The Effect of Antennae Movement Restriction on Odor Discrimination in Honeybees

(*Apis Mellifera*)

by

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ABSTRACT

Honeybees require the use of their antennae to perceive different scents and pheromones, communicate with other members of the colony, and even detect wind vibrations, sound waves, and carbon dioxide levels. Limiting and/or removing this sense makes bees much less effective at acquiring information. However, how antennal movements might be important for olfaction has not been studied in detail. The focus of this work was to evaluate how restriction of antennae movements might affect a bee's ability to detect and perceive odors. Bees were made to learn a certain odor and were then split up into a control group, a treatment group that had their antennae fixed with eicosane, and a sham treatment group that had a dot of eicosane on their heads in such a way that it would not affect antennae movements but still add the same amount of weight. Following a period of acclimation, the bees were tested with the conditioned odor, one that was perceptually similar to it, and to a dissimilar odor. Using proboscis-extension duration and latency as response measures, it became clear that both antenna fixation and sham treatments affected the conditioned behavior. However, these treatment effects did not reach statistical significance. Briefly, both fixation of antennae as well as the sham treatment reduced the discriminability of the conditioned and similar odors. Although more data can be collected to more fully evaluate the significance of the treatments, the behavior of the sham group could indicate that mechanoreceptive hairs on the head play an important role in olfaction. It is also possible that there are other factors at play, possibly induced by the fixed bees' increased stress levels.

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In addition, a thank you to Professor Majid Ghaninia, who helped devise the differing quality Brassica odors by selecting the different components to be used in each mixture, as well as calculating the appropriate amounts needed. I also thank Shawn Mahoney and Aaron Valencia for their work in developing the MATLAB antennal tracking program. The results displayed later in this paper could not have been obtained without them.

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INTRODUCTION

Olfaction takes place when odor molecules move through the environment and bind to receptors that transmit signals throughout the olfactory system (R. W. S. Schneider, Lanzen, et al., 1998). Although before that can happen, odor molecules must first cross a boundary layer, which is a turbulent region of changing flow velocity surrounding any surface (Crimaldi & Koseff, 2001). In mammals, the boundary layer in question lies near the surface of the olfactory epithelium in the nose. For insects, the boundary layer lies along the surface of the antennae (R. W. S. Schneider, Price, et al., 1998). In order for organisms to smell more efficiently, odors have to pass through this boundary layer quickly. Accordingly, many organisms deploy various methods of *active sensing*, which involve moving the relevant sensory organs in order to optimally sample areas of interest (Wachowiak, 2011). Mammals can sniff, which pulls odor particles through the boundary layer at a much higher velocity (Wachowiak, 2011). As insects lack the ability to sniff, their analogue consists of moving their antennae as odorants pass by in an airstream (Lent & Kwon, 2004), hypothetically increasing the effective speed of the particles going through the boundary layer.

Olfactory perception in insects takes place in the antennae, through the olfactory receptor neurons (ORNs) located on them. In honeybees, the antennae consist of two basal segments, scape and pedicle, and a flagellum sometimes composed of many similarly shaped segments (D. Schneider, 1964). Lining the antennae are sensilla, which are tiny plate-like structures that are each connected to around 50,000 ORNs through

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numerous pores (Elgar et al., 2018). Between the dendrites of the ORNs and the pore plate lies an aqueous phase of hemolymph, which odorants cross with the assistance of odorant-binding proteins (Galizia et al., 2012). The whole process of transduction, from boundary layer to pore plate to aqueous layer to ORN, takes place within less than 2 milliseconds (Szyszka et al., 2014).

Honeybees move their antennae and associated sensors, which could enhance detection of odors in a complex, dynamic environment. Antennal movement is facilitated through muscles in the antennal socket, where the base of the scape is located. There are also muscles governing the elbow-like movement where the scape meets the pedicle. Aside from these two joints, honeybees can slightly move their flagellum independently. This wide variation of movement allows bees to resolve fast changes in odor concentration and enables them to resolve temporal differences in the arrival of a target odor versus background odors (Szyszka et al., 2014). This allows bees to triangulate the direction and distance of the odor.

If antennal movement helps bees to detect and discriminate odor, it stands to reason that restriction of the antennal movement should reduce the bees' olfactory capabilities. To put this to the test, three treatment groups will be compared. One group will be left unchanged as a control group, one group will have both of their antennal joints fixed, and another group will be sham treated so as to not restrict antennal movements. The bee groups will be trained to learn a conditioned odor, and then tested twice with three odors including the conditioned odor, an odor that is perceptually similar

to it, and one that is dissimilar to it. After conditioning trials, bees normally show strongest proboscis extension reflex (PER) to the conditioned odor, slightly less strongly to the similar odor, and lowest to the dissimilar odor. These graded PER responses are described as a pattern of generalization (Perez et al., 2016), and previous studies have shown this testing procedure to be sensitive to slight changes in odor perception (Stopfer et al., 1997). We predict that antennal restriction will lessen the generalization pattern, i.e., reduce discriminability, as evident in responses to these odors. This experiment will isolate the effect of antennal movement and may provide new insights on what role they play in odors, possibly akin to sniffing in mammals. If the hypothesis is supported, then the bees with fixed antenna will have shorter response durations and longer response latencies than the reference group, with the sham treatment being equal to the control (untreated) bees.

MATERIALS & METHODS

Research Animals

Honeybees were collected from hives on the days they were to be tested. Every day, the honeybees were subjected to one of three treatments (fixed antennae, sham, or control), and then to a battery of PER testing to assess learning and generalization/discrimination.

Odor Preparation

Throughout the course of this experiment, 3 different odor mixtures were utilized: a "full" Brassica mixture, a "half" Brassica mixture, and a "non" Brassica mixture. The basis for the odors chosen for the full Brassica mixture was the identification of these odors as a subset of the odor composition in inflorescences of Brassica rapa (Wright et al., 2002). The full Brassica mixture contained 330μl each of the following components: Nonanal, Decanal, α-Farnesene, Phenylacetyldehyde, Acetophenone, and Methyl salicylate. The non-Brassica mixture continued to use the same volume ratios for each of its respective components, but the components used were Benzaldehyde, Ethyl butyrate, methyl butyrate, Myrcene, 4-Hydroxy-4-methyl-2-pentanone, and 1-hexanol. In the case of half Brassica, 330μl of each of the 6 following components were used: Decanal, Phenylacetyldehyde, Methyl salicylate, Ethyl butyrate, Myrcene, and 1-hexanol. In essence, half Brassica was a blend of the 2 other mixtures, as it contained 3 components from both of them.

To condition honeybees to the full Brassica odor, we collected bees from the colony using glass scintillation vials. We then brought the bees into the lab, and placed them on ice until they stopped moving, in what was no longer than a few minutes each. Cooling was necessary to "anesthetize" the bees so that they could be handled. The bees were then immediately strapped into harnesses and left to acclimate to their surroundings in a plastic box, itself enclosed in a cabinet for 45 minutes. To determine which bees were responding to sucrose, we used 0.5 M sucrose in a Gilmont syringe to stimulate their antennae in order to determine which bees even responded to the stimulus of sugar. However, we did not feed them, as this test served to narrow them down to the responsive bees to be used in subsequent conditioning and testing. Following that, the responsive bees were subjected to a PER test (See Testing of Bees section for detailed procedures.)

PER Conditioning

Honeybees in particular have been frequently evaluated in the PER conditioning protocol (Bitterman et al., 1983; Kanazawa et al., 2005; Smith & Burden, 2014), and that protocol was used here to evaluate learning curves for treatment classes of bees. To perform PER, a single bee was placed into a testing arena consisting of clear acrylic glass to reduce outside disturbance and was allowed to acclimate for 20 seconds (Cook et al., 2019). After acclimation, an odorized airflow was blown directly at the bee through a glass syringe. Odor was presented for 4 seconds. If a bee extended its proboscis within the first 3 s, it was rewarded with a 0.4 μl droplet of 1.5 M sucrose solution using a

Gilmont syringe and scored as a positive response (Cook et al., 2019). If the bee did not

extend its proboscis to the odor in this time, a 0.4 μl droplet of 1.5 M sucrose solution

was touched to the antenna to elicit proboscis extension, then to the proboscis for reward,

during the last second of the odor delivery (Cook et al., 2019). This was marked as a non-

response. After odor delivery and reward, bees remained in the testing arena for 30

seconds. They were then removed and replaced with the next bee. Each individual trial

lasted for 60 seconds. As training progressed and bees began responding to odors, they

received the reward directly to their proboscis, thus earning more responses. All bees

were first conditioned to the full Brassica odor. Timing was tracked using a stopwatch,

and the precise odor and feeding interval was tracked by a programmed PLC that

signaled the experimenter to feed the bee with a tone, which is inaudible to the bee. For

visualization of this procedure see Smith & Burden (2014). The intervals between trials

for each individual were always at least 6 minutes.

Testing of Bees

In this experiment, there were two distinct phases of testing, acquisition trials and

two identical sets of testing trials, the latter of which was further broken down into

"before" and "after" treatments. The acquisition phase consisted of 6 PER trials for each

bee, all of which utilized full Brassica as the training odor. During acquisition, only the

honeybees that displayed at least 3 positive responses during the last 4 trials were

selected to be used further in the next phase of testing. (Responses were recorded as

described above in PER Conditioning.) After the acquisition trials, bees were left to rest for 15 minutes before the treatment tests began.

This part of the experiment was broken down into two sequences. The first sequence, named "before treatment", saw the bees return from their 15-minute respite to be tested with a battery of 4 odors: full Brassica, half Brassica, non-Brassica, and hexane (the latter is the solvent used for all test odors). To illustrate, if 10 bees made it to this phase on a given day, all of the bees would be tested on one odor first, then all would be tested on the second odor, then the third and the fourth. To eliminate a particular bias for odors and/or a simple learning of patterns, the order of the odors was randomized for the second part of the treatment tests. Here, both positive and negative responses were noted and used for statistical analysis, and videotaping allowed for offline scoring of latency and duration of responses. (See Videotaping methodology below). After this sequence of four odors, the bees were placed into one of three treatment groups. As only one treatment group would be tested per testing day, the bees either received fixed antennae via eicosane fixation (Figure 1), a dot of eicosane on the head (Figure 1) or were left untreated as a control group (Figure 1). For the purposes of this paper, they will simply be referred to as fixed, sham, and reference groups. The bees were then left to acclimate to their treatment for another 15 minutes, after which they were subjected to another battery of the same 4 odors, albeit in a different sequence. This final test was named "after treatment" and also featured video recordings for every single bee response.

Fig. 1. The image demonstrates the different treatment of the bee groups. (A) Control bee "Reference" with antennae unrestricted; (B) The "Sham" group with eicosane placed so as to not restrict antennal movements; (C) The "Fixed" group with antennal movements restricted. (Note in C, the fixation of both antennae joints.)

Videotaping

Responses in the treatment tests were video recorded for later offline video analysis, with the camera being positioned above the bee to provide a clear top-down view of the proboscis extension, as well as to include in-frame the light that indicated the flow of odor. The raw videos were then imported into a program called SwarmSight, which measured all fluctuations within 4 sensor fields, including the light, proboscis and both antennae (Birgiolas et al., 2017). Those values were then imported into MATLAB, which generated a peak analysis graph for the light and proboscis fields. With correct user interpretation, MATLAB then generated a spreadsheet of how long the proboscis was extended for (duration), as well as how long it took from odor presentment to the initial extension of the tongue (latency). Frame-by-frame antennal positions were also recorded for calculation of different movement parameters.

Statistical Analysis

As touched on above, duration and latency values were generated via MATLAB and then compiled into individual spreadsheets. These spreadsheets were then reorganized and streamlined in Excel, only to be plugged into MATLAB again for generation of latency and duration graphs, as seen in Figure 6 and Figure 7 below. Additionally, antennal movement graphs were generated in MATLAB using bees across all three treatment groups, but only using individuals tested on the conditioned stimulus, full Brassica. This was done to eliminate the variable of odor and isolate the effect of fixation on antennae velocity, position, and fluctuation. Antennae movements are displayed in Figures 2 & 3.

RESULTS

Antenna Movement to Odors and Effects of Antenna Restriction

We hypothesized that antenna movement affects how honeybees perceive environmental odors. Based on this hypothesis, we predicted that the bees would alter the way they move antennae upon odor stimulation. Through quantifying the angular velocity, coverage area, sector location and changing of directions (see Methods), we observed that the bees indeed showed some changes in antenna movement on both left and right antenna upon odor stimulation. Noticeably, both the left (Fig.2) and right (Fig.3) antenna moved closer to the proboscis (downward trajectory in Sector Location) and changed moving direction more frequently (upward trajectory in Direction Change) during odor presentation in the reference and sham groups. However, to know if these behavioral changes have any active role in perceiving odors, it's necessary to restrict the movement of antennae.

Antennae fixation reduced movement of the antennae relative to unrestrained and sham treatment conditions (Figures 2 & 3). The first three rows of each figure demonstrate the major movements of the antennae, with the first row showing how fast each antennae moved, the second row showing how much area each antennae covered, and the third row showing what sector (general area) the antennae are primarily located in. These sectors are numbered 1-5, and feature sector 1 located directly in front of the bee and subsequent sectors being located adjacently all the way to sector 5 behind the bee. These figures demonstrate the effectiveness of the antennae fixation, as the fixed

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bees have notably reduced velocity and coverage area after their treatment. Note the

relatively diminished amplitude of the purple spikes (after treatment) compared to the

blue spikes (before treatment). The fixed bees also demonstrate a much more stable

sector location, again because of the diminished amplitude seen in the purple line. The

last row of each figure then demonstrates the finer movements of the antennae and

illustrate how many times the antennae changed direction over the course of time,

resembling wave-like lines. Here, fixed bees still demonstrate movement, which can be
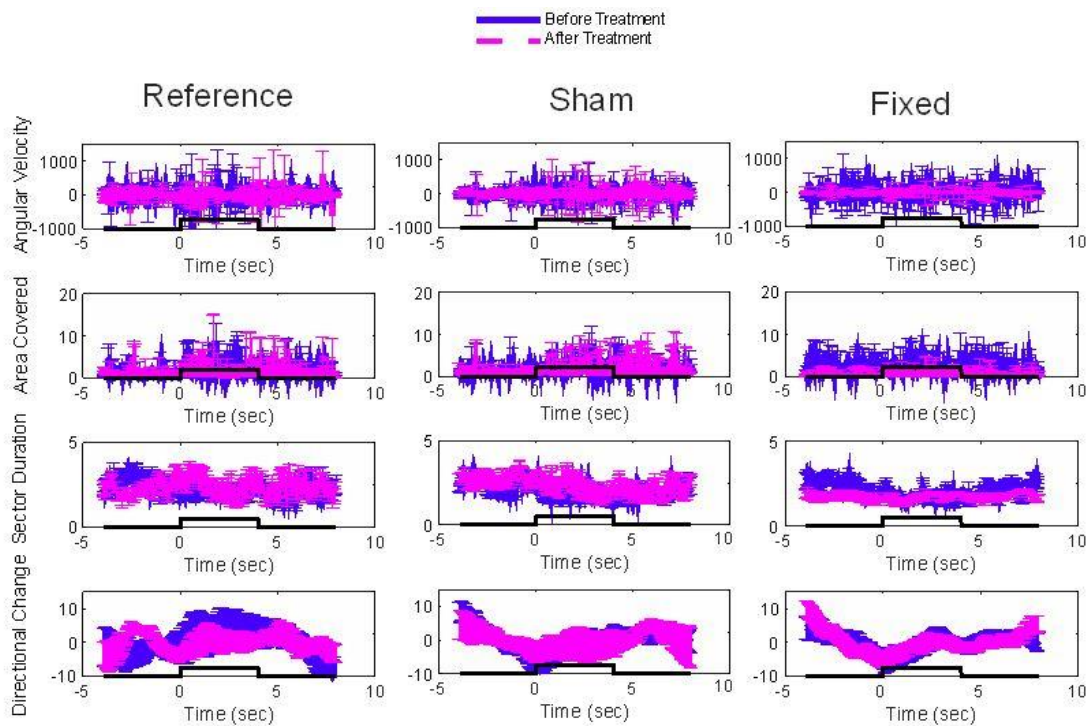

Fig.2. Quantification of left-antenna movement under different treatments in context of full Brassica stimulation. The raised black bar on each graph stands for the period in which the odor was presented. Treatment groups are arranged in columns, and the four parameters (angular velocity, area covered, sector duration, directional change) are arranged in rows. Purple lines represent values before treatment, and blue lines represent those after treatment. The curves show mean +/- standard error from an average of 8 bees per group.

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accounted for on the basis that the bees can still move their flagellum, thus registering

movement. Fixation here causes delayed movement in response to the odor presentation

in contrast to reference and sham treated bees, as noted by the rightwards shift of the
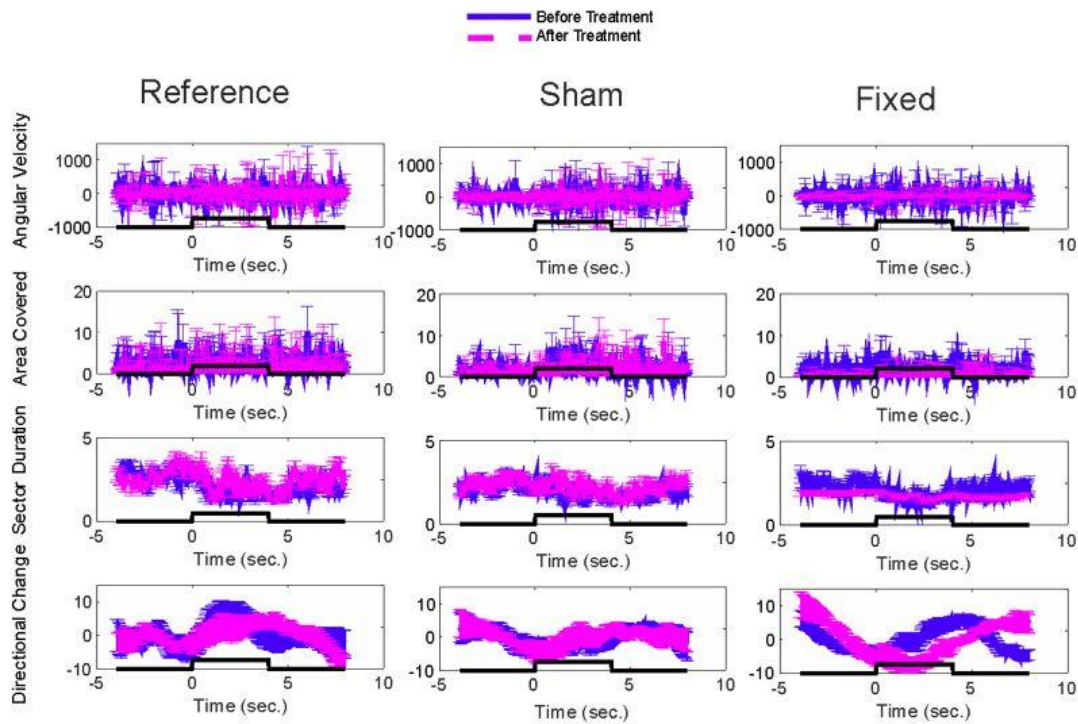
purple wave in comparison to the blue one.

Fig.3. Quantification of right-antenna movement under different treatments in context of full Brassica stimulation. The raised black bar on each graph stands for the period in which the odor was presented. Treatment groups are arranged in columns, and the four parameters (angular velocity, area covered, sector duration, directional change) are arranged in rows. Purple lines represent values before treatment, and blue lines represent those after treatment. The curves show mean +/- standard error from an average of 8 bees per group.

Associative learning and odor-generalization pattern

To assess how antenna restriction may affect odor perception, we first needed to

train honeybees to associate an odor (CS) with sucrose (US) reward. Using the standard

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training protocol (see Methods), we observed that about 70% of the bees learned the full

Brassica odor (CS) by the third trial, and 100% of the bees learned the association by the

5th trial (Fig. 4). This robust procedure allowed us to test if and how the bees would

generalize the learned association to odors other than full Brassica; more importantly,

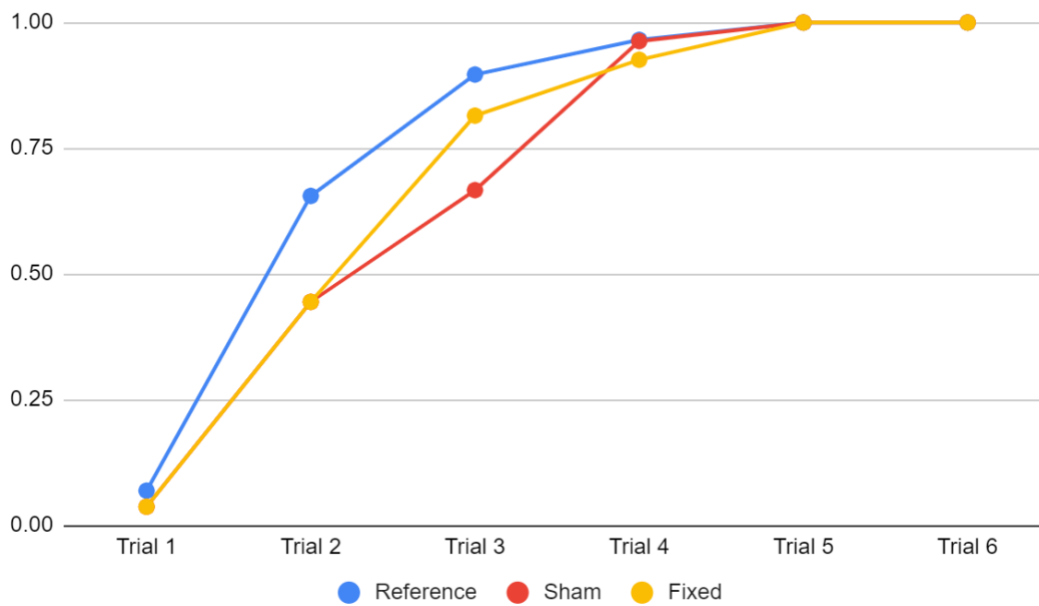how such generalization pattern would be affected by antenna fixation.

Fig.4. Acquisition curves. All bees used in the testing phase learn the full Brassica odor by the end of the acquisition phase. The different treatment groups are not representative of actual treatment groups as such, and only serve to signify that the bees comprising the different acquisition curves will be separated into those treatment groups after acquisition but prior to the start of the testing phase.

Among the bees that successfully learned the full Brassica odor, about 80%

showed PER response to the full Brassica odor and slightly less percentage to the half

Brassica odor (Fig.5, blue and orange bars on left panel). Only less than 20% of bees

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showed PER to non-Brassica odor and the solvent control (Fig.5, yellow and green bars on left panel). Clearly, the bees' capability of generalizing the learned association to other odors is tightly linked to the similarity between the learned odor and test odors. The higher similarity between odors, the closer behaviors bees show.

Next, we wondered if the pattern of generalization would be disrupted by antenna fixation. As shown on the right panel of Fig.5, there was an overall decrease of percentage even including the reference group where bees' antennae were not treated. This was possibly due to memory extinction. In spite of the general decline in responses, the generalization gradient described before treatment remained intact. There was no difference in the shape of the generalization gradient between Reference, Fixed, and Sham operated groups.
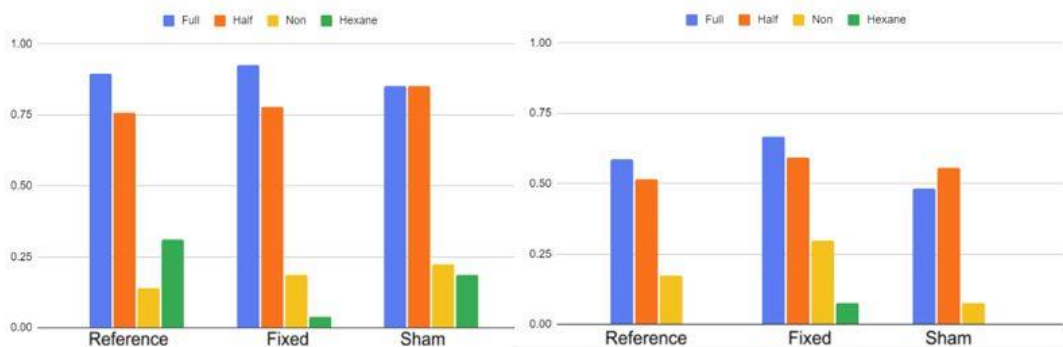

Fig.5. Effects of antenna treatment on generalization pattern. The two graphs presented above illustrate how many bees in each group responded in the testing phase after acquisition. The first test on the left was performed prior to treatment. Here, the response to the conditioned odor was the strongest, and the response generalized to the half Brassica odor was slightly less in two of the groups. Non-Brassica and hexane (control) responses were significantly lower. The second test was performed after treatment and is featured in the graph on the right.

PER strength affected by antenna treatment

In Fig.5, the bees were not distinguished by their behavioral strength. In other words, a bee extended proboscis for a full time-course was scored the same as a bee showed PER just briefly. We went ahead to measure the duration and latency of PER in each bee. Before antenna treatment, the pattern of generalization was apparent (bottom left, Fig.6). There is a clear decrease of PER duration across odor similarity, suggesting a strong odor effect. Indeed, we conducted a 2-way ANOVA on PER duration, considering the odors as one factor and test phases as the other factor. The p value for odors is significant (p=.0075, F=5.06, N=156). These results confirmed the generalization pattern observed in Fig.5. After antenna fixation, the pattern of generalization was disrupted. The averaged PER duration was about equal between full Brassica and half Brassica (bottom left, Fig.7). This phenomenon is consistent when including bees that did not show PER during test, i.e., non-responders (Fig.6, Fig.7, top left panels). PER latency, however, did not show a clear pattern of generalization before treatment (Fig.6, Fig.7, right column).
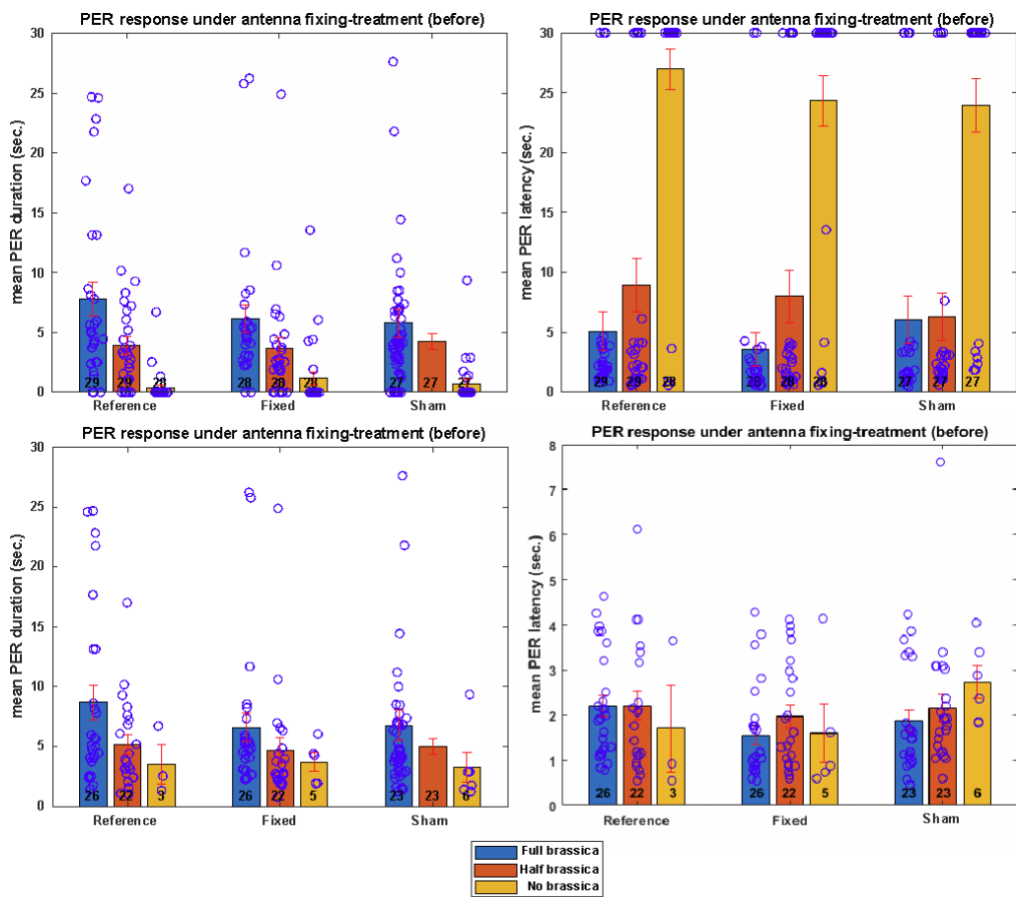
Fig.6. Effects of antenna fixation on PER duration. Each of the 4 pictured graphs features three treatment groups, Reference, Sham, and Fixed. The two measures here are response duration and response latency, with the former signifying how long the proboscis was extended for, and the latter signifying how long it took for the bees to extend their proboscis following odor presentation. Response duration is the left column of graphs and response latency is in the right column of graphs. Blue circles signify individual response values. The top row of graphs demonstrates all bees tested, including the bees who did not respond. The non-responsive bees were given a max latency value of 30 (seconds) and a minimum duration of 0 (seconds). In the bottom row of graphs, only bees that provided a response are included. The non-responders do not significantly affect the generalization gradient present in the duration graphs, as the response to the conditioned odor remained the longest, followed by half Brassica and then by no Brassica.
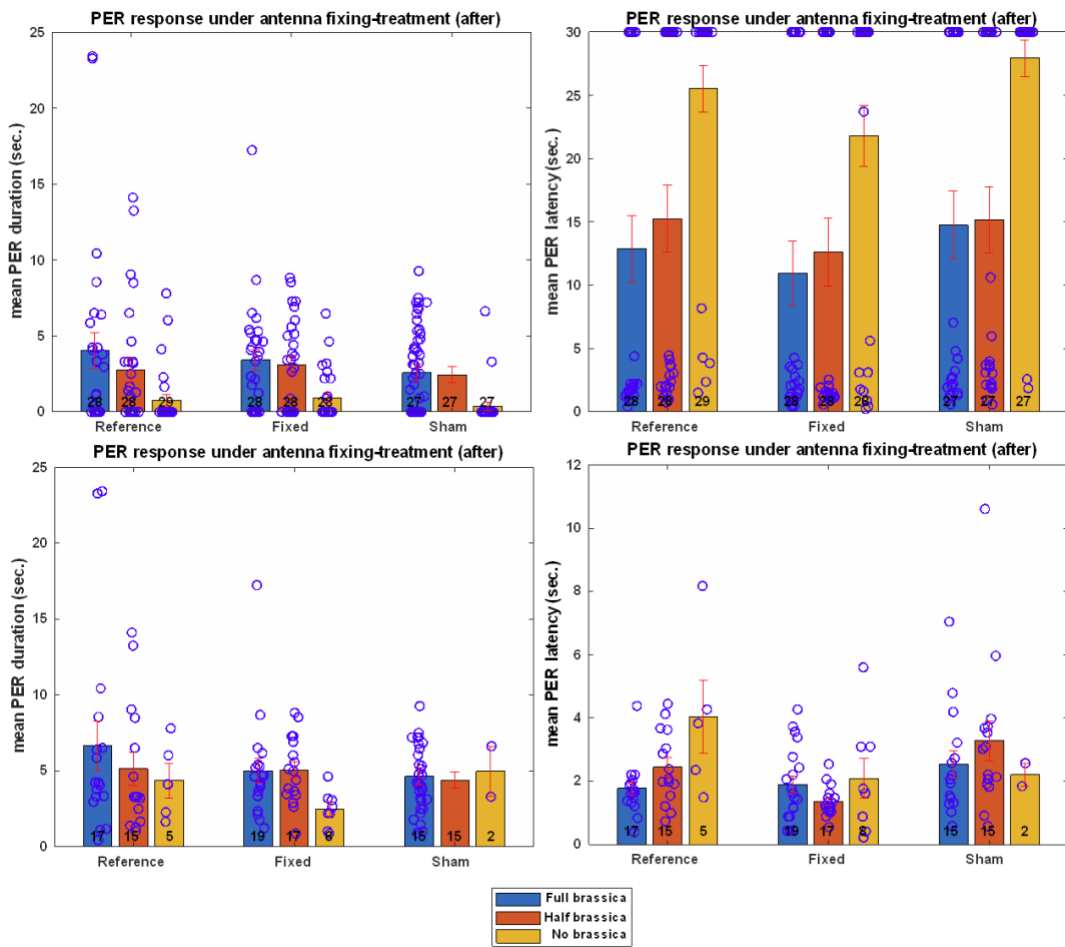
Fig.7. In the testing phase after treatment, duration becomes shorter in each treatment group as the odor becomes more different from the conditioned stimulus. The treatment itself is shown to affect response latencies, with fixed bees having the shortest, followed by the sham treatment. Once more, the first row of graphs show that the non-responders flatten the durations while inflating the latencies of each treatment group, masking the differences now present between the groups.
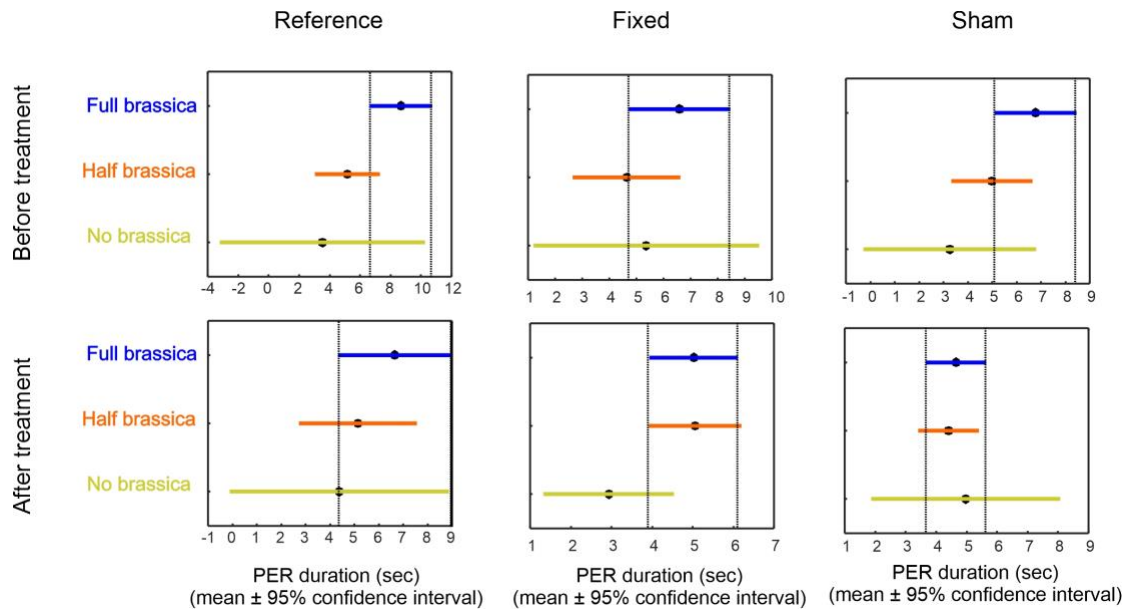
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Fig.8. Effects of antenna treatments on PER duration. Colored lines represent the mean duration +/- 95% of confidence level. Overlapped confidence levels indicate non-significance.

To summarize, before antenna treatment, the bees showed the longest PER duration when they were presented with the conditioned odor, i.e., full Brassica. The PER duration became shortened when presented with half Brassica. This graded response is due to the change in odor composition from full to half mixture. In other words, the bees distinguished half Brassica from the full Brassica. This capability is, however, disrupted by antenna treatment. After fixing antennae or with sham treatment, the bees recognized full Brassica and half Brassica equally well (Fig.8), suggesting that antenna movement facilitates fine odor discrimination after associative learning.

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DISCUSSION

Our results provide an indication that antennal movements are in some way related to odor perception. The use of a generalization gradient was two-fold, in that the first series helped identify baseline pattern for bees learning odor qualities and the second series allowed us to identify slight changes in perception with different treatments. To allow for a gradient based on similarity, odor mixtures were used instead of single odors. The molecular structures of the odor components present in the conditioned odor (*full Brassica*) were totally different than those in *no Brassica*, and as a result, the bees demonstrated significantly reduced responses with the latter mixture before and after treatment. However, the *half Brassica* mixture contained half of the learned molecular structures, and accordingly, the bees' responses were only slightly lower than responses to *full Brassica*, thus forming a gradient. The reduction of this gradient after treatment reveals that treatment did have a significant effect on the bees' perception, as the gradient would have remained unchanged otherwise. Of note, the *overall* reduction seen in the responses after treatment suggests another factor, like stress induced from handling or behavioral extinction from the first test series.

As seen in figures 6 and 7, the conditioned (full Brassica) and similar (half Brassica) odor mixture response ratios are the most important indicators of perception. In those figures, the blue (conditioned) and orange (similar) bars in the reference group maintained the same ratio even after their treatment, notwithstanding the overall reduced response level after treatment. The ratio of blue bars being larger than the orange bars is

not present in the sham and fixed groups after treatment, with the bars being almost equivalent in those groups. Once more, this serves to indicate that treatment affected the bees' ability to perceive differences in odor.

Albeit surprisingly, the sham treatment was found to lower the bees' odor perception ability (blue and orange bars at similar levels) in the same capacity as the fixed treatment. This cannot be explained from antennal movement alone, as all four parameters were close to equivalent between the sham and reference groups. The sham treatment only showed a closer similarity with the fixed group within the PER responses, which suggests the sham bees might have been affected by another factor.

Possible explanations for the sham phenomenon lie within the integration of mechanosensation and olfaction. Along a bee's head, there are sensory hairs which are known to be important in flower detection, as exemplified in a previous study by Sutton (2016), which focused on the hairs' ability to detect electric fields via mechanical deflection of the hair. Thus, it is possible that mechanosensation from hairs near the antennae on the head, which were occluded by the sham treatment, augment odor detection and/or perception.

The results in this paper, although not statistically significant, reveal trends that suggest new experiments to follow up with. While this could simply entail acquiring more data using the established protocol, other options include altering the placement of the sham treatment on the bees' heads, to determine if there are specific areas of hairs that are more important for olfaction. Leaving the mechanosensory hairs intact would

also serve to further isolate the collateral effect of stress induced from eicosane placement in general. As antennal movements have been shown to be governed by combined visual and mechanosensory inputs (Roy Khurana & Sane, 2016), another experiment to consider moving forward involves electrophysiology. This would involve measuring the responses of first-order brain interneurons to observe the neurochemical effects of obstructing mechano-hairs and what relation that has with antennal movement.

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