Available online at www.sciencedirect.com
SCIENCE DIRECT®
Behavioural Brain Research 145 (2003) 135–143
BEHAVIOURAL BRAIN RESEARCH
www.elsevier.com/locate/bbr

Research report

Non-elemental processing in olfactory discrimination tasks needs bilateral input in honeybees

Bernhard Komischke [a], Jean-Christophe Sandoz [b,*], Harald Lachnit [c], Martin Giurfa [b]

[a] *Neurobiology, Institute of Biology, Free University of Berlin, Königin-Luise-Str. 28/30, D-14195 Berlin, Germany*
[b] *Centre de Recherches sur la Cognition Animale, Université Paul-Sabatier, 118, Route de Narbonne, 31062 Toulouse cedex 04, France*
[c] *Department of Psychology, Philipps-University of Marburg, Gutenbergstr. 18, D-35032 Marburg, Germany*

Received 21 October 2002; received in revised form 24 March 2003; accepted 24 March 2003

Abstract

In patterning discriminations, animals have to differentiate a compound stimulus AB from each of its elements A and B. In positive patterning (PP), the compound is reinforced whilst the single elements are non-reinforced. In negative patterning (NP), single elements are reinforced whilst the compound is non-reinforced. Using olfactory conditioning of the proboscis extension response (PER), we asked whether honeybees (*Apis mellifera*) can solve these patterning problems when odorants are given unilaterally as well as bilaterally to the antennae. Separating the olfactory input space of bees into two independent zones using plastic walls placed between the antennae, we conditioned bees in PP and NP procedures, with input on one side, on both sides, or in an ambiguous problem where bees had to solve PP on one side and NP on the other side. We found that bees with simultaneous bilateral input solve both patterning tasks efficiently. In contrast, PP but not NP was learned by bees receiving unilateral olfactory input. Bees subjected to the ambiguous NP/PP problem only solved PP. As PP can be solved through mere elemental processes, but NP is critically dependent on the use of non-elemental learning processes, our results suggest that bilateral olfactory input is necessary for non-elemental processing to take place in the bee brain.

Keywords: Negative patterning; Positive patterning; Olfaction; Side-specific learning; Non-elemental learning; Honeybee

1. Introduction

Most sensory systems are bilaterally symmetrical, a fact that allows for better integration of information from the surroundings. In general, the information perceived on one sensory input side is slightly different from that perceived on the contralateral side, and the brain uses such differential information to build internal representations with additional dimensions. The two brain sides thus collaborate to build a representation of the animals' environment. With regard to olfaction, bilateral sensory input was shown to be mainly beneficial through the difference in information content obtained from each input side [2,24]. But is the use of the two brain sides also beneficial when animals receive identical sensory input on each side?

Apis mellifera honeybees can learn to associate odor stimuli with sucrose reward. While foraging, they use their learning capacity to efficiently exploit food sources [25]. Using the paradigm for conditioning the proboscis extension reflex (PER) [4,18,44], bees were shown to be able to build odor-reward associations and to differentiate between numerous odor stimuli [46]. In this paradigm, harnessed honeybees are conditioned to olfactory stimuli associated with a reinforcement of sucrose solution. When the antennae of a hungry bee are touched with sucrose solution, the animal reflexively extends its proboscis to reach out toward and to lick the sucrose. Odors presented to the antennae do not usually release such a reflex in naive animals. If an odor is presented immediately before sucrose solution (forward pairing), an association is formed and the odor will subsequently trigger the proboscis extension response (PER) in a subsequent test. This effect is clearly associative and involves classical conditioning [4]. Thus, the odor can be viewed as the conditioned stimulus (CS) and sucrose solution as the reinforcing unconditioned stimulus (US). Simple elemental tasks can be efficiently achieved by bees receiving olfactory input from only one antenna [8,22,23,38,39]. Interestingly, even when receiving unilateral odor input, the formation of the olfactory memory appears to rely on the use of both brain sides, at least at the level of second-order integration centers like

* Corresponding author. Tel.: +33-561-55-65-03; fax: +33-561-55-61-54.
E-mail address: sandoz@cict.fr (J.-C. Sandoz).

0166-4328/$ – see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0166-4328(03)00105-0

the mushroom bodies: localized cooling of the contralateral mushroom bodies was found to significantly impair performance in retention tests after single-trial conditioning [8]. After a retention period of 3–24 h, bees also respond to a unilaterally learned odor when this odor is presented on the contralateral side, thus showing inter-hemispheric transfer of the olfactory memory trace [38]. Beyond unilateral conditioning tasks, bees can also be trained in a side-specific manner, with ambiguous discrimination problems. For instance, when explicitly trained, bees can learn opposite information on the two brain sides, i.e. learning a differential discrimination A+B− on one side, and the opposite discrimination B+A− on the other side [38]. All these studies show that associative olfactory learning in the honeybee relies on both unilateral and bilateral processes. Recently, reduced learning of odor component B in a mixture of two odorants (AB) after learning A (the "blocking" effect) was described as requiring olfactory input from both antennae [45]. This suggests that bilateral olfactory input, even if identical on each body side, could provide the bees with increased processing capacity (note that the existence of the blocking phenomenon in honeybees is highly controversial [5,10,12,42,43]).

Honeybees are also able to solve complex learning problems [26,27]. Among these, patterning discrimination tasks are particularly interesting, because they raise an ambiguity problem for the animal [6,7,35]. In such learning problems, animals have to differentiate a compound stimulus AB from its elements A and B. In positive patterning (PP), the single elements are nonreinforced whilst the compound is reinforced (A−, B−, AB+). Conversely, in negative patterning (NP), single elements are reinforced whilst the compound is nonreinforced (A+, B+, AB−). Although they may appear symmetrical, these two tasks differ in the kind of processing needed to solve them: a PP discrimination can be solved by animals using elemental processing, i.e. if the animal treats the compound AB as the simple sum of its elements A and B. The associative strengths of each of the elements could be below the threshold needed for inducing a conditioned response, but once added during compound presentation they might result in an associative strength above this threshold. This could explain why the animals respond more to the compound than to either element. By contrast, a NP discrimination cannot be solved in this way, because the associative strength upon compound presentation would always be higher than each of the associative strengths of the elements. Therefore, it is not possible to explain higher responses to the elements than to the compound based on pure elemental processing. Solving NP can only be explained if the animals are capable of non-elemental processing, i.e. if they treat the compound as being different from the simple sum of its elements A and B.

In the present work, we ask whether honeybees can solve such patterning problems with unilateral olfactory input, or whether identical bilateral olfactory input allows additional processing capacity, as was suggested for blocking [45]. In particular, it was observed in a wide range of species, including honeybees, that NP is more difficult to solve than PP [3,6,13,16,17,19,32,33,48]. We were therefore interested in possible differences in the extent to which PP and NP could be solved with unilateral olfactory input. We thus compared performances of bees in unilateral PP and NP conditioning with performances in bilateral NP and PP conditioning. We also evaluated bees' performance when confronted with a contradictory patterning problem, having to simultaneously solve PP on one side and NP on the opposite side (A−B−AB+/A+B+AB−). This group allowed us to test whether bilateral but non-simultaneous input to both antennae is sufficient to solve both patterning tasks.

2. Materials and methods

2.1. Subjects

Free-flying honeybee foragers, *A. mellifera*, were caught at the entrance to outdoor hives in the morning of each experimental day. They were placed in small glass vials and immobilized by brief cooling. The bees were then harnessed in small metal tubes so that they could only move their antennae and mouthparts, including the proboscis [4,44]. To separate the olfactory input space of the bee into two independent zones, we used thin plastic walls placed between the two antennae (Fig. 1A). The walls were made of a 40 mm × 50 mm piece of overhead transparency plastic, cut precisely to the shapes of the bee holder and the bee's head. Each wall was then attached with low-temperature melting wax in order to fill in any remaining open spaces between sides, under the proboscis to the front, on the head, or on the back of the tube. The wall was placed slightly to one side, so that the proboscis could move freely [38]. Afterwards bees were kept in the dark at high humidity for 2 h. Fifteen minutes before starting the experiments, each subject was checked for intact proboscis extension reflex (PER) by touching both antennae lightly with a toothpick soaked with 30% sucrose solution (w/w) without subsequent feeding. Extension of the proboscis beyond a virtual line between the open mandibles was counted as a PER (unconditioned response). Animals that did not show the reflex (<10%) were discarded.

2.2. Stimulation apparatus

During conditioning, each bee was placed individually in front of a bilateral odor-supplying device (Fig. 1B). An identical airstream was provided on each side. Before reaching the bee, each air stream was directed through one (or two—see compound presentations) of three channels, each guarded by a valve controlled by the experimenter via a computer. Each channel contained a cartridge obtained from the cut end of a 1 ml-syringe. The first channel, where the air stream flowed continuously when no odor stimulation was given (most of the time), contained a cartridge filled with a piece of filter paper. The second and third channels

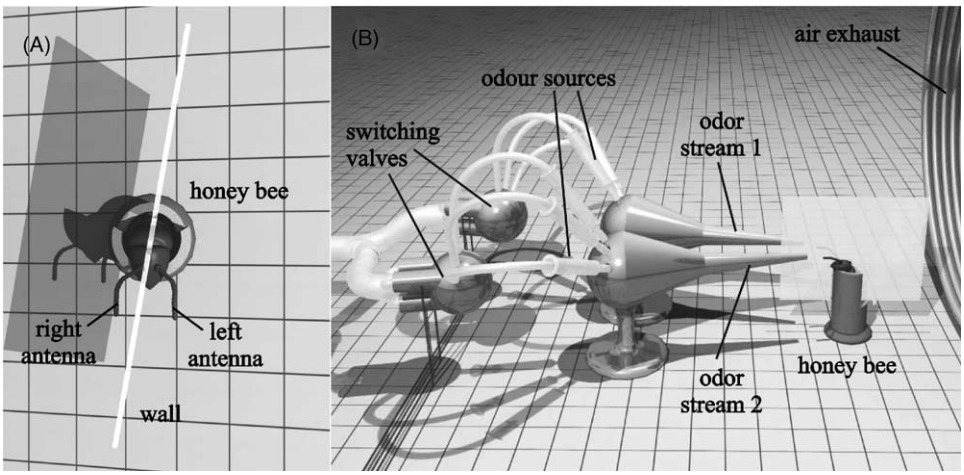

Fig. 1. Schematic view of the stimulation system used for unilateral and bilateral odor stimulations. (A) Honeybee placed in a holder with a wall between its antennae. The wall is placed slightly to one side to allow the proboscis to move freely. (B) A computer-controlled stimulation device allows delivery of individual odors or mixtures to the bee, on either one or both sides of the wall. Airflows are constant and equal on both sides. An exhaust fan behind the bee removes odorants from the lab.

each contained an odor source, i.e. a piece of filter paper with 4 μl of odor substance (one source with limonene and one with 2-nonanone on each side—both odors obtained from Sigma–Aldrich—Deisenhofen, Germany). The computer allowed the experimenter to apply side-specific or bilateral odor stimulations, switching valves unilaterally or bilaterally. Single-odor presentations were carried out by switching the airflow from the odorless channel to one of the odor channels. For compound stimulations, the airflow went through both odor channels. The overall airflow reaching the bee was identical on both sides and for all types of stimulations (element or compound presentations). Each air stream was precisely directed to each antenna (parallel to the wall on each side) with 4 cm pieces of Teflon tubing (5 mm internal diameter). The distance between the outlet of the odor-supplying device and each antenna was 1 cm. An exhaust placed behind the bee's head (Fig. 1B) removed the released odorants from the lab.

2.3. Training trials

Each trial lasted 60 s. At the beginning of each trial one subject was placed in front of the odor-supplying device for 26 s to allow familiarization with the training situation. The CS was then presented for 4 s (unilaterally or bilaterally). For reinforced trials, the US (30% sucrose solution) was applied 3 s after CS onset. We applied a compound-US, first lightly touching one antenna (or both—see below) with a toothpick soaked with the sucrose solution and after proboscis extension, the bee was allowed to feed for 3 s. In groups receiving a unilateral CS (Single-PP, Single-NP, NP/PP), the antenna ipsilateral to the CS received the US. For bees receiving a bilateral CS (Double-PP and Double-NP groups, see below), both antennae were simultaneously touched with two toothpicks and sucrose solution was given to the proboscis for 3 s. After completing each 60-s trial, animals were returned to their resting positions. Non-reinforced trials were identical to reinforced trials, except that the US was omitted altogether.

2.4. Experimental design

Five experimental groups were subjected to either one or two patterning discrimination problems. Two groups, Double-NP and Double-PP, served as control groups to evaluate the ability of bees to solve PP or NP when they receive bilateral olfactory input (as in previous studies [6,7]), but have a plastic wall on the head. Two other groups, Single-NP and Single-PP, were designed to evaluate the ability of honeybees to solve PP or NP with unilateral olfactory input. Finally, the NP/PP group was used to evaluate the effect of providing contradictory information to the two brain sides. This group received an NP schedule on one side, and, interleaved within the same experimental session, a PP schedule on the opposite side. The experimental design is summarized in Table 1.

Each experimental procedure consisted of 24 trials (12 reinforced and 12 non-reinforced) with 12 min inter-trial intervals. In PP (groups Double-PP, Single-PP and side 2 of NP/PP), bees received 12 reinforced trials with AB, and 12 non-reinforced trials, 6 with A and 6 with B. In NP (groups Double-NP, Single-NP and side 1 of NP/PP), bees received 12 reinforced trials, 6 with A and 6 with B, and 12 non-reinforced trials with AB. In all groups, four possible trial sequences were used (see Table 1), so that bees received in alternating order a reinforced and a non-reinforced trial, and the sequence began with AB (in 50% of the cases), with A (25%), or with B (25%). NP/PP bees were stimulated every 6 min, alternating between one side (NP) and the other (PP). They thus received twice as many learning trials as

Table 1
Protocol of the five experimental groups

Group	Side 1	Side 2	Trial sequences
Double-PP	6A− 6B− 12AB+	6A− 6B− 12AB+	#1
Double-NP	6A+ 6B+ 12AB−	6A+ 6B+ 12AB−	#2
Single-PP	6A− 6B− 12AB+	–	#1
Single-NP	6A+ 6B+ 12AB−	–	#2
NP/PP	6A+ 6B+ 12AB−	6A− 6B− 12AB+	#1 and #2

Each group received 24 trials, except the NP/PP group, which received 48 trials (24 on each side). In each group, four possible trial sequences were used. On each experimental day, side 1 was the left side for half the bees in each group, and the right side for the other half. (#1) Four possible sequences: A−, AB+, B−, AB+ or B−, AB+, A−, AB+ or AB+, A−, AB+, B− or AB+, B−, AB+, A−. For one given bee, the sequence of four trials was repeated six times. (#2) Four possible sequences: A+, AB−, B+, AB− or B+, AB−, A+, AB− or AB−, A+, AB−, B+ or AB−, B+, AB−, A+. For one given bee, the sequence of four trials was repeated six times.

the other groups (48 trials with 6 min ITIs), but they were identical to the other groups with respect to the number of trials received on one given side (24 trials with 12 min ITIs).

2.5. Response measurement

We recorded whether or not a bee extended its proboscis after onset of the CS and, in the reinforced trials, before presentation of the sucrose solution (US). Multiple responses during a CS were counted as a single PER. After completing the experiments, bees were again checked for the proboscis extension reflex. All bees used in the experiments ($n = 160$) responded to this test and were therefore kept for further analysis.

2.6. Statistical analysis

In Figs. 2 and 4, data are presented in six blocks of four trials: each block corresponds to one A trial, one B trial and two AB trials. Because in one experimental group (Double-PP) bees treated the two odors differently (they responded significantly more to 2-nonanone than to limonene), we could not pool their responses to the elements. Therefore, Figs. 2 and 4 show the responses to limonene as odor A and to 2-nonanone as odor B.

In the experiments, we measured the proportions of conditioned responses (%PER). Analyses of variance (ANOVAs) were used for between-group as well as within-group comparisons. Although parametric analysis of variance is usually not allowed in the case of dichotomous data such as those for the PER, Monte Carlo studies have shown that it is permissible to use ANOVAs for dichotomous dependent variables under certain conditions [20], which are met by our data: equal cell frequencies and at least 40 degrees of freedom of the error term. The alpha level was set to 0.05 (two-tailed) for all analyses. Stated probability levels are based on the Greenhouse–Geisser [11] adjustment of degrees of freedom where appropriate.

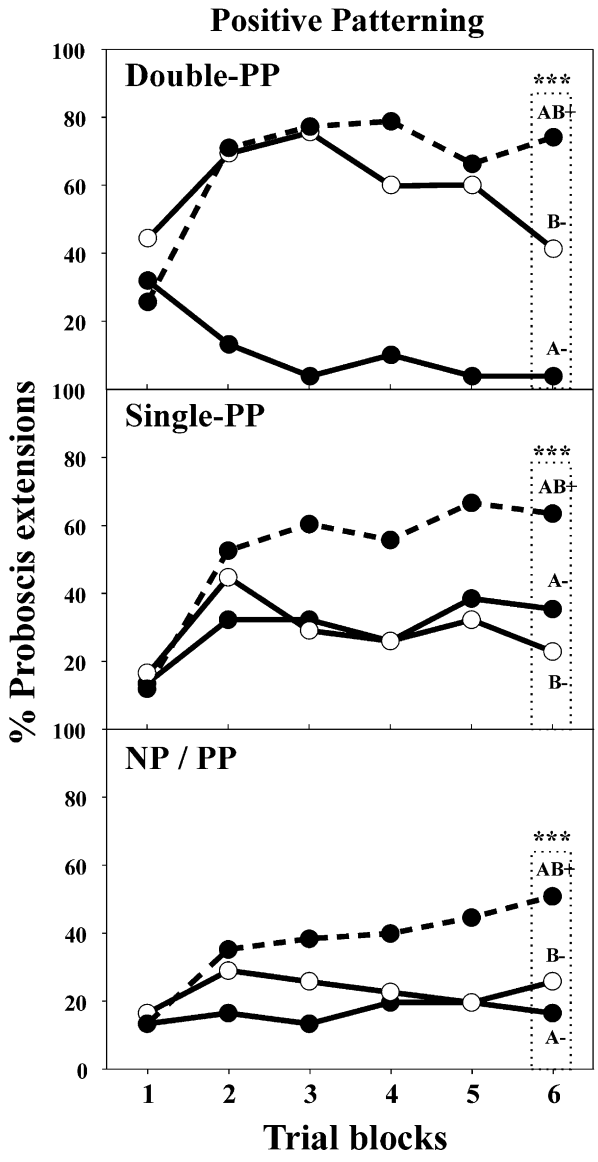

Fig. 2. Positive patterning groups. Three groups of 32 bees were subjected to positive patterning training with olfactory input on both sides (Double-PP), on a single side (Single-PP), or on one side, the other side being subjected to negative patterning (NP/PP). For each group, we present the % proboscis extension across blocks, each block consisting of one presentation of each of the elements A and B and of the mean of two compound presentations (AB). In all groups, bees learned to respond significantly more strongly to the compound than to the elements (*** $P < 0.001$, ANOVA).

To evaluate the performances of bees at the individual level, we counted the number of errors made in the last block of trials, which contained one trial with each of the single odors and two trials with the compound. For PP (AB+, A−, B−), an error was counted each time a bee either responded to the individual elements, or did not respond to the compound. By contrast, for NP (AB−, A+, B+), an error was counted each time a bee responded to the compound, or did not respond to one of the two elements. Theoretically, since

there were four trials per block, the number of errors could vary between 0 and 4. However, most bees made between 0 (they responded correctly) and 2 errors (they either responded to every stimulation, or to none). A few bees made three errors. Because ANOVAs could not be computed in this case, the comparisons of errors between groups were performed using a Kruskal-Wallis test (2 d.f.). When significant, pair-wise comparisons were done using the Noether method, including a correction for multiple comparisons [40]. Of particular importance were bees which made 0 errors, since only such bees could be considered as having solved the NP or the PP task, effectively differentiating the compound from each of its elements. The comparison between groups of the number of individuals making 0 errors was performed using a *G*-test (2 d.f.).

3. Results

3.1. Positive patterning

Fig. 2 illustrates for the three experimental groups trained in a PP schedule the percentage of proboscis extension responses (%PER) across six blocks of trials to stimuli A, B, and AB. All three groups solved the PP discrimination. At the end of training, bees responded more to the compound AB than to each of the elements A and B. However, the amount of differentiation differed between groups. The statistical analysis (Group × Stimulus × Block ($3 \times 3 \times 6$) ANOVA) confirmed this view: overall, bees responded differently to the stimuli (main effect stimulus: $F_{2,186} = 50.7$, $P < 0.001$) and this differentiation varied significantly both across trials (Stimulus × Block interaction: $F_{10,930} = 11.8$, $P < 0.001$) and among groups (Group × Stimulus interaction: $F_{4,186} = 11.8$, $P < 0.001$). To analyze more precisely how each group differentiated the three stimuli at the end of training, we focused on block 6. We found heterogeneity between groups in their responses to the stimuli (Group × Stimulus (3×3) ANOVA, main effect stimulus: $F_{2,186} = 37.5$, $P < 0.001$; Group × Stimulus interaction: $F_{4,186} = 4.8$, $P < 0.002$). We thus examined the responses of each group separately. All three groups showed significantly different responses to the stimuli (ANOVA, Double-PP group: $F_{2,186} = 29.33$, $P < 0.001$; Single-PP group: $F_{2,186} = 10.26$, $P < 0.001$; NP/PP group: $F_{2,186} = 7.48$, $P < 0.001$). Multiple comparisons using Tukey tests showed that in group Double-PP bees responded more to AB than to either A or B. Unexpectedly, responses to B were higher than to A. In the Single-PP group, responses to AB exceeded both responses to A and to B. In the NP/PP group, responses to AB were higher than to A and nearly significantly higher than responding to B. The three groups differed in responses to A (ANOVA, $F_{2,186} = 4.41$, $P < 0.02$), but not to B ($F_{2,186} = 1.80$, $P > 0.16$), and only marginally in responses to AB ($F_{2,186} = 2.45$, $P > 0.08$). A post-hoc Tukey test showed that responses to A in the Double-PP group were lower than in the Single-PP group, with the NP/PP group falling in between.

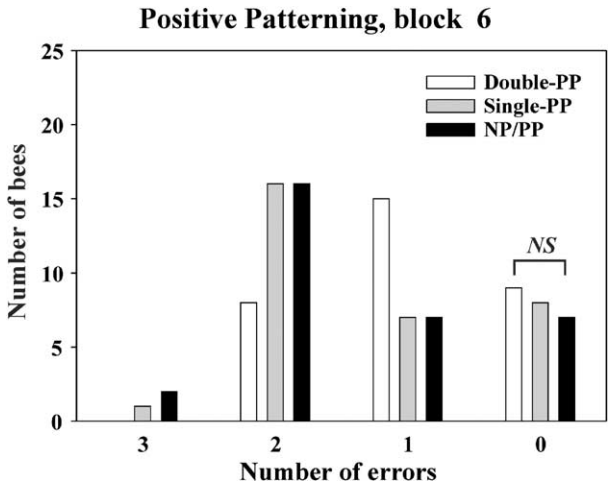

Fig. 3. Positive patterning groups. Number of errors made by bees in the last block of trials. No significant difference in the overall distribution between groups. In particular, in all groups, from 7 to 9 bees did not make any error in this last block and were considered as having solved the PP problem, with no difference between groups (NS, *G*-test).

We next analyzed the errors committed by bees in the last block of trials at the individual level. This analysis complements the previous statistics, which focused on group performance. For each bee, an error was counted whenever it responded to A or to B, or when it did not respond to AB. Fig. 3 shows the distribution of errors committed by bees from the three groups. Bees subjected to PP thus made between 0 and 3 errors, and showed a similar distribution of errors in the different groups (Kruskal-Wallis test, $H = 5.0$, NS, 2 d.f.). Bees which did not make any errors in this last block were particularly interesting to us (see right-hand bars in Fig. 3) since they efficiently solved the PP task (they responded to the compound but not to the elements). Thus, 9, 8 and 7 bees were in this category for the groups Double-PP, Single-PP and NP/PP respectively, without any difference between groups (*G*-test, $G = 0.33$, NS, 2 d.f.). Taken together, these results show that bees solved PP with the same efficiency when receiving olfactory input on one side, on both sides, or when subjected to PP on one side together with NP on the contralateral side.

3.2. Negative patterning

Fig. 4 illustrates for the three experimental groups trained in an NP schedule the percentage of proboscis extension response (%PER) across six blocks of trials to stimuli A, B, and AB. Observation of the data suggests that the Double-NP group, but not the Single-NP group, was able to solve the discrimination task. Group NP/PP showed some response differentiation between the compound AB and the elements A and B. These impressions were confirmed by the statistical analysis (Group × Stimulus × Block

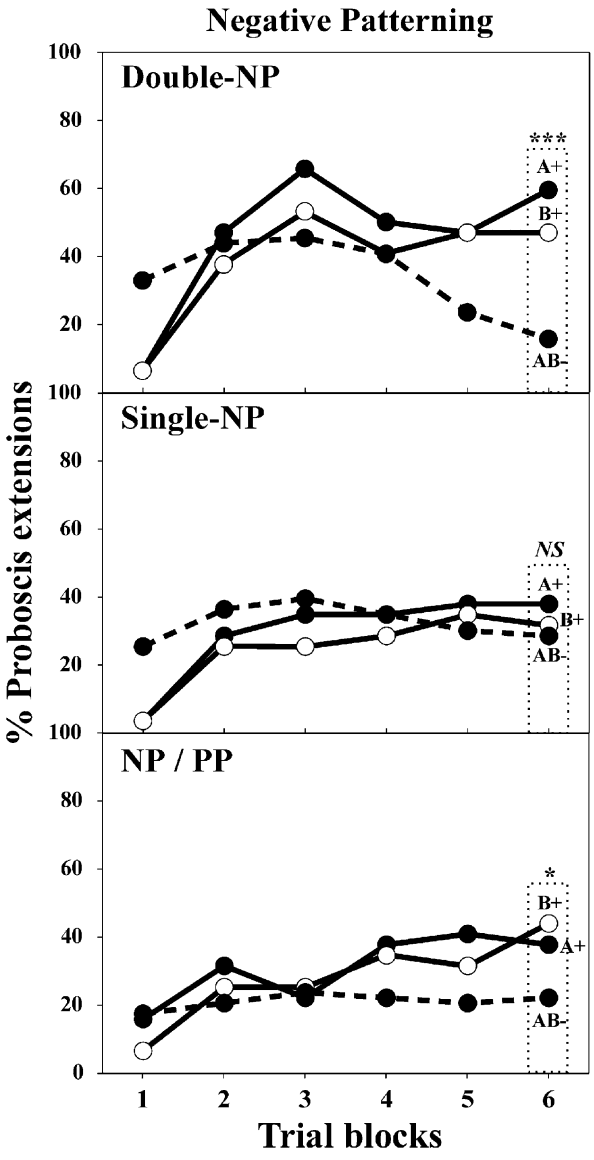

Fig. 4. Negative patterning groups. Three groups of 32 bees were subjected to negative patterning training with olfactory input on both sides (Double-NP), on a single side (Single-NP), or on one side, the other side being subjected to positive patterning (NP/PP). For each group, we present the % proboscis extension across blocks, each block consisting of one presentation of each of the elements A and B and of the mean of two compound presentations (AB). Only Single-NP and NP/PP group bees significantly differentiated among stimuli at the end of training ($^{*}P < 0.05$, $^{***}P < 0.001$, NS: not significant, ANOVA).

($3 \times 3 \times 6$) ANOVA): overall, bees differentiated the stimuli (main effect stimulus: $F_{2,186} = 4.29$, $P < 0.02$) and this differentiation varied significantly both across trials (Stimulus × Block interaction: $F_{10,930} = 6.54$, $P < 0.001$) and among groups (Group × Stimulus interaction: $F_{4,186} = 2.74$, $P < 0.04$). As for the PP groups, we next analyzed how each group differentiated the three stimuli at the end of training (block 6). We found heterogeneity between groups in their responses to the stimuli (Group × Stimulus (3×3) ANOVA, main effect stimulus: $F_{2,186} = 15.24$, $P < 0.001$ and Group×Stimulus interaction: $F_{4,186} = 3.59$, $P < 0.008$). We thus examined the responses of each group separately. The Double-NP ($F_{2,186} = 17.31$, $P < 0.001$) and NP/PP ($F_{2,186} = 4.33$, $P < 0.03$) groups but not the Single-NP group ($F_{2,186} < 1$) showed significant differentiation among the three stimuli. Multiple comparisons using Tukey tests showed that in the Double-NP group responses to AB were lower than responses to both A and B. In the NP/PP group, however, responses to AB did not differ from responses to A, and were only nearly significantly lower than those to B. No difference appeared between groups in their responses to any of the stimuli (A: $F_{2,186} = 2.45$, $P > 0.08$; B: $F_{2,186} = 1.05$, AB: $F_{2,186} < 1$, NS).

We next analyzed the errors committed by bees in the last block of trials at the individual level. For each bee, an error was counted whenever it responded to AB, or when it did not respond to A or to B. Fig. 5 shows the distribution of errors made by bees from the three groups. Bees made between 0 and 3 errors, but the overall distribution of errors differed between groups (Kruskal-Wallis test, $H = 9.6$, $P < 0.01$, 2 d.f.). More precisely, the number of bees making no errors in the last trial (i.e. bees which solved the NP task) differed between groups ($G = 10.45$, $P < 0.01$, 2 d.f.). Nine such bees were present in the Double-NP group, versus one and two bees in the Single-NP and NP/PP groups, respectively.

Taken together, these results show that bees could only solve the NP task when they received simultaneous olfactory input on both brain sides. When receiving unilateral olfactory input, they could not solve the task at all. When experiencing A+, B+, AB− training at one antenna interleaved with A−, B−, AB+ trials at the other antenna (NP/PP group), bees showed some amount of differentiation between AB and A and B (Fig. 4), but at the individual level,

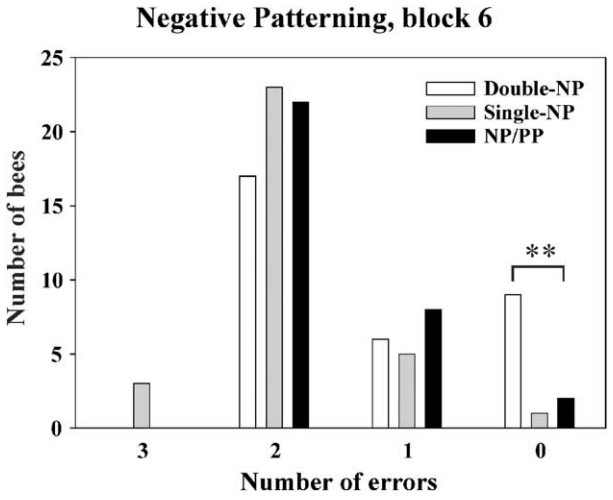

Fig. 5. Negative patterning groups. Number of errors made in the last block of trials. The distribution of errors showed heterogeneity among groups. In particular, 9 bees from the Double-NP group vs. 2 (NP/PP group) and 1 (Single-NP group) did not make any error in this last block and were considered as having solved the NP problem. This difference was significant ($^{**}P < 0.01$, G-test).

only two bees made no error, a proportion which could be coincidental.

4. Discussion

Our work shows that bees can solve PP (A−, B−, AB+) with olfactory input on one side, on both sides or on one side, when the other side was subjected to an NP task in an interleaved fashion. In contrast, NP (A+, B+, AB−) can only be solved if bees receive simultaneous bilateral olfactory input. Although the patterns of stimulus presentations in these two patterning tasks seem to be symmetrical, previous work has suggested that they may rely on different processes. It is a general observation in a wide range of species, including honeybees, that NP is more difficult to solve than PP [3,6,13,16,17,19,32,33,48]. Theoretically, the major difference between the two tasks is that PP could potentially be solved based on a mere elemental summation principle (according to the Rescorla–Wagner model [34]), whereas NP cannot. To solve NP, additional non-elemental processing has to take place (see Section 1). The fact that PP could be solved with unilateral olfactory input is perfectly in line with previous work, which showed that elemental processing can be achieved with input to only one brain side [8,22,23,38,39]. Our most interesting result however is the fact that NP could not be solved unilaterally, since it suggests that the kind of non-elemental processing needed by honeybees to solve NP requires simultaneous bilateral olfactory input to the brain.

4.1. Solving the ambiguous NP/PP problem

We subjected one group of bees to both NP and PP problems interleaved within the same experimental session, each patterning schedule being applied to one input side (NP/PP group). The results showed that bees solved the PP task with the same efficiency in this case as when presented with the PP problem on only one side (Single-PP). On the contralateral side (NP side), bees showed slightly higher responses to the reinforced elements A and B than to the non-reinforced compound AB. However, when looking at individual responses, we found that only two bees out of 32 could be viewed as having really solved the task (i.e. having made no error at the end of training), which could be coincidental. This discrepancy between individual error rates and overall percentages is due to the fact that a number of individuals made only one error in the last block, either responding to the compound once or not responding to one of the elements. Although these individuals show higher proportions of responses to the elements than to the compound, they could not be viewed as having solved the task completely. They could be in the course of differentiating between elements and compound. Thus, although we insist that bees did not solve NP efficiently in this group, olfactory input on both sides but not simultaneously could lead to the use of non-elemental processing, as in the Double-NP group. To shed more light on this question, further NP/PP experiments should be performed, which would include a higher number of learning trials. Alternately, another explanation could also account for higher responses to the elements than to the compound on the NP side. In a previous study, we showed that bees could learn odors with reversed contingencies on the two brain sides (A+B−/B+A− training [38]), thus responding to each odor on the side where it was reinforced, and not on the side where it was non-reinforced. These bees apparently learned side-specific rules of the type A+/A− (Sandoz, Galizia, Menzel, in press). The NP/PP group of the present experiment is in principle a more complex version of the A+B−/B+A− problem, where bees need to give opposite values to three classes of stimuli between sides (A, B and AB). It is possible that the use of side-specific rules could have induced slightly better results in the NP/PP group than were obtained in the Single-NP group. To exclude this possibility, a bilateral NP/PP experiment with a different set of odors on each side (A+B+AB−/C−D−CD+) could be performed, in order to exclude the use of side-specific rules.

4.2. Non-elemental processing theories

An unexpected result was obtained in the Double-PP group as we showed that the two elements were first processed unequally. In the first half of the procedure (Fig. 3, blocks 1–3), bees seemed to first reduce the complexity of the problem by treating it as a differential conditioning problem (AB/B+ versus A−); B and the compound AB being treated as one odor. In the second half (blocks 4–6), bees then differentiated the element B from the compound AB, when responses to A were already low. In the Double-NP group, unequal responses to the two elements were also found, differentiation progressing more rapidly between A and the compound AB than between B and AB. Such inequalities between elements in an NP task can be readily explained by conventional learning models, based only on differences in the salience of each element for the animal [31]. Briefly, two main classes of theories have been developed so far, which can explain how organisms learn to solve non-elemental learning tasks like NP. The unique-cue hypothesis, based on the Rescorla and Wagner model [34], assumes that a compound is perceived as being the sum of each of its elements, plus an additional stimulus U, which is unique to the compound and results from the joint presentation of the elements [33,47]. According to this idea, the A+, B+, AB− discrimination corresponds in fact to an A+, B+, ABU− discrimination. By contrast, the configural theory presented by Pearce [29,30] views each compound as a new stimulus, distinct from its elements. Solving patterning tasks then corresponds to learning a discrimination between three different but similar stimuli. Our previous work in honeybees has concentrated on the ability of these theories to explain non-elemental discrimination tasks [6,7,41]. Until now, our results have provided extensive evidence that non-elemental

olfactory processing in the bee follows the rules of a modified unique-cue model (MUC) (Deisig, Lachnit, Sandoz, Lober, Giurfa, in press). The MUC is based on the precepts of the unique cue theory, but also takes into account the fact that the salience of stimuli is reduced when they appear in a compound (see also [14]). In particular, the MUC model predicts that in NP, if the two elements A and B have different saliencies, the discrimination between the compound and the less salient element will progress more rapidly than that between the compound and the more salient element (note that the usual unique-cue model makes the opposite prediction, see [31]). Additional data (B. Komischke, unpublished results) show that for bees, 2-nonanone (stimulus B) is more salient than limonene (stimulus A), both alone and in a compound. The results of the Double-NP group thus follow the prediction of the MUC model hypothesis, since differentiation between B and AB was slower than between A and AB (for PP, both unique cue and MUC models predict that the most salient stimulus will show intermediate responses between the less salient stimulus and the compound; the data from the Double-PP group show exactly this response pattern). Therefore, the present asymmetry in response to the elements confirms our previous studies, suggesting that compound processing in the honeybee follows the rules of the modified unique-cue hypothesis. We thus believe that an odor compound is processed as its elements plus an additional internal stimulus (or unique-cue).

4.3. Neural substrates of patterning discrimination in the honeybee brain

If the presentation of an olfactory compound generates a unique cue, which allows the brain to solve patterning tasks efficiently, the present study suggests that either generating or using such a unique cue can only take place when bees simultaneously receive olfactory input from both antennae. What advantage could bilateral olfactory input confer over unilateral input, particularly when the information obtained on each side is identical? In the honeybee brain, odor processing involves different stages and is symmetrical between sides. Axons of the chemoreceptors on each antenna project to the 160 glomeruli of each antennal lobe, the primary olfactory center, where they synapse with about 4000 local interneurons and about 800 projection neurons [1,28]. The projection neurons further convey the information to higher brain centers, the mushroom body calices and the lateral protocerebral lobes [1,28]. In the antennal lobe, optical imaging techniques have shown that odors elicit glomerular response patterns [15] based on a code, which is conserved between individuals [9,37]. We think that the antennal lobes could provide the substrate for the generation of a unique cue upon compound presentation. When presenting a blend of two odours A and B, glomerular activation patterns in the antennal lobe resemble the summation of the patterns of A and B, but are slightly different. Such a difference increases with the number of elements present in the compound [15]. The difference appearing between the summed patterns generated by elemental odours and that observed on compound presentation could act as a unique cue. Such information would thus be available to higher brain centers, which would then need to extract it so that it can receive associative strength in the same way as any external stimulus [33]. Within this frame, the dependency of NP on bilateral olfactory input would indicate the necessity of concerted processing of the mushroom bodies on both brain sides. Our working hypothesis is that the mushroom bodies are involved in the extraction of the neural representation of the unique cue. A means to test this hypothesis is to make use of a technique that uses hydroxyurea at the larval stage to generate adult bees with specific lesions at the level of the mushroom bodies [21,22]. As a result of this treatment, bees often show unilateral ablations of the median calyx [21]. According to our hypothesis, we expect that unilateral mushroom body ablations would make NP, but not PP, more difficult to solve for bees. At the anatomical level, the olfactory pathways of the two brain hemispheres are mainly connected at the level of the output of the MBs, the α-lobes. Placed at an intensive information crossway in the bee brain, each α-lobe receives direct information from the antennal lobe [1], as well as processed information from the MB calyces ipsilaterally and from the contralateral α-lobe [28,36]. This interesting property could allow the comparison of neural representations of individual odorants and their compound, and possibly the computation and extraction of the unique cue representation. The challenge of future work will be to specifically address the role of the α-lobes in non-elemental olfactory processing and to understand why bilateral input is needed to solve such tasks as negative patterning.

Acknowledgements

We thank T. Franke for Fig. 1. J.C. Sandoz was funded by a research fellowship from the Alexander von Humboldt Foundation and by the Human Frontier Science Program. Martin Giurfa was supported by the ‘Programme Action Cognitique’ of the French Research Ministry, the ‘Fondation pour la Recherche Médicale’ and the Human Frontier Science Program (Young Investigator Grant). H. Lachnit was supported by grant La 564/10-3 from the German Science Foundation (Deutsche Forschungsgemeinschaft: DFG). We are grateful to an anonymous referee and to Mary Wurm for helping to improve the manuscript.

References

[1] Abel R, Rybak J, Menzel R. Structure and response patterns of olfactory interneurons in the honeybee, *Apis mellifera*. J Comp Neurol 2001;437:363–83.

[2] Basil JA, Hanlon RT, Sheikh SI, Atema J. Three-dimensional odor tracking by *Nautilus pompilius*. J Exp Biol 2000;203:1409–14.

[3] Bellingham WP, Gillette Bellingham K, Kehoe EJ. Summation and configuration in patterning schedules with the rat and rabbit. Anim Learn Behav 1985;13:152–64.
[4] Bitterman ME, Menzel R, Fietz A, Schäfer S. Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). J Comp Psychol 1983;97:107–19.
[5] Couvillon PA, Campos AC, Bass TD, Bitterman ME. Intermodal blocking in honeybees. Q J Exp Psychol B 2001;54:369–81.
[6] Deisig N, Lachnit H, Giurfa M, Hellstern F. Configural olfactory learning in honeybees: negative and positive patterning discrimination. Learn Memory 2001;8:70–8.
[7] Deisig N, Lachnit H, Giurfa M. The effect of similarity between elemental stimuli and compounds in olfactory patterning discriminations. Learn Memory 2002;9:112–21.
[8] Erber J, Masuhr T, Menzel R. Localization of short-term memory in the brain of the bee, *Apis mellifera*. Physiol Entomol 1980;5:343–58.
[9] Galizia CG, Sachse S, Rappert A, Menzel R. The glomerular code for odor representation is species specific in the honeybee *Apis mellifera*. Nat Neurosci 1999;2:473–8.
[10] Gerber B, Ullrich J. No evidence for olfactory blocking in honeybee classical conditioning. J Exp Biol 1999;202:1839–54.
[11] Greenhouse SW, Geisser S. On methods in the analysis of profile data. Psychometrika 1959;24:95–112.
[12] Hosler JS, Chandra SB, Smith BH. Heritable variation for latent inhibition and its correlation to reversal learning in the honeybee, *Apis mellifera*. J Comp Psychol 2000;114:86–97.
[13] Hull CL. Exploration in the patterning of stimuli conditioned to the GSR. J Exp Psychol 1940;27:95–110.
[14] James JH, Wagner AR. One-trial overshadowing: evidence of distributive processing. J Exp Psychol: Anim Behav Processes 1980;6: 188–205.
[15] Joerges J, Küttner A, Galizia CG, Menzel R. Representations of odours and odour mixtures visualized in the honeybee brain. Nature 1997;387:285–8.
[16] Kehoe EJ, Graham P. Summation and configuration: stimulus compounding and negative patterning in the rabbit. J Exp Psychol: Anim Behav Processes 1988;14:320–30.
[17] Kinder A, Lachnit H. Responding under time pressure: testing an animal learning model and a model of visual categorization. Q J Exp Psychol 2002;55A:173–93.
[18] Kuwabara M. Bildung des bedingten reflexes von pavlovs typus bei der honigbiene, *Apis mellifera*. J Fac Sci Hokkaido Univ (Zool) 1957;13:458–64.
[19] Lachnit H, Kimmel HD. Positive and negative patterning in human classical skin conductance response conditioning. Anim Learn Behav 1993;21:314–26.
[20] Lunney GH. Using analysis of variance with a dichotomous dependent variable: an empirical study. J Educat Meas 1970;7:263–9.
[21] Malun D. Early development of mushroom bodies in the brain of the honey bee *Apis mellifera* as revealed by BrdU incorporation and ablation experiments. Learn Memory 1998;5:90–101.
[22] Malun D, Giurfa M, Galizia CG, Plath N, Brandt R, Gerber B, Eiserman B, et al. Hydroxyurea-induced partial mushroom body ablation does not affect acquisition and retention of olfactory differential conditioning in honeybees. J Neurobiol, vol. 53. in press.
[23] Macmillan CS, Mercer AR. An investigation of the role of dopamine in the antennal lobes of the honeybee, *Apis mellifera*. J Comp Physiol A 1987;160:359–66.
[24] Martin H. Zur Nahorientierung der Biene im Duftfeld. Zugleich ein Nachweis zur Osmotropotaxis bei Insekten. Z Vergl Physiol 1964;48:481–533.
[25] Menzel R, Greggers U, Hammer M. Functional organization of appetitive learning and memory in a generalist pollinator, the honey bee. In: Papaj D, Lewis AC, editors. Insect learning: ecological and evolutionary perspectives. New York: Chapman and Hall; 1993. p. 79–125.
[26] Menzel R, Giurfa M, Gerber B, Hellstern F. Elementary and configural forms of memory in an insect: the honeybee. In: Friederici A, Menzel R, editors. Learning: rule extraction and representation. New York: Walter de Gruyter & Co; 1999, p. 259–82.
[27] Menzel R, Giurfa M. Cognitive architecture of a mini-brain: the honeybee. TICS 2001;5:62–71.
[28] Mobbs PG. The brain of the honeybee *Apis mellifera* I. The connections and spatial organization of the mushroom bodies. Phil Trans R Soc Lond B 1982;298:309–54.
[29] Pearce JM. A model for stimulus generalization in pavlovian conditioning. Psychol Rev 1987;94:61–73.
[30] Pearce JM. Similarity and discrimination: a selective review and a connectionist model. Psychol Rev 1994;101:587–607.
[31] Redhead ES, Pearce JM. Stimulus salience and negative patterning. Q J Exp Psychol 1995;48:67–83.
[32] Rescorla RA. "Configural" conditioning in discrete-trial bar pressing. J Comp Physiol Psychol 1972;79:307–17.
[33] Rescorla RA. Evidence for unique stimulus account of configural conditioning. J Comp Physiol Psychol 1973;85:331–8.
[34] Rescorla RA, Wagner AR. A theory of Pavlovian conditioning: variations in the effectiveness of reinforcement and non-reinforcement. In: Black A, Prokasy WF, editors. Classical conditioning II, New York: Appleton-Century-Crofts; 1972. p. 64–99.
[35] Rudy JW, Sutherland RJ. Configural association theory and the hippocampal formation: an appraisal and reconfiguration. Hippocampus 1995;5:375–89.
[36] Rybak J, Menzel R. Anatomy of the mushroom bodies in the honey bee brain: the neuronal connections of the alpha-lobe. J Comp Neurol 1993;334:444–65.
[37] Sachse S, Rappert A, Galizia CG. The Spatial representation of chemical structures in the antennal lobe of honeybees: steps towards the olfactory code. Eur J Neurosci 1999;11:3970–82.
[38] Sandoz JC, Menzel R. Side-specificity of olfactory learning in the honeybee: generalization between odors and sides. Learn Memory 2001;8:286–94.
[39] Sandoz JC, Hammer M, Menzel R. Side-specificity of olfactory learning in the honeybee: US input side. Learn Memory 2002;9:337–48.
[40] Scherrer B. Biostatistiques. Quebec: Gaëtan Morin; 1984.
[41] Schubert M, Lachnit H, Francucci S, Giurfa M. Nonelemental visual learning in honeybee. Anim Behav 2002;64:175–84.
[42] Smith BH. An analysis of blocking in odorant mixtures: an increase but not a decrease in intensity of reinforcement produces unblocking. Behav Neurosci 1997;111:57–69.
[43] Smith BH, Cobey S. The olfactory memory of the honeybee *Apis mellifera* II. Blocking between odorants in binary mixtures. J Exp Biol 1994;195:91–108.
[44] Takeda K. Classical conditioned response in the honey bee. J Insect Physiol 1961;6:168–79.
[45] Thorn RS, Smith BH. The olfactory memory of the honeybee *Apis mellifera*, III. Bilateral sensory input is necessary for induction and expression of olfactory blocking. J Exp Biol 1997;200: 2045–55.
[46] Vareschi E. Duftunterscheidung bei der Honigbiene—Einzelzell-Ableitungen und Verhaltensreaktionen. Z Vergl Physiol 1971;75:143–73.
[47] Whitlow JW, Wagner AR. Negative patterning in classical conditioning: summation of response tendencies to isolable and configural components. Psychonom Sci 1972;27:299–301.
[48] Woodbury CB. The learning of stimulus patterns by dogs. J Comp Psychol 1943;35:29–40.