Assessment of pollen rewards by foraging bees

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Summary

1. The removal of pollen by flower-visiting insects is costly to plants, not only in terms of production, but also via lost reproductive potential. Modern angiosperms have evolved various reward strategies to limit these costs, yet many plant species still offer pollen as a sole or major reward for pollinating insects.
2. The benefits plants gain by offering pollen as a reward for pollinating are defined by the behaviour of their pollinators, some of which feed on the pollen at the flower, while others collect pollen to provision offspring.
3. We explore how pollen impacts on the behaviour and foraging decisions of pollen-collecting bees, drawing comparisons with what is known for nectar rewards. This question is of particular interest since foraging bees typically do not ingest pollen during collection, meaning the sensory pathways involved in evaluating this resource are not immediately obvious.
4. Previous research focussed on whether foraging bees can determine the quality of pollen sources offered by different plant species, and attempted to infer the mechanisms underpinning such evaluations, mainly through observations of collection preferences in the field
5. More recent experimental research has started to focus on if pollen itself can mediate the detection of, and learning about, pollen sources and associated floral cues.
6. We review advancements in the understanding of how bees forage for pollen and respond to variation in pollen quality, and discuss future directions for studying how this ancestral floral food reward shapes the behaviour of pollinating insects.

Introduction

Insect pollination is considered the oldest form of pollen transfer (Labandeira & Currano 2013), and the vast majority of modern angiosperms benefit from visitation by insects (Ollerton, Winfree & Tarrant 2011), investing heavily in attractive floral displays and rewards for pollinators. Despite a widespread switch during angiosperm evolution from rewarding with pollen to the provision of nectar for insect visitors, pollen nevertheless remains an important food resource for consumption and collection by flower-visiting insects. While insects wish to maximize the amount of pollen they consume or collect during a flower visit, for plants, pollen removal also comes at a cost, both energetic and in terms of lost reproductive potential (Westerkamp 1997; Hargreaves, Harder & Johnson 2009). Compared to pollen, nectar is considered to be a more convenient pollinator reward for the plant to produce (Simpson & Neff 1983; Heil 2011). From an insects perspective, harvesting nectar requires fewer morphological and behavioural adaptations than pollen collection (Thorp 1979), and is easier to digest (Huber & Mathison 1976). In addition, nectar often contains solutes such as amino acids, meaning pollinators are able to meet a range of nutritional demands with this reward (for reviews see Nicolson 2011; Nepi 2014).

The emergence of nectar-producing organs during the late Cretaceous period, a time characterized by a fast succession of radiation bouts in both plants and the insects that pollinate them (Grimaldi 1999), likely led to the recruitment of novel pollinator clades. However the manner in which pollinator behaviour may have changed in response to this new floral reward is rarely discussed. Most likely behavioural changes exerted new selective pressures that resulted in further co-evolutionary changes in both flowers and insects. One idea that has received little attention is that due to the relative ease with which nutritional quality can be assessed, nectar may be more effective at rewarding learning than pollen, and thus may exert greater control over the behaviour of pollinators. If true, then nectar may also better promote constancy to the flowers visited by insects, enhancing out-crossing potential. In order to compare, we need to know how each reward type affect movement patterns, learning and foraging decisions, and whether this varies between reward types, leading to differential effects on plant-pollinator relationships. The assessment of pollen rewards is yet not fully understood, but with recent advances in research concerning pollen-foraging behaviour, sensory processing and learning in pollinators it is becoming more feasible to evaluate the influence of reward type in shaping plant-pollinator interactions. In this review we will largely focus on bees and include examples and references to work with both social and solitary species that has thus far provided most of the relevant facts and insights.

For bees and many other flower visitors, pollen is an important source of nutrition for larval development, adult maintenance and sexual maturation. The dietary needs of these insects and their various life stages are diverse, as is the nutritional 'quality' of pollen provided by different plant families, species and even individual plants within a population (reviewed by Roulston & Cane 2000). Bee species differ in their ability to digest different pollen types and to cope with the presence of toxins or protective compounds. Pollen type has been shown to dramatically affect both the development and survival of young bees and larvae (e.g. Standifer 1967; Schmidt, Thoenes & Levin 1987; Schmidt *et al.* 1995; Genissel *et al.* 2002; Roulston & Cane 2002; Tasei & Aupinel 2008; Sedivy, Müller & Dorn 2011; Di Pasquale *et al.* 2013), and so it has often been postulated that bees would stand to benefit by being selective in the pollen they choose to collect.

In the case of nectar foraging it is well established that bees evaluate the nutritional value of this reward instantaneously and over the duration of the foraging trip, accurately assessing the flow rate and sugar content of nectar provided by flowers (Núñez 1970). Pollen is diverse in form and the proportions of key nutrients vary considerably, which is likely to make foraging choices and the assessment of profitability a more complex task. One solution would be to establish foraging selectivity by specialising on pollen of particular plants, and indeed the majority of early bees were oligolectic (Michez *et al.* 2008; Wappler *et al.* 2015). However, among modern bees only a few truly oligolectic species remain, as over evolutionary time increases in the breadth of pollen diets have become more common (Müller 1996; Danforth, Conway & Ji 2003). Generalist collection strategies ensure that bees consume a diverse range of nutrients while also diluting plant protection products and toxins (Eckhardt *et al.* 2014). Yet selectivity seems to persist even in highly polylectic species, such as honeybees and bumblebees, which do not collect pollen from all plant species available. Rather, individual foragers concentrate their foraging efforts on a selection of plant species, showing preferences for one pollen type over another (e.g. Schmidt 1982; Müller 1995; Cook *et al.* 2003; Requier *et al.* 2015; Vaudo *et al.* 2016) and a capacity for flower constancy during pollen collection (e.g. Heinrich 1979; Minckley & Roulston 2006). However, whether such preferences are based on individual foragers' assessment of nutritional differences between pollen rewards remains a major outstanding question.

So far studies attempting to address this issue have yielded mixed results. Many are correlational, relating bees' foraging preferences in the field to the levels of a particular nutrient(s) found in the pollen provided by different plant species (Robertson *et al.* 1999; Hanley *et al.* 2008; Leonhardt & Blüthgen 2012; Somme *et al.* 2015). Since pollen is the major source of protein for bees, levels of this macronutrient, and/or the relative abundance of amino acids have frequently been proposed as cues relevant to bees, but results are not consistent, and there appears to be no simple relationship between collection preferences and the nitrogen content of pollen (Levin & Bohart 1955; Schmidt 1982; Schmidt & Johnson 1984; Schmidt 1984; van der Moezel *et al.* 1987; Pernal & Currie 2002). For example, when offered a source of protein, in the form of de-fatted soybean flour, diluted to varying degrees with alpha cellulose, a non-nutritional, inert powder, Pernal and Currie (2002) observed no difference in the weight of pollen loads collected by honeybees, suggesting that foragers did not discriminate between pollen samples on the basis of protein content alone. Similarly, Roulston and Cane (2002) reported that sweat bee foragers did not vary how much pollen they provisioned when offered pollen sources enriched in protein content to varying degrees via the addition of soybean meal, even though pollen protein content was shown to affect offspring body size. As such, evidence is lacking for the bees' ability to discriminate between floral pollen on the basis of crude protein content alone, particularly within the range of naturally occurring variation. Further studies have suggested that other macronutrients such as lipids, are equally or more important (Singh, Saini & Jain 1999; Schmidt & Hanna 2006; Avni *et al.* 2014; Vaudo *et al.* 2016), or that bees may be guided by the presence of toxins or distasteful compounds (Sedivy, Müller & Dorn 2011).

The lack of consensus among these studies likely arises from the method of investigation. In the first instance, pollen is a complex substance, varying between species and individual plants in a multitude of respects. Though sometimes acknowledged, this is frequently unaccounted for in field studies. However this is perhaps not surprising, given it is impossible to simultaneously control all the dimensions along which pollen varies without the use of artificial pollen surrogates. Furthermore, accurate measurements of the chemical composition of pollen are hampered by methodological limitations arising from the use of fresh plant samples or bee-collected pollen that has been altered through the addition of nectar by foraging corbiculate bees (Roulston & Cane 2000; Campos *et al.* 2008; Nicolson 2011). Finally, such studies often do not consider the sensory experience of an individual forager, as well as their prior experience and other floral cues and environmental factors which may play a role in guiding collection preferences. We argue that in order to determine which component(s) of the pollen reward may be guiding bees' foraging preferences, it is important to consider pollen collection from a behavioural perspective. In this review we examine current evidence regarding what bees can sense during pollen collection, considering which cues are salient and what role learning, prior experience and in the case of social bees, feedback from the nest, might play in determining preferences. We also evaluate to what extent current experimental evidence, and comparisons with nectar foraging behaviour, might explain the factors that guide pollen collection and the formation of associations between floral cues and pollen rewards. We hypothesise that rather than simply detecting and basing foraging decisions on the presence or concentration of particular nutrients, pollen collecting bees are likely to make an overall sensory assessment during foraging, utilising a suite of cues and recalling prior experience.

Do foraging bees taste pollen?

Pollen-collecting bees typically do not ingest pollen at the flower, instead transport it back to the nest for consumption by their offspring, or in the case of social bees, the colony as a whole. Nevertheless, foraging bees may have ample opportunity to sample grains pre-ingestively with their gustatory organs, the mouthparts and antennae, which frequently come into contact with pollen during collection. Bees often probe flowers with the antennae (Ribbands 1949; Lunau 2000) and in some cases, grasp and scrape pollen from the anthers with their mandibles (Thorp 1979). Some species even have specialised hairs on the mouthparts, designed for collecting pollen from flowers with protected anthers. (Parker & Tepedino 1982; Müller 1995). To facilitate adherence of the pollen grains to each other and the pollen baskets, corbiculate bees add regurgitated fluids to the grains, thus potentially providing further opportunities for gustatory sampling through contact between the pollen-covered body and the mouthparts. But what can bees taste?

Compared to what is known about both vision and olfaction, the gustatory sense of bees is still poorly understood. Honeybees possess only 10 gustatory receptor genes (Robertson & Wanner 2006; Jung *et al.* 2015); bumblebees have 23 (Sadd *et al.* 2015). This is substantially fewer than found in other insects (68 genes in fruit flies, (Liman, Zhang & Montell 2014); 52 genes in mosquitoes (Hill *et al.* 2002)), and has been taken as an indication of bees' limited ability to detect gustatory compounds in their environment. Taste responses are recorded extracellularly at the tip of sensory sensillae and assigned to functional classes of gustatory receptors (GRN). 'Sweet' and 'bitter' receptors, genes and pathways (in analogy to the human sense of taste) are well described in *Drosophila*, as well as receptors that respond to salt, water and carbonation (Yarmolinsky, Zuker & Ryba 2009). *Drosophila* is quite insensitive to amino acids and proteins in their food, which occur only at low concentrations in their diet. To date it is the best understood gustatory system amongst insects, and importantly this work shows that taste perception arises from the combined activity of different GRN. GRN sensitive to sugar are found on the antennae, mouthparts and the distal segment (tarsi) of the forelegs in honeybees (Whitehead & Larsen 1976). Some honeybee GRN are sensitive to salts or particular toxins, either when presented alone or in combination with sucrose stimulation (Wright *et al.* 2010; de Brito Sanchez 2011; Kessler *et al.* 2015). Honeybees presumably possess an additional receptor type on their mouthparts that mediates responses to either protein (Dethier 1961) or amino acids (Shiraishi & Kuwabara 1970), though this is yet to be tested at the physiological level in bees. In hoverflies (*Eristalis tenax*), a pollinator which consumes pollen at the flower, extracts of pollen diluted in water stimulate the labellar salt receptor cells but not sugar receptors (Wacht, Lunau & Hansen 2000). More studies characterising the response profiles of gustatory receptors and neural pathways in bees and other pollen-collecting insects are certainly needed.

Behavioural experiments have provided further insights into the gustatory pathways that could be relevant to the assessment of pollen. Bees are sensitive to the presence of amino acids in nectar. When offered the choice, bees preferentially imbibe those containing amino acids over pure sucrose solution, presumably differentiating between the two rewards through pre-ingestive mechanisms (e.g. Inouye & Waller 1984; Simcock, Gray & Wright 2014). In restrained bees, when the antennae of unsatiated bees are touched with nectar or artificial sucrose solution, a reflexive extension of the proboscis (PER) is observed, a behaviour characterised as an unconditioned, appetitive response to stimulation with a food reward (Bitterman *et al.* 1983). Such a response can be elicited following a single or few repeated pairings with olfactory, visual or tactile stimuli, and is frequently utilised as a paradigm for studying learning with sucrose rewards in harnessed bees (PER conditioning). Reflexive PER responses have also been observed in honeybees stimulated at the antennae with hand-collected almond pollen (Scheiner, Page & Erber 2004) and bee-collected pollen (Grüter, Arenas & Farina 2008; Nicholls & Hempel de Ibarra 2013), supporting the idea that pre-ingestive gustatory pathways are involved in the assessment of pollen rewards. Very few individuals respond with PER to inert alpha-cellulose powder, used to dilute pollen in experiments and as a pollen surrogate, which suggests that bees are able to detect phago-stimulatory compounds in pollen through the antennae. The presence of additional sugars in dry honeybee-collected pollen does not seem to be perceived by honeybees, at least not at the level of the antennae, the most sucrose-sensitive sensory organ. When pollen was delivered to the antennae of honeybees with a small sponge during an attempt to condition the pollen-PER to an odour (Nicholls & Hempel de Ibarra 2013), bees failed to form an association between the odour and reward, responding no differently from a control group that was stimulated with a clean sponge (Fig. 1A). Since bees readily form an association between this odour and sugars presented in solution with water, this suggests that any sugar present in the dry pollen was not detected by bees, as no association was formed.

More recently, Ruedenauer et al. (2016) trained bumblebees in a different PER conditioning paradigm, in which pollen and a pollen surrogate were paired with a sucrose reward. Pollen and casein were mixed in various concentrations with cellulose and water to form a thick, wet paste that was presented on a small copper plate which bees touched with their antennae. The sucrose reward was delivered to one of the antenna while the other was still in contact with the humid paste. Using chemo-tactile cues, bees learnt to distinguish between pollen and pollen-surrogate stimuli differing in absolute protein concentration, though only when the concentration differences between the two stimuli were sufficiently large. Though it is unclear how these differences might compare to naturally occurring variations in crude protein between pollen species (2-60% protein, Roulston & Cane, 2000), the study offers new methods and insights that are yet another demonstration of the rich sensory capabilities of bees and the multisensory nature of the information extracted from pollen rewards. The numerous controls that were conducted alongside these experiments reflect the difficulties that experimenters face when trying to reliably separate tactile and chemical stimulation (Scheiner, Erber & Page Jr 1999; Giurfa & Malun 2004; Nicholls & Hempel de Ibarra 2013).

The importance of olfactory cues

Pollen is both fragrant and also often conspicuously coloured, providing additional, potentially highly salient, cues. Indeed it has been suggested that in early angiosperms, prior to the appearance of a well-developed perianth, the androecium itself may have served as the original advertisement for attracting pollinating insects (Faegri & Pijl 1971; Crepet *et al.* 1991). In general, floral odours provide important cues that can guide pollinator foraging decisions (Raguso 2008; Wright & Schiestl 2009) and are undoubtedly salient sensory stimuli for pollinators. Bees are renowned for their extraordinary ability to detect, discriminate and learn odours (e.g. Laska *et al.* 1999), although they are poor at detecting the odours of amino acids, which as previously discussed, are considered an important nutritional component of the pollen reward (Linander, Hempel de Ibarra & Laska 2012). Most likely insects learn and rely on the overall olfactory signature of pollen-rewarding flowers. For example, bees have been shown to be capable of distinguishing pollen odours from that of the whole flower (von Aufsess 1960; Dobson, Danielson & Wesep 1999; Carr *et al.* 2015), perhaps unsurprising given pollen, particularly the outer pollenkitt layer, emits odour bouquets that differ strikingly in their composition from other floral odours (Dobson & Bergström 2000). Bees in controlled choice experiments have been found to be guided by the presence of previously experienced pollen odours (Hohmann 1970; Pernal & Currie 2002; Konzmann & Lunau 2014; Beekman, Preece & Schaerf 2016), preferring pollen-containing samples that are rich in odour, over odour-poor surrogates, or learning the odour bouquets of different pollen species when rewarded with sucrose (von Aufsess 1960; Cook *et al.* 2005; Ruedenauer, Spaethe & Leonhardt 2016).

In natural settings it is more difficult to measure how pollinators respond to variation in odour concentrations, and to test the significance of pollen odour cues for finding flowers or predicting the amount of pollen available (Galizia *et al.* 2005; Raguso 2008; Carr *et al.* 2015), especially when pollen odours are simultaneously presented with other strong sensory cues in the form of floral odour bouquets, colours or patterns. In experimental tests we found that pollen-foraging bumblebees did not utilise a considerable contrast in odour concentration to distinguish between pollen samples, and instead based their choices on differences in visual appearance (Nicholls & Hempel de Ibarra 2014).

Studies testing olfactory learning where pollen itself serves as the reward can provide further insights. Arenas and Farina (2012) concluded from their experiments with scented pollen feeders that honeybees learn to associate a particular odour with the presence of pollen, although it cannot be fully ruled out that their preferences could have been determined by their earlier olfactory experience (Arenas & Farina 2014). It is more suitable to train less experienced foragers and test under more controlled conditions for demonstrating if and what bees learn when pollen alone serves as the reward.

The PER conditioning paradigm offers the advantage of simply applying pollen to specific sensory organs to condition bees to an unfamiliar odour under highly controlled conditions. As previously mentioned, the PER paradigm has previously proven extremely valuable for examining the sensory and neural pathways underlying sucrose-rewarded learning in bees and other insects (e.g. Hammer & Menzel 1995; Burke & Waddell 2011). Pollen elicits reflexive proboscis extensions when applied to the antennae, as required for the paradigm, however multiple pairings of odour and pollen presentation do not result in a conditioned response to the odour. This suggests bees are not able to form an association between an odour and a pollen reward under these conditions (Nicholls & Hempel de Ibarra 2013). An earlier study by Grüter, Arenas and Farina (2008) prematurely reported that honeybees could learn to associate a reward mixture of pollen and water (70% pollen w:w) with an odour following three PER training trials. However, without indispensable controls it is not possible to conclude that an observed increase in responsiveness to the odour is the result of bees learning a predictive relationship between the odour and pollen reward. It could be potentially caused by other factors, such as an increase in sensitivity due to repeated antennal stimulation or clogging of the antennae with a sticky substance.

Pollen-rewarded learning of visual cues

While PER conditioning paradigms permit tight control over the delivery of conditioned and contextual odour stimuli and rewards, it can be challenging to select appropriate stimuli and obtain necessary controls, especially when both the conditioned stimulus and the reward provide cues in the same sensory modality. Furthermore bees are restrained in these experiments, which may negatively impact on the learning process. Visual conditioning of freely-behaving bees thus appears to be a more advantageous method for examining the reward properties of pollen in associative learning.

Bees and most other pollinating insects have excellent visual capabilities, even though their eyes are small and have low spatial resolution (von Frisch 1967; Kevan & Baker 1983; Hempel de Ibarra, Vorobyev & Menzel 2014). When pollen is displayed openly by the flower, it often contributes to flower patterns, though as with pollen odour, visual cues cannot be seen in detail from a distance, but are resolved only once a pollinator has arrived at the flower (reviewed by Hempel de Ibarra, Langridge & Vorobyev 2015). If cues are learnt, it is most likely that foragers are guided by sensory cues displayed by the whole flower or by joint displays of inflorescences and across co-located plants.

In learning experiments we showed that naïve bumblebees (*Bombus terrrestris*) not only learn the colour of pollen samples, but are also able to form an association between the pollen reward and a coloured stimulus surrounding it (Nicholls & Hempel de Ibarra 2014). Bees were offered two colours in combination with two pollen samples that differed in pollen concentration (Fig. 1B). After a short training period, bees shifted their initial preference for the coloured stimulus paired with the low concentration of pollen towards the alternative colour associated with the more concentrated pollen mixture. This was demonstrated for different colour pairings, suggesting that the bees' learning abilities with pollen rewards are not limited to particular colours that might frequently occur in petals of pollen-displaying flowers. Muth, Papaj and Leonard (2016) further observed that bumblebees (*Bombus impatiens*) are able to form long-lasting associations of up to seven days between pollen and a coloured stimulus, using artificial flowers with both a coloured 'corolla' and an 'anther', a small chenille brush from which pollen was collected (Fig. 1C). Interestingly, when both flower parts indicated the presence of a reward, bees seemed to attend more closely to the colour of the corolla than the colour of the anther. Again, this can be explained by the poor resolution of bee eyes. Given its larger size, the corolla would be more suited to attracting and guiding the approach of bees to the flower than the smaller anthers.

Mechano-sensory feedback during pollen collection

The lack of learning with pollen rewards observed in restrained bees in the PER paradigm discussed above may indicate that some component intrinsic to the active collection of pollen is necessary for reinforcing behaviour during pollen foraging, most likely through the activation of specific motor patterns and mechano-sensory feedback during pollen collection. Studies of buzz pollination, where bees use vibrational movements to shake pollen from poricidal anthers, show that both bumblebees and carpenter bees adjust their flower handling time according to the amount of pollen released by a flower (Buchmann & Cane 1989; De Luca *et al.* 2013; Burkart, Schlindwein & Lunau 2014), though this is not necessarily true for all flowers with this type of anthers (Nunes-Silva *et al.* 2013). The vibrational movements can be varied both in duration and amplitude, forming part of a mechano-sensory feedback system that might have the capacity to modulate buzzing behaviour in response to signals about the state and type of flower.

Mechano-sensory feedback is also likely to be involved in learning during non-buzzing pollen collection. It has been suggested that grain size and shape may influence the manner in which grains pack in to the corbiculae (Vaissiere & Vinson 1994; Pernal & Currie 2002; Lunau *et al.* 2015), thus bees may select pollen species in order to maximise packing efficiency. Interestingly, grain size correlates with protein content in a number of species (Baker & Baker 1979; Roulston, Cane & Buchmann 2000). Physical cues could therefore serve as reliable indicators of pollen identity, which in turn could influence the selection of pollen species.

Recently a hitherto unknown sensory capability of bees was discovered, the detection and discrimination of electric fields that stimulate mechano-sensory hairs located on the bee's body (Clarke *et al.* 2013; Sutton *et al.* 2016). Electrostatic forces can aid pollen transfer (Gan-Mor *et al.* 1995; Vaknin *et al.* 2001), and insect visitation, pollen removal and pollination status all alter the electric potential of a flower. Electric fields may be another important, yet understudied, cue utilised by pollen-collecting bees.

Efficiency of pollen harvesting behaviour in bees, including handling of the flower to access anthers and grooming of pollen from the body surface, which depend both on the pollen deposition mode and the pollen packing behaviour itself, needs to be studied further to understand under which circumstances the evaluation and learning about pollen rewards is based on the handling requirements for different pollen and flower types. When designing behavioural experiments and field observations it thus seems essential to include more measures and controls that account for the possibility that pollen packing might influence bee foraging decisions.

Pollen is a multi-modal stimulus

Taking into account their diverse sensory capabilities, from a bees' perspective pollen represents a multi-modal stimulus, simultaneously providing foragers with gustatory, olfactory, visual and mechano-sensory cues, all of which could be used to guide their foraging choices. Different pollen species are likely to provide a widely varying array of sensory signals, making it difficult to address the functions and interactions of sensory modalities or to determine which cues are most salient for bees. Salience may vary depending on context, or bees might rely on multi-modal associations; perceptual information,may vary according to relative saliences, experience and spatio-temporal constraints on their foraging movements.

There are methodological difficulties to be considered when using pollen or pollen surrogates in experiments that aim to isolate the various dimensions of pollen as a multi-modal stimulus. Different substances vary in both their nutritional and physical properties. Fresh, hand-collected pollen of a single plant species seems to most closely resemble the natural state of pollen encountered by bees at the flower, but it is very difficult to obtain in sufficient quantities and to maintain in a fresh state over the duration of behavioural experiments. Usually experimenters revert to commercially-collected pollen that can be purchased either as single- or mixed-species pollen from different geographic locations. While single-species pollen has the advantage of controlling a particular cue, such as grain size, mixed-species pollen offers a diverse range of nutrients and can be useful for masking potential confounding cues, or diluting the presence of toxins and unpalatable compounds. Bee-collected pollen is easier to obtain in large quantities, and so far there is no evidence to suggest that in the dry form, the sucrose added by foragers is sensed by bees. Pesticide load is likely to be lower than in commercially collected single-species pollen, typically harvested from intensively farmed crops such as fruit orchards, given that honeybee colonies placed in agricultural landscapes typically utilise a range of wild flowers in addition to crops. Hand-collected pollen may also contain anthers and other plant material, and it is often not clear whether experimenters take steps to remove such plant tissue prior to testing. All pollen that is not freshly picked from a plant is usually dried to prolong longevity, and in the case of commercially available hand-collected pollen from crop plants, additionally treated to improve effectiveness in crop plant fertilisation. Sometimes pollen is washed by experimenters to remove surface sugars before being presented to bees as either a dried powder or wet paste. There is a risk that washing may place grains under osmotic pressure, bursting them and expelling their content whilst simultaneously removing other important phago-stimulatory compounds present in the pollenkitt. Details of washing procedures should be reported and assessed, as it can change pollen properties quite substantially (e.g. Ruedenauer, Spaethe & Leonhardt 2015; Nicholls, Chow and Hempel de Ibarra, personal observations). Using surrogates, such as alpha-cellulose and casein, can be very advantageous for manipulating particular chemical and tactile cues in isolation, but are limited in their potential to simulate the diversity and variability of pollen cues present in real flowers. The above mentioned are all challenges that need to be considered when studying the sensory mechanisms underlying pollen foraging and reward assessment.

The role of experience in pollen evaluation

The act of removing pollen from flowers involves motor patterns that are hard-wired (e.g. Russell *et al.* 2016), though some aspects of this behaviour can be fine-tuned with experience (Raine & Chittka 2007; Morgan *et al.* 2016). Furthermore, individual collection preferences have been shown to be affected by prior foraging experience. Cook *et al.* (2003) found that honeybees preferred pollen species containing a higher concentration of essential amino acids only when they had previous experience of foraging on this pollen type. This suggests that bees undertake an experience-based assessment of pollen quality. Supportive evidences comes from experiments where distinctive responses were recorded in bumblebees offered a choice between pollen mixes diluted to varying degrees with cellulose (Nicholls & Hempel de Ibarra 2014). Some individuals had a preference for the more familiar pollen type, even if it had a lower protein concentration. Preferences changed over time or even disappeared, with bees accepting variable pollen rewards (Konzmann & Lunau 2014; Nicholls & Hempel de Ibarra 2014). It remains open which sensory cues may be involved in this familiarity effect.

In honeybees, interpretation of the waggle dance offers a unique opportunity to gain insight into individual foraging preferences. When foragers aim to recruit nest mates to a profitable food source, they decide whether to dance and, in the case of nectar sources, how vigorously to perform their dance (Lindauer 1948; von Frisch 1967; Seeley, Mikheyev & Pagano 2000). When pollen stores are low, pollen foragers have been observed to dance not only for flower pollen but also for a range of pollen surrogates such as dry milk, potato powder, wheat or soy flour and for pollen from wind-pollinated plants (e.g. hazel) (Lindauer 1948). Lindauer (1948) also offered potato flower mixed with bitter-tasting wormwood (*Artemisia absinthium*) pollen which contrary to his expectation did not diminish, but increased the dancing activity of bees, with some bees switching to preferentially collect it. Waddington, Nelson and Page (1998) observed that honeybees were less likely to perform a dance to alert their hive-mates to the location of a pollen source diluted with alpha-cellulose, presumably because it was perceived as inferior. However such a conclusion has been disputed by a more recent study. Beekman et al. (2015) found that long-term exposure to a particular pollen type at known locations affects how bees respond to changes of pollen qualities. More research is needed to clearly establish whether individual assessment of pollen resources affects reward evaluation and the propensity to dance.

Comparing sucrose- and pollen-rewarded learning

Comparisons of nectar and pollen foragers, their behavioural adjustments and similarities or differences of learning processes during nectar and pollen collection can provide interesting insights to understand the assessment of pollen rewards by bees. Recent work has centred on the question of how pollen-rewarded sensory assessment and learning of floral features compare to the associations that are acquired during nectar collection. Studies with sucrose-rewarded bees have found that learning is impaired when individuals are prevented from imbibing the reward (Sandoz, Hammer & Menzel 2002; Wright *et al.* 2007), so it is reasonable to expect that in pollen-collecting insects, pollen may be a less effective behavioural reinforcer than nectar. Another major difference between the two types of learning is the handling time required to collect the reward. The location and extraction of nectar generally takes less time and provides direct pre- and post-ingestive feedback for bees, which might enhance learning and re-learning, speed up decision-making and strengthen flower constancy. On the other hand, longer pollen handling times could influence the perception of reward quality. Once learned, bees might be slower in extinguishing memories, less prone to fully switch to new flower types and therefore possibly show lower levels of flower constancy. Such questions remain unanswered, and only very recently have attempts been made to compare the two types of learning.

Nicholls, Ehrendreich and Hempel de Ibarra (2015) compared learning and memory recall of naïve pollen and nectar-foraging honeybees trained under similar conditions in the laboratory (pollen odours were present in each condition inside the reward box, Fig. 1D). In simple colour association tasks, pollen and nectar-rewarded bees performed equally well. When bees were required to repeatedly re-learn which colour (Blue or Yellow) was paired with the reward, pollen-rewarded bees initially exhibited longer search times to find the reward following a switch in rewarding colour. Evidence for a difference in the strength of memories formed for the two colours between sucrose- and pollen-rewarded bees comes from their differing responses in a memory test performed one hour after training. Whilst pollen-rewarded bees exhibited an equal preference for both learnt colours, nectar-rewarded bees preferred the colour that was reinforced first, presumably because this association was consolidated rapidly and formed a more robust memory, which could have interfered with the recall of subsequently learnt colour pairings. This is first evidence to suggest that differences might exist in the mechanisms underlying pollen and sucrose-rewarded learning, an idea that needs to be investigated further.

Muth, Papaj and Leonard (2015) examined how bumblebees' behaviour might be modulated when foraging for both reward types simultaneously. Nectar and pollen were provided in artificial flowers, the colour of which signalled the type of reward provided. Interestingly, while half of the bees tested chose to forage for both types of reward in the same foraging bout and readily learnt both colour associations simultaneously, the rest preferred to collect only one reward both within and across multiple foraging trips. Here also bees learnt each colour-reward association easily, once more supporting the notion that pollen-rewarded learning is fast and establishes robust colour memories (Nicholls & Hempel de Ibarra 2014; Muth, Papaj & Leonard 2016). These fast associations are likely to form the basis for individual pollen constancy within and between foraging trips of bees.

One difficulty that arises when comparing learning in pollen- and nectar-rewarded bees is in controlling the visual and olfactory cues provided by the different types of reward. This can be somewhat overcome by scenting feeders (Arenas & Farina 2012) or constraining bees to collect pollen in the dark (Nicholls, Ehrendreich & Hempel de Ibarra 2015). The visual appearance of pollen may impact on initial colour preferences and/or the acquisition and recall of colour-reward associations (Nicholls & Hempel de Ibarra 2014; Muth, Papaj & Leonard 2015). Furthermore, it is important to establish whether foragers specialising on nectar or pollen may inherently differ in their cognitive abilities.

It has been proposed that pollen-foraging honeybees form better sucrose-rewarded olfactory associations (Scheiner, Page & Erber 2004) as a result of variation in sucrose sensitivity between forager types leading to differences in individual perception of reward quality (Scheiner *et al.* 2005). It may at first appear paradoxical that bees which forage for pollen are more sensitive to sucrose than those which collect nectar, but Page *et al.* (2006) argue that such specialisation could be adaptive for the colony, since nectar foragers would collect only from flowers producing highly concentrated nectar, thus returning to the hive the best quality resource currently available. Scheiner, Page and Erber (2004) also suggest that sucrose responsiveness is unlikely to be directly responsible for the differences in pollen and nectar forager behaviour, rather that variation in sucrose response thresholds may represent general differences in sensory processing. This view is supported by the fact that sucrose sensitivity is also known to correlate with sensitivity to other modalities such as pollen (Scheiner, Page & Erber 2004) and light (Tsuruda & Page 2009; Scheiner *et al.* 2014). Differences in sensitivity to external stimuli have been demonstrated to have an impact on differences in learning between forager types. Scheiner, Erber and Page Jr (1999) found that pollen foragers learned a tactile PER conditioning task more rapidly, reached a higher asymptote and greater resistance to extinction than nectar foragers. An analogous result was found for olfactory PER conditioning (Scheiner, Barnert & Erber 2003), though differences in the learning performance of foragers reinforced with their respective rewards, has yet to be tested.

Social cues and colony feedback

While in this review we have advocated a focus on the individual sensory experience of a pollen-collecting bee, the role that social cues may play in guiding pollen foraging behaviour should not be overlooked, especially considering the majority of studies reported here have used social bees as their subjects. For honeybees and bumblebees, levels and quality of collective pollen storage as well as feedback from nest mates may also be important, adding an additional layer of complexity to the process of determining the relative importance of various factors guiding the evaluation of pollen rewards by foraging bees. Pernal and Currie (2001) observed that honeybees altered foraging effort in response to fluctuations in the protein content of stored pollen. There was no difference in the quality or breadth of species collected under either manipulation, which suggests that individual honeybee foragers lack the ability, or at least do not solely attend to the protein content of pollen whilst collecting, and rely on the feedback about colony demand from the nurse bees which unload their pollen sacs. Indeed, young honeybees at the age when they typically engage in nursing, change their feeding behaviour to compensate for protein deprivation (Paoli *et al.* 2014). This mechanism could potentially contribute to the regulation of in-hive interactions between hive bees and pollen foragers.

Bumblebees are also able to adjust colony collection rates over time to compensate for changes in colony stores and responding to variation in pollen concentrations at artificial feeders (Kitaoka & Nieh 2009). Since bumblebees unload their own pollen baskets, and individually assess brood levels and stored pollen (Dornhaus & Chittka 2005), one might predict that it would be more efficient for bumblebees to possess the ability to individually assess some aspect of pollen quality directly at the flower, supplementing information gained inside the colony.

Concluding remarks

Multiple floral cues have the potential to influence bees' pollen collection behaviour and perception of pollen rewards, in addition to their own experiences and in the case of social bees, the feedback they receive directly or indirectly from their nest mates. Since most foragers can combine pollen and nectar foraging, either on the same trip or throughout their life (Robinson 1992; Wcislo & Cane 1996; Hagbery & Nieh 2012; Konzmann & Lunau 2014), this may add to their experience base and further influence navigational and foraging decisions during pollen collection. Nonetheless, what is clearly established is that pollen-foraging bees individually prefer some flowers over others and have the ability to detect differences between pollen(-like) samples of different chemical, colour and/or mechano-sensory qualities.

Pollen is a complex and diverse food substance and floral reward. Although much effort has been made, we are still lacking answers to fundamental questions necessary to fully understand the adaptive value of floral pollen rewards, regarding their differences in nutritional quality, within the limits set by requirements for plant fertilisation (Roulston, Cane & Buchmann 2000), and sensory cues that mediate pollen foraging and pollen reward evaluation in pollinating insects. How pollen rewards may influence the foraging decisions of pollen-collecting insect pollinators is thus far best studied in bees, since many aspects of their behaviour, the neural pathways and mechanisms are well understood with regards to nectar rewards.

Still little is known about how nutrients in food rewards, other than sucrose, are encoded and processed pre- and post-ingestively by bees, how and which molecular pathways are shared or diverge, which brain regions are involved in turning reward value into foraging decisions. Preliminary investigations suggest that, as observed in mammals, encoding of reward type in the bee brain may involve a subset of the molecular pathways implicated in a generalized food-based response, though particular brain regions and populations of nerve cells were observed to be uniquely responsive to differences in food type (McNeill *et al.* 2015). Transcriptional changes in the mushroom bodies, the main centres of sensory integration in the insect brain, vitally important for learning and cognitive processes, appear to play an important role in encoding differences in both reward type and value.

Pollinators will accept a range of pollen rewards of varying nutritional value within a bracket of cost-benefit assessment that considers various aspects – floral and pollen cues, handling requirements, availability of pollen sources and individual experience. To understand the reward functions of pollen, it is important to separate these different factors and describe the varied mechanisms that are involved in the perception of pollen rewards. More studies addressing sensory and learning mechanisms in pollen-foraging bees, and comparisons with nectar-foraging modes in the same individuals or with nectar-foraging conspecifics, are needed for continuing the quest of uncovering the mechanistic basis of pollen foraging. Recent advances in research technologies and genome sequencing provide new avenues for gaining interesting insights in the evolution and functions of flower pollen as a reward for pollinators.

Data Accessibility

This manuscript does not use data.

Figure 1: Methods for experimental testing of pollen collection and pollen-rewarded learning in bees. (A) When stimulated with pollen bees spontaneously respond with a proboscis extension (PER). In the olfactory PER conditioning paradigm, the typical sucrose reward was substituted with pollen in an attempt to train bees to associate an unfamiliar odour with pollen reward (Nicholls & Hempel de Ibarra 2013). Small cosmetic sponges were dusted in dry pollen and frequently replaced during conditioning. Bees in the control group were trained to the same unfamiliar odour but 'rewarded' with a clean sponge that was attached to a pollen-coated sponge to provide pollen scent. (B) Bees accept pollen presented in petri dishes, which can be placed on a coloured background (Nicholls & Hempel de Ibarra 2014). (C) Sophisticated pollen feeders, where the pollen is dusted onto small chenille brushes (Muth et al. 2016). The brushes are placed inside of differently shaped artificial flowers or attached to a coloured base to form anther-like structures (photos courtesy of A. Russel; from Russell & Papaj 2016). (D) Bees can be trained to collect sucrose or pollen rewards inside of dark boxes. One colour marked the entry tube that led to the inside of the reward box (Nicholls, Ehrendreich & Hempel de Ibarra 2015). The entrance marked by the alternative colour was blocked at the end by a mesh that would still allow pollen odour to diffuse.

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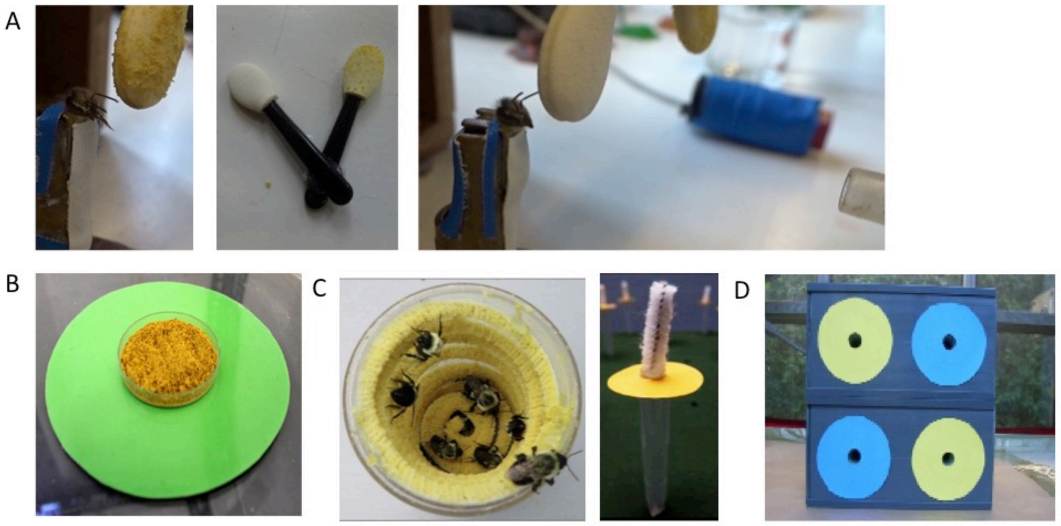

Figure 1