

Odor information transfer in the stingless bee *Melipona quadrifasciata*: effect of in-hive experiences on classical conditioning of proboscis extension

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Abstract A recent study showed that the stingless bee *Melipona quadrifasciata* could learn to discriminate odors in a classical conditioning of proboscis extension response (PER). Here we used this protocol to investigate the ability of these bees to use olfactory information obtained within the colony in an experimental context: the PER paradigm. We compared their success in solving a classical differential conditioning depending on the previous olfactory experiences received inside the nest. We found that *M. quadrifasciata* bees are capable of transferring the food-odor information acquired in the colony to a differential conditioning in the PER paradigm. Bees attained higher discrimination levels when they had previously encountered the rewarded odor associated to food inside the hive. The increase in the discrimination levels, however, was in some cases unspecific to the odor used indicating a certain degree of generalization. The influence of the food scent offered at a field feeder 24 h before the classical conditioning could also be seen in the discrimination attained by the foragers in the PER setup, detecting the presence of long-term memory. Moreover, the improved performance of recruited bees in the PER paradigm suggests the occurrence of social learning of nectar scents inside the stingless bees' hives.

Keywords Associative learning · Odor discrimination · Information transfer · Stingless bees · Social learning

Introduction

Within the learning process, the ability to retrieve information that has been acquired in a different context is a key attribute that contributes to the plasticity of animal behavior (Rescorla et al. 1985). This transfer of information can optimize the use of stored memories making them less dependent on where they were obtained and using the learned information wherever it is necessary. Regarding foraging behavior, the ability to transfer associations is essential to improve its efficiency in an always-changing environment (Balsam 1985).

Social insects have been studied in this sense since it is possible to analyze their behavior under different and controlled contexts. There are many examples showing that olfactory experiences acquired within a social context can affect posterior individual behaviors and vice versa (e.g., honeybees von Frisch 1967; Wenner et al. 1969; Gerber et al. 1996; Sandoz et al. 2000; Chaffiol et al. 2005; bumblebees, Dornhaus and Chittka 1999; ants, Rocas 1990; wasps, Jandt and Jeanne 2005). The use in the field of the previously encountered food odors implies that the scent information gained in the nest can be evoked later in a different context as when looking for a food source. Social interactions amongst nestmates appear as a key feature during the acquisition of food-related information. Learning that is influenced by a conspecific or its product is defined as *social learning* (Heyes 1994), and it has been described in various insect species (reviewed by Leadbeater and Chittka 2007). The social information can be inadvertently provided (cues) or based on traits specifically designed by

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selection to convey information (signals) (Danchin et al. 2004). Particularly bees show remarkable abilities to learn from conspecifics (Leadbeater and Chittka 2007) and to acquire simple and even complex associations (Menzel 1999; Giurfa 2003).

Issues regarding social learning have been studied in honeybees, for example, by means of the classical conditioning with the proboscis extension response (PER) paradigm (Farina et al. 2005; Gil and De Marco 2005; Grüter et al. 2006) that is a highly standardized learning procedure. The PER protocol has been thoroughly used to study learning dynamics in several insect species (in honeybees Takeda 1961; in bumble bees Laloi et al. 1999; in *Drosophila* Chabaud et al. 2006; in mosquitoes Tomberlin et al. 2006), and it has recently been developed for the stingless bee *Melipona quadrifasciata* (Mc Cabe et al. 2007). The possibility to use this controlled learning procedure with stingless bees allows us to state the question whether *Melipona quadrifasciata* bees have the ability to transfer associations from a given context to another, focusing specifically in the passage of information from a social context to an individual one.

Regarding the use of olfactory information in stingless bee species, there are several examples that show that when bees are alerted about a profitable food source by nestmates some species do not inform the location of the food source (e.g. *Trigona iridipennis* Lindauer 1956), while others appear to inform direction but not distance (e.g. *Melipona scutellaris* and *M. quadrifasciata* Jarau et al. 2000; *Plebeia tica* Aguilar et al. 2005). In both cases alerted bees fly out looking for the smell the recruiting bee carried and land on any food source along the way that has that same smell. These studies, however, have not analyzed how specific is the information about the floral odor the recruits carry when looking for the advertised food source, or how the previous odor experiences affect the learning performance at an individual level.

We aimed to study if the stingless bee *Melipona quadrifasciata* is capable of transferring olfactory information between different contexts and how in-hive experiences with scented food affect their performance during classical differential olfactory conditioning. Specifically, we analyzed the influence of pre-stimulation with (1) scented food artificially introduced inside the hive and (2) when the food scent was carried by the foraging nestmates. In addition, we evaluated the influence of the food scent encountered during foraging 24 h before performing the learning assay.

Materials and methods

The experiments were performed on the campus of the University of São Paulo, Ribeirão Preto, Brazil. The Experimental

Series I (see below for details) was performed between August and December 2005. The Experimental Series II and III were carried out between July and November 2006. We used four colonies of *Melipona quadrifasciata anthidioides* (subsequently referred to as *M. quadrifasciata*). The bee colonies used had been established in this environment for 1 year already before starting the experiments. All colonies contained a queen, brood and stored food.

The three experimental series were always evaluated by means of a differential conditioning with the proboscis extension response (PER) paradigm (Takeda 1961; Bitterman et al. 1983).

Differential PER conditioning

To perform a differential PER conditioning (Takeda 1961; Bitterman et al. 1983) worker bees were first harnessed. They were immobilized by chilling and mounted in appropriately cut pipette tips that restrained the body movement but allowed free movement of the antennae and mouthparts (Frings 1944). The protocol used here with *M. quadrifasciata* is the same we had already established in a previous study (Mc Cabe et al. 2007). We placed the bees in front of a continuous airflow, and to apply the odorant stimulus, by means of an electronic valve, the air flux was redirected to pass through a syringe with a filter paper imbibed in 4 µl of odor. Both pure odors used, Linalool (LIO) and Phenylacetaldehyde (PHE), are natural components of flower scents (Knudsen et al. 1993) and were obtained from Sigma–Aldrich, Steinheim, Germany. They were presented either as a rewarded odor (conditioned stimulus, CS+) immediately prior to the presentation of a 50% w/w sucrose solution (unconditioned stimulus, US) or as a non-rewarded odor (CS−). Both odors were presented five times each in a pseudo-randomized order (CS−, CS+, CS+, CS−, CS−, CS+, CS−, CS+, CS+, CS−). The inter-trial interval lasted 10–15 min between CS presentations. The same interval was maintained between the last trial and the testing phase that consisted of the non-rewarded presentation of both odors. Only bees that showed the unconditioned response (the reflexive extension of the proboscis after applying a 50% w/w sucrose solution to the antennae; unconditioned response, UR) and that did not respond to the mechanical airflow stimulus were used. Each trial lasted for 46 s and consisted of 20 s of airflow, 6 s of odor (CS) and 20 s of airflow. During rewarded trials, the sugar solution (US) was delivered during the last 3 s of the CS, when the bees had extended the proboscis (PER) as a response of contacting their antenna with the sucrose solution. A conditioned response (CR) was considered only if the bee responded by fully protruding its proboscis during the first three-seconds of odor presentation without need of touching her antenna with the sucrose solution. Due to the limitations of the PER

protocol for this bee species (Mc Cabe et al. 2007), we only recorded very low proportions of spontaneous responses (SR). This meant that we could not rely just on the bees' spontaneous PER towards the odors to assess the influence of the previous olfactory experience as it has been done in studies performed with honeybees (Farina et al. 2005, 2007; Grüter et al. 2006; Arenas and Farina 2008). A way to go beyond this is to perform a differential conditioning, which is a typical within-subject control and implies discrimination and to explicitly not respond to the non-rewarded odor. That is why we considered that the best way to analyze learning abilities was during differential conditioning. The bees that presented a SR to an odor were removed from the analysis and did not continue with the following PER conditioning protocol allowing all the experimental bees to start from the same baseline.

Experimental series

Experimental Series I: offering of scented food within the hive

To study the influence of previous olfactory experiences in the performance of *M. quadrifasciata* bees during a differential conditioning with the PER paradigm, we placed inside three hives a single small plastic cup (20 ml approx) filled with 50% w/w sucrose solution. The cups were refilled every 2 days to ensure a constant supply of solution. In one colony we placed unscented sugar solution (CONTROL hive), inside the second hive we placed sugar solution scented with 50 µl of LIO per liter of solution (LIO-treated hive), and in the third hive the sugar solution was scented with the same concentration of PHE (PHE-treated hive). The odor concentration used is the lowest registered to elicit a behavioral response in the honeybee (Gil and De Marco 2005). The solution was placed inside the hives 2 days before starting the experiment to allow most of the bees to get in contact with the scented food either by ingesting the sucrose solution directly at the in-hive feeder or by its distribution through trophallaxis between nestmates. Data collection lasted for around 20 days.

Bees were collected at the hives' entrances using plastic tubes and placed in an incubator (28°C, 60% relative humidity, and darkness). After 40 min in captivity, bees were harnessed. Once they regained motility of their head and mouth parts after being chilled during harnessing they were fed 50% w/w sucrose solution for about 2 s before being kept in the incubator for at least 2 h. Harnessed bees from each colony were separated into two groups and subjected to a standard differential PER conditioning protocol: one group received LIO as CS+ and the other group received PHE as CS+.

Experimental Series II: transfer of scented food during recruitment inside the hive

In the previous experiment, bees had received sugar solution scented with one of the odors used during the differential conditioning inside the hive. We now wanted to evaluate if stingless bees *M. quadrifasciata* can learn the association between food and odor specifically during the social interactions within the colony. We trained 3–4 bees to collect 50% w/w un-scented sucrose solution from an artificial ad libitum feeder placed 20 m away from the hive. These bees were marked with a color dot on their thoraxes. Once these forager bees flew between the feeder and the hive 3–4 times, we replaced the feeder by a clean one filled with 50% w/w sucrose solution either un-scented or scented with a pure odor (either LIO 50 µl/l or PHE 50 µl/l). The recruited bees that arrived at the feeder (un-marked bees) were captured with plastic tubes as soon as they landed, before they contacted the sucrose solution. If any bee had direct contact with the sucrose solution at the feeder, she was left aside in a plastic tube and not considered in the following stages of the experiment. In this way any previous contact they could have had with the sucrose solution must have occurred inside the hive, where the most plausible contacts with the incoming scented food is through trophallaxis with the active forager (Hrnčir et al. 2000; Farina et al. 2005, 2007; Gil and De Marco 2005). The recruited bees were collected for 45 min. The bees in the plastic tubes were placed for 15 min in the incubator. Then, they were harnessed and placed in the incubator again for at least 1.5 h. The bees recruited to unscented solution were divided in two groups and subjected to a differential PER conditioning: one group with LIO as the rewarded conditioned stimulus (CS+) and the other with PHE as CS+. The bees recruited to the LIO solution were subjected to a differential PER conditioning where LIO was the CS+ and the ones recruited to PHE-scented solution went through a differential PER conditioning with PHE as CS+. To achieve a sufficient number of bees in each group, each series was repeated during 4–5 days. To avoid pseudo-replication at the end of the conditioning protocol the bees were always marked with color dots on their thoraxes and were not used for any further experiments.

Experimental Series III: classical conditioning 24 h after foraging scented food

Our aim was to evaluate if *M. quadrifasciata* bees possess olfactory memory that can last for 24 h. For that we placed an ad libitum feeder with unscented 50% w/w sucrose solution in the meliponary and let the bees feed freely. Once there were at least five bees collecting from the feeder we

replaced it for a clean one filled with scented sucrose solution. We performed two experiments: in the first one the feeder offered sugar solution scented with LIO 50 μ l/l and in the second the feeder offered PHE 50 μ l/l diluted in the solution. All arriving bees were allowed to feed for 1 h and marked with a color dot on their thoraxes. A total of 24 h after foraging the scented solution they were offered unscented 50% w/w sucrose solution in the same location where they had fed the day before. As soon as a marked bee arrived she was captured with a plastic tube. Capturing lasted for 45 min. The bees in the tubes were then placed in the incubator for 15 min. They were harnessed and placed in the incubator again for at least 1.5 h. The bees that had ingested a LIO solution the day before were subjected to a differential PER conditioning where LIO was the CS+. The bees that had drunk a PHE-scented solution went through differential PER conditioning with PHE as CS+.

Statistical analysis

The SR-values obtained during the differential conditioning were compared using a *G* test and multiple comparisons were performed using the Dunn–Sidak correction (Sokal and Rohlf 1995). Performance during conditioning was analyzed using a discrimination index (DI) that was calculated as the difference between the bees' response to the CS+ minus the response to the CS−. The DIs were calculated for each bee at each of the five pair of trials of the differential conditioning. The index could take values of −1, if the bee responded only to the CS−, 0 if the bee responded equally to both odors, or 1, if it only responded to the rewarded odor CS+. To analyze the performance during the conditioning within and between groups, a two-way ANOVA for repeated measures analysis was performed. Monte Carlo studies have shown that it is permissible to use ANOVA on dichotomous data only under certain conditions (Lunney 1970), which are met by our experiments (at least 40 degrees of freedom of the error term). One of the factors taken into account was the successive trials and the other one the previous olfactory treatment. If we detected statistical differences in the principal factors we carried out Scheffé post hoc comparisons. If statistical differences were detected in the interaction between factors we computed simple effects using the corresponding error and afterwards performed Tukey comparisons to the control.

Regarding the testing phase, we compared the proportion of bees that responded to the rewarded odor with the non-rewarded ones by means of Fisher's exact test. To compare proportions of response between groups at the testing phase we used *G* test.

Since the comparison of the performance between the bees recruited to scented odor (Series II) and the bees that ingested scented solution 24 h prior to the conditioning

(Series III) was made against the same control group the significance level was modified to $\alpha' = 0.025$ (Sokal and Rohlf 1995).

Results

Experimental Series I: offering of scented food within the hive

The idea of placing in-hive feeders with scented food was to answer how the encounter with a rewarded odor inside the hive affects the performance of *M. quadrifasciata* bees in an associative learning task. During the olfactory differential conditioning, the first time an odor is presented and the bee responds by protruding her proboscis is considered as the SR. This response could be influenced by prior-odor experiences (Gerber et al. 1996; Farina et al. 2005). Then, we compared the proportion of SR to each odor of the bees from the three different hives. The SR to LIO was higher in the bees of the LIO-treated hive than the bees from the control hive [*G* test: $G_h = 19.67$, $P < 0.05$, $N = 258$; multiple comparisons ($\alpha' = 0.025$): control hive vs. LIO-treated hive: $G_h = 14.14$, $P < 0.025$; control hive vs. PHE-treated hive: $G_h = 0.56$, $P = 0.45$; Fig. 1, left panel]. When comparing the SR to PHE, it becomes clear that bees from both olfactory-stimulated hives, LIO-treated hive and PHE-treated hive, responded more to PHE than did the bees from the control hive [*G* test: $G_h = 6.53$, $P < 0.05$, $N = 258$; multiple comparisons ($\alpha' = 0.025$): control hive vs. LIO-treated hive: $G_h = 5.50$, $P < 0.025$; control hive vs. PHE-treated hive: $G_h = 5.50$, $P < 0.025$]. All these bees that responded spontaneously to any of the odors were removed from the analysis. Since the number of bees spontaneously responding was very low we went ahead with the differential conditioning.

To analyze if the acquisition dynamics suffered any modifications due to the previous olfactory stimulation we compared the performance of the three colonies during the differential conditioning when they had the same odor as rewarded stimulus (CS+). When PHE was the CS+ (Fig. 1, middle panel) a two-way ANOVA for repeated measures revealed statistical differences for all source of variation: there is an effect of the successive trials ($F = 25.53$, $df = 4$, $P < 0.001$), an effect of the olfactory stimulation in each hive ($F = 5.48$, $df = 2$, $P = 0.01$) and the interaction of both factors was also significant ($F = 2.39$, $P = 0.02$). Thus, we computed simple effect analyses and Tukey post hoc comparisons that revealed that: (1) at the second pair of trials the PHE-treated bees showed higher discrimination levels than the Control bees; (2) at the fourth pair of trials the PHE-treated bees discriminated more than the LIO-treated ones; and (3) at the last (fifth) pair of trials the bees from

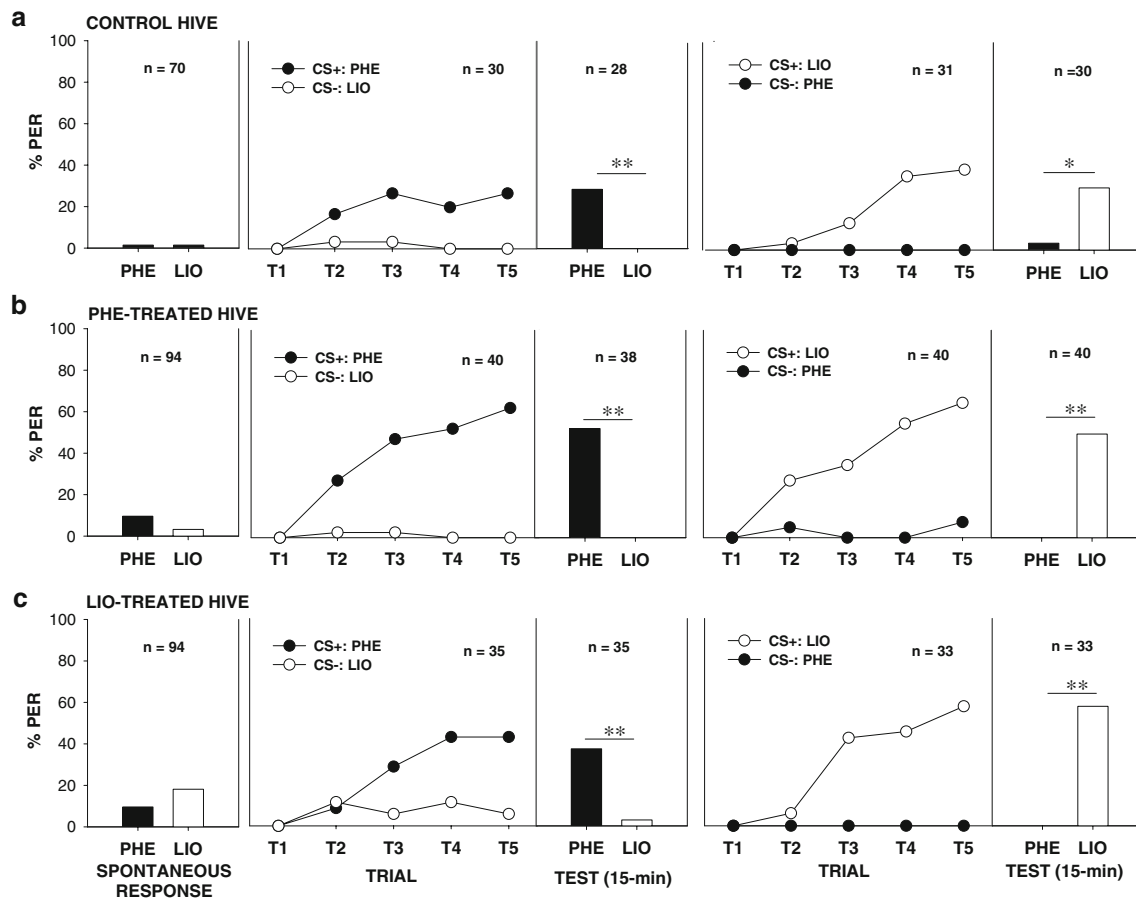


Fig. 1 Percentage of bees that extended the proboscis (% PER) during the first presentation of the odor (spontaneous response), percentage of bees that responded during five pairs of trials (training), and bees that responded during a testing period 15 min after training (test) during the differential PER conditioning experiment: **a** CONTROL HIVE an in-hive feeder offered unscented sucrose solution; **b** PHE-TREATED

HIVE an in-hive feeder offered sucrose solution scented with phenylacetaldehyde *PHE*; **c** LIO-TREATED HIVE an in-hive feeder offered sucrose solution scented with Linalool *LIO*. Asterisks indicate statistical differences in Fisher exact Test (* $P < 0.05$, ** $P < 0.01$, see “Results” for details)

the PHE-treated hive discriminated more than those from either the Control hive or the LIO-treated hive. These results show that when bees had prior contact with sugar solution scented with PHE inside the hive, they ended up discriminating that odor better than those bees that had had contact with food either un-scented or scented with a different odor (LIO in this case).

Regarding the series where LIO was the rewarded odor (CS+) (Fig. 1, right panel) the two-way ANOVA for repeated measures revealed statistical differences for each of the factors (successive trials: $F = 37.9$, $df = 4$, $P < 0.001$ and olfactory stimulation: $F = 3.33$, $df = 2$, $P = 0.04$), but it did not appear to be an effect of the interaction of both ($F = 1.62$, $df = 8$, $P = 0.12$). Scheffe post hoc comparisons showed that the bees from the LIO-treated hive discriminated better than the ones from the control hive ($P = 0.04$), but not more than the bees from the PHE-treated hive ($P = 0.83$). These bees from the PHE-treated hive in turn did not discriminate LIO better as the CS+ than the bees

that belonged to the control hive ($P = 0.19$). This would leave the discrimination level of the bees of the PHE-treated hive in an intermediate position between the bees from the LIO-treated hive and the bees from the control hive. Fifteen minutes after the end of the protocol we tested the bee’s response to both odors (testing phase). We can see that in all the groups the bees responded more to the odor that had been rewarded during the differential conditioning than to the one not associated to a sucrose reward (Fisher’s exact test: control hive with PHE as CS+: $P < 0.01$; control hive with LIO as CS+: $P < 0.05$; PHE-treated hive with PHE as CS+: $P < 0.01$; PHE-treated hive with LIO as CS+: $P < 0.01$; LIO-treated hive with PHE as CS+: $P < 0.01$; LIO-treated hive with LIO as CS+: $P < 0.01$). When comparing the proportion of conditioned response to PHE during the testing phase between the three different hives (Fig. 1, middle panel, black bars), there were no differences among them (G test: $G_h = 4.94$, $P = 0.08$, $N = 100$). The same was the case when the proportion of response to LIO

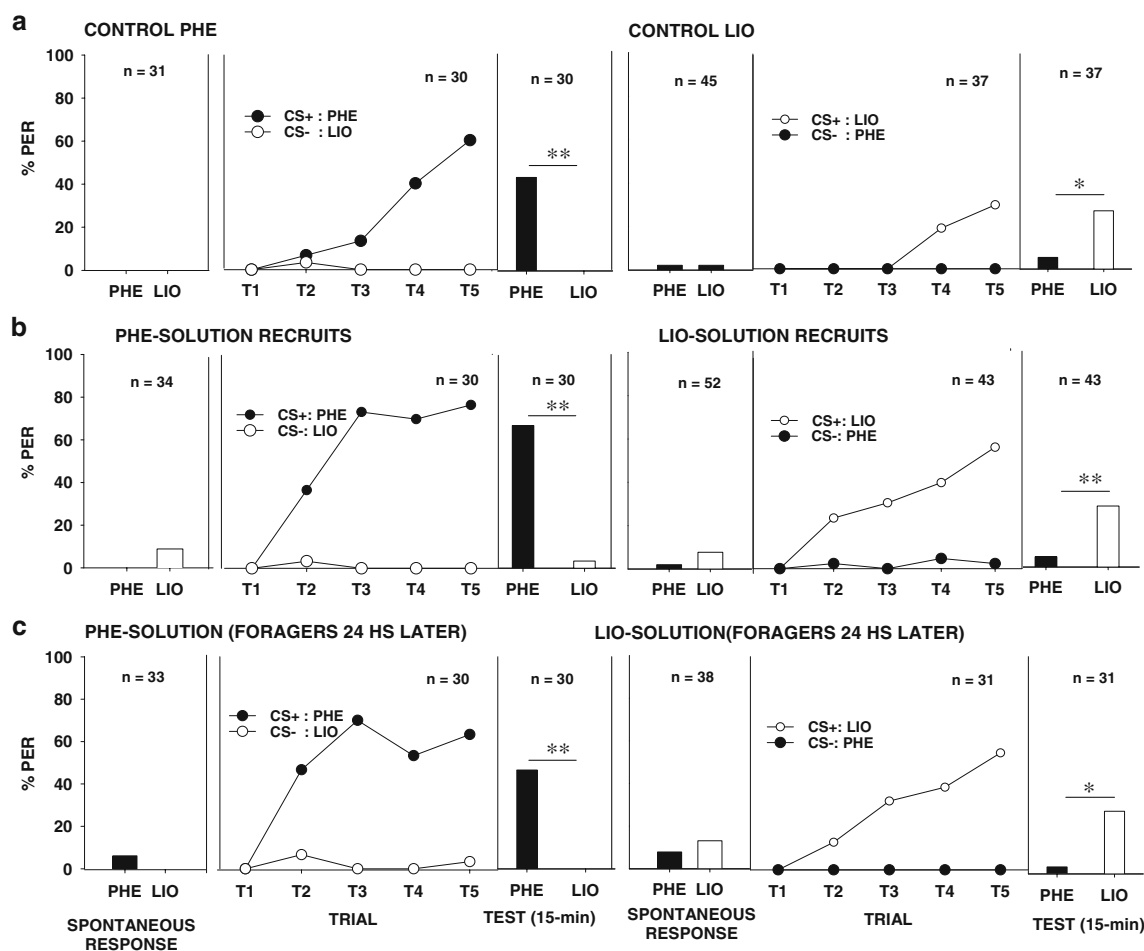


Fig. 2 Percentage of bees that extended the proboscis (% PER) during the first presentation of the odor (spontaneous response), percentage of bees that responded during five pairs of trials (training), and bees that responded during a testing period 15 min after training (test) during the differential PER conditioning experiment: **a** bees recruited to an un-scented-sucrose-solution feeder with Phenylacetaldehyde as CS+ (CONTROL PHE) or Linalool as CS+ (CONTROL LIO); **b** bees re-

cruited to a feeder with sucrose-solution scented either with *PHE* (PHE-SOLUTION RECRUITS) or *LIO* (LIO-SOLUTION RECRUITS); **c** FORAGERS 24-HS LATER bees that have collected scented sucrose solution 24 h before (*left panel*, solution scented with PHE; *right panel*, solution scented with LIO). Asterisks indicate statistical differences in Fisher exact Test (* $P < 0.05$, ** $P < 0.01$, see “Results” for details)

between hives (G test: $G_h = 5.22$, $P = 0.07$, $N = 103$; Fig. 1, right panel, white bars) was compared. The retention of the information acquired during the differential conditioning appeared to be independent of the bees’ performance during the learning protocol.

Experimental Series II: transfer of scented food during recruitment inside the hive

Since stingless bees during recruitment alert nestmates and transmit the odor of the encountered food source, we wanted to evaluate at an individual level if the recruited bees can evoke the odor transferred by a nest mate inside the hive in an artificial learning paradigm.

A small number of bees were trained to feed from artificial feeders with either un-scented or scented sucrose

solutions and allowed to recruit nestmates to it. Of the recruited bees that arrived to the feeder with any of the sucrose solutions the proportion that responded spontaneously to the odors presentation in the PER paradigm was very low (Fig. 2, left panel), so no statistical analysis was performed.

We then analyzed if the discrimination process of the bees recruited to a scented solution was different from those recruited to an un-scented solution. For PHE as the rewarded odor (Fig. 2a, b; left panel) bees that were recruited to a PHE-scented solution showed higher discrimination levels than the ones recruited to un-scented solution at the second, third and fourth pair of trials (two-way ANOVA for repeated measures: successive trials: $F = 49.24$, $df = 4$, $P < 0.001$; olfactory stimulation: $F = 8.89$, $df = 2$, $P < 0.001$; interaction of both factors: $F = 5.18$, $df = 8$, $P < 0.001$;

analysis of the interaction by means of simple effects and the corresponding Tukey comparisons to the control). When LIO was the rewarded odor bees recruited to LIO-scented solution showed higher discrimination indexes than the control hive at trials 2 to 5 (two-way ANOVA for repeated measures: successive trials: $F = 28.57$, $df = 4$, $P < 0.001$; olfactory stimulation: $F = 7.3$, $df = 2$, $P < 0.005$; interaction of both factors: $F = 1.86$, $df = 8$, $P = 0.06$; Scheffe test $P < 0.005$). These results show that the encounter with the odor of the discovered food inside the nest helps the recruits to solve the discrimination between rewarded and non-rewarded odors better than the ones that lack that olfactory experience.

During the testing phase, in which the presentation of both odors was un-rewarded, in each of the four experimental groups the bees had higher proportions of response to the odor that had been rewarded during the conditioning (Fisher's exact test: un-scented with PHE as CS+: $P < 0.01$; un-scented with LIO as CS+: $P < 0.05$; PHE-scented with PHE as CS+: $P < 0.01$; LIO-scented with LIO as CS+: $P < 0.01$). When we compared the proportion of response at the testing phase between olfactory stimulated groups and their respective Control groups, we found no significant differences (G test: recruits to un-scented solution using PHE as CS+ vs. recruits to PHE-scented solution: $Gh = 3.33$, $P = 0.07$, $N = 60$; recruits to unscented solution with LIO as CS+ vs. recruits to LIO-scented solution: $Gh = 0.01$, $P = 0.93$, $N = 80$).

Experimental Series III: classical conditioning 24 h after foraging a scented food

To determine whether the association between odor and reward is maintained for at least 24 h, we analyzed the performance of bees that had fed on scented sucrose solution 24 h prior to the conditioning. When presented for the first time with two odors, the one they had ingested and a new one, the spontaneous response to both of them was very low, and when they had ingested LIO-scented solution it was also quite indiscriminate (most bees that responded did so to both odors). During the differential conditioning, as it was seen with the bees recruited to scented solution, the bees that had ingested the scented solution 24 h before presented higher discrimination indexes than the bees that had not encountered the odors before (Fig. 2, panels a, c). For PHE as CS+, the foragers that had collected PHE-scented solution 24 h earlier had higher discrimination indexes at the second and third pair of trials (two-way ANOVA for repeated measures: successive trials: $F = 49.24$, $df = 4$, $P < 0.001$; olfactory stimulation: $F = 8.89$, $df = 2$, $P < 0.001$; interaction of both factors: $F = 5.18$, $df = 8$, $P < 0.001$; analysis of the interaction by means of simple effects and the corresponding Tukey comparisons to the

control). For LIO as the CS+ the bees that had foraged LIO-scented solution also presented higher discrimination indexes than the ones that had foraged un-scented sugar solution at trials 2 to 5 (two-way ANOVA for repeated measures: successive trials: $F = 28.57$, $df = 4$, $P < 0.001$; olfactory stimulation: $F = 7.3$, $df = 2$, $P < 0.005$; interaction of both factors: $F = 1.86$, $df = 8$, $P = 0.06$; Scheffe test $P < 0.01$).

During the testing phase the response to the conditioned odor of these bees was always higher than to the non-rewarded odor (Fisher's exact test: PHE-treated with PHE as CS+: $P < 0.01$; LIO-treated with LIO as CS+: $P < 0.05$). When we compared the proportion of response at the testing phase between the bees that had foraged scented food 24 h before and their respective control groups, we found no significant differences (G test: recruits to un-scented solution using PHE as CS+ vs. foragers of PHE-scented solution: $Gh = 0.07$, $P = 0.79$, $N = 60$; recruits to un-scented solution with LIO as CS+ vs. foragers of LIO-scented solution: $Gh = 0.01$, $P = 0.91$, $N = 68$).

Discussion

The aim of this work was to analyze if *M. quadrifasciata* bees are capable of transferring information between different behavioral contexts, and more specifically how olfactory experiences inside the colony influence posterior associative learning within a classical (Pavlovian) context. Although there was not an even effect in all the trials of the differential classical conditioning and there were differences between the pure odors used, the ability to discriminate between the odor previously encountered in a different context from the PER conditioning (inside the hive or while foraging 24 h before) was improved during this learning procedure compared to the bees that lacked this kind of stimulation. This improved effect was seen in the case with an in-hive feeder or when bees were activated presumably by the foragers returning from a scented food source. The latter result, in which the encounter with the food must have been mediated by another individual, shows that *M. quadrifasciata* bees are capable of social learning, as it has been found for honeybees (Farina et al. 2005). We also detected an improvement of the learning performance in foragers that had ingested a scented sucrose solution 24 h prior to the conditioning with that odor, which indicated the formation of stable and long-lasting memories about floral odors in these stingless bees.

Our results show that the placement of scented food inside the colony improved the bees' performance during the conditioning protocol. One explanation for this result is the participation of non-associative processes such as pseudo-conditioning (that is an increase in the response just

by the repeated presentation of the US) or sensory priming. However, we have also observed that the increased response appears to be odor specific. When PHE was introduced inside the hive the bees that were taught to associate PHE with a reward during the conditioning ended up with higher discrimination indexes than the bees from the control hive and the LIO hive. When bees with the same previous stimulation were taught to associate a novel odor (LIO) with the reward, they learned that association in the same way as the bees from the Control hive. Besides, when LIO was used to scent the food that was introduced into the hive and was also used as CS+ during conditioning, the bees showed higher discrimination indexes than the Control bees that lacked this olfactory stimulation. Bees that had encountered LIO-scented food inside the hive learned the association PHE-reward in the same way as the bees from the Control hive. Even though PHE-stimulated bees did not learn the association between LIO and the reward differently from the Control ones, PHE-stimulated bees did not differ from the LIO-stimulated ones with the same conditioning procedure, which were able to discriminate the odors in an improved way. This fact suggests some generalization of the odors under some circumstances. Taking all this into account, it is plausible that associative learning has been established inside the hive. This odor-specific learning was similar to both odors used in our experiments and, contrary to conspicuous asymmetries in the discrimination found for bumblebees within the same PER paradigm (Laloi and Pham-Delegue 2004), we did not detect any differences in *Melipona* (Mc Cabe et al. 2007, this study).

However, the improvements we detected in the discrimination levels of the bees that previously fed on a sucrose solution scented with the rewarded odor cannot be seen at the testing phase. At that point (unrewarded presentation of both odors 15 min after the end of the learning protocol), and despite finding some tendencies in Experimental Series I, the proportion of responses to the rewarded odor did not differ among treatments. Even more, responses neither at pre-training (SR) nor at post-training (CR at the testing phase) seem to be suitable variables to evaluate quantitatively learning abilities in differences in *Melipona* bees. Only during the training phases, differences amongst treatments could be found. In this sense, it is worth mentioning the fact that the PER paradigm has been especially designed and optimized for honeybees (Takeda 1961; Bitterman et al. 1983). Thus, after these results and others already published (Mc Cabe et al. 2007) we can conclude that the PER conditioning is still a limited procedure to evaluate learning abilities in stingless bees species.

Stingless bees display social foraging strategies that include different types of interactions inside the hive such as food sharing by trophallaxis (Hrncir et al. 2000) or vibratory signals (Aguilar and Briceño 2002; Hrncir et al. 2004;

Jarau et al. 2000; Nieh 2004). These social interactions might be relevant features during collective foraging since they allow the transfer of resource-related cues in an inadvertent way, i.e., as a by-product of the actions or traits of animals (Danchin et al. 2004; Dall et al. 2005). Particularly *M. quadrifasciata* bees do not seem to give precise information about the food source location, at least during the first 90 min of recruitment (Jarau et al. 2000). They appear to jostle around the hive simply alerting nest mates and giving food samples while the recruits go out in a random search. At this point, the odor of the food source could be especially important; particularly after discarding scent marking along the way, piloting flight and mechano-sensory-communication as channels for encoding information about the location of food sources (Hrncir et al. 2000). It has been shown in honeybees that trophallaxis allows the receiver bee to learn the association between the floral odor and the reward (Gil and De Marco 2005; Grüter et al. 2006; Farina et al. 2007). The ability of the honeybee *Apis mellifera*, to transfer olfactory information between different contexts: a natural environment (during foraging or inside the colony) and a controlled laboratory one (classical PER paradigm) (Gerber et al. 1996; Farina et al. 2005, 2007; Grüter et al. 2006), has also been described. Yet, we cannot be sure if it was by trophallaxis that the recruits received the information about the floral odor since no in-hive observations were made. The fact that these bees appear to discriminate better than those recruited to un-scented solution gives the idea that odor information was transferred during the scented-food circulation and that *M. quadrifasciata* bees are able to evoke this information in the PER paradigm. It has been shown in honeybees (von Frisch 1967; Wenner et al. 1969; Arenas et al. 2007, 2008) and bumblebees (Dornhaus and Chittka 1999) that the in-hive acquired information can bias the foragers' preference of different food sources. Even though there are some results that would indicate that this is also the case for stingless bees (Lindauer 1956; Slaa et al. 1998), food choice experiments should be done to conclusively prove this idea.

The acquisition of information inside the hive is crucial for the coordinated responses of individuals at the colony level (Seeley 1995), allowing a much better adjustment to the environments' offer at that particular moment. For honeybees, it has been showed that this kind of olfactory information associated to food can rapidly spread between individuals inside the hive (Grüter et al. 2006), affecting the activated forager mates in their food search (Grüter et al. 2008) and when the bees fly within the close range of the feeding site (von Frisch 1967; Wenner et al. 1969; Arenas et al. 2007, 2008). The evidence we present here that shows that the olfactory associations made inside the nest, either by ingesting stored food or by interacting with nestmates, together with the fact that odor-rewarded memories can last

for at least 24 h, can help us to begin elucidating the importance of floral scent information in the stingless bee foraging strategies.

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