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RESEARCH ARTICLE

Can honey bees discriminate between floral-fragrance isomers?

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ABSTRACT

Many flowering plants present variable complex fragrances, which usually include different isomers of the same molecule. As fragrance is an essential cue for flower recognition by pollinators, we ask whether honey bees discriminate between floral-fragrance isomers in an appetitive context. We used the olfactory conditioning of the proboscis extension response, which allows training a restrained bee to an odor paired with sucrose solution. Bees were trained under an absolute (a single odorant rewarded) or a differential conditioning regime (a rewarded versus a non-rewarded odorant) using four different pairs of isomers. One hour after training, discrimination and generalization between pairs of isomers were tested. Bees trained under absolute conditioning exhibited high generalization between isomers and discriminated only one out of four isomer pairs; after differential conditioning, they learned to differentiate between two out of four pairs of isomers but in all cases generalization responses to the non-rewarding isomer remained high. Adding an aversive taste to the non-rewarded isomer facilitated discrimination of isomers that otherwise seemed non-discriminable but generalization remained high. Although honey bees discriminated isomers under certain conditions, they achieved the task with difficulty and tended to generalize between them, thus showing that these molecules were perceptually similar to them. We conclude that the presence of isomers within floral fragrances might not necessarily contribute to a dramatic extent to floral odor diversity.

KEY WORDS: Olfaction, Isomers, Learning, Discrimination, Generalization, Proboscis extension response, Honey bees

INTRODUCTION

Flowers advertise their presence and identity to pollinators via salient sensory cues that can be innately attractive or that can be learned and memorized in association with the food reward they provide (nectar and/or pollen). In this way, flowering plants obtain fertilization services while pollinators obtain food necessary for survival (Faegri and van der Pijl, 1978; Kevan and Baker, 1983). Floral fragrances constitute essential cues in insect–flower interactions (Raguso, 2008). They can be learned in association with food so that pollinators may recognize rewarding flowers by their scent (Menzel, 1985; Riffell et al., 2008; Sandoz, 2011). In this way, flower constancy, i.e. the successive foraging visits to the same flower species as long as it remains profitable (Chittka et al., 1999; Waser, 1986), can be maintained, thereby ensuring plant fertilization.

Floral fragrances can include many odorant molecules and appear as olfactory bouquets of variable complexity (Dudareva and Pichersky, 2006; Raguso, 2008). Among these odorants, isomers of the same molecule can be found in floral bouquets (Knudsen et al., 1993). This co-occurrence is frequent in the particular case of food-deceptive orchids. These plants do not provide food reward and nevertheless lure pollinators using different strategies in order to ensure their fertilization (Jersáková et al., 2006; Wright and Schiestl, 2009). Food-deceptive orchids have highly variable intraspecific fragrances (Dormont et al., 2014; Salzmann and Schiestl, 2007; Salzmann et al., 2007a,b), a fact that can be advantageous for a deceptive plant. As visiting and handling a non-rewarding flower can be a costly negative experience (Gaskett, 2011; Jersáková et al., 2006), and the pollinator could learn thereby to avoid that flower based on its distinctive traits, the high variability in floral traits might disrupt avoidance learning and maintain pollinator visits. In other words, learning the association between floral stimuli and absence of reward would be more difficult when flower information is highly variable (Heinrich, 1975; Juillet and Scopece, 2010). As some pollinators such as bees can detect and learn minimal variations in the proportions of odor components in a blend (Locatelli et al., 2016), the presence of different isomers in the floral fragrance of food-deceptive orchids could provide a way to increase olfactory variability and disrupt avoidance learning.

The capacity of pollinators to distinguish between isomers has been studied in the case of the honey bee, which is a representative pollinator and a standard model for the study of olfactory perception, learning and memory (Giurfa, 2007; Giurfa and Sandoz, 2012; Sandoz, 2011). Honey bees are appealing animal models to address this question as they can be easily trained to land on a rewarded odor target (in the case of free-flying bees) or to respond to an odorant by extending their proboscis (PER or proboscis extension response, in the case of harnessed bees), following its association with sucrose solution. In an experiment with free-flying honey bees, 26 odorants were simultaneously presented in adjacent bottles disposed on a vertical arrangement and bees were trained to land on the bottle presenting an odorant associated with sucrose reward. Bees were able to discriminate some floral-fragrance isomers, such as the optical forms of limonene and α-pinene, but not others, such as α-terpineol, indicating that discrimination was substance specific (Laska and Galizia, 2001). Yet, as all 26 odorants were presented simultaneously and distributed over a large area, it is difficult to determine whether bees had indeed access to all information while flying over the bottle arrangement, and thus whether performances reflect discrimination based on comparative evaluation of odorant alternatives.

The olfactory conditioning of PER, which provides a more controlled assessment of odor discrimination and generalization between pairs of odorants (Bitterman et al., 1983; Giurfa and Sandoz, 2012; Matsumoto et al., 2012), was also used to study isomer discrimination in harnessed bees. Using this approach, it was

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Received 15 March 2018; Accepted 18 May 2018

Journal of Experimental Biology (2018) 221, jeb180844. doi:10.1242/jeb.180844

shown that drones can be trained to discriminate between the isomers of 4-methyl-hexanoic acid (Kafka et al., 1973) and that workers can be trained to distinguish cresol isomers, which are indicators of air, water and soil pollution. (Blažytė-Čereškienė and Būda, 2007). None of these odorants is common in floral bouquets: the latter can be typically found in mammalian urine and feces whereas the former has a cheesy smell to humans. Discrimination of isomers present in floral fragrances was studied in the case of the terpenoid carvone (Lensky and Blum, 1974) for which high discrimination was reported. Yet, no information about the PER conditioning and testing procedures was provided, which makes it difficult to evaluate the robustness of this result.

Here, we focused on floral-fragrance isomers with the perspective of understanding the interaction between honey bees and deceptive orchids. The choice of honey bees is justified because these insects are also known as visitors of these orchids (Aguiar, 2014; Gumbert and Kunze, 2001; Suetsugu and Fukushima, 2014) and physiological responses to the fragrance components of deceptive orchids have been characterized in these insects (Galizia et al., 2005; Salzmann et al., 2007a,b). Specifically, we aimed at determining whether these insects learn to discriminate between isomers commonly found within the floral fragrances of these orchids. We used the olfactory PER conditioning, and novel odor-releasing machines with the highest temporal odor resolution as well as state-of-the-art conditioning and testing methods (Matsumoto et al., 2012; Szyszka et al., 2014). We trained bees under two different conditioning regimes: absolute conditioning in which a single odorant is paired with sucrose reward, and differential conditioning in which two odorants are trained, i.e. a positive one associated with sucrose reward, and a negative one associated with absence of reward or with an aversive taste. Although in both cases bees learn to respond to the odorant paired with sucrose, discrimination abilities are usually boosted by differential conditioning, which is thought to enhance attentional processes and thus stimulus discrimination, not only in bees, but also in other species (Avarguès-Weber and Giurfa, 2014; Barth et al., 2014; Perez et al., 2016). As discrimination learning is also increased if the intensity of the penalty associated with the negative odorant is increased (de Brito Sanchez et al., 2015), we also studied differential conditioning of isomers when the negative odorant was associated with an aversive taste (either concentrated saline or quinine solution) (de Brito Sanchez et al., 2015). In other words, we attempted to push olfactory discrimination to its limits to determine the real capacity of bees to distinguish floral-fragrance isomers.

MATERIALS AND METHODS

Bees

Honey bees (*Apis mellifera* Linnaeus 1758) were obtained from the apiary of the Research Center on Animal Cognition located at the campus of the Université Paul Sabatier, Toulouse, France. Female foragers, typically 2–3 weeks old, were collected upon landing on a gravity feeder containing 1 mol l^{-1} sucrose solution and before they started feeding. In this way, we ensured that bees caught for experiments had a high appetitive motivation. Bees were placed on ice for 5 min in order to reduce their activity, then mounted in individual holders and fed with 5 µl of 1 mol l^{-1} sucrose solution. Behavioral tests started 3 h after harnessing the bees. This period is of standard use to keep the bees hungry and ensure a high appetitive motivation for conditioning (Matsumoto et al., 2012). During this time, bees were kept in a dark and humid chamber, following the standard procedure of PER conditioning (see Matsumoto et al., 2012).

Stimuli

Isomers can be classified as structural or spatial. Structural isomers (constitutional isomers) have the same molecular formula but different bonding patterns and atomic organization; spatial isomers (stereoisomers) have the same sequence of atoms, which differs in their orientations in space but not in connectivity or bonding (IUPAC, http://goldbook.iupac.org). We used four pairs of isomers. Three pairs were stereoisomers and the fourth pair included structural isomers (Fig. 1). As structural isomers, we used α-pinene and β-pinene, which were obtained by the 1:1 racemic mixture of their respective stereoisomers. As stereoisomers, we used *R*-(+)-limonene and *S*-(−)-limonene, (+)-α-pinene and (−)-α-pinene, and (+)-β-pinene and (−)-β-pinene. We chose these isomers as they are commonly found in the highly variable bouquet of food-deceptive orchid species but also in the fragrance of many other flower species (e.g. Salzmann et al., 2007a,b; Dormont et al., 2014). Thus, the performance of bees in well-controlled olfactory-discrimination experiments should reveal the role of these odorants in the pollination of these plants. All stimuli used were purchased

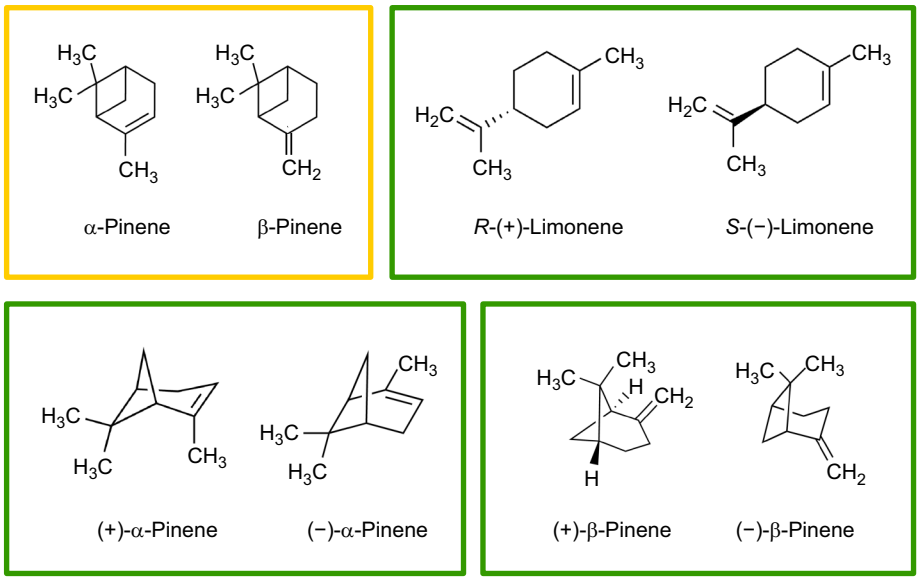

Fig. 1. Isomers used as conditioning stimuli. Each box encloses a pair of isomers, which were used in our experiments. The yellow box contains the structural isomers whereas the green boxes contain the stereoisomers (spatial isomers).

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Table 1. Purity of the odorants used

Odorant	Purity (%)
(+)-α-Pinene	98.0
(−)-α-Pinene	98.0
(+)-β-Pinene	99.0
(−)-β-Pinene	99.0
(*R*)-(+)-Limonene	97.0
(*S*)-(−)-Limonene	96.0

Purity values are from gas chromatograph measurements (commercial description of the product).

from Sigma–Aldrich® (Saint-Quentin Fallavier, France). Table 1 provides the purity of the odorants used.

Olfactory conditioning

Odorants were used as conditioned stimuli in the olfactory PER conditioning, an appetitive Pavlovian conditioning protocol that allows the training of harnessed honey bees to associate odorants, the conditioned stimulus (CS), with a reward of sucrose solution, the unconditioned stimulus (US).

The experimental procedure of the olfactory PER conditioning was the standard one described by Matsumoto et al. (2012). Before conditioning, bees were tested for intact unconditioned PER response by presenting 1 mol l^{-1} sucrose solution to their antennae (which induces PER). Bees that did not show PER during this initial stimulation were either satiated, in poor physical conditions (exhausted) or physically hampered (their proboscis was probably stuck in the harnessing tube). Irrespective of the reason for an absence of PER, bees non-responding to sucrose were removed from the experiment. Indeed, an absence of PER to sucrose will also result in an absence of PER to the conditioned odor, which in these cases does not correspond necessarily to an absence of learning (Matsumoto et al., 2012).

The odorants to be conditioned were inside glass vials and were delivered by an automated odor-releasing machine controlled by a microcomputer (Arduino® Uno, Tilburg, North Brabant, The Netherlands). The harnessed bee was placed in front of the machine. The set-up released a continuous flow of clean air to the bee antennae and the airflow could be diverged upstream to the vials containing the odorants, to provide the conditioned stimulus for 4 s. An air extractor was placed behind the bee to prevent odorant accumulation. Following the odor stimulation, a 1 mol l^{-1} sucrose solution (US) was delivered to the antennae and proboscis for 3 s, with 1 s overlap with the odorant in the case of the CS+ (rewarded CS). Thus, the inter-stimulus interval was 2 s. Each trial lasted 1 min and started with the setting of the bee in the conditioning device for 26 s, which was followed by the CS–US stimulation (for a total of 6 s); finally, the bee was left in the set-up for another 28 s until it was removed and replaced by the next bee. One hour after conditioning, a retention test was performed in which the CS was presented without reward. In this test, the non-conditioned isomer of a pair was also presented in order to assess generalization or discrimination, depending on the experiment performed. Test odors were presented in a sequence that was randomized from bee to bee.

Experiment 1: absolute conditioning

In absolute conditioning, a single odorant was paired with sucrose reward. To this end, three conditioning trials spaced 10 min apart were used, as this ensures high acquisition and retention (Giurfa, 2007; Giurfa and Sandoz, 2012; Menzel, 1999). Two groups of bees were conditioned in parallel, each one with one of the two isomers of a given pair as CS+. One hour after conditioning, both groups had a retention test, in which the CS was presented, and a generalization test with the alternative isomer of the pair. No reward was delivered during the tests.

Experiment 2: differential conditioning

In differential conditioning, two odorants were used as CS: one paired with sucrose reward (CS+), and another that was not associated with reward (CS−). Bees had to learn the discrimination between the CS+ and the CS−. This conditioning form is particularly useful to determine whether individuals can indeed distinguish between two stimuli as it improves discrimination in various species and sensory modalities due to the different outcome of the conditioned stimuli (Avarguès-Weber and Giurfa, 2014; Barth et al., 2014; Desmedt et al., 2017; Dyer and Chittka, 2004; Giurfa, 2004; Giurfa et al., 1999; Hanson, 1959; Josens et al., 2009; Perez et al., 2016).

The two isomers of a given pair were used as conditioned stimuli to determine whether bees could indeed discriminate them. Ten conditioning trials spaced 10 min apart were used (i.e. five CS + and five CS− trials). The presentation of the CS+ and CS− during conditioning was pseudorandomized. As in the previous experiment, bees had a retention test 1 h after conditioning. In this test, they were presented again with the two trained odorants, both in the absence of reinforcement.

Experiment 3: differential conditioning with higher penalty on the CS–

In order to potentially boost discrimination learning of isomers, we enhanced the penalty associated with the CS− as this procedure has been shown to enhance discrimination (Avarguès-Weber and Giurfa, 2014). While the CS+ continued to be paired with sucrose reward, the CS− was now paired with 60 mmol l^{-1} quinine solution or with 3 mol l^{-1} salt (NaCl) solution, which affect olfactory differential conditioning (de Brito Sanchez et al., 2015). This experiment was performed using the pair (+)-α-pinene and (−)-α-pinene as the CS. We chose this pair of isomers as bees were unable to discriminate between them in Experiments 1 and 2 (see the Results section for further details).

Data analysis and statistics

During the tests and conditioning trials, PER was recorded as a binomial response (0 for absence of PER and 1 for occurrence of PER). We calculated and represented the proportion of bees exhibiting PER to the conditioned odorants (i.e. conditioned responses) in any conditioning trial and test. For the acquisition, the proportion of bees that chose the CS+ (absolute conditioning) or the CS+ and the CS− (differential conditioning) was analysed by means of a generalized linear mixed model (GLMM) for binomial family in which 'Trial' was considered a continuous factor (trial effect) and the 'Individual Identity' (Bee) and 'Date' (replicate) were random factors (individual effect). Test proportions were also analysed using GLMM for binomial family, in which the 'Individual Identity' (Bee) and 'Date' (replicate) were considered random factors (individual effect). This procedure showed that there was no effect of the identity of the CS+ within each pair of isomers, i.e. that learning was the same irrespective of the isomer of the pair that was associated with sucrose reward. Thus, we were able to pool these data for both the acquisition and the tests. Multiple comparisons were performed using Tukey's method (*z*-values reported throughout the article).

All statistical analyses were performed with R 3.4.2 (http://www.R-project.org/). Packages *lme4* (Bates et al., 2014; Bretz et al.,

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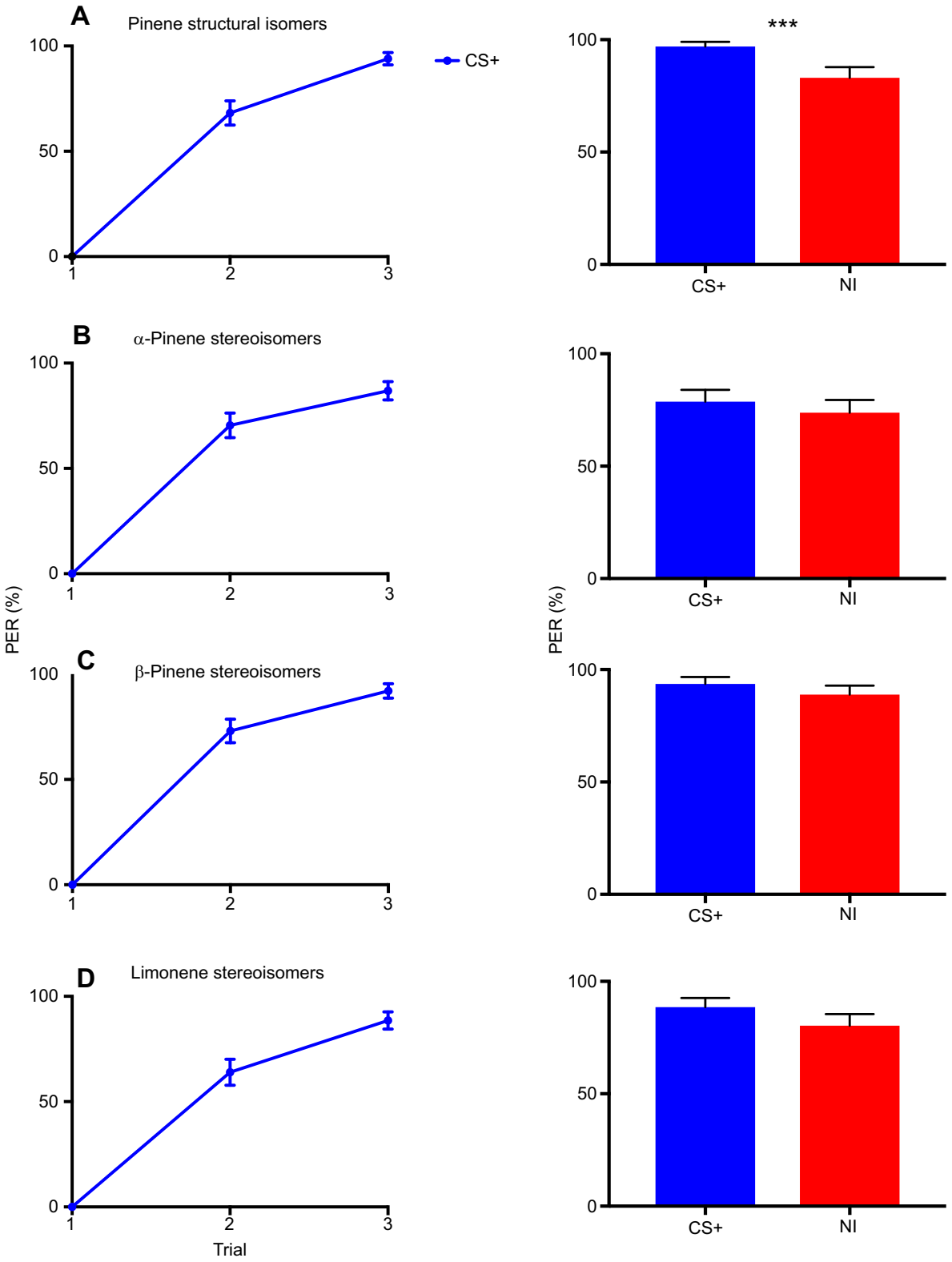

Fig. 2. Experiment 1: absolute conditioning. The left panels show the pooled acquisition curves for the two subgroups of each isomer pair (each subgroup was trained with one of the isomers of the pair) during absolute conditioning. Bees were trained to associate a given isomer (CS+) with sucrose solution during three conditioning trials. The right panels show the pooled retention (blue bar, response to the CS+) and generalization performances [red bar, response to the non-trained isomer (NI)] of the two subgroups of each isomer pair tested 1 h after conditioning in the absence of reward. A significant discrimination between the CS+ and the NI was only found for the pair of structural isomers of pinene (P<0.001, ANOVA). Yet, in all cases, including the pinene structural isomers, generalization to the NI was very high, thus showing that bees perceived isomers of a pair as highly similar. Error bars represent the standard error of the mean (s.e.m.). (A) Pinene structural isomers, N=65; (B) α-pinene stereoisomers, N=61; (C) β-pinene stereoisomers, N=63; (D) limonene stereoisomers, N=61. PER, proboscis extension response. ***P<0.001.

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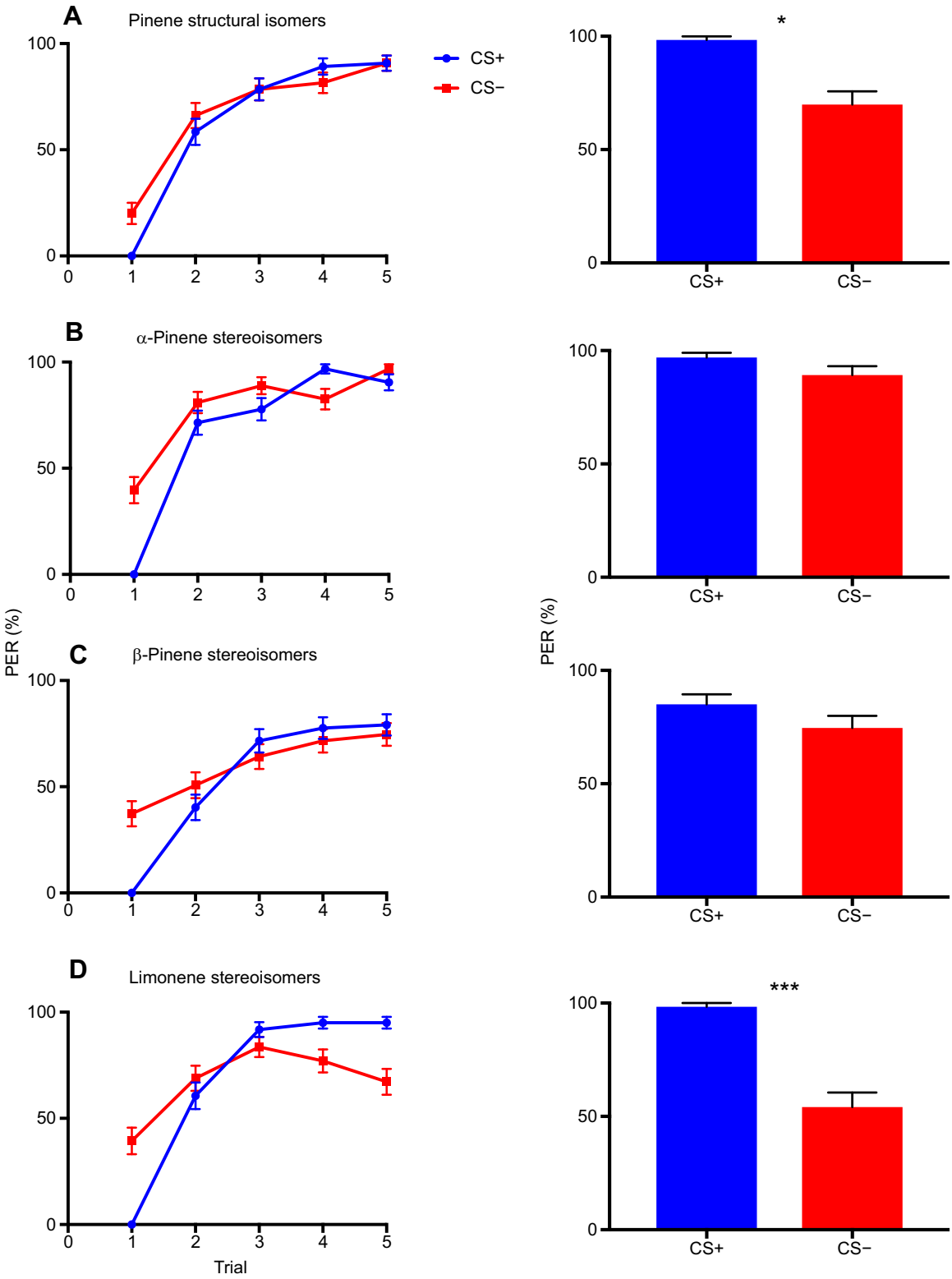

Fig. 3. Experiment 2: differential conditioning. The left panels show the pooled acquisition curves for the two subgroups of each isomer pair trained to discriminate the two isomers during differential conditioning. Bees were trained to associate a given isomer (CS+) with sucrose solution and the alternative isomer with absence of reward (CS−) during 10 conditioning trials (five CS+ and five CS− trials). Bees learned to differentiate the limonene stereoisomers (P<0.001, Tukey's test) but were unable to learn to discriminate the other three pairs of isomers. The right panels show the pooled retention performance (blue bar, response to the CS+; red bar, response to the CS−) of the two subgroups of each isomer pair tested 1 h after conditioning in the absence of reward. During retention, discrimination was again visible for the limonene stereoisomers (P<0.001, ANOVA) but also for the pinene structural isomers pair (P=0.03, ANOVA). In all cases, there was a high level of generalization between CS+ and CS−. Error bars represent the standard error of the mean (s.e.m.). (A) Pinene structural isomers, N=65; (B) α-pinene stereoisomers, N=63; (C) β-pinene stereoisomers, N=67; (D) limonene stereoisomers, N=61. PER, proboscis extension response. *P<0.05; ***P<0.001.

2016) and *lsmeans* were used for GLMMs and Tukey's method for multiple comparisons, respectively. Data are available upon request to the senior author M. Giurfa.

RESULTS

Experiment 1: absolute conditioning

We trained bees to associate a given isomer with a reward of sucrose solution. Retention was tested 1 h after conditioning by presenting the training isomer without reward. The alternative isomer of the pair was also presented in a generalization test 1 h after conditioning. Learning was similar for the two isomers of a pair so that a single curve is shown in Fig. 2 for a given pair. For all isomers trained, there was a trial effect (pinene structural isomers: d.f.=2, χ^2=45.814, P<0.001, N=65; α-pinene stereoisomers: d.f.=2, χ^2=55.483, P<0.001, N=61; β-pinene stereoisomers: d.f.=2, χ^2=62.136, P<0.001, N=63; limonene stereoisomers: d.f.=2, χ^2=49.857, P<0.001, N=61), indicating that bees learned across trials to associate the isomer trained with sucrose reward (Fig. 2).

During the retention tests, bees responded to the CS+ at a level that was similar to that reached in the last acquisition trial (Fig. 2, blue bars); thus, showing efficient mid-term retention. The response to the alternative, non-trained isomer (NI) was also high and comparable to that of the CS+; thus, showing high generalization or lack of discrimination between isomers of a pair. This was particularly evident for stereoisomer pairs (α-pinene stereoisomers: CS+=78.68%, NI=73.77%; choice effect, d.f.=1, χ^2=1.9467, P=0.16; β-pinene stereoisomers CS+=93.65%, NI=88.88%; choice effect, d.f.=1, χ^2=1.0374, P=0.30; limonene stereoisomers CS+=88.52%, NI=80.32%; choice effect, d.f.=1, χ^2=3.6709, P=0.06). In the case of the pinene structural isomers, generalization was also very high, yet significantly different from the response to the CS+ (CS+=96.92%, NI=83.07%; choice effect, d.f.=1, χ^2=14.1759, P<0.001), indicating that 1 h after absolute conditioning, bees could differentiate between these two structural isomers (Fig. 2A), even if they treated them as highly similar. Thus, both structural and stereoisomers were perceptually similar to bees. In the case of stereoisomers, the question remains of whether they can be indeed distinguished at all. The next experiment aimed at answering this question.

Experiment 2: differential conditioning

We trained bees to discriminate the two isomers of a given pair by rewarding one with sucrose solution and presenting the other without sucrose. In this way, we aimed to determine whether bees can indeed discriminate those isomers that elicited similar responses as the CS+ in the previous experiment. Retention was tested 1 h after conditioning by presenting both the rewarded and the non-rewarded isomer without reward. For all isomer pairs, acquisition was similar, irrespective of which isomer was rewarded or non-rewarded. Thus, the results of the two subgroups of an isomer pair were pooled and presented as CS+ versus CS− discrimination curves (Fig. 3, left panels).

For all isomer pairs, we found a trial effect as responses varied along trials (pinene structural isomers: d.f.=4, χ^2=125.899, P<0.001, N=65; α-pinene stereoisomers: d.f.=4, χ^2=128.510, P<0.001, N=63; β-pinene stereoisomers: d.f.=4, χ^2=119.829, P<0.001, N=67; limonene stereoisomers: d.f.=4, χ^2=124.908, P<0.001, N=61). Yet, for all isomer pairs, responses increased both for the CS+ and the CS− so that in three out of four cases (α-pinene stereoisomers, β-pinene stereoisomers and pinene structural isomers), bees were unable to discriminate the two isomers of a pair during acquisition (CS+ versus CS−: pinene structural isomers: z_{1298}=−1.910, P=−0.66; α-pinene stereoisomers: z_{1258}=1.403, P=0.92; β-pinene stereoisomers: z_{1398}=0.339, P=1). Only in the case of limonene stereoisomers (Fig. 3D), discrimination was significant (CS+ versus CS−: z_{1218}=−4.334, P<0.001) but, even in this case, response levels to the CS− remained high at the end of training.

In the retention tests, bees again responded to the CS+ at a level that was similar to that reached in the last acquisition trial (Fig. 3, blue bars). Responses to the CS− remained high and discrimination was again visible for the limonene stereoisomers (CS+=98.36%, CS−=54.09%; choice effect, d.f.=1, χ^2=13.1679, P<0.001) but also for the pinene structural isomers (CS+=98.41%, CS−=69.84%; choice effect, d.f.=1, χ^2=8.5088, P=0.03). The other pairs were not discriminable (α-pinene stereoisomers: CS+=96.92%, CS−=89.23%; choice effect, d.f.=1, χ^2=3.0227, P=0.08; β-pinene stereoisomers CS+=85.07%, CS−=74.62%; choice effect, d.f.=1, χ^2=1.3716, P=0.24). Thus, after differential conditioning, discrimination was found for the structural isomers of pinene and stereoisomers of limonene (Fig. 3A,D). Yet, even if these isomers could be discriminated, high levels of generalization were observed between members of a pair. In the other cases, bees did not show significant discrimination between isomers (Fig. 3B,C).

Experiment 3: the effect of quinine and saline solutions on discrimination learning

In this experiment, we aimed at improving discrimination by increasing the penalty associated with the CS− in the differential conditioning procedure. While the CS+ continued to be paired with sucrose reward, the CS− was now paired with 60 mmol l^{-1} quinine solution or with 3 mol l^{-1} NaCl solution. This experiment was performed with the α-pinene stereoisomers, for which no discrimination was found in the previous experiment (Fig. 3B).

When quinine solution was used as the negative reinforcement paired with the CS− (Fig. 4A), acquisition did not change compared with that obtained when the CS− had no reinforcement (Fig. 3B). The group trained with quinine increased its responses to odorants along trials (trial effect: d.f.=4, χ^2=64.328, P<0.001, N=60) but did not learn to discriminate the CS+ from the CS− (CS+ versus CS−: z_{858}=−1.553, P=0.87). Only in the retention test, a significant discrimination was found (CS+=90.47%, CS−=80.95%; choice effect, d.f.=1, χ^2=4.6378, P=0.03) but responses both to the CS+ and the CS− were very high (Fig. 4A, blue and red bars); thus, showing a high level of generalization between isomers.

When saline solution was used as the negative reinforcement paired with the CS− (Fig. 4B), bees also increased their responses along trials (trial effect: d.f.=4, χ^2=97.373, P<0.001, N=60) but, this time, they were able to discriminate the two α-pinene stereoisomers at the end of training (Fig. 4B, CS+ versus CS−: z_{1198}=−4.399, P<0.001).

During the retention tests, bees responded significantly more to the CS+ than to the CS− (CS+=90.00%, CS−=71.66%; choice effect, d.f.=1, χ^2=19.879, P<0.001); thus, confirming the significant discrimination observed at the end of training (Fig. 4B, blue and red bars). Again, high percentages of generalization were observed between the two isomers, as in the previous two experiments. It thus seems that under certain reinforcement conditions (here the use of saline solution as aversive US), discrimination between isomers can be improved. Yet, even in these cases, the task remains difficult for bees due to the high levels of generalization between the odorants tested.

DISCUSSION

We performed controlled odor learning and retention experiments to evaluate the capacity of the honey bee to discriminate isomers

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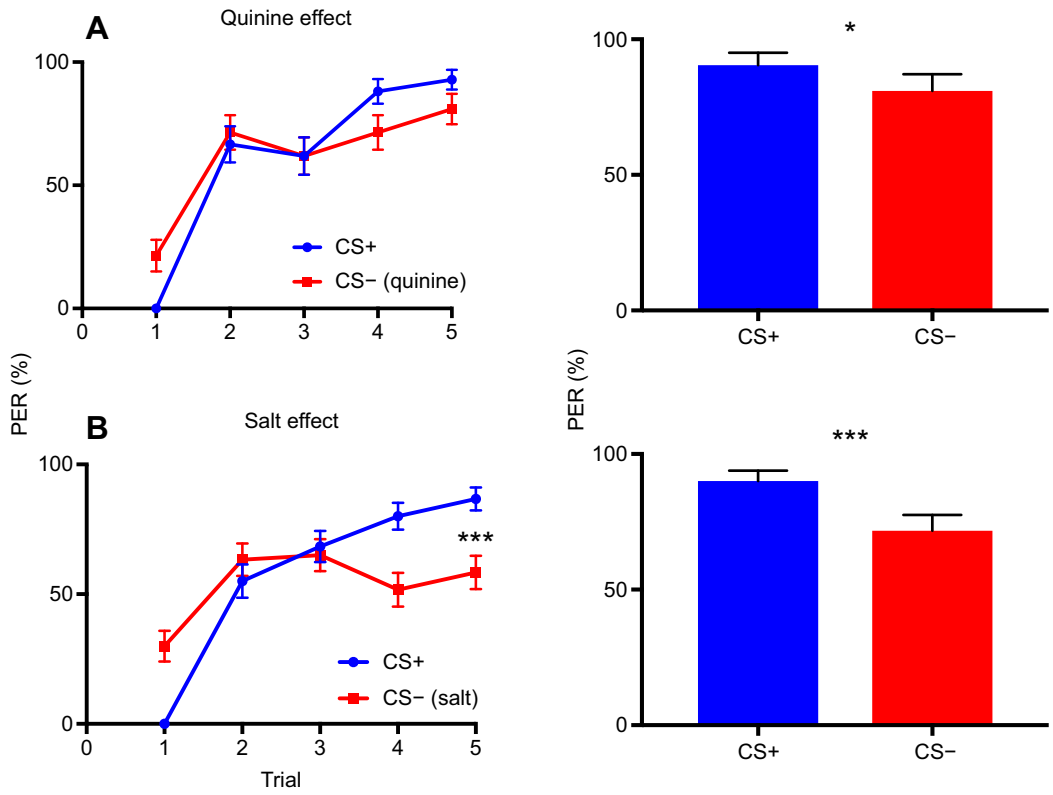

Fig. 4. Experiment 3: the effect of quinine and saline solutions on discrimination learning. The left panels show the pooled acquisition curves for the two subgroups trained to discriminate the two α-pinene stereoisomers during differential conditioning. (A) Bees (*N*=60) were trained to associate a given isomer (CS+) with sucrose solution and the alternative isomer with 60 mmol l^{-1} quinine solution during 10 conditioning trials (five CS+ and five CS− trials). (B) Bees (*N*=60) were trained to associate a given isomer (CS+) with sucrose solution and the alternative isomer with 3 mol l^{-1} NaCl solution during 10 conditioning trials (five CS+ and five CS− trials). Only bees trained with concentrated NaCl solution learned the discrimination (*P*<0.001, Tukey's test). In A and B, the right panels show the pooled retention performance (blue bar, response to the CS+; red bar, response to the CS−) of the two subgroups tested 1 h after conditioning in the absence of reward. Both the quinine and the saline groups responded significantly more to the CS+ than to the CS− (quinine group: *P*=0.03; saline group: *P*<0.001, ANOVA). In both groups, a high level of generalization between CS+ and CS− was observed. Error bars represent the standard error of the mean (s.e.m.). PER, proboscis extension response. **P*<0.05; ****P*<0.001.

commonly found in the floral fragrance of food-deceptive orchids. If isomers were easily discriminated, their variable presence within floral fragrances could contribute to the diversity of floral bouquets of deceptive orchids, thus rendering the association between the absence of reward and a predictable odorant signature difficult. We found that although bees easily learn to associate floral isomers with sucrose reward, and remember them well 1 h later, they can hardly discriminate between pairs of isomers or, if they do it, they exhibit high levels of generalization between these similar stimuli. This indicates that the olfactory isomers used in our study appear very similar to them. In other words, if orchid deception relies in part on the diversity of floral fragrances, the presence of isomers in these fragrances might not contribute to a dramatic extent to this diversity.

Olfactory discrimination is not a fixed perceptual capacity depending strictly on structural properties of the odorants considered. It varies significantly depending on the conditioning procedure employed and thus on the way experimenters ask questions to their experimental subject (Giurfa, 2004; Li et al., 2008; Linster et al., 2002). In particular, differential conditioning enhances odor discrimination as it requests that animals learn to respond to a rewarded odorant and not to a non-rewarded odorant (Barth et al., 2014; Perez et al., 2016). This results in two independent processes being generated: an excitatory generalization gradient around the CS+, and an inhibitory gradient around the CS− (Avarguès-Weber and Giurfa, 2014; Hanson, 1959). Additive and multiplicative interactions between these gradients have been proposed to explain discrimination performances (Perez et al., 2016). In particular, it has been suggested that differential conditioning enhances attentional processes; thus, enhancing discrimination between the CS+ and the CS− (e.g. Carrillo et al., 2000). Insects are not an exception to this situation: differential conditioning enhances discrimination and decreases the level of generalization between olfactory stimuli in fruit flies (Barth et al., 2014) and carpenter ants (Desmedt et al., 2017; Josens et al., 2009; Perez et al., 2016). In honey bees, a similar phenomenon has been observed in the case of visual discriminations (Avarguès-Weber and Giurfa, 2014). In our experiments, differences between absolute and differential conditioning can be seen by focusing on retention performances after training, which were obtained under identical test conditions. After absolute conditioning, discrimination was only found for the structural isomers of pinene (α- and β-pinene). After differential conditioning, it was found both for this pair of isomers and for the stereoisomers of limonene [*R*-(+)-limonene and *S*-(+)-limonene]. The differentiation improved between absolute and differential conditioning from 13.85% to 25.57% for the pinene isomers and from 8.20% to 44.27% for limonene stereoisomers. Note that the 8.20% differentiation was close to significance (*P*=0.06), which shows that changing the conditioning procedure provided the framework for discrimination to be achieved in the case of these odorants. Yet, in both cases, the level of responses to the

CS− after differential conditioning remained high. These responses were even higher in the case of the other stereoisomer pairs [(+) and (−)-α-pinene; (+) and (−)-β-pinene] in which no differentiation was found after differential conditioning.

Increasing the penalty associated with the CS− during differential conditioning can also boost stimuli discrimination (Avarguès-Weber and Giurfa, 2014; de Brito Sanchez et al., 2015). In the honey bee, associating the CS− with an aversive taste, such as concentrated (3 mol l^{-1}) NaCl solution and, to a minor extent, concentrated (60 mmol l^{-1}) quinine solution, increases the discrimination between CS+ and CS− in olfactory PER conditioning (de Brito Sanchez et al., 2015). Our results show that for the pair of stereoisomers of α-pinene that could be differentiated neither after absolute nor after differential conditioning, differentiation was possible if the CS− was paired with quinine. This was further improved when the association was with saline solution (Fig. 4). This difference in inhibitory strength between quinine and NaCl solutions was also found by de Brito Sanchez et al. (2015), and indicates that for honey bee taste, concentrated quinine solution is probably not as aversive as for humans (de Brito Sanchez, 2011) given that its effect was relatively low (3% improvement) compared with saline solution (11% improvement). These results can be explained based on enhanced or diminished attentional processes resulting from the presence or absence of the higher penalty associated with the CS− (Avarguès-Weber and Giurfa, 2014). The absence of penalty or the presence of a low-intensity penalty (quinine solution) would not increase attention, thus rendering the stereoisomers of α-pinene hardly discriminable. On the contrary, the presence of the highly aversive saline solution would enhance attention upon discriminative choices; thus, improving differentiation. Note, however, that even under this condition, bees continued responding to a very high level to the CS−, thus showing that the task was particularly difficult in perceptual terms.

Previous studies showed that free-flying and harnessed bees achieve better discrimination performances when trained to differentiate different types of isomers. Using free-flying honey bees, Laska and Galizia (2001) trained bees to land on three bottles presenting a rewarded odorant in an array of 48 similar bottles disposed in a 70×80 cm vertical rack of six rows of eight bottles each. The other bottles contained alternative, non-rewarded odorants. In a test without reward, bees had to detect the only bottle containing the previously rewarded odorant among the non-rewarded ones presented in the other bottles. The stimuli included 10 pairs of enantiomers. The bees were able to discriminate between the stereoisomers of limonene, α-pinene, β-citronellol, menthol and carvone but failed to distinguish between the (+) and (−) forms of α-terpineol, camphor, rose oxide, fenchone and 2-butanol. In our study, we used the olfactory conditioning of PER and harnessed bees, which provides a more controlled scenario than that of free-flying bees. We found differentiation between the structural isomers of pinene and the stereoisomers of limonene after differential conditioning and between the stereoisomers of α-pinene when NaCl was paired with the CS− in differential conditioning. However, in all cases high rates of generalization between odorants were observed, while Laska and Galizia (2001) observed bees choosing with more than 90% of accuracy. This difference can be explained by considering the experimental design used in the two cases. First, it is probably more costly for a bee to fly and choose a target than to only extend its proboscis. Therefore, accuracy could be enhanced in a free-flying scenario. However, our olfactory PER conditioning compared explicitly responses with the two isomers of a pair that were temporally adjacent (i.e. they were presented one after the other), whereas the design of Laska and Galizia (2001) did not control for the adjacency between rewarded and non-rewarded isomers. Odorants perceived immediately after choosing the trained isomer could be very different; thus, facilitating perceptual contrast and discrimination. Moreover, given the wide range over which bees had to fly, the kind of information used during their choices and the odorants they compared successively while flying over the experimental array remains unknown. Finally, due to the large number of odorants trained in this experiment (N=26), only three bees were trained and tested per rewarded stimulus (Laska and Galizia, 2001), a sample size that could distort conclusions and that contrasts with the high number of individuals (at least 30 per conditioned odorant) trained in our PER protocol.

In addition to the free-flying bee study, three previous studies used the PER conditioning to study isomer discrimination in bees. One of them showed that drones can be trained to discriminate between the isomers of 4-methyl-hexanoic acid (Kafka et al., 1973) whereas another reported that honey bee workers can be trained to distinguish cresol isomers, which are indicators of pollution. (Blažytė-Čereškienė and Būda, 2007). These odorants, however, are not found in the fragrances of flowers pollinated by bees. In fact, cresol isomers are known to be repellent for bees (Jürgens et al., 2006; Kite, 1995). The only known study that used olfactory PER conditioning to determine whether bees distinguish isomers present in floral fragrances (Lensky and Blum, 1974) reported a high discrimination between the stereoisomers of carvone – an odorant present in many flowers pollinated by Euglossine bees (Armbruster et al., 1989; Whitten et al., 1986). However, that study did neither provide information about the PER conditioning procedure nor about data analysis, which makes it impossible to compare with our results. Our study is the first detailed study analysing the olfactory discrimination of floral fragrance isomers by honey bees using the olfactory PER conditioning procedure.

We found that isomer discrimination is substance specific. While it was more difficult for bees to discriminate the pinene isomers, discrimination of the limonene stereoisomers was possible under differential conditioning. The latter seems to be a simple task for other species, such as humans and squirrel monkeys (Laska and Galizia, 2001; Laska and Teubner, 1999; Laska et al., 1999). It has been shown that odorant discrimination and generalization depend on the concentration of the odorants provided (Wright and Smith, 2004). Very low concentrations (e.g. 0.0002 mol l^{-1}) result in high generalization whereas higher concentrations (0.2 mol l^{-1} and 2.0 mol l^{-1}) promote discrimination. In our case, we provided almost pure odorants (Table 1), which excludes the possibility of generalization due to low detectability.

A simple explanation for the difficulty exhibited by bees in discriminating between isomers may be that the degree of purity of the molecules used in our experiments was not high enough to avoid contamination by the alternative isomer. Yet, in all cases, we selected the highest purity available on the market, which was high enough (98–99%) to ensure selective learning, except for limonene isomers whose purity was 96–97%. This is precisely the pair that could be discriminated after differential conditioning, thus showing that the potential presence of impurities, including that of the alternative limonene isomer, did not affect discrimination. It may be argued that even impurities smaller than 1% may be detected by bees and result in generalization. Calcium imaging of the fruit fly antennal lobe showed that an impurity of 0.0006% in a chemical sample was entirely responsible for a sizable response in olfactory receptor cells (Paoli et al., 2017). Yet, a single receptor-cell response does not necessarily translate into a behavioral response. For

instance, in experiments in which bees were trained to discriminate two pure odorants, their response to 90:10 ratios of these odorants was very similar as that elicited by the pure odorants (Fernandez et al., 2009). Thus, a difference of 10% was not detected as inducing a drastic perceptual difference. We thus conclude that potential contaminations with the non-trained isomer around 1–2% were not driving the generalization responses recorded in our tests.

In an ecological scenario, pollinators typically visit two to five flowers of a same deceptive orchid before abandoning the inflorescence (Aguiar, 2014; Jersáková and Johnson, 2006; Johnson et al., 2004; Tuomi et al., 2015). This would be closer to absolute conditioning, as the animal would experience the same stimulus several times, without a different stimulus in between. Also, given the cost of visiting and handling a flower without nectar or pollen reward, a deceptive flower can be seen as a negative experience for a pollinator searching for nectar (Gaskett, 2011; Jersáková et al., 2006). In differential conditioning, we improved discrimination by using appetitive reward (sucrose solution) and punishment (saline solution); yet, deceptive orchid species do not present an aversive taste to its pollinator. Therefore, the natural pollination scenario differs from differential conditioning in the absence of mechanisms to improve discrimination. It is thus plausible to assume that different isomers do not have a major role for the perceptual variability of floral fragrance as in a pollination context it would be difficult for a bee to discriminate between such similar odorants.

Answering questions on pollination using a cognitive approach as the one used in our study allows accessing a pollinator's perspective from an insect–plant interaction. Several studies reported the great floral fragrance variability found in food-deceptive orchids (Salzmann et al., 2007a,b; Salzmann and Schiestl, 2007; Dormont et al., 2014), and this floral trait polymorphism is thought to have an important role on deceiving the pollinators (Heinrich, 1975). However, few studies take into consideration the pollinators' point of view to elucidate this question. Here, we were able to determine to what extent honey bees can differentiate isomers of the same molecule, and how these odorants contribute to the perceptual variability of floral fragrances. The fact that bees find it difficult to discriminate between the isomers tested does, however, not disprove the hypothesis that floral polymorphism could have an important role for deceptive pollination as isomers are only part of fragrance variability. Other sensory cues such as colors and shapes could be highly variable in deceptive orchids (Ackerman et al., 2011), and contribute through their variability to the deception phenomenon. This hypothesis needs to be tested by considering flowers from the insect's perspective and not from the human experimenter's perspective (Juillet and Scopece, 2010; Vorobyev et al., 1997). Our study shows that the information we obtain from plants, as the variable fragrance profiles obtained from gas chromatography analysis, can have a different meaning when the pollinators' cognitive abilities are taken into account.

Acknowledgements
We thank David Baracchi for very useful comments and corrections on a previous version of our manuscript. We also thank Maria Eugenia Villar and Paul Marchal for help with PER experiments, Alexis Buatois for help with data analysis and statistical support, and Lucie Hotier for beekeeping support during the experiments.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: M.G.; Methodology: J.M.R.B.V.A., M.G.; Formal analysis: J.M.R.B.V.A.; Investigation: J.M.R.B.V.A., M.G.; Resources: M.G.; Writing - original draft: J.M.R.B.V.A., M.G.; Writing - review & editing: J.M.R.B.V.A., M.G.; Supervision: A.R., M.S., M.G.; Project administration: M.G.; Funding acquisition: M.G.

Funding
We thank the French Research Council (Centre National de la Recherche Scientifique) and the Université Toulouse III - Paul Sabatier for support. J.M.R.B.V.A. was funded by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) [fellowship 2016/17128-8].

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