

RESEARCH ARTICLE

Bees associate colour cues with differences in pollen rewards

Elizabeth Nicholls* and Natalie Hempel de Ibarra†

ABSTRACT

In contrast to the wealth of knowledge concerning sucrose-rewarded learning, the question of whether bees learn when they collect pollen from flowers has been little addressed. The nutritional value of pollen varies considerably between species, and it may be that bees learn the features of flowers that produce pollen best suited to the dietary requirements of their larvae. It is still unknown, however, whether a non-ingestive reward pathway for pollen learning exists, and how foraging bees sense differences between pollen types. Here we adopt a novel experimental approach testing the learning ability of bees with pollen rewards. Bumblebees were reared under controlled laboratory conditions. To establish which pollen rewards are distinguishable, individual bees were given the choice of collecting two types of pollen, diluted to varying degrees with indigestible α -cellulose. Bees preferentially collected a particular pollen type, but this was not always the most concentrated sample. Preferences were influenced by the degree of similarity between samples and also by the period of exposure, with bees more readily collecting samples of lower pollen concentration after five trials. When trained differentially, bees were able to associate an initially less-preferred contextual colour with the more concentrated sample, whilst their pollen preferences did not change. Successful learning of contextual cues seems to maintain pollen foraging preferences over repeated exposures, suggesting that fast learning of floral cues may preclude continuous sampling and evaluation of alternative reward sources, leading to constancy in pollen foraging.

KEY WORDS: Pollen, Learning, Preferences, Evaluation, Bumblebees

INTRODUCTION

The ability to learn the floral features of plants providing the best nectar rewards, and preferentially visit these flowers over other available sources of nectar (a behaviour termed flower constancy), has been well studied in bees (Ribbands, 1949; von Frisch, 1967; Waser, 1986). However, it is unclear whether bees form associative relationships between floral features and pollen quality and how this may be linked to the development of individual pollen foraging preferences, given that bees do not ingest this reward whilst foraging. Previous studies indicate that bees learn to manipulate pollen-rewarding flowers (Raine and Chittka, 2007), and demonstrate that they can associate pollen odours with a sucrose reward (von Aufsess, 1960; Cook et al., 2005). Whilst bees can sense pollen with their antennae (Scheiner et al., 2004), attempts to condition the proboscis extension response (PER) with pollen as the unconditioned stimulus have proved unsuccessful (Nicholls and

Hempel de Ibarra, 2013), suggesting that under restrained conditions at least, pollen does not reinforce learning. If a reward pathway for pollen exists in the bee brain, it may be quite distinct from that for sucrose and may operate within a more restricted behavioural context than sucrose-reinforced PER. In contrast, Arenas and Farina (Arenas and Farina, 2012) reported that bees can recall odours that have been paired with a pollen reward; however, it is possible that prior foraging experience on nectar sources could account for this. In the present study we used naïve bumblebee (*Bombus terrestris* L.) foragers, housed under controlled conditions, to test whether bees learn during pollen collection. Specifically, we investigated whether bees can distinguish between pollen types, and whether they can associate contextual colour cues with differences in pollen rewards.

Foragers of generalist bee species, such as honeybees and bumblebees, collectively bring in pollen from a wide range of plant species. Nevertheless, they do not exploit all available pollen sources, exhibiting foraging preferences (Wahl, 1966; Boch, 1982; Schmidt, 1982; Levin and Bohart, 1955; Doull, 1966; Robertson et al., 1999; Cook et al., 2003; Hanley et al., 2008), which suggests that they are able to distinguish between pollen sources and locations when collecting from different plant species. Field experiments with bumblebees have shown that individual foraging preferences correlate with the availability, and in some cases the protein and amino acid concentration (typically used as a proxy for nutritional quality), of pollen provided by flowers (Cook et al., 2003; Hanley et al., 2008). Robertson et al. (Robertson et al., 1999) observed bumblebee visits to *Mimulus guttatus*, a species polymorphic with regards to the quality of pollen it provides. In dual-choice field assays, the best predictor of foraging preference was found to be protein content, and bees were also more likely to forage, and foraged for longer in patches of *Mimulus* varieties producing better quality pollen.

In previous experiments pollen has been successfully manipulated by adding indigestible α -cellulose (Waddington et al., 1998; Pernal and Currie, 2001). Honeybees have been shown to be less likely to dance to advertise the availability of diluted pollen (Waddington et al., 1998), and bumblebees reduce colony foraging rates, relative to those for pure pollen (Kitaoka and Nieh, 2009). So far, evidence from field and laboratory experiments confirms that bees are sensitive to differences in pollen rewards, but the processes leading to the formation of pollen preferences in an individual remains unknown. Previous studies have not distinguished the effects of prior foraging experience and exposure to contextual cues and/or social information from the non-digestive assessment of nutritional value by individual foragers during pollen collection (Schmidt, 1982; Waddington et al., 1998; Pernal and Currie, 2001; Pernal and Currie, 2002; Kitaoka and Nieh, 2009).

Carefully controlling the foraging experience of naïve bumblebees under laboratory conditions, we evaluated preferences for pollen samples diluted with indigestible α -cellulose during single foraging bouts and after multiple exposures to determine whether bees could distinguish them. To rule out the influence of social information,

Centre for Research in Animal Behaviour, School of Psychology, University of Exeter, Perry Road, Exeter EX4 4QG, UK.

*Present address: Department of Evolution, Behaviour and Environment, School of Life Sciences, University of Sussex, Falmer, Brighton BN1 9QG, UK

†Author for correspondence (n.hempel@exeter.ac.uk)

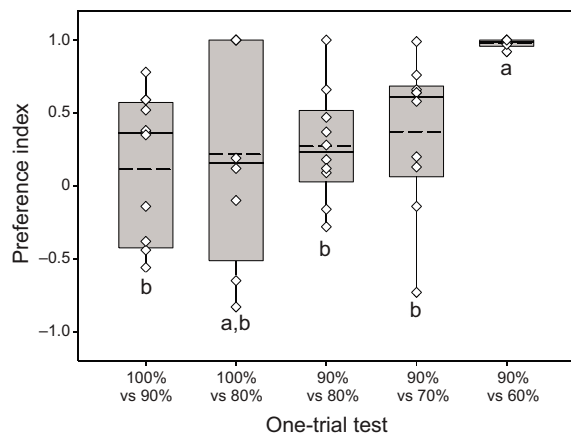


Fig. 1. Preference index scores (open diamonds) based on time spent foraging on each pollen sample during a single exposure to two pollen types. Scores greater than zero indicate a preference for the sample with the higher pollen content. Solid lines represent the median preference score, dashed lines the mean. A one-way ANOVA revealed an effect of sample pairing on the strength of the preference index; letters denote significant differences between groups (Dunnett's T3 *post hoc* test).

bees were tested individually. Subsequently, we examined whether bees could associate differences in pollen quality with contextual coloured cues, which could potentially allow individuals to make foraging decisions prior to alighting on a food source, as seen in nectar-foraging bees.

RESULTS

Bees were able to distinguish between pollen samples very quickly, foraging preferentially on one of two pollen types within a single trial (Fig. 1). The time bees spent collecting one sample over another was significantly different (paired *t*-test, 100% versus 90%, $t_{11}=6.180$, $P<0.001$; Wilcoxon test, 90% versus 80%, $Z=-2.803$, $P=0.005$; 90% versus 70%, $Z=-2.803$, $P=0.005$). Not all individuals preferred the more concentrated sample, and when offered two novel samples (100% and 80% pollen) individual collection preferences were spread between both concentrations ($Z=-2.521$, $P=0.012$; Fig. 1). Overall, bees made a greater proportion of re-landings to their preferred sample (90% versus 100%, $Z=-3.059$, $P=0.002$; 90% versus 80%, $t_8=2.714$, $P=0.026$; 90% versus 70%, $t_6=3.457$, $P=0.007$). Bees offered 90% versus 60% showed a strong uniform preference for the higher quality pollen ($Z=-2.201$, $P=0.028$), and made exceptionally few landings and no re-landings on 60%, suggesting the use of pollen-derived visual and possibly olfactory cues to inform foraging decisions prior to landing.

Adding α -cellulose changes the appearance and olfactory intensity of the pollen samples. We therefore evaluated the discriminability of the samples by measuring the pollen spectra and testing a new group of bees directly to determine whether they could distinguish between samples on the basis of olfactory cues alone. By manipulating the mass of pollen provided in the test dishes covered with pierced lids, it was possible to keep visual cues identical, whilst varying the olfactory intensity of the samples to match the concentrations used in the previous experiment. Bees were offered Petri dishes either containing 3 g of 90% pollen or 2.33 g of 90% pollen, which equates to the same mass of pollen and therefore olfactory intensity of 3 g of a 70% pollen- α -cellulose mixture. To simulate the olfactory difference between 90% and 60%, bees were offered dishes containing either 3 or 2 g of 90% pollen. Bees showed

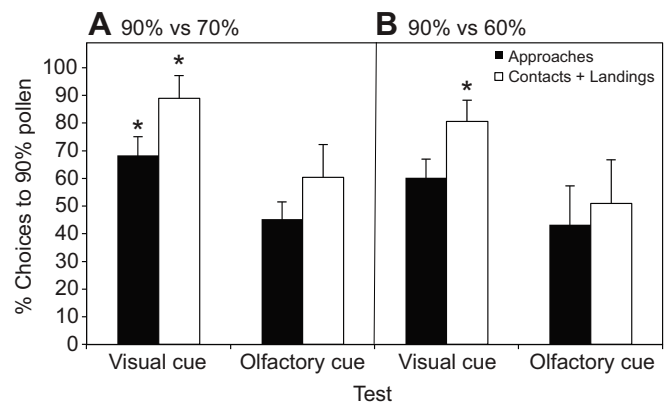


Fig. 2. Discrimination performance of bees in unrewarded tests after a short pre-exposure to 90% pollen. Pollen contents in the dish were manipulated such to simulate olfactory differences between samples whilst keeping the visual appearance the same, or vice versa, presenting visually different samples that were matched in their olfactory intensity. Four groups of bees were offered either (A) 90% versus 70% pollen (visual cue $N=6$, olfactory cue $N=6$) or (B) 90% versus 60% pollen (visual cue $N=7$, olfactory cue $N=5$). Black bars represent the proportion of approaches to 90% pollen and white bars the proportion of contacts. Asterisks denote a significant deviation from equal choice ($*P\leq 0.05$).

no preference in terms of their approaches or contacts to one dish over another (Fig. 2A), suggesting that they were not simply more attracted to the stronger smelling samples (90% versus 70% olfactory intensity: approaches, $t_5=0.479$, $n=94$, $P=0.470$; contacts, $t_5=0.547$, $n=87$, $P=0.607$; 90 versus 60%: approaches, $t_4=0.550$, $n=66$, $P=0.612$; contacts $t_4=0.394$, $n=72$, $P=0.713$).

When this experiment was repeated, using samples diluted to varying degrees but manipulating the mass provided in order to match odour intensity, bees did show a preference in the test between samples on the basis of visual appearance alone (Fig. 2B). When provided with a choice between 3 g of 90% pollen and 3.86 g of 70% pollen (matching the mass of pollen present in 90% pollen), bees showed a clear test preference for 90% pollen ($t_5=3.144$, $n=76$, $P=0.026$). Likewise, in a choice between 3 g of 90% and 4.5 g of 60% pollen, there was a visible trend towards 90% pollen in the number of approaches and a significant difference between the number of contacts (approaches, $t_6=1.543$, $n=57$, $P=0.174$; contacts, $t_6=2.594$, $n=59$, $P=0.041$).

To understand how bees' collecting preferences may change with increasing foraging experience, we selected two pairs of pollen samples based on the results of the previous experiments: a pair that yielded uniform collection preferences for the higher concentration across all bees during the first trial (90% against 60%), and the pair closest to it in concentration, but that resulted in more scattered collection preferences (90% against 70%). We predicted that collection preferences would persist or possibly even strengthen with increasing experience, but in fact the collection preference for 90% over 70% pollen shifted by the fifth trial, with some individuals switching to preferentially collect 70% pollen (trial 5, $t_5=3.277$, $P=0.022$; Fig. 3). The same was observed for 90% versus 60% pollen, despite the fact that bees had a strong initial collection preference for 90% (trial 5, $t_5=2.238$, $P=0.075$).

Importantly, in experiments with either single or repeated exposure to two types of pollen, collection preferences were not predicted by the first dish encountered, and individuals made a large number of switches throughout the entirety of each trial (mean \pm s.e. = 12.6 ± 1.2). On average, bees in the first experiment spent 3 min 35 s (± 23 s s.e.m.) handling pollen in the dishes, and there was no

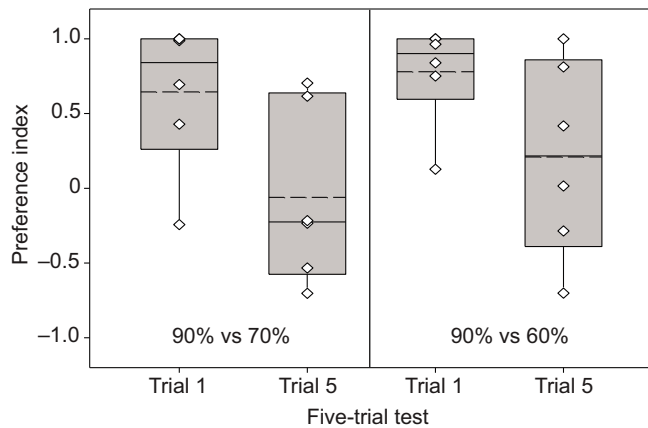


Fig. 3. Preference index scores (open diamonds) based on time spent foraging on each pollen sample on the first and last trial, following five exposures to pollen samples. (A) Bees given the choice between 90% and 70% pollen. (B) Bees given the choice between 90% and 60% pollen. Scores greater than zero indicate a preference for the sample with the higher pollen content. Solid lines represent the median preference score, dashed lines the mean.

significant difference in handling time between treatments ($H_4=5.812$, $P=0.214$). Furthermore, there was no correlation between preference scores and total time spent collecting pollen in the dishes (handling time), indicating that bees that preferentially collected the more diluted samples did not compensate for the lower concentration of pollen by collecting more.

Given that the bees distinguished between pollen samples, we could use these as rewards to train bees differentially. In a final set of experiments, we examined whether bees could associate contextual coloured cues with differences in the pollen samples. During an unrewarded test, individual bees were presented with a pair of coloured stimuli (blue versus green; orange versus white) and their spontaneous colour preference was recorded. Subsequently, during training, the higher pollen concentration (90%) was paired with the non-preferred colour (blue) and the lower pollen concentration (either 60 or 70%) was paired with the preferred colour (green). If bees were able to form associations between coloured cues and the differences in pollen samples, after training their colour choices should differ from the spontaneous level, when tested again in the unrewarded colour preference test. Indeed, bees were able to associate contextual coloured cues with the 90% and 60% pollen (Fig. 4B). Their initially significant test preference for green ($Z=-2.207$, $P=0.027$, $n=87$ approaches) vanished following five trials of differential training, and in the unrewarded post-training test, bees chose the higher-rewarding blue equally as often as green ($Z=-1.219$, $P=0.223$, $n=66$). The number of contacts did not change significantly, presumably because of a reduction of contacts in the post-training test ($n=17$ compared with $n=57$ prior to training). Throughout training trials, bees collected only 90% pollen and did not make a single landing on the 60% sample, suggesting that contextual cues assisted in the maintenance of their original preference for 90%. This result is in stark contrast with the previous experiment where, in the absence of coloured contextual cues, bees foraged on both 60% and 90% pollen by the fifth trial (Fig. 3).

When the difference between pollen samples was smaller (90% versus 70%), however, bees did visit the more diluted sample during training trials and maintained their preference for green in the unrewarded post-training test ($Z=-2.201$, $P=0.028$, $n=137$; Fig. 4A). The distribution of contacts did not change between the unrewarded

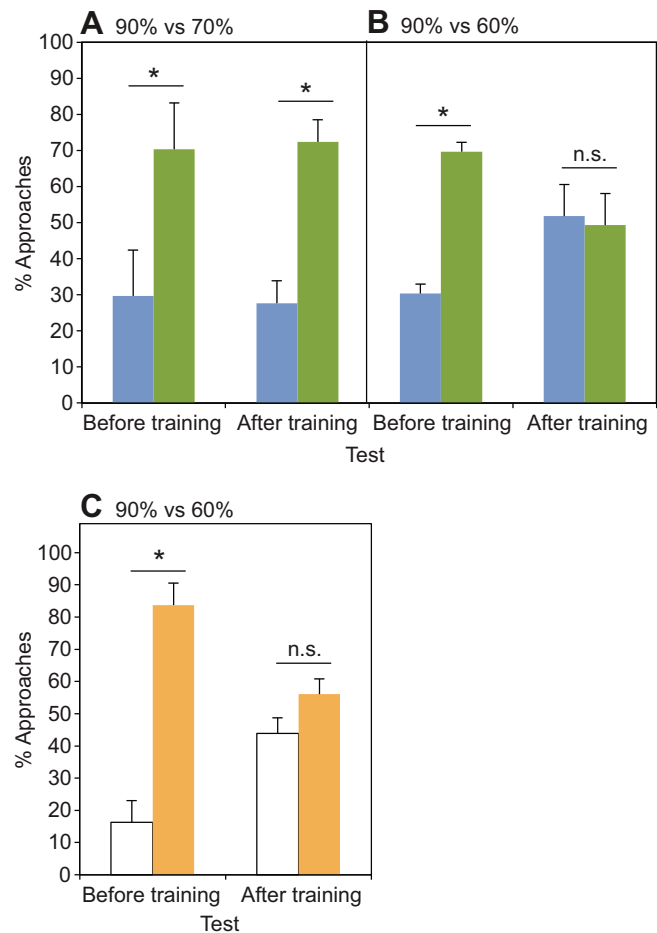


Fig. 4. Mean proportion of approaches made to blue (paired with 90% pollen) and green (paired with either 70% or 60% pollen) stimuli in unrewarded tests. (A) 90% versus 70%. (B) 90% versus 60%. (C) Same as B, but with a different colour pair (white paired with 90% pollen versus orange paired with 60% pollen). Error bars denote standard error. Asterisks denote a significant deviation from equal choice ($*P\leq 0.05$).

tests (pre-training test $n=24$, post-training test $n=14$). When the 90% versus 60% discrimination was repeated with a new group of bees and another combination of coloured cues (white versus orange), results confirmed that the shift in colour preference is not dependent on the particular set of coloured stimuli used (Fig. 4C). Prior to training, bees displayed a significant test preference for orange ($Z=-2.023$, $N=5$, $n=54$, $P=0.043$), whereas after training bees shifted their test preference towards white (paired with 90% pollen during training), making a similar number of approaches to both colours ($t_4=0.623$, $n=158$, $P=0.567$). The number of contacts changed in a manner similar to that of the approaches, but the sample size was too small to support any conclusions with statistical significance (pre-training test $n=15$, post-training test $n=15$).

DISCUSSION

Our results show that bumblebees can individually assess pollen samples and discriminate between them during collection, quickly forming preferences, though these may alter with experience. In support of previous field observations (Cresswell and Robertson, 1994; Robertson et al., 1999; Hanley et al., 2008), our work further demonstrates that bumblebees have the capacity to associate differences in pollen quality with floral features such as petal colour,

thus enabling bees to discriminate between flowers prior to alighting and efficiently exploit the best available pollen rewards.

Previous studies of pollen preferences have either observed the behavior of naturally foraging bees (e.g. Schmidt, 1982; Cresswell and Robertson, 1994; Robertson et al., 1999; Hanley et al., 2008) or monitored fluctuations in colony foraging effort, depending on pollen availability (e.g. Fewell and Winston, 1992; Pernal and Currie, 2001; Pernal and Currie, 2002; Kitaoka and Nieh, 2009), and have yielded contradictory conclusions regarding whether bees are able to assess differences in the nutritional quality of pollen. Waddington et al. (Waddington et al., 1998) showed that honeybee foragers were less likely to dance, or danced less vigorously, for pollen that had been diluted with indigestible α -cellulose, suggesting that they deemed diluted pollen to be a poorer resource than pure pollen. Pernal and Currie (Pernal and Currie, 2001), however, maintain that honeybee foragers lack the ability to individually assess pollen protein content and must rely on feedback from nurse bees. When the quality of pollen stored in honeybee hives was manipulated, they observed that honeybees responded by increasing foraging effort, rather than opting to collect pollen from plants with more protein rich pollen. The results of the present study suggest that a number of factors besides direct assessment of nutritional quality may influence pollen preferences, such as prior experience, evaluation of the range of qualities available and learning of contextual floral cues.

Prior experience may have a substantial effect on initial foraging preferences. For example, many individuals had an initial preference for 90% pollen over the higher quality pure pollen, presumably because they had learnt during pre-training to recognize the sensory and/or physical characteristics of this particular reward. However, we also observed that rather than maintaining or strengthening preferences, repeated exposure to two types of pollen led to bees more readily collecting poorer quality samples. Though diluted, the poorer sample still contained a large amount of pollen, and given the size of our experimental setup, there was no difficulty to bees exploring the alternative pollen source during repeated trials, presumably allowing them to reassess the quality of the poorer sample relative to the familiar one. A lack of substantial differences in foraging costs between the samples, which may be required to lead to accurate discrimination in the absence of additional cues, may have meant that bees accepted the more diluted pollen more readily because it still provided a sufficiently high reward. Why this might result in a shift of preferences in some individuals remains unclear. When foraging on sucrose solutions, bees evaluate food sources not purely on the basis of absolute differences in nutritional content, but also account for other conditions that determine the temporal, metabolic and social costs of collection (e.g. Núñez, 1982). This is probably also true for pollen collection.

We used a mixture of pollen species to balance non-nutritional sensory and/or physical characteristics of pollen that can differ between plant species. Some studies claim that bees may be sensitive to features such as grain size, moisture content and electrostatic charge, all of which could affect packing efficiency (Stanley and Linskens, 1974; Vaissière and Vinson, 1994; Vaknin et al., 2000); however, the experimental evidence is very weak, considering that previous experience was not controlled for, bees were exposed to several pollen types, and comparisons with multi-species pollen in between tests with single-species pollen were allowed during the experiments. Nevertheless, we cannot completely rule out that in our experiments more diluted mixes of pollen and α -cellulose possessed some physical advantages for being packed into the pollen baskets, making collection more efficient and thus

potentially driving the switch in preference observed in some bees, or weakening of preference observed in others, once the sensory cues of the alternative sample were evaluated and learnt over several trials. Importantly, however, handling time was the same across treatments, and the variation in preferences across all experiments evinces that the discriminability of samples was not simply based on preferences for a higher packaging quality, with lower dilutions being easier to pack.

Providing the difference between the pollen samples was sufficiently large, bees succeeded in learning to identify pollen samples via contextual coloured cues and did not collect more diluted pollen following repeated exposures. Likewise, honeybees display reward-matching choices when differences in sugar rewards are large and contextual cues permit learning of the features of the food source (Banschbach, 1994; Greggers and Muelshagen, 1997), forming the basis for flower constancy. Given the variation in the suitability of pollen from different plant species for rearing larvae (Schmidt et al., 1995; Roulston and Cane, 2000; Tasei and Aupinel, 2008), a similar mechanism may lead to flower-constant behaviour during pollen collection, which could conceivably result in selection on the quality of pollen produced by insect-pollinated flowers. This is of particular significance for flowers that do not provide a nectar reward, though the primary role of pollen as an agent for gamete transmission likely imposes constraints on the degree of selection by pollinators (Roulston et al., 2000).

We never observed bees eating pollen during collection. This is in line with other work that suggests that foraging honeybees do not consume pollen (Michener et al., 1978; Thorp, 1979; Thorp, 2000). Though Smeets and Duchateau (Smeets and Duchateau, 2003) report lower survivorship in adult bumblebees prevented from accessing pollen, this is not evidence that foragers eat pollen. Those bees were raised on an artificial nectar solution, and therefore would have also been lacking access to amino acids and proteins found naturally in floral nectar (Baker and Baker, 1986).

In conclusion, pollen foraging behaviour involves learning and individual decision-making, which influences the formation of preferences. Additional studies will be required to fully understand the sensory processes underpinning reward evaluation and learning during pollen collection.

MATERIALS AND METHODS

Naïve bumblebees (*Bombus terrestris* L., Koppert, Suffolk, UK) were kept within an indoor flight net (100×100×80 cm), thus maintaining a constant sensory and foraging environment throughout experiments. Bees were fed *ad libitum* with black sucrose feeders (50% w/w). Commercially available pollen (Werner Seip, Ebersgöns, Germany), was presented to bees in Petri dishes (9 cm diameter), finally ground and diluted to 90% (w/w) with α -cellulose. Pollen-collecting bees were individually marked with numbered tags.

Experimental setup

The flight net was connected to a test arena of the same size, via a Perspex corridor with several doors to ensure that only one bee at a time was permitted to enter the test arena. Pollen was presented to bees in Petri dishes (5 cm diameter) placed randomly on a disc (75 cm diameter) of grey paper, with a transparent Perspex cover for ease of cleaning. Between trials, spilt pollen was removed and dishes were moved frequently to prevent positional bias. All surfaces were cleaned with ethanol to remove any potential markings. Only one bee was present in the arena during each training trial and test. Behaviour was video-recorded with a camera suspended from the ceiling of the flight cage.

Experiment 1

The quality of pollen was manipulated by diluting samples with α -cellulose (Waddington et al., 1998; Pernal and Currie, 2001). Following *ad libitum*

access to 90% pollen (w/w) in the living cage, and two training trials with 90% pollen in the experimental arena, bees were given a single rewarded trial in which they were presented with four pollen dishes (5 cm diameter), two of each sample type. Individual bees were tested only once with one of the following sample pairs. Five groups of new bees were tested with the following sample pairs: 100% versus 90% ($N=12$), 90% versus 80% ($N=10$), 90% versus 70% ($N=10$) and 90% versus 60% ($N=6$). One group of bees ($N=8$) was also tested with undiluted and 80% pollen, neither of which were experienced during pre-training, to determine their preference for two novel samples. Each dish contained 3 g of a particular sample type.

Experiment 2

We predicted individual preferences for the higher quality pollen sample to strengthen with prolonged exposure, as bees should become better at discriminating between samples over time. To test this, two new groups of bees collected pollen in five trials with 90% versus 70% ($N=6$) or 90% versus 60% ($N=6$), the sample pairings for which bees had the clearest preference for the higher quality pollen in the previous experiment. An individual preference index was calculated by normalizing the time difference between foraging on the less and the more diluted sample to the overall time spent collecting pollen in a trial. As an additional measure of active discrimination by bees, we also calculated the proportion of re-landings made to each individual's preferred and non-preferred sample.

Experiment 3

We tested whether bees could distinguish between samples of differing quality prior to alighting by relying on information provided by the odour and/or appearance of the pollen samples. After being pre-trained to 90% pollen, bees were presented with four Petri dishes, which were covered with transparent lids to prevent access to the pollen. Small holes permitted the odour of the pollen to diffuse through. By manipulating the mass of the different pollen samples it was possible to alter the visual or olfactory characteristics of the samples independently.

In the olfactory discrimination test, bees were exposed to dishes containing 90% pollen to keep the visual cues identical. To vary the olfactory cues, two of the dishes contained 3 g and two contained 2.33 g, matching the odour intensity of 3 g of 70% pollen. In the visual discrimination test, two dishes contained 3 g of 90% pollen and two dishes contained 3.86 g of 70% pollen, to match the odour intensities of the samples. Tests lasted until bees gave up their search and were limited to a maximum of 5 min. The number of approaches, contacts and landings made to each dish during the first minute of testing was recorded. The order in which the two tests were presented was varied between individuals. Bees received a reminder trial between the two tests, during which they were permitted to collect 90% pollen, as in pre-training.

This experiment was subsequently repeated to determine whether bees could discriminate between 90% and 60% pollen on the basis of visual or olfactory cues alone. In the olfactory discrimination test, dishes contained either 3 or 2 g of 90% pollen, the latter matching the odour intensity of 3 g of 60% pollen. In the visual discrimination test, dishes contained either 3 g of 90% pollen or 4.5 g of 60% pollen.

Spectra of the illuminating light, stimuli and pollen samples were measured with a calibrated photospectrometer (Avaspec2048, Avantes, Surrey, UK) (see supplementary material Fig. S1) to estimate differences in visual appearance between pollen samples (Peitsch et al., 1992; Vorobyev et al., 2001). The 90%, 80% and 70% pollen samples were quite similar in colour but differed in chromatic contrast (supplementary material Table S1), which bees may have used for discrimination (Hempel de Ibarra et al., 2002; Niggebrügge and Hempel de Ibarra, 2003). Samples differed also in brightness, another cue which bees could have used for discrimination (Hempel de Ibarra et al., 2000), with the undiluted and 90% pollen being the dimmest samples, and the more diluted samples being brighter (supplementary material Table S1).

Experiment 4

Prior to training, spontaneous colour preferences were individually recorded by presenting a bee with four discs (15 cm diameter), two of each colour to be discriminated (blue/green or orange/white). A Petri dish of pollen was

placed in the centre of each disc, covered with a transparent, perforated lid, to prevent bees from collecting pollen during testing, but maintaining the olfactory/visual cues of the pollen reward. An approach was counted when a bee's whole body crossed from the grey background to the coloured stimulus. Testing lasted between 2 and 5 min, until the bee gave up its search, and the number of approaches to each disc was recorded.

Bees were trained in five trials of differential conditioning. The preferred colour (green/orange) was paired with the more diluted sample (either 70% or 60% pollen, $N=6$ per group) and the less preferred colour (blue/white) with the less diluted sample (90% pollen).

Colour preferences were re-tested following training. Covered dishes now contained yellow-dyed (yellow food colour, Silver Spoon, Peterborough, UK) α -cellulose to remove any confounding pollen-derived cues, ensuring that choices were made on the basis of the colour of the contextual stimulus only. The colour of the yellow-dyed α -cellulose differed strongly from all pollen samples (supplementary material Fig. S1), representing a novel 'pollen' colour.

Statistical analysis

For rewarded trials, differences in the time spent collecting each pollen type within a trial (Experiment 1) and between trials (Experiment 2) were compared using a two-tailed paired t -test. In addition, a preference index was calculated for each individual. The duration of time spent foraging on the more diluted pollen sample was subtracted from the time spent foraging on the less diluted sample and divided by the total amount of time spent foraging. Negative scores indicate a preference for the more diluted pollen sample, whereas positive scores represent a preference for the less diluted sample. Preference indices were also calculated based on the number of landings. These scores were highly correlated with the duration preference scores in all cases (Spearman's rank, 100% versus 90%, $r=0.860$, $P<0.001$; 100% versus 80%, $r=0.975$, $P<0.001$; 90% versus 70%, $r=0.879$, $P<0.001$; 90% versus 60%, $r=1.000$, $P<0.001$). A one-way ANOVA (with Dunnett's T3 pairwise comparison), performed on arc-sin transformed data was used to test whether a difference in the pollen content of the two samples (i.e. treatment group) had an effect on the overall strength of bees preferences.

In unrewarded tests (Experiments 3 and 4), paired t -tests (or Wilcoxon's signed-rank test where data were not normally distributed) were used to compare the number of approaches to each dish (90 versus 70/60%) or each stimulus type (blue versus green or orange versus white). Colour choices were tested both prior to training, to determine initial preferences (two-tailed test), and after training, to determine whether training resulted in a preference for the colour paired with the more concentrated (90%) pollen sample (one-tailed test).

To rule out any influence of positional biases on foraging decisions, the number of approaches or the duration of foraging at each dish location were compared for every experiment using repeated-measures ANOVA. In all cases there was no significant difference between locations.

Acknowledgements

We are grateful to K. Langridge, I. P. L. McLaren, J. Isden and T. S. Collett for helpful discussions.

Competing interests

The authors declare no competing financial interests.

Author contributions

E.K.N. and N.H. designed the experiments. E.K.N. performed the experiments and analysed the data. E.K.N. and N.H. drafted and revised the article.

Funding

We are grateful to the Biotechnology and Biological Sciences Research Council for financial support (BB/I009329/1).

Supplementary material

Supplementary material available online at <http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.106120/-DC1>

References

Arenas, A. and Farina, W. M. (2012). Learned olfactory cues affect pollen-foraging preferences in honeybees, *Apis mellifera*. *Anim. Behav.* **83**, 1023-1033.

- Baker, H. G. and Baker, I.** (1986). The occurrence and significance of amino acids in floral nectar. *Plant Syst. Evol.* **151**, 175-186.
- Banschbach, V.** (1994). Colour association influences honeybee choice between sucrose concentrations. *J. Comp. Physiol. A* **175**, 107-114.
- Boch, R.** (1982). Relative attractiveness of different pollens to honeybees when foraging in a flight room and when fed in the hive. *J. Apic. Res.* **21**, 104-106.
- Cook, S. M., Awmack, C. S., Murray, D. A. and Williams, I. H.** (2003). Are honey bees' foraging preferences affected by pollen amino acid composition? *Ecol. Entomol.* **28**, 622-627.
- Cook, S. M., Sandoz, J.-C., Martin, A. P., Murray, D. A., Poppy, G. M. and Williams, I. H.** (2005). Could learning of pollen odours by honey bees (*Apis mellifera*) play a role in their foraging behaviour? *Physiol. Entomol.* **30**, 164-174.
- Cresswell, J. E. and Robertson, A. W.** (1994). Discrimination by pollen-collecting bumblebees among differentially rewarding flowers of an alpine wildflower, *Campanula rotundifolia* (Campanulaceae). *Oikos* **69**, 304-308.
- Doull, K. M.** (1966). The relative attractiveness to pollen-collecting honeybees of some different pollens. *J. Apic. Res.* **5**, 9-14.
- Fewell, J. H. and Winston, M. L.** (1992). Colony state and regulation of pollen foraging in the honeybee, *Apis mellifera* L. *Behav. Ecol. Sociobiol.* **30**, 387-393.
- Greggers, U. and Muelshagen, J.** (1997). Matching behavior of honeybees in a multiple-choice situation: the differential effect of environmental stimuli on the choice process. *Anim. Learn. Behav.* **25**, 458-472.
- Hanley, M., Franco, M., Pichon, S., Darvill, B. and Goulson, D.** (2008). Breeding system, pollinator choice and variation in pollen quality in British herbaceous plants. *Funct. Ecol.* **22**, 592-598.
- Hempel de Ibarra, N., Vorobyev, M., Brandt, R. and Giurfa, M.** (2000). Detection of bright and dim colours by honeybees. *J. Exp. Biol.* **203**, 3289-3298.
- Hempel de Ibarra, N., Giurfa, M. and Vorobyev, M.** (2002). Discrimination of coloured patterns by honeybees through chromatic and achromatic cues. *J. Comp. Physiol. A* **188**, 503-512.
- Kitaoka, T. and Nieh, J.** (2009). Bumblebee pollen foraging regulation: role of pollen quality, storage levels, and odor. *Behav. Ecol. Sociobiol.* **63**, 501-510.
- Levin, M. D. and Bohart, G. E.** (1955). Selection of pollen by honeybees. *Am. Bee J.* **95**, 392-393.
- Michener, C. D., Winston, M. L. and Jander, R.** (1978). *Pollen Manipulation and Related Activities and Structures in Bees of The Family Apidae*. Lawrence, KS: University of Kansas.
- Nicholls, E. and Hempel de Ibarra, N.** (2013). Pollen elicits proboscis extension but does not reinforce PER learning in honeybees. *Insects* **4**, 542-557.
- Niggebrügge, C. and Hempel de Ibarra, N.** (2003). Colour-dependent target detection by bees. *J. Comp. Physiol. A* **189**, 915-918.
- Núñez, J. A.** (1982). Honeybee foraging strategies at a food source in relation to its distance from the hive and the rate of sugar flow. *J. Apic. Res.* **21**, 139-150.
- Peitsch, D., Fietz, A., Hertel, H., de Souza, J., Ventura, D. F. and Menzel, R.** (1992). The spectral input systems of hymenopteran insects and their receptor-based colour vision. *J. Comp. Physiol. A* **170**, 23-40.
- Pernal, S. F. and Currie, R. W.** (2001). The influence of pollen quality on foraging behavior in honeybees (*Apis mellifera* L.). *Behav. Ecol. Sociobiol.* **51**, 53-68.
- Pernal, S. F. and Currie, R. W.** (2002). Discrimination and preferences for pollen-based cues by foraging honeybees, *Apis mellifera* L. *Anim. Behav.* **63**, 369-390.
- Raine, N. E. and Chittka, L.** (2007). Pollen foraging: learning a complex motor skill by bumblebees (*Bombus terrestris*). *Naturwissenschaften* **94**, 459-464.
- Ribbands, C. R.** (1949). The foraging method of individual honeybees. *J. Anim. Ecol.* **18**, 47-66.
- Robertson, A. W., Mountjoy, C., Faulkner, B. E., Roberts, M. V. and Macnair, M. R.** (1999). Bumblebee selection of *Mimulus guttatus* flowers: the effects of pollen quality and reward depletion. *Ecology* **80**, 2594-2606.
- Roulston, T. H. and Cane, J. H.** (2000). Pollen nutritional content and digestibility for animals. *Plant Syst. Evol.* **222**, 187-209.
- Roulston, T. H., Cane, J. H. and Buchmann, S. L.** (2000). What governs protein content of pollen: pollinator preferences, pollen-pistil interactions, or phylogeny? *Ecol. Monogr.* **70**, 617-643.
- Scheiner, R., Page, R. E. and Erber, J.** (2004). Sucrose responsiveness and behavioral plasticity in honey bees (*Apis mellifera*). *Apidologie (Celle)* **35**, 133-142.
- Schmidt, J.** (1982). Pollen foraging preferences of honeybees (Hymenoptera, Apidae). *Southwest. Entomologist* **7**, 255-259.
- Schmidt, L. S., Schmidt, J. O., Rao, H., Wang, W. and Xu, L.** (1995). Feeding preference and survival of young worker honeybees (Hymenoptera: Apidae) fed rape, sesame, and sunflower pollen. *J. Econ. Entomol.* **88**, 1591-1595.
- Smeets, P. and Duchateau, M. J.** (2003). Longevity of *Bombus terrestris* workers (Hymenoptera: Apidae) in relation to pollen availability, in the absence of foraging. *Apidologie (Celle)* **34**, 333-337.
- Stanley, R. G. and Linskens, H. F.** (1974). *Pollen: Biology, Biochemistry, Management*. Berlin: Springer-Verlag.
- Tasei, J. N. and Aupinel, P.** (2008). Nutritive value of 15 single pollens and pollen mixes tested on larvae produced by bumblebee workers (*Bombus terrestris*, Hymenoptera: Apidae). *Apidologie (Celle)* **39**, 397-409.
- Thorp, R. W.** (1979). Structural, behavioral, and physiological adaptations of bees (Apoidea) for collecting pollen. *Ann. Mo. Bot. Gard.* **66**, 788-812.
- Thorp, R. W.** (2000). The collection of pollen by bees. *Plant Syst. Evol.* **222**, 211-223.
- Vaissière, B. E. and Vinson, S. B.** (1994). Pollen morphology and its effect on pollen collection by honeybees, *Apis mellifera* L. (Hymenoptera: Apidae), with special reference to upland cotton, *Gossypium hirsutum* L. (Malvaceae). *Grana* **33**, 128-138.
- Vaknin, Y., Gan-Mor, S., Bechar, A., Ronen, B. and Eisikowitch, D.** (2000). The role of electrostatic forces in pollination. *Plant Syst. Evol.* **222**, 133-142.
- von Aufsess, A.** (1960). Geruchliche nahorientierung der biene bei entomophilen und ornithophilen blüten. *Z. Vgl. Physiol.* **43**, 469-498.
- von Frisch, K.** (1967). *The Dance Language and Orientation of Bees*. Cambridge, MA: Harvard University Press.
- Vorobyev, M., Brandt, R., Peitsch, D., Laughlin, S. B. and Menzel, R.** (2001). Colour thresholds and receptor noise: behaviour and physiology compared. *Vision Res.* **41**, 639-653.
- Waddington, K. D., Nelson, C. M. and Page, R. E.** (1998). Effects of pollen quality and genotype on the dance of foraging honey bees. *Anim. Behav.* **56**, 35-39.
- Wahl, O.** (1966). Besitzen hieselnde bienen einen spursinn für den nährwert des sammelgutes. *Z. Bienenforsch.* **8**, 229-235.
- Waser, N. M.** (1986). Flower constancy: definition, cause, and measurement. *Am. Nat.* **127**, 593-603.

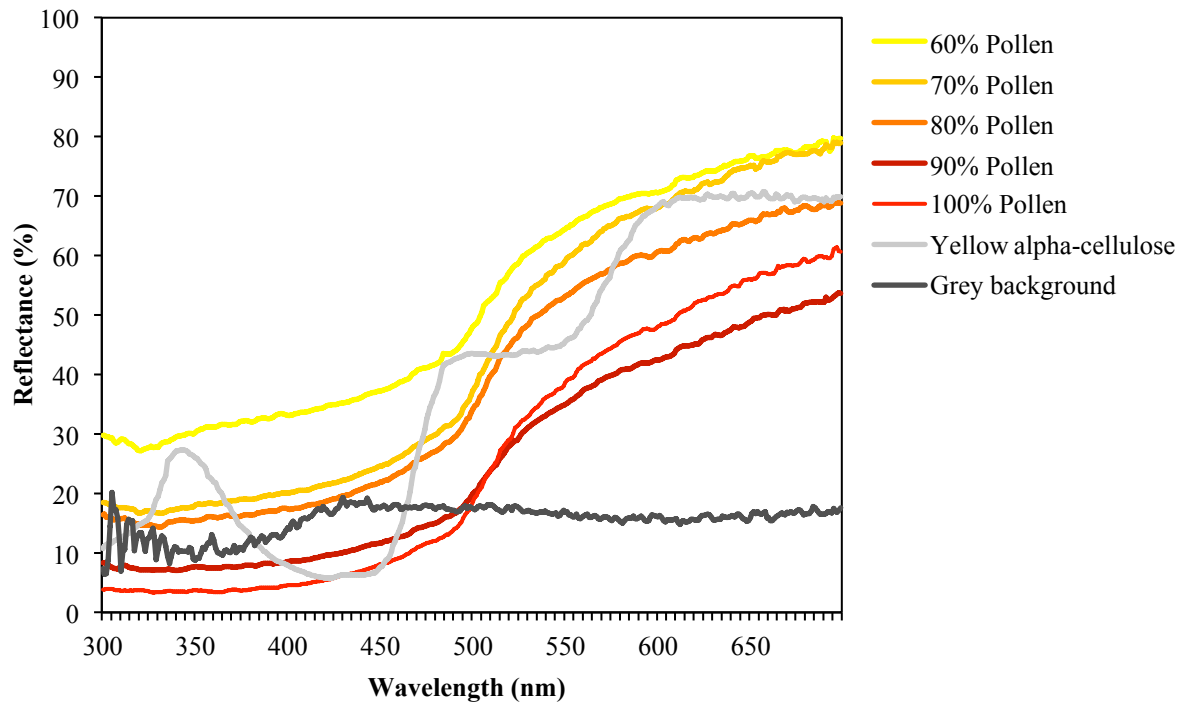


Fig. S1 Reflectance spectra of the pollen samples. Yellow-dyed alpha-cellulose was used in the unrewarded test of the differential conditioning experiment and strongly differed in colour from all pollen samples with colour distances of 4-6.6 RNL units [receptor-noise limited model of bee colour vision (Vorobyev, et al., 2001)].

Stimulus	Chromatic	L-receptor
	contrast to grey background	
90% pollen (training)	7.1	1.8
100% pollen	10.3	1.8
80% pollen	5.7	2.7
70% pollen	5.6	3.0
60% pollen	3.6	3.5
Yellow alpha-cellulose (test)	10.1	2.6
Blue disc	2.1	2.9
Green disc	12.2	3.1

Table S1. Differences in colour and brightness of the pollen samples and colour discs. Perception of achromatic brightness cues is mediated by the L-receptor in bees. The contrast has been calculated relative to the grey background using bumblebee photoreceptor sensitivities (Peitsch et al. 1992). Chromatic contrasts were quantified using the Receptor Noise Limited model of colour vision, also known as the Vorobyev-Osorio model (Vorobyev et al. 2001).