

Conditioned Behavior in *Drosophila melanogaster*

(learning/memory/odor discrimination/color vision)

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ABSTRACT Populations of *Drosophila* were trained by alternately exposing them to two odorants, one coupled with electric shock. On testing, the flies avoided the shock-associated odor. Pseudoconditioning, excitatory states, odor preference, sensitization, habituation, and subjective bias have been eliminated as explanations. The selective avoidance can be extinguished by retraining. All flies in the population have equal probability of expressing this behavior. Memory persists for 24 hr. Another paradigm has been developed in which flies learn to discriminate between light sources of different color.

Because the hereditary mechanics of *Drosophila melanogaster* are understood in detail, the behavioral repertoire of this organism and the neural system that specifies it are amenable to genetic analysis. Many flies of identical genotype are readily produced, so that behavioral measurements can be made on populations rather than individuals, yielding instant statistics. If a mutation is found in a gene affecting behavior, methods using genetic mosaics exist for localizing the site of the gene's action to a specific region ("focus") in the fly (1). Anatomical or biochemical changes at the foci of various mutants may then be correlated with alterations in behavior.

One aspect of behavior that so far has been inaccessible to this form of analysis is learning. Conditioning experiments in *Drosophila* and other dipterans are fraught with complications, and most such studies have been inconclusive. A major problem is pseudoconditioning, in which the training schedule nonspecifically alters the state of the organism, producing changes in behavior that can be misinterpreted as associative learning. An example is the "central excitatory state" (2) in the blowfly *Phormia regina*; exposure of a hungry fly to sucrose solution arouses it so that afterward it extends its proboscis in response to a variety of unrelated stimuli. This probably accounts (3) for the results of Frings (4). The proper control for pseudoconditioning is to disassociate the reinforcement in time from the stimulus; if the response results from true learning it should depend on simultaneous or near-simultaneous presentation of stimulus and reinforcement.

Another pitfall is the possibility of odor cues laid down by the flies. Our early experiments indicated that a stimulus, presumably an odor, was left in the apparatus by flies when shocked and later used by them as a cue for avoidance. The presence of odor trails may have affected the results of Murphy (5) on T-maze learning by *Drosophila*; these have recently been contradicted by Yeatman and Hirsch (6).

Habituation is the decrease in a response on repeated presentation of the same stimulus. Although it can be considered a rudimentary form of learning, in some cases it occurs at the sensory receptors (7), so it is not necessarily related to higher learning in the central nervous system. Exposure of *Drosophila*

larvae to odor altered their behavior as adults (8). This was interpreted as associative learning (9), but has since been shown to result from habituation (10).

Nelson (11) has published a convincing report of classical conditioning in the blowfly *Phormia regina*, training and testing individual flies with taste cues. In the present study we have sought to demonstrate learning unequivocally in *Drosophila* and to devise a paradigm suitable for mutant isolation, in which flies can be trained and tested *en masse*. All our experiments are variants of one experimental design. During training, flies are exposed to two different stimuli—either two odorants or two colors of light—one of which is associated with a negative reinforcement, such as electric shock. The flies are then removed and tested in a new apparatus, similar to the training arrangement but without reinforcement, and their avoidance of each of the two stimuli is measured. The reciprocal experiment is also done with a second group of flies, with shock coupled to the other stimulus. In each case the flies selectively avoid the stimulus that had been associated with shock during their training.

Using this type of experiment and related controls, we have been able to demonstrate olfactory and visual discriminative learning in *Drosophila*, eliminating the complications discussed above as explanations for our results. The selective avoidance behavior has the properties expected of conventional learning; it is extinguishable or reversible by later training and is an individual rather than a collective property of the flies.

Recently K. G. Götz (personal communication) has trained individual *Drosophila* to turn toward light or dark portions of the visual field. H. C. Spatz, A. Emanns, and H. Reichert (personal communication) have also found visual discriminative learning with populations of *Drosophila*.

MATERIALS AND METHODS

D. melanogaster of the Canton-Special (C-S) wild-type strain were used. A mutant, *yellow*², was the second strain in mixed-population experiments. To make the genetic background of the mutant similar to the normal C-S strain, crosses were done to replace the autosomes and about 50% of the X-chromosome with C-S material. Stocks were maintained as usual (12). Three-day-old flies were transferred to fresh food bottles to allow them to clean themselves for 10-30 min before training.

Olfactory Learning. An apparatus originally designed for behavioral countercurrent distribution (12) was used in these experiments (Fig. 1). Polystyrene test tubes, 17 × 100 mm (no. 2017, Falcon Plastics; Oxnard, Calif.) were aired for a

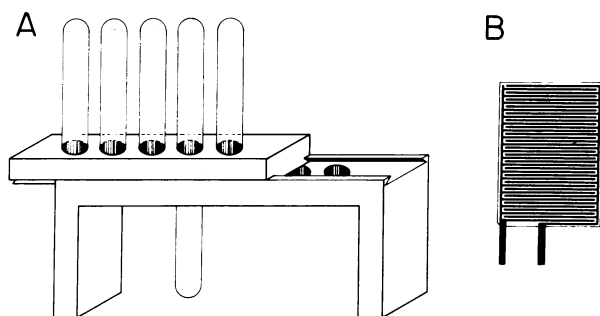


FIG. 1. (A) Apparatus used in the olfactory learning experiments. Two plastic blocks can be slid past each other on a dovetail joint. Holes running through each block are fitted with Teflon O-rings, to grip plastic tubes. (B) Printed circuit grid for shocking flies. The grid is rolled up and inserted into a plastic tube, which is plugged into the apparatus. Conductive tabs for applying voltage are bent around the tube rim to the outside.

week and used for only one experiment. "Rest" tubes had about 20 perforations at the closed end, made with a hot 26-gauge wire before airing. Grids for shocking flies had alternately connected copper strips 1 mm wide, 1 mm apart on an epoxy backing (Fig. 1B). They were made from 0.0025-inch printed-circuit material (Mica Corp., Century City, Calif.), using DCR photoetch materials (Dynachem Corp., Santa Fe Springs, Calif.). Grids were cleaned before experiments by two 24-hr washes in 95% ethanol. They were rinsed in water, ethanol, and ethyl ether and aired for at least 12 hr.

3-Octanol (no. 16449), 4-methylcyclohexanol (no. 16954), *cis* 4-methylcyclohexanol (no. 25155), and *trans*-4-methylcyclohexanol (no. 25168) were from K & K Laboratories, Hollywood, Calif. Stearic acid (no. 2733) and quinine sulfate (no. 6970) were from Matheson, Coleman, and Bell, Inc., Norwood, Ohio). Solutions of the odorants in ether were 1 ml:100 ml (1 g:100 ml for stearic acid). Odorant solution (0.2 ml) was spread over a grid surface, and the ether was allowed to evaporate (1 min). Such grids were usable for at least 2 hr.

The shock reinforcement on the grids was 90 V ac, 60 Hz. When quinine sulfate was used as a negative reinforcement, the dry powder was applied to the grids with a no. 3 artist's brush, and the excess was tapped off, leaving 8–10 mg on each grid. The conditioning experiments were carried out in a darkened room at 22°. A 15-W fluorescent lamp, General Electric cool white, F15T8-CW, was the light source for phototaxis. Flies were etherized and counted after each experiment was completed.

Visual Learning. The apparatus was a black Lucite Y-maze (Fig. 5). Entry was from a 17 × 100-mm polystyrene test tube covered with black masking tape. The arms were polystyrene tubes with the closed ends cut off and replaced with epoxy-cemented glass cover slips for more uniform illumination. For experiments with ultraviolet light, unaltered tubes were used. The arms contained standard grids without odorants. Quinine sulfate, when used, was applied as above. The only illumination was by white fluorescent light filtered through Balzers interference filters (half-width 10 nm). Intensity was adjusted with Wratten neutral density filters. The experiments were carried out at 22°.

Statistical Significance Levels were determined by the Wilcoxon signed-rank tests (13) (one-tailed distribution). Con-

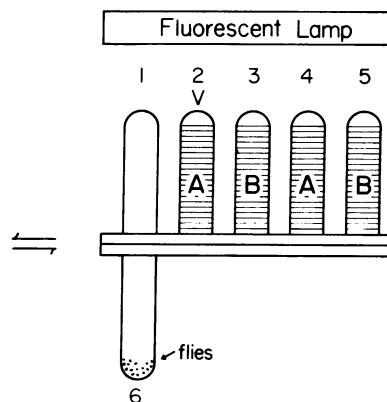


FIG. 2. Basic olfactory paradigm. Tube 1 is the rest tube, 2 and 3 are for training, 4 and 5 are for testing. Tube 6 is the start tube. Horizontal stripes in tubes indicate grids. A and B denote odorants 3-octanol and 4-methylcyclohexanol, respectively. V indicates voltage on the grid. See text for training and testing sequences.

fidence limits, where given, were determined by computing the relevant index for each experiment of a series and calculating the variance of the distribution of these values. The limits given here are standard errors of the mean. The experiments reported were run in consecutive series, with all the experiments in a series included.

RESULTS

Olfactory learning

Basic Paradigm and Controls. The paradigm required the flies to discriminate between an odor coupled with shock and another odor presented without reinforcement. The apparatus in Fig. 1A has two arrays of tubes which slide past each other so that a tube in one array can abut any tube in the other. For each experiment, appropriate tubes are fitted with grids. About 40 flies are placed in the starting tube. A run is started by holding the apparatus vertically and shaking the flies to the bottom of the start tube by tapping the apparatus on a rubber pad. The start tube is shifted into register with the proper grid tube. The apparatus is then laid horizontally before a fluorescent lamp which induces the phototactic response; the flies run from the start tube towards the grid.

In the basic paradigm (Fig. 2) tube 1 is a "rest" tube with holes at the end to allow odor to escape. Tubes 2–5 contain grids with odorants: tubes 2 and 4 each have 3-octanol on their grids; tubes 3 and 5 have 4-methylcyclohexanol. Tubes 2 and 3 are used for training, tubes 4 and 5 for testing. Voltage is applied to tube 2 only. The use of separate tubes for training and testing removes the flies from any odors they may have left on the grids during training, so that during testing the chemical odorants are the only possible cues for selective avoidance.

For training, the sequence of runs was: rest tube (60 sec), tube 2 (15 sec), rest tube (60 sec), tube 3 (15 sec). This cycle was repeated three times. (A tendency to avoid tube 2 was already evident by the second cycle.) The flies were then tested in the same sequence with tubes 4 and 5 instead of 2 and 3. The number of flies avoiding the grid on each run was counted visually. More flies avoided tube 4 than tube 5. Tube 4 contained 3-octanol, which had been presented simultaneously with shock during training.

TABLE 1. *Olfactory avoidance learning (ten experiments; pooled data)*

Specific odor paired with shock in training	Total flies	No. avoiding		Fraction avoiding	
		OCT	MCH	OCT	MCH
3-Octanol (OCT)	369	210	68	0.57	0.18
4-Methylcyclohexanol (MCH)	397	73	193	0.18	0.49

As a control for odor bias, the paradigm was repeated with a second population of flies, but with the voltage on tube 3. This time, on testing, more flies avoided grid 5. Table 1 shows the pooled results from a series of 10 such experiments. The data given are for the first test run to each odor. In all 20 cases, the flies selectively avoided the shock-associated odor. The difference in avoidance was significant ($P < 0.001$) for both reciprocal halves of the experiment.

The experimental design rules out pseudoconditioning as an explanation for the results, since the second part of the experiment serves as a control for the first and vice versa. To eliminate experimenter bias, the experiments in this series were run blind. The order of the training tubes and the order of the testing tubes were determined by separate coin tosses, and the experimenter did not know the odor in each tube. In about half the experiments, the sequence of odors during testing was the reverse of that during training. Thus the flies' behavior cannot be explained by a stereotyped order of responses or by nonspecific excitatory effects.

Not all odors work. Of 40 tested, only five gave consistently good results. The 4-methylcyclohexanol used in Table 1 was a mixture of *cis* and *trans* isomers. Either isomer can be used for training against 3-octanol or against the other isomer.

The results so far could be explained in terms of sensory habituation instead of learning, if one assumes that the flies are nonspecifically sensitized by shock to avoid all odors. During training they progressively avoid the tube with grid voltage; therefore, they spend more time in the presence of the control odorant. They might then become habituated to it and avoid it less during the subsequent testing. However, if this were true the flies would avoid the control odor used in their training less than an entirely new odor. In fact, this is not so (Table 2). Temporal association of an odor with shock is necessary for avoidance.

Learning Index. It is convenient to define a quantitative index of the specific odor avoidance attributable to learning. A simple measure is the fraction of the population avoiding the shock-associated odor minus the fraction avoiding the

TABLE 2. *Avoidance of odors by trained flies*

Odor	Fraction of flies avoiding
Shock-associated	0.40 ± 0.04
New	0.12 ± 0.02
Control	0.15 ± 0.02

3-Octanol, 4-methylcyclohexanol, and stearic acid were the odors used in this series. Each was used as shock-associated, new, or control odor in different experiments, with the six possible permutations equally represented. Nine experiments (with reciprocal halves).

control odor. For example, in a typical experiment, of 33 flies trained to avoid 3-octanol, 17 (a fraction 0.51) avoided 3-octanol, while 2 flies (a fraction 0.06) avoided 4-methylcyclohexanol. Therefore, the learning index (λ_d) for this trial is 0.45. Similarly, for the reciprocal half of the experiment the index λ_b was 0.27. The learning index (Λ) for the experiment is defined as the average of the values for the two halves (0.36 in this case). Its theoretical range is $-1 \leq \Lambda \leq 1$. If flies always avoid the shock-associated odor, never the control ("perfect learning"), $\Lambda = 1$. If association with shock does not affect the flies' odor preference (no learning), $\Lambda = 0$. If the population runs preferentially to the shock-associated odor ("masochism"), $\Lambda < 0$. For the ten experiments in Table 1, the average value $\bar{\Lambda}$ was 0.34 ± 0.02 .

Extinction, Reversal, and Persistence. Extinction of the selective avoidance behavior becomes evident when the odor cues are presented without shock reinforcement. In each of the experiments of Table 1 the flies were actually tested three times. The selective avoidance response decreased in successive tests: $\bar{\Lambda}_1 = 0.34 \pm 0.02$; $\bar{\Lambda}_2 = 0.23 \pm 0.03$; $\bar{\Lambda}_3 = 0.13 \pm 0.03$. The differences are significant: $P(\bar{\Lambda}_2 \geq \bar{\Lambda}_1) < 0.01$; $P(\bar{\Lambda}_3 \geq \bar{\Lambda}_2) < 0.01$. This decreased response was not due to diffusion or degradation of the odor cues in the tubes, since the flies could be retrained in the same apparatus. Nor was it the result of a lessened "alertness" in the population due to lack of shock; shock alone in the absence of odor cues did not restore selective avoidance.

It is also possible to reverse the flies' odor preference with extinction followed by reverse training (Fig. 3).

If not extinguished by testing, the learned behavior persists longer. Separate groups of flies were trained as in the basic paradigm, but kept undisturbed in the rest tube for various times up to 1 hr before testing. Fig. 4 shows the results. It is clear that memory persists for an hour, although some decay is evident. If the usual training procedure is repeated four times at 2-hr intervals, some selective avoidance behavior is demonstrable 24 hr after the last training session [six experiments: $\bar{\Lambda} = 0.12 \pm 0.02$; $P(\bar{\Lambda} \leq 0) < 0.001$].

Independence and Homogeneity. Experiments are necessary to test whether the selective avoidance is a property of individual flies or a collective "stampede" effect. One way to decide this is to train two populations to avoid different odors, mix the flies, then see whether they separate according to their different training experiences. About 50 *yellow* mutant flies were trained to avoid 3-octanol, and about 50 normal flies were trained simultaneously to avoid 4-methylcyclohexanol. The two groups were then mixed in one of the start tubes and run to a rest tube for 60 sec. The mixed population was then run into a fresh grid tube containing 3-octanol. After 15 sec the tube array was shifted so that flies that entered the grid tube were separated from those that avoided it. Flies of each class were collected and etherized, and their genotypes were scored. The entire procedure was repeated with two fresh groups of flies of the same two genotypes, except that they were tested with a grid tube containing 4-methylcyclohexanol.

To rule out any effect of genotype on odor preference, a reciprocal pair of procedures was also carried out. *Yellow* flies were trained to avoid 4-methylcyclohexanol, normal flies to avoid 3-octanol. Five complete experiments were performed. In 17 of the 20 test runs, the flies that avoided the grid tube were enriched in the genotype that had experienced

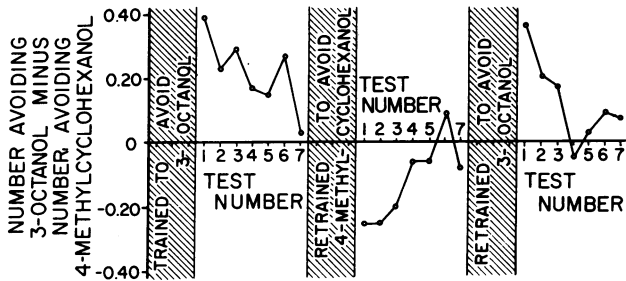


FIG. 3. Extinction and reversal of the learned response. A population of 36 flies was trained to avoid 3-octanol, then tested repeatedly without reinforcement. They were reverse-trained to avoid 4-methylcyclohexanol and retested, then reverse-trained and tested again.

that odor simultaneously with shock ($P < 0.01$). The pooled results are shown in Table 3. Both genotypes showed selective avoidance, but the learning index for each was smaller than that in the basic paradigm: ($\bar{\Lambda}$ [yellow] = 0.17 ± 0.08 ; $\bar{\Lambda}$ [normal] = 0.23 ± 0.06). This reduction indicates that there is some stampede effect, with flies of one persuasion tending to drag along those of the other. Nevertheless, the fact that the two types will separate indicates that the information for the proper choice resides in the individual flies.

In the basic paradigm (Table 1), the difference in avoidance corresponding to learning represents only a third of the population. Does this "fractional learning" arise from some inhomogeneity in the population, or is it due to a stochastic component in the behavior of all the flies? To answer this question, flies that avoided the shock-associated odor were separated from those that did not, and 24 hr later each group was retrained and retested (half to the same odor, half to the other). The performance of both groups was the same [ten experiments: $\bar{\Lambda}$ (avoiders) = 0.31 ± 0.02 ; $\bar{\Lambda}$ (nonavoiders) = 0.34 ± 0.05]. This result suggests that the expression of learning is probabilistic in every fly. There is no evidence for an "intelligent" subset of the population.

TABLE 3. Separation of normal and yellow flies with different training

Aversive training	Ratio on testing (normal/yellow)			
	OCT on test grid		MCH on test grid	
	Enter-ing	Avoid-ing	Enter-ing	Avoid-ing
Normal against OCT, yellow against MCH	0.69	1.65	1.17	0.65
Normal against MCH, yellow against OCT	1.41	0.55	0.68	3.18

Two populations of different genotype, trained to avoid different odors, were mixed and tested against one of the odors. Those flies that entered the odor tube were separated from those that avoided it. Each class was etherized and scored for genotype. The ratios given here are normalized; they represent the fraction of the normal population in the specified class (e.g., avoiders) divided by the fraction of the yellow population in the same class. All the enrichment ratios are in the direction to be expected if flies of each genotype express their learned behavior independently. (OCT, 3-octanol; MCH, 4-methylcyclohexanol.)

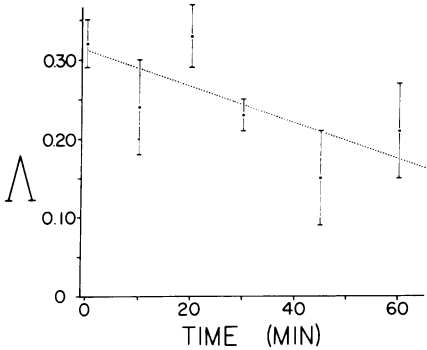


FIG. 4. Persistence of memory. Between training and testing the flies were left in the rest tube for up to 60 min. Each point represents three to seven experiments with reciprocal halves. The flies performed erratically on their first run after the long rest; therefore, each population was run once to 3-octanol before testing was begun. Λ = learning index.

Learning with Quinine Reinforcement. It was found that flies tend to avoid surfaces coated with fine quinine sulfate powder. Accordingly, flies were trained and tested in the usual manner, but with quinine sulfate replacing shock as the aversive reinforcement on one of the training grids. On testing, the flies selectively avoided the odor previously associated with quinine (ten experiments: $\bar{\Lambda} = 0.24 \pm 0.03$). This result demonstrates that the flies' learning is not restricted to a single mode of reinforcement. It also rules out artifacts due to electric shock.

Visual learning

To determine whether a sensory modality other than olfaction can be used, we developed a paradigm based on different colors of light. In addition, it requires a choice by the flies rather than simple avoidance. Fig. 5 shows the apparatus. Forty to 100 flies were placed in a stoppered plastic test tube coated with black tape. After 60 sec, the tube was placed at the entrance to a Y-maze; one arm was illuminated with 610-nm red light, the other with 450-nm blue light. Light intensities were balanced so that naive flies ran equally to both arms. During training, negative reinforcement was administered in one of the arms by coating the grid with quinine sulfate powder. The flies were allowed 30 sec to run phototactically into the arms, then shaken back into the start tube, which was removed, stoppered, and kept in darkness for 60 sec. This training procedure was repeated twice more. After the final 60-sec rest, the flies were tested in a second Y-maze, identical to the training maze but without quinine. After 30 sec the start tube was removed and a foam stopper pushed up to the fork of the maze, holding the flies in the arms that they had chosen. The flies in each arm were etherized and counted. To rule out induced color bias unrelated to learning, a second population was trained to avoid the other color. Twenty such

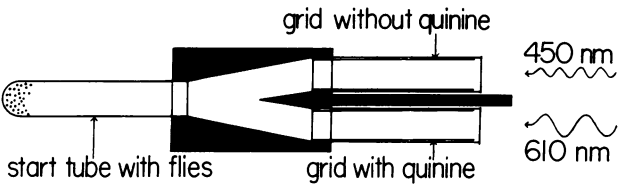


FIG. 5. Apparatus for visual training.

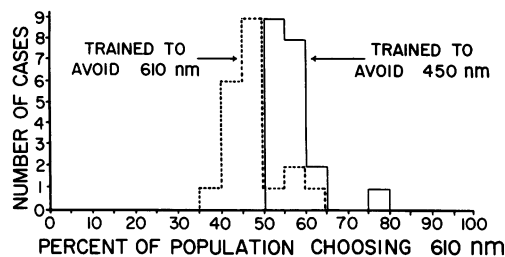


FIG. 6. Visual learning histograms, showing distributions of Y-maze choices.

reciprocal pairs were done. On testing, the flies selectively avoided the light of the color that had been associated with quinine. Fig. 6 shows the influence of training on color choice.

The learning index used here is analogous to that for olfactory learning. λ_a is the fraction of flies entering the arm with the control color minus the fraction entering the arm with the quinine-associated color. The reciprocal experiment gives λ_b . The learning index Λ for the experiment is defined as the average of λ_a and λ_b . In 19 of the 20 cases, Λ was positive. $\bar{\Lambda} = 0.09 \pm 0.01$; $P(\Lambda \leq 0) < 0.001$.

Control experiments ruled out left-right and brightness discrimination as explanations for these results (Table 4). Electric shock, when used instead of quinine, was also effective [ten experiments, $\bar{\Lambda} = 0.09 \pm 0.03$; $P(\bar{\Lambda} \leq 0) < 0.01$]. Flies were successfully trained with another pair of colors, 350 nm (ultraviolet) and 470 nm (blue), corresponding to the sensitivity maxima of the two photoreceptor systems in the *Drosophila* eye (14) [ten experiments: $\bar{\Lambda} = 0.08 \pm 0.02$; $P(\bar{\Lambda} \leq 0) < 0.01$].

DISCUSSION

Drosophila can be trained to avoid specific olfactory or visual cues. This behavior has characteristics typical of learning. It can persist for a day, but is rapidly extinguished or reversed by retraining. Various possible effects have been controlled for in the olfactory paradigm. While these might be relevant under other conditions, their influence on the experiments described here is negligible. Although the visual experiments are less extensive, their symmetrical design and their similarity to the olfactory paradigm make explanations other than learning improbable.

The learned behavior shown by the flies is fairly sophisticated, requiring sensory discrimination and (in the visual situation) choice. However, the effect is not strong. Under the most favorable conditions, only a third of the population demonstrates learning. Nevertheless, all the flies have the same apparent capability. It is likely that we have not yet found the optimal cues or the most suitable task. Conditioning has been demonstrated with two sensory modalities, sight and smell, and two forms of reinforcement, electric shock and quinine sulfate. These can be used in the four possible combinations to give similar avoidance behavior. Therefore, it is plausible that the association of stimulus and reinforcement occurs in the central nervous system.

TABLE 4. Visual discrimination learning

Training and test choice in Y-maze	No. of cases	
	$\Lambda > 0$	$\Lambda < 0$
A. 450 nm versus 610 nm	19	1
B. 350 nm versus 470 nm	9	1
C. Left versus right (control)	9	11
D. Bright versus dim light (control)	11	9

Flies were trained in a Y-maze, with quinine sulfate as reinforcement in one of the arms and the stimulus pairs listed. The flies discriminated successfully between both light wavelength pairs (experiments A and B). In experiments C and D, white light was used in both arms. Intensities were equal in C, 10:3 in D.

This work may be useful in the analysis of *Drosophila*'s sensory systems, since discriminative learning proves that the stimuli in question can be distinguished by the fly. The visual learning paradigm provides an example. Anatomical, physiological, and indirect behavioral experiments have shown that *Drosophila* has two visual receptor systems, with maximum sensitivities at different wavelengths (14). The present experiments suggest that the fly may use the color information it is equipped to detect.

The demonstration of conditioned behavior in *Drosophila* and the development of procedures in which flies can be trained and tested in populations may permit the isolation of mutants with altered abilities to learn, consolidate, or remember. This would permit the genetic techniques available in *Drosophila* to be applied to these problems.

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