

## Odour aversion after olfactory conditioning of the sting extension reflex in honeybees

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### SUMMARY

In Pavlovian conditioning, an originally neutral stimulus (conditioned stimulus or CS) gains control over an animal's reflex after its association with a biologically relevant stimulus (unconditioned stimulus or US). As a consequence, a conditioned response is emitted by the animal upon further CS presentations. In such a situation, the subject exhibits a reflex response, so that whether the CS thereby acquires a positive or a negative value for the animal is difficult to assess. In honeybees, *Apis mellifera*, an odour (CS) can be associated either with sucrose solution (US) in the appetitive conditioning of the proboscis extension reflex (PER), or with an electric shock (US) in the aversive conditioning of the sting extension reflex (SER). The term 'aversive' may not apply to the latter as bees do not suppress SER as a consequence of learning but, on the contrary, start emitting SER to the CS. To determine whether the CS acquires a positive or a negative value in these conditioning forms, we compared the orientation behaviour of freely walking honeybees in an olfactory-cued Y-maze after training them with an odour–sucrose association (PER conditioning) or an odour–shock association (SER conditioning). We show that the same odours can acquire either a positive value when associated to sucrose, or a negative value when associated to an electric shock, as bees respectively approach or avoid the CS in the Y-maze. Importantly, these results clearly establish the aversive nature of SER conditioning in honeybees.

Key words: appetitive learning, aversive learning, classical conditioning, avoidance, olfaction, honeybee.

### INTRODUCTION

Animals learn to associate sensory stimuli or their own behavioural responses to particular outcomes, which often possess a positive or negative hedonic value for the animal. In Pavlovian conditioning, in particular, an originally neutral stimulus (the conditioned stimulus or CS) gains control over a reflex response of the animal after its association with an appetitive (food, drink) or an aversive (air puff, electric shock, inedible food) stimulus (the unconditioned stimulus or US). Because conditioned responses are automatically emitted without control by the animal, it is not always clear whether the CS acquires thereby a positive (attractive) or negative (repelling) value despite the supposed appetitive or aversive nature of conditioning protocols. To solve this issue, it is necessary to place the subjects in an experimental situation in which they can freely behave in response to the learned stimulus. Typically, orientation towards the CS reflects its positive value, whereas avoidance indicates its negative value.

Honeybees are a well known model for the study of associative learning (Menzel, 1999; Giurfa, 2007). In the laboratory, they learn to associate odours with sucrose reward in a Pavlovian conditioning protocol, termed the olfactory conditioning of the proboscis extension reflex (PER) (Takeda, 1961; Bitterman et al., 1983). The PER is a reflex exhibited by bees when antennal, tarsal or proboscis chemoreceptors are stimulated with a sucrose solution (US) (Frings, 1944). After pairing of an originally neutral odour (CS) with the US, the odour gradually gains control over the PER, so that the bee then extends its proboscis in response to the odour alone. Besides this behavioural measurement, it is possible to evaluate the bees' choice between two different odours using the head-turning response evinced even under harnessing conditions (Shafir et al., 1999). However, harnessing precludes the study of

avoidance/approach responses to the odours. The use of freely moving animals is therefore necessary to evaluate the associations established during conditioning. In the case of PER conditioning, the positive (attractive) quality acquired by the odour after pairing with sucrose was clearly shown by experiments in which bees previously conditioned in a PER protocol demonstrated an increased orientation towards the odour, either when walking in a four-armed olfactometer (Sandoz et al., 2000) or when flying in a wind tunnel (Chaffiol et al., 2005). These and other experiments in bees (e.g. Gerber et al., 1996) have shown that changing the context in the framework of studies on olfactory learning and retention is a useful procedure, as olfactory memories are extremely resistant to context changes.

Learning abilities of bees are not limited to appetitive associations. Recently, a novel Pavlovian conditioning protocol was developed, in which bees learn to associate an initially neutral odour (CS) with a mild electric shock (US) (Vergoz et al., 2007). Bees fixed individually on a metallic holder reflexively extend their sting (sting extension reflex, SER) to the application of an electric shock to the thorax (Núñez et al., 1983; Núñez et al., 1998; Balderrama et al., 2002). This is a typical defensive response of bees to potentially noxious stimuli (Breed et al., 2004). Pairing the odour with the electric shock results in the odour gradually gaining control over the SER. As for PER conditioning, since the animals are restrained in individual holders their avoidance/approach behaviour cannot be assessed. This novel conditioning paradigm was nevertheless termed 'aversive conditioning', in comparison with similar odour–electric shock associations performed in *Drosophila* (Tully and Queen, 1985; Schwärzel et al., 2003) or rodents (Okutani et al., 1999; Kilpatrick and Cahill, 2003), i.e. based on the aversive nature of the unconditioned stimuli delivered. In *Drosophila*, the aversive nature

of conditioning is clear because after successful conditioning the animals clearly avoid the CS in choice tests. In the case of olfactory SER conditioning, the term 'aversive' could be considered inappropriate given that no response inhibition is observed during conditioning (the bees learn to produce SER to the CS) and that the orientation behaviour of honeybees towards to the CS was never evaluated. Would conditioned honeybees explicitly avoid the odour CS, showing that the odour acquired an aversive value? This question is not trivial, as in natural conditions honeybees display stereotyped behaviours for the defence of the colony (Winston, 1987; Seeley, 1995). For instance, bees are known to attack intruders at the hive entrance, and may be found not to avoid the CS in a choice test, but on the contrary to quickly approach the CS and attempt to attack it.

In the present work, we asked whether olfactory SER conditioning in the honeybee does indeed constitute a case of aversive learning, by analyzing the orientation behaviour of freely walking honeybees presented with odours in a Y-maze. To provide a comparative framework with appetitive conditioning, we also tested PER-conditioned bees in the same setup. We explicitly asked whether SER-conditioned bees avoid the odour associated with the aversive US (electric shock), whereas PER-conditioned bees approach the odour associated with the appetitive US (sucrose).

## MATERIALS AND METHODS

### Insects

A total of 468 honeybees, *Apis mellifera* L., were used in these experiments. Worker bees were captured from an indoor hive, and were chilled on ice for 5 min until they stopped moving.

### PER conditioning

The experimental procedure for conditioning the proboscis extension response was the standard one used in previous studies on olfactory learning in honeybees (Bitterman et al., 1983; Sandoz et al., 1995; Guerrieri et al., 2005). Bees were mounted individually in metal holders leaving their antennae and mouthparts free. Ten minutes after recovery from cooling, honeybees were fed 5 µl sucrose solution (50% w/w). Then, animals were deprived of food for 3 h before conditioning.

### SER conditioning

The experimental procedure for conditioning the sting extension was the same as that developed by Vergoz et al. (Vergoz et al., 2007). Each bee was individually fixed on holders consisting of two brass plates fixed to a Plexiglas base. The bee's petiole and neck were respectively placed into notches in each of the brass plates, so that the bee closed the electric circuit. A Scotch Tape girdle maintained the thorax. The brass plates were connected to the output of a stimulator (60 Hz AC current). The notches were smeared with an electroencephalogram gel (Spectra 360 Electrode Gel, Parker Laboratories, Fairfield, NJ, USA) to ensure good contact between the plates and the bee. After fixation, bees were fed 5 µl 50% sucrose solution and were then left to accommodate to the experimental situation for 2 h before conditioning.

### Stimuli

Two odours, 1-hexanol and 1-nonanol, were used as CSs. They were chosen because they are well discriminated by the bees and induce low generalisation (Guerrieri et al., 2005). Five microlitres of pure odorants (1-hexanol or 1-nonanol; Sigma Aldrich, Deisenhofen, Germany) were applied to 1 cm<sup>2</sup> pieces of filter paper, which were placed into 20 ml syringes. Odours were manually delivered to the

antennae at a distance of 2 cm, paying attention to eject the whole volume of the syringe in a homogeneous flow throughout the 5 s of odour presentation. An air extractor placed behind the bee prevented odour accumulation, as well as possible contamination by pheromone release. The appetitive US was a sucrose solution (50% w/w) applied to the antennae and proboscis for 2 s. The aversive US was an electric shock of 7.5 V applied to the thorax for 2 s.

### Conditioning

Bees were individually subjected to a differential conditioning procedure, in which one odour (the CS+) is associated with the US (i.e. reinforced) and another odour (the CS-) is presented explicitly without US (i.e. non-reinforced). Such a protocol is helpful because it contains an internal control, as animals that efficiently learned the CS-US association will respond to the CS+ but not to the CS-. The US is sucrose in the case of PER conditioning and a mild electric shock in the case of SER conditioning. On each experimental day, half of the bees received 1-nonanol (A) reinforced and 1-hexanol (B) non-reinforced and *vice versa* for the other half of the bees. Odorants were presented in a pseudo-random sequence of six reinforced and six non-reinforced trials (ABBA BAAB ABBA) starting with odorant A or B in a balanced manner, so that no effect of a particular odorant could influence the results. Each trial lasted 30 s. The bee was placed in the stimulation site in front of the air extractor and left for 15 s before being presented with the odorant for 5 s. In the case of reinforced trials, the US was applied 3 s after odorant onset and finished with the CS. The bee was then left in the setup for 10 s and removed. The inter-trial interval was always 10 min.

In all experiments, responses to the CS were measured during the 3 s in which the CS preceded the US. Multiple responses were counted as a single conditioned response. Responses to the US were measured throughout conditioning during US presentations. Only bees that consistently (i.e. at all trials) showed a PER to the sucrose solution or a SER to the electric shock were kept for further analysis, corresponding to 94.6% for PER and 85.5% for SER conditioning.

### Choice test in a Y-maze

The aim of our study was to observe the orientation and behaviour of freely moving bees in a Y-maze presenting both the CS+ and the CS- after successful PER or SER conditioning. Only bees that efficiently learned the PER or SER task, i.e. that responded to the CS+ but not to the CS- in the two last blocks of trials, were used in the Y-maze experiment. As a control for possible odour or side preference, naïve bees were also subjected to a test in the Y-maze presenting the two odours.

We used an acrylic Y-maze which allows recording decisions of a walking insect confronted with two odours, each presented in one arm of the maze (Fig. 1) (Dupuy et al., 2006). The device was positioned under homogeneous red light, provided by a cold light source in a dark room, which prevented the bees from using visual cues for orientation and from trying to fly. The entrance channel and the arms of the maze were 1.9 cm high, and 8 cm and 6 cm long, respectively. The arms were at a 90° angle, each at 135° from the entrance channel. The maze was placed on a rectangular supporting base (13.5 cm × 14.5 cm) from which it could be removed to be cleaned. The base was supported by four acrylic cylinders (10 cm high). The maze was covered by a glass plate (10 cm × 15 cm). The floor of the maze was covered by a piece of filter paper, which was replaced with a clean one after each visit of a honeybee to the maze, to avoid the use of pheromonal cues.

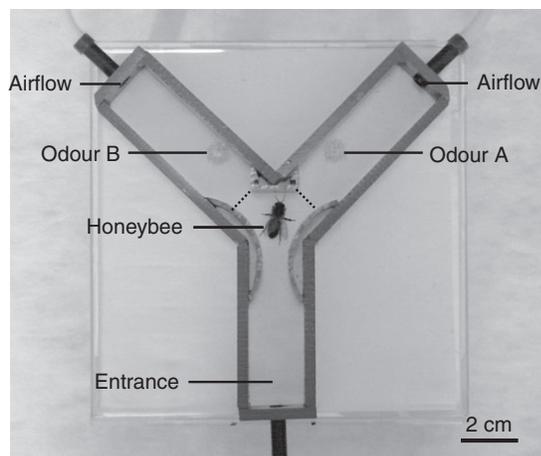


Fig. 1. The Y-maze for testing honeybees' choice behaviour. Top view of the acrylic Y-maze. Each bee was placed in the entrance channel of the maze, where it was released. The bee moved towards the decision area, where it had to choose between the two arms containing the odours (delimited by dashed lines). The airflows ensured odour diffusion. The first choice of the bee for one of the arms, and the time spent in each arm were recorded.

The entrance to each arm was defined at its narrowest point, connecting the arms to the entrance channel (Fig. 1, dotted lines). In each arm, a micropipette tip containing a piece of filter paper (1 mm × 20 mm) loaded with 15 µl odour substance was inserted into a hole in the floor. The tips were sealed at their base and the top was covered with a plastic net hood to avoid direct contact with the chemicals. Each tip was placed 1.5 cm from the arm entrance, so that honeybees entering an arm experienced the odour emanating from it. An air stream (15 ml min<sup>-1</sup>) filtered by active charcoal was humidified and driven from the back of each arm by means of plastic tubes. This allowed the odours to be driven towards the decision area of the maze. The glass cover allowed better concentration of odours. An air extractor was situated above the maze to eliminate odours escaping from the maze throughout the experiment.

After the conditioning procedure, bees were allowed to rest for 1 h in the dark room. Good learners (see above) were carefully removed from their holder, paying attention to avoid unnecessary stress for the animal. They were then tested in the Y-maze with one arm containing the CS+ and the other arm containing the CS-. From one bee to the next, the placement of the odours 1-hexanol and 1-nonanol in each arm was swapped, so that no effect of the sides could influence the results.

Bees were individually introduced at the proximal end of the entrance channel and their location was recorded for 180 s with a video camera (JVC Everio, GZ-HD7E). This duration was chosen after preliminary experiments which showed that it is long enough for most bees to choose one of the arms, but short enough to avoid non odour-dependent exploration of the Y-maze. In parallel, possible proboscis or sting extensions in the Y-maze were visually recorded in relation to the visited arm. A bee was considered in one of the arms when its head and thorax were beyond the virtual line at the arm's entrance (Fig. 1, dotted line). Each bee was subjected to only one test in the maze.

#### Statistics

The percentage of individuals showing a PER or SER at each trial was used to plot acquisition curves. For each conditioning type,

the two equal subgroups receiving 1-hexanol and 1-nonanol as CS+ were pooled. To analyse the variation of performance during acquisition, we used repeated measures analyses of variance (ANOVAs) with trial (from 1 to 6) and odour (CS+ vs CS-) as within-group factors. Monte Carlo studies have shown that it is permissible to use ANOVA on dichotomous data only under controlled conditions, which are met by our experiments (equal cell frequencies and at least 40 degrees of freedom of the error term) (Lunney, 1970). Video recordings of bee activity in the Y-maze were analysed at a frequency of 1 frames<sup>-1</sup> using custom software (M. Combe, CRCA, Toulouse, France). To this end, we focused on three maze areas: the entrance channel and the two arms. We measured the first arm visited by each bee, as well as the total amount of time spent in the CS+ and in the CS- arm. We used a binomial test to compare the proportion of first choices to the CS+ with a random choice (50%). A Wilcoxon matched-pairs test was used to compare the relative time spent in the CS+ and CS- arms. Lastly, a McNemar  $\chi^2$ -test was applied to compare the percentage of proboscis or sting extensions exhibited by bees in the two arms. The significance threshold for all analyses was  $P < 0.05$ . Statistical tests were performed with STATISTICA 5.5 (Statsoft, Tulsa, USA) and R 2.6.2 (Foundation for Statistical Computing, Vienna, Austria).

## RESULTS

### Conditioning

#### PER conditioning

Honeybees efficiently learned to differentiate the odorant that preceded sucrose solution (CS+) from the non-reinforced odorant (CS-) in the course of training ( $N=142$ , ANOVA for repeated measurements: trial × odour interaction,  $F_{5,705}=118.4$ ,  $P < 0.0001$ ; Fig. 2A). They significantly increased their proboscis extension responses to the CS+ ( $F_{5,705}=139.9$ ,  $P < 0.0001$ ) and decreased their responses to the CS- ( $F_{5,705}=4.4$ ,  $P < 0.001$ ). At the end of conditioning (trial 6), honeybees responded significantly more to the CS+ than to the CS- (McNemar test,  $\chi^2=102.1$ ,  $P < 0.0001$ ). Overall, 69.7% of the bees (99 out of 142) performed correctly in the last two blocks of trials, responding only to the CS+ and not to the CS-. Of these, 79 (55.6%) were afterwards tested in the Y-maze.

#### SER conditioning

Honeybees also learned to differentiate the odorant that preceded the electric shock (CS+) from the non-reinforced odorant (CS-) in the course of training ( $N=238$ , ANOVA for repeated measurements: trial × odour interaction,  $F_{5,1180}=44.2$ ,  $P < 0.0001$ ). They significantly increased their sting extension responses to the CS+ ( $F_{5,1180}=18.8$ ,  $P < 0.0001$ ) and decreased their responses to the CS- ( $F_{5,1180}=21.3$ ,  $P < 0.0001$ ; Fig. 2B). At the end of conditioning (trial 6), honeybees responded significantly more to the CS+ than to the CS- (McNemar test,  $\chi^2=101.1$ ,  $P < 0.0001$ ). As observed in previous work (Vergoz et al., 2007), performance in SER conditioning was lower than in PER conditioning. Consequently, only 36.5% of the bees (87 out of 238) responded correctly in the two last blocks of trials. As learning success was lower in SER conditioning compared to PER conditioning, we trained more bees in the former to reach a similar number of individuals tested in the Y-maze. Thus, 72 bees (30.2% of total) were tested in the Y-maze.

#### Choice tests in the Y-maze

Conditioned bees were observed for 180 s in the Y-maze presenting both the CS+ and the CS- and their first choice, as well as the time spent in each arm, were recorded. In this setup, naive bees ( $N=39$ )

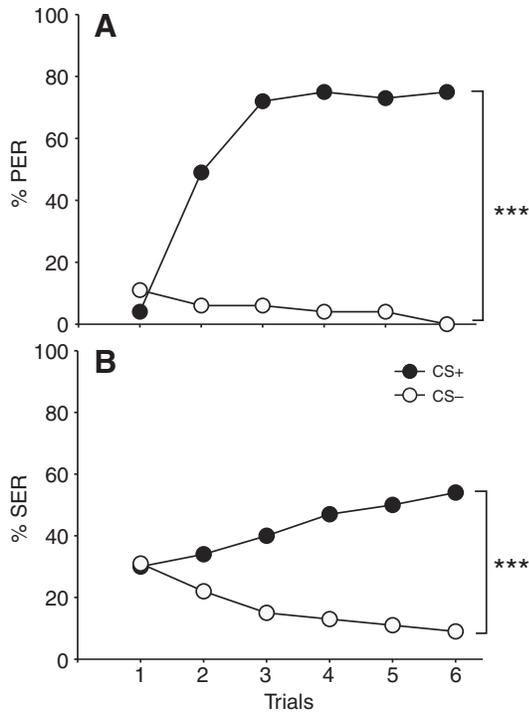


Fig. 2. Associative olfactory conditioning in honeybees. (A) Conditioning of the proboscis extension reflex (PER). Percentage of PER in bees trained with an odorant explicitly reinforced with sucrose solution [CS+ (positive conditioned stimulus), black circles;  $N=142$ ] and with a non-reinforced odorant (CS-, white circles). Bees learned to differentiate between CS+ and CS- in the course of training ( $***P<0.001$ ). (B) Conditioning of the sting extension reflex (SER) in bees trained with an odorant explicitly reinforced with an electric shock (black circles,  $N=238$ ) and with an odorant explicitly non-reinforced (white circles). Bees learned to differentiate between CS+ and CS- in the course of training ( $***P<0.001$ ).

chose with equal probability the right or the left arm (respectively, 43.6% and 56.4%, binomial test:  $P=0.52$ , NS) or the odours 1-hexanol or 1-nonanol (respectively, 53.8% and 46.2%, binomial test:  $P=0.75$ , NS). They also spent an equal amount of time in both arms, considering the sides (Wilcoxon test:  $z=0.88$ , NS) or the odours (Wilcoxon test:  $z=0.88$ , NS). We conclude that bees have neither a spontaneous preference for one side of the Y-maze, nor for one of the tested odorants.

#### PER conditioning

Honeybees that learned to associate an odour with a sucrose reward (CS+) chose this odour in the Y-maze over a previously non-reinforced odour (CS-,  $N=79$ ; Fig. 3A). Thus, 64.6% of the bees chose the arm containing the CS+, a proportion which was significantly higher than a random choice (binomial test:  $P=0.013$ ). In addition, bees spent significantly more time in the arm containing the CS+ (64.4% of the total time spent in the odour arms) than in the one containing the CS- (35.6%; Wilcoxon test:  $z=3.47$ ,  $P<0.001$ ; Fig. 3B). When observing conditioned responses produced by bees in the Y-maze, we found that 49% of the bees showed proboscis extensions in the CS+ arm and only 6% in the CS- arm (McNemar test,  $\chi^2=32.2$ ,  $P<0.0001$ ; Fig. 4). Most proboscis extensions occurred within 1 cm of the micropipette tip containing the odour source. Thus, PER-conditioned bees preferentially chose the CS+ arm, spent more time in it and extended the proboscis in the vicinity of the

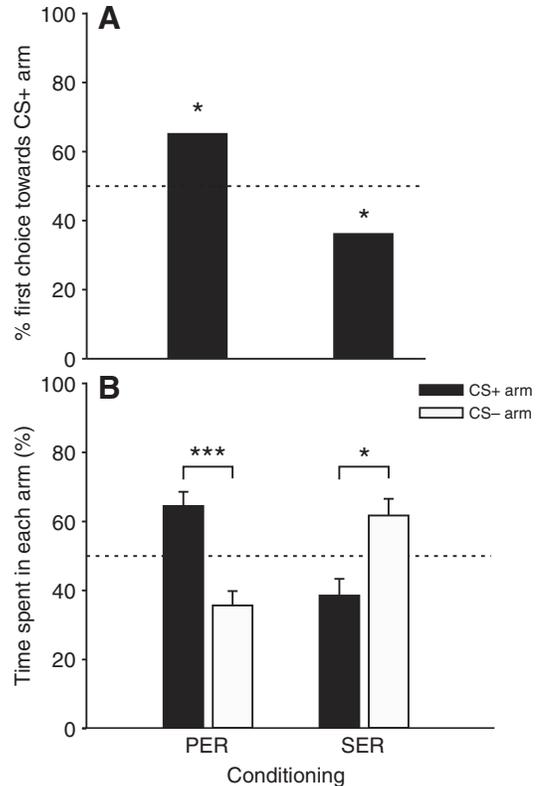


Fig. 3. Orientation of honeybees in the Y-maze, 1 h after associative olfactory conditioning. (A) First choice towards the arm containing the CS+, after PER conditioning ( $N=79$ ) and SER conditioning ( $N=72$ ). The dashed line at 50% indicates random choice between the CS+ and CS- arms. After PER conditioning, honeybees significantly chose the CS+. By contrast, after SER conditioning, honeybees significantly avoided the CS+ ( $*P<0.05$ ). (B) Amount of time spent in each Y-maze arm, after PER conditioning ( $N=79$ ) and SER conditioning ( $N=72$ ). After PER conditioning, honeybees spent significantly more time in the CS+ arm. By contrast, after SER conditioning, honeybees spent significantly more time in the CS- arm than in the CS+ arm ( $*P<0.05$ ;  $***P<0.001$ ).

CS+, thereby showing the excitatory, attractive nature of the learned CS.

#### SER conditioning

Honeybees that learned an odour associated with an electric shock (CS+) avoided this odour in the Y-maze, thus preferring the previously non-reinforced odour (CS-,  $N=72$ ; Fig. 3A). Only 36.1% of the bees chose the arm containing the CS+, a proportion that was significantly lower than a random choice (binomial test:  $P=0.024$ ). In addition, bees spent significantly less time in the arm presenting the CS+ (38.4% of the total time spent in the odour arms) than in the one presenting the CS- (61.6%; Wilcoxon test:  $z=2.19$ ,  $P<0.05$ ; Fig. 3B). SER-conditioned bees never showed sting extensions in the Y-maze. Despite this absence of SER in freely walking bees, the inhibitory, aversive nature of the learned CS is revealed by the fact that SER-conditioned bees avoided the arm presenting the CS+ and spent more time in the arm containing the non-reinforced odour.

#### DISCUSSION

The present work shows that honeybees subjected to a Pavlovian conditioning procedure can use the learned information in a novel

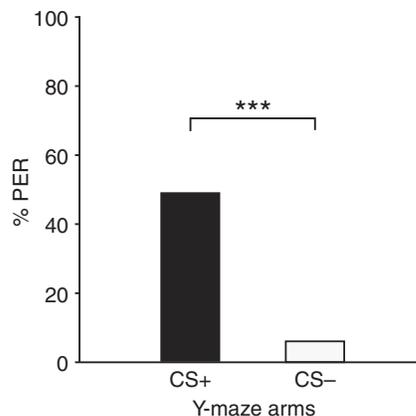


Fig. 4. Proboscis extensions in the Y-maze arms after PER conditioning. Honeybees ( $N=79$ ) that learned an odour–sucrose association, showed significantly more proboscis extensions in the CS+ than in the CS– arm ( $***P<0.001$ ).

operant context, i.e. in a context in which choice outcome depends on their behaviour. Honeybees that learned an odour–sucrose association in a harnessed condition were afterwards attracted by this odour in a Y-maze and spent more time in its vicinity. Conversely, honeybees that learned an odour–electric shock association in a harnessed condition clearly avoided this odour and spent more time in the opposite arm of the maze, thus demonstrating the aversive nature of SER conditioning in honeybees.

Previous studies investigated the ability of bees to transfer olfactory information gained in a given experimental situation to novel situations. Using free-flying bees, Reinhard et al. (Reinhard et al., 2004) showed that an odour that was previously learned at a sucrose-reinforced feeder, induced foragers to fly to and revisit this feeder when blown into the hive. Thus, the odour induced retrieval of navigational and/or visual memories associated to the feeder despite being delivered in the different context of the bee hive. Gerber et al. (Gerber et al., 1996) studied proboscis extension responses of harnessed bees that had previously foraged on basswood trees (*Tilia* sp.). These bees showed initial responses to the basswood tree odour as high as 60% compared with naïve bees that had low spontaneous response levels. This suggested a possible transfer of information learnt in a foraging situation (operant context) to the restrained PER situation (Pavlovian context). However, it could be argued that in this case, Pavlovian conditioning could have taken place at the moment when bees were sucking nectar from the basswood, so that, strictly speaking this observation suggests, but does not demonstrate, a transfer between an operant and a Pavlovian situation. Adopting the reverse experimental design, Bakchine-Huber et al. (Bakchine-Huber et al., 1992) and Sandoz et al. (Sandoz et al., 2000) used the Pavlovian conditioning of PER in harnessed bees and then showed increased orientation responses of conditioned bees towards the CS when walking freely in a four-armed olfactometer. Chaffiol et al. (Chaffiol et al., 2005) confirmed these observations with bees flying in a wind tunnel. These results showed that information gained in appetitive Pavlovian conditioning induced orientation towards the CS in a choice situation involving operant components. In this case, harnessed bees could not have learned to approach the odour during conditioning, thus showing that transfer between situations did indeed exist. The possible natural function of this transfer ability may be related to the observation that bees do indeed learn to associate odours and

nectar reward within the hive during trophallaxis with returning foragers (Farina et al., 2005; Gil and De Marco, 2005), and subsequently choose the odour learnt within the hive in a foraging context (von Frisch, 1946; Arenas et al., 2007). Most importantly, these experiments also showed that after the formation of an odour–sucrose association, the learnt odour had acquired a positive/attractive nature for the animal. In the present study, our PER-conditioned bees were attracted by the CS+ in the Y-maze, thus confirming previous accounts and validating our experimental setup for studying bees' choice behaviour. Bees also manifested conditioned responses (proboscis extensions) in the vicinity of the odour. This behaviour, also observed by Sandoz et al. (Sandoz et al., 2000) in a four-armed olfactometer, indicates beyond doubt that the association learnt while restrained was indeed retrieved while walking in the Y-maze. This situation probably recapitulates what recruited foragers may experience in a natural context when visiting flowers that release the odour learnt within the hive. Taken together these results show the high resistance of olfactory memories to changes in context (Gerber et al., 1996) and validate the use of transfer procedures to assess the nature of olfactory associations established during different forms of olfactory learning.

By contrast, nothing was known until now about the possibility that bees trained using SER conditioning transfer the learnt information to a novel situation involving operant components. We show here that bees trained to associate an odour with an electric shock did indeed transfer the learned association to a choice situation in a Y-maze. We had hypothesized that bees may either avoid the odour that was previously associated to a noxious stimulus, demonstrating a typical case of aversive conditioning, or on the contrary, that they may orient towards the odour and display aggressive behaviours, as they do at the colony entrance. Our results clearly show that bees avoid the arm containing the odour previously associated to the electric shock, and spend significantly less time in the arm of the maze containing this odour. These results demonstrate that SER conditioning in honeybees is a case of aversive conditioning, the association of an odour with the electric shock bestowing the odour a negative hedonic value. The avoidance response displayed by bees in our experiment is thus similar to what is typically observed after aversive conditioning in *Drosophila* (Tully and Quinn, 1985; Schwärzel et al., 2003) and rats (Okutani et al., 1999; Kilpatrick and Kahill, 2003). However, honeybees show division of labour, with some workers involved in within-hive tasks such as cell cleaning, brood and queen tending, comb building etc., while others are engaged in outside tasks like guarding and foraging (Winston, 1987; Seeley, 1995). In this work, we used in-hive bees, the caste of which was not known. It is possible that different castes would show different transfer behaviours in the Y-maze, in particular guard bees, which are involved in colony defence, and could approach the odour associated with the shock and attempt to sting it. Comparing the responses of different worker castes in the Y-maze will be the goal of future experiments.

One advantage of studying orientation behaviour after both PER and SER conditioning in a unifying paradigm is that we could explicitly show that the same odours can be associated with sucrose or with an electric shock (see also Vergoz et al., 2007) and that the orientation behaviour of the bees dramatically changes according to the associated unconditioned stimulus. In Y-maze tests performed on naïve individuals no difference was found in the choice of the two odours used in our study, thus showing that these odours were neutral (neither positive nor negative) prior to conditioning. Both odours became clearly attractive after PER conditioning but repulsive after SER conditioning. These data fit with a module-based view of

honeybee behaviour, based on the existence within each individual of at least two modules, a foraging and a defensive module, which would control appetitive learning on the one hand, and aversive learning on the other (Roussel et al., 2009). It is well established that learning in a PER task, involving sucrose as reinforcement, is highly dependent on the sucrose response threshold of the bees (e.g. Scheiner et al., 1999; Scheiner et al., 2001). Thus, bees that are more responsive to sucrose learn and memorize better in olfactory conditioning using sucrose as reinforcement. Likewise, we have recently shown that bees that are more responsive to electric shocks also learn and memorize better in olfactory SER conditioning, which uses electric shock as reinforcement. However, contrary to current theories (Page et al., 2006), we found that responsiveness to sucrose and to shocks are not correlated, that is, a bee that has a low response threshold for sucrose does not automatically have a low response threshold for electric shocks (Roussel et al., 2009). This suggests that SER and PER conditioning could belong to two different modules determining bees' behaviour, and could be related, respectively, to defensive and foraging tasks. Interestingly, there may be a neural basis for the dichotomy between aversive and appetitive learning. Appetitive reinforcement, in particular sucrose reinforcement, is mediated by octopaminergic neurons in the insect brain. For instance, injection of octopamine into the bee brain substitute for sucrose reward and mediate the formation of an appetitive olfactory memory (Hammer and Menzel, 1998). Similarly, repressing the function of octopamine receptors impairs olfactory learning performance (Farooqui et al., 2003), probably because it prevents detection of the sucrose reward at the central level. By contrast, dopamine is necessary for aversive olfactory learning in insects [*Drosophila* (Schwärzel et al., 2003; Schroll et al., 2006); crickets (Unoki et al., 2005; Unoki et al., 2006); bees (Vergoz et al., 2007)]. In particular, bees subjected to pharmacological blocking of their dopaminergic system are unable to learn to differentiate between CS+ and CS- in SER conditioning (Vergoz et al., 2007). The current model for explaining that bees can show both attraction or repulsion to the same odours depending on the US is based on convergence of the olfactory pathway both with octopaminergic neurons mediating appetitive reinforcement (positive hedonic value) and dopaminergic neurons mediating aversive reinforcement (negative hedonic value). Concomitant activation of odour-specific neurons with one or the other reinforcement system during conditioning would strengthen specific output connectivity linked to particular behavioural routines, triggering approach or avoidance, respectively (Gerber et al., 2004).

The orientation behaviour of bees was clearly symmetrical between PER and SER conditioning situations, as bees respectively chose or avoided the CS+ arm, and spent more or less time in this arm of the maze. However, we observed an important difference between the two situations. As indicated above, after PER conditioning, many bees extended their proboscis in the CS+ arm. By contrast, after SER conditioning, we never observed any sting extension in the Y-maze. Sting extension is certainly more elusive than proboscis extension, but during the Y-maze experiments we also never saw any of the movements that usually accompany sting extension, such as abdomen flexion, opening of the sting chamber, etc. This observation exemplifies the dissociation of the different associations taking place in Pavlovian conditioning (Rescorla, 1988; Kirsch et al., 2004). First, through CS-US pairing, the CS gradually gains control over the conditioned response, so that when presented alone, it triggers the response. In parallel, the CS also acquires a positive or negative hedonic value depending on the US, inducing different types of behaviours, such as approach or avoidance. In the case of PER conditioning, both associations were clearly retrieved

in the Y-maze. After SER conditioning, however, only the negative value of the odour appeared to control the behaviour of the bees. Retrieval of memories is dependent on the experimental context (Haney and Lukowiak, 2001) and on the motivation of the animals (Lewis and Takasu, 1990). Thus, we may hypothesize that this difference is due to the context change and to differences in bees' motivation when placed in the Y-maze after each type of conditioning. As proposed above, after PER conditioning, a free-walking hungry bee is in a context that could correspond to food searching, so that the retrieval of the CS-PER association is facilitated. Conversely, after SER conditioning, we believe that the bee's motivation is to escape the prior situation in which it has received noxious stimuli. In addition, in nature, defensive behaviour takes place at the hive entrance, i.e. in a highly social context. Therefore, a lone bee in a Y-maze is probably not in a defensive context and thus retrieval of the CS-SER association is more difficult. In other terms, asymmetry in bees' motivation after SER and PER conditioning may contribute to such differential retrieval of olfactory memories when placed into the Y-maze. In general, it should be noted that transfer performance of the bees in the Y-maze was lower than could be expected, as only a portion of the bees placed in the Y-maze – and which had learned efficiently the CS-US association – actually chose the expected arm. This again can be explained by context differences between the two experimental situations and the possibility that a non-negligible proportion of bees explore the maze rather than making odour-mediated choices.

This study establishes the aversive nature of SER conditioning in honeybees, showing that originally neutral odours paired with an electric shock acquire a negative hedonic value. Moreover, the same odours can take either positive or negative values providing additional evidence for a module-based view of insect reinforcement systems. Future work should attempt to track down the neuronal counterparts of these modules in the bee brain, in particular pre-motor systems giving rise to avoidance *versus* attraction responses.

#### LIST OF ABBREVIATIONS

ANOVA	analysis of variance
CS	conditioned stimulus
CS+	reinforced conditioned stimulus
CS-	non-reinforced conditioned stimulus
PER	proboscis extension response
SER	sting extension response
US	unconditioned stimulus

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#### REFERENCES

- Arenas, A., Fernandez, V. M. and Farina, W. M. (2007). Floral odor learning within the hive affects honeybees' foraging decisions. *Naturwissenschaften* **94**, 218-222.
- Bakchine-Huber, E., Pham-Delègue, M. H., Patte, F. and Masson, C. (1992). Modification d'une préférence olfactive après apprentissage chez l'abeille: influence de la nature du signal appris. *C. R. Acad. Sci. III* **314**, 325-330.
- Balderrama, N., Núñez, J., Guerrieri, F. and Giurfa, M. (2002). Different functions of two alarm substances in the honeybee. *J. Comp. Physiol. A* **188**, 485-491.
- Bitterman, M. E., Menzel, R., Fietz, A. and Schäfer, S. (1983). Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *J. Comp. Psychol.* **97**, 107-119.
- Breed, M. D., Guzman-Novoa, E. and Hunt, G. J. (2004). Defensive behavior of honey bees: organization, genetics, and comparison with other bees. *Annu. Rev. Entomol.* **49**, 271-298.
- Chaffiol, A., Laloi, D. and Pham-Delègue, M. H. (2005). Prior classical olfactory conditioning improves odour-cued flight orientation of honeybees in a wind tunnel. *J. Exp. Biol.* **208**, 3731-3737.
- Dupuy, F., Sandoz, J. C., Giurfa, M. and Josens, R. (2006). Individual olfactory learning in *Camponotus* ants. *Anim. Behav.* **72**, 1081-1091.

- Farina, W. M., Grüter, C. and Diaz, P. C. (2005). Social learning of floral odours inside the honeybee hive. *Proc. Biol. Sci.* **272**, 1923-1928.
- Farooqui, T., Robinson, K., Vaessin, H. and Smith, B. H. (2003). Modulation of early olfactory processing by an octopaminergic reinforcement pathway in the honeybee. *J. Neurosci.* **23**, 5370-5380.
- Frings, H. (1944). The loci of olfactory end-organs in the honey-bee, *Apis mellifera* L. *J. Exp. Zool.* **97**, 123-134.
- Gerber, B., Geberzahn, N., Hellstern, F., Klein, J., Kowalsky, O., Wüstenberg, D. and Menzel, R. (1996). Honey bees transfer olfactory memories established during flower visits to a proboscis extension paradigm in the laboratory. *Anim. Behav.* **52**, 1079-1085.
- Gerber, B., Tanimoto, H. and Heisenberg, M. (2004). An engram found? Evaluating the evidence from fruit flies. *Curr. Opin. Neurobiol.* **14**, 737-744.
- Gil, M. and De Marco, R. J. (2005). Olfactory learning by means of trophallaxis in *Apis mellifera*. *J. Exp. Biol.* **208**, 671-680.
- Giurfa, M. (2007). Behavioral and neural analysis of associative learning in the honeybee: a taste from the magic well. *J. Comp. Physiol. A* **193**, 801-824.
- Guerrieri, F., Schubert, M., Sandoz, J. C. and Giurfa, M. (2005). Perceptual and neural olfactory similarity in honeybees. *PLoS Biol.* **3**, 1-14.
- Hammer, M. and Menzel, R. (1998). Multiple sites of associative odor learning as revealed by local brain microinjections of octopamine in honeybees. *Learn. Mem.* **5**, 146-156.
- Haney, J. and Lukowiak, K. (2001). Context learning and the effect of context on memory retrieval in Lymnaea. *Learn. Mem.* **8**, 35-43.
- Kilpatrick, L. and Cahill, L. (2003). Modulation of memory consolidation for olfactory learning by reversible inactivation of the basolateral amygdala. *Behav. Neurosci.* **117**, 184-188.
- Kirsch, I., Lynn, S. J., Vigorito, M. and Miller, R. R. (2004). The role of cognition in classical and operant conditioning. *J. Clin. Psychol.* **60**, 369-392.
- Lewis, W. J. and Takasu, K. (1990). Use of learned odors by a parasitic wasp in accordance with host and food needs. *Nature* **348**, 635-636.
- Lunney, G. H. (1970). Using analysis of variance with a dichotomous dependent variable: an empirical study. *J. Educ. Meas.* **7**, 263-269.
- Menzel, R. (1999). Memory dynamics in the honeybee. *J. Comp. Physiol. A* **185**, 323-340.
- Núñez, J., Maldonado, H., Miralto, A. and Balderrama, N. (1983). The stinging response of the honeybee: effects of morphine, naloxone and some opioid peptides. *Pharmacol. Biochem. Behav.* **19**, 921-924.
- Núñez, J., Almeida, L., Balderrama, N. and Giurfa, M. (1998). Alarm pheromone induces stress analgesia via an opioid system in the honeybee. *Physiol. Behav.* **63**, 75-80.
- Okutani, F., Yagi, F. and Kaba, H. (1999). Gabaergic control of olfactory learning in young rats. *Neurosci.* **93**, 1297-1300.
- Page, R. E., Scheiner, R., Erber, J. and Amdam, G. V. (2006). The development and evolution of division of labor and foraging specialization in a social insect (*Apis mellifera* L.). *Curr. Top. Dev. Biol.* **74**, 253-286.
- Reinhard, J., Srinivasan, M. V., Guez, D. and Zhang, S. W. (2004). Floral scents induce recall of navigational and visual memories in honeybees. *J. Exp. Biol.* **207**, 4371-4381.
- Rescorla, R. A. (1988). Behavioral studies of Pavlovian conditioning. *Annu. Rev. Neurosci.* **11**, 329-352.
- Roussel, E., Carcaud, J., Sandoz, J. C. and Giurfa, M. (2009). Reappraising social insect behavior through aversive responsiveness and learning. *PLoS ONE* **4**, e4197.
- Sandoz, J. C., Roger, B. and Pham-Delègue, M. H. (1995). Olfactory learning and memory in the honeybee: comparison of different classical conditioning procedures of the proboscis extension response. *C. R. Acad. Sci. III* **318**, 749-755.
- Sandoz, J. C., Laloi, D., Odoux, J. F. and Pham-Delègue, M. H. (2000). Olfactory information transfer in the honey bees: compared efficiency of classical conditioning and early exposure. *Anim. Behav.* **59**, 1025-1034.
- Scheiner, R., Erber, J. and Page, R. E., Jr (1999). Tactile learning and the individual evaluation of the reward in honey bees (*Apis mellifera* L.). *J. Comp. Physiol. A* **185**, 1-10.
- Scheiner, R., Page, R. E., Jr and Erber, J. (2001). The effects of genotype, foraging role, and sucrose responsiveness on the tactile learning performance of honey bees (*Apis mellifera* L.). *Neurobiol. Learn. Mem.* **76**, 138-150.
- Schroll, C., Riemensperger, T., Bucher, D., Ehmer, J., Völler, T., Erbguth, K., Gerber, B., Hendel, T., Nagel, G., Buchner, E. et al. (2006). Light-induced activation of distinct modulatory neurons triggers appetitive or aversive learning in *Drosophila* larvae. *Curr. Biol.* **16**, 1471-1477.
- Schwärzel, M., Monastirioti, M., Scholz, H., Friggi-Grelin, F., Birman, S. and Heisenberg, M. (2003). Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in *Drosophila*. *J. Neurosci.* **23**, 10495-10502.
- Seeley, T. D. (1995). *The Wisdom of the Hive*. Cambridge, MA: Harvard University Press.
- Shafir, S., Wiegmann, D. D., Smith, B. H. and Real, L. A. (1999). Risk-sensitive foraging: choice behaviour of honeybees in response to variability in volume of reward. *Anim. Behav.* **57**, 1055-1061.
- Takeda, K. (1961). Classical conditioned response in the honeybee. *J. Insect Physiol.* **6**, 168-179.
- Tully, T. and Quinn, W. G. (1985). Classical conditioning and retention in normal and mutant *Drosophila melanogaster*. *J. Comp. Physiol. A* **157**, 263-277.
- Unoki, S., Matsumoto, Y. and Mizunami, M. (2005). Participation of octopaminergic reward system and dopaminergic punishment system in insect olfactory learning revealed by pharmacological study. *Eur. J. Neurosci.* **22**, 1409-1416.
- Unoki, S., Matsumoto, Y. and Mizunami, M. (2006). Roles of octopaminergic and dopaminergic neurons in mediating reward and punishment signals in insect visual learning. *Eur. J. Neurosci.* **24**, 2031-2038.
- Vergoz, V., Roussel, E., Sandoz, J. C. and Giurfa, M. (2007). Aversive learning in honeybees revealed by the olfactory conditioning of the sting extension reflex. *PLoS One* **3**, e288.
- von Frisch, K. (1946). Die Tänze der Bienen. *Öst. Zool. Zeitschr.* **1**, 1-48.
- Winston, M. L. (1987). *The Biology of the Honey Bee*. Cambridge, MA: Harvard University Press.