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Bees remember flowers for more than one reason: pollen mediates associative learning

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Ever since Karl von Frisch's Nobel Prize-winning work in the early 1900s, bees have served as an important model system for the study of learning, memory and foraging behaviour. Bees can learn about floral features including colour, scent, texture and electrostatic charge, and show surprisingly sophisticated forms of learning. However, nearly every study of bee cognition and foraging to date has used a sole reward: nectar, most often in the form of a simple sucrose solution. Plants also offer a number of other rewards to pollinators, the most prevalent being pollen that bees collect as their primary source of protein. Indeed, a significant proportion of angiosperm species are nectarless, rewarding bees with pollen alone. Surprisingly, whether free-flying bees can learn visual features based solely on floral pollen rewards is unknown. Here we show that bees can learn to associate multiple floral features with a pure pollen reward. Furthermore, these associations are remembered long term, comparable to bees' memory for nectar associations. These findings raise new questions about bee learning and the evolutionary history between plants and bee pollinators.

Pollination mutualisms play a key role in our understanding of angiosperm evolution, and are drivers of both ecological and agricultural processes. How floral traits affect pollinators' visits to flowers has long intrigued researchers of plant–pollinator interactions. In the century since von Frisch's discovery of their colour vision, bees have served as tractable models for the study of pollinator behaviour and floral morphology (reviewed in Leonard & Masek, 2014). For example, to entice naïve bees to visit them, flowers may exploit sensory biases for particular colours, patterns or scents (Lunau & Maier, 1995; Schiestl, 2010). To encourage repeated visits to the same species (and thus conspecific pollen transfer), plants also offer rewards, the most common being nectar. Bees rapidly learn associations between nectar and floral features (e.g. colour, pattern, scent, texture, heat and iridescence: Clarke, Whitney, Sutton, & Robert, 2013; Dyer, Whitney, Arnold, Glover, & Chittka, 2006; von Frisch, 1967; Whitney et al., 2009), and use these features to locate both flowers from a distance and nectar after landing. Investigations of nectar-based learning in bees have revealed impressive cognitive abilities (Giurfa, 2007) making them a model of learning and its neural underpinnings (Fahrbach, 2006; Menzel, 2012; Wright, Mustard, & Simcock, 2010). However, nectar is not the only reward offered by flowers (Armbruster, 2011; Renner, 2006); bees also forage intensively for pollen. Although a substantive body of work has addressed nectar versus pollen foraging in the context of division of labour (e.g. Scheiner, Page, & Erber, 2004), the dynamics of learning with pollen rewards is remarkably understudied. This is surprising, because bees collect pollen from a wide variety of plant species, including more than 10% of all angiosperm species (including representatives from some 27 families: Vogel, 1978) that offer only pollen as a reward (e.g. *Solanum*, *Papaver*, *Dodecatheon*: Buchmann, 1983).

Nectar and pollen differ from each other in both their chemical composition and in their function for both pollinators and plants. Nectar is composed primarily of carbohydrates (sucrose, fructose and glucose), but can also contain amino acids, protein, lipids and secondary compounds that can make it toxic or repellent to some animals (Adler, 2000; Richardson et al., 2015). Honeybee and bumblebee foragers imbibe nectar and use it to fuel flight, as well as taking it back to their colonies to feed other workers and larvae. On the other hand, pollen is a bee's primary source of protein (while it also contains free amino acids, starches, sterols and lipids: Baker & Baker, 1979; Roulston & Cane, 2000; Speranza, Calzoni, & Pacini, 1997). Many social bees collect pollen from the anthers of flowers, packing it into pollen baskets (corbiculae) before returning

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with it to the colony. There, it is fed to developing larvae and is critical for their growth (Schulz, Huang, & Robinson, 1998). From the plant's perspective, pollen rewards represent a trade-off between the benefit of offering a reward of high enough quality that it induces pollinators to transfer their pollen to conspecifics (e.g. Hanley, Franco, Pichon, Darvill, & Goulson, 2008; Leonhardt & Blüthgen, 2012) and the cost of male fitness lost by its consumption (Hargreaves, Harder, & Johnson, 2009).

In contrast to nectar foraging, little is known about what floral features bees learn when foraging for pollen. Unlike nectar, which is usually concealed from view, pollen rewards are often conspicuously coloured or displayed on colourful anthers (Lunau, 1992). This raises the question of whether, as is the case for nectar, bees associate floral cues with pollen presence, or instead evaluate pollen-related visual stimuli directly. Pollen visual cues are so potent that nectar-rewarding plants may use pollen or anther mimics to attract bees (Heuschen, Gumbert, & Lunau, 2005; Lunau & Maier, 1995; Tang & Huang, 2007) and they can even interfere with the learning of nectar–colour associations (Pohl, Watolla, & Lunau, 2008). Although volatiles strongly guide bees' pollen foraging (e.g. Dobson, 1987), the colours of pollen, anther and/or corolla may also predict pollen presence. Understanding what floral visual features, if any, are learned and remembered in a pollen-foraging context would help clarify the sources of selection on visual displays produced by pollen-rewarding plants and raise new questions about the mechanics of bee learning in relation to multiple reward types.

Few studies have addressed bee learning in relation to pollen foraging (e.g. Raine & Chittka, 2007a), much less associative learning linking pollen rewards and floral stimuli. For example, harnessed honeybees (in a proboscis extension reflex, PER, protocol) can learn associations between scent and pollen (Arenas & Farina, 2012; Grüter, Arenas, & Farina, 2008; but see Nicholls & Hempel de Ibarra, 2013), and free-flying bees learn to associate honeybee-collected pollen with scent and ('corolla') colour (Arenas & Farina, 2012; Nicholls & Hempel de Ibarra, 2014). However, most of these studies have used honeybee-collected pollen, which differs from floral pollen in a number of respects. When packing pollen into their corbiculae, honeybees add regurgitated nectar, resulting in a pollen load that is a mixture of pollen, nectar, digestive enzymes secreted by bees (Roulston & Cane, 2000) and in some cases foreign material (Davis, 1996). These pollen loads contain much higher quantities of sugar than pollen sampled directly from flowers (Human & Nicolson, 2006; Leonhardt & Blüthgen, 2012; Qian, Khan, Watson, & Fearnley, 2008). Indeed, half or more of the mass of honeybee-collected pollen may consist of regurgitated nectar-derived sugars (Roulston, Cane, & Buchmann, 2000). One analysis using pollen from *Aloe greatheadii* var. *davyana* showed that the honeybee's pollen load contained significantly more water (13–21% wet weight), more carbohydrates (35–61% dry weight) and less protein (28–51% dry weight) than the same pollen that had not been processed by honeybees. Therefore, in studies where honeybee-collected pollen has been used, learning may conceivably have been mediated by pollen, regurgitated nectar, or both resources. We therefore used exclusively floral-collected pollen in this study to determine its effects as a potential reinforcer.

We asked whether bumblebees (*Bombus impatiens*) form associations between pollen and visual features of both the anther and the corolla. After assessing their baseline colour preferences, we trained free-flying bees on arrays of artificial flowers to examine whether (1) corolla colour, (2) anther colour, or (3) both corolla and anther colour predicted the presence of pollen (Fig. 1). These treatments mimic a variety of ecological scenarios, as across different plant species, the colour of the anther and/or corolla may be the best indicator of pollen presence (Fig. 2). After training, we then tested recall shortly thereafter in an unrewarded test phase. To assess whether bees remembered associations long term, we trained an additional group of bees to one of two corolla colours (as in treatment 1) and then tested their memory retention 1 day and 7 days after training.

METHODS

Subjects

We used 72 bees from four colonies of *B. impatiens* (Koppert Biological Systems, Howell, MI, U.S.A.) for treatments 1–3. Of these 72 bees, all were used in colour preference tests but only 60 ($N = 20$ in each treatment) went on to training as 12 bees did not return to forage after their preference test. The four colonies were represented across different treatments (Supplementary Table S1). To assess long-term memory, we then used an additional 20 bees taken from two of the previously used colonies as well as a fifth colony (Supplementary Table S1). Colonies were connected sequentially to a central foraging arena (122 × 59 × 59 cm high) where all training and testing took place (Fig. S1a). The arena was lit from above by an LED light strip (2100 lumens, 4000K, Lithonia Lighting, Conyers, GA, U.S.A.) and the room was illuminated by both fluorescent and natural light. Prior to experiments we maintained bees on honeybee-collected pollen (~0.5 g/day, Koppert Biological Systems) but used flower-collected cherry pollen (*Prunus* sp., Firman Pollen Co., Yakima, WA, U.S.A.) throughout experiments.

During experiments, colonies had ad libitum access to 30% (w/w) sucrose solution but no access to pollen apart from what they collected during the experiment. This food regime kept foragers motivated for pollen foraging, because, in bumblebees, most individuals collect both nectar and pollen according to colony needs (Free, 1955). We marked foragers that collected pollen from a 'pretraining' array with numbered tags (Apinaut, Steißlingen, Germany). For the corolla-only and corolla-and-anther treatments, the pretraining array consisted of flowers with grey corollas and beige anthers and for the anther-only treatment, the pretraining array consisted of flowers with white corollas and beige anthers. Thus, the pretraining array always offered a stimulus visually distinct from the flower part subsequently rewarded in training but matched it in other regards. After tagging foragers that visited this pretraining array, these bees were then individually presented with the two flower types they would later encounter during training in a 'colour preference test' (Fig. 1). This array was identical to the training array with the exception that all flowers contained ~10 (± 2) mg of pollen on their anthers. Bees were given individual access to the colour preference array over a single foraging bout and were allowed to collect pollen before leaving the array.

Floral Arrays

During training and testing, bees encountered an array of artificial flowers arranged in a 5 × 4 grid. Flowers were spaced 7 cm apart at the base (5 cm apart at the top) and consisted of three-dimensional print-outs of 5 cm diameter disks (Makerbot, New York, NY, U.S.A.) placed on inverted plastic tubes (3 × 8 cm), with a coloured circle (the 'corolla') printed on waterproof paper (National Geographic Adventure Paper, Margate, FL, U.S.A.) and laminated. 'Anthers' were chenille stems (Creatology, Mountain View, CA, U.S.A.), protruding 25 mm vertically from the corolla. Pollen (10 ± 2 mg) was placed near the top of the anther, always on the same side (i.e. facing away from the colony; see Supplementary Fig. S1b for a diagram of the flower and Video S1 for images of foraging array).

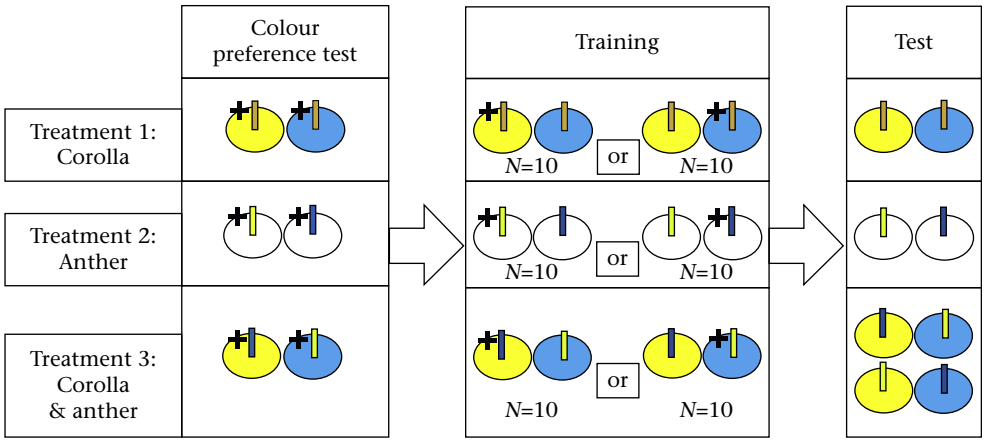

Figure 1. Protocol for training bees to floral features associated with pollen rewards across three treatments. To assess initial colour preferences, bees were allowed to individually forage upon 20 rewarding flowers of two types. After 24–48 h, they were trained over two trials on an array of flowers where only one type was rewarding, before being given a test where no flowers contained pollen rewards.

Figure 2. Flowers have a number of visual features that pollen-foraging bees might learn. Corolla and anther colour can vary both between species, as in (a) *Solanum dulcamara* and (b) *Dodecatheon alpinum*, and within species, as in (c) *Erythronium americanum*, and even over time, as in (d) *Borago officinalis*, where corolla colour signals availability of both nectar and pollen rewards. Photographs: (a, b): Anne Leonard; (c): Donald Drife; (d): Hans Bernhard.

Anthers on unrewarding flowers had been stored overnight in a sealed container of pollen, separated from the pollen by mesh, which we presumed would reduce the difference in scent between rewarding and unrewarding anthers. While we do not know whether bees can differentiate between rewarding and unrewarding anthers based on scent alone, our preliminary observations indicated that naïve pollen-foraging bees did not land on anthers that were not treated as described above. In the current experiment at least, bees used floral features rather than presence of pollen to determine which flowers to visit (see Results). Between trials, all anthers were replaced and fresh pollen was added to rewarding flowers. Corollas were wiped with 70% ethanol. We randomized the position of the flowers on the array across all trials and between bees.

We measured the reflectance spectra of the four floral colour cues and two pretraining colours used in the experiment and plotted the colours into bee colour space taking into account the photoreceptor spectral sensitivities of *B. impatiens* (Chittka, 1992; Skorupski & Chittka, 2010; see Supplementary Fig. S2). As expected, the yellow anther was most similar in colour to the yellow corolla and the blue anther was most similar in colour to the blue corolla (Supplementary Table S2).

Experimental Training and Testing

Twenty-four to 48 h after having the colour preference test, we gave an individual bee two experimental training trials and one test trial (Fig. 1). We assigned bees to three experimental treatment groups where (1) the colour of the corolla, (2) the anther, or (3) the corolla and anther together predicted a pollen reward during training. In treatment 1, we trained bees that blue-corolla flowers were rewarding and yellow-corolla flowers were unrewarding ($N = 10$), or the reverse ($N = 10$). All anthers in this treatment were beige. In treatment 2, we trained bees that blue anthers were rewarding and yellow anthers were unrewarding ($N = 10$), or the reverse ($N = 10$). All corollas in this treatment were white. In treatment 3, we trained bees that rewarding flowers had a blue corolla and a yellow anther (BCYA) and unrewarding flowers had a yellow corolla and a blue anther (YCBA) ($N = 10$), or the reverse ($N = 10$). Throughout all training trials, the rewarding anther offered pollen whereas the unrewarding anther was scented but unrewarding. We stopped the trial when the bee returned to the colony, or after she had not visited a flower for 10 min. Between the

training trials and test trial bees returned to the colony and removed their pollen loads (taking ~10 min).

In the test for treatments 1 and 2, bees encountered the same 20 flowers but with no pollen rewards. For treatment 3, bees encountered 16 unrewarding flowers representing all four combinations of anther and corolla colours (Fig. 1).

Long-term memory

Using a second group of bees from three colonies, we trained eight subjects to yellow corollas and 12 to blue corollas following the procedure outlined above. We did not film these training visits, but we observed foragers to ensure that they visited flowers and collected pollen during training. We then tested bees that returned to forage on an unrewarding array 1 day after training ($N = 8$ yellow-trained bees, $N = 9$ blue-trained bees), and again 7 days after training ($N = 8$ yellow-trained bees, $N = 7$ blue-trained bees).

Behaviour recorded

Bumblebees use a variety of both passive and active strategies to collect pollen from flowers (e.g. sonication; 'milking', 'stroking', 'drumming', 'striking': reviewed in Buchmann, 1983; Thorp, 2000). In the case of our artificial flowers, all bees collected pollen by active 'scrabbling', using their legs to scrape pollen off of the anther surface (see Supplementary Video S1). To pack the pollen on to their corbiculae, a bee extends its proboscis (regurgitating nectar), grooms its proboscis with its front legs, grooms pollen from its body and packs it using its legs; in our assay, this usually occurred on the flower's anther or in flight, but occasionally the bee would land to groom elsewhere (on a flower's corolla or the arena wall).

All colour preference tests and training and testing trials were filmed using an HD Sony camcorder (30 frames/s) mounted on a tripod placed on top of the test arena facing downwards. We video recorded all training and test trials because bees' pollen-collecting visits to different flowers occurred in rapid succession and therefore events were difficult to quantify live. We viewed recorded videos frame by frame (QuickTime Player v.10.3) and coded the colour of the flowers visited and whether the bee gained a reward. For an example of the recording used to quantify behaviour, see Supplementary Video S1.

As bees only collected and searched for pollen from the flowers' anthers, we defined a 'visit' to a flower type as the bee touching the anther with its antennae or legs (either by landing or hovering in front of the top of the anther). If the anther contained pollen and the bee was seen to scrabble with its legs, the visit was recorded as 'rewarded'. If the anther had no pollen on it and the bee touched it with its antennae or legs, the visit was recorded as 'unrewarded' (see Supplementary Video S1). Around 30% of the time, bees briefly touched rewarding anthers with their antennae or legs, but they did not scrabble to collect pollen from them. We excluded these 'visits' from analyses, because if a bee did not collect pollen from the rewarding flower colour (for reasons that could not be determined), then it was not clear whether this 'visit' would reinforce, or even inhibit, colour learning. Unlike in nectar-foraging experiments, anthers containing pollen were not emptied on a single visit. Instead, bees would generally collect pollen from all (or at least 8/10) rewarding anthers, and then return to anthers they had already visited to collect more pollen; these visits were also scored as 'rewarding'. Even though each training trial contained just 10 rewarding flowers, bees made on average (±SD) 61 ± 24, 50 ± 27 and 50 ± 27 rewarded visits per trial (for treatments 1, 2 and 3, respectively).

Data Analyses

To estimate bees' initial floral preferences in the colour preference test, we used the first 10 flowers a bee visited. To determine whether a bee preferred a particular flower type over chance, we compared the mean number of each flower type chosen to chance (5/10 flower choices) using one-sample *t* tests.

To determine whether bees made more visits to the rewarding flower type across successive visits (i.e. learned which flower type contained pollen), we carried out linear mixed models (LMMs) (one for each treatment) where the response variable was 'number of correct choices in 10 successive visits' and the explanatory variables were 'flower type that was rewarding' (e.g. yellow-corolla or blue-corolla), the continuous variable 'visit number' (blocked in groups of 10) and the random factor 'bee' nested within the random factor 'colony'.

To determine whether bees had learned the floral association with pollen, visiting more of the previously rewarding flower type in the unrewarded test than predicted by chance, we used the proportion of visits to the rewarding flower type in the bees' first 10 visits of the test (or fewer if the bee made only 7–9 visits in total) as a measure of their retention of preference for the rewarding flower type. We then compared the mean of this value (across all bees within a treatment) to a baseline preference for a particular flower type (the proportion of bees in the treatment group that visited the rewarding flower type on their first training visit) using one-sample *t* tests (e.g. blue-trained treatment 1 bees had a baseline preference of 0.6; see Results, Fig. 3a). Because the test phase results of treatment 3 involved novel stimuli with no baseline data, we instead determined whether there was a difference in the number of visits made to the four flower types by fitting an LMM with the response variable 'number of visits' and the explanatory variable 'flower type' (1 of 4 possible types) with the random factors 'bee' nested within 'colony'. For the long-term retention tests, we compared the mean proportion of blue flowers visited between the two training groups after 1 day and after 7 days.

All data were analysed using R v.3.1.1. (R Core Team, 2014). All LMMs were carried out using the lme() function in nlme package, (Pinheiro, Bates, DebRoy, & Sarkar, 2012) specifying type III sums of squares. Maximal models were run initially, and if the interaction was nonsignificant, the model was run again with the interaction removed. In cases of significant interactions, simplified models were run to determine the significance of the individual factors in these interactions.

RESULTS

Initial Colour Preferences

When foraging for pollen, bees preferred flowers with blue corollas (treatment 1) and yellow anthers (treatment 2) above chance; accordingly, they also preferred flowers with the combination of blue corolla/yellow anther (treatment 3) above chance (one-sample *t* tests: corolla: $t_{24} = 4.359$, $P < 0.0005$; anther: $t_{19} = 4.543$, $P < 0.0005$, corolla and anther: $t_{24} = 7.185$, $P < 0.0001$; Fig. S3).

Learning of Floral Features

In all three treatments, bees rapidly learned the floral feature associated with the pollen reward (Fig. 3). For treatments 1 and 2, bees made more correct flower visits across successive visits to the rewarding flower type and this did not differ depending on which floral colour was rewarding (LMMs: treatment 1: trial: $F_{1,169} = 12.862$, $P < 0.0005$; treatment: $F_{1,16} = 2.856$, $P = 0.110$; treatment 2: trial:

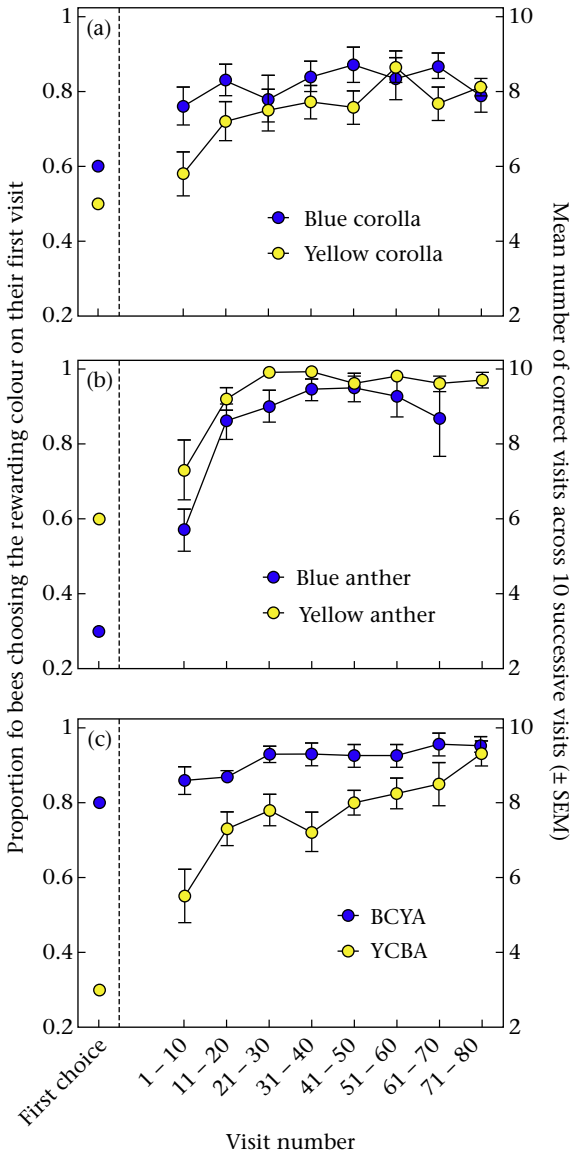

Figure 3. First choice of bees during training and the number of correct (rewarded) visits to the rewarding flower type, averaged (±SE) across blocks of 10 visits, for (a) corolla colour (treatment 1), (b) anther colour (treatment 2) and (c) corolla and anther colour (treatment 3). ◯: yellow stimulus; ●: blue stimulus.

$F_{1,103} = 34.502$, $P < 0.0001$; treatment: $F_{1,18} = 2.920$, $P = 0.105$; Fig. 3a,b). In treatment 3, both BCYA-trained and YCBA-trained bees made more correct flower landings across successive visits in training; this effect was larger for YCBA-trained bees than for BCYA-trained bees (Fig. 3c); (LMM: visit number∗treatment interaction: $F_{1,117} = 11.531$, $P < 0.0001$; visit number: $F_{1,117} = 39.207$, $P < 0.0001$; treatment: $F_{1,18} = 28.779$, $P < 0.0001$).

Test Performance

Bees that were trained to a particular corolla colour (treatment 1) or anther colour (treatment 2) visited flowers of the type that had previously been rewarding more often than what their initial preference would predict (one-sample t tests: corolla colour, blue-trained: $t_6 = 6.148$, $P < 0.001$; corolla colour, yellow-trained: $t_6 = 5.203$, $P < 0.005$; anther colour, blue-trained: $t_7 = 14.33$; $P < 0.0001$; anther colour, yellow-trained: $t_8 = 22.00$; $P < 0.0001$; Fig. 4). Bees in treatment 3 visited the flower type they were trained to more than the three other flower types, showing that bees had learned both the anther and corolla colour during training (LMMs: BCYA: flower type: $F_{3,24} = 48.406$, $P < 0.0001$; YCBA: $F_{3,24} = 9.123$, $P < 0.0001$; Fig. 5).

To determine whether BCYA-trained bees visited blue-corolla flowers with blue anthers more than either of the two yellow-corolla flower types, we also conducted two post hoc pairwise comparisons, setting the Bonferroni-corrected α value at 0.025. BCYA bees visited blue-corolla flowers with blue anthers more than either of the two yellow-corolla flower types (paired t tests: comparison with yellow-corolla, blue anther: $t_8 = 5.080$, $P < 0.001$; comparison with yellow corolla, yellow anther: $t_8 = 10.39$, $P < 0.0001$). Pairwise comparisons also showed that YCBA-trained bees visited the three flower types they were not trained to in equal proportions ($P > 0.3$ in all cases).

Long-term Memory

Bees trained to blue or yellow corollas differed in which flowers they visited after 24 h, with blue-trained bees visiting more blue flowers than yellow-trained bees (unpaired t test: $t_{15} = 7.113$, $P < 0.0001$; Fig. 6). This difference persisted 7 days later ($t_{13} = 4.536$, $P < 0.001$).

DISCUSSION

For nearly a century, the study of how bees learn floral signals, the ecological consequences of that learning and the evolutionary processes that shape these signals have mainly concerned a single floral reward, nectar. On one hand, this focus is understandable, as floral nectar commonly functions as a food reward for visitors and is highly tractable for use in experiments (Nicolson, Nepi, & Pacini, 2007). In particular, sucrose solution has served as a simple, widely accepted surrogate for nectar, facilitating studies of bee learning (reviewed in Giurfa, 2007; Menzel & Muller, 1996). On the other hand, pollen is also essential for bee survival and reproduction; the two nutrient sources are not interchangeable (e.g. Nicolson, 2011). Moreover, pollen rewards are taxonomically widespread (Vogel, 1978) and pre-date the evolution of nectar (Crepet, Friis, Nixon,

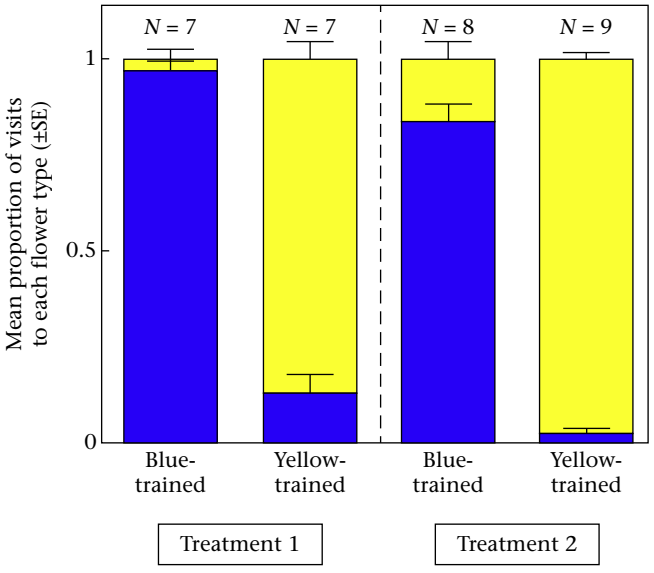

Figure 4. Flower choices of bees in the unrewarding test phase across the first two treatments. These choices were measured as the average (±SE) proportion of flowers visited in the first 10 visits to flowers on the test array. ■: blue stimulus; □: yellow stimulus.

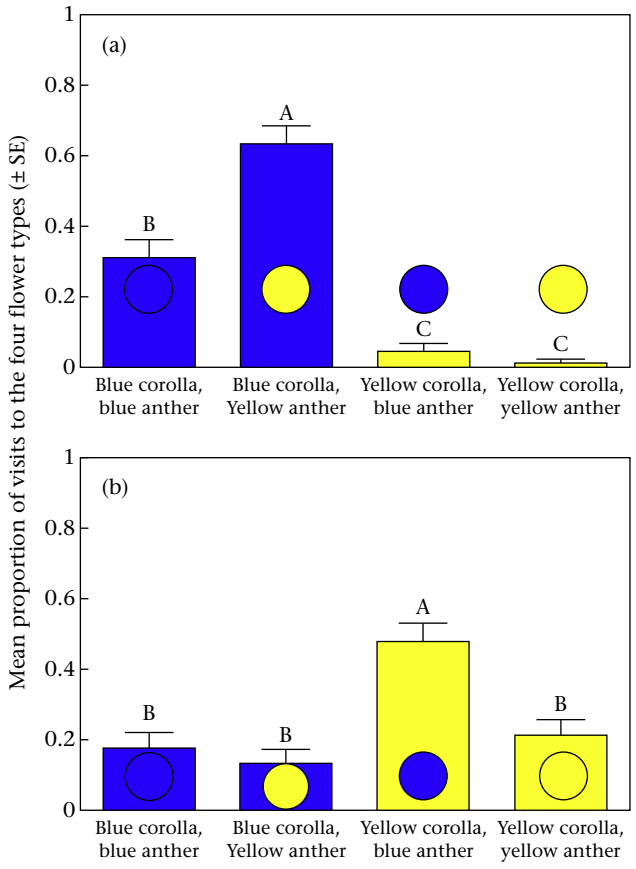

Figure 5. Flower choices of bees in the unrewarding test phase from the 'corolla and anther' treatment 3, where bees were trained to (a) blue corollas (■) with yellow anthers (○) and (b) yellow corollas (□) with blue anthers (●). These choices were measured as the average (±SE) proportion of flowers visited in the first 10 visits to flowers on the test array. Bars indicate corolla colour, and circles indicate anther colour.

Lack, & Jarzembowski, 1991; Labandeira, Kvaček, & Mostovski, 2007; Willmer, 2011). Plant species that offer only pollen as a reward vary tremendously in their floral displays, including anther and corolla colours, suggesting that such visual cues might be useful for bees to learn; if so, bees' learned responses could account in part for the diversity of pollen and anther colours. Despite the obvious importance of pollen for bee nutrition, questions about how pollen-foraging bees initially respond to flowers, what they learn about them and how learned and unlearned responses compare to those of nectar-foraging bees have largely gone unanswered.

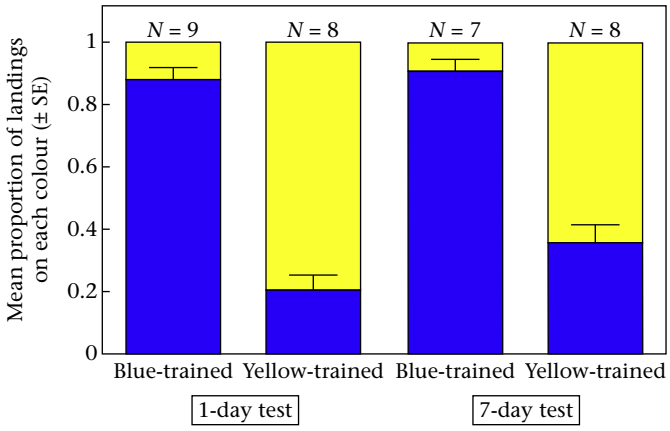

Figure 6. Flower choices of bees trained to blue and yellow corollas when tested on an unrewarding test array, 1 day and 7 days after being trained. Shown is the proportion of the first 10 visits (touches to the flower's anther with antennae or legs) bees made to a particular flower type (■: blue stimulus; □: yellow stimulus).

Our results point to a role for both colour biases and learned associations in guiding pollen foraging behaviour, similar to the role both play in nectar-foraging behaviour. We found that, when searching for pollen, bees initially visited flowers based on the colour of particular floral features: they favoured blue corollas, which are also attractive to nectar-foraging bees (e.g. Ings, Raine, & Chittka, 2009; Raine & Chittka, 2007b) and yellow anthers, which are innately attractive to bees and potentially used by flowers as a visual signal of pollen (Heuschen et al., 2005; Lunau & Maier, 1995; Tang & Huang, 2007). The preference for blue corollas may conceivably be explained by bees' generalizing from the pretraining array (the grey colour used was more similar to the blue corollas than to the yellow corollas; 0.041 and 0.205 hexagon units, respectively; see Supplementary Material). However, the beige training anthers were more similar in colour to the blue anthers than to the yellow anthers (0.090 and 0.109 hexagon units), yet the bees initially preferred yellow anthers (as has been found in previous work; Heuschen et al., 2005). Therefore, the preference for yellow anthers transcended the bees' experience during pretraining in the current experiment. The initial colour preferences shown in the colour preference test were further supported by the first flower type that bees visited when they first encountered the training array, as in all three treatments, bees trained to the two different flower types showed a floral preference on their first landing similar to preferences in the colour preference test.

From these initial colour preferences, bees then learned to associate floral colour cues with pollen in all cases, visiting the correct flowers more frequently across training and in the test phase. Between the first flower visit and the average performance across the first 10 visits, the number of correct flower visits increased dramatically, especially in cases where bees were being trained to the less preferred flower type. This may have been due to rapid learning, or it is also possible that after failing to find pollen on a flower type that was previously rewarding in the colour preference test, bees attended more to the pollen itself to guide foraging behaviour (Dobson & Bergström, 2000; Lunau, 1992). However, direct cues from the pollen were clearly not the main determinant in guiding bee behaviour, as bees visited many unrewarding flowers throughout training (especially when they were the preferred type) as well as in the test phase.

We found that bees not only formed associations between pollen presence and visual features of the anther and corolla, but they even learned to discriminate a rewarding colour pattern (i.e. a combination of stimuli) from unrewarding colour patterns, a capacity only shown previously in nectar foraging (e.g. Pohl et al., 2008). Although our findings show that both anther and corolla colours may allow bees to discriminate among pollen-rewarding plant species, bees seemed to attend to the corolla colour more than to the anther colour: BCYA-trained bees visited flowers with the 'correct' corolla colour (blue) and the 'incorrect' anther colour (yellow) over flowers with the 'correct' anther colour (yellow) and the 'incorrect' corolla colour (blue). YCBA-trained bees did not preferentially visit yellow-corolla flowers with the 'incorrect' anther colour over the two 'incorrect' corolla colour (blue) flowers, but given that bees had a strong initial preference for blue corollas, the lack of discrimination between these three flower types suggests that these bees also attended to corolla colour. Further study of this apparent bias towards learning corolla colours, using multiple colour combinations and anther sizes (combined with information about the relative saliency of natural anther colours versus corolla colours in plants) would pinpoint its generality.

Bees not only learned floral pollen–colour associations, but they also remembered these associations long term. This ability to learn and remember based on pollen cues alone may have ecological consequences, as it demonstrates that decisions throughout a forager's lifetime of which flowers to visit depend not only on memories of nectar rewards, but also on memories of pollen rewards. This is of relevance for understanding the success and persistence of pollen-only pollination syndromes (e.g. buzz-pollinated species, 8–10% of angiosperms: Buchmann, 1983), because such learning would provide a path to floral fidelity by increasing the chances of effective conspecific-to-conspecific pollen transfer.

Our results support findings from previous studies showing that honeybee-collected pollen reinforces choice behaviour (Arenas & Farina, 2012; Grüter et al., 2008; Nicholls & Hempel de Ibarra, 2014), as well associative learning between odours and pollen in harnessed honeybees (Arenas & Farina, 2012). Whether learning differs between bees that are reinforced with floral pollen versus honeybee-collected pollen is not clear. Given recent evidence that bees can detect differences between types and qualities of pollen (Ruedenauer, Spaethe, & Leonhardt, 2015) and that bees have taste receptors on their antennae and tarsi (Mommaerts, Wäckers, & Smagghe, 2013), it seems at least plausible that they can detect the nectar present in honeybee-collected pollen and that this might alter behaviour.

Our finding that bees learn to associate floral features with pollen is not globally surprising given that learning is often involved in selecting resources with which to provision offspring (Papaj & Prokopy, 1989), for example in parasitoid wasps (Dukas & Duan, 2000; Lewis & Takasu, 1990; Lewis & Tumlinson, 1988) and butterflies (Weiss & Papaj, 2003). Bees probably learn a number of associations between stimuli and different rewards, and how multiple rewards (such as nectar and pollen) interact is a clear avenue for future study. For example, do individuals that collect both nectar and pollen simultaneously show stronger fidelity to plant species whose flowers offer both rewards? Furthermore, do bees that collect nectar from one plant species and pollen from another possess cognitive mechanisms for keeping learning of one species' floral features from interfering with that of the other? Understanding the potential for such interference could help explain how bees partition foraging effort either across trips or across individuals (Page, Scheiner, Erber, & Amdam, 2006). Given that disruptions to the pollen foraging routines of both native and managed bees have been highlighted as a major factor in their declines (Goulson, Hanley, Darvill, Ellis, & Knight, 2005), taking into account this key aspect of bee foraging behaviour and how it interacts with nectar foraging is imperative.

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Supplementary Material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.anbehav.2015.09.029.

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