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Francismeire J.
Telles da Silva

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## A behavioural approach

**Doctoral Thesis**

**Francismeire Jane Telles da Silva**

**Granada, 2015**

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## A behavioural approach

Thesis presented by Francismeire Jane Telles da Silva to obtain the degree of Doctor of Philosophy (PhD) in the Faculty of Science, Department of Ecology, University of Granada.

**Granada, 2015**

**La doctoranda Francismeire Jane Telles da Silva y el director de la tesis Miguel Angel Rodríguez Gironés Arbolí, garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por la doctoranda bajo la dirección del director de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.**

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*“I have a friend who's an artist ... He'll hold up a flower and say "look how beautiful it is," and I'll agree. Then he says "I as an artist can see how beautiful this is but you as a scientist take this all apart and it becomes a dull thing," and I think that he's kind of nutty. First of all, the beauty that he sees is available to other people and to me too, I believe... At the same time, I see much more about the flower than he sees…I mean it's not just beauty at this dimension, at one centimeter; there's also beauty at smaller dimensions, the inner structure, also the processes. The fact that the colors in the flower evolved in order to attract insects to pollinate it is interesting; it means that insects can see the color. It adds a question…All kinds of interesting questions which the science knowledge only adds to the excitement, the mystery and the awe of a flower. It only adds. I don't understand how it subtracts.”*

**— Richard Feynman**

**The Pleasure of Finding Things Out**

**BBC, Horizon, 1981.**

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# RESUMEN

*El color de las flores transmite, en última instancia, información para ojos diferentes a los nuestros*

Las flores y los sentidos de los polinizadores interactúan. Las plantas representan fuentes de nutrición - polen y néctar – para los insectos, y anuncian las recompensas por medio de distintas señales, representadas en las flores. Para explotar una flor, el visitante floral requiere de capacidades sensoriales específicas para detectar las señales florales, y asociarlas con la recompensa ofrecida. Una de las modalidades sensoriales más importantes y exploradas en el contexto planta-visitante floral es la visión. Al igual que las flores diversifican sus colores, muchos polinizadores poseen el sistema visual adecuado para detectarlas. A parte de la abeja de la miel (*Apis mellifera*), el conocimiento sobre el sistema visual y capacidades cognitivas de otros visitantes florales es escaso. Para entender correctamente la relación entre el sistema visual de los visitantes florales y las propiedades espectrales de las flores, debemos primero entender qué significa un color para un animal, y cómo funciona su sistema visual.

En la presente tesis, constituida de cuatro capítulos, hemos estudiado diferentes aspectos del sistema visual de dos especies de polinizadores: *Bombus terrestris* (abejorro) y *Macroglossum stellatarum* (esfinge colibrí). Algunos estudios han investigado el sistema visual, el comportamiento y la cognición de ambas especies en diferentes niveles. Sin embargo, aún existen muchas cuestiones por resolver. En los cuatro capítulos de la tesis, estudiamos las respuestas comportamentales de ambas especies, con variaciones cuando al sistema sensorial y la metodología aplicada. En los dos primeros capítulos investigamos el comportamiento de *Bombus terrestris* frente a tareas de discriminación y detección, utilizando flores artificiales. Mientras que en los dos últimos capítulos estudiamos aspectos básicos y fundamentales del sistema visual de *Macroglossum stellatarum*: la sensibilidad espectral y la capacidad de discriminar colores. A pesar

de que los abejorros (*Bombus terrestris*) y la abeja de la miel (*Apis mellifera*) son especies cercanas, estudios comparativos indican diferencias en muchos aspectos de su comportamiento relacionados con el uso de la información visual. Así como las flores son diversas, también lo es el comportamiento de forrajeo de los polinizadores. Éstos pueden emplear estrategias complejas, y modular su comportamiento, para seleccionar, detectar y discriminar las flores más gratificantes. *Macroglossum stellatarum* es considerada un importante polinizador de muchas especies de plantas en Europa, aun así, la información básica sobre el sistema visual de esta especie se desconocía hasta el desarrollo de esta tesis.

Los visitantes florales a menudo tienen que hacer comparaciones entre flores basándose en diferencias entre ellas, principalmente visuales. Los modelos de visión en color intentan predecir la facilidad con la que los visitantes florales pueden discriminar entre colores con diferentes propiedades espectrales. Las predicciones de los modelos se basan en la distancia perceptual entre dos colores, donde distancias perceptuales pequeñas indican que la discriminación será difícil, mientras que distancias perceptuales grandes indican que la discriminación será fácil. En principio, no hay ninguna razón obvia para usar un modelo u otro, sin embargo debido a la formulación y asunciones de los modelos, las predicciones pueden divergir. En el **primer capítulo** de esta tesis, estudiamos las predicciones de diferentes modelos de visión del color, testando cómo se acercan las predicciones de los modelos al comportamiento del abejorro durante una tarea de discriminación de flores artificiales. Para ello, seleccionamos cuatro pares de flores artificiales para que las distancias perceptuales (información cromática) entre las flores de cada par fuesen distintas según dos de estos modelos, y similares según un tercer modelo de visión. También medimos e incorporamos a los análisis los contrastes acromáticos (brillo y el contraste producido en el fotorreceptor verde) entre las flores de un par. Los abejorros fueron divididos entre los pares de flores artificiales y entrenados a discriminar entre los dos colores del par seleccionado. Uno de

los colores llevaba la recompensa azucarada, mientras que el otro una solución amarga (quinina). Medimos el tiempo que los abejorros tardaron en encontrar las flores con recompensa, así como la precisión de las elecciones (medida como la proporción de respuestas correctas). El tiempo que las abejas necesitaron para seleccionar una flor y la proporción de aciertos difirió entre los pares de colores: los tiempos de decisión disminuyeron con el aumento del contraste acromático (brillo y contraste en el fotorreceptor verde), y la proporción de aciertos aumentó con el aumento del contraste acromático y la distancia perceptual. Los resultados de este capítulo ponen de manifiesto, en primer lugar, que tanto los contrastes cromáticos como los acromáticos afectaron la discriminabilidad de los pares de colores y en segundo lugar, que las predicciones de los modelos no siempre están de acuerdo con el comportamiento de los visitantes florales. El color de las flores afecta a las elecciones de las abejas durante la actividad de forrajeo y las elecciones durante el forrajeo afectan el éxito reproductivo de las plantas, por ello, una mejor comprensión de qué modelo es el más exacto es necesario para predecir el comportamiento de las abejas y las implicaciones ecológicas de la elección de flores y color.

Así como el color, el tamaño de las flores afecta el comportamiento de los abejorros durante la actividad de forrajeo cuando las flores están presentes en fondos homogéneos. Si las flores son grandes, el tiempo de búsqueda se correlaciona con el contraste cromático entre éstas y el fondo donde están. Mientras que si el contraste cromático es pequeño o las flores son pequeñas, las abejas tardan más tiempo para detectarlas en un fondo verde homogéneo, ajustando su comportamiento de varias maneras. Además de la visión, el olfato es otra modalidad sensorial importante que las abejas utilizan durante la actividad de forrajeo. Por lo tanto, en el **segundo capítulo** hemos estudiado el efecto del color, el tamaño, el entrenamiento y la presencia o ausencia de una segunda señal (olor) sobre el comportamiento del abejorro durante la detección y discriminación de flores presentadas contra un fondo complejo. Los abejorros buscando flores

azules fueron más precisos, volaron más rápido, siguieron rutas más directas entre las flores (viajando menores distancias) y necesitaron menos tiempo para encontrarlas, en comparación con los abejorros que buscaban flores rojas. Al explotar flores rojas, los abejorros se equivocaron más veces en la elección y requirieron más tiempo para encontrarlas, aunque el rendimiento incrementó con la experiencia y con la presencia de olor. El tamaño de las flores por sí solo no afectó el comportamiento de los abejorros, pero sí al interactuar con el color. Los abejorros tardaron más tiempo para encontrar las flores rojas grandes. Estas flores presentaban los menores contrastes en color (cromático) con el fondo, y aunque el contraste acromático excedía aquél de las flores azules, la eficiencia en la detección no es comparable entre ambos canales (cromático y acromático). La percepción de los estímulos fue afectada principalmente por el contraste en color entre el fondo y la flor. La dificultad impuesta por esta combinación pudo ser compensada con la presencia de un segundo estimulo sensorial (olor), o con el entrenamiento. Nuestros resultados difieren de los que se han encontrado en un experimento similar, usando un fondo verde homogéneo, por ello presentamos posibles razones en la discusión del capítulo.

En los últimos años, muchos estudios se han realizado sobre el sistema visual de algunos visitantes florales (especialmente abejas y algunas mariposas y sirfídeos), lo que ha generado un aumento en la comprensión sobre los mecanismos y funciones implicados en la percepción visual. Sin embargo, para la mayoría de especies muchos aspectos básicos siguen siendo poco claros o desconocidos. *Macroglossum stellatarum* es un visitante floral diurno - de la familia de polillas mayoritariamente crepusculares Sphingidae – con demostrada capacidad de aprendizaje. *Macroglossum stellatarum* puede ser fácilmente criada en el laboratorio, lo que hace que esta polilla sea una excelente candidata para estudios en ambientes bajo condiciones controladas. Experimentos previos han sugerido que *M. stellatarum* posee visión en color, con fotorreceptores sensibles en las zonas del ultravioleta (UV), azul y verde. La polilla tiene preferencias innatas y

una amplia capacidad de discriminación para algunos colores dentro del espectro visible hipotético para esta especie. Sin embargo, la sensibilidad espectral de los fotorreceptores sigue siendo desconocida, así como sus picos de absorbancia. Para entender el comportamiento de un animal mediante la realización de experimentos de visión en color, primero tenemos que comprender cómo la especie en cuestión percibe su entorno y distingue diferentes estímulos visuales. Por lo tanto, en el **tercer capítulo**, hemos caracterizado la sensibilidad espectral de *Macroglossum stellatarum* y establecido la absorbancia máxima de sus fotorreceptores, mediante pruebas de comportamiento y electrorretinograma (ERG). Mediante la medición de ERG vimos que *M. stellatarum* tiene sensibilidades máximas en el ultravioleta (UV-349 nm), azul (440 nm) y verde (521 nm). Para determinar la sensibilidad espectral con base en el comportamiento (espectro de acción), entrenamos a las polillas para que fuesen capaces de asociar un disco iluminado con una recompensa, y un disco oscuro con la ausencia de recompensa. Mientras que las posiciones espectrales de máxima sensibilidad encontradas en pruebas de comportamiento están de acuerdo con las predicciones del modelo basadas en los datos de ERG, la sensibilidad a la luz azul fue 30 veces mayor de lo esperado. Relacionamos esta mayor sensibilidad con la preferencia innata de la polilla por el color azul y presentamos una discusión general sobre los resultados dentro del capítulo.

A pesar de la preferencia innata por determinados colores, *M. stellatarum* se alimenta de una variedad de especies de flores y prefiere el color a la fragancia cuando se le da la oportunidad de elección. Dada la importancia de las propiedades espectrales de las flores para la polilla, y conociendo la sensibilidad espectral de los fotorreceptores, la capacidad de la polilla de discriminar entre longitudes de onda similares resulta interesante. La discriminación entre longitudes de onda se define como la capacidad que presenta un organismo en discriminar entre estímulos monocromáticos perceptualmente parecidos. En el contexto del forrajeo, esta capacidad

puede resultar ventajosa, especialmente para insectos que demandan altas cantidades de energía, como *M. stellatarum* por su comportamiento de revolotear frente a las flores mientras se alimenta. En el **cuarto capítulo** pusimos a prueba la capacidad de *M. stellatarum* para discriminar entre longitudes de onda similares. Para tal, entrenamos a las polillas para asociar luces monocromáticas específicas con una recompensa. La selección de las longitudes de onda se basó en los picos de los fotorreceptores y sus respectivas áreas superpuestas, determinados en el capítulo anterior. Una vez que las polillas aprendieron a asociar una luz monocromática con la recompensa, testamos su capacidad de discriminación en una prueba de doble elección. En esta prueba la longitud de onda asociada a la recompensa fue presentada junto con una longitud de onda sin recompensa, inicialmente 20 nanómetros más larga o más corta que la longitud de onda del entrenamiento. Además de los dos mínimos pronunciado que se esperaban (en el área donde los fotorreceptores se superponen), *M. stelattarum* presentó un tercer mínimo entre el pico del fotorreceptor azul y la curva de inflexión del fotorreceptor verde. Utilizamos un modelo de visión del color para predecir la capacidad de discriminación de la polilla y comparamos las predicciones con los datos de comportamiento. Las distancias mínimas encontradas en el test de comportamiento se asemejaron a las predichas por el modelo, pero el modelo no predijo un tercer mínimo de discriminación encontrado en el test de comportamiento. Los mínimos de discriminación de las longitudes de onda en *M. stellatarum* fueron menores que aquellos encontrado para la abeja de la miel (*Apis mellifera*), acercándose a los valores encontrados para una mariposa tetracromática (*Papilio xuthus*). En este capítulo, discutimos sobre las diferencias en la capacidad de discriminación entre las especies, aparte de incorporar una nueva perspectiva sobre estos análisis: el uso de la información acromática.

La capacidad sensorial de los visitantes florales ha ayudado, al menos en parte, a moldear y aumentar la variabilidad de las señales florales. Sin embargo, para muchos de estos visitantes, la

capacidad del sistema sensorial así como sus límites no están del todo determinados, y para muchos otros, sigue estando inexplorado y desconocido. Resulta difícil interpretar el comportamiento de un visitante floral en una comunidad de plantas, por ejemplo, si no entendemos adecuadamente los mecanismos que modulan su percepción, lo que en última instancia afecta a la relación planta-polinizador.

La presente tesis aumenta la comprensión sobre el sistema visual de dos especies de polinizadores importantes en Europa, el abejorro (*Bombus terrestris*) y la esfinge colibrí *Macroglossum stellatarum*. Los experimentos utilizando la abeja de la miel han ayudado a entender parcialmente la visión de insectos a lo largo de los años, pero similar información para la gran mayoría de los visitantes florales permanece desconocida. Esta tesis demuestra que la capacidad visual de cada especie es única. Los procesos evolutivos que han moldeado la capacidad sensorial de los visitantes florales no son claros, pero la importancia de los estudios comportamentales dentro del contexto planta-polinizador es esencial, no sólo por el valor económico del servicio de polinización, pero para entender adecuadamente los mecanismos que gobiernan esta relación.

# SUMMARY

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*Flower colour ultimately conveys information for eyes different from ours*

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Flowers and the senses of pollinators interact. Plants represent sources of nutrition – pollen and nectar – for insects, and announce the rewards though different signals, presented in the flowers. To exploit a flower, the floral visitor needs specific sensory and cognitive abilities to detect the floral signals and to associate it with the reward. One of the most important and studied sensory modalities, in the context of plant-floral visitor, is the vision. Just as many flowers are differently coloured, many pollinators are known to possess the adequate visual system to detect them. Except for the honeybee (*Apis mellifera*), the knowledge about the visual system and cognition of floral visitors is scarce. To properly understand the relationship between the visual system of floral visitors and the spectral properties of flowers, we must first understand what colour means to an animal, and how its visual system works.

In the present thesis, composed of four chapters, we studied different aspects of the visual system of two pollinator species: *Bombus terrestris* (bumblebee) and *Macroglossum stellatarum* (hummingbird hawkmoth). Some studies have already investigated the visual system, behaviour and cognition of both species at different levels. Yet, much information is missing. In the four chapters of this thesis we studied the behaviour of both species, with differences concerning the sensorial system and the applied methodology. In the first two chapters we investigated the behaviour of *Bombus terrestris* while performing tasks related with discrimination and detection of artificial flowers. In in the last two chapters we studied basic and fundamental aspects of the visual system of *Macroglossum stellatarum*: the spectral sensitivity and the colour discrimination capacity. Despite the fact that bumblebees (*Bombus terrestris*) and honeybees (*Apis mellifera*) are related species, comparative studies have shown differences in many aspects of their behaviour

related with the use of visual information. Flowers are diverse and so is the foraging behaviour of pollinators. These can employ different strategies and modulate their behaviour to select, detect and discriminate the most rewarding flowers. *Macroglossum stellatarum* has been considered as an important pollinator of many plants in Europe; yet, basic information about its visual system remained unknown until the development of this thesis.

Floral visitors are often required to make comparative judgments between flowers, based on, mainly, visual differences between them. Models of colour vision attempt to predict the ease with which animals can discriminate between colours with different spectral properties. The predictions of models are based on the perceptual distance between two colours, where small perceptual distances indicate that discrimination will be difficult, while large perceptual distances mean that discrimination will be easy. There is, in principle, no obvious reason to use one model over the other, but due to models' formulation and assumptions, predictions can diverge. In the **first chapter**, we focused on the predictions of different colour vision models and tested how well they adjust to the bumblebee behaviour during a flower discrimination task. We selected four pairs of artificial flowers differing in their perceptual distances (chromatic information) according to two models, while being similar according to a third one. Achromatic contrasts (brightness and green contrast) between flowers were also measured and incorporated into the analyses. Bumblebees were divided between pairs of artificial flowers and trained to discriminate between the two colours of the pair. One of the colours carried the reward (sucrose solution), while the other a punishment (quinine). We measured the time bumblebees took to find the rewarding flowers, as well as the accuracy (measured as the proportion of correct choices). The time that bees required to select a flower and the proportion of correct choices differed between pairs: decision times decreased as achromatic contrast increased, and the proportion of correct choices increased with achromatic contrast and perceptual distance. First, these results suggest that both chromatic and achromatic contrasts affected the discriminability of colour pairs and second, that model predictions are not always in agreement with the animal behaviour. Since flower colour affects the foraging choices of bees and foraging choices affect the reproductive success of plants, a better understanding of which model is more accurate is required to predict bee behaviour and the ecological implications of flower choice and colour.

As well as colour, the size of the flowers constrains bumblebee behaviour during the foraging activity when flowers are presented in homogeneous backgrounds. When flowers are big, search time correlates with the colour contrast between flowers and background. When colour contrast or flowers are small, bees take longer to detect these flowers against a homogeneous green background and adjust their behaviour in several ways. Besides vision, olfaction is another important sensory modality that bees use during the foraging activity. Therefore, in the **second chapter** we studied the effect of colour, size, training and the presence or absence of a second cue (odour) on bumblebee behaviour while detecting and discriminating flowers presented against a complex background. Bumblebees looking for blue flowers were more accurate, flew faster, followed more direct paths between flowers (travelling less distances) and needed less time to find them, than bumblebees looking for red flowers. When exploiting red flowers they made more errors and required more time to find flowers, although performance increased with experience and with the presence of odour. The size of flowers alone did not directly affect the bumblebee behaviour; however, when combined with colour it had an effect. Bumblebees took longer to find big red flowers. These flowers, had the smallest chromatic contrast with the background, and although the achromatic contrast exceeded that of the blue flowers, the efficiency in detection is not comparable between channels (chromatic and achromatic). The perception of stimuli was mainly affected by the chromatic contrast between the flowers and the complex background, but the difficulty imposed by this combination could be compensated by the presence of a second cue (odour), or the amount of received training. Our results differ from those found in a similar experiment using homogeneous green background and we present possible reasons in the discussion of the chapter.

In recent years many studies have been done on the visual system of some floral visitors (especially bees and to some extent butterflies and Syrphids), raising the understanding about mechanisms and functions involved in visual perception. Nevertheless, for most species many basic aspects remain unclear. *Macroglossum stellatarum* is a diurnal floral visitor – of the otherwise mainly crepuscular Sphingidae family – with demonstrated learning abilities. *Macroglossum stellatarum* can easily be raised in laboratory, which makes this moth an excellent candidate for studies in environments under controlled conditions. Previous experiments have suggested that *M. stellatarum* possesses true colour vision, with receptors sensitive in the ultraviolet (UV), blue and green areas of the spectrum. The moth has demonstrated innate preferences and broad colour discrimination capacity over some ranges of the hypothetical visible spectrum. However, the spectral sensitivity of the retinal photoreceptors remains unknown, as well as their peak absorbance. If we aim to understand an animal's behaviour by carrying out colour vision experiments, we first have to comprehend how animals perceive their environment and distinguish different visual stimuli. In the **third chapter** we therefore characterised the spectral sensitivity of the hummingbird hawkmoth *Macroglossum stellatarum* and established the peak absorbance of its photoreceptors, using behavioural tests and electroretinogram (ERGs) analyses. By measuring ERGs we determined that *M. stellatarum* has ultraviolet (UV), blue and green receptors maximally sensitive at 349, 440 and 521 nm. To determine the behavioural spectral sensitivity (action spectrum), we trained moths to associate an illuminated disk with a food reward, and a dark disk with no reward. While the spectral positions of sensitivity maxima found in behavioural tests agree with model predictions based on the ERG data, the sensitivity to blue light was 30 times higher than expected. We relate this higher sensitivity with the moth's innate preference for blue colour and present a general discussion about results within the chapter.

Despite the innate preference for particular colours, *M. stellatarum* feeds from a variety of flower species and prefers colour over scent when given the opportunity to choose. Given the importance of the spectral properties of flowers for this moth, and knowing the spectral sensitivity of its photoreceptors, the moth's capacity to discriminate between similar wavelengths is of interesting. Wavelength discrimination is defined as the ability of an organism to discriminate between perceptually close monochromatic stimuli. In the context of foraging, this capacity can result advantageous, especially for insects demanding high energy intakes such as *M. stellatarum* due to its hovering behaviour while feeding. In the **fourth chapter** we tested the capacity of *M. stellatarum* to discriminate between similar wavelengths. We trained moths to associate specific monochromatic lights with a reward. Wavelength selection was based on the moth's photoreceptors peaks and overlapping areas, determined in the chapter three. Once a monochromatic light was associated with a reward, moths were tested in a dual choice experiment, in which the rewarding wavelength was presented together with an unrewarding wavelength, initially 20 nm longer or shorter than the trained wavelength. Besides two expected pronounced minima (where photoreceptors overlap), *M. stelattarum* presented a third minimum between the peak of blue receptor and the inflexion curve of the green receptor. We used a colour vision model to predict the discrimination capabilities of the moth and to compare it with the behavioural data. Moth´s behavioural minima were similar to those predicted by the colour vision model, but the model did not predict a third less pronounced minimum of discrimination found during the behavioural tests. Minima of wavelength discrimination in *M. stellatarum* were smaller than those found for the honeybees (*Apis mellifera*), approaching values found for a tetrachromatic butterfly (*Papilio xuthus*). In that chapter, we discuss the differences in the discrimination capacity of those floral visitors tested so far, while integrating new perspectives for this type of experiments: the use of achromatic information.

The sensory capacity of floral visitors has helped to shape and increase the variability of the flower signals. However, for many floral visitors, the sensorial system capacity and also its limits are not well understood, and for many others, it remains largely unexplored. It results difficult to interpret the behaviour of a floral visitor within a plant community if we do not properly understand the mechanisms modulating this relationship.

The results of the present thesis raise the understanding of the visual systems of two important pollinator species in Europe, the bumblebee (*Bombus terrestris*) and the diurnal hummingbird hawkmoth (*Macroglossum stellatarum*). For years, experiments using the honeybee helped to build an understanding of insect vision, but similar information for the vast majority of floral visitors remains unknown. The present thesis shows that the visual capacity of each species is unique. The evolutionary processes that have shaped the sensory ability of floral visitors are unclear, but the importance of behavioural studies in the plant-pollinator context is essential, not only because of the economic value of the pollination service, but also to properly understand the mechanisms linking the plant-pollinator relationship.

# GENERAL INTRODUCTION

*"Pollination is an ancient and crucial ecosystem service"* (Kevan and Menzel 2012)

The evolutionary success and the ecological dominance of the angiosperms have been associated with a number of features. Perhaps the feature most often considered is the use of animals to transport pollen between flowers (Armbruster 2014). Almost 90% of flowering plant species, including many important crop species, rely on animal pollination (Klein et al 2007; Ollerton et al 2011; Burkle et al 2013). Beyond the practical significance, the interaction between floral traits and floral visitors has been an important force in the studies of biology, from evolution and ecology to animal learning and behaviour (Willmer 2011).

This usually, but not always, mutualistic communication between plants and animals has been successful because floral signals and pollinator senses interact. The interaction consists of two major components: floral traits and the neural and sensory systems of pollinators. The complex nature of floral traits reflects a combination of selective pressures, both historical and contemporary, among which are those mainly, but not solely, exerted by the sensory abilities of pollinators. Plants can also converge their signals to exploit pollinator senses and to diverge from co-occurring species to ensure pollinator constancy (Balamurali et al 2015).

To be more attractive, flowers can offer multisensory signals, such as visual, olfactory, tactile and so on. Visual signals are by far the most explored ones in the context of plant-pollinators interaction. Just as flowers are brightly coloured, many floral visitors are known to possess colour vision (Waser et al 1996; Briscoe and Chittka 2001). Colour is an important signal cue, and in most cases effectively makes the flower visitors respond to it (Balamurali et al 2015).

Next, we are briefly going to describe general aspects of the visual system of insects to better understand the main sensory channel studied in the present thesis, to then place the thesis in context and present the general and specific aims.

# 1. PIONEER STUDIES OF INSECT VISION

> *"The world that any organism experiences is a product of the specific sensory filters that the animal has acquired during its evolution"* (Chittka and Wells 2004)

Human colour science and psychophysics became established during the nineteenth century, and people began to ask whether animals see colours (Kelber et al 2003). It was a hymenopteran species that first provided insight about the topic. More than 125 years ago, Lubbock (1889) discovered that ants have ultraviolet sensitivity, demonstrating for the first time a sensory capacity not held by humans (Chittka and Wells 2004). The concept of the flower colour being adapted to their floral visitors rather than to our esthetic perception added an entirely new aspect to the theory of the plant-animal relationship.

A few decades after Lubbock's discovery, Karl von Frisch published his pioneering work on the colour vision of the European honeybee, *Apis mellifera* (Frisch 1914). In his study, von Frisch first trained bees to a blue coloured card by rewarding them with sucrose solution. Subsequently, in unrewarded tests the coloured card was presented together with grey cards of different intensities, but with one of the grey cards having similar intensity compared to the trained coloured card. If honeybees relied on the intensity of stimulus, they would not be able to discriminate a particular shade of grey from the training colour. The experiment concluded that bees are guided by the colour rather than the brightness of stimuli (Frisch 1914). As a consequence of the discovery, colour vision in honeybees (*Apis mellifera*) has been extensively studied and research in honeybee vision has laid the foundations for the understanding of insect colour vision (Hempel de Ibarra et al 2014).

## 2. Perceiving the World: Compound Eyes

> *"Insects obtain visual information about their environment via their compound eyes"* (Stavenga 2002)

Insects have compound eyes, which are by far the most common eye type in the animal kingdom (Cronin et al 2014). Compound eyes are constructed of many individual optical units (Fig. 1), known as ommatidia (unit eye = ommatidium). These units are tubular in shape and consist of one or more lenses – typically an outer transparent cuticular "corneal lens" and an inner "crystalline cone" (Cronin et al 2014). The number of ommatidia varies greatly, depending on the species or even caste system.

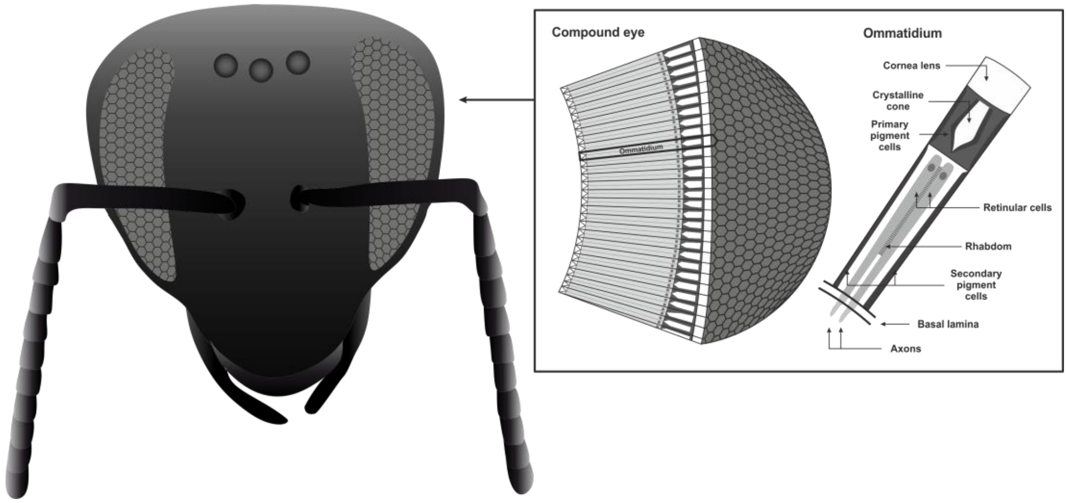

**Fig. 1** Insect compound eye and the ommatidium detail. Artwork of Dolores Ruiz Lupión.

Inside the ommatidia we find the rhabdoms, which are made up of retinular cells (variable across taxa, but between six and nine depending on the insect). Each of the retinular cells (or photoreceptors) possess a microvillous photosensitive rod-like region known as a "rhabdomere"; this it contributes, together with the ommatidium's other retinular cells, to a collective rhabdom. The rhabdom is a light-guiding structure that houses the rhodopsin molecules and receives and absorbs the incoming light (more details below). Each ommatidium receives light from a small region of space. Thus, the greater the number and density of ommatidia in a compound eye, the more finely sampled is visual space (Cronin et al 2014).

Compound eyes fall into two broad subtypes: apposition (those in which no gap or clear zone exists between the retinula cells and the crystalline cones) and superposition (those with a clear space between the crystalline cones and the retinula cells) eyes (Exner 1891). We also know that within each subtype there are several further subtypes (for details see Land and Nilsson 2002), but here we are briefly going to discuss about the two main subtypes to refresh the reader with the main functional and anatomical differences between them.

## 2.1. APPOSITION AND SUPERPOSITION EYES

From the outside, apposition and superposition eyes are almost indistinguishable. Both are convex structures with facets of similar dimensions, and clearly variants of the same general design. Superposition eyes are frequently encountered in crepuscular and nocturnal arthropods, while apposition eyes, the more common, in diurnal species (Hariyama et al 2001).

An essential difference between apposition and superposition eyes lies in the degree of optical isolation between adjacent ommatidia. This is greatest in the apposition type of eye, in which the rhabdoms and crystalline cones touch and there is absorptive screening pigment between the ommatidia (Fig. 2a). These two features reduce the amount of light that can reach a rhabdom from facets other than the one above it. In superposition eye, a pigment-free clear zone is interposed between facets and the rhabdoms (Fig. 2b), so that light entering the eye through different facets can became focused on one rhabdom (Bruce et al 2010). This arrangement increases the photon catch, making superposition eyes more sensitive than apposition eyes.

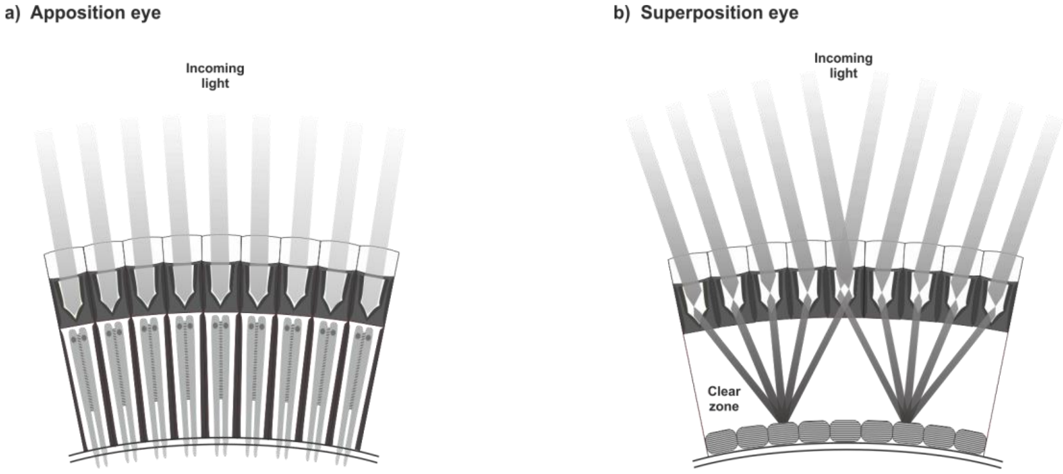

**Fig. 2** Schematic representation of (a) apposition and (b) superposition eyes. Artwork: Dolores Ruiz Lupión

## 3. PERCEIVING THE WORLD: LIGHT AND COLOUR INFORMATION

> *"Eyes are devices for extracting useful information from the light reflected or emitted from objects in the world around us"*
>
> *(Land and Nilsson 2002)*

Light entering an ommatidium is absorbed in the light-guiding rhabdom. The next step is to understand how rhabdoms transduce the light signals into electrical changes in nerve cells, which can ultimately modulate behaviour. First, we will shortly define light and the information it carries.

Light is a form of electromagnetic radiation, in a narrow band of the electromagnetic spectrum. One way to think of electromagnetic radiation is as an electromagnetic wave. Another way – and here more suitable – is to describe light as a stream of tiny wave-like particles, called photons (Backhaus et al 1998). Each photon consists of a quantum of energy - the shorter the wavelength of the light the larger the energy quantum (Bruce et al 2010). For animal vision, the range of visible radiations extends from about 300 to 700 nm (Dyer 2012).

Photoreceptors' major task is to perform measurements of the photon fluxes rather than of the light energy (Backhaus et al 1998). Once absorbed, the physical entity of photon is lost, so no single photoreceptor can distinguish a change in wavelength of light from a change in its intensity (Solomon and Lennie 2007). This is the principle of univariance (Rushton 1972). Differences in the spectral composition of light falling on two neighbouring receptors containing the same visual pigment may cause a difference in their electrical response, but a difference in just the intensity of light can have exactly the same effect. In order to detect differences in the spectral composition of light, an animal must possess pigments with different absorption spectra (Bruce et al 2010).

The spectral sensitivity of photoreceptors is mainly determined by the rhodopsin pigment molecules in the rhabdomeres (the spatial arrangements of visual pigments, chromophores and screening pigments in the insect eye may also modify the spectral sensitivity of photoreceptor, Briscoe and Chittka 2001). The rhodopsins are a family of light-sensitive molecules, each made up of two components linked together: a protein, opsin, and a smaller molecule, retinal (Bruce et al 2010).

Photoreceptors do not absorb all wavelengths of light with equal efficiency (Warrant and Nilsson 1998). The wavelength most efficiently absorbed is the absorbance peak wavelength of the particular rhodopsin molecule resident in the photoreceptor (Warrant and Nilsson 1998). Thus, for each type of photoreceptor, there is a different relationship between wavelength of light and the probability of absorption, or absorption spectrum (Bruce et al 2010). Knowledge about the peak of absorption of a visual pigment ($\lambda_{max}$) allows us to calculate its theoretical absorbance spectrum according to existing templates, for example those proposed by Govardovskii et al (2000) and Stavenga (2010) (Kemp et al 2015). Information about absorbance spectrum can be used to calculate aspects of the visual capacities of an animal. Most insects so far studied have receptors maximally sensitive at the ultraviolet (UV~350 nm), blue (~440 nm) and green (~530 nm) areas of the visible spectrum (Briscoe and Chittka 2001).

When a pigment molecule absorbs light, its chemical structure changes. This, in turn, is coupled to an alteration in the structure of the cell membrane, so that the membranes' permeability to ions is modified, which in turns leads to a change in the electrical potential across the membrane (Bruce et al 2010). It is this change in potential that is signalled to the brain, and which ultimately leads to the perception of light. The magnitude and the relative stimulation of the photoreceptor classes convey information relative to the brightness (achromatic) and the wavelength (chromatic) of the impinging light (Srinivasan 2010).

## 3.1. COLOUR VISION AND PROCESSING

Many experiments have demonstrated that the existence of multiple spectral types of photoreceptors is not sufficient for colour vision, and subsequent neural stages are necessary (Kelber et al 2003). Colour processing typically requires the comparison of signals between photoreceptors types (Paulk et al 2009). An individual receptor can determine the amount of light it absorbs, but not the spectral composition of that light. Depending on the number of receptors used, visual systems are classified as monochromatic, dichromatic, trichromatic, tetrachromatic and so on.

The presence of opponent channels is then an essential prerequisite for colour vision, since receptor information must be compared to detect differences between light stimulation.

Opponent processing of visual information, broadly defined, refers to any mechanism that extracts chromatic signals by comparing input channels from different photoreceptors, or different combinations of photoreceptors (Skorupski and Chittka 2008). The existence of such interactions can be inferred from numerous physiological experiments (Vorobyev and Brandt 1997) but the exact number of opponent neurons, localization and how they interact is still not clear.

Visual signals detected by photoreceptors in the retina are transmitted to the lamina, the first visual neuropil, which then inputs to the second and largest neuropil in the insect brain's optic lobe, the medulla. A crucial stage in the insect visual processing pathway is the medulla, containing the largest number of neurons and neural types of the optic neuropil (Paulk et al 2009). Information generated in these two previous ganglia is believed to be further processed by colour-opponent coding neurons of the lobula (the third visual neuropil) (Backhaus et al 1998), although new insights about the complexity of colour processing indicate that an initial stage of colour processing could occur in the medulla (Paulk et al 2009).

Visual neurons can also process information by means of non-opponent interactions (Vorobyev and Brandt 1997). While the term chromatic is used to specify those aspects of colour which are coded by opponent mechanism, the term achromatic refers to aspects of colour coded by non-opponent mechanisms, or by a single receptor type (Vorobyev and Brandt 1997; Brandt and Vorobyev 1997). In principle, achromatic signals might be derived by summing the outputs of any number of different spectral types of photoreceptor, but normally, outputs of a single type of photoreceptor (the green receptor) are used for tasks such as motion perception and form vision (Osorio and Vorobyev 2005).

There is ample evidence that animals use chromatic and achromatic signals for different purposes (Osorio and Vorobyev 2008) and at least for bees, achromatic and chromatic information are processed in different and segregated channels or pathways. The terms channel and pathway are used indistinctly to refer to the fact that many animals are thought to process colour and luminance information separately, even though they are perceived simultaneously (Kemp et al 2015). The activation or use of one channel or other depends on the visual angle of the object (Giurfa et al 1996; Giurfa et al 1997). For instance, in bees, the achromatic pathway (mediated by the green photoreceptor) mediates detection and discrimination of objects of small angular sizes, while the chromatic visual pathway is used to detect and discriminate objects subtending large visual angles (Giurfa et al 1996; Giurfa et al 1997).

Regardless of the signal (chromatic or achromatic), flowers need to be detectable and/or recognizable against their background where they grow (Osorio and Vorobyev 2008). Animals can pay more attention to chromatic cues when they are presented simultaneously with achromatic ones, and can also respond to achromatic cues when they are presented alone (Kelber 2005), or when they convey more useful information.

### 3.2. MODELLING PHOTORECEPTOR SENSITIVITY AND EXCITATION

As we have previously mentioned, light can be described in terms of relative power across the spectrum, but for colour vision, it is the quantum emissions produced by each wavelength that matters. The colour vision system analyses the light stimulus at first by absorbing photons in the different types of photoreceptors according to their spectral sensitivities. Thus, photoreceptor spectral sensitivity is generally the minimum requirement for a sensory-based analysis of colour (Kemp et al 2015). The spectral sensitivity of a visual pigment is determined by its peak ($\lambda_{max}$) and for most insects this information is available (Peitsch et al 1992; Briscoe and Chittka 2001; Théry and Gomez 2010), which makes it easy to model receptor sensitivities using available templates (Govardovskii et al 2000; Stavenga 2010, but see Skorupski and Chittka 2010).

For any compound eye viewing the surface of an object, the number of photons going to be absorbed can be quantified by a set of receptor quantum catches ($Q_i$) according to the following expression:

$$Q_i = k_i \int_{\lambda} D(\lambda) S(\lambda) R_i(\lambda) \mathrm{d}\lambda \qquad (1),$$

where λ indicates the wavelength range (for many insects from 300 to 700 nm), $D(\lambda)$ the spectral light intensity distribution, $S(\lambda)$ the reflectance spectrum of the viewed object and $R_i(\lambda)$ spectral sensitivity function of receptor i (for a trichromatic i = UV, blue and green). Integration is performed over the spectral range where the visual system is sensitive (Vorobyev and Brandt 1997).

In the course of the day the intensity of the illumination changes. A simple change in weather conditions or shades of light could make the colour of an object to be differently perceived. This would be quite a challenge for a colour vision system since the colours would always look different, and a reliable system of object detection would not work (Menzel 1990). Animals foraging under variable illumination conditions must be able to adapt to such changes, and several experimental studies have shown that they do compensate for changes of colour caused by changes of illumination spectra. How this is achieved is not completely understood (Chittka et al 2014; Kemp et al 2015), but there is a valid approximation demonstrated to predict such adaptation state (Dyer 1999; Vorobyev et al 2001), making a visual system to be colour constant: the von Kries approximation. Colour constancy is the ability of a visual system to identify a stimulus by its spectral properties, independent of the spectral distribution of the illuminant (Kevan et al 2001). The von Kries approximation assumes that outputs of photoreceptors are scaled with the intensity and distribution of the incoming light (Kries 1905;

Chittka et al 2014). The approximation relies in the fact that receptors increase their sensitivity when they absorb few photons, and decrease their sensitivity when they are strongly stimulated (Chittka and Wells 2004). Such an algorithm can be implemented by receptor adaptation, and so invokes the simplest physiological mechanism; no special-purpose neural circuitry is required (Vorobyev et al 2001). The sensitivity factor $k_i$ in equation 1 is determined by:

$$k_i = \frac{1}{\int_{\lambda} D(\lambda) I_B(\lambda) R_i(\lambda) \mathrm{d}\lambda}. \qquad (2),$$

where $I_B(\lambda)$ is the spectral reflection function of the background to which receptors are adapted.

Instead of a receptor space, a graphical representation of colour, known as chromaticity diagram, is often used to place the coloured stimuli along the coordinate axes. A chromaticity diagram is a graphical representation of how similar two colours are to the eyes of a viewer (Renoult et al 2015). It incorporates relevant information (previous steps above) into the calculation of a colour's spatial position (Pike 2012). For some diagrams, the coordinates axes are obtained from the information encoded in the photoreceptor excitation levels $E_i$, ($i$ = $E_{uv}$, $E_{blue}$ and $E_{green}$ for a trichromatic system), which are calculated as follows:

$$E_i = \frac{Q_i}{Q_i + 1}. \qquad (3)$$

where $Q_i$ is the receptor quantum catch (equation 1). For more details see Backhaus (1991) and Chittka (1992).

In chromaticity diagrams, the intensity, or achromatic dimension, is removed so that the location of a stimulus does not depend on its intensity. Consequently, chromaticity diagrams have one dimension less than the corresponding colour space (Kelber et al 2003). Colour spaces are analysed and discussed in the first chapter of this thesis.

## 4. THE NEXT STEP: COGNITION AND BEHAVIOUR

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*"Insects have sophisticated abilities that allow them to cope efficiently with their environment"* (Giurfa and Menzel 1997)

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Cognition can be defined as the neuronal processes concerned with the acquisition, retention, and use of information (Dukas 2004). Cognition determines behavioural traits that affect animal ecology and evolution (Dukas 2004). Sensory modalities allow floral visitors to perceive the world around them, but without cognition, acquired information loses power.

Cognitive capacities are modulated by evolution and environment (Dukas and Ratcliffe 2009). The tiny brains of the floral visitors are capable of resolving amazing tasks: perception, learning, navigation, orientation and decision-making are examples of everyday life activities of floral visitors. Although this thesis does not study cognition *per se*, it is implicit in the animal behaviour. The acquisition and processing of useful information, restricted here to the visual system, is translated in behavioural responses, which are in turn dependent on cognitive mechanisms.

Animals can respond to ecologically relevant stimuli either by means of innate preferences or learned abilities. Although initial behaviour is expected to be modulated by innate preferences, many of these preferences can be easily overcome. We can train naïve animals to associate a reward with a specific colour or pattern, even when the stimulus does not correspond to a preferred colour or the pattern is not related to a floral shape.

Results from different experiments have demonstrated that floral visitors can modulate behaviour in response to the difficulty of the task, making adjustments in different ways. For instance, floral visitors seem to "specialize", being constant to a determined floral resource as long as it is available, which means that they can "learn" to "associate" a floral resource with a reward and "return" to visit it again. Limitation at the brain level seems to be a plausible answer for such behaviour (the floral constancy), although there appears to be no single and simple explanation for flower constancy (Waser 1986; Chittka et al 1999; Chittka et al 2014).

Simple behavioural studies in the context of foraging have helped scientist to elucidate more complex questions about processing of visual information (Giurfa 2013; Hempel de Ibarra et al 2014). Experiments testing floral visitors in the foraging context offer a wide possibility of questions to be explored, since finding food is a never-ending and crucial task for them. The performance of subjects can be measure under controlled conditions and information about decision making, reaction time, individual strategies, accuracy and trade-offs can be obtained.

Increasing the information about visual capacities and abilities in different species of floral visitors can help us better to understand the similarities and differences between them. It also expands our knowledge of how communication with plants changes from species to species. Outputs can give insights to a vast number of areas, as conservation, ecology, physiology and neurobiology. For instance, only by assuming the presence, type and number of photoreceptors one cannot make a confident conclusion about an animal's visual capacity. To properly understand what a signal means to an animal, we have to study the behavioural outcomes employed in day-to-day activities. Our results indicate the extent to which data obtained from honeybees can be compared to a related hymenoptera species (*Bombus terrestris*) and how the visual and cognitive capacity of a diurnal hawkmoth (*Macroglossum stellatarum*) differs from the two former.

# 5. PLACING THE THESIS IN CONTEXT

*Vision, learning and behaviour*

Since von Frisch's finding, we have already accumulated a great deal of knowledge of the psychophysics, ecology and neural bases of insect colour vision. Many of these findings were obtained by testing the honeybee capacities and abilities of finding and discriminating colours, as well as aspects of its behaviour, learning and social communication. Honeybee colour vision has been investigated in more detail than any other animal except humans (Vorobyev and Menzel 1999). Because information for other floral visitors is still scarce, much of what was obtained using the honeybee is extrapolated to other hymenopteran taxa (Chittka et al 1992) or at least assumed to be similar to some extent.

Vision plays a key role in the detection of resources by diurnal pollinators. The anatomy of the eyes, the nature of the photoreceptors and the neural connectivity of the visual system differ between insect groups. This suggests that each insect group could see the world in different shapes and colours, a result that could have important implications for the ecology and evolution of plant-pollinator networks. Only recently, studies of insect vision with different floral visitors are coming to light, as for butterflies (Kinoshita and Arikawa 2000; Frentiu et al 2007; Koshitaka et al 2008; Blackiston et al 2011), flies (Fukushi 1989; Troje 1993; for a general revision see Lunau 2014), eusocial bees (Lunau 1990; Lunau 1991; Gumbert 2000; Dyer 2006; Lunau et al 2009; Skorupski and Chittka 2010a) and solitary bees (Menzel et al 1988; Fauria and Campan 1998; Anfora et al 2010).

Despite the huge amount of information available for *Apis mellifera* (doing a simple search on SCOPUS using as key words "honeybee", "color/colour" and "vision", a total of 162 papers were found, compared with 38 for *Bombus terrestris* and 14 of *Macroglossum stellatarum*) at the behavioural and (to a lesser but still good degree) visual processing levels, information about how pollinators other than honeybee use visual floral cues to detect and discriminate objects is still limited and the literature has many gaps. All floral visitors analysed so far have proved to have receptors capable of acquiring and processing visual information from their environment (Peitsch et al 1992; Briscoe and Chittka 2001), and many experiments have demonstrated that colour affects the behaviour of these floral visitors.

We aimed to study different aspects of the visual system of two species: an eusocial bee, *Bombus terrestris*, and a diurnal moth, *Macroglossum stellatarum*. The selection of species was based on the easiness with which we could obtain animals to perform experiments under controlled conditions. Both *Bombus terrestris* and *M. stellatarum* are known to have trichromatic colour vision, and it seems that at least *B. terrestris* is similar to *A. mellifera* in having two parallel channels for processing visual information. However, the related species have shown differences in other aspects of colour discrimination, perception and behaviour (Dyer et al 2008; Morawetz and Spaethe 2012; Orbán and Plowright 2014; Sherry and Strang 2014). Despite the efforts of Almut Kelber in studying the species, little is known about the visual system of *M. stellatarum*. For instance, we know that *M. stellatarum* has true colour vision and can be trained to discriminate between colours, but the spectral sensitivity and the fine colour discrimination remained unknown before our experiments.

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# GENERAL AIM

The present thesis, based on four chapters, investigates the ability of two floral visitors to visually detect and discriminate colours, and their visual capacities and limits by means of (mainly) behavioural responses. Our ultimate aim is to contribute to the advance of the understanding of the visual system of important floral visitor species, aside from *Apis mellifera*, filling existent gaps of information, as well as to understand the visual constraints among floral visitors.

# MOTIVATION AND SPECIFIC AIMS

Questions to be resolved were dependent on the species and on the available knowledge of the visual system. The motivation and specific aims of the thesis are presented below.

**a)** Models of colour discrimination attempt to predict the ease with which animals can discriminate between stimuli of different colours. From the point of view of an ecologist trying to understand how an animal sees the world, colour discrimination models are often treated as black boxes that, once fed with certain input data, provide a "perceptual distance" between two colours. The ability to predict in which colours the bee sees the world is therefore an important step in understanding their foraging choices under certain conditions. In **chapter one**, we investigate which of the three contending colour vision models predicts bumblebee (*Bombus terrestris*) behaviour the best in a colour discrimination task under controlled laboratory conditions.

**b)** Many flowers have evolved displays that make them more conspicuous to their pollinators. Colour and odour are two important cues that plants use to attract their floral visitors. The size of a flower has been proved to affect the search time in a homogeneous background. How the presence of either both cues (colour and odour) or only one of them affects the behaviour of floral visitors when seeking food is still not clear, especially when searching for inconspicuous stimulus (i.e. reddish flowers of reduced size) against a complex background. By focusing on single modalities, we may be underestimating bee performance during a foraging activity, and missing opportunities to understand multisensory perceptual linkages. In **chapter two** we aim to understand how colour, size, training and the presence or absence of a second cue, as odour, affect the foraging activity of bumblebees seeking nectar in a semi-natural environment. We also compare the obtained results with those from a similar experiment using a homogeneous background.

**c)** Spectral sensitivity of photoreceptors strongly influence colour vision. Although the compound eyes of *Macroglossum stellatarum* have been assumed to present three receptors types similar to those of the related crepuscular species *Manduca sexta*, the presence, peaks ($\lambda_{max}$) and overall sensitivity of these receptors have not been experimentally determined. In **chapter three** we investigate the sensitivity of *M. stellatarum* using monochromatic lights in the context of flower visits; we also quantify the number and determine the peaks ($\lambda_{max}$) of photoreceptors.

**d)** Results obtained in chapter three led us to think about colour discrimination capacity of *Macroglossum stellatarum*. Fine colour discrimination when foraging must be advantageous for a floral visitor since many co-occurring flowering species can present similar colours (a very common strategy used by plants). What is the minimum colour difference between similar flowers to be perceived as different on this moth' eyes? Distinguishing between similar colours could enhance the foraging activity and increase performance when foraging in a natural context. In **chapter four** we behaviourally investigate the minima of discrimination achieved by *M. stellatarum* when choosing between similar wavelengths, and compare these minima values with those obtained for the honeybee and a tetrachromatic butterfly.

# CHAPTERS AT A GLANCE

CHAPTER ONE: Insect vision models under scrutiny: what bumblebees (*Bombus terrestris terrestris* L.) can still tell us.

**Authors**: Francismeire Jane Telles and Miguel A. Rodríguez-Gironés

**Published in:** The Science of Nature: Naturwissenschaften, 102(4): 1-13, 2015.

CHAPTER TWO: Enhancing foraging efficiency: use of colour and odour during flower detection in a complex background.

**Authors**: Francismeire Jane Telles, Guadalupe Corcobado, Alejandro Trillo and Miguel A. Rodríguez-Gironés

CHAPTER THREE: Out of the blue: the spectral sensitivity of hummingbird hawkmoths.

**Authors**: Francismeire Jane Telles, Olle Lind, Miriam Judith Henze, Miguel Angel Rodríguez-Gironés, Joaquin Goyret, Almut Kelber

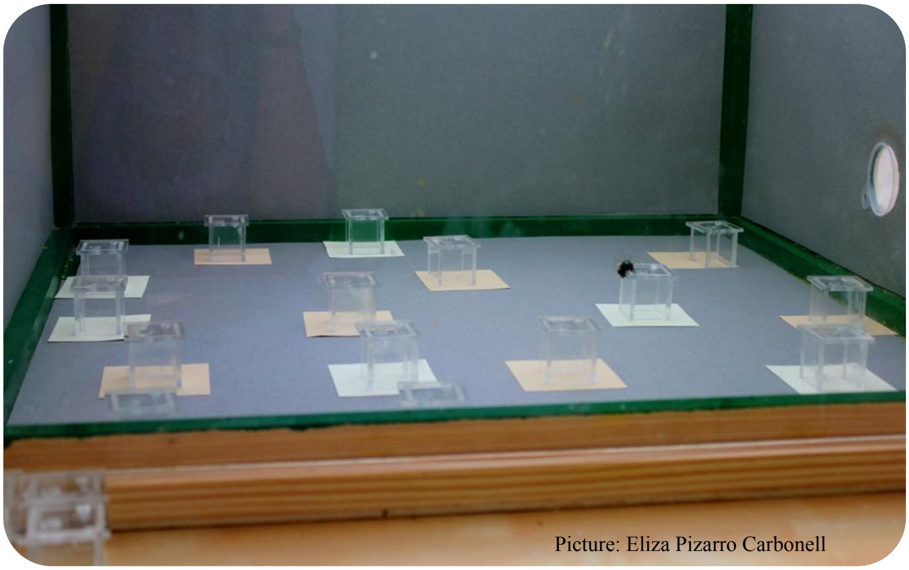
**Published in:** Journal of Comparative Physiology A, Jun; 200(6):537-46, 2014.

CHAPTER FOUR: Wavelength discrimination in the hummingbird hawkmoth *Macroglossum stellatarum*.

**Authors**: Francismeire J. Telles, Almut Kelber and Miguel A. Rodríguez-Gironés.

**Under review:** The Journal of Experimental Biology

# CHAPTER ONE


Picture: Eliza Pizarro Carbonell

# Insect vision models under scrutiny: what bumblebees (*Bombus terrestris terrestris* L.) can still tell us

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## Abstract

Three contending models address the ability of bees to detect and discriminate colours: the colour opponent coding model (COC), the colour hexagon model (CH) and the receptor noise-limited model (RN), but few studies attempt to determine which model fits experimental data best. To assess whether the models provide an accurate description of bumblebee colour space, we trained bees to discriminate four colour pairs. The perceptual distance between the colours of each pair was similar according to the CH model, but varied widely according to the COC and RN models. The time that bees required to select a flower and the proportion of correct choices differed between groups: decision times decreased as achromatic contrast increased, and the proportion of correct choices increased with achromatic contrast and perceptual distance, as predicted by the COC and RN models. These results suggest that both chromatic and achromatic contrasts affected the discriminability of colour pairs. Since flower colour affects the foraging choices of bees, and foraging choices affect the reproductive success of plants, a better understanding of which model is more accurate under each circumstance is required to predict bee behaviour and the ecological implications of flower choice and colour.

**Keywords**: colour discrimination, colour hexagon, colour opponent coding, achromatic contrast, receptor noise-limited, search time

## Introduction

Like most pollinators, bumblebees use visual information to detect and recognize landmarks and food sources. To maximize foraging efficiency, they require an effective visual system and the ability to associate visual cues with rewards. Several models have been developed to understand how colour vision is processed. Three of them are currently used to study colour-discrimination by bees and other hymenoptera: the colour-opponent coding (COC; (Backhaus 1991), colour hexagon (CH; Chittka 1992) and receptor noise-limited (RN; (Vorobyev and Brandt 1997; Vorobyev and Osorio 1998; Vorobyev et al 2001)) models. The COC model was originally formulated for honeybees, *Apis mellifera*, while the CH model was developed for trichromatic hymenopteran species and the RN model for a much wider range of taxa, including species with di- and tetrachromatic colour vision. In the remainder of this paper, RN refers to the trichromatic version of the receptor noise-limited model.

The three models assume that colour information is processed via two sets of colour-opponent coding neurons. The COC model used the least-squares method to obtain the set of opponent mechanisms that best fitted honeybee data from a multidimensional scaling experiment (Backhaus et al 1987). Using this set of opponent mechanisms we can plot the colour loci of arbitrary colours on a plane. According to the COC model, the perceptual distance between two colours is proportional to the distance between their loci, calculated with the city-block metric (Backhaus 1991). The COC model should provide a reasonably good description of the honeybee colour space, but if different hymenopteran species are endowed with different sets of colour-opponent coding neurones, the COC model might not be applicable to other species. The CH model therefore assumed that the two opponent mechanisms were orthonormal and that perceptual distance between two colours was proportional to the Euclidean distance between their loci (Chittka 1992). With these assumptions, perceptual distances are independent of the specific choice of opponent mechanisms. The rationale of the CH model was that, when we ignore the set of colour opponent mechanisms used by a species, it might be preferable to describe its colour space using rather non-specific sets of mechanisms than those of another species (Chittka et al 1992). Finally, the RN model assumes that it is noise at the receptor level, and not the specific information-processing rule, that sets the discrimination limit, its parameters are inferred from electrophysiological recordings in photoreceptor cells (Vorobyev et al 2001) and the model has been used in different experiments to predict chromatic distances between spatially separated stimuli (Hempel de Ibarra et al 2001; Hempel de Ibarra et al 2002; Dyer and Neumeyer 2005; Niggebrügge et al 2009; Martínez-Harms et al 2014). One common point between the COC and CH model is the way they deal with the non-linearity of phototransduction. Signals from receptor cells are nonlinearly related to the quantum flux that forms the input to the receptor (Naka and Rushton 1966a; Chittka 1996a) and both models assume that the phototransduction process is well described by the Naka-Rushton equation (Naka and Rushton 1966a; Naka and Rushton 1966b; Backhaus 1991). By contrast, the RN model assumes linear phototransduction in its linear version (Vorobyev and Osorio 1998) and logarithmic phototransduction in the logarithmic version of the model (Vorobyev et al 2001).

The three models predict the main features of the honeybee spectral sensitivity data (Vorobyev and Brandt 1997; Brandt and Vorobyev 1997) and explain a number of experimental results (Giurfa et al 1997; Hempel de Ibarra et al 2002; Lotto and Chittka 2005; Dyer and Neumeyer 2005; Arnold and Chittka 2012), but the plurality of assumptions of available models could be confusing and lead to erroneous conclusions in ecology and vision research. For example, the ability of pollinators to locate flowers should affect how floral resources are partitioned among pollinator groups (Rodríguez-Gironés and Santamaría 2005). Hence, flower colour may influence both resource partitioning and the selective pressures to which flowers are subject. However, to understand the effect of colour on resource partitioning, we must know with which ease different pollinator species will locate flowers. Likewise, as long as we accept several colour discrimination models, their different assumptions remain putative mechanisms for visual information processing. Falsifying one or more of these models would evidence the models' underlying assumptions to be incorrect, narrowing down the search of putative mechanisms and therefore contributing to our understanding of the bee's visual system.

Due to differences in their underlying assumptions the models can make different predictions, and it is possible to select a set of colour pairs in such a way that the different models rank their perceptual distances in different orders. If we train bees to discriminate between the two colours of each pair, the proportion of correct choices indicates their perceptual distances, and therefore we can use these results to evaluate the models.

To select the model that best describes bumblebee (*Bombus terrestris terrestris* L.) colour discrimination, we trained bumblebees to discriminate four pairs of coloured stimuli in the laboratory. The pairs had similar perceptual distances according to the CH model, but varying perceptual distances according to the COC and RN models. Although the COC and RN models have parameters inferred from honeybee behavioural and neurophysiological data, respectively, all three models are commonly used to estimate perceptual colour distances in bumblebees (Gumbert 2000; Kunze and Gumbert 2001; Spaethe et al 2001; Dyer and Chittka 2004c; Dyer and Chittka 2004a; Dyer and Chittka 2004b; Lunau et al 2006; Dyer et al 2008; Martínez-Harms et al2010; Arnold and Chittka 2012; Rohde et al 2013) and it seems reasonable to ask which model is more accurate under our experimental condition.

## Materials and Methods

### *Rationale for the selection of colour pairs*

Let us draw, in the CH plane, a circle of radius **r** and centre **P**. According to the CH model, the perceptual distance between two colours is proportional to the Euclidean distance between their loci in the colour hexagon (Chittka 1992). Therefore the perceptual distance between colours represented by **P** and **Q**, where **Q** is any point on the circle, should be equal to **r**, regardless of the angle **α** between the vertical axis and the segment **PQ** (Fig. 1a).

Knowing the loci of two colours on the CH model, it is straightforward to calculate their perceptual distance according to the COC and RN models (see Supplementary material 1). This distance depends on the angle **α** (Fig. 1b), and the change can be as large as five-fold for the RN model. It follows from Fig. 1b that, if we have two colour pairs, ($\mathbf{P}, \mathbf{Q_1}$) and ($\mathbf{P}, \mathbf{Q_2}$), such that $\boldsymbol{\alpha}_1$ is slightly greater than 0º and $\boldsymbol{\alpha}_2$ around 100º, the chromatic distance between **P** and $\mathbf{Q_1}$, according to the COC and RN models, should be much higher than the chromatic distance between **P** and $\mathbf{Q_2}$. Assume that we train a group of bees to discriminate between **P** and $\mathbf{Q_1}$, and a second group to discriminate between **P** and $\mathbf{Q_2}$. According to the predictions of the CH model, no difference in the performance of bees between groups should be found. By contrast, if the COC or RN models were correct, performance should be better for bees of the (**P**, $\mathbf{Q_1}$) group than for bees of the (**P**, $\mathbf{Q_2}$) group.

Based on these considerations, we have selected four colour pairs in such a way that, when plotted on the CH colour space, all pairs had the same distance, but the line segments joining the two loci of a pair formed different angles with the vertical axis (see below). Figure 1 represents a hypothetical example – not the colours used for the experiment, these are specified in the following.

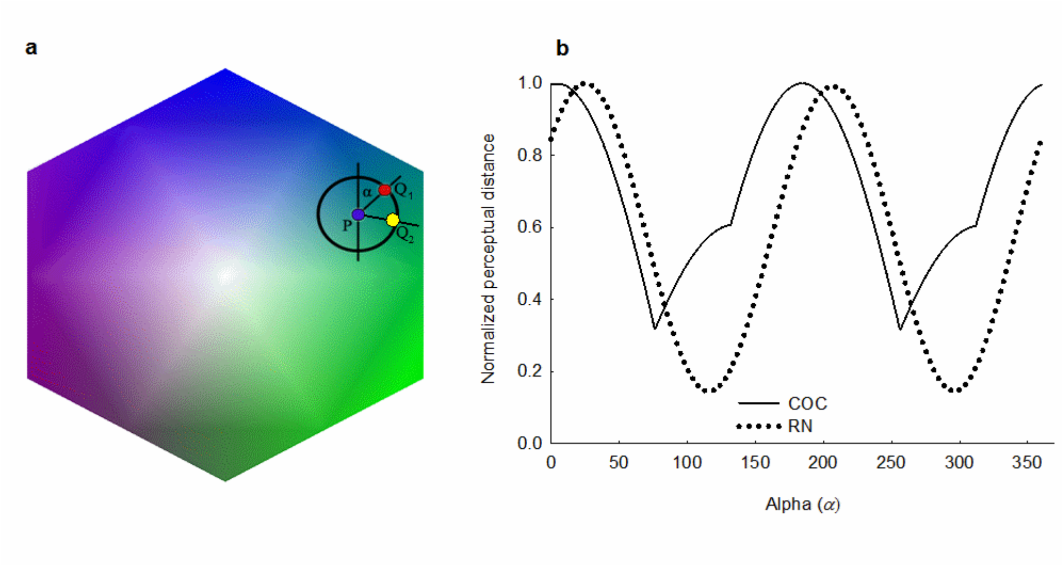

**Fig. 1 a** According to the colour hexagon (CH) model, the perceptual distance between colours represented by points P and Q, where Q lies on a circle of radius *r* centred at P, is independent of the angle, α, between the PQ segment and the vertical axis. **b** Colour distance between points P and Q (α) according to the colour-opponent coding (COC, solid line) and receptor noise-limited (RN, dashed line) models, plotted vs. the angle that the PQ segment makes with the vertical axis, α. Note that the perceptual distance between P and Q changes with the orientation of the PQ segment. The COC and RN models use different perceptual scales. For comparison, distances have been normalised. Colours (both P and Q (α)) are chosen in such a way that $E_{UV} + E_B + E_G = 1$ when bees are habituated to the background. For this hypothetical example, calculations assume D65 illumination function and green background colour (HKS coloured paper 54N).

## *General setup*

Bumblebees, *Bombus terrestris terrestris*, kept indoors in a single-chamber nesting box (length, width, height: 30 x 20 x 25 cm), were trained to collect 60% (weight/weight) sucrose solution from ultraviolet (UV) transparent artificial flowers (hollow Plexiglass cubes: 4 x 4 x 4 cm, with 3 mm thick walls) inside a flight cage (70 x 70 x 35 cm) connected to the hive by a gated tunnel. Bees had *ad libitum* access to pollen within their nest box and were allowed to collect 20% (weight/weight) sucrose solution from a transparent feeder, positioned inside the flight cage, outside experimental sessions. The flight cage was lined with UV-reflecting grey cardboard paper (Canson Mi-Teintes – 431, Fig. 2). Incoming light (Fig. 2) was provided by two Philips TL-D90 Graphica 36w/965 white light tubes and one Philips TL-D 36w BLB UV light tube, 75 cm above the cage floor. Light flicker was converted to 1.200 Hz, diffused and homogenized by a single sheet of Rosco 216 (Rosco, Germany) UV-transmitting white diffusion screen.

## *Computation of colour distances*

We measured the spectral irradiance of the light inside the cage and the reflectance spectra of stimuli and background in the range of 300 - 700 nm (Fig. 2) with an Ocean Optics USB 4000 spectrometer (Dunedin, FL, USA). For all computations, we used the average of three measurements. The absolute irradiance (photons$\cdot$s$^{-1}\cdot$cm$^{-2}\cdot$nm$^{-1}$) was measured using a cosine corrector (CC-3-UV-S, Ocean Optics, Dunedin, FL, USA) to collect light, which was transmitted through an optical fibre to the spectrometer. A lamp (LS-1-CAL-220, Ocean Optics) of known output was used to calibrate the spectrometer. For the measurements of the background and stimuli, the spectrometer was calibrated with a standard white (Ocean Optics WS-1) and measures were taken using a reflexion probe at 45° to the surface.

Supplementary material 1 specifies how we calculated perceptual distances according to the three models. For the computations, we used the photoreceptor spectral sensitivity of *B. terrestris dalmatinus* (Skorupski et al 2007), as Chittka et al (2001) found no difference between the behavioural preferences of this subspecies and *B. terrestris terrestris*. Green and brightness contrast, contrast of target colours against background and spectral purity were calculated as specified in Supplementary material 1 and the results are presented in Supplementary material 2 (Table S1).

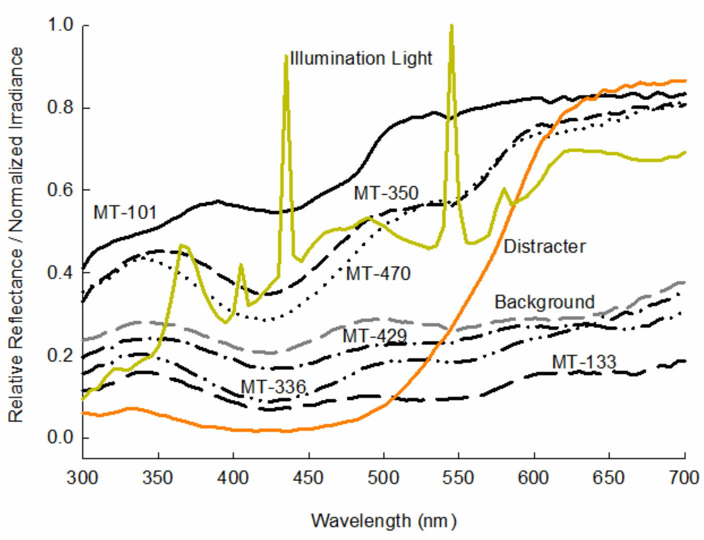

**Fig. 2** Spectral reflectance of stimuli and background, and normalized irradiance. (Irradiance reached a maximum of $5.33756 \cdot 10^{11}$ photons$\cdot$s$^{-1}\cdot$cm$^{-2}\cdot$nm$^{-1}$ for 545 nm).

## *Stimuli and perceptual colour distances*

Colour stimuli were 7 x 7 cm squares cut from Canson Mi-Teintes cardboard (98lb/160 gsm series) and set under the transparent Plexiglass flowers. By combining six different colours (references 133, 429, 336, 350, 101 and 470; Fig. 2) we formed four pairs (Table S2, Supplementary material 2). Within each pair, one colour was arbitrarily designed as the "A" colour and the other as the "B" colour. According to CH model (Chittka 1992), perceptual distances were similar for all four pairs (mean ± s.e.m. = 0.0549 ± 0.0007 hexagon units). However, according to the COC (Backhaus 1991) and RN (Vorobyev and Osorio 1998; Vorobyev et al 2001) models, there were differences in perceptual distances (Supplementary material 2, Table S2, Fig. 3) – note that, because all colour pairs had similar distances (coefficient of variation = 0.06) according to the logarithmic version of the RN model (Vorobyev et al 2001), we only consider the linear version of this model (Vorobyev and Osorio 1998). For our choice of colour pairs, the predictions of the logarithmic version are indistinguishable from those of the CH model.

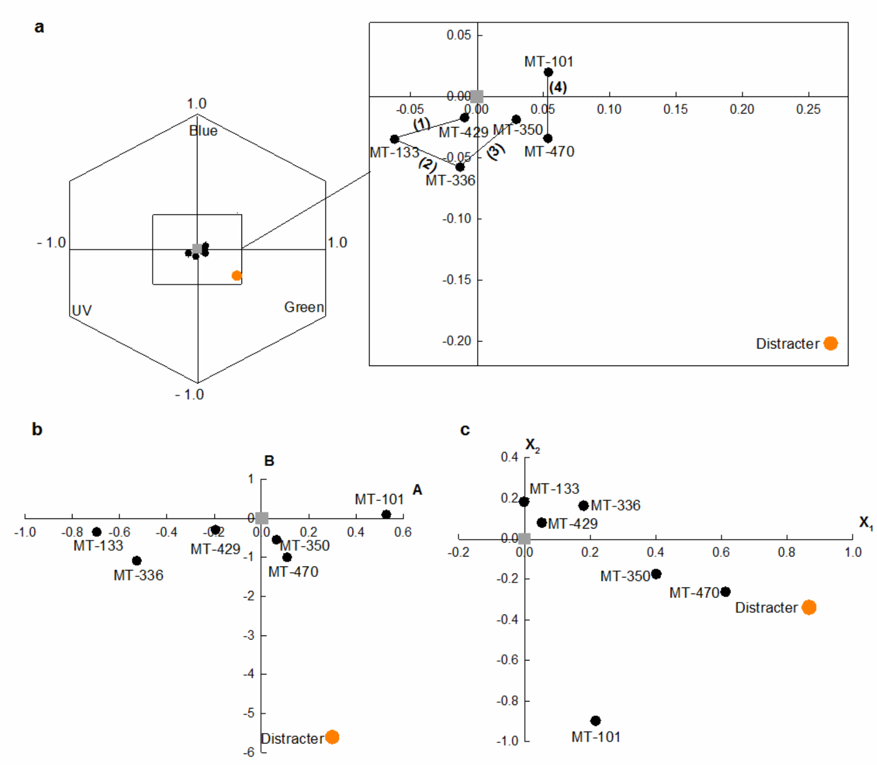

**Fig. 3** Loci of stimuli (black circles), background (grey square) and orange distracter presented during phase I (orange circle), plotted on the **a** CH, **b** COC and **c** RN linear colour spaces. Note the detailed magnification of the CH colour space showing the stimuli and background distribution with black lines connecting the two colours of each pair (bold numbers in parenthesis indicate the group to which they belong). A and B in panel **b** represent the two colour opponent coding channels used in the COC model. $X_1$ and $X_2$ in panel **c** correspond to orthogonal axes (for detailed information see Hempel de Ibarra et al 2001), calculated with coefficients values of A = 1.104, B = 1.154, a = 0.453 and b = 0.547, assuming noise to be $e_{UV} = 0.74$, $e_B = 0.67$ and $e_G = 0.61$ (obtained from Fig. 3c, Skorupski and Chittka 2010).

## *Experimental procedure*

During sessions, only the experimental subject was allowed to enter the flight cage. The trajectory hive-cage-hive was considered as a foraging bout and any contact with the surface of a stimulus, regardless of whether the bee only touched it with its front legs or landed on it, was considered as a choice. After every foraging bout, flowers were replaced with new ones and cleaned with 30% ethanol to remove olfactory cues. To rule out position learning, the spatial arrangement of the flowers was randomized before each foraging bout.

The experiment was divided in three phases: a pre-training phase was used to discard individuals not using colour as a cue (phase I); during training (phase II), individuals learnt to discriminate between perceptually similar colours; and finally (phase III) their performance was tested over a non-rewarded foraging bout. A total of five individuals were rejected after failing to meet the selection criteria of phase I (see below). Discarding these individuals should not bias the results for two reasons: (1) the task used to discard bees was not the task they had to perform during the training and testing phases and (2) the five bees had been pre-assigned to the four experimental pairs (two bees to group 3, one bee to each of the other groups).

We successfully trained and tested 64 bumblebees (16 per pair), from five different colonies, approximately matched in size (mean ± SD length of the left eye, measured as the distance of the longest surface perimeter through the centre: 2.35 mm ± 0.13 mm) due to the relationship between eye size, optical quality and behavioural ability at target detection and discrimination (Macuda et al 2001; Spaethe and Chittka 2003; Wertlen et al 2008). All selected bumblebees did not differ in eye size between groups (GLM analysis: $X^2 = 0.02$; df = 3; p = 1). Of the 16 bees tested for each colour pair, eight bees were trained to seek nectar in colour A, and the remaining ones in colour B. Bees were assigned to a colour pair in a random order and therefore there was no association between colony and colour pair.

Upon entering the flight cage, bees encountered eight target and eight distracter flowers as specified below. From any flower, the nearest neighbours subtended an average angle greater than 8º (bumblebees use chromatic cues to process visual information when objects subtend a visual angle greater than 2.7º; Dyer et al 2008). The average distance between nearest and most distant flowers was 10.63 cm and 52.15 cm, respectively. Target and distracter flowers differed in the reward they offered as well as in their colour. In order to habituate bees to encountering empty flowers before the extinction test (phase III), during phases I and II four of the target flowers contained 30µl of 60% sucrose solution and the other four were empty. All eight distracter flowers contained 30µl of 0.12M quinine hydrochloride dehydrate (SIGMA) solution during phases I and II.

### *Phase I: pre-training*

Within each group, target flowers were of colour A for half of the bees and of colour B for the other half. Target colours remained the same throughout the experiment (phases I, II and III), while distracter colours changed. During phase I distracter flowers had the same colour for all bees (Canson Mi-Teintes 553, orange to humans – Fig. 2). This colour was sufficiently dissimilar to all others (mean ± s.e.m. distance: 0.33± 0.013 hexagon units, 5.81 ± 0.21 COC units, 0.85 ± 0.12 RN units, Table S3, Supplementary material 2) so that any bee should have been able to discriminate between it and the target colour to which it was being trained. Phase I aimed on eliminating bees that were not using chromatic information to locate nectar. In this phase, bees were allowed to complete as many foraging bouts as needed to visit 60 flowers. Individuals that chose target flowers on at least 70% of visits entered phase II.

### *Phase II: training*

In phase II bees had to discriminate between the two colours of the pair to which they were assigned. Now, bees pre-trained with colour A were going to find the complementary opponent colour, B, as the distracter flower, and *vice-versa*, with a total of 16 flowers inside the flight cage. Bees completed 15 foraging bouts during phase II. During each bout, bees were allowed to visit as many flowers as they wanted, but we recorded only the first six flowers visited, ignoring revisits to the same previous flower, only being considered a new visit after a different visitation to another flower (already empty or not). Once satisfied, bees flew back to the hive. Upon completion of training, bumblebees were tested in a final non-rewarded foraging bout.

### *Phase III: testing*

Phase III lasted for a single foraging bout with bees visiting as many flowers as they wanted. Flowers had the same colours as in phase II, but they were all clean and empty. This foraging bout was recorded using a video camera for subsequent analysis. Using the Picture Motion Browser program (PMB, ver.5.8.02, Sony) we calculated the average time that bees spent in choosing flowers. For each flower visit, we measured the time elapsed since the bee left a flower until it landed on the following one. Dividing the overall time by the number of flowers (including the final one) that the bee approached and inspected during its flight trajectory, we obtained the average time spent per flower during each visit. Taking the median of these values over the duration of phase III, we obtained the "decision time" – or time spent travelling to and inspecting a flower before deciding whether to land on or reject it.

### *Statistical analyses*

A Mann–Whitney U analysis, with Bonferroni correction for multiple comparisons ($\alpha_{adjusted}$ = 0.012), revealed that, for each colour pair, the proportion of correct choices (Table S4, Supplementary material 2) was independent of whether target flowers were of colour A or B (all p > $\alpha_{adjusted}$). We therefore pooled the data from bees trained to seek nectar at A or B flowers, ignoring this factor in subsequent analyses.

We explored how the acquisition of the discrimination task (changes in the proportion of correct responses through phase II) and final performance (proportion of correct choices during phase III) changed with decision time, colour pair and other parameters that might affect bee choices: brightness (Hempel de Ibarra et al 2000; Reisenman and Giurfa 2008), green contrast (Giurfa et al 1996; Giurfa et al 1997; Giurfa and Vorobyev 1998; Hempel de Ibarra et al 2001; Hempel de Ibarra et al 2002; Hempel de Ibarra and Giurfa 2003; Martínez-Harms et al 2010), colour contrast to the background and spectral purity (Lunau 1990; Lunau 1993; Lunau et al 1996; Lunau et al 2006; Rohde et al 2013; Papiorek et al 2013).

To analyse the acquisition of the discrimination task, we divided phase II (15 foraging bouts) in three blocks of five foraging bouts each and calculated, for each bee, the proportion of correct choices in each block. Because there were too many explanatory variables to include them all in a single model, we performed several groups of repeated-measures analyses of variance on these data. Each group included a subset of explanatory variables. Within each group, we started with the full model, which included all the explanatory variables of the group and interaction terms, and systematically removed non-significant interactions and variables to find the model with the lowest value of Akaike's information criterion (AIC - Akaike 1973).

In the first group, we started with a model having block (1 to 3) as within-subject repeated measure, colour pair as between-subjects categorical factor, and log-transformed decision time as continuous variable (decision time was log transformed to improve the linearity of the relationship). This model also included the interactions between block and colour pair and (log-transformed) decision time. The process was then repeated with eight additional groups. For the initial model of these groups we replaced colour pair with a continuous measure of perceptual distance (as predicted by the COC or RN model – half of the groups with each variable) and added a variable related to the achromatic properties of the pair (brightness contrast, green contrast, spectral purity or colour contrast against the background – each of these variables was combined with the two measures of perceptual distance).

Table 1 specifies the full model and the most parsimonious model for each group. Note that this exercise was not repeated with the CH and logarithmic RN models because there was not sufficient variability in perceptual distances of the four colour pairs when calculated with these models (coefficients of variation of 0.04 and 0.06, respectively). From all the models tested, we selected the one with the lowest AIC value and those within 2 AIC units, and these were the models we used for hypothesis testing. In the selected model, we estimated *p* values with type II tests.

**Table 1** Model selection for the repeated-measures analysis of task acquisition (phase II). CCB = Colour contrast against the background.

| **Initial Model** | **Selected Model** | **$AIC_{Initial}$** | **$AIC_{Final}$** |
|---|---|---|---|
| Block * ColourPair + logTime + Block : logTime | Block + ColourPair | -195.66 | -257.41 |
| Block * COC + logTime + Brightness + Block : logTime + Block : Brightness | Block + COC + Brightness | -216.82 | -265.10* |
| Block * COC + logTime + GreenContrast + Block : logTime | Block + COC + | -215.71 | -264.32* |

| | | | |
|---|---|---|---|
| + Block : GreenContrast | GreenContrast | | |
| Block * COC + logTime + SpectralPurity + Block : logTime + Block : SpectralPurity | Block + COC + SpectralPurity | -208.09 | -260.12 |
| Block * COC + logTime + CCB + Block : logTime + Block : CCB | Block + COC | -203.22 | -261.57 |
| Block * RN + logTime + Brightness + Block : logTime + Block : brightness | Block + RN + Brightness | -218.67 | -264.82* |
| Block * RN + logTime + GreenContrast + Block : logTime + Block : GreenContrast | Block + RN + GreenContrast | -219.23 | -265.34* |
| Block * RN + logTime + SpectralPurity + Block : logTime + Block : SpectralPurity | Block + RN + SpectralPurtiy | -205.89 | -256.66 |
| Block * RN + logTime + CCB + Block : logTime + Block : CCB | Block + RN | -201.92 | -261.26 |

* most parsimonious models

For the analysis of final performance, we determined, for each bee, the number of correct choices over the first 15 flower visits in the extinction test (phase III). Subsequent visits were discarded because bee behaviour becomes haphazard as the number of empty flowers visited increases (Lotto and Chittka 2005). These data were analysed with generalized linear models (GLM) with binomial distribution and logit link function. As in the previous case, we fitted nine sets of models to the data, with the same structure described for the repeated-measures ANOVAs, except that in the GLMs we removed the variable “block” (as we only included data from the last trial). In each of these sets, we systematically removed interaction terms and variables to search for the most parsimonious (lowest AIC value) model. Table 2 specifies the full model and the most parsimonious model for each group. We used the most parsimonious models to determined statistical significance, based on type II log-likelihood ratio tests and used planned contrasts to compare performance on specific colour pairs. Note that, in these models, decision time was not log transformed. The reason for this is that we used a non-linear (logit) link function.

**Table 2** Model selection for the generalized linear models (GLM) of the extinction test (phase III). CCB =Colour contrast against the background.

| Initial Model | Selected Model | $AIC_{Initial}$ | $AIC_{Final}$ |
|---|---|---|---|
| ColourPair * Time | ColourPair * Time | 284.45 | 284.45* |
| COC + Brightness | COC + Brightness | 285.6 | 285.6* |
| COC + GreenContrast | COC + GreenContrast | 288.42 | 288.42 |
| COC + SpectralPurity | COC + SpectralPurity | 295.45 | 295.45 |
| COC + CCB | COC + CCB | 311.92 | 311.92 |
| RN + Brightness | RN + Brightness | 288.1 | 288.1 |
| RN + Green Contrast | RN + GreenContrast | 286.5 | 286.5* |
| RN + Spectral Purity | RN + SpectralPurity | 304.87 | 304.87 |
| RN + CCB | RN | 315.47 | 313.49 |

*most parsimonious models

Finally, we used GLM’s with Gaussian distribution and identity link function to investigate whether decision times differed between groups. In the first analysis, we used pair as categorical variable. In the following analyses, which were followed by a *post hoc* Tukey’s HSD test for comparisons among groups, we used perceptual distance (calculated with the COC or RN model) and achromatic (brightness or green) contrast. Statistical analyses were performed with R software (R Core Team 2013).

## Results

In the analysis of task acquisition during training, the most parsimonious models contained block of trials, perceptual distance and either brightness or green contrast (Table 1). We obtained the same results regardless of whether we used the COC or RN models to estimate perceptual distances. Although the model with RN distance and green contrast had the lowest AIC, the difference in AIC value between this model and those with COC and brightness was less than two units (Table 1). As a result, we cannot conclude that one model fits the data significantly better than the others (Burnham and Anderson 2002), and therefore we used these four models for hypothesis testing (Table 3).

**Table 3** Hypothesis-testing for the repeated-measures analysis of task acquisition (phase II). Only the most parsimonious models are described here.

| **Model** | **Variables** | $X^2$ | **d.f.** | ***p*** |
|---|---|---|---|---|
| **Block + COC + Brightness** | | | | |
| | Block | 89.19 | 2 | **<0.0001** |
| | COC | 11.45 | 1 | **0.0007** |
| | Brightness | 10.90 | 1 | **0.001** |
| **Block + COC + GreenContrast** | | | | |
| | Block | 89.19 | 2 | **<0.0001** |
| | COC | 13.97 | 1 | **0.0002** |
| | GreenContrast | 10.13 | 1 | **0.0015** |
| **Block + RN + Brightness** | | | | |
| | Block | 89.19 | 2 | **<0.0001** |
| | RN distance | 10.39 | 1 | **0.0013** |
| | Brightness | 10.88 | 1 | **0.001** |
| **Block + RN + GreenContrast** | | | | |
| | Block | 89.19 | 2 | **<0.0001** |
| | RN | 14.31 | 1 | **0.0001** |
| | GreenContrast | 11.49 | 1 | **0.0007** |

We obtained qualitatively similar results for the four most parsimonious models. The proportion of correct responses increased with experience (effect of block – Fig. 4), with perceptual distance (as calculated with the COC or RN model) and with achromatic (brightness or green) contrast (Table 3).

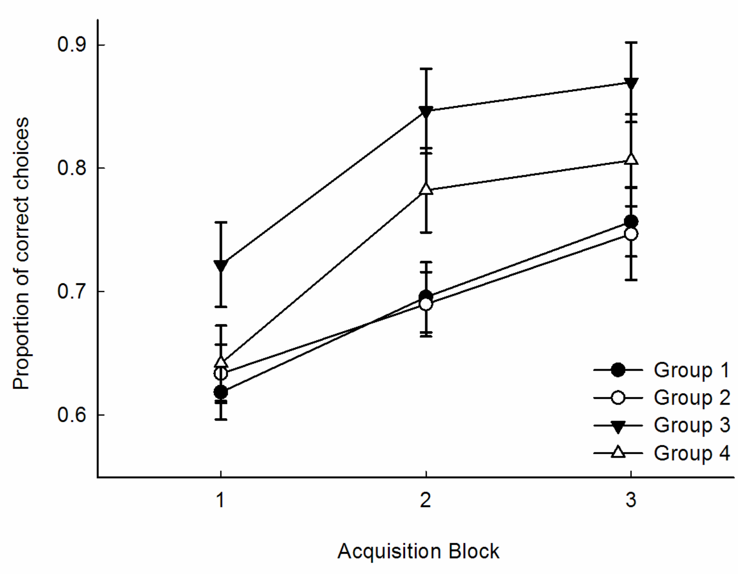

**Fig. 4** Acquisition of colour discrimination task during training (phase II): Change in the proportion of correct choices as training progresses for the different treatment groups. Error bars denote standard errors

Overall, the most parsimonious model for the extinction test (phase III) included colour pair as a categorical factor, decision time and their interaction (Table 2). The model with COC distance and brightness was within 2 AIC units (ΔAIC = 1.15), and the model with RN distance and green contrast was just beyond the 2-units limit (ΔAIC = 2.05). Since the “2-units rule” is an arbitrary criterion and was infringed only by 0.05 units, we used all three models for hypothesis testing.

In the most parsimonious model there was a significant effect of colour pair, and the proportion of correct choices increased with decision time, while the interaction between the two variables was marginally non-significant (Table 4). In the alternative models, the proportion of correct choices increased with (COC or RN) perceptual distance, as well as with the (brightness or green) achromatic contrast (Table 4, Fig. 5). There was a strong correlation between brightness and green contrast (Pearson´s correlation r = 0.95, t = 40.27, p = < 0.001), thus the two variables are interchangeable in the analyses and figures.

**Table 4** Results of the generalized linear models (GLM) for the extinction test analysis. Only the selected models and their variables are described here.

| **Model** | **Variables** | $X^2$ | **d.f.** | ***p*** |
|---|---|---|---|---|
| **ColourPair * Time** | | | | |
| | ColourPair | 41.71 | 3 | **<0.0001** |
| | Time | 4.21 | 1 | **0.04** |
| | ColourPair : Time | 6.88 | 3 | 0.08 |
| **COC + Brightness** | | | | |
| | COC | 28.10 | 1 | **<0.0001** |
| | Brightness | 28.52 | 1 | **<0.0001** |
| **RN + GreenContrast** | | | | |
| | RN | 35.29 | 1 | **<0.0001** |
| | GreenContrast | 28.98 | 1 | **<0.0001** |

The contrast analysis revealed that the proportion of correct choices of group 3 was significantly higher than for the other groups (t > 3.26, d.f. = 60, p < 0.002).

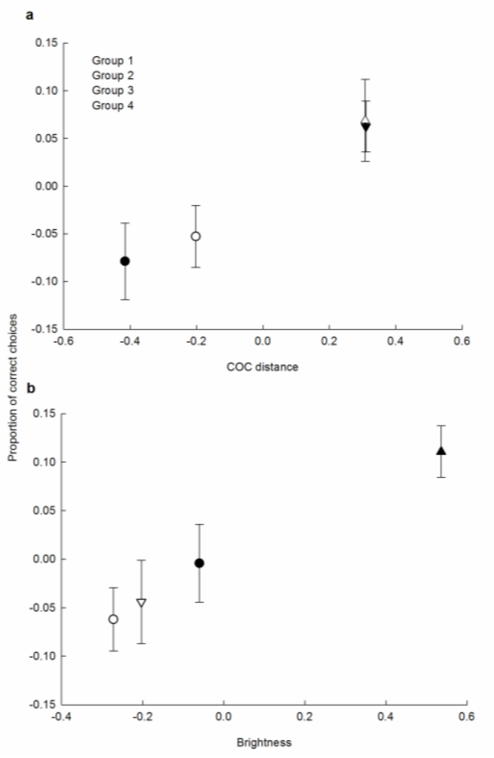

**Fig. 5** Partial regression plots showing the relationship between the proportion of correct choices during phase III and **a** COC distance and **b** brightness contrast. In the vertical axis, we show the residuals of the regression of the proportion of correct choices on **a** brightness contrast or **b** COC distance. In the horizontal axis, the residuals of the regression of **a** COC distance on brightness contrast or **b** brightness contrast on COC distance (Moya-Laraño and Corcobado 2008). Error bars are standard errors.

Concerning the effect of decision time on the proportion of correct choices, after controlling for colour pair, bees with longer decision times were more likely to choose flowers of the correct colour. Since the interaction between colour pair and decision time approached statistical significance, it is important to ask how the relationship between decision time and proportion of correct choices changed between groups. Inspection of the regression coefficients (Table S5, Supplementary material 2) showed that the proportion of correct choices increased with decision time for all colour pairs (Fig. S1, Supplementary material 2), with the exception of group

3 – the group where discrimination reached its maximum value – where increasing decision time had no effect on the proportion of correct choices.

We now ask how the properties of the stimuli affected the decision time (Fig. 6). The most parsimonious model included a single variable: green contrast. Nevertheless, the model with brightness contrast and RN distance was within 2 AIC units (Table S6, Supplementary material 2) and must also be considered.

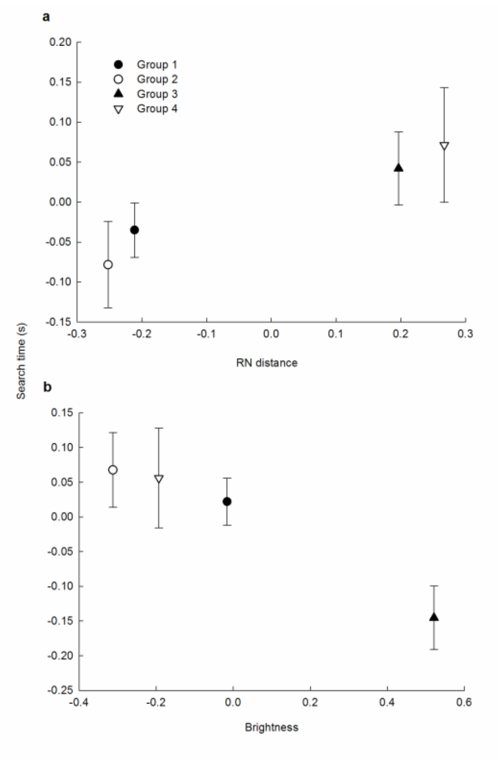

**Fig. 6** Partial regression plots showing the relationship between search time and **a** RN distance and **b** brightness contrast. In the vertical axis, we show the residuals of the regression of the search time on **a** brightness contrast or **b** RN distance. In the horizontal axis, the residuals of the regression of **a** RN distance on brightness contrast or **b** brightness contrast on RN distance. Error bars are standard errors.

Decision time decreased as achromatic contrast increased (Fig. 6b) but, somewhat surprisingly, it increased with RN perceptual distance (Fig. 6a), and these effects were statistically significant (Table S7, Supplementary material 2). A Tukey HSD test revealed that decision times were higher for bees in group 4 than for all other groups (all $p < 0.014$), while there were no significant differences in the decision times of bees trained with colour pairs 1, 2 and 3 (all $p > 0.92$).

## Discussion

The COC, CH and RN models are often used in the literature to estimate chromatic distances as perceived by bees (Hempel de Ibarra et al 2002; Reisenman and Giurfa 2008; Benard and Giurfa 2008; Niggebrügge et al 2009; Martínez-Harms et al 2010; Rodríguez-Gironés et al 2013; Wang et al 2013; Nityananda and Pattrick 2013). Nevertheless, although the models make different predictions, few attempts have been made to determine which model provides the best description of the data (Vorobyev and Brandt 1997; Vorobyev et al 1999; Hempel de Ibarra et al 2000), and even less effort has been devoted to determine whether different models should be applied in different circumstances.

Models' predictions can be in agreement with behavioural data in some cases for a particular subset of coloured stimuli during a detection or discrimination task, but there are also instances in which they will fail (Hempel de Ibarra et al 2014).

Most of the studies that use the models to estimate perceptual distances were meant to elucidate the (fine and large) colour discrimination ability of bees (Dyer and Chittka, 2004a), the detectability of colours against complex and neutral backgrounds (Spaethe et al 2001; Forrest and

Thomson 2009), the effect of conditioning protocol on learning rate (Giurfa 2004; Reser et al 2012), or the effect of aversive stimuli on learning ability (Chittka et al 2003; Avarguès-Weber et al 2010). There was sporadic interest in testing model predictions about the detectability of bright and dim objects (Hempel de Ibarra et al 2000) or under different light conditions (Arnold and Chittka 2012; Dyer and Chittka 2004b), while other studies address questions about innate and spontaneous colour preferences (Giurfa et al 1995; Lunau et al 1996; Raine and Chittka 2007; Papiorek et al 2013) and different learning forms in bees (Giurfa and Sandoz 2012).

In this context, the present experiment constitutes the first attempt to determine which model provides the best description of perceptual chromatic distances in bumblebees, much as Hempel de Ibarra et al (2000) attempted to determine which model should be used to estimate the detectability of stimuli and Brandt and Vorobyev (1997), using spectral sensitivity data (von Helversen 1972; Bobeth 1979), to test hypotheses about the physiology underlying honeybee spectral sensitivity. Our results show that the ability of free-flying bumblebees to discriminate colour pairs is not well predicted by their perceptual distance, as calculated with the CH model. At the end of the experiment, the proportion of correct choices for different colour pairs ranged from 70% (groups 1 and 2) to 90% (group 3), although all colour pairs had the same CH distance (mean ± s.e.m.= 0.0549 ± 0.0007; Table S2, Supplementary material 2). While the predictions of the COC and RN models were somewhat better, they too failed to predict which colour pair bees would find it easier to discriminate: bumblebees were significantly better at discriminating between the two colours of group 3 ($d_{COC} = 1.13$; $d_{RN} = 0.40$) than of group 4 ($d_{COC} = 1.51$; $d_{RN} = 0.78$). These findings demonstrate experimentally that, although current models may be used to obtain rough estimates of perceptual distances, they cannot be used to predict whether bumblebees will discriminate one colour pair better than another.

It has repeatedly been stated that bees ignore brightness differences when choosing target colours subtending a known visual angle (Backhaus et al 1987; Backhaus 1991; Chittka et al 1992; Lehrer and Bischof 1995; Vorobyev and Brandt 1997; Giurfa and Vorobyev 1997; Giurfa et al 1997; Niggebrügge and Hempel de Ibarra 2003; Reisenman and Giurfa 2008; Papiorek et al 2013). Besides, the idea prevails that honeybees and bumblebees use an achromatic channel when stimuli subtend a small visual angle and a chromatic channel when they subtend a large one (Lehrer and Bischof 1995; Giurfa et al 1996; Giurfa et al 1997; Giurfa and Vorobyev 1998; Dyer and Griffiths 2012). Supposedly, bees use the chromatic or achromatic channel depending on the task, but they do not use both channels simultaneously. In our setup, with 16 flowers randomly distributed on a 70 x 70 cm surface, the visual angle subtended by the nearest flowers when a bee departed from a flower was typically greater than 8º – and it increased as the bee approached the flower, but also decreased for flowers farther away. Because bumblebees use chromatic cues to process visual information when objects subtend an angle greater than 2.7º (Dyer et al 2008), we expected them to use chromatic cues in the discrimination task. Nevertheless, both chromatic and achromatic contrasts affected the discriminability of colour pairs – indicating that bees can use the achromatic channel since it is available to the visual system even for subtended large visual angles. It is impossible, however, to infer here whether bumblebees were using both channels at the same time or in a sequence since they were flying around the arena. Because of the strong correlation between green and brightness contrasts among our stimuli pairs, however, it is impossible to determine which achromatic cue bees used.

Careful psychophysics experiments will be required to produce detailed descriptions of the bumblebee colour space. At least within the region of colour space we used, however, we can conclude that the COC and RN models provide a better description than the CH model of bumblebees' perceptual distances, and that bumblebees can use chromatic and achromatic cues to solve a discrimination task even for stimuli that subtend a large visual angle.

### *Implications for colour processing mechanisms*

The visual processing mechanisms that enable colour discrimination are not completely understood. For example, models assume that bee colour discrimination results from comparing the output of photoreceptors using two colour opponent mechanisms. The topology of the colour space results from the choice of unspecified orthogonal colour opponent mechanisms according to the CH model (Chittka 1992), from the choice of a specific pair of colour opponent mechanisms according to the COC model (Backhaus 1991), and from the level of noise of the different receptor types according to the RN model, limiting performance of colour opponent mechanisms (Vorobyev and Osorio 1998; Vorobyev et al 2001). Our data clearly show that postulating a pair of orthogonal mechanisms (CH model) is not enough to describe the colour space of bumblebees, but differences in the goodness of the fits of the COC and RN models are insufficient to rule out an exclusive effect of receptor noise limiting colour discrimination or the specificity of the colour opponent mechanism adopted by the COC model in the topology of colour space.

If the finding that the COC model provides a good fit to our data suggests that the nature of colour opponent mechanisms must be taken into account to understand colour perception, this suggestion should be taken with care: bees possess several types of colour opponent neurons (Yang et al 2004) distributed along different regions in the bee brain (Yang et al 2004; Paulk and Gronenberg 2008) and the implication of these regions in chromatic information processing is still not clear (Mota et al 2013) and so, how the different colour opponent neurons are used to produce what seems to be a two-dimensional colour space (Backhaus et al 1987). On the other hand, the

RN model also predicted the bumblebee performance well for the set of colour pairs used in this work, and it could also be that discrimination was limited by the receptor noise mechanism. It is not clear, however, that such limitation is set only by receptor noise, with neural noise being negligible and with that, the opponent interactions between receptor signals (Vorobyev et al 2001).

Both the COC and RN models were originally based on experimental honeybee data. Because honeybees and bumblebees perform differently in colour discrimination and detection tasks (Dyer et al 2008; Wertlen et al 2008; Morawetz and Spaethe 2012), and it has been suggested that the two species could differ in the way they process ommatidia signals at the neural level (Wertlen et al 2008), it is somewhat surprising that both models provide a relatively good description of the bumblebee colour space. These findings suggest that the two species could process colour information in a similar way. Since honeybees and bumblebees belong to the same subfamily (Apinae), it is possible that the CH model provides a better description of the colour space of more distantly related species. Nevertheless, as the COC and RN models made similar predictions for our colour pairs, we cannot rule out the possibility that noise at the receptor level has a strong effect on perceptual distances. If this were the case, the goodness of fit of the COC model to our data would be a spurious consequence of our choice of colours, since the RN model assume that the noise in receptor mechanisms is dominant and discrimination of signals does not depend on how the receptor signals are combined to form opponent mechanisms (Vorobyev et al 2001).

Regardless of whether perceptual distances are determined by the choice of colour opponent mechanisms, receptor noise or a combination of both, the role of chromatic and achromatic cues in discrimination tasks may have to be reconsidered and more data should be collected before attempting to produce a new descriptive model of bee colour space and topology.

The positive correlation between decision time and performance has been demonstrated by many authors (Spaethe et al 2001; Chittka et al 2003; Dyer and Chittka 2004b; Skorupski et al 2006; Chittka and Spaethe 2007; Rodríguez-Gironés et al 2013), suggesting that bees face a trade-off between increasing accuracy and speed (Chittka et al 2003; Chittka and Spaethe 2007). Our results are consistent with the existence of this trade-off: within colour pairs, the proportion of correct choices increased with decision time – with the exception of group 3, where discrimination was easiest. Between groups, however, decision times were shortest when the achromatic contrast between target and distracter flowers was largest (Fig. 6b), but they increased with perceptual chromatic distance (Fig. 6a).

The behaviour of a bee is the result of a decision-making process that operates on memory and perception, and in order to predict how bees will respond to different environments we need to understand perception, learning and decision making. Likewise, if we are to infer the properties of perception from the results of behavioural experiments, we need to know the decision-making process linking perceptual input to behaviour. In addition, the study of recently discovered colour opponent neurons could give some new insights about the visual information process, within distinct bee brain regions.

### *Ecological implications*

Size and shape can affect the foraging choices of bees and other insects because they can constrain access to the reward (Inouye 1980; Stang et al 2006; Santamaría and Rodríguez-Gironés 2007). Flower colour has also been proposed to affect the foraging choices of pollinators (Raven 1972; Chittka and Waser 1997; Rodríguez-Gironés and Santamaría 2004). Also, the combination of flower size and colour have been shown to affect the time that bees need to detect flowers (Spaethe et al 2001) in a homogenous background. As we have seen, both the probability of selecting the correct flower colour and the time required to select a flower depend on the chromatic and achromatic parameters of the target and distracter flowers. Both factors affect foraging efficiency: inaccurate foragers will waste precious time exploiting suboptimal flowers, and increasing decision time reduces the number of flowers that a bee can visit per unit of time. The optimal balance between increasing accuracy and decreasing decision time will depend on the available options and the need to track changes in resource availability (Dyer and Chittka 2004b; Chittka and Spaethe 2007). At the same time, the balance achieved by foraging bees has important consequences for the reproductive success of plant: flower constancy promotes conspecific pollen flow and reduces pollen loss and stigma clogging (Chittka et al 1999).

Hence, because flower colour affects the foraging choices of bees, and foraging choices affect the reproductive success of plants, and thus the selective pressures to which a plant is exposed within a community (Rodríguez-Gironés and Santamaría 2005; Rodríguez-Gironés and Santamaría 2010), in order to understand and predict changes in flower aspect and abundance through time and space and their ecological implications, we first need to understand how the ability of bees to discriminate between flowers and the time they require to accomplish this task depend on the spectral properties of the flowers, and the learning mechanisms that modulate their behavioural flexibility.

## Conclusions

Developing a colour-difference formula valid throughout the colour space of bumblebees may be an impossible quest (Chittka and Kevan 2005). This, however, does not mean that we should use available models blindly, without trying to elucidate which model provides the most accurate description in each context. This information will greatly increase the performance and power of available models. The present paper is only a small contribution in this direction, but one that can easily be extended in future experiments.

## Chapter Acknowledgements

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## Supplementary material

Additional supplementary material can be found at the respective section of this thesis.

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# CHAPTER TWO

## Enhancing foraging efficiency: use of colour and odour during flower detection in a complex background

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## Abstract

We ignore how floral visitors exploit and integrate visual and olfactory cues when seeking food and how background complexity affects flower detection. Here, we aimed to understand the use of visual and olfactory information for bumblebees (*Bombus terrestris terrestris* L.) during the foraging activity. To explore this issue, we evaluated the effect of flower colour (red and blue), size (8, 16 and 32 mm), scent (presence or absence) and the amount of received training on the foraging strategy of bumblebees seeking nectar in a complex visual environment. Globally, flower colour had the strongest effect on the foraging strategy. Bumblebees looking for blue flowers were more accurate, flew faster, followed more direct paths between flowers and needed less time to find them, than bumblebees looking for red flowers. In turn, training and the presence of odour helped bees to find red flowers. When bees looked for red flowers, search time increased with flower size; but search time was independent of flower size when bees looked for blue flowers. Our results differ from those found in a similar experiment using homogeneous green background. Bumblebees will make use of all available sensory inputs to enhance the foraging activity.

**Keywords**: complex background, sensory signals, red flowers, visual cue, odour, *Bombus terrestris*.

## Introduction

Visual cues strongly influence foraging efficiency in bees (Skorupski et al 2006; Chittka and Spaethe 2007). For example, Spaethe et al (2001) demonstrated that colour and size of flowers affect search time and speed during the foraging activity in a homogeneous background.

In noisy environments, flowers and their visitors can benefit by the presence of multimodal signals, given that on one hand, visitors can explore these signals and use them as cues to find the suitable food resource at close and long distances and, on the other hand, plants can benefit from an efficient visit. In this context, the use of multimodal cues, as odour and colour, might enhance foraging decisions by providing bees with additional sources of information (Leonard and Masek 2014), or by influencing the foraging behaviour (Knudsen et al 1999; Odell et al 1999; Kunze and Gumbert 2001).

Most experiments on the colour vision of free-flying bees use simple arenas with homogeneous backgrounds (for an exception see Forrest and Thomson 2009). This design allows full control of the variables involved and is appropriate to answer questions about the psychophysics of colour vision. Nevertheless, background complexity affects the colour preferences of bumblebees (Forrest and Thomson 2009) and it might have an effect on flower detectability (Rodríguez-Gironés and Santamaría 2004). Because bees forage in a highly textured, complex visual environment, answering ecological questions may require different approaches.

In a homogeneous environment, flower detectability increases with colour contrast against background when flowers are large, and with green contrast when flowers are small (Spaethe et al 2001). To assess the effect of flower size, colour and scent on the foraging strategy and efficiency of bumblebees (*Bombus terrestris terrestris* L.) in a complex visual environment, we followed bees as they searched for nectar at blue and red flowers of different sizes, in the presence or absence of olfactory cues, in a large flight cage with a complex visual background.

## Materials and Methods

### *General setup and procedure*

The experiment was done between late May and early August of 2012 in an outdoor flight arena (length, width, height: 5 x 2.50 x 2.40 m), with the long axis in the east-west direction. The arena was built with wire-mesh and its roof was covered by dark green shading net. The south wall was overlaid with expanded polystyrene (EPS), to a height of 1.5 m. The EPS panel was painted with yellowish, brownish and greenish colours, and covered with ivy plants (*Hedena* sp.) to simulate a natural foraging environment (Fig. S1a, Supplementary data). In the north-west corner of the arena sat a bumblebee nesting box (30 x 20 x 25 cm), connected through a gated tunnel to a small feeding cage (38 x 42 x 40 cm), where bees could obtain 20% (weight/weight) sucrose solution from an uncoloured feeder outside experimental sessions. Bees had ad libitum access to pollen inside the nesting box. Colour-naïve bumblebees flew into the flight arena only during bee selection and experimental sessions.

We attached 60 green EPS cubes (2 x 2 x 2 cm), with an Eppendorf tip inserted in the upper face, to the EPS panel. The Eppendorf tips contained 10 µl sucrose solution (60% weight/weight) in half of the cubes, and were empty in the other half. Bees could identify rewarded cubes by the presence of a coloured paper square 8, 16 or 32 mm in side, hereafter referred to as flower (Fig. S1b, Supplementary data). The squares could be blue (R: 0, G: 135, B: 255) or red (R: 255, G: 0, B: 0), printed with an Epson Stylus Photo R3000 (EPSON) colour printer onto Ilford Galerie, Smooth Pearl 290 gsm (grams/square meter) paper (ILFORD Imaging Switzerland GmbH). EPS cubes and Eppendorf tips were cleaned with ethanol 30% and haphazardly rearranged after each foraging bout – defined as a trip hive - flight cage - hive.

### *Illumination and colour measurements*

We measured illumination (vector irradiance impinging the EPS panel) and reflectance spectra of stimuli and background within the range of 300 – 700 nm (Fig.1) using a spectrometer (DT-MINI-2-GS Light Source, Ocean Optics USB 4000, Dunedin, FL, USA). Spectral irradiance was measure using a cosine corrector (CC-3-UV-S, Ocean Optics, Dunedin, FL, USA) coupled to the optical fiber connected to the spectrometer, after spectrometer calibration with a lamp of known output (LS-1-CAL-220, Ocean Optics). To cover the natural light variation along the day, we took five measurements of irradiance at each of three different positions within the cage at 12:00, 15:00 and 17:00 h and averaged all 45 values.

Reflectance spectra were measured relative to a white standard (WS-1 diffuse reflectance standard, Ocean Optics). For all computations we used the normalized average of five reflectance measurements. For the red and blue colour stimuli, we used the spectral sensitivity of bumblebees (Skorupski et al 2007) to compute achromatic green and brightness contrasts relative to the average background (as in Spaethe et al 2001) and chromatic contrasts according to the colour opponent coding (Backhaus 1991), colour hexagon (Chittka 1992) and receptor noise models (Vorobyev and Osorio 1998; Vorobyev et al 2001) (Table S1, Supplementary material).

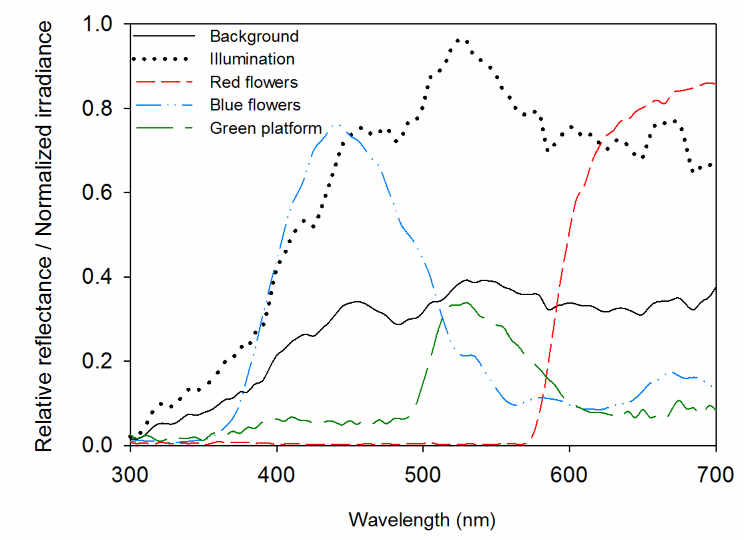

**Fig. 1** Spectral properties of stimuli, irradiance and background. Normalized irradiance and relative reflectance of background and colour stimuli within the range of 300 nm to 700 nm.

## *Experimental procedure*

We randomly assigned bumblebees to two odour treatments: unscented (UC, n=24) and scented (SC, n=24). Within each odour treatment, 12 bees were trained and tested with blue and the other 12 with red flowers. For the scented treatment, we added 5µl of lavender oil (*Lavandula officinalis*, from Marnys®, Aroma Therapy World) solution (2:100 in pentane) onto rewarded EPS cubes immediately before each foraging bout. Because a highly concentrated scent could result in an aversive behaviour (Kunze and Gumbert 2001), we had previously established the concentration with a detection test, in which bees had to find rewarded EPS cubes using only the olfactory cue.

Each bumblebee experienced a single colour-odour combination, but all three flower sizes (8, 16 and 32 mm) in a pseudorandom order – each possible order was experienced by two bees for each colour-odour combination. This design uncouples the effects of stimulus size and experience.

### *Bee selection and pre-training session*

If the next bee had to be trained with flowers of a given treatment (UC or SC, blue or red) and starting with a given size (small, medium or large), we arranged the arena with flowers of the corresponding size and treatment and allowed five bumblebees to explore it. Once one bee started foraging, we tagged it and removed the other four. Without changing flower type or size, we allowed the tagged bee to make five foraging bouts to familiarize itself with the foraging environment. After those five foraging bouts, the experimental session started.

### *Experimental session*

We divided the experimental session in three rounds of ten, six and six foraging bouts. Flower size changed from round to round so that each bee experienced the three flower sizes – one size per round. During each foraging bout, we recorded the total number of visited flowers, correct (coloured rewarded platforms) and incorrect (unrewarded green platforms) choices and the time bumblebees spent flying from flower 2 to 6 (regardless of whether they were rewarded or unrewarded). We excluded the first flower to avoid variation due to the distance between the feeding cage and the flower. For each round, size and bee, we calculated the average time and divided it by the number of visited flowers (five) to obtain the "search time" – an estimate of the time bees required to find one flower. We considered a choice when a bumblebee touched the top of the platform with its front legs, regardless of whether it landed or not on the flower.

### *Flight behaviour*

To test whether bumblebees adapted their flight pattern to the foraging task, during the last three foraging bouts of each round we recorded bees – using a Sony video camera (DCR-SR47, Sony Hand Cam) – whenever they foraged within a framed 130 x 80 cm rectangle in the centre of the EPS panel (Fig. S1a, Supplementary data).

We developed a Matlab program (BeeTracker, available upon request) to extract from the videos the travel time (time elapsed from takeoff to flower choice), total path length and average flight speed (path length divided by search time) – using the rectangular frame to convert pixels to distances. This analysis was restricted to the 1054 trajectories that did not leave the framed area: 636 for the UC treatment and 418 for the SC treatment.

Because we recorded bees with only one camera, path length and speed refer to the components of movement along the EPS panel, and ignore displacements towards or away from the camera. Because bumblebees flew within 20 cm of the EPS panel, movement along this plane provides a good approximation to 3D displacement and speed.

### *Novel colour test*

After the third round, we performed a novel colour test to evaluate how bumblebees trained with blue flowers would perform when seeking nectar in red flowers and vice-versa. This test consisted of a single foraging bout, during which bees encountered 16 mm flowers of the unfamiliar colour. These flowers were scented for bees in the SC treatment, and unscented for bees in the UC treatment. We recorded the search time as in the training sessions and the number of correct and incorrect choices.

### *Statistical analysis*

We used generalized linear mixed models (GLMMs) with binomial distribution family and *logit* link function to examine the influence of colour, the presence or absence of odour, size or round on the proportion of correct choices. By contrast, we used linear mixed effect models (LMMs), with normal distribution, to test the effect of the same predictors over the search time, flight speed and total path length of bees. Round and size were never analyzed together in a same model. Instead, all the analyses were performed twice, using either size or round. To analyze the performance of bees during the novel learning test, we used a generalized linear model (GLM) with binomial distribution for the proportion of correct choices (*logit* link) and a linear model (LM) for the search time.

For the mixed models, we selected the most parsimonious random terms as suggested by Zuur et al (2009, pages 121-122). We tested all possible combinations of random terms as well as the model without random terms, and selected the model with lowest AIC value (Akaike 1973).

All analyses were performed using the R software, version 3.0.3 (R Core Team 2013). For the mixed models, we used functions GLMM and LMER, belonging to the *lme4* package (Bates and Maechler 2013).

# Results

## *Training data*

### *Proportion of correct choices (Table 1)*

Bees looking for blue flowers seldom landed on empty flowers (8 incorrect choices out of 11.761), regardless of the odour treatment, round and size (Fig. 2). Bumblebees looking for red flowers, on the other hand, started with low accuracy and their performance improved with round or in the presence of scent (Fig. 2), although the positive effect of scent decreased with round (Table 1, odour treatment x round interaction). Search time also affected the proportion of correct choices: bumblebees that spent more time inspecting flowers were more accurate (Table 1).

**Table 1** Coefficient values with standard errors and hypothesis testing for the proportion of correct choices analyses. OT = odour treatment; SE = standard error.

| | ***Model parameters*** | | ***Hypothesis testing*** | | |
|---|---|---|---|---|---|
| ***Model: Round. Random term = (1\|BeeID)*** | | | | | |
| *Variables* | *Coefficients* | *SE* | $X^2$ | d.f. | *p* |
| Intercept | 4.83 | 1.00 | | | |
| Colour | -4.23 | 1.05 | 16.26 | 1 | **<0.0001** |
| OT | 1.04 | 0.97 | 1.14 | 1 | 0.28 |
| Round | 1.34 | 0.66 | 4.08 | 1 | **0.04** |
| SearchTime | 0.96 | 0.29 | 10.69 | 1 | **0.001** |
| Colour:OT | 0.66 | 1.05 | 0.40 | 1 | 0.53 |
| Colour:Round | -0.73 | 0.67 | 1.19 | 1 | 0.27 |
| OT:Round | -0.28 | 0.12 | 5.63 | 1 | **0.02** |
| ***Model: Size. Random term = (Size\|BeeID)*** | | | | | |
| *Variables* | *Coefficients* | *SE* | $X^2$ | d.f. | *p* |
| Intercept | 5.18 | 1.41 | | | |
| Colour | -2.22 | 1.47 | 36.63 | 1 | **<0.0001** |
| OT | 0.38 | 1.10 | 10.44 | 1 | **0.001** |
| Size | 0.21 | 0.13 | 0.70 | 1 | 0.40 |
| SearchTime | -0.24 | 0.33 | 0.53 | 1 | 0.47 |
| Colour:OT | 0.82 | 1.22 | 0.45 | 1 | 0.50 |
| Colour:Size | -0.20 | 0.13 | 2.59 | 1 | 0.11 |
| OT:Size | 0.003 | 0.02 | 0.02 | 1 | 0.90 |

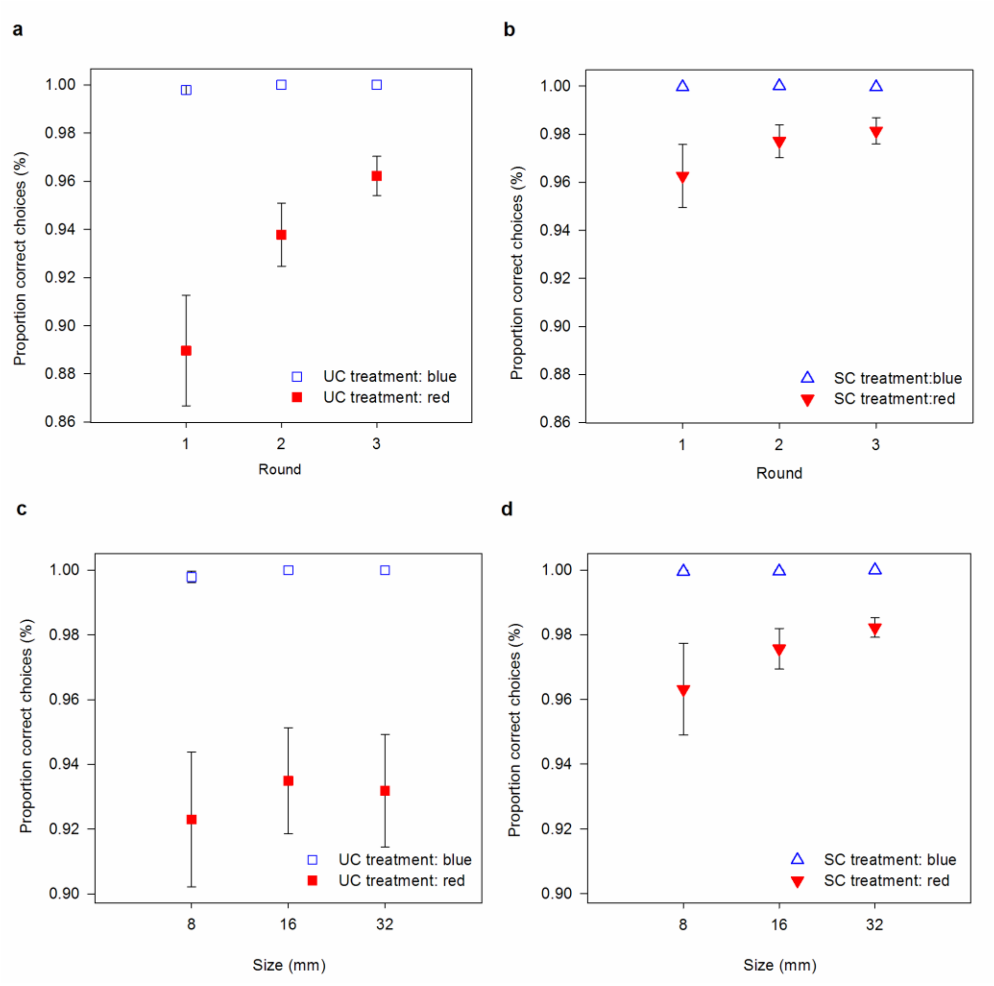

**Fig. 2** Percentage of correct choices versus round (a, b) and size (c, d) for the unscented (a, c) and scented (b, d) treatments. Error bars are standard errors.

To understand better the odour treatment x round interaction, we analyzed separately the performance of bees from the two odour treatments, using the same selected random structure for the model. In both analyses, accuracy increased with round (UC: $X^2 = 84.49$, df = 1, p = <0.0001; SC: $X^2 = 14.09$, df = 1, p = 0.0002), but the effect was greater for the UC (slope= 0.60; SE=0.06) than for the SC (slope=0.36; SE=0.1) treatment.

In the analyses including flower size instead of round, standard errors increased and, as a result, only colour and odour treatment affected accuracy: bees were more accurate when looking for blue than red flowers, or when looking for scented than unscented flowers, but flower size had no effect on accuracy (Table 1; Fig. 2c, d).

### *Search time (Table 2)*

Search time was lower for bumblebees looking for blue flowers than for those looking for red flowers (Fig. 3). For bees looking for red flowers, search time decreased with round (Table 2, Fig. 3a, b).

**Table 2** Coefficient values with standard errors and hypothesis testing for the search time analyses. OT = odour treatment; SE = standard error.

| | ***Model parameters*** | | ***Hypothesis testing*** | | |
|---|---|---|---|---|---|
| ***Model: Round. Random term = (1\|BeeID)*** | | | | | |
| *Variables* | *Coefficients* | *SE* | $X^2$ | d.f. | *p* |
| Intercept | -0.65 | 0.09 | | | |
| Colour | 0.82 | 0.11 | 205.18 | 1 | **<0.0001** |
| OT | 0.09 | 0.11 | 0.02 | 1 | 0.88 |
| Round | -0.02 | 0.03 | 5.03 | 1 | **0.02** |
| Colour:OT | -0.03 | 0.11 | 0.07 | 1 | 0.79 |
| Colour:Round | -0.01 | 0.04 | 0.13 | 1 | 0.71 |
| OT:Round | -0.04 | 0.04 | 1.18 | 1 | 0.28 |
| ***Model: Size. Random term = (1\|BeeID)*** | | | | | |
| *Variables* | *Coefficients* | *SE* | $X^2$ | d.f. | *p* |
| Intercept | -0.59 | 0.08 | | | |
| Colour | 0.64 | 0.09 | 42.30 | 1 | **<0.0001** |
| OT | -0.06 | 0.09 | 0.38 | 1 | 0.54 |
| Size | -0.005 | 0.003 | 3.29 | 1 | 0.07 |
| Colour:OT | -0.03 | 0.11 | 0.07 | 1 | 0.79 |
| Colour:Size | 0.008 | 0.003 | 6.14 | 1 | **0.01** |
| OT:Size | 0.004 | 0.003 | 1.20 | 1 | 0.27 |

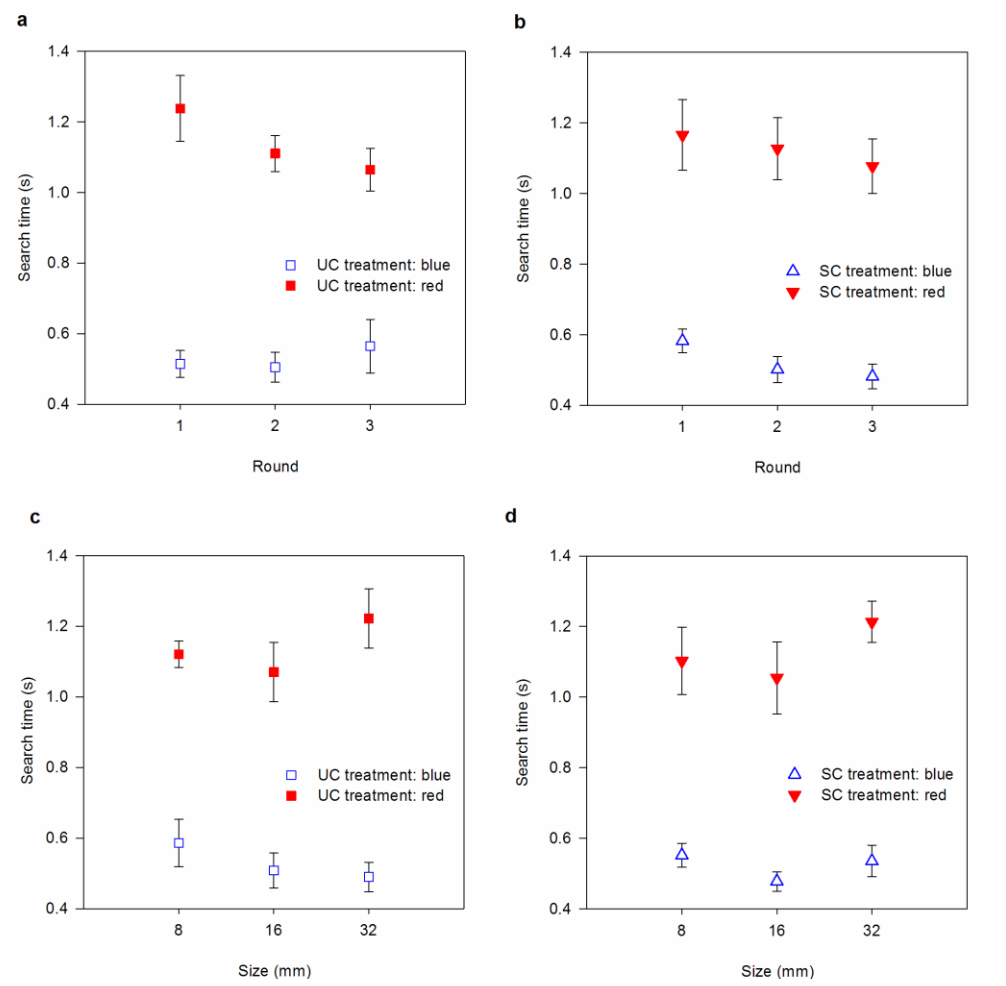

**Fig. 3** Search time (s) versus round (a, b) and size (c, d) for the unscented (a, c) and scented (b, d) treatments. Error bars are standard errors.

Flower size itself did not have a significant effect on search time, but its interaction with colour did (Table 2, p = 0.01). To study this interaction, we reanalyzed colours independently. When bees were looking for red flowers, search time increased with size (slope = 0.005, SE = 0.002; $X^2$ = 4.59, df = 1, p = 0.03). For blue flowers, in turn, the slope of the regression was negative (slope = -0.002, SE = 0.001), although not statistically different from zero ($X^2$ = 1.22, df = 1, p = 0.27).

### *Average flight speed (Tables 3)*

Round and its interaction with flower colour significantly affected average flight speed (Table 3). We therefore reanalyzed the flight speed separately for each colour. Bees looking for blue flowers flew slowly during the first round, increasing their flight speed as training progressed (Fig. 4a, b. Slope = 0.10, SE = 0.03; $X^2$ = 9.20, df = 1, p = 0.002), while bumblebees looking for red flowers flew at the same speed throughout the experiment ($X^2$ = 0.0001, df = 1, p = 0.99). Average flight speed was not affected by odour treatments (Fig. 4a, b).

**Table 3** Coefficient values with standard errors and hypothesis testing for the flight speed analyses. OT = odour treatment; SE = standard error.

| | ***Model parameters*** | | ***Hypothesis testing*** | | |
|---|---|---|---|---|---|
| ***Model: Round. Random term = (1\|BeeID)*** | | | | | |
| *Variables* | *Coefficients* | *SE* | $X^2$ | d.f. | *p* |
| Intercept | 5.27 | 0.10 | | | |
| Colour | 0.20 | 0.12 | 3.13 | 1 | 0.08 |
| OT | -0.02 | 0.11 | 0.05 | 1 | 0.82 |
| Round | 0.10 | 0.04 | 5.41 | 1 | **0.02** |
| Colour:OT | 0.03 | 0.09 | 0.10 | 1 | 0.75 |
| Colour:Round | -0.10 | 0.05 | 4.37 | 1 | **0.04** |
| OT:Round | -0.003 | 0.05 | 0.004 | 1 | 0.95 |
| ***Model: Size. Random term = (1\|BeeID)*** | | | | | |
| *Variables* | *Coefficients* | *SE* | $X^2$ | d.f. | *p* |
| Intercept | 5.48 | 0.08 | | | |
| Colour | -0.05 | 0.10 | 0.33 | 1 | 0.57 |
| OT | -0.005 | 0.10 | 0.24 | 1 | 0.62 |
| Size | -0.001 | 0.003 | 0.07 | 1 | 0.79 |
| Colour:OT | 0.04 | 0.09 | 0.20 | 1 | 0.65 |
| Colour:Size | 0.002 | 0.004 | 0.56 | 1 | 0.45 |
| OT:Size | -0.002 | 0.004 | 0.27 | 1 | 0.60 |

In the analysis with flower size, none of the factors affected average flight speed (Fig. 4c, d. Table 3).

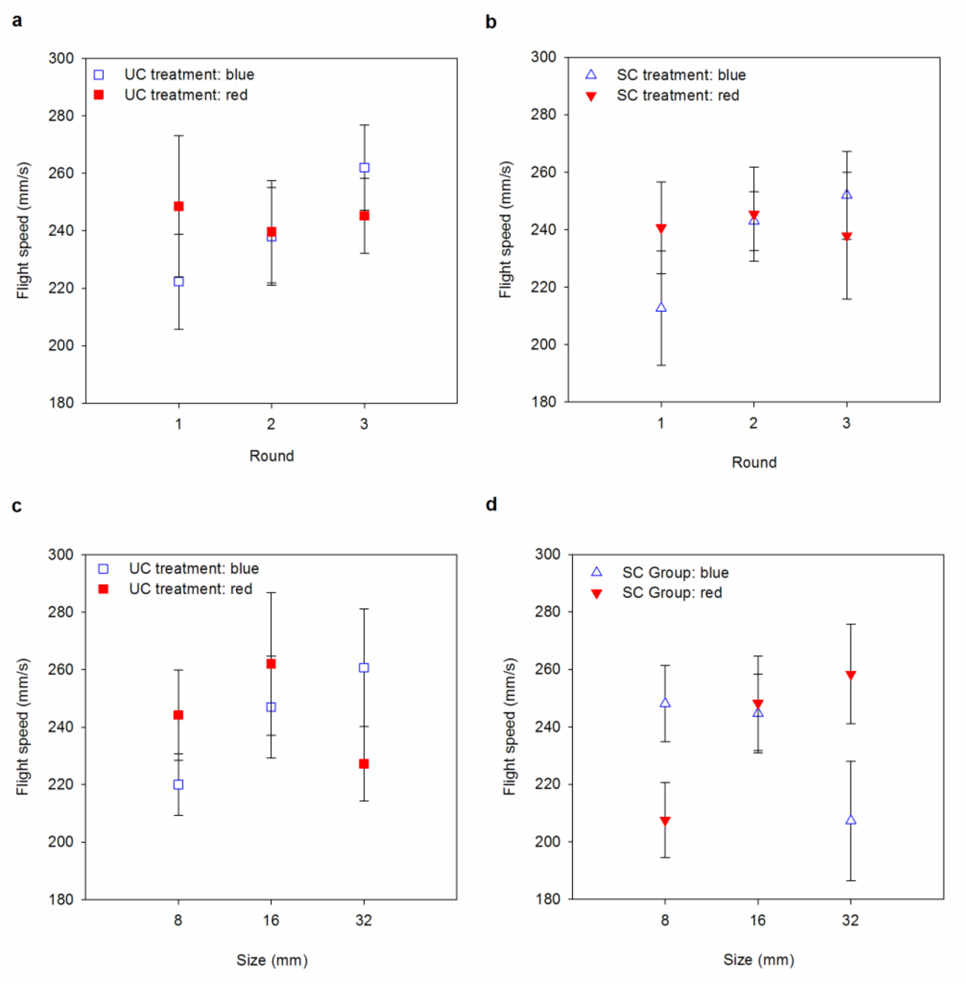

**Fig. 4** Averaged flight speed (mm/s) versus round (a, b) and size (c, d) for the unscented (a, c) and scented (b, d) treatments. Error bars are standard errors.

### *Path length (Tables 4)*

Regardless of whether we included round or flower size in the model, colour and its interaction with odour treatments significantly affected path length (Table 4). Path length was shorter when bees were looking for blue rather than for red flowers, although the difference decreased in the presence of scent (Fig. 5).

**Table 4** Coefficient values with standard errors and hypothesis testing for the total path length analyses. OT = odour treatment. SE = standard error.

| | ***Model parameters*** | | ***Hypothesis testing*** | | |
|---|---|---|---|---|---|
| ***Model: Round. Random term = (1\|BeeID)*** | | | | | |
| *Variables* | *Coefficients* | *SE* | $X^2$ | d.f. | *p* |
| Intercept | 6.01 | 0.12 | | | |
| Colour | 0.74 | 0.14 | 27.94 | 1 | **<0.0001** |
| OT | 0.12 | 0.14 | 0.77 | 1 | 0.38 |
| Round | 0.04 | 0.05 | 0.57 | 1 | 0.45 |
| Colour:OT | -0.25 | 0.11 | 5.78 | 1 | **0.02** |
| Colour:Round | -0.07 | 0.06 | 1.67 | 1 | 0.20 |
| OT:Round | -0.007 | 0.06 | 0.01 | 1 | 0.91 |
| ***Model: Size. Random term = (1\|Size)*** | | | | | |
| *Variables* | *Coefficients* | *SE* | $X^2$ | d.f. | *p* |
| Intercept | 6.08 | 0.12 | | | |
| Colour | 0.67 | 0.11 | 36.46 | 1 | **<0.0001** |
| OT | 0.16 | 0.11 | 2.01 | 1 | 0.15 |
| Size | 0.0006 | 0.006 | 0.01 | 1 | 0.90 |
| Colour:OT | -0.23 | 0.10 | 5.70 | 1 | **0.02** |
| Colour:Size | -0.005 | 0.005 | 0.99 | 1 | 0.32 |
| OT:Size | -0.003 | 0.005 | 0.39 | 1 | 0.53 |

When colours were analysed separately, the presence of odour reduced path length when bumblebees were looking for red flowers, although the difference was not statistically significant (slope = -0.14, SE = 0.09; $X^2$ = 1.82, df = 1, p = 0.09). Somewhat surprisingly, when bumblebees looked for blue flowers the presence of odour increased path length, although once again the difference was not statistically significant (slope = 0.10, SE = 0.06; $X^2$ = 2.74, df = 1, p = 0.09).

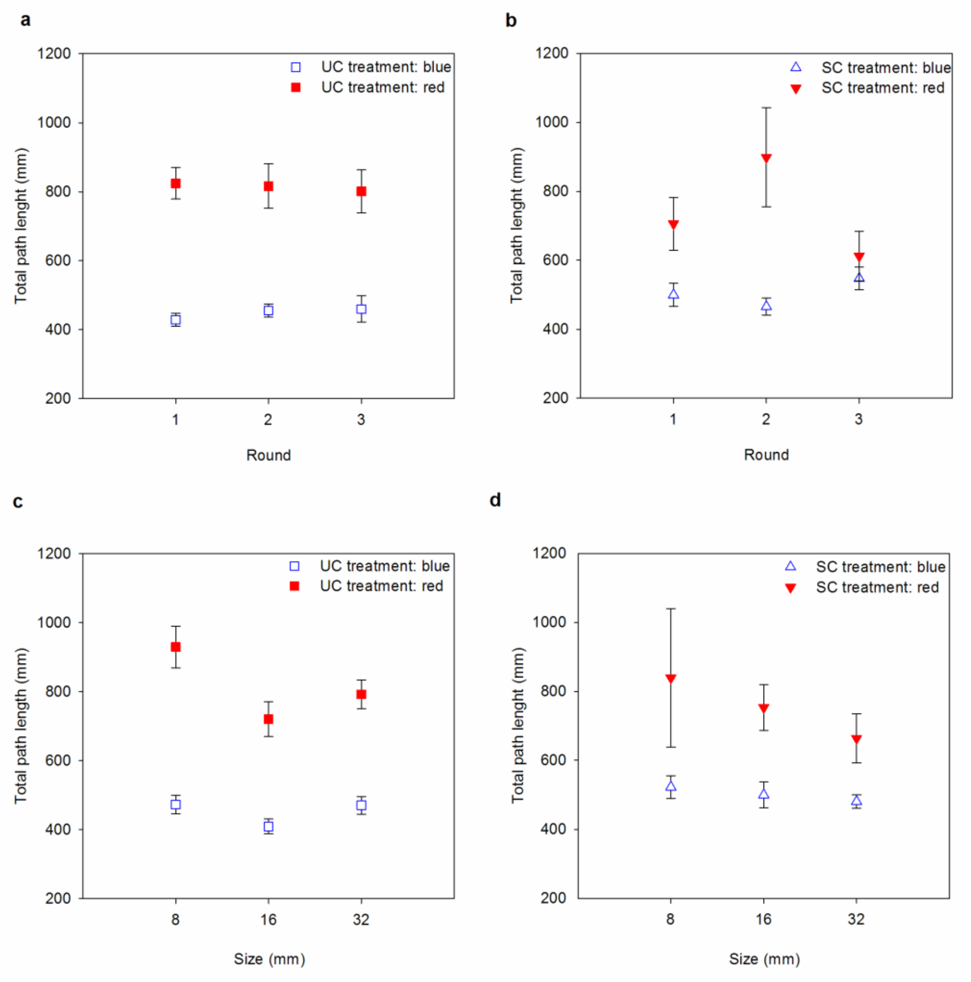

**Fig. 5** Total path length of bumblebees (mm) versus round (a, b) and size (c, d) for the unscented (a, c) and scented (b, d) treatments. Error bars are standard errors.

### *Novel colour test: proportion of correct choices and search time*

The proportion of correct choices was greater when bees trained with red flowers had to look for blue flowers than when bees trained with blue flowers had to look for red flowers and, regardless of the colour treatment, the proportion of correct choices increased in the presence of scent (Fig.

6a, Table 5). Search time was also greater when bees trained with blue flowers had to look for red flowers, and decreased in the presence of scent (Fig. 6b, Table 5).

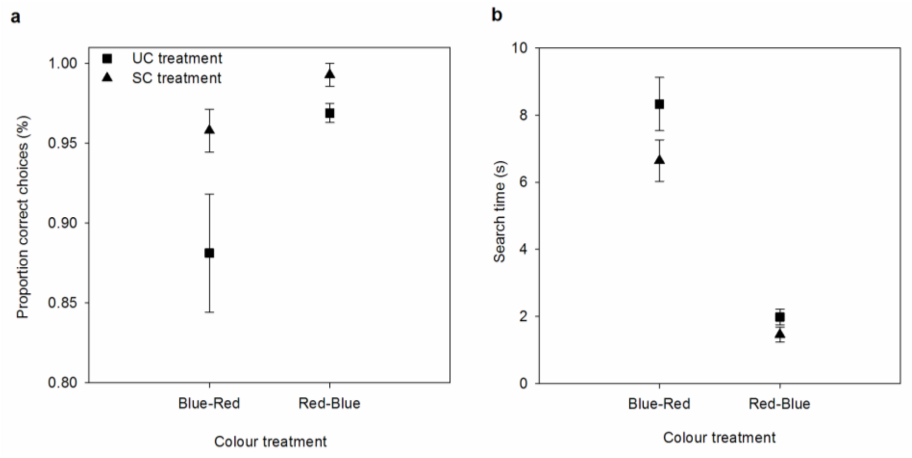

**Fig. 6** Proportion of correct choices (a) and search time (b) during the novel colour experiment for the unscented (squares) and scented (triangles) odour treatments.

**Table 5** GLM (correct choices) and LM (search time) models with hypothesis-testing for the novel colour analyses. OT = odour treatment.

| **Model** | **Variables** | | | |
|---|---|---|---|---|
| ***Correct Choices*** | | ***$X^2$*** | **d.f.** | ***p*** |
| | OT | 10.43 | 1 | **0.001** |
| | ColourTreatment | 19.16 | 1 | **<0.0001** |
| | | | | |
| ***SearchTime*** | | ***SS/F*** | **d.f.** | ***p*** |
| | OT | 0.74/5.56 | 1 | **0.02** |
| | ColourTreatment | 19.62/147.20 | 1 | **<0.0001** |

## Discussion

Colour was more important for bees than any other factor. There were strong differences between correct choices, search time, flight speed and total path length between colours. The performance of bumblebees looking for red flowers improved with training, especially in the absence of odour, which had a positive effect on performance even in the absence of training. Flower size had little effect on bee behaviour. Only its interaction with flower colour had a significant impact on search time. Given that we had only two replicates of each size-round combination, however, it is possible that a weak effect of flower size was masked by the effect of training, and that such effect could be revealed by increasing sample size.

Bees looking for red flowers maintained or even reduced their flight speed as training progressed, adjusting their behaviour to minimize the risk of missing flowers, while bees looking for blue flowers increased the flight speed with training. Despite these speed differences, path length was greater when bees looked for red flowers than when they looked for blue flowers, although the difference decreased in the presence of scent.

### *The background effect*

Spaethe et al (2001) found a significant correlation between search time, colour and size when using a homogeneous green background and odourless flowers. In that study, flowers of three sizes (circles 28, 15, and 8 mm in diameter) were presented in a descending order, and search time increased as flower size decreased. In our background, flower size had little effect on bumblebees' performance or behaviour. Only search time was affected by the interaction between flower colour and size: bumblebees looking for red flowers took longer to find big flowers (32 mm) than medium (16 mm) and small (8 mm) flowers, while search time was independent of flower size (and had a tendency to decrease as size increased) when flowers were blue.

We plotted together the results from Spaethe at al. (2001) experiment and ours (ignoring odour treatments - no effect during search time analysis) regarding search time and flower size (Fig. 7) to better understand similarities and differences across experiments. In order to make the data comparable, search times from both experiments were normalized (details in the figure's legend). In a homogeneous background, the effect of flower size on search time was stronger than the effect of flower colour. In our setup, however, it was the other way around (Fig. 7).

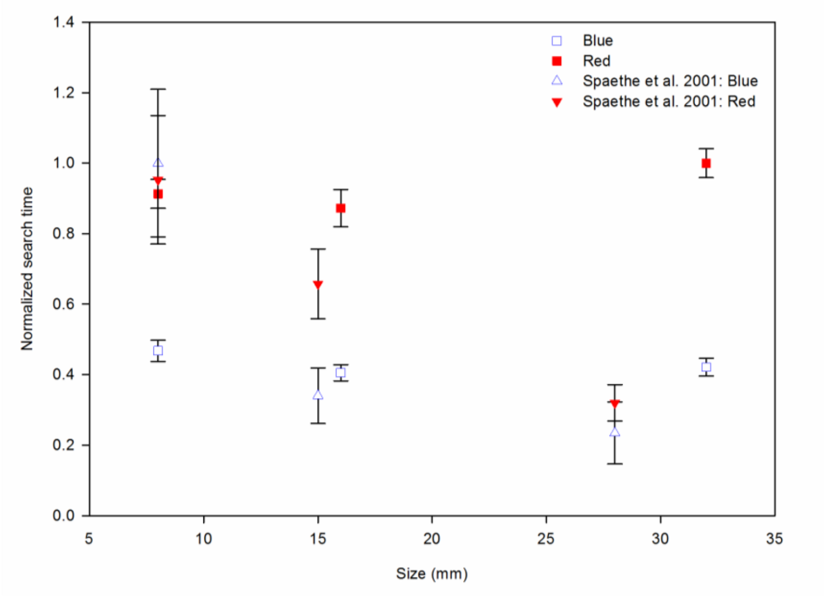

**Fig. 7** Normalized search times of bumblebees looking for flowers of different sizes and colours in homogeneous (triangles) and complex (squares) backgrounds. For normalization, search times were divided by the maximum search time of their dataset (44.4 s the data reported by Spaethe et al 2001 and 1.22 s for this experiment).

In the experiment performed by Spaethe et al (2001), all bees started looking for large flowers, and then went to medium and small flowers. Search time might have been expected to decrease as the experiment progressed, but it increased: the effect of flower size was strong enough to erase any effect of training in the homogeneous background.

Normalized search time differences between experiments could not be explained by the chromatic contrast of stimuli against backgrounds, since these contrasts were similar across experiments (Table 1 of Spaethe et al (2001) and Table S1 in Supplementary material of this study).

### *The chromatic – achromatic information use in flower detection and discrimination*

Bees possess two separate but interacting visual pathways for flower detection and discrimination (Giurfa et al 1996; Giurfa et al 1997). The chromatic pathway is used when targets subtend a large visual angle, while the achromatic pathway, mediated by the green receptors, is used when targets subtend a small visual angle (Giurfa et al 1996; Giurfa et al 1997; Dyer et al 2008). Although bees can detect stimuli subtending a large visual angle in the absence of chromatic information, presumably using the achromatic pathway, such detection is difficult (Hempel de Ibarra et al 2000; Reisenman and Giurfa 2008). This could explain why bumblebees looking for red flowers needed more time to find large than small and medium sized flowers.

Another striking point is that bees are very fast at learning tasks based on chromatic contrasts, while they require extended learning to perform tasks based on achromatic contrasts (Giurfa and Vorobyev 1998). In agreement with this finding, bumblebees exploiting red flowers improved their performance with training, while the performance of bees exploiting blue flowers was excellent from the beginning.

### *Flight speed and total path length: visual and olfactory modulation*

Different studies have reported the existence of the trade-off between speed and accuracy (Chittka et al 2003; Dyer and Chittka 2004; Chittka et al 2009; Rodríguez-Gironés et al 2013; Telles and Rodríguez-Gironés 2015), and bees adjust their behaviour to the difficulty of the task. Bees looking for blue flowers increased their flight speed as they became more experienced – either because the task became easier or because they learnt that they could increase their speed without making more errors. Bees looking for red flowers, however, had to maintain their flight speed throughout the experiment.

Bumblebees exploiting red flowers travelled less distance in the presence of scent. It remains unclear whether bumblebees visiting red flowers used scent to guide landing when approaching flowers (Lunau 1991; Lunau 1992; Dobson et al 1999), or as a long-distance cue (Kunze and Gumbert 2001; Vereecken and Schiestl 2009).

### *Novel colour learning behaviour*

When bees form elemental associations between a reward, scent, and colour, these cross-modal relationships are linked in memory (Leonard and Masek 2014) and apparently used during new information acquisition. During the novel task experiment, bumblebees trained with blue and red flowers in the presence of odour had a high proportion of correct choices and spent less time looking for the novel flowers than those bees trained with the same colours but in the absence of odour (Fig. 6). The presence of scent by itself helped bumblebees to find the novel stimulus in both colour treatments.

Because bees found it easier to find blue than red flowers, bees trained with red flowers and looking for blue flowers during the novel task experiment performed better, being faster and more accurate, easily switching from red to the novel blue flowers (Red-Blue treatment) than bees facing the opposite transition. This behaviour was consistent between odour treatments. Forrest and Thompson (2009) tested bumblebees preference for blue and red flowers when these flowers were presented in a homogeneous and complex backgrounds. Bumblebees showed no preference between blue and red flowers when presented against a homogeneous green background, but strongly preferred the blue flowers, the most conspicuous stimuli, when presented against a complex background (Forrest and Thomson 2009). When given the opportunity, bumblebees are going to prefer the colour that allows for a better balance between speed and accuracy.

## Concluding remarks

Perception of a stimulus is affected by the colour of its background (Kinoshita et al 2008). When flowers appear against a complex background, flower size and colour affect bumblebee behaviour and performance differently from what was found in an earlier experiment using homogeneous green background (Spaethe et al 2001).

Floral odour is important when the visual task is difficult, as with our UV-absorbing red flowers or as it might be for the UV-reflecting white flowers naturally visited by bumblebees (Chittka and Waser 1997; Lunau et al 2011). Multimodal stimuli allow pollinators to use different sensory channels when foraging in different contexts, and at different scales (Goyret et al 2009). Bumblebees, generalist flower visitors, benefit from their capacity of using one or more sensory modalities to improve target detection when relying on a single sensory modality is inefficient.

Bumblebees adjust their behaviour to facilitate target detection and discrimination, and they might make use of all available sensory inputs and neural pathways, as long as they can enhance the foraging activity.

## Chapter Acknowledgements

We thank Shikhar Mann for the initial code of bee flight analysis and Andreas Kämpgen for the development of the BeeTracker program and assistance during video analyses. This work was supported by the Spanish Ministerio de Ciencia e Innovación/Fondo Europeo de Desarrollo Regional - FEDER (grant number CGL2010-16795 to M.A.R.G.) and a PhD studentship to F.J.T. from the Spanish National Research Council (CSIC), co-funded by the European Social Fund (grant number JAE: JAEPre033).

## Supplementary material

Additional supplementary material can be found at the respective section of this thesis.

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# CHAPTER THREE

# Out of the blue: the spectral sensitivity of hummingbird hawkmoths

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# Abstract

The European hummingbird hawkmoth *Macroglossum stellatarum* is a diurnal nectar forager like the honeybee, and we expect similarities in their sensory ecology. Using behavioural tests and electroretinograms (ERGs), we studied the spectral sensitivity of *M. stellatarum*. By measuring ERGs in the dark-adapted eye and after adaptation to green light, we determined that *M. stellatarum* has ultraviolet (UV), blue and green receptors maximally sensitive at 349, 440 and 521 nm, and confirmed that green receptors are most frequent in the retina. To determine the behavioural spectral sensitivity (action spectrum) of foraging moths, we trained animals to associate a disk illuminated with spectral light, with a food reward, and a dark disk with no reward. While the spectral positions of sensitivity maxima found in behavioural tests agree with model predictions based on the ERG data, the sensitivity to blue light was 30 times higher than expected. This is different from the honeybee but similar to earlier findings in the crepuscular hawkmoth *Manduca sexta*. It may indicate that the action spectrum of foraging hawkmoths does not represent their general sensory capacity. We suggest that the elevated sensitivity to blue light is related to the innate preference of hawkmoths for blue flowers.

**Keywords:** spectral sensitivity, *Macroglossum stellatarum*, Sphingidae, insect colour vision, action spectrum

## Introduction

A hundred years ago, Karl von Frisch convinced his sceptical contemporaries that even the humble honeybee had a sensory capacity that was thought to be specific for “higher animals” including humans: colour vision (Frisch 1914). Only eight years later, Friedrich Knoll published his careful and detailed observations on the diurnal European hummingbird hawkmoth, *Macroglossum stellatarum*, mostly based on the moths’ innate preferences for flower features and dark roosting places (Knoll 1922).

Like other members of the sphingid family, hummingbird hawkmoths are acrobatic flyers that feed “on the wing” while hovering in front of a flower. Unlike workers of the social honeybee, solitary moths rely solely on their innate preferences when searching for their first nectar meal, and we know from a series of experiments, that they prefer blue, radial patterns and a contrasting nectar guide on their very first foraging flight (Kelber 1997; Kelber 2005; Kelber and Balkenius 2007). After a successful flower visit, hummingbird hawkmoths can easily learn to associate flower features with a reward. However, while honeybees are central-place foragers and can be trained to visit a food source frequently, hawkmoths only feed for their own needs, making training more demanding and testing slow. Still, we have discovered that they are more responsive to colour than to odour (Balkenius and Kelber 2006), that they can learn to discriminate flower colours and colour patterns (Kelber 1996; Kelber 2002; Kelber 2005), and that they use colour to control precise proboscis movements when searching for the entrance to the nectar reservoir of a flower (Goyret and Kelber 2011; Goyret and Kelber 2012).

In all previous experiments, we have assumed that the photoreceptors of *M. stellatarum* have sensitivities similar to those of the crepuscular or nocturnal hawkmoths *Manduca sexta* and *Deilephila elpenor*. These species, similar to honeybees, have colour vision based on three spectral types of receptor with maximal sensitivity to the ultraviolet (345-357nm), blue (440-450 nm) and green (520-525 nm) part of the spectrum (Höglund et al 1973; Schwemer and Paulsen 1973; Bennett and Brown 1985). While many diurnal butterflies have evolved additional receptor types for colour vision (e.g. Koshitaka et al 2008), behavioural tests have suggested the absence of an additional receptor type sensitive to longer wavelengths (seen as red by human observers) in *M. stellatarum* (Kelber and Hénique 1999). An early study (Hasselmann 1962), however, found sensitivity in the long-wavelength range, but recently, only three opsin genes have been identified in *M. stellatarum* (Xu et al 2013). A study on a species of leafhopper demonstrated nicely that insect green receptors can have some sensitivity at rather long wavelengths (Wakakuwa et al 2014).

We have now tested the sensitivity of the hummingbird hawkmoth, *M. stellatarum*, to spectral lights in the context of flower visits. This allowed us to determine the action spectrum of the species for foraging. We also performed electroretinograms (ERGs) to determine the spectral sensitivity of the photoreceptors. We compare the action spectrum of *M. stellatarum* with receptor sensitivity and with data from *Manduca sexta* and the honeybee.

## Materials and Methods

### *Experimental animals*

For all experiments, we used *M. stellatarum* bred in the laboratory from our own colony. The gene pool of this colony is regularly refreshed with wild-caught animals. Larvae were kept at room temperature indoors and fed fresh *Galium mollugo* until pupation. Shortly before eclosure, pupae were transferred to a flight cage with a 12:12 light: dark cycle.

For ERGs, we used moths that had been flying and foraging for several days indicating that they had normal vision. For behavioural experiments, naïve adult moths were introduced to experiments 24 h after eclosure, without any previous experience with flowers, and tested for up to 8 weeks.

### *ERGs*

We recorded ERGs from seven hummingbird hawkmoths during spring 2013. A moth was inserted into a tight plastic tube on a holder connected to a lockable ball-and-socket joint. The protruding head, the proboscis and the antennae were firmly glued to the tube with a 1:1 mixture of melted beeswax and resin. In a Faraday cage, an electrolytically sharpened tungsten electrode was advanced into the ventral or dorsal margin of one eye using a piezo-driven micromanipulator (PM10 DC3-K, Märzhäuser, Wetzlar, Germany), and a reference electrode was positioned in the contralateral side of the head.

Light from a 200 W Xenon lamp (Cermax LX175F ASB-XE-175EX, SP Spectral Products, Putnam, Con- necticut, USA) was directed to the eye via the central, 400 µm-wide fibre of a forked light guide (QR400-7-SR/BX, Ocean Optics, Dunedin, Florida, USA). Seen from the posi- tion of the moth, this provided a 5° stimulus, which illuminated the entire eye, when a shutter was opened. The spectral content of the stimulus could be changed from ultraviolet (330 nm) to red (700 nm) in 10 or 20 nm steps by passing the white light through one of 22 narrow-band interference filters (10–12 nm full width at half maximum; Melles Griot, Rochester, NY, USA). To achieve stimuli of equal quantum flux at all wavelengths, neutral density filters (fused silica, Melles Griot) were inserted in the light path.

Constant light from a green light emitting diode (LED; dominant wavelength 521, 34 nm full width at half maxi- mum; LXHL-MM1D Green Luxeon Star, Quadica Devel- opments Inc., Brantford, Ontario, Canada) was presented for spectral adaptation via the six outer fibres of the forked light guide (each 400 µm in diameter). This adaptation light covered a visual angle of 14° and provided between 4 × 1012 and 2 × 1015 quanta cm−2 s−1 at the position of the eye, depending on the operating current of the LED. Recorded ERGs were amplified (P15 AC amplifier, Natus Neurology Incorporated—Grass Technologies, Warwick, Rhode Island, USA) and digitized using custom-made LabView code (National Instruments Corporation, Austin, Texas, USA).

Prior to recording ERGs, the eye was dark-adapted for about 30 min. For stimulation, we presented flashes of 40 ms duration, separated by 5 s interflash intervals. The spectral sensitivity was measured six times, alternating between series starting with short and proceeding to long wavelengths, and series in the reverse order. Before and after each spectral series, a response–intensity (*V*−log *I*) relationship was determined to control for changes in recording quality and to establish the saturation level of responses (around 15 mV hyperpolarisation, in the dark- adapted retina). To isolate responses of short wavelength receptors, we repeated the recordings following the protocol described above, while the eye was illuminated with constant green adaptation light. The adaptation light was switched on 10 min before a series of recordings started, and presented with increasing intensities during subsequent series. After all light adaptation experiments were finished, the eye was dark adapted again and a last spectral series was recorded to control whether the initial results under dark adaptation could be reproduced.

Based on the sigmoidal *V*−log *I* relationship determined before and after each spectral series, response amplitudes *V* to stimuli of equal quanta were converted into sensitivities *S* and normalized to the maximal spectral sensitivity by:

$$S = 10^{(\log I - \log Imax)}, \tag{1}$$

where $I$ is the intensity of light eliciting a response of amplitude $V$, and $I_{max}$ is the intensity of light that elicits the maximal response amplitude within the spectral series. We averaged all spectral series from one animal recorded under the same adaptation condition and used an established pigment absorbance template (Govardovskii et al 2000) to estimate the number and sensitivity maxima ($\lambda_{\text{max}}$) of receptor types contributing to the ERG. All analyses were performed using custom-made programs in Matlab (R2012b, The MathWorks, Natick, Massachusetts, USA). In a first step, the templates were fitted to the spectral sensitivity curves of the dark-adapted eye using a non-linear least squares approach, in which amplitude and wavelength of alpha and beta absorbance peaks of the pigment were varied independently. We calculated the relation between alpha and beta peak of the green receptor and adapted a template (Govardovskii et al 2000) for each animal. In a second step, we fitted a sum of the adapted formulae for multiple pigments to the spectral sensitivity curves of the light adapted eye by non-linear least squares. To get the best estimate for $\lambda_{\text{max}}$ of a specific receptor type, we selected curves, in which the contribution of the other receptors was minimal. Finally, we averaged the $\lambda_{\text{max}}$ values of all animals and $R^2$ values for the fits used to determine the respective $\lambda_{\text{max}}$.

## *Behavioural experiments*

In behavioural tests, we trained hummingbird hawkmoths to associate a narrow-band light stimulus with a sucrose reward, and a dark stimulus with absence of the reward. By lowering the intensity of the monochromatic light until the moths could no longer distinguish both stimuli, we established the spectral sensitivity threshold between 360 and 640 nm.

Experiments were performed with free-flying moths during the summers of 2011 and 2012 in Lund, Sweden. The experimental flight cage (60 cm in height, 74 cm in width and 61 cm in depth, Fig. 1a) was illuminated from above using white light emitting diodes (LEDs) and fluorescent tubes (Osram Biolux, 18 Watt). The intensity in the cage was adjusted to 40 lux measured at the height of the stimuli (ILT1700 radiometer with SPM068 photomultiplier, International Light). The ceiling was made from thin soft plastic foil, the walls of the cage were covered with grey cardboard and both were painted with black stripes to facilitate a detection of the flight limits by the moths. The spectrum of the light reflected from this background is presented in Fig. 1c.

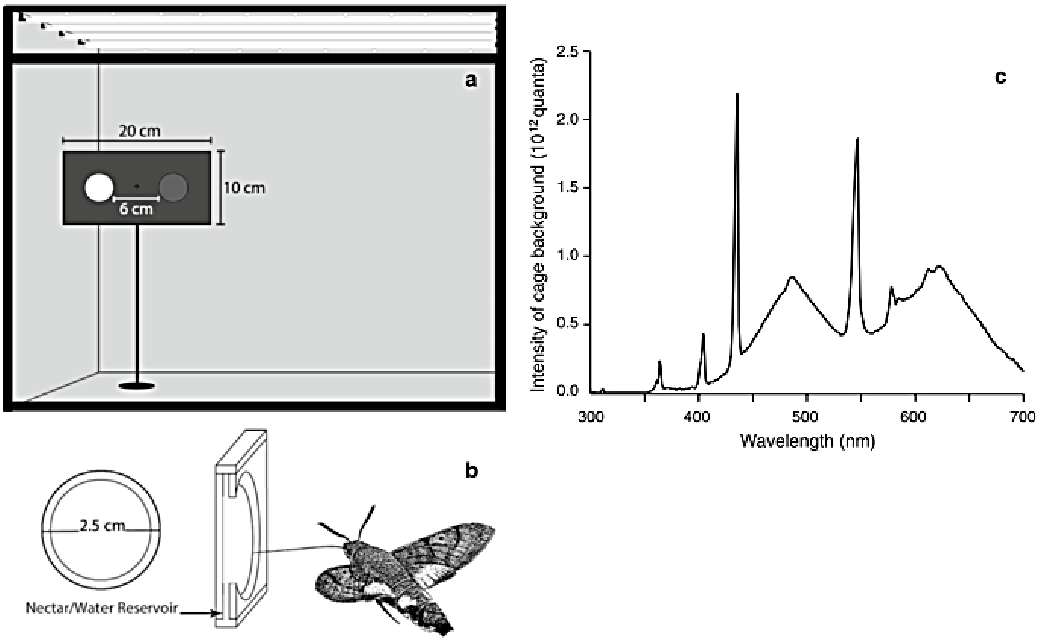

**Fig. 1** (a) Flight cage used for behavioural tests of *M. stellatarum*. (b) Feeders with a circular groove to present sucrose solution or water. (c) The spectral composition of the cage illumination as reflected from the background.

Narrow-band stimuli were provided by a monochromator (TILL Polychrome V, Till Photonics GmbH, Germany). Wavelength and intensity of the light were controlled using the manufacturer's software (PolyCon 3.0 version 3.0.12, Till photonics GmbH, Germany), and additional neutral density filters (fused silica, Melles Griot) were used to adjust the intensity within a range of five orders of magnitude (between 6 × 107 and 1013 quanta cm−2 s−1 for different wavelengths, measured at 3 cm distance from the stimuli with an ILT1700 radiometer). We used 14 wavelengths ranging from 360 to 620 nm, in steps of 20 nm, and in addition 370 nm. All wavelengths were presented at 15 nm full width at half maximum with exception of 440 nm that was presented at 10 nm.

During 2011, we tested moths with wavelengths between 420 and 620 nm, and during 2012 we completed the data in the UV (360–400 nm) range and repeated experiments with some wavelengths (420, 540, 560, 580, 600 and 620 nm) to compare the results obtained in both years. This comparison allowed us to control for variation in intensity measurements between the first and the second year.

The narrow-band light illuminated one of two circular UV-transparent Plexiglas disks (2.5 cm in diameter, separated by 6 cm) inserted into a vertical rectangular black plate (20 cm wide and 10 cm high) 36 cm above the floor (Fig. 1a). A reward of 3 µl of 20 % (w/w) sucrose solution was presented in an annular groove (invisible to the moth, see Fig. 1b) making the illuminated disk the positive (rewarded) stimulus, while the second disk was not illuminated and served as negative (unrewarded) stimulus presenting the same amount of water. Each newly eclosed animal was placed in an individually numbered moth container and assigned to a first training wavelength. For each experimental session, a single moth was released from its container, allowed to fly inside the experimental cage and given 90 s to make a first choice. An approach to the illuminated disk (rewarded stimulus) that ended in proboscis contact was considered a correct choice, and an identical approach to the dark disk (unrewarded stimulus) an incorrect choice. After every correct choice, the moth was allowed to feed for 5 s. After any choice, both stimuli were covered by the experimenter manually with a piece of cardboard of the same colour as the background, for 5 s. This caused the moth to keep some distance from them (8–12 cm) until the next stimulus presentation (trial).

The position of rewarded and unrewarded stimulus was changed between trials in a pseudorandom order to rule out learning of spatial cues. An experimental session was completed when the animal had made 10 choices or stopped flying. At the end of each session, a moth was allowed to drink sucrose solution ad libitum using the wavelength and intensity presented during the session. A satisfied moth usually sat down on the wall of the flight cage and was caught, placed inside its container and stored in the dark until the next day.

Using a rewarded light stimulus has the consequence that the animal perceives two negative (dark) stimuli when stimulus intensity is below detection threshold. As similar experiments with moths have not been reported, we introduced a control procedure to find out whether a moth that did not approach the stimuli was still motivated to feed but unable to detect the light, or simply lacked motivation, for instance, because it was not hungry. The control procedure was performed if 90 s elapsed without any choice. A light of 440 nm (2 × 1010 quanta cm−2 s−1) was then presented to the moth. Naïve moths have a strong preference for light of this wavelength, and after a rewarded visit, they are more responsive to other colours (Kelber 1997). If the moth responded to the control light, it was allowed to feed on it. We recorded a ‘no-choice’ response for the previous trial and assumed that the moth was motivated to feed but had failed to detect the light stimulus. After a positive control, a second attempt was made with the stimulus tested in the respective experimental session. If the moth still did not respond, we repeated the control procedure up to four times. After four ‘no-choice’ trials with positive controls, we finished the experimental session and tested the moth again the next day. If a moth did not respond to the control procedure, we assumed that it was satiated and finished the experimental session.

In the first session with each wavelength, animals were trained to respond to light at the highest intensity (ranging from 2 × 1010 to 1013 quanta cm−2 s−1 depending on wave- length). Once a moth had reached 80 % correct choices in 10 consecutive trials (usually after a single session), stimulus intensity was reduced in several steps in subsequent sessions, and up to 10 choices were collected from each moth for each intensity, until we reached an intensity for which moths made 50 % or less correct choices.

After finishing all trials with one wavelength, responsive animals were trained and tested with other wave- lengths. From earlier studies, we know that moths re-learn new colours with one or very few training trials (Kelber and Hénique 1999). In total, 55 animals were trained and tested during both years. No animal could be tested with all 15 wavelengths but single animals were tested with up to 6 different wavelengths. At least seven and on average ten animals contributed to the data at any wavelength–intensity combination.

### *Analysis of behavioural data*

For statistical analysis, we pooled data from all animals tested at a single wavelength. We assumed that the choice distribution followed binomial statistics, and that the relation between correct choice frequency and stimulus intensities at each wavelength can be described by a logistic psychometric function:

$$\psi(x) = \gamma + (1 - \gamma - \varepsilon)\frac{1}{1+e^{\frac{a-x}{b}}}, \tag{2}$$

where $\psi(x)$ is the fraction of correct choices at intensity $x$, $\gamma$ is the lower asymptote that was fixed to 0.5, $\varepsilon$ is the lapse rate (the difference between the upper asymptote and 1), which was restricted not to exceed 0.2 (equivalent to the chosen criterion of 80 % correct choices before testing started), and a and b are unrestricted parameters that deter- mine slope position and steepness, respectively (Wichmann and Hill 2001). We used maximum likelihood to fit the psychometric function to the measured spectral sensitivity data at each wavelength and evaluated the robustness of the fits by resampling the measured data using non-parametric bootstrapping (500 simulations). Calculations were carried out using the programme Palamedes (v. 1.5.0, Prins and Kingdom 2009) in Matlab (R2011a, MathWorks, Natick, MA, USA). The threshold was defined as the intensity, for which equation 2 predicted 75 % correct choices.

We fitted equation 2 to two sets of data, excluding and including ‘no-choice’ trials. In the latter analysis, we interpreted ‘no-choice’ trials as failures to detect the stimulus and, therefore, assumed a 0.5 probability of making a correct choice in these trials (random choice).

### ***Procedure to fit model predictions based on ERGs to the action spectrum***

To evaluate the relation between the spectral sensitivity of the eye determined by ERGs and the action spectrum of the animals, we used the receptor noise limited (RNL) model (Vorobyev and Osorio 1998) that has been developed using honeybee spectral sensitivity data (Brandt and Vorobyev 1997; Vorobyev et al 2001) and proven successful in describing spectral sensitivity for dichromatic, trichro- matic, and tetrachromatic animals (Vorobyev and Osorio 1998; Goldsmith and Butler 2003; Lind et al 2014).

The quantum catch, $q$, of a receptor, $i$, is given by:

$$q_i = k_i \int R_i(\lambda) I_s(\lambda) d\lambda, \tag{3}$$

where $R$ is the sensitivity of the receptor, $I$ is the quantum radiance of the stimulus, $s$, and integration is carried out over the spectrum from 300 to 700 nm. The scaling factor, $k$, is given by von Kries transformation, in which receptor responses are normalized to the quantum catch for the background spectrum $b$ (Fig. 1c):

$$k_i = \frac{1}{\int R_i(\lambda) I_b(\lambda) d\lambda}, \tag{4}$$

In tests of spectral sensitivity, the difference in quantum catch between the adaptive background and a superimposed monochromatic stimulus of wavelength $\lambda$ is:

$$\Delta q_i = k_i R_i(\lambda) I_s(\lambda), \tag{5}$$

The spectral sensitivity as a function of wavelength is given by:

$$\Delta S = \frac{e_1^2(\Delta q_3 - \Delta q_2)^2 + e_2^2(\Delta q_3 - \Delta q_1)^2 + e_3^2(\Delta q_2 - \Delta q_1)^2}{(e_1 e_2)^2 + (e_1 e_3)^2 + (e_2 e_3)^2}, \tag{6}$$

where $e$ is the standard deviation of receptor noise and the unit of $S$ is JND (just noticeable difference) with 1 JND representing threshold spectral sensitivity. The standard deviation of noise is here treated as a limiting Weber frac- tion $\omega$, and we assume that this fraction is inversely proportional to the number of receptors contributing to each receptor mechanism, $\eta$, by:

$$e = \omega = \frac{v_i}{\sqrt{\eta_i}}, \tag{7}$$

where $v$ is the noise within one single receptor cell (Vorobyev and Osorio 1998). By the use of equation 7, we account for receptor pooling (the procedure of summing receptor outputs in one mechanism), which increases signal-to-noise ratio and thus signal robustness.

# Results and discussion

## *ERGs*

We recorded complete sets of ERG data from the eyes of two male and five female hummingbird hawkmoths. In one male and two females the recording electrode was inserted into the ventral half of the eye, in the remaining animals it was inserted into the dorsal half. No obvious differences between sexes or eye regions were noticed, thus the wavelengths of maximal sensitivity ($\lambda_{max}$) of each receptor type were averaged for all animals. Lepidopterans have 3-hydroxyretinal visual pigments (Vogt 1989), the absorbance spectra of which can be approximated well by established template formulae (Stavenga 2010). Figure 2 presents measurements from one animal and absorbance spectra fitted using the pigment template (Govardovskii et al 2000).

Optimal fits to the data recorded in the dark-adapted retina were obtained assuming a green-sensitive visual pigment with $\lambda_{max}$ at 521 ± 3.6 nm (goodness of fit: $R^2 = 0.99$, n = 7; see Fig. 2a). The finding that responses of green receptors make by far the largest contribution to the ERG in the dark-adapted eye is consistent with the frequency of the different receptors types in the retina of other sphingid moths (Schlecht et al 1978; White et al 2003). Spectrally adapting the eye to green light (521 nm) allowed us to determine the spectral sensitivity of blue and UV receptors. In the most extreme adaptation state, the contribution of green receptor signals to the ERG was marginal, and the data were best fitted by the absorbance spectra of a blue- and a UV-sensitive visual pigment with $\lambda_{max}$ at 440 ± 3.5 nm ($R^2 = 0.98$, n = 7) and 349 ± 2.9 nm ($R^2 = 0.97$, n = 7) respectively (see Fig. 2b). Intermediary spectral adaptation states were more variable and confirmed the presence of three spectral receptor types in the eyes of hummingbird hawkmoths (Xu et al 2013; see Fig. 2c).

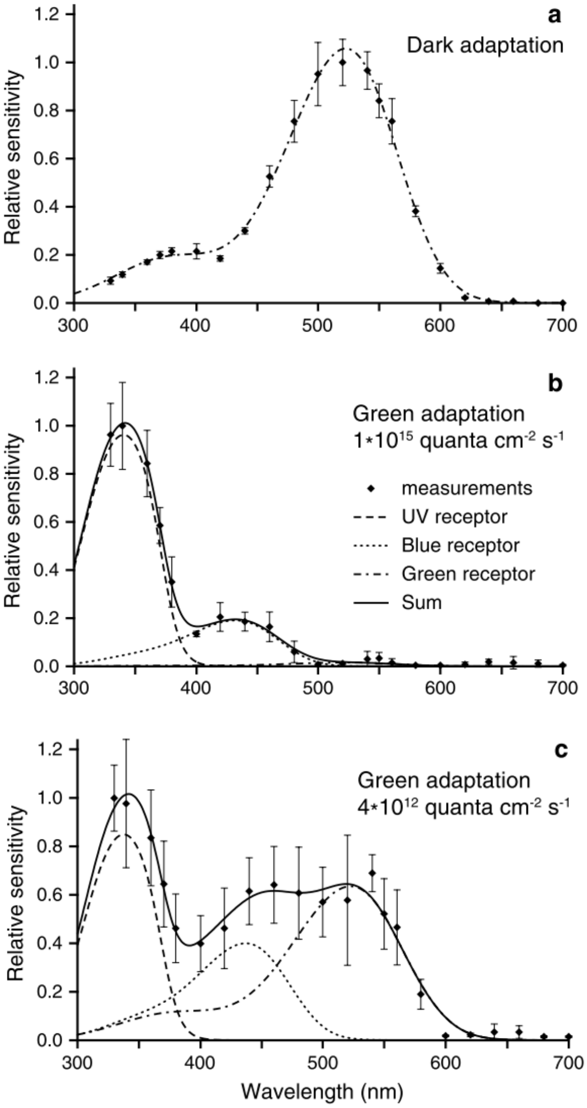

**Fig. 2** Spectral sensitivities of a female *M. stellatarum* derived from ERGs. Symbols denote measurements (average ± standard deviation, n = 6) under different adaptation states. Dashed, dotted and mixed lines indicate absorbance spectra of UV-, blue- and green-sensitive visual pigments, respectively, fitted to the data using the Govardovskii template (Govardovskii et al 2000). Solid lines give the summed curve of all absorbance spectra. Data collected under (a) dark adaptation, (b) adaptation to the brightest green light (10$^{15}$ quanta cm$^{-2}$ s$^{-1}$) and (c) to an intermediate intensity of green light (4*10$^{12}$ quanta cm$^{-2}$ s$^{-1}$).

### *Behavioural tests*

We successfully trained and tested animals to all 15 used wavelengths and obtained choice frequencies for at least five intensities with each wavelength. Moths learned fast to associate the reward with the brightest light stimuli, often reaching the criterion of 80% correct choices within a single training session, but this depended on the wavelength. The error rate increased as the intensity of stimuli was decreased. Although moths continued to make choices when light intensities decreased below their detection threshold, ‘no choice’ behaviour (see Methods section for definition) occurred, and was more prevalent at lower light intensities. For this reason, we determined detection thresholds for each wavelength in two ways: using only those trials in which moths made a choice (see Supplementary Fig. S1) and counting “no choice” trials as detection failures (Fig. 3).

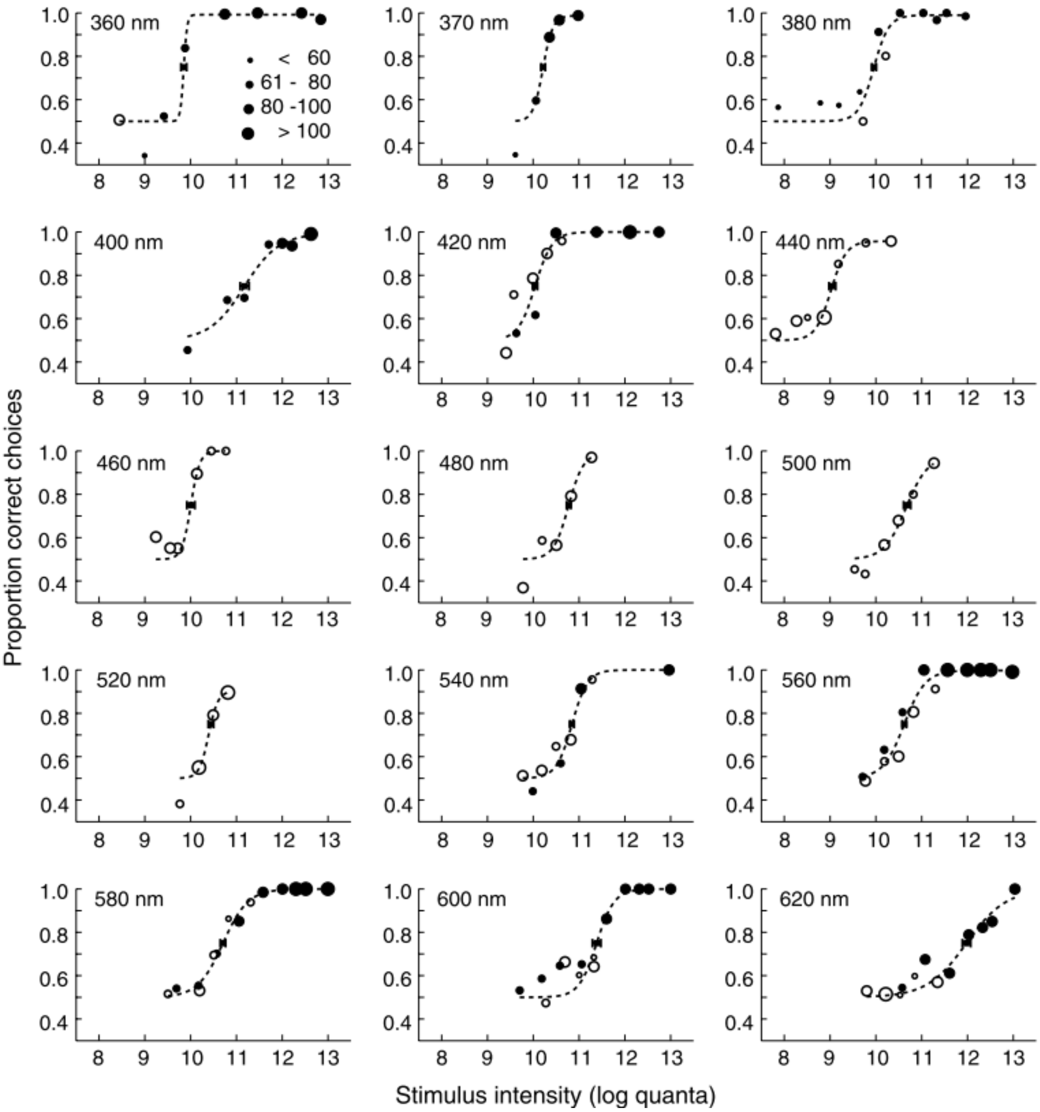

**Fig. 3** Behaviourally determined sensitivity thresholds for 15 wavelengths. Spectral sensitivity data and fitted logistic functions. Data include 'no-choice' behaviour (for details see text). Error bars indicate the robustness of the fit of the psychometric function to the data, obtained by non-parametric bootstrapping (500 simulations) evaluated at threshold (75% correct choices). Open circles show data collected 2011, filled circles show data collected 2012, differently sized data points represent different numbers of choices, equivalent to different weight of the data in the fitting procedure. Dashed lines represent the logistic function.

Both methods yielded similar results (Fig. 4). The action spectrum – defined as the inverse of the threshold intensity – has a prominent peak at 440 nm, and two shallower peaks in the ultraviolet (360 and 380 nm) and at long wavelengths (520 to 580 nm). The sensitivity was lower at 400 nm, 480 and 500 nm, and strongly decreased at longer wavelengths (600 and 620 nm).

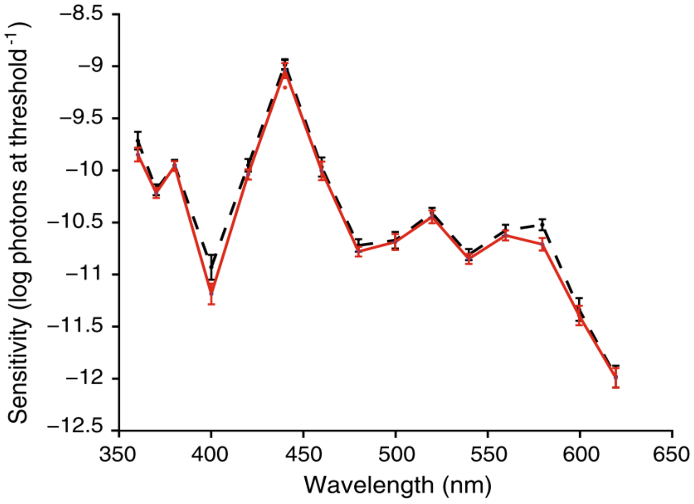

**Fig. 4** Spectral sensitivity threshold of *M. stellatarum*. Behaviourally determined thresholds for feeding behaviour using two evaluation methods, solid line: including 'no choice' behaviour, dotted line excluding 'no-choice'. For details see text.

### *Comparing ERG and behavioural data using a colour vision model*

Next, we compared the behavioural data with expectations from the sensitivities determined for UV, blue and green receptors by ERGs (Fig. 5a), using the RNL model (Vorobyev and Osorio 1998). We assumed that signals from all receptors in one ommatidium are pooled, and that the relative frequency of receptor types in *M. stellatarum* is similar to that in *M. sexta* and *D. elpenor*, with a receptor ratio of 1:1:7 (UV:blue:green receptors; see Schlecht et al 1978; White et al 2003).

To fit the spectral sensitivity function to the measured data, we used a least squares approach with only receptor noise, *v*, as free parameter. Generally, we found that the spectral positions of maxima in the behavioural spectral sensitivity curve agreed fairly well with expectations but amplitudes did not (solid curve in Fig. 5b).

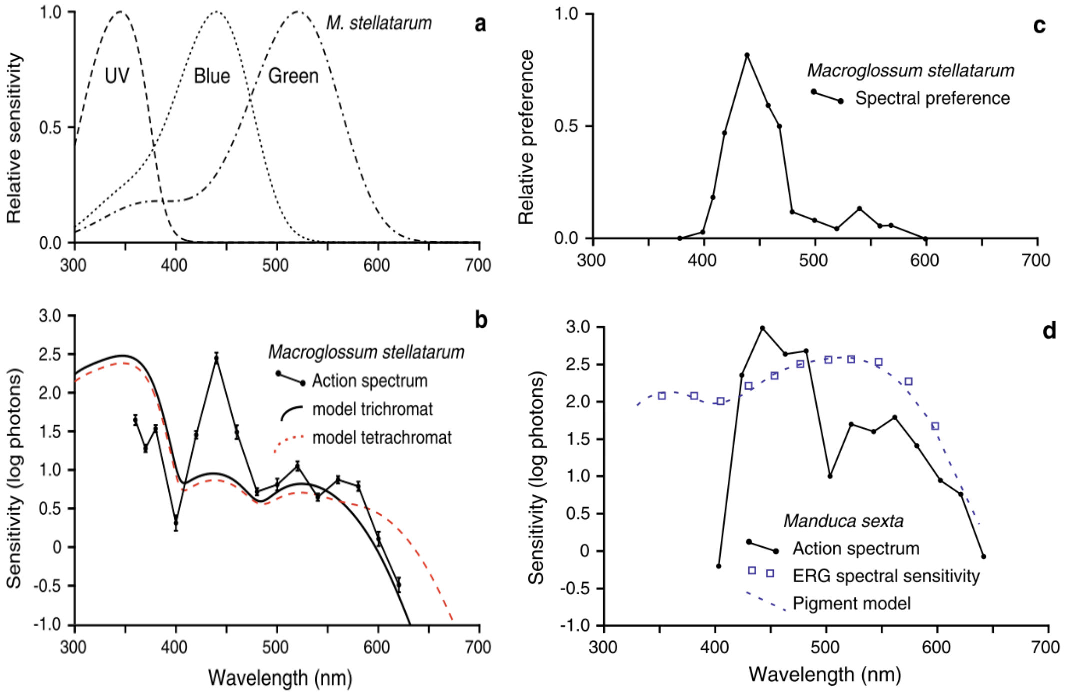

**Fig. 5** Spectral sensitivity of *M. stellatarum* in comparisons. (a) Normalized average sensitivity of photoreceptors of 7 moths maximally sensitive to light of 349 nm, 440 nm and 521 nm. (b) Behaviourally determined sensitivity (line with filled circles, including 'no choice') and two RNL model fits. Solid line: assuming three receptor types as shown in (a), dashed line: assuming an additional receptor type with maximal sensitivity at 560 nm. For details, see text. (c) Behaviourally determined preference of naïve *M. stellatarum* given the choice between lights of 13 wavelengths and 470 nm (original data from Kelber 1997). (d) Spectral sensitivity of *Manduca sexta* determined behaviourally (line with filled circles) and based on ERGs (empty squares), and a fit (dashed line) using known receptor sensitivities (adapted from Cutler et al 1995).

The highest peak of the action spectrum coincided with the physiologically determined sensitivity peak of the blue receptor at 440 nm. However, the behaviourally established sensitivity at this wavelength was 30 times higher than expected from the assumed abundance of blue receptors in the ommatidia of *M. stellatarum* and from physiological recordings (Fig. 5b). The low sensitivity to light of 400 nm agreed well with expectations, but we did not use wavelengths shorter than 360 nm in behavioural tests, thus we could not observe a reduction of sensitivity at wavelengths shorter than the peak of the UV receptor at 349 nm. The behavioural results at longer wavelengths reflected the physiologically determined sensitivity of the green receptor (peaking at 521 nm) but the sensitivity dip at 540 nm and the relatively high sensitivity at 560 and 580 nm could not be explained.

Although our ERG data are consistent with the presence of three receptor types, and earlier behavioural data (Kelber and Hénique 1999) suggest that *M. stellatarum* does not use a fourth, red-sensitive receptor for colour vision, the old study by Hasselmann (1962) seemed to indicate a fourth receptor type. Therefore we tested whether including a receptor with maximal sensitivity at 560 nm improved the agreement between model expectations and behavioural results. This was clearly not the case (dashed curve in Fig. 5b), as a tetrachromat should have considerably higher sensitivity to long wavelengths. We therefore exclude the possibility that an additional receptor sensitive to longer wavelengths contributed to the behaviour. Despite some inconsistencies (see below), the behavioural data and model calculations agree with the conclusion that M. stellatarum is a trichromat, just like M. sexta, Deilephila elpenor and the honeybee.

### *An unexpectedly high sensitivity to blue light*

Left with the behavioural response to unexpectedly low intensities of blue light (440 nm), we can think of several reasons for this mismatch with model expectations. First, as no noise measurements of hawkmoth photoreceptors have ever been performed, it is theoretically possible that blue receptors have a much lower noise level than both green and UV receptors. Still, we cannot account for the 1.5 log units difference in sensitivity, even if we assume an unrealistically large difference in noise levels between blue receptors and the other receptors, a lower number of green receptors contributing to the behaviour, or a degree of pooling of blue receptor signals that is highly unlikely given the species' rather fine spatial resolution for colour patterns (Goyret and Kelber 2012).

Second, the used RNL model may not be suited to describe the kind of data we measured. However, as Brandt and Vorobyev (1997) demonstrated, the RNL model describes the action spectrum of honeybees better than other models that also assume opponent mechanisms. A model that does not take opponent mechanisms into consideration does not describe the position of the minima in the action spectrum of *M. stellatarum* at 400 and 480 - 500 nm (not shown). However, even in such a model, we would have to assume unrealistically high frequencies of blue receptors in the retina, if we wanted to fit the amplitude of the peak at 440 nm.

Alternatively, it is possible that our behavioural tests, performed in the context of foraging, did not measure the general sensory capability of the species, which is determined by receptor sensitivities and limited by receptor noise only. Instead, we assume that filter processes at later stages in the visual pathway or at central stages involved in decision-making in the brain, give different weight to information from different receptor channels, or even control sensitivities by feedback to the peripheral visual system. We consider it likely that such processes are related to innate preferences of the moths for flower colours, thus we compare our behavioural results with spectral preferences of the species (Kelber 1997). Sensitivity changes on a peripheral level, caused by the motivational state of insects, have been found in the olfactory system, where sensitivity to pheromones, host odours and oviposition substrate odours differs depending on the internal state of the animal (see, for instance, Siju et al 2010; Barrozo et al 2011). Similar differences have not been described for visual sensitivity, as far as we know.

### *Comparing behavioural sensitivity with spectral preferences in the context of foraging*

In tests of spectral preference, flower-naïve *M. stellatarum* moths were given the choice between two narrowly tuned lights of equal quantum flux (Kelber 1997). One of these stimuli was kept constant at 470 nm, while the second one was varied between 380 and 600 nm. Results obtained with a background illumination similar to that in the present experiment are re-plotted in Fig. 5c. It shows a very strong maximum at 440 nm, a minor but significant maximum at 540 nm and a dip between these two maxima. Light of 380 nm or 600 nm wavelength was not chosen at all. Experiments with reflecting colours confirmed the strong innate preference and high salience of blue stimuli for eliciting feeding in *M. stellatarum* (Kelber 1997; Kelber and Balkenius 2007).

### *Comparison with other nectar foragers*

Knoll (1926) observed a preference for blue colours in the hawkmoth *Hyles livornica*, but the only other hawkmoth species studied in detail is *Manduca sexta*. Cutler et al (1995) tested *M. sexta* in a way that is intermediate between the two methods described above for *M. stellatarum*. Moths were given the choice of a broadband green (520 nm maximum, 95 nm full width at half maximum) stimulus and one of 13 narrowband stimuli (20 nm full width at half maximum). Both stimuli could be varied in intensity, and the authors used preference data to establish an action spectrum.

Cutler et al (1995) also found a pronounced maximum of the action spectrum in the blue range and compared their results to ERG data, with very much the same result that we obtained now for the hummingbird hawkmoth (Fig. 5d). A more recent study on colour preferences in *M. sexta* confirmed the strong preference for blue (Goyret and Kelber 2008).

The situation is different in honeybees. Helversen (1972) determined the action spectrum of two workers of *Apis mellifera*, in the context of foraging. His results – very much in contrast to our results and those of Cutler et al (1995) – could nicely be described using the RNL model, taking into account only the spectral sensitivity and known noise level of honeybee photoreceptors (Vorobyev et al 2001).

## Conclusions

Our experiments determined the spectral sensitivity maxima of the three types of photoreceptor that *Macroglossum stellatarum* uses for trichromatic colour vision. Based on our results on *M. stellatarum* and their similarity to earlier results on *M. sexta*, we suggest that not only the sensitivity of photoreceptors but also the relevance of blue in the context of foraging is reflected in the action spectrum of hawkmoths. Sensitivity to blue light is high although blue receptors are much rarer in the retina than green receptors, as ERG data confirm. We hypothesize that the sensitivity of UV and green receptors may be down-regulated in the visual pathway carrying the signals used for flower detection, in the context of foraging.

Further studies are needed to better understand the spectral sensitivity at different stages in the visual and motor control system of hawkmoths, and its regulation by the motivational state of the animals. While bees use colour vision mostly to detect flowers, moths use this sensory modality also to detect suitable substrates for oviposition. Thus, we can speculate that female *M. stellatarum* motivated to lay eggs, may give higher weight to the green receptor signals, which could serve them in the search for the green leaves of the larval host plant.

## Chapter Acknowledgements

We thank Alejandro Trillo for catching wild moths and collecting eggs in Spain and Michael Pfaff and Samantha Goyret for enduring help with breeding *Macroglossum stellatarum*. We are grateful to Michael Pfaff for help with building the flight cage, to Eric Warrant for sharing electrophysiology equipment, to the Lund Vision Group for inspiring discussions and to two referees for constructive comments. We gratefully acknowledge financial support from the Swedish Research Council (grant 621-2009-5683) and the Wenner-Gren foundation to AK, from CSIC Studentship JAE PRE (2011: ESTCSIC – 6715 and 2012: ESTCSIC – 7853) to FJT and from the Spanish Ministerio de Ciencia e Innovación/FEDER (project CGL2010-16795) to MARG.

## Supplementary material

Additional supplementary material can be found at the respective section of this thesis.

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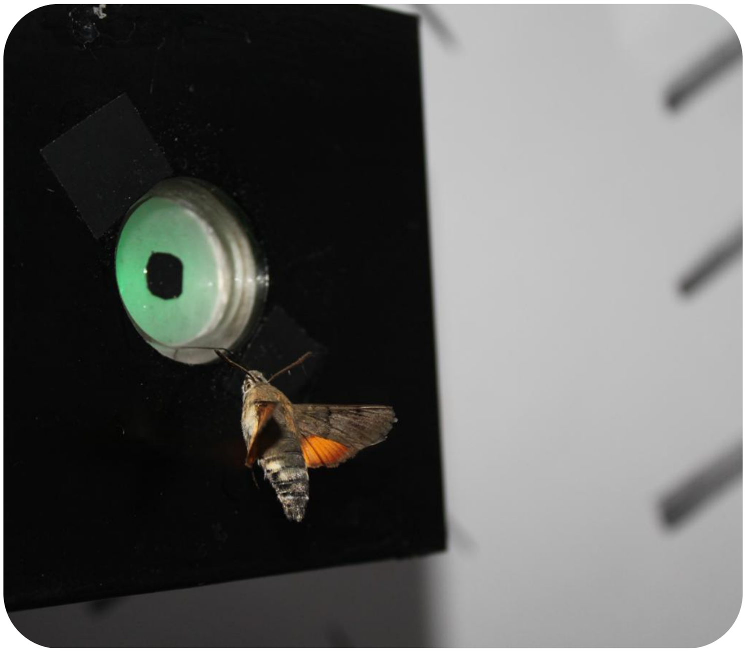
# CHAPTER FOUR



# Wavelength discrimination in the hummingbird hawkmoth *Macroglossum stellatarum*

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## Abstract

Despite the strength of the relationship between insect vision and the spectral properties of flowers, only in very few species of floral visitors the visual system has been studied in some detail. For instance, wavelength discrimination was only tested in two species of floral visitors: the honeybee (*Apis mellifera*) and the butterfly *Papilio xuthus*. Here, we present the results of wavelength discrimination for the hawkmoth *Macroglossum stellatarum*, and compare these data with those found for the former two species and the predictions of a colour discrimination model. We trained moths do feed from a disk, illuminated with monochromatic light and presenting a reward, and tested them in a dual choice situation where they had to choose between the rewarded wavelength and a novel unrewarded wavelength. To characterise the wavelength discrimination threshold we increased (or decreased) the wavelength of the test stimulus until we found the discrimination thresholds for each rewarded wavelength. In agreement with model predictions, we found two expected minima of wavelength discrimination thresholds where photoreceptor sensitivities overlap. Although the model correctly predicted the minima of discrimination, we found a minor third, unpredicted, minimum around the peak of the blue photoreceptor. The best discrimination thresholds of *M. stellatarum* are around 1 to 2 nm and thus comparable to those found in the tetrachromatic butterfly *P. xuthus,* and better than those found for the honeybee. Although studies of wavelength discrimination focus on the chromatic properties of stimuli, we also varied light intensity to test its effect on the discrimination capacity.

**Keywords:** wavelength discrimination, *Macroglossum stellatarum*, visual system, chromatic and achromatic cues.

# Introduction

Flower colour ultimately conveys information to eyes different from ours, with plants exploring the visual system of potential pollinators by attracting them to visit their flowers. Once the visual information is associated with the presence of a reward (nectar and/or pollen), insects learn to return to the flowers. To be flower-constant, the floral visitor must be able to continuously perceive the difference between the selected flower and distracters. If discrimination is based on visual cues and the difference in colour between co-occurring flowers is smaller than a threshold value, discrimination by colour only is impossible.

The minimum required differences between colours to be discriminated, also known as wavelength discrimination thresholds, have only been studied in the honeybee (von Helversen 1972), and the butterfly *Papilio xuthus* (Koshitaka et al 2008) across all taxa of insect flower visitors. Studies of wavelength discrimination are generally interested in the chromatic mechanisms (Kelber et al 2003), and stimulus intensities are adjusted to prevent achromatic differences. Honeybees, for instance, can discriminate narrow-banded colours in the blue-green region with a minimum wavelength difference of 4.5 nm (von Helversen 1972) when the threshold is set at 70% of correct choices, and 3 nm in the same region when threshold is set as 60% (Koshitaka et al 2008), while *Papilio xuthus* can discriminate even finer differences, approaching 1 nm at 430 and 560 nm (Koshitaka et al 2008) at a threshold of 60% of correct choices.

Recently, the spectral sensitivity of the European hummingbird hawkmoth *Macroglossum stellatarum* was determined (Telles et al 2014), with previous experiments demonstrating other aspects of the visual system and revealing remarkable learning abilities (Kelber 1996; Kelber 1997; Kelber and Henique 1999; Kelber 2002; Balkenius and Kelber 2004; Kelber 2005). Based on its trichromatic visual system, with receptors maximally sensitive in the ultraviolet (UV – 349 nm), blue (440 nm) and green (521 nm) range, *M. stellatarum* can discriminate between colours of different spectral properties (Kelber 1996; Kelber and Henique 1999). These moths can discriminate monochromatic lights differing 15 nm in the ultraviolet range and 30 nm in the blue and the blue-green range (Kelber and Henique 1999). However, it was not clear whether this discrimination was based on chromatic or achromatic cues for some wavelengths. Besides, it has being suggested that when there is a wavelength difference sufficient to allow for discrimination by chromatic cues, intensity plays a minor role for discrimination for the moths (Kelber and Henique 1999; Kelber 2005), but colour discrimination thresholds have remained unknown for this species.

Here, we measure the limits of wavelength discrimination in the entire visible spectrum of *M. stellatarum*, determining discrimination thresholds from the data with the approach of Koshitaka et al (2008). Specifically, we selected six monochromatic wavelengths to cover the peaks and overlapping areas of photoreceptor sensitivities and trained moths during consecutives days to associate one of the six wavelengths with a reward, to finally test moths' abilities to discriminate between monochromatic wavelengths. We compare wavelength discrimination thresholds of *M. stellatarum* with predictions of a colour vision model and with those values experimentally obtained for other flower visitors.

## Materials and Methods

### *General procedure and setup*

*Macroglossum stellatarum* were bred on their natural food plant in the lab (for details see Telles et al, 2014). Eclosed moths were individually trained and tested in a flight cage (60 cm x 74 cm x 61 cm, Fig. 1a) to feed from a feeder disk illuminated by spectral lights. Six wavelengths were selected as the rewarded wavelengths according to the determined spectral sensitivity for these moths (Fig. 1b): 380 nm, 400 nm, 440 nm, 480 nm, 520 nm and 560 nm. With these wavelengths we covered the sensitivity peaks of ultraviolet, blue and green receptors and the areas were receptor sensitivities overlap. We assigned newly hatched, flower-naïve moths to one of the rewarded wavelengths, forming six groups (with initially six to seven moths per group). We kept each moth in an individual box in a dark environment for 24 hours to increase feeding motivation (Goyret and Kelber 2011) before training and subsequent testing sessions. Moths were trained to associate the light of the chosen wavelength with the reward and remained with the same rewarded wavelength until the end of experiments. The illumination intensity of the cage was 40 lux (for details see Telles et al, 2014) during the experiment. A grey background was used to cover the walls of the flight cage (Fig. 1a). The spectral composition of the cage illumination as reflected from the background is given in Fig. 1c.

Monochromatic visual stimuli were produced by two light sources (TILL Polychrome V, Till Photonics GmbH, Germany) that were used individually during the training session and simultaneously during the testing session (details below). We kept the bandwidth constant (full width at half maximum of 15 nm), and varied the intensity of the stimuli between $1.98 \times 10^{10}$ and $2.92 \times 10^{11}$ quanta/cm$^2$/s. We varied the relative intensity between rewarded and novel wavelengths such that the rewarded wavelength had either the same, a lower or a higher number of photons than the novel wavelength (detailed information about intensities can be found in Table S1, Supplementary material). We did not vary intensity in all wavelength combinations since in earlier experiment intensity was demonstrated to not play a major role in discrimination (Kelber and Henique 1999). Two circular UV-transparent Plexiglas disks (2.5 cm in diameter, separated by 6 cm) inserted into a vertical rectangular black plate (20 cm wide and 10 cm high), 36 cm above the floor positioned on the left side of the cage (Fig. 1a), were used to present the reward and unrewarded stimuli.

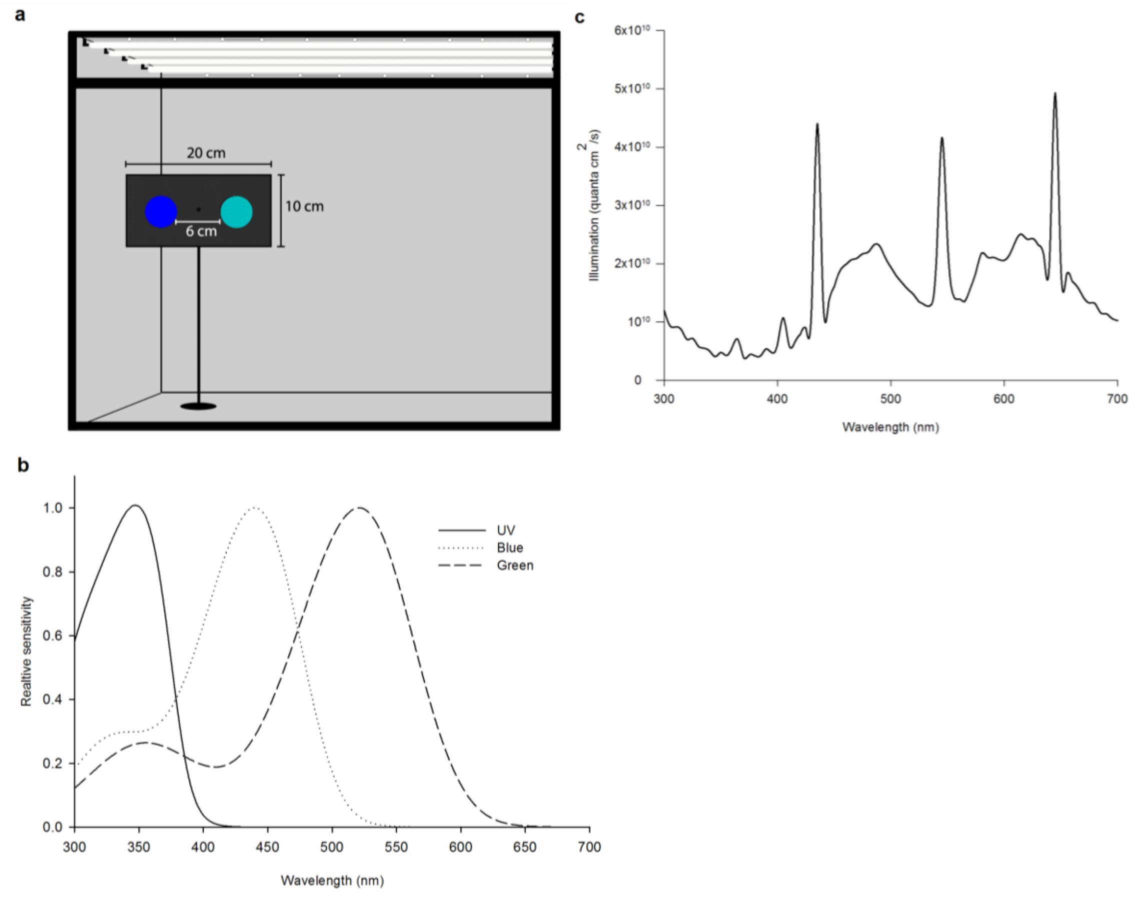

**Fig. 1 Experimental flight cage, *Macroglossum stellatarum* spectral sensitivity and ambient illumination**. (a) Flight cage and disks arrangement exemplifying a typical moth's view of stimuli during test sessions, during training one of the disks were unilluminated. (b) Normalized sensitivity of photoreceptors calculated using the Govardovskii template (Govardovskii et al 2000). (c) Spectral composition of the cage illumination as reflected from the background.

### *Stimuli and experimental sessions*

Monochromatic light of one of the six wavelengths was presented to the moths as the rewarded stimulus, and a dark disk was used as the unrewarded stimulus, during training. Once moths reached 80% of correct choices the testing sessions started. In testing sessions both disks were illuminated. The rewarded training wavelength was offered together with the light of a novel (unrewarded) wavelength and moths had to choose one of them. The rewarded disk carried 3 µl of sucrose solution at 20% (weight/weight) concentration, while the unrewarded disk carried the same amount of water. Both solutions were presented in an annular groove surrounding the disks (invisible to the moth, see Fig. 1b in Telles et al 2014).

Based on a previous experiment (Kelber and Henique 1999), novel wavelengths were initially chosen to be 20 nm longer or shorter than the rewarded wavelength (Table 1). Tested differences ($\Delta\lambda$) between rewarded and novel wavelengths did not follow a decreasing order. They were established according to the moth discrimination capacity and could assume different values for different wavelength combinations. If the initial distance of 20 nm between wavelengths was not sufficient for moths to reach our threshold criterion (80%) at the end of the first testing session, the distance was increased by 10 nm, and this could be repeated until reaching a maximum of 50 nm. When moths could not discriminate a novel wavelength 50 nm longer or shorter than the rewarded wavelength, we stopped tests with these wavelength combinations. If a moth reached our threshold criterion with one novel wavelength difference, tests continued with more similar wavelengths. One testing session was performed with each moth every day until discrimination thresholds for shorter and longer wavelengths were determined. This procedure took between 15 and 24 days, for a single moth, depending on the rewarded wavelength and individual performance.

**Table 1** Rewarded wavelengths used during training, initially proposed (initial) and final novels wavelengths used during testing sessions. $\lambda$- = shorter wavelengths; $\lambda$+ = longer wavelengths. In bold: rewarded wavelengths that changed from initial distances.

| **Rewarded (nm)** | **Novel $\lambda$-** | | **Novel $\lambda$+** | |
|---|---|---|---|---|
| | *Initial* | *final* | *initial* | *final* |
| 380 | 360 | **340** | 400 | 400 |
| 400 | 380 | 380 | 420 | **440** |
| 440 | 420 | 420 | 460 | **470** |
| 480 | 460 | **450** | 500 | 500 |
| 520 | 500 | 500 | 540 | **570** |
| 560 | 540 | **510** | 580 | **585** |

An approach to the disk of the rewarded wavelength that ended in a proboscis contact was considered a correct choice. An identical approach to the unrewarded disk was considered an incorrect choice. Moths were allowed to feed for five seconds after a correct choice. After this time or after an incorrect choice, both stimuli were immediately covered for five seconds with a piece of cardboard of the same colour as the background, and the setup was prepared for the next trial. The position of rewarded and unrewarded stimuli was changed between trials in a pseudorandom order to rule out position learning.

An experimental session ended after 15 trials, or when the moth stopped flying (because of lack of motivation or because it got satiated after some trials). We then offered sucrose solution *ad libitum* in the disk with the rewarded wavelength to the moth. A satisfied moth usually sat down on the wall of the flight cage, where it was caught and stored in its individual dark box until the next day.

### *Statistical analysis of intensity match and wavelength discrimination*

To inspect the possible effect of intensities on wavelength discrimination, we used generalized linear models (GLM) in R, version 3.0.3 (R Core Team 2013), with binomial distribution and logit transformation of the proportions of correct choices, for those wavelength pairs where intensities varied. Using the same function, we also tested whether the choice distribution between rewarded and novel wavelengths was different from chance and presented the results in Table S1, Supplementary material. GLM belongs to the default *car package*. Significance intensity values were corrected ($p_{corrected}$) using the Bonferroni approach.

### *Behavioural wavelength discrimination thresholds*

The total numbers of visits, number of correct and incorrect choices of moths within a group during the testing sessions were used to determine the wavelength discrimination ability of *Macroglossum stellatarum*. We used the same approach that Koshitaka et al (2008) applied on *Papilio xuthus*, where the proportion of correct choices was plotted for each group of moths trained with one of the six rewarded wavelengths. A line set at the level of the established threshold intersecting both sides of the plot (shorter and longer wavelengths) was used to determine $\Delta\lambda$ (nm) for each combination of rewarded and novel wavelengths. Distances between the central wavelength and that corresponding to the intersection point towards shorter and longer wavelengths were determined at 70% and 60% criteria of correct choices (Figure 2). These threshold criteria were selected such that data could be compared with those of the honeybee at 70% (von Helversen 1972) and *Papilio xuthus* at 60% (Koshitaka et al 2008). We applied the same method used by von Helversen when studying the honeybee (1972) to plot the values obtained at thresholds towards longer and shorter wavelengths, which consists in a plot of $\Delta\lambda$ (nm) as a function of the middle wavelength between the rewarding wavelength and that corresponding to the minimum discriminable wavelength. For instance, with a rewarding wavelength of 440 nm, and a threshold at 445 nm, the middle wavelength would be determined as 442.5 nm.

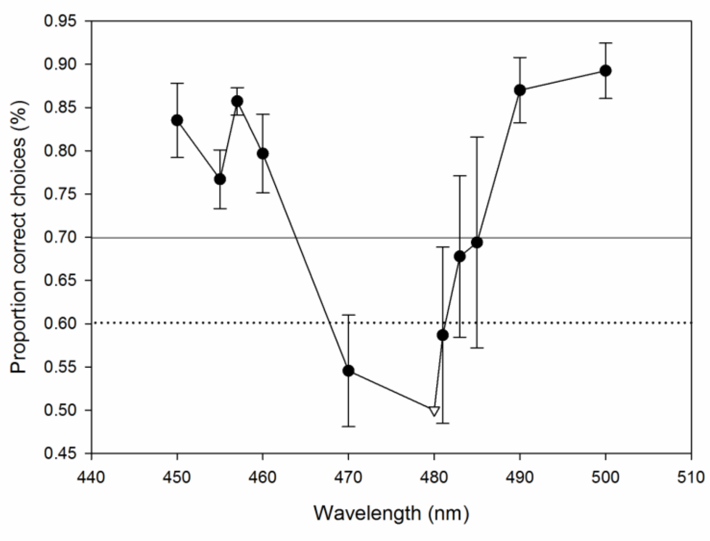

**Fig. 2 Discrimination of shorter and longer novel wavelengths**. Central wavelength of 480 nm (triangle down) and moths' accuracy depending on the distance between wavelengths (black circles). The dashed line indicates the 60% criterion, the black line, 70% criterion. Error bars are standard errors of moths.

## *Theoretical wavelength discrimination thresholds*

We compared the behaviourally determined Δλ with values predicted by the receptor noise limited model (Vorobyev and Osorio 1998; Vorobyev et al 2001). Adapting the method proposed by Koshitaka et al (2008) to the trichromatic system of *M. stellatarum* (their equation 1), the wavelength discrimination threshold can be calculated from the noise in each receptor channel, $\omega$, and the derivatives of photoreceptor signals, $\left(\frac{df_i}{d\lambda}\right)$, as:

$$\Delta\lambda = \sqrt{\frac{(\omega_1\omega_2)^2 + (\omega_1\omega_3)^2 + (\omega_2\omega_3)^2}{(\omega_1)^2\left(\frac{df_2}{d\lambda} - \frac{df_3}{d\lambda}\right)^2 + (\omega_2)^2\left(\frac{df_1}{d\lambda} - \frac{df_3}{d\lambda}\right)^2 + (\omega_3)^2\left(\frac{df_1}{d\lambda} - \frac{df_2}{d\lambda}\right)^2}} \quad (1)$$

The noise parameters, $\omega_i$, were calculated from the relative number of UV, blue and green receptors present in one moth ommatidium (1:1:7 respectively, Telles et al, 2014). According to Koshitaka et al (2008) and Vorobyev and Osorio (1998) (equation 2),

$$\omega_i = v_i/\sqrt{n_i}, \quad (2)$$

where $v_i$ is the noise level of a single photoreceptor of type $i$ (assumed to be independent of photoreceptor type, and arbitrarily set so that for the long-wavelength photoreceptors $\omega_L = 0.05$; Koshitaka et al 2008) and $n_i$ is the number of receptors of a type $i$. This leads to $\omega_{UV} = \omega_B = 0.132$ and $\omega_G = 0.05$.

We assumed a logarithmic relationship between photoreceptor signal and quantum catch (Koshitaka et al 2008):

$$\frac{df_i}{d\lambda} = \frac{k_i}{1 + k_i P_i}\frac{dP_i}{d\lambda}, \quad (3)$$

where $P_i$ is the spectral sensitivity of photoreceptor $i$ (UV, blue or green) and

$$k_i = c/\int P_i(\lambda)I(\lambda)d\lambda \quad (4)$$

where $I(\lambda)$ is the background light intensity distribution as a function of wavelength $\lambda$, and the parameter $c$ is chosen such that, for the long wavelength receptor, $k_G = 1$ (Koshitaka et al 2008). For the spectral sensitivities of the photoreceptors we adopted the template of Govardovskii et al (2000), with maximum sensitivities at 349 nm, 440 nm and 521 nm for the UV, B and G photoreceptors, respectively (Telles et al 2014).

## Results

### *Wavelength discrimination and intensity matches*

For most pairs of wavelengths for which light intensities were varied, we found no significant difference between choice distributions in the tests with different light intensities. Choice distribution for single tested pairs and for those pairs where intensity varied for a same wavelength combination can be found in Table S1, Supplementary material. When testing for differences between two intensities that varied for a same wavelength combination, we only found statistically significant differences for two of the six rewarded wavelengths: 480 and 560 nm.

For the rewarded wavelength of 480 nm, intensity had an effect in tests with two novel wavelengths, 460 nm ($X^2$=13.74; df=1; $p_{corrected}$= <0.001) and 483 nm ($X^2$=11.69; df=1; $p_{corrected}$= 0.002). With 460 nm, a decrease in intensity (from $1.5x10^{11}$ to $7.8x10^{10}$ quanta/cm$^2$/s) of the rewarded wavelength resulted in a decrease in the proportion of correct choices from 89% to 68%. Note, however, that both choice distributions differ significantly from chance (p <0.001, Table S1). In tests with 483 nm, a decrease in intensity (from $2.9x10^{11}$ to $1.5x10^{11}$ quanta/cm$^2$/s) of the rewarded wavelength resulted in an increase on the proportion of correct choices, from 68% to 85%. Again, choice distributions in both tests differ significantly from chance (p<0.001, Table S1). It is also important to note that stimulus intensity did not affect the proportion of correct choices when moths discriminated between 480 nm and 485 nm.

For the rewarded wavelength of 560 nm, differences between intensities were found in tests with the novel 570 nm wavelength ($X^2$=7.98; df = 1; $p_{corrected}$= 0.01). Reducing the intensity of the rewarded wavelength (from $1.6x10^{11}$ to $4.8x10^{10}$ quanta/cm$^2$/s) resulted in a decrease of correct choices (p=0.006) from 68% to 49%. Consequently, the choice distribution analysis showed that with reduced intensity, choices were not different from chance (Table S1).

### *Behavioural wavelength discrimination thresholds*

Moths were successfully trained to all rewarded wavelengths, but during tests the performance depended on the novel wavelengths. When stimulus intensity affected performance, we calculated one discrimination threshold for each intensity (Table 2). The threshold discrimination function (Fig. 3) plots the average of the intensity-specific thresholds or, when performance was independent of stimulus intensity, threshold values calculated pooling the data from all intensities.

**Table 2** Different Δλ (nm) values for rewarded wavelengths at lower and higher intensities. Values change depending on the criterion (70% or 60%).

| **Wavelength (nm)** | | **Δλ (nm)** | |
|---|---|---|---|
| **rewarded** | **novel** | **60%** | **70%** |
| 480 lower | 483 | 1.1 | 1.8 |
| 480 higher | 483 | 1.2 | 2.1 |
| 480 average | 483 | 1.1 | 2 |
| | | | |
| 480 lower | 460 | 13.9 | 20.2 |
| 480 higher | 460 | 11.6 | 14.4 |
| 480 average | 460 | 12.3 | 16.3 |
| | | | |
| 560 lower | 570 | 14.1 | 15.8 |
| 560 higher | 570 | 6.9 | 7 |
| 560 average | 570 | 11.2 | 17 |

The Δλ function (Fig. 3) has two pronounced minima at approximately 380 nm and 480 nm showing the best wavelength discrimination areas. The first minimum corresponds to the region where the spectral sensitivities of the UV, blue and (minimally) green receptors overlap, while the second minima corresponds to steep flanks in the sensitivity curves of the blue and green receptor (Fig. 1b). A third small minimum was found around 435 nm, which is between the blue receptor peak and the inflection point of the green receptor.

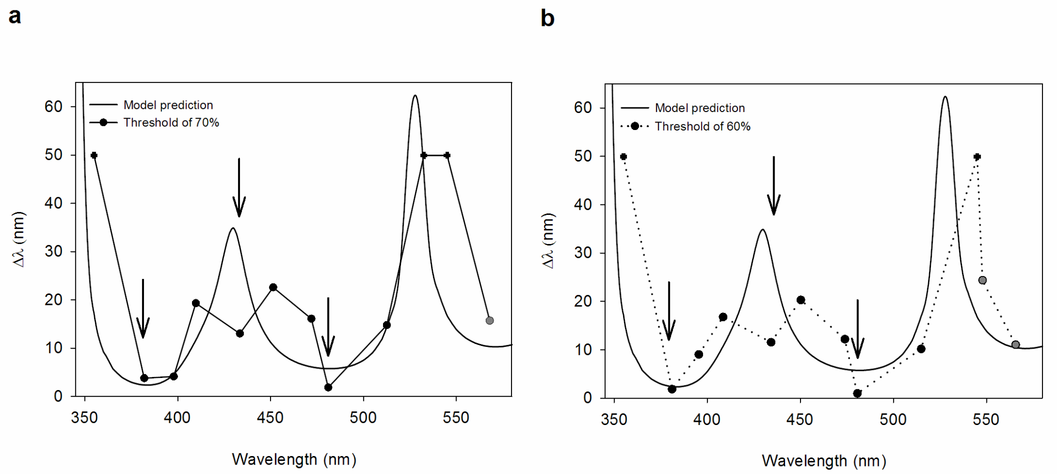

**Fig. 3 Behavioural and predicted Δλ (nm) functions**. The smooth curves represent model results, with two minima of wavelength discrimination (for model details see text). Behavioural results (connected black circles) exhibit three minima of discrimination (arrows) at **(a)** 382, 433 and 481 nm for a threshold of 70%, and **(b)** 381, 434, and 480 for 60%. Crosses represent tested wavelengths for which discrimination did not occur. We used the maximum differences between wavelengths (50 nm) for graphical representation. Grey circles represent wavelength discrimination based on achromatic cues.

### *Chromatic and achromatic thresholds*

Discrimination thresholds for each rewarded wavelength are given in Tables 3 and 4, and the Δλ functions using thresholds of 60% and 70% are presented in figure 3. The overall shape of Δλ function does not change much if we limit accuracy at the criterion of 70% or 60% of correct choices (Fig. 3). The main difference lies in the green range of the Δλ function. For wavelengths between 520 and 570 nm, moths did not reach the threshold of 70% of correct choices (Table 3) and a value of 50 nm (the maximum difference between wavelengths applied during experiments) was used for graphical representation. However, using a threshold of 60%, Δλ (nm) could be determined for almost all rewarded wavelengths (Table 4).

**Table 3** Δλ (nm), rewarded and minimum discriminable wavelengths (nm) for both lower and higher distances at the level of 70% of correct choices.

| **Rewarded wavelength** | **Discrimination of shorter wavelengths** | | **Discrimination of longer wavelengths** | |
|---|---|---|---|---|
| | threshold λ | Δλ (nm) | threshold λ | Δλ(nm) |
| 380 | - | - | 384.3 | 4.3 |
| 400 | 397.8 | 4.2 | 419.4 | 19.42 |
| 440 | 426.8 | 13.1 | 462.7 | 22.7 |
| 480 | 463.8* | 16.2 | 482* | 2 |
| 520 | 505.1 | 14.9 | - | - |
| 560 | - | - | 575.8* | 15.8 |

* Intensity-dependent averaged value

At 60% of correct choices, moths discriminated 560 nm as the rewarded wavelength from both shorter and longer wavelengths. To better understand the differences when compared with the thresholds of 70%, we calculated the quantum catches of receptors produced for all the monochromatic lights of wavelength *λ* and intensity *Io*, presented in combination with the rewarded 560 nm wavelength applying equation 2.3 from Koshitaka et al (2008).

Discrimination of 560 nm from shorter wavelengths was better explained by the use of chromatic information provided by the blue, green, and minimally UV receptors (Table S2, Supplementary material). Discrimination of 560 nm from longer wavelengths, however, could only be explained assuming that moths responded to the achromatic contrast of green receptors (in this spectral range, the input produced by the UV and blue receptors were insignificant). Indeed, reducing the difference between longwave receptors quantum catches hinders discrimination (Table S2, Supplementary material).

Moths could not discriminate between a disk illuminated with 380 nm light and another one illuminated by light of shorter wavelength. Even when the wavelength of the test light was 340 nm (Δλ= 40 nm), the proportion of correct choices was only 46%. Otherwise, when combined with longer wavelengths, moths chose correctly at 20 nm of difference (400 nm). Discrimination thresholds for the rewarded wavelength of 380 nm was Δλ= 3.9 at 70% criterion, and Δλ= 2 nm at 60% (Tables 3 and 4).

**Table 4** Δλ (nm), rewarded and minimum discriminable wavelengths (nm) for both lower and higher distances at the level of 60% of correct choices.

| **Rewarded wavelength** | **Discrimination of shorter wavelength** | | **Discrimination of longer wavelength** | |
|---|---|---|---|---|
| | threshold λ | Δλ (nm) | threshold λ | Δλ (nm) |
| 380 | - | - | 382 | 2 |
| 400 | 390.8 | 9.2 | 416.9 | 16.9 |
| 440 | 428.3 | 11.7 | 460.4 | 20.4 |
| 480 | 467.7* | 12.3 | 481.1* | 1.1 |
| 520 | 509.7 | 10.3 | - | - |
| 560 | 535.5 | 24.5 | 571.2* | 11.2 |

* Intensity-dependent averaged value

All moths trained to the rewarded wavelength of 400 nm presented an unexpected behaviour during test sessions. They consistently preferred shorter wavelengths to the rewarded wavelength of 400 nm, repeatedly visiting unrewarded shorter wavelengths. This behaviour was not observed when 400 nm was presented with longer wavelengths, or when the 400 nm stimulus was paired with a dark disk during training. We did not observe any behaviour related with escape:

moths naturally fed from shorter wavelengths without being previously trained to do it. Because discrimination occurred, for this set of data we used the inverse proportion of correct choices to calculate thresholds (incorrect choices assumed to be correct and *vice versa*. For details of choices see Table S1 in Supplementary material).

### *Theoretical wavelength discrimination thresholds*

Figure 3 shows the comparison of the behaviourally determined Δλ functions, for the thresholds of 70% (Fig. 3a) and 60% (Fig. 3b) and the values predicted by the receptor-noise limited model of colour discrimination (Vorobyev and Osorio 1998). The model correctly predicted the presence of threshold minima near 380 nm, 480 nm and 560 nm, but predicted a threshold maximum around 440 nm, where a dip was observed instead. Behavioural thresholds found at the 60% criterion between 510 and 580 nm agree reasonably well with thresholds predicted by the model.

## Discussion

### *Discrimination minima and comparison with other floral visitors*

Behavioural wavelength discrimination thresholds have been measured only in a small number of insect species (von Helversen 1972; Neumeyer 1992; Fratzer et al 1994; Koshitaka et al 2008; Camlitepe and Aksoy 2010). Among the flower visitors tested so far, *Macroglossum stellatarum* presents minima of wavelength discrimination similar to those of the tetrachromatic butterfly (*Papilio xuthus*) when threshold is set at 60% of correct choices (Fig. 4a) and that of the honeybee (von Helversen 1972) when threshold is set at 70% (Fig. 4b).

Koshitaka et al (2008) suggested that *Papilio xuthus* uses four spectral classes of photoreceptors for colour discrimination. Minima in the Δλ function of this butterfly were found approximately at 430, 480 and 560 nm. At these wavelengths they could discriminate a difference of only 1 nm. At the criterion of 60%, *M. stellatarum* could discriminate a wavelength difference of 1.1 nm at 480 nm and a difference of 2 nm at 380 nm wavelength. Compared with the honeybee, at a criterion of 70% correct choices, *M. stellatarum* could discriminate smaller differences (2 nm) at 480 nm than the minimum of 4.5 nm at 495 nm found by von Helversen (1972), using the same criterion.

Moths trained to associate a reward with a wavelength of 380 nm, discriminated and preferred the illuminated disk compared to the dark unrewarded one, but when confronted in a dual choice task using the rewarded wavelength and shorter wavelengths, they chose randomly. Failure to discriminate colours in the UV range was also found during experiments with dichromatic ants (Camlitepe and Aksoy 2010), which the authors related to the ants' use of UV light for orientation in the habitat and not feeding. However, when UV light was presented to the ants during absolute conditioning, they showed a significant preference for the rewarded UV-illuminated disk (340 nm) over the unrewarded dark disk. The same occurred during our tests. It therefore seems likely that *M. stellatarum*, in earlier experiments, used intensity-related cues when discriminating between 365 and 380 nm stimuli (Kelber and Henique 1999).

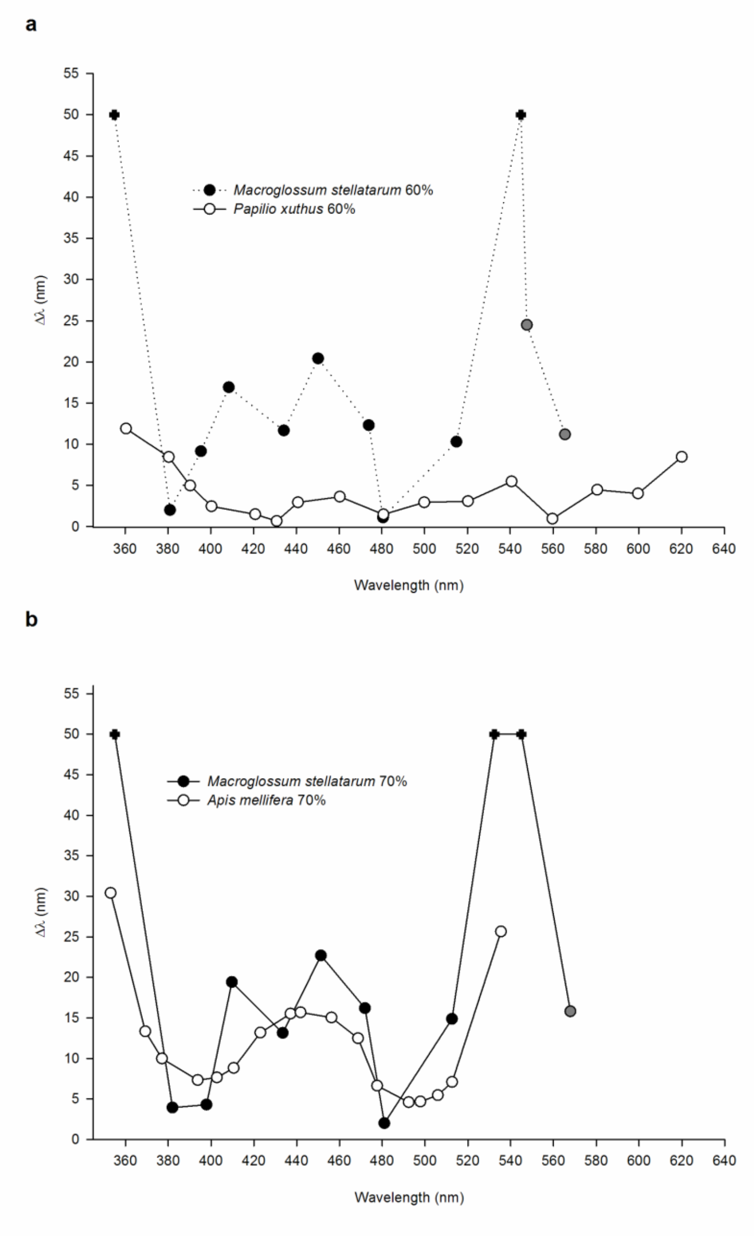

**Fig. 4 The Δλ (nm) comparison between floral visitors.** (a) Δλ (nm) functions of *Macroglossum stellatarum* compared to *Papilio xuthus* established at a criterion of 60% of correct choices, and (b) Δλ (nm) functions of *Macroglossum stellatarum* compared to *Apis mellifera*, determined at a criterion of 70% of correct choices. Crosses represent tested wavelengths for which discrimination did not occur. We used the maximum differences between wavelengths (50 nm) for graphical representation. Grey circles represent wavelength discrimination based on achromatic cues.

### Preference switch

Moths strongly preferred the unrewarded wavelength of 380 nm when offered together with the rewarded wavelength of 400 nm during tests, although during training, moths clearly discriminated between dark and illuminated disks. We know from the spectral sensitive curves (Telles et al 2014) that *Macroglossum* needs higher intensity for the wavelength of 400 nm as compared to 380 nm and 420 nm. Differences in quantum catches between light of 400 nm and shorter novel wavelengths cannot explain the preference for shorter wavelengths. Overall quantum catches of photoreceptors are not better for 380 nm (Table S2, Supplementary material). Our recent study has shown an influence of innate colour preference on spectral sensitivity (Telles et al 2014). Maybe to moths, shorter UV lights can release innate responses associated with foraging behaviour under natural conditions. Despite of this peculiar preference, model predictions agree fairly well with the observed discrimination threshold in this range.

### The use of chromatic and achromatic cues for wavelength discrimination

Although most experiments are centred in the chromatic aspect of wavelength discrimination, we also considered the achromatic capacity of *M. stellatarum* in perform wavelength discrimination as being of relevance. The role of achromatic information on the perception of stimuli cannot be disregarded, as long as achromatic differences can be noticed by the visual system of floral visitors. Evaluating the potential effect of achromatic cues could be interesting to understand the way animals use available information during the foraging activity.

For all wavelengths up to 560 nm we demonstrated that the moths used chromatic cues during discrimination (Table S1 and S2). Generally, wavelength discrimination for longer lights is not measured, because most insects cannot rely on chromatic mechanism to compare stimuli. Here we show that at long wavelengths, to which only a single receptor is sensitive, moths - just as humans - can still discriminate differences, but now based on the achromatic signals, i.e. based on how much darker the light of long wavelengths appears to their eyes.

Despite the significant differences in accuracy between intensities of the rewarded wavelength of 480 nm and shorter (460 nm) and longer (483 nm) wavelengths, moths still relied mostly in chromatic signals to perform discrimination in this spectral range (Table S2, Supplementary material). It is interesting that the achromatic aspect of colour is learned more slowly when chromatic information is available. Kelber (2005) reported that it took several days to train moths do choose the dimmer of two 440 nm lights that differed in intensity by a factor of 10. In another experiment moths trained to discriminate between wavelengths of 590 nm and 630 nm (Kelber and Henique 1999) were faster in comparison with the aforementioned experiment (Almut Kelber personal observation). It seems less difficult for moths to discriminate long wavelengths based only in the available achromatic information, but the task becomes very difficult when moths have to discriminate between wavelengths using the achromatic aspect of lights that also offer chromatic cues.

The $\Delta\lambda$ function for a threshold of 60% better shows the properties of chromatic and achromatic cues used by *M. stellatarum* when discriminating between stimuli. It is not surprising that the hummingbird hawkmoth can use chromatic and achromatic cues depending on the task during foraging activity (Kelber 2003; Kelber 2005; Goyret and Kelber 2012). They also pay less attention on achromatic cues (as differences between light intensities) when the wavelength difference is large enough for chromatic discrimination (Kelber and Henique 1999). Because the moths' accuracy when discriminating lights using achromatic cue has proven to be low (Kelber and Henique 1999; Kelber 2005), when using a threshold of 60%, more Δλ values in the green range could be obtained.

The intensity of colour stimuli corresponds to the achromatic aspect of colour, while the spectral composition, to the chromatic aspect (Kelber 2005). The discrimination between 560 nm and 570 nm wavelengths was clearly based on achromatic cues. Light was more salient when the moth was looking at the 560 nm at high and lower intensities. Although the proportion of correct choices decreased when light intensity was reduced, the absorption of 560 nm light by moths at its lower intensity was still superior to that of 570, 580 and 585 nm novel wavelengths and moths could use the achromatic aspect of the rewarded wavelength to discriminate it from darker lights. Comparing the absorbed number of photons and those minima required to evoke a response (Telles et al 2014), under our experimental setup the wavelength of 560 nm at high intensity was fivefold more intense than the minimum threshold.

In a previous experiment, Kelber and Henique (1999) trained moths to discriminate between lights of two long wavelengths (590 and 630 nm). Both stimuli were adjusted to have equal physical intensities (Kelber and Henique 1999). However, authors noticed that discrimination was based on the achromatic different between lights, since variation in light intensity yielded different performances. When the 630 nm stimulus was presented with a higher intensity than the 590 nm stimulus, moths were unable to choose the correct colour. This result clearly indicates that discrimination was based on the achromatic difference between both stimuli (Kelber and Henique 1999).

In the wavelength discrimination experiments with *Papilio xuthus* (Koshitaka et al 2008) and honeybees (von Helversen 1972), wavelength pairs were presented with the same intensity. Perceived brightness differences were thus not excluded as discrimination cues, but even these species learn chromatic cues better than achromatic cues (Hempel de Ibarra et al 2000; Kinoshita et al 2008; Kinoshita and Arikawa 2014).

## Final remarks

The capacity to discriminate between similar colours has advantages for a flower visitor, especially when the environment is complex and energy demands are high, as in hovering moths (Farina et al 1994; Kelber and Balkenius 2007; Willmer 2011). Moreover, animals must be capable to compare previously stored information with the multiple visual stimuli that are simultaneously present.

We have found that *Macroglossum stellatarum* can discriminate very small wavelength differences in two ranges of the spectrum. Honeybees, that have three receptor types, like *M. stellatarum*, can perform extraordinary discrimination tasks under differential conditioning after extended training periods (Giurfa 2004) or in the presence of an aversive solution (Avarguès-Weber et al 2010), even though they require larger wavelength differences for discrimination than those found for the hummingbird hawkmoth. The butterfly *Papilio xuthus*, with four receptor types, in contrast, can discriminate wavelengths in a broader spectral range with smaller differences than both trichromats.

Differences between discrimination capacities of moths and bees can be discussed from two perspectives: differences in the visual system and differences in the general ecology of solitary hummingbird hawkmoths and eusocial honeybees. Nonetheless, we have to be aware that experiments were not performed under the same conditions and that differences in thresholds may not necessarily reflect real differences in discrimination abilities.

Despite being day-active, *Macroglossum stellatarum* has a superposition compound eye (a design more typical of nocturnal insects) which confers higher absolute sensitivity when compared to apposition compound eyes, as those of *Apis mellifera*. If sensitivity is limited by receptor noise, the visual signal-to-noise ratio can improve with increasing photon catch. Hence, if sensitivity is higher in superposition eyes (because light reaches photoreceptors in each ommatidium through several hundred facets), with the same amount of light, the eye of *Macroglossum* can absorb more photons per integration time and perhaps better discriminate light with lower noise levels than the eye of *Apis mellifera*. Another possibility to explain discrimination differences between both species is related to their general ecology. Because *Macroglossum stellatarum* is a solitary insect, it could have higher motivation to perform better colour discrimination than honeybees (which, after an unsuccessful foraging trip, can return to the hive and feed from reservoirs). This difference is not dependent on the visual system, but on the motivation to perform a task.

It becomes increasingly clear that an animal's ability to exploit information based on the spectral properties cannot be inferred from the mere presence in the eye of photoreceptors with differing spectral sensitivities (White et al, 1994); that potential must be properly exploited by studying the animal behaviour and the neural processing of information.

## Chapter Acknowledgements

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## Supplementary material

Additional supplementary material can be found at the respective section of this thesis.

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# GENERAL DISCUSSION

*Differences in the sensory abilities between floral visitors are ecologically interesting and important.*

Plants exhibit an impressive variability of signals to attract the attention of floral visitors. The colourful, texturized and fragrant market of flowers exists under apparently similar ecological conditions (Barrett and Harder 1996; Barrett 1998), with flowers species not only differing in their nectar and pollen rewards, but also in their appearance (Raine and Chittka 2012), with close relatives reproducing in different ways and with unrelated taxa sharing similar floral adaptations (Barrett 1998; Barrett 2003).

The intimate relationship between the colour of flowers and the visual system of floral visitors provides a series of testable hypothesis. Since the discovery that an important pollinator, the honeybee, could perceive the world in colour (Frisch 1914), and in a fashion different of us (Kühn 1927; Daumer 1956; Autrum and Zwehl 1964), the topic became more and more attractive. Ecologists investigating this relationship are incorporating new vistas, such as the floral visitor capacity to select, recognize, learn and discriminate the most suitable rewarding flower, and the mechanisms behind these capacities.

Particularly important for a proper understanding of the communication between senders and receives is to know basic aspects of each other. In the present thesis, we investigated the visual system of two species: *Bombus terrestris* and *Macroglossum stellatarum*. Experiments were based on behavioural responses under the foraging context. Exceptions were the use of the electroretinogram technique in chapter three, and the additional measured effect of odour in chapter two. Although the aim of the thesis was to explore the visual system of other pollinator species beyond the *Apis mellifera* model, it is worthwhile to compare the results of experiments with similar information available for the honeybee.

Many species with different requirements living in similar and different ecological contexts acquire and process the environmental information according to their capacities and needs. Received signals contain information that forms the basis for decision making, and animals can tune specific channels or modulate the behaviour in different ways to achieve better results. Inferences about the ecology and evolution of the communication between plants and animals are not accurate when the analysis is simply based on the assumed number and peaks of photoreceptors (**chapter three**), because spectral sensitivities and distribution of photoreceptors in an animal's eyes are only one component of colour information processing (Dyer et al 2011); neither by making general assumptions about the mechanisms behind the processing of the visual information (**chapter one**), or the weight animals give to the outputs (chromatic and achromatic cues) of photoreceptors (**chapter one and four**). To predict whether a species is capable of a task, it is necessary to understand how visual signals modulate behaviour under the specific context in which they are operating, and how environmental complexity affects the insect behaviour, as much as sensory signals do (**chapter two**). New insights, as some of those presented in this thesis, enrich the plant-pollinator field of study: first by increasing the knowledge for species that are not well studied (**chapters three and four**); second by offering new explanations and perspectives on questionable general results of basic aspects of the visual system, colour processing and behaviour (**chapters one and two**).

Knowledge of perception and cognition of floral visitors comes mainly from studies with the honeybee, *Apis mellifera*, followed by studies with the bumblebee and to a smaller degree from studies with butterflies, flies and moths. Moreover, decades of research with the honeybee have accumulated a number of consistent information with respect to its visual system, the existence of colour constancy and neural processing of visual information (Frisch 1914; Daumer 1956; Autrum and Zwehl 1964; Menzel 1967; von Helversen 1972; Kien and Menzel 1977a; Kien and Menzel

1977b; Neumeyer 1980; Neumeyer 1981; Menzel et al 1986; Backhaus et al 1987; Menzel and Backhaus 1989; Backhaus 1992; Srinivasan 2011; Giurfa 2012; Hempel de Ibarra et al 2014; Martínez-Harms et al 2014).

Although research using honeybees has made a significant contribution to the study of animal vision and cognition (Sherry and Strang 2014), it remains unclear to what extent these findings can be extrapolated to the rest of Hymenoptera species. Do we expect all bee species to behave in the same way, or to have the same visual and cognitive capabilities? The species-specific differences identified in many studies (Dyer et al 2008; Lunau et al 2009; Townsend-Mehler et al 2011; Moreno et al 2012; Morawetz and Spaethe 2012; Orbán and Plowright 2014; Sherry and Strang 2014) clearly show that the ecological context and hence foraging strategy of a species has a strong impact on the mechanisms of visual information (Menzel 1985; Menzel et al 1988; Menzel et al 1989; Morawetz and Spaethe 2012).

If we simply measure the number of taxa under the same or different environmental conditions that remain to be studied, we can realize that the way to properly understand the relation between plants and floral visitors is long. There are about 250 species of *Bombus* and less than ten *Apis* species worldwide (Sherry and Strang 2014). Yet, most of all the available information about insect colour vision and behaviour has been addressed using basically three Hymenoptera species: the over-represented *Apis mellifera* and, not more than two bumblebees (*B. terrestris* and *B. impatiens*). Most bee species are solitary, a behaviour considered to be the primitive state (Danforth et al 2011), but both honeybees and bumblebees present some level of sociality, either primitive or advanced (Cardinal and Danforth 2011; Danforth et al 2011).

Vision is one of the most important modalities under the foraging context and different species are expected to weight the visual information in different ways. The affirmation was proved to be true in chapter four, when we compared the capacity of discrimination of the solitary *M. stellatarum* with the eusocial honeybee. However, it is easy to understand why studies with social insects are more reliable and so numerous: social insects, as honeybees and bumblebee, have an organized system inside their colonies and can be easily kept under controlled conditions. Indeed, it is thanks to their social behaviour with labour division, including offspring care, and their generalist nature as floral visitors (Sherry and Strang 2014), that workers can be trained to perform tasks under the most important context to them: foraging.

Both honeybees and bumblebees have a trichromatic colour vision system based on ultraviolet, blue and green photoreceptors (Peitsch et al 1992; Skorupski and Chittka 2010a), with similar spectral sensitivities (Peitsch et al 1992). In addition, both species learn to associate a colour with a reward (Dyer and Chittka 2004c; Giurfa 2004; Wertlen et al 2008; Reser et al 2012; Hempel de Ibarra et al 2014). Although honeybees (*Apis mellifera*) and bumblebees (*Bombus terrestris*) are typical in terms of photoreceptors (Peitsch et al 1992; Briscoe and Chittka 2001), behavioural and visual differences between these species were found under the foraging context, when detecting and discriminating either dissimilar or similar colours (Dyer et al 2008).

A number of reasons for the observed differences in colour discrimination and object detection between bumblebees and honeybees are provided by Dyer et al (2008), among which are differences in the distribution of the three photoreceptor classes within the ommatidia, differences in the spatial summation of photoreceptor signals, which affects visual acuity, and differences in colour coding neurons (Dyer et al 2008). In their overall hypothesis, the authors highlight visual acuity differences between species as being more relevant in the studied context (Dyer et al 2008). Visual acuity has been demonstrated to present substantial variance among and across insect eyes, a finding which is ultimately associated with the lifestyle and ecology of species (Land 1997). Bumblebee eye resolution is better than that of the honeybee (Macuda et al 2001) and the processing properties of their visual pathways may also differ (Dyer et al 2008; Wertlen et al 2008). Honeybee has good colour discrimination capacity but poor colour detection capabilities, whereas for bumblebees it is the other way around (Dyer et al 2008).

Honeybees and bumblebees also differ in attentional processes. Morawetz and Spaethe (2012) found that selective attention differently influenced bumblebee and honeybee behaviours. When examining the impact of distracting visual information on search performance, they found that bumblebees were significantly less affected by distracting objects than honeybees (Morawetz and Spaethe 2012). The authors also measured the strategy applied by both species when solving the proposed task and identified a speed–accuracy trade-off: whereas bumblebees displayed slow but correct decision-making, honeybees exhibited fast and inaccurate decision-making (but see Burns 2005 and Burns and Dyer 2008). Honeybees deploy a more impulsive search, while bumblebees are less risky and more accurate in their choices (Morawetz and Spaethe 2012).

The species also differ in the choice of plant taxa they select for visiting even under the same conditions. Leonhardt and Blüthgen (2012) placed colonies of both bumblebees and honeybees at the same habitat with the same resource abundance and heterogeneity. They found consistent differences across colonies in the species' foraging patterns, with bumblebees collecting pollen from at least twice as many plant species than honeybees, resulting in little overlap between explored plants (Leonhardt and Blüthgen 2012). The conclusion of the authors is based on the capacity of *Bombus* to recognize the quality of the floral resource (pollen). Bumblebees have the ability to perceive and judge food quality and optimize their foraging behaviour accordingly. Thus they manage to collect pollen with significantly higher pollen protein content and more essential amino acids. Honeybees tend to exploit large resource patches, focusing on quantity instead of quality (Leonhardt and Blüthgen 2012). In this context, bumblebees and honeybees clearly exploit different plants, which could be taken as indirect evidence that resource partitioning occurs between species. However, it remained unknown whether flower colour or shape also influences the selective detected behaviour.

Plants and their floral visitors have to communicate in order to have a mutual benefit. To understand the communication system stablished between a flower and its pollinator, we need to address the question of how reliable the communication system is, how receivers perceive the visual stimulus and discriminate it from the rest. Several colour vision models have been proposed to answer these questions. However, the predictions of models have been tested only few times against the behavioural response of animals (chapter one). These models incorporate sensory information and follow calculations based on experimental (Backhaus 1991; Vorobyev and Osorio 1998; Vorobyev et al 2001) or theoretical analyses (Chittka 1992). Although the prediction of models can be consistent under certain circumstances and for a specific subset of coloured stimuli, they can also contrast from each other (Vorobyev and Brandt 1997; Brandt and Vorobyev 1997; Telles and Rodríguez-Gironés 2015)(Vorobyev and Brandt 1997; Brandt and Vorobyev 1997; Telles and Rodríguez-Gironés 2015)(Vorobyev and Brandt 1997; Brandt and Vorobyev 1997; Telles and Rodríguez-Gironés 2015), failing to correctly predict the detection and discrimination performance of bees (chapter one).

In chapter one, we showed that bumblebee performance, during a colour discrimination task, only reasonably agrees with the predictions obtained using the colour opponent coding model (COC, Backhaus 1991) and the receptor noise limited model (RN, Vorobyev and Osorio 1998; Vorobyev et al 2001), while the colour hexagon model (Chittka 1992) failed to predict discrimination (Telles and Rodríguez-Gironés 2015). According to the overall predictions, bumblebees followed a general pattern: when the distances between stimuli increase, the easiness of differentiability also increases, but the relationship between discrimination ability and predicted perceptual distance was not monotonic. Also, not only chromatic differences between stimuli, but also achromatic signals provided by the use of the green receptor, and the intensity between stimuli, significantly affected the bumblebee behaviour (Telles and Rodríguez-Gironés 2015).

The differences in the predictions of models found in chapter one, can be discussed here by the differences in the models' assumptions and formulation. Despite the fact that both models (COC and RN) are based on experimental evidences, they were not specifically designed to represent different species and sets of colours, although the RN model claims to be more general.

The COC model was proposed after some behavioural (Backhaus et al 1987) and neurophysiological data (Kien and Menzel 1977a; Backhaus and Menzel 1987) had been collected for the honeybee. Backhaus (1991) used the results from a multidimensional scaling (MDS) experiment performed by training individual bees to one out of twelve colour stimuli from the same blue-green region (for more details see Backhaus et al 1987). These results were generalized to colours not originally included in the MDS experiment. Also, the colour opponent mechanism applied in the model was inferred from early results of neurophysiological experiment (Kien and Menzel 1977a) where only two types of neurons were found (for a critical comment see Chittka and Kevan 2005). Today, it is known that bees possess several types of colour opponent neurons distributed along different regions in the bee brain (Yang et al 2004), but the implication of these regions in chromatic information processing is still not clear (Mota et al 2013). It is premature to conclude that the colour difference formula applied in the MDS analysis and the initial set of neurons found for the honeybee are the same for all Hymenoptera species and colours. The validity of this statement would have to be demonstrated by testing different species with different samples from their colour space, a further project that needs to be addressed. There may be significant differences between species in the way the information is acquired and processed, and only by measuring the behaviour of the animal in question we can truly validate predictions, and increase our general understanding of animal colour perception.

The RN model is a more general model, accounting for all types of photoreceptor interactions, as long as information about the specific receptor noise is known, but receptor noise is difficult to quantify and thus this information is not available for many species (Vorobyev et al 2001; Skorupski and Chittka 2010b). The model was designed to explain and predict near-threshold detection of colour. However, following common usage, we used it to compare colours with distances varying from below-threshold to above-threshold discrimination of bees (Dyer and Chittka 2004c; Dyer and Chittka 2004a). The model assumes that colour discrimination is constrained by the noise of the photoreceptors. If the discrimination performance is limited only by receptor noise, the distance in a colour space does not depend on the specific coding of the receptor signals (Osorio and Vorobyev 1996). This model, as the COC and the CH, does not predict detection or discrimination when luminance mechanisms (achromatic signals) are of relevance (Vorobyev and Osorio 1998), and contrary to the COC, the nature of the colour opponent coding mechanisms that compare receptor signals is ignored.

Since both the COC and RN models gave similar predictions of discrimination for our tested pairs (chapter one), we cannot claim either models' assumptions to be incorrect, but instead, to be incomplete. Regardless of whether perceptual distances are determined by the nature of colour opponent mechanisms, receptor noise or a combination of both, the role of achromatic cues in discrimination tasks has to be tested during behavioural experiments. Finally, it is without a doubt that colour vision models have increased our understanding of the ecological significance of floral colour and vision of floral visitors (Chittka 1996a; Chittka 1997; Chittka and Waser 1997). But we believe that general assumptions are valid when questions come from a general context, given the complexity of visual perception, models' prediction cannot be taken as true when we want to test specific mechanisms, or account for specific evolutionary relationships. Models are useful tools, which can be applied more accurately when we understand the basis of the visual system they are modelling. More validation involving ecologically realistic tasks are necessary and in the chapter one of this thesis, we presented the first attempt to contribute in this direction, showing a methodology that can be improved and easily be extended in future experiments.

Most natural scenes are set against visually complex backgrounds and flowers must show salient features in order to be detected. Despite the importance of backgrounds on the detection of stimuli, there are surprisingly few experiments testing the effect of background on the floral visitor behaviour during a foraging activity (Hempel de Ibarra et al 2000; Forrest and Thomson 2009). In this context, we designed the third chapter of this thesis. We expect a communication in multiple modalities to be beneficial when environment is noisy or when visual stimuli are not salient, because the presence of different signals directed to different sensory channels supposedly reduce the perceptual errors of receivers under a complex scenario (Rowe 1999). Multimodal stimulation is common in plant-animal communication. Flowers usually send multiple signals or cues in different sensory modalities, such as vision and olfaction (Schaefer and Ruxton 2011). Many flowers are coloured, and just as many are scented (Dobson 1994; Raguso 2008b).

Chemical compounds present in many floral scents evolved earlier than flowers (Schiestl 2010). The theory is that plants exploited the communication system among insects by using those compounds that insects were already pre-adapted to respond to (Schiestl 2010), just as they did by investing in flower colouration (Chittka 1996b). Although the final message (“visit me”) is essentially the same, vision and olfaction differ in many ways (Chittka and Brockmann 2005). It is not well understood how floral visitors perceive multimodal stimuli in a general context (Kunze and Gumbert 2001; Balkenius et al 2006; Balkenius and Kelber 2006; Raguso 2008b; Raguso 2008a; Goyret et al 2009; Raguso 2009; Leonard et al 2011; Katzenberger et al 2013; Leonard and Masek 2014), nor how efficiently the signal can be transmitted and used in a complex visual environment.

A simple way to study the weight given by an animal to different sensorial modalities is to combine them in preference tests or learning experiments (Kelber and Balkenius 2007). In chapter two, we demonstrated how colour and odour modulated the behaviour of bumblebees, and how receivers relied on one or both cues when stimuli were presented in a complex background. It turned out from our experiment that, bumblebees are able to perceive and strongly rely on both cues when stimuli are not salient against the complex background, as it is the case for red flowers. In the absence of scent, the amount of training also makes bees improve detection. Bees are known to rely on flower colour for the detection of stimulus by evaluating the green contrast of the target flower against the background (Lehrer and Bischof 1995). Thus, against a complex background with stimuli differing in conspicuousness, chromatic contrast is more important for detecting flowers, independent of the size (chapter two). When background is invariant, as in homogeneous backgrounds, colour contrast and flower size are positively correlated with detection time (Spaethe et al 2001). But for bumblebees, the detection of big red flowers resulted difficult in our complex scenario. These flowers presented the smallest chromatic contrast with the background, and although achromatic contrast exceeded that of blue flowers, it is not comparably efficient for short distance detection (Hempel de Ibarra et al 2001), especially for large surfaces. Complex backgrounds directly affect the detection of stimuli by increasing the noise fluctuation (Goulson 2000) and have been demonstrated to modulate the bee behaviour and preferences (chapter two and Forrest and Thomson 2009).

Overall, the role of olfactory cues on floral visitor behaviour is not as well-known as the role of visual cues (Raguso 2008a; Riffell 2011; Schaefer and Ruxton 2011). Yet, most pollinators use both visual and olfactory signals to locate and identify flowers (Goyret et al 2007; Katzenberger et al 2013; Riffell and Alarcón 2013), with floral discrimination being enhanced by the presence of scent (Giurfa 1994; Kunze and Gumbert 2001), uncertainty about the rewarded colour being reduced (Leonard et al 2011) and floral constancy increased (Gegear 2005; Gegear and Laverty 2005). The benefit of using both scent and colour to locate flowers was also apparent under changing environmental conditions. After training bumblebees to discriminate rewarding and unrewarding flowers that differed in colour and scent, and testing the bees at different levels of illumination, Kaczorowski et al (2012a) found that accuracy declined among bees when flowers were unscented, but not among bees whose flowers differed in both colour and scent (Kaczorowski et al 2012a). Communication systems are very often studied in isolation, but floral cues are presented in conjunction with other floral signals. The relative importance of one signal over another during flower detection is more likely to be variable across pollinators (Schaefer and Ruxton 2011).

As we have seen, the perception of a stimulus is affected by the complexity of the background, but such complexity can also be compensated by the conspicuousness of the stimulus or by the presence of a second cue. Floral odour is important when the visual task is difficult (Giurfa 1994), as it was for our tested bumblebees exploiting UV-absorbing red flowers as well as it might be for the UV-reflecting white flowers naturally visited by bumblebees (Chittka and Waser 1997; Lunau et al 2011). Bumblebees are going to adjust their behaviour to facilitate target detection and discrimination, and they might make use of all available sensory inputs, as long as they can enhance the foraging activity. In addition, we also have demonstrated that odour information previously acquired can be used during a novel task in a complex background. Familiar odours are known to trigger navigational and visual memories in experienced bees when the trained scents are blown into the hive (Reinhard et al 2004), but how learned scent acts during a novel learning task had not been tested. During the novel task experiment in chapter two, bumblebees trained with blue and red flowers in the presence of odour had a high proportion of correct choices and spent less time looking for the novel flowers than those bees trained with the same blue and red colours, but in the absence of odour. It seems that learning to combined colour–odour cues led to a better memory formation and retrieval compared with single modality (Kulahci et al 2008) and that in the absence of the trained colour, odour acts facilitating detection and discrimination of novel colours presenting the same odour.

By far, bees are arguably the most important group of angiosperm-pollinating insects, comprising nearly 20.000 described species (Danforth et al 2011). But a broad range of angiosperm taxa are pollinated by insects other than the bees (Coleoptera, Diptera, and Lepidoptera), and in comparison, much less is known about the sensory attributes and learning abilities that guide their behaviours (Weiss 2001). In comparison with social insects, raising and studying solitary insects is simply more difficult, and motivating them to perform behavioural tasks is a challenge as they do not have to constantly provide food to their congeneric. We can speak for ourselves. We have tried to perform colour discrimination experiments with two species of Syrphids (*Sphaerophoria rueppellii* and *Eristalis tenax*), and one solitary bee (*Osmia caerulescens*) as part of the project in which this thesis was framed. The whole process of data acquisition was excessively slow, time-consuming and laborious, and the data were exceedingly noisy, so we had to abandon the experiments.

Some Lepidoptera species have been studied more extensively in the last 20 years, specially butterflies, who are now considered an important group of subjects in the study of insect vision (for a revision see Kinoshita and Arikawa 2014). But most of the lepidopteran families are moths (Stavenga and Arikawa 2006), which are relatively underrepresented in the literature (Cutler et al 1995; Kelber and Henique 1999; Raguso and Willis 2002; Goyret et al 2008; Goyret et al 2009; Kaczorowski et al 2012b). Most species in the Lepidoptera order appear to possess UV, blue, and green receptors with limited variability in wavelength positioning, although there is one intriguing difference in comparison with the Hymenoptera (Briscoe and Chittka 2001): while only a few species of bees and wasps have red receptors, such receptors are far more common in the Lepidoptera, which in turn, is not correlated neither with diurnal nor nocturnal lifestyles (Briscoe and Chittka 2001; Arikawa 2003; Stavenga and Arikawa 2006; Kinoshita and Arikawa 2014). For instance, the diurnal hummingbird hawkmoth, *Macroglossum stelattarum*, possesses sensitivity in the ultraviolet, blue and green areas of the visible spectrum, which are comparable with its crepuscular and nocturnal relatives, the *Manduca sexta* and *Deilephila elpenor*.

The first step in interpreting the effect of a visual signal on the floral visitor is to know the receptor types of the animal in question and their sensitivity. Built on this fact, we developed the chapter three. There are pronounced differences between species (and sometimes individuals) and the use of a surrogate visual system sensitivity to explain another one is a risky endeavour, but very often undertaken. Previous experiments have shown cognitive and visual capacities of *M. stellatarum* (Kelber 1996; Kelber 1997; Kelber 2002; Kelber 2005) and now, information about the peaks and sensitivity of photoreceptors are known (chapter three).

Spectral sensitivity is given by the minimum intensity of monochromatic light that can be detected, with sensitivity being the inverse of threshold intensity (Kelber et al 2003). In chapter three we tested the sensitivity of *Macroglossum*'s photoreceptors and showed that the sensitivity of the moth is comparable to those of the bees and relative moth species, but their capacities to sense colours are higher (Telles et al 2014). During analyses, neither electroretinograms measurements, nor the model prediction of sensitivity could describe and predict the behaviour of moths. Nonetheless, because receptor noise of moths was not measured, the robustness of the model prediction cannot be totally questioned. What we have learned from this experiment is that differences in colour sensitivity cannot necessarily be explained by the number of photoreceptors and their spectral sensitivities. Behaviour needs to be studied to accurately understand the relationship between animal vision and the performed task (Menzel 1985; Telles et al 2014). The scenario can change depending on whether moths are tested under the mating paradigm or in the oviposition context (Kelber 1999). For instance, *M. stellatarum* seems to give more weight to vision during the foraging activity, what is related to our findings of extra spectral sensitivity in the blue range, ignoring odour during a first choice, while the nocturnal relative, *Deilephila elpenor*, respond preferably to the odour (Balkenius et al 2006), despite its ability to discriminate colours in very dim light (Kelber 2003), and the crepuscular *Manduca sexta* requires both visual and olfactory stimuli to release a feeding behaviour (Raguso and Willis 2002), although the colour is the ultimate indicator of a nectar source (Goyret et al 2007).

Following the same rationality, for instance, *Melipona quadrifasciata* learns colours at the feeding place faster and more accurately than when the same task has to be performed at the hive entrance, where performance is slower and less accurate (Menzel et al 1989). The authors of this experiment conclude from the findings an existence of context-specific colour differences in discrimination: *Melipona* discriminates UV-blue colours better at the feeding place than at the hive entrance. Moreover, at the hive entrance the best discriminated colours were those from the blue-green region of the spectrum (Menzel et al 1989). These differences cannot result from differences in photoreceptors signals, because exactly the same training and test procedure were applied (Menzel et al 1989). Differences were a clear result of how information was weighted under a specific motivational state.

Although inferences about an animal's vision and its capacity of discrimination can be done by measuring its spectral sensitivity, additional evidence is required to show that two or more photoreceptor spectral classes are actually involved in colour discrimination (Kelber et al 2003), and these assumptions frame the rationality of the forth chapter of this thesis. The weight an animal gives to changes in the information contained in the light that reaches the eyes, after being coded, implies indirect information about the number and use of photoreceptors during the task.

Translating the sentence to a more ecological context: the capacity of an animal to detect and discriminate flowers on the basis of reflected wavelengths, independent of potential brightness differences, is the result of having colour vision.

Colour discrimination requires the presence of at least two types of photoreceptors with different (but overlapping) spectral sensitivities (Skorupski and Chittka 2011), and opponent (chromatic) interactions between them (as explained in the introduction to this thesis). Thus, to specify the quality of a visual system with a particular set of photoreceptors, one needs to estimate the number of different signals the animal can discriminate for a specific threshold (or likewise find indistinguishable signals) (Vorobyev and Menzel 1999). With this information we can compare minima and maxima values with those of animals with similar and different sets of photoreceptors, tested in the same behavioural context.

*Macroglossum stellatarum* strongly relies on visual information for feeding (Kelber and Pfaff 1997; Balkenius et al 2006; Goyret and Kelber 2012). The ability of the moth to discriminate between dissimilar lights have been previously tested (Kelber and Henique 1999). Kelber and Henique (1999) demonstrated that moths possess true colour vision by discriminating dissimilar lights in the range of UV, blue and blue-greenish areas of the spectrum (Kelber and Henique 1999). Distances between monochromatic lights were not short (20 nm in the UV and 30 nm for the aforementioned ranges), and moths performed well when discriminating between them. In chapter four we tested the discrimination ability of *Macroglossum stellatarum* for dissimilar as well as similar perceptual colours. When distances were of 20 nanometres moth performed well just as they did in the previous experiment of Kelber and Henique (1999). With one exception that in our setup moths could not discriminate a wavelength light of 380 nm from short wavelengths up to 340 nm (chapter four). Despite the fact that authors of the previous experiment adjusted lights to have the same intensity (quanta per second per square centimetre), information about the real differences between intensities for the moth (measured as quantum catches), were not analysed, because spectral sensitivities were unknown (Kelber and Henique 1999). We believe moths relied on achromatic information to perform discrimination between 380 nm and 360 nm in the earlier experiment (Kelber and Henique 1999).

Using monochromatic lights and varying intensities, we found (chapter four) that *M. stellatarum* can discriminate very small wavelength differences in two ranges of the spectrum (UV and blue areas). Among the flower visitors tested so far, *Macroglossum stellatarum* presents minima of wavelength discrimination comparable to those of the tetrachromatic butterfly *Papilio xuthus* (Koshitaka et al 2008) and that of the honeybee (von Helversen 1972), when thresholds of discrimination were adjusted to be equal for the different species (chapter four). Comparing the curves of discrimination, *M. stelattarum* seems to be more accurate than the honeybee. However, we cannot claim that the differences in thresholds reflect real differences in discrimination abilities. It must be considered that experiments were not performed in the same setup and, although animals were tested in the feeding context, that they could present different motivational state.

The intensity produced by a colour stimuli corresponds to the achromatic aspect of colour, while the spectral composition corresponds to the chromatic aspect (Kelber 2005). *Macroglossum stellatarum* is capable of using both visual cues to discriminate between monochromatic lights (chapter four and Kelber 2005). Surprisingly and despite the fact that insects tested so far use achromatic cues to detect and even discriminate flowers (Hempel de Ibarra et al 2000; Hempel de Ibarra et al 2002; Telles and Rodríguez-Gironés 2015), experiments testing the ability to used achromatic information during a discriminatory task are few and incomplete. The use of achromatic information when performing an important task such as the discrimination between rewarding and unrewarding flowers seems plausible in a natural context. As long as the visual information is available and the visual system capable of perceiving it, making use of chromatic or achromatic cues seems a valid option (Giurfa et al 1997; Kelber 2005; Telles and Rodríguez-Gironés 2015).

Several results of the present thesis offer new insights about insect vision (chapters three and four), adding new perspectives (chapter two) to old and controversial questions (chapters one). The impact of insect pollinators on flower colour can be affected by their visual capacity (Thairu and Brunet 2015). While the assessment of details of floral colour (as spectrometry) and photoreceptor number and type is straightforward (Peitsch et al 1992; Briscoe and Chittka 2001; Kelber et al 2003), experimental work about insect colour vision and behaviour is available for only a few species (Lunau 1991; Peitsch et al 1992; Kelber 1996; Stavenga and Arikawa 2006; Hempel de Ibarra et al 2014; Kinoshita and Arikawa 2014; Lunau 2014). A better understanding of the visual system of non-model pollinators (i.e., pollinators other than *Apis mellifera*) could add considerable explanatory power to the observation of flower colour variation in many plant species (Van Der Niet et al 2014). Also it might be helpful for the understanding of the evolution and adaption of certain flower colours in specific environments (Chittka 1997; Campbell et al 2010; Dyer et al 2012; Bischoff et al 2013; Shrestha et al 2014; Dyer et al 2015). Although similarities in the way insects acquire (photoreceptor input level) and process (spectral opponency at the neural level) visual information have been found, this does not imply that colour vision is based on similar neural strategies, resulting in similar perception of the environment (Menzel and Backhaus 1991). Thus, it is worthwhile to study and compare the colour vision system of different species.

If we want to move on to the next level of complexity in understanding the relationship between plants and floral visitors' sensorial system, it requires to include the plasticity, environmental adaptations and realistic information about the sensory system and behaviour of insects under different contexts (Hebets 2011; Dyer 2012b; Dyer et al 2015; Kemp et al 2015).

Insect eyes and neural processing of information are well understood for few species only. The way other flower visitors work, can drastically differ depending on the context. Insects are flexible and capable to modulate their behaviour according to the task. With the exception of some very basic reflexes, it is still not possible to link an insect's visual input to its behavioural output (Borst 2009). By extrapolating information from well-characterized model organisms, we will lose analytical power, given the complexity of visual perception (Kemp et al 2015).

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# CONCLUSIONES GENERALES

1. Los modelos de visión en color pueden predecir diferentes distancias perceptuales para un mismo conjunto de estímulos. Estos mismos modelos no pueden describir con precisión la capacidad de los abejorros (*Bombus terrestris*) para discriminar entre colores perceptualmente similares. Dado que los modelos de visión en color son herramientas poderosas, un mejor entendimiento sobre las limitaciones de cada modelo, y las diferencias sensoriales entre especies, se hace necesario antes de decidir qué modelo utilizar a la hora de hacer inferencias sobre la capacidad de discriminación o percepción de un visitante floral.

2. Los colores de las flores ofrecen información valiosa y estudios empleando ensayos de comportamiento sobre cómo los visitantes florales usan la información espectral durante una tarea relevante para ellos, como la alimentación, puede ayudar a aumentar la información para diferentes especies, así como validar las predicciones y observaciones derivadas de especies modelo, como *Apis mellifera*. Se hace necesario entender cómo la habilidad de las abejas para discriminar entre flores y el tiempo que requieren para realizar esta tarea, dependen de las propiedades espectrales de las flores, de los mecanismos de aprendizaje y de la flexibilidad comportamental de cada especie.

3. Las flores usan señales visuales y olfativas para anunciar su presencia. Si una flor es conspicua, en términos de visión, los abejorros pueden fácilmente encontrarla en un fondo complejo, dispongan o no de fragancia. La fragancia floral es importante cuando la tarea visual es difícil. Cuando explotan flores inconspicuas en un fondo complejo, los abejorros son menos precisos, aunque el rendimiento puede aumentar con la experiencia, o con la presencia de una fragancia.

4. Una fragancia familiar parece desencadenar un proceso de atención durante la adquisición de nueva información en los abejorros, aumentando el rendimiento y ayudando a reducir el tiempo empleado al buscar por nuevas flores.

5. El estado motivacional de una especie y sus preferencias innatas por determinados colores, parecen superar la sensibilidad espectral medida al nivel fisiológico. Por ejemplo, *Macroglossum stellatarum* parece pesar la información visual de una manera distinta cuando los datos de electrofisiología y comportamiento son comparados.

6. Los visitantes florales ajustan su comportamiento para facilitar la detección y discriminación de flores, pudiendo hacer uso de toda la información sensorial disponible, siempre que resulte ventajoso durante la actividad de forrajeo. Una revisión sobre el uso de información acromática durante el desarrollo de una tarea relevante para especies con diferentes estilos de vida y capacidades visuales se hace recomendable.

7. Las generalizaciones sobre los principios considerados fundamentales para la visión en color y el procesamiento de la información por los insectos deben ser puestos a prueba con diferentes especies, así como deben realizarse esfuerzos para adquirir información fundamental para otras especies de visitantes florales durante tareas realizadas en la naturaleza. La comprensión de la forma en que los visitantes florales perciben y aprenden sobre su mundo puede ayudar a hacer inferencias acerca de la evolución de la diversidad de señales en las flores.

# GENERAL CONCLUSIONS

1. Models of colour vision can predict different perceptual distances from the same set of stimuli. These models cannot accurately describe the capacity of bumblebees (*Bombus terrestris*) to discriminate between perceptually similar colours. Because colour vision models are powerful tools, a better understanding of the limitations of models and the sensorial differences between species is necessary before deciding which model to use when making inferences about the capacity of discrimination and perception of a floral visitor.

2. Flower colours offer valuable information and studies employing behavioural assays about how floral visitors use spectral information during a relevant task, as foraging, help to increase the information for other species, as well as to validate predictions and observations derived from model-species, as *Apis meliffera*. It is necessary to understand how the ability of pollinators to discriminate between flowers and the time that they require to accomplish this task, depend on the spectral properties of the flowers, the learning mechanisms and the behavioural flexibility of species.

3. Flowers use visual and olfactory cues to advertise their presence. If a flower is salient, in terms of vision, bumblebees can readily find it in a complex background, regardless of the presence or absence of scent. Floral scent however is important when the visual task is difficult. When exploiting inconspicuous flowers against a complex background, bumblebees are less accurate, but performance can increase with experience or with the presence of scent.

4. A familiar floral scent seems to trigger bumblebee attention during acquisition of new information, increasing the accuracy of bees when looking for novel flowers, as well as helping to reduce the search time for the novel target.

5. The motivational state of a species and its innate preferences for certain colours seems to overcome the spectral sensitivity measured at the physiological level. For instance, *Macroglossum stellatarum* seems to weight colour information in a different way, when electrophysiological and behavioural data are compared.

6. Floral visitors adjust their behaviour to facilitate flower detection and discrimination, and they make use of all available sensory inputs and neural pathways, as long as they can enhance the foraging activity. A revision of the use of achromatic information during a relevant task for species with different lifestyles and visual capacities is recommended.

7. Generalizations about principles considered as fundamental to colour vision and information processing for insects, must be tested with different species. Additional efforts are required to acquire fundamental information for different floral visitors and commonly performed tasks in nature. Understanding the way floral visitors perceive and valuate the world can help to ultimately make inferences about the evolution of floral signals.

# SUPPLEMENTARY INFORMATION

**Supplementary material 1**: Calculating Perceptual Distances, Colour and Achromatic Contrasts

The quantum catch of a photoreceptor, $q_i$ (where i = UV, B or G), is an estimate of the number of impinging photons that the photoreceptor absorbs under specific illumination conditions. If $R_i(\lambda)$ is the spectral sensitivity of type-i photoreceptors, then its quantum catch is given by:

$$q_i = k_i \cdot \int_\lambda R_i(\lambda) \cdot I(\lambda) \cdot D(\lambda) \cdot d\lambda, \tag{1}$$

where $I(\lambda)$ and $D(\lambda)$ are the reflectance spectrum of the stimulus and the spectral distribution of incident light, respectively, and $k_i$ is a scaling factor chosen so that quantum catches equal 1 for the background spectrum, $I_b(\lambda)$, to which photoreceptors are adapted:

$$k_i = \frac{1}{\int_\lambda R_i(\lambda) \cdot I_b(\lambda) \cdot D(\lambda) \cdot d\lambda} \tag{2}$$

For the colour hexagon, CH (Chittka 1992), and colour-opponent coding, COC (Backhaus 1991) models we must also calculate the excitation level of photoreceptor neurons, $E_i$, according to the equation

$$E_i = \frac{q_i}{1 + q_i} \tag{3}$$

## COC distance

To calculate the colour distance between two stimuli according to the COC model, for each colour of the pair we first calculate the colour-opponent mechanisms (Backhaus 1991) A and B

$$A = -9.86 \cdot E_{UV} + 7.70 \cdot E_B + 2.16 \cdot E_G \tag{4}$$

$$B = -5.17 \cdot E_{UV} + 20.25 \cdot E_B - 15.08 \cdot E_G \quad (5)$$

If colours 1 and 2 produce excitation values ($E_{UV1}$, $E_{B1}$, $E_{G1}$) and ($E_{UV2}$, $E_{B2}$, $E_{G2}$), from which we can compute ($A_1$, $B_1$) and ($A_2$, $B_2$) using equations (4) and (5), then the perceptual colour distance between colours 1 and 2 according to the COC model, $d_{12}$, is given by (Backhaus 1991)

$$d_{12} = |A_1 - A_2| + |B_1 - B_2| \quad (6)$$

## CH distance

From the photoreceptor excitations (equation 3), we calculate the hexagon coordinates X and Y,

$$X = \sqrt{3} \cdot (E_G - E_{UV})/2 \quad (7)$$

$$Y = E_B - 0.5 \cdot (E_{UV} + E_G) \quad (8)$$

The CH distance between two colours, $E_{12}$, is the Euclidean distance between the loci of the colours on the hexagon (Chittka 1992):

$$E_{12} = [(X_1 - X_2)^2 + (Y_1 - Y_2)^2]^{1/2} \quad (9)$$

## RN distance

To obtain the RN perceptual distance we do not need to obtain the loci of the two colours of a pair on a hypothetical colour space. Rather, the RN model assumes that perceptual distances are determined by the level of noise at the different photoreceptor channels, $e_i$. For the particular case of *Bombus terrestris* habituated to daylight, these parameters are $e_{UV} = 0.74$, $e_B = 0.67$ and $e_G = 0.61$ (see Fig. 3c of Skorupski and Chittka 2010). With these noise parameters and the quantum catches, the perceptual distance, $(\Delta S^t)^2$, between colours 1 and 2 is given by

$$(\Delta S^t)^2 = \frac{{e_{UV}}^2 \cdot (\Delta q_G - \Delta q_B)^2 + {e_B}^2 \cdot (\Delta q_G - \Delta q_{UV})^2 + {e_G}^2 \cdot (\Delta q_{UV} - \Delta q_B)^2}{(e_{UV} \cdot e_B)^2 + (e_{UV} \cdot e_G)^2 + (e_B \cdot e_G)^2} \quad (10)$$

Two versions of this model have been proposed, corresponding to different definitions of the Δq. For the linear version (Vorobyev and Osorio 1998),

$$\Delta q_i = q_{2i} - q_{1i} \tag{11}$$

while for the logarithmic version (Vorobyev et al 2001),

$$\Delta q_i = \log(q_{2i}) - \log(q_{1i}) \tag{12}$$

## Calculating COC from CH Distances

It is possible to express the colour-opponent mechanisms A and B of the COC model (equations 4 and 5) as linear combinations of the colour-opponent mechanisms X and Y of the CH model (equations 7 and 8). Specifically,

$$A = 6.94\cdot X + 7.70\cdot Y \tag{13}$$

$$B = -5.72\cdot X + 20.25\cdot Y \tag{14}$$

Given P = (x, y), let Q = (x’, y’) be a point a distance r from P, at an angle α with the vertical axis. The coordinates of Q on the (X, Y) plane of the colour hexagon are therefore

$$x' = x + r\cdot\sin(\alpha) \tag{15}$$

$$y' = y + r\cdot\cos(\alpha) \tag{16}$$

From equations 13 and 14, the A and B colour-opponent mechanisms for points P and Q are:

$$A_P = 6.94\cdot x + 7.70\cdot y \tag{17}$$

$$B_P = -5.72\cdot x + 20.25\cdot y \tag{18}$$

and

$$A_Q = 6.94\cdot(x + r\cdot\sin(\alpha)) + 7.70\cdot( y + r\cdot\cos(\alpha)) \tag{19}$$

$$B_Q = -5.72\cdot(x + r\cdot\sin(\alpha)) + 20.25\cdot( y + r\cdot\cos(\alpha)) \tag{20}$$

From equation 6, it follows that

$$d_{PQ} = r\cdot(|6.94\cdot\sin(\alpha)+7.70\cdot\cos(\alpha)| + |-5.72\cdot\sin(\alpha)+20.25\cdot\cos(\alpha)|) \qquad (21)$$

## Calculating RN from CH Distances

A given point on the CH plane corresponds to infinitely many different reflectance spectra (essentially, the same hue with different brightness; Chittka 1992). To calculate the correspondence between CH and RN distances we must therefore make additional assumptions. In particular, we will assume that colours (both P and Q (α)) are chosen in such a way that

$$E_{UV} + E_B + E_G = 1 \qquad (22)$$

For each point (X, Y) on the colour hexagon, this constraint, together with equations 7 and 8, define a system of three linear equations with three unknowns ($E_{UV}$, $E_B$ and $E_G$) which can be solved for the photoreceptor excitation values. In other words, given the coordinates of a point on the colour hexagon, equation 22 allows us to calculate the photoreceptor excitation values and, from equation 3, the corresponding quantum catches produced by a colour stimulus. We can therefore calculate the quantum catches corresponding to points P = (x, y) and Q = (x', y'), a distance r from P and at an angle α with the vertical axis. Once we have calculated the quantum catches, the RN distance between the colours corresponding to P and Q is simply obtained from equation 10.

## Calculating brightness and green contrast

We calculated brightness and green contrasts as specified by Reser et al (2012). Let $q_{ic}$ be the quantum catch of photoreceptor i (i = 1, 2, 3 for photoreceptors UV, B and G) when the eye of the bee is stimulated with colour c (equation 1).

***Brightness contrast between colours 1 and 2:***

$$Br_{12} = \frac{\sum_{i=1}^{3} q_{i1}}{\sum_{j=1}^{3} q_{j2}} \tag{23}$$

***Green contrast between colours 1 and 2:***

$$G_{12} = \frac{q_{31}}{q_{32}} \tag{24}$$

## Chromatic contrast of target colours against background

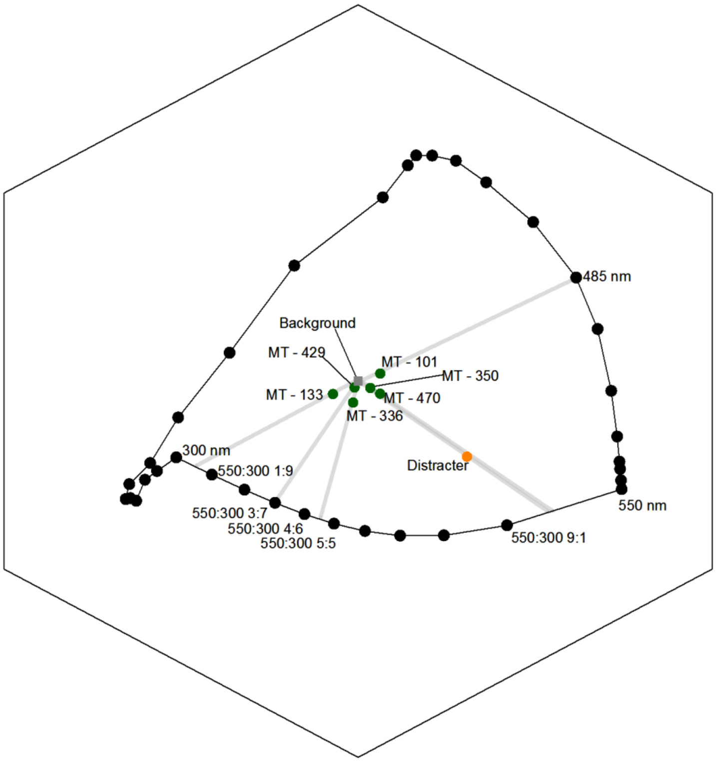
Let ($X_c$, $Y_c$) be the colour hexagon coordinates of colour c (equations 7 and 8). The chromatic contrast of colour c agains its background is the euclidean distance between the loci of the colour and the background. Because the coordinates of the background are (0, 0), this is simply $\sqrt{X_c^{\ 2} + Y_c^{\ 2}}$. The contrast between the chromatic contrasts of colours 1 and 2 against the background is therefore

$$CCB_{12} = \frac{\sqrt{X_1^{\ 2} + Y_1^{\ 2}}}{\sqrt{X_2^{\ 2} + Y_2^{\ 2}}} \tag{25}$$

## Spectral purity

The spectral purity of colour c is the ratio between its chromatic contrast against the background (as defined above) and the chromatic contrast against the background of its dominant wavelength (Lunau et al 1996; Rohde et al 2013) – where the dominant wavelength of colour c is the intersection of the spectral line with the straight line through the centre of the hexagon and the locus of colour c (Fig. S1). Given colours 1 and 2, their spectral purity contrast equals the ratio of their spectral purities.

**Fig. S1** Loci of stimuli (green and orange circles), background (grey square) and spectral locus (black circles connected by a line) plotted in the CH space. The stimuli are indicated by their reference names and the corresponding hues are highlighted in grey. The continuous line represents the spectrum locus for bumblebees with the illumination and background colour used in the experiment



**Supplementary material 2:** Additional information about chromatic and achromatic parameters of stimuli used in the experiment, regression coefficients for the proportion of correct choices, model selection and hypothesis testing for the search time and the relationship between proportion of correct choices, decision time (s) and the COC distance during the extinction phase

**Table S1** Quantum catch, Green contrast (GC), Brightness contrast, Colour contrast against the background (CCB) and Spectral purity contrast (SP) of target colours used in the experiment.

| **Group** | **Colour reference** | **Quantum catch** | | | **GC** | **Brightness** | **CCB** | **SP** |
|---|---|---|---|---|---|---|---|---|
| | | **UV** | **Blue** | **Green** | | | | |
| Background | MT - 431 | 1 | 1 | 1 | | | | |
| Distracter | MT - 553 | 0.180 | 0.117 | 0.903 | 1.108 | 2.500 | 0.334 | 0.574 |
| 1 | MT-133 | 0.573 | 0.416 | 0.414 | 2.426 | 2.146 | 3.639 | 3.026 |
| 1 | MT-429 | 1.047 | 0.957 | 1.005 | | | | |
| 2 | MT-336 | 0.934 | 0.719 | 0.882 | 2.129 | 1.807 | 1.205 | 1.018 |
| 2 | MT-133 | 0.573 | 0.416 | 0.414 | | | | |
| 3 | MT-350 | 2.005 | 1.991 | 2.355 | 2.669 | 2.506 | 1.659 | 2.690 |
| 3 | MT-336 | 0.934 | 0.719 | 0.882 | | | | |
| 4 | MT-101 | 2.348 | 3.038 | 3.234 | 1.468 | 1.560 | 1.107 | 1.094 |
| 4 | MT-470 | 1.672 | 1.649 | 2.203 | | | | |

**Table S2** Colour opponent mechanisms according to the CH (X and Y), COC (A and B) and $RN_L$ ($X_1$ and $X_2$) models, for the different colours used, perceptual distance for each pair, calculated using the CH, COC and RN (lineal and logarithmic versions) models, and angle α that the segment linking the loci (in the colour hexagon) of the two colours of the pair makes with the vertical axis. Dis = distracter; CR = colour reference.

| **Group** | **CR** | **Colour opponent mechanisms** | | | | | | **Angle (α)** | **Perceptual distance** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **X** | **Y** | **A** | **B** | **$X_1$** | **$X_2$** | | **CH** | **COC** | **$RN_L$** | **$RN_{Log}$** |
| Dis | MT -553 | 0.278 | -0.208 | 0.331 | -5.815 | 0.867 | -0.339 | | | | | |
| 1 | MT-133 | -0.062 | -0.035 | -0.697 | -0.354 | -0.001 | 0.182 | 71° | 0.055 | 0.55 | 0.11 | 0.29 |
| 1 | MT-429 | -0.009 | -0.017 | -0.195 | -0.299 | 0.053 | 0.079 | | | | | |
| 2 | MT-336 | -0.012 | -0.058 | -0.528 | -1.094 | 0.180 | 0.163 | 294° | 0.054 | 0.90 | 0.18 | 0.29 |
| 2 | MT-133 | -0.062 | -0.035 | -0.697 | -0.354 | -0.001 | 0.182 | | | | | |
| 3 | MT-350 | 0.030 | -0.019 | 0.063 | -0.554 | 0.401 | -0.174 | 227° | 0.057 | 1.13 | 0.40 | 0.27 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 3 | MT-336 | -0.012 | -0.058 | -0.528 | -1.094 | 0.180 | 0.163 | | | | |
| 4 | MT-101 | 0.054 | 0.019 | 0.527 | 0.090 | 0.217 | -0.898 | 180° | 0.054 | 1.51 | 0.78 | 0.32 |
| 4 | MT-470 | 0.053 | -0.034 | 0.108 | -1.002 | 0.612 | -0.263 | | | | | |

**Table S3** Perceptual distances between stimuli and distracter during phase I.

| Group | Stimuli reference | Perceptual distance between distracter and stimuli | | | |
|---|---|---|---|---|---|
| | | CH | COC | $RN_L$ | $RN_{Log}$ |
| 1 | MT-133 | 0.38 | 6.49 | 1.01 | 2.44 |
| 1 | MT-429 | 0.34 | 6.04 | 0.91 | 2.29 |
| 2 | MT-336 | 0.33 | 5.58 | 0.85 | 2.17 |
| 2 | MT-133 | 0.38 | 6.49 | 1.01 | 2.44 |
| 3 | MT-350 | 0.31 | 5.53 | 0.50 | 2.12 |
| 3 | MT-336 | 0.33 | 5.58 | 0.85 | 2.17 |
| 4 | MT-101 | 0.32 | 6.10 | 0.86 | 2.20 |
| 4 | MT-470 | 0.28 | 5.03 | 0.27 | 1.98 |

**Table S4** Proportion of correct choices and search time for target colours A and B within groups during the extinction phase (III).

| Group | Colour Reference | Target colour | Proportion correct choices (%) | Search time(s) |
|---|---|---|---|---|
| 1 | MT-133 | A | 69 | 1.57 |
| 1 | MT-429 | B | 74 | 1.68 |
| 2 | MT-336 | A | 67 | 1.73 |
| 2 | MT-133 | B | 72 | 1.73 |
| 3 | MT-350 | A | 94 | 1.58 |
| 3 | MT-336 | B | 89 | 1.55 |
| 4 | MT-101 | A | 88 | 1.83 |
| 4 | MT-470 | B | 71 | 2.17 |

**Table S5** Regression coefficients of the GLM for proportion of correct choices (phase III) with group, decision time and their interaction as explanatory variables. Baseline is group 4.

| Coefficients | Estimate | Std. Error | z value | Pr (>\|z\|) |
|---|---|---|---|---|
| Intercept | -0.648 | 1.046 | -0.620 | 0.535 |

| | | | | |
|---|---|---|---|---|
| Group 1 | -2.503 | 2.099 | -1.193 | 0.233 |
| Group 2 | 2.243 | 1.494 | 1.501 | 0.133 |
| Group 3 | 0.765 | 2.354 | 0.325 | 0.745 |
| Time | 1.159 | 0.602 | 1.925 | 0.054 |
| Group 1 : Time | 1.674 | 1.399 | 1.197 | 0.231 |
| Group 2: Time | -1.630 | 0.912 | -1.787 | 0.074 |
| Group 3 : Time | 0.392 | 1.642 | 0.239 | 0.811 |

**Table S6** Generalized linear models with Gaussian distribution to investigate the effect of colour pair, distance (COC and RN) brightness and green contrast on the search time (s).

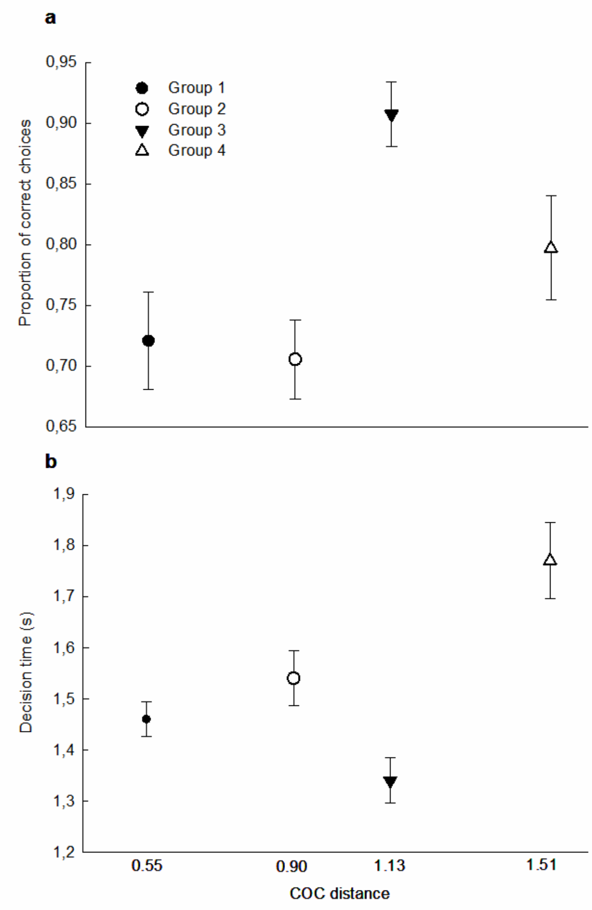
| **Initial Model** | **Selected Model** | **AIC$_{Initial}$** | **AIC$_{Final}$** |
|---|---|---|---|
| ColourPair | ColourPair | -10.734 | -10.734 |
| COC + Brightness | COC + Brightness | -11.374 | -11.374 |
| COC + GreenContrast | GreenContrast | -12.593 | -14.333* |
| RN + Brightness | RN + Brightness | -12.499 | -12.499* |
| RN + Green Contrast | GreenContrast | -12.708 | -14.333* |

*****most parsimonious models

**Table S7** Hypothesis-testing for the GLM analysis with Gaussian distribution of chromatic and achromatic parameters affecting the search time (s). Only the most parsimonious models are described here.

| **Model** | **Variables** | **$X^2$** | **d.f.** | ***p*** |
|---|---|---|---|---|
| **GreenContrast** | | | | |
| | GreenContrast | 27.04 | 1 | <0.0001 |
| **RN + Brightness** | | | | |
| | RN | 4.87 | 1 | 0.03 |
| | Brightness | 10.30 | 1 | 0.001 |

**Fig. S1** Relationship between proportion of correct choices, decision time (s) and the COC distance during extinction phase (III): Effect of perceptual distance, as predicted by the COC model, on **a** the proportion of correct choices and **b** decision time during phase III. Error bars are standard errors



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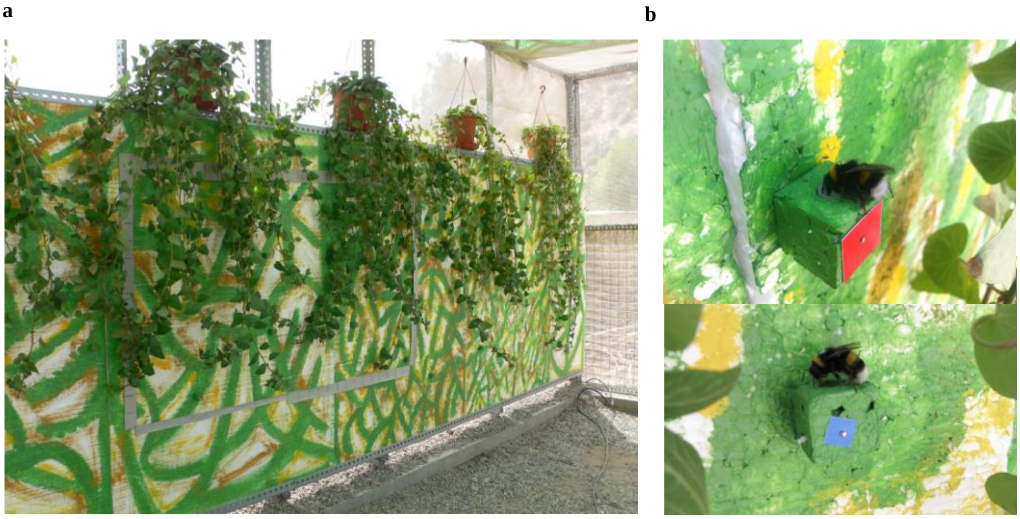
# SUPPLEMENTARY INFORMATION: CHAPTER TWO

## **Supplementary material 1:** Quantum catches and overview of the experimental setup

**Table S1** Quantum catches, chromatic and achromatic properties of stimuli. Colour contrast against the average background (CCB), according to the colour hexagon (CH, Chittka 1992), the colour opponent coding (COC, Backhaus 1991) and the receptor-limited (RN, Vorobyev and Osorio 1998) models and achromatic green (GC) and brightness (BG) contrasts, calculated as specified by Spaethe et al (2001).

| | **Quantum catches** | | | **CCB** | | | **GC** | **BC** |
|---|---|---|---|---|---|---|---|---|
| **Stimulus** | **UV** | **Blue** | **Green** | **CH** | **COC** | **RN** | | |
| Blue | 0.93 | 2.19 | 0.91 | 0.21 | 5.78 | 0.56 | -0.02 | 0.14 |
| Red | 0.05 | 0.03 | 0.10 | 0.05 | 0.94 | 0.07 | -0.41 | -1.33 |

**Figure S1** (a) Overview of the EPS panel and flight cage. (b) Detail of platform with red (32 mm) and blue (8 mm) stimuli.



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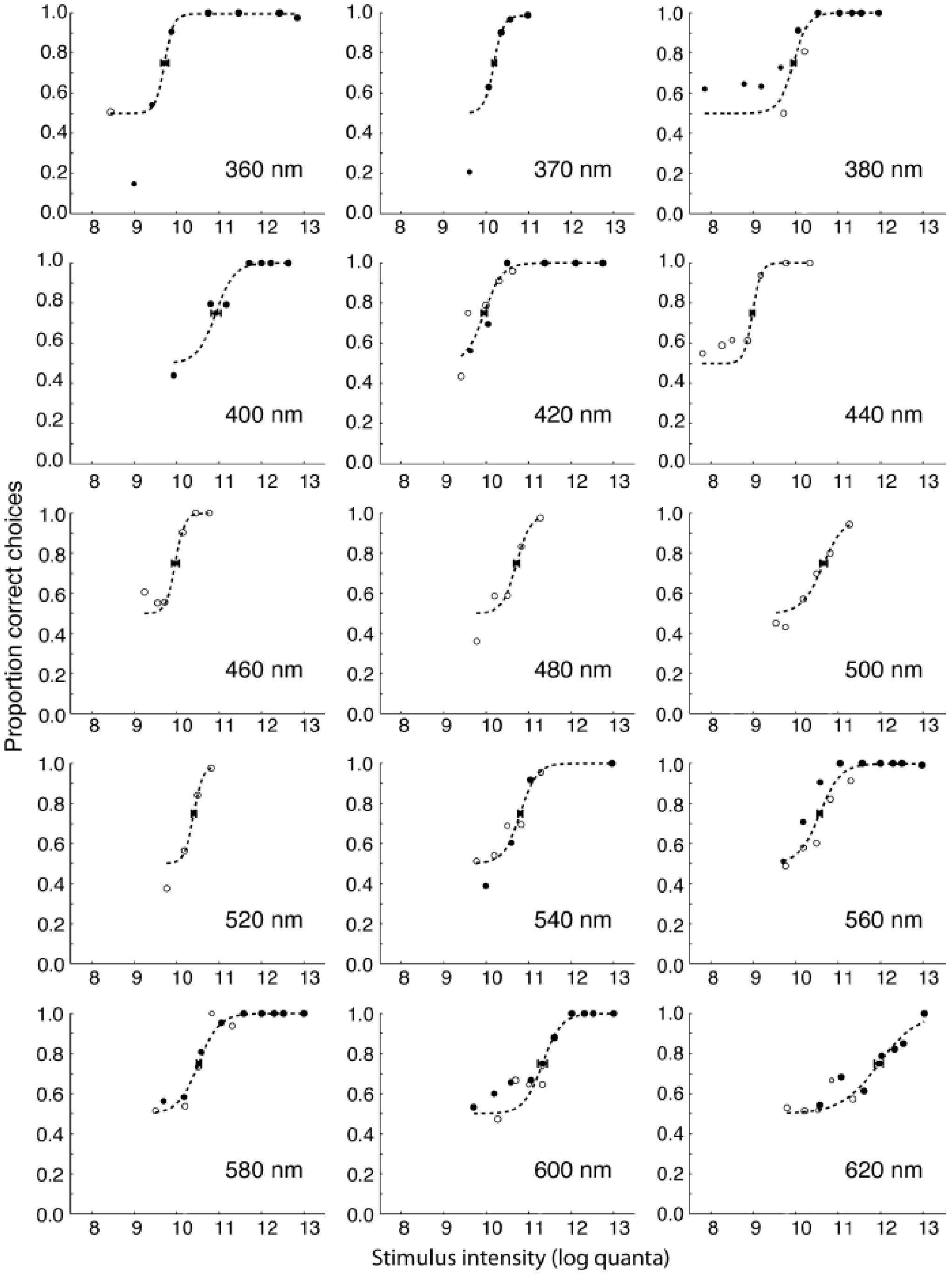
# SUPPLEMENTARY INFORMATION: CHAPTER THREE

**Supplementary Material 1:** Analysis of “no choice behaviour” to determine the spectral sensitivity of *Macroglossum stellatarum*

**Figure S1** Behaviourally determined sensitivity thresholds for 15 wavelengths. Spectral sensitivity data and fitted logistic functions. Data disregard ‘no choice’ behaviour (for details see text). Error bars indicate the robustness of the fit of the psychometric function to the data, obtained by non-parametric bootstrapping (500 simulations) evaluated at threshold (75% correct choices). Open circles show data collected 2011, filled circles show data collected 2012, differently sized data points represent different numbers of choices, equivalent to different weight of the data points in the fitting procedure. Dashed lines represent the logistic function.


360 nm
370 nm
380 nm
400 nm
420 nm
440 nm
460 nm
480 nm
500 nm
520 nm
540 nm
560 nm
580 nm
600 nm
620 nm
Proportion correct choices
Stimulus intensity (log quanta)
0.0
0.2
0.4
0.6
0.8
1.0
8
9
10
11
12
13

# SUPPLEMENTARY INFORMATION: CHAPTER FOUR

**Supplementary material 1:** Light intensity and quantum cacthes of tested mochocromatic lights

**Table S1 Total number of moths, correct and incorrect choices for each wavelength combination and significance values (*p*) for wavelength discrimination.** Wavelengths in bold represent those wavelengths where performance was dependent on intensity (see main text). Nph = number of photons; λ+ = rewarded wavelength; λ- = novel wavelength; *ns* = not significant (discrimination not different by chance).

| **Rewarded (λ+)** | **Novel (λ-)** | **Nph ($cm^2/s$)** | | **Choices** | | **Number of moths** | ***p*** |
|---|---|---|---|---|---|---|---|
| | | **(λ+)** | **(λ-)** | **correct** | **incorrect** | | |
| 380 | 340 | 8.33E+10 | 2.33E+10 | 41 | 49 | 6 | *ns* |
| | 350 | 8.33E+10 | 3.63E+10 | 89 | 79 | 6 | *ns* |
| | 360 | 8.33E+10 | 3.58E+10 | 33 | 39 | 6 | *ns* |
| | 370 | 8.33E+10 | 4.76E+10 | 43 | 36 | 6 | *ns* |
| | 370 | 1.33E+11 | 6.78E+10 | 36 | 38 | 5 | *ns* |
| | 380 | 8.33E+10 | 8.33E+10 | - | - | - | |
| | 381 | 1.33E+11 | 7.40E+10 | 49 | 39 | 6 | *ns* |
| | 382 | 8.33E+10 | 8.01E+10 | 45 | 32 | 6 | *ns* |
| | 382 | 1.33E+11 | 8.01E+10 | 44 | 28 | 6 | *ns* |
| | 383 | 8.33E+10 | 8.04E+10 | 37 | 33 | 5 | *ns* |
| | 383 | 1.33E+11 | 8.04E+10 | 42 | 34 | 6 | *ns* |
| | 385 | 8.33E+10 | 8.23E+10 | 51 | 8 | 5 | < 0.001 |
| | 385 | 1.33E+11 | 8.23E+10 | 52 | 14 | 6 | < 0.001 |
| | 390 | 8.33E+10 | 8.73E+10 | 71 | 8 | 6 | < 0.001 |
| | 400 | 8.33E+10 | 9.77E+10 | 75 | 3 | 6 | < 0.001 |
| 400 | 380 | 1.78E+11 | 5.37E+10 | 77 | 13 | 6 | < 0.001 |
| | 390 | 1.78E+11 | 6.06E+10 | 70 | 20 | 3 | < 0.001 |
| | 395 | 1.78E+11 | 6.37E+10 | 64 | 26 | 6 | < 0.001 |
| | 397 | 1.78E+11 | 9.50E+10 | 96 | 57 | 6 | 0.03 |
| | 399 | 1.78E+11 | 9.82E+10 | 91 | 62 | 5 | 0.03 |
| | 400 | 1.78E+11 | 1.78E+11 | - | - | - | |
| | 410 | 1.10E+11 | 1.03E+11 | 27 | 32 | 6 | *ns* |
| | 415 | 1.10E+11 | 1.06E+11 | 45 | 45 | 6 | *ns* |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 420 | 1.10E+11 | 1.08E+11 | 47 | 16 | 6 | < 0.001 |
| | 425 | 1.10E+11 | 1.08E+11 | 51 | 17 | 6 | < 0.001 |
| | 430 | 1.10E+11 | 1.07E+11 | 58 | 14 | 6 | < 0.001 |
| | 440 | 1.10E+11 | 7.04E+10 | 69 | 19 | 6 | < 0.001 |
| 440 | 420 | 1.36E+11 | 3.25E+10 | 70 | 14 | 6 | < 0.001 |
| | 420 | 1.36E+11 | 1.08E+11 | 56 | 9 | 6 | < 0.001 |
| | 425 | 1.36E+11 | 1.08E+11 | 42 | 7 | 5 | < 0.001 |
| | 427 | 1.36E+11 | 1.08E+11 | 78 | 37 | 5 | < 0.001 |
| | 430 | 1.36E+11 | 1.07E+11 | 34 | 32 | 4 | *ns* |
| | 435 | 1.36E+11 | 1.06E+11 | 57 | 41 | 6 | *ns* |
| | 438 | 1.36E+11 | 1.07E+11 | 52 | 38 | 6 | *ns* |
| | 440 | 1.36E+11 | 1.36E+11 | - | - | - | |
| | 455 | 1.36E+11 | 1.07E+11 | 55 | 60 | 6 | *ns* |
| | 460 | 1.36E+11 | 1.13E+11 | 41 | 29 | 6 | *ns* |
| | 463 | 1.36E+11 | 1.16E+11 | 41 | 22 | 5 | 0.02 |
| | 465 | 1.36E+11 | 1.17E+11 | 56 | 7 | 5 | < 0.001 |
| | 470 | 1.36E+11 | 1.15E+11 | 43 | 4 | 5 | < 0.001 |
| 480 | 450 | 7.84E+10 | 1.04E+11 | 76 | 15 | 6 | < 0.001 |
| | 455 | 1.55E+11 | 1.07E+11 | 79 | 24 | 7 | < 0.001 |
| | 457 | 1.55E+11 | 1.10E+11 | 66 | 11 | 6 | < 0.001 |
| **480** | **460** | 7.84E+10 | 1.13E+11 | 126 | 58 | 7 | <0.001 |
| **480** | **460** | 1.55E+11 | 3.17E+10 | 67 | 8 | 7 | <0.001 |
| | 470 | 7.84E+10 | 1.15E+11 | 42 | 35 | 6 | *ns* |
| | 480 | 2.92E+11 | 2.92E+11 | - | - | - | |
| | 481 | 2.92E+11 | 9.59E+10 | 44 | 31 | 5 | *ns* |
| **480** | **483** | 1.55E+11 | 1.03E+11 | 157 | 27 | 6 | <0.001 |
| **480** | **483** | 2.92E+11 | 1.03E+11 | 61 | 29 | 7 | <0.001 |
| | 485 | 1.55E+11 | 1.02E+11 | 189 | 79 | 6 | <0.001 |
| | 485 | 2.92E+11 | 6.84E+10 | 49 | 26 | 5 | 0.009 |
| | 490 | 1.55E+11 | 9.80E+10 | 87 | 13 | 7 | <0.001 |
| | 500 | 7.84E+10 | 8.84E+10 | 83 | 10 | 7 | <0.001 |
| 520 | 500 | 1.17E+11 | 2.25E+10 | 58 | 4 | 5 | <0.001 |
| | 500 | 1.17E+11 | 7.74E+10 | 99 | 12 | 5 | <0.001 |
| | 505 | 5.70E+10 | 8.45E+10 | 50 | 20 | 5 | <0.001 |
| | 507 | 5.70E+10 | 6.15E+10 | 39 | 36 | 5 | *ns* |
| | 510 | 5.70E+10 | 8.24E+10 | 134 | 92 | 5 | 0.005 |
| | 515 | 5.70E+10 | 8.24E+10 | 64 | 47 | 4 | *ns* |
| | 517 | 5.70E+10 | 7.99E+10 | 55 | 47 | 4 | *ns* |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 520 | 1.17E+11 | 1.17E+11 | - | - | - | |
| | 540 | 5.70E+10 | 6.74E+10 | 48 | 42 | 5 | *ns* |
| | 540 | 5.70E+10 | 4.61E+10 | 76 | 69 | 5 | *ns* |
| | 550 | 5.70E+10 | 6.29E+10 | 105 | 84 | 4 | *ns* |
| | 560 | 5.70E+10 | 6.26E+10 | 33 | 32 | 5 | *ns* |
| | 570 | 5.70E+10 | 5.45E+10 | 33 | 35 | 5 | *ns* |
| 560 | 510 | 4.83E+10 | 8.24E+10 | 76 | 37 | 4 | <0.001 |
| | 520 | 4.83E+10 | 7.74E+10 | 60 | 42 | 4 | *ns* |
| | 520 | 9.66E+10 | 7.74E+10 | 61 | 52 | 4 | *ns* |
| | 530 | 4.83E+10 | 7.24E+10 | 37 | 18 | 4 | *ns* |
| | 530 | 9.66E+10 | 4.91E+10 | 34 | 21 | 4 | 0.008 |
| | 540 | 9.66E+10 | 1.98E+10 | 94 | 71 | 6 | *ns* |
| | 560 | 9.66E+10 | 9.66E+10 | - | - | - | |
| **560** | **570** | 4.83E+10 | 5.45E+10 | 125 | 129 | 6 | *ns* |
| **560** | **570** | 1.55E+11 | 2.30E+10 | 57 | 27 | 6 | 0.002 |
| | 580 | 4.83E+10 | 5.07E+10 | 65 | 24 | 6 | <0.001 |
| | 585 | 4.83E+10 | 4.93E+10 | 71 | 47 | 4 | 0.03 |

**Table S2 Quantum catches and green contrasts of rewarded and novel wavelengths according to the intensity**. λ+ = rewarded wavelength; λ- = novel wavelength; GC = green contrast.

| **λ (nm)** | | **Quantum Catches (*q*)** | | | | | | **GC** |
|---|---|---|---|---|---|---|---|---|
| | | **UV** | | **Blue** | | **Green** | | **q(λ-)/q(λ+)** |
| λ+ | λ- | λ+ | λ- | λ+ | λ- | λ+ | λ- | |
| 400 | 380 | 6.59E+09 | 1.95E+10 | 1.13E+11 | 2.21E+10 | 3.46E+10 | 1.25E+10 | 3.61E-01 |
| 400 | 390 | 6.59E+09 | 8.03E+09 | 1.13E+11 | 3.10E+10 | 3.46E+10 | 1.28E+10 | 3.70E-01 |
| 400 | 395 | 6.59E+09 | 4.56E+09 | 1.13E+11 | 3.65E+10 | 3.46E+10 | 1.29E+10 | 3.72E-01 |
| 400 | 397 | 6.59E+09 | 9.70E+09 | 1.13E+11 | 5.74E+10 | 3.46E+10 | 1.88E+10 | 5.44E-01 |
| 400 | 399 | 6.59E+09 | 8.53E+09 | 1.13E+11 | 6.09E+10 | 3.46E+10 | 1.93E+10 | 5.57E-01 |
| 480 | 483 | 5.18E+05 | 1.46E+05 | 1.30E+11 | 4.11E+10 | 2.03E+11 | 7.49E+10 | 3.69E-01 |
| 480 | 483 | 2.75E+05 | 1.46E+05 | 6.89E+10 | 4.11E+10 | 1.08E+11 | 7.49E+10 | 6.94E-01 |
| 480 | 460 | 1.39E+05 | 1.82E+06 | 3.85E+10 | 9.64E+10 | 5.46E+10 | 5.26E+10 | 9.64E-01 |
| 480 | 460 | 2.75E+05 | 5.09E+05 | 7.61E+10 | 2.70E+10 | 1.08E+11 | 1.47E+10 | 1.36E-01 |
| 560 | 510 | 6.17E+01 | 7.43E+03 | 3.64E+07 | 6.90E+09 | 3.08E+10 | 8.01E+10 | 2.61E+00 |
| 560 | 520 | 1.23E+02 | 2.79E+03 | 7.27E+07 | 2.76E+09 | 6.15E+10 | 7.75E+10 | 1.26E+00 |
| 560 | 520 | 6.17E+01 | 2.79E+03 | 3.64E+07 | 2.76E+09 | 3.08E+10 | 7.75E+10 | 2.52E+00 |
| 560 | 530 | 1.23E+02 | 7.32E+02 | 7.27E+07 | 6.86E+08 | 6.15E+10 | 4.81E+10 | 7.81E-01 |
| 560 | 530 | 6.17E+01 | 1.08E+03 | 3.64E+07 | 1.01E+09 | 3.08E+10 | 7.09E+10 | 2.30E+00 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 560 | 540 | 1.23E+02 | 1.26E+02 | 7.27E+07 | 1.04E+08 | 6.15E+10 | 1.79E+10 | 2.91E-01 |
| 560 | 570 | 6.17E+01 | 3.25E+01 | 3.64E+07 | 1.59E+07 | 3.08E+10 | 2.62E+10 | 8.53E-01 |
| 560 | 570 | 1.98E+02 | 1.37E+01 | 1.17E+08 | 6.72E+06 | 9.89E+10 | 1.11E+10 | 1.12E-01 |
| 560 | 580 | 6.17E+01 | 1.45E+01 | 3.64E+07 | 5.91E+06 | 3.08E+10 | 1.72E+10 | 5.59E-01 |
| 560 | 585 | 6.17E+01 | 9.83E+00 | 3.64E+07 | 3.67E+06 | 3.08E+10 | 1.37E+10 | 4.44E-01 |

*“Everything is theoretically impossible, until it is done.”*

**— Robert A. Heinlein**

**The Rolling Stones**

**1952**