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Bias to pollen odors is affected by early exposure and foraging experience

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ABSTRACT

In many pollinating insects, foraging preferences are adjusted on the basis of floral cues learned at the foraging site. In addition, olfactory experiences gained at early adult stages might also help them to initially choose food sources. To understand pollen search behavior of honeybees, we studied how responses elicited by pollen-based odors are biased in foraging-age workers according to (i) their genetic predisposition to collect pollen, (ii) pollen related information gained during foraging and (iii) different experiences with pollen gained at early adult ages. Bees returning to the hive carrying pollen loads, were strongly biased to unfamiliar pollen bouquets when tested in a food choice device against pure odors. Moreover, pollen foragers' orientation response was specific to the odors emitted by the pollen type they were carrying on their baskets, which suggests that foragers retrieve pollen odor information to recognize rewarding flowers outside the hive. We observed that attraction to pollen odor was mediated by the exposure to a pollen diet during the first week of life. We did not observe the same attraction in foraging-age bees early exposed to an artificial diet that did not contain pollen. Contrary to the specific response observed to cues acquired during foraging, early exposure to single-pollen diets did not bias orientation response towards a specific pollen odor in foraging-age bees (i.e. bees chose equally between the exposed and the novel monofloral pollen odors). Our results show that pollen exposure at early ages together with olfactory experiences gained in a foraging context are both relevant to bias honeybees' pollen search behavior.

1. Introduction

Honeybees choose the flowers from which they forage for nectar and pollen. The differential visitation of flowers for nectar is governed, on the one hand, by the profitability of the food sources (Seeley, 1985) and on the other hand, by the presence of previously learned floral cues that provide bees with relevant information about the resources and facilitate their discovery during foraging flights (von Frisch, 1918, 1919; Ribbands, 1955; Menzel, 1999; Dukas, 2008). Among the flowers that offer pollen some species are visited more frequently than others (Free 1963, 1993; Nye and Mackensen, 1965; Olsen et al., 1979) probably because their pollen differs in chemical composition, nutritional value and/or amount of attractant components (Robertson et al., 1999; Kitaoka and Nieh, 2009; Hanley et al., 2008). In contrast, the influence of previous experiences on pollen foraging behavior of honeybees, a topic relevant for understanding pollination interactions, is less well known (Arenas and Farina, 2012).

Whilst most foraging honeybees are able to collect nectar, pollen-foraging behavior is restricted to the most sensitive members of the colony, those who are able to respond to gustatory stimuli, such as sugar or fatty acids, present at very low concentrations (Page et al., 1998; Scheiner et al., 2004; Arenas and Farina, 2012). Pollen is mainly collected in order to supply brood and young bees with a source of protein, but it is rarely consumed by foragers, who have a limited capacity to digest it (Winston, 1987; Crailsheim, 1998).

Most flowers emit highly complex, species-specific bouquets of volatile organic chemicals (Knudsen et al., 1993; Raguso, 2008; Schiestl, 2010). The composition of these floral scents may vary according to different plant parts and organs (Flamini et al., 2002, 2003, 2007). Maximal pollinator activity often coincides with high floral emissions (Hoballah et al., 2005), which suggests that floral signals play an adaptive role in guiding pollinators to flowers (Dobson et al., 1999; Raguso, 2008). However, how bees select

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flowers for pollen collection is not completely understood. Previous results suggest that pollen foraging is strongly elicited by pollen odor (van der Pijl, 1960), in which steroid substances and free fatty acids are the main components mediating the attraction (Hügel, 1962; Lepage and Boch, 1968). Recent evidence shows that learning of olfactory cues might also be involved (Grüter et al., 2008; Arenas and Farina, 2012) as honeybee foragers are able to learn odors associated with pollen as the only reward stimulus. Interestingly enough, foragers that gathered pollen performed better than non-pollen foragers during olfactory conditioning, which suggests that a differential response towards pollen related stimuli is a key component in task allocation and division of labor between these two functional sub-castes (Robinson, 1992).

In honeybee colonies, young workers are highly prone to perceive (and probably learn) cues whilst they feed on or manipulate pollen in the hive (Winston, 1987; Crailsheim, 1998). Early experiences with pollen odors might provide future foragers with key information about how exploited pollen sources smell (Arenas et al., 2013). In this regard, initial preferences for pollen odors in a solitary-pollen-specialist bee (*Colletes fulgidus longi plumosus*) were observed to be shaped by the pollen diet on which bees were reared as larvae (Dobson, 1987). In honeybees, whether pollen odors experienced early in life bias later pollen foraging response or not is still under debate (Boelter and Wilson, 1984).

In order to provide a framework for the interpretation of honeybee plasticity regarding pollen foraging activities, we aimed to study honeybees' pollen search images. We wondered how searching is modified according to: (i) foraging functional sub-castes (i.e. genetic predisposition to collect pollen); (ii) species-specific pollen related information acquired during foraging and (iii) different experiences with pollen which occur at early adult stages. In a first experiment, we tested whether pollen foraging bees (carrying pollen) and non-pollen (presumably foraging for nectar) foraging bees were equally attracted to novel pollen bouquets. In a second experiment, we tested the influence of species-specific pollen odors experienced previously on bees' pollen preference. We captured pollen loaded bees and let them choose between the odor emitted by the same pollen type that they had been carrying in their baskets and the odor emitted by other pollen that was being simultaneously exploited by workers of the same colony. In a third experiment, we investigated if attraction to pollen odors was shaped by the exposure to pollen during the first week after adult emergence. Responses to pollen bouquets and pure odors were measured in foraging-bees that had been reared with a multifloral pollen diet. Finally, in a fourth experiment, we tested the hypothesis that species-specific experiences gained at early adult ages are memorized and bias later foraging behavior. Experimentally, we fed newly emerged bees with monofloral diets and measured their preferences between the experienced and an unfamiliar pollen odor when they reached foraging ages.

2. Materials and methods

2.1. Study site and animals

The experiments were carried out during the summer seasons of 2011 and 2012 in the experimental field of the School of Exact and Natural Sciences of the University of Buenos Aires (34°32′S, 58°26′W). Worker bees from 4 colonies of European honeybees (*Apis mellifera*), containing about 25,000 bees each, were used in the different experiments.

In the experiments that required foragers (Experiments 1 and 2), bees were captured as they entered the hive. Incoming bees carrying pollen loads were considered pollen foragers whilst those bees that entered without pollen loads were defined as non-pollen foragers. All the experimental bees were tested within 30 min of being captured.

For the experiments that required control of the bees' previous olfactory experiences (Experiments 3 and 4), we used workers that were kept in the laboratory from the time of their emergence. They were obtained from sealed brood frames maintained under controlled conditions inside an incubator (36 °C, 55% relative humidity, no light). Newly emerged bees were collected in groups of about 150 individuals and placed in wooden cages (''lab-caged bees''). The cages ($10 \times 10 \times 10$ cm) were maintained inside a second incubator (30 °C, 55% relative humidity, no light). Bees were provided with water and fed with 1.8 M sugar solution *ad libitum* until they were tested at 17 days of age, at which age workers commonly initiate foraging tasks (Rösch, 1925; Lindauer, 1952; Seeley, 1982). Rearing conditions did not affect the general constitution of the bees.

2.2. Y-maze and odor stimulation

We used an acrylic Y-maze (Arenas and Farina, 2012) to quantify honeybees' preferences towards two odors. We measured each bee's *first choice* and the *proportion of time spent in each arm* of the maze. Whilst the former variable indicates the animal's orientation towards a preferred cue, the latter reflects how meaningful this cue is according to the time the animal invests searching the area.

Before testing for the bees' orientation responses in the Y-maze, each bee was individually allowed to explore the unscented set-up for 5 min (for details, see Arenas and Farina, 2012). After this period of familiarization, the bee was gently removed and the maze was cleaned with ethanol (96% v/v). Ten minutes later, the bee was released again in the Y-maze in order to measure its orientation response in the presence of the odors. We focused on three maze areas: the entrance channel, the scented arms. If the bee's *first choice* took longer than 3 min, the test was interrupted and the bee was excluded from the experiment. Less than 8% of the tested animals (24 bees) were excluded. The time spent in each area of the maze was recorded for 2 min after the first choice. The *proportion of time spent in each arm* was calculated as the relative time that the bee spent in that area of the maze. Although we recorded the time spent in the three areas of the maze, we just presented and compared the mean score of the proportion of time the bees spent in the arms. Each bee was tested only once.

Olfactory stimuli used to scent the arms were (i) pure odors, (ii) a multifloral pollen bouquet or (iii) monofloral pollen bouquets. In all cases, preferences were tested between two olfactory cues. We did not test odors (either pure or pollen-related) against a blank because bees showed a strong bias towards the scented option in preliminary experiments. Linalool (LIO), Phenylacetaldehyde (PHE) and 1-hexanol (1-HEX) were used as pure odors to control for olfactory stimulation during testing. We decided to use pure odors since they are more likely to be unknown for free-flying bees than complex floral blends. A small piece of filter paper was soaked with 4 μl of pure odor and placed inside a syringe. Pollen bouquets were obtained by placing pollen samples of 0.7 mg of bee-collected pollen inside a syringe. The filter papers with the pure odor and the pollen samples were replaced every two hours to ensure that the odor intensity did not vary greatly. For multifloral pollen bouquets were used bee-collected pollen samples gathered in a distant apiary (about 1150 km away, provided by Cabaña Brandsen, Santiago del Estero, Argentina). The plant species available in Santiago del Estero differ from those surrounding our apiary. Thus, we assume that multifloral pollen bouquets were unfamiliar for our bees, although no headspace measurements were performed to confirm the identity of the samples. For monofloral pollen odors we used pollen samples of *Hypochaeris* sp, *Taxodium* sp and a Solanaceae.

Each olfactory stimulus was delivered by means of a constant air flow (15 ml/s) that passed through the 1 ml syringes that contained the odorant source. 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. The bee was introduced at the proximal end of the entrance channel. The entrance channel of the maze and the arms were 8 cm and 6 cm long, respectively, and 1.9 cm high. These dimensions enabled the bees to walk, but not to fly. The arms were at a 90° angle, each at 135° from the entrance channel. The maze was placed on a rectangular supporting base (13.5 × 14.5 cm), from where it was removed and cleaned after each recording. The maze was covered by a glass rectangle (10 × 15 cm), which prevented the bees from flying and escaping.

2.3. Experimental series

2.3.1. Experiment 1. Testing preferences in bees returning with and without pollen loads

In this experiment we tested the effect that pollen odors have on the orientation response of foraging bees that are actively involved in pollen collection and of non-pollen foragers (i.e. free-flying bees that were not handling pollen at the time). Bees were tested for their odor preferences to unfamiliar scents: a multifloral pollen bouquet vs. a pure odor (LIO, PHE, or 1-HEX). Each bee was confronted with only one of the three pure odors. We then evaluated whether orientation to pollen-related cues is set according to foraging functional sub-castes or not.

2.3.2. Experiment 2. Testing pollen odor memory of free-flying pollen foragers

We tested whether pollen odor cues experienced whilst foraging on a specific pollen type provide reliable information to guide a forager towards the food source. We focused on bees returning to the hive carrying pollen loads collected from a Solanaceae and from *Hypochaeris* sp. Immediately after capture, the pollen loads were removed by means of soft forceps, observed under the microscope (Labomed microscope CXR III) and identified using palynological techniques (Hodges, 1984; Kearns and Inouye, 1993). For the test, foraging bees were placed inside the maze and one of the arms of the maze was scented with the pollen the forager brought back to the hive (*Hypochaeris* sp or Solanaceae) and the other arm with the alternative (Solanaceae or *Hypochaeris* sp).

We assumed that free-flying bees carrying pollen had collected it from natural sources and hence they had experienced its odor while foraging. We did not rule out the possibility that pollen-odor memory in pollen-foraging bees was established through several foraging bouts or even inside the hive (e.g. in the recruiting context). Since we did not control for the previous experiences of these bees, we can only be sure that they experienced the carried pollen at least once prior to being captured.

2.3.3. Experiment 3. Testing olfactory preference in bees exposed to pollen at an early age

To answer whether responses to pollen odors in foraging-age bees are affected by the exposure of bees to pollen at early adult ages or not, we compared the orientation responses of individuals reared in lab-cages with or without pollen as a component of their diet. The first group was offered an *ad libitum* mixture of bee-collected pollen (provided by Apícola Calandri, Buenos Aires, Argentina). The second group was given artificial food for honeybees (NutriApis; Apilab, Buenos Aires, Argentina). This product contained: vitamins A, B2, B6, B12, C, D3, E and K; biotin; methionine; lysine; arginine; choline; nicotinic acid; calcium pantothenate; folic acid and sucrose. Food intake, mortality, and locomotor activity at 17 days of age were normal in bees exposed to the artificial food, which suggests that all bees, independently of the diet to which they were exposed, had a similar physiological state. Bee-collected pollen was mechanically crushed with a mortar and then hydrated before being offered. The pollen and the artificial food (henceforth: surrogate) were offered during the first 7 days of the bees' adult lives and removed from the cages on the 8th day. After this time, the bees were offered water and 1.8 M sugar solution.

Caged-bees were tested for their olfactory orientation at 17 days of age. A novel multifloral pollen bouquet and a pure odor (LIO, PHE or 1-HEX) were presented in the Y-maze. Because experimental bees were kept in captivity their whole lives, we are sure that both stimuli were not experienced previously.

2.3.4. Experiment 4. Testing pollen odor memory in early exposed bees

Here we tested memory to the odors of pollen offered as food to lab-caged bees during the first week of life. We evaluated the hypothesis that newly (naive) foragers are more attracted to a scent reminiscent of pollen odors experienced at young adult ages. Lab-caged bees were fed with a single-pollen diet containing pollen from one of the following: *Hypochaeris* sp, a Solanaceaeor *Taxodium* sp. All three pollen types were collected and processed as in Experiment 2.

Preferences were tested between the experienced monofloral pollen odor and a second novel monofloral pollen odor. Bees that experienced Solanaceae pollen were tested to Solanaceae vs. *Taxodium* sp pollen odors, bees that experienced *Taxodium* sp pollen were tested to *Taxodium* sp vs. *Hypochaeris* sp pollen odors and those which experienced *Hypochaeris* sp pollen were tested to *Hypochaeris* sp vs. Solanaceae pollen odors.

2.4. Statistics

We applied a chi-square test for goodness of fit to the bees' first choices to determine whether the observed frequencies deviated significantly from random (50% for each; Sokal and Rohlf, 1995). To compare the orientation responses between the different odors used along the series (LIO, PHE and 1-HEX), we applied G tests to the proportions (Sokal and Rohlf, 1995). Since the homogeneity of variance assumption was met, we compared the means of the *proportion of time spent in each arm* of the maze using repeated measures ANOVA (RM-ANOVA). LSD tests were performed for *post hoc* comparisons to determine differences among the pure odors used in different series. When significant interactions between factors were detected, we applied simple effects to evaluate the effect of one factor separately for each level of the other factor (Quinn and Keough, 2002).

3. Results

3.1. Experiment 1. Testing preferences in bees returning with and without pollen loads

In this experiment we tested the response to pollen odors of foraging bees actively involved in pollen collection and of bees that were not handling pollen at the time. Since no differences for the *first choices* were detected among the 3 series of this experiment (LIO, PHE or 1-HEX), data within pollen and non-pollen groups were pooled (G-test$_{\text{First choice/pollen foragers}}$: $G_2 = 0.300$, $p = 0.860$, $N = 43$; G-test$_{\text{First choice/non-pollen foragers}}$: $G_2 = 1.259$, $p = 0.532$, $N = 43$). Pooled data revealed on the one hand, that pollen foraging bees were more attracted to a pollen bouquet than to a pure odorant, even when pollen bouquets were unfamiliar for the bees (Chi-square test$_{\text{First choice/non-pollen foragers}}$: $\chi^2_1 = 14.534$, $p < 0.001$, $N = 43$; Fig. 1A). On the other hand, non-pollen foragers were equally attracted to pollen than to pure odors (Chi-square test$_{\text{First choice/pollen foragers}}$: $\chi^2_1 = 3.842$, $p = 0.445$, $N = 43$, Fig. 1B). Significant differences in odor preferences were detected between pollen and non-pollen foragers (G-test$_{\text{non-pollen forager/pollen forager}}$: $G_2 = 5.384$, $p = 0.020$, $N = 86$).

We applied a 3-way repeated measures ANOVA to compare the means of the *proportion of time spent in each arm.* The *odors* used in the 3 series of the experiment (LIO, PHE or 1-HEX) and the *forager type* (pollen and non-pollen foragers) were the independent factors, whereas the *time spent in each arm* (pollen-scented or pure odor-scented arm) was the repeated measure. We found significant differences among the 3 series of the experiment (RM-ANOVA$_{\text{odor}}$: $F_{2,80} = 6.692$, $p = 0.002$, $N = 86$). *Post hoc comparison* revealed longer times spent in the pollen odor arm in the LIO and PHE-series than in the 1-HEX series. In addition, the interaction between the *time spent in each arm* and *forager type* revealed that pollen foragers stayed relatively longer in the pollen scented arm than non-pollen foragers (RM-ANOVA$_{\text{time spent in each arm*forager type}}$: $F_{1,80} = 6.422$, $p = 0.013$, $N = 86$; Fig. 1C and D). Thus, these results show that although the orientation behavior of bees towards pollen odors can vary depending on the identity of the tested stimuli, it is nevertheless influenced by the bees' predisposition to collect pollen.

3.2. Experiment 2. Testing pollen odor memory of free-flying pollen foragers

With the results of Experiment 1 in mind, we investigated whether sensitivity for pollen in pollen foraging bees also leads to type-specific memory for pollen odors. To do this, we tested foragers for memories of the odor of the pollen loads that they had carried back to the hive. No differences were detected between the responses for the two types of pollen carried by the foragers (Solanaceae or *Hypochaeris* sp), which allowed us to pool the data (G-test$_{\text{First choice/pollen type}}$: $G_2 = 0.2333$, $p = 0.629$, $N = 51$; Fig. 2A). Pooled data revealed that the foragers strongly preferred the odor

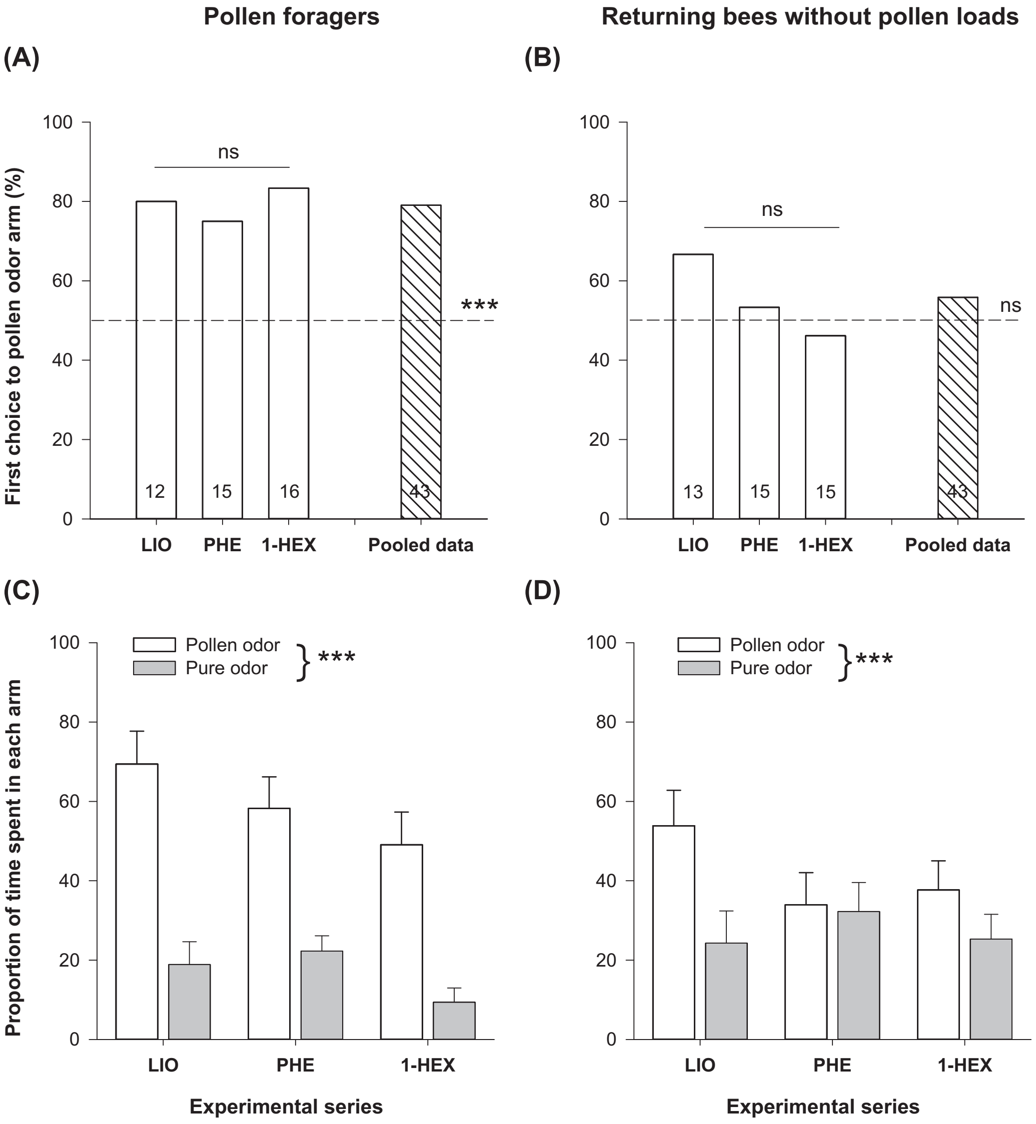

Fig. 1. Orientation preferences of pollen foragers (A and C) and bees returning without pollen loads (B and D) in the Y-maze. The maze contained a novel pollen bouquet in one arm and a pure odorant (LIO, PHE or 1-HEX) in the other. Presentation of the stimuli was randomized. A and B indicate *First choice* towards the pollen odor arm. The dashed line at 50% indicates random choice between the pollen odor and the pure odor arm. C and D indicate the *proportion of time spent in each arm* ($\bar{X}$+SE). Asterisks indicate statistical differences ($^{***}p < 0.001$). The number of subjects is indicated at the bottom of the bars in A and B.

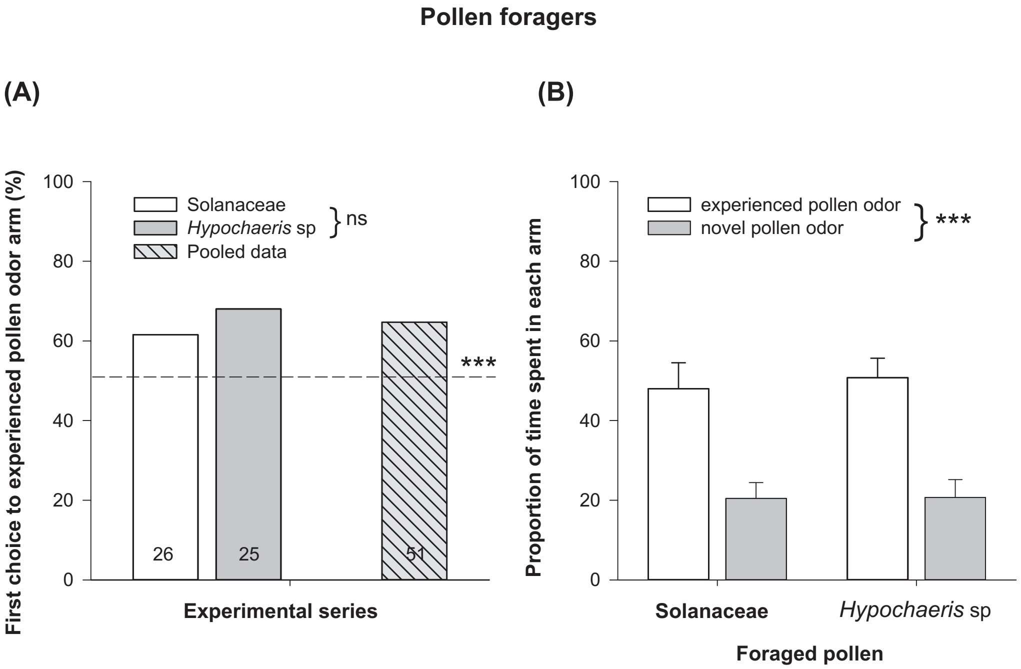

Fig. 2. Transference of olfactory memory to Y-maze. Pollen-odor memory was acquired under natural uncontrolled conditions. The orientation response was quantified in the Y-maze, which contained single natural pollen odors from a Solanaceae in one arm and from *Hypochaeris* sp in the other. A indicates the *First choice* towards the previously learned pollen odor arm. The dashed line at 50% indicates random choice between the learned stimuli and the unfamiliar pollen odor arm. B indicates the *proportion of time spent in each arm* ($\bar{X}$+SE). Asterisks indicate statistical differences ($^{***}p < 0.001$). The number of subjects is indicated at the bottom of the bars in A.

of the pollen that they had carried (Chi-square test$_{\text{First choice}}$: $\chi^2_1 = 3.841$, $p = 0.035$, $N = 51$; Fig. 2A) and that they could accurately discriminate between the pollen odors.

The *time spent in each arm* was irrespective of the identity of the pollen type. However, foragers spent significantly more time in the arm with the scent of the pollen they had carried than in the arm scented with the alternative pollen odor (RM ANOVA$_{\text{time}}$: $F_{1,46} = 4.475$, $p < 0.039$, $N = 51$). These results indicate that odors of specific pollen types could be accurately learned under natural conditions, transferred and retrieved within a different context (Gerber et al., 1996).

3.3. Experiment 3. Testing olfactory preference in bees exposed to pollen at an early age

To investigate early exposure effects of pollen on bees of foraging age, we reared animals in the lab with or without pollen as a component of their diet. Both groups responded equally regarding the 3 series (G-test$_{\text{choice/odor}}$: $G_2 = 2.880$, $p = 0.236$, $N = 61$, Fig. 3A; G-test$_{\text{choice/odor}}$: $G_2 = 0.146$, $p = 0.929$, $N = 62$; Fig. 3B). Therefore the data were pooled for both bees early exposed to pollen and for naive bees. Bees reared on pollen preferred the arm scented with the pollen bouquet (Chi-square test$_{\text{choice}}$: $\chi^2_1 = 27.557$, $p < 0.001$, $N = 61$; Fig. 3A). In contrast, bees reared in absence of pollen exhibited no preferences (Chi-square test$_{\text{choice}}$: $\chi^2_1 = 3.161$, $p = 0.075$, $N = 62$; Fig. 3B). Comparison between exposed and naïve groups revealed that *choice* responses differed significantly (G-test$_{\text{choice/exposed bees vs. naïve bees}}$: $G_1 = 27.348$, $p < 0.001$, $N = 123$), which indicates that exposure to pollen at early adult ages affects decisions taken later in the life.

Next, we assayed the *time spent in each arm* of the maze by pollen-exposed or naïve bees by means of a 3-way repeated measures ANOVA. The 3 *odors* used during tests and the *exposure* at early stages of life (diet with pollen or without pollen) were the independent factors. The *time spent in each arm* (novel pollen bouquet or pure odor-scented arm) was the repeated measure. Fig. 3C and D show the overall performance of both groups of bees. As we already found in previous experiments, the *time spent in each arm* was affected by the identity of the pure odorant (RM-ANOVA$_{\text{time spent in each arm}*\text{odor}}$: $F_{2,118} = 3.887$, $p < 0.023$, $N = 123$; Fig. 3C and D). ANOVA also revealed that pollen exposed bees stayed longer on the pollen-scented arm than bees without any prior pollen experience (RM-ANOVA$_{\text{time spent in each arm}*\text{exposure}}$: $F_{1,118} = 23.204$, $p < 0.001$, $N = 123$). In concordance with the *first choice* response, these results highlight the role of early pollen exposure in biasing later pollen-odor mediated behavior.

3.4. Experiment 4. Testing pollen odor memory in early exposed bees

To further investigate the exposure effects of pollen on foragers' orientation response, we tested memory to the odors of pollen (Solanaceae, *Taxodium* sp and *Hypochaeris* sp) offered as food during the first week of life. We first observed that *choice* responses were equally distributed among the different pollen types (G-test$_{\text{choice/pollen type}}$: $G_2 = 3.648$, $p = 0.161$, $N = 69$; Fig. 4A). Although biases induced by pollen of *Taxodium* sp appeared to be quite strong and might denote the presence of a long-term pollen-based odor memory, overall analysis did not detect particular preferences towards any of the odors experienced in the diet (Chi-square test$_{\text{First choice}}$: $\chi^2_1 = 0.014$, $p = 0.904$, $N = 69$; Fig. 4A).

In contrast, pollen odor of *Taxodium* sp did bias the *time spent in each arm*. The interaction between *time spent in each arm* and *pollen type* confirmed that not all three pollen olfactory stimuli produced the same outcome (RM ANOVA$_{\text{time spent in each arm}*\text{pollen type}}$: $F_{2,66} = 3.543$, $p = 0.0345$, $N = 69$; Fig. 4B). Simple effect analysis revealed that foraging-age bees fed on *Taxodium* sp pollen stayed significantly longer in the *Taxodium* sp pollen odor arm than in the arm with a different pollen odor ($F_{1,132} = 13.120$, $p < 0.001$). Divergence in results indicates that although young adult workers were able to learn some pollen odor as early as a few days after emergence and to recall it at foraging ages, learning and retrieval is strongly influenced by the identity of the pollen.

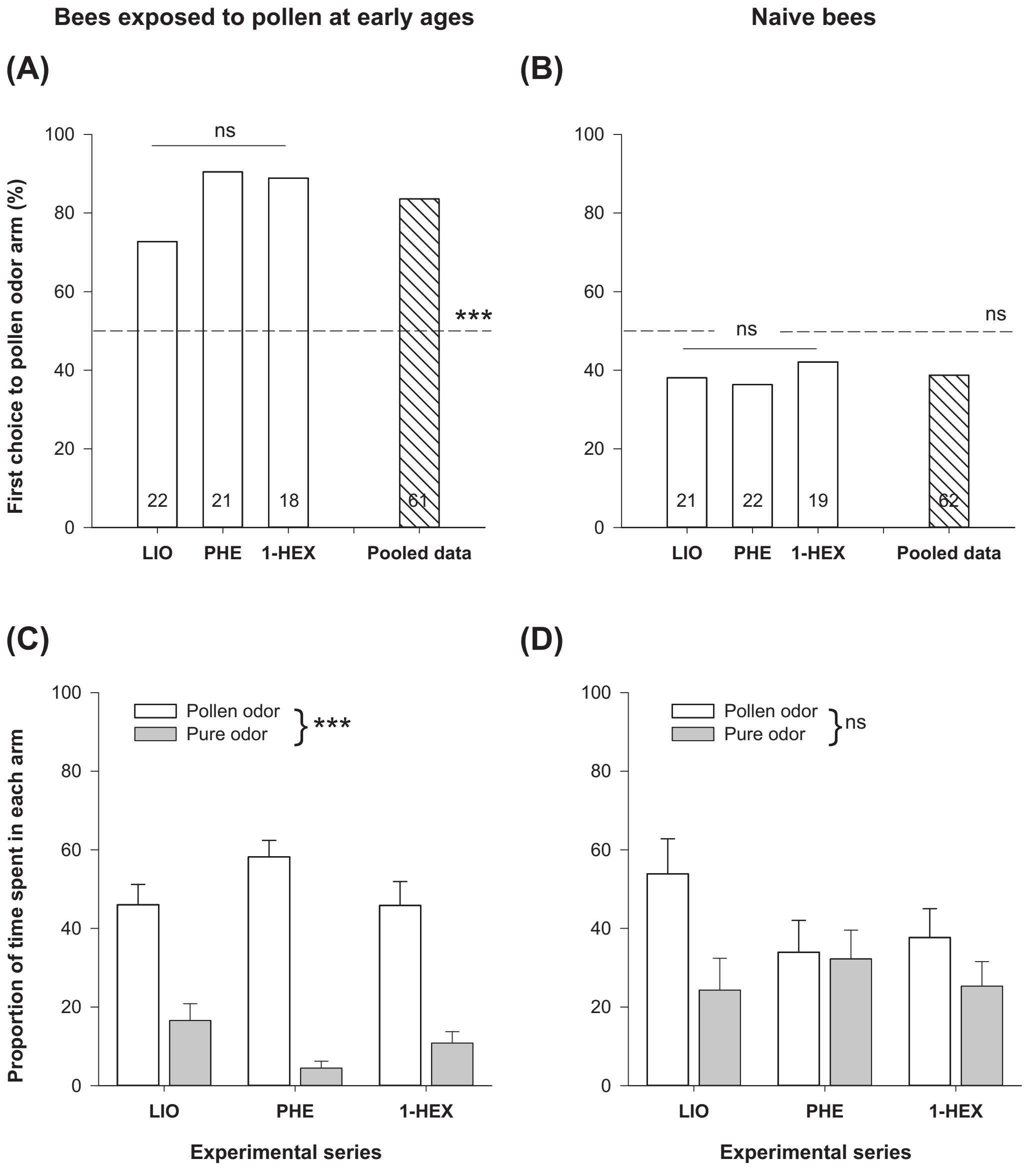

Fig. 3. Orientation preferences of foraging-age bees after early pollen exposure. The bees were reared in laboratory conditions and exposed to different *diets* during the first week of adulthood: with pollen (A and C) or with a surrogate (B and D). Bees were tested at the age of 17 days in a maze which contained a novel pollen bouquet and a novel pure odor (LIO, PHE or 1-HEX). A and B indicate *First choice* towards pollen odor arm. The dashed line at 50% indicates random choice between the pollen odor and pure odor arm. C and D indicate the *proportion of time spent in each arm* ($\bar{X}$+SE). Asterisks indicate statistical differences ($^{***}p < 0.001$). The number of subjects is indicated at the bottom of the bars in A and B.

4. Discussion

We confirmed that pollen odor is an important cue that influences the orientation of pollen foraging honeybees in a Y-maze, suggesting that it might similarly do so when pollen-foraging bees are selecting flowers in the field (van der Pijl, 1960). In addition, we showed that choice behavior of bees to pollen odors is influenced by their predisposition to collect pollen (pollen or non-pollen foragers). Orientation was not biased in bees that were not exposed to pollen stimuli in the rearing environment, suggesting that exposure soon after emergence might play a role in setting up later odor-mediated responses, at least in bees predisposed to collect pollen. Moreover, we found that species-specific olfactory cues from the pollen carried by foragers returning to the hive were significantly preferred as shown by the bees' orientation in the Y-maze. Whereas experiences of free-flying bees with specific pollen odors biased the orientation of the bees when tested in the Y-maze, olfactory orientation responses by lab-caged bees exposed to specific single-species pollen diets were not affected by the pollen type.

During this study we used bee-collected pollen, which is not pure pollen as presented by flowers but is mixed with oral substances produced by the worker bees themselves. Therefore, we cannot exclude the possibility that bees responding to pollen odors are actually responding to the composite of plant- and bee-produced constituents. However, using bee-collected pollen as stimuli for all our experiments and for comparing different groups of bees, enables us to draw conclusions regarding bees' genetic predisposition to collect pollen, their previous foraging-related experiences, etc. Moreover, and even if bees were responding to added products, this might be biologically relevant for the transfer of information within the hive, as was suggested by von Frisch (1923).

Linalool, PHE, 1-HEX are common components of headspace volatiles of flowerheads (Knudsen et al., 1993). Less frequently, they can also be found in pollen (Flamini et al., 2002, 2003, 2007). Since these pure odors are not exclusively found among pollen volatiles,

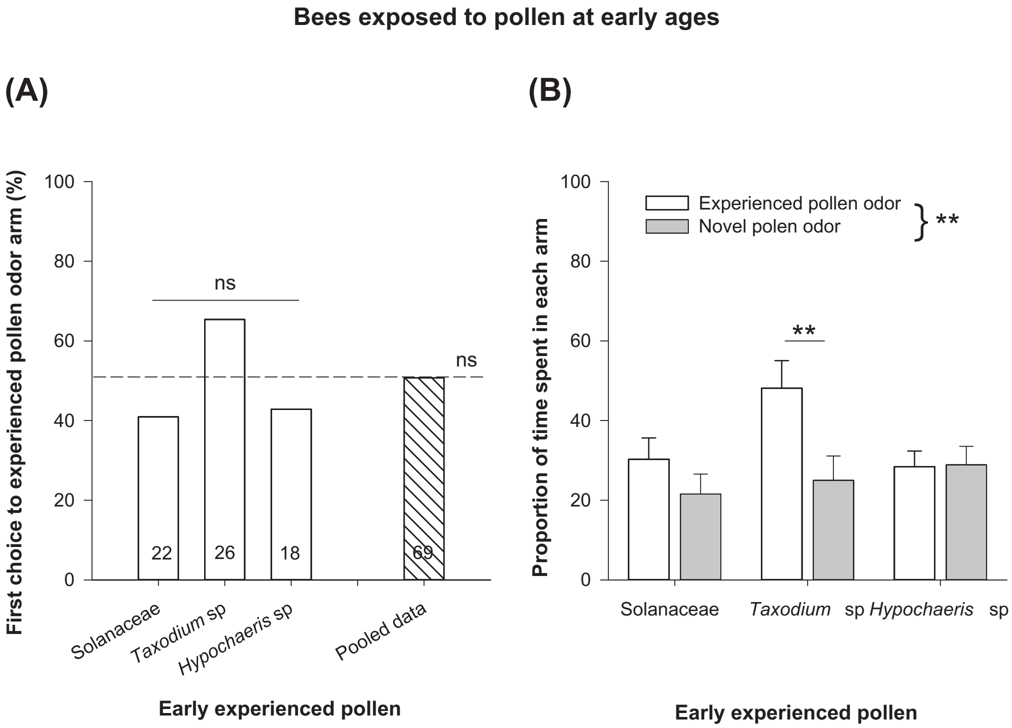

Fig. 4. Transference of olfactory memory to Y-maze. Pollen-odor memory acquired from the food offered during the first week of life was tested. The orientation response was quantified in the Y-maze, which contained single natural pollen odors from a Solanaceae, from *Taxodium* sp or from *Hypochaeris* sp in the arms. A and B indicate the *First choice* towards the learned pollen odor arm. The dashed line at 50% indicates random choice between the learned stimuli and novel pollen odor arm. C and D indicate the *proportion of time spent in each arm* ($\bar{X}$+SE). Asterisks indicate statistical differences ($^{**}p < 0.01$). The number of subjects is indicated at the bottom of the bars in A and B.

they are less likely to elicit a distinct innate behavioral response than whole-flower fragrances, as they do not provide specific information for recognition of pollen quantity and quality (Pernal and Currie, 2001). The fact that we obtained similar responses in free-flying bees and in lab-reared-bees, which were certainly naive to pure odors, suggests that honeybees are not innately predisposed to these cues and that they had not been learnt in the field by tested foragers.

4.1. Orientation response to pollen odors by pollen foraging bees

Recently it has been suggested that in plant families where pollen is exposed and easily available, the final and ultimate host recognition occurs upon landing through pollen contact chemicals (Piskorski et al., 2011). Our results, however, confirm that pollen odor is sufficient to bias orientation in pollen foraging honeybees (Louveaux, 1959; Hügel, 1962; Lepage and Boch, 1968). In the absence of previously encountered pollen odors, foragers were able to orientate towards unfamiliar pollen bouquets (Fig. 1A and C). Such strong orientation responses seem to depend on the maturity and foraging tasks of each bee, since incoming bees without pollen loads exhibit no orientation preferences towards pollen odors (Fig. 1B). This might reflect that pollen odor cues, though relevant to foragers involved in pollen gathering, do not attract mature bees involved in other tasks. Whether or not a honeybee's response to pollen odors depends on the bee's current role is not trivial and indicates that the development and/or expression of pollen-odor mediated responses might contribute to separate bees into nectar- and pollen-foraging tasks (Page et al., 1998; Scheiner et al., 2004). Furthermore, different preferences towards pollen odors by pollen and non-pollen foragers could be related to different response thresholds that are under genetic control (Page et al., 1998) and/or influenced by the condition of the colony (Pernal and Currie, 2001; Pankiw et al., 2002; Fewell, 2003). Responsiveness to pollen-based odors, as well as to other pollen-related cues such as fatty acids (Arenas and Farina, 2012) might represent a key component in task allocation and division of labor between these two functional sub-casts (Robinson, 1992).

Our results further suggest that pollen foragers learn species-specific pollen odors (Fig. 2). In Experiment 2 we observed that bees that had handled a particular pollen type could recall this previous experience when the odor was tested against other pollen types that had been simultaneously available in the surroundings and were being exploited by the colony at the time (Fig. 2A and B). The accuracy of the choice according to the type of pollen brought back to the hive is consistent with learning abilities of foragers to associate odors and pollen reward (Arenas and Farina, 2012). Recently, we showed that honeybee foragers are able to learn neutral odor cues (LIO and PHE) associated with pollen as the only reward stimulus. In those experiments, olfactory memory established while bees foraged on a scented feeder were accurately retrieved in a dual-choice test (Y-maze), suggesting that the same process is involved in the acquisition and transfer of non-pollen floral odors (LIO and PHE) and pollen odors. In this case, similarly to what occurred with scented nectars, pollen may provide a rewarding stimulus (US), whereas its species-specific aroma (Dobson et al., 1996) might act as the conditioned stimulus (CS; Cook et al., 2005).

Cook et al. (2003) showed that foraging honeybees with prior experience of oilseed rape pollen (*Brassica napus*) preferred to land on and collect this pollen over that of field bean (*Viciafaba*). Despite the lack of controls for visual and tactile stimulation, these experiments suggest that memory established whilst a bee exploits a pollen source can be helpful for guiding the forager towards the learned stimulus later on. Such a process should increase foraging efficiency, as it would mean that a bias is established towards food sources (von Frisch, 1918, 1919; Ribbands, 1955; Menzel, 1999).

Generalization on the part of the bees towards pollen olfactory cues (Ghirlanda and Enquist, 2003; Guerrieri et al., 2005) might direct foragers to new pollen types by allowing the bees to extract information from common features of pollen aromas. Controlled experiments assaying bees' olfactory perception showed that foraging bees broadly generalized pollen odors from different species during an absolute classical conditioning (Cook et al., 2005). However, these odors could be successfully distinguished if they were learned in a discrimination conditioning, a protocol that forced bees to respond differentially to the odors since one of them is paired with reward whilst the other remains unrewarded (Bitterman et al., 1983; Cook et al., 2005). These previous tests support our findings that honeybees can distinguish specific pollen odors and may use them in recognition/discrimination of floral species. Finally, generalization might help foragers to find potential food sources when previously learned cues are no longer available in the surroundings.

4.2. Early olfactory experiences affect pollen odor preferences

Within the hive, young bees may have the chance to perceive and even learn many odors whilst performing tasks such as nursing or food processing. Nurses consume stored pollen to cover their own protein requirement and to produce a protein enriched fluid called brood food in their hypopharyngeal glands (Winston, 1987). Our study (Experiments 3 and 4) suggests that early experiences are responsible for the positive orientation response towards pollen by bees of older ages (Figs. 3 and 4). Thus, pollen odors in the food or in the rearing environment during the in-hive period might prepare workers for later tasks, providing individuals, at least those genetically predisposed to collect pollen, with key information about how pollen sources might smell. Pollen odors perceived from the diet might interact with odors perceived through other in-hive experiences, such as pollen odors clinging to the bodies of dancing bees (Diaz et al., 2007), which enables the formation of a positive bias in novice foragers.

Memory that resists metamorphosis has been suggested to be a mechanism by which feeding preferences are established in solitary-pollen-specialist bees (Dobson, 1987), whereby the bees become imprinted to pollen odor during pre-imaginal stages and subsequently as adults show a preference for the same scent (Dobson, 1994; Masson and Arnold, 1984). On the contrary, previous studies on honeybees showed that bees reared on a single pollen diet did not prefer the pollen types on which they had been fed as they developed (Boelter and Wilson, 1984). Our results are consistent with these studied, as we demonstrated that early experiences with specific pollen odors could only be retrieved in 1 out of 3 cases (Fig. 4A and B).

Young adult workers can learn odors diluted in sugar solution as early as a few days after emergence and can recall this memory at foraging ages (Arenas and Farina, 2008; Arenas et al., 2009). However, it seems that a process other than associative learning must be involved in the development of pollen-odor mediated response in young workers. Whatever the process, our results suggest that the olfactory system of young bees needs to be primed by pollen-related inputs to change and enhance its responsiveness to pollen odors.

5. Conclusion

Our results led us to conclude that exposures at early ages and the olfactory experiences gained in the foraging context interact with pollen foragers' genetic predisposition to bias their search behavior towards pollen sources.

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