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SHORT COMMUNICATION

Honeybees learn floral odors while receiving nectar from foragers within the hive

Walter M. Farina · Christoph Grüter · Luis Acosta · Sofía Mc Cabe

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Abstract Recent studies showed that nectar odors brought back by honeybee foragers can be learned associatively inside the hive. In the present study, we focused on the learning abilities of bees, which directly interact via trophallaxis with the incoming nectar foragers: the workers that perform nectar-receiving tasks inside the hive. Workers that have received food directly from foragers coming back from a feeder offering either unscented or scented sugar solution [phenylacetaldehyde (PHE) or nonanal diluted] were captured from two observational hives, and their olfactory memories were tested using the proboscis extension response paradigm. Bees that have received scented solution from incoming foragers showed significantly increased response frequencies for the corresponding solution odor in comparison with those that have received unscented solution. No differences in the response frequencies were found between food odors and colonies. The results indicate that first-order receivers learn via trophallaxis the association between the scent and the sugar solution transferred by incoming foragers. The implications of these results should be considered at three levels: the operational cohesion of bees involved in foraging-related tasks, the information propagation inside the hive related to the floral type exploited, and the putative effect of these memories on future preferences for resources.

Keywords Honeybees · Floral odorants · Foragers · Phenylacetaldehyde · Nonanal

W. M. Farina (✉) · C. Grüter · L. Acosta · S. Mc Cabe
Departamento de Biodiversidad y Biología Experimental,
Facultad de Ciencias Exactas y Naturales,
Grupo de Estudio de Insectos Sociales,
IFIBYNE-CONICET, Universidad de Buenos Aires,
Pabellón II, Ciudad Universitaria (C1428EHA),
Buenos Aires, Argentina
e-mail: walter@fbmc.fcen.uba.ar

C. Grüter
Division of Behavioural Ecology,
University of Bern,
Ethologische Station Hasli,
Wohlenstrasse 50a,
3032 Hinterkappelen, Switzerland

Introduction

Nectivorous insects, such as honeybees, use floral odorants to search for and identify food sources (von Frisch 1919). Odor cues present in nectar and pollen can be learned during the first foraging trips and help bees to return to the recently discovered feeding places (von Frisch 1967). These olfactory memories can be retained during several days (Beekman 2005), being retrieved either when bees fly in the close range of a known floral patch or inside the hive when scents of known food sources reactivate experienced foragers to resume collecting tasks (Ribbands 1954; Johnson and Wenner 1966).

Olfactory learning can also happen inside the colonies while the incoming scented nectar is shared among hive mates and through the food odor clinging on the returning forager's body (von Frisch 1967; Wenner et al. 1969; Farina et al. 2005; Grüter et al. 2006). Recent studies demonstrated that floral scents present in the nectar brought back by foragers can be learned by hive mates that later will be recruited to the advertised flower type (Farina et al. 2005; Grüter et al. 2006). In this social context, trophallaxis would be the most plausible mechanism by which the liquid food and its odors are associated. In fact, it is already known that associative learning occurs among caged honeybees through single mouth-to-mouth trophallactic contacts (Gil and De Marco 2005).

Olfactory conditioning could not only be relevant for the recruitment to specific floral species but also for the organization of foraging-related tasks within the hive. Accordingly, a recent study reported that the occurrence of a transfer of food with a given scent between nectar foragers and hive mates is not random but is affected by olfactory experiences made during previous food exchanges (Goyret and Farina 2005).

As nectar receivers initiate nectar distribution within the colony (Seeley 1995), the analysis of their learning abilities is crucial for understanding how chemosensory information related to the incoming nectar can be propagated at the social level. With this in mind, first-order nectar receivers that interacted with a group of trained foragers were captured, and their proboscis extension responses (PERs) to odors diluted in sugar solution were assessed in the laboratory. We also tested whether nectar receivers showed differences in their odor responses depending on their odor experience. Among the odors tested, we presented those diluted in the nectar previously brought back by foragers and which were experienced by receivers during trophallactic contacts.

Materials and methods

The experiment was performed at the end of the nectar flow season (February–April) at the experimental field of the University of Buenos Aires. We used two two-frame observation hives (henceforth: H1 and H2) containing a colony of about 3,200 European honeybees (*Apis mellifera ligustica*) each. Colonies had a queen, brood, and reserves.

Experimental procedure

A group of foragers was trained to collect a 2.0-M unscented sugar solution at a small plate feeder (about 8 cm diameter), placed at a distance of about 30 cm from the hive entrance for about 30 min. During the training period, foragers were marked with a colored spot onto the thorax. After this period, we offered at the feeder a solution having the same sucrose concentration and that was either unscented (day 1) or scented (days 2 and 9) for 60–90 min. During this period, we marked (with a new color) the hive bees that received the solution via trophallactic contacts for at least 5 s from the color-marked foragers (Fig. 1). This duration guarantees an effective passage of food during trophallaxis (Farina and Wainselboim 2001). To mark the receiver bees, we used a sliding acrylic wall that was partly covered with a mosquito screen, which allowed us to paint the receivers' thorax while they interacted with the marked incoming foragers. This device could be moved horizontally, from side to side, allowing us to scan the whole area of the exposed face of the hive. Afterward, the mosquito-screen piece of the sliding wall was replaced by an acrylic one (3×27 cm) with an opening in the center (2.5-cm diameter) that allowed the insertion of a suction tube to capture the marked receiver bees (Fig. 1c). This new sliding wall allowed us to move the opening in two dimensions. The capture of the marked hive bees, i.e., those that received food from marked foragers, lasted 30–45 min. After the capture, the bees were anesthetized with CO_2 and harnessed in plastic tubes (Fig. 1d), allowing the antennae and the proboscis to move freely (Bitterman et al. 1983). The bees were then kept in the dark (25°C, 55% relative humidity) for 1 h.

Odors used

Each colony was exposed to a solution-odor sequence: in H1, the unscented solution was presented on day 1, phenylacetaldehyde (PHE) in solution (all scented solutions contained 50 μl of pure odor per liter of solution) on day 2,

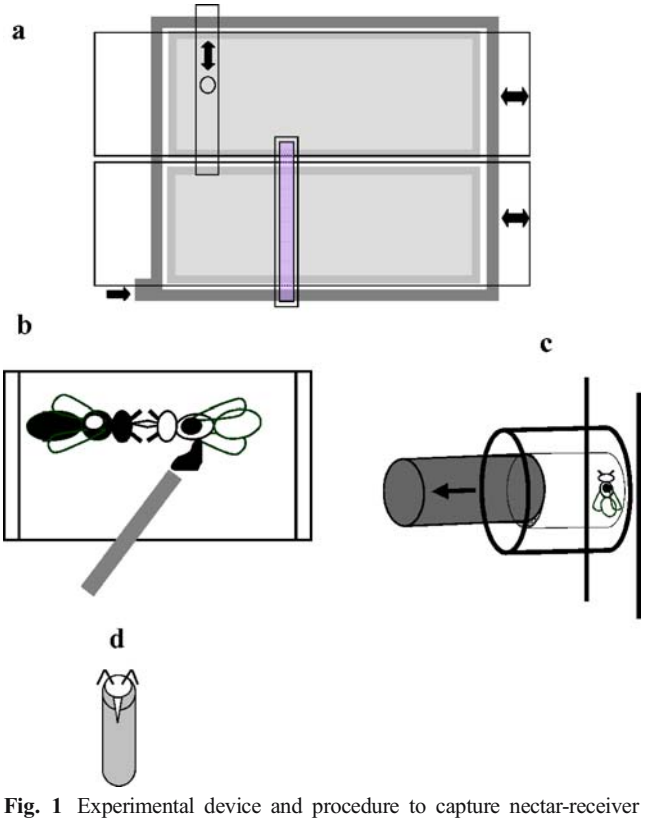


Fig. 1 Experimental device and procedure to capture nectar-receiver bees inside the hive to test their olfactory memories in a PER assay. **a** The experimental hive with its sliding acrylic walls. One of them (the lower one) was partly covered with a mosquito screen. This allowed us to paint the receivers' thorax (*white bee*) during the trophallaxis with a marked forager (*black bee*). **b** Afterward, the mosquito-screen piece was replaced by an acrylic one with an opening in the center [see the upper comb in (**a**)] that allowed us the insertion of a suction tube to capture the marked receivers (**c**). The captured receiver was then anesthetized and harnessed in plastic tubes, allowing the antennae and the proboscis to move freely (**d**)

and nonanal in solution on day 9; in H2, it was unscented solution on day 1, nonanal in solution on day 2, and PHE in solution on day 9. Three odors were presented in the PER paradigm: PHE, nonanal, and 2-octanol. The odors tested presented a similar carbon-chain length (eight or nine carbons) and relative low vapor pressures (Table 1). With this combination, we compared PER values to: (1) the same test odors for the different conditions and (2) the different solution odors. All odors used were natural flower compounds (Knudsen et al. 1993) and were obtained from Sigma-Aldrich, Steinheim, Germany.

PER testing

We tested the receiver PERs to the test odors. Bees that showed the unconditioned response (UR, the reflexive extension of the proboscis after applying a 1.0-M sucrose solution to the antennae) and did not respond to the mechanical airflow stimulus were used. For both colonies, the bees were allocated in equal numbers to the six possible odor sequences, e.g., one-sixth of the bees captured were tested in the sequence PHE–nonanal–2-octanol and the remaining five groups in the rest of the possible sequences.

The PER of the bees that had received unscented sugar solution gave us a general picture of spontaneous response frequencies for the test odors. Hive bees receiving scented solution were exposed to the solution odor collected by the forager mates (nonanal or PHE) and to the test odors (2-octanol or the alternative solution odor, either nonanal or PHE). After odor presentations, the bees were tested again for the UR, and bees not responding (less than 5%) were excluded from the analysis. The interval between the odor presentations lasted about 15 min. A device that delivered a continuous airflow was used for odorant application (for details of this setup, see the work of Guerrieri et al. 2005). Test trials lasted for 46 s. During the first 20 s, the bees received clean air followed by 6 s of odor stimulation and then 20 s of clean air again.

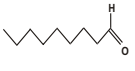
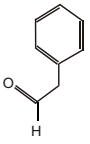
Statistics

We compared PER frequencies using log-linear models (Zar 1999). Interactions between PER frequencies, presence or absence of solution odors, and colonies were tested. We ran three models, one for each solution odor separately and one to compare the two solution odors. We adjusted alpha levels of the mutual independence tests for multiple comparisons using the Dunn–Sidak correction (Sokal and Rohlf 1981). All significant *P*-values remained significant after the correction.

Results

The percentages of responses for the three test odors in H1 are shown in Fig. 2a. From 49 bees receiving unscented solution in the hive, 4% responded only to PHE, 2% responded only to nonanal, and about 8% responded only to 2-octanol. From 22 bees receiving PHE solution in the hive, 32% responded only to PHE, and 5% responded only to nonanal. From 30 bees receiving nonanal solution in the hive, 33% responded only to nonanal, while 3% responded to the three test odors. Fig. 2b shows the percentage of responses in H2 to the same three odors tested in H1. From 37 bees receiving unscented solution in the hive, 8% of the bees responded only to PHE, and about 3% responded to nonanal. From 39 bees receiving nonanal solution in the hive, 26% responded only to nonanal, 3% responded only to PHE, and about 10% responded to more than one test odor. Seven days later, we used PHE in the solution. From

Table 1 Functional groups, chemical structures, carbon-chain lengths, and vapor pressures of the odors used in the experiment. The compounds are general floral odorants (after Knudsen et al. 1993)

Functional group	Compound	Structure	Carbon-chain length	Vapour Pressure (mm Hg; 25°C)
Aldehyde	Nonanal		9	0.37
Aldehyde	PHE		8	0.392
Secondary alcohol	2-Octanol		8	0.24

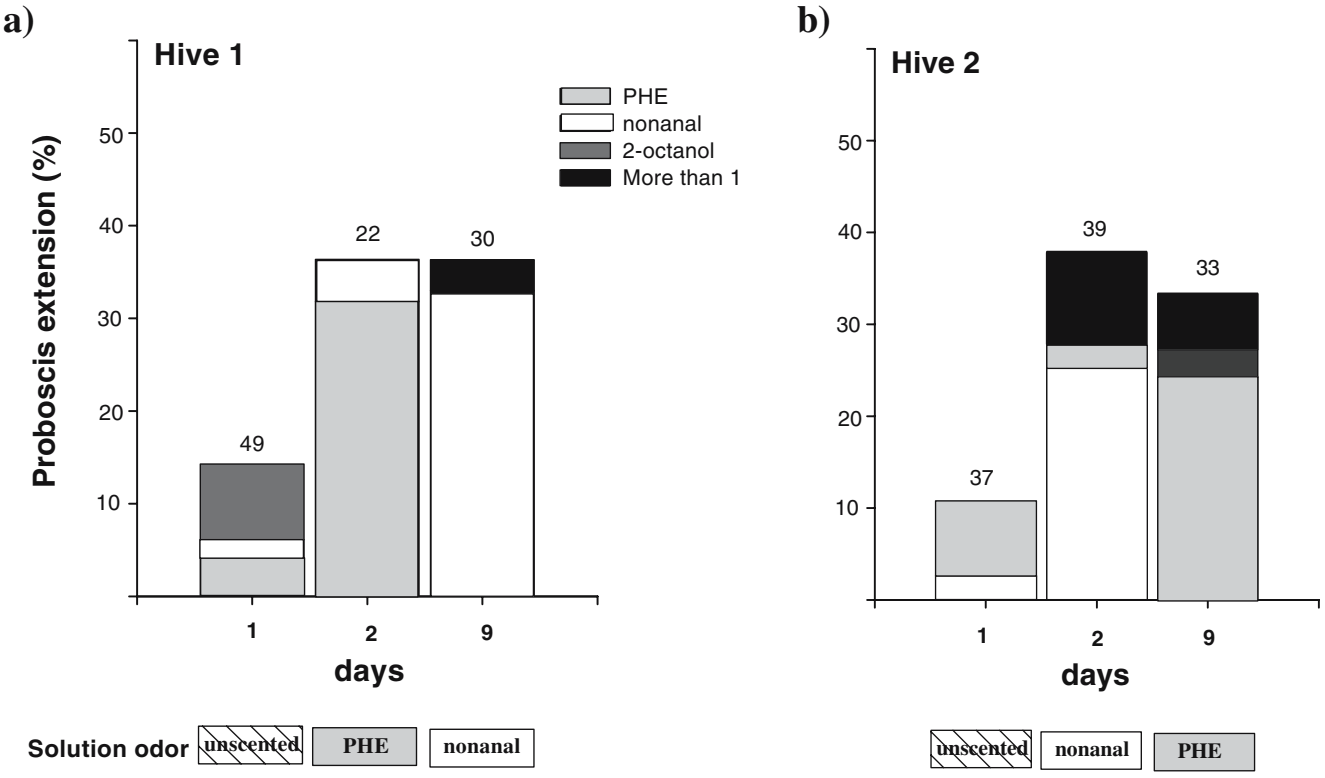


Fig. 2 Proboscis extension response (PER) percentages for food-receiver bees that extended the proboscis on the first presentation of an odor in two observation hives. **a** Responses from the hive 1 during the experimental period. Its corresponding odor condition was: unscented solution, PHE (phenylacetaldehyde) in solution, and nonanal in solution were collected by a group of trained forager mates at a feeder offered with a 2.0-M sucrose solution during days 1, 2, and 9, respectively. **b** Responses from the hive 2 during the experimental period. Its corresponding odor condition was: unscented solution, nonanal in solution, and PHE (phenylacetaldehyde) in solution were collected by a group of trained forager mates at a feeder offered with a 2.0-M sucrose solution during days 1, 2, and 9, respectively. A random presentation of test odors had been performed during the PER test (for details, see "Materials and methods"). Responses for PHE (*gray*), nonanal (*white*), 2-octanol (*dark gray*), and for more than one test odor (*black*). Number of tested bees *above bars*

33 bees receiving PHE solution in the hive, 24% responded only to PHE, 3% responded only to 2-octanol, and 6% responded to more than one test odor.

We tested interactions between PER frequencies (PER, variable 1) for PHE, the presence or absence of PHE in the previously collected solution (odor, variable 2), and the two colonies (hive, variable 3). A global test of mutual independence among the three variables using log-linear models revealed significant mutual dependence (G=17.42, df=4, N=141, P=0.002). Partial independence tests suggested that there is a significant interaction between the presence of odor in solution and the PER frequencies (odor vs hive and PER: G=17.14, df=3, P<0.001; PER vs hive and odor: G=13.53, df=3, P=0.004) but no effect of hive (hive vs odor and PER: G=4.88, df=3, P=0.18). This was tested using a two-dimensional contingency table (G test: G=12.53, df=1, P<0.001).

Similar results were found in the case of nonanal. After finding mutual dependence between the variables (G=28.87, df=4, N=155, P<0.001), we tested for partial independence. Again, the results suggested a significant interaction between the odor presence in solution and PER frequencies (odor vs hive and PER: G=27.87, df=3, P<0.001; PER vs hive and odor: G=25.07, df=3, P<0.001) but no effect of hive (hive vs odor and PER: G=3.32, df=3, P=0.35). A subsequent G test showed a significant effect of odor presence on PER frequencies (G test: G=24.55, df=1, P<0.001).

These results show that both odors present in the solution had a significant effect on PER frequencies, but that there was no difference in PER frequencies for the odors between the two hives. We then tested if the PER frequencies for PHE and nonanal were different when they were in the solution and if there were colony effects, but found no significant mutual dependence (G=1.06, df=4, N=124, P=0.90).

Discussion

Hive bees that received scented solution from incoming foragers showed a significant increase in PER for the corresponding solution odor compared to those that received unscented solution. These differences were found when foragers collected a scented solution, irrespective of

the identity of the odorant used. The lack of differences found between colonies also suggests that the order of food odor presentation did not affect the olfactory learning abilities for the different odor compounds diluted in the solution.

These results suggest that first-order receiver honeybees can learn via trophallaxis the nectar odor brought back by foragers inside the hive. The fact that this odor triggers the appetitive response (PER) of nectar receivers clearly shows an odor–reward association. It has already been reported that olfactory learning occurs within honeybee colonies (von Frisch 1967; Wenner et al. 1969; Farina et al. 2005); however, there was, until now, no direct evidence that effective food receivers learn the association between reward and its odor.

Our nectar receivers most likely received solution during one trophallactic contact (even though trophallaxes involving already marked receivers were observed in a few instances) which would be the equivalent of a single learning trial and tested 2 h later (corresponding to the temporal window of a medium-term memory, Menzel 1999). Although the PER values were significant for the odors received via trophallaxis, the responses found were lower than expected for single learning trials in laboratory studies (Menzel 1999; Gil and De Marco 2005). This could be explained by the effect of the dramatic change of context suffered by the hive bees after the capture (from the hive to the harnessing tubes in the laboratory; see Bouton and Moody 2004 for a review). A recent study showed that honeybees captured inside the hive presented increasing PERs with increasing foraging time at a scented food source (Grüter et al. 2006). Therefore, it is possible that the rather low response frequencies found in this study are due to the small amount of collected solution and/or the short time in which the hive bees were exposed to the scented food. Furthermore, the complex in-hive environment could lead to an unpredictable olfactory training, which is difficult to control experimentally.

The acquired olfactory information may be especially important at two different stages during adult life. First, because nectar receivers perform tasks inside the nest exclusively (Seeley 1995), the capability to learn the scent of the incoming nectar might affect decision-making of receivers once they return to the delivery area of the hive to unload new samples of fresh nectar. In this sense, it was recently reported that the probability of trophallactic interactions among incoming foragers and receivers is affected by olfactory experiences established during previous interactions inside the hive (Goyret and Farina 2005). Therefore, it is likely that the olfactory memories formed by receivers will affect the occurrence of subsequent trophallaxes with nectar foragers.

On the other hand, these memories may affect the behavior of bees once they become foragers. Foraging follows nectar receiving and processing tasks (i.e., receivers are normally younger than foragers; Seeley 1995). Because long-term olfactory memories can be established inside the hive (Farina et al. 2005), the olfactory information acquired by receivers is likely to cause preferences for food sources once these bees initiated foraging tasks.

In summary, hive bees can learn the contingency between odor and reward during unloading contacts with nectar foragers. This fact will be crucial for the olfactory information management at the social level. In the short-term, this capability will affect the operational cohesion of bees involved in foraging-related tasks and the propagation of olfactory information within the hive. In the long-term, it could affect putative preferences for resources once hive bees initiate foraging flights.

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