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Antennal movements can be used as behavioral readout of odor valence in honey bees

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A B S T R A C T

The fact that honey bees have a relatively simple nervous system that allows complex behaviors has made them an outstanding model for studying neurobiological processes. Studies on learning and memory routinely use appetitive and aversive learning paradigms that involve recording of the proboscis or the sting extension. However, these protocols are based on all-or-none responses, which has the disadvantage of occluding intermediate and more elaborated behaviors. Nowadays, the great advances in tracking software and data analysis, combined with affordable video recording systems, have made it possible to extract very detailed information about animal behavior. Here we describe antennal movements that are elicited by odor that have no, positive or negative valence. We show that animals orient their antennae towards the source of the odor when it is positive, and orient them in the opposite direction when the odor is negative. Moreover, we found that this behavior was modified between animals that had been trained based on protocols of different strength. Since this procedure allows a more accurate description of the behavioral outcome using a relatively small number of animals, it represents a great tool for studying different cognitive processes and olfactory perception.

Introduction

Odor perception studies in restrained honey bees have fruitfully employed the proboscis extension reflex (PER) appetitive conditioning (Bitterman et al., 1983; Guerrieri et al., 2005). Other paradigms have employed aversive conditioning, in which the conditioned odor is associated with heat, electric shocks or deterrent substances; in these protocols, evidence of learning arises as the sting extension or as the withholding of the proboscis extension (Junca et al., 2014; Smith et al., 1991; Vergoz et al., 2007; Wright et al., 2010). Although all these paradigms have allowed us to broaden our knowledge on how bees learn associations and recognize odors, they present a limitation: the responses used to quantify behavior at the individual level are measured as binary parameters. That is, both sting or proboscis extension are recorded as "yes or no", which can hide graded and more elaborated responses. Furthermore, there is still no measurable behavior in restrained bees that, along a single dimension, can provide information about the appetitiveness or aversiveness of a given stimulus. This is because the extension of the proboscis plays a role only in case of stimuli related to food intake and sting extension constitutes a defensive response upon dangerous or nociceptive stimuli. These limitations do not only affect the study of memory, but also of neurobiology of perception and behavior in general, since the extension of the sting or proboscis is practically the only behavioral measure that can be obtained from restrained bees.

Nowadays, combining high speed cameras and automatic algorithms it is possible to precisely track animal movements or parts of their body without interfering with their behavior (Mathis et al., 2018). This possibility allows recording, tracking and deciphering antennal movements in restrained bees in search of patterns that might indicate how animals detect and perceive different stimuli (Claverie et al., 2021; Lei et al., 2022). The rationale behind this search is that honey bees use their antennae to sense their environment, responding to tactile, gustatory and olfactory stimuli (Erber et al., 1998; Kevan & Lane, 1985; Scheiner et al., 2001; Suzuki, 1975). Recently, Cholé and collaborators studied whether antennal movements elicited upon odor stimulation are modified by conditioning in ways that could be used as evidence of olfactory learning (Cholé et al., 2015). They found that, after appetitive conditioning, honey bees actively redirect their antennae pointing towards the source of the conditioned odor. In contrast, they did not disclose any change after aversive olfactory conditioning. Thus, it still remains elusive whether honey bees show different antennal scanning behavior

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upon stimulation with odors that have different biological values and if this variable serves as an informative and sensitive measurement to evaluate odor valence.

Pheromones represent key molecules to perform studies on odor perception, since they are chemical messages with both appetitive and aversive values, produced by animals, which can be sensed by conspecifics and potentially change their behavior (Wyatt, 2017). These molecules have been found in mammals, birds, insects, fishes and amphibians. Particularly, social insects release pheromones in several contexts: foraging, colony defense, kin and social status recognition and sexual behaviors. Moreover, when disturbed, honey bees release deterrent substances through the mandibular glands and the sting chamber, marking a specific place, which is then avoided by other individuals (Balderrama et al., 1996). These substances play an important role during foraging, since they allow marking flowers that have been recently visited and probably depleted of reward (Giurfa & Núñez, 1992; Giurfa & Núñez, 1993; Núñez, 1967).

In the present study, we analyzed how honey bees use their antennae to sense the environment and if the antennal orientation behavior changes depending on whether the animals face neutral, aversive or appetitive odors. To do that, we recorded honey bees with a high frequency camera and tracked antennal movements using DeepLabCut software. We confirmed previous results in regards to changes in antennal direction upon stimulation with an appetitive learned odor (Cholé et al., 2015), and also revealed that an alarm pheromone as 2-heptanone elicits an antennal response that looks opposite to the one elicited by the appetitive odor. Furthermore, we found that trained honey bees show a differential antennal behavior when placed in the training context and before odor stimulation, which can be interpreted as an active sensing behavior and an attention-like process in search of the learned odor.

Materials and methods

Animals

Honey bee (*Apis mellifera*) pollen foragers were captured at the entrance of a hive located at the campus of University of Buenos Aires (Argentina). Once in the laboratory, we anesthetized bees on ice and restrained in individual metal harnesses, allowing movements of their mouthparts and antennae. The head was fixed with a wax drop to prevent any movement. After recovery from anaesthesia, bees were fed with two droplets of a 1 M sucrose solution and remained undisturbed until the evening, when they were fed ad libitum. Honey bees were housed in a humid box at 18ºC in a 12:12 h light:dark cycle. We carried out both training and test sessions between 10 AM and 1 PM, after 18–20 h of food deprivation. Thirty minutes before the training session, the animals were stimulated at the antennae with sucrose and only bees that showed a rapid and conspicuous proboscis extension reflex were used for the experiment.

Olfactory Stimulation

During conditioning and testing, bees were stimulated using an odor delivery device that allowed the experimenter to control composition, concentration and duration of the odor stimulus. This device provided a continuous airflow of 500 ml/min. During stimulation, a second airflow of 50 ml/min that passed through the vials with a saturated headspace of the odorants was added to the carrier flow. Either acetophenone, 2-heptanone or a 1:1 mixture of both were used as odorants (diluted 1:10 in mineral oil in the liquid phase of the vials, all reagents from Sigma-Aldrich). The airflow was pointed to the bee's head through a teflon tube, kept at a distance of 3 cm. A gentle air exhaust located 10 cm behind the bee continuously removed odors from the training or testing arena.

Appetitive conditioning protocol

In order to count with an odor that has a clear appetitive value, honey bees were subjected to appetitive olfactory conditioning of the proboscis extension reflex using the odor acetophenone (Bitterman et al., 1983). During the training trials, an animal was positioned in the training arena in front of the air flow and remained 30 s before odor stimulation in order to get adapted to the context. After that, stimulation with acetophenone started and lasted for 4 s. One second before the end of the odor stimulation, the animal was stimulated on their antennae with a droplet of 0.6 µl of sucrose 2 M, eliciting the proboscis extension. The droplet was moved to the proboscis, allowing the animal to ingest it. Two groups of honey bees were conditioned. One group received a strong training protocol formed by 5 conditioning trials. A second group received only 3 conditioning trials. In all cases the interval between trials was 10 min. A third group of bees remained untrained.

Test

Test sessions were carried out 24 h after conditioning and consisted in three trials: animals were stimulated in pseudo-random order with acetophenone, 2-heptanone or the binary mixture of both odorants in a 1:1 proportion without receiving any reward. Each test trial had a duration of 70 s and was divided in three phases. During the first 30 s, bees were positioned in the training arena in front of the clean airflow. Then, they were stimulated with one odor for 10 s. Finally, bees stayed 30 s more in the context in front of the clean airflow.

Antennal behavior analysis

In order to analyze antennal movements, test trials were recorded from a top view with a Flir Blackfly S USB-3 camera at 60 fps. Antennae position was tracked off-line using the DeepLabCut software (Mathis et al., 2018). A total of 174 videos from 58 bees ($n_{untrained} = 12$; $n_{3trials} = 13$; $n_{5trials} = 33$) were loaded into the software. Between 3 and 10 frames from each video were selected and landmarks were labeled as a training data set for the algorithm. We used as landmarks the tip, pedicel and base of both antennae, the tip of both mandibles, the base of the mandible and the proboscis. In order to configure the tracking network, we used default parameters proposed by Deep Lab Cut (Nath et al., 2019). After a first software training round the tracked videos were analyzed. We evaluated the results through carefully watching the videos in search for tracking errors. A few videos had some errors in certain frames (mostly due to shadows or appendices like legs interfering with the image). All errors found were also identified by the network, since they showed a low likelihood value (parameter calculated by the network) for the wrong tracked body parts in those frames. Some of these frames were manually re-labeled and the network was re-trained in order to diminish the number of errors. We repeated this procedure three times, until the number of errors by watching the videos and looking for frames with a low likelihood was minimal. At the end, we obtained the position of the marked parts frame by frame, along with the likelihood parameter (Nath et al., 2019). We discarded all the frames with a less than 0.8 likelihood in at least one of the labels (2,2% of all data). With this data we calculated the angle between each antenna and the middle axis of the head as shown in Fig. 1A. Because the camera and the bee head were fixed, the position of the base of the antenna was averaged between all frames in order to avoid variability between frames. The line marking 0 degrees was traced from the antennal base orthogonal to the line connecting the base of both antennae. We also calculated the extension of both antennae, measuring the distance between the base and the tip of the antenna, and the angle between the two antennae, being 0 degrees when they were both pointing straight forward and 360 degrees when they were pointing straight backwards.

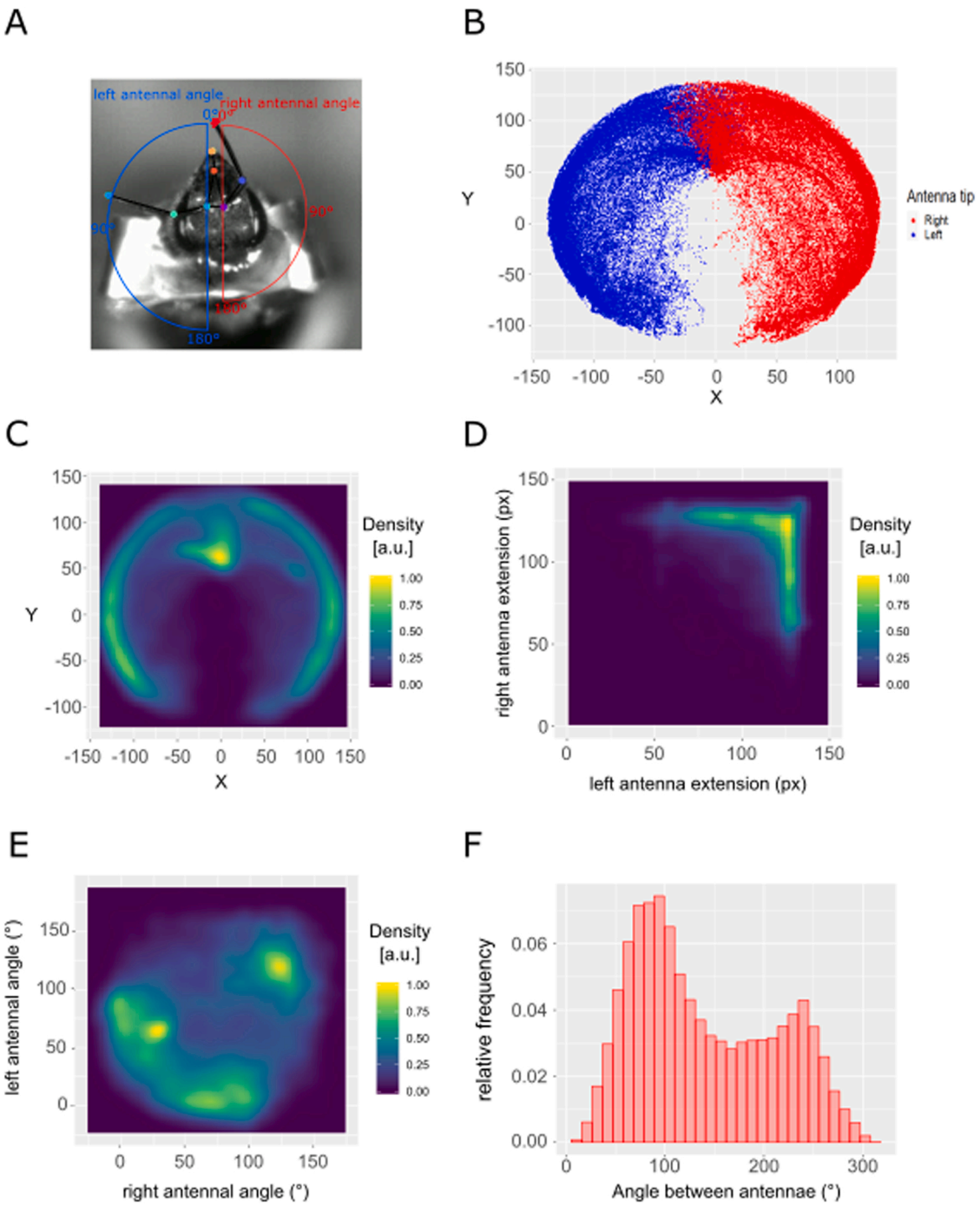

Fig. 1. Antenna track and biophysical description of antennae movements. Honey bees were placed in individual metal harnesses with their antennae, mandibles and proboscis free to move. Video recordings were performed from the top at 60 Hz acquisition frequency. Deep Lab Cut was used to track the antennae position in each video frame. A) The figure shows an example bee with the body parts labeled and tracked by the trained network. B) Antenna tips position for all the frames and all the animals (N = 12) during 30 s facing a clean air flow in absence of odor. The origin of the plot (0,0) corresponds to the base of each antenna. Tip of the right antenna in red and tip of the left antenna in blue. C) Density distribution of the antennae tip. D) Linear distance between the base and the tip of the antenna. The figure shows the distance measured for the left antenna vs the distance measured for the right one in each frame. E) Density plot, of the angle of the antenna and middle axis vs. the angle of the right antenna and the middle axis in each frame. F) Frequency histogram of angles between the two antennae. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

Statistical analyses

To analyze the response to odors, we calculated the median angle between antennae during the time window between second 2 and second 5 of odor stimulation for each animal and then we analyzed it by repeated-measures Generalized Linear Models (RM-GLMM), using experimental groups and testing odor as fixed factors and a gamma link function (Yu et al., 2022). When appropriate, planned comparisons between groups were performed.

To analyze the response before odor stimulation we calculated the median angle between antennae during the 30 s of the first of the three tests for each animal and then analyzed them by Generalized Linear Models (GLM), using the experimental group as fixed factor and a gamma link function.

To analyze the change in antennal orientation induced specifically by the odor we compared the median angle between antennae in the time window between second 2 and second 5 of acetophenone stimulation with the median angle between antennae during 30 s before the odor stimulation (pre stimulation phase) for the animals trained with 5 trials using a Wilcoxon matched-paired signed rank. In order to avoid any bias induced by the repeated stimulation we considered in this analysis only the animals that received acetophenone as the first test odor. Figures were designed with Graphpad RStudio 3.6.1 and Inkscape™ and statistical analysis were performed with RStudio 3.6.1.

Results

Non-odor elicited antennal responses

With the aim of characterizing the antennal movement pattern by which honey bees sense the environment, we video-recorded the antennae at high-speed (frame rate 60fps) and tracked antennae position during periods of rest and odor stimulation. Initially, we characterized the antennal movements in untrained bees that faced a continuous flow of clean air. Fig. 1 A shows the camera-view and landmarks that were used to train the antennae tracking software (see methods). The analysis provided in this study was focused on the tip of the right and left antennae (red and blue dots). Fig. 1B shows the sum of all positions taken by the tip of the antennae along all recordings. As observed, antennae tip positions demarcate an ellipse around the base of the antennae, with exception of a narrow stripe from the center to the back of the head. The area covered by each antenna is strictly circumscribed to a semicircle at the ipsilateral side of the antenna, and a minor region of overlap at the medial region of the animal. After performing a two-dimensional kernel density estimation (kde2D) (Fig. 1 C) we found that not all positions are equally frequent in this ellipse. On the contrary, the tip of the antennae tends to move along the distal border of this ellipse which corresponds to the extended antennae, and along this border the antennae were more frequently oriented pointing towards the left and right lateral sides of the head. In order to describe to what extent honey bees scan these areas with the antennae fully extended or flexing or raising them, we measured the distance between the base and the tip of the antenna as a measurement of the extension of the antenna. Fig. 1D shows a density map that describes the extension of the right antenna as a function of the extension of the left one. The upper-right corner of this map corresponds to both antennae fully extended independently of the direction to which they were oriented. As it is revealed by the figure, when one antenna shows a shorter extension, either because it is flexed or raised, the other one shows its full extension. Thus, the antennal scanning behavior is constituted by sequences of antennal positions in which at least one of the antennae is always extended in this plane. Then we performed a similar analysis but in relation to the direction in which the antennae are pointing. Fig. 1E, shows a density map of the angles in which left and right antennae are pointing at the same time. This analysis tells us which one is the position of the left antenna as function of the position of the right one. This analysis does not focus on the position of both antennae as independent behaviors, rather it helps to establish if there are more frequent configurations taken by both antennae as left-right coordinated behavior. The Fig. 1E shows three main angular combinations in which it was more frequent to find the tip of the antennae. One of them is with both antennae pointing symmetrically backwards, forming each one an angle of approximately 125° with the middle axis of the honey bee. Two other more frequent combinations, symmetric among them, were one antenna pointing forward forming an angle between 0° and 25° with the middle axis of the honey bee and the second antenna pointing laterally forming an angle of approximately 65° with the middle axis. Fig. 1 F shows the angle determined between the two antennae. As observed in the frequency histogram and consistently with Fig. 1E, the angle determined between the two antennae goes from 0° to 300° with two more frequent separations among them that peak at 90 and 250° which correspond to the three antennal configurations disclosed in Fig. 1E.

Odor elicited antennal responses

As a next step, we analyzed whether antennal movements are different when honey bees are stimulated with odors of different valence. Thus, we compared antennal responses elicited by an appetitively learned odor and responses elicited by the alarm pheromone 2-heptanone, assuming they should evoke differential responses. We trained bees using the standard olfactory appetitive conditioning protocol of the proboscis extension (Bitterman et al., 1983). A group of bees underwent a conditioning protocol of 5 trials separated by 10 min intervals using acetophenone as conditioned odor and sucrose applied to the antennae and proboscis as reward. A second group of bees underwent only 3 training trials that we used as a weaker training protocol. A third group of bees remained untrained. One day after the training session all animals were tested using acetophenone, 2-heptanone and a mixture 1:1 of both odors. Antennal movements were recorded and analyzed as in the previous section.

As exploratory analysis we focused first on the more distinct conditions that we had: they were 2-heptanone in untrained honey bees that had not received any appetitive reward in the experimental context, and acetophenone in honey bees that had undergone 5 appetitive conditioning trials with this odor. Fig. 2 provides a detailed analysis of the antennal responses during three seconds of odor stimulation starting one second after odor onset. As observed in Fig. 2Ai the conditioned odor acetophenone elicited an orientation of both antennae to the medial region of the animal and pointing towards the odor source. As shown by the representation in figure 2Bi, both antennae took specular orientations in relation to the middle axis. In addition, while stimulated with the conditioned odor, both antennae were most of the time fully extended (figure 2 Ci). As a consequence, the angle between the two antennae was reduced. The frequency histogram showing the angle between the two antennae in figure 2Di differs drastically from the distribution observed during stimulation with clean air in Fig. 1 F. This result based on acetophenone as appetitive conditioned odor is in agreement with a previous study that used 1-hexanol and 1-nonanol as conditioned odors (Cholé et al., 2015), and are important two-fold: first because they validate our setup and possibility to use antennal movements as evidence that the honey bee recognizes and appetitive learned odor, and second, they extend the previous observation to a new odor, strengthening the conclusion that antennal movements can be monitored as olfactory conditioned response regardless of odor identity.

Stimulation with the pheromonal component 2-heptanone elicited a completely different pattern of antennal movements. Figure 2Aii shows the density map indicating the antenna tip position during stimulation with the alarm pheromone. This time, the bees extended both antennae pointing backward and in the opposite direction of the odor source (Figure 2Aii). Figure 2Bii shows that both antennae moved symmetrically in regard to the middle axis. The heat map shows a dense spot around 140° for both antennae, and, as a consequence there is a peak in 280° for the angle between the two antennae (Fig 2Bii). It is important to remark that both antennae responded completely extended in the group of untrained animals that were tested with 2-heptanone and in the group of trained animals that were tested with acetophenone (Fig. 2C i and ii). Therefore, despite that animals oriented their antennae in opposite directions, the extension was similar in both cases and, importantly, was different from the exploratory behavior shown in the absence of odors when bees alternated extensions and flexions of the antennae.

Graded antennal responses

The possibility to clearly discriminate among three patterns of antennal responses encouraged us to extend the analysis to all groups and odors, and, in parallel we monitored the proboscis extension elicited by the odors (Fig. 3). First, we analyzed the antennal response of the untrained animals to the presumably neutral odor acetophenone. As

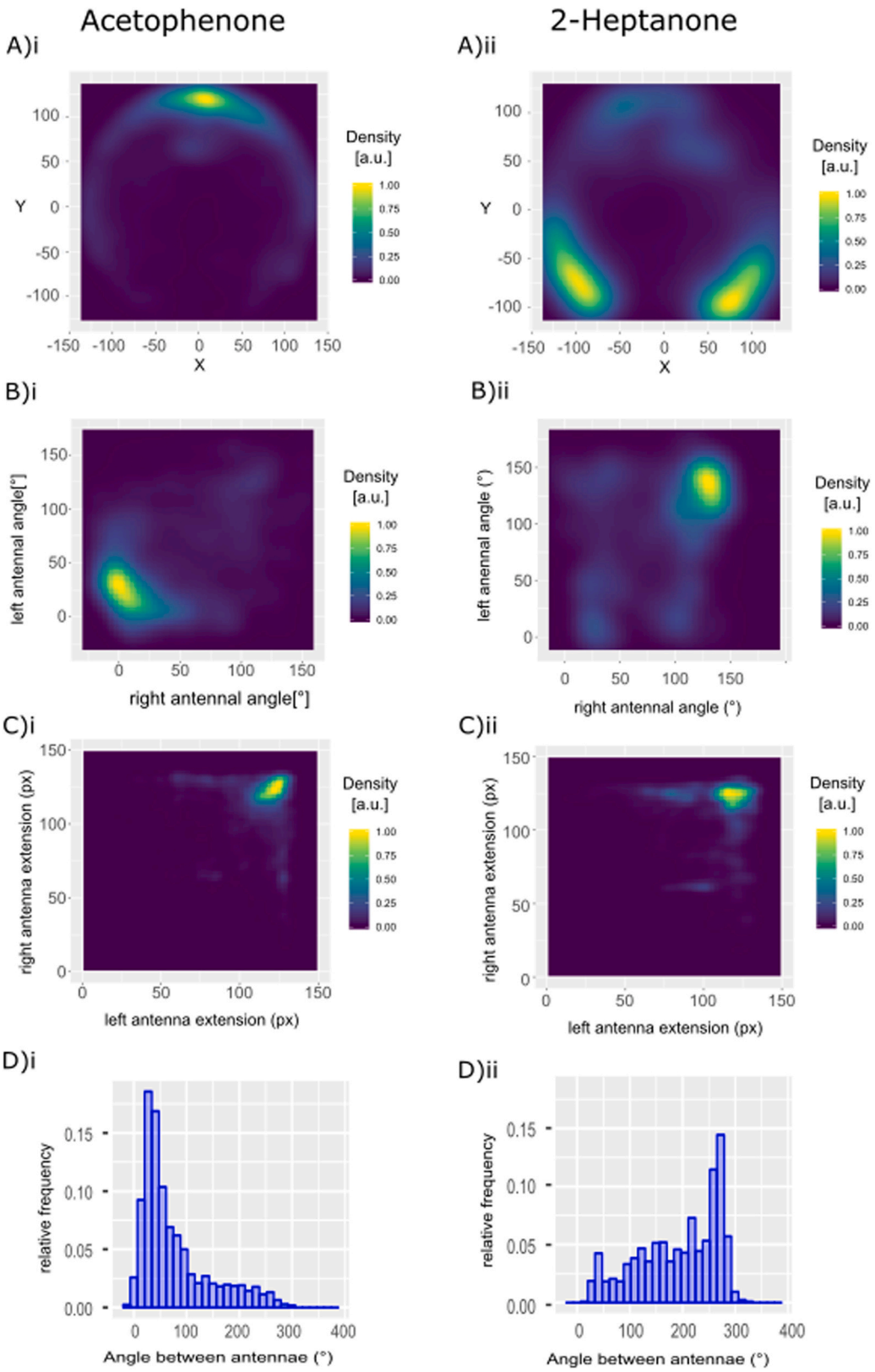

Fig. 2. Antennae orientation during odor stimulation. Animals were exposed to two odors with definite appetitive or aversive value. A, B, C, and D correspond to test trials with i) the appetitive conditioned odor acetophenone in 5-trials trained bees, ii) the alarm pheromone component 2-heptanone in untrained bees. A) Antennae tip density position upon stimulation with the odorant. B) Density plot of the angle determined between the right antenna and the middle axis vs. angle determined between the left antennae and the middle axis. C) Extension of the right vs left antennae. D) Frequency histogram of the angle between the two antennae during stimulation with the odorant.

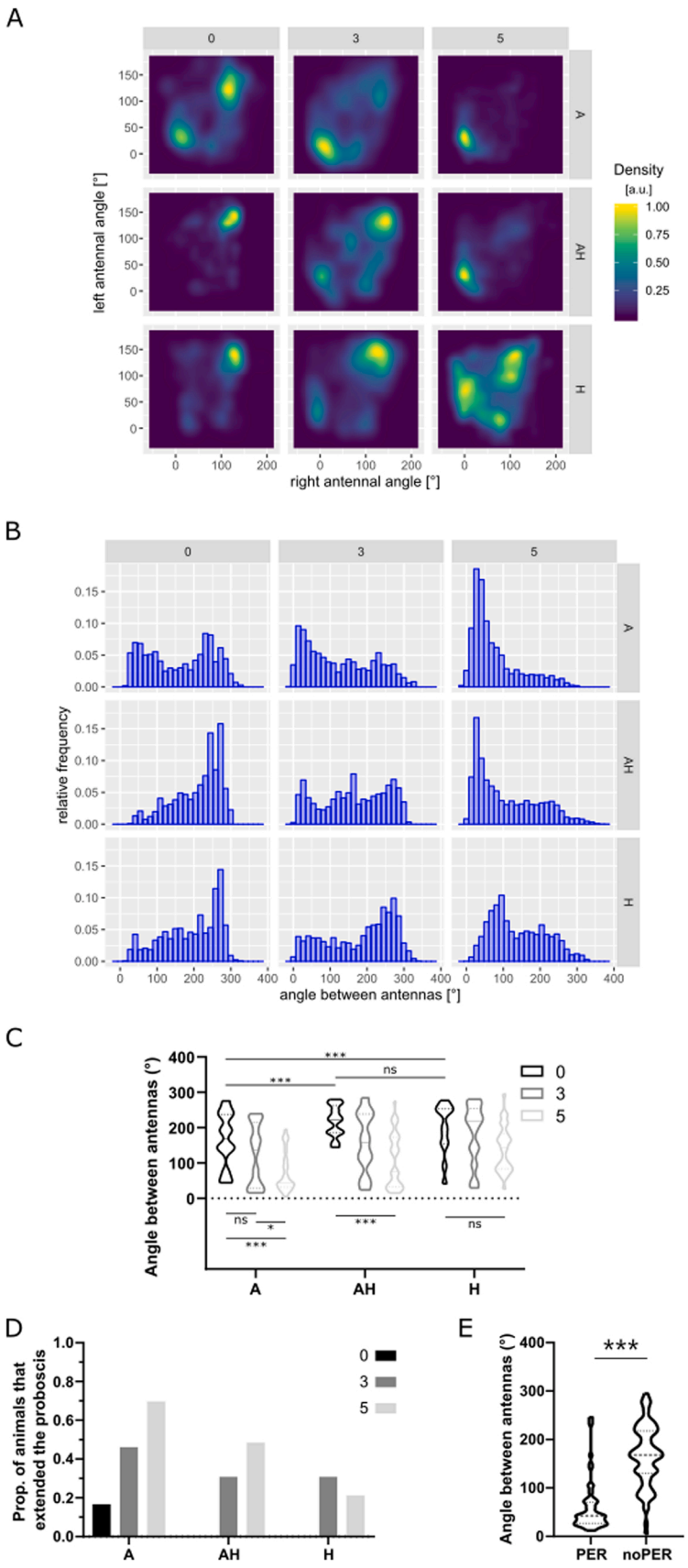

Fig. 3. Antennae orientation upon stimulation with odors of graded or mixed valence. A) Three groups of honey bees; untrained (0 trials-left column), 3-trials (middle column), and 5-trials (left column) trained with acetophenone as conditioned odor. A: Density plots of right and left antennae angular configurations during odor stimulation. Test odors: A: acetophenone (upper panel), AH: 1:1 mixture of acetophenone and 2-heptanone (middle panel), H: 2-heptanone (lower panel). B) Frequency histogram of the angle between the two antennae for the same bee groups and odors as in A. C) Median and quartiles of the angle between the two antennae from the groups of bees and odors described in A and B. Wald test: training factor $X^2 = 19.05$, $p < 0.001$, odor factor: $X^2 = 31.08$, $p < 0.001$, odor x training: $X^2 = 5.77$, $p = 0.22$. Same bee groups and odors as in A. D) Proportion of bees that responded with the extension of the proboscis upon odor stimulation for the same bee groups and odors as in A. E) Median and quartiles of the angle between the two antennae in the subgroup of 5-trials trained bees that did not extend the proboscis during test (noPER) and the subgroup of the 5-trials trained bees that extended the proboscis during test (PER). $n_{noPER} = 78$, $n_{PER} = 60$. Mann-Whitney test: $U = 462$, $p < 0.001$.* $p < 0.05$, *** $p < 0.001$.

observed in Fig. 3A, acetophenone elicited a "mixed" behavior in untrained bees. We found two more frequent antennal configurations. One of them corresponded to both antennae pointing forward, similar to the behavior elicited by appetitive odors, and a second one that corresponded to both antennae pointing backwards, as it was described for the alarm pheromone. An inspection at the individual level revealed differences across animals since some of them oriented their antennae pointing forward, others did it pointing backwards, and others scanned the whole area back and forward. The frequency histogram in Fig. 3B shows a distribution of the angles between the two antennae that is consistent with the antennae at these two extreme positions and also with intermediate angles. Thus, in naive bees acetophenone elicited different antennal response patterns across animals that were distinct from the pattern observed upon stimulation with clean air. This result indicates that animals react to the stimulation with the odor, however the odor does not have a uniform valence across animals. Remarkably, there were only a minor proportion of animals that extended their proboscis in response to acetophenone, which also indicates that acetophenone is not appetitive for untrained bees (Fig. 3D, black columns).

Next, we compared this performance with the one elicited by acetophenone in the groups that had received 3 and 5 conditioning trials. As observed in Fig. 3B, the frequency of cases in which animals adopted the backward configuration was reduced in bees with three appetitive conditioning trials and did almost disappear after the 5-trials conditioning. Statistical analysis based on the angular distance between the two antennae (Fig. 3 C) revealed significantly smaller angles in the 5-trials group vs the 3-trials ($p < 0.05$) and the untrained group ($p < 0.001$), and a trend in the 3-trials vs the untrained group ($p = 0.10$). It is important to note that when we quantified PER, we observed the same tendency. The proportion of bees that responded positively after three conditioning trials was 0.46, and after five trials was 0.70 (Fig. 3D).

Another observation resulted from the analysis of the antennal behavior elicited by the alarm pheromone in trained bees. The three lower panels in Fig. 3A show the orientation adopted by antennae during stimulation with 2-heptanone after the different training conditions. While 2-heptanone elicited only "backward" antennal response in naive bees, movements of the antennae to the medial region of the animal became more frequent as the training protocol became stronger. Such an effect might be explained by a certain degree of generalization of the conditioned response based on contextual cues that are common between the training and testing context. This effect was however not consistent in all bees and thus it resulted not statistically significant (untrained group vs 5 trials group: $p = 0.13$). We found a similar scenario when we analyzed the extension of the proboscis. While no untrained bee extended its proboscis in response to 2-heptanone, a proportion of trained bees responded positively to this odor (Fig. 3D). Importantly, the recording of the proboscis extension failed to discriminate between the response to a neutral odor as acetophenone in untrained bees, from the response to the aversive pheromone component 2-heptanone.

After that, we analyzed the antennal behavior when we challenged honey bees with a mixture that contained both, the alarm pheromone and the appetitive learned odor acetophenone. The question behind this analysis was to determine whether honey bees assign or not a definite valence to such a mixture and if it was possible for us to determine it by focusing on the antennal movements. Fig. 3A middle panels show the orientation adopted by the antennae during stimulation with the mixture after the different training conditions. As observed, the performance of the untrained group (left column) was fully consistent with the presence and detection of the alarm pheromone. Right and left antennae oriented backwards producing the same response pattern that was observed with 2-heptanone alone. The corresponding panel in Fig. 3B shows that the distribution of angles formed between the two antennae does not differ from the distribution observed when the stimulus was 2-heptanone alone ($p = 0.64$). In contrast, the distribution was significantly different from the one elicited upon stimulation with acetophenone ($p < 0.01$). The response pattern upon stimulation with the mixture changed markedly when acetophenone became an appetitive rewarded odor. We observed a graded response from the untrained to the 3-trials and the 5-trials trained groups. After three conditioning trials the response to the mixture included orientations of the antenna forward as well as backward and intermediate angular combinations, resembling the antennal response elicited by acetophenone in untrained bees. Strikingly, the antennal response changed drastically after the strong training protocol. After 5-trials conditioning with acetophenone, the mixture that contained acetophenone and the alarm pheromone elicited an antennal orientation that is different from the response of untrained bees ($p < 0.001$) and more similar to the response elicited by acetophenone alone. In summary, untrained bees behaved according to the presence of the alarm pheromone, and trained bees behaved according to the presence of the appetitive learned odor. These results show that training changed the valence of the mixture and that monitoring antennal movements resulted in a sensitive parameter to show it. In regards to the PER, we also found a proportion of the trained bees that responded positively upon stimulation with the mixture. However, the proportion was lower than when they were tested with acetophenone alone, which discloses that the alarm pheromone interferes with the appetitive response.

Finally, we evaluated if the extension of the proboscis and the pattern of antennal movements were correlated at the individual level. For this aim, we separated the bees that extended the proboscis upon stimulation with the conditioned odor and bees that did not, and calculated in each group the angle between antennae upon stimulation with the odor. As it is shown in Fig. 3E, honey bees that extended the proboscis also pointed the antennae forward which is revealed as significant lower angles compared with the animals that did not extend the proboscis ($p < 0.001$).

Training context and anticipation of the conditioned odor

All recordings of antennal movements lasted 70s that comprised 30 s of placement in the experimental context facing a continuous flow of clean air, 10 s of odor stimulation, and 30 s after odor offset. Fig. 4A shows the frequency distribution of the angle between the two antennae during the 70 s that lasted the test trials in trained and untrained bees, for the pure odors and the mixture. Remarkably, we found differences between groups in the antennal behavior during the 30 s of placement in the context and before odor onset. As it is evident in Fig. 4A this effect was especially clear in the group of bees that underwent the strong 5-trials training and was independent of the odor that was used in the next 10 s of odor stimulation. The Fig. 4B summarizes the distribution of angles between the two antennae during the 30 s of the first time that bees were installed in the context for the test. As observed, honey bees that had the strong-training protocol pointed their antennae forward towards the continuous airflow already before the odor onset (5 trials group vs 3 trials group: $p < 0.001$, 5 trials group vs untrained group: $p < 0.001$). We did not observe this effect in the animals that received a weaker training protocol composed of 3 conditioning trials (untrained group vs 3 trials group: $p = 0.56$). This result indicates that either the exposure to the training context, the mechanical stimulation with the carrier air, or both, evoked an appetitive memory and the orientation of the antennae into the direction from which the conditioned odor should come. This anticipatory behavior of the antennal movements constitutes a sensitive parameter that allows evidencing a contextual memory that is normally not observed with the proboscis extension.

Next, we asked whether the antennal orientation that in the previous sections we considered elicited by the conditioned odor acetophenone, could have been only an effect of the testing context. Thus, we performed a final comparison between antennae position before and during stimulation with acetophenone in the 5-trials trained bees. Fig. 4C shows the median angle between the antennae before and during odor stimulation in all 5-trials trained bees that were stimulated with acetophenone during their first test trial. As observed, honey bees that had already

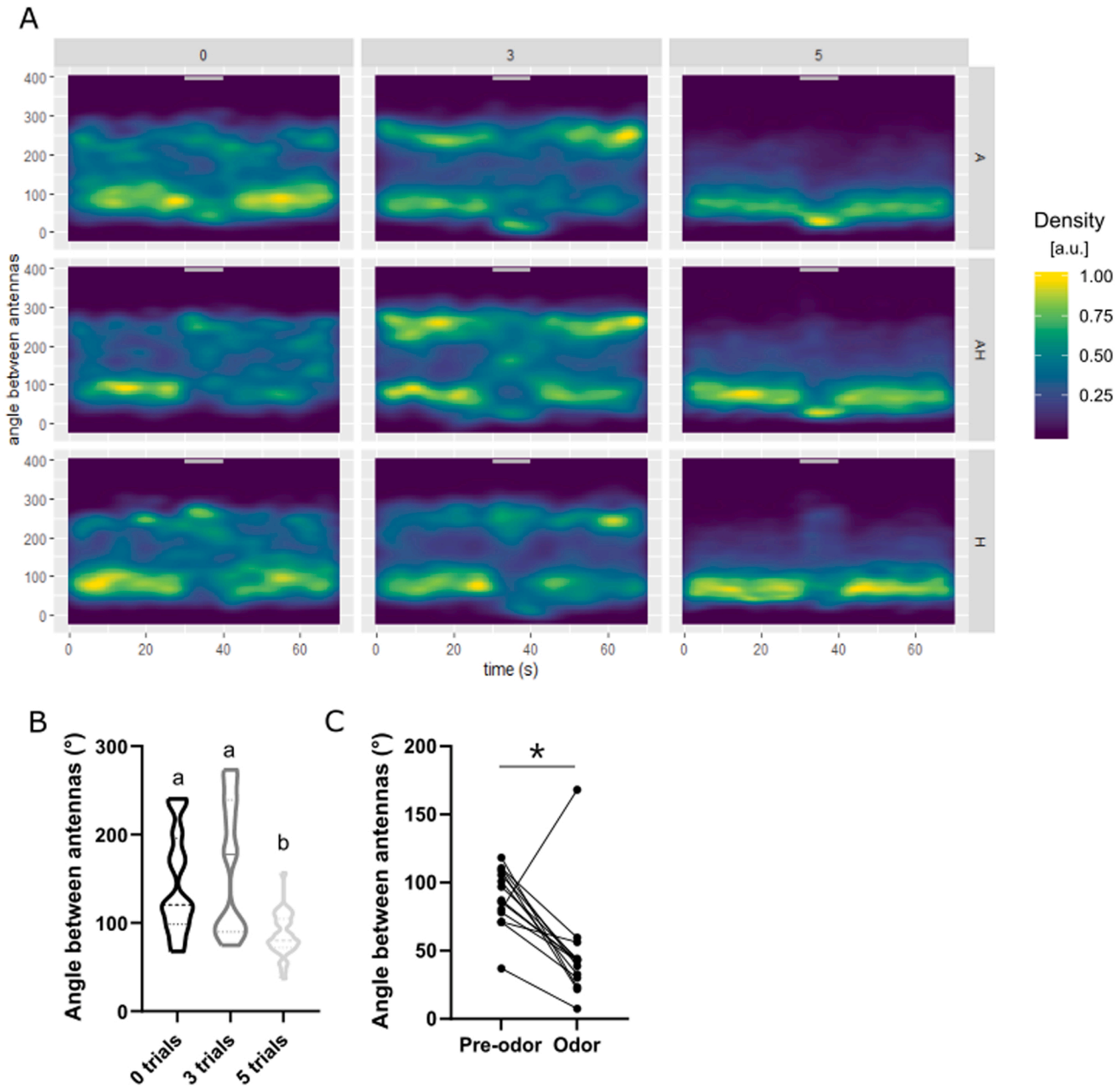

Fig. 4. Peristimulus analysis of the angle between the two antennae. Testing trials comprised 30 s exposure to the context, 10s odor stimulation and 30 s of context after odor offset. A) Color coded frequency of the angles between the two antennae adopted during the 70s of each trial. Honey bee groups and odors are the same as in Fig. 3. B) Median, quartiles and distribution of the angles adopted by animals in each group during the 30 s in the context and before odor onset. Only data of the first of the three test trials was considered for analysis. Wald test: training factor $X^2 = 16.50$, $p < 0.001$. Different letters stand for significant differences between groups. C) Comparison of the median angle between the two antennae adopted by each animal before and during stimulation with acetophenone in bees of the 5-trials trained group. Only cases in which acetophenone was the test odor used during the first test trial were considered. Wilcoxon test: $W = -79$, $p < 0.05$. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

reduced the angle between the two antennae before odor onset (Fig. 4B), reduced this angle even more when the odor was present ($W = -79$, $p < 0.05$). Thus, both effects, context and odor, contributed to the performance of the trained group. Strikingly, we did not find differences in antennal movements during the pre-odor phase between animals that extended the proboscis towards the odor and the subpopulation that did not extend it.

Discussion

In this work we analyzed the response of honey bees toward odors of different valence. By video tracking antennal movements, we first described antennal movements upon stimulation with a clean air flow. Afterwards, we distinguished different antennal orientations that correlated with the valence assigned to the odors. Importantly, this methodology allowed us to reveal graded responses that might normally be hidden in all-or-nothing responses, such as the sting or proboscis extension.

Since honey bees use their antennae to sense the environment, the description of the antennal behavior is relevant to understanding sensory processing. Spontaneous antennal movements are even a good readout of animal motivation and health condition as it was shown that it is affected during sickness (Kazlauskas et al., 2016). In line with a recent study in bumble bees (Claverie et al., 2021), we observed that

antennae do not move randomly back and forward, rather they show preferred regions towards which they are oriented most of the time (Fig. 1). The more frequent angles between the two antennae were 90° and 270°. How the scanning behavior and the preferred angular separations among the two antennae optimize the information that animals extract from the environment is an interesting question raised from this study that should be further analyzed from a more biophysical perspective (Claverie et al., 2021).

Only a few former studies have measured antennal movements in response to odors in honey bees and provided contradictory results. Originally it was reported that upon odor stimulation bees orient their antennae towards the odor source (Erber et al., 1993; Suzuki, 1975). Later, in more recent studies it was reported that bees orient their antennae in backward direction when stimulated with a novel odor (Cholé et al., 2015; Lei et al., 2022). The discrepancies may arise from differences in the set of odors that were used. Erber et al., 1993 and Suzuki et al., 1975 used floral components that might be slightly appetitive. Indeed, Suzuki et al., reported some events of proboscis extension concomitant to the forward antennal movements. In our results with acetophenone in untrained bees, we obtained evidences that honey bees detected the odor, since the pattern of the antennal movements differed from the movements elicited by the clean airflow, however we did not observe a uniform antennal orientation across bees, which might indicate interindividual differences in regards to the innate value of the odor or differences in motivation across animals (Fig. 3). In contrast, the orientations adopted by the antennae were highly consistent across animals when we tested odors with a definite valence. First, we did confirm the results obtained by Cholé et al., 2015 in regards to the changes in antennae orientation produced after appetitive conditioning: animals that underwent 5-trials appetitive conditioning showed a clear orientation towards the conditioned odor 24 h after training. Remarkably, it was possible to reveal differences between trained and untrained animals using a relatively small number of animals which represent a great advantage of the present protocol. Second, we found a clear backwards orientation of the antennae when we tested an aversive odor. This result seems to contradict the study by Cholé et al., in which no change in antennal orientation was found in response to aversive conditioned odors (Cholé et al., 2015). However, recently, Cholé et al. have deepened their former study extending their analysis to different odors, reporting that aversive pheromones (including 2-heptanone) elicit backward movements, in agreement with our present work (Cholé et al. 2022). Although it might be inaccurate to differentiate innate from learned behaviors (Gorostiza, 2018; Mameli & Bateson, 2006), it should be noted that 2-heptanone is an odor with a strong innate relevance in the wild and, therefore, is possible that honey bees have been previously exposed to it before their capture. The molecule 2-heptanone has been described as a deterrent compound produced by the mandibular glands, that when released elicits aggressive behavior in guardian bees (Shearer & Boch, 1965) acting as an alarm pheromone in combination with isopentyl acetate (Boch et al., 1970; Free & Simpson, 1968). Later, the production and release of 2-heptanone was also found in forager bees (Robinson, 1985) and it was postulated that 2-heptanone acts as an innate deterrent substance that would be used to mark depleted flowers (Free et al., 1985; Rieth et al., 1986). Thus, even though the precise role of 2-heptanone is not yet completely clear and might be context-dependent, there is no doubt that its release occurs in aversive contexts and must enhance the alertness of the receiver.

In Fig. 4 we showed that trained animals show different antennae orientation already from the moment in which they are placed in the context and before odor stimulation, revealing a conditioned response towards the training context. Different aspects of the context and sensory modalities other than olfaction might have played a role in this phenomenon. For example, visual and mechanical stimuli from the training context might have entered in association with the conditioned odor and with the reward. Indeed, there is previous evidence that proboscis extension response can be conditioned in harnessed honey bees using visual stimuli (Avarguès-Weber & Mota, 2016). Furthermore, it was shown that honey bees discriminate between different colors, and they can use this information to recognize a particular context in which a classical olfactory conditioning was carried out (Mota et al., 2011). In our experiment, probably because the training protocol included a 30 s wait before the CS-US happened, no animal extended its proboscis upon placement in the context. Thus, trained and untrained bees could not have been differentiated before odor onset if their antennae had not been observed. Importantly, the present analysis demonstrates that beyond the olfactory CS-US association, also a contextual association is established that can be disclosed based on the analysis of the antennal movements. This observation raises at least two important issues. First, an obvious but often underestimated phenomenon: animals do not learn only what they are trained for. In this case a complex context, that includes the operator, room illumination, set-up, are integrated as reward predictors. Second, it is relevant to note that this contextual memory does not trigger the proboscis extension but is revealed by a change in antennal movements. These results highlight the relevance of the present approach, since it allowed disclosing phenomena that otherwise would stay unobserved. However, since we did not disclose differences during the pre-odor phase between animals that later extended the proboscis and animals that did not, we cannot discard that the familiarization with the context instead of an association between the context and the reward could explain the forward movements in the trained group.

In summary, we have observed that honey bees orient their antennae forward or backwards depending on stimulation with two clearly different odors. Which one is the reason to adopt these orientations might be related with strategies to obtain additional or different information about the odor source. We consider the study of the antennal movements as complementary to the more classic proboscis and sting extension. As observed here, it resulted in a powerful parameter to reveal context memory in anticipation of the conditioned odor. Similarly, it provided a graded measure to describe how a mixture composed by two odors is differentially perceived by animals with different experiences. Interestingly, the appetitive or aversive quality of given stimulus could be measured based on one behavior and along a single dimension. Future studies must test more odors with different values and different intensities, and test them in combination with different experiences and motivational states to decipher what antennal movements can tell about sensory perception and processing.

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References

Avarguès-Weber, A., Mota, T., 2016. Advances and limitations of visual conditioning protocols in harnessed bees. J. Physiol. Paris 110 (3 Pt A), 107–118.

Balderrama, N., Núñez, J., Giurfa, M., Torrealba, J., de Albornoz, E.G., Almeida, L.O., 1996. A deterrent response in Honeybee (Apis mellifera) foragers: dependence on disturbance and season. J. Insect Physiol. 42 (5), 463–470.

Bitterman, M.E., Menzel, R., Fietz, A., Schäfer, S., 1983. Classical conditioning of proboscis extension in honeybees (Apis mellifera). J. Comp. Psychol. 97 (2), 107–119.

Boch, R., Shearer, D.A., Petrasovits, A., 1970. Efficacies of two alarm substances of the honey bee. J. Insect Physiol. 16 (1), 17–24.

Cholé, H., Junca, P., Sandoz, J.-C., 2015. Appetitive but not aversive olfactory conditioning modifies antennal movements in honeybees. Learn. Memory 22 (12), 604–616.
Cholé, H., Merlin, A., Henderson, N., Paupy, E., Mahé, P., Arnold, G., Sandoz, J.-C., 2022. Antenna movements as a function of odorants' biological value in honeybees (Apis mellifera L.). Researchsquare. https://doi.org/10.21203/rs.3.rs-1342433/v1.
Claverie, N., Buvat, P., Casas, J., 2021. Active sensing in bees through antennal movements is independent of odor molecule. bioRxiv. https://doi.org/10.1101/2021.09.13.460114.
Erber, J., Kierzek, S., Sander, E., Grandy, K., 1998. Tactile learning in the honeybee. J. Comp. Physiol. A Sens. Neural Behav. Physiol. 183 (6), 737–744. https://doi.org/10.1007/s003590050296.
Erber, J., Pribbenow, B., Bauer, A., Kloppenburg, P., 1993. Antennal reflexes in the honeybee: tools for studying the nervous system. Apidologie 4 (3), 283–296. https://doi.org/10.1051/apido:19930308.
Free, J.B., Pickett, J.A., Ferguson, A.W., Simpkins, J.R., Smith, M.C., 1985. Repelling foraging honeybees with alarm pheromones. J.Agric. Sci. 5 (2), 255–260. https://doi.org/10.1017/s0021859600056318.
Free, J.B., Simpson, J., 1968. The alerting pheromones of the honeybee. Zeitschrift für Vergleichende Physiologie 61 (3), 361–365. https://doi.org/10.1007/bf00428008.
Giurfa, M., Núñez, J.A., 1992. Honeybees mark with scent and reject recently visited flowers. Oecologia 89 (1), 113–117.
Giurfa, M., Núñez, J., 1993. Visual modulation of a scent-marking activity in the honeybee Apis mellifera L. Naturwissenschaften 80 (8), 376–379. https://doi.org/10.1007/bf01138797.
Gorostiza, E.A., 2018. Does cognition have a role in plasticity of "innate behavior"? a perspective from. Front. iPsychol. 9, 1502.
Guerrieri, F., Schubert, M., Sandoz, J.-C., Giurfa, M., 2005. Perceptual and neural olfactory similarity in honeybees. PLoS Biol. 3 (4), e60 https://doi.org/10.1371/journal.pbio.0030060.
Junca, P., Carcaud, J., Moulin, S., Garnery, L., Sandoz, J.-C., 2014. Genotypic influence on aversive conditioning in honeybees, using a novel thermal reinforcement procedure. PloS One 9 (5), e97333.
Kazlauskas, N., Klappenbach, M., Depino, A.M., Locatelli, F.F., 2016. Sickness behavior in honey bees. Front. Physiol. 7, 261.
P.G. Kevan , M.A. Lane , Flower petal microtexture is a tactile cue for bees Proceedings of the National Academy of Sciences of the United States of America 82 14 1985 4750 4752.
Lei, H., Haney, S., Jernigan, C., Guo, X.J., Cook, C.N., Bazhenov, M., Smith, B.H., 2022. Novelty detection in early olfactory processing of the honey bee Apis mellifera. Plos One 17 (3). https://doi.org/10.1371/journal.pone.0265009.
Mameli, M., Bateson, P., 2006. Innateness and the sciences. Biol. Phil. 21 (2), 155–188.
Mathis, A., Mamidanna, P., Cury, K.M., Abe, T., Murthy, V.N., Mathis, M.W., Bethge, M., 2018. DeepLabCut: markerless pose estimation of user-defined body parts with deep learning. Nat. Neurosci. 21 (9), 1281–1289.
Mota, T., Giurfa, M., Sandoz, J.-C., 2011. Color modulates olfactory learning in honeybees by an occasion-setting mechanism. Learn. Memory 18 (3), 144–155.
Nath, T., Mathis, A., Chen, A.C., Patel, A., Bethge, M., Mathis, M.W., 2019. Using DeepLabCut for 3D markerless pose estimation across species and behaviors. Nat. Protocols 14 (7), 2152–2176.
Núñez, J.A., 1967. [Collecting bees label dried food sources according to fragrance]. Die Naturwissenschaften 54 (12), 322–323.
Rieth, J.P., Wilson, W.T., Levin, M.D., 1986. Repelling honeybees from insecticide-treated flowers with 2-heptanone. J. Apicult. Res. 25 (2), 78–84.
Robinson, G.E., 1985. Effects of a juvenile hormone analogue on honey bee foraging behaviour and alarm pheromone production. J. Insect Physiol. 31 (4), 277–282.
Scheiner, R., Page Jr., R.E., Erber, J., 2001.). The effects of genotype, foraging role, and sucrose responsiveness on the tactile learning performance of honey bees (Apis mellifera L.). Neurobiol. Learn. Memory 76 (2), 138–150.
Shearer, D.A., Boch, R., 1965. 2-Heptanone in the mandibular gland secretion of the honey-bee, 530–530 Nature 206 (4983). https://doi.org/10.1038/206530a0.
Smith, B.H., Abramson, C.I., Tobin, T.R., 1991. Conditional withholding of proboscis extension in honeybees (Apis mellifera) during discriminative punishment. J. Comp. Psychol. 105 (4), 345–356. https://doi.org/10.1037/0735-7036.105.4.345.
Suzuki, H., 1975. Antennal movements induced by odour and central projection of the antennal neurones in the honey-bee. J. Insect Physiol. 21 (4), 831–847. https://doi.org/10.1016/0022-1910(75)90012-8.
Vergoz, V., Roussel, E., Sandoz, J.-C., Giurfa, M., 2007. Aversive learning in honeybees revealed by the olfactory conditioning of the sting extension reflex. PloS One 2 (3), e288.
Wright, G.A., Mustard, J.A., Simcock, N.K., Ross-Taylor, A.A.R., McNicholas, L.D., Popescu, A., Marion-Poll, F., 2010. Parallel reinforcement pathways for conditioned food aversions in the honeybee. Curr. Biol. CB 20 (24), 2234–2240.
Wyatt, T.D., 2017. Pheromones. Curr. Biol. CB 27 (15), R739–R743.
Yu, Z., Guindani, M., Grieco, S.F., Chen, L., Holmes, T.C., Xu, X., 2022. Beyond t Test and ANOVA: Applications of Mixed-effects Models for more Rigorous Statistical Analysis in Neuroscience Research 110 (1), 21–35. https://doi.org/10.1016/j.neuron.2021.10.030.