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## RESEARCH ARTICLE

# Configural perception of a binary olfactory mixture in honey bees, as in humans, rodents and newborn rabbits

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## ABSTRACT

How animals perceive and learn complex stimuli, such as mixtures of odorants, is a difficult problem, for which the definition of general rules across the animal kingdom remains elusive. Recent experiments conducted in human and rodent adults as well as newborn rabbits suggested that these species process particular odor mixtures in a similar, configural manner. Thus, the binary mixture of ethyl isobutyrate (EI) and ethyl maltol (EM) induces configural processing in humans, who perceive a mixture odor quality (pineapple) that is distinct from the quality of each component (strawberry and caramel). Similarly, rabbit neonates treat the mixture differently, at least in part, from its components. In the present study, we asked whether the properties of the EI.EM mixture extend to an influential invertebrate model, the honey bee *Apis mellifera.* We used appetitive conditioning of the proboscis extension response to evaluate how bees perceive the EI.EM mixture. In a first experiment, we measured perceptual similarity between this mixture and its components in a generalization protocol. In a second experiment, we measured the ability of bees to differentiate between the mixture and both of its components in a negative patterning protocol. In each experimental series, the performance of bees with this mixture was compared with that obtained with four other mixtures, chosen from previous work in humans, newborn rabbits and bees. Our results suggest that when having to differentiate mixture and components, bees treat the EI.EM in a robust configural manner, similarly to mammals, suggesting the existence of common perceptual rules across the animal kindgdom.

KEY WORDS: *Apis mellifera*, Odor mixture, Configural learning, Appetitive conditioning

## INTRODUCTION

Natural sensory stimuli mostly occur in combination with other stimuli and thus constitute compound stimuli, both within and across sensory modalities. When and how animals perceive the elements of a stimulus compound or treat the compound as an entity different from its elements have been the subject of intense research in a wide range of animal models. Experimental approaches mostly based on associative learning gave rise to different theoretical accounts that can be framed in terms of elemental and configural modes of perceptual processing. On the one hand, elemental theories (e.g. Rescorla and Wagner, 1972) consider that compound

stimuli would be treated as a sum of elemental units, each of which potentially enters in association with reinforcement during learning. On the other hand, configural theories (e.g. Pearce, 1987, 1994) treat compound stimuli as distinct configurations, and when mixtures are presented, these configural units directly enter in association with reinforcement. Intermediate accounts also exist and propose the existence of both elemental and configural units (e.g. Rescorla, 1972; Rescorla, 1973; Whitlow and Wagner, 1972).

The complexity of compound stimulus perception is well illustrated by the case of olfactory mixtures, which mostly happen in nature as complex blends of many volatile components and may allow the recognition of certain components (individual odorants) but can also be perceived as distinct odor entities. Experimental results support the existence of these different types of perception in humans and other mammals (Laing and Francis, 1989; Laska and Hudson, 1993; Jinks and Laing, 2001; McNamara et al., 2007; Sinding et al., 2013). Generally, in humans, the more components in a mixture, the more configurally it is perceived, so that a binary mixture (two components) will usually allow recognition of the qualities of its elements (Laing and Francis, 1989). Remarkably, however, it has been observed that the smell of a particular binary mixture, EI.EM, may be more representative (typical) of a target odor, in this case a pineapple odor, than either of the two components, which rather evoke a strawberry (EI, ethyl isobutyrate) and a caramel odor (EM, ethyl maltol) (Le Berre et al., 2008b; Barkat et al., 2012). This observation fits with configural processing, which leads to the perception of a novel odor in addition to (i.e. weak configural perception), or in place of (i.e. robust configural perception), the odor of each component. Remarkably, this effect appears within rather tight boundaries. For instance, the mixture must contain quite specific proportions of each odorant (Le Berre et al., 2008a). Interestingly, weak configural processing of the same mixture has been demonstrated in another mammal species, the European rabbit (*Oryctolagus cuniculus*), at another stage of development (shortly after birth) and using a different protocol than in humans (associative conditioning, mainly). Rabbit pups can learn to associate an initially neutral odorant (conditioned stimulus, CS) with the presentation of the rabbit mammary pheromone (unconditioned stimulus, US). After conditioning, the rabbit pups exhibit head-searching oral grasping responses, which are usually displayed by pups during nursing, to the CS (Coureaud et al., 2006). Using this protocol, it was shown that pups conditioned to the EI.EM mixture respond to the components in unrewarded tests, but when conditioned to one of the elements, EI or EM, they do not generalize to the mixture (Coureaud et al., 2008). As in humans, the effect is quite specific to this particular mixture. For instance, it does not happen with mixtures of either element with a third odorant, guaiacol (G) (Coureaud et al., 2009). It also depends on the relative ratio of the components in the mixture (Coureaud et al., 2011; Schneider et al., 2016). These results suggest that the rabbits perceive in the EI.EM mixture not only the odors specific to components EI and EM, but also a third percept, different and specific to the mixture (EI.EM configuration). This

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hypothesis has been confirmed by a pharmacological approach demonstrating that after conditioning to EI.EM, the pup memory of EI and EM can be erased while they continue to respond to the EI.EM odor quality (Coureaud et al., 2014). Recent results obtained in rodents show that the same mixture is also perceived configurally, at least in part, in adult rats and mice (Wilson et al., 2020). If the EI.EM mixture supports configural processing in four vertebrate species, one may ask whether these properties extend to other animal groups, and possibly even to invertebrates.

The honey bee *Apis mellifera* is an influential invertebrate model for the study of olfactory perception, learning and memory (Galizia and Menzel, 2001; Sandoz, 2011; Galizia, 2014). Honey bees are constantly exposed to olfactory mixtures both within and outside of the hive. Within the colony, these insects employ a rich repertoire of pheromones to communicate in many behavioral contexts such as brood rearing, swarming or colony defense (Free, 1987; Slessor et al., 2005; Sandoz et al., 2007). Honey bee pheromones are complex mixtures of multiple odorants, and different compositions of these mixtures convey different meanings. For instance, the brood pheromone is a mixture of esters of common fatty acids, the proportions of which carry information about a larva's age to nursing bees (Le Conte et al., 1994). While foraging, honey bees use floral cues, and especially odor cues, to find pollen and nectar. Floral scents are mixtures of odorants that vary in composition depending on a plant's genotype and developmental stage and/or with local environmental conditions (Pham-Delègue et al., 1989; Dobson, 1994). Floral scents are also an integral part of the colony's scent, emanating from food stores (nectar, pollen) or being brought back by foragers (Arenas et al., 2008). Honey bees are thus confronted with the problem of discriminating among complex mixtures but also of recognizing the same floral source although its odorant composition varies. Indeed, honey bees are able to differentiate between very subtle differences in odor mixtures, as for instance between two genotypes of the same species or between flowering stages (Pham-Delègue et al., 1989; Wright et al., 2002). In contrast, many of the variations in plant volatile emissions are unrelated to resource quality, and therefore, another key ability is olfactory generalization, i.e. the ability to treat as equivalent stimuli that are perceived as different (Shepard, 1987; Ghirlanda and Enquist, 2003).

In honey bees, olfactory mixture perception has been studied experimentally using the conditioning of the proboscis extension response (PER), a protocol in which the bees learn to associate an initially neutral odor (CS) with a sucrose reward (US) applied to the antennae and then to the proboscis (Bitterman et al., 1983; Giurfa and Sandoz, 2012). When the antennae of a hungry bee are touched with sucrose solution, the animal reflexively extends its proboscis to suck the sucrose. Olfactory stimuli presented to the antennae do not normally release a PER in naive animals. However, if an odor is presented immediately before the sucrose solution (forward pairing, usually with a 3 s inter-stimulus interval), an association is formed. Following conditioning, successful learners extend their proboscis in response to the odor alone (Takeda, 1961; Bitterman et al., 1983). This protocol was applied to attempt to understand complex mixture processing using whole floral extracts (Pham-Delègue et al., 1986; Le Metayer et al., 1997) or synthetic mixtures of 6-14 components (Pham-Delègue et al., 1993; Wadhams et al., 1994; Laloi et al., 2000; Reinhard et al., 2010). The general finding of these studies was that after learning a complex mixture, bees respond only to some of their components, which have been termed key components (Wadhams et al., 1994; Laloi et al., 2000; Reinhard et al., 2010). The perceptual salience of a component may support its ability to become a key component, but the identity of the other components in the mixture

also play a role, which renders predictions difficult (Laloi et al., 2000; Reinhard et al., 2010). Owing to the inherent complexity of mixture processing, research has strongly focused on binary mixtures (Getz and Smith, 1987, 1990, 1991; Chandra and Smith, 1998; Smith, 1998; Deisig et al., 2001). Usually, when learning a binary mixture, bees respond to both of its components (Getz and Smith, 1987, 1990). However, one component is often learned better than the other, a phenomenon known as overshadowing (Smith, 1998). Again, overshadowing is difficult to predict, but a recent study showed that odorant salience is not the main determinant, the effect of cumulative experience and the generalization profile of an odorant appearing to play a larger role in this process (Schubert et al., 2015).

Inspired by theoretical concepts mentioned above with regards to elemental versus configural processing of compound stimuli, experiments in bees have attempted to understand how a mixture is represented in the bee brain using the so-called patterning experiments (Chandra and Smith, 1998; Deisig et al., 2001). In such experiments, bees have to differentiate between two single odorants A and B and the mixture AB. In negative patterning, the single elements are both reinforced when presented alone (A+, B+), while the mixture is non-reinforced (AB−). The experiments performed in bees (Deisig et al., 2001, 2002, 2003; Lachnit et al., 2004) suggested that the best model for explaining mixture learning was at the interface between elemental and configural models, called the unique cue hypothesis (Rescorla, 1972, 1973; Rescorla and Wagner, 1972). In addition to the representations of the elements, the mixture would give rise to a supplementary (internal) representation, the unique cue. Bees can thus attribute novel qualities to the joint presentation of two odorants, and this effect can be detected using differential conditioning procedures such as negative patterning. However, negative patterning tasks are notoriously difficult for bees and differentiation success is usually limited (Chandra and Smith, 1998; Deisig et al., 2001, 2007; Komischke et al., 2003; Devaud et al., 2015). If a particular mixture, such as the EI.EM mixture tested in humans, rabbits and rodents, also supports strong configural properties in bees, then differentiation success in a negative patterning experiment with this mixture should be higher than with other mixtures commonly tested in this species.

In this study, we used PER conditioning to evaluate how bees perceive the EI.EM mixture known to be perceived in a configural manner by humans, rabbits and rodents. In a first experiment, we evaluated bees' spontaneous configural processing, asking how similar they perceived mixture and components. We used a standard generalization protocol (e.g. Guerrieri et al., 2005; Schubert et al., 2015), in which bees were trained to associate one of the elements or the mixture with sucrose reward. Afterwards, they experienced a test phase in which each element and the mixture were presented without reward. Spontaneous configural perception can be measured as a lack of generalization between the mixture and the elements. In a second experiment, we evaluated bees' ability for configural learning, by explicitly training them to differentiate the mixture from its elements. To this aim, we used the negative patterning differential training procedure detailed above (Deisig et al., 2001). Here, configural learning is observed when bees start to respond to the elements but not to the mixture in the course of training. In each of these experiments, the performance of the bees with the EI.EM mixture was compared with that obtained with four other mixtures, chosen from previous work in humans, newborn rabbits and bees.

## MATERIALS AND METHODS
### Insect preparation
Honey bee (*Apis mellifera* Linnaeus 1758) workers of unknown age were caught at the hive entrance on the CNRS campus in Gif-sur-

Yvette (France). The bees were anesthetized on crushed ice for approximately 3 min and were then harnessed in individual metal tubes, leaving their antennae and mouthparts free. Two adhesive strips were placed behind the head and the abdomen to keep the bees in position. Bees were fed with 3 µl of sugar solution (50% w/w) to homogenize their satiety level and they were left to rest for 2 h in a dark and humid box before conditioning.

## Olfactory stimuli

This study tested the hypothesis that the EI.EM mixture supports configural perception in honey bees, as was observed in humans, rabbits and rodents (e.g. Le Berre et al., 2008b; Coureaud et al., 2020; Wilson et al., 2020). This mixture was composed of ethyl isobutyrate (EI, CAS number: 97-62-1) and ethyl maltol (EM, CAS number: 4940-11-8), in a 50/50 v/v dilution of pure EI and 10% EM in ethanol. The performances of bees with this mixture were compared with those obtained with four other mixtures. Two of these mixtures were previously shown to be perceived in an elementary fashion by humans and rabbits (Coureaud et al., 2009; Sinding et al., 2011; Schneider et al., 2016): EI.G was a 50/50 mixture of pure EI and 50% guaiacol in ethanol (G, CAS number: 90-05-1); and EM.G was a 50/50 mixture of 10% EM and 50% G in ethanol. Finally, two mixtures of floral odorants used in previous studies on honey bee learning and perception were chosen (Guerrieri et al., 2005; Schubert et al., 2015). One was a 50/50 mixture of 1-hexanol (henceforth '6ol', CAS number: 111-27-3) and 1-nonanol (henceforth '9ol', CAS number: 143-08-8). The second one was composed of 2-octanone (henceforth '8one', CAS number: 111-13-7) and octanal (henceforth '8al', CAS number: 124-13-0).

The olfactory stimulation apparatus produced a constant airflow of 52.5 ml s$^{-1}$. This flow, composed of a principal airflow of 50 ml s$^{-1}$ and a secondary flow of 2.5 ml s$^{-1}$, was directed to the bee through a glass tube (0.5 cm diameter) at a distance of 2 cm. The secondary airflow could be directed to one of two sub-circuits (one containing an odorant source, and another without any odorant) before being injected again into the main airflow. Most of the time, air flowed through the odorless sub-circuit. Olfactory stimulation induced a switch of the secondary flow to the odorant sub-circuit for 5 s, through a Pasteur pipette containing a piece of filter paper (20×2 mm) soaked with 5 µl of odorant solution. The other sub-circuit included an identical Pasteur pipette with a clean piece of filter paper. An air extractor placed behind the bee prevented odorant accumulation.

## Experiment 1 – Generalization

In a first experiment, we studied bees' generalization performances between each mixture and its components. Bees were thus subjected to a conditioning phase in which they associated one stimulus (component or mixture) with a sucrose reward, followed by a test phase, in which they received unrewarded presentations of the components and of the mixture.

## Acquisition

For each mixture AB, three groups of bees were formed. Two groups were conditioned with one of the components (A+ and B+) and a third group was conditioned with the mixture (AB+). Conditioning consisted of five trials with 10 min inter-trial intervals (ITIs). The CS was an odorant stimulation delivered for 5 s (see Olfactory stimuli above). The US was a 50% w/w sucrose solution delivered to the antennae and to the proboscis. A conditioning trial lasted 30 s. Fifteen seconds after the bee was placed in front of the airflow, the odor CS was presented for 5 s. After 3 s, the sucrose US was applied for the last 2 s of CS presentation. Then the bee was left in front of

the airflow until the 30 s were over. At each trial, bees' responses to the CS alone (during the first 3 s) and to the US were recorded. This allowed us to measure the bees' performances during acquisition (CS), as well as to follow their appetitive motivation (US) throughout the experiment. Bees that did not respond to the US at least once during the acquisition phase were discarded from the analysis (13% overall).

## Test phase

One hour after the conditioning phase, bees were subjected to the test phase. They were presented with component A, component B, the mixture AB and the solvent as control, in a random order, with 10 min ITIs. A test trial lasted 30 s. Fifteen seconds after the bee was placed in front of the airflow, the odor stimulus was presented for 5 s. Then the bee was left in front of the airflow until the 30 s were over. The bee's response to each stimulus was recorded. Bees that responded to the solvent could not be considered as conditioned to the CS, and were thus discarded from the analyses. They represented 3% overall. At the end of the test phase, a last test with the sucrose US was conducted to verify that the bees were still able to show a PER.

## Experiment 2 – Discrimination (negative patterning)

In a second experiment, we tested bees' ability to differentiate between each mixture and its components when explicitly trained to this end. We applied a negative patterning protocol, in which the animal learns to respond to the single elements (A+, B+) but not to the mixture (AB–). A conditioning session was composed of six blocks of four trials, in all 24 trials, with 10 min ITIs (overall duration 240 min). In each block, bees received one presentation of each element alone with a reward and two unrewarded presentations of the mixture (1A+, 1B +, 2AB–). The order of presentations within a block was changed for each bee.

## Statistics

Bees' PER were scored in dichotomous form, with bees extending the proboscis (1) or not (0) when stimuli were presented during conditioning or testing. Only full extensions of the proboscis were counted as responses.

## Experiment 1 (generalization)

For each mixture, acquisition performances to the elements and the mixture were analyzed using generalized linear mixed models (GLMMs) with a repeated-measures design and a binomial family, with stimulus (each element and the mixture) and trial (from 1 to 5) as fixed factors, and the individual bees as random factor (R version 3.6.2, 'glmer' function, 'lme4' package). An interaction term between stimulus and trial was included. Non-significant terms were dropped sequentially and the significance of each factor was assessed with likelihood ratio tests ('anova' function, 'stats' package). When a significant effect of the stimulus factor appeared, it was followed by pairwise comparisons using Tukey's multiple contrasts ('emmeans' function, 'emmeans' package), which includes a correction for multiple testing. Comparisons of conditioning success among groups, as measured by responses to the CS at the last (fifth) conditioning trial, were performed using Fisher's exact test ('fisher.test' function, 'stats' package).

As necessary for testing our hypotheses, bees' responses in the test phase were only analyzed within each conditioning group. A GLMM was performed to test whether bees responded differently to the mixture and the two elements, with stimulus as fixed factors and the individual bee as a random factor. As the stimulus factor was significant in all cases (see Results), it was followed by pairwise

comparisons using the exact McNemar test ('mcnemar.exact' function, 'exact2×2' package). Bonferroni corrections were applied for these multiple comparisons. Because responses to each stimulus were compared with two other stimuli, the alpha level was corrected as follows: $\alpha'=\alpha/2=0.025$.

To compare bees' configural processing with the different mixtures, we measured the proportion of two categories of individuals: (1) in the groups conditioned to one of the mixture components, configural responders were defined as the bees that responded to this odorant in the tests but did not generalize to the mixture; and (2) in the groups conditioned to a mixture, configural responders were defined as all the bees that responded to this mixture in the tests but did not generalize to any of the components. The proportions of configural responders were first compared among the five groups using Fisher's exact test (4 d.f.) and, when significant, were followed by pairwise Fisher's exact tests (1 d.f.). As each group was compared with four other groups, the alpha level was corrected as follows: $\alpha'=\alpha/4=0.0125$.

### Experiment 2 (negative patterning)

We measured the percentage of conditioned responses observed in the 12 CS+ (six with each component) and 12 CS– (mixture) trials. Data were then grouped to obtain six blocks of two CS+ and six blocks of two CS– trials. For all groups, cumulative link mixed models (CLMMs), adapted for ordinal variables, were used to analyze the blocked data, with block (from 1 to 6) and stimulus (CS+/CS–) as fixed effects and the individual bee as a random factor. An interaction between block and stimulus was included. Differentiation success at any given block was evaluated with a CLMM including the stimulus as fixed effect and the random factor. To visualize the time course of differentiation success among the different mixture types, we calculated for each bee at each block of trials a discrimination index: $\Delta_{discrimination}=$(number of responses to CS+)–(number of responses to CS–). This index thus potentially ranged from −2 to +2. For clarity, and to simplify comparisons with the learning curves, it was transformed to %PER by multiplying it by 50%. Thus, the $\Delta_{discrimination}$ curve corresponds to the difference between the CS+ and CS– curves. Comparison of bees' discrimination curves with the five tested mixtures was performed using a CLMM, with mixture and block (from 1 to 6) as fixed factors and the individual bee as the random factor. An interaction between mixture and block was included in the model. Comparison of $\Delta_{discrimination}$ at the last (sixth) block of trials was performed with a cumulative link model (CLM) including the stimulus as a fixed effect, followed by pairwise comparisons using Tukey's multiple contrasts as above.

### RESULTS

The aim of this study was to evaluate whether honey bees process the EI.EM mixture in a configural manner, either spontaneously or after explicit training. The performance of bees with this mixture was compared with that obtained with four other mixtures either that were made with each of the EI.EM components and a third component, or that were previously used in studies of olfactory perception in honey bees. The first experiment tested whether any of these mixtures gives rise to spontaneous configural perception. To address this question, we used a generalization procedure, which evaluated the propensity of bees to treat mixtures and components as different or similar stimuli. Configural perception would be observed here as a lack of generalization between the mixtures and their components in this experiment.

### Experiment 1 – Generalization

A total of 933 bees divided in 15 groups were conditioned. For each of the five binary mixtures, one group of bees was conditioned to the mixture and two groups were conditioned to each of its components. The performance of bees in the acquisition phase is presented in Fig. S1. Bees from all groups learned to associate the olfactory stimulus with sucrose reinforcement as shown by a significant increase in PER to the presentation of the CS along the five trials of the conditioning procedure (GLMM, trial effect, $\chi^2>638.1$, 4 d.f., $P<0.001$). In two out of the five mixtures (EI.EM and EM.G), a significant effect of the group was observed during the acquisition phase (GLMM, stimulus effect, $\chi^2>5.99$, 2 d.f., $P<0.05$). For EI.EM, the effect was slight, and multiple comparisons did not yield any significant pairwise contrast, owing to threshold correction. For EM.G, multiple comparisons showed that the difference was due to more rapid acquisition with the mixture EM.G than with the component EM, the component G falling in between (Tukey contrasts, corrected $P=0.030$). No significant interaction was found between stimulus and trial for any of the studied mixtures (GLMM, stimulus×trial effect, $\chi^2<7.10$, 8 d.f., $P>0.52$). In the end, for all five mixture groups, the same level of performance was reached at the fifth conditioning trial between mixture and components (in all cases, Fishers' exact test, $P>0.21$, 2 d.f.). We thus compared in the test phase the generalization performance of bees that had learned all CSs efficiently, without any difference between each mixture and its components.

The responses of bees during the test phase are presented in Fig. 1. For all five mixture groups, responses in the test phase showed a significant interaction between the odorant used for conditioning and the odorants used in the test phase (GLMM, stimulus×trial interaction, $\chi^2>62.0$, 4 d.f., $P<0.001$). Generally, individuals that were conditioned to a single component responded strongly to that component and generalized much more to the mixture than to the other component. For example, in the case of the EI.EM mixture, EI+ bees responded strongly to EI, generalized but with a response decrement to the EI.EM mixture and responded very little to EM, the other component (a novel odor for them). Similarly, EM+ individuals responded strongly to EM, partially generalized to EI.EM and showed very low responses to EI. The same general pattern was observed for the five mixtures. Remarkably, the response decrement during generalization from the components to the mixture was significant in nine out of 10 cases (exact McNemar test, $P<0.01$). Only when bees were conditioned to 8al did they fully generalize to the 8al.8one mixture (exact McNemar test, $P=0.074$).

In contrast, individuals that were trained with a binary mixture responded most strongly to the conditioned mixture, but also generalized to the components, albeit at a lower level. The response decrement during generalization from the mixture to the components was significant in nine out of 10 comparisons (exact McNemar test, $P<0.004$). The exception was again for bees trained with the 8one.8al mixture, which generalized fully to 8al (exact McNemar test, $P=0.074$). In these mixture-conditioned bees, we next evaluated whether the phenomenon of overshadowing (see Introduction) was observable, i.e. if bees conditioned to each mixture responded more to one of the components than to the other. Qualitative differences were observed for all mixtures, but the difference was significant in only one case: bees conditioned to the EM.G mixture responded much more strongly to G than to EM (exact McNemar test, $P<0.001$).

These results revealed that generalization was relatively strong between the mixtures and their components, irrespective of whether bees were conditioned to the mixture or to one of its components. This
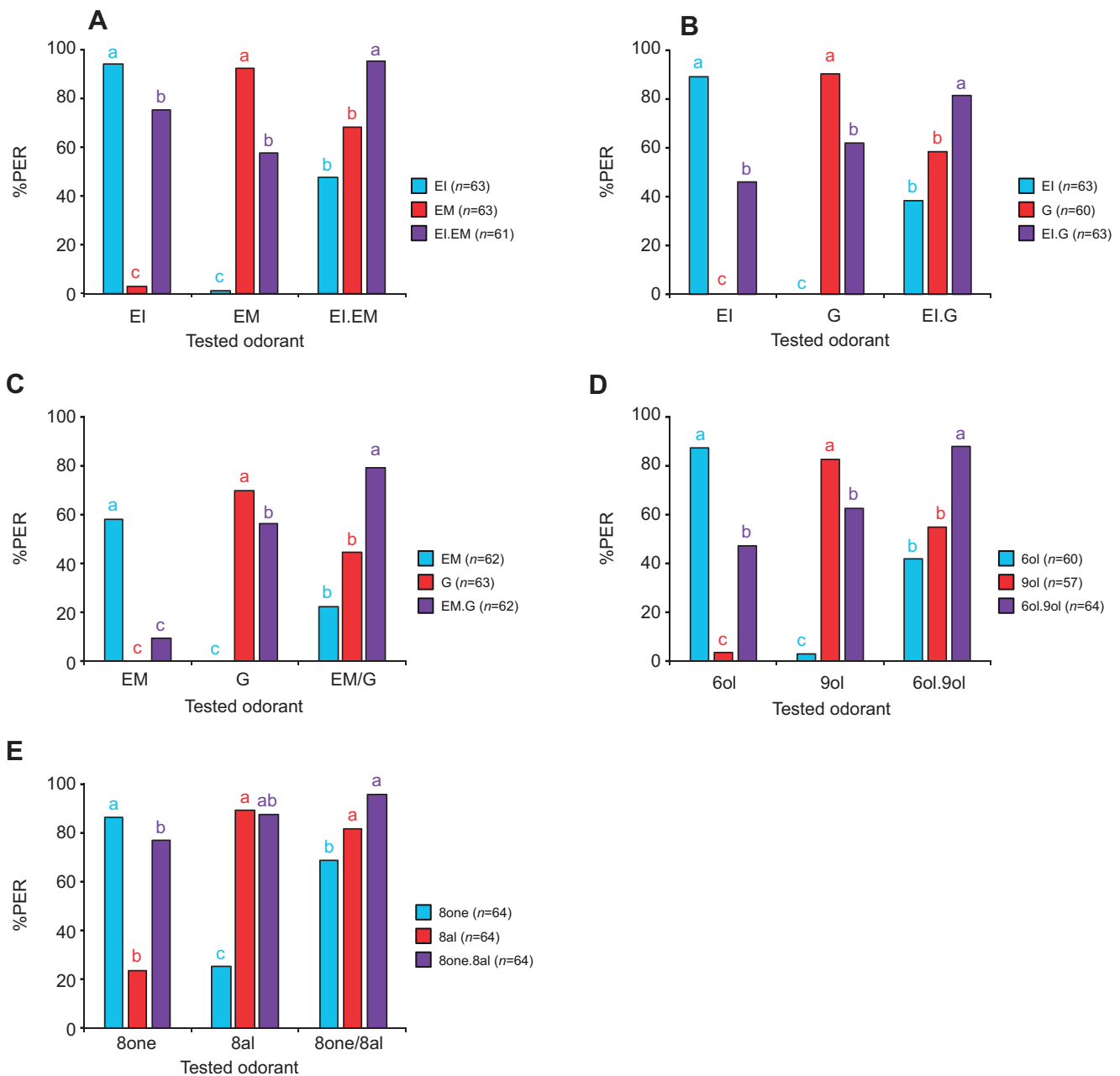
**Fig. 1. Generalization experiment – performances during the test phase.** Five mixtures were studied: (A) EI.EM); (B) EI.G; (C) EM.G; (D) 6ol.9ol; (E) 8one.8al. The graphs show the percentage of proboscis extension responses (%PER) observed at the unrewarded presentation of each element and the mixture (presented in a random order at 10 min intervals). For each conditioning group, pairwise comparisons were made among the three test stimuli because the overall comparison was significant (GLMM, stimulus effect, $P<0.05$). They are indicated with different colors for each conditioning group (blue and red for bees conditioned to each of the elements, violet for bees conditioned to the mixture). Different letters indicate statistical difference in the pairwise comparisons (exact McNemar test, corrected $P<0.025$). Individuals that responded to the solvent in the test phase were discarded. Odorant abbreviations: EI: ethyl isobutyrate; EM: ethyl maltol; G: guaiacol; 6-ol: 1-hexanol; 9-ol: 1-nonanol; 8al: octanal; 8one: 2-octanone. n.s.: non-significant.

means that none of the mixtures was perceived as intrinsically different from its elements, i.e. evidence for configural processing was rather low. Was this tendency different among the tested mixtures? To answer this question, we quantified within each group the proportion of individuals showing a response pattern compatible with configural perception (Fig. 2). They corresponded to the bees that responded to the component they learned during the test phase but not to the mixture, after learning of the components (Fig. 2A) or individuals that responded to the mixture but to none of the components during the test phase, after learning of the mixture (Fig. 2B). Concerning the bees

that had learned a component and responded to that component in the test phase ($80<N<117$), between 16.6% and 50.0% did not respond to the mixture and thus produced a response pattern compatible with configural processing (Fig. 2A). A significant heterogeneity was observed among these proportions (Fisher's exact test, $P<0.001$, 4 d.f.). This was due to the 8one.8al mixture showing a lower proportion of configural-like responses than the other four mixtures (Fisher's exact test, $P<0.0125$, 1 d.f. each). The EI.EM mixture did not produce a higher proportion of such responses than the rest of the mixtures (Fisher's exact tests, $P>0.30$, 1 d.f. each).
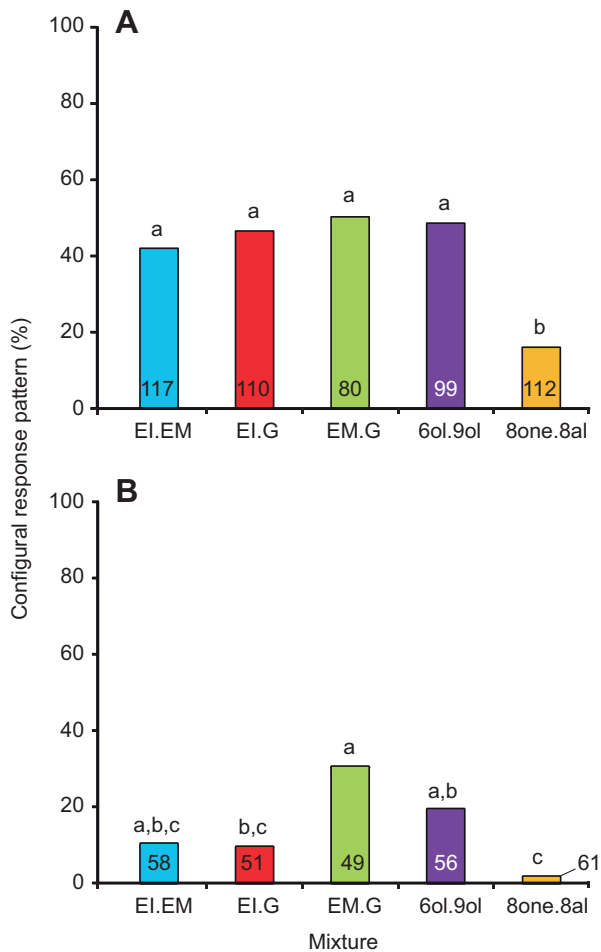
**Fig. 2. Generalization experiment – proportions of individuals showing a configural response pattern.** (A) Individuals conditioned to one of the elements (two groups for each mixture type), which respond to this element but not to the mixture in the test phase. (B) Individuals conditioned to a mixture, which respond to this mixture but to none of its elements in the test phase. Numbers of individuals are indicated within each bar. Different letters indicate statistical difference in the pairwise comparisons (Fisher's exact test, $P<0.0125$). Odorant abbreviations are as in Fig. 1.

Among the bees that had learned a mixture and responded to that mixture in the test phase ($51<N<61$), between 1.6% and 30.6% did not respond to any of the components, and thus produced a response pattern compatible with configural processing (Fig. 2B). A statistical heterogeneity appeared among these proportions (Fisher's exact test, $P<0.001$, 4 d.f.), with a significant difference between the EM.G mixture and the EI.G and 8one.8al mixtures (Fisher's exact test, $P<0.0125$, 1 d.f. each) and between the 6ol.9ol mixture and the 8one.8al mixture (Fisher's exact test, $P<0.0125$, 1 d.f.). Note that, here, the higher proportion found for the EM.G mixture is not attributable to configural processing, but rather to overshadowing, because we observed that EM is overshadowed by G in the mixture. Therefore, EM learners generalized very little to the mixture. As regards the EI.EM mixture, it did not stand out as supporting more configural processing than other mixtures (Fisher's exact tests, $P>0.05$, 1 d.f. each).

We conclude from this experiment that the EI.EM mixture is not processed in a spontaneous configural manner by honey bees when presented in a simple generalization protocol. The next experiment asked whether explicit differential training between mixtures and components allows the bees to treat these mixtures as a configuration.

**Experiment 2 – Discrimination (negative patterning)**
A total of 300 bees from five groups were conditioned in a 24-trial negative patterning procedure (A+, B+, AB−) to evaluate the ability of bees to differentiate between a mixture AB and its components A and B. Fig. 3 presents the responses of bees to the reinforced elements (CS+, two trials per block) and to the non-reinforced mixture (CS−, two trials per block) in the course of six blocks of trials. For reference, the responses to each of the reinforced elements and to the non-reinforced mixture in the course of six blocks of trials are shown in Fig. S2. For all mixtures, the beginning of the conditioning procedure is controlled by 'elemental summation', corresponding to the summation of associative strengths of the elements when the mixture is presented. Accordingly, at the start of conditioning, bees respond more to the unrewarded mixture (CS−) than to the elements (CS+). It generally took several blocks of trials until bees managed to reverse this tendency and to solve the learning task. For all mixtures, we found a significant stimulus×block interaction (CLMM, $\chi^2>71.04$, 5 d.f., $P<0.001$), showing that responses to CS+ and CS− evolved differently in the course of conditioning. Also for all mixtures, bees responded significantly more to the CS+ than to the CS− in the last (sixth) block of trials (CLMM, stimulus effect, $\chi^2>4.97$, 1 d.f., $P<0.05$). However, dramatic differences appeared among mixtures with respect to the bees' efficacy in solving the task. Over the whole procedure, only two mixtures, EI.EM and 6ol.9ol, showed significantly more responses to the CS+ than to the CS− (CLMM, stimulus effect, EI.EM : $\chi^2>65.4$, 1 d.f., $P<0.001$; 6ol.9ol, $\chi^2>4.05$, 1 d.f., $P<0.05$), while for the other three mixtures, bees responded more often to the CS− than to the CS+, showing that elemental summation prevailed (CLMM, stimulus effect, $\chi^2>7.73$, 1 d.f., $P<0.01$). The moment when bees started to respond significantly more to the CS+ than to the CS− was different among mixtures. For EI.EM and 6ol.9ol, this happened at block 4 (CLMM, stimulus effect, $\chi^2>10.4$, 1 d.f., $P<0.01$), while it was only at block 6 for all the other mixtures (CLMM, stimulus effect, $\chi^2>4.97$, 1 d.f., $P<0.05$).

We next directly compared the discrimination performance of bees between each mixture and its components, by calculating at each block of trials the difference ($\Delta_{discrimination}$) between responses to the CS+ and to the CS− (Fig. 4). The performance of bees differed greatly depending on the mixtures (Fig. 4A). Accordingly, statistical analysis revealed a significant mixture effect (CLMM, $\chi^2=94.3$, 4 d.f., $P<0.001$), a significant block effect ($\chi^2=562.8$, 5 d.f., $P<0.001$), as well as a significant interaction between these factors (mixture×block interaction, $\chi^2=243.4$, 20 d.f., $P<0.001$). This last effect shows that differentiation speed differed among mixtures. Observation of the curves shows that discrimination appeared first and reached higher levels for the EI.EM mixture than for all the other mixtures. Indeed, we found significantly higher performances for EI.EM over the whole procedure than for any of the other mixtures (Fig. 4B, Tukey multiple contrasts, corrected $P<0.001$). The 6ol.9ol mixture achieved the second best performances, which were significantly better than those observed for the other three mixtures (Tukey multiple contrasts, corrected $P<0.05$). Likewise, the discrimination level reached at the end of the procedure was heterogeneous among mixtures (CLM, mixture effect, $\chi^2=64.7$, 4 d.f., $P<0.001$) and was significantly higher for the EI.EM mixture than for all the other mixtures (see letters in Fig. 4A, Tukey multiple contrasts, corrected $P<0.05$). With lower performances, the EI.G mixture reached better differentiation than both EM.G and 8one.8al mixtures (Tukey multiple contrasts, corrected $P<0.05$).

We conclude from this experiment that the EI.EM mixture supported better negative patterning performances than the other
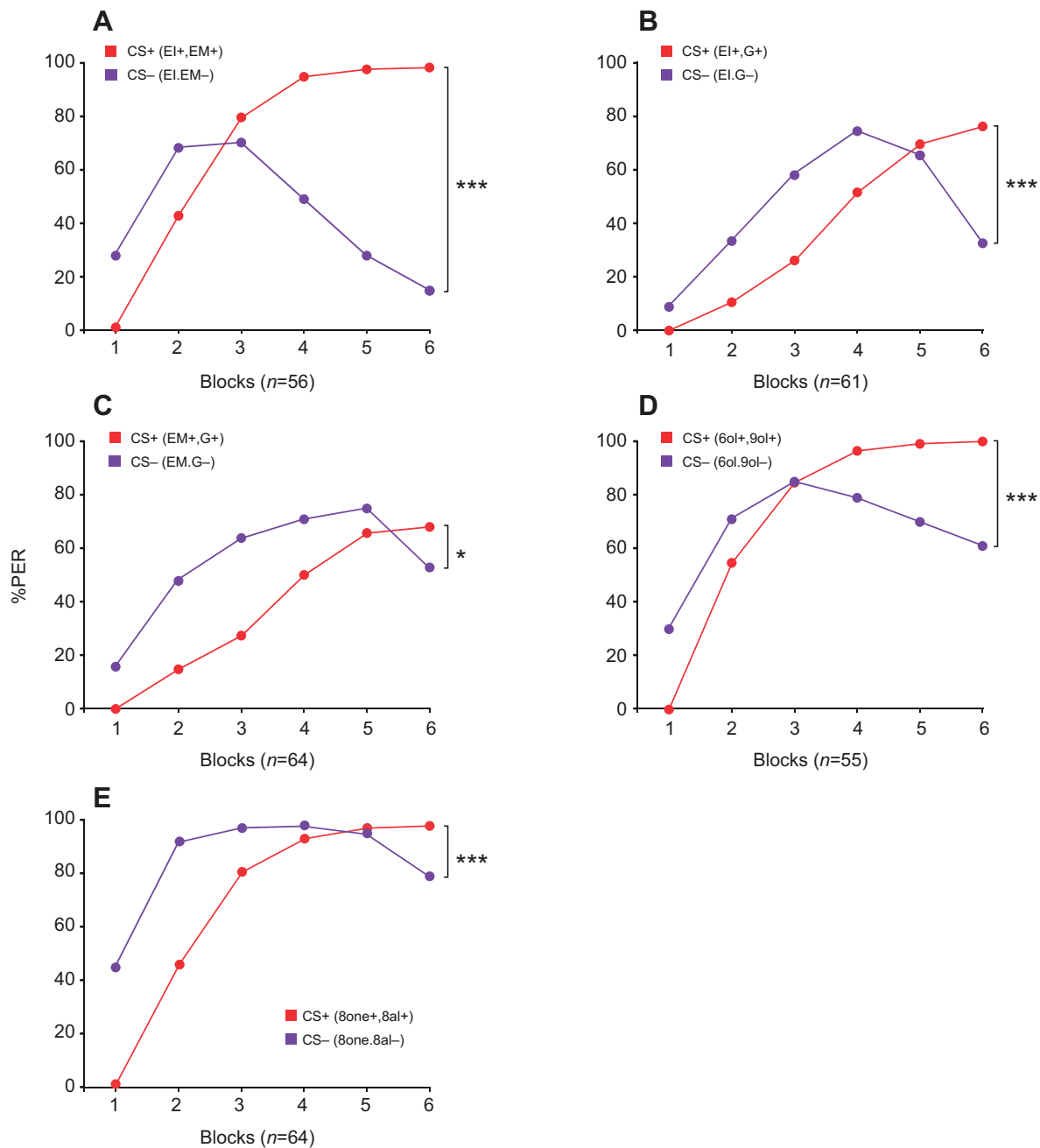
**Fig. 3. Discrimination (negative patterning) experiment – conditioning performances.** Five mixtures were studied: (A) EI.EM; (B) EI.G; (C) EM.G; (D) 6ol.9ol; (E) 8one.8al. The performance (%PER) of bees is shown for the rewarded elements (CS+) and for the unrewarded mixture (CS−), along six blocks of four trials (one with each element, two with the mixture, as many rewarded as unrewarded trials). The same data are shown for each element and the mixture in Fig. S2. Asterisks indicate significant differences at the last (sixth) block of trials (CLMM, stimulus effect). Odorant abbreviations are as in Fig. 1. n.s.: non-significant; *$P<0.05$; ***$P<0.001$.

mixtures used in the present study, suggesting that differential training allowed the development of configural processing of this mixture.

## DISCUSSION

In this study, PER conditioning was used to evaluate how bees perceive and learn the EI.EM binary mixture, previously shown to be perceived in a configural manner by human and rodent adults, and by newborn rabbits. Four other mixtures were used as controls. In experiment 1, we studied spontaneous configural processing by measuring perceptual similarity between each mixture and its components. We found generally strong generalization between mixtures and their components, without any particular specificity for

the EI.EM mixture. In experiment 2, we evaluated configural learning and the ability of bees to discriminate between the mixture and both of its components in a negative patterning protocol. Although bees quickly differentiated the EI.EM mixture from its elements, performances with the four control mixtures were significantly poorer. In this experiment, the EI.EM mixture supported particular performances compatible with configural processing.

### Generalization versus discrimination between mixture and components

We found clearly different responses of bees in the two experiments. In the first experiment, most bees conditioned to a mixture tended to
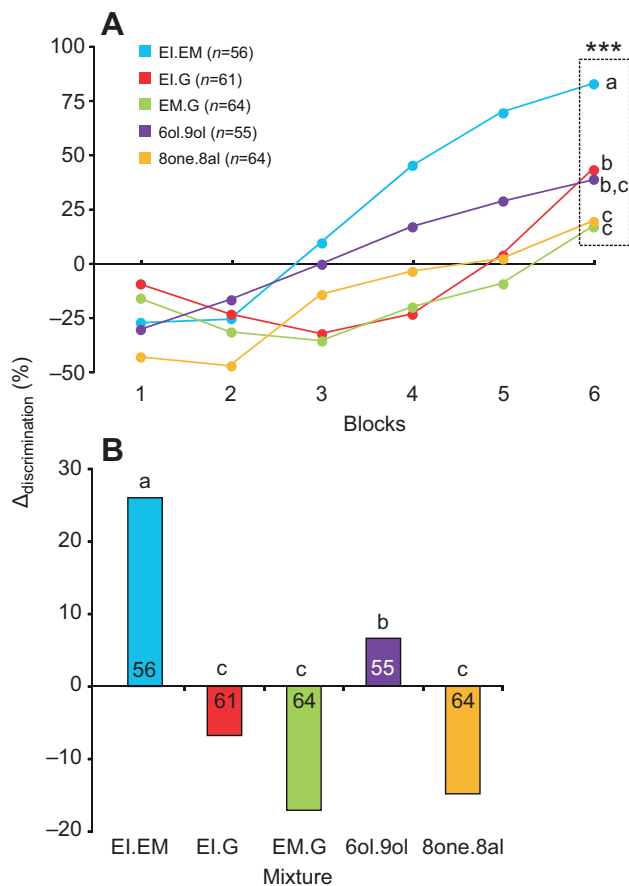
**Fig. 4. Discrimination (negative patterning) experiment – discrimination performances.** (A) Bees' discrimination success [$\Delta_{discrimination}$=(number of responses to CS+)–(number of responses to CS−)] shown as %PER, along the six blocks of four trials. Asterisks indicate significant difference at the last block of trials (CLM, stimulus effect, $P<0.001$). Different letters indicate statistical difference in the pairwise comparisons (Tukey contrasts, corrected $P<0.05$). (B) Discrimination success over the whole procedure (six blocks, i.e. 24 trials). Different letters indicate statistical difference in the pairwise comparisons (Tukey contrasts, corrected $P<0.05$). Odorant abbreviations are as in Fig. 1. n.s.: non-significant.

respond to the components and vice versa. This experiment was designed to evaluate spontaneous configural perception, i.e. how similar bees perceived both types of stimuli (Smith and Menzel, 1989; Guerrieri et al., 2005). Such a protocol does not indicate whether bees respond to both stimuli because they cannot discriminate between them or because they simply treat them as equivalent stimuli, although they perceive them as different (Shepard, 1987). With this protocol, the EI.EM mixture did not stand out compared with the four other tested mixtures: it showed about the same level of configural responders as three other mixtures after conditioning to the elements or even a lower level when conditioned to the mixture (Fig. 2). The only noticeable effect was found for the 8one.8al mixture, which was remarkably non-configural, almost all animals generalizing between mixtures and components. It is also in this mixture that no overshadowing was found. These observations fit with those made by Schubert et al. (2015) with the same mixture, in particular regarding the lack of overshadowing. We do not have any solid hypothesis to explain this particular effect. In experiment 2, we evaluated configural learning by explicitly training the bees to differentiate between mixture and components. Interestingly, the 8one.8al mixture, which had shown low configural properties in

experiment 1, was also very difficult to train in negative patterning in experiment 2. On the contrary, the EI.EM mixture, which was unremarkable in experiment 1, supported rapid and efficient differentiation between mixture and components in experiment 2. This result is important because it suggests that the EI.EM mixture develops particular configural properties compared with the other mixtures, when differential training is involved.

Two types of patterning tasks exist, based on a differential conditioning between a binary mixture and its components. In negative patterning, the single components are reinforced when presented alone (A+, B+), while the mixture is non-reinforced (AB−). The symmetric task, termed positive patterning, reverses the reinforcement schedule, with the mixture being reinforced (AB+) and the elements being non-reinforced when presented alone (A−, B−). Although bees have been shown to solve both tasks with olfactory stimuli, they find negative patterning more difficult than positive patterning (Deisig et al., 2001, 2002, 2007; Komischke et al., 2003; Devaud et al., 2015). The reason for this is that positive patterning can theoretically be solved using a purely elemental model, in which the mixture is the simple sum of its elements. In such a model, when each component is reinforced, a mixture would elicit, through elemental summation, a higher level of responses than to each component, which is exactly what is expected in a positive patterning task. Note that elemental summation is clearly visible at the beginning of negative patterning in all our experiments, bees starting to respond more to the mixture than to the elements (Fig. 4, see the performances at block 2 for instance). In a purely elemental model, however, animals would never manage to inhibit response to the AB mixture in a negative patterning task (as seen at the end of each experiment in Fig. 4). Successful differentiation in negative patterning is thought to rely on some level of configural processing. As detailed in the Introduction, mixture processing in honey bees has been shown to best fit with an expansion of elemental models, called the unique cue hypothesis (Rescorla, 1972, 1973; Whitlow and Wagner, 1972). In the brain, in addition to the representations of the components (elements), the mixture would give rise to a supplementary (internal) representation, the unique cue. During negative patterning, the unique cue U would build inhibitory associations with the US, while the elements A and B would build excitatory associations. All previous observations as well as our own data suggest that different mixtures support different levels of differentiation in a negative patterning task (Deisig et al., 2001; Komischke et al., 2003; Devaud et al., 2015). This observation can be explained in the framework of the unique cue model, if the unique cue has a different salience compared with the elements for the different mixtures. Our results suggest that the EI.EM mixture would give rise to a remarkably salient unique cue (see below).

## Neural processing of mixture information

Can we explain these observations based on our current knowledge of the neural substrates of olfactory processing and learning in the bee brain? Extensive knowledge of the honey bee olfactory system allows the proposal of neural models subtending behavioral performances (Galizia and Menzel, 2001; Sandoz, 2011; Galizia, 2014). In short, odorants are detected by ~60,000 olfactory receptor neurons (ORNs) on each antenna. ORNs project into the antennal lobe (AL), the primary olfactory center, analogous to the vertebrate olfactory bulb, made of 160 morphological and functional units, the glomeruli. Each ORN expresses one type of olfactory receptor (OR) protein, and all ORNs expressing the same OR project to the same glomerulus. Glomeruli are interconnected by local, mostly inhibitory interneurons (~4000) (Fonta et al., 1993). Processed olfactory information is

further conveyed by ∼900 projection neurons (PNs) to higher-order brain centers [mushroom bodies (MBs) and lateral horn; Abel et al., 2001; Rybak, 2012].

Several studies used *in vivo* calcium imaging to unravel the coding of individual odorants in the AL based on their chemical nature (Joerges et al., 1997; Sachse et al., 1999; Carcaud et al., 2012, 2018) as well as of olfactory mixtures (Joerges et al., 1997; Deisig et al., 2006, 2010; Stierle et al., 2013; Locatelli et al., 2016; Jernigan et al., 2019). These studies show that individual odorants evoke multi-glomerular activity patterns, which are different for different odorants. Similarity among activity patterns in the antennal lobe follows chemical similarity (Sachse et al., 1999) and correlates with how similar honey bees treat these odorants in behavioral experiments (Guerrieri et al., 2005; Carcaud et al., 2012, 2018). Therefore, optical imaging captures a behaviorally meaningful aspect of olfactory coding. When two components are presented in a mixture, the evoked pattern resembles the sum of the individual patterns at AL input, but local networks within the AL induce the emergence of configural properties at the level of AL output (Deisig et al., 2006, 2010; see also Silbering and Galizia, 2007 in *Drosophila*). These configural properties have been proposed as a possible neural substrate for the unique cue (Lachnit et al., 2004).

This neural model of mixture processing in the first relay of the olfactory pathway can be used to propose hypotheses explaining the two main findings of our study: (1) different mixtures differ in their propensity to be treated in a configural manner by honey bees; and (2) the IE.EM mixture displays stronger configural properties when used in the discrimination (negative patterning) protocol than in the generalization protocol. The first observation can be explained by focusing on the role of lateral inhibition within the AL. It is known to play a major role in shaping the representation of mixtures that will be transmitted to higher-order brain centers. Lateral inhibition is thought to rely on a two-tiered network in the AL (Sachse and Galizia, 2002; Silbering and Galizia, 2007; Deisig et al., 2010), corresponding to two main anatomical types of LNs; roughly 13% of the 4000 LNs diffuse homogeneously in the AL (homoLNs), whereas the rest show a high branching density in one particular glomerulus (heteroLNs; Flanagan and Mercer, 1989; Fonta et al., 1993). HomoLNs would be involved in homogeneous inhibition, playing the role of a gain control (avoiding saturation of the system), while heteroLNs would apply asymmetrical lateral inhibition between glomeruli. Both computational modeling based on optical imaging data (Linster et al., 2005) and neurophysiological experiments (Girardin et al., 2013) indeed confirm that inhibition in the AL is spatially patchy and its weight highly heterogeneous among glomeruli. This means that inhibition between two glomeruli A and B can be asymmetrical. Consequently, predicting the representation of a particular mixture based on those of its components can be tricky at AL output (Silbering and Galizia, 2007; Fernandez et al., 2009; Deisig et al., 2010). It is thus not difficult to understand that different combinations of glomerular patterns (i.e. different types of components) will trigger different levels of inhibitory interactions, and thus different departures between mixture and component representations.

Concerning the second observation, a rather simple explanation can be proposed. It is well established that different training schedules have different effects on the way bees treat sensory stimuli, especially when evaluating whether they can differentiate between similar stimuli (Giurfa, 2004; Avarguès-Weber et al., 2010). In particular, differential conditioning (A+, B−) allowed a previous study to show discrimination of similar visual stimuli, which were treated indifferently by bees after absolute conditioning (A+) (Giurfa, 2004; see Dyer and Chittka, 2004 for a similar

demonstration in bumble bees). In a way, our two experiments provided the same situation: in experiment 1, bees were subjected to absolute conditioning with the components alone and discovered the mixture in the unrewarded tests, whilst in experiment 2, they were trained in a differential conditioning schedule and the rewarded presentations of the elements were coupled with explicit non-rewarded presentations of the mixture. As for the visual studies presented above, experience-dependent modulation of perception and/or the recruitment of different attentional processes could explain such differences in the behavioral performance of bees. Experimental data already support the former explanation involving an experience-dependent plasticity of sensory processes. Several studies found a plasticity of olfactory response maps in the antennal lobe following appetitive conditioning of the PER (Faber et al., 1999; Sandoz et al., 2003; Fernandez et al., 2009; Rath et al., 2011; Locatelli et al., 2016). These studies generally found that differential training tends to decorrelate the maps of CS+ and CS− odorants (but see Peele et al., 2006), making them potentially more discernible after training than before. Using highly similar complex mixtures, a study compared the effect of a differential conditioning procedure with a situation in which the CS+ was a mixture and the CS− the undetectable presentation of a solvent control (Locatelli et al., 2016). In the first case, the bees were explicitly trained to differentiate two odors, while the second case corresponded to an absolute training with a single odor. Their results clearly showed that similarity between odor response maps in the differential conditioning group was reduced compared with the absolute conditioning group. Because the amount of reward was the same in both groups, it is the multiple presentations of the unrewarded odor that allowed this decorrelation to occur. It is still unclear how this plasticity takes place, but it is currently thought that it involves octopaminergic modulation of inhibitory synapses from LNs (Hourcade et al., 2009; Rein et al., 2013; Sinakevitch et al., 2013; Chen et al., 2015). As above, because of the patchiness and heterogeneity of LN networks (Linster et al., 2005; Girardin et al., 2013) it is not difficult to imagine that plasticity on these local networks may produce different results with different mixtures, for instance producing more salient unique cues for some mixtures. In other words, differential training would increase the configural part of certain mixture representations, allowing particularly efficient differentiation in a negative patterning protocol, as found here for the EI.EM mixture. It would thus be particularly interesting now to use optical imaging to study the maps evoked by different mixtures and their components before and after negative patterning training.

Importantly, configural olfactory learning is known to involve higher-order centers of the honey bee brain, especially the MBs, known as learning, memory and multimodal integration centers (Heisenberg, 1998; Menzel, 2012). Indeed, a recent study demonstrated that bees with anesthetized MBs learn and distinguish individual odorants normally, but are defective in learning higher-order discrimination tasks such as the negative patterning used in our experiment 2 (Devaud et al., 2015). Interestingly, this study could show that blockade of GABAergic signaling (typically performed by recurrent neurons from MB output lobes to the calyces in this insect; Grünewald, 1999) recapitulated this effect. This suggests that recurrent inhibition at this stage of the olfactory pathway may play a crucial role, probably together with lower-level processes described above, in allowing bees to learn rules helping them to distinguish between mixtures and their elements. The possible attentional effects mentioned above could typically involve this structure. Clearly, more work is needed to determine the respective roles of each neural level in the configural perception and learning of olfactory mixtures.

## Evolutionary convergence for mixture processing?

The negative patterning performances observed with the EI.EM mixture suggest configural processing of this mixture in bees. This result fits with previous observations made (i) in humans, in which the EI.EM was more typical of a 'pineapple' percept than any of the components (Le Berre et al., 2008b); (ii) in adult rodents, which clearly discriminate the EI.EM mixture from its individual components (Wilson et al., 2020); and (iii) in rabbit neonates, which rarely or never generalized from the components to the mixture (Coureaud et al., 2008, 2014, 2020). Thus, the same mixture is able to support configural processing in widely different species, both mammals and insects, triggering the question of the possible origin of such similarities. Although the architectures of the olfactory nervous systems of vertebrates and insects show fascinating similarities (Hildebrand and Shepherd, 1997; Ache and Young, 2005; Su et al., 2009), especially concerning the modular (glomerular) organization of their primary olfactory centers [AL and olfactory bulb (OB)], it is well established that OR proteins in insects and vertebrates are unrelated (Benton et al., 2006; Benton, 2015). Thus, the sensory equipment for detecting odorants at the periphery is rather different in the four species that showed configural processing of the EI.EM mixture. Despite these differences in peripheral receptors, general odor coding rules were shown to be remarkably similar. In both systems, each odorant is detected by a combination of peripheral receptors, in turn activating a combination of neural units (glomeruli) in the primary centers (AL and OB) (Su et al., 2009). One may propose that convergent evolution owing to similar ecological/environmental constraints throughout evolutionary times (on ORs, lateral inhibition systems, etc.) may have granted the olfactory systems of insects and mammals with comparable functional properties. The highly correlated inter-odor similarity relationships observed in different mammals and insects fits with this idea (Haddad et al., 2008; Dupuy et al., 2010). Similar functional properties may produce similar effects on the perception of odor mixtures, i.e. more mixture interactions for the EI.EM mixture than for other types of mixtures. Extensive work is now needed to understand how the configural properties of the EI.EM mixture appear in the different animal models, both mammals and insects. The range of neurophysiological techniques available in honey bees will be helpful in this endeavor.

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