

Prior classical olfactory conditioning improves odour-cued flight orientation of honey bees in a wind tunnel

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Summary

Odours are key cues used by the honey bee in various situations. They play an important role in sexual attraction, social behaviour and location of profitable food sources. Here, we were interested in the role of odours in orientation at short distance, for instance the approach flight to a floral patch or in close proximity to the hive entrance. Using a newly designed wind tunnel, we investigated the orientation behaviour of the bee towards two different odours: a social odour and a floral component, linalool. We then tested the effect of prior olfactory conditioning (conditioning of the proboscis extension reflex) on subsequent flight orientation. We showed that both stimuli induced orientated behaviour

(orientated flights, circling around the odour source) in up to 70% of the worker bees, social odour being slightly more attractive than the linalool. We found thereafter that orientation performance towards the floral compound can be significantly enhanced by prior classical olfactory learning. This type of information transfer, from a Pavlovian associative context to an orientation task, might allow future foragers to acquire, within the hive, relevant information about the odours and food they will encounter during their later foraging bouts.

Key words: honey bee, orientation in flight, olfactory cue, wind tunnel, olfactory conditioning, information transfer.

Introduction

Honey bees use odours as key cues to orientate in many contexts: in sexual attraction and courtship behaviour mediated by the queen pheromone (Winston, 1987; Loper et al., 1996), in social attraction and orientation during swarming mediated by the Nasonov pheromone (Free, 1987; Schmidt, 1994, 1999), in nest recognition during returning flight (Butler and Free, 1952), in defence of the colony (Free, 1961) and in location and recognition of food sources using floral scents (Menzel et al., 1993; Smith and Getz, 1994). The ability of the bees to learn and recognise olfactory cues has been widely studied since the founding work by von Frisch (1967), especially with regard to the role of odours in foraging behaviour. Many experimental approaches have been used, all relying on the analysis of various behavioural responses: orientation and olfactory choice of free-flying foragers visiting artificial scented feeders (Couvillon and Bitterman, 1980; Giurfa and Nuñez, 1992; Greggers and Mauelshagen, 1997), walking orientation towards odours using laboratory devices like locomotion compensators (Kramer, 1976) or olfactometers (Getz and Smith, 1990; Bakchine et al., 1990), and classical olfactory learning by conditioning of the proboscis extension reflex (Takeda, 1961; Vareschi, 1971; Bitterman et al., 1983;

Hammer and Menzel, 1995; Laloi et al., 2000; Guerrieri et al., 2005). Most of these reports addressed either orientation during long-range flights or the response of bees under highly controlled conditions, with particular interest in the olfactory learning and memory processes. By contrast, little is known about the role of odours during short-range orientation in flight such as that involved in the approach to a floral patch or in proximity to the nest entrance. It is nevertheless often recognised since von Frisch (1967) that scent plays a predominant role when the bee is close enough to the target (one or a few metres), for instance inducing the bee to land or enter a flower (Williams, 1983; Galizia et al., 2005).

Honey bees experience a wide range of olfactory information throughout their life. This information can be gained inside the hive, for example from the stored pollen and nectar or during dances, as recruited bees make antennal contact with the dancing bee that is impregnated with floral scents. It can also be acquired outside the hive during the foraging bouts. The ability to use olfactory information acquired in various contexts during subsequent orientation tasks might contribute to the enhancement of orientation performance. Reporting that flower volatiles, carried by

returning foragers from the sites they have recently visited, could be used by the recruits to search for food, von Frisch (1967) and Wenner et al. (1969) were the first to demonstrate that olfactory information acquired in the hive can be used in an orientation context. Since then, some studies have shown that bees are able to transfer information from a foraging situation to an associative context (Gerber et al., 1996) and from an associative context to a walking orientation response in an olfactometer (Backchine et al., 1992; Sandoz et al., 2000). Recently, Reinhard et al. (2004) showed that a scent blown into the hive can trigger trajectory memories of a site the bees had previously visited. This impressive finding of an associative recall also confirms that orientation of the bees can take into account various memorised information.

In the present work, we investigated the role of odours in the orientation behaviour during short-range flights, and we tested the effect of a prior olfactory experience on subsequent flight orientation. One goal in investigating such types of orientation behaviour is to measure not only the final outcome but also how an individual actually succeeds in the task. Indeed, quantifying the number of visits to a site gives little information on the difficulty the bees had in finding it, since it does not document the method they used to achieve the task, and it might underestimate some types of exploratory strategy based on trial-and-error repetitions. Wind tunnel devices enable the investigator to detail the individual approach flight and to control for the various cues that could be used by the insects under natural conditions. This type of device has been extensively used to study olfactory orientation in flying insects (e.g. Baker et al., 1984; Fadamiro et al., 1998; Jang et al., 2000). It was shown to be particularly suitable for analyzing the orientation mechanisms involved in sexual attraction, in the search for plants by phytophagous insects or in host research by parasitoids. A wind tunnel was previously used to study the energetics of flight on suspended honey bees (Hanauer-Thieser and Nachtigall, 1995) but it has never been exploited for the study of flight orientation in this species, although some types of flight room refer to a similar approach (Poppy and Williams, 1999; Laloi et al., 2000). Consequently, we adapted a wind tunnel originally designed for the observation of moths and parasitoids for use with honey bees. We established reliable flight descriptors prior to using this device to study how worker bees can orientate in flight towards two different volatiles: an odour of nestmates, which can be part of the olfactory cues driving the returning flight of a bee to the hive, and linalool, a common floral odour. We then investigated whether olfactory cues acquired through classical olfactory conditioning of the proboscis extension reflex (Bitterman et al., 1983) could influence a bee's orientation within a 2-m range.

Materials and methods

Biological material

Experiments were conducted on worker honey bees, *Apis mellifera* L., reared under commonly used laboratory conditions (e.g. Sandoz et al., 2000; Laloi et al., 2000).

Emerging bees were collected from brood combs of two outdoor hives during summer. They were caged in groups of ~60 individuals and maintained in an incubator (32–34°C, 55% relative humidity, in darkness). Bees were provided with sugar and water *ad libitum* and with pollen as a source of protein until 8 days of age. They were reared under these conditions until testing at the age of 14–15 days, when honey bees generally become foragers (Seeley, 1982).

Wind tunnel

The wind tunnel (Fig. 1) constituted a transparent vault (Plexiglas), 62 cm central height, on a 200×72 cm floor. An input fan created an airflow by driving the air of the experimental room into the flight chamber, and an extraction fan drove the air outdoors. A fine mesh net was positioned at the entrance of the flow to obtain a laminar airflow in the flight chamber (as checked using white NH₄Cl vapour). The following conditions were set up to ensure satisfactory honey bee flight: the input and output flow speed was adjusted to 0.3 m s⁻¹; the temperature was set to 23±1°C, which was shown to induce good honey bee flight activity (Hanauer-Thieser and Nachtigall, 1995); the light was adapted to the high flicker fusion of 150 Hz (van Praagh, 1972) by using four fluorescent neon lights (36 W, 120 cm length each) set on high-frequency connectors (Mazda ref 136 HFR). The lights were arranged in pairs, 38 cm above the top of the flight chamber vault, the two pairs being separated by a distance of 60 cm. To reduce phototropism, indirect light was obtained by setting a piece of opaque cardboard under the neon lights so that most of the light was reflected onto the walls of the experimental room. Under these conditions, the luminosity was of 6.6 μmol photons m⁻² s⁻¹ (400 lux) within the whole flight chamber. In our experimental conditions, the UV light component was highly reduced. This may affect the flight structure although little is known about the role of UV in short-range flight. Nevertheless, since our experiments were always based on comparisons between a test group and a control

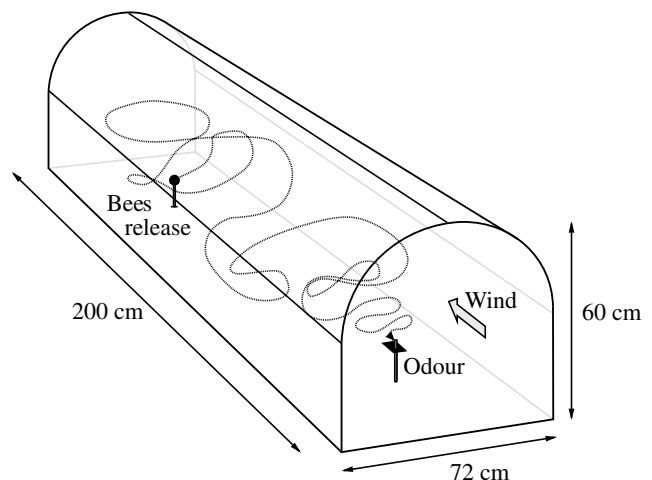


Fig. 1. General characteristics of the wind tunnel, with an example of a typical honey bee oriented flight.

group, both exposed to the same tunnel conditions, a putative change in the flight structure would have been balanced between groups and would not affect our conclusions. Our tunnel could be improved by using UV-transmitting Plexiglas instead of normal Plexiglas and by using lights with a UV component. Preliminary experiments, intended to improve displacement in flight in the tunnel, showed that visual marks were useful to facilitate the orientation of the bees, since bees need image motion to estimate distances (Kirchner and Srinivasan, 1989). Thus, vertical bands (black paper, 2 cm width \times 40 cm length) were added to the sides of the Plexiglas vault. Trap doors on the tunnel floor allowed manipulations inside the flight chamber.

Volatiles were delivered to the flight chamber through a glass tube (diameter 0.5 cm) emerging 18 cm above the tunnel floor. To obtain a regular flow of volatiles, the odour was delivered through a constant outflow using a pump (1085 ml min⁻¹). The output of the odour source was positioned in the middle of a metallic cardboard disk (5 cm diameter) that could be used as a visual cue and a possible landing area.

General procedure for flight activity recording

Each bee was placed in a small individual cage (3 \times 4 \times 5 cm) for at least 1 min before introduction into the flight chamber. The cage was positioned on a platform set 18 cm above the floor and 120 cm from the odour source (Fig. 1). Once the trap doors were locked, the cage could be opened from the outside. After the air flow was switched on, we let the honey bee get familiarized with the tunnel conditions for at least 15 s. The experiment started when the bee was released from the cage. Behaviour was observed for 5 min. Bees that took more than 1 min to fly out of the cage were excluded from further analysis.

Two types of parameters were recorded during the experiments. First, we recorded the total flight duration of each bee and its flight duration around the source, i.e. in a volume of 20 \times 25 \times 72 cm centred at the odour source. This volume was chosen after preliminary experiments, since it corresponded to the distance where pre-landing behaviour was obvious. Appropriate marks on the Plexiglas vault allowed the visualization of this zone. Second, we recorded the proportion of bees performing orientated flights (upwind zigzag flight) and the proportion of bees circling (i.e. bees exhibiting circling flights or stationary flights around the source for more than 2 s).

Conditioning of the proboscis extension reflex

The experimental procedure for classical conditioning of the proboscis extension was the standard procedure detailed elsewhere (Bitterman et al., 1983; Sandoz et al., 1995). Each bee was mounted in a glass holder and starved for four hours. Bees were then individually subjected to three conditioning trials (C1, C2, C3) with 15-min inter-trial intervals. Before each trial, bees were positioned for 15 s in the airflow to familiarise them with the mechanical stimulation. Then the

odour stimulus (conditioned stimulus) was presented for 6 s and then, 3 s after the onset of the odour, the antennae were touched with a 30% sucrose solution (unconditioned stimulus). The subsequent proboscis extension was rewarded with a drop of the same sucrose solution. Bees that showed spontaneous responses at the first presentation of the odour were discarded from the following steps since later responses of such individuals could not be interpreted as purely associative.

Experiment 1: orientation toward nestmate odour and linalool

The first experiment was designed to study worker bees' orientation towards two different volatiles, an odour of nestmates and a floral odour. The nestmates' odour source consisted of a sealed box (11.2 \times 17.5 \times 13 cm) containing ~50 bees originating from the same rearing cage as the tested individual. To avoid visual and acoustic cues, this box was placed outside the tunnel, and the stimulating air flow passed through the box before being released in the flight chamber. Thus, only volatiles were delivered into the tunnel. The floral odour source was linalool (Sigma, St Quentin Fallavier, France; 95–97% purity), a common floral compound (Knudsen et al., 1993). The stimulating flow passed through a glass vial containing 200 μ l of pure linalool. Three groups of bees were thus subjected to the following stimulations: bees exposed to nestmates' odour ($N=25$), bees exposed to linalool ($N=25$) and bees exposed to no odour (control group; $N=25$).

Experiment 2: effect of odour conditioning on the orientation task

A second experiment was designed to analyse the influence of prior olfactory learning on the olfactory orientation of the bees in the wind tunnel. Bees ($N=25$) were conditioned to linalool using the proboscis extension paradigm. In parallel, control bees ($N=25$) were subjected to the whole procedure (identical conditions of harnessing, starvation and stimulation) but without odour delivery. At the end of the conditioning session, bees were gently removed from the glass holders, with special care given to their wings, and then caged individually with food for one hour. They were then tested in the flight tunnel in the presence of linalool, following the conditions described above.

Data recording and statistical analysis

During the observations, the different flight parameters were recorded using The Observer software version 3.0 (Noldus Inc., Wageningen, The Netherlands). After control for normality and homoscedasticity, total flight durations were compared amongst groups using a one-way analysis of variance (ANOVA). With respect to flight duration around the source, data did not meet the assumptions of normality and homoscedasticity. Thus, this variable was analysed using non-parametric tests: a Mann–Whitney test was used to compare two groups (experiment 2: conditioned bees and control bees) and a Kruskal–Wallis test was used to compare three groups (experiment 1: bees stimulated with linalool, bees stimulated with nestmate odour, and control bees). In the cases in which

the Kruskal–Wallis test indicated a significant difference among groups, non-parametric pairwise comparisons (Conover, 1980) were applied in order to identify the groups that differed at the experimentwise alpha level of 0.05. With respect to the number of bees that performed orientated flights and the number of bees that performed circlings, groups were compared using a χ^2 test. When the proportion of individuals was found to differ amongst more than two groups (experiment 1), pairwise comparisons were conducted using χ^2 tests with 1 d.f., in order to identify the groups that differed. To ensure that the experimentwise alpha level was 0.05, the alpha level of each pairwise test was adjusted downward according to the Dunn–Sidak correction (Sokal and Rohlf, 1995).

Results

Experiment 1: orientation towards nestmate odour and linalool

The total flight duration (Fig. 2A) varied from 135.9 ± 13.0 s for the control group, i.e. bees presented with no odour, to 149.2 ± 12.3 s for the bees stimulated with nestmate odour.

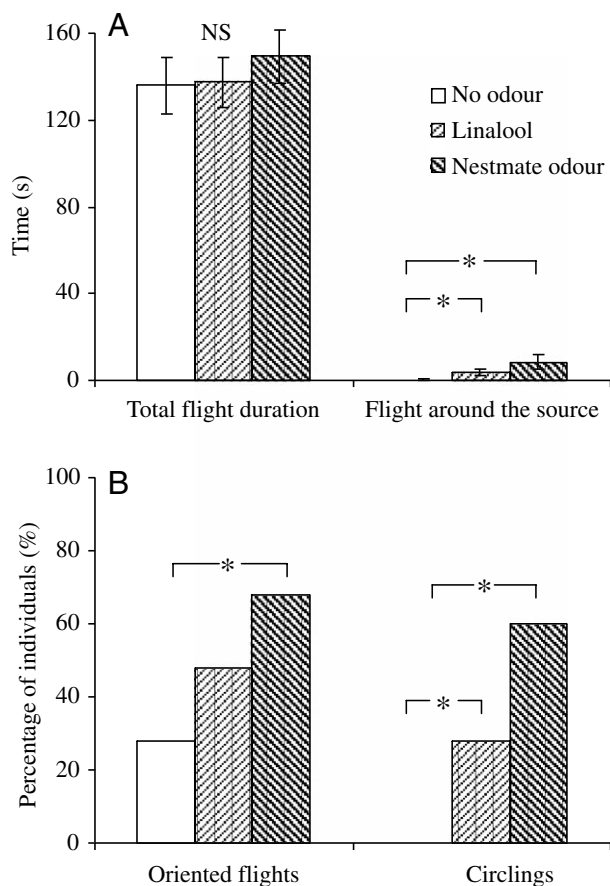


Fig. 2. Orientation of worker bees in the wind tunnel according to the odorous stimulation: nestmates' odour, linalool and no odour (control group). (A) Mean duration \pm S.D. of the total flight and of the flight around the odour source. (B) Percentages of bees exhibiting orientated flights (upwind zigzag flight) and circling behaviour. Asterisks indicate significant differences: $P < 0.05$; NS, not significant.

Total flight duration did not differ significantly according to the odour source (ANOVA, $F=0.35$, $P > 0.05$, $N=25$), bees flying on average for 47% of the 5 min observation period. In contrast to the total flight, the flight around the source (Fig. 2A) varied significantly according to the nature of this source (Kruskal–Wallis test, $H=13.58$, $P < 0.01$, $N=25$). Pairwise comparisons indicated that bees of the control group spent less time around the source (0.32 ± 0.15 s) than bees stimulated with nestmate odour (8.44 ± 3.14 s) and linalool (4.00 ± 1.42 s). These two latter groups did not differ significantly. The proportion of bees that performed orientated flights (Fig. 2B) differed significantly according to the odour source ($\chi^2=8.01$, $P < 0.01$, $N=25$). Pairwise comparisons indicated that bees stimulated with nestmate odour exhibited more orientated flights than the control group (68% and 28% of the bees, respectively). Other comparisons did not yield significant differences. The proportion of circlings (Fig. 2B) also differed significantly according to the odour source ($\chi^2=21.74$, $P < 0.001$, $N=25$). Pairwise comparisons indicated that bees of the control group (exhibiting no circlings) differed significantly from bees stimulated with both nestmate odour and linalool (60% and 28% of the bees exhibiting circlings, respectively). Thus, while the total flight duration was not influenced by the presence of an odour, both linalool and nestmate odour induced orientation responses, the nestmate odour tending to be more attractive than the floral component.

Experiment 2: effect of odour conditioning on the orientation task

Bees previously conditioned to linalool in the proboscis extension procedure were compared with control bees. The total flight duration (Fig. 3A) did not differ significantly between the two groups (ANOVA, $F=0.36$, $P > 0.05$, $N=25$), flight durations being similar to those observed in the first experiment (136.3 ± 9.6 s for the conditioned bees, 127.3 ± 11.7 s for the control bees). By contrast, the two groups differed with respect to their flight duration around the source (Fig. 3A; Mann–Whitney test, $z=4.75$, $P < 0.001$, $N=25$), conditioned bees spending more time around the odour source (7.00 ± 1.86 s) than control bees (0.36 ± 0.14 s). Conditioned bees exhibited significantly more orientated flights than control bees (Fig. 3B; $\chi^2=5.56$, $P < 0.05$, $N=25$). The proportion of circlings also differed between the two groups (Fig. 3B; $\chi^2=9.44$, $P < 0.01$), conditioned bees exhibiting more circlings (40%) than control bees (4%). Thus, there was a strong effect of conditioning on the flight behaviour in the wind tunnel, conditioned bees showing an increase in the orientation response towards the odour.

Discussion

We investigated olfactory cued orientation at short distance in the honey bee using a wind tunnel. Wind tunnels were first designed to study sexual attraction in non-social insects (Miller and Roelofs, 1978; Willis and Arbas, 1998), and they also proved powerful for investigating host location in parasitoids

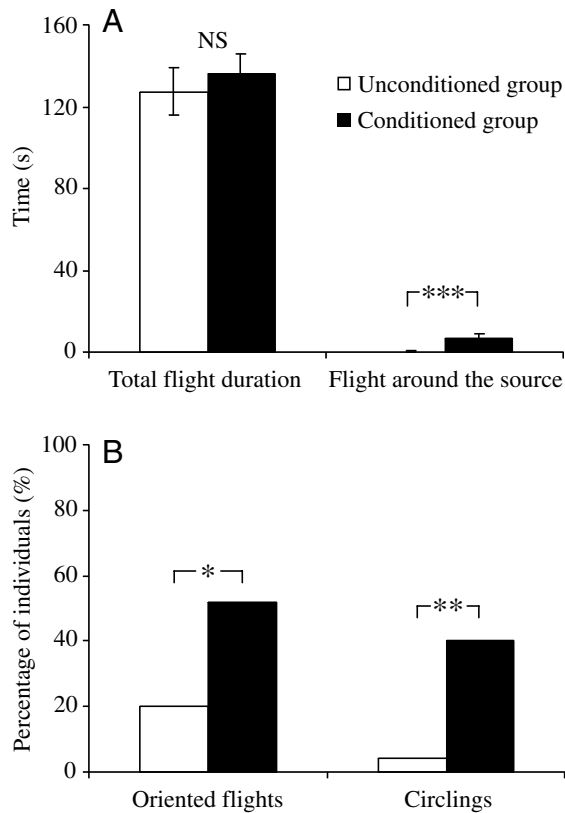


Fig. 3. Effect of a proboscis extension conditioning procedure on the orientation towards linalool. (A) Mean duration \pm s.d. (seconds) of the total flight and of the flight around the odour source. (B) Percentages of bees exhibiting orientated flights (up-wind zig-zag flight) and circling behaviour. Asterisks indicate significant differences: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not significant.

(Kerguelen and Cardé, 1997) and plant location in phytophagous insects (Prystupa et al., 1988; Reed and Landolt, 2002). Surprisingly, wind tunnels have not previously been used in the study of flight orientation in the honey bee, probably because flight characteristics and social behaviour of this species have discouraged use of this type of experimental device based on the observation of single individuals in an enclosed space. Nevertheless, when appropriate conditions are established, worker bees can produce exploratory and orientated flights. Indeed, we were able to apply common flight descriptors to the honey bee: total flight duration, flight duration around the source, percentage of individuals exhibiting orientated flight and percentage of individuals circling close to the source. Moreover, the rearing procedures ensured that all the tested bees had experienced the same environmental context from emergence until testing. Thus, the overall procedure allowed us to study olfactory short-range orientation of the bees under highly standardised conditions.

We first analysed the behaviour of the bees in response to two types of olfactory stimuli: (1) linalool, a common floral compound (Knudsen et al., 1993) that is not known to have any pheromonal value to the honey bee, and (2) a kin odour

from nestmates. In all experiments, bees placed in the tunnel without odour (control groups) and bees subjected to odour stimulations did not differ with regard to the total flight duration, all of them flying for approximately half the observation period (5 min). This suggests that the mere presence of an odour stimulus did not enhance flight willingness. In the first experiment, the two odours induced orientated flights but they differed slightly with regard to the extent of this effect. Nestmate odour induced orientated flights in 68% of the workers, and circling behaviour around the source in 60% (compared with only 28% and 0%, respectively, for the control group), thereby demonstrating a strong influence of this social odour. Under the same conditions, linalool induced orientated flights in 48% of the bees and circlings in 28%. One can question whether the vapour pressure and the diffusion range of the odours might affect the performance of the bees. With regard to the nature of the social odour, the volatiles produced by caged worker bees contained at least Nasonov pheromone, the main active components of which are geraniol, citral and nerol. All these components, as well as linalool, have approximately the same volatility (boiling point at 760 mmHg; linalool 198–200°C; geraniol 229–230°C; citral 228–229°C; nerol 225–228°C). We can thus assume that the diffusion of the tested odours within the tunnel did not differ significantly and that this factor did not affect our results.

The social odour produced by caged nestmates was supposed to mimic the social cues that can drive the approach flight of a bee returning to the hive. Nest olfactory recognition mainly relies on the orientation pheromone produced by the Nasonov gland (Pickett et al., 1980; Ferguson and Free, 1981), which could be the basis of the attraction observed in our experiments. Studies on the production of geraniol, a main active component of the Nasonov pheromone, have shown high variation with age (Boch and Shearer, 1963): very young bees do not produce geraniol, but appreciable amounts are produced after the bees are more than 12 days old. Thus, our caged bees actually released Nasonov pheromone. Consequently, they might have experienced this odour (or other compounds belonging to their nestmates) before testing, in an imprinting-like process. Indeed, the recognition of the nest odour is known to involve early learning of various odours such as cuticular hydrocarbons and odours of stored food (Breed and Stiller, 1992; Breed et al., 1995, 1998). Thus, the observed attraction might rely on an innate response to the compounds and/or on a previous experience during the development of the bee. With the exception of the nestmate odours, we can assume that our laboratory rearing conditions led to a much reduced olfactory exposure, ensuring no exposure to linalool at least from the time of emergence. By contrast, we cannot reject the possibility that the bees had experienced linalool during their development. Indeed, the effects of passive exposure to environmental cues during development are well documented in insects. This exposure most often occurs at the early adult stage, but some work has also indicated preimaginal experiences (Isingrini et al., 1985; Dobson, 1987; Carlin and

Schwartz, 1989). In the honeybee, Sandoz et al. (2000) have found an effect of early adult exposure but no effect of preimaginal exposure. An exposure to linalool, before we collected and caged the bees, could thus explain why this floral compound induced orientated flights. More generally, similarities between the responses to nestmate odour and linalool also suggest that olfactory orientation at short range might be first based on a non-specific response to the presence of an odour, which could be modulated according to the biological value of the odorant stimulation (e.g. floral or pheromonal volatiles) or to the previous experience of the bee.

The second experiment was designed to consider whether a previous experience (classical olfactory conditioning of the proboscis extension) could actually modify odour-cued orientation at short range. Some bees exhibited spontaneous proboscis extension to linalool at the first presentation of this odour. For our purpose, these bees were discarded from the experiment since their later responses could not be attributed to associative learning alone. The bees that responded spontaneously represented 20% of all the tested bees, which is similar to the values reported in the literature (Bhagavan et al., 1994; Sandoz et al., 1995; Laloi et al., 2001). Our results showed that, with linalool diffused upwind, only 20% of the non-conditioned bees made orientated flight, and only 4% of them exhibited circling behaviour. The orientation performance towards this floral odour was strongly enhanced (up to 52% of orientated flights and 40% of circlings) by prior classical olfactory associative learning. Moreover, the patterns of response observed in the first experiment are similar to those obtained after olfactory conditioning. This suggests that the results of the first experiment could also reflect some kind of associative experience.

This result demonstrates, under controlled conditions, that honey bees can use olfactory information gained in a previous classical conditioning procedure in a subsequent flight orientation context. This complements previous studies in the honey bee on information transfer from one context to another. First, Jakobsen et al. (1995) showed that free-flying bees could be attracted to artificial feeders by an odour added in the hive. Thereafter, Gerber et al. (1996) showed a possible information transfer of learning in a free-flying foraging situation (instrumental context) to the reflex response of proboscis extension (Pavlovian context). In situations more closely related to our experiment, two studies have shown that olfactory information acquired in a classical conditioning of the proboscis extension reflex can influence the walking orientation performance of a bee in an olfactometer device. Bakchine et al. (1992) found that conditioning to geraniol, a pheromonal/floral compound, increased the orientation response of bees towards this odour in a four-armed olfactometer. Sandoz et al. (2000) obtained similar results with two floral compounds, linalool and phenylacetaldehyde. More generally, these results suggest that a forager could gain information about the odours it can use to navigate and search for food not only during foraging (von Frisch, 1967; Menzel et al., 1993) but also within the hive before its first foraging

bout. Indeed, worker bees returning to the hive bring odours and food that constitute, at least for a certain time period, a good indication of the available resources. Future foragers should thus have already acquired some kind of olfactory preferences that could improve their subsequent foraging performance.

Our results show the necessity of considering the influence of learning when addressing the question of olfactory orientation even at short distance. For this, the wind tunnel could be a powerful tool for examining precisely the variation of flight structure and orientation performance in response to various factors. These factors could be the nature and the complexity of odorous stimuli, the distance at which compounds are detected, as well as various types of learning procedure such as differential conditioning in which bees are conditioned to discriminate a rewarded odour from a non-rewarded one. The wind tunnel could also be particularly suitable for studying the role of odours in honey bee decision making during foraging.

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