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Asymmetrical generalisation between pheromonal and floral odours in appetitive olfactory conditioning of the honey bee (*Apis mellifera* L.)

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Abstract The capacity to generalise between similar but not identical olfactory stimuli is crucial for honey bees, allowing them to find rewarding food sources with varying volatile emissions. We studied bees' generalisation behaviour with odours having different biological values: typical floral odours or alarm compounds. Bees' behavioural and peripheral electrophysiological responses were investigated using a combined proboscis extension response conditioning-electroantennogram assay. Bees were conditioned to pure linalool (floral) or to pure isoamyl acetate (alarm) and were tested with different concentrations of both compounds. Electrophysiological responses were not influenced by conditioning, suggesting that the learning of individual compounds does not rely on modulations of peripheral sensitivity. Behaviourally, generalisation responses of bees conditioned to the alarm compound were much higher than those of bees conditioned to the floral odour. We further demonstrated such asymmetrical generalisation between alarm and floral odours by using differential conditioning procedures. Conditioning to alarm compounds (isoamyl acetate or 2-heptanone) consistently induced more generalisation than conditioning to floral compounds (linalool or phenylacetaldehyde). Interestingly, generalisation between the two alarm compounds, which are otherwise chemically different, was extremely high. These results are discussed in relation to the neural representation of compounds with different biological significance for bees.

Keywords Olfactory learning · Generalisation · Electroantennogram · Proboscis extension response conditioning · Alarm pheromones

Abbreviations *CS* conditioned stimulus · EAG electroantennogram · *GI* generalisation index · *PER* proboscis extension response · *US* unconditioned stimulus

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Introduction

At the individual level, the foraging behaviour of honey bees is based on learning a variety of floral stimuli, including odours (Koltermann 1969), colours (Menzel 1967), patterns and shapes (Wehner 1981). Of these cues, olfactory signals play a major role in the recognition of food sources (Kriston 1973; Menzel et al. 1993). Bees are able to differentiate a large number of olfactory signals from their environment (Vareschi 1971; Laska et al. 1999) and learn which predict food rewards and which do not (Menzel et al. 1993). However, under natural conditions honey bees confront floral odours, which are blends containing tens to hundreds of components. The composition of such floral odours varies in quality or in quantity both over time and in space (Pham-Delègue et al. 1989, 1992). Therefore, generalisation, defined as the tendency of animals to respond behaviourally to stimuli which differ from a learnt stimulus (Pearce 1987) is a fundamental process for bees' survival: it allows bees to find fruitful food sources in spite of fluctuations in those sources' volatile emissions.

In theory, animals generalise between stimuli because these stimuli activate similar neural representations (Pearce 1987; Shepard 1987). The more the presented stimulus differs from the learnt one, i.e. the more distant neural representations of stimuli are in the psychological space of animals, the less generalisation is observed. The

study of generalisation is thus a means of understanding how animals perceive the stimuli of their environment. In bees, olfactory generalisation was mainly studied in an appetitive learning context, where workers are trained to associate an odour to a sugar reward. Bees conditioned to individual compounds ('compound' being defined as a given chemical entity) or to mixtures were shown to generalise their responses to a wide range of other olfactory stimuli (Smith and Menzel 1989; Smith 1991; Pham-Delègue et al. 1993). In particular, they generalise more between compounds belonging to the same chemical class (i.e. with the same functional group) than between compounds of different classes (Smith and Menzel 1989; Getz and Smith 1990). Within a given chemical class, they seem to generalise more between compounds with similar carbon chain lengths than with dissimilar lengths (Laska et al. 1999). In terms of stimulus intensity, generalisation between two doses of the same compound decreases with the dose difference, and more along decreasing than along increasing concentrations (Pham-Delègue et al. 1993; Bhagavan and Smith 1997). Thus, considerable knowledge about the rules governing olfactory generalisation in bees has been gained in the last decade. Recently, optical imaging studies of neural activity at the level of the glomeruli of the antennal lobe, the primary olfactory neuropile of the bee brain, revealed the essential principles of olfactory coding and a first step toward understanding the neural representation of odours (Joerges et al. 1997; Sachse et al. 1999; Galizia and Menzel 2001). However, no effort has yet been invested in understanding what effect the functional value of different chemical entities, i.e. their biological significance for bees, could have on their neural representation.

Beside floral odours which mediate foraging, other olfactory stimuli play a major role in the social life of bees: pheromones are used for reproduction, social communication and cohesion and the defence of the society (Free 1987). Although floral odours elicit highly plastic behaviours, pheromonal compounds give way to relatively fixed behavioural patterns. The neural processing of pheromonal and food odours is generally believed to be based on two subsystems: one, a very specific system, limited to the recognition of pheromonal compounds (the specialist system) and a second, more broadly tuned, responding to a wide range of food-associated odours (Masson and Mustaparta 1990; Hildebrand and Shepherd 1997). Now, both type of odours can be learnt by honey bees in an appetitive context (Pham and Masson 1985; Marfaing et al. 1989; Getz and Smith 1990). Optical imaging studies of olfactory coding at the level of the antennal lobes led Joerges et al. (1997) to suggest that both kinds of substances differ in their neural coding. Pheromonal odours would yield activation patterns which remain constant among different individuals, whilst floral odours would generate more variable patterns. Galizia et al. (1999) contested this view such that it is unclear whether the neural coding of pheromones and floral odours follows the same principles. In order to improve our understanding of the neural representation of such compounds in honey bees, we compared bees' learning and generalisation performances with pheromonal odours to those obtained with common floral odours.

To study learning and generalisation in an appetitive context, we used the procedure for conditioning the proboscis extension response (PER) of honey bees (Kuwabara 1957; Bitterman et al. 1983). As differential treatment of pheromonal and floral odours may already take place at the peripheral level, and since previous work showed modulations of peripheral sensitivity during olfactory conditioning with floral odours (De Jong and Pham-Delègue 1991; Wadhams et al. 1994), we combined PER conditioning with electroantennogramm (EAG) recordings on live bees. We thus studied the patterns of behavioural and electrophysiological responses during generalisation behaviour with both floral and alarm odours.

Materials and methods

Bees

Emerging Italian worker bees, *Apis mellifera ligustica*, were collected from outdoor hives. They were caged in groups of about 50 individuals, maintained in an incubator at 33°C, 55% RH, and fed ad libitum with sugar, pollen and water. Fourteen- to sixteen-day-old bees were used in the experiments since workers usually become foragers at this age (Sakagami 1953; Seeley 1982) and give the most consistent performances in the PER conditioning assay (Pham-Delègue et al. 1990). Before the experiments, bees were starved for 2 hours in the cage, and were individually mounted in glass holders, leaving only their antennae and mouth parts free.

Odour stimuli

In experiment 1, odour stimuli used for the conditioning procedure were pure +/– linalool (Sigma, 97%) or pure isoamyl acetate (Jannsen Chimica, 99%). The former is a constituent of floral odours (Knudsen et al. 1993) whilst the latter is the main component of the sting alarm pheromone of honey bees (Boch et al. 1962). Doses used for conditioning (i.e. contained in 10 µl) were 8.65 mg and 8.76 mg, respectively (Stecher et al. 1968). For the testing procedure, serial dilutions were made up in hexane (Prolabo, 95%) in order to obtain doses applied onto the filter paper (i.e. contained in 10 µl) of 10^{-7} g, 10^{-6} g, 10^{-5} g and 10^{-4} g. The stimuli used for testing were therefore approximately the equivalent of 0.001–1% of the dose used for conditioning. In experiment 2, all odour stimuli were presented in their pure form. Two more compounds were used, phenylacetaldehyde (Sigma, 95%) and 2-heptanone (Sigma, 99.8%). The former is a common floral volatile (Knudsen et al. 1993) and the latter is the unique component of the alarm pheromone produced by the mandibular glands of bees (Shearer and Boch 1965). The doses of these compounds used for conditioning (i.e. contained in 10 µl) were 10.23 mg and 8.20 mg, respectively.

Behavioural and electrophysiological responses of bees conditioned to a floral or a pheromonal compound (experiment 1)

Bees were subjected to a PER conditioning procedure with either a pure floral odour (linalool) or a pure alarm compound (isoamyl

acetate). After 3 h of rest, two to three bees, selected at random, were used in coupled EAG-PER recordings with different concentrations of both compounds.

PER conditioning

The PER is produced naturally by foragers visiting flowers and contacting nectar with their antennae, mouth parts or tarsi. It usually leads to a food uptake, and the learning of olfactory cues associated with the nectar. This process can be reproduced on restrained bees under laboratory conditions (Kuwabara 1957; Takeda 1961). During a conditioning trial, an odour (conditioned stimulus – CS) is presented to the bee in temporal association with a stimulation of the antennae with a sugar solution (unconditioned stimulus – US) and a reward at the level of the proboscis (food uptake). After conditioning, the CS alone can trigger the PER in bees.

In this experiment, bees were subjected to three conditioning trials with 10 min inter-trial intervals. Prior to each conditioning trial, bees were placed for 15 s in an air stream (50 ml s^{-1}) delivered through a glass tube (1 cm in diameter) positioned approximately 1 cm from the bees's head. The odour-delivery system utilised a disposable Pasteur pipette cartridge. The odour stimulus (10 µl of either pure linalool or pure isoamyl acetate) was applied to a piece of filter paper (40 mm × 3 mm) which was inserted into the cartridge. Vapour from the cartridge was delivered for a 6-s period into the air stream passing continuously over the bee by means of a secondary air stream (2.5 ml s^{-1}) controlled by a solenoid valve. Three seconds after the beginning of the odour stimulation, the antennae were contacted with a 30% sucrose solution (w/w), and the ensuing proboscis extension was rewarded by a 3-s sucrose uptake. For each trial, the bee's responses during the first 3 s of odour presentation were noted. Bees showing spontaneous responses to the CS were discarded. Only bees that responded to the CS after the first conditioning trial (i.e. that responded at the second and third trial) were considered properly conditioned and were used for coupled EAG-PER recordings. Conditioning was equally efficient with linalool and with isoamyl acetate, and such individuals represented 71.6% ($n=113$) and 67.1% ($n=76$) of total bees, respectively. Naive individuals, i.e. bees that had been stimulated with neither odour nor sucrose solution, were used as controls.

Coupled EAG-PER recordings

EAG responses were obtained using glass electrodes filled with saline solution (NaCl 9 g; KCl 0.2 g; glucose 4.36 g in 1 l of water). The left antenna was attached to the rim of the glass holder using two thin (0.3 mm) tape strips. The recording electrode was inserted into a prepunctured hole in the tip of the fixed antenna. The right antenna was cut at the level of the scape and the reference electrode was inserted into the remaining part of the scape. The signal generated by the antenna was passed through an amplifier (Neurolog System, bandpass d.c. to 500 Hz, gain 1000) and displayed on a trace-oscilloscope so that EAGs could be measured after the stimulation on the oscilloscope screen. PERs were recorded by the experimenter during the stimulations.

The testing procedure began 15 min after the bee was placed in the stimulation apparatus, in order to familiarise it with the electrodes and with the permanent air flow (25 ml s^{-1}). The bee was then subjected to 20 olfactory stimulations (secondary air flow of 8.3 ml s^{-1}): each compound (linalool and isoamyl acetate) was presented twice at four stimulus doses (from 10^{-7} g to 10^{-4} g). After every fourth stimulation, bees were stimulated with a 10-µl hexane control. To avoid any sensory adaptation of the antenna during the procedure, stimulations were carried out in an increasing dose gradient, and with increasing intervals between presentations (intervals of 2, 2, 3 and 5 min for doses of 10^{-7} g,10^{-6} g,10^{-5} g and 10^{-4} g, respectively). Samples (10 µl) of the solutions of test compounds were applied to a filter paper strip (40 mm×3 mm), and the solvent was allowed to evaporate for 30 s before the filter paper strip was inserted into a Pasteur pipette cartridge. A fresh cartridge was prepared prior to each stimulation. Stimulus delivery was 1 s.

Asymmetrical generalisation responses between floral and pheromonal compounds (experiment 2)

In order to test the idea that alarm pheromones provoke increased generalisation behaviour in an appetitive context, we subjected bees to a differential conditioning procedure (Bitterman et al. 1983; Smith et al. 1991). In such a procedure, two compounds are presented alternately, one rewarded (CS+) and the other not (CS–). Bees typically start generalising between the two compounds presented, responding to the CS– as well as to the CS+. After several presentations of both compounds, if they are able to differentiate between them, they will eventually stop responding to the CS–. Thus, both generalisation behaviour and discrimination ability can be studied. Four compounds were used, two floral odours (linalool and phenylacetaldehyde) and two alarm odours (isoamyl acetate and 2-heptanone).

After being mounted in the holders, bees were starved another 3 h before being conditioned. For each bee, the procedure consisted of 4 CS+ trials and 4 CS– trials with inter-trial intervals of 7.5 min. On CS+ trials, bees received the CS+ in association with a sugar reward (30% sucrose solution), and on CS– trials, they were stimulated with the CS– odour alone. The procedure always began with a CS– trial, so that spontaneous responses to both odours could be recorded (spontaneous responses to the CS+ were recorded during the first 3 s of the first CS+ trial, i.e. before any US stimulation was given). Individuals presenting spontaneous responses to either CS+ or CS– were discarded from the analysis. As we were interested in cross-compound generalisation in all possible pairs of the four compounds, 12 experimental groups (i.e. 12 pairs of compounds) were formed.

Statistical analysis

In experiment 1, the comparison of EAG dose-response curves for conditioned and naive bees was performed with a two-way repeated measurements ANOVA (dose × group). As bees were stimulated twice with each dose of compound, the mean value of these two EAGs was used in the test. Responses to the solvent at different times throughout the testing procedure were compared between groups using a two-way repeated measurements ANOVA (time × group). Thresholds for EAG responses were determined by comparing responses for each dose of compound to that obtained at the next solvent presentation, using paired *t*-tests.

Behavioural responses were recorded for each bee as the number of proboscis extensions over two presentations of each dose of compound. They are represented in the figures as overall percentages of responses. The comparison of behavioural responses between conditioned and naive bees to each dose of each compound was carried out using a Mann-Whitney test on the individual number of responses.

In experiment 2, to compare the level of generalisation among the different compound pairs, we calculated a generalisation index (GI). For each bee, GI was calculated according to the number of responses obtained to the CS+ (R^+) and to the CS– (R^-):

$$GI = 1 - \frac{R^+ - R^-}{R^+ + R^-} \quad (1)$$

The normal GI values are between 0 (when bees respond only to the CS+, thus showing *no generalisation*) and 1 (when bees respond equally to the CS+ and CS–, thus generalising totally between stimuli), although theoretically values above 1 could be attained if bees responded more to the CS– than to the CS+ (maximum value: 2). To compare GIs obtained in the different groups, we used a

Kruskal-Wallis test with 3 *df*, followed by two-by-two comparisons by means of the Noether method, which includes a correction for multiple comparisons (Scherrer 1984).

Results

Behavioural and electrophysiological responses of bees conditioned to a floral or a pheromonal compound (experiment 1)

Electrophysiological responses

Conditioning to linalool

After conditioning to linalool (Fig. 1a) the dose-response curve for linalool showed a strong increase in EAG levels over the range of doses tested, for bees conditioned to linalool ($n = 14$) and naive bees ($n = 12$). The ANOVA comparing the responses to the four different doses of linalool showed a strong dose effect ($F_{3,72} = 295$, $P \ll 0.001$) but no group effect ($F_{1,24} = 0.001$, NS). Responses to isoamyl acetate showed a lower level of increase, from 0.41 mV (10^{-7} g) to between 0.68 mV and 0.84 mV at 10^{-4} g. There again, a significant dose effect ($F_{3,72} = 57.6$, $P \ll 0.001$) appeared, but no group effect ($F_{1,24} = 1.03$, NS). EAG responses to the solvent (dotted lines) were stable throughout the testing procedure ($F_{3,72} = 0.40$, NS), and similar in conditioned and naive bees ($F_{1,24} = 0.52$, NS).

Conditioning to isoamyl acetate

After conditioning to isoamyl acetate (Fig. 2a) EAG responses to linalool increased from 0.49 mV (10^{-7} g) to 1.68 mV at 10^{-4} g for conditioned bees ($n = 12$) and to 1.83 mV for naive bees ($n = 12$). The ANOVA comparing the responses to linalool between the two groups showed a strong dose effect ($F_{3,66} = 394$, $P \ll 0.001$), but no group effect ($F_{1,22} = 0.92$, NS). For isoamyl acetate, the CS, EAG responses increased from 0.40 mV (10^{-7} g) to between 0.78 mV and 0.83 mV (10^{-4} g). The ANOVA showed a dose effect ($F_{3,66} = 31.7$, $P \ll 0.001$) but no group effect ($F_{1,22} = 0.08$, NS). As before, EAG responses to the solvent were low, stable throughout the procedure ($F_{1,22} = 2.26$, NS) and similar in conditioned and naive groups ($F_{3,66} = 1.96$, NS).

EAG response thresholds

The EAG threshold, defined as the dose eliciting EAG responses significantly higher than the solvent, was 10^{-7} g for linalool in all groups (*t*-test, $t > 3.3$, $P < 0.015$ for all doses) and 10^{-6} g for isoamyl acetate (*t*-test, $t > 2.15$, $P < 0.05$ for all doses except for 10^{-7} g, $t < 1.0$, NS).

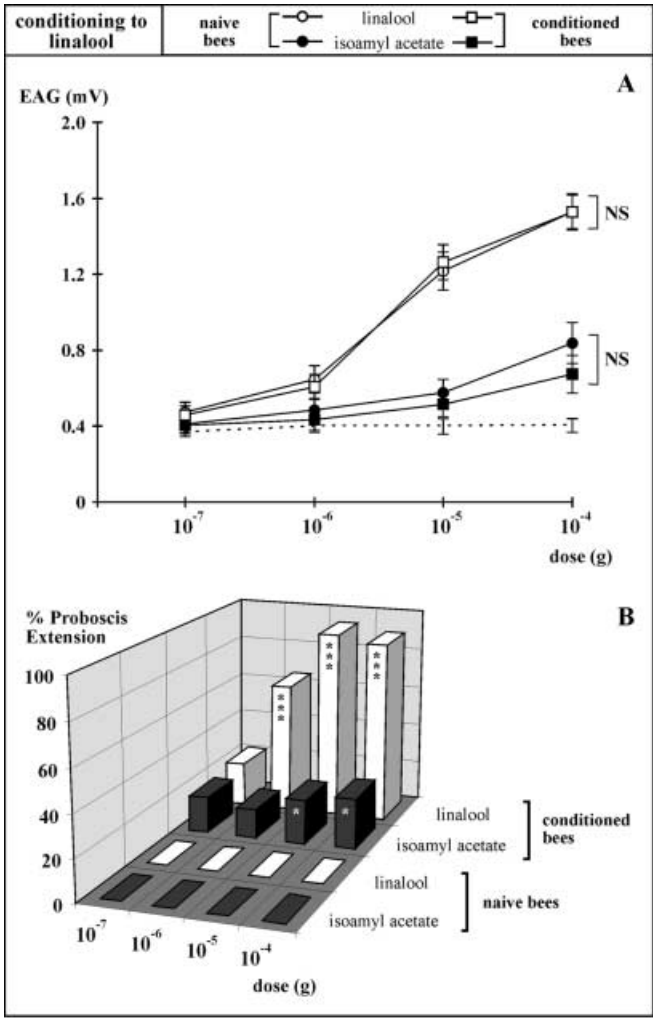

Fig. 1 a Electrophysiological [electroantennogram (EAG) in mV ± SE] and behavioural responses (% proboscis extensions) to four different concentrations of linalool and isoamyl acetate for bees conditioned to linalool ($n = 12$) and naive bees ($n = 14$). EAG responses were not modified by conditioning, neither for the conditioned stimulus, CS (linalool) nor for a novel odorant (isoamyl acetate). The comparison of dose-EAG response curves between groups was carried out using a two-way repeated measurements ANOVA (NS: non-significant). The *dotted line* shows mean responses to the solvent (in mV ± SE) throughout the procedure. **b** Proboscis extension responses (PERs) were mainly elicited for different concentrations of linalool, the CS. Low generalisation to isoamyl acetate was recorded. Comparisons of responses between conditioned and naive groups were made at each dose of each compound with Mann-Whitney tests (1 *df*). The result is represented on the bars of the conditioned group when significant ($^{*}P < 0.05$; $^{***}P < 0.001$)

Behavioural responses

Conditioning to linalool

In conditioned bees, responses to linalool increased from a level of 21% (10^{-7} g dose) to more than 90% (10^{-5} g and 10^{-4} g) (Fig. 1b). Responses of conditioned bees to linalool were significantly greater than those of naive bees at doses between 10^{-6} g and 10^{-4} g

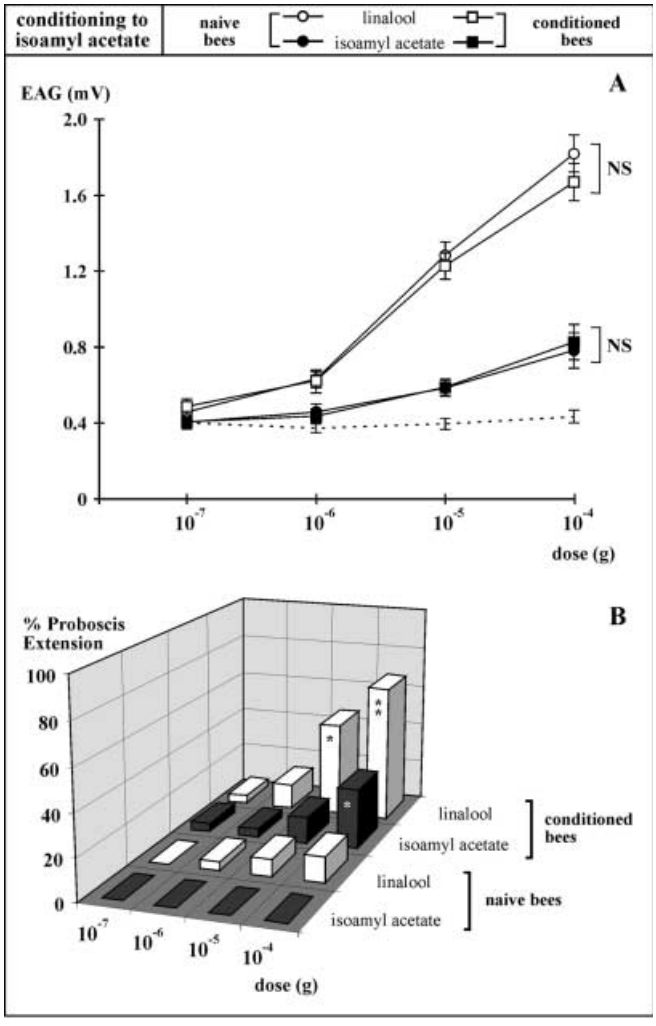

Fig. 2 **a** Electrophysiological (EAG in mV ± SE) and behavioural responses (% proboscis extensions) to four different concentrations of linalool and isoamyl acetate for bees conditioned to isoamyl acetate ($n = 12$) and naive bees ($n = 12$). EAG responses were not modified by conditioning, neither for the conditioned stimulus (CS; isoamyl acetate) nor for the novel odorant (linalool). The comparison of dose-EAG response curves between groups was carried out using a two-way repeated measurements ANOVA (NS: non significant). The *dotted line* shows mean responses to the solvent (in mV ± SE) throughout the procedure. **b** PERs were observed for the highest concentration of isoamyl acetate, the CS. High levels of qualitative generalisation were obtained to linalool, here a novel odorant. Comparisons of responses between conditioned and naive groups were carried out at each dose of each compound with Mann-Whitney tests (1 *df*). The result is represented on the bars of the conditioned group when significant ($^{*}P < 0.05$; $^{**}P < 0.01$)

(Mann-Whitney test, $z > 3.56$, $P < 0.001$). In contrast, responses to isoamyl acetate were low at all doses tested (between 14% to 25%), with a significant difference between conditioned and naive bees at 10^{-5} g and 10^{-4} g levels ($z > 2.2$, $P < 0.05$). Responses to the solvent were low in the conditioned group (14%) and zero in the naive group, with no statistical difference between groups (Fig. 1b).

Conditioning to isoamyl acetate

Proboscis extension responses of conditioned and naive bees were very low (below 12%) for all presentations of low doses (10^{-7} g and 10^{-6} g) of both linalool and isoamyl acetate (Fig. 2b), with no significant difference between groups (Mann-Whitney test, $z < 0.9$, NS). In the conditioned group, responses to isoamyl acetate (CS), increased slightly at higher doses, reaching 29% (10^{-4} g dose), which was significantly higher than in naive bees ($z = 2.1$, $P < 0.05$). An additional test with pure isoamyl acetate was performed on conditioned individuals at the end of the procedure. It yielded a 67% response level ($n = 12$), showing that the level of responses elicited by 10^{-4} g was not the highest level possible.

In the conditioned group, responses to linalool, which is not the CS in this case, strongly increased at the dose of 10^{-5} g (46%) and eventually reached 67% (10^{-4} g). In naive bees, responses to linalool were low (below 13%), and significantly lower than those of conditioned bees ($z > 2.11$, $P < 0.05$). Responses to the solvent were below 7%, with no difference arising between groups ($z = 0.92$, NS).

Asymmetrical generalisation responses between floral and pheromonal compounds (experiment 2)

Good conditioning was obtained to all four compounds used as CS+ (between 87% and 96% responses at the fourth CS+ trial), without any difference among odours (linalool $n = 118$, phenylacetaldehyde $n = 112$; isoamylacetate $n = 102$, 2-heptanone $n = 89$; Kruskal-Wallis test, 3 *df*, $H = 4.0$, NS). In contrast, strong differences appeared in responses to the CS– (generalisation) : when the CS+ was a floral compound (Fig. 3a, $n = 42$–44; Fig. 3b, $n = 31$–38), low responses to the CS– were observed (below 18%). When the CS+ was one of the alarm compounds and the CS– a floral compound (Fig. 3c, $n = 27$–36), higher levels of responses were obtained to the CS– at the beginning of the procedure (between 26% and 50%), showing high generalisation of conditioned responses to these unrewarded stimuli. Responses then decreased quickly during repeated presentations of both CS+ and CS– (to below 17%), proving that bees have the ability to discriminate these compounds. Finally, when both CS+ and CS– were pheromones (Fig. 3d, $n = 29$–30), very high responses were obtained to the CS– (as high as 70% at the third CS– trial), showing dramatically high generalisation between the two compounds. Performances improved during the procedure, but responses to the CS– remained high (23–45%). The analysis of generalisation indexes (Fig. 4) showed a clear statistical heterogeneity between groups (Kruskal-Wallis test, 3 *df*, $H = 106.6$, $P < 0.0001$). Two-by-two comparisons showed a significant difference between situations where the CS+ was a floral compound (A, B: index of 0.114 and 0.112), situations where the CS+ was a pheromonal compound and the CS– a floral compound (C: index of 0.313) and

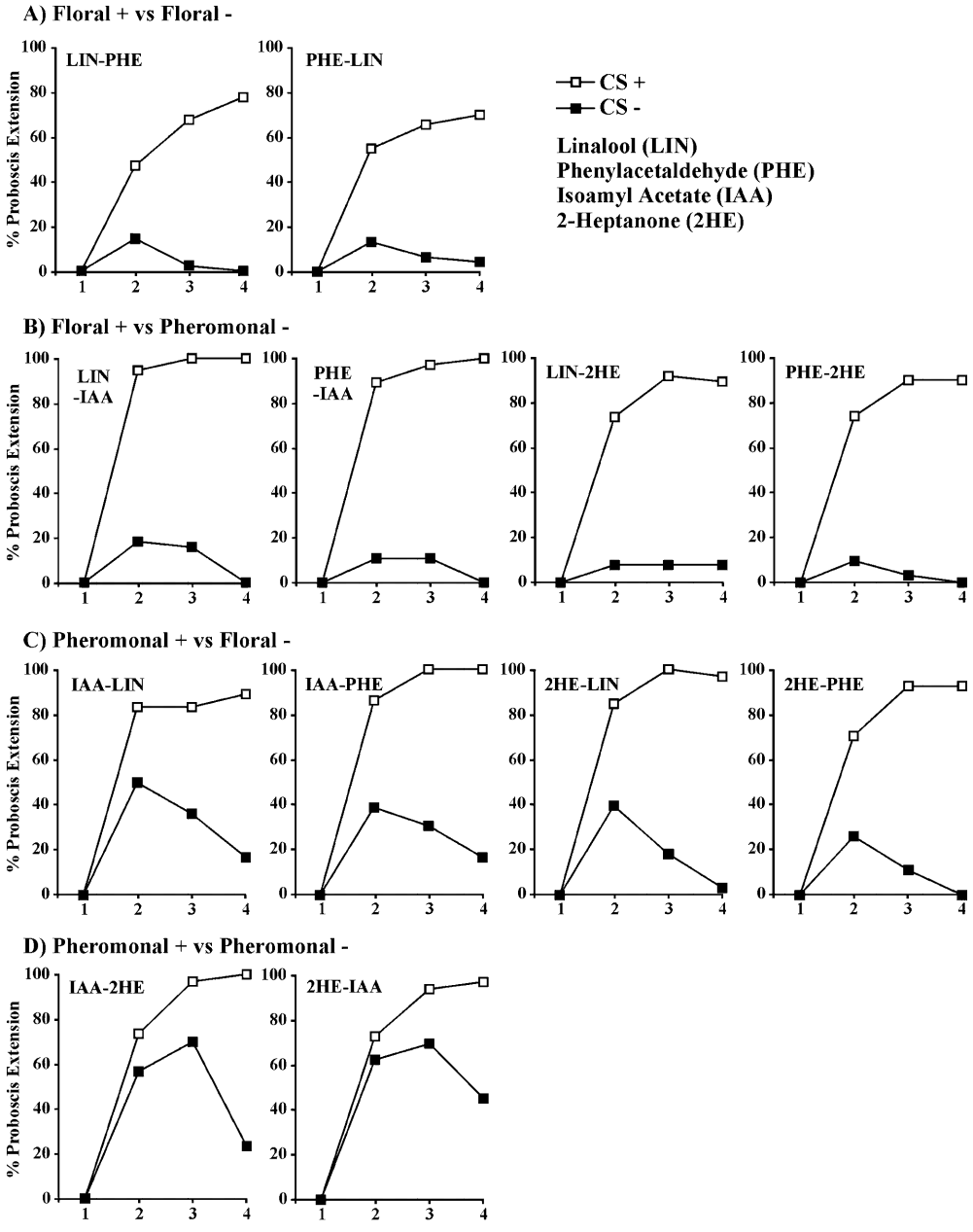

Fig. 3a–d Differential conditioning procedures where two compounds are alternately presented to the bees, one being rewarded (CS+) and the other being unrewarded (CS–). Among four compounds, two of floral origin (linalool and phenylacetaldehyde) and two of alarm origin (isoamyl acetate and 2-heptanone), all possible pairs of compounds were tested. They are presented according to the origin of the compounds: **a** Both CS+ and CS– were floral compounds (two groups : $n = 42$ and 44). **b** Floral odours were rewarded and pheromonal odours unrewarded (four groups: $n = 38$, 37, 37, 31). **c** Pheromonal odours were rewarded and floral odours unrewarded (four groups: $n = 36$, 36, 33, 27). **d** Both CS+ and CS– were pheromonal compounds (two groups: $n = 30$, 29). Low generalisation was obtained when bees were conditioned to the floral compounds (**a**, **b**), whilst strong generalisation to unrewarded odours was obtained when the alarm compounds were rewarded (**c**, **d**), demonstrating asymmetrical generalisation patterns between floral and alarm pheromone compounds. Generalisation was extremely high between the two alarm compounds (**d**)

situations where both CS+ and CS– were pheromones (D: index of 0.673) (corrected significance threshold of 0.0042).

Discussion

Peripheral modulations during learning and generalisation?

At the electrophysiological level, no difference between conditioned and naive bees appeared in EAG responses to the conditioning odour or to a novel odour, independent of their floral or pheromonal origin. This result appears to contradict previous findings with honey bees where increases in EAG responses were found after conditioning (De Jong and Pham-Delègue 1991; Wadhams et al. 1994). In these studies, olfactory mixtures were used as conditioning stimuli (essential oils and a synthetic 6-component mixture, respectively). In contrast, using individual compounds as in our study, Bhagavan and Smith (1997) obtained no such increase in EAG levels, but in the case of one odorant tested, a decrease in EAG responses to the conditioning odour. However, this decrease was shown to be due to an olfactory adaptation of the antenna and was thus not related to associative learning phenomena. Such adaptation has not occurred in our study, probably because we recorded EAGs on live bees instead of on isolated heads as Bhagavan and Smith (1997) did, allowing longer inter-trial intervals between stimulations (2–5 min). A common conclusion from our work and

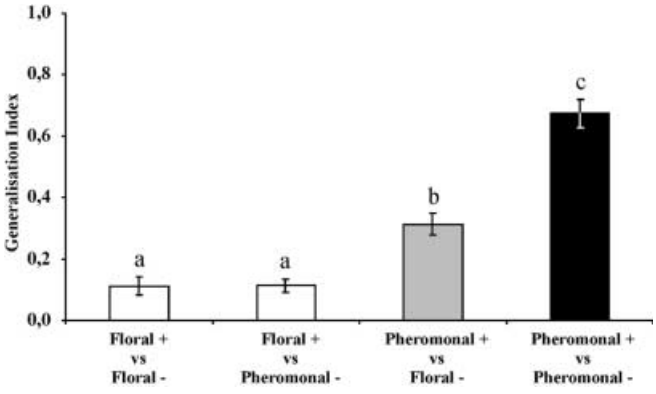

Fig. 4 Generalisation index (GI) for the four situations in the differential conditioning experiment. The index was low and identical for groups where the floral odours were rewarded, but was significantly higher when alarm pheromone compounds were rewarded. Generalisation was even higher between the two alarm compounds. Comparison of GIs obtained in the four conditioning situations was done with a Kruskal-Wallis test ($H = 106.6$, $P < 0.0001$, 3 *df*), followed by two-by-two comparisons by means of the Noether method (Scherrer 1984). Different letters indicate significant differences in this test (corrected significance threshold $\alpha' = 0.0042$)

from the study by Bhagavan and Smith (1997) is that, with individual compounds, modulations in EAG responses due to associative learning are unlikely. As for olfactory mixtures, the situation may be different. It has already been postulated that at the antennal level, *sensilla placodea*, which contain up to 35 olfactory receptor neurons, may behave as integrated processing units (Getz and Akers 1994). These receptor neurons, each reacting to different components of a mixture, would not fire independently of each other, but due to inhibitions within the placode, would provide an integrated signal to central areas through co-ordinated firing patterns (Getz and Akers 1995). If associative learning phenomena are responsible for observed changes in EAG levels, some central process should be implicated, since olfactory (CS) and gustatory (US) neural pathways involved in the conditioning of proboscis extension only meet in central areas such as the antennal lobes, the lateral protocerebral lobe or the mushroom bodies (Hammer 1997). We may thus hypothesise that mixtures have a particular effect on the afferent signal transmitted to central areas, which might in turn induce a centrally-mediated modulation process of sensory sensitivity. However, in the studies which found significant modifications of peripheral sensitivity with mixtures (De Jong and Pham-Delègue 1991; Wadhams et al. 1994), controls for non-associative effects (such as US only, CS only, or unpaired CS-US groups) were not included. Therefore, a direct implication of associative learning phenomena is as yet not verified. Future studies in this direction should therefore focus on mixture conditioning and include control experiments for non-associative modulations of sensory responses.

Quantitative generalisation patterns with floral and pheromonal odours

According to EAG thresholds (as compared to the solvent – experiment 1), linalool and isoamyl acetate are perceived by bees at doses of 10^{-7} g and 10^{-6} g respectively, i.e. 0.001% and 0.01% of the dose used for conditioning. At the behavioural level, linalool elicited high responses even at 0.01% of the dose used for conditioning (almost the perceptual threshold), whilst isoamyl acetate was only poorly recognised at a dose of 1%. Linalool was thus, in comparison to isoamyl acetate, more widely recognised along decreasing dose gradients. This result is in agreement with several previous studies where quantitative generalisation was poor with isoamyl acetate (recognition at doses between 1 and 5%: Pham and Masson 1985; Marfaing et al. 1989) and more important with linalool (recognition at doses of 0.1%: Waller et al. 1973). The nature of the compounds used in our study might be responsible for the difference observed in quantitative generalisation. Flower volatiles are key cues mediating the choice and orientation behaviour of honey bee foragers and linalool is one of the few floral compounds responsible for the recognition of oilseed rape aroma by honey bees (Blight et al. 1997). The recognition of such key compounds over a wide dose range could provide increased foraging efficiency. On the other hand, isoamyl acetate is used for the defensive behaviour of the bee colony. Boch et al. (1970) have shown that alarm reactions of bees to isoamyl acetate are restricted to a certain dose window. Consistently, N. Balderrama et al. (unpublished observations) found that two different doses of isoamyl acetate can have antagonistic effects on the stinging responses of bees stimulated with electric shocks. Different doses of isoamyl acetate may thus be perceived by bees as different biological signals. In this case, behavioural quantitative generalisation would be restricted to a narrow range of doses near the one experienced by bees. Further experiments where bees would be conditioned and tested with a wider range of doses of isoamyl acetate would be needed to document this question. In parallel, applying differential conditioning procedures with different doses of isoamyl acetate would allow testing whether bees actually can differentiate between different doses of this compound.

Qualitative generalisation patterns between floral and pheromonal compounds

In experiment 1, generalisation was strong from isoamyl acetate to linalool but low from linalool to isoamyl acetate, suggesting asymmetrical generalisation patterns between these two compounds with different functional values. To extend the validity of this result, we added two compounds in experiment 2, phenylacetaldehyde (floral odour) and 2-heptanone (alarm

pheromone) and tested generalisation patterns between all possible pairs of odours. We found generalisation to be weak when bees were rewarded with the floral compounds, but much higher when they were rewarded with the pheromonal compounds, all compounds otherwise being as efficiently learnt and clearly discriminated by bees. One could attribute such asymmetrical responses to generalisation asymmetries between chemical classes, as was already observed by Smith and Menzel (1989). However, in the work cited, bees conditioned to acetates (like isoamyl acetate) did not show higher generalisation to terpene alcohols (like linalool) or to aldehydes (like phenylacetaldehyde) than in the reversed situation. Bees conditioned to 2-ketones (such as 2-heptanone), showed increased responses to alcohols, but not to terpenes or to aldehydes. We therefore assume that the phenomenon we observed is rather due to the pheromonal value of isoamyl acetate and 2-heptanone.

Pheromones are known to have general physiological effects on bees (Free 1987), which could affect the perception of presented stimuli or the overall responsiveness of bees. For instance, stimulations with isoamyl acetate were shown to trigger an endogenous opioid system in bees, resulting in a stress analgesia (Núñez et al. 1998). However, we do not think that such general opioid response could explain our results, since the analgesia would rather induce a generally *decreased* responsiveness to all kinds of stimuli. Besides, a recent study suggests that 2-heptanone does not trigger such an opioid system (N. Balderrama et al., unpublished observations). Furthermore, increased generalisation appeared only when bees were stimulated with the pheromones in temporal association with a sucrose reward, i.e. as CS+ and not as CS–. We therefore think that the effect we obtained is specific to the alarm odour -appetitive reward association. Now, isoamyl acetate and 2-heptanone are known deterrents to foraging (Free et al. 1985) which seems to indicate that they are surprising stimuli for bees in an appetitive context. Our hypothesis is that increased generalisation, as found in our study, is due to the association of these alarm compounds with an unexpected outcome. The responsiveness of animals to the stimuli of their environment depends on their attention to these stimuli (Mackintosh 1975; Holland and Gallagher 1995; Robbins and Everitt 1996). The attention of bees could have been increased during conditioning by the relative 'inconsistency' between an alarm odour presentation and a sugar reward. Bees would then be more attentive and more likely to respond to other olfactory stimuli in this case than when conditioned to floral odorants, which are not as 'surprising' stimuli in this context. Such increased attention could be a long-lasting phenomenon, since asymmetrical generalisation was observed in experiment 1 about 3 h after conditioning. Future experiments would therefore have to study the time course of such phenomenon. Moreover, since asymmetrical generalisation seems to depend on the alarm-CS/sugar-US association, varying the inter-stimulus (CS-US) interval would provide interesting insight into the properties of such association.

Qualitative generalisation between alarm compounds

An exciting finding of our study is that generalisation was extremely high between the two alarm compounds. The level of generalisation obtained between the two pheromones was even significantly higher than in the situation where only the CS+ was an alarm pheromone (see Fig. 3c, d). Interestingly, the two molecules have very different chemical structures. Furthermore, isoamyl acetate is released by the sting chamber (Boch et al. 1962) and 2-heptanone is produced by the mandibular glands (Shearer and Boch 1965). Both compounds can elicit aggressive behaviour from bees, but 2-heptanone appears to be less active than isoamyl acetate (Boch et al. 1970; Balderrama et al. 1987) and serves a more restricted purpose, mostly labelling intruders for directing the attacks of other bees (Free 1987). These two compounds thus have a number of important differences, but normally occur in the same context, the defence of the colony. We think that these two compounds with a similar function, when occurring in a context very different from an alarm reaction (an appetitive context in our case) could be perceived by bees as relatively similar. More generally, this could mean that the similarity in the neural representation of two odorants for bees is not restricted to their chemical similarity, but is also dependent on their functional value. This fact would be in agreement with current theories stating that the neural representations of stimuli are also assembled in the 'psychological space' of animals according to their normal outcome or function (Shepard 1987). However, before one can satisfyingly conclude to such a phenomenon in honey bees, new studies, including components from other pheromones, such as the Nasanov or the Queen pheromone, should be conducted. In parallel, the neural representation of odorants can be approached by studying neural activity in the brain by way of optical imaging preparations (Joerges et al. 1997; Galizia et al. 1997). Odorants appear to be coded according to spatio-temporal activity patterns in the glomeruli of the antennal lobes (Galizia et al. 1999). As these patterns are known to be modified by conditioning (Faber et al. 1999), the next step in this work will be to follow the evolution of such activity patterns during learning and generalisation experiments with a range of pheromonal and floral compounds. This could eventually lead to a better understanding of the way animals like bees perceive their olfactory environment and extract important cues for their survival.

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