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Computerised video tracking, movement analysis and behaviour recognition in insects

Lucas P.J.J. Noldus *, Andrew J. Spink, Ruud A.J. Tegelenbosch

Noldus Information Technology b.v., P.O. Box 268, 6700 AG Wageningen, The Netherlands

Abstract

The need for automating behavioural observations and the evolution of systems developed for that purpose are outlined. Automatic video tracking systems enable behaviour to be studied in a reliable and consistent way, and over longer time periods than if it is manually recorded. To overcome limitations of currently available systems and to meet researchers' needs as these have been identified, we have developed an integrated system (EthoVision) for automatic recording of activity, movement and interactions of insects. The system is described here, with special emphasis on file management, experiment design, arena and zone definition, object detection, experiment control, visualisation of tracks and calculation of analysis parameters. A review of studies using our system is presented, to demonstrate its use in a variety of entomological applications. This includes research on beetles, fruit flies, soil insects, parasitic wasps, predatory mites, ticks, and spiders. Finally, possible future directions for development are discussed.

Keywords: Video tracking; Movement analysis; Behaviour recognition; EthoVision

1. Introduction

1.1. Automated behavioural observation

The behaviour of insects and arachnids[1] is commonly recorded in either a manual or semi-automated way. Traditionally, a researcher observes the insect, and if he

* Corresponding author
E-mail address: l.noldus@noldus.nl (L.P.J.J. Noldus).

[1] Throughout this paper, the term 'insects' is meant to include arachnids (spiders, mites and ticks) as well as true insects.

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considers that a certain behaviour pattern is displayed, this pattern is noted—either by writing it down, or by entering the data into an event-recording program (Noldus, 1991; Noldus et al., 2000). Visual observation and manual recording can be implemented with a relatively low investment, and for some behaviours it may be the only way to document their occurrence. However, automated observation systems can often provide very significant advantages: in particular, events are recorded more reliably because the computer algorithm always works in the same way, and the system does not suffer from observer fatigue or drift, so observations can continue almost indefinitely (Spruijt et al., 1998; Noldus et al., 2001).

Technology for automated detection and recording of animal behaviour and movement has evolved dramatically in the past decade. For example, early systems, using hard-wired electronics, were able to track only a single animal in highly artificial environments. Examples are the use of a grid of infrared beams, either as the sole detector (Clarke et al., 1985; Robles, 1990; Kirkpatrick et al., 1991) or in combination with other methods such as strain gauge transducers placed under the arena (Gapenne et al., 1990). Many detection methods have been tried (Reynolds and Riley, 2002), including ultrasound (Akaka and Houck, 1980), Doppler radar (Martin and Unwin, 1988), and more recently a microwave-based 'actometer' able to detect very small movements (Knoppien et al., 2000).

1.2. Video tracking

Video tracking systems were introduced in the early 1990s, offering clear advantages of flexibility, spatial resolution and temporal precision for many applications. An early method of automatic video tracking, still used in some commercially available systems, is to feed the analogue video signal to a dedicated tracking unit, which detects peaks in the voltage of the video signal (indicating a region of high contrast between the tracked animal and background). The position of the peak in the line scan, and the line number are then used to produce the x, y co-ordinates of the animal's position, and the co-ordinates are fed to the serial port of a computer (Vorhees et al., 1992). These analogue systems have the disadvantage of being relatively inflexible (dedicated to particular experimental set-ups) and can normally track only one animal, in rather restricted lighting and background conditions. Greater flexibility is achieved by the use of a video digitizer (frame grabber), which enables real-time conversion of the entire video image to a high-resolution grid of pixels. This allows pattern analysis to be carried out on video images and so yields quantitative measurements of the observed animals' behaviour. The functionality of such a system is limited mostly by the sophistication of the video tracking software.

Several digitiser-based video tracking systems are commercially available. However, most have severe limitations. They can track only one animal per arena (if multiple moving targets are present they cannot be individually identified); backgrounds with only simple grey-scale values are needed; only a limited range of experimental set-ups can be handled, and colour video is not utilised.

2. The EthoVision system

2.1. Development history

Our system was developed to overcome the limitations of the techniques mentioned in Section 1.2, and to provide a general-purpose video tracking, movement analysis, and behaviour recognition tool. Based on a high-resolution colour video frame grabber and flexible software, it is a versatile image processing system designed to automate behavioural observation and movement tracking on multiple animals simultaneously against a variety of complex backgrounds.

Development, deployment and support of the concept has continued for almost a decade, in collaboration with universities (in particular Utrecht University, Wageningen University and the University of Neuchâtel) and with industrial research laboratories. The first DOS-based implementation was released in 1993, but the algorithms have recently been implemented in a new software package for Windows 98, NT and 2000. The description in this paper is based on EthoVision 2.3, the latest version at the time of writing.

2.2. Operation principle

A CCD video camera records the area in which the insects are (that is, the scene), and its video output (direct or recorded) is digitised by a frame grabber and passed directly on to the computer's memory. The software then analyses each frame in order to distinguish the object(s) to be tracked from the background, on the basis of either their grey scale (brightness) or hue and saturation (colour) values. Having detected the objects, the software extracts the co-ordinates of the geometric centre and surface area of each one. Calculations are carried out on the features to produce quantified measurements of the insects' behaviour. For instance, if the position of an insect is known for each video frame, and the whole series of frames is analysed, the average speed of locomotion of an insect during an experiment can be calculated. If multiple insects are present in one arena, the distance between several individually identified insects can be computed for each frame. In addition, if certain regions are identified as being of interest (the centre and edges of a circular arena, for example), the proportion of time spent by the insects in those regions can be determined.

In contrast to some other video analysis systems (Spooner et al., 1994; Pan et al., 1996), EthoVision was designed as a generic tool that can be used in a wide variety of different set-ups and applications. Most studies using EthoVision are carried out using laboratory rats and mice (for a review, see Spink et al., 2001), but the system is also used in research on insects, fish, birds, primates, farm animals and other mammals. The EthoVision software has been described in detail by Noldus et al. (2001). The following paragraphs focus on those aspects of the software that are of special relevance for the study of insect movement and behaviour.

2.3. File management

Information is organised at several levels (see Fig. 1). The highest level is called a *workspace*, i.e. a container for one or more experiments. A workspace can be used to keep together experiments that have a specific relation (belonging to one project, using the same apparatus, etc.). An *experiment* embodies a series of *trials* carried out with a particular experimental design. The data from one insect collected during a trial, the *x*,*y* co-ordinates and body surface, is referred to as a *track*. In addition to the data files generated by EthoVision, the user can create setting files called *profiles*, which can be used to easily switch between different set-ups.

2.4. Experiment design

As well as tracking objects, a professional video tracking system allows the researcher to define a complete experimental protocol, in terms of the independent variables of an experiment, and their values. In EthoVision, up to 99 independent variables (such as treatment, ambient temperature or genetic strain) can be defined. These independent variables can be used to select, sort and group data, both when plotting tracks and analysing the data. For instance, one can select to plot all the

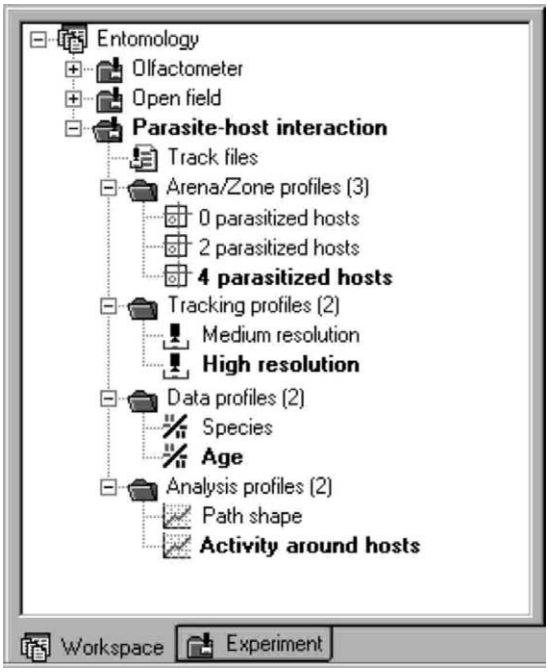

Fig. 1. The EthoVision workspace explorer. The workspace 'Entomology' contains three experiments. Experiment 'parasite–host interaction' is active (shown in bold), and it contains a series of different profiles, illustrating the different uses to which these stored settings may be put.

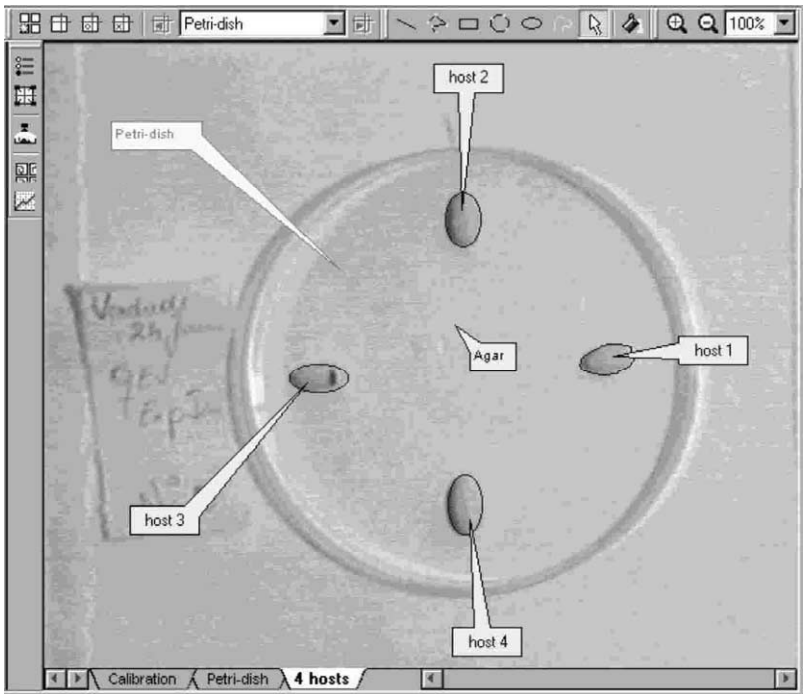

Fig. 2. An EthoVision arena definition. The video image of the set-up is used to draw and define the arena and zones of interest. The circular arena is divided into different zones: the beans with host insects (hosts 1, 2, 3 and 4) and the agar substrate. Video material courtesy of J.P. Monge.

tracks from parasitoids of a particular treatment group, or calculate the mean time taken to reach the odour bait by female cockroaches compared with males.

Prior to data acquisition one can predefine the values of the independent variables for a series of individual trials one plans to run in an experiment. In this way, the design of the experiment, and also the testing, can be done before the actual trials are performed. The system can thus be used to schedule the trials, i.e. assist the experimenter in applying the correct sequence of treatments and selecting the correct individuals. During data acquisition, the values of the independent variables are displayed on the computer monitor, providing immediate feedback on the characteristics of the trial (which insect is currently being tested, what is the treatment, etc.).

2.5. Arena and zone definition

In EthoVision, up to 16 enclosures can be placed under one camera, and each enclosure can be treated as a separate independent replicate (called an *arena*; see Fig. 2). The individuals in these arenas can be tracked simultaneously. By defining an arena in the video image in which object tracking takes place, parts that do not

belong to the defined arena are ignored during data acquisition. Different geometrical shapes (rectangle, circle, polygon, curve, line, and freehand) allow the user to draw the outline of any experimental set-up. One can thus use the system for a Petri dish test, olfactometer experiment, wind tunnel, or any other standard test. Because the signal from the video camera is directly displayed on the computer screen, the arena outlines can be traced with high accuracy.

An essential aspect of all modern video tracking systems is the possibility to define regions of interest (in EthoVision called *zones*). These can be used in the analysis (e.g. to compute the time spent in different parts of a test chamber) and for automatic start and stop conditions (see Section 2.9). Zones can be combined to make a *cumulative zone* (e.g. if a test apparatus has more than one target area), or defined as *hidden zones*. The concept of hidden zones allows the system to deal with instances when the insect is obscured by something between it and the camera. The system assumes that when a walking insect disappears from view and it was last seen adjacent to a hidden zone, it is inside the hidden zone. A hidden zone can also double as a normal zone: if the insect is visible within the boundaries of the zone, EthoVision assumes that it is on top of it and not inside. This option is useful, for instance, in studies of orientation behaviour around traps, where the trap can be defined as a normal zone (the insect can be on top of it) as well as a hidden zone (the insect can enter it). Zones can be defined and altered either before or after data acquisition, allowing iterative exploratory data analysis of the effects of changing zone positions and shapes. Points of interest can also be defined (e.g. the odour dispenser in a wind tunnel).

2.6. Object detection

During a data acquisition run, EthoVision grabs video images at a user-defined rate (up to 30 Hz). Subsequently, image processing algorithms are applied to detect the insect(s) against the background, to identify individuals (when tracking more than one insect per arena) and to extract relevant image features. The tracking process is displayed on the screen (Fig. 3). The software offers three different object detection methods:

Grey scaling defines all connecting pixels within a defined range of grey-scale values as the animal, and all other pixels as the background (or the other way round, per the user's choice). The upper and lower thresholds can be set either manually by the user, or calculated automatically by the program. Grey scaling is a fast detection method (allowing the highest sample rate), but cannot be used if the same grey scale values are present both in the insect's image and the background.

The second method, *subtraction*, first (before the start of the trial) stores a reference image, with no insects present, then (during the trial) subtracts the grey scale value of each pixel of the reference image from the equivalent pixel of the live image. Any pixels that belong to objects larger than those defined as noise and have a subtracted value other than zero represent changes that are likely to be due to the

presence of the insect. The user can define whether the insect has to be lighter or darker than the background, or just different. This method tolerates more differences in light intensity across the scene. It thus becomes possible to separate an insect with a light body colour on a greyish background from its (black) shadow. The method is also suitable for insects with a heterogeneous coloration, such as wasps with a striped thorax or abdomen.

The third detection method, *colour tracking*, uses the colour of the insect (or a marker painted on it) to identify and track it. EthoVision uses the hue and saturation components of the hue-saturation-intensity (HSI) colour space model to track objects (Spink et al., 2000; Noldus et al., 2001). By using both hue and saturation, EthoVision can distinguish objects which are more similar in colour to each other than if only using hue (e.g. objects with the same hue but differing saturation values), which is why the system can track as many as 16 different colours in each arena at once. Note that this number depends on light conditions and available colours; in practice the maximum is usually smaller than 16. In addition, the use of these two complementary detection descriptors makes the object identification more robust, so that, for instance, objects can be tracked more reliably if the light intensity (brightness) is uneven across the arena (Noldus et al., 2001).

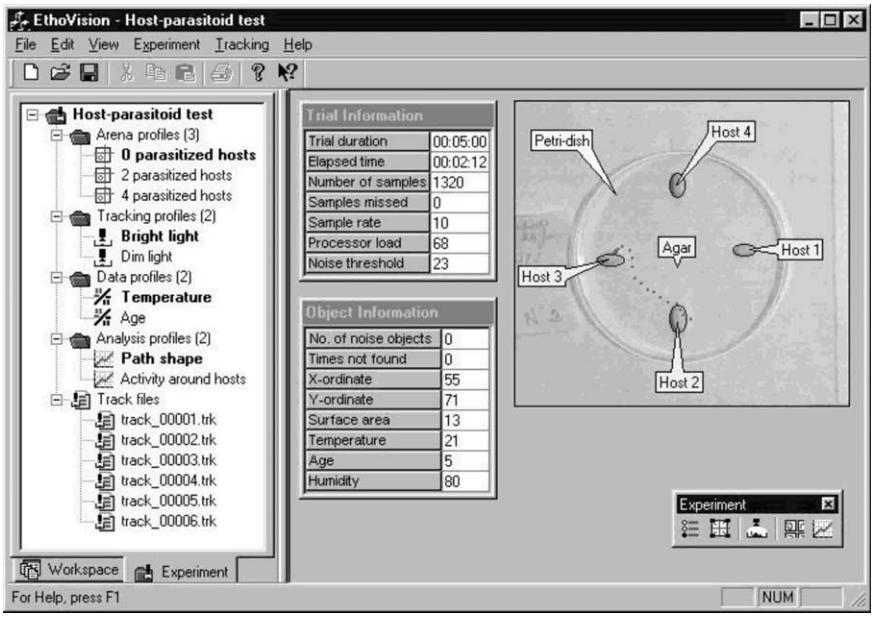

Fig. 3. During data acquisition, EthoVision displays the live video image, tracking statistics (elapsed time, number of samples, processor load, etc), measurement values of the object (x, y co-ordinates and surface area) and characteristics of the trial (independent variables). Video material courtesy of J.P. Monge.

With all three methods, objects that are either smaller than the insect (such as frass particles) or larger (such as reflections) can be excluded on the basis of their size. For details about object identification, see Noldus et al. (2001).

2.7. Image resolution

Data acquisition can be further fine-tuned by modifying the image resolution. The frame grabber used by EthoVision has a resolution of 768×576 pixels. Using a rule of thumb that an object in a digitised image should occupy at least three adjacent pixels in order to be reliably distinguished from noise, this implies that the system can track an insect in an arena up to ca. 200 times its size (e.g. a 1 cm beetle in a 2×2 m arena, or a 0.5 mm wasp in a 10 cm diameter leaf cage). Note that: (a) to obtain the maximum arena size one should take the smallest dimension of the insect's body (i.e. the width of an elongated insect, and not its length); and (b) the number 200 assumes optimal illumination and contrast; in practice the maximum is usually lower. By choosing between low, medium and high resolution, the user can pick the optimum compromise between tracking detail and computational load (which translates to maximum sample rate). If processor load becomes a limiting factor, one can let EthoVision use a moving *scan window* rather than search the entire arena for the object. With this option, only a limited area around the object's last position is searched. As a result, the maximum sample rate goes up and tracking is not disturbed by occasional moving objects elsewhere in the arena (e.g. fluid reflections). When an insect is temporarily out of view, the software can be set to resume tracking by automatically repositioning the scan window.

2.8. Image filtering

EthoVision gives the mathematical centre of the digitised picture of the insect's body, including any extremities (e.g. the antennae), as its location. This means this point may be further towards the animal's anterior than is correct. Furthermore, the system will still detect movement of the insect when only the antennae are moving. In order to prevent this, the antennae can be removed from the image, using image filtering (erosion). The wire mesh of a cage can also be filtered out using a similar technique (dilation). These filters also help to avoid accidental mix-up of the insect with background noise.

2.9. Experiment control

EthoVision has a facility for automatic experiment control. This can be used to start or stop data acquisition depending on the location of the animal. For instance, the system can be set to start a trial when a wasp has crawled up the central tube of a 4-way olfactometer and stop the trial after it has been in one of the arms for more than a user-defined length of time (i.e. a spatial and temporal criterion combined). One can also let the system perform trials in series, with the number of

trials and the inter-trial interval (e.g. 72 consecutive trials, each with 5 min duration and separate by 55 min intervals) defined by the user. Such series are useful in, for instance, studies of movement rhythms.

2.10. Data analysis

The user can specify data sets at several hierarchical levels for track analysis and visualisation. The data can be selected, sorted and grouped by independent variables. In addition the track files can be split up using time windows (e.g. 1-min intervals), spatial zones (Section 2.5) or behavioural states (detected automatically by the system or recorded manually by the user). This way a selection of the tracks in an experiment can be plotted on a single screen, for instance in a matrix of compound by dosage. Track plots can be displayed with or without the arena and zones or the background image of the experimental set-up. EthoVision can replay the acquired tracks on the screen, allowing a detailed and interactive visual analysis of the data.

For numerical analysis, one can group tracks by the values of independent variables, e.g. the mean velocity can be calculated for all insects that were reared on a particular diet. Data can be smoothed by activating a 'minimal distance moved' filter (to eliminate slight 'apparent' movements) and down-sampling steps (to eliminate redundant data if a lower sample rate describes the path equally well or better). This is especially important when an accurate quantification of the path shape is required (Bell, 1991). A wide range of quantitative measures of behaviour is available in EthoVision, including parameters for location and time, path shape, individual behavioural states and social interactions, and these can be described with a full range of descriptive statistics. Parameters, statistics and raw data can be exported in a variety of formats for making graphs and carrying out further analysis in third-party programs such as The Observer (Noldus et al., 2000), Microsoft Excel, SAS or SPSS. More detailed information about these parameters is given in Appendix A.

3. Examples of the use of EthoVision in research on insect (and arachnid) movement and behaviour

3.1. Behavioural interactions between male and female ticks

Dr R. Zemek and co-workers have studied the effect of female status on interactions between male and female *Ixodes ricinus* ticks (Bouman et al., 1999; Zemek et al., 2002). Pairs of ticks were observed on a glass arena made of a Petri dish (diameter 8 cm) placed bottom up in the centre of a larger Petri dish (diameter 15 cm) filled with distilled water. The ticks' movements were observed from above by a colour CCD camera connected to a computer outside the room. This set-up was designed to allow all personnel to remain outside the room during the experiment, which was essential because the ticks respond strongly to the presence

of humans. The ticks were tracked with EthoVision at a rate of 5 samples/s, using grey scaling as the object detection method. Because males are always smaller than females, the system could distinguish the ticks from each other on the basis of a difference in size. The latency to contact between male and female and the speed and duration of movement of male towards female were interpreted as indicators of female attractiveness over short distances, whereas the mean distance between the sexes was interpreted as an index of the overall attractiveness of the female. They found that the males did not walk randomly, but were attracted to the females and that males were more attracted to females that were engorged on guinea-pig blood.

3.2. Orientation behaviour of ticks near liquid water

Kröber and Guerin (1999) also studied ticks with EthoVision. They made a detailed analysis of the path moved by *Boophilus microplus* and *I. ricinus* in the vicinity of a patch of water. In general, ticks showed a strong border response: as soon as one of the legs touched the water, the tick rotated and walked away from the water in a one-step turn. However, if ticks could not regain contact with the dry surface in a one-step turn, they either made a multi-step turn to bring both legs back to the dry surface or walked astride the border, eventually rotating only so much that unilateral contact with the dry surface was not lost (border walks) or even walking onto the wet surface (Kröber and Guerin, 2000). The authors suggest the existence in the tick's central nervous system of a type of counter that determines when the avoidance reaction is to be given up.

3.3. Movement characteristics as predictors of searching efficiency of parasitic wasps and predatory mites

Parasitic wasps such as *Encarsia formosa* are of great commercial value in biological control of greenhouse pests. Video tracking can be a useful tool in selecting species suitable under given conditions for controlling certain pests. For example, Drost et al. (2000) measured the velocity (to identify the best natural enemy) and turning rate (to characterise the searching pattern) of parasitoids of *Bemisia argentifolia* whitefly. The wasps ($<$ 1 mm length) were tracked on a leaf disc in a Petri dish (diameter 5 cm) lit from below to give a good visual contrast. A 3 mm band at the edge of the leaf disc was defined as a zone and excluded from analysis (because the boundary causes 180° turns that are unrelated to the presence of hosts). The data will be used to create a simulation model of host-searching behaviour for each of the species studied.

Krips et al. (1999) have carried out movement studies to determine the effects of leaf hair density on the searching efficiency of *Phytoseiulus persimilis*, a predatory mite used for biological control of spider mites. *P. persimilis* searches randomly within a prey patch and the rate at which they encounter prey depends on the speed with which they search the leaf surface. Krips et al. (1999) compared three cultivars of the ornamental crop *Gerbera jamesonii*, differing largely in the density of leaf hairs on the lower surface of the leaves. Predatory mites (0.7–0.9 mm long) were

tracked individually on leaf discs of 16 cm^2 placed above a circular fluorescent tube and under a video camera. Walking speed turned out to be highest on the cultivar with the lowest hair density, whereas walking activity, defined as the percentage of time spent walking, was not dependent on leaf hair density. These results suggest that biological control of spider mites on gerbera may be hampered by leaf hairs.

Whereas, host location has been extensively studied in parasitic wasps foraging for hosts in open spaces such as fields or forests, little is known about the way parasitoids locate their hosts in confined complex spaces such as granaries. A.M. Cortesero and J.P. Monge (personal communication) used EthoVision to study close-range host location in *Eupelmus vuilleti*, a tropical ectoparasitoid which attacks larvae and pupae of the seed-eating beetle *Callosobruchus maculatus*. This host occurs in granaries where cowpea (*Vigna unguiculata*) seeds are stored. Female parasitoids (3.5×0.75 mm) were observed in a 20×20 cm arena consisting of a glass plate on which a 1 cm high frame was placed. Approximately 400 contiguous cowpea seeds were fixed with adhesive paper onto the bottom glass plate. In order to prevent females from going under the seed layer, the spaces between the seeds were filled with fine sand. A glass plate rested on the frame to close the experimental arena. At the beginning of the experiment, one *E. vuilleti* wasp was introduced from the side of the arena. The wasp's movement was video-tracked at a rate of 2 samples/s. The arena was filled with uninfested seeds only, or uninfested seeds and 3, 9 or 12 infested seeds placed at the centre of the arena, respectively. The following parameters were used to quantify host-searching behaviour: total distance moved, host location time (latency to contact with host in infested seed), velocity, proportion of the arena visited, and proportion of time spent in different zones of the arena. The results show that host presence as well as host number influence *E. vuilleti*'s locomotor behaviour when foraging among cowpea seeds. When no host was present or when only three hosts were present, the distance covered by the females was high and the females explored most of the experimental arena. When nine or 12 hosts were present, the distance covered decreased. In that situation, females reached their hosts faster and limited the proportion of the arena explored. The wasp's adaptive behaviour indicates that this species is able to perceive host presence at fairly low densities (nine infested seeds among 400 uninfested). The video tracking set-up can be used to study many other aspects of the host-searching strategy of species such as *E. vuilleti* foraging for hosts in complex environments.

In crop systems where biological control agents are mass-reared, movement parameters have the potential to become a standard measurement in insect quality control (van Schelt et al., 1995).

3.4. Behavioural response of aphids to plant volatiles

A video tracking system can be used as part of a behavioural bioassay to determine the responses of herbivores to plant volatiles. B. Donato and J. Hardie at Imperial College (Silwood Park, UK) used EthoVision to track and analyse the

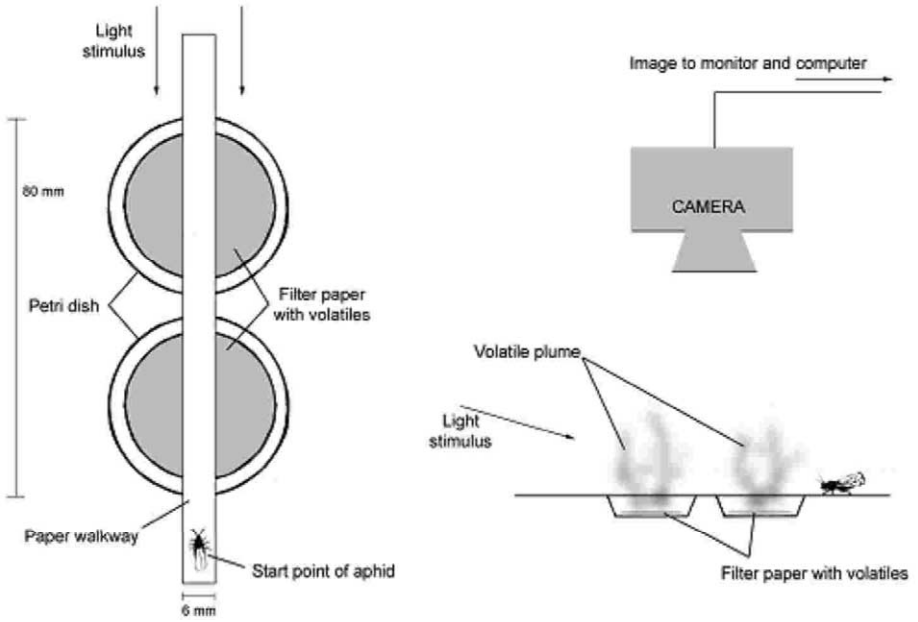

Fig. 4. Schematic diagram of a behavioural bioassay to determine the responses of aphids to plant volatiles. Image courtesy of B. Donato.

movement of winged *Aphis fabae* while walking through odour fields (personal communication). Their test arena (see Fig. 4) consisted of a 6 mm wide paper strip laid over the top of two, open, 3.5 cm Petri dishes. In the base of each Petri dish was a circle of filter paper (Whatman no. 1) cut to fit the Petri dish, to which 50 μl of diluted volatile in paraffin was added. The width of the walkway was chosen to be wide enough to allow the aphid to behave normally but narrow enough that volatile airflow would be relatively unhindered. The aphids were drawn along the paper walkway and over both Petri dishes by illuminating it with a narrow beam of light. The light source was placed 1 m from the end of the walkway and the insects moved towards it phototactically. A video camera looked down into the arena and the position of the aphid was recorded 4.2 times/s. For a run to be considered complete the test aphid needed to pass over both the test and control Petri dishes whilst walking. Runs where the aphids flew, passed under the paper strip or failed to cross both zones were noted but not included in the analysis. Readings were only taken while the aphids were within the illuminated area, and runs where the aphid passed out of the illuminated area for more than 50 readings (12 s) were discarded. Each trial consisted of a total of 40 runs, 20 with the aphid passing through the test zone first, and 20 passing through the control zone first. The velocities and absolute meander of each aphid as they passed through the two zones were then analysed and compared.

3.5. Photo-responsive behaviour of deathwatch beetles

Video tracking has proven a suitable technique to quantify beetle movements in a choice bioassay, in which the insect could move between a light and a dark area. In a study on the deathwatch beetle, *Xestobium rufovillosum*, Belmain et al. (2000) placed eight transparent polyethylene cylinders, each 2.5 cm in diameter and 19 cm long, under a single video camera. One end of each test tube was covered in black plastic (the dark end) and the other end was covered in clear plastic to allow light to enter (the light end). Using fluorescent light, the light intensity ranged from 1.32 lux at the light end to < 0.002 lux at the dark end. Individual insects were placed through a central hole in each tube, and their movement was recorded over an 8-h period. EthoVision was used to divide each behavioural chamber into three zones of equal size: light, middle and dark (Fig. 5). Parameters calculated included the latency time to the light or dark zone, the mean total distance moved in each zone, and the mean total distance moved. The results showed that both male and female insects 1–12 days old prefer the light zone. Insects more than 12 days old demonstrated sexually differential photo-responsive behaviour. Males showed a preference for the light, while females preferred the dark. These differences in behaviour have implications for monitoring and trapping of these wood-boring insects.

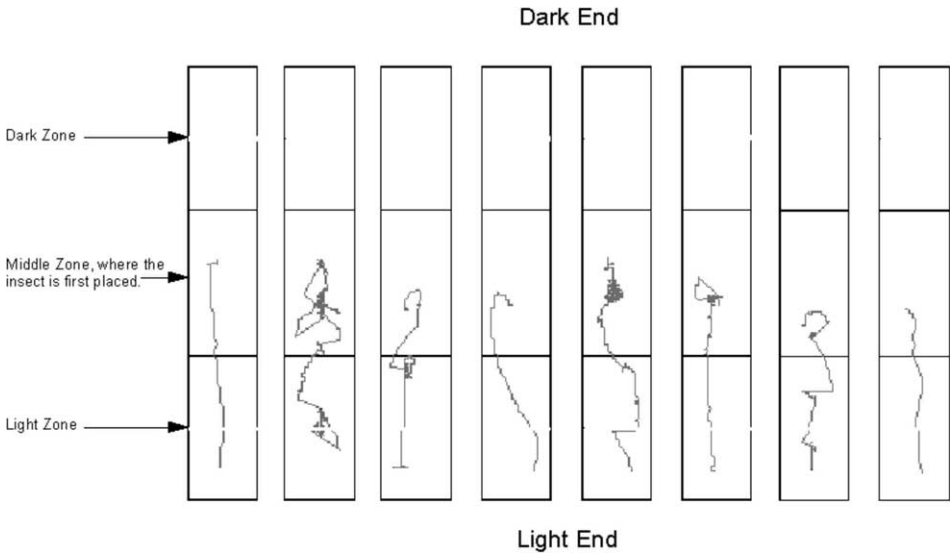

Fig. 5. Tracks showing the first 45 min of an 8-h experiment with male deathwatch beetles in glass chambers, showing the initial orientation of beetles towards the light zone of the tube. From Belmain et al. (2000), reprinted with permission.

3.6. Movement patterns as a measure of behavioural ageing

Spontaneous locomotor activity and patterns of movement can be used as a measure of behavioural ageing. Le Bourg and Minois (1999) studied the effect of exposure to hypergravity on the ageing process in *Drosophila*. Individual flies, either submitted to 2 weeks of hypergravity in a centrifuge (3 or $5 \times g$) or kept at $1 \times g$, were released in the centre of a black square arena (side length: 32.5 cm) surrounded by black vertical walls, illuminated by a circular fluorescent tube above the arena. Flies had a 2 mm^2 white paper fastened to the thorax. This paper covered a part of the wings to inhibit any attempt to fly, and it allowed a high contrast with the back ground of the arena. The path of the fly was digitised from the moment of release until either a 3 min interval had lapsed or when the insect left a virtual 25 cm arena around the release point (i.e. a zone defined in EthoVision). The following analysis parameters provided most useful: the maximum distance in cm from the centre (see 'Distance to zone center' in Appendix A), the latency time to departure from the 25 cm zone, and the velocity in cm/s. The first two parameters were used as an index of diffusion. The experiment showed that hypergravity-exposed flies were impaired at young ages when compared to 1-*g* flies (lower velocity and diffusion) but this gravity effect was erased (velocity) or even reversed (diffusion) at later ages.

3.7. Small-scale movement patterns of Collembola

Bengtsson et al. (2001) have used EthoVision to measure small-scale movement patterns of *Onychiurus armatus* (Collembola). The objective is to develop a dispersal model of these soil insects. The researchers studied the movement pattern of *O. armatus* on a plane surface with and without physical heterogeneity, i.e. obstacles. They also tested the effect of food deprivation on the movement pattern. Glass Petri dishes (diameter 20 cm) were filled with clay that was coloured black by adding Fe_3O_4. The obstacles consisted of glass tubes placed vertically in the clay, with tube size and the number per unit area varying between experiments. Since *O. armatus* lack pigmentation and the background was black, image subtraction was used as the detection method. One individual (1.5–2 mm) at the time was tracked for 1 hour at a rate of 1 sample/s. In order to diminish the disturbance of light and to prevent insects from disappearing from view, illumination was provided by four red darkroom lamps placed around the arena. Analysis of calculated velocity and turn angle (both absolute and relative) showed that *O. armatus* link periods of irregular walking with periods of looping. Some individuals made almost all their loops clockwise, some counter-clockwise and the rest showed no preference. Physical heterogeneity did not change the movement pattern but 'hungry' animals tended to make loops that were closer to being perfect circles (i.e. loops with a larger area/circumference ratio) and moved more tortuously than those that had recently fed. The track in Fig. 6 shows the movement pattern over 1 h of an individual in an arena with obstacles.

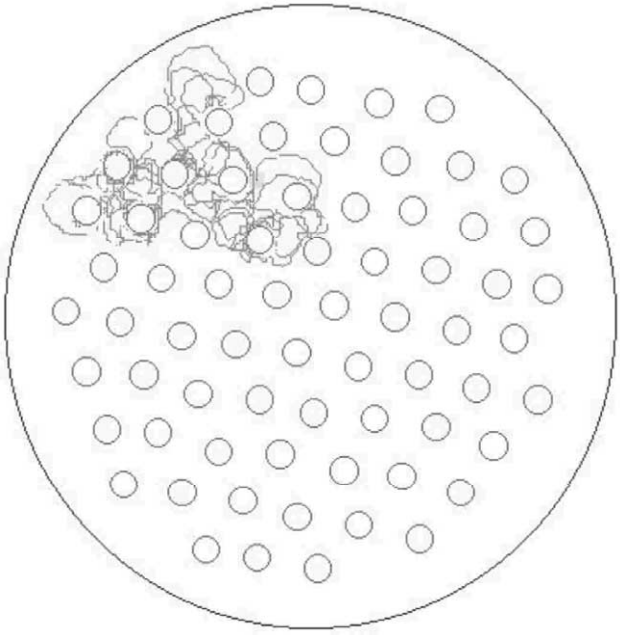

Fig. 6. Movement pattern of an individual *O. armatus* (Collembola) in an arena with obstacles. Image courtesy of A.M. Bengtsson.

3.8. Olfactory jump reflex habituation in mutant Drosophila strains

To increase the effectiveness of typically very labour-intensive screening procedures of *Drosophila* mutants, Panchal et al. (2002) have developed a semi-automated olfactory jump reflex habituation (simple learning) assay, based on video tracking technology. In this procedure, flies are housed individually in 16 glass chambers and receive odour pulses periodically. They first show a jump escape response, but this wanes after repeated odour presentations.

The apparatus consists of a metal frame holding 16 vertical glass chambers connected to two common bubblers. One bubbler holds benzaldehyde solution in mineral oil, the other contains mineral oil only (control). A continuous airflow of 1 l/min per chamber is drawn through the chambers and one of the bubblers at a time. The position of a computer-driven 3-way valve determines if the air travels through the control or the benzaldehyde solution. Usually a 4-s odour pulse is delivered to the 16 chambers simultaneously every minute. The flies show an escape response (jump) to benzaldehyde as long as they are not habituated to it. The fly movement is detected by a high-resolution monochrome CCD camera, digitised at 12.5 Hz and analysed with EthoVision software (using background subtraction and a scan window of 50 pixels). The 16 chambers cover an area of 24×14 cm, 50 cm from the camera lens.

According to Panchal et al. (2002) the illumination and the image enhancement settings in the software are crucial for detecting the small flies. They use five flicker-free cold light sources: a low-intensity (high intensity would obscure the flies) flat light source at the back of the chambers to provide good contrast for the black flies; two vertical linear light sources placed to the back sides of the chambers to illuminate the edges of the cylindrical chambers (otherwise black stripes are formed where the flies are obscured); and two horizontal linear light sources placed at the front bottom and top position to avoid formation of dark areas at the ends of chambers.

The system makes it possible to distinguish between walking and jumping of a *Drosophila* fly after stimulus delivery. This allows the researchers to perform mass habituation mutant screens at the speed of 1000–2000 potential mutant lines a year.

3.9. Locomotory activity of predatory beetles

In a study on the extent of life history variation in three staphylinid beetle species (*Stenus* spp.), showing different habitat preferences, Betz and Fuhrmann (2002) used video tracking to measure locomotory activity. Adult specimens (5–6 mm long, 1.5–2 mm wide) were individually placed in a circular arena (diameter 18 cm), surrounded by water to prevent the beetles from leaving it. The beetles were recorded with a video camera from above during their movement through the arena while searching for prey. The total distance moved (per 5 min) and the speed of movement during locomotory runs were computed as estimates of locomotory activity. In order to provide for possible interspecific differences in preferences for differently structured surfaces, the experiment was performed in two different types of arenas: (a) a plain surface without any additional structure; (b) a structured surface regularly pasted with small pieces of leaves. Both arena types allowed only horizontal movements of the beetles. *Stenus juno* beetles proved to be the least active concerning both total distance moved and mean velocity during runs, whereas *S. comma* clearly exhibited the highest locomotory speed. Generally, locomotory activity was reduced in the arenas with structure.

3.10. Identification of the neural bases of locomotor activity in Drosophila

Dr Jean-René Martin and co-workers study the neural bases of locomotor activity in *Drosophila melanogaster*, to understand how different parts of the brain interact to generate and regulate locomotor activity. They follow a neuroethological approach, using different ways to disturb various parts of the brain, including mutations, chemical ablation and toxigenic methods. Following treatment, the flies' locomotor performance and defects are measured (Martin et al., 1998). Until recently, these studies were performed using a light-gate detection method (Martin et al., 1999), which is an indirect measurement of locomo-

tor activity. It does not tell one what a fly does between two light-gate crosses. For that reason, the researchers have begun to use a video tracking system to more precisely analyse and characterise *Drosophila* locomotor activity.

The recording set-up consists of six square glass chambers (4 × 4 cm, 4 mm high to limit vertical movement) placed on a glass plate and illuminated from below by a back-light unit (15 × 20 cm). Each chamber holds one *Drosophila* fly (about 4 mm long). Movement is recorded at 5 samples/s during 7 h. Flies are tracked using the image subtraction method in EthoVision, detecting only objects that are darker than the background. Several parameters are calculated:

- Total distance moved (about 200 m for a 7-h recording).
- Number of bouts of movement. Wobbling of the fly is filtered out using the following parameters in the movement detection algorithm: averaging interval = 3 samples, start velocity = 3 mm/s, stop velocity = 1 mm/s.
- Duration of movement.
- Velocity of the fly (mm/s).
- Number of entries into a zone (diameter 3.5 cm) in the middle of the arena.
- Total time spent in this zone.

Other analysis parameters are planned, including heading, turning angle, angular velocity, and meander. According to Dr Martin (personal communication) the video tracking system is a useful tool to precisely reveal different components of *Drosophila* locomotor activity, in order to subsequently dissect and map the brain structures that generate and/or regulate it.

3.11. Spatial orientation in wolf spiders

Video tracking is also used to study the spatial orientation behaviour of wolf spiders. Dr J. Ortega-Escobar (personal communication) investigated how *Lycosa tarentula* is able to find its nest when it has been displaced, either in daylight or in darkness. Spiders were placed in a rectangular terrarium with a nest in the middle of one long side. After 5 days of habituation, the spider was gently pushed from the burrow, placed into a transparent open glass container and transferred to the centre of an open field (diameter 90 cm). It was left in the centre of it, in a different direction from that of the burrow. The illumination at the floor of the open field was about 300–325 lux. For tests in darkness, an IR light source and IR-sensitive video camera were used. Tracking was done at 5 samples/s, using image subtraction and an erosion filter of 2 pixels (to remove the extremities from the digitised image). Parameters calculated included the length of the path when the spider had crossed a user-defined circle (diameter 20 cm) around the centre, speed and the direction of movement at the point where the spider crossed the circle. The results showed that *L. tarentula* is not able to find its nest under these artificial conditions, whereas it is able to under natural conditions (Ortega-Escobar and Muñoz-Cuevas, 1999). Furthermore, it adopts a strategy of turning a fixed angle, i.e. the angle that would carry it back to the burrow. In darkness, this spider is not able to turn this fixed angle, so it seems that it needs visual information to accomplish this strategy.

3.12. Visual stimuli in mate location by the Colorado potato beetle

The nature and relative importance of stimuli affecting recognition of the sexes and spatial manoeuvring towards a potential mate by Colorado potato beetles (CPB, *Leptinotarsa decemlineata*) are still controversial. To assess the role of visual stimuli in locating another individual, Szentesi et al. (2001) have studied CPB adults' responses to different hues, stationary objects and to a pattern resembling the elytra. They tested if CPB can differentiate among surfaces reflecting in different bands of the visible spectrum, and if adults react to beetle-sized stationary coloured objects.

The tests took place in a circular arena (diameter 52 cm) made of white paper board. The arena radius corresponded to the distance from which behavioural responses among beetles were observed in the fields. The height of the arena was ca. 50 cm to avoid external visual disturbance. The arena was illuminated from above by two fluorescent light sets, placed 20 cm apart. In addition, there was a circular fluorescent tube (diameter 20 cm) placed around and above the CCD camera. The light sources produced ca. 2700 lux on the surface on which the insects walked. The paths of individual beetles were tracked with EthoVision until the beetle reached the target (coloured board or object) or the arena wall, or until 10 min had lapsed. Track parameters were calculated (track duration and length as well as speed of movement) and the tape-recorded paths were further processed for behavioural analysis using The Observer (Noldus et al., 2000) and for obtaining mean angular directions by circular statistics. The beetles showed a strong preference for a yellow–black striped board of 10×20 cm size and were also attracted to other boards coloured yellow, yellow–green and light-green.

4. Discussion

4.1. Suitability of video tracking for automated observation

Automated observation using video tracking is particularly suitable for measuring three types of behaviours: locomotor behaviour, expressed as spatial measurements (distance, speed, turning, etc.) that the human observer is unable to accurately estimate (Buresova et al., 1986; Spruijt et al., 1990, 1998), behaviours that occur briefly and are then interspersed with long periods of inaction (Martin et al., 1992), and behaviours that occur over many hours, such as diurnal variation in behaviour (Spruijt and Gispen, 1983; Olivo and Thompson, 1988).

The image resolution of modern frame grabbers, usually over 500×500 pixels, allows movement tracking at a spatial resolution more than 10–15 times that of a typical grid of photo-beams (e.g. one with 32×32 beams). This allows small insects to be tracked in arenas 100–200 times their size. On the other hand, this also sets an upper limit to the observation area. The temporal resolution, up to the video frame rate set by TV standards (PAL: 25 frames/s, NTSC: 30 frames/s) is much higher than that of other tracking techniques such as radio tracking, radar tracking

or GPS tracking. This makes video tracking ideally suited for detailed studies of movement patterns and interactions between insects. On the other hand, video tracking using CCD cameras has the inherent drawback of requiring a minimum amount of light (visible, IR, or UV irradiating fluorescent pigments) and being sensitive to occlusion of the object being tracked. As a result, video tracking inside a dense plant canopy is not feasible.

As can be seen from Section 3 of this paper, one of the strengths of EthoVision is that it is sufficiently flexible to be able to be used in a wide variety of different experimental set-ups, applications and with a wide-ranging assortment of species. Other video tracking software has been specifically designed for a particular experiment, such as the Morris water maze for rodents (Spooner et al., 1994; Mukhina et al., 2001), whereas EthoVision can work with any shape of arena, with a large variety of different backgrounds and lighting conditions. Although there are, of course, practical limitations, in principle EthoVision can be used to track insects in any situation where the insect is within sight of a camera and in a delimited and constant area which is no more than 200 times its size.

The ability of EthoVision to track multiple objects in an arena means that interactions between animals can be identified (Spruijt et al., 1992; Sams-Dodd, 1995; Rousseau et al., 1996; Sgoifo et al., 1998). Entomologists can use this technique in studies on sexual behaviour, predator–prey and parasitoid–host relationships. Marking and tracking separate parts of animals independently, as done by Šustr et al. (2001) in their research on pigs, is possible with insects as well, provided that they are sufficiently large for dots of paint to be applied well apart. This could be used to study orientation responses of insects to a variety of stimuli.

4.2. The importance of illumination

Proper illumination is an essential requirement for any video tracking set-up. For small insects (e.g. *Drosophila* flies, parasitic wasps) and species with minimal contrast against the background (e.g. predatory mites) backlight is the recommended solution. In this situation, the arena (glass plate, Petri dish, paper sheet or leaf disc) is placed between the camera and the light source; the light shines through the arena, creating maximum contrast between the insect and the substrate. This also inhibits strongly phototactic species from flying up from the arena.

Overhead light is used when sufficient contrast between insect and background is present, when the background is not translucent, and when colour information is part of the measurement. In such set-ups, attention must be paid to prevent reflection of light into the camera, as this may interfere with object detection.

For movement tracking of nocturnal insects, an IR light source in combination with an IR-sensitive camera works well. In fact, most modern monochrome CCD cameras are sufficiently sensitive in the near-IR (up to 1000 nm). If tracking needs to proceed around the clock, in daylight as well as in darkness, the camera should be fitted with a band-pass filter adapted to the IR light source used. This way, the camera does not 'see' the daylight anymore and—with the IR light left on continuously—EthoVision will track undisturbed for 24 h/day. This technique was

used in a recent study on diel activity patterns of the western flower thrips, *Frankliniella occidentalis* (W.D.J. Kirk, personal communication).

4.3. Future developments

Despite the fact that video tracking technology has clearly proven its use for the study of insect behaviour and movement, there is still room for further development and improvement. The following are a few aspects, of particular relevance to entomological research, which might possibly be developed in the future. Whether or not such functionality will be implemented in a commercial video tracking system such as EthoVision will depend on the demands of the scientific community.

4.3.1. Using digital video input

Current video tracking systems take their input from an analogue video camera, the signal of which is digitised by a frame grabber. A logical development is that systems would be able to take direct input either from digital cameras or digital video files. A digital camera eliminates the constraints on spatial and temporal resolution set by TV standards (see the Sections 4.3.2 and 4.3.3 for more details). Storage of video images in digital files on disk has many advantages over the use of videotape, including duplication without loss of quality and fast random access. The latter is especially useful if video files are to be inspected visually with an observational tool such as The Observer Video-Pro (Noldus et al., 2000), thus synchronising movement tracking with behavioural event recording. Digital video files also allow for more advanced data management and project administration, as well as the possibility of combining the animation of a movement track with playback of the corresponding video clip.

4.3.2. Increasing the temporal resolution

Most video tracking systems use CCD cameras designed for use in close-circuit TV systems. As mentioned in Section 4.1, TV standards set an upper limit to the temporal resolution. A frame rate of 30 images/s is sufficient for most studies of walking insects but not for fast flying species, especially if insects are observed at close range. One way to overcome this is by using a de-interlaced video signal, i.e. analysing the even and odd fields of the video signal separately. This doubles the maximum temporal resolution to 60 samples/s. However, at the same time the spatial resolution is halved (since only half the number of TV lines is present in each field), and not every camera/frame grabber combination offers this possibility. If even 60 samples/s is insufficient, one is dependent on specialised high-speed cameras, which exist with speeds up to 1000 Hz. These units are usually part of dedicated motion capture or machine vision systems.

4.3.3. Increasing the size of the arena

Given the small size of many insects and the limitations imposed by the image resolution of standard CCD cameras and frame grabbers, the arena observed by a single camera is limited to a maximum size of about 200 times the size of the insect.

This usually limits movement studies to the confines of the laboratory, using arenas that do not represent the natural foraging space of the animal. There are several ways to enable tracking over a larger area. One option is to use a high-resolution video camera. CCD sensors are now available at resolutions of 1280×1024 pixels. This quadruples the maximum observation area, but the amount of data to be processed increases proportionately. Real-time tracking with a standard PC will probably only be possible with scan window techniques (Section 2.7).

An alternative to the use of an (expensive) high-resolution camera is connecting an array of (inexpensive) standard CCD cameras to a multiplexer. By doing so, surveillance over a much larger area becomes possible. Meyhöfer (2001) used this technique in field studies of aphids and their predators, using 16 cameras and a time-lapse VCR. Whereas Meyhöfer analysed the videotapes manually (using The Observer Video-Pro; see Noldus et al., 2000) it should be possible to let a video tracking system perform this task automatically, provided of course that background, light conditions, contrast, etc. are adequate for video tracking.

K.R. Hopper (personal communication) has designed an ingenious solution for the same problem, by connecting a standard CCD camera to two arms (in x and y direction) that are moved by servo motors driven by the tracking computer. By letting the camera follow the insect, movements in an arena several metres long and wide can be tracked automatically.

Instead of letting the camera move over the arena, one can also let the substrate move under a stationary camera, creating an arena of theoretically unlimited size. This is the operating principle of the locomotion compensator, whereby the insect walks on a servosphere which is moved in the opposite direction by a feedback loop in response to movement detected by a sensor. The original design (Kramer, 1976) uses an optomechanical detector and requires a reflector to be attached to the insect, a rather invasive technique that interferes with normal walking behaviour. Recently, van der Pers (2001) presented a newly designed system, based on a CCD sensor and image processor. This apparatus, which can be fitted with spheres of different diameters, no longer requires a reflector on the animal and can track arthropods as small as predatory mites. Sakuma (2002) has taken a similar approach.

4.3.4. Tracking large and indeterminate numbers of insects

EthoVision, using object identification techniques, can track up to 16 identified animals in a single enclosure. However, tracking a large and continually varying number of insects, especially if they are crowded close together, requires a quite different approach. Some progress has been made using track segmentation and reconstruction (Buma et al., 1998), and active modelling and prediction of the animals' shapes (Sergeant et al., 1988; Bulpitt et al., 2000). Once it becomes possible to track large numbers of unidentified insects, a video tracking system can be utilised to monitor insect movements around traps, and to analyse the behaviour of ants, termites, honeybees, and other social insects.

4.3.5. Automatic detection of behaviours and movement patterns

In its current implementation, EthoVision is able to detect whether an insect is moving or whether two individuals are approaching each other, but (in common with other commercial systems) it cannot automatically detect other behaviours or movement patterns (unless, of course, it is possible to define them in terms of the existing parameters). Considerable progress has been made with the use of model-based pattern recognition, statistical classification and neural networks, to automatically detect body postures and behavioural sequences in rats (van Lochem et al., 1998; Rousseau et al., 2000; Heeren and Cools, 2000; Twining et al., 2001) and pigs (Šustr et al., 2001). Some of these techniques may be applied to insects as well, e.g. for automatic classification of movement patterns in *Drosophila* flies. Further development and refinement of these algorithms is necessary before they can be incorporated into a standard video tracking system.

4.4. Availability

EthoVision is commercially available from Noldus Information Technology and various international distributors. Readers can contact the first author for more information or visit the EthoVision homepage on the web (www.noldus.com/products/ethovision/).

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Appendix A. EthoVision's analysis parameters

Each selected parameter (e.g. velocity) is calculated for each individual sample. The user selects which statistic is required (e.g. mean), and the combination of the two is displayed (e.g. mean velocity) per track or per group (e.g. the mean of the mean track velocities for all insects that received a given treatment). Input filters can be applied to the data to either down-sample the points and/or exclude samples where there is just an apparent displacement due to pivoting on the spot, etc. Most of the parameters (such as distance moved and velocity) are continuous variables, but some are state variables (in zone, proximity, relative movement, moving, and manually recorded behaviours).

A.1. Location and time

- *Distance moved:* The length of the vector connecting two sample points, i.e. the distance of the centre of gravity of the insect between one sample and the next.
- *Velocity:* Distance moved per time unit (i.e. speed).
- *Distance to point:* The distance between the centre of gravity of the insect and a location inside or outside the arena defined by the user.
- *In zone:* Whether or not the insect is in a particular zone. Zones can be redrawn at any time. They can also be added together to form cumulative zones and defined as a hidden zone for use with distinct areas where the insect can disappear from view (e.g. a trap).
- *Distance to zone centre:* The shortest distance between the centre of gravity of the insect and the centre of a user-defined zone.
- *Distance to zone border:* The shortest distance between the centre of gravity of the insect and the border of a user-defined zone.

These basic parameters are used to calculate a series of derived parameters (see below).

A.2. Path shape

This category describes the geometrical shape of the path travelled by an insect. Some of these parameters can be based on unsigned degrees (absolute) as well as signed degrees (relative).

- *Heading:* Direction of movement in relation to a user-defined reference line.
- *Turn angle:* Angle between the movement vectors of two consecutive sample intervals (absolute or relative).
- *Angular velocity:* Average speed of change in direction of movement, i.e. amount of turning per unit of time (absolute or relative). This equals the turn angle divided by time.
- *Meander:* Change in direction of movement relative to the distance moved, i.e. amount of turning per unit distance (absolute or relative). This equals the turn angle divided by distance.

A.3. Individual behavioural states

Activities of the insect can be assigned to behavioural states.

- *Moving:* Whether or not the insect's velocity exceeds a user-defined level. The user can set thresholds for both 'starting moving' and 'stop moving', and the parameter is calculated over a running average of a user-defined number of samples.
- *Manually recorded behaviours:* Whilst EthoVision is tracking the insect(s), the user can manually record (by keystroke or mouse-click) behaviours that cannot be detected automatically (such as feeding, oviposition or mating behaviour). These can be analysed in exactly the same way as any of the state variables automatically measured by EthoVision.

A.4. Social interactions

On the basis of relative movement between pairs of simultaneously tracked insects, distances and movements are classified and assigned to particular parameters of social behaviour ('social' here referring to any interaction between two individuals).

- *Distance between objects:* The distance between the centre of gravity of two insects.
- *Proximity:* Whether or not the insect is closer than a defined distance from another tracked insect. The user can define threshold values for both 'in proximity with' and 'not in proximity with', to ensure that the frequency of changes of state are biologically meaningful.
- *Relative movement:* Whether or not an insect shows a relative displacement towards ('moving to') or away from ('moving from') another tracked insect. EthoVision does not just measure whether two objects get closer to or further away from each other, but the speed and direction of movement is taken into account so that it is possible to correctly assess situations where insect A is moving towards insect B, but B is moving away from A (Spruijt et al., 1992).
- *Speed of moving to and from:* The distance-weighted speed at which an insect moves towards or away from another insect. Therefore closer objects are given a heavier weighting and movements between objects a long way from each other have a slower speed of movement. 'Speed of moving to' is only calculated if the relative movement is 'moving to'.
- *Net relative movement:* The signed, distance-weighted change in distance between two insects. Net relative movement combines the two parameters 'speed of moving from' and 'speed of moving to'.

The parameters of social interactions, based on the relative movement between pairs of objects, can also be calculated for the relative movement between insect(s) and zones/points of interest (i.e. a static position inside or outside the arena). This allows the user, for example, to calculate the speed with which an insect moves towards an odour source or when an animal is in proximity with the centre of a trap.

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