Individuality in Phototactic Preference in Tethered Adult *Drosophila melanogaster*

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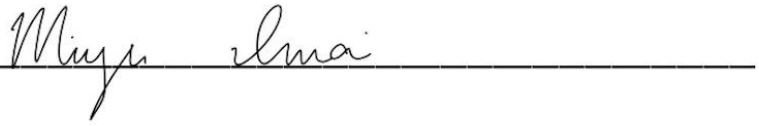
Signature: 

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List of Contributions

I completed this thesis under the guidance of Dr. de Bivort and my mentor, Danylo Lavrentovich. The current research project was conceived by Dr. de Bivort but jointly designed by Miyu Imai and Danylo Lavrentovich. Behavioral assays and statistical analysis were done independently by Miyu Imai. The Tunnel Assay Schematic presented in Figure 2 and the Calcium imaging setup for odor stimuli presented in Figure 30 was taken from a study by Churgin et al. 2021. The Fly Visual System Schematic presented in Figure 3 was taken from a study by Melnattur and Lee 2011. Computer modeling was done jointly by Miyu Imai with Danylo Lavrentovich and results were interpreted by Miyu Imai with assistance and guidance from Danylo Lavrentovich.

Abstract

Individuals, even when inbred and raised in similar environments, have variable behaviors. However, the mechanistic underpinnings of individuality remain poorly understood. *Drosophila melanogaster* is a useful model system for studying individuality, as flies have short lifespans and thus can be raised very quickly, and they are small/easy to handle, allowing us to collect behaviors for many individuals. Genetically similar flies raised in the same environments display individual variability in behaviors such as turn bias, temperature difference, olfaction, and other behaviors. This thesis set out to identify signals of individuality in tethered flies walking on a ball, which is a paradigm that is especially useful for the simultaneous measurement of circuit activity and walking behavior. I built a fly-on-a-ball setup that yields robust walking behavior and includes a visual assay that I use to analyze choices flies make when faced with boundaries between lights on and lights off. My experiments showed that individual flies display variable behavioral preferences in response to light stimuli and that the extent of variation exceeds that of blind control flies that do not perceive differences between lights on and off. I calculated several different light preference metrics and found significant signs of individuality by comparing these scores. Establishing individuality in this tethered setting will aid future investigations that measure calcium activity with behaviors simultaneously.

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Chapter I: Introduction

Individuality is a fundamental aspect of behavior. Individuals, even when inbred and raised in similar environments, have variable behaviors. Genetically similar flies raised in the same environments display individual variability in behaviors such as turn bias, temperature preferences, olfactory preferences, and other behaviors (Buchanan, Kain, and de Bivort 2015). This reflects the abundance of stochasticity in biological systems. It is impossible for two biological systems to function identically because of various characteristics, such as the countless number of nonlinear inter-molecular interactions, and thermodynamic instability (Honegger and de Bivort 2018). The mechanistic underpinnings of individuality remain poorly understood.

Drosophila melanogaster is a useful model system for studying individuality, as flies have short lifespans and thus can be raised very quickly, and they are small/easy to handle, allowing for the collection of behaviors for many individuals. This is very helpful for studying individuality, since making measurements over many individuals is required. Projects previously done in the de Bivort lab have assayed on the order of dozens to hundreds of individual flies (Churgin et al. 2021) (Werkhoven et al. 2021). The Decathlon project, where each fly underwent ten different behavioral assays, used over 200 flies. *Drosophila* usually takes about ten days to grow to adults, allowing us to raise young, active flies without time-intensive care. The immense genetic toolkit in *Drosophila* allows for testing a wide variety of mutants.

Connecting circuit-level variation to behavioral variation in fly olfaction

A major question addressed by the de Bivort lab is whether correlates of behavioral individuality can be found in circuit activity. Fly olfaction is a useful system to study this question since the olfactory circuit is well characterized and generally stereotyped across individuals.

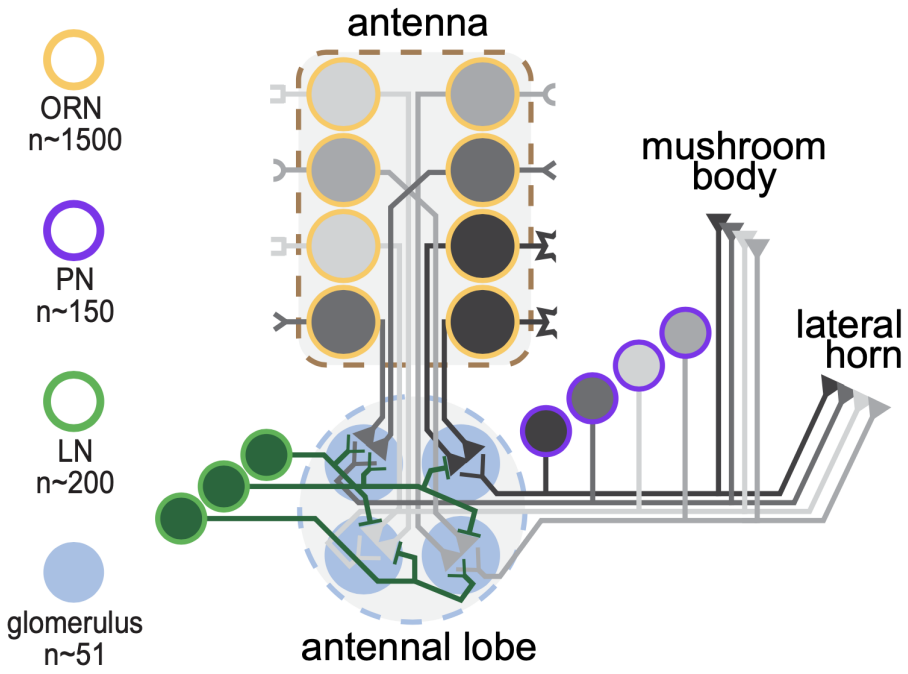


Figure 1: Antennal lobe schematic (Churgin et al. 2021)

The antennal lobe is the first site of olfactory information processing, and much has been understood about the cellular and synaptic mechanisms of neural odor representations. A schematic of the circuit is shown in Figure 1. Odorant molecules bind to receptors in the dendrites of olfactory receptor neurons (ORNs) in the antennae and maxillary palps, and ORNs have axons that project into the brain into microcircuits called glomeruli (Wilson 2013). All of

the ORNs that express a given odorant receptor converge into the same glomerulus. In this area, they make excitatory synapses with second-order neurons called projection neurons, PNs, which project into structures further into the brain, the lateral horn, and the mushroom body (Wilson 2013). Glomeruli are interconnected by a network of mostly-inhibitory local neurons, LNs (Shang et al. 2007) (Wilson and Laurent 2005). Activity can be measured in neurons in the circuit by measuring the fluorescence intensity of GCaMP, a calcium reporter. Imaging calcium activity while presenting odors has enabled the de Bivort lab to quantify ORN and PN responses to odors in dozens of individual flies (Honegger and de Bivort 2018) (Churgin et al. 2021).

Olfactory preference behaviors can be readily recorded, for instance, through tracking the motion of flies in an odor tunnel assay in which each half of each tunnel is perfused with one of two odors. As visualized in Figure 2 below, odor preference scores can be computed for each individual as the fraction of time each fly spent in one half of the chamber versus the other.

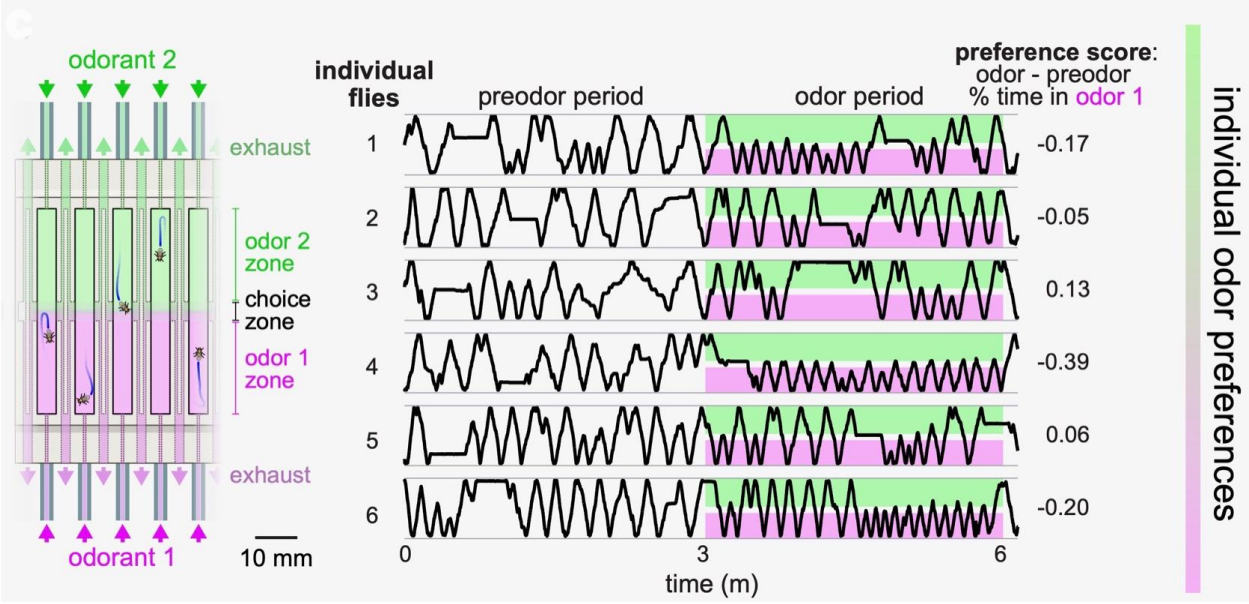


Figure 2: Tunnel Assay Schematic (Adapted from Churgin et al. 2021)

This shows the schematic for tunnel assay for measuring odor preferences, along with an example of an individual fly's ethogram.

Recent work in the de Bivort lab has combined behavioral preference recordings via the odor tunnel assay and neural activity recordings via calcium imaging to identify sites in the brain in which neural activity variation can predict behavioral variation among individuals (Churgin et al. 2021). An individual's PN responses were found to be predictive of behavioral preference between two aversive odorants, 4-methylcyclohexanol (MCH) and 3-octanol (OCT). However, an individual's ORN responses were most predictive of preference between a single aversive odorant (OCT), and air (Churgin et al. 2021). Thus, we have a promising system for identifying sites in the brain where variation predicts preference and behavioral variation. Circuits can encode different aspects of individuality in different places in different cell types. Therefore, fly olfaction is a very good system to determine the circuit underpinnings of behavioral variation.

Establishing individuality in walking behavior in response to visual stimuli

Though we understand that neural representations vary, the mechanism of this pattern remains poorly understood. An ongoing interest in the lab is to identify circuit elements predictive of moment-to-moment behavioral variability. Focusing on the olfactory circuit as an example, there is a myriad of complexity in the odor tunnel behavior – the example data in the top subpanel of Figure 2 shows that flies make many choices, especially at the boundaries between odors – sometimes they sample the boundary and turn back, or continue straight through the boundary.

What kinds of patterns in activity are visible in the antennal lobe as a fly makes choices at the boundary? How variable are these patterns across flies? In order to assess this, we need to measure neural activity and behavior simultaneously. A widely used paradigm – the fly-on-a-ball assay – is well suited for this purpose, in which a fly is mounted underneath a microscope enabling calcium imaging, or in a patch clamp setup enabling electrophysiology recordings, while also exhibiting walking behavior on an air-supported foam ball. Such a device has been used notably in the navigation field. For example, a study by Fisher et al. studies visual cue positions on *Drosophila* retina neurons using a fly-on-a-ball assay. They propose a model that correlated synaptic activity triggers long-term synaptic depression of visually stimulated inhibition (Fisher et al. 2019).

In the broader goal of combining behavior and neural activity measurement to identify circuit correlates of trial-to-trial variability, we first must establish behavioral individuality in behaviors as the fly walks on a ball. The goal of my thesis is to establish individuality in tethered flies in the fly-on-a-ball assay. I chose to focus on responses to visual stimuli, because adult flies are known to have variable light preference behaviors (J. S. Kain, Stokes, and de Bivort 2012), and setting up LEDs for visual stimuli enabled immediate behavioral tracking. The setup I constructed can be later adapted to test odor stimuli for eventual odor-related investigations.

Drosophila **visual circuit**

Phototaxis is widely studied and is a fruitful model system for studying individuality. Light is detected by photoreceptors, neurons containing light-sensing Rhodopsin proteins. These signals are relayed to optic lobes, where they are processed and then sent to further structures in the

brain (Behnia and Desplan 2015). The *Drosophila* visual system is composed of a combination of the eye and four optic ganglia: the lamina, the medulla, the lobula and the lobula plate (Figure 3) (Meinertzhagen and Hanson 1993). A fly perceives the visual world through activation of photoreceptor neurons, which are organized as arrays called ommatidia. Each ommatidium contains eight photoreceptor neurons, which are divided into three classes, based on their position and opsin gene expression. Figure 3 shows a schematic of the fly visual system. R1-R6, which are the six outer photoreceptors, respond to a broad spectrum of visible light, and express Rh1-opsin. They are comparable to vertebrate rod cells, which also respond to a broad spectrum of low-intensity light. R7 and R8, the two inner photoreceptors, respond to more specific wavelengths. They are comparable to vertebrate code cells, which respond to colorless stimuli. The R7 and R8 cells can be further categorized based on their opsin gene expression pattern; R7 photoreceptors respond to ultraviolet light, and express Rh3 or Rh4 opsin, while R8 photoreceptors respond to blue or green light, and express Rh5 or Rh6 opsin, respectively. These elements come together to conduct retinotopy, where neurons in the lamina and medulla respond to their respective photoreceptors from the visual spectrum of light, to accurately visualize and process the visual world (Melnattur and Lee 2011).

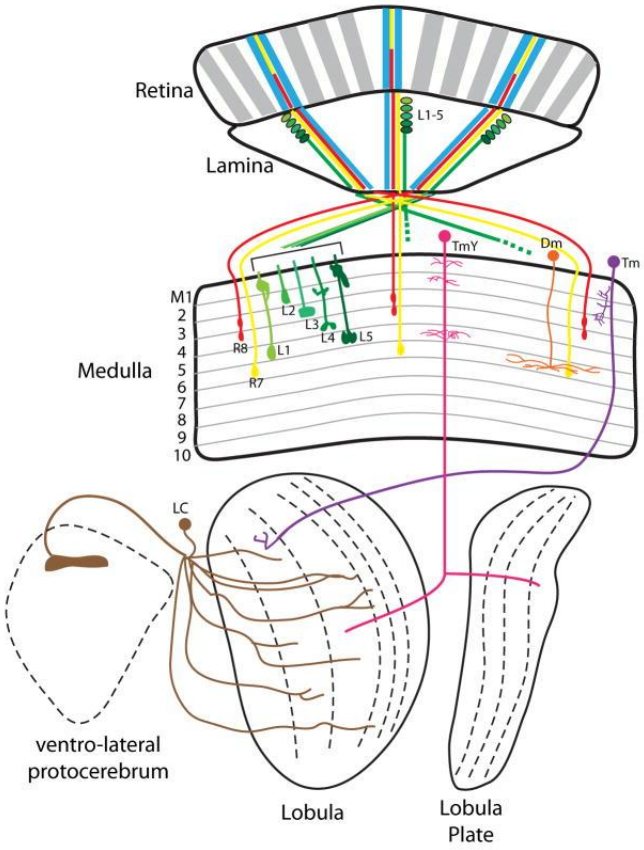


Figure 3: Fly Visual System Schematic (Taken from Melnattur and Lee 2011**)**

The fly visual system consists of the retina and four optic ganglia; the lamina, medulla, lobula and lobula plate. R1–R6 (shown in blue) sends axons from the retina to the lamina, which connect with lamina neurons (L). R7 cells (yellow) and R8 cells (red) connect with M6 and M3 layers of the medulla, respectively. L1–L5 (green) connect with layers M1–M5. The motion pathways are moderated by the lamina neurons L1 and L2, which relay R1–R6 input to a set of medulla neurons (striped circles) (Melnattur and Lee 2011).

Light preference in adult flies

In adults, it is known that *Drosophila* typically moves toward light when startled. Kain et al. developed "FlyVac," an assay collecting phototactic responses in single flies in parallel, to study

the neurobiological factors underlying individual-to-individual behavioral differences. The flies were loaded onto a start tube and exposed to an LED stimulus when they reached a choice point with an LED on either end. The fly's choice was recorded, and subsequently, the fly was pulled back to the start tube with a vacuum trap. The study found that Canton S. strain *Drosophila melanogaster* exhibits photopositive behavior, choosing the light side of the tunnel on average 80% of the time. The distribution over individuals was wide, and was consistent with a model assuming uniform phototactic preferences across individuals. However, in the w1118 strain, in which the flies were genetically mutated in a gene *white*, the fly's phototactic preference distribution was broader. Kain et al. also demonstrated that this variability in phototactic preference can be scaled up or down by manipulating serotonin levels, implicating a neuromodulator in the level of behavioral individuality in this circuit (J. S. Kain, Stokes, and de Bivort 2012).

Larvae and checkerboard assay

Phototaxis of fly larvae is a common paradigm for understanding *Drosophila* behavior and their mechanism, and allows us to investigate the circuits for orientation behavior from sensory input to motor output. It is also known that *Drosophila* larvae have the opposite preference as the adults, and are negatively phototactic during most of their development (Kane et al. 2013). Studies by Kane et al. studied the sensorimotor structure in Canton S. *Drosophila* larvae, using a checkerboard style board with light and dark zones. They examined the larva's motion as they navigated transitions between dark and light squares, as well as turning angles at the boundaries.

Adult *Drosophila* is also known to navigate based on patterns of linearly polarized light, and to positions of the sun. The nervous system detects and processes sensory information to direct steering maneuvers, which allow them to navigate direction (Warren, Giraldo, and Dickinson 2019). Recent studies by Giraldo et al. showed that adult *Drosophila* uses the position of a simulated sun to fly straight (Giraldo et al. 2018). This indicates strong promise in identifying individual signatures in *Drosophila* and is a good paradigm in vision that can be implemented on adults.

In our project, our overall goal is to establish individuality in walking behavior on the ball. We started by adopting paradigms from the assay in Kane et al. 2013, the checkerboard altering light-dark zone assay used to study larval phototaxis, but for adults. We utilize the FicTrac software, a technology that converts the walking behavior of flies tethered onto a ball into fictive motion in a 2-dimensional plane. This software is used widely for the fly-on-a-ball assay, and collects data on the fly's speed, direction, fictive position, rotation, etc (Moore et al. 2014).

I have built a fly-on-a-ball setup that yields good walking behavior, and I have set up a visual assay that I use to analyze choices flies make when faced with boundaries between lights on and lights off. I was directly involved in engineering the device, including designing how to mount flies, how to record/interpret behaviors while the fly walks the ball, and how to present stimuli that depend on the fictive position of the fly. I first established good walking behavior from the fly, and verified them using quantifiable values, such as speed. I then set up the presentation of a visual stimulus (an LED controlled by an Arduino) based on real-time outputs of the fly's motion from FicTrac.

Chapter II: Materials and Methods

Fly Care

48-72 hour post-eclosion adult female CantonS *Drosophila* were used in all experiments, as females are easier to glue for needle mounting than males, and 48-72 hour old young adult flies are consistently active during trials, as old flies spent most of their time grooming, while 0-1 day old flies were often less active on the ball. All flies were grown at room temperature under laboratory conditions.

For control flies, we used Bloomington *Drosophila* Stock Center (Indiana University) #5685 norpA mutated flies, which have eliminated phospholipase-C required for phototransduction in all photoreceptors (Hardie et al. 2002). Compared to CS Wild-Type flies, these mutations cause a stark decrease in visual input, effectively making the norpA mutated flies blind. For another set of control flies, we used the Canton S. strain *Drosophila* as in our experimental group, but without the LED visual stimulus.

Fly-on-a-Ball Setup

We designed a setup modeled from (Loesche and Reiser 2021), a resource that outlines building a compact, low-cost fly-on-a-ball setup, with 3-D printed parts replacing specialized equipment. Figure 5 shows the assay we built for the fly-on-a-ball setup. The LED light is positioned directly in front of the ball and the fly, while 3 IR lights illuminate the ball for the IR camera and FicTrac to track.

Pre-Mounting

Prior to experiments, flies were anesthetized using ice. Under cool conditions, flies are rapidly immobilized, and can also rapidly wake up once off ice, which is desirable for our experiments. In addition, icing does not interfere with their other senses, unlike CO2 anesthetization (Nilson, Sinclair, and Roberts 2006). After containing flies into a vial, they were placed in ice for around 15 minutes, before fixing them on a needle. Any less, the flies would not be anesthetized long enough for fixing, while long exposure to chill coma might result in longer recovery time (Nilson, Sinclair, and Roberts 2006).

Needle Mounting

Before the flies are taken off ice, a BSTEAN 34 dispensing needle (diameter 0.06mm) is mounted onto a micromanipulator, and glue is applied on the tip of the needle. A drop of glue sufficient to stick to the thorax of the fly, but not enough to stick to other body parts or slide into the groove, was applied to the tip of the needle.

The flies were placed on a 3D-printed plate with grooves, which were then placed on a slanted metal pedestal that was cooled using a chiller. This is a 40 x 40 mm thermoelectric module mounted between a 40 x 60 mm aluminum plate and a 90 x 90 aluminum heat-sink with a fan for cooling. This design was provided by (Loesche and Reiser 2021). When this device is powered with a 12V 5A supply, the top aluminum plate can reach a temperature below 0 degrees, cooling the grooved plate placed above. This allows the fly to be cooled even after getting off the ice, as it is being positioned for needle mounting.

The fly is oriented facing downward into one of the grooves, with their head positioned at the top for a higher elevation. Once the fly is fitted inside, a needle is placed directly above, and is gradually lowered onto the fly thorax using a micromanipulator. The micromanipulator ensures that the needle is precisely centered on the fly thorax from all directions along the x-axis, y-axis, and z-axis. For a clear and focused visualization of the fly, we used a Plugable USB2-MICRO-250X USB Digital Microscope, with a diffused 4x 5050 SMD LED, on 250x magnification.

After positioning the needle close to the fly, a minute amount of glue was gently transferred to the thorax using the tip of fine forceps. UV light was briefly shined for 3-5 seconds to harden the glue. Exposure for a longer duration may harm the fly or shock the fly and deter the fly's activity. Once the glue is properly secured, a micromanipulator is used to carefully elevate the fly from the groove, as the needle's sudden movement may cause the needle to "bounce" into the groove, impaling the fly.

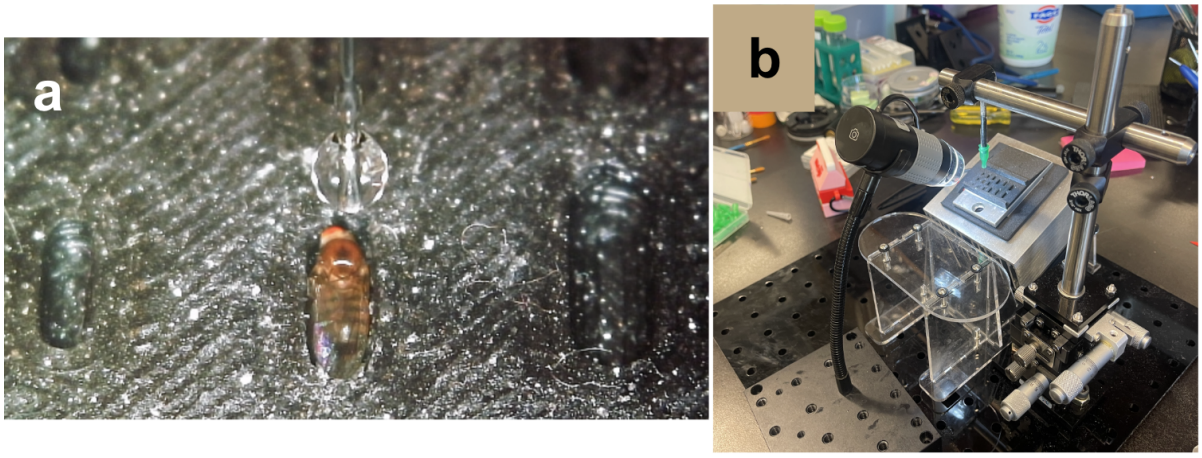


Figure 4: Setting up a fly on a needle

(A) A view of the fly-setup assay, with a needle tip positioned on a micromanipulator ready for gluing on a fly. The USB microscope can be seen pointing at the 3D printed grooves, with the micromanipulator positioned directly above the grooves.

(B) A view from the USB microscope while gluing the fly on a needle. The drop of glue on the needle is above the thorax of the fly.

Fly Positioning and Walking

After positioning and gluing the fly onto the needle, the fly is then ready to be placed onto the fly-on-a-ball device. The needle with the fly attached is affixed to another micromanipulator positioned above the ball. To allow for proper acclimation, the flies are left on the ball for at least 30 minutes – flies do not exhibit robust walking sooner than this. All walking experiments are conducted at a controlled temperature of 28 C degrees – this had a large impact on walking performance. This optimal temperature setting was observed to be critical for robust walking behavior, as deviations from this threshold resulted in suboptimal walking behavior. This finding is consistent with the observations reported in a similar study by (Loesche and Reiser 2021).

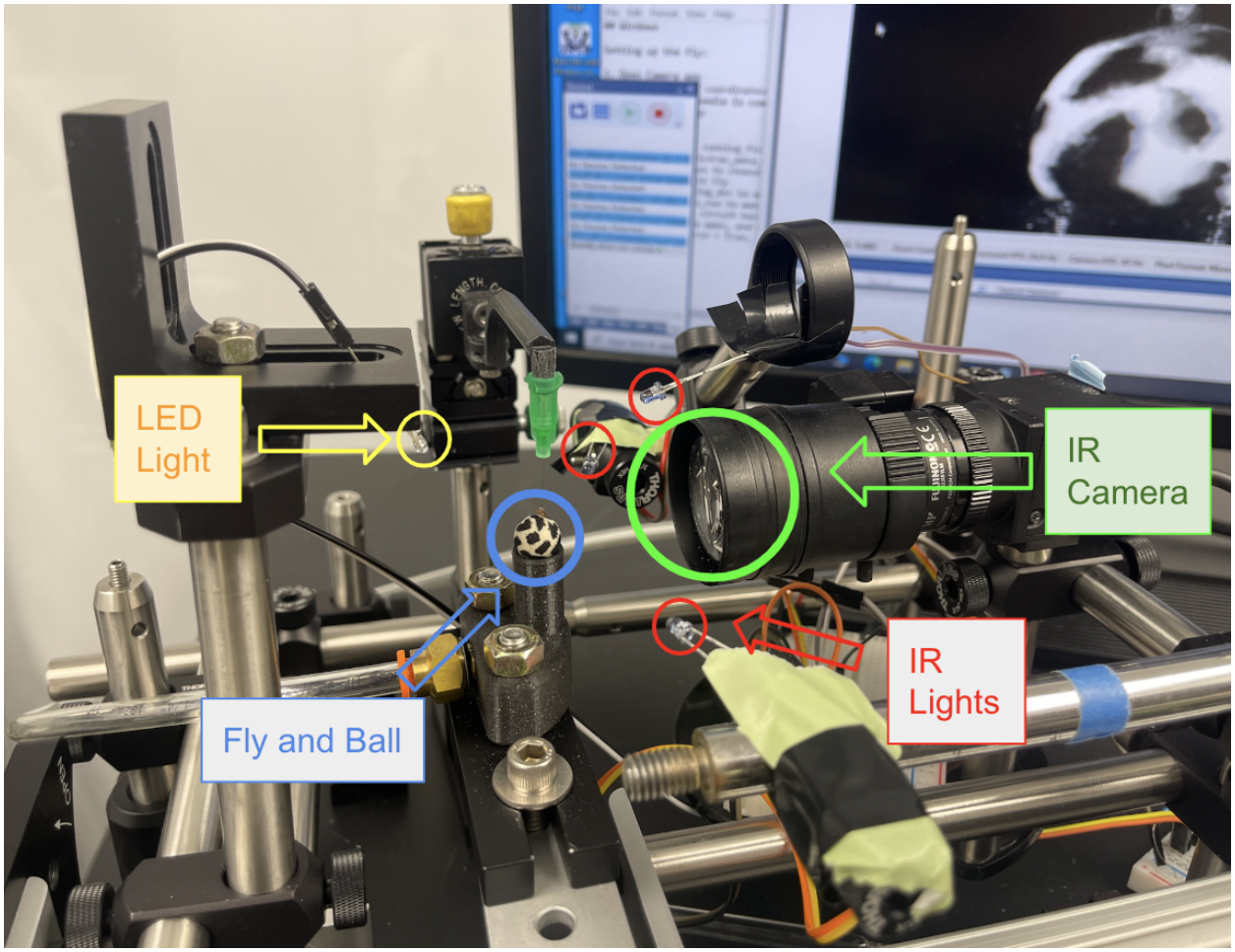


Figure 5: Assay for fly-on-a-ball setup

Light stimulus:

To investigate the behavioral individuality of the fly in response to exposure to a visual stimulus, a single visible light LED was positioned directly in front of the fly. An Arduino was employed to control the LED, which was triggered by real-time tracking outputs from FicTrac.

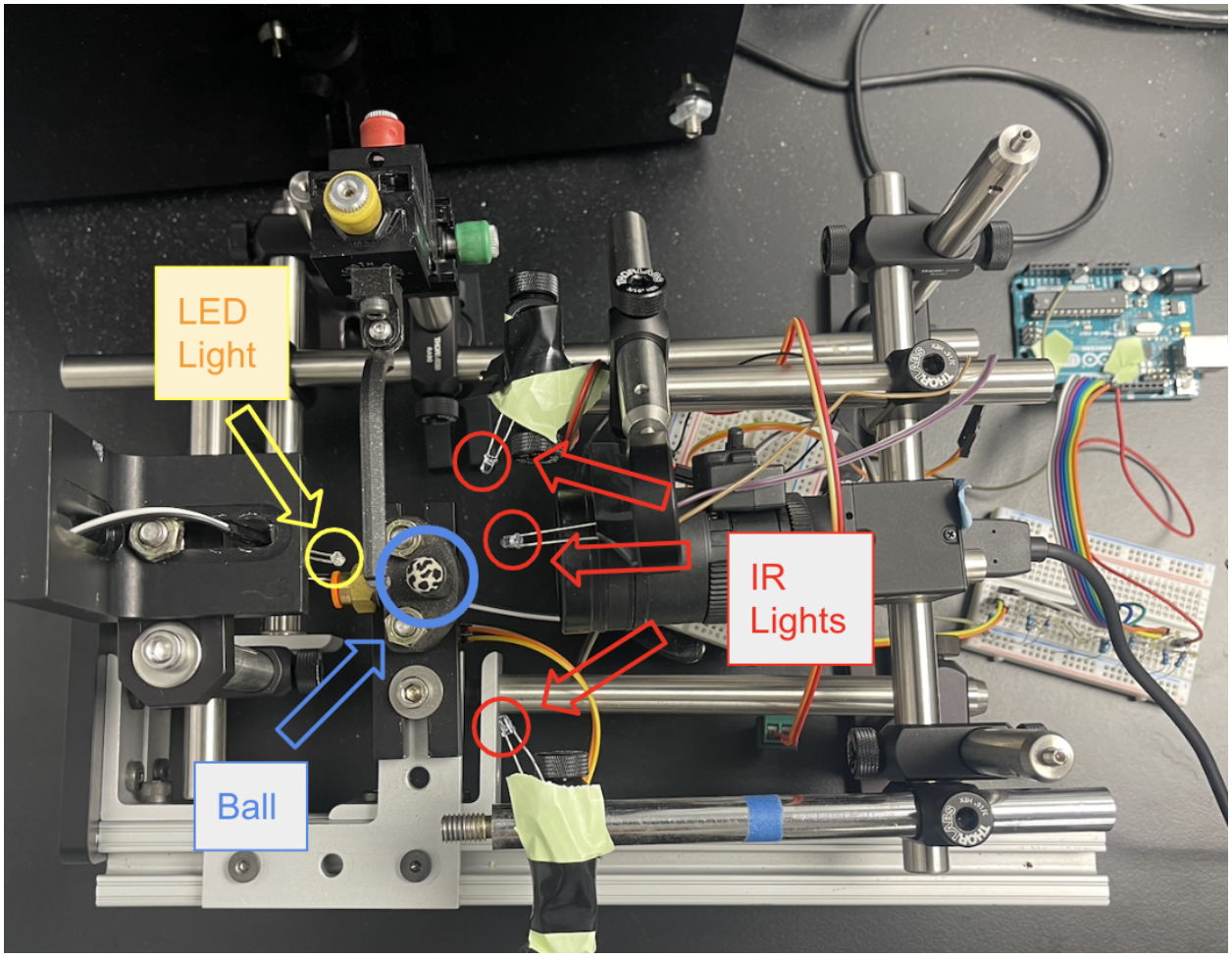


Figure 6: LED location

Figure 6 shows a birds-eye-view of the assay. From the camera side, three infrared (IR) lights are positioned around the IR camera to ensure consistent illumination of the ball without any shadows that might interfere with FicTrac tracking. The lights are amenable to the placement of various stimuli that face the fly from the other side. On the fly and ball side, an LED stand is employed to firmly anchor the LED in a position directly in front of the fly, while remaining hidden from the IR camera. The LED stand can be shifted horizontally along the assay, enabling easy relocation of the LED for future experiments, without disrupting other elements in the assay.

IR ball tracking

To have the most accurate and reliable experimental conditions, all experiments were conducted in complete darkness. The ball was illuminated by infrared LEDs, and its movement was captured using an infrared camera. Three infrared light bulbs were secured on poles to uniformly illuminate the ball from behind, upper left, and upper right. This arrangement prevents dark shadows or overlapping light on the sphere's surface, which could lead to flawed measurements, calculations, or registration issues with the FicTrac software. In previous trials, FicTrac was unable to distinguish between dark markings on the ball from dark shadows, underscoring the significance of an even layer of illumination.

Once the ball was uniformly illuminated, an infrared camera was secured directly behind the fly, to foster the most precise positioning adjustments for the fly. This optimization of the fly's position allows for the most accurate FicTrac measurements possible.

Foam ball

We decided to use Last-A-Foam FR-7120 (General Plastics Manufacturing Company, Tacoma, WA, USA) based on recommendations from (Loesche and Reiser 2021) and constructed a sphere with a 9.79mm diameter. The ball floats by receiving air from a tube connected to the air duct from the wall – we set the airflow to 1.6 l/min so that the ball smoothly rotates upon air delivery and does not jiggle around. The ball is situated in a 3D printed ball-holder.

For optical tracking of the spherical rotations with FicTrac, we painted unique, distinct patterns that help FicTrac pick up and analyze the movement of the ball. We used BLK 3.0, a matte acrylic black paint, so that both IR camera and FicTrac can easily identify the markings. The BLK 3.0 was less reflective and had better contrast than a black permanent marker, and worked much better for both IR illumination tracking and regular visual tracking using FicTrac (Loesche and Reiser 2021).

Behavioral tracking with FicTrac

To perform real-time behavioral tracking and exposure to visual stimuli, we used FicTrac, a software specifically designed for inferring fictive paths of animals based on movements of a patterned ball. It has been used in fly-on-a-ball experiments in the past (Loesche and Reiser 2021) (Grabowska et al. 2020) (Aragon et al. 2022). FicTrac enables us to collect frame-by-frame sphere orientations, fictive 2-D positions of the animal as it is walking, heading orientation, instantaneous speed, and many other measures, which is outputted to a file for later analysis. FicTrac also stores a video of the full experiment as well.

FicTrac can estimate the instantaneous rotation of the sphere, using individual frames of a patterned ball and its previously constructed template of the sphere's pattern. Then, using each frame estimate for the sphere's orientation, FicTrac can reconstruct the fly's virtual trajectory both on the sphere, and in a fictive 2D map. From there, it can calculate the fly's axes of rotations and the speed along different axes, as well as the inferred location on the fictive 2D map. This inferred position is useful and important because it is the basis for all of our visual

stimulus experiments, and is what we use to understand where the fly is positioned on the fictive map.

We used an IR camera and IR lights to illuminate the fly and enable tracking without interfering with the fly's visual stimuli. We used a BlackFLY-U3-13S2M-CS USB 3.0 Monochrome Camera, with a 33ms frame rate.

FicTrac saves all coordinate information post-experiment and also outputs them in real-time, allowing us to deliver light-based stimuli as the fly is behaving on the ball.

FicTrac Configuration

Before starting a run, the FicTrac software must be properly configured to enable the software to precisely compute data based on the inputs from the camera. The inputs of the configuration are set interactively into the ConfigGUI file, which is then utilized to monitor and track the ball's movement accurately. First, the edges of the sphere are manually selected by determining the sphere's boundaries, as depicted in Figure 7A. Next, the ignore regions are identified (Figure 7B) to specify the stationary portions and preclude them from being incorrectly interpreted as moving elements of the sphere. Lastly, we set the rotation axis and the position of the fly (Figure 7C), which in our setup would be directly situated in front of the camera.

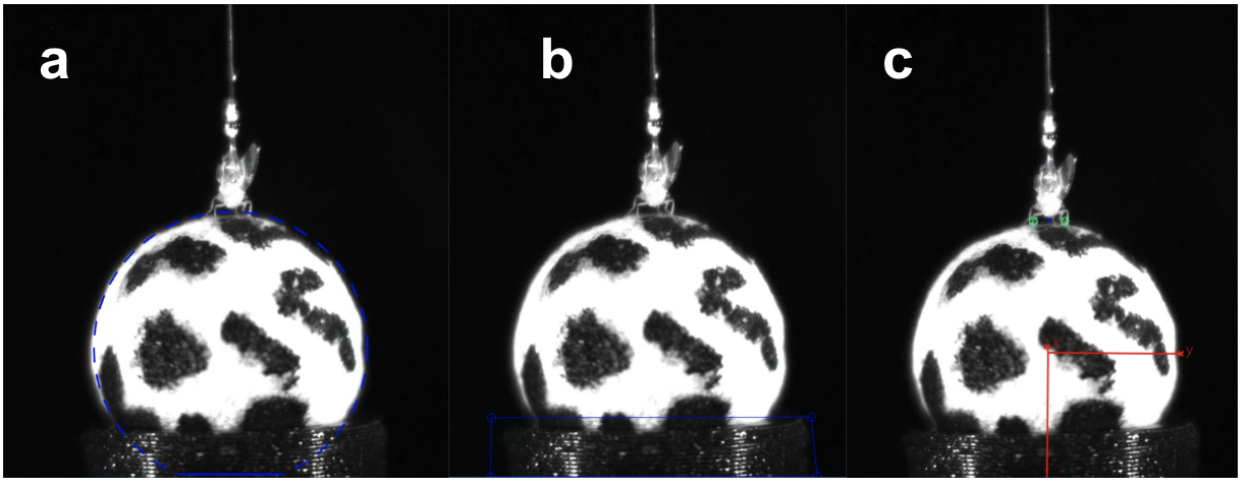


Figure 7: FicTrac Configuration

(A) Setting sphere boundaries (blue). The blue dotted region specifies the points around the circumference of the trackball in the input image.

(B) Setting ignore regions (navy). The blue region specifies the polygon regions that should be ignored during the tracking.

(C) Setting X-Y-Z axis (red). The red vectors indicate the body axes inferred by the orientation of the animal on the ball.

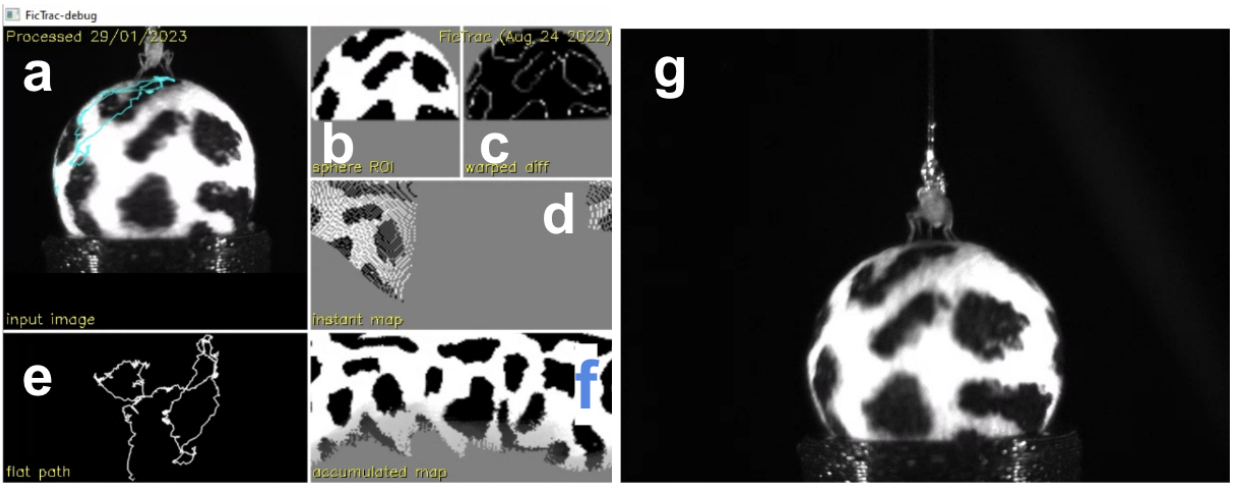


Figure 8: FicTrac panels

(A) The real-time image with a superimposed path of the fly that will be the input for FicTrac to calculate by, lit by IR camera

(B) Instantaneous image of FicTrac's calculated sphere and the marks.

(C) Instantaneous image of FicTrac's calculated outline of the marks on the sphere

(D) Real-time image of 2D map of FicTrac's input from the camera

(E) The fictive 2D path the fly has taken in the past 30 seconds

(F) FicTrac's accumulated map for the markings on the sphere

(G) A picture of a fly on the ball with needle attached from directly above

Checkerboard details

All flies were experimented on a virtual checkerboard, in the fictive two-dimensional space, and their movements were tracked using a FicTrac software. In this setup, when the fly entered a "light" square, the LED positioned directly in front of the fly was turned on. When the fly entered a "dark" square, the LED was turned off to ensure darkness. The squares were set to be 25 mm by 25 mm in the fictive space. A larger width resulted in boxes too wide to measure the fly's activities, while a smaller width resulted in the fly passing through too many boxes too often. The FicTrac software calculated the fly's fictive coordinates, triggering the activation or deactivation of the LED light bulb via a coded Arduino with an LED circuit. The frame-by-frame position of the fly in the fictive 2-D space was output by custom Python scripts, as well as the frames at which the LED transitioned between on and off.

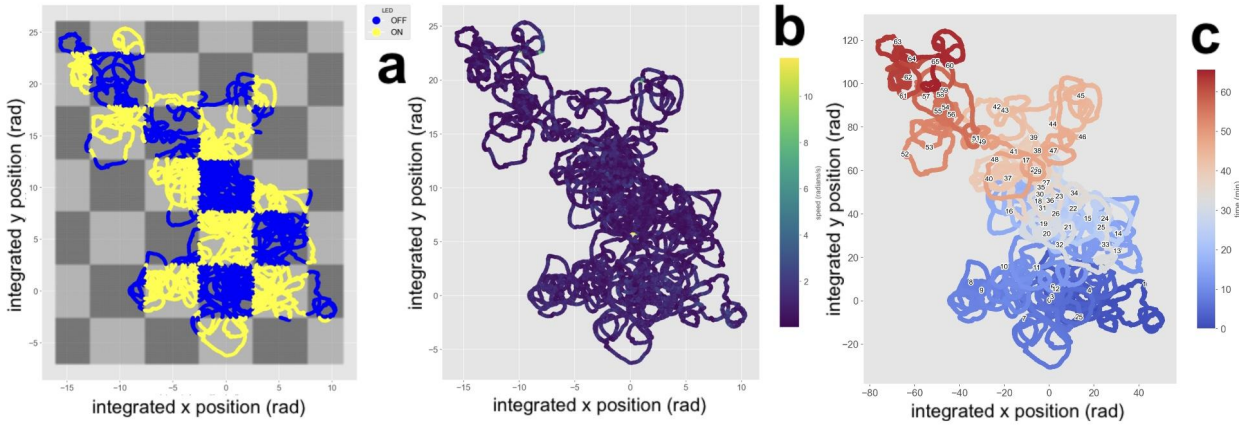


Figure 9: Checkerboard

(A) Checkerboard map showing the fly's fictive 2D map, and the LED status on its path

(B) The fly's fictive 2D map, color-coded by its speed

(C) The fly's fictive 2D map, color-coded by the overall time, blue indicating the start of the experiment, red indicating the end of the experiment

Statistical Techniques

95% confidence intervals for all statistics (means, standard deviations) were estimated by bootstrapping sample data 1000 times and listing the 2.5th and 97.5th percentiles of the statistic among bootstraps.

Chapter III: Results

Establishing Walking Behavior

In the goal of establishing individuality in walking behavior in tethered flies on the fly-on-a-ball assay, we first focused on establishing robust and reliable walking behavior on the ball. We designed and constructed a stage in which flies are tethered on a needle and walking on an air-supported foam ball. Female flies aged between 48 and 72 hours post-eclosion were used for all tracking experiments. Fly walking behavior was recorded for ~60 minutes in temperature-controlled rooms set at 28 C degrees, since lower temperatures did not yield robust walking.

We designed a fly-on-a-ball assay that was suitable for an LED to serve as a visual stimulus for the fly. Figure 10A presents a birds-eye view of the assay, in which an LED is positioned securely in front of the fly, and three IR lights are positioned behind the fly to illuminate the ball for IR tracking. Figure 10B and 10C show a side view and close-up, respectively, while Figure 10D shows an illustrated assay highlighting key components.

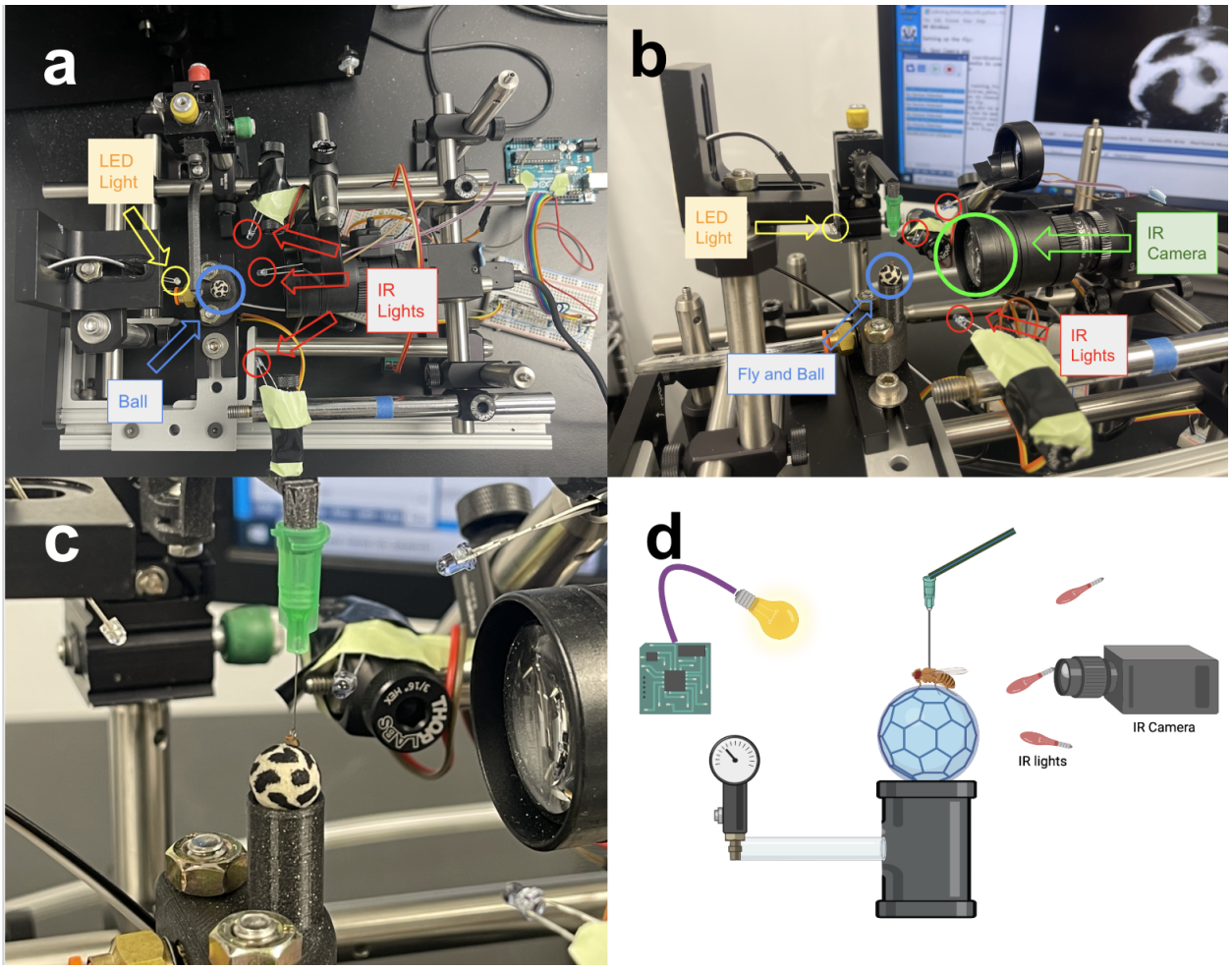


Figure 10: Constructed assay
(A) Birds-eye view of the assay. The LED light is oriented directly in front of the ball, with 3 IR lights illuminating the ball for the IR camera in the dark.

(B) View of the assay from the side

(C) Close-up view of the assay with the fly is placed on the ball

(D) Illustrated assay with key components

Optimal fly walking behavior is characterized by all six legs making clear contact with the ball, a smooth and prolonged path, and distinct separation between walking and grooming behaviors. Mendes et al. studied and compiled a comprehensive analysis of *Drosophila* locomotion

parameters, utilizing optical touch sensors and video imaging to capture adult fly walking. They found that flies exhibiting good walking behavior typically move at speeds ranging from 7.2mm/s to 44.7mm/s, and specific foot placement on the surface (Mendes et al. 2012). We visually assessed videos of fly walking and their behavior seemed comparable to non-ball walking, as well as what is usually observed on other fly-on-ball assays. The speed observed was within the range given in the study by Mendes et al., notably after running flies at a controlled temperature of 28 C degrees.

After confirming good walking, we initiated real-time light stimulus presentations that were activated or deactivated based on the fly's fictive position computed by FicTrac. We first considered a single example Canton S. strain fly in order to test good walking behavior by using a fictive checkerboard assay, in which a fly in a "light" square would be exposed to visible light from an LED, while the fly in a "dark" square would be in darkness. Each square was set to 25mm x 25mm, and we ensured that light stimuli were properly administered at each frame with custom Python scripts.

We first analyzed the behavior of a single Canton S. fly to characterize walking behavior in the assay and assess first-order visual stimulus behaviors. The fly's walking path is shown in Figure 11A, showing a representative fly path that traverses multiple light and dark boxes during the run, allowing us to examine its behavior as it walks through borders across light-to-dark transitions, or across dark-to-light transitions. The fly explored various boxes rather than remaining still, which indicates an active walking behavior. Figure 11B shows a fly's full run, with its speed in either light or dark regions. Analysis of this fly example reveals that the fly's

speed slows down to around 3mm/s in the dark, while speeding up to 25 mm/s in the light, demonstrating that this particular fly is slower in the dark. A speed of 25mm/s is consistent with a study by Mendes et al., which established Drosophila running speed ranging from 7.2 mm/s to 44.7 mm/s, with an average of approximately 28 mm/s (Mendes et al. 2012). This graph gives us confidence in establishing good walking behavior, as a walking speed of 25mm/s is a hallmark of good walking behavior, independent of any light-based behaviors. Figure 11C depicts a histogram of the log velocity for this fly, revealing two modes corresponding to pausing/grooming and running behaviors, respectively. This pattern is consistent with walking behavior observed in other fly-on-a-ball experiments (J. Kain et al. 2013). Figure 11D shows a bar plot demonstrating the proportion of time the same fly spends in light versus dark regions. The bar plot indicates that this fly spends significantly more time in the dark than in the light, justifying its low fraction in light, at only 0.098. Figure 11E presents the fly's (natural log of the) seconds spent in each box during the fly's run. The distribution has an average of 5 seconds, but is skewed with a long tail, indicating many short visits in a box and some long dwellings. This suggests this fly does not spend excessive time in a single square and moves quickly between the checkerboard boxes, demonstrating good walking behavior. Figure 11R and 11G display the (natural log of) seconds this fly spent in a light and dark box, respectively. Both graphs indicate excellent walking behavior, with brief visits to most boxes. Finally, Figure 11H displays the fly's angle (yaw) velocity through the run in light or dark conditions. Yaw is the twist along a vertical axis, and can be interpreted as the change in heading the fly would achieve by turning left or right.

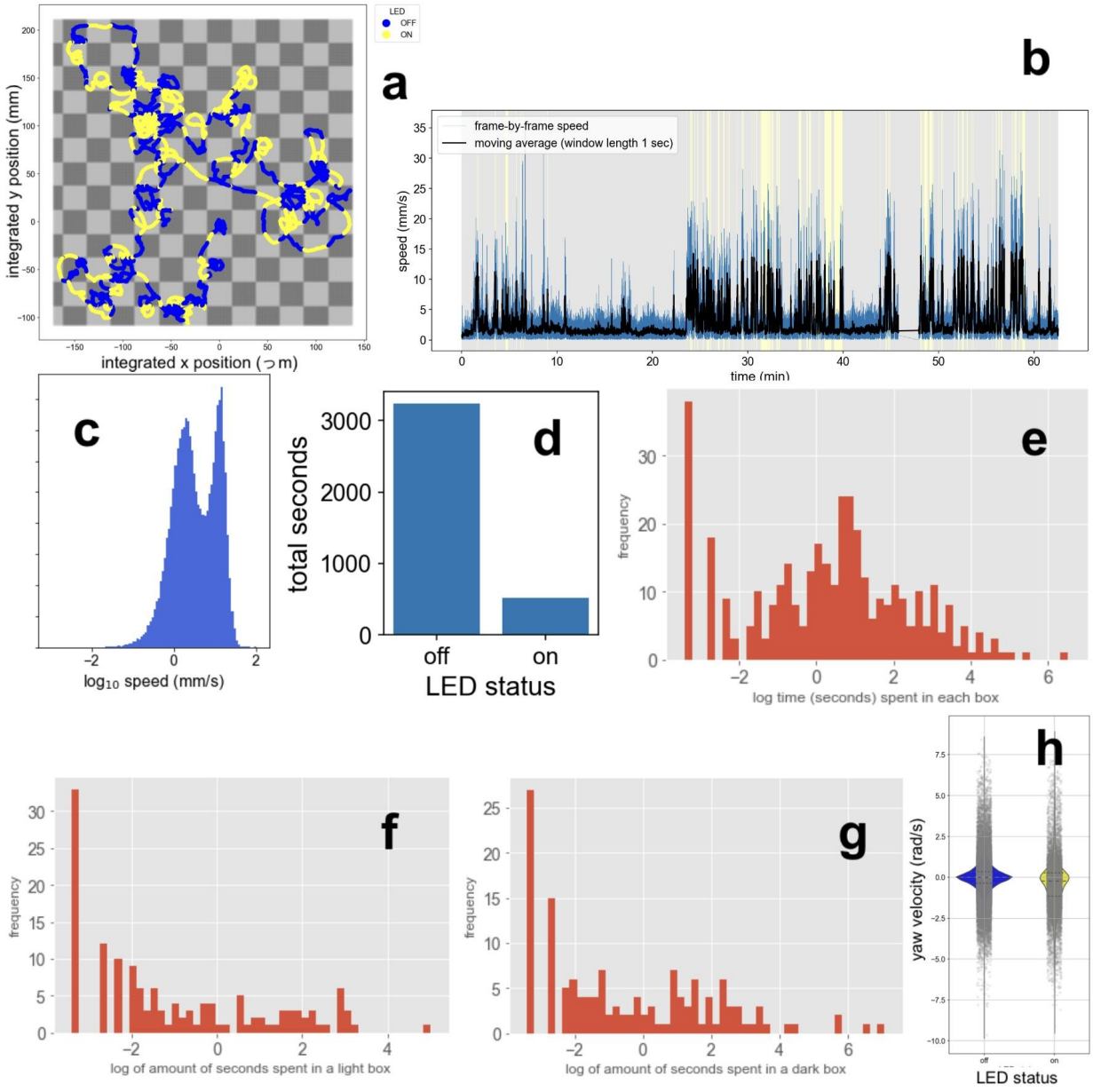


Figure 11: Demonstrating walking behavior for a single fly

(A) The fictive path of an individual fly on a virtual checkerboard, with the path of the fly shown in yellow when walking through a light box, and blue when walking through a dark box.

(B) Speed trace through complete experiment. The blue lines represent the speed at each frame, and the black line represents a smoothened version, averaging the speed a frame before and after. The gray background represents times in which the fly was in a dark square, while the yellow background represents the times in which the fly was in a light region.

(C) Speed log histogram, displaying density of the log of the speed.

(D) Histogram of amount of time spent in light vs. dark

(E) Histogram for the amount of time, in seconds, the fly was in a single box.

(F) Histogram for the natural log amount of seconds spent in a dark box.

(G) Histogram for the natural log amount of seconds spent in a light box

(H) Yaw velocity depending on the LED status

Metrics

After establishing good walking behavior and ensuring proper light stimulus presentations, we devised several metrics to quantify phototactic preferences and compared the distribution of experimental Canton S flies with those of two control groups: genetically mutated blind flies (NorpA) and Canton S. flies without a visual stimulus (fictive fly positions were recorded in the same checkerboard field, but the LED would not turn on in the light regions). 33 experimental flies, 16 NorpA mutated blind flies, and 15 Canton S. flies without LED were tracked on the ball for an hour each. We analyzed time spent in the light/dark conditions, and behaviors at borders between light/dark squares, such as speed and heading angles, and we evaluated the extent of behavioral variability between the three groups.

Metric 1: Fraction of time in the light

We first started with one of the simplest scores – the fraction of time a fly spent in a light region throughout the experiment. To calculate this, we divided the total time that the fly spent in a light region by the total time of the run. The resulting percentage represents the fraction of time the fly spent in the light. The example fly shown in Figure 11D had a low fraction of time in the light. We computed this fraction for all flies and constructed histograms for the experimental group and the blind/CS no-LED controls. Figure 12 shows both control groups, blind flies and Canton S. strain flies without LED stimulus, tightly clustered around their average value of 0.51 (95% CI 0.49-0.53) and 0.51(95% CI 0.49-0.54), as expected. The control groups are indifferent between light and dark conditions, hence spending equal time in both conditions, resulting in a light fraction of 0.5 for spending half of their time in the light. In contrast, the experimental group

showed a lower average value of 0.40 (95% CI 0.35-0.46), and significantly larger standard deviation of 0.16 (95% CI 0.12-0.19). This clear drop in the average value for the experimental group is further reinforced as the 95% confidence interval does not overlap at all with the average value of either of the control groups. The findings suggest two effects. Firstly, there is an average photonegative tendency among the experimental CS flies exposed to light stimuli. Secondly, the variety in output and larger standard deviation gives us confidence for a diverse range in individual preference and a clear impact of the visual stimulus on the experimental flies.

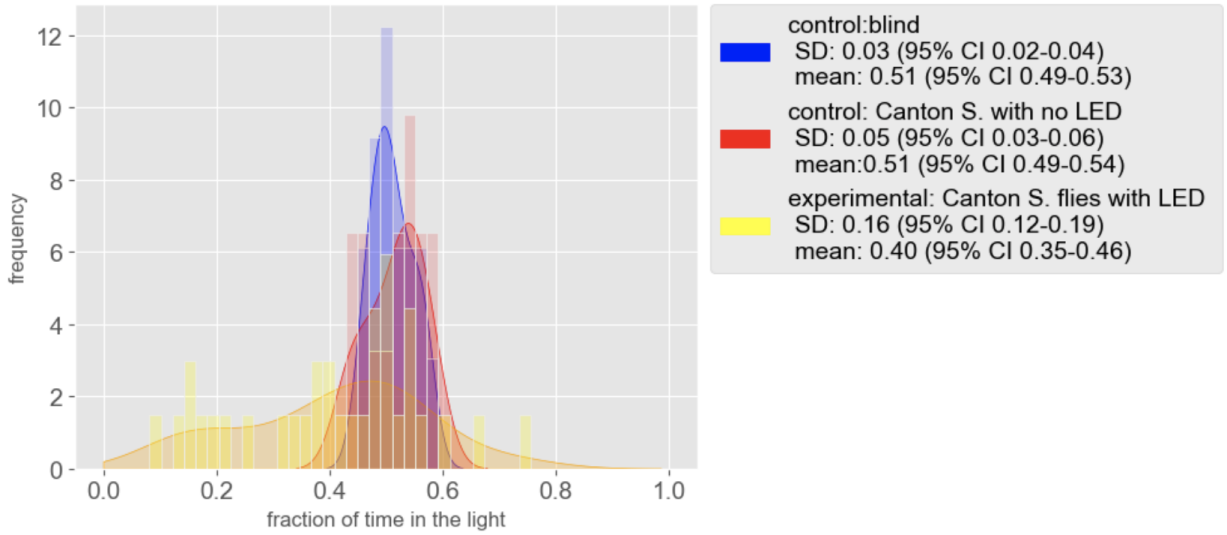


Figure 12: Histogram for the fraction of time spent in a light region

Metric 2: Time spent in a light/dark box

Based on data presented earlier, we observed that flies varied in the amount of time spent in light/dark regions over the entire span of the experiment. We next sought to understand walking dynamics on a finer scale by analyzing the duration of time spent in each light or dark square.

In Figure 13A, we present a histogram of the duration an individual fly spent in each box, which indicates that the average time spent in a single box is approximately 5 seconds. To compare this metric across the three groups, we devised a box score, defined as the mean time spent in each box, for the 64 flies across all groups. Figure 13B illustrates the distribution of the time spent by flies in each box. The blind control group exhibited the most pronounced peak, with the lowest standard deviation of 1.23 (95% CI 0.71-1.76), and an average value of 5.18 (95% CI 4.61-5.84) seconds per box. The no LED control group presented a wider distribution, with a larger standard deviation of 2.39 (95% CI 1.53-3.28) and an average value of 4.97 (95% CI 3.77-6.26) seconds per box. The experimental group displayed the most substantial distribution and spread, with a significantly greater standard deviation of 5.51 (95% CI 2.27-8.13), and also a greatest average value of 7.95 (95% CI 6.39-10.03) seconds. This increase in average value for the experimental group is strengthened by the lack of overlap of average values for both control groups in the 95% confidence interval. These results indicate that while the majority of flies in all groups spent approximately 5-7 seconds in each box, the experimental group spent the most time in a box and also had the most considerable variety in their box-stay duration.

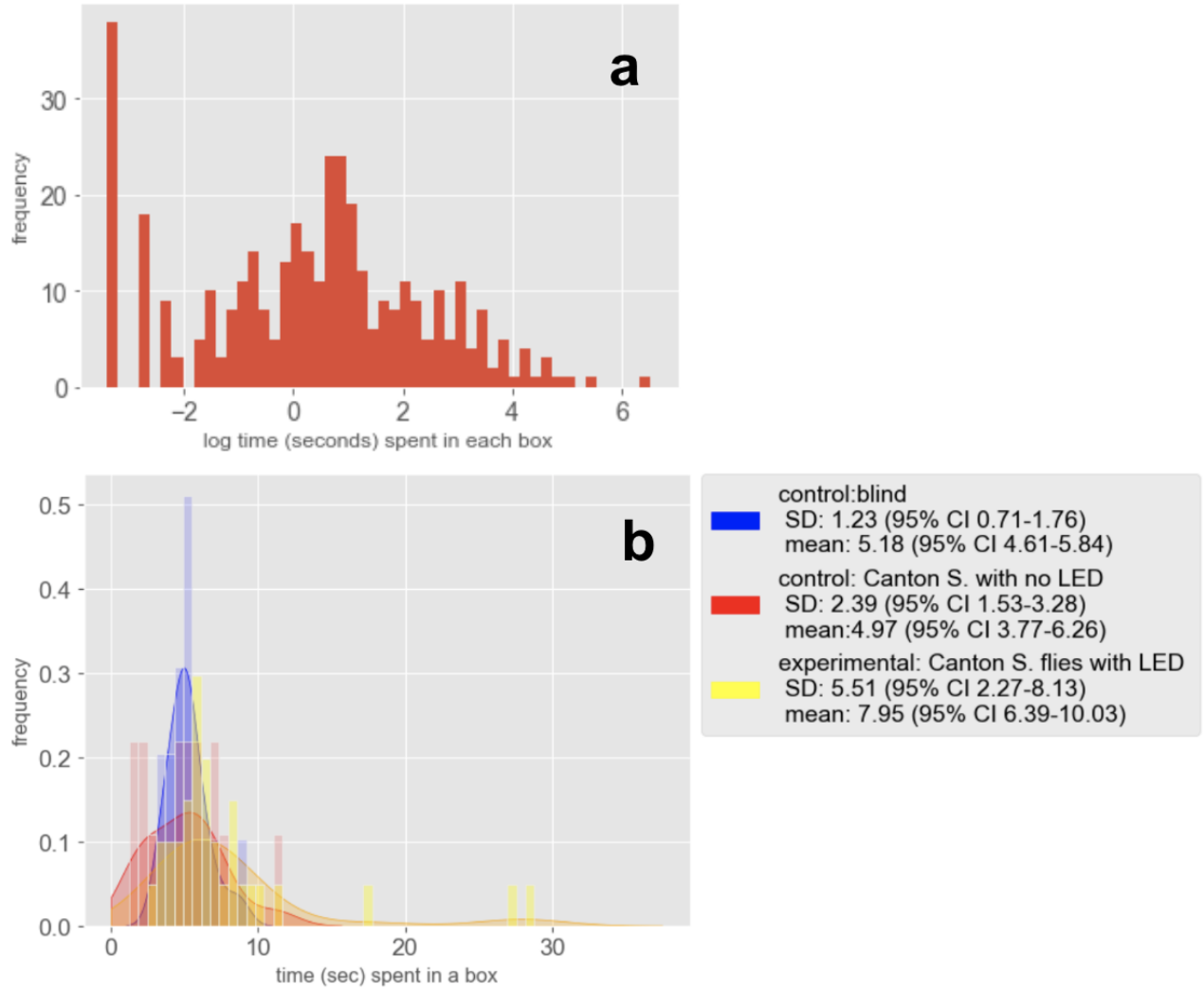


Figure 13: Time spent in any box

(A) Histogram of the seconds spent in each box for an individual fly

(B) Histogram of the seconds spent in each box for multiple flies

A natural question that arises connects to the duration of time flies spend in light and dark regions. Specifically, we are interested in determining the length of time that flies remain in each light box or dark box before moving out. Figure 14A depicts a histogram of the natural logarithm of seconds spent by an individual fly in each light box. To compare this metric across three groups, we devised a light box score, calculated by taking the natural logarithm of the average

time, in seconds, that a fly spent in each light box. It is important to note that this is distinct from the light fraction, which measures the overall time the fly spent in light, relative to the total duration of the run. Rather, the light box score focuses on the time that flies spent in each individual light box. Consequently, even if the fly spent the majority of the run in the light, it is possible for it to have a short stay in a light box, if it was particularly active. The histogram is expected to include many small values for flies that were very active, although they may have spent the majority of the time in the light.

Once a quantification method was set up for measuring the time spent by flies in a box, we then want to compare the scores across the three groups. For this purpose, we devised a light box score for each fly, defined by the average seconds spent in each light box and taking the natural log of the average. The resulting histogram of light box scores, across all three groups, is shown in Figure 14B, revealing that all groups spent an average 5 seconds in each light box. However, the blind control flies exhibited a very narrow distribution with the lowest standard deviation of 0.22 (95% CI 0.14-1.73), while the experimental group and no LED control group exhibited a similar and wide distribution of light box scores, with a standard deviation of 0.53 (95% CI 0.39-0.68) and 0.53 (95% CI 0.39-0.66), respectively. These results suggest that while the scores across three groups averaged out to a comparable value, the experimental group exhibited a wider distribution than the blind control flies, but not significantly wider than the no LED control flies.

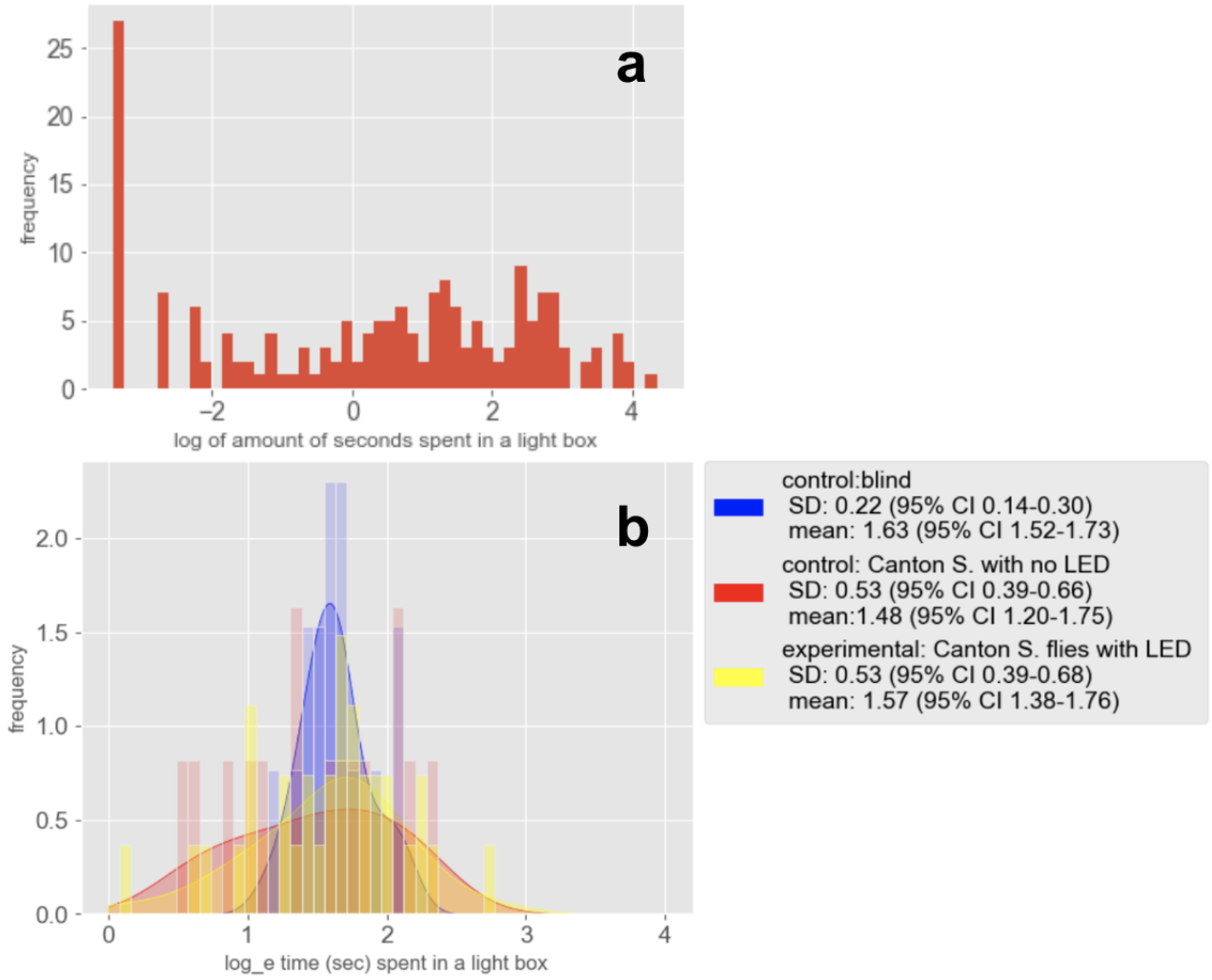


Figure 14: Light Box Score

(A) Histogram for the log time (seconds) spent in a light box for an individual fly

(B) Histogram for the log time (seconds) spent in a light box for all individual flies across the three groups

After obtaining the light box score, we proceeded to evaluate the corresponding score for dark boxes. Figure 15A illustrates the distribution of the time spent by flies in a box. To compare this metric across the three groups, we devised a dark box score, defined as the mean time spent in each dark box, for the 64 flies across all groups. Figure 15B displays a distribution of time spent

by a single fly in each of the dark boxes. Similar to the general trend from Figure 14B, the blind control group exhibited the strongest peak, with the smallest standard deviation of 0.25 (95% CI 0.16-0.34). The no LED control and experimental group displayed a similar and wider distribution, with a larger standard deviation of 0.56 (95% CI 0.38-0.73) and 0.60 (95% CI 0.39-0.79), respectively. However, the average time spent in a dark box is significantly larger in the experimental group with a score of 2.09 (95% CI 1.89-2.31), compared to both the no LED control group, at 1.42 (95% CI 1.12-1.71), and blind control group, at 1.60 (95% CI 1.48-1.73). The significance of this increase in average time spent in a dark box for the experimental group is supported by the lack of overlap with either of the control groups at the 95% confidence interval. These results indicate that while the no LED control group and experimental groups displayed similar distributions and exhibited variety in their dark box-stay, the experimental group spent the longest duration in each dark box compared to both control groups.

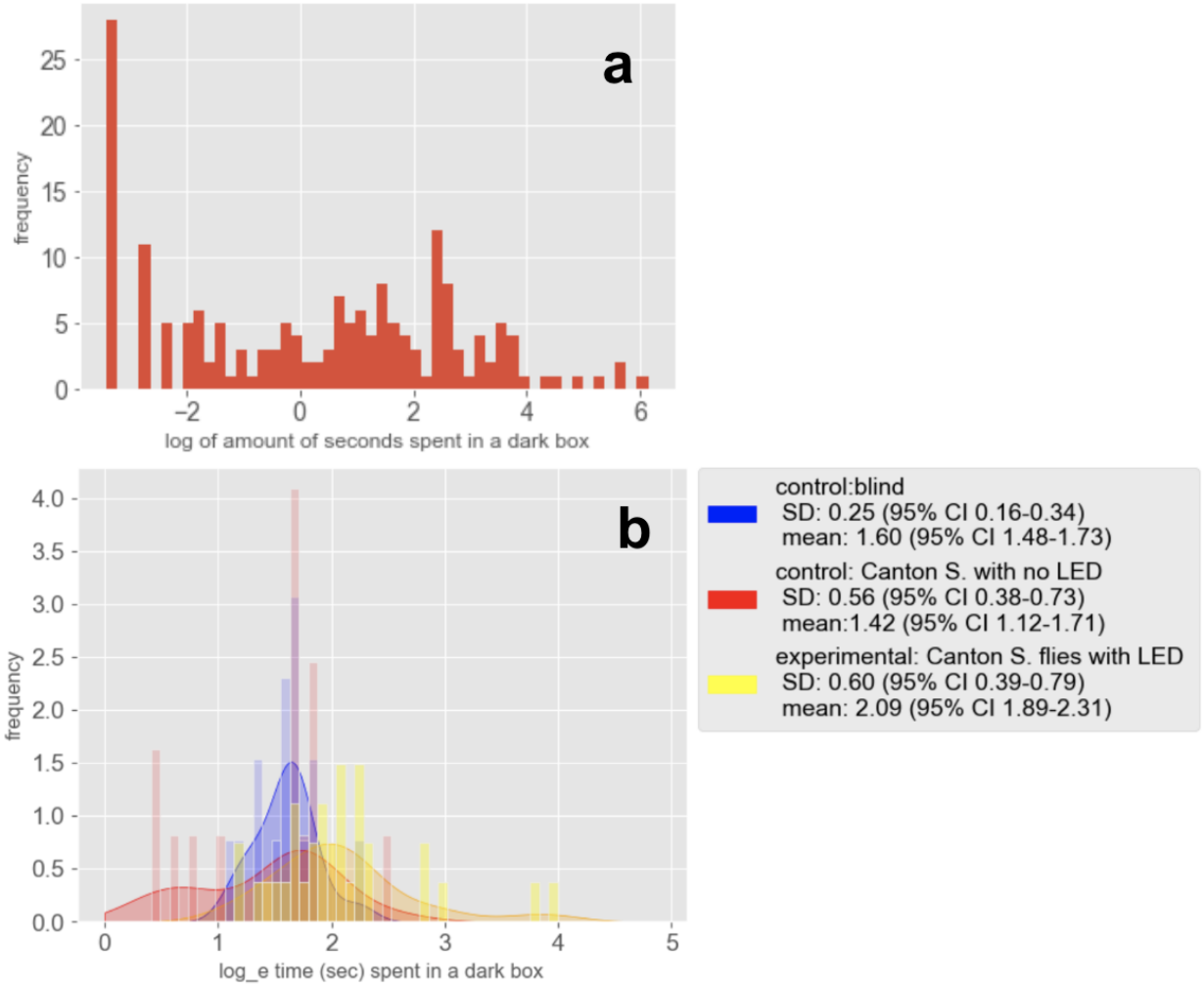


Figure 15: Dark Box Scores

(A) Histogram for the log time (seconds) spent in a dark box for an individual fly

(B) Histogram for the log time (seconds) spent in a dark box for all individual flies across the three groups

Based on our previous findings, a natural next step aims to explore potential correlations between the average time spent in dark and light regions across the three groups. In Figure 16, we present a scatter plot of the light box score versus the dark box score for each fly across all three groups. We observe a positive linear correlation of 0.86 (95% CI 0.72-0.96) and 0.94 (95%

CI 0.86-0.98) for the blind and no LED control groups, respectively, with a very low p-value at 0.00 (testing the null hypothesis of no correlation). These results indicate a strong positive linear correlation for the control groups, suggesting that the flies spent equal amounts of time in the respective boxes regardless of the presence of a light stimulus. This makes intuitive sense, as we expect a fly to exhibit an almost perfect correlation if they do not perceive differences between light and dark regions. In contrast, the experimental group showed a wider distribution of scores, with a correlation coefficient of 0.30 (95% CI -0.01-0.55), and a p-value of 0.09 against the null hypothesis of no correlation. The confidence interval ranges from -0.01 to 0.55, indicating that there are promising signs of individual differences between the experimental fly's behavioral response in light and dark. The results indicate a positive but weak correlation between the time spent in light and dark boxes for the experimental group compared to both control groups. The prevalence of data points above the diagonal positive slope for the experimental group indicates a tendency for experimental flies to spend less time in each light box and more time in each dark box. Taken together, these results suggest two findings: that on average, the experimental flies tend to avoid light and prefer spending more time in the dark, and that experimental flies displayed a wide variability across individuals in their time spent in each box.

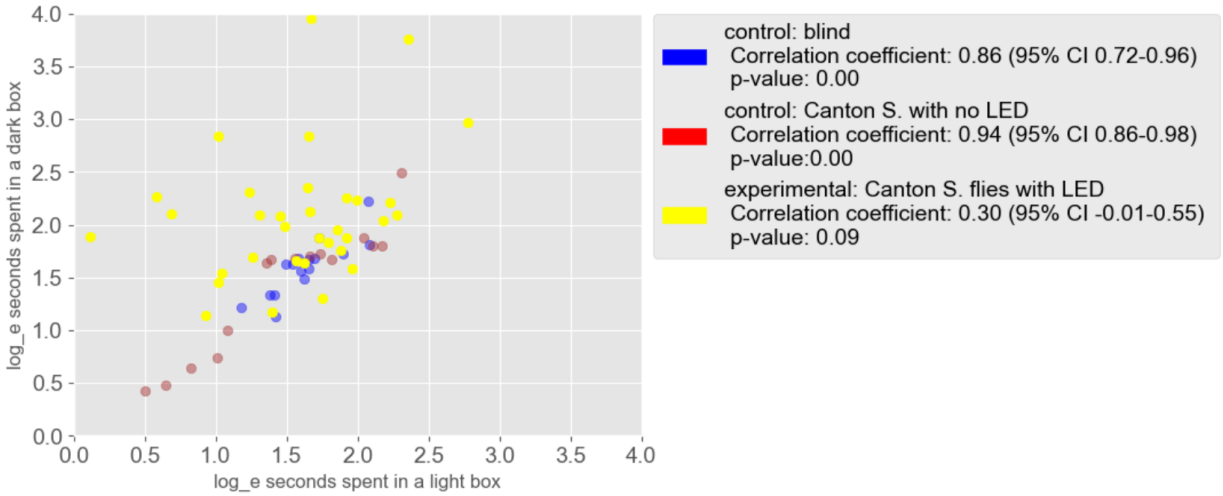


Figure 16: Scatter plot comparing dark box score and light box score

A comparison of individual flies between a blind control fly and an experimental fly demonstrates striking differences in how the flies differ in their time spent in each light or dark squares. Figures 17A and 17B show the histograms representing a blind fly, which appear to be nearly identical. In contrast, Figures 17C and 17D show the histograms representing an experimental individual fly, and display noticeably distinct distributions, indicating different behavioral preferences in the light and dark. This striking contrast emphasizes the effectiveness of the control groups in distinguishing the experimental groups' responses to visual stimuli.

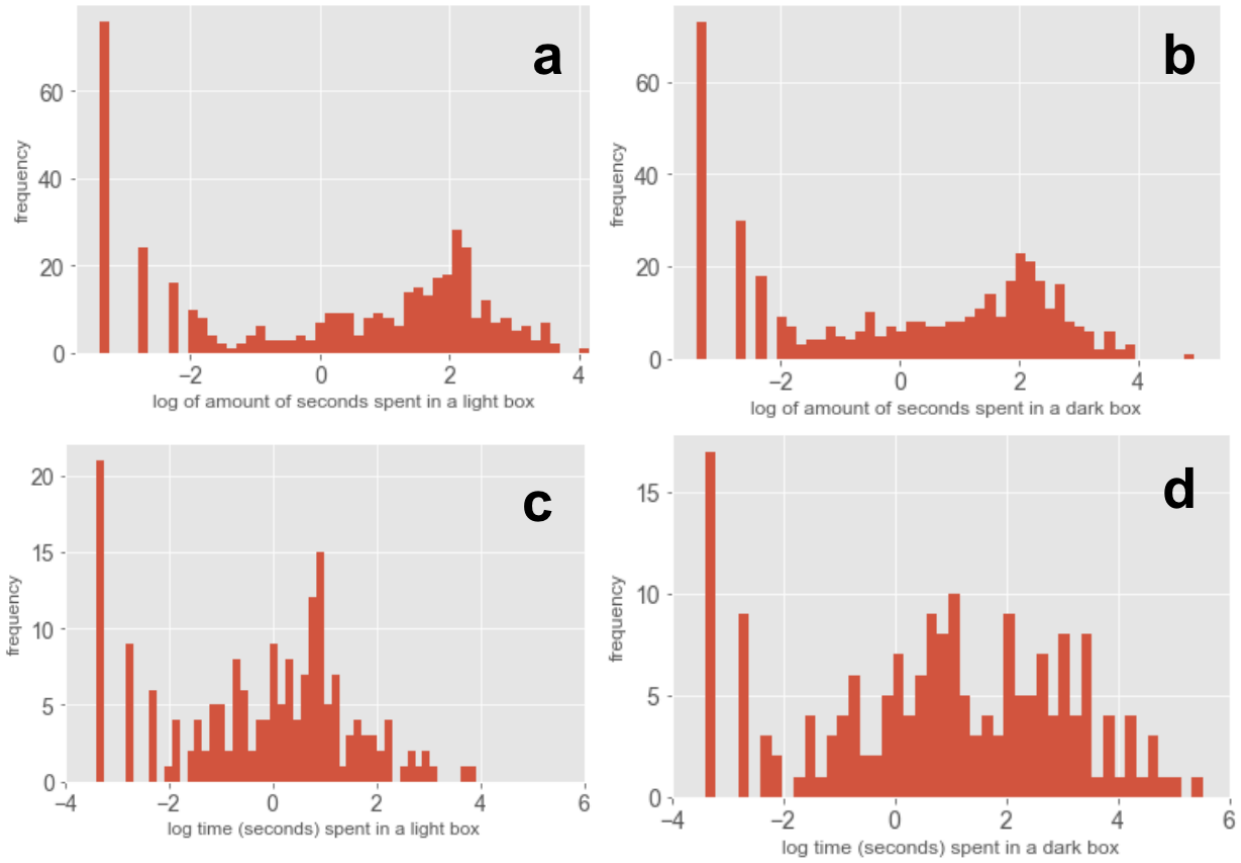


Figure 17: Comparison of individual plots

(A) Histogram of the log time (sec) spent in each light box for blind control flies

(B) Histogram of the log time (sec) spent in each dark box for blind control flies

(C) Histogram of the log time (sec) spent in each light box for experimental flies with LED visual stimulus

(D) Histogram of the log time (sec) spent in each dark box for experimental flies with LED visual stimulus

Metric 3: Speed

After observing behavioral variability among individuals with their time spent in each box, our next objective is to analyze the velocity of each fly during the experiment. We focused on the speed outputted by the FicTrac software, which captures the fly's speed at each frame of approximately 30 ms per frame, allowing for a detailed analysis of each run for every individual fly.

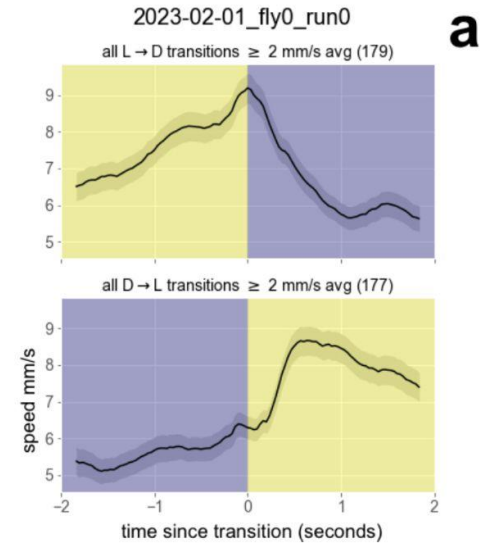
Given the fictive checkerboard map with light and dark regions, our focus centered on characterizing the behavioral response of *Drosophila* as it crosses the border between the two regions. We first examined an example fly's speed for 2 seconds before and after the transition across a border. This is where most of the discernible speed increases and decreases above baseline were apparent. In Figure 18A, we present the speed profile for an individual fly in response to a transition from a light region to a dark region, by representing the average speed of the fly at each frame during the 4 second transition period for each type of transition. The top panel of Figure 18A displays the average speed of the fly during a light-to-dark transition, while the bottom panel illustrates the average speed during a dark-to-light transition. Our analysis reveals that this example fly tends to slow down after transitioning to a dark region, and speeds up after transitioning from a dark region, indicating that it exhibits an overall decrease in speed in the dark and an increase in speed in the light.

In our findings so far, we found individual differences in fly speed during transitions between light and dark conditions. We aim to determine whether these differences exhibited signs of

individuality. To assess this, we computed a speed score, by calculating the (average speed for two seconds after the transition - average speed for 2 seconds before the transition) / average speed for 2 seconds before the transition). This yielded a score that could indicate whether the final condition was faster (positive score) or slower (negative score) than the initial condition, with a score of 0 indicating no change in speed.

Figure 18B, illustrating the distribution of average change in speed across light-to-dark transitions, displayed blind control groups forming the tightest distribution around 0, with the smallest standard deviation at 0.01 (95% CI 0.01-0.02) around an average value of 0.00 (95% CI 0.00-0.01). The no LED control group showed a wider distribution with a standard deviation of 0.03 (95% CI 0.02-0.05), and a greater average value at 0.02 (95% CI 0.00-0.04). Finally, the experimental group showed the widest distribution with a standard deviation of 0.06 (95% CI 0.04-0.09) and also the smallest average value of -0.02 (95% CI -0.04-0.01). There is moderate evidence that the average light-to-dark relative change in speed is lower in experimental flies compared to control flies, because even the largest value in the 95% confidence interval for the experimental group barely overlaps with the lowest values in the 95% confidence interval for both of the control groups. However, this effect is not as strong as that seen in the fraction of time spent in the light. Figure 18C, which illustrates the distribution of average change in speed across a dark-to-light transition, reveals a similar outcome. Both the blind and Canton S. no-LED control groups demonstrate a tight distribution around 0, with a standard deviation of 0.03 (95% CI 0.02-0.04) and 0.03 (95% CI 0.01-0.05), around an average 0.00 (95% CI -0.02-0.02) and 0.02 (95% CI 0.01-0.04), respectively. The LED experimental group exhibited greater variability, with the largest standard deviation of 0.10 (95% CI 0.05-0.14), and a slightly larger average at

0.03 (95% CI 0.00-0.07). The control groups showed an average score close to 0, which aligns with our expectations, given that the control groups should exhibit no preference before or after the transitions, resulting in an average change in speed across a transition of 0. The experimental group displayed the widest distribution and variability in the average change in speed across individuals, but averaged to a positive value, indicating that the flies are slightly faster in light than in the dark. The positive average speed change across a dark-to-light transition indicates that, similar to the results from Figure 18B, the flies exhibited faster speed in light than in the dark. These findings suggest that while the transitions across all groups average to around 0, there exist individual differences in speed response to visual stimuli in the experimental group, thus emphasizing the need for further investigation into the underlying mechanisms driving these differences.


2023-02-01_fly0_run0
a
all L → D transitions ≥ 2 mm/s avg (179)
all D → L transitions ≥ 2 mm/s avg (177)
speed mm/s
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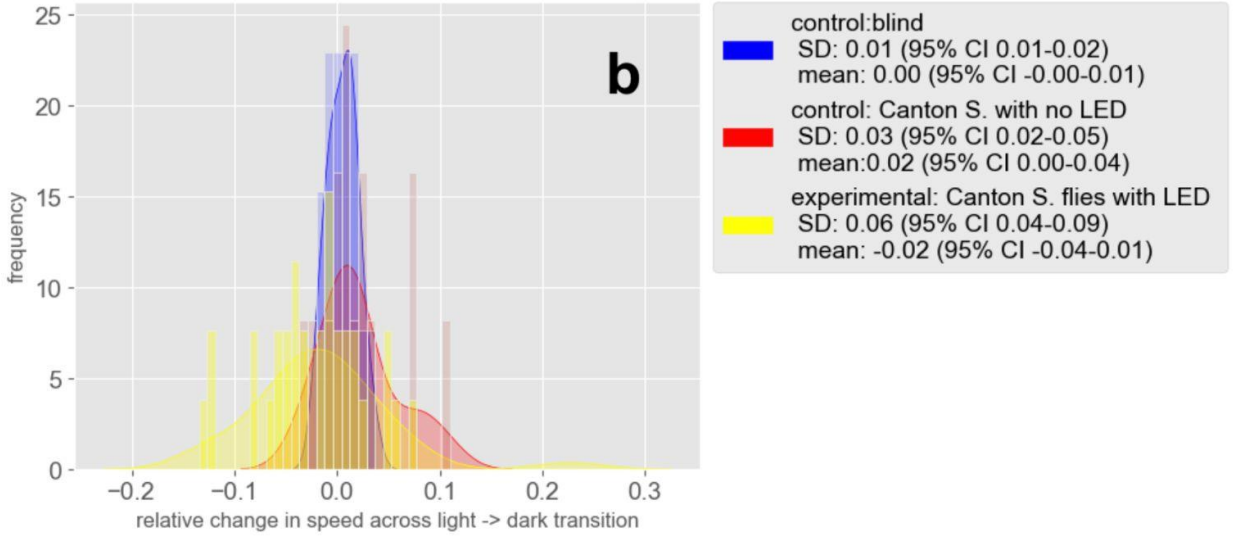

b
frequency
relative change in speed across light -> dark transition
control:blind
SD: 0.01 (95% CI 0.01-0.02)
mean: 0.00 (95% CI -0.00-0.01)
control: Canton S. with no LED
SD: 0.03 (95% CI 0.02-0.05)
mean:0.02 (95% CI 0.00-0.04)
experimental: Canton S. flies with LED
SD: 0.06 (95% CI 0.04-0.09)
mean: -0.02 (95% CI -0.04-0.01)

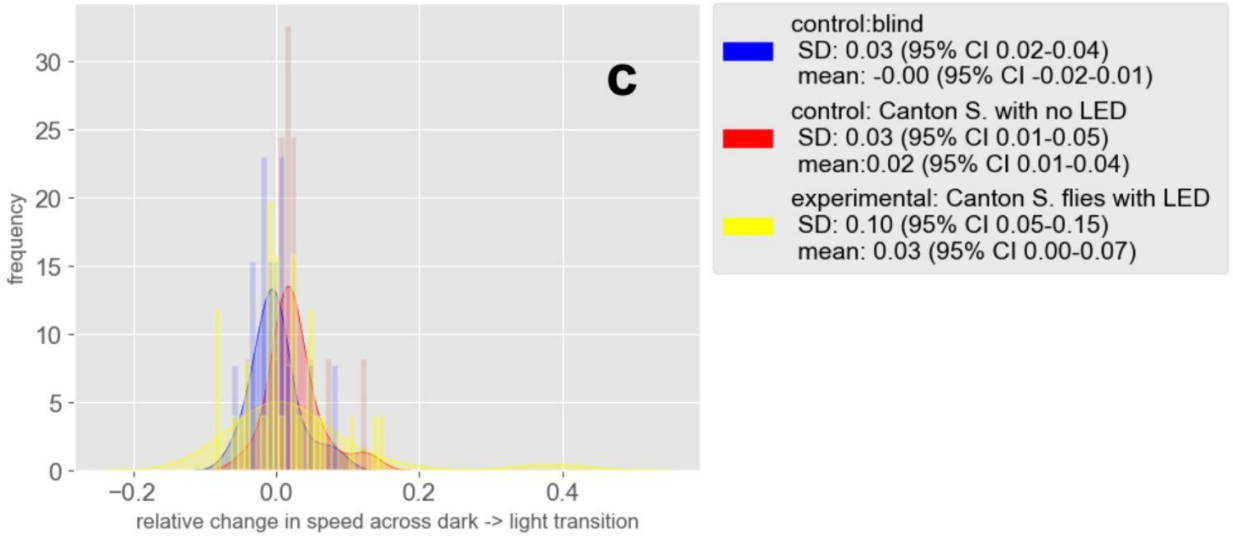

c
frequency
relative change in speed across dark -> light transition
control:blind
SD: 0.03 (95% CI 0.02-0.04)
mean: -0.00 (95% CI -0.02-0.01)
control: Canton S. with no LED
SD: 0.03 (95% CI 0.01-0.05)
mean:0.02 (95% CI 0.01-0.04)
experimental: Canton S. flies with LED
SD: 0.10 (95% CI 0.05-0.15)
mean: 0.03 (95% CI 0.00-0.07)

Figure 18: Speed transition

(A) Average speed transitions with all transitions calculated for individual flies

(B) A histogram of the average speed change across a light-to-dark transition for flies across the three groups

(C) A histogram of the average speed change across a dark-to-light transition for flies across the three groups

After obtaining two speed scores for both transitions, a comparative analysis was performed to evaluate the speed score between the flies, across the three groups. Figure 19A illustrates the comparison between the speed score for light-to-dark transitions and dark-to-light transitions. Both blind and no LED control groups exhibited proximity around the (0,0) point, indicating no relative change in speed across either transition, whereas the no-LED flies were dispersed along a weak positive slope line. The correlation coefficient for the blind control is 0.26 (95% CI -0.06-0.53) with a p-value of 0.35 against a null of no correlation, while the correlation coefficient for the no LED control was 0.70 (95% CI 0.20-0.94) with a p-value of 0.00. The insignificant correlation for the blind controls might be explained by their extreme proximity to the expected point at (0,0), as they were too close to even exhibit a positive slope line. The high correlation for the no LED control follows our null hypothesis of no correlation, as we expect a direct positive line to indicate no difference in speed across transitions. On the other hand, the experimental group exhibited a correlation coefficient of 0.16 (95% CI -0.56-0.77), with a p-value of 0.20 against a null of no correlation. We observe that the confidence interval range is broad, including 0, consistent with the observations of wide variability among flies in the relative speed change across light-dark and dark-light transitions. The experimental flies exposed to visual stimuli displayed dispersed data points below 0 for the light-to-dark transition speed score

while showing uniform distribution for the dark-to-light transition speed score. The average light-to-dark speed score of -0.015 indicated a higher speed in the initial condition, in the light than in the dark. In contrast, the average dark-to-light speed score of 0.033 suggested a higher speed in the final condition, in the light, than in the dark. The results suggest two findings: that on average, the experimental flies are faster in the light than in the dark, and that the experimental flies have a wide distribution in their average change in speed across both transitions.

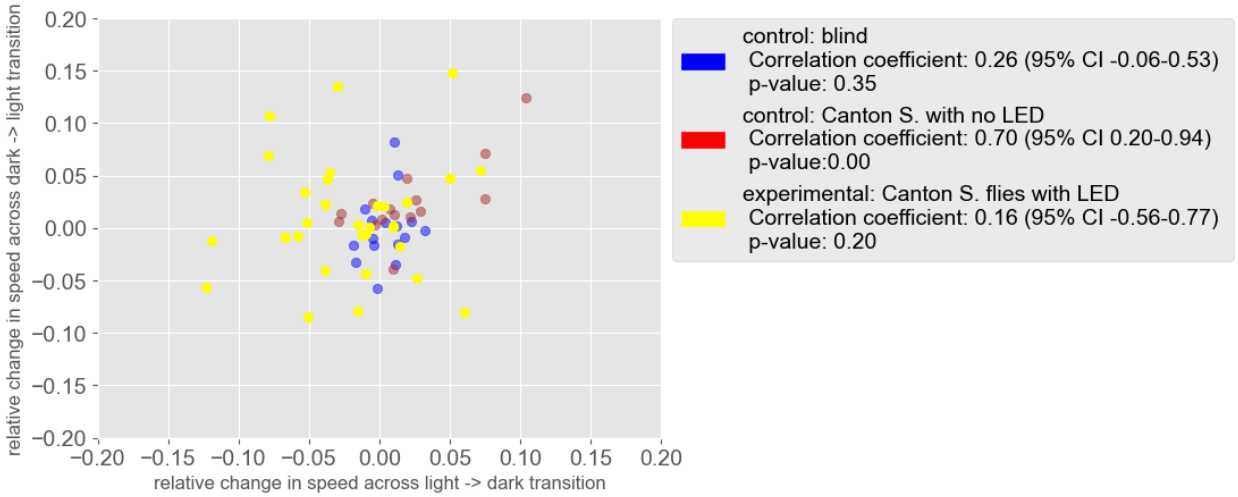


Figure 19: Scatter plot of the change in speed across transitions

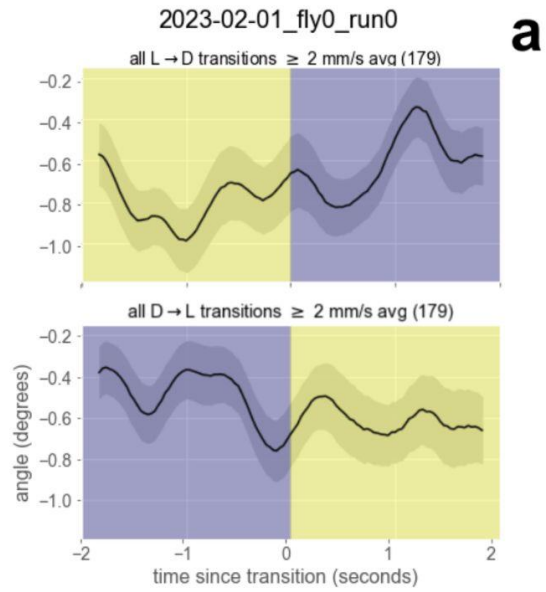
Metric 4: Yaw

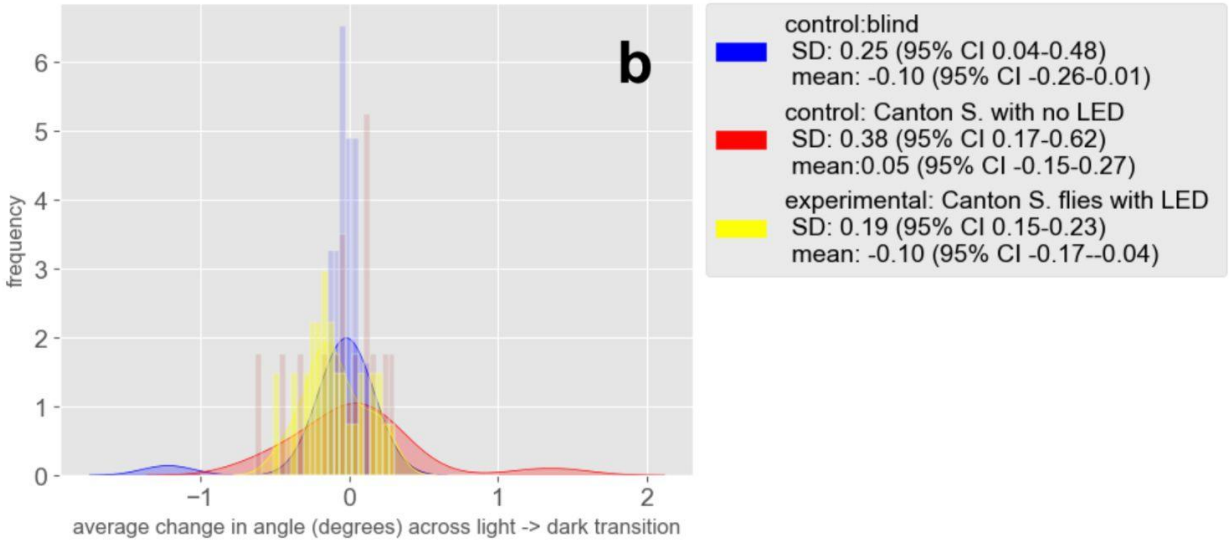
One of our key metrics of interest is a fly's yaw, its turn size about the z-axis, as it crosses the border. A positive yaw is a clockwise turn to the right, while a negative yaw is a counterclockwise turn to the left. To quantify this metric, as shown in Figure 20A, we plotted an individual fly's average yaw, in degrees, at each frame during the 4-second transition period for each type of transition, similar to Figure 18B with the speed score. To compare the angle transitions across the three groups, we devised a yaw score, calculated as the degree difference between the average yaw 2 seconds after and before a transition. A negative score would therefore indicate that the heading change after the transition was more counterclockwise than before the transition, and a positive score would indicate that the heading chance after the transition was more clockwise than before the transition, and a score of 0 would indicate that there was no heading change across the transition.

Figure 20C illustrates the average change in yaw across light-to-dark transitions. We observe that the blind control group has a standard deviation of 0.25 degrees (95% CI 0.04-0.48) and a low average value of -0.10 (95% CI -0.26-0.00). The no LED control group has the largest standard deviation of 0.39 degrees (95% CI 0.16-0.61), and also the greatest average value at 0.05 (95% CI -0.15-0.28). The experimental group surprisingly has the lowest standard deviation at 0.19 degrees (95% CI 0.15-0.23), and a low average value of -0.10 (95% CI -0.17-0.02). These results indicate that while both blind control and experimental groups have the same average value, the experimental group has the tightest distribution. Although the slightly negative score indicates

that the experimental group had on average a larger turn in the light than in the dark, this effect is negligible given that the score is very close to 0, and is the same value as the blind control group.

Figure 20B reveals a similar outcome to Figure 20A, but for dark-to-light transitions. The blind control group has a standard deviation of 0.26 degrees (95% CI 0.05-0.49), and the lowest average value of -0.11 (95% CI -0.28-0.00). The no LED control group, on the other hand, has the greatest standard deviation at 0.35 degrees (95% CI 0.17-0.57), as well as the greatest average value at 0.08 (95% CI -0.10-0.29). Finally, the experimental group has the lowest standard deviation at 0.17 degrees (95% CI 0.13-0.22), and an average value at -0.07 (95% CI -0.13-0.00). The slightly negative yaw score suggests that the experimental flies had a larger turn in the dark than in the light, though this effect is negligible given that the value is still very close to 0, and the blind control group has an even lower score. These results indicate that the experimental group in fact did not exhibit much variability in behavioral preference in yaw across dark-to-light transitions. Both Figure 20B and Figure 20C suggest that the experimental group has the lowest standard deviation, indicating that overall, the experimental group does not exhibit considerable variability in the turn across both transitions, implying few individual differences in turn response to visual stimuli in this group.


2023-02-01_fly0_run0
a
all L → D transitions ≥ 2 mm/s avg (179)
all D → L transitions ≥ 2 mm/s avg (179)
angle (degrees)
time since transition (seconds)


b
frequency
average change in angle (degrees) across light -> dark transition
control:blind
SD: 0.25 (95% CI 0.04-0.48)
mean: -0.10 (95% CI -0.26-0.01)
control: Canton S. with no LED
SD: 0.38 (95% CI 0.17-0.62)
mean:0.05 (95% CI -0.15-0.27)
experimental: Canton S. flies with LED
SD: 0.19 (95% CI 0.15-0.23)
mean: -0.10 (95% CI -0.17--0.04)

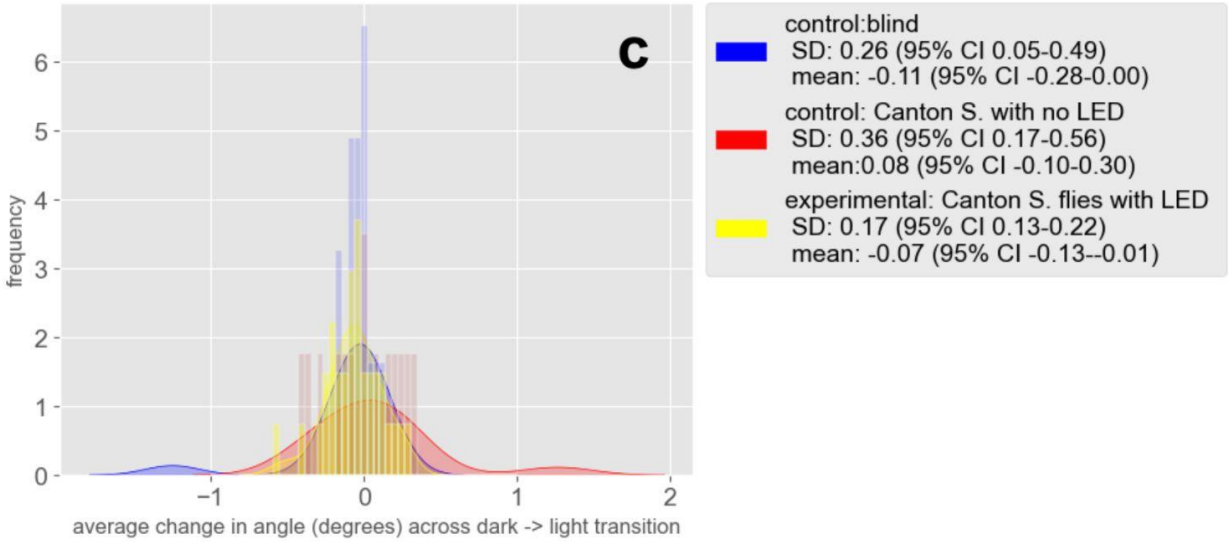

c
frequency
average change in angle (degrees) across dark -> light transition
control:blind
SD: 0.26 (95% CI 0.05-0.49)
mean: -0.11 (95% CI -0.28-0.00)
control: Canton S. with no LED
SD: 0.36 (95% CI 0.17-0.56)
mean:0.08 (95% CI -0.10-0.30)
experimental: Canton S. flies with LED
SD: 0.17 (95% CI 0.13-0.22)
mean: -0.07 (95% CI -0.13--0.01)

Figure 20: Yaw Score

(A) Example of the angle (yaw) of a fly responding to LED stimulus, 2 seconds before and 2 seconds after the transitions. Similarly to above, the top panel shows the transition from a light region to a dark region. The bottom panel shows the transition from a dark region to a light region.

(B) Histogram of the average change in angle (degrees) across a light-to-dark transition for the three groups

(C) Histogram of average change in angle (degrees) across a dark-to-light transition for the three groups

After quantifying a metric for turn for individual flies, we sought to compare the light-to-dark and dark-to-light yaw scores for each fly to gain insights into their behavior. Figure 21 illustrates a scatter plot of the light-to-dark yaw score against the dark-to-light yaw score for the three groups. Both blind and no LED control groups are prominent around (0,0), which is the expected value for controls as we do not expect heading turns across a border. As expected, they exhibit strong positive linearity, with a correlation coefficient of 0.90 (95% CI 0.50-1.00) and 0.98 (95% CI 0.95-1.00), respectively, both with a very small p-value of 0.00 against the null hypothesis of no correlation. A strong correlation approaching 1 was expected for the control groups, as they do not distinguish between light or dark regions. The confidence interval ranging from 0.50-1.00 for the blind control is likely due to their extreme proximity to the expected (0,0) point, as they are too close to a single point to form a perfect positive slope. The experimental group displays a wider distribution of data points, with a correlation coefficient of 0.56 (95% CI 0.16-0.83) and a p-value of 0.00. This is a significant correlation, but is weaker than both control groups, as the confidence level ranges from 0.16 to 0.83. The fact that there is a weaker correlation between the

average heading change among the two transition types for the experimental flies than among the CS controls may hint at variability among individuals, but further investigation is needed to characterize it deeper. Taken together, these results indicate that there is weak evidence for individual behavioral preference among the experimental flies for this measure of yaw angle across transitions.

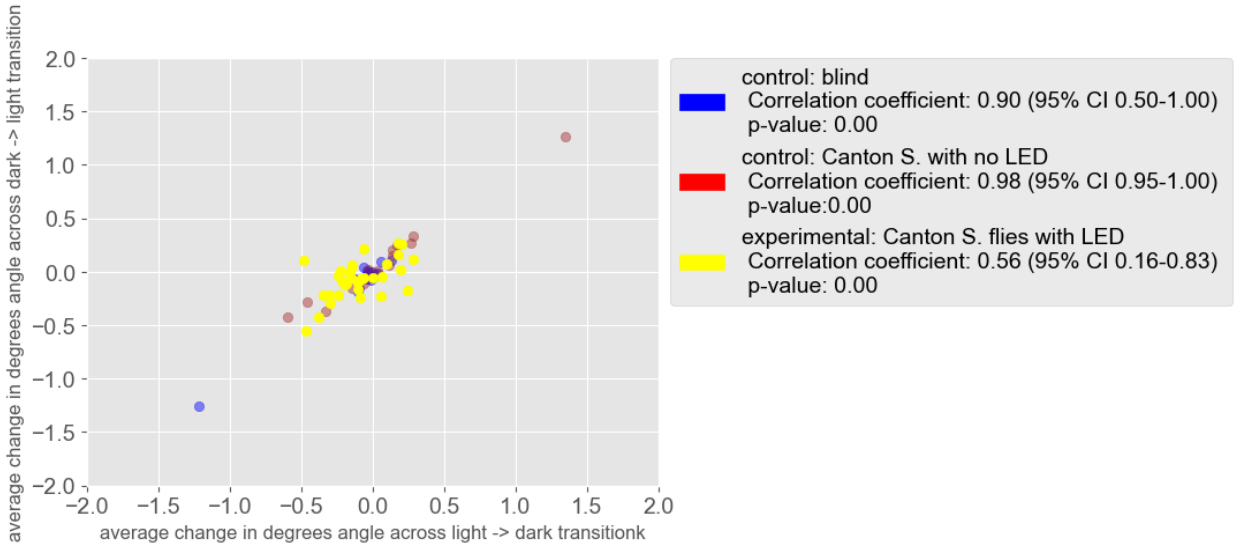


Figure 21: Scatter plot comparing light-to-dark yaw score to dark-to-light yaw score.

Metric 5: Fraction of time paused vs running

As we examine and contrast fly speed, it raises the question of what accounts for the prolonged duration of time in the dark. Are flies pausing more frequently in the dark, or are they moving more slowly in the dark? We next investigated how each individual fly behaves in terms of pausing/grooming, or running throughout the course of the experiment.

To differentiate between pausing and running behavior in flies, we utilized a histogram of log speeds as shown in Figure 12B. The histogram displayed two distinct peaks, indicating a clear separation between fly speed during pausing and running. We set a cutoff value between the two modes at 5.62mm/s, establishing paused behavior as fly speed below 5.62mm/s, and running behavior as speed above 5.62mm/s.

We conducted a comprehensive analysis of the pausing behavior of flies in light and dark environments. We quantified the fraction of time spent paused (below 5.62 mm/s) in both light and dark environments for each fly during each run. Figure 22A depicts the distribution of the fraction of time spent paused in light for the three groups. The blind control group had the tightest distribution with the lowest standard deviation of 0.06 (95% CI 0.04-0.08) and also the largest average value of 0.39 (95% CI 0.35-0.42). The no LED control group had a broader distribution of 0.11 (95% CI 0.08-0.14) and a lower average value of 0.35 (95% CI 0.29-0.40). Finally, the experimental group showed the widest distribution with the largest standard deviation of 0.16 (95% CI 0.12-0.20) and also the lowest average value of 0.33 (95% CI 0.28-0.38). This indicates that the experimental flies exhibit both the largest variability in

pausing behavior in the light and the least amount of time spent pausing in the light, compared to both control groups.

Figure 22B illustrates the fraction of grooming and pausing behavior in the dark, and displays a similar result. The blind control group exhibited the tightest distribution with the lowest standard deviation of 0.07 (95% CI 0.05-0.09), and an average value of 0.37 (95% CI 0.33-0.40). The no LED control group has a wider distribution with a standard deviation of 0.11 (95% CI 0.07-0.13) and the lowest average value of 0.33 (95% CI 0.27-0.39). Finally, the experimental group has both the broadest distribution with the largest standard deviation of 0.16 (95% CI 0.12-0.19), and the largest average value of 0.50 (95% CI 0.45-0.55). This substantial increase in average value is statistically significant, as even the lowest value in the 95% confidence interval for the experimental group is greater than the highest values in the 95% confidence interval for both control groups. The results indicate that the experimental group exhibits a significantly higher fraction of time pausing in the dark compared to both control groups, and exhibit the widest variability of pausing behavior in both light and dark. Taken together, our findings suggest that experimental flies display a tendency for spending more time engaging in grooming and pausing behaviors in the dark compared to the light.

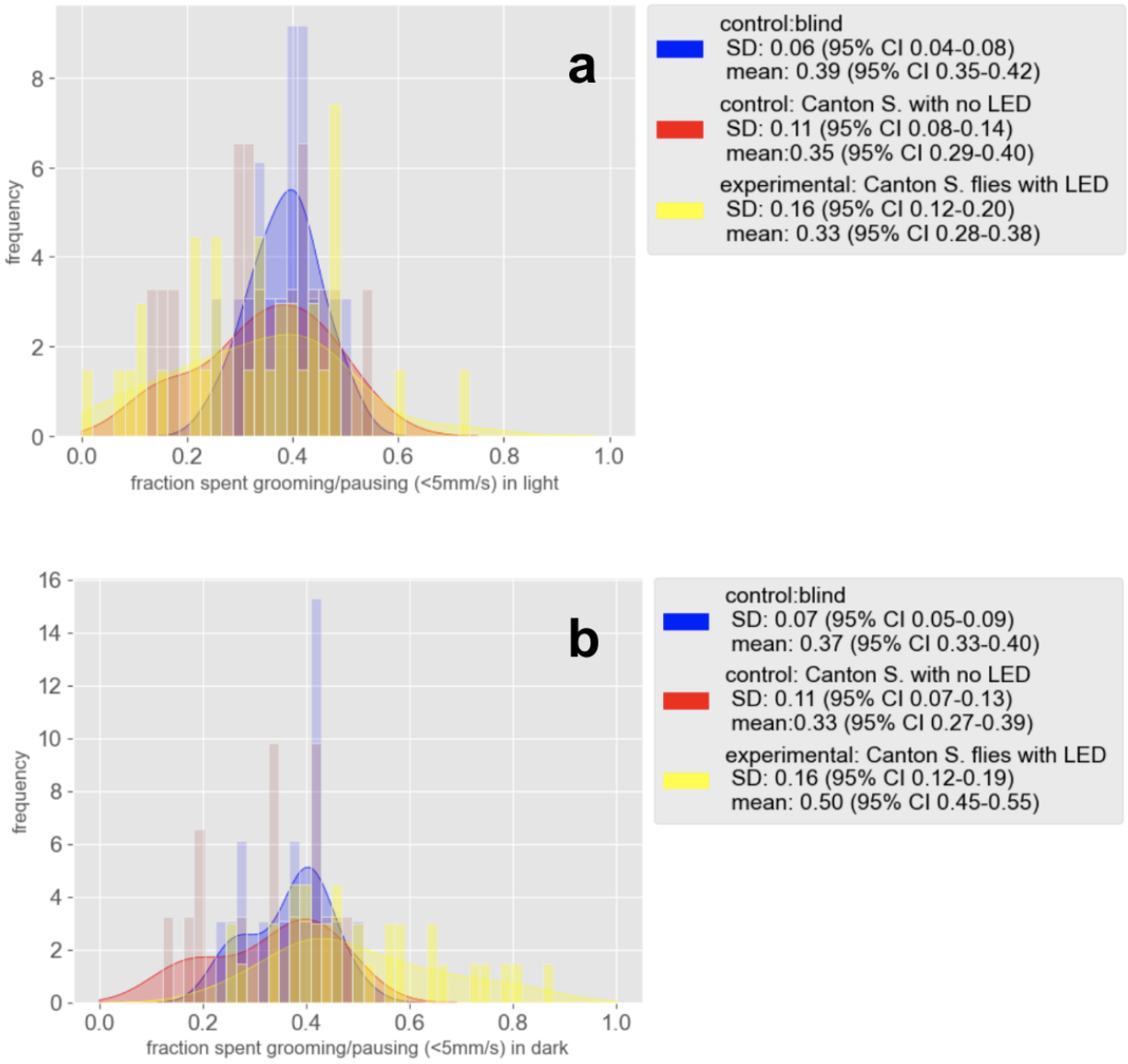


Figure 22: Grooming/Pausing in light and dark

(A) Histogram of a fly's fraction of time spent grooming/pausing in light

(B) Histogram of a fly's fraction of time spent grooming/pausing in dark

Now with a quantification method for grooming and pausing behaviors, we aim to investigate grooming and pausing behavior of flies in light and dark regions. Figure 23 illustrates a scatter

plot of the fraction a fly spends on pausing behavior in the light versus the dark. Both control groups, blind and no LED, follow a strong positive linear line, with a correlation coefficient of 0.64 (95% CI 0.23-0.86) and 0.68 (95% CI 0.27-0.90), and a small p-value of 0.01 and 0.00, respectively, against the null hypothesis of no correlation. This direct positive correlation observed among both control groups between the fraction spent pausing in the light and in the dark is consistent with our expectation that flies would pause at similar rates in light and dark regions when there is no differing perception of light. On the other hand, the experimental flies exhibit strong negative linearity, with a correlation coefficient of -0.80 (95% CI -0.90–0.67) and a small p-value of 0.00. These results indicate that the experimental flies have a strong and direct negative correlation between the fraction spent pausing in light and in the dark, strengthened by the narrow confidence interval from -0.90 to -0.67.

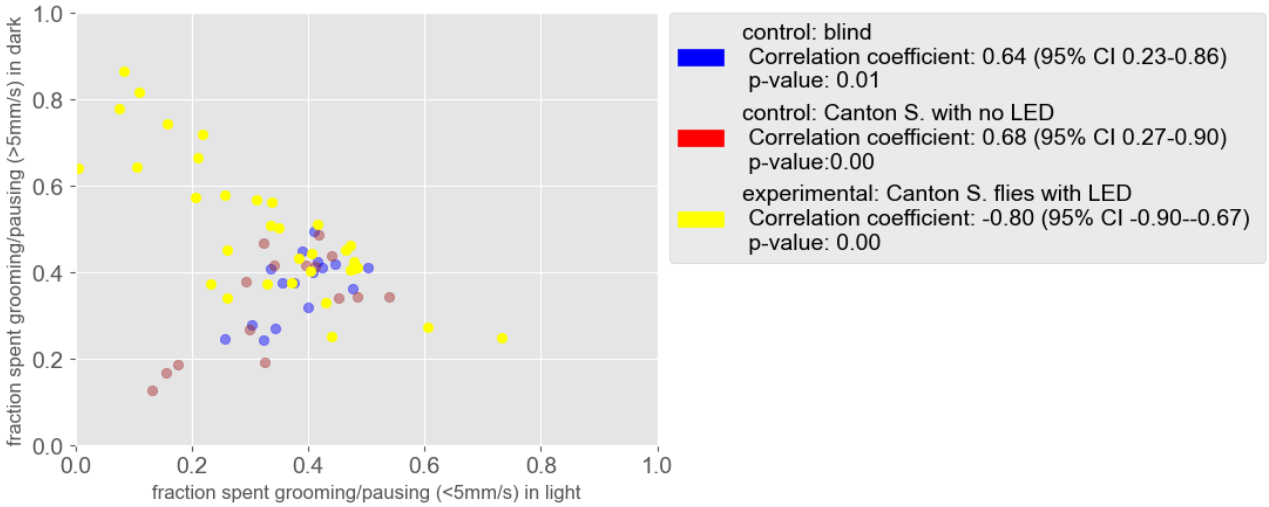


Figure 23: Scatter plot of fraction spent grooming/pausing in the light versus the dark

Taken together, these results suggest three findings. First, the experimental flies have a wide distribution on their fraction spent pausing in both light and the dark. Second, that on average, experimental flies spend more time pausing in the dark than in the light. Third, that the fraction spent pausing in light is inversely correlated with the fraction spent pausing in the dark.

Comparisons

Through establishing various metrics, we have investigated the flies variability and preferences among each metric across the three groups. We are now interested in examining potential correlation between the various metrics. One key comparison of interest is the relationship between a fly's speed and its time spent in light, to understand the relationship between a behavioral response when exposed to light and the overall preference through the experiment. To investigate this, we analyzed the average speed of each fly while in the light and plotted it against the light fraction, the proportion of time the fly spent in the light throughout the experiment. Figure 24 displays an almost vertical distribution for the experimental group, and a horizontal distribution for the control groups. The correlation coefficient for the blind control group and no LED control group was 0.05 (95% CI -0.54-0.63) and 0.21(95% CI -0.16-0.61), with p-values of 0.88 and 0.45, respectively. This suggests no correlation for both control groups for the fly's average speed and fraction of time in the light, strengthened by their very range in confidence level. On the other hand, the experimental group displayed a negative correlation coefficient of -0.41 (95% CI -0.62–0.08), with a p-value of 0.02, indicating a negative relationship between speed and time spent in light. The fact that the 95% CI overlaps with 0 indicates that the fraction spent in light is not well-explained by the average speed in the light.

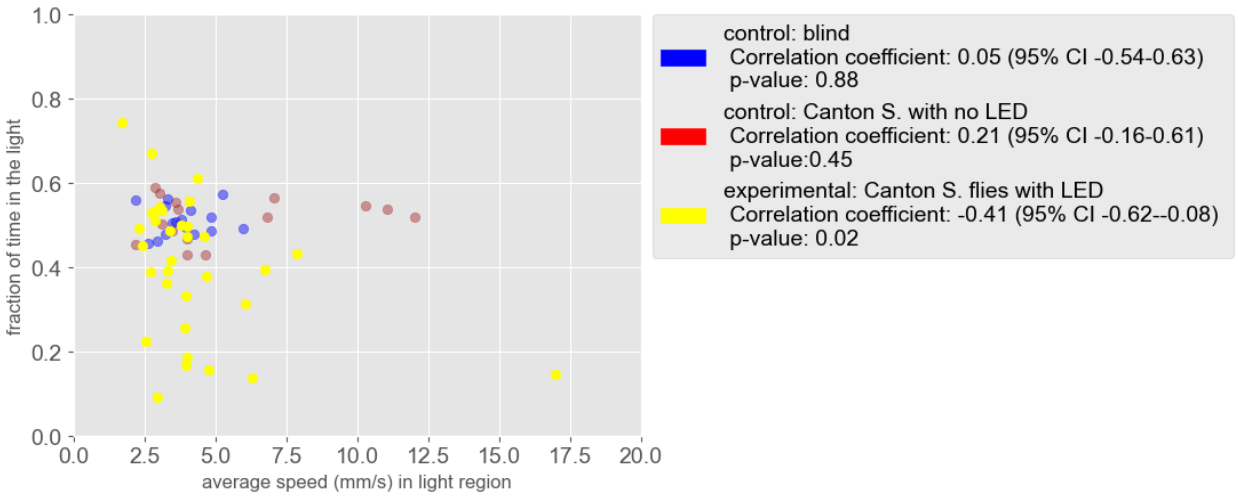


Figure 24: Scatter plot speed in the light vs. time spent in light

With the previous comparison across the average speed in light and fraction of time spent in light, it would be of interest to compare the light-to-dark speed score, the average change in a fly's speed across a transition, against the light fraction. A scatter plot shown in Figure 25 shows that the blind and no LED control groups exhibited the expected behavior, with close proximity around the expected value of (0,0.5), indicating an average speed difference of 0 and a light fraction of 50%. The blind and no LED control group exhibited no discernible correlation, with a correlation coefficient of -0.06 (95% CI -0.54-0.49) and 0.48 (95% CI 0.07-0.79), and p-values of 0.86 and 0.08, respectively. This result could be explained by their extreme proximity to the expected value point, since not many points deviate away from the point to create a slope, and is strengthened by the broad range in confidence intervals. The experimental group also displayed no correlation, with a correlation coefficient of 0.27 (95% CI -0.05-0.58) and a p-value of 0.14. With a broad range in confidence levels, there is little evidence for an association between the relative change in speed across the transitions and the fraction spent in light. The data also indicates a lower average light fraction, at 0.4, suggesting that the flies spend more time in the dark, and that the average change in speed across light-to-dark transitions is -0.02, suggesting

that most flies had higher speeds in the light compared to the dark. The vertical distribution of data points suggests that despite observed individual behavioral differences in the fraction of time spent in light, flies generally do not exhibit significant changes in speed across light-to-dark transitions, with a slightly higher speed in light than in the dark. This finding is consistent with our previous findings, as lower speed scores indicate that the fly has higher speed in light than in dark, which in turn explains their reduced time spent in light.

Figure 25B displays similar results, with data points indicating the blind and no LED control groups in close proximity around the expected value of (0,0.5). They exhibit no association between the relative change in speed across transitions and the fraction spent light, with a correlation coefficient of -0.14 (95% CI -0.60-0.38) and -0.13 (95% CI -0.50-0.26), and p-values of -.55 and 0.69, respectively. Their wide range in confidence interval supports our claim for no correlation, which could be caused by the close proximity to the expected value of (0, 0.5). Similarly to Figure 25A, the experimental group also shows negligible correlation, with a coefficient of -0.20 (95% CI -0.56-0.18) and a p-value of 0.27, though the range of confidence interval indicates a stronger association than the control groups. With an average value of 0.03, the positive score indicates that the flies had a higher speed in light than it did in the dark, consistent with our findings from Figure 25A. The vertical wide distribution suggests that the observed variability in light fraction does not interfere with the general tendency of flies to move faster in light than in the dark. Taken together, these results suggest there is little evidence for a correlation between a fly's fraction of time spent in light and its relative speed change across light/dark transitions.

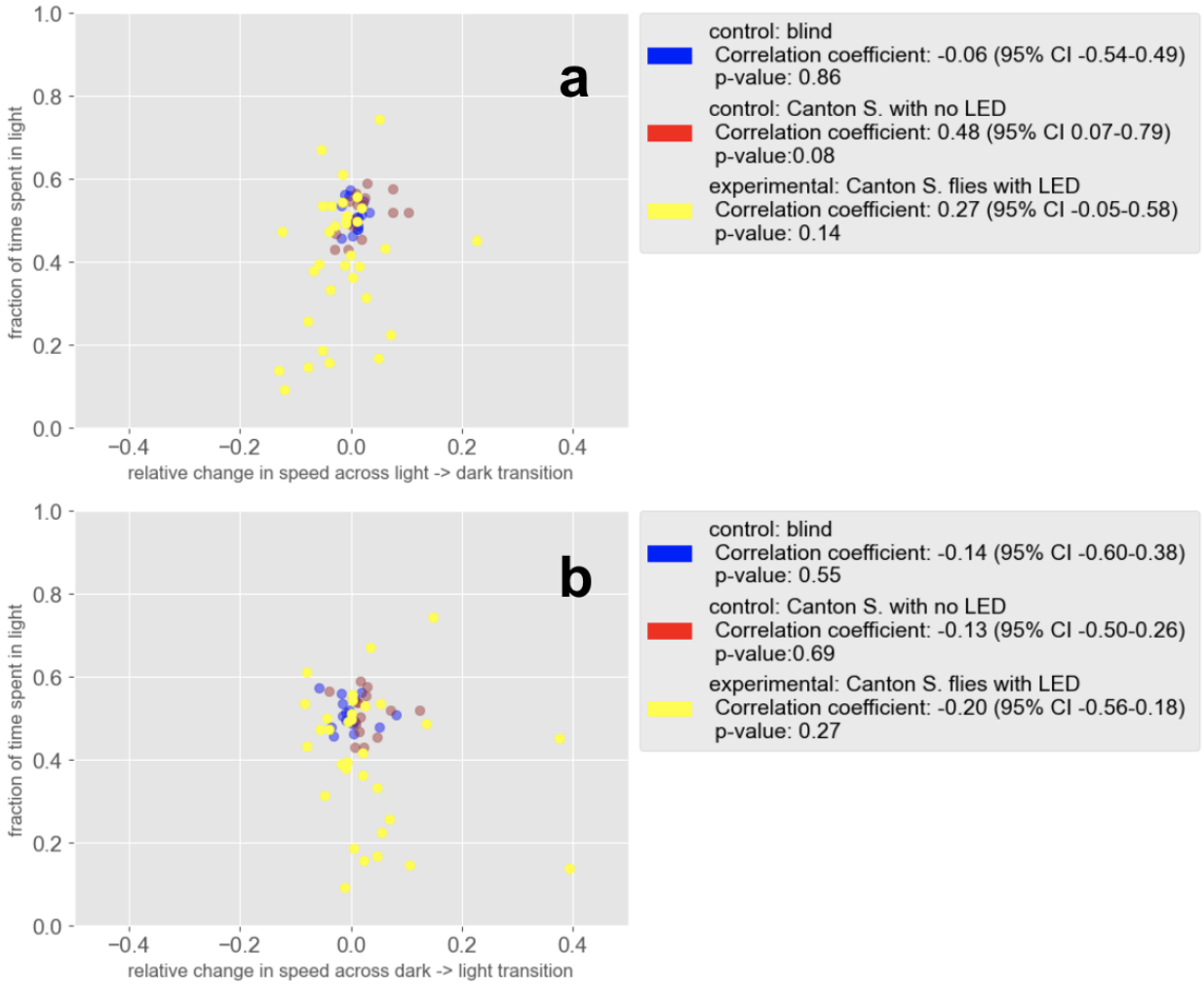


Figure 25: Scatter plot of relative change in speed across light-to-dark transitions and dark-to-light transitions against the fraction of light

Through comparisons between pausing and grooming behavior in light and dark regions, we aim to investigate how individual flies grooms throughout the experiment. Figure 26 presents a comparison of a fly's pausing fraction in a light region, against their average speed in the light. We observed a significant negative correlation in the blind and no LED control groups, with a correlation coefficient of -0.87 (95% CI -0.96--0.69) and -0.91 (95% CI -0.97--0.78),

respectively, and a very small p-value of 0.00 for both control groups, against the null hypothesis of no correlation. This indicates a direct correlation between a higher fraction spent pausing and a lower average speed in the light, strengthened by the narrow range in confidence interval. The experimental group also showed a negative correlation, though of smaller magnitude, with a correlation coefficient of -0.59 (95% CI -0.75--0.38) and a small p-value of 0.00. The datapoint distribution among experimental group flies also appeared to be horizontally spread, with most flies exhibiting a uniformly low average speed in light, regardless of their behavioral variability with the fraction spent pausing in light. These results suggest that the experimental flies have significant but weaker negative association compared to the blind and no LED control groups when comparing their pausing behavior and average speed in light.

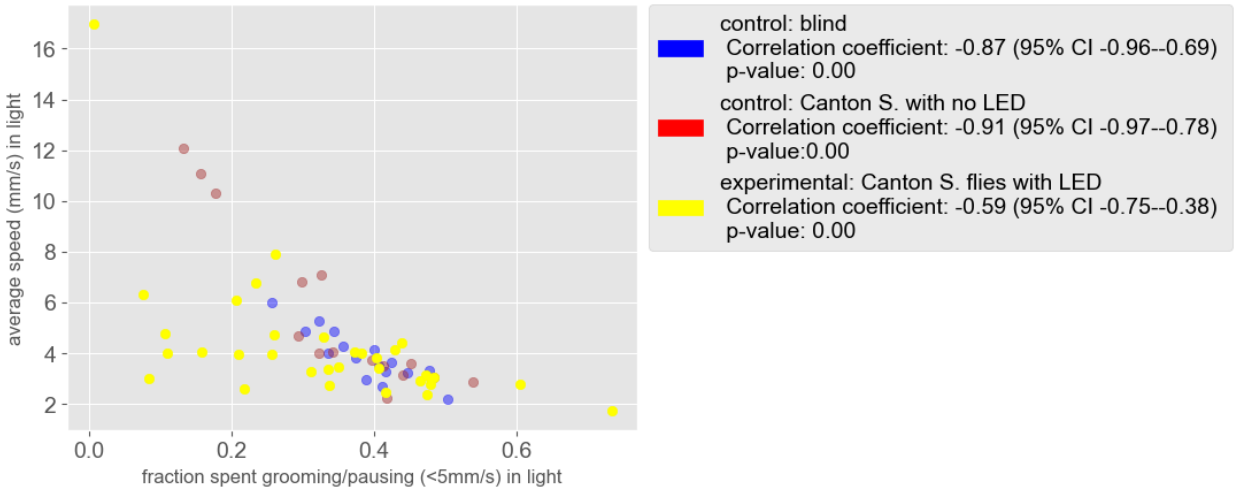


Figure 26: Scatter plot of the fraction a fly spends grooming/pausing in light versus the average speed in light

With the previous comparison across the fraction spent pausing in the light and in the dark, it would be of interest to compare the fraction spent pausing in the light and the fraction spent in the light. A scatter plot shown in Figure 27 illustrates the fraction a fly spends paused in light against the fraction a fly spends in light, and shows that the blind control group and no LED control groups exhibited very weak correlation between the two values, with a correlation coefficient of 0.35 (95% CI -0.20-0.76) and 0.16 (95% CI -0.36-0.59), and p-values of 0.19 and 0.53, respectively. Their weak correlation could be explained by their proximity to the point around (0.5, 0.4), as both control groups exhibited narrow distributions for each metric. On the other hand, the experimental group displayed a very strong positive correlation, with a correlation coefficient of 0.96 (95% CI 0.93-0.98) and a very low p-value of 0.00, suggesting that there is a strong direct association between the fraction a fly spent pausing and the fraction a fly spent in light.

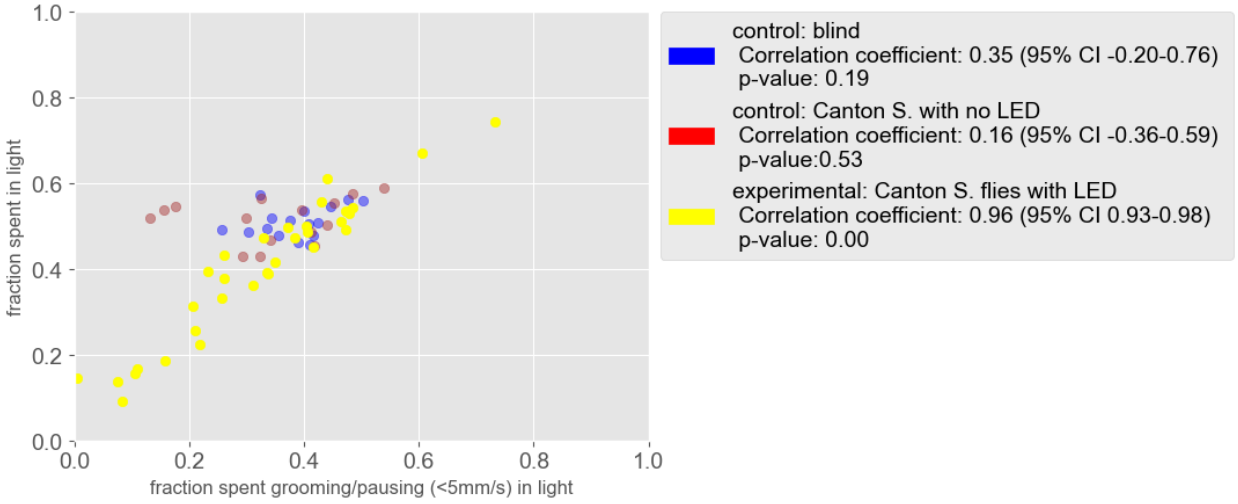


Figure 27: Scatter plot of the fraction a fly spends grooming/pausing in light versus the fraction spent in light

Taken together, these results suggest two findings; that the fraction spent pausing is also inversely correlated with their average speed in light, and that the fraction spent pausing is strongly and directly correlated with their fraction spent in light. This is consistent with our findings from Figure 24, which indicates that the average speed in light is inversely correlated with the fraction in light.

Summary Table:

The key findings of this thesis are summarized in the following table.

Metric	Effect of Individuality among experimental flies compared to controls	Extent of Individuality
Light fraction	- Experimental flies exhibited lower fraction spent in light and a broader distribution - Standard deviation is significantly larger for experimental flies compared to both control groups, suggesting variability in individual behavior in response to visual stimulus.	Strong
Time spent in box	- Experimental flies exhibited on average less time spent in each light box, and more time spent in each dark box. - Standard deviation was the largest for the experimental group compared to both control groups. - Experimental flies exhibited no correlation between time spent in light and time spent in dark, while both controls displayed a strong positive correlation	Strong
Speed transitions	- Experimental flies were faster in the light than in the dark - Experimental flies displayed widest distribution in relative change in speed across both transitions, indicating individual preference - Experimental flies exhibited no correlation between speed changes from light-to-dark and from dark-to-light transitions, while both controls displayed a strong positive correlation	Strong
Yaw transitions	- The standard deviation was smallest for experimental flies compared to both control flies, indicating that experimental flies did not show considerable variability in heading changes at boundaries - Experimental flies exhibited a positive correlation between yaw turn from light-to-dark and from dark-to-light transitions, while both controls displayed a strong positive correlation. However, the weaker correlation suggests that flies are behaving differently between the transitions	Weak

Pause fraction	- Experimental flies paused more in the dark than in the light relative to both control groups - Standard deviation was greatest in experimental flies for both light and dark environments, compared to both control groups - Experimental flies exhibited very strong negative correlation, while both controls exhibited strong positive correlation.	Very Strong
Comparisons		
Average Speed in light vs. light fraction	- Experimental flies exhibited strong negative correlation, while both control groups exhibited no correlation	Strong
LD score vs. light fraction	- Experimental flies exhibited no correlation (a vertical distribution), while both control groups also exhibited no correlation	Intermediate
Pause vs. speed in light	- Experimental flies exhibited a strong negative correlation, while both control groups also exhibited a stronger negative correlation.	Intermediate
Pause vs. fraction in light	- Experimental flies exhibited very strong positive correlation, while both control groups exhibited no correlation	Very Strong

Chapter IV: Discussion

We have successfully identified behavioral individuality among *Drosophila melanogaster* in response to visual stimuli. Five distinct metrics were devised to gauge behavioral individuality, including 1) light fraction, 2) time in light or dark boxes, 3) the average change in speed across transitions, 4) the average change in turn across transitions, and 5) the fraction spent pausing. Strong signs of individuality were observed in light fraction, time in boxes, and fraction spent pausing, while speed transition and yaw transition metrics gave weaker signals of behavioral individuality. We identified photonegative tendencies in flies, characterized by a decreased time spent in a light box, an increased speed in light, increased pauses in dark, and reduced pauses in a light environment.

1) An analysis of the light fraction showed that the average light fraction of the experimental group was lower than that of both control groups, suggesting an average photonegative tendency among the experimental CS flies exposed to light stimuli, and a much wider distribution compared to controls.
2) Analysis of the time spent in boxes indicates that the experimental flies tend to spend less time in each light box and prefer spending more time in each dark box. This might be caused by a fly's decision to leave a box when they realize they are in an unpreferred box, and stay in a box when they like the environment. As we saw from the light fraction that many flies preferred dark environments, which might cause them to leave a light box quickly. For future studies, we could track flies on a larger checkerboard with wider boxes, to see if the ratio of the time they spend in each dark and light box stays the same,

to check this effect. We would expect the time spent in each box to be longer, as it would take longer to transverse across the box, but the ratio should stay similar.

3) Analysis of the average change in speed across transitions indicates that on average, the experimental flies are faster in the light than in the dark, but displayed a wide variability across their average speed change in speed across transitions. We first thought the speed changes could be caused by a fly's reaction to the environment, but found this to be unlikely because of the lack of association between the relative change in speed across light-to-dark or dark-to-light transitions. This would indicate that velocity changes are not causing the photonegative tendency seen in light fraction, as we do not see an observable correlation between both transitions. However, we did see considerable variability in speeds across flies, which indicate that flies behave differently in light and dark environments.
4) The yaw metric did not show significant change across transitions or a greater standard deviation compared to control flies. This might be caused by the yaw score being quantified in directional metrics, making it less clear to assess the true head turn. We could look at a single-sign metric such as turn rate, in which 0 is no turning and more positive values represent more turning, because the metric used in this study could involve CW and CCW turns canceling each other out. Future paths could entail measuring the yaw rate against head turn angle, in order to quantify the fly's behavioral reaction to a visual transition.
5) Finally, an analysis of pausing behavior in light or dark environments showed great promise for individuality, among the strongest of our signals. The fraction spent pausing in light and dark were inversely correlated, indicating that a fly will not spend a lot of

time pausing in both environments, but will pause in one or the other. This serves as strong evidence for behavioral individuality, as long pauses did not indicate an inactive fly, but directly correlates with their preference for the environment. A future experiment could entail a light that turns on if the fly is detected to be paused in the dark, and see how it reacts, and another that would turn off it paused in the light. If the fly truly has a preference for an environment, we would expect to see them move immediately after the light turns on or off, to move back to a place they prefer. If the fly continues their pausing behavior, they might not have a preference between light and dark.

We saw great promise for individuality through investigating variability and preferences among the metrics. Four comparisons were made across the metrics to further gauge behavioral individuality. Through analysis of the comparisons across various metrics, we strengthened our claim of photonegative tendencies in flies, and also that the rate of pausing underlies this, instead of the speed. The results suggest four findings; that the fly's average speed in light is inversely correlated to the light fraction, that the light-to-dark speed score is not correlated with the light fraction, that the fraction spent pausing is also inversely correlated with their average speed in light, and that the fraction spent pausing is strongly and directly correlated with their fraction spent in light.

We set out to find what is causing wide variability in light fraction and the overall photonegative tendency among flies. We first tried to explain the light fraction with speed, by relating the fraction spent in light with various speed metrics. However, a comparison with the fly's relative change in speed across a light-to-dark transition revealed that there is no correlation between the

two values. We saw that the average speed for flies was quite consistent regardless of their fraction spent in light. This indicates it is difficult to use speed changes to predict the fraction of light.

To further investigate what causes light fraction, we compared the fraction spent in light with our pause/grooming metrics. We first compared the fraction a fly spends pausing in light against a fly's average speed in light, which revealed an inverse relationship weaker than both controls. The results indicate that most flies exhibit a uniformly low average speed in light, regardless of their behavioral variability with the fraction spent pausing in light, suggesting that their photonegative tendency in light fraction is not explained by their speed differences, but instead, by their preference to pause in the dark. It seems like instead of varying their speed, the fly is choosing to pause in their preferred environment to raise or lower their fraction spent in light and fraction spent pausing. The next comparison of the fraction in light against the fraction pausing in light demonstrated that a fly's fraction pausing in light is incredibly informative of their light fraction. Fractions in light against fraction pausing in light displayed a strong direct correlation, indicating that while flies show photonegative tendency, the strongest driver for their fraction spent in light is their fraction of pausing in light.

In our project, our overarching goal was to establish individuality in walking behavior on a ball for *Drosophila*, by drawing on paradigms from the larval study of phototaxis on tethered flies done by Kane et al. 2013. Instead of Kane's checkerboard illumination pattern, we created our own fictive virtual checkerboard map, which the fly was free to cross through as it moved on the floating ball (Kane et al. 2013). While Kane's assay studied the larvae's track path, turn angle,

and speed, we ran the checkerboard assay with *Drosophila* young adults, and created scores to compare them across groups. Unlike Kane's study, we faced challenges with observing yaw turns, as flies can easily manipulate the ball in the yaw-direction with one leg or during grooming. A future direction could entail analyzing turn rate relative to heading changes at the boundary, as done in the larval study of tethered flies by Kane et al.. We would expect no correlation between the turn rate and heading changes for either of the control groups, while expecting a positive correlation for the experimental group, as we expect flies with high preference for an environment to turn faster and with more magnitude.

Effects of serotonin and other neuromodulators on individuality

In adults, it is known that neuromodulators affect variability in behavioral preferences. Neuromodulators are chemicals with a broad range of targets and roles that can trigger large shifts in behavior, allowing organisms to adjust their behaviors based on environmental context (Maloney 2021) (Bargmann and Marder 2013). Neuromodulators are a critical element in defining behavioral variability and individuality, as they allow the animal to adapt and change their behavior in response to evolutionary pressure, and by allowing for shifts in their neural circuits, instead of forming new behavioral circuits (Katz and Lillvis 2014) (Skutt-Kakaria et al. 2019).

The study by Kain et al 2012 found that the extent of individuality in phototactic preference was impacted in *white* mutants, who have decreased concentrations of neurotransmitters serotonin, dopamine, and histamine (J. S. Kain, Stokes, and de Bivort 2012). They demonstrate that

serotonin is involved in suppressing behavioral variability and suggest that serotonin alters the wiring that diversifies phototactic polarity, by suppressing the developmental noise that occurs during neurogenesis. Neurogenesis leads to physiological differences in the circuit, which then diversifies phototactic polarity (J. S. Kain, Stokes, and de Bivort 2012). In addition, studies by Sitaraman et al. show that serotonin is essential in *Drosophila* for reinforcing associative learning and place memory. Their study genetically altered serotonin and dopamine levels in the *Drosophila* by manipulating the neurons that create them, and found that serotonin is necessary for memory formation (Sitaraman et al. 2008). This could also affect our study and Drosophila behavior as well, and future studies could be done involving altering serotonin levels. A study by Sampson et al. also indicates that serotonin modulates voltage dependence of potassium channels in photoreceptors. A recording of honeybee single cells on lobula neurons showed that serotonergic signaling reduces background activity, directional, selectivity, and amplitude of field potentials from moving stripes (Sampson et al. 2020). Given these results, we would expect an increase of serotonin to increase light fraction, as well as an increased time spent in a box, due to a reduction in background activity. We might also see a decreased average speed and yaw degree change across transitions, due to a reduced directional selectivity, and an increased fraction spent grooming, due to the reduced amplitude of field potentials. Moreover, we would expect increased serotonin to decrease variability in light preference, consistent with the effects shown in the study by Kane et al. 2012. (J. S. Kain, Stokes, and de Bivort 2012)

Photonegative Tendency

Through our analysis, we identified a photonegative tendency in flies, characterized by a decreased time spent in a light box, an increased speed in light, increased pauses in the dark, and reduced pauses in the light. In a study done by Werkhoven et al., they implemented a Decathlon experiment, where each fly underwent 10 different behavioral assays, using over 200 flies. They studied phototaxic behavior and preferences through a couple of these assays, such as the LED Y-Maze photo bias test, the Spatial Shade-light photo occupancy test, and the Temporal Shade-light photo occupancy test (Werkhoven et al. 2021). The LED Y-maze had flies turn toward or away from a lit LED, and scored the fraction of turns toward the lit arm of the arena. In the Spatial Shade-Light assay, flies chose to stand in lit or shaded regions of an arena that changed every 4 minutes, and scored the fraction of time spent in the light. In the Temporal Shade-Light test, the fly traveled into virtual zones that triggered illumination of the arena, and scored the fraction of time spent in the light.

A behavior summary of the three assays illustrates the behavioral outcomes and the fraction of time the flies spent in light, for inbred and outbred batches of flies. The flies in the decathlon study had an average phototactic positive behavior for the LED Y-Maze photo bias showing a preference for light, indicated by a score greater than 0.5. Specifically, the LED Y-Maze photo bias seems to show a distribution that peaks around 0.6 for the inbred batches, while showing more flattened curve distribution for the outbred batch. Overall, the average seems to lie above 0.5, showing that the fly chose to turn to the light more times than it turned away. However, the Spatial Shade-light photo occupancy and the Temporal shade-light photo occupancy, which most resembles our experiment here, shows a phototactic negative behavior, with a score average

below 0.5. Specifically, the Spatial Shade-light photo occupancy shows a distribution that averages below 0.5 for both inbred and outbred batches, indicating photonegative tendency among all fly groups. These results indicate that in this assay with a half lit, half dark arena, the flies generally occupied the half that was dark. The results for the Temporal shade-light photo occupancy assay, which most resembles our assay, were even more striking, with averages around 0 for both inbred and outbred batches. The results suggest that in this assay with an invisible virtual boundary, the fly spent a significant majority of their time in the dark. Taken together, the results suggest that when the fly can travel to virtual zones that trigger illumination, flies tend to exhibit photonegative behavior, avoiding light, which is consistent with our findings.

Fly selection for data collection

Prior to experimentations, only active flies were selected for data collection. Flies that exhibited generally high levels of activity, such as those that rapidly ascended to the top of the vial after being knocked down, or those that displayed high leg movement on the needle and during acclimation, were prioritized to gather data. Inactive flies would often remain stationary on the ball for the entire duration of the experiment, rendering them unsuitable for data collection on transitions. Some control group Canton S. strain flies without visual stimulus were omitted from transition data collection if they did not display sufficient levels of activity, and were replaced with another fly. Figure 28 illustrates the cumulative distance traveled by the fly against the full duration of the experiment, for the flies that were included in the analysis presented here. Among these flies, more active flies displayed constant traveling, creating a positive slope, while inactive flies displayed sporadic movements, traveling inconsistently. Some control group flies exhibited

lower levels of activity as compared to those in the experimental group, possibly due to the absence of stimuli. This observation is consistent with our findings in Figure 22A, where the blind and no LED controls exhibited the most narrow distribution around the largest fraction spent pausing in light, and 15B, where the blind and no LED controls also exhibit the most narrow distribution around the longest time staying in a dark box.

The slope of the lines in Figure 28 correspond with the speed of the fly. Therefore, a steeper slope indicates a faster fly, while a flatter slope indicates a slower fly. Our results from Figure 22 suggest that experimental flies display a tendency for spending more time engaging in grooming and pausing behaviors in both dark and light, compared to both control groups. Consistent with this finding, the blind and no LED control lines in Figure 28 to be "faster," with a steeper slope than many of the experimental lines. This is also consistent with our results from Figure 13, which displays that the experimental group exhibited the greatest time spent in any box, compared to both control groups.

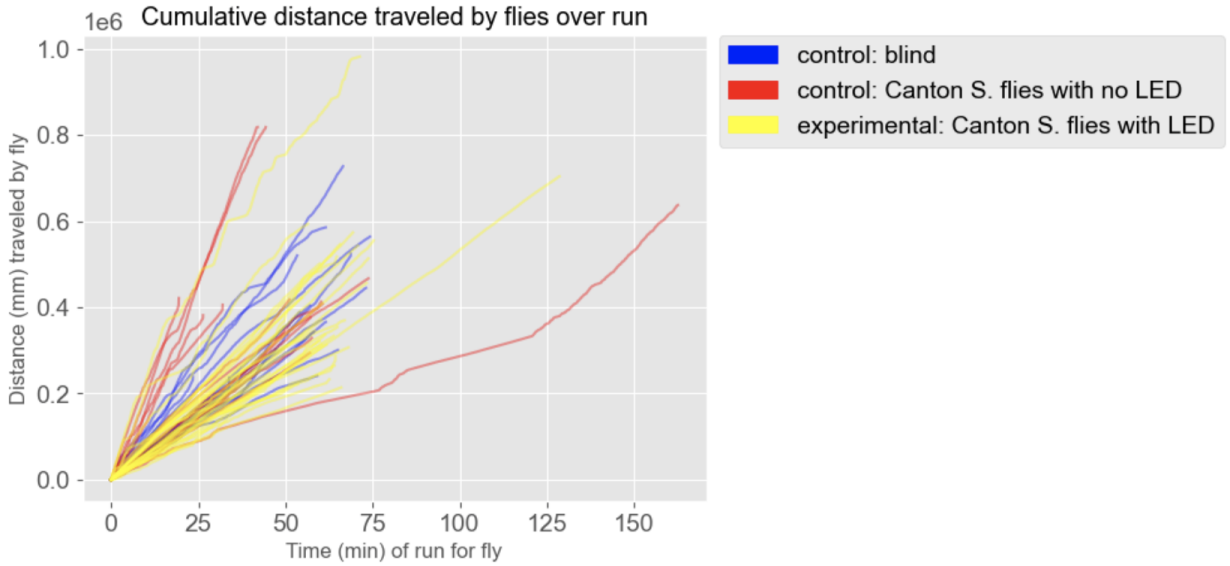


Figure 28: Graph on cumulative distance traveled (mm) vs. time (min) across all individual flies

Future Directions

Testing different fictive geometries and maps

Our fly-on-a-ball assay is able to accommodate different light stimulus patterns, and are not limited to a checkerboard. A straightforward-to-implement assay to assess individuality in fly behavior is to evaluate their fictive lane preference, instead of the checkerboard map we used in this project. It would be interesting to determine if adult *Drosophila* can 'learn' and stick to their preferred regions in a fictive lane map. This study has established that flies exhibit behavioral preferences, and generally exhibit photonegative tendencies. We conducted preliminary experiments on six flies on a lane map and evaluated their metrics. Figure 29A illustrates the walking path of a single individual fly, indicating a relatively vertical trajectory. Figure 29B displays the fraction of time a fly spent in a light region and off region, and we observe that the same fly has spent almost twice as much time in an off region as in a light region, suggesting that its movements were influenced by behavioral preferences. Figure 29C illustrates the fraction in light across flies from all groups and we observe that most flies, not just the individual shown in 29A and 29B, have an overall lower light fraction compared to all groups from the checkerboard assay. This might be caused by the lane map being more easily learned by the fly, so that the individual can more easily stay in their preferred environment. Lane maps provide an unbounded

direction for flies to walk in and remain in the same visual stimulus condition, and might allow the fly to learn and navigate the borders more easily.

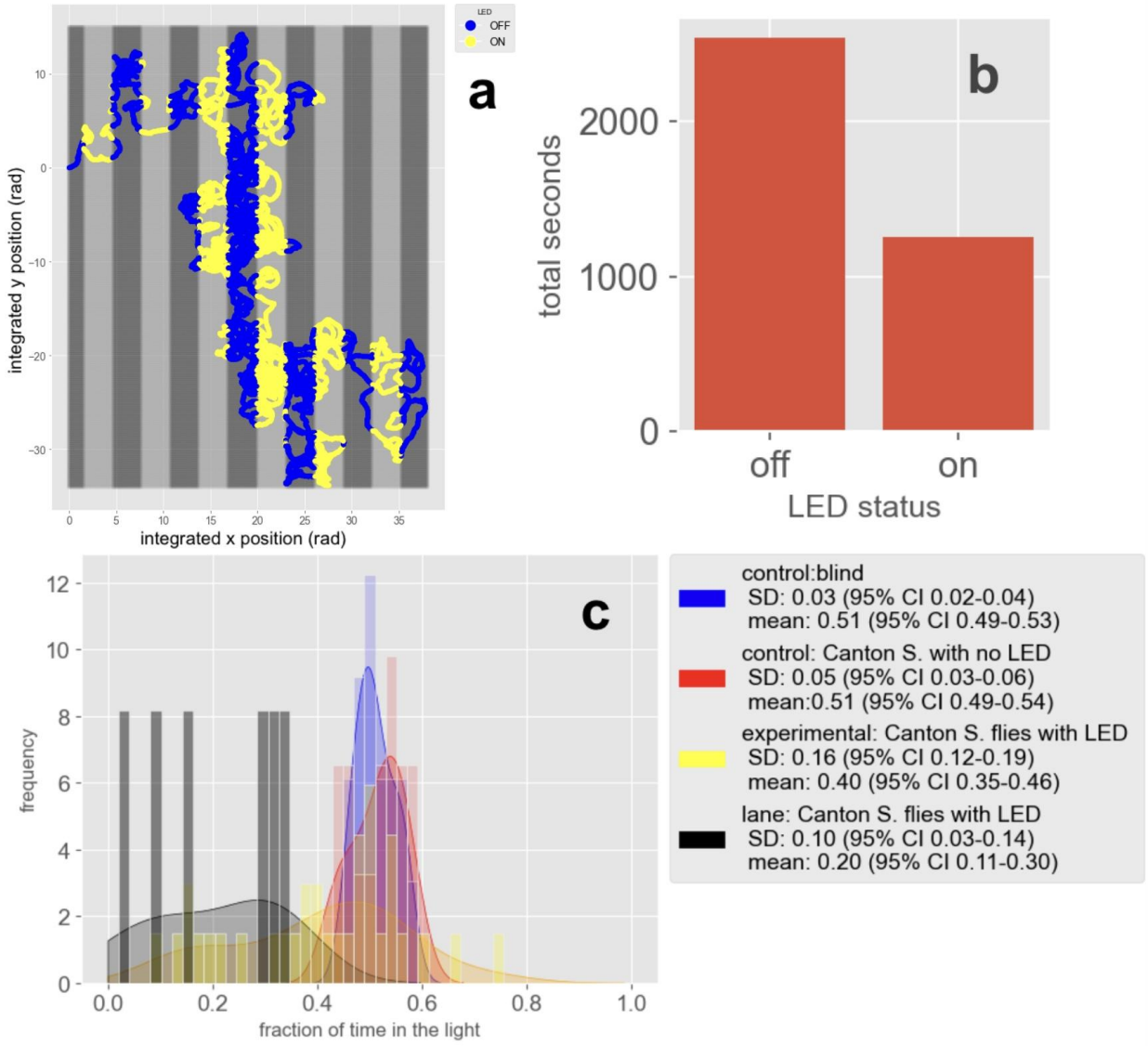


Figure 29: Lane map

(A) The path of the fly in a lane map, for an individual fly

(B) The LED status for an individual fly during the run

(C) Histogram for the fraction of time in light, for 6 lane flies, experimental group, and two control groups.

Individuality in odor preference behaviors in the fly-on-a-ball assay

For future directions, we hope to interface this assay with odor stimuli. Previous work in the lab has combined behavioral measurements with neural activity measurements by recording odor preference behaviors in individual flies and then recording calcium activity. Churgin et al. demonstrated that a fly's odor representations in the brain are predictive of its behavioral odor preferences (Churgin et al. 2021). The preference score considered in the previous work is a single number representing the fraction of time a fly spends in one half vs another in the odor tunnel assay. However, as seen in Figure 2, there is complexity in the behavior, as flies make varying choices across the odor boundaries, such as sampling the boundary and turning back, or walking straight through. An interest in the lab is to record calcium activity as the fly makes decisions moment-to-moment, which will require a fly on a ball assay. My assay is well-suited for this kind of project, because of the infrastructure it established for recording walking behavior and measuring individuality, and also because it is straightforward to change stimuli and accommodate neural activity recording. In particular, one can replace the needle mounting and glue a fly into a plate underneath a 2-photon microscope objective (an example is shown in Fig 30). Surgically cutting the cuticle enables optical access to the brain, without disrupting walking behavior, thus allowing for simultaneous walking behavior collection and neural activity recording by calcium imaging. Such a design has been implemented in numerous other studies, as seen in the methodology employed by Seelig et al. in their study (Seelig et al. 2010).

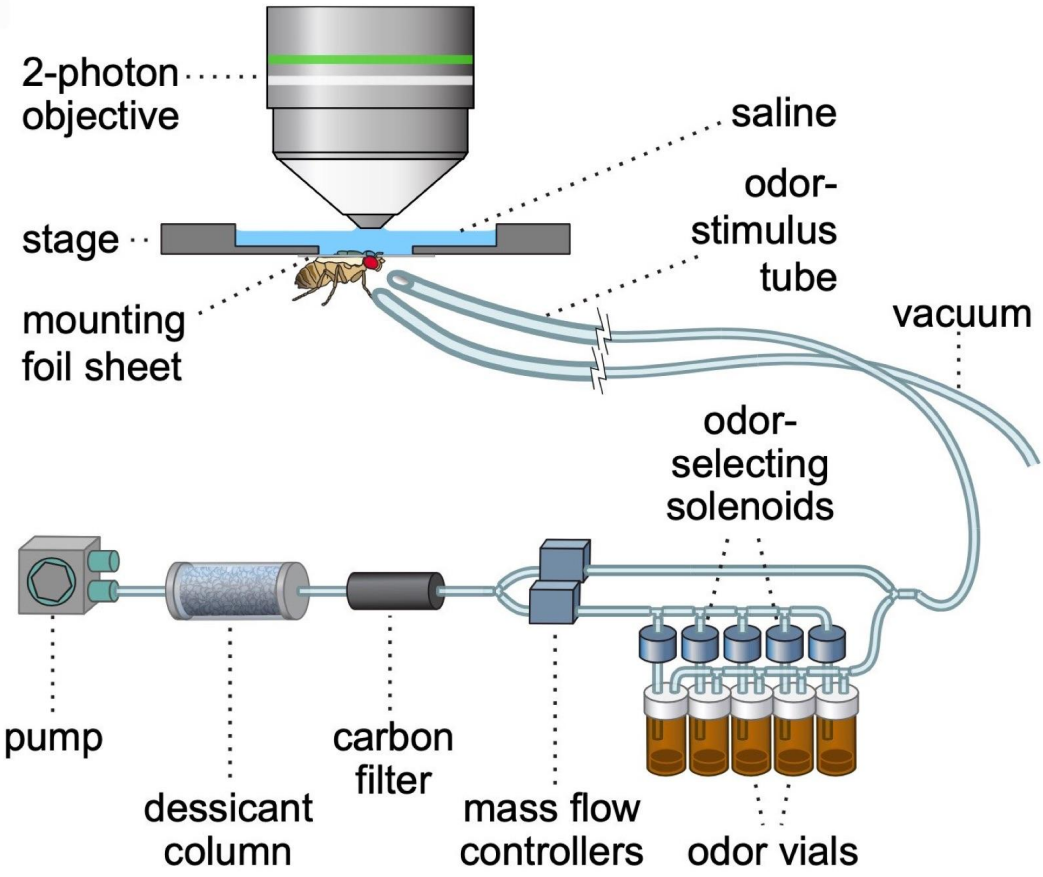


Figure 30: Calcium imaging setup for odor stimuli (taken from Churgin et al. 2021)

This figure illustrates a head-fixed calcium imaging setup with odor delivery. Behavior could be tracked simultaneously by recording motion on a ball with my assay.

Testing varying LED positions to investigate individuality signals in yaw

It is also informative to evaluate the impact of varying LED positions on fly behavior, as the current assay was conducted with the LED positioned directly above and in front of the fly. We hypothesize that altering the position of the LED could result in changes to the fly's path, angle transitions, and speed transitions. *Drosophila* navigates to patterns of linearly polarized light and to the position of the sun. The nervous system detects and processes sensory information to

control steering maneuvers, which allow them to navigate (Warren, Giraldo, and Dickinson 2019). Recent studies by Giraldo et al. showed that *Drosophila* uses the position of a simulated sun to fly straight (Giraldo et al. 2018). Their studies on tethered flies saw that flies adapted to the arbitrary headings set by the simulated sun stimulus. We therefore anticipate that flies might turn left if the LED were placed on that side, as they adapt the yaw signal in the direction of the light source. Further research incorporating LED stimuli positioned to the left and right of the fly would provide further insights into how *Drosophila* process and respond to visual stimuli. .

Conclusion

Our project set out to identify signals of individuality in tethered *Drosophila melanogaster,* on a fly-on-a-ball assay we built for behavioral analysis. We calculated several light preference metrics, including the light fraction, time spent in a box, speed transition, yaw transition, and pause fraction, and found significant signs of individuality through comparing correlation coefficients and standard deviations between these scores, and across metrics. Our results suggest that while individual flies display variable behavioral preferences in response to light stimuli, flies generally display photonegative tendencies. In addition, this light fraction is not caused by the fly's speed, but instead, its pauses in their preferred environment. Establishing phototactic individuality in this tethered fly assay will be helpful to further understand the visual and olfactory circuitry in *Drosophila*, and can extend to other organisms.

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