowering of *O. mascula*.

Floral volatiles

Sampling of floral volatiles Floral volatiles were monitored using solid-phase microextraction (SPME), a nondestructive, solvent-free sampling technique permitting the sampling of volatiles *in situ* on living plant individuals. A total of 36 *O. mascula* individuals were randomly selected and sampled for scent collection between 11:00 h and 16:00 h. We sampled four individuals of each colour morph in each of the sites 1 and 2 during 2006, and 10 individuals of each colour in site 2 during 2007. 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, that is, between 11:00 and 16:00 h.

Sampling by SPME was performed using 65 μm polydimethylsiloxane/divinylbenzene (PDMS-DVB) fibres (Supelco, Sigma-Aldrich, Bellefonte, PA, USA). The whole inflorescence was enclosed in a bag made from polyethylene terephthalate (Nalophan; Kalle Nalo GmbH, Wursthüllen, Germany), a nonreactive plastic. After the equilibration time, the fibre was introduced with a manual holder into the Nalophan bag containing the inflorescence. The fibre was exposed for 45 min in close proximity (2 cm) to flowers. For each sampled population of *O. mascula* individuals, 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

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 (Varian, Palo Alto, CA, USA) 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 to 220°C at 4°C min^{-1}. Mass spectra were recorded in electronic impact (EI) at 70 eV, and identified by comparison with data of the NIST 98 software library (Varian, Palo Alto, CA, USA). Floral volatiles were identified based on retention time of external standards, and with GC-MS analyses. Peaks were quantified using Star Chromatography Software (Varian, Palo Alto, CA, USA). The relative importance of each compound was expressed with respect to total volatiles in order to compare the volatile profile of the samples.

Data analysis

The chemical compositions of floral volatiles from purple- and white-flowered *O. mascula* were compared using principal-component analysis (PCA, covariance matrix, STATBOX 6.6; Logi Labo, Paris, France). Relative proportions of compounds emitted by whole inflorescences were used for these multivariate analyses and only the compounds which accounted for > 1% of total volatiles were included in these analyses. We then tested the effect of colour and population on the relative proportion of volatile compounds using a multivariate analysis of variance (MANOVA, PROC GLM, type 3, SAS V9.1; SAS, Cary, USA) followed by univariate (i.e. sequential) analyses on each dependent variable to test which compounds contributed to the overall significance in this analysis

(Stevens, 1992) with Sidak's corrections for multiple comparisons.

Measuring reproductive success

Natural fruit set The total numbers of white- and purple-flowered individuals were counted in the two sites to establish the proportion of each colour morph within each observed population of *O. mascula* individuals. The reproductive success of plants was assessed by comparing the mean fruit set among populations of three types, all within an area of *c.* 2 ha: (i) 12 natural populations with only purple-flowered individuals (n = 255); (ii) 14 natural populations of purple-flowered individuals together with a few white-flowered individuals (n = 258); and (iii) 13 experimental populations with only purple-flowered individuals occurring naturally (n = 292) and to which white lures mimicking white-flowered individuals were added (see next paragraph below). In all populations and for each individual, at the end of April (4 wk after placing the visual lures in the experimental populations) we counted the number of mature fruits and the total number of flowers in each inflorescence, to establish the fruit set. Individual fruit sets were pooled for each of the three types of populations (populations of pure purple plants; populations of mixed white/purple plants; populations including visual lures), as fruit set values were not significantly different within a population type. In this study, the fruit set was used to estimate plant fitness, but further experiments will need to consider seed viability or seed germination as well. To test for the effect of the three types of populations on the number of flowers per inflorescence and fruit set values, we performed a Poisson regression analysis of these data with a log link function (Genmod analysis; SAS v9.1).

Use of white lures The role of visual cues in pollinator attraction to *O. mascula* flowers was estimated by using visual lures during field bioassays. We used white ping-pong balls to mimic white-flowered morphs within a population of purple-flowered individuals. This white, spherical standardized object is roughly similar in size to an *O. mascula* inflorescence. Each white ping-pong ball was fixed on a wire supporting a shaft made of dark green metal, adjusted so that the height of the lure was equal to the mean height of surrounding *O. mascula* purple inflorescences. We placed these lures within populations of purple-flowered individuals (one lure for 22 ± 5 purple-flowered individuals) at frequencies similar to the mean natural frequency of white inflorescences recorded in mixed populations (one white-flowered individual for 21.7 ± 9.8 (mean ± SD) purple-flowered individuals). The populations of purple plants used for this experiment were selected at random within all the populations of only purple-flowered individuals. Visual lures were placed at random within each sampled population, at the very beginning of the flowering period, and were left during the whole flowering period.

We measured the exact distance between each purple individual and the nearest white individual or white lure, in order to estimate whether proximity to a white morph affects the probability that a purple individual is pollinated. For each 20 cm distance class, we used a Mann–Whitney *U*-test to compare the reproductive success of purple-flowered individuals in the two different situations, that is, in populations with white individuals or lures and in pure purple populations.

Results

Chemical composition of floral volatiles

The volatile profile of *O. mascula* inflorescences showed very little variation between the two colour morphs. A total of 61 volatile compounds were identified in the floral volatiles of *O. mascula*: 51 and 43 compounds, respectively, were detected in the volatile emissions from purple-flowered and white-flowered individuals (Table 1). In both colour morphs, the volatile profile was largely dominated by terpene products: 27 of the 43 compounds (62.8%) in the white-morph emissions, and 34 of the 51 compounds (66.6%) in the purple-morph emissions were terpene products. The major components (> 10% of the profile) in both morphs were (E)-ocimene, limonene, (Z)-3-hexenyl acetate, and linalool. No difference was observed between populations for the same colour morph.

A few qualitative differences were observed between the two colour morphs. Eighteen compounds were found only in the volatile emissions from purple inflorescences, while 10 other compounds present in volatiles from white inflorescences were not detected in any of the purple samples. All these 'specific' components were found only as traces (< 1%) and were found only in a few individuals (fewer than half of the individuals for each colour morph). Half of the compounds found exclusively in white-morph volatiles originated from the shikimic pathway (five of the 10 components specific to white inflorescences, e.g. methyl cinnamate), whereas all compounds specific to purple-morph profiles were related to the two other (lipid and terpenoid) pathways.

A PCA analysis conducted on the relative proportions of the most abundant compounds (compounds occurring at > 1% in the profile) showed no clear separation between the volatiles from the two colour morphs (Fig. 1). Three components – (E)-ocimene, linalool and limonene – explained 68% of the variance. Considering the compounds observed in both colour morphs, the overall composition of the bouquet (20 compounds) did not differ significantly between the two colour morphs and among populations (MANOVA

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Table 1 Mean composition of *Orchis mascula* floral volatiles

		Purple (n = 18)				White (n = 18)			
Compound	RT	Mean	SE	CV	O	Mean	SE	CV	O
Fatty acid derivatives									
(Z)-3-hexenol	6.20	0.65	0.63	0.78	4	1.26	0.42	0.37	12
Methylheptenone	10.61	3.49	1.93	1.03	10	4.16	1.20	0.59	13
Decane	11.24	–	–	–	–	0.88	0.88	0.94	4
(Z)-3-hexenyl acetate	11.74	7.46	2.44	0.89	16	14.20	3.89	1.03	17
Octatrienal	13.86	0.28	0.28	0.53	3	–	–	–	–
Methyl octanoate	14.82	0.53	0.39	0.54	4	–	–	–	–
Methyl caprilate C6	14.97	0.11	1.00	3.02	3	–	–	–	–
2-Decanone	15.18	–	–	–	–	0.22	0.22	0.47	2
(Z)-hexenyl butyrate	17.44	1.44	0.59	0.49	9	0.29	0.14	0.26	11
Octatrienone	17.63	0.12	0.12	0.35	5	0.77	0.77	0.88	7
Hexenyl valorate	17.71	0.16	0.16	0.40	2	–	–	–	–
Dimethyl octadiene diol	23.50	0.34	0.34	0.58	3	–	–	–	–
Methyl caprate C10	23.97	0.93	0.31	0.32	6	–	–	–	–
Alcanal	24.75	0.17	0.15	0.36	4	–	–	–	–
Methyl dodecanoate	28.04	0.37	0.37	0.61	6	–	–	–	–
Cyclopentanol (derived)	28.17	0.83	0.83	0.91	5	0.36	0.36	0.60	8
Methyl laurate	28.24	1.32	1.32	1.15	5	0.90	0.90	0.95	7
Benzenoids									
Acetophenone	13.8	0.64	0.49	0.61	9	2.68	1.33	0.81	14
2-Phenylethanol	14.74	0.93	0.64	0.66	12	1.86	1.59	1.17	13
Phenyl ethanol	15.42	–	–	–	–	0.29	0.29	0.54	3
Cinnamaldehyde	17.25	–	–	–	–	0.87	0.35	0.38	8
4-Phenylbutanone	23.51	–	–	–	–	0.36	0.17	0.28	7
Methyl cinnamate (Z)	23.72	–	–	–	–	0.89	0.61	0.65	4
Methyl cinnamate (E)	24.14	–	–	–	–	0.15	0.11	0.28	4
Terpenoids									
α-Pinene	8.29	2.52	0.64	0.40	17	4.12	0.78	0.38	18
Sabinene	9.61	2.89	0.67	0.39	17	3.41	0.65	0.35	17
β-Pinene	9.98	2.55	0.76	0.48	15	3.84	1.91	0.97	13
Myrcene	11.19	3.88	1.220	0.62	16	5.23	2.00	0.87	18
Limonene	11.59	11.67	4.26	1.25	16	12.88	2.74	0.76	16
1.8-Cineole	11.75	3.95	2.86	1.44	13	3.52	0.75	0.40	17
(Z)-ocimene	11.77	2.35	0.50	0.33	17	1.87	0.52	0.38	16
(E)-ocimene	12.17	26.68	7.20	1.39	15	16.30	3.60	0.89	17
β-Terpineol	12.80	0.73	0.43	0.50	8	0.30	0.16	0.29	5
Sabinene hydrate	13.13	0.12	0.83	2.40	4	0.29	0.29	0.54	10
Linalool oxide	13.71	4.75	1.27	0.58	17	9.30	2.20	0.72	18
Linalool	14.15	13.15	5.89	1.62	17	3.46	0.78	0.42	17
Allo-ocimene 1	15.13	2.52	0.86	0.54	9	1.74	0.71	0.54	12
Dimethyloctatetraene	15.24	–	–	–	–	0.73	0.58	0.68	3
Allo-ocimène 2	15.56	0.53	0.22	0.30	10	0.62	0.23	0.29	6
4-Oxoisophorone	15.76	0.65	0.47	0.58	3	0.35	0.27	0.46	7
Terpinene-4-ol	16.01	0.82	0.69	0.76	3	–	–	–	–
p cymen-8-ol	16.87	0.12	0.12	0.35	2	–	–	–	–
Cinerone	17.12	0.75	0.75	0.87	4	0.38	0.38	0.62	6
Pinocarvone	16.16	0.61	0.49	0.63	8	0.48	0.33	0.48	2
Hydroxy cineole	16.30	0.55	0.55	0.74	5	–	–	–	–
Carvone	16.76	0.36	0.36	0.60	2	–	–	–	–
Pinocarveol	17.45	0.18	0.18	0.42	9	0.78	0.53	0.60	5
α-Terpineol	17.63	1.19	0.52	0.48	10	1.39	0.48	0.41	14
Terpenyl acetate	18.65	0.81	0.81	0.90	5	–	–	–	–
Hydroxy linalool	21.43	–	–	–	–	0.79	0.79	0.89	5
Thujopinene	22.15	0.17	0.13	0.32	2	–	–	–	–
α-Copaene	23.67	0.22	0.22	0.47	3	–	–	–	–
β-Bourbonene	23.99	0.44	0.38	0.57	8	0.13	0.72	2.00	3
β-Caryophyllene	25.13	0.23	0.19	0.40	5	0.26	0.28	0.55	5

Table 1 (Continued.)

Compound	RT	Purple (n = 18)				White (n = 18)			
		Mean	SE	CV	O	Mean	SE	CV	O
Trans-α-bergamotene R	25.44	0.15	0.13	0.34	5	1.12	0.71	0.67	2
β-Farnesene	25.62	1.38	0.79	0.67	4	–	–	–	–
Geranylacetone	25.95	–	–	–	–	0.89	0.44	0.47	3
Germacrene D	26.87	0.84	0.84	0.92	5	–	–	–	–
α-Curcumene	26.98	0.72	0.50	0.59	9	0.50	0.42	0.59	5
α-Muurolene	27.56	0.69	0.69	0.83	2	0.73	0.73	0.85	6
α-Longipinene		0.12	0.83	2.40	8	0.84	0.58	0.63	3
δ-Cadinene	27.98	0.47	0.47	0.69	3	0.55	0.47	0.63	7
(E.E)-α-farnesene	28.18	0.23	0.18	0.38	7	–	–	–	7

Values are expressed as a percentage relative to total volatile compounds.
RT, retention time; SE, standard error; CV, coefficient of variation; O, occurrence (i.e. the number of individuals in which the compound was detected; the total of sampled individuals for each colour morph is 18).

analysis: $F_{1,32}$ = 0.08, P = 0.78, and $F_{2,32}$ = 1.08, P = 0.35, respectively). For most compounds, there was considerable variation among individuals of each colour morph. For example, the concentration of (E)-ocimene varied from 0 to 81.3% of the profile between the purple-morph individuals, and from 0 to 47.7% between the white-morph individuals. (Z)-3-hexenyl acetate, limonene, α-pinene, linalool, linalool oxide and methyl heptenone also showed very large differences in their relative proportions among orchid individuals, within each of the two morphs.

Reproductive success

White morphs were consistently rare; the observed frequencies of white-flowered individuals, calculated by considering all the populations, including pure purple populations, were 1.03% in site 1 in 2006 (n = 1563), and 0.87 and 0.91% in site 2 in 2006 (n = 1145) and 2007 (n = 1324), respectively. In other neighbouring populations, white-flowered individuals never accounted for > 1.4% of the population (B. Schatz, pers. obs.). Plant density within a population was not significantly different between populations of pure purple individuals and populations of mixed-colour individuals (Student's test, P = 0.97). The mean number of flowers per inflorescence was significantly different between the two colour morphs (χ^2 = 7.6; d.f. = 1, P = 0.006), and was 11.95 ± 0.89 (mean ± SE) in the purple-coloured morph (n = 805) and 14.55 ± 0.15 in the white-coloured morph (n = 21; the small sample size is to the result of the low frequency of this morph within the studied populations).

A total of 805 orchid individuals and 11 709 flowers were surveyed to evaluate fruit set. For the three types of population, the average fruit set of purple-flowered individuals was 6.16 ± 0.40% (mean ± SE) in populations with only purple-flowered individuals, 27.22 ± 1.38% in populations displaying a few white-flowered individuals, and 26.35 ± 1.20% in populations with white lures (Fig. 2). The mean number of fruits produced per inflorescence was significantly different among these three types of population (Genmod, χ^2 = 44.68, d.f. = 2, P< 0.0001). Fruit sets of purple-flowered individuals were not significantly different in the populations with white flowers or with experimental lures (χ^2 = 0.00; d.f. = 1, P > 0.99), but fruit sets in both these kinds of populations were more than four times higher than that of purple-flowered individuals in populations where the latter comprised the sole morph (χ^2 = 32.93; d.f. = 1, P < 0.001). The average fruit set of white-flowered individuals (n = 13) was 6.67 ± 2.11%, which was not significantly different from those of purple-flowered individuals in populations where they were the sole morph (χ^2 = 0.02; d.f. = 1, P = 0.88).

Fruit set of purple individuals was highly significantly dependent on whether they were near a white-flowered individual or a white lure (Fig. 3). This effect was significant for plants at distances of between 10–20 cm and 150–160 cm from the white individual or lure (Fig 3). The effect was similar in naturally polymorphic populations and in experimental populations with lures, except for the 110–120 cm and 130–140 cm distance classes (which were the only distance classes in which fruit set of purple individuals was significantly different between these two kinds of population; Mann–Whitney U-test, P < 0.01). Within the range of 10–160 cm, fruit set also decreased with increasing distance from the white individual or lure. Maximum mean fruit sets were observed at 10–20 cm, reaching 43.9% in naturally polymorphic populations and 47.4% in experimental populations with white lures.

Discussion

Three important and surprising results emerge from this study; first, colour polymorphism was not associated with very marked differences in floral scents, in contrast to studies in other plant species (Majetic *et al.*, 2007; Salzmann & Schiestl, 2007); secondly, the presence of rare white-flowered morphs of *O. mascula* resulted in increased

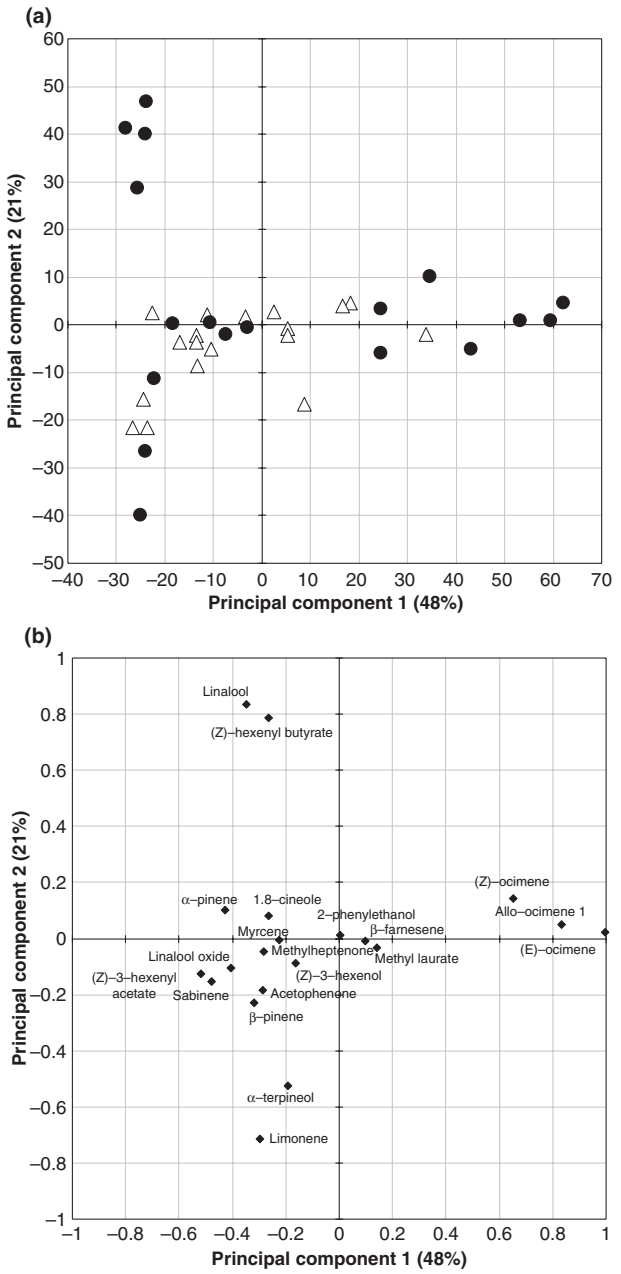

Fig. 1 (a) Principal-component analysis (PCA) of *Orchis mascula* floral volatiles emitted by inflorescences of purple morph (closed circles) and by inflorescences of the white morph (open triangles); (b) Factor loadings of the main volatile compounds from *O. mascula* flowers.

reproductive success of neighbouring purple-flowered individuals in mixed purple/white populations; and thirdly, visual signals, rather than olfactory signals, were responsible for increased pollinator visitation of purple individuals.

Influence of floral signals on reproductive success

As reported in previous studies (Nilsson, 1983; Van der Cingel, 1995; Jacquemyn *et al.*, 2008), the overall average fruit set of *O. mascula* was low (mean fruit set 6%), both for purple-morph individuals in pure purple populations and for white-morph individuals. Surprisingly, our study showed that the presence of co-occurring white-flowered individuals led to significantly higher reproductive success of nearby purple-flowered individuals (mean fruit set 27%), while white-flowered plants themselves had the same low fruit set (6%). These results are incompatible with the hypothesis of NFDS frequently assumed to explain floral colour polymorphism in nonrewarding orchids (Gigord *et al.*, 2001), as we found that the presence of the rare white *O. mascula* morph resulted in an advantage for the common purple morph.

Do differences in olfactory cues provided by the two *O. mascula* colour morphs help to explain differences in the number of pollinator visits to purple and white morphs? Floral volatiles emitted by the two *O. mascula* colour morphs showed no clear differences in chemical composition. Most volatile compounds were common to both purple and white inflorescences, and their relative proportions were similar in the two. The few qualitative differences detected between the two morphs involved minor compounds that were present in only few individuals and in very low amounts. Our results deviate from those of recent studies that have found flower colour–flower scent associations, with different colour morphs in polymorphic species showing different floral scent profiles (Olesen & Knudsen, 1994; Flamini *et al.*, 2002; Zucker *et al.*, 2002; Salzmann & Schiestl, 2007). Interestingly, white-coloured flower morphs have been reported in other systems to emit volatile blends clearly different from that of the purple morph (e.g. by emitting more benzenoid products (Li *et al.*, 2006; Majetic *et al.*, 2007)). We also found a larger number of aromatic compounds in the volatiles of white morphs, in *O. mascula* (Table 1), as well as in other orchid species showing white/purple floral polymorphism (L. Dormont & B. Schatz, unpublished). Because of the high variability of *O. mascula* floral volatiles (Table 1; Nilsson, 1983; Salzmann *et al.*, 2007a) and the absence of significant differences between purple and white morphs, we hypothesize that odour signals from *O. mascula* flowers probably do not help pollinators to distinguish between the two colour forms. However, further olfactory tests (e.g. GC-EAD experiments that examine pollinator responses to the minor compounds specific to each colour morph) are needed to explore in greater detail whether there is any variation in the olfactory cues for pollinators between purple and white morphs.

It seems reasonable to expect that visual signals alone probably play the key role in colour morph discrimination by insects in this case. Our experiments with visual lures confirmed this presumption: the presence of white ping-pong balls that mimic white *O. mascula* inflorescences enhanced the reproductive success of purple-flowered individuals located near the white lure. The effect was virtually

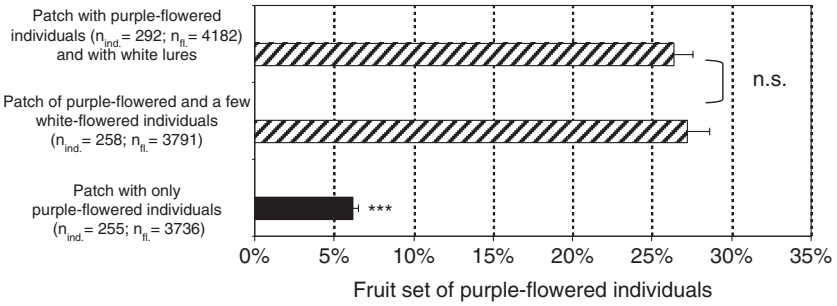

Fig. 2 Fruit set (mean ± SE) of purple-flowered *Orchis mascula* individuals in the three population types. Results from the Genmod analysis are indicated in the figure (n.s., non significant; ***, $P < 0.001$).

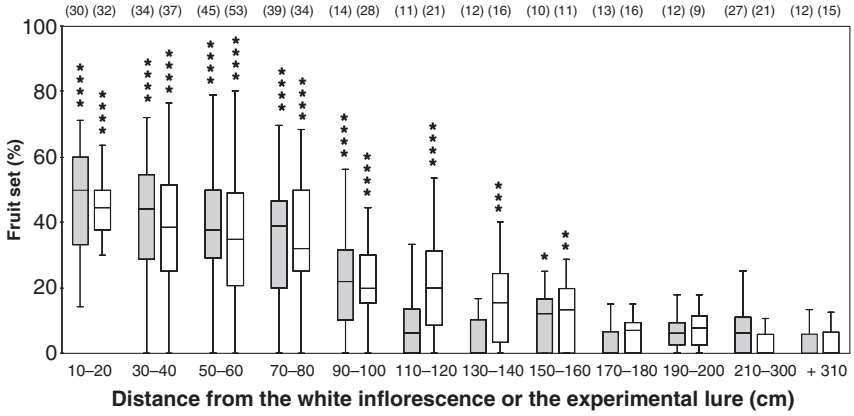

Fig. 3 Box plot representations of the relationship between fruit set (mean ± SE) of purple-flowered *Orchis mascula* individuals and the distance to the white inflorescence or the experimental lure, in either natural populations with a few white-coloured individuals (grey boxes) or in populations with white lures (white boxes). For a population type, each distance class was compared with those of the control population (i.e. the population with only purple-coloured individuals) by Mann–Whitney *U*-test (****, $P < 0.0001$; ***, $P < 0.0005$; **, $P < 0.005$; *, $P < 0.05$). For each population type and for each distance class, the sample size is indicated in brackets.

identical in magnitude (fruit set increased from 6 to 27%), whether the nearby white-coloured object was an *O. mascula* inflorescence or a ping-pong ball. This last result demonstrates that the association in nature between presence of white flowers and increased seed set is causal, and not the result of some unmeasured abiotic or biotic factor that simultaneously increases seed set and affects flower coloration. Also, in four populations of mixed white/purple plants, the white inflorescences were experimentally removed from the populations; we observed in these populations low fruit set for all purple-flowered individuals, similar to those recorded in natural populations of pure purple-morph plants (B. Schatz, unpublished).

Two main questions emerge from these results: why did the presence of white-flowered *O. mascula* result in increasing pollinator visits to nearby purple-flowered plants; and how can we explain the maintenance of the rare white morph, which was less frequently visited and pollinated than the purple morph?

Role of the white/purple colour contrast in pollinator attraction

Many authors have reported differences in reproductive success between floral colour morphs. In particular, in plant species where rare white-flowered individuals occur in populations dominated by coloured flowers, white flowers are often less frequently pollinated than coloured flowers, and consequently have lower reproductive success (Waser & Price, 1981; Brown & Clegg, 1984; Odell *et al.*, 1999). All these studies deal with species that produce nectar rewards for floral visitors. The absence of reward in *O. mascula* flowers may have a dramatic effect on pollinator behaviour. After visiting a flower without nectar, bumblebee pollinators have been observed to switch between colour morphs and to orient to flowers with distinct colour characters (Smithson & MacNair, 1997; Gigord *et al.*, 2001). The presence of contrasting colours within single populations has also been shown to be an attractive signal for bumblebees (Spaethe *et al.*, 2001; Lunau *et al.*, 2006). It seems plausible to suppose that after unrewarding visits to purple flowers, naïve pollinators probably avoid homogeneous populations of purple flowers, and may then preferentially orient to a different colour or to a colour contrast such as a mix of white and purple flowers. Our results with white ping-pong balls fit this hypothesis: the presence of an artificial colour contrast that mimics the white/purple colour contrast of natural mixed populations led to increased visitation of those populations compared with pure purple populations. The use of visual lures showing no colour

contrast (purple- or dark green-coloured ping-pong balls placed within pure purple populations) had no effect on the reproductive success of neighbouring purple *O. mascula* (R. Delle-Vedove, unpublished). Moreover, we demonstrated a clear effect of the distance from the white flower or lure on the fruit set of purple individuals. Fruit set decreased with increasing distance from the white flower or lure, strongly supporting our hypothesis that increased visitation of purple individuals resulted from the presence of a colour-contrasted object (inflorescence or lure). This effect was significant at distances of 10–160 cm from the white object, resulting in increased fruit set of most purple individuals within a population (*c.* 80–90% of the total individuals).

Maintenance of the rare white-flowered *O. mascula*

Another question remains unanswered: how can the maintenance of rare white inflorescences be explained in evolutionary time? Is the presence of rare white-flowered *O. mascula* merely the product of repeated spontaneous mutations, or is it a consequence of pollinator-mediated selection that maintains the frequency of the white morph at a value greater than can be explained by repeated mutations? Spontaneous mutations affecting floral pigmentation genes, and which may produce white-flowered variants, have been reported to explain the presence of low frequencies (0.1%, on average, in these studies) of white individuals in natural populations of pigmented flowers (Waser & Price, 1981; Epperson & Clegg, 1987; Levin & Brack, 1995). In *O. mascula*, however, the frequency of white-flowered variants in the whole population (1%) is *c.* 10 times higher than that observed in these studies, and it is unlikely that such high frequencies could be the result of repeated spontaneous mutations alone. Moreover, the deceptive strategy helps to reinforce the key role of pollinators in plant reproductive success, and we also demonstrated by using visual lures that floral colour had a dramatic effect on reproductive success through pollinator-imposed selection.

If recurrent mutation alone cannot explain the persistence of white-flowered morphs, then what mechanisms can? Three plausible hypotheses can be suggested: if expression of white flowers is a recessive trait, white-flowered morphs, and the attendant phenomena we observed, may be unselected secondary consequences of inbreeding; white-flowered morphs may persist because they have higher male fitness; white-flowered morphs are maintained by kin selection, increasing the fruit set of nearby, and related, purple-flowered individuals. Each of these hypotheses will be discussed in turn.

One hypothesis to explain the maintenance of the white morph in *O. mascula* is that it is a consequence of inbreeding. Self-fertilization in flowers is likely to produce more homozygotes, including more homozygotes of the white recessive allele (Clegg & Durbin, 2000), and higher inbreeding rates (either through selfing or through biparental inbreeding) might thus produce higher frequencies of white-flowered individuals. Although the frequency of white-flowered genotypes may be reduced through deleterious pleiotropic effects of colour genes (Coberly & Rausher, 2008), some recessive alleles producing white genotypes (e.g. the *a* allele in *Ipomoea purpurea*) have been shown in some cases to have a transmission advantage that can increase their frequency (Fehr & Rausher, 2004). In this context, possible specific advantages of the homozygous white-flowered genotypes in some micro-environmental conditions are likely to reduce fitness differences between the two *O. mascula* colour morphs.

The presence of white-coloured flowers might also be explained by a greater male reproductive success of the white morph. Because many pollinator species are attracted to the white colour or to a colour contrast including white (Chittka & Raine, 2006; Lunau *et al.*, 2006), it can be hypothesized that pollinators may primarily visit the white *O. mascula* morph in a mixed population of white/purple morphs, resulting in a higher rate of pollinia removal for the white morph. However, preliminary records of pollinia removal in *O. mascula* showed that male fitness, estimated by the rate of pollinia removal, did not differ between the two morphs (B. Schatz & L. Dormont, unpublished).

Another possible explanation for the maintenance of white *O. mascula* variants is kin selection. Kin selection refers to individuals increasing their inclusive fitness (i.e. the sum of direct fitness and indirect fitness effects through impact on the fitness of social partners) through behaviour or other traits that increase the fitness of related individuals (Hamilton, 1964). In *O. mascula*, the presence of white-flowered variants might be regarded as an adaptation that benefits the purple-flowered relatives of white-flowered morphs, rather than providing a direct benefit to white-flowered individuals. The postulated mechanism of kin selection could work only if the neighbouring individuals that benefit from proximity to a white-flowered individual are related to it. Spatial genetic structure, with aggregation of related individuals, has already been demonstrated in populations of other species of the genus *Orchis* (Chung *et al.*, 2005). The mechanism we postulate in *O. mascula* could thus be plausible. This population structure, upon which kin selection depends, could also lead to high levels of inbreeding, which, as seen earlier in this section, could produce the homozygous white-flowered individuals essential for the kin-selection mechanism.

Whatever the process involved, the increase of fruit set of pigmented flowers in the vicinity of white-flowered variants in *O. mascula* represents a new mechanism different from those that have so far been postulated to maintain colour polymorphism in plants. We are currently examining the hypotheses discussed – for example, by analysing the genetic relatedness of individuals within *O. mascula* populations

and by conducting parallel studies in other orchid species on the effects of white inflorescences on the reproductive success of individuals within the population.

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References

Ackerman JD, Carromero W. 2005. Is reproductive success related to color polymorphism in a deception pollinated tropical terrestrial orchid? *Caribbean Journal of Science* **41**: 234–242.

Bond AB. 2007. The evolution of color polymorphism: crypticity, searching images, and apostatic selection. *Annual Review of Ecology, Evolution and Systematics* **38**: 489–514.

Bournérias M, Prat D. 2005. *Les orchidées de France, Belgique, et Luxembourg*. Mèze: Coll. Parthénope, Biotope.

Brown BA, Clegg MT. 1984. Influence of flower color polymorphism on genetic transmission in a natural population of the common morning glory, *Ipomoea purpurea*. *Evolution* **38**: 769–803.

Chittka L, Raine NE. 2006. Recognition of flowers by pollinators. *Current Opinion in Plant Biology* **9**: 428–435.

Chung MY, Nason JD, Chung MG. 2005. Spatial genetic structure in populations of the terrestrial orchid *Orchis cyclochila* (Orchidaceae). *Plant Systematics and Evolution* **254**: 209–219.

Clegg MT, Durbin ML. 2000. Flower color variation: a model for the experimental study of evolution. *Proceedings of the National Academy of Sciences, USA* **97**: 7016–7023.

Coberly LC, Rausher MD. 2008. Pleiotropic effects of an allele producing white flowers. *Evolution* **62**: 1076–1085.

Cozzolino S, Schiestl F, Muller A, De Castro O, Nardella AM, Widmer A. 2005. Evidence for pollinator sharing in Mediterranean nectar-mimic orchids: absence of premating barriers? *Proceedings of the Royal Society of London B* **272**: 1271–1278.

Dickson CR, Petit S. 2006. Effect of individual height and labellum colour on the pollination of *Caladenia* (syn. Arachnorchis) behrii (Orchidaceae) in the northern Adelaide region, South Australia. *Plant Systematics and Evolution* **262**: 65–74.

Epperson BK, Clegg MT. 1987. Instability at a flower color locus in the morning glory. *Journal of Heredity* **78**: 346–352.

Fehr C, Rausher MD. 2004. Effects of variation at the flower-color A locus on mating system parameters in *Ipomoea purpurea*. *Molecular Ecology* **13**: 1839–1847.

Flamini G, Cioni PL, 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. *Flavor and Fragrance Journal* **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 LDB, Macnair MR, Smithson A. 2001. Negative frequency-dependent selection maintains a dramatic flower color polymorphism in the rewardless orchid *Dactylorhiza sambucina* (L.). *Proceedings of the National Academy of Sciences, USA* **98**: 6253–6255.

Hamilton WD. 1964. The genetical evolution of social behavior, I, II. *Journal of Theoretical Biology* **7**: 1–52.

Jacquemyn H, Brys R, Honnay O, Hermy M. 2008. Effects of coppicing on demographic structure, fruit and seed set in *Orchis mascula*. *Basic and Applied Ecology* **9**: 392–400.

Jersakova J, Johnson SD, Kindlmann P. 2006a. Mechanisms and evolution of deceptive pollination in orchids. *Biological Reviews* **81**: 219–235.

Jersakova J, Kindlmann P, Renner SS. 2006b. Is the color dimorphism in *Dactylorhiza Sambucina* maintained by differential seed variability instead of frequency-dependent selection? *Folia Geobotanica* **41**: 61–76.

Johnson SD, Peter CI, Nilsson LA, Ågren J. 2003. Pollination success in a deceptive orchid is enhanced by co-occurring rewarding 'magnet' plants. *Ecology* **84**: 2919–2927.

Jones KN, Reithel JS. 2001. Pollinator-mediated selection on a flower color polymorphism in experimental populations of *Antirrhinum* (Scrophulariaceae). *American Journal of Botany* **88**: 447–454.

Knudsen JT. 2002. Variation in floral scent composition within and between populations of Geonoma macrostachys (Arecaceae) in the western Amazon. *American Journal of Botany* **89**: 1772–1778.

Koivisto A-M, Vallius E, Salonen V. 2002. Pollination and reproductive success of two colour variants of a deceptive orchid, *Dactylorhiza maculata* (Orchidaceae). *Nordic Journal of Botany* **22**: 53–58.

Levin DA, Brack E. 1995. Natural selection against white petals in *Phlox*. *Evolution* **49**: 1017–1022.

Li ZG, Lee MR, Shen DL. 2006. Analysis of volatile compounds emitted from fresh *Syringa oblate* flowers in different florescence by headspace solid-phase microextraction-gas chromatography-mass spectrometry. *Analytica Chimica Acta* **276**: 43–49.

Lunau K, Fieselmann G, Heuschen B, van de Loo A. 2006. Visual targeting of components of floral colour patterns in flower-naive bumblebees (*Bombus terrestris*; Apidae). *Naturwissenschaften* **93**: 325–328.

Majetic CJ, Raguso RA, Tonsor SJ, Ashman T-L. 2007. Flower color-flower scent associations in polymorphic *Hesperis matronalis* (Brassicaceae). *Phytochemistry* **68**: 865–874.

Majetic CJ, Raguso RA, Ashman T-L. 2009. The sweet smell of success: floral scent affects pollinator attraction and seed fitness in *Hesperis matronalis* (Brassicaceae). *Functional Ecology* **23**: 480–487.

Moya SJD, Ackerman JD. 1993. Variation in the floral fragrance of *Epidendrum ciliare* (Orchidaceae). *Nordic Journal of Botany* **13**: 41–47.

Nilsson LA. 1983. Anthecology of *Orchis mascula* (Orchidaceae). *Nordic Journal of Botany* **3**: 157–179.

Odell E, Raguso RA, Jones KN. 1999. Bumblebee foraging responses to variation in floral scent and color in snapdragons (*Anthirrhinum*: Scrophulariaceae). *American Midland Naturalist* **142**: 257–265.

Olesen JM, Knudsen JT. 1994. Scent profiles of colour morphs of *Corydalis cava* (Fumariaceae) in relation to foraging behaviour of bumblebee queens (*Bombus terrestris*). *Biochemical Systematics and Ecology* **22**: 231–237.

Raguso RA. 2008a. Start making scents: the challenge of integrating chemistry into pollination ecology. *Entomologia Experimentalis et Applicata* **128**: 196–207.

Raguso RA. 2008b. Wake up and smell the roses: the ecology and evolution of floral scent. *Annual Review of Ecology, Evolution and Systematics* **39**: 549–569.

Raguso RA, Levin RA, Fooze SE, Holmberg MW, McDade LA. 2003. Fragrance chemistry, nocturnal rhythms and pollination 'syndromes' in *Nicotiana*. *Phytochemistry* **63**: 265–284.

Rausher MD. 2008. Evolutionary transitions in floral color. *International Journal of Plant Sciences* **169**: 7–21.

Salzmann CC, Schiestl FP. 2007. Odour and colour polymorphism in the food-deceptive orchid *Dactylorhiza romana*. *Plant Systematics and Evolution* **267**: 37–45.

Salzmann C, Cozzolino S, Schiestl F. 2007a. Floral scent in food-deceptive orchids: species specificity and sources of variability. *Plant Biology* **9**: 720–729.

Schemske DW, Bierzychudek P. 2007. Spatial differentiation for flower color in the desert annual *Linanthus parryae*: was Wright right? *Evolution* **61**: 2528–2543.

Schiestl F. 2005. On the success of a swindle: pollination by deception in orchids. *Naturwissenschaften* **92**: 255–264.

Schiestl F, Ayasse M, Paulus HF, Erdmann D, Francke W. 1997. Variation of floral scent emission and postpollination changes in individual flowers of *Ophrys sphegodes* subsp. *sphegodes*. *Journal of Chemical Ecology* **23**: 2881–2895.

Smithson A, MacNair MR. 1997. Negative frequency-dependent selection by pollinators on artificial flowers without rewards. *Evolution* **51**: 715–723.

Smithson A, Juillet N, MacNair MR, Gigord LD. 2007. Do rewardless orchids show a positive relationship between phenotypic diversity and reproductive success? *Ecology* **88**: 434–442.

Spaethe J, Tautz J, Chittka L. 2001. Visual constraints in foraging bumblebees: flower size and color affect search time and flight behaviour. *Proceedings of the National Academy of Sciences, USA* **98**: 3898–3903.

Stanton ML, Snow AA, Handel SN, Bereczky J. 1989. The impact of a flower-color polymorphism on mating patterns in experimental populations of wild radish (*Raphanus raphanistrum* L.). *Evolution* **43**: 335–346.

Stevens J. 1992. *Applied multivariate statistics for the social sciences*. Hillsdale, NJ, USA: Lawrence Erlbaum Associates.

Strauss SY, Whittall JB. 2006. Non-pollinator agents of selection on floral traits. In: Harder LD, Barrett SCH, eds. *The ecology and evolution of flowers*. Oxford, UK: Oxford University Press, 120–138.

Van der Cingel NA. 1995. *An atlas of orchid pollination-European orchids*. Rotterdam: Balkema.

Warren J, Mackenzie S. 2001. Why are all colour combinations not equally represented as flower-colour polymorphisms? *New Phytologist* **151**: 237–241.

Waser NM, Price MV. 1981. Pollinator choice and stabilizing selection for flower color in *Delphinium nelsonii*. *Evolution* **35**: 376–390.

Weiss MR. 1995. Floral color-change – a widespread functional convergence. *American Journal of Botany* **82**: 167–185.

Zucker A, Tzfira T, Ben-Meir H, Ovadis M, Shklarman E, Itzhaki H, Forkmann G, Martens S, Neta-Sharir I, Weiss D *et al.* 2002. Modification of flower color and fragrance by antisense suppression of the flavonone 3-hydroxylase gene. *Molecular Breeding* **9**: 33–41.