

**Color vision of insect pollinators – photoreceptor properties,
behavioral bases and its implications for the evolution of
angiosperms**

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Author's contribution:

R. Menzel, M. Vorobyev and I discussed the design of the experiments presented on the first chapter. I wrote the article the first chapter is based on, considering the suggestion of all co-authors. I wrote the manuscripts of the other two chapters. R.M. and I designed the geographical survey of flower colors reported on the second chapter. M.V. and I designed the experiments presented on the third chapter. With the only exception of the intracellular recordings reported on the first chapter, which were done by J Schorn, I carried out all the experiments, measurements and data analysis presented in the present thesis.

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Summary

In the present thesis I studied the spectral properties of photoreceptors as well as the behavioral bases of color vision in representative insect pollinators and evaluated this data in the context of the reproductive ecology and evolutionary biology of representative plant species. In the work presented in the first chapter I examined the visual mechanisms involved in the apparent ecological specialization of glaphyrid beetles to red flowers described for the South-East Mediterranean region. I characterized the photoreceptors spectral sensitivity of *Pygopleurus israelitus* (Glaphyridae: Scarabaeoidea: Coleoptera). In addition, using chromaticity diagrams to calculate the distribution of beetle-visited flower, I evaluated whether chromatic discrimination differs between *P. israelitus* and the honeybee. The measurements revealed the presence of three types of photoreceptors, maximally sensitive in the UV, green and red areas of the spectrum. The distribution of flower colors revealed a high level of correspondence between the receptor-based color vision of *P. israelitus* and the reddish flowers they encounter in nature and suggests differences in the coding of flower colors between *P. israelitus* and the honeybee. In the work presented in the second chapter I studied flower color evolution in *Papaver rhoeas*. According to Archeological and Taxonomic evidence this species was introduced into Central Europe from the Levant region about 5 thousand years ago. Intra-specific color differences were evaluated by comparing flower color composition of populations along its distribution range in Central Europe and the Levant region. The data revealed that populations of this species have diverged with respect to their flower color composition. While populations in Central Europe are composed exclusively by individuals having UV-reflecting flowers, populations in the Levant region seem to be composed almost exclusively by individuals having UV-absorbing flowers. In order to evaluate whether *P. rhoeas* has diverged with respect to its flower color appearance for its main insect pollinator groups, I compared the distribution of flower color loci in the chromaticity diagram of *P. israelitus* and the honeybee. The results of this evaluation indicates that *P. rhoeas* has diverged with respect to its flower color appearance for insect pollinators and suggest that the absence of UV-absorbing flowers in Central European populations would be congruent with the historical shift from beetle to bee pollination undergone by this species as it was introduced northwards into Europe. In the last chapter, I evaluated how differential learning affects color generalization in the honeybee. In particular, I evaluated how the position in the color space of a novel color relative to a positively

reinforced color (CS+) and an unrewarded color (CS-) affects the occurrence of experience induced behavioral biases. My results revealed that when the novel color varied from CS- in the same way as CS+ but to a lower extent, subjects maintained their stronger response towards the former CS+. In contrast, when a novel color varied from CS- in the same way as CS+ but to a greater extent, subjects shifted their strongest response away from CS- towards the novel stimulus. These results revealed the occurrence of “peak shift” in the honeybee color vision and indicate that honeybees can learn color stimuli in relational terms based on their chromatic properties. The findings presented on this thesis are discussed in the context of the theoretical implications derived from the historical nature of plant pollinator interactions.

Zusammenfassung

In der vorliegenden Arbeit analysierte ich sowohl die spektralen Rezeptoreigenschaften wie auch die Verhaltensgrundlagen des Farbsehens repräsentativer Arten von bestäubenden Insekten. Diese Daten werden in den Kontext der Blütenökologie und Evolutionsbiologie repräsentativer Pflanzenarten gestellt. Im ersten Kapitel untersuchte ich die spektralen Rezeptoreigenschaften der Glaphyriden, einer Blumen besuchenden Käfergruppe, in Bezug auf rote Blumen in der südöstlichen Mittelmeerregion, die zu einer ökologischen Spezialisierung geführt haben. Ich verwende die spektrale Empfindlichkeit der Fotorezeptoren von *Pygopleurus israelitus* (Glaphyridae: Scarabaeoidea: Coleoptera) um mit Hilfe eines Farbsehmodells die Verteilung der Farbborte in einem chromatischen Diagramm von Käfer-besuchten Blumen zu berechnen. Die Ergebnisse vergleiche ich mit entsprechenden Analysen an der Honigbiene. Meine Analysen weisen die Existenz von drei Fotorezeptoren mit spektralen Empfindlichkeitsmaxima im Ultraviolett, Grün und Rot nach. Die Verteilung der Blumenfarben im chromatischen Diagramm macht deutlich, dass Farbunterscheidung von *P. israelitus* an rote Blumen mit und ohne UV Reflexion optimal angepasst ist und suggeriert außerdem, dass es Unterschiede in der Kodierung von Farben zwischen *P. israelitus* und der Honigbiene gibt. Im zweiten Kapitel untersuchte ich die Evolution der Blumenfarben von *Papaver rhoeas*. Laut archäologischer und taxonomischer Beweise kam diese Art vor ungefähr 5000 Jahren aus der levantischen Region nach Mitteleuropa. Ich wertete arttypische

Farbunterschiede über den Vergleich von Farborten verschiedener Populationen in Mitteleuropa und in der levantischen Region aus. Meine Daten ergaben, dass sich Populationen dieser Art in ihren Farbmustern unterscheiden. Während die Populationen Mitteleuropas fast ausschließlich UV-reflektierende Blumen enthalten, haben die Populationen der levantischen Region vornehmlich UV-absorbierende Blumen. Um herauszufinden, ob sich das farbliche Erscheinungsbild von *P. rhoeas* gegen über seinen Bestäubern verändert hat, verglich ich die Verteilung der Farborte im chromatischen Diagramm für diese Populationen für *P. israelitus* und die Honigbiene. Meine Ergebnisse stützen die Interpretation, dass sich das farbliche Erscheinungsbild von *P. rhoeas* verändert hat. Außerdem führen meine Ergebnisse zu der Annahme, dass das Fehlen von UV-absorbierenden Blumen in den Populationen in Mitteleuropa mit dem Wechsel von der Käfer- zur Bienenbestäubung zusammenhängt, die sich durch das Ausbreiten dieser Bestäuberart in den nördlichen Regionen ergab. Im letzten Kapitel untersuchten ich, wie sich das Erlernen von Farbmarken durch die Honigbiene auf die Generalisierung zwischen Farbmarken auswirkt. Dazu wurde auf einer belohnten Farbe (CS+) in Gegenwart einer nicht belohnten Farbe (CS-) dressiert und systematisch überprüft, wie sich dies auf die Unterscheidung von neuen Farbpaaren auswirkt. Meine Ergebnisse wiesen eine neuartige Leistung bei den Bienen nach, den sogenannten „Peak shifts“. Wenn die Testfarbe im chromatischen Diagramm näher am CS- war als der CS+, gaben die Versuchstiere immer der CS+ Farbe den Vorrang. Wenn allerdings die Testfarbe im chromatischen Diagramm weiter entfernt vom CS- war als der CS+, wählten die Bienen die Testfarbe. Diese Ergebnisse weisen nach, dass die Honigbiene aufgrund ihrer chromatischen Wahrnehmung Farbstimuli im direkten Vergleich unterscheiden können. Die Ergebnisse meiner Arbeit werden vor den Hintergrund der coevolutiven Entwicklung von Pflanzen und Bestäubern diskutiert.

General Introduction

The relationship between flowering plants and animal pollinators represents a paradigm to study evolutionary processes and patterns of diversification. The interest on plant-pollinator interactions can be traced back to the work by Sprengel (1793), Müller (1873), and Robertson (1895), on floral mechanisms and natural history of plant-pollinator interactions, and to the work by Darwin (1862, 1876, 1877), who focused on the evolutionary processes influenced by pollination. Since those early studies onward, many authors have argued that specialized animal pollination plays a crucial role in the diversification of angiosperms (Darwin 1876; Grant 1949; Stebbins 1974; Crepet 1984). Along with this, some authors have proposed that specialization would represent a general evolutionary trend among pollination systems (Stebbins 1970; Crepet 1983, 1984), with tight co-evolution between plants and pollinators (Gilbert and Raven 1975). This view is implicit in the concept of “pollination syndromes”, defined as suite of floral traits promoting visits of specific groups of animal pollinators. At the level of individuals, this view assumes that pollinators tend to specialize on the flowers they visit. Through their selective behaviors pollinators would mediate a process of floral isolation (“ethological isolation” sensu (Grant 1949), which could potentially lead to the origin and maintenance of reproductive barriers to hybridization (Grant 1952; Levin 1971; Grant 1994). Several sources of evidence support this view. Dramatic specialization does occur in some pollination systems, and specialization together with tight co-evolution seems to explain the rapid diversification of angiosperms and animal pollinators during the mid-Cretaceous, 90 to 125 million years ago (Crepet 1983; Kiester et al. 1984; Eriksson and Bremer 1992; Cardinal et al. 2010; Labandeira 2010).

However, the idea of specialization as a general evolutionary trend among pollination systems has been questioned mainly because plant-pollinator interactions have repeatedly been demonstrated to be more generalized than previously assumed (e.g. Grant 1994; Waser et al. 1996; Waser 1998). The theoretical difficulties to explain sympatric speciation under incomplete assortative mating have also raised doubts about the role of animal pollinators in the speciation of flowering plants (Grant 1994; Waser 1998). Considering that the relationship between plants and animals fluctuate in time and space, some authors have stressed the need of studying plant-animal interactions from a historical perspective (Gould 1986; Futuyma 1988; Donoghue 1989; Armbruster 1992). In line with this, it has been proposed that the role of pollinators in the evolution of flowering plants needs to be judged through careful assessments of the pollinator

environment of representative plant species, extended through time and space (Waser et al. 1996). Given that taxonomically different groups of pollinators differ in aspects so diverse as their morphology, physiology and sensory and cognitive capacities, such assessments should also include the characterization of those structural aspects of pollinators affecting the way they relate with plants. Along this line, a historical perspective is particularly relevant if one aims to understand animal behavior, which takes place in the epigenesis of individuals as a continuous process of conservation of their adaptation to the medium (Maturana-Romesin and Mpodozis 2000). Considering the niche of an organism as the part of the medium the organism encounters moment after moment in the realization of its living (Maturana-Romesin and Mpodozis 2000), each one, plants and pollinators, represent important features of the niche of the other, interacting at the level of individuals and species in a continuously recursive manner. In accordance to this definition, which corresponds substantially to what von Uexküll (1957) refers to as “Umwelt”, every new interaction displaces the circumstances under which following interactions will take place. From this it can be said that every new interaction is function of a history of previous interactions and that along their recursive encounters, plants and pollinators change congruently as part of the niche of the other. Considering the recursive nature of plant-pollinator interactions, a more comprehensive understanding of pollinators’ biology together with insights into the ecological contingencies under which the relationships between plants and pollinators develop and persist over time can provide a better understanding about how such relationships evolve. In the present work I have considered these aspects to study the relationship between flowering plants and pollinators. Particularly, I studied the spectral properties of photoreceptors as well as the behavioral bases of color vision in representative insect pollinators and evaluated this data in the context of the reproductive ecology and evolutionary biology of representative plant species.

In the work presented on the first chapter I studied the relationship between glaphyrid beetles and red-bowled shaped flowers in the South-East Mediterranean region. 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), have 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). 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), several reports indicate, however, that beetles also rely on color cues to find flowers (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 South-East 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 South-East Mediterranean region (Dafni et al. 1990). 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. 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 I addressed the question of how color coding mediates the apparent ecological specialization of *P. israelitus* to red flowers.

In the work presented on the second chapter I studied the evolution of flower color in *Papaver rhoeas*. Fossil evidence suggests that *P. rhoeas* was introduced from the eastern border of the Mediterranean westwards into Europe as consequence of the spread of agriculture about 5 thousand years ago (Zohary and Hopf 1994). Ever since Lotmar (1933) measured its spectral reflection, *P. rhoeas* has served as an example of a species having UV-reflecting flowers (Kugler 1947; Daumer 1958; Menzel and Shmida 1993). A report by Dafni et al. (1990) suggests, however, that populations of this species might differ with respect to their flower color composition. Intraspecific flower color differences within this species would result intriguing considering that its introduction into Europe caused a shift in the type of pollinators this species interacts with. While in Central European populations hymenopterans represent *P. rhoeas*' main visitors (McNaughton and Harper 1960; Dobson et al. 1996), Mediterranean populations are mainly visited by glaphyrid beetles (Dafni et al. 1990; Bosch et al. 1997). These two pollinator groups appear to differ fundamentally with respect to their receptor based color vision (Martínez-Harms et al. 2012). Accordingly, flower color

differences within *P. rhoeas* could affect their appearance for glaphyrid beetles and bees differently. I examined potential color differences within *P. rhoeas* by evaluating flower color composition in populations along its distribution range in the Mediterranean region and Central Europe. In addition, I evaluated differences in flower appearance for insect pollinators by comparing the distribution of flower color loci in the chromaticity diagram of *Pygopleurus israelitus* and the honeybee *Apis mellifera*.

Finally, in the work presented in the third chapter I evaluated how the honeybee *Apis mellifera* generalize colors after being differentially trained with perceptually similar colors. Stimuli generalization accounts for the cognitive act of treating different stimuli as equivalent. Given that under natural conditions flowers will seldom appear twice identical, generalization represents a fundamental cognitive ability of pollinators. In experiments of generalization after differential learning, the response to novel stimuli is usually tested in subjects trained to discriminate a positively reinforced stimulus (CS+) from an unrewarded (or negatively reinforced) stimulus (CS-). A very well established form of generalization in animal and human perception following differential learning is the so call “peak shift” phenomenon (Thomas et al. 1991; Wills and Mackintosh 1998; Ghirlanda and Enquist 2003; Lynn et al. 2005). This refers to a change in the strongest response away from the higher training value stimulus (CS+) toward a novel stimulus that differ from CS- in the same way as the former but to a greater extent. This form of generalization has received considerable attention in evolutionary biology because of its potential relevance in the evolution of natural signals. In the context of pollination, its occurrence has led to the hypothesis that “peak shift” could be involved in floral evolution (Lynn et al. 2005; Wright et al. 2009). In the present work I evaluated how honeybee foragers generalize colors after being differentially trained to perceptually similar colors and in particular, the effect of varying the position in the color space of a novel stimulus relative to a rewarded (CS+) and unrewarded stimulus (CS-) in the occurrence of “peak shift”.

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

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. The 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 I 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 to one side of the locus for green foliage background, whereas in the honeybee the flower color loci surround the locus occupied by green foliage. These 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.

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, allow 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; Vorobyev et al. 2001b; Stavenga and Arikawa 2006). Regardless of the lifestyle of the particular species, 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; Correia 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), have 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 separate 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 South-East Mediterranean region between red bowl-shaped flowers and beetles from the family Glaphyridae (Scarabaeoidea: Coleoptera) (Fig. 1.1). Several species of these beetles, which strongly rely on visual cues to find flowers, are dominant pollinators of

red flowering plants in the South-East Mediterranean region (Dafni et al. 1990). Red flowering plants occur in large populations in the South-East 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).

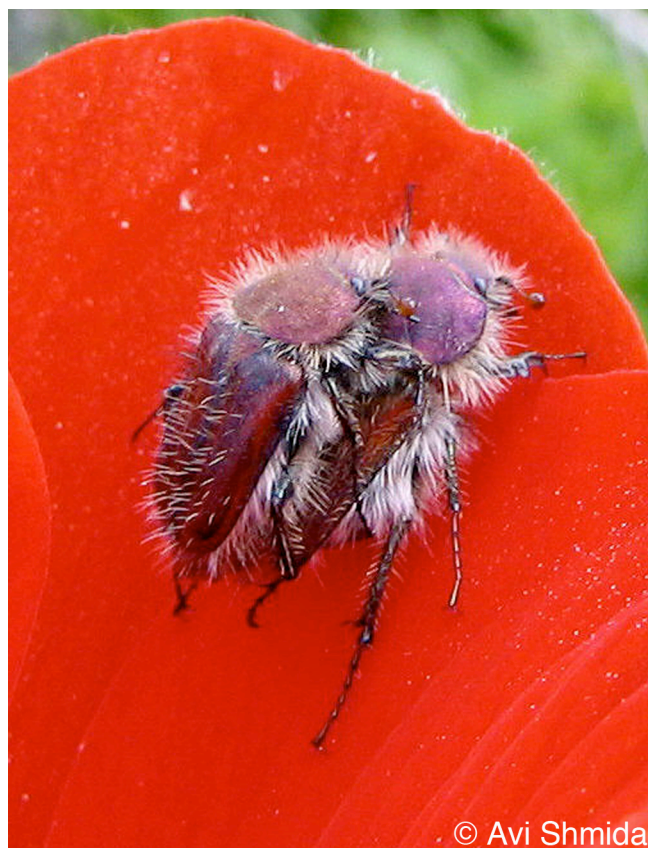


Fig. 1.1 Photograph of a couple of *Pygopleurus sp* (Glaphyridae: Coleoptera) mating on a red flower.

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. The results revealed the presence of three photoreceptor types with maximal sensitivity in the UV, green and red part of the spectrum. To my knowledge this represents the first

report in 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 I addressed the question of how color coding mediates the apparent ecological specialization of *P. israelitus* to red flowers. The 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. In order 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 nm and 700 nm. Animals were dark adapted for 30min 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 vs. 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-second 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 I 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. The ERG was modeled as an absolute value of the linear combination of receptor sensitivities as:

$$ERG(\lambda) = \left| \sum_{i=1}^n k_i R_i(\lambda) \right|, \quad (2)$$

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, both positive and negative weights of receptor inputs were allowed. 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 the analysis in order 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.

The color opponent receptor noise-limited model (Vorobyev and Osorio 1998; Vorobyev et al. 2001a) was used 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 is 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). In order 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, levels of noise set to the values measured in the honeybee were used 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 the quantum catch q_k of corresponding photoreceptor k was calculated,

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

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 I 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

$$f_k = \ln(q_k), \quad (4)$$

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, I 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 the axes corresponding to the respective L-M and S-[L+M] direction in the chromatic diagram were used (Kelber et al. 2003):

$$\begin{aligned} X_1 &= A(f_L - f_M), \\ X_2 &= B(f_S - (af_L + bf_M)), \end{aligned} \quad (5)$$

where:

$$\begin{aligned} A &= \frac{1}{\sqrt{(\omega_L)^2 + (\omega_M)^2}}, \\ B &= \sqrt{\frac{(\omega_L)^2 + (\omega_M)^2}{(\omega_L)^2(\omega_M)^2 + (\omega_S)^2(\omega_L)^2 + (\omega_S)^2(\omega_M)^2}}, \\ a &= \frac{(\omega_M)^2}{(\omega_L)^2 + (\omega_M)^2}, \\ b &= \frac{(\omega_L)^2}{(\omega_L)^2 + (\omega_M)^2}, \end{aligned}$$

The noise values were set to $\omega_S = 0.13$, $\omega_M = 0.06$ and $\omega_L = 0.12$ (Vorobyev et al. 2001a). The distance in the color space can be expressed as:

$$\Delta S^2 = \Delta X_1^2 + \Delta X_2^2, \quad (6)$$

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. 1.2). 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 receptor as indicated by the overlap in the UV and green area of the spectrum (data not shown).

The spectral sensitivities can be approximated as a sum of Gaussian functions (Eq. 1, Methods). Parameters of the model are given in Table 1.1. 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.

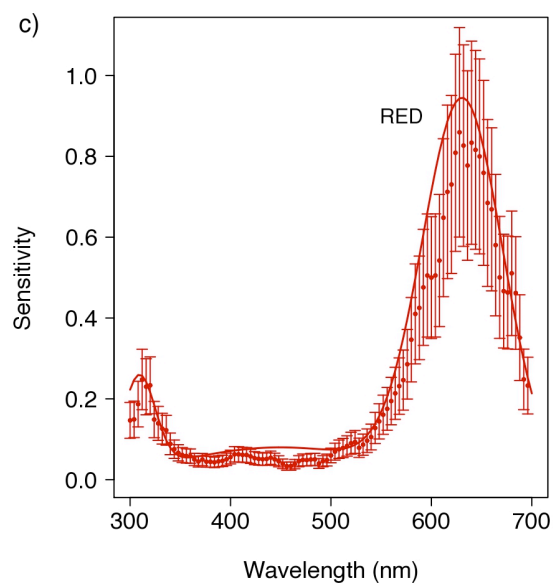
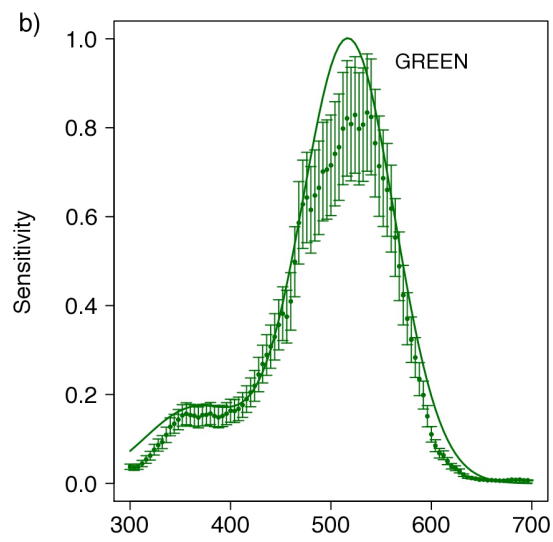
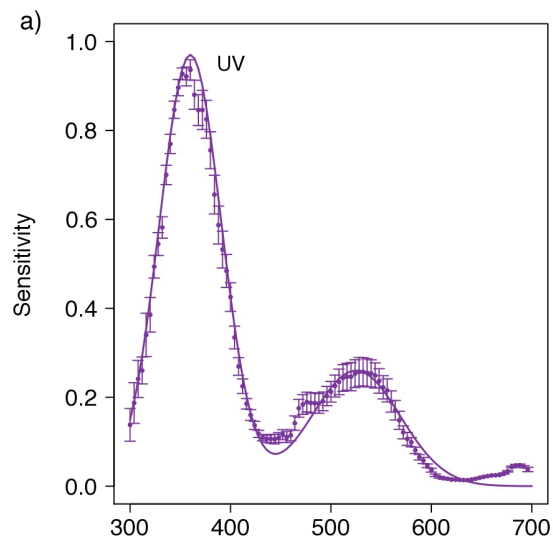


Fig. 1.2 Spectral sensitivities of three classes of photoreceptors found in *Pygopleurus israelitus*. The dots in the figure represent the mean spectral sensitivity function of a) UV (S), b) green (M) and c) red (L) receptors measured by intracellular recordings. The continuous line represents the photoreceptor 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 is given as (n, m): S (4, 2); M (14, 4); L (4, 3)

Table 1.1 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.

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

	A	λ^0 [nm]	δ [nm]	B	λ^1 [nm]	σ [nm]	C	λ^2 [nm]	γ [nm]
UV (S)	0.97	360	31	0.26	525	44	-	-	-
Green (M)	1	517	46	0.17	365	50	-	-	-
Red (L)	0.93	631	40	0.23	309	16	0.08	449	100

The results from the ERG recordings indicate that photoreceptor contributions differ between the ventral and dorsal portions of *P. israelitus*'s eye (Fig. 1.3a, c). While in the ventral portion of the eye the S, M and L photoreceptors characterized in the intracellular recordings seem to contribute to spectral the 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. 1.3, Table 1.2). 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 the intracellular recordings, I 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. 1.3b, d). To quantify the effect of adaptation on the sensitivity of the ventral portion of the eye in the blue region of the spectrum, I 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 \pm SE); post-adaptation ratio of ERG(420 nm)/ ERG(360 nm) = 0.56 ± 0.27 (mean \pm 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*.

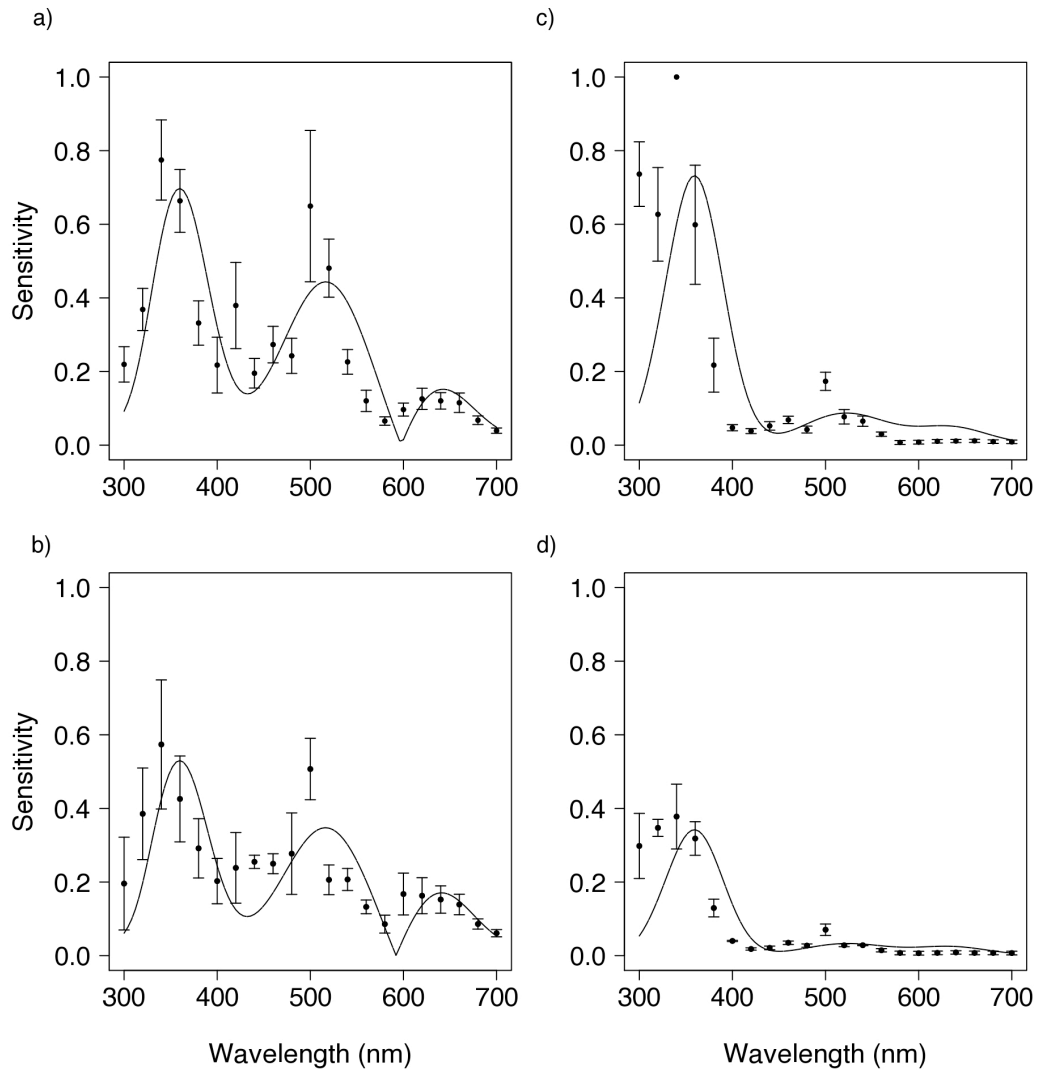


Fig. 1.3 Spectral sensitivity function as measured by ERG recordings from the ventral portion of the eye (a and b) and the dorsal portion of the eye (c and d). Black dots in **a** and **c** show the measured spectral sensitivity after dark adaptation while in figures **b** and **d** the dots represent 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 1.2 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. The values of the parameters were adjusted to provide a least square approximation of the whole eye spectral sensitivity.

	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 plant species evaluated with respect to their spectral properties revealed a diversity of flower reflectance curve types. The flowers of the different species could be categorized by their levels of reflectance in different areas of the spectrum (Fig. 1.4). 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.

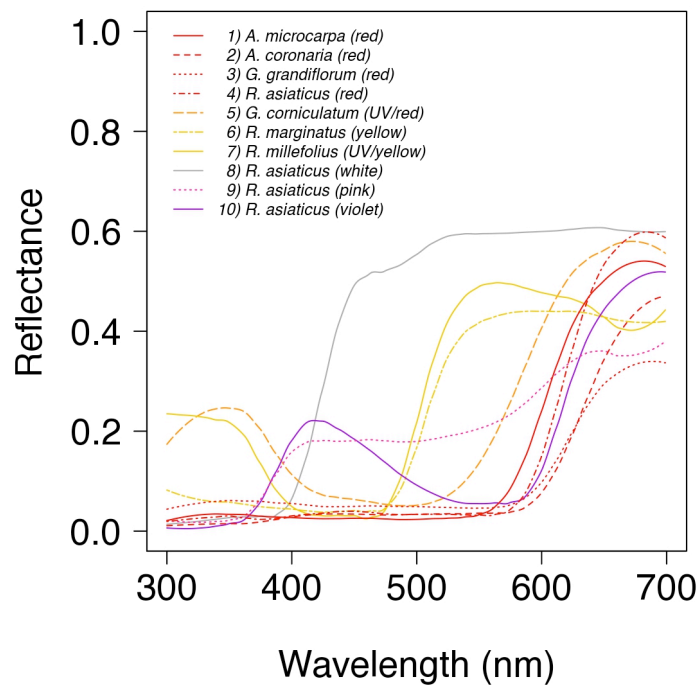


Fig. 1.4 Spectral reflectance functions of flowers from the plant species included in the analysis. The spectra correspond to the flowers categorized as red (*A. microcarpa*, *A. coronaria*, *G. grandiflorum*, *R. asiaticus*), UV/red (*G. corniculatum*), yellow (*R. marginatus*), UV/yellow (*R. millefolius*), and white, purple and pink (*R. asiaticus*).

Color loci of flowers and leaves were plotted in the chromaticity diagrams of *P. israelitus* and the honeybee (Fig. 1.5a, b). In these chromaticity diagrams the Euclidean distance corresponds to color distance as calculated according to the receptor noise-limited color opponent model (see 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. 1.5a), i.e. compared to leaves flowers provide a stronger positive red-green signal. In the chromaticity diagram of the honeybee (Fig. 1.5b), 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.

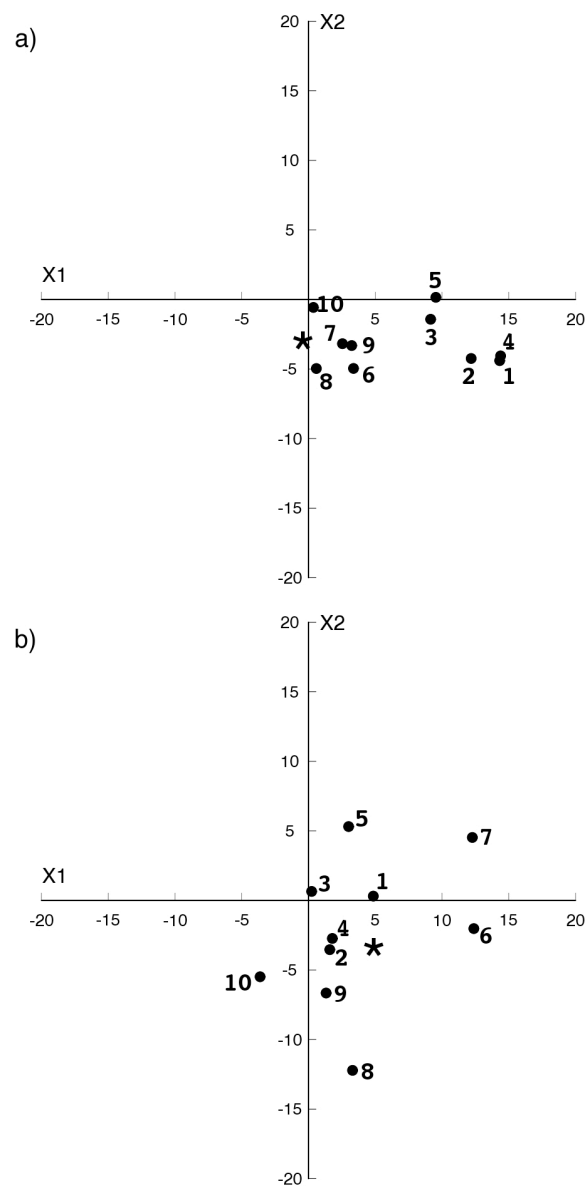


Fig. 1.5 Loci of flowers (dots) and the locus calculated from the average of all leaf spectra (asterisk) in *Pygopleurus israelitus* (a) and *Apis mellifera* (b) chromaticity diagrams. For each point a number was given to identify the species. The loci correspond to: 1-*Adonis microcarpa* (red), 2-*Anemone 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)

To evaluate the effect of different spectral sensitivities on the ability to detect different flower colors against a background of leaves, I 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 1.3). 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 1.3). 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.

To quantify the difference between beetles and honeybees with respect to their ability to discriminate flower colors, I 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*.

Table 1.3 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). Distances are given in standard units.

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

Discussion

Structural basis for color vision in *Pygopleurus israelitus*

The 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 do 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 receptor 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. The data does not allow 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. 1.3a, 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 portion 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 the 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, the results suggest that *P. israelitus* do not possess 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, reveal 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, the 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. 1.2c), making some form of filtering a likely mechanism influencing the spectral sensitivity of *P. israelitus*' red photoreceptor. To my knowledge this represents the first report in 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 modeling presented here 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. Additionally, 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 suggest 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 South-East 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*, the results presented here 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 South-East 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: Sacarabaoidea: 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 South East 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 overlap 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 (Kearse et al. 2010) (Fig. 1.1). 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, represent 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.

Floral color divergence associated to *Papaver rhoeas*' (Papaveraceae) historical range expansion: implications on flower appearance for insect pollinators

Abstract

Papaver rhoeas is a widespread agricultural weed, which according to Archeological and Taxonomic evidence was introduced into Central Europe from the Levant region about 5 thousand years ago. In the present work I present data showing that populations of *P. rhoeas* have diverged with respect to their flower color composition. While spectral measurements taken in Central Europe revealed populations composed exclusively by individuals having UV-reflecting flowers, measurements taken along its native distribution range revealed populations composed almost exclusively by individuals having UV-absorbing flowers. The likely introduction of *P. rhoeas* into Central Europe induced a shift from beetle (Glaphyridae) to bee pollination. These pollinators appear to differ basically with respect to their receptor-based color vision. The impact of *P. rhoeas*' intra-specific flower color differences on the appearance of flowers for insect pollinators was evaluated by comparing the distribution of flower color loci in the chromaticity diagram of *Pygopleurus israelitus* (Glaphyridae) and the honeybee *Apis mellifera* (Hymenoptera). The results of this evaluation revealed that the differences in UV reflectance found within *P. rhoeas* would be sufficient as to allow insect pollinators to discriminate between color morphs. The distribution of flower color loci in the color space of the glaphyrid beetle shows that both types of color morphs occupy loci well separated from the loci occupy by leaves. In the chromaticity diagram of the honeybee, on the other hand, while UV-reflecting flowers occupy loci well separated from leaves, the loci occupy by UV-absorbing flowers largely overlap the loci occupy by green foliage. These results indicate that *P. rhoeas* has diverged with respect to its flower color appearance for insect pollinators and suggest that the absence of UV-absorbing flowers of *P. rhoeas* in Central Europe is congruent with the historical shift from beetle to bee pollination undergone by populations that established in this region.

Introduction

Animal pollinators have long been considered to play a major role in speciation of flowering plants (Darwin 1876; Grant 1949; Levin 1971; Grant 1994). Central to this idea is the assumption that based on their preferences and their capacities to perceive and learn floral attributes pollinators would tend to specialize on the flowers they visit. Under such scenario, speciation is thought to occur through initially flower polymorphic variants, which would present combinations of floral attributes promoting visits of different type of pollinators (Grant 1949; Grant 1994). The process of floral isolation mediated by pollinators (“ethological isolation”, sensu Grant 1949) would in turn lead to population divergence and would contribute to the origin and maintenance of ethological reproductive barriers to hybridization (Grant 1952; Levin 1971; Grant 1994). Despite the fact that much of the research on pollination has based on this theoretical framework, the role of pollinators in speciation of angiosperms is still controversial (see Waser 1998; Wasser 2001; Fenster et al. 2004). The rather generalized interactions reported for many pollination system together with the theoretical difficulties to explain sympatric speciation under incomplete assortative mating, has contributed to the skepticism with respect to the role of ethological isolation in plant evolution (Grant 1994; Waser et al. 1996; Waser 1998). Considering that plant-pollinator interactions fluctuate in time and space, floral variations need to be evaluated under a historical perspective in order to get insights about the contingencies under which such variability might have evolved. A main issue along the idea of pollinator-mediated speciation is whether pollinator preferences (or sensory constraints) for alternative floral traits would be consistent enough as to drive floral isolation. The lack of studies examining the correlation between phenotypic divergence in natural populations and pollinators biases can be explain by the difficulties associated with conducting experiments with plants and pollinators under natural conditions and over evolutionary relevant time periods (but see Gegeer and Burns 2007). Introduced plants allow overcoming these difficulties. Due to human-induced geographical expansion, introduced plants are exposed to fluctuating and some times dramatic changes in their pollinator environments, providing natural experiments to evaluate the influence of such changes on flower divergence over defined geographical, ecological and temporal scale.

Within this context I have studied flower color evolution in *Papaver rhoeas* (Papaveraceae), a widespread agricultural weed inside and outside of Europe. Based on taxonomic and archaeological evidence (Godwin 1975; Willerding 1986), *P. rhoeas* has

been postulated as a synanthropic species (human-related) (Kadereit 1990), both with respect to its distribution as well as its origin. Fossil evidence, which goes back to the Middle Neolithic in Central Europe, suggests that *P. rhoeas* was introduced in this region from the eastern border of the Mediterranean westwards into Europe as consequence of the spread of agriculture about 5 thousand years ago (Zohary and Hopf 1994). Ever since Lotmar (1933) measured its spectral reflection, *P. rhoeas* has served as an example of a species having UV-reflecting flowers (Kugler 1947; Daumer 1958; Menzel and Shmida 1993). A report from Dafni (et al. 1990) suggests, however, that populations of *P. rhoeas* might differ with respect to their flower color composition. Color represents an important floral attribute affecting flower/pollinator interactions. It represents the most important flower property for long distance detection, recognition and discrimination (Proctor and Yeo 1972; Waser 1983; Giurfa et al. 1996; Giurfa and Menzel 1997; Menzel et al. 1997; Vorobyev et al. 1997) and both intra- and inter-specific color variations have been documented to influence the selective behaviors of pollinators (e.g., Levin 1972; Kay 1976; Waser and Price 1981; Gumbert et al. 1999; Schemske and Bradshaw 1999). In the case of *P. rhoeas*, intra-specific flower color differences would result intriguing considering that the introduction of this species into Europe caused a shift in the type of pollinators this species interacts with. While hymenopterans represent the main visitors of *P. rhoeas* in Central Europe (McNaughton and Harper 1960; Dobson et al. 1996), Mediterranean populations are mainly visited by glaphyrid beetles (Dafni et al. 1990; Bosch et al. 1997). These two types of pollinator groups appear to differ fundamentally with respect to their receptor based color vision. The compound eyes of the glaphyrid beetle *Pygopleurus israelitus* (Coleoptera, Glaphyridae) were found to contain photoreceptors maximally sensitive in the UV, green and red regions of the spectrum (Martínez-Harms et al. 2012, Fig. 1.2). Hymenopterans, on the other hand, are known to have a trichromatic color vision system with photoreceptors maximally sensitive in the UV, blue and green region of the spectrum (Peitsch et al. 1992). Accordingly, flower color differences within *P. rhoeas* might affect their appearance for glaphyrid beetles and bees differently.

In the present study I have examined potential color differences within *P. rhoeas* by evaluating population flower color composition along its distribution range in the Mediterranean and Central Europe. Additionally, differences in flower appearance for insect pollinators were evaluated by comparing the distribution of flower color loci in the chromaticity diagram of *Pygopleurus israelitus* and the honeybee *Apis mellifera*.

Methods

Flower reflectance spectra

Flowers of *P. rhoeas* were collected from 10 different sites along its distribution range in the Levant region and from 8 different sites along its distribution range in Central Europe. Flower color was evaluated by means of spectral reflectance measurements and photographic techniques. Spectral reflectance measurements were carried out on flowers and leaves of samples collected in the field. The samples were kept fresh until spectral reflectance measurements were made. The reflectance spectra of flowers and foliage were measured using a spectrometer (model SD2000; Ocean Optics, Dunedin, FL) and measurements were taken between 300 and 700 nm. A white reflectance standard (Spectralon, 99%; Labsphere, North Sutton, NH) was used to calibrate the spectrometer. Sample patches were illuminated by a xenon lamp through a fiber optic under an angle of 45 ° to the optical axis of a fiber optic that collected the light reflected by the sample. Flowers were additionally evaluated with respect to their spatial patterns of ultraviolet light (UV) reflectance by means of photographic techniques. UV exposures of whole flowers were taken using a digital camera EOS 10D (Canon USA Inc., Lake Success, NY, USA) especially modify for this purposes. The combination of a quartz lens (105 mm, UV-Nikkor, Nikon, Tokyo, Japan) with two types of band-pass filter allowed exposing the digital sensors of the camera to either UV or visible light. For UV exposures a narrow band-pass filter was used (Baader Venus U-Filter, Baader Planetarium, Mammendorf, Germany), which consisted in a optically polished Schott UG11 substrate with dielectric coating which totally blocked wavelengths in the visible and infrared parts of the spectrum while transmitting between 320 and 380 nm with a half-band-width of 60 nm. For exposures in the visible spectrum, I used a broad band-pass filter that transmitted light between 400 and 700 nm.

Modeling insect color perception.

I 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 is limited by the noise generated by the photoreceptors. The model does not make any assumptions about color opponent mechanisms and assumes that intensity cues (brightness) 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). In order to investigate how the spectral sensitivity of photoreceptors affects the distribution of colors in chromaticity diagrams and considering that noise levels for *P. israelitus*' receptors are not known, levels of noise set to the values measured in the honeybee were used to model the color vision of the glaphyrid beetle. This allows a comparison of the chromaticity diagram of the honeybee with the chromaticity 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 the quantum catch q_i of corresponding photoreceptor i was calculated,

$$q_i = k_i \int_{\lambda} I(\lambda) S(\lambda) R_i(\lambda) d\lambda , \quad (1)$$

where $R_i(\lambda)$ is the spectral sensitivity of receptor of type i , $S(\lambda)$ is the reflectance spectrum, $I(\lambda)$ is the illumination spectrum and k_i is a scaling factor. In the case of trichromatic vision, $i = S, M, L$ (corresponding to short-, medium-, and long-wavelength receptors respectively). Here I assume that illumination is a standard D65 daylight (Wyszecki and Stiles 1982). The scaling factor (k_i) was set so that the quantum catches for a green foliage background are equal to unity, giving a receptor contrast space (Cole et al. 1993).

$$k_i = 1 / \int_{\lambda} I(\lambda) S^b(\lambda) R_i(\lambda) d\lambda , \quad (2)$$

where $S^b(\lambda)$ is the mean spectrum calculated from the spectral measurements of leaves collected in the field.

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.

$$f_i = \ln(q_i), \quad (3)$$

To plot color stimuli, I use 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 axis 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 axis of chromatic diagram (Vorobyev and Osorio 1998). Moreover, color opponent mechanisms are generally not orthogonal to each other, while the axes of chromatic diagram 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 the chromaticity diagram 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 I use the axes corresponding to the respective L-M and S-[L+M] direction in the chromatic diagram (Kelber et al. 2003):

$$\begin{aligned} X_1 &= A(f_L - f_M), \\ X_2 &= B(f_S - (af_L + bf_M)), \end{aligned} \quad (4)$$

where:

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

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

$$a = \frac{(\omega_M)^2}{(\omega_L)^2 + (\omega_M)^2},$$

$$b = \frac{(\omega_L)^2}{(\omega_L)^2 + (\omega_M)^2},$$

The noise values were set to $\omega_S = 0.13$, $\omega_M = 0.06$ and $\omega_L = 0.12$ (Vorobyev et al. 2001a). The distance in the color space can be expressed as:

$$\Delta S^2 = \Delta X_1^2 + \Delta X_2^2, \quad (5)$$

Results

Spectral measurements were carried out on flowers from a total of 127 individuals along the geographical distribution range of *P. rhoeas* in the Levant region and from a total of 117 individuals along its geographical range in Central Europe. Measurements in populations from Central Europe revealed only individuals having flowers that reflected light in the UV range of the spectrum. The reflectance curve measured from these flowers show a reflectance peak between 300 and 400 nm, absorption between 400 and 550 nm and strong reflectance above 600 nm (Fig 2.1a). In contrast to what was found in Central Europe, measurements in populations from the Levant region revealed, almost exclusively, individuals having flowers that absorb UV light. Only one UV-reflecting flower was observed after analyzing UV exposures taken to large patches of flowers (data not shown). These flowers had reflectance functions characterized by strong absorption between 300 and 550 nm, while reflecting exclusively above 600 nm (Fig 2.1b). In order to evaluate the spatial patterns of flowers' UV reflectance, UV exposures were taken in the field. The images revealed that petals from flowers collected in Central Europe reflect UV light over the whole surface of the portion of the petals appearing red to the human eye (Fig 2.2a). Flowers collected in the Levant region, on the other hand, showed UV absorbance throughout the whole surface of the human-red portion of the petals (Fig. 2.2b). The results on the spectral properties of *P. rhoeas*' flowers indicate that populations of this species have diverged with respect to their flower color composition.

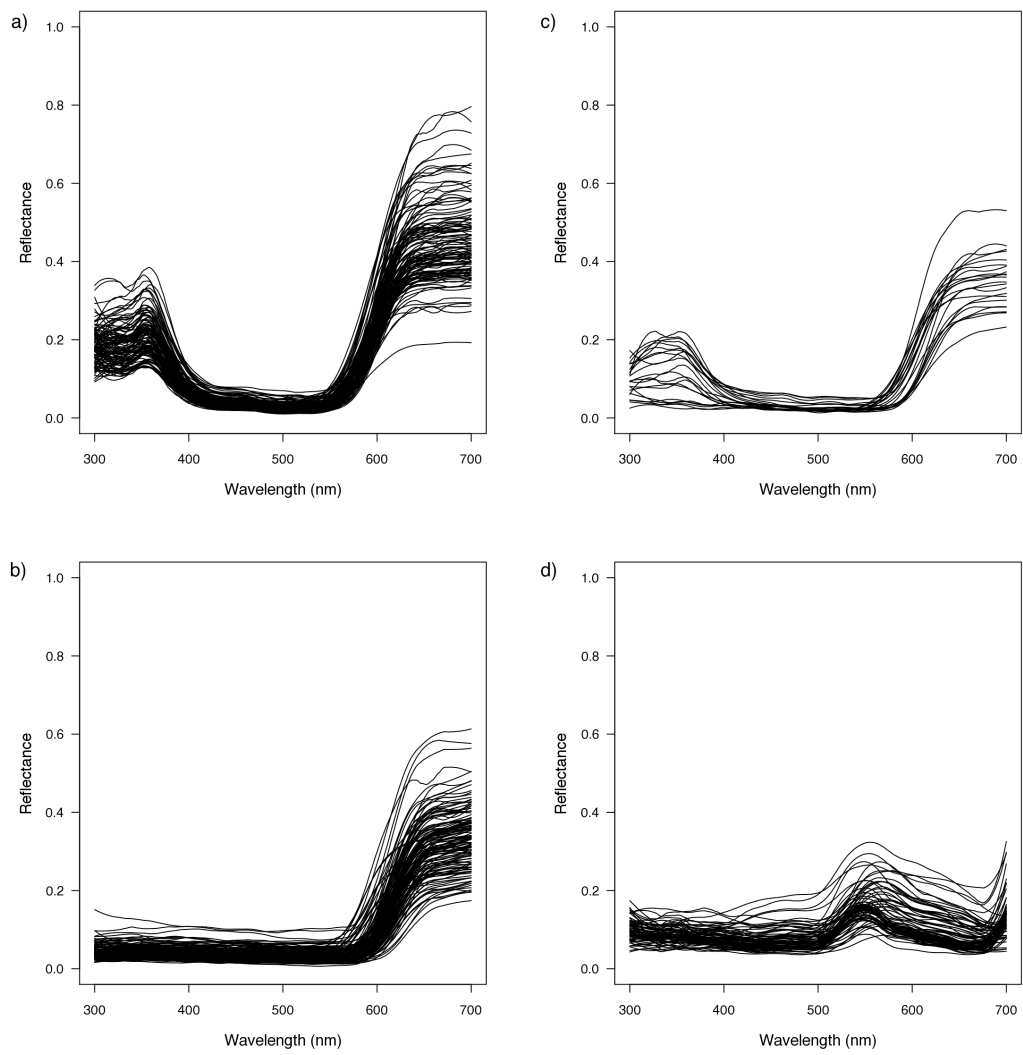


Fig. 2.1 Spectral reflectance functions of flowers and Leaves (c) of *Papaver rhoeas* from individuals collected along its distribution range in Central Europe (a), the Levant region (b), and Greece (c).

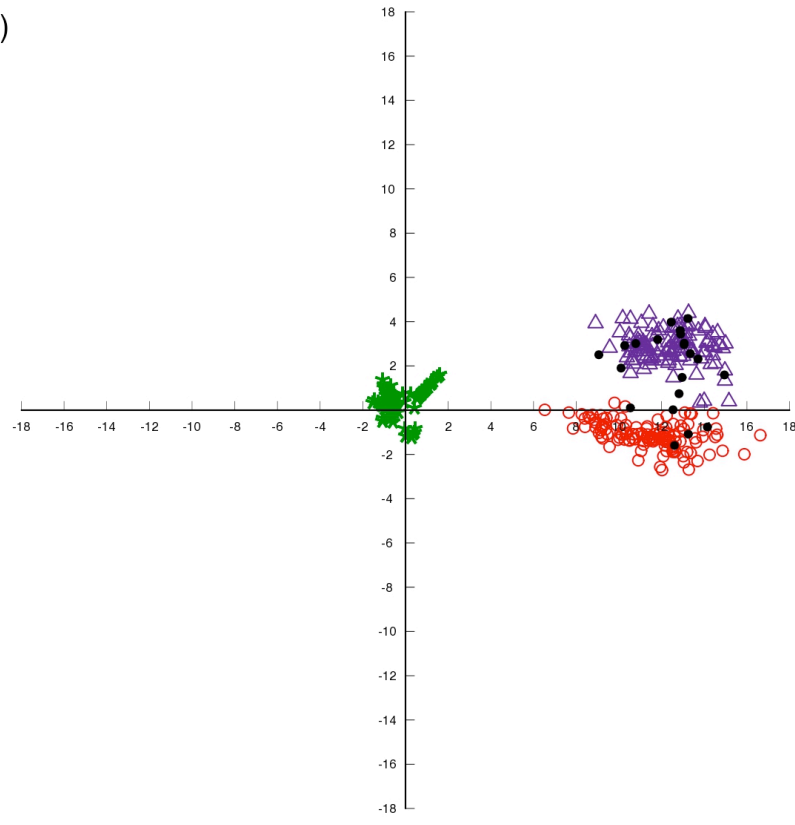


Fig. 2.2 Exposures of flowers of *P. rhoeas* collected in populations in Central Europe (a) and the Levant region (b). The images on the left correspond to flowers as perceived by the human eye, while the images on the right correspond to flowers as seen under UV light.

In order to evaluate how different the color morphs of *P. rhoeas* would appear for its insect pollinators, I compared the distribution of flower colors in the chromaticity diagram of glaphyrid beetles and bees. 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 perceptual space of beetles, the distribution of flower color loci revealed that UV-reflecting and UV-absorbing color morphs occupy loci well separated from the loci occupy by green foliage (Fig 2.3a). In both cases, the loci of flowers lie to the right

of the loci occupy by leaves, indicating that both cases flowers would provide a stronger red-green signal. Along the X_2 direction, on the other hand, the loci of the two morphs lie at different quadrants of the chromaticity diagram, with the loci of UV-reflecting flowers located in the upper quadrant. This difference indicates that UV-reflecting flowers induce a stronger UV signal than UV-absorbing flowers. The separation between the color loci of flowers and leaves indicate that a receptor-based color vision like the one found in *P. israelitus* would allow to discriminate both color morphs from a green foliage background based on their color. On the other hand, the separation between the loci of the different color morphs indicate that a receptor-based color vision like the one found in *P. israelitus* would allow to discriminate UV-reflecting from UV-absorbing flowers of *P. rhoeas* on the bases of their chromatic differences. In the chromaticity diagram of the honeybee, the distribution of flower color loci revealed that the two color morphs differ with respect to their separation from the loci occupy by leaves (Fig 2.3b). On the one hand, the loci occupy by UV-reflecting flowers are well separated and lie above the loci occupy by leaves, indicating that they provide stronger positive UV signal than leaves. This separation indicates that honeybees' would discriminate UV-reflecting flowers from a green foliage background on the bases on their color. The distribution of the loci occupy by UV-absorbing flowers, on the other hand, tend to overlap with the loci occupy by leaves. This overlap indicates that UV-absorbing flowers of *P. rhoeas* would appear similar in color to a green foliage background. The analysis of flower color loci distribution indicates that the receptor-based color vision characterizing *P. israelitus* and the honeybee would allow them to chromatically discriminate the two color morphs of *P. rhoeas*, i.e. the two morphs would appear as different for both type of pollinators. These results indicate that populations of *P. rhoeas* have diverged with respect to their flower color appearance for its insect pollinators. In addition, the results indicate that the differences in flower color found within *P. rhoeas* would affect flowers appearance for glaphyrid beetles and bees differently.

a)



b)

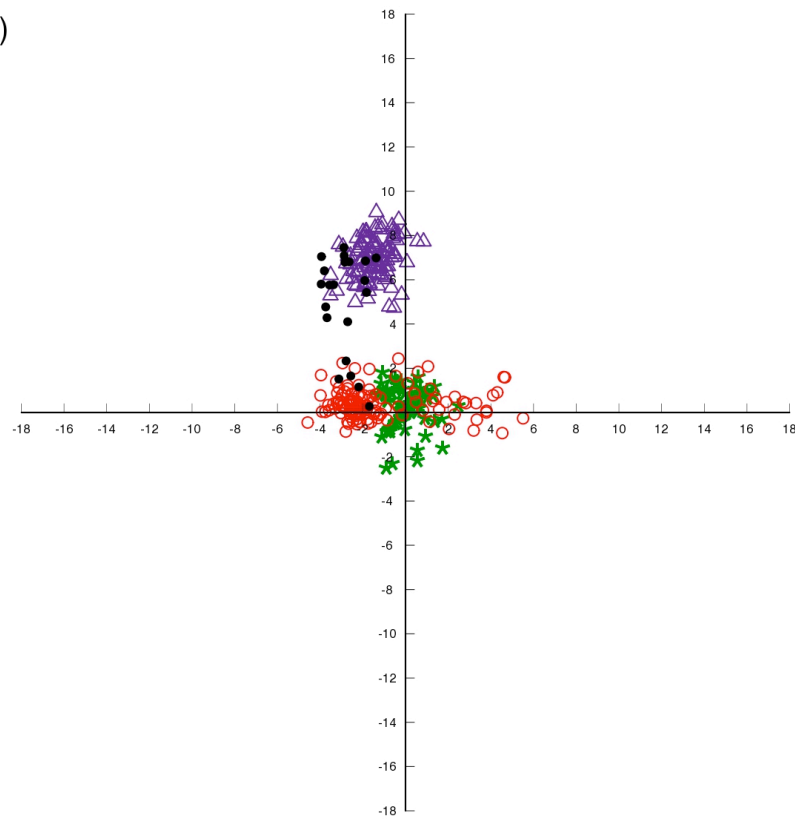


Fig. 2.3 Loci of leaves (green asterisk) and flowers of *P. rhoeas* from individuals collected along its distribution range in Central Europe (violet triangles), the Levant region (red circles), and Greece (black dots) in the chromaticity diagrams of *Pygopleurus israelitus* (a) and *Apis mellifera* (b).

Discussion.

Archeological and taxonomic evidence convincingly supports the hypothesis that *P. rhoeas* was introduced into Central Europe from the South East Mediterranean region as an agricultural weed about 5 thousand years ago (Godwin 1975; Willerding 1986; Zohary and Hopf 1994). The results presented here indicate that since then, populations of *P. rhoeas* have diverged with respect to their flower color composition. Spectral measurements carried out along *P. rhoeas*' distribution range in Central Europe confirmed earlier reports, revealing populations composed exclusively by individuals having UV-reflecting flowers. Spectral measurements taken in the South-East Mediterranean region, on the other hand, revealed populations composed almost exclusively by individuals having UV-absorbing flowers. In the survey carried out along this region, only one UV-reflecting flower was observed after analyzing UV exposures taken to large patches of flowers (data not shown), indicating that UV-reflecting flowers occur in low proportions in the South East Mediterranean. Additionally, it was observed that when cultivated in Central Europe (Berlin), individuals grown from seeds collected in the Mediterranean region developed UV-reflecting and UV-absorbing flowers, the later occurring in a much higher proportion than observed in the geographical survey of flower color presented here. In contrast to both previous cases, measurements taken in Greece revealed a scenario in which both type of color morphs were present in the same populations. Altogether, these results suggest the existence of an east/west cline of UV flower reflectance associated to *P. rhoeas*' historical distribution range expansion. Furthermore, and considering that populations of *P. rhoeas* in the South East Mediterranean region would resemble its ancestral condition, the results suggest that populations in Central Europe were initially polymorphic with respect to their flower color composition, and that the UV-absorbing flower morph of *P. rhoeas* was lost in this region.

As mentioned above, the introduction of *P. rhoeas* into Central Europe induced a shift from beetle to bee pollination in populations that established in this region. In

order to evaluate whether *P. rhoeas* has also diverged with respect to its flower color appearance for insect pollinators, the distribution of flower color loci was evaluated in the color space of *Pygopleurus israelitus* and the honeybee *Apis Mellifera*. These species differ basically with respect to their receptor-based color vision and were used as representatives of Glaphyridae and Hymenoptera, which represent the two main insect pollinator groups of *P. rhoeas*. While *P. israelitus* was found to have photoreceptors maximally sensitive to UV, green and red, the honeybee has a set of photoreceptors with sensitivity peaks in the UV, blue and green. The distribution of flower color loci revealed that flowers from Central European populations are well separated from flowers collected in populations from the Levant region, both in the chromaticity diagram of the glaphyrid beetle and the honeybee. This separation indicates that populations of *P. rhoeas* have diverged with respect to their flower color appearance for its main insect pollinator groups. Furthermore, the results indicate that differences in flower color within *P. rhoeas* would affect their appearance for glaphyrid beetles and bees differently. As seen in the chromaticity diagram of *P. israelitus* (Fig 2.3a), both color morphs occupy loci well separated from the loci occupy by leaves. This separation indicates that a receptor-based color vision like the one found in this beetle species would allow good discrimination of both color morphs from a foliage background based on their chromatic properties. In the chromaticity diagram of the honeybee (Fig 2.3b), on the other hand, the loci occupy by UV-absorbing flowers tend to overlap the loci occupy by leaves, indicating that red UV-absorbing flowers and leaves would appear similar in color for bees. This result is not surprising considering that honeybees lack a receptor sensitive in the red range of the spectrum and that the detection of pure red flowers by trichromatic bees seems to involve mechanisms other than their color vision (Martínez-Harms et al. 2010). In contrast to the overlap between UV-absorbing flowers and leaves, red UV-reflecting flowers occupy loci well separated from the loci occupy by leaves, indicating that petal reflectance in the UV part of the spectrum adds a chromatic component that would allow bees to discriminate these flowers from a green background through chromatic mechanisms. From this it can be argued that bees, at least with respect to their color vision, would present a sensory bias towards UV-reflecting flowers of *P. rhoeas*. These results also suggest that the historical scenario presented by the divergence in flower color within *P. rhoeas* would resemble the scenario under which pollinator-mediated speciation is thought to occur. As indicated by the results presented here, Central European populations of *P. rhoeas*

seem to have originated from initially color polymorphic populations, composed by individuals having flowers with different levels of UV reflectance. Considering bees' sensory constraints, the transition from flower polymorphic populations to populations composed exclusively by individuals having UV-reflecting flowers would be congruent with the transition from beetle to bee pollination undergone by this species as it was introduced northwards into Europe.

Central to the view of pollinator-mediated speciation is the assumption that pollination systems tend towards specialization. This view has been questioned because studies in pollination have repeatedly demonstrated that animal-plant interactions, even when assumed to be specialized, tend to be more generalized than previously thought (e.g. Grant 1994; Waser et al. 1996; Waser 1998). Despite the fact that dramatic specialization in pollination systems does occur, the theoretical difficulties to explain sympatric speciation under incomplete assortative mating have raised doubts about the role of pollinators in plant speciation (Grant 1994; Waser 1998). To resolve this apparent paradox, Ollerton (1996) suggested that present floral diversity would represent periods of specialization to taxonomically restricted range of pollinators in which most floral evolution would take place. Accordingly, the history of a particular genus or species would include periods of specialization and generalization disrupted by fluctuations in pollinator environment. Considering that taxonomically different groups of pollinators might differ in aspects so diverse as their behavior, sensory capacities, physiology and morphology, the extent to which a floral attribute can be interpreted as promoting either specialized or generalized interactions needs to be evaluated under defined pollinator environments. Fluctuations in pollinator environment along the distribution range of *P. rhoeas* together with the difference in color vision between its main insect pollinator groups, allows evaluating flower color under different ecological scenarios. As discussed above, while both bees and glaphyrids have the receptor-based color vision to perceive red UV-reflecting flowers based on their color, only glaphyrid beetles would have the receptor-based color vision to perceive red UV-absorbing flowers of *P. rhoeas* as colorful. From this it could be argued that when beetles and bees form part of the pollinator environment, reflectance in the UV by flowers of *P. rhoeas* could favor generalized plant-pollinator interactions. This scenario corresponds to the one populations in the Mediterranean region are exposed to. The distribution of glaphyrid beetles and hymenopterans overlap along this region and both type of pollinators can be found visiting flowers of *P. rhoeas*. In contrast, under an ecological scenario in which

only hymenopterans interact with populations of *P. rhoeas*, as in the case of populations in Central Europe, flower reflectance in the UV could be interpreted as a trait promoting specialized interactions between those flowers and bees. These different scenarios suggest that the geographical expansion of *P. rhoeas* exposed this species to fluctuating pollinator environments under which either generalized or specialized plant-pollinator interactions could have been established. In line with this, the differences in flower color diversity between populations revealed by the results presented here would reflect the different ecological contexts under which populations have developed along *P. rhoeas*' current distribution range. These contrasting scenarios suggest that the contingencies provided by pollinator environments could determine whether a pollination system would tend towards specialization or generalization, indicating that these do not represent evolutionary tendencies by them self, but can result as consequence of the historical contingencies undergone by pollination systems.

Despite being considered one of the major threats to biodiversity (Wilcove et al. 1998; Pimentel et al. 2001; Pauchard and Shea 2006), exotic plant species provide natural experiments that enable researchers to study ecological and evolutionary processes across defined spatial and temporal scales. Within this context, and given that sexual reproduction represents an important aspect determining the establishment of plants into new environments, it has been argue that reproductive traits may be subject to evolutionary changes. Evidence of divergence in reproductive traits included reports of divergence of floral morphological characters promoting self-pollination and reproductive phenology (Barrett et al. 2008). The results presented here represent the first evidence of evolutionary changes in floral morphology with potential consequences in flower-pollinator interactions. These findings highlight *P. rhoeas* as a unique model to study the role of mutualisms in biological invasions and the role of pollinators in the evolution of flowering plants.

Color generalization after differential learning in the honeybee *Apis mellifera*.**Abstract**

Stimuli generalization accounts for the cognitive act of treating different stimuli as equivalent. It is often found that similarities along one or various perceptual dimensions, combined with knowledge about how stimuli vary across those dimensions, determines the degree of generalization. Using the receptor noise limited model of color vision to define a perceptual continuum, generalization of perceptually similar colors after differential learning was investigated in the honeybee *Apis mellifera*. To this end, honeybee foragers were differentially trained to discriminate between a rewarded (CS+) and an unrewarded (CS-) stimulus, and then tested with respect to their response towards the former CS+ when presented against a novel color. In particular, I evaluated how the position in the color space of a novel color relative to a CS+ and a CS- affects generalization. The results revealed that when the novel color varied from CS- in the same way as CS+ but to lower extent, subjects maintained their stronger response towards the former CS+ stimulus. In contrast, when the novel stimuli varied from CS- in the same way as CS+ but to a greater extent, subjects shifted their strongest response away from CS- towards the novel stimuli. These results revealed the occurrence of ‘peak shift’ in the honeybees’ color vision and indicate that the honeybee can learn color stimuli in relational terms based on their chromatic properties.

Introduction

Stimulus generalization can be defined as “the behavioral fact that a conditioned response formed to one stimulus may also be elicited by other stimuli which have not been used in the course of conditioning” (Hilgard and Marquis 1940). Similarity along one or various perceptual dimensions, combined with knowledge about how stimuli vary along those dimensions, seems to correlate reliably with the degree of generalization between stimuli (Shepard 1987). After training subjects with a single exemplar on a physical or sensory continuum (absolute learning) it is often found that the farther a novel stimuli is from the training exemplar the less likely it is to be treated as equivalent (Spence 1937; Shepard 1987). In natural conditions, however, animals do not encounter isolated stimuli, but rather arrays of multiple stimuli that seldom appear twice identical. Under such scenarios and depending on the history of interactions with those objects, animals can learn to respond distinctively to different stimuli (differential learning). In experiments of generalization after differential learning, the response to novel stimuli is usually tested in subjects trained to discriminate a positively reinforced stimulus (CS+) from an unrewarded (or negatively reinforced) stimulus (CS-). Borrowing the terms of excitation and inhibition from neurophysiology, Pavlov (1927) proposed that this kind of learning induces both excitatory learning for the CS+ and inhibitory learning for CS-. Spence (1937) extended this idea and proposed that the response to novel stimuli after differential learning would be jointly influenced by the generalization for CS+ and CS-.

A very well established form of generalization in animal and human perception following differential training is the so call “peak shift” phenomenon (Thomas et al. 1991; Wills and Mackintosh 1998; Ghirlanda and Enquist 2003; Lynn et al. 2005). This refers to a change in the strongest response away from the higher training value stimulus (CS+) towards a novel stimulus that differs from CS- in the same way as the former but to a greater extent. Köhler (1918/1938) carried out the first experiments describing this behavioral phenomenon. His earlier experiments consisted of training subjects (first done with chickens and later with chimpanzees) to discriminate between a rewarded lighter shade of grey vs. an unrewarded darker shade of grey. After subjects had learned the discriminatory task, the response towards the former rewarded stimulus was tested against a novel, even lighter shade of grey. Köhler reported that subjects selected the novel shade in over 70% of the trials, and suggested that animals learned to respond to the lighter of the two shades (i.e. to the relationship between stimuli) rather

than to the absolute shade value of the stimuli. Accordingly, when the absolute value of the stimuli was varied, but the relationship between them was held constant, animals transposed their preference to the “relationally correct” stimulus. Since then, this psychological phenomenon has been reported to occur in a number of different animals and for a variety of sensory modalities (e.g. Thomas et al. 1991; Wills and Mackintosh 1998; Ghirlanda and Enquist 2003). An important aspect to consider when establishing the occurrence of a cognitive phenomenon such as “peak shift” is to define a perceptual continuum for specifying this form of generalization (Shepard 1987; Baddeley et al. 2007). To investigate high-level sensory processes, such as generalization or categorization, it is desirable to have a measure of stimuli that is independent of the process itself. Given that spectral stimuli form a physical and perceptual continuum and that noise in photoreceptors ultimately sets sensory thresholds (Kelber et al. 2003), color is an appropriate subject to investigate generalization.

The honeybee *Apis mellifera* represents a useful model for studying generalization of color stimuli. They rely strongly on color cues to find flowers and due to their remarkable learning and memory capabilities, it is relatively easy to train honeybee foragers by using a single or a set of colored targets and test their response to stimuli they have not experienced during the course of training. These characteristics have motivated extensive behavioral and physiological studies that have allowed extracting the dimensions and quantitative interactions of parameters characterizing honeybees’ color vision (Backhaus and Menzel 1987; Backhaus et al. 1987). Honeybees have trichromatic color vision with photoreceptors maximally sensitive in the ultraviolet (S-receptor, $\lambda_{\max}=344\text{nm}$), blue (M receptors, $\lambda_{\max}=436\text{nm}$) and green (L receptors, $\lambda_{\max}=544\text{nm}$) regions of the spectrum (Peitsch et al. 1992). Based on these photoreceptors and depending on the angular size subtended by a visual stimulus, bees can discriminate colored targets through chromatic or achromatic cues. While discrimination of targets subtending small visual angles (<15 deg.) is mediated exclusively by bees’ L-receptor achromatic pathway, chromatic discrimination is only possible at visual angles larger than 15 deg. (Giurfa et al. 1996). Given that color discrimination threshold in honeybees is well predicted by a model assuming that discrimination performance (excluding brightness) is limited by photoreceptor noise (Vorobyev and Brandt 1996; Vorobyev et al. 2001a), receptor excitation represents a parsimonious way to account for spectral data in terms of relatively low-level processes.

I have used this knowledge to specify a continuum in the color space of honeybees in order to study color generalization.

Previous studies have focused on how differential learning affects color discrimination capacities in honeybees (Giurfa 2004; Avargues-Weber et al. 2010). Although, studies of generalization after differential conditioning reported the occurrence of peak shift in bumblebees color vision (Lynn et al. 2005), no study has evaluated color generalization after differential learning in honeybees. In the present work I evaluated how honeybees generalize colors after being differentially trained to discriminate perceptually similar colors and in particular, the effect of varying the position in the color space of a novel stimulus relative to a rewarded (CS+) and unrewarded stimulus (CS-) in the occurrence of peak shift.

Materials and Methods

Experimental setup and training procedure

A group of free-flying honeybees, *Apis mellifera L.*, were trained to collect 30% sucrose solution in a feeder located at 30m from the hive. Foragers were individually marked and trained to enter an experimental setup to collect from then on 50% sucrose solution from vertically presented colored targets. Only one bee was trained and tested at a time. The experimental setup consisted in a wooden Y-shaped maze with a UV-transparent plexiglas ceiling to ensure daylight conditions within the maze. A sliding door ensured that only one bee at a time could enter the maze. Bees were trained to fly through an entrance hole in the middle of the frontal panel to enter a decision chamber, once in the decision chamber they were able to see both back walls of the maze simultaneously. The back walls measured 20 X 20 cm² and had a 0.6 cm diameter central hole, through which sucrose solution could be delivered. The stimuli were presented vertically on the center of the back walls and their position was constantly exchanged in a pseudorandom way to ensure that the bees did not associate the reward with any particular side of the maze. This experimental setup presents the advantage of allowing controlling the angular size of the stimuli. In the experiments I used colored targets with a diameter of 8 cm, which located at 15 cm from the decision chamber subtended an angular sizes of 30°. Foragers were trained to discriminate a rewarded colored target (CS+) from an

unrewarded one (CS-), presented over a grey background. The training consisted in allowing the bees to enter the decision chamber and approach the training stimuli. When the bee chose the arm containing the CS+ they were rewarded with sugar water delivered at the stimulus. Once it filled its crop it returned to the hive, which finished the learning trail. If the bee entered the arm containing the CS-, it was gently pushed out of the maze by opening the ceiling and hat to enter the maze again. This procedure was repeated until the bee made a correct choice, then it was rewarded and allowed to return to the hive.

Defining a color perceptual continuum

I used the color opponent receptor noise-limited model (Vorobyev et al., 2001a; Vorobyev and Osorio, 1998) to estimate similarities between colors. This model is based on the assumption that detection and discrimination of light stimuli is limited by the noise generated by the photoreceptors. Intensity cues (brightness) are assumed to be ignored. The model predictions agree with the results of behavioral experiments in a number of animals (Vorobyev et al., 2001a; Vorobyev and Osorio, 1998; Koshitaka et al., 2008). The parameters of the model are the photoreceptor noise levels (Vorobyev et al., 2001a). For each color stimuli I calculated the quantum catch q_i of corresponding photoreceptor i ,

$$q_i = k_i \int_{\lambda} I(\lambda) S(\lambda) R_i(\lambda) d\lambda , \quad (1)$$

where $R_i(\lambda)$ is the spectral sensitivity of receptor of type i , $S(\lambda)$ is the reflectance spectrum, $I(\lambda)$ is the illumination spectrum and k_i is a scaling factor. In the case of trichromatic vision, $i = S, M, L$ (corresponding to short-, medium-, and long-wavelength receptors respectively). Here I assume that illumination is a standard D65 daylight (Wyszecki and Stiles 1982). The scaling factor (k_i) was set so that the quantum catches for the grey background are equal to unity, giving a receptor contrast space (Cole et al. 1993).

$$k_i = 1 / \int_{\lambda} I(\lambda) S^b(\lambda) R_i(\lambda) d\lambda , \quad (2)$$

where $S^b(\lambda)$ is the spectrum of the grey background.

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,

$$f_i = \ln(q_i), \quad (3)$$

To plot color stimuli, I use a chromaticity diagram where the Euclidean distance between the points corresponds to the predicted ability to discriminate the stimuli. Here I use the axes corresponding to the respective L-M and S-[L+M] direction in the chromatic diagram (Kelber et al., 2003):

$$\begin{aligned} X_1 &= A(f_L - f_M), \\ X_2 &= B(f_S - (af_L + bf_M)), \end{aligned} \quad (4)$$

where:

$$\begin{aligned} A &= \frac{1}{\sqrt{(\omega_L)^2 + (\omega_M)^2}}, \\ B &= \sqrt{\frac{(\omega_L)^2 + (\omega_M)^2}{(\omega_L)^2(\omega_M)^2 + (\omega_S)^2(\omega_L)^2 + (\omega_S)^2(\omega_M)^2}}, \\ a &= \frac{(\omega_M)^2}{(\omega_L)^2 + (\omega_M)^2}, \\ b &= \frac{(\omega_L)^2}{(\omega_L)^2 + (\omega_M)^2}. \end{aligned}$$

The noise values were set to $\omega_S = 0.13$, $\omega_M = 0.06$ and $\omega_L = 0.12$ (Vorobyev et al. 2001a). The distance in the color space can be expressed as:

$$\Delta S^2 = \Delta X_1^2 + \Delta X_2^2, \quad (5)$$

Test

After completing 20 training trials, bees' choices were tested under conditions of extinction (no reward presented) in a dual choice situation. During a period of 2 minutes I recorded the cumulative number of approaches to the targets that ended with the bee contacting the targets. All tests were done using fresh stimuli to avoid the influence of scent marks on test performance. Side asymmetries were compensated by testing each pair of colors twice and exchanging their position within the maze. Two reinforcement trials between tests of the same color pair and eight between tests of different color pairs were provided to avoid extinction of the response towards the stimuli. Two tests were carried out per bee. In a control test (pre-test) the level of response for the stimuli used for training was evaluated by presenting the CS+ and the CS- in the Y-maze. In a second test the color previously used as CS+ was presented in the Y-maze together with a novel stimulus. Three sets of colors, containing three colors each (Fig. 3.1), were used to study the effect of varying the position of a novel stimulus in the color space with respect to a CS+ and a CS- stimulus on generalization. Colors were chosen so they would lie approximately in a line in the honeybee color space (Fig. 3.2). By maintaining the identity of the CS- color fixed and exchanging the identity of the other two colors as novel and CS+ stimuli, each set of colors was used for two types of experiments. Values of chromatic distance between color stimuli and their respective CS- stimulus, as well as their loci in the color space of the honeybee are given in Table 3.1 and Fig. 3.2 respectively. In the type I experiment, corresponding to experimental condition 1, 2 and 3, the stimuli whose loci lied farther away in the color space from their respective CS- were used as novel stimuli (S_3, S_6, S_9), while the stimuli whose loci lied closer from CS- were used as CS+ (S_2, S_5, S_8) (Fig. 3.1, Fig. 3.2, Table 3.1); in the type II experiment, corresponding to experimental condition 1', 2' and 3', the stimuli located farther away in the chromaticity diagram from their respective CS- were used as CS+ (S_3, S_6, S_9), while the stimuli whose loci lied closer from CS- were used as novel stimuli (S_2, S_5, S_8) (Fig. 3.1, Fig. 3.2, Table 3.1).

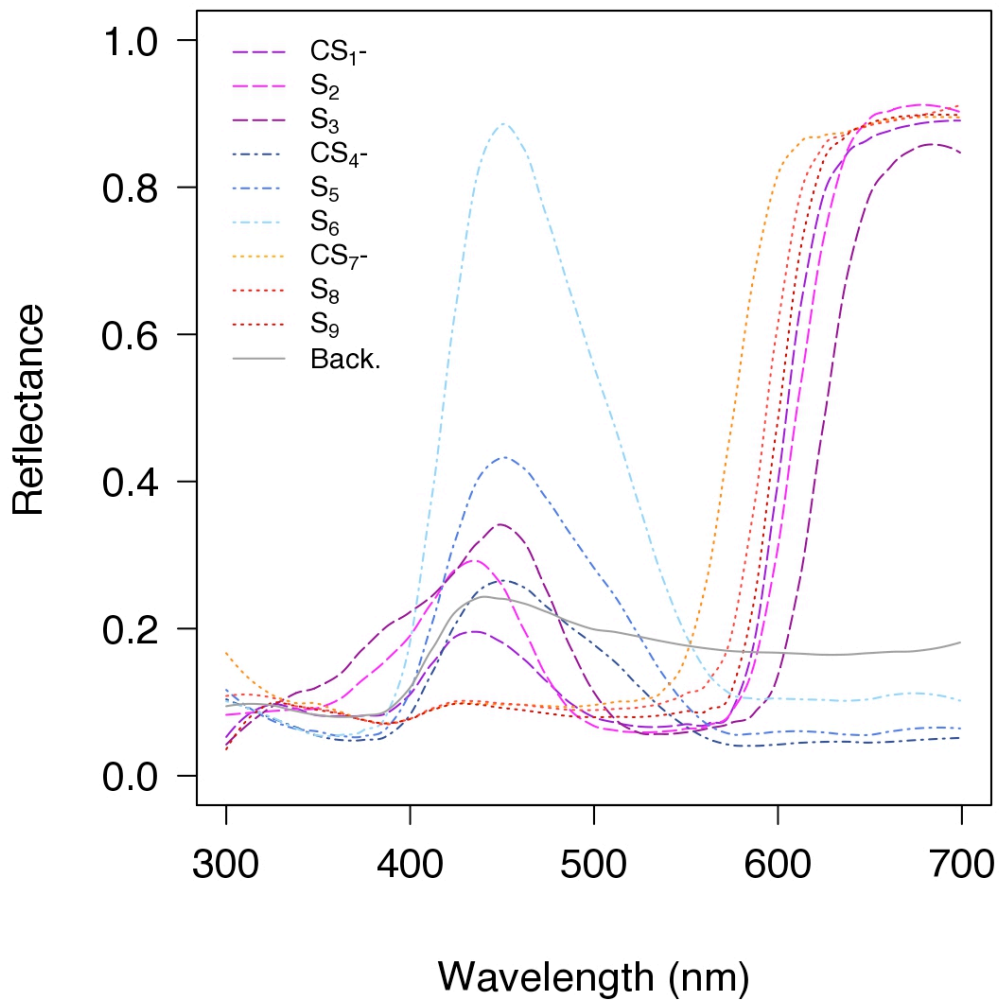


Fig. 3.1 Spectral reflectance functions of the grey background (Back.)(HKS-92N) and the three set of colored papers used in the experiments. The color of the curves illustrate the colors as they appear to humans. The colored stimuli CS₁- (HKS-24N), S₂ (HKS-26N) and S₃ (HKS-29N) were used under the experimental condition 1 and 1'. The colored stimuli CS₄-, S₅ and S₆ were produced by means of a color printer and were used under experimental condition 2 and 2'. The colored stimuli CS₇- (HKS-8N), S₈ (HKS-12N) and S₉ (HKS-14N) were used under experimental condition 3 and 3'.

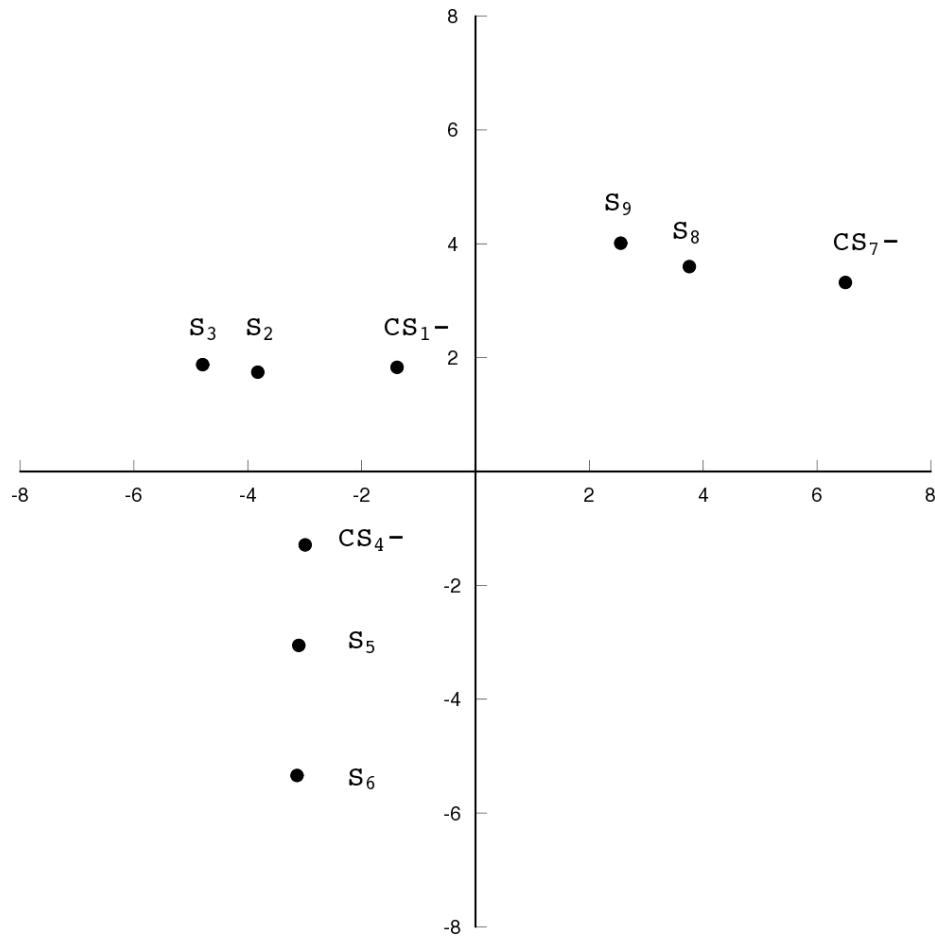


Fig. 3.2 Loci of color stimuli used in the experiments in the chromaticity diagram of *Apis mellifera*.

Table 3.1. Photoreceptor specific contrast to the grey background, chromatic coordinates, and chromatic distances (ΔS) to the respective CS- stimuli for the colors used in the experiments. Values were calculated according to the receptor noise-limited model (Vorobyev and Osorio 1998; Vorobyev et al. 2001a). Chromatic distances are given in standard units.

Color	qS	qM	qL	X_1	X_2	ΔS
CS ₁ -	0.95	0.76	0.63	-1.38	1.83	
S ₂	1.24	1.08	0.64	-3.82	1.74	2.44
S ₃	1.53	1.34	0.70	-4.79	1.88	3.42
CS ₄ -	0.75	0.97	0.65	-2.99	-1.29	
S ₅	0.91	1.52	1.00	-3.10	-3.05	1.77
S ₆	1.30	3.00	1.97	-3.13	-5.34	4.05
CS ₇ -	0.91	0.48	1.14	6.50	3.32	
S ₈	0.88	0.48	0.79	3.76	3.60	2.76
S ₉	0.86	0.46	0.64	2.55	4.01	4.01

Statistics

The accumulative number bees' choices for each pair of colors were pooled after testing for homogeneity (heterogeneity G-test) and used to test the null hypothesis of random choice between colors of a pair by means of a log-likelihood ratio test for goodness of fit (G -test) (Sokal and Rohlf 1995). The level of response towards the rewarded color in the pre-test and test was compared by means of an ANOVA for repeated measures (Sokal and Rohlf 1995). The level of significance was set at 0.05 in all the analyses.

Results

The experiments tested how the position in the color space of a novel stimulus relative to a CS- and a CS+ affects generalization by bees in a dual choice situation. Two types of experiments were carried out: In the type I experiments, bees were trained to select a rewarded vs. an unrewarded color. Animals were then subjected to two unrewarded tests. A test in which the training colors were presented to the bees (pre-test), and a generalization test in which the former CS+ was tested against a novel color whose locus in the color space was located towards the same direction from CS- as CS+ but farther away (i.e. the novel color subtended higher chromatic contrast to CS- than CS+) (experimental condition 1, 2 and 3) (Fig. 3.3a, c and e); in the type II experiments, individuals were trained to a rewarded vs. an unrewarded color and then subjected to two unrewarded tests. A pre-test in which the training colors were presented to the bees and a generalization test in which subjects' response to the former CS+ was evaluated when presented against a novel color whose locus in the color space was between the locus of the CS- and the locus of the CS+ (i.e. the novel color subtended lower chromatic contrast to CS- than CS+) (experimental condition 1', 2' and 3') (Fig. 3.3b, d and f). Each type of experiment was performed on three different experimental groups ($n=4$ for each group).

Type I experiment

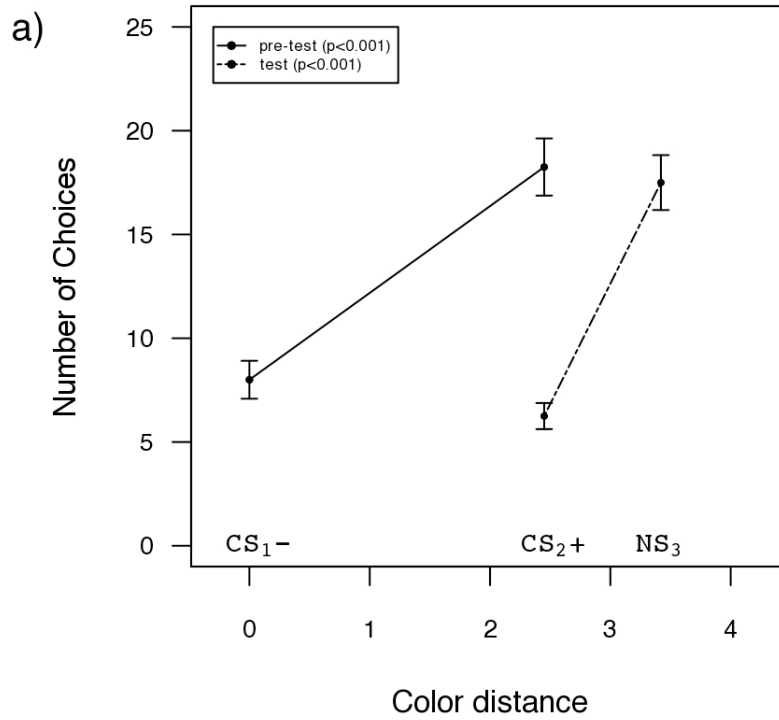
The experimental groups in condition 1, 2 and 3 were evaluated under the type I experiment. In condition 1, individuals were trained to CS₁- and CS₂+ and tested using S₃ as novel stimulus (Fig 3.2, Table 3.1). In condition 2, CS₄- and CS₅+ were used as training stimuli and S₆ as novel stimulus (Fig 3.2, Table 3.1). In condition 3, bees were trained to CS₇- and CS₈+ and tested using S₉ as novel color (Fig 3.2, Table 3.1). The results of the pre-test showed that after 20 training trials subjects gave their stronger response to the stimulus previously rewarded during training (Fig. 3.3a, c, e). The responses differed significantly from random for the three experimental groups (see Fig. 3.3 for P values), indicating that bees learned to respond differentially to the neutral and positive reinforced stimuli. In the generalization tests, the responses to the stimuli differed significantly from random in the three treatment groups (condition 1: $G=22.19$, $P<0.001$; condition 2: $G=7.02$, $P<0.01$; condition 3: $G=17.86$, $P<0.001$). An ANOVA for repeated measures revealed that in the generalization tests the responses towards the

former CS+ were significantly different to the responses towards the same stimulus in the pre-test (condition 1: $F_{1,3}=37.57, P<0.01$; condition 2: $F_{1,3}=12.51, P<0.05$; condition 3: $F_{3,9}=37.4, P<0.01$). Under this experimental condition, the responses towards the color stimuli revealed that when the novel stimulus subtended higher chromatic differences to CS- than CS+, subjects treated the novel and CS+ stimulus as different but shifted their stronger response away from CS- towards the novel color.

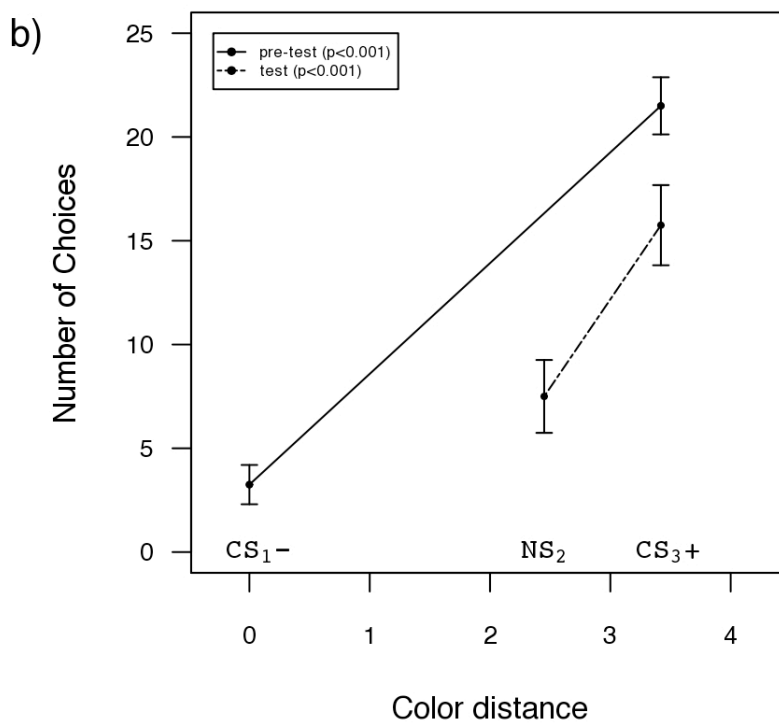
Type II experiment

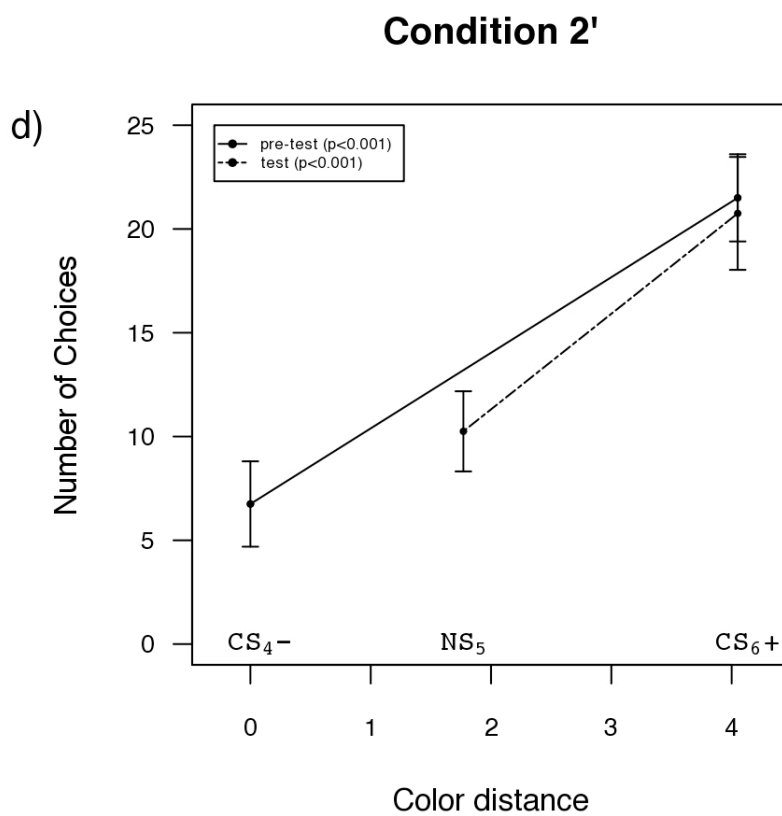
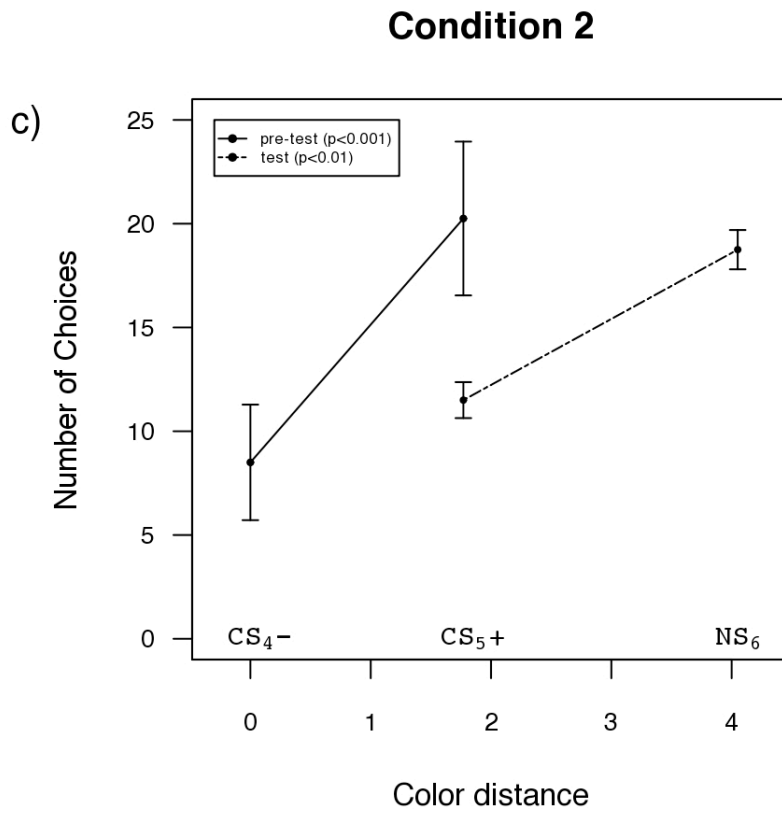
The experimental groups in condition 1', 2' and 3' were evaluated under the type II experiment. In condition 1', individuals were trained to CS₁₋ and CS₃₊ and tested using S₂ as novel stimulus (Fig 3.2, Table 3.1). In condition 2', CS₄₋ and CS₆₊ were used as training stimuli and S₅ as novel stimulus (Fig 3.2, Table 3.1). In the condition 3', bees were trained to CS₇₋ and CS₉₊ and tested using S₈ as novel color (Fig 3.2, Table 3.1). The results of the pre-test showed that after 20 training trials, subjects responded maximally to the stimulus previously rewarded during training (Fig. 3.3b, d, f). The responses differed significantly different from random for the three experimental groups (see Fig. 3.3 for *P* values), indicating that bees learned to discriminate the stimuli. In the generalization tests, the responses to stimuli differed significantly from random (condition 1': $G=11.97, P<0.001$; condition 2': $G=14.51, P<0.001$; condition 3': $G=18.42, P<0.001$). However, in contrast to the results in the type I experiments, the level of preference for the former CS+ did not differ between the pre-test and the generalization tests (ANOVA for repeated measures: condition 1': $F_{1,3}=5.04, P=0.11$; condition 2': $F_{1,3}=0.05, P=0.84$; condition 3': $F_{3,9}=4.55, P=0.12$). Under this experimental condition, the results revealed that when the novel stimulus subtended lower chromatic contrast to CS- than CS+, subjects maintained their preference for the stimulus previously rewarded during training.

Condition 1

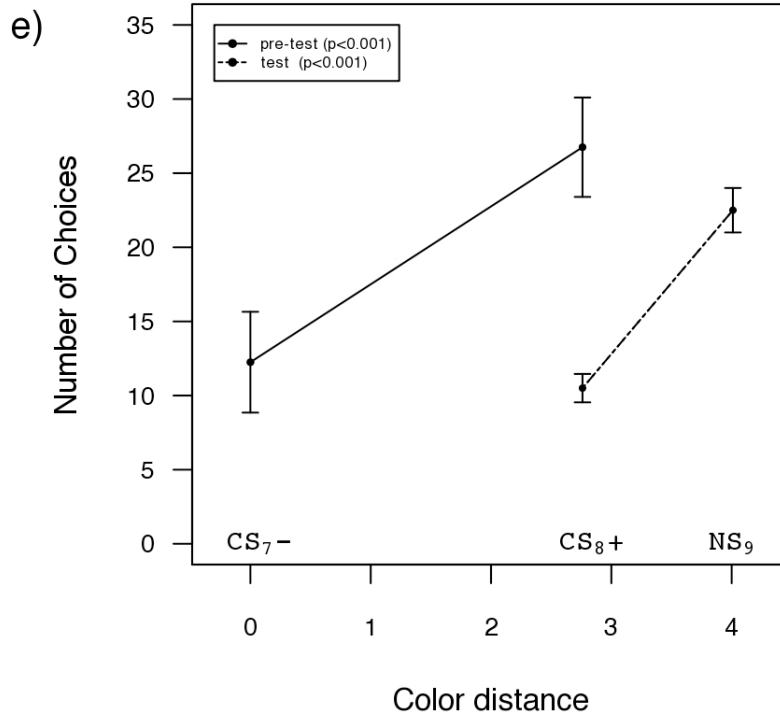


Condition 1'





Condition 3



Condition 3'

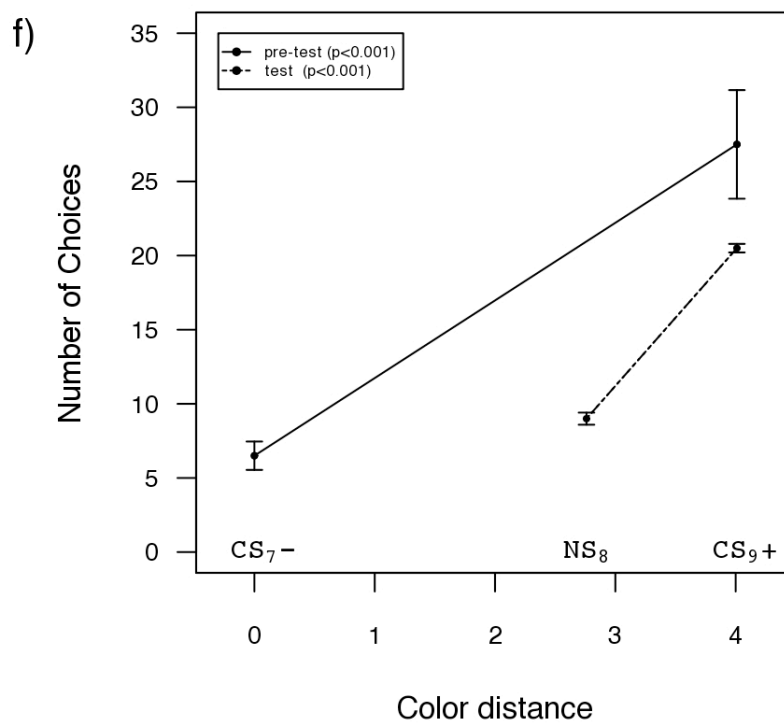


Fig. 3.3 Results of experiments of color generalization under the experimental conditions in which the test was done using; a novel color located farther away from CS- relative to CS+, corresponding to condition 1 (a), 2 (c) and 3 (e); and a novel color that lied between CS- and CS+, corresponding to condition 1' (b), 2' (d) and 3' (f) (n=4 for each experimental condition). The figure shows the mean number of choices for each color during the extinction tests vs. the color distance from the respective CS-stimulus. The solid line corresponds to the response toward each color in the pre-test, aimed to evaluate the response towards the training stimuli. The dotted line corresponds to the level of response towards each color in the generalization test, aimed to evaluate the response towards the former CS+ stimulus when presented against a novel stimulus.

Discussion

In the present study I evaluated how honeybees generalize colors over a perceptual continuum after being differentially conditioned to discriminate between perceptually similar colors. By training honeybee foragers to discriminate a rewarded color (CS+) from an unrewarded one (CS-), subjects' preference for the former CS+ was tested against a novel color, which depending on the experimental condition occupied a locus in the color space whose position differed from CS- in the same way as CS+ but that lied either closer or farther away from CS- relative to CS+. The results revealed that when the response towards CS+ was tested against a novel color whose locus was located closer to CS- than CS+, corresponding to experimental condition 1', 2' and 3' (Fig. 3.3b, d and f), subjects maintained their preference for the previously rewarded color. In contrast, subjects tested with a novel color located farther away from CS- relative to CS+, corresponding to experimental condition 1, 2 and 3 (Fig. 3.3a, c, and e), shifted their stronger response away from CS- towards the novel color. These results indicate that individuals learned to respond differentially to the training stimuli based on the chromatic properties of colors relative to each other and that they later generalized their response in a way that was congruent with the position of the novel stimuli in the color space relative to the rewarded and unrewarded color. In accordance with this, when the novel color differed from CS- in the same way as CS+ but occupied a locus located farther away from CS- relative to CS+, the relationship between the novel color and the CS+ conserved the relationship between stimuli encountered during training, inducing a peak shift in the response of the bees. The congruence between the

level of response towards the novel stimuli and their position in the color space relative to the rewarded and unrewarded color indicates that bees can extract relationships between stimuli based on their chromatic properties.

Peak shift is a taxonomically widespread psychological phenomenon reported to occur in a variety of sensory modalities (e.g. Thomas et al. 1991; Wills and Mackintosh 1998; Ghirlanda and Enquist 2003). Among hymenopterans, peak shift was reported after differential conditioning with binary mixtures of odors in honeybees (Wright et al. 2009) and after differential conditioning with color stimuli in bumblebees (Lynn et al. 2005). Although, my results are consistent with previous evidence of peak shift in bumblebees, differences in the experimental procedures between studies need to be discussed. One difference has to do with the experimental setup. Previous experiments in bumblebees were carried out in a less constrained experimental arena, with various color discs presented horizontally over a grey background. In comparison, the Y-maze presents the advantage of allowing to control the size of the visual angle subtended by targets and thus the type of visual cue used in a given discrimination task (Giurfa et al. 1997). Considering that bees' color perception is constrained to objects subtending relatively large visual angles ($<15^\circ$ in honeybees) (Giurfa et al. 1996), controlling the angular size of colored targets ensures that chromatic cues are available to the bees as they choose between stimuli. This represents a crucial aspect if one aims to study generalization over a color perceptual continuum in bees. In the present study the visual angles subtended by colored targets were large enough to allow chromatic discrimination by honeybees (30° from the decision chamber). Given that behavioral evidence has shown that bees ignore achromatic cues when chromatic cues are available, controlling the angular size of colored targets also allows to disregard the use of achromatic signals (Giurfa et al. 1997). An additional difference between the present study and the previous study with bumblebees is the circumstance provided by the different experimental designs. While in an arena multiple choice situations can be provided, the Y-maze provides either a single or a dual choice situation. Given that the response towards stimuli is defined a priori by the experimenter in both experimental designs, differences between designs influence the performance of bees. For instance, under the dual choice situation of the experiments presented here, learning to fly towards the rewarded color also implies learning to explicitly avoid the unrewarded stimulus. Köhler (1918/1938) proposed that the simultaneous presentation of stimuli

was critical for the observed transposition because it allows a direct comparison between stimuli and because allows treating the two stimuli in the discrimination task as a whole. In the present study, and in accordance with Köhler's earlier reports, when the absolute value of the stimuli was varied but the relationship between colors was held constant, honeybee foragers transposed their maximal response to the "relationally correct" stimulus. This suggests that subjects responded to the pair of stimuli as a whole.

Empirical evidence and theoretical accounts have shown that peak shift is not only influenced by prior reinforcement history as proposed by Spence (1937), but also by the similarity between the stimuli used in generalization testing and the overall similarity of the testing pair with the training pair (for review see Lazareva 2012). In relation to the first of these two factors, previous studies have shown that the spacing between CS- and CS+ affects the occurrence of peak shift. A peak shift is more likely to be induced using stimuli close to one another on a sensory dimension, while distant stimuli may fail to induce such forms of generalization (Hanson 1959; Thomas 1962; Thomas et al. 1991; Derenne 2006). With respect to the second factor, previous studies using multiple testing pairs of stimuli have shown that as the testing pairs move farther away from the positively reinforced stimulus, subjects' response shifts from preferring the "relationally correct" stimulus to responding primarily to the "relationally incorrect" stimulus. As the stimuli move even farther apart, this preference declines towards chance level (Honig 1962). In the case of honeybees and in agreement with the idea that they can respond to pairs of stimuli as a whole, previous studies on visual generalization suggest that honeybees can extract and link different visual features together. On those studies, which used complex visual stimuli composed by sets of multiple features, it was shown that honeybees can build "generic pattern representations" of complex stimuli and respond appropriately to novel stimuli in accordance to the basic layout learned during training (Stach et al. 2004). Furthermore, given that these results indicate that honeybees are able to generate a large set of object description from a finite set of elements, Benard et al. (2006) argued that this type of generalization performance reflects certain levels of stimuli categorization. In line with this interpretation, the experiments presented here showed that the way honeybee foragers transposed their response to novel situations resembled a categorization of testing colors as either neutral or positive reinforced stimulus. Given that under my experimental conditions colored targets subtended angular sizes at which bees appear to ignore achromatic cues (Giurfa

et al. 1997) and that bees' differential response to colored targets was congruent with the chromatic properties of the stimuli relative to each other, the present work represents the first evidence of categorization like performance based on chromatic cues in honeybees.

In the context of bees' color discrimination capacities, differential learning has been shown to lead to higher levels of color discrimination. It has been argued that as a consequence of differential training, bees learn stimuli in relational terms by attending to those aspects that distinguish them from each other (Giurfa 2004, Avargues-Weber 2010). Consistent with these studies, the results presented here also revealed fine color discrimination. The values of chromatic distance between colors were below the discrimination threshold value of 2.4 predicted by the RNL model of color vision for several of the color pairs tested in the experiments presented here (S_4 vs. S_5 , $\Delta S=1.77$, S_2 vs. S_3 , $\Delta S=0.98$; S_5 vs. S_6 , $\Delta S=2.29$; S_8 vs. S_9 , $\Delta S=1.27$). Despite their similarity, the response towards stimuli in the pre-test and generalization test revealed that subjects discriminated between all the color pairs tested. These results provide evidence favoring the idea that bees can learn colors in relational terms and reveal that differential learning does not only affect color discrimination capacities, but also the way honeybees generalize color stimuli.

Due to the response bias inherent to peak shift, this form of generalization has received considerable attention in evolutionary biology because of its potential relevance in the evolution of natural signals. In the context of pollination biology, its occurrence in bees has led to the hypothesis that peak shift could be involved in floral evolution (Lynn et al. 2005; Wright et al. 2009). Natural scenarios have been proposed under which these type of behavioral biases could be induced and under which peak shift could influence the divergence of floral traits (Wright et al. 2009). Central to the idea of pollinator-mediated speciation is the assumption that pollinators tend to specialize on the flowers they visit. Their selective behaviors would in turn lead to a process of floral isolation ("ethological isolation", sensu Grant 1949) and eventually to floral divergence in sympatric populations. However, considering that a main issue along this idea is whether pollinator preference for alternative floral traits would be consistent enough as to drive floral isolation, the influence of a phenomenon as peak shift in the evolution of floral characters will depend on the stability over time of such a behavioral bias. A study in birds showed that subjects rapidly modify their preferences

as a consequence of changes in stimuli-reinforcement contingencies, indicating that caution is needed when relating peak shift to the evolution of natural signals (Baddeley et al. 2007). Flowers provide a fluctuating scenario as food resource and certainly the capacities of pollinators to actively learn floral attributes and to transfer that learning to new situations, plays a crucial role in coping with these fluctuations. However, considering that animal behavior takes place as the result of a continuous process of conservation of their adaptation, the ecological contingencies provided by flowers will determine the stability of a cognitive phenomenon such as peak shift in animal pollinators.

General discussion

Considering that the relationship between animals and plants are highly fluctuating in time and space, some authors have stressed the need to approach their study from a historical perspective (Gould 1986; Futuyma 1988; Donoghue 1989; Armbruster 1992). In line with, Waser et al. (1996) proposed that the role of pollinators in the evolution of flowering plants needs to be judge through careful assessments of the pollinator environment of representative plant species, extended through time and space. Due to the taxonomic diversity of pollinators, these assessments should include the characterization of those structural aspects of pollinators affecting the way they relate with plants, such as their morphology, physiology and sensory and cognitive capacities. Along this line, an important aspect to consider about pollinators is their ability to actively learn floral attributes in accordance to stimuli-reinforcement circumstances. Flowers provide a fluctuating scenario as food resource. Therefore, knowledge about how pollinators learn and later generalize that learning to new situations, combined with knowledge about the contingencies experienced by pollinators along their ontogeny, can provide a better understanding of the conditions that can lead pollinators to discriminate or generalize floral forms. In the present work I have considered these aspects to study the implications of pollinators' color vision for the reproductive ecology and evolutionary biology of angiosperms.

Main questions

Chapter I

In the first chapter of the present thesis I focused on the relationship between glaphyrid beetles and red-bowled shaped flowers in the South-East Mediterranean region. Particularly, I investigated the mechanisms by which *Pygopleurus israelitus* (Glaphyridae) perceives flower colors and how color coding might mediate its apparent specialization to red flowers.

Chapter II

In the second chapter, I evaluated flower color evolution within *Papaver rhoeas*. Particularly, I examined intra-specific color differences by comparing flower color composition of populations along its distribution range in the Mediterranean region and Central Europe. In addition, differences in flower appearance for insect pollinators were evaluated by comparing the distribution of flower color loci in the chromaticity diagram

of *Pygopleurus israelitus* and the honeybee *Apis mellifera*.

Chapter III

In the work presented in the third chapter I evaluated how the honeybee *Apis mellifera* generalize colors over a perceptual continuum after being differentially trained to discriminate perceptually similar colors. In particular, I evaluated the effect of varying the position in the color space of a novel stimulus relative to a rewarded (CS+) and unrewarded stimulus (CS-) in the occurrence of experience-induced behavioral biases.

Main findings

Chapter I

The results presented in the first chapter revealed the presence of three types of photoreceptors in the eyes of *Pygopleurus israelitus*, 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. No receptor with peak sensitivity in the blue part of the spectrum was found in the 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, the results suggest that *P. israelitus* do not possess blue-sensitive receptors.

The analysis of flower color 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 the color space of *P. israelitus* suggest that when seen against a green foliage background red flowers would be more conspicuous to beetles than flowers of other colors. Additionally, 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 suggest 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 in this work indicates that *P. israelitus* have the receptor-based color vision to chromatically perceive red flowers.

Chapter II

The results presented in the second chapter revealed that populations of *P. rhoeas* have diverged with respect to their flower color composition. Spectral measurements carried out along *P. rhoeas*' distribution range in Central Europe revealed populations composed exclusively by individuals having UV reflecting flowers. Spectral measurements taken in the South East Mediterranean, on the other hand, revealed populations composed almost exclusively by individuals having UV-absorbing flowers. In the survey along this region only one UV-reflecting flower was observed after analyzing UV exposures taken to large patches of flowers, indicating that UV-reflecting flowers occur in very low proportions in the South East Mediterranean. Additionally, it was observed that when cultivated in Central Europe (Berlin), individuals grown from seeds collected in the Mediterranean region developed UV-reflecting and UV-absorbing flowers, the later occurring in a much higher proportion than observed in the geographical survey of populations flowers composition. In contrast to both previous cases, measurements taken in Greece revealed a scenario in which both type of color morphs were found in the same populations. Altogether, these results suggest the existence of an east/west cline of UV flower reflectance associated to *P. rhoeas*' historical range expansion. Furthermore, considering that populations of *P. rhoeas* in the South East Mediterranean would resemble its ancestral condition, the results suggest that populations of this species in Central Europe were initially polymorphic with respect to their flower color composition, and that the UV-absorbing flower morph have been lost in this region.

To evaluate how the differences in color affect flower appearance for insect pollinators, I compared the distribution of flower color loci in the color space of *Pygopleurus israelitus* (Coleoptera: Glaphyridae) and *Apis Mellifera* (Apidae: Hymenoptera). These species differ basically with respect to their receptor-based color vision and were used as representatives of *P. rhoeas*' main insect pollinator groups. The distribution of flower color loci revealed that flowers from Central European populations are well separated from flowers collected in populations from the Levant

region, both in the chromaticity diagram of the glaphyrid beetle and the honeybee. This segregation indicates that populations of *P. rhoeas* have diverged with respect to their flower color appearance for insect pollinators. The results also revealed that differences in flower color would affect their appearance for beetles and bees differently. As seen in the chromaticity diagram of *P. israelitus* (Fig 2.3a), both color morphs occupy loci well separated from the loci occupy by leaves. This separation indicates that a receptor-based color vision like the one found in this beetle species would allow them to discriminate both color morphs from a green background based on their chromatic properties. In the chromaticity diagram of the honeybee, on the other hand, the loci occupy by UV-absorbing flowers tend to overlap the loci occupy by leaves, indicating that red UV-absorbing flowers and leaves would appear similar in color for bees. In contrast, red UV-reflecting flowers occupy loci well separated from the loci occupy by leaves, indicating that petal reflectance in the UV part of the spectrum adds a chromatic component that would allow bees to discriminate these flowers through chromatic mechanisms. From this it can be argue that bees, at least with respect to their color vision, would present a sensory bias towards UV reflecting flowers of *P. rhoeas*. Considering bees' sensory constrains, it can be argue that the transition from populations compose by polymorphic variants to populations compose exclusively by individuals having UV reflecting flowers would be congruent with the transition from beetle to bee pollination undergone by this species as it was introduced northwards into Europe.

Chapter III

For the experiments presented in the third chapter honeybee foragers were trained to discriminate a rewarded color (CS+) from an unrewarded one (CS-). After a fixed number of training trails, subjects' response towards the former CS+ was tested against a novel color that depending on the experimental condition occupied a locus in the color space whose position differed from CS- in the same way as CS+ but that lied either closer or farther away from CS- relative to CS+. The results from these experiments revealed that when the response towards CS+ was tested against a novel color whose locus was located closer from CS- than CS+, subjects maintained their preference for the color previously rewarded during training. In contrast, when subjects were tested with a novel color whose locus was located farther away from CS- relative CS+, subjects shifted their stronger response away from CS- towards the novel color.

These results indicate that subjects learned to respond differentially to the training stimuli based on the chromatic properties of the colors relative to each other and that later they generalized their response in a way that was congruent with the position in the color space of the novel stimuli relative to the rewarded and unrewarded color. In accordance with this, when the novel color differed from CS- in the same way as CS+ but occupied a locus located farther away from CS- than CS+, the relationship between the novel color and the former CS+ conserved the relationship between stimuli encountered during training, inducing a peak shift in the response of the bees. The congruence between the level of response towards the novel stimuli and their position in the color space relative to the rewarded and unrewarded color indicates that bees can extract relationships between stimuli based on their chromatic properties.

Evolutionary implications

Chapter I

The results presented on the first chapter revealed a high level of congruence between receptor-based color vision of *P. israelitus* and the red and orange flowers they seem to prefer. 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. Furthermore, considering that red flowers are very prominent features of the landscape in the South-East Mediterranean region, it can be argued that this ecological scenario present conditions under which specialized interactions between beetles and red flowers could be favored.

Chapter II

Fossil and taxonomic evidence indicate that *Papaver rhoeas* was introduced into Central Europe about 5 thousand years ago. The results presented on this chapter revealed that since then, populations of *P. rhoeas* have diverged with respect to their flower color composition. This divergence seems to be congruent with the shift from beetle to bee pollination undergone by *P. rhoeas* as it was introduced into Central Europe. Considering the differences in color vision between glaphyrids and bees, this presents the opportunity to evaluate changes in flower color under different pollinator environments and over defined spatial and temporal scale. My results revealed that

while both type of pollinators have the receptor-based color vision to perceive red UV-reflecting flowers by their color, only glaphyrid beetles have the receptor-based color vision to chromatically perceive red UV-absorbing flowers. These results suggest contrasting scenarios with respect to the type of relationship that *P. rhoeas* could established with pollinators on the bases of its flower color. From the results it can be argue that when beetles and bees form part of *P. rhoeas*' pollinator environment, reflectance in the UV could be interpret as a floral trait favoring generalized plant-pollinator interactions. This scenario correspond to the one populations around the Mediterranean region are expose to. In this region both type of insect pollinators can be found visiting flowers of *P. rhoeas*. In contrast, under an ecological scenario in which only hymenopterans interact with populations of *P. rhoeas*, as in the case of populations in Central Europe, flower UV reflectance can be interpret as a floral trait promoting specialized interactions between those flowers and bees. These different scenarios suggest that the human-induced geographical expansion of *P. rhoeas* exposed this species to fluctuating pollinator environments under which either generalized or specialized plant-pollinator interactions might have been favored. In line with this, differences in flower color diversity between populations of *P. rhoeas* would reflect the different pollinator environments under which populations have developed.

Chapter III

Due to the response bias inherent to peak shift, this form of generalization has receive considerable attention in evolutionary biology because of its potential relevance in the evolution of natural signals. In the context of pollination biology, its occurrence in bees has led to the hypothesis that peak shift could be involved in floral evolution (Lynn et al. 2005; Wright et al. 2009). Central to the idea of pollinator-mediated speciation is the assumption that pollinators tend to specialize on the flowers they visit. Their selective behaviors would in turn lead to a process of floral isolation (“ethological isolation”, sensu Grant 1949) and eventually to floral divergence in sympatric populations. However, and considering that a main issue along this idea is whether pollinator preference for alternative floral traits would be consistent enough as to drive floral isolation, the influence of a phenomenon as peak shift in the evolution of flowers will depend on the stability of such a behavioral bias over time. A study in birds showed that subjects rapidly modify their preferences as consequence of changes in stimuli-

reinforcement contingencies, indicating that caution is needed when relating peak shift to the evolution of natural signals (Baddeley et al. 2007). Flowers provide a fluctuating scenario as food resource and certainly the capacities of pollinators to actively learn floral attributes and transfer that learning to new situations plays a crucial role in coping with these fluctuations. However, considering that animal behavior takes place as the result of a continuous process of conservation of their adaptation, the ecological contingencies provided by flowers will determine the stability of a cognitive phenomenon such as peak shift in animal pollinators.

Conclusions

As mentioned above, specialization has been proposed as a general evolutionary tendency among pollination systems (Stebbins 1970; Crepet 1983, 1984). Despite the longstanding of this idea and the fact that it enjoys several sources of support, studies in pollination have repeatedly demonstrated that plant-pollinator interactions tend to be more generalized than previously thought (e.g. Grant 1994; Waser et al. 1996; Waser 1998). Based on this, some authors have elaborated on the idea that generalization rather than specialization would be favor in pollination systems (Waser et al. 1996). The same authors recognized, however, the limitations derived from the dichotomy form by specialization and generalization, and argue in favor of replacing this implied dichotomy with a continuum (Waser et al. 1996). I propose that the causes of these limitations do not lie on the dichotomy presented by these views but on the theoretical implications of considering specialization and generalization as evolutionary tendencies. Common to these contrasting views is the fact that they both imply a pre-established directionality in the evolution of pollination systems. The idea of a pre-established directionality in evolutionary processes results from the methodology of constructing history from an observable, small-scale present (Gould 1986). Through this approach, evolution appears to the observer that looks back from the present forms as having followed a directed course of changes from their ancestral form. However, given that historical processes occur as a becoming in which every moment is originated as a transformation of a preceding one, they do not follow a pre-established course or direction (Maturana-Romesin and Mpodozis 2000). The apparent directionality of historical processes result from the fact that every new situation restricts the domain of possible changes that can follow (Maturana-Romesin and Mpodozis 2000). In the case of historical processes resulting from the interaction between plants and pollinators, any

shift in their relation that becomes epigenetically conserved generation after generation will constitute a constraint for future ontogenic shifts. The result will be that lineages of plants and pollinators will indeed follow a directional and congruent course of evolutionary changes. However, the direction of these changes do not follow a pre-established aim or purpose, but they arise moment after moment in the phylogenic co-drift between plants and pollinators. Based on these theoretical considerations and the results presented on this thesis, I propose that neither generalization nor specialization represent evolutionary tendencies by them self. Instead, I proposed that generalization and specialization occur as consequence of the historical contingencies under which the relationship between plants and animal pollinators take place.

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