METHODS & DESIGNS

Automatic stimulus presentation for the proboscis extension reflex in diptera

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An apparatus is described that automates the elicitation of the proboscis extension reflex (PER) in dipterans. The PER has been used as a measure of both classical conditioning and central excitation. Most studies of PER have employed manual stimulation, which adds inter- and intraexperimenter differences in technique as another source of variance. The advantages of this apparatus are (1) elimination of individual differences in manual techniques of experimenters as a possible source of error variance and (2) capability of stimulating several flies individually during a single session. An experiment is reported comparing the manual and automated methods.

The dipteran proboscis extension reflex (PER) (Vargo & Hirsch, 1982b; Vaysse & Médioni, 1973) has been used as a measure of excitatory classical conditioning (Hirsch & McCauley, 1977; McGuire & Hirsch, 1977; Nelson, 1971; Tully, Zawistowski, & Hirsch, 1982), conditioned suppression of proboscis extension (Médioni, Cadieu, & Vaysse, 1978; Médioni & Vaysse, 1975), and central excitation (Dethier, Solomon, & Turner, 1965; McGuire, 1981; Tully & Hirsch, 1982a, 1982b, 1983; Vargo & Hirsch, 1982a).

All of the above-referenced studies, except Médioni's, involved the experimenter's stimulating the animals manually. In our work, this has always been a matter of concern because experimenters vary in their technique (i.e., personal equations), making comparisons among studies by different experimenters difficult to interpret.

For example, a member of our laboratory conducted an experiment that involved videotaping the testing technique of four experimenters (Ricker, Note 1). From these tapes, it was noted that the experimenters differed in (1) the length of time they administered the stimuli to the animals, (2) where they applied the stimuli on the animals, and (3) the amount of shaking and mechanical stimulation they gave each fly. It was also noted that the stimulating procedure for each experimenter was not consistent over trials of the test.

The Médioni group avoided this problem by develop-

ing an apparatus that stimulates an individual fly without the participation of the experimenter. Briefly, the Médioni apparatus consists of a vertically positioned kymograph. Felt rectangles, each soaked with an appropriate stimulus solution, are positioned on the kymograph drum. The fly is mounted on a needle clamped to a support stand and adjusted so that its legs come in contact with the felt. As the drum rotates, the fly walks over the felt pads and its tarsal chemoreceptors are stimulated with the solution. A serious limitation, however, is that this placement of the stimulus pads can accommodate the observation of only one individual, making tedious the collection of large data sets.

We have adapted the Médioni apparatus so that, depending on the procedure, instead of one, several individuals can be tested together. Our adaptation and use of the apparatus permits the collection of larger data sets and eliminates variations in experimenter technique. Besides describing the apparatus, we report the results of an experiment comparing the manual and automated methods for studying the frequency of proboscis extension in *Drosophila melanogaster* (Vargo & Hirsch, 1982a, 1982b).

DESCRIPTION OF APPARATUS

The basic components of the apparatus (Figure 1) consist of an electrically powered kymograph (Bird Kymograph, No. 70-060, Phipps and Bird, Inc., Richmond, Virginia) connected in series with a rheostat for regulating drum speed. The kymograph drum is made by bolting together from the inside two 16 cm (diameter) x 15.5 cm drums and spray painting them white. An 18-in. fluorescent lamp (two 15-W tubes) is placed, vertically, approximately 30 cm from the drum surface for illumination. Finally, if *Drosophila* are used on the

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Figure 1. Basic equipment and their relative positions during a test. The kymograph is depicted with the two drums bolted together (A), and the stimulus strips (B) placed obliquely across the drum. The fluid reservoirs (C) feed stimulus solutions to the strips. The solution runoff is collected in a drip pan (D) that empties into a larger container (E). The dowel rods (F) are attached to the support stand (G) with clay (H). On the ends of the dowel rods are the pipet tips containing the flies. The fluorescent light and stereomicroscope are also shown. The stereomicroscope is attached to the horizontal bar (I) by means of a pivotal arm (J) that allows the experimenter a range of 6 cm to observe individual flies.

Figure 2. The pretest stimulus strip (A) upon which the fly is walking is twice as thick (high) as the sucrose stimulus strip (B) to allow the proboscis to come in contact with the pretest solution but not the sucrose solution. The two stimulus strips are separated by a rubber band (C). The fly in the pipet tip is mounted with a small amount of plasticene (D) on the dorsal surface to reduce escape.

apparatus, a stereomicroscope mounted on an upright adjustable stand is used to view the animals. For observing larger diptera, a stereomicroscope is unnecessary. This apparatus can also accommodate the blowfly, *Phormia regina*; however, *D. melanogaster* was the subject of the study to be reported.

Flies are mounted in micropipet tips¹ (Vargo & Hirsch, 1982a, 1982c). The head and forelegs of a mounted fly protrude through a hole cut in the micropipet tip (Figure 2). The micropipet tips, cut to 2.5 cm in length, are then placed on 1/16 in. (diameter) x 6 in.

wooden dowels that are attached with clay to a support stand. The clay provides the flexibility in three dimensions to adjust the pitch, yaw, and extension of a pipet tip so that each individual fly can be positioned as desired.

The major adaptation permitting the kymograph to accommodate more than one animal involves the placement of the stimulus pads. In Médioni's arrangement, the stimulus pads were cut in rectangles and placed with the long side parallel to the rim of the drum. On our apparatus, the stimulus pads consist of 3 mm x 57 cm strips of filter paper (Whatman No. 3) that are placed obliquely along the drum. With the oblique placement of the strips, individual flies positioned vertically along the drum can be observed sequentially as the drum turns and the stimulus strips approach each fly.

The length of time that an individual walks over one strip and is, thereby, stimulated by the solution soaked into it is dependent on (1) rotational speed of the drum, (2) width of the stimulus strip, and (3) angle the strip is placed along the drum. Therefore, depending on the duration of stimulation desired, any combination of the above-mentioned parameters can be used.

Our stimulus strips require no adhesive or mechanical connection to stay in place on the drum. When saturated, the liquid adhesion is sufficient to hold the strip in place. To maintain the dampness of the strips, solutions are fed to them through capillary tubes connected to polyethylene reservoirs (diameter = 4.5 cm, height = 1.25 cm, volume = 15 ml) placed on top of the drum. The ends of the capillary tubes are pulled to approximate .1-mm openings, resulting in fluid's flowing along the strips at 1.0 ml/min.

As fluid flows along the stimulus strips, the strips tend to act as wicks, preventing a solution's running over onto other strips that may be positioned elsewhere on the kymograph drum. However, for two strips close together, leaching may occur between them, resulting in contamination of the solutions. To prevent this, a rubber band (Arco, Size 18, 3 in. x 1/16 in.) is cut open, stretched between the two strips, and fastened to screws at the top and bottom of the drum. The rubber band acts as a barrier to the solutions and enables two strips to be nearly contiguous. We have tested the effectiveness of the rubber band to prevent leaching by dyeing one of the solutions. After 1 h, no dye could be detected on the adjacent stimulus strip using a stereomicroscope to observe the strips. A drip pan (19 cm in diameter) with a 6-cm hole in the center is placed under the drum to catch the solution runoff.

For observing *Drosophila*, the stereomicroscope is attached to a stand and can be moved vertically over a range of approximately 30 cm. It is attached to the stand by an arm that can pivot the body of the scope in the vertical direction, allowing the observer to view flies within an approximate 6-cm range. This pivotal movement is easy, requires little time, and does not interfere with the observation of individual flies.

Since the total length of the drum is 31 cm, the

horizontal bar of the stand must be moved in order to observe sequential squads of flies (see Figure 1). Grouping and placement of flies is specific to the procedure and stimulus schedule employed. Movement of the horizontal bar takes approximately 3 sec and, again, does not interfere with the observation of animals. Stands with rack-and-pinion drive require less effort to move the arm in a vertical position and can likewise be employed.

COMPARISON OF TECHNIQUES

Both methods, manual and automated, were used to screen *Drosophila melanogaster* for central excitatory state (CES) (Vargo & Hirsch, 1982a, 1982b). The procedure for testing CES consists of a 5-sec water pretest followed immediately by stimulation for 5 sec with .25-M sucrose. After sucrose stimulation, 45 sec elapse with no stimulation; then, a 5-sec water posttest is administered. All stimuli are administered to the prothoracic tarsi of individuals food deprived for 36 h. CES, initiated by sucrose stimulation, is measured as an increase in response to posttest over that given to the pretest. Proboscis extension is scored zero or one at each stimulus presentation. Total scores for pretest, sucrose, and posttest represent the sum of responses over 12 trials; therefore, the maximum score attainable is 12. The intertrial interval (ITI; time from onset of pretest in one trial to onset of pretest in the succeeding trial) is 6 min. When performed manually, the solutions are presented in small test tubes (5 x .5 cm) to the prothoracic tarsi.

To test CES on the kymograph, the drum was rotated at a speed of approximately 8.3 cm/min (circumference = 50 cm, ITI = 6.02 min). Stimulus strips of approximately 3 mm x 57 cm were placed on the drum at an angle of 26 deg. At these values, a fly traverses approximately 7 mm of strip length in 5 sec. The leading edge of the posttest strip is placed 6.2 cm from the side of the sucrose strip, a distance traversed in approximately 45 sec.

Two subtle features of the manual CES method that had to be simulated on the drum were that (1) during the test, the tarsi should not touch anything except the stimulus strips and (2) the animals should be allowed to imbibe during the water pre- and posttests, but not during sucrose stimulation. To accomplish this, the animals were positioned above the drum so that they were unable to touch its surface, when they were not over a stimulus strip and, when they were over the stimulus strips, their tarsi but not their proboscises could touch the strips. This satisfies one condition. To satisfy the other condition, a second stimulus strip was laid over the pre- and posttest strips, thus doubling their height (i.e., thickness, Figure 2), allowing the extended proboscis to touch the strips in order to imbibe the fluid.

EXPERIMENTAL RESULTS

Flies were tested with one method and then immediately with the second. To balance the experiment, both orders were run; one group was tested first manually and then with the automated method (M-A), and the other group was tested in the reverse order (A-M). The results of this experiment are presented in Table 1. Using order, method, and individuals within order as factors, a nested analysis of variance was performed on pretest, sucrose, and posttest scores.

The analysis indicated that (1) order was not significant for any of the three stimuli, (2) individual differences within order were significant for all three stimuli, (3) the method of testing flies had a significant effect on sucrose and posttest, but not on pretest, scores, and (4) the Order by Method interaction was significant for pretest and posttest, but not sucrose, scores (all significance levels were set at $p = .05$).

From previous studies (Tully & Hirsch, 1983; Vargo & Hirsch, 1982a), pretest has been shown to be a measure of general water responsiveness in the animal. The pretest results indicate that both manual and automatic methods of testing give somewhat comparable results for water responsiveness; however, because of the presence of interaction, no further interpretation is possible without further experiments.

The sucrose results indicate that the automatic method consistently gives lower scores than the manual method. One explanation for this difference has its basis in a previously unreported behavior observed on the automated apparatus: fly participation. As the strips approach, most animals extend their tarsi to walk over them; that is, unless an animal participates, it is never stimulated. With the manual method, participation was never a factor, since the solutions were brought to the tarsi. Table 2 lists the mean number of trials (of 12) that animals did not participate using the automated method (data presented for both orders). Using a two-factor mixed/repeated design (or split-plot design; Keppel, 1973, p. 433), the analysis indicated that order, stimulus (pretest, sucrose, or posttest), and the Order by Stimulus interaction were not significant ($p > .05$).

Table 1
Comparison of Automated (A) and Manual (M)
Testing Methods: Proboscis Extension Scores

Method	Pretest		Sucrose		Posttest	
	Mean	SE	Mean	SE	Mean	SE
A-M Order						
A	4.28	.672	10.78	.499	6.62	.702
M	5.75	.655	11.59	.375	8.94	.624
M-A Order						
A	4.44	.599	10.87	.455	7.62	.630
M	3.25	.624	11.53	.384	7.62	.689

Note— $N = 32$ in each condition.

Table 2
Nonparticipatory Behavior Scores From
the Automated Procedure

Order	N	Pretest		Sucrose		Posttest	
		Mean	SE	Mean	SE	Mean	SE
A-M	32	1.22	.396	.75	.336	.88	.391
M-A	32	1.16	.436	1.09	.454	.81	.379

The participation therefore does not appear to be affected by the type of stimulus presented or whether the animals are previously stimulated manually. This participatory behavior is interesting because it offers an opportunity to study anticipatory or operant-like behavior in this species.

Finally, the posttest results indicate that method has an influence on the results, the nature of which is not easily interpreted because of the presence of interaction.

From the data, the advantages or disadvantages of employing one method over another are not entirely clear. Contrary to our expectations, the automatic method does not significantly decrease the observed variance; however, the use of the automatic method ensures that the variance observed is a function of the animals' behavior and participation and not of experimenter differences in testing techniques, as mentioned earlier. This is an extremely important consideration when one is concerned with the reliable measurement of individual differences in behavior.

REFERENCE NOTE

1. Ricker, J. Personal communication, February 1981.

REFERENCES

- DETHIER, V. G., SOLOMON, R. L., & TURNER, L. H. Sensory input and central excitation in the blow fly. *Journal of Comparative and Physiological Psychology*, 1965, **60**, 303-313.
- HIRSCH, J., & McCAULEY, L. A. Successful replication of, and selective breeding for, classical conditioning in the blowfly *Phormia regina*. *Animal Behaviour*, 1977, **25**, 3.
- KEPPEL, G. *Design and analysis: A researcher's handbook*. Englewood Cliffs, N.J.: Prentice-Hall, 1973.
- MCGUIRE, T. R. Selection for central excitatory state (CES) in the blow fly *Phormia regina*. *Behavior Genetics*, 1981, **11**, 331-338.
- MCGUIRE, T. R., & HIRSCH, J. Behavior-genetic analysis of *Phormia regina*: Conditioning, reliable individual differences, and selection. *Proceedings of the National Academy of Sciences*, 1977, **74**, 5193-5197.
- MÉDIONI, J., CADIEU, N., & VAYSSE, G. Sélection divergente pour la rapidité d'acquisition d'un conditionnement chez la Drosophile (*Drosophila melanogaster*). *Comptes Rendus des Séances de la Société de Biologie*, 1978, **172**, 961-967.
- MÉDIONI, J., & VAYSSE, G. Suppression conditionnelle d'un réflexe chez la Drosophile (*Drosophila melanogaster*): Acquisition et extinction. *Comptes Rendus des Séances de la Société de Biologie*, 1975, **169**, 1386-1391.
- NELSON, M. C. Classical conditioning in the blowfly (*Phormia regina*). *Journal of Comparative and Physiological Psychology*, 1971, **77**, 353-368.
- TULLY, T., & HIRSCH, J. Behavior-genetic analysis of *Phormia regina*: I. Isolation of pure breeding lines for high and low levels of the central excitatory state (CES) from an unselected population. *Behavior Genetics*, 1982, **12**, 395-416. (a)
- TULLY, T., & HIRSCH, J. Behavior-genetic analysis of *Phormia regina*: II. Detection of a single, major-gene effect from behavioural variation for central excitatory state (CES) using replicate hybrid crosses. *Animal Behaviour*, 1982, **30**, 1193-1202. (b)
- TULLY, T., & HIRSCH, J. Two new nonassociative components of the proboscis extension reflex in the blow fly, *Phormia regina*, which may affect measures of conditioning and of the central excitatory state (CES). *Behavioral Neuroscience*, 1983, **97**, 146-153.
- TULLY, T., ZAWISTOWSKI, S., & HIRSCH, J. Behavior-genetic analysis of *Phormia regina*. III. A phenotypic correlation between the central excitatory state (CES) and conditioning remains in replicated F₂ generations of hybrid crosses. *Behavior Genetics*, 1982, **12**, 181-191.
- VARGO, M., & HIRSCH, J. Central excitation in the fruit fly (*Drosophila melanogaster*). *Journal of Comparative and Physiological Psychology*, 1982, **96**, 452-459. (a)
- VARGO, M., & HIRSCH, J. Components of the proboscis extension reflex. *Drosophila Information Service*, 1982, **58**, 149-150. (b)
- VARGO, M., & HIRSCH, J. A mounting technique to observe proboscis extension. *Drosophila Information Service*, 1982, **58**, 174. (c)
- VAYSSE, G., & MÉDIONI, J. Premières expériences sur la gustation tarsale chez *Drosophila melanogaster*. Stimulation par le saccharose. *Comptes Rendus des Séances de la Société de Biologie*, 1973, **167**, 560-564.

NOTE

1. Since the Vargo and Hirsch (1982a, 1982b, 1982c) articles, the method of mounting *Drosophila* has been improved by Mark Holliday. Instead of the notched pipet as explained in the references, pipet tips cut without a notch are used and the fly is held in place by a small piece of plasticene, as portrayed in Figure 2. This method makes mounting the flies much easier and reduces the number of flies that escape.

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