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Olfactory learning and memory in the honeybee: comparison of different classical conditioning procedures of the proboscis extension response

Apprentissage et mémoire olfactifs chez l'abeille : comparaison de différentes procédures de conditionnement classique de la réponse d'extension du proboscis

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RÉSUMÉ

L'apprentissage olfactif a été étudié chez l'abeille en employant le conditionnement de l'extension du proboscis sur des individus en contention. Nous avons comparé, dans les mêmes conditions expérimentales, les procédures de conditionnement les plus communément employées, c'est-à-dire en 1 essai, en 3 essais en masse (intervalles entre essais de 1 min) et en 3 essais espacés (intervalles entre essais de 10 min), en utilisant du linalol comme stimulus conditionnel. Deux expériences ont été réalisées, dans lesquelles des abeilles étaient soumises à : (1) un unique test à différents temps (30 s à 14 jours) après la procédure de conditionnement ; (2) un premier test dans les 3 h suivant le conditionnement, les abeilles étant retestées ensuite chaque jour (jusqu'à 5 tests). La trace mnésique d'un stimulus odorant appris peut durer toute la vie de l'abeille, et cela même après une unique association avec du sucre. La répétition des tests à 1 jour d'intervalle induit une forte baisse du niveau de réponse, cet effet étant plus prononcé après 1 seul essai de conditionnement. ▲

Mots clés : *abeille, mémoire olfactive, apprentissage, conditionnement de l'extension du proboscis.*

ABSTRACT

Olfactory learning in the honeybee was investigated using the conditioned proboscis extension reflex on restrained individuals. We compared, under the same experimental conditions, the most commonly used conditioning procedures, i.e. *1 trial, 3 massed trials (1 min inter-trial intervals), and 3 spaced trials (10 min inter-trial intervals) procedures, using linalool as the conditioned stimulus. Two experiments were performed in which worker bees were subjected to: (1) a single test at different times (30 s to 14 days) after the conditioning procedure; (2) a first test within 3 h after the conditioning procedure, and were then retested daily (up to 5 tests). The memory trace of a learnt odorant stimulus could last for the lifetime of the bee, even after a single association with sugar. Repeated tests with 1 day inter-test duration induced a strong decrease of the response level, this effect being more pronounced after a 1-trial conditioning.* ▲

Key words: honeybee, olfactory memory, learning, proboscis extension conditioning.

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VERSION ABRÉGÉE

L'apprentissage olfactif chez l'abeille domestique a été étudié en utilisant le conditionnement de l'extension du proboscis sur des individus en contention. Cet essai biologique présente l'avantage de pouvoir contrôler strictement les conditions expérimentales, mais les modalités du conditionnement varient beaucoup en fonction des auteurs. Ainsi, le nombre d'associations odeur-nourriture effectuées et la durée des intervalles entre les essais de conditionnement peuvent varier. Afin de réaliser une comparaison entre différents types de conditionnement sur la rétention de l'odeur apprise et la résistance à l'extinction, nous avons choisi 3 procédures couramment employées, c'est-à-dire en 1 seul essai, en 3 essais espacés de 10 min (3 essais espacés) et en 3 essais espacés de 1 min (3 essais en masse). Dans une première expérience, des abeilles de 14-15 jours, montées dans des tubes de contention en verre, reçoivent l'un de ces 3 conditionnements, puis sont testées 1 seule fois à un temps compris entre 30 s et 24 h après le dernier essai de conditionnement. Les abeilles testées jusqu'à 4 jours après le conditionnement restent en contention tout ce temps; les abeilles testées à 7 et 14 jours sont remises en cagettes de 20 individus entre le conditionnement et le test car des durées de contention supérieures à quelques jours les épuisent. Dans une deuxième expérience, des abeilles du même âge et conditionnées selon le même protocole que précédemment subissent un test dans les 3 h suivant le conditionnement et sont retestées chaque jour, dans une limite de 5 tests au total.
Concernant la durée de rétention de l'odeur apprise (lorsque les abeilles sont testées 1 seule fois), nous obtenons pour toutes les procédures de conditionnement employées un taux de réponse à l'odeur très élevé (supérieur à 70 %) et qui ne décroît pas significativement au cours du temps. Jusqu'à 14 jours après le conditionnement, ce qui correspond à la durée de vie d'une ouvrière en été (un mois), même après 1 seule association odeur-nourriture, l'odeur est reconnue à plus de 50 %. Aucune différence entre procédures de conditionnement n'apparaît. Ces résultats contredisent le modèle de cinétique temporelle de la mémoire olfactive de l'abeille décrit par Menzel. Cet auteur met en évidence une décroissance temporaire (située entre 1 et 10 min après le conditionnement) du niveau de réponse conditionnée, alors que nous obtenons un niveau de réponse stable au cours du temps. Cependant la qualité et/ou la quantité du stimulus odorant utilisé pourraient être responsables de la différence de cinétique observée.
En ce qui concerne la résistance à l'extinction (lorsque les abeilles sont testées de manière répétée), nos données montrent une décroissance significative du niveau de réponse à l'odeur apprise au cours des tests, allant de 80 % à 20-40 %. Cette décroissance est plus forte après 1 seul essai de conditionnement qu'après 3 essais. L'intervalle court ou long entre les essais n'influe pas sur ce processus d'extinction.
En conclusion, dans nos conditions expérimentales, le nombre d'essais de conditionnement influe sur la résistance de la mémoire à l'extinction au cours de tests répétés. En revanche, la rétention à long terme de l'odeur apprise, évaluée par 1 seul test réalisé à des durées variables après le conditionnement, est peu affectée. Aucun effet d'une présentation plus ou moins rapprochée des essais de conditionnement n'a été mis en évidence. ▲

In the honeybee, olfactory learning and memory have been investigated using different types of biological assays (see [1] for a review) including the observation of free-flying bees visiting food sources *e.g.* [2-5], and the conditioned proboscis extension response on restrained bees *e.g.* [6-9]. The use of restrained bees appeared to be particularly adequate to control learning-relevant parameters and this approach was applied to the study of behavioural, genetic, and neurobiological bases of olfactory learning in the honeybee *e.g.* [10-12].
The most common paradigm is the classical odour conditioning of the proboscis extension reflex based on the paired association of an odour (conditioned stimulus CS) and a sucrose reward (unconditioned stimulus US) delivered to the antennae and proboscis [13-17]. Various conditioning procedures have been used. Frings [13] subjected the bees to the highest number of conditioning trials possible (*i.e.* as long as the bees took food), and Takeda [15] carried out 10 trials. Later, Bitterman *et al.* [7] and Menzel and Bitterman [18] used procedures with 4 or 8 trials. More recently, 1-trial conditioning procedures were preferred [8, 9, 19]. Indeed, acquisition curves showed that after 1 conditioning trial, up to 60% of the individuals already exhibited the conditioned response, a steady state being reached after 3 trials [7, 17].
Different conditioning paradigms were also used: in parallel to the standard procedure with paired US-CS presentations, unpaired procedure (successive presentation of CS and US [7]), differential or discriminative conditioning (alternative presentation of a rewarded CS and an unrewarded CS [18, 20]), mass-presentation of paired US-CS (with 1 min inter-trial instead of 10-15 min inter-trials in the standard procedure [7]), aversive conditioning (paired US-CS associated to an electric shock [21]), were used. However, with the exception of the work by Bitterman *et al.* [7] who showed that the acquisition rate was similar for standard, mass and discriminative conditioning procedures, few studies were conducted to compare the efficiency of these different procedures.
In addition, using classical conditioning procedures, the time dependence of retention was studied, and led to the development of a model of sequential organisation of olfactory memory in the honeybee [9]. These authors differentiated 3 types of memory: (1) a short-term memory, lasting a few minutes with a prevalent non-associative component; (2) an intermediate memory which takes place after a consolidation process; and (3) a long-term memory, obtained after several US-CS associations.
In this study, we aimed to evaluate on the time course of olfactory memory, the effects of different classical conditioning procedures, with various numbers of conditioning trials and inter-trial intervals, on restrained bees. We compared the most common procedures usually applied, *i.e.* conditioning with 1 or 3 trials, either massed or spaced. For a direct evaluation of the relative efficiency of the 3 procedures, we carried out the 3 types of experiments daily, using the same conditioning odour. The recorded parameters to estimate learning performances were the time dependence of retention over days, and the resistance of the conditioned response to repeated presentations of the unrewarded conditioning stimulus.

Materials and methods

Biological material

Most authors have used foragers of unknown age collected at the hive entrance and starved for various periods. Only Brandes *et al.* [19] standardised these conditions by using individuals reared in a flight room until 14 days old, and receiving a controlled amount of food 3 h before conditioning. In order to control experimental conditions, the experiments were done with Italian worker bees *Apis mellifera ligustica* reared under caged conditions and tested at a known age. Emerging bees were collected from combs of outdoor hives and caged in groups of about 100 individuals, maintained in an incubator at 33° C, 55% RH and fed *ad libitum* with sugar, pollen and water. The bees were used at the age of 14-15 days, since it has been shown that most worker bees become foragers at that age [22] and give the most consistent performances in the conditioned proboscis extension assay [23].

Stimulation method

Each bee was positioned in a constant and steady airflow of 52.5 ml/s delivered through a 1 cm diameter glass tube placed 1 cm from the head of the bee. This flow was composed of a main airflow of 50 ml/s and a secondary airflow of 2.5 ml/s used for the stimulus delivery. The odour source, 10 µl of pure linalool, was applied onto a 40 x 3 mm filter paper strip inserted in a disposable Pasteur pipette. The secondary flow was delivered continuously into the main airflow, through either the pipette containing the odour source or an identical empty pipette, with a solenoid valve control. A fan was set opposite to the airflow, to withdraw the released odour.

Protocol

The conditioning and testing procedures were developed according to the works by Bitterman *et al.* [7]. Bees were individually mounted in glass holders, with their antennae and mouth parts free, and were starved for 4 h to standardise parameters that might affect learning. After that time, 22% of the workers did not show a clear proboscis extension reflex after stimulation of the antennae with a 30% sucrose solution and were discarded from our experiments.

• *Conditioning.* At the beginning of each conditioning trial, the bee was placed in the airflow for 15 s to be familiarised to the mechanical stimulation. The odour stimulus (CS) was then delivered for 6 s; after 3 s, the antennae were stimulated with a 30% sucrose solution (US); the proboscis extension was then rewarded by a food uptake (with the same sucrose solution).

Three different conditioning procedures were compared in this work. The bees were conditioned with either: (1) 1 conditioning trial *(1-trial)*; (2) 3 trials with 10 min inter-trial intervals *(spaced trials)*; and (3), 3 trials with 1 min inter-trial intervals *(mass trials)*.

• *Testing.* Bees were placed in the airflow for 15 s to familiarise them with the mechanical stimulation. The odour stimulus (CS) was then presented for 6 s. The occurrence of a proboscis extension (conditioned response – CR) during the stimulation time was recorded. Two different experiments were conducted according to the testing procedure.

Experiment 1. Each bee was subjected to a single test at various times after the conditioning procedure:

(1) Short-term tests. The bees were tested once in a range of 9 different times, from 30 s to 3 h after the last conditioning trial.

(2) Middle-term tests. The bees were tested 1, 2, 3 or 4 days after the conditioning procedure. We showed in a preliminary experiment (not detailed here) that bees could survive up to 7 days in a glass holder, if they were fed regularly. We kept these bees in the glass holders for 1 to 4 days, feeding them every night with a sugar solution (25% fructose) different from the one used in the conditioning procedure. With these conditions, we obtained a 95% survival rate, and 82% of the surviving bees still showed the proboscis extension reflex (checked after the test by contacting the antennae with the US). The other bees were discarded.

(3) Long-term tests. The bees were tested 7 or 14 days after the conditioning procedure. Since few bees would survive in the glass holders for such long durations, they were removed from the holders and were returned to their rearing cages in groups of 20 individuals between conditioning and testing. At the time of the tests, the survival rate was 67%, with 97% of the surviving bees showing a proboscis extension reflex.

Experiment 2. Each bee was tested once during the 3 h immediately following the conditioning procedure (9 testing times from 30 s to 3 h) and was retested after 1, 2, 3 and 4 days. In this experiment, the animals were kept in glass holders under the same conditions used in the middle-term tests of *Experiment 1.* The survival rate was 87% (83% of the surviving bees showing a proboscis extension reflex).

Data Treatment

The time-dependence of the responses for each conditioning procedure in *Experiment 1* was assessed by comparison of the distribution of the conditioned responses along time with a theoretical flat distribution, using a homogeneity χ^2 test with 14 df. Pairwise comparisons of the conditioned responses obtained during the tests between the different conditioning procedures were then made with a χ^2 test, 1 df. When conditions of application of the χ^2 test were not fulfilled according to Cochran's rule [24], we used Fisher's exact method [25]. The significance threshold was 5% divided by n, with n being the number of comparisons in which each data set was used. In this case, for 3 pairwise comparisons, $n = 2$. In addition, we compared the conditioned responses obtained in *Experiment 2* to those of *Experiment 1* recorded in the middle-term tests. The statistic used was a χ^2 test with 1 df and a 5% significance threshold.

Results

Of the 755 bees conditioned in our experiments, 17% showed a spontaneous response to the linalool (*i.e.* spontaneous proboscis extension to the CS at the first conditioning trial). All bees, including those that gave a spontaneous response, were kept.

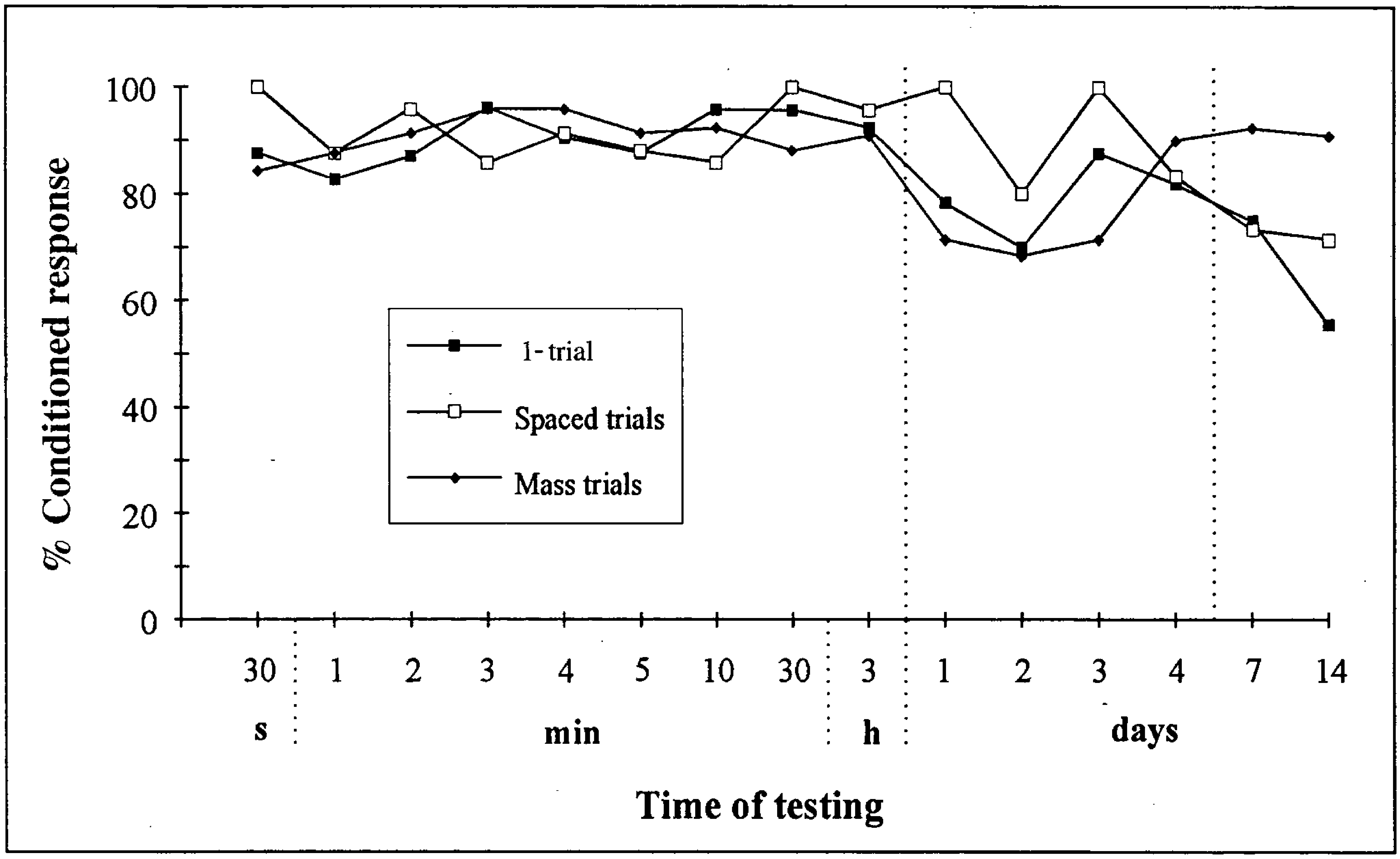


Figure 1. ***Time course of the memory trace (retention) after 3 different conditioning procedures*** *(in* 1-trial, *3 trials with 10 min inter-trial intervals* – spaced trials, *3 trials with 1 min inter-trial intervals* – mass trials*) as measured in* Experiment 1.

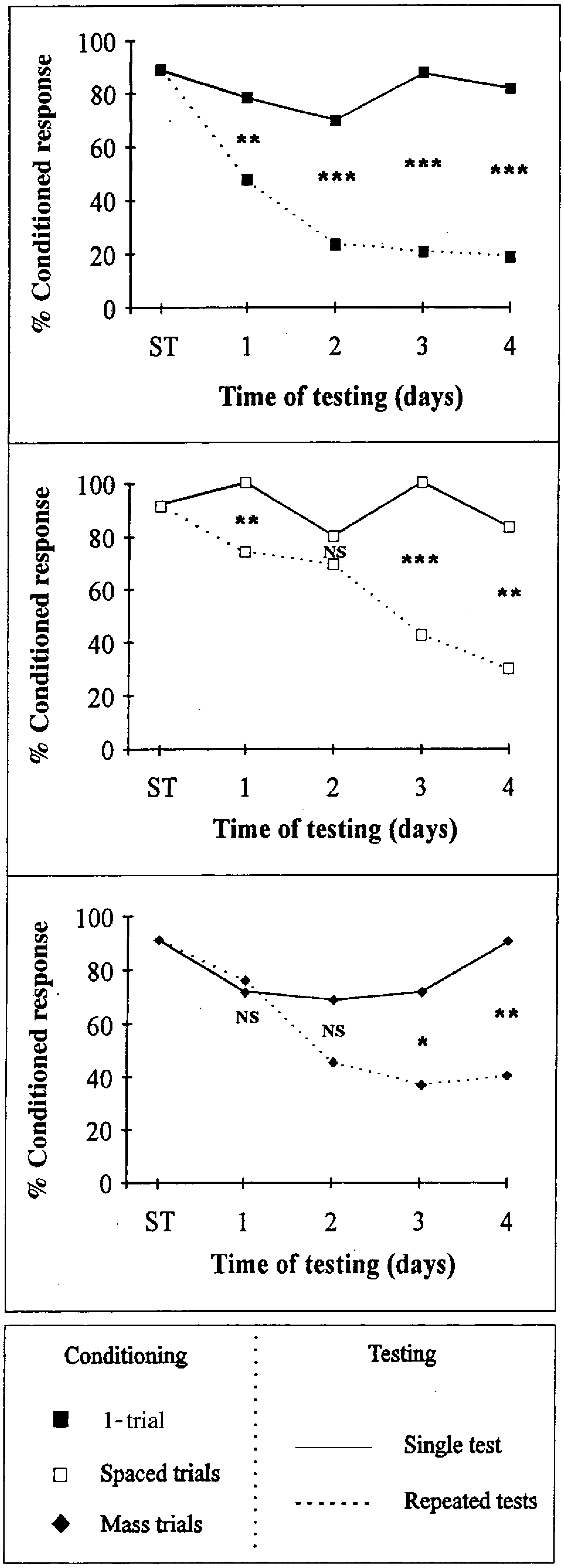
Experiment 1

The percentages of bees which showed a conditioned response during the test at different times following the 3 conditioning procedures are presented in *Figure 1*. The number of bees in each independent group was comprised between 19 and 26 for the short-term, 10 and 23 for the middle-term, 7 and 16 for the long-term tests.
In the short-term tests (when bees were tested within 3 h after conditioning), the level of response was high (above 80%) and constant regardless of the conditioning procedure. Statistical analysis showed no difference between the 3 conditioning procedures at any testing time.
In the middle-term tests (from 1 to 4 days), the percentage of conditioned responses remained high (from 70% to 100%) but was more variable than in the short-term tests. The *spaced trials* procedure gave slightly higher levels of response than the *1-trial* and the *mass trials* procedures, this difference being significant only when bees are tested 1 day after conditioning (P = 0.008 and 0.021 for pairwise comparisons between the *spaced trials* procedure and the *mass trials* and *1-trial* procedures respectively).
In the long-term tests (7 and 14 days), the level of response was still very high (90%) for the *mass trials* procedure. The *1-trial* and the *spaced trials* procedures elicited lower levels of response, although the differences were not significant.
The homogeneity χ^2 test consistently showed that the level of conditioned responses did not statistically differ from a flat distribution (P = 0.053, P = 0.085 and P = 0.153 for the *spaced trials*, *1-trial* and *mass trials* respectively). Therefore we can consider that the memory trace remained stable over a period of several days for all conditioning procedures considered.

Experiment 2

For each type of conditioning procedure, the percentages of conditioned responses obtained with a single or repeated tests performed from 1 to 4 days after the conditioning procedure are compared in *Figure 2*. The bees tested once were the same as those of the middle-term tests in *Experiment 1*.
In the repeated tests, the number of bees tested the first

Figure 2. ***Comparison of single and daily repeated tests (extinction) performed after 3 different conditioning procedures*** *(in* 1-trial, *3 trials with 10 min inter-trial intervals* – spaced trials, *3 trials with 1 min inter-trial intervals* – mass trials*) as measured in* Experiments 1 *and* 2 *respectively. Plotted at ST is the level of response obtained during the first test, taking place within 3 h after the conditioning, i.e. at short-term. The levels of significance for pairwise comparisons are given as follows: *** P < 0.001; ** P < 0.01; * P < 0.05; NS non significant.*



day (*i.e.* for the second time, because the first testing took place within 3 h after conditioning) was comprised between 168 and 189 individuals according to the conditioning procedure used. The following days these numbers decreased to 93 to 144 bees tested on the second day (third test), 68 to 86 tested on the third day (fourth test) and 20 to 34 bees tested on the fourth day (fifth test). Indeed, not all individuals were kept for all 4 days, essentially for contingent difficulties (*i.e.* feeding duration).

Repeated tests induced a decrease in the level of conditioned response from an initial value of about 90% when bees were tested at short term (first test) down to 20%, 30% and 40% at the fourth testing day, respectively for the *1-trial*, *spaced trials* and *mass trials* procedures. The decrease due to unrewarded repeated presentations of the odour was greater after conditioning with the *1-trial* procedure. Differences between single and repeated tests were significant from the second test, *i.e.* day 1 (P was comprised between 9.9×10^{-4} and 5.4×10^{-3}, 1 df, from day 1 to day 4). For the *spaced trials* and the *mass trials* conditioning, the decrease in the rate of conditioned response was significant from day 3, *i.e.* the fourth test.

Discussion

The effects of different conditioning procedures on learning and memory capabilities and on the resistance to extinction process have been studied with parallel trials using the same conditioning stimulus. By testing the bees once at different times after the conditioning procedure, we investigated the retention process. Our data showed that the level of conditioned response remained high and stable, and especially within the 3 h following the conditioning. This contradicts previous work which showed a decrease in the level of conditioned response from 80% down to less than 50%, between 1 and 10 min after the conditioning [26]. These results were obtained for a *1-trial* conditioning procedure, with orange scent, and was confirmed later with carnation [27]. The time dependence of the olfactory response levels shown by these authors was similar to that found for 1-trial colour learning of free flying bees [28]. Thus, they interpreted this temporary decrease as a transition phase between a short-term memory, where the non-associative component related to sensitisation is prevalent, and an intermediate memory, where the associative component rises during the consolidation process [27, 29]. Reversal of learned behaviour is also facilitated during this transition phase [30]. We did not observe this decrease under our experimental conditions with the *1-trial* conditioning, which might be due to the odour stimulus chosen. We may assume that linalool, which appeared to be particularly salient in floral blend recognition [31], could induce a strong associative component already immediately after conditioning. Therefore, with linalool, the associative component might prevail in the response level, and mask the biphasic time-course of the conditioned response. Consistent with this assumption, Smith [8] showed different temporal dynamics of olfactory retention by conditioning and testing with a range of pure odorants.

The quality of the conditioned stimulus may thus affect learning performances, some odours inducing a stronger appetitive response than others in the same conditioning procedure [32]. Usually pure odorants of pheromonal or floral origin were used as conditioned stimulus. To keep close to natural conditions of foraging, we used linalool, a common floral component. Although floral volatiles are complex chemical blends, it has been shown that the recognition of oilseed rape flower extract was based on a limited range of key components among which linalool was particularly active [31]. However, to understand the responses to natural blends, further experiments are in progress using other chemicals either pure or in mixtures, and at different concentrations, to test their efficiency in the different conditioning procedures.

In considering long-term responses, independently of the conditioning procedures, we showed that the level of conditioned response was still higher than 50% when the bees were tested once, even 14 days after conditioning, *i.e.* at an age of 28 days, being the average life span of spring and summer bees according to Sakagami [22]. Such stability of the olfactory memory trace has not previously been demonstrated. Data on long-term retention obtained from visual conditioning in free-flying bees, showed a progressive decrease in the response probability to reach a random level 8 days after a 1-trial conditioning [9, 17].

In our work, no significant difference were observed in the levels of conditioned responses, when tested once, between the different conditioning procedures. Menzel [27] found that the 3-trials conditioning protocol elicited a stronger consolidation process than a 1-trial procedure, and thus the temporary decrease reflecting the transition from short-term to intermediate and long-term memory was less detectable. Also, visual memory trace remained strong at long-term after 3 conditioning trials but not after 1 trial [17]. With linalool, differences between procedures could be slight because of the strong associative component already obtained after 1 conditioning trial, which was not noticeably improved after a 3-trials conditioning. In addition, under our conditions, we did not find significant differences between spaced and mass presentation of 3 conditioning trials. This seems consistent with the results obtained by Bitterman *et al.* [7], who observed the same levels of response after massed and spaced paired trainings. However, these data were collected by measuring the acquisition rate and not the retention performance as we did. These results contradict those of Erber [33] who reported that mass learning was less effective than spaced due to weaker consolidation of the information stored, but these data were gained from colour learning in free-flying bees.

Differences between procedures were observed when testing the resistance of the memory trace to extinction by repeated presentation of the unrewarded conditioning stimulus. Thus, we showed a decrease related to an extinction process, that was stronger after conditioning with 1 than with 3 trials. Although such extinction processes have been described by several authors, the slope of the decrease seemed to be largely dependent on the inter-trial testing intervals. Mercer and Menzel [26] obtained stable response levels with intervals ranging from 5 to 60 min, whilst Bitterman *et al.* [7] observed a strong decrease in the probability of response for 1 min inter-trial intervals; they also emphasized the occurrence of a spontaneous recovery when an interval of 35 min was given after a session of extinction trials. Such spontaneous recovery corroborates the work by Brandes [11] who did not observe pronounced extinction for intervals longer than 30 min. In our study, with 1 day intervals, the level of response faded, which suggests that what we observed was a forgetting process rather than an extinction process which would be more reversible after a time of recovery. The possible difference or interaction between a rather temporary process of extinction and a more definitive process of memory fading requires further investigation. From our data on repeated tests over several days, it may be assumed that for a 1-trial conditioning, resistance to extinction – or to forgetting – would be lower than for a 3-trials conditioning. The weaker consolidation of memory trace induced by 1-trial conditioning, although not noticeable when testing the retention time course with linalool, nevertheless appeared when testing the resistance of the conditioned response to repeated stimulus presentation.

In a conclusion, we found that the memory trace might remain stable over the life span of the bee, at least for pure linalool as the conditioned stimulus. Under our experimental conditions, 1-trial conditioning appeared to induce high learning performances and long-term retention at the same level as 3-trials conditioning either spaced or massed. However, the resistance of the conditioned response to repeated tests was lower for a 1-trial conditioning. Further experiments are needed to investigate the effects of the quality and concentration of the odour stimulus on the dynamics of olfactory memory in the honeybee. ▼

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