Research

Color modulates olfactory learning in honeybees by an occasion-setting mechanism

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A sophisticated form of nonelemental learning is provided by occasion setting. In this paradigm, animals learn to disambiguate an uncertain conditioned stimulus using alternative stimuli that do not enter into direct association with the unconditioned stimulus. For instance, animals may learn to discriminate odor rewarded from odor nonrewarded trials if these two situations are indicated by different colors that do not themselves become associated with the reward. Despite a growing interest in nonelemental learning in insects, no study has so far attempted to study occasion setting in restrained honeybees, although this would allow direct access to the neural basis of nonelemental learning. Here we asked whether colors can modulate olfactory conditioning of the proboscis extension reflex (PER) via an occasion-setting mechanism. We show that intact, harnessed bees are not capable of learning a direct association between color and sucrose. Despite this incapacity, bees solved an occasion-setting discrimination in which colors set the occasion for appropriate responding to an odor that was rewarded or nonrewarded depending on the color. We therefore provide the first controlled demonstration of bimodal (color–odor) occasion setting in harnessed honeybees, which opens the door for studying the neural basis of such bimodal, nonelemental discriminations in insects.

The capacity to learn relationships between events in the environment is of central importance for adapting successfully to a complex and changing world. Animals can learn that an originally neutral stimulus (the conditioned stimulus, or CS) acts as a predictor for a biologically significant stimulus (the unconditioned stimulus, or US). This elemental association constitutes the basis of classical (Pavlovian) conditioning (Pavlov 1927). Another form of associative learning consists of learning that a specific behavior is followed by a biologically relevant reinforcement. This kind of association is the basis of operant (or instrumental) conditioning (Skinner 1935). Skinner additionally established that animals can learn to emit the operant response in the presence of a "discriminative stimulus" that informs when the association between behavior and reinforcement is valid. He suggested, therefore, that discriminative stimuli do not themselves elicit the operant behavior but simply "set the occasion" for the operant behavior to occur (Skinner 1938). Yet, occasion setting is not a prerogative of operant conditioning (Holland 1983, 1992). Indeed, a given stimulus (the occasion setter; Schmajuk and Holland 1998) may also indicate the temporal validity of the relation between a CS and a US (Skinner et al. 1998).

Positive occasion setters signal that a CS will be reinforced, while negative occasion setters signal that a CS (potentially the same CS) will not be reinforced. For instance, an animal may learn to respond to a CS that directly predicts the occurrence of the US in the presence of a positive occasion setter A (A→CS→US). In addition, the same animal may learn not to respond to the same CS in the presence of a negative occasion setter B, which informs that no US will be available upon CS presentation (B→CS→No US). The CS may therefore gain associative strength, while stimuli A and B inform the animal about the validity of a contingency (CS–US or CS–No US), but are not directly associated with the US. After conditioning, neither A nor B, presented alone, will be able to elicit a conditioned response. Researchers interested in nonelemental forms of learning may focus on occasion setting as a paradigm to understand how animals solve tasks without relying on a simple, elemental link between CS and US. As occasion setting consists of disambiguating an otherwise uncertain CS, it provides an appropriate framework for analyses of nonelemental learning and its underlying neural bases.

Occasion setting has been studied in a variety of animals (rats: Bouton and Swartzentruber 1986; pigeons: Rescorla et al. 1985; mollusks: Colwill et al. 1988; nematodes: Law et al. 2004; flies: Brembs and Wiener 2006) including humans (Palmatier and Bevins 2008). However, in the case of one of the main models for the study of learning and memory, the honeybee *Apis mellifera* (Giurfa 2007), occasion setting has not been explicitly studied in controlled laboratory conditions. For almost four decades, honeybee learning and memory have been mainly studied by means of a Pavlovian conditioning protocol, the olfactory conditioning of the proboscis extension reflex (PER) (Bitterman et al. 1983). When the main chemosensory organs of a hungry bee, the antennae, are touched with sucrose solution (US), the insect reflexively extends its proboscis (PER) to reach out to and lick the sucrose. Pairing an odor (the CS) with sucrose reward leads to the formation of an odor–sucrose (CS–US) association, which results in the animal responding with PER to a subsequent presentation of the odor alone. This protocol offers unique advantages as it allows studying appetitive learning and retention in individually harnessed honeybees, which despite immobilization, exhibit fast and robust acquisition and retention performances, as three pairings of odor and sucrose are enough to generate an olfactory memory that may last the bees' entire life (Menzel 1999). Immobilization especially offers the possibility of accessing the honeybee brain by means of a variety of invasive techniques, including pharmacology, lesions, electro-, and optophysiological recordings. It is therefore possible to couple conditioning

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protocols and the study of the mechanisms underlying learning and memory.

Beside simple odor–sucrose associations, bees can also master nonelemental discrimination problems in which odors have ambiguous valences (Deisig et al. 2001, 2002, 2003; Sandoz and Menzel 2001; Komischke et al. 2003). Despite a growing interest for the analysis of nonelemental learning in miniature brains (Giurfa 2007), no study has attempted so far to study multimodal learning, and more specifically, occasion setting of odor learning by other sensory cues in the framework of PER conditioning. A possible explanation for this fact may reside in the almost exclusive nature of the CS in PER conditioning. Indeed, although olfactory cues are extremely efficient as CS in this protocol, this is not the case for all sensory modalities, especially visual cues. Indeed, visual stimuli such as color or motion may act as CS in PER conditioning protocols, but to do so they require the drastic procedure of antennal ablation, thereby precluding multimodal learning (Hori et al. 2006, 2007; Niggebrügge et al. 2009). For reasons that are so far not understood, harnessed honeybees can learn an association between visual cues and sucrose reward only if their antennae have previously been cut, a procedure that eliminates the possibility of olfactory stimulation. Moreover, ablated bees exhibit low acquisition levels (usually 30%–40% conditioned responses) and require many trials (around 20) to learn to respond with PER to colors or motion cues (Hori et al. 2006, 2007). Clearly, these problems have limited the study of multimodal learning in harnessed bees.

Interestingly, the fact that harnessed, intact bees (bees whose antennae have not been ablated) do not learn a direct association between visual cues and sucrose reward does not exclude (1) that they could perceive these cues, and (2) that these cues could act, despite their lack of direct association with the US, as occasion setters to disambiguate conflictive olfactory information. In fact, the definition of an occasion setter (see above) precisely underlines its modulatory capacity and its lack of association with the US. We asked here, therefore, whether color cues can modulate olfactory learning via an occasion-setting mechanism. This question is addressed within the framework of nonelemental, multimodal learning and the search for protocols in which simple links between CS and US do not provide the solution to a discrimination task. Specifically, we first verified that intact bees are incapable of exhibiting conditioned PER to colors covering the entire bee visual spectrum, ranging from UV to red. We then studied the capacity of different colors to set the occasion for responding or not to the same odor depending on its pairing with sucrose reward. We finally analyzed the nature of the associations established within this protocol, by systematically varying the temporal relationship between the occasion setters (colors), and the CS target (odor). Our results show that despite their lack of direct association with sucrose reward, colors do indeed set the occasion for appropriate responding to an odor that could be rewarded or nonrewarded. We therefore provide the first controlled demonstration of bimodal (color–odor) occasion setting in harnessed, intact honeybees, which opens the door for addressing in further work the neural basis of such nonelemental discrimination.

Results

Experiment 1: Color conditioning of PER in harnessed bees

This experiment was performed to determine whether, in restrained honeybees, monochromatic lights in different regions of the bees' visual spectrum can enter into association with sucrose reward delivered to the proboscis. Bees that learn the color–sucrose association should afterward exhibit the appetitive PER upon color presentation. In addition, we determined whether at all these wavelengths the presence of the antennae interferes with color learning, as suggested previously using a single wavelength as CS ($\lambda = 618$ nm) (Hori et al. 2006).

We chose four different wavelengths as conditioned stimuli. The first three were chosen to provide high-level excitation of the S, M, and L photoreceptor types (monochromatic lights of $\lambda =$ 350, 439, and 540 nm), and the fourth one was that used in a previous report ($\lambda = 618$ nm) (see Hori et al. 2006). For each of the four wavelengths used as CS, two groups of bees were compared, one whose antennae were ablated and another that kept intact antennae. Figure 1 shows the acquisition performance of all eight groups along 20 conditioning trials (20 pairings color–sucrose) performed over 2 d (10 trials per day), following the procedure described by Hori et al. (2006). Within each day, conditioning trials were separated by an intertrial interval (ITI) of 10 min.

For all conditioned wavelengths the number of bees that exhibited color-induced PER increased significantly along trials in the antennae-deprived groups (one-way repeated-measure ANOVA, trial effect; 350 nm: $F_{(19,513)} = 4.99$, $P < 0.0001$; 439 nm: $F_{(19,513)} = 3.98$, $P < 0.0001$; 540 nm: $F_{(19,551)} = 6.26$, $P < 0.0001$; 618 nm: $F_{(19,551)} = 8.28$, $P < 0.0001$). In contrast, no significant learning was found in the groups with intact antennae for three out of four wavelengths used as CS (439 nm: $F_{(19,570)} = 0.96$, NS; 540 nm: $F_{(19,532)} = 1.12$, NS; 618 nm: $F_{(19,570)} = 1.09$, NS). Only in the case of 350 nm was a significant increase observed ($F_{(19,513)} = 1.98$, $P = 0.008$), but this effect appearing at the end of conditioning should be taken with caution as it results from the responses of only two bees out of 28.

For all conditioned wavelengths, ablated bees learned significantly better than intact bees, as indicated by group × trial repeated-measure ANOVAs (group effect, 350 nm: $F_{(1,54)} = 5.51$, $P < 0.05$; 439 nm: $F_{(1,57)} = 5.84$, $P < 0.05$; 540 nm: $F_{(1,56)} = 7.24$, $P < 0.01$; 618 nm: $F_{(1,58)} = 11.26$, $P < 0.005$). In all four cases the group × trial interaction was highly significant ($P < 0.0001$), thus showing that ablated and intact groups responded differently over time. This result demonstrates that the presence of antennae impairs color associative learning in harnessed bees, and that this effect is robust and similar over the whole visual spectrum of the bees.

All four wavelengths were learned similarly by ablated bees as shown by a comparison of their acquisition curves (wavelength × trial repeated-measure ANOVA; wavelength effect: $F_{(3,112)} = 0.88$, NS). The interaction was also not significant ($F_{(57,2128)} = 0.80$, NS). These results show that all four wavelengths had the same salience as conditioned stimulus and were learned as efficiently by harnessed bees.

Experiment 2: Bimodal (color–odor) conditioning of harnessed bees

The previous results raise an intriguing question, namely, why are harnessed bees with intact antennae not able to learn a color-reward association? Are harnessed bees with intact antennae "blind"? This hypothesis does not seem tenable, as even in classical olfactory PER conditioning, harnessed bees sometimes learn to respond to visual cues in the environment, such as hand movements of the experimenter delivering the sucrose reward. Rather, it could be that in a restrained situation intact bees are tuned to favor odor–sucrose associations—given the facility with which they learn them—and use colors (or other visual cues) as contextual information in which the odor–sucrose association is embedded. In that sense, although they would definitely perceive color differences, they would not be prone to directly associate them with the presence or absence of reward.

We tested this idea by training intact bees to achieve a bimodal discrimination in which different colors informed whether or

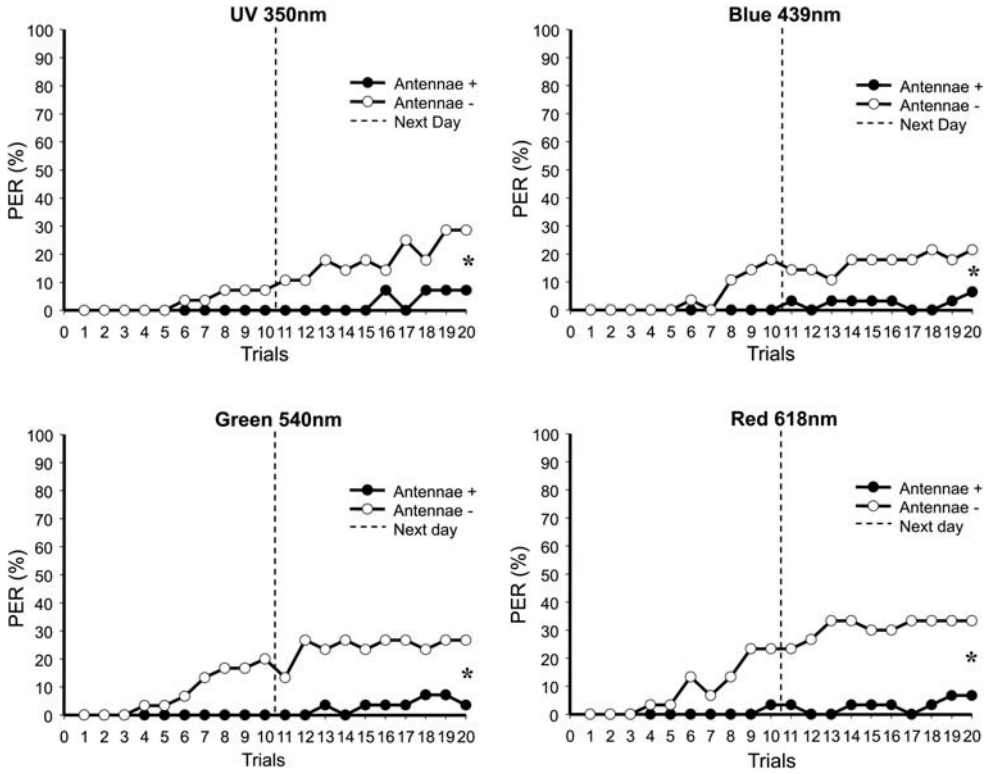

Figure 1. Color conditioning of the proboscis extension reflex (PER) and the effect of antennae deprivation. Performance (percentage of PER) of intact or antennae-deprived bees over 2 d, with 10 conditioning trials per day and an intertrial interval (ITI) of 10 min. Color-induced PER was studied in bees trained to monochromatic lights in the UV ($\lambda = 350$ nm), blue ($\lambda = 439$ nm), green ($\lambda = 540$ nm), and red range ($\lambda = 618$ nm). For all conditioned wavelengths the number of bees that exhibited color-induced PER increased significantly along trials in the antennae-deprived group, but not in the groups with intact antennae. Asterisks indicate significant differences between antennae-deprived group and intact group in two-way repeated-measure ANOVA (group effect). $n = 28–31$ bees per group (antennae-deprived or intact) for each wavelength (UV, blue, green, or red).

not a given odor was followed by sucrose reward. As the same odor could be either rewarded or nonrewarded, and thus represented an ambiguous stimulus, the only way to achieve the discrimination was to pay attention to the colors that defined the appropriate context for responding. We trained bees in a differential conditioning procedure (see Fig. 2) in which one odor (O), 1-nonanol, was either rewarded (O+, where + stands for presence of reward or nonrewarded (O–, where – stands for absence of reward), and colors X and Y defined which contingency was valid. Colors were provided by the monochromatic lights UV, blue, and green used in Experiment 1. Three combinations of two wavelengths were established (UV vs. blue, blue vs. green, UV vs. green).

In other words, bees were trained in a XO+ vs. YO– discrimination (Fig. 2). Training consisted of 10 XO+ trials and 10 YO– trials (20 trials in total), which were presented in a pseudorandom sequence, with an ITI of 10 min. Each color stimulus was presented for 12 sec; 3 sec after color onset, odor O was presented for 6 sec. Thus, after odor offset, color stimulation continued for 3 sec. In rewarded trials, sucrose was delivered to the antennae and proboscis during 3 sec, 3 sec after odor onset (and thus, 6 sec after color onset). In nonrewarded trials the 12-sec color and the 6-sec odor stimulations were provided without any sucrose. For each color combination, two subgroups of bees were always trained to balance the experiment, one in which X predicted the O+ association (XO+) and Y the O– association (YO–), and another in which contingencies were inversed (e.g., UV/O+ vs. blue/O– and UV/O– vs. blue/O+).

Within each color combination, the performance of the two subgroups of bees trained with inverse contingencies did not differ significantly from each other, so that acquisition data were pooled (*subgroup* × *stimulus* × *trial* ANOVA, *subgroup* effect: UV vs. blue: $F_{(1,42)} = 2.30$, NS; blue vs. green: $F_{(1,42)} = 1.29$, NS; UV vs. green: $F_{(1,36)} = 1.30$, NS). Figure 2 shows the pooled performance of bees for each of the three color combinations. It should be noted that we never observed any response to the colors that preceded the odor presentation. Figure 2 therefore shows PER elicited by the odor before sucrose presentation (conditioned responses). In all three cases, bees learned to discriminate between rewarding and nonrewarding odor trials (*stimulus* × *trial* ANOVA, *stimulus* effect; UV vs. blue: $F_{(1,42)} = 9.94$, $P < 0.01$; blue vs. green: $F_{(1,42)} = 4.97$, $P < 0.05$; UV vs. green: $F_{(1,36)} = 10.83$, $P < 0.01$). The interaction was significant for all three color combinations (UV vs. blue: $F_{(9,378)} = 4.32$, $P < 0.0001$; blue vs. green: $F_{(9,378)} = 2.40$, $P < 0.05$; UV vs. green: $F_{(9,324)} = 5.58$, $P < 0.0001$), showing that bees did indeed develop different responses for rewarding and nonrewarding odor trials during conditioning.

Careful observation of Figure 2 shows that for each color combination, discrimination started after or around the fifth XO+/YO– pair of trials. Indeed, an analysis of discrimination performed over the first five trials with each stimulus (trials 1–5) showed that within each color combination bees did not distinguish between rewarding and nonrewarding odor trials (*stimulus* effect: UV vs. blue: $F_{(1,42)} = 0.06$, NS; blue vs. green: $F_{(1,42)} = 1.05$, NS; UV vs. green: $F_{(1,36)} = 1.71$, NS). On the contrary, if the same analysis is performed over the last five trials (trials 6–10),

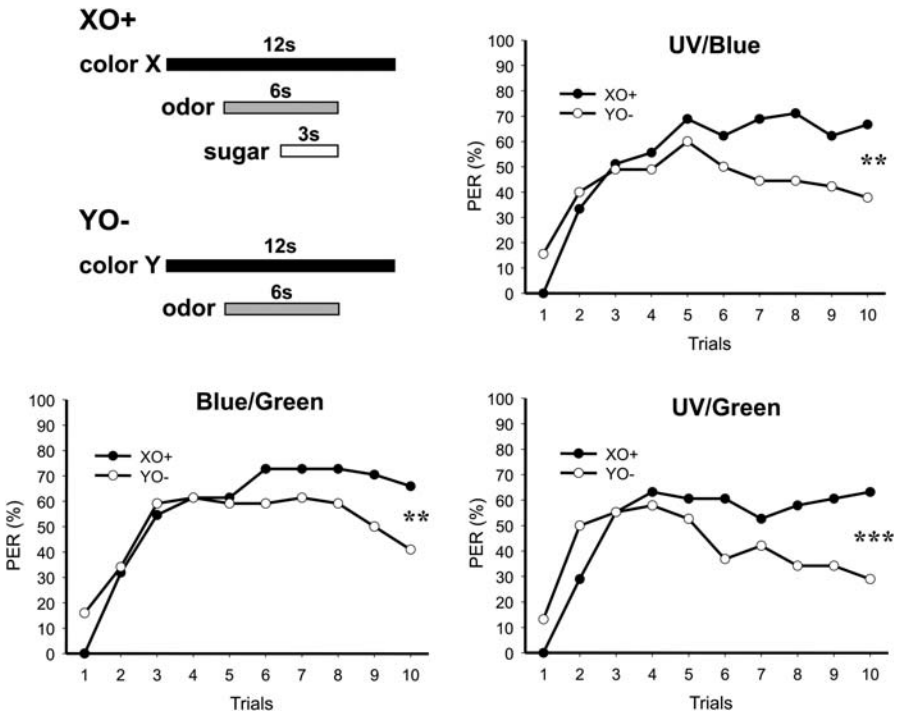

Figure 2. Bimodal (color–odor) conditioning of harnessed bees: colors as modulators of olfactory learning. Bees were trained in a differential conditioning procedure in which one odor (O), 1-nonanol, was either rewarded (O+) or nonrewarded (O–), and colors X and Y defined which contingency was valid. Training consisted of 10 XO+ trials and 10 YO– trials, which were presented in a pseudorandom sequence and with an ITI of 10 min. Each color stimulus was presented for 12 sec. Odor O was presented for 6 sec, 3 sec after color onset; thus, after odor offset color stimulation continued for 3 sec. In rewarded trials, sucrose was delivered to the antennae and proboscis for 3 sec, 3 sec after odor onset. In this experiment, in which the color started 3 sec before the odor, we never observed any PER to any of the colors. The percent of PER presented in the graphs was measured during the 3 sec before US delivery, when color and odor presentations occur simultaneously. For all color pairs tested (UV vs. blue: $n = 45$ bees; blue vs. green: $n = 44$ bees; UV vs. green: $n = 38$ bees), bees learned to discriminate between rewarded (XO+) and nonrewarded (YO–) trials. Asterisks indicate significant difference between PER rates for XO+ and YO– in the last five conditioning trials (two-way repeated-measure ANOVA —stimulus effect).

significant discrimination was found for all color combinations (UV vs. blue: $F_{(1,42)} = 17.13$, $P < 0.001$; blue vs. green: $F_{(1,42)} = 17.48$, $P < 0.001$; UV vs. green: $F_{(1,36)} = 24.90$, $P < 0.0001$), thus showing that the global discrimination effect we found developed mostly during the last five trials.

Before concluding from these results that colors act as occasion setters for disambiguating the odor–sucrose association, a number of control experiments are needed. First, we should rule out the possibility that honeybees could have used any other cue than color or learned the sequence of trials, even though a pseudorandomized order was used (Fig. 3A). We thus trained intact bees in an experiment exactly like the bimodal experiment above, except that the colors were omitted. Bees were subjected to a differential conditioning protocol, in which the odor (O) was either rewarded (O+) or nonrewarded (O–). Bees experienced 10 rewarding and 10 nonrewarding trials (20 trials in total), which were presented in the same pseudorandom sequence as above, with an ITI of 10 min. The odor stimulus was presented for 6 sec, and in rewarded trials, sucrose was delivered to the antennae and proboscis during 3 sec, 3 sec after odor onset. In nonrewarded trials, only the 6-sec odor stimulation was provided. Thus, this experiment was exactly like the bimodal experiment above, except that the colors were omitted. Under these conditions, bees increased their responses to the odor along trials (*stimulus* × *trial* ANOVA, *trial* effect, $F_{(9,405)} = 7.89$, $P < 0.0001$) as a consequence of the excitatory effect of sucrose reward, but could not discriminate between rewarding and nonrewarding trials (*stimulus* effect, $F_{(1,45)} = 0.42$, NS) because the same odor was used in both cases. This confirms that in the bimodal experiment, bees could not have used uncontrolled cues or the sequence of trials to learn the discrimination.

As a second control, we wanted to rule out that bees could have solved the discrimination by associating the colors to the reward. This was not expected to be the case, because bees with intact antennae were unable to learn a color as CS in Experiment 1 (see above). However, contrary to Experiment 1, Experiment 2 involved a differential conditioning procedure, and bees received the US on both antennae and proboscis. Moreover, the durations of and intervals between color and sucrose stimulations were different. For these reasons, a specific control experiment was needed (Fig. 3B). Intact, harnessed bees were trained in a differential conditioning to discriminate a rewarded color X+ from a different nonrewarded color Y–. As in the bimodal experiment, three color combinations were established: UV vs. blue, blue vs. green, and UV vs. green. Within each combination, experiments were fully balanced, as two subgroups of bees were always trained, one for which one color was chosen as X+ and the other as Y–, and another for which color identities were reversed (e.g., UV+ vs. blue– and UV– vs. blue+). Bees experienced 10 X+ and 10 Y– trials (20 trials in total), which were presented in a pseudorandom sequence and with an ITI of 10 min. Each color stimulus was presented for 12 sec; in rewarded trials, sucrose was delivered to the antennae and proboscis during 3 sec, 6 sec after color onset (see Fig. 3B). After sucrose offset, color stimulation continued for 3 sec. The interstimulus interval was, therefore, 6 sec and stimulus overlap 3 sec. In nonrewarded trials only the 12-sec color stimulation was provided. Thus, this experiment was exactly like the bimodal experiment above, except that the odor was omitted.

Irrespective of the color combination, bees never responded significantly to the color that was rewarded and were therefore unable to learn the color discrimination between X and Y (Fig. 3B). Within each color combination, the two subgroups of bees trained with inverse contingencies exhibited the same performances, so that their acquisition data were pooled (*subgroup* × *stimulus* × *trial* ANOVA, *subgroup* effect: UV vs. blue: $F_{(1,46)} = 1.86$, NS; blue vs. green: $F_{(1,44)} = 0.29$, NS; UV vs. green: $F_{(1,45)} = 2.64$, NS). Figure 3B shows the pooled performance of bees for each of the three color combinations. In all three cases, bees did not learn the discrimination (*stimulus* effect; UV vs. blue: $F_{(1,46)} = 1.86$, NS; blue vs. green: $F_{(1,44)} = 1.12$, NS; UV vs. green: $F_{(1,45)} = 2.64$, NS) and their responses to the rewarded color were practically nonexistent. These results show that harnessed bees with intact antennae are unable to learn a color discrimination problem, and thus

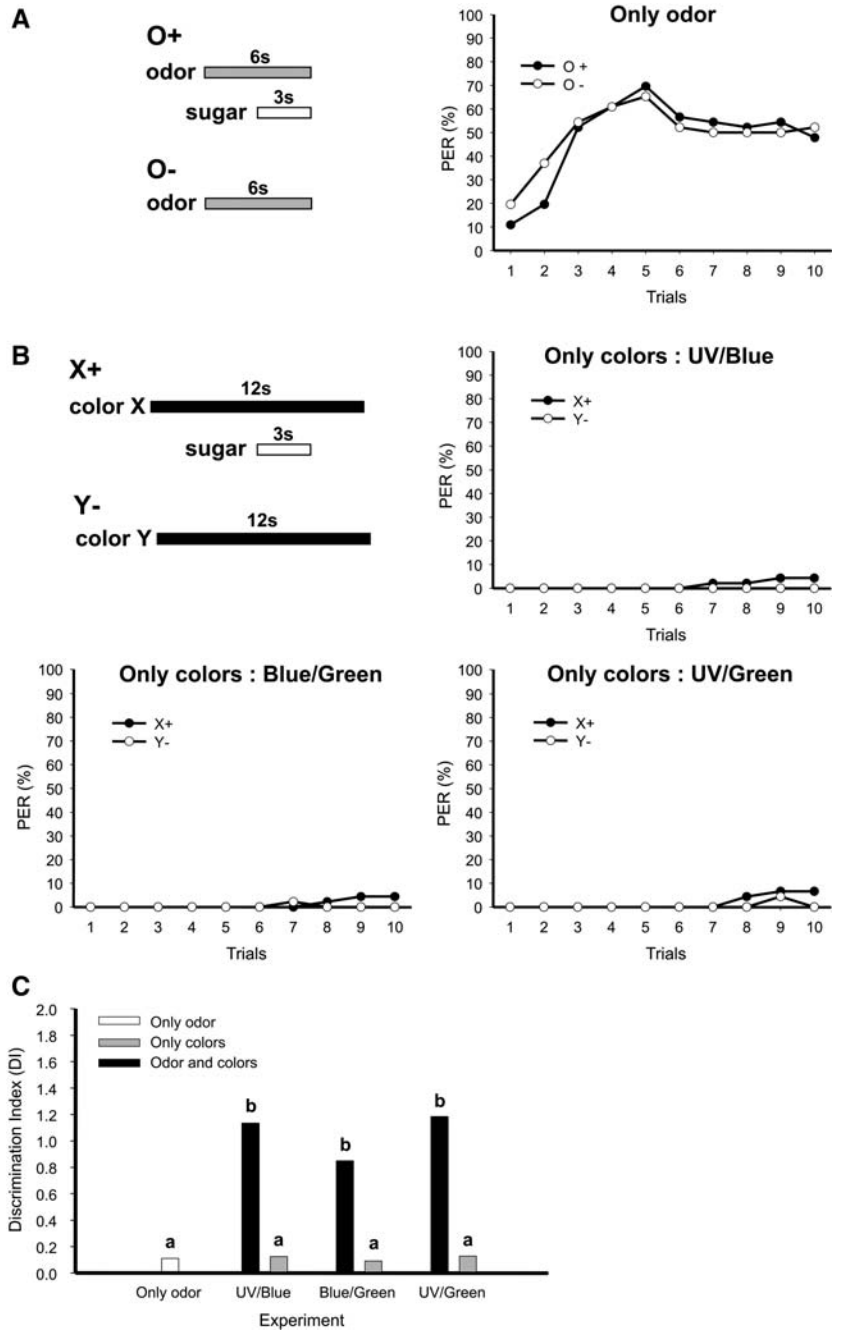

Figure 3. Control groups for bimodal (color–odor) conditioning. (*A*) To check that bees could not learn a pseudorandom sequence of rewarded and unrewarded odor trials, a control group "only odor" was trained in 10 rewarded and 10 nonrewarded trials with the same odor (O+/O–). The odor stimulus (1-nonanol) was presented for 6 sec. In rewarded trials, sucrose was delivered to the antennae and proboscis for 3 sec, starting 3 sec after odor onset. Bees increased their responses (%PER) to the odor in the course of training, but could not discriminate between rewarded and nonrewarded trials. $n = 46$ bees (*B*) To verify that bees with intact antennae are unable to learn a color discrimination as expected from Experiment 1, control groups "only colors" were trained in 10 rewarded trials of a chromatic stimulus (X+) and 10 nonrewarded trials of another chromatic stimulus (Y–). Each color stimulus was presented for 12 sec. In rewarded trials, sucrose was delivered to the antennae and proboscis for 3 sec, 6 sec after color onset. After sucrose offset, color stimulation continued for 3 sec. Irrespective of the color combination (UV vs. blue: $n = 48$ bees, blue vs. green: $n = 47$ bees; UV vs. green: $n = 47$ bees), bees almost never responded to the color that was rewarded and were therefore unable to learn the color discrimination between X and Y. (*C*) Discrimination index ($DI = [\Sigma_{XO+}] - [\Sigma_{YO-}]$ calculated in the last five trials for experimental groups (bimodal color–odor conditioning) and control groups (only odor and only colors). Different letters represent statistical differences (Kruskal–Wallis test) between the DI calculated for each experimental or control group.

could not respond correctly in the bimodal experiment based only on color-reward associations.

To compare the results of the bimodal and of the control experiments, we calculated for each group a discrimination index (DI), taking into account the bees' performance in the last five trials. For instance, in the bimodal experiment, this index results from subtracting the sum of the last five responses to YO– from the sum of the last five responses to XO+. It ranges from five (if bees only responded to XO+ in the last five trials) to −5 (if bees only responded to YO– in the last five trials). A zero value corresponds to a lack of discrimination, with an equal number of responses to XO+ and YO–. Figure 3C shows a comparison of this index for all three experimental series (odor and colors: Fig. 2; only odor: Fig. 3A; only colors: Fig. 3B). Discrimination was not visible when the odor was presented alone ($DI = 0.11$) nor when colors had to be discriminated alone (UV vs. blue: $DI = 0.13$; blue vs. green: $DI = 0.09$; UV vs. green: $DI = 0.13$). The discrimination index DI did not differ between these four cases (Kruskal–Wallis test; $H_3 = 0.34$; NS). In contrast, discrimination increased significantly within each color combination when color and odors were presented together (Mann-Whitney test; UV vs. blue: $Z_{adj.} = 3.83$, $P < 0.001$; blue vs. green: $Z_{adj.} = 2.96$, $P < 0.01$; UV vs. green: $Z_{adj.} = 4.55$, $P < 0.0001$). The enhanced DI values did not differ between all three color combinations (Kruskal–Wallis; $H_2 = 1.06$, NS), thus showing that irrespective of the color pair used, bees learned equally well to distinguish odor-rewarding from odor-nonrewarding trials when colors indicated the valid contingency at each trial. Even if harnessed bees with intact antennae did not make any direct association between color and reward, as shown by the fact that they never exhibited conditioned PER to colors during acquisition, it is clear that they are not blind, but perceive and discriminate colors, which can predict odor-reward contingency.

Experiment 3: Temporal relationship between color and odor in bimodal conditioning

The results of Experiment 2 suggested that colors set the occasion for appropriate responding to an odor that may or may not be rewarded, but are not themselves directly associated with the sucrose reward. In this experiment, we determined the temporal relationship

that is needed between colors and odor to allow appropriate responses to the odor. We also explicitly verified that colors do not bear any direct relationship to the US.

As in Experiment 2, we trained bees with a XO+ vs. YO− discrimination, where 1-nonanol was the odor (O) that could be (+) or not (−) associated with sucrose reward and X and Y the colors that predicted odor contingency. The colors used were UV and green with two balanced subgroups (UV/O+ and green/O− or UV/O− and green/O+). Both color and odor were each presented for 6 sec. The interval between color and odor onset was varied systematically in order to create four experimental groups (Fig. 4A–D): (1) Group 9-sec: the odor started 9 sec after color onset, so that there was a 3-sec gap between the two stimulations; (2) Group 6-sec: the odor started 6 sec after color onset, i.e., when color ended, so that there was no gap between the two stimulations; (3) Group 3-sec: the odor started 3 sec after color onset so that there was an overlap of 3 sec between stimulations; (4) Group 0-sec: odor and color started simultaneously, so that they fully overlapped for 6 sec. Thus, color and odor overlapped in Groups 3-sec (Fig. 4C) and 0-sec (Fig. 4D), but not in Groups 9-sec (Fig. 4A) and 6-sec (Fig. 4B). One hour after conditioning, bees were subjected to nonrewarded tests in which the two colors and the odor used during conditioning were presented separately and in a random sequence, varying from bee to bee. With these tests, we aimed to determine the relative amount of excitatory associative strength gathered by colors and odors as a result of conditioning.

Figure 4 (A–D) shows the performance of the four groups of bees (Groups 9-, 6-, 3-, and 0-sec). Within each group, the performance of the balanced groups in which color contingencies were reversed was not significantly different, so that their results were pooled (*subgroup* × *stimulus* × *trial ANOVA*, *subgroup* effect; Group 9-sec: $F_{(1,39)} = 0.22$, NS; Group 6-sec: $F_{(1,40)} = 0.45$, NS; Group 3-sec: $F_{(1,40)} = 3.53$, NS; Group 0-sec: $F_{(1,39)} = 0.32$, NS). An analysis over the entire number of conditioning trials showed that bees managed to discriminate rewarding from nonrewarding trials only in Groups 3-sec (Fig. 4C; $F_{(1,40)} = 13.42$, $P < 0.001$) and 0-sec (Fig. 4D; $F_{(1,39)} = 12.62$, $P < 0.01$). In Groups 9-sec (Fig. 4A) and 6-sec (Fig. 4B), no discrimination was achieved at the end of training (Group 9-sec: $F_{(1,39)} = 0.99$, NS; Group 6-sec: $F_{(1,40)} = 1.32$, NS), thus suggesting that overlap between odor and color was necessary to solve the task. An analysis over the last five conditioning trials similar to that

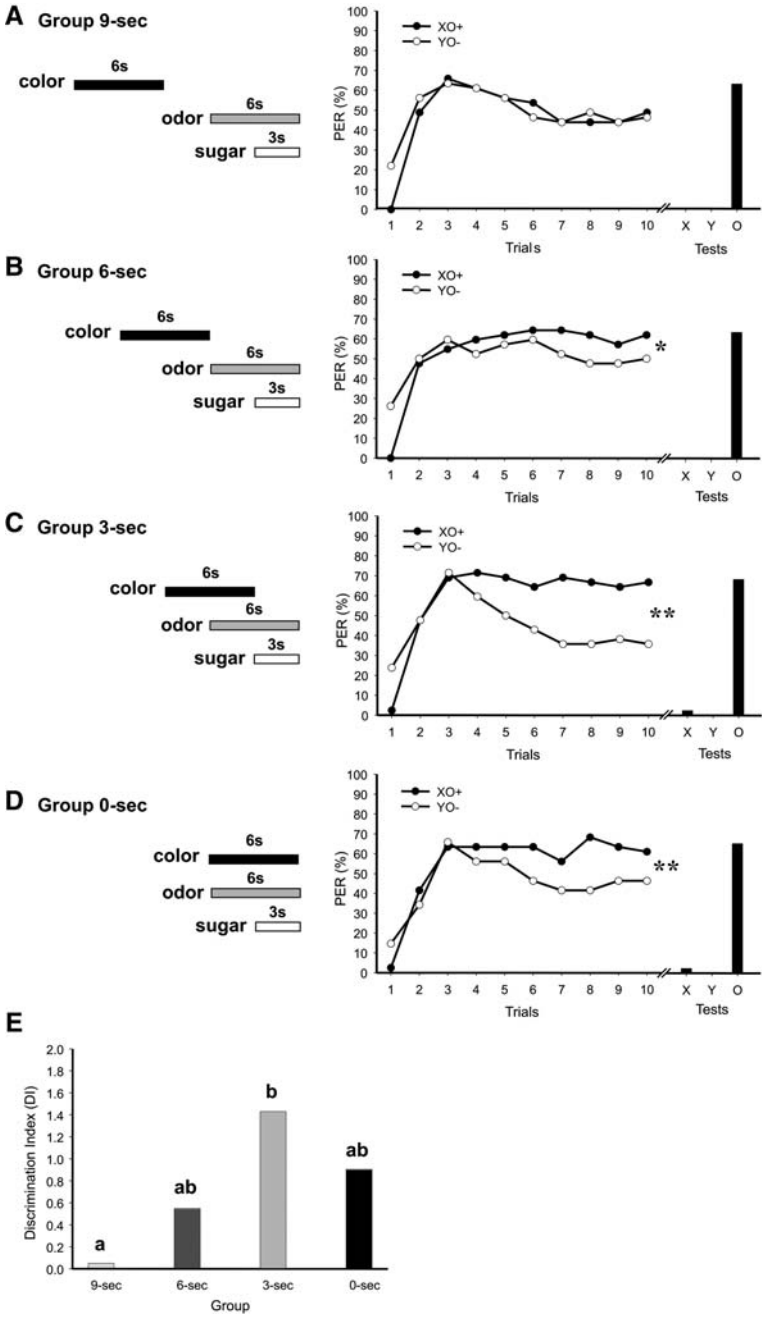

Figure 4. Temporal relationship between color and odor in bimodal conditioning of harnessed bees. Four independent groups were trained in bimodal conditioning with a constant duration of 6 sec for color and 6 sec for odor stimuli, but with a different interval between these stimuli. (*A*) Group 9-sec ($n = 41$ bees): the odor started 9 sec after color onset, so that there was a 3-sec gap between the stimulations. In this case, no discrimination was achieved at the end of training. During retention tests, bees responded to the odor O, but not to colors X and Y. (*B*) Group 6-sec ($n = 42$ bees): the odor started 6 sec after color onset, so that there was no gap between stimulations. In this case, bees managed to discriminate XO+ from YO− trials at the end of training. During retention tests, bees responded to the odor O, but not to colors X and Y. (*C*) Group 3-sec ($n = 42$ bees): the odor started 3 sec after color onset so that there was an overlap of 3 sec between stimulations. Along training, bees significantly discriminated XO+ from YO− trials. During retention tests, bees responded to the odor O, but not to colors X and Y. (*D*) Group 0-sec ($n = 41$ bees): the odor and the color started simultaneously. Bees showed significantly more responses to OX+ than to OY− along training. During retention tests, bees responded to the odor O, but not to colors X and Y. Asterisks in *B*, *C*, and *D* indicate significant differences in PER responses to OX+ and OY− in the last five conditioning trials (two-way repeated-measure ANOVA—*stimulus* effect). (*E*) Discrimination index (DI = $[\Sigma_{XO+}]-[\Sigma_{YO-}]$) calculated in all experimental groups (9-, 6-, 3-, and 0-sec). Letters represent statistical differences (Kruskal–Wallis test) between the DI calculated for the different groups.

performed in Experiment 2 showed that beside Groups 3-sec and 0-sec where discrimination was confirmed (Group 3-sec: $F_{(1,40)} = 18.37$, $P < 0.001$; Group 0-sec: $F_{(1,39)} = 13.45$, $P < 0.001$), Group 6-sec (Fig. 4B) also showed a discrimination that bordered on significance (Group 6-sec: $F_{(1,40)} = 4.04$, $P = 0.0501$). This suggests that although more trials would probably induce significant discrimination also in the 6-sec group, it is certainly not facilitated compared with groups presenting an overlap between colors and odor. Consistent with this idea, Group 9-sec (Fig. 4A), presenting a gap between color and odor stimulations, did not show any discrimination in the five-trial analysis.

The discrimination index DI calculated over the five last XO+ and YO− trials (Fig. 4E) differed significantly among the four groups of bees (Kruskal–Wallis test, $H_3 = 12.3$, $P < 0.01$). In particular, a significant difference was found between Groups 9- and 3-sec (Dunn's multiple comparisons; $P < 0.01$), but not between the other groups. This result indicates that although overlap between color and odor favors discrimination between odor-rewarding and odor-nonrewarding trials (Groups 3- and 0-sec), a color stimulation that is adjacent to the odor (Group 6-sec) can also induce some discrimination.

One hour after conditioning, bees of the four groups responded strongly to the odor in nonrewarded tests (Fig. 4A–D: between 60% and 70% conditioned responses). The percentage of conditioned responses did not differ among the four groups ($\chi^2 = 0.28$, df: 3, NS), thus showing that in all cases the odor acquired the same excitatory associative strength based on its direct association with the US. When colors were tested, no conditioned responses could be recorded in either group, neither for the color that predicted an odor–sucrose association nor for the color that predicted an odor-no sucrose association (Fig. 4 A–D). Thus, contrary to the odor, colors did not acquire any excitatory associative strength, even in those cases in which they were either directly adjacent to (Group 3-sec) or overlapping with sucrose reward (Group 0-sec). This confirms the findings of Experiment 2, in which no conditioned PER to colors was observed during training and demonstrates that intact, harnessed bees directly associate an odor, but not colors, with sucrose reward. These results indicate that the bimodal color–odor conditioning of intact, harnessed bees constitutes a case of occasion setting in which colors determine appropriate responding to an odor. Such an occasion setting was not possible when colors and odor were temporally dissociated (Group 9-sec, presenting a gap between the two kinds of stimuli), but was established whenever they were adjacent or overlapping.

Experiment 4: Stimulus adjacency vs. overlap in bimodal conditioning of harnessed bees

In this experiment we attempted to analyze the contribution of color–odor adjacency and color–odor overlap to the occasion-setting mechanism. Given that discrimination between odor-rewarding and odor-nonrewarding trials was possible when colors were adjacent to (Group 6-sec in Experiment 3) or overlapped with the odor (Groups 3- and 0-sec in Experiment 3), and that the highest discrimination performance (i.e., highest DI) was found in the Group 3-sec, where colors anticipated but also overlapped with the odor, we aimed at determining the temporal component that was more important for the modulation of olfactory learning by colors.

To answer this question, we trained two independent experimental groups of bees with a XO+ vs. YO− discrimination, where 1-nonanol was the odor (O) that could be (+) or not (−) associated with sucrose reward, and X and Y the colors that predicted odor contingency. The training procedure was the same as in Experiment 2. Colors used were UV and green with two balanced subgroups (UV/O+ and green/O− and UV/O− and green/O+). Colors were presented for 3 sec, while odor was presented for 6 sec. In Group "Adjacent," the interstimulus interval between color onset and odor onset was 3 sec and there was no overlap between the stimulations (Fig. 5A). In Group "Overlap," the interstimulus interval was 0 sec as color and odor started simultaneously, so that overlap was 3 sec, i.e., the duration of the color stimulation (Fig. 5B). In both groups sucrose reward was delivered for 3 sec, 3 sec after odor onset.

Figure 5 (A–B) shows the performance of the "Adjacent" and "Overlap" groups. In both cases there were no significant differences between the performances of the balanced groups in which color contingencies were inverse, so that their results were pooled (*subgroup* × *stimulus* × *trial* ANOVA, *subgroup* effect, Adjacent Group: $F_{(1,46)} = 0.012$, NS; Overlap Group: $F_{(1,46)} = 0.001$, NS). An analysis over the entire conditioning procedure showed that when colors and odor were presented simultaneously (Overlap Group; Fig. 5B) bees achieved discrimination between odor-rewarding and odor-nonrewarding trials (*stimulus* × *trial*

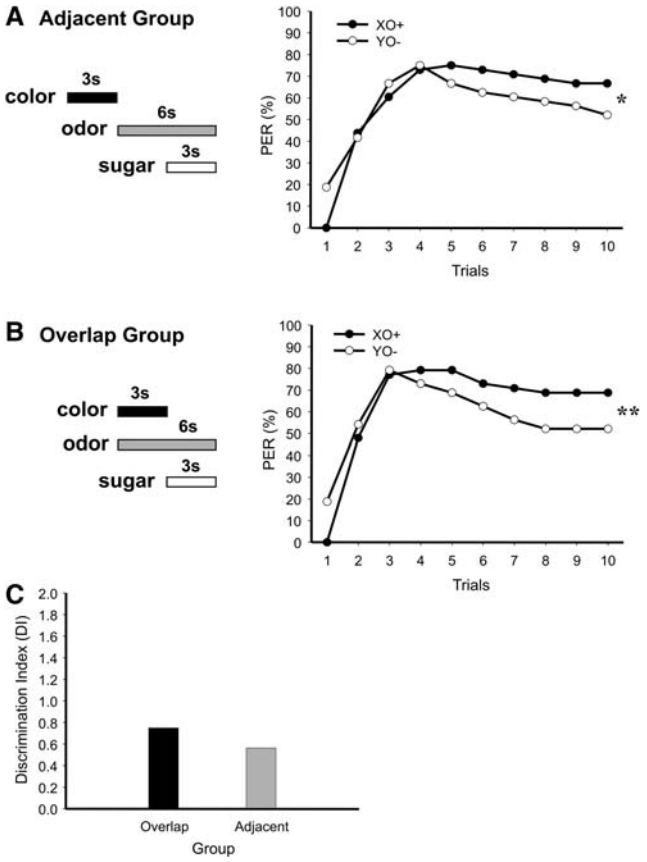

Figure 5. Stimulus adjacency vs. overlap in bimodal conditioning of harnessed bees. Two independent groups were trained in bimodal conditioning with a constant duration of 3 sec for color and 6 sec for odor stimuli, but with a different interval between these stimuli. (*A*) Adjacent Group ($n = 48$ bees): color started 3 sec before odor onset. Color offset was simultaneous with odor onset, so that there was no overlap between color and odor. (*B*) Overlap group ($n = 48$ bees): color and odor started simultaneously, so that they overlapped during 3 sec. In both groups (*A*,*B*) bees showed significantly more responses to OX+ than to OY− along training, thus discriminating between rewarded and nonrewarded trials. Asterisks indicate significant differences in PER responses to OX+ and OY− in the last five conditioning trials (two-way repeated-measure ANOVA —*stimulus* effect). (*C*) Discrimination indices calculated for both experimental groups show no difference.

ANOVA, *stimulus* effect, $F_{(1,46)} = 5.53$, $P < 0.05$). On the contrary, when colors preceded the odor (Adjacent Group, Fig. 5A), discrimination was not significant ($F_{(1,46)} = 1.38$, NS). Observation of Fig. 5B nevertheless suggests that discrimination also developed in the Adjacent Group. Focusing the analysis on the last five XO+/YO− trials confirmed this impression (Adjacent Group: $F_{(1,46)} = 4.2$, $P < 0.05$; Overlap Group: $F_{(1,46)} = 12.8$, $P < 0.001$), thus showing that both stimulus overlap and adjacency contribute to the occasion setting mechanism. This conclusion was confirmed by analysis of the discrimination index, which showed that DI did not differ significantly between the two groups (Fig. 5C, Mann-Whitney test; $Z_{adj.} = 0.04$, NS). These results confirm that colors can modulate olfactory learning both when presented in a forward-paired, nonoverlapping fashion, and when overlapping with the odor.

Discussion

Our results constitute the first account of occasion setting in the framework of studies on conditioning of the proboscis extension reflex. We show that bimodal conditioning of the proboscis extension reflex is possible using colors as occasion setters and an odor as the stimulus that needs to be disambiguated. Colors modulated responding to a partially reinforced conditioned odor presented in close temporal proximity, thus setting the occasion for responding (or not responding) to the odor. Despite the contiguity of both kinds of stimuli with sucrose reward, colors did not enter into direct association with this US, while odors did. By showing this, we refuted the idea that antennal amputation of harnessed bees is required for observing experience-dependent changes in behavior when colors are used as stimuli and proboscis extension reflex as the read-out of such plasticity. Although antennae-ablated bees learned a direct association between color and sucrose reward, bees with intact antennae never exhibited conditioned PER to colors, but learned to use colors as occasion setters for appropriate odor responding.

Antennal presence interferes with color conditioning of proboscis extension reflex

In harnessed bees, antenna deprivation is required for successful visual conditioning of PER (Hori et al. 2006, 2007; Niggebrügge et al. 2009; this study). Thus, sensory input from the antennae somehow interferes with the formation of associations between visual stimuli and sucrose reward. Indeed, the antennae are very important organs for bees, as they are involved in many sensory processes such as olfactory, mechano-sensory, gustatory, and auditory perception (Frings 1944; Lacher and Schneider 1963; Esslen and Kaissling 1976; Dreller and Kirchner 1993; Goodman 2003). These modalities, which are most relevant when not flying (for instance when communicating with nestmates within the hive or when collecting nectar and/or pollen from flowers) may actively interfere with visual processing when the insect is immobile. We know from our experiments that restrained intact bees perceive colors, since our bees used colors successfully as modulators of olfactory learning (Experiments 2–4). Thus, in restrained bees sensory input from the antennae might simply convey more salient information than that apprehended through visual channels. In such a scenario, competition between sensory modalities for attention and/or for entering into an association with sucrose reward might be biased toward antennal cues, and could interfere with visual conditioning.

According to this hypothesis, the number of conditioning trials required to reach a plateau in the learning curve and the level of conditioned responses reached at the end of training are dramatically different between color and odor PER conditioning. While only a few trials (usually three) are required for successful odor conditioning of PER, and levels of 80%–90% of conditioned PER are usually reached in intact bees (Bitterman et al. 1983; Sandoz et al. 1995; Guerrieri et al. 2005), a much higher number of conditioning trials is required for visual conditioning of PER in antennae-ablated bees (Hori et al. 2006, 2007; this study) and acquisition levels are generally lower (Masuhr and Menzel 1972; Hori et al. 2006; this study). However, such a direct comparison between the success of olfactory conditioning in intact bees and of visual conditioning in antennae-ablated bees is probably not pertinent. In our view, PER conditioning following antenna ablation is not adequate for studying color perception and learning, as antennal ablation may have severe consequences on bees' fitness, motivation, and behavior. Antennae-ablated bees have been found to respond significantly less to tarsal sucrose stimulation than intact bees, thus showing that antennae deprivation can disrupt sucrose responsiveness and possibly the reinforcing function of the sucrose US (de Brito Sanchez et al. 2008). Moreover, we observed in our laboratory that antennae-deprived bees present distorted flight ability, aggressive behaviors against nest mates, and engage in excessive grooming (data not shown). For these reasons, we attempted to demonstrate color-dependent learning in intact bees and we could show that colors cues can indeed control olfactory learning performance via an occasion setting mechanism.

Visual-olfactory conditioning in honeybees: From free-flying to harnessed conditions

Multimodal appetitive learning has been mostly studied in freely flying bees, but results obtained so far yielded different conclusions. On the one hand, several studies indicated a synergistic effect between color and odor within a bimodal compound, so that combined color–odor cues led to better memory formation and retrieval compared with single modality cues (Reinhard et al. 2004, 2006; Kulahci et al. 2008). For instance, in bumble bees, Kunze and Gumbert (2001) found that the mere presence of an odor enhances the discriminability of a pair of colors. These authors suggested that color discrimination may be limited by attention, which may be increased in the presence of an odor. On the other hand, other studies have reported inhibitory effects within a color–odor compound, so that odors tend to overshadow colors based on differences in salience (Couvillon and Bitterman 1982, 1988, 1989; Couvillon et al. 1983; Funayama et al. 1995; Greggers and Mauelshagen 1997). These contradictory results reveal an important limitation of studies on multimodal perception and learning in freely flying bees: the influence of the respective temporal characteristics of the two signals on learning performance, or the type of process involved in the use of multimodal stimuli cannot be dissected. When bees approach a color–odor cued feeder, color may act as a far-distance signal, and odor as a close-up signal. It is thus difficult to interpret bees' performance, given that sequential rather than simultaneous stimulus processing may occur during the approach to the target. These two scenarios, sequential vs. simultaneous stimulus processing, may determine dramatic differences in performance, such as those supporting synergistic vs. inhibitory within-compound processing.

Contrary to experiments with freely flying bees, experiments with harnessed bees allow a precise control of visual and olfactory stimulations and constitute, therefore, a promising model for studying the behavioral and neurophysiological basis of multimodal learning in bees. Such experiments are, nevertheless, rare, probably because of the difficulty of training intact harnessed bees with visual cues (see Experiment 1). In the only report available to our knowledge on bimodal (odor–color) conditioning of

proboscis extension reflex in bees, Gerber and Smith (1998) studied potential blocking of odor learning by color preconditioning. They showed that, contrary to what was expected, a pretrained color did not block odor when delivered in a compound but facilitated olfactory learning. Interestingly, despite this facilitatory effect exerted by color, bees did not react to color after compound training similarly to what we found. In our case, we took advantage of the harnessing situation to propose a bimodal learning task in which the temporary dynamics of color and odor were fully controlled. Contrary to the experiments by Gerber and Smith (1998) in which the odorant was always paired with sucrose reward, the odor used as CS in our experiments was partially reinforced, so that bees had ambiguous experiences with respect to this CS. Under these circumstances, bees resolved this ambiguity by focusing on the extent to which the odor reinforcement contingencies were dependent on color cues. In our protocol, color cues constituted the only feature allowing to categorize odor reinforcement and nonreinforcement, and thus modulated responses to the target odor.

Occasion setting in harnessed bees: The nature of associations between colors, odor, and sucrose

In occasion setting, the feature (also called the occasion setter or the modulator) indicates the actual relationship between a target CS and the US, but does not acquire associative strength by itself (Rescorla et al. 1985; Bouton and Swartzentruber 1986; Schmajuk et al. 1998; Pearce and Bouton 2001). In our experimental conditions, this would be consistent with the odor but not the color entering into association with the US. This is precisely what we found. In Experiments 2–4, for instance, bees did not exhibit PER to the presentation of colors during training, but responded with PER to odor presentations. Moreover, in Experiment 3, bees strongly responded to the odor but not to the colors in retention tests performed 1 h after conditioning. In addition, the level of responses to the odor in the tests was not affected by varying the interval between color and odor during conditioning. The success of discrimination was thus not based on changes in the associative strength of odor or colors, but corresponded to an occasion-setting mechanism as defined above: colors qualified as occasion setters of the odor–US association because they modulated odor responses without directly being associated with the sucrose US. More precisely, one color set the occasion for responding to the odor (positive occasion setter), whereas the other color set the occasion for not responding to the odor (negative occasion setter), in a situation corresponding to an occasion-setting discrimination. Honeybees, therefore, rejoin the list of organisms capable of occasion setting, such as humans (Palmatier and Bevins 2008), rats (Bouton and King 1983; Bouton and Swartzentruber 1986), pigeons (Rescorla et al. 1985), *Aplysia californica* (Colwill et al. 1988), *Drosophila melanogaster* (Brembs and Wiener 2006), and *Caenorhabditis elegans* (Law et al. 2004).

Temporal relationship between colors and odor in occasion setting

In Pavlovian learning, the same stimulus can be directly predictive, indirectly predictive, or nonpredictive of the US when presented in compound with other stimuli, depending on the temporal arrangement of the stimuli. For instance, in appetitive-positive occasion-setting experiments with rats, Ross and Holland (1981) showed that occasion setting was only promoted by a feature (OS) that preceded a target (CS) in a serial relation. In contrast, there was no occasion setting when the feature was presented simultaneously with the target, and in this case the feature directly entered in association with the US. We thus attempted to test whether the success of occasion setting and the associative strengths acquired by color and odor stimuli were modified depending on the temporal arrangement of the two stimuli. Our results showed that occasion setting of colors on odor learning was not possible when colors preceded odor onset and left a trace interval of 3 sec between color offset and odor onset. However, if color preceded the odor and finished just before odor onset without any trace interval, occasion-setting discrimination was achieved. This observation is very important because it shows that colors can act as signals for the odor–US association on their own, without being in a color–odor compound. However, it must be emphasized that the best occasion-setting discrimination performance was achieved when color onset preceded odor onset and when both stimuli overlapped for 3 sec, i.e., when presenting both a forward-pairing component and a compound component of color and odor. Comparing the performance of this group with those in Experiment 4 suggests that both forward and overlap components contribute to overall group performance. It may be, however, that subtle differences in response latencies exist between the forward-preceding (Group Adjacent) and the simultaneous situation (Group Overlap) of Experiment 4. Latencies, which were not measured in our work, could be slightly longer in the simultaneous situation, which would suggest that the bees would need more time to evaluate the color in order to respond afterward to the odor in an appropriate way.

A natural context for occasion setting in honeybees

The modulation of olfactory learning by color cues found in our work may reflect specific adaptations of honeybee learning in the context of natural foraging. In nature, honeybees collect food on flower patches, in which many different floral species can coexist. Advertising cues used by flowers such as color, shape, and odor may be uninformative or even ambiguous when considered alone; however, they provide a unique, distinctive label when considered in a multimodal compound, which reduces the chances of mistaking the rewarding species for another one. The optimal forward-pairing relationship between color and odor found in Experiment 3 could correspond to the normal sequence of sensory stimuli perceived by bees when flying toward a flower. As suggested by von Frisch (1967), bees would first perceive the flower's color and then its odor. After landing on a particular flower, visual learning would be hindered by processing and learning of close-up cues such as odors and tactile information (Kevan et al. 1985; Menzel 1985). In other words, floral colors could set the occasion for specific appetitive responses elicited by odor cues perceived during close-up recognition. In this scenario, proboscis extension responses to odors would reflect flower recognition in the short range, while other behavioral responses related to in-flight orientation, which are not expressed in our harnessed situation, could reflect recognition of visual cues.

Neural bases of occasion setting in honeybees

Our study illustrates a cross-modal interaction between olfactory and visual cues during appetitive learning in the honeybee. This result indicates that the neural circuits involved in the processing of these signals should, at a certain stage, interact. Although visual and olfactory processing have been intensively studied in the honeybee, little is known about the integration of these two sensory modalities in the bee brain. Visual and olfactory processing pathways converge in each brain hemisphere onto a multimodal structure called the mushroom body. Sensory input to this structure (visual, olfactory, mechanosensory, and gustatory) is spatially segregated, except in a specific region termed the basal ring, which receives afferences from the olfactory and the visual circuits (Mobbs 1982; Ehmer and Gronenberg 2002). The mushroom

bodies, and more specifically the basal ring, therefore appear as a possible substrate where color–odor interactions could take place. In the fruit fly, the mushroom bodies confer sensory gain control and inhibitory gating, allowing processing of salient signals as well as filtering of background noise and irrelevant signals (Guo and Guo 2005; Liu et al. 2007; Xi et al. 2008). In honeybees, the higher salience of olfactory over visual cues could also be modulated by the mushroom bodies. Alternative regions for cross-talk between visual and olfactory circuits in the bee brain have also been suggested in the median, lateral, and posterior protocerebrum and in the dorsal lobe (Erber and Menzel 1977; Maronde 1991). Future work should attempt to determine the nature of the interactions between odor and color pathways in the mushroom bodies and in further areas of the bee brain.

Materials and Methods

Honeybees *Apis mellifera* were collected from an outdoor hive and brought to the laboratory where they were placed in small glass vials and cooled on ice until they ceased their movements.

Experiment 1: Color conditioning of PER in harnessed bees

Bees were prepared and conditioned with monochromatic light stimuli following the procedure described in Hori et al. (2006). Briefly, in half of the bees both antennae were cut with fine scissors at the base of the scapus. The other half conserved the antennae intact. Bees with leaking hemolymph were discarded from the experiments. Both groups of bees were then individually harnessed in small metal tubes so that they could only move their antennae (if present) and mouthparts, including the proboscis. For 2 d, bees were kept in the dark at high humidity and fed 10 μL of 50% (weight/weight) sucrose solution every morning and evening. Thereafter (48 h after harnessing) (Hori et al. 2006), each subject was checked for intact PER by lightly touching the proboscis with a toothpick soaked with the sucrose solution without subsequent feeding. Animals that did not show the reflex were discarded. During conditioning, each bee was placed in the center of the experimental arena, located in a room illuminated with 40 W fluorescent neon lamps. The arena consisted of a tube holder ($10 \times 7 \times 2.5$ cm) and a sheet of translucent (UV-Visible transmitting) tracing paper that was attached as a roof providing a 5-cm-high ceiling in the center of the arena (Hori et al. 2006). Monochromatic lights (UV, $\lambda = 350$ nm; blue, $\lambda = 439$ nm, green, $\lambda = 540$ nm, or red, $\lambda = 618$ nm) were provided by a Polychrome V System (TILL Photonics GmbH), equipped with a 150 W Xenon lamp. Wavelength and intensity selection were controlled by a computer program (TILL visION 4.0.1.3). Each monochromatic light was adjusted to have an irradiance of 84 ± 2 photon counts $\times$ msec^{-1} at the level of the bee's head, measured using a fixed grating spectrometer (Ocean Optics S2000). In experiments requiring ultraviolet light (350 nm), the experimenter used UV protective eyewear (Ultra-Violet Products Ltd.) and Sol-Vex Premium protective gloves (Ansell Edmont Healthcare). A quartz light guide was placed above the roof of the arena, and the exit pupil of the light guide was set 20 cm above the head of the harnessed bee so that the entire tracing paper roof was illuminated with the chosen wavelength.

Bees were trained to associate a monochromatic light (UV, blue, green, or red) as CS with a reward of 50% (weight/weight) sucrose solution as US during 10 conditioning trials per day. Trials were separated by an intertrial interval (ITI) of 10 min. Conditioning proceeded during two days (20 trials in total). Each trial lasted 60 sec. At the beginning of each trial the subject was placed inside the arena for 30 sec to allow familiarization with the experimental context. Thereafter, the monochromatic light was presented for 7 sec. Four seconds after the onset of the CS, 1 μL of 50% sucrose solution was delivered to the bee by means of a micropipette, which directly touched the proboscis to evoke PER. The bee was then fed for 3 sec. Thus, the interstimulus interval (ISI) was 4 sec and the overlap between CS and US was 3 sec. After stimulation, the bee was left in the setup for 23 sec and then removed. The beginning and end of each trial, as well as the onset and offset of CS and US, were controlled and signaled by a computer programmed to emit tones of different frequencies for each event. Once the 10 trials of a conditioning session were completed, bees were kept in the dark and high humidity without being fed until the next conditioning session on the second day (i.e., for about 22 h). During each trial, we recorded whether or not bees extended the proboscis within the 4 sec of CS stimulation (conditioned responses to color) before the US presentation. Multiple responses during a CS were counted as a single PER.

Experiment 2: Bimodal (color–odor) conditioning of harnessed bees

Honeybees with intact antennae were individually harnessed and kept in the dark and high humidity for 2 h. Fifteen minutes before starting the experiment, each subject was checked for intact PER by lightly touching the antennae with a toothpick soaked with 50% sucrose solution without subsequent feeding.

Training consisted of 10 rewarded trials using a compound stimulus (see Fig. 2) with a visual (X) and an olfactory (O) component (XO+) and 10 nonrewarded trials using a compound stimulus with a different visual component (Y) but the same odor (YO−). Thus, bees were conditioned along 20 trials (10 reinforced and 10 nonreinforced) in which odor–color combinations (XO+ and YO−) were presented in a pseudorandom sequence starting with combination XO+ or YO− in a balanced way. At most, two reinforced/nonreinforced trials followed each other within a conditioning phase.

Visual stimuli (X and Y) consisted of two of the three wavelengths used in the previous experiment (350, 439, or 540 nm). The odorant used (O) was 1-nonanol (Sigma Aldrich). Five microliters of pure odorant were applied onto a 1-cm^2 stripe of filter paper placed into a 20-mL syringe, which allowed odorant delivery to the antennae. Each trial lasted 60 sec. At the beginning of each trial the subject was placed inside the set-up during 25 sec to allow familiarization with the experimental context. An air extractor placed behind the bee prevented odorant accumulation. Thereafter, the monochromatic light was presented for 12 sec. Three seconds after color onset, the odor was presented for 6 sec (Fig. 2). In rewarded trials, 3 sec after odor onset, 1 μL of 50 % sucrose solution (US) was delivered for 3 sec to the bees' antennae and proboscis by means of a micropipette. Afterward, the bee was kept in the setup for 23 sec before being removed. The ITI was always 10 min. Two independent groups of bees were trained in order to balance the contingencies of the two wavelengths used as X and Y. A control group was trained to discriminate 10 rewarded trials with the odor (O+) from 10 nonrewarded trials with the same odor (O−) in the absence of colors (Fig. 3A). Additional control groups were trained to discriminate 10 rewarded trials with a chromatic stimulus (X+) from 10 nonrewarded trials with another chromatic stimulus (Y−) in the absence of any odor (Fig. 3B). In the controls, odor and colors were presented following the same dynamics as in the experimental groups. In the control with two colors, two independent groups of bees were trained in order to balance the contingencies of the two wavelengths used as X and Y.

The beginning and the end of each trial, as well as the onset and offset of visual, olfactory, and sucrose stimulation were controlled and signaled by a computer programmed to emit tones of different frequencies for each event. We quantified PER during visual and/or olfactory stimulation. Figures 2 and 3A–B represent the percent of PER during the 3 sec before US delivery.

Experiment 3: Temporal relationship between color and odor in bimodal conditioning of harnessed bees

Bees prepared as in the previous experiment (Experiment 2) were trained using a compound stimulus with a visual (X) and an olfactory (O) component (XO+) that had to be discriminated from a compound stimulus with a different visual component (Y) but

the same odor (YO−). Bees were conditioned along 20 trials (10 XO+ and 10 YO− trials) in which odor–color combinations (XO+ and YO−) were presented in a pseudorandom sequence starting with combination XO+ or YO− in a balanced way. Color and odor stimulations lasted 6 sec each. Ultraviolet (350 nm) and green (540 nm) were used as stimulating wavelengths and 1-nonanol as olfactory stimulus (O). The training procedure was the same as in the previous experiment (Experiment 2) with respect to the number, sequence, and duration of rewarded (XO+) and nonrewarded (YO−) trials and the intertrial interval (ITI).

Four independent groups differing in the interval between color and odor stimulation were trained and tested: (1) Group 9-sec (Fig. 4A): the odor started 9 sec after color onset so that there was a 3-sec gap between the stimulations; (2) Group 6-sec (Fig. 4B): the odor started 6 sec after color onset so that there was no gap between the two stimulations; (3) Group 3-sec (Fig. 4C): the odor started 3 sec after color onset so that there was an overlap of 3 sec between the stimulations; (4) Group 0-sec (Fig. 4D): odor and color started simultaneously.

Retention tests were performed 1 h after the last conditioning trial and consisted in a randomized presentation of the odor (1-nonanol), green (540 nm), and ultraviolet light (350 nm) without reward. The interval between tests was 10 min.

Experiment 4: Stimulus adjacency vs. overlapping in bimodal conditioning of harnessed bees

Bees prepared as in the two previous experiments (Experiments 2 and 3) were trained using a compound stimulus with a visual (X) and an olfactory (O) component (XO+) that had to be discriminated from a compound stimulus with a different visual component (Y) but the same odor (YO−). This experiment was identical to Experiment 3 (training along 10 XO+ and 10 YO− trials) except for the duration of color stimulation, which lasted 3 sec instead of 6 sec. Odor stimulation lasted 6 sec. In the "Adjacent Group" (Fig. 5A), color started 3 sec before odor onset; color offset was simultaneous with odor onset, so that there was no overlap between color and odor. In the "Overlap group" (Fig. 5B), color and odor started simultaneously, so that they overlapped for 3 sec.

Statistical analysis

In Experiment 1, ANOVA for repeated measures was used for within-group comparisons (one-way ANOVA) to determine whether performance increased in the course of training. Two-way ANOVA (*group* × *trials*) was used to compare acquisition performance between intact and antennae-deprived bees. In Experiments 2, 3, and 4, two-way repeated measure ANOVA (*stimulus* × *trial*) was used to determine whether bees learned to discriminate XO+ from YO− both during the entire conditioning procedure and during the last five XO+ and YO− trials. Studies based on Monte Carlo simulations have shown that ANOVA may be used on dichotomous data like PER under controlled conditions, which were met by our experiments (Lunney 1970).

To compare discrimination success among experimental groups, we used a discrimination index (Di). For each bee, we computed the difference between its responses to the odor in XO+ trials and in YO− trials ($DI = [\Sigma_{XO+}] - [\Sigma_{YO-}]$). This analysis was performed over the whole training procedure (10 trials with XO+ and 10 with YO−) or only during the last five presentations of XO+ and YO−. Depending on the number of experimental groups, we used Mann–Whitney or Kruskal–Wallis tests to compare DI values between groups. χ^2 tests were used to compare retention performances between groups.

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