Learned olfactory cues affect pollen-foraging preferences in honeybees, *Apis mellifera*

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Honeybees, *Apis mellifera*, show learned odour preferences for flowers that provide nectar as a reward. However, little is known about such behavioural plasticity when bees exploit pollen sources. Furthermore, the question about whether nectar and pollen foragers use the same learned strategy to improve foraging efficiency remains untested. Here, we demonstrate that honeybee foragers are able to learn odour cues associated with pollen as a reward. This was tested in free-flying bees in a dual-choice feeding device after the bees had gathered pollen from a scented feeder. Free-flying bees that associated odour with pollen successfully recalled these memories in olfactometer odour choice tests in a Y-maze, but they failed to show extension of the proboscis to learned odour cues when restrained (proboscis extension reflex, PER, assay). In addition, odour cues associated with pollen at the feeding site induced foraging reactivation when bees were blown into the hive. In PER assays, after fatty acids were applied to the bees' antennae, pollen foragers were more responsive than nectar foragers. This, in turn, allowed pollen foragers in the PER assay to associate an odour cue with pollen in some trials. On the other hand, the unconditioned response (UR) and the odour-conditioned response (CR) to sucrose and amino acids were similar for both types of foragers. Pollen foragers also showed more URs to fresh pollen of different flower species and even performed better during conditioning with some pollen types as the reward than did nectar foragers. By studying biases in pollen-foraging responses after learning, we provide new insights to help comprehend and characterize the search for food between pollen and nonpollen honeybee foragers.

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Insects rely on innate behaviour to respond to a variety of factors that change unpredictably in space and time (Giurfa et al. 1995; Gumbert 2000). Many environmental stimuli, however, need to be learned to expand the type and amount of information to which animals can respond (Dukas 2008). Honeybees, *Apis mellifera*, strongly rely on learning (the process that adaptively changes the individual behaviour as a consequence of experience) to predict environmental features. This behavioural plasticity has been extensively shown for exploitation of nectar sources (Wehner 1967; Koltermann 1969; Menzel & Erber 1978) but has seldom been shown for exploitation of pollen sources (Cook et al. 2003).

While foraging for nectar, which is a rewarding stimulus that is rapidly metabolized, providing energy supplies, bees assess the value of the food and subsequently associate the surrounding cues to such a perception (Scheiner et al. 1999, 2001). As long as the bee

has repeated stimulus–reward experiences, these associations turn into memories. These memories improve foraging efficiency by directing the bee towards the learned stimulus when it is present (Ribbands 1955; Koltermann 1969; Barth 1985; Wenner & Wells 1990; Menzel 1999). In particular, olfactory memories influence a bee's orientation (Chaffiol et al. 2005) and landing response (Free 1969; Arenas et al. 2007, 2008) by eliciting discrete behaviours such as extension of the proboscis after conditioning (Takeda 1961) and by reactivating experienced foragers to visit past profitable sources that were scented with the memorized odour (Ribbands 1954; Johnson & Wenner 1966; Reinhard et al. 2004).

In contrast, the influence of learning and memory on pollen-foraging behaviour, a topic that has broad relevance for understanding pollination interactions and task partitioning among members in a social insect colony, is less well known. The extent to which honeybees are able to assess pollen quality locally (Waddington et al. 1998; Pernal & Currie 2002) and learn incidental floral cues (Scheiner et al. 2004; Grüter et al. 2008) from plants that do not offer nectar (Vogel 1983) has not been studied in depth.

Honeybees that forage for pollen prefer to visit certain floral species over others (Free 1963; Nye & Mackensen 1965; Olsen et al.

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1979; Free 1993), and they make more frequent visits to plants producing high-quality pollen (Hanley et al. 2008). When tested under experimental conditions, however, pollen foragers showed a bias towards pollen irrespective of the nutritional value of the pollen source, suggesting that pollen-based cues have a greater influence on pollen-foraging behaviour than does the nutritional value of the source (Pernal & Currie 2002). Pernal & Currie (2002) found that foragers were incapable of making foraging decisions based on the protein content of the source, but the bees could discriminate among sources by evaluating the intensity of the pollen odour, a cue that might provide information about pollen availability and abundance (Dobson et al. 1999).

Other than spontaneous preferences elicited by pollen-based stimuli, pollen-foraging preferences may also rely on learning. Cook et al. (2003) showed that foragers with prior experience of oilseed rape pollen preferred to land on and collect oilseed rape rather than a different pollen type (field bean) when tested in a dual-choice device. This preference for oilseed rape pollen did not seem to be due to an innate response for its pollen-based cues (such as the colour or odour), but seemed to be learned while the bees were collecting this specific resource. In the alternative situation, however, bees that experienced the field bean pollen showed no preference towards it, suggesting that decision making based on information acquired from pollen still remains elusive.

Besides the influence of pollen-based cues in terms of acquired behaviour (Cook et al. 2003, 2005), restrained bees can also learn neutral pure odours associated with bee-collected pollen (Grüter et al. 2008). Bees extend their proboscises when sugar solution is applied to their antennae (Takeda 1961), but also when either hand-collected or bee-collected pollen is applied to the antennae (Page et al. 1998; Scheiner et al. 2004). The contingency created between the reward (unconditioned stimulus, US) and the odour (conditioned stimulus, CS) then enables the bees to extend the proboscis to the odour alone, and hence, to predict the oncoming pollen reward (Grüter et al. 2008). However, it is unknown whether bees assess hand-collected pollen and learn incidental floral cues associated with it.

In the present study, we address the question of how forager bees learn and retain stimuli associated with pollen. To this end, we first focused on the retrieval of putative olfactory memories acquired while foraging for pollen or nectar on an artificial feeder and measured the bees' responses to these resources in terms of orientation, information transfer and foraging reactivation (i.e. acquired behaviours). Orientation responses were tested to reveal the role of olfactory memories in guiding bees towards the scent previously learned at the pollen source. Retrieval of memories acquired in free-flying bees were then tested under laboratory conditions (i.e. Y-maze and proboscis extension reflex, PER, paradigm) to evaluate and compare pollen and nonpollen foragers' abilities to transfer and extend the use of acquired information to a broader range of environmental features. We also examined whether memories established with pollen could be recalled inside the hive and lead experienced foragers to resume activity at previously profitable pollen-foraging sites.

Finally, we tested the learning abilities of pollen and nonpollen foragers towards hand-collected pollen from different species used as reward in the PER assay. Likewise, we examined the role of various pollen components (sugars, fatty acids and amino acids) as excitatory (appetitive) stimuli during classical PER conditioning.

METHODS

Study Site and Animals

The experiments were carried out during the summer seasons of 2010 and 2011 in the experimental field of the School of Exact and Natural Sciences of the University of Buenos Aires (34°32′S, 58°26′W). Ten colonies of European honeybees, *Apis mellifera*, were used in the different experiments. The experimental colonies, containing about 40 000 bees each, were commercial hives housed in our apiary. Honeybee foragers were initially trained to visit, ad libitum within a few hours (2–3 h), a feeder that offered a dilute sugar solution (15% w/w) 50 m from the apiary. Then, the feeder was replaced by a petri dish containing 10 g of crushed pollen. The bees that shifted to pollen collection were colour coded with acrylic paint on the thorax or abdomen. Different colour-marked bees were unambiguously identified, allowing us to track the changes in individual behaviours according to a given experience. Although experiments were carried out near the apiary, spontaneous visits to the artificial pollen-feeders were rare, allowing us to readily capture and remove unmarked bees from the experimental setting. In most of the experiments, we used a mixture of bee-collected pollen (Apícola Calandri, Buenos Aires, Argentina); however, we used hand-collected pollen to control for the extra sugars that bees commonly add during pollen packing.

The experiments complied with the animal care guidelines of the National Institutes of Health (1985) and the current laws of Argentina.

Experiment 1

Olfactory conditioning in free-flying honeybees

Here we studied the possibility that bees exploiting a pollen source can learn neutral odorant cues associated with it, and in turn, establish associative memories that, once retrieved, are helpful for guiding the forager towards the feeder scented by the learned odour. By testing landing preferences, we quantified the role of learned odour in guiding foraging flights near the artificial feeder and in controlling landing responses towards it. Moreover, these experiments attempted to clarify the role of pollen as a rewarded stimulus (unconditioned stimulus, US) in olfactory conditioning when free-flying bees experienced a neutral odorant while collecting pollen.

Before starting the conditioning procedure, we unambiguously marked bees while they were foraging ad libitum at a pollen feeder (pretraining feeder) that was placed in the middle of a wooden platform (160 cm long). There were two phases to the conditioning: a training phase and a testing phase. The training phase began when the feeder (i.e. training feeder) was scented by a piece of filter paper (5 × 2 cm) soaked in a pure odorant (100 µl). The piece of paper was placed inside a plastic mesh cylinder (7 cm high, 2.5 cm diameter) similar to a 'hair curler' that rested inside the feeder. We used two pure odorants that are commonly found in melliferous flora (Knudsen et al. 1993), linalool (LIO) and phenylacetaldehyde (PHE), as the conditioned stimulus (CS).

We performed three experiments (experiments 1a, 1b, 1c). In the first and second experiments, training lasted for 1 h, during which foragers had free access to the training feeders and completed several foraging bouts (see below). The third experiment was performed to test for bees' landing response after a single conditioning trial (i.e. only one foraging bout).

For all three experiments, the training feeder was removed and the platform remained empty for 1 h before testing phase began. Then, two similar feeders (i.e. testing feeders) were placed equidistant (130 cm) from each other on the platform (65 cm from the original position of the training feeder). At this point the feeders were scented: one with the conditioned odour offered during training (LIO or PHE) and the other with a novel testing odour (PHE or LIO). We recorded the bees' landings at both feeders. Bees were captured after the first landing (i.e. each bee was tested only once). If the landing occurred at the feeder scented by the conditioned odour (CS), the response was considered as correct. Each bee was

tested alone. The positions of the feeders were exchanged pseudorandomly. In experiments 1a and 1c, the testing feeders offered pollen, whereas in experiment 1b they offered powdered chalk. The rationale behind this was to offer a mimic for the bees to collect that neither represented a putative reward nor presented odours of pollen. Additionally, we quantified the proportion of bees that actually gathered what the feeders offered.

To determine the number and extent of trials that enabled learned responses in experiments 1a and 1b, we counted visits to the training feeder and measured inter- and intratrial intervals during successive foraging visits. The intertrial intervals were defined as the time between two successive foraging bouts (from the time the bee abandoned the feeder up to the time it returned to the feeding site), whereas the intratrial intervals were defined as the time the bee took to gather its load (from the time the bee landed at the rewarded training feeder up to the time it left the feeder).

Experiment 2

Transfer of information acquired during pollen foraging to behavioural responses in the laboratory

Here we challenged bees' abilities to transfer and extend the use of the olfactory information acquired in a pollen-gathering context to a broader range of environmental features. We tested whether retrieval of the putative olfactory memories established during pollen foraging were independent of the retrieval context and could be transferred to (1) an orientation response in a Y-maze or (2) a PER assay in the laboratory. By doing so we focused on retrieval of CS-specific memory and discarded the putative effect of nonspecific memories that might have been established during training, such as visual and tactile memories.

The honeybee foragers were initially trained to visit, ad libitum, a pollen feeder (a petri dish containing 10 g of commercial bee-collected crushed pollen mix), which was later scented with a neutral olfactory stimulus (conditioned stimulus; CS (LIO or PHE)) as described for experiment 1. Bees that arrived at the scented feeder were unambiguously marked with different coloured paints. This allowed us to control the number of trials per bee. Colour-marked bees were captured after five consecutive conditioning trials. Half of these bees were tested in a Y-maze in which one arm was scented with the conditioned odour (LIO or PHE) and the other arm was scented with a novel odour (PHE or LIO). The other half of the bees were harnessed and tested once for their PER to the CS and once for their PER to the novel odour, as described below (experiment 4).

As an additional control for analysing the ability of the honeybee to transfer olfactory memories between different contexts, we repeated the experiment using sucrose solution (rather than pollen) as the US (50% w/w sucrose solution). That is, an ad libitum feeder offered an unscented sucrose solution while glass petri dishes (1 cm high, 15 cm diameter) containing filter paper circles (5.5 cm diameter) soaked in a pure odorant (100 µl of pure odorant) were placed below the feeder to deliver the odorant to the feeding surroundings. Before analysing the orientation response in the Y-maze (for details, see Supplementary Material), the bees were individually allowed to explore the device to familiarize them with the set-up. To obtain orientation responses in an appetitive context, the bees were fed a small drop of sugar solution (20% w/w) in the main chamber of the maze (entrance). The entrance led to two arms separated by 90°, which remained unscented during this phase. After 5 min, each bee was gently removed and the maze was cleaned with ethanol (96% v/v). Then, 10 min later, the focal bee was released again at the entrance channel to measure its preference. At this time, the Y-maze contained the CS on one arm and a novel

odorant on the other arm. The odorants were delivered in a constant airflow (15 ml/s) that passed through a 10 ml syringe containing 4 µl of LIO or PHE on a small piece of filter paper. Both airstreams were driven from the bases of the two arms by means of an air pump. Laminar airflows carried the odorants towards the intersection of both arms: the decision area of the maze.

Once in the Y-maze, we recorded bees' decisions and times using custom-made software (Grupo de Estudio de Insectos Sociales, INSSOC, Facultad de Ciencias Exactas Y Naturales, Buenos Aires, Argentina). To this end, we focused on three maze areas: the entrance channel and the two arms. We measured each bee's first choice and the proportion of time that it spent in each arm (henceforth: percentage of time spent in each arm, 'stay behaviour') for up to 2 min. Both variables are appropriate for studying olfactory memories in ants and honeybees (Provecho & Josens 2009; Carcaud et al. 2009), and represent two main components of food-searching behaviour: first choice enables the bee's orientation towards the learned cue, and stay behaviour enhances the bee's chances of finding the food once it recognizes the learned cue. Both measurements are expected to be similarly influenced by olfactory memories; however, slight differences have been reported (Provecho & Josens 2009), suggesting that specific processes underlying memory recall are operating in each case. The time spent in each arm was recorded once the first choice was made, although if this took longer than 3 min, the subject was excluded from the experiment. For this variable we obtained the relative time that the bee spent in each area of the maze (the entrance channel and the two arms). As a consequence, we obtained the average proportion of time spent in each area. Afterwards we reported the percentage of time spent in the CS and in the novel odour arm. Each bee was subjected to only one test in the maze and no reinforcement was provided during the recording phase.

We expected odour–pollen associations to bias the orientation response positively towards the CS in the Y-maze as well as to elicit CS-specific responses in the PER paradigm.

Experiment 3

Odour-triggered reactivation of experienced bees to a past pollen-foraging site

In this experiment we investigated reactivation of experienced foragers to past profitable pollen sources. To this end, we tested whether putative learned olfactory cues acquired at the source can trigger navigational memories that assist foragers in flying back to a known pollen source. We predicted, therefore, that learned odours would induce foragers to return to the feeder's former location when we merely introduced the scent into the hive, even when the destination no longer bore the scent.

For this, bees belonging to the focal colonies were trained to forage for pollen from a training-scented feeder located 50 m from the hives. The training pollen feeder contained 10 g of bee-collected pollen scented with either LIO or PHE as described for experiment 2. The training period lasted 3 h, and each bee that arrived at the feeder during this period was unambiguously paint-marked. After the 3 h training period, the training feeder was removed and the platform remained empty for 3 h; this phase was called the extinction phase. After the extinction phase, the testing phase began. For testing, we placed an unscented (no CS or novel odour) testing feeder containing pollen in the same location where the training feeder had been. The testing feeder contained pollen to ensure that the arriving bees were actually engaged in pollen gathering.

The testing phase lasted 6 h, during which time we performed 6 min testing events each hour. Such a short testing event (6 min) reduced the possibility of measuring chance arrivals. During each

testing event, the CS or the novel odorant was blown into the hive. Olfactory stimulations (100 μl of pure odorant applied to a 5 × 2 cm piece of filter paper) were blown (58 ml/s) into the hive through the hive entrance. In addition, we introduced clean air into the hive as a control stimulus for arrivals to the odourless treatment. The testing feeder was removed during nonstimulation periods. We captured each bee that arrived at the feeder during the 6 min testing period, but we considered only those bees that could be identified on the bases of their colour marks. Since only the colour-marked individuals experienced the odour cue with pollen (reward), we excluded all unmarked bees from the analysis. To obtain sufficient data, we repeated the entire procedure (training, extinction and testing phases) 16 times (eight times each for LIO and PHE as the trained odour) using new locations for the feeders. In this experiment, we did not control for the number of trials that each bee experienced during the training phase.

Experiment 4

Olfactory PER conditioning using fresh pollen and its main constituents as the US

The PER assay is based on the reflexive responses of the proboscis elicited by a US applied to the antennae and it has been extensively used during olfactory conditioning with sugar solution as a reward (Bitterman et al. 1983). Interestingly, forager bees extend their proboscises when both hand-collected and bee-collected pollens are applied to the antennae (Page et al. 1998; Scheiner et al. 2004). We then tested the learning abilities of pollen and nonpollen (presumably nectar) foragers towards different hand-collected pollen and some of its components (sugars, fatty acids and amino acids) that were used as reward in the PER assay. Four conditioning trials (intertrial interval of 10 min; thus, four spaced trials) were performed using LIO as a CS.

Bees captured at the entrance of the hive were anaesthetized with CO_2 and harnessed in metal tubes in such a way that they could still freely move their mouthparts and antennae. They were kept in an incubator (30 °C, 55% RH and darkness) for about 2–3 h before being tested in the PER assay. Only bees that showed an unconditioned response (UR) to application of fresh pollen, amino acids or fatty acids onto the antennae and that showed no response to a mechanical stimulus (airflow) were used. Moreover, we ruled out the alternative possibility that the presentation of pollen aromas alone could elicit a PER. For this, we quantified the PERs after presenting aromas blown from a natural pollen sample (50 g) inside a 10 ml syringe. High levels of PER in this regard would indicate a spontaneous response towards odours of pollen or the existence of olfactory memories related to this stimulus.

A device that delivered a continuous airflow was used for the odorant application. During conditioning, a constant airflow of 50 ml/s was delivered to the head of the bees through a tube (1 cm diameter) placed 2 cm in front of the bees. Filter paper was impregnated with the odour (4 μl of pure odorant on 30 × 3 mm piece of filter paper) and placed inside a syringe. The odour was delivered through a secondary airstream (6.25 ml/s) injected in the main airflow during delivery of the odour. During the PER experiment, a fan extracted the released odours to avoid contamination. Each learning trial lasted 40 s. Before odour presentation, the bees were rested for 15 s in the airflow to allow familiarization of the set-up as well as to test the bees' response towards the mechanical stimulus. For the classical conditioning training procedure, we presented the CS for 6 s. Reinforcement was placed on the bee's proboscis (mouthparts) for 3 s, 3 s after the onset of the CS. During testing, we considered the PER during the first 3 s of presentation of the test odour. The presentation order of the odours during the tests was balanced.

Fresh (hand-collected) pollen was obtained from dandelion (*Taraxacum officinale*) and drunken stick (*Chorisia speciosa*) flowers by brushing the pistils with a soft paintbrush. Pollen grains were kept at 5 °C until used. We obtained amino acids from Sigma-Aldrich (St Louis, MO, U.S.A.; Amino Acid Standards physiological, basics; A6282). This solution contained 15 amino acids (g-amino-n-butyric acid, ammonium chloride, L-anserine, L-arginine, L-carnosine, creatinine, ethanolamine, L-histidine, L-homocystine, d-DL-hydroxylysine, L-lysine, 1-methyl-L-histidine, 3-methyl-L-histidine, L-ornithine and L-tryptophan at 2.5 μmol/ml ± 4% in 0.1 N HCL) that are commonly found in pollen samples. We obtained myristic, palmitic, stearic, oleic, elaidic, linoleic, linolelaidic, linolenic, arachidic and behenic acids from Supelco Analytical (Sigma-Aldrich, F.A.M.E. Mix C14-C22; 18917-1Amp), which are also likely to occur in pollen samples.

Statistics

We applied a chi-square test for goodness of fit to the results of the bees' landing responses at the testing feeder in the dual-choice device (experiment 1) and to the bees' first choices in the Y-maze (experiment 2) to determine whether the observed frequencies significantly deviated from random expected frequencies (50% for each; Sokal & Rohlf 1995). We also used this analysis in experiment 3 to examine differences between the number of bees reactivated by the CS and novel odorant or air stimulation. To carry out these multiple comparisons (bees reactivated to the CS versus novel odorants and bees reactivated to the CS versus air stimulation), we reduced the risk of type I errors by adjusting the probability per comparison to $\alpha' = 1 - (1 - \alpha)^{1/k}$, with $k = 2$, according to Dunn–Sidák correction (Sokal & Rohlf 1995). We also applied this correction in experiments 2 and 4. To compare the PER levels and orientation responses between the different odours and forager types (experiment 2), we applied *G* tests to the proportions (Sokal & Rohlf 1995). We compared the percentage of time spent in each arm of the maze (experiment 2) and the CR and UR profiles achieved in the PER assay (experiment 4) using repeated measures ANOVA. When significant interactions between factors were detected in the ANOVAs, we applied simple effects to describe the relation between these factors (Quinn & Keough 2002).

RESULTS

Experiment 1

Olfactory learning in free-flying bees

As a general result from experiments 1a, 1b and 1c, we observed that, after training, bees preferred to land on the pollen feeder scented with the odour previously offered in the training pollen feeder (LIO and PHE) rather than on the testing feeder scented with a novel odour. In experiment 1a, where the testing feeders also contained bee-collected pollen, the number of landings on the conditioned-odour feeder was significantly higher than on the novel-odour feeder (chi-square test: LIO: $\chi_1^2 = 12.25$, $N = 16$, $P < 0.001$; PHE: $\chi_1^2 = 4.17$, $N = 24$, $P = 0.041$; Fig. 1a). These preferences indicated that, despite the presence of pollen, the neutral odour (CS) was learned and influenced the landing response on the correct feeder.

Similar preferences were observed in experiment 1b, where the testing feeders offered powdered chalk instead of pollen (chi-square test: LIO: $\chi_1^2 = 31.410$, $N = 39$, $P < 0.001$; PHE: $\chi_1^2 = 8.0$, $N = 32$, $P = 0.004$; Fig. 1b). Surprisingly, the conditioned odour itself was enough to bias landings on the correct feeder. However, only a few bees actually collected the powdered chalk and packaged it into small loads. In fact, most of the conditioned bees
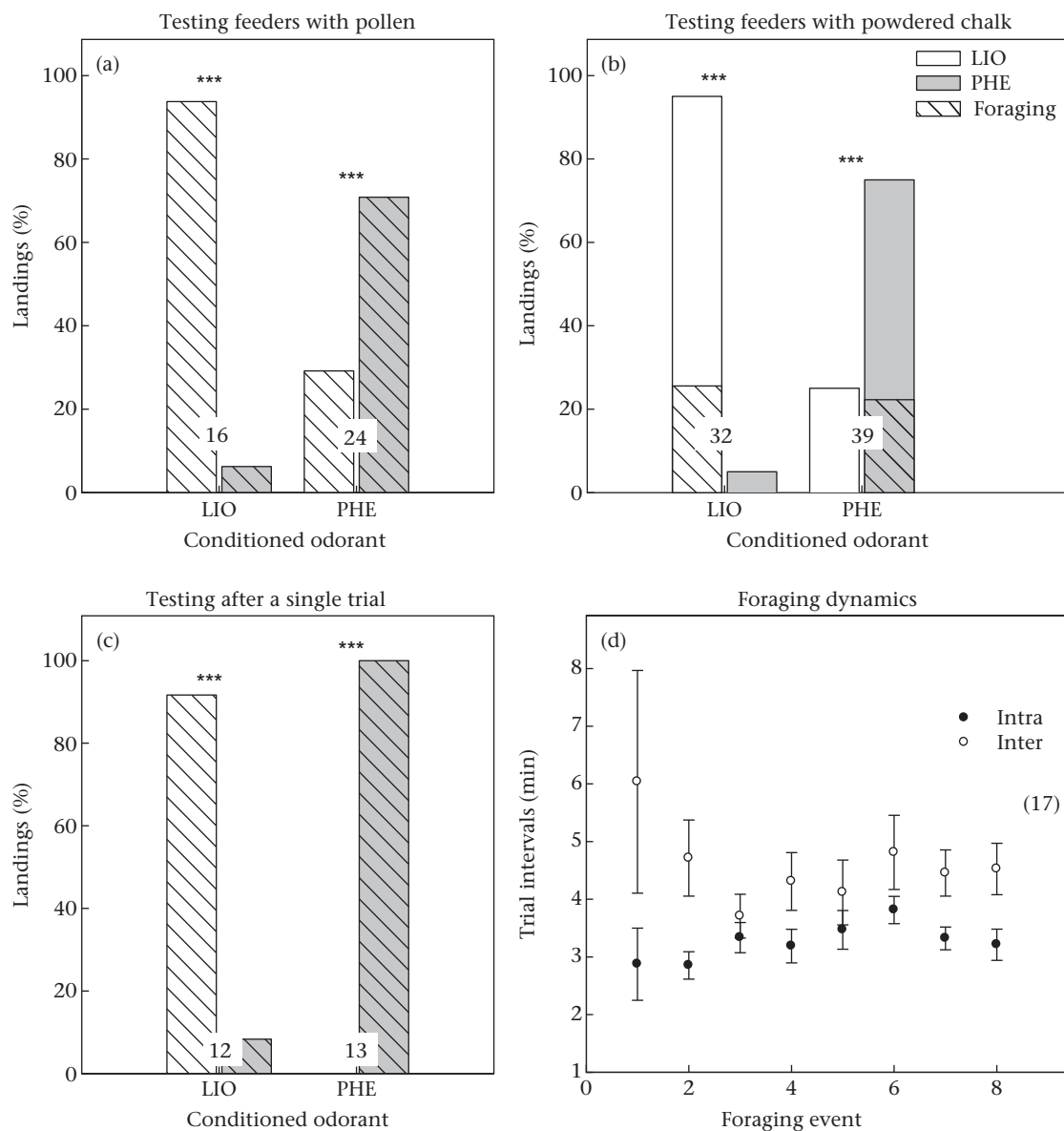
Figure 1. Percentages of landings on testing feeders after honeybees collected pollen from a linalool (LIO)-scented or phenylacetaldehyde (PHE)-scented training feeder: (a) testing feeder offered pollen after multiple foraging bouts; (b) testing feeder contained powdered chalk after multiple-trial conditioning; (c) testing feeder offered pollen after a single visit to the training feeder. Asterisks indicate statistical differences for the landing response (***$P < 0.001$; see Results for details). Number of landings is shown at the bottom of each bar. □: landings at the LIO-scented testing feeder; ▨: landings at the PHE-scented feeder; ▨: proportion of bees that collected pollen or powdered chalk from the testing feeders. (d) Dynamics of foraging bouts ($\overline{X} +$ SE intra- and intertrial conditioning intervals recorded over successive foraging bouts).

abandoned the feeders immediately after touching the powdered chalk. The former results indicated that, in the absence of pollen aromas, which unequivocally indicated the presence of pollen, the bees' choices relied on the odours that were previously paired with the resource. A strong preference for landing on the correct feeder was also measured after a single learning event (experiment 1c; chi-square test: LIO: $\chi^2_1 = 9.308$, $N = 13$, $P = 0.002$; PHE: $\chi^2_1 = 10.286$, $N = 14$, $P = 0.001$; Fig. 1c), showing that pollen foragers could learn neutral odours associated with pollen within a single foraging bout, as has been observed with sugar as a reward (Menzel 1999).
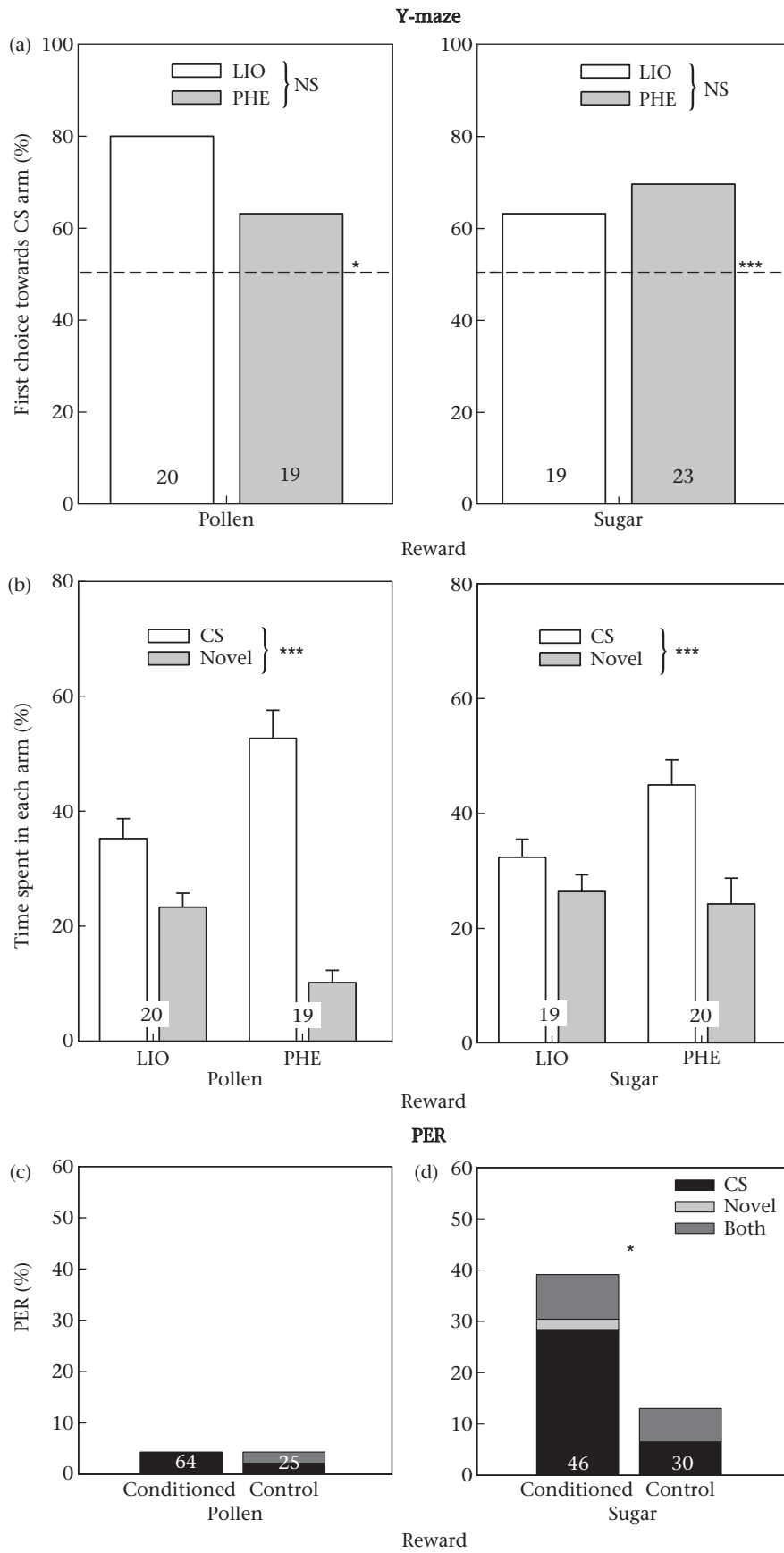
Figure 1d shows the dynamics of foraging behaviour over 1 h, where the honeybees completed seven (at most eight) foraging bouts at the training site located 50 m from the hives. Both inter- and intratrial intervals remained constant over successive visits (RMANOVA: intertrial interval: $F_{7, 112} = 1.843$, $N = 17$, $P < 0.092$;

intratrial interval: $F_{7, 112} = 1.732$, $N = 17$, $P < 0.108$; Fig. 1d) and hence were not affected by learning. In spite of this, dispersion of the data was clearly reduced after the first visit.

Experiment 2

Transfer of information acquired during pollen foraging to behavioural responses in the laboratory

Olfactory memories established during pollen foraging at an artificially scented feeder were successfully retrieved in the device for analysing orientation responses (i.e. the Y-maze). As expected, memories established with sugar were also retrieved. For the first choice, there were no differences between the conditioned odours (LIO and PHE), so we pooled the data (G test: first choice/pollen reward: $G_1 = 1.376$, $N = 39$, $P = 0.240$; first choice/sugar reward: $G_1 = 0.191$, $N = 42$, $P = 0.661$; Fig. 2a). Pollen–odour and sugar–odour

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Figure. Y-maze and PER results. (a) First choice towards CS arm (%) for Pollen and Sugar rewards; (b) Time spent in each arm (%) for LIO and PHE in Pollen and Sugar; (c, d) PER (%) for Conditioned and Control in Pollen and Sugar.

associations were similarly transferred (G test first choice/pollen versus sugar reward: $G_1 = 0.250$, $N = 81$, $P = 0.617$). In both cases, more bees chose the CS as their first choice (chi-square test: first choice/pollen reward: $\chi_1^2 = 4.666$, $N = 42$, $P = 0.030$; first choice/ sugar reward: $\chi_1^2 = 7.410$, $N = 39$, $P < 0.001$; Fig. 2a). For the percentage of time spent in each arm, we found that the conditioned bees stayed relatively longer on the CS-scented arm (Fig. 2b; note that the percentages in Fig. 2b do not add up to 100% because the experimental bees also spent time at the entrance channel of the maze, which is not shown in the figure). The percentage of time spent in each arm differed according to the identity of the CS and the nature of the reward (RMANOVA: time*odour: $F_{1, 77} = 14.675$, $N = 81$, $P < 0.001$; time*reward type: $F_{1, 77} = 5.549$, $N = 81$, $P < 0.021$; Fig. 2b). Simple effect analyses revealed that pollen foragers and sugar foragers spent similar amounts of time on the CS-scented arm ($F_{1, 154} = 2.965$, $P = 0.085$) and that PHE was better transferred as the CS than was LIO ($F_{1, 154} = 18.465$, $P < 0.001$).

Olfactory memories established with sugar as a reward were successfully transferred to the PER paradigm, but memories established with pollen failed. Responses to the CS did not differ and were pooled (G test: LIO versus PHE/sugar reward: $G_1 = 0.641$, $N = 46$, $P = 0.423$; LIO versus PHE/pollen reward: $G_1 = 2.694$, $N = 64$, $P = 0.100$). Bees showed high levels of PER to the odour associated with the sugar solution feeder (G test: PER/sugar reward: $G_1 = 3.945$, $N = 76$, $P = 0.046$; Fig. 2d), but they showed low levels of PER (no transference) to odours associated with pollen as a reward (LIO versus PHE/pollen reward: $G_1 = 0.040$, $N = 89$, $P = 0.839$; Fig. 2c).

Experiment 3

Odour-triggered reactivation of experienced bees to a past pollen-foraging site

Olfactory cues associated with pollen at the feeding site induced foraging reactivation when these cues were blown into the hive (Fig. 3). The conditioned odour triggered more visits than the novel odour irrespective of the stimulus identity (chi-square test: LIO: $\chi_1^2 = 5.77$, $N = 39$, $P = 0.0163$; PHE: $\chi_1^2 = 10.76$, $N = 41$, $P = 0.001$; Fig. 3). Similarly, the number of visits made in response to CS stimulations and air stimulations also differed (chi-square test: LIO: $\chi_1^2 = 10.31$, $P = 0.001$, $N = 35$; PHE: $\chi_1^2 = 15.16$, $N = 38$, $P < 0.001$; Fig. 3). All of these comparisons were in reference to $\alpha' = 0.025$.

Experiment 4

Olfactory PER conditioning using fresh pollen and its main constituents as the US

The percentage of bees that showed an unconditioned response (UR) and the percentage of bees that were successfully conditioned during the four spaced trials in the PER assay are shown in Fig. 4. Because many bees stopped responding to the US after the fourth trial, in some cases, we did not have sufficient conditioned responses (CRs) for statistical analysis.

As expected, both nonpollen and pollen foragers showed high levels of PER to the sugar solution (30% w/w; Fig. 4a). These responses remained stable over successive presentations, and both

groups showed excellent learning abilities for associating the odours with this reward (Fig. 4a). In contrast, the bees' UR to fatty acids showed a steady decline over successive presentations (RMANOVA: trial: $F_{3, 168} = 45.757$, $N = 118$, $P < 0.001$), which was similar in the nonpollen and pollen foragers (trial*forager type: $F_{3, 168} = 1.487$, $N = 118$, $P = 0.217$, $N = 118$; Fig. 4b). However, the proportion of pollen foragers that responded to the US was significantly higher than that of nonpollen foragers (forager type: $F_{1, 56} = 11.927$, $N = 118$, $P < 0.001$). Cue acquisition, only observed in pollen foragers, was low but consistent with a rapid response in the second trial (trial: $F_{3, 33} = 11.927$, $N = 118$, $P = 0.06$). The amino acid solution did not elicit a UR, so nonpollen foragers ($N = 31$) and pollen foragers ($N = 31$) could not be conditioned (Fig. 4c). Both pollen and non-pollen foragers showed high levels of URs to hand-collected pollen at the beginning, a response that decreased rapidly in nonpollen foragers for dandelion pollen (trial*forager type: $F_{3, 204} = 43.625$, $N = 78$, $P < 0.001$; Fig. 4d). Despite the fact that most of the pollen foragers continued to respond to dandelion pollen after the fourth presentation (Fig. 4d), associative learning was weak (trial: $F_{3, 51} = 1.369$, $N = 18$, $P = 0.265$). Furthermore, the proportion of pollen foragers that responded to the drunken stick pollen was significantly greater than that of nonpollen foragers (forager type: $F_{1, 76} = 27.016$, $N = 78$, $P < 0.001$; Fig. 4e), and the rate at which both groups ceased responding differed significantly (trial: $F_{3, 228} = 17.950$, $N = 78$, $P < 0.001$; Fig. 4e). Pollen foragers performed well during conditioning and showed significant improvement across trials (trial: $F_{3, 60} = 9.469$, $N = 21$, $P < 0.001$), whereas nonpollen foragers failed to do so (trial: $F_{3, 15} = 1.752$, $N = 6$, $P = 0.199$).

In summary, the UR and CR profiles for the hand-collected pollen and its constituents differed and varied depending on the type of forager: nonpollen or pollen foragers. As a general observation, the pollen foragers always responded more than nonpollen foragers and for longer periods (more trials). Conditioned responses were successfully achieved with the sugar solution, the two types of fresh pollen and the fatty acids. We detected no PERs towards the odour of pollen when it was held near the antenna, just before touching it.

DISCUSSION

Here, we clearly demonstrate that preferences during pollen foraging involve learned behaviours. Bees that forage for pollen might take advantage of their ability to learn and retain olfactory information to perform their tasks efficiently. Bees exploiting a pollen source can learn the surrounding odorant cues within a few visits and in turn establish memories that, once retrieved, can be helpful for guiding the forager towards the learned stimulus encountered in the field. Other than spontaneous preferences elicited by pollen-based stimuli, the ability of bees to learn about other features of the source, such as floral odours, expands the type and amount of information to which foragers can respond and, consequently, their behavioural repertoire. Memorization of olfactory information acquired with pollen as the only rewarding stimulus is relevant since it could improve foraging efficiency by reducing the time spent searching for flowers (Menzel 1999).

Our results show that the memory retrieval of odour−pollen associations is independent of contextual parameters as long as

Figure 2. Transfer of olfactory memories from an artificial feeder outside the hive to (a, b) a Y-maze and (c, d) a proboscis extension response (PER) paradigm. Transfer was measured after ad libitum foraging on pollen or a sugar solution from a feeder scented with linalool (LIO) or phenylacetaldehyde (PHE). Orientation response was quantified in the Y-maze, which contained the conditioned odorant (LIO or PHE) on one arm and a novel odorant (PHE or LIO) on the other: (a) first choice towards the arm containing the conditioned stimulus (CS); the dashed line at 50% indicates random choice between the CS and the novel odour; (b) percentage of time spent in each arm ($\overline{X} + SE$). PER assay: pooled proboscis extension responses to the CS (LIO or PHE) and the novel odour (LIO or PHE) after the bees foraged on LIO- or PHE-scented (c) pollen or (d) sugar solution. Control bees always foraged on unscented food at the testing feeder. Asterisks indicate the statistical differences between the pooled data ($^*P < 0.05$; see Results for details). Number of bees tested is shown inside each bar.
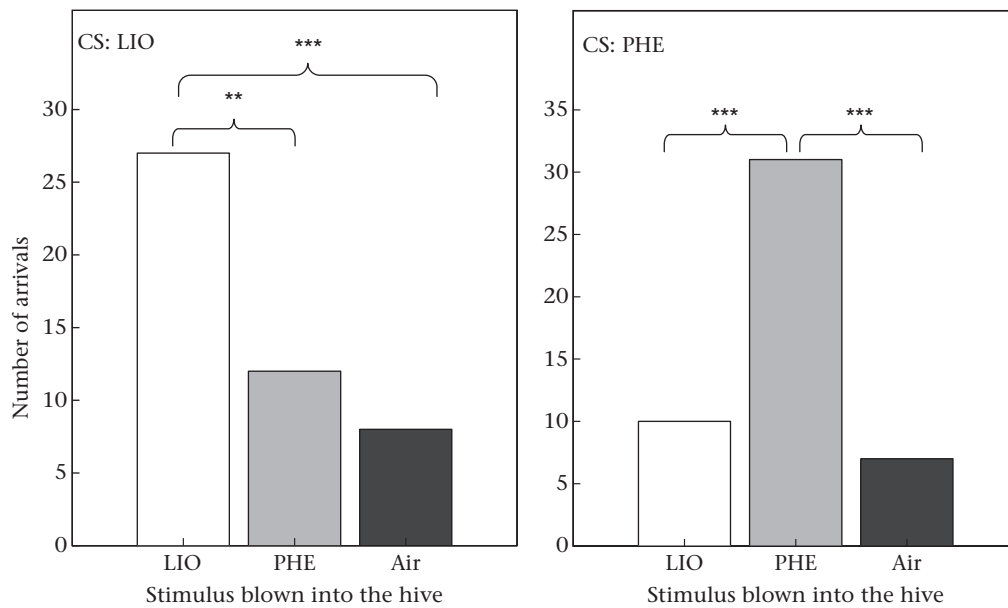
Figure 3. Odour-triggered reactivation of experienced bees to a past pollen-foraging site that carried (a) linalool (LIO) or (b) phenylacetaldehyde (PHE) as the conditioned stimulus (CS). Arrivals of experienced-marked bees to a pollen feeder after LIO, PHE or air had been blown into the hive. Blowing of the stimuli was randomized. **$P < 0.01$; ***$P < 0.001$ (where $\alpha' = 0.025$).

these contexts are biologically relevant to the pollen forager. In addition, we show that the ability to transfer and retrieve such memories, irrespective of the behavioural context, can be useful for reactivating pollen foragers towards past profitable sources,

a phenomenon that might have a strong impact on recruitment and searching for resources. The conditioned responses previously obtained with bee-collected pollen as the reward (Grüter et al. 2008) were successfully replicated for different types of hand-collected
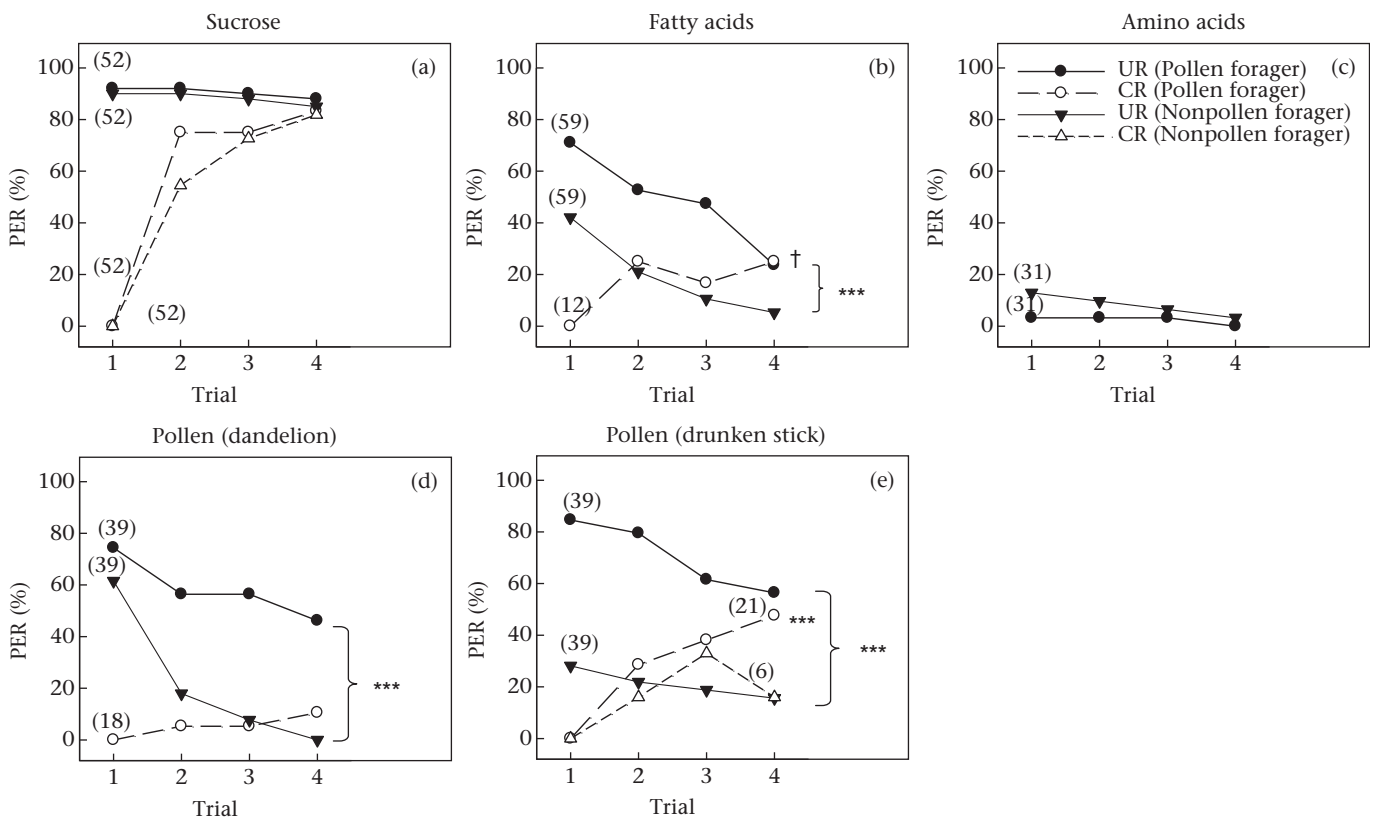

Figure 4. Proboscis extension response (PER) using different stimuli as a reward for pollen and nonpollen honeybee foragers. PER levels for unconditioned responses (UR, continuous lines) and conditioned responses (CR, dashed lines) in nonpollen foragers (triangles) and pollen foragers (circles) when (a) sucrose solution, (b) fatty acids, (c) amino acids, or (d) hand-collected pollen from dandelion or (e) drunken stick flowers were offered as excitatory stimuli. For the URs, the asterisks indicate statistical differences between forager types (***$P < 0.001$); for the CRs, the asterisks indicate statistical differences within trials (†$P = 0.06$; ***$P < 0.001$). In all cases the bees were conditioned to linalool. Number of subjects is indicated on each curve.

pollen. Interestingly, the olfactory memories established during odour–pollen associations do not seem to rely on amino acids, but most probably on sugars (Bitterman et al. 1983) and/or fatty acids.

Pollen-acquired Information Biases Foraging Behaviour

In the natural gathering situation recreated in experiment 1, we demonstrated that bees were able to learn neutral floral odorants associated with a pollen source. When tested for their landing preferences, the bees accurately chose the feeders scented with the learned odour. This response was first achieved in bees that visited the foraging site seven or eight consecutive times. A similar pattern of choice was quantified after a single learning event, suggesting that odour–pollen associations can be quickly established within the proper foraging context. One-trial associative learning of colours or odours with nectar rewards has been well documented in honeybees (Menzel 1999), but has never been established for pollen rewards. Pollen, mainly collected as a protein source for the brood, is generally scarce in terms of sugars (see below), which suggests that inner pollen-related factors might interact for such a prompt acquisition.

In experiments 1a and 1c, where the testing feeders contained pollen, all bees gathered pollen, indicating that olfactory memories were used, as expected, in the pollen-foraging context. Interestingly, and in spite of the role of pollen-based odours in making foraging decisions (Pernal & Currie 2002), we found that landing responses can also be induced by the presence of the CS alone (experiment 1b). In addition, by replacing the pollen with powdered chalk, we tested the hypothesis that learned stimuli not only elicit a landing response, but also trigger other behaviours such as the gathering of pollen-like resources. In this regard, Roces (1990) found that leaf-cutting ants, *Acromyrmex lundi* C., gathered filter paper disks more frequently when the disks were impregnated with the odour that previously scented the food in the foraging arena. In our experiment, most of the bees stopped gathering powdered chalk immediately after first few touches. However, 20% of the foragers that approached the testing feeder managed to load the powder successfully, but the amount loaded was never as much as when loading natural pollen. This low acceptance of the pollen mimic might indicate that the CS alone was not enough to trigger gathering. Nevertheless, other reasons such as differences in the size of the particles, which is known to affect the ease of pollen collection (Baker & Baker 1979; Simpson & Neff 1983), or the absence of any palatable factors might have caused this breakdown.

Apart from the role in close-range orientation and landing, odour–pollen associations also influenced the orientation response in the Y-maze (experiment 2), where neither visual nor tactile cues were involved. This finding highlights and strengthens the importance of nonpollen-based cues for making decisions in a pollen-foraging context. The ability to transfer information between contexts is a key feature of behavioural plasticity that allows the animal to extract relevant information from a particular situation to be used in another situation, thus optimizing the use of stored memories (Sandoz et al. 2000). As has been shown for odour–nectar (Gerber et al. 1996) and odour–sugar associations (Sandoz et al. 2000; Arenas et al. 2007), we found that memories established with pollen as reward could be transferred to a different behavioural context. Transfer was clearly shown for memories that elicited the same type of response, such as flying and walking towards the stimulus in the maze (i.e. orientation), but not for memories that elicited different responses such as flying and protruding the proboscis. The fact that odour memories failed to be retrieved in a classical (Pavlovian) context might indicate that PER is not an appropriate behavioural response for predicting an imminent pollen reward. This asymmetry led us to speculate that

although the processes involved in the acquisition, retention and retrieval of odour information with pollen and nectar are similar, there are still differences in behavioural plasticity of pollen and nectar foragers that need to be clarified.

Consistent with the bees' ability to transfer and retrieve pollen odour memories irrespective of the behavioural context, we observed that a group of bees trained to forage at a pollen-scented feeder could be triggered to return to the feeder's former location merely by injecting the CS into the hive. Reactivation to foraging sites has previously been shown in nectar foragers (Ribbands 1954; Johnson & Wenner 1966; Reinhard et al. 2004). For instance, Reinhard et al. (2004) found that learned cues could trigger navigational and visual memories (i.e. vector memories) in bees that experienced a sugar feeder carrying different scents or colours. Similarly, we found that learned odorants triggered the recall of memories associated with the past pollen-foraging site, which might mediate reactivation of experienced bees to that site.

Foraging reactivation by odorants could be extremely functional for recruitment inside the hive (Reinhard et al. 2004); for example, if one of the potential recruits had already foraged at this feeding location in the past. The smell of the floral odour clinging to the bodies of incoming bees could trigger the recruit's memory and induce her to return to the feeding location. In our experiment, the blown CS resembled the previous situation in which the odours might rapidly indicate to a recruit and/or an inactive forager that the food source she used to visit is productive again.

Proboscis Extension Response and Pollen Gathering

We conducted several assays to explore mandible and hindleg movements after application of gustatory stimuli to the antennae or tarsi of restrained bees (A. Arenas, unpublished data). Up to now, none of these assays had shown a conspicuous behavioural outcome susceptible to being conditioned. On the other hand, although PER does not seem to be the most appropriate behavioural outcome for determining olfactory experiences with pollen in the field (experiment 2), PER could be conditioned with pollen, hence it is useful for detecting differences in perceptual capabilities that are relevant for decision making (Scheiner et al. 2001). Previous studies have shown that the proportion of bees that respond to bee-collected pollen differs between pollen and nonpollen foragers (Page et al. 1998; Grüter et al. 2008). We also found these variations between forager types for hand-collected pollens and fatty acids (experiment 4). These differences were certainly related to thresholds of response (Page et al. 1998). Indeed, there is evidence that the response threshold could be responsible for separating nectar- and pollen-foraging tasks. Pollen foragers are more likely to show higher sensitivity to sugar, extending the proboscis to solutions of a lower sugar concentration, than do nectar foragers (Page et al. 1998; Pankiw & Page 2000). In addition, our results support the hypothesis that pollen foragers are more sensitive to other gustatory stimuli such as fatty acids. Pollen foragers' high sensitivity to other gustatory stimuli might even enable the perception of pollen constituents (including sugar) when these constituents are scarce or present at low rates or concentrations, thus permitting the establishment of memories that would not be acquired by animals with lower sensitivity (hence, most nectar foragers).

Pollen foragers in the present study differed in their preference for hand-collected pollen types. Similar differences in preference have also been reported under free-flying conditions between oilseed rape and field bean pollens (Cook et al. 2003).

Amino acids did not elicit a PER in either the nonpollen foragers or the pollen foragers, which is consistent with a lack of chemoreceptors on the antennae for detecting free amino acids, soluble

enzymes or other indicators of dietary proteins (Bernays & Chapman 1994). With regard to this matter, previous studies have shown that foraging preferences are not biased by crude protein content of a resource (Pernal & Currie 2002). Still, a modulator effect of amino acids on learning and retention with pollen could be plausible since an improvement in the conditioning performance was achieved when glycine was added to a rewarding sugar solution (Kim & Smith 2000).

How Is Olfactory Information Acquired?

Unlike nectar, what the bee perceives as a reward from pollen during learning is poorly understood. Pollen is a complex stimulus that presents different proportions of amino acids, peptides, fatty acids, vitamins, minerals and carbohydrates, which are generally restricted by two very resistant pollen wall layers: the exine and the intine (Roulston & Cane 2000). These two layers make the nutrient-rich cytoplasm inaccessible once the pollen is stored inside the hive due to biochemical changes resulting from the addition of microbial organisms in the hive (Roulston & Cane 2000). It is unlikely that rewarding stimuli are in the cytoplasm of the grain; most likely they are on the outermost layer instead, the pollenkitt. The pollenkitt is the most common adhesive material found around pollen grains and contains a mixture of lipids, hydrocarbons and proteins. It provides colour (Pacini & Hesse 2005) and odour (Dobson et al. 1996) to the grain and enables adhesion and packaging (Pacini & Hesse 2005). Most relevant in this context, pollenkitt would provide a reward for pollinators (Pacini & Hesse 2005).

Conclusions

Our experiments led us to conclude that neutral floral odours can be learned by honeybees using pollen as a reward stimulus. We clearly demonstrate that preferences during pollen foraging involve learned behaviours, and we show that nectar and pollen foragers use the same strategy for learning to improve odour-mediated responses. Many studies have focused upon foragers' responses to sugar to improve our knowledge about the partitioning of foraging tasks. Instead, we focused on pollen-mediated responses to describe how these responses influence the decisions of both pollen and nonpollen foragers (i.e. nectar foragers).

Supplementary Material

Supplementary material for this article is available, in the online version, at doi:10.1016/j.anbehav.2012.01.026.

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