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## Simultaneous and successive colour discrimination in the honeybee (*Apis mellifera*)

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**Abstract** The colour discrimination of individual free-flying honeybees (*Apis mellifera*) was tested with simultaneous and successive viewing conditions for a variety of broadband reflectance stimuli. For simultaneous viewing bees used form vision to discriminate patterned target stimuli from homogeneous coloured distractor stimuli, and for successive discrimination bees were required to discriminate between homogeneously coloured stimuli. Bees were significantly better at a simultaneous discrimination task, and we suggest this is explained by the inefficiency with which the bees' brain can code and retrieve colour information from memory when viewing stimuli successively. Using simultaneous viewing conditions bees discriminated between the test stimuli at a level equivalent to 1 just-noticeable-difference for human colour vision. Discrimination of colours by bees with simultaneous viewing conditions exceeded previous estimates of what is possible considering models of photoreceptor noise measured in bees, which suggests spatial and/or temporal summation of colour signals for fine discrimination tasks. The results show that when behavioural experiments are used to collect data about the mechanisms facilitating colour discrimination in animals, it is important to consider the effects of the stimulus viewing conditions on results.

**Keywords** Colour vision · Receptor noise · Colour space · Just-noticeable-difference · Photoreceptors

**Abbreviations** s.e.: Standard error · HSB: Hue-saturation-brightness · RN: Receptor noise · Jnd: Just-noticeable-difference

### Introduction

In many cases colour discrimination in human subjects is significantly affected by the display technique used to present stimuli (Newhall et al. 1957; Uchikawa and Ikeda 1981; Uchikawa 1983; Romero et al. 1986; Kulikowski and Walsh 1991). For example, Newhall et al. (1957) showed that the size of uncertainty ellipses was approximately six times greater for subjects discriminating stimuli viewed successively as compared to simultaneously. In a typical simultaneous viewing condition, human subjects are required to view stimuli in a split field with the fovea whilst the stimuli are adjusted so that a just-noticeable-difference (jnd) can be discriminated (MacAdam 1942; Newhall et al. 1957). However, in a successive viewing condition stimuli colour must be coded and retrieved from memory before a behavioural response can be elicited (Uchikawa and Ikeda 1981; Romero et al. 1986; Kulikowski and Walsh 1991). Uchikawa and Ikeda (1981) showed that the time course for deterioration in discrimination ability with successive viewing conditions in humans is as short as 60 ms after a stimulus has been removed from the visual field. Whilst successive viewing conditions may be ecologically more relevant to many real world discrimination tasks as it takes into account the additional complexity of memory formation and retrieval, the trade-off is that discrimination thresholds obtained with successive presentation techniques may be less precise for determining mechanisms of visual performance (Kulikowski and Walsh 1991). Thus, to understand the mechanisms of colour vision that might exist in animals, it is important to know whether different results are ob-

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tained for discrimination tasks depending upon whether stimuli are viewed simultaneously or successively.

Bees use colour vision to both detect flowers (Hempel de Ibarra et al. 2001; Spaethe et al. 2001) and to discriminate between rewarding and non-rewarding flowers (Chittka et al. 1997; Dyer and Chittka 2004a). Honeybees have trichromatic vision with three classes of photoreceptors maximally sensitive at 344, 436 and 544 nm (Menzel and Backhaus 1991; Peitsch et al. 1992), and a number of colour spaces have been proposed to allow for representations of bee colour perception (see Vorobyev and Brandt 1997 for review). Several studies have attempted to understand the mechanisms that might facilitate the perception of colour in honeybees by examining processes mediated at receptor and post receptor levels of the visual system (Backhaus and Menzel 1987; Backhaus et al. 1987; Backhaus 1991; Brandt and Vorobyev 1997; Vorobyev et al. 2001). For example, Vorobyev et al. (2001) found that receptor noise in the honeybees' visual system closely predicted colour discrimination thresholds determined through behavioural studies.

The visual acuity of bees is relatively poor compared with human vision (Land 1997), and therefore, bees must fly very close to different flowers meaning that most stimuli colours will be viewed successively. It is also likely that many previous behavioural studies evaluating colour discrimination in bees (e.g. von Helversen 1972; Backhaus and Menzel 1987; Backhaus et al. 1987; Backhaus 1991; Vorobyev et al. 2001; Dyer and Chittka 2004a, b, c; Giurfa 2004) have tested successive colour discrimination because stimuli were spatially separated. One potential way to evaluate simultaneous colour discrimination in bees is to test their ability to discriminate differently coloured patterns, which has previously been used to evaluate possible regional specialisations of the bee's visual system (Menzel and Lieke 1983; Srinivasan and Lehrer 1988; Lehrer 1998, 1999; Hempel de Ibarra et al. 2001, 2002). For example, Hempel de Ibarra et al. (2001) evaluated the detection angle of coloured patterns by bees and showed that the detection threshold varied depending upon the specific combination of chromatic and achromatic cues. Hempel de Ibarra et al. (2002) then evaluated the ability of honeybees to discriminate between differently coloured patterns and showed that bees are able to use form vision to discriminate between patterns that only varied in chromatic contrast. The studies by Hempel de Ibarra et al. (2001, 2002) thus used simultaneous colour discrimination, because to detect or discriminate a pattern a bee was viewing a bi-coloured stimulus at exactly the same point in time. Recent studies have shown that bumblebees (Dyer and Chittka 2004b, c) and honeybees (Giurfa 2004) learn to discriminate between colours differently depending upon whether differential or absolute conditioning is used, although those studies only considered homogeneous colour stimuli. In this study, we use differential conditioning to evaluate whether simultaneous or successive viewing conditions affect the accuracy of honeybee

colour discrimination, and we thus measure the perceptual limit of colour discrimination capabilities in honeybees.

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## Materials and methods

### Stimulus generation

#### *Software generation of stimuli*

Corel Draw 7.3 software was used to create 31 differently coloured stimuli using the Hue-Saturation-Brightness (HSB) computer model of colour space. The colours were selected in such a way that they were located on a line between human blue and yellow colours, which lies between blue and green in a colour space of the honeybee (Neumeyer 1980, 1981) and is a range of stimuli for which bee discrimination is best (von Helversen 1972). The difference between the colour steps was controlled with the fountain-fill-colour-blend function so that there was predicted to be an equal step of colour difference between the 31 stimuli. The colours were given the names B1 to B15 for the 15 different blue stimuli, Y1 to Y15 for the 15 different yellow stimuli, and G0 for the stimulus that was neutral in colour for a human observer (Table 1). The colours B1 and Y1 were most similar in colour to G0. Stimuli were created considering colour distances that bees experience difficulty discriminating based upon previous studies of fine discrimination capabilities in bees (Dyer and Chittka 2004a, b; Giurfa 2004), and so that the relative brightness of the different stimuli was consistent (Table 1).

#### *Printing of stimuli and spectrophotometer measurements*

Stimuli were printed with an Epson Stylus Photo 870 colour printer using new colour (T008) and black and white (T007) cartridges at 1,440 dpi onto Epson 167 g/m<sup>2</sup> matte paper (97% ISO brightness). A thin layer of contact adhesive was applied to stimuli so that these could be cleaned with ethanol and water.

For the simultaneous colour discrimination task, target stimulus was a five-point star with a distance of 35 mm separating points opposing on the 144° angle of the star (Fig. 1 insert). The colour of the star was the designated target colour for a given discrimination task, and the coloured star was presented on a grey background of colour G0 so that the total size of each target stimulus was 50 mm square. The distractor stimulus was a homogeneous 50 mm square card of colour G0.

For successive colour discrimination 35 mm square homogeneous colours were used as target stimuli, and 35 mm square homogeneous G0 grey stimuli were used as distractors.

Spectral reflectance of stimuli was measured with an Instrument Systems (Spectro 100) optical spectral analyser calibrated against a magnesium oxide standard (Fig. 1).

**Table 1** Lists the stimuli used in the study, the colour values in a HSB computer model of colour space, subjective bee brightness, and the colour distance between a particular colour and G0 considering various models of bee and human colour space. Hex units are from a hexagon colour space for bees (Chittka 1992), RN units are for the receptor noise model for bees (Vorobyev et al. 2001), and jnd are just-noticeable-differences for human vision considering MacAdams ellipse equations (MacAdam 1985). Stimuli shown in bold were the stimuli with which bees were actually tested. Using simultaneous colour discrimination conditions bees were able to discriminate all colours from the stimulus G0 with greater than 70% accuracy

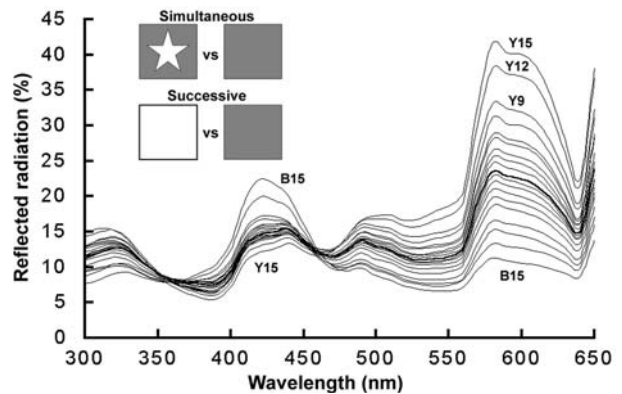
Colour	HSB (H)	HSB (S)	HSB (B)	Bee contrast	Hex $\Delta C$ (Bee)	RN $\Delta C$ (Bee)	jnd (Human)
<b>B1</b>	44	16	64	0.998	0.008	0.23	1
<b>B2</b>	41	12	62	1.006	0.017	0.58	2
<b>B3</b>	32	8	61	0.985	0.023	0.72	3
<b>B4</b>	9	4	59	0.960	0.032	1.05	4
<b>B5</b>	300	3	58	0.914	0.035	1.14	4
B6	267	7	58	0.893	0.044	–	–
<b>B7</b>	254	11	60	0.892	0.063	2.04	8
B8	250	15	60	0.867	0.066	–	–
<b>B9</b>	248	18	61	0.874	0.076	2.48	10
B10	245	22	62	0.879	0.087	–	–
B11	244	25	63	0.922	0.095	–	–
<b>B12</b>	244	29	64	0.899	0.102	3.41	17
B13	242	32	65	0.874	0.109	–	–
B14	242	35	66	0.878	0.119	–	–
<b>B15</b>	242	38	67	0.895	0.132	4.47	23
G0 (Grey)	47	19	65	1.000	0.000	0.00	0
<b>Y1</b>	49	22	67	1.007	0.010	0.32	1
<b>Y2</b>	49	25	69	1.020	0.017	0.56	2
<b>Y3</b>	51	28	70	1.023	0.025	0.77	3
<b>Y4</b>	52	31	72	1.048	0.035	1.04	4
<b>Y5</b>	52	34	73	1.055	0.040	1.21	4
Y6	52	36	75	1.085	0.041	–	–
<b>Y7</b>	52	39	76	1.109	0.047	1.38	6
<b>Y8</b>	53	42	78	1.152	0.053	–	–
<b>Y9</b>	53	44	80	1.131	0.059	1.77	7
Y10	53	46	81	1.149	0.067	–	–
Y11	54	48	83	1.174	0.082	–	–
<b>Y12</b>	54	50	84	1.178	0.090	2.79	10
Y13	54	52	86	1.191	0.100	–	–
Y14	54	54	87	1.183	0.110	–	–
<b>Y15</b>	54	56	89	1.191	0.115	3.56	13

## Discrimination task rational

### Simultaneous colour discrimination for blue stimuli

To test simultaneous colour discrimination, it is important that the stimuli to be discriminated are both viewed at exactly the same time, as it is known in humans the deterioration of colour discrimination ability begins to decline 60 ms after the removal of a stimulus (Uchikawa and Ikeda 1981). Pilot experiments indicated that if bees were presented with split-field stimuli they would often fly to a part of a stimulus similar to the training colour, but not use the edge to facilitate discrimination. Therefore, to achieve simultaneous viewing conditions we used a procedure where a bee was trained to use form vision to discriminate a five point coloured star presented on a grey (colour G0) background from a stimulus that was a homogeneous grey background. A star shape was used for the discrimination task as this is similar to the types of patterns for which bees show innate preferences (Lehrer et al. 1995).

A bee was first trained to discriminate an easy task of B15 star on G0 background versus a homogeneous G0 card, and then once a bee had learnt this task it was trained progressively to discriminate more difficult simultaneous tasks (B12, B7, B5, B4, B3, B2 and finally B1) by always rewarding the star pattern. To solve this type of visual problem the bee must use form vision to



**Fig. 1** Spectral reflectance of the nine blue (B1-B5, B7, B9, B12 and B15) and the nine yellow (Y1-Y5, Y7, Y9, Y12 and Y15) stimuli used in the behavioural experiments with bees. The stimulus G0 is shown in bold and there is a sequential progression of the reflectance curves for each colour series. The insert shows an example of simultaneous and successive discrimination tasks for bees. In the simultaneous task, the star is filled with a designated target colour and the bee learns to discriminate the star shape from the homogeneous grey stimulus (this ensures simultaneous colour discrimination when shape can be detected). In the successive discrimination task, the bee must learn homogeneous colours and thus, there is potentially a memory component to the discrimination task

detect the star, but it must also use its colour vision to be able to discriminate the star from the background colour, which ensures a simultaneous viewing condition. To

assess whether a bee had learnt the different colour discrimination tasks, we used the preconditions described in Dyer and Chittka (2004a), which are described in detail in the bee training section below.

The sequential training procedure to progressively smaller colour distances was done for two reasons. First, by training the bees to what were essentially different colour discrimination tasks the bee was being taught to use its form vision to discriminate between the stimuli, and the bee had to also use its colour vision to actually discriminate the star shape (ensuring simultaneous colour discrimination). Second, a number of recent studies have shown that for fine colour discrimination tasks, it is important to use differential conditioning so that the bee can learn to solve very difficult colour discrimination problems (Dyer and Chittka 2004a, b, c; Giurfa 2004), and in particular bees take a longer time to learn more difficult colour discrimination tasks (Dyer and Chittka 2004a). The sequential training was, thus, used to allow each bee a long enough time to first learn how to use form vision to identify stars, and then to use simultaneous discrimination for the very fine colour discrimination tasks. At the completion of the training to the smallest colour distance (B1 versus G0) each bee was given a non-rewarded test to evaluate discrimination ability.

#### Successive colour discrimination for blue stimuli

For the successive discrimination task bees had to discriminate between homogeneous target colours and homogeneous distractor colour (G0). In this case discrimination is successive (Dyer and Chittka 2004a) as bees must fly a short distance to look at the different colours. The range of colours tested (B15, B12, B7, B5, B4, B3, B2 and finally B1) was the same as for the blue simultaneous discrimination task described above, and we used the same method of sequentially training bees. At the completion of the training to the smallest colour distance (B1 versus G0) each bee was given a non-rewarded test to evaluate discrimination ability.

#### Simultaneous colour discrimination for yellow stimuli

This method is essentially the same as described above for blue stimuli. A bee was first trained to discriminate an easy task of Y15 star on G0 background versus a homogeneous G0 card, then once a bee had learnt this task it was trained progressively to discriminate more difficult simultaneous tasks (Y12, Y7, Y5, Y4, Y3, Y2 and finally Y1). When each bee learnt the smallest colour discrimination task (Y1 versus G0) then it was given a non-rewarded test to evaluate discrimination.

#### Successive colour discrimination for yellow stimuli

This method is essentially the same as described above for blue stimuli. For the successive discrimination task

bees had to discriminate between homogeneous target colours and homogeneous distractor colour (G0). The range of colours tested (Y15, Y12, Y7, Y5, Y4, Y3, Y2 and finally Y1) was the same as for yellow simultaneous discrimination, and we used the same method of sequentially training bees to progressively smaller colour distances.

At the completion of the training to the smallest colour distance (Y1 versus G0), each bee was given a non-rewarded test to evaluate discrimination ability.

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## Colorimetry

For the illumination spectrum, we considered the standard D65 function converted to quantum flux units (Wyszecki and Styles 1982), and for photoreceptors, we use data for the honeybee (Menzel and Backhaus 1991; Peitsch et al. 1992).

#### Stimulus brightness

To control for brightness of stimuli it is important to know the visual angle as bees use different photoreceptor combinations depending upon the visual angle of the stimulus (Giurfa et al. 1996, 1997; Giurfa and Lehrer 2001). Pilot experiments showed that when 35 mm square colour stimuli were presented on a horizontal surface honeybees flew at a distance of about 4.1 cm  $\pm$  1.9 cm s.d. above the surface when making decisions about which colour to choose, which represents a visual angle of about 46°. To control relative brightness of stimuli for the visual system of the honeybee, we considered the bees were evaluating stimuli from a visual angle of greater than 15° and the brightness was mediated by the three spectral classes of the photoreceptors (Giurfa et al. 1996, 1997; Giurfa and Lehrer 2001). We, thus, calculated a contrast index (Eq. 2) with the sum of the quantum catch of the three photoreceptor classes for each stimulus relative to the quantum catch of the three-photoreceptor classes for the grey stimulus (Table 1). The variation in the CI for the different stimuli is relatively small, and since brightness does not appear to play a major role in colour discrimination tasks for bees (Backhaus and Menzel 1987; Giurfa and Vorobyev 1998; Lehrer 1999), we concluded that it would not be an important factor in the ability of bees to discriminate between stimuli in the experiments.

The quantum catch of each photoreceptor ( $Q_i$ ) is given by:

$$Q_i = \int_{300}^{650} S_i(\lambda)I(\lambda)D(\lambda)d\lambda, \quad (1)$$

Where  $S_i(\lambda)$  is the spectral sensitivity of the UV, Blue and Green receptor classes, respectively,  $I(\lambda)$  is the spectral reflectance function of the stimulus and  $D(\lambda)$  is the spectral distribution of the illuminant.



The contrast index (CI) is given by:

$$CI = \frac{\Sigma Q^t i}{\Sigma Q^g i}, \quad (2)$$

where  $\Sigma Q^t i$  and  $\Sigma Q^g i$  are the sum of quantum catches for the target and grey stimuli, respectively.

Calculation of colour distance for honeybees in hexagon colour space

Colour distance between target stimuli and the distractor grey stimulus was calculated in a hexagon colour space model (Chittka 1992).

The relative amount of light absorbed by each photoreceptor class is given by  $P$ :

$$P = R \int_{300}^{650} Si(\lambda)I(\lambda)D(\lambda)d\lambda \quad (3)$$

and the variable  $R$  is the coefficient of adaptation to the background stimulus ( $I_B$ ), where the visual system of the honeybee was assumed to be adapted to a spectrally neutral stimulus reflecting 15% radiation at all wavelengths, which matches the mean reflectance value for absolute brightness of the stimuli used in the experiment.

$$R = 1 / \int_{300}^{650} Si(\lambda)I_B(\lambda)D(\lambda)d\lambda. \quad (4)$$

The transduction of photoreceptor absorption ( $P$ ) into receptor excitations ( $E$ ) is given by:

$$E = \frac{P}{(P + 1)}. \quad (5)$$

Coding is performed by two unspecified colour opponent mechanisms and colour distance is calculated as the Euclidean distance between stimuli loci in colour space (Chittka 1992).

Calculation of colour distance for honeybees in receptor noise space

We also calculated colour distances between the various stimuli and the grey stimulus with the receptor noise model (RN) of colour specification used by Vorobyev et al. (2001) for honeybees. This was done using Eqs. 2 and 3 given in Hempel de Ibarra et al. (2001), and the calculated values are presented in Table 1.

Calculation of just-noticeable-differences in colour for a standard human observer

To allow a numeric comparison of the relative discrimination capabilities of honeybees and humans, we calculated human standard observer jnd steps between the G0 colour and the respective target colours. This was

done with MacAdams ellipses (MacAdam 1942) as described by MacAdam (1985). These equations are also available in Dyer (2001). MacAdams ellipses are an experimentally determined set of ellipses for different regions of a CIE colour space and can be used to represent the colour vision of a CIE1931 standard observer (MacAdam 1985). This method, thus, allows a numeric representation of the colour difference between stimuli for human vision.

## Bee training

### General conditions

Five individually marked honeybees were trained to discriminate between surface reflectance colours (see Sect. 1 Stimulus generation) presented on a horizontally mounted rotating circular board of 50-cm diameter. A horizontal presentation of stimuli was used as this ensured that the bees used the lower half of the frontal eye region which allows for finer colour discrimination (Lehrer 1999). Experiments were conducted outdoors using natural daylight at a distance of 30 m from the hive, and 5 m from a feeding station where bees collected 10% sugar water. One bee was trained to the testing location at a time and marked, and if any other bees arrived at the testing location these were caught in a small net so they could not interfere with the training of the marked bee. The board was covered with dark grey card so stimuli could be easily detected. The board could be rotated to avoid bees using spatial cues to discriminate between stimuli during training. Pilot experiments showed that with this system bees chose identical stimuli at 50% correct choice when half of the stimuli were rewarded with 25% sugar water (and deemed targets) and the other half of the stimuli contained a drop of 0.12% quinine hemisulphate salt in water (and deemed distractors).

Each of the five bees was trained and tested in the same order (detailed below), which included simultaneous discrimination of blue stimuli, successive discrimination of blue stimuli, simultaneous discrimination of yellow stimuli, and finally successive discrimination of yellow stimuli. The bees were tested with blue stimuli on one day and yellow stimuli on the next day. One of the bees was only tested on blue stimuli, as it did not return to either the testing site nor the feeding station after its initial day of training and we had to assume that it had perished.

Bee training and testing of simultaneous and successive colour discrimination

On the rotating board three coloured target stimuli and three distractor stimuli were arranged at spatially randomised coordinates for each foraging bout. For the smallest colour distances a reference system was used by

the experimenter as these colour distances were at the limit of what could be perceived by the human colour visual system. During training the target colour was rewarded with a small drop of 25% sugar water, and the distractor colour contained a small drop of 0.12% quinine hemisulphate salt in water as this bitter tasting solution acts as a punishment for incorrect choices and leads to more accurate decision making in bees (Chittka et al. 2003). If a bee landed on a target flower and drank the sugar water, the sugar water was replenished using a pipette when the bee next landed and drank from another target stimulus. A bee typically visited between 6 and 9 target stimuli per foraging bout to fill its stomach. During a bout we scored the number of visits to target and distractor stimuli where the bee clearly made contact with a stimulus. Each bee was trained sequentially on the colours which became progressively more similar to the distractor colour. To decide whether a bee had learnt a colour discrimination task, we used the pre-conditions described in Dyer and Chittka (2004a). When a bee discriminated between stimuli with 100% accuracy in two consecutive bouts or with greater than 50% accuracy in five consecutive bouts it was deemed that the colour discrimination task had been learnt. The next colour discrimination task in the sequence was then presented to the bee.

When a bee finally learnt the smallest colour distance used for the blue (B1 versus G0) and then yellow (Y1 versus G0) sequence of colours, the bee was given a

non-rewarded test where we scored the number of visits to either the target or distractor colour. This was done for both simultaneous and successive viewing conditions and it was these results that were used for statistical analysis.

#### Calculation of uncertainty circles

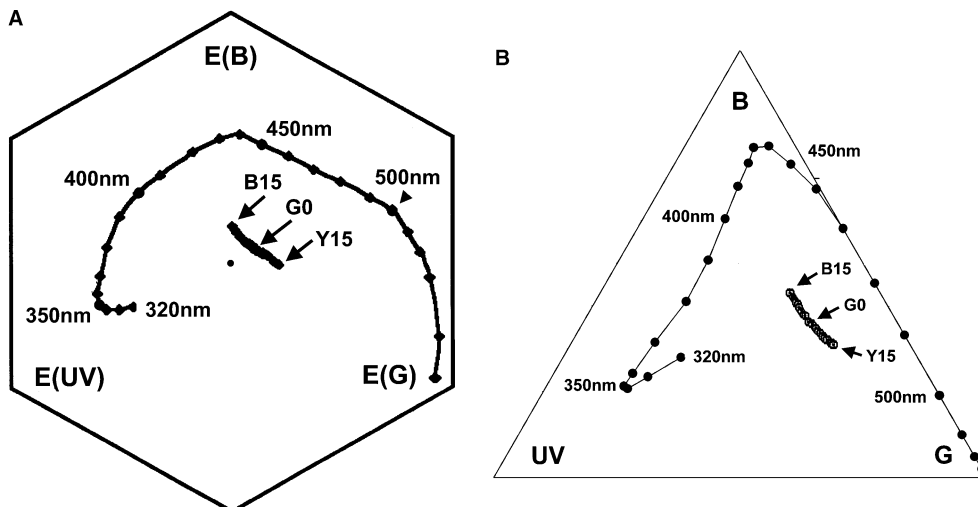
The approximate size of uncertainty zones that included colour stimuli that were so perceptually similar that these could not be discriminated by the bees' colour visual system was defined by the surface area of a circle in a hexagon colour space. By choosing the criteria of 75% correct choices it is possible to determine the surface area of the circle whose radius is the perceptual colour distance between stimuli that can just be discriminated. The relative size of the uncertainty zone depending upon successive and simultaneous viewing conditions was then calculated by dividing the surface areas of the circles for the respective conditions.

## Results

### Colorimetry

Table 1 lists the stimuli used (specified with our naming system), their HSB colour model values and the predicted hexagon colour distance between each stimulus and the grey stimulus. A representation of colour loci in a hexagon colour space is shown in Fig. 2a, and a representation in a colour triangle in Fig. 2b. To allow for a convenient specification system for the different stimuli, we tested the correlation between the colour steps generated in Corel Draw 7.3 and the colour distances calculated in hexagon colour space for the printed stimuli. For both blue and yellow stimuli, there was a very strong correlation between colour step and actual colour distance ( $R^2 = 0.9953$  and  $0.9849$  for blue and yellow stimuli, respectively)

**Fig. 2** Plots of stimuli loci in a hexagon colour space (Chittka 1992) to represent honeybee colour vision. The corners of the hexagon show the excitations of the Ultraviolet  $E(UV)$ , Blue  $E(B)$  and Green  $E(G)$  bee photoreceptors, and the locus shows spectrally pure colour stimuli. The stimuli are also shown plotted in a triangle colour space for honeybee photoreceptors (see Neumeyer 1980, 1981 for details) to allow a visual comparison of loci positions in a different model of colour space (Lehrer 1999). The stimuli sequence used in training bees were very small steps in colour difference, and consequently, the loci are very close together. Precise hexagon colour space distances between individual colours and G0 are provided in Table 1



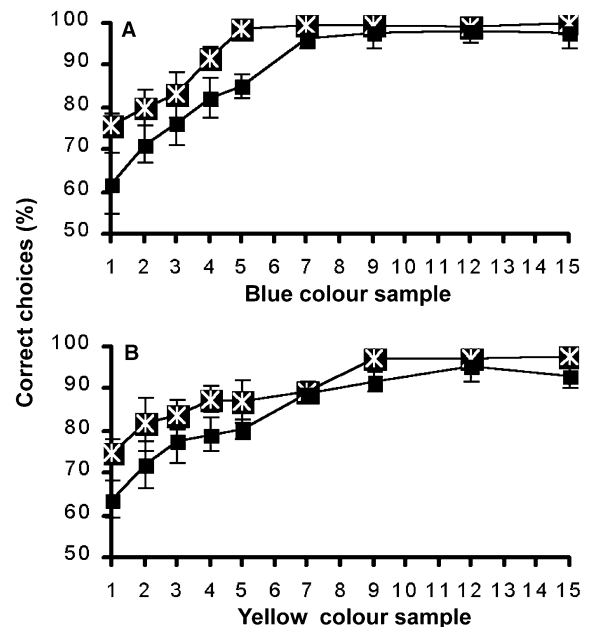
showing that this is an accurate method of producing stimuli for behavioural experiments on bee colour vision. Hence, stimuli are described by their colour step name since this is simpler than specifying stimuli differences, which can be obtained directly from Table 1. Since the HSB colour model values provide a good correlation to colour distance in a hexagon model of colour space, the values in Table 1 should also provide a reasonable guide for generating colour stimuli for future experiments. Table 1 also lists the colour distance between the respective stimuli and colour G0 for a RN model of colour space for bees (Vorobyev et al. 2001), and for a standard human observer considering CIE1931 colour space and MacAdam's ellipses (MacAdam 1985).

### Discrimination performance during learning phases

Figure 3 shows the frequency of correct choices to target stimuli during the rewarded bouts for the various target colours. For both blue (Fig. 3a) and yellow stimuli (Fig. 3b) the colour discrimination is close to ceiling level for large colour distances, but discrimination gradually decreases with a reduction in colour separation between the target and the distractor stimuli. Figure 3 suggests that colour discrimination is poorer for successive compared with simultaneous colour discrimination. For example, for the blue coloured stimuli B1 through to B5 there is 2 s.e. higher frequency of correct choices for simultaneous compared with successive discrimination (Fig. 3a), and for the yellow coloured stimuli Y1 through to Y5 there is more than 2 s.e. higher frequency of correct choices for simultaneous compared with successive discrimination (Fig. 3b). The goal of the experiment design was to test simultaneous and successive discrimination close to the discrimination threshold when bees had received an extensive amount of differential training to coloured stimuli. Thus to collect data totally free of any possible influence from olfactory or spatial learning effects, we conducted a non-rewarded test immediately following the training to the smallest colour distance.

### Calculation of uncertainty circles

Figure 3a and Table 1 show that for blue stimuli viewed simultaneously, a colour distance of 0.008 hexagon units is required for 75% discrimination, whilst for successive viewing conditions the colour distance needs to be 0.023 hexagon units. By calculating the size of the uncertainty circles for each of these viewing conditions, and then dividing the surface area for the successive condition by the surface area for the simultaneous condition the relative size of the zone describing uncertain colours is approximately 8.3 times larger for successive viewing conditions. For yellow stimuli (Fig. 3b), the relative size



**Fig. 3** The frequency of bees correctly choosing a target colour depending upon the colour distance between the target colour (specified by colour number) and the distractor colour G0. There was a very strong correlation between colour steps (represented here by number) and their perceptual colour distance in hexagon colour space ( $R^2=0.9953$  and  $0.9849$  for blue and yellow stimuli, respectively), and thus, colour steps on the X-axis can be taken as representative of a linear increase in perceptual colour distance. Error bars show  $\pm 1.0$  s.e. **a** Choices by bees on blue stimuli for simultaneous (asterisk) and successive viewing conditions (filled boxes). **b** Choices by bees on yellow stimuli for simultaneous (asterisk) and successive viewing conditions (filled boxes). The ability of bees to discriminate between colours significantly better when viewed simultaneously was confirmed with non-rewarded trials for the smallest colour distance tested (result not shown, see text for statistics)

of the zone describing uncertain colours is approximately 6.2 times larger for successive viewing conditions.

### Non-rewarded test for blue stimuli

Pilot experiments showed that honeybees made decisions about the stimuli when flying at a height of 4.1 cm ( $\pm 1.9$  cm s.d.). During the non-rewarded tests the bees flew at a height of less than 10 cm above the stimuli, ensuring that the visual angle was greater than  $15^\circ$ . This indicates that choices were mediated by spectral input to the bees' three photoreceptor classes.

For the blue stimulus B1 discriminated simultaneously from G0 the mean frequency of correct choice with simultaneous presentation was 76.6% ( $\pm 3.0$  s.e.), and for successive presentation it was 61.8% ( $\pm 2.7$  s.e.). The data for these non-rewarded tests is at a very similar level to data recorded when rewards were presented to bees (Fig. 3), but the non-rewarded tests allow for a comparison of data totally free from influence of olfaction or spatial learning effects. To statistically evaluate

the simultaneous and successive colour discrimination conditions for the data collected in the non-rewarded trials we use a two-tailed paired samples *t*-test ( $n=5$ ,  $t=5.520$ , d.f. = 4,  $P=0.005$ ), showing that the ability of honeybees to discriminate the blue coloured stimulus was significantly better when viewed simultaneously.

#### Non-rewarded test for yellow stimuli

For the yellow stimulus Y1 discriminated simultaneously from G0 the mean frequency of correct choice with simultaneous presentation was 73.1% ( $\pm 2.7$  s.e.), and for successive presentation it was 62.8% ( $\pm 3.4$  s.e.). To statistically evaluate the two presentation methods, we use a paired samples *t*-test ( $n=4$ ,  $t=4.232$ , d.f. = 3,  $P=0.024$ ) showing that the ability of honeybees to discriminate yellow colours is significantly better when the colours are simultaneously viewed. The  $n$  value is lower for yellow stimuli because one bee perished before these tests, however, there was still sufficient statistical power to yield a significant result.

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## Discussion

### Simultaneous and successive viewing conditions in honeybees and humans

The findings of this study show that the type of stimuli presentation method has a significant effect on the quality of data that can be obtained from honeybees for colour discrimination functions when testing results close to the discrimination threshold. To our knowledge, this is the first comparative evaluation of simultaneous and successive colour discrimination capabilities in an animal study. The finding is consistent with data for human colour vision studies where successive viewing conditions yield poorer colour discrimination than simultaneous viewing conditions (Newhall et al. 1957; Uchikawa and Ikeda 1981; Uchikawa 1983; Romero et al. 1986; Kulikowski and Walsh 1991), which is due to the inefficiency with which memory can code and retrieve information about colour for successive viewing conditions (Kulikowski and Walsh 1991). Thus, a likely explanation for why successive colour discrimination is poorer than simultaneous colour discrimination in bees is because of the inefficiency with which colour information can initially be coded and then retrieved from memory. Our pilot experiments showed bees presented with split-field stimuli would fly to a part of a stimulus similar to the training colour, but not use the edge to facilitate discrimination. A similar conclusion can be drawn from the results of Lehrer (1999), where bee discrimination of split-field stimuli was actually lower than for homogeneous stimuli. Thus, to test simultaneous colour discrimination in animals, it is important that the test animal is first trained to a shape discrimination task as we did here.

### Implications for comparing colour discrimination capabilities in animals

The effect of simultaneous viewing conditions on the behavioural results is important for discrimination capabilities in honeybees. For example, Fig. 3 shows that to achieve the same behavioural response the colour distance between target and distractor stimuli needs to be between two and three times as large for stimuli viewed successively as compared with simultaneously. For blue stimuli, the size of the uncertainty circle is 8.3 times larger for successive viewing conditions, and for yellow stimuli, it is 6.2 times larger for successive viewing conditions. This equates well with the findings of Newhall et al. (1957) who showed that the size of the uncertainty region in colour space was about six times greater for human subjects discriminating stimuli viewed successively as compared with simultaneously. Previous studies on non-human species have usually demonstrated that colour discrimination is poorer than that which has been shown for the human visual system. For example, one way to assess colour discrimination ability is to measure the ability to discriminate between two spectrally different stimuli at different spectral wavelengths, which is referred to as the  $\Delta\lambda/\lambda$  function (Neumeyer 1991). In humans, this discrimination function is usually determined using simultaneous viewing conditions with two half test fields, and the region of best discrimination allows stimuli differing by 1-nm wavelength to be discriminated (Hurvich 1981; Wysecki and Stiles 1982). The  $\Delta\lambda/\lambda$  function has been determined for several animals and the region of best discrimination is poorer than 1 nm; for example, honeybees 4 nm (von Helversen 1972), goldfish (*Carassius auratus*) 5 nm (Neumeyer 1986), turtle (*Pseudemys scripta elegans*) 4 nm (Arnold and Neumeyer 1987), squirrel monkey (*Saimiri sciureus*) 4 nm (Jacobs and Neitz 1985), pigeon (*Columba livia*) 4 nm (Emmert and Delius 1980) and the Weston grey squirrel (*Sciurus griseus*) 3 nm (Jacobs 1981). The ability of these animals to discriminate between spectral stimuli may thus be poorer than what has been reported for human observers due to the successive viewing conditions, as in all the non-human studies the animals were trained to two spatially separated test fields.

### Implications for understanding mechanisms of colour vision

To understand the mechanisms that facilitate colour vision in bees, it is necessary to test theories with behavioural experiments (Neumeyer 1980, 1981; Backhaus et al. 1987; Srinivasan and Lehrer 1988; Backhaus 1991; Lehrer 1999; Vorobyev et al. 2001; Dyer and Chittka 2004a). Vorobyev et al. (2001) report that receptor noise in the honeybees' visual system closely predicted colour thresholds determined through behavioural studies, and by using this model it has been estimated that bees are not capable of discriminating colour distances less than about 2.3 RN units (Hempel



de Ibarra et al. 2001, 2002). In the experiment design of Vorobyev et al. (2001), the stimuli were spatially separated by 180 mm, meaning that it is unlikely that bees could make simultaneous discriminations between the stimuli. In the current study, we provided bees with extensive levels of differential conditioning to colour stimuli with simultaneous viewing conditions and found that bees are capable of discriminating approximately 0.3 RN units (Table 1). The results indicate a level of colour discrimination in bees that exceeds levels that are expected from theoretical evaluations of how photoreceptor noise might limit colour discrimination reported by Vorobyev et al. (2001). Discrepancies in equating receptor noise to behavioural experiments have been reported in previous studies. For example, Kaissling and Priesner (1970) showed that the measurement of the olfactory behavioural responses by the butterfly *Bombyx mori* is approximately one order of figure better than what might be expected based upon measurements of signal detection by single cells considering receptor noise. Furthermore, measurements of dark noise in single macaque monkey (*Macaca fuscata*) cones (Schnapf et al. 1990) yield results that would preclude light detection that has actually been obtained with human subjects (Donner 1992). These discrepancies may be due to underlying assumptions about how variations in receptor noise are integrated with spatial and/or temporal summation to allow signals to be discriminated by animals (Donner 1992; Vorobyev et al. 2001). Thus, it appears that direct predictions between receptor noise determinations and behavioural responses appear to be difficult to precisely equate, which may partially explain why we were able to achieve better colour discrimination in the honeybee than what might be expected based upon measurements of photoreceptor noise. Three other reasons may also explain the high level of colour discrimination we observed in honeybees. First, the bees were provided with extensive levels of differential conditioning, which has recently been shown to be important for fine colour discrimination tasks (Dyer and Chittka 2004b, c; Giurfa 2004). Second, bees may not always solve difficult discrimination tasks to the limit of their ability, and instead may sacrifice accuracy for foraging speed (Chittka et al. 2003; Dyer and Chittka 2004c). We attempted to control for this by punishing choices made to the incorrect stimuli, which has the effect of bees improving accuracy and sacrificing speed (Chittka et al. 2003). Third, the findings of this current study shows that it is important to use simultaneous viewing conditions to avoid the complexity of coding and retrieving information from memory.

Why simultaneous colour discrimination might yield a sigmoidal type discrimination function

Dyer and Chittka (2004a) showed that when bumblebees (*Bombus terrestris*) discriminate colours successively, the task becomes progressively more difficult as there is a

reduction in the colour distance for bees between target and non-target colours, even for colour distances well above the discrimination threshold. We had anticipated that for simultaneous colour discrimination that there might be a step-type function describing coloured stimuli that could, or could not be discriminated. However, the shape of the function for simultaneous discrimination is sigmoidal and similar in shape to the successive discrimination curve (Fig. 3), although the functions are separated by about 2 s.e. for colours 1 to 5. The explanation for why there is not a step-type function for simultaneous discrimination may be because of the probabilistic nature with which colour neurons respond to similar colours. For example, Komatsu and Ideura (1993) showed the discharge rates of individual colour coding neurons in the macaque monkey (*Macaca fuscata*) correlate with the relative colour similarity of presentation stimuli considering a human colour space. It is also known that visual attention in humans is often mediated by the colour salience map mediating differences in colour (Parkhurst et al. 2002). Then two possibilities for the finding of a relatively shallow sigmoidal-type function for honeybee colour discrimination with simultaneous viewing conditions could be: (1) the probability of individual neurons in the bee responding to differences in stimuli is quite variable, even for colour distances well above discrimination threshold; and/or (2) even with the extensive training bees were provided with in the experiments it is possible that for free-flying bees detecting smaller differences in a colour pattern may place increased cognitive load on attention requirements, thus, leading to an increased error rate in colour discriminations even for colours well above the discrimination threshold. For example, in tasks where colours must be detected against a background, which is a form of simultaneous colour discrimination, honeybees (Niggebrugge and Hempel de Ibarra 2003) and bumblebees (Spaethe et al. 2001) detect colour targets less reliably when there is a reduction in colour contrast between target colour and background colour, even though colour distances are clearly above the colour discrimination threshold for bees.

Implications for minimal cognitive capacity required for high levels of colour discrimination

In the human brain a large amount of neural tissue is devoted to the processing of colour information (Kandel et al. 2000). The finest colour discrimination task that we could get honeybees to discriminate with 75% accuracy was a colour distance that represents about 1 jnd for a standard human observer (Table 1), which is consistent with the difficulty which we experienced in discriminating stimuli B1 or Y1 from G0. It, thus, appears that the brain of the honeybee, which contains only about 0.01% the number of neurons as the human brain (Zhang and Srinivasan 1994), is capable of discriminating between colours that are at the limit of human colour discrimi-

nation. This does not mean that in real world scenarios bees receive sufficient differential conditioning to be able to actually use these fine colour discrimination abilities, and indeed bees often generalise on similar colours (Gumbert 2000; Dyer and Chittka 2004b, c; Giurfa 2004) which is why there has been evolutionary pressure for plants to produce distinctive flower colours (Dyer and Chittka 2004a, b). However, our current results show that with the correct conditioning the visual and neural processing capabilities of the honeybee are able to discriminate between colours that are at the limit of human colour discrimination, and that great care needs to be taken in designing presentation of stimuli for experiments that attempt to understand the limits of colour vision in bees or other animals.

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