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Relationship of visual and olfactory signal parameters in a food-deceptive flower mimicry system

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Pollinators such as bees are attracted to flowers by their visual display and their scent. Although most flowers reinforce visits by providing pollen and/or nectar, there are species—notably from the orchid family—that do not but do resemble rewarding species. These mimicry relationships provide ideal opportunities for investigating the evolution of floral signals and their impact on pollinator behavior. Here, we have reanalyzed a case of specialized food mimicry between the orchid *Orchis israelitica* and its model, the lily *Bellevalia flexuosa*. Based on current knowledge of insect sensory physiology, we were able to characterize both the visual and olfactory signals of model and mimic, as well as of two phylogenetically related orchids. By using a color vision model, we mapped each species' visual signals to the perceptual space of honeybees and found an apparent shift of the mimic's visual signals towards the model. We confirm that visual mimicry is present. We analyzed the flower odors by using gas chromatography/mass spectroscopy. We related these signals to the perceptual space of the pollinators by testing the scent extracts physiologically, using in vivo brain imaging. We found no evidence of olfactory mimicry. The results indicate that evolutionary pressure acts on the visual, but not olfactory, traits of *O. israelitica* toward a higher similarity to its model. Apparently, odor mismatch does not prevent a bee from landing on a flower that has the expected visual display. The results therefore argue for the dominance of visual stimuli in short-distance flower choice. The orchid may still depend on long-distance olfactory attraction originating from neighboring model plants. *Key words:* flowers, mimicry, olfactory signals, *Orchis israelitica*, visual signals. *[Behav Ecol]*

Plants pollinated by animals need to attract their pollinators in order to reproduce. This creates a double challenge. First, they must use signals that are detectable by the pollinator, and second, they must provide the pollinators with reward to ensure their repeated visits. Mimicry by plants can reduce the cost of attracting pollinators if no reward is given, but in order to be effective, the plant must change appropriate floral signals to match those of a model plant offering rewards (Macior, 1971; Rathcke, 1983). By comparing the signaling strategies of a mimic with its model, and with close relatives that do not rely on the same model, we can investigate the functional role that these strategies play in the communication between the plant and its pollinator, and the strategies used by the mimic for deceiving the pollinator.

Colors are among the most important floral signals (Menzel and Shmida, 1993; von Frisch, 1914). In most studies of floral mimicry, colors were judged by using human color vision (Dafni and Ivri, 1981; Fritz, 1990; Vöth, 1982). Other studies compared the petals' spectral reflectance (Nilsson, 1983b). However, similar color appearances may be caused by different spectral inputs to the color vision system (metameric colors), or contrary, very small differences in spectral reflectance may cause quite different color percepts. Because perceived similarity of stimuli is a central neural process, comparing physical reflectance properties of the stimulus alone is not sufficient for judging the similarity of visual displays for a receiver system. For honeybees, a mathematical model of color vision has been developed that allows quantifying the similarity of two colors if their reflectance spectrum is known (Backhaus, 1991). Such a model does not account for the stimulus' intensity. Experimentally, it has been shown that target detection in the honeybee is driven by both color and intensity cues. The underlying chromatic and achromatic visual systems differ in their spatial resolution. Thus, the resolution for images is higher if these are processed by an achromatic channel (L-receptor contrast) than if they are processed by chromatic vision, so that the two have to be analyzed separately (Giurfa and Vorobyev, 1998; Giurfa, et al. 1996, 1997, 1999; Hempel de Ibarra, et al. 2001).

Odors are also very important floral signals. In particular, they have been shown to be critically involved in sexual mimicry pollination systems. In the orchid genus *Ophrys*, male solitary bees are lured to the flowers that resemble female mating partners (Borg-Karlson, 1990; Borg-Karlson and Tengö, 1986; Stowe, 1988; Wiens, 1978). The males are attracted to visit these flowers and pseudocopulate with them (Kullenberg, 1961; Paulus and Gack, 1990; Schiestl et al., 1999). Extracts of *Ophrys lutea* flowers elicited as strong an attraction as did the mandibular gland secretion of the solitary bee *Andrena fuscipes* (Borg-Karlson and Tengö, 1986). Flower extract and mandibular gland secretion both contained geraniol, nerol, and *E,E*-farnesol, indicating that the orchid mimics the bee's secretion. Signal similarity was shown on a chemical level by using gas chromatography, measuring receptor neuron responses, and investigating behavioral responses in a bioassay (Ågren and Borg-Karlson, 1984; Schiestl et al., 1999). In all

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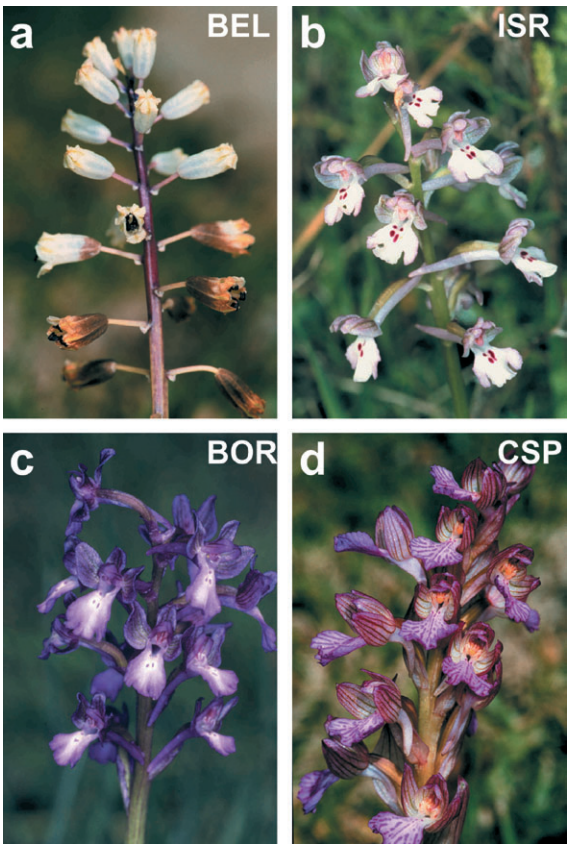

Figure 1
The model species *Bellevalia flexuosa* (Liliaceae) (a, BEL) and the nonrewarding mimic *Orchis israelitica* (Orchidaceae) (b, ISR) form a mimicry system (Dafni and Ivri, 1981). We compared floral signals of mimic versus model. Floral signals were also compared with those of the nearest relative to *O. israelitica*, *O. boryi* (c, BOR) and a more distantly related species of the genus, *O. caspia* (d, CSP).

cases of nonappetitive mimicry, the signaling odor was one or a combination of very few "key substances," which makes them comparable to the pheromone situation. The situation is different for floral odors advertising nectar, as is the case for the *Orchis-Bellevalia* mimicry system analyzed in the present study. These odors typically consist of hundreds volatile substances that form a compound scent note (Dobson, 1987, 1994), posing a fundamentally different problem in a mimicry situation. So far, floral odor mimicry has never been shown in food-deceptive mimicry systems (Dettner and Liepert, 1994).

Several species in the genus *Orchis* are thought to attract flower visitors from rewarding plants, thus fooling the insect that visits the flowers with the expectation of a reward (Dafni, 1983; Dafni and Ivri, 1979, 1981; Fritz, 1990; Nilsson, 1983a,b, 1984; Vöth, 1982; for reviews, see Dafni, 1987; Little, 1983; van der Cingel, 1995). It is estimated that one third of the Orchidaceae, that is, between 6500 and 10,000 species, are deceptive in some way (Ackermann, 1986), but only a part of these are food-deceptive.

Floral signals are addressed to the perceptual system of the respective pollinator (Proctor and Yeo, 1972; Rathcke, 1983; von Frisch, 1914). With the growing knowledge of the behavioral physiology and neurobiology of bees, the main pollinator of many food-deceptive orchids, it is now possible to investigate mimicry systems that had been described in the past by field observations integrating modern techniques and concepts. These aim at similarity measures that are not based on the sensory systems of humans or on the physical properties of the signals but are based on the signal perceived by the receiver in the mimicry system. Dafni and Ivri (1981) observed bees foraging on the nectar-rich Liliaceae *Bellevalia flexuosa* Boiss (Figure 1a) and then switching to the nonrewarding orchid *Orchis israelitica* Baumann and Dafni (Figure 1b). The latter showed a higher seed set when coflowering near *B. flexuosa* stands; the striking similarity of the appearances of the plants to the human eye suggested that *O. israelitica* practices signal mimicry, deceiving flower visitors of *B. flexuosa* (Dafni and Ivri, 1981). All signal parameters were described without considering the perception of the pollinators, and no olfactory signals were included. We measured the olfactory and visual signals of mimic and model. We also considered the mimic's sibling species, *O. boryi* Reichenb. Fil. (Figure 1c), and a more distantly related orchid, *O. caspia* Trautv (Figure 1d), which frequently coflowers in the proximity to *B. flexuosa*, in order to detect any possible shift of the signal characteristics of the perceived mimic from an ancestral status toward the signal characteristics of the model. We evaluated the perceptual properties of the signals by referring to a detailed color vision model of bees and by measuring odor-induced neural activity patterns in the bee's brains. From these studies, we propose a specific model for how the bee is deceived by the orchid's signaling strategy.

METHODS

Study species

We examined a case of specific mimicry between the nonrewarding orchid *Orchis israelitica* Baumann and Dafni (often referred to as mimic in this article) and its rewarding model, the lily *Bellevalia flexuosa* Boiss (Liliaceae) (Figure 1a,b). To test the hypothesis that the floral signals of the mimic converged to those of the model, we compared the signals of the mimic to those of its nearest relative, *Orchis boryi* Reichenb. Fil. (Figure 1c) and to a more distantly related species of the same genus, *O. caspia* Trautv (Figure 1d). We based species relatedness on a genetic analysis and phylogenetic reconstruction of the genus *Orchis* (Cozzolino et al., 2001). *B. flexuosa*, *O. israelitica*, and *O. caspia* were sampled in northern Israel, near Amirim (Mt. Meron region, 500 mNN) in 1996 and 1999. *O. boryi*, an endemic species of Greece, was sampled in central Greece near Nafpaktos (300–600 mNN) in 1997–1999. All species are pollinated predominantly by bees. Dafni and Ivri (1981) intensively studied the pollination biology of *B. flexuosa* and *O. israelitica* and suggested a specific mimicry between *B. flexuosa* as the model and *O. israelitica* as the mimic. Dafni (1983) described a generalized mimicry for *O. caspia* flowering in Israel, with bees switching from several different food plants to the orchid. *O. boryi* in central Greece is also a generalized mimic: bees visiting this orchid were observed foraging on several different food plants in the direct vicinity (Gumbert and Kunze, 2001).

Visual signals

Color loci

Petal reflectance spectra were measured from 300–700 nm by using a flash photometer (Groebel SR01, resolution of 1 nm). For further details, see Backhaus (1991) and Menzel and Shmida (1993). Recordings were taken directly in the field in order to avoid any deterioration in color. Sample sizes were 148 (*B. flexuosa*), 161 (*O. israelitica*), 200 (*O. boryi*), and 138 (*O. caspia*). For all visual signals, statistical analyses (ANOVA and post-hoc *t* tests) were done in JMP (SAS). To assess color similarity and variability among flowers of rewarding and nonrewarding plants, we used a model of bee color vision that

takes into account the spectral sensitivity of bee photoreceptors as well as the basic neural mechanisms of color opponent coding (COC; Backhaus, 1991). The spectral sensitivity functions used for the three photoreceptor types of *Apis mellifera* are based on physiological measurements (Peitsch et al., 1992). The COC model allows us to quantify perceptual differences between colors by measuring the distance of their loci in a two-dimensional color space. Because the two color-opponent dimensions are perceptually independent, color distance is calculated by adding the distance for each dimension (city-block metric), a measure henceforth called color distance (Backhaus, 1991). Flower colors with loci that are close to each other are perceived as more similar, whereas those with a larger color distance appear less similar (Gumbert et al., 1999; Menzel and Shmida, 1993). This model has been shown to accurately predict color choice behavior with respect to color similarity in the honeybee (Backhaus and Menzel, 1992; Brandt and Vorobyev, 1997). Similar color vision models were successfully used to quantify floral color distributions in flower communities (Chittka et al., 1997; Menzel and Shmida, 1993;). Such models also apply to a large range of bee species that possess a similar set of spectral photoreceptor types (Chittka et al. 1992; Peitsch et al., 1992).

L-receptor contrast

Visual orientation toward a target is guided not only by color vision but also by achromatic vision (Giurfa and Vorobyev, 1998; Giurfa et al., 1996, 1997, 1999). The achromatic system is mediated exclusively by the long-wavelength photoreceptors (L-receptor signal). Chromatic and achromatic visions in honeybees have different spatial resolution. Achromatic signals have higher spatial resolution and are detected from a further distance than are chromatic (color) signals. Chromatic and achromatic vision is tuned to different angular sizes of objects. If a colored object subtends a visual angle larger than 15 degrees, L-receptor contrast is not used for detection, and these objects are detected on the basis of their chromatic properties (Giurfa and Vorobyev, 1998, Niggebrügge and Hempel de Ibarra, 2003). Objects subtending visual angles smaller than 15 degrees and down to 5 degrees are detected on the basis of their L-receptor contrast to the background. In this angular range, chromatic properties are not perceived anymore. Finally, objects subtending an angle less than 5 degrees cannot be detected irrespective of the L-receptor contrast value. In the present study, L-receptor contrast provided by the signal against the background was calculated for each flower from the spectral data as the excitation values of the long wave receptor for the flower stimulus. Sample sizes were as for color loci.

Display size

The size of a flower and both its achromatic and chromatic contrasts to the background define the distance over which it can be detected. Because both the far-distance L-receptor signal and the close-up chromatic signal are used in sequence during an approach flight to a particular flower, the size of the object represents an important character (Giurfa et al., 1996). The similarity of model and mimic with respect to display size of the four species was measured. Images of 25 randomly chosen plants were taken as photographs of the inflorescence with a black background and a printed scale. These images were digitized, and the display area of this view for each inflorescence was calculated. Sample sizes were 25 inflorescences for each species.

Color variability

Color variability within a population was measured as the mean color distance (in COC units, see above) between the flower colors of all individuals of a species and the mean color for that species. The higher the mean color distance, the more variable the population is colored to a bee. Sample size and statistical analysis correspond to the color loci analysis.

Olfactory signals

Odor sampling

Volatile substances (referred to as headspaces) emitted by the intact inflorescences were collected in the habitat (Kaiser, 1993). For each sample, one inflorescence was enclosed in a plastic bag (polyethylene terephthalate, Toppits, Melitta GmbH). Air was drawn through the bag by suction (SKC Ltd. personal battery-driven pump SSA-86027x) at a constant flow rate of 40 ml/min controlled by a flow meter (Supelco) for 6–8 h during daytime (approximately 0900–1700 h, depending on altitude and weather conditions). Activated charcoal was used as adsorbent (Precision charcoal filter, 5 mg) in a glass tube (5 × 65 mm). The filter was extracted with 20 µl hexane (pa, Sigma-Aldrich). We cleaned the adsorption material in the filter tubes by rinsing with methanol, dichloromethane, and pentane/hexane (pa, Sigma-Aldrich). Samples were stored in the dark at −18°C until analysis. The blank was treated in the same way as the samples, but no plant material was included in the plastic bag.

Chemical analysis

All samples were analyzed by gas chromatography/mass spectroscopy (GC/MS) injecting 1 µl of the sample in a Varian 3400GC connected to a Finnigan SSQ 7000 mass spectrometer, with electronic ionization (EI). An HP-wax column (Hewlett Packard), 30-m 0.25-mm ID (0.25-µm film thickness) with helium as carrier gas was used. The injector was a split/splittless type, closed for 45 s at isothermal 225°C. The temperature program was 40°C (1 min), followed by an increase of 10°C/min to 200°C, remaining at 200°C for 10 min. The constituents were identified by comparison of their mass spectra and GC retention values with those of synthetic and authentic reference compounds and those given in the Finnigan Nist library. Only the main fragrance-constituents emitted by the flowers were identified and chosen for comparison between the plant species. Because of the minute amounts of each sample, it was, unfortunately, not possible to separate the enantiomers of the chiral constituents present in the odor samples.

Neural activity patterns

Unlike for the visual system, there is no model of olfactory coding that would predict olfactory perception on the basis of a chemical analysis of the stimulus. To relate a given stimulus to the animal's perception, odor-evoked activity has to be measured physiologically within the brain. By using calcium imaging techniques, the neural responses to odors can be measured in the first olfactory brain center, the AL. Odors lead to patterned activity in the functional units of the AL, the olfactory glomeruli (Galizia and Menzel, 2001; Joerges et al., 1997). We measured odor-responses in the honeybee ALs following published methods (Galizia et al., 1998; Sachse et al., 1999).

Preparation and calcium imaging

Adult worker honeybees were caught from four different hives. Bees were either free flying or kept in a flight chamber at a constant night/day rhythm. After cooling, the bees were harnessed in a Plexiglas stage by using dental wax. The antennae were fixed with silicone (Kwik-Sil, WPI) at their scapus. A window was cut into the cuticle of the head. Glands and tracheae were removed; the esophagus was cut. The brain

was then washed in Ringer solution (130 mM NaCl, 6 mM KCl, 4 mM $MgCl_2$, 5 mM $CaCl_2$, 160 mM sucrose, 25 mM glucose, 10 mM HEPES at pH 6.7, 500 mOsmol) in order to remove proteases and other enzymes that may have been released by glands or by the cut esophagus. Fifty microliters Calcium Green AM dye (Molecular Probes) were first dissolved in 50 µl Pluronic in DMSO and then diluted in 950 µl Ringer solution. The brain was stained by bath applying the dye for 1 h.

The stained bees were placed under an epifluorescent microscope (20×, NA 0.6 air objective) with constant Ringer perfusion (1 ml/1 min) at room temperature (22°C). Images were taken at a rate of about two frames per second, with 240-ms exposure time per image and measured with a 12-bit CCD camera (Photometrics CH250A). For each odor, 4 µl of the pure substance or 20 µl of the natural flower odor extract were placed on a filter paper (1 cm^2) in a 1-ml glass syringe. The stimuli were delivered by puffing 0.8 ml odor-laden air (duration, 2 s). Interstimulus interval was 45 s. Natural flower odors were taken from headspace samples, dissolved in 20 µl hexane. The solvent was allowed to evaporate for 20 s before filling the syringe. Neural activity patterns, including between two and seven measurements of each odor sample, were analyzed in three honeybees. The following odors were included in the present study: head space samples of the four study species (BEL indicates *B. flexuosa*; ISR, *O. israelitica*; BOR, *O. boryi*; and CSP, *O. caspia*), blanks taken together with the head space samples in the field (BLK) and pure hexane as the main sample solvent (HXT; Sigma-Aldrich, pa). In addition, a sample was prepared following the procedure for the flower odor sample, but using pure hexane alone. The hexane droplet was applied to the filter paper, and we waited for approximately 20 s before filling the syringe. In this case the solvent was already evaporated, and no signal was measured (data not shown). This indicates that also in the case of the flower odors, most of the solvent was already evaporated before filling the syringe with the odor-loaded filter paper. As an additional control stimulus, we tested a syringe plus filter paper without sample. There were no responses to the controls. To facilitate the identification of individual glomeruli of the AL, the response to the odor 1-nonanol (NO1; Sigma-Aldrich 99%) was also measured, which consists of glomeruli T1-33 and T1-17 (Sachse et al., 1999).

Mapping of glomerular responses

In honeybees, glomeruli can be identified on the basis of their size, shape, and relative position. They are labeled with a number and one out of the four antennal tracts (T1 through T4) that innervate them, for example, T1-33, T2-4 or T3-45 (Flanagan and Mercer, 1989). Because the glomerular structure is not visible during calcium imaging, we stained the glomeruli after functional imaging. The neural sheath was digested with protease (Protease Type XIV, Sigma) for 5 min, and then the AL was stained with the lipophilic dye RH795 (Molecular Probes). After 30–45 min, the glomerular structure became visible in epifluorescent light and could be imaged at different focal depths (see Figure 5, interplane distance = 10 µm). The images were sharpened with Photoshop (Adobe) in order to reconstruct the glomerular borderlines for each animal measured. The glomeruli were identified by using their morphology and relative position to other glomeruli by comparison with a computer atlas of the AL (Galizia et al., 1999b) (see Figure 5a). Most glomeruli in the visible region receive input by the T1 tract of the antennal nerve. For these glomeruli, we omitted the T1 in the name, that is, glomerulus T1-17 is simply referred to as glomerulus 17 in this article. In each preparation we could identify from 25–32 glomeruli. We measured the coordinate position of the middle of each identified glomerulus in the anatomical photo. A defined area of 5 × 5 pixels (corresponding to 25 × 25 µm fully within the selected glomerulus) around this central point was used for integrating that glomerulus' response to each stimulus.

Responses were calculated as relative changes in fluorescence intensity ($\Delta F/F$, where F is calculated as the mean of frames 4 through 7, before stimulus onset at frame 9). The time courses were corrected for bleaching in the following way: for each frame (point in time), the average signal was calculated. This gives a single function of signal intensity against time. A log-function was fitted to this time course, and the values of the log-function detracted from each frame. This treatment removes global bleaching and/or photoisomerization effects without changing the spatial components of odor responses. Repeated measurements were averaged in order to reduce signal-to-noise ratio. Response strength for each glomerulus was measured as the maximum signal after stimulus onset. Responses from different animals were compared with the basis of identified glomeruli (Galizia et al., 1999a).

RESULTS

Visual signals

The color signals of ISR (Figure 2) were more similar for bees to those of the model species BEL than those of its nearest relative BOR and other coflowering food-deceptive orchids (CSP, which was not included in Figure 2; the values for *O. caspia* uniformly overlap with those for *O. boryi*). All species covered a relatively broad range in the color space.

The color of the mimic was closest to the model compared with the other orchids. We calculated the average color similarity to the mean color of the model BEL (Figure 3a). These were (in COC units, see Methods) 0.73 ± 0.04 for BEL, 1.03 ± 0.03 for ISR, 2.82 ± 0.07 for BOR, and 2.86 ± 0.07 for CSP (mean ± SEM). The differences between groups were highly significant (ANOVA, $F_{3,644} = 385.6$, $p < .0001$), and a post-hoc *t* test gave only CSP and BOR as nonsignificantly different. Thus, even though the mimic's color is more similar to that of the model, the population mean is still significantly different from it. However, looking at the distribution of the values, it is clear that the two, model and mimic, have a strong overlap; that is, many individual *O. israelitica* flowers have a color locus that many individual *B. flexuosa* have (see Figure 3a, where the means are given with the standard deviation, and not the SEM.)

The specific mimic *O. israelitica* had a color variability that was statistically not distinguishable from its model, BEL, but was significantly lower compared with the relatively high variability seen in other orchids. Color variability was assessed as the mean distance of individual flower color loci to the mean color locus of that species (Figure 3b). These were (in city-block COC units ± SEM) 0.73 ± 0.04 for BEL, 0.68 ± 0.04 for ISR, 1.49 ± 0.06 for BOR, and 1.31 ± 0.10 for CSP. The differences between the groups were highly significant (ANOVA $F_{3,644} = 48.1$, $p < .0001$), and a post-hoc *t* test gave significant differences between the means of all pairs, with exception of the model and the mimic.

The model had the highest L-receptor contrast, followed by the orchids. Within the orchids, L-receptor contrast similarity to the model was highest for the mimic. L-receptor contrast, a value important for long-distance detection of inflorescences, was measured as 2.29 ± 0.02 for BEL, 1.57 ± 0.03 for ISR, 1.00 ± 0.03 for BOR, and 1.19 ± 0.03 for CSP (Figure 3c). The ANOVA gave a highly significant difference between the

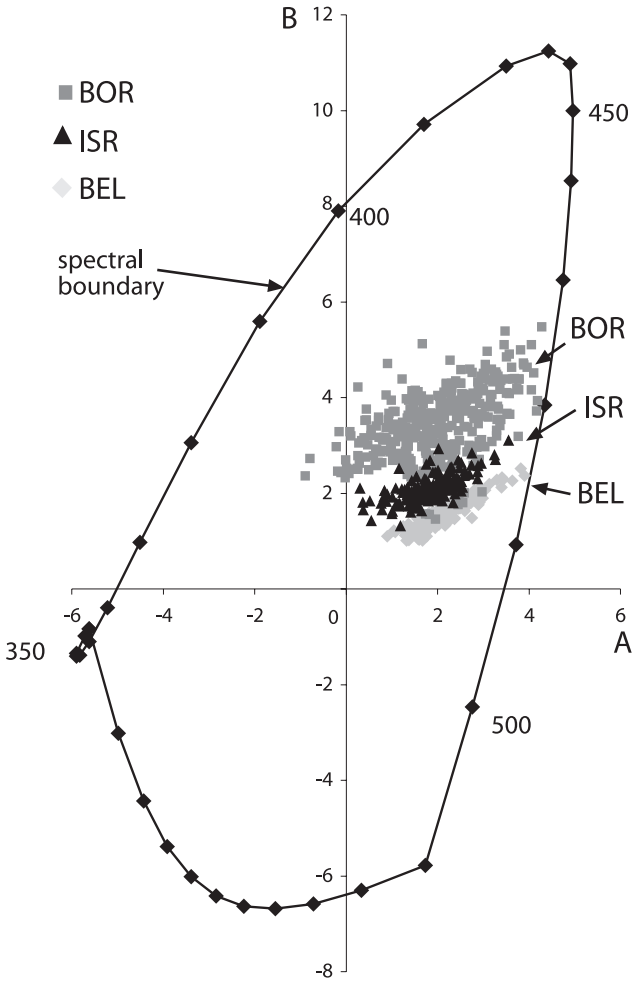

Figure 2
Color loci of model (*B. flexuosa*, BEL), mimic (*O. israelitica*, ISR), and its nearest relative (*O. boryi*, BOR) in the bee color space defined by the color opponent coding (COC) diagram (Backhaus, 1991). The diagram represents the excitation values in a scale of two COC channels, A (abscissa) and B (ordinate), that correspond to COC neurons identified in the visual pathway of the bee. Channel A receives positive input from the long wavelength (L-receptor or green) photoreceptor type and negative inputs from the short (UV) and medium (blue) wavelength photoreceptor types. Channel B receives positive input from the blue photoreceptor type and negative from the UV and green photoreceptor types. A color distance of 1.4 units measured in a city-block metric corresponds to 75% discriminability between two colors. The color space is limited by the line that connects the loci of spectral lights (values shown from 300–510 nm) as well as a mixture of short (300 nm) and long wavelength (510 nm) in different ratios. Color loci of less saturated colors, such as floral colors, are situated more toward the center of the color space.

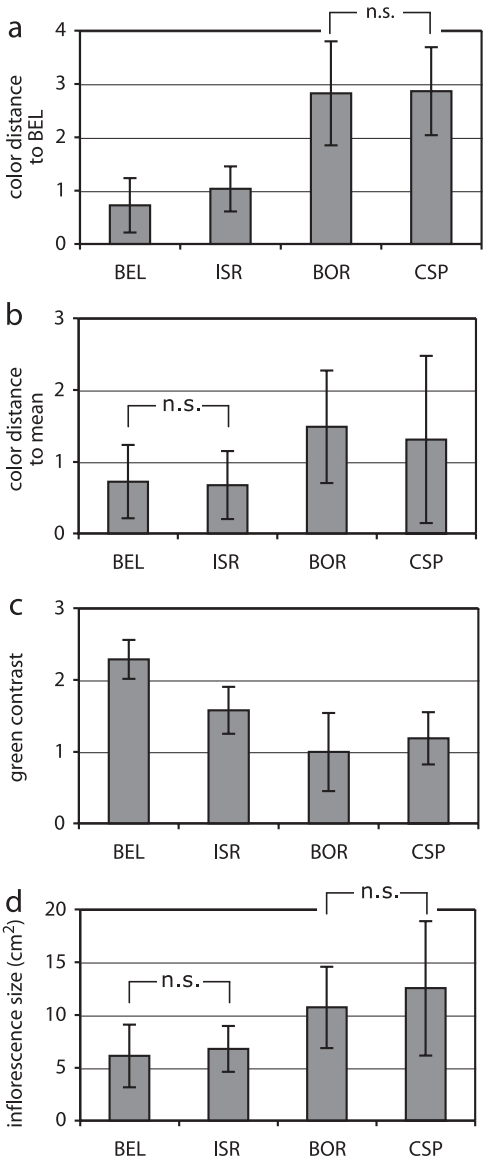

Figure 3
Comparison of visual signal parameters for *B. flexuosa* (BEL), *O. israelitica* (ISR), *O. boryi* (BOR), and *O. caspia* (CSP). Error bars show standard deviation. In all four parameters, differences are significant to $p < .0001$ (ANOVA). Number of flowers measured 149 (BEL), 161 (ISR), 200 (BOR), and 138 (CSP). (a) Color distance in COC units between flowers of a target species to the mean flower color of the model, BEL. This measure quantifies color similarity to the model. (b) Color distance in COC units between flowers and their mean color. This measure quantifies color variability for each species. (c) L-receptor contrast of flowers against a standard background. (d) Inflorescence display sizes measured in cm^2. Twenty-five inflorescences of each species were sampled. n.s.= not significant in post-hoc *t* test ($p \gg .05$).

groups ($F_{3,644} = 331.1$, $p < .0001$), and a post-hoc *t* test analysis showed that the means of all groups differed significantly. Nevertheless, the distributions of L-receptor contrast were highly overlapping (see Figure 3c, which shows standard deviation), and the overlap between model and mimic was greater than that of any of the other two orchid species.

Also, a higher similarity of the mimic's signals to those of the model than to those of the other two orchids was seen in the inflorescence size (Figure 3d). Mean optical display size for *B. flexuosa* inflorescences was 6.12 ± 0.59 cm^2; for *O. israelitica*, 6.79 ± 0.44 cm^2; for *O. boryi*, 10.72 ± 0.77 cm^2; and for *O. caspia*, 12.53 ± 1.27 cm^2. The difference between the group means was highly significant (ANOVA, $F_{3,96} = 13.8$, $p < .0001$). A post-hoc *t* test revealed that there was no statistical difference between the means of the model and the mimic and also no difference between *O. boryi* and *O. caspia* at a $p = .05$ level. Thus, the similarity of the mimic *O. israelitica* to the model was higher with regard to the display size than those of *O. boryi* and *O. caspia*.

Inflorescence size determines the maximal distance at which a bee can detect the plant, which can be calculated

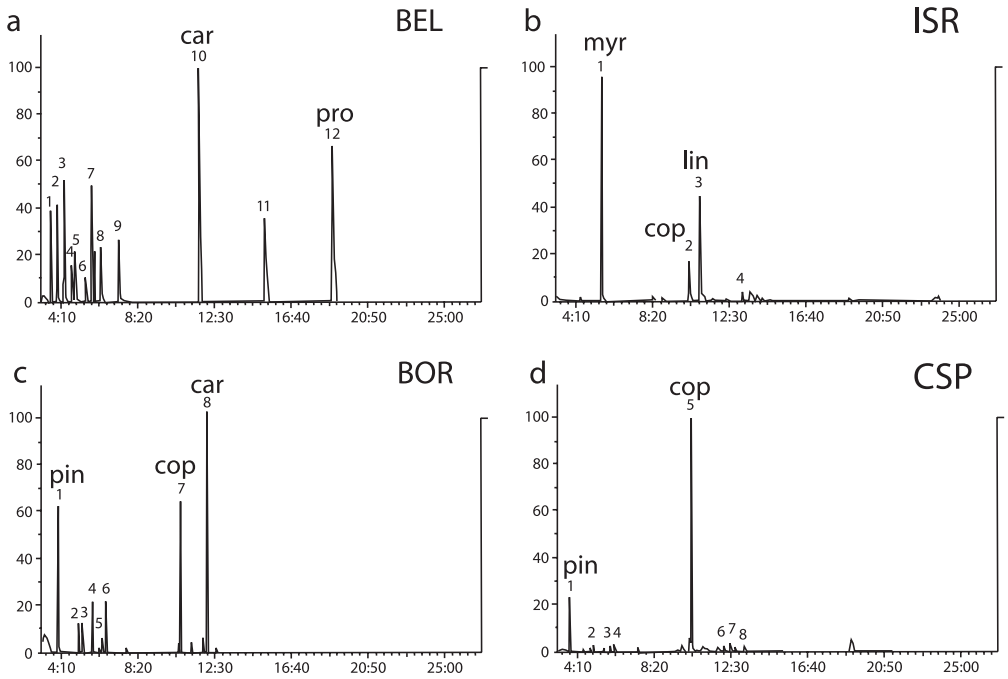

Figure 4
Gas-chromatograms for floral head space samples of (a) model *B. flexuosa*, (b) mimic *O. israelitica*, (c) the nearest relative to the mimic *O. boryi*, and (d) a more distantly related coflowering orchid *O. caspia*. For details of sampling and chemical analysis see Methods. Main peaks were identified (relative amounts given in parenthesis). BEL: 1, α-pinene (7.7); 2, solvent artifact; 3, methyl isobutylketone (10.3); 4, sabinene (4.4); 5, unidentified; 6, cyclopentanon (10.4); 7, limonene (8.2); 8, 2-hexanol (4.5); 9, 5-ethyl cyclopentanon (5.17); 10, β-caryophyllene (49.3); 11, plasticizer; and 12, propyl tetradecanoate. ISR: 1, myrcene (37.3); 2, α-copaene (13.5); 3, linalool (22.8); and 4, unsaturated ester (2.3). BOR: 1, α-pinene (12.4); 2, β-pinene (6.3); 3, sabinene (6.1); 4, myrcene (8.3); 5, limonene (3.4); 6, 1,8-cineol (7.9); 7, α-copaene (13.7); and 8, β-caryophyllene (28.4). CSP: 1, α-pinene (6.3); 2, β-pinene (0.5); 3, sabinene (1.0); 4, 1,8-cineole (1.1) 5, α-copaene (38.2); 6, β-caryophyllene (3.1); 7, unidentified sesquiterpene (3.8); and 8, unidentified sesquiterpene (4.3).

trigonometrically. With an inflorescence size less than 8 cm, as found for the mimic and model in this study, bees may recognize its color at a distance closer than 30 cm (15-degree visual angle for chromatic vision), and its L-receptor contrast at distances closer than 90 cm (5-degree visual angle for achromatic vision).

Olfactory signals

Chemical characterization

The main compound emitted by the model BEL was the sesquiterpene β-caryophyllene (peak 10) (Figure 4a). Minor compounds were the monoterpenes α-pinene (peak 1), limonene (peak 7), and sabinene (peak 4). Two ketones (methyl isobutyl ketone and 5-ethyl cyclopentanone) were also identified. The main compounds in the mimic *O. israelitica* were the terpenes myrcene (peak 1), linalool (peak 3), and α-copaene (peak 2) (Figure 4b). Small amounts of 3-hexanol, hexanal, heptanal, and nonanal were present, as well as the aromatic 2-phenylethanol. Two aliphatic dienic aldehydes (base peak 81) were characteristic constituents of the odor. Main peaks in *O. boryi* were identified as β-caryophyllene (peak 8) and α-copaene (peak 7) (Figure 4c). Monoterpenes present in *O. boryi* were α-pinene, β-pinene, sabinene, myrcene, limonene, and 1,8-cineol. The odor of *O. caspia* was rich in sesquiterpenes (Figure 4d). The main peak was identified as α-copaene (peak 5). Three other sesquiterpenes, β-caryophyllene, humulene, and α-cadinene were present. α-Pinene was the main monoterpene (peak 1). Oxygenated constituents were 1,8-cineol, cyclopentanone, and 5-ethylcyclopentanon.

Based on the enrichment technique used for collecting the volatiles, no particular similarity between the odors of model and mimic can be concluded from the identification of the main components in the odor samples. For technical reasons, we only collected and analyzed the volatile fraction ranging from C5 through C21. Therefore, constituents of low volatility could not be considered even though they may have importance in short-range attraction or discrimination in visiting bees, and may influence the differences between the species considered. A certain overlap in compounds was observed for the odors of the orchid species. This was particularly evident for the monoterpenes and for some sesquiterpenes such as α-copaene and β-caryophyllene.

Neural activity patterns

The inflorescence odor of the model and the mimic elicited clearly distinct activity patterns in the bee brain. We measured odor responses in the olfactory glomeruli of the AL, the primary brain structure involved in odor processing. Calcium imaging resulted in clear intracellular calcium increases in the AL when stimulating with the floral extracts and the control odor 1-nonanol (Figure 5). No responses were found for the control stimuli, which were evaporated hexane, the blank headspaces, and filter-laden syringe. When the solvent hexane was not allowed to evaporate before charging the syringe, strong glomerular responses were observed (HXT in Figure 5c). Response intensities in the strongest glomeruli were in the range of 1–2.5% $\Delta F/F$ for floral extracts compared with responses of 3–4% to pure nonanol. This difference reflects the differing stimulus concentration.

After staining the ALs with the membrane-selective dye RH795, the borderlines of olfactory glomeruli became visible (Figure 5a). We could thus morphologically reconstruct the glomerular map and identify individual glomeruli based on

their shape and relative position (Figure 5a). We superimposed these maps to the calcium activity maps (Figure 5b). We found that the extract from the model BEL elicited strongest responses in glomerulus T3-62 and weaker responses in several other glomeruli. The response in T3-62 was found in all animals investigated ($n = 3$). The mimic, *O. israelitica*, elicited responses in glomerulus T1-54, a response also found in *O. caspia*. In the latter species, additional responses in other glomeruli, in particular in T1-56, were observed (Figure 5b). Thus, the strongest glomeruli were clearly different for the mimic and the model. Responses in the weaker glomeruli were partially overlapping, but the identity of which glomerulus was weakly active differed between individuals. Variability between individuals may be owing to differences in previous odor experience of the bees, because our experimental animals were not naive. Alternatively, or additionally, weaker signals also have a lower signal/noise ratio, which leads to higher variability in the inter-individual comparison. The responses were also different between the two orchids, *O. israelitica* and *O. caspia*, but for these two samples the overlap in the patterns was more prominent, also encompassing strongly active glomeruli.

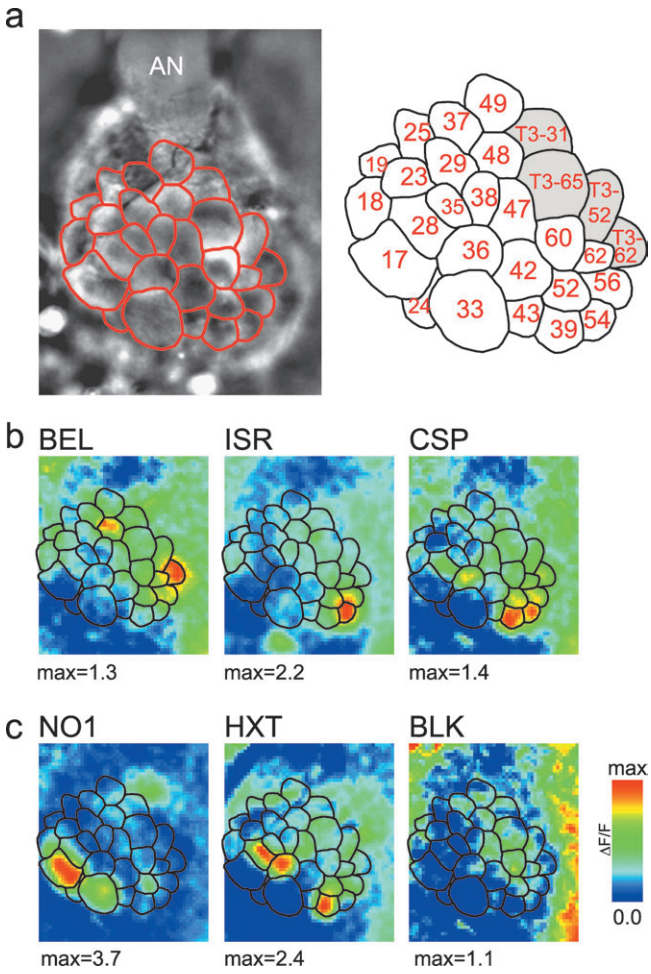

Figure 5
Calcium imaging of odor-evoked activity patterns. (a) Anatomical photo of antennal lobe with superimposed the glomerular borders as revealed in the staining with the membrane-selective dye RH795. The glomerular wire mesh is repeated, enlarged, to the right, with each glomerulus labeled with its identity. All glomeruli with just a number are innervated by the T1 tract of the antennal nerve (i.e., the glomerulus labeled 54 is T1-54); glomeruli innervated by T3 are shaded. AN indicates antennal nerve. (b) Responses to extracts from *B. flexuosa* (BEL), *O. israelitica* (ISR), and *O. caspia* (CSP) in an exemplary animal. The glomerular borders are superimposed to the activity images in order to allow attribution of activity spots to identified glomeruli. Each image is scaled to the range between zero and the maximum response in that image. This value is given below each frame. The false-color sequence is given in panel c. BEL has its strongest response in glomerulus T3-62; ISR, in T1-54; and CSP, in T1-54 and T1-39. (c) False-color coded images from calcium recordings from the same animal to the control odor 1-nonanol (NO1, strongest responses in T1-17 and T1-33), to the solvent used, hexane (HXT, responses in T1-28 and T1-36, as well as T1-39), and to the blank control, showing no response. Images are scaled as in panel b.

DISCUSSION

The function of floral signals is to attract pollinators. In a mimicry system, one may think that a perfect match of the mimic with its model may be the best evolutionary solution. However, the costs for such a match may outweigh the benefits, if an imperfect match proves sufficient for allowing secure reproduction. We show here that such an imperfect match is realized by the orchid *O. israelitica* with respect to its model, *B. flexuosa*: mimicry occurs for visual signals but not for olfactory signals. The deficiency in olfactory mimicry may be balanced by two developments: the orchid displays only a weak own odor and is reproductively most efficient when growing close to its model, suggesting that it profits from the model's proximity.

We compared floral signals by using the pollinator's perception, rather than physical quantification. We applied a color vision model developed for the honeybee, and we measured odor-induced neural activity patterns in the antennal lobe of the honeybee. One may argue that honeybees are not the principal pollinator in the mimicry system studied here. However, it was shown for the color vision system of many pollinating Apoidea that their color vision is very similar (Chittka et al., 1992; Peitsch et al., 1992; Vorobyev and Menzel, 2000). The situation is more difficult for the olfactory system, because no other species of pollinating Apiodea has been tested for its neural odor integration or its odor discrimination so far. We argue here that if neural coding in the honeybee antennal lobe would indicate similarity between odors (although they might be different in physical/chemical composition), we could assume perceptual similarity also for other pollinating Apoidea. If the neural code would indicate differences between the representations of these odors in honeybee, it would be unlikely that other Apoidea would perceive them as equal odors.

Visual similarity for bees is increased

The analysis of the model's and the mimic's visual signals suggests that there is evolutionary pressure on the orchid for similarity with the lily. In 1981 Dafni and Ivri first described this similarity. They also noted the dark violet dots on the orchid's labellum, possibly mimicking the anthers of *B. flexuosa*, which are also dark colored. We did not consider these small patterns in our analyses but measured the color of the labellum, accounting for the main floral color: a mixture of the human cream white and a human rosé hue. We show that *O. israelitica*, compared with its relatives, produces flower colors in a strongly overlapping range with the model (Figure 3a). Also the achromatic L-receptor contrast corresponds to that of the model (Figure 3c). This may play an even more important role than does the color, because bees use this uncolored visual parameter as a long-distance cue for detecting and discriminating an object against its background. Objects covering visual angles from 5–15 degrees on the retina of a bee are only evaluated by its long-wave receptor

signal against the background (Giurfa et al., 1996). Only when an object appears larger to a bee, because of either closer proximity or a larger display, color information can be used for discrimination. Because the visual angle is of major importance during detection and discrimination of an object, we also considered the display size of model and mimic. There was no difference between the inflorescence size of model and mimic, whereas the mimic was significantly different from its closely related orchids. Both *O. caspia* and *O. boryi* produced larger inflorescences, which may be relevant in attracting visitors from different coflowering food plants (Figure 3d). In contrast, *O. israelitica* has a reduced display size, increasing its resemblance to the model.

Together, floral color, L-receptor contrast and display size are the visual parameters that are relevant for an approaching insect to identify a flower at intermediate and close range, forming its "search image." All three signals show significant evolutionary convergence to the model, which in combination may have a considerable effect for a bee on a foraging flight. Although the most likely scenario appears to be that the orchid's display has converged to the model, the "status quo" data collected here do not exclude other possibilities, for example, that a stronger divergence in the closely related orchids has led to them being more distinct.

Color variability is reduced

Visual mimicry between *O. israelitica* and *B. flexuosa* is also supported by the analysis of floral color variability. In this case of specific mimicry, the mimic relies on its signal similarity to the model. Therefore, a high variability might decrease the probability of repetitive visits. Thus, a specific mimic should show low variation, aiming for a consistent and strong overlap with the specific model. This corresponds to our observations for *O. israelitica* (Figure 3b). The situation is different for most other orchids, in which color variability is found to be especially high (Ackermann and Galarza-Pérez, 1991; Darwin, 1862; Heinrich, 1975). Here, variability in floral signals may serve as a tool to impair the avoidance learning of deceived insect flower visitors (Ackermann, 1986, Dafni, 1984; Heinrich, 1975; but see also Smithson and Macnair, 1997), because bees are good learners in both appetitive and aversive paradigms (Giurfa, 2003; Menzel and Giurfa, 2001). However, this is only profitable for species following a generalized mimicry strategy by attracting pollinators from several plants in their vicinity. In that case, nonrewarding flowers may profit from high variability in two ways: (1) because of their variable colors, they might increase the probability of being similar to one of the surrounding food plants; and (2) once they have been visited, the effect of inhibitory learning by their pollinators may be reduced, thus increasing the probability of visiting other individuals of the deceptive species.

In other words, color display variability directly influences the error rate of the pollinator. A specific mimic, such as the one investigated here, profits from a low error rate and has reduced variability. The resulting cost (aversive learning by the bee) is balanced by other adaptations (rare occurrence, proximity to the rewarding model).

Odor similarity is not realized

We took headspace samples of flower odors under natural conditions and identified them by gas chromatography and mass spectrometry. The chemical characterization indicated that the chemicals released by the model were rather different from those released by the mimic.

Perception of chemically different scents might be similar if the receptors of the bee were selective for those parts of the flower bouquet that are overlapping, or if neural coding was to create similar percepts for different component mixtures. In the absence of a valid model for olfactory coding, similarity in odor perception must be tested directly. We could not collect sufficient amounts of headspace extract to perform behavioral experiments, for example, differential appetitive conditioning of the bee with the model's and the mimic's odor using a proboscis extension paradigm, or assays testing the degree of generalization between the two odors. We therefore reverted to a physiological approach by measuring odor representations in the first olfactory center of the bees' brains, the ALs. Such measurements visualize at least part of the neural code of odors and are known to correlate with behaviorally determined discrimination values (Galizia and Menzel, 2000). Stimulating honeybees with odors sampled from the flowers did not reveal any similarity of the mimic's odor with that of the model. Although low activity levels were found in similar areas of the AL, there was no overlap in strongly activated glomeruli excited by the collected headspaces of mimic and model. The only overlap in strongly activated glomeruli was found among the orchid species. Thus, chemical mimicry involving the floral volatiles is unlikely for this mimicry pair, even when analyzed by a method that relates the chemical stimuli to odor representation in the bee brain. This implies that a honeybee could easily distinguish the two plants on the basis of their odors. Consequently, it must be assumed that the chemical stimulus does not affect the bee's choice behavior sufficiently to impair the reproductive success of the orchid.

Alternatively, appetitive attraction may be based solely on one or a few key substances that act irrespective of the presence of additional substances. In that case, the additional substances may be responsible for the different odor responses observed in the antennal lobe, whereas mimicry would possibly be based on a key substance that elicits a response in a glomerulus or a group of glomeruli that would not have been accessible in our measurements. However, learning experiments with bees involving appetitive olfactory conditioning give strong evidence against this view: bees can learn almost any odor, quickly and efficiently, and there is no evidence for "release substances" that elicits feeding behavior. Note that this is fundamentally different from a system involving sexual mimicry. In fact, all chemical mimicry systems described so far involve only one or a few key compounds, most of them relying on sexual deception (Dettner and Liepert, 1994; Schiestl et al., 1999). Therefore, we rather assume that it may be an impossible task to imitate a complex mixture of several different compounds, owing to the complex web of biochemical processes involved.

Another perspective comes from signaling theory. What is the error rate that a pollinating system can tolerate? From the point of view of the pollinator, an occasional visit to a nonrewarding orchid does not cause a high cost, suggesting that the evolutionary pressure to minimize error rate is limited. From the point of view of the orchid, however, visits by the pollinator to a conspecific flower are imperative, arguing for a strong evolutionary pressure to avoid aversive learning in the honeybee. Therefore, we have to ask how aversive learning of the orchid odor is prevented.

In our experiments we had to accumulate the headspaces over a long sampling time in order to obtain reasonable amounts of odor for analysis and bioassay. Therefore, the strength of the response is a function of the sampling method rather than of the amount of substance released by the plant. The relative response magnitudes found in the present study, in which *O. israelitica* shows responses comparable in magnitude to those elicited by *B. flexuosa*, does not indicate that both plants emit equally strong odors. Many nonrewarding orchids

do not provide scent cues or provide them only in negligible amounts (Dafni, 1987; Kaiser, 1993). It may well be that the physiological task, for the bee, consists in an odor–no odor choice rather than comparing the two activity patterns that we have measured. A hypothetical additional or alternative strategy would be that odor variability might be increased in deceptive orchids (compare with the parallel argument about visual displays in nonspecific mimicry systems, above, where a good visual match predicts low variability). The rational would be that given the difficulty in mimicking a complex odor bouquet, high variability would avoid that negative olfactory associations might reduce the landing-probability on another orchid. We did not sample enough probes to allow for analysis of variance in either of our two measures of odor, GC/MS and imaging of odor responses. More research is needed to address this question.

Visual and olfactory attraction

Our findings substantiate the hypothesis that pollination mimicry may be restricted to visual parameters (Roy and Raguso, 1997). Visual signals are well known for guiding bees to flowers (Menzel et al., 1997) and play an important role in the bees' choice behavior. Visual cues are used for orientation from a distance toward objects. Scent has been suggested to function at close-distance, for example, inducing the visitor to land and enter a flower (von Frisch, 1914; von Aufsess 1960; Williams, 1983), but our data suggest that the close-distance function of odors may be subordinate to visual cues. For the orchid/lily investigated here, close range is 30 cm (color vision) or 90 cm (achromatic vision). However, odors may play a prominent role in long-distance attraction, that is, farther than 90 cm. It is likely that *O. israelitica* gains from the long-range olfactory display of *B. flexuosa*, by flowering in close proximity to it. Indeed, higher seed sets are found for the orchid stands that are close to the model patches (Dafni and Ivri, 1981). The relatively weak scent of the orchid may be an adaptation compatible with this explanation. In addition to allowing long-distance odor "hitch-hiking," weaker odor cues may make inhibitory learning and a later avoidance behavior more difficult for the flower visitors (Dafni, 1984; Heinrich, 1975, 1979; Kunze and Gumbert, 2001). Bees that have learned to avoid a nonrewarding flower type occasionally revisit it, either as a consequence of this putative variability or in order to test for any changes in the reward situation (Kunze and Gumbert 2001). *O. israelitica* is an endemic species in Northern Israel and is a rare plant even in its typical habitat. In rare orchids, even few visits may determine reproductive success during one flowering period, owing to their highly specialized system of pollen transport in large pollen packages (pollinia). Therefore, sporadic visits to nonrewarding flowers, even after discrimination learning took place, may suffice for pollen transfer in orchids. Considering these circumstances, the absence of olfactory mimicry in food-deceptive orchids may not be surprising.

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