

## Fast learning but coarse discrimination of colours in restrained honeybees

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Accepted 10 February 2009

### SUMMARY

**Colours are quickly learnt by free-moving bees in operant conditioning settings. In the present study, we report a method using the classical conditioning of the proboscis extension response (PER) in restrained honeybees (*Apis mellifera*), which allows bees to learn colours after just a few training trials. We further analysed how visual learning and discrimination is influenced by the quality of a stimulus by systematically varying the chromatic and achromatic properties of the stimuli. Using differential conditioning, we found that faster colour discrimination learning was correlated with reduced colour similarity between stimuli. In experiments with both absolute and differential conditioning, restrained bees showed poor colour discrimination and broad generalisation. This result is in strong contrast to the well-demonstrated ability of bees to finely discriminate colours under free-flight conditions and raises further questions about the temporal and perceptual processes underlying the ability of bees to discriminate and learn colours in different behavioural contexts.**

Key words: PER, classical conditioning, colour vision, honeybee, insect learning.

### INTRODUCTION

Visual information is crucial for bees that leave their hive for foraging, exploring new nest sites or simply learning the general landscape. Colour is a particularly helpful feature in learning and identifying foraging targets and landmarks (von Frisch, 1965; Cheng et al., 1986; Chittka and Menzel, 1992; Vorobyev and Menzel, 1999). Accordingly, free-flying bees readily learn to associate a coloured stimulus with a reward (von Frisch, 1914; Kühn, 1924; Daumer, 1956; Daumer, 1958; Menzel, 1967; Menzel, 1968; Menzel, 1969). A single exposure to a sucrose reward on a colourful plate mediates learning and the formation of short-term memories in free-flying bees, and experiencing a few colour-reward pairings leads to long-term colour memories (Menzel, 1967; Menzel, 1968; Menzel, 1979; Menzel and Erber, 1972) (for a review, see Menzel et al., 1974). By contrast, restrained bees seem to learn with much greater difficulty to associate colours with a sucrose reward in a classical conditioning procedure, the proboscis extension response (PER) (Masuhr and Menzel, 1972). Kuwabara (Kuwabara, 1957) succeeded in training restrained bees to colours after cutting off the antennae; however, in his experiments bees needed to experience many colour–reward pairings before showing a consistent PER response. Recently, Hori and colleagues (Hori et al., 2006) replicated those early experiments, applying more rigid conditioning procedures and appropriate controls. Training was extended over two days to achieve stable levels of performance, and at least five conditioning trials were required before bees learnt the association. Again these experiments indicated that colour conditioning of PER is rather difficult. For comparison, olfactory conditioning of PER requires only a single trial for the bees to learn an odour and approximately three training trials to reach reliable, high levels of performance (Vareschi, 1971; Bitterman et al., 1983) (for a review, see Menzel, 1999).

Since classical conditioning of PER is a very valuable tool to investigate sensory and learning mechanisms at the behavioural and neuronal level, it was considered worthwhile to continue searching

for methods which might be used for visual learning in restrained bees, in particular PER conditioning using colours as conditioned stimuli.

Colour vision and learning has been well-characterised in studies with free-flying honeybees (e.g. Daumer, 1956; Menzel, 1967; Menzel, 1968; Menzel, 1969; von Helversen, 1972; Menzel and Greggers, 1985; Menzel and Backhaus, 1991; Neumeyer, 1980; Neumeyer, 1981; Backhaus et al., 1987; Werner et al., 1988; Srinivasan and Lehrer, 1988; Giger and Srinivasan, 1996; Giurfa et al., 1996; Giurfa et al., 1997; Brandt and Vorobyev, 1997; Lehrer, 1999; Hempel de Ibarra et al., 2000; Hempel de Ibarra et al., 2001; Niggebrügge and Hempel de Ibarra, 2003). Psychophysical models were developed to predict how bees discriminate colours (e.g. Backhaus, 1991; Vorobyev et al., 2001). Neurophysiological studies attempted to elucidate neural post-receptor mechanisms underlying colour vision (e.g. Menzel, 1974; Kien and Menzel, 1977a; Kien and Menzel, 1977b; Hertel, 1980; Riehle, 1981; Hertel and Maronde, 1987; Ehmer and Gronenberg, 2002; Yang et al., 2004); however, many questions still remain unanswered, in particular with respect to higher-order colour processing and learning and the respective neural substrates. Behavioural paradigms with fixed bees are therefore highly valuable because they offer new opportunities for studying colour vision at both the behavioural and neurophysiological level. Our current conditioning protocol proved to lead to fast acquisition and allowed us to advance the study of how colours are learnt by restrained bees. We used both absolute and differential conditioning and quantitatively assessed the discrimination and generalisation of coloured stimuli that were varied systematically in their chromatic and achromatic properties. Contrary to our expectations we found that bees discriminated colours poorly and also showed broad colour generalisation which raises further important questions as to how colour information is processed in the honeybee brain in different behavioural contexts.

## MATERIALS AND METHODS

### Procedure

Honeybees, *Apis mellifera carnica* L., were immobilised by cooling briefly and then harnessed in small metal tubes (Bitterman et al., 1983). The animals were fed *ad libitum* 20 h before training with 30% sucrose solution. 2 h before the experiment, the last antennal segments (the flagellae) were cut.

During training, bees were placed in the centre of a cylinder (7 cm in diameter) constructed from standard printer paper. A 5×5 cm window in the cylinder permitted handling and observation of the bees. The setup was located in a dark room with constant temperature (22°C). The visual stimulus was projected from behind on the cylinder wall opposite the observation window creating a circular light spot with slightly diffuse borders (cold light lamp Highlight 3001, Olympus, Hamburg, Germany). The spot was large (~70 deg.) and presented to the right eye of the bees. Prior to and in-between training trials, bees were placed in a chamber illuminated by a UV-white fluorescent lamp (Arcadia Bird Lamp Compact, Arcadia, Redhill, UK).

After placing a bee in the cylinder and allowing for 15–20 s for adaptation, the conditioned stimulus (CS) was displayed for 7 s. The sugar reward [50% sucrose solution, unconditioned stimulus (US)] was applied directly to the proboscis 4 s after CS onset and the bee was allowed to feed for 3 s. We recorded separately whether or not a bee extended its proboscis during the initial CS presentation and whether it responded to the US application. Bees that did not respond to the US application for three subsequent trials were withdrawn from the experiment. The inter-trial interval (ITI) was 12±2 min.

### Stimuli

Several colour and grey filters (e-colour, Rosco, Stamford, CT, USA) were used to create differently coloured stimuli (human yellow, green, blue). Spectral irradiance of the light stimuli was measured with a SD2000 spectrometer (Ocean Optics, Dunedin, FL, USA) calibrated with a Tungsten-Halogen Light Source (LS-1-CAL, Ocean Optics). For each training-test stimuli pair, we calculated relative receptor excitation (quantum catches) and colour distances ( $\Delta S$ ) in the perceptual colour space of bees using the RNL (Receptor Noise Limited) model of bee colour vision (Vorobyev et al., 2001). This was used as a measure for colour similarity (Table 1). Brightness cues were estimated as relative differences in contrast mediated by the long-wavelength sensitive receptor (L-receptor), which is the input channel for the achromatic visual system used for object and pattern recognition (Giger and Srinivasan, 1996; Giurfa et al., 1997; Giurfa and Vorobyev, 1998; Hempel de Ibarra et al., 2001). We also estimated the differences in the activation of all three receptor types, which mediates the phototactic response in bees (overall quantum catch) (Menzel and Greggers, 1985).

### Experiments 1 and 2 (absolute and differential conditioning)

During absolute conditioning, the bees were presented with 10 stimulus–reward pairings [rewarded colour stimulus (CS+)] whereas during differential conditioning, bees experienced a sequence of 10 CS+ interspersed with 10 presentations of an unrewarded colour stimulus (CS–), maintaining the same ITIs. The sequence was kept constant for all animals experiencing alternating CS+ and CS– and always began with a CS+. Following the final training trial, the bees were confronted with several unrewarded tests in which the novel colour stimuli were presented that differed from the training stimuli in either chromatic or achromatic properties. Tests were delivered continuing the same ITI but the order of presentation was randomised between animals.

Table 1. Chromatic and achromatic properties of the stimuli used

Chromatic properties		Achromatic properties		
		Stimulus	Relative quantum catch	
Stimulus pairs	$\Delta S$		L-receptor	All receptors
Blue – green	25.8	White	1	1
Blue – yellow	24.1	Yellow	0.81	0.69
Blue – white	11.4	Green	0.28	0.24
Blue – d. blue	1.6	Blue	0.13	0.21
Yellow – white	13.6	D. yellow	0.11	0.09
Yellow – d. yellow	6.0	D. green	0.04	0.03
Yellow – green	5.8	D. blue	0.02	0.03
Yellow – d. green	5.1	Arena	0.002	0.005
D. yellow – blue	24.5			
D. yellow – green	11.8			
Green – d. green	10.9			

Left columns: chromatic distance ( $\Delta S$ ) between stimulus pairs as a measure of perceptual similarity between colours using a honeybee colour vision model [Receptor Noise Limited (RNL) model (Vorobyev et al., 2001)]. Right columns: relative difference in L-receptor quantum catch and in overall quantum catch of all three receptor types, normalised on the brightest stimulus (white).

### Experiment 3 (memory test and extinction)

To investigate how long the learnt colour was retained, we trained one group to a yellow stimulus for three trials and tested their response to the yellow CS after 1 h of rest under the daylight lamp. Two other groups were tested for extinction and were exposed to the CS without reward several times after an initial training period consisting of either three or seven trials.

### Statistics

We compared the difference between response rates in the first and the final training trial using a Wilcoxon matched-pairs test ( $\alpha$ -level 0.05). This test was also used to assess whether during tests bees discriminated or generalised the learnt colour stimulus by comparing the response probability between the last rewarded training trial and the unrewarded test trials. In order to analyse whether bees discriminated colour in differential conditioning procedures, we also used the Wilcoxon matched-pairs test, comparing responses towards CS+ and CS– in the final training trials. The generalisation strength for different unrewarded test stimuli was assessed by comparing the reactions with a McNemar  $\chi^2$  test (within groups; with Bonferroni adjustment for multiple comparisons where necessary) or a G-test for contingency tables (between groups).

## RESULTS

### Experiment 1: colour discrimination and generalisation after absolute conditioning

In the first experiment, we investigated how bees discriminate a particular colour from other similar or dissimilar colours. Bees were trained in parallel with either a blue or a yellow stimulus during 10 trials (Fig. 1). Responses to the light stimulus increased rapidly for both groups (Wilcoxon matched-pairs test,  $Z=2.8$  and  $3.5$ , respectively,  $P<0.05$ ). In the subsequent unrewarded tests, bees conditioned with the blue stimulus ( $N=30$ ) responded significantly less to the yellow and green test stimuli (Wilcoxon matched-pairs test,  $Z=2.87$  and  $2.77$ , respectively,  $P<0.05$ ). Both colours were very dissimilar to the training colour (Table 1) and therefore we expected such a result. Bees in the test responded to the dim blue stimulus that was similar in colour to the training stimulus but much dimmer (Table 1). This result indicated that they did not use the brightness

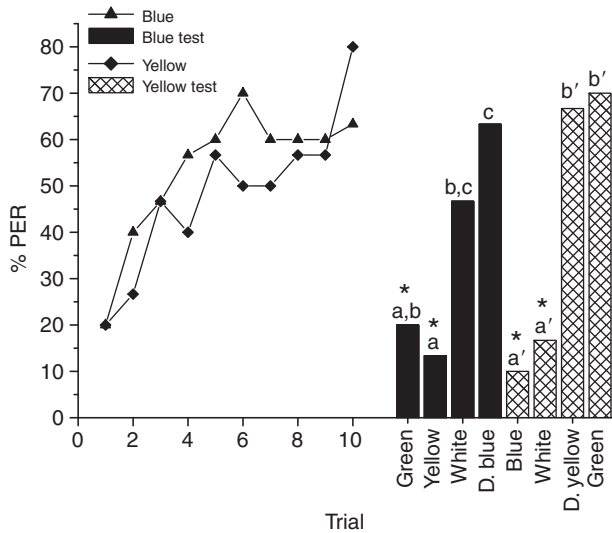


Fig. 1. Colour discrimination and generalisation after absolute conditioning. Bees were exposed to either a blue or yellow spot and rewarded with sucrose solution during 10 training trials. The graph depicts the rate of proboscis extensions to the trained stimulus before delivering the reward. Following acquisition, each bee trained with the blue stimulus (triangles) was tested subsequently with a yellow, dim blue (d. blue), green and white unrewarded stimulus (black bars). Similarly, bees trained to a yellow stimulus (diamonds) were tested with a blue, dim yellow (d. yellow), green and white test stimulus (chequered bars). Bars for test responses are arranged according to the chromatic distance between the test stimulus and the respective rewarded colour stimulus (CS+) (descending order). Asterisks depict significant differences between responses during the last acquisition trial and the test trial (Wilcoxon matched-pairs test,  $P < 0.05$ ). Differences in responses to test stimuli were assessed for the groups trained to blue (black bars) and yellow (chequered bars) separately (McNemar  $\chi^2$  test; bars with different letters differ significantly from each other,  $P < 0.05$ ). PER, proboscis extension response.

cue of the coloured stimulus but relied instead on its chromatic aspect. Bees showed a generalised response to the white stimulus, which was much more similar to the training stimulus than the green and yellow test stimuli (Wilcoxon matched-pairs test,  $Z = 0$  and  $1.22$ , respectively, for dim blue and white, n.s.) (Fig. 1).

Bees conditioned with the yellow stimulus ( $N = 30$ ) showed a similar pattern of responses. They did not extend the proboscis when presented with the blue and white test stimuli, which were very dissimilar in colour (Wilcoxon matched-pairs test,  $Z = 4.01$  and  $3.63$ , respectively,  $P < 0.05$ ). However, they did respond to the dim yellow and green stimuli (Wilcoxon matched-pairs test,  $Z = 1.26$  and  $1.01$ , respectively, n.s.), i.e. test stimuli, which were more similar in colour to the training stimulus.

When comparing the level of generalisation and discrimination in the unrewarded tests, we found some evidence for a graded response that roughly correlates with colour similarity. The comparison of the responses to test stimuli for the bees trained to blue revealed that the scores were not significantly different between yellow and green, between green and white and between white and dim blue (McNemar  $\chi^2$  test, adjusted for multiple testing,  $\chi^2 = 0.49$ ,  $4.9$  and  $1.6$ , n.s.). Given the high spontaneous response rates in this experiment, we analysed the results again, scoring only the choices of those bees that did not display a spontaneous response in the first CS presentation. Results for learning and tests were not qualitatively different, i.e. led to the same significant or non-significant test results. This applies to all the experiments presented in this paper

suggesting there was no influence of spontaneous choices on the learning and discrimination performance.

To summarise, we found a consistent pattern of responses to similar and dissimilar colours following an absolute conditioning procedure (Table 1). Green proved to be more similar to yellow than blue or white, white was more similar to blue than to yellow. During the tests, bees responded to colours that were perceptually similar to the trained CS in terms of their chromatic aspects. To determine whether bees might have learnt the brightness of the CS and used it to discriminate between dim and bright colours in the tests, we compared the performance against the relative brightness differences of the stimuli (Table 1). On inspection, it seems unlikely that bees might have relied simply on brightness differences given that they responded to test colours that were both brighter and dimmer than those trained. This conclusion is further supported by the observation that acquisition of dim and bright colours trained in parallel [yellow and blue (Fig. 1); yellow and dim yellow (data not shown,  $N = 29$  and  $31$ , respectively)] was identical.

### Experiment 2: colour discrimination and generalisation after differential conditioning

Generalisation performance can be strongly influenced by experimental procedures. Therefore, we wished to explore whether a differential conditioning procedure would induce accurate colour discrimination. Based on evidence from modelled thresholds for colour discrimination in free-flying bees (Vorobyev et al., 2001), we expected that bees would now be able to differentiate between similar colours which they did not discriminate in the previous experiment, e.g. yellow and green. We also asked whether bees might use differences in brightness when trained to discriminate similar colours. Both questions could be addressed using differential conditioning.

A new set of bees were trained in a differential conditioning paradigm (10 CS+/CS- blocks) to a yellow CS+ and either a green ( $N = 20$ ) or a blue ( $N = 20$ ) CS- (Fig. 2A). As expected, bees discriminated yellow from blue easily at the end of the training (Wilcoxon matched-pairs test,  $Z = 3.06$  and  $3.0$ , respectively,  $P < 0.05$ ) but did not discriminate when the yellow CS+ stimulus was trained against the green CS- or when green was presented as novel test colour to the bees trained with the blue CS- (Wilcoxon matched-pairs test,  $Z = 0.91$  and  $0$ , respectively, n.s.). The same effect was observed when bees were trained to the reversed set of stimuli (Fig. 2B); they easily discriminated the blue CS+ from a yellow CS- stimulus ( $N = 17$ ; Wilcoxon matched-pairs test,  $Z = 2.55$ ,  $P < 0.05$ ) but failed to show discrimination between the green CS+ and the yellow CS- stimuli ( $N = 18$ ; Wilcoxon matched-pairs test,  $Z = 0$ , n.s.).

The conditioned colour stimuli differed not only in terms of their chromatic aspect but also in their relative brightness. For example, the blue stimulus may have appeared dimmer to the bees than the yellow rewarded stimulus (Table 1). We therefore compared across all groups (Fig. 2A,B) whether bees might have learnt the relative brightness difference by measuring their response to a test stimulus that was similar in colour but dimmer than the respective CS+. The response towards training and test stimulus did not differ when bees were trained to a yellow CS+ and tested with a dim yellow stimulus (Fig. 2A) (Wilcoxon matched-pairs test,  $Z = 0$  and  $1.6$ , respectively, n.s.), trained to a blue CS+ and tested with a dim blue stimulus (Fig. 2B) (Wilcoxon matched-pairs test,  $Z = 0$ , n.s.) or trained to a green CS+ and tested with a dim green stimulus (Fig. 2B) (Wilcoxon matched-pairs test,  $Z = 1.83$ , n.s. ( $P = 0.07$ )). Furthermore, some bees were trained to a green CS+ versus a yellow CS-, meaning that here the unrewarded colour was brighter than the rewarded one.

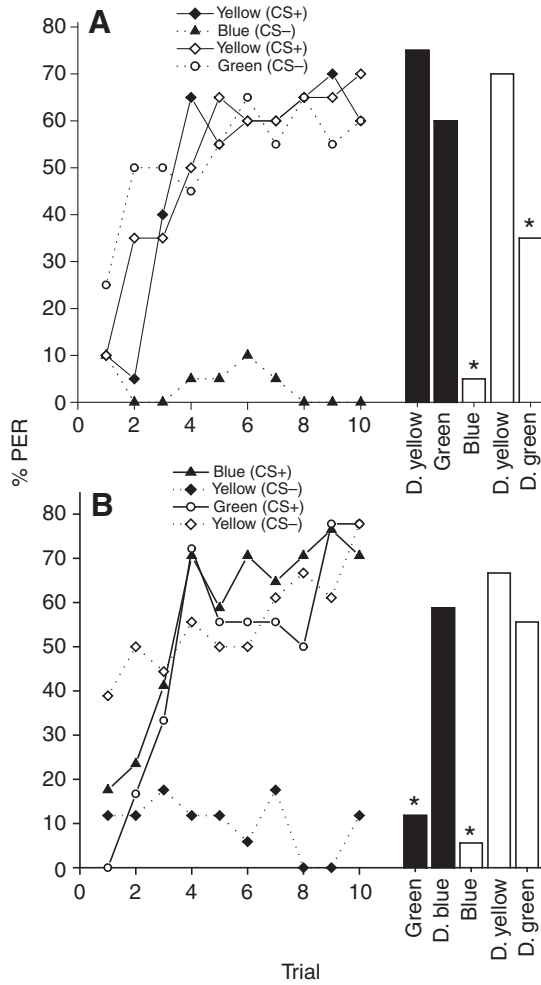


Fig. 2. Colour discrimination and generalisation after differential conditioning. (A) Some bees were trained to the yellow stimulus as rewarded colour stimulus (CS+) (closed diamonds) versus the blue stimulus as unrewarded colour stimulus (CS-) (closed triangles) and tested with dim yellow (d. yellow) and green stimuli (black bars). Other bees were also trained to the yellow stimulus as CS+ (open diamonds) but experienced a green CS- (open circles). They were tested with blue, dim yellow and dim green (d. green) test stimuli (white bars). (B) In the reversal group, bees were exposed to a yellow CS+ versus a blue CS+ (closed diamonds and triangles, respectively) or a green CS+ (open circles) (corresponding yellow CS-: open diamonds). The former bees were tested with a green and dim blue (d. blue) stimulus (black bars). Bees rewarded on the green stimulus were tested with a dim green, dim yellow and blue stimulus (open bars). Asterisks depict significant differences between responses during the last acquisition trial and the test trial (Wilcoxon matched-pairs test,  $P < 0.05$ ). PER, proboscis extension response.

However acquisition was not different in this group as compared with the others, and bees responded to the dim yellow test stimulus (Fig. 2B) (Wilcoxon matched-pair test,  $Z = 0.9$ , n.s.).

These results so far also indicate that bees are unlikely to use brightness differences for colour discrimination and generalisation under our experimental conditions. The only deviation from these observations was a significantly reduced test response to the dim green following training bees to a (brighter) yellow CS+ versus a (dimmer) green CS- (Fig. 2A) (Wilcoxon matched-pairs test,  $Z = 2.07$ ,  $P = 0.04$ ). We cannot rule out that in this particular test, bees might have reacted less to the dim green test stimulus because it was much dimmer. Perhaps if the brightness of stimuli is extremely different,

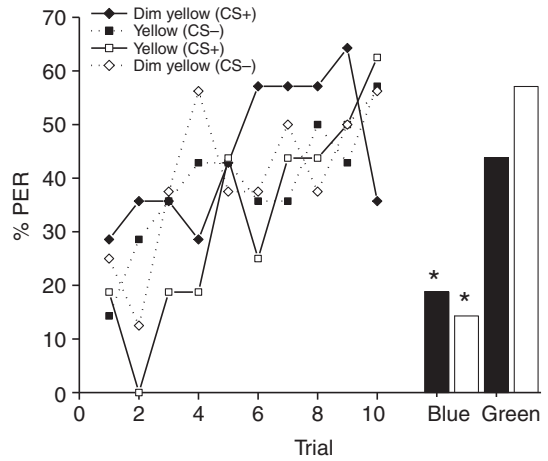


Fig. 3. Differential conditioning of different intensities. Bees were trained to discriminate a dim yellow (d. yellow) rewarded colour stimulus (CS+) (closed diamonds) from a yellow unrewarded colour stimulus (CS-) (closed squares;  $N = 14$ ) or vice-versa (open symbols;  $N = 16$ ). Response rates are depicted separately for CS+ (solid lines) and CS- (broken lines). After training, they were tested with a similar and dissimilar colour, green and blue, respectively (black bars, bees trained to dim yellow CS+; white bars, bees trained to yellow CS+). Asterisks depict significant differences between responses during the last acquisition trial and the test trial (Wilcoxon matched-pairs test,  $P < 0.05$ ). PER, proboscis extension response.

bees might perceive such differences, which is a question of interest for future work. However, in all other colour combinations tested in the present study, bees were not able to use brightness differences.

Finally, we repeated the experiment using another set of similar colours with a large difference in brightness (yellow and dim yellow stimuli) (Fig. 3). In this case, both groups were tested with the same set of test stimuli (blue and green) to evince potential asymmetries in colour discrimination between the reciprocal training arrangements that might be undetectable during acquisition.

Acquisition was similar in both groups trained in parallel with reciprocal colour arrangements reaching significant increases in response probability (Fig. 3) (Wilcoxon matched-pairs test,  $Z = 2.52$  and  $2.02$ , respectively,  $P < 0.05$ ). Bees, however, failed to use the brightness difference to respond exclusively to the rewarding stimulus during 10 blocks of successive CS+ and CS- presentations; they did not discriminate between a bright and a dim yellow stimulus and vice-versa (Fig. 3) (Wilcoxon matched-pairs test,  $Z = 0.53$  and  $1.60$ , respectively, n.s.). Brightness differences were not attended during tests either. For example, bees conditioned to dim yellow CS+ did not respond in the test to the blue that was similar in brightness but responded towards the chromatically similar green (Wilcoxon matched-pairs test,  $Z = 0.6$ , n.s. and  $2.4$ ,  $P < 0.05$ , respectively). Bees trained to a yellow CS+ also responded in the same way during test (Wilcoxon matched-pairs test,  $Z = 1.0$ , n.s., and  $2.4$ ,  $P < 0.05$ , respectively).

Since bees failed to discriminate between similar colours such as yellow and green, we trained a new group of bees to a blue CS+ versus a white CS-, a colour pair with an intermediate chromatic similarity (see Table 1; Fig. 4). A second group was trained in parallel to a blue CS+ versus a yellow CS- that was dissimilar in colour. As expected from the previous experiment (see Fig. 2B), bees were able to quickly inhibit their response to a yellow CS- ( $N = 22$ ) (Fig. 4). The response to the white CS- was initially not different to that of the blue CS+ (Fig. 4). However, at the end of 10 training blocks

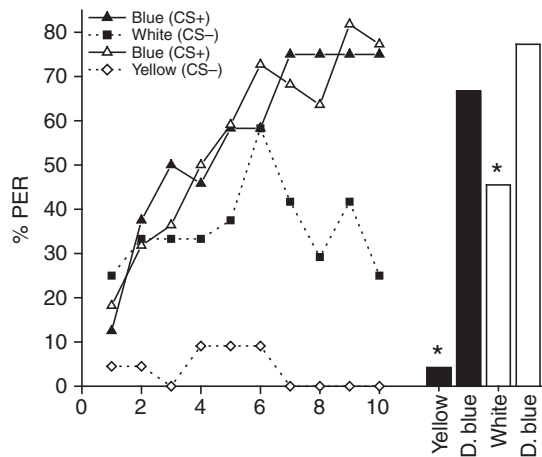


Fig. 4. Differential conditioning of intermediate colours. Bees were trained to discriminate a blue rewarded colour stimulus (CS+) (closed triangles) against a white unrewarded colour stimulus (CS-) (closed squares) or a yellow CS- (open diamonds) (corresponding blue CS+: open triangles), respectively. The first group was tested with a yellow and dim blue (d. blue) test stimulus (black bars), while the other group that experienced the yellow CS- was tested with a white and dim blue test stimulus (open bars). Asterisks depict significant differences between responses during the last acquisition trial and the test trial (Wilcoxon matched-pairs test,  $P < 0.05$ ). PER, proboscis extension response.

bees showed a significantly reduced response to the white CS- ( $N = 24$ ; Wilcoxon matched-pairs test,  $Z = 3.41$ ,  $P < 0.05$ ).

Interestingly, the conditioning procedure seemed to have affected the bees' responses towards these two colour stimuli of an intermediate similarity. Following absolute conditioning with the blue stimulus (first experiment; Fig. 1), bees responded strongly to white whilst after a differential conditioning, they were able to discriminate these colours. Furthermore, bees did not respond to white as a novel test colour after being conditioned to a blue CS+ versus a yellow CS- (Fig. 4) (Wilcoxon matched-pairs test,  $Z = 2.37$ ,  $P = 0.02$ ).

### Experiment 3: strength of acquisition after an absolute colour conditioning

How strong are the associations formed during visual PER conditioning with colours? In order to approach this question of interest when investigating learning mechanisms underlying classical PER conditioning, we trained bees to the yellow stimulus during three trials ( $N = 42$ ) (Fig. 5). One hour after the last training trial, bees were found to respond to the learnt colour with the same response probability as in the final training trial (Wilcoxon matched-pairs test,  $Z = 0.8$ , n.s.). Such a result ensured that the training and testing procedures of our experiments described above were performed in a reliable learning setting.

To test whether learnt associations were resistant to extinction as proposed by Kubawara (Kubawara, 1957), we continued to present the CS without sugar reward after an initial conditioning period of either three ( $N = 24$ ) or seven trials ( $N = 32$ ) (Fig. 5). The response rate dropped significantly between the last training trial and the third extinction trial in the group that had been rewarded seven times (Wilcoxon matched-pairs test,  $Z = 2.52$ ,  $P < 0.05$ ). Later extinction trials revealed no further decrease in performance. In the group trained in three trials, there was no significant drop in performance.

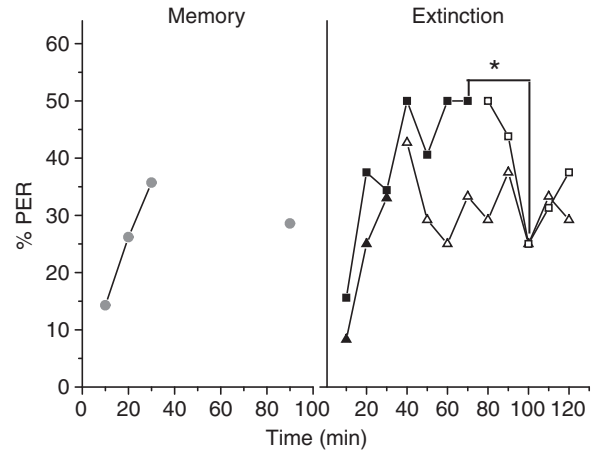


Fig. 5. Memory test and extinction trials after absolute conditioning. The memory recall for a previously rewarded yellow stimulus was tested after one hour of rest following a three trial conditioning period (grey circles). To test the response to extinction trials, bees were trained during three (black triangles) or seven learning trials (black squares) with a yellow stimulus. The training was followed by nine or five subsequent unrewarded stimulus presentations (open symbols), respectively. The asterisk depicts significant differences between responses during the last acquisition trial and unrewarded trials (Wilcoxon matched-pairs test,  $P < 0.05$ ).

## DISCUSSION

In the current study, we explored the ability of bees to learn, discriminate and memorise colours by conditioning the extension of the proboscis in restrained bees. Previous studies have shown variable, low or no success in classically conditioning bees to light stimuli (Kubawara, 1957; Masuhr and Menzel, 1972; Gerber and Smith, 1998), leading some authors to argue that the learning of visual cues in bees might be mediated by stimulus-response rather than stimulus-reward associations (Mauelshagen and Greggers, 1993). In the present study, we observed fast and stable acquisition that occurred within the first few trials for all of the five coloured stimuli used. Bees responded to a specific colour rather than to a general light stimulus. Colour-specific associations were retained for at least one hour. Our data support previous evidence that bees use colour information for the proboscis extensions towards a conditioned visual stimulus in expectation of reward (Kubawara, 1957; Daumer, 1958; Hori et al., 2006).

An interesting outcome of this study was the poor colour discrimination ability of bees. Colours that were predicted to be distinguishable for the bee eye on the basis of bee colour vision models and on evidence obtained in numerous studies with free-flying bees (e.g. von Helversen, 1972; Backhaus et al., 1987; Vorobyev et al., 2001; Giurfa, 2004) were not discriminated. The sensitivity functions of the three types of bee photoreceptors are relatively narrow and well-separated within their visible spectrum, allowing for an excellent colour vision with a high resolution of colours (von Helversen, 1972; Vorobyev, 1997; Vorobyev and Menzel, 1999). Still under the current experimental conditions, bees were not able to learn to discriminate between similar colours, although they did show discrimination for colour pairs that were very dissimilar or of intermediate similarity (the latter only following differential conditioning). We found that the least similar stimulus pair, blue and yellow, were discriminated easily from the second training trial onwards whereas the more similar stimuli, blue and

white, were discriminated only following the sixth trial onwards (Fig. 4). The most similar yellow–green and yellow–dim yellow stimulus combinations were not discriminated within the 10 blocks of CS+/CS– training provided (Figs 2 and 3). In the current experiments, the degree of difficulty reflected in the length of training required to achieve discrimination was clearly related to the degree of chromatic similarity. The question remains open as to whether, under the current experimental conditions using PER, conditioning similar colours, such as green and yellow, were below the threshold of discrimination. If this in fact was not the case, we hypothesise that a training period of more than 10 CS+/CS– blocks may have eventually resulted in differential responses between even similar colours. It would then be a case to provide bees with an extended training before they could perform well in a difficult discrimination task as shown for free-flying bees (Dyer and Chittka, 2004a).

We found different responses at testing to a colour of intermediate similarity (white) for bees experiencing either absolute or differential training to blue (Figs 1 and 4). The bees generalised strongly to white when blue had been trained alone whereas the response to white was significantly reduced when blue had been trained in differential conditioning *versus* yellow. This is in line with results from experiments comparing the performance of bees after absolute and differential conditioning to visual and olfactory stimuli (Giurfa et al., 1999; Giurfa, 2004; Dyer and Chittka, 2004b; Wright et al., 2008). These results provide support for the hypothesis that coarse colour discrimination in PER-mediated visual learning may be an effect of the strong generalisation of colours. More experiments are required to uncover the perceptual basis of the observed colour discrimination performance in the PER-conditioning paradigm and whether this improves with either extended or perhaps more frequent training.

Most results pointed to the use of chromatic rather than brightness-related similarity or difference between stimuli during learning. Bees were not using brightness differences within the brightness range tested in the present study. However, we cannot fully exclude the possibility that these could be perceived and perhaps even learnt. Under certain experimental conditions, animals may fail to demonstrate their ability to discriminate. For instance, fruit flies trained in a flight simulator (Ernst and Heisenberg, 1999) were able to discriminate some patterns, such as differently oriented bars and crosses or crosses and circles, as proven by distinct preferences during the pretraining phase. But they did not use this discrimination to solve the subsequently conditioned discrimination task with the same two stimuli. However, since free-flying bees are rather insensitive to brightness differences of stimuli of a similar or larger size than the spots we used here (Backhaus et al., 1987; Brandt and Vorobyev, 1997; Giurfa et al., 1997; Giurfa and Vorobyev, 1998; Hempel de Ibarra et al., 2000; Niggebrügge and Hempel de Ibarra, 2003), we conclude that it is most likely that in our current experiments bees did not rely on the brightness of stimuli.

We tested the extinction of the learnt colour and found that the response rate declined during the first three extinction trials after pairing the yellow stimulus with reward seven times. Kuwabara (Kuwabara, 1957) reported that bees that had learnt to respond to a colour still reacted after 30 consecutive unrewarded stimulus presentations. In this early work, he did not use a strict timing protocol for conditioning. Animals were trained during two days with ITIs ranging from 15 to 60 min, which may explain the difference between their study and our results. In our experiments, performance did not drop beyond a level that was similar to the

initial acquisition responses after three extinction trials. This result resembles the recovery effects that have been reported for olfactory PER conditioning one hour following the final of five extinction trials (Sandoz and Pham-Delègue, 2004). A direct comparison, however, is difficult and further studies are required to address the dynamics of extinguishing visually conditioned PER.

Based on our current results, we propose that the PER might be relevant for behavioural contexts in which colour information is not required to be as precise as it is known to be for visually guided behaviours during free flight. Olfactory and mechanosensory cues may be more important to bees in non-flight behaviour, such as orienting and detecting sugar reward whilst on a flower or communication in the hive, and therefore processed more thoroughly in such PER-related behavioural contexts. It remains to be studied further whether colour discrimination is indeed poor in a PER-conditioning context, and how it relates to the behavioural role of visually conditioned PER and to properties of sensory information processing pathways linked to the PER-eliciting network in the honeybee brain.

#### LIST OF ABBREVIATIONS

CS	conditioned stimulus
CS+	rewarded colour stimulus
CS–	unrewarded colour stimulus
ITI	inter-trial interval
PER	proboscis extension response
US	unconditioned stimulus

C.N. was funded by a fellowship of the Graduate School 'Functional Insect Science' (DFG, Germany), G.L. by the DFG (LE 1809/1-2) and N.H.I. by the Royal Society, UK. We thank E. Nicholls for helpful comments.

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