Floral colour signal increases short-range detectability of a sexually deceptive orchid to its bee pollinator

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SUMMARY

Orchids of the genus *Ophrys* are pollinated by males of solitary bees and wasps through sexual deception. The flowers mimic the behaviourally active compounds of the sex pheromone of receptive females and thus attract males that seek to copulate. Odour is the main attractant while visual stimuli have been assumed so far to play only a minor role. In contrast to most species of the genus, Heldreich's orchid *Ophrys heldreichii*, which is pollinated by males of the long-horned bee *Tetralonia berlandi*, possesses a bright pink perianth that appears conspicuous to a human observer. We investigated the role of this floral colour signal in pollinator attraction. We filmed approach flights of male bees to flowers in which we removed the original perianth and in which we substituted the perianth with an artificial one of a particular selected colour. At distances >30 cm, male search time correlated only with wind speed but not with the spectral parameters of the perianth, i.e. chromatic and green receptor-specific contrast. By contrast, in the close range (<30 cm), where the perianth subtends a visual angle of at least 5 deg. to the bee's eye, search time decreased with increasing green receptor contrast between perianth and background; however, no correlation with chromatic contrast or wind speed was found. Our results indicate that pollinators are first attracted by olfactory signals from a distance. Once in the vicinity of the flower where spatial vision of the males is sufficient, they are guided exclusively by vision. However, it can be expected that possession of a 'non-private' colour signal would increase the risk of pollen loss in sexually deceptive orchids by accidentally attracting non-specific flower visitors. We therefore discuss the occurrence of colour signals in the genus *Ophrys* in respect to the species-specific visual system of the pollinators.

Key words: colour vision, pollination, sexual deception, orchids, signal evolution, *Ophrys heldreichii*, *Tetralonia berlandi*.

INTRODUCTION

Pollination by deception is a widespread phenomenon in angiosperm plants (Renner, 2006) but it is most pronounced in the Orchidaceae family. The proportion of deceptive species in this worldwide family is about 30% whereas in the Western Palearctic it makes up probably more than half of all species (Dafni, 1984; Paulus, 2005; Paulus, 2006). To attract potential pollinators, deceptive orchids imitate their models by exploiting key signals used in plant–pollinator relationships or sexual communication (Jersáková et al., 2006). Two major strategies are found in orchids, food deception and sexual deception, which differ in the quality of involved signals in respect to their species-specific perceptibility and targeted audience. Food deceptive species imitate the colour and shape of rewarding models and thus allow the orchids to achieve visits by pollinators foraging on the model plants (Vogel, 1972; Dafni, 1987; Gumbert and Kunze, 2001; Galizia et al., 2005). The involved sensory channel, namely vision, is 'non-private' and the signals can be perceived by both the targeted pollinators and a broad range of unspecific flower visitors (Kevan and Baker, 1983; Rosenthal and Ryan, 2000; Schaefer et al., 2004). Sexually deceptive orchids, however, exploit 'private' communication channels to lure pollinators. They produce the behaviourally active components of the sex pheromone of receptive females of the imitated insect species to attract males and elicit mating behaviour. During the subsequent copulation attempt, the so-called pseudocopulation, the pollinaria become attached to the male's body and pollen is transferred upon visitation of subsequent flowers (Kullenberg, 1961; Ayasse et al., 2003; Schiestl et al., 2004; Schiestl, 2005). Pollinator attraction in these orchids is very specific

as only males of the target species are attracted while other flower visitors do not respond to the odour bouquet. Due to the highly specific attraction mechanism, the flowers of sexually deceptive orchids usually do not possess conspicuous colour signals to avoid accidental attraction of unspecific pollinators (Alcock, 2005; Delforge, 2006).

Nearly all species of the Mediterranean orchid genus *Ophrys* are sexually deceptive (Kullenberg, 1961; Paulus and Gack, 1990; Paulus, 2005; Paulus, 2006). They mimic the sex pheromone of insect females, usually solitary bees and wasps, to attract males that seek to copulate (Kullenberg, 1961; Paulus and Gack, 1990). The labellum of an *Ophrys* flower resembles, in part, features of the body of the female bee or wasp for males of the respective species and serves as the substrate on which the attracted males perform pseudocopulations (Paulus and Gack, 1990; Paulus, 1997). The three sepals (herein referred to as perianth for simplification) are usually inconspicuously greenish and foliage-like. To insects they thus appear achromatic (Delforge, 2006). It is noteworthy that of the more than 200 described *Ophrys* species, *ca*. 30% possess a visually conspicuous perianth, which is pink or white and at least partially matches the spectral reflectance patterns of other co-flowering plants (Delforge, 2006; Spaethe et al., 2007). However, the functional significance of this colour signal with respect to pollinator attraction is unknown. Recently we showed that the pollinator of the Heldreich's bee orchid *Ophrys heldreichii*, which are the males of the long-horned bee *Tetralonia berlandi*, prefer flowers with a pink perianth over flowers with removed perianth in a dual choice test (Spaethe et al., 2007). Whether this choice behaviour is determined

by constraints imposed by the sensory capacities of the pollinator has yet to be tested. Also, the relative importance of such colour signals compared with odour signals for male attraction and guidance towards the flower is unknown.

In the present study, we address the question whether the coloured perianth functions as a close range signal in pollinator attraction and how it interacts with the odour signal (the mimetic sex pheromone). We used artificial perianths of various colours attached to real *Ophrys heldreichii* flowers to test the effect of visual parameters (chromatic and receptor specific contrast and size) on the pollinator's detection capability.

MATERIALS AND METHODS
Study site, orchid and pollinator

Experiments were carried out in 2007 and 2008 on Crete, about 5 km northeast of Neapolis (N35 deg.15′13″, E25 deg.38′03″) with the Heldreich's bee orchid *Ophrys heldreichii* Schlt. and its pollinator, males of the long-horned bee *Tetralonia berlandi* Dusmet [syn. *Eucera* (*Synhalonia*) *berlandi* Dusmet, syn. *Eucera ruficollis* Brullé] (Apoidea, Apidae, Eucerini) (Paulus and Gack, 1990). *Ophrys heldreichii* shows a conspicuous pink perianth and is the only representative of the *Ophrys oestrifera* group found on Crete (Delforge, 2006; Kretzschmar et al., 2002). For the experiments, intact flowers were collected from various sites within an area of about 20 km around the experimental site. The orchid does not occur at the experimental site and thus all tested males were naïve to *O. heldreichii* flowers.

Behavioural experiments

Males of *T. berlandi* approaching *O. heldreichii* flowers were filmed from above with a digital video camera (Sony DCR-SR50, Tokyo, Japan) at a rate of 25 frames s^{-1}. The camera was mounted at a height of 180 cm and the viewed area subtended approximately 110×70 cm. To account for perspective distortion we included a 10×10 cm grid that was used as a reference when calculating the flight path and flight distance (see below).

A single flower of *O. heldreichii* was placed in the lower centre of the filmed area. Approaching bees were immediately caught after contact with the flower and individually marked with a colour marker. Only approaches of single males that resulted in contact with or landing on the flower labellum were included in the analysis. All individuals re-visiting the same flowers were excluded.

For each approach we measured wind speed at flower level by means of an anemometer (Windmaster 2, Kaindl Electronic, Rohrbach, Germany). Males are attracted from a distance by the odour produced by the flower (Kullenberg and Bergström, 1976) and thus they usually approach upwind guided by the odour plume. Therefore, we continuously rotated the entire setup to allow the wind to come from behind the flower.

In the first experiment, we tested whether the presence or absence of the coloured perianth has any effect on the flight behaviour of the males. We first recorded approaches towards an intact flower for 30 min. Then we removed the perianth and recorded approaches to the same flower for another 30 min. Using the same flower minimises variance that originates from differences of odour strength and quality between individual flowers (Ayasse et al., 2000). To prevent any bias due to tissue injury, we incised all three sepals of the intact flowers before its initial use (Spaethe et al., 2007). Altogether, 14 individual flowers were tested.

In the second experiment, we tested for those spectral parameters of the perianth that determine flower detection. The original perianth was replaced by an artificial one cut from coloured cardboard, which resembled the original in size and shape. We tested five different colours: pink, green, blue, yellow and an UV-absorbing grey (see below). The perianth was randomly exchanged every 15 min, and flowers were replaced every 2 h.

Video files were transferred to a personal computer and individual approach flights were analysed frame-by-frame using free software (DGeeMe 1.0 beta version, www.geeware.com). The position of the bee's head and the position of the orchid were marked in every frame. We used the reference grid to remove perspective distortion by applying projective transformation (Wolf and Ghilani, 1997). The final coordinates were used for calculating the distance between the head of the male and the orchid. Positional errors that resulted from changes of flight altitude were assumed to be negligible because bees usually flew at a constant height close above the ground when approaching a flower (M.S., H.F.P. and J.S., personal observation).

Bees possess apposition compound eyes, which provide them with a relatively coarse image of their environment (Land, 1997). For honeybees, the distance threshold for detection of a single object similar to the size of an *Ophrys* flower (*ca.* 2.5 cm in diameter) is about 30 cm, which is the distance at which the object subtends a visual angle of 5 deg. to the bee's eye (Giurfa et al., 1996; Dyer et al., 2008). According to this known limitation in honeybees, we defined an inner circle (close range; 30 cm around the flower) within which the males should be able to use their visual system for detecting the flower and an outer circle (mid range; between 30 and 60 cm) in which the males should be able to use olfactory but not visual information for orientation. We calculated the amount of time a male needed once it entered the outer circle to reach the inner circle ('mid range search time') and once it entered the inner circle to touch or land on the flower's labellum ('close range search time').

In the third experiment, we tested whether the size of the perianth influences the choice behaviour of males by means of a dual choice experiment. To completely eliminate odour-variance-induced bias, we used both artificial orchid labellae and artificial perianths. The labellum models were cast in latex and painted according to the original labellum. Artificial perianths of three different sizes (normal size and 0.5 and 1.5 times the original size in respect to area) were cut from pink cardboard (see below). The flower models were presented in a paired design (small *vs* normal, normal *vs* large size) at a height of 25 cm and at a distance of 10 cm from each other facing the same direction. As visual properties alone do not suffice to attract pollinators (Kullenberg, 1961; Paulus, 1988; Spaethe et al., 2007), we channelled air through an acrylic glass jar, containing about 2–5 inflorescences of *O. heldreichii*, *via* a Y-shaped tube equally to both flower models by means of an air pump (SCHEGO optimal, Offenbach am Main, Germany) (Spaethe et al., 2007). Choices of individual male bees were recorded. The bees were caught and marked after each visit. Bees approaching from the side and re-visiting bees were not included in the analysis. The position of the flowers and the perianths were interchanged in a random order to exclude position biases.

Stimuli spectral properties

Spectral reflection of the *O. heldreichii* perianth and coloured cardboards were measured by means of an USB 2000 spectrometer with a deuterium/halogen light source between 300 and 700 nm (Fig. 1; Ocean Optics B.V., Duiven, The Netherlands). Measurements were conducted on small *ca.* 0.25 cm^2 areas and calibrated with a white standard (Diffuse Reflectance Standard WS-1, Ocean Optics). To estimate bee specific receptor contrasts and perceptual colour distances between the perianth and the
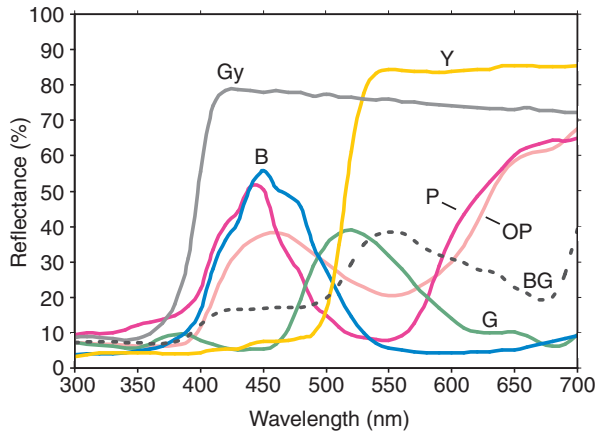
Fig. 1. Spectral reflectance curves of the original perianth of *Ophrys heldreichii*, the artificial ones used in the experiments and the background (B, blue; BG, background; G, green; Gy, UV-absorbing grey; OP, original perianth; P, pink; Y, yellow).

Table 1. Green receptor excitation and chromatic contrast in relation to background colour

Colour	Chromatic contrast (hexagon units)	Green contrast (dimensionless)
Original perianth	0.17	−0.02
Pink	0.44	−0.11
Green	0.11	0.00
Blue	0.36	−0.12
Yellow	0.51	0.16
Grey (−UV)	0.09	0.22

Values were calculated using the colour hexagon model and the spectral sensitivity curves of the UV, blue and green receptor of the honeybee (Chittka, 1992). Note that for the regression model the absolute contrast values were used (Spaethe et al., 2001).

background, we applied the bee hexagon model following standard procedures (Chittka, 1992; Chittka and Kevan, 2005). Most hymenopterans are found to have three photoreceptor types with phylogenetically conserved spectral sensitivities (Briscoe and Chittka, 2001). It is thus likely that *T. berlandi* has receptor sensitivities similar to other bees. We therefore used spectral receptor curves from the honeybee for calculations (Peitsch et al., 1992). In addition to colour information, honeybees and bumblebees also use an achromatic visual channel for flower detection that relies only on the green receptor signal as input (Giurfa et al., 1996; Dyer et al., 2008). Both channels are deployed depending on the visual angle of the object. If the subtended visual angle of the object is large (*ca.* 15 deg.), colour contrast is used; for smaller visual angles, bees deploy the green contrast alone (Giurfa et al., 1996; Giurfa and Vorobyev, 1998; Dyer et al., 2008). Therefore, we quantified colour contrast and green contrast of the perianths to the background [Table 1; for details of calculation, see Chittka and Kevan and Spaethe et al. (Chittka and Kevan, 2005; Spaethe et al., 2006)].

Statistics

Statistical analyses were performed with SPSS 11.5 (SPSS Inc., Chicago, IL, USA). All *P* values above 0.05 were considered as statistically not significant. Search times did not differ between the

years 2007 and 2008 and were therefore pooled (data not shown). For the first experiment, we applied a nonparametric Mann–Whitney *U*-test to compare search times between flowers with intact and removed perianths.

For the second experiment, we used a multiple linear regression analysis with a forward stepwise addition of independent variables in order to identify the factors affecting search time. We included wind speed, the square of wind speed (to account for non-linear effects, see below) (see also Brady et al., 1995), chromatic contrast and green-receptor contrast as predictors. For the third experiment, a two-tailed binomial test ($\pi=0.5$) was used to test whether males' visitation rate differed significantly from random.

RESULTS

Nearly all males approached upwind from the presented flower. They flew in a typical zig–zag flight pattern (Fig. 2) that is known from various insects that orient themselves by means of an odour plume (Murlis et al., 1992; Mafra-Neto and Cardé, 1994). When we compared search time of males in the mid range (30–60 cm distance), we found no differences between approach flights to intact flowers and to flowers in which the perianth was removed ($Z=-0.65$, $P>0.05$) (Fig. 3A). By contrast, at close range (<30 cm) search time was almost three times higher when males approached flowers with removed perianths compared with intact flowers ($Z=-3.13$, $P<0.01$) (Fig. 3B), indicating that the males used visual flower features for detection at short distances.

When presenting flowers with artificial perianths, we discovered that chromatic and green receptor contrast had no effect on search time at mid range; however, the search time was significantly
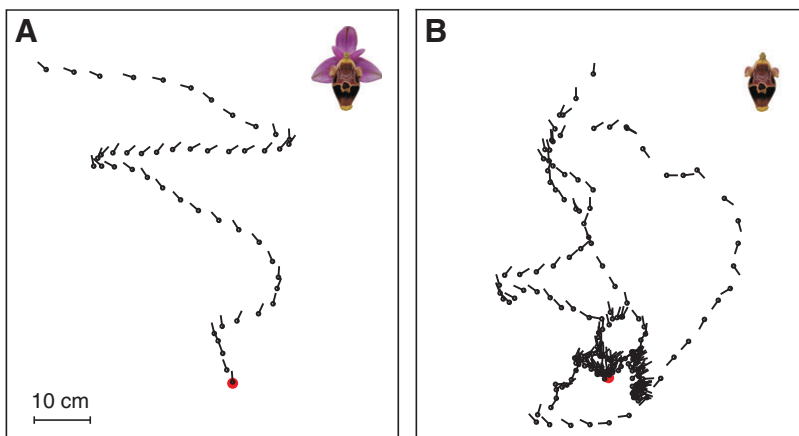

Fig. 2. Typical approach flights of *Tetralonia berlandi* males to (A) an intact *Ophrys heldreichii* flower and (B) a flower in which the pink perianth was removed. The position of the bee's head (open circle) and the body axis (line) are shown every 40 ms. The red dot marks the position of the orchid. The scale bar applies to both graphs.
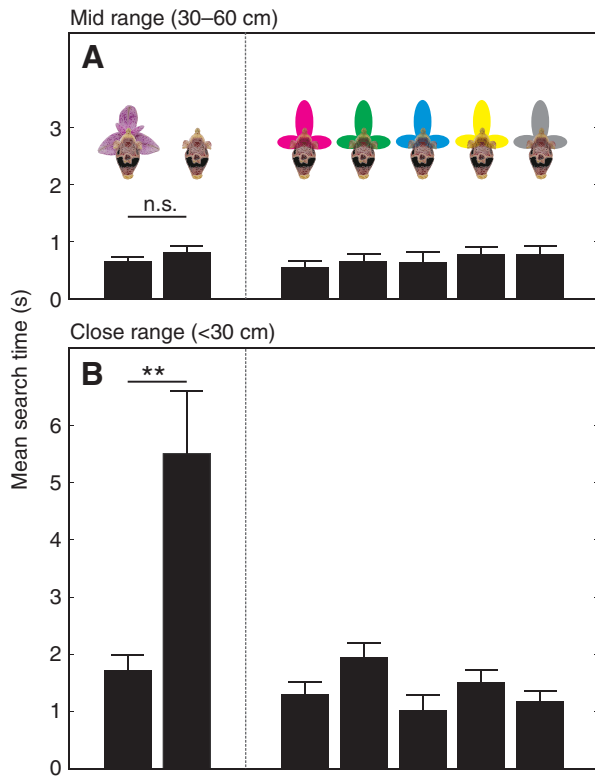
Fig. 3. Search times of *T. berlandi* males approaching *O. heldreichii* flowers in (A) the mid range (30–60 cm) and (B) close range (<30 cm). In Experiment 1 (left), we compared original flowers with those in which the perianth was removed ($N_{original}=33$; $N_{w/o}=32$), and in experiment 2 (right), with flowers in which the original perianth was replaced with different artificial perianths ($N_{pink}=22$; $N_{green}=25$; $N_{blue}=15$; $N_{yellow}=18$; $N_{grey}=11$). Spectral properties of the artificial perianths are given in Fig. 1; Means±s.e.m.; n.s. $P>0.05$; **$P<0.01$; Mann–Whitney U-test.

time negatively correlated with wind speed. However, with wind speed above $2.7\,\mathrm{m\,s}^{-1}$, search time increased with increasing wind speed, indicating that higher wind speed impedes orientation probably due to increasing turbulences. To summarise, at close range the search time of males was correlated with green receptor contrast but not with chromatic contrast or wind speed. By contrast, at distances >30 cm, search time was affected by wind speed but not by the visual properties of the perianth (Table 2, Figs 3 and 4).

When males were allowed to choose between two flowers with identical labellum and odour bouquet but different perianth size, they mostly chose the larger perianth, even when the size was 1.5 times larger than the original one (small *vs* original size: $P<0.001$, $N=14$; original *vs* large size: $P<0.001$, $N=20$; 2-tailed binomial test). Thus males obviously do not prefer a particular perianth size or flower shape but seem to prefer the largest flower.

DISCUSSION

Our data clearly show that *T. berlandi* males employ different sensory modalities at various distances from the *O. heldreichii* flower for orientation during approach flight. At large distances (>30 cm), where models of target detection derived from studies with honeybees and bumblebees predict that compound eye spatial resolution is not sufficient to rely on vision (Giurfa et al., 1996; Dyer et al., 2008), search time was found to correlate only with wind speed, indicating that males exclusively depend on olfactory cues. At these distances, males exhibited the typical zig–zag flight pattern that is found in insects that orient themselves by means of an odour plume (Fig. 2) (Kennedy, 1983). With increasing wind speed, the odour plume usually narrows and becomes more directed, allowing the males to orient themselves more precisely (Fig. 4) (Brady et al., 1995). However, when wind speed exceeds a certain level, it might become more difficult for the approaching males to steer and keep track of the odour plume due to increased turbulences, which results in an increase in search time (Fig. 4). As soon as *T. berlandi* males come close enough to visually detect a flower, they change their behaviour. At distances where the flower subtends at least 5 deg. visual angle, search time was correlated only with the green contrast between the perianth and the background but not with wind speed (Table 2). Thus, our field observations nicely match the behaviour of bumblebees and honeybees tested in dual choice experiments in the laboratory. Under controlled conditions, bees trained to detect a rewarding stimulus, e.g. a coloured disc or a real flower that provides sufficient green contrast, can perceive the

correlated with green contrast (but not chromatic contrast) at close range (Fig. 3), i.e. males were faster at finding the flower when green contrast was high (Table 2). Interestingly, wind speed had a strong effect on search time at mid range but no effect at close range. We found that a nonlinear regression best explains the variance in search time (Table 2, Fig. 4). For winds between 0 and $2.7\,\mathrm{m\,s}^{-1}$, search

Table 2. Effects of wind speed, colour contrast and green contrast on search time (dependent variable) in the mid (top) and close range (bottom)

Dependent variable	Explanatory factor	Beta	F	P	R^2
Search time (mid range)	**Intercept**	**1.274**	**14.869**	**<0.001**	**0.253**
	Wind speed	**−0.656**		**<0.001**	
	(Wind speed)²	**0.120**		**<0.01**	
Factors not included					
	Colour contrast	−0.015		0.873	
	Green contrast	−0.156		0.116	
Search time (close range)	**Intercept**	**1.850**	**5.544**	**<0.001**	**0.059**
	Green contrast	**−3.633**		**<0.05**	
Factors not included					
	Colour contrast	−0.054		0.637	
	Wind speed	−0.202		0.065	
	(Wind speed)²	−0.162		0.131	

Multiple linear regression with stepwise addition of independent variables was applied to the data. Variables that did not significantly improve the model were not included. Significant variables are shown in bold; ($N_{pink}=22$; $N_{green}=25$; $N_{blue}=15$; $N_{yellow}=18$; $N_{grey}=11$).
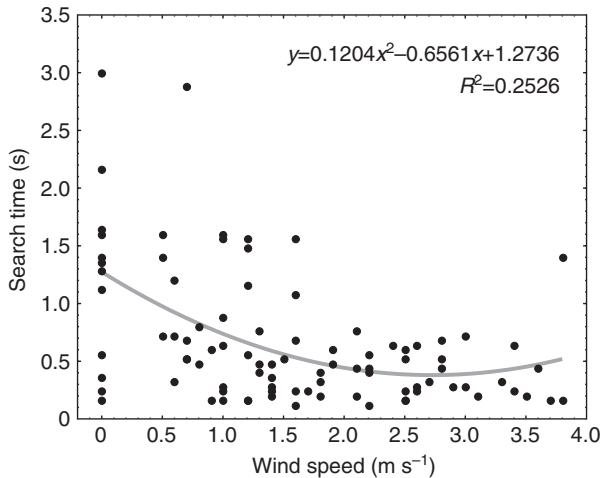
$$y=0.1204x^2-0.6561x+1.2736$$
$$R^2=0.2526$$

Fig. 4. The effect of wind speed on the search time in the mid range (30–60 cm distance from the flower) (*N*=91). Every data point corresponds to an individual approach of a male *T. berlandi* to an *O. heldreichii* flower. The light grey line indicates the nonlinear regression curve (see Results).

stimulus when it subtends a visual angle between 3 deg. and 5 deg. (Giurfa et al., 1996; Spaethe et al., 2001; Dyer et al., 2007; Dyer et al., 2008). However, when a stimulus provides only chromatic and no green receptor contrast, bee spatial resolution deteriorates and detection threshold increases to 15 deg. visual angle (Giurfa et al., 1996; Dyer et al., 2008). *Tetralonia berlandi* males thus initially detect the flowers by their green-receptor specific contrast. However, they may also perceive the colour of the perianth when they come closer to the flower but it is unlikely that the colour affects the final approach and the landing behaviour. We found that all males which were naïve to *O. heldreichii* flowers and which approached close enough to perceive the flower, also landed on the labellum irrespective of the perianth colour (M.S., H.F.P. and J.S., personal observation).

Our results together with recent findings (Spaethe et al., 2007) provide strong evidence that the pink perianth in *O. heldreichii* increases short-range detection and attractiveness for its pollinator, *T. berlandi* males. As visitation rate limits reproductive success, one would assume a strong selection pressure acting on bee-pollinated *Ophrys* flowers to increase attractiveness by means of imitating the sex pheromone of the pollinator's female and by providing a visual signal for close range attraction and detection. Surprisingly, the perianth is coloured in only about 30% of all *Ophrys* flowers. In all other species it is green and thus provides neither chromatic nor green contrast for bees (Delforge, 2006). We thus speculate that the presence of a colour signal is related to the visual system of the pollinator. Males from the tribes Eucerini and Anthophorini are frequently the pollinators of *Ophrys* species (Kullenberg, 1961; Kullenberg and Bergström, 1976; Paulus and Gack, 1990). They are fast fliers and exhibit a distinct visual and olfactory system that is employed to detect receptive females (Michener, 2000). In a search of the literature, we discovered that 74% (42 out of 57) of all *Ophrys* species, in which the known pollinator has been identified to be an Eucerini or Anthophorini male, possess a coloured perianth (Deforge, 2006; Paulus and Gack, 1990). By contrast, only 7% (4 out of 58) of species that are pollinated by males of the large genus *Andrena* (Andreninae) show a coloured perianth. Andrenine bees usually lack a distinct sexual

dimorphism of their sensory system. The presumably small benefit of possessing a coloured perianth for species pollinated by members of this genus might be outweighed by the disadvantage of accidentally attracting non-specific flower visitors and thus increasing the risk of pollen loss. Even though we did not account for phylogenetic relationships, the data suggest that the occurrence of a coloured perianth in *Ophrys* is likely to be correlated with the importance of the visual system for mate detection in the pollinating males.

The flower colour and shape and the pattern of the labellum in *Ophrys* species are often bizarre, and numerous authors have interpreted the appearance of the flowers as visual mimicry of the body of the pollinator's female (Detto, 1905; Kullenberg, 1961; Kullenberg and Bergström, 1976; Paulus and Gack, 1990). However, we must be cautious when regarding the visual appearance of flowers to not misinterpret visual similarities or analogies with the expected female bees. Hymenopteran compound eyes differ from human eyes in both spatial resolution and spectral sensitivity (Land, 1997; Briscoe and Chittka, 2001). Bees and wasps can perceive ultraviolet light, a part of the light spectrum to which humans are insensitive, and they use different receptor-specific visual channels, depending on the angular size of the targeted object. For example, in this study we showed that close range detection of the *Ophrys* flower is improved by the visually conspicuous perianth. However, search time does not correlate with the colour *per se* as we perceive it. Rather search time mainly depends on the green-receptor specific contrast, which is an important brightness channel for bees but is not perceivable by humans. Therefore, to arrive at an understanding of the functional significance and evolution of flower patterns and colourations in sexually deceptive orchids, we must take into account the specific properties and limitations of the pollinator's visual system.

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