Learned suppression of photopositive tendencies in *Drosophila melanogaster*

ÉRIC LE BOURG and CHRISTIAN BUECHER
Université Paul-Sabatier, Toulouse, France

A task was designed to teach individual flies to avoid a lighted area after they had displayed an initial preference for it. The flies walked in a T-maze and chose between a lighted and a darkened alley leading, respectively, to a lighted and a darkened vial. Flies that were photopositive on a first trial were subjected to an aversive stimulus (a filter paper inserted into the lighted vial and wetted with a quinine solution), and they performed 16 training trials; they learned to avoid the lighted vial. The flies trained with water instead of quinine in the lighted vial still displayed avoidance of the lighted vial, but to a lesser extent. The flies trained with a dry filter paper in the lighted vial did not show any increase in avoidance during training. Like the flies trained with no quinine at all, those trained to avoid the lighted vial under a partial reinforcement condition (one half of the trials with quinine, the other half with a dry vial) did not master the task. Finally, removal of the quinine after an avoidance acquisition criterion was reached resulted in an extinction process.

Due to numerous advantages, *Drosophila melanogaster* has been used for many years in studies on learning and memory. Among other advantages, this fly offers the possibility of applying genetic and neurological tools to generate learning- and memory-deficient mutants (see, e.g., Tully & Gold, 1993), as well as flies with morphological brain alterations (see, e.g., Heisenberg, Borst, Wagner, & Byers, 1985). The genetic and neural substrates of learning and memory can thereby be identified. Other advantages, such as short generation time and life span, are of interest in studies of the effects of aging on learning and memory (see, e.g., Brigui, Le Bourg, & Médioni, 1990; Savvateeva et al., 2000). The purpose of this article is to describe a new learning task that can easily be used in individuals of various ages, and with minimal equipment.

Some learning procedures for *D. melanogaster* have been described in the last decades. Unfortunately, some of them are questionable with respect to the occurrence of learning. For instance, Hay (1975) reported that flies learn to make a right or left choice in a maze to reach light. However, the right-left choice was determined from the experimenter's viewpoint, irrespective of the position of the fly. Mariath (1985) claimed that a tethered fly put on a platform could learn to move right or left when a heat stimulus was presented on the left or the right side of the

fly, respectively. However, learning as well as reversal learning were immediate, and the learning-memory mutants *dunce*, *rutabaga*, and *amnesiac* learned as well as the wild-type Canton-S strain did. Therefore, the flies in this setup may have simply escaped heat by moving the platform, without involvement of any operant learning.

Some learning tasks provide only group data (see, e.g., Quinn, Harris, & Benzer, 1974; Tully & Quinn, 1985) because the flies are tested en masse. Whether it is preferable to study learning at the individual or at the group level has been a matter of debate (e.g., Hirsch & Holliday, 1988; Tully, 1984), and each of the flies tested en masse must have actually been subjected to the reinforcer (Wallace & Sperlich, 1988). The use of individual data would ensure that all subjects have been subjected to the same conditions, particularly in terms of contact with the reinforcer. Moreover, individual data would allow different phenotypes to be measured in the same animals, whereas group data do not. For instance, the search for a possible relationship between learning ability and longevity implies that individual scores must be obtained. For these reasons, a learning task providing individual data is essential, even though "measuring differences among individuals . . . for behavioral phenotypes is time-consuming. . . . [L]earning and memory, in particular, require excessive time to assay," as was pointed out by Tully et al. (1990, p. 204).

Wolf and Heisenberg (1991) used a flight simulator to condition individual tethered flies. However, this procedure requires a lot of equipment that is not necessarily available to other laboratories. Wustmann and Heisenberg (1997) also designed a learning task for individual nontethered flies. This procedure requires that flies be willing to move for memory testing, which can be a problem in older flies. Memory of conditioned courtship could

This article is dedicated to Jean Médioni, who died in August 2001. Many thanks are due Martin Giurfa for his helpful comments on previous versions of the article. This work was supported by Santé-Société Grant 98N72/0048 from the French Centre National de la Recherche Scientifique (CNRS). Correspondence concerning this article should be addressed to E. Le Bourg, Laboratoire d'Éthologie et Cognition Animale, FRE CNRS 2382, Université Paul-Sabatier, 118 route de Narbonne, 31062 Toulouse cedex 4, France (e-mail: lebourg@cict.fr).

be tested in 1- to 4-week-old males (Savvateeva et al., 2000) but, obviously, conditioned courtship cannot be used in females.

Learned inhibition of the proboscis-extension response (PER) has also been studied (see, e.g., Médioni & Vaysse, 1975). This procedure imposes at least one day of starvation of individual tethered flies. Starvation is detrimental, particularly to old flies, and Minois and Le Bourg (1997) argued that the contrasted effects of age on habituation (no effect: Fresquet, 1997; Le Bourg, 1983; slower habituation with age: Fois, Médioni, & Le Bourg, 1991; Minois & Le Bourg, 1997) were linked to resistance to starvation.

The PER learning procedure designed in our lab (Médioni & Vaysse, 1975) has a few drawbacks (preliminary fasting, selection of starvation-resistant flies, lengthy procedure: up to 1 h per fly). It is therefore useful to design a new learning task in *D. melanogaster* that would allow testing of individual untethered flies of both sexes and various ages. This article describes a rapid-learning task (about 15 min per fly) based on avoidance of a lighted area, which does not require preparation of flies before the experiments.

Le Bourg and Badia (1995) have reported that phototaxis slightly decreases with age. In their design, flies had to choose between a lighted vial and a darkened vial set at the exit of the two arms of a T-maze. If the lighted vial was wetted with a bitter quinine hydrochloride solution, the flies that had reached it had a greater, although not significant, tendency to go toward the darkened vial on a second trial than did the flies that had reached a lighted vial wetted with only water. The authors thus hypothesized that flies, if subjected to more than two trials, could learn to avoid the lighted area. The present article explores this idea using a procedure derived from that of Le Bourg and Badia. We report that individual flies can learn to avoid a vial associated with an aversive stimulus. Furthermore, the procedure allows the study of extinction when the aversive stimulus is removed.

All experiments reported below were carried out at the individual level, which means that each fly received a learning score and that the flies were not tested en masse. Moreover, all experiments involved both sexes, unlike some previously cited studies that used only males (e.g., most studies on PER). For each fly, all learning trials were run on the very same day, with no delay between trials. In other words, these experiments dealt with short-term memory (STM).

GENERAL METHOD

Flies

The experimental flies were adult males and females of the wild strain Meyzieu. This strain had been maintained since 1976 by mass mating on the standard medium (agar, sugar, corn meal, and dead yeast) enriched with live yeast. The experimental flies were obtained as follows: Eggs laid during a 15-h period (50 five-day-old pairs) were transferred in batches of 25 to 80-ml glass vials containing the medium described above. Upon emergence, flies with 9 days of preimaginal development were transferred under ether

anesthesia, in groups of 15 same-sex flies, to 20-ml polystyrene vials (Model 5111, Polylabo, France) containing the standard medium with a drop of live yeast. Vials were replaced twice a week. The flies spent their lives in an incubator (Maxi-Artic model, Jouan, France); the rearing temperature was $25^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$; the light (fluorescent lamp) was on from 08:00 to 20:00.

Learning Procedure

In the learning task, individual flies made successive choices between a lighted and a darkened alley in a T-maze to avoid a punishment. The flies choosing the lighted alley reached a lighted vial, where they could be subjected to an aversive stimulus.

Apparatus

The apparatus consisted of two 3-ml transparent polystyrene vials (Model 5088, Polylabo, France) set vertically at the exit of the arms of a T-maze (Figure 1). Light was provided within the lighted vial by one vertical optical fiber (150-W halogen lamp). The intensity of the light, as measured on the table below the maze, was around 5,800 lx. The inside of the lighted vial was covered with white filter paper, except at its opening (1 cm left free of paper) and at the opposite end, and a solution could be deposited on the filter paper. The darkened vial, free of filter paper, was inserted into another, opaque gray vial that prevented any light source from entering. A 1-ml syringe containing a single fly was connected to the entrance of the maze.

The maze was drilled into a block of opaque gray plastic (PVC). The width of the alley was 2 mm and the height 1.4 mm, which prevented the flies from walking on the walls. The ceiling was made of transparent Plexiglas, and its bottom side was partially covered with a red filter (Kodak Wratten 1497072 gelatin filter). Flies are nearly blind to red light (Médioni, 1955; Schümperli, 1973) and could not perceive the light transmitted through the ceiling, but the experimenter could observe each fly while it crossed the maze. At the exits of the T-maze, two opaque gray PVC disks were inserted into the ceiling. The darkened alley was covered by an opaque disk up to the choice point, in order to avoid any light in the arm leading to the darkened vial. The light of the optical fiber was transmitted through the lighted vial to the maze and could be perceived by the fly crossing it. When the fly reached the choice point, it could choose between the lighted and the darkened alley leading to the lighted and the darkened vial, respectively. Two ceilings were made, the first one with placement of the lighted vial on the right exit of the maze, and the second, symmetrical one with the lighted vial on the left exit.

Experimental Procedure

The temperature of the experimental room was $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$; this room was lit only with the optical fiber above the maze (Figure 1). For each fly, the experiment was as follows. The fly was transferred to the syringe without anesthesia and was gently pushed to the start of the maze, where it made a choice between the two alleys. If the fly returned to the start of the maze before reaching one of the vials, it was allowed to resume the trial. The fly could walk either on the floor or on the ceiling of the maze, but most of the observed flies walked on the floor. The flies that entered the lighted vial were considered photopositive, and those that entered the darkened vial were considered photonegative; the trial ended when the fly reached a vial. If the fly reached the darkened vial on the first trial, it was discarded. In other words, only flies showing a photopositive choice on the first trial were used, and were trained to suppress this tendency. The filter paper in the lighted vial was wetted before the experiment with 130 μl of either distilled water or 10^{-1} M quinine hydrochloride solution (Fluka 22630, Switzerland), or it was dry, depending on the experiment. When the fly reached a vial, the vial was separated from the maze, and the fly was transferred to the syringe without anesthesia. This transfer procedure required a strong mechanical stimulation, which is known to induce an increase in positive phototaxis (Médioni, 1961). Therefore, any learning that

Figure 1. Schematic diagram of the apparatus used in the learning task, showing both top and front views (see explanations in the Apparatus subsection of the General Method section). The figure shows the ceiling of the maze with the lighted vial set on the right exit. Another ceiling, symmetrical to that shown in the figure, was built with the lighted vial set on the left exit.

occurred not only had to suppress spontaneous positive phototaxis, but also had to inhibit induced phototaxis. The fly could walk on the wet or dry filter paper during the interval between the time when the vial was reached and the transfer to the syringe (usually for 10–15 sec), which means that the intertrial interval was about 30 sec. After transfer to the syringe, a new trial was run.

Unless otherwise noted (Experiment 3), 16 training trials were run for each fly. For one half of the trials, the lighted vial was on the right side of the maze (R), and a double alternation procedure was used throughout the experiment (RLLRLLRLLRLL and LLRLLRLLRLLRLL). This procedure ensured that flies made

a choice between the alleys mainly on the basis of their light contrast, and not because they followed their own odorous traces on successive trials or had a left- or right-turning bias. Two mazes were used: one with the ceiling designed to place the lighted vial on the right side, and the other for placement of the lighted vial on the left side. After each fly was tested, mazes and ceilings were cleaned with distilled water and dried; the darkened and lighted vials were discarded, and new ones were used for the next fly.

Experiments involved both sexes and were performed from about 9:00 a.m. to 5:00 p.m. The details of each experiment will be described below.

Statistical Data

The percentage of flies reaching the lighted vial on the first trial was computed. As in a previous study (Le Bourg & Badia, 1995), this percentage indicates the initial phototaxis tendency.

In flies that completed the 16 training trials, the following variables were computed: (1) number of photonegative choices (i.e., choice of the darkened vial) in four blocks of four trials; (2) time interval between the first and the last trials; (3) number of trials during which flies returned to the start of the maze. This number may thus vary between 0 (no return observed) and 16 (at least one return at each trial).

A variation in the number of photonegative choices during the four blocks, hereafter called the learning score, indicates the tendency toward increased avoidance of the lighted vial. It can be hypothesized that the time to complete training will vary depending on whether the lighted vial is associated with an aversive stimulus or not. Similarly, the number of returns to the start of the maze could vary according to the presence or absence of the aversive stimulus in the lighted vial. Given that the present results did not point to such an effect, this number of returns has not been analyzed in this article, except in the extinction phase of Experiment 3 and in Experiment 4.

Dichotomized data were analyzed with a logistic model (DataDesk 6.1, Data Description Inc., www.datadesk.com). A logistic model is a generalized linear model fit to binary data. Like the classic analysis of variance (ANOVA), a logistic model is used to compute main effects of various factors (e.g., sex), as well as interactions between them. However, chi-square tests were used when a logistic model could not be used because there was an empty cell, which implies that interactions between factors could not be computed.

EXPERIMENT 1

Le Bourg and Badia (1995) reported that most of their flies choosing between a lighted and a darkened vial set at the exit of the two arms of a T-maze entered the lighted vial. If the lighted vial was wetted with a bitter quinine hydrochloride solution, flies that reached it had a larger (but nonsignificant) tendency to go toward the darkened vial on a second trial than did flies that reached a lighted vial humidified with water. The authors thus hypothesized that flies could learn to avoid the lighted area if they were subjected to more trials. However, for technical reasons, it was not easy to use their maze in a study involving several trials. Therefore, it was necessary to design a new maze, which is used in the present study. The present experiment tested whether flies can learn to avoid a lighted vial associated with an aversive stimulus if more than two trials are run.

Method

One half of the flies were subjected to training with quinine solution in the lighted vial as an aversive stimulus (quinine group). The other half were trained with distilled water in the lighted vial (water group). It can be expected that flies trained with quinine will progressively display a learned preference for the darkened vial, not observed in those trained with water. Ten 5- to 7-day-old flies of each sex completed training for each reinforcement condition.

Results and Discussion

The phototaxis tendency in the first trial was analyzed with a logistic model for dichotomized data using flies that had completed the first trial ($n = 67$, i.e., 20 flies photonegative on the first trial, 7 photopositive flies lost

before completion of training, and 40 photopositive ones that completed training). Sex, reinforcement, and their interaction had no significant effect on phototaxis (F s close to 1, data not shown), and 70.15% of the flies reached the lighted vial on the first trial.

A three-way ANOVA (two independent factors, sex and reinforcement; one dependent factor, blocks of trials) showed that the flies trained with quinine made a larger number of photonegative choices than did those trained with water [Figure 2; $F(1,36) = 8.52$, $p = .006$]. The number of photonegative choices increased with the order of blocks [$F(3,108) = 9.60$, $p < .0001$], and the nearly significant interaction between blocks and reinforcement showed that during the last block the quinine group tended to display more photonegative choices than the water group did [$F(3,108) = 2.57$, $p = .0577$]. The sex effect and other interactions were not significant.

A two-way ANOVA showed that the effects of sex and reinforcement, as well as their interaction, on the time to complete the 16 trials were not significant (log transformation applied to the times, data not shown; mean $\pm$ SEM: 13.48 ± 0.56 min).

These results show that the flies trained with quinine learned to prefer the darkened vial with training. In other words, it seems that the flies learned to avoid the lighted vial because it was associated with an aversive stimulus. However, the flies trained with water also displayed an increasing number of photonegative choices with training (if the ANOVA is restricted to flies trained with water, the block effect is still significant; data not shown). Therefore, it could be that a portion of the results of flies trained with quinine was due to a tendency to avoid light, even in the absence of an aversive stimulus, and despite the mechanical stimulations used after each trial that should promote positive phototaxis. Alternatively, it could be that water itself is an aversive stimulus, at least in the context of this experiment. However, water is probably a weaker reinforcer than quinine, because lower learning scores are observed with water than with quinine (Figure 2).

The next experiment was conducted to test whether water may be considered an aversive stimulus, and whether there is a tendency to avoid the lighted vial with training when it does not contain water.

EXPERIMENT 2

In Experiment 1, it was shown that water may be an aversive stimulus, an unexpected result. The purpose of the present experiment was twofold. In the first part (Experiment 2A), we tested the flies' spontaneous preference for staying on a filter paper soaked with quinine or water or on a dry paper. In the second part (Experiment 2B), we tested whether the flies learned to avoid the lighted vial when no water was present on the filter paper.

Method

Experiment 2A. To test for a spontaneous preference between quinine and water or between a wet and a dry environment, two

Figure 2. Experiment 1: number of photonegative choices (learning score $\pm$ SEM) as a function of blocks of four trials. For each block, the learning score may vary between 0 and 4. For instance, flies of the quinine group increased their tendency to go to the darkened vial with successive blocks (less than once in four trials in the first block and more than twice in the last block). One half of the flies were trained with quinine solution as an aversive stimulus in the lighted vial (quinine group), and the other half were trained with distilled water (water group). Because the sex effect was not significant, the sexes were pooled in the figure. Ten males and 10 females were used in each reinforcement condition.

3-ml vials were internally covered with a filter paper up to the opening, a 1-mm space being left free of paper. In a first experiment, one of the vials was wetted with water and the second with a 10^{-1} M quinine hydrochloride solution (160 μ l in both cases). Ten 1-week-old flies of each sex were placed individually into one of the vials, which was then connected to the other vial (new vials were used for each fly), and time spent in each vial during a 5-min period was immediately recorded. The flies spent 142.65 ± 8.53 sec in the quinine vial [t test of the difference with the theoretical mean of 150 sec: $t(19) = 0.86$, n.s., no sex effect; data not shown]. In a second experiment, ten 1-week-old flies of each sex had to choose between a dry and a wet vial: The flies spent 231.95 ± 8.49 sec in the dry vial [$t(19) = 9.65$, $p < .0001$, no sex effect; data not shown]. These experiments show that the flies prefer to stay on a dry rather than on a wet paper and that there is no obvious preference between quinine and water. These results confirm that walking on wet paper is unpleasant to flies. However, Experiment 1 has shown that a difference between the quinine group and the water group appears during training. It may thus be concluded that water is a less aversive stimulus than quinine.

Experiment 2B. Because flies do not like to stay on wet paper, the increased tendency with trials to go to the darkened vial when the lighted vial contains water (Experiment 1) could be due to an avoidance of that vial, and not to a decrease in positive phototaxis. Experiment 2B thus tested whether flies would avoid the lighted vial when no water is present on the filter paper—that is, whether positive phototaxis decreases with trials. Therefore, flies entering the lighted vial could walk on a filter paper that was either dry (dry group) or wetted with quinine (quinine group).

To test for a possible effect of the reinforcement schedule, a partial reinforcement condition was used for one half of the flies trained with quinine (partial group). These flies walked on a filter paper wetted with quinine in 50% of the trials. The latter condition used a double alternation of the reinforced (quinine: Q) and non-reinforced (dry: D) trials combined with the double alternation of the position of the lighted vial (on the right [R] or left [L] side of the maze). The order of trials in each block of four was RQ RD LD LQ for about one half of these flies, and LQ LD RD RQ for the other half. For the other half of the flies trained with quinine, the lighted vial was wetted with the quinine solution on each trial (quinine group).

Ten 7- to 9-day-old flies of each sex completed training in each group (quinine, partial, dry).

Results and Discussion

Because the logistic model cannot be used if there is an empty cell, it was necessary to pool the three reinforcement groups (no male in the dry group reached the darkened vial). The phototaxis tendency on the first trial was thus analyzed with a chi-square test using the 78 flies that had completed the first trial. Sex had no significant effect on phototaxis ($p > .20$), and 80.77% of the flies reached the lighted vial on the first trial.

A three-way ANOVA (two independent factors, sex and reinforcement; one dependent factor, blocks of trials) showed that the flies of the quinine group made more

photonegative choices than did those of the partial and dry groups [Figure 3; $F(2,54) = 9.47, p = .0003$]. The number of photonegative choices increased with the order of blocks [$F(3,162) = 7.45, p < .0001$], and the interaction between blocks and reinforcement showed that the quinine group displayed more photonegative choices during the last two blocks [$F(3,162) = 7.07, p < .0001$]. The sex effect and other interactions were not significant.

Males in the three groups and flies of both sexes in the quinine group were slightly slower to complete training [two-way ANOVA, sex effect: $F(1,54) = 3.94, p = .0003$; reinforcement effect: $F(1,54) = 5.70, p = .0205$, no significant interaction]. However, although significant, the effects were of little importance because the highest mean difference between groups did not exceed 1 min (grand mean $\pm$ SEM: 8.32 ± 0.10 min).

These results show that the flies trained with quinine on each trial chose the darkened vial more often than both the flies trained with quinine on half of the trials and those trained with no quinine at all did. Contrary to what happened when the lighted vial was associated with water (Experiment 1), there was no increase in the number of photonegative choices with training in the dry condition. This result shows that the tendency to avoid

light with trials did not increase, provided that the lighted vial was dry. The results also show that, contrary to what happened with the constant reinforcement condition (quinine group), a partial reinforcement with a double alternation of reinforced and nonreinforced trials (partial group) did not result in learning. A previous study on the conditioned inhibition of the PER (Brigui et al., 1990) also showed that the flies faced with partial reinforcement in a double alternation of reinforced and nonreinforced trials had some difficulties in conditioning. However, in the study of Brigui et al., the flies subjected to partial reinforcement with a regular alternation of one reinforced and one nonreinforced trial had scores similar to those trained under the constant reinforcement condition.

The results of Experiment 1, which showed that flies have an increased tendency with trials to prefer the darkened vial if the lighted vial is wet, are due to the fact that water is an aversive stimulus, as was confirmed by Experiment 2A. The results of Experiment 2B show that there is no tendency to avoid the lighted vial with training if this vial is dry. Therefore, it can be concluded that the strong photopositive tendency of flies can be suppressed only if the lighted vial is always associated with

Figure 3. Experiment 2: number of photonegative choices (learning score $\pm$ SEM) as a function of blocks of four trials. One third of the flies were trained with quinine solution as an aversive stimulus in the lighted vial on each trial (quinine group), a second third were trained with quinine on two out of four trials (partial reinforcement group), and the last third were trained with a dry, lighted vial (dry group). For the last group, the inside of the lighted vial was covered with filter paper. Because the sex effect was not significant, the sexes were pooled. Ten males and 10 females were used in each reinforcement condition.

an aversive stimulus. Indeed, the photopositive tendency is so strong that even an association of the lighted vial with quinine in two out of four trials (partial reinforcement) is insufficient to result in an avoidance of the lighted vial.

Because the present experiment has shown that flies can learn to inhibit their positive phototaxis, there is now a need to test whether they also show an extinction of learning when the aversive stimulus is removed.

EXPERIMENT 3

Previous experiments have shown that flies can learn to avoid a lighted vial associated with an aversive stimulus. Removal of the reinforcement in the lighted vial is therefore expected to decrease the avoidance score, particularly because the mechanical stimulation used before each trial to transfer the fly into the syringe (see the General Method section) is known to increase positive phototaxis (Médioni, 1961).

Extinction can be studied only in flies with similar levels of acquisition, as Brigui et al. (1990) did, studying the extinction of the conditioned inhibition of the PER. In our procedure, we used a criterion of acquisition of three photonegative choices on four successive trials. If the flies chose randomly on each trial between the lighted and the darkened vials (i.e., with a 50% probability), the probability of this criterion's being observed at any time during training should be .5625 (binomial test). However, all of the flies involved in the training were photopositive in the first trial. If we used as the random probability that observed in the first trial at the group level (about 80% of the flies were photopositive on this trial; see the previously reported results), the probability that the criterion will be observed at any time during training would be .0336.

Method

The flies were subjected to the training procedure, the lighted vial being associated with quinine on each trial. The flies were trained up to the criterion of acquisition, or to the 16th training trial if the criterion was not reached. One third of the flies that reached criterion were then immediately subjected to 16 extinction trials, while the filter paper covering the inside of the lighted vial was dry (extinction group). Another third were subjected to 16 more training trials (quinine group). The last third were subjected to 16 trials, in which the inside of the lighted vial was covered with a filter paper humidified with 130 μ l of distilled water (water group). Ten flies (5–9 days old) of each sex completed the experiment in each group. The extinction group was expected to display a low avoidance of the lighted vial, the quinine group to display a strong avoidance, and the water group to have intermediate scores.

Results and Discussion

The phototaxis tendency on the first learning trial was analyzed with a chi-square test using the 117 flies that had completed the first trial. Sex had no significant effect on phototaxis ($p > .20$), and 67.52% of the flies reached the lighted vial on the first trial.

A three-way ANOVA was used to analyze the 16 trials after the acquisition criterion was reached (two independent factors, sex and procedure: extinction, quinine, water; one dependent factor, blocks of trials). As was expected, flies of the extinction group avoided the lighted vial less than did those of the quinine and water groups, and no clear difference was observed between the latter two groups [Figure 4; $F(2,54) = 96.37, p < .0001$]. The sex effect was not significant ($F < 1$). The number of photonegative choices increased slightly with order of blocks [$F(3,162) = 7.84, p < .0001$], even in the extinction group; this point will be discussed below. All interactions were not significant (F 's close to 1).

The effects of procedure and sex on time to complete the 16 trials were analyzed with a two-way ANOVA, a log transformation being applied to times. The females (mean $\pm$ SEM: 12.90 $\pm$ 0.72 min) were slower to complete the 16 trials than were the males [mean $\pm$ SEM: 10.20 $\pm$ 0.36 min, $F(1,54) = 14.12, p = .0004$]. The flies of the extinction group were faster (mean: 9.90 $\pm$ 0.45 min) than were those of the quinine and water groups [means: 12.50 $\pm$ 0.96 min and 12.25 $\pm$ 0.66 min, respectively; $F(2,54) = 5.89, p = .0048$]. This result is in accordance with that of Experiment 2B, which also showed that the flies trained with quinine were slower to complete the 16 learning trials than were those trained with a dry vial. The sex $\times$ procedure interaction was not significant.

A two-way ANOVA (two independent factors, procedure and sex; a square-root transformation was applied to the returns) showed that the number of returns to the start of the maze was higher for the females than for the males [$F(1,54) = 8.09, p = .0063$, mean of females $\pm$ SEM: 3.37 $\pm$ 0.44 returns; mean of males: 1.70 $\pm$ 0.26 returns]. The flies of the extinction group made fewer returns than did those of the quinine and water groups [$F(2,54) = 8.96, p = .0004$, respectively; mean of extinction, quinine, and water groups: 1.25 $\pm$ 0.30 returns, 2.95 $\pm$ 0.59 returns, and 3.40 $\pm$ 0.36 returns, respectively). The interaction between sex and procedure was not significant. Not unexpectedly, the number of returns was positively correlated with the time to complete the 16 trials (computations using the square root of returns and the log of times, $r = .58, n = 60, p < .0001$). Positive correlation coefficients were observed in each sex and procedure group, but not all of them were significant.

Finally, we tested whether the first nonavoidance (i.e., the first trial on which flies reached the lighted vial) varied with the procedure. It was impossible to compute an ANOVA, because of the very high number of ties—that is, of flies reaching the lighted vial on the first trial. The flies were thus dichotomized into two groups, those that reached the lighted vial in the first trial, and those that reached it in any other trial (all of the flies showed one nonavoidance or more). This variable was analyzed with a logistic model using the 60 flies that had completed the experiment. Flies of the extinction group (85% reached

Figure 4. Experiment 3: number of photonegative choices (extinction/learning score $\pm$ SEM) as a function of blocks of 4 trials during the 16 trials after an acquisition criterion is reached. The flies were trained with quinine as an aversive stimulus in the lighted vial, and those that failed to reach an acquisition criterion of three photonegative choices on 4 successive trials in 16 training trials were discarded. One third of the flies that reached criterion (10 males and 10 females) were subjected to 16 training trials (lighted vial with quinine: quinine group), whereas another third were subjected to 16 trials with a lighted vial wetted with distilled water (water group). The final third were subjected to 16 extinction trials, the inside of the lighted vial being covered with a dry filter paper (extinction group). Because the sex effect was not significant, the sexes were pooled.

the lighted vial in the first trial) were faster than those of the quinine (35%) or water (60%) groups to show non-avoidance [$F(2,54) = 4.07, p = .0226$]. A Scheffé post hoc test showed that this effect was due to the difference between the extinction and the quinine groups, the difference between the extinction and the water groups and that between the quinine and the water groups being non-significant. Sex and its interaction with the procedure had no significant effect (F s close to 1).

The effect of the procedure suggests that the flies chose between the lighted and the darkened alleys by relying on more than the light differences between them. The flies of the quinine and water groups could also use humidity differences between the lighted and the darkened vials because, for these groups, the lighted vial was wet. Thus, it could be that there was more relative humidity in the alley leading to the lighted vial than in the darkened alley. It can be hypothesized that the flies of the quinine and water groups detected that humidity difference and tended to avoid the lighted vial. By contrast, the flies of the extinction group did not detect humidity, in that both the darkened and the lighted vials were dry,

and chose the lighted vial early. In other words, if humidity differences are used as a conditioned stimulus by flies during the training phase, the absence of humidity in the lighted vial can help to disrupt the learned association during the extinction phase. However, within each group (extinction, quinine, water), reaching the lighted vial on the first trial is not predictive of a low avoidance score, because the mean avoidance scores are similar for flies that had reached the lighted vial on the first trial and for those that had reached it on a later trial (extinction group: 4.06 and 4.00 avoidances, respectively; quinine group: 10.14 and 11.46 avoidances, respectively; water group: 9.83 and 10.50 avoidances, respectively). Finally, it is worth noting that the partial reinforcement condition of Experiment 2B (partial group), in which the flies were subjected to sequences of two trials with a dry lighted vial and two trials with quinine, did not result in a greater avoidance of quinine vials than of dry ones (data not shown). In other words, the flies here were unable to detect a difference between quinine and dry alleys by relying on humidity differences between them. It could be that completing acquisition is a prerequisite for using

humidity differences as a cue. The following experiment tested whether flies use humidity differences as a conditioned stimulus to learn to avoid the lighted vial associated with an aversive stimulus.

Completing acquisition has some consequences for the probability of choosing the darkened vial during the extinction phase. The flies subjected to extinction had a significantly greater tendency with successive trials to choose the darkened vial (extinction group in Figure 4). In contrast, the flies trained with a dry lighted vial (dry group in Figure 3) did not show such a tendency. Replicate studies of acquisition and extinction that will be reported elsewhere confirmed these results. It seems, thus, that flies that have learned to avoid the lighted vial have not forgotten this previous experience when subjected to extinction. These flies still have a tendency to avoid the lighted vial, after a huge decrease of said tendency in the first trials. In other words, flies subjected to extinction cannot be considered as naive flies that have never been confronted with the aversive stimulus, and the persistence of low avoidance of the lighted vial is probably linked to the previous association between this vial and the aversive stimulus. When long-term memory (LTM) is studied in the future, it would be useful to know the time needed to abolish the trace of a previous acquisition.

The main result of Experiment 3 is that the flies of the quinine group showed a high avoidance of the lighted vial after reaching an acquisition criterion of three avoidances on four trials, whereas the flies subjected to extinction showed a fast recovery of photopositive choices. The flies of the water group did not show an extinction process but, on the contrary, had an avoidance score very slightly lower than that of the flies of the quinine group. The main conclusion of Experiment 3 is that our learning procedure allows observation of an extinction process after an acquisition criterion has been reached.

The last experiment of this study focuses on the use of humidity differences as a conditioned stimulus, considering that the present experiment has shown that they can be used by flies, at least at the beginning of the extinction phase.

EXPERIMENT 4

Method

In this experiment, we tested whether flies would use humidity cues in the maze to avoid the lighted vial associated with an aversive stimulus. Three groups of flies were used. Flies entering the lighted vial could walk on a filter paper that was either dry (dry group) or humidified with quinine (quinine group). The flies of the dry-quinine group, which entered the lighted dry vial, were immediately transferred to a vial containing a filter paper soaked with quinine. Therefore, the flies of this group could not use humidity differences between the lighted and the darkened vials, but were subjected to a delayed aversive stimulus after they had reached the lighted vial. Given that there was a short delay in the fly's transfer from the dry vial to the quinine vial, the time to complete training was not analyzed.

For each of the three groups, ten 5- to 9-day-old flies of each sex completed the training.

Results and Discussion

Because the logistic model cannot be used if there is an empty cell, it was necessary to pool the three reinforcement groups (no female used in the dry-quinine condition reached the darkened vial). The phototaxis tendency on the first trial was thus analyzed with a chi-square test using the 71 flies that had completed the first trial. Sex had no significant effect on phototaxis ($p > .25$), and 85.92% of the flies reached the lighted vial on the first trial.

A three-way ANOVA (two independent factors: sex and reinforcement; one dependent factor: blocks of trials) showed that the flies of the quinine group made more photonegative choices than did those of the two other groups, which displayed similar scores [Figure 5, $F(2,54) = 23.05$, $p < .0001$]. The number of photonegative choices increased with the order of blocks [$F(3,162) = 11.20$, $p < .0001$]. Neither the sex effect nor any of the interactions was significant. There was a tendency for the flies of the dry group to display more photonegative choices in successive blocks, but this effect was due to the very low score on the first block (compare the flies trained with a dry lighted vial in Figures 3 [dry group] and 5 [dry group]).

A two-way ANOVA (two independent factors: sex and reinforcement; a square-root transformation was applied to the returns) showed that the number of returns to the start of the maze was larger in females than in males [$F(1,54) = 5.33$, $p = .0247$; mean of females $\pm$ SEM: 2.43 ± 0.51 returns; males: 1.13 ± 0.29 returns]. The flies of the quinine group made more returns than did those of the dry and dry-quinine groups, which did not differ from each other [$F(2,54) = 19.79$, $p < .0001$; means of quinine, dry, and dry-quinine groups: 3.60 ± 0.51 returns, 1.40 ± 0.53 returns, 0.35 ± 0.13 returns, respectively]. The interaction between sex and the reinforcement procedure was not significant.

The results of this experiment indicate that the flies probably used humidity cues as a conditioned stimulus. Flies probably learn that humidity is a predictor of the aversive stimulus, and learning is impaired if humidity is absent. However, the procedure we used in this experiment is far from perfect, because the flies of the dry-quinine group did not walk on quinine when they reached the vial, which did not help them make an association between the aversive stimulus and the choice of the lighted vial. Attempts were made not to use a dry vial, but rather to place a vial wetted with quinine at the exit of the maze when the fly reached it. However, in many cases, flies escaped before the experimenter put the vial in place and, thus, this procedure could not be used.

GENERAL DISCUSSION

Our experiments have shown that flies learn to inhibit their strong positive phototaxis if the choice of the lighted vial is associated with an aversive stimulus, and they are again photopositive once the aversive stimulus

Figure 5. Experiment 4: number of photonegative choices (learning score $\pm$ SEM) as a function of blocks of four trials. The lighted vial of the quinine group contained a filter paper soaked with quinine. The lighted vial of the dry group contained a dry filter paper. The lighted vial of the dry-quinine group contained a dry filter paper, but flies that had reached the lighted vial were immediately transferred to a vial containing a filter paper soaked with quinine. Therefore, this group could not use humidity differences between the lighted and the darkened vials when crossing the maze, but were subjected to an aversive stimulus if they reached the lighted vial. Because the sex effect was not significant, the sexes were pooled. Ten males and 10 females were used in each reinforcement condition.

is removed. Learning occurred with either quinine or water as the aversive stimulus (Experiment 1). Experiment 2A showed that flies prefer to stay on a dry paper rather than on a wet one, and Experiment 2B showed that no learning occurred if the lighted vial was dry. The decreased tendency to choose the lighted vial is thus not due to decreased positive phototaxis with trials, and Experiment 2B showed that, likewise, no learning occurred under a partial reinforcement condition. In other words, positive phototaxis is a robust behavior that can be disturbed only if it is associated with a punishment. Experiment 3 showed that extinction is easy, and Experiment 4, that flies use humidity cues as a conditioned stimulus.

This study showed no sex-related difference with regard to learning or extinction abilities, despite some differences in the time needed to complete the experiment and the number of returns to the start of the maze. Both sexes can thus be studied, and a forthcoming article will show that older flies can also be tested. Given that training involved 16 trials in a few minutes, this procedure could also be used to test whether STM is intact, for instance, in mutants.

The purpose of this project was to design a learning task that can be performed simply and rapidly and that lacks the drawbacks of the PER conditioning procedure used in our lab for many years (see the introduction). The learning task described here seems to fulfill these requirements. The procedure does not require any preparation of flies prior to the experiment, as is the case with PER (Brigui et al., 1990) and conditioned visual flight orientation procedures (Wolf & Heisenberg, 1991). Flies can thus be transferred directly from their rearing vials to the maze, and about 15 min are needed to complete 16 training trials.

Some points concerning our procedure have to be emphasized. First, avoiding the lighted vial is not linked to activity level, because flies have to walk for the same length in the maze. In other words, there is no confusion between a fly's possibly resting and its avoidance score, because the fly has to choose actively between two alleys, and not passively to avoid the lighted alley. Second, the internal tiny dimensions of the maze (similar to those of another maze used to study alternation behavior; see Le Bourg, 1987) inhibit the fly's possible tendency to

follow one of the walls throughout the maze, because the fly is in contact with both sides. Under such conditions, one-side thigmotaxis and centrifugal swing do not bias the choice between the lighted and darkened alleys (Grosslight & Harrison, 1961). Third, observing individual flies ensures that they had been in contact with the aversive stimulus each time they chose the lighted vial reinforced with quinine. Lastly, it can be concluded that flies actually associated the aversive stimulus and the choice for the lighted alley, because flies trained with no quinine and no water in the lighted vial display low avoidance during training. In other words, flies did not show a decreased positive phototaxis with training or an increased randomization of their choices in the T-maze.

Our procedure, however, is far from perfect, and we list below possible criticisms and possible responses to them.

First, flies stopping within the maze can be a problem, because they can rest for a long time (see, e.g., Hendricks et al., 2000). To provoke flies to walk after they have stopped, the experimenter provided mechanical stimuli to the maze (e.g., tapping with the syringe on the maze or with a finger on its ceiling). However, if older flies were tested, one could face the risk of higher inactivity and lower responsiveness to these mechanical stimuli.

Second, the experiment is done only with flies that show positive phototaxis on the first trial—that is, about 80% of all tested flies. It could thus be asked whether changes of illumination could increase the proportion of flies that are discarded because they are photonegative on the first trial. A preliminary experiment involving several light intensities (120, 670, 3,850, and 5,800 lx, 50 males and 50 females per intensity) and a less elaborated maze ceiling (no gray disks; see Figure 1) showed that the percentage of photopositive choices on a single trial did not vary with light intensity. However, it was slightly higher in males (84.50%) than in females (74.50%). The percentage of photopositive flies on the first trial is therefore stable and does not vary with illumination.

Third, an increased avoidance of the lighted vial during training could nevertheless be due to a loss of positive phototaxis. However, this explanation can be discarded, because flies trained with a dry lighted vial did not show increased avoidance during training (Figure 3). Furthermore, the study of extinction has shown that, once the aversive stimulus is removed from the lighted vial, the avoidance of that vial is low again. It seems, thus, that the only cause for avoidance of the lighted vial is its association with an aversive stimulus. If a fly reaches the lighted vial wetted with quinine, its tendency to avoid that vial on the next trial will increase, or at least it will not decrease. In contrast, if that vial is dry, the learned association rapidly wanes, as is the case during extinction after training (Figure 4), or it is not strengthened, as is the case during partial reinforcement training (Figure 3). This difficulty in increasing avoidance under a partial reinforcement condition could be due to the mechanical stimulation used after each trial because, when

quinine is absent, the stimulatory effect on positive phototaxis (Médioni, 1961) is not counterbalanced by the inhibitory effect of association with quinine.

Lastly, the duration of contact with the aversive stimulus may vary from trial to trial. The experimenters regularly observed that flies climbed up the side of the lighted vial during the first training trials. However, if the filter paper in that vial was wetted with quinine, many flies rapidly avoided walking on it. In other words, at this time of training, flies were unable to avoid the lighted vial but had already learned to avoid the quinine in it. Obviously, this behavior reduced time spent in the presence of quinine. In order to ensure that any fly reaching the lighted vial had contact with quinine, the experimenters verified that, before transfer to the syringe, all of the flies that had reached the lighted vial had walked on quinine, at least for a few seconds.

On the whole, the learning procedure described here provides a fast and simple means to study learning and extinction at the individual level in flies, using an aversive stimulus. One of the advantages of an aversive stimulus is that, contrary to what happens with sucrose as a reinforcer of PER in fasting bees (see, e.g., Menzel, Hammer, Braun, Mauerlshagen, & Sugawa, 1991), its repetitive use is possible. In contrast, each reinforcement with sucrose decreases the probability of a new PER, because satiated bees do not show any PER. Our results are, thus, a new example of a learning procedure using aversive stimuli in flies. In other studies at the individual level, heat has been used successfully (Wolf & Heisenberg, 1991; Wustmann & Heisenberg, 1997), and, in our lab, electric shocks (Médioni & Vaysse, 1975) and quinine were used to inhibit PER in fasting flies (see, e.g., Brigui et al., 1990). However, PER conditioning is not the best learning procedure, because it cannot be used to study LTM, given that fasting can kill animals, especially old flies. Our procedure does not suffer from such a flaw, and preliminary studies show that studying LTM is possible and easy in young and old flies alike. A forthcoming article will report on acquisition, extinction, and LTM in flies of different ages.

A final point of interest in this discussion is the question of what has been learned by flies. It is difficult to know whether they avoided the lighted vial and went to the darkened one simply because there was no other choice, or whether they acquired a preference for the darkened vial. This is the reason why the number of returns to the start of the maze was recorded. If the first hypothesis were true, one could expect to observe a large number of returns to the start of the maze, because that is another way to try to avoid the lighted vial. Except in Experiments 3 and 4, the number of returns was not significantly higher in flies tested with the aversive stimulus, which could indicate that flies reaching the choice point learned to go to the darkened vial, rather than only to avoid the lighted one. However, returning to the start of the maze does not terminate the trial, given that the fly has to exit into one of the vials; flies could eventually

learn to reach the darkened vial even if they preferred to return to the start of the maze.

Although there is thus an operant component in the learning task, a classical component is also present because, before the first avoidance, conditioned stimuli (CS: light, and probably humidity) are a predictor of an unconditioned stimulus (US: aversive stimulus). However, this learning task is not an example of classical conditioning, because flies do not consistently encounter the association CS-US—they can perceive the CS and avoid the US. A classical conditioning procedure would imply blocking the darkened alley in order to force the fly into the lighted vial on each trial and, after some trials, to test the fly in a maze with both alleys open. Classical conditioning would be observed if experienced flies went to the darkened vial more than naive flies did. A yoked-control experiment would be useful in deciding whether the learning task is an instance of operant conditioning (on the difference between classical and operant components in a learning task, see Brembs & Heisenberg, 2000). However, it seems difficult to perform such an experiment using our procedure. Furthermore, a yoked control would probably not display any learning, simply because the partial reinforcement group (Figure 3) showed that, when the lighted vial was not consistently associated with quinine, no learning occurred.

REFERENCES

- BREMB, B., & HEISENBERG, M. (2000). The operant and the classical in conditioned orientation of *Drosophila melanogaster* at the flight simulator. *Learning & Memory*, **7**, 104-115.
- BRIGUI, N., LE BOURG, E., & MÉDIONI, J. (1990). Conditioned suppression of the proboscis-extension response in young, middle-aged and old *Drosophila melanogaster* flies: Acquisition and extinction. *Journal of Comparative Psychology*, **104**, 289-296.
- FOIS, C., MÉDIONI, J., & LE BOURG, E. (1991). Habituation of the proboscis-extension response as a function of age in *Drosophila melanogaster*. *Gerontology*, **37**, 187-192.
- FRESQUET, N. (1997). Effects of aging on inhibitory learning and short-term memory in *Drosophila melanogaster*. *International Journal of Comparative Psychology*, **10**, 90-109.
- GROSSLIGHT, J. H., & HARRISON, P. C. (1961). Variability of response in a determined turning sequence in the meal worm (*Tenebrio molitor*): An experimental test of alternative hypotheses. *Animal Behaviour*, **9**, 100-103.
- HAY, D. A. (1975). Strain differences in maze-learning ability in *Drosophila melanogaster*. *Nature*, **257**, 44-46.
- HEISENBERG, M., BORST, A., WAGNER, S., & BYERS, D. (1985). *Drosophila* mushroom body mutants are deficient in olfactory learning. *Journal of Neurogenetics*, **2**, 1-30.
- HENDRICKS, J. C., FINN, S. M., PANCKERI, K. A., CHAVKIN, J., WILLIAMS, J. A., SEHGAL, A., & PACK, A. I. (2000). Rest in *Drosophila* is a sleep-like state. *Neuron*, **25**, 129-138.
- HIRSCH, J., & HOLLIDAY, M. (1988). A fundamental distinction in the analysis and interpretation of behavior. *Journal of Comparative Psychology*, **102**, 372-377.
- LE BOURG, E. (1983). Aging and habituation of the tarsal response in *Drosophila melanogaster*. *Gerontology*, **29**, 388-393.
- LE BOURG, E. (1987). Alternation behavior, aging and longevity in *Drosophila melanogaster*: Cross-sectional and longitudinal studies. *Biology of Behaviour*, **12**, 163-174.
- LE BOURG, E., & BADIA, J. (1995). Decline in photopositive tendencies with age in *Drosophila melanogaster* (Diptera: Drosophilidae). *Journal of Insect Behavior*, **8**, 835-845.
- MARIATH, H. A. (1985). Operant conditioning in *Drosophila melanogaster* wild-type and learning mutants with defects in the cyclic AMP metabolism. *Journal of Insect Physiology*, **31**, 779-787.
- MÉDIONI, J. (1955). Précision de l'orientation phototropique et sensibilité spectrale chez *Drosophila melanogaster* Meig. (race sauvage) [Accuracy of phototropic orientation and spectral sensitivity in *Drosophila melanogaster* Meig. (wild strain)]. *Comptes Rendus des Séances de la Société de Biologie*, **149**, 2248-2251.
- MÉDIONI, J. (1961). *Contribution à l'étude psychophysique et génétique du phototropisme d'un insecte: Drosophila melanogaster Meigen*. [Contribution to the psychophysiological and genetic study of the phototropism of an insect: *Drosophila melanogaster* Meigen]. Unpublished doctoral dissertation, University of Strasbourg.
- MÉDIONI, J., & VAYSSE, G. (1975). Suppression conditionnelle d'un réflexe chez la drosophile (*Drosophila melanogaster*): Acquisition et extinction [Conditional suppression of a reflex in *Drosophila melanogaster*: Acquisition and extinction]. *Comptes Rendus des Séances de la Société de Biologie*, **169**, 1386-1391.
- MENZEL, R., HAMMER, M., BRAUN, G., MAUELSHAGEN, J., & SUGAWA, M. (1991). Neurobiology of learning and memory in honeybees. In L. J. Goodman & R. C. Fisher (Eds.), *The behaviour and physiology of bees* (pp. 323-353). Wallingford, CN: C.A.B. International.
- MINOIS, N., & LE BOURG, E. (1997). Hypergravity and aging in *Drosophila melanogaster*: 9. Conditioned suppression and habituation of the proboscis extension response. *Aging Clinical & Experimental Research*, **9**, 281-291.
- QUINN, W. G., HARRIS, W. A., & BENZER, S. (1974). Conditioned behavior in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences*, **71**, 708-712.
- SAVATEEVA, E., POPOV, A., KAMYSEV, N., BRAGINA, J., HEISENBERG, M., SENTZ, D., KORNUBER, J., & RIEDERER, P. (2000). Age-dependent memory loss, synaptic pathology and altered brain plasticity in the *Drosophila* mutant *cardinal* accumulating 3-hydroxykynurenine. *Journal of Neural Transmission*, **107**, 581-601.
- SCHÜMPERLI, R. A. (1973). Evidence for colour vision in *Drosophila melanogaster* through spontaneous phototactic choice behaviour. *Journal of Comparative Physiology*, **86**, 77-94.
- TULLY, T. (1984). *Drosophila* learning: Behavior and biochemistry. *Behavior Genetics*, **14**, 527-557.
- TULLY, T., BOYNTON, S., BRANDES, C., DURA, J. M., MIHALEK, R., PREAT, T., & VILLELLA, A. (1990). Genetic dissection of memory formation in *Drosophila melanogaster*. In *Cold Spring Harbor symposia on quantitative biology: Vol. LV* (pp. 203-211). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- TULLY, T., & GOLD, D. (1993). Differential effects of *dunce* mutations on associative learning and memory in *Drosophila*. *Journal of Neurogenetics*, **9**, 55-71.
- TULLY, T., & QUINN, W. G. (1985). Classical conditioning and retention in normal and mutant *Drosophila melanogaster*. *Journal of Comparative Physiology A*, **157**, 263-277.
- WALLACE, B., & SPERLICH, D. (1988). Conditioning the behavior of *Drosophila melanogaster* by means of electric shocks. *Proceedings of the National Academy of Sciences*, **85**, 2869-2872.
- WOLF, R., & HEISENBERG, M. (1991). Basic organization of operant behavior as revealed in *Drosophila* flight orientation. *Journal of Comparative Physiology A*, **169**, 699-705.
- WUSTMANN, G., & HEISENBERG, M. (1997). Behavioral manipulation of retrieval in a spatial memory task for *Drosophila melanogaster*. *Learning & Memory*, **4**, 328-336.