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A New Behavioral Paradigm for Visual Classical Conditioning in *Drosophila*

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Abstract

Visual learning in animals is a remarkable cognitive ability that plays a crucial role in their survival and adaptation. Therefore, the ability to learn is highly conserved among animals. Despite lacking a centralized nervous system like vertebrates, invertebrates have demonstrated remarkable learning abilities. Here, we describe a simple behavioral assay that allows the analysis of visual associative learning in individually traceable freely walking adult fruit flies. The setup is based on the simple and widely used behavioral assay to study orientation behavior in flies. A single wing-clipped fly that has been starved for 21 h is placed on a platform where two unreachable opposite visual sets are displayed. This visual learning protocol was initially developed to study the cognitive ability of fruit flies to process numerical information. Through the application of the protocol, flies are able to associate a specific visual set with an appetitive reward. This association is revealed 2 h later during the testing session where we observed a change in their preference upon learning (i.e., change in their spontaneous preference). Moreover, this protocol could potentially be used to associate any other visual object/property to the reward, expanding the opportunities of studying visual learning in freely walking fruit flies at individual level.

Keywords: *Drosophila melanogaster*, Appetitive learning, Visual conditioning, Short-term memory, Cognitive ability

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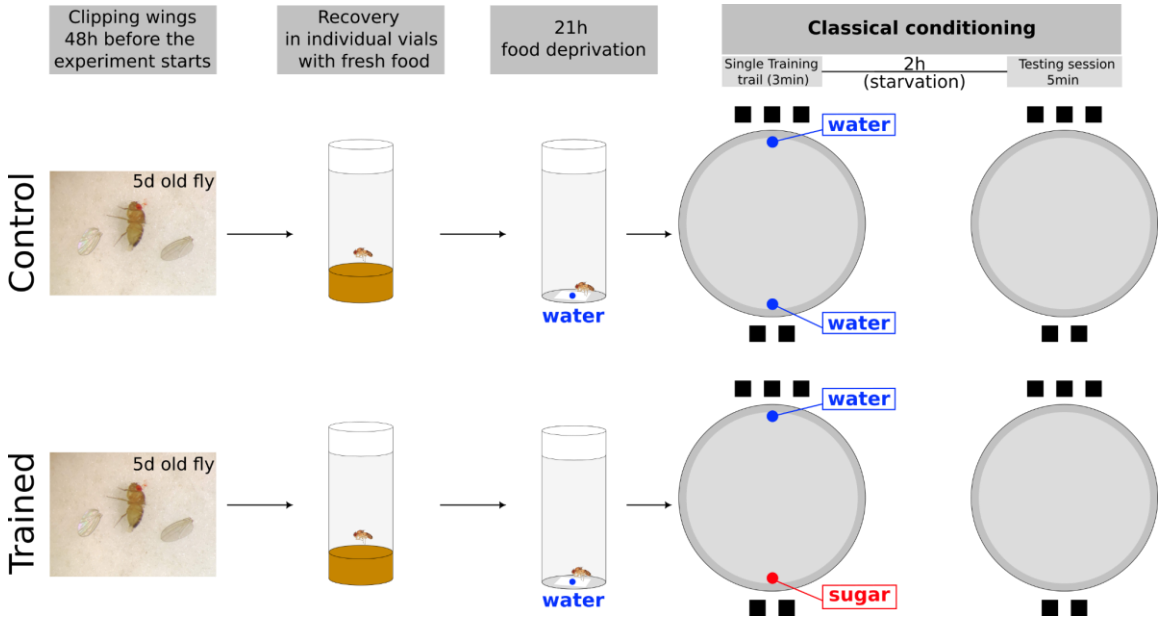
Graphical overview



Graphical overview of conditional learning protocol. Forty-eight hours before conditioning, the wings of the flies are clipped, and individual flies are left to recover in a fresh food vial. Twenty-one hours before the conditional learning starts, flies are transferred to a starvation vial containing wet paper. The training session consists in placing a drop of sugar next to the place with the lower number of objects (numerosity) and a drop of water next to the larger numerosity. The fly is placed in the arena and left to freely walk for three minutes. Once the session is finished, the fly is placed back in their empty vial for 2 h until the testing session starts.

Background

Associative learning enables animals to prepare for behaviorally relevant events, thus being highly conserved. Learning to predict events in the environment and which stimuli tend to occur together help us interact effectively with our environment. This cognitive process has been shown to play a role in a wide diversity of behaviors, including interactions with predators, prey, rivals, and mates. Thus, learning is expected to be essential for survival and reproduction in many species. The study of such associative learning in simple model systems, like the fruit fly *Drosophila melanogaster*, facilitates its understanding especially at multiple levels of analysis. This model allowed researchers to uncover principles and mechanisms of learning and memory as it offers a wide range of powerful genetic tools to dissect intrinsic molecular mechanisms (McGuire et al., 2005; Pitman et al., 2009).

During Pavlovian classical conditioning, animals learn to associate a salient unconditioned stimulus with a neutral stimulus. Olfactory classical conditioning is a well-studied form of associative learning in the fruit fly *D. melanogaster* (see Busto et al., 2010). Subsequently, other methods to measure various types of learning and memory have been proposed, including courtship conditioning (Koemans et al., 2017), aversive phototaxic suppression assay (Ali et al., 2011), and wasp-exposure conditioning (Kacsoh et al., 2019).

Regarding visual learning, it has been extensively studied using the so-called flight simulator (Wolf and Heisenberg, 1991). Here, a tethered fly is flying stationarily in the center of a cylindrical virtual arena panorama where different visual stimuli are displayed. Combined with heat punishment, the setup changes into an operant conditioning paradigm in which the fly learns to control the appearance of the punisher. Like this, animals have to learn from the consequences of their own and voluntary actions in order to avoid the punishment. Other studies show that populations of flies are able to associate the color of the illumination alone (Vogt et al., 2015) or in combination with odors (Vogt et al., 2014; Thiagarajan et al., 2022; Okray et al., 2023) with an appetitive or aversive stimulus.

Despite the relative abundance of aversive learning assays in which flies operantly avoid heat by choosing certain orientations relative to landmarks, only a few behavioral paradigms for appetitive visual learning in adult *Drosophila* have been reported to date (Heisenberg, 1989; Schnaitmann et al., 2010; Vogt et al., 2014). Here, we established a new behavioral paradigm for visual classical conditioning in freely-walking adult *Drosophila*. In their natural environment, fruit flies primarily move by walking rather than flying. Studying them in a walking state allows us to better understand their visual learning abilities in a context that aligns with their ecological niche. This assay allows analysis of the behavior of individual flies while they make a choice, learn, and memorize (short-term memory) visually rewarded associations. The paradigm described here is based on a simple behavioral assay to study orientation behavior in flies (Buridan paradigm), which can be easily assembled with a few available supplies (Figure 1). Particularly, this visual learning is based on the spontaneous numerical preference of flies, which manifests as a larger occupancy next to the higher numerosity in a two-choice test (Bengochea et al., 2023). The setup consists of presenting two visual sets of different numbers of objects that are located opposite to each other, in order to be processed individually and not treated as a whole. Since flies have a spontaneous preference for larger sets of objects, the appetitive reward is placed next to the *non-preferred* numerical set (lower numerosity), while the innately preferred one is water-paired (Figure 1B). A single fly is placed on the platform (Figure 1C). During the 3-min training trial, the fly explores the arena and eventually reaches the sugar and water. Two hours after training, the learned preference of the fly is evaluated during a 5-min testing phase (Graphical overview). By analyzing the walking occupancy in the arena, we are able to observe a decrease or change in their natural tendency to prefer the larger quantities due to the association of the sugar with the visual set. The representative results of this assay reveal that the application of the protocol impacts on the fly's numerical preference (Figure 2, Bengochea et al., 2023). A single training trial of 3 min is sufficient to teach flies to associate an appetitive reward with a visual set that lasts at least two hours and to partially or totally reverse a spontaneous tendency.

Materials and reagents

Fly stocks

Wildtype CantonS flies (BL#64349, 7 days old at the moment of the training session)

Materials

1. Eight circular fluorescent tubes (Philips, L 40 w, 640C circular cool white)
2. Lamp holder and connectors G10Q (Vossloh, catalog number: 101528)
3. Two electrical transformers (Osram Quicktronic QT-M 1×26–42)
4. Eight power cables with switch (Tibelec, catalog number: 163910)
5. Diffuser paper (Canson, Translucent paper 180 g/m^2)
6. Ten squares of black paper (31 mm × 31 mm)
7. Filter paper (Whatman, catalog number: 1001-110)
8. Paint brush (Size 1; Boesner, model: Da Vinci Nova Serie 1570, catalog number: D15701))
9. Empty vials (Polystyrene; Dutscher, catalog number: 789001B)
10. Invisible tape (Scotch Magic 3M, catalog number: 7100027389)
11. Seamstress ruler
12. Pencil

Reagents

1. Distilled water
2. Mineral water (Evian)
3. Glucose 1.5 M (Sigma-Aldrich, catalog number: G8270), prepared with Evian water
4. 70% ethanol (VWR, catalog number: 83801)

5. Agar (Genesee Scientific, catalog number: 66-103)
6. Cornmeal (Genesee Scientific, catalog number: 62-100)
7. Yeast (Genesee Scientific, catalog number: 62-106)
8. Glucose (Sigma-Aldrich, catalog number: 68270)
9. Molasses (Genesee Scientific, catalog number: 62-117)
10. Ethanol (VWR, catalog number: 83801)
11. Nipagin (Thermo Fisher Scientific, catalog number: A14289)
12. Propionic acid (Sigma-Aldrich, catalog number: P5561)
13. *Drosophila* standard cornmeal/agar food (see Recipes)

Recipes

1. ***Drosophila* standard cornmeal/agar food**
   8 g of agar
   60 g of cornmeal
   50 g of yeast
   20 g of glucose
   50 g of molasses
   19 mL of ethanol 70%
   1.9 g of Nipagin
   10 mL of propionic acid
   Bring up to 1 L of final volume with distilled water

Equipment

1. Scissor (Vannas Spring Scissors, 3 mm Blades; Fine Science Tool, catalog number: 15000-00)
2. CO_2 pads (Dutscher, catalog number: 789183)
3. CO_2 pistol (Dutscher, catalog number: 789096)
4. Two Buridan's Paradigm: include baseplate with platform and chamber, acrylic cylinder, and posts to hold the lamps (Peira Scientific Instruments, https://www.peira.be/)
5. Two webcams (Logitech HD Pro c920 webcam full HD)
6. Two PCs
7. Fly incubator (Panasonic, MIR-554-PE; fluorescent lamp: Panasonic FL15D 15W)
8. Microscope (Leica, M80)

Software

1. Buritrack (http://buridansourceforge.net)
2. R Core Team (https://www.R-project.org/)
3. RStudio (https://support--rstudio-com.netlify.app/products/rstudio/)

Procedure

A. Prepare flies for behavioral recording

1. Raise flies at 12:12 h light/dark cycle, 60% humidity and 25 °C.

2. Hatched flies are disposed of in case their age is not controlled (including those that transiently stick to the food or walls).
3. Collect all newly hatched flies into a new *experimental* vial (no more than 20 flies per vial). Flies should be 0–1 day old.
4. Collect 5-day-old flies (at least 60 flies: 30 for control group and 30 for trained group) by tapping and transferring them to a new vial with fresh food.
5. Anesthetize the flies by holding the vial upside down and inserting the CO_2 pistol. Fill in the CO_2 for a few seconds until flies are asleep (flies do not move anymore and fall into the vial cap).
6. Transfer the flies to a CO_2-dispensing porous pad.
7. Clip both wings using scissors under the microscope. The wings need to be shortened to at least 1/3 of the original length and must be straight to the crossing line of both wings. The flies must not be anesthetized for more than 5 min; therefore, it is useful to portion the flies.
8. Save each fly in individual vials with food (1 cm full) at 25 °C. Split and store them in two different racks—one for the control group (CT) and one for the trained group (TR).
9. Seven-day-old flies are used for the experiment. Twenty-one hours before running the experiment, place each fly in a starving vial containing a piece of filter paper watered with Evian water. Store them at 25 °C until running the experiment.

B. Prepare behavioral arena for learning experiment

Two setups will be prepared in parallel to run the control (CT) and trained (TR) group at the same time. It is important to switch the setups, so that the two groups are run in both assays. For half of the animals in the experiment, the acrylic cylinder must be rotated 180° to exclude any uncontrolled and systematic influence of other stimuli of the surroundings.

1. Place the visual diffuser paper on the inside of the acrylic cylinder.
2. Tape the black squares with scotch tape: on one side of the cylinder, place the three squares at 40 mm from the bottom with a distance between them of 22 mm. Tape the remaining two squares in the opposite side of the cylinder (180° apart) using the same distances. It is important that the sets are well aligned. The center of the sets must be 180° apart (Figure 1).
3. Open the Buritrack software and place the visual sets in the top and bottom part of the image captured by the webcam (Figure 1B). The contrast and luminosity of your camera may need to be adjusted, such that the arena is visible.
4. Add distilled water to the Buridan chamber.
5. Darken the room: close blinds and curtains and switch off the room lights.
6. Switch on the Buridan fluorescent lights.
7. Clean the platform with ethanol 70%.
8. Place the round filter paper on the platform.
9. In the Buridan planned to run the trained group, add two drops (~100 µL) of glucose solution next to the visual stimulus to be conditioned (e.g., 2 squares set) and two drops (~100 µL) of Evian water to the other visual stimulus (e.g., 3 squares set). For the control group, in the other Buridan, place two drops of water next to each stimulus (Figure 1B).

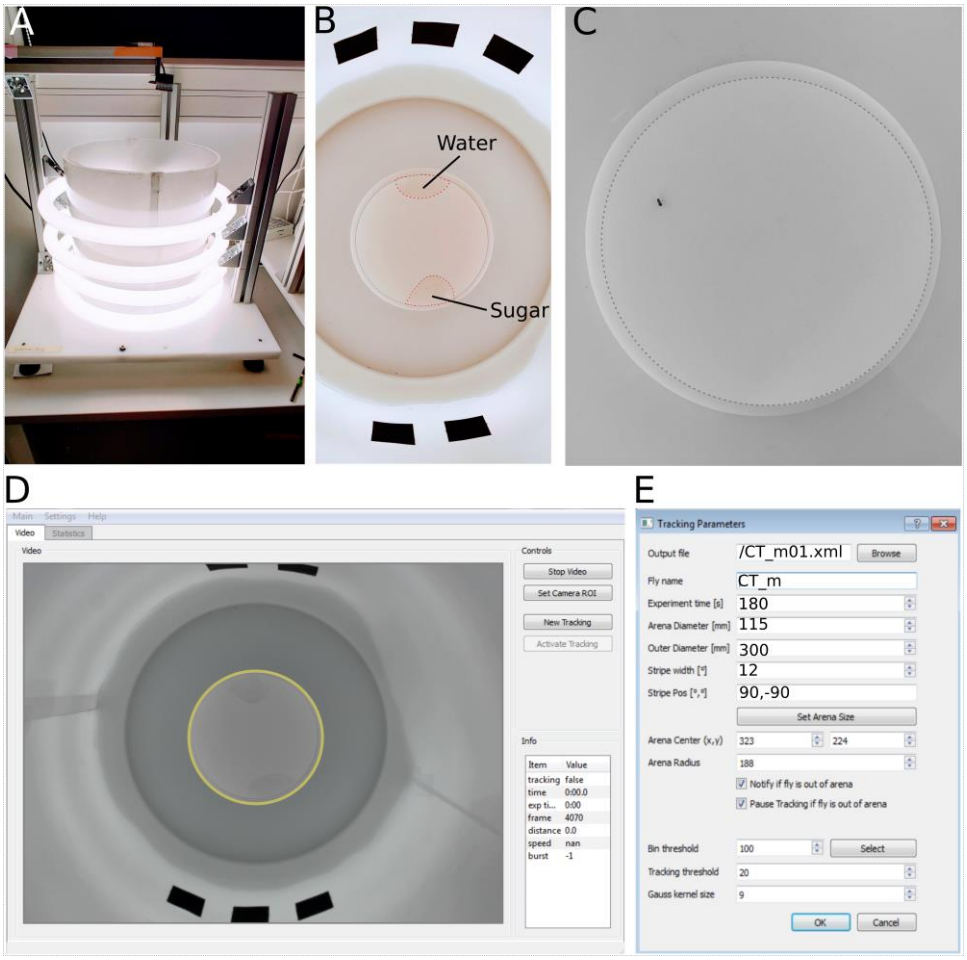


Figure 1. Behavioral apparatus and walking recording. A. Outside view of the Buridan paradigm. B. Top view of the assay, where a filter paper (gray dash line) is placed in the platform to add water or sugar next to the visual stimulus (red dashed lines). C. Picture of the fly performing during the testing session. D, E. Image of the Buritrack software to set the parameters of the experiment.

C. Run the training session

1. Set the temperature of the platform to 25 °C.
2. Press the Button *New Tracking* and adjust all the parameters in the *Tracking* dialog (Figure 1D). Generate a folder named "Training" to save your recording. Open Buritrack and set the parameters of the recording. Browse the Training folder, select the name of the file (e.g., TR_f01 for the case of TR group female #1; CT_m01 for the case of CT group male #1) and the fly group (e.g., TR_f, for female TR flies; CT_m for CT male flies), and the recording time (in seconds).
3. Set the size of the arena. While setting the platform position, left-click on three points on the platform edge. Check that the drawn circle fits with the platform; if not, you can right-click to erase one point (one per click) (yellow line in Figure 1D).
4. Gently place the fly on the platform. You may want to adjust the luminosity of your camera again.
5. Activate tracking in Buritrack to record the walking behavior (Figure 1D).
6. If the fly jumps off of the platform, the tracker stops and plays a sound alarming the experimenter. Put the fly back on the platform using a brush and click *Activate Tracking* again to continue with the experiment.
7. After 3 min the session is over. Gently remove the fly from the platform and place it back in their starving vial.

8. Clean the platform with 70% ethanol, prepare a new filter paper with sucrose and water for the next fly, and set the new file name in Buritrack. Once it is done, place the following fly and start a new recording by clicking the *New Tracking* button.
9. Continue running each fly individually by cleaning the platform with 70% ethanol between flies and using a new clean filter paper each time. Half of the animals will be trained and tested with one stimulus orientation (e.g., three squares on the top part of the image in the Buritrack software), and the other half will be trained and tested with the opposite orientation of the visual sets (e.g., three squares on the bottom part of the image).

D. Run the testing session

1. Right after finishing the training session, clean the platform with abundant water and 70% ethanol to remove any trace of glucose.
2. Two hours after finishing the training session, place a clean filter paper on the platform.
3. Place the fly to record the testing session.
4. Generate a folder named "Testing" to save your recording. Open Buritrak and set the parameters of the recording. Browse the Testing folder and select the name of the file, fly group, and the recording time (300 s). It is important to use the same names as in the training session (e.g., TR_f01, CT_m01) to then analyze the behavior of the very same fly during the training and testing sessions.
5. Set the size of the arena.
6. Activate tracking in Buritrack to record walking behavior.
7. After running the recording, remove the filter paper, clean the platform with 70% ethanol, and place a new round filter paper for testing the next fly.

Data analysis

1. Once the experiment is finished (at least 30 animals per group), store the data of the training and testing sessions in different folders by pooling the data collected in each setup.
2. Open RStudio.
3. Invert the x-y position of half of the animals (that were run with inverted configuration of the visual stimuli) by using the change_orientation_xy.R file (https://github.com/HassanLab/Bengochea_etal_2023/blob/main/change_orientation_xy.R).
4. Analyze the training and testing sessions by running the Analysis.R code (https://github.com/HassanLab/Bengochea_etal_2023/blob/main/Analysis_Learning.R).
5. Check if all flies (from trained and control groups) reached the sugar and water. Flies that did not visit either the sucrose or the water must be discarded.
6. After running the Analysis.R code, you will find the following output files:
   a. Individual trajectories: In a single PDF you will find the trajectory of each fly. The color and the size of the path changes depending on the amount of time spent in each position in the platform.
   b. Individual transition plots: Transition plots were done as described before (Colomb et al., 2012). Briefly, the platform was divided in 60 × 60 hexagons and the fly's position raised the count of each hexagon by one in the arena. The scale starts at 0 (blue) and goes up until a value calculated by the 95% quantile of the count-distribution (red).
   c. YPosition density plot: Population Y position density plot by group (Figure 2).
   d. Transition plots by group/group and sex: population transition plots depending on the group of interest.
   e. PI Comparison: Final boxplot to compare the performance of each group. Each dot indicates the PI for each fly tested. To calculate the preference index, the arena was divided into three zones. We sum the density of passage of the hexagons within zones close to the visual stimuli while the center part of the arena was not analyzed. Values indicate mean ± SD. The preference index was calculated as:

$$PI = \frac{totaldensity(largersetarea) - totaldensity(smallersetarea)}{totaldensity(largersetarea) + totaldensity(smallersetarea)}$$

For the statistical analyses, you should check for normal data distribution using the Shapiro-Wilk normality test. Then, we chose the appropriate parametric or non-parametric test. We statistically compared groups with the non-parametric Wilcoxon rank sum test (Figure 2).

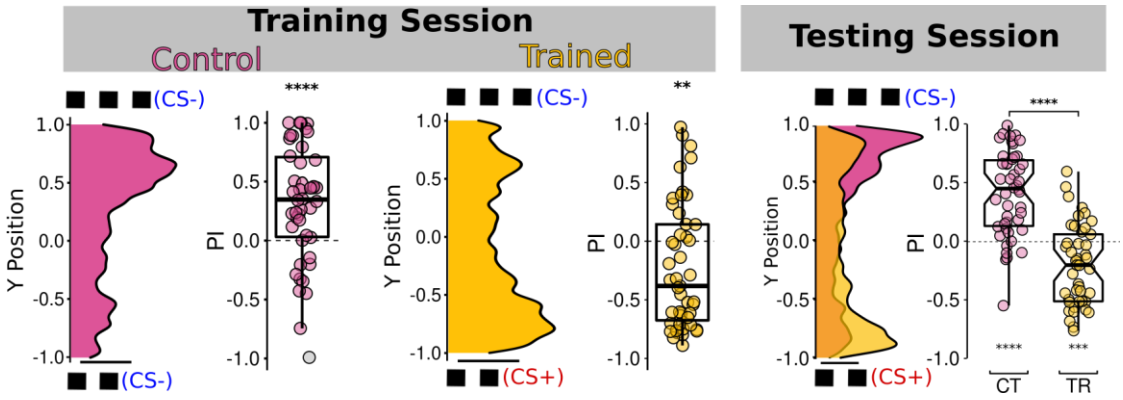
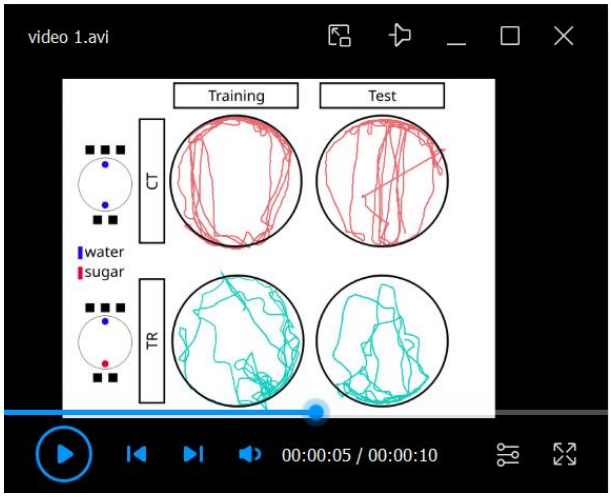


Figure 2. Flies trained in a 2 vs. 3 squares contrast showed a preference for the two squares during the testing session. Kernel density plots on the left denote the position permanence of the fly along y-axis for each group and session. On the right, boxplots show median population preference index. Trained flies significantly preferred the smaller set of squares [n = 45, PI = -0.20 ± 0.34, t(44) = -3.93, p = 2.9e-04, One sample *t*-test], opposite to the control group that shows a preference for the three squares set [n = 42, PI = 0.39 ± 0.36, t(41) = 7.19, p = 8.87e-09, One sample *t*-test; comparison between groups: t(84) = 7.9, p = 7.94e-12, Welch two sample *t*-test]. Box limits: upper (75) and lower (25) quartiles, and whiskers, 1.5× inter quartile range. Each dot corresponds to a single fly performance. Gray dots indicate outliers.



Video 1. Trajectories of two different flies: one that belongs to the control group (red traces) and one to the trained group (blue traces) during the training and testing sessions. This visual representation shows that trained flies tend to spend significantly more time in the proximity of the set of two squares when compared to the control group of untrained flies.

Limitations

Since flies are placed in the platform to freely explore the arena, one important limitation of the protocol is that we cannot control the amount of time each fly spends in contact with the sugar or water. Furthermore, we cannot control which stimulus will first reach the fly.

Discussion and conclusions

Studying the cognitive abilities and learning processes of animals is crucial for understanding their behavior and adaptation in their respective ecological niches. Visual learning, in particular, plays a vital role in an animal's survival and decision-making abilities.
Here, we established a new behavioral paradigm for visual classical conditioning in freely walking individual adult *Drosophila*. Our protocol, with a single training trial of 3 min, generates a visual memory that lasts at least 2 h, allowing the study of short-term visual memories. However, it remains to be analyzed whether flies can form long-term visual memories by applying longer training protocols. This protocol for studying visual learning in fruit flies has a significant impact on the field of research. Due to the flexibility of visual objects that can be presented, the protocol can be adapted to study other properties of visual learning and will allow to delve deeper into the intricate mechanisms underlying visual learning in insects.
This protocol adapts the well-standardized methodology for studying orientation behavior in freely walking flies, enabling other researchers to easily replicate the experiments. This contributes to the cumulative knowledge in the field and facilitates the validation of findings across different laboratories.
Studying invertebrate learning not only provides insights into the cognitive capacities of these animals but also contributes to our understanding of the evolution and mechanisms of learning across different species. By unraveling the intricacies of invertebrate learning, we can gain valuable knowledge about the fundamental principles of learning and cognition in general.

General notes and troubleshooting

Under optimal conditions, the results of experiments conducted on different days, seasons, and by different experimenters are reproducible. However, fluctuations in room temperature and humidity can impact behavioral performance. Therefore, it is crucial to maintain stable room conditions. When clipping the wings, it is important to handle the flies carefully to avoid causing harm. It is advisable to clip more flies than necessary for the experiment in case of any losses due to clipping or starvation. The remaining wing length should not exceed 1/3 of the original length to prevent excessive jumping from the platform. Strict timing is essential for both the training and testing sessions, and it can be challenging to run parallel groups. Efficiently placing and removing the flies from the platform is crucial to ensure consistent session durations for all flies. Prior training and practice are recommended before starting the experiment.

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Competing interests

The authors have no conflict of interest to declare.

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