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RESEARCH ARTICLE

Pheromones modulate responsiveness to a noxious stimulus in honey bees

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ABSTRACT

Pheromones are chemical substances released into the environment by an individual, which trigger stereotyped behaviors and/or physiological processes in individuals of the same species. Yet, a novel hypothesis has suggested that pheromones not only elicit innate responses but also contribute to behavioral plasticity by affecting the subjective evaluation of appetitive or aversive stimuli. To test this hypothesis, we exposed bees to three pheromonal components whose valence was either negative (i.e. associated with aversive events: isopentyl acetate and 2-heptanone) or positive (i.e. associated with appetitive events: geraniol). We then determined the effect of this exposure on the subjective evaluation of aversive stimuli by quantifying responsiveness to a series of increasing electric shock voltages before and after exposure. Two experiments were conducted varying the time lapse between shock series (15 min in experiment 1, and 24 h in experiment 2). In experiment 1, we observed a general decrease of shock responsiveness caused by fatigue, due to the short lapse of time between the two series of shocks. This decrease could only be counteracted by isopentyl acetate. The enhancing effect of isopentyl acetate on shock responsiveness was also found in experiment 2. Conversely, geraniol decreased aversive responsiveness in this experiment; 2-heptanone did not affect aversive responsiveness in any experiment. Overall, our results demonstrate that certain pheromones modulate the salience of aversive stimuli according to their valence. In this way, they would affect the motivation to engage in aversive responses, thus acting as modulators of behavioral plasticity.

KEY WORDS: Behavioral plasticity, Alarm pheromones, Aggregation pheromone, Aversive responsiveness, Sting extension response, *Apis mellifera*

INTRODUCTION

Pheromones are intraspecific chemical messengers playing a fundamental role in animal communication (Karlson and Lüscher, 1959; Wyatt, 2014). These signals are usually released into the environment, which trigger stereotyped behaviors and/or physiological processes in individuals of the same species that perceive them. Besides this well-documented pheromonal action, a novel hypothesis suggests that pheromones not only elicit innate responses but also contribute to behavioral plasticity by modulating innate responsiveness to reinforcement stimuli and thus the learning and memorization of cues predicting such reinforcements (Baracchi et al., 2017).

Honey bees are appropriate study organisms for testing this hypothesis. Their social lifestyle relies on a highly efficient division of labor among castes (Page et al., 2006; Wilson, 1971; Winston, 1987) and on sophisticated communication codes. The latter includes dances used to signal the presence of profitable food sources or nest sites (von Frisch, 1967), and a rich spectrum of pheromones, which regulate multiple social interactions and individual behaviors (Free, 1987). Several pheromones have been identified in *Apis mellifera*, and the neural circuits devoted to pheromone processing in the bee brain have also been studied (Carcaud et al., 2015; Roussel et al., 2014; Sandoz et al., 2007; Wang et al., 2008). Furthermore, innate responses to appetitive stimuli (Page and Erber, 2002; Scheiner et al., 2004) and aversive stimuli (Roussel et al., 2009; Tedjakumala and Giurfa, 2013) have been thoroughly characterized through standardized protocols in this insect, thus enabling the study of reinforcement responsiveness and the impact of pheromones on these responses.

Here, we focused on aversive responsiveness, which can be quantified through the sting extension response (SER) to electric shocks (Roussel et al., 2009; Tedjakumala et al., 2014; Tedjakumala and Giurfa, 2013), and on three pheromone components, which differ in valence and social context: geraniol, 2-heptanone (2H) and isopentyl acetate (IPA). Geraniol is the main component of the Nasanov gland, which elicits attraction and aggregation of receiver honey bee workers (Boch and Shearer, 1962; Butler and Calam, 1969). As this pheromone component signals valuable resources, triggers attraction and relates to an appetitive searching motivation, we characterize it as a 'positive-valence pheromone'. The alarm substance 2H is released by the mandibular glands of workers and exerts a repellent action on intruders and robbers from other hives (Shearer and Boch, 1965). Isopentyl acetate (also called isoamyl acetate) is the main component of the sting alarm pheromone released by the Koschevnikov gland of workers, which causes receiver bees to sting, attack (Boch et al., 1962) and stop foraging (Butler and Free, 1952; Free et al., 1985). As 2H and IPA signal potential noxious or aversive situations/stimuli, and trigger attack or avoidance behaviors, we characterize them as 'negative-valence pheromones'.

Aversive responsiveness is quantified via the propensity to exhibit SER to a series of increasing voltages. SER can be systematically triggered in harnessed bees by the delivery of mild electric shocks (Lenoir et al., 2006; Núñez et al., 1997, 1983; Vergoz et al., 2007). Sting responsiveness to shocks varies among bees within a colony (Lenoir et al., 2006; Roussel et al., 2009). For instance, foragers exhibit higher sting extension responsiveness than guards when stimulated with a series of increasing voltages. Sensitivity to noxious stimulation determines behavioral specializations within the hive, thus contributing to the social

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List of abbreviations

2H	2-heptanone
5-HT	serotonin
DA	dopamine
IPA	isopentyl acetate
OA	octopamine
PER	proboscis extension response
SER	sting extension response

organization of the colony (Roussel et al., 2009; Tedjakumala and Giurfa, 2013).

We studied the impact of geraniol, 2H and IPA on responsiveness to electric shocks in honey bee foragers. We measured shock responsiveness, exposed the same bees to pheromones and then re-measured their shock responsiveness. We hypothesized that negative- and positive-valence pheromones exert different modulatory effects on responsiveness assessed via SER: the former would increase SER by providing further aversive contextual cues while the latter would decrease it, as appetitive signals may detract the bees from aversive behaviors (Nouvian et al., 2015). According to this view, pheromones (and their main components) would modulate the bees' subjective evaluation of aversive stimuli, thus contributing to behavioral plasticity.

MATERIALS AND METHODS

Experiments were conducted at the Research Center on Animal Cognition, at the campus of the University Paul Sabatier (43°33′N, 1°28′E; 150 m above sea level). We used European honey bee female workers *Apis mellifera* L., typically 2–3 weeks old, collected at the apiary of our institute. Only nectar foragers caught at an artificial feeder containing 30% (w/w) sucrose solution were used as these bees are highly responsive to electric shocks (Roussel et al., 2009). Bees were captured in glass vials upon landing on the feeder and before they started feeding to control for the volume of liquid contained in their crop, which may influence electric conductivity and thus the subjective strength of electric shocks. They were then brought to the laboratory, which was maintained at a constant temperature of 25°C. Each bee was its own reference as aversive responsiveness was measured before and after pheromone exposure. Two experiments were performed in which the period of time between the two measurements of aversive responsiveness was varied: in experiment 1, it was 15 min, and in experiment 2, it was 24 h. Experiment 2 thus allowed for a recovery of aversive responsiveness between the two shock series and controlled for a possible effect of fatigue and/or sensory adaptation in the aversive responses measured after pheromone exposure. Fig. 1 summarizes the experimental procedure for the two experiments, which was the same except for the time elapsed between the two shock series.

Preparation of the bees

In the laboratory, bees were rapidly cooled on ice until they showed the first signs of immobility. Subsequently, they were harnessed with tape in holders consisting of two copper plates fixed to a plastic base, as previously described (Núñez et al., 1997; Vergoz et al., 2007). The bee's body thus made a bridge between the two plates, which facilitated the delivery of the electric shocks; 0.05 ml of EEG gel (Spectra 360 Electrode Gel, Parker Laboratories, Fairfield, NJ, USA) was placed between the copper plates to obtain a good contact between the plates and the thorax of the bee (neck and propodeum fitted into the notches of the plates). The bees were then fed with 5 µl of 50% (w/w) sucrose solution and placed in an incubator (at 28°C and 48% relative humidity) in the dark for 2 h. This resting time ensured that the bees adapted to the new harnessed situation. They were randomly assigned either to a control group that did not experience pheromone exposure or to an experimental group that was exposed to a given pheromone (one group per pheromone).

Measurement of shock responsiveness

Two identical set-ups were used in parallel, one for the control group and the other for the experimental group. Each set-up consisted of a Plexiglas box where a holder containing a bee could be connected to the output of an electric stimulator (50 Hz AC current). An air extractor was placed behind each holder to avoid the potential accumulation of alarm pheromone released by the bee upon shock delivery. When the holders were plugged into the set-ups, a timer was triggered and a series of 2 s electric shocks of increasing voltage was delivered to the bee, with a 2 min inter-shock interval to avoid sensitization. Voltages followed an ascending log series of 0.25, 0.5, 1, 2, 4 and 8 V (Roussel et al., 2009). Between and during shocks, the occurrence of SER was recorded as a binary variable (1 when the sting length exceeded that of the last two segments of the abdomen and 0 when this was not the case). If the bee responded several times during a single shock, only one response was noted. Bees that did not respond to any of the six voltages (7 out of 472 bees, i.e. 1.48%) were excluded from the analyses (pre-established, standard criterion). From these bees, only 4 did not respond after a specific treatment: 2 after 2H exposure, 1 after IPA exposure and 1 after mineral oil exposure.

In experiment 1, where the lapse of time between the two shock series was 15 min, bees were exposed to the pheromone immediately at the end of the first shock series (Fig. 1). Thus, pheromone exposure occupied the 15 min lapse of time between shock series. In experiment 2, where the lapse of time was 24 h, bees were placed back in the incubator after the end of the first shock series. At the end of the day, they were released and individually placed in boxes with water and 50% (w/w) sugar solution *ad libitum*; boxes were then placed in the incubator. The following day, bees were cooled on ice and harnessed again. Harnessing was followed by a subsequent resting period in the incubator, which lasted 2 h. Bees were exposed to pheromone or mineral oil after this rest period. Then, the second series of shocks took place. Care was taken to ensure that shocks were delivered during the same hours as the previous day to avoid any circadian effect on responsiveness. In all cases, we kept track of the identity of each bee.

In both experiments, once the second series of electric shocks was finished, bees were killed by placing them in the freezer (−22°C). At the end of the day, glass vials were cleaned with detergent and water, and holders and set-ups were cleaned with ethanol to avoid odor marks.

Pheromone exposure

Bees belonging to the control group were exposed to 25 µl of mineral oil while experimental groups were exposed to one of the three pheromone components: geraniol, IPA or 2H. All chemicals were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Bees were individually confined for 15 min in a 137 ml glass vial containing a filter paper (1×5 cm) soaked with the pheromone component placed under a hood (Baracchi et al., 2017). The entire exposure process took place under a hood to avoid contamination between controls and pheromone-exposed bees. All pheromone substances were diluted to 24% (6 µl pheromone+19 µl mineral oil) (Baracchi et al., 2017; Urlacher et al., 2010). For IPA,

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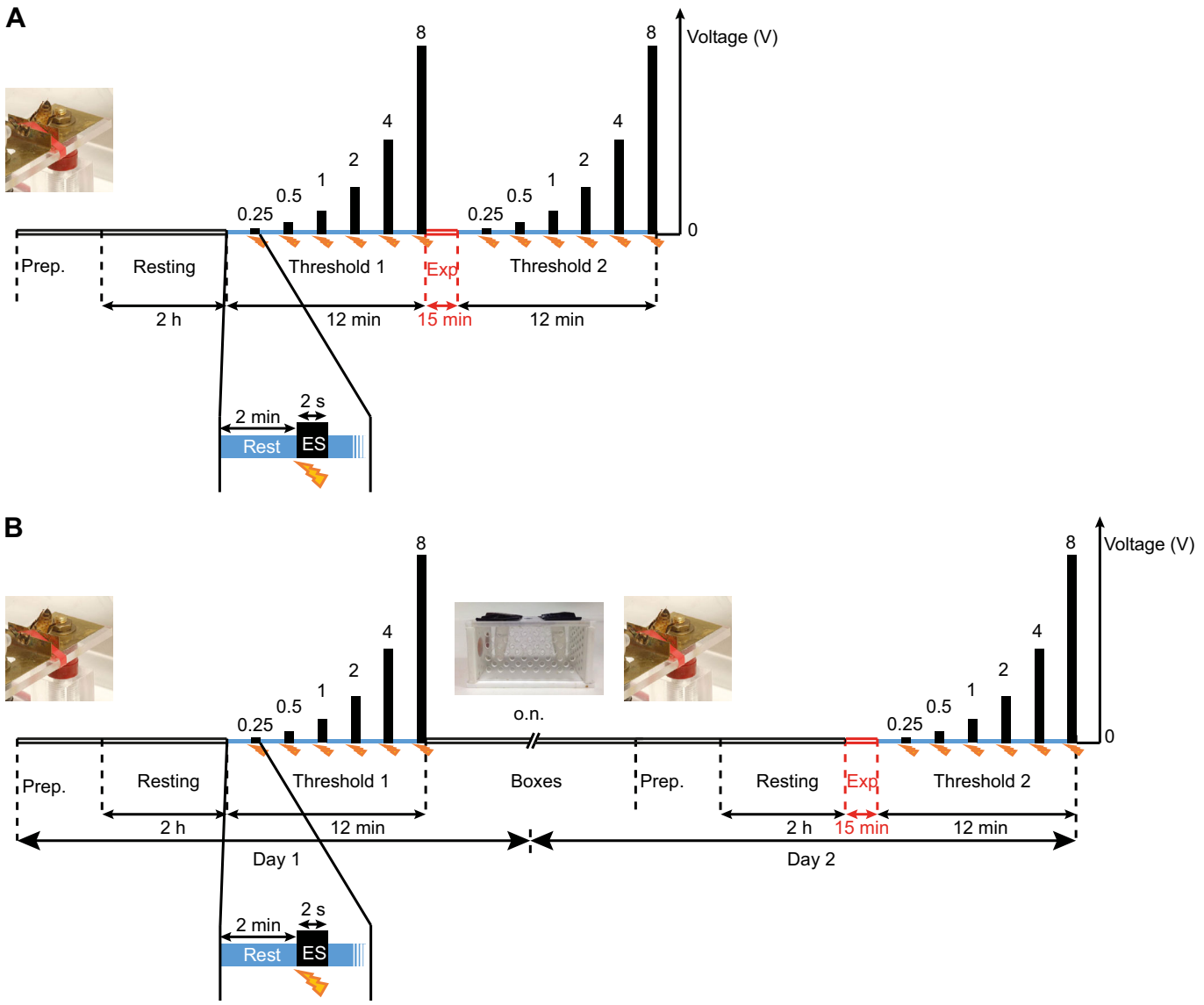

Fig. 1. Experimental schedule of experiments 1 and 2. (A) Experiment 1; (B) experiment 2. In both cases, bees were captured at the beginning of the experiment and randomly assigned either to a control or to an experimental group run in parallel (see Materials and methods for more details). Prep.: preparation of the bees; ES: electric shock; o.n.: overnight; Exp.: exposure (to pheromone in the experimental group and to mineral oil in the control group). The pictures show a harnessed bee in the shock delivery setup (Vergoz et al., 2007) and a beekeeping box in which bees stayed overnight in experiment 2.

this volume corresponded to the amount of IPA contained in 3–10 sting glands (Hunt et al., 2003). For 2H, we used the amount corresponding to that found in 1–3 mandibular glands of foragers (Vallet et al., 1991). In the case of geraniol, which is produced by the Nasanov gland, we used the same amount as for the other two pheromones as this gland has no reservoir (Snodgrass, 1956). In all cases, the amount of pheromone chosen corresponds to natural aversive or appetitive situations recruiting several bees at the same time.

In both experiments, a treatment consisted of a pheromone-exposed group and of its control run in parallel. In experiment 1, six replicates were performed for the geraniol treatment (n=86 bees; 43 for geraniol exposed and 43 for mineral oil exposed), 2H treatment (n=96 bees; 48 for 2H exposed and 48 for mineral oil exposed) and IPA treatment (n=96 bees; 49 for IPA exposed and 47 for mineral oil exposed). In experiment 2, we performed four replicates for geraniol treatment (n=48 bees; 25 for geraniol exposed and 23 for mineral oil exposed) and 2H treatment (n=56 bees; 28 for 2H exposed and 28 for mineral oil exposed) and six replicates for IPA treatment (n=83 bees; 42 for IPA exposed and 41 for mineral oil exposed). After the 15 min of pheromone/mineral oil exposure, bees were directly placed in their respective set-ups for assessment of aversive responsiveness.

Statistical analysis

We performed between-group comparisons to determine whether differences existed between bees exposed to mineral oil (control group) and bees exposed to one of the three pheromone components (experimental group). Furthermore, we performed within-group comparisons to determine whether differences could be detected before and after exposure in the same group of bees. We conducted three distinct analyses for each treatment (geraniol, 2H, IPA). The response data acquired from SER during both shocks and inter-shock intervals were fitted to general linear mixed models (GLMMs) using the *glmer* function of the *lme4* package (Bates et al., 2015). SER served as a binary-response variable (binomial

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family, 'logit' link), while group (control/experimental) and exposure (before/after) were entered as fixed effects and voltage as covariate. We included the bees' identity as a random effect, to account for the repeated measurements performed, and nested it into the replicates to account for the fact that bees tested within a given replicate were probably more affected by similar conditions (weather, pressure, etc.) than those tested in different replicates. Previous papers have shown that SER increases with voltage (Balderrama et al., 2002; Núñez et al., 1997; Roussel et al., 2009; Tedjakumala et al., 2014), an effect that was found in all groups of our experiments (Figs 2 and 3). Therefore, we did not focus on the interaction of voltage with other factors but instead focused on the interaction of group with exposure in order to achieve between-group and within-group comparisons (see above). To this end, we

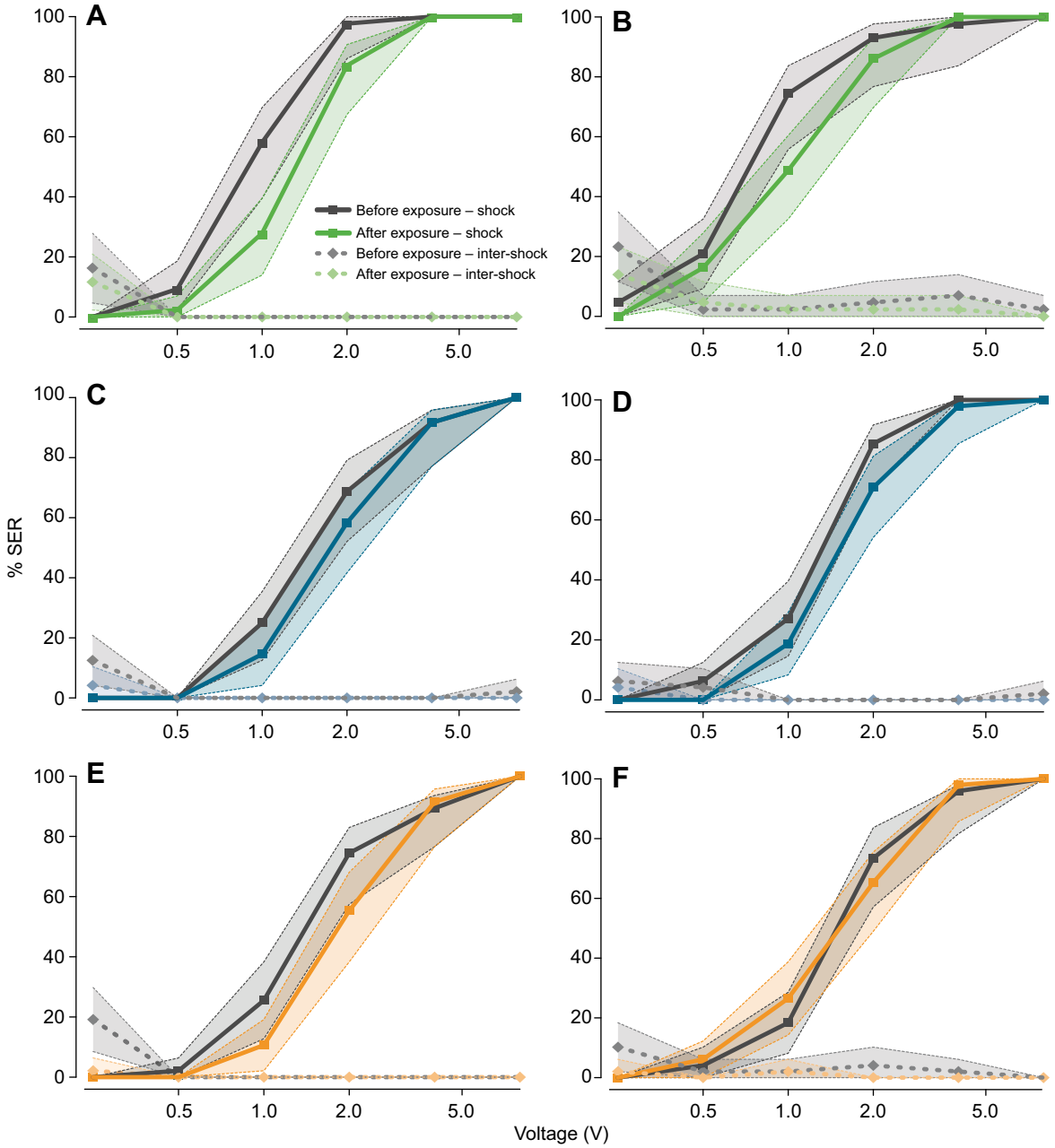

Fig. 2. Experiment 1: effect of pheromone exposure on population shock responsiveness with 15 min between shock series. Shock responsiveness was recorded as the number of bees responding with SER to a given voltage. The abscissa is represented on a logarithmic scale. Solid lines represent responses to electric shocks (dark gray: before exposure; colored: after exposure). Dashed lines represent responses during inter-shock intervals (i.e. in the absence of shock; gray: before exposure; colored: after exposure). Curves are shown with their bootstrapped 95% confidence intervals (*BCa* function of the bootBCa package). (A, C,E) Control groups exposed to mineral oil between the two shock series; (B,D,F) groups exposed to pheromone between the two shock series. (A,B) Control (n=43, A) and geraniol (n=43, B). (C,D) Control (n=48, C) and 2-heptanone (2H, n=48, D). (E,F) Control (n=47, E) and isopentyl acetate (IPA, n=49, F). While inter-shock responsiveness remained low and was not affected by pheromone/mineral oil exposure, shock responsiveness varied between the two series of shocks. The short lapse of time between these two series induced a general decrease of responsiveness in both control (A,C,E) and pheromone-exposed groups (B,D), which was due to fatigue. Only in the case of IPA (F) did shock responsiveness remain unchanged, thus showing that this pheromone component was able to counteract the fatigue effect, restoring responsiveness to original levels.

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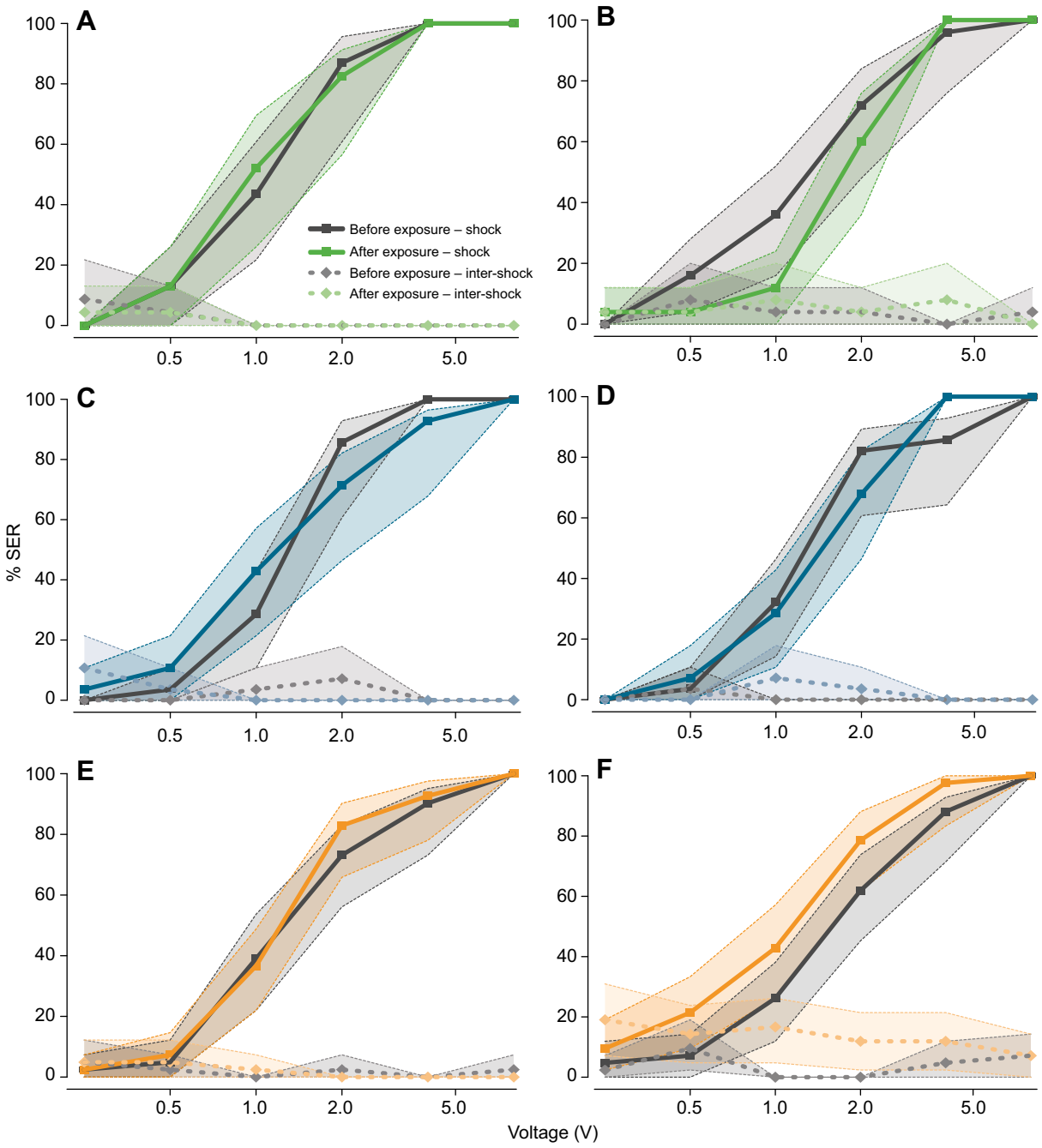

Fig. 3. Experiment 2: effect of pheromone exposure on population shock responsiveness with 24 h between shock series. Shock responsiveness was recorded as the number of bees responding with SER to a given voltage. The abscissa is represented on a logarithmic scale. Solid lines represent responses to electric shocks (dark gray: before exposure; colored: after exposure). Dashed lines represent responses during inter-shock intervals (i.e. in the absence of shock; gray: before exposure; colored: after exposure). Curves are shown with their bootstrapped 95% confidence intervals (*BCa* function of the bootBCa package). (A,C,E) Control groups exposed to mineral oil between the two shock series; (B,D,F) groups exposed to pheromone between the two shock series. (A, B) Control (n=23, A) and geraniol (n=25, B). (C,D) Control (n=28, C) and 2H (n=28, D). (E,F) Control (n=41, E) and IPA (n=42, F). Inter-shock responsiveness remained low and was not affected by mineral oil, geraniol or 2H exposure (A–E). However, IPA significantly enhanced responsiveness in the absence of shock (F). Responsiveness to electric shocks varied depending on the pheromone to which the bees were exposed. After geraniol exposure (B), bees responded less to electric shocks, while they increased their responses after IPA exposure (F).

used a least-squares means (LSM) *post hoc* procedure with Bonferroni correction for multiple comparisons (*lsmeans* function from R package *lsmeans*; Lenth, 2016).

In all cases, data met the assumptions of the tests used. All statistical analyses were performed with the open software R-3.3.1 (http://www.R-project.org/). The entire datasets are available upon request from the corresponding author (M.G.).

RESULTS

Experiment 1

We evaluated SER responsiveness to a series of increasing voltages before and after pheromone or mineral oil exposure. Bees were exposed to their respective substance immediately after the end of the first shock series and the lapse of time between the two shock series was 15 min. Fig. 2 shows the

responses of bees exposed to geraniol, 2H and IPA, and of their respective controls.

As expected, SER to electric shocks (Fig. 2, solid lines) increased significantly in all bees as voltage increased (Fig. 2A,B: geraniol treatment including experimental and control groups; χ^2=60.63, d.f.=1, P<0.001; Fig. 2C,D: 2H treatment including experimental and control groups; χ^2=90.72, d.f.=1, P<0.001; Fig. 2E,F: IPA treatment including experimental and control groups; χ^2=138.99, d.f.=1, P<0.001). A comparison of responses between the first and second series of shocks (dark-gray versus colored solid lines) revealed a decrease of responsiveness during the second series in both the geraniol treatment (Fig. 2A,B: χ^2=16.48, d.f.=1, P<0.001) and the 2H treatment (Fig. 2C,D: χ^2=4.13, d.f.=1, P=0.04). For these bees, the interaction between group and exposure was not significant (geraniol treatment: χ^2=1.15, d.f.=1, P=0.28; 2H treatment: χ^2=0.13, d.f.=1, P=0.72), thus showing that control and experimental bees exhibited the same decrease of responsiveness between the two shock series. In the case of IPA treatment (including experimental and control groups), the interaction between group and exposure was significant (Fig. 2E,F: χ^2=6.25, d.f.=1, P=0.01). Significance was due to the fact that control bees decreased their responsiveness during the second series of shocks (LSM *post hoc* with Bonferroni correction, before versus after: P=0.01), while IPA-exposed bees maintained the same responsiveness (LSM *post hoc* with Bonferroni correction, before versus after: P=1).

During inter-shock intervals (i.e. in the absence of shock), bees exhibited a low responsiveness (Fig. 2, dashed lines). However, this responsiveness was not the same at each inter-shock interval (GLMM, χ^2=93.33, d.f.=5, P<0.001). A high percentage (up to 20%) of bees responded during the 2 min before the first shock, which corresponded to the stressful period following placement in the set-up. Thereafter, SERs decreased significantly during the other inter-shock intervals (1st versus 2nd: P<0.001, 1st versus 3rd: P<0.001, 1st versus 4th: P<0.001, 1st versus 5th: P<0.001, 1st versus 6th: P<0.001). None of the treatment groups exhibited a significant interaction between group (control/experimental) and exposure (before/after) (Fig. 2A,B: χ^2=0.14, d.f.=1, P=0.71; Fig. 2C,D: χ^2=0, d.f.=1, P=0.97; Fig. 2E,F: χ^2=0.08, d.f.=1, P=0.78), thus showing that pheromones did not affect inter-shock responsiveness.

Taken together, the results of experiment 1 show that the short lapse of time between the two shock series induced a general decrease in shock responsiveness, which may have been due to fatigue. Only IPA was able to counteract this effect by keeping general responsiveness at the same level as that observed prior to pheromone exposure.

Experiment 2

In this experiment, bees were exposed to their respective treatment after a lapse of time of 24 h. Fig. 3 shows the responses of bees exposed to geraniol, 2H and IPA, and of their respective controls exposed to mineral oil. As in the previous experiment, all bees exhibited a significant increase of SER with voltage (Fig. 3A,B; geraniol treatment including experimental and control groups; χ^2=109.47, d.f.=1, P<0.001; Fig. 3C,D; 2H treatment including experimental and control groups; χ^2=96.63, d.f.=1, P<0.001; Fig. 3E,F: IPA treatment including experimental and control groups; χ^2=97.27, d.f.=1, P<0.001). A comparison of responses between the first and second series of shocks (Fig. 3; dark-gray versus colored solid lines) revealed that exposure to mineral oil did not affect responsiveness (LSM *post hoc* with Bonferroni correction, before versus after, P=1 for geraniol, 2H and IPA controls). Thus, in the control groups, the 24 h lapse of time allowed recovery from the first series of electric shocks. In the experimental groups exposed to pheromones, different patterns of responses were observed. Bees exposed to 2H did not change their shock responsiveness, as shown by a non-significant interaction between group and exposure (Fig. 3C,D; solid lines; χ^2=0.02, d.f.=1, P=0.89). In contrast, bees exposed to geraniol and IPA varied their shock responsiveness and in consequence the interaction between group and exposure was significant (Fig. 3A,B; geraniol: χ^2=4.26, d.f.=1, P=0.04; Fig. 3E,F; IPA: χ^2=5.20, d.f.=1, P=0.02). Specifically, in the geraniol treatment, control and experimental groups behaved differently after exposure (LSM *post hoc* with Bonferroni correction, P<0.05) as experimental bees tended to respond less after geraniol exposure than before (LSM *post hoc* with Bonferroni correction, P=0.07). In the case of IPA, experimental bees responded more after exposure than before (LSM *post hoc* with Bonferroni correction, P<0.001). However, control and experimental bees reached similar levels of response after exposure (LSM *post hoc* with Bonferroni correction, P=1).

The analysis of responsiveness during the inter-shock intervals (Fig. 3, dashed lines) revealed again that responsiveness was low and decreased in the absence of shock (χ^2=11.85, d.f.=5, P=0.04). In control bees (Fig. 3A,C,E), inter-shock responsiveness was not affected by mineral oil exposure (gray versus colored dashed lines; LSM *post hoc* with Bonferroni correction, before versus after, P=1 for geraniol, 2H and IPA controls). Similarly, exposure to geraniol and 2H (Fig. 3B,D) did not change inter-shock responsiveness as shown by the non-significant interaction between group and exposure (geraniol: χ^2=0.51, d.f.=1, P=0.48; 2H: χ^2=0.37, d.f.=1, P=0.54). This interaction was only significant for IPA (Fig. 3F; χ^2=4.32, d.f.=1, P=0.04). Bees exposed to IPA behaved differently after exposure when compared with their control group (LSM *post hoc* with Bonferroni correction, P<0.01). Moreover, experimental bees increased their responsiveness after exposure to IPA (LSM *post hoc* with Bonferroni correction, P<0.001).

Taken together, the results of experiment 2 show that the long lapse of time (24 h) between the two shock series restored shock responsiveness and that IPA and geraniol exerted opposite effects on aversive responsiveness; IPA enhanced it and geraniol decreased it.

DISCUSSION

Our study aimed at investigating the role of pheromones as modulators of bees' subjective evaluation of aversive stimuli and thus at uncovering a non-canonical function of pheromones as key components of behavioral plasticity. To this end, we exposed bees to three pheromonal components of different valence (two negative, i.e. associated with aversive events, and one positive, i.e. associated with appetitive events) and determined the effect of this exposure on shock responsiveness using a within-group approach (comparison of SER responsiveness before and after exposure to two electric shock series of increasing voltage). As SER responsiveness to electric shocks provides a reliable readout of the bees' subjective evaluation of punishment (Roussel et al., 2009; Tedjakumala et al., 2014; Tedjakumala and Giurfa, 2013), changes in responsiveness following pheromone exposure show that pheromones are capable of behavioral modulation beyond the specific context in which they are released.

Two experiments were conducted to assess this effect with time lapses of either 15 min (experiment 1) or 24 h (experiment 2) between the two shock series. In both experiments we found

a consistent enhancing effect of IPA on shock responsiveness. This enhancement even affected inter-shock responsiveness in experiment 2. Conversely, geraniol decreased aversive responsiveness in experiment 2 but not in experiment 1, although this may have been hidden by a fatigue effect due to the short lapse of time (15 min) between the two series of shocks. In this experiment, the only group not showing a decrease of responsiveness between shock series was the one exposed to IPA, thus indicating that this pheromone was able to counteract the fatigue-based decrease through its enhancement of aversive responsiveness. In both experiments, no effect of 2H on aversive responsiveness was found.

The effect of a positive-valence pheromone on the SER

Our results reveal the novel finding that geraniol, an appetitive pheromone component, has the capacity to modulate the subjective evaluation of aversive stimuli. Exposure to this substance decreased aversive responsiveness to electric shock, thus showing that it diminished the perceptual impact of shock in bees.

A recent study also found that innate appetitive floral odors (linalool and 2-phenylethanol), but not citral (another component of the Nasanov gland), diminish defensive responses (attack of a moving dummy) of honey bees (Nouvian et al., 2015). This could have been due to the lower concentration of citral used or to the caste employed (guards in their case, foragers in ours) as the function of the Nasanov gland changes with age (Boch and Shearer, 1963). Yet, the coincident fact is that an innate appetitive signal, geraniol in our case or two floral odors in Nouvian et al. (2015), down-regulated aversive responsiveness.

At first sight, this detraction of aversive behaviors by appetitive signals may appear counter-adaptive. Indeed, even though a food shortage might affect colony fitness on the long term, an alarm pheromone indicates an immediate danger, which might affect colony survival. It was thus suggested that appetitive floral odors, which are usually encountered away from the colony during foraging, could act as markers of distant foraging locations, thus detracting bees from their aggressiveness (Nouvian et al., 2015). This hypothesis provides a partial account of the geraniol effect, as this pheromone component is indeed released at attractive food sources (Free, 1987) but also at the nest entrance to orient returning foragers (Ribbands and Speirs, 1953) and swarms (Schmidt, 1994). Thus, rather than a location effect, the conflict between an appetitive signal (attractive floral odors, geraniol) and an aversive signal or context (enemy, electric shock) seems to be responsible for down-regulating aversive responsiveness.

The effect of negative-valence pheromones on the SER

IPA and 2H are released in response to potential aversive situations (Boch et al., 1962; Shearer and Boch, 1965), although alternative functions have been reported for 2H (see below). It could be expected, therefore, that unlike geraniol, both pheromones provide a relevant alarm context enhancing aversive responsiveness. This hypothesis was only confirmed for IPA but not for 2H: the former increased shock responsiveness (or restored it to basal levels against fatigue) while the latter did not influence shock responsiveness.

The enhancement of shock responsiveness induced by IPA is similar to the one observed in Africanized honey bees (*Apis mellifera scutellata*) exposed to small amounts of this substance (0.3 µl versus 6 µl in our experiments; Balderrama et al., 2002). However, Africanized bees also decreased their shock responsiveness after being exposed to larger amounts of IPA (2.5, 5, 10 and 12.5 µl; Balderrama et al., 2002; Núñez et al., 1997). These values underline the known differences in aversive sensitivity between Africanized and European bees (Collins et al., 1982): the former are more defensive and react faster to smaller amounts of IPA while the latter are slower and require higher amounts to respond defensively. The fact that we observed an enhancement of aversive responsiveness with 6 µl of IPA in our European bees, while only 0.3 µl was required in Africanized bees to induce a similar effect, is consistent with the reported variation in defensive behavior between these two races. As amounts above 2.5 µl induce an opposite effect (i.e. decreased shock responsiveness) in Africanized bees, amounts above a threshold value higher than 6 µl could produce a similar effect in European bees. Such a decrease has been explained by the activation of an opioid-like system by IPA, which would induce an analgesia-like state, depressing responsiveness to a noxious stimulus (Núñez et al., 1997). According to Núñez et al. (1997), 'the resulting stress-induced analgesia in the defender bee would reduce its probability of withdrawal thus increasing its efficiency against enemies'. This would be of particular importance in the context of a massive attack where all forces should be mobilized.

Unlike IPA, 2H did not affect shock responsiveness in our experiments. In the case of Africanized bees, Balderrama et al. (2002) found that large amounts (12.5 µl) of 2H increased shock responsiveness while small amounts (0.3 µl) did not affect it. Given the different sensitivity of Africanized and European bees to alarm signals, the intermediate amount of 2H we used (6 µl) could correspond to the small amounts assayed in Africanized bees. Furthermore, these values suggest that 2H is not directly associated with stinging responsiveness except if provided in massive doses. This is consistent with the results of Boch et al. (1970), who found that IPA is 20–70 times more efficient than 2H in eliciting alarm behavior at the hive entrance. Our results thus confirm the conclusion that IPA and 2H have different functions (Balderrama et al., 2002). IPA would act as a 'true' alarm pheromone, triggering SER, while 2H could act as an alarm signal, which would be insufficient to trigger SER. Interestingly, alternative functions have been suggested for 2H; it has been identified as an eventual paralyzing agent of enemies bitten by the bees (Papachristoforou et al., 2012) and as a potential negative scent mark to label recently visited and depleted food sources (Giurfa, 1993; Vallet et al., 1991). This multiple functionality could attenuate the impact of 2H on shock responsiveness.

Pheromone modulation contributes to behavioral plasticity

Our findings underline the role of pheromones as potential modulators of different behaviors, depending on their valence and dose. Such modulation could take place at two basic levels: the perceptual one, thus affecting the evaluation of the shock, and/or the motor-output one, thus affecting the production of SER. Distinguishing between these alternatives is difficult based on behavioral evidence; neural analyses would be necessary to determine whether and how their corresponding neural circuits are affected by pheromone exposure. In a recent study, Nouvian et al. (2018) analyzed the stinging attacks of bees towards a rotating dummy, which could be in part assimilated to the stinging response measured here. This response is triggered by IPA, which is consistent with the enhancement of SER found in our work. Nouvian et al. (2018) quantified the levels of biogenic amines in the brain of stinging bees exposed to IPA and found that serotonin (5-HT) and dopamine (DA), but not octopamine (OA), were increased upon IPA exposure (Nouvian et al., 2018). As these two biogenic amines have been related to aggression and attentional processes (Tedjakumala et al., 2014; Tedjakumala and Giurfa, 2013), this finding can be linked to a modulatory effect of IPA on noxious-

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stimulus perception. At the motor-output level, analyses performed on isolated terminal abdominal ganglia of bees have shown that OA is a crucial modulator of SER (Burrell and Smith, 1995). This ganglion receives innervation from dorsal and ventral unpaired neurons, which are major releasers of OA (Stevenson and Sporhase-Eichmann, 1995). Not surprisingly, therefore, OA modulates several motor components of SER (Burrell and Smith, 1995). The fact that IPA exposure does not affect brain levels of OA (Nouvian et al., 2018) seems to favor the hypothesis that the modulatory effect of pheromones found in our work occurs at the perceptual rather than the motor level. Alternatively, the two levels could be affected sequentially with extremely short delays. Whether and how the increase in 5-HT and DA found upon IPA exposure translates into a major release of OA for motor control of SER remains to be determined.

The pheromonal modulation of noxious-stimulus perception is consistent with a new model describing the decision-making process underlying the defensive response of bees (Nouvian et al., 2015). In this model, an individual defensive score resulting from the integration of intrinsic (e.g. genetic traits, caste, age, etc.) and extrinsic (e.g. weather, season, available resources, etc.) factors would be weighed against an internal threshold to determine whether the bee engages in colony defense (Fig. 4A). We suggest that pheromones change this score, and that this change depends on pheromone valence. Negative pheromones, associated with aversive, dangerous events, would move the score closer to the threshold that needs to be overcome to elicit defensive responses, while positive pheromones would move the score away from the threshold, thus detracting bees from defensive behaviors (shaded arrow and red bar in Fig. 4A). A similar scheme can be proposed for appetitive behaviors such as foraging (Fig. 4B). In this case, an appetitive score determined by intrinsic and extrinsic factors would be weighed against an internal threshold to decide whether a bee engages in appetitive search behavior. In this case, positive pheromones would move the score closer to the threshold value, thus facilitating foraging, while negative pheromones would move it away from the threshold, thus inhibiting foraging (shaded arrow and green bar in Fig. 4B).

The appetitive scenario proposed (Fig. 4B) is consistent with the findings of a recent paper, which reported the effect of the same pheromones used in our work (at the same concentration) on an appetitive innate response, the proboscis extension response (PER), which is triggered by the contact of sucrose receptors on the antennae with sucrose solution (Baracchi et al., 2017). The authors investigated whether geraniol, 2H and IPA modulate appetitive responsiveness to sucrose and habituation to sucrose stimulation. Pheromones associated with an aversive context induced a significant decrease of sucrose responsiveness as 40%

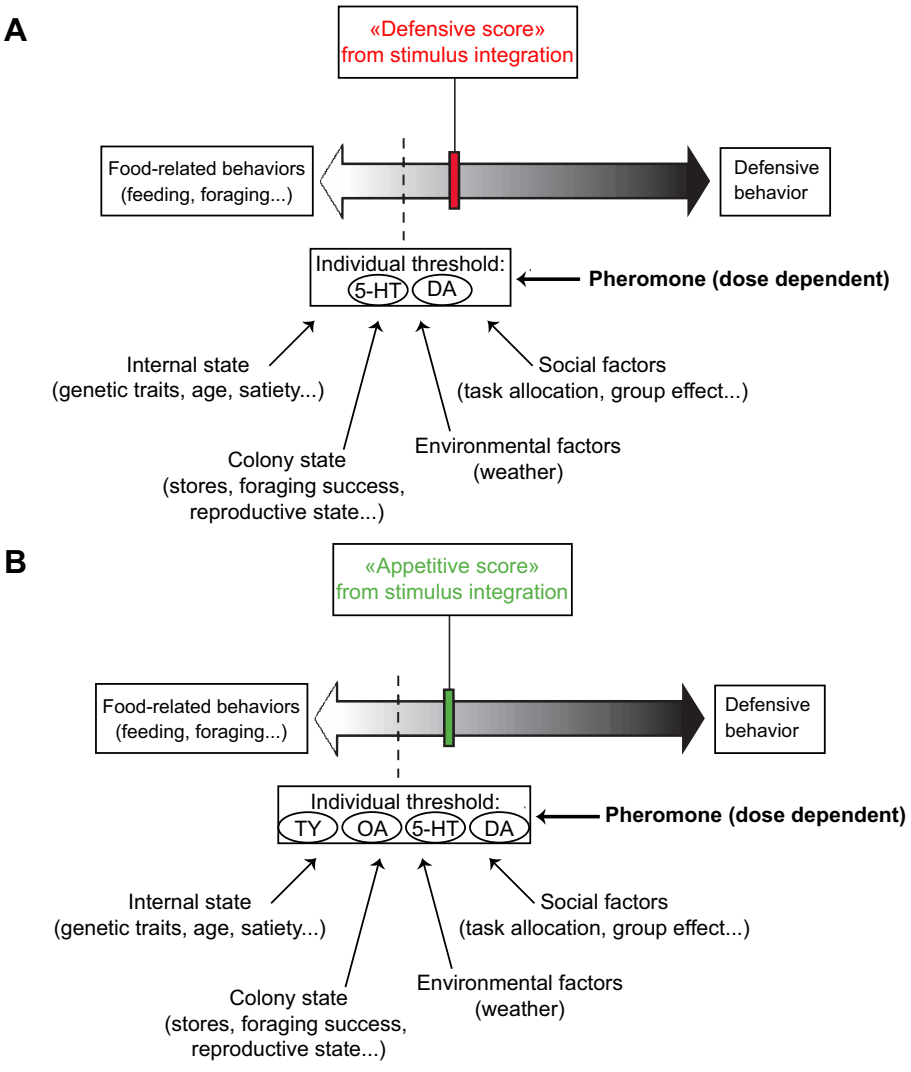

Fig. 4. A model accounting for the modulatory effect of pheromones on decision making in honey bees. The model (adapted from Nouvian et al., 2015) postulates that each individual is characterized by a defensive and an appetitive score, which are determined by extrinsic and intrinsic factors. Pheromones may act on this score, moving it away from or towards a threshold that needs to be overcome to elicit a specific behavior. (A) Defensive score and its relationship with a defensive-response threshold. Pheromones modify the score depending on their valence (shaded arrow and red bar). Positive, appetitive pheromones move it away from the threshold, thus decreasing the probability of a defensive response. Negative, aversive pheromones have the opposite effect, moving the score towards the threshold and thus increasing the probability of a defensive response. (B) Appetitive score and its relationship with an appetitive-response threshold. Pheromones modify the score depending on their valence (shaded arrow and green bar). Positive, appetitive pheromones move the score towards the threshold, thus increasing the probability of an appetitive response. Negative, aversive pheromones have the opposite effect, moving the score away from the threshold, thus decreasing the probability of an appetitive response. 5-HT, serotonin; DA, dopamine; TY, tyramine; OA, octopamine.

and 60% of bees exposed to IPA and 2H, respectively, did not respond to any sucrose concentration. In bees that responded to sucrose, geraniol enhanced sucrose responsiveness while 2H, but not IPA, had the opposite effect. Taken together, our results and those of Baracchi et al. (2017) show that IPA increases shock responsiveness and suppresses sucrose responsiveness. In contrast, geraniol enhances sucrose responsiveness and decreases aversive responsiveness. These results demonstrate that the same pheromone, at the same concentration, can have different effects according to the context (i.e. appetitive or aversive) in which it is released. The case of 2H seems more complex because of the possible multiple roles of this pheromone (see above): Baracchi et al. (2017) found that 2H suppressed sucrose responsiveness in 60% of the bees and down-regulated this responsiveness in the remaining 40%; in our case, no effect on aversive responsiveness was detected.

The modulatory effect of pheromones might be based on the action of these chemicals on different aminergic circuits modulating behavior. In the honey bee, several studies have shown that OA acts as a crucial neuromodulator of appetitive responses (Hammer, 1993; Scheiner et al., 2002) while DA and 5-HT are involved in aversive responses (Tedjakumala et al., 2014; Vergoz et al., 2007). Recent studies in the bee have cast doubt about the validity of such a clear separation between OA and DA in appetitive and aversive reinforcement signaling, respectively (Klappenbach et al., 2013). Irrespective of this, pheromones could regulate the balance of the biogenic amines contained in the bee brain, enhancing or depressing responsiveness to different kinds of stimuli according to their valence and context of release.

Through this non-canonical action (in the sense of not being associated directly with the response modulated, like the effect of geraniol on SER or of 2H on PER), pheromones would act on an animal's motivation to engage in a given behavior. Moreover, as pheromones change the subjective perception of stimuli, being attractive (sucrose) or aversive (electric shock), they may also have an impact on the capacity to learn about these stimuli. Bees that exhibit high responsiveness to sucrose solutions of variable concentration are better learners in olfactory and tactile conditioning protocols that use sucrose solution as a reward (Scheiner et al., 2001a,b). Similarly, the more sensitive bees are to an electric shock, the better they learn about that shock (Roussel et al., 2009). Therefore, the effect of pheromones might not only be restricted to responsiveness and motivation but also could affect learning and memory via the modulation of the salience of an unconditioned stimulus. Thus, besides conveying stereotyped messages, pheromones have an important role as modulators of behavioral plasticity.

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The authors declare no competing or financial interests.

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