Structural and behavioral aspects of *Bombus dahlbomii* associated with its role as a visitor of red flowers.

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por

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FACULTAD DE CIENCIAS

UNIVERSIDAD DE CHILE

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TESIS DE MAGISTER

Se informa a la Escuela de Postgrado de la Facultad de Ciencias que la Tesis de Magíster presentada por el candidato.

Jaime Andres Alonso Martínez Harms

Ha sido aprobada por la comisión de Evaluación de la tesis como requisito para optar al grado de Magíster en Ciencias con mención en Ecología y Biología Evolutiva, en el examen de Defensa de Tesis rendido el 8 de Agosto de 2007.

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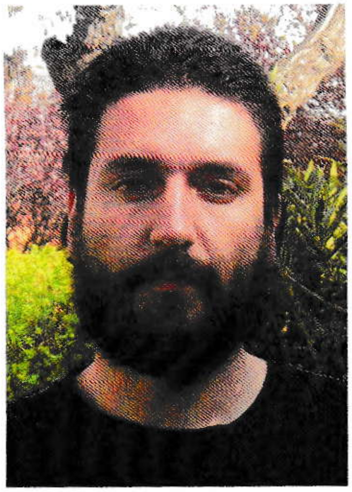
dedicada a Marta y a mis hijos Eru y Luna



Biografía

Jaime Martínez nació en Concepción el 29 de Octubre de1978. El año 1997 entra al programa de Bachillerato en Ciencias de la Universidad de Chile desde el cual se cambia finalmente a la carrera de Licenciatura en Ciencias con mención en Biología. Durante sus estudios de pregrado nace el año 2000 Eru, hijo de Jaime y Natalia. Con la compañía de ellos egresa el año 2002 de pregrado. El año 2004 entra al Magíster en Ciencias Biológicas, mención Ecología y biología evolutiva, donde desarrolla la tesis detallada en las próximas paginas. Durante sus estudios de Magíster nace además en el año 2005 Luna, quien junto a su hermano y padres partirán a vivir a Alemania durante el año 2008.

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Resumen

La coloración roja ha sido considerada por algunos biólogos de la polinización como un rasgo que evoluciono en flores polinizadas por aves como una adaptación para excluir a las abejas. Sin embargo, la literatura revela varios casos de flores rojas visitadas por abejas. Para entender la relación entre flores rojas y abejas es necesario tomar en cuenta tanto el sistema visual de las abejas como las propiedades de reflectancia de las flores. En general, las abejas poseen un sistema visual con tres tipos de fotorreceptores selectivos espectralmente, máximamente sensible en las regiones del espectro correspondientes al UV (receptor-S), azul (receptor-M) y verde (receptor-L). En teoría, estos fotorreceptores permiten a las abejas discriminar flores rojas sobre un fondo verde mediante mecanismos acromáticos. Sin embargo, algunas flores rojas para el ojo humano, además de reflejar luz en la parte roja del espectro, también reflejan suficiente luz en el UV o azul como para ser percibidas mediante el sistema de visión de color de las abejas. Los Bosques templados de Sudamérica presentan un gran numero de especies de plantas con flores rojas ornitofilas, muchas de las cuales también son visitadas por el abejorro nativo, *Bombus dalhbomii.*

Evaluamos la reflectancia espectral de ocho de estas plantas, representantes de siete familias, como también el loci que ocuparían estas flores en el espacio cromático de la abeja. Se midió la sensibilidad espectral de *Bombus dalhbomii* a través de registros electroretinograficos, se evaluó su preferencia innata por colores y mediante experimentos de entrenamiento evaluamos los mecanismos involucrados en la discriminación de estímulos rojos por parte de la abeja nativa.

Los resultados muestran que cuatro de las especies estudiadas tienen flores que reflejan exclusivamente en el rojo y que cuatro tienen flores rojas con un leve máximo de reflectancia en el azul. Dos de estas especies ocupan una región no cromática en el espacio de color de la abeja, mientras que seis ocupan regiones cromáticas. La evaluación de la sensibilidad espectral no revela una sensibilidad extendida a longitudes de onda largas que pudieran favorecer la discriminación cromática del rojo por parte de el abejorro nativo. Los experimentos de preferencia innata muestran que esta abeja nativa presenta una fuerte preferencia por el azul y una preferencia secundaria por el violeta y el rojo. Los experimentos de entrenamiento sugieren que Bombos dalhbomii se basa en contrastes acromáticos inducidos en el receptor-L para detectar flores puramente rojas. Los resultados confirman que las abejas perciben flores rojas dando luces de los mecanismos involucrados.

Abstract

Red coloration has been considered by some pollination biologists as a trait that evolved in bird-pollinated flowers as an adaptation to exclude bees. However, the literature reveals several cases of red flowers visited by bees. In order to understand the relation between red flowers and bees it is necessary to take into account the bee's visual system on one hand and flower spectral reflectance properties on the other. In general, bees have a visual system with three kinds of spectrally selective photoreceptors, maximally sensitive at the UV (S-receptor), blue (M-receptor) and green (L-receptor) part of the spectrum. Theoretically, these photoreceptors allow a bee to discriminate red flowers over a green background through achromatic mechanisms. Some flowers that appear red to humans however, besides reflecting light in the red part of the spectrum, also reflect sufficient UV or blue light to be useful in bee color vision. The South American temperate forests contain a large number of plant species with ornithophyllus red flowers, many of which are also visited by the native bumblebee, *Bombus dahlbomii.* The spectral reflectance curves from eight of these plant species representing seven plant families were evaluated, along with the loci occupied by these flowers in bee color space. *B. dahlbomii*'s spectral sensitivity was measured through electroretinogram recordings, it's innate preference for colors was tested and from training experiments the visual mechanisms involved in the discrimination of pure red flowers by the native bee were evaluated. Results show that four of the plant species studied have pure red-reflecting flowers and four have red flowers with a small peak in the blue part of the spectrum. Two of these species occupy an uncolored region in bee's color space while six occupy colored regions. The spectral sensitivity evaluation field to reveal extended sensitivity to long-wavelength that could favor chromatic discrimination of red by the native bumblebee. The innate preference experiments showed a strong preference for blue and a secondary preference for purple and red in the native bee. The training experiments suggest that *Bombus dahlbomii* relies on achromatic contrast induced on the L-receptor type to detect pure red flowers. Results further confirm that bees can perceive red flowers and shed light on possible mechanisms.

Introduction

According to the pollination syndrome concept, particular combinations of flower attributes, such as color, form, depth, and nectar composition, arise as the product of co-evolution between plants and their pollinators, considering the latter's sensory capacities, dietary needs, activity, mobility, ability to handle flowers and learning capacities as selective forces on the part of pollinators (Kevan & Baker, 1984). Under this concept, red coloration is typically considered by pollination biologists as a characteristic trait of bird-pollinated (ornithophyllus) flowers. Raven (1972) suggested that "red is the only color of the spectrum that is at once inconspicuous to most insects and also an excellent "signal" of a high caloric reward for birds". Nevertheless, at least in the case of bees, caution is required when discarding red as a perceptual cue for pollinator attraction. Indeed there are several records in the pollination literature of bees visiting red flowers. In order to fully understand the relation between bees and red flowers it is necessary to take into account the visitor's visual system on one hand and flower spectral properties on the other (Menzel and Shmida, 1993).

Most bee species have trichromatic colour vision (Peitsch et al. 1992) with three kinds of spectrally selective photoreceptors (Peitsch et al. 1992), maximally sensitive in the ultraviolet (S-receptor; λmax = 344 nm), blue (M-receptor; λmax = 436 nm), and green regions of the spectrum (L-receptor; λmax = 544 nm). Based on these photoreceptors bees perceive light from 300 to around 650 nm. But because color discrimination through chromatic differences, independently of intensity, requires at least two kinds of photoreceptors being active during the visual task, chromatic discrimination only becomes possible within the range where the spectral sensitivity curves of the different photoreceptor types overlap, which in bees lies approximately between 350 and 550 nm (Daumer, 1956, Von Helversen 1972, Menzel 1967).

Achromatic vision, on the other hand, also occurs in bees whenever only one spectral type of receptor is involved in a visual task. For honeybees two types of achromatic pathways have been described: E-vector analysis for navigation driven by the S-receptor (Wehner and Rossel 1985) and an achromatic channel driven by the L-receptor which enables bees to perform various visually guided tasks related to motion as well as distance estimation and edge detection. Because a single spectral type of photoreceptor cannot code for color, these performances are color blind (Giurfa et al. 1997, for review see Giurfa and Lehrer 2001).

Considering that bees alternatively use both their chromatic and L-receptor mediated achromatic visual pathway in the detection of colored stimuli, knowing the spectral reflectance characteristics of flowers becomes extremely important to understand how bees perceive them. Particularly in the case of red flowers, it has been shown that some flowers that appear red to the human eye, besides reflecting light in the red part of the spectrum, also reflect sufficient UV or blue light to be useful in bee color vision (Menzel and Shmida, 1993, Chittka and Waser 1997). A pure red reflecting flower over a green background, on the other hand, will show chromatic differences within a range where only bee's L-receptor type is sensitive, reason why bees probably perceive them through the achromatic differences induce by this colors on bee's L-receptor type. However, the visual mechanisms involve in the discrimination of red targets by bees have not been evaluated.

The 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 as to how red coloration in these flowers relates to the native bumblebee's perceptual capacities. Such flowers might reflect some of the shorter wavelengths visible to typical bees, as indeed, has been reported for some red flowers (Menzel and Shmida, 1993, Chittka 1994). Alternatively, capacity to chromatically perceive the longer red wavelength might have evolved in bee species inhabiting an environment replete with red flowers having abundant nectar, as proposed by Chittka et al. (2001). Additionally such red bee-visited flowers might provide sufficient achromatic contrast against the green foliage background of their forest habitat so as to be detectable by bees. Under such conditions red flowers would appear as dark targets over a bright background to the bees. This possibility has been suggested by Chittka (1997) but to our knowledge has not been evaluated. Finally, considering the abundance of red flowers visited by *Bombus dahlbomii* in the SATF, it would be interesting to evaluate to what extend *Bombus dahlbomii*'s innate color preference reflects red flower richness in this particular ecosystem. In contrast to the low variability observed among bee species at the receptor level, bees innate color preference have showed variable patterns that some authors have associate to differences on the chromatic environments occupy by different bee species (Chittka et al. 2001).

Hypothesis

Red flowers visited by *Bombus dalhbomii* are expected to either reflect light in the UV or blue parts of the spectrum or exhibit levels of achromatic contrast detectable by *Bombus dalhbomii.*

Bombus dalhbomii has a trichromatic color vision that allows it to discriminate pure red reflecting flowers through achcromatic contrast induced on the L-receptor type.

General Objectives.

The aim of this thesis was to investigate how the spectral properties of red flowers from the SATF relate to *Bombus dahlbomii*'s perceptual capacities.

Specific Objectives.

1) Measure the spectral reflectance curves of eight plant species derived from seven different families with human red-looking flowers that have been reported in the literature or have been observed by us to be visited by *B. dahlbomii*;

2) Measure *Bombus dahlbomii*'s spectral sensitivity through electroretinogram (ERG) recordings, so as to evaluate possible extended spectral sensitivity to the long wavelengths as a response to the abundance of pure red reflecting flowers;

3) Using the spectral sensitivity curves of a closely related bee, determine the achromatic properties of all the red patterns for the eight plant species studied. Using the "receptor noise limited model" (Vorobyev et al. 2001) for color vision, determine the chromatic contrast of the red patterns with respect to the background, along with the loci these colors would occupy in bee's color space;

4) Behaviorally evaluate the role of the chromatic and achromatic contrast in the discrimination of red-colored targets by *Bombus dahlbomii*.

(5) Behaviorally evaluate color preference of naïve *Bombus dahlbomii* workers.

Materials and Methods

Plant species studied

The focal species were *Asteranthera ovata* and *Mitraria coccinea* (Gesneriaceae) (Smith-Ramírez 2005), *Crinodendron hookeranum* (Eleocarpaceae) (personal observation), *Embothrium coccineum* (Proteaceae) (Rovere et al., 2006), *Lapageria rosea* (Philesiaceae) (Humaña & Riveros, 1994), *Desfontainia spinosa* (Desfontaineaceae) (personal observation), *Eccremocarpus scaber* (Bignoniaceae) (Belmonte et al. 1994), and *Tristerix verticillatus* (Loranthaceae) (personal observation), all reported or observed by us to be visited in natural populations by *Bombus dahlbomii* as well as hummingbirds. *Asteranthera ovata, Crinodendron hookeranum, Embothrium coccineum, Lapageria rosea, Mitraria coccinea and Tristerix 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 in 5 yellow lobes. Table 1 details the localities where the samples from the different species were collected. Images of flowers for the plant species considered are shown in Figure 1.

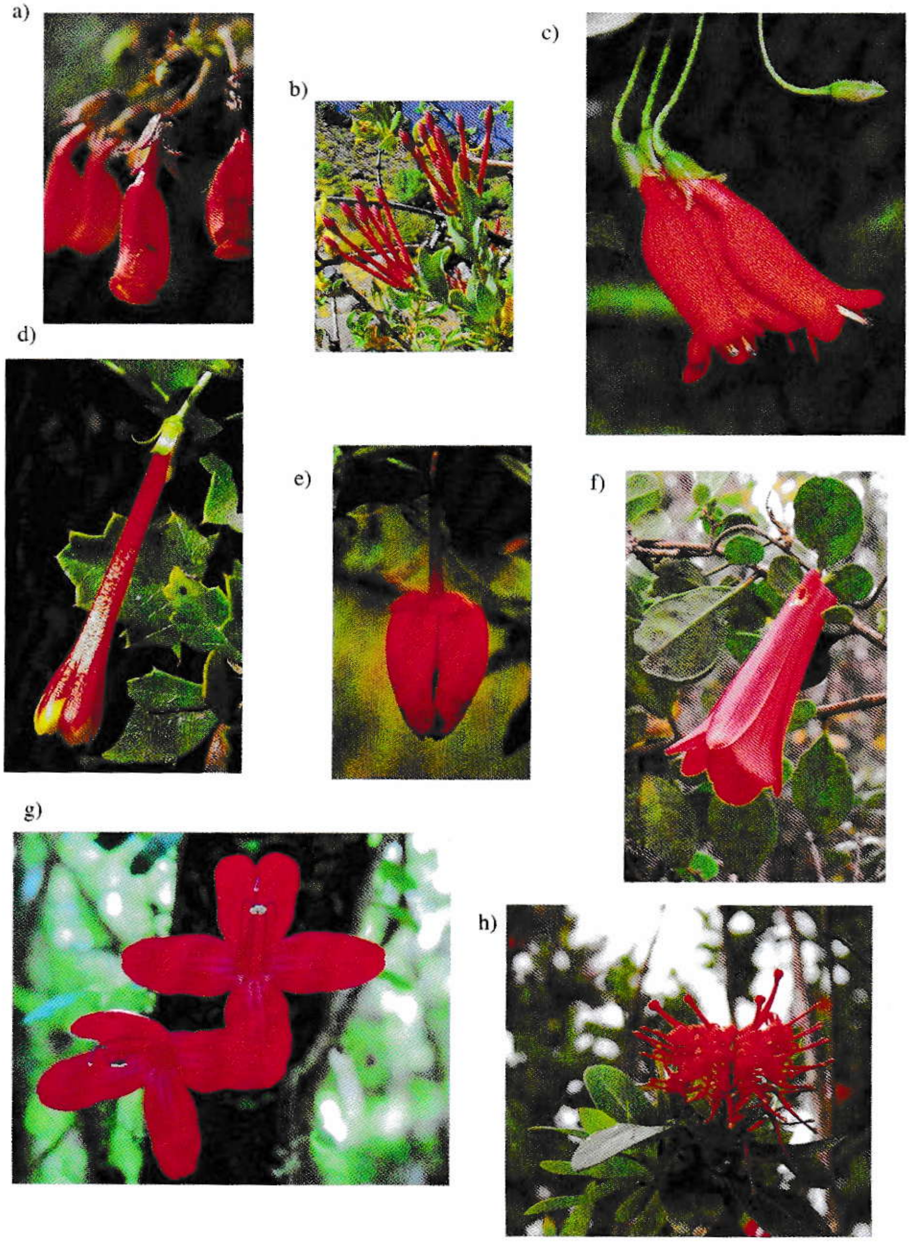
Table 1.

Localities where material of the different plant species considered in this study was collected.

Plant Species	Collect location	Region	Altitude (mts)
Eccremocarpus scaber	Cuesta Barriga	RM	678
Tristerix verticillatus	Camino Farellones	RM	1910
Mitraria coccinea	Niebla	VIX	101
Desfontainia spinosa	Los Ruiles	VII	230
Crinodendron hookeranum	Chiloe	XI	27
Lapageria rosea	Los Ruiles	VII	248
Asteranthera ovata	Chiloe	XI	28
Embothrium coccineum	Niebla	VIX	29

Figure 1

Images of the flowers of the plant species considered in this study; a) *Eccremocarpus scaber* (Photo by Pablo Necochea); b) *Tristerix verticillatus*; c) *Mitraria coccinea* (Photo by Rocio Jaña); d) *Desfontainia spinosa* (Photo by Natalia Marquez); e) *Crinodendron hookeranum* (Photo by Ana Maria Humaña); f) *Lapageria rosea* (Photo by Camila Villavicencio); g) *Asteranthera ovata* (Photo by Fernanda Salinas); h) *Embothrium coccineum* (Photo by Juan Luis Celis).



A.Measurement and categorization of flower reflectance spectra

Intact samples of flowers of each species borne on branches were collected in the field and kept fresh until reflectance spectra measurements were made. The reflectance spectra of the flowers and foliage were measured by means of a fiber-optics spectrometer (model S200; Ocean Optics, Dunedin, FL) between 300 and 700 nm through a data-acquisition input/output card (12-bit 100 ks; DAQCard-700; National instruments, Austin, TX) fitted into a computer. A white reflectance standard (Spectralon, 99%; Labsphere, North Sutton, NH) 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 to 2 cm from the sample allowed measurements of a surface area of 0.1 to 0.4 cm^2. In case the flowers displaying more than one color, as in *Eccremocarpus scaber* and *Desfontainia 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, 400-500 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 plateous) of the slope.

B. Measurements of spectral sensitivity by Electroretinogram recordings

ERG recordings where made for workers of *Bombus dahlbomii* captured in the wild. For the recordings the insect's bodies were immobilized leaving only the head free. The ERG was recorded under photopic conditions by keeping the bee's eye adapted with a white background light. The optical system consisted of a stabilized power supply with a quartz lamp, a monochromator, and 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 into the eye (for more details on the optical system see Chavez et al, 2003). An electronic Shutter set the flash duration and an optical quartz wedge (0-4 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 700 nm in 20-nm steps. The ERG signals were recorded with a pair of Ag/AgCl electrodes, amplified, and low- and high-pass filtered (100 Hz an 300 Hz) with a high-gain amplifier (model DP-301; Warner Instruments, Hamden, CT). Before each experiment, the photon flux emission from the lamp, between 300 to 800 nm, was measured with a calibrated photocell positioned at the eye level (Optometry S370; UDT Instruments, Hawthorne, CA).

The ERG response was evoked by an increasing number of photons per flash (with 1 to 2-seconds intervals between the flashes) at fixed wavelength(s). The response amplitudes were measured between baseline and peak. The amplitude response was normalized by $r/r_{\max} = i/i + \sigma$, where i is the incident photon number at the eye, $r/r_{\max}$ is the normalized response (b-wave), and σ is the half-saturation response. For the spectral sensitivity experiments, the amplitude of the signal was measured from the average response (n = 10-50 trials) to dim flashes, covering the range of 300 to 700 nm. The spectral sensitivity (S_λ) function was measured as $S_\lambda = r_{\text{peak}}/I$, where I is the flash photon flux, and r_{peak} is the maximum peak response for a dim flash.

C. Determination of the spectral properties of flower's red coloration considered for a trichromatic bee.

Using the spectral reflectance curves of flowers red coloration together with the spectral sensitivity curves of *Bombus morio* photoreceptors, receptor-specific and chromatic contrast to their foliage was established. For this purpose receptor quantum catches, Qi, were calculated as

$$Qi = \int_{300}^{700} S_i(\lambda)R_i(\lambda)d\lambda, \qquad \text{Eq. 1}$$

where i denotes the spectral type of receptor (S, M, L), $S_i(\lambda)$ is the spectral sensitivity function of receptor i (Peitsch et al, 1992), and $R_i(\lambda)$ is the reflectance spectrum of the flowers or background color. The receptor-specific contrast of the red pattern to its background was calculated as the quantum catch ratio of the photoreceptor relative to it's green foliage background:

$$qi = Q_i / Q^b{}_i, \qquad \text{Eq. 2}$$

where Q_i and Q^{b}_i denotes the receptor quantum catch for the red pattern and the background respectively. Only the L-receptor contrast has been found to mediate an achromatic visual channel related to a variety of visual tasks (for review see Giurfa and Lehrer 2001). Based on the three receptor-specific contrast, determination of chromatic contrast of the red color in a flower in relation to its green background was undertaken using the receptor noise limited model (RNL model, Vorobyev and Osorio 1998; Vorobyev et al. 2001).

-The receptor noise limited model (RNL).

The RNL model assumes that color is coded by two chromatic (color opponent) mechanisms. The distance in the color space (chromatic distance) was calculated as:

$$\Delta S = \sqrt{\frac{\omega^2_S(\Delta f_L - \Delta f_M)^2 + \omega^2_M(\Delta f_L - \Delta f_S)^2 + \omega^2_L(\Delta f_S - \Delta f_M)^2}{(\omega_S\omega_M)^2 + (\omega_S\omega_L)^2 + (\omega_M\omega_L)^2}} \qquad \text{Eq. 3}$$

where ω_i denotes the standard deviation of the noise in the receptor i=S,M,L, $f_i = \mathrm{Ln}(q_i)$ is the receptor-specific and Δf_i the difference in receptor signals between two stimuli, in this case between the flower's color and the green background. The ω_i values were obtained from electrophysiological recordings in single photoreceptor cells (Vorobyev et al. 2001). According to this estimate $\omega_s=0.13$, $\omega_M=0.06$, $\omega_L=0.12$.

Comparisons of behavioral threshold with the RN model predictions indicates that in most cases the stimuli are not discriminable if $\Delta S<2.3$ (Vorobyev et al. 2001). A two-dimensional color opponent diagram corresponding to RN model can be obtained by considering a plane, whose co-ordinates are related to receptor signals *fi* by

$$X = A\,(f_L - f_M) \qquad Y = B\,(f_S - (a\,f_L + b\,f_M)),$$

where
$$A = \sqrt{\frac{1}{\omega^2_M+\omega^2_L}}, \qquad B = \sqrt{\frac{\omega^2_M+\omega^2_L}{(\omega_S\omega_M)^2+(\omega_S\omega_L)^2+(\omega_M\omega_L)^2}},$$

$$a = \frac{\omega^2_M}{\omega^2_M+\omega^2_L}, \qquad b = \frac{\omega^2_L}{\omega^2_M+\omega^2_L}$$

Euclidean distance in this *Y*- *X* plane is equal to that given by Eq. 3.

D. Behavioral evaluation of the role of the chromatic and achromatic contrast in the discrimination of pure red reflecting colored targets by Bombus dahlbomii.

-Experimental set-up and procedure

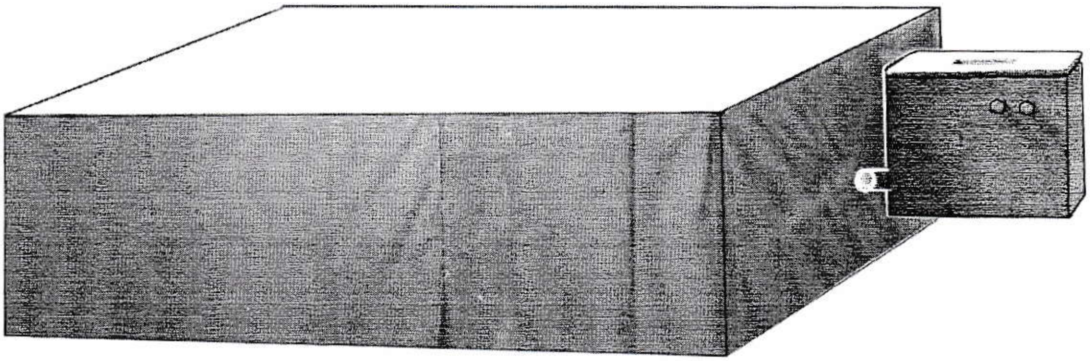
A colony of *Bombus dahlbomii* was collected in Farellones, RM, 2200 m.a.s.l., and transferred directly to the laboratory for the behavioral experiments. We used an experimental area of 120cmx120cmx40 cm (Fig. 2), illuminated with natural light and an artificial standard white light. This arena was connected to a nest-box which contained the colony through a clear plastic tunnel that could be selectively closed. Workers were trained to enter the arena to collect a sucrose solution from circular pure red reflecting discs, 10 cm diameter, located flat on the arena floor, using green a the background color.

In a first experiment a total of four trained workers where marked and exposed to a situation where they had to choose between the training stimuli/background configuration (Tr) and an alternative one that was equivalent with respect to Tr at the chromatic or achromatic level.

In a second experiment the same four trained workers were able to choose between Tr and three color configurations chromatically equivalent to the background, but with different levels of L-receptor contrast respect to Tr. In both experiments, only Tr was rewarded. Bee's trials in the arena were video taped to later analyze the bees choices. After each trial the colored targets were cleaned with alcohol to eliminate any possible odor cue left by the bees.

Figure 2.

Schematic drawing of basic setup for laboratory behavioral experiments reported here. A nestbox, which contained the bee colony, was connected to a flight arena via a clear tunnel. The arena contained the color stimuli, which were positioned flat on the arena floor. The color stimuli were plastic circles of 10 cm diameter. Figure design courtesy of N. Marquez, U. de Chile.



-Stimuli design.

The colors used for constructing the training and experimental stimuli/background configurations were colored papers bought in the local market combined with neutral filters of variable optical densities. The spectral reflectance curves of the different colors (Fig. 3) and the spectral properties of the different color configurations were obtained as described above for the flower patterns (Table 2, Fig 4). The colors and filters were chosen to independently evaluate the role of the chromatic and achromatic contrast in the discrimination of red colored targets by *Bombus dahlbomii*. In the first experiment the training configuration (Tr) consisted of a red target over a green background (Red/background 1); this configuration yielded a negative achromatic contrast value of 0.25 and a chromatic contrast value of 11.85. The alternative configuration (Alt. 1) was a blue target over a green background (Blue/background1); this configuration was different both at the achromatic and chromatic levels with contrast values of 0.72 and 13.2 respectively. Alt. 2 consisted in a dark green target chromatically different respect to Tr, with a contrast value of 1.06 (Dark-green 1/background 1) and equivalent at the achromatic level respect to Tr with a contrast value of 0.3. The Alt. 3 configuration consisted of the same red color used for Tr but against a dark green background (Red/background 2); this configuration was chromatically equivalent respect to Tr, but different at the achromatic level with a L-receptor contrast value of 1.0.

In the second experiment we evaluated the correct choices for Tr when tested against Alt. 2 (Green 1/background 1), Alt. 4 (Green 2/background 1) and Alt 5 (Green 3/ background 1). All of these stimuli are chromatically equivalent to the background but with variable levels of achromatic contrast respect to Tr. Alt. 2, as mentioned above, is quite similar to Tr on its L-receptor contrast. Alt. 4 has a chromatic contrast of 0.71 and an achromatic contrast of 0.62. Alt. 5 has a chromatic contrast of 0.61 and an achromatic contrast of 0.75.

-Statistics

The first and total number 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).

Figure 3.

Spectral reflectance curves of the colors used in the behavioral experiments: **a)** colors used in the first experiment; **b)** colors used in the second experiments.

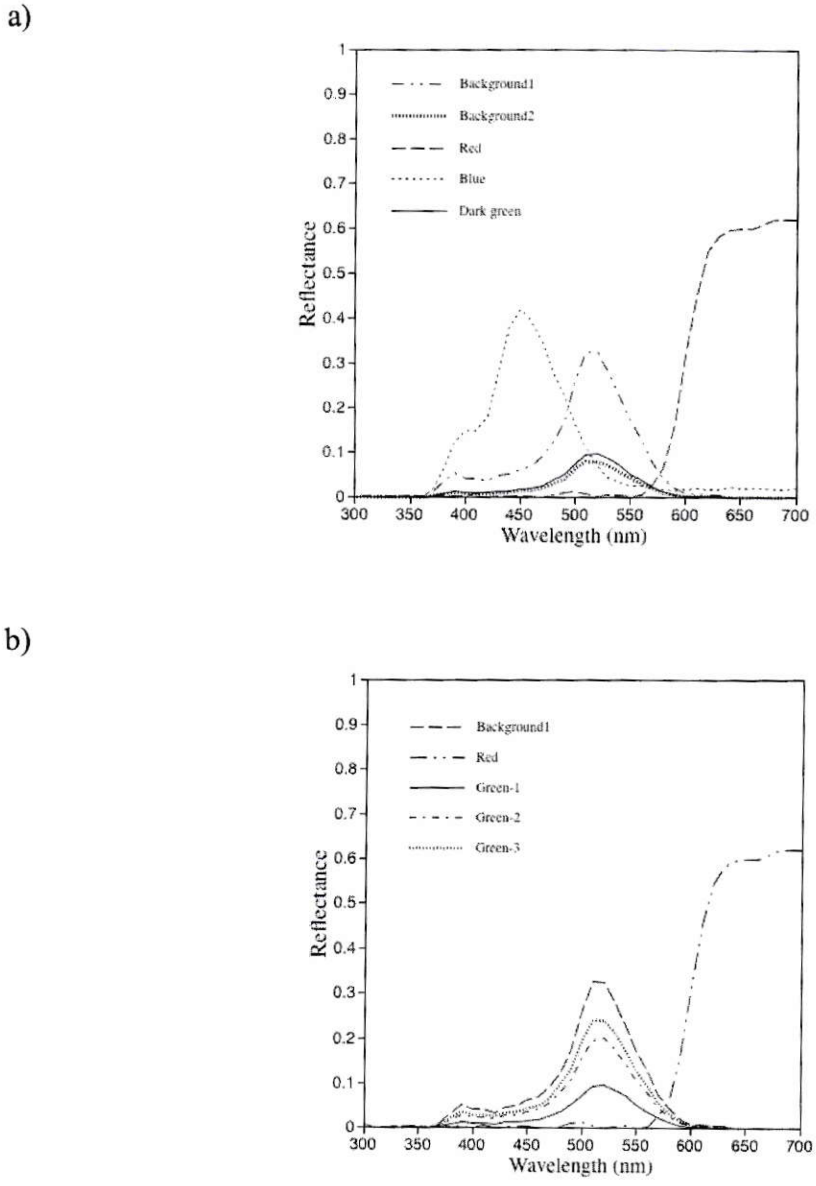


Figure 4.

Loci of different color configurations chosen for the experiments in the color diagram representing bee's color space. From the relative quantum catch of each photoreceptor the chromatic coordinates and distances between stimuli in the color space has been calculated for each color according to the Receptor Noise Limited model (RN model, Vorobyev et al. 1998, 2001; see Table1). The unity distance corresponds to one standard deviation of the noise. The color locus of the background is by definition at origin of the color diagram (0, 0). *Inset*: L-receptor contrast values of the training and alternative configurations used in the experiments.

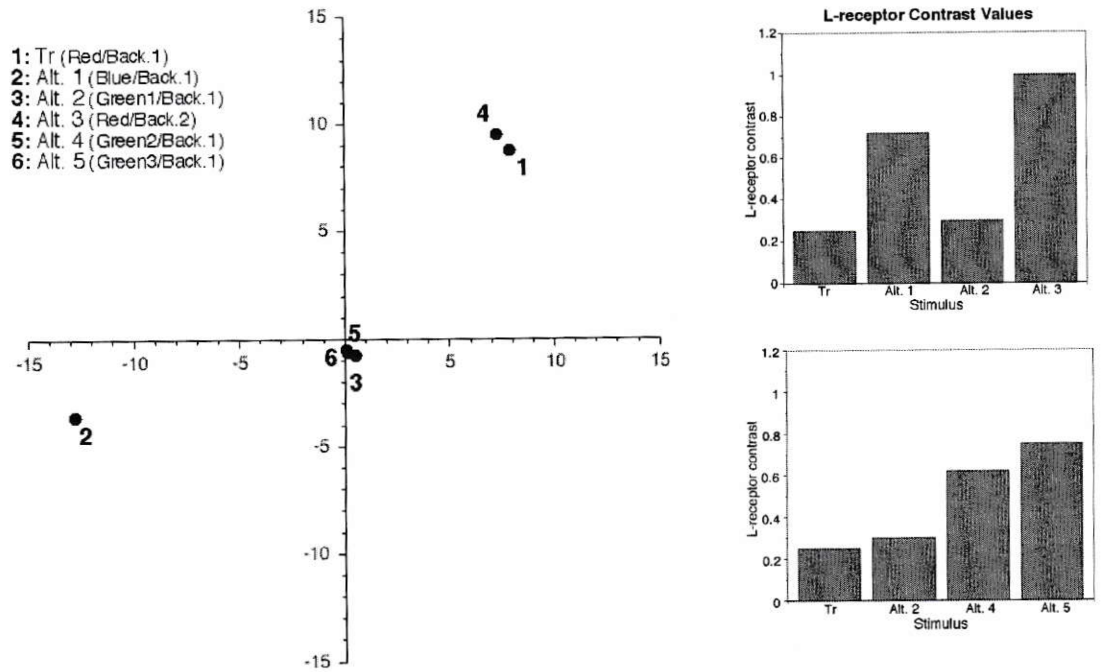


Table 2.

Spectral properties of the color configurations used in the behavioral experiments. 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 et al. 1998, 2001) and are given in standard units. Colors are not discriminable for bees if ΔS<2.3

Chromatic configuration	Chromatic distance (ΔS) to the background	Receptor specific contrast to the background		
		S	M	L
Tr (Red/Back.1)	11.85	0.35	0.08	0.25
Alt.1 (Blue/Back.1)	13.2	1.69	3.95	0.72
Alt.2 (Green-1/Back.1)	1.06	0.25	0.27	0.3
Alt.3 (Red/Back.2)	12.01	1.72	0.37	1.0
Alt.4 (Green-2/Back.1)	0.71	0.54	0.6	0.62
Alt.5 (Green-3/Back.1)	0.61	0.68	0.73	0.75

E. Evaluation of "Bombus dahlbomii" innate color preference.

-Experimental set-up and procedure

For this experiments we used color naïve *Bombus dahlbomii* workers from colonies reared under laboratory conditions into nest-boxes with pollen and sucrose solution ad-libhitum. The experiments were done with the same set up describe above. Bees were trained to collect sucrose solution in transparent 10 cm diameter discs located flat over a green background. After training bees were offered five spectrally different colored targets over the same green background used in the training procedure (Fig 5). Only one bee at a time was allowed to enter the arena for the experiments and each bee was tested only on their first bout into the arena. Bee trials into the arena were recorded to analyze bee's first and total number of choices for each of the different color offered. After each trial the colored targets were cleaned up with alcohol to eliminate any possible odor cue left by the bees.

-Stimuli

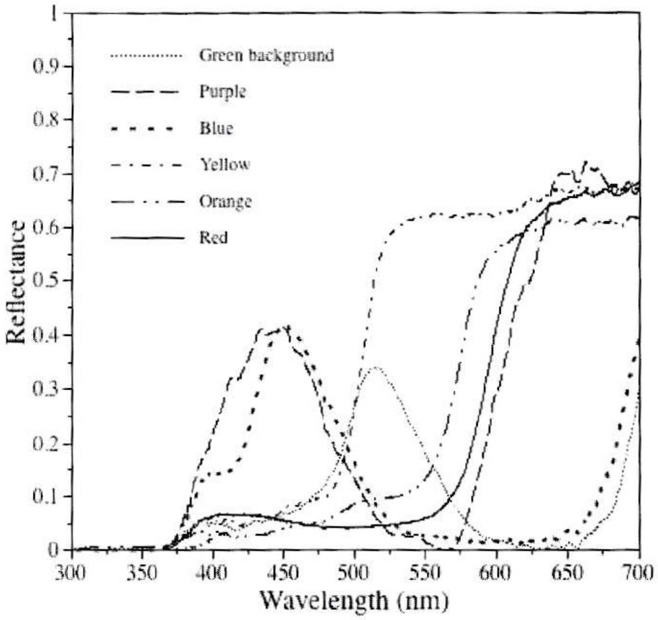
The five spectrally different colors used to evaluate *B. dahlbomii's* innate preference were presented simultaneously over a green background and were chosen to cover the range from blue to red. The spectral reflectance curves of the colors used are given in figure 5.

-Statistics.

The colors of the first and total targets visited were compared with expected random visits using a G-test for goodness of fit.

Figure 5.

Spectral reflectance curves of the target and background colors used in the innate preference experiments.



Results.

A. Measurement and categorization of flower reflectance spectra

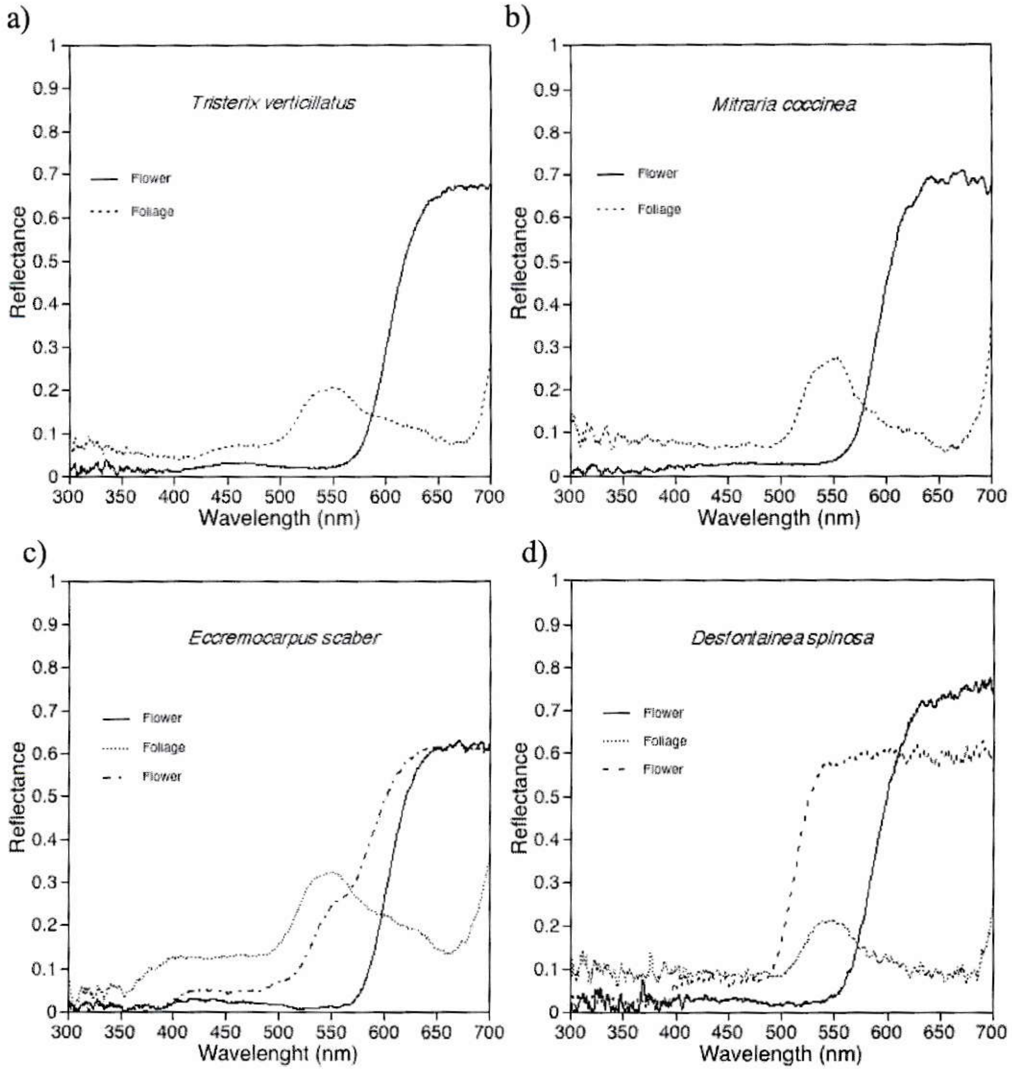
The spectral reflectance curves allow distinguishing two main categories of flowers:

A1. Pure red reflecting type.

This type of reflectance curve characterized *Tristerix verticillatus* and *Mitraria coccinea* with uniformly human red looking flowers (Fig 6 a,b), and *Eccremocarpus scaber* (Fig 6 c) and *Desfontainia spinosa* (Fig 6 d) having red flowers combined with pure orange or pure yellow patterns. All these red flowers absorb light strongly between 300 and 590 nm. There is a sharp step at around 600, whence all light above this step is reflected.

Figure 6.

Spectral reflectance curves of flowers classified a pure red reflecting flowers. a) *Tristerix verticillatus*; b) *Mitraria coccinea*; c) *Eccremocarpus scaber*; d) *Desfontainia spinosa.*

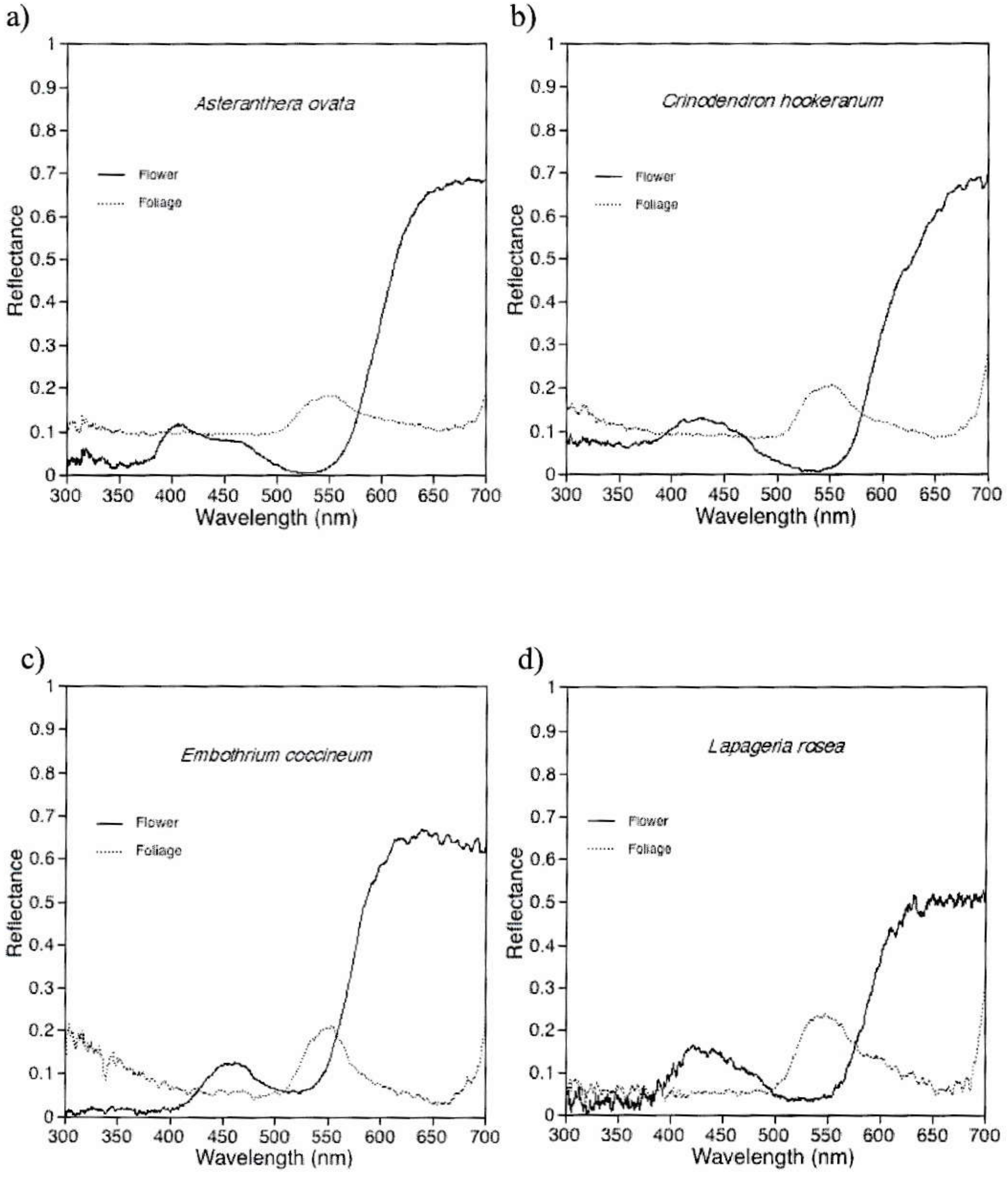


A2. Blue/red reflecting type.

The flowers in this category are characterized by a small peak in the blue part of the spectrum and a sharp step at around 600 nm, after which all light is reflected. Flowers in this category vary with respect to the amount of reflectance in the UV and green parts of the spectrum. The flowers of *Asteranthera ovata* and *Lapageria 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 and disappears at around 480 nm and a sharp step at around 595 nm with reflectance over the entire range above this step (Fig 7 a,d). The flowers of *Crinodendron hookeranum* show a peak in the blue part of the spectrum that occurs at 400 nm and disappears at 480 nm, moderate reflectance in the UV and green parts of the spectrum and sharp step at around 600 nm, with reflectance over the entire long wavelength part of the spectrum (Fig 7 b). *Embothrium coccineum* absorb UV, has a small peak in the blue part of the spectrum that appears at 430 nm to disappear at around 490 nm, reflects at the green part of the spectrum and shows a sharp step at around 570 nm reflecting all light above this step (Fig 7 c). All flowers in this category are unicoloured.

Figure 7.

Spectral reflectance curves of flowers classified as blue-red reflecting flowers. a) *Asteranthera ovata*; b) *Crinodendron hookeranum*; c) *Embothrium coccineum*; d) *Lapageria rosea*.

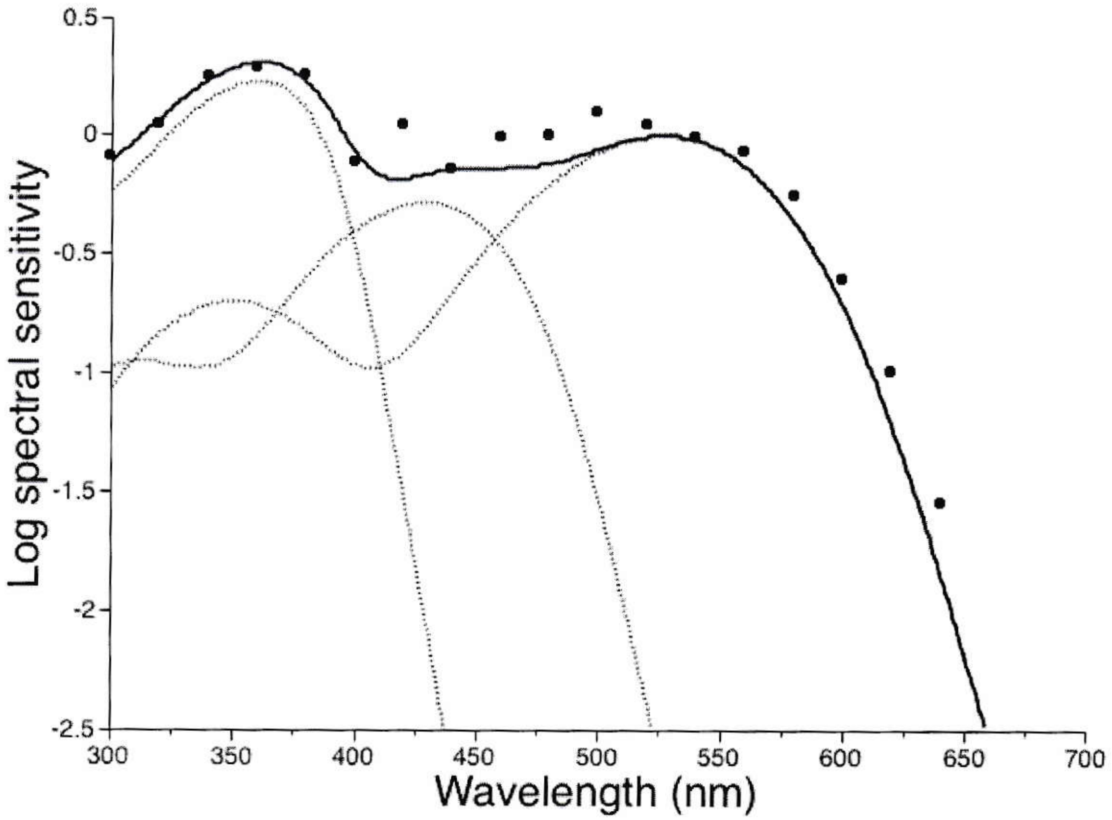


B. Measurements of spectral sensitivity by Electroretinogram recordings

Figure 8 shows the spectral sensitivity function for *B. dahlbomii*'s (n=6vv) obtained with a white light background of photopic intensity. *B. dahlbomii*'s photopic spectral sensitivity function extends from 300 to 640 nm. The spectral sensitivity function shows a clear peak at the UV part of the spectrum, with a maximal value at 360 nm. The sensitivity at the blue and green parts of the spectrum does not show a clear separation between the two spectrum domains, showing sensitivity peaks at around 420 nm and 510 nm. These results do not unreveal an extended sensitivity to the long wavelengths by *Bombus dahlbomii*. They match other results obtained for trichromatic bees through the same methods (Goldsmith 1960, 1958; Menzel 1971).

Figure 8.

Spectral sensitivity curve for *Bombus dahlbomii* measured through ERG recordings. The intensity-response function was obtained with a white light background of photopic intensity.



C. Determination of red pattern's spectral properties.

Different levels of chromatic and achromatic contrast were predicted for pure red and blue/red flowers (Table 3). In the case of blue/red reflecting flowers, all show an important chromatic component, yielding ΔS values above the threshold. *E. coccineum*, which yielded a ΔS value of 6.29, is the specie with the lowest L-receptor contrast, with a value of 1.14. For *A. ovata*, *C. hookeranum* and *L. rosea*, also with blue/red reflecting flowers, the evaluation gave ΔS values above the threshold, but with L-receptor contrasts values of 0.51, 0.5 and 0.7 respectively.

For the pure red reflecting flowers, *E. scaber* and *T. verticillatus* fall into an uncolored category. *E. scaber* yielded a ΔS value of 1.52 while *T. verticillatus* yields a ΔS value of 0.36. At the achromatic level, these flowers show high negative L-receptor contrast to the background with values of 0.28 for *E. scaber* and 0.35 for *T. verticillatus*. The remaining two species of pure red reflecting flowers, *D. spinosa* and *M. coccinea*, yielded ΔS values above the chromatic discriminable threshold. *D. spinosa* yielded ΔS values of 2.58 and an L-receptor contras of 0.61, while the corresponding values for *M. coccinea* were 2.98 and 0.51, respectively.

The loci occupied by the red coloration of each flower may be represented in a diagram of bee's color space (Fig 9). This illustrates that that pure red flowers occupy a different loci with respect to blue/red flowers. Two of the pure red reflecting flowers fall into an uncoloured region in bee's color space, while blue/red reflecting flowers tend to be located further from the center of the diagram, which represents the locus occupy by the background.

Table 3.

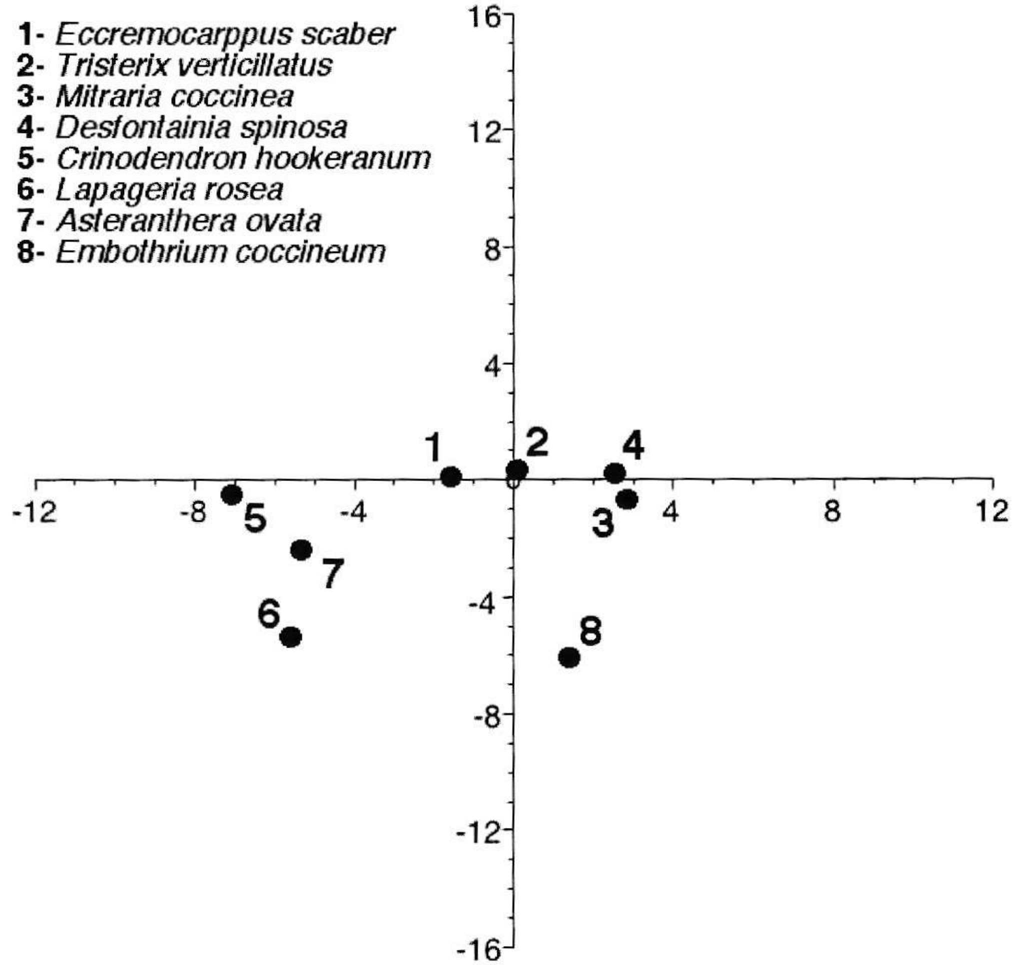
Spectral properties of red patterns from eight plants species with respect to their green foliage background.

Plant Species	Chromatic distance (ΔS) to the foliage background	Sing of the L-receptor contrast	Receptor-specific contrast		
			S	M	L
Eccremocarpus scaber	1.52	(-)	0.34	0.35	0.28
Tristerix verticillatus	0.36	(-)	0.36	0.35	0.35
Mitraria coccinea	2.98	(-)	0.34	0.35	0.51
Desfontainia spinosa	2.58	(-)	0.47	0.43	0.61
Crinodendron hookeranum	7.04	(-)	0.99	1.28	0.5
Lapageria rosea	7.76	(-)	0.59	1.47	0.7
Asteranthera ovata	5.84	(-)	0.64	1.03	0.51
Embothrium coccineum	6.29	(+)	0.41	0.94	1.14

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.

Figure 9.

Loci of a flower's red coloration pattern for eight different plant species in the color diagram representing the bee's color space. From the relative quantum catch of each photoreceptor the chromatic coordinates and distances between stimuli in the color spaces has been calculated for each color according to the Receptor Noise Limited model of honeybee color vision (RN model, Vorobyev et al. 1998, 2001; see table1). The unity distance corresponds to one standard deviation of the noise. The color locus of the background is by definition at origin of the color diagram (0, 0).



D. Behavioral evaluation of the role of the chromatic and achromatic contrast in the discrimination of pure red reflecting colored targets by "Bombus dahlbomii".

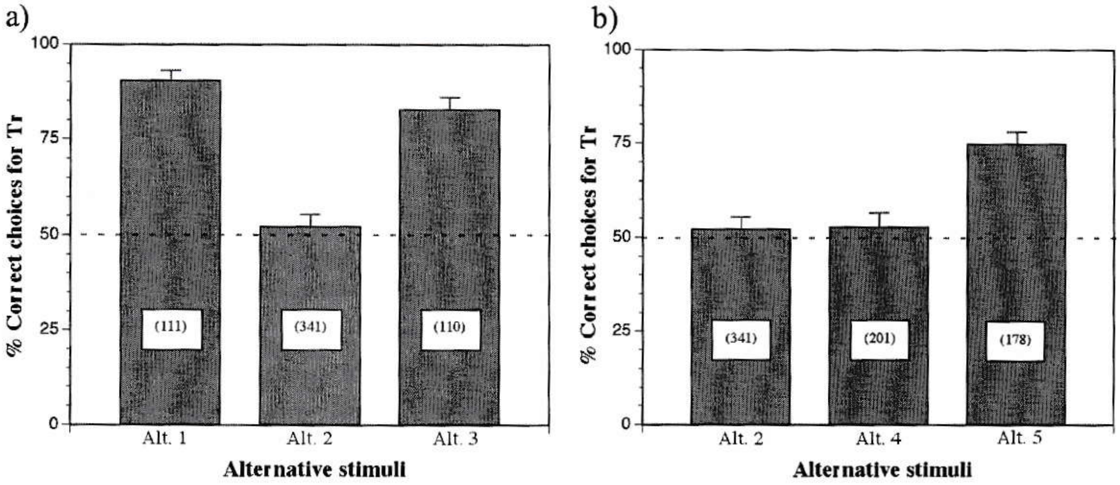
When bees were tested to choose between Tr and Alt1, the latter of which was chromatically and achromatically different than Tr, bees correctly chose the rewarded Tr configuration (Fig. 10 a). When Tr was presented along with Alt2, the bees randomly selected the two stimuli. Note that Tr and Alt2 had similar L-receptor contrast but were different chromatically. When Tr was tested against Alt3, which was equivalent chromatically but different at the L-receptor contrast level respect to Tr, bees correctly chose the Tr configuration. These results suggest that under our experimental conditions *Bombus dahlbomii* detected and discriminated the red targets over the green background based on achromatic differences induce on the L-receptor type.

In the second experiment we compared the correct choices for Tr when presented against Alt. 2, Alt. 4 and Alt. 5 (Fig. 10 b). As we mentioned above when Tr was tested against Alt 2 bees chose randomly between them. When Tr was tested against Alt. 4, bees also chose randomly between stimuli, even though Alt. 4 had a L- receptor contrast value of 0.62 compared to 0.25 showed for Tr. When Tr was tested against Alt. 5 however, which had a L-receptor contrast of 0.75, bees chose correctly the Tr configuration. According to this result the native bumblebee perceives colored targets with negative levels of achromatic contrast as equivalent within a wide range of L-receptor contrast. Given the contrast values shown by Alt. 4 and Alt. 5, our results suggest an achromatic discrimination threshold that lies between 0.62 and 0.75.

The results from first and total number of choices were statistically indistinguishable. Thus in Figure 10 only the total number of choices for each color were plotted.

Figure 10.

Results from Experiment 1 (a) and Experiment 2 (b): percentages of choices for the trained pure red stimuli (Tr) (mean ± SE) as function of the alternative stimulus. The broken line at 50% indicates random choice level. Values in parenthesis indicate the total number of choices recorded in each test situation: Tr vs. alternative 1: G=52; P<0.05; Tr vs. alternative 2: G=1.7; NS; Tr vs. alternative 3: G=37; p<0.05; Tr vs. Alternative 4: G= 0.04; NS; Tr vs alternative 5 : G= 27; P<0.001; n=4 bees.

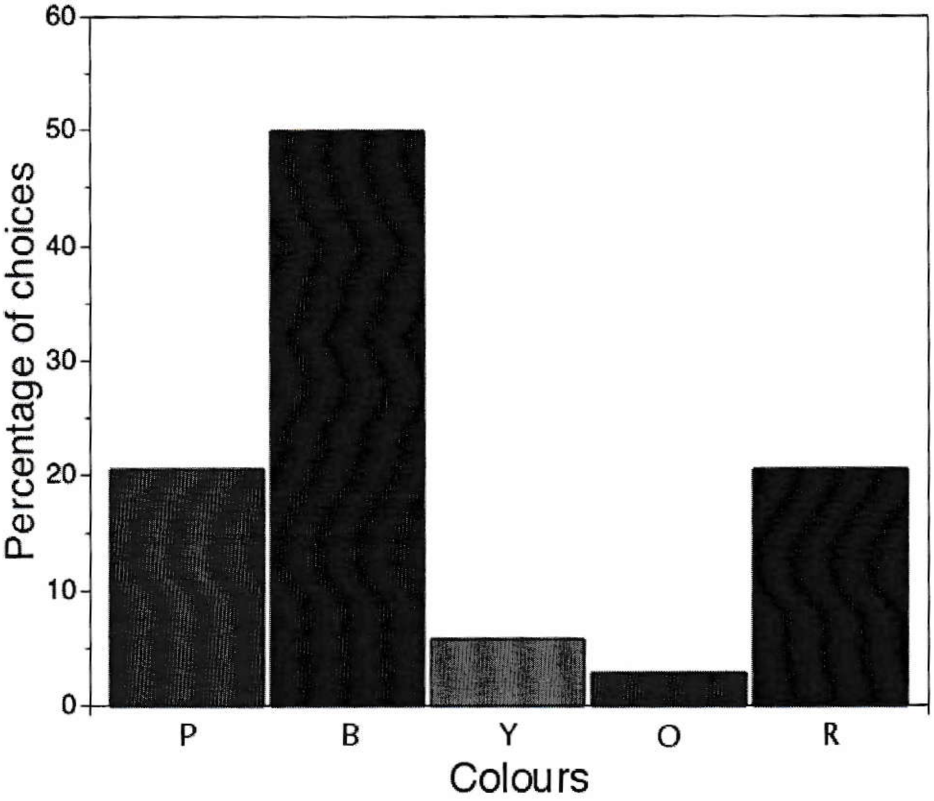


D. Evaluation of "Bombus dahlbomii" innate color preference.

From the five colors offered, naïve workers of *Bombus dahlbomii* showed a strong innate preference for blue and a secondary preference for purple and red (Figure 11). The distribution of colors chosen by the bees differed significantly from random, for both the first (G= 22,9; P< 0,001; n=34) and total (G= 70,6; P< 0,001) number of choices for each color. Results from the first choice showed that blue targets received 50% of the total choices while the red and purple targets received around 20% each.

Figure 11.

Percentage of firsts choices of color naïve *Bombus dahlbomii* workers on different colored targets from a total of 34 trials. The distribution of colors chosen by the bees differed significantly from random (G= 22,9; P< 0,001; n=34)



Discussion

The eight ornithophyllous red-flowered species derived from seven plant families revealed a diversity of situations with respect to their bee specific coloration. Half of the species evaluated (*Crinodendron hookeranum, Lapageria rosea, Asteranthera ovata* and *Embothrium coccineum*), representing four plant families, have sufficient spectral reflection in the blue part of the spectrum to be useful for bees color discrimination. Therefore, even if reflection above 650 nm per se is invisible for bees, these flowers could be discriminated by the bees based on their color vision. The other species studied (*Eccremocarpus scaber, Tristerix verticillatus, Mitraria coccinea* and *Desfontainia spinosa*) representing four families, show reflection exclusively in the long wavelength part of the spectrum, above 650 nm, raising the question as to whether *Bombus dahlbomii* might see this part of the spectrum and use it for color discrimination. Although this possibility cannot be disregard from our results, bee's color vision has been shown to be rather conservative at the receptor level. In all *Bombus* species studied so far, none has revealed a long wave receptor beyond the usual green receptor with λmax at 540 nm and a half band width of about 110 nm (Peitsch et al. 1992, Skorupski et al 2007). Our results based on ERG recordings did not unravel an extended sensitivity to long wavelengths, suggesting that *B. dahlbomii* probably perceives colors through the same mechanisms as most bees evaluated so far.

With respect to the spectral properties of flowers, considered for a trichromatic bee, differences at the chromatic and achromatic levels were found among the plant species studied. At the achromatic level, differences on the L-receptor contrast were seen both in the pure red and blue/red reflecting flowers. Interestingly, the pure red flowers gave the highest negative contrast for L-receptor, opening up the possibility that achromatic contrast might be involved in the detection of these flowers by bees. At the chromatic level, highest contrast values were found among blue/red reflecting flowers, with values above the discrimination threshold in all of these flowers. Among them, *E. coccineum* showed the lowest L-receptor contrast with a value very close to 1, equivalent to no contrast at the L-receptor. These results interesting considering the great diversity of insect visitors reported for this specie, which include a total of five hymenopteran species (Smith-Ramirez 2005, Rovere et al. 2006), suggesting that the blue component found in these flowers may play a role in their chromatic detection by trichromatic insects.

Pure red-reflecting flowers, on the other hand, can be divided into those with ΔS values below the chromatic discrimination threshold (*T. verticillatus* and *E. scaber*) and those with ΔS values above the threshold (*D. spinosa* and *M. coccinea*). The chromatic contrast values below the threshold obtained for *T. verticillatus* and *E. scaber* are in agreement with Chittka (1994), who speculated that pure red-reflecting flowers may fall into an uncolored region in bees color space. Our results situate *D. spinosa* and *M. coccinea* into a chromatic discriminable region in bee's color space, with ΔS values above threshold, but as we argued above, a trichromatic bee could detect these red flowers over a green foliage background through an achromatic mechanism.

The behavioral evaluation carried out in *Bombus dalhbomii* supports the above notion. Our experiments (Fig. 10 a) showed that *Bombus dalhbomii* workers did not discriminate Tr from Alt. 2, which is chromatically different with respect to Tr but equivalent at the achromatic level. However, they did discriminate between Tr and Alt. 3, where the latter is chromatically equivalent with respect to Tr but different at the L-receptor contrast level. These results suggest that the native bumblebee detects and discriminates pure red reflecting flowers over a green foliage background based on achromatic differences induced on the L-receptor type. The levels of achromatic contrast shown by Tr and Alt. 2 on the other hand, are comparable to the levels shown by some of the pure red flowers evaluated. But, because of the lack of data on the minimal level of achromatic contrast required by a bee to achromatically discriminate a stimuli, lower levels of L-receptor contrast, as the one shown by *D. spinosa*, might be harder to detect for the native bumblebee. The results from the second experiment helps to clarify this point (Fig. 10 b), showing again that, bees were not able to discriminate Tr from Alt. 2 but interestingly neither from Alt. 4, which had an achromatic contrast value of 0.62 compare to 0.21 and 0.3 showed by Tr and Alt. 2 respectively. However, bees did discriminate Tr from Alt. 5, which had a L-receptor contrast value of 0.75. The wide range of achromatic contrast that was perceive as equivalent by the native bumblebee in experiment 2, shows that under our experimental condition bee's achromatic L-receptor channel was quite insensitive to mild differences in the contrast level. The correct discrimination of Tr from Alt. 5 on the other hand, suggests that the minimal level of L-receptor contrast required by the native bumblebee to discriminate a red colored target, or to discriminate the alternative stimuli as different from Tr, probably lies between 0.62 and 0.75, in correspondence with the L-receptor contrast values of Alt. 4 and Alt. 5 respectively. These results allow us to speculate that a pure red flower with an L-receptor contrast value as that shown by *D. spinosa* could be discriminated by *B. dahlbomii* achromatically.

Raven's (1972) proposed that red coloration was acquired in hummingbird-pollinated plants as an adaptation for excluding bees. Based on Spaethe et al's (2001) observation that bees take a longer time to detect red targets in relation to those of other colors, Rodríguez-Gironés & 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 (Kevan, 1983; Vickery, 1992; Daumer, 1958, Menzel and Shmida, 1993), including for the four new species studied here, that bees are capable of actively detecting and visiting pure red flowers, very probably, as we showed here, by their high levels of achromatic contrast. Furthermore, the abundance of red flowers in the SATF seems to have a positive impact over the innate preference for colors of *B. dahlbomii,* which, as in the case of other *Bombus* species reported in the literature (Chittka et al. 2001), showed a secondary preference for red. Our results on the visual mechanisms involve in the discrimination of red allow us to speculate that this preference would be for the negative achromatic contrast that a red target subtends against a green background.

To fully understand relationship between red flowers and their visitors it is essential to consider the historical context in which such flowers and their pollinator relationships evolved. Hummingbirds (Apodiformes, Trochilidae) first appeared in the late Paleocene (58.5 Mya) (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 only distributed today. The genus *Bombus* originated in the late Eocene to early Oligocene (around 30 Mya). Historical biogeographic assessments reveal an Old World origin followed by multiple dispersal events into the New World occurring after 21 Mya, with movements into South America estimated after 10 Mya, in concordance with the formation of the Panamanian land bridge (Hines and Cameron 2006). 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 bumble bees, 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's (1997) on the need to lay to rest the notion that red coloration evolved as 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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