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Original article

Antennal movements as indicators of odor detection by worker honeybees[1]

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Abstract – Detecting olfactory perception in animals is a difficult task requiring indirect investigation at the peripheral level (e.g., using electroantennograms) or at the behavioral level (e.g., using choice behavior assays). We show in restrained honeybees that variations in antennal movements can be used to analyze odor detection. Two methods, based on the analysis of digital recordings during odor stimulations, have been used: manual or automatic (with image processing) data collection of the successive positions of the antennae. The results were similar for the two methods and showed that citral induced an increase in the velocity of the antennal movements. Comparing the olfactory stimulation (citral) to air alone stimulation and to stimulation consisting of air saturated with water or quinine solution, we conclude from these observations that only volatile molecules induce detectable movements of the antennae in the honeybee.

***Apis mellifera* / antennal movements / olfaction / video frame analysis / automatic records**

1. INTRODUCTION

Olfaction is commonly involved during experiments studying associative learning, for example, the olfactory conditioning of the Proboscis Extension Reflex (PER) in the honeybee. This paradigm uses the fact that a neutral odorant, associated with a sugar reward, can elicit a PER after only one pairing of the two stimulations (Bitterman et al., 1983).

The analysis of antennal movements can be used as a sensory-motor test to control odor detection. The recording of odor-induced antennal movements enables us to study odor perception without using a behavioral paradigm (Bakchine et al., 1990). It allows us to test thresholds for different odors and to compare appetitive and aversive olfactory stimuli.

It is important to precisely define olfactory detection when studying olfactory learning processes in insects, to distinguish detection from other phenomena. For example, the bee's response to olfactory stimuli can decrease over time. This decrease can be due to a loss of olfactory sensitivity or to a disturbing effect on associative processes following, for example, the application of a neurotoxic agent like an insecticide or pharmacological agent (Cano Lozano et al., 1996, 2001). Previous research has linked odor detection with antennal scanning movements. Electromyograms and motoneuronal activities of antennae can be recorded after olfactory stimulation (Suzuki, 1975). Erber et al. (1993) demonstrate ipsilateral antennal movements after one-sided odor presentation to a bee. In this work, they registered

[1] Two videos and a script are available at http://www.edpsciences.org/apido
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antennal crossings of two phototransistors beams. The antennal scanning movements (when the scapus is immobilized) have been studied during odorant airflow (Peteraitis and Vaitkevièienë, 1995) and these movements are supposed to increase the deposition of odor molecules on chemoreceptors (Peteraitis, 1999).

During tactile motor learning, Erber et al. (1997) measured the frequency of antennal contacts of a bee when scanning a metal plate using an image analyzing system. However, the detection of the precise antennal location required the use of a small dot of ink covering the tip of the antenna.

Therefore, we present a relatively simple, rapid and more efficient method to demonstrate odor detection by insects. The first aim of our work was to compare two methods based on frame analysis of digital recordings when bees perceived an odor (citral). In the first method, successive antennal positions were manually recorded during the video playback. In the second one, antennal movements were quickly registered after automatic frame analysis by computer. Analysis of the antennal movements showed no significant difference between the two methods. Thus, in the second part of the work, we compared antennal movements induced by different stimuli using the more rapid and practical automatic method.

2. MATERIALS AND METHODS

2.1. Setting up of the animal

Worker honeybees (*Apis mellifera* L.) were collected from a single hive. The experiments were conducted from April to June with foragers. Individual honeybees were mounted in small tubes so that the head protruded, allowing free movement of the antennae. Before recording, the insects were held in the experimental set up for over 10 minutes with continuous neutral airflow. White sticky tape, placed behind the bee's head, was used to make a white background to obtain well-contrasted pictures of the antennae. Each insect was tested once in one condition of stimulation. A total of 120 honeybees were used, equally distributed among 4 groups, corresponding to the 4 experimental situations (see below).

2.2. Stimulating apparatus

The continuous airflow, received ventrally by the honeybee, was shunted with a solenoid valve system to a removable odor cartridge (a tube of 4 cm in length, and 0.5 cm in diameter) containing a filter paper. A computer drove the stimulating set up and switched the airflow for 6 s during the recordings. The flow was tuned so that the odor puff did not modify the characteristics of the airflow (13 mL per s delivered at 4 cm). A volume of 7 µL of water or a chemical compound was deposited on the filter paper in the cartridge. Quinine hydrochloride was used at 0.1M; citral was not diluted. The filter paper was also tested alone to verify the accuracy of the stimulating system.

2.3. Recordings

Antennal movements were recorded from the front of the head of each bee (Fig. 1). The ambient light level was 300 lux (no additional source). The camera (webcam USB, Philips "ToUcam"), was connected to a personal computer (MacIntosh G3, 233 MHertz, or G4, 800 MHertz)) and centered between the left and right scapus of the antennae. This reference was used as the zero point for the measurements of angle formed by one antenna and the sagittal plane. The lens of the webcam was unscrewed to adjust the focus on the insect head.

Each frame was 320 X 240 pixels and the recording speed was 10 frames per second, which limited the number of frames to analyze while still allowing a good description of antennal trajectories. Each movie comprised 12 s before stimulation, 6 s of stimulation and 12 s after stimulation for a total duration of 30 seconds. The digitized movie files were stored on a hard drive for frame-by-frame analysis. These records were made with low cost camera with classic USB interface. Sophisticated devices or interface card were not necessary. The characteristics of the current computers are compatible with digital video recordings with 10 frames per second.

2.4. Antennal tracking

Each antenna is composed of three parts. The basal segment is the scapus articulated with the head. Four muscles permit multidirectional movements in front or on all sides. The distal segments, the pedicellus and the attached flagellum, have a hinge joint with 2 antagonistic muscles. There is no joint between the scapus and the pedicellus. Hence the antennae were approximated as two straight lines originating from the center of the head, adjacent to the right and left sockets of the scapus, respectively.

Two methods were compared to analyze antennal movements. In the first one, the positions of the end of both right and left antennae were manually (and visually) located. Each frame of the digital recording was successively displayed on the computer screen. By clicking on the mouse, the XY coordinates of the

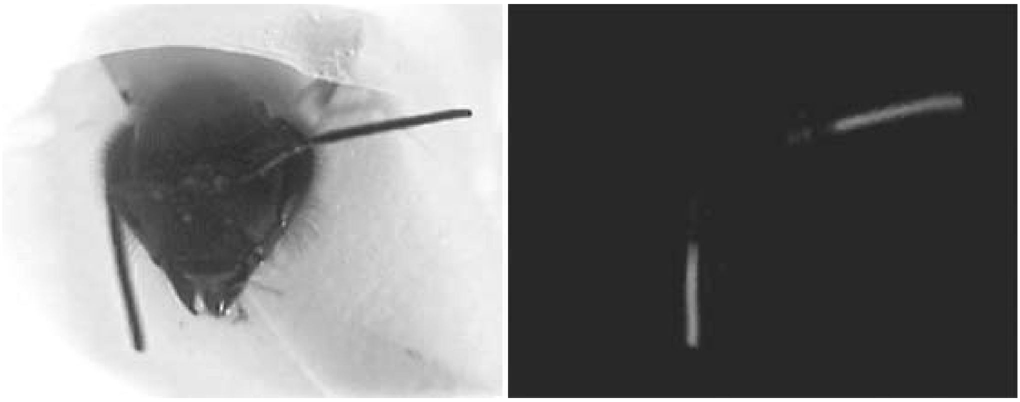

Figure 1. Comparison between a normal video frame (at left) and an automatically reconstructed one (right). On the resulting image, the remaining pixels corresponded to the changes from the preceding frame of the movie, which highlights the antennal movements. The position of the brightest pixels, left and right gave the directions of each antenna (calculated by the image processing software).

tip of each antenna were recorded. From these values, the angular positions of the antennae (relative to the sagittal plane) were calculated and the results showed the variations of the angular movements of the antennae between two successive images.

In the second method, each image was automatically compared to the next one using a freeware loaded on the computer (Fig. 1). A new film was constructed based on differences between two successive frames. This image processing was made with VideoScript application (public release: http://www.videoscript.com). This video and image-processing tool for Windows and Mac OS allowed the authors to set up the suitable scripts (On request, the authors can convey the scripts).

On both the right and left halves of each frame, the coordinates of the pixel with maximal intensity were automatically recorded. These points defined the new angular positions of the antennae. The computations of the angular positions of the antennae and of the angular movements between two frames were the same for the two methods and correspond to the difference between the angle of the antenna and the sagittal plane (left and right).

Each antenna could change its position during the 100 milliseconds duration between 2 successive video frames. This angular movement was expressed as angular velocity. For most of the results, these values (absolute values) were added for each recorded second (ten images = one second) and the results were expressed in degrees per second.

3. RESULTS

3.1. Effect of citral odor on antennal movements studied by both methods

Preliminary tests showed that there was no significant difference between left and right antennal movements. ANOVA with both stimulation and side factors showed significant effect ($P < 0.0001$) for "Odor" factor (Citral), no significant effect for "Side" factor ($P = 0.4977$) and "interaction" factor ($P = 0.9244$). So, the right and left values were pooled. The graphic and statistic comparisons were stated from five successive 6 s blocks: two blocks before, one block during and two blocks after odor presentation. The stimulation period could be compared to two initial periods and two aftereffect periods.

The shape of the two curves of angular velocity, (Fig. 2) were similar but the values were globally higher for the automatic method.

The angular velocity had a constant value before odor presentation. The olfactory stimulation was correlated with an increase in angular velocity ($P < 0.01$** in Fig. 2), which persisted for at least 6 seconds after stimulation.

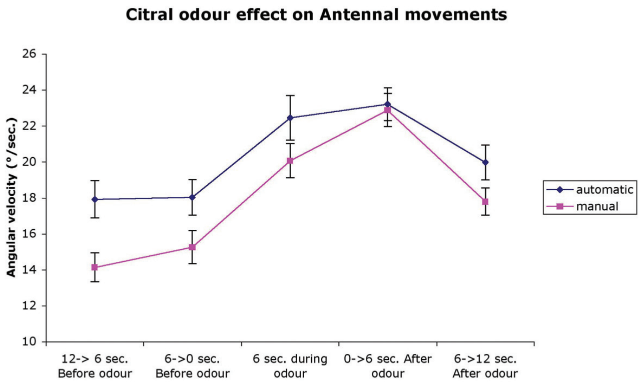

Figure 2. Angular velocity of antennal movements recorded with automatic or manual method, for the same digital movies. Five blocks of 6 s were compared for 30 s of film analysis (means of 30 bees ± standard error). The significant differences between the successive points are stated: * if $P < 0.05$; ** if $P < 0.01$.

The difference between the maximal and minimal points was larger in the manual method; so, a slight difference, between the two methods, appeared just after the odor flow (continuation of the increasing, $P < 0.05$ * in Fig. 2 for the manual record and stability for the automatic record).

The global difference between the results of the two methods disappeared if 2 degrees were uniformly added to all the values of the manual method: F = 14.538, $P < 0.001$ with exact values, F = 0.224, NS with corrected values. There was no significant interaction between method and stimulation (F = 0.885, NS).

Using the results of each frame by frame change of the antennal position, the exact difference, between the two methods of recordings, was plotted (Fig. 3), for all antennal angular movements, for each corresponding frame (automatic value – manual value, 17940 values).

Most of the distributed values were near zero. In this case, the results were similar for the two methods; more than 70% of the differences were less than 10 degrees. With the raw data, the mean value was effectively of 2.025 ± 0.204 (SD).

However some values were very different because the automatic record could fail in the detection of the antennae. Several hypotheses could explain the cases where the registered values differed between the two methods. Some movements of the buccal appendages were sometimes detected by the automatic frame analysis when antennae were motionless. On the other hand, visual observation of the frames allowed us to record some positions of the antennae, directed near the optical axis of the video camera. For example, when the dark shapes of the antennae were in front of the dark head of the insect, and not enough contrasted, they were not detectable by the automatic system.

The distal point of the straight line that schematized the antenna differed for the two methods. We visually detected the tip of the antenna but the automatic image processing detected the highlight point of the flagellum. This point of maximal contrast was located more or less distally on the antenna.

We concluded that the differences between the angular values recorded by the two methods did not notably influence the characteristics of the citral effect on the angular velocity of the antennae.

3.2. Controlling the effect of non-odorant parameters on antennal movements

Four conditions were tested with the automatic method to check the effect of the odorant molecules. (1) The airflow was always constant but the switching operation used to drive the odor in the air stream could induce a mechanosensory stimulation of the antennal receptors. Thus, the effect of the airflow was controlled on the antennal movements. (2) Quinine was sometimes used as a repellent compound in learning experiments. A gustatory effect was

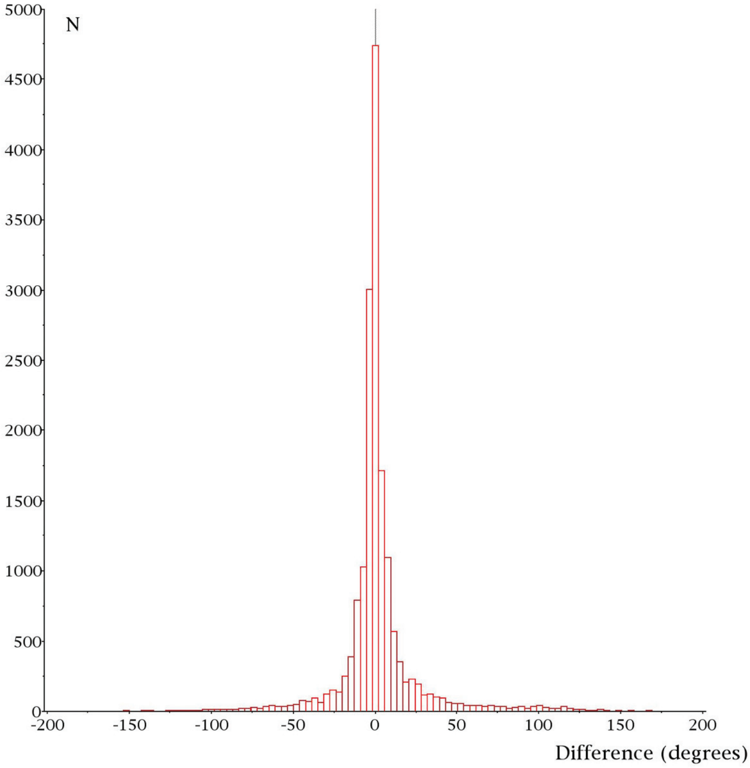

Figure 3. Frequency distribution of the differences between the values recorded by the two methods. All the angular changes between each pair of successive frames were taken into account (N = 17940).

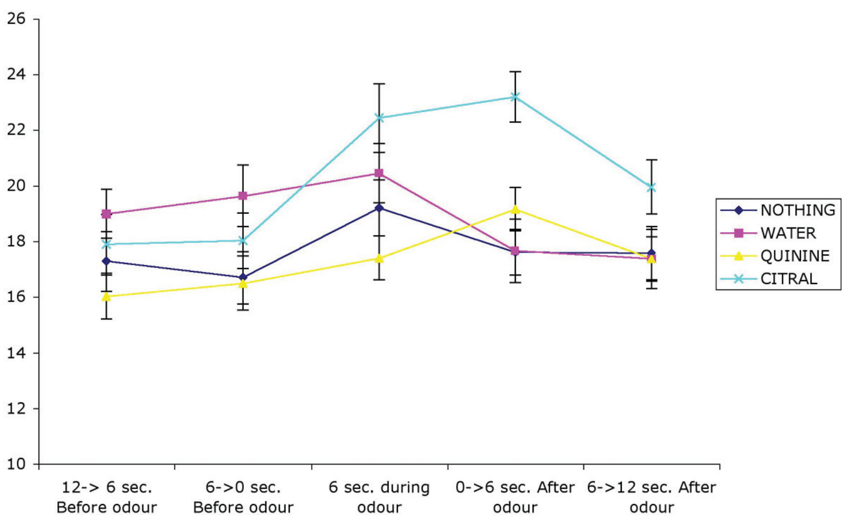

Figure 4. Angular velocity (in °/s) for three conditions of stimulation: control (nothing in the airflow), water or quinine hydrochloride 0.1M in the stimulation chamber; comparison with the effect of a volatile odorant compound (citral).

attributed to quinine but a volatile action could be detected with this study. (3) Water usually used as a solvent was also tested. We tested the water vapor detection by the antennae. (4) Results obtained with citral were used as the reference for the effect of odor on antennal tracking (Fig. 4).

The differences between citral odor and each of the other stimulating conditions were always significant (t = 4.08, 2.201, 4.664 with control, water or quinine respectively).

The results were not significantly different between these last three conditions, neither for their eventual stimulating effect (comparison between the empty cartridge, water or quinine; ANOVA: F = 2.893; NS) nor for their "dynamic" effect (comparison between the five blocks, before, during and after stimulation; F = 1.412, NS).

These results allowed us to control the accuracy of the stimulating apparatus. They also confirmed the efficiency of the automatic method to demonstrate that the honeybee had effectively detected the odor and responded by active antennal movements.

4. DISCUSSION

Many approaches have been developed to determine if an insect has actually detected an odor. Electrophysiological studies of the olfactory sensilla or of the whole antenna (electroantennogram) give information about the sensitivity of sensory organs but do not prove that perception processes are really involved. On the other hand, behavioral studies based on odor choices in a Y maze or in an olfactometer (Bakchine et al., 1990; Cano Lozano et al., 1996) suppose that cognitive and motor mechanisms do not interfere with olfactory detection.

Antennal movements could be the simplest indication of sensory detection by insects. To actively take information from the visual or tactile environment, free moving insects use their antennae (Honegger, 1981; Lambin, 1984; Kevan and Lane, 1985; Camhi and Johnson, 1999; Dürr et al., 2001).

Some authors have already demonstrated that scanning antennal movements of the worker bee can be elicited by odors. However, the methods previously used were more complicated. Electromyograms were recorded from flexor and extensor muscles, from the immobilized scapus of bees by Suzuki (1975). Suzuki's work also described the responses of motoneurons controlling the antennal movements induced by odors. In such experiments, animals were disturbed.

In many experimental studies with olfactory stimulation, antennal movements have been recorded and described with more or less precision. The optical method (light beam scanning) allowed researchers to evaluate amplitude, duration, frequency and linear speed of the flagellar movements (the scapus being immobilized) during odor stimulation (Peteraitis and Vaitkevièienë, 1995). The aim was to evaluate the volume of space scanned by the antennae but the antennal movements were restrained by the immobilized scapus.

Erber and Schildberger (1980) and Erber et al. (1993) showed that in honeybees, antennal responses could be specifically directed to vertically moving visual patterns, mechanical stimuli and to chemical compounds. Five distinct odors were compared and the effect of the concentration was studied. For these records of antennal movements, the scapus was free but the passages of each antenna were counted by a single phototransistor and did not give a good description of the movements.

A better method to evaluate modification of antennal scanning, induced by sucrose stimuli and modulated with microinjection of serotonin or octopamine, was used by Pribbenow and Erber (1996). The scapus was fixed and the authors counted the antennal contacts with a silver wire and they recorded the muscle potentials. Kisch and Erber (1999) have made operant tactile conditioning with a similar device. A precise description of the successive antennal positions was shown in the work of Erber et al. (1997). The image analyzing system of video frames presented 7500 x–y coordinates for periods of five minutes. The scapus was free but the small drop of ink put on the flagellum to allow detection of the antennal positions could slightly hamper the bee and alter the movements.

Our method based on video film analysis has a better accuracy even if 3-D information is generally lost. The manual analysis seems to be the most efficient but it is also slightly tedious.

On the other hand, the implementation of the automatic method is easy and fast. Digital records and image processing to detect successive antennae positions require just a personal computer equipped with a webcam. The comparison of the results obtained with the two methods shows that qualitative results are quite similar.

The use of a webcam for shooting can be easily coupled with experiments on fixed insects like the one designed for conditioning purposes. Olfactory capacities are controlled even if toxic or pharmacological agents can affect memory or cognitive functions. The film analysis takes a few minutes. It requires neither modification of the experimental conditions, nor colored ink on the antennae and a normal light level is sufficient.

The present results do not show any effect of the airflow or compound solution.

The only condition with an increase in the antennal angular velocity is met with odor (citral) stimulation. We have recorded similar results after preliminary tests with odors of coffee and limonene. So, antennal movements can really be compared to the movements made by vertebrates to scent, and their variations can be very good indicators of odor detection.

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Résumé – Les mouvements antennaires en tant qu'indicateurs de la détection d'odeur chez les ouvrières d'abeilles domestiques. Les capacités mnésiques et cognitives des abeilles sont souvent évaluées à l'aide d'épreuves comportementales mettant en jeu la perception olfactive. De nombreux travaux étudient les modifications de l'apprentissage olfactif induites par des substances pharmacologiques. Dans ces conditions, il est difficile d'attribuer une baisse de la performance à un effet sur la sensibilité olfactive ou sur les processus cognitifs proprement dits. Ce travail repose donc sur l'hypothèse que la perception d'une odeur est liée à une augmentation de l'activité motrice des antennes, tendant à augmenter la probabilité de rencontre entre les récepteurs olfactifs et les molécules odorantes. Les mouvements antennaires peuvent alors être considérés comme un indice simple de la détection d'une odeur par l'abeille. L'analyse de films, obtenus à l'aide d'une simple caméra vidéo numérique (type « webcam »), montre qu'une odeur, telle que le citral, provoque une nette augmentation de la vitesse des mouvements antennaires (Fig. 2). Cette méthode n'a d'intérêt que si son utilisation est relativement simple, rapide et fiable. C'est pourquoi les résultats obtenus par le pointage manuel « image par image » des extrémités des antennes ont été comparés à ceux recueillis grâce à une détection automatique des mouvements antennaires, grâce à un logiciel (freeware) d'analyse d'images. La validité de la méthode automatique a été démontrée dans un premier temps (Figs. 2 et 3) puis cette méthode a été utilisée pour comparer la stimulation olfactive (citral) à la stimulation provoquée par un flux d'air seul ou saturé en vapeur d'eau ou par une solution de quinine. Cette automatisation du recueil des positions successives des antennes permet la comparaison des résultats obtenus en vue de confirmer que l'augmentation des mouvements est effectivement d'origine olfactive. L'effet de la variation du courant d'air dans la capsule vide mais contenant habituellement l'odeur a ainsi pu être testé. L'éventuelle variation du flux d'air n'entraîne aucun résultat significatif (Fig. 4). À la différence de l'odeur de citral, la vapeur d'eau ou la solution de quinine, qui ont des effets gustatifs, ne modifient pas l'activité motrice des antennes (Fig. 4). Ces résultats nous permettent donc de conclure que ce sont bien les stimulations olfactives induites par des molécules volatiles qui sont à l'origine de l'augmentation des mouvements antennaires. Grâce au recueil automatique des données que nous proposons, cette réponse comportementale simple, basée sur l'augmentation des mouvements antennaires, peut être appliquée à des animaux soumis à divers apprentissages olfactifs.

***Apis mellifera* / mouvement antennaire / olfaction / analyse de film vidéo / enregistrement automatisé**

Zusammenfassung – Bewegungen der Antennen als Indikator für die Erkennung eines Duftes durch Arbeiterinnen der Honigbienen. Das Gedächnis und die Dufterkennung bei Honigbienen sind oft mit Verhaltensreaktionen auf Grund olfaktorischer Wahrnehmung bestimmt worden. Es gibt viele Arbeiten, die eine Beeinflussung eines erlernten olfaktorischen Reizes durch pharmakologische Substanzen zeigen. Bei diesen Konditionierungen ist es schwierig zu unterscheiden, ob die Verringerung der Reaktion durch Änderung der olfaktorischen Empfindlichkeit oder dem Verlauf eines Erkennungsprozesses zuzuordnen ist. Hier wird die Hypothese aufgestellt, dass die Wahrnehmung eines Duftes an den Geruchsrezeptoren mit einer verstärkten Bewegungsaktivität der Antennen einhergeht. Diese ist abhängig von der Erhöhung der Wahrscheinlichkeit, dass Duftmoleküle auf einen

Geruchsrezeptor treffen. Demnach können die Bewegungen der Antennen als einfacher Index für die Wahrnehmung eines Duftes durch die Biene betrachtet werden. Die Analyse von Filmen, die mit einer einfachen digitalen Videokamera (Typ "webcam") aufgenommen wurde, zeigt, dass ein Duft, wie z.B. Citral, eine Nettoerhöhung der Schnelligkeit der Antennenbewegung bewirkt (Abb. 2). Diese Methode ist von Interesse, weil ihre Anwendung relativ einfach und schnell ist. Die Bewegung der Antennenspitzen (Flagellum) wurde mit manuell gesteuerten Einzelbildern ausgewertet. Die Ergebnisse wurden mit den Werten verglichen, die mit einem automatisierten Filmprotokoll der Antennenbewegung und mit Hilfe einer freeware Bildanalyse gesammelt wurden. Zunächst wurde das Ergebnis der automatischen Bildverarbeitung im ersten Zeitabschnitt gezeigt (Abb. 2 und 3). Dann wurde mit dieser Methode der Vergleich einer olfaktorischen Stimulation (Citral) mit Stimulationen durch einen reinen Luftstoß oder durch mit Wasserdampf oder mit Quininlösung gesättigter Luft durchgeführt. Die Automatisierung der Auswertung aufeinander folgender Positionen der Antennen erlaubte einen Vergleich der Ergebnisse. Es bestätigte sich, dass die erhöhten Bewegungen der Antennen nur auftraten, wenn Duft gegeben wurde. Der Effekt der Variation des Luftstroms in der leeren Duftkapsel, der aber einen habituellen Duft enthielt, konnte so getestet werden. Zufällige Variationen des Luftstroms ergaben keine signifikanten Änderungen (Abb. 4). Im Unterschied zum Citralduft, konnten Wasserdampf oder Quininlösung, die Geschmacksreaktionen hervorrufen, die Bewegungsaktivität der Antennen nicht modifizieren (Abb. 4). Aus diesen Ergebnissen schließen wir, dass nur olfaktorische Stimulationen mit flüchtigen Molekülen die Bewegung der Antennen erhöhen. Dank der automatisierten Sammlung der Einzelereignisse können wir vorschlagen, diese vergleichsweise einfache Reaktion der Erhöhung der Antennenbewegungen bei diversen olfaktorischen Lernprozessen anzuwenden und sie mit Bewegungen anderer Tieren zu vergleichen.

***Apis mellifera* / Antennenbewegung / Geruchswahrnehmung / Video-Filmanalyse / automatisiertes Bildprotokoll**

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