RESEARCH ARTICLE

Wavelength discrimination in the hummingbird hawkmoth *Macroglossum stellatarum*

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ABSTRACT

Despite the strong relationship between insect vision and the spectral properties of flowers, the visual system has been studied in detail in only a few insect pollinator species. For instance, wavelength discrimination thresholds have been determined in two species only: the honeybee (*Apis mellifera*) and the butterfly *Papilio xuthus*. Here, we present the wavelength discrimination thresholds (Δλ) for the hawkmoth *Macroglossum stellatarum*. We compared the data with those found for the honeybee, the butterfly *P. xuthus* and the predictions of a colour discrimination model. After training moths to feed from a rewarded disc illuminated with a monochromatic light, we tested them in a dual-choice situation, in which they had to choose between light of the training wavelength and a novel unrewarded wavelength. To characterise the Δλ function, we decreased the difference between wavelengths in subsequent tests. We also varied the light intensity to test its effect on the discrimination capacity. In agreement with the predictions of the model, we found two expected minima of discrimination where photoreceptor sensitivities overlap, as well as a third, minor, unpredicted minimum around the peak of the blue photoreceptor. *Macroglossum stellatarum* is capable of discriminating between lights with a wavelength difference of 1–2 nm. These discrimination minima are similar to those found for the tetrachromatic *P. xuthus*, and are better than those of the honeybee. The moth is also capable of using achromatic information to discriminate between lights of long wavelengths.

KEY WORDS: Wavelength discrimination, *Macroglossum stellatarum*, Insect vision, Visual system, Chromatic information, Achromatic information

INTRODUCTION

Flower colour conveys information to eyes different from ours. Plants exploit 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 flower visitor must be able to continuously perceive the difference between the selected flower and alternative species. If the difference in colour between co-occurring flowers is smaller than a threshold value, discrimination based on visual information is impossible.

Across all taxa of insects pollinators, the minimum wavelength differences required for discrimination of monochromatic lights, also known as wavelength discrimination thresholds, have only

been studied in the honeybee, *Apis mellifera* (von Helversen, 1972), and the butterfly *Papilio xuthus* (Koshitaka et al., 2008).

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 the threshold is set at 60% (Koshitaka et al., 2008), while *P. xuthus* can discriminate even finer differences: 1 nm at 430 and 560 nm (Koshitaka et al., 2008) at a threshold of 60% of correct choices.

Recently, we determined the spectral sensitivity of the European hummingbird hawkmoth *Macroglossum stellatarum* (Linnaeus 1758) (Telles et al., 2014), and previous experiments revealed remarkable vision-related learning abilities in this species (Balkenius and Kelber, 2004; Kelber, 1996, 1997, 2002, 2005; Kelber and Henique, 1999). Based on its trichromatic visual system, with receptors maximally sensitive in the ultraviolet (UV, 349 nm), blue (440 nm) and green (521 nm) range of the spectrum, *M. stellatarum* can discriminate between colours of different spectral properties (Kelber, 1996; Kelber and Henique, 1999). For instance, moths can discriminate between monochromatic lights differing by 15 nm in the UV and 30 nm in the blue and the blue–green ranges (Kelber and Henique, 1999). Yet, it was not clear whether discrimination was based on chromatic or achromatic cues for some wavelengths. Besides, it has been suggested that when there is a wavelength difference large enough to allow for discrimination by chromatic cues, intensity plays a minor role (Kelber, 2005; Kelber and Henique, 1999).

In this study, we measured the limits of wavelength discrimination of *M. stellatarum*. We determined discrimination thresholds using the approaches taken by von Helversen (1972) and Koshitaka et al. (2008). Specifically, we selected six monochromatic wavelengths to cover the peaks and overlapping areas of photoreceptor sensitivities and trained moths to associate one of the six wavelengths with a reward. After that, we tested the moths' ability to discriminate between monochromatic lights, as well as the effect of stimulus intensity. We compared wavelength discrimination thresholds of *M. stellatarum* with the predictions of a colour vision model and with the values experimentally obtained for other flower visitors.

MATERIALS AND METHODS
Setup and general procedure

Experiments were performed in a flight cage (60×74×61 cm; Fig. 1A), with an illuminance of 40 lx (for details, see Telles et al., 2014). The walls of the cage were covered with grey cardboard (Fig. 1A). The spectral composition of the cage illumination as reflected from the background is given in Fig. 1C.

Six wavelengths (380, 400, 440, 480, 520 and 560 nm) were selected to cover the spectral regions where the sensitivity of *M. stellatarum* photoreceptors peak and overlap (Fig. 1B). We assigned newly hatched, flower-naive moths (bred on their natural food plant in the laboratory) to one of the selected wavelengths, forming six groups (with initially six to seven moths per group). Each moth was

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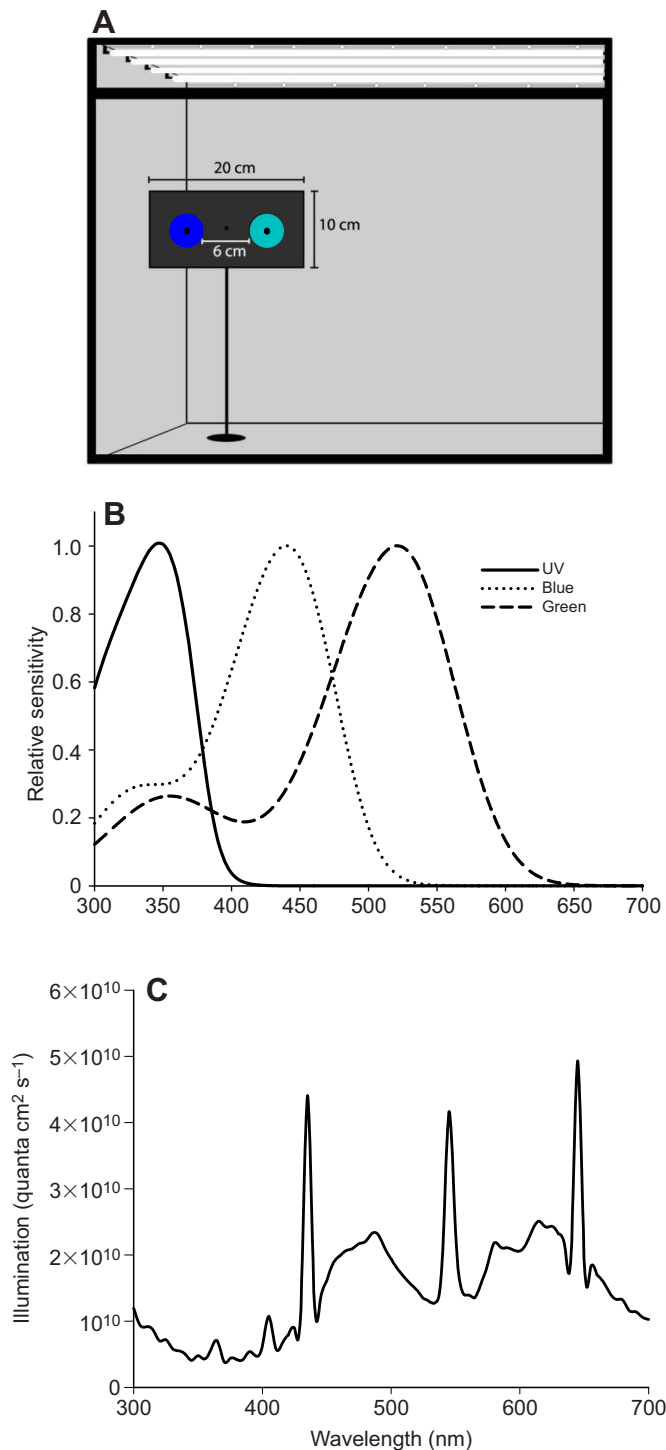*Author for correspondence (meirecuesta@gmail.com)

Fig. 1. Experimental setup and ~~procedure~~. (A) Flight cage and disc arrangement exemplifying a typical moth's view of stimuli during test sessions. During training, one of the discs was unilluminated. (B) Normalised spectral sensitivity of photoreceptors calculated using the Govardovskii template (Govardovskii et al., 2000) and the maximal sensitivity (λ_{max}) of each receptor type. (C) Spectral composition of the cage illumination as reflected from the background.

kept in an individual box in a dark environment for 24 h to increase feeding motivation (Goyret and Kelber, 2011) before training and subsequent testing sessions. Only after moths reached 80% of correct choices (see below) were the testing sessions started. Moths were individually trained and tested.

Stimuli

Monochromatic lights (half bandwidth=15 nm) were projected from behind by two light guides (1000 µm, Ocean Optics) onto two circular UV-transparent Plexiglas discs (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). These discs were used to present the rewarded (sucrose) and unrewarded (water) stimuli. The monochromatic stimuli were produced by two monochromators (TILL Polychrome V, Till Photonics GmbH, Germany), used individually during the training session and simultaneously during the testing session (details below). The light distribution on the discs followed a Gaussian distribution, and to reduce the perceived difference between the centre and edges, we painted a black spot (0.5 cm) in the centre of both discs (Fig. 1A).

Experimental sessions

During training, a monochromatic light of one of the six wavelengths was presented to the moths as the rewarded stimulus (Fig. S1), and a dark disc was used as the unrewarded stimulus. In testing sessions, both discs were illuminated and the rewarded training wavelength was offered together with the light of a novel (unrewarded) wavelength, and moths had to choose one of them. We considered as a correct choice any approach to the disc of the rewarded wavelength that ended in a proboscis contact. An identical approach to the unrewarded dark or novel disc was considered an incorrect choice. The rewarded disc carried 3 µl of sucrose solution at 20% (weight/weight) concentration, while the unrewarded disc carried the same amount of water.

Moths were allowed to feed for 5 s after a correct choice. Afterwards, or after an incorrect choice, we covered both stimuli for 5 s with a piece of cardboard of the same colour as the background. During this time, the setup was prepared for the next trial. We changed the position of rewarded and unrewarded stimuli 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* to the moth in the disc with the rewarded wavelength. A satisfied moth usually landed on the wall of the flight cage, where it was caught and stored in its individual dark box until the next day.

Wavelength distance and intensity differences

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 discrimination capacity of the moth, and could assume different values for different wavelength combinations. If the initial distance of 20 nm was not large enough for moths to reach our threshold criterion (80%) at the end of the first testing session, the distance was increased until reaching a maximum of 50 nm. If moths could not discriminate a novel wavelength 50 nm longer or shorter than the rewarded wavelength, we stopped tests with the respective combination. If a moth reached our initial threshold criterion, tests continued with closer wavelengths.

During the testing sessions, we varied the relative intensity (from 1.98×10^{10} to 2.92×10^{11} quanta cm^{-2} s^{-1}) between rewarded and novel wavelengths, such that the rewarded wavelength had the same, a lower or a higher intensity than the novel wavelength (detailed information about intensities can be found in Table S1). We did not vary intensity for all wavelength combinations because in an earlier

Table 1. Rewarded and novel wavelengths used during training and testing sessions

Rewarded (nm)	Novel λ− (nm)		Novel λ+ (nm)	
	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**

λ−, shorter wavelengths; λ+, longer wavelengths.
Note the adjustment from the initial proposed novel wavelengths to the final novel wavelengths used during testing. Novel wavelengths that changed from the initial wavelengths are in bold.

experiment with *M. stellatarum*, intensity did not play a major role in discrimination (Kelber and Henique, 1999).

Testing sessions lasted until two discrimination thresholds, one for shorter and one for longer wavelengths, were determined for each moth. This took between 15 and 24 days for a single moth, depending on the rewarded wavelength and individual performance.

Statistical analysis of intensity match and wavelength discrimination

To inspect the possible effect of intensity 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 proportion 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. We presented the results in Table S1. GLM belongs to the default car package. Significance intensity values were corrected ($P_{corrected}$) using the Bonferroni approach.

Determination of behavioural wavelength discrimination thresholds

We used the number of correct and incorrect choices of moths during the testing sessions to determine the wavelength discrimination ability of *M. stellatarum*, following the same approach as Koshitaka et al. (2008) on *P. 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 threshold intersecting both sides of the plot (shorter and longer wavelengths) was used to determine two Δλ values (nm) for each rewarded wavelength, one for longer and one for shorter wavelengths. Wavelength differences between the rewarded wavelength and those corresponding to the intersection point towards shorter and longer wavelengths were determined at 70% and 60% criteria of correct choices (Fig. 2). These threshold criteria were selected such that the data could be compared with those obtained for the honeybee at 70% (von Helversen, 1972) and *P. xuthus* at 60% (Koshitaka et al., 2008). To plot the thresholds towards longer and shorter wavelengths, we applied the method used by von Helversen (1972), when studying the honeybee. Δλ (nm) is presented as a function of the wavelength halfway between the rewarded and the threshold wavelengths. For instance, with a rewarded wavelength of 440 nm, and a threshold at 445 nm, this wavelength would be determined as 442.5 nm.

Theoretical wavelength discrimination thresholds

We compared the behaviourally determined Δλ with values predicted by the receptor noise limited model (Vorobyev and
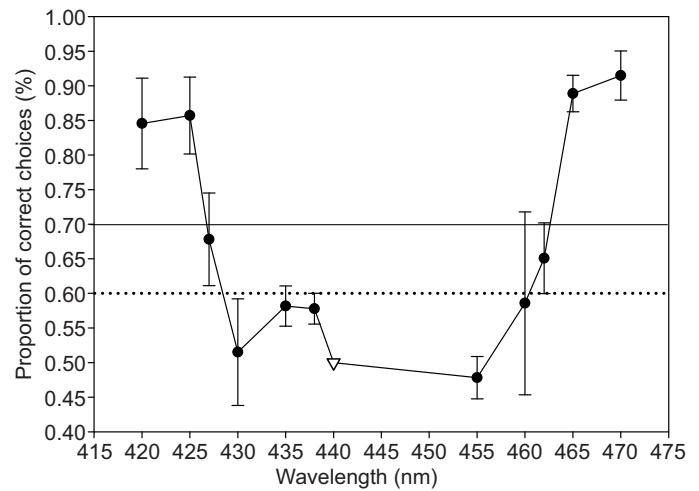

Fig. 2. Discrimination of shorter and longer novel wavelengths. Trained rewarded wavelength of 440 nm (triangle) and moth accuracy (for the number of moths tested at each wavelength combination, see Table S1) depending on the difference between wavelengths (circles). The dashed line indicates the 60% criterion; the solid line indicates the 70% criterion. Error bars are standard errors.

Osorio, 1998; Vorobyev et al., 2001). Adapting the method proposed by Koshitaka et al. (2008) to the trichromatic system of *M. stellatarum* (see their eqn 1), the wavelength discrimination threshold can be calculated from the noise in each receptor channel, ω, and the derivatives of photoreceptor signals, $(df_i/d\lambda)$ 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, ω_i, were calculated from the relative number of UV, blue and green receptors present in one moth ommatidium (1:1:7; see Schlecht et al., 1978; White et al., 2003). According to Koshitaka et al. (2008) and Vorobyev and Osorio (1998):

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

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 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), so that:

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

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 λ, and the parameter c is chosen such that, for the long-wavelength receptor, $k_G=1$ (Koshitaka et al., 2008). For the
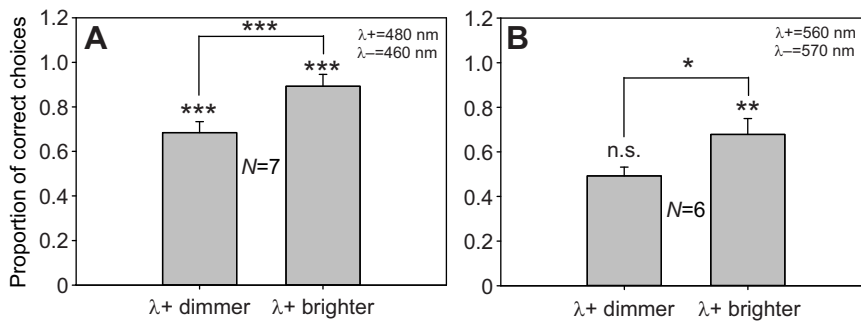
Fig. 3. Intensity-dependent performance. Averaged proportion of correct choices between trained ($\lambda+$) and novel ($\lambda-$) wavelengths of (A) 480 nm versus 460 nm and (B) 560 nm versus 570 nm. Bars represent accuracy when the trained light was brighter or dimmer than the novel stimulus (in terms of the number of photons emitted per cm^2 per s). Asterisks denote levels of significance for the comparison between observed response rates and chance level, and for the comparison between intensity levels (*$P<0.05$, **$P<0.01$, ***$P<0.001$, n.s.=$P>0.05$). N=number of individuals used for each intensity. Error bars are standard errors.

spectral sensitivities of the photoreceptors, we adopted the template of Govardovskii et al. (2000), with maximum sensitivities at 349, 440 and 521 nm for the UV, B and G photoreceptors, respectively (Telles et al., 2014).

RESULTS

Moths were successfully trained to all rewarded wavelengths and performed choices with all tested wavelength combinations. We determined thresholds for all wavelengths, as illustrated for one example in Fig. 2.

Wavelength discrimination and intensity matches

For most pairs of wavelengths, we found no significant effect of stimulus intensity on the proportion of correct choices, but for the rewarded wavelengths of 480 and 560 nm, performance varied with intensity (Fig. 3; Table S1). When the stimulus intensity affected performance, we calculated one discrimination threshold for each intensity, as well as the average value (Table 2).

For the rewarded wavelength of 480 nm, intensity had an effect in tests with two novel wavelengths: 460 nm (χ^2=13.74, d.f.=1, $P_{corrected} \leq 0.001$) and 483 nm (χ^2=11.69, d.f.=1, $P_{corrected}$=0.002). With 460 nm, a decrease in intensity (from 1.5×10^{11} to 7.8×10^{10} quanta cm^{-2} s^{-1}) of the rewarded wavelength resulted in a decrease in the proportion of correct choices from 89% to 68% (Fig. 3A). Note, however, that both choice distributions differed significantly from chance (Fig. 3A; Table S1). In tests with 483 nm, a decrease in intensity (from 2.9×10^{11} to 1.5×10^{11} quanta cm^{-2} s^{-1}) of the rewarded wavelength resulted in an increase in the proportion of correct choices, from 68% to 85%. Again, choice distributions in both tests differed 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 and 485 nm (Table S1).

For the rewarded wavelength of 560 nm, differences between intensities were found in tests with the novel wavelength of 570 nm (χ^2=7.98, d.f.=1, $P_{corrected}$=0.01). Reducing the intensity of the rewarded wavelength (from 1.6×10^{11} to 4.8×10^{10} quanta cm^{-2} s^{-1}) relative to the intensity of the novel light (Table S1) resulted in a decrease of correct choices from 68% to 49% (Fig. 3B). Consequently, the choice distribution analysis showed that, with reduced intensity contrast, choices were not different from chance (Fig. 3B; Table S1).

Behavioural wavelength discrimination thresholds

During testing sessions, the performance was dependent on the novel wavelength (Fig. 2). Discrimination thresholds are given in Tables 3 and 4, and the $\Delta\lambda$ functions using thresholds of 60% and 70% are presented in Fig. 4. The $\Delta\lambda$ function has two pronounced minima at approximately 380 and 480 nm, showing the best discrimination areas for *M. stellatarum*. The first minimum corresponds to the region where the spectral sensitivities of the UV, blue and (minimally) green receptors overlap, while the second minimum 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 peak of the blue receptor and the steep flank of the green receptor.

The overall shape of the $\Delta\lambda$ function did not change if we used the criterion of 70% or 60% of correct choices (Fig. 4). The main difference was in the long-wavelength range. 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, when using a threshold of 60%, $\Delta\lambda$ (nm) could be determined for almost all rewarded wavelengths (Table 4). To establish whether moths were choosing on the basis of chromatic or achromatic information in this range, we calculated – applying eqn 2.3 from Koshitaka et al. (2008) – the relationship between stimulus wavelength, intensity and

Table 2. Different wavelength discrimination threshold ($\Delta\lambda$) values for rewarded wavelengths at lower and higher intensities

Wavelength (nm)		$\Delta\lambda$ (nm)	
Rewarded	Novel	60%	70%
480 lower	483	1.1	2.1
480 higher	483	1.2	1.8
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	16.2
560 lower	570	14.1	15.8
560 higher	570	6.9	6.9
560 average	570	11.2	17

Values change depending on the criterion (70% or 60%).

Table 3. Rewarded wavelength, minimum discriminable wavelength and $\Delta\lambda$ values for both lower and higher combinations at the level of 70% of correct choices

Rewarded wavelength (nm)	Discrimination of shorter wavelengths (nm)		Discrimination of longer wavelengths (nm)	
	Threshold λ	$\Delta\lambda$	Threshold λ	$\Delta\lambda$
380	–	–	383.9	3.9
400	395.7	4.3	419.4	19.4
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.

Table 4. Rewarded wavelength, minimum discriminable wavelength and Δλ values for both lower and higher combinations at the level of 60% of correct choices

Rewarded wavelength (nm)	Discrimination of shorter wavelengths (nm)		Discrimination of longer wavelengths (nm)	
	Threshold λ	Δλ	Threshold λ	Δλ
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.

photoreceptor quantum catch (Table S2). For wavelengths above 560 nm, quantum catches for UV and blue receptors are insignificant, indicating that moths used achromatic cues for discrimination.

On the short wavelength range of the spectrum, moths could not discriminate between a disc illuminated with 380 nm light and another one illuminated with light of shorter wavelengths. For the combination of 340 and 380 nm (Δλ=40 nm), the proportion of correct choices was only 46%. However, for the combination of 380 and 384 nm, the proportion of correct choices was 70% (Tables 3 and 4).

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 disc 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).

Theoretical wavelength discrimination thresholds

Fig. 4 shows the comparison of the Δλ function predicted by the receptor–noise limited model of colour discrimination (Vorobyev and Osorio, 1998) and the behaviourally determined thresholds

using the criterion of 70% (Fig. 4A) and 60% (Fig. 4B) correct choices. The model correctly predicted the presence of the threshold minima near 380 and 480 nm, but predicted a maximum at around 440 nm, where a small dip was observed instead. Behavioural thresholds found at the 60% criterion between 510 nm (Fig. 4B) and 580 nm (see Fig. S2 for a detailed explanation) agree reasonably well with those 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 (Camlitepe and Aksoy, 2010; Fratzer et al., 1994; Koshitaka et al., 2008; Neumeyer, 1992; von Helversen, 1972). Among the flower visitors tested so far, *M. stellatarum* presents minima of wavelength discrimination similar to those of the tetrachromatic butterfly *P. xuthus* when threshold was set at 60% of correct choices (Fig. 5A) and that of the honeybee (von Helversen, 1972) when threshold was set at 70% (Fig. 5B).

The large Δλ values in the threshold curve of *M. stellatarum*, compared with those of *P. xuthus* (Fig. 5A), could possibly result from methodological differences. In particular, the separation between the two stimuli differed between the experiments. They were 1.5 cm apart in the experiment with *P. xuthus* (Koshitaka et al., 2008) and 6 cm apart in our setup (Fig. 1A). In the experiments with *A. mellifera*, the distance between stimuli was probably 20 cm or more (inferred from fig. 3 of von Helversen, 1972). The small (1.5 cm) distance in the *P. xuthus* experiment might have made simultaneous comparison possible, facilitating discrimination.

Koshitaka et al. (2008) suggested that *P. xuthus* uses four spectral classes of photoreceptors for colour discrimination. Minima in the Δλ function of this butterfly were found at approximately 430, 480 and 560 nm. At these wavelengths, they could discriminate differences as small as 1 nm. Using the criterion of 60% of correct choices, *M. stellatarum* could also discriminate a wavelength difference of 1.1 nm at 480 nm and a difference of 2 nm at 380 nm. Compared with the honeybee, at a criterion of 70% of 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.
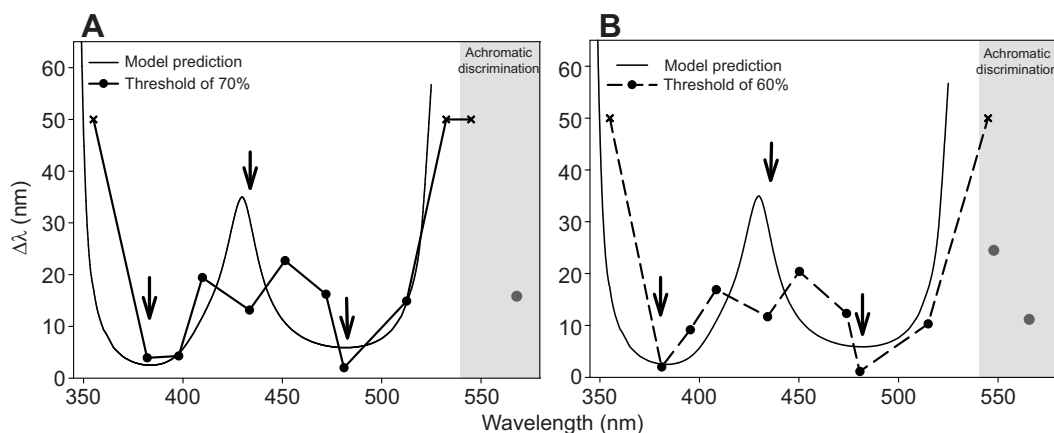

Fig. 4. Behavioural and theoretical wavelength discrimination threshold (Δλ) functions. The smooth curves represent the model prediction, with two minima of wavelength discrimination (for model details, see Results). Behavioural results (connected black circles) exhibit three minima of discrimination (arrows) at (A) 382, 433 and 481 nm for a threshold of 70%, and (B) 381, 434 and 480 for a threshold of 60% (following von Helversen, 1972). Crosses represent tested wavelengths for which discrimination did not occur. We used the maximum differences between wavelengths (50 nm) for graphical representation. The grey area and circles represent the range where wavelength discrimination was based on achromatic information. For the number of moths tested, see Table S1.
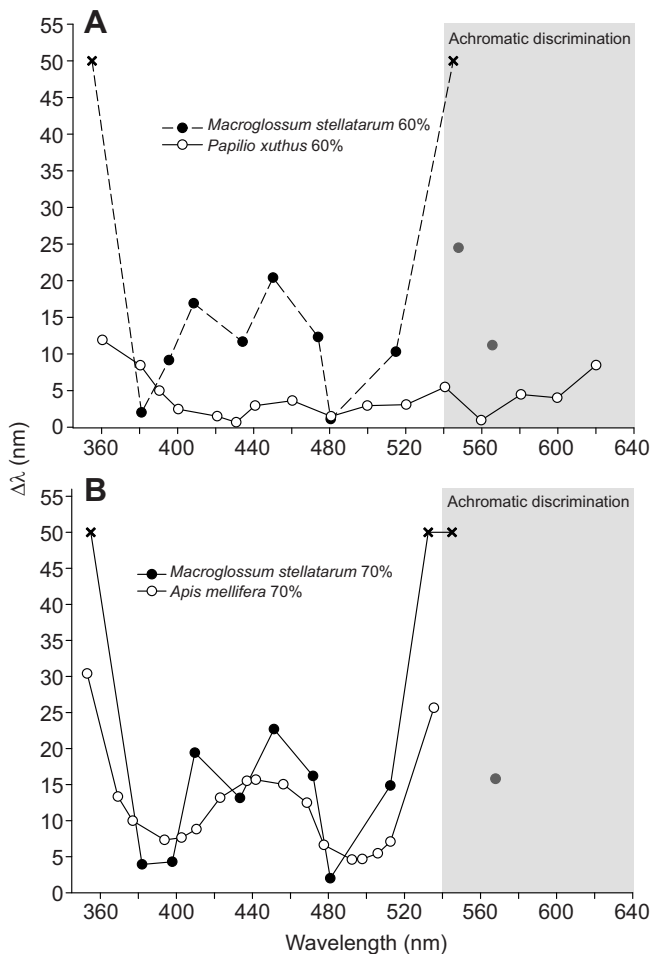
Fig. 5. The Δλ comparison between floral visitors. (A) Δλ functions of *M. stellatarum* compared with those of *Papilio xuthus* established at a criterion of 60% of correct choices. (B) Δλ functions of *M. stellatarum* compared with those of *Apis mellifera*, determined at a criterion of 70% of correct choices. Crosses represent tested wavelengths for which discrimination did not occur. We used the maximum differences between wavelengths (50 nm) for graphical representation. The grey area and circles represent the range where wavelength discrimination was based on achromatic information. For the number of moths tested, see Table S1.

The moths that were trained to the rewarded wavelength of 380 nm discriminated between and preferred the illuminated disc compared with the dark unrewarded disc, but in a dual-choice task with stimuli of the rewarded wavelength and shorter (UV) wavelengths, they chose randomly. Failure to discriminate between colours in the UV range was also found during feeding experiments with dichromatic ants (Camlitepe and Aksoy, 2010). The authors related the finding to the fact that the studied ant species uses the 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 disc (340 nm) over the unrewarded dark disc, similar to our moths. It thus seems likely that *M. stellatarum*, in earlier experiments, used intensity-related cues when discriminating between 365 and 380 nm stimuli (Kelber and Henique, 1999).

Preference switch

Moths strongly preferred the unrewarded novel wavelength of 380 nm when it was offered together with the rewarded wavelength

of 400 nm during tests, although during training, moths clearly discriminated between dark and illuminated discs. We know from the behaviourally determined spectral sensitive curves (Telles et al., 2014) of *M. stellatarum* that the moth needs higher intensity for the wavelength of 400 nm as compared with 380 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 were not higher for the 380 nm light (Table S2). Our recent study has shown an influence of innate colour preference on spectral sensitivity (Telles et al., 2014). Maybe to moths, shorter wavelength UV light can release innate responses associated with foraging behaviour under natural conditions. Despite this peculiar preference, the prediction of the colour vision model agrees 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 on the chromatic aspect of wavelength discrimination – wavelength discrimination above 560 nm is seldom measured, except for insects with red receptors – we also considered the capacity of *M. stellatarum* to perform achromatic wavelength discrimination. The intensity of stimuli corresponds to the achromatic aspect of colour, and the spectral composition to the chromatic aspect (Kelber, 2005). Given that achromatic differences can be perceived by the visual system of flower visitors, there is no justification for ignoring the role of achromatic information. Evaluating the potential effect of achromatic cues could help us to understand the way in which animals use available information during their foraging activity.

The discrimination between 560 and 570 nm wavelengths by our moths was most probably based on achromatic information. For 560 nm, the normalised spectral sensitivity of the UV, blue and green receptors was 1.3×10^{-9}, 7.5×10^{-4} and 0.63, respectively. It seems therefore unlikely that moths used the output from UV and blue receptors for discriminations between 560 nm and longer wavelengths. The green receptor quantum catch was greater for the 560 nm light than for the 570, 580 and 585 nm light in all tested intensities, so the moths could use the green receptor contrast between stimuli to select the rewarded wavelength. In agreement with this suggestion, in the test with 560 and 570 nm, the proportion of correct responses increased with the green contrast (Fig. 3B; Tables S1 and S2).

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

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 mostly relied on chromatic signals in this range (Table S2).

The Δλ function for a threshold of 60% shows more clearly the properties of chromatic and achromatic cues used by *M. stellatarum* when discriminating between wavelengths (Fig. 4B). It is not surprising that hummingbird hawkmoths can use chromatic and achromatic information depending on the task during the foraging

activity (Goyret and Kelber, 2012; Kelber, 2003, 2005). They also pay less attention to achromatic information (as differences between light intensity) when the wavelength difference is large enough to allow easy chromatic discrimination (Kelber and Henique, 1999). The moths choose the correct stimulus less frequently when using achromatic information for discrimination (Kelber, 2005; Kelber and Henique, 1999), so that when using a threshold of 60%, more $\Delta\lambda$ values in the green range could be obtained.

Final remarks

The capacity to discriminate between similar colours is important for a flower-visiting insect, 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).

We found that *M. 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 after extended training periods (Giurfa, 2004), or in the presence of an aversive solution (Avarguès-Weber et al., 2010). Yet, they require larger wavelength differences for discrimination compared with the hummingbird hawkmoth. In contrast, the butterfly *P. xuthus*, using four receptor types, can discriminate wavelengths in a broader spectral range with smaller differences than both trichromats.

Differences between the discrimination capacities of moths and social bees can be discussed from two perspectives: differences in the visual system and differences in the general ecology of solitary and eusocial insects. 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, *M. stellatarum* has a superposition compound eye (a design more typical of nocturnal insects), which confers high absolute sensitivity when compared with apposition compound eyes, such as those of *A. mellifera*. If sensitivity is limited by photoreceptor noise, the signal-to-noise ratio can improve with increasing photon catch. Hence, if sensitivity is high in superposition eyes (because light reaches photoreceptors in each ommatidium through several hundred facets), with the same amount of light, the eye of *M. stellatarum* can absorb more photons per integration time, compared with *A. mellifera*, allowing a higher discrimination capacity. Another possibility to explain differences between the two species is related to their general ecology. Because *M. stellatarum* is a solitary insect, it could have a 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 motivational state to perform a task.

It is increasingly clear that an animal's ability to exploit information based on spectral properties cannot be inferred from the mere presence in the eye of photoreceptors with different spectral sensitivities (Telles et al., 2014; White et al., 1994); that potential must be properly exploited by studying the behavioural performance and the neural processing of information.

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Summary: *Macroglossum stellatarum* is the third flower-visiting insect for which the wavelength discrimination thresholds have been determined. Minima of discrimination were remarkable when compared with the honeybee and a tetrachromatic butterfly.

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 than expected by chance).

Rewarded (λ+)	Novel (λ-)	Nph (cm²/s) (λ+)	Nph (cm²/s) (λ-)	Choices correct	Choices incorrect	Number of moths	*p*
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
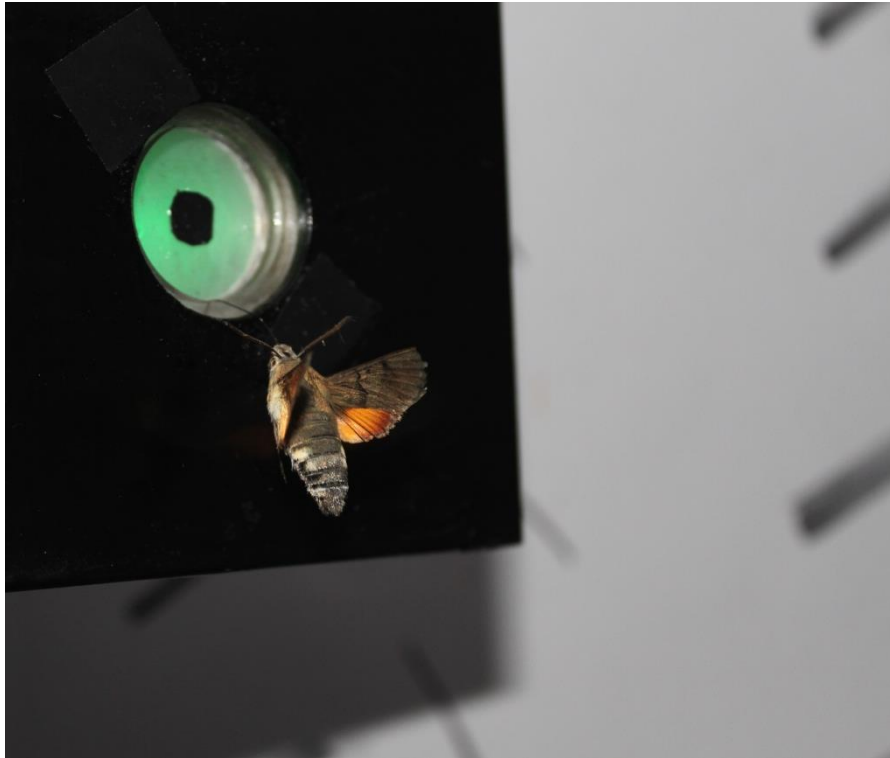
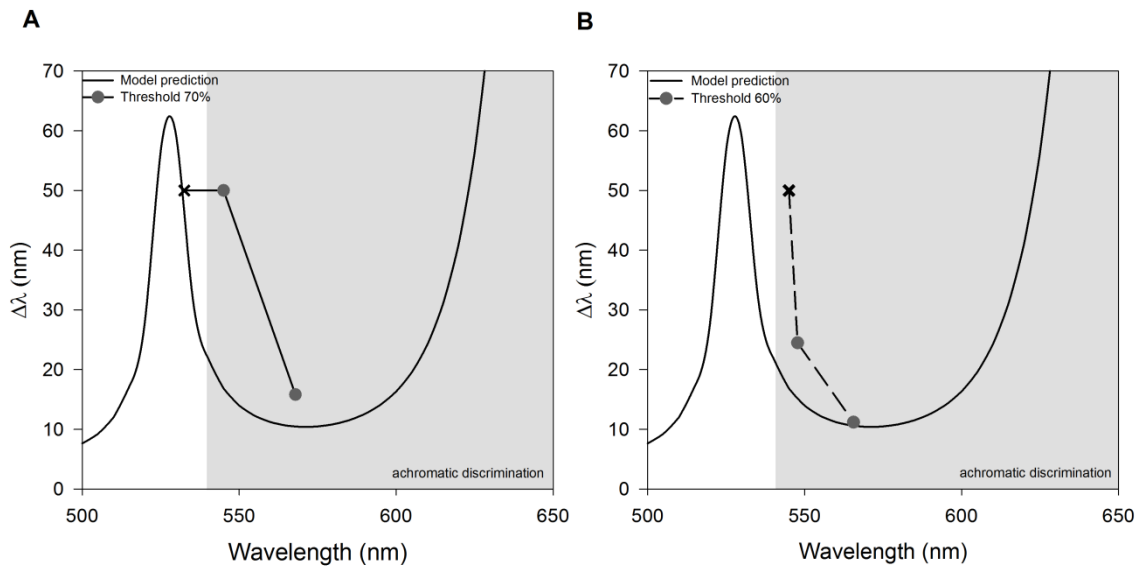**Figure S1.** *Macroglossum stellatarum* **visiting the rewarded feeder during a training session.**

Figure S2. Model prediction and behavioural long wavelength discrimination based on achromatic information. (A) Δλ (nm) function when threshold is set at 70% and (B) 60% of correct choices. The model used to predict the discrimination capacity of *M. stellatarum* explicitly assumes that achromatic mechanisms are disregarded (Vorobyev and Osorio, 1998) and yet, it predicts a region of high wavelength discrimination ability at 565 nm (and another one in the UVB range - data not shown). Mathematically, these regions of high wavelength discrimination are probably artefacts of the structure of equation (1) in the main text. Because the numerator is constant, low values of Δλ result when the denominator is large. This, in turn, happens when the slope of the spectral sensitivity curve of one receptor type is markedly different from the slope in another receptor type. At 565 nm, the UV and blue receptors absorb no light and their spectral sensitivity curves have flat slopes (Fig. 1B), while the spectral sensitivity of the green receptors is decreasing fast (negative slope), leading to low values of Δλ. Similar dips in predicted wavelength discrimination threshold, below 350 nm and above 600 nm, would appear for *Papilio xuthus* if the figure was extended (Fig. 4 of Koshitaka et al., 2008).