

# Olfactory learning in the stingless bee *Tetragonisca angustula* (Hymenoptera, Apidae, Meliponini)

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**Abstract** *Tetragonisca angustula* stingless bees are considered as solitary foragers that lack specific communication strategies. In their orientation towards a food source, these social bees use chemical cues left by co-specifics and the information obtained in previous foraging trips by the association of visual stimuli with the food reward. Here, we investigated their ability to learn the association between odors and reward (sugar solution) and the effect on learning of previous encounters with scented food either inside the hive or during foraging. During food choice experiments, when the odor associated with the food was encountered at the feeding site, the bees' choice is biased to the same odor afterwards. The same was not the case when scented food was placed inside the nest. We also performed a differential olfactory conditioning of proboscis extension response with this species for the first time. Inexperienced bees did not show significant discrimination levels. However, when they had had already interacted with scented food inside the hive, they were able to learn the association with a specific odor. Possible olfactory information circulation inside the hive and its use in their foraging strategies is discussed.

**Keywords** Associative learning · Proboscis extension response · Operant response · *Tetragonisca angustula* · Stingless bee

## Introduction

Within the Apidae family, there are two tribes that comprise the eusocial bees: *Apini* and Meliponini (Michener 2000). The *Apini* tribe includes the honeybee *Apis mellifera*. These bees have been the focus of numerous studies not only because of their intrinsic characteristics as a social species with sophisticated communication and learning skills (von Frisch 1967; Seeley 1995), but also as a successful model to study high-order cognitive functions in a relatively small and simple brain (Menzel 1999; Giurfa 2007). The Meliponini tribe includes the stingless bees, which comprise several hundred species distributed through more than 36 genera (Camargo and Pedro 1992; Michener 2000). This diversity is manifest not only in the number of genera and species, but also in the wide spectrum of behaviors they display regarding their feeding habits (Kerr 1960; Biesmeijer and Slaa 2004). They present varied foraging strategies (Johnson 1983) combined with diverse communication behaviors for information transfer either intra or inter-specifically (Nieh 2004; Hrncir et al. 2006; Barth et al. 2008; Hrncir 2009; Jarau 2009, Slaa and Hughes 2009). This makes the stingless bees a particularly interesting group to study communication and learning because of the comparative options they offer both within their group and also with the closely related *A. mellifera*.

Among the stingless bees, *Tetragonisca angustula* presents a particularly wide spread distribution (Schwarz 1938). In the American continent, they are found from southern Mexico to the northern part of Argentina (Camargo and Pedro 1992; Moure 2008; Yáñez-Ordóñez et al. 2008). They have been catalogued as poor recruiters (Lindauer and Kerr 1958; Aguilar et al. 2005) and as solitary foragers (Johnson 1983) that rely mainly on intrinsic information (Biesmeijer and Slaa 2004), but lack sophisticated

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communication strategies to inform a food source's location (Slaa et al. 2003). Some of the cues they use in the foraging context are the presence of a nest mate at a food source (local enhancement, Slaa et al. 2003), the chemical cues deposited by co-specifics (nest mates or not) around or near to the feeding site (Villa and Weiss 1990) and visual information learned during previous foraging trips (familiar landmarks, Villa and Weiss 1990).

In other Neotropical bee species, the floral odors are known to play an important role during foraging (Roubik 1989; Michener 2000). This olfactory information can be picked up either during foraging trips (Biesmeijer and Slaa 2004) or within the hive as the scent of the food brought in by a successful nest mate (von Frisch 1967; Farina et al. 2005; Mc Cabe and Farina 2009). To use these olfactory cues, the bees must learn the association between the sensory stimulus (odor) and food reinforcement. This kind of association has been studied in standardized laboratory conditions in several insect species using different procedures.

In search of a suitable approach to study associative learning in *T. angustula* bees, we begun with a free-flying choice procedure. This protocol enables the bees to perform in a natural environment and provides qualitative information about the putative effect of olfactory experiences on subsequent foraging decisions. Because their choice behavior was affected by the ingestion of scented food, we used the proboscis extension response (PER) protocol to assess these bees' olfactory learning abilities. The PER classical conditioning is the best standardized associative protocol used with various insect species (honeybees: Takeda 1961; bumble bees: Laloi et al. 1999; flies: Tully and Quinn 1985; Chabaud et al. 2006; mosquitoes: Tomberlin et al. 2006; cockroaches: Watanabe et al. 2003; moths: Daly and Smith 2000; hymenopterous parasitoids: Kaiser et al. 2003), and it has recently been adapted for the stingless bee *Melipona quadrifasciata* (Mc Cabe et al. 2007; Mc Cabe and Farina 2009).

Our results could have implications not only determining the learning abilities of a non-conventional model species to study these issues, but also to deepen the knowledge of the social organization of stingless bees during resource exploitation.

## Materials and methods

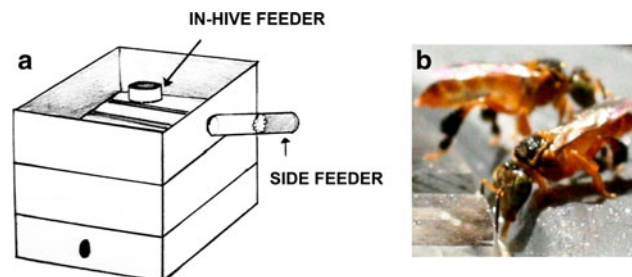
The experiments were performed with the native stingless bee *T. angustula*. The experimental hive was brought to the laboratory from the province of Misiones located on the northeast of Argentina in December 2005. The hive was kept inside the laboratory with a plastic tube through the wall that allowed the free circulation of the bees to the out-

side. During the winter season, it was kept warm by means of an electric pillow surrounding the nest. To provide sucrose solution as food during the winter and to introduce scented food during the experiments, we used two types of feeder. An in-hive feeder consisted of a plastic cup with a capacity of 20 ml that was placed inside the hive (Fig. 1) and contained small wooden sticks to prevent the bees from drowning in it. We also used a side feeder that consisted of a transparent plastic test tube inserted laterally on one of the hive's walls (Fig. 1). This feeder had a cotton cap at the opening of the tube to allow the bees to drink the sucrose solution from the cotton and avoided spilling inside the hive. This side feeder had the advantage that we could see the bees that were drinking the sucrose solution and allowed us to remove the tube and isolate those particular bees. Both feeders were placed at the upper part of the hive, over a wooden floor made of rectangular tables that were 1 cm apart from one another to allow the bees to go through them and access the feeders. The experiments were performed during the summer seasons (January–March 2007, 2008 and 2009) at the experimental field of the University of Buenos Aires (34°32'S, 58°26'W).

## Influence of olfactory appetitive experiences in the bees' food choice

### Training

*Tetragonisca angustula* bees were trained to forage at an artificial ad libitum feeder, consisting of an acrylic cup (6.5 cm high, 4 cm diameter) inverted over a Plexiglas plate 10-mm thick, 5.5 cm diameter, and with 16 grooves (1 × 1 × 10 mm) cut in a radial arrangement that contained 50% w/w unscented sucrose solution (Fig. 1). This feeder was placed on a photographic tripod at the hive's entrance at midday and, as the bees began to feed, was gradually moved further away until its final location 3 m away from the entrance. Once this location was reached, the bees were allowed to fly back and forth between the hive and the feeder for 30 min and feed freely. This period was equivalent



**Fig. 1** **a** Schematic drawing of the placement of the in-hive feeder and side feeders in the hive. **b** *Tetragonisca angustula* bees at an ad libitum feeder outside the hive

to 10 foraging trips approximately according to the previous observations.

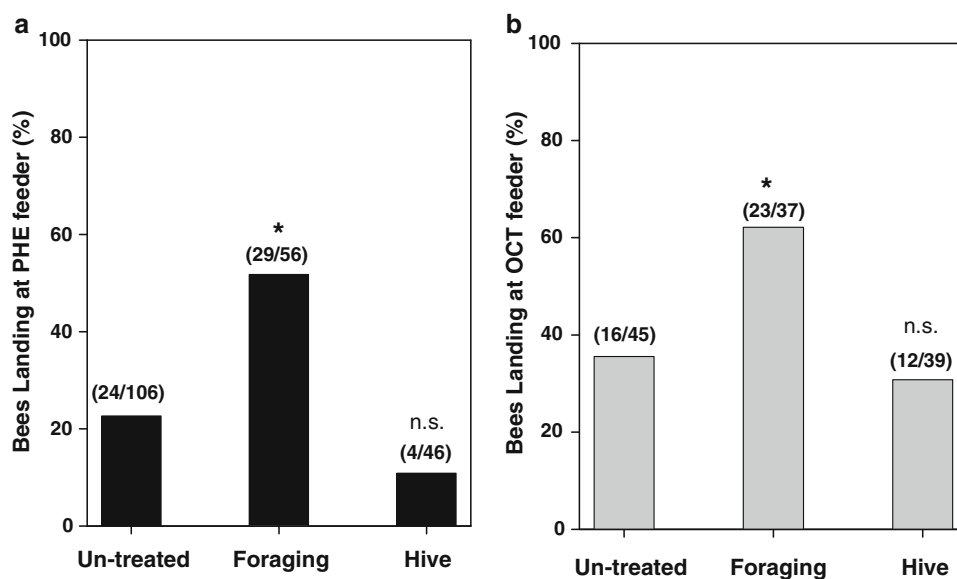
### Spontaneous odor choice

Once the bees had fed freely for 30 min from the unscented sucrose solution, the training feeder was removed and a Plexiglas transparent rectangular plate of  $20 \times 45$  cm was placed on top of the tripod. Two clean identical feeders similar to the one used for the training phase were placed on each end of this plate. Each feeder contained 50% weight/weight unscented sucrose solution and a  $2 \times 2$  cm filter paper attached to the top imbibed with 50  $\mu$ l pure odor: in series (1) the odors used were Linalool (LIO) and phenylacetaldehyde (PHE) and in series (2) we used 2-octanol (OCT) and nonanal (NON). All four odors are natural components of flower scents (Knudsen et al. 1993) and were obtained from Sigma-Aldrich, Steinheim, Germany. For honeybees, the odors in each pair are known to have similar intrinsic salience and they show low generalization between those odors indicating they can effectively discriminate between them (Sandoz et al. 2001; Guerrieri et al. 2005). Bees were allowed to choose and land on the feeder device for 5 min. As soon as they landed they were captured with plastic tubes. Bees that landed on the feeder while another nest mate was present were captured and left aside, but were not considered because they could have been guided by it as a visual cue during landing (Slaa et al. 2003). After these 5 min, the feeders were switched from

one end to the other of the Plexiglas plate to avoid any side biases due to light or any other visual cue. After another 5 min of bees' capture, the test feeders were removed and the training feeder was replaced in a central position for another 30 min between trials so as not to loose potential foragers. The choice feeders were alcohol cleansed to avoid the bees' footprints or any other pheromone mark to interfere with the following 10 min of choice offer. All the bees captured at the test feeders were killed to avoid recording the same bee's choice more than once.

### Olfactory experiences in a foraging context

To assess the influence of prior appetitive olfactory experiences during foraging, we allowed the bees to drink scented sugar solution at an ad libitum feeder outside the hive. We trained the bees as described before to a central feeder offering unscented food located 3 m away from the hive. However, once this position was reached, the feeder was cleaned and filled with 50% w/w scented sucrose solution. During the spontaneous odor choice, when the bees had collected unscented sugar solution, *T. angustula* bees had shown preference for the LIO (in series 1) and NON (in series 2) scented feeders (Fig. 2). Therefore, we offered sugar solution scented with the odors to which they had shown the lower preference to see whether we could bias their foraging choice after ingesting scented food. In series (1), the solution was scented with 50  $\mu$ l of PHE per liter of sugar solution and in the series and (2) the sugar solution



**Fig. 2** Choice behavior in a dual food choice device. **a** Proportion of bees that landed on the PHE-scented feeder from the control group (un-treated), from the group that had ingested PHE-scented solution during foraging (foraging) and from the group that had ingested PHE-scented solution at an in-hive feeder (hive). **b** Proportion of bees that

landed on the OCT-scented feeder from the control group (un-treated), from the group that had ingested OCT-scented solution during foraging (foraging) and from the group that had ingested OCT-scented solution at an in-hive feeder (hive). Asterisks indicate statistical differences from the control group, *G* test (\**P* < 0.025, see “Results” for details)

was scented with OCT 50  $\mu\text{l/l}$ . They were allowed to drink the scented sugar solution for 30 min, which is the equivalent of approx 10 feeding trips, and marked during that time. Then, they were offered the choice between two clean feeders as described before, i.e. filled with unscented solution and with filter papers with the respective pure odors for 10 min.

#### *Olfactory experiences inside the hive*

To study whether the bees' odor choice could be affected by the olfactory experiences perceived in a social context, we placed plastic cups (approx. 20 ml) filled with scented sucrose solution inside the upper part of the hive (in-hive feeder, Fig. 1). The solution was placed 2 days before starting the experiment to allow circulation of the scented food. In this way, the bees could get in contact with the scented food either by direct ingestion or by its distribution through trophallaxis between nest mates as it happens in other social bees (Farina et al. 2005; Mc Cabe and Farina 2009). The feeder was refilled each day to ensure a constant supply of scented sucrose solution for as long as the data collection took place (between 3 and 4 days for each of the series). The series with PHE inside the hive was performed in January 2009 and the series with the OCT stimulation was performed in March 2009.

To assess the bees' choice, they were evaluated following the same protocol as the one described before, i.e. training to a central un-scented feeder and then the choice between two scented feeders.

#### *Olfactory classical conditioning of proboscis extension*

To study associative learning in honeybees, the best standardized and simple procedure is the classical Pavlovian conditioning of proboscis extension (Kuwabara 1957; Takeda 1961). In this protocol the reflex response of extending the proboscis, which is released after contacting the subject's antennae with a sugar solution (Frings 1944), can be associated with a neutral stimulus, such as an odorant (Takeda 1961; Bitterman et al. 1983) after successively pairing this stimulus with the reward. This learning assay allows a very precise control of the olfactory stimulation and of the individual's response.

#### *Bees' capture and harnessing*

To capture the bees to perform the differential conditioning, we placed a small amount (2 ml) of unscented sucrose solution in the side feeder (Fig. 1). Placing such a small volume of food made the cotton cap remain at the bottom of the tube and the bees had to walk inside of it to access the sucrose solution. This allowed us to remove the tube gently

and have a variable number of bees (around 20) to work with. These bees were placed individually in small plastic tubes and allowed to rest for at least 40 min. To perform the PER classical olfactory conditioning (Takeda 1961; Bitterman et al. 1983) subjects needed to be 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). Then, they were placed in an incubator (28°C, 55% relative humidity, darkness) and allowed to rest for at least 2 h until they presented the unconditioned response (UR), i.e. the reflexive extension of the proboscis after applying a 50% w/w sucrose solution onto the antennae.

#### *Differential PER conditioning*

Only bees that showed the UR were used for the conditioning. Each bee was placed between the airflow generated by an air pump (25 ml/seg) and an extractor that removed the air. In this way, we ensured a gentle continuous flow of clean air. This bee species did not present any PERs to the mechanical airflow stimulus. To apply the odorant stimulus, the air pump flux was redirected by means of an electronic valve to pass through a syringe with a filter paper imbibed in 4  $\mu\text{l}$  of odor. We used two natural components of flower scents (Knudsen et al. 1993): LIO and PHE. The bees were presented with the odors either rewarded with 50% w/w sucrose solution (rewarded conditioned stimulus, CS+) or non-rewarded (non-rewarded conditioned stimulus, CS−) five times each in a pseudo-randomized order (CS−, CS+, CS+, CS−, CS−, CS+, CS−, CS+, CS+, CS−). The intertrial interval lasted for 10–15 min between CS presentations depending on the number of bees being trained. Each trial lasted for 46 s and consisted of 20 s of airflow, 12 s of odor (CS) and 14 s of airflow. We chose to offer 12 s of olfactory stimulation assuming this duration could improve the bees' learning performance based on the studies with bumblebees (Laloi et al. 1999) and *Scaptotrigona* stingless bees (Mc Cabe et al. 2007). During rewarded trials, the sugar solution (unconditioned stimulus, 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 when the bee responded fully protruding its proboscis during the first 9 s of odor presentation without the need of touching her antenna with the sucrose solution. Bees that responded to the first presentation of the CS (spontaneous response, SR) were left apart and did not continue with the following PER conditioning protocol allowing all the experimental bees to start from the same baseline. The test phase consisted on the non-rewarded presentation of both odors 15 min after

the end of the conditioning: odors were presented randomly; some individuals received first LIO and then PHE and others the other way round.

### Olfactory pre-stimulation

Our aim was to study the influence of previous olfactory experiences in the learning performance at the PER setup in *T. angustula* bees. To do so, we placed 1.5 ml of scented sugar solution (50  $\mu$ l of scent, either LIO or PHE/l sugar solution) at the side feeder and allowed the bees to feed for 1 h. After that the feeder was separated from the hive and the bees that were walking inside of it were harnessed as previously described. Owing to the small size of this species (body length 4 mm, Schwarz 1938) that complicated its manipulation and the fact that the bees were collected at a feeder directly connected to the hive, we had no possibility of individually marking the experimental individuals. However, capturing the subjects at a feeder containing scented sugar solution allows assuming these bees had had contact with the odor diluted in the food. In honeybees, it has been described that the SR is well correlated with the previous olfactory experiences of the subjects (Gerber et al. 1996; Sandoz et al. 2000; Farina et al. 2005; Grüter et al. 2006; Arenas and Farina 2008). Because we practically did not observe any SRs to the odors, we performed the differential conditioning protocol to analyze if the influence of the previous experiences could be detected in the bees' learning dynamics (Chandra et al. 2000, Farina et al. 2005; McCabe et al. 2007, Fernández et al. 2009). We established four experimental groups: bees fed with LIO scented sugar solution that went through the differential conditioning with LIO as CS+ or with PHE as CS+, and bees fed with PHE scented sugar solution with each of the odors as CS+ as well.

### Statistical analysis

To compare the odor choices during the free-flying choice experiment, we used a *G* test of goodness of fit in each of the experimental series to compare the choice of naïve bees with the choice of the bees that had ingested scented sucrose solution either during foraging or at the in-hive feeder. Multiple comparisons were performed using the Dunn–Sidak correction (Sokal and Rohlf 1995).

Performance during the classical olfactory 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 in each group, an 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). The repeated measures were the successive training trials. If we detected statistical differences in this analysis, we carried out Tukey's HSD tests to detect differences between the trials. Regarding the testing phase, we compared the proportion bees that responded to the rewarded odor and to the non-rewarded one by means of Fisher's exact test.

## Results

### Influence of olfactory appetitive experiences in the bees' food choice

The idea of performing this experiment was to find out if free-flying *T. angustula* bees could associate a floral odor and a sugar reward either during foraging or during the circulation of scented food inside the hive that could later on bias their subsequent food choices. The data from the experimental series (1) were collected in different years: we had a control group for the spontaneous choice between LIO and PHE and the stimulation at the outside feeder done in 2008 and another control group and the stimulation inside the hive done in 2009. Because both control groups showed no statistical differences (Fisher's exact test, two tailed,  $P = 0.818$ ), we grouped them together and compared both pre-stimulation treatments with that control group only, in the same way that we compared the data of the experimental series (2). When naïve bees were offered a free choice between an LIO or a PHE scented feeder, only 23% of the bees went to the PHE-scented one (Fig. 2, left panel). To see if the bees' spontaneous preference could be biased after ingesting PHE-scented sugar solution during previous foraging trips, we fed a group of bees with PHE-scented solution at an outdoor feeder. Their choice of the PHE-scented feeder increased to 53% (*G* test:  $G_h = 23.624$ ,  $P < 0.05$ ,  $N = 208$ ; multiple comparisons: control vs. PHE at feeder,  $\alpha' = 0.025$ :  $G_h = 13.85$ ,  $P < 0.001$ ). However, when the PHE-scented food was located inside the hive, the bees' choice did not show statistical differences from the control group (multiple comparisons: control vs. PHE inside the hive,  $\alpha' = 0.025$ :  $G_h = 3.13$ ,  $P = 0.076$ ).

To make sure, this results were not a specific effect of this particular set of odors, we chose a second pair of odors, OCT and NON, and repeated the experiment. Here, we observed that OCT was the least preferred odor for the control group (Fig. 2, right panel). As seen with the other pair



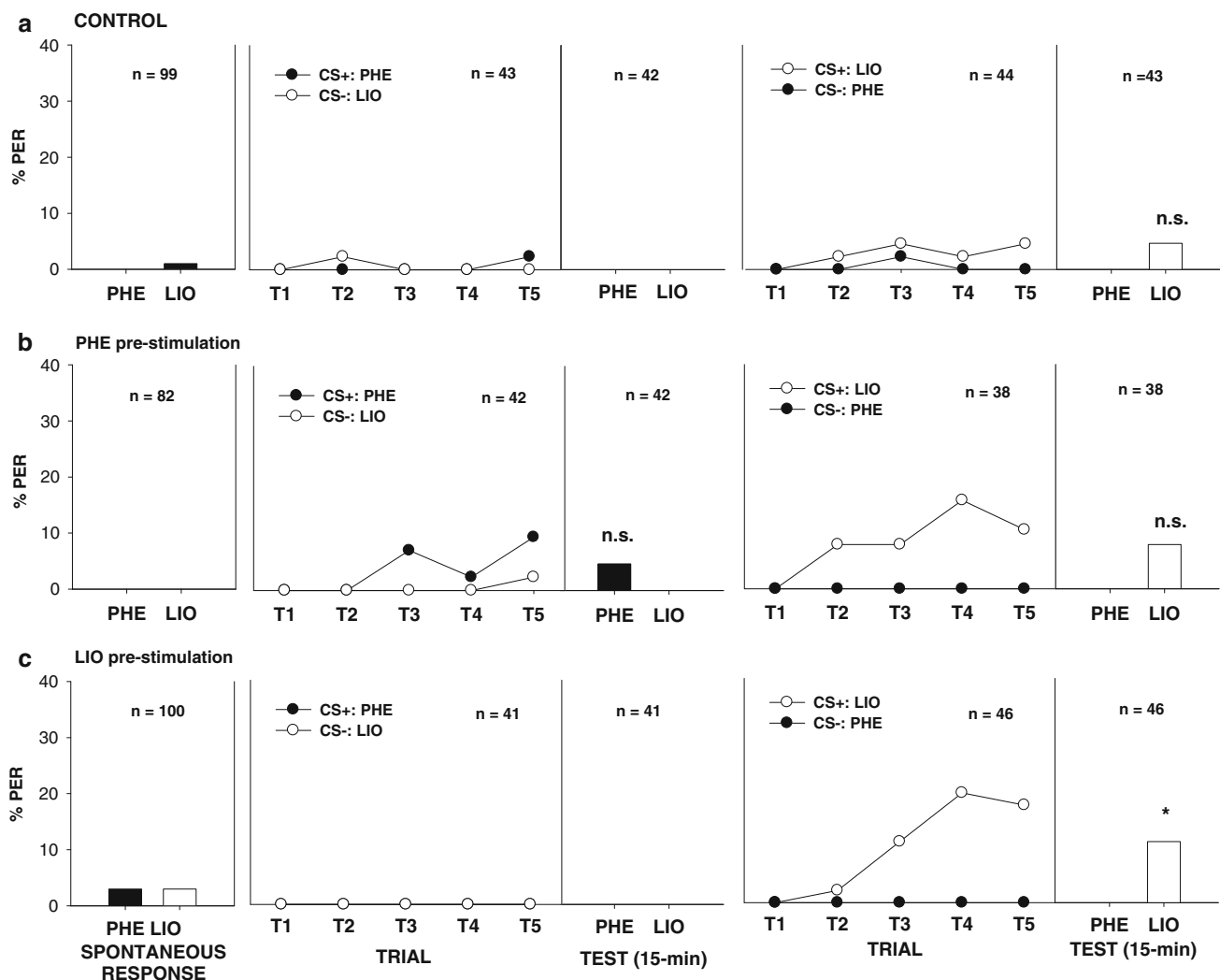
of odors, when the bees ingested the OCT-scented solution at the outdoor feeder during foraging they increased their choice of that odor, but their preference was unchanged when the scented solution was offered inside the hive ( $G$  test:  $G_h = 8.94$ ,  $P < 0.05$ ,  $N = 121$ ; multiple comparisons: control vs. OCT at feeder,  $\alpha' = 0.025$ :  $G_h = 5.83$ ,  $P < 0.025$ ; control vs. OCT inside the hive,  $\alpha' = 0.025$ :  $G_h = 0.22$ ,  $P = 0.639$ ).

### Classical PER conditioning

To perform a classical PER conditioning with *T. angustula*, the first requisite is to show that bees present a clear PER. Of the 407 harnessed bees, 69% of them showed the reflexive response of proboscis extension when their antennae were contacted with a 50% w/w sucrose solution. These

responding bees were used to perform the olfactory differential conditioning. In this protocol, the first time, an odor is presented and the bee responds protruding her proboscis is considered as a SR. This response is related to previous olfactory experiences. We hardly recorded any SR. Only one of the 99 bees of the control group responded to the odors spontaneously (Fig. 3a, left panel). In both other pre-stimulated groups, even though they had had previous encounters with the odors at the side feeder, only five of the LIO fed bees showed some SRs, and they responded indiscriminately to both the familiar (LIO) and the novel (PHE) odor (Fig. 3c, left panel).

With bees from the three groups (control, PHE pre-stimulation and LIO pre-stimulation), we performed a differential conditioning assay with PHE as the rewarded conditioned stimulus (CS+) and a second conditioning



**Fig. 3** 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 side

feeder offered unscented sucrose solution, **b** PHE pre-stimulation: a side feeder offered sucrose solution scented with phenylacetaldehyde, PHE, **c** LIO pre-stimulation: a side feeder offered sucrose solution scented with Linalool, LIO. Asterisks indicate statistical differences in Fisher's exact test, one tail (\* $P < 0.05$ , see "Results" for details)

procedure using LIO as CS+. In the first case (CS+ = PHE, Fig. 3 middle panel) none of the groups showed any significant discrimination throughout the five conditioning trials (repeated measures ANOVA: control with PHE as CS+:  $F = 1.25$ ,  $df = 4$ ,  $P = 0.289$ ; PHE pre-stimulation with PHE as CS+:  $F = 1.67$ ,  $df = 4$ ,  $P = 0.160$ ; LIO pre-stimulation PHE as CS+: we did not record any CRs). On the other hand, when LIO was CS+ (Fig. 3 right panel), we found that the bees that had had some previous olfactory experiences could now discriminate between the rewarded and non-rewarded odors (repeated measures ANOVA: control with LIO as CS+:  $F = 0.49$ ,  $df = 4$ ,  $P = 0.739$ ; PHE pre-stimulation with LIO as CS+:  $F = 2.43$ ,  $df = 4$ ,  $P = 0.049$ ; LIO pre-stimulation LIO as CS+:  $F = 4.47$ ,  $df = 4$ ,  $P = 0.002$ ). For the LIO pre-treated group, both the fourth and fifth trials of the conditioning showed higher discrimination levels than the initial trial (Tukey's HSD test). The PHE pre-stimulation group showed higher discrimination in the fourth trial than in the first trial (Tukey's HSD test).

These results indicate that when LIO was the odor being associated with a sugar reward during the conditioning and the bees had had previous access to scented food, even though the final level of discrimination was low, the bees did indeed discriminate between the odors and responded more to the rewarded one. Apparently, the effect of the pre-stimulation was not specific since having contact with scented solution either with PHE or with LIO had a similar effect of enhancing the discrimination of LIO as the rewarded odor.

During the test phase, that is the non-rewarded presentation of both odors 15 min after the conditioning protocol, only the bees with LIO pre-stimulation that had a protocol with LIO as CS+ during the conditioning showed a specific higher response to that odor instead of towards the non-rewarded one (Fisher's exact test, one tail: control hive with LIO as CS+:  $P = 0.247$ ; PHE-treated hive with LIO as CS+:  $P = 0.120$ ; PHE-treated hive with PHE as CS+:  $P = 0.247$ ; LIO-treated hive with LIO as CS+:  $P = 0.028$ ; Fig. 3, right panel).

## Discussion

This study shows that *T. angustula* bees can use the information of the food odor they had previously collected to bias their choices later on, which implies associating that odor with food and remembering that association in subsequent foraging trips. However, when the scented food was placed inside the hive, the bees' choice was unbiased. When they performed a complex task such as a differential classical conditioning they initially showed no responses to the rewarded odor, but when the bees had access to the odors in an appetitive context prior to the learning assay, their

performance improved. This is a first step to clarify *T. angustula*'s individual use of olfactory information, but there are still questions about the use of social information that need to be studied in more detail to unveil this species use of food odor during foraging decisions.

## Effect of previous olfactory experiences on food choice

Foraging animals make several decisions regarding food obtainment based on the information acquired in different contexts. The one source of information that is common to any one of them is the individual itself, that is, its spontaneous preferences and memory of previous events (Biesmeijer and Slaa 2004). During the food choice experiment, inexperienced bees tended to bias towards one of the two odors offered simultaneously. This initial tendency, however, was susceptible of being modified. After several foraging bouts collecting sugar solution scented with the "least preferred" odor, the bees showed a higher proportion of choice of that odor as compared to the naïve individuals. This means that successful previous foraging experiences can be taken into account when deciding the choice between different food sources.

Besides, the information about their own previous foraging success, honey bees are known to use the food odor information acquired inside the hive to choose among food sources (Free 1969; Arenas et al. 2007, 2008). Bumble bees have also been described to bias their choices in the foraging context influenced by the odor both brought in by a successful forager and introduced inside the honey pots (Molet et al. 2009). In our study, however, we found that placing scented food inside the hive was not enough to bias *T. angustula* bees' initial preference.

## Classical conditioning in the PER protocol

The PER classical conditioning is a standard learning assay commonly used to study honey bees' and bumble bees' associative learning abilities (Bitterman et al. 1983; Laloi et al. 1999) and it has also been successfully used with stingless bees (*Melipona quadrifasciata*, Mc Cabe et al. 2007). However, it is a protocol specially tailored for honeybees (Toda et al. 2009). Both bumble bees and stingless bees show low final discrimination values as compared to honeybees that reach levels of 70–100% PER after a single conditioning trial (see Bitterman et al. 1983; maximum 44% bumble bees: Laloi et al. 1999 60% stingless bees: Mc Cabe et al. 2007). In this study, we show that inexperienced foragers of *T. angustula* were not able to discriminate between a rewarded and a non-rewarded odor in this procedure. They hardly showed any conditioned responses at all. However, when these bees had had previous contact with a scented sugar solution inside the nest, their discrimination

levels were still very low (maximum 20%), but could now be detected, suggesting that prior olfactory experiences within the social context can improve the learning performance of *Tetragonisca*.

There were asymmetries in the discrimination performance with both odors: bees were able to discriminate LIO as the CS+, but not PHE. A similar asymmetry has also been shown in bumble bees which discriminate LIO much better than they do PHE (Laloi and Pham-Delegue 2004). This higher salience of LIO could be related to the fact that even though both LIO and PHE are typical components of floral odors, LIO appears to be more common in the mellifera flora (Knudsen et al. 1993). Other authors have proposed that LIO could have intrinsic communication value as a pheromone (Sandoz et al. 2001; Laloi and Pham-Delegue 2004). In fact *T. angustula* bees showed a clear spontaneous preference to LIO when having to choose between LIO and PHE during our food choice experiments: nearly 80% of the inexperienced foragers landed on the LIO scented feeder. The differences between LIO and PHE were also evident when the bees were offered scented food before the differential conditioning assay. The discrimination of LIO as CS+ only became evident after the bees had ingested scented sugar solution at the side feeder. However, the effect of this previous contact was not the same for both food odors: after the stimulation with PHE-scented sugar solution the discrimination of LIO became scarcely significant ( $P = 0.049$ ), but was lost at the test phase. Conversely, when it was LIO itself that the bees ingested before the conditioning with LIO as CS+, not only they clearly discriminated between the rewarded and non-rewarded odors during the training protocol, but they also showed a higher specific response to LIO at the test phase. This could be an indication that the food odor not only has a general unspecific effect, but that a specific association could be established inside the nest between the food and its scent and this association can later on be evoked in the PER paradigm. This phenomenon of olfactory information transfer between the context where the information is acquired and the context where that same information is recalled has been described for the stingless bee *Melipona quadrifasciata* (Mc Cabe and Farina 2009) and also in honeybees (Gerber et al. 1996).

#### Food/information distribution inside the nest

When a bee returns from a successful foraging trip, she unloads the nectar. Honeybees unload it to other nest mates who are in charge of nectar processing and also share short trophallactic contacts with other foragers (von Frisch 1967; Farina 1996; De Marco and Farina

2003; Grüter et al. 2008; Farina and Grüter 2009). These contacts within the hive have proven to transfer the food's odor to the receiver bee (Farina et al. 2007) and allow rapid transfer of olfactory information throughout the whole colony (Grüter et al. 2006). In bumblebees, the crop unloading is done directly into the honey pots by the foraging bee (Dornhaus and Chittka 2005). These honey pots are the source of the olfactory information stored inside the colony: a bumble bee probes the nectar contained in them and then goes out to forage (Dornhaus and Chittka 2005). There is not a detailed description for *T. angustula* of the food circulation dynamics inside the nest. They do indeed perform trophallactic contacts (Ferreira Grosso and Bego 2002), but the rest of the food processing circuit has not yet been described. We had three kinds of feeders. Both at the side feeder (before the PER conditioning) and at the foraging feeder outside the nest, the individuals had direct contact with the scented food. In both these cases, we saw an effect of the previous olfactory experiences. On the other hand, when we placed the scented food at the in-hive feeder, we assumed it would circulate through all the nest mates, either by direct feeding or by trophallaxis. In this case, the bees' choice behavior was unaffected by this olfactory experience. The in-hive feeder was placed in the upper part of the colony, above the brood cells. It is possible that the group of bees in charge of taking the sugar solution and processing it to place it in the honey pots did not interact directly with the bees that were in charge of foraging outside the nest. In this way, it could be that the foraging bees did not have previous contact with the scented food inside the hive, and that is why their food choice was unbiased. When scent is placed inside bumble bee nests not paired with food, it has almost no effect on the bees' choice (Molet et al. 2009). In the LIO versus PHE series, there is even a small tendency to avoid the feeder with the scent that had been placed inside the hive. There is evidence in honeybees (Arenas et al. 2008) that the worker bees that perceive an odor not paired with food tend to avoid that scent in a food choice device.

#### Foraging strategies

The food source's odor is one of the most common sources of information for foraging bees. Among the stingless bees, there are some species to which the food odor is the only cue used to find the food source in their random search, others rely on recruitment cues (mainly chemical cues deposit near or at the feeding site) and only use the food odor in the final recognition of the food source or when some of the cues are absent (Aguilar 2004). When *T. angustula* bees return from a successful



foraging trip they display a general alerting behavior conformed by random zig-zag running and sound production (Kerr 1969; Aguilar 2004). They are solitary foragers (Johnson 1983) that do not show successful recruitment activity measured as an increase of nest mates at a rich food source (Aguilar et al. 2005). Taking this into account, it is likely that they rely more on their own individual prior experiences than on the information provided by nest mates. This could explain why only the direct contact with the scented food had some effect on their subsequent choice behavior and not the circulation of scented food inside the hive. However, the use of self-acquired information appears as extremely important regardless of the possession of sophisticated communication strategies: Honey bees, who have the most sophisticated and finely tuned communication channel among insects (the waggle dance, see von Frisch 1967) may ignore occasionally the information acquired inside the nest from their nestmates' waggle dance and favor their own previous experience with food odors in their search for food (Grüter et al. 2008).

Another trait to be considered is that the food's scent gains importance in the final approach to the food source inducing the bee to land on the flower (von Frisch 1914). *T. angustula* bees are known to leave strong pheromone marks on the food sources that promote the bees' choice, even if it contradicts the individuals' previous experience with the food source's color (Villa and Weiss 1990). In this sense, it could be possible that the food odor may be not particularly relevant to these bees. Besides, the fact that the PER protocol is not perfectly suited for small stingless bees (Mc Cabe et al. 2007 this study), this could be an additional reason for their poor performance in the PER protocol. Similar results have been seen in other stingless bees: *Scaptotrigona aff depilis* bees, who leave strong pheromone marks along the path to the food source during foraging (Lindauer and Kerr 1958; Schmidt et al. 2003), are unable to learn to discriminate floral odors in the PER protocol (Mc Cabe et al. 2007). On the other hand *Melipona quadrifasciata* bees, who have been described to leave pheromone marks at the food source but also provide information about the food source's distance to the hive and direction (Jarau et al. 2000), appear to perform better in this protocol (Mc Cabe et al. 2007).

Stingless bees are especially interesting because of their diversity of foraging and recruitment strategies within the social bee species (Kerr 1969; Michener 2000). The incorporation of *T. angustula*, which has a broad distribution in the American continent, as a putative model to perform quantitative studies in this behavioral framework, opens a promising field for comparative research on learning in Neotropical social bees.

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