egg batch of *H. pomatia* may be very small for *A. arbustorum*, since *H. pomatia* lays its eggs in a hole about 6 cm deep[8,17]. In contrast, batches of *A. arbustorum* eggs are often laid in grass tufts or in moss, i.e., at sites where hatchlings of *H. pomatia* often forage[5]. In spite of this, the rarity of either of these two species encountering other eatable eggs (egg shells of sympatric *Cepaea nemoralis, C. hortensis* and *Perforatella incarnata* are not eatable[9]), as well as the restriction of egg cannibalism to a short period of life (viz. the hatchling stage[11]), may prevent the evolution of interspecific egg predation of cannibalistic hatchlings.

Acknowledgment. This study was supported by the Swiss National Science Foundation. I am grateful to H. Dobson and S. Ulfstrand for comments on the manuscript.

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0014-4754/88/030276-02$1.50 + 0.20/0

A semi-automatic computerized analysis of tracks of ciliates

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Received 8 October 1987; accepted 4 December 1987

Summary. The behavior of Protozoa can be studied by using the ethogram as a conceptual tool capable of giving an almost complete picture of the motor biology of these microorganisms. A new semi-automatic, computerized method for drawing ethograms is described here: it allows a time-saving of about 70%, in comparison with the similar manual procedure. Microorganism movements are photographed by a Pentax LX camera from the screen of a TV monitor, connected to the stereomicroscope by a TV camera, and resolved into single images using a stroboscopic apparatus. The pictorial data are introduced into the computer by means of a digitizing tablet, and the track analysis is performed semi-automatically. The measurements recorded are then processed using a commercial statistics package in order to obtain a general view of the quantitative parameters of each ethogram.
Key words. Computer analysis; ethogram; ethology; Protozoa.

After Jennings' masterpiece[1], the behavior of Protozoa has been long disregarded, and investigated rather as cell motility than as true behavior, so that quantitative descriptions of the actual movements of protozoan organisms are today almost nonexistent, as has been clearly stated by different authors[2-4]. The first ethogram as defined by Eibl-Eibesfeldt[5], for a protozoon was drawn by Ricci[6,7], using both the dark field time exposure technique[8] and TV recording, for *Oxytricha bifaria,* Ciliata, Hypotrichida, according to Corliss[9]; its behavior was described by means of 9 basic elements. A far more complete standard ethogram (45 quantitative and qualitative elements) is now used; it makes it possible to gain more insight into the adaptative strategies of the ciliates, to monitor the effects of experimental treatment and to analyze, in terms of both qualitative and quantitative alterations of the standard ethogram, the effects of experimental treatment on several cellular targets (ciliary engines, membrane potential, cell shape, cell adhesion, etc.). So far, however, the extreme complexity of the process of drawing a single ethogram (4–5 weeks, for an expert operator) has discouraged the frequent use of this tool and strongly reduced the opportunity of testing its potentialities. On the other hand, it has already been shown[10] that microorganism tracks can be analyzed by means of fully automatic procedures. If the speed of the microorganism is low, the analysis is performed in real time; in this case the hardware system consists of a TV camera mounted on a microscope and connected to an image-digitizer plugged into a computer bus. If the speed of the microorganism is high, the analysis is performed off-line; for this purpose a videorecorder is inserted between the TV camera and the image-digitizer; the computer controls the feed of the videorecorder and thus the speed of the analysis. By means of this technique, the microscopical images are analyzed and the cell bodies are recognized and followed in time and space by the computer; in the semi-automatic technique, on the contrary, the positions of the microorganisms are introduced by the operator by means of an input device such as a digitizing tablet. A fully automatic analysis of the tracks requires very expensive equipment: therefore, we adopted a semi-automatic procedure that requires a hardware set-up which is within the reach of every laboratory.

Materials and methods. The microorganisms creep and swim freely in a droplet of physiological medium between a slide and a coverslip, kept at a distance of 3 mm from each other. A Hitachi TV camera is mounted on a microscope Wild M 420 and is connected to a Sony monitor. The microorganism's movements are photographed either by means of a Wild MPS 51 camera directly mounted on the dark-field equipped Stereomicroscope (the camera shutter is kept open for 4 s of 6 s) or from the screen of the TV monitor, by a Pentax LX camera; in this case, the tracks previously recorded under dark field conditions by a tape recorder Sony Betamax, are resolved into a series of single images by a strobo-

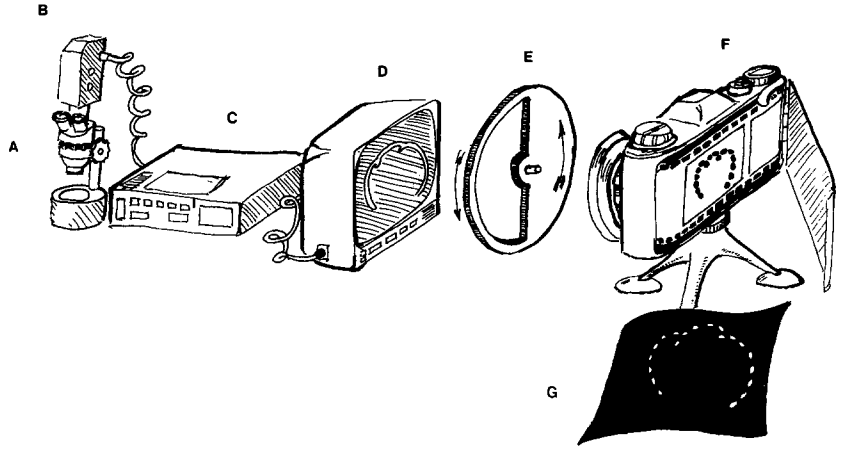

Figure 1. The apparatus used for the optical acquisition of the tracks; the microscope M 420 (*A*) is coupled to a TV camer (*B*), a videotape recorder (*C*) and a TV screen (*D*). Dark-field recordings are photographed (*F*), through a stroboscopic disk (*E*), to get a track in which the successive positions of the cell are represented by single images (*G*).

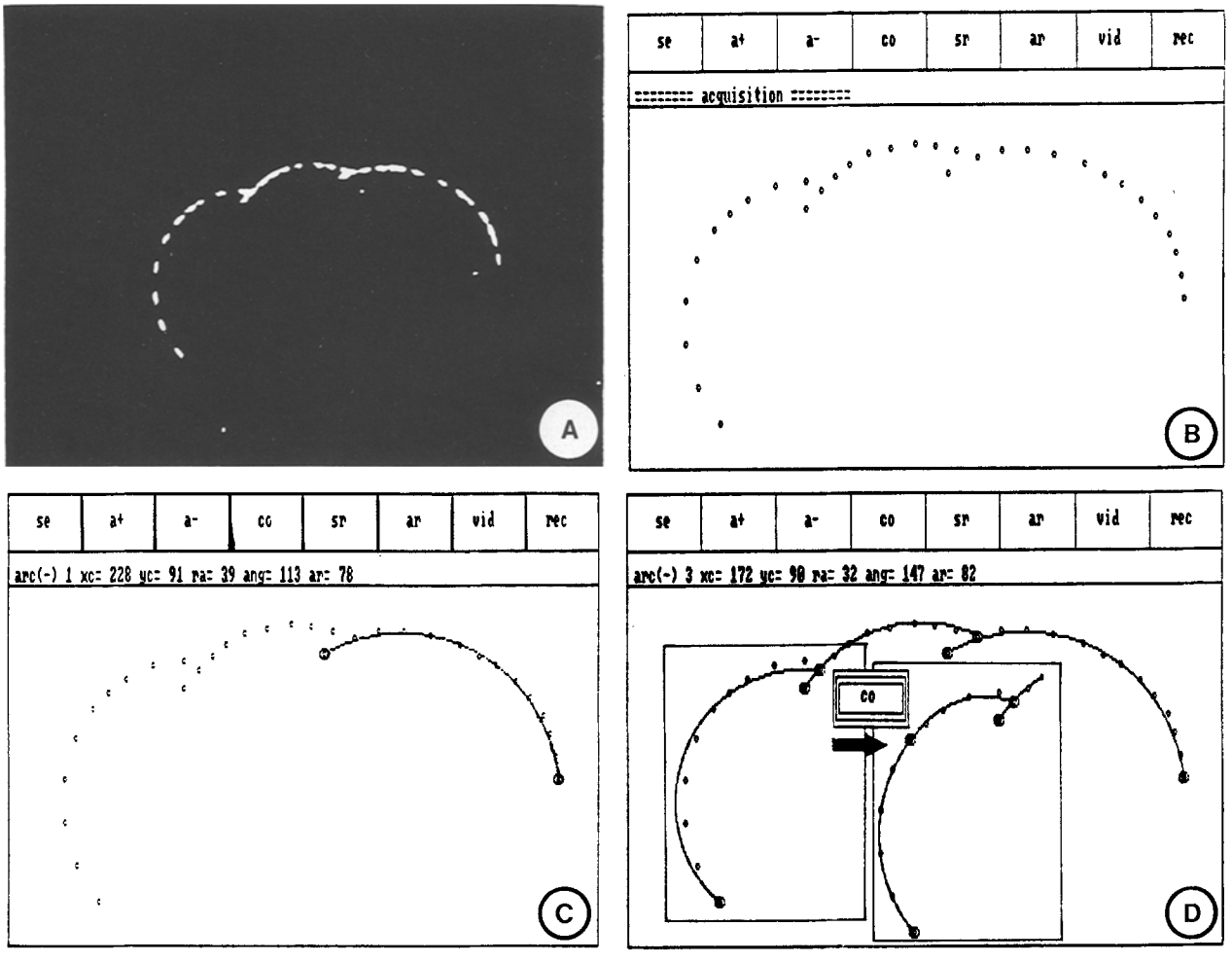

Figure 2. *A* The photographic, stroboscopic image of a track; *B* the result of the track acquisition as shown on the computer monitor, in a template of the digitizing tablet: each circlet represents one cell body; *C* the arc drawn is the result of the interpolation between two chosen points of the track, by means of the selected geometric element. In the command line, the selected element (arc-), the progressive number (1), the center coordinates (xc, yc), the length of the radius (ra), the width of the subtended angle (ang) and its length (ar) are shown; *D* example of a possible mistake in the choice of the limiting points of the track; the third arc actually proved to consist of two different arcs, shown in the central panel: such a correction is possible after selecting 'CO', in the upper part of the tablet.

scopic disk, inserted in the optical path of the camera itself: the rotating speed of the disk determines the number of cellular bodies describing the track itself (fig. 1). The picture obtained is placed on a digitizing tablet Graphtec KD 4030, connected via a serial interface to a personal computer IBM XT. The tablet has been divided into two parts: the upper part is used as a command keyboard, while the lower part is used for data introduction. The specific zones of the tablet are activated by the pressure of its magnetic pen. The whole of the software for the semi-automatic procedure has been written ad hoc, in Basic language and it is menu-driven. A template of the tablet structure is displayed on the computer monitor Hantarex Boxer 12, thus allowing the operator visually to control the command selection and the data introduction and processing. A commercial statistical package Stat Pal, from Marcel Dekker Inc, is used for the final mathematical treatment of the data.

Track analysis. In figure 2A, the photograph of a track is shown: when it is placed on the digitizing tablet, the operator has to select the origin of the coordinates and the X and Y ranges, before acquiring the track itself, by pinpointing sequentially each of the cellular bodies forming the track (fig. 2B); the X and Y coordinates of these data points are introduced into a bidimensional array. Typically, the track of a ciliate consists of a sequence of three geometric elements, randomly following each other: segments ('SE'), rightward arcs ('A +') and leftward arcs ('A −'). In figure 2 only leftward arcs are represented, because *O. bifaria* creeps only along leftward arcs.

In order to analyze the tracks, the operator has to select, on the upper part of the tablet, the proper type of track element (namely 'SE', 'A +', 'A −'), whose parameters are to be calculated: for example, for *O. bifaria,* the A − zone of the tablet must be pinpointed (fig. 2B, upper part). The points apparently representing the extremes of that particular part of the track to be interpolated by means of the pre-selected element, are pinpointed now by the operator and, soon after, highlighted on the screen (fig. 2C). The result of these operations is an arc drawn by the computer between the selected points: if this curve does not fit all the points between the two extremes, the operation can be repeated, by selecting 'CO' (for 'correction') on the tablet, in order to choose different limiting points individuating more appropriately the part of the track to be described by that particular geometric element. In our example, figure 2D, the operator corrects the third arc by replacing it with two other arcs fitting the experimental points better. The procedure can be repeated until the fitting arc describes properly the real track.

For each element the computer calculates, visualizes and stores the following parameters: for leftward and rightward arcs coordinates of the center, radius (microns) and width (degrees) of the subtended angle and length, for the segments, length (figs 2C, 2D). Once acquired and analyzed completely the whole track is recorded by the computer, by selecting 'REC' (for 'Record'), on the tablet; thus, the screen image is stored in a scratch zone of the computer memory (R.A.M.). Whenever the operator needs to look at that track, he can select 'VID' (for 'Video') on the tablet and the

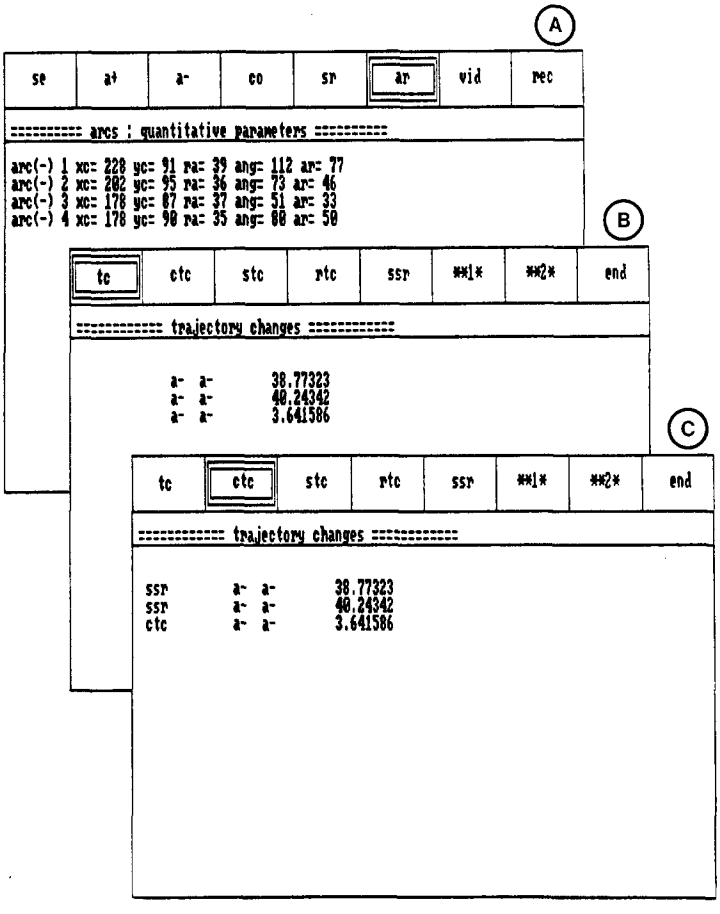

Figure 3. *A* The monitor shows the parameters of 4 different arcs; *B* the monitor shows the three couples of elements (leftward arcs) and the angles formed by them; *C* the operator, after labeling as SSR the first two trajectory changes, has just labeled as CTC the third one.

track, recalled from RAM, will appear on the monitor again. At the end of the analyzing phase, all the values of the different parameters measured for segments or arcs can be shown on the monitor, by selecting either 'SR' (for 'Segment Results') or 'AR' (for 'Arc Results'), respectively (fig. 3 A).

Now, to complete the drawing of the ethogram, the correction angles (namely the geometric relationships between the successive elements of the track) have to be analyzed, so that the 4 different types of motor patterns, which are known to describe the general direction of the movement can be recognized and measured. These types of trajectory changes are the following: a) the C.T.C. (Continuous Trajectory Change); the cell passes from one element of the track to the next without any clear-cut spatial discontinuity; b) the S.T.C. (Smooth Trajectory Change); the cell slows down, correcting its trajectory by a small angle; c) the R.T.C. (Rough Trajectory Change); the cell stops for about 0.1 s and turns around its barycenter like the needle of a compass, before moving forward in the new direction; d) S.S.R. (Side-Stepping Reaction); after stopping, the cell creeps backwards for a small distance, about as long as its body, and then rotates clockwise through a certain angle, before creeping forwards again. When the TC (Trajectory Change) zone of the tablet is pressed, the values of the correction angles of that track are shown ordinately on the monitor, together with the two linear elements forming each angle; for example, the track of figure 2 A shows three successive angles, all formed by two leftward arcs, and their widths are given on the screen as indicated in figure 3 B. At this point the operator selects on the tablet the proper kind of trajectory change to refer to a certain angle, and his choice appears on the monitor, on the left of the elements forming that angle. Figure 3 C represents the monitor as it appears, for example, when the third correction is labeled as CTC.

By selecting 'FILE' on the tablet, one lets the computer record all the data describing arcs, segments, angles and trajectory changes of one track in one single file: the file name (in this case OBCONT.09) recalls the species studied (*Oxytricha bifaria*), the experimental conditions ('controls') and the progressive number of the track ('the ninth track'). Before analyzing a new track, one has to select 'END' on the tablet, thus resetting the computer. An analytical study follows the track analysis. The files stored in the computer are processed by means of the commercial package mentioned in 'Materials and methods', to get the statistical elaboration of the data, which is necessary for a proper understanding of the ethogram (means, standard deviations, linear correlation, χ^2, curve fitting, frequency distributions, and graphic representations).

Conclusions. The ethogram has been shown to represent a basic tool not only to describe exhaustively the motor patterns of a certain ciliate, but also to analyze the effects of certain kinds of experimental treatment, such as starvation or ionic perturbation of the environment. The method also makes it possible to ask the right questions in the proper way[11]. How is the creeping underneath the meniscus regulated? What is the role played by the number, type and topographic insertion of the ciliary engines in determining the geometry, in a broad sense, of the creeping and/or of the swimming pathway? How does the body shape itself affect the general motility of a certain species[3, 11]? On the other hand, the ethogram represents the outcome of a long and complex piece of analytic work, and this fact, so far, has hindered any extensive use of the ethogram itself. The semi-automatic analysis of the motor pattern of a certain species, as described here, seems to offer the opportunity of overcoming this handicap, in as much as it allows a time saving of about 70%; while an ethogram drawn by the manual technique needs some weeks of hard work, the semi-automatic analysis allows us to fulfil the same task in less than six days. Furthermore, in our opinion, the type of semi-automatic analysis described here, which allows a fast and precise processing of the ethographic data, requires equipment which should not be beyond the means even of simply-equipped laboratories, making it possible for anyone to carry out good studies of Protozoa behavior, both as an end in itself and as a biological trait of organisms undergoing experimental treatments.

Acknowledgment. This paper was supported by grants from CNR and from MPI.

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