

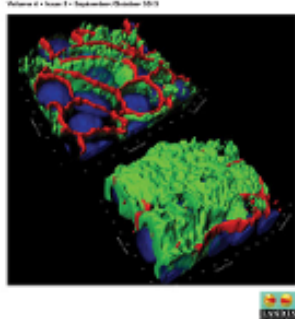
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Differences in colour learning between pollen- and sucrose-rewarded bees

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Abstract

What bees learn during pollen collection, and how they might discriminate between flowers on the basis of the quality of this reward, is not well understood. Recently we showed that bees learn to associate colours with differences in pollen rewards. Extending these findings, we present here additional evidence to suggest that the strength and time-course of memory formation may differ between pollen- and sucrose-rewarded bees. Colour-naïve honeybees, trained with pollen or sucrose rewards to discriminate coloured stimuli, were found to differ

in their responses when recalling learnt information after reversal training. Such differences could affect the decision-making and foraging dynamics of individual bees when collecting different types of floral rewards.

Key Words

bee, pollen, sucrose, reward, learning, memory, foraging

Abbreviations

Generalized Estimating Equations (GEE)

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The ability of bees to learn the location of profitable flower patches and the features of highly rewarding flowers is an adaptation to efficiently exploit flowers as a variable food source¹⁻⁵. This learning-based flower constancy has been widely studied in nectar-foraging pollinators⁵⁻⁷ and is likely to have been a powerful evolutionary driver in the dominance of nectar as the main reward type in modern angiosperms⁸⁻¹⁰. However, insect pollination pre-dates the emergence of nectar-producing organs, and not all flowers provide a nectar reward^{8, 9, 11-13}. This raises the question whether pollen alone is sufficient to reinforce learning in pollinating insects, and whether such learning processes differ from those which occur during reinforcement with sucrose solution.

Social bees collect pollen as a protein source for the brood and queen, and species such as honeybees and bumblebees are polylectic, meaning they forage for pollen from a range of different plant species. However, despite being pollen generalists, honeybees and bumblebees are known to preferentially collect the pollen of certain plants over others¹⁴⁻¹⁷. Such preferences can develop during the lifetime of a foraging individual¹⁸, although the sensory and neural mechanisms involved are currently unknown. Recently we demonstrated a role for learning in the development of pollen preferences, finding that individual bumblebees are able to discriminate pollen that has been artificially diluted with alpha-cellulose to varying degrees, and can associate such differences with the presence of a contextual coloured cue¹⁹. This kind of learning may be mediated by reward mechanisms other than those implicated in sucrose-rewarded learning, since pollen is not ingested but is actively packed into the leg corbiculae during collection. In further experiments we compared the learning and recall of colour memories in both sucrose and pollen-rewarded bees trained under the same experimental conditions. Given that colour learning in bees is fast, and strong long-term memories can already be formed after three colour-reward pairings, we opted to use a colour-

reversal conditioning paradigm^{20,21}. We expected that bees would learn better when rewarded with sucrose than with pollen, since it is known from studies with sucrose-rewarded bees that individuals prevented from imbibing a reward show lower levels of acquisition²²⁻²⁴.

Small colonies were housed within a flight net¹⁹. Colour-naïve honeybees were hatched in an incubator and kept in a colour-neutral environment within the flight net to prevent the formation of foraging-related colour experiences and thus achieve equal levels of colour exposure during the experiment. Pollen was provided in the dark, inside grey opaque boxes which prevented exposure to the colour of pollen. Bees were observed entering the boxes to collect pollen, or feeding from a grey sucrose feeder, and were marked individually. Subsequently they were conditioned with their respective feeders to fly back and forth between the flight net and a testing arena connected by a wide Plexiglas corridor with sliding doors. To begin with, a bees' initial colour preferences were tested in the absence of rewards, by recording the number of approaches an individual bee made to two coloured stimuli (Blue/Yellow) mounted on the surface of a grey box (Fig. 1B). Two-tailed paired *t*-tests were used to compare bees' choices to each stimulus. In the colour-learning task that followed, stimuli were mounted on the surface of two grey boxes (Fig. 1A), and pollen-rewarded bees had to learn to enter and leave the box via entrance tubes positioned at the centre of the rewarding colour (e.g. Blue). The end of the tubes in the centre of the unrewarding colour (e.g. Yellow), and all tubes used when training sucrose-rewarded bees, were covered in mesh to prevent access to the box. The mesh still permitted the odour of pollen to diffuse out, meaning both pollen and sucrose-rewarded bees were exposed to pollen odour during learning. Sucrose-foraging bees were rewarded with droplets of sucrose (30% w:w) at a distance of approximately 1 cm from the entrance of the tube. Tubes of the unrewarded colour contained a drop of water to prevent bees using water vapour as a cue. All bees

returned to the hive after each training trial to unload the rewards and returned to the entrance of the testing arena voluntarily to continue foraging in subsequent trials. After each test trial bees also returned to the flight net and could enter the hive. We therefore assume that bees of each forager type were equally satiated and motivated to forage.

In the first experiment, pollen- ($n=7$) and sucrose-foraging ($n=6$) bees were individually trained in a bout of five trials in which Blue was the rewarding colour (Blue⁺), followed by a further five Yellow-rewarded (Yellow⁺) trials. Colour preferences were re-tested following each five trial bout, revealing that under our experimental conditions, naïve honeybees could learn to associate either a pollen or sucrose reward with a coloured stimulus, and that their preferences shifted quickly following a reversal in the rewarding colour (Fig. 1C). We did not find any striking differences in the choices of each forager type during unrewarded colour tests. Interestingly, the time taken to enter a correctly rewarding tube (search time) during acquisition differed to some extent (Fig. 1D). General estimating equation (GEE) modelling was used to test whether bees adjusted their search behaviour towards the rewarding colour within each 5-trial bout, with search time as the response variable, and forager type and trial or bout number as factors. Pollen foragers exhibited significantly longer latencies in finding the correct rewarding stimulus than sucrose-rewarded foragers during training (GEE, Forager type, $X^2_1=16.176$, $p<0.001$). However, for both groups, latency decreased within each bout (Least significant difference (LSD) contrast, Pollen, T1-T5 $p=0.028$; T6-T10 $p=0.001$; Sucrose T1-T5 $p=0.009$; T6-T10 $p=0.020$), and by the fifth trial of each bout foragers from both groups exhibited similar search times.

In a second experiment, a new cohort of bees ($n=12$) were trained for twenty trials in a colour-reversal learning task, where bouts of five Blue⁺ and five Yellow⁺ trials were alternated (Fig. 2A). Bees' colour preferences were tested before training to assess

spontaneous preferences. After training, memory recall of the two colours was tested; immediately after the fourth training bout and following a delay of one hour to distinguish interference effects arising from the most recently trained colour (Yellow⁺).

Both pollen and sucrose foragers had a strong initial preference for Blue (Fig. 2A, Pre-Test, 2-tailed paired *t*-test, Pollen-reward $t_{14}=3.262$ $p=0.006$; Sucrose-reward $t_{11}=5.836$, $p<0.001$).

When re-tested after the fourth training bout (Yellow⁺) of the colour-reversal task, both pollen and sucrose-rewarded bees showed a strong preference for Yellow (Fig. 2A, Test 1, 1-tailed paired *t* test, Pollen $t_5=3.376$, $p=0.020$; Sucrose $t_5=4.417$, $p=0.010$), which indicates that the most recent colour task (Yellow⁺) determined immediate memory recall in both groups. Though overall search times did not differ (GEE, Forager type $\chi^2_1=0.808$, $p=0.369$), during the reversal task pollen and sucrose-rewarded bees responded differently to switches in rewarding colour. Whilst sucrose-rewarded bees exhibited little difference in search times between the first trial of a new bout and the last trial of the preceding bout (Fig. 2B, LSD contrast, ns), pollen rewarded bees exhibited a significant increase in time taken to find the rewarding colour in all but the final switch (LSD contrast, T₅-T₆ $p=0.009$, T₁₀-T₁₁ $p=0.031$, T₁₅-T₁₆ $p=0.493$). By the fifth trial of each bout, there was no difference in the search times of the two groups.

After Test 1, which immediately followed the fourth training bout, bees returned to the colony flight net, but all food sources were removed from the net to prevent foraging. Following a one hour rest, bees were allowed to return to the test arena for a second unrewarded preference test (Test 2). Two completely different choice patterns were observed between pollen and sucrose-rewarded bees. Whilst pollen foragers chose both colours equally (Fig 2A, Test 2, $t_5=1.452$, $p=0.210$), sucrose-rewarded bees strongly preferred Blue ($t_5=5.531$, $p=0.033$). This was not the result of a lowered motivation to forage as bees in both groups

were equally fast in finding the rewarding stimulus in the first trial of a final Yellow⁺ training bout (LSD contrast, $T_{21} p=0.424$) and showed a strong preference for Yellow in the final test (Fig. 2A, Test3, Pollen $t_5=4.596$, $p=0.030$; Sucrose $t_5=4.992$, $p=0.035$). It is also unlikely that the sucrose-rewarded bees experienced memory loss and reverted to the spontaneous preference. Previous studies have shown that in sucrose-rewarded bees, reversal conditioning within the same context leads to the formation of memories for both trained colours²³⁻²⁵. We conclude that these bees formed associations of different strengths for the Blue and Yellow stimuli which influenced their choices in a test situation that was ambiguous with respect to the given colour task. It is possible that the first-learned colour was consolidated rapidly during the initial training trials, forming a more robust memory and influencing memory formation for the second colour in the next phase of the reversal training²⁵⁻²⁷. Menzel²⁵ showed that for sucrose-rewarded bees, between one to five trials of learning one colour were sufficient to interfere with subsequent acquisition of a second colour association in the same context. These interferences disappeared after extended training and bees chose both colours equally. After a long delay, where the setup was removed for the whole of the next day and installed again on the second day after training, he observed that bees still chose both colours equally, though interference effects reappeared during the initial bouts of the continued reversal training. Whilst our results do not fully mirror those findings, as our experiments differed to some degree in procedures, and we used naïve bees, they are nevertheless consistent with this and other previous work showing that successive conditioning of two colours leads to varying interactions during learning and memory recall^{25, 27}.

The present findings provide preliminary evidence to suggest that differences exist in the learning mechanisms of bees rewarded with sucrose or pollen. Whilst there are no indications that pollen-rewarded colour learning is much slower or worse than in sucrose-rewarded bees,

the distinct performances in memory recall hint at variations in the time course or strength of memory formation with these two reinforcers. This could influence how flexible pollen and sucrose foragers may be when responding to variations in floral resources. One could speculate that learning two colours equally might enable pollen foragers to more flexibly switch between several learnt flower colours when a preferred flower becomes temporarily unavailable or rare which would be advantageous given the costs of learning to handle new flowers. Since handling is generally considered to be less important in determining foraging success in nectar foragers, the temporarily stronger fixation on a particular colour due to faster consolidation of the first-established memory could be less costly than for pollen foragers. To be able to test such scenarios, however, future studies should elucidate the learning mechanisms underlying pollen rewards further and how pollen-rewarded learning influences decision-making, the foraging dynamics of individual foragers and potentially the regulation of task partitioning in bee colonies.

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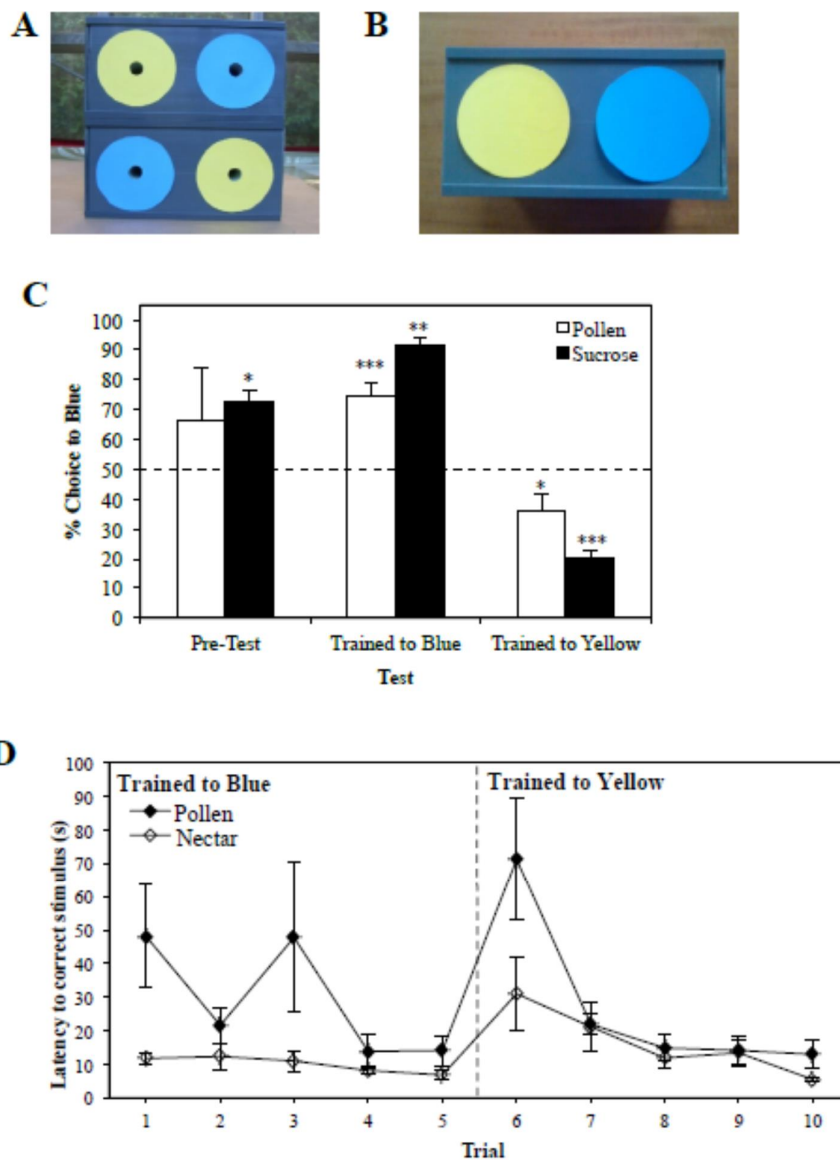


Figure 1: Test apparatus. (A) Bees had to learn to enter the boxes via a tube positioned in the centre of the correct colour to access the reward. For sucrose-rewarded bees, a drop of sucrose was provided inside the entrance tubes. For the unrewarded colour and sucrose-rewarded bees, the ends of the entrance tubes were covered with mesh permit the odour to diffuse out. Arrangement of the stimuli was changed after each trial. (B) Prior to and following training bees colour preferences were tested in the absence of rewards.

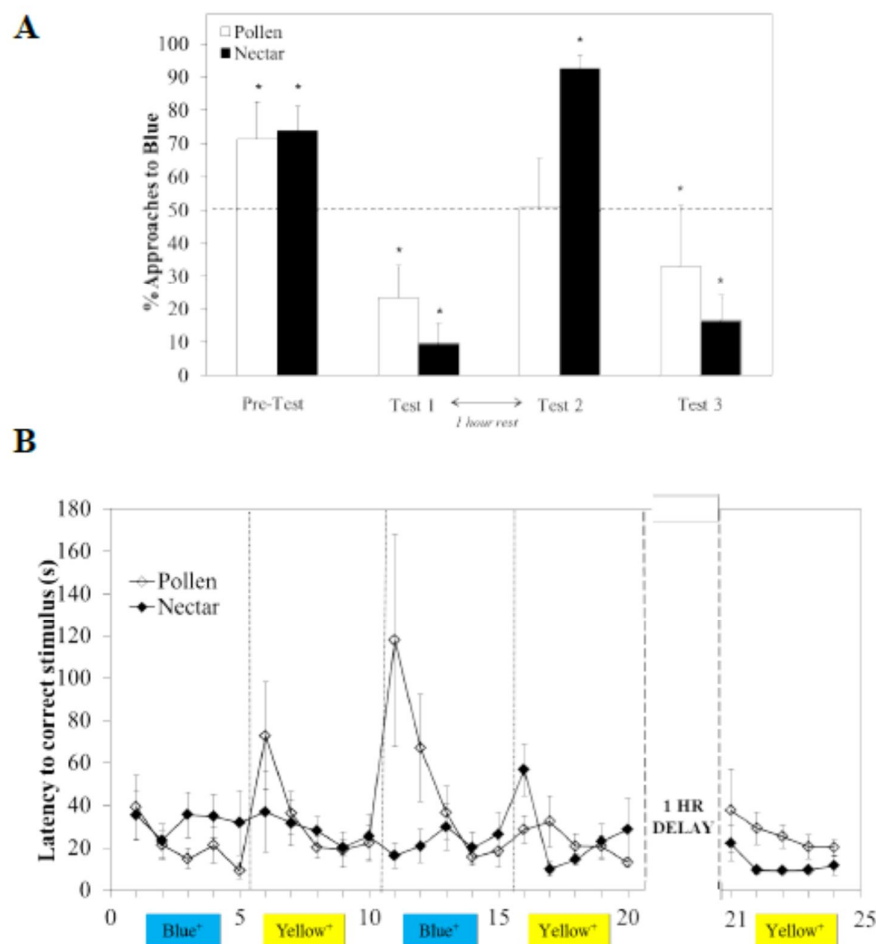


Figure 2: Pollen (white bars, mean % approaches to blue \pm SE, N=15) and sucrose-rewarded (black bars, N=12) bees showed a strong preference for blue prior to training (A). The rewarded color was switched every five trials, for twenty trials, after which bees from both groups had a preference for Yellow, the last rewarded color (Test 1). After a one-hour rest however, pollen rewarded bees chose both colors equally (N=6), whereas sucrose-rewarded bees (N=6) strongly preferred blue (Test 2). In the final bout of training to yellow, both groups preferred this color (Test 3). During each five trial bout, both pollen (white diamonds, mean latency \pm SE) and sucrose-rewarded bees (black diamonds) showed a reduction in search time between the first and last trial (B), though pollen-rewarded bees exhibited longer search times immediately following a color reversal.