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Olfactory learning by means of trophallaxis in *Apis mellifera*

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Summary

Early reports indicate that trophallaxis, i.e. the exchange of liquid food by mouth, may allow honeybees to assign nectar odours with predictive values to anticipate biological meaningful reward stimuli. Nevertheless, this type of learning has not been addressed directly. In the present study, pairs of animals were isolated to induce trophallaxis under controlled conditions and, afterwards, the honeybee proboscis extension reflex was used to investigate the possible role of trophallaxis in learning olfactory cues. The results demonstrate unambiguously that associative learning actually occurs by means of trophallaxis. Animals associate the odour (as the conditioned stimulus or CS) and the sucrose (as the unconditioned stimulus or US) present in the solution they receive through trophallaxis. Moreover, this particular kind of learning leads to long-term olfactory memories after a single learning trial, even when trophallaxis is brief. In addition, we found that the strength of association is clearly affected by CS and US intensity as well as the recent previous foraging experiences of the animals. Comparisons are presented among several features of the learning during trophallaxis and the classical conditioning of the proboscis extension reflex with restrained subjects. Finally, the relevance of learning through trophallaxis in the task of successful foraging is discussed.

Key words: honeybee, *Apis mellifera*, trophallaxis, olfactory conditioning, associative learning.

Introduction

Honeybees learn which environmental stimuli predict biological meaningful reinforcing stimuli and benefit from well-developed learning abilities to gather energy efficiently from the environment. As a consequence, associative learning becomes an essential component of the honeybee foraging behaviour (Menzel, 1985; Gould, 1993). Thus, for instance, forager honeybees learn visual and chemical cues to search and recognize their foraging targets (von Frisch, 1965). Hence, their choice behaviour is not random but guided by specific memories that usually lead to an optimisation of relative profits (Greggers and Menzel, 1993).

In 1923, Karl von Frisch (von Frisch, 1923) demonstrated that floral scents clinging to the body of an experimental bee (returning to the nest from a natural nectar source) can stimulate other individuals of the same colony to leave the nest and visit the prospective source. Yet, within the broad spectrum of the floral scents nectar is also perfumed with specific fragrances. In view of that, von Frisch (1946) extended his analyses by considering nectar odours as possibly also being olfactory cues that might enhance recruitment. Specifically, he took into account that each forager carries a scented crop of nectar in its honey stomach each time it enters the nest after a successful bout. Thus, since nectar is distributed rapidly among colony members by means of trophallaxis, i.e. the exchange of liquid food by mouth (Doolittle, 1907; Rösch, 1925; Nixon and Ribbands, 1952; Free, 1956), its distribution might enhance recruitment on the basis of olfactory learning occurring during trophallaxis. In other words, forager-mates receiving nectar samples inside the nest might learn (and later recognize) the odour of the nectar being collected and search for the prospective nectar source by using its particular olfactory cues in their subsequent flights.

Thus, von Frisch (1946) presented marked bees with sugar solutions scented with the particular fragrance of a given flower species ‘A’. In doing this, however, he used an artificial feeder designed in such a way that bees contacted the offered solution only with their tongues (i.e. animals gathered the offered reward without exposing their bodies to the scented solution). In addition, this feeder was externally enwreathed with scented petals of a second flower species ‘B’. As a result, the marked foragers returning to the nest carried sugar solutions that contained the odour ‘A’ (the nectar scent belonging to the first flower species) and, at the same time, their bodies carried externally odour ‘B’ (the floral scent

belonging to the second flower species). Afterwards, he analysed the effects of both odours (A and B) on recruitment by placing two different outdoor flower dishes (each presenting flowers of the first or the second flower species) and then counting the number of recruited bees arriving at each one of the dishes. He found that recruited bees searched preferentially for the flower species whose odour (A) was diluted in the offered solution, especially when the training feeder was placed at a distance several hundred meters from the nest. Following a series of complementary experiments, von Frisch concluded that, in addition to the floral odours attached externally to the forager bodies, specific olfactory information (about the flower species being exploited) is transferred to nest-mates on the basis of nectar scents. Subsequent experiments also demonstrated that foragers usually receive nectar samples before being recruited (Dirscheld, 1960) and that learned scents blown artificially inside the nest can trigger visual memories of specific locations that trained bees have previously visited (Reinhard et al., 2004).

Indeed, trophallaxis, the behaviour through which returning foragers transfer their nectar crops to other members of the colony (Doolittle, 1907; Rösch, 1925; Nixon and Ribbands, 1952; Wilson, 1971), might allow nest-mates to learn the specific olfactory cues of the nectar they receive. During a single trophallactic interaction, a food-donor opens its mandibles broadly, keeping its antennae downward and close to the head, while a variable number of recipient nest-mates start contacting its prementum with their protruded proboscis to sip the nectar it proffers, also moving their antennae towards the donor (Free, 1957, 1959). Thus, the recipients receive both olfactory and gustatory stimulation. However, although early reports indicated that trophallaxis may allow honeybees to assign nectar odours with predictive values (Butler, 1951; Ribbands, 1955; von Frisch, 1965), the prospective olfactory learning involved in trophallaxis has been never addressed directly. As a consequence, the idea that foragers transfer nectar-related olfactory information by delivering scented nectar inside the nest relies basically on indirect evidence (Ribbands, 1955; von Frisch, 1965). That is, the role of trophallaxis in learning nectar-related olfactory cues has not been analysed by measuring the trophallactic behaviour of the foragers and its possible correlation with well-quantifiable learning performances. Instead, it has been inferred from the ensuing choice behaviour of the animals (von Frisch, 1946). Obviously, the latter perspective relies on the fact that nectar is distributed by means of trophallaxis within honeybee colonies. However, it is a complex set of visual and chemical stimuli which, alongside innate strategies improving the gathering of energy as well as specific memories, determines the choice behaviour of free-flying honeybees (von Frisch, 1965). Hence, to address directly the possible olfactory learning involved in trophallaxis and its function in the context of the foraging task requires, initially, a detailed quantification of both trophallaxis and learning under controlled experimental situations that resemble natural conditions as closely as possible. Such an analysis does not yet exist. Furthermore, the effects of both the odour and sugar concentration present in the transferred nectar on the possible olfactory learning occurring during trophallaxis are entirely unknown. In addition, if honeybees acquire specific olfactory memories by means of trophallaxis, it would be extremely important to identify whether these are short- or long-term memories. This distinction might have important implications on the foraging strategies arising both at the individual and the group-level.

The first aim of the present study was therefore to examine whether a single trophallactic interaction might serve a forager to associate the odour (as the conditioned stimulus or CS) and the sugar (as the unconditioned stimulus or US) present in the sucrose solution it receives through trophallaxis. During the experiments, pairs of animals were first isolated to induce trophallaxis under controlled conditions. Afterwards, classical olfactory conditioning of the honeybee's proboscis extension response (PER conditioning), a well-developed method used extensively to analyse different aspects of appetitive learning and memory formation (Kuwabara, 1957; Takeda, 1961; Bitterman et al., 1983; Menzel, 2001), was used to investigate the possible role of trophallaxis in learning olfactory cues. We argued that if a honeybee perceives an odour stimulus diluted in the sucrose reward it receives during trophallaxis (immediately before sucrose or even simultaneously), it must form an association between the two stimuli such that the odour may trigger the animal's proboscis extension in subsequent tests (as the conditioned response or CR). In addition, we addressed three further important questions: (i) the effect of the odour concentration (CS intensity), (ii) the effect of the sugar concentration (US intensity) and (iii) the time course of the olfactory learning by means of trophallaxis. Finally, the relevance of learning through trophallaxis in the task of successful foraging is discussed.

Materials and methods

Methods

We first examined whether the proboscis extension response of a forager honeybee can be conditioned to the odour present in the sugar solution it receives during a single trophallactic interaction. Next, we examined the effects of both CS and US intensity (i.e. the odour concentration and the sugar concentration, respectively, of the solution received through trophallaxis) on the olfactory learning occurring during trophallaxis. We used *Apis mellifera* L. foragers from a colony of approximately 20 000 individuals. Experiments were conducted between February and April in the Experimental Field of the Faculty of Exact and Natural Sciences of the University of Buenos Aires (34°33′S, 58°26′W).

Experimental subjects

Foragers were labelled and trained to collect unscented sucrose solution at an *ad libitum* feeder placed 10 m away from the hive. At the beginning of each trial, two arriving foragers were captured at the feeder (without allowing them to make contact with the offered solution) and carried to the laboratory

by using individual plastic tubes. Afterwards, one of the foragers (the donor bee) was fed with sucrose solution (up to satiation) while the other one (the recipient bee) remained unfed inside the plastic tube. Each donor was fed through the end of the plastic tube, which was covered by a soft mesh with slots whose diameters (~1 mm) were larger than the cross section of the honeybee proboscis. Thus, donors placed individually inside the tubes were easily able to extend their proboscis through the mesh in order to get the sugar solution offered (from outside) *via* a graded capillary tube. In this way, they contacted the offered solutions only with their proboscis. During single trials, the time elapsed between both animals being captured and the donor being fed was approximately 5 min. The sugar solutions offered to donors presented different odours and sucrose concentrations according to the experimental series described below.

Recording trophallaxis during a single experimental session

Once the donor had been fed, both donor and recipient were placed inside a transparent experimental arena to induce trophallaxis (for a detailed description of the arena, see Farina and Núñez, 1991). Once in the arena, animals were initially separated by a sliding door placed in the centre of the arena. Each session started when the door was removed (allowing the animals to get in contact with each other) and finished when a single trophallaxis had occurred or when no trophallaxis occurred after the first 10 min following the beginning of the session. Following trophallaxis, the recipient only was further used, testing its proboscis response as described below. If no trophallaxis occurred the animals were removed and the procedure was repeated with a different pair of animals. The duration of trophallaxis was recorded. We obtained 10–20 animals per day that received sucrose solution during a single trophallactic event.

Harnessing

Once a single trophallaxis occurred between donor and recipient, the recipient was induced to enter a small plastic harness (a cone-shaped tube 4 cm long with an open end 4 mm in diameter) from which its head remained protruded, by exploiting the natural positive phototaxis of honeybees. A light bulb was placed 30 cm above the arena and the plastic roof of the arena was covered with a sheet of paper while the harness remained illuminated. Following trophallaxis (when both donor and recipient remained separated by the sliding door), the light was turned on and the recipient rapidly moved from the inner arena into the illuminated harness. Afterwards, it was fixed in the harness by two pieces of thin tape, one placed on the top between the head and the thorax and the other horizontally behind the thorax. Thus, the animal could freely move its antennae, mandibles and proboscis. Once fixed in the harnesses, recipients were placed in racks within a dark humidified chamber. In the evening following trophallaxis, they were fed up to satiation (unscented 1.8 mol l^{-1} sucrose solution) and kept inside the chamber until tested.

Testing the proboscis extension response of the recipient bees

By testing the subsequent proboscis extension of the recipients we could determine whether an association had been established between the odour (CS) and sucrose (US) present in the sugar solution received through trophallaxis. Each recipient was tested 21, 27 and 46 h following trophallaxis (first, second and third test, respectively). During each of the three different tests, animals were presented with two different odours. Hence we always tested their responses to both linalool, the odour added (or not) to the solution they had received during trophallaxis (see the experimental series described below), and eugenol, a second odour to which the animals were never exposed.

Half of the bees were presented with the sequence linalool–eugenol and the remaining half with the sequence eugenol–linalool. 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 20 s accommodation period, after which the respective 5 s stimulation started. After stimulation bees remained at the test site for other 15 s and were then placed back in the racks.

Prior to the beginning of each of the three tests (30 min), animals were stimulated by applying sucrose solution (1.8 mol l^{-1}) to their antennae to determine whether or not they responded to the US. Recipients that failed to respond were excluded from the analysis. These trials were performed outside the test room to avoid possible associations among unspecific features of the test room and the US. Spontaneous responses to the air stream were also tested prior to odour stimulation. Animals that responded positively to the air stream were excluded from the analysis as well. In between successive tests, bees were kept in the dark humidified chamber and only fed up to satiation (as described above) in the evening following the first two tests.

Measuring the learning performances

Throughout the experiments, each animal was considered to show a conditioned response (CR) when it only responded to linalool. Animals that responded to eugenol, i.e. a second control odour (see above) and not to linalool, as well as those that responded to both odours (0 and 4.6%, respectively, throughout all the experiments described here) were excluded from the analysis.

Next, for each of the three different tests, we calculated the percentage of positive proboscis extensions (%PE_1, %PE_2 and %PE_3, corresponding to the first, second and third tests, respectively) as the proportion of animals that showed a CR, as calculated from the total number of tested animals after excluding (1) animals that responded to the control odour, (2) animals that failed to respond to the US prior to the test and (3) animals that responded to the air stream prior to the test.

In addition, we calculated a general percentage of positive

proboscis extensions (%PE_G) as the proportion of animals that showed a CR in any of the three different tests, calculated from the total number of tested animals, after excluding (1) animals that responded to the control odour in any of the single tests, (2) animals that failed to respond to the US in all the single tests and (3) animals that responded to the air stream in all the single tests.

Experimental series

Three different experimental series were performed to vary both the odour concentration and sucrose concentration of the sugar solution that recipients received during trophallaxis, and the sucrose concentration they had experienced previously at the feeder (where animals were captured at the beginning of the experiments). After donors had been fed (see above), the experimental procedure was identical in all series.

Series 1: Olfactory conditioning by means of trophallaxis

In this experimental series we addressed whether olfactory conditioning occurs during a single trophallactic interaction. First, foragers were allowed to collect unscented 1.8 mol l^{-1} sucrose solution at the feeder. Next, donors were fed with either scented (50 µl of linalool per litre of sucrose solution) or unscented 1.8 mol l^{-1} sucrose solution. Finally, following trophallaxis, the conditioned responses of the recipients were tested as described above.

Series 2: Effect of CS intensity

In this experimental series we analysed the effects of CS intensity on the subsequent CR. As in the previous series, foragers collected unscented 1.8 mol l^{-1} sucrose solution at the feeder. Next, six different experimental groups were defined based on the odour concentration of the sugar solution (1.8 mol l^{-1} sucrose solution) used to feed the donors, i.e. 0 (unscented), 0.1, 1, 5, 50 and 100 µl linalool l^{-1} solution. Donors were offered the different solutions quasi-randomly over successive experimental days. As before, the conditioned responses were tested following trophallaxis.

Series 3: Effect of US intensity

We also analysed the effects of US intensity on the subsequent CRs. In this experimental series, foragers collected unscented 0.5 mol l^{-1} sucrose solution at the feeder. Next, donors were fed with scented solutions that presented sucrose concentrations of either 0.5 mol l^{-1} or 1.8 mol l^{-1}, employing an odour concentration of 5 µl linalool l^{-1} solution, which had elicited intermediate response levels in the second series (see Results). In addition, a third group of donors (i.e. control group) was fed with unscented 0.5 mol l^{-1} sucrose solution. The different solutions were used quasi-randomly. As before, the conditioned responses were tested following trophallaxis.

Statistical analysis

The percentages of positive proboscis extensions were compared using *G*-tests (comparisons among groups) and McNemar tests (comparisons among different tests). Data on the duration of trophallaxis were analysed using *t*-tests, analysis of variance (ANOVA) and Tukey–Kramer comparisons (Zar, 1984).

Results

Series 1: Olfactory learning by means of trophallaxis

In the first experimental series we compared the learning performances from two different groups of animals that received either unscented or scented (50 µl linalool l^{-1} solution) 1.8 mol l^{-1} sucrose solution during a single trophallactic interaction. Fig. 1 shows the %PE_G obtained for each of these groups. The results clearly show a higher proportion of CR for animals that received scented solutions during trophallaxis (68.75% and 2.5% for bees that received either scented or unscented sucrose solution, respectively; $G_{(1)}$=57.9, P<0.0001, N=112, G-test). Obviously, only bees that have received the scented solution during trophallaxis respond to the scent in the test. The corresponding response levels obtained for the different tests (not illustrated) were: %PE_1=38.7, %PE_2=77.3 and %PE_3=66.7 for the animals that received scented solutions and %PE_1=0, %PE_2=1.8 and %PE_3=4,4 for those that received unscented solutions.

Series 2: Effect of CS intensity

Next we extended the analysis in order to evaluate the effects of CS intensity on the subsequent CRs. We thus compared responses from six different groups of animals defined on the basis of the odour concentration present in the sugar solution they received during trophallaxis (ranging from 0, i.e. unscented, up to 100 µl linalool l^{-1} solution, see Materials and methods). As shown in Fig. 2A (see the insert, hatched bars), the higher the odour concentration the higher the

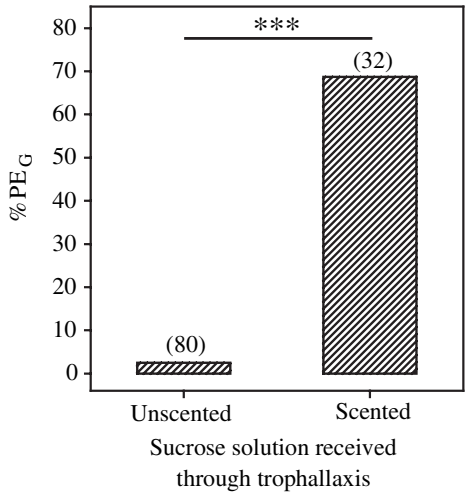

Fig. 1. General percentage of proboscis extensions (%PE_G) from two different groups of animals that received either unscented or scented (50 µl l^{-1}) 1.8 mol l^{-1} sucrose solution during a single trophallactic interaction. Asterisks indicate statistical differences (G-test, ***P<0.001; see Results for details). The number of animals is given in parentheses.

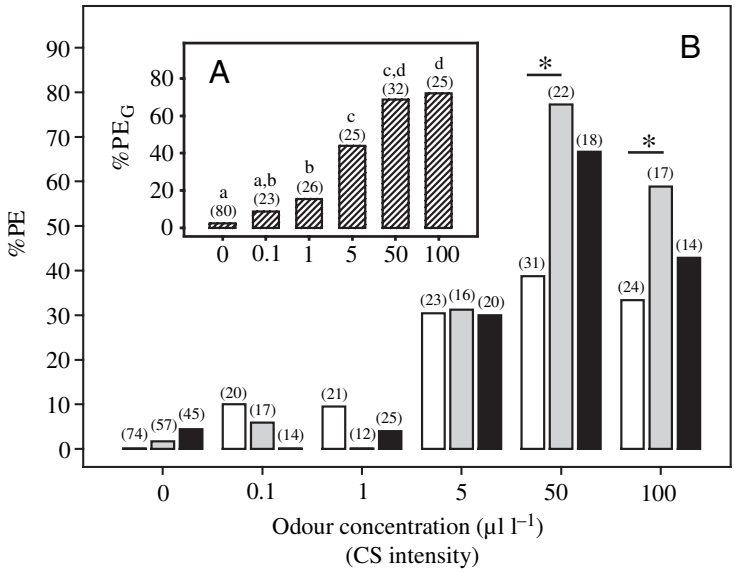

Fig. 2. Conditioned responses from six different groups of animals, defined on the basis of the odour concentration present in the 1.8 mol l^{-1} sucrose solution they received during a single trophallactic interaction. (A) General percentage of proboscis extensions (%PE_G). Letters indicate statistical differences among the different odour concentrations (*G*-test, $P<0.001$; see Results for details): the results of two groups that do not differ significantly are denoted by the same letter. (B) Percentage of proboscis extensions (%PE) corresponding to the first (%PE_1, white bars), the second (%PE_2, grey bars) and the third test (%PE_3, black bars). Animals were tested 21, 27 and 46 h following trophallaxis in a cumulative fashion. Asterisks indicate statistical differences among tests (McNemar test, $*P<0.05$). The number of animals is given in parentheses. In B, differences in the sample size within a given odour concentration are due to differences in mortality and US responsiveness prior to the tests.

general response level (% PE_G: $G_{(5)}=91.8$, $P<0.0001$, $N=211$; data from 0 µl l^{-1}, unscented, and 50 µl l^{-1} correspond to the first experimental series). Hence, an odour concentration of 0.1 µl l^{-1} gave response levels that did not differ statistically from the spontaneous responses elicited by the animals that received unscented solutions. Odour concentrations of 1 and 5 µl l^{-1} gave increasing intermediate values and, finally, the highest odour concentrations assayed (50 and 100 µl l^{-1}) gave maximum response levels.

Fig. 2B shows the effects of CS intensity on the more specific response levels obtained for each of the three different tests (%PE_1: $G_{(5)}=43.7$, $P<0.0001$, $N=193$; %PE_2: $G_{(5)}=71.6$, $P<0.0001$, $N=141$; %PE_3: $G_{(5)}=41.6$, $P<0.0001$, $N=136$; *G*-test). Interestingly, for the highest odour concentrations (50 and 100 µl l^{-1}), responses increased significantly between the first and the second tests (performed 21 and 27 h following trophallaxis, respectively). Since animals were tested in a cumulative fashion (see Materials and methods), we expected that the first extinction test would lead to a similar or even a reduced response in the second test, but instead we found an increase (see Fig. 2B, white and grey bars, 50 µl l^{-1} group: $\chi^2=6.13$, $P=0.01$, 100 µl l^{-1} group: $\chi^2=5.14$, $P=0.02$; McNemar test). In the third test, the responses did not differ significantly from the first or the second test for both 50 and 100 µl l^{-1} concentrations (see Fig. 2B, black bars, 50 µl l^{-1}: %PE_1 *vs* %PE_3, $\chi^2=1.5$, $P=0.2$, %PE_2 *vs* %PE_3, $\chi^2=0.12$, $P=0.9$; 100 µl l^{-1}: %PE_1 *vs* %PE_3, $\chi^2=3.13$, $P=0.8$, %PE_2 *vs* %PE_3, $\chi^2=0.25$, $P=0.6$; McNemar test).

Series 3: Effect of US intensity

To analyse the effects of US intensity we compared responses from two different groups of animals that received different concentrations of scented sucrose solution during trophallaxis (either 0.5 mol l^{-1} or 1.8 mol l^{-1}). A control group of animals (which received unscented 0.5 mol l^{-1} sucrose solution) was included in the analysis (see Materials and methods). Results showed that the general response level increases together with the sucrose concentration (Fig. 3A, %PE_G: $G_{(1)}=8.7$, $P=0.003$, $N=64$; *G*-test). No responses were

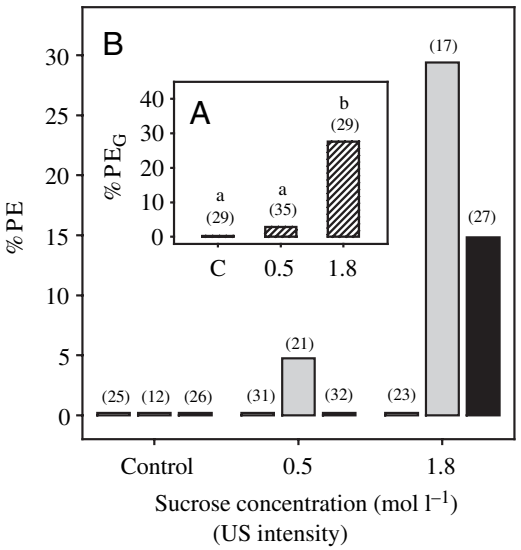

Fig. 3. Conditioned responses from two different groups of animals that received different concentrations of scented (5 µl l^{-1}) sucrose solution during trophallaxis (either 0.5 mol l^{-1} or 1.8 mol l^{-1}). A control group of animals (C) received unscented 0.5 mol l^{-1} sucrose solution. (A) General percentage of proboscis extensions (%PE_G). Letters indicate statistical differences among the different odour concentrations (*G*-test, $***P<0.001$; see Results for details): the results of two groups that do not differ significantly are denoted by the same letter. (B) Percentage of proboscis extensions (%PE) corresponding to the first (%PE_1, white bars), second (%PE_2, grey bars) and third tests (%PE_3, black bars). Animals were tested 21, 27 and 46 h following trophallaxis in a cumulative fashion. The number of animals is given in parentheses. In B, differences in the sample size within a given group are due to differences in mortality and US responsiveness prior to the tests.

found for the control group throughout the different tests (Fig. 3A,B). The performances of animals that received scented 0.5 mol l^{-1} sucrose solution did not differ statistically from the performances of the control group (%PE_G: $G_{(1)}$=1.2, P=0.3, N=64; G-test) but those that received scented 1.8 mol l^{-1} sucrose solution gave higher responses in comparison to the control group (% PE_G: $G_{(1)}$=12.4, P=0.0004, N=58; G-test).

With respect to the responses obtained in the different tests (Fig. 3B), animals did not respond to CS presentation in the first test. In the second test (%PE_2), response levels were 4.8% and 29.4% for animals that received 0.5 mol l^{-1} and 1.8 mol l^{-1} scented sucrose solutions, respectively ($G_{(1)}$=4.5, P=0.03, N=38; G-test). In addition, in the third test (%PE_3) results gave values of 0% and 14.8% for animals that received 0.5 mol l^{-1} and 1.8 mol l^{-1} scented solutions, respectively ($G_{(1)}$=6.6, P=0.01, N=59; G-test). The performances of the animals that received either unscented (i.e. the control group) or scented 0.5 mol l^{-1} sucrose solution did not differ when the second (%PE_2) and the third (%PE_3) tests are compared (%PE_2: $G_{(1)}$=0.9, P=0.3, N=33; G-test, %PE_3: animals did not respond in either one condition or the other). By contrast, the performances of the animals that received scented 1.8 mol l^{-1} sucrose solution were significantly higher than those of the control group in the case of the same two tests (%PE_2: $G_{(1)}$=6.1, P=0.01, N=29; %PE_3: $G_{(1)}$=5.7, P=0.02, N=53; G-test). In this series, data did not allow within-test comparisons, due to the high proportion of animals that did not show conditioned responses.

Effects of the recent previous foraging experience

Next, we evaluated possible effects of previous recent foraging experiences, i.e. the sucrose concentration offered at the training feeder, on the subsequent learning performances of the recipients (data correspond to the second and the third experimental series). We thus compared responses from two different groups of animals that collected either 0.5 mol l^{-1} or 1.8 mol l^{-1} unscented sucrose solutions (see Materials and methods) prior to their trophallactic interactions. Once in the arena, all the animals received scented (5 µl linalool l^{-1} solution) 1.8 mol l^{-1} sucrose solution by means of trophallaxis. As Fig. 4 shows, animals that collected 0.5 mol l^{-1} sucrose solution prior to trophallaxis did not respond to CS presentation during the first test, whereas the %PE_1 was significantly higher (30.4%) for animals that collected 1.8 mol l^{-1} sucrose solution (Fig. 4, white bars, %PE_1, $G_{(1)}$=10.9, P<0.001, N=46; G-test). The responses of both groups did not differ statistically in the second and the third tests, although a tendency was found, suggesting higher response levels for animals that had collected 1.8 mol l^{-1} sucrose solution at the feeder (Fig. 4, grey bars, %PE_2=29.4% (0.5 mol l^{-1}) *vs* 31.2% (1.8 mol l^{-1}), $G_{(1)}$=0.15, P=0.7, N=33; black bars, %PE_3=14.8% (0.5 mol l^{-1}) *vs* 30% (1.8 mol l^{-1}), $G_{(1)}$=1.57, P=0.2, N=47). Likewise, the general response level (%PE_G, not illustrated) did not differ statically between both groups ($G_{(1)}$=1.59, P=0.2, N=54; G-test), although a tendency also suggested higher response levels for the animals that collected 1.8 mol l^{-1} sucrose solution prior to trophallaxis (27.6% and 44.0% for 0.5 and 1.8 mol l^{-1}, respectively).

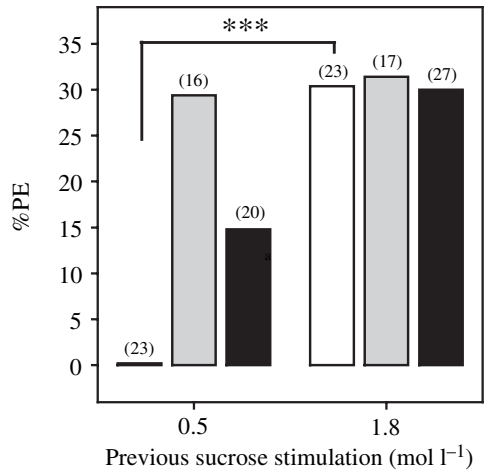

Fig. 4. Conditioned responses from two different groups of animals that collected different unscented sucrose concentrations at the training feeder (either 0.5 mol l^{-1} or 1.8 mol l^{-1}) prior to trophallaxis. Once in the arena, all the animals received scented (5 µl l^{-1}) 1.8 mol l^{-1} sucrose solution during trophallaxis. Data are presented for the first (%PE_1, white bars), the second (%PE_2, grey bars) and the third test (%PE_3, black bars). Animals were tested 21, 27 and 46 h following trophallaxis in a cumulative fashion. Asterisks indicate statistical differences among tests (G-test, ***P<0.001). The number of animals is given in parentheses. Differences in the sample size within each group are due to differences in mortality and US responsiveness prior to the tests.

The duration of trophallaxis

The duration of trophallaxis ranged from 1.2 s up to 23.6 s throughout the totality of the experiments (11.7±0.6 s, mean ± S.E.M.). In the second experimental series, analysing the effects of CS intensity, no differences were found among groups ($F_{(5,223)}$=0.88, P=0.5, one-way ANOVA). By contrast, in the third experimental series, addressing the effects of US intensity, differences appeared among groups ($F_{(5,241)}$=10.1, P<0.001, one-way ANOVA after log transformation). The higher the sucrose concentration the lower the duration of trophallaxis when animals exchange scented sugar solutions (P<0.001, Tukey–Kramer comparison). No differences appeared between these groups and the control group (unscented 0.5 mol l^{-1} *vs* scented 0.5 mol l^{-1}: P=0.1, unscented 0.5 mol l^{-1} *vs* scented 1.8 mol l^{-1}: P=0.06, Tukey–Kramer comparisons).

We also tested for a possible correlation between the duration of trophallaxis and the subsequent learning performances of the recipients, using a *post-hoc* analysis. To this end, we considered the trophallactic interactions of the animals that showed subsequent conditioned responses. Thus, for all the series assayed, two different groups of animals were defined according to the responses they showed in the various tests: (1) bees that showed conditioned responses and (2) bees

that did not. Afterwards, the durations of their respective trophallactic interactions were compared. In the second experimental series, no differences were found among groups (interaction term: $F_{(5,216)}$=0.56, P=0.7; PE response factor: $F_{(1,217)}$=0.53, P=0.5, odour concentration factor: $F_{(5,217)}$=0.79, P=0.6, two-way ANOVA). After pooling data from the totality of the odour concentrations assayed in this series, durations (mean ± S.E.M.) were 11.8±0.7 s and 12.2±0.5 s for animals that responded and animals that did not respond during the various tests, respectively. In the third series, only the situation in which animals received scented 1.8 mol l^{-1} sucrose solution was analysed (sample sizes did not allow comparisons for the remaining treatments). As before, no differences were found in the mean duration of trophallaxis ($t_{(1,27)}$=1.43, P=0.2, t-test). Values (mean ± S.E.M.) were 13.4±1.2 and 10.4±1.3 for animals that responded and those that did not respond, respectively.

Discussion

Olfactory learning by means of trophallaxis

Early reports suggested that trophallaxis allows honeybees to learn nectar-related olfactory cues (Butler, 1951; Ribbands, 1955; von Frisch, 1965). However, a simultaneous quantification of both trophallaxis and learning was never directly addressed. As a result, there is no conclusive analysis on the possible role of trophallaxis in learning nectar-related olfactory cues. We therefore took advantage of the olfactory conditioning of the proboscis extension response in honeybees (Menzel, 2001) to test the proboscis extension responses of animals that received either scented or unscented sugar solution during a single trophallactic interaction. We found that responses were markedly higher when the animals received scented solutions (Fig. 1). This definitely proves olfactory learning during trophallaxis. Hence, the exchange of scented liquid food by mouth leads to associative learning in honeybees after only one single event and one may view the donor bee as a 'teacher' for the recipient bee whenever the olfactory information carried by the donor reduces the recipient's level of uncertainty.

Associative learning is usually characterized as either classical (Pavlovian) conditioning (stimulus–stimulus and stimulus–response associations) or operant (instrumental) conditioning (response–contingent reinforcing; Pavlov, 1927; Colwill and Rescorla, 1986). In classical conditioning, conditioned stimuli (CS) become predictive for unconditioned stimuli (US). After conditioning (forward-pairing of CS and US) CS elicit conditioned responses (CR). The CR can be considered as anticipatory responses, as a training of behavioural habits, or as conditioned motivations and emotions that are appropriate to the unconditioned reward stimulus (Pavlov, 1927; Colwill and Rescorla, 1986). In operant conditioning, the animal's spontaneous responses are strengthened by response–contingent reinforcement (Skinner, 1938; Hebb, 1956; Rescorla, 1994). During trophallaxis, honeybees display active behaviour. Hence, one might ask whether or not olfactory learning through trophallaxis constitutes operant conditioning; however, trophallaxis represents an instinctive behaviour that neither occurs by chance nor needs be learned. Hence, the idea of olfactory learning through trophallaxis representing a classical associative conditioning is undoubtedly more likely.

Under our experimental conditions, both stimuli (CS and US) are perceived during trophallaxis. In addition, the odour (CS) is diluted in the sucrose solution (US) that animals receive. Thus, although the temporal relationship between CS and US is rather fixed it may vary with the performance of both animals during trophallaxis. Yet the CS (odour) will always either precede the US or occur at the same time as the US. In classical PER conditioning the highest learning rates are observed when the CS is presented a few seconds before the US (forward conditioning), but simultaneous paring of both stimuli is also effective (Menzel, 1969, 1990). In addition, conditioned responses frequently develop with repetition of conditioning trials, although single trial learning is known from a few examples. In the classical PER conditioning, a single pairing of CS and US raises the animals' responses from a very low spontaneous level (<10%) up to mostly >50% (Bitterman et al., 1983). In trophallaxis, a single learning trial raised the responses from 3% (spontaneous responses) up to 70%, indicating a fast and robust form of learning. All our experiments were carried out with forager bees. By using classical PER conditioning, however, Ray and Ferneyhough (1999) reported differences in learning performances when foragers and younger bees are compared. Since trophallaxis also occurs between young bees (nurse and guard bees), it will be interesting to study learning through trophallaxis in younger workers.

Effect of CS intensity

We assume that the amount of odour diluted in the sucrose solution that animals receive during trophallaxis is directly related to CS intensity. We found that olfactory learning through trophallaxis improved with higher CS intensities (Fig. 2). In the classical PER conditioning, odour concentration also affects conditioned responses (Pelz et al., 1997), although odour application differed in our situation from that applied normally in PER conditioning experiments. Pelz et al. (1997), for instance, used 10 µl of an odour per litre of solvent as the lowest initial dilution. In our experiments, the minimal odour concentration that allowed learning unambiguously was 5 µl of odour per litre of sugar solution (Fig. 2). In the classical PER experiments, however, the odours presented during conditioning were always dissolved in air, but in our experiments they were mixed with water (i.e. dissolved in the solution transmitted during trophallaxis). It is unknown how these different procedures affect the final concentrations that reach the chemoreceptors at the antenna, and thus a quantitative comparison is not possible.

The effects of CS intensity on learning through trophallaxis might be explained from two different perspectives. On the one hand, a low concentration of diluted odour might not be

detectable. As a result, the corresponding pairing of CS–US cannot be achieved. On the other hand, although perceived, a highly diluted odour might lead to an insufficient olfactory stimulation, i.e. it might lie below a certain threshold value that must be exceeded to assign the odour with a predictive value according to associative learning processing. Based on several earlier results, the latter possibility is more likely. First, it has been shown that honeybees can perceive scents even when greatly diluted and, accordingly, their ensuing threshold of odour perception is comparable to that of a man (von Frisch, 1919, 1965; Ribbands, 1954). In this study, the lowest odour concentration ($0.1\ \mu l\ l^{-1}$) was weakly perceived by the researcher. Thus, it is reasonable to assume that the animals were able to perceive all the odour concentrations used during the second experimental series. Secondly, recent theories on associative learning have introduced the concept of 'salience', i.e. an experience-independent feature of a conditioned stimulus that determines the rate at which it can enter into associations with a given reward (Rescorla and Wagner, 1972; Sutton and Barto, 1990). Thus, learning depends on stimulus salience (for alternative views, see Durlach, 1989; Spear et al., 1990). Higher concentrations of an odour may lead to higher salience and thus better learning. Intensity and salience effects on learning can only be separated if the perceptual intensity is controlled as, for example, in the study by Pelz et al. (1997), and if odours of equal subjective strength are compared.

Memory over time

Animals were tested at three different times following trophallaxis: 21, 27 and 46 h. According to the well-studied temporal dynamics of memory formation after PER conditioning (Menzel, 1999), animals were tested during early and later long-term memory. Under our conditions, a single trophallactic interaction leads to high levels of memory in both memory phases. Thus, a single trial of trophallaxis induces long-term olfactory memories. This is different from PER conditioning. In PER conditioning the memory after trial has usually already begun to decay several hours after acquisition. Multiple conditioning trials, however, induce a stable, long-lasting memory (Menzel, 1999). It thus appears that the transition to long-term memory may not always require multiple learning trials, as believed so far. Interestingly, our data also show that responses increased over time for the highest CS intensities but not for lower CS intensities. Improvement of retention over time is usually interpreted as indicating a consolidation process. Accordingly, it might be concluded that memory consolidation may be stronger for high than for low CS intensities. In addition, although not statistically significant, a tendency was found indicating lower response levels in the latter test (only for the highest CS intensities). Further experiments employing single tests distributed over time are required to evaluate whether extinction underlies this tendency.

Effect of US intensity

Stronger US intensities usually lead to better learning (Rescorla and Wagner, 1972). The experiments of series 3 constitute the first attempt to investigate the role of sucrose concentration (US intensity) in a task involving learning through trophallaxis. We found that higher US intensity enhances conditioned responses after a single conditioning trial. In addition, in evaluating the effects of US intensity, we always employed an odour concentration ($5\ \mu l\ l^{-1}$) that elicits intermediate response levels, and it will be interesting to consider different combinations of both US and CS intensities for further research.

In the honeybee, previous work also analysed the effects of US intensity (sucrose solution) on appetitive learning. By means of classical PER conditioning, for instance, Bitterman et al. (1983) found similar learning performances when sucrose solutions of 7, 20 and 40% w/w (i.e. mass of sucrose/mass of solution) were used as US intensities, although the lowest US intensity reduced the rate of acquisition. In addition, no differences in retention were found when $0.5\ mol\ l^{-1}$ and $2.5\ mol\ l^{-1}$ sucrose solutions were used as US in classical PER conditioning (Menzel et al., 2001). The effects of US intensity on associative learning were also tested in the case of tactile learning and, interestingly, foragers differed with respect to their responsiveness to different concentrations of sucrose. Hence, in honeybees, the value of a sucrose concentration as the US has a relative quality (Scheiner et al., 1999; Scheiner, 2004).

Effect of the recent previous foraging experience

Interestingly, under our experimental conditions, learning was also affected by the recent previous foraging experiences of the animals, i.e. the sucrose concentration they experienced at the training feeder prior to trophallaxis (see Materials and methods). The higher the sucrose concentration they experienced previously the higher the percentage of subsequent conditioned responses, especially during the first test (Fig. 4). It is well known that sucrose modulates ongoing activities in honeybees. That is, it affects the motivational level of the animals and may enhance the probability or even the strength of several responses to other stimuli, leading to a status of 'arousal', i.e. a short-lived behavioural state that may accelerate the gathering of information required for the formation of specific associative memories (Hammer and Menzel, 1995). Thus, a stronger sucrose stimulation at the feeder may arouse the animal and lead to better learning because of higher sensitivity to the stimuli that will be perceived shortly during trophallaxis.

The duration of trophallaxis

The duration of the trophallactic interactions that led to learning ranged from 1.2 s to 23.6 s. Thus, olfactory learning occurs even when trophallaxis is very short. Experiments with free-flying honeybees raised the question whether the strength of association increases with the duration of sucrose stimulation (Buchanan and Bitterman, 1988; Couvillon et al., 1991; Menzel and Erber, 1972). Only small effects of US duration were found. In PER conditioning, the duration of

sucrose stimulation (even beyond 1 s) appeared not to affect the strength of the association, but more rigorous experiments are necessary to be certain (Hoban et al., 1996). Our results similarly provide no indication that the duration of trophallaxis might influence the strength of the response. These findings are in agreement with the notion that CS/US pairing is the major determinant of associative learning, independent of the duration of either stimulus. Additional support comes from the finding that different stimuli perceived throughout feeding are not associated with reward, but only those experiences at the onset of reward (Opfinger, 1931, 1949; Menzel, 1968).

Olfactory learning through trophallaxis and foraging behaviour

We showed that associative learning of an odour occurs during a behavioural performance, trophallaxis, which is very common in social insects. This kind of learning leads to long-term olfactory memories. According to these findings, employed foragers may train nest-mates by means of trophallaxis and will therefore influence the subsequent search behaviour of bees flying out to forage. Moreover, we found that olfactory learning through trophallaxis occurs after a single conditioning trial, even when trophallaxis is brief. Previous results indicate that foragers increase the number of their offering contacts (i.e. the brief interactions in which they act as food donors during trophallaxis) after experiencing an increase in reward (De Marco and Farina, 2001). Together with the present results, this means that highly rewarding nectar sources may exhibit a high probability that their chemosensory cues will be learned through trophallaxis by potential newly recruited foragers. Furthermore, within the colony, nectar foragers perform offering contacts as well as brief begging contacts (acting as food-receivers during trophallaxis; von Frisch, 1965). Recently, De Marco and Farina (2003) showed that an increased resource uncertainty enhances the foragers' begging behaviour. If an increased resource uncertainty enhances proboscis extensions (as potential learning trials) and long-term olfactory memories can be formed (or even retrieved) by means of trophallaxis, it will be then interesting to study how the nectar-related chemosensory information transmitted during trophallaxis (at any time within the colony) might affect the initial choice behaviour of newly recruited foragers and the ongoing foraging process of employed foragers. In addition, since trophallaxis also occurs between a dancing bee and its followers, it is likely (but has not yet been proven) that recruited bees seek the odour learned by imbibing samples from the dancer (Lindauer, 1961; von Frisch, 1965).

According to the present results, the strength of the associative learning involved in trophallaxis increases together with both CS and US intensity as well as the sucrose stimulation experienced previously by the animals. Nectar-bearing flowers offer a variety of nectar odours as well as sugar concentrations under natural environmental conditions. Since the combination of the olfactory and gustatory stimuli provided by a given nectar source constitute a primary source of guiding cues, olfactory learning through trophallaxis may be crucial as long as nectar foragers use odours and sucrose concentrations to optimise their foraging choices (von Frisch, 1965; Gould, 1993). Our results predict that high levels of nectar-scent concentrations as well as sugar rewards will both enhance the number of aroused forager-mates and guide them to the productive sources.

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