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Real-Time Detection and Analysis of the Exploratory Behavior of Small Animals

An Application to the Study of the Olfactory Behavior of Honeybees in a Four-Choice Device

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The analysis of exploratory behavior of small arthropods is an invaluable tool to screen for biologically relevant chemicals such as pheromones [1] or allelochemicals [2]. In most situations, the analysis consists in recording the consecutive space coordinates of the animal in an experimental area (detection) and then extracting behaviorally relevant parameters from these data (analysis).

Detection has been addressed either manually [3] with computer-assisted data entry techniques [4], or with sophisticated hardware tracking techniques such as the compensatory bowl [5, 6]. Another approach consists in using video computerized systems to record two-dimensional [7] or three-dimensional movements [8] which allow more flexibility, longer recordings and should limit experimental analysis errors. However, hardware specifications usually restrict the experimental conditions to an homogeneous background, and limit the data-sampling rate.

We have already reported a computer-assisted method for data recording and analysis of small arthropods in a four-armed device [9]. We have shown that the density of visits was a good parameter to estimate the attractiveness of an odor stimulus. Furthermore, this parameter allowed us to compare large sets of data in order to take into account interindividual variability of the exploratory behavior. However, this method involved manual recording of the animal's coordinates.

We report here a method using a PC-based image analysis system* which allows automatic recording of the data, real-time tracking (up to 25 points/s) of objects moving on a nonhomogeneous background and possible application to various experimental devices and sizes of objects. The sofware approach developed is outlined here. As an example, we describe the use of this system in an experimental protocol designed to test the behavioral responses of honeybees to a scent according to their age.

The standard configuration of the system (STARWISE*) is based on a PC-compatible computer (PC/AT3) with a digital imaging board (512 × 512 points, 256 levels of gray), a high-resolution RGB graphic monitor, and a printer with graphics capabilities (Fig. 1). During experiments, data were recorded on a video tape recorder (U-MATIC) through a B&W CCD camera (512 × 512) and controlled using a standard monitor. Data recording can be either done during experiments or off-line, which allow the adjustment of different detection parameters.

The camera should be provided with a lens fitting the size of the experimental device and the size of the target animal. The experimental device can be set on a lighting table for better contrast and to avoid the problems of shadows cast when using a point source of light.

The high-resolution monitor is used to visualize the detection, the resulting trajectory, and the analysis of visit frequency.

Data acquisition software has been designed to track a moving object in a restricted area, the limits of which are defined by the user. The contour of the device outlined by the user limits the research to a probable area and can be reused for later processing. Areas of special interest can also be outlined. A unique capability of the frame grabber makes it possible to discard background static intensity variations and to use a constant gray threshold value to detect a moving object.

In order to speed up the detection process, the search is restricted to a small window which tracks the object by linear prediction. Track coordinates are sampled at a maximal rate, thus yielding uneven time intervals. The raw data are stored in a separate file and can be edited.

False detections are interactively discarded off-line by the operator. An off-

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line procedure is used to generate a regular time sampling with a linear interpolation algorithm.

These data can be used to extract classical track description parameters such as distance covered, distance between two points, instantaneous and average speed and direction, instantaneous and average acceleration. In addition, the analysis may be extended to the study of parameters such as angles between an axis (wind direction, for example) and the direction of the animal, or tracking angles (successive direction changes).

We have focused on computing global parameters specific to the shape of the device such as the distance and the time spent in each region of special interest [10]. We have extended this last approach by computing these variables for individual squares obtained by superimposing a grid onto the experimental area. The density of presence can therefore be displayed according to a gray scale (or false colors). A hard-copy procedure** allows the mapping of the distribution to be printed on a laser or thermic printer. Individual maps can be summated over a population to take into account interindividual variability. Individual or population data are saved for further statistical analysis.

Emerging worker bees (*Apis mellifera* L.) were caged in groups of 50 individuals and reared under controlled conditions (23 °C, 50% r.h., 16:8 h L:D) until testing at ages ranging from 2 to 20 days. The exploratory behavior of individual bees was observed in a four-choice airflow device previously designed for parasitoids [10] and adapted to honeybees [11]. One field was scented with geraniol diluted in paraffin oil (20 μl, 10^{-2} vol/vol) diffusing at 15 l/h, the three other control fields being supplied with paraffin oil only at the same flow rate. Rotation of the location of the scented field for every tested bee permitted the reduction of artifacts. The device was positioned on a light table providing red light (160 lx) and the temperature was 25 °C at 55% r.h. For each age group, eight bees were tested. Bees were individually introduced into the central area of the observation chamber and their movement recorded for 5 min on videotape.

Off-line analysis was performed to reconstruct experimental tracks and measure the time spent in each field. For each age group, the time spent in the scented field was compared to the time spent in the unscented fields using a Friedman test (3 df). To test the age effect between experimental groups on the response to geraniol and to the controls, we used a one-way analysis of variance, 9 df, for multiple comparison analysis applied to the individual times spent in each field, followed by a least significant difference for multiple range analysis (Statgraphics 4.0, STCS Inc.).

The mean time spent in the scented field versus the unscented fields for the different age groups is reported in Table 1. A significant attraction toward geraniol appeared in the 6-, 12-, and 14-day groups, corresponding to 41, 37, and 37.5% time spent in the scented field. The mean times allocated to the control fields ranged from 51 to 99 s, i.e., 17 to 33% of the observation period.

Among the age groups, an age effect appeared for the response to geraniol (F = 3; df = 9:70; P = 0.004) but not for the controls. The response to geraniol fluctuated according to age (Fig. 2), with the strongest difference occurring between the 6-day group, the most attracted to geraniol (average 123.5 s), and the 2-day (66.4 s) and the 8-day (68.5 s) groups. However, the response of the 6-day group did not differ significantly from those of the 4-, 12-, 14-, 18-day groups, while the responses of the 2- and 8-day groups are similar to those of the 10-, 16-, 20-day groups.

The strong response to geraniol of an individual of the 6-day group is vi-

Table 1. Mean time (s) and standard deviations (between parentheses) spent in the scented field and in the controls according to the age of the worker bees. The level of significance of the Friedman analysis of the mean time spent in the 4 fields is given for each age group (N.S. no significance,* $0.01 < P \leq 0.05$,** $0.001 < P \leq 0.01$)

	Age [days]									
	2	4	6	8	10	12	14	16	18	<
Geraniol	66.4 (28.9)	114.4 (39.5)	123.5 (29.9)	69.5 (24.3)	87.9 (35.8)	110. (41.2)	112.5 (19.4)	86.3 (16.3)	103.4 (34.9)	89.1 (40.5)
Control 1	99. (31.6)	70.3 (39.5)	57.7 (32.2)	79.1 (15.7)	67.9 (13.9)	75.6 (32.6)	66.2 (20.1)	75.4 (14.9)	72.3 (27.3)	63.3 (32.8)
Control 2	83.5 (32.7)	54.9 (24.8)	54.9 (21.9)	84.4 (23.4)	75.6 (23.5)	55. (14.4)	54.1 (9.1)	70.4 (17.7)	66. (17.7)	82. (54.6)
Control 3	51.1 (15.9)	60.4 (18.8)	63.9 (34.4)	67. (15.3)	68.6 (15.8)	59.4 (27.9)	67.2 (19.7)	67.9 (17.6)	58.3 (8.5)	65.6 (14.7)
P	0.16 N.S.	0.15 N.S.	0.017 *	0.4 N.S.	0.01 N.S.	0.01 **	0.003 **	0.25 N.S.	0.06 N.S.	0.12 N.S.

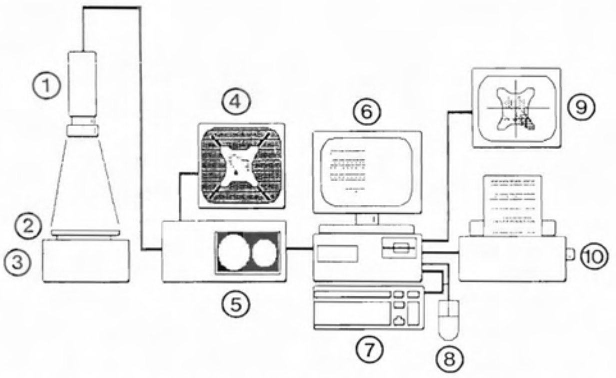

Fig. 1. The real-time tracking system. *1* CCD camera, *2* experimental device, *3* lighting table, *4* control monitor, *5* video tape recorder, *6* PC-compatible computer (PC/AT3), *7* key board, *8* mouse, *9* high-resolution RGB graphic monitor, *10* printer

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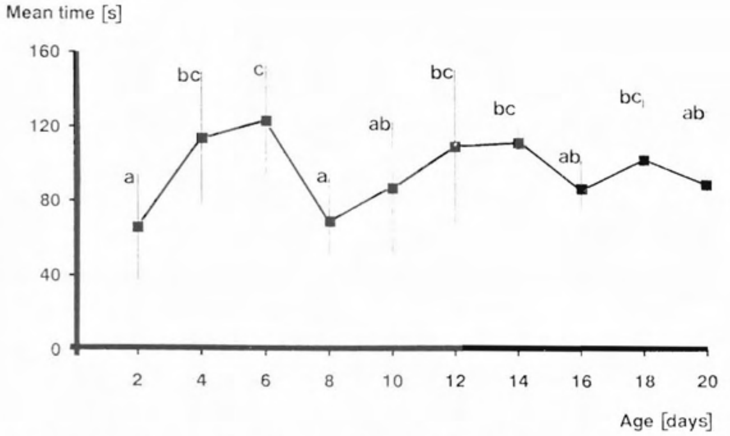

Fig. 2. Mean times and standard deviations ($N = 8$ bees per group) spent in the scented field according to the age of the worker bees Statistics: one-way analysis of variance (9 *df*) for multiple comparison analysis followed by a least significant difference for multiple range analysis. Following the multiple range analysis, values were classified from the lowest (*a* indexation) to the highest (*c* indexation) Values indexed *a* differ significantly from values *bc* and *c*, but not from values indexed *ab*. Values indexed *bc* do not statistically differ from those indexed *ab* and *c*

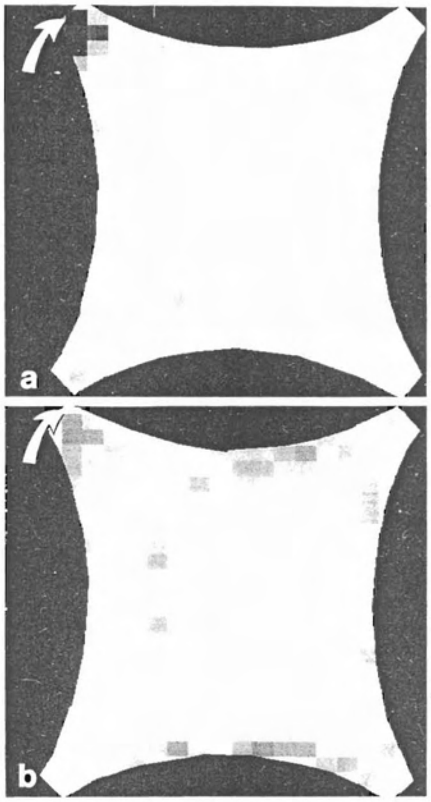

Fig. 4. Density of frequenting of the observation chamber pooled over 8 individuals. The gray levels refer to the number of positions recorded in each unit square, ranging over 12 classes from white (6 positions) to black (over 72 positions). A strong attraction is shown to the scented field (*arrow*) for the 6-day group (a), whereas the observation chamber is visited at random in the 8-day group (b)

sualized in Fig. 3, where the track appears to be nearly restricted to the scented area (A). The distance covered and the time spent in each zone were 163.1 cm and 251 s in field A, 43 cm and 30 s in field B, 0 cm and 0 s in field C, 4.7 cm and 4 s in field D. The maximum distance between two successive points was 2 cm and the total distance covered was 219.5 cm including 11.4 cm transition distance between zones.

Fig. 3. Track of a 6-day-old worker bee showing a strong attraction to the scented field (A) versus the controls (B, C, D)

Distance covered [cm]	Time spent [s]
A: 163.1	251
B: 43	30
C: 0	0
D: 4.7	4
Transit 11.4	15

The average instant speed was 0.92 cm $\mathrm{s^{-1}}$ (S.D. 0.85 cm $\mathrm{s^{-1}}$). The average speed in each zone was 0.65 cm $\mathrm{s^{-1}}$ in field A, 1.43 cm $\mathrm{s^{-1}}$ in field B and 1.2 cm $\mathrm{s^{-1}}$ in field D.

The pooled responses of the 6-day group in terms of density of visits showed a significant response to geraniol compared to the unscented fields (Fig. 4a) while the responses of the 8-day group were randomly distributed (Fig. 4b).

The present tracking system has distinctive characteristics and advantages over comparable systems:

- robustness of detection: the detection works properly even when the object's size decreases to 2 – 3 pixels such as with parasitoids [12] or mites [13] tested in bidimensional areas;
- background independence: as far as a contrast exists between the object and the background, detection algorithm efficiency is not impaired; this allows more flexibility in designing an experimental area with landmarks or static objects, or in choosing the shape of the device. For instance, it could be adapted to devices widely used in studies of animal responses to semiochemicals, such as wind tunnels. Under our experimental conditions, animals can be detected even with low light intensities or red light. This allows good viability of the behavior measurements since there is no disturbance resulting from the reflection of additional light;
- freely moving animals can be observed without impairing their movements with labels (painted spots, paper, or magnetic marks) as is necessary for the use of movement compensator systems [6];
- spatial analysis of the behavior: tracks sampled during an experiment can be subjected to path analysis (speed, length, and as an option, track angles, tortuosity) and frequency density mapping which may be required in other experimental situations such as the analysis of free access to alimentary resources. Analysis of activities recorded from restricted areas of special interest is also available;
- standard image analysis hardware: the hardware configuration is standard on PC-based image analysis; the same equipment is routinely used to perform

image analysis on insect brain microscopic slides [14]. The possible offline or on-line analysis of tracks leads to better management of the image analysis system for multi-use purposes.

In this paper we demonstrate the use of this system in the study of an olfactory-mediated behavior in honeybees. A spontaneous attraction to geraniol, the major constituent of the Nasanov pheromone [15], has been observed. This attraction is consistent with data obtained in other experimental situations such as foraging on an artificial flower device in a flight room [16] or outdoors [17]. Also, crop pollination can be improved by the use of a synthetic blend mimicking the Nasanov pheromone [18].

The response to geraniol appeared to fluctuate with age. The strongest attraction was not elicited in bees at a potential foraging age, but rather was observed in 6-day-old worker bees, that is, when bees are still supposed to be devoted to hive duties [19]. However, the polyethism of tasks according to age is largely flexible [20] and these results may be related to the fact that the highest antennal sensitivity was shown to occur in the first days of adult life [21]. The influence on the behavioral response to an odor stimulus of other factors such as prior experience or rearing conditions is currently under investigation using the automatized real-time analysis system.

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