biology letters

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Biol. Lett. 2006 **2**, 98-100
doi: 10.1098/rsbl.2005.0361

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doi:10.1098/rsbl.2005.0361
Published online 18 October 2005

Apis mellifera bees acquire long-term olfactory memories within the colony

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Early studies indicate that *Apis mellifera* bees learn nectar odours within their colonies. This form of olfactory learning, however, has not been analysed by measuring well-quantifiable learning performances and the question remains whether it constitutes a 'robust' form of learning. Hence, we asked whether bees acquire long-term olfactory memories within the colony. To this end, we used the bee proboscis extension response. We found that within-the-nest bees do indeed associate the odour (as the conditioned stimulus) with the sugar (as the unconditioned stimulus) present in the incoming nectar, and that the distribution of scented nectar within the colony allows them to establish long-term olfactory memories. This finding is discussed in the context of efficient foraging.

Keywords: *Apis mellifera*; olfactory learning; proboscis extension response (PER); conditioning; nectar foraging

1. INTRODUCTION

Already Karl von Frisch (1946) has reported evidence indicating that *Apis mellifera* bees learn nectar-related olfactory cues within their colonies. This form of learning, however, has not been analysed by measuring well-quantifiable learning performances. Instead, it has been inferred from the ensuing choice behaviour of the animals (Von Frisch 1946, 1965). Thus, if bees acquire specific olfactory memories within the colony, the question remains whether these are short- or long-term memories (Menzel 1999). This distinction might have important implications for both foraging and pollination. We asked whether bees acquire long-term olfactory memories within the colony. To this end, we took advantage of the classical olfactory conditioning of the bee proboscis extension response (PER conditioning, Takeda 1961; Bitterman *et al.* 1983). Our experimental approach was straightforward; we first presented a group of foragers with scented sugar solution during a flowering-like foraging period. Next, within-the-nest bees were randomly collected from the hive and tested for long-term olfactory memories derived from the odour diluted in the offered reward. We then found that both foragers and younger bees had already established long-term olfactory memories, even when they had never foraged on the training feeder. The relevance of this finding is discussed in the context of efficient foraging.

2. MATERIAL AND METHODS

A colony of *Apis mellifera ligustica* (Spinola) bees (without a queen) was obtained from a larger colony (henceforth, original hive) and placed indoors in a one-frame observation hive. Foragers were marked and trained to collect 1.8 M scented (i.e. 50 µl of pure 1-nonanol (Sigma) per litre of solution) sucrose solution from an artificial feeder placed 15 m away from the hive. The feeder offered 50 µl of sugar solution per minute during four different foraging periods distributed over four successive days (one foraging period per day). Each foraging period began at 9.00–11.00 h and lasted approximately 270 min. Twenty-four hours after the end of the latest foraging period, unmarked bees (i.e. animals that had never foraged on the training feeder; henceforth, test bees) were randomly collected from the inner observation hive. A second group of bees (henceforth, control bees) was simultaneously collected from the original hive (i.e. descendents of the same queen). Bees from this colony had never foraged on the training feeder. Animals from both groups were restrained in metal harnesses. Each animal could freely move its antennae, mandibles and proboscis (Bitterman *et al.* 1983). Once fixed in the harnesses, they were placed in racks in a dark humidified chamber. On the evening following capture, they were fed up to satiation (unscented 1.8 M sucrose solution) and kept inside the chamber until tested. Next, 48 h after the beginning of the latest foraging period, bees from both groups were tested for long-term olfactory memories (Menzel 1999) derived from the odour diluted in the offered solution. Tests began at 10.00 h. We assume that if a bee, either a forager or a younger bee, perceives the odour diluted in the offered reward (as the conditioned stimulus or CS) immediately before sucrose solution (as the unconditioned stimulus or US), or even simultaneously, it must establish an association between the two stimuli such that the odour may trigger the bee's PER in a subsequent test (as the conditioned response or CR). During the test, animals were presented with two different odours. We tested their responses to both 1-nonanol, i.e. the odour added to the solution that had been offered at the feeder, and carnation oil (purchased pure from the pharmacy), i.e. a second odour to which the animals had never been exposed. Half of the bees were presented with the sequence 1-nonanol—carnation and the remaining half with the sequence carnation—1-nonanol. Odours were presented via an air stream delivered through a 20 ml plastic syringe that contained a piece of filter paper soaked with 4 µl of pure odorant (the odour source). A fan placed behind the animal extracted the odours released in the test room. Each of these trials lasted approximately 40 s. Removing bees from the racks to the test site was followed by a 20 s accommodation period after which the respective 5 s stimulation started. After stimulation the bees remained at the test site for another 15 s and were then returned to the racks. Prior to the test, bees were stimulated by applying sucrose solution (1.8 M) to their antennae to determine whether or not they responded to sucrose stimulation. Bees that failed to respond were excluded from the analysis. Spontaneous responses to the air stream were also tested prior to odour stimulation. Animals that responded positively to the air stream were also excluded from the analysis. We determined that each animal showed a CR when it only responded to 1-nonanol. Bees that responded to carnation (the control odour) and not to 1-nonanol (0.9 and 1% for the test and the control bees, respectively; $G_{(1)}=0.007$, $p=0.9$, $n=215$, G-test), as well as those that responded to both odours (9.6 and 9.9% for the test bees and the control bees, respectively; $G_{(1)}=0.004$, $p=0.9$, $n=215$, G-test) were excluded from the analysis. We then calculated the percentage of positive proboscis extensions (% PE) as the proportion of animals that showed CRs as calculated from the total number of tested animals (after excluding: (i) animals that responded to the control odour, (ii) animals that failed to respond to the US prior to the test and (iii) animals that responded to the air stream prior to the test). The % PE were compared by means of G-tests (Zar 1984). Experiments were conducted in February 2003 in the School of Exact and Natural Sciences of the University of Buenos Aires (34°33′ S, 58°26′ W).

Received 20 April 2005
Accepted 24 June 2005

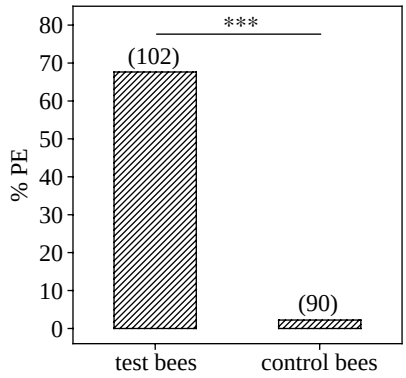

Figure 1. Percentage of proboscis extensions (% PE) recorded for test bees and control bees. Animals (i.e. within-the-nest bees) were tested for long-term memories 48 h after the latest foraging period (see §2 for details). Asterisks indicate statistical differences (*G*-test, $p<0.001$; see §3 for details). The number of animals is given in parentheses.

3. RESULTS

An increasing number of marked foragers collected a total amount of 43 ml of sugar solution during the 4 successive foraging days (18 h). Test bees were presented with the CS via the nectar crops of the marked foragers. We then compared the learning performances of two different groups of animals (test bees and control bees) that had been exposed, or not, to the scented 1.8 M sucrose solution previously offered at the feeder. Figure 1 shows the % PE obtained for each group. Results show a higher % PE for the test bees (67.6% and 2.2% for the test bees and the control bees, respectively; $G_{(1)}=105.4$, $p<0.0001$, $n=192$, *G*-test).

4. DISCUSSION

We found that test bees (see above) showed a high percentage of CRs (figure 1). According to the temporal dynamics of memory formation after PER conditioning (Menzel 1999), they exhibited already consolidated olfactory memories. Hence, our results indicate that within-the-nest bees associate the odour (CS) with the sugar (US) present in the incoming nectar and that the distribution of scented nectar within the colony allows them to establish a robust form of learning under natural conditions. In a honeybee colony, 75% of the whole population corresponds to young bees involved in different within-the-nest tasks (i.e. food-receivers, nurse and guard bees; Seeley 1995). According to the observed percentage (68%) of CRs, it is reasonable to assume that both foragers and younger bees acquired long-term olfactory memories, though the relative proportions of both groups remain unknown and their particular learning performances cannot be compared. Indeed, both foragers and younger bees learn olfactory cues under controlled laboratory conditions (Ray & Ferneyhough 1999; Ichikawa & Sasaki 2003).

It has been recently reported that trophallaxis, the exchange of liquid food by mouth (Wilson 1971), allows bees to learn nectar scents and leads to long-term olfactory memories under controlled laboratory conditions, i.e. bees associate the odour (CS) with the sugar (US) present in the nectar they receive by means of trophallaxis (Gil & De Marco 2005). Trophallaxis most likely also underlies acquisition of long-term olfactory memories within the colony, mainly because of two reasons: (i) the nectar collected in the field is rapidly distributed among colony members via trophallaxis (Wilson 1971) and (ii) bees inexorably perceive nectar odours during trophallaxis (Gil & De Marco 2005). Moreover, olfactory conditioning in honeybees strongly depends upon CS–US contingency (Bitterman *et al.* 1983; Menzel 1999). Thus, if that were the case, analysis must still be done upon the effects of CS and US intensity (Gil & De Marco 2005) on the olfactory learning occurring within the colony. Furthermore, both the rate and the duration of the whole CS–US stimulation provided by a given nectar source might determine the strength of the association achievable at the colony level. In addition, the area where most trophallaxes occur is relatively large and contains other bees, so that foragers and receivers need to search for a partner, usually antennating several other bees before a partner is found (Seeley 1995). Interestingly, unfamiliar odours present in the mouthparts of a possible partner decrease the occurrence of trophallaxis (Gil & Farina 2003). Moreover, familiar odours seem to elicit trophallaxes when foragers face increased resource uncertainty (De Marco & Farina 2003). If the perception of nectar odours affects the occurrence of trophallaxis, both foragers and receivers might benefit from learned odours in searching for a transfer partner, eliciting trophallaxis or even avoiding it. This might have important implications in the organization of foraging within the colony. According to this hypothesis, however, the effect of a given CS must be closely related to its relative prevalence inside the nest (as calculated from the relative intake rates of the different types of nectar being simultaneously exploited by the colony) in order to provide a given group of foragers and receivers (experiencing an increasing cohesion based on the type of nectar they exchange) with the necessary plasticity to gradually 'move' their interactions into a different group.

Although the colony simultaneously exploits different flower species, individual bees tend to forage on a single flower species (Von Frisch 1965; Seeley 1995; Chittka *et al.* 1999). This kind of flower fidelity seems to improve individual foraging strategies by reducing search and handling times (Heinrich 1975; Keban & Baker 1983). Presumably, both the recognition of specific flowers (which involves learning) and their manipulation are sharpened during flower fidelity, enhancing the rate of nectar gathering (Heinrich 1975). Indeed, bees not only exhibit flower fidelity, but also benefit from olfactory (and visual) long-term memories acquired during foraging in order to optimize their choices (Von Frisch 1965; Chittka *et al.* 1999; Menzel 1999). Olfactory memories established within the colony might play a critical role during the early development of flower fidelity. This means, for instance, that currently unemployed foragers (and even non-experienced foragers) might benefit from a highly prevalent olfactory CS present

within the colony in order to elicit their later foraging bouts. According to this hypothesis, the higher the rate of encounter with the rewarded CS the higher the probability of flying out to search for the prospective nectar source. This might, in turn, enhance the rate of nectar gathering during flowering, particularly in the case of flower species in which flowering begins abruptly and diminishes (Rathcke & Lacey 1985). Moreover, the flowering periods of most species of plants do not allow closing species-specific foraging fidelity and colonies must track different blossoms throughout the season (Heinrich 1975; Seeley 1995). It would be interesting to investigate how these olfactory memories are integrated into the context of the foraging task, especially when two or more combinations of CS–US stimuli are simultaneously perceived within the colony. This might have important implications for the study of foraging and pollination.

This study was supported by funds from the Argentinean Research Council (CONICET). The authors thank Dr W. M. Farina and H. Verna (University of Buenos Aires) for logistic support of this project. The present experiments comply with the Principles of Animal Care (publication No. 86-23, revised 1985) of the National Institutes of Health and the corresponding national current laws.

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