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Should food-deceptive species flower before or after rewarding species? An experimental test of pollinator visitation behaviour under contrasting phenologies

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Abstract

Many plant species reward their pollinators, whereas some species, particularly among orchids, do not. Similarity of floral cues between co-flowering species influences how rapidly pollinators learn to avoid deceptive plants. This learning process, which affects the reproductive success of deceptive plants, may additionally depend on relative timing of flowering of sympatric rewarding and deceptive species. We tested the combined effects of corolla colour similarity and flowering order of rewarding and deceptive artificial inflorescences on visitation by naïve bumblebees. When deceptive inflorescences were offered after rewarding inflorescences, bumblebees visited them four times more often if both species were similar compared with when they were dissimilar. Pollinator visitation rate to deceptive inflorescences offered before rewarding inflorescences was intermediate and independent of similarity. Thus, early-flowering deceptive species avoid the costs of dissimilarity with rewarding species. This mechanism may favour adaptive evolution of flowering phenology in deceptive species and explain why temperate deceptive orchids usually flower earlier than rewarding ones.

Keywords

artificial inflorescences; *Bombus terrestris*; corolla colour similarity; deceptive pollination; flowering phenology; pollinator behaviour; pollinator learning.

Introduction

Flowering phenology is a crucial life-history trait that strongly affects plant reproductive success and population structure through its effects on sexual reproduction and pollen flow (Rathcke & Lacey, 1985; Elzinga *et al.*, 2007). Populations of single plant species may have divergent flowering phenologies across local environments (Quinn & Wetherington, 2002; Antonovics, 2006; Franks *et al.*, 2007). As flowering phenology is at least partly under genetic control (Elzinga *et al.*, 2007), this variation may reflect adaptive evolution in response to environment-driven selection (Wolfe *et al.*, 2004). In addition to environmental conditions (Ollerton & Lack, 1992), biotic interactions can also impose selection on flowering schedules. Relevant biotic interactions may involve other plant species, pollinators, seed dispersers, plant predators or vectors of diseases (Elzinga *et al.*, 2007).

In predominantly out-crossing angiosperm species whose reproductive success is limited by pollinators, selection is expected to favour patterns of flowering time that maximize pollinator visits and pollen exchange among conspecifics (Rathcke, 1983). Because orchids often exhibit low fruit sets (Darwin, 1877; Ackerman, 1986; Gill, 1989; Neiland & Wilcock, 1998) due to pollinator limitation (Tremblay *et al.*, 2005), selection for optimal pollinator visitation may be particularly strong on their flowering phenology. Food-deceptive orchids, which offer no reward to their pollinators (Van der Pijl & Dodson, 1966; Ackerman, 1986), present a special case. Flowering phenology may evolve not only to match the peak of pollinator abundance, but also towards optimal flowering overlap with rewarding co-flowering species.

Pollinator visitation rates of deceptive plants strongly depend on pollinator learning. Generalist pollinators, such as bees, learn to avoid food-deceptive plants

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(avoidance learning, Gigord *et al.*, 2002; Ollason & Ren, 2002) and to visit preferentially rewarding ones (associative learning, Dukas & Real, 1993; Gumbert, 2000) by associating floral cues to the presence or absence of reward. However, avoidance learning slows down when pollinators are unlikely to encounter deceptive and rewarding plants in short sequences (Dukas & Real, 1993; Internicola *et al.*, 2006), as happens when both species do not flower at the same time. Therefore, deceptive species may benefit from flowering at a time when few or no rewarding sympatric species flower. In addition, pollinator avoidance learning usually increases with foraging experience (Internicola *et al.*, 2007). Decreasing visitation rates as a consequence of pollinator learning may exert sufficient selective pressures on flowering phenology to explain why, in many temperate deceptive orchids, individuals that flower in early spring are pollinated at a higher rate than late-flowering ones (Tremblay *et al.*, 2005). Higher reproductive success for early-flowering individuals has been observed in *Calypso bulbosa* (Ackerman, 1981), *Anacamptis morio* (Nilsson, 1984), *Orchis mascula* (Nilsson, 1983) and *Dactylorhiza sambucina* (Nilsson, 1980). Thus, pollinator learning must be understood in the context of the plant communities in which deceptive and rewarding species co-occur in space and time.

In natural plant communities, pollinator learning may be further modified by specific traits of co-flowering deceptive and rewarding species (Johnson *et al.*, 2003; Internicola *et al.*, 2006, 2007). Among these traits, corolla colour plays a major role for flower recognition (Menzel & Shmida, 1993) and pollinator learning (Heinrich *et al.*, 1977). If deceptive and rewarding co-flowering plants have similar corolla colours, pollinators may be less efficient at discriminating them, which would result in higher visitation rate to the deceptive plants (Gigord *et al.*, 2002; Dyer & Chittka, 2004a; Internicola *et al.*, 2007). Interestingly, corolla colour similarity between a rewarding and a deceptive species may affect pollinator visitation rates to the deceptive plants even if they do not flower at the same time, via carry-over and memory effects. For instance, bumblebees usually visit flowers that are similar in corolla colour to those they previously experienced as rewarding (Gumbert, 2000; Gigord *et al.*, 2002). Consequently, the extent to which corolla colour similarity influences pollinator behaviour may depend on pollinators' previous experience and on the temporal order in which both types are encountered. Thus, we expect that both pollinators and sympatric plant species are likely to exert strong selective pressures on the flowering phenology of deceptive orchids.

We experimentally investigated how the temporal sequence of flowering and corolla colour similarity of deceptive and rewarding species influence pollinator visitation rate to the deceptive species. We mimicked a natural plant community with deceptive and rewarding orchid-looking artificial inflorescences. Inflorescences were either similar or dissimilar for corolla colour. Deceptive inflorescences were offered either before or after the rewarding inflorescences and their flowering phenology partly overlapped. We exposed naïve bumblebees to these inflorescences and monitored flower visitation behaviour over time. We predicted that deceptive inflorescences receive more visits when occurring alone than when co-flowering with the rewarding inflorescences. We also predicted that pollinators would learn more slowly when deceptive inflorescences are offered before than after the rewarding inflorescences, the deceptive plants benefiting from pollinator naivety. Finally, we predicted an interaction between colour similarity and phenology. Deceptive inflorescences should receive more visits when exposed to pollinators after similar than after dissimilar rewarding inflorescences. However, pollinator visitation rate to deceptive inflorescences should be independent of corolla colour similarity when deceptive inflorescences are offered before the rewarding ones.

Methods

Artificial inflorescences

We used artificial inflorescences that consisted of 10 orchid-looking zygomorphic flowers giving access to wells containing 3 μL of either nectar (30% sucrose solution, rewarding flower) or water (deceptive flowers). Inflorescence height, floral shape and display were design to mimic a typical European deceptive orchid (see Internicola *et al.*, 2007 for details). Flowers were either made of yellow (Y), dark yellow (DY) or blue (B) paper. We assessed the spectral reflectance functions of colour traits (Fig. 1a) by spectrophotometric analysis (High Sensitivity Spectrophotometer S2000; New Electro-Optical Concept, Beaufays, Belgium). We calculated the position of colour traits in the colour space as defined by Chittka (1992) (Fig. 1b) accounting for the spectral sensitivity functions of *Bombus terrestris* photoreceptors (Peitsch *et al.*, 1992) and the spectral distribution of the light in the experimental environment. This calculation was refined by assuming that the bee visual system was adapted to the green background in the experiment. This approach yields pairwise colour similarity values, given by the Euclidean distances between the positions of the colour traits in the colour space. These distances can be expressed in 'hexagon units' (Chittka, 1992). Bumblebees that experienced differential conditioning (i.e. bumblebees that learn rewarding flowers in the presence of deceptive flowers) are able to discriminate colours that differ by 0.062 hexagon units (Dyer & Chittka, 2004a; Dyer, 2006). In our experiment, the blue tone was clearly distinct from both yellow tones (Y/B distance = 0.418 hexagon units; DY/B distance = 0.417 hexagon units; Fig. 1b). Yellow and dark yellow were more similar, but distinguishable for bumblebees according to colour

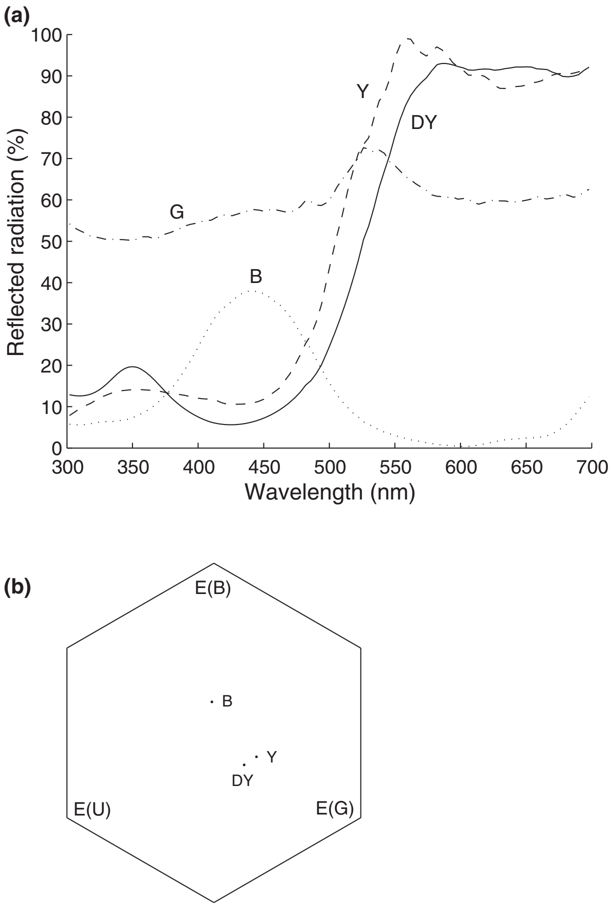

Fig. 1 (a) Spectral reflectance functions of the green background (G) and of yellow (Y), dark yellow (DY) and blue (B) flowers. (b) Hexagon representing the colour space defined by the trichromatic colour vision (photoreceptor excitation) of *Bombus terrestris* in ultraviolet (E(U)), blue (E(B)) and green (E(G)). Points show the relative position of yellow (Y), dark yellow (DY) and blue (B) colours as used in the experiment, i.e. under indirect natural sunlight within a greenhouse and assuming adaptation of the visual system of bumblebees to the green background. Distances between points represent similarity between colours.

distance (Y/DY distance = 0.087 hexagon units) and to a preliminary set of behavioural tests (see Internicola *et al.*, 2007 for details).

Study animals and behavioural observations

We used one *B. terrestris* (L.) hive (Natupol©; Koppert B.V., Berkel en Rodenrijs, The Netherlands). All individuals hatched in captivity and were naïve. We connected the hive to a flight cage (2.4 m × 3.6 m and height = 1.5 m) with a transparent plastic tube, through which bumblebees were visible. The cage base bore a 24 × 36 grid square system, with grid size 9 × 9 cm^2, identifying 864 positions to randomly allocate the artificial inflorescences. We fed bees *ad libitum* with pollen and for 1h day^{-1} with sucrose syrup, to encourage bees to forage for nectar in the cage. To habituate bees to experimental conditions, we allowed them to enter the cage containing 10 randomly placed flowerless leaf-green inflorescences containing 6 μL of nectar per well for 1–4 h day^{-1}. Bees that consecutively probed a minimum of five wells on an inflorescence were caught, marked and released back into the cage. In experiments, we used only marked bees (i.e. bees having learned to feed on the artificial inflorescences) and allowed them to forage in the cage where we randomly placed 45 artificial inflorescences. We refer to each sequence of visits (defined as the bee landing on a flower and probing the well) of a given bumblebee to the artificial inflorescences from the moment it left the hive until it returned to it, as to an experimental bout.

We recorded the sequence of visited inflorescences for each bumblebee. From this, we calculated the total number of visits to rewarding and deceptive inflorescences for each bout and bee. We ran the experiment in a greenhouse (indirect natural sunlight, temperatures 21 to 24 °C) at the University of Lausanne from 13 October to 14 November 2006, daily between 08:30 and 17:30 hours.

Experimentally manipulated factors

We investigated the effect of two fully crossed factors (corolla colour similarity and temporal pattern of flowering) on the foraging behaviour of *B. terrestris* on spatially interspersed rewarding and deceptive inflorescences. We tested for the effect of corolla colour similarity between deceptive and rewarding inflorescences with pairs of deceptive vs. rewarding inflorescences which bore yellow vs. dark yellow flowers (similar), or yellow vs. blue flowers (dissimilar). In both cases, the yellow inflorescences were deceptive. We tested two different patterns of flowering order (flowering phenologies) of deceptive and rewarding inflorescences. In the treatment simulating early flowering of the deceptive inflorescences (early), all 45 inflorescences present in the first bout were deceptive and progressively replaced by rewarding inflorescences in later bouts. In the treatment simulating late flowering of deceptive inflorescences (late), all 45 inflorescences present in the first bout were rewarding and progressively replaced by deceptive inflorescences in later bouts. At each successive bout, we replaced nine of the inflorescences by an equal number of inflorescences of the other type. Thus, the frequency of the deceptive inflorescences varied between 0% and 100% within each treatment, decreasing (early) or increasing (late) by 20% at each successive bout, whereas the total number of inflorescences remained constant (‘replacement series’ design). Rewarding and deceptive inflorescences overlapped in four of six bouts. This led to a total of four treatment combinations (similar/dissimilar × early/late,

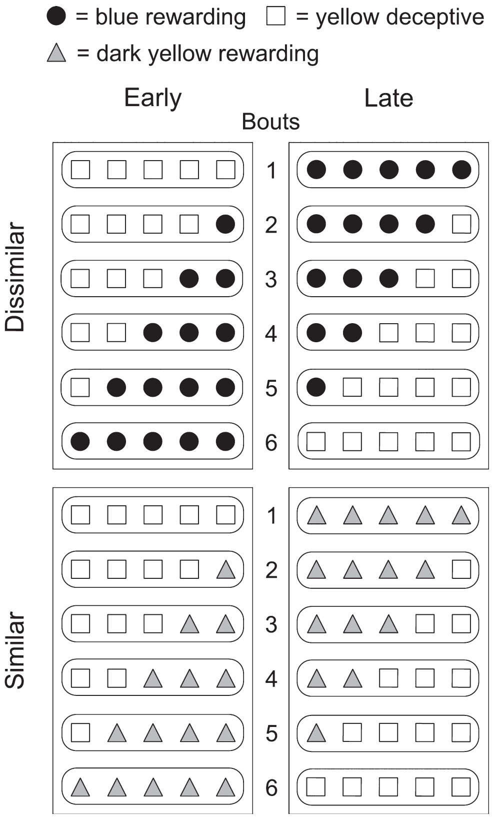

Fig. 2 Experimental design to test for the combined effects of flowering order (early vs. late) and corolla colour similarity (dissimilar vs. similar) of deceptive relative to rewarding inflorescences on bumblebee visitation. Shown are the proportions of inflorescence types offered to the bees in the six consecutive bouts (rounded boxes) for each of the four treatment combinations (rectangular boxes). White squares: yellow deceptive; grey triangles: dark yellow rewarding; black circles: blue rewarding.

Fig. 2). After each bout, we cleaned and refilled all wells with either water or nectar and randomly re-allocated inflorescences within the cage. Each bee experienced only one treatment combination. For each treatment combination, we tested 15 bumblebees. No individual bumblebee was re-used. We only included in the analysis bumblebees that completed the six bouts in a single day, whatever the number of inflorescences they visited in each bout, to avoid possible confounding by over-night memory decay (Keasar *et al.*, 1996).

Data analysis

We analysed separately:

1. bouts in which the bumblebees were exposed to deceptive inflorescences flowering alone, either at the beginning or end of their flowering period (i.e. the initial bout no. 1 at the start of the learning sequence for early treatments, and the final bout no. 6 for late treatments);
2. bouts in which bumblebees could simultaneously encounter both rewarding and deceptive inflorescences at different frequencies, when the flowering phenologies of rewarding and deceptive inflorescences overlapped (bouts 2–5 of all treatment combination).

For bouts in which only deceptive inflorescences were available, we calculated the number of deceptive inflorescences visited by each bumblebee. We used a mixed-model ANOVA to test for differences in the number of visits to deceptive inflorescences with colour treatments (similar or dissimilar) and phenology treatments (early or late) as descriptive variables. Because no transformation met the assumptions of normality and homoscedasticity, we performed permutation tests on mean squares (Manly, 1997). We tested the effects of corolla colour similarity and flowering phenology by separately permuting the levels of these factors in the data set, whereas we tested for the interaction between colour and phenology by simultaneously permuting these factors. We calculated *P*-values as the proportion of permutated mean-square estimates larger than or equal to the observed mean-square over 1000 permutations.

For bouts in which rewarding and deceptive inflorescences co-occurred, we calculated – for each bumblebee and bout – the difference between the observed proportion of visits to the deceptive inflorescences and the expected proportion of visits to the deceptive inflorescences under random visitation (i.e. the frequency of the deceptive inflorescences). This value corresponds to the observed deviation from random visitation and should be equal to zero if bumblebees visited inflorescences at random, negative if bumblebees undervisited the deceptive inflorescences and positive if they overvisited them. We analysed the effects of corolla colour similarity (similar or dissimilar), phenology (early and late) and bout on the deviation from random visitation with a mixed-model ANOVA and permutation tests on mean squares. We accounted for the effect of individual bees to avoid pseudo-replication. We estimated the effects of colour and phenology treatments by separately permuting the levels of these factors and imposing the same level values of the permutated factor within each bee. The effect of bouts was estimated by permutations within each bee. Pairwise and three-way interactions were tested by simultaneously permuting the interacting factors. We conducted all statistical analyses with R 2.6.1 software (R development Core Team, 2007). Results are given as estimated mean values ± standard errors ($\bar{x}$ ± SE).

Results

Bumblebee visitation behaviour was significantly affected by flowering phenology in interaction with corolla colour similarity (MS = 487.35, d.f. = 1, *P* = 0.041). The deceptive inflorescences that occurred alone after the rewarding inflorescences received four times more visits when both species had flowers of similar colour than when those were of dissimilar colour (Fig. 3). By contrast, when the deceptive inflorescences were offered before the rewarding inflorescences, there was no significant effect of corolla colour (Fig. 3).

We further investigated the tendency of pollinators to avoid the deceptive inflorescences when both species were simultaneously present by estimating the deviation from random visitation. This deviation was defined as the signed difference between the proportion of deceptive inflorescences visited and the proportion of deceptive inflorescences available. Over time, the deviation from random visitation became significantly more pronounced (Table 1, Fig. 4), implying that bumblebees increasingly avoided the deceptive inflorescences with increasing experience. In particular, the rate at which deceptive inflorescences were increasingly avoided was faster when the deceptive inflorescences were offered after the rewarding ones (late) than in the reverse case (early, Table 1, Fig. 5). Thus, over time, the bumblebees avoided the deceptive inflorescences more efficiently when they first experienced a plant community with a higher proportion of rewarding inflorescences, than when they were first confronted with a plant community with a higher proportion of deceptive inflorescences.

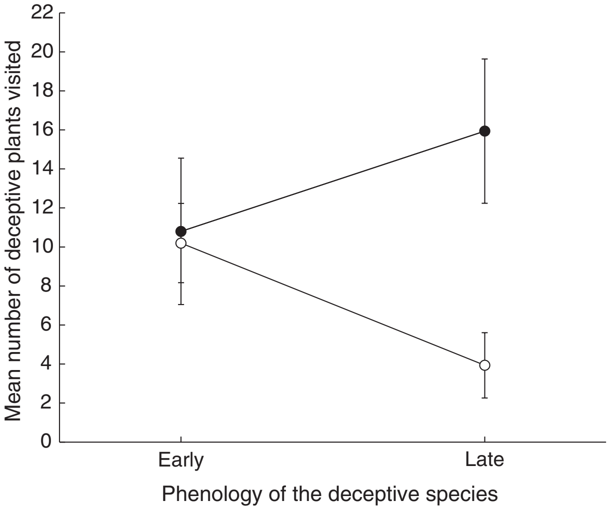

Fig. 3 Mean number of bumblebee visits to deceptive artificial inflorescences in bouts 1 and 6 according to phenology and colour treatments. The deceptive species frequency was in every case of 100%. Closed symbols = deceptive and rewarding inflorescences were of similar corolla colour (yellow/dark yellow); open symbols = deceptive and rewarding inflorescences were of dissimilar corolla colour (yellow/blue). Early = the deceptive inflorescences were present before rewarding inflorescences; late = the deceptive inflorescences were present after rewarding inflorescences.

Table 1 ANOVA table for the effects of corolla colour similarity, flowering phenology, bout and their interaction on the deviation from random visitation (difference between the observed and the expected number of visits to the deceptive inflorescences) in bouts 2–5.

	d.f.	MS	*P*
Source of variation (error: between bees)			
Colour similarity	1	0.178	0.1
Flowering phenology	1	0.528	0.002
Colour similarity × flowering phenology	1	0.02	0.596
Residuals	56	0.054	
Source of variation (error: within bees)			
Bout	3	0.707	< 0.001
Colour similarity × bout	3	0.041	0.43
Flowering phenology × bout	3	1.785	< 0.001
Colour similarity × flowering phenology × bout	3	0.016	0.625
Residuals	168	0.023	

The effect of individual bumblebee was taken into account in the model. Colour similarity and flowering phenology have only one level per bee, so that these factors and their interactions are grouped in the first part of the table (error: between bees). As bout is the only factor that has different levels within each bee, the effect of this factor and its interactions are shown in the second part of the table (error: within bees).

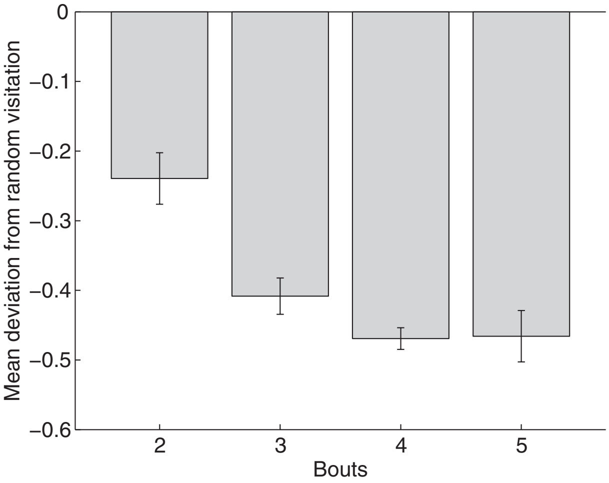

Fig. 4 Mean deviation from random visitation (i.e. signed difference between the proportion of deceptive inflorescences visited and the proportion of deceptive inflorescences available), illustrating increasing avoidance of the deceptive inflorescences over time in bouts 2–5.

Discussion

In a controlled manipulative experiment using artificial inflorescences, we tested for the joint effects of temporal patterns of flowering and corolla colour similarity of deceptive and rewarding inflorescences on the number of bumblebee visits to deceptive inflorescences. The novel finding is that corolla colour similarity significantly

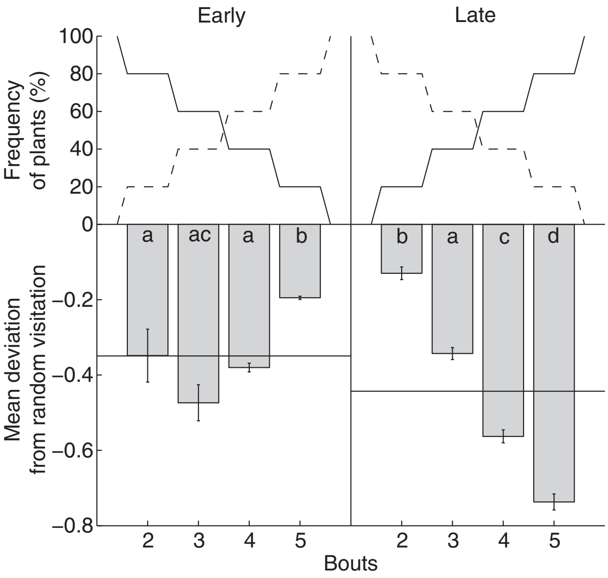

Fig. 5 *Top*: mean frequency of rewarding (*dashed line*) and deceptive inflorescences (*solid line*) over time in the experiment. Deceptive inflorescences were offered either before (early) or after (late) the rewarding inflorescences. *Bottom*: deviation from random visitation (i.e. signed difference between the proportion of deceptive inflorescences visited and the proportion of deceptive inflorescences available), illustrating avoidance of the deceptive inflorescences over time in bouts 2–5. Horizontal lines = mean across bouts 2–5. The letters show significant ($P < 0.05$) differences between treatment groups (Tukey's *post hoc* comparison test).

interacted with temporal variation in the occurrence of rewarding and deceptive inflorescences, crucially modifying both foraging success and avoidance learning of pollinating insects. On the whole, an early-flowering deceptive species should receive more visits irrespective of whether it flowers before similar or dissimilar rewarding sympatric species, whereas for late-flowering deceptive species a benefit should accrue but only if they have similar corolla colour to rewarding sympatric species. Considering this result, the safest strategy for deceptive plants may be to flower before sympatric rewarding species because the species composition of plant communities is rarely stable over time (Elzinga *et al.*, 2007). By flowering early, deceptive plants are likely to benefit from pollinator naivety and reduce the variance in reproductive success compared with late-flowering individuals. Reduced variance in reproductive success may increase fitness (Gillespie, 1977) and population persistence. As flowering phenology can be heritable (Elzinga *et al.*, 2007), selection may favour deceptive plant individuals that flower before rewarding species, especially when similarity to sympatric species is unpredictable. In agreement with this, a recent comparison of the starting date of flowering in European orchid species showed that deceptive orchids flower significantly earlier than their rewarding counterparts (L. Pellissier, P. Vittoz and L.D.B. Gigord, unpublished work).

When deceptive and rewarding inflorescences were simultaneously present, bumblebees avoided the deceptive inflorescences more efficiently when those were offered after rather than before the rewarding inflorescences. For instance, when the frequency of the deceptive inflorescences was 80%, bumblebees discriminated more strongly against the deceptive inflorescences if these were present after (fourth bout in the late phenology treatment) than before (second bout in the early phenology treatment) the rewarding inflorescences. This indicates that bumblebees avoid the deceptive inflorescences more efficiently when their relative frequency is high (Smithson & Gigord, 2003), only if they acquired sufficient experience (Heinrich, 1976). This is consistent with evidence that learning is faster if reinforced through a reward. The variation in foraging costs with the frequency of deceptive inflorescences may additionally explain our result: bumblebees avoided more efficiently the deceptive inflorescences when their frequency increased in successive bouts (late), compared with when their frequency decreased in successive bouts (early). Irrespective of the mechanism (i.e. avoidance learning and/or costs of rewardless visits), deceptive inflorescences received most pollinator visitations in the early phenology treatment, strongly suggesting that under natural conditions reproductive success of deceptive plants will be affected by the phenology of sympatric rewarding species.

Our experimental approach has a number of limitations. First, the selective pressures for specific flowering phenology arising from interspecific interactions depend on which animals are the primary pollinators of a plant species (Heinrich, 1975). Our approach may be most relevant for deceptive orchids that are primarily pollinated by bumblebees, such as *Hymantoglossum robertianum* (Smithson & Gigord, 2001), *A. morio* (Johnson & Nilsson, 1999) or *D. sambucina* (Nilsson, 1980), whereas deceptive species that rely more on specialist pollinators may suffer less from competition with sympatric rewarding species. Second, some sympatric plant species may not flower every year, as observed in several orchid populations (Kindlmann & Balounová, 2001), or different plant species may respond in discordant manners to environmental fluctuations. Thus, the selective pressures exerted by sympatric plant species on flowering phenology may change among years. Third, the timescale of our experiment is hours and involves short-term behavioural changes and learning of individual bumblebees. Although inexperienced bees may be available throughout the season, as the mortality of foraging individual bees is high, the rate of learning and naivety of pollinators may change over time. Bumblebees that experienced differential conditioning (i.e. exposed to both deceptive and rewarding plants during learning) rapidly learn and acquire a long-term memory that can last for about

10 days (Dyer & Chittka, 2004b). Consequently, even if naïve bumblebees continuously join the pool of foraging individuals, those that emerge later in the season (when both deceptive and rewarding species co-flower) may learn more rapidly and for a longer time, so that the effective proportion of inexperienced bumblebees is likely to decrease throughout the flowering season. Moreover, several temperate deceptive orchid species are mainly pollinated by newly emerged bumblebee queens (Nilsson, 1980, 1984), which have no foraging routines as long as they have not established nests (Nilsson, 1983, 1984). During early spring, bumblebee queens may be lured more frequently than later in the season. Thus, our experimental conditions are consistent with natural conditions for some deceptive orchid species. In addition, antagonistic biotic interactions, such as predation (Pilson, 2000), or other mutualistic interactions, such as those favouring seed dispersal (Zimmerman *et al.*, 1989; Aizen, 2003) and germination, may conflict with those induced by sympatric rewarding species and pollinators, and on other components of flowering phenology, such as floral longevity and the timing of scent emission during anthesis. For these reasons, measures of reproductive success (i.e. pollinia removal and deposition; fruit set) at a fine temporal scale throughout the flowering period of deceptive species in their natural environment, coupled with pollinator foraging behaviour observations, are necessary to fully validate our results.

In conclusion, our experiment indicates that pollinator avoidance learning is simultaneously affected by corolla colour similarity and temporal flowering order of rewarding and deceptive species. As a result, it should be beneficial for deceptive plants to flower early and before rewarding plants. Early flowering may ensure that visitation rates are independent of the similarity in corolla colour to any co-flowering rewarding species. By contrast, a late flowering strategy should only be beneficial for deceptive plants if rewarding and deceptive species have similar floral cues, but may result in the lowest visitation rates when co-flowering rewarding species have dissimilar floral cues. Early flowering may be additionally advantageous to deceptive species, because bumblebee learning depends on the frequency with which rewarding plants are encountered when it acquires experience. Biotic interactions of deceptive orchids in natural plant communities may have important evolutionary implications on their flowering phenology, which in turn may affect their maintenance. In particular, the widespread early-flowering phenology of temperate deceptive orchids may represent an adaptive strategy to escape avoidance learning and ensure reproduction through reduced variance in visitation rates.

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