

## Basic Neuroscience

## Revisiting olfactory classical conditioning of the proboscis extension response in honey bees: A step toward standardized procedures

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## HIGHLIGHTS

- ▶ The honey bee *Apis mellifera* is a robust model for the study of Pavlovian conditioning.
- ▶ The olfactory conditioning of the proboscis extension response (PER) is a fundamental tool for the study of Pavlovian learning in bees.
- ▶ We revisit olfactory PER conditioning and define a standardized framework for using this behavioral protocol.
- ▶ We present all the methodological details necessary for successful implementation of olfactory PER conditioning.

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## ABSTRACT

The honey bee *Apis mellifera* has emerged as a robust and influential model for the study of classical conditioning thanks to the existence of a powerful Pavlovian conditioning protocol, the olfactory conditioning of the proboscis extension response (PER). In 2011, the olfactory PER conditioning protocol celebrated its 50 years since it was first introduced by Kimihisa Takeda in 1961. In this protocol, individually harnessed honey bees are trained to associate an odor with sucrose solution. The resulting olfactory learning is fast and induces robust olfactory memories that have been characterized at the behavioral, neuronal and molecular levels. Despite the success of this protocol for studying the bases of learning and memory at these different levels, innumerable procedural variants have arisen throughout the years, which render comparative analyses of behavioral performances difficult. Moreover, because even slight variations in conditioning procedures may introduce significant differences in acquisition and retention performances, we revisit olfactory PER conditioning and define here a standardized framework for experiments using this behavioral protocol. To this end, we present and discuss all the methodological steps and details necessary for successful implementation of olfactory PER conditioning.

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## 1. Introduction

Associative learning is a fundamental property of nervous systems, which is governed by conserved rules both across species and across modalities. In a classical conditioning procedure, the animal is presented with two types of stimuli, the unconditioned stimulus (US) and the conditioned stimulus (CS). The US is a stimulus which innately evokes a response, while the CS is usually a neutral

stimulus without any initial connection with a response. Through forward-pairing of CS and US, the animal learns that the CS predicts US delivery and starts responding to the CS. The response evoked by the CS is termed ‘conditioned response’ (Pavlov, 1927).

Insects constitute successful models for the study of learning and memory due to their remarkable learning abilities mediated by relatively simple neural systems containing lower numbers of neurons compared to vertebrates (Menzel, 1999; Mizunami et al., 2004; Davis, 2005; Giurfa, 2007). Among insects, honey bees (*Apis mellifera*) are reported to have the highest and broadest range of learning abilities (Menzel, 1999; Menzel and Giurfa, 2001; Giurfa, 2003, 2007; Sandoz, 2011; Giurfa and Sandoz, 2012). Honey bees are able to associate food reward with different sensory stimuli such as odors, colors, visual patterns, tactile or thermal stimuli

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(Menzel, 1999; Giurfa, 2007). However, studies on learning and memory in honey bees have mostly used harnessed individuals and olfactory learning protocols when the goal was to achieve a full control of behavior by the experimenter. The most popular protocol used to this end is the olfactory conditioning of the proboscis extension response (PER), which is a case of classical (Pavlovian) conditioning (Takeda, 1961; Vareschi, 1971; Bitterman et al., 1983; Giurfa and Sandoz, 2012).

The PER is a reflexive response of hungry bees which is part of their feeding behavior while foraging or within the hive (Frings, 1944; Frings and Frings, 1949). It occurs when the antennae, tarsi or mouth parts come in contact with sucrose solution; the bee then reflexively extends its proboscis (PER) to reach the sucrose solution and drink it (Fig. 1a). Odors generally do not evoke the PER in bees naïve to the experimental conditions. During conditioning, an odor (CS) is presented in close temporal association with sucrose solution (US). At the end of training, the odor alone elicits the PER, indicating that the bee has learned the odor-sucrose association (Takeda, 1961; Bitterman et al., 1983). PER is usually recorded as a dichotomous response (1 or 0), which can thus be used as an index for learning and memory performances.

The protocol of olfactory PER conditioning, first established by Takeda (1961), was later standardized by Bitterman et al. (1983). Since then, numerous studies have used it to study the behavioral, neural and molecular bases of olfaction, learning and memory formation (Giurfa and Sandoz, 2012). Yet, throughout the years, a series of procedural variants and deviations from the original procedure have arisen, which render comparative analyses of behavioral performances difficult. Moreover, because even slight variations in conditioning procedures may introduce significant differences in acquisition and retention performances, such procedural variations are not trivial and need to be considered carefully. In many cases, researchers willing to use olfactory PER conditioning for the first time complain that Section 2 is usually not detailed enough to achieve successful conditioning in a straightforward way. A first attempt to overcome these problems has focused on neuropharmacological experiments which address the molecular bases of olfactory PER conditioning (Felsenberg et al., 2011). Here we provide a broader approach and we detail and discuss all the procedural steps required for efficient olfactory PER conditioning. We focus on absolute conditioning in which a single odorant is paired with sucrose solution, a case of olfactory PER conditioning, which can be established in the laboratory with minimal investment.

## 2. Materials and methods

### 2.1. Insects

An explicit control of the bee caste used for conditioning is recommended because foragers are the individuals that exhibit the highest appetitive motivation for sucrose within the hive (Scheiner et al., 2001) and that are, therefore, more appropriate for appetitive olfactory conditioning. Capture of bees departing from the hive in the morning or late afternoon (avoiding mid-day times when young bees perform their first orientation flights) enhances the probability of obtaining empty foragers for experiments. Empty foragers (i.e. with empty crop) are necessary to ensure highest appetitive motivation for the experiments.

When possible, a better alternative is placing an artificial feeder a few meters away from the hive and training bees to collect food on it, which ensures that real foragers can be regularly captured. Another possibility is to capture returning pollen foragers, which can be recognized easily by their filled pollen baskets. Pollen foragers display a high motivation for sucrose and learn, therefore, very well in appetitive conditioning protocols (Scheiner et al.,

2004). To capture them, a wire mesh to block the hive entrance and glass vials to catch individual bees can be used. In any case, the procedure sometimes mentioned of capturing bees *within* the hive has to be avoided as it confounds potential foragers with other castes, like nurses and guards which may not exhibit an equally significant appetitive motivation.

### 2.2. Catching bees

A pyramid (height 24.5 cm, apex 3.5 cm × 3.5 cm, base 18 cm × 18 cm) made of UV-translucent Plexiglas is useful to catch bees when they depart from the hive and fly toward the sky. The Plexiglas has to be UV-transparent, in order to offer a complete view of celestial cues and thus lure the departing bees into the pyramid (Fig. 1b). The pyramid is closable at the apex and at the base. For catching bees, the pyramid is held at a frontal distance of about 10–20 cm from the hive entrance, with the base open and the apex closed. In doing this, abrupt movements and standing directly in front of the hive entrance (instead of laterally) should be avoided to prevent arousing guard bees. When enough bees have been caught, the base is closed and the pyramid is taken to the laboratory.

In the laboratory, the pyramid is darkened (except for its apex), for instance by placing thick cloth over it. Because of their positive phototactic behavior, bees will then tend to leave the pyramid one by one through the apex. They can thus be individually captured into glass vials.

Vials are then placed in crushed ice as long as it is necessary to render the bees motionless (usually between 3 and 5 min) so that they can be harnessed individually. Cooling time should be kept to a minimum as extended cooling could impair learning performances (Frost et al., 2011) and survival in the harness.

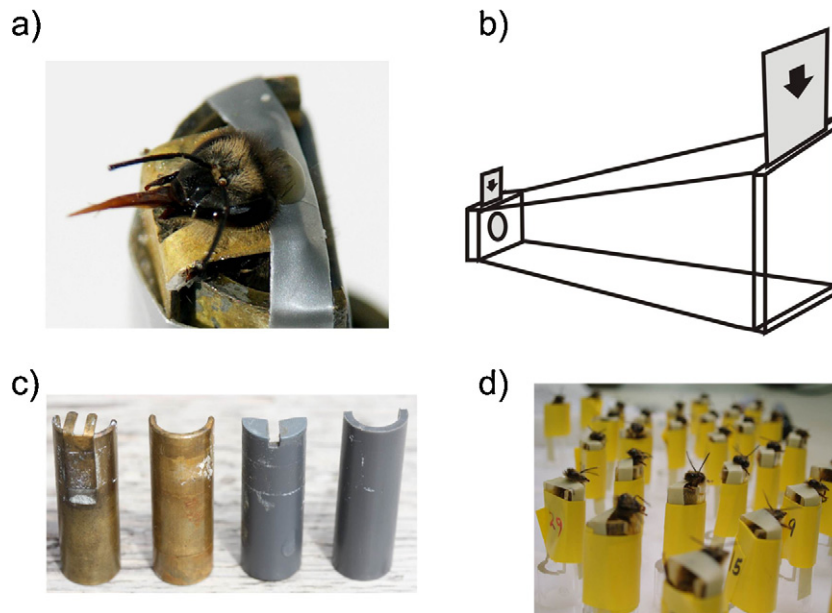
### 2.3. Harnessing bees

As soon as the bees cease their movements, they can be placed in the harnessing tubes. Harness tubes (outer diameter 10 mm, inner diameter 8 mm, height 32 mm) can be made of metal, plastic, acrylic, etc. (Fig. 1c). Plastic tubes are preferable since they drag less temperature from the harnessed bee than metal tubes. Each bee is fixed within a tube using a piece of adhesive tape placed at the level of the neck, the rest of the body being concealed within the tube. Low-temperature melting wax can also be used to further immobilize the posterior part of the head. Once fixed in the tube, the bee should only be able to freely move its mouthparts and antennae, thus hiding other body parts from possible contacts with sucrose stimulation. The forelegs of the bees, for instance, should not be able to move freely but should remain enclosed within the tube to avoid interference with olfactory and sucrose stimulation. A U-shaped support for the head could be fixed onto the upper ring of the tube, and a slit in the tube could be cut such that the proboscis does not get stuck (Fig. 1a and c).

Each harness tube should be numbered to allow individual identification of the bees throughout the experiment. A rack with numbered boreholes is useful for handling and identifying harnessed bees (Fig. 1d).

### 2.4. Feeding bees

Harnessed bees should be fed a drop (~5 µl) of sucrose solution approximately 30 min after fixation to avoid excessive starvation and subsequent mortality before the start of the experiment. Sucrose solution (usually 50%, weight/weight, i.e. 1.80 M) is usually used to this end and as the US in conditioning trials. The choice of sucrose concentration is crucial for the experimental success. Diluted sucrose concentrations (below 15–20%, i.e. 0.45–0.63 M)



**Fig. 1.** (a) The proboscis extension response (PER) of a harnessed honey bee in response to antennal stimulation with sucrose solution. (b) Pyramid (lateral perspective) for capturing bees at the hive entrance and transporting them to the laboratory. Arrows indicate sliding doors. (c) Different models of harnessing tubes; two of them (left) are made of copper, the other two (right) from PVC. Note the supports for stabilizing the neck of the bee on the leftmost version and the slit cut in the PVC version (third from left to right) to avoid that the proboscis gets stuck in the tube and (d) Numbered bees in a rack.

are suboptimal for the appetitive motivation of the bees while highly concentrated solutions (i.e. >60% or 2.26 M) are also suboptimal due to their high viscosity, which renders ingestion through the proboscis difficult (Farina and Núñez, 1991). Thus, concentration in the range of 30–50% (1.00–1.80 M) should be used in the experiments.

Different methods have been used to deliver sucrose solution to a harnessed bee. Two basic options can be retained here, which vary in their accessibility and simplicity: a low-cost one, which consists in presenting a toothpick soaked in sucrose solution to the bee, and a more sophisticated one, which consists in delivering sucrose solution by means of a micropipette or a syringe calibrated to deliver small volumes (e.g. 0.2  $\mu$ l increments). The latter has the advantage of allowing precise control of volumes ingested.

When sucrose solution touches the antennae of a hungry bee, PER will be elicited and the bee will lick the solution with its proboscis. Bees that do not show PER are either satiated or in poor physical condition. Such bees should be removed from the experiment due to the lack of appetitive motivation and unconditioned response. Also, the lack of response may be due to the proboscis being stuck in the tube if the bee was not fixed appropriately.

Capturing the bees in the evening of the previous day is the preferable procedure. Captured, harnessed bees should be fed to satiation (i.e. until they no longer respond with PER upon stimulation with sucrose solution, or – if ingested volumes can be measured – until reaching the maximal crop capacity which in the case of European bees is between 50 and 60  $\mu$ l (Núñez, 1966)) and kept overnight in a dark, humid container at a temperature around 20 °C. This procedure has the advantage of habituating the bees to the restrained condition of the tubes and of standardizing their level of satiation. It should therefore be chosen whenever it will be possible. Alternatively, bees can be caught early in the morning of the experiment's day. In this case, if conditioning starts only 3–4 h after capture, feeding may be unnecessary; if however, conditioning starts in the afternoon, a drop (~5  $\mu$ l) of sucrose solution is fed approximately 30 min after fixation (i.e. when all bees have been fixed) to avoid excessive starvation and resulting mortality before the start of the experiments.

It should be kept in mind that these are general rules of thumb, which should be adapted depending on the season, local conditions, etc. Feeding will reduce appetitive motivation and thus unconditioned responses (PER) to sucrose. Yet, potentially harmful procedures such as squeezing the abdomen in order to empty the crop should be avoided as other internal organs may also be damaged by this procedure. Therefore, a good balance should be kept between starvation and feeding, to keep bees with good appetitive motivation and sufficient vitality.

### 2.5. Odorants and olfactory stimulation

Even for the simplest case of absolute conditioning presented here, at least two odors are necessary. To control for odor-specific biases in conditioning and retention, it is recommended to run two groups in parallel, each one trained with a different odor as the CS. In the experiments presented here, two groups of bees are conditioned, each to one of the two odors (here 1-nonanol and 2-hexanol) used as CSs. These two odors are well distinguished from each other and are easily associated with sucrose solution (Guerrieri et al., 2005b).

Using two odors has the additional advantage of allowing a test of memory specificity after conditioning: each bee trained with one odor (say CS1) is tested afterwards with two odors, the conditioned odor (CS1) and the unknown, novel odor (the CS2 used in the other group, henceforth 'NOD'). In this way, it is possible to distinguish memories that are odor-specific (CS-specific) and only evoked by the CS1 from unspecific PER responses which are also elicited by the NOD. The difference between these two response categories (responses to the CS and to the NOD) estimates the CS-specific memory. As mentioned above, to balance the effect of the two odorants chosen in the experiments, half of the bees should be conditioned with the CS1 and the other half with the CS2.

As for sucrose delivery (see above), different options can be used to deliver the odors to a harnessed bees. Two basic options will be mentioned here; one which represents a low-cost method,

accessible to everyone, and another one which constitutes a more sophisticated method allowing better control of the olfactory stimulus. The first option relies on a simple plastic syringe containing a filter paper impregnated with the odorant to be conditioned. The second option implies achieving olfactory stimulation by means of computer-controlled odor stimulation devices, which allow efficient control of temporal properties of the odor stimulus. Here we will focus on the syringe method of olfactory stimulation to favor spreading of PER conditioning as a low-cost, accessible procedure.

Two 20 ml syringes, each containing a piece of filter paper (10 mm × 30 mm) soaked with 5  $\mu$ l of odorant (2-hexanol in syringe 1 and 1-nonanol in syringe 2) are used in the experiments presented here. If syringes are chosen for odor stimulation, then using two odors in any absolute conditioning experiment is mandatory, even if just one odor is conditioned in such protocol. The mechanic stimulation of the air puff could act as a confounding CS in conditioning trials, i.e. bees could learn the air puff instead of the odorant. The test with the NOd is therefore of fundamental importance to verify that such mechanic stimulation is not driving the bee's responses alone, in which case PER will be specific to the learned odor. Using laboratory gloves during syringe preparation is important to avoid odor residuals on the experimenter's hands during the experiments.

## 2.6. Odor removal

When carrying out olfactory PER conditioning, odors presented to the bees have to be exhausted from the experimental site as soon as the stimulation ends. Therefore, a ventilation hood ('exhaust') should be placed at the experimental site, with the other end connected to a standard air extraction. During conditioning and test trials, honey bees are set individually in front of the odor stimulation device, with the exhaust at their back. Ventilation should not be too strong to avoid unintended mechanical stimulation.

## 2.7. Mixing odor (CS) and sucrose solution (US) should be avoided

A deviation of the standard procedure is the mixing of the odor substance with the sucrose solution (Rogers and Vallortigara, 2008; Anfora et al., 2010; Frasnelli et al., 2010). This procedure goes against the original experimental design of having separate and controllable CS and US stimulations as desired in Pavlovian conditioning designs (Pavlov, 1927). It is inappropriate because there is no control by the experimenter of olfactory stimulus onset and offset. At some undefined point, when the syringe comes closer to the bee (without any possibility to know what 'closer' means) the odor will be perceived. Thus, fundamental parameters which affect memory formation in a dramatic way, such as the inter-stimulus interval (ISI), i.e. the separation between CS onset and US onset (here sucrose delivery), can never be controlled in this design. Typically, for optimal learning the CS should precede the US by 1–5 s (Menzel, 1990 – see below), which is not possible when mixing the CS and the US. Furthermore, this mixing does not allow performing an elemental control of associative learning (see below), the unpaired control, because no separate presentation of CS and US is possible. Furthermore, as the odor remains around the bee for some undefined period after the animals got the sucrose reward because it is in the syringe that is retreated from the bee after US delivery, this form of conditioning may induce inhibitory learning in honey bees, resulting from the CS being perceived after the US (Hellstern et al., 1998) or may result in the CS losing part of its anticipatory power with respect of the US. Lastly, sucrose containing odor will accumulate in the social stomach and gut of the bee

providing an uncontrolled odor stimulus outlasting odor stimulation and possibly adapting the animal to the odor stimulus.

## 2.8. Absolute conditioning

In the present study, we focus on absolute conditioning, which is a simple form of associative conditioning in which an animal has to learn that a single CS (odor) predicts a US (sucrose solution).

As explained above, we use one conditioning odor (CS, either 1-nonanol or 2-hexanol) and an additional odor in memory retention tests (the novel odor or NOd; 1-nonanol for bees conditioned with 2-hexanol and vice versa). For multiple-trial conditioning, parameters such as the number of trials and the duration of the intertrial interval (ITI) can be varied according to the purpose of the experiment. In the present study, we perform the standard procedure of absolute conditioning with 5 CS–US pairings with an average ITI of 10 min (Fig. 2a). This procedure yields a robust and stable long-term memory that can be retrieved several days after conditioning (>4 days) and that is dependent on protein synthesis (Menzel, 1999).

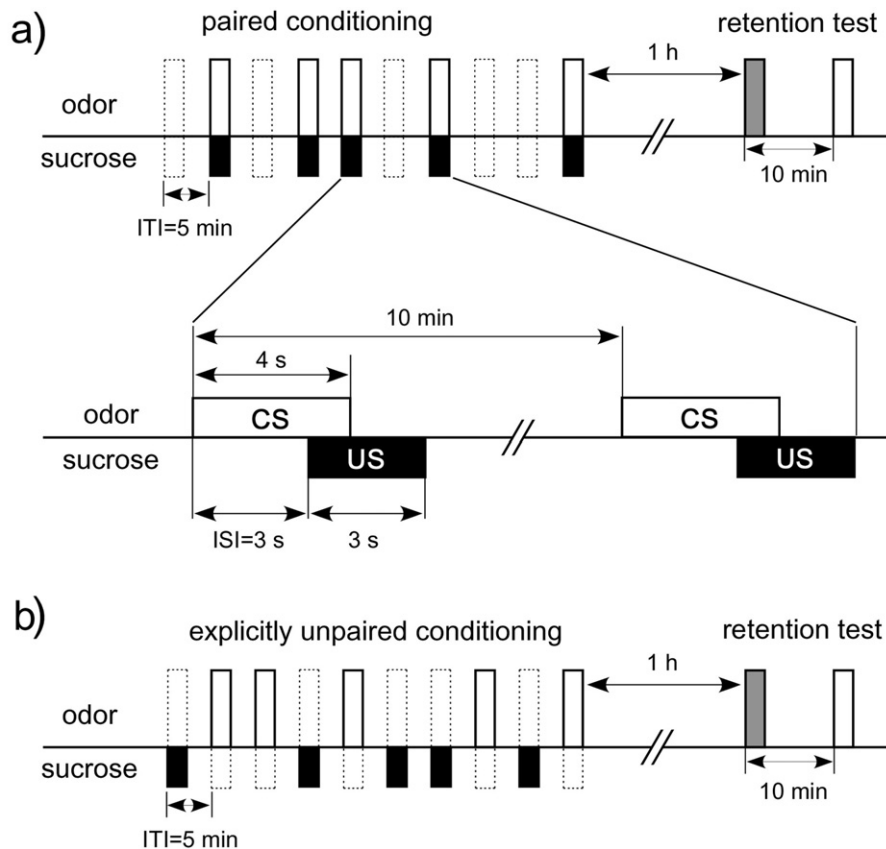
The first honey bee is set in the conditioning place in front of the exhaust and is left there for at least 25 s before applying any stimulation, to familiarize it with the contextual situation and temporally uncouple visual and possibly mechanosensory (from the exhaust pipe) from the conditioning procedure. The bee is then presented with a 4-s odor stimulation (CS) and a subsequent 3-s sucrose stimulation (US) with an interstimulus interval (ISI) of 3 s (1 s overlap). This ISI is preferred as it yields robust retention performances (Menzel, 1990). Controlling the ISI is extremely important as seemingly small variations in the relationship between CS and US can yield vastly different conditioning and retention results.

After CS–US pairing, the bee is left in the conditioning place for other 25 s. This is important, because during this period, the contextual cues around the setup will lose any anticipating, predictive link with the US. The whole trial lasts therefore 1 min. Shorter trials (e.g. 30 s) can be used taking care of balancing the pre- and post CS–US pairing periods. In this case these periods may last 12 s each.

The presence (+) or absence (–) of PER in the 3 s between odor onset and sucrose delivery (conditioned response to the odor) is recorded. If the bee responds with PER to the odor, the antennae and then the proboscis are touched with the toothpick soaked in sucrose solution and the bee is allowed to drink for 3 s. If no PER to the odor occurs, the same compound US is delivered, i.e. first to the antennae and then to the extended proboscis. Touching the antennae and the proboscis with sucrose is important because such compound US yields significantly better acquisition and retention performances (Bitterman et al., 1983).

Once the trial is finished, the bee is removed from the experimental setup and the next bee is installed to start a new conditioning trial. If a trial length of 30 s is chosen, conditioning 20 bees in a row ensures an ITI of 10 min from the 1st to the 2nd trial. If trial length is 1 min, conditioning 10 bees in a row ensures an ITI of 10 min. In all cases, the experimenter should use a laboratory timer or clock to ensure a well controlled ITI in all bees. The conditioning procedure presented here contains five trials per bee and thus lasts 50 min.

Several kinds of experimental controls can be performed in parallel to the *paired group* described above. One of them is the *explicitly unpaired group* (Lavond and Steinmetz, 2003), which has to include a number of bees equivalent to that used in the paired group. The results of both groups, always run in parallel, are compared to determine whether increases in conditioned responses in the absolute-conditioning group are the consequence of real associative learning. In the explicitly unpaired group, bees receive unpaired presentations of the CS and of the US (5 odor-only and 5 sucrose-only presentations in a pseudo-randomized sequence;



**Fig. 2.** Experimental procedure of absolute olfactory classical PER conditioning. (a) Paired group: conditioning consists of a pairing of a 4-s odor stimulation (CS: white bars) and a subsequent 3-s stimulation of sucrose solution (US: black rectangles) with 1 s overlap (ISI=3 s). Bees were conditioned along 5 trials of paired CS-US presentations, with an average ITI of 10 min between CS presentations, and with interspersed blank trials (ITI of 5 min) used to equate placements in the experimental setup with respect to the control group (explicitly unpaired group). Retention tests were performed 1 h after the last conditioning trial and consisted of presentations of the CS (white bar) and of a novel odor (gray bar), both without US and (b) Explicitly unpaired group: bees received explicitly unpaired presentations of the CS and of the US (5 odor-only and 5 sucrose-only presentations, 5 min apart in a pseudo-randomized sequence). Thus, this group has exactly the same sensory experience as the paired group but has no possibility of establishing associative links between CS and US. The average ITI between CS presentations is also 10 min.

Fig. 2b). Thus, both the paired and the explicitly unpaired groups have exactly the same sensory experience (5 CS and 5 US presentations), the difference being in the pairing or absence of pairing between odor and sucrose.

An additional factor that needs to be controlled for is the fact that the number of placements in the setup is twice as high (if not controlled) in the explicitly unpaired group as in the paired group. This can be corrected by inserting 5 blank trials between the conditioning trials in the paired group (Fig. 2a). In such blank trials, the bee is simply placed in the setup, and remains there for 30 s without any stimulation. In doing so, the average ITI between two CS presentations has to be equated between the paired and the explicitly unpaired group. In Fig. 2, for instance, the ITI between CS trials is variable (15, 10, 5 and 10 min) yet its mean value is 10 min for both groups.

### 2.9. Memory retention tests

Memory retention is assessed by presenting two odors to the trained bees: the conditioned odor (CS) and the novel odor (NOD) delivered by two different odor syringes. The timing of retention tests is identical to that of conditioning trials, with the only difference that no US is delivered. The order of presentations of the two odors should be randomized between bees to avoid sequential effects. Thus, half of the subjects are tested with the CS first and the NOD second, while the other half is tested with the reversed

sequence. As for conditioning, the ITI between odor stimulations is 10 min.

After the last odor stimulation performed in the retention test, the unconditioned response (PER to sucrose) is tested in all animals by applying sucrose solution to the antennae. Bees that do not show the unconditioned response have to be discarded from the data, as their lack of response to the odors cannot be necessarily ascribed to a lack of memory but is most probably due to a low physical condition or problems to move the proboscis.

In the design presented here, the retention test is performed 1 h after conditioning. The lapse between the last conditioning trial and the retention test is defined by the experimenter based on the experiment's goal. When studying memory formation, a typical design involves testing bees at different post-conditioning times (e.g. 1 h, 24 h and 72 h after conditioning). In this case, independent groups of bees have to be used (and conditioned) for each time interval. Testing the same group of bees repeatedly at several retention tests may induce a process of extinction (Eisenhardt and Menzel, 2007), because bees are exposed to repeated presentations of the CS without any US, as well as fatigue, which results in progressive decrease of conditioned responses that is not related to memory dynamics.

If bees are tested several minutes to a few hours after conditioning, they are kept in their harnessing tubes in a dark, humid container at room temperature until the test to maintain their physical condition. Bees tested 1 or a few days after conditioning can also

be kept in the harnessing tubes because mortality is usually low in these conditions; yet, bees should be fed to satiation with 50% sucrose solution, in the late afternoon at least after an interval of 60 min after the end of conditioning. Feeding should be performed under conditions as different as possible from the training or testing conditions (e.g. bees should not be removed from the rack contrarily to what occurs during conditioning and testing). If the bees need to be kept for longer periods of time (one or a few weeks), one can also try keeping them in tubes but mortality can be high in such harnessing conditions. One possible way to ensure survival until very late retention tests is to release the bees from the harnessing tubes and keep them in a small cage where they can move and socially interact. To this end, bees are individually identified by means of color marks painted on the thorax with watercolors, following a code that allows later recognition. Bees are then placed in groups in small cages (e.g. 65 mm × 70 mm × 25 mm) supplied with water and a diet of 50% sucrose and 50% honey mixture ad libitum. The cages should be kept in a dark and humid container at room temperature. On the morning of the test day, bees are transferred from the cages into glass vials, cooled on ice and placed again individually in the harness tubes. Retrieval tests are usually performed after 5 h of food deprivation to ensure adequate appetitive motivation.

## 2.10. Spontaneous responses

Although the definition of a conditioned stimulus is that it should be neutral at the beginning of conditioning (Pavlov, 1927), sometimes bees may respond to the odorant in the first conditioning trial before sucrose delivery. These responses are usually termed 'spontaneous responses' and may be due to prior experience with the odorant, either in the field (Gerber et al., 1996) or in the hive (Arenas et al., 2007; Farina et al., 2007), or to enhanced appetitive motivation triggering PER to neutral stimuli (Harris and Woodring, 1992). The question of whether bees exhibiting spontaneous responses have to be discarded from the analyses can be answered from two different viewpoints: on one hand, one might discard these bees arguing that the strict definition of a CS requires that bees do not respond to the odor in the first conditioning trial; on the other hand, one might keep these bees considering that even if the CS is not strictly neutral to them, it is an important information to provide an estimation of the population's spontaneous tendency to respond to the odorant. Yet, if most of the bees exhibit such spontaneous responses, a change in the odorant chosen as CS is recommended.

## 2.11. Data analysis

The occurrence of PER to the odorant (conditioned response) has to be measured during conditioning trials and retention tests. The basic procedure consists in recording whether or not a bee extends its proboscis after onset of the odor (CS) and before presentation of the sucrose solution (US) in the case of reinforced trials, so that the anticipatory response recorded could only have been evoked by the CS. Multiple responses during a CS have to be counted as a single PER. Extension of the proboscis beyond a virtual line between the open mandibles is counted as PER.

Besides simple counting of PER occurrence as dichotomous response (response or absence of it), it is also possible to achieve quantification of behavior in a continuous scale by videorecording the bee from above during conditioning/retention and offline digitizing videotapes in order to dispose from data on PER duration, latency, etc. Obtaining these measures is time-consuming but may be more sensitive to differences across treatments in certain circumstances (Hosler and Smith, 2000).

For each conditioning trial, the percentage of conditioned responses (% of CR) is calculated as the number of bees showing

PER to the conditioned odor with respect to the total number of bees assayed. Percent data do not give an idea about the reliability of the CR estimated. To indicate such reliability, 95% confidence intervals may be computed and represented both for acquisition and retention performances.

All experiments are performed with two odors, 1-nonanol and 2-hexanol, in a balanced protocol (see above). When examining the effect of the odor used as CS, the null hypothesis is that there are no significant differences between groups in their acquisition/retention levels. The same null hypothesis applies to the comparison of acquisition/retention levels between explicitly unpaired and paired groups. When examining acquisition within each group, the null hypothesis is that there are no significant differences in conditioned responses to the CS along conditioning trials; for retention tests, the null hypothesis is that there are no significant differences between CS and NOD responses.

### 2.11.1. Acquisition

Acquisition performance within each group can be tested using Cochran's *Q* test, which is a non-parametric test applied to the analysis of randomized block designs (repeated measurements) with a binary response variable (1/0 as in PER measurements).

To compare acquisition performances between two groups (e.g. paired vs. explicitly unpaired groups, see above), Mann-Whitney *U* tests can be used on the sum of responses to the CS observed during conditioning [so, in the case of 5 conditioning trials, each bee has a score between 0 (no response) and 5 (responses at all trials)]. If more than two groups are to be compared for the same variable (sum of CS responses during conditioning), then a Kruskal-Wallis test can be used. Yet, both methods have the disadvantage of losing the dynamics of acquisition as they both reduce it to a single data point (the sum of CS responses). A similar criticism applies to the use of Fisher's exact tests and/or  $\chi^2$  tests to compare the summed CS responses between groups. Yet, as always in statistics, the test chosen may be appropriate depending on the question raised. For instance, one may want to focus only on the last acquisition trial as a measure of acquisition and compare between groups the level of correct responses; in this case, Fisher's exact tests and/or  $\chi^2$  tests may be appropriate.

Cochran's *Q* test cannot be used for comparing acquisition curves between groups as it is a within-group test. Thus, repeated-measures analyses applicable for between-group comparisons have to be used. A solution to this problem is the use of a standard two-factor analysis of variance (ANOVA) for repeated measurements, with one factor being the *treatment* to be analyzed (e.g. paired vs. unpaired) and the other factor being the response along *trials* (e.g. trials 1–5). The interaction between the two factors is also computed, which allows detecting specific *treatment* × *trial* effects. ANOVA procedures are in principle not applicable in the case of binary response variables; yet Monte Carlo studies have shown that it is possible to use ANOVA for this kind of data if comparisons imply equal cell frequencies and at least 40° of freedom of the error term (Lunney, 1970). By fulfilling these conditions, the use of repeated-measurement ANOVA allows not only between-group comparisons but also within-group analysis as achieved by the Cochran test. Alternatively, a Generalized Linear Mixed Model (GLMM) (Baayen et al., 2008) can be used with binomial error structure and logit link function, including trial number, conditioning group (paired or unpaired) and conditioned stimulus as fixed factors.

In any case, large sample sizes (40–50 bees per group) that ensure statistical power are mandatory. Even if the ANOVA procedure is robust enough to tolerate smaller sample sizes, working with around 10 individuals per group (or even less), which has often been done in past but also in some present studies, should definitely be avoided.

### 2.11.2. Retention

The difference in response levels to the CS and to the NOd is evaluated by means of a McNemar test, which is applicable for paired-sample testing of binomial response variables. Differences in CS or NOd responses between groups are assessed using  $\chi^2$  or Fisher's exact tests.

As we systematically tested the CS and the NOd, we also compared memory specificity for the CS between groups. We calculated the proportion of individuals responding to the CS and not to the novel odor ('Specific Memory'; see Hourcade et al. (2009)). This proportion is compared between groups using  $\chi^2$  or Fisher's exact tests.

## 3. Results

Two groups of bees were conditioned in an explicitly paired manner, either with 1-nonanol ( $n=46$ ) or with 2-hexanol as CS ( $n=46$ ). Both groups did neither differ in acquisition (ANOVA for repeated measurements; factor odorant:  $F_{1,90}=0.01$ ,  $P=0.92$ ) nor in retention performances ( $F_{1,90}=0.23$ ,  $P=0.64$ ) so that the data could be pooled and presented as a single paired group (Fig. 3; white circles, white bars). Similarly, the two groups of bees conditioned in an explicitly unpaired manner either with 1-nonanol ( $n=51$ ) or with 2-hexanol ( $n=47$ ) did neither differ in acquisition ( $F_{1,96}=0.14$ ,  $P=0.70$ ) nor in retention ( $F_{1,96}=0.79$ ,  $P=0.38$ ) so that data were also pooled and presented as a single explicitly unpaired group (Fig. 3; black circles, black bars).

### 3.1. Acquisition

Fig. 3a shows the acquisition (% PER to the CS) of both groups. Bees from the paired group increased their responses to the CS during trials (repeated measure ANOVA  $group \times trial$ ,  $trial$  effect,  $F_{4,364}=119.38$ ,  $P<0.0001$ ), while bees from the explicitly unpaired group did not show such increase ( $F_{4,388}=1.97$ ,  $P=0.10$ ), thus showing the associative nature of performance variation in the paired group. There was a significant difference in acquisition between both groups (repeated measure ANOVA  $group \times trial$ ,  $group$  effect,  $F_{1,188}=449.13$ ,  $P<0.0001$ ) and the  $group \times trial$  interaction was also significant, thus underlining the different response patterns of both groups during conditioning (repeated measure ANOVA  $group \times trial$ ,  $interaction$  effect  $F_{4,752}=82.47$ ,  $P<0.0001$ ).

### 3.2. Retention

Fig. 3b shows retention performances 1 h after conditioning (% of PER both to the learned odor, CS, and to the novel odor, NOd) for both the paired (white bars) and the unpaired group (black bars). While the paired group exhibited significantly higher responses to the CS than to the NOd (McNemar test:  $\chi^2=52.02$ ,  $P<0.0001$ ), revealing the presence of a CS-specific memory, the explicitly unpaired group responded neither to the CS nor to the NOd ( $\chi^2=0.00$ ,  $P=1$ ). We thus conclude that only the paired group formed an olfactory memory that was specific for the CS.

### 3.3. CS-specific memory

CS-specific memory can be compared between both groups by computing the percentage of individuals responding to the CS and not to the NOd. Fig. 3c shows the level of CS specific memory within each group. There was a highly significant difference between the explicitly unpaired and the paired group ( $\chi^2=73.27$ ;  $df: 1$ ;  $P<0.0001$ ), thus showing that only the latter formed a CS-specific memory.

## 4. Discussion

### 4.1. Conditioning variants

In the present study, we have focused on absolute conditioning with 5 conditioning trials separated by an ITI of 10 min. This is a simple form of conditioning in which bees have to learn that a single odorant predicts the outcome of sucrose reward. Despite its apparent simplicity, we have shown that control groups are required which demand a thoughtful conception. For instance, the use of an explicitly unpaired group as control leads to a modification of the paired group, reflected in the introduction of blank trials necessary to equate the number of placements in the conditioning setup. Another control for the absolute conditioning procedure is the backward-conditioning group in which bees experience the same numbers of CS and US presentations as in the paired group but in all cases the US antecedes the CS so that theoretically the CS cannot predict the US. Yet caution is necessary in the case of backward conditioning as inhibitory learning (i.e. the CS is no longer neutral but inhibitory) can be induced depending on the ISI (Hellstern et al., 1998). This again underlines the importance of parameters such as the ISI, the ITI, the number of conditioning trials and the intensities of CS and US, whose importance we have detailed in Section 2.

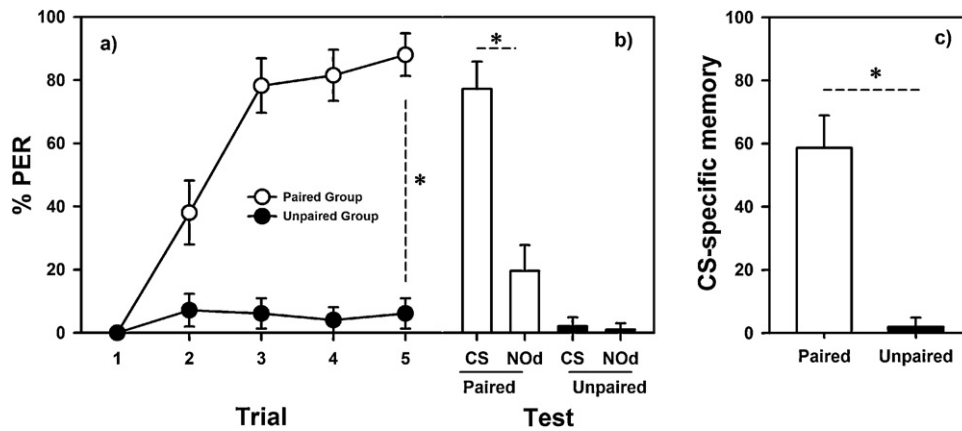
Several conditioning protocols exist, which are variants of the simple case presented here. Their use depends on the specific questions raised by the experimenter and in all cases the basic principles detailed here should also apply. A typical example is the differential conditioning of PER (Bitterman et al., 1983), which is widely used as it has the advantage of providing a within-group control of the associative nature of bees' performance. In differential PER conditioning, a bee has to learn to respond to a rewarded odorant (CS<sup>+</sup>) but not to a non-rewarded odorant (CS<sup>-</sup>). Each bee acts therefore as its own control, as different responses are expected to two odorants differing in their outcome.

Differential conditioning may be used to evaluate the bees' capacities to discriminate between odorants. It is therefore useful for the analysis of perceptual problems. Note, however, that depending on the conditioning protocol adopted, distinct perceptual performances can be induced. For instance, while in absolute conditioning bees are trained to respond to a unique odorant, without discriminative requirements being implicit in the training, differential conditioning may enhance discrimination capabilities as bees are explicitly trained to discriminate between stimuli. Perceptual measures derived from one protocol or the other may, therefore, differ.

Many PER conditioning variants have been used to study perception, learning and memory in bees: blocking (Smith and Cobey, 1994; Hosler and Smith, 2000; Guerrieri et al., 2005a), overshadowing (Smith, 1998), second-order conditioning (Hussaini et al., 2007), reversal learning (Komischke et al., 2002; Devaud et al., 2007; Mota and Giurfa, 2010), sensory preconditioning (Müller et al., 2000), latent inhibition (Chandra et al., 2000, 2010; Fernández et al., 2009), and negative and positive patterning (Deisig et al., 2001, 2002, 2003) are just some examples of a battery of protocols that have been adapted to bees and that implied establishing appropriate conditioning schedules and controls. Despite the apparent complexity of some of these protocols, we are confident that the basic procedures explained in this article will help researchers to perform these protocols and design still other variants of PER conditioning.

### 4.2. Environmental factors

Other factors such as circannual, circadian, environmental conditions (e.g. weather, season, etc.) and foraging duration may also influence PER conditioning and olfactory memory formation (Scheiner et al., 2003; Hadar and Menzel, 2010). Weather



**Fig. 3.** Absolute olfactory PER conditioning of honey bees. (a) Acquisition curves (% of PER to the CS) of honey bees of the paired group (white circles) and of the explicitly unpaired group (black circles); 95% confidence intervals are shown. (b) Retention performances (right panel) of the paired (white bars) and the explicitly unpaired group (black bars) 1 h after conditioning. Bars represent % of PER to the CS and to the novel odor (NOd); 95% confidence intervals are shown and (c) CS-specific memory (% of bees responding to the CS and not to the NOd) in the paired and the explicitly unpaired groups; 95% confidence intervals are shown. \* $P < 0.05$ .

conditions may change within a season or between consecutive days and will affect PER conditioning performance. A given experiment should thus be performed over several days to minimize daily effects, and most importantly, all groups to be compared need to be run in parallel over the whole period of data collection. In this way, performances in the different experimental groups will be equally affected by environmental variations thus excluding this confounding variable.

#### 4.3. Interpreting the results

Given the dichotomous nature of the response measured (PER: 1 or 0), the gradually changing acquisition curve (Fig. 3a) does not reflect the stepwise nature of individual bee responses (Pamir et al., 2011); yet it provides a basis to assess learning at the population level and to promote analyses of individual performances with respect of group ones. Indeed, the learning curve presented in Fig. 3a may hide inter-individual differences in learning rate, related, for instance, to the fact that the population of bees used may be heterogeneous in terms of their sensitivity to the sucrose used as US (Scheiner et al., 1999, 2004, 2005). Thus, besides considering conditioned responses at a group level, it may be interesting to carry separate analyses at a subgroup level, pooling individuals that exhibit statistically similar response patterns. Two recent studies, one using olfactory PER conditioning (Rath et al., 2011) and another using an aversive protocol (Roussel et al., 2010), the olfactory conditioning of the sting extension response (SER) (Vergoz et al., 2007; Giurfa et al., 2009) have applied this method to individuals that were treated identically and that exhibited nevertheless different learning successes ('learners' and 'non-learners'). Both studies analyzed whether these subgroups of bees exhibited different levels of neural activation in the antennal lobe, the primary olfactory center in the insect brain, in order to relate neural activation and learning success. This example shows how consideration of inter-individual differences in acquisition, which may be hidden by a global acquisition curve, may reveal interesting phenomena at a cellular level.

## 5. Conclusion

Olfactory PER conditioning in bees is a well-established protocol originally conceived for the study of learning and memory (Takeda, 1961), which has repeatedly proven its value over the 50 years since it was conceived (Giurfa and Sandoz, 2012). Its versatility has promoted its use in areas as diverse as psychological, neurobiological and ecological studies, as well as in applied research on pesticides,

among others. Yet, although the protocol was standardized 30 years ago (Bitterman et al., 1983), deviations from the original procedures have appeared, which have added confusion and contradictory results. We decided therefore to revisit olfactory PER conditioning and detail to an extent that admits no doubts about the procedures, the methodology underlying this conditioning protocol. A previous attempt, which focused on the neuropharmacological study of learning and memory combining PER conditioning and drug injections in the bee nervous system, was certainly inspiring (Felsenberg et al., 2011) but remained specific to the neuropharmacological approach. Here, we aimed to offer a more general analysis of all the steps of the behavioral procedure in order to make a standardized procedure accessible to researchers from diverse scientific fields. We hope that scientists who are unfamiliar (or partially familiar) with this protocol will become attracted to its easiness and robustness, and will therefore join the efforts toward novel breakthroughs by means of olfactory PER conditioning.

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