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).