

RESEARCH ARTICLE

Honeybee drones are attracted by groups of conspecifics in a walking simulator

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ABSTRACT

During the mating season, honeybee males, the drones, gather in congregation areas 10–40 m above ground. When a receptive female, a queen, enters the congregation, drones are attracted to her by queen-produced pheromones and visual cues and attempt to mate with the queen in mid-air. It is still unclear how drones and queens find the congregations. Visual cues on the horizon are most probably used for long-range orientation. For shorter-range orientation, however, attraction by a drone-produced aggregation pheromone has been proposed, yet so far its existence has not been confirmed conclusively. The low accessibility of congregation areas high up in the air is a major hurdle and precise control of experimental conditions often remains unsatisfactory in field studies. Here, we used a locomotion compensator-based walking simulator to investigate drones' innate odor preferences under controlled laboratory conditions. We tested behavioral responses of drones to 9-oxo-2-decenoic acid (9-ODA), the major queen-produced sexual attractant, and to queen mandibular pheromone (QMP), an artificial blend of 9-ODA and several other queen-derived components. While 9-ODA strongly dominates the odor bouquet of virgin queens, QMP rather resembles the bouquet of mated queens. In our assay, drones were attracted by 9-ODA, but not by QMP. We also investigated the potential attractiveness of male-derived odors by testing drones' orientation responses to the odor bouquet of groups of 10 living drones or workers. Our results demonstrate that honeybee drones are attracted by groups of other drones (but not by workers), which may indicate a role of drone-emitted cues for the formation of congregations.

KEY WORDS: *Apis mellifera*, Mating, Congregation area, Orientation, Pheromone, Behavior

INTRODUCTION

The domesticated honeybee *Apis mellifera* has become a mainstream animal model for scientific research in ethology, neurobiology and animal cognition because of its rich behavioral repertoire and astonishing cognitive abilities (Von Frisch, 1965; Michener, 1974; Winston, 1987; Seeley, 1996; Menzel, 1999; Giurfa, 2007; Sandoz, 2011; Menzel, 2012). Honeybees are globally the most economically valuable pollinator for a majority of fruit, vegetable and seed crops, and thus play a crucial role in providing sufficient food supplies for today's more than 7 billion people worldwide (United Nations Environmental Programme, 2010). Yet, for all the knowledge acquired on this model organism, crucial

aspects of its reproductive behavior, which are essential for optimization of beekeeping strategies, still remain elusive.

Honeybees display a particularly striking mating behavior, which has long fascinated beekeepers and researchers alike (Butler, 1609; Jean-Prost, 1957; Ruttner, 1957; Ruttner and Ruttner, 1972; Koeniger et al., 1979; Baer, 2005). During the mating season, sexually mature drones fly out on warm and sunny afternoons and gather high in the air at discrete congregation areas located usually 10–40 m above ground, with a diameter of 30–200 m (Loper et al., 1987; Loper et al., 1992; Koeniger and Koeniger, 2004). Drone congregations may contain at any one time as many as 11,000 drones from up to 240 different colonies (Free, 1987; Baudry et al., 1998; Koeniger et al., 2005b). When a virgin queen enters a congregation area, many drones are attracted to her, both by olfactory signals (pheromones) and by visual cues at shorter range (Gries and Koeniger, 1996). Drones follow the virgin queen in a comet-like swarm and engage in a scramble competition, each individual struggling for the most promising position to approach and mate with the queen (Gries and Koeniger, 1996). Within 15–30 min, the queen mates with 10–20 drones, which die directly after copulation (Baudry et al., 1998; Palmer and Oldroyd, 2000). Hence, drones are organisms specially adapted for mating and are tuned to the queens' pheromones. Pheromones are volatile chemicals used for communication between individuals of the same species (Karlson and Lüscher, 1959). Honeybees, like many insects, employ a rich repertoire of pheromones to ensure intraspecific communication in many behavioral contexts (Free, 1987; Sandoz et al., 2007; Le Conte and Hefetz, 2008). The queen, the only fertile female in the colony, communicates her presence and manifests her influence by means of a mixture of substances released mainly from her mandibular glands. This queen mandibular pheromone (QMP) reinforces social cohesion within the hive by attracting young workers and enticing them to lick and antennate the queen (Winston, 1987; Slessor et al., 1988; Slessor et al., 2005). It also ensures the reproductive monopole of the queen by inhibiting the development of the workers' ovaries (Hoover et al., 2003).

QMP was originally considered to be a unique substance, 9-oxo-(E)-2-decenoic acid (9-ODA) (Barbier and Lederer, 1960; Callow and Johnston, 1960; Butler et al., 1962). Later studies revealed the existence of at least four additional components (Slessor et al., 1988), including two enantiomers of 9-ODA's biosynthetic precursor, (R)- and (S)-9-hydroxy-(E)-2-decenoic acid (9-HDA), and two other compounds, methyl *p*-hydroxybenzoate (HOB) and 4-hydroxy-3-methoxyphenylethanol (HVA). Whereas the odor bouquet of virgin queens is strongly dominated by 9-ODA, the ratio of QMP components changes after mating, leading to a more balanced mixture with proportionally less 9-ODA in mature queens (Pankiw et al., 1996; Plettner et al., 1997).

Accordingly, 9-ODA was shown to be the major queen-produced sex pheromone, attracting drones to virgin queens in congregation areas from a distance of 60 m (Gary, 1962; Pain and Ruttner, 1963;

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List of symbols and abbreviations

9-HDA	9-hydroxy-(E)-2-decenoic acid
9-ODA	9-oxo-2-decenoic acid
10-HDA	10-hydroxy-(E)-2-decenoic acid
HOB	methyl p-hydroxybenzoate
HVA	4-hydroxy-3-methoxyphenylethanol
QMP	queen mandibular pheromone

Butler and Fairey, 1964) and potentially even larger distances (Loper et al., 1993). However, 9-ODA alone does not always reproduce the effect of a complete queen extract in attraction bioassays (Pain and Ruttner, 1963). Some recent data suggest that 9-HDA and an additional component, 10-hydroxy-(E)-2-decenoic acid (10-HDA), increase the numbers of contacts made by drones on baited queen dummies when presented in a blend with 9-ODA (Brockmann et al., 2006). The queen sex pheromone may therefore be a complex blend that is most effective when all components are present in appropriate ratios in the mixture. Thus, while 9-ODA is clearly the main attractant for drones, the question of co-attractants is still unresolved.

Until now, it has still not been fully understood how drones – and virgin queens – find the congregation areas in the first place. Even though the life span of a drone is limited to a few weeks (Fukuda and Ohtani, 1977), drone congregation areas are surprisingly constant in location from year to year, and some congregations have been reported to form consistently at the same place over decades (Jean-Prost, 1960; Ruttner and Ruttner, 1968; Ruttner, 1985; Koeniger and Koeniger, 2004). Whereas the presence of a queen is not necessary (Jean-Prost, 1957; Ruttner and Ruttner, 1965; Koeniger and Koeniger, 2004), visual cues on the horizon, such as mountains, valleys and tree tops in less mountainous regions, have been shown to be important for the formation of a drone congregation area and are used for long-range orientation (Ruttner and Ruttner, 1966; Ruttner and Ruttner, 1972; Ruttner, 1985; Pechhacker, 1994). However, horizon cues cannot explain orientation at the area itself and the clear-cut dimensions of a drone congregation, as the borders of a congregation are intriguingly well-defined: when a virgin queen leaves the congregation area, drones rapidly stop their pursuit and return to their conspecifics in the congregation (Ruttner and Ruttner, 1965; Ruttner, 1985; Loper et al., 1992). The existence of possible drone-produced aggregation pheromones in honeybees has been proposed, but its existence needs experimental confirmation (Free, 1987; Gerig, 1972). Recently, an attractive effect of male-derived odors has been demonstrated in drone congregations of a stingless bee species (Galindo López and Kraus, 2009), and male aggregation pheromones have been identified from the mandibular glands of some hymenopteran species (Ayasse et al., 2001). However, the mandibular glands of honeybee drones are extremely reduced and glandular secretory production terminates at the time when drones begin leaving the hive for nuptial flights (Ruttner, 1985; Lensky et al., 1985). Although these findings potentially contradict a prominent role of honeybee drones' mandibular glands in the formation of drone congregation areas, Lensky et al. suggested in the same study that the glands may still contain minor quantities of compounds, which are attractive to other drones (Lensky et al., 1985).

So far, all behavioral experiments on innate odor preferences of honeybee drones suffer from the limited accessibility of drone congregation areas, which are located high up in the air. In previous studies, long poles or helium balloons have been used to present stimuli to drones within the congregation (Gary, 1962; Butler and Fairey, 1964; Ruttner and Ruttner, 1966; Gerig, 1971;

Koeniger et al., 2005a; Brockmann et al., 2006). However, such field studies are arduous and experimental conditions can be difficult to control in a satisfactory manner. The aim of the present study was to establish a new laboratory attraction assay that allows testing innate odor preferences of drones under strictly controlled experimental conditions. To this end, we used a locomotion compensator-based walking simulator and designed two specific experimental procedures: (1) a bidirectional orientation test, in which drones were presented with odorants either from their right or their left side; (2) a quadrant choice test, in which drones were given control over odor stimulation. We measured behavioral responses of drones to stimulation with a panel of biologically relevant odors: these included 9-ODA and QMP to validate the functionality of our setup and experimental procedures and to test whether drones generally respond in a uniform manner when 9-ODA is presented either as a single component or as part of a mixture. We also investigated the possible existence of attractive male-derived odors in honeybees by testing drones' behavioral responses to stimulation with the odor bouquet from groups of living drones or workers.

RESULTS

A total of 347 drones were tested in our walking simulator setup. When mounted on the ball, drones usually directly started walking and turning to the left and to the right. In the longest version of our experiments, each drone was kept on the ball for 15 min (odor quadrant test). In such an experiment, we observed that the drones' activity slowly decreased over time, as shown by their average walking speed (see supplementary material Fig. S1). However, walking speed at the end of the experiment still remained at about 60% of the initial value. Therefore, in this work, no exclusion of individuals based on their walking activity was performed.

Bidirectional odor orientation test

In the bidirectional odor orientation test (Fig. 1A), we tested whether stimulation with a 1 s odor pulse of 9-ODA ($N=24$) or QMP ($N=25$) from either the right or the left side resulted in drones changing their walking speed or turning toward the side of odor stimulation. When evaluating possible changes in walking speed, we found a significant heterogeneity among drones' responses when the tested odorant was 9-ODA (Friedman test, $\chi^2=11.4$, $P=0.0095$; Fig. 2A), but not when the stimulus was QMP (Friedman test, $\chi^2=1.05$, $P=0.78$; Fig. 2B). More specifically, bees significantly increased their walking speed when 9-ODA was presented on their right side, compared with the respective control (Wilcoxon test, $Z=3.24$, $P=0.0012$; Fig. 2A). However, this effect was not found when 9-ODA was presented on the left side (Wilcoxon test, $Z=0.47$, $P=0.64$). When evaluating possible changes in turning direction (Fig. 2C,D), we also found a significant heterogeneity among drones' responses when the tested odorant was 9-ODA (Friedman test, $\chi^2=8.57$, $P=0.036$; Fig. 2C), but not when the stimulus was QMP (Friedman test, $\chi^2=1.85$, $P=0.60$; Fig. 2D). More specifically, bees turned in opposite directions – and toward the odorant – when 9-ODA was presented on the right or on the left side (9-ODA right versus 9-ODA left, Wilcoxon test, $Z=2.03$, $P=0.042$; Fig. 2C), but did not do so when unscented air was presented (control left versus control right: Wilcoxon test, $Z=0.17$, $P=0.86$). Thus, only 9-ODA tended to induce a change in drones' behavior, increasing somewhat their walking speed and their turning direction. No significant effect of QMP appeared in this experiment. Albeit significant, these effects were small and we next endeavored to provide a more adequate orientation test allowing a clearer behavioral readout for innate odor preferences of drones.

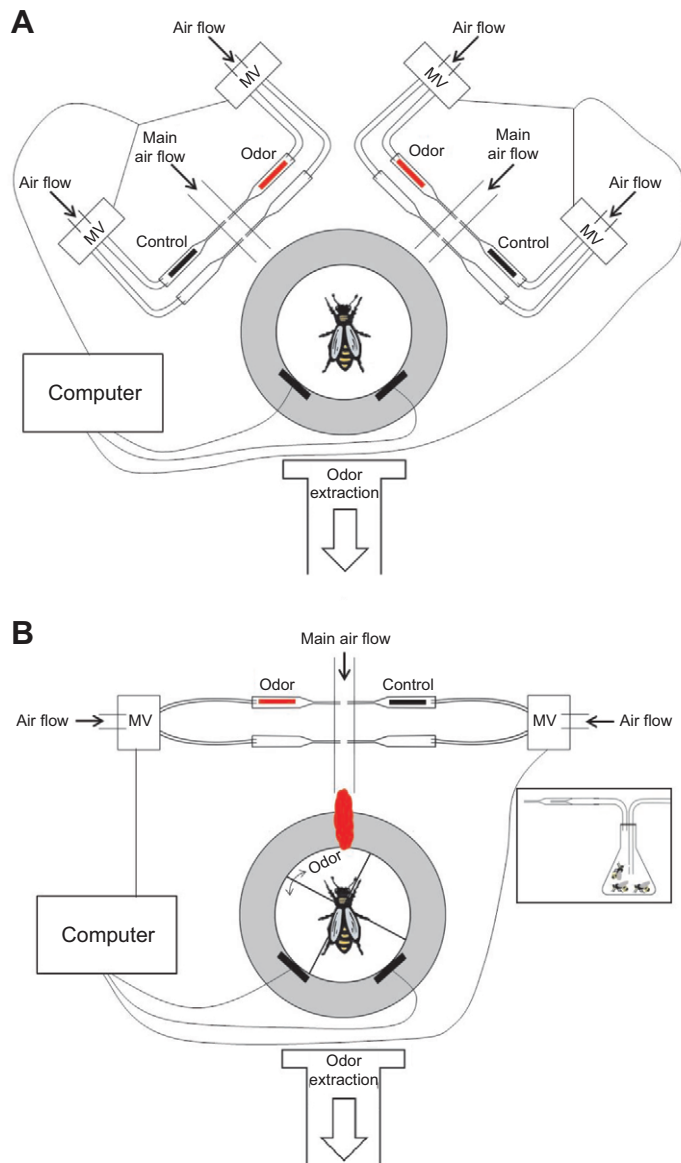


Fig. 1. Walking simulator setup. A tethered honeybee drone is allowed to walk freely on an air-supported ball (in white). Ball displacement is recorded via two computer-mouse sensors (black bars close to the ball), which allows reconstruction of the drone's walking path. Odor stimulation is provided via constant air streams directed at the drone. Odors are quickly removed from the setup by an exhaust behind the drone. All experiments were conducted in complete darkness. (A) System used for the bidirectional odor orientation test. For stimulus delivery, two glass tubes are directed at the antennae of the drone, one from the left, the other from the right (45 deg from the drone's axis). Odor stimulation from one or the other side was given at precisely defined time points using computer-controlled magnetic valves (MV) switching between odor-laden and empty pipettes. This allowed us to measure whether odor stimulation results in a directed behavioral response toward odor origin. For stimulation, we used 9-oxo-2-decanoic acid (9-ODA), queen mandibular pheromone (QMP) and solvent control. (B) System used for the odor quadrant choice test. For stimulus delivery, a single glass tube is directed frontally at the drone's antennae. The ball is divided into four virtual quadrants, one of which is designated as the odor quadrant. After a stimulation-free accommodation phase of 5 min, stimulus control is granted to the drone for 5 min: whenever the drone is heading toward the odor quadrant, odor stimulation is activated using the computer-controlled magnetic valves (stimulus control phase). This allowed us to quantify whether the animal preferred receiving odor stimulation. For stimulation, we used either odorants (9-ODA or QMP) or groups of 10 living drones or workers and respective controls. The inset shows the glass vial used for the presentation of living insects.

Odor quadrant choice test

We reasoned that drones may have difficulties finding the origin of the odor source in our setup and that we may need to give drones some control over the odor stimulation to be able to measure a clear attraction toward the presented odorants. Based on the same locomotion compensator as above, we designed the odor quadrant choice test (Fig. 1B), in which the odor is presented to the drone whenever it is heading toward a particular quadrant of the ball. Therefore, the odor quadrant choice test allowed quantifying whether drones preferred receiving odor stimulation. For quantification, we measured the time drones spent heading toward the odor quadrant before, during and after the stimulus control phase, during which odor stimulation was coupled with the drones' heading direction (Fig. 3). For stimulation, we used 9-ODA ($N=43$), QMP ($N=41$), groups of 10 living drones ($N=62$) or workers ($N=48$) and respective controls ($N=98$). Before the stimulus control phase (Fig. 3A), drones spent approximately one quarter of their time in the odor quadrant irrespective of the group to which they were assigned. Accordingly, we did not find any statistical difference among groups (Kruskal–Wallis test, $H_{\text{before}}=3.35$, $P_{\text{before}}=0.5$). During the stimulus control phase (Fig. 3B), however, a clear heterogeneity appeared in the time spent by the different groups in the odor quadrant ($H_{\text{during}}=13.5$, $P_{\text{during}}<0.0089$). Dunn's multiple comparisons test showed that drones spent significantly more time in the odor quadrant when the odor bouquet of 10 drones ($q=2.48$, $P=0.013$) or 9-ODA ($q=2.04$, $P=0.040$) was presented compared with the control stimulation. This effect was not observed for the QMP mixture or for the odor bouquet of 10 workers ($q=0.61$, $P=0.54$ and $q=0.52$, $P=0.60$, respectively). After the stimulus control phase (Fig. 3C), no difference among groups appeared anymore in the time spent in the odor quadrant ($H_{\text{after}}=7.36$, $P_{\text{after}}=0.12$). We conclude that when given control over odor stimulation, drones can display an odor preference in a laboratory assay. From the proposed stimuli, only 9-ODA and the bouquet of living drones were found to be attractive to drones. QMP and the bouquet from living workers did not induce any change in the drones' behavior.

DISCUSSION

In this study, we established a new laboratory assay to study innate odor preferences of honeybee drones under controlled experimental conditions. In our walking simulator, drones were attracted to 9-ODA, the main queen-produced sex pheromone, but not to QMP, a blend of 9-ODA and other components. Using the odor bouquet of groups of living animals for stimulation revealed that drones are attracted by groups of other drones but not by workers. This is the first evidence under controlled laboratory conditions for a honeybee drone-produced attractive odor cue, which may be important for the formation of drone congregations.

In our first experimental approach, odorants were presented to drones either from the left side or from the right side, expecting the drones to display a clear turning response toward the side on which an attractive odor was presented (bidirectional odor orientation test, Fig. 1A). The results showed that clear odor-specific turning responses of drones were extremely rare and the large majority did not show any obvious reaction to odor stimulation, even though some significant effects of 9-ODA, the major active component of queen sex pheromone, could be measured. Upon presentation of 9-ODA, drones tended to increase their walking speed, but this effect was only significant for stimulations coming from one side. We do not know the reason for this observation. Despite our careful design of the setup, it could be due to an uncontrolled asymmetry in odor stimulation. Alternatively, it might be related to sensory

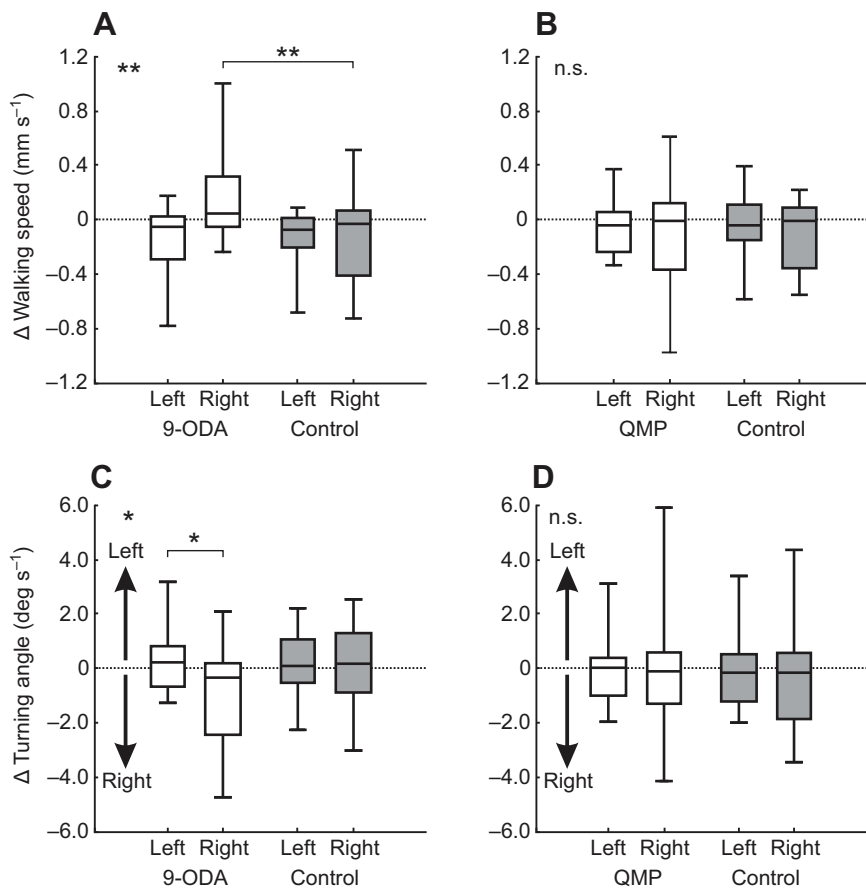


Fig. 2. Bidirectional odor orientation test. Change in walking speed (A,B, in mm s^{-1}) and turning direction (C,D, in deg s^{-1}) comparing 20 s before and 20 s after stimulation with a 1 s pulse from the left or the right side with 9-ODA (A,C, $N=24$) or QMP (B,D, $N=25$) and respective solvent controls. For walking speed, a positive value indicates that drones walked more quickly. For turning direction, a positive value indicates a turn to the left side and a negative value a turn to the right side. Boxes show the median and interquartile ranges, while whiskers represent the 10th and 90th percentiles. 9-ODA but not QMP induced behavioral responses from drones, as shown by a difference in walking speed and turning direction among stimulations (Friedman test, upper left corner of each panel; $*P < 0.05$; $**P < 0.01$; n.s., not significant). Drones walked more quickly when stimulation with 9-ODA was provided from the right (A, Wilcoxon test, $**P < 0.01$), and turned in opposite directions when 9-ODA came from the left or the right side, orienting toward the odor (C, Wilcoxon test, $*P < 0.05$).

asymmetries of drones between sides, as suggested recently for honeybee workers that were shown to harbor more olfactory sensilla on the right antenna than on the left (Letzkus et al., 2006; Frasnelli et al., 2010). A possible explanation for drones' difficulty in showing a clear turning response toward the odorants may be that it was hard for them to determine from which side the odor stimulus originated. In an earlier study, Kramer demonstrated that walking honeybees use positive anemotaxis for odor orientation (Kramer, 1976). Thus, when encountering an attractive odor, bees first orient upwind, toward the airflow. However, in our bidirectional orientation test, drones were not able to walk upwind because of the lateral positions of the two air flows. Another drawback of this protocol is that even a turning response toward the stimulus did not change stimulus intensity or duration. By contrast, in their natural environment drones receive direct sensory feedback in response to their behavior and the lack of feedback in the bidirectional odor orientation test might have strongly impaired their behavioral performance.

We designed the odor quadrant test to overcome these different problems. First, the air flow was provided frontally from only one direction, so that drones always walked upwind. Second, the insects were given full control over the odor stimulation, thus providing direct feedback in response to their behavior. Indeed, in this case, drones spent significantly more time in the odor quadrant during the stimulus control phase when 9-ODA or the odor bouquet of 10 living drones was presented. The effects were relatively weak, albeit statistically robust, and required testing many individuals. Because of its location in the laboratory, our experimental procedure did not provide the context in which drones usually depart for their mating flights as it was not designed to imitate the natural situation of mating flights in the best possible way, but to provide clear criteria

for measuring whether a drone is attracted by an odorant and to allow maximal control over experimental procedure. Accordingly, we could not control the behavioral and physiological state of drones at the time of the experiment, which may have impacted their performance. It should be noted that this is true for most laboratory assays because of their reductive design, as for instance in the widely used proboscis extension conditioning paradigm (Bitterman et al., 1983), where tethered bees are neither in a foraging context nor most likely in the same behavioral state as a departing forager in the wild. However, drones' mating behavior may be much more sensitive to context changes than, for instance, foraging behavior in workers.

We found that drones discriminate between 9-ODA, which strongly dominates the odor bouquet of virgin queens, and QMP, which rather resembles the odor bouquet of a mated queen. This differential treatment by drones of 9-ODA and QMP could be evolutionarily adaptive, as it would not be beneficial for a drone to approach and try to mate with an already mated queen when they meet in the hive or during swarming. Interestingly, some observations have indicated that drones can be attracted to mated queens in free flight, when the latter were artificially introduced into drone congregation areas in field experiments (Pain and Ruttner, 1963). Naturally, mated queens are highly unlikely to enter congregation areas on their own initiative and the initial approach of drones exemplifies the extremely competitive character of a congregation area, where even the slightest chance for successful mating is seized. In this case, the visual modality initially plays a crucial role: reportedly, drones even respond to stones thrown into the congregation (Ruttner, 1985). In any case, the fact that drones can discriminate between 9-ODA and QMP has interesting

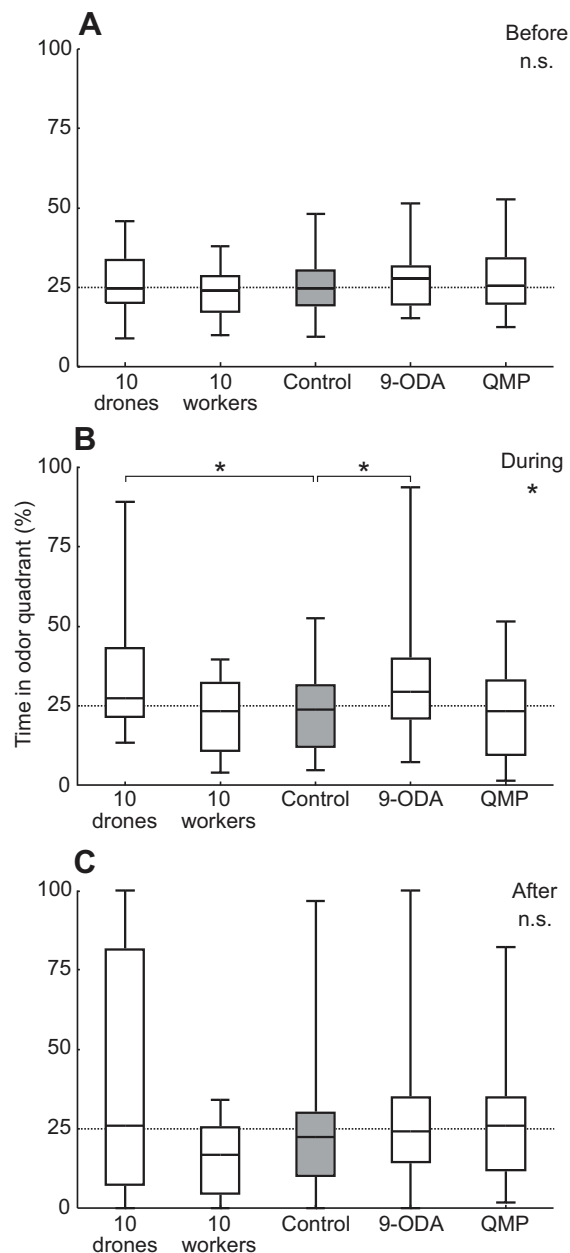


Fig. 3. Odor quadrant choice test. Box plots showing the median time drones spent in the odor quadrant before (A), during (B) and after (C) the stimulus control phase. Boxes show the interquartile ranges, while whiskers represent the 10th and 90th percentiles. Kruskal–Wallis tests revealed significant differences between responses to different stimuli during ($P < 0.01$), but not before or after the stimulus control phase ($P = 0.5$ and $P = 0.12$, respectively). Drones spent significantly more time in the odor quadrant when 9-ODA ($N = 43$) or the odor bouquet of 10 drones ($N = 62$) was presented compared with control stimulation ($N = 98$; Dunn's multiple comparisons). Stimulation with QMP ($N = 41$) or the odor bouquet of 10 workers ($N = 48$) had no effect.

implications for the neuronal processing of queen sex pheromone. The honeybee drone olfactory system is specially adapted for the detection and processing of mating-relevant olfactory cues. The antennae of drones feature an extremely high number of 9-ODA-receptive sensilla placodea (Kaissling and Renner, 1968; Esslen and Kaissling, 1976; Brockmann et al., 1998; Brockmann and Brückner, 2005) and the first olfactory neuropile of the drone brain, the antennal lobe, contains several hypertrophied glomeruli (termed

macroglomeruli), one of which responds specifically to 9-ODA (Arnold et al., 1985; Sandoz, 2006). Our behavioral results indicate that information on 9-ODA is not processed in a pure labeled line manner, where detection of 9-ODA would always elicit a stereotypic behavior, independently of other odorants presented with it. Rather, information on additional components of the odor bouquet is taken into account and integrated, leading to an adapted, flexible behavioral response. Thanks to the advent of optical imaging in the drone brain, the study of such integration is now accessible (Sandoz, 2006).

Queens arrive at congregation areas ~1 h after drones (Jean-Prost, 1957; Ruttner, 1985; Koeniger and Koeniger, 2004). Hence, the formation of a drone congregation area cannot depend on the presence of queen-produced pheromones. Our result that drones are attracted by the odor bouquet of other drones provides the first statistically robust evidence under controlled experimental conditions for a drone-produced attractive odor cue in honeybees. Within the hive, drones are known to cluster together on some parts of the comb (Ohtani, 1974). Such behavior may involve an attractive olfactory cue, as suggested by our experiments. Outside of the hive, two previous studies provided some indications for the existence of a drone-produced attractive odor cue (Gerig, 1972; Lensky et al., 1985). Unfortunately, because of the difficulty of testing such effects in nature, these studies provided low numbers of replicates and did not evaluate the results statistically. Even more, Lensky's report on the drones' behavioral responses to the putative drone-produced attractive odor is contradictory: drones were described to approach the odor sources presented directly at apiaries in a comet-like swarm, which rather corresponds to their behavior when following a queen in a congregation. Such apparently abnormal behavior may have been induced by an extremely high concentration of odor on the baits. As a possible source for the putative drone-produced attractive signal, Lensky et al. suggested the mandibular glands of drones (Lensky et al., 1985). In a comprehensive review, Ayasse et al. described male-produced pheromones and attractants and their source of origin for a large variety of insect species (Ayasse et al., 2001). In numerous genera of ants and bees, the males' mandibular glands have been suggested as the source of sex attractants, although in most cases the active components have not yet been conclusively identified. For honeybees, a major role of the drones' mandibular glands remains debatable, because they begin to degenerate at an age of 9 days, i.e. just around the time when drones start leaving the hive for nuptial flights and before drones are fully sexually mature (Ruttner, 1985; Lensky et al., 1985). Alternately, honeybee drones present apparently functional antennal glands (Romani et al., 2003). In addition, in more than 30 species of bumblebees, the labial glands were identified as the source of male-produced attractive components (Ayasse et al., 2001). Identification of the honeybee drone-produced active component thus requires thorough chemical analyses of the content of the different candidate glands followed by attraction bioassays. The walking simulator presented in the present study may constitute an ideal tool for testing candidate pheromonal molecules. Considering the highly specialized olfactory system of drones, the question arises whether one or more of the macroglomeruli of the antennal lobe are not specific for queen-produced but rather for drone-produced odor cues, and further neurophysiological approaches may be helpful for narrowing down the range of putative candidate glands and identifying male-produced sex pheromones.

How do our results aid in our understanding on the formation and coherence of drone congregation areas? As described previously, drones and queens use cues on the horizon for far-range orientation,

following flyways between prominent landmarks such as mountains or high tree tops (Ruttner and Ruttner, 1966; Ruttner and Ruttner, 1972; Ruttner, 1985; Pechhacker, 1994). Based on radar observations, Loper et al. reported that drone congregations form preferably at intersections and branching points of these flyways and suggested that this may be due to a prolonged stopping time when drones reorient at these intersections (Loper et al., 1992). Our experiments showed that drones are attracted by the odor bouquet of other drones and one might speculate that because drones accumulate at intersections and branching points, their odor bouquet may build up and, following a virtuous circle, more and more drones may be attracted to this location, resulting over time in the formation of a drone congregation area. Furthermore, the accumulated odor bouquet of all present drones would provide a good explanation for the clear-cut boundaries of a congregation area (Ruttner and Ruttner, 1965; Ruttner, 1985; Loper et al., 1992). Identification of the active component of the male-produced attractive odor cue will allow testing this hypothesis in the field. Furthermore, it will be interesting to see in future experiments whether queens are likewise attracted by olfactory cues emitted from groups of drones. Our laboratory approach is a useful tool for dissecting behavioral responses of honeybee drones, queens and workers to different pheromone cues and a meaningful complement to field assays at congregation areas. Unlocking the details of honeybee mating behavior will be a key to optimizing beekeeping strategies and may be instrumental in our enduring effort to cover the food requirements of an ever-growing world population.

MATERIALS AND METHODS

Animals

Honeybees *Apis mellifera* L. were caught from outdoor hives on the CNRS campus in Gif-sur-Yvette, France, between April and August 2012. At the beginning of the drone season, drones were caught from inside the hive (bidirectional odor orientation test). During the main season, drones were caught at the hive entrance in the afternoon, when they departed on or returned from nuptial flights (odor quadrant choice test). The drones were placed in a plastic box containing a piece of wax comb and provided honey and water *ad libitum*. They were kept in an incubator at 34°C for at least one night before experiments started. During periods of bad weather conditions, age-marked drones were caught from inside the hives and only drones that were at least 8 days old were used for experiments, as drones usually start leaving the hive for nuptial flights at this age (Ruttner, 1985) ($N=23$, corresponding to 7.1% of all drones tested in the odor quadrant choice test). Drones and workers that were used for odor stimulation were caught either at the hive entrance or from inside the hive, depending on weather conditions. They were also kept in plastic boxes inside an incubator for at least one night before being used in the experiments.

Experimental setup

Walking simulator

In order to test drones' odor preferences, we built a walking simulator based on a locomotion compensator system (Buchner, 1976; Kramer, 1976; Dahmen, 1980). Basically, the walking simulator setup consists of an air-supported ball, on which a tethered honeybee drone was allowed to freely walk in any direction by turning the ball below it (supplementary material Movie 1). As a ball holder, we used a custom-made Plexiglas block with a hemispherical cavity slightly larger than ball diameter. An air inlet at the bottom of the cavity allowed the ball to float on an air cushion. Because of the custom-made ball holder design, only a weak air stream was needed to support the ball sufficiently and, hence, no disturbing air currents were detectable in the vicinity of the drone. Air flow was precisely controlled using a pressure regulator (Air Liquide REC BS 50-1-2, Paris, France). The air was filtered using activated charcoal (Sigma-Aldrich Norit RB1, Steinheim, Germany).

During the course of our experiments, we developed two walking simulator systems that were identical except for the size of the ball. One

system used a ping-pong ball (Cornilleau Competition, Breteuil, France; 40 mm diameter, 2.7 g mass) while another used a larger Styrofoam ball (Opitec, Vincennes, France; 100 mm diameter, 10.2 g mass). Pilot experiments showed that drones walk well on both ball types and subsequent statistical analyses of angular speed confirmed that the drones' ability to turn the ball and control their heading direction, which were the criteria tested in our experiments, was not affected by ball type (ping-pong ball median angular speed=20.4 deg s⁻¹, Styrofoam ball median angular speed=20.0 deg s⁻¹; Mann-Whitney *U*-test: $N=144$ ping-pong balls, $N=261$ Styrofoam balls, $Z=0.58$, $P=0.56$). Bidirectional tests (see Fig. 1A, Fig. 2) used the ping-pong ball, while odor quadrant tests (Fig. 1B, Fig. 3) predominantly used the Styrofoam ball (89.7% of tested drones).

To record ball movement, two highly sensitive optical sensors from laser mice were used (Logitech G500, Morges, Switzerland; resolution: 5700 dpi, signal rate: 1000 Hz). They were attached to the Plexiglas block at the horizontal equator of the ball and at a relative angle of 90 deg to each other (Fig. 1). The body axis of the insect was always precisely aligned at an angle of 45 deg with respect to both mouse sensors. Mouse signals were integrated and recorded via custom-written software programmed in LabView 2011 (National Instruments, Nanterre, France) using ManyMouse to separately handle the signals of both mouse sensors (source code by Ryan C. Gordon; <http://icculus.org/manymouse>). From the recorded ball movements, custom-written software directly calculated drones' walking paths, and provided throughout the experiment several parameters such as walking speed, turning direction and heading. Drones were tethered to the system with a very small insect needle (minutens 3.20, Ento Sphinx, Pardubice, Czech Republic), which was glued to the thorax using UV-reactive glue (3M ESPE Sinfony dentique opaque 3, Cergy-Pontoise, France) and a curing light (Woodpecker LED.B, Guilin, Guangxi, PR China). For this, drones were shortly anesthetized on ice and allowed to recover for at least 10 min prior to the experiment. All experiments were performed in complete darkness under an opaque cage protecting the setup from light and undesired air currents.

Experimental procedure

For evaluation of odor attraction, two different experimental procedures were used (Fig. 1).

In a first experiment, we asked whether drones can orient toward a biologically relevant odor source coming from its left or right side. We thus designed a setup allowing odor presentation either from the left side or from the right side of the animal. The drones placed in the walking simulator were subjected to two permanent air flows, which were placed at an angle of 45 deg on each side of their walking direction (bidirectional odor orientation test; Fig. 1A). Air flows were directed at the drone's antennae via two inert and easy to clean glass tubes (inner diameter: 7 mm). Each air flow consisted of a main air flow (1 l h⁻¹) and a secondary air flow (0.2 l h⁻¹), which were filtered by activated charcoal (Sigma-Aldrich Norit RB1) and regulated by flow-meters (Brooks Instrument Model 1355E Sho-rate, R-2-15-D and R-2-15-AAA, respectively, Hatfield, PA, USA). An odor stimulation could be applied using computer-controlled magnetic valves (Lee LFAA1200118H, Voisins Le Bretonneux, France; controlled via a BMCM R8 relay and USB-PIO, Maisach, Germany), switching the secondary air flow from an empty Pasteur pipette to a pipette loaded with an odor source (odor cartridge). Because of the fast-switching magnetic valves, total air flow to the bee was held at a constant rate of 1.2 l h⁻¹. The two identical odor stimulation air flows on each side allowed presentation of odors at precisely defined time points either from the left or the right side of the drone. Hence, we could measure whether drones are orienting toward (or away from) an odor upon stimulation.

After being placed in the setup, the drone was left in the dark without stimulation for 5 min to accommodate to the experimental conditions. During this time, the drone could freely walk on the ball. Odor pulses of 1 s were then presented with an inter-stimulus interval of 1 min according to the following stimulation sequence: 3×(control right, odor A right, control left, odor A left), 3×(control right, odor B right, control left, odor B left). The sequence of odor A and odor B and the sequence of stimulations from the left and the right side were pseudo-randomized between animals. In this experiment, we used 9-ODA and QMP (Pherotech, now Contech, Victoria,

BC, Canada) as odors A and B, and respective solvent controls (2-propanol; Sigma-Aldrich). Odor sources consisted of 10 μl of diluted odorant (50 $\mu\text{g } \mu\text{l}^{-1}$) loaded onto filter paper ($\sim 1 \text{ cm}^2$) and placed in an odor cartridge. After the solvent evaporated (2 min), the odor cartridge was closed. During the experiment, odors used for stimulation were quickly removed from the setup by an air extractor placed behind the bee and the walking simulator.

In a second experiment, we gave drones full control over the odor stimulation. In this setup, only one air flow (identical to those described above) was placed directly in front of the drone. After a habituation phase of 5 min as above, control over odor stimulation was granted to the drone. To this end, the ball was divided into four virtual quadrants, and one was designated as the odor quadrant (the odor quadrant changed in a pseudo-randomized manner between drones). During the 5 min stimulus control phase, odor stimulation was activated whenever the drone was heading toward the odor quadrant (odor quadrant choice test; Fig. 1B). By this, drones received a clear feedback to their own behavior, allowing us to measure whether the insect preferred to receive odor stimulation, i.e. how long the insect remained in the odor quadrant. To signal the presence of an odor cue in the setup at the beginning of the stimulus control phase, a 1 s pulse was given to the drone with the tested odor. After the stimulus control phase, drones were left to move freely in the setup for another 5 min, without any odor stimulation.

For odor stimulation in the stimulus control phase, we used 9-ODA or QMP in odor cartridges. To avoid possible olfactory adaptation that may be caused by potentially prolonged periods of stimulation in this protocol (if the animal remains in the odor quadrant), odor presentation was pulsed with an on/off phase of 100 ms each. In addition, odor concentration was reduced to 5 $\mu\text{g } \mu\text{l}^{-1}$, which is sufficient for eliciting neuronal activity in drones (Sandoz, 2006). Besides these odorants, we also presented the odor bouquets from groups of 10 living drones or 10 living workers. Stimulation animals were placed in a 100 ml vial that was used in place of pipettes. Because of the lower odor concentration and the higher volume of headspace in the vial containing the living animals, continuous air flow was used in these cases. Respective controls supplemented each experiment using either solvent-only cartridges or empty containers. A given experimental drone was used in only one experiment, with only one stimulus type (9-ODA, QMP, 10 living drones, 10 living workers or control stimulation).

The performed experiments comply with the current laws of the French Republic.

Data evaluation

To test for odor-induced behavioral changes in the bidirectional odor orientation test, we calculated for each animal the mean change in turning direction (in deg s^{-1}) and walking speed (in mm s^{-1}) between windows of 20 s before and 20 s after odor stimulation. Trials with 9-ODA and with QMP were analyzed separately, as not all drones were present in both trials. We used a Friedman ANOVA to compare the change in walking speed or the change in turning angle among stimulations with odorant or control coming from the left or the right side of the animal. For instance, a typical trial testing the effect of 9-ODA contained four stimulations: 9-ODA right, control right, 9-ODA left and control left. When the Friedman test indicated a significant heterogeneity among these values, specific Wilcoxon tests were carried out. For walking speed, we expected a possible change for the odorant compared with the control. Thus, each value obtained for the odorant on one side was compared with the value obtained for the control on the same side (i.e. 9-ODA right versus control right; 9-ODA left versus control left). For turning direction, we expected an opposite change in direction when the odorant came from the left or the right side, but no difference for the control stimulations. Therefore, the values obtained for the odorant were compared between sides (9-ODA right versus 9-ODA left), as were control values (control right versus control left).

For the odor quadrant choice test, we first excluded all individuals that never crossed the odor quadrant during the stimulus control phase and, hence, never received odor stimulation in response to their own behavior ($N=30$, corresponding to 9.3% of all drones tested in the odor quadrant choice test). We pooled data of the respective control stimulations of odor cartridges and living animals, as there was no significant difference in the

time spent in the 'odor' quadrant (odor quadrant time) before, during and after the stimulus control phase (Mann-Whitney U -test: $N=44$ control odor cartridges, $N=54$ control living animals, $Z_{\text{before}}=-0.075$, $P_{\text{before}}=0.94$, $Z_{\text{during}}=-0.41$, $P_{\text{during}}=0.68$, $Z_{\text{after}}=1.09$, $P_{\text{after}}=0.27$). We tested for statistical differences in the time spent in the odor quadrant before, during and after the stimulus control phase for 9-ODA, QMP, control and groups of 10 living drones or workers using a Kruskal-Wallis test. When significant, responses to odors were compared with the control using the Dunn method for non-parametric multiple comparisons (Zar, 1999). Statistical analyses and plotting of graphs were performed with R Studio 0.97.311 (based on R 2.15.2, The R Foundation for Statistical Computing) and Statistica 8.0 (StatSoft, Tulsa, OK, USA).

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Competing interests

The authors declare no competing financial interests.

Author contributions

A.S.B. and J.-C.S. conceived the experiment and designed the walking simulator setup. A.S.B. built the experimental setup and programmed the recording software. A.S.B. and F.B. collected and analyzed the data. A.S.B., F.B. and J.-C.S. interpreted the results. A.S.B. and J.-C.S. wrote the manuscript. All authors read and approved the final version of the manuscript.

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Supplementary material

Supplementary material available online at <http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.094292/-DC1>

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