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RESEARCH ARTICLE

Visual conditioning of the sting extension reflex in harnessed honeybees

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SUMMARY

Visual performances of honeybees have been extensively studied using free-flying individuals trained to choose visual stimuli paired with sucrose reward. By contrast, harnessed bees in the laboratory were not thought to be capable of learning a Pavlovian association between a visual stimulus (CS) and sucrose reward (US). For reasons as yet unknown, harnessed bees only learn visual cues in association with sucrose if their antennae are ablated. However, slow acquisition and low retention performances are obtained in this case. Here, we established a novel visual conditioning protocol, which allows studying visual learning and memory in intact harnessed bees in the laboratory. This protocol consists of conditioning the sting extension reflex (SER) by pairing a visual stimulus (CS+) with an electric shock punishment (US), and a different visual stimulus (CS–) with the absence of shock. Bees with intact antennae learned the discrimination between CS+ and CS– by using chromatic cues, achromatic cues or both. Antennae ablation was not only unnecessary for learning to occur but it even impaired visual SER conditioning because of a concomitant reduction of responsiveness to the electric shock. We thus established the first visual conditioning protocol on harnessed honeybees that does not require injuring the experimental subjects. This novel experimental approach opens new doors for accessing the neural correlates of visual learning and memory in honeybees.

Key words: vision, learning, memory, conditioning, sting extension reflex, honeybee.

INTRODUCTION

Honeybees represent an attractive model for studying the perception, learning and memorization of visual cues by a relatively simple brain (Menzel and Giurfa, 2001; Giurfa, 2007). Visual cues are detected by compound eyes made of ~5400 ommatidia, which host nine photoreceptor cells. Three types of photoreceptors, S, M and L (for short-, mid- and long-range wavelength, respectively), peaking in the UV, blue and green regions of the spectrum, respectively, have been identified in the honeybee retina (Peitsch et al., 1992; Wakakuwa et al., 2005). Bees possess trichromatic color vision (Daumer, 1956) in addition to achromatic vision, which is mediated by the L-photoreceptor type and is mainly used for motion perception and detection of distant objects (Giurfa et al., 1996; Giurfa et al., 1997).

Vision in honeybees has been extensively studied using behavioral experiments that exploit the fact that free-flying bees can be easily trained to associate a visual stimulus with sucrose reward (Giurfa, 2007). Pairing a visual target with a drop of sucrose solution induces the formation of a visual memory that, depending on the number of trials, may be consolidated from labile to long-term forms (Menzel, 1968). Yet, experiments with free-flying bees neither allow a precise temporal control of visual stimulations nor grant simultaneous access to the animal's brain for investigating the neural bases of visual learning and perception (Avarguès-Weber et al., 2011). Immobilizing bees in the laboratory may solve these problems, but immobilization should in no case prevent bees from learning visual cues in a way comparable to free-flying bees in natural conditions. Studies towards this goal are rare, probably because of the difficulty of training harnessed bees with visual cues when compared with free-flying bees (Masuhr and Menzel, 1972). For this reason, the study of visual learning and memory in bees has rarely progressed beyond the behavioral level.

By contrast, studies on honeybee olfactory learning and memory span a broader spectrum, going from behavioral analyses to cellular and molecular dissections of experience-dependent olfactory plasticity (Menzel and Müller, 1996; Menzel, 1999). Such success is due to the existence of a classical conditioning protocol, the olfactory conditioning of the proboscis extension reflex (PER) (Takeda, 1961; Bitterman et al., 1983), which has been repeatedly used for the study of appetitive olfactory learning in immobilized bees in the laboratory. In this protocol, individually harnessed bees, which extend the proboscis if their antennae and/or mouthparts are contacted with sucrose solution [the unconditioned stimulus (US)], learn to associate an odorant [the conditioned stimulus (CS)] with a sucrose reward if the odorant precedes the sucrose by a few seconds. As a consequence, conditioned bees exhibit PER to presentations of the odor alone (Takeda, 1961; Bitterman et al., 1983). As bees are immobilized and yet exhibit robust learning, it has been possible to use invasive procedures to access the brain and identify cellular correlates of the CS (odor), the US (sucrose) and their association (Hammer, 1993; Hammer, 1997; Menzel, 1999; Menzel, 2001; Giurfa, 2003; Giurfa, 2007).

Attempts to develop similar visual conditioning protocols in harnessed bees have not been so successful. Kuwabara found that bees learn to associate colored lights with sucrose reward, but

learning performances were low (Kuwabara, 1957). The aspect necessary for this conditioning to work is the so far inexplicable necessity of sectioning the antennae for learning to occur (Kuwabara, 1957; Hori et al., 2006). Indeed, recent reports on color and visual motion conditioning in honeybees (Hori et al., 2006; Hori et al., 2007; Niggebrügge et al., 2009; Mota et al., 2011a) repeatedly showed that it is necessary to cut the antennae for the bees to learn the trained visual cues. Even after ablation of the antennae, learning success in these experiments was limited. In the majority of the cases, poor and slow acquisition performances were obtained (Kuwabara, 1957; Hori et al., 2006; Hori et al., 2007; Mota et al., 2011a). Furthermore, when conditioned responses were reported to be fast and to reach high levels, discrimination performances were coarse and retention performances were poor (Niggebrügge et al., 2009).

These results raise serious doubts about the validity of antennae-ablated bees as an experimental model for studying visual perception, learning and memory. Indeed, antennae ablation may have severe consequences on bees' fitness and appetitive motivation. It has been shown that sucrose responsiveness (measured *via* tarsal stimulation with sucrose solution and subsequent PER quantification) decreases dramatically when the antennae of honeybees are sectioned (de Brito Sanchez et al., 2008). This suggests that antennae ablation decreases the subjective value of the reward, probably because the bees are damaged and appetitive motivation has been partially lost. It seems, therefore, crucial to conceive visual learning protocols in which bees preserve their antennae. Despite the fact that harnessed intact bees are unable to directly associate visual stimuli with sucrose reward, they can perceive and discriminate colors as shown by the fact that colors can act as occasion setters for appropriate responding to an odor that could be either rewarded or non-rewarded (Mota et al., 2011a). Nevertheless, up to now there has been no conditioning protocol allowing conditioning behavioral responses to visual stimuli in intact harnessed honeybees.

In the present work we introduce a novel visual conditioning protocol in which intact harnessed honeybees are trained to form aversive associations between a visual CS and an electric shock as the US. We made use of a recently established protocol for the study of aversive olfactory learning and memory, the olfactory conditioning of the sting extension reflex (SER) (Vergoz et al., 2007; Carcaud et al., 2009; Giurfa et al., 2009; Roussel et al., 2009), which we adapted for the visual modality. In its original version, individually harnessed bees are exposed to pairings of neutral odors as the CS with a mild electric shock as the US. Bees, which innately extend the sting upon stimulation with the noxious electric shock (Núñez et al., 1997), learn the odor–shock association so that the odor that was originally neutral elicits the SER at the end of training. Here we subjected bees to a differential conditioning procedure, in which a visual stimulus was paired with the electric shock (CS+) and a second visual stimulus was presented without shock (CS–). We asked whether intact bees learn to discriminate different visual stimuli and whether they remember this information 1 h after conditioning. Moreover, we analyzed whether antennae ablation affects visual learning and discrimination. We aimed, therefore, at providing the first report on visual conditioning of SER in honeybees, in order to open new research avenues for controlled laboratory studies of visual learning in this insect.

MATERIALS AND METHODS

Animals

Honeybees, *Apis mellifera* Linnaeus 1758, collected from an outdoor hive were brought to the laboratory and chilled on ice for 5 min until they stopped moving. They were then harnessed on individual holders designed for aversive conditioning (Vergoz et al., 2007) (see Experimental setup) and fed 5 µl of 50% (w/w) sucrose solution. Bees were then placed in a dark chamber for 2 h to allow familiarization with the experimental situation.

Experimental setup

The holder for aversive conditioning (Vergoz et al., 2007) (Fig. 1) consisted of two brass plates fixed to a Plexiglas® plate. The petiole and the neck of the bee were tightly fitted into small notches in the brass plate. These notches, as well as the space between the two brass plates, were smeared with EEG gel (Spectra 360 Electrode Gel, Parker Laboratories, Fairfield, NJ, USA) to ensure good contact between the brass plates and the bee. A girdle of adhesive tape clamped the bee's thorax in the space between the two brass plates. Both brass plates were connected to the output of the stimulator (60 Hz AC current). The resistance measured between the brass plates in the presence of the bee was 200–300 KΩ. An air extractor placed behind the bee prevented possible contamination by pheromone release. All experiments were performed in a dark room with very weak illumination measured and adjusted to 10 lx using a luminance meter (LX-107, LUTRON Electronic, Taipei, Taiwan). Visual stimulation (see Stimuli) was provided by a

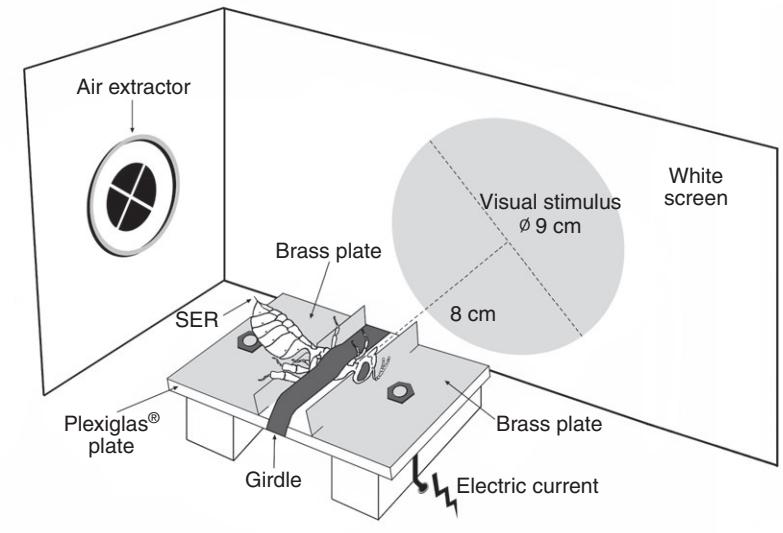

Fig. 1. Experimental setup for visual conditioning of the sting extension reflex (SER). A honeybee was individually harnessed in a holder allowing the delivery of a mild electric shock (Vergoz et al. 2007). The visual stimulus was presented on a white screen to the right eye of the harnessed bee. See Materials and methods for details.

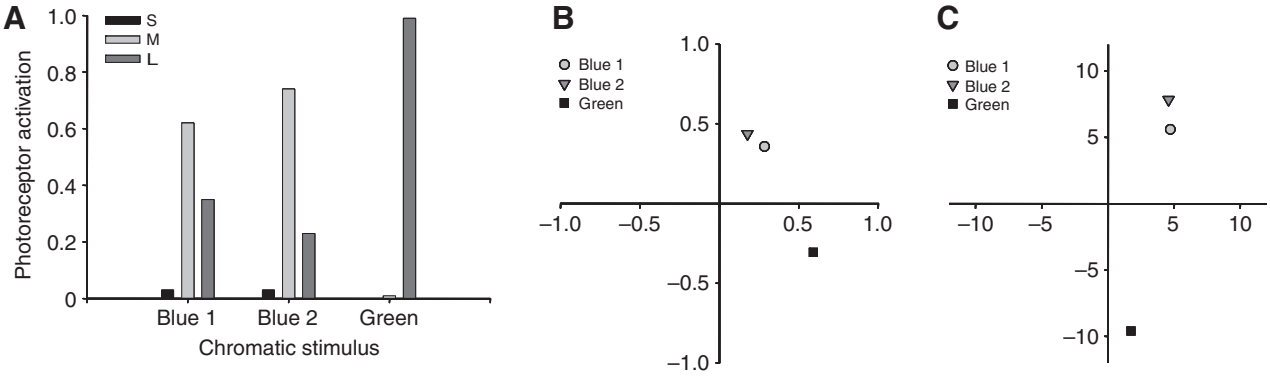

Fig. 2. Chromatic and achromatic cues of visual stimuli used in visual SER conditioning experiments. (A) Photoreceptor activation (proportion) of the short- (S), medium- (M) and long-range (L) wavelength photoreceptor types of the honeybee (Peitsch et al., 1992) as induced by Blue 1 (440 nm peak, 25 nm bandwidth), Blue 2 (439 nm peak, 10 nm bandwidth) and Green (540 nm peak, 10 nm bandwidth). (B) Loci of the three stimuli in the hexagon color space of honeybees (Chittka, 1992). (C) Loci of the three stimuli in the color opponent coding (COC) space of honeybees (Backhaus, 1991).

Polychrome V System (TILL Photonics GmbH, Gräfelfing, Germany), equipped with a 150 W Xenon lamp. Visual stimuli were projected from the back of a white screen made of standard printer paper (Niggebrügge et al., 2009), which was placed to the right of the bee, thus directly stimulating its right eye (Fig. 1).

Stimuli

Unconditioned stimulus

The aversive US was an electric shock of 7.5 V applied for 2 s (Vergoz et al., 2007).

Conditioned stimuli

Spectral irradiance of the light stimuli was measured in photon counts $cm^{-2} s^{-1}$ at the level of the bee eye using a fixed grating spectrometer (SD2000, Ocean Optics, Ostfildern, Germany) with a DT1000 mini light source (200–1100 nm) and R400-7 UV/VIS optical fiber (Ocean Optics). The sensitivity of the spectrometer in the range 400–650 nm is estimated as 86 photons/count/ms. Light intensity and spectral bandwidth were adjusted by setting the aperture of entrance and exit slits of the Polychrome V System using motors controlled by software (PolyCon/Poly V FW, TILL Photonics GmbH). Light from the monochromator was conducted by a quartz light guide to the back of the white screen, thus producing a 9 cm diameter colored disc visible by the right eye of the harnessed bee (Fig. 1). The white screen was placed at a distance of 8 cm from the bee eye (Fig. 1), so that the colored disc subtended a visual angle of 59 deg to the right eye of the bee. Under these experimental conditions, we expected that chromatic channels would be mainly solicited for the processing of such a large-field chromatic stimulation (Giurfa et al., 1996; Giurfa and Vorobyev, 1997; Hempel de Ibarra et al., 2001; Hempel de Ibarra et al., 2002).

Three different chromatic stimuli were used as CS: a green light peaking at 540 nm (10 nm bandwidth; henceforth Green) and two blue lights, one peaking at 440 nm (25 nm bandwidth; henceforth Blue 1) and another peaking at 439 nm (10 nm bandwidth; henceforth Blue 2). Taking into account the spectral sensitivities of the three types of honeybee photoreceptors (Peitsch et al., 1992) and the spectral curves of each chromatic stimulus, we calculated the photoreceptor excitation induced by each visual stimulus (Fig. 2A). To this end, we took into account the particular situation of our experimental setup (see above) and calculated the spectral light that reached the bee eye upon stimulation with the monochromator. As the experiments were performed in a dark room with very weak illumination, spectral irradiance functions of the three chromatic stimuli were not convolved with a daylight function, as is usually done for experiments performed with free-flying animals trained under daylight (Backhaus, 1991; Chittka, 1992). The reference background defined for evaluation of chromatic and achromatic signals was the reflectance spectrum of the white screen as measured under the weak illumination of the dark room.

We calculated the perceptual chromatic distance (ΔS) between stimuli in two theoretical models of color vision proposed for honeybees, the color hexagon space (Chittka, 1992) (Fig. 2B) and the color opponent coding (COC) space (Backhaus, 1991) (Fig. 2C). For both spaces, results were very similar as both Green and Blue 1 and Green and Blue 2 were different from each other whereas Blue 1 and Blue 2 were similar in chromatic terms (Table 1).

We also calculated achromatic cues in two different ways. On the one hand, we used light flux (photon counts $cm^{-2} s^{-1}$) as a physical measurement (Menzel and Greggers, 1985; Werner et al., 1988; Lotto and Chittka, 2005; Lotto and Wicklein, 2005). On the other hand, we considered the spectral sensitivity curves reported for each photoreceptor type (Peitsch et al., 1992) to calculate photoreceptor excitations (photon catches) (Table 2). The achromatic cues considered for each stimulus were its overall intensity and its L-receptor contrast (Giurfa et al., 1996; Giurfa et al., 1997). Overall intensity was measured either as the light flux corresponding to that stimulus or as the sum of all calculated photoreceptor excitations with respect to the background (Table 2). L-receptor contrast was calculated as the L-receptor excitation obtained upon stimulation with a given stimulus relative to that yielded by the background (Table 2). For both overall intensity and L-receptor contrast, photon catches were normalized with respect to the highest value obtained.

Conditioning procedure

We performed a differential conditioning in which bees had to learn to discriminate two visual stimuli, one (CS+) that was reinforced (i.e. paired with the electric shock) and one that was not (CS–). Visual stimuli (A and B) were presented in a pseudo-random sequence of six reinforced and six non-reinforced trials (e.g. ABBABAABABBA) starting with stimulus A or B in a balanced fashion. Trials were separated by an inter-trial interval (ITI) of 10 min and each trial lasted 60 s. At the beginning of each trial, the bee was placed for 30 s in the experimental setup (Fig. 1) to allow

Table 1. Perceptual chromatic distances (ΔS) between the pairs of stimuli used in the differential conditioning experiments, calculated for the hexagon and color opponent coding (COC) spaces

Stimulus pair	Hexagon	COC
Blue 1/Green	0.73	18.17
Blue 2/Green	0.85	20.27
Blue 1/Blue 2	0.12	2.36
Blue 2_L/Blue 2_H	0	0

For both spaces, results were very similar as both Green and Blue 1 (Experiment 1) and Green and Blue 2 (Experiments 2 and 4) were different from each other whereas Blue 1 and Blue 2 (Experiment 3) were similar in chromatic terms. Neither color space takes into account intensity differences between stimuli; therefore, the same stimulus (Blue 2) presented at two different light fluxes, low (Blue 2_L) and high (Blue 2_H), occupies the same locus in these spaces (Experiment 3).

familiarization with the experimental context. A visual stimulus was then presented for 5 s. In reinforced trials, the electric shock started 3 s after visual stimulus onset and lasted 2 s. Thus, the inter-stimulus interval (ISI) was 3 s and the overlap between the CS and the US was 2 s. In non-reinforced trials, the CS– was delivered alone. After stimulation, the bee was left in the setup for 25 s and then removed. The beginning and the end of each trial, as well as the onset and offset of CS and US, were either directly controlled or signaled by a computer programmed to emit tones of different frequencies for each event. Retention tests were performed 1 h after the last conditioning trial and consisted of 5 s presentations of the visual stimuli used for conditioning but without reinforcement. Stimulations were separated by an ITI of 10 min and the sequence of stimulus presentation (AB or BA) varied from bee to bee.

Throughout the procedure we recorded the bees' SER, both to the CS (conditioned response) and to the US (unconditioned response). Only bees that consistently reacted to the US (electric shock) at the last conditioning trial and at the end of the tests (unconditioned response) were kept for further analysis. The percentage of bees that did not fulfill this criterion was never higher than 5% of the sample size of each experiment.

Experiments

Experiment 1: can bees learn a visual discrimination based on aversive reinforcement?

We trained two groups of bees in parallel. For one group, Blue 1 was the reinforced stimulus and Green was the non-reinforced stimulus (Blue 1+ *versus* Green–), whereas for the other group the contingencies were reversed (Blue 1– *versus* Green+). The conditioned stimuli differed both in their chromatic and achromatic properties (Tables 1 and 2). However, the large visual angle subtended by the visual target, and the fact that the stimuli to be discriminated were very different in their chromatic properties, made it highly probable that mainly chromatic cues would be used for solving this task (Giurfa et al., 1997).

Table 2. Achromatic cues of stimuli used in our experiments

Stimulus	Photon flux (photon counts $cm^{-2}\,s^{-1}$)	Photon catch	
		All receptors	L-receptor
Blue 1	2.1×10^6	0.48	0.25
Blue 2_H	7.6×10^6	1.00	0.34
Blue 2_L	2.1×10^6	0.25	0.09
Green	7.6×10^6	0.69	1.00

The achromatic cues considered for every stimulus were its overall intensity and its L-receptor contrast (Giurfa et al., 1996; Giurfa et al., 1997). As a physical measurement of overall intensity, the photon flux of each stimulus was measured at the level of the bee eye using a fixed grating spectrometer. Overall intensity was also measured in terms of photon catches as the sum of all calculated photoreceptor excitations with respect to a black background. L-receptor contrast was calculated as the L-receptor excitation obtained upon stimulation with a given stimulus with respect to the same black background. For both overall intensity and L-receptor contrast, photon catches were normalized with respect to the highest value obtained.

Experiment 2: can bees learn to discriminate visual stimuli based on chromatic differences?

The two visual stimuli used in the previous experiment (Blue 1 and Green) differed in their chromatic and/or achromatic properties (Tables 1 and 2) so that both kinds of cues were at the bees' disposal even if we considered that the use of achromatic cues was less probable. In this experiment we asked whether bees could solve the visual discrimination using mainly the chromatic properties of the stimuli. To this end, we trained bees to discriminate between Blue 2 and Green. Again, two groups were conditioned in parallel: Blue 2+ *versus* Green– and Blue 2– *versus* Green+.

We calibrated the physical overall intensity of these stimuli so that they presented the same light flux level (7.6×10^6 photon counts $cm^{-2}\,s^{-1}$; Table 2). At the physiological level (photon catches), however, Blue 2 presented a higher overall intensity than Green (1.00 *versus* 0.69, respectively), whereas the trend was reversed in the case of the L-receptor contrast (0.34 and 1.00, respectively). If bees relied on achromatic differences assessed through these physiological channels, then performances could be asymmetric between the two groups Blue 2+ *versus* Green– and Blue 2– *versus* Green+ because overall intensity and L-contrast yield opposite predictions.

To verify that the association formed in our conditioning protocol did indeed link the visual stimuli with their respective outcome, we performed a control experiment in which we used a single visual stimulus (Blue 2) both as CS+ and as CS–. This control, in which exactly the same stimulus is as often reinforced and non-reinforced and for which no discrimination is expected, allows us to ensure that bees did not use uncontrolled cues or the sequence of trials to learn visual discriminations involving different stimuli (Mota et al., 2011a).

Experiment 3: can bees learn to discriminate visual stimuli based on achromatic differences?

In this experiment, we asked whether bees could discriminate between two visual stimuli based on achromatic differences. To answer this question we performed two subexperiments. In the first one, we trained bees to discriminate two stimuli, Blue 1 and Blue 2, that presented a small chromatic difference but significant differences in achromatic cues (Tables 1 and 2). They differed in their physical (2.1 and 7.6×10^6 photon counts $cm^{-2}\,s^{-1}$, respectively) and physiological overall intensities (0.48 and 1.00, respectively). Their L-receptor values were 0.25 (Blue 1) and 0.34 (Blue 2). Two groups were conditioned in parallel: Blue 1+ *versus* Blue 2– and Blue 1– *versus* Blue 2+.

In the second subexperiment, we aimed to rule out the argument that the small chromatic difference existing between Blue 1 and Blue 2 could support differentiation. To this end, we used the same chromatic stimulus, Blue 2, presented at two different overall physical intensities, corresponding to the intensities used in the previous experiment (Blue 2_L, 2.1 photon counts $cm^{-2}\,s^{-1}$; Blue 2_H, 7.6×10^6 photon counts $cm^{-2}\,s^{-1}$; Table 2). Blue 2_L and Blue 2_H obviously differed in their physiological intensities (0.25 and 1.00, respectively) and L-receptor values (0.09 and 0.34, respectively). Two groups were trained in parallel: Blue 2_L+ *versus* Blue 2_H– and

Blue 2_L– *versus* Blue 2_H+. This experiment was therefore aimed at establishing whether, in the absence of chromatic differences, harnessed intact bees could be trained to differentiate the same visual stimulus varying only in the achromatic dimension.

Experiment 4: effect of antennae deprivation on visual aversive conditioning

In Experiments 1 to 3, bees had intact antennae, as opposed to previous appetitive visual conditioning experiments on harnessed bees (Hori et al., 2006; Hori et al., 2007; Niggebrügge et al., 2009; Mota et al., 2011a), in which antennae ablation was crucial for obtaining visual learning. In the present experiment, we evaluated the effect of antennae deprivation on visual SER conditioning.

We established two groups of bees, one in which the whole flagella (10 last antennal segments) of both antennae were cut with fine scissors ('bees without antennae'), and another that conserved intact antennae ('bees with antennae'). Antennae ablation was performed immediately after chilling and harnessing, 2 h before conditioning. Bees with hemolymph leaking from the amputated end of their antennae were discarded from the experiments. Bees with and without antennae were trained to discriminate between Blue 2 and Green. Each group (with or without antennae) was divided in two subgroups trained with reversed stimulus contingencies (Blue 2 + *versus* Green– and Blue 2– *versus* Green).

Experiment 5: effect of antennae deprivation on US responsiveness

We asked whether differences in visual aversive learning between intact and ablated bees could be due to differences in how these two groups of animals perceived and responded to the electric shock (US). To answer this question, we quantified the responsiveness of harnessed bees with and without antennae to a series of six electric shocks of increasing voltage (0.25, 0.5, 1, 2, 4 and 8 V) corresponding to a logarithmic series (Núñez et al., 1983; Roussel et al., 2009). During each stimulation, we recorded whether bees emitted the SER. In order to avoid sensitization of SER along the shock sequence, placement trials were interspersed between shock trials. During placement trials, bees were placed in the stimulation setup without receiving any shock. In this case we recorded whether bees exhibited SER during the 2 s corresponding to the timing of the electric shock in voltage trials. Consecutive tests were separated by at least 2 min. Bees starting to respond to a given voltage and not responding to higher subsequent voltages were not included in the analyses (<3%), as their responses could not be interpreted in terms of sensitivity to the electric shock.

Statistics

We analyzed the conditioned responses to the visual stimuli used as CS, i.e. the SER occurring after visual stimulus onset and before electric-shock delivery. The bees' responses were scored as 0 (no response) or 1 (sting extension). Multiple responses during a CS presentation were counted as a single SER. The percentage of SER recorded during acquisition was used to plot acquisition curves and retention performances. Repeated-measures ANOVA was used to analyze the variation of responses to the CS+ and to the CS– in the course of training. ANOVAs were used for both between-group and within-group comparisons. Monte Carlo studies have shown that it is permissible to use ANOVA on dichotomous data under controlled conditions, which are met by our experiments (Lunney, 1970). Possible differences between responses to the CS+ and to the CS– in retention tests were analyzed by means of a McNemar test.

We quantified differentiation during acquisition and/or test by computing for each bee and each trial a delta (Δ) value resulting from the difference between its responses to the CS+ and those to the CS–. Thus, Δ could take values of –1, 0 or 1. ANOVAs were used to analyze the variation of Δ values in the course of training and for comparing Δ curves between groups. Mann–Whitney tests were used to evaluate differences between groups in each conditioning trial. In shock responsiveness assays, Fisher's exact tests were used to evaluate differences in SER responses between independent groups (with *versus* without antennae) in each placement trial. In all cases, the alpha level was set at 0.05.

RESULTS

Experiment 1

We trained bees in a differential conditioning procedure to discriminate a visual stimulus paired with an electric shock (CS+) from another visual stimulus presented without reinforcement (CS–). To this end we used two chromatic stimuli, Blue 1 and Green, which differed in their chromatic and achromatic properties (Fig. 2). For one group of bees, Blue 1 was the reinforced stimulus and Green was the non-reinforced stimulus (Blue 1+ *versus* Green–), whereas for a second group the contingencies were reversed (Blue 1– *versus* Green+). Because there were no significant differences between the two groups, data were pooled (group × stimulus × trial effect, ANOVA, $F_{5,255}$=0.51, P=0.77). The resulting learning curves (Fig. 3A, N=53) show that in the course of training, bees learned to respond to the CS+ and to inhibit their response to the CS–. Accordingly, we found a significant effect of the stimulus (repeated-measures ANOVA, $F_{1,52}$=35.03, P<0.001) as well as a significant interaction between stimulus and trials ($F_{5,260}$=5.51, P<0.001) showing that the evolution of responses in the course of training varied depending on the type of CS considered. Indeed, responses to the CS+ increased significantly in the course of training ($F_{5,260}$=3.17, P<0.01) whereas responses to the CS– decreased significantly ($F_{5,260}$=2.29, P<0.05). One hour after the last acquisition trial, bees were presented with both visual stimuli without reinforcement in retention tests (Fig. 3A). In these tests, bees responded significantly more to the CS+ than to the CS– (McNemar test, χ^2=19.36, P<0.001). Thus, intact harnessed bees learned to extend the sting differentially to the two visual stimuli and were able to retrieve the learned information 1 h after conditioning.

Experiment 2

The two visual stimuli used in the previous experiment (Blue 1 and Green) differed in both their chromatic and achromatic properties (Tables 1 and 2) so that both kinds of cues could have been used by the bees for the discrimination, although the rather large visual angle subtended by the stimuli (59 deg) made the use of chromatic cues more probable (Giurfa et al., 1996; Giurfa et al., 1997).

Here we asked whether bees could solve the visual discrimination based mainly on the chromatic properties of the stimuli. To this end we trained bees to discriminate between Blue 2 and Green, which were calibrated to have the same physical overall intensity (Fig. 2E). The performances of the two experimental groups (Blue 2+ *versus* Green– and Blue 2– *versus* Green+) were symmetric despite the fact that the two stimuli had diverging values as achromatic cues (overall physiological intensity was higher for Blue 2 than for Green, but the relationship was the inverse for L-receptor contrast; see Table 2). These differences did not affect acquisition of the discrimination (group × stimulus × trial effect, ANOVA, $F_{5,205}$=0.84, P=0.52), thus the performances of both groups were pooled. All in all, the symmetry of the performance of both groups and the fact that the stimuli were

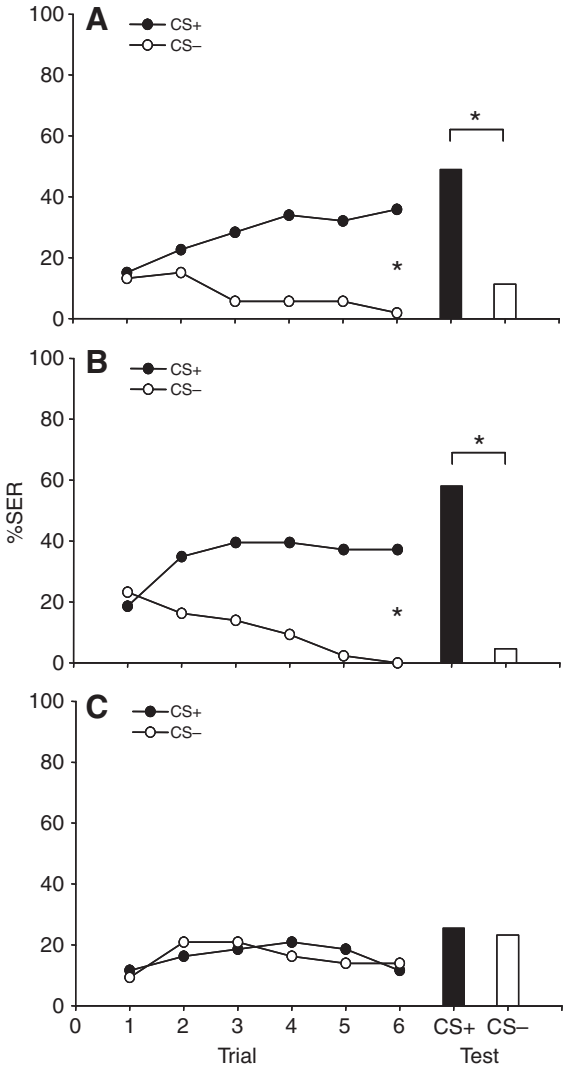

Fig. 3. Visual SER conditioning in harnessed bees: visual discrimination based on aversive reinforcement. (A) Percentage of SER responses during six blocks of conditioning trials and in retention tests for bees trained to discriminate between Blue 1 and Green, which differed in both their chromatic and achromatic properties (N=53). Bees significantly increased SER to the punished stimulus (CS+) and decreased SER to the non-punished stimulus (CS–) in the course of conditioning. During retention tests, they also responded significantly more to the CS+ than to the CS–. (B) Bees were trained to discriminate between Blue 2 and Green, which differed in their chromatic information (N=43) and were calibrated to the same overall physical intensity (7.6×10^6 photon counts $cm^{-2}\,s^{-1}$; Table 2). Bees significantly increased SER to the CS+ and decreased SER to the CS– in the course of conditioning. During retention tests, they also responded significantly more to the CS+ than to the CS–. (C) Control group using a single visual stimulus (Blue 2) both as CS+ and as CS– (N=43). Bees did not discriminate between the same stimulus reinforced (CS+) and non-reinforced (CS–).

presented at a large visual angle in order to recruit chromatic channels suggest that harnessed bees differentiated between both CS based on their different chromatic properties.

As shown by the resulting learning curves (Fig. 3B, N=43), bees quickly learned to respond to the CS+ and to inhibit their response to the CS–, so that a significant stimulus effect ($F_{1,42}$=26.25, P<0.001) and a significant trial × stimulus interaction ($F_{5,210}$=5.87, P<0.001) were found showing that the evolution of responses in the course of training varied depending on the type of CS considered. Indeed, responses to the CS+ increased significantly in the course of training ($F_{5,210}$=2.30, P<0.05) whereas responses to the CS– decreased significantly ($F_{5,260}$=4.54, P<0.001). Likewise, in retention tests 1 h after conditioning, bees responded significantly more to the CS+ than to the CS– (McNemar test, χ^2=19.36, P<0.001).

To verify that the association formed in our conditioning protocol did indeed link the visual stimuli with their respective outcome, we performed a control experiment in which we used a single visual stimulus (Blue 2) both as CS+ and as CS–. In such conditions (Fig. 3C, N=43), bees did not differentiate between reinforced and non-reinforced trials. Accordingly, neither the stimulus effect ($F_{1,42}$=0.075, P=0.79) nor the trial × stimulus interaction ($F_{5,210}$=0.64, P=0.67) were significant. This control shows, therefore, that in the absence of chromatic and/or achromatic differences the discrimination was not possible. Thus, in our protocol bees acquired a new conditioned response based on the perceptual differences between the visual stimuli used as CS.

Experiment 3

We asked whether bees could discriminate between two visual stimuli based on achromatic differences. To answer this question we performed two experiments. In the first one, we trained bees to discriminate between two stimuli, Blue 1 and Blue 2, that were similar in terms of their chromatic properties (see their respective loci in both perceptual color spaces, Fig. 2B,C), but that clearly differed in their achromatic cues (Table 2). The performances of the two experimental groups (Blue 1+ *versus* Blue 2– and Blue 1– *versus* Blue 2+) did not differ significantly and thus were pooled (group × stimulus × trial effect, ANOVA, $F_{5,210}$=1.49, P=0.19). The resulting learning curves showed that bees efficiently learned to discriminate between the two stimuli (Fig. 4A, N=44). Over successive trials, bees learned to respond more to the CS+ so that a significant stimulus effect ($F_{1,43}$=35.08, P<0.001) and a significant trial × stimulus interaction were found ($F_{5,215}$=3.61, P<0.01). Such an effect was justified by a significant variation of CS+ responses in the course of training ($F_{5,215}$=2.65, P<0.05) whereas CS– responses remained low and did not vary significantly ($F_{5,215}$=1.12, P=0.35). In retention tests 1 h after conditioning, bees also showed a successful differentiation between CS+ and CS– (McNemar test, χ^2=16.06, P<0.001; Fig. 4A).

As the difference between Blue 1 and Blue 2, though low, may have been enough to facilitate discrimination in chromatic terms, we performed a second experiment in which we used the same chromatic stimulus, Blue 2, presented at two different overall intensities (low and high, Blue 2_L and Blue 2_H, respectively). In this way we aimed at ruling out the argument that the small chromatic difference between Blue 1 and Blue 2 accounted for the discrimination achieved in the previous experiment.

The two groups trained with reversed stimulus contingencies (Blue 2_L+ *versus* Blue 2_H– and Blue 2_L– *versus* Blue 2_H+) did not differ significantly and were therefore pooled (group × stimulus × trial effect, ANOVA, $F_{5,200}$=0.78, P=0.57). The resulting curves (Fig. 4B, N=42) show that bees successfully learned to differentiate between Blue 2_L and Blue 2_H so that a significant stimulus effect ($F_{1,41}$=20.58, P<0.001) was found, although no significant trial × stimulus interaction was found ($F_{5,205}$=2.04, P=0.07). Specifically, bees did not significantly increase their CS+ responses in the course of training ($F_{5,205}$=0.42, P=0.83) but they significantly reduced their CS– responses ($F_{5,205}$=2.49, P<0.05). One hour after the last conditioning trial, bees still responded to the CS+ and not to the CS– (McNemar test; SER: χ^2=13.07, P<0.001).

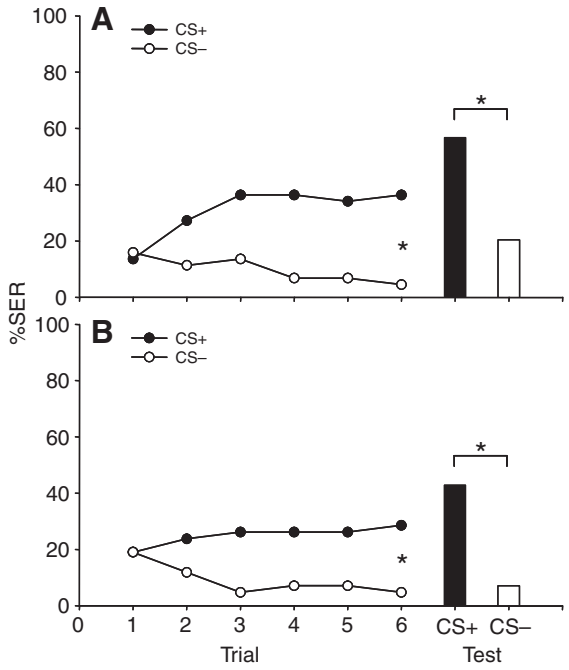

Fig. 4. The use of achromatic differences in visual SER discrimination by harnessed bees. (A) Bees were trained to discriminate between Blue 1 and Blue 2, two stimuli that were very similar in terms of their chromatic properties (N=44), but which clearly differed in their achromatic cues. Bees significantly increased SER to the CS+ and decreased SER to the CS– in the course of conditioning. During retention tests, they also responded significantly more to the CS+ than to the CS–. (B) Bees were trained to discriminate the same chromatic stimulus, Blue 2, presented at two different overall intensities (low and high, Blue 2_L and Blue 2_H, respectively; N=42). Bees significantly increased SER to the CS+ and decreased SER to the CS– in the course of conditioning. During retention tests, they also responded significantly more to the CS+ than to the CS–.

The two groups presented in Fig. 4 (Blue 1/Blue 2 and Blue 2_L/Blue 2_H) differed neither in their acquisition performance (group × stimulus × trial effect, ANOVA, $F_{5,420}$=0.31, P=0.91) nor in their Δ values in the retention tests (Mann–Whitney test, Z_{adj}=0.06, P=0.95). This experiment thus shows that, when no other visual cues are available, intact harnessed bees learn to discriminate visual stimuli in our aversive conditioning protocol based solely on achromatic differences.

Experiment 4

In Experiments 1 to 3, bees had intact antennae, as opposed to previous appetitive visual conditioning experiments on harnessed bees (Hori et al., 2006; Hori et al., 2007; Niggebrügge et al., 2009; Mota et al., 2011a), in which antennae ablation was crucial for obtaining visual learning. In our previous experiments, bees with intact antennae learned to associate visual stimuli with an electric shock and to respond to these visual stimuli with a SER. In the present experiment, we evaluated the effect of antennae deprivation on visual SER conditioning. Bees with and without antennae were trained to discriminate between Blue 2 and Green. Each group (with or without antennae) was divided in two subgroups trained with reversed stimulus contingencies (Blue 2+ *versus* Green– and Blue 2– *versus* Green+). No differences appeared between subgroups and thus their data were pooled (bees without antennae: group × stimulus × trial effect, ANOVA, $F_{5,480}$=0.39, P=0.86; bees with antennae: group × stimulus × trial effect, ANOVA, $F_{5,485}$=0.19, P=0.97).

Again, bees with antennae (Fig. 5B, N=98) quickly learned to discriminate between visual stimuli as shown by a significant stimulus effect ($F_{1,98}$=50.76, P<0.001) and a significant trial × stimulus interaction ($F_{5,490}$=10.65, P<0.001). One hour after the last conditioning trial, bees still responded more to the CS+ than to the CS– (McNemar test, χ^2=25.04, P<0.001). Bees without antennae (Fig. 5A, N=99) seemed to have more difficulties in learning the discrimination. However, in the course of training, these bees also learned to respond more to the CS+ than to the CS–, thus yielding a significant stimulus effect ($F_{1,97}$=17.24, P<0.001) and a significant trial × stimulus interaction ($F_{5,485}$=4.77, P<0.001). After 1 h, they also responded significantly more to the CS+ than to the CS–, even if response levels were low (McNemar test, χ^2=14.06, P<0.001).

To determine whether antennae ablation affected acquisition and retention, we compared the performances of the groups with and without antennae. We computed for each bee and each trial a Δ value (in %) resulting from the difference between the bee's response to the CS+ and the bee's response to the CS–. Fig. 5C shows the Δ values obtained for bees with and without antennae, both for acquisition and retention. Bees with antennae had significantly higher performance values for the discrimination task than bees without antennae, as shown by a significant group effect ($F_{1,195}$=5.90, P<0.05). Significant differences were observed for trials 4, 5 and 6 (Mann–Whitney test; trial 4, Z_{adj}=3.29, P<0.001; trial 5, Z_{adj}=2.30, P<0.05; trial 6, Z_{adj}=2.32, P<0.05), thus confirming that intact bees learned to discriminate better than ablated bees. However, Δ values did not differ between these two groups in the retention tests (Mann–Whitney test, Z_{adj}=1.85, P=0.064). Taken together, these results show that antennae ablation impairs visual aversive learning, and does not improve it as observed in visual appetitive learning of restrained bees (Hori et al., 2006).

Experiment 5

We then asked whether the differences in visual aversive learning we observed between intact and ablated bees could be due to differences in sensitivity and responsiveness to the US. We thus quantified the responsiveness of bees with (N=57) and without antennae (N=54) to a series of electric shocks of increasing voltage (from 0.25 to 8 V). In both groups (Fig. 5D), the percentage of SER to the electric shocks increased with increasing voltages (shock effect: intact bees, $F_{5,280}$=77.62, P<0.001; ablated bees, $F_{5,265}$=90.55, P<0.001). In both groups, responses to the electric shocks were significantly higher than responses to the placements (treatment effect: intact bees, $F_{5,280}$=238.26, P<0.0001; ablated bees, $F_{5,265}$=175.28, P<0.0001). However, shock responsiveness was significantly lower in ablated bees than in intact bees (group effect: $F_{1,109}$=18.28, P<0.001). We thus conclude that bees without antennae learned less efficiently in the visual SER conditioning protocol because they were less responsive to the electric shock acting as the US.

Interestingly, a significant difference between intact and ablated bees was also found in their SER responses to the placements (group effect: $F_{1,109}$=7.76, P<0.01). Nevertheless, this difference only occurred in the first placement trial, not in the other five trials (trial 1, Fisher's exact test, P<0.01). Thus, in the first trial, bees without antennae were less responsive to placement than bees with antennae.

DISCUSSION

In the present study, we established a novel visual conditioning protocol that allows studying visual learning and memory in intact

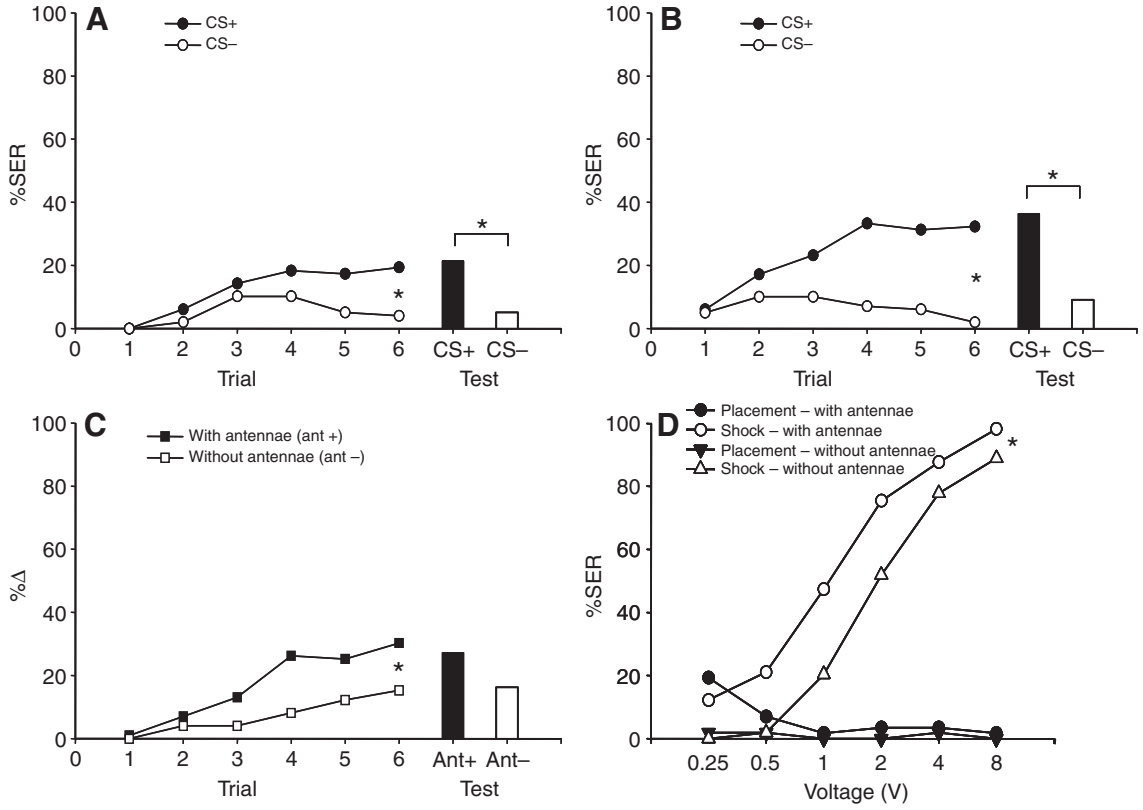

Fig. 5. Effect of antennae deprivation on visual SER conditioning and shock responsiveness. (A) Bees deprived of their antennae were trained to discriminate between Blue 2 and Green, as in Experiment 2 (*N*=99). Bees without antennae responded more with SER to the CS+ than to the CS– during both acquisition trials and retention tests. (B) Bees with intact antennae were also trained to discriminate between Blue 2 and Green (*N*=98). Bees with intact antennae responded more with SER to the CS+ than to the CS– during both acquisition trials and retention tests. (C) Delta value (Δ; %) resulting from the difference between the bees' response to the CS+ and to the CS– calculated for the group of bees with (B) and without (A) antennae. The Δ values obtained for bees with antennae were significantly higher than those obtained for bees without antennae during acquisition trials, thus showing that bees with antennae had significantly better learning performance for visual discrimination than bees without antennae. Yet, Δ values did not differ between these two groups in retention tests. (D) Responsiveness (%SER) of bees with (*N*=57) and without antennae (*N*=54) to a series of electric shocks of increasing voltage (0.25 to 8 V). The graphs also show the percentage of SER in placement trials in which bees were placed in the stimulation setup without receiving any shock. In both groups (with and without antennae), SER to the electric shocks increased with increasing voltage, and responses to the electric shocks were significantly higher than responses to the placements. However, shock responsiveness was significantly lower in ablated bees than in intact bees.

harnessed bees in the laboratory. The protocol consists of conditioning the SER, which is normally elicited by noxious stimuli (Núñez et al., 1997) *via* repeated pairings between a visual stimulus (CS) and an electric shock (US). In this aversive visual protocol, bees with intact antennae learn to discriminate between two visual stimuli (CS+ and CS–) associated with different outcomes. To this end, bees can use chromatic cues, achromatic cues or both. In contrast to previous reports on appetitive visual learning in harnessed bees (Hori et al., 2006; Hori et al., 2007; Mota et al., 2011a), antennae ablation was not necessary for learning to occur. On the contrary, we showed that sectioning the antennae of harnessed bees impaired visual SER conditioning because of a reduction of responsiveness to the electric shock acting as US. More generally, we established the first visual conditioning protocol on harnessed honeybees in the laboratory that does not require injuring the experimental subjects.

Experimental features of the visual conditioning of SER

In the appetitive protocol of the olfactory conditioning of the PER, intact immobilized bees learn an association between an odor and a reward of sucrose solution delivered to the antennae and then to the proboscis (Takeda, 1961; Bitterman et al., 1983). As a consequence, bees extend their proboscis to the odorant that anticipates the sucrose reward. Acquisition is usually fast and results in high levels of conditioned responses (bees normally reach a plateau of 80 to 90% conditioned responses after three to five trials). On the contrary, visual conditioning of PER is slow and generally results in low levels of conditioned responses (bees normally reach a plateau of 30 to 40% conditioned responses after 20 trials or more, performed during 2 days) (see Hori et al., 2006; Hori et al., 2007). Furthermore, the bee antennae have to be sectioned to enable visual conditioning of PER.

In olfactory SER conditioning, learning levels are often lower than those obtained in PER conditioning (see Vergoz et al., 2007; Carcaud et al., 2009; Giurfa et al., 2009; Roussel et al., 2009; Roussel et al., 2011), and are coincident with those found for visual SER conditioning in the present work. Furthermore, acquisition rates are similar in both cases and no sectioning of the antennae is required, as this procedure impairs rather than improves the bees' performance. It is remarkable that in both visual and olfactory SER conditioning, conditioning success does not reach the typical levels observed for olfactory PER conditioning (80 to 90%). Two main factors may explain this observation. First, the fact that bees experience a succession of noxious stimulations during acquisition

may lower their responsiveness to a CS. We have observed, for example, that following a rest period after conditioning, retention levels are sometimes higher than those observed at the last conditioning trial, thus reflecting response recovery. Second, the rather unnatural position of bees in the conditioning setup may reduce learning performance. Although the same argument applies to vertical fixation in individual tubes for PER conditioning, bees in SER conditioning are fixed on their back, which may increase stress levels. A change in the holding method can be envisaged in order to determine whether this factor is in part responsible for not reaching higher conditioning levels.

Here we chose ITI and ISI (between CS and US) values that were derived from olfactory SER conditioning (Giurfa et al., 2009), but systematic studies should be performed in order to determine optima for both variables in the visual domain. Furthermore, the fact that retention performances were significant 1 h after conditioning reveals that acquisition of an association between a visual CS and an electric shock leads to the formation of mid-term memories. Such memories may also consolidate in long-term memories retrievable several days after conditioning, as is the case for appetitive (Menzel, 1999) and aversive olfactory memories (Giurfa et al., 2009). If this is the case, one can finally answer a pending question in studies on visual learning and memory, namely whether long-term visual memories depend on protein synthesis in honeybees (Menzel et al., 1993; Wittstock et al., 1993). In this case, our protocol offers the advantage of facilitating the use of invasive procedures such as targeted injections of transcription and translation inhibitors in the bee brain, compared with previous studies that attempted similar approaches in free-flying bees (Menzel et al., 1993; Wittstock et al., 1993).

Other protocols may allow the use of invasive procedures to study the neural bases of visual performances. Luu et al. have recently studied the orientation of the body axis of a tethered honeybee suspended in a visual arena made up of four LCD monitors, which displayed a moving panorama centered on the suspended bee (Luu et al., 2011). This protocol was conceived to measure visual control of in-flight behavior in bees, i.e. how image motion perceived through the bee eyes affects fly performance. There is no associative learning component in this protocol, which results in an open-loop situation, i.e. the animal cannot influence the stimulus it perceives. A combination of a closed-loop version of this protocol coupled with US delivery would possibly constitute an appropriate, alternative strategy to study visual learning and memory in bees.

Visual discrimination learning in aversive conditioning of the SER

In our new aversive conditioning protocol, intact harnessed honeybees learned to discriminate between: (1) dissimilar colors with different achromatic cues (Blue 1/Green and Blue 2/Green; Fig. 3); (2) similar colors with different achromatic cues (Blue 1/Blue 2; Fig. 4A); and (3) the same color differing in achromatic cues (Blue 2_L/Blue 2_H; Fig. 4B). In all cases, discrimination was based exclusively on visual differences and not on uncontrolled cues, as shown by the control in which a single identical stimulus was used both as CS+ and CS– (Blue 2/Blue 2; Fig. 3C). Interestingly, we found no obvious differences in performances between situations in which chromatic or achromatic cues could be used, thus suggesting that, under our experimental conditions, discrimination of visual stimuli on the basis of their spectral quality or on achromatic signals was equally possible.

All three pairs of colors used in our study (Blue 1/Green, Blue 2/Green and Blue 1/Blue 2) are predicted to be distinguishable by bees on the basis of color vision models [COC (Backhaus, 1991); hexagon (Chittka, 1992)]. In the case of the more similar colors Blue 1 and Blue 2, experiments on color discrimination thresholds in free-flying bees showed that colors that are even closer than our Blue 1/Blue 2 pair in the hexagon space can still be distinguished by bees (Dyer and Neumeyer, 2005). Thus, all the color pairs used, similar or dissimilar, were possibly above the discrimination threshold and, therefore, yielded equivalent discrimination performances. An interesting question is whether such thresholds and color vision models are tenable in the case of aversive conditioning, given that they were all obtained in an appetitive framework in which bees were rewarded with sucrose solution for every correct choice. Does the use of an aversive US enhance discrimination of similar color stimuli to a level higher than that predicted by current color vision models? In free-flying bees, visual discrimination of two similar colors is dramatically improved if, besides rewarding the CS+ with sucrose solution, the CS– is punished with quinine solution (Avarguès-Weber et al., 2010). Similarly, the use of the electric shock could facilitate visual discrimination and promote finer discrimination performances.

Niggebrüge et al. performed experiments on appetitive PER conditioning of harnessed honeybees using visual stimuli as CS (Niggebrüge et al., 2009). They reported coarse visual discrimination and broad generalization gradients after appetitive differential conditioning, contrasting with the fine discrimination performances obtained in the present study. Moreover, harnessed bees seemed unable to discriminate light intensity differences in the appetitive protocol (Niggebrüge et al., 2009), whereas in our case bees achieved visual discriminations based on achromatic cues only. Because the same visual stimulation procedure (lateral projection of a circular light spot) was used in our study and in that by Niggebrügge et al. (Niggebrüge et al., 2009), the observed results can only be attributed to differences in the way restrained bees use visual information in appetitive and aversive learning protocols, or to antennae amputation (see below). In any case, our protocol yields results that correspond better with visual performances measured in free-flying bees (Giurfa and Menzel, 1997; Vorobyev and Brandt, 1997; Hempel de Ibarra et al., 2001; Chittka and Wells, 2004) and provides, therefore, an interesting tool for studying visual perception and learning in restrained honeybees.

An interesting result of the present study is the demonstration that restrained bees can use achromatic differences to discriminate between otherwise identical chromatic targets (Blue 2_L/Blue 2_H; Fig. 4B). Achromatic cues are typically disregarded for calculations of perceptual distances in color space models [COC (Backhaus, 1991); hexagon (Chittka, 1992)] because it has been shown that color discrimination does not involve the use of achromatic cues in bees (Giurfa et al., 1997). However, when large-field color information does not help bees to achieve a visual discrimination, they may use achromatic cues available to them, such as differences in L-contrast available at the borders of visual targets (Hempel de Ibarra et al., 2000). In our case, we conclude that achromatic signals can be used for visual discrimination if no alternative cue is available, even when such signals subtend a rather large visual angle as in our experiments (59 deg).

Effect of antennae deprivation on aversive and appetitive visual learning

We found that both intact and antennae-ablated bees are able to learn an aversive association between a visual stimulus and an electric shock. Thus, sensory input from the antennae does not impair visual aversive learning as it does for visual appetitive learning. On

the contrary, we found that sectioning the antennae impairs rather than improves aversive visual learning, contrary to what has been reported for appetitive visual learning in harnessed bees (Hori et al., 2006; Hori et al., 2007; Niggebrügge et al., 2009; Mota et al., 2011a). This impairment can be interpreted as a decrease in the subjective value of the punishment mediated by the electric shock in antennae-ablated bees relative to intact bees. We found that bees deprived of their antennae are less sensitive to electric shocks than intact bees, which means that they would perceive the provided punishment (a 7.5 V electric shock) as a milder US [see Scheiner et al. (Scheiner et al., 2005) for a discussion of subjective reward in appetitive conditioning]. The direct correlation we found here between responsiveness to the US and visual learning performance coincides with the results reported for aversive olfactory SER conditioning (Roussel et al., 2009) and for appetitive olfactory PER conditioning (Scheiner et al., 2001a; Scheiner et al., 2001b; Scheiner et al., 2004; Scheiner et al., 2005). In both cases, bees that were less sensitive to the US (shock and sucrose, respectively) were less efficient in olfactory learning tasks involving this US. Thus, antennae ablation decreases responsiveness to the shock, which in turn decreases visual learning performance involving that US.

How could antennae deprivation affect responsiveness to an electric shock provided to the thorax? A straightforward explanation is that antennae amputation has deleterious consequences on bees' fitness, thus reducing their general responsiveness to external stimuli. Similarly to the effect we found on aversive-US (shock) responsiveness, antennae-ablated bees respond significantly less to tarsal sucrose stimulation than intact bees (de Brito Sanchez et al., 2008). Thus, antennae deprivation can disrupt appetitive-US (sucrose) responsiveness, thereby affecting appetitive learning. Another explanation is that antennae amputation induces a general neurohormonal response of the animal, which then acts on its general state, including its sensitivity to sensory stimuli. For instance, one could imagine that antennae amputation may induce the release of opioid-like substances in the bee brain, which are known to decrease the responsiveness of bees towards electric shocks (Núñez et al., 1983). In other words, the poor visual learning performance in appetitive training of antennae-ablated bees could result from the effect of impaired sucrose (US) perception or from the inhibitory physiological processes resulting from antennae amputation, or from a combined effect of both.

All in all, the different results showing the disruption of (aversive and appetitive) US responsiveness after antennae ablation reveal the severe consequences of antenna ablation on bees' fitness, motivation and behavior. Therefore, from our point of view, even if harnessed bees deprived of their antennae allow the quantification of some behavioral performances, they do not constitute an adequate model for studying visual perception and learning.

Visual SER conditioning: a way towards studies on behavioral and neural bases of bimodal learning

Our novel visual conditioning protocol represents a very useful tool for studies aiming to analyze multimodal learning in harnessed honeybees. Because the bees retain their antennae, they can be conditioned with compound stimuli made of both visual (the present study) and olfactory cues (Vergoz et al., 2007; Roussel et al., 2009; Carcaud et al., 2009; Giurfa et al., 2009; Roussel et al., 2011) in a SER conditioning paradigm. In addition, the fact that intact bees are harnessed in this protocol offers the possibility of accessing the honeybee brain with a variety of invasive techniques to understand the neural bases of visual and bimodal (visual–olfactory) learning. Injections of pharmacological agonists or antagonists could thus be used to elucidate the necessity and sufficiency of different brain structures for solving bimodal discriminations of varying complexity (Giurfa, 2003). Additionally, electrophysiological or optophysiological recordings of neural activity could be coupled with our conditioning protocol to study the learning of visual or bimodal stimuli. Calcium-imaging recordings of brain activity were recently coupled with olfactory SER conditioning in order to study experience-dependent changes in neural activity during aversive conditioning (Roussel et al., 2010). Moreover, a novel protocol for performing optophysiological calcium-imaging recordings of visual-circuit activity in the honeybee brain upon visual stimulation of the compound eye was recently established in our laboratory (Mota et al., 2011b). Using these novel experimental approaches would, for the first time, enable research on experience-dependent changes in the bee brain related to visual and/or multimodal learning, a goal that has remained elusive until now.

In conclusion, we have shown that bees successfully learn to differentiate between reinforced and non-reinforced visual stimuli through visual conditioning of the SER and that such differentiation can be achieved by means of chromatic and/or achromatic cues, which may serve as CS. Our results also show that antennae ablation impairs visual aversive learning performance by reducing the bees' sensitivity to the electric shock. Our work thus constitutes the first report on visual conditioning of SER in honeybees and will hopefully open new research avenues for controlled laboratory studies of visual learning and memory in this insect.

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