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Can red flowers be conspicuous to bees? *Bombus dahlbomii* and South American temperate forest flowers as a case in point

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SUMMARY

It has been argued that trichromatic bees with photoreceptor spectral sensitivity peaks in the ultraviolet (UV), blue and green areas of the spectrum are blind to long wavelengths (red to humans). South American temperate forests (SATF) contain a large number of human red-looking flowers that are reported to be visited by the bumblebee *Bombus dahlbomii*. In the present study, *B. dahlbomii*'s spectral sensitivity was measured through electroretinogram (ERG) recordings. No extended sensitivity to long wavelengths was found in *B. dahlbomii*. The spectral reflectance curves from eight plant species with red flowers were measured. The color loci occupied by these flowers in the bee color space was evaluated using the receptor noise-limited model. Four of the plant species have pure red flowers with low levels of chromatic contrast but high levels of negative L-receptor contrast. Finally, training experiments were performed in order to assess the role of achromatic cues in the detection and discrimination of red targets by *B. dahlbomii*. The results of the training experiments suggest that the bumblebee relies on achromatic contrast provided by the L-receptor to detect and discriminate red targets. These findings are discussed in the context of the evolutionary background under which the relationship between SATF species and their flower visitors may have evolved.

Key words: color vision, red flowers, bee, chromatic contrast, achromatic contrast.

INTRODUCTION

Red coloration is typically considered by pollination biologists as a characteristic trait of bird-pollinated flowers (ornithophyllous) aimed to exclude bees as pollinators (Raven, 1972; Rodríguez-Gironés and Santamaría, 2004). Nevertheless, the large number of red flowers known to be visited by bees (for a review, see Chittka and Waser, 1997), along with behavioral evidence showing that bees can learn to detect and differentiate different types of orange-red stimuli (Reisenman and Giurfa, 2008), indicates that caution is required when discarding red as a perceptual cue for bee pollinator attraction. In order to fully understand the relationship between bees and red flowers it is necessary to take into account the spectral properties of the flower on one hand and the color vision system of the pollinator on the other hand (Menzel and Shmida, 1993).

Most hymenopteran species evaluated so far have trichromatic color vision with three kinds of spectrally selective photoreceptors, maximally sensitive (λ_{max}) in the UV (S-receptor; λ_{max}=344 nm), blue (M-receptor; λ_{max}=436 nm) and green regions of the spectrum (L-receptor; λ_{max}=544 nm) (Peitsch et al., 1992). Visual discrimination by bees can be either chromatic or achromatic depending on the angular size subtended by a visual stimulus (Giurfa et al., 1996; Giurfa et al., 1997). While detection and discrimination of objects subtending small visual angles (5–15 deg.) is mediated by the L-receptor achromatic pathway of bees, detection and discrimination of objects subtending visual angles larger than 15 deg. can be mediated by bees' chromatic visual pathway (Giurfa et al., 1996) or by the L-receptor achromatic pathway when stimuli provide no or little chromatic cues (Hempel De Ibarra et al., 2000).

South American temperate forests (SATF) contain a large number of hummingbird-pollinated plant species with flowers that are usually referred to as ornithophyllous red flowers (Armesto et al., 1996; Aizen et al., 2002). Many of these flowers besides being visited by birds are also visited by hymenopterans and especially by the only native bumblebee in SATF, *Bombus dahlbomii* (Smith-Ramirez, 1993; Smith-Ramirez et al., 2005). This raises the question of how the red coloration in these flowers relates to the native bumblebee's perceptual capacities. One possibility is that, as in the case of some solitary bees (Peitsch et al., 1992), *B. dahlbomii* might have evolved a red λ_{max} receptor. Human red-looking flowers, however, might reflect sufficient UV or blue light to be seen as color by bees (Menzel and Shmida, 1993; Chittka and Waser, 1997). Alternatively, pure red-reflecting flowers seen against a green foliage background might provide sufficient achromatic contrast to be detected by the L-receptor system of the common trichromatic bee visual system (Chittka and Waser, 1997).

In order to test these alternative hypotheses we measured *B. dahlbomii*'s spectral sensitivity through electroretinogram (ERG) recordings and estimated the contribution of different visual pigments to the overall spectral sensitivity. The spectral reflectance of eight plant species with human red-looking flowers visited by the native bumblebee were measured, and their chromatic and achromatic properties were estimated using the receptor noise-limited (RNL) model of honeybee color vision (Vorobyev and Osorio, 1998; Vorobyev et al., 2001). Finally, considering the findings by Reisenman and Giurfa (Reisenman and Giurfa, 2008) showing that bees trained to detect pure red-reflecting stimuli fail to discriminate them from chromatically different stimuli when L-receptor differences between stimuli are absent, we trained *B. dahlbomii* to pure red targets and asked whether it discriminates them from fully achromatic stimuli

displaying comparable levels of L-receptor contrast. We find that the achromatic contrast characterizing red flowers when seen against a green foliage background provide sufficient L-receptor contrast as to allow their detection and discrimination by *B. dahlbomii*.

MATERIALS AND METHODS

Measurements of spectral sensitivity by ERG recordings

ERG recordings were made from a total of seven workers of *Bombus dahlbomii* Guérin-Méneville (Hymenoptera, Apidae) captured in the wild. For these recordings the insects' bodies were immobilized leaving only the head free. The ERG was recorded under photopic conditions by keeping the bee's eye adapted to a white background light from a quartz tungsten lamp (150 W). The optical system consisted of a stabilized power supply with a quartz lamp, a monochromator and a short-pass, long wave-absorbing filter to eliminate stray light at short wavelengths from the monochromator. A series of quartz lenses were used to focus the stimulus on to the eye [for more details on the optical system, see Chavez et al. (Chavez et al., 2003)]. An electronic shutter set the flash duration, and an optical quartz wedge [0–4 OD (optical density)] attenuated the incident number of photons. The monochromator, optical wedge and shutter were under computer control and adjusted to deliver 10 ms flashes at wavelengths from 300 to 800 nm in 20 nm steps. The ERG signals were recorded with a pair of Ag/AgCl electrodes placed on the surface of the bee's eyes, the signals were low- and high-pass filtered (1 kHz and 1 Hz) with a high-gain amplifier (model DP-301; Warner Instruments, Hamden, CT, USA). Before each experiment, the photon flux of the lamp was measured with a calibrated photocell (Optometry S370; UDT Instruments, Hawthorne, CA, USA) positioned at the location of the eye.

To determine an intensity response function the ERG response was evoked by increasing the number of photons per flash (with 1–2 s intervals between the flashes) at fixed wavelength(s) from 300 to 680 nm. The spectral sensitivity (S_λ) as function of λ was determined as $S_\lambda = r_{peak}/I$, where I is the flash photon flux, and r_{peak} is the average of the maximum peak response for dim flashes (N=10–50 trials), where the amplitude of the response, at a particular wavelength, increases linearly with respect to the intensity. In our optical setup the light stimuli was designed to cover the complete area of the eye. For more details on the methods, see Chavez et al. (Chavez et al., 2003).

In order to estimate the contribution of visual pigments to the spectral sensitivity function, we applied an iterative modeling program to simulate several possible combinations of different visual pigments (for details, see Herrera et al., 2008). To recover the properties of the α absorption band we used Lamb's (Lamb, 1995) formulation (see also Govardovskii et al., 2000):

$$\log a(\lambda) = \log \frac{1}{\exp\left[a\left(A-\frac{\lambda_{max}}{\lambda}\right)\right]+\exp\left[b\left(B-\frac{\lambda_{max}}{\lambda}\right)\right]+\exp\left[c\left(C-\frac{\lambda_{max}}{\lambda}\right)\right]+D}, \quad (1)$$

where a is the λ_{max} bandwidth dependency, defined as:

$$a = 0.8795 + 0.0459 e^{\frac{(\lambda_{max}-300)^2}{11940}}. \quad (2)$$

The other parameters are: A=69.7, B=28, C=–14.9, D=0.674, b=0.922, c=1.104. The β-band absorption properties follow a log-normal function (Stavenga et al., 1993), and the β-band's λ_{max} is related to α λ_{max} (Palacios et al., 1998) by:

$$\beta - \lambda_{max} = 0.429\alpha\lambda_{max} + 123. \quad (3)$$

A non-linear fit was use to minimize the least-square function (Nelder and Mead, 1965). As a starting point for the fitting model we use α-band mean values from *Bombus* species already evaluated with respect to their UV, blue and green pigment sensitivities (Peitsch et al., 1992; Skorupski et al., 2007).

Measurement and categorization of flower reflectance spectra

The focal species were *Asteranthera ovata* Hanst. and *Mitraria coccinea* Cav. (Gesneriaceae) (Smith-Ramirez et al., 2005), *Crinodendron hookeranum* Gay. (Eleocarpaceae) (J.M.-H., personal observation), *Embothrium coccineum* Forst. (Proteaceae) (Rovere et al., 2006), *Lapageria rosea* R. et Pav. (Philesiaceae) (Humaña and Riveros, 1994), *Desfontainia spinosa* R. et Pav. (Desfontaineaceae) (J.M.-H., personal observation), *Eccremocarpus scaber* R. et Pav. (Bignoniaceae) (Belmonte et al., 1994) and *Tristerix verticillatus* R. et Pav. (Loranthaceae) (J.M.-H., personal observation). All these species were reported or observed by us to be visited in natural populations by *B. dahlbomii* as well as by hummingbirds. *Asteranthera ovata*, *C. hookeranum*, *E. coccineum*, *L. rosea*, *M. coccinea* and *T. verticillatus* have unicolored red corollas. *Eccremocarpus scaber* has a tubular corolla that shows a gradient from red, on the proximal part of the corolla, to orange on the distal part of the corolla. *Desfontainia spinosa* has long tubular red corollas distally divided into five yellow lobes.

Intact samples of flowers of each species borne on branches were collected in the field and kept fresh until reflectance spectra were measured by a fiber-optics spectrometer (model S2000; Ocean Optics, Dunedin, FL, USA) between 300 and 700 nm using a data-acquisition input/output card (12-bit 100 ks; DAQCard-700; National instruments, Austin, TX, USA) fitted to a computer. A white reflectance standard (Spectralon, 99%; Labsphere, North Sutton, NH, USA) was used for calibration. Sample patches were illuminated by a flash xenon lamp (Ocean Optics) through a silica-fused fiber optic (400 μm diameter) with six external concentric fibers. The reflected light was collected with a single central internal fiber. The light radiance sensor at a distance of 1–2 cm from the sample allowed measurements of a surface area of 0.1–0.4 cm^2. In the case of flowers displaying more than one color, as in *E. scaber* and *D. spinosa*, measurements of each color were made separately.

The categorization of the flower reflection spectra was based on the area under the normalized function calculated for the spectral regions between 300–400 nm, 400–500 nm and 500–600 nm. Additionally, in order to characterize a prominent slope in the reflection function we determined the wavelength value at which the reflection function crosses the 50% value between the two adjacent extremes (maxima, minima or plateaus) of the slope.

Determination of the spectral properties of red coloration in flowers

Using the spectral reflectance curves of flower's red coloration together with the photoreceptor spectral sensitivity curves simulated for *B. dahlbomii*, the receptor-specific contrast and chromatic contrast with respect to an average foliage background were established. For this purpose receptor quantum catches were calculated as:

$$Q_i = \int_{300}^{700} S_i(\lambda) I(\lambda) R_i(\lambda) d\lambda, \quad (4)$$

where i denotes the spectral type of receptor (S, M, L), $S_i(\lambda)$ is the spectral sensitivity function of receptor i, $I(\lambda)$ is the illumination spectrum and $R_i(\lambda)$ is the reflectance spectrum of the flowers or background color. Standard D65 daylight was assumed as illumination light (Wyszecki and Stiles, 1982). The receptor-specific contrast (q_i) of the red pattern to the average background was calculated as the quantum catch ratio of the photoreceptor relative to the average foliage background:

$$q_i = \frac{Q_i}{Q_i^b}, \quad (5)$$

where Q_i and Q_i^b denote the receptor quantum catch for the red pattern and the background, respectively.

The RNL model

The RNL model assumes that detection and discrimination of color is limited by the noise originated in the photoreceptors. Intensity (brightness) cues are ignored in the model. Predictions of the model agree with the results of behavioral experiments in a number of animals including the honeybee (Vorobyev and Osorio, 1998; Vorobyev et al., 2001; Koshitaka et al., 2008). Chromatic contrast (ΔS) for each flower was calculated as:

$$\Delta S = \sqrt{\frac{(\omega_L)^2(\Delta f_S - \Delta f_M)^2 + (\omega_M)^2(\Delta f_L - \Delta f_S)^2 + (\omega_S)^2(\Delta f_L - \Delta f_M)^2}{(\omega_S\omega_M)^2 + (\omega_S\omega_L)^2 + (\omega_M\omega_L)^2}}, \quad (6)$$

where ω_i denotes the standard deviation of the noise in the receptor i=S,M,L, f_i=Ln(q_i) is the receptor-specific contrast and Δf_i is the difference in receptor signals between two stimuli, in this case between the color of the flower and the green background. The only Hymenopteran for which receptor noise has been measured is the honeybee (Vorobyev et al., 2001); therefore, we used these values to construct the chromatic diagram for the set of receptors simulated for *B. dahlbomii*. The ω_i values were obtained from electrophysiological recordings in single photoreceptor cells. According to these measurements ω_S=0.13, ω_M=0.06, ω_L=0.12.

Theoretical considerations based on the ideal observer theory show that stimuli are discriminable at the level of 75% correct choices when ΔS>2.3.

A two-dimensional color opponent diagram corresponding to RNL model can be obtained by considering a plane, whose co-ordinates are related to receptor signals f_i by:

$$X = A\left(f_L - f_M\right) \qquad Y = B\left(f_S - (af_L + bf_M)\right),$$

$$A = \frac{1}{\sqrt{(\omega_L)^2 + (\omega_M)^2}}, \quad B = \sqrt{\frac{(\omega_L)^2 + (\omega_M)^2}{(\omega_L\omega_M)^2 + (\omega_S\omega_L)^2 + (\omega_S\omega_M)^2}},$$

where:

$$a = \frac{(\omega_M)^2}{(\omega_L)^2 + (\omega_M)^2}, \qquad b = \frac{(\omega_L)^2}{(\omega_L)^2 + (\omega_M)^2}. \quad (7)$$

Euclidean distance in the color space given by Eqn 6 can be expressed as:

$$\Delta S^2 = \Delta X^2 + \Delta Y^2. \quad (8)$$

Behavioral evaluation of the role of achromatic contrast in the discrimination of pure red-reflecting colors

Experimental setup and procedure

A colony of *B. dahlbomii* was collected in the field and transferred directly to the laboratory for the training experiments. The experiments were performed in an experimental flight arena of 120×120×40 cm, illuminated with natural light together with an artificial standard white light. The arena was connected to a nest-box, which contained the colony, through a clear plastic tunnel that could be selectively closed. Bees were trained to detect a pure red stimulus (Tr) against a green background. During the learning trials only red targets were presented in the experimental arena, a procedure that resembles absolute conditioning. The targets used in the training and test phase of the experiment were 10 cm diameter circular discs, and the sucrose solution was applied directly on the center of the disc.

For the experiments a total of four trained worker bees were marked and tested in a discrimination test. First we tested if the bees had learned Tr by presenting this stimulus in the arena together with a blue stimulus, which differed with respect to Tr both at the chromatic and achromatic level. In the three subsequent tests Tr was presented in the arena together with an unrewarded alternative stimuli chromatically equivalent to the background but with variable levels of achromatic contrast. Stimuli with three different levels of L-receptor contrast were chosen for these tests. During the test phase only one stimulus was simultaneously presented together with Tr in the experimental arena. The behaviors of the bees in the arena were videotaped and later analyzed with respect to their choices. All of the approach flights that ended up with landing or contact of the bee with the colored targets were considered as choices. After each trial the targets were cleaned with alcohol to eliminate any possible odor cue left by the bees and the position of the targets was randomly changed.

Stimuli design

The colors used for constructing the training and alternative stimuli were colored papers combined with neutral filters of variable densities. The spectral reflectance curves of the different colors (Fig. 1) and the spectral properties of the different stimuli were measured as described above for flower colors (Table 1). Chromatic and achromatic contrasts were calculated with respect to the green background color. The red-training stimulus (Tr) had a negative L-receptor contrast value of 0.13 and a chromatic contrast value of 6.94. The first alternative stimulus was a blue target with chromatic and achromatic contrast values of 12.34 and 0.64, respectively. The other three alternative stimuli were chromatically equivalent to the background and we named them achromatic 1, 2 and 3 (Achr.1, Achr.2 and Achr.3, respectively). The chromatic contrast values of all three achromatic stimuli were below the discrimination threshold (ΔS<2.3) (Table 1). At the achromatic level, Achr.1 had the L-receptor contrast closest to that of Tr, with a value of 0.3. Achr.2 and Achr.3 had L-receptor contrast values of 0.62 and 0.74, respectively.

Statistics

The first and total numbers of choices for each configuration were pooled, and the null hypothesis of random choice between the different chromatic configurations was tested by means of a log-likelihood ratio test for goodness of fit (G-test) (Sokal and Rohlf, 1995).

RESULTS

Measurements of spectral sensitivity by ERG recordings

The spectral sensitivity function of *B. dahlbomii* (N=7) was measured under photopic conditions (white light background) (Fig. 2). It extends

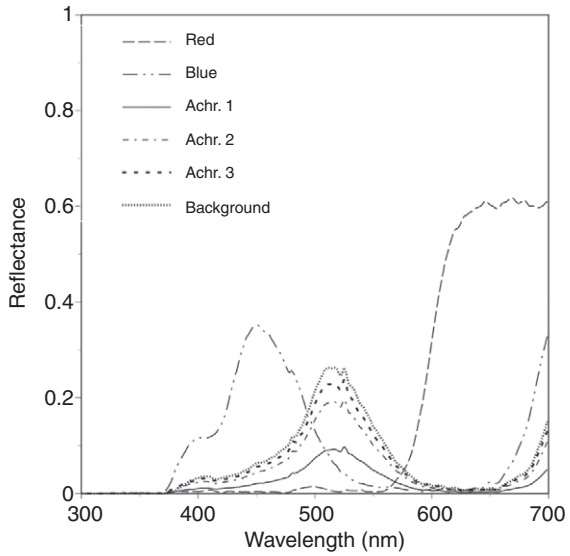

Fig. 1. Spectral reflectance curves of the colors used in the behavioral experiments. Achr. 1–3, achromatic 1–3.

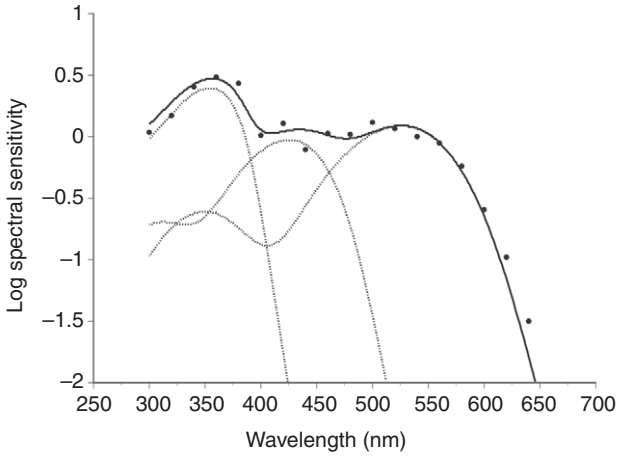

Fig. 2. Photopic spectral sensitivity curve for *Bombus dahlbomii* measured through electroretinogram (ERG) recordings (black dots) using dim flashes (averaging N=10–50), every 20 nm between 300–640 nm (N=7). The continuous line represents the global function fitted to our data, while the dotted line function corresponds to the modeled pigments' sensitivities. The simulation gave λ_{max} values and their relative participation at 355 nm (53%), 425 nm (20%) and 526 nm (27%) (r_2=0.99).

from 300 to 640 nm. A clear peak is found in the UV part of the spectrum, with a maximal sensitivity value at 360 nm. As expected from the overlap of the two corresponding rhodopsins the sensitivity in the blue and green parts of the spectrum does not show a clear separation, indicating underlying sensitivity peaks at around 420 nm and 510 nm, respectively. These results do not show an extended sensitivity to long wavelengths in *B. dahlbomii*, and are in agreement with results obtained for other trichromatic hymenopteran species utilizing similar electroretinographical methods (Goldsmith, 1958; Goldsmith, 1960; Menzel, 1971). The simulation performed to estimate the sensitivity peaks of the visual pigments that contribute to the ERG signal is also included on Fig. 2. The mean values locate the sensitivity peaks at 355, 425 and 526 nm (r_2=0.99), close to the values found by intracellular recordings for other *Bombus* species (Peitsch et al., 1992; Skorupski et al., 2007).

Measurement and categorization of flower reflectance spectra

The spectral reflectance curves distinguish two main categories of flowers: pure red-reflecting type and blue/red-reflecting type.

Pure red-reflecting type

This type of reflectance curve was found in *T. verticillatus* and *M. coccinea* (uniformly human red-looking flowers, Fig. 3A,B), and *E. scaber* (Fig. 3C) and *D. spinosa* (Fig. 3D) that have red flowers combined with pure orange or pure yellow patterns. All these red flowers absorb light strongly between 300 and 590 nm and have a sharp increase of reflectance at around 600 nm.

Blue/red-reflecting type

The flowers in this category are characterized by a small peak in the blue part of the spectrum and a sharp increase of reflectance around 600 nm. Flowers in this category vary with respect to the amount of reflectance in the UV and green parts of the spectrum. The flowers of *A. ovata* and *L. rosea* absorb in the UV and reflect moderately in the blue and green parts of the spectrum; these flowers show a small peak that appears at 390 nm, a trough at around 480 nm and a sharp rise at around 595 nm from where on reflectance stays high (Fig. 3E,H). The flowers of *C. hookeranum* show a peak in the blue part of the spectrum that occurs at 400 nm (a trough at 480 nm) and sharp rise around 600 nm from where on reflectance stays high (Fig. 3F). *Embothrium coccineum* absorbs UV, has a small peak in the blue part of the spectrum at 430 nm (with a trough around 490 nm). This flower also reflects in the blue-green and red parts of the spectrum, with a sharp rise around 570 nm (Fig. 3G). All flowers in this last category are unicolored.

Table 1. Spectral properties of the color configurations used in the behavioral experiments

Color paper	Chromatic contrast (ΔS) to the background	Receptor specific contrast to the background		
		S	M	L
Red	6.94	0.09	0.05	0.13
Blue	12.34	1.6	3.22	0.64
Achr. 1	1.02	0.25	0.27	0.3
Achr. 2	0.5	0.56	0.59	0.62
Achr. 3	0.4	0.7	0.73	0.74

Receptor-specific contrasts represent the quantum catches normalized to the background for each receptor type.
Chromatic distances to the background were calculated according to the receptor noise-limited model (Vorobyev and Osorio, 1998; Vorobyev et al., 2001) and are given in standard units. Colors are not discriminable for bees if ΔS<2.3. Achr. 1–3, achromatic 1–3.

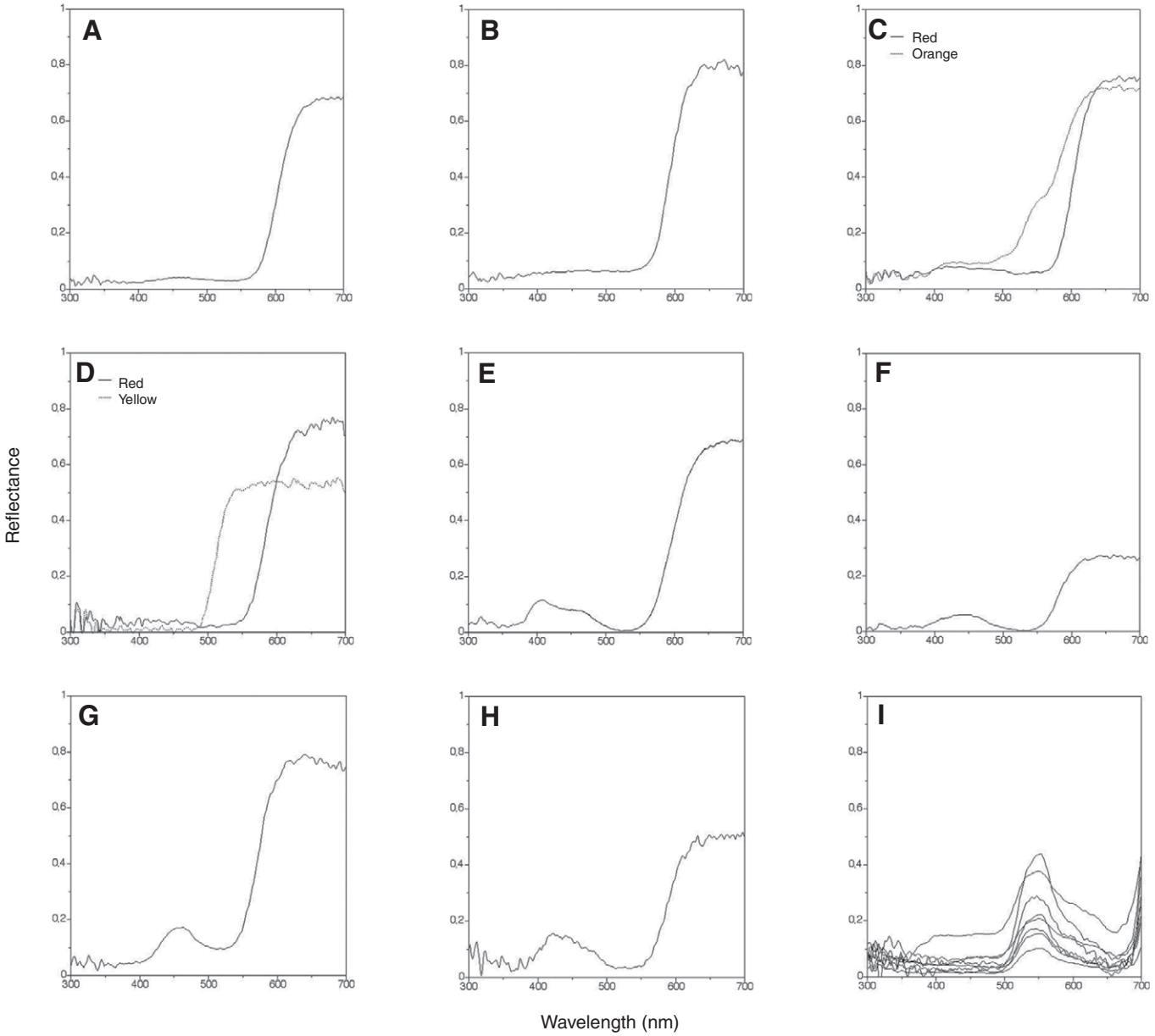

Fig. 3. Spectral reflectance curves of human red-looking flowers from eight plant species from the temperate forests of southern South America. (A) *Tristerix verticillatus*, (B) *Mitraria coccinea*, (C) *Eccremocarpus scaber* and (D) *Desfontainia spinosa* belong to the pure red category, while (E) *Asteranthera ovata,* (F) *Crinodendron hookeranum,* (G) *Embothrium coccineum* and (H) *Lapageria rosea* belong to the blue/red category. The spectral reflectance curves of the leaves (I) for all the species evaluated are included in the figure. For (C) *Eccremocarpus scaber* and (D) *Desfontainia spinosa* the secondary colors present in the flowers were also plotted.

Determination of the spectral properties of red coloration in flowers

Different levels of chromatic and achromatic contrast are predicted for pure red and blue/red flowers (Table 2). In the case of blue/red-reflecting flowers, all show a chromatic component, and should be discriminated by their chromatic contrast according to the RNL model of color vision. *Embothrium coccineum*, which yielded an ΔS value of 6.86, is the species among blue/red flowering species with the lowest L-receptor contrast, with a value of 1.38. For *A. ovata*, *C. hookeranum* and *L. rosea*, also species with blue/red-reflecting flowers, ΔS values are above the color discrimination threshold, and L-receptor contrasts values of 0.44, 0.27 and 0.63, respectively, were found.

The pure red-reflecting flowers from *D. spinosa* fall into a, so called, uncolored region in *B. dahlbomii*'s color space with a ΔS value of 1.79. The uncolored region is given this name because colors occupying this region subtend chromatic distance values, with respect to the background (center of the diagram), below the discrimination threshold and would then appear achromatic to the bees. *Mitraria coccinea*, *T. verticillatus* and *E. scaber*, however, yield ΔS values of 3.34, 3.6 and 5.87, respectively. At the achromatic level, these flowers show negative L-receptor contrast to the background with values of 0.54 for *D. spinosa*, 0.73 for *M. coccinea*, 0.39 for *T. verticillatus* and 0.61 for *E. scaber*.

The respective loci of the red coloration in each flower are plotted in the bee's color space diagram (Fig. 4). Pure red flowers occupy

Table 2. Spectral properties of red patterns of eight plant species respect to an average green foliage background

Color paper	Chromatic distance (ΔS) to the foliage background	Receptor specific contrast to the background		
		S	M	L
Desfontainia spinosa	1.79	0.63	0.68	0.54
Eccremocarpus scaber	5.87	0.98	1.32	0.61
Mitratia coccinea	3.34	0.8	1.08	0.73
Tristerix verticilatus	3.6	0.49	0.62	0.39
Asteranthera ovata	9.76	0.73	1.49	0.44
Crinodendrom hookeranum	9.86	0.32	0.83	0.27
Embothrium coccineum	6.86	0.76	1.98	1.38
Lapageria rosea	9.78	0.96	2.11	0.63

Receptor-specific contrasts represent the quantum catches normalized to the background for each receptor type. Chromatic distances to the background were calculated according to the receptor noise-limited model (Vorobyev and Osorio, 1998; Vorobyev et al., 2001) and are given in standard units. Colors are not discriminable for bees if ΔS<2.3.

Note that *Eccremocarpus scaber*, *Tristerix verticillatus*, *Mitraria coccinea* and *Desfontainia spinosa* belong to the pure red category, while *Crinodendron hookeranum*, *Lapageria rosea, Asteranthera ovata* and *Embothrium coccineum* belong to the blue/red category.

different loci than blue/red flowers. One of the pure red-reflecting flowers falls into an uncolored region in the bee's color space while the other three occupy a cluster on the periphery of this region. Blue/red-reflecting flowers tend to be located further from the center of the diagram, indicating that they are particularly well discriminated from the background.

Behavioral evaluation of the role of achromatic contrast in the detection and discrimination of pure red-reflecting colored targets by *B. dahlbomii*

When bees were tested to discriminate between Tr and a blue target, the latter of which was chromatically and achromatically different from Tr, bees correctly chose the rewarded Tr configuration (G=52; P<0.05) (Fig. 5), indicating that the bees learned to detect Tr and were able to discriminate it from the blue target. In the subsequent three tests Tr was tested against fully achromatic stimuli with variable levels of L-receptor contrast. When Tr was presented along with Achr. 1, the bees randomly selected the two stimuli (G=1.7; NS). Note that Achr. 1 is the stimulus with the L-receptor contrast value closest to the one of Tr. When Tr was tested against Achr. 2, bees also chose randomly between these stimuli (G=0.04; NS), even though Achr. 2 had a L-receptor contrast value of 0.62 compared with 0.13 for Tr. When Tr was tested against Achr. 3, however, which had a L-receptor contrast of 0.74, bees correctly chose the Tr configuration. The results from the first and total number of choices were statistically equivalent. Thus, in Fig. 5, we plotted only the total number of choices for each color. According to these results bumblebees trained to detect pure red targets discriminated them on the basis of achromatic L-receptor contrast, and that the animals required rather high L-receptor contrast differences between stimuli in order to discriminate them.

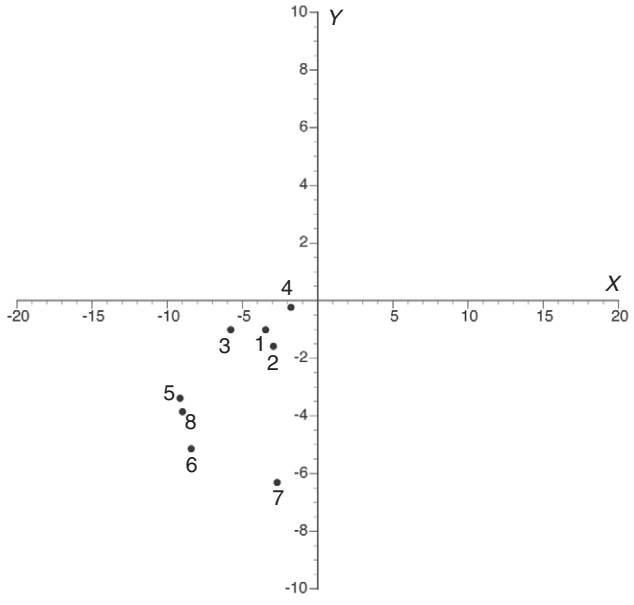

Fig. 4. Loci of flowers' red coloration pattern in the color diagram representing the bees' color space. (1) *Tristerix verticillatus*, (2) *Mitraria coccinea,* (3) *Eccremocarpus scaber*, (4) *Desfontainia spinosa*, (5) *Asteranthera ovata*, (6) *Crinodendron hookeranum*, *(*7) *Embothrium coccineum* and (8) *Lapageria rosea.* The chromatic coordinates were calculated for each color according to the receptor noise-limited model of honeybee color vision (Vorobyev and Osorio, 1998; Vorobyev et al., 2001). The unity distance corresponds to one standard deviation of the noise. The color locus of the background (foliage average) is by definition at origin of the color diagram (0, 0).

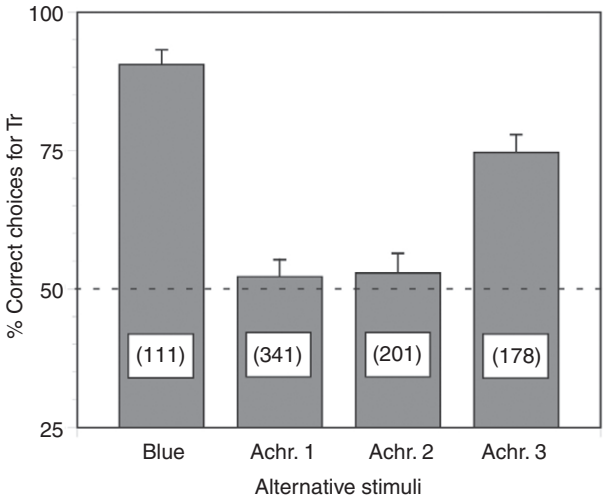

Fig. 5. Results of training experiment: percentages of choices for the pure red training stimuli (Tr) (means ± s.e.) as function of the alternative stimulus. The broken line at 50% indicates random choice level. Values in parentheses indicate the total number of choices recorded in each test situation: Tr *vs* Blue: G=52: P<0.05; Tr *vs* Achr. 1: G=1.7; NS; Tr *vs* Achr. 2: G=0.04; NS; Tr *vs* Achr. 3: G=27; P<0.001; N=4 bees. Achr. 1–3, achromatic 1–3.

DISCUSSION

The eight ornithophyllous red-flowered species evaluated in this study revealed a diversity of conditions with respect to their bee-specific coloration. Half of the species evaluated (*C. hookeranum*, *L. rosea*, *A. ovata* and *E. coccineum*), representing four plant families, have sufficient spectral reflection in the blue part of the spectrum to be suitable for bee color discrimination. The other species studied (*E. scaber*, *T. verticillatus*, *M. coccinea* and *D. spinosa*), representing four families, show reflection exclusively in the long wavelength part of the spectrum with a sharp increase around 600 nm, raising the question about the visual mechanism implicated in the discrimination of these flowers by bees.

One possibility is that *B. dahlbomii* might posses extended long-wave sensitivity. However, considering that the only example of tetrachromatic color vision including a red receptor was found in a solitary bee (Peitsch et al., 1992), bee's color vision is considered to be a rather conserved trait at the receptor level. In all 11 *Bombus* species studied so far, none have revealed a long-wave sensitivity beyond the usual range of the green receptor with a λ_{max} at around 540 nm and a half-bandwidth of about 110 nm (Peitsch et al., 1992; Skorupski et al., 2007). Our results based on ERG recordings do not support the possibility of an extended sensitivity at longer wavelengths, indicating that *B. dahlbomii* discriminates colors through the same mechanisms as most bees evaluated so far.

The flowers studied here provide both chromatic and achromatic contrast for the color vision system of *B. dahlbomii*. Blue/red-reflecting flowers are well separated from the green foliage background color with high values of chromatic contrast (Fig. 4, Table 2). Pure red-reflecting flowers, however, tend to cluster close to the uncolored region in the bees' color space with some species subtending chromatic contrast above and others below the discrimination threshold (Table 2). At the achromatic level, differences in the L-receptor contrast were seen both in the pure red and in the blue/red-reflecting flowers. Due to the lower intensities by which red flowers would stimulate the L-receptor relative to the green foliage background, most of these flowers yield negative values of L-receptor contrast. Thus, red flowers seen only by bees' L-receptor achromatic channel will appear as dim targets against a bright green foliage background.

A critical parameter determining visual stimuli detectability by bees is the spatial distribution of L-receptor contrast (Giurfa et al., 1996; Hempel De Ibarra et al., 2001). Patterns having a central disc weak in L-receptor contrast (dim) surrounded by a ring strong in L-receptor contrast (bright) yield detection limits at lower visual angles than a pattern having a bright central disc surrounded by a dim color (Hempel De Ibarra et al., 2001). When viewed through the low spatial resolution eyes of bees, stimuli having a dim-center/bright-surround L-receptor contrast distribution will have enhanced edges, while bright-center/dim-surround stimuli will have blurred edges (Hempel De Ibarra et al., 2001). It has been suggested that the structural substrate explaining the impairment of stimuli with blurred edges are center surround organization neurons (Giurfa and Vorobyev, 1998). Considering that all the pure red flowers studied here have negative values of L-receptor contrast, it can be argued that this characteristic of red flowers might represent a salient cue, which would increase their detectability, facilitate their learning and determine the strategy by which bees would discriminate them from other flowers.

A recent study shows that honeybees can discriminate between several pairs of long wavelength-reflecting stimuli subtending large visual angles (30 deg.), and that the bees' discrimination performances correlate both with the chromatic and L-receptor contrast (Reisenman and Giurfa, 2008). As pointed out by Reisenman and Giurfa (Reisenman and Giurfa, 2008) the correlation between the bees' performance and the chromatic contrast might be due to the fact that some of the stimuli used in their study stimulated the M-receptor as well, and therefore neural integration could in principle evaluate the differences between two types of input channels. Furthermore, the same study shows that bees, which learned to discriminate red stimuli with $\lambda_{ste}{}_{P}>570$ nm, were not able to discriminate red stimuli from chromatically different stimuli when both the red and alternative stimuli were equivalent at the L-receptor contrast level. These findings suggest that stimuli with $\lambda_{ste}{}_{P}>570$ nm are learnt on the basis of its L-receptor contrast, and that the achromatic cues are also used to discriminate them. In order to address this hypothesis we conducted behavioral experiments aiming to test if a trichromatic bee like *B. dahlbomii* discriminates a red target from fully achromatic ones displaying comparable levels of L-receptor contrast. In our experiments the stimuli were displayed horizontally on the surface of a flight arena, using green as background color. This experimental setup provides less constrained (the bees could approach the target from any direction and view it under varying visual angle) and more naturalistic conditions than the setup used in the experiments reported by Reisenman and Giurfa (Reisenman and Giurfa, 2008) in which bees were trained to vertically arranged stimuli within a Y-maze in order to control the angular sizes of the stimuli. Our results show that *B. dahlbomii* workers discriminated the pure red-training stimulus from a blue stimulus. The trained bees could not, however, discriminate the pure red-training stimuli from fully achromatic ones within a wide range of L-receptor contrast, indicating that bees learned to detect the red targets using their L-receptor-mediated visual pathway.

Raven proposed that red coloration was acquired in hummingbird-pollinated plants as an adaptation for excluding bees (Raven, 1972). Based on Spaethe et al's (Spaethe et al., 2001) observation that bees take a longer time to detect red targets in relation to those of other colors, Rodríguez-Gironés and Santamaría (Rodríguez-Gironés and Santamaría, 2004) argued that such low relative efficiency might explain the avoidance of red flowers by bees. However, it is becoming clear from the many reports of bees visiting pure red flowers (Daumer, 1958; Kevan, 1983; Menzel and Shmida, 1993; Vickery, 1992), including the four new species studied here, that bees are capable of actively detecting and visiting pure red flowers by their high levels of negative achromatic contrast.

To fully understand the relationship between red flowers and their visitors it is essential to consider the historical context in which such flowers and their pollinators evolved. Hummingbirds (Apodiformes, Trochilidae) first appeared in the late Paleocene (58.5 million years ago) (Bleiweiss, 1998). Although a recent fossil has surfaced in the Old World (Mayr, 2004), it is generally agreed that the hummingbirds arose in the New World (and specifically South America) (Bleiweiss, 1998), where they are restricted today. The genus *Bombus* originated in the late Eocene to early Oligocene (around 30 million years ago). Historical biogeographical assessments reveal an Old World origin followed by multiple dispersal events into the New World occurring after 21 million years ago, with migration into South America estimated after 10 million years ago, in accordance with the formation of the Panamanian land bridge (Hines, 2008). This historical scenario suggests that red hummingbird-pollinated flowers in southern South America would have pre-dated the appearance of bumblebees to the extent that native bumblebees would have colonized successfully into an already rich red-flower environment. These circumstances suggest that red coloration in hummingbird-pollinated flowers in southern South

America probably evolved independently of any interaction with bumblebees, although clearly this claim cannot be made with respect to other kinds of bees. In any case, all other things being equal, our results tend to support Chittka and Waser (Chittka and Waser, 1997) on the need to lay to rest the notion that red coloration evolved as a way to exclude bee visitors. Solving the enigmatic relationship between red-colored flowers and hummingbirds requires a deeper understanding of the historical evolutionary context under which this relationship evolved.

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