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RESEARCH ARTICLE

Honeybees generalize among pollen scents from plants flowering in the same seasonal period

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ABSTRACT

When honey bees (*Apis mellifera*) feed on flowers, they extend their proboscis to absorb the nectar, i.e. they perform the proboscis extension response (PER). The presence of pollen and/or nectar can be associated with odors, colors or visual patterns, which allows honey bees to recognize food sources in the environment. Honey bees can associate similar, though different, stimuli with the presence of food; i.e. honey bees discriminate and generalize among stimuli. Here, we evaluated generalization among pollen scents from six different plant species. Experiments were based on the PER conditioning protocol over two phases: (1) conditioning, in which honey bees associated the scent of each pollen type with sucrose, and (2) test, in which honey bees were presented with a novel scent, to evaluate generalization. Generalization was evinced by honey bees extending their proboscis to a novel scent. The level of PER increased over the course of the conditioning phase for all pollen scents. Honey bees generalized pollen from *Pyracantha coccinea* and from *Hypochaeris radicata*. These two plants have different amounts of protein and are not taxonomically related. We observed that the flowering period influences the olfactory perceptual similarity and we suggest that both pollen types may share volatile compounds that play key roles in perception. Our results highlight the importance of analyzing the implications of the generalization between pollen types of different nutritional quality. Such studies could provide valuable information for beekeepers and agricultural producers, as the generalization of a higher quality pollen can benefit hive development, and increase pollination and honey production.

KEY WORDS: *Apis mellifera*, Conditioning, Palynology, Proboscis extension response, Similarity

INTRODUCTION

Learning can be defined as a change in behavior owing to an individual's experience. Indeed, behavioral plasticity is crucial for an animal, as it enables adaptation to a constantly changing environment (Giles and Rankin, 2009). In this context, the ability to both discriminate and generalize among different stimuli is a key factor for survival, for instance, when facing changes in food availability. Discrimination allows the animal to distinguish different stimuli, whilst generalization allows the animal to classify similar, though different, stimuli into the same category (Getz and Smith, 1987; Laska et al., 1999; Gumbert, 2000; Ghirlanda and Enquist, 2003; Guerrieri et al., 2005; Robazzi Bignelli Valente Aguiar et al., 2018). The western honey bee (*Apis mellifera*) represents one of the principal model organisms in the study of learning (Menzel and Erber, 1978; Menzel, 1999, 2001; Giurfa, 2003, 2007, 2015). During their foraging lifetime, honey bees are confronted with different stimuli, such as floral scents, colors and textures, some of which are associated with the presence of food (i.e. nectar, pollen). Honey bees perceive and remember different types of stimuli (i.e. visual, tactile, chemical) and can associate them with other stimuli. For instance, an odor can be associated with a reward, e.g. sucrose solution (von Frisch, 1967; Hammer and Menzel, 1995; Tedjakumala and Giurfa, 2013; Giurfa, 2015), or with a punishment, e.g. an electric shock (Vergoz et al., 2007; Bos et al., 2013). Here, we investigated how honey bees generalize among scents from different pollen types (i.e. pollen from different plant species) and which characteristics allowed pollen types be perceived as similar by individuals. Many studies on honey bee learning have been performed observing the proboscis extension response (PER). This response occurs when honey bees land on flowers and detect the presence of nectar with their antennae. Thus, individuals associate the flower shape, color and odor with the presence of food, i.e. associative learning is induced. This behavior can be reproduced under laboratory conditions using harnessed honey bees that spontaneously extend their probosces when their antennae are stimulated with sucrose solution. An odor (conditioned stimulus, CS) forward-paired with the sucrose reward (unconditioned stimulus, US) becomes a predictor stimulus of reward. Thus, an association is built between both stimuli; this association is known as PER conditioning and has been extensively used in studies of learning for the last six decades (Takeda, 1961; Bitterman et al., 1983; Hammer and Menzel, 1995; Gerber et al., 1996; Giurfa, 2007; Avarguès-Weber et al., 2011; Matsumoto et al., 2012). Using this methodology, honey bees have been assayed following a great variety of learning paradigms allowing researchers to unravel how they interact with their environment (Giurfa, 2003; Bos et al., 2013; Giurfa and Sandoz, 2015; Baracchi et al., 2018).

Honey bees follow a 'flower constant' foraging strategy, meaning that once an individual recognizes the flower of a certain plant species as a convenient food source, it will preferentially forage on flowers of that plant species as long as those flowers are available (see Chittka et al., 1999). Honey bees collect nectar and pollen during their foraging trips. Nectar mainly provides carbohydrates, while pollen provides proteins and lipids, which constitute the food supply for the

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colony (Winston, 1987; Fewell and Winston, 1992; Roulston and Cane, 2000; Requier et al., 2017). Floral nutritional resources (nectar and pollen) vary in quality and quantity among plant species (Scheiner et al., 2004; Di Pasquale et al., 2013; Vaudo et al., 2015) and in the course of the season. Therefore, honey bees forage on a wide diversity of plants to fulfill their nutritional needs, including in human-disturbed landscapes such as croplands (Requier et al., 2015). Honey bees discriminate among nectar types based on nutritional attributes, while different types of pollen are distinguished via both gustatory and olfactory cues. For instance, several authors have shown the honey bees' ability to generalize among visual or olfactory stimuli (Wehner, 1967, 1971; Smith and Menzel, 1989; Giurfa et al., 1996; Deisig et al., 2002, 2003; Stach et al., 2004; Guerrieri et al., 2005; Benard et al., 2006; Horridge, 2009). Concerning the olfactory modality, it has been observed that honey bees are able to generalize among both pure odors (Guerrieri et al., 2005) and odor blends (Reinhard et al., 2010). Nonetheless, the criteria by which honey bees generalize among pollen types are not yet fully understood (Pernal and Currie, 2001; Cook et al., 2005; Nicolson, 2011; Ruedenauer et al., 2015, 2018; Beekman et al., 2016; Muth et al., 2016).

Under natural conditions, flowers release mixtures of volatile substances, thus producing complex and diverse odor cues. The composition of the mixture varies from one flower to another, even among flowers of the same plant species. To identify different flowers as conspecific, a flower constant forager must be able to discriminate among flowers coexisting in the field to identify the preferred plant species (Menzel, 1985). Furthermore, a flower constant forager must be able to generalize among flowers, i.e. to recognize that different flowers correspond to the preferred plant species (Free, 1963; Wright and Schiestl, 2009). Under laboratory conditions, some studies have evaluated the perceptual ability of honey bees to identify resources found in nature (Pernal and Currie, 2001, 2002; Cook et al., 2003, 2005; Arenas and Farina, 2012; Nicholls and Hempel de Ibarra, 2017). Pollen, unlike nectar, is neither collected with the proboscis nor stored in the crop. However, it is adaptive for honey bees to estimate the quality of pollen and hence to generalize based on nutritional characteristics (Nicholls and Hempel de Ibarra, 2017). For instance, honey bees were able to generalize among pollen types by evaluating the intensity of the pollen scent as well as the composition of the odorant mix, a trait that might provide information about pollen availability and abundance (Dobson et al., 1999). Honey bees generalize among aliphatic molecules with similar carbon chain lengths or carrying the same functional group (Guerrieri et al., 2005). However, natural floral scents (including pollen scents) are not pure substances, but complex mixtures of a great variety of molecules in different proportions. Consequently, honey bees must face the dilemma of generalizing among similar floral blends whose exact composition varies from one individual flower to another (Sandoz, 2011).

In the present study, we examined how honey bees generalize among scents from pollen grains of different plant species. Specifically, we asked whether this generalization depended on plant species that are taxonomically related, nutritional characteristics of pollen (e.g. similar amounts of protein) or flowering period. To answer these questions, we performed PER conditioning and evaluated generalization among the different pollen scents presented.

MATERIALS AND METHODS

Determination of botanical origin, protein content and grain size of pollen types

Several pollen samples were collected from the honey bee (*Apis mellifera* Linnaeus 1758) hives at the field station of the National Institute of Agricultural Technology – Bariloche Research Lab (EEA INTA Bariloche, San Carlos de Bariloche, Province of Río Negro, Patagonia, Argentina, 41°7022.36″S, 71°1504.94″W). For pollen collection, we installed pollen traps (Apipolen®, Madrid, Spain) in three hives during 24 h on three austral summer dates (23 November 2015, 29 December 2015 and 22 January 2016). Subsequently, nine samples (three hives×3 days) of mixtures of honey bee-collected pollen from different botanical origins were obtained. These samples were left to dry at room temperature (22°C) over 48 h. Afterwards, we selected sub-samples of 30 g each and classified the different types of pollen loads according to their color, shape and texture, following Filipiak's method for identification of pollen pellet morphospecies (PPMs; Filipiak et al., 2017). Finally, we weighed and calculated the percentage of each PPM within the sub-samples and selected the six most abundant PPMs (i.e. those with the highest percentages, values that vary between 30 and 50% of the subsample). The sub-samples per PPM type were divided into three lots: one to determine the botanical origin, a second for the analysis of protein content and the third to be used as conditioned stimulus during the experimental design.

To determine the botanical origin of the six types of PPM previously selected, we performed observations using (1) an optical microscope (OM) with samples mounted on slides (plus agar and safranin diluted at 90%) (Louveaux et al., 1978; Von Der Ohe et al., 2004) and (2) a scanning electron microscope (SEM). The OM observations allowed a preliminary identification of the botanical origin of the PPM types at the family level. Afterwards, we followed the SEM procedure developed by Pernal and Currie (2001), consisting of very high-resolution photographs of the pollen structures (Fig. S1). Photographs were captured at the Atomic Center of Bariloche (Centro Atómico Bariloche, Depto. Caracterización de Materiales – Servicio de Microscopia y Rayos X, San Carlos de Bariloche, Río Negro, Argentina). In these photographs, we observed and compared the morphological characteristics of the pollen with referenced material (Markgraf and D'Antoni, 1978; Valdés et al., 1987; González-Romano and Candau, 1989; Tellería and Forcone, 2002; Forcone et al., 2006). In addition, a post-identification validation was carried out via comparison between the plant species identified and their presence in the landscape surrounding the hives. Taxonomic identification of the pollen types allowed us to establish which types of pollen could be grouped to evaluate the effect of taxonomical family and flowering period.

In order to measure the protein content of each selected pollen, we took a sub-sample of 0.15 g (weighed with an Acculab® scale) and performed a semi-micro Kjeldahl method with a block digester (Bremner, 1996; Campos et al., 2008; Forcone et al., 2011). Protein content was estimated by multiplying the percentage amount of nitrogen by 6.25 (Van Soest, 1967). More details on the protein content of the selected pollen are available in Table S1.

Honey bees

A total of 310 worker honey bees were captured during March 2016. They were captured using transparent plastic containers placed at the entrances of 15 hives at the field station of the National Institute of Agricultural Technology, Bariloche Research Station. Between 10 and 30 individuals were captured per day from randomly selected hives, in order to carry out the experiments (see below). The collection was carried out in the early hours of the morning, as soon as the honey bees began their foraging activity. Once captured, honey bees were transported to the laboratory in plastic containers. Honey bees were anesthetized by placing the container in a fridge at

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4°C for 5 min and then kept on ice for no longer than 10 min. Subsequently, each individual was placed into an Eppendorf tube (5 ml), the tip of which had been cut off to enable the head of the honey bee be gently pushed through the hole. Each individual was then harnessed with tape on the back of the head, allowing free movements of the antennae and mouthparts. After 3 h recovery from the anesthesia and prior to beginning the learning protocol, we tested the motivation of each individual to respond to sucrose by touching each honey bee's antennae with a toothpick soaked in sucrose solution (50% w/v). This procedure was performed at 20.4±2°C and 39.6±10% relative humidity. Those individuals that did not extend their proboscis when stimulated with sucrose solution were discarded (n=42). To avoid any effect of external odors, the Eppendorf rack with the harnessed honey bees was placed inside an air extraction hood, between the extraction aperture of the air extraction and the syringe that released the olfactory stimuli (i.e. pollen scents). During the experiments, the airflow was maintained at a minimum and the uniformity of the airflow of the extraction hood was controlled.

Conditioning phase

During the conditioning phase, honey bees were trained to associate different pollen scents (CS) with a reward of sucrose solution (US). This procedure consisted in three conditioning trials (C1, C2 and C3); each trial lasted 1 min and there was an inter-trial interval (ITI) of 10 min. Twenty-five seconds after the trial onset, we released the scent of a PPM (CS). The pollen scent was produced using 30 mg of a PPM type introduced via a 10-ml syringe, the piston was pulled back and air was blown on to the antennae over 5 s. Three seconds later, sucrose solution was presented to the honey bee by touching both the antennae and proboscis with the soaked toothpick (US). US presentation lasted for 5 s (Fig. S2). As a control, a similar protocol to the conditioning treatment was performed but no pollen was put inside the syringe (in this case we expected no PER). We randomly assigned individuals to the different treatments (six treatments: one treatment per PPM). Those individuals that performed a PER in to the US at the first conditioning trial or those that did not respond to the CS in any trial were discarded for subsequent data analysis (n=55).

Among the total number of individuals used (n=310), 55 honey bees were discarded for the entire data analysis procedure because they did not show PER during all of the conditioning trials or did not respond immediately to sugar water (n=42), or responded positively to pollen in the first trial before the US (n=13). We performed this selection, considering three aspects. (1) The absence of PER performance by individuals in any of the conditioning trials could be related to an inadequate recovery of the anesthesia. (2) The absence of response to sucrose solution as a reward could be related to the honey bees being assayed as nectar foragers. Reade and Naug (2016) demonstrated that foraging decisions in honey bees were determined by individual requirements of carbohydrates and that pollen foragers have a higher carbohydrate intake than nectar foragers. (3) PER to pollen scent in the first trial could indicate a previous association between pollen scent and sucrose. In those cases, we would not induce any learning during our experiments. Therefore, those individuals that extended their probosces when presented with pollen scent in the first conditioning trial were excluded from the data analyses; those cases were distributed among pollen types 3, 4, 5 and 6 (n=13; Fig. S3). Additionally, 18 honey bees were used for control treatment and all responded positively only to the US. Thus, the remaining honey bees (n=237; 76%) were used for the experiments.

Test phase

The test phase began 10 min (ITI) after performing the conditioning phase. During the test phase, honey bees were presented with four different pollen scents, in a sequence of four trials with ITI of 10 min. No reward was presented during the test. The goal of this phase was to evaluate whether honey bees would respond to conditioned and to novel stimuli (i.e. a type of pollen different from the one used for conditioning). Honey bees would extend their probosces when presented with novel stimuli if those stimuli were perceived as similar to the CS, thus providing evidence of generalization. To avoid any bias owing to the sequence in which scents were to be presented, the order of test stimuli was established following a randomized sequence. For example, we used pollen 1 for conditioning and pollens 3, 5, 1 and 4 for test. Given the randomness of the sequence, in some situations, within the pollen types used in the test phase, the same pollen used for conditioning may or may not have been present. Therefore, the combinations have different sample size (for details, see Guerrieri et al., 2005). By performing a random sequence of four trials to test the six types of pollen, the duration of the protocol and the fatigue of the experimental individual were reduced, preventing the honey bee from diminishing its response capability.

Data analyses

All statistical analyses were performed using R version 3.5.1 (https://www.r-project.org/).

Conditioning phase

The correlative link between the proportion of successful PER at C2 and C3 was tested by a Pearson correlative test. By means of an ANOVA within the general linear model (GLM) framework, we analyzed whether there were differences between the success of PER for each PPM type at trial 3. Following the same analytic approach, we also evaluated whether different characteristics of pollen part-explained variation in the level of PER at C3. We analyzed the effect of two pollen traits, size of pollen grain and protein amount, and (based on the characterization of botanical origin) we also considered taxonomic classification and flowering period. Differences in the levels of PER at C2 (fixed effect) and C3 (response variable) were tested by binomial GLM with a logit link function. Model residuals were extracted and inspected against fitted values (residuals versus fitted plot and normal Q–Q plot) to ensure residual normality and homoscedasticity assumptions were fulfilled.

Test phase

The correlative link between the proportion of successful PER at C3 (fixed effect) and test (response variable) was evaluated as a learning validation test, using binomial GLM with a logit link function. Model residuals were also extracted and inspected against fitted values. In a similar way to data analysis carried out for conditioning, four ANOVAs were performed to evaluate the possible effect of pollen traits (size of pollen grains and amount of protein), taxonomic family and temporal distance of flowering (i.e. lapse of time between end of flowering and the execution of the experiment in March) on the level of PER. It should be noted that for this analysis we only used the results obtained when a given individual was conditioned and tested with the same type of pollen.

Generalization

In order to determine the perceptual similarity among different pollen scents, we successively renamed each pollen identity

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following a matrix of simplification (see Table S5). For instance, pollens 1, 2 and 3 were first named 'A, B, C' as their original identity and then simplified for the similarity test and renamed 'A, B, B', 'A, A, B', 'A, B, A' and 'A, A, A' (the similarity between the three pollens is tested with the latter scenario, e.g. 'A, A, A'=all types of pollen are perceived as equal, 'A, A, B'=pollen 1 and 2 are generalized; 'A, B, C'=no generalization). Thus, the six pollen identities were renamed along all the possible 56 simplified combinations of renamed identity scenarios. Binomial GLMs were then used to compare the proportion of successful PER (response variable) to the renamed pollen identity (fixed factor) (Table S6). Following a heuristic approach, all the possible combinations of scenarios were evaluated. The candidate models were ranked according to the Akaike information criterion (AIC) to find the best compromise between fit and complexity (i.e. models with $\Delta AIC<10$). From the selected model, differences between each pollen type were evaluated with *a posteriori* multiple pairwise comparisons (Tukey's HSD test).

RESULTS

Determination of botanical origin, protein content and grain size of pollen types

From the six pollens selected, the following botanic origins were determined: pollen 1: *Pyracantha coccinea* Roemer (Rosales: Rosaceae); pollen 2: *Capsella bursa-pastoris* (L.) Medik (Brassicales: Brassicaceae); pollen 3: *Carduus thoermeri* Linnaeus (Asterales: Asteraceae); pollen 4: *Hypochaeris radicata* Linnaeus (Asterales: Asteraceae); pollen 5: *Diplotaxis tenuifolia* Linnaeus (Capparales: Brassicaceae); and pollen 6: *Salix humboldtiana* Willdenow (Malpighiales: Salicaceae). Fig. S1 shows the morphology of each selected pollen. Most of these plant species are exotic in Patagonia, Argentina (Table S1), where the experiments were performed. Because we were only interested in identifying the pollen grains of the dominantly harvested plants, we did not deepen in the taxonomical identification of the rest of the pollen loads.

Among the pollen grains identified, the percentage of protein content varied from 16.87% in pollen 3 (*C. thoermeri*) to 24.37% in pollen 2 (*C. bursa-pastoris*) (Table S1). With regard to the size of pollen grain, pollen 3 was the largest (polar axis=43–52 µm; equatorial diameter=49–55 µm) whereas pollen 6 (*S. humboldtiana*) was the smallest (polar axis=18–22 µm; equatorial diameter=15–19 µm). To evaluate the effect of taxonomic family four categories were established: Rosaceae (pollen 1), Brassicaceae (pollens 2 and 5), Asteraceae (pollens 3 and 4) and Salicaceae (pollen 6). We also established two categories of flowering period according to the end the blooming period: spring and early summer (pollens 1, 2 and 6; which end their flowering in November–December), and summer and early autumn (pollens 3, 4 and 5; which end their flowering in February–March). More details on flowering and pollen traits are available in Table S1.

Conditioning phase

The level of PER increased during the conditioning trials for each pollen type (Fig. 1A). The proportion of PER varied from 0.52 to 0.90 at C2 and from 0.72 to 1.0 at C3. PER level was highest when honey bees were trained with pollen 1, followed by pollen 6 and pollen 2. The level of PER in the third conditioning trial (C3) was positively correlated with the PER level in the second conditioning trial (C2) (Pearson's rank correlation coefficient r_P=0.938, P=0.006, t=5.409, d.f.=4; Fig. 1B). This suggested that honey bees would be able to associate the scent of pollen in the second trial and there is no loss of learning (due to fatigue) in the next trial. The last conditioning trial (C3) showed high PER levels for each pollen

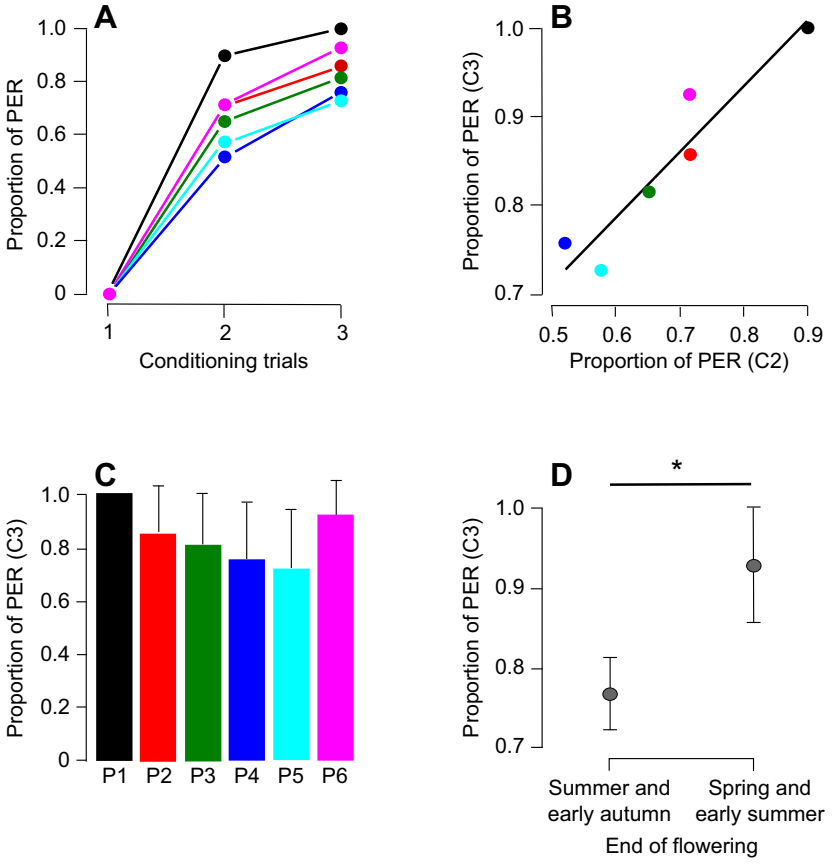

Fig. 1. Behavioral responses of honey bees during the conditioning phase. (A) Proportion of proboscis extension response (PER) success to each type of pollen in each conditioning trial (pollen 1, n=28; pollen 2, n=39; pollen 3, n=47; pollen 4, n=64; pollen 5, n=29; pollen 6, n=30). (B) Pearson's correlation between the proportion of PER of C2 and C3. There was a significant correlation between the variables (Pearson's rank correlation coefficient r_P=0.938). (C) Proportion of PER for each type of pollen at C3. The vertical lines indicate the standard error of the mean. There were no significant differences between pollen types. (D) The effect of two periods of flowering on the proportion of PER at C3. The asterisk indicates a significant difference (ANOVA, *P<0.05). Mean±s.e.m. values are shown.

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Table 1. The effect of pollen characteristics on the level of the proboscis extension response (PER) during the conditioning phase

Pollen characteristics	*F*-value	*P*-value
End of flowering	10.86	0.030*
Grain size	0.38	0.569
Taxonomical family	0.84	0.410
Protein amount	0.21	0.667

The asterisk indicates a significant difference (ANOVA, *P<0.05). d.f.=1 for each model run.

type (i.e. 0.72–1), without a significant species effect on learning success (binomial GLM, Z=1.905, P=0.342; Table S2, Fig. 1C). The ANOVAs performed between the particular traits of each type of pollen and PER at C3 showed no significant differences, unlike the effect of pollen grouped by flowering period on PER (F=10.86. P=0.030, d.f.=1; Table 1, Fig. 1D). Those types of pollen that end their flowering period early in the season (pollens 1, 2 and 6) exhibited a higher level of PER.

Test phase

Honey bees showed a lower level of PER during the test phase as compared with the conditioning phase, varying from 0.52 (pollen 6) to 0.66 (pollen 1). Similarly to the conditioning phase, no effect of pollen type was detected on the level of PER at test (GLM, P=0.264; Table S3, Fig. 2A). The level of PER at test was not correlated with the level of PER at C3 of the conditioning phase (r_P=0.195, t=0.398, d.f.=4, P=0.711; Fig. 2B). There was a decrease in the PER level in the test phase and this is perhaps a consequence of the honey bee's fatigue owing to the duration of the experimental protocol. The variability in the level of PER among the presented pollen types was not significantly explained by the pollen traits ($P_{\text{Grain size}}$=0.723, $P_{\text{Taxonomic family}}$=0.275, $P_{\text{Protein amount}}$=0.260) or pollen grouped by flowering period (P=0.734; see statistics in Table 2).

Generalization

The generalization matrix (which related the level of PER between both phases of the learning protocol for each combination of pollen types; Fig. 3A, Table S4) showed that higher levels of PER corresponded to those trials in which the same pollen type was used in both phases (main diagonal). The matrix structure was asymmetric, showing that honey bees did not respond in the same way when a pair of pollen types from conditioning to test were interchanged. For example, when pollen 1 was used for conditioning and pollen 3 for test, we observed a proportion of 0.57 of PER, but for the inverse combination (pollen 3 for conditioning and pollen 1 for test), the level of PER decreased to 0.5.

According to the mean values of PER in the generalization matrix, there were pollen pairs that showed higher levels of PER, suggesting that both pollen types were perceived as similar: pollen 2–pollen 1 (0.73), pollen 1–pollen 3 (0.57) and pollen 4–pollen 1 (0.67). However, because two or more types of pollen could be perceived as similar, 56 models (binomial GLMs) were performed to analyze different combinations (Table S5). Five models were thus selected (Table S5). These models included the same four types of pollen (pollens 1, 2, 3 and 4) that exerted the higher levels of PER at the generalization matrix. The first model selected (ΔAIC=0, Z=5.273, P<0.01) established that honey bees perceived pollen 1 and pollen 4 as equals, while the second model suggested that pollen 2 is also similar to pollen 1 and pollen 4 (ΔAIC=5.723, Z=4.719; P<0.01).

These results were in agreement with those obtained by the simple models (in which it is established that one type of pollen differs from the rest), as there were not significant differences for pollen 2 (model 50, ΔAIC=27.587, Z=−0.867, P=0.386) and pollen 4 (model 43, ΔAIC=26.221, Z=1.463, P=0.144). In contrast, pollens 1, 3, 5 and 6 were perceived as different from the rest (pollen 1: model 27, ΔAIC=21.074, Z=−2.707, P<0.01; pollen 3: model 39, ΔAIC=24.469, Z=1.964, P<0.05; pollen 5: model 24, ΔAIC=19.464, Z=2.938, P<0.01; pollen 6: model 33, ΔAIC=23.028, Z=2.289, P<0.05; Fig. 3B).

The *a posteriori* analysis of the level of PER for each pollen type assuming pollen 1=pollen 4 (selected model) shows non-significant differences (Fig. 4A, Table S6). These results were confirmed by the estimates of Akaike weights (Fig. 4B).

DISCUSSION

We studied honey bees' ability to generalize among pollen scents from different plant species. The majority of the pollen types identified belonged to exotic plant species in Patagonia, southern Argentina, and had different nutritional and phenological characteristics (Forcone et al., 2005; Tellería and Forcone, 2000).

During the conditioning phase of the experiment, some stimuli induced greater levels of PER than others, e.g. pollen 1 (*P. coccinea*), pollen 2 (*C. bursa-pastoris*) and pollen 6 (*S. humboldtiana*). Overall, the level of PER was independent of the amount of protein, grain size or taxonomic family. This observation is consistent with previous studies that showed similar results and demonstrated that these variables are unlikely to serve as recognition cues (Schmidt and Johnson, 1984; Schmidt et al., 1987). However, when we grouped pollen types according to the end of the flowering period, we found that pollen from flowers of early ending produce higher levels of PER (e.g. pollen 6, *S. humboldtiana*). Arenas and Farina (2014) demonstrated that early experiences might have a fundamental role for the preferences towards certain types of pollen by honey bees of older ages (17 days of age). They argued that within the hive, young honey bees

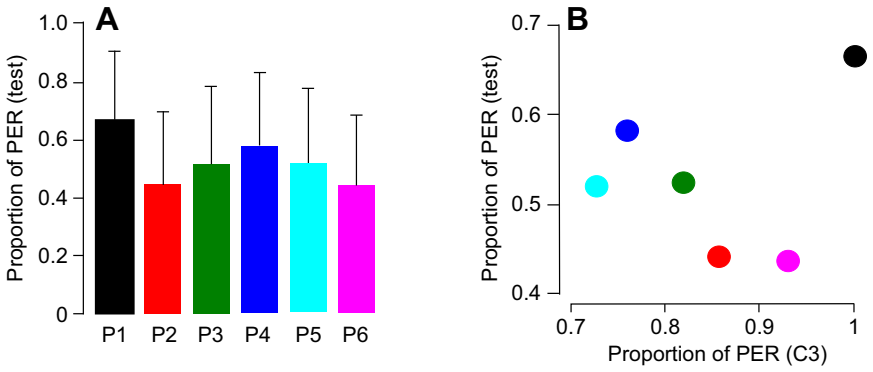

Fig. 2. Behavioral responses of honey bees during the test phase. (A) Proportion of PER for each type of pollen in those trials in which bees were conditioned and tested with the same type of pollen (pollen 1, n=15; pollen 2, n=18; pollen 3, n=38; pollen 4, n=48; pollen 5, n=23; pollen 6 n=23). The vertical lines indicate the standard error of the mean. There were no significant differences for the behavioral responses between pollen types. (B) Pearson's correlation between the proportion of PER at C3 and test. There was no correlation between the variables (Pearson's rank correlation coefficient r_P=0.195).

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Table 2. The effect of pollen characteristics on the level of PER during the test phase

Pollen characteristics	*F*-value	*P*-value
End of flowering	0.13	0.734
Grain size	0.14	0.723
Taxonomical family	1.59	0.275
Protein amount	1.71	0.260

The evaluated characteristics show no effect on the behavioral response of bees (ANOVA, $P>0.260$). d.f.=1 for each model run.

perceive and may even learn many odors whilst performing tasks such as nursing or food processing, which enables the formation of pollen preferences in foragers (Arenas and Farina, 2014; Cholé et al., 2019). Moreover, several studies showed that the honey 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). Based on these reports, we assume that, in our experiments, honey bees performed a higher level of PER toward pollen of flowers available in early summer (November–December) and that those scents may remind the honey bees the odors perceived in the hive during their first days as adults, before becoming foragers. In fact, we used forager individuals (over 21 days), and when we performed the experiment, the early seasonal flowers were not available in the environment. In future work it would be interesting to study learning in adult honey bees of different ages and their ability to memorize olfactory stimuli such as pollen scents.

At the test phase, despite there being no significant differences in the level of PER among the different pollen scents, pollen 1 (*P. coccinea*) continued to induce the highest level of PER. Neither phylogenetic relatedness, protein content of the pollen nor grouped pollen had any influence on the level of PER. Similar results were obtained in other studies in which it had been determined that size and protein content of pollen were unlikely to serve as recognition cues in honey bees during foraging behavior (Levin and Bohart, 1955; Schmidt and Johnson, 1984; Schmidt et al., 1987; Pernal and Currie, 2002). Possibly, other chemical compounds, regardless of the nutritional value of the pollen source (as phago-stimulants or phago-deterrents, secondary metabolites, the presence of pollenkitt or volatile compounds) as well prior experience, may influence honey bee preferences (Cook et al., 2003; Nicholls and Hempel de Ibarra, 2017). Numerous studies have provided evidence for a chemical source for pollen recognition cues (Doull, 1966; Lepage and Boch, 1968; Robinson and Nation, 1968; Doull and Standifer, 1969; Schmidt, 1985; Hanna and Schmidt, 2004; Pacini and Hesse, 2005), but none of them have successfully identified any compounds in pollen that serve this role.

When we analyzed (with the generalization matrix) which pollen scents honey bees perceived as similar, we observed that those individuals conditioned to pollen 4 (*H. radicata*) generalized to pollen 1 (*P. coccinea*). However, honey bees conditioned to pollen 1 did not generalize towards pollen 4 with the same level of PER, they did so in a smaller proportion. Therefore, generalization was asymmetric, indicating that the level of PER between any pair of stimuli depended on the order in which the stimuli were presented. Generalization asymmetry is a common phenomenon and has been reported in previous studies (Guerrieri et al., 2005; Schubert et al., 2015; see also an analogous case in ants reported by Bos et al., 2013), suggesting that our observation is an ordinary outcome. Chemical characteristics of pollen that we evaluated explained neither the differences among the levels of PER at conditioning and test, nor why certain pairs of pollen were generalized. The main feature to be noticed is the fact that both species were founded simultaneously during early summer (November–December).

Ghirlanda and Enquist (2003) proposed that all animals are able to generalize if the stimuli have similar ecological value. Moreover, Guerrieri et al. (2005) demonstrated that the chemical group and chain length of odor molecules determined the honey bees' generalization responses. This led us to hypothesize that the pair of pollen types generalized by honey bees possibly possesses a similar chemical composition of volatile compounds, which emit olfactory cues with the predominance of certain molecules that promote generalization (e.g. aldehydes such as hexanal). According to Wright and Schiestl (2009), unrelated plant species may have similar floral scents with common volatile compounds owing to selection pressure from a specific pollinator.

Guerrieri et al. (2005) also demonstrated that honey bees conditioned to aldehydes generalized very little to odors belonging to other functional groups, and in contrast, honey bees conditioned to other functional groups generalized highly to aldehydes. This showed that generalization between aldehydes and molecules containing other functional groups was asymmetrical; analogous results could be visualized in our generalization matrix. Therefore, we suggest that the high level of PER in the test when honey bees were presented with a novel pollen was due to the novel pollen scent containing a great proportion of aldehydes.

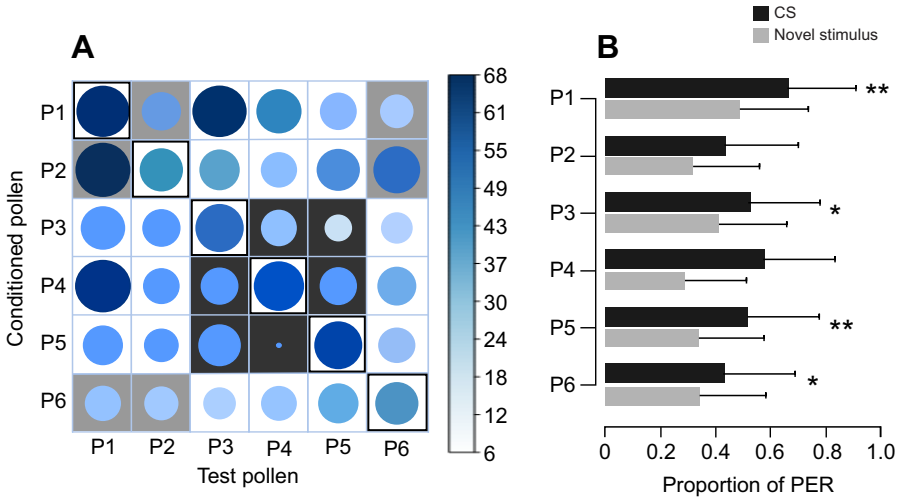

Fig. 3. Generalization matrix, which relates the level of PER at conditioning to the level of PER at test for each combination of pollen types. (A) The perception matrix represents the mean value of PER success during the conditioning versus test (n=237). Dark blue, maximal response; white, minimal response. Light gray background: flowering ends in spring and early summer; dark gray background: flowering ends in summer and early autumn. (B) Proportion of PER between both phases for each type of pollen. The black bars represent the PER success in those trials in which we always used the same pollen in both phases (CS), while the gray bars indicate the PER success of the trials in which a different pollen was used in the test phase (novel stimulus). The horizontal lines indicate the standard error of the mean. Asterisks indicate significance of the binomial GLM (*P<0.05; **P<0.01).

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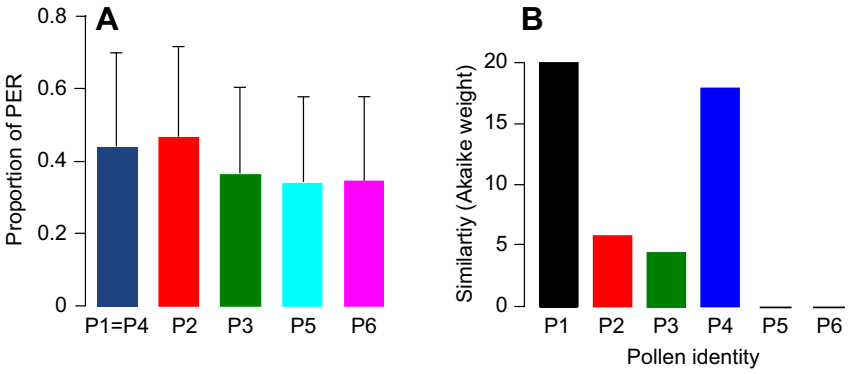

Fig. 4. Perception of pollen similarity based on the selected model. (A) Proportion of PER (conditioning and test) for each pollen type, and assuming P1=P4. There were no significant differences. Mean±s.e.m. values are shown. (B) Similarity value estimated by Akaike weights.

Pollen scents are species-specific and honey bees may recognize inter-specific pollen differences and infer the quality based on the content of only some amino acids, using this information for their foraging decisions (Dobson and Bergström, 2000; Cook et al., 2003; Piskorski et al., 2011; Ruedenauer et al., 2019). It remains unclear which perceptual cues determined honey bees generalizing among plants present in Patagonia. In any case, it will be interesting to analyze the adaptive implications of the generalization from one type of pollen with low protein content (e.g. pollen 4) to another with medium protein content (e.g. pollen 1). This behavior possibly benefits the hive development; in fact, the ability to generalize plays a key role in reducing the cost of foraging, where different flowers can give similar rewards in the form of nectar and pollen (Waser et al., 1996). However, we also demonstrated that generalization could occur inversely – from pollen with high protein content (e.g. pollen 2, *C. bursa-pastoris*) to another with medium protein content (pollen 1, *P. coccinea*).

Wright et al. (2008) argued that olfactory generalization is a mechanism used by animals to adjust their sensitivity to differences in complex olfactory stimuli in a context-dependent manner. Consequently, this ability plays a key role in reducing the cost of foraging, as it allows honey bees to successfully forage in a changing environment, decreasing the distance, duration and number of flights (Waser et al., 1996). Therefore, identifying the types of pollen that were perceived as similar (but have different nutritional qualities and/or flowering period) can provide valuable information for beekeepers and agricultural producers, as it allows them to manage the floral offer (selecting flowers of high nutritional resource) to obtain a greater production of honey or a better pollination service. In-depth study of honey bee selection behavior will enhance the sustainable management of beehives. Our results contribute to the background of knowledge about the complex cognitive performances of honey bees, and are in agreement with those of previous studies, which evidenced how pollen scent could be a crucial cue of honey bee foraging behavior (Wright and Smith, 2004; Cook et al., 2005; Arenas and Farina, 2012; Balamurali et al., 2015). The ability to generalize pollen odor could exert an important selective pressure determining plant reproductive success and plant co-evolution; however, our knowledge about how olfactory learning in pollinators determines the expression of these floral cues remains relatively poor (Wright and Schiestl, 2009). As we observed, perceptual similarity among pollen scents relies not only on chemical cues, but also on the temporality of the flowering season. Therefore, the role of temporality as a dimension in perceptual spaces should be taken into account in future cognitive studies.

We also suggest that it would be of great interest to conduct studies using an integrated approach (between behavioral ecology and neuroscience) to understand the mechanisms by which pollen scents are processed in the honey bee neural system. This information could improve our understanding about the pollinator–plant interaction as well about the processes that underlie the evolution of floral signals (Balamurali et al., 2015; Rush et al., 2016).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.L.P., F.R., V.F.-A., F.J.G.; Methodology: A.L.P., F.J.G.; Validation: J.W., F.J.G.; Formal analysis: A.L.P., F.R.; Investigation: A.L.P., J.W., F.J.G.; Resources: G.H., F.J.G.; Data curation: A.L.P., F.R., J.W., F.J.G.; Writing - original draft: A.L.P., F.R., V.F.-A., G.H., F.J.G.; Visualization: G.H.; Supervision: V.F.-A., F.J.G.; Funding acquisition: F.J.G.

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Supplementary information
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