

Characterizing Choice Behavior in *Drosophila* Olfactory Classical Conditioning

By

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ABSTRACT

Drosophila melanogaster is a powerful model organism to understand the neural and molecular mechanisms underlying learning and memory. However, memory is usually studied by assessing choice behavior at only one point in time in *Drosophila*, which limits our understanding of how flies respond to a learned cue. We developed a simple open-field style assay that records the continuous behavioral dynamics of flies as they form preferences between two competing olfactory stimuli. We trained flies to associate odors with either a reward (sucrose) or punishment (electric shock) and found that within 30 seconds, flies learned to approach the sugar-associated odor and avoid the shock-associated odor. Their collective behavior dynamics suggest the flies became more decisive over time; they traveled more on the preferred side, stayed closer to the source of the preferred cue, made more stops there, and consistently turned away from alternative cue. We confirmed these results by showing that a learning-deficient mutant strain displayed no choice behavior. This assay provides enhanced resolution for understanding decision-making as a continuous, dynamic process in *Drosophila*.

INTRODUCTION

The ability of an animal to recall cues associated with aversive stimuli, such as predators, or appetitive stimuli, like food, enhances survival in complex and dynamic environments (Dukas, 2007; Shettleworth, 2009; Redish and Mizumori, 2015; Morand-Ferron, 2017). This highly conserved ability is demonstrated in evolutionarily diverse animals, from sea slugs to humans (Walters, Carew and Kandel, 1981; Molchan *et al.*, 1994). Use of *Drosophila melanogaster* in particular has helped uncover conserved genetic and circuit-level components of associative memory during formation, maintenance, and suppression (Davis, 1993, 2011, 2023; Dubnau *et al.*, 2001, 2003; Heisenberg, 2003; Schwaerzel *et al.*, 2003; Waddell, 2013; Aso *et al.*, 2014; Cognigni, Felsenberg and Waddell, 2018).

The association between an odor and a reinforcer is the most well-characterized form of memory in *Drosophila* and remains a benchmark of learning and memory in contemporary research. (Quinn, Harris and Benzer, 1974; Tabone and de belle, 2014). Particularly, the introduction of a simple T-maze assay in which flies chose between two odor options to avoid the one previously paired with intermittent shock marked a significant advancement in the field (Tully and Quinn, 1985; Pitman *et al.*, 2009). The use of population-based memory assays, including the *Drosophila* T-maze, has accelerated the discovery of genes, signaling pathways, and neural circuits involved in encoding aversive and appetitive memory (Dudai *et al.*, 1976; Dudai, 1983; Dévav *et al.*, 1986; Folkers, Drain and Quinn, 1993; Schwaerzel *et al.*, 2003; Guven-Ozkan and Davis, 2014; Lee, 2015; Boto, Stahl and Tomchik, 2020; Davis, 2023). Consequently, *Drosophila* olfactory conditioning methodology has remained largely unchanged for over 30 years (Dwijesha *et al.*, 2024).

Decision-making is a dynamic process (Wang, 2012; van Breugel, 2021; Harris and Hutcherson, 2022), yet maze-based assays typically restrict analysis of choice behavior to the distribution of an entire population at a single point in time, which limits the ability to understand

the decision-making process. It's essential to measure decision-making as it occurs since distinct patterns of locomotion and transitions between stimuli occur across different stages of memory (Chabaud, Preat and Kaiser, 2010; Ichinose and Tanimoto, 2016). However, current olfactory conditioning assays cannot measure behaviors that individual flies within a group perform while choosing between a paired and unpaired odor, leaving the behavioral strategies *Drosophila* exhibit during and after making a decision poorly understood.

To better characterize choice-dependent behaviors in flies expressing associative olfactory memory, we introduce a high-throughput two-choice assay that consists of an open-field style circular arena in which groups of flies can continuously choose between two odors streaming from opposite sides of the arena. For scalability, the apparatus consists of four circular arenas capable of testing multiple groups simultaneously, while also recording the data of individual animals over time to capture individual variation in choice behavior. During assessment of olfactory memory two minutes after training, we compared locomotor and positional metrics between arena sides during continuous access to conditioned stimuli. Here, we describe the timeline of decision-making between stimuli following appetitive (sucrose) and aversive (shock) conditioning, as well as choice-dependent behavioral metrics that may better characterize decision-making in *Drosophila*.

METHODS

Fly husbandry

The following *Drosophila melanogaster* strains were used for appetitive and aversive short-term memory experiments: *Canton-S* (RRID:BDSC_64349), *w¹¹¹⁸*, (RRID:BDSC_3605), and *dunce* (Pde4[dnc-1], RRID:BDSC_6020). Flies were reared on standard cornmeal-agar food with tegosept antifungal and propionic acid antibacterial substrates inside 40 mL plastic vials and housed in a 24 °C incubator at 70% humidity with a 14:10 Light:Dark cycle with lights on at 7:00 am. Vials were flipped every three days to prevent crowding. 1- to 2-day-old males were collected under humidified CO₂ for < 5 minutes and given 24 hours to recover. Following CO₂ recovery, all flies underwent 24 hours of wet starvation to homogenize hunger state prior to training. Vials housed 30 collected flies to ensure 20 flies per vial could be tested following starvation. All flies were 3-5 days old at the time of testing. Behavior trials were conducted between 2 and 9 hours after lights on.

Two-choice assay components and fabrication

All components for the assay and airflow equipment are listed in Table S1. The Two-Choice apparatus consists of stacked Teflon (CS Hyde, Lake Villa, Illinois) and white acrylic (Acrylic-TL-White-7328, Plastics 2000, Modesto, California) sheets that form a four-arena behavioral chamber (for dimensions see Fig. 1B). Two removable clear Plexiglas lids (TAP plastics) cover the arenas (Fig. S1). A PDF schematic for laser cutting each acrylic arena layer and Plexiglas lids is available at <https://github.com/kaun-lab>. Each circular arena contains two port entrances on opposite sides for simultaneous introduction of odors. Nylon mesh port screens cut to 6 x 2 mm rectangles (McMaster-Carr, Elmhurst, Illinois) are fitted within slits covering the port entrances. Two perforated parallel lines surrounding the midline in each circular arena allow vacuum of air or odor to prevent odor accumulation over time. Sheets were

glued (Loctite, 681925, Rocky Hill, Connecticut) and lids fastened with nylon socket head screws (95868A261, McMaster-Carr). 3mm diameter Teflon tubes (F015090, Fluorostore, Parsippany, New Jersey) were pushed into each entrance and secured. Tubes and screens were covered with PTFE FEP tape (CS Hyde). Teflon tubes were capped with 1/8" male luer to hose barb adapters (MFLX30800-24, Masterflex, Vernon Hills, Illinois) for connection to odor source tubes. The vacuum tube was inserted and secured in the vacuum chamber of the Two-Choice (Fig. S1C).

Air and odor delivery

Two air and one vacuum flowmeters were set at 50 and 150 mL/min, respectively, on flowmeters (air flowmeters 112-02 Glass, Cole-Parmer; vacuum flowmeter 092-04 Glass, Cole-Parmer), such that each 50 mL/min of air flowed into both ports of an arena at once and would not build up within the arenas during trials. A 3-way valve (411L3212HV, Asko, Suwanee, Georgia) was connected via swivel-elbow tubes (7880T189, McMaster-Carr) to a flowmeter (Cole Parmer, Vernon Hills, Illinois) and two 40 mL glass vials with polypropylene caps (Fisher); one filled with 3mL odor and the other vial with 10 mL ddH₂O. Two Plastic Luer Lock Tube Coupling (51525K222, McMaster-Carr) were tapped into each odor vial cap and connected to Teflon tubes leading to each arena. A custom switchboard was wired to a 3-way valve to control the airflow to vials with ddH₂O or odors. The tube exiting the ddH₂O vial and the tube exiting the odor were joined into a single tube by a Wye connector (5111K542, McMaster-Carr), and the exiting single tube was connected to another Wye connector to lead the lines to the two ports of arena. Each of the air flowmeters supply air to the connected odor vials.

This assay requires that neither odor used induces a significant aversion or preference. Thus, to ensure no inherent odor preference, we measured preference between varying concentrations of Ethyl Acetate (EA) (Fisher Science Education, Hanover Park, Illinois) and Isoamyl Acetate (IAAc) (Fisher Science Education) in odor-naïve Canton-S and *w*¹¹¹⁸ flies. Prior

to odor balancing experiments, flies were wet-starved for 24 hours via placement in 10 mL vials prefilled with 2 mL of 1% agar after an initial 24 hours of CO₂ recovery. Two concentrations EA and IAAc were diluted in mineral oil (Fisher Science Education). The pairs of concentrations evaluated were [1:512 EA; 1:512 IAAc], [1:1024 EA; 1:1024 IAAc] and [1:512 EA; 1:1024 IAAc] (Fig. 2). After dilution, 3 mL of each odor solution was pipetted into the glass vials attached to the two-choice tubes.

During odor balancing experiments, flies were placed in Two-Choice arenas in groups of 10 and exposed to odorless airflow for 15 seconds on each arena side to evaluate baseline distribution across arena sides. After this initial phase, flies were exposed to EA and IAAc emitted from opposite arena sides for 90 seconds. Preference for an arena side following odor exposure was then compared to baseline side preference without odor stimuli (Fig. 2E). For memory experiments, concentrations of 1:512 EA and 1:1024 IAAc were used as these concentrations did not invoke a biased preference on either arena side (Fig. 2J-M). Odors were replaced every other trial to ensure consistent potency.

Behavior environment

All behavior experiments were conducted inside a dark temperature- and humidity-controlled environment. The chamber consisted of a 68 x 53 x 43 cm³ box was composed of 5 mm thick black corrugated plastic boards (CorrugatedPlastics.Net) with an accessible entrance at the front that, when closed, provided a dark environment. A SMART 200W electric flat panel radiant heating plate with adjustable thermostat (SUMLGIT, Shenzhen Jinhui Technology Co., Ltd) with two attachable 12x12x2.5 cm 12V low-noise 3-Pin 1400 rpm computer fans (UMLIFE), was placed at the back of the box to ensure a consistent temperature of 24°C throughout the interior. Humidity was controlled by flowing humidified air directly into the circular behavior arenas via Teflon tubing. A circular port at the top of the box fed directly to an exhaust arm to prevent potential odor accumulation within the box.

Odor-shock training

Shock arenas were composed of layered acrylic materials using dimensions as described in (Scaplen *et al.*, 2019) with a custom designed copper grid with 36 0.5 mm width copper (BasicCopper) strips spaced 0.5 mm apart fixed to the arena floor (Fig. S2). A PDF schematic for laser cutting each acrylic arena layer and Plexiglas lids is available at <https://github.com/kaunlab>. Connecting wires from the copper grid were attached to each arena floor and fed into a single-pin cable mount (Switchcraft, Chicago, IL), leading to the Pulse Stimulator.

All shock-trained flies were wet-starved for 24 hours via placement in 10mL vials prefilled with 2 mL of 1% agar. To condition shock memory, flies within agar vials were taken from the incubator and aspirated into shock arenas in the dark at 24° C and 70% humidity and exposed to either EA (1:512) or IAAc (1:1024) without shock for two minutes. Flies were then exposed to the reciprocal odor while receiving 80 V shocks (S48 Square Pulse Stimulator, Astro-Med, Warwick, RI) lasting 1.5 seconds every 5 seconds for two minutes. Because shock conditioning took place in arenas with the same dimensions as the two-choice assay, the same flow rates were used (50 mL/min). The two-minute exposures to each odor and condition were repeated a total of three times in one training session.

Odor-sucrose training

Sucrose training tubes consisted of a 40mL clear polypropylene tube (Fisher) containing a filter paper dampened with 3 mL ddH₂O or 2 mM sucrose, then placed into a 1280 cm³ plexiglass box where either EA (1:512) or IAAc (1:1024) was streamed for 2 minutes. The ddH₂O and sucrose filter papers were set in the training vials the previous evening and left to dry overnight. Training vials included a rubber cap with a 24 mm diameter circular hole covered with nylon mesh screen to expose flies to odor stimuli within the plexiglass boxes. Because the

volumes of each plexiglass box were much greater than the two-choice arenas, odor flow rates to each box were set at 1.2 L/m by flowmeters (092-04 Glass, Cole-Parmer). Airflow proceeded from 1/2" inner diameter Teflon tubing (5239K17, McMaster-Carr) to a glass bubbler containing 200 mL ddH₂O. Humidified air reached both glass vials containing each odor simultaneously by forking into separate tubes, then flowed to each plexiglass box. A passive vacuum port at the opposite end of each box directed the air/odor out at the same flow rate as incoming air/odor.

All sucrose-trained flies were wet-starved for 24 hours by being placed in 10mL vials prefilled with 2 mL of 1% agar. To condition sucrose memory, the agar-filled vials were brought from the incubator to a plexiglass box set to 24° C and 70% humidity. Flies were first aspirated into training vials with H₂O and paired with an odor (unpaired condition). Following two minutes of odor exposure in the H₂O training vial, flies were immediately transferred into a separate training vial containing filter paper dampened with 2 mL of 2 mM sucrose in ddH₂O and exposed to the reciprocal odor in a second plexiglass box for two minutes. The two-minute exposures to each odor and condition were repeated a total of three times in one training session. Sucrose training occurred under red light for experimenter visibility as vials were transferred between training boxes.

Odor-choice memory test

Two minutes after shock or sucrose memory conditioning (Fig. 1A), flies were aspirated into the Two-Choice apparatus (10 flies per arena). Two arenas of a single genotype/condition were tested simultaneously in each trial. All trials for a respective genotype/condition took place on the same day in a counterbalanced order by which odor was paired with unconditioned stimuli. Each memory trial lasted 135 seconds; flies received only air for the first 15 seconds, then were exposed to 1:512 EA and 1:1024 IAAC simultaneously from opposite ports for the remainder of the trial. Arenas were wiped down with ddH₂O and dried between each trial. Naive flies did not receive odor conditioning before testing.

Data collection and analysis

All data files and recordings are available for download in the Brown Digital Repository (<https://doi.org/10.26300/4bv0-xd14>). Videos were recorded at 30 frames per second and saved as .AVI files using FlyGrAM software and arena stand (Scaplen *et al.*, 2019). In the FlyGrAM GUI, the “Experimental Duration” parameter was set to 135 seconds, the “Time until Stimulus Onset” parameter was set to 15 seconds, and “Stimulus Duration” parameter was set to 120 seconds. “Write .AVI Video” and “Write .CSV File” checkboxes were also selected. Recordings were captured with a USB camera (USBFHD01M-L21, ELPCCTV, China) capped with a 6mm focal length F2.0, M12 adaptor lens (PT-0620, M12 Lenses, Maitland, Florida). An infrared filter (54–664 (RG-850), Edmund Optics, Barrington, New Jersey) was applied to the lens to record trials while avoiding the confounding effects of light exposure (Han et al., 2025). Raw data was retrieved from video files using TRex software (Walter and Couzin, 2021). Detection lighting threshold value was set to 15.

The position of each fly was represented as the center of mass or “centroid” point in TRex, and the midline separating arena sides was defined by a 2-dimensional line, such that a fly could not be classified as part-way or “in between” arena sides. If Trex lost track of a fly’s position, their data was supplanted by the average of values directly before and after the period during which tracking was lost. All raw behavioral metrics output by TRex were aggregated using a custom-written PANDAS script (annotated code and instructions available at <https://github.com/kaun-lab>).

The behaviors of Midline U-turns and stops close to odor port behaviors adapted the parameters of a sharp turn and stop behavior described in Ctrax software (Branson, Alice A. Robie, *et al.*, 2009) as well as additional parameters; U-turns began within 0.5 cm of the midline with the fly facing the midline (head at a $<|90|^\circ$ angle from the midline) and ended with the head’s orientation at a greater angle from the midline than at the beginning of the U-turn (Fig.

S5). Stops close to odor port occurred within 0.9 cm of the port, closer to the odor port than the arena midline. To quantify discrete behaviors that do not occur continuously (midline U-turns, stops close to odor port, and arena side transitions) the average number of each behavior made per-fly was binned every five seconds. The bin size of five seconds was chosen to avoid reporting rates of discrete behaviors in trivially small units (e.g. average U-turns made per-frame would be a small decimal) while retaining the ability to visualize change over time when plotted on a line graph. Cumulative distance travelled over time was also averaged across flies and binned every five seconds as binning would not affect the average of cumulative values. The average length of time taken to transition from each respective arena side, hereafter referred to as bout duration, was instead binned every 15 seconds as flies in all groups made an average of fewer than one transition every ten seconds (Fig. S4).

Memory was quantified with a Preference Index (PI); the proportion of flies avoiding shock-paired odor (or approaching sucrose-paired odor) minus the proportion that did not (Quinn, Harris and Benzer, 1974). To visualize both choice dynamics across time and variability across flies, we calculated PI in two ways, by group and by individual. Line graphs plot group PI over time and bar graphs plot the average individual PI, with individuals represented by dots. Group PI was calculated at each frame by subtracting the number of flies on the unpaired odor side from the number of flies on the paired odor side and dividing by the total number of flies (Ichinose and Tanimoto, 2016). The group PI at time t is;

Equation 1)

$$PI_{group}(t) = \frac{N_+(t) - N_-(t)}{N_+(t) + N_-(t)}$$

where $N_+(t)$ and $N_-(t)$ denote the number of flies occupying each arena side in one frame, respectively.

To determine individual PI values per-fly, the time a fly spent on the unpaired odor side was subtracted from the time spent on the paired odor side out of a total time interval (T), expressed as;

Equation 2)

$$PI_f = \frac{1}{T} \sum_{t=1}^T F_f(t)$$

Where $F_f(t)$ indicates the PI value of each fly (f) at each frame (t) as;

$$F_f(t) = \begin{cases} +1 & \text{if in CS +} \\ -1 & \text{if in CS -} \end{cases}$$

and averaged across individuals as

Equation 3)

$$\overline{PI}_{indiv} = \frac{1}{N} \sum_{f=1}^N PI_f$$

Flies that did not make at least one transition across arena sides during the trial were excluded for all measures, including group and individual PI, and were not considered as Ns in calculated averages. Thus, all included flies must have an individual PI of >-1 , <1 when calculated over the entire trial. However, when individual PI is calculated over a portion of a trial, such as before odors are introduced, included flies may still have a value of -1 or 1 , indicating that they did not transition within that portion, but at a different time in the trial.

Statistical tests were performed in GraphPad Prism 10.5.0. A minimum of eight arenas (10 flies per arena) were ran per genotype/condition, equivalent to eight independent samples (as arenas) and $N = 80$ flies tested per group. Assumptions of parametric statistics were assessed with D'Agostino–Pearson, Shapiro–Wilk, and Kolmogorov–Smirnov tests. Two-Way

ANOVAs compared effects of (arena side $\times$ experimental phase), One-Way ANOVAs compared effects between conditions, and individual comparisons following ANOVAs were evaluated by Tukey's multiple comparisons post-hocs. Within each condition, comparisons between arena sides and between experimental phases were performed using paired t-tests. Comparisons between genotypes were performed using unpaired t-tests. Asterisks denote significant differences * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. N counts and statistical tests per-figure are shown in Table S2.

RESULTS

A simple two-choice assay to test olfactory memory

Using the two-choice assay, we assessed decision-making over time in two genetic backgrounds commonly used in memory assays (*Canton-S* and *w¹¹¹⁸*). Our first goal was to find odor concentrations that did not produce a strong bias towards one odor over the other. This is key to this assay since innate odor bias can easily obscure learned preference. Flies were exposed to concentrations of Ethyl Alcohol (EA) and Isoamyl Acetate (IAAc) that were streamed from opposite arena sides and tracked for 2 minutes.

Since transitions between regions containing different stimuli have effectively been used to measure choice (Chabaud, Preat and Kaiser, 2010), we hypothesized that a stabilization in average transitions would indicate when a choice was made. Naïve *Canton-S* flies increase transitions when odors permeate the arenas (~5 seconds after odors begin flowing), but transitions significantly decrease after 25 seconds (Fig. 2B-C, $p = 0.0007$). Within the remaining 90 seconds of the experiment, average transitions did not significantly change (Fig. 2D, $p = 0.06$). These results suggest that a collective choice of arena side is made within 30 seconds of odor exposure and maintained for the remainder of the experiment. Henceforth, these intervals will be referred to as the “exploration” and “choice” phases, respectively (Fig. 2B).

We then compared the average preference index per-fly (Fig. 2E) between the odor-off and choice phases to evaluate odor bias for EA or IAAc at differing concentrations. Naïve *Canton-S* flies showed a preference for 1:512 EA over 1:512 IAAc (Fig. 2F-G, $p < 0.0001$). They also preferred 1:1024 EA over 1:1024 IAAc (Fig. 2H-I, $p = 0.02$) but did not prefer 1:512 EA over 1:1024 IAAc (Fig. 2J-K, $p = 0.33$). To confirm a lack of odor bias between 1:512 EA and 1:1024 IAAc, we tested naïve *w¹¹¹⁸* flies and found that they also did not prefer EA over IAAc at these concentrations (Fig. 2L-M, *w¹¹¹⁸* $p = 0.80$). These results collectively demonstrate

that naïve flies do not show significant bias between 1:512 ethyl alcohol and 1:1024 isoamyl acetate, suggesting they are reasonable odor concentrations to use for olfactory conditioning.

Memory for an odor previously paired with sugar or shock

We next evaluated memory for electric shock or sucrose on the Two-Choice apparatus by testing whether flies would avoid an odor previously paired with shock or move towards an odor previously paired with sugar. Flies were trained to associate an odor with three successive 2-minute sessions of 80mV shock, or three successive 2-minute sessions of 2M sucrose (Fig 3A). After training, flies were tested for preference for the unconditioned stimulus paired odor relative to the unpaired odor (Fig. 3B). Naïve flies did not receive any previous experience with odors, shock, or sucrose.

Group preference index was plotted continuously over time (Fig. 3C-D), and an individual preference index was averaged across odor-off and choice phases to quantify learning within each condition. The average preference index of Naïve *Canton-S* flies did not differ between odor-off and choice phases (Fig. 3E, $p = 0.19$). Shock-trained *Canton-S* exhibited greater avoidance of the shock-paired odor during the choice phase (Fig. 3F, $p < 0.0001$), while sucrose-trained *Canton-S* flies exhibited greater preference for the sucrose-paired odor during the choice phase (Fig. 3G, $p < 0.0001$).

Similar to their *Canton-S* counterparts, naïve w^{1118} flies did not develop an arena side preference (Fig. 3H, $p = 0.11$). However, shock-trained w^{1118} showed greater avoidance of the side containing shock-paired odor (Fig. 3I, $p < 0.0001$), and sucrose-trained w^{1118} showed greater preference for the side containing sucrose-paired odor (Fig. 3J, $p = 0.007$). These results demonstrate robust aversive and appetitive memory in both wild-type *Canton-S* and w^{1118} flies.

To determine whether flies were forming associations between each odor and the unconditioned stimulus similarly, we plotted memory for each odor individually (Fig. S3). Shock-trained *Canton-S* (Fig. S3A) flies avoided shock paired with either odor, but the effect size was slightly larger in those that received shock paired with ethyl alcohol (Fig. S3B-C, EA shock-paired $p < 0.0001$; IAAc shock-paired $p = 0.02$). Shock-trained w^{1118} (Fig. S3D) also avoided shock paired with either odor, but the effect size was slightly larger in those that received shock paired with isoamyl acetate (Fig. S3E-F, EA shock-paired $p = 0.02$; IAAc shock-paired $p = 0.0004$).

Interestingly, sucrose-trained *Canton-S* flies that received sucrose paired with EA preferred the sucrose-paired odor (Fig. S3G-H, $p < 0.0001$) while *Canton-S* flies that received sucrose paired with IAAc did not prefer IAAc during testing (Fig. S3I, $p = 0.22$). Similarly, only sucrose-trained w^{1118} flies that received sucrose paired with IAAc preferred the sucrose-paired odor (Fig. S3J-K, $p = 0.004$) while those that received sucrose paired with EA did not prefer EA during testing (Fig. S3L, $p = 0.18$). Together, these results suggest that behavioral expression of memory may depend on which odors are used in the assay and how they interact with both the unconditioned stimulus and the genetic background of the flies being tested.

Associative memory conditioning impacts locomotion during odor choice

Flies exhibit freezing behavior following exposure to aversive or threatening stimuli (Gibson *et al.*, 2015; Zacarias *et al.*, 2018; Ferreira and Moita, 2020; Sato and Takahashi, 2025), while sucrose ingestion induces locomotion and goal-directed foraging behavior (Bell *et al.*, 1985; Bazzell *et al.*, 2013; Murata, Brockmann and Tanimura, 2017; Rajagopalan *et al.*, 2023). We therefore hypothesized that shock-training would reduce locomotor activity, and sucrose-training would increase locomotor activity (Fig. 4). We recorded the average cumulative distance travelled by each group continuously over time (Fig. 4 A-D) and compared the average

cumulative distance travelled between naïve and trained flies during the odor-off and post-choice phases.

Shock-trained *Canton-S* travelled less overall than naïve *Canton-S* (Fig. 4E, $p < 0.0001$) but not during the odor-off phase (Fig. 4F, $p = 0.59$), whereas shock-trained w^{1118} travelled less than naïve w^{1118} overall (Fig. 4G, $p < 0.0001$) and less during the odor-off phase (Fig. 4H, $p < 0.0001$). Sucrose-trained *Canton-S* flies also travelled more overall than naïve *Canton-S* flies ($p < 0.0001$) and travelled more during the odor-off phase ($p = 0.002$). Sucrose-trained w^{1118} flies additionally travelled more than naïve w^{1118} flies overall ($p = 0.002$) and more during the odor-off phase ($p = 0.04$). These results reflect that sucrose-training increased locomotor activity and shock-training decreased activity.

Distance moved is associated with learned preference

Regional locomotor activity has previously been used as a measure of deliberate engagement with conditioned stimuli in rodents (Totty *et al.*, 2021; Iglesias *et al.*, 2023) and *Drosophila* (Chabaud, Preat and Kaiser, 2010). Therefore, despite our previous findings that sucrose and shock conditioning have opposite effects on distance travelled, we surmised that both conditioned groups would travel more in the arena side they preferred. We hypothesized that shock-trained flies would exhibit lower activity in the presence of shock-paired odor compared to the no-shock-paired odor, and sucrose-trained flies would exhibit greater locomotor activity when in the presence of sucrose-paired odor compared to the no-sucrose-paired odor (Fig. 5A-B). To evaluate this, we analyzed the mean distance travelled per five-second bin on each arena side. To account for unequal distribution of flies over time, per-frame means were normalized to the number of flies occupying each side before binning.

In naïve flies, distance travelled did not differ between arena sides (Fig. 5C-D, *Canton-S* $p = 0.23$; Fig. 5E-F, w^{1118} $p = 0.28$). Following shock training, both genotypes travelled less on

the side containing shock-paired odor (Fig. 5G-H, *Canton-S* $p < 0.0001$; Fig. 5I-J, w^{1118} $p < 0.0001$). Shock-trained *Canton-S* travelled more on the no-shock-paired odor side during the odor-off phase (Fig. 5H, $p = 0.04$ exploration phase ($p < 0.0001$) and choice phase ($p < 0.0001$). Shock-trained w^{1118} also travelled more on the no-shock-paired odor side during the odor-off phase (Fig. 5J, $p = 0.003$), exploration phase ($p = 0.003$), and choice phase ($p < 0.0001$). While effects during the odor-off phase could reflect a response to trace amounts of odor residue or an overall reduction in activity in shock-trained flies, differences were greatest in the choice phase for both genotypes, suggesting a distinct reduction in activity in the presence of the conditioned odor.

Following sucrose training, both genotypes travelled more on the side containing sucrose-paired odor (Fig. 5K-L, *Canton-S* $p < 0.0001$; Fig. 5M-N, w^{1118} $p < 0.0001$). When compared at each phase, *Canton-S* flies travelled more on the no-sucrose paired odor side during the odor-off phase (Fig. 5L, $p = 0.02$), but travelled more on the sucrose-paired odor side during the exploration phase ($p = 0.006$) and choice phase ($p < 0.0001$). w^{1118} flies did not travel more on either side during the odor-off phase (Fig. 5N, $p = 0.23$) but travelled more on the sucrose-paired odor side during the exploration phase ($p < 0.0001$) and during the choice phase ($p = 0.01$). These results indicate that sucrose-trained flies travel more on the side containing the sucrose-paired odor. Overall, distinct locomotion responses in the presence of the conditioned odor reflect a learned behavioral response that may be expressed as the choice to engage with one stimulus over another.

Latency to transition between sides is associated with learned preference

Previous work has also used counts of arena side transitions to represent choice in flies (Chabaud, Preat and Kaiser, 2010), but did not measure transitions continuously over time. We hypothesized that trained flies would make fewer average transitions in the choice period compared to naive flies, indicating learned preference. To measure this, we averaged the

number of transitions between arena sides binned every five seconds in the choice phase (Fig. S4A-H). Compared to naïve *Canton-S*, shock-trained *Canton-S* made fewer average transitions in the choice phase (Fig. S4I $p < 0.0001$) and fewer overall transitions (Fig. 4SJ $p < 0.0001$) in the choice phase than naïve *Canton-S*. However, sucrose-trained *Canton-S* did not make fewer average transitions in the choice phase (Fig. S4I, $p = 0.99$) or total transitions (Fig. S4J, $p = 0.99$).

Shock-trained w^{1118} flies made fewer average transitions per time bin in the choice phase ($p < 0.0001$) and fewer overall transitions ($p < 0.0001$) than naïve w^{1118} . Like *Canton-S* flies, sucrose-training did not reduce average transitions in the choice phase (Fig. S4K, $p = 0.99$) or total transitions made (Fig. S4L, $p = 0.99$), but did decrease the overall number of transitions made ($p = 0.03$). Given that sucrose-trained flies showed a clear preference for sucrose-paired odors (Fig. 3), the lack of difference between sucrose-trained and naïve flies in number of transitions may simply reflect the number of flies that are moving.

To further assess transitions as a metric of choice, we next evaluated the average latency to transition from each arena side, hereafter referred to as “bout durations” (Fig. 6A-B). Because flies in all groups made an average of less than one transition every 10 seconds throughout the experiment (Fig. S4J-L), average bout durations over time were binned every 15 seconds. We hypothesized that over time, trained flies would wait longer to transition out of their preferred arena side. As predicted, average bout duration throughout the experiment did not differ between arena sides in naïve flies (Fig. 6C-D, *Canton-S* $p = 0.39$; Fig. 6E-F, w^{1118} $p = 0.83$). Following shock training, average bout durations were reduced on the side containing the shock-paired odor (Fig. 6G-H, *Canton-S* $p < 0.0001$; Fig. 6I-J, w^{1118} $p < 0.0001$). When compared at each experimental phase, there was no difference in bout duration between sides in either genotype during the odor-off phase (Fig. 6H, *Canton-S* $p = 0.29$; Fig. 6J, w^{1118} $p = 0.26$), but both genotypes had a reduced average bout duration on the side containing shock-

paired odor during the exploration phase (*Canton-S* $p < 0.0001$; w^{1118} $p = 0.008$) and the choice phase (*Canton-S* $p < 0.0001$; w^{1118} $p < 0.0001$).

Following sucrose training, bout duration was greater on the side containing sucrose-paired odor in both genotypes (Fig. 6K-L, *Canton-S* $p < 0.0001$; Fig. 6M-N, w^{1118} $p < 0.0001$). When compared at each phase, *Canton-S* flies exhibited lower bout durations on the sucrose-paired odor side during the odor-off phase (Fig. 6L, $p = 0.04$), but greater bout durations on the sucrose-paired odor side during the exploration phase ($p = 0.006$) and choice phase ($p < 0.0001$). In sucrose-trained w^{1118} , bout duration did not differ between sides during the odor-off phase (Fig. 6N, $p = 0.67$) but was greater in the sucrose-paired odor side during the exploration phase ($p < 0.0001$) and choice phase ($p < 0.0001$).

These results indicate that during the exploration and choice phases, flies transition readily between odors but have longer bouts in the side they prefer. Over time, they will more rapidly exit the odor previously paired with shock and linger with the odor previously paired with sucrose. Together with regional distance travelled, mean bout duration may reflect the choice between different stimuli in associative memory assays.

Behaviors that reflect that a decision has been made

We next sought to identify metrics within the choice phase that indicate the maintenance of a decision (Fig. 7). We first evaluated midline U-turns, instances where flies approach the center of the arena then turn back around to remain on the same side (Fig. 7A; for parameters see Fig. S5). Since the number of U-turns made depends on locomotor activity, the metric was normalized as average U-turns per cm travelled. Naïve *Canton-S* flies did not make more U-turns per cm travelled on one arena side over the other (Fig. 7B, $p = 0.16$), but shock-trained *Canton-S* made fewer U-turns per cm travelled on the side containing shock-paired odor (Fig. 7C, $p < 0.0001$), and sucrose-trained *Canton-S* made more U-turns per cm travelled on the side

containing sucrose-paired odor (Fig. 7D, $p = 0.03$). Similarly, naïve w^{1118} flies did not make more U-turns per cm travelled on either side during the post-choice phase (Fig. 7E, $p = 0.32$). Shock-trained w^{1118} also did not make more U-turns per cm travelled on either side (Fig. 7F, $p = 0.16$), perhaps due to a greater reduction in overall locomotion than shock-trained *Canton-S* flies (Fig. 4. E-H). Sucrose-trained w^{1118} also made more U-turns per cm travelled on the side containing sucrose-paired odor (Fig. 7G, $p = 0.003$). These results suggest that once flies decide between stimuli, they actively reject the stimulus they did not choose.

The average proximity flies stayed from the odor port while occupying each arena side was also calculated during the choice phase (Fig. 7H). We hypothesized that, when occupying their respective arena sides, flies would stay closer to the preferred stimulus than they would to the other stimulus. Naïve *Canton-S* did not get closer to ports emitting either odor (Fig. 7I, $p = 0.13$), but shock-trained *Canton-S* stayed farther from the port emitting shock-paired odor (Fig. 7J, $p < 0.0001$), and sucrose-trained *Canton-S* stayed closer to the port emitting sucrose-paired odor (Fig. 5K, $p < 0.0001$). Similarly, naïve w^{1118} also did not get closer to either odor port (Fig. 7L, $p = 0.39$), whereas shock-trained w^{1118} stayed farther from the port emitting shock-paired odor (Fig. 7M, $p < 0.0001$) and sucrose-trained w^{1118} stayed closer to the port emitting sucrose-paired odor (Fig. 7N, $p = 0.04$)

Given that flies stay closer to their chosen stimulus in the post-choice phase, we hypothesized that they would also stop more frequently close to the odor port emitting the preferred odor during the choice phase. A stop behavior met the per-frame parameters of a stop as specified previously (Branson, Alice A Robie, *et al.*, 2009), where proximity to the odor port was defined as being within 0.9 cm of the odor port (Fig. 7O). In naïve *Canton-S* flies, there was no difference in the total number of close-to-port stops between ports on either side (Fig. 7P, $p = 0.052$), while shock-trained *Canton-S* made fewer stops close to the port emitting shock-paired odor (Fig. 7Q, $p < 0.0001$) and sucrose-trained *Canton-S* made more stops close to the port

emitting sucrose-paired odor (Fig. 7R, $p < 0.0001$). Similarly, naïve w^{1118} flies also did not make more stops close to either port (Fig. 5S, $p = 0.90$) while shock-trained w^{1118} made fewer stops close to the port emitting shock-paired odor (Fig. 7T, $p = 0.0002$) and sucrose-trained w^{1118} made more stops close to the port emitting sucrose-paired odor (Fig. 7U, $p = 0.0002$).

The evaluation of U-turns, distance to port, and number of stops close to port collectively suggests that flies exhibit differences in discrete behaviors when navigating between two conditioned odor stimuli that reflect their choice of preferred odor.

***dunce* mutants exhibit reduced learning and choice-related behaviors in the assay**

To test whether the two-choice assay was effective in identifying mutations known to reduce memory, we tested whether *dunce*, a gene that encodes a cAMP phosphodiesterase, impacts learned odor choice (Dudai *et al.*, 1976; Byers, Davis and Kiger, 1981; Qiu and Davis, 1993). *dunce* mutants express a truncated version of cAMP phosphodiesterase that does not inactivate cAMP, leading to chronic cAMP overexpression that fails to regulate synaptic plasticity (Byers, Davis and Kiger, 1981; Davis and Kiger, 1981; Davis, 1996; Gervasi, Tchénio and Preat, 2010). As a result, *dunce* mutants exhibit reduced appetitive and aversive learning and memory (Dudai *et al.*, 1976; Tempel *et al.*, 1983).

We assessed whether *dunce* reduced learned preference for an odor associated with either shock or sugar compared to its genetic control (*Canton-S*) by recording preference index and analyzing post-choice behaviors (Fig. 8A-G). While *dunce* mutants were able to learn to avoid the shock-paired odor (Fig. S6E, $p = 0.004$), their memory was significantly reduced compared to *Canton-S* (Fig. 8H, $p < 0.0001$). *dunce* mutants did not significantly prefer the sucrose-trained odor (Fig. S6F, $p = 0.20$), which resulted in a significant reduction in appetitive memory compared to *Canton-S* controls (Fig. 8L, $p = 0.001$).

We next tested if the strength of choice behaviors was greater in *Canton-S* than *dunce* flies. Shock-trained *Canton-S* flies made more U-turns per cm travelled on the side containing odor without shock pairing (Fig. 8I, $p = 0.02$), remained closer to the no-shock paired odor port (Fig. 8J, $p < 0.0001$) and made more stops close to the no-shock paired port (Fig. 8K, $p < 0.0001$) than shock-trained *dunce* flies. Sucrose-trained *Canton-S* flies also made more U-turns per cm travelled on the side containing sucrose-paired odor (Fig. 8M, $p = 0.002$), remained closer to the port emitting sucrose-paired odor (Fig. 8N, $p < 0.0001$), and made more stops close to the port emitting sucrose-paired odor (Fig. 8O, $p < 0.0001$) than their *dunce* counterparts. These metrics indicate that the *dunce* mutation significantly impacts memory as indicated by a lack of robust choice behavior, suggesting the two-choice assay and identified behaviors demonstrated in the presence of the paired odor are useful for identifying genes and circuits that impact memory formation.

Discussion

Decision-making has been the subject of increasing interest in *Drosophila* research (Jovanic, 2020; Buchert *et al.*, 2022; Lebovich *et al.*, 2024; Ladd and Simpson, 2025), and olfactory classical conditioning assays remain a popular method for assessing choice. However, traditional maze-based conditioning assays were not designed to capture the behavioral dynamics that occur throughout decision-making. We introduce a high-throughput olfactory Two-Choice assay of associative learning and memory that captures individual and group behavior over time to better characterize behavioral choice.

Using the assay, we show that conditioned flies exhibit a sustained collective preference between odor stimuli within 30 seconds of odor introduction. This interval mirrors the speed of decision-making previously reported in shock-trained flies (Ichinose and Tanimoto, 2016) and suggests that choice occurs in the same timeframe following appetitive conditioning. Furthermore, we found that flies exhibit distinct locomotor dynamics between each odor stimulus that reflect their decision. The largest differences between stimuli typically occur following the first 30 seconds of odor exposure (the choice phase), coinciding with the latency for collective decision-making. Within the choice phase, we observed additional behaviors that indicate active avoidance of an odor previously paired with an aversive stimulus, and engagement with an odor previously paired with a rewarding stimulus, including the length of bouts near the paired odor, number of U-turns toward the paired odor, proximity to the odor port, and number of stops near the odor port. We identified that the number of U-turns towards a rewarding odor or away from a punishing odor is an effective metric for identifying learned choice. Moreover, we found that when occupying each respective arena side, trained flies would get closer on average to the port emitting the preferred odor, as well as make more stops close to the preferred odor port. Given our previous findings that exhibit greater locomotor activity on the preferred odor side, the close average proximity and more frequent stops close to the

preferred port provide further evidence that sucrose-trained flies are engaging in foraging or seeking behavior towards the source of the sucrose-paired odor, while the shock-trained flies may be trying to get as far as possible from the shock-trained odor source. Thus, rather than a single static measurement, our results reflect decision-making as a series of behavioral strategies exhibited across stimulus exposure.

To our surprise, our data revealed that trained flies continuously transitioned between arena sides throughout the experiment, although the overall number of transitions was decreased in shock-trained flies (Fig. S4). This suggests that even after choosing between stimuli, flies will still not strictly limit their exposure to the preferred stimulus. Indeed, one study showed that flies' expression of olfactory memory depends on access to comparison between both odors during testing (Modi *et al.*, 2023), suggesting that by transitioning between odors over the course of the experiment, flies are engaging in comparison between the odors in the process of remembering. We additionally found that both sucrose and shock-trained flies reduce their bout time on the side with an odor they want to avoid and increase their bout time on the side with an odor they prefer, suggesting that comparison between odors decreases but does not stop once a collective choice is made.

The ability to measure multiple behavioral metrics of individual flies within a group provides a powerful tool to identify individual variation in memory formation and behavioral choice. Although our results show that a greater proportion of flies spent more time on the preferred odor side, we observed a subset of flies in both conditions and genotypes that preferred the side containing odor without sucrose-pairing or the odor paired with shock. Similarly, there was a subset of flies that spent an approximately equal amount of time on both arena sides. Flies, like humans, exhibit inherent variability in behavior across individuals (Werkhoven *et al.*, 2021; De Bivort *et al.*, 2022; Smith *et al.*, 2022; Godesberg, Bockemühl and Büschges, 2024). The study of behavioral variability has revealed physiological mechanisms

that contribute to innate preference (Maloney, 2021; Rihani and Sachse, 2022) and is important to understanding how neural circuits maintain flexibility while producing stable behavioral outputs (De Bivort, 2025). Moreover, decisions are processed and ultimately acted upon differently as a group compared to as an individual (Conradt and Roper, 2005; van der Meer, Kurth-Nelson and Redish, 2012; Suzuki *et al.*, 2015; Gall *et al.*, 2017). By averaging preference index per-fly and per-arena, we were able to assess within-subject memory and visualize continuous preference dynamics without filtering out individual variability. Future studies could apply similar continuous preference methodologies to explore complex decision tasks in *Drosophila* and other model organisms.

Taken together, our findings show that flies exhibit a range of behaviors to exhibit a choice between stimuli that parallels findings in rodent (Izquierdo and Belcher, 2012; Ahmed, 2018; Roy *et al.*, 2021) and human studies (Camchong, Endres and Fein, 2014; Cabeza *et al.*, 2020; Midroit *et al.*, 2021). As a model organism with high genetic tractability and unique resources such as the whole-brain connectome (Zheng *et al.*, 2018; Dorkenwald *et al.*, 2024), increasing the scope of behavioral analysis in *Drosophila* will accelerate efforts to understand general neurobiological principles underlying decision-making. We demonstrated that this simple two-choice behavioral tracking assay effectively identifies mutations that impact memory and provides additional metrics to understand how genes and circuits contribute to behavioral choice. Thus, it presents a sensitive high-throughput method for capturing the dynamic process of memory-based decision making with enhanced resolution.

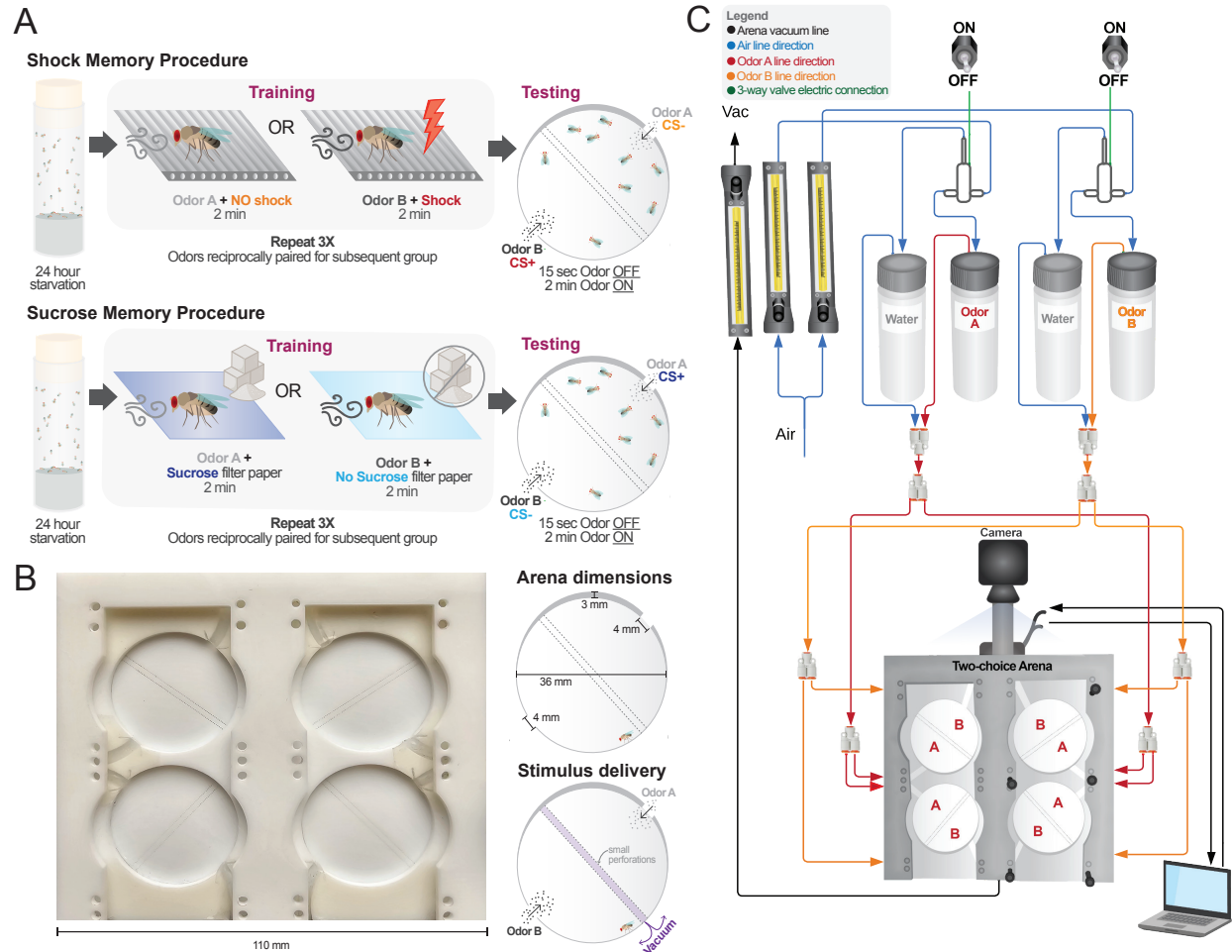


Fig. 1. Two-Choice setup and experimental procedures (A) Graphic of shock and sucrose memory experimental procedures. Food-deprived flies were trained to associate an odor (ethyl acetate or isoamyl alcohol) with 80V electric shock or 2M sucrose. Flies were initially exposed to an odor without sucrose or shock for two minutes, then exposed to the other odor paired with sucrose or shock over the following two minutes. This training cycle was repeated a total of three times. Immediately after training, 10 flies per arena were aspirated into the apparatus and exposed to both odors for two minutes to test odor preference. **(B)** The behavioral arenas from above (left), along with arena dimensions (top right) and features for stimulus delivery (bottom right). **(C)** Odor delivery setup. Air and vacuum flow rates are adjustable by separate flowmeters. Airflow can be switched from a 3-way valve to pass through either an odor or water vial before reaching the arenas. After passing through a vial, air is forked into two tubes to reach two arenas at once. Forked tubes can be detached and reconnected to switch the odor emitted at each arena side between trials.

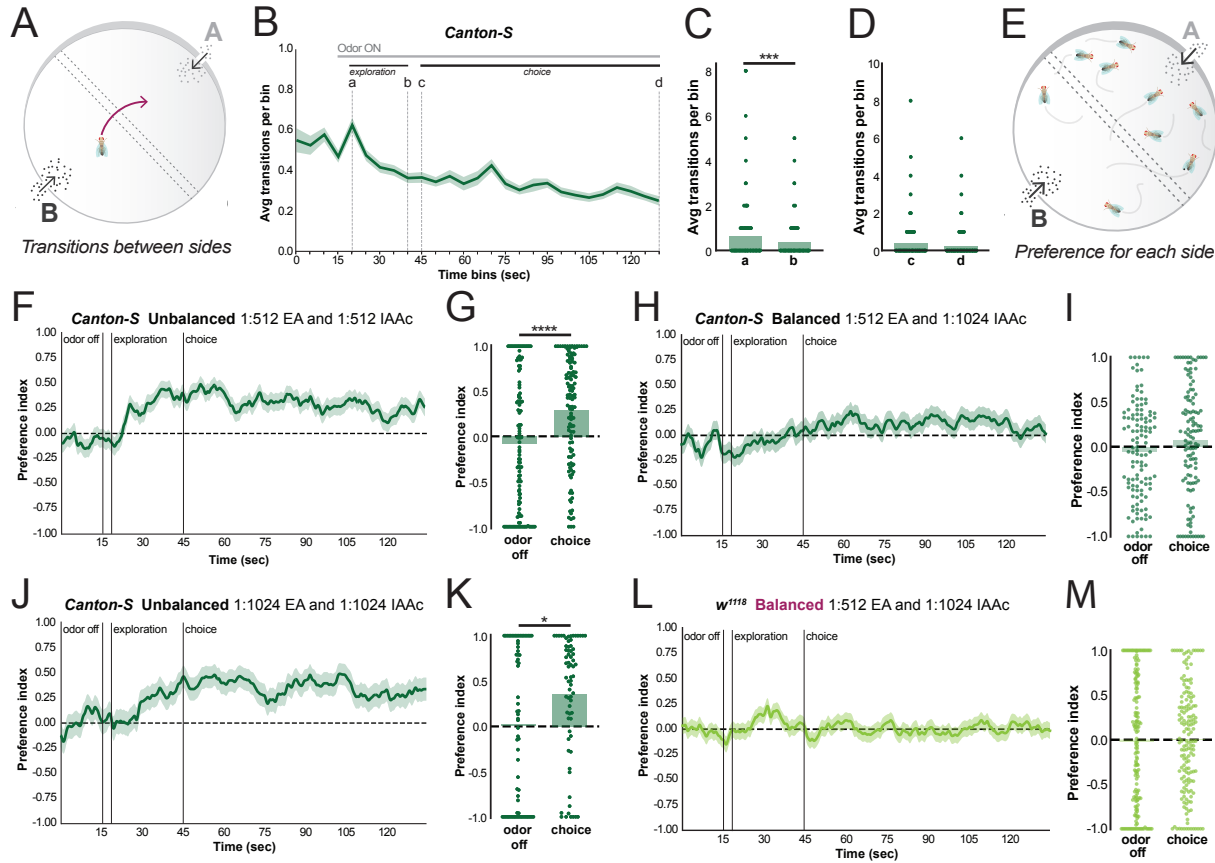


Fig. 2. Transitions and preference index reflect time to decision.

Naïve flies were exposed to differing concentrations of EA and IAAC following starvation to find a pair of concentrations that did not evoke an odor bias. **(A)** Graphic depiction of a transition between arena sides. **(B)** Average transitions made per 5-second bin in naïve *Canton-S* flies. **(C)** Once odors reached the center of the arenas, average transitions made between arena sides decreased within 25 seconds. **(D)** After 25 seconds of odor exposure, average transitions did not decrease for the remainder of the experiment. **(E)** Graphic depiction of per-group preference index as the distribution of flies across each arena side over time. **(F-G)** Naïve *Canton-S* flies show a preference for 1:512 EA over 1:512 IAAC after 25 seconds of odor exposure. **(H-I)** Naïve *Canton-S* flies show a preference for 1:1024 EA over 1:1024 IAAC within 25 seconds of odor exposure. **(J-K)** Naïve *Canton-S* flies show no preference for 1:512 EA over 1:1024 IAAC after 25 seconds of odor exposure. **(L-M)** *w¹¹¹⁸* flies show no preference for 1:512 EA over 1:1024 IAAC after 25 seconds of odor exposure.

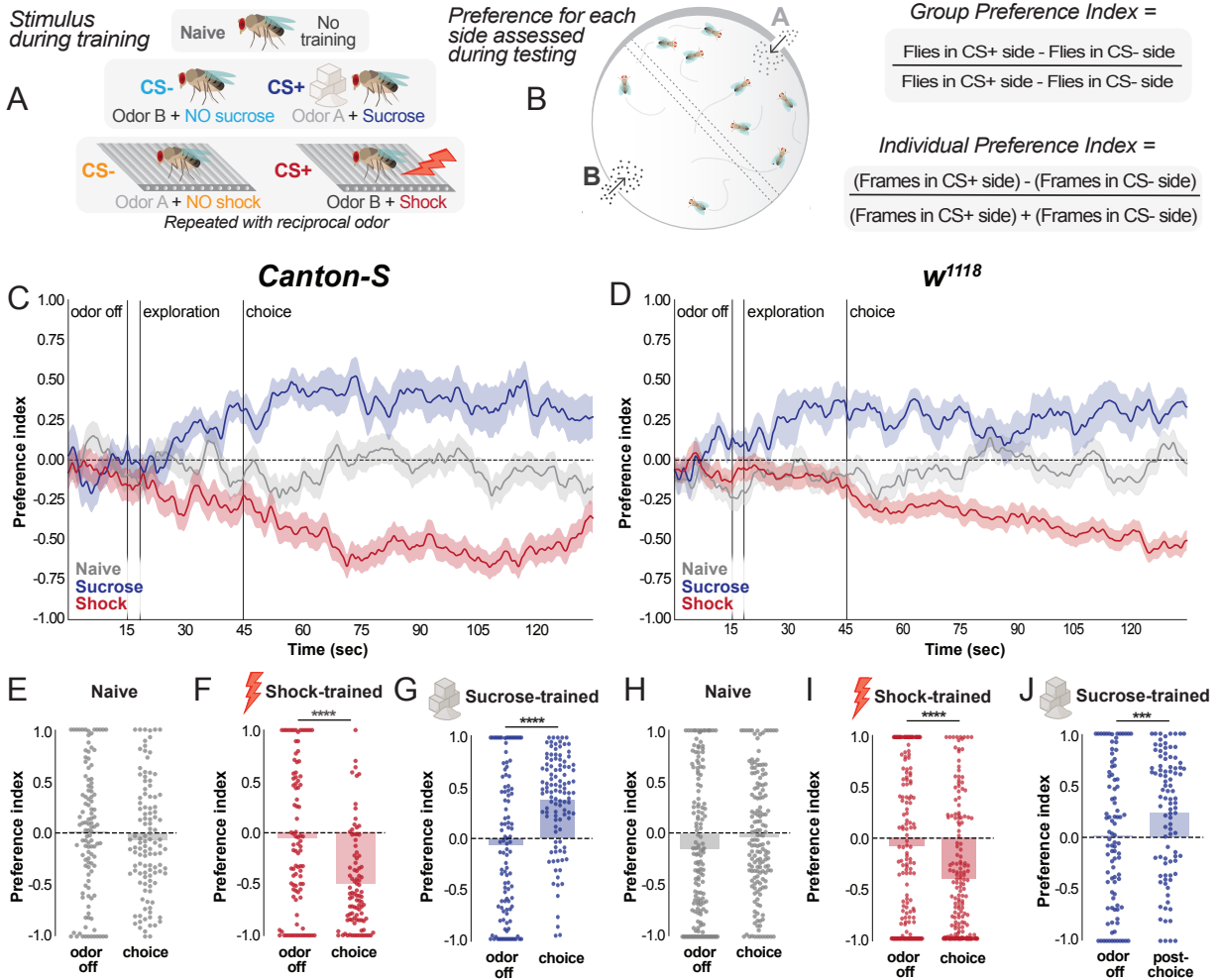


Fig. 3. Olfactory memory in *Canton-S* and *w¹¹¹⁸* flies.

(A) Appetitive and aversive conditioning procedures. (B) The per-group preference index visualizes stimulus preference continuously over time. A positive preference index in trained flies indicates a preference for the conditioned stimulus (sucrose or shock). Preference index for naïve flies is plotted relative to arena side A. In (C) *Canton-S* and (D) *w¹¹¹⁸* flies, sucrose training increased the preference for the paired odor and shock training decreased the preference for the paired odor following the first 30 seconds of odor exposure. (E) Naïve *Canton-S* did not develop a side preference regardless of which odor was presented on each side. (F) Shock-trained *Canton-S* avoided the shock-paired odor. (G) Sucrose-trained *Canton-S* preferred odor paired with sucrose. (H) Naïve *w¹¹¹⁸* did not develop a side preference. (I) Shock-trained *W1118* avoided the shock-paired odor. (J) Sucrose-trained *w¹¹¹⁸* preferred the sucrose-paired odor.

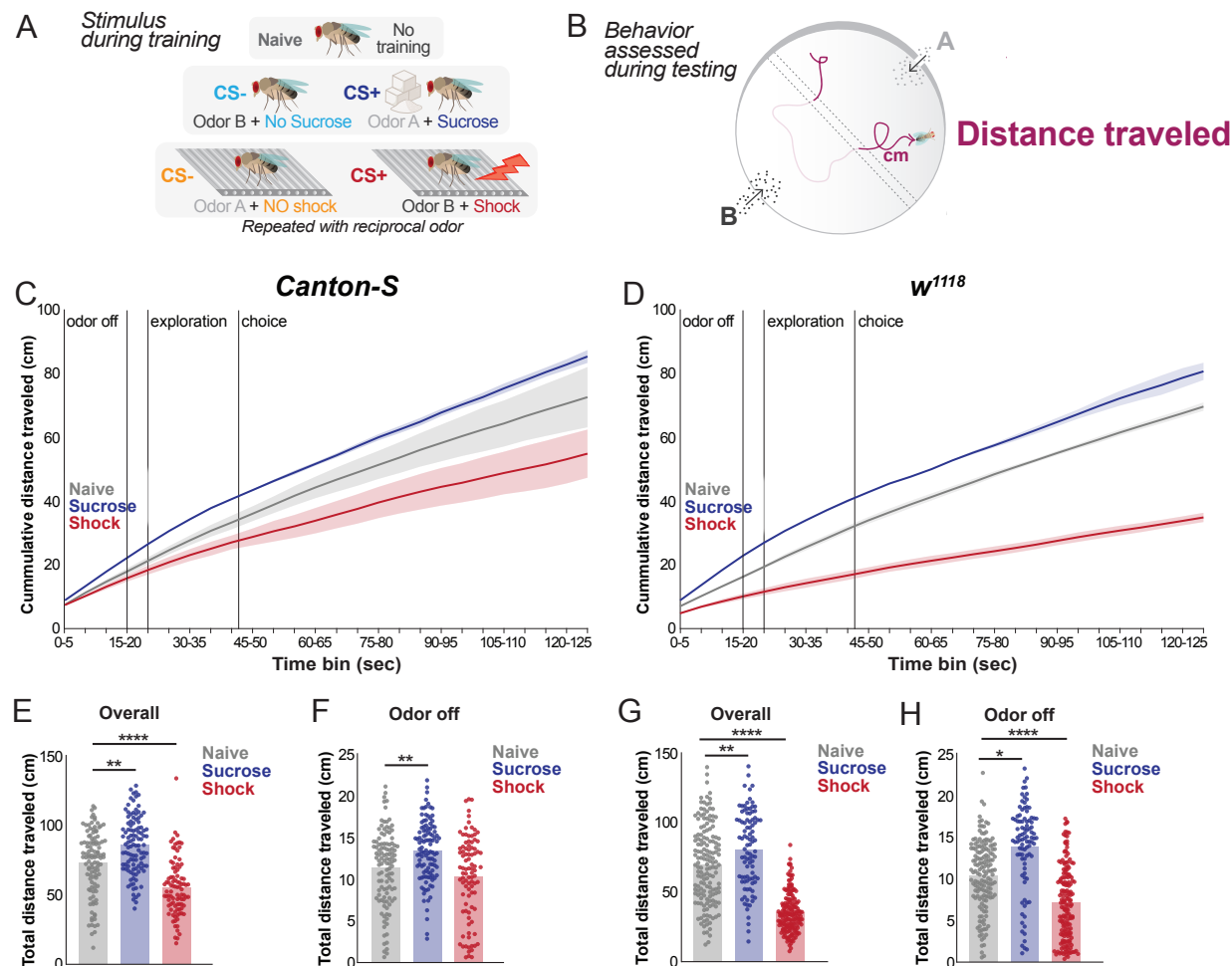


Fig. 4. Locomotor activity depends on conditioning

(A) Appetitive and aversive conditioning procedures. (B) Depiction of the regional distance travelled for a single fly. Distance travelled per region was normalized to the number of flies occupying each region across each video frame. (C) Shock-trained *Canton-S* and (D) w^{1118} flies travel less over time than their naïve counterparts, while sucrose-trained flies travel more than their naïve counterparts. Compared to naïve *Canton-S* flies, shock-trained *Canton-S* traveled less overall (E) but not during the odor-off phase (F), and sucrose-trained *Canton-S* traveled more overall and during the odor-off phase. Compared to naïve w^{1118} flies, shock-trained w^{1118} travelled less and sucrose-trained w^{1118} travelled more overall (G) and during the odor-off phase (H).

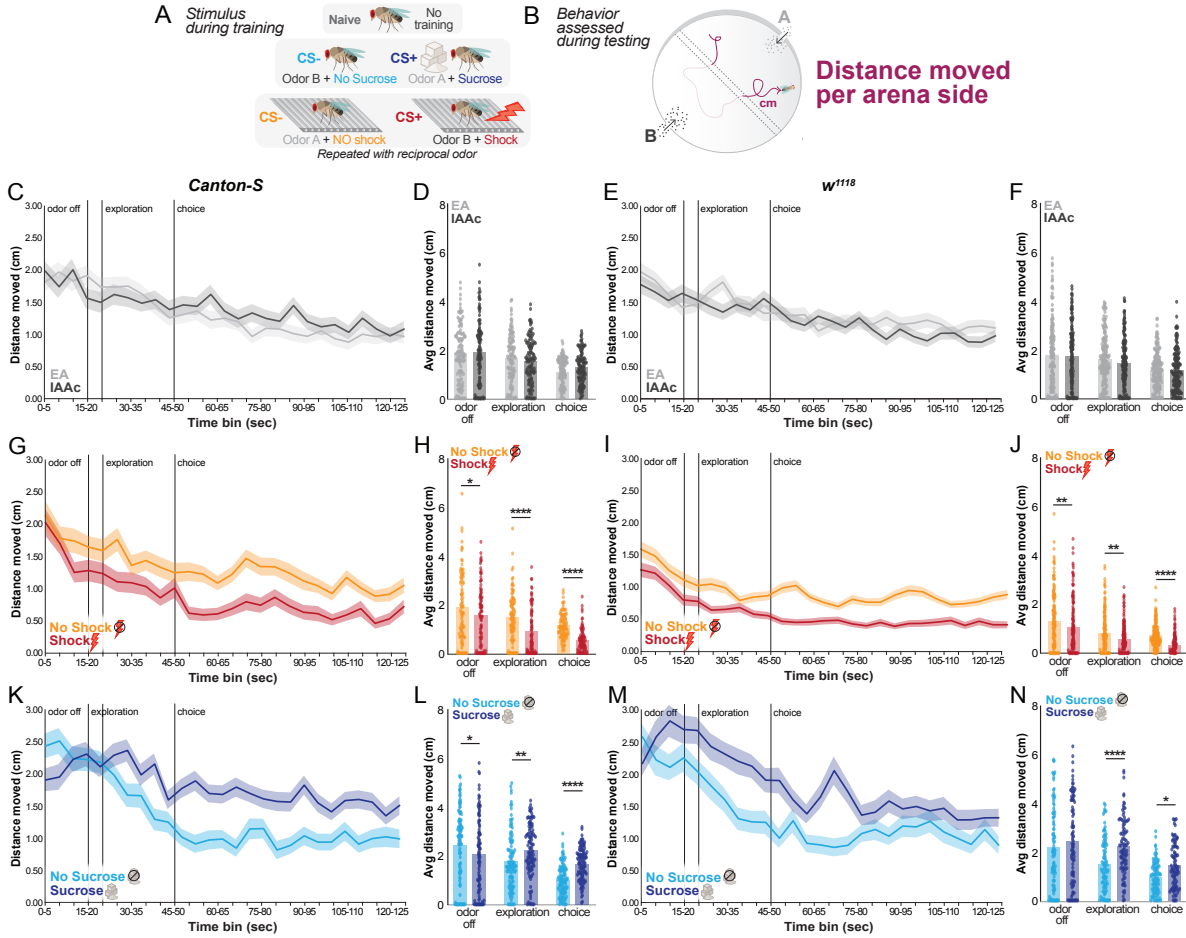


Fig. 5. Regional distance travelled reflects memory.

The average distance travelled between arena sides over time was normalized to the number of flies occupying each arena side per-frame. **(A)** Appetitive and aversive conditioning procedures. **(B)** Graphic of regional distance travelled. **(C)** Naïve *Canton-S* did not travel more on either side over time, or at any experimental phase **(D)**. **(E)** Naïve *w¹¹¹⁸* did not travel more on either side over time, or at any experimental phase **(F)**. **(G-H)** Shock-trained *Canton-S* travelled less on the side containing the shock-paired odor over time. **(I-J)** Shock-trained *w¹¹¹⁸* travel less on the side containing shock-paired odor over time. **(K-L)** Sucrose-trained *Canton-S* travelled more on the side containing sucrose-paired odor over time. **(M-N)** Sucrose-trained *w¹¹¹⁸* travelled more on the side containing sucrose-paired odor over time.

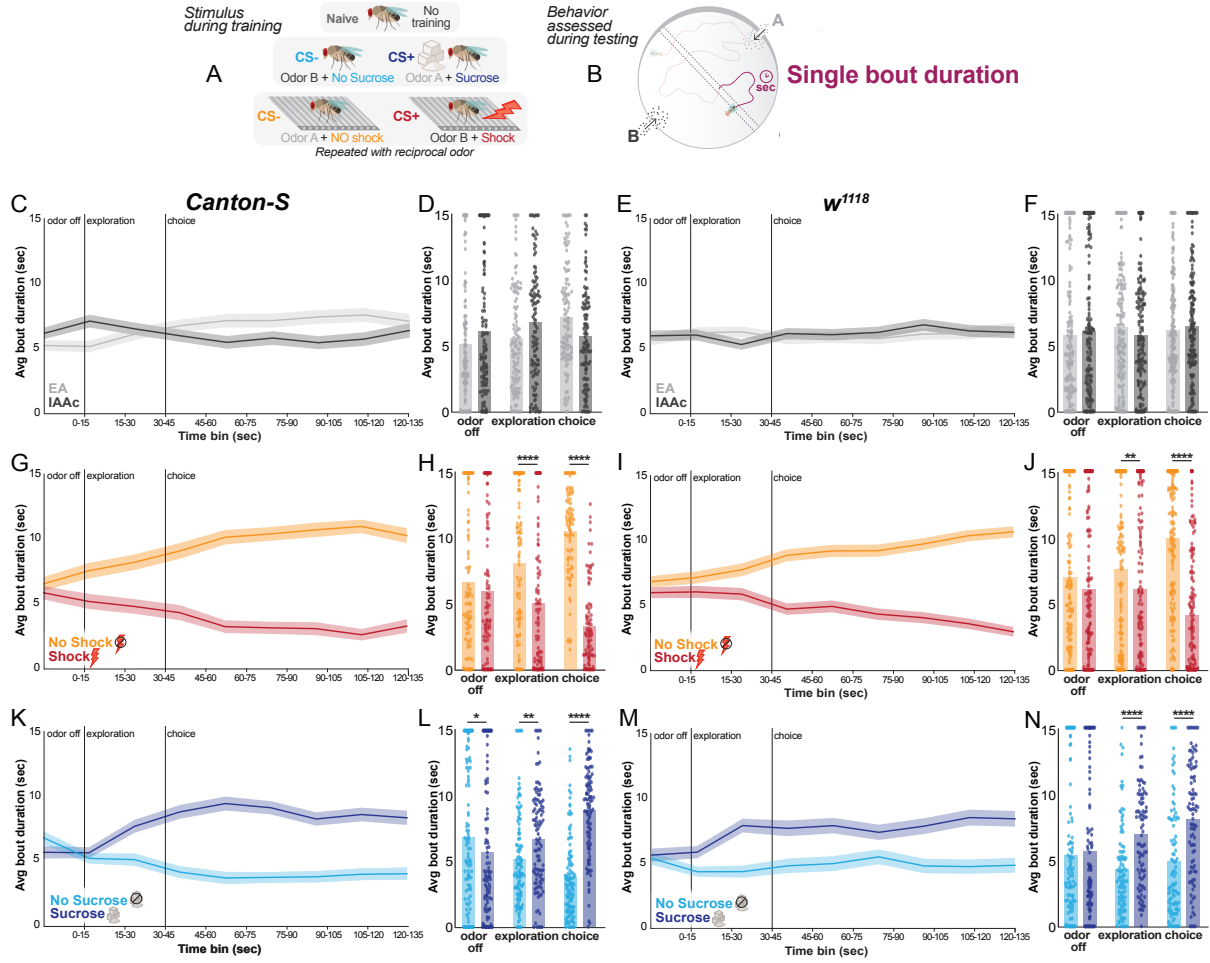


Fig. 6. Bout duration reflects memory.

Average bout duration on each arena side was binned every 15 seconds. **(A)** Appetitive and aversive conditioning procedures. **(B)** Graphic of a single bout duration on one arena side. **(C-D)** Naïve *Canton-S* did not wait longer to transition from either side over time. **(E-F)** Naïve *w¹¹¹⁸* did not wait longer to transition from either side over time. **(G-H)** Shock-trained *Canton-S* waited longer to transition out of the odor without shock-pairing over time. **(I-J)** Shock-trained *w¹¹¹⁸* waited longer to transition out of the odor without shock-pairing over time. **(K-L)** Sucrose-trained *Canton-S* waited longer to transition out of the sucrose-paired odor over time. **(M-N)** Sucrose-trained *w¹¹¹⁸* waited longer to transition out of the sucrose-paired odor over time.

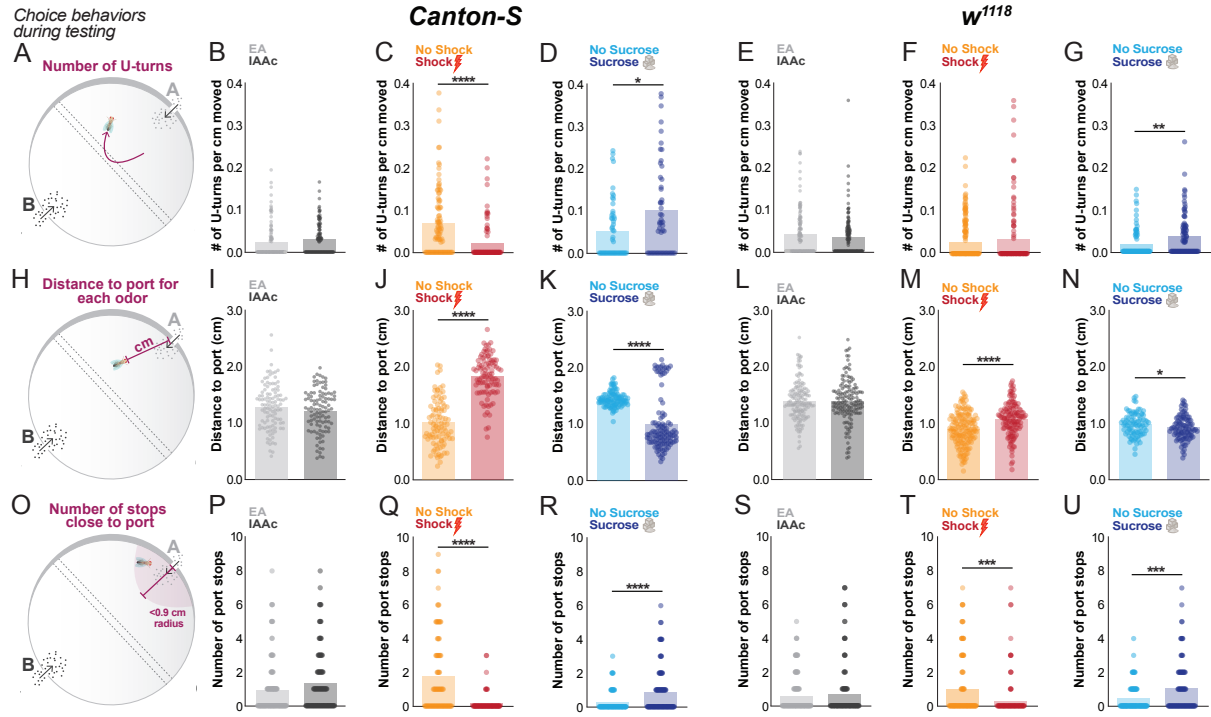


Fig. 7. Behaviors that reflect memory-based decisions.

(A) Graphic depiction of a U-turn. **(B-G)** Naïve flies did not make more U-turns per cm travelled on either side, while trained flies made more U-turns per cm travelled when occupying the arena side containing the preferred odor stimulus (sucrose-paired or no-shock-paired), except shock-trained w^{1118} flies. **(H)** Graphic depiction of the distance to an odor port on a given arena side. **(I-N)** Naïve flies did not stay closer to ports emitting either odor, but trained flies stayed closer to the port emitting the preferred odor. **(O)** Graphic depiction of a stop close to an odor port. **(P-U)** Naïve flies did not make more stops close to either odor port, but trained flies made more stops close to the preferred odor stimulus.

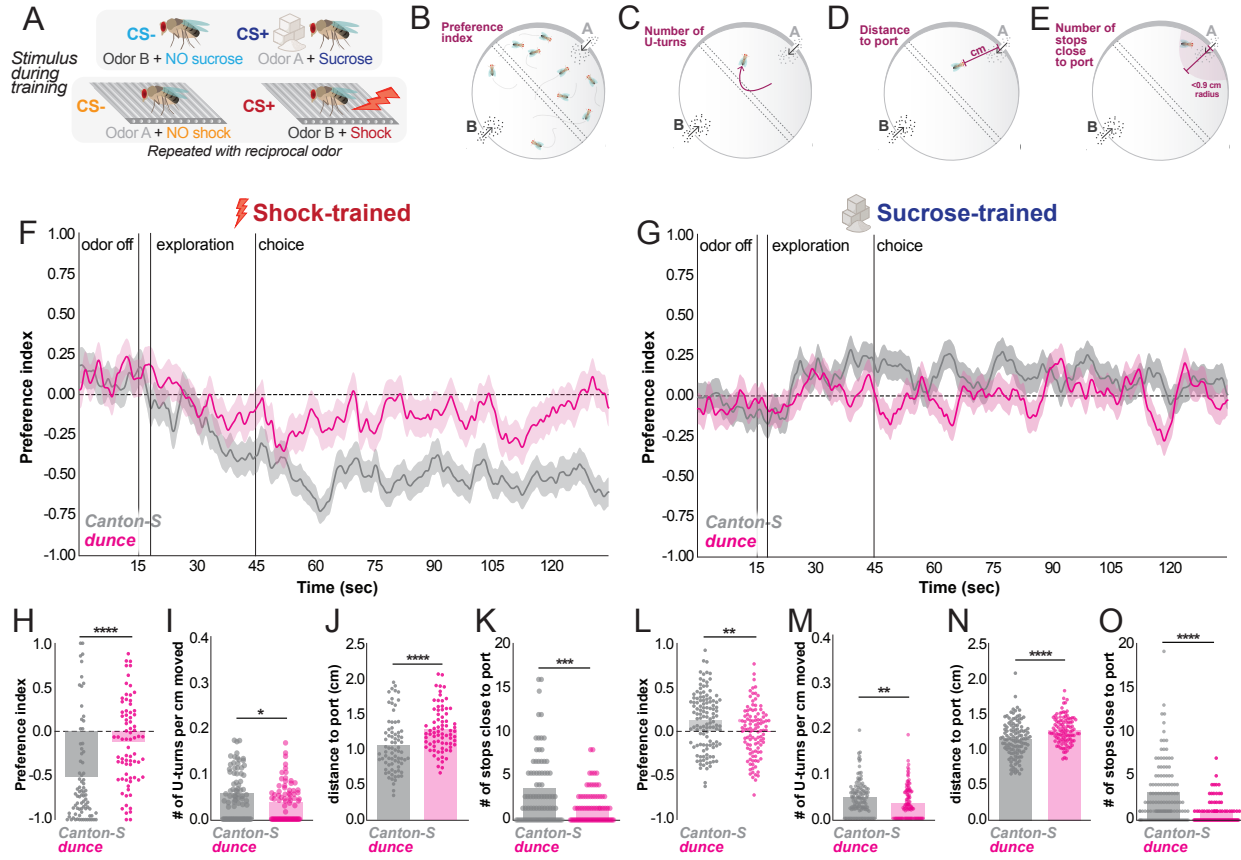
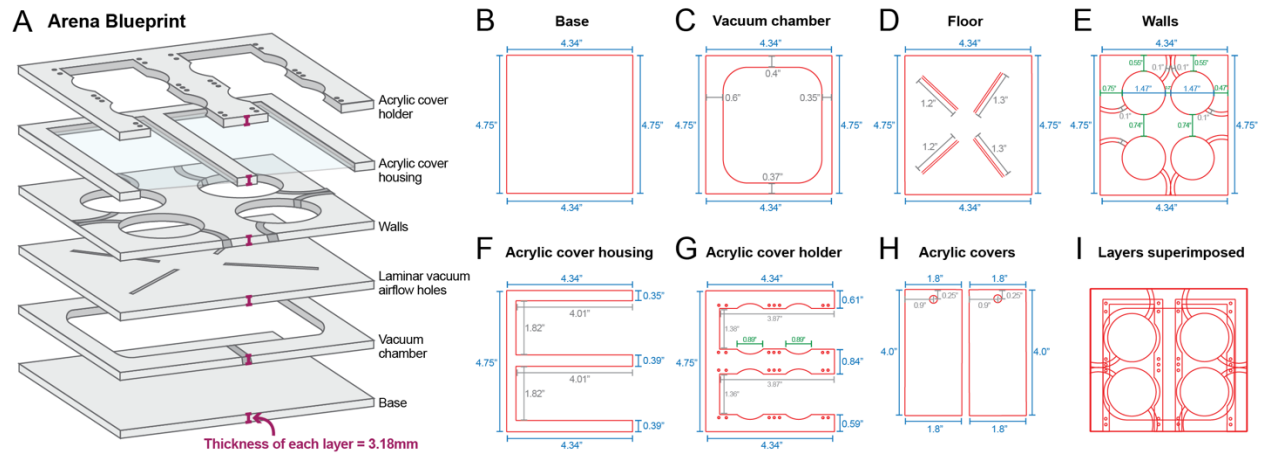


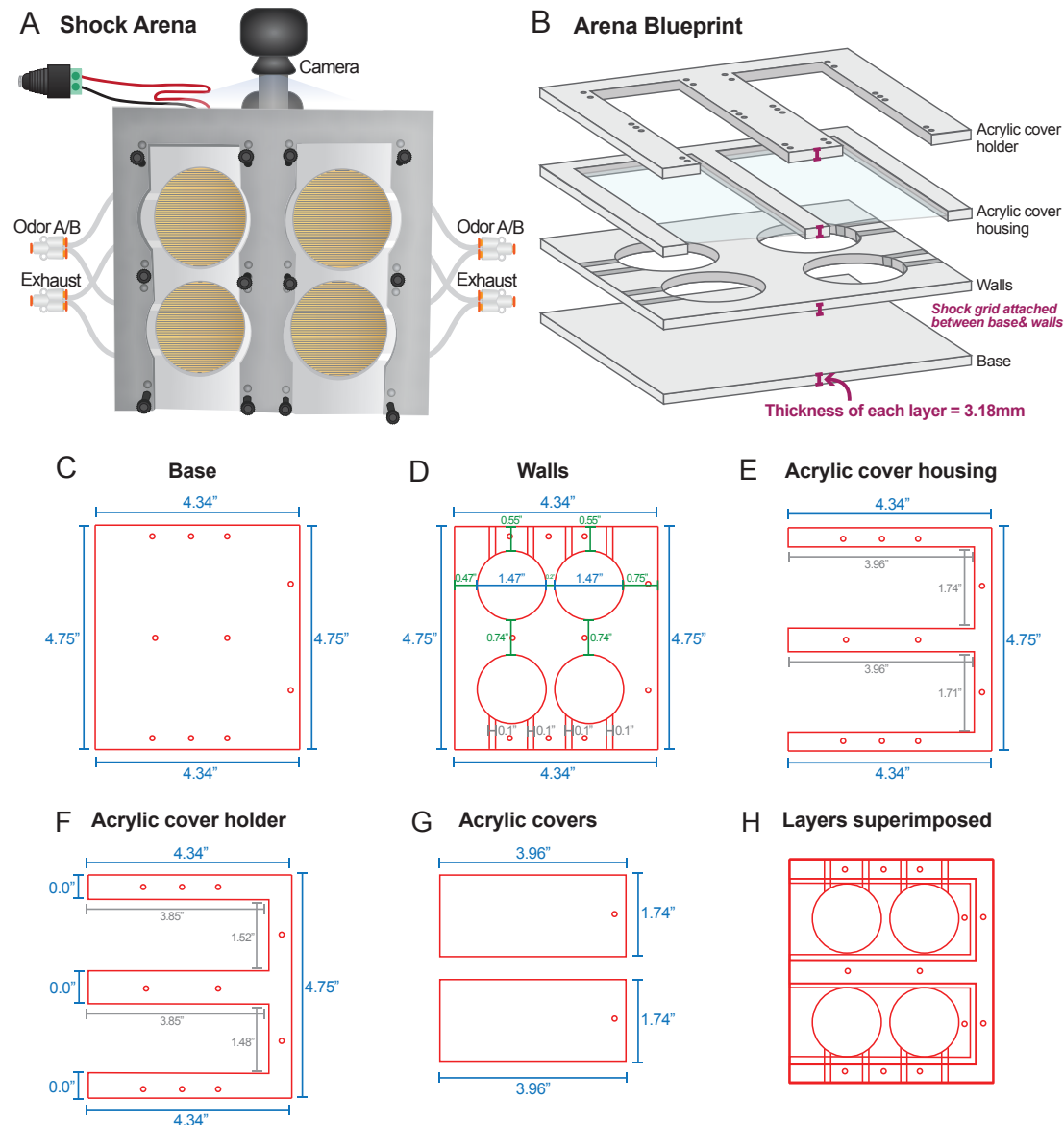
Fig. 8. *Duncle* mutants exhibit blunted learning and post-choice behaviors.

Preference index in the post-choice phase and post-choice behaviors were compared between Wild-Type *Canton-S* and *duncle* mutants. **(A)** Appetitive and aversive conditioning procedures. **(B-E)** Graphics of behaviors are compared between genotypes. **(F-G)** Average preference index over time for the shock-paired **(F)** and sucrose-paired **(G)** odors by each genotype. **(H-K)** Shock-trained *Canton-S* flies exhibited greater preference for the odor without shock-pairing and more robust post-choice behavior **(H)** by making fewer U-turns in the shock-paired odor side **(I)**, staying farther away from the source of shock-paired odor **(J)**, and making fewer stops close to the shock-paired odor port **(K)** than shock-trained *duncle* flies. **(L)** Sucrose-trained *Canton-S* flies exhibit greater preference for the sucrose-paired odor and more robust post-choice behavior by making more U-turns on the sucrose-paired odor side **(M)**, staying closer to the source of sucrose-paired odor **(N)**, and more frequently stopping close to the sucrose-paired odor port **(O)** than sucrose-trained *duncle* flies.



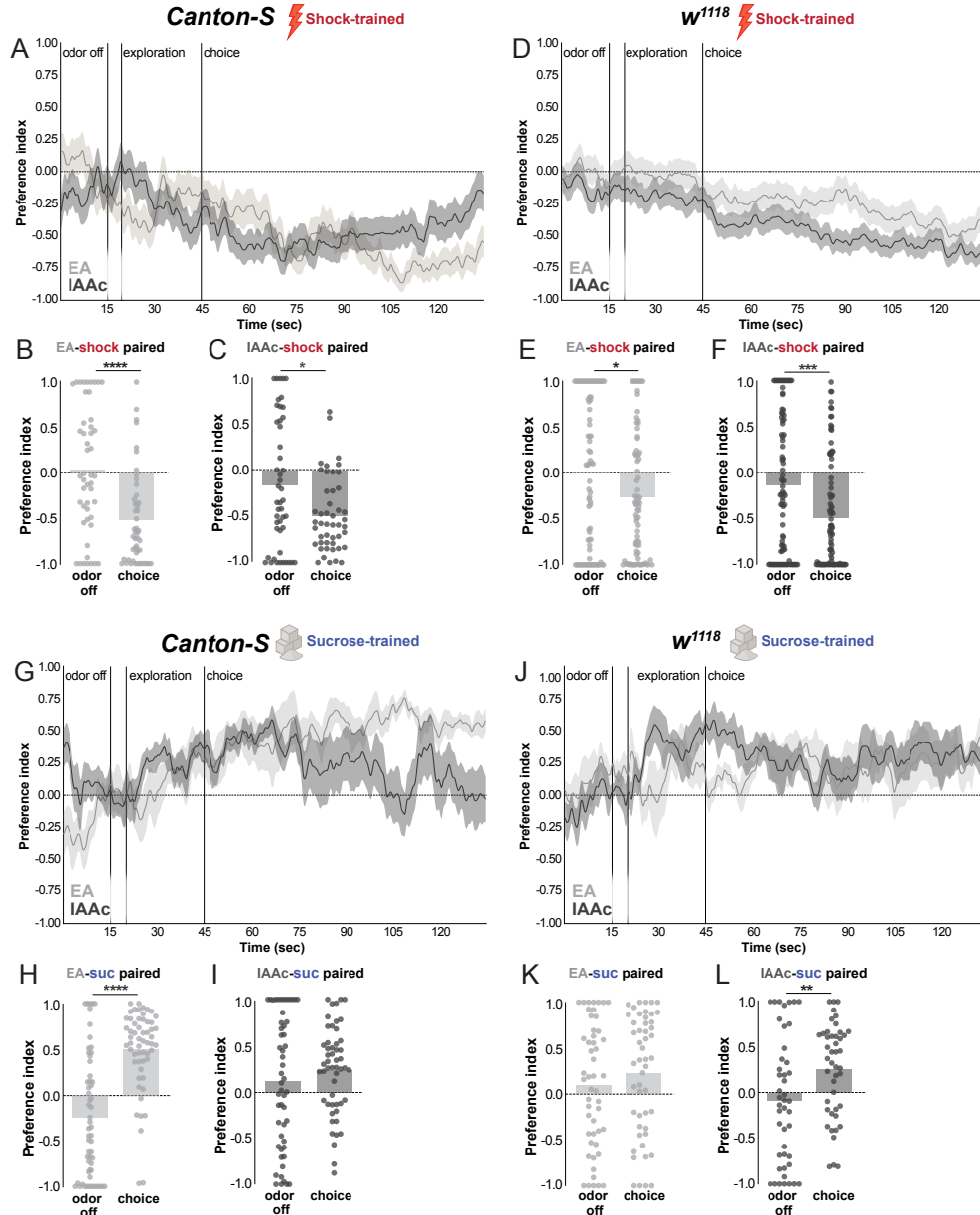
Supplementary Figure 1. Layered diagram of the Two-Choice apparatus.

(A) The two-choice apparatus consists of six separately laser-cut Teflon sheets sealed with plastic adhesive (Loctite, 681925, Rocky Hill, Connecticut). The bottom two layers (B-C) form a pocket of air sucked out of the arenas by the vacuum tube attached to the vacuum chamber layer. The middle two layers (D-E) form the floor and walls of the four arenas featured in the assay. The top two layers (F-G) contain the housing for the removable plexiglass arena covers (H) and screw holes to fasten the covers in place, forming the superimposed apparatus (I). A PDF of the laser printing schematics is available for download at <https://github.com/kaunlab>.



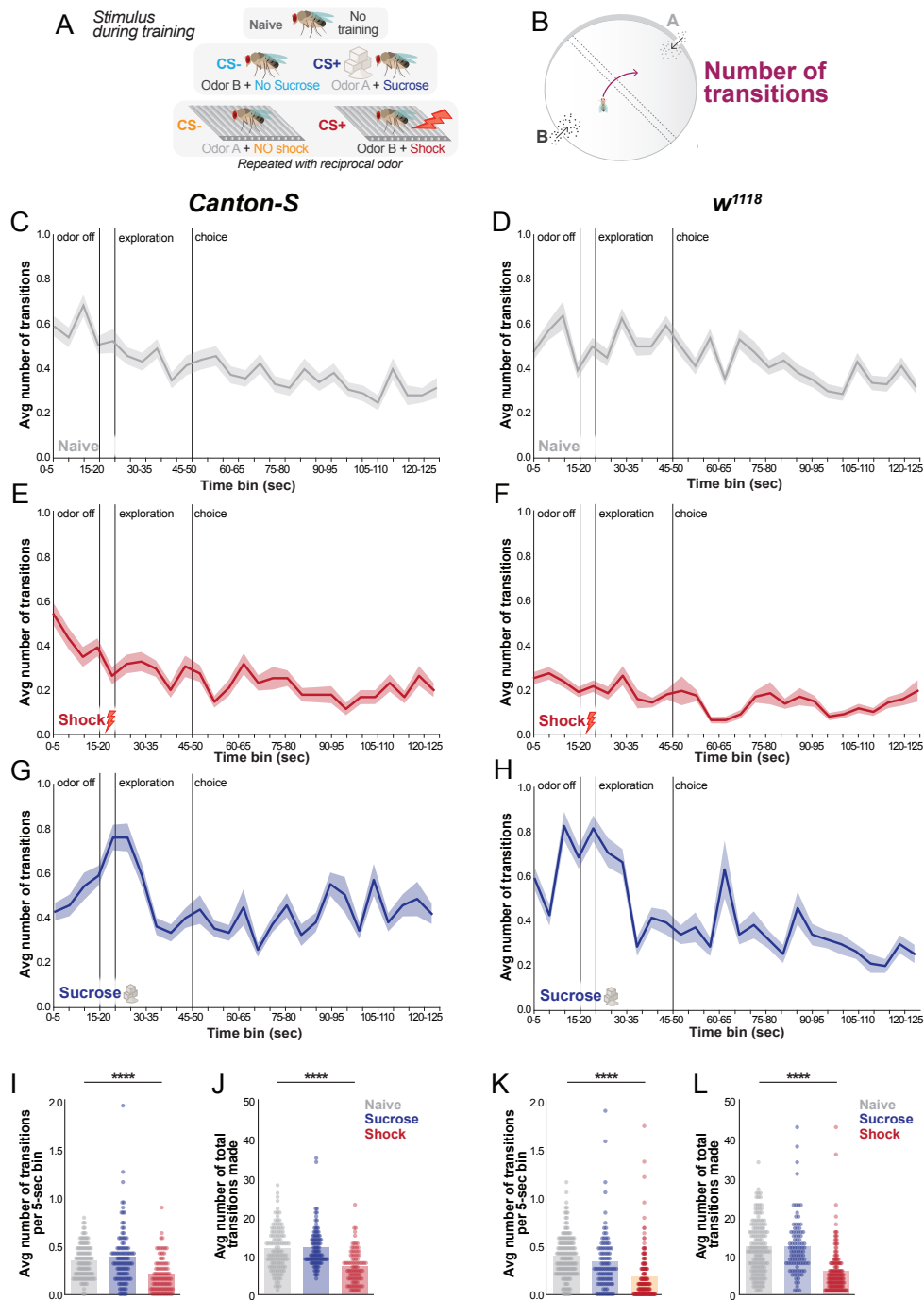
Supplementary Figure 2. Shock training arena.

(A) Illustration of shock arena used during training, adopted from (Scaplen *et al.*, 2019). Herein, odors flow one at a time through single tubes to each arena. Each electrode surface beneath the copper grating is connected individually to hookup wires fed into a single connector that can be plugged into the pulse stimulator. **(B)** Layard diagram of the shock arena. **(C-G)** Individual layer dimensions. **(H)** Final Behavioral arena dimensions match the two-choice assay.



Supplementary Figure 3. Strength of exhibited associative learning depends on odor.

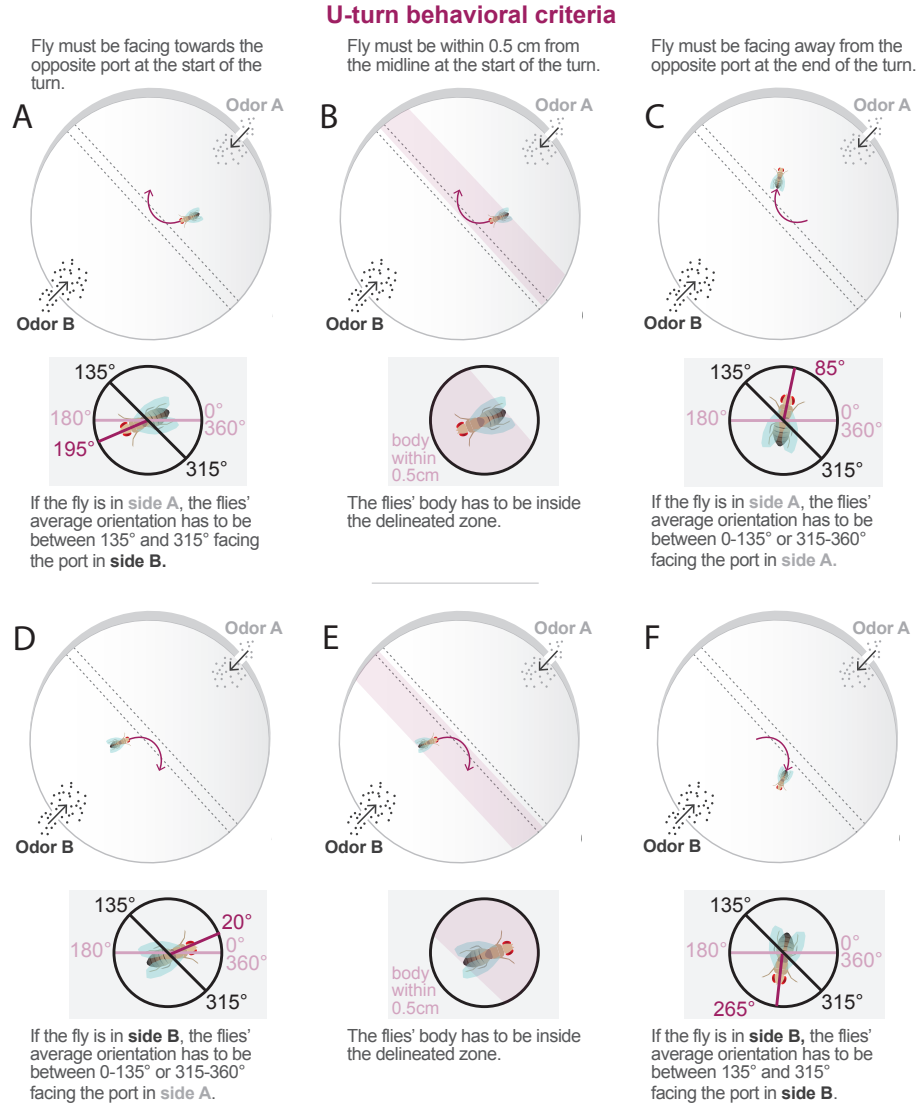
(A) Shock-trained *Canton-S* preference index over time grouped by shock-paired odor. **(B-C)** Effect size of preference index was greater *Canton-S* that received shock paired with EA than in those that received shock paired with IAAC. **(D)** Shock-trained *w¹¹¹⁸* preference index over time grouped by shock-paired odor. **(E-F)** Effect size of preference index was greater in *w¹¹¹⁸* flies that received shock paired with IAAC than in those that received shock paired with EA. **(G)** Sucrose-trained *Canton-S* preference index over time grouped by sucrose-paired odor. **(H-I)** Sucrose pairing with EA but not IAAC resulted in associative memory in *Canton-S* flies. **(J)** Sucrose-trained *w¹¹¹⁸* preference index over time grouped by sucrose-paired odor. **(K-L)** Sucrose pairing with IAAC but not EA resulted in associative memory in *w¹¹¹⁸* flies.



Supplementary Figure 4. Shock but not sucrose training decreased average side transitions.

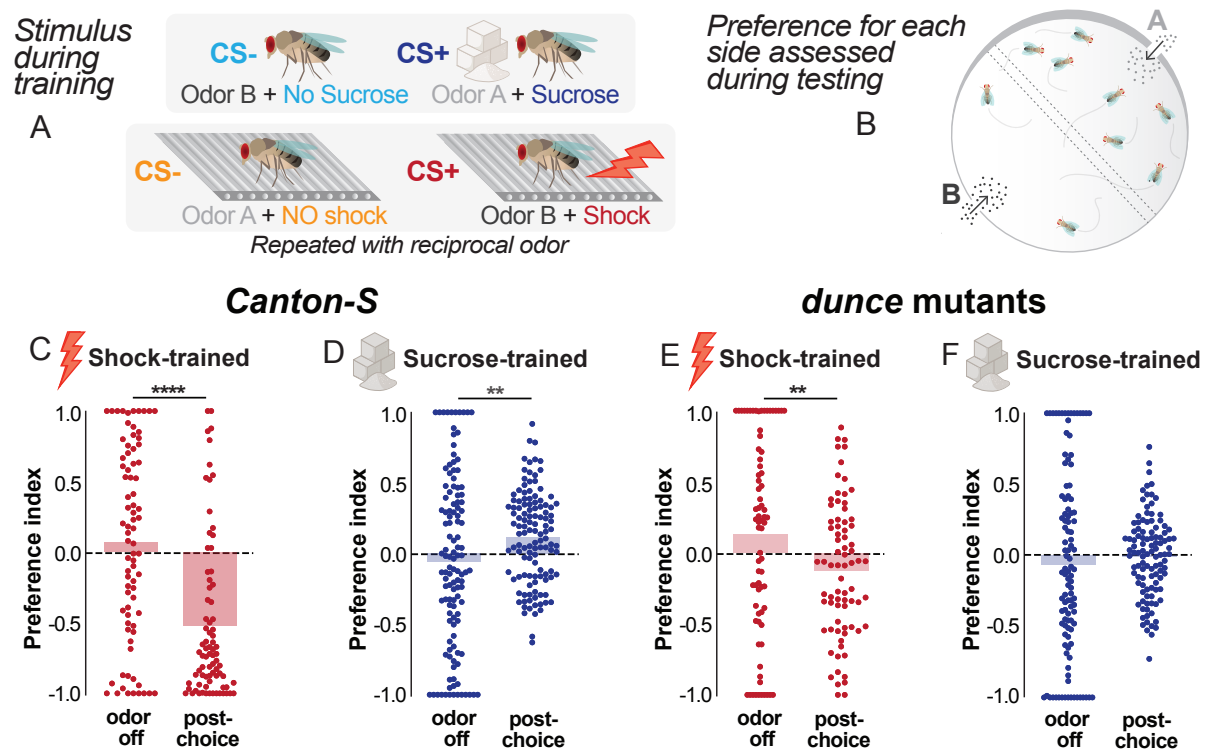
Transitions between arena sides were averaged per 5-second bin over time. **(A)** Sucrose and shock-association procedures. **(B)** Graphic of a transition between arena sides. **(C-H)** Average transitions over time for each genotype and treatment group (naïve, sucrose-trained, and shock-

trained). In *Canton-S* flies, shock reduced the average number of transitions made per time bin during **(I)** the post-choice period and **(J)** the total transitions made during the experiment. Shock-trained w^{1118} flies also exhibited decreased average transitions per bin in the during **(K)** the post-choice period, and **(L)** fewer overall transitions made.



Supplementary Figure 5. U-turn away from midline parameters on each arena side.

An example arena where the midline is at a 135° angle from the x axis is illustrated. A U-turn must first meet the per-frame criteria of a sharp turn as delineated by (Branson *et al.*, 2009) for at least five frames. In order for a fly on the top arena side to make a U-turn, it must **(A)** be oriented towards the midline (135°-315°) in the five frames preceding the start of the turn, **(B)** start the turn within 0.5 cm of the midline, and **(C)** be oriented away from the midline (0°-135°)U(315°-360°) on the side the turn was initiated for at least five frames. In order for a fly on the bottom arena side to make a U-turn, it must **(D)** be oriented towards the midline (0°-135°)U(315°-360°) in the five frames preceding the start of the turn, **(E)** start the turn within 0.5 cm of the midline, and **(F)** be oriented away from the midline (135°-315°) on the side the turn was initiated for at least five frames.



Supplementary Figure 6. Within-subject learning in Canton-S and *dunce* strains.

(A) Sucrose and shock-association procedures. (B) Graphic of preference behavior. *Canton-S* flies exhibited both aversive (C) and appetitive (D) olfactory associative memory. (E) Shock-trained *dunce* mutants exhibited shock memory, but aversive memory was significantly stronger in *Canton-S* than *dunce* flies (Fig. 7L). (F) Sucrose-trained *dunce* mutants did not exhibit appetitive memory.

COMPONENT	PART ID	VENDOR
PTFE Teflon sheet	3.18 mm thick sheet	CS Hyde
White Acrylic sheet	TL 7328	Various
Clear Plexiglas sheet	3.18 mm thick sheet	Various
Nylon mesh	9318T28	McMaster-Carr
Plastic bonding adhesive	681925	Loctite
Nylon socket head screws	95868A261	McMaster-Carr
Teflon tubes	F015090	Cole Parmer
Teflon tape	23-FEP-.5-5	CS Hyde
Luer to hose barb adapters	MFLX30800-24	Masterflex
Air flowmeter	112-02	Cole-Parmer
Vacuum flowmeter	092-04	Cole-Parmer
3-way valve	411L3212HV	Asko
Swivel-elbow tubes	7880T189	McMaster-Carr
Starvation vials	AS507	Fisher
Luer lock tube coupling	51525K222	McMaster-Carr
Wye connector	5111K542	McMaster-Carr
Threaded lid glass vials	01-672-735	Fisher
Ethyl acetate	S25305	Fisher
Isoamyl acetate	AC150662500	Fisher
Mineral oil	BP26291	Fisher
flyGrRAM Components	N/A	Parts table provided in (Scaplen <i>et al.</i> , 2019)

Table 1. Two-Choice assay components list.

Figure	Comparison description		Statistical test	Posthoc analysis	N	P-value	Statistic	Significance
2	C	Canton-S naïve average transitions between first and last time bins of exploration phase	Wilcoxon matched-pairs signed rank test	N/A	(1:512 EA 1:512 IAAC) N = 118, (1:1024 EA 1:1024 IAAC) N = 69, (1:512 EA 1:1024 IAAC) = 113	0.0007	W = -3240	***
	D	Canton-S naïve average transitions between first and last time bins of choice phase				0.0568	W = -1131	ns
	G	(1:512 EA 1:512 IAAC) Canton-S average preference index between odor off and choice phases				<0.0001	W = 3361	****
	I	(1:1024 EA 1:1024 IAAC) Canton-S average preference index between odor off and choice phases				0.0158	W = 735	*
	K	(1:512 EA 1:1024 IAAC) Canton-S average preference index between odor off and choice phases				0.3343	W = 668	ns
	M	(1:512 EA 1:1024 IAAC) W1118 average preference index between odor off and choice phases				0.8015	W = 286	ns
3	E	Naïve Canton-S average preference index during odor-off vs. after choice	Wilcoxon matched-pairs signed rank test	N/A	Canton-S naïve = 113, sucrose-trained = 109, shock-trained = 94, W1118 naïve = 158, sucrose-trained = 95, shock-trained = 172	0.1919	W = -901	ns
	F	Shock-trained Canton-S average preference index during odor-off vs. after choice				< 0.0001	W = -2481	****
	G	Sucrose-trained Canton-S average preference index during odor-off vs. after choice				< 0.0001	W = 2915	****
	H	Naïve W1118 average preference index during odor-off vs. after choice				0.1069	W = 1824	ns
	I	Shock-trained W1118 average preference index during odor-off vs. after choice				< 0.0001	W = -5294	****
	J	Sucrose-trained W1118 average preference index during odor-off vs. after choice				0.0068	W = 1313	**
4	E	Canton-S total total distance travelled between condition	Kruskal-Wallis test	Dunn's test	Canton-S naïve = 113, sucrose-trained = 109, shock-trained = 94, W1118 naïve = 158, sucrose-trained = 95, shock-trained = 172	< 0.0001	H = 79.97	****
	F	Canton-S distance travelled during the odor-off phase between conditions				< 0.0001	H = 22.19	****
	G	W1118 total total distance travelled between condition				< 0.0001	H = 184.5	****
	H	W1118 distance travelled during the odor-off phase between conditions				< 0.0001	H = 108.2	****
5	C	Canton-S naïve average distance travelled on each arena side over time	Repeated measures ANOVA	N/A	Canton-S naïve = 113	Arena side factor $p = 0.2326$, time factor $p < 0.0001$, interaction $p = 0.7303$	Arena side factor F(1, 112) = 1.441, time factor F(14.17, 1587) = 18.66, interaction F(17.73, 1986) = 10.7746	Arena side factor (ns), time factor (****), interaction (ns)
	D	Canton-S naïve average distance travelled between each arena side during odor off choice and post-choice	Ordinary 2-way ANOVA	N/A		Arena side factor $p < 0.7095$, phase factor $p < 0.0001$, interaction $p = 0.2688$	Arena side factor F(1, 672) = 0.1388, phase factor F(2, 672) = 28.35, interaction F(2, 672) = 1.210	Arena side factor (ns), phase factor (****), interaction (ns)
	E	W1118 naïve average distance travelled on each arena side over time	Repeated measures ANOVA	N/A		Arena side factor $p = 0.5172$, time factor $p < 0.0001$, interaction $p = 0.8984$	Arena side factor F(1, 157) = 0.2124, time factor F(14.42, 2284) = 11.80, interaction F(14.72, 2311) = 0.5579	Arena side factor (ns), time factor (****), interaction (ns)
	F	W1118 naïve average distance travelled between each arena side during odor off choice and post-choice	Ordinary 2-way ANOVA	N/A	W1118 naïve = 158	Arena side factor $p < 0.2837$, phase factor $p < 0.0001$, interaction $p = 0.8047$	Arena side factor F(1, 942) = 1.151, phase factor F(2, 942) = 23.82, interaction F(2, 942) = 0.2174	Arena side factor (****), phase factor (****), interaction (ns)
	G	Canton-S shock-trained average distance travelled on each arena side over time	Repeated measures ANOVA	N/A		Arena side factor $p < 0.0001$, time factor $p < 0.2056$	Arena side factor F(1, 93) = 59.01, time factor F(26, 2418) = 15.68, interaction F(26, 2418) = 1.219	Arena side factor (****), time factor (****), interaction (ns)
	H	Canton-S shock-trained average distance travelled between each arena side during odor off, choice and post-choice	Ordinary 2-way ANOVA	Tukey's multiple comparisons test		Arena side factor $p < 0.0001$, phase factor $p < 0.0001$, interaction $p = 0.3114$	Arena side factor F(1, 558) = 34.78, phase factor F(2, 558) = 37.04, interaction F(2, 558) = 1.263	Arena side factor (****), phase factor (****), interaction (ns)
	I	W1118 shock-trained average distance travelled on each arena side over time	Repeated measures ANOVA	N/A	Canton-S shock-trained = 94	Arena side factor $p < 0.0001$, time factor $p < 0.0001$, interaction $p = 0.4276$	Arena side factor F(1, 171) = 53.48, time factor F(8.124, 1566) = 27.49, interaction F(11.26, 1929) = 1.018	Arena side factor (****), time factor (****), interaction (ns)
	J	W1118 shock-trained average distance travelled between each arena side during odor off, choice and post-choice	Ordinary 2-way ANOVA	Tukey's multiple comparisons test		Arena side factor $p < 0.0001$, phase factor $p < 0.5862$	Arena side factor F(1, 1026) = 35.13, phase factor F(2, 1026) = 58.09, interaction F(2, 1026) = 0.5344	Arena side factor (****), phase factor (****), interaction (ns)
	K	Canton-S sucrose-trained average distance travelled on each arena side over time	Repeated measures ANOVA	N/A		Arena side factor $p < 0.0001$, time factor $p < 0.0001$, interaction $p = 0.0022$	Arena side factor F(1, 108) = 34.28, time factor F(12.85, 1388) = 24.77, interaction F(15.63, 1688) = 2.338	Arena side factor (****), time factor (****), interaction (**)
	L	Canton-S sucrose-trained average distance travelled between each arena side during odor off, choice and post-choice	Ordinary 2-way ANOVA	Tukey's multiple comparisons test	Canton-S sucrose-trained = 109	Arena side factor $p = 0.0104$, phase factor $p < 0.0001$, interaction $p < 0.0001$	Arena side factor F(1, 648) = 6.601, phase factor F(2, 648) = 36.68, interaction F(2, 648) = 11.70	Arena side factor (*), phase factor (****), interaction (****)
	M	W1118 sucrose-trained average distance travelled on each arena side over time	Repeated measures ANOVA	N/A		Arena side factor $p < 0.0001$, time factor $p < 0.0001$, interaction $p < 0.2007$	Arena side factor F(1, 94) = 23.26, time factor F(13.49, 1268) = 28.37, interaction F(14.34, 1348) = 1.289	Arena side factor (****), time factor (****), interaction (ns)
	N	W1118 sucrose-trained average distance travelled between each arena side during odor off, choice and post-choice	Ordinary 2-way ANOVA	Tukey's multiple comparisons test		Arena side factor $p < 0.0001$, phase factor $p < 0.0001$, interaction $p = 0.1455$	Arena side factor F(1, 564) = 30.34, phase factor F(2, 564) = 36.45, interaction F(2, 564) = 1.934	Arena side factor (****), phase factor (****), interaction (ns)
6	C	Canton-S naïve average bout duration on each arena side over time	Repeated measures ANOVA	N/A	Canton-S naïve = 113	Arena side factor $p = 0.3905$, time factor $p = 0.3885$, interaction $p = 0.0070$	Arena side factor F(1, 112) = 0.7433, time factor F(2.984, 334.2) = 4.116, interaction F(5.487, 614.5) = 1.052	Arena side factor (ns), time factor (ns), interaction (**)
	D	Canton-S naïve average bout duration between each arena side during odor off choice and post-choice	Ordinary 2-way ANOVA	N/A		Arena side factor $p = 0.4545$, phase factor $p = 0.1078$, interaction $p = 0.0012$	Arena side factor F(1, 672) = 0.5600, phase factor F(2, 672) = 2.235, interaction F(2, 672) = 6.764	Arena side factor (ns), phase factor (ns), interaction (**)
	E	W1118 naïve average bout duration on each arena side over time	Repeated measures ANOVA	N/A		Arena side factor $p = 0.8317$, time factor $p = 0.0007$, interaction $p = 0.8474$	Arena side factor F(1, 157) = 0.04531, time factor F(8, 1256) = 3.423, interaction F(8, 1256) = 0.5129	Arena side factor (ns), time factor (ns), interaction (ns)
	F	W1118 naïve average bout duration between each arena side during odor off choice and post-choice	Ordinary 2-way ANOVA	N/A	W1118 naïve = 158	Arena side factor $p = 0.9444$, phase factor $p = 0.5660$, interaction $p = 0.3423$	Arena side factor F(1, 942) = 0.00486, phase factor F(2, 942) = 0.5696, interaction F(2, 942) = 1.073	Arena side factor (ns), phase factor (ns), interaction (ns)
	G	Canton-S shock-trained average bout duration on each arena side over time	Repeated measures ANOVA	N/A		Arena side factor $p < 0.0001$, time factor $p = 0.0642$, interaction $p < 0.0001$	Arena side factor F(1, 93) = 85.60, time factor F(2.129, 198) = 2.730, interaction F(4.844, 450.5) = 8.384	Arena side factor (****), time factor (ns), interaction (****)
	H	Canton-S shock-trained average bout duration between each arena side during odor off, choice and post-choice	Ordinary 2-way ANOVA	Tukey's multiple comparisons test		Arena side factor $p < 0.0001$, phase factor $p = 0.4608$, interaction $p < 0.0001$	Arena side factor F(1, 588) = 87.25, phase factor F(2, 588) = 0.7758, interaction F(2, 588) = 23.85	Arena side factor (****), phase factor (ns), interaction (****)
	I	W1118 shock-trained average bout duration on each arena side over time	Repeated measures ANOVA	N/A	W1118 shock-trained = 172	Arena side factor $p < 0.0001$, time factor $p = 0.0005$, interaction $p < 0.0001$	Arena side factor F(1, 171) = 50.86, time factor F(8, 1368) = 3.488, interaction F(8, 1368) = 11.72	Arena side factor (****), time factor (****), interaction (****)
	J	W1118 shock-trained average bout duration between each arena side during odor off, choice and post-choice	Ordinary 2-way ANOVA	Tukey's multiple comparisons test		Arena side factor $p < 0.0001$, phase factor $p = 0.5457$, interaction $p < 0.0001$	Arena side factor F(1, 1026) = 59.06, phase factor F(2, 1026) = 0.6057, interaction F(2, 1026) = 19.36	Arena side factor (****), phase factor (ns), interaction (****)
	K	Canton-S sucrose-trained average bout duration on each arena side over time	Repeated measures ANOVA	N/A		Arena side factor $p < 0.0001$, time factor $p = 0.0009$, interaction $p < 0.0001$	Arena side factor F(1, 108) = 61.07, time factor F(3.805, 411) = 4.892, interaction F(5.679, 613.4) = 7.795	Arena side factor (****), time factor (****), interaction (****)
	L	Canton-S sucrose-trained average bout duration between each arena side during odor off, choice and post-choice	Ordinary 2-way ANOVA	Tukey's multiple comparisons test	Canton-S sucrose-trained = 109	Arena side factor $p < 0.0001$, phase factor $p = 0.5179$, interaction $p < 0.0001$	Arena side factor F(1, 648) = 31.20, phase factor F(2, 648) = 0.6587, interaction F(2, 648) = 30.59	Arena side factor (****), phase factor (ns), interaction (****)
	M	W1118 sucrose-trained average bout duration on each arena side over time	Repeated measures ANOVA	N/A		Arena side factor $p < 0.0001$, time factor $p < 0.0001$, interaction $p = 0.0981$	Arena side factor F(1, 94) = 16.67, time factor F(4.235, 398.1) = 9.936, interaction F(6.671, 627) = 1.753	Arena side factor (****), time factor (****), interaction (ns)
	N	W1118 sucrose-trained average bout duration between each arena side during odor off, choice and post-choice	Ordinary 2-way ANOVA	Tukey's multiple comparisons test		Arena side factor $p < 0.0001$, phase factor $p = 0.0536$, interaction $p = 0.0029$	Arena side factor F(1, 564) = 30.65, phase factor F(2, 564) = 2.941, interaction F(2, 564) = 5.912	Arena side factor (****), phase factor (ns), interaction (**)

Table 2.1 Statistics tests per-figure, figures 2-6.

7	B	Canton-S naïve average U-turns per cm between arena sides after choice	Wilcoxon matched-pairs signed rank test	N/A	Canton-S naïve = 113	0.1592	W = 473	ns
	C	Canton-S shock-trained average U-turns per cm between arena sides after choice			Canton-S shock-trained = 94	< 0.0001	W = -1538	****
	D	Canton-S sucrose-trained average U-turns per cm between arena sides after choice			Canton-S sucrose-trained = 109	0.028	W = 966	*
	E	W1118 naïve average U-turns per cm between arena sides after choice			W1118 naïve = 158	0.324	W = -585	ns
	F	W1118 shock-trained average U-turns per cm between arena sides after choice			W1118 shock-trained =172	0.1583	W = -397	ns
	G	W1118 sucrose-trained average U-turns per cm between arena sides after choice			W1118 sucrose-trained = 95	0.0032	W = 753	**
	I	Canton-S naïve average distance to odor port on each arena side after choice	paired T-test (two-tailed)		Canton-S naïve = 98	0.1272	t(97) = 1.538	ns
	J	Canton-S shock-trained average distance to odor port on each arena side after choice	paired T-test (one-tailed)		Canton-S shock-trained = 94	< 0.0001	t(93) = 10.62	****
	K	Canton-S sucrose-trained average distance to odor port on each arena side after choice	Wilcoxon matched-pairs signed rank test		Canton-S sucrose-trained = 104	< 0.0001	W = -3784	****
	L	W1118 naïve average distance to odor port on each arena side after choice	paired T-test (two-tailed)		W1118 naïve = 138	0.3878	t(137) = 0.8664	ns
	M	W1118 shock-trained average distance to odor port on each arena side after choice	paired T-test (one-tailed)		W1118 shock-trained =124	< 0.0001	t(123) = 4.298	****
	N	W1118 sucrose-trained average distance to odor port on each arena side after choice	paired T-test (one-tailed)		W1118 sucrose-trained = 88	0.0358	t(87) = 1.824	*
	P	Canton-S naïve total stops close to port on each arena side after choice	Wilcoxon matched-pairs signed rank test		Canton-S naïve = 113	0.052	W = 686	ns
	Q	Canton-S shock-trained total stops close to port on each arena side after choice			Canton-S shock-trained = 94	< 0.0001	W = -1342	****
	R	Canton-S sucrose-trained total stops close to port on each arena side after choice			Canton-S sucrose-trained = 109	< 0.0001	W = 853	****
	S	W1118 naïve total stops close to port on each arena side after choice			W1118 naïve = 158	0.9043	W = -53	ns
	T	W1118 shock-trained total stops close to port on each arena side after choice			W1118 shock-trained =172	0.0002	W = -976	***
	U	W1118 sucrose-trained total stops close to port on each arena side after choice			W1118 sucrose-trained = 95	0.0002	W = 821	***
	8	H	Shock-trained Canton-S vs. Duncie average post-choice preference index		Mann-Whitney test	Canton-S sucrose-trained = 120, Duncie sucrose-trained = 108, Canton-S shock trained = 79, Duncie shock trained = 76	< 0.0001	U = 1554
I		Shock-trained Canton-S vs. Duncie average post-choice U-turns per cm. travelled	0.019	U = 2439			*	
J		Shock-trained Canton-S vs. Duncie average distance to sucrose paired odor side after choice	< 0.0001	U = 1787			****	
K		Shock-trained Canton-S vs. Duncie total stops close to sucrose-paired port after choice	0.0009	U = -1032			***	
L		Sucrose-trained Canton-S vs. Duncie average post-choice preference index	0.0014	U = 4998			**	
M		Sucrose-trained Canton-S vs. Duncie average post-choice U-turns per cm. travelled	0.0026	U =5100			**	
N		Sucrose-trained Canton-S vs. Duncie average distance to sucrose paired odor side after choice	< 0.0001	U = 4397			****	
O		Sucrose-trained Canton-S vs. Duncie total stops close to sucrose-paired port after choice	< 0.0001	U = 3591			****	
S.3	B	EA-shock paired Canton-S preference index	Wilcoxon matched-pairs signed rank test	N/A	CS shock-trained EA sucrose paired =48	< 0.0001	W = -814	****
	C	IAAc-shock paired Canton-S preference index			CS shock-trained IAAc sucrose paired = 46	0.0212	W = -371	*
	E	EA-shock paired W1118 preference index			W1118 shock-trained EA sucrose paired = 80	0.0156	W = -846	*
	F	IAAc-shock paired W1118 preference index			W1118 shock-trained IAAc sucrose paired = 92	0.0004	W = -1645	***
	H	EA-suc paired Canton-S preference index			CS sucrose-trained EA sucrose paired = 57	< 0.0001	W = 1235	****
	I	IAAc-suc paired Canton-S preference index			CS sucrose-trained IAAc sucrose paired = 52	0.2213	W = 166	ns
	K	EA-suc paired W1118 preference index			W1118 sucrose-trained EA sucrose paired = 50	0.179	W = 187	ns
	L	IAAc-suc paired W1118 preference index			W1118 sucrose-trained IAAc sucrose paired = 45	0.0041	W = 463	**
S.4	I	Canton-S average transitions per bin in the choice phase	Kruskal-Wallis test	Dunn's test	Canton-S naïve = 113, sucrose-trained = 109, shock-trained = 94, W1118 naïve = 158, sucrose-trained = 95, shock-trained = 172	< 0.0001	H = 38.31	****
	J	Canton-S total transitions				< 0.0001	H = 53.68	****
	K	W1118 average transitions per bin in the choice phase				< 0.0001	H = 102.9	****
	L	W1118 total transitions				< 0.0001	H = 92.84	****
S.6	C	Canton-S shock-trained average preference index compared between odor off and post-choice	Wilcoxon matched-pairs signed rank test	Dunn's test	Canton-S shock-trained = 79, Canton-S sucrose-trained = 120, Duncie shock-trained = 76, Duncie sucrose-trained = 108,	< 0.0001	W = -1840	****
	D	Canton-S sucrose-trained average preference index compared between odor off and post-choice				0.0089	W = 1804	**
	E	Duncie shock-trained average preference index compared between odor off and post-choice				0.0037	W = -1028	**
	F	Duncie sucrose-trained average preference index compared between odor off and post-choice				0.2034	W = 544	ns

Table 2.2. Statistics tests per-figure, figures 7-8, supplementary figures S.3-S.6.

4	naïve vs. sucrose $p = 0.0019$, naïve vs. shock $p < 0.0001$, sucrose vs. shock $p < 0.0001$	naïve vs. sucrose $z = 3.423$, naïve vs. shock $z = 5.666$, sucrose vs. shock $z = 8.885$	naïve vs. sucrose **, naïve vs. shock ****, sucrose vs. shock ****
	naïve vs. sucrose $p = 0.0021$, naïve vs. shock $p = 0.5920$, sucrose vs. shock $p < 0.0001$	naïve vs. sucrose $z = 3.385$, naïve vs. shock $z = 1.289$, sucrose vs. shock $z = 4.507$	naïve vs. sucrose **, naïve vs. shock ns, sucrose vs. shock ****
	naïve vs. sucrose $p = 0.0019$, naïve vs. shock $p < 0.0001$, sucrose vs. shock $p < 0.0001$	naïve vs. sucrose $z = 3.423$, naïve vs. shock $z = 5.666$, sucrose vs. shock $z = 8.885$	naïve vs. sucrose **, naïve vs. shock ****, sucrose vs. shock ****
	naïve vs. sucrose $p = 0.0416$, naïve vs. shock $p < 0.0001$, sucrose vs. shock $p < 0.0001$	naïve vs. sucrose $z = 2.461$, naïve vs. shock $z = 10.89$, sucrose vs. shock $z = 11.89$	naïve vs. sucrose *, naïve vs. shock ****, sucrose vs. shock ****
5	odor off $p = 0.0363$, exploration $p < 0.0001$, choice $p < 0.0001$	odor off $q (558) = 2.968$, exploration $q (558) = 5.633$, choice $q (558) = 5.840$	odor off (*), choice (****), post-choice (****)
	N/A	N/A	N/A
	odor off $p = 0.0027$, exploration $p = 0.0028$, choice $p < 0.0001$	odor off $q (1026) = 4.253$, exploration $q (1026) = 4.232$, choice $q (1026) = 6.033$	odor off (**), exploration (**), choice (****)
	N/A	N/A	N/A
	odor off $p = 0.0172$, exploration $p = 0.0062$, choice $p < 0.0001$	odor off $q (648) = 3.378$, exploration $q (648) = 3.882$, choice $q (648) = 5.789$	odor off (*), exploration (**), choice (****)
	N/A	N/A	N/A
	odor off $p = 0.2288$, exploration $p = < 0.0001$, choice $p = 0.0106$	odor off $q (564) = 1.704$, exploration $q (564) = 5.637$, choice $q (564) = 3.625$	odor off (ns), exploration (****), choice (*)
6	odor off $p = 0.2883$, exploration $p < 0.0001$, choice $p < 0.0001$	odor off $q (558) = 1.503$, exploration $q (558) = 6.264$, choice $q (558) = 15.11$	odor off (ns), exploration (****), choice (****)
	N/A	N/A	N/A
	odor off $p = 0.2600$, exploration $p = 0.0078$, choice $p < 0.0001$	odor off $q (1026) = 1.594$, exploration $q (1026) = 3.769$, choice $q (1026) = 13.46$	odor off (ns), exploration (**), choice (****)
	N/A	N/A	N/A
	odor off $p = 0.0394$, exploration $p = 0.0058$, choice $p < 0.0001$	odor off $q (648) = 2.919$, exploration $q (648) = 3.916$, choice $q (648) = 12.69$	odor off (*), exploration (**), choice (****)
	N/A	N/A	N/A
	odor off $p = 0.6688$, exploration $p < 0.0001$, choice $p < 0.0001$	odor off $q (564) = 0.6053$, exploration $q (564) = 5.904$, choice $q (564) = 7.051$	odor off (ns), exploration (****), choice (****)
S.4	naïve vs. sucrose $p = >0.9999$, naïve vs. shock $p < 0.0001$, sucrose vs. shock $p < 0.0001$	naïve vs. sucrose $z = 0.05715$, naïve vs. shock $z = 5.429$, sucrose vs. shock $z = 5.439$	naïve vs. sucrose ns, naïve vs. shock ****, sucrose vs. shock ****
	naïve vs. sucrose $p = >0.9999$, naïve vs. shock $p < 0.0001$, sucrose vs. shock $p < 0.0001$	naïve vs. sucrose $z = 0.6004$, naïve vs. shock $z = 6.153$, sucrose vs. shock $z = 6.675$	naïve vs. sucrose ns, naïve vs. shock **, sucrose vs. shock ****
	naïve vs. sucrose $p = >0.9999$, naïve vs. shock $p < 0.0001$, sucrose vs. shock $p < 0.0001$	naïve vs. sucrose $z = 0.1148$, naïve vs. shock $z = 9.147$, sucrose vs. shock $z = 7.768$	naïve vs. sucrose ns, naïve vs. shock ****, sucrose vs. shock ****
	naïve vs. sucrose $p = 0.0325$, naïve vs. shock $p < 0.0001$, sucrose vs. shock $p < 0.0001$	naïve vs. sucrose $z = 2.548$, naïve vs. shock $z = 9.461$, sucrose vs. shock $z = 5.568$	naïve vs. sucrose *, naïve vs. shock ****, sucrose vs. shock ****

Table 2.3. Post-hoc statistics tests per figure; figures 7-8, supplementary figures S.3-S.6.

REFERENCES

- Ahmed, S.H. (2018) "Individual decision-making in the causal pathway to addiction: contributions and limitations of rodent models," *Pharmacology, Biochemistry, and Behavior*, 164, pp. 22–31. Available at: <https://doi.org/10.1016/j.pbb.2017.07.005>.
- Aso, Y., Hattori, D., Yu, Y., Johnston, R.M., Iyer, N.A., Ngo, T.-T., Dionne, H., Abbott, L., Axel, R., Tanimoto, H., *et al.* (2014) "The neuronal architecture of the mushroom body provides a logic for associative learning," *eLife*, 3, p. e04577. Available at: <https://doi.org/10.7554/eLife.04577>.
- Bazzell, B., Ginzberg, S., Healy, L. and Wessells, R.J. (2013) "Dietary composition regulates *Drosophila* mobility and cardiac physiology," *The Journal of Experimental Biology*, 216(Pt 5), pp. 859–868. Available at: <https://doi.org/10.1242/jeb.078758>.
- Bell, W.J., Cathy, T., Roggero, R.J., Kipp, L.R. and Tobin, T.R. (1985) "Sucrose-stimulated searching behaviour of *Drosophila melanogaster* in a uniform habitat: modulation by period of deprivation," *Animal Behaviour*, 33(2), pp. 436–448. Available at: [https://doi.org/10.1016/S0003-3472\(85\)80068-3](https://doi.org/10.1016/S0003-3472(85)80068-3).
- Boto, T., Stahl, A. and Tomchik, S.M. (2020) "Cellular and circuit mechanisms of olfactory associative learning in *Drosophila*," *Journal of Neurogenetics*, 34(1), pp. 36–46. Available at: <https://doi.org/10.1080/01677063.2020.1715971>.
- Branson, K., Robie, Alice A., Bender, J., Perona, P. and Dickinson, M.H. (2009) "High-throughput ethomics in large groups of *Drosophila*," *Nature Methods*, 6(6), pp. 451–457. Available at: <https://doi.org/10.1038/nmeth.1328>.
- Branson, K., Robie, Alice A., Bender, J., Perona, P. and Dickinson, M.H. (2009) "High-throughput ethomics in large groups of *Drosophila*," *Nature Methods*, 6(6), pp. 451–457. Available at: <https://doi.org/10.1038/nmeth.1328>.
- van Breugel, F. (2021) "Correlated decision making across multiple phases of olfactory-guided search in *Drosophila* improves search efficiency," *The Journal of Experimental Biology*, 224(16), p. jeb242267. Available at: <https://doi.org/10.1242/jeb.242267>.
- Buchert, S.N., Murakami, P., Kalavadia, A.H., Reyes, M.T. and Sitaraman, D. (2022) "Sleep correlates with behavioral decision making critical for reproductive output in *Drosophila melanogaster*," *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology*, 264, p. 111114. Available at: <https://doi.org/10.1016/j.cbpa.2021.111114>.
- Byers, D., Davis, R.L. and Kiger, J.A. (1981) "Defect in cyclic AMP phosphodiesterase due to the dunce mutation of learning in *Drosophila melanogaster*," *Nature*, 289(5793), pp. 79–81. Available at: <https://doi.org/10.1038/289079a0>.
- Cabeza, L., Giustiniani, J., Chabin, T., Ramadan, B., Joucla, C., Nicolier, M., Pazart, L., Haffen, E., Fellmann, D., Gabriel, D., *et al.* (2020) "Modelling decision-making under uncertainty: A direct comparison study between human and mouse gambling data," *European Neuropsychopharmacology: The Journal of the European College of Neuropsychopharmacology*, 31, pp. 58–68. Available at: <https://doi.org/10.1016/j.euroneuro.2019.11.005>.

- Camchong, J., Endres, M. and Fein, G. (2014) "Decision making, risky behavior, and alcoholism," *Handbook of Clinical Neurology*, 125, pp. 227–236. Available at: <https://doi.org/10.1016/B978-0-444-62619-6.00014-8>.
- Chabaud, M.-A., Preat, T. and Kaiser, L. (2010) "Behavioral Characterization of Individual Olfactory Memory Retrieval in *Drosophila Melanogaster*," *Frontiers in Behavioral Neuroscience*, 4. Available at: <https://doi.org/10.3389/fnbeh.2010.00192>.
- Cognigni, P., Felsenberg, J. and Waddell, S. (2018) "Do the right thing: neural network mechanisms of memory formation, expression and update in *Drosophila*," *Current Opinion in Neurobiology*, 49, pp. 51–58. Available at: <https://doi.org/10.1016/j.conb.2017.12.002>.
- Conradt, L. and Roper, T.J. (2005) "Consensus decision making in animals," *Trends in Ecology & Evolution*, 20(8), pp. 449–456. Available at: <https://doi.org/10.1016/j.tree.2005.05.008>.
- Davis, R.L. (1993) "Mushroom bodies and *Drosophila* learning," *Neuron*, 11(1), pp. 1–14. Available at: [https://doi.org/10.1016/0896-6273\(93\)90266-t](https://doi.org/10.1016/0896-6273(93)90266-t).
- Davis, R.L. (1996) "Physiology and biochemistry of *Drosophila* learning mutants," *Physiological Reviews*, 76(2), pp. 299–317. Available at: <https://doi.org/10.1152/physrev.1996.76.2.299>.
- Davis, R.L. (2011) "Traces of *Drosophila* memory," *Neuron*, 70(1), pp. 8–19. Available at: <https://doi.org/10.1016/j.neuron.2011.03.012>.
- Davis, R.L. (2023) "Learning and memory using *Drosophila melanogaster*: a focus on advances made in the fifth decade of research," *GENETICS*. Edited by J. Carlson, 224(4), p. iyad085. Available at: <https://doi.org/10.1093/genetics/iyad085>.
- Davis, R.L. and Kiger, J.A. (1981) "Dunce mutants of *Drosophila melanogaster*: mutants defective in the cyclic AMP phosphodiesterase enzyme system.," *The Journal of cell biology*, 90(1), pp. 101–107. Available at: <https://doi.org/10.1083/jcb.90.1.101>.
- De Bivort, B., Buchanan, S., Skutt-Kakaria, K., Gajda, E., Ayroles, J., O'Leary, C., Reimers, P., Akhund-Zade, J., Senft, R., Maloney, R., *et al.* (2022) "Precise Quantification of Behavioral Individuality From 80 Million Decisions Across 183,000 Flies," *Frontiers in Behavioral Neuroscience*, 16, p. 836626. Available at: <https://doi.org/10.3389/fnbeh.2022.836626>.
- De Bivort, B.L. (2025) "The Developmental Origins of Behavioral Individuality," *Annual Review of Cell and Developmental Biology*, 41(1), pp. 331–352. Available at: <https://doi.org/10.1146/annurev-cellbio-101323-025423>.
- Dévav, P., Pint'er, M., Yalcin, A.S. and Friedrich, P. (1986) "Altered autophosphorylation of adenosine 3',5'-phosphate-dependent protein kinase in the dunce memory mutant of *Drosophila melanogaster*," *Neuroscience*, 18(1), pp. 193–203. Available at: [https://doi.org/10.1016/0306-4522\(86\)90188-0](https://doi.org/10.1016/0306-4522(86)90188-0).
- Dorkenwald, S., Matsliah, A., Sterling, A.R., Schlegel, P., Yu, S., McKellar, C.E., Lin, A., Costa, M., Eichler, K., Yin, Y., *et al.* (2024) "Neuronal wiring diagram of an adult brain," *Nature*, 634(8032), pp. 124–138. Available at: <https://doi.org/10.1038/s41586-024-07558-y>.

Dubnau, J., Chiang, A.-S., Grady, L., Barditch, J., Gossweiler, S., McNeil, J., Smith, P., Buldoc, F., Scott, R., Certa, U., *et al.* (2003) “The staufen/pumilio pathway is involved in *Drosophila* long-term memory,” *Current biology: CB*, 13(4), pp. 286–296. Available at: [https://doi.org/10.1016/s0960-9822\(03\)00064-2](https://doi.org/10.1016/s0960-9822(03)00064-2).

Dubnau, J., Grady, L., Kitamoto, T. and Tully, T. (2001) “Disruption of neurotransmission in *Drosophila* mushroom body blocks retrieval but not acquisition of memory,” *Nature*, 411(6836), pp. 476–480. Available at: <https://doi.org/10.1038/35078077>.

Dudai, Y. (1983) “Mutations affect storage and use of memory differentially in *Drosophila*,” *Proceedings of the National Academy of Sciences*, 80(17), pp. 5445–5448. Available at: <https://doi.org/10.1073/pnas.80.17.5445>.

Dudai, Y., Jan, Y.N., Byers, D., Quinn, W.G. and Benzer, S. (1976) “dunce, a mutant of *Drosophila* deficient in learning,” *Proceedings of the National Academy of Sciences of the United States of America*, 73(5), pp. 1684–1688. Available at: <https://doi.org/10.1073/pnas.73.5.1684>.

Dukas, R. (ed.) (2007) *Cognitive ecology: the evolutionary ecology of information processing and decision making*. Nachdr. Chicago: Univ. of Chicago Press.

Dwijesha, A.S., Eswaran, A., Berry, J.A. and Phan, A. (2024) “Diverse memory paradigms in *Drosophila* reveal diverse neural mechanisms,” *Learning & Memory (Cold Spring Harbor, N.Y.)*, 31(5), p. a053810. Available at: <https://doi.org/10.1101/lm.053810.123>.

Ferreira, C.H. and Moita, M.A. (2020) “Behavioral and neuronal underpinnings of safety in numbers in fruit flies,” *Nature Communications*, 11(1), p. 4182. Available at: <https://doi.org/10.1038/s41467-020-17856-4>.

Folkers, E., Drain, P. and Quinn, W.G. (1993) “Radish, a *Drosophila* mutant deficient in consolidated memory,” *Proceedings of the National Academy of Sciences*, 90(17), pp. 8123–8127. Available at: <https://doi.org/10.1073/pnas.90.17.8123>.

Gall, G.E.C., Strandburg-Peshkin, A., Clutton-Brock, T. and Manser, M.B. (2017) “As dusk falls: collective decisions about the return to sleeping sites in meerkats,” *Animal Behaviour*, 132, pp. 91–99. Available at: <https://doi.org/10.1016/j.anbehav.2017.08.001>.

Gervasi, N., Tchénio, P. and Preat, T. (2010) “PKA dynamics in a *Drosophila* learning center: coincidence detection by rutabaga adenylyl cyclase and spatial regulation by dunce phosphodiesterase,” *Neuron*, 65(4), pp. 516–529. Available at: <https://doi.org/10.1016/j.neuron.2010.01.014>.

Gibson, W.T., Gonzalez, C.R., Fernandez, C., Ramasamy, L., Tabachnik, T., Du, R.R., Felsen, P.D., Maire, M.R., Perona, P. and Anderson, D.J. (2015) “Behavioral Responses to a Repetitive Visual Threat Stimulus Express a Persistent State of Defensive Arousal in *Drosophila*,” *Current Biology*, 25(11), pp. 1401–1415. Available at: <https://doi.org/10.1016/j.cub.2015.03.058>.

Godesberg, V., Bockemühl, T. and Büschges, A. (2024) “Natural variability and individuality of walking behavior in *Drosophila*,” *Journal of Experimental Biology*, 227(22), p. jeb247878. Available at: <https://doi.org/10.1242/jeb.247878>.

- Guven-Ozkan, T. and Davis, R.L. (2014) "Functional neuroanatomy of *Drosophila* olfactory memory formation," *Learning & Memory (Cold Spring Harbor, N.Y.)*, 21(10), pp. 519–526. Available at: <https://doi.org/10.1101/lm.034363.114>.
- Harris, A. and Hutcherson, C.A. (2022) "Temporal dynamics of decision making: A synthesis of computational and neurophysiological approaches," *Wiley Interdisciplinary Reviews. Cognitive Science*, 13(3), p. e1586. Available at: <https://doi.org/10.1002/wcs.1586>.
- Heisenberg, M. (2003) "Mushroom body memoir: from maps to models," *Nature Reviews Neuroscience*, 4(4), pp. 266–275. Available at: <https://doi.org/10.1038/nrn1074>.
- Ichinose, T. and Tanimoto, H. (2016) "Dynamics of memory-guided choice behavior in *Drosophila*," *Proceedings of the Japan Academy. Series B, Physical and Biological Sciences*, 92(8), pp. 346–357. Available at: <https://doi.org/10.2183/pjab.92.346>.
- Iglesias, A.G., Chiu, A.S., Wong, J., Campus, P., Li, F., Liu, Z. (Nemo), Bhatti, J.K., Patel, S.A., Deisseroth, K., Akil, H., *et al.* (2023) "Inhibition of Dopamine Neurons Prevents Incentive Value Encoding of a Reward Cue: With Revelations from Deep Phenotyping," *The Journal of Neuroscience*, 43(44), pp. 7376–7392. Available at: <https://doi.org/10.1523/JNEUROSCI.0848-23.2023>.
- Izquierdo, A. and Belcher, A.M. (2012) "Rodent models of adaptive decision making," *Methods in Molecular Biology (Clifton, N.J.)*, 829, pp. 85–101. Available at: https://doi.org/10.1007/978-1-61779-458-2_5.
- Jovanic, T. (2020) "Studying neural circuits of decision-making in *Drosophila* larva," *Journal of Neurogenetics*, 34(1), pp. 162–170. Available at: <https://doi.org/10.1080/01677063.2020.1719407>.
- Ladd, C.E. and Simpson, J.H. (2025) "Behavior choices amongst grooming, feeding, and courting in *Drosophila* show contextual flexibility, not an absolute hierarchy of needs," *The Journal of Experimental Biology*, p. jeb.250826. Available at: <https://doi.org/10.1242/jeb.250826>.
- Lebovich, L., Alisch, T., Redhead, E.S., Parker, M.O., Loewenstein, Y., Couzin, I.D. and De Bivort, B.L. (2024) "Spatiotemporal dynamics of locomotor decisions in *Drosophila melanogaster*." *Animal Behavior and Cognition*. Available at: <https://doi.org/10.1101/2024.09.04.611038>.
- Lee, D. (2015) "Global and local missions of cAMP signaling in neural plasticity, learning, and memory," *Frontiers in Pharmacology*, 6. Available at: <https://doi.org/10.3389/fphar.2015.00161>.
- Maloney, R.T. (2021) "Neuromodulation and Individuality," *Frontiers in Behavioral Neuroscience*, 15, p. 777873. Available at: <https://doi.org/10.3389/fnbeh.2021.777873>.
- van der Meer, M., Kurth-Nelson, Z. and Redish, A.D. (2012) "Information processing in decision-making systems," *The Neuroscientist: A Review Journal Bringing Neurobiology, Neurology and Psychiatry*, 18(4), pp. 342–359. Available at: <https://doi.org/10.1177/1073858411435128>.
- Midroit, M., Chalençon, L., Renier, N., Milton, A., Thevenet, M., Sacquet, J., Breton, M., Forest, J., Noury, N., Richard, M., *et al.* (2021) "Neural processing of the reward value of pleasant

odorants,” *Current Biology*, 31(8), pp. 1592-1605.e9. Available at: <https://doi.org/10.1016/j.cub.2021.01.066>.

Modi, M.N., Rajagopalan, A.E., Rouault, H., Aso, Y. and Turner, G.C. (2023) “Flexible specificity of memory in *Drosophila* depends on a comparison between choices,” *eLife*, 12, p. e80923. Available at: <https://doi.org/10.7554/eLife.80923>.

Molchan, S.E., Sunderland, T., McIntosh, A.R., Herscovitch, P. and Schreurs, B.G. (1994) “A functional anatomical study of associative learning in humans,” *Proceedings of the National Academy of Sciences*, 91(17), pp. 8122–8126. Available at: <https://doi.org/10.1073/pnas.91.17.8122>.

Morand-Ferron, J. (2017) “Why learn? The adaptive value of associative learning in wild populations,” *Current Opinion in Behavioral Sciences*, 16, pp. 73–79. Available at: <https://doi.org/10.1016/j.cobeha.2017.03.008>.

Murata, S., Brockmann, A. and Tanimura, T. (2017) “Pharyngeal stimulation with sugar triggers local searching behavior in *Drosophila*,” *Journal of Experimental Biology*, p. jeb.161646. Available at: <https://doi.org/10.1242/jeb.161646>.

Pitman, J.L., DasGupta, S., Krashes, M.J., Leung, B., Perrat, P.N. and Waddell, S. (2009) “There are many ways to train a fly,” *Fly*, 3(1), pp. 3–9. Available at: <https://doi.org/10.4161/fly.3.1.7726>.

Qiu, Y. and Davis, R.L. (1993) “Genetic dissection of the learning/memory gene *dunce* of *Drosophila melanogaster*,” *Genes & Development*, 7(7B), pp. 1447–1458. Available at: <https://doi.org/10.1101/gad.7.7b.1447>.

Quinn, W.G., Harris, W.A. and Benzer, S. (1974) “Conditioned behavior in *Drosophila melanogaster*,” *Proceedings of the National Academy of Sciences of the United States of America*, 71(3), pp. 708–712. Available at: <https://doi.org/10.1073/pnas.71.3.708>.

Rajagopalan, A.E., Darshan, R., Hibbard, K.L., Fitzgerald, J.E. and Turner, G.C. (2023) “Reward expectations direct learning and drive operant matching in *Drosophila*,” *Proceedings of the National Academy of Sciences*, 120(39), p. e2221415120. Available at: <https://doi.org/10.1073/pnas.2221415120>.

Redish, A.D. and Mizumori, S.J.Y. (2015) “Memory and decision making,” *Neurobiology of Learning and Memory*, 117, pp. 1–3. Available at: <https://doi.org/10.1016/j.nlm.2014.08.014>.

Rihani, K. and Sachse, S. (2022) “Shedding Light on Inter-Individual Variability of Olfactory Circuits in *Drosophila*,” *Frontiers in Behavioral Neuroscience*, 16, p. 835680. Available at: <https://doi.org/10.3389/fnbeh.2022.835680>.

Roy, N.A., Bak, J.H., International Brain Laboratory, Akrami, A., Brody, C.D. and Pillow, J.W. (2021) “Extracting the dynamics of behavior in sensory decision-making experiments,” *Neuron*, 109(4), pp. 597-610.e6. Available at: <https://doi.org/10.1016/j.neuron.2020.12.004>.

Sato, D.X. and Takahashi, Y. (2025) “Neurogenomic and behavioral principles shape freezing dynamics and synergistic performance in *Drosophila melanogaster*,” *Nature Communications*, 16(1), p. 5928. Available at: <https://doi.org/10.1038/s41467-025-61313-z>.

- Scaplen, K.M., Mei, N.J., Bounds, H.A., Song, S.L., Azanchi, R. and Kaun, K.R. (2019) "Automated real-time quantification of group locomotor activity in *Drosophila melanogaster*," *Scientific Reports*, 9(1), p. 4427. Available at: <https://doi.org/10.1038/s41598-019-40952-5>.
- Schwaerzel, M., Monastirioti, M., Scholz, H., Friggi-Grelín, F., Birman, S. and Heisenberg, M. (2003) "Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in *Drosophila*," *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 23(33), pp. 10495–10502. Available at: <https://doi.org/10.1523/JNEUROSCI.23-33-10495.2003>.
- Shettleworth, S. (2009) "Learning: Introduction and Pavlovian Conditioning," in *Cognition, Evolution, and Behavior*. 2nd ed. New York, NY: Oxford Academic, pp. 96–135.
- Smith, M.A.-Y., Honegger, K.S., Turner, G. and De Bivort, B. (2022) "Idiosyncratic learning performance in flies," *Biology Letters*, 18(2), p. 20210424. Available at: <https://doi.org/10.1098/rsbl.2021.0424>.
- Suzuki, S., Adachi, R., Dunne, S., Bossaerts, P. and O'Doherty, J.P. (2015) "Neural mechanisms underlying human consensus decision-making," *Neuron*, 86(2), pp. 591–602. Available at: <https://doi.org/10.1016/j.neuron.2015.03.019>.
- Tabone, C. and de belle, S. (2014) "Olfactory learning and memory assays," in *Behavioral Genetics of the Fly (Drosophila Melanogaster)*. Cambridge University Press, pp. 231–249.
- Tempel, B.L., Bonini, N., Dawson, D.R. and Quinn, W.G. (1983) "Reward learning in normal and mutant *Drosophila*," *Proceedings of the National Academy of Sciences of the United States of America*, 80(5), pp. 1482–1486. Available at: <https://doi.org/10.1073/pnas.80.5.1482>.
- Totty, M.S., Warren, N., Huddleston, I., Ramanathan, K.R., Ressler, R.L., Oleksiak, C.R. and Maren, S. (2021) "Behavioral and brain mechanisms mediating conditioned flight behavior in rats," *Scientific Reports*, 11(1), p. 8215. Available at: <https://doi.org/10.1038/s41598-021-87559-3>.
- Tully, T. and Quinn, W.G. (1985) "Classical conditioning and retention in normal and mutant *Drosophila melanogaster*," *Journal of Comparative Physiology. A, Sensory, Neural, and Behavioral Physiology*, 157(2), pp. 263–277. Available at: <https://doi.org/10.1007/BF01350033>.
- Waddell, S. (2013) "Reinforcement signalling in *Drosophila*; dopamine does it all after all," *Current Opinion in Neurobiology*, 23(3), pp. 324–329. Available at: <https://doi.org/10.1016/j.conb.2013.01.005>.
- Walter, T. and Couzin, I.D. (2021) "TRex, a fast multi-animal tracking system with markerless identification, and 2D estimation of posture and visual fields," *eLife*, 10, p. e64000. Available at: <https://doi.org/10.7554/eLife.64000>.
- Walters, E.T., Carew, T.J. and Kandel, E.R. (1981) "Associative Learning in *Aplysia* : Evidence for Conditioned Fear in an Invertebrate," *Science*, 211(4481), pp. 504–506. Available at: <https://doi.org/10.1126/science.7192881>.

Wang, X.-J. (2012) “Neural dynamics and circuit mechanisms of decision-making,” *Current Opinion in Neurobiology*, 22(6), pp. 1039–1046. Available at: <https://doi.org/10.1016/j.conb.2012.08.006>.

Werkhoven, Z., Bravin, A., Skutt-Kakaria, K., Reimers, P., Pallares, L.F., Ayroles, J. and De Bivort, B.L. (2021) “The structure of behavioral variation within a genotype,” *eLife*, 10, p. e64988. Available at: <https://doi.org/10.7554/eLife.64988>.

Zacarias, R., Namiki, S., Card, G.M., Vasconcelos, M.L. and Moita, M.A. (2018) “Speed dependent descending control of freezing behavior in *Drosophila melanogaster*,” *Nature Communications*, 9(1), p. 3697. Available at: <https://doi.org/10.1038/s41467-018-05875-1>.

Zheng, Z., Lauritzen, J.S., Perlman, E., Robinson, C.G., Nichols, M., Milkie, D., Torrens, O., Price, J., Fisher, C.B., Sharifi, N., *et al.* (2018) “A Complete Electron Microscopy Volume of the Brain of Adult *Drosophila melanogaster*,” *Cell*, 174(3), pp. 730-743.e22. Available at: <https://doi.org/10.1016/j.cell.2018.06.019>.