# The repellent scent-mark of the honeybee
# *Apis mellifera ligustica* and its role as communication cue during foraging

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## Summary

Experimental evidence for flower-marking in honeybees (*Apis mellifera ligustica*), using pairs of workers from the same colony foraging on an artificial patch of flowers, is reported. Workers marked artificial flowers with scent and strongly rejected all flowers they had recently visited. The same rejection behavior, in a lower although significant proportion, was observed when bees visited flowers just abandoned by the other individual of the pair. The repellent nature of this scent-mark was demonstrated with the use of an air extractor connected to the patch of artificial flowers. When the apparatus was turned on, the rejection behavior disappeared and bees accepted both flowers just abandoned by themselves and flowers just abandoned by the other bee. Differences in the response level of bees to their own marks or to the partner's marks suggest that the repellent scent-mark applied by a bee during foraging would basically be a self-use signal, although it certainly has value in communicating with other workers.

## Introduction

Honeybees guide their foraging behavior towards potential food sources by means of visual cues at longer distances and olfactory cues at shorter distances (Frisch, 1967). Among olfactory cues, pheromones play an outstanding role in foraging orientation. Several attractive pheromones have been reported in relation to honeybees' foraging behavior (Ribbands, 1955; Frisch, 1967; Free, 1968; Butler et al., 1969; Free and Williams, 1972; Ferguson and Free, 1979; Free et al., 1982). These marks have also been reported for bumblebees (Cameron, 1981; Schmitt and Bertsch, 1990; Schmitt et al., 1991) and stingless bees (Lindauer and Kerr, 1958; Kerr et al., 1963,

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1981; Villa and Weiss 1990). The common principle of these substances is that they are employed to signal and mark rewarding food sources and that they have a considerable persistence due to their chemical structures and low volatility (Schmitt et al., 1991). For this reason, they are considered "long-term signals" (Schmitt and Bertsch, 1990).

Honeybees, however, do not rely exclusively on such attractive pheromones: they also use a repellent scent-mark that promotes the rejection of exhausted (Núñez, 1967) and recently-visited rewarding flowers, not yet replenished with nectar (Giurfa and Núñez, 1992). Thus, this scent-mark has a different meaning when compared with the attractants cited above and it is therefore applied in a different context, i.e. in the non-rewarding flowers rather than in the rewarding ones.

It has been demonstrated that this mark can be used to avoid revisiting a flower the bee has just left. Giurfa and Núñez (1992) thus cautiously employed the term "pheromone", which usually implies communication among at least two partners of the same species (Karlson and Lüscher, 1959).

In the present work, new evidence is given on the repellent scent-mark and the question is discussed whether the mark left by a bee is recognized by another individual of the same colony.

## Materials and methods

*Apis mellifera ligustica* from a hive located 50 m from the laboratory were used. Experiments were performed in the apiary of the Argentine Beekeeper Society, González Catán, Buenos Aires (lat 34.6° South).

### Apparatus

A food-source simulator (Núñez, 1971, Giurfa and Núñez, 1992) was placed near the laboratory window. It consisted of an acrylic disc 50 cm in diameter with twelve (12) identical, numbered, yellow flowers, spaced 10 cm apart (Fig. 1). Each flower received a 50% sucrose solution (w/w) through a cannula connected to an automatic microburette of the Scholander type, driven by a synchromotor. Sucrose solution is considered odorless to bees (Schwarz, 1955; Frisch, 1967). Previous calibrations were made in order to ensure that all the flowers were actually equally rewarding in terms of sucrose flow. Bees could reach the sucrose solution through a PVC access tube on each flower. An air extractor was connected to the food source simulator as shown in Fig. 1. Each flower had two perforations adjacent to the sugar cannula so that odor marks could be carried away by activating the fan. Previous controls (Giurfa and Núñez, 1992) showed that the air extractor itself did not influence the behavior of the bees: the number of landings on flowers per minute and the movement pattern were the same with the air extractor on or off.
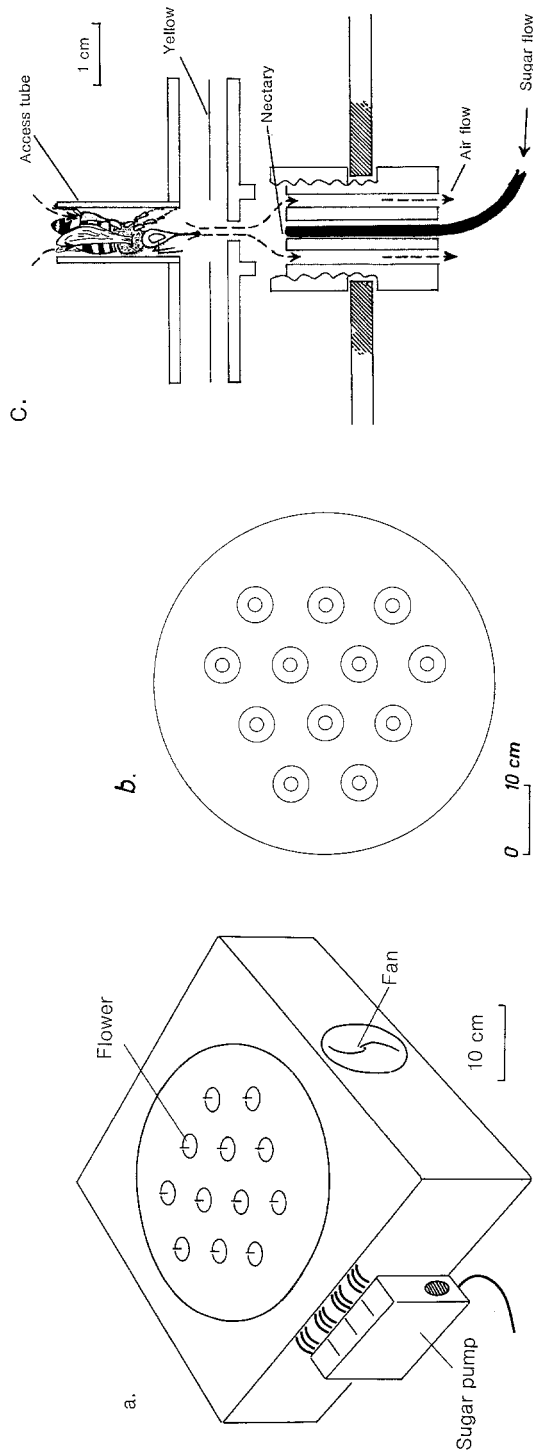
**Figure 1.** Food-source simulator (a) general view; (b) upper view of the artificial patch with 12 numbered flowers; (c) detail of an artificial flower and a bee's acceptance (see Materials & Methods for definition of acceptance)

*Procedure*

Pairs of worker bees were marked with color spots on the thorax and trained to collect sucrose solution from the artificial flowers on the simulator. After two pretraining visits, where 1 μl/min total flow was provided (i.e. each flower received 1/12 of this total flow), sucrose flow was suppressed in six of the twelve flowers. At the same time, the total flow was changed to 2 μl/min. Thus, the resulting total flow at the simulator was again 1 μl/min. Since foraging bees evaluate not the instantaneous sucrose flow velocity (μl/min), but its average over a certain period of time (Varjú and Núñez, 1991), a perceived decrease in the total flow average of the patch, resulting from the introduction of 6 non-rewarding flowers, would lead to a decrease in the foraging motivation (Núñez, 1970). That is, the bees would cease to forage on the patch, a fact that would impede the execution of the experiment. The procedure described above allowed not only the introduction of six non-rewarding flowers, but also the maintenance of the same total sucrose flow, ensuring the return of the experimental bees.

The disposition of rewarding and non-rewarding flowers was established following a random number series, to ensure aleatory distribution of places in the simulator when both kinds of flowers were present. The apparatus presented both kinds of flowers in order to facilitate better observation of the scent-marking strategy assuming that bees might avoid the "wrong" flowers in a situation in which flower position was not a reliable cue due to the patch being irregularly rotated to avoid training to flower positions.

Bees were tested on the simulator in pairs. Measurements began when the second member of the pair arrived at the artificial patch and ended when one of the members left for the hive. In other words, measurements only took place when both bees were present in the patch of artificial flowers. In some cases, one bee was still working when the measurement ended, but the first visit from one bee never overlapped with second visit of the other bee. Bees could arrive and depart out of phase although they frequently arrived at the same time.

We defined two categories of temporal foraging sequences: "Event 0", defined as the immediate return of the focal bee to a flower just abandoned by itself (the companion bee has not landed at this flower before the focal be returns), and "Crossed Event 0", defined as the immediate visit to a flower just abandoned by the companion bee (Fig. 2). In both cases, only events occurring within an interval of less than 5 sec were considered. Event 0 normally occurred within this interval and Crossed Event 0 occurring after a longer interval were not considered because differences in the observed behavior could be due to a greater evaporation time of the repellent scent-mark. By choosing the 5-sec interval, we made both events comparable.

The number of these two events in which Acceptances (landings in which bees fully entered the access tube of artificial flowers; see Fig. 1 c) and Rejections (landings in which bees introduced only the antennae into the access tube and immediately flew away) occurred were recorded. As the edge of the access tube was 2 cm away from the "nectary" of the artificial flowers, bees showing a rejection behavior neither sucked nectar nor tried to do so.
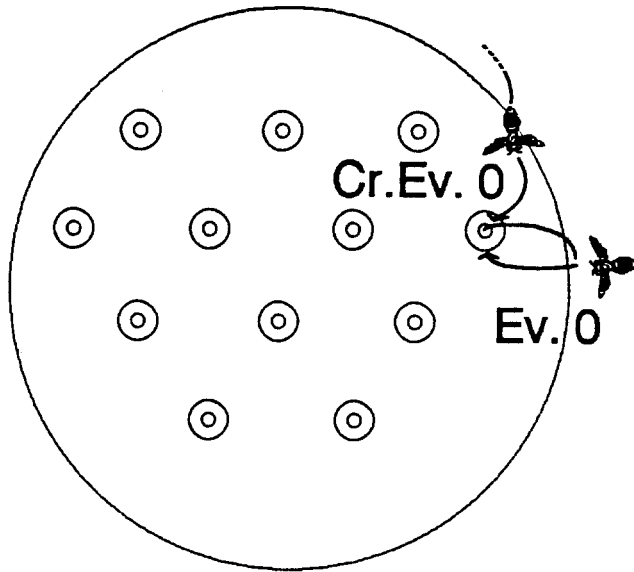
**Figure 2.** Two possible cases of "Event 0" and "Crossed Event 0". In "Event 0" the focal bee abandons a flower and then returns to the same one without visiting other flowers (the companion bee has not landed at this flower before the focal bee returns). In "Crossed Event 0" the focal bee abandons a flower and then directly enters another one that was just left by the companion bee. In both cases, only events occurring within an interval shorter than 5 sec were considered

In the next measurement, the distribution of rewarding and non-rewarding flowers was inverted, the air extractor was activated and the same parameters were measured with the same pair of bees. Twenty pairs of bees were measured in this way. As a result, 210 Event 0 and 402 Crossed Event 0 were analyzed.

**Results**

Table 1 shows that when the air extractor was off, bees rejected flowers just abandoned by themselves, whereas they accepted these flowers, when the air extractor was activated. Rejections significantly disappeared and acceptances significantly increased when the air extractor was activated. The interaction between rows and columns proved to be significant after a "2 × 2 G Test of Independence" (Sokal and Rohlf, 1981), i.e. the kind of behavior evinced on Event 0 depended on whether the air extractor was on or off ($G_{adj} = 146.70$; df: 1; $p < 0.001$).

Table 2 shows that bees recognized the partner's mark as they significantly rejected flowers just abandoned by the other bee. When the air extractor was activated, however, rejections disappeared and bees accepted flowers just abandoned by the partner. In this event category, the interaction between rows and columns was also significant ($G_{adj} = 212.79$; df: 1; $p < 0.001$).

**Table 1.** Number of Event 0 (immediate return of a bee to an artificial flower just abandoned by itself) in the two behavioral categories (Rejection and Acceptance) in both experimental situations (air extractor off and on, respectively); $G_{adj} = 146.7$; $df : 1$; $P < 0.001$

|                   | Rejection | Acceptance | $\Sigma$ |
|-------------------|-----------|------------|----------|
| Air extractor off | 95        | 28         | 123      |
| Air extractor on  | 1         | 86         | 87       |
| Total             | 96        | 114        | 210      |

**Table 2.** Number of Crossed-Event 0 (immediate visit to an artificial flower just abandoned by the other bee) in the two behavioral categories (Rejection and Acceptance) in both experimental situations (air extractor off and on, respectively); $G_{adj} = 212.8$; $df : 1$; $P < 0.001$

|                   | Rejection | Acceptance | $\Sigma$ |
|-------------------|-----------|------------|----------|
| Air extractor off | 141       | 76         | 217      |
| Air extractor on  | 3         | 182        | 185      |
| Total             | 144       | 258        | 402      |

**Table 3.** Analysis of Event 0 and Crossed Event 0 situations considering proportions. Ho: Proportion of Rejections in Event 0 = Proportion of Rejections in Crossed Event 0; Ha: Proportion of Rejections in Event 0 > Proportion of Rejections in Crossed Event 0

|                 | Rejection | Acceptance | Total | Prop. Reject.    | Prop. Accep.     |
|-----------------|-----------|------------|-------|------------------|------------------|
| Event 0         | 95        | 28         | 123   | $\hat{p}_1 = 0.772$ | $\hat{q}_1 = 0.228$ |
| Crossed Event 0 | 141       | 76         | 217   | $\hat{p}_2 = 0.650$ | $\hat{q}_2 = 0.350$ |
| Total           | 236       | 104        | 340   | $\bar{p} = 0.694$   | $\bar{q} = 0.306$   |

$Z = 2.23$
$P < 0.025$ (one-tailed)

Finally, we analyzed whether the proportion of rejections was greater in Event 0 than in Crossed Event 0, i.e. if bees more strongly rejected their own scent-marks or their partner's mark (Ha; one-tailed). We performed a statistical test for comparing two proportions (Zar, 1984, p. 395) and the results are presented in Table 3. We can reject the null hypothesis of the proportion of rejections being equal whenever bees visited a flower just abandoned by another bee compared to whenever bees visited a flower just abandoned by themselves ($Z = 2.23$; p < 0.025; one tailed).

## Discussion

The present work demonstrates that honeybees use a repellent scent to mark artificial flowers. We showed that bees use such a mark to avoid revisiting the flowers they just abandoned. In this sense, the repellent mark is a self-use cue. Individual scent-marks associated with foraging orientation have also been reported for workers of the

ponerine ant, *Pachycondyla tesserinoda* (Jessen and Maschwitz, 1986) and the myrmicine ant, *Leptothorax affinis* (Maschwitz et al., 1986). In these species leader ants profit from the recognition of their own trail-marks since they must unravel the confusion of trails occurring near the nest and its surroundings in order to avoid unknown areas where they might easily get lost. In the case of the repellent scent-marking of honeybees, considering that bees normally perform more than a hundred acceptances during a visit (Giurfa and Núñez, 1992), this strategy would save the time/energy required by a bee to crawl completely into the access tube of recently depleted or exhausted flowers and then to leave it by backing out. The energetic advantages of such an individual marking strategy have been discussed elsewhere (Giurfa and Núñez, 1992).

The repellent nature of the bees' scent-mark was demonstrated by the use of an air extractor, an experimental device that carries the scent-mark away from the artificial flowers. When activated, it increased the relative level of acceptances of the artificial flowers (Tab. 1 and 2). Had the scent-mark been attractive, it was predicted that the bees would reject all the flowers when the olfactory cue was absent, a fact not observed in our experiments.

We also demonstrate that bees actually recognize and therefore respond to the repellent scent-mark of another bee of the same colony by rejecting the flower just abandoned by the latter. It would then be correct to use the expression "repellent pheromone" for the reported repellent scent-mark. The variation in the level of response according to whether an Event 0 or a Crossed Event 0 took place (Tab. 3) deserves, nevertheless, a comment. The proportion of Acceptances/Rejections in the Event 0 situation was 1:3.39, while in the Crossed Event 0 situation, it was 1:1.85. The former was significantly greater than the latter, i.e., even if a significant tendency was observed in the direction of rejecting flowers marked by a partner, the effect was not as strong. Thus, it is possible that the original function of the repellent mark is self-orientation in a flower patch, although it certainly has value in communicating with other workers. It would thus act as a cue in the sense that bees might be responding not to a specially designed pheromone signal produced by other foragers, but to a chemical cue that foragers cannot help but leave behind as they forage.

The source and chemical nature of such olfactory cues are at present unknown. Nevertheless, recent experiments by Giurfa on Africanized honeybees (unpublished results) and of Vallet et al. (1991) indicate that 2-heptanone could be involved in the scent-marking of depleted flowers. Two functions were originally attributed to this substance produced by the mandibular glands of workers: alarm (Maschwitz, 1964; Shearer and Boch, 1965) and release of stinging behavior (Free and Simpson, 1968). Interestingly, the level of 2-heptanone reaches its peak in foragers rather than in guard bees (Simpson, 1961; Boch and Shearer, 1967; Crewe and Hastings, 1976; Vallet et al., 1991), a fact that could indicate that 2-heptanone is more closely related to the repellent scent-marking by honeybees.

Foragers would benefit if they could avoid visiting flowers for a short period after depletion; that is until secretion replenishes the nectar and during which time evaporation attenuates the repellent scent-mark. In the experimental situation prevailing in this work, one may ask how the repellent mark affects the partner's foraging efficiency. The half-life of the mark has been estimated to be around 1 min

(Giurfa and Núñez, 1992) in an experimental situation where each of the twelve artificial flowers provided sucrose solution with a flow of 0.17 μl/min. As the mark has a very short duration, the bees could increase their foraging efficiency by arriving together (or in groups) at the patch. Although not quantified, this fact was sometimes observed during our experiment and was also observed by Kevan and Lack (1985) and by Kevan (personal communication).

Considering the properties of the repellent substance, one may ask in which natural context is this substance likely to play a role in the foraging behavior of honeybees. If the mark is applied to avoid revisiting of the same flower within a short time interval, one should search for a natural context where this fact (a very fast revisiting, due to shorter interfloral distances, for example) could occur. A possible answer may be provided by the Compositae inflorescences, where a great number of individual florets are densely packed and where bees forage intensively, walking over the available florets of the capitula. Further studies are necessary to study whether inflorescences of some Compositae could be a possible context for the use of the repellent scent-mark.

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