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ORIGINAL PAPER

Evidence of red sensitive photoreceptors in *Pygopleurus israelitus* (Glaphyridae: Coleoptera) and its implications for beetle pollination in the southeast Mediterranean

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Abstract A very well-documented case of flower-beetle interaction is the association in the Mediterranean region between red bowl-shaped flowers and beetles of the family Glaphyridae. The present study examines the visual mechanisms by which *Pygopleurus israelitus* (Glaphyridae: Scarabaeoidea: Coleoptera) would perceive the colors of flowers they visit by characterizing the spectral sensitivity of its photoreceptors. Our measurements revealed the presence of three types of photoreceptors, maximally sensitive in the UV, green and red areas of the spectrum. Using color vision space diagrams, we calculated the distribution of beetle-visited flower colors in the glaphyrid and honeybee color space and evaluated whether chromatic discrimination differs between the two types of pollinators. Respective color loci in the beetle color space are located on one side of the locus for green foliage background, whereas in the honeybee the flower color loci surround the locus occupied by green foliage. Our results represent the first evidence of a red sensitive photoreceptor in a flower-visiting coleopteran species, highlighting Glaphyridae as an interesting model group to study the role of pollinators in flower color evolution.

Keywords Coleoptera · Pollination · Color vision · Flower colors · Color space

J. Martínez-Harms (✉) · J. Schorn · R. Menzel
FU Biologie, Institut für Biologie-Neurobiologie,
Freie Universität Berlin, Königin-Luise Str. 28/30,
14195 Berlin, Germany
e-mail: j.martinez-harms@fu-berlin.de

M. Vorobyev
Department of Optometry and Vision Science,
University of Auckland, Private Bag 92019,
Auckland 1142, New Zealand

A. Shmida
Department of Ecology, Evolution and Behavior,
Center for the Study of Rationality,
The Hebrew University, 91904 Jerusalem, Israel

T. Keasar
Department of Biology and Environment,
University of Haifa-Oranim, 36006 Tivon, Israel

U. Homberg · F. Schmeling
Fachbereich Biologie-Neurobiologie/Ethologie,
Philipps-Universität Marburg, Karl von Frisch Str. 8,
35032 Marburg, Germany

Introduction

According to the concept of pollination syndromes, combinations of floral traits reflect specialization to certain type of pollinators. Along this idea, some authors have proposed that the tendency of pollination systems to specialize (Stebbins 1970; Crepet 1983, 1984) leads to tight co-evolution between plants and pollinators (Gilbert and Raven 1975). Floral colors play a key role in flower/pollinator interactions and under a scenario of mutual specialization, some authors have speculated on the potential for adaptation of pollinators' color vision to optimize flower detection (Chittka 1996; Vorobyev and Menzel 1999). Despite the fact that the color vision of the two most extensively studied insect pollinator groups, hymenopterans and lepidopterans, allows good discrimination of a wide range of colors, systematic studies have not revealed clear trends of spectral tuning of their color vision to the spectral properties of the flowers they visit (Chittka and Menzel 1992; Vorobyev and Menzel 1999; Briscoe and Chittka 2001; Vorobyev et al. 2001a, b; Stavenga and Arikawa 2006). Regardless of the lifestyle of the particular species,

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most hymenopteran species evaluated so far have three types of photoreceptors with spectral sensitivities very similar to those found in honeybees (sensitivity peaks at 340, 440 and 540 nm, respectively) (Menzel and Blakers 1976; Peitsch et al. 1992; Briscoe and Chittka 2001; Skorupski et al. 2007). Lepidopterans, on the other hand, have additional photoreceptors covering a broader range of spectral sensitivities (Briscoe 2002; Stavenga and Arikawa 2006)—a diversity that has been related to intraspecific communication rather than to their role as pollinators (Arikawa et al. 2005; Stavenga and Arikawa 2011).

While there is a large amount of data available on color vision in Hymenoptera and Lepidoptera, relatively little is known about spectral receptor types and color vision in other insect pollinator groups (Menzel 1979; Briscoe and Chittka 2001; Kelber et al. 2003). Coleopterans represent an extraordinarily diverse insect group known to act as predominant pollinators of a large number of angiosperms (Bernhardt 2000). Phylogenetic studies in Coleoptera have resulted in the classification of 4 suborders, 17 superfamilies and 168 families. Species belonging to 11 of these families are known to act as flower visitors (van der Pijl 1960; Gottsberger 1989; Dafni et al. 1990; Hawkeswood 1990; Correira et al. 1993; Englund 1993; Singer and Cocucci 1997; Gibernau et al. 1999; Sakai and Inoue 1999; Sakai et al. 1999; Mawdsley 2003; Thien et al. 2009). The likely interaction between beetles and flowering plants very early in the history of angiosperms (Grant 1950; van der Pijl 1960; Thien et al. 2009) has long influenced the use of beetle-pollinated flowers as model systems for studies on the origin and evolution of angiosperms (Bernhardt and Thien 1987; Endress 1987; Takhtajan 1991). Despite the importance attributed to beetle pollination, regarded as one of the earliest modes of floral specialization (Bernhardt 2000), information on color vision in coleopterans is in general rather limited and in the case of beetle pollinators is restricted to only few dichromate scarab species (Scarabaeoidea: Coleoptera). Representatives of well-separated lineages of Coleoptera have been evaluated with respect to their spectral sensitivity with results indicating that differences in the receptor-based color vision between members of this group do exist. Studies on coleopteran color vision have revealed three different types of spectral sensitivity. In two species, *Carabus nemoralis* and *C. auratus* (Carabidae: Geadephaga: Coleoptera), electroretinographic (ERG) recordings suggest a tetrachromatic color vision with photoreceptors maximally sensitive to UV, blue, green and red (Hasselmann 1962). Trichromacy with photoreceptors having sensitivity peaks in the UV, blue and green range of the spectrum, considered as the basal condition among insects (Chittka 1996; Briscoe 2000; Briscoe and Chittka 2001; Spaethe and Briscoe 2004), has been reported for species belonging to 3 different families (Lall et al. 1982; Lin 1993; Doring and Skorupski 2007). The third group, which contains the only four species having flower-visiting habits studied so far, corresponds to dichromatic species with photoreceptors maximally sensitive to UV and green reported in members of 6 different families of Coleoptera (Gribakin 1981; Warrant and McIntyre 1990; Lin and Wu 1992; Jackowska et al. 2007; Lall et al. 2010; Maksimovic et al. 2011). Consistent with the lack of sensitivity to blue light in dichromatic beetles, the only coleopteran species for which the genome has been sequenced, *Tribolium castaneum* (Tenebrionidae: Coleoptera) shows the absence of a blue opsin within its genome (Richards et al. 2008).

Pollination by beetles has classically been thought to be guided by scent rather than by color (van der Pijl 1960; Fægri and van der Pijl 1979; Bernhardt 2000). Although many beetle taxa do appear to depend on odor to reach flowers (Pellmyr and Patt 1986; Young 1986; Eriksson 1994), several reports indicate that beetles also rely on color cues (Dafni et al. 1990; Steiner 1998; van Kleunen et al. 2007). Within this context, a very well-documented case of flower-beetle interaction is the association in the southeast Mediterranean region between red bowl-shaped flowers and beetles from the family Glaphyridae (Scarabaeoidea: Coleoptera) (Fig. 1). Several species of these beetles, which strongly rely on visual cues to find flowers, are dominant pollinators of red flowering plants in the southeast Mediterranean region (Dafni et al. 1990). Red flowering plants occur in large populations in the southeast Mediterranean region and during their flowering time (February–April) represent prominent features of the landscape in this region. It has been observed that glaphyrid beetles tend to visit red flowers almost exclusively when they are present (Tamar Keasar and Avi Shmida personal observations). Colored trap experiments indicate that red coloration alone would explain this preference (Dafni et al. 1990; Keasar et al. 2010).

To evaluate the mechanisms by which glaphyrid beetles perceive flower colors, the spectral sensitivity of *Pygopleurus israelitus* (Glaphyridae: Scarabaeoidea: Coleoptera) was studied using intracellular recordings and ERG measurements. Our results revealed the presence of three photoreceptor types with maximal sensitivity in the UV, green and red parts of the spectrum. To our knowledge, this represents the first report on insects of spectral range of color vision extended to the long wavelength part of the spectrum with only three spectral receptor types and constitutes the first evidence of red sensitive photoreceptors in a flower-visiting beetle. The photoreceptor spectral sensitivity data were used to model color vision in *P. israelitus*. By comparing the receptor-based chromatic discrimination of the beetle with the well-supported color vision model in the honeybee, we addressed the question of how color

Fig. 1 Photograph of a couple of *Pygopleurus sp* (Glaphyridae: Coleoptera) mating on a red flower

coding mediates the apparent ecological specialization of *P. israelitus* to red flowers. Our results indicate that the receptor-based color vision of *P. israelitus* is well suited to allow chromatic discrimination of the reddish flowers they encounter in nature and suggests differences in the coding of flower colors between *P. israelitus* and the honeybee.

Materials and methods

Electrophysiological recording and stimulation

For electrophysiological experiments, animals were captured in the field and brought to the lab where they were kept at 8–12 °C. Intracellular recordings were performed in two females and two males of *P. israelitus*. Photoreceptors were evaluated with respect to their spectral sensitivity using conventional methodology (Peitsch et al. 1992). Receptor spectral sensitivity functions $R(\lambda)$ were determined by a light-clamp technique, which makes it possible to establish an $R(\lambda)$ function within a few seconds at 4 nm spectral resolution (Menzel et al. 1986). Briefly, a grid monochromator was used to scan the spectrum between 300 and 700 nm. To clamp the response of the receptor cell to a preselected receptor potential, the light flux at each wavelength (4 nm steps) was automatically adjusted using a circular neutral density wedge. Therefore, only the tonic component of the receptor potential contributed to the response while the cell became slightly light adapted (see Menzel et al. 1986 for a more detailed description of the method). The specimen was dark adapted prior to taking the spectral measurements. The illuminating light was calibrated with a radiation meter following the procedures described in Peitsch et al. (1992). Once a photoreceptor was impaled, a spectral scan from 300 to 700 nm followed by a scan from 700 to 300 nm was performed. The quality and stability of the intracellular recordings were assured by the usual set of criteria observed in our lab including intracellular potential drop by penetrating the cell of at least −40 mV, stable intracellular potential throughout the measurements (drift of less than ±5 mV), saturating light responses above 25 mV, only depolarizing components of light responses including strong stimuli off axis, and accurate alignment of the optical axis by a perimeter (visual angle of the opening of the light guide of 0.5°). Usually, the spectral scans were recorded more than once in the same cells and the values from forward and backward scans were averaged. All intracellular measurements were done in the ventral part of the eye.

ERG measurements were performed in a total of six females and four males of *P. israelitus*. ERG responses were recorded differentially by inserting a silver electrode in each eye of the animals, using an AC pre-amplifier (P55, Grass-Telefactor, West Warwick, RI, USA). A computer-controlled light stimulator was used, consisting of a xenon arc lamp source, a shutter, six quartz neutral density filters with optical densities covering 4.6 log units, and a monochromator (Omni-λ 150, LOT-Oriel Group Europe, Darmstadt, Germany). The light from the optical set up was focused on one end of a quartz optical fiber, while the other end was directed to one of the eyes of the animals. A radiometer (Optometer P-2000, Gigahertz-Optik GmbH, Türkenfeld, Germany) was used to calibrate the light stimulus to isoquantal flux at 20 nm steps between 300 and 700 nm. Animals were dark adapted for 30 min prior to the onset of the experiments. For the adaptation experiments, the animals were exposed to a blue light obtained from a combination of a normal white light source and a BG12 interference filter; the blue light ranged from 400 to 500 nm with a peak at 450 nm. The light was directed at the animals' eyes for 5 min prior to the ERG measurements, and during the experiments light pulses were applied in addition to the blue adapting light. Glaphyrid beetles have distinct dorsal and ventral eye regions. Potential differences in the spectral sensitivities of the dorsal versus ventral portions of the eyes were determined by selectively stimulating only one eye region with the 21 equiquantal monochromatic flashes, using 50 ms light

pulses at 5 s inter-pulse intervals. The stimulus intensity response function was measured over a range of 3-log unit attenuation at the wavelengths eliciting higher responses. The response amplitude curve $V(I)$ was later fitted using a least square optimization method with a Hill sigmoid $V(I) = V_P\ I^h/(R^h + I^h)$, where the independent variable I represents the light intensity, V_P the peak response, h represents the Hill's slope and R the intensity for the half maximal response (Laughlin 1981). The inverse function $I(V) = R[V/(V_P - V)]^{1/h}$ was used to estimate the effective intensities $I(V)$ of tested stimuli evoking response amplitudes in the range (0–V_P).

Modeling electrophysiological receptor spectral sensitivities and ERG responses

Due to the lateral spectral filtering effects in fused rhabdoms and electrical interactions between photoreceptor cells within an ommatidium, insect spectral sensitivities generally have complicated shapes with secondary maxima (Menzel and Snyder 1975). As a result, modeling spectral sensitivity by applying standard visual pigment spectra is difficult (Govardovskii et al. 2000). To approximate the spectral sensitivities, we used a sum of Gaussian functions model (Koshitaka et al. 2008). After averaging the recordings from different cells, the spectral sensitivities were approximated as:

$$R_i(\lambda) = A_i \exp\left(-\frac{(\lambda - \lambda_i^0)^2}{2\delta_i^2}\right) + B_i \exp\left(-\frac{(\lambda - \lambda_i^1)^2}{2\sigma_i^2}\right) + C_i \exp\left(-\frac{(\lambda - \lambda_i^2)^2}{2\gamma_i^2}\right), \quad (1)$$

where index i corresponds to the spectral type of the sensitivity and A_i, B_i, C_i, λ_i^0, δ_i, λ_i^1, σ_i λ_i^2, γ_i are parameters, whose values were adjusted to provide a least square approximation of measured photoreceptor spectral sensitivities using the 'FindMinimum' procedure in Mathematica 5.

ERG responses result from the electrical response of photoreceptors and from interactions between receptor responses. We modeled the ERG as an absolute value of the linear combination of receptor sensitivities as:

$$\mathrm{ERG}(\lambda) = \left|\sum_1^n k_i R_i(\lambda)\right|, \quad (2)$$

where $R_i(\lambda)$ is a spectral sensitivity of a receptor of type i given by Eq. 1, k_i is a weight of the contribution of this photoreceptor and n is the number of spectral types of photoreceptors. The model has n parameters whose values were obtained using a least square procedure and the 'FindMinimum' procedure in Mathematica 5. To account for both excitatory and inhibitory receptor inputs, we allow both positive and negative weights of receptor inputs. It is important to note that more sophisticated nonlinear modeling may provide a much better fit to experimental data, because the ERG is generally a nonlinear function of receptor inputs. However, any nonlinear model requires a larger number of parameters, which cannot be accurately determined with the given accuracy of the experimental data.

Flower reflectance spectra

The spectral reflectance functions of beetle-visited flowers were measured with a spectral photometer over the range of 300–700 nm as described in Menzel and Shmida (1993). The plant species included in the present study were selected on the basis of observations indicating that glaphyrid beetles visit their flowers. Only the dominant color, corresponding to the spectral reflectance function type occupying the largest area within the flower, was considered in the analysis. Spectra from the following species were measured; *Adonis microcarpa* (Ranunculaceae), *Anemone coronaria* (Ranunculaceae), *Glaucium corniculatum* (Papaveraceae), *Glaucium grandiflorum* (Papaveraceae), *Ranunculus asiaticus* (Ranunculaceae), *Ranunculus marginatus* (Ranunculaceae) and *Ranunculus millefolius* (Ranunculaceae). Domesticated varieties of *R. asiaticus* show flower color polymorphism (red, white, pink and purple) and glaphyrid beetles have been reported to visit only the red morph. However, considering observations of glaphyrid visits to non-red color morphs of species having similar color polymorphism (e.g., *A. coronaria*, H. Tzohari personal communication), non-red phenotypes of *R. asiaticus* were included in our analysis to evaluate the distribution of such flower colors in the beetles and bees color space. Spectral reflectance from leaves was also measured for several of the above species. The ecological distribution of the species included in this study overlaps with that of *P. israelitus.*

Modeling insect color perception

We used the color opponent receptor noise-limited model (Vorobyev and Osorio 1998; Vorobyev et al. 2001a) to describe the distribution of flower colors in the chromaticity diagram of the honeybee and *P. israelitus*. This model is based on the assumption that detection and discrimination of light stimuli are limited by the noise generated by the photoreceptors. The model does not make any assumptions about color opponent mechanisms and assumes that intensity (brightness) cues are ignored. The model predictions agree with the results of behavioral experiments in a number of animals including the honeybee (Vorobyev and Osorio 1998; Vorobyev et al. 2001a) and the swallowtail butterfly, *Papilio xuthus* (Koshitaka

et al. 2008). The parameters of the model are the photoreceptor noise levels. Levels of photoreceptor noise have been measured for several hymenopterans (Vorobyev et al. 2001a; Frederiksen et al. 2008). To investigate how the spectral sensitivity of photoreceptors affect the distribution of colors in chromaticity diagrams and considering that noise levels for *P. israelitus*' receptors are not known, we used levels of noise set to the values measured in the honeybee to model the color vision of the glaphyrid beetle. This allows a comparison of the chromatic diagram of the honeybee with the chromatic diagram corresponding to the spectral sensitivities of *P. israelitus* (Vorobyev et al. 2001b). Because of the lack of information on photoreceptor noise levels for *P. israelitus*, it is important to note that this version of the model does not allow any conclusions about the ability of *P. israelitus* to discriminate colors. However, the model does allow a qualitative comparison of the distribution of colors by considering the effect of variation of photoreceptor spectral sensitivity alone. For each flower reflectance, we calculated the quantum catch q_k of corresponding photoreceptor k,

$$q_k = c_k \int_{\lambda} I(\lambda) S(\lambda) R_k(\lambda) d\lambda \tag{3}$$

where $R_k(\lambda)$ is the spectral sensitivity of receptor of type k, $S(\lambda)$ is the reflectance spectrum, $I(\lambda)$ is the illumination spectrum and c_k is a constant describing the absolute sensitivity of each receptor type. In the case of trichromatic vision, k = S, M, L (corresponding to short-, medium-, and long-wavelength receptors, respectively). Here, we assume that illumination is a standard D65 daylight (Wyszecki and Stiles 1982).

According to the log-linear version of the receptor noise-limited model (Vorobyev and Osorio 1998), receptor signals are related to receptor quantum catches by

$$f_k = \ln(q_k). \tag{4}$$

It is important to note that in this version of the model, the distance between colors does not depend on the absolute sensitivity of photoreceptors described by parameters c_k. To plot color stimuli, we used a chromaticity diagram where the Euclidean distance between the points corresponds to the predicted ability to discriminate the stimuli. The distance between points does not depend on the choice of coordinate axes because any set of orthogonal axes can be used to describe and calculate the distance, i.e., the metric of Euclidean space is invariant with respect to rotation of coordinates. It follows from the assumptions of the receptor noise-limited model that the actual orientation of color opponent mechanisms is not related to the axes of chromatic diagrams (Vorobyev and Osorio 1998). Moreover, color opponent mechanisms are generally not orthogonal to each other, while the axes of chromatic diagrams are (Vorobyev and Osorio 1998; Kelber et al. 2003). For example, a visual system may have L–M and L–S opponent mechanisms, while the orthogonal to L–M direction is S − [aL + bM], where the values of parameters a and b depend on the noise of receptor mechanisms. The actual orientation of color opponent mechanisms does not affect color discrimination, given that thresholds are set by noise originating in photoreceptor mechanisms, and, therefore, the orientation of color opponent mechanisms cannot be inferred from color thresholds (Vorobyev and Osorio 1998). Because the axes of chromaticity diagrams are not related to color opponent mechanisms, it is important to pay attention to mutual distribution of points in the diagram, rather than to the positions of the points with respect to the axes. Here, we use the axes corresponding to the respective L–M and S − [L + M] direction in the chromatic diagram (Kelber et al. 2003):

$$\begin{aligned} X_1 &= A(f_{\mathrm{L}} - f_{\mathrm{M}}), \\ X_2 &= B(f_{\mathrm{S}} - (af_{\mathrm{L}} + bf_{\mathrm{M}})), \end{aligned} \tag{5}$$

where:

$$A = \frac{1}{\sqrt{(\omega_{\mathrm{L}})^2 + (\omega_{\mathrm{M}})^2}},$$

$$B = \sqrt{\frac{(\omega_{\mathrm{L}})^2 + (\omega_{\mathrm{M}})^2}{(\omega_{\mathrm{L}})^2(\omega_{\mathrm{M}})^2 + (\omega_{\mathrm{S}})^2(\omega_{\mathrm{L}})^2 + (\omega_{\mathrm{S}})^2(\omega_{\mathrm{M}})^2}}$$

$$a = \frac{(\omega_{\mathrm{M}})^2}{(\omega_{\mathrm{L}})^2 + (\omega_{\mathrm{M}})^2},$$

$$b = \frac{(\omega_{\mathrm{L}})^2}{(\omega_{\mathrm{L}})^2 + (\omega_{\mathrm{M}})^2}.$$

The noise values were set to $\omega_{\mathrm{S}} = 0.13$, $\omega_{\mathrm{M}} = 0.06$ and $\omega_{\mathrm{L}} = 0.12$ (Vorobyev et al. 2001a). The distance in the color space can be expressed as:

$$\Delta S^2 = \Delta X_1^2 + \Delta X_2^2. \tag{6}$$

Results

Intracellular recordings revealed three different kinds of receptor spectral sensitivity functions with peaks in the UV (S for short-wavelength), green (M for middle-wavelength), and red (L for long-wavelength) areas of the spectra (Fig. 2). A photoreceptor with sensitivity peaks in the UV and in the green area of the spectrum was measured only on two occasions. The spectral sensitivity of these two cells represented a composite of both the S and the M receptors as indicated by the overlap in the UV and green area of the spectrum (data not shown).

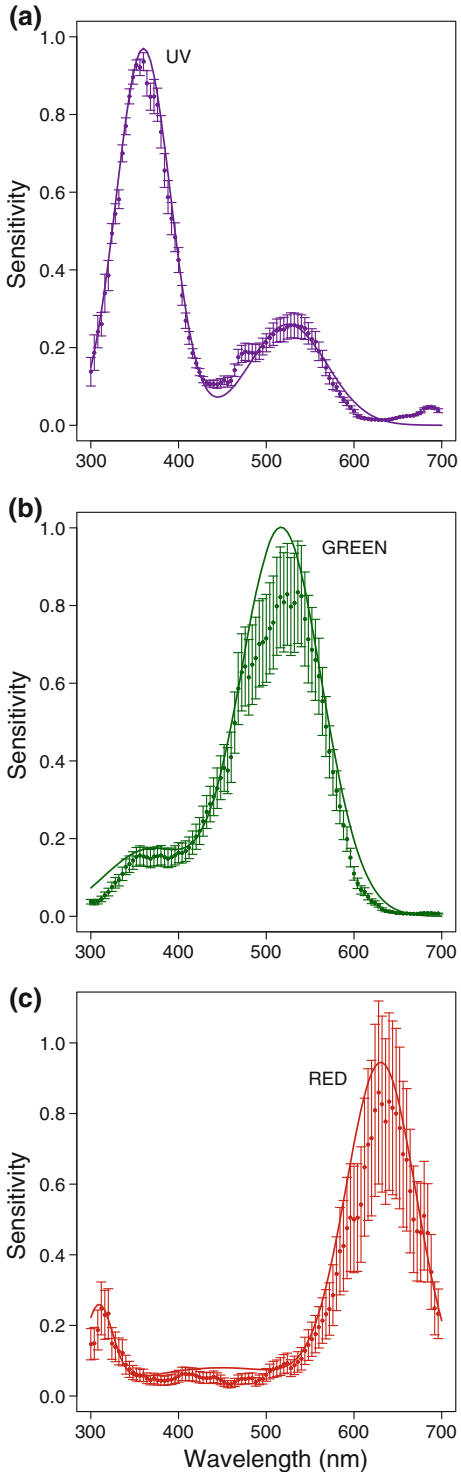

Fig. 2 Spectral sensitivities of three classes of photoreceptors found in *Pygopleurus israelitus*. The *dots* in the figure represent the mean spectral sensitivity function of **a** UV (S), **b** *green* (M) and **c** *red* (L) receptors measured by intracellular recordings. The *continuous line* represents the photoreceptor spectral sensitivity approximated as a sum of Gaussian functions. Number of measured cells of each class (n) and number of animals (m), each cell class was recorded from, are given as (n, m): S (4, 2); M (14, 4); L (4, 3)

The spectral sensitivities can be approximated as a sum of Gaussian functions (Eq. 1, "Materials and methods"). Parameters of the model are given in Table 1. The spectral sensitivities of the S and M receptors were approximated by the sum of two Gaussian functions with secondary peaks roughly corresponding to the peak of M and S receptors, respectively. The L receptor was approximated by the sum of three Gaussian functions; the secondary peaks were not obviously related to the primary peaks of the S and M receptors.

The results from the ERG recordings indicate that photoreceptor contributions differ between the ventral and dorsal portions of *P. israelitus*'s eye (Fig. 3a, c). While in the ventral portion of the eye, the S, M and L photoreceptors characterized in our intracellular recordings seem to contribute to spectral sensitivity; in the dorsal region, the response to UV (S photoreceptor) was predominant with apparently little contribution from the other receptors. Results of spectral sensitivity obtained by ERG recordings can be reasonably approximated as a linear combination of the inputs of the three receptor types found in single cell recordings (Fig. 3; Table 2). In the ventral portion of the eye, the ERG at 420 nm is higher than that predicted by a linear combination of receptor inputs. To test whether this can be attributed to the contribution of a fourth type of receptor peaking in the blue part of the spectrum (420 nm) and missed in our intracellular recordings, we repeated ERG recordings under adaptation by blue light. If a separate blue-sensitive receptor is present in the eye, the adaptation to blue light should significantly decrease sensitivity in the blue area of the spectrum and leave the sensitivity in other parts of the spectrum largely unaltered. Adaptation to blue light decreased the sensitivity in UV, blue and green areas of the spectrum (Fig. 3b, d). To quantify the effect of adaptation on the sensitivity of the ventral portion of the eye in the blue region of the spectrum, we considered the ratio of ERG (420 nm), which corresponds to the maximum in the blue to ERG (360 nm), corresponding to the maximal sensitivity of UV receptors. This ratio was practically unaffected by the adaptation; pre-adaptation ratio of ERG (420 nm)/ERG (360 nm) = 0.57 ± 0.18 (mean ± SE); post-adaptation ratio of ERG (420 nm)/ERG (360 nm) = 0.56 ± 0.27 (mean ± SE). This indicates that even if a separate receptor with sensitivity in the blue area of the spectrum were present in the eyes of *P. israelitus*, its contribution is not great enough to be detected in the ERG. Neither the intracellular recordings nor the ERG measurements revealed differences in the spectral sensitivity between males and females of *P. israelitus*.

The plant species evaluated with respect to their spectral properties revealed a diversity of flower reflectance curve

Table 1 Parameters used to model the spectral sensitivities of the UV (S), green (M) and red (L) receptors found in single cell recordings as a sum of Gaussian functions

	A	λ^0 [nm]	δ [nm]	B	λ^1 [nm]	σ [nm]	C	λ^2 [nm]	γ [nm]
UV (S)	0.97	360	31	0.14	514	50	–	–	–
Green (M)	1	517	46	0.17	365	50	–	–	–
Red (L)	0.93	631	40	0.23	309	16	0.08	449	100

The values of the parameters were adjusted to provide a least square approximation of measured photoreceptor spectral sensitivities

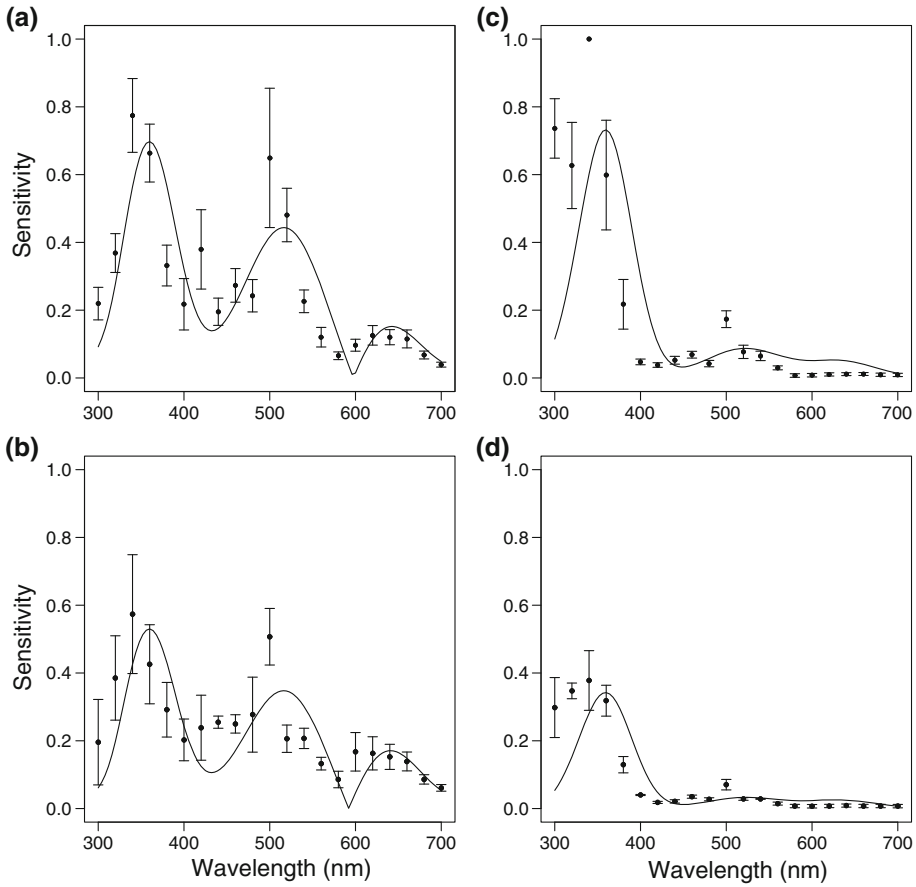

Fig. 3 Spectral sensitivity function as measured by ERG recordings from the ventral portion of the eye (**a**, **b**) and the dorsal portion of the eye (**c**, **d**). *Black dots* in **a** and **c** show the measured spectral sensitivity after dark adaptation while in figures **b** and **d** the *dots* represent the spectral sensitivity measured after 5 min blue light adaptation. The *continuous line* represents the spectral sensitivity approximated as an absolute value of the linear combination of the three types of receptors found in single cell recordings

Table 2 Parameters (k_i) used to model the spectral sensitivity measured in ERG as an absolute value of the linear combination of the three types of receptors found in single cell recordings

	k_{UV}	k_{green}	k_{red}
Dorsal	71	33	−21
Dorsal, blue adapted	54	27	−23
Ventral	81	−15	6
Ventral, blue adapted	38	−8	3

The values of the parameters were adjusted to provide a least square approximation of the whole eye spectral sensitivity

types. The flowers of the different species could be categorized by their levels of reflectance in different areas of the spectrum (Fig. 4). Red flowers from *A. coronaria, A. microcarpa, G. grandiflorum* and *R. asiaticus* have spectral reflectance curves characterized by strong absorbance between 300 and 550 nm while reflecting all light above 600 nm. *G. corniculatum* also has reddish flowers with strong reflectance above 600 nm but with additional reflectance in the UV range. *R. marginatus* have yellow flowers with strong reflectance above 500 nm absorbing all light between 300 and 460 nm. *R. millefolius* has yellow flowers with additional reflectance in the UV range. The human-white variety of *R. asiaticus* reflects all light above 400 nm while *F. densiflora* and the violet and pink varieties of *R. asiaticus* have spectral curves with different levels of reflectance between 380 and 700 nm corresponding to the range of blue, green and red.

Color loci of flowers and leaves were plotted in the chromaticity diagrams of *P. israelitus* and the honeybee (Fig. 5a, b). In these chromaticity diagrams, the Euclidean

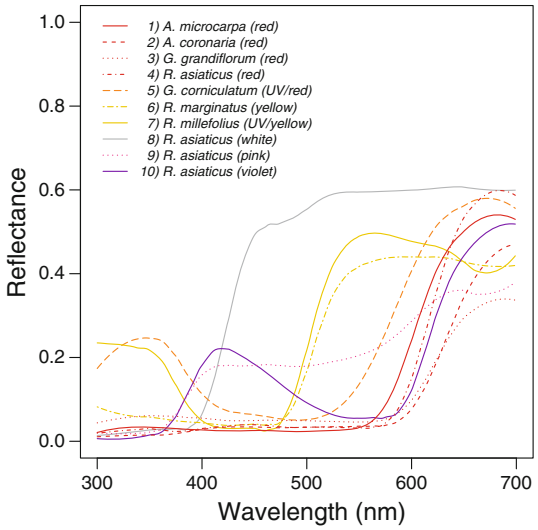

Fig. 4 Spectral reflectance functions of flowers from the plant species included in the analysis. The spectra correspond to the flowers categorized as *red* (*A. microcarpa, A. coronaria, G. grandiflorum, R. asiaticus*), *UV/red* (*G. corniculatum), yellow* (*R. marginatus*), *UV/yellow* (*R. millefolius),* and *white, purple and pink* (*R. asiaticus*)

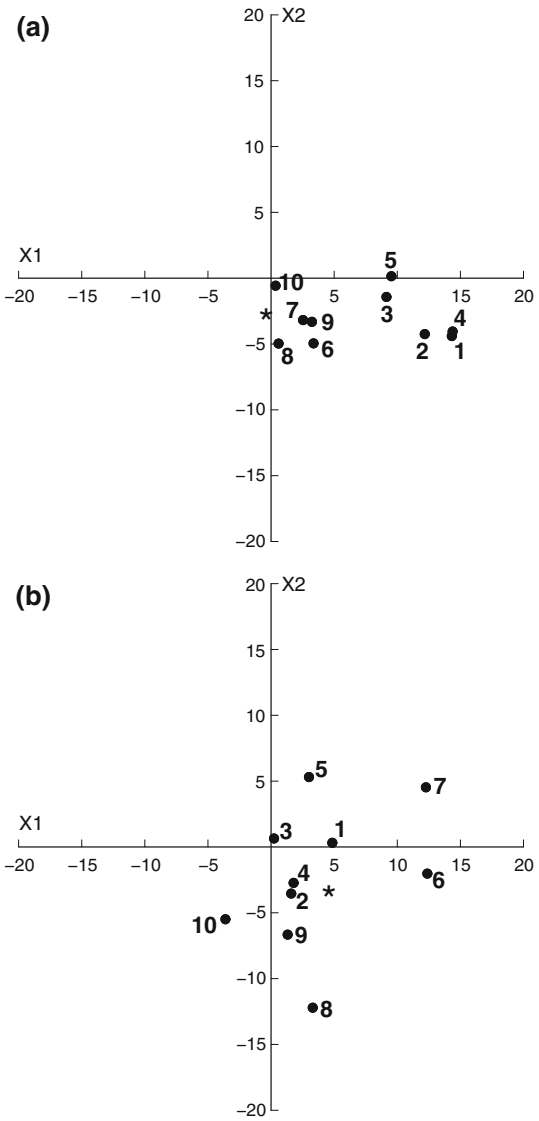

Fig. 5 Loci of flowers (*dots*) and the locus calculated from the average of all leaf spectra (*asterisks*) in *Pygopleurus israelitus* **a** and *Apis mellifera* **b** chromaticity diagrams. For each *point* a number was given to identify the species. The loci correspond to: *1—Adonis microcarpa* (*red*), *2—Anemone coronaria* (*red*), *3—Glaucium grandiflorum* (*red*), *4—Ranunculus asiaticus* (*red*), *5—Glaucium corniculatum* (*UV/red*), *6—Ranunculus marginatus* (*yellow*), *7—Ranunculus millefolius* (*UV/yellow*), *8—Ranunculus asiaticus* (*white*), *9—Ranunculus asiaticus* (*pink*), *10—Ranunculus asiaticus* (*violet*)

distance corresponds to color distance as calculated according to the receptor noise-limited color opponent model (see "Materials and method" section, Vorobyev et al. 2001a). Both diagrams use the values of noise measured in the photoreceptors of the honeybee (Vorobyev et al. 2001a). Therefore, the diagram does not predict *P. israelitus*' ability to discriminate colors, but it does allow us to compare the distribution of colors in the chromaticity diagram of *P. israelitus* with that of the honeybee. The X_1 axis of the diagram corresponds to the L–M opponent direction in the color space (red–green for the beetle and green–blue for the bee), the X_2 direction corresponds to the S − [M + L] opponent direction in the color space. In the beetle's chromaticity diagram, flower colors lie to the right of the points corresponding to leaves (Fig. 5a), i.e., compared to leaves flowers provide a stronger positive red-green signal. In the chromaticity diagram of the honeybee (Fig. 5b), on the other hand, the points occupied by flower colors surround the locus occupied by leaves, spreading much more along the S − [M + L] opponent direction than in the beetles' color space.

To evaluate the effect of different spectral sensitivities on the ability to detect different flower colors against a background of leaves, we calculated distances for each flower color to the mean leaf color. Large distances correspond to better discrimination, short distances to worse or no discrimination (Table 3). In the beetle's color space, the loci of red and orange flowers are further away from those of leaves than the loci of flowers of other colors (see list of flowers in Table 3). Compared to the respective distances in the honeybee diagram, red flowers yielded higher chromatic distances to leaves in the beetle's diagram. In contrast, flower colors resulting from reflectance in the UV, blue and green range of the spectrum yielded higher color distances in the honeybee color space as compared to the respective distances in the color space of beetles. These results indicate that the color vision of *P. israelitus* is well suited to chromatically discriminate the colors of the reddish flowers they seem to prefer.

Table 3 Chromatic distances (ΔS) of flower colors to the mean leaf locus according to the receptor noise-limited model (Vorobyev and Osorio 1998; Vorobyev et al. 2001a)

Plant species	Chromatic distance (ΔS) to the mean leaf color loci	
	Beetle	Honeybee
Adonis microcarpa (red)	14.82	3.65
Anemone coronaria (red)	12.64	3.31
Glaucium grandiflorum (red)	9.66	6.13
Ranunculus asiaticus (red)	14.78	3.17
Glaucium corniculatum (UV/red)	10.39	8.85
Ranunculus marginatus (yellow)	4.28	7.6
Ranunculus millefolius (UV/yellow)	2.95	10.78
Ranunculus asiaticus (white)	2.25	9.02
Ranunculus asiaticus (pink)	3.65	4.88
Ranunculus asiaticus (violet)	2.48	8.78

Distances are given in standard units

To quantify the difference between beetles and honeybees with respect to their ability to discriminate flower colors, we consider the spread of flower color loci in the respective chromaticity diagrams (Vorobyev and Brandt 1997; Vorobyev and Menzel 1999). The spread of points in two dimensions can be characterized by ellipses of scatter, which are calculated from the variance of their coordinates. The main radii of the ellipse of scatter are equal to the standard deviation of the spread of data along the main axis of the ellipse. The area of an ellipse is equal to $\pi r_a r_b$, where r_a and r_b denote the ellipse radii. The area occupied by flowers color loci in the chromaticity diagram of beetles and bees was 32.18 and 114.47, respectively. The larger area occupied by flowers and leaves in the honeybee diagram indicates that the photoreceptor types characterizing bees allow better discrimination of the flower colors evaluated here than the set of photoreceptors found in *P. israelitus*.

Discussion

Structural basis for color vision in *Pygopleurus israelitus*

Our intracellular measurements of spectral sensitivities in *Pygopleurus israelitus* revealed three types of photoreceptors, maximally sensitive in the UV (S), green (M) and red (L) parts of the spectrum with λ max values at 352, 536 and 628 nm, respectively. Whereas the spectral sensitivity function of the UV and green receptors does not show major variations in comparison to the ones found in other insect species, the spectral sensitivity function of the red receptor reveals a λ max value among the longest wavelengths recorded in insects and represents the longest value outside Lepidoptera (Menzel 1979; Briscoe and Chittka 2001; Stavenga and Arikawa 2006). A rather large scatter was observed in the spectral sensitivity data for the M and L receptors. Since the responses recorded intracellularly from the M and L receptors were equally variable as the one from the S receptors, this may indicate variability of spectral absorption by M and L receptors. Variability of spectral absorption could result from many causes including waveguide effects, screening pigment effects, or expression of multiple visual pigments. Our data do not allow us to distinguish between these possibilities. An additional kind of receptor with sensitivity peaks in the UV and green areas of the spectrum was also recorded on two occasions. Such double peaked spectrally sensitive receptors can result from the co-expression of S and M wavelength sensitive opsins within photoreceptors (Szel et al. 2000; Arikawa et al. 2003) but could also result from functional or artificial coupling between the membranes of adjacent photoreceptors by the recording electrode (Menzel 1979). The ERG spectral sensitivity measurements indicate differences in the contribution of photoreceptors to the ERG signal between the ventral and dorsal portions of *P. israelitus*' eyes (Fig. 3a, c). While the three photoreceptor types characterized intracellularly seem to contribute to spectral sensitivity in the ventral portion of the eye of *P. israelitus*, the dorsal portion of the eye showed predominant sensitivity to UV with apparently little contribution from other receptor types. These results suggest specialization of the ventral and dorsal portions of the eye to different aspects of the ecology of these beetles. Perhaps the high sensitivity to UV in the dorsal region resembles findings in the flightless desert scarab *Pachisoma striatum* (Scarabaeidae: Scarabaeoidea: Coleoptera), showing that an extensive part of the dorsal eye of *P. striatum*, having UV and UV/green sensitive receptors, is equivalent to the dorsal rim area used for polarized light navigation in other insects (Dacke et al. 2002).

Differences and similarities to other coleopterans

No receptor with peak sensitivity in the blue part of the spectrum was found in our intracellular recordings and the analysis of the ERG did not reveal a channel that could be independently adapted by blue light. Although a small contribution by blue receptors cannot be ruled out, our results suggest that *P. israelitus* do not posses blue-sensitive receptors. The absence of a blue receptor in *P. israelitus* coincides with findings in other Coleopteran species. The spectral sensitivity of additional species of Scarabaeoidea suggests that the absence of blue receptors is a common condition within this group. Spectral sensitivity

measurements in *Lethrus apterus* Laxm (Geotrupidae: Scarabaeoidea: Coleoptera), *Cetonia aurata, Liocola brevitarsis, Onitis alexis,* and *Potosia metallica* (Scarabaeidae: Scarabaeoidea: Coleoptera) revealed the presence of UV and green receptors while blue-sensitive receptors were lacking in all five cases (Mazokhin-Porshnyakov 1962; Gribakin 1981; Warrant and McIntyre 1990; Lin and Wu 1992). The difference in spectral sensitivity between *P. israelitus* and the four species from Scarabaeidae, which are analogous to *P. israelitus* with respect to their flower-visiting habits, reveals that differences exist in the color vision of beetle pollinators. These differences open up the question of how diverse beetle pollinators might be with respect to their color vision. Given that beetle pollination is regarded as one of the earliest mode of flower specialization (Bernhardt 2000), answering this question would provide insights into the role flower colors could have played on such early modes of flower/pollinator interactions. In the case of flower-visiting beetles belonging to Scarabaeoidea, the evidence available so far suggests that dichromacy might reflect the ancestral condition within this group. Furthermore and considering trichromacy with receptors maximally sensitive in the UV, blue and green parts as the basal condition among insects (Menzel 1979; Chittka 1996; Briscoe 2000; Briscoe and Chittka 2001; Spaethe and Briscoe 2004), the presence of a red receptor in *Pygopleurus israelitus* suggests a secondary re-gain of trichromacy.

Evidence of red sensitivity in two species of Carabinae (Geadephaga: Coleoptera) (Hasselmann 1962), together with the fact that Glaphyridae and Carabinae belong to well-separated lineages of coleopterans (Hunt et al. 2007), suggests that red sensitive photoreceptors might have evolved independently more than once within Coleoptera. Such evolutionary processes might resemble those found in butterflies, which have developed multiple photoreceptors as the result of spectral filtering and opsin gene duplication (Qiu and Arikawa 2003; Wakakuwa et al. 2004; Stavenga and Arikawa 2011). For the butterfly *Pieris rapae crucivora*, there is convincing evidence that various spectral filters cause the diversification of the spectral sensitivities of long-wavelength sensitive photoreceptors (Qiu and Arikawa 2003; Wakakuwa et al. 2004). Consistent with this, our results on *P. israelitus* show that the measured spectral sensitivity function of red photoreceptors is much narrower than the one predicted by a pigment template (Fig. 2c), making some form of filtering a likely mechanism influencing the spectral sensitivity of *P. israelitus*' red photoreceptor. To our knowledge, this represents the first report on insects of spectral range of color vision extended to the long-wavelength part with only three spectral receptor types and constitutes the first evidence of red sensitive photoreceptors in a flower-visiting beetle.

Modeling color perception

Keeping in mind that the lack of information on photoreceptor noise values for *P. israelitus* in our modeling provides qualitative rather than quantitative considerations, the analysis of flower loci distribution in the receptor-based color space of *P. israelitus* and *Apis mellifera* suggests that the presence of a red receptor determines how colors resulting from extreme long-wavelength reflectance are perceived. Inspection of the chromaticity diagrams shows that flower colors are in general well separated from leaves in the chromaticity diagram for both beetles and bees. The separation between the color loci of flowers and leaves in the chromaticity diagram of *P. israelitus* may be utilized by the beetle to discriminate flowers from leaves using chromatic neural mechanisms. Flower colors occupied a greater area in the honeybee color space, suggesting that bees discriminate flower colors better than beetles. In the case of red flowers, on the other hand, values of flower color distance in *P. israelitus'* color space suggest that when seen against a green foliage background red flowers would be more conspicuous to beetles than flowers of other colors. In addition, the lower distance yielded by red flowers in the color space of bees as compared to the respective distances in the chromaticity diagram of the beetles suggests that the visual strategy used by honeybees to find red flowers differs from that used by beetles. While trichromatic bees seem to perceive red flowers through achromatic mechanisms (Martínez-Harms et al. 2010), the evidence presented here indicates that *P. israelitus* have the receptor-based color vision to chromatically perceive red flowers. The capacity to chromatically perceive red could mediate the learning of red flowers by their color and thus the apparent specialization on such flowers reported for glaphyrids in the southeast Mediterranean region.

Ecological and evolutionary implications

The idea that plant–pollinator interactions tend toward specialization, implicit in the concept of "pollination syndromes", is a matter of controversy among pollination biologists (e.g., Waser et al. 1996; Fenster et al. 2004). This view has been mainly questioned because pollination systems have repeatedly been demonstrated to be more generalized than previously thought (Grant 1994; Waser et al. 1996; Waser 1998). Considering that dramatic specialization in pollination systems does occur, Ollerton (1996) suggested that the history of plant-pollinator interactions includes periods of specialization and generalization, and that during periods of specialization most evolution would occur. In the case of *P. israelitus*, our results reveal a high level of congruence between its receptor-based color vision and the red and orange flowers they seem to prefer. As

mentioned above, red flowers are very prominent features of the landscape in the southeast Mediterranean region, presenting a scenario under which specialization could be favored. However, further evaluations are required to establish the extent to which this sensory congruence can be explained on the basis of glaphyrids' relationship with red flowers, or whether it corresponds to an inherited condition common within Glaphyridae. High levels of sensory congruence to natural signals have been reported in other coleopteran species as well. Striking examples are fireflies (Lampyridae; Coleoptera), which appear to have spectral sensitivities narrowly tuned to the bioluminescent emission spectra of conspecifics (Cronin et al. 2000). As in fireflies, the visual system of glaphyrids might also mediate behaviors outside the context of feeding (e.g., detection of conspecifics), indicating that aspects other than their role as pollinators need to be considered if one aims to understand the evolution of the receptor-based color vision of *P. israelitus*. Indeed, glaphyrid beetles are often characterized as being brightly colored and many species exhibit color polymorphism, conditions under which the presence of a red receptor might also be involved. A more extensive evaluation of Glaphyridae is required to establish the extent to which a red receptor could be considered a characteristic of this group.

Beetles are considered to have played a major role as pollinators of early angiosperms, using flowers as food sources and mating places. The oldest known record of Glaphyridae, a specimen of the genus *Glaphyrus* (Glaphyridae: Scarabaeoidea: Coleoptera) from the Yixian Formation in China (Upper Jurassic or Lower Cretaceous) (Nikolajev and Ren 2011), suggests that these insects were already present by the time of angiosperm's earliest diversification (dated to <140 Ma). Despite the fact that glaphyrid habits as flower visitors could have been established very early in the evolution of angiosperms, their interaction with red flowers in the southeast Mediterranean region is considered a more derived form of beetle pollination (Bernhardt 2000). Nevertheless and independent from the evolutionary origin of the receptor-based color vision of *P. israelitus*, the way these beetles perceive colors might have direct implications on the persistence of their interaction with red flowers over time. In the east Mediterranean region, the phenology of glaphyrid beetles and red flowers overlaps in a temporal succession of species that conserve this mode of plant-pollinator interaction (Dafni et al. 1990). While beetles mediate the reproduction of the plants, flowers provide food resource, mating site and shelter for the beetles (Keasar et al. 2010) (Fig. 1). Considering the niche of an organism as the part of the medium it encounters moment after moment in the realization of its living (Maturana-Romesin and Mpodozis 2000), each one, beetles and flowers, represents important features of the niche of the other, interacting as individuals and as species in a continually recurring manner (i.e., recursively). Given the importance of floral color as cue for glaphyrids, the presence of a red sensitive photoreceptor in their visual system can be understood as a determinant character involved in the recursive processes that conserve this form of plant–pollinator interaction. Information on color vision of Glaphyridae, although limited, highlights these insects as an interesting model group in the study of color vision evolution in general and pollination biology in particular.

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