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# Drosophila Passive Avoidance Behavior as a New Paradigm to Study Associative Aversive Learning

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**TITLE:**

*Drosophila* Passive Avoidance Behavior as a New Paradigm to Study Associative Aversive Learning

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**KEYWORDS:**

Passive avoidance, aversive learning, associative learning, memory, *Drosophila*, western diet, flight exercise

**SUMMARY:**

This work describes a simple behavioral paradigm that allows the analysis of aversive associative learning in adult fruit flies. The method is based on suppressing the innate negative geotaxis behavior due to the association formed between a specific environmental context and an electric shock.

**ABSTRACT:**

This protocol describes a new paradigm for analyzing aversive associative learning in adult flies (*Drosophila melanogaster)*. The paradigm is analogous to passive avoidance behavior in laboratory rodents in which animals learn to avoid a compartment where they have previously received an electric shock. The assay takes advantage of negative geotaxis in flies, which manifests as an urge to climb up when they are placed on a vertical surface. The setup consists of vertically oriented upper and lower compartments. On the first trial, a fly is placed into a lower compartment from where it usually exits, and then within 3-15 s, steps into the upper compartment where it receives an electric shock. During the second trial, 24 h later, the latency is significantly increased. At the same time, the number of shocks is decreased compared to the first trial, indicating that flies formed long-term memory about the upper compartment. The recordings of latencies and number of shocks could be performed with a tally counter and a stopwatch or with an Arduino-based simple device. To illustrate how the assay can be used, the passive avoidance behavior of *D. melanogaster* and *D. simulans* male and female were characterized here. Comparison of latencies and number of shocks revealed that both *D. melanogaster* and *D. simulans* flies efficiently learned the passive avoidance behavior. No statistical differences were observed between male and female flies. However, males were a little faster while entering the upper compartment on the first trial, while females received a slightly higher number of shocks in every retention trial. The Western diet (WD) significantly impaired

learning and memory in male flies while flight exercise counterbalanced this effect. Taken together, the passive avoidance behavior in flies offers a simple and reproducible assay that could be used for studying basic mechanisms of learning and memory.

**INTRODUCTION:**

Learning and memory is an evolutionarily ancient adaptation mechanism to the environment, conserved from *Drosophila (D.)* to human[1]. The fruit fly is a beautiful model organism to study fundamental principles of learning and memory as it offers a wide range of powerful genetic tools to dissect intrinsic molecular mechanisms[2]. The pioneering genetic screening studies, which identified *rutabaga*[3], *amnesiac*[4], and *dunce*[5] genes critical for learning and memory[2], took advantage of olfactory conditioning as the fruit flies rely on their keen sense of smell to find food, potential mates, and to avoid predators[6].

Olfactory conditioning has become a popular paradigm to study the mechanism of learning and memory, thanks to the introduction of olfactory T-maze by Tully and Quinn[7,8]. Subsequently, other methods to measure various types of learning and memory have been proposed, including visual conditioning[9], courtship conditioning[10], aversive phototaxis suppression assay[11], and wasp-exposure conditioning[12]. However, most of these assays have a complex setup that must be custom-built at a university workshop or purchased through a vendor. The paradigm described here is based on a simple behavioral assay to study aversive associative learning in flies that can be easily assembled with a few available supplies.

The described paradigm is equivalent to passive (or inhibitory) avoidance behavior in laboratory mice and rats in which animals learn to avoid a compartment where they have previously received electric foot shock[13]. In murids, the procedure is based on their innate avoidance of bright light and preference for darker areas[14]. On the first trial, the animal is placed into the bright compartment, from where the animal quickly exits, stepping into a dark compartment, where an electric foot shock is delivered. Usually, a single trial is sufficient to form a solid long-term memory, resulting in significantly increased latency 24 h later. The latency is then used as an index of the ability of the animal to remember the association between the aversive stimulus and the specific environment[15].

This work describes an analogous procedure using *D.* as a model system which offers several advantages over rodent models including cost-effectiveness, larger sample size, the absence of regulatory oversight, and access to powerful genetic tools[16,17]. The procedure is based on negative geotaxis behavior, which manifests in flies' urge to climb up when they are placed on a vertical surface[18]. The setup consists of two vertical chambers. On the first trial, a fruit fly is placed into a lower compartment. From there, it usually exits within 3-15 s, stepping into the upper compartment where it receives an electric shock. During a 1 min trial, some flies may occasionally re-enter the upper compartment, which results in an additional electric shock. During the testing phase, 24 h later, the latency is significantly increased. At the same time, the number of shocks is decreased compared to the first day indicating that flies formed aversive associative memory about the upper compartment. The latency, number of shocks, and the duration and frequency of grooming bouts are then used to analyze the animal behavior and the ability to form and

remember the association between the aversive stimulus and the specific environment. The representative results reveal that exposure to the Western diet (WD) significantly impairs passive avoidance behavior in male flies, suggesting that the WD profoundly impacts the fly's behavior and cognition. Conversely, flight exercise alleviated the negative effect of the WD, improving passive avoidance behavior.

**PROTOCOL:**

## 1. Preparation of passive avoidance apparatus

1.1. Drill a 4 mm hole perpendicular to the wall surface of the 14 mL polypropylene culture tube and 8 mm away from the tube bottom.

NOTE: Use an electric drill and 5/32 drill bit for best results.

1.2. Using a steel utility knife, cut off the upper part of the 14 mL polypropylene culture tube to create a 45 mm long tube bottom fragment. The bottom fragment serves as the lower compartment.

1.3. Cut off the tip of 1,000 µL blue pipette tip using a single edge razor blade to make the opening wide enough for a passage of a single fly. Cut off the narrowing part of the blue tip to create a 12 mm fragment. Insert this fragment firmly into a 4 mm hole of the lower compartment. This is used as a loading dock for transferring the flies.

1.4. Cut a 15 mm piece of transparent vinyl tubing 5/8" ID (see **Table of Materials**) to create a coupling. Insert upper and lower compartment into the coupling from opposite ends to securely attach the lower compartment to the upper compartment.

1.5. Using a 2-prong adjustable clamp, attach the assembly to a vertical stand. Orient the assembly vertically with the shock tube as an upper compartment.

1.6. Connect the shock tube wires to an electrical stimulator (see **Table of Materials**) to deliver electric shocks. The duration of the training period is 1 min.

NOTE: To facilitate observation, position a piece of white paper behind the shock tube to serve as a white background of the apparatus. Put a lamp with a 75 W equivalent soft light bulb above the shock compartment. Place an adjustable arm magnifier lamp in front of the setup. A representation of the passive avoidance apparatus is shown in **Figure 1**.

## 2. Preparation of the flies for the passive avoidance procedure

2.1. Immobilize 3–4 day old *D. melanogaster* or *D. simulans* flies using ice-cold block and transfer them into individual vials with food 24 h before the experiment (1 fly per vial) following the procedure described previously[19].

NOTE: The experiments described here compared 3-4 day-old male *vs*. female flies in *D. melanogaster* and *D. simulans*.

2.2. Before the behavioral experiments, code all the vials. For this, assign each group a letter, for example, "A", "B", "C", etc., and each fly a number. Reveal this code only after all data have been recorded and analyzed. Use at least 20 flies per genotype or treatment to counter individual variations.

NOTE: Performing the experiments and analyses "blind" allows excluding a bias in assessing the performance of the fly and data analysis.

## 3. Performing the first trial

3.1. Using a fly mouth aspirator (see **Table of Materials**) described previously[20], gently transfer a fly from the individual vial into the lower compartment *via* the loading dock. Gently suck one fly into the mouth aspirator by sucking air. Deposit the fly by lightly blowing into the loading dock.

NOTE: Avoid stressing the animal during catching and loading.

3.2. Immediately after the fly is loaded into the lower compartment, start a 1 min timer and stopwatch.

NOTE: The stopwatch is used to measure latencies and tally counter to count the number of shocks.

3.3. Press the stopwatch to record the first latency when the fly enters the shock tube by placing all paws on the grid. Turn on the stimulator to deliver an electric shock to the fly. The stimulation parameters are 120 volts, 1000 ms duration, 1 pulse/s (PPS), train duration 2000 ms.

3.4. Deliver additional shocks if fly re-enters shock tube. Record the number of received shocks during a 1 min trial with a tally counter or an Arduino-based counter for each fly (see **Table of Materials**). If using the Arduino-based counter, please follow the steps below.

NOTE: An optional Arduino-based device AKM-007 (see **Table of Materials**) can be used to measure time, latency, the number of shocks, and the frequency and duration of grooming bouts for each animal by pressing and releasing the corresponding buttons on the device. The buttons on the device are assigned to measure latency, administer and record the number of shocks, and measure the frequency and duration of grooming bouts.

3.4.1. Press the **Start** button at step 3.2., and press the **Shock** button at step 3.3.

3.4.2. To record the duration of a grooming bout, press the **Grooming** button at the beginning of a grooming bout on the device and release this button at the end of the grooming bout.

NOTE: The grooming bouts were measured throughout 1 min trial. Extensive grooming could be indicative of animal stress[21,22]. The Arduino-based device saves all data as CSV file to a memory card.

3.5. At the end of a 1 min trial, gently transfer the fly back to an individual vial. Write down the latency, the number of received shocks, and any notable changes in the behavior.

3.6. Clean the lower and shock compartment with 70% ethanol, wipe with a lint-free cleaning tissue (see **Table of Materials**), and dry with the hairdryer. Repeat the trial with the next fly.

3.7. After the behavioral experiments, clean the lower compartment with water and odorless detergent. Wipe the lower compartment and the shock compartment with 70% ethanol, and air-dry overnight.

## 4. Performing the second trial

4.1. Perform the second trial by repeating the procedure described above (step 3) 24 h later. Test the flies in the same sequence as in the previous day.

## 5. Analysis of the results

5.1. Calculate the average latency, the average number of shocks, and duration of grooming bouts for trial 1 and trial 2 for each experimental group of animals. Perform student t-test for two-group comparison or ANOVA for multiple comparisons with post-hoc analyses using Tukey's test[23].

## REPRESENTATIVE RESULTS:

The passive avoidance was studied in *D. melanogaster* (Canton-S) and *D. simulans*. The experiments compared the latencies number of received shocks between consecutive trials. Initially, the experiments were performed with 3-4 day old male *D. melanogaster* flies. Flies were maintained on the standard Bloomington Formulation diet in a climate-controlled environment at 24 °C under a 12 h light-dark cycle, 70% humidity, and controlled population density. The density was controlled by keeping breeding conditions constant for all groups. 15 males and 15 females were bred for 48 h in a bottle at 24 ˚C, 70% humidity, and a 12 h light cycle to generate the offspring. Passive avoidance behavior in a fruit fly was studied in four 2 min trials spaced 24 h apart. The trials were performed at the same time of the day. A fly was gently aspirated from an individual vial during a trial and transferred into the lower compartment *via* a loading dock (**Figure 1**). The experiments revealed that *D. melanogaster* could successfully learn and memorize passive avoidance behavior. In the first trial, the naive fly would enter the upper compartment on average within 16 s (16.15±2.64) and would often re-enter into it, receiving on average 2 shocks (2.18±0.19). During consecutive trials, the latency would significantly increase while the number of shocks would decrease, indicating that flies learned association between the upper compartment and an electric shock (**Table 1**, **Figure 2 A,B**). Analysis of frequency distribution

showed that negative geotaxis is a powerful motivation. During the first trial, most naive flies would enter the upper compartment within 5-15 s (bin 10, **Figure 2C**) and receive 1-3 shocks (**Figure 2D**). In contrast, most flies would not enter the upper compartment in the fourth trial and would correspondingly not receive any shocks (**Figure 2E,F**).

To verify that the passive avoidance assay works in other fruit fly species, the experiments were repeated in *D. simulans* male and female flies kept under the same housing conditions as *melanogaster* flies described above. The trial duration was reduced to 1 min. The frequency distribution for latencies clearly showed that if the fly does not enter the upper compartment within 60 s in the first trial, it usually does not enter at all (**Figure 3C**). The number of trials was reduced to three as the above-described experiments reliably demonstrated that even a single trial was sufficient for a fly to form aversive associations. *D. simulans* males and females flies were both effective at learning the passive avoidance behavior, as evident from the data in the graphs and tables (**Figure 3**, **Figure 4**; and **Table 2, Table 3**).

Comparison of latencies and number of shocks between males and females did not reveal any statistically significant differences (**Figure 5 A,B**). However, male flies were a little faster entering the upper compartment on the first trial, while females received a slightly higher number of shocks in every trial (**Figure 4A,B**). The difference was particularly evident in the graphs illustrating frequency distributions (**Figure 4C-F**). The frequency distribution of latencies in the third trial indicated that most flies entered the upper compartment within 7.5-12.5 s (**Figure 4E**). The frequency distribution for the number of shocks in the third trial shows that female flies received on average 1-2 shocks in the last trial (**Figure 4F**).

The total duration of grooming behavior was also recorded during trials. Fly grooming behavior consists of repeated coordinated movement of forelegs and hindlegs sweeping over the wings, head, and body[24]. Self-grooming, also known as auto grooming, has been previously linked to behavioral stress in flies[24], laboratory rodents[25], and humans[22]. Therefore, studies of self-grooming behavior —can provide insights into the anxiety-related behaviors[26]. The analysis of the total duration of grooming bouts during passive avoidance trials revealed that self-grooming was significantly increased in female flies in trials two and three (**Figure 5C**, **Table 4**).

Growing evidence suggests an association between high-calorie WD and impaired cognitive performance[27]. Experimentally, the WD produces adverse effects on mitochondrial brain function, neurogenesis, and synaptic plasticity[28-30]. A recent study documented that the WD increases triglycerides and shortens lifespan in D., while flight exercise counterbalances the detrimental effect of the WD[31]. To begin to explore the effects of the WD and flight exercise on passive avoidance behavior, *D. simulans* male flies were subjected to 5 days WD, flight exercise, or a combination of WD and flight exercise according to protocols described previously[31]. The WD, containing 15% Nutiva USDA Certified Organic, non-GMO, Red Palm Oil, 15% Sucrose, and 0.1M NaCl, was prepared as published elsewhere[31]. Flight exercise was performed on groups of sixty male flies housed in 1-gallon clear plastic drums strapped to a horizontal platform attached to an electrical motor and operated by a timer. The exercise was performed daily for 7 h for 5 days as published previously[31]. At the end of the 5-day diet and exercise regimen, flies were

sorted into individual vials under cold anesthesia and subjected to two trials of passive avoidance behavior. The experiments showed that the WD increases the number of shocks received in trials one and two and decreases latency in trial two, indicating that the WD significantly impairs aversive associative learning in flies (**Figure 6**, **Table 5**). There was also a downward trend in the number of shocks in trial two in a group with the WD and exercise combination, suggesting that exercise may mitigate the impact of caloric overload.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Schematic illustrating passive avoidance assay.** The lower compartment dimensions are 45 mm in length and 17 mm in outer diameter. The upper compartment dimensions are 80 mm in length and 18 mm in outer diameter.

**Figure 2: Passive avoidance behavior in *D. melanogaster* males.** (**A**) Average latency (s) per trial. The graph shows that the latencies increase significantly with the number of trials. Several flies are indicated on each bar. (**B**) An average number of received shocks per trial. The graph shows that the number of shocks decreases significantly with the number of trials. Several flies are indicated on each bar. (**C**) Frequency distribution displaying the number of flies within a bin of latencies in the first trial. Numbers on the x-axis are bin centers. The bins are intervals (0-5 s, 5-15 s, 15-25 s, etc.). The graph indicates that most flies enter the upper compartment within 5-15 s. (**D**) Frequency distribution displaying the number of flies within a bin of shocks in the first trial. Numbers on the x-axis are bin centers. The graph indicates that most flies receive 1-3 shocks in the first trial. Few flies did not enter the shock compartment on the first trial and received zero shocks. They were excluded from the subsequent trials. (**E**) Frequency distribution displaying the number of flies within a bin of latencies in the fourth trial. The graph indicates that most flies do not enter the upper compartment. (**F**) Frequency distribution displaying the number of flies within a bin of shocks in the fourth trial. The graph shows that most flies receive 0 shocks in the last trial. Abbreviations: ns- nonsignificant, *- $P<0.05$, **- $P<0.01$, ***-$P<0.001$, ****- $P<0.0001$. One-way ANOVA with Tukey's multiple comparisons test.

**Figure 3: Passive avoidance behavior in *D. simulans* males.** (**A**) Average latency (s) per trial. The graph shows that the latencies increase significantly with the number of trials. Several flies are indicated on each bar. (**B**) An average number of received shocks per trial. The graph shows that the number of shocks decreases significantly with the number of trials. Several flies are indicated on each bar. (**C**) Frequency distribution displaying the number of flies within a bin of latencies in the first trial. The graph indicates that most flies enter the upper compartment within 2.5-12.5 s. Numbers on the x-axis are the bin centers. (**D**) Frequency distribution displaying the number of flies within a bin of shocks in the first trial. The graph indicates that most flies receive 1-3 shocks in the first trial. Numbers on the x-axis are the bin centers. (**E**) Frequency distribution displaying the number of flies within a bin of latencies in the third trial. The graph indicates that most flies do not enter the upper compartment. (**F**) Frequency distribution displaying the number of flies within a bin of shocks in the third trial. The graph shows that most flies receive 0 shocks in the last trial. Abbreviations: *- $P<0.05$, ****- $P<0.0001$. One-way ANOVA with Tukey's multiple comparisons test.

**Figure 4: Passive avoidance behavior in *D. simulans* females.** (**A**) Average latency (s) per trial. The graph shows that the latencies increase significantly with the number of trials. Several flies are indicated on each bar. (**B**) An average number of received shocks per trial. The graph shows that the number of shocks decreases significantly with the number of trials. Several flies are indicated on each bar. (**C**) Frequency distribution displaying the number of flies within a bin of latencies in the first trial. The graph indicates that most flies enter the upper compartment within 2.5-12.5 s. Numbers on the x-axis are the bin centers. (**D**) Frequency distribution displaying the number of flies within a bin of shocks in the first trial. The graph indicates that most flies receive 2 shocks in the first trial. Numbers on the x-axis are the bin centers. (**E**) Frequency distribution displaying the number of flies within a bin of latencies in the third trial. The graph indicates that most flies enter the upper compartment within 7.5-12.5 s. (**F**) Frequency distribution displaying the number of flies within a bin of shocks in the third trial. The graph shows that most female flies received 1-2 shocks in the last trial. Abbreviations: **- P<0.01, ***-P<0.001, ****- P<0.0001. One-way ANOVA with Tukey's multiple comparisons test.

**Figure 5: Comparison of passive avoidance and grooming behavior in *D. simulans* males and females.** (**A**) Average latency (s) per trial. The graph shows no statistically significant differences between males and females in the latencies. (**B**) An average number of received shocks per trial. The graph shows no statistically significant differences between males and females in the number of received shocks. (**C**) The total duration of grooming bouts in trials 1-3. While there were no statistically significant differences between males and females, the female flies showed a considerable increase in grooming behavior during trials 2 and 3 compared to trial 1. Abbreviations: *- P<0.05. One-way ANOVA with Tukey's multiple comparisons test.

**Figure 6: Effect of the WD on *D. simulans* male's passive avoidance behavior.** (**A**) Average latency (s) per trial. The graph shows that the WD significantly increased latency in WD and WDE groups. (**B**) An average number of received shocks per trial. The graph shows a statistically significant increase in the number of shocks received by flies on the WD in trials 1 and 2. Interestingly, flight exercise significantly negated the effect of the WD according to the Student t-test. Abbreviations: CD- control diet, CDE- control diet with exercise, WD- western diet, WDE- western diet with exercise. *- P<0.05, **- P<0.01. One-way ANOVA with Tukey's multiple comparisons test. #- P<0.05, Student t-test.

**Table 1: Effect of training on avoidance of electric shock in *D. melanogaster* males.** The data for latencies and received shocks are presented as means ± SEM. One-way ANOVA with Tukey's multiple comparisons test.

**Table 2: Effect of training on avoidance of electric shock in *D. simulans* males.** The data for latencies and received shocks are presented as means ± SEM. One-way ANOVA with Tukey's multiple comparisons test.

**Table 3: Effect of training on avoidance of electric shock in *D. simulans* females.** The data for latencies and received shocks are presented as means ± SEM. One-way ANOVA with Tukey's

multiple comparisons test.

**Table 4: Effect of passive avoidance on *D. simulans* grooming behavior.** The data are presented as means ± SEM. One-way ANOVA with Tukey's multiple comparisons tests.

**Table 5: Effect of the WD and exercise on avoidance of electric shock in *D. simulans* males.** The data for latencies and received shocks are presented as means ± SEM. One-way ANOVA with Tukey's multiple comparisons test. Abbreviations: CD- control diet, CDE- control diet with exercise, WD- western diet, WDE- western diet with exercise.

**DISCUSSION:**

Avoidance of threatening stimuli is a crucial characteristic of adaptive behavior in various species from C*. elegance* to human[32]. Avoidance learning procedures which typically entail the escaping of an aversive event, are commonly used behavioral tasks to investigate learning and memory processes in laboratory rodents[13] since the 1970's[32]. In active avoidance procedures, an indifferent stimulus or conditioned signal (CS) is followed by an aversive event or unconditioned signal (US), which animals learn to avoid by performing a specific behavioral task. In passive avoidance procedures, an animal needs to avoid the aversive US by associating a previously punished behavior with a specific environmental context[33]. Longer retention test latencies indicating better memory suggest that the animal developed a detailed representation of the training experience[13]. Passive avoidance training consists of a single trial; however, the brain mechanisms underlying the acquisition of this task are complex as the animal learns to associate various pieces of information, including environmental, spatial-positional, and aversive stimuli[13]. Altering these stimuli allows studying of episodic and contextual types of memory[13].

The paradigm described here adopted passive avoidance behavior in rodents to adult fly as a model system. While several procedures have been developed to study learning and memory in flies, including olfactory[7,8], visual[9], and courtship conditioning[10], these assays are rather time-consuming and require a complex setup. The protocol described here is a simple behavioral assay to study associative aversive learning in flies, which consists of 1 min trials and can be easily set up with a few available supplies. The protocol potentially allows for additional manipulation of the environmental cues, with the addition of olfactory, visual, and other contextual stimuli, by changing colors or geometrical patterns for the lower compartment or adding olfactory cues into the cap of the upper chamber. In addition, this assay can be easily adapted to study short-term memory, middle-term memory, and anesthesia-resistant memory by manipulating intervals between trials and subjecting flies to anesthesia.

The assay worked equally well in *D. melanogaster* and *D. simulans* male and female flies, demonstrating that the paradigm could be adapted to different *D.* species. The changes in fly behavior characterized by increased latencies and decreased number of shocks were statistically significant in the second trial and would strengthen with subsequent trials. Interestingly, if naïve flies were habituated to the apparatus without electric shock, they would enter the upper compartment a little faster on the second and the third trials. However, the decrease in latencies was not statistically significant (data not shown). No statistically significant differences were

observed between sexes, although female flies had somewhat longer latencies and received slightly more shocks. This difference could be due to a combination of factors, including females' failure to associate the shock with the upper compartment, a stronger geotaxis, or possibly because females are slightly larger and slower than males. The total duration of grooming bouts was significantly higher in the second and third trials in female flies, which draws a parallel between *D.* and rodent anxiety-like behaviors[26].

Interestingly, as shown in **Figures 3E** and **Figure 4E**, the latencies in the third trial were split into two extremes (less than 30 s and 60 s). These differences, however, were not observed in the 4$^{th}$ trial indicating that the majority of the flies eventually acquired the task (**Figure 2E**). While the exact reasons for the individual differences in developing passive avoidance tasks are unclear, natural populations may contain polymorphism and mutations influencing learning and memory[3,5,34,35]. A recent study showed that polymorphism in the *foraging* (*for*) locus encoding a cGMP-dependent protein kinase (PKG) might mediate alternative strategies in learning foraging behavior[34]. Flies with the "rover" allele and higher activity have better short-term memory, while flies with the "sitter" allele and sedentary behavior show better long-term memory[34].

The assay revealed that the WD significantly impaired learning and memory in flies, which was evident by shorter latencies and receiving more shocks in the second trial. The higher number of shocks in the first trial after the WD could indicate deficits in short-term memory and a failure to make associations between specific environmental context and an electric shock. Interestingly, flight exercises partially mitigated the negative effect of the WD as the WDE flies had received significantly fewer shocks in the second trial. This suggests that flight exercise could reverse the impact of metabolic overload and improve cognitive performance. These data are supported by our previous observation on the beneficial effects of flight exercise on fly physiology, reproduction, and behavior[31].

One of the critical steps in the described protocol is transferring the flies from the individual vial into the lower chamber. Stressed flies would either enter the upper compartment too quickly or won't enter at all. The flies that did not enter the upper compartment on the first trial could still enter the upper compartment after being transferred back to an individual vial and given a 5 min break. However, it is better to exclude these flies from the experiment. Fly age is another crucial factor as the negative geotaxis became significantly weaker in 30-day old flies (data not shown). Thus more flies would not enter the upper chamber on the first trial and would be excluded from the experiment.

The advantage of the described assay is that it is a simple behavioral task that produces reproducible data in both *D. melanogaster* and *D. simulans* male and female flies. The assay could help the study of basic mechanisms underlying learning and memory impairments resulting from various genetic, pharmacological, and dietary manipulations. Moreover, since the retention trials can be performed at different times after initial training, short-term, intermediate, and long-term memory could be potentially interrogated separately.

There are also potential limitations with this one-trial task, including inter-subject variability and

age differences (discussed above). The learning and memory could also be influenced by various factors, including the behavioral state at the time of learning, experimental manipulations, and responses to stress[36]. Since the US is given in the compartment with a copper grid, the entry of animals is sometimes difficult to assess unless the photo beams and infrared cameras could be installed for video tracking in the future.

Taken together, the assay presented here is a simple, reliable, and reproducible procedure that allows for studying memory mechanisms. It could potentially open new avenues for detailed analysis of memory consolidation in flies, including gene-drug interactions, in both contextual and episodic-like aspects of memory.

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**DISCLOSURES:**
The authors declare no conflicts of interest.

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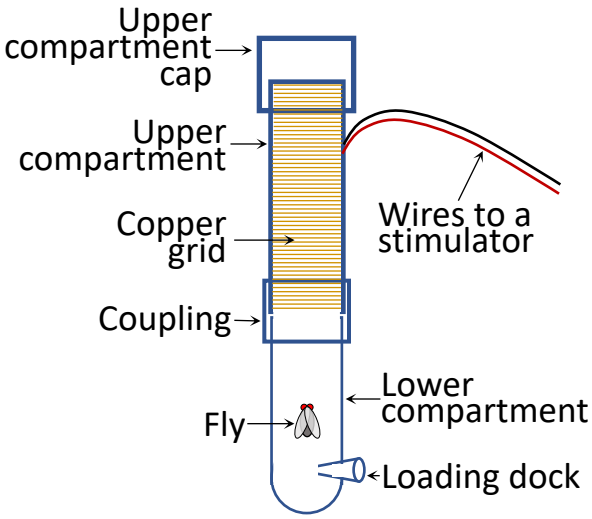



Fig. 1.

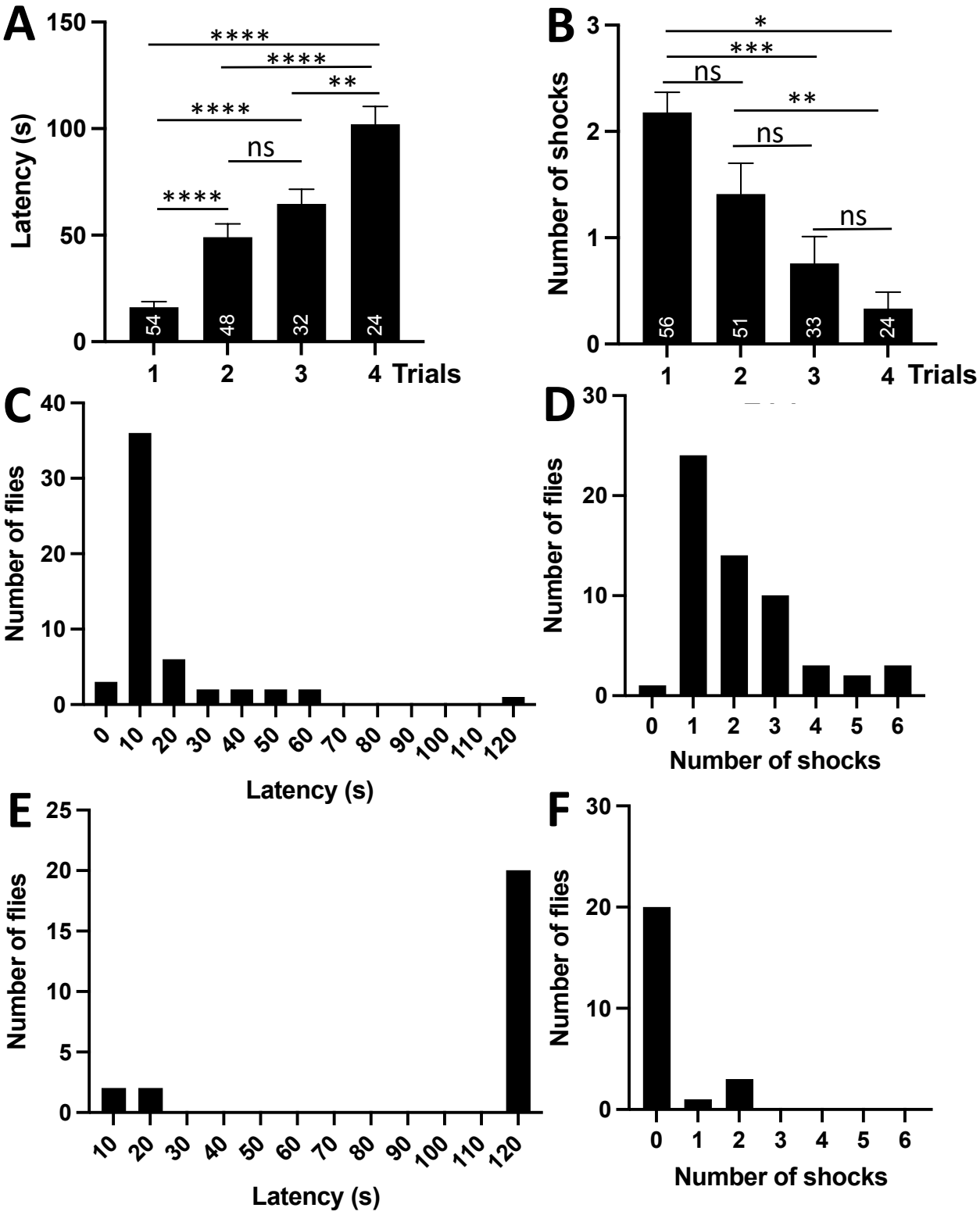



Fig. 2.

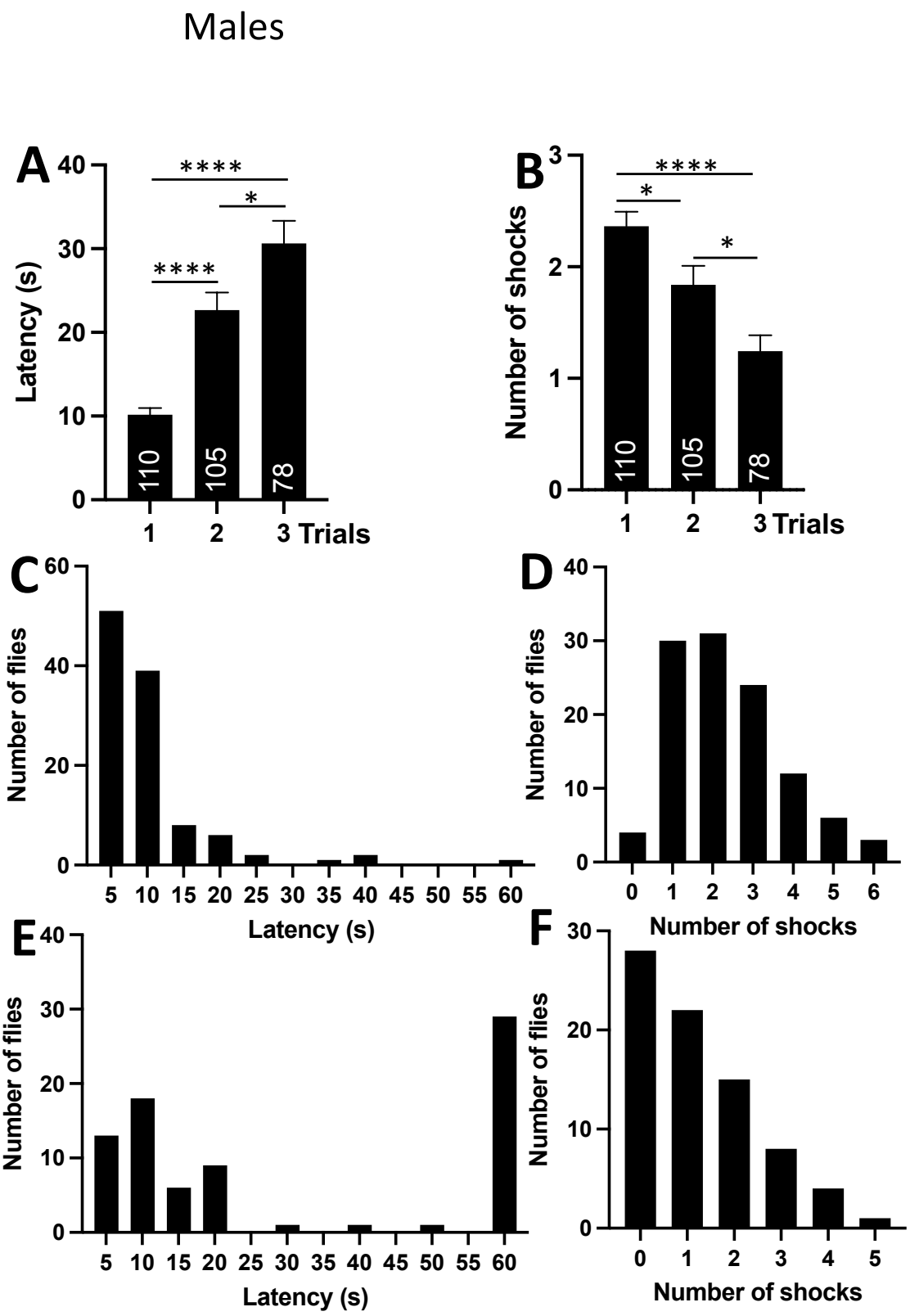



Fig. 3.

Females

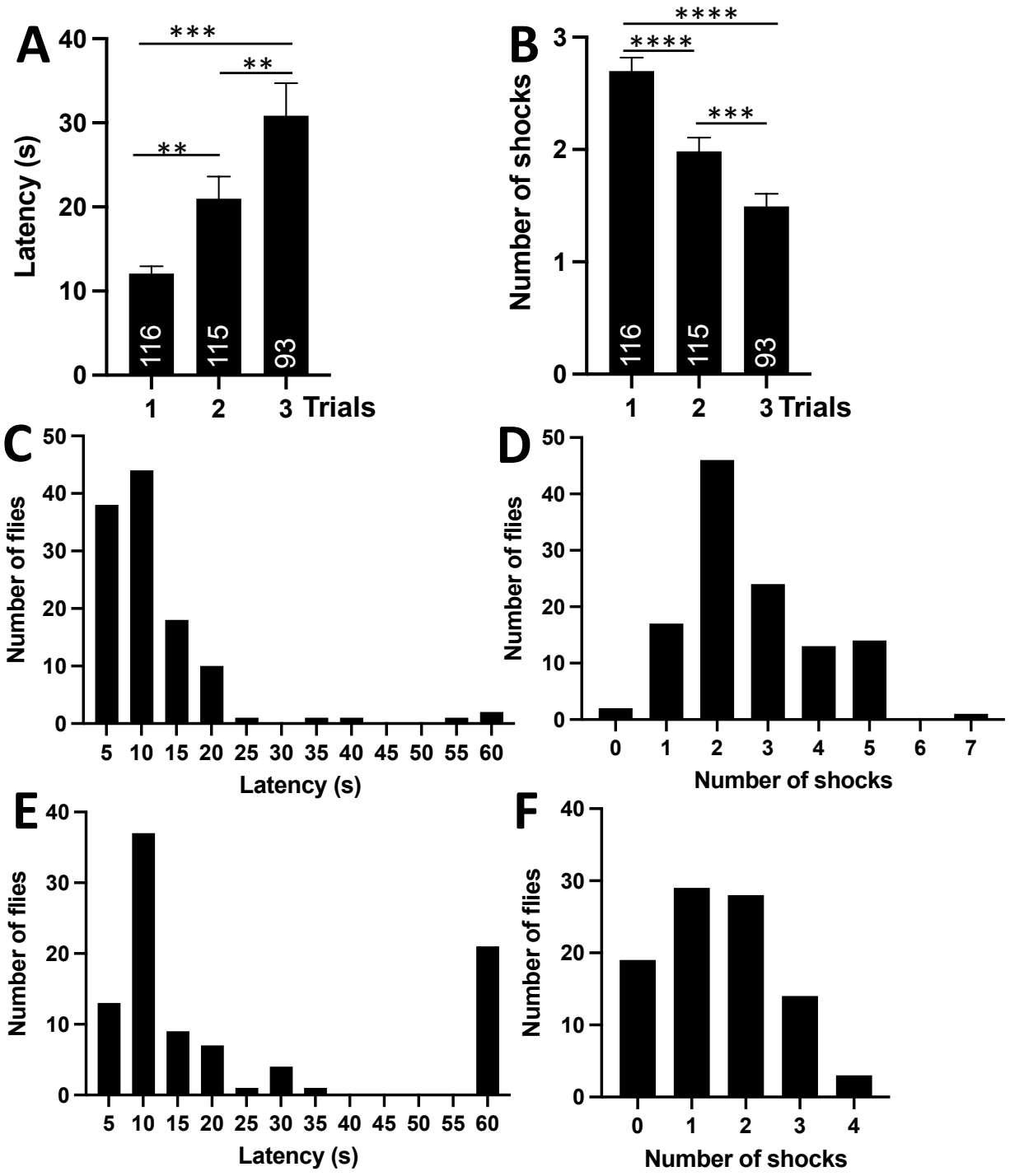



Fig. 4.

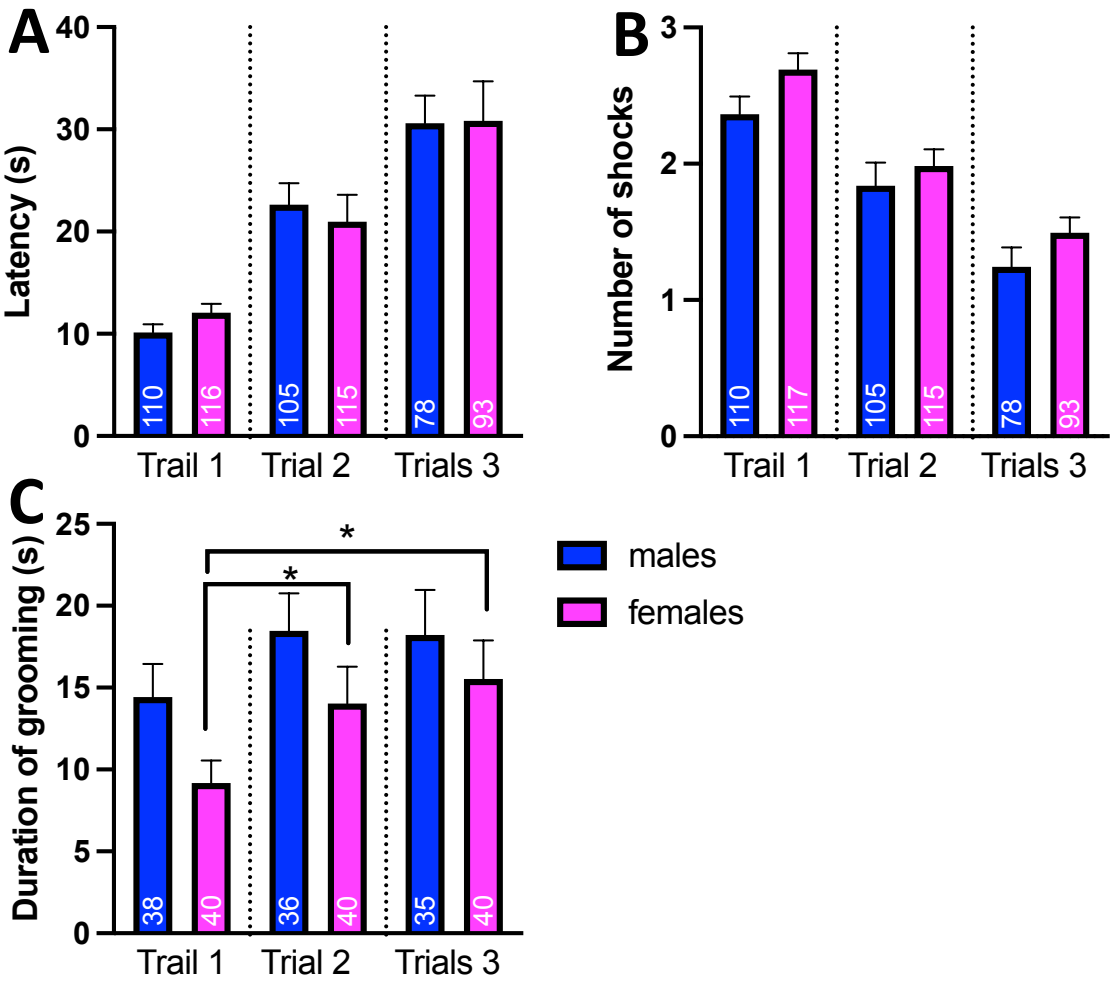



Fig. 5.

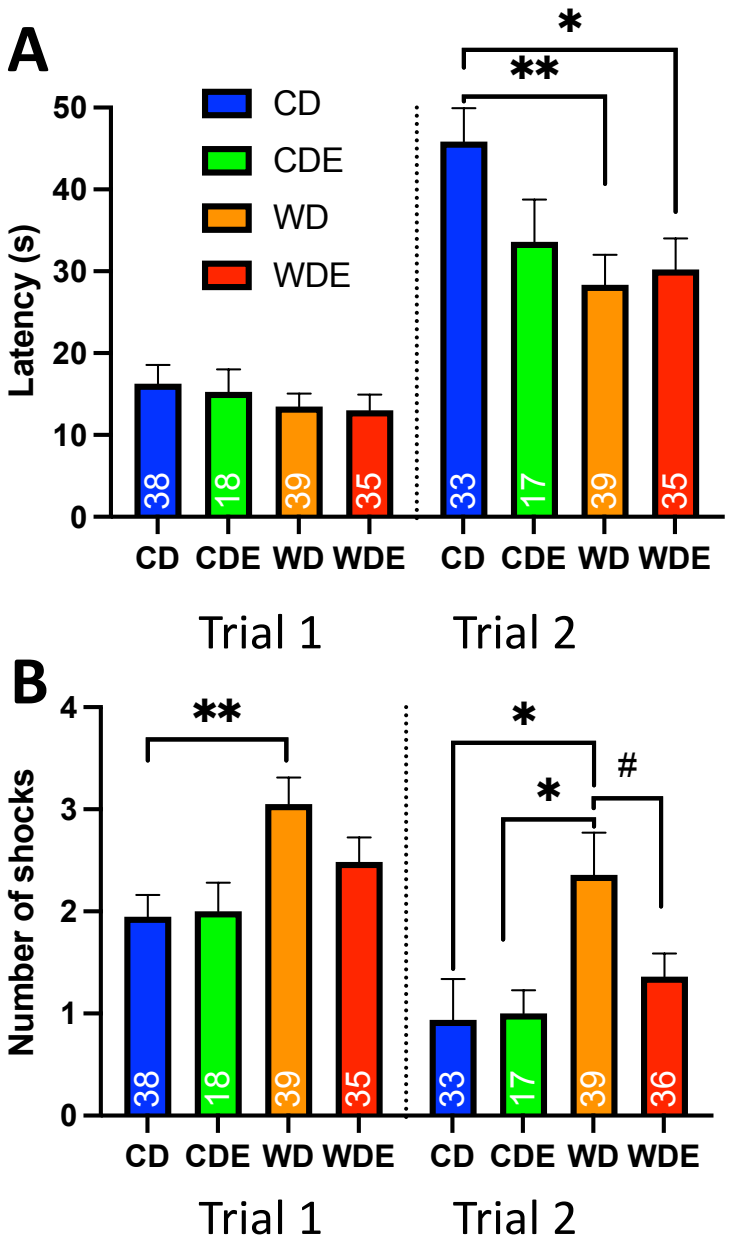



Fig. 6.

Table 1



| Trials | Trial 1 | Trial 2 |
|---|---|---|
| **Latency (s)** | 16.15±2.64 | 49.08±6.2 |
| **Shocks** | 2.18±0.19 | 1.41±0.29 |
| **Latency Tukey's multiple comparisons between trials** | **Summary** | **A** |
| 1 vs. 2 | **** | |
| 1 vs. 3 | **** | |
| 1 vs. 4 | **** | |
| 2 vs. 3 | ns | |
| 2 vs. 4 | **** | |
| 3 vs. 4 | ** | |
| **Shocks Tukey's multiple comparisons between trials** | **Summary** | **A** |
| 1 vs. 2 | ns | |
| 1 vs. 3 | *** | |
| 1 vs. 4 | **** | |
| 2 vs. 3 | ns | |
| 2 vs. 4 | * | |
| 3 vs. 4 | ns | |

| Trial 3 | Trial 4 |
|---|---|
| 64.72±6.8 | 102.1±12.1 |
| 0.76±0.25 | 0.33±0.22 |

| djusted P Value |
|---|
| <0.0001 |
| <0.0001 |
| <0.0001 |
| 0.2043 |
| <0.0001 |
| 0.0099 |
| **djusted P Value** |
| 0.0646 |
| 0.0004 |
| <0.0001 |
| 0.2569 |
| 0.0253 |
| 0.694 |

Table 2 Click here to access/download;Table;63163_Table 2.xlsx

| Trials | Trial 1 | Trial 2 |
|---|---|---|
| **Latency (s)** | 10.16±0.78 | 22.64±2.1 |
| **Shocks** | 2.36±0.13 | 1.84±0.17 |
| **Latency Tukey's multiple comparisons between trials** | **Summary** | **Adjusted** |
| 1 vs. 2 | **** | <0.0 |
| 1 vs. 3 | **** | <0.0 |
| 2 vs. 3 | * | 0.0 |
| **Shocks Tukey's multiple comparisons between trials** | **Summary** | **Adjusted** |
| 1 vs. 2 | * | 0.0 |
| 1 vs. 3 | **** | <0.0 |
| 2 vs. 3 | * | 0.0 |

| Trial 3 |
| --- |
| 30.63±2.69 |
| 1.24±0.14 |

| l P Value |
| --- |
| )001 |
| )001 |
| 229 |

| l P Value |
| --- |
| 166 |
| )001 |
| 172 |

Table 3



| Trials | Trial 1 | Trial 2 | Trial 3 |
|---|---|---|---|
| **Latency (s)** | 12.07±0.89 | 20.98±2.63 | 23.20±2.16 |
| **Shocks** | 2.65±0.12 | 1.98±0.12 | 1.49±0.11 |
| **Latency Tukey's multiple comparisons between trials** | **Summary** | **Adjusted P Value** | |
| 1 vs. 2 | ** | 0.0023 | |
| 1 vs. 3 | **** | <0.0001 | |
| 2 vs. 3 | ** | 0.0028 | |
| **Shocks Tukey's multiple comparisons between trials** | **Summary** | **Adjusted P Value** | |
| 1 vs. 2 | **** | <0.0001 | |
| 1 vs. 3 | **** | <0.0001 | |
| 2 vs. 3 | *** | 0.0003 | |

Table 4 Click here to access/download;Table;63163_Table 4.xlsx

| Trials | Trial 1 | Trial 2 |
|---|---|---|
| **Male's grooming (s)** | 14.42±1.02 | 18.47±2.82 |
| **Female's grooming (s)** | 9.15±1.39 | 14.03±2.26 |
| **Tukey's multiple comparisons between trials males** | **Summary** | **Adjusted** |
| 1 vs. 2 | ns | 0.4 |
| 1 vs. 3 | ns | 0.4 |
| 2 vs. 3 | ns | 0.9 |
| **Tukey's multiple comparisons between trials females** | **Summary** | **Adjusted** |
| 1 vs. 2 | * | 0.0 |
| 1 vs. 3 | * | 0.0 |
| 2 vs.3 | ns | 0.8 |

| Trial 3 |
| --- |
| 18.23±2.75 |
| 15.50±2.38 |

| d P Value |
| --- |
| 384 |
| 873 |
| 971 |

| d P Value |
| --- |
| 299 |
| 426 |
| 481 |

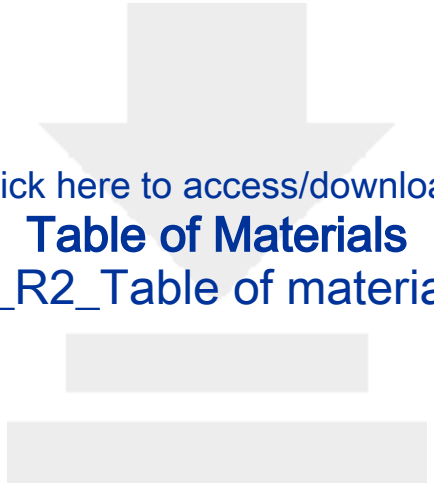
Table 5 Click here to access/download;Table;63163_Table 5.xlsx

| Latencies | Trial 1 |
|---|---|
| CD | 16.26±2.28 |
| CDE | 15.28±2.75 |
| WD | 13.49±1.58 |
| WDE | 13.00±1.95 |
| **Latency Tukey's multiple comparisons between groups Trial 1** | **Summary** |
| CD vs. CDE | ns |
| CD vs. WD | ns |
| CD vs. WDE | ns |
| CDE vs. WD | ns |
| CDE vs. WDE | ns |
| WD vs. WDE | ns |
| **Latency Tukey's multiple comparisons between groups Trial 2** | **Summary** |
| CD vs. CDE | ns |
| CD vs. WD | ** |
| CD vs. WDE | * |
| CDE vs. WD | ns |
| CDE vs. WDE | ns |
| WD vs. WDE | ns |

| Shocks | Trial 1 |
|---|---|
| CD | 1.95±0.21 |
| CDE | 2.0±0.28 |
| WD | 3.05±0.26 |
| WDE | 2.49±0.24 |
| **Shocks Tukey's multiple comparisons between groups Trial 1** | **Summary** |
| CD vs. CDE | ns |
| CD vs. WD | ** |
| CD vs. WDE | ns |
| CDE vs. WD | ns |
| CDE vs. WDE | ns |
| WD vs. WDE | ns |
| **Shocks Tukey's multiple comparisons between groups Trial 2** | **Summary** |
| CD vs. CDE | ns |
| CD vs. WD | * |
| CD vs. WDE | ns |
| CDE vs. WD | * |
| CDE vs. WDE | ns |
| WD vs. WDE | ns |

| Trial 2 |
| --- |
| 45.85±4.09 |
| 33.59±5.19 |
| 28.33±3.7 |
| 30.23±3.79 |
| **Adjusted P Value** |
| 0.9916 |
| 0.7369 |
| 0.6475 |
| 0.9523 |
| 0.9122 |
| 0.9981 |
| **Adjusted P Value** |
| 0.2785 |
| 0.0082 |
| 0.0283 |
| 0.8578 |
| 0.9594 |
| 0.9844 |

| Trial 2 |
| --- |
| 0.94±0.4 |
| 1.0±0.23 |
| 2.36±0.41 |
| 1.36±0.23 |
| **Adjusted P Value** |
| 0.9992 |
| 0.005 |
| 0.3765 |
| 0.0523 |
| 0.6446 |
| 0.3268 |
| **Adjusted P Value** |
| 0.9996 |
| 0.0194 |
| 0.8246 |
| 0.0122 |
| 0.9306 |
| 0.1508 |



Dear Dr. Saha,

Please find attached answers to the editorial and reviewers' comments. We made every effort to address all questions and concerns. As a result of these changes, this manuscript became stronger and more focused. Thank you for your consideration!

Sincerely,

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Greenville, NC 27834
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Fax: 252-744-3460
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**Editorial comments:**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
A: We thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

2. Please revise the following lines to avoid previously published work: 48-49, 217-220, 339-342, 385.
A: We revised the lines: 48-49, 217-220, 339-342, 385 to avoid previously published work.

3. Please revise the abstract to be between 150-300 words, clearly stating the goal of the protocol. Here the word limit is exceeding.
A: The abstract was revised to fit word limitations.

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
A: We revised the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

5. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol.
A: We revised phrases containing "could be," "should be," and "would be" throughout the protocol.

6.Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?
Step 1.5: How was the coupling done, and how was the attachment to the shock tube done and secured?
Step 2.1: How was the anesthesia given, and how was proper anesthetization confirmed? Which fly genotype was used here? Was there any age, sex-specific bias?
Step 3.1: How was this done? Please provide all details as to how the flies were caught and transferred. Alternatively, add references to published material specifying how to perform the protocol action.

Step 3.7 NOTE: Does the grooming bout refer to the additional shocks given after the first electric shock. If yes, please specify this in the protocol. Also, please describe in detail how the frequency and duration of grooming bouts are recorded and calculated.
A: More details were added to your protocol steps, including Steps 1.5, 2.1, 3.1, and 3.7.

7. Please do not abbreviate journal names in the References.
A: Journals are cited with full names in References.

**Reviewer #1:**
We thank the reviewer for the very thoughtful comments, which helped to improve the manuscript.

Major Concerns: None

Minor Concerns: After the manuscript - the comments/description section was incoherent and full of errors that needs to be rectified in the publication version
Answer to Minor concerns:
The comments/description section was revised.

**Reviewer #2:**
We thank the reviewer for the very thoughtful comments, which helped to improve the manuscript.

Major and minor Concerns:
Throughout the manuscript, authors used Drosophila many times. Except for the first appearance in the manuscript, an abbreviation (D.) should be used. 'simulans' was often used alone, but it should be written D. simulans.
A: Drosophila is now abbreviated as D. throughout the text.

line-82: I don't understand 'regulatory oversight'. Please clarify it.
A: By regulatory oversight, we meant IACUC approval of animal protocols for vertebrate animals.

line-109: it is unclear what 'a 12-mm fragment' means. Please clarify it.
A: We clarified in the text that we cut off the narrowing part of the blue tip to create a 12mm long piece.

line-150: please clarify 'home vial'. Do you keep the flies individually after each trial?
A: By home vials, we meant individual vials. It is now corrected in the text.

line-160: it is not clear at what time points the grooming bouts were measured (after flies returned to the lower compartment in response to e-shock?)
A: Grooming bouts were measured throughout the 1 min trials. It is now explained in the text.

line-190: 'within 10 sec'. this is in contrast to 16 sec (line 185).
A: “within 10 sec” were referring to the frequency distribution of the latencies. For example, a 10-sec bin comprises all flies with latencies from 5 to 15 sec. This is different from the average latency. We now explained this in the text.

Figure 1: what is the top rectangle? what is the dimension (diameter x length) of the 14-mL tube?
A: the top rectangle is a cap of the shock tube. The corresponding labeling was added to the figure. We now also indicate the dimensions of the 14 ml tube in the legend (45mm length x 17mm outer diameter).

All tables are just alternative presentations of the histograms. I suggest deletion of these, since it does not provide new information.
A: We believe the tables provide valuable numerical information for the means, SEMs, and P-values, which many readers especially those who would like to replicate the protocol might find useful.

Figures 2C, D: Is it possible to have precisely 'zero', '10', '20' etc. latencies? (see also Fig. 3C, 4C). According to line-144, 'zero' sec latency is impossible. I believe the x-axis should be presented as intervals (for instance, 1-5 sec, 6-10 sec, 11-15 sec, etc.)
I was also puzzled by 'zero' number of shocks (see also Fig. 3D, 4D). Shouldn't flies get at least one shock (during the first trial)?
A: The frequency distribution graphs show the number of flies within certain ranges of latencies or shocks, which are called “bins”. For example, bin 0 will have flies with latencies from up to 5 sec, bin 10 will have flies with latencies between 5 and 15 sec, and so on. While we agree that it might be useful to present bins as intervals, unfortunately, the statistical program GraphPad Prism does not allow this option. The x-axis in distribution graphs is automatically formatted as bin centers. As far as zero shocks are concerned, some flies never entered the shock tube on the first trial and were removed from subsequent trials. We now explain all this in the text.

Figures 3E, and 4E: averages of latency were presented in 3A and 4A, respectively. interestingly however, the latencies were split into two extremes (less than 20 and 60). Some of the flies barely learned or failed to memorize (less than 20 sec), while the remaining ones did (60 sec). Therefore, simply averaging values doesn't seem to be appropriate for this case. Authors should provide more insightful discussion about such a split event.
It was also unclear why authors tested D. simulans.
A: This is a very good observation. Indeed, we noticed that if a fly does not enter the shock compartment within 30 sec it will not enter it at all. However, this distribution while well visible in the third trial disappears in the 4$^{th}$ trial, indicating that all flies eventually learn the task. We do not know the reason for the distribution in the third trial, but individual variations in fly learning ability may be explained by some genetic polymorphisms which control alternative strategies of learning. A recent study showed that polymorphism in the foraging (for) locus encoding a cGMP-dependent protein kinase (PKG) may mediate alternative strategies in learning foraging behavior. Flies with the "rover" allele and higher activity have better short-term memory while flies with the "sitter" allele and sedentary behavior show better long-term memory. (Mery et al., PNAS 2007). We now discuss this in the text.

line-224: regarding WD. Is there anything known about the WD-fed flies? For instance, do they weigh more? body size? it is also helpful if authors provide a hint why this test is important.
A: Yes, our recently published paper has documented effects of a western high-calorie diet (WD) in flies (Murashov et al., FASEB Bioadvances 2021). Among many effects, the WD increases the level of triglycerides and shortens lifespan in D. which can be mitigated by flight exercise. We now explain this in the text.

line-350: perhaps authors need to provide an example how this can be done.

A: it can be easily done by changing colors or geometrical patterns for the lower compartment or adding olfactory cues via the cap of the upper shock tube. The shock tube is a commercially acquired part of olfactory T-maze and can be easily adapted for olfactory conditioning.
I suggest additional experiments as follows:
1) For the Figure 2, please do the same without e-shock. This will be a control experiment. The latencies are not expected to change in this control.
2) Please do the same with a known learning-memory mutant (such as rutabaga or dunce). This will provide an additional validation of this protocol.
A: 1) We have done repeated measurements in naïve flies. Without electric shock, they would enter the upper compartment a little faster in the second and third trials. However, these changes were not statistically significant.
2) While we agree that testing rutabaga or dunce flies would be extremely interesting, and we plan to do it in the future, we believe it is outside the scope of this protocol paper.

Reviewer #3
We thank the reviewer for the very thoughtful comments, which helped to improve the manuscript.

Major concerns:
1. Drosophila olfactory learning and memory formation can be genetically dissected into five distinct phases: learning, short-term memory, middle-term memory, anesthesia-resistant memory and long-term memory. The authors stated that their passive avoidance apparatus can be used for studying memory mechanisms, but no data in the article shows this feature.
A: We stated that the current paper describes a protocol for testing learning and long-term memory in drosophila. However, this assay can easily be adapted to study short-term memory, middle-term memory, anesthesia-resistant memory by manipulating intervals between trials and subjecting flies to anesthesia. We have now added this to the discussion.
2. Figure 1 should more clearly show the steps of assembling the equipment and the final physical equipment including a 2-prong adjustable clamp, a vertical stand, an electrical stimulator, etc.
A: While we agree that more details on fig.1might be helpful, however, we believe it would be redundant as the video provide a detailed explanation of all the steps of assembling the equipment.
3. In figure 6, the authors stated that the WD significantly increased latency in WD and WDE groups, but it seems that there is no such result from the figure. In addition, other studies have pointed out that WDE is better than WD in memory, but the author's experimental results seem to be different.
A: We are a little confused by this comment as our data in fig.6 clearly show that the WD is detrimental to memory while exercise (WDE) counterbalanced this effect.

Minor Concerns:
1. On page 25 in the pdf file, the word "optioonal" should be revised to "optional".
A: We do not have page 25 and we cannot find the misspelled word. However, we want to assure you that we carefully checked the manuscript for all grammatical errors and typos.
2. On pages 26-29 in the pdf file, garbled characters appear.
We do not have pages 26-29 in our version of PDF and do not see any garbed characters.