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Research report

The trial-spacing effect in olfactory patterning discriminations in honeybees

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Abstract

Harnessed bees conditioned to associate odors and sucrose reward learn to discriminate between olfactory mixtures and their odor components in negative (NP: A+, B+, AB−) and positive (PP: A−, B−, AB+) patterning experiments. They thus extend the proboscis to the reinforced (CS+) but not to the non-reinforced (CS−) stimuli. Using the same protocol, we studied whether or not trials, which are spaced in time, are more effective in supporting patterning discrimination than massed trials which succeed fast to each other ('trial-spacing effect'). Training followed a NP (4 A+, 4 B+, 8 AB−) or a PP (4 A−, 4 B−, 8 AB+) schedule, with a 1:1 ratio between CS+ and CS− trials (8 CS+ and 8 CS− trials). ITIs of 1, 3, 5 and 8 min were used in both tasks. Increasing ITI resulted in better differentiation between reinforced and non-reinforced CSs in both NP and PP tasks. However, whereas only the longest ITI of 8 min allowed discrimination in NP, PP could already be solved with an ITI of 5 min. This difference might be due to the fact that NP, but not PP, would require the formation of a unique cue and thus longer processing times. We thus show that the trial-spacing effect, previously demonstrated for single stimulus conditioning, also determines performance in patterning tasks in which three different stimuli (A, B, AB) alternate so that elements have to be discriminated from their compound.

Keywords: Learning; Conditioning; Positive and negative patterning; Intertrial-interval; Honeybees; Olfaction

1. Introduction

Stimuli in the natural environment may appear as multimodal compounds, thus raising the question of how animals process and respond to these compounds. From pure elemental processing, in which the compound is perceived as the sum of its components [34] to configural processing, in which the compound is perceived as a whole new configuration [30,31], different from its components, several models have been postulated to account for compound processing and learning in animals. To discern between these possibilities, specific discrimination problems – the so-called patterning experiments – were conceived. In these experiments, animals are trained to differentiate between two single elements (e.g. A and B) and the compound composed of both of them (AB). In *negative patterning* (NP), the single elements A and B are both reinforced when presented alone (A+, B+), while the compound is non-reinforced (AB−). Conversely, in *positive patterning* (PP), the two elements are both non-reinforced when presented alone (A−, B−), while their compound is reinforced (AB+). These two problems are not equivalent with respect to the processing models mentioned above. NP cannot be solved if pure elemental processing is applied. Since in NP each component is reinforced, a compound would elicit, through elemental summation, twice as much responding as each component. In this case, an animal would never learn to inhibit its reaction to the non-reinforced compound. PP, on the other hand, could be solved if pure elemental processing is applied. If each non-reinforced component elicits low responding, the compound could elicit consistent responding through elemental summation. In this case, the animal solves the discrimination as it scarcely responds to the components while it responds to the compound.

Honeybees, *Apis mellifera*, are a standard model for the study of compound processing and learning in the olfactory domain [6–9,13,20,27,38]. Olfactory compounds (henceforth, mixtures)

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play a relevant role in the bees' natural life, both for kinship and for food source recognition. Bees learn the odors of the flowers on which they obtain nectar and pollen as rewards, which are indispensable for their individual and collective survival. This learning can be studied in the laboratory by means of the olfactory conditioning of the proboscis extension reflex (PER) [4,39]. When the antennae of a hungry bee are touched with sucrose solution, the animal reflexively extends its proboscis to reach out and suck the sucrose. Odors to the antennae do not release such a reflex in naive animals. If, however, an odor is presented immediately before sucrose solution, an association is formed which enables the odor to release the PER in a following test. This learning relies on Pavlovian associations, the odor being the conditioned stimulus (CS), and the sucrose solution the unconditioned stimulus (US) [4]. Using this protocol, we have previously showed that honeybees can successfully solve NP and PP problems in which single odors and their binary mixtures were associated with different outcomes with respect to sucrose reward [7–9].

In a previous work [7], we observed that a variation of reinforcement density (i.e. the number of reinforced trials divided by the total number of trials) affected the amount of differentiation between reinforced (CS+) and non-reinforced (CS−) stimuli in both patterning tasks. In PP (A−, B−, AB+), three reinforcement densities were used: 0.17 (Group 1/5 in [7]: 10 A−, 10 B−, 4 AB+, i.e. 4 CS+/24 trials), 0.33 (Group 1/2: 8 A−, 8 B−, 8 AB+, i.e. 8 CS+/24 trials) and 0.50 (Group 1/1: 6 A−, 6 B−, 12 AB+, i.e. 12 CS+/24 trials). In NP (A+, B+, AB−), three reinforcement densities were also used: 0.25 (Group 1/3: 3 A+, 3 B+, 18 AB−, i.e. 6 CS+/24 trials), 0.33 (Group 1/2: 4 A+, 4 B+, 16 AB−, i.e. 8 CS+/24 trials) and 0.50 (Group 1/1: 6 A+ trials, 6 B+ trials and 12 AB− trials, i.e. 12 CS+/24 trials). Discrimination between CSs+ and CSs− at the end of both NP and PP training varied significantly with reinforcement density. This variation was mainly due to variation in CS+ responses since responses to CSs− at the end of training remained low and did not vary with reinforcement density. Responses to CSs+, on the other hand, were significantly affected by reinforcement density. Both for NP and PP, we found that CS+ responses were low for the lowest reinforcement density (0.17 for PP and 0.25 for NP). Increasing reinforcement density to 0.33 resulted in a significant enhancement of responses to CSs+ both for PP and NP. When reinforcement density was further increased to 0.5, CS+ responses remained at a similar level in PP whilst they decreased in NP [7].

These results were intriguing because the so-called "trial-spacing effect" [1] posits that reinforced trials, which are spaced in time, are more effective in supporting learning than massed trials which succeed fast one after the other. Usually, this effect is observed in retention tests performed at a longer term and has been attributed to the induction by spaced trials of a protein-synthesis dependent memory termed long-term memory (LTM) (Drosophila: [40]; mice: [37]; honeybees: [26]). In the honeybee, however, the trial-spacing effect is visible both at the level of acquisition and long-term retention [26]. Thus, when honeybees are trained with a single reinforced odor in PER conditioning, short intertrial-intervals (ITIs) of only 30 s or 1 min, induce slower acquisition and lower retention than longer ITIs of 5 and 10 min [24]. Whether this conclusion applies to NP and PP learning is an open question because these more complex problems do not involve a single reinforced stimulus (CS+), which is repeatedly presented, but instead a succession of interspersed reinforced (CS+) and non-reinforced (CS−) stimuli. Nevertheless, if the total number of trials is kept constant and the number of CS+ trials is varied to change reinforcement density (as in the PP and NP experiments of [7]), the average ITI between CS+ trials will vary dramatically. In cases of low reinforcement density, rewarding trials will be obviously more spaced than in the case of high reinforcement density. Low reinforcement density, due to the reduced number of CS+ trials and concomitant longer ITIs between such trials, should support higher CS+ responses than higher reinforcement densities where CS+ trials would be rather massed in terms of ITI. We did not find such a trend in our previous experiments [7]. Instead, CS+ responses, and eventually differentiation, were lower for the lowest reinforcement densities (0.17 for PP and 0.25 for NP) both for PP and NP.

A possible argument to explain why low reinforcement densities did not support high CS+ responses in our PP and NP experiments may be that the number of reinforced trials was too low to result in successful CS+ learning and differentiation. Thus, 4 and 6 CS+ trials might have been insufficient in a total of 24 trials to support strong CS+ responses and differentiation both in PP and NP, respectively. To elucidate this point, we explicitly studied the effect of the temporal separation between CS trials on the acquisition of negative and positive patterning in this work. Contrarily to our previous work [7], in which the number of CS+ and CS− trials was varied while leaving the ITI constant, in the present work we explicitly varied the ITI while leaving constant the number of CS+ and CS− trials. Our results show that increasing the ITI between conditioned trials results in better differentiation between reinforced and non-reinforced CSs in both olfactory NP and PP in honeybees.

2. Materials and methods

Honeybees (*A. mellifera* L.) were caught at the entrance of outdoor hives at the beginning of each experimental day. Each bee was immobilized by cooling in a freezer and then mounted into restraining harnesses such that it could only move the antennae and mouthparts, including the proboscis [4,39]. Animals were then kept undisturbed in the experimental room in front of a small fan delivering a constant airflow comparable to that of the odor-supplying device, for approximately 2 h. This treatment guaranteed that the mechanical airflow stimulation during training could not act as a predictor for the US.

Thirty minutes before the start of training, each subject was checked for intact proboscis extension reflex by touching one antenna with a toothpick imbibed with sucrose solution without subsequent feeding. Extension of the proboscis beyond a virtual line between the open mandibles was counted as PER (unconditioned response). Animals that did not show the reflex (<10%) were discarded for the experiments.

The US consisted of 1.25 M sucrose solution. The CSs were the odorants limonene and 2-octanol (SIGMA, Deisenhofen, Germany). On each experimental day, 4 μl of pure odorant were applied onto a fresh strip of filter paper. The paper strips were placed into a 1 ml plastic syringe and mounted in an odor-supplying device (for description see [7]). When located in front of the device, each experimental bee received a gentle, constant flow of clean air provided by a standard aquarium pump. Computer-driven solenoid valves (Lee Company, Essex, CT) controlled airflow delivery. During periods of odorant delivery, the airflow was shunted through a syringe containing the odorant. In that way, a single odorant or a mixture of two odorants could be delivered to the bee. In

the latter case, the valves corresponding to two different syringes were opened simultaneously such that the airflow arriving at the antennae of the bee contained the two odors as a mixture. An exhaust system behind the bee removed odor-loaded air. Between conditioning trials, bees were replaced in front of the small fan besides the odor-delivering device.

At the beginning of each conditioning trial, the experimental bee was placed in front of the odor-supplying device for 15 s to allow familiarization with the training situation. Thereafter the CS was presented for 6 s. In reinforced trials, the US onset occurred 3 s after CS onset. Both antennae were lightly touched with a toothpick imbibed with the sucrose solution and after proboscis extension the bee was allowed to feed for 3 s. Therefore, the interstimulus interval was 3 s and the overlap between CS and US was also 3 s. Non-reinforced trials consisted of 6 s CS presentation without reward. At the end of each trial, animals remained 8 s in front of the odor-supplying device before being returned to their resting position, which usually lasted 1 s (total trial duration: 30 s).

During acquisition, we recorded whether a bee extended its proboscis during the 3 s after onset of the odor (both for the CS+ and for the CS−) and in the absence of the US. Thus, the responses recorded were conditioned responses not directly evoked by the US. The criterion for the occurrence of a conditioned response was the same as for the unconditioned one (extension of the proboscis beyond a virtual line between the open mandibles) except that it should occur in response to the olfactory stimulation. Multiple responses during a CS were counted as single PER. We also monitored responses to the US throughout the experiment. Animals that did not respond to the US more than twice out of the eight reinforced trials during the course of acquisition were discarded from the analyses of data (3.1%).

2.1. Experimental design

Training was performed according to a NP (4 A+, 4 B+, 8 AB−) or a PP (4 A−, 4 B−, 8 AB+) schedule. Limonene and 2-octanol were randomized as odorants A and B. In order to equate the number of reinforced and non-reinforced trials in both kinds of experiments, the ratio between CS+ and CS− trials was always 1:1 (8 CS+ and 8 CS− trials). Thus, reinforcement density (the number of reinforced trials divided by the total amount of trials) was always 0.50. We could show in an earlier work [7] that PER differentiation with such a 1:1 ratio and an ITI of 8 min was significant for both PP and NP. The sequence of CS+ and CS− trials was pseudo-randomized to avoid that bees either learn a given stimulus sequence (alternated CS−/CS+ presentations) or experience too long sequences without reinforcement (fully randomized presentations). Each day, new training sequences were drawn randomly using a computer program. Sequences chosen for the experiments did not include more than three subsequent reinforced or non-reinforced trials. NP and PP experiments were alternated, i.e. 1 day was assigned to NP while the next day was assigned to PP.

To study the effect of the temporal separation between CS trials on NP and PP, we explicitly varied the ITI between trials while leaving constant the number of CS+ (8) and CS− (8) trials. Both in PP and NP, four groups of bees were trained with ITIs of 1, 3, 5, or 8 min, respectively (and were thus assigned groups “NP-1, NP-3, NP-5, NP-8 and PP-1, PP-3, PP-5 and PP-8”). A total of 40 bees were trained for each group. On each experimental day, groups with different ITIs were trained successively. As appetitive learning depends on the motivational state of bees, which can be related to the amount of sucrose solution in the bee’s crop [29], bees in all groups experienced on average the same starvation time (166 min) between cooling and the beginning of the first conditioning trial.

Although the four ITIs refer to the interval between two consecutive trials, independently of their outcome (i.e. CS+ or CS−), longer ITIs correspond, on average, to longer intervals between CS+ trials. For instance, if the ITI is 1 min, an experiment with 8 CS+ and 8 CS− will last 16 min and 8 CS+ will occur in 16 min. On average, and given that stimulus order was randomized, this corresponds to 1 CS+ every 2 min. If the ITI is 8 min, on the other hand, an experiment with 8 CS+ and 8 CS− will last 128 min, which corresponds, in average, to 1 CS+ every 16 min.

2.2. Data transformation and statistical analyses

We measured the percentage of conditioned responses observed in the 8 CS+ and 8 CS− trials. Data were then grouped to obtain four blocks of two CS+ and four blocks of two CS− trials. For all groups, repeated-measure analyses of variance (ANOVAs) were used to analyze the blocked data. Although ANOVA is usually not allowed in case of dichotomous data such as those of the PER, Monte Carlo studies have shown that it is permissible to use it under certain conditions [22], which were met by the two experiments reported here (equal cell frequencies and at least 40 degrees of freedom of the error term). To reach equal cell frequencies (sample size of 40 bees per group), and because at the end of conditioning, some groups had a disparate sample size (between 40 and 49 bees per group), we randomly removed between 0 and 9 bees per group. The alpha level was set to 0.05 for all analyses.

3. Results

We studied the role of the interval between trials in NP and PP problems. To this end, we explicitly varied the ITI between consecutive trials (1, 3, 5, or 8 min) while leaving constant the number of CS+ and CS− trials. The resulting average interval between USs was 2, 6, 10 and 16 min, respectively.

3.1. Negative patterning

In negative patterning, bees were trained with 4 A+, 4 B+ and 8 AB− presentations. A Group × Element (4 × 2) ANOVA showed no significant difference in the responses to each reinforced element A or B (main effect element: $F<1$) nor a significant Group × Element interaction ($F<1$). Therefore, the responses to the elements were pooled and blocked. Fig. 1 shows, for each ITI, the averaged course of conditioned responses (% PER) to the CSs+ (pooled responses to elements A+ and B+; black circles) and to the CS− (responses to mixture AB−; white circles) across four blocks of conditioning trials.

At the beginning of training (Block 1), responses to the non-reinforced mixture (CS−) were always significantly higher than responses to the reinforced single odors (CSs+) in all groups (repeated measures ANOVA; group NP-1: $F_{1,39}=23.93$, $p<0.001$; group NP-3: $F_{1,39}=9.82$, $p<0.01$; group NP-5: $F_{1,39}=37.52$, $p<0.001$; group NP-8: $F_{1,39}=14.25$, $p<0.01$). This might be explained by assuming that at the beginning of training, when the bees have no information about the outcome of the single odors and mixture, they would not respond to the first presentation of one of the reinforced odors (A+ or B+) because up to this point they did not experience that an odor may be followed by a reward. After such an experience, they would exhibit a high tendency to generalize responding to any odor, single element or mixture, even if the mixture is in fact non-reinforced. This empirical rule (‘respond to a CS only after experiencing reward on it, generalize further responses to any other CS’) may result in the response asymmetry, which was observed at the beginning of training and which was later reversed during the course of training.

At the end of training (Block 4), differentiation between CSs+ and CS− was not equal for all groups (Fig. 1). Differentiation between CSs+ (A+, B+) and CS− (AB−) was not significant in groups NP-1, NP-3 and NP-5 ($F<1$). However, bees trained with an ITI of 8 min (group NP-8) responded significantly more to the CS+ than to the CS− in the last block ($F_{1,39}=14.25$, $p<0.01$). Thus, only the group subjected to the longest ITI (group

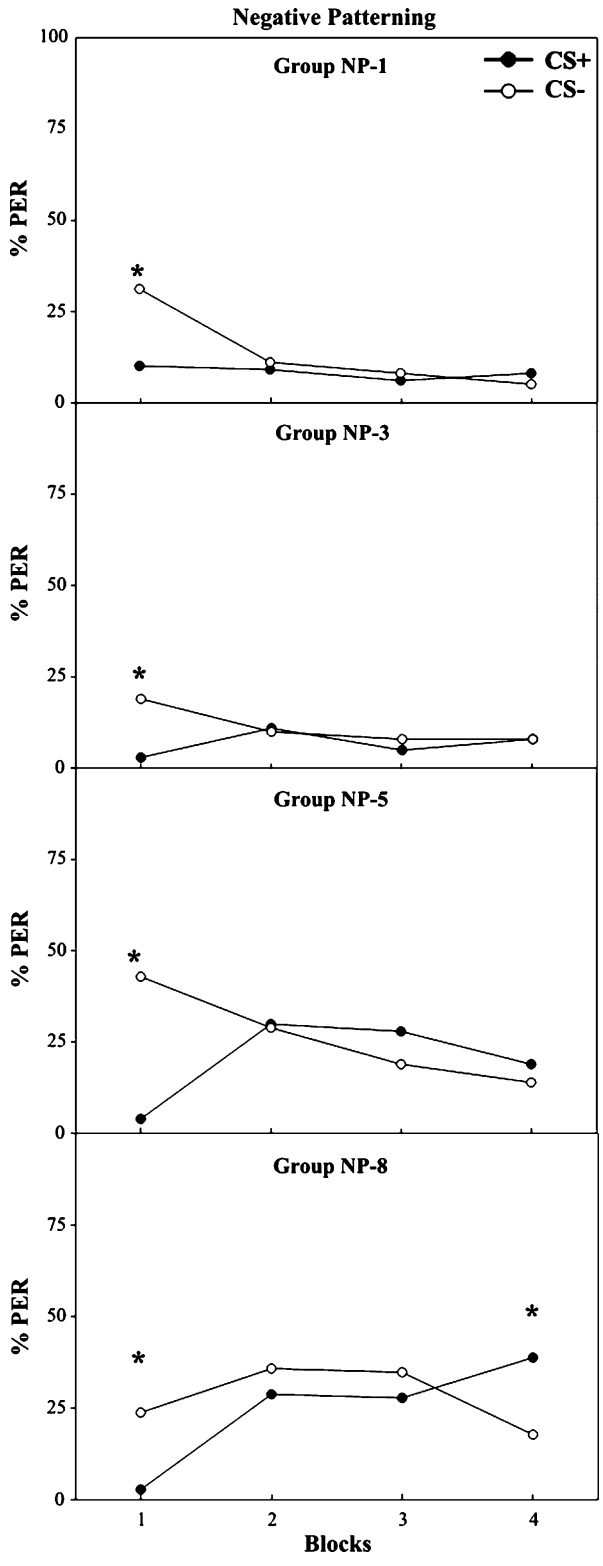

Fig. 1. Percentage of PER (proboscis extension reflex) of bees trained in a negative patterning discrimination (A+, B+, AB−) across four blocks of training trials with different intertrial-intervals (ITIs). Black circles indicate pooled responses to the reinforced single odors A and B (CSs+), white circles indicate responses to the non-reinforced mixture AB− (CS−). First panel (group NP-1): ITI = 1 min; second panel (group NP-3): ITI = 3 min; third panel (group NP-5): ITI = 5 min; fourth panel (group NP-8): ITI = 8 min. Significant differentiation between CSs+ and CS− was found only in the group trained with an ITI of 8 min (group NP-8).

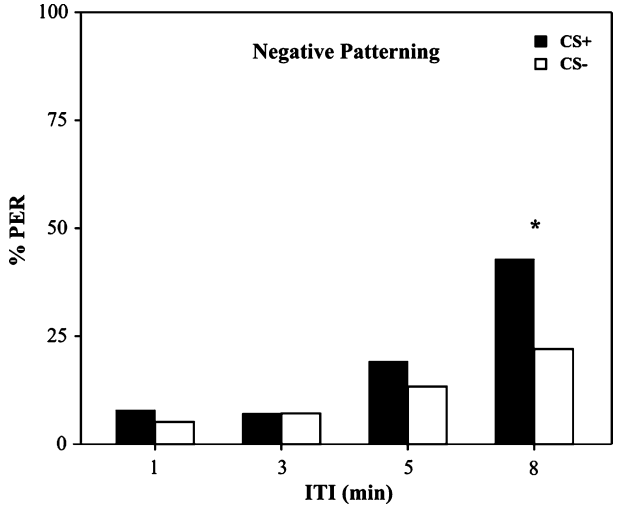

Fig. 2. Percentage of PER (proboscis extension reflex) in the last block of training in a negative patterning discrimination (A+, B+, AB−) with different intertrial-intervals (ITIs). Black bars indicate pooled responses to the reinforced single odors A and B (CSs+), white bars indicate responses to the non-reinforced mixture AB (CS−). Both the responses to the CSs+ and to the CS− increased with increasing ITI. Most importantly, differentiation between CSs+ and CS− also increased with increasing ITI.

NP-8) developed successful differentiation on olfactory NP discrimination.

In order to detect variation trends depending on ITI for responses to the CS+ and CS− at the end of training, we present only the results obtained in Block 4 in Fig. 2. The pooled responses (% PER) to the reinforced odors A and B (CSs+: black bars) and the responses to the non-reinforced mixture AB (CS−: white bars) are depicted as a function of ITI. Comparisons of these results across groups with linear trend analyses showed that both responses to the CSs+ ($F_{1,156} = 26.18$, $p < 0.001$) and to the CS− ($F_{1,156} = 5.72$, $p < 0.02$) increased with increasing ITI. Most importantly, differentiation between CSs+ and CS− responses also increased with increasing ITI ($F_{1,156} = 11.34$, $p < 0.001$). These results clearly show that increasing the ITI, thereby decreasing reinforcement density, leads to an increase in responding to both types of stimuli, CSs+ and CS−, and to better differentiation in olfactory NP discrimination in honeybees.

3.2. Positive patterning

In positive patterning, bees were trained with 4 A−, 4 B− and 8 AB+ presentations. A Group × Element (4 × 2) ANOVA showed no significant difference in the responses to each non-reinforced odor A or B (main effect Element: $F_{1,312} = 2.37$, NS) nor a significant Group × Element interaction ($F < 1$). Therefore, the responses to the two single odors A and B were pooled and blocked. Fig. 3 shows, for each ITI, the averaged course of conditioned responses (% PER) to the CS+ (responses to AB+; black circles) and to the CSs− (pooled responses to A− and B−; white circles) across four blocks of conditioning trials.

At the beginning of training (Block 1), responses to the non-reinforced odors (CSs−) were always higher than responses to the reinforced mixture (CS+). This difference was significant in

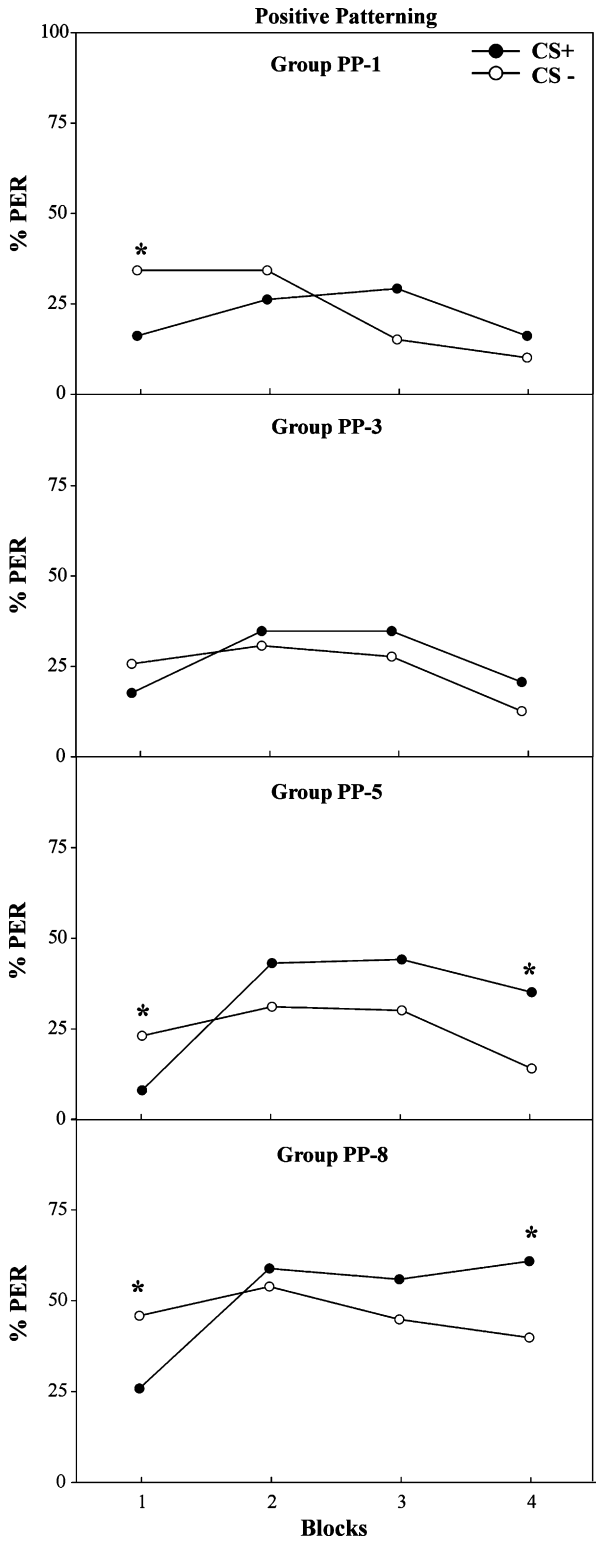

Fig. 3. Percentage of PER (proboscis extension reflex) of bees trained in a positive patterning discrimination (A−, B−, AB+) across four blocks of training trials with different intertrial-intervals (ITIs). Black circles indicate pooled responses to the reinforced mixture AB (CS+); white circles indicate responses to the non-reinforced single odors A and B (CSs−). First panel (group PP-1): ITI = 1 min; second panel (group PP-3): ITI = 3 min; third panel (group PP-5): ITI = 5 min; fourth panel (group PP-8): ITI = 8 min. Significant differentiation between CS+ and CSs− was found only in the groups trained with ITIs of 5 and 8 min (groups PP-5 and PP-8, respectively).

groups PP-1, PP-5 and PP-8 (PP-1: $F_{1,39} = 10.00$, $p < 0.01$; PP-5: $F_{1,39} = 13.50$, $p < 0.01$; PP-8: $F_{1,39} = 14.18$, $p < 0.01$;), but not in group PP-3 ($F_{1,39} = 3.47$, NS). In the latter group, however, the tendency was also to respond more to the CSs− than to the CS+. This effect may be explained by the same empirical rule described for NP ('respond to a CS only after experiencing reward on it, generalize further responses to any other CS'). In this case, fewer responses would be observed to the reinforced compound at the beginning of training while single odors would elicit more generalized responses. This pattern of responses was later reversed during the course of training.

At the end of training (Block 4), differentiation between CS+ and CS− was not equal for all groups (Fig. 3). Differentiation between CSs+ (AB+) and CSs− (A−, B−) was not significant in groups PP-1 and PP-3 ($F < 3.82$). However, bees trained with an ITI of 5 and 8 min (groups PP-5 and PP-8) responded significantly more to the CS+ than to the CSs− in the last block (PP-5: $F_{1,39} = 9.46$, $p < 0.01$; PP-8: $F_{1,39} = 15.85$, $p < 0.001$). Thus, both PP-5 and PP-8 groups developed successful differentiation in olfactory PP discrimination.

In order to detect variation trends depending on ITI for responses to the CS+ and CSs− at the end of training, we present only the results obtained in Block 4 in Fig. 4. The pooled responses (% PER) to the non-reinforced odors A and B (CS−: white bars) and the responses to the reinforced mixture AB (CS+: black bars) are depicted as a function of ITI. Comparison of these results across groups by means of linear trend analyses showed that both responses to the reinforced mixture CS+ ($F_{1,156} = 32.14$, $p < 0.001$) and to the non-reinforced single odors CSs− ($F_{1,156} = 19.86$, $p < 0.001$) increased significantly with increasing ITI. Most importantly, differentiation between CS+ and CSs− responses increased significantly with increasing

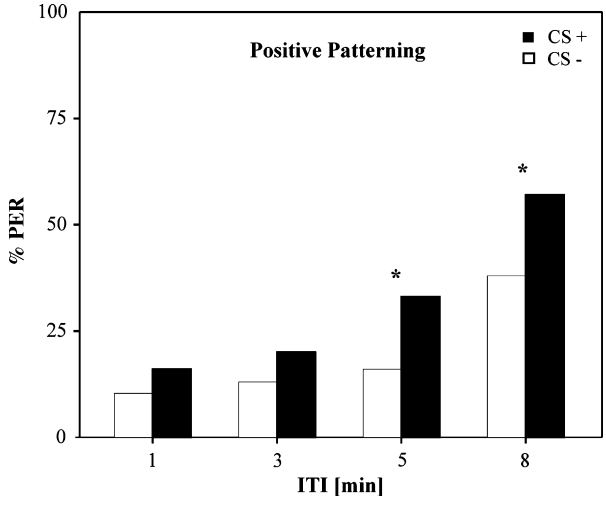

Fig. 4. Percentage of PER (proboscis extension reflex) in the last block of training in a positive patterning discrimination (A−, B−, AB+) with different intertrial-intervals (ITIs). Black bars indicate pooled responses to the reinforced mixture AB (CS+); white bars indicate responses to the non-reinforced single odors A and B (CSs−). Both responses to the reinforced mixture CS+ and to the non-reinforced single odours CSs− increased significantly with increasing ITI. Most importantly, differentiation between CS+ and CSs− increased significantly with increasing ITI.

ITI ($F_{1,156} = 5.31$, $p < 0.03$). Taken together, these results show that increasing the ITI, thereby decreasing reinforcement density, leads to an increase of responding to both types of stimuli, CS+ and CSs−, and to better differentiation in olfactory PP discrimination in honeybees.

3.3. Comparisons between negative and positive patterning

To provide a direct comparison between NP and PP, we compared for each ITI differentiation success between the two tasks, NP and PP. For ITIs of 1 and 3 min, as bees did not learn any of these tasks, differentiation in the last block did not differ significantly between NP and PP ($F_{1,78} = 0.71$ and 1.60 for 1 and 3 min, respectively; NS in both cases). For an ITI of 5 min, differentiation was significantly better in the PP group than in the NP group ($F_{1,78} = 4.18$, $p < 0.05$) as bees managed to solve PP but not NP with this particular ITI. Finally, for an ITI of 8 min, no difference was found between tasks ($F_{1,78} = 0.00$, NS) as bees managed to solve both NP and PP with similar success with this particular ITI. This analysis confirms that PP can be solved with a shorter ITI of 5 min compared to NP.

4. Discussion

Our present work shows that increasing the intertrial-interval (ITI) between conditioned trials leads to better differentiation between reinforced and non-reinforced conditioned stimuli (CSs) in olfactory negative (NP) and positive patterning (PP) discrimination tasks in honeybees. In both cases, short ITIs (1 and 3 min) did not allow the bees to differentiate between single odors and their binary mixture. For PP, an ITI of 5 min allowed successful differentiation while this was not the case for NP. However, increasing the ITI to 8 min allowed successful differentiation in both NP and PP. Such observation is in line with the trial-spacing effect [1], which posits that when animals are trained with a single reinforced stimulus, CS trials that are temporally more spaced lead to better learning. In the present work, in which reinforced and non-reinforced CS trials were interspersed, we explicitly varied the ITI between CSs, while the total number of trials was kept constant for all groups. Our results show that the distribution of reinforced trials in time is a critical factor for successful discrimination. Based on these results, however, we cannot assess the impact of the number of reinforced trials *per se* because this variable was kept constant (8 CS+ among 16 trials) in all our experimental groups. In a previous work [7], we found that lower reinforcement densities (0.17 in PP and 0.25 in NP, 4 and 6 CS+ trials among 24 trials, respectively) did not support higher CS+ responses and differentiation as the trial-spacing effect would have predicted. We suggested that for the trial-spacing effect to be valid, a minimum number of reinforced trials should be available. In the present work, we show that when such an amount is reached, longer ITIs do indeed support better CS+ acquisition and better differentiation between CS+ and CS−.

Our work confirms that, in honeybees, the trial-spacing effect is visible already during acquisition (see Section 1 and [24]). Analysis of the trial-spacing effect on acquisition is usually avoided because of the possible confounding factors automatically associated to such endeavour: since the same number of training trials is given over different periods of time in the different groups, several time-dependent effects could take place, which deserve a separate discussion.

4.1. The effect of overall experimental time

The goal of our experiments was to vary systematically the ITI between groups in order to study the effect of this variation on NP and PP discrimination. The procedure employed implied that the total time spent in each experiment also varied between groups. It can be, therefore, argued, that overall experimental time generates the differences evinced between groups both in NP and PP. This conclusion is, in fact, difficult to separate from our conclusion on ITI as the variable generating differences between groups. Because longer ITIs correspond to longer overall experimental times, both variables are necessarily confounded. Note, however, that all groups were trained with 8 CS+ and 8 CS− such that differences between groups can only be ascribed to temporal aspects of our conditioning procedure.

To avoid confounding factors, one could suggest an additional control, which would consist of a retention test performed at a variable interval after the last conditioning trial so that it would equate all groups in terms of overall experimental time. In such a retention test, bees would be presented with the CS+ and the CS− without reward. If, for instance, for all groups the test were to be performed 3 h after conditioning start, it would be performed 52 min after the last conditioning trial for the groups having an ITI of 8 min (overall experimental time: 128 min), 100 min after the last conditioning trial for the groups having an ITI of 5 min (overall experimental time: 80 min), 132 min after the last conditioning trial for the groups having an ITI of 3 min (overall experimental time: 48 min) and 164 min after the last conditioning trial for the groups having an ITI of 1 min (overall experimental time: 16 min). If, despite having the same overall experimental time (180 min), all groups exhibit the same differences that we found in acquisition, it could be concluded that differences between groups can be attributed to ITI. However, this experiment is also questionable. Note that in this case, another confounding variable remains, namely the different post-conditioning periods of the ITI groups. Differences between these groups in the retrieval test could thus reflect differences in post-conditioning period. Thus, overall experimental time and ITI are difficult to decorrelate. In the light of this situation, our results provide a valuable contribution to the question of trial-spacing effect and olfactory patterning discriminations in honeybees.

4.2. The effect of appetitive motivation

Given the differences in overall experimental time mentioned above, it could be argued that differences in appetitive motivation could influence the differences between ITI groups found in our work. In other words, because all groups receive eight times sucrose reward but during different overall experimental times (i.e. 16 min in ITI 1 min versus 128 min in ITI 8 min), bees

may exhibit different levels of satiation and therefore different motivations to respond to the US and to learn the discriminations. This argument can, however, be dismissed because of the low amount of reward provided during our experiments. Before conditioning, bees were starved for approximately 2 h in order to ensure a higher motivation to respond to the US (see Section 2). During the experiments they received eight times the US, and the amount received each time can be estimated to be around 3 μl or less (because bees drink for 3 s and it has been shown that their ingestion rate is 1 μl/s; see [29]). Thus, bees at the end of conditioning have approximately 20 μl of sucrose solution in their crops, having started with a relatively empty crop. This volume represents only one third of their crop capacity [29] so that it cannot introduce significant variations in responsiveness between groups. Such variations could be assumed if, for instance, bees were satiated along experiments and after 128 min (group ITI 8) energetic consumption would have reduced sucrose solution to basal levels, something that would not be expected after only 16 min (group ITI 1 min). Because in all cases, the amount of sucrose solution remains low, this factor can be discarded. Note that even if motivation reduced learning rates to some extent in short ITI groups, comparisons between NP and PP at each ITI are unbiased because both groups had exactly the same number of rewarded trials over the same period of time. In particular, comparison of both tasks with 5 min ITIs showed unambiguously that PP was significantly better solved than NP.

4.3. The effect of non-associative phenomena

The question of the possible impact of non-associative phenomena on the trial-spacing effect on olfactory PER conditioning in the honeybee has been discussed in great detail [24]. Habituation to either CS and/or US can be discarded, because this non-associative phenomenon occurs after many stimulus presentations, implying more trials than what we used in our experiments (US > 20 [5], CS > 200 [3]). Furthermore, our results are not compatible with interpretations invoking a role for sensitization in the bees' response as this non-associative phenomenon should occur in the shorter ITI groups, thus enhancing the general level of responsiveness. Figs. 2 and 4 show that higher levels of responding and discrimination were reached in the longer but not in the shorter ITI groups, thus discarding a possible effect of sensitization in our results.

4.4. The effect of associative phenomena

As for associative effects, acquisition could be low in groups with short ITIs because of backward conditioning, a phenomenon demonstrated in olfactory PER conditioning in honeybees [14]. If trials follow each other in a fast succession, an inhibitory association can take place between the US of trial n, and the CS of trial $n+1$ (backward association). However, previous work [14] showed that such inhibitory backward associations occur mainly at an interval of 15 s between US and CS and that longer intervals (i.e. 30 s) do not support backward learning. In our case, the shortest ITI was 1 min, which is well above this limit. We therefore interpret our findings as being mediated by associative effects taking place within each conditioning trial. The questions that arise are thus why do bees need longer ITIs to perform patterning tasks, and why is the minimum duration longer for NP than for PP?

4.5. Levels of complexity in NP and PP discriminations

The fact that NP could only be solved with the longest ITI of 8 min, while PP could be already solved with ITIs of 5 min and longer is in line with the ubiquitous observation that PP is more easily learned than NP (e.g. [2,16,32]). For instance, NP solving requires longer stimulus duration in rabbits [17], as well as longer ITIs [18] and longer processing time in humans [21]. In bees, we found recently that olfactory PP, but not NP, can be solved with odor stimulation of only one antenna while NP solving requires bilateral olfactory stimulation [19], indicating that the kind of processing necessary for NP is only available when both brain sides get direct olfactory input. These differences refer to distinct degrees of complexity in PP and NP processing. PP admits elemental processing (the total associative strength of a compound stimulus is equal to the sum of the associative strengths of its elements; [34]) because responses to the non-reinforced, single elements may be low while responses to the reinforced compound may be significant due to summation of the elements' associative strengths. NP, on the other hand, can only be solved through a processing that is different from the pure elemental summation of the elements' associative strengths. Such summation would always result in the animals responding more to the compound than to each element and therefore in the impossibility of solving NP. In the case of olfactory PP and NP in bees, we recently showed that specific processes are involved in such discriminations, which rely on the formation of a unique cue [7–9]. The unique cue hypothesis retains the summation principle of elemental theories but assumes in addition, that a specific representation (the 'unique cue') emerges from the combined presentation of the elements in compounds [32,33,41]. In olfactory NP, in which the presentations of the single odors are followed by reinforcement but mixture presentations are not, differentiation between the two classes of stimuli can only occur if the unique cue gathers enough negative associative strength to counteract the summed associative strength of the mixture. Our previous works show that the formation of a unique cue is essential for olfactory NP solving in bees [7–9]. The formation of a unique cue may as well be involved in PP, although theoretically it is not essential [7,8]. From this perspective, a unique cue would be more relevant for solving NP, compared to PP solving.

4.6. The formation of a unique cue in NP and PP discriminations

The formation of a unique cue in olfactory patterning discriminations in bees is a process that may require longer processing times due to the inherent ambiguity of these tasks when compared to simpler forms of olfactory learning. Single-stimulus conditioning (also called absolute conditioning), for instance, is an unambiguous task in which only one stimulus is repeatedly reinforced (A+). In honeybee olfactory learning, success-

ful acquisition is reached in single-stimulus conditioning even with very short ITIs (massed training: ITIs of 30 s or 1 min; [4,12,26,36]). Differential conditioning represents a higher level of complexity as it involves two stimuli with different outcomes (A+, B−), which have to be discriminated. In bee differential olfactory conditioning, successful discrimination has also been reported with ITIs as short as 1 min [11]. However, patterning tasks are even more complex because of their inherent ambiguity at the level of single elements, which are as often reinforced as non-reinforced [13]. We therefore suggest that they need more processing time to be successfully resolved by the animals. If the formation of a unique cue requires time, shorter ITIs and high trial density would counteract this process and would thus impair rather NP than PP. This hypothesis is consistent with the fact that an ITI of 5 min allows solving of PP but not of NP.

4.7. Memory consolidation and NP and PP discriminations

Successful differentiation in tasks like NP and PP relies on the formation of memories, which are known to go through several sequential or parallel stages. Time intervals are known to be critical in determining memory phases, their duration and their underlying physiological processes [10,23,25]. The different sequential or parallel memory stages support behavior at different times after learning, from early, labile forms (short-term memory, STM) to later, consolidated forms (long-term memory, LTM). In honeybee olfactory learning, early memory after a single learning trial is particularly sensitive to extinction and reversal learning, while a consolidated memory (LTM), only reached after multiple learning trials, is much more resistant [24,25]. Recently, a comparison between massed and spaced olfactory conditioning in the honeybee demonstrated that longer ITIs do not only lead to better acquisition, but also to better memory consolidation during acquisition and better performance later at long-term stages [26]. It was suggested that this finding might reflect the time-dependent interaction of constructive (long-term strengthening) and destructive (short-term forgetting) phenomena during acquisition [26]. Destructive effects would dominate acquisition in massed conditioning, whereas constructive effects would dominate acquisition in spaced conditioning [15,28]. Massed conditioned bees would thus show reduced performance during acquisition because consolidation is interfered by close temporal consecutive learning trials. This principle could very well explain our results. At each trial in our NP or PP tasks, a consolidation process would be started, which would take longer than for simple conditioning tasks because of the task's complexity (two single odors and a compound involved, CS+ versus CS− discrimination involving ambiguity at the level of the single odors). Each new trial, coming after 1, 3, or even 5 min in the case of NP, would disturb ongoing consolidation, and thus prevent the formation of the relevant associations. Following this line of thoughts, the range of time-intervals corresponding to "massed conditioning", i.e. supporting reduced performance due to blocked consolidation, would be dependent on the task. It would be of about 1 min for simple forms of conditioning, but would extend to 3–5 min for more complex learning tasks, like patterning discriminations. To test this idea, other complex learning procedures, involving more than two stimuli and being ambiguous at the level of the elements, should be studied using different intertrial-intervals. A potential candidate is the biconditional discrimination (AB+, CD+, AC−, BD−; [35]).

5. Conclusion

In other animal models, the trial-spacing effect is mostly studied in the long-term retention tests and seems to depend on the specific formation of a protein-dependent long-term memory [37,40]. Here we show a clear trial-spacing effect at the level of the acquisition of NP and PP tasks. Increasing ITI resulted in better differentiation between reinforced and non-reinforced CSs in both NP and PP tasks. However, whereas only the longest ITI of 8 min allowed discrimination in NP, PP could already be solved with an ITI of 5 min. This difference might be due to the fact that NP, but not PP, would require the formation of a unique cue and thus longer processing times. Future work should evaluate the effect of different ITIs on differentiation performances both in NP and PP but in the long-term range, in particular when protein-synthesis dependent LTM is active (about 3 days after training [24]).

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