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ORIGINAL PAPER

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 8 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, 2005; Kelber and Balkenius 2007). After a successful flower visit, hummingbird hawkmoths can easily learn to associate flower

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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 et al. 2006), that they can learn to discriminate flower colours and colour patterns (Kelber 1996, 2002, 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, 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 (345–357 nm), 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, Connecticut, 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 position 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 maximum; LXHL-MM1D Green Luxeon Star, Quadica Developments 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 4×10^{12} and 2×10^{15} 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

$$S = 10^{(\log I - \log I_{max})}, \quad (1)$$

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 (λ_{max}) of receptor types contributing to the ERG. All analyses were performed using custom-made programmes 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 λ_{max} of a specific receptor type, we selected curves, in which the contribution of the other receptors was minimal. Finally, we averaged the λ_{max} values of all animals and R^2 values for the fits used to determine the respective λ_{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

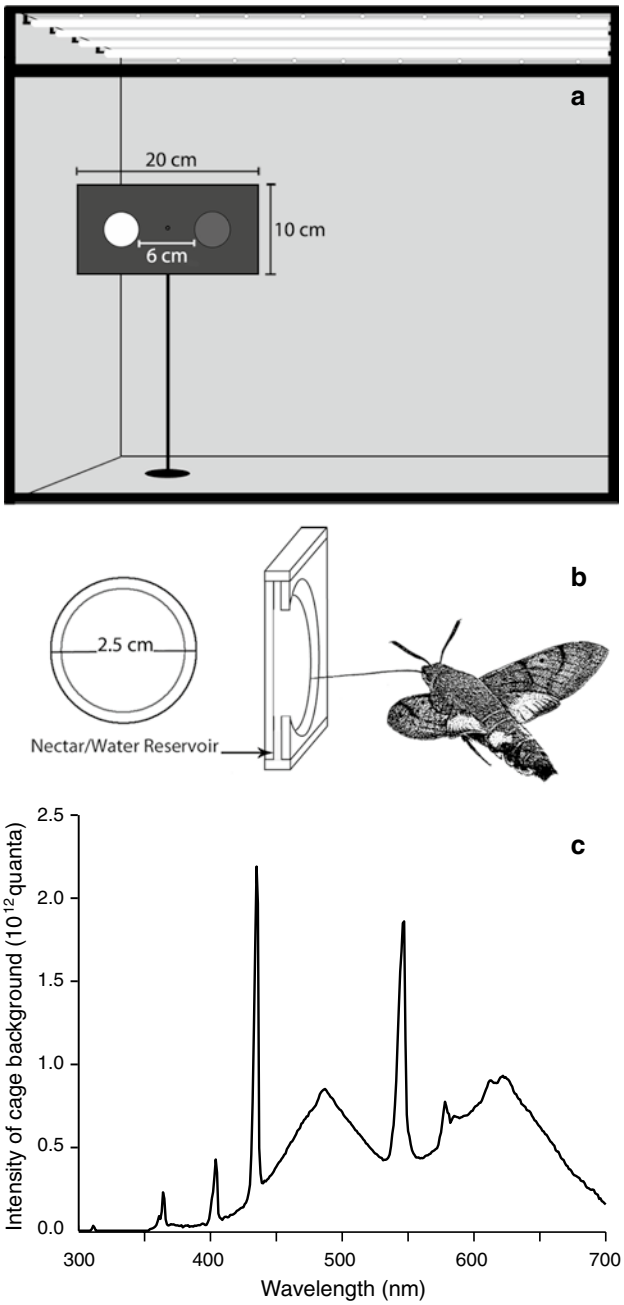

Fig. 1 **a** Flight cage used for behavioural tests of *M. stellatarum*. **b** Feeders with a circular groove to present sucrose solution or water. **c** The spectral composition of the cage illumination as reflected from the background

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.

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 6×10^7 and 10^{13} 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 (2×10^{10} 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 2×10^{10} to 10^{13} quanta cm^{-2} s^{-1} depending on wavelength). 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 wavelengths. 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:

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

where ψ (x) is the fraction of correct choices at intensity x, γ is the lower asymptote that was fixed to 0.5, ε 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 determine 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, trichromatic, 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:

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

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):

$$k_i = \frac{1}{\int R_i(\lambda) I_b(\lambda) \mathrm{d}\lambda}. \quad (4)$$

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

$$\Delta q_i = k_i R_i(\lambda) I_s(\lambda), \quad (5)$$

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

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

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 fraction ω, and we assume that this fraction is inversely proportional to the number of receptors contributing to each receptor mechanism, η, by:

$$e = \omega = \frac{v_i}{\sqrt{\eta_i}}, \quad (7)$$

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 (λ_{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 fitted absorbance

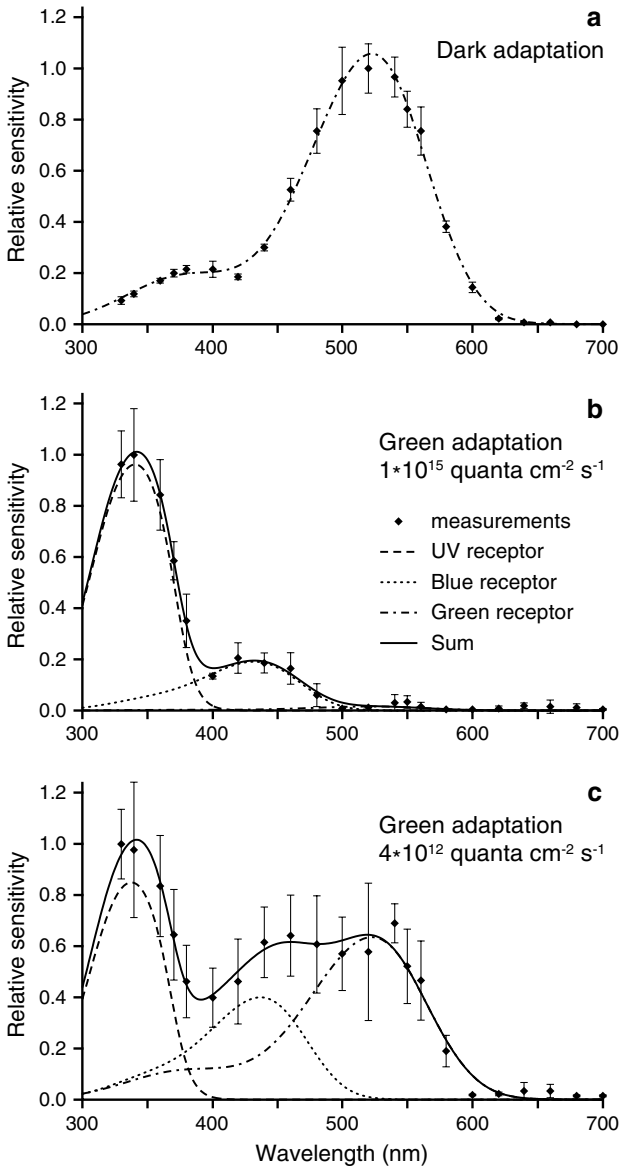

Fig. 2 Spectral sensitivities of a female *M. stellatarum* derived from ERGs. *Symbols* denote measurements (average ± standard deviation, $n = 6$) under different adaptation states. *Dashed*, *dotted* and *mixed lines* indicate absorbance spectra of UV-, *blue-* and *green*-sensitive visual pigments, 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})

spectra based on the Govardoskii 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 λ_{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 λ_{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).

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).

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).

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

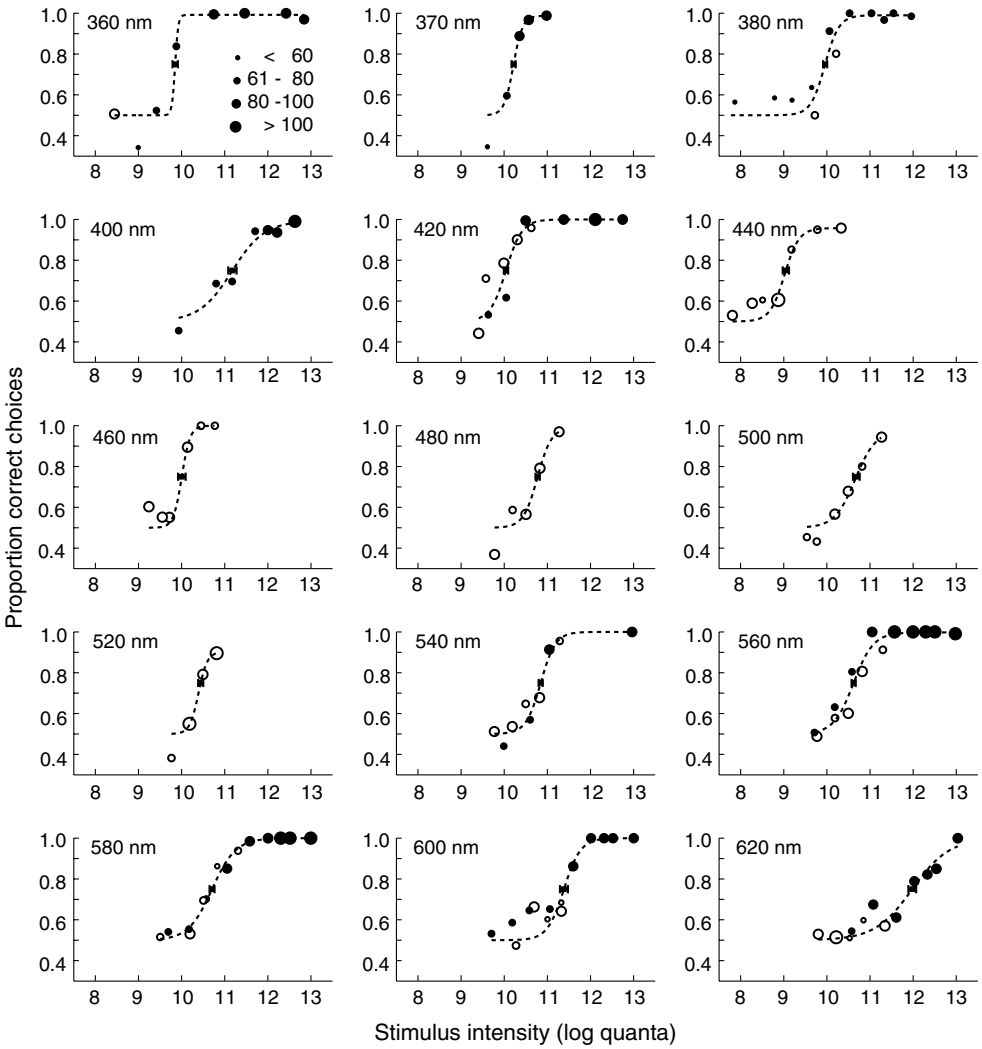

Fig. 3 Behaviourally determined sensitivity thresholds for 15 wavelengths. Spectral sensitivity data and fitted logistic functions. Data include 'no-choice' behaviour (for details see text). *Error bars* indicate the robustness of the fit of the psychometric function to the data, obtained by non-parametric 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 (see inset in the left uppermost graph), equivalent to different weight of the data in the fitting procedure. *Dashed lines* represent the logistic function.

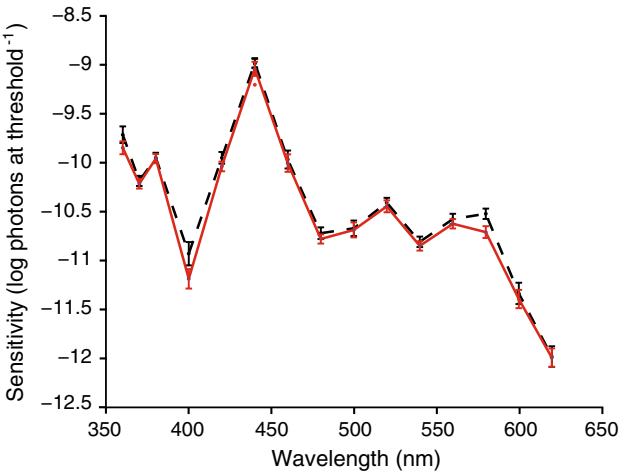

Fig. 4 Spectral sensitivity threshold of *M. stellatarum*. Behaviourally determined thresholds for feeding behaviour using two evaluation methods, *solid line* including 'no-choice' behaviour, *dotted line* excluding 'no choice'. For details see text

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).

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.

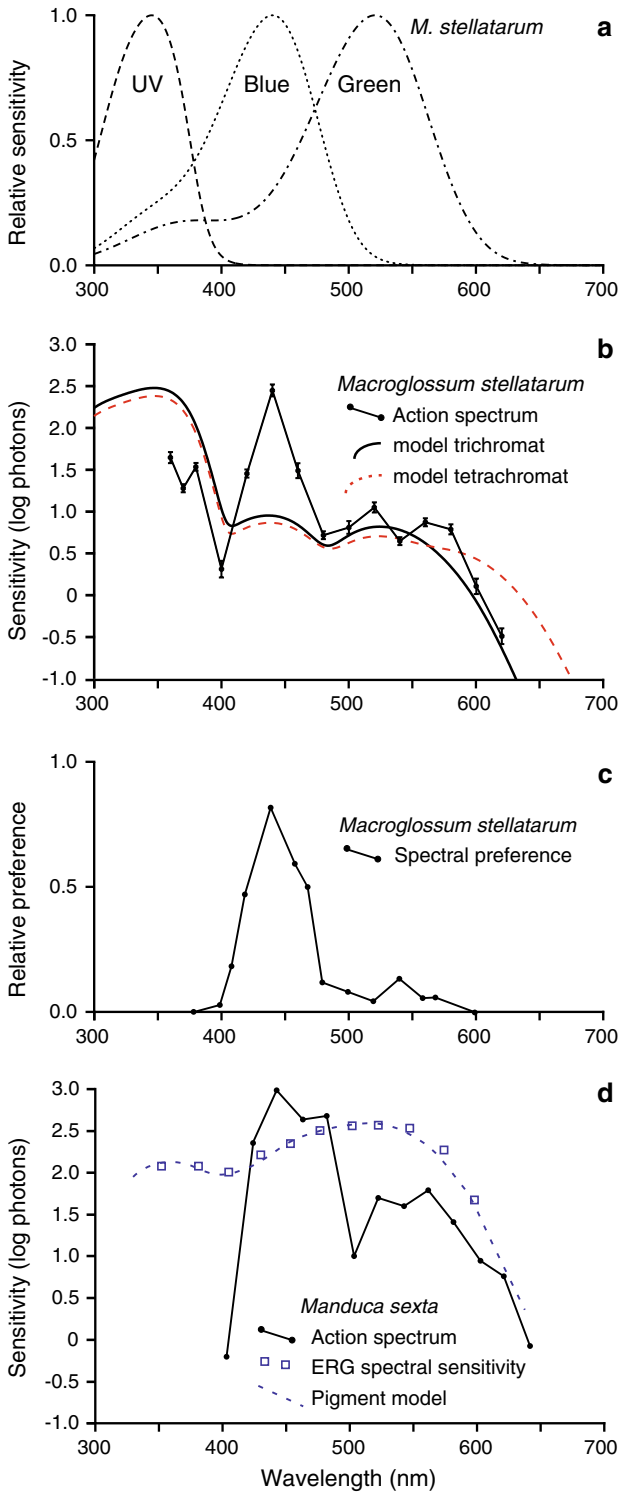

Fig. 5 Spectral sensitivity of *M. stellatarum* in comparisons. **a** Normalized average sensitivity of photoreceptors of seven moths maximally sensitive to light of 349, 440 and 521 nm. **b** Behaviourally determined action spectrum (*line with filled circles*, including 'no choice') and two RNL model 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** Behaviourally determined action spectrum of *Manduca sexta* (*line with filled circles*), spectral sensitivity based on ERGs (*empty squares*), and a fit (*dashed line*) using known receptor sensitivities (adapted from Cutler et al. 1995)

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 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 *M. 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 et al. 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 *M. 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.

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