

Optimising virtual reality for visual behaviour experiments in tethered walking flies

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Work conducted under the guidance of

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Certificate

This is to certify that this dissertation titled "*Optimising virtual reality for visual behaviour experiments in tethered walking flies*" towards the partial fulfilment of the BS-MS dual degree program at the **Indian Institute of Science Education and Research, Pune** represents work carried out by **Omika Wadhwa** at the **Janelia Research Campus** under the supervision of **Dr. Michael Reiser**, Senior Group Leader during the academic year 2022-2023.



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Declaration

I hereby declare that the matter embodied in the report entitled “Optimising virtual reality for visual behaviour experiments in tethered walking flies” is the result of work carried out by me at the Janelia Research Campus of the Howard Hughes Medical Institute in Ashburn, VA, USA under the supervision of Dr. Michael Reiser and the same has not been submitted elsewhere for any other degree.



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Abstract

When shown a rotating grating pattern, *Drosophila melanogaster* moves in the direction of the pattern. This is known as the optomotor response. It is a robust response and has been observed over multiple experimental conditions. Here, I use the robust optomotor response to replicate the optomotor responses of the flies on an inexpensive fly-on-ball setup. I also characterise the intensity of the stimuli at which flies show optomotor responses on the setup. The characterisation of the setup increases the replicability of experiments by having multiple fly-on-ball setups using a low budget. Experimenters have used virtual reality environments to solve neuroscience problems. LED arenas and projectors have been used to show virtual reality environments to study behaviour. Here, I used a single tablet to stimulate flies visually and study their responses on the inexpensive setup. I attempted to optimise the virtual reality of two tablets to replicate optomotor experiments on the inexpensive fly-on-ball setup. This increases the field of view of the stimuli on the fly eye and opens up a repertoire of experiments that researchers can do on inexpensive setups to study visual circuitry. In the single tablet setup, I obtained symmetric responses in the case when I placed the tablet in the front and asymmetric responses when I placed it at the side of the fly. In the two-tablet setup, I obtained asymmetric responses where I expected symmetric responses and vice versa.

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Contributions

Contributor Name	Contributor Role
Michael Reiser	Conceptualisation ideas
Omika Wadhwa, Michael Reiser, Frank Loesche	Methodology
Frank Loesche, Omika Wadhwa	Software
Omika Wadhwa	Validation
Omika Wadhwa	Formal analysis
Omika Wadhwa	Investigation
Michael Reiser, Edward Rogers, Frank Loesche	Resources
Omika Wadhwa, Frank Loesche	Data Curation
Omika Wadhwa	Writing - original draft preparation
Omika Wadhwa, Frank Loesche	Writing - review and editing
Omika Wadhwa	Visualisation
Michael Reiser	Supervision
Michael Reiser	Project administration
Michael Reiser	Funding acquisitions

Table 1. List of contributors to the work presented in this thesis.

Introduction

Many animals when shown whole field rotating visual stimuli show a behavioural response known as the optomotor response (Götz, 1968). *Drosophila melanogaster*, with its wide genetic repertoire, serves as an ideal model organism to study the neural circuitry behind this response (Benzer, 1967). The optomotor response has been hypothesised to reduce the relative motion between the visual surround and the image formed on the eye in a stationary animal. This response also prevents the insect from unplanned perturbations in self-motion, such as gusts of wind or internal perturbations, such as bilaterally asymmetric motor output, and thus helps in course stabilisation (B. J. Duistermars et al., 2007; Theobald et al., 2010). For example, flies, when shown a progressive optic flow field, tend to move in the direction of the optic flow field. Accordingly, flies, when shown a rotating clockwise (counterclockwise) grating pattern, move clockwise (counterclockwise). This is a robust behaviour and has been extensively characterised in the past (Götz & Wenking, 1973).

The optomotor response has been studied in tethered animals. Tethering serves two advantages - one, it separates the response of the animal due to the visual component of the stimulus from the proprioceptive, vestibular one, and second, it allows the allocation of the visual stimulus onto the animal's retina with great precision (Borst et al., 2010). Experiments done by Hassenstein and Reichardt (1956) using the beetle *Chlorophanus viridis* walking on a spherical Y maze built with straws and measuring turning tendencies to moving visual stimuli led them to build the Hassenstein Reichardt correlator (HRC) model of elementary motion detectors (Hassenstein & Reichardt, 1956). Efforts have been made to find the neurobiological correlates of elementary motion detectors. Recent advances in microscopic techniques to image the activity of the neurons in the fly brain while it is shown visual stimuli have made it possible to identify the T4 and T5 neurons as the first substrate for direction computation in the fly visual system (Seelig et al., 2010). There is a whole field studying the neural substrates and models related to motion computation in the fly eye (Bahl et al., 2013; Gruntman et al., 2018, 2019, 2021; Maisak et al., 2013; Strother et al., 2014, 2017).

Anatomy of fly visual system

The compound eyes of insects consist of ommatidia, single hexagonal optical units of detection of light, that contain photoreceptors (Nérier & Desplan, 2016). There are two classes of photoreceptors in the fly visual system - the R1-6 class and the R7/R8 class of photoreceptors (Sharkey et al., 2020). The R1-6 class of photoreceptors are arranged at the outer end of the hexagon, while the R7/R8 photoreceptors are stacked up in the middle.

The R7 photoreceptor is located distal to the R8 photoreceptor. Due to the anatomical position of the receptors, the R8 photoreceptor receives ambient light through the R7 photoreceptor. Each photoreceptor contains as a photosensitive organelle an optically isolated rhabdomere which extends through the whole depth of the eye. Since the R7/R8 are stacked together, their rhabdomeres are arranged in tandem fashion with one another (Chou et al., 1996; Heisenberg & Wolf, 1984).

The visual system in the fly's brain has a special kind of wiring principle called the neural superposition principle, which is typical of cycloraphan flies (*Drosophila*, *Musca*, *Calliphora*). The neural superposition principle is the wiring principle where different photoreceptors from adjacent ommatidia that receive inputs from the same point in space converge onto similar synaptic units in the lamina called a "cartridge".

The pigment Rh1 found in photoreceptors R1-6 throughout the eye has two absorption peaks - one in the ultraviolet and the other in the green. Four other pigments with single absorption peaks are expressed in R7 or R8, forming a stochastic matrix for colour vision (Heisenberg & Wolf, 1984).

The resolving power of the movement detectors is determined by the angular spacing ($\Delta\Phi$) between the optical axes of neighbouring ommatidia. The angular spacing is different from the angular sensitivity of the ommatidia. The angular sensitivity of the visual elements is dependent upon the size of the rhabdomere tip and the optical properties of the cornea lens. The R1-6 photoreceptors have a larger angular sensitivity as compared to the R7/R8 photoreceptors due to the smaller distal endings of the R7/R8 rhabdomere as compared to the R1-6 rhabdomeres. This leads to distinct contrast functions for the R1-6 photoreceptors and the R7/R8 photoreceptors. The R1-6 photoreceptors mediate high sensitivity of the visual system, while the R7/R8 photoreceptors mediate high acuity of the visual system (Heisenberg & Wolf, 1984).

Due to this dichotomy, it was earlier assumed that at very low light levels, optomotor reactions were mediated predominantly by retinula cells R1-6 if low spatial frequency patterns (broad stripes) were used for stimulation (Kirschfeld, 1972). Responses to sufficiently regular and narrow gratings were hypothesised to be mediated by R7/R8 retinula cells due to their better contrast transfer properties. This was known as the “two subsystems hypothesis” (Eckert, 1973; Götz, 1964). However, this hypothesis was later falsified by experiments done by Martin Heisenberg and Erich Buchner (Heisenberg & Buchner, 1977).

Anatomy of the fly optic lobe

The fly optic lobe consists of four neuropils - lamina, medulla, lobula and lobula plate. The columnar structure of these layers is similar to the columnar structure of the retina in mammals (Fischbach & Dittrich, 1989). This adheres to retinotopy, the principle where neighbouring points in space are processed by neighbouring facets in the eye and represented by neighbouring columns in the brain. The axons from photoreceptors R1-6 terminate in the lamina while the axons from photoreceptors R7 and R8 terminate in the medulla. At the lobula plate level, neurons sensitive to motion and optic flow are found. These are the lobula plate tangential cells. The horizontal (HS) and vertical (VS) system cells of the lobula plate tangential cells have been found to be essential in the computation of directional motion (Borst & Haag, 2002).

There are two main chiasms in the optic lobe. The first one occurs between the lamina and the medulla, and this causes an inversion of the front-to-back retinotopic map between the lamina and the medulla. The second optic chiasm is between the medulla, lobula and lobula plates.

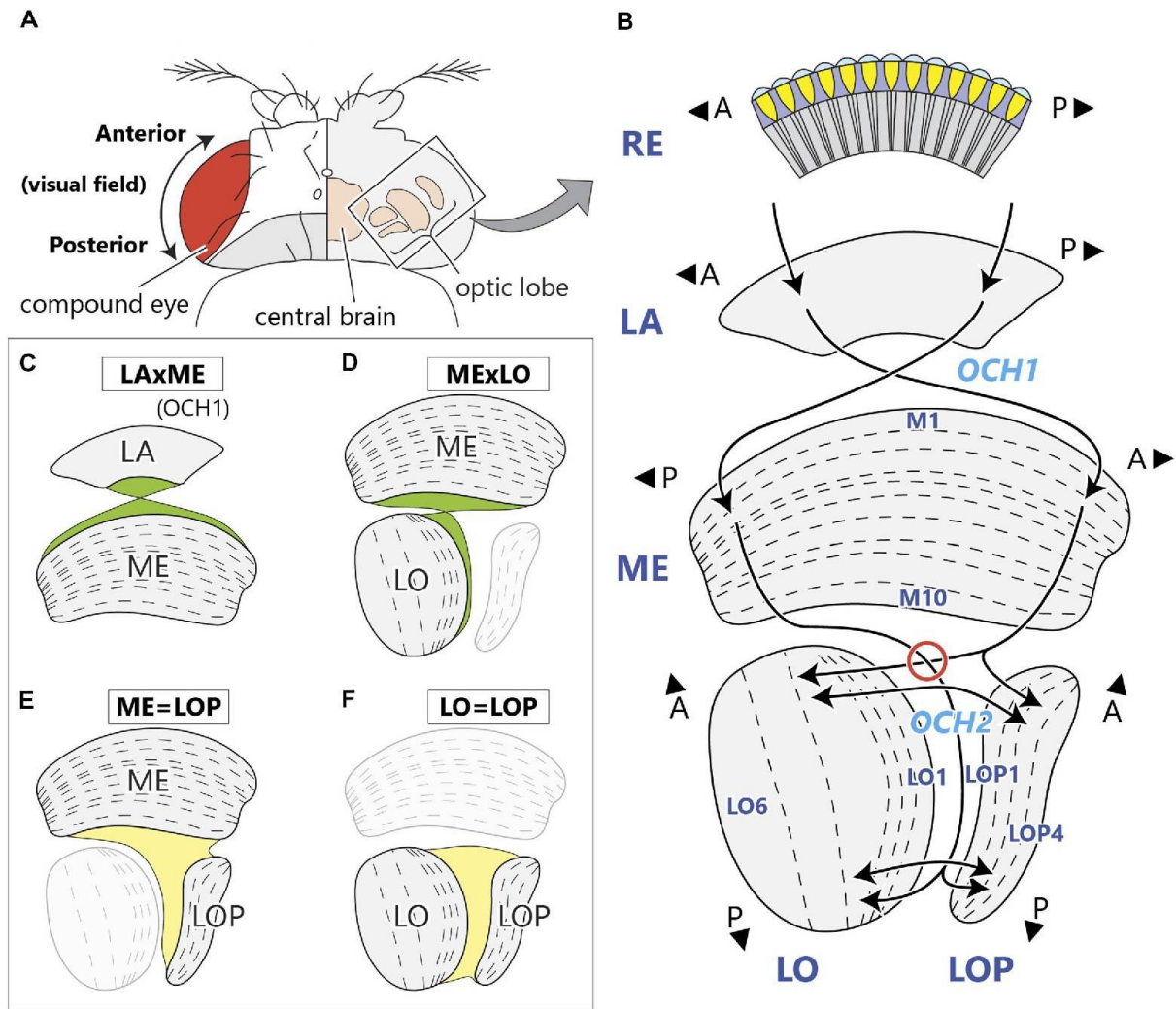


Figure 1. Anatomy of the optic neuropils and their chiasmata.

(A) Dorsal view of the fly's head and the optic lobe. The optic lobe forms a large part of the fly's brain. It is located just beneath the retina of the compound eye.

(B) The retina and optic lobe and the topological projection patterns between the neuropils. Four stratified neuropils of the optic lobe: the lamina (LA), medulla (ME), lobula (LO) and the lobula plate (LOP). The outermost retina (RE) is home to clusters of photoreceptors located in the ommatidia, that project directly to the lamina. OCH1 is the first optic chiasm and represents the inversion of the antero-posterior (A-P) retinotopic axis between the lamina and the medulla. The second optic chiasm (OCH2) relays information from the medulla to the proximal part of the lobula and the lobula plate. **(C-F) Projection between neuropils.** (C) Chiasm OCH1 between LA and ME, (D) OCH2 between ME and LO, (E) Straight projection between ME and LOP, (F) Straight projection between LO and LOP. Figure and text adapted from (Shinomiya et al., 2019)

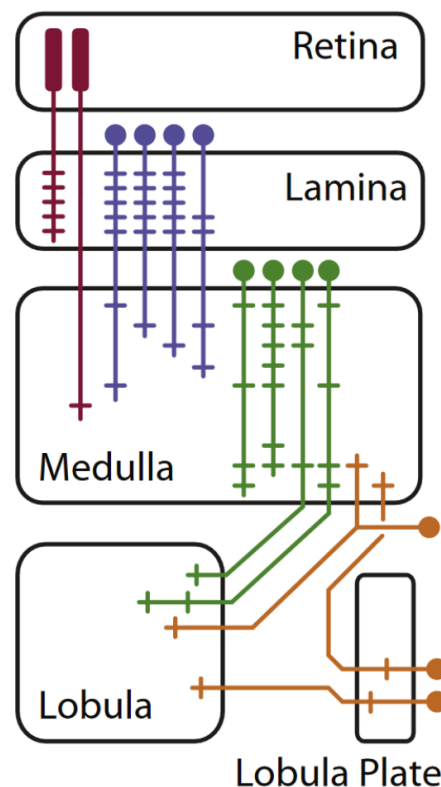


Figure 2. Columnar representation of the fly optic lobe and retina.

The cell body of representative columnar neuron types is indicated by a disc, while short bars represent the main arborisation. Red represents the photoreceptors, blue represents the lamina monopolar cells, green represents the medulla intrinsic neurons and transmedullary neurons, while orange represents the T cells. Figure obtained from Michael Reiser.

Experiments done with tethered walking flies to study the optomotor response

The optomotor response has been studied in walking flies. Using a tread compensator to keep flies in place, the optomotor response was initially studied using behavioural mutants like *white apricot* (w_a), *ebony* and *opm*. It has been observed that fruit flies reverse their optomotor response at spatial periods lying between Φ and 2Φ (Götz & Wenking, 1973). There are two types of optomotor responses - the translatory optomotor response and the rotatory optomotor response. The translatory optomotor response is evoked when a fly is shown

progressive (front to back) or regressive motion (back to front), while the rotatory optomotor response is evoked when a fly is shown rotating gratings. The response to both of these optic flow fields is similar in magnitude, but opposite in direction.

In fact, walking speed has been shown to be controlled by the visual signal and optic flow generated by this visual signal (Creamer et al., 2018). Recently, the The HS cells of the lobula plate tangential cells (LPTCs) have been shown to be involved in the selection of directional selectivity in tethered walking flies (Busch et al., 2018; Chiappe et al., 2010).

Using computer science to solve neuroscience problems

Recent advancements in the field of neuroscience have incorporated machine learning and computer vision techniques to quantify behaviour of the animal. These include tools to track flies and classify behaviours (Branson et al., 2009; Kabra et al., 2013). These advancements have pointed towards the advent of an ethomics era in the field of neuroscience (M. Reiser, 2009).

Virtual reality environments have been used in order to study optomotor responses (B. Duistermars et al., 2012; Robinson, 1981), object fixation (Green et al., 2019; Mongeau & Frye, 2017), landing behaviours (Schuster et al., 2002; Tammero & Dickinson, 2002b), and a lot of other behaviours. Virtual reality environments simulate real environments presented to animals while they are head restrained (Dombeck & Reiser, 2012). This is advantageous for the dissection of neural circuits underlying animal behaviour because it allows for the imaging of neural activity in the brain and making causal inferences with respect to behaviour.

Behaviour is studied both in open loop as well as closed loop. An open loop environment is one where the stimulus is completely decoupled from the movement of the animal. A closed loop environment is where the stimulus changes with respect to the response of the animal. Closed loop environments are especially useful in studying object fixation behaviours as well as menotaxis. Controlling the feedback gain in closed loop environments also provides more control to the repertoire of experiments that can be done by the experimenter in order to perturb the behaviour

of the animal by providing unnatural stimuli and understand its underlying neural circuitry.

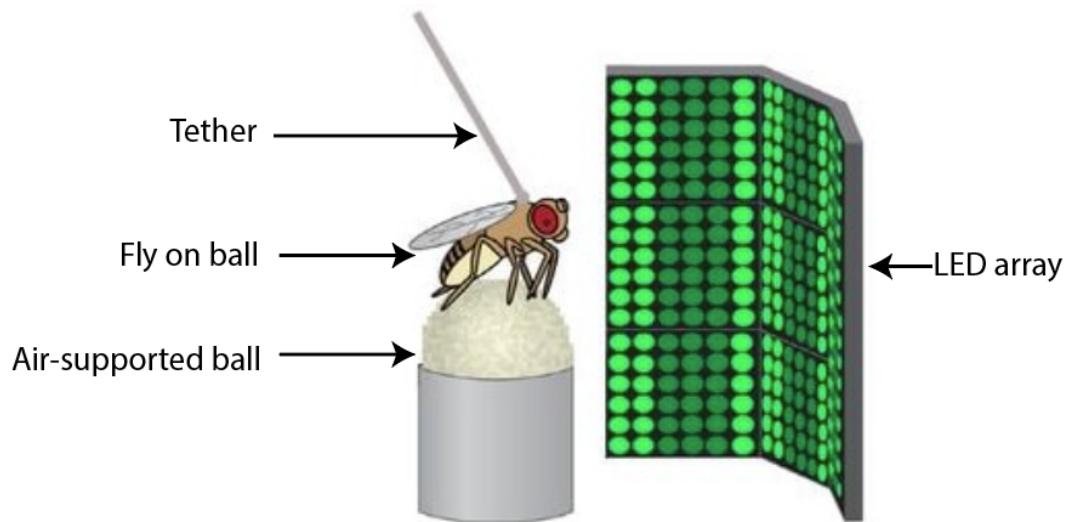


Figure 3. LED arrays for fly-on-ball experiments. *Figure adapted from (Strother et al., 2018), reproduced with permission from Elsevier (attached as a supplement).*

Virtual reality environments have been simulated using LED arrays (M. B. Reiser & Dickinson, 2008) with the fly walking on a ball (Figure 3) (Strother et al., 2018). However, the cost of these fly-on-ball setups is around 16000 USD (Loesche & Reiser, 2021) per setup, and oftentimes, only a few setups can be used in labs with high funding. In order to increase reproducibility, an inexpensive fly-on-ball setup has been built by Frank Loesche (Loesche & Reiser, 2021). This setup uses 3D printed components and tablets in order to show stimuli to the animal. However, tablets are tuned to the human eye and not the fly eye. Also, the spectrum of wavelengths of light emitted by LEDs is much smaller than the spectrum of light emitted by the tablets in order to generate a certain colour. This gives us more control over the exact colour of the visual stimuli being displayed to the fly in an LED arena than a display which uses tablets.

In the cylindrical LED arenas, one has control over the rotational and the translational component of the optomotor response along with coverage of the lateral and posterior parts of the fly eye which receive which stimulus. However, such type of control is limited by the flat screen of the tablet, which can only simulate circular motion of the stimuli in the anterior part of the fly eye through a program. It is currently not used to stimulate the lateral and the posterior regions of the fly eye.

These issues bring up the question of validation of the setup using the tablets. The tablets have been validated in a previous study (Loesche & Reiser, 2021). Here, I reproduced the optomotor response of the fly on the inexpensive fly-on-ball setup. I also built a binary classifier to discriminate between “walking” and “not walking” behaviours of the fly. Then, I replicated the optomotor response on the inexpensive fly-on-ball setup using open loop stimuli. I further performed intensity analysis by showing gratings of increasing intensity of the fly in order to characterise the threshold at which the fly shows optomotor responses on the inexpensive setup. After this, I wanted to increase the field of view of stimuli that one can show to the fly using tablets. This requires the use of two tablets in synchrony displaying visual stimuli. In order to achieve this goal, I first looked at responses to a tablet placed horizontally in front of the fly and at an angle of 45 degree with respect to the vertical axis (Figure 13). I then looked at responses of the fly after placing two tablets at 90 degrees to each other in front of the fly. While the single tablet experiments gave results as expected for the tablets in the two positions, the two tablet experiments were a little weird. Optimisation of the two tablet setup should increase the field of view of visual stimuli shown to the fly and increase the repertoire of optomotor experiments done on the fly.

Materials and Methods

The Inexpensive Setup

I used an inexpensive fly-on-ball setup. It is made out of 3D printed components and readily available consumer products (Loesche & Reiser, 2021). It consists of a 3D printed micromanipulator arm to hold the tether in place, a micromanipulator in order to position the tether in place, and an omnidirectional treadmill (sphere). Air supports the sphere. The spheres are made of foam and are cut to 9 mm in diameter. The spheres need to be nearly perfect in shape, not too smooth, light enough to float via the air, but not so light that the flies can pick them up. The spheres were cut from Last-A-Foam FR-7120 (General Plastics Manufacturing Company, Tacoma, WA, USA). In order to optically track the sphere with near-infrared (NIR) illumination, I paint the NIR reflective foam of the sphere with BLK 3.0 paint (Stuart Semple Studio, Dorset, UK). This provides high contrast features of the ball. I illuminate the ball using near infrared light. The camera used is the Flir Firefly S FFy-U3-042SM-S camera (FLIR Integrated Imaging Solutions, Inc.). I then feed the video of the ball moving into FicTrac, the software used to measure animal movement on the ball.

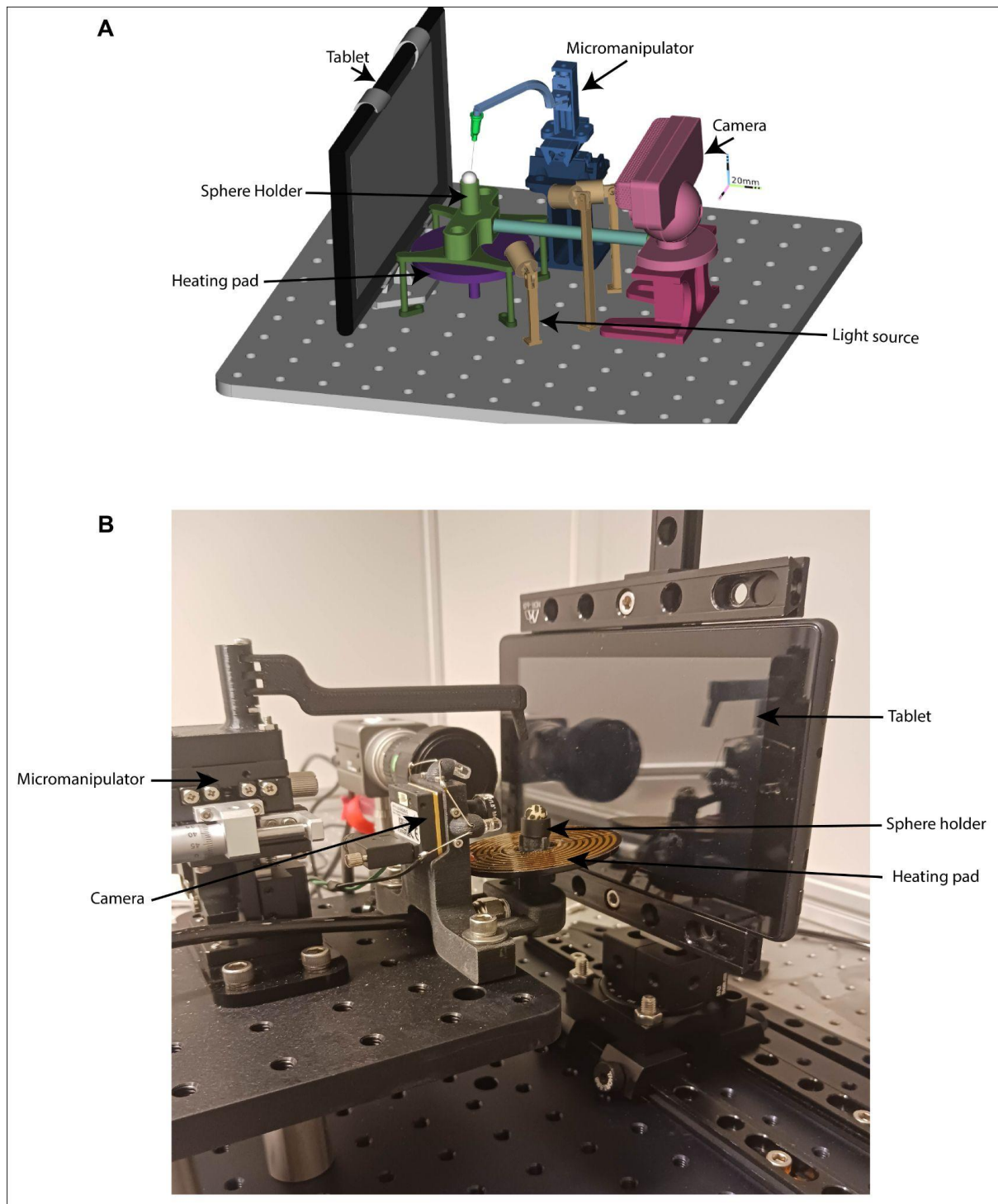


Figure 4. The inexpensive fly-on-ball setup.

(A) Schematic of the setup. Adapted from (Loesche & Reiser, 2021).

(B) Picture of the setup.

Measurement of animal behaviour

FicTrac (Moore et al., 2014) is a software that estimates tethered animals' fictive walking tracks in an infinite two dimensional plane while they are fixed in space and walking on a rotating sphere. Using a single video stream as input, FicTrac derives several measurements of the movement, for example, the trajectory, heading direction, and speed. To achieve this, the software generates a map of the unique patterns on the sphere. It then matches the visible pattern from each frame to the map to estimate the orientation of the sphere. The change of sphere rotation over time allows to record the fly trajectory and heading direction.

Calibration of FicTrac

In order to accurately measure the animal movement, FicTrac needs to be correctly calibrated such that it gives an accurate output of reality. Calibration of FicTrac depends on various parameters, including:

1. The region of interest of the camera image which contains the ball
2. The regions within the camera frame to be ignored during tracking via FicTrac
3. Body axis reference: Used in order to convert the coordinates from the camera's coordinates to the coordinates with respect to the body axis of the animal. These are four dots that mark a virtual square aligned with the animals' axes and determine the transformation between the camera angle and the animal movement.
4. Vertical field of view: The angle made by the region of interest onto the camera (Figure 5).

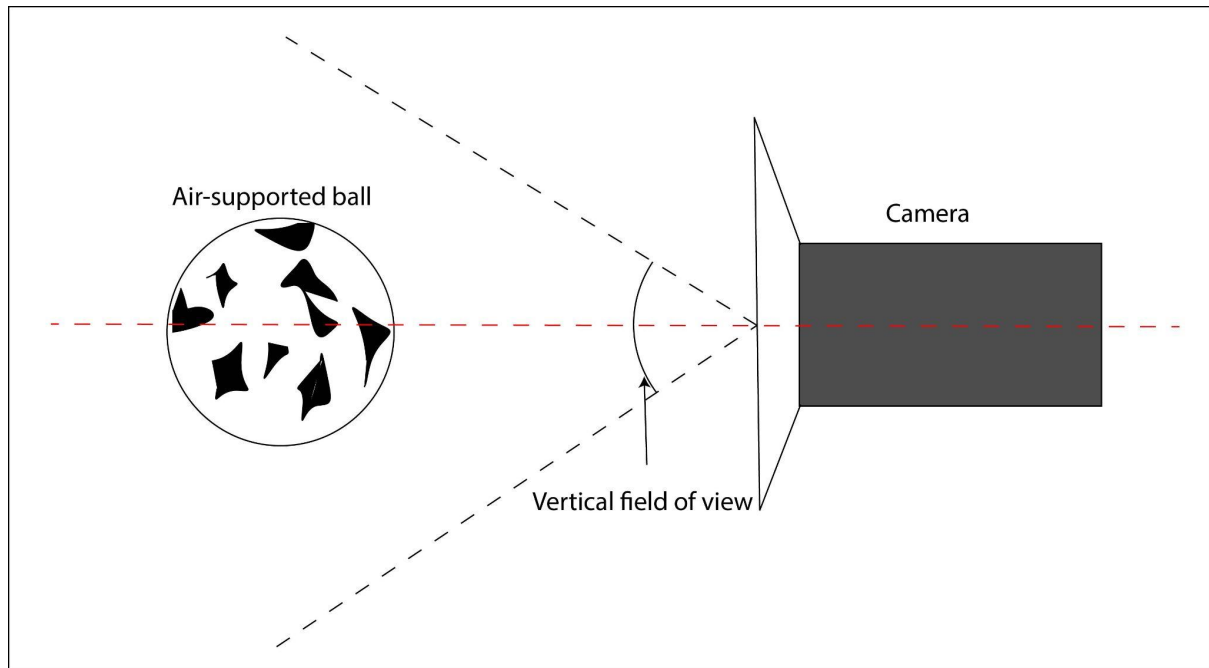


Figure 5. Vertical field of view.

Definition of the vertical field of view, as the angle made by the camera frame on the camera.

I optimised two parameters of this problem here in order to configure FicTrac correctly: the body axis reference and vertical field of view.

In order to optimise the body axis reference, I built an alternative setup similar to the experimental setup. I controlled the ball rotation using a servo motor, whereby it could rotate along only one of the cardinal axes in space - roll, pitch, or yaw axis. I then plotted the recorded FicTrac movement for different FicTrac parameters (Figure 6). I found that FicTrac is very sensitive to the body axis reference parameter and small changes in the parameter result in large changes in output.

Rotation around the Roll Axis

The sphere rotates around the roll axis when the tethered animal moves sideways in a straight line without any forward movement or change in orientation. If configured correctly, FicTrac should show a straight line parallel to the Y axis in the trajectory plot and not change for the heading direction. In Figure 6(A), the trajectory does not match these expectations. Figure 6(B) shows measurements close to the expected

output of FicTrac, with the trajectory almost parallel to the Y axis and little change in heading direction.

Rotation around the Pitch Axis

The expectation is that the plot shows forward-backward movement with no change in heading direction. In Figure 6(C), the output of FicTrac closely matches the expectations.

Rotation around the Yaw axis

When the virtual fly turns around its yaw axis, there should be no movement in the trajectory plot, but the heading direction should change. In Figure 6(D), there is very little distance moved by the fly as indicated by its trajectory, but a large change in heading direction.

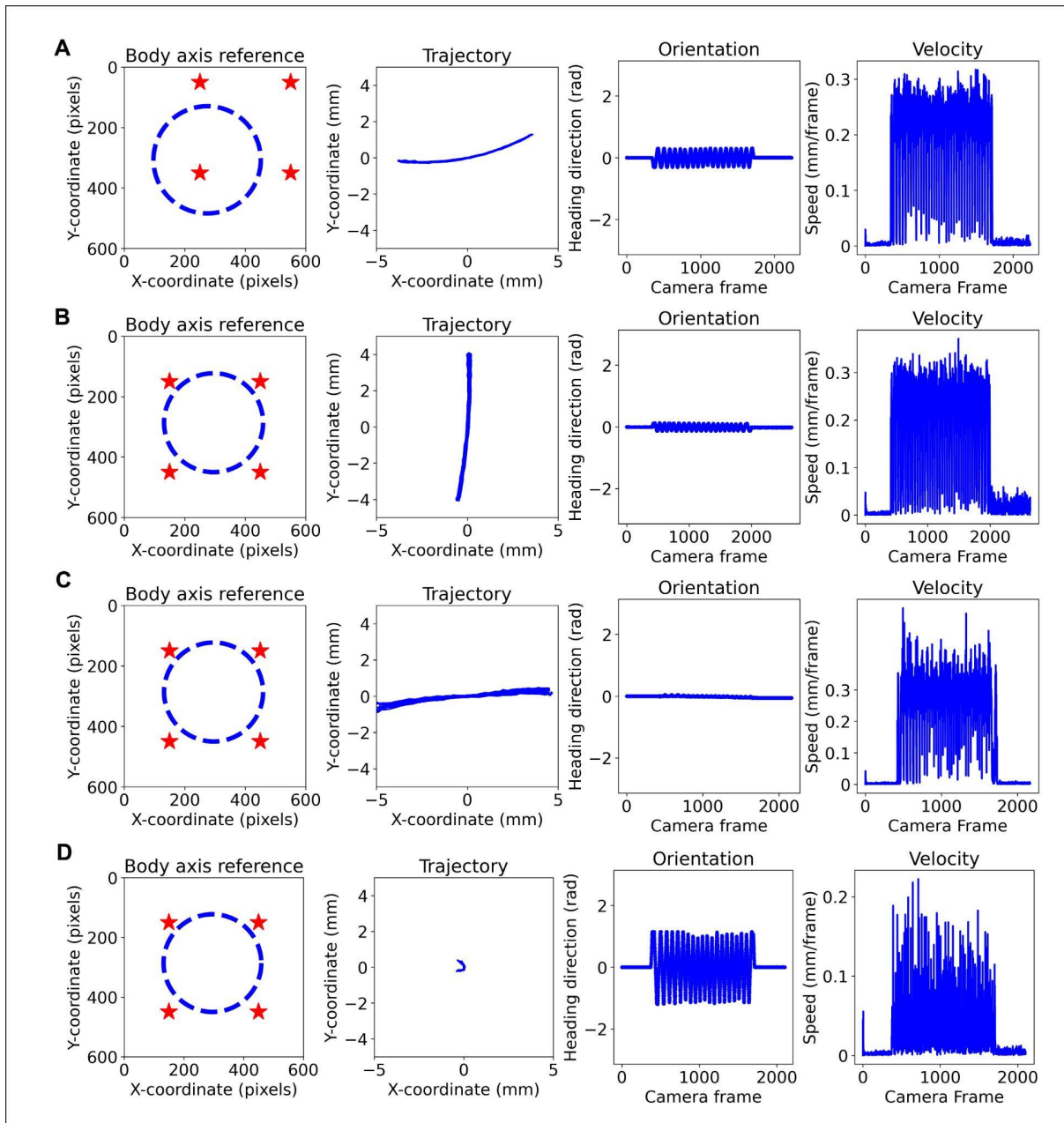


Figure 6. Calibration of FicTrac by plotting its output for different body axis references.

(A) Incorrect configuration of FicTrac causes Incorrect responses of FicTrac when the sphere rotates around the roll axis.

(B) Correct configuration of FicTrac causes correct responses of FicTrac when the sphere rotates around the roll axis

(C) Correct responses of FicTrac when the sphere rotates around the pitch axis

(D) Correct responses of FicTrac when the sphere rotates around the yaw axis

The next FicTrac parameter of interest is the vertical field of view.

I measured the accuracy of the parameter using the delta rotation error score of the output of FicTrac. FicTrac logs the delta rotation error score at each camera frame. I then plot the mean of the delta rotation error score of each pre-recorded video along different vertical field of views, and obtained a “tuning curve” of the vertical field of views (Figure 7). I found the optimal vertical field of view to be 43 degrees. I recorded 5 different videos of the fly walking on the ball, and performed offline tracking of these videos with different vertical fields of view in order to plot the tuning curve. If FicTrac incorrectly tracked the ball, then that would give rise to a higher delta rotation error score, than when FicTrac correctly tracked the ball.

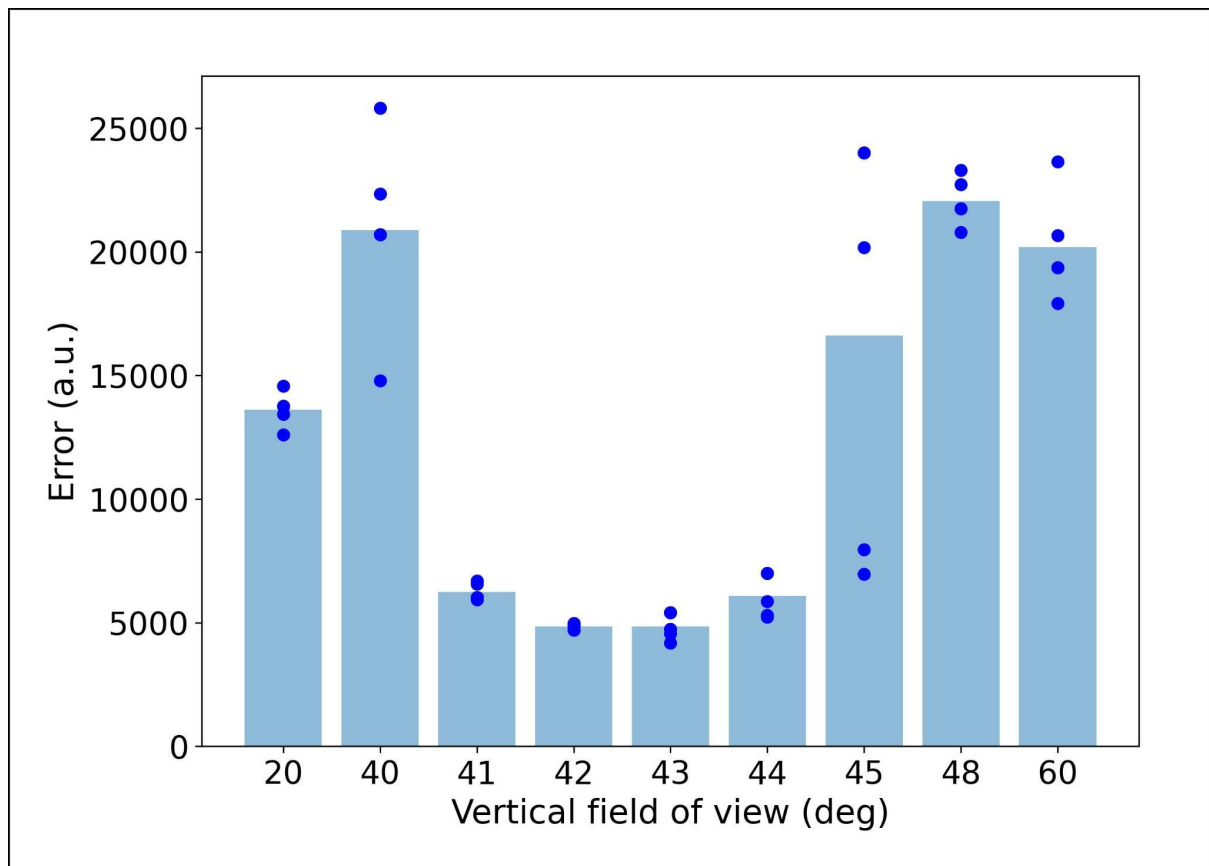


Figure 7. Optimisation of the vertical field of view.

The plot shows the mean error against the vertical field of view. The mean error reaches a minimum when the vertical field of view equals 43 degrees. The units of error on the y axis indicate arbitrary units (a.u.).

Fly rearing and stocks

I used *Drosophila melanogaster* of the wild type strain Dickinson Lab (DL) (Tammero & Dickinson, 2002a) for the experiments. I regularly flipped the fly stocks in order to prevent overcrowding and contamination of mites. I reared the flies in plastic vials at Janelia Research Campus containing standard cornmeal media. In order to maintain a record of the age of the flies, I flipped new flies out of the stock vial every 24 hours. I reared the flies on standard Janelia cornmeal food. I used unstarved and starved flies in Protocol 1. In contrast, I used only starved flies for the rest of the Protocols 2-5. In the case of using starved flies, I placed them in starvation media containing nutrient free 1% agarose to prevent desiccation. I starved the flies for 2 to 6 hours before the experiment. I reared the flies in an incubator, whose light was set to 16 hour day and 8 hour night cycle for the flies. Frank set the conditions of humidity of the incubator to be 65% and the temperature at 22°C.

Animal Preparation and Tethering

I used 3 to 6 day old female flies for the experiments. I used female flies because they are bigger in size and easier to tether than male flies. I cold anaesthetised the flies below 4°C, and then separated them based on sex. I then tethered the flies at a tethering station. The tethering station consists of a sarcophagus (a cavity on the cooling plate) to place the fly (Figure 8), and a micromanipulator to place the tether on the fly at the appropriate angle of 90 degrees between the tether and the anteroposterior axis of the body of the fly. I attached the tether to the thorax of the fly using resins that polymerise upon UV illumination, converting from liquid to solid within seconds. The glue used in the lab is KOA 300 (Kemxert, Poly-Lite, York, PA, USA) that requires UV light to cure. I recorded the time of anaesthetisation and waking up of the fly. I then took the flies to the behaviour room and placed them successively on the ball. I then followed the protocol of the respective experiment to collect data from the fly.

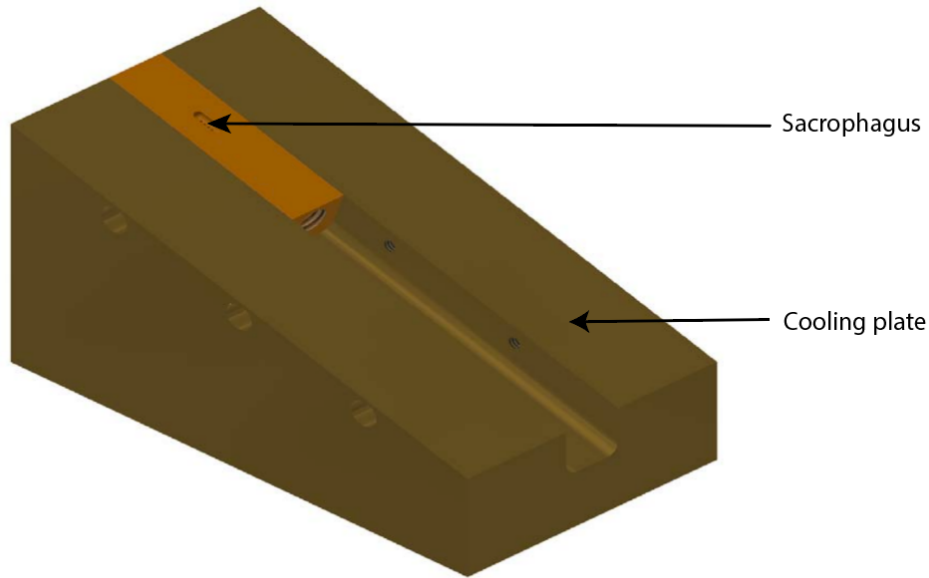


Figure 8. The cooling plate and sarcophagus. *Figure adapted from (Component Design, n.d.).*

Behaviour experiments

I performed all experiments in a dark box whose temperature was between 24°C and 26°C. The air conditioning in the room maintained its humidity at 18-19%. I kept the target temperature of the ball at 32°C in order to increase fly walking behaviours. A closed-loop system maintained the target temperature that detected the current temperature of the ball and either heated it or decreased its temperature to the target by stopping heating of the ball. Flies walk more often and faster at elevated temperatures. Flies walking at higher temperatures, below 35°C, which is considered noxious for the flies, have similar walking behaviours as compared to flies walking at room temperature, except for the fact that they walk more consistently and do not pause as often to groom (Huey et al., 1992; Soto-Padilla et al., 2018). In case of using starved flies, I starved them for 2 to 6 hours before the experiments. I conducted all the experiments with the flies enclosed in a box, in order to prevent light from external sources from confounding the experimental results.

Protocol 1: Data analysis of walking behaviours

Flies walked on the ball for 20 minutes without the display of a visual stimulus. The objective was to measure the walking behaviour of the fly. I built a binary classifier

over the displacement in order to discriminate between “walking” and “not walking” behaviours. I used x and y coordinate data from FicTrac to calculate displacements between consecutive frames. If plotted as a histogram, the data was highly skewed towards very low values close to zero, and it was hard to define a boundary between “walking” and “not walking” due to an unclear difference in displacements between “walking” and “not walking”. I determined that calculating displacements over a sliding window would be useful to classify between “walking” and “not walking”. This smoothed the histogram and provided a gentler transition from lower to higher values of displacement. A very small length of the sliding window (200 ms) would give rise to the problem just highlighted. However, a very large sliding window (1000 ms) would over smooth the data and classify turns made by the fly as false negatives when it came to looking at walking behaviours (Figure 10). Hence, I used a sweet spot between these two extremes and used a sliding window of 600 ms in order to classify the behaviour of the fly as “walking” or “not walking”. I collected data from 6 flies, and calculated the median of the displacements. I used a percentage of the median as a threshold to classify “walking” from “not walking”. A very small threshold of 5 percent would give rise to false positives of walking behaviour, while a very large threshold of 45 percent would give rise to false negatives (Figure 9). After using different values of the threshold, I determined this sweet spot between the two extremes to be 30 percent of the median. The program used this as a threshold to classify walking behaviours from not walking behaviours. The program binary classified each frame using this threshold into walking or not walking based on the sliding window it was associated with. A schematic of this process is shown in Figure 9. I then calculated the percentage of walking.

$$\text{Percentage of walking} = \text{Number of frames where fly walked} / \text{Total number of frames}$$

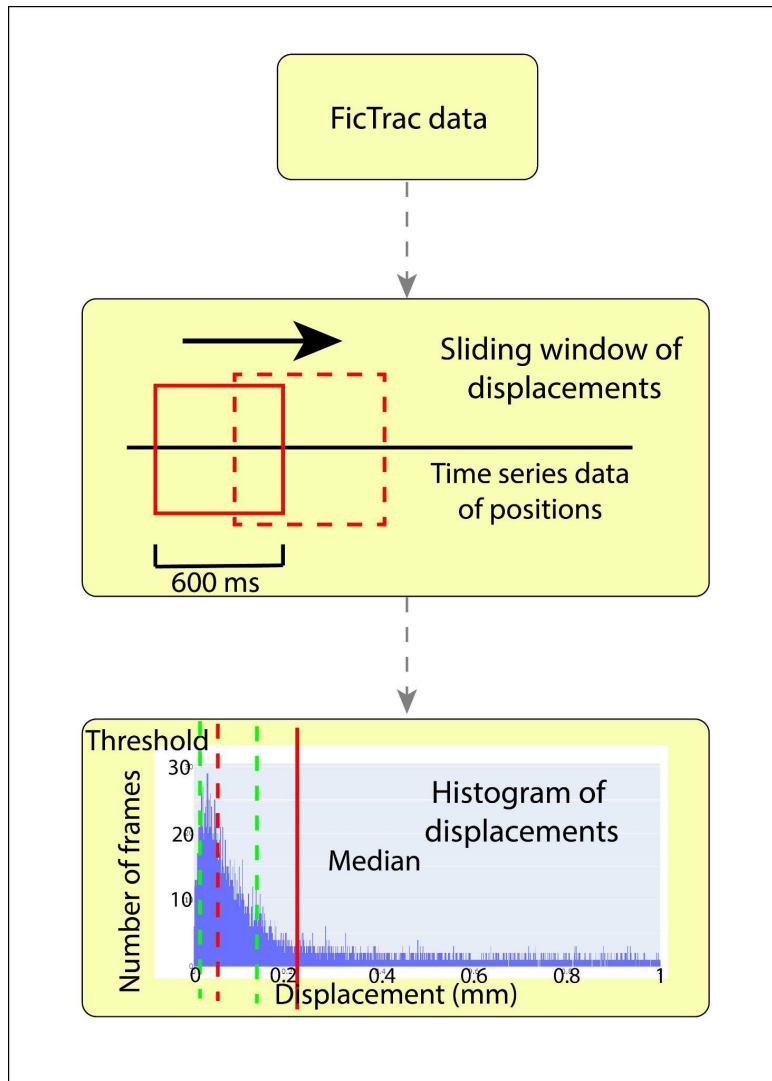


Figure 9. The data analysis pipeline.

FicTrac (top) is the software that measures animals' trajectories while they walk on a ball. A sliding window of 600 ms (middle) smoothes FicTrac data, and the algorithm calculates the histogram of displacements (bottom). The classifier uses a threshold of 30% of the median (red dashed line) to classify "walking" and "not walking" behaviours. The green dashed line on the left represents the threshold if the classifier uses 5% of the median to classify behaviours, and the green dashed line on the right represents the threshold if the classifier uses 45% of the median as the threshold.

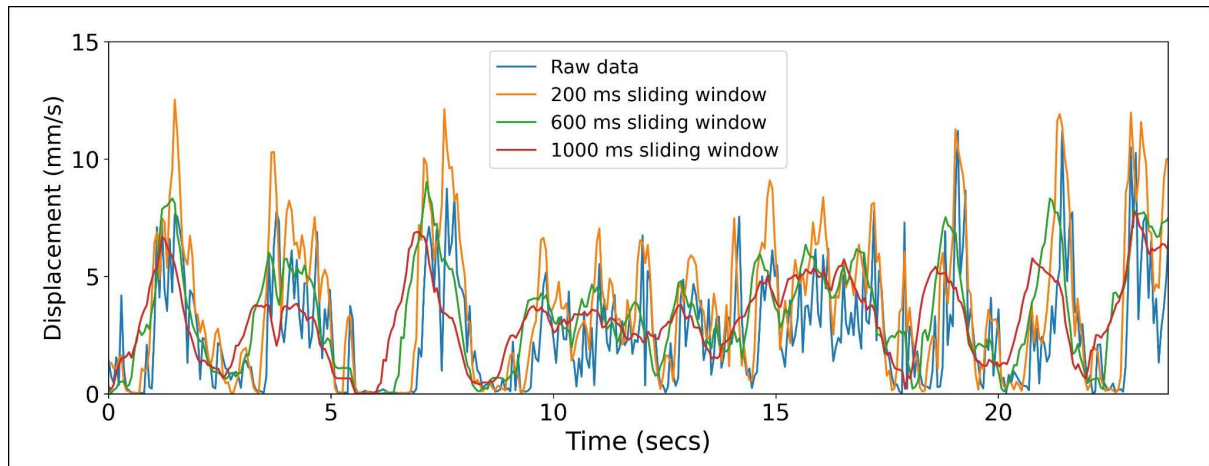


Figure 10. Different sliding windows used to smoothe the data.

Displacement smoothed over three different sliding windows. A 1000 ms sliding window over-smoothes the data, while a 200 ms sliding window under-smoothes the data.

Statistical analyses of walking behaviours

I used a non-parametric test, the Wilcoxon rank sum test, to determine whether starved flies walked more as compared to non-starved flies. I then used the scipy.stats library (Virtanen et al., 2020) to run the Wilcoxon rank sum test.

Display of visual stimuli to the fly

The software used to display the stimulus to the fly is called FlyFlix (Loesche & Reiser, 2021). It uses Flask-SocketIO communication between a client and a server in order to display visual stimuli via the Amazon tablets. FlyFlix uses the JavaScript client ThreeJS to render the visual stimuli onto the tablet. FlyFlix also logs the data of the stimulus condition in a comma-separated value (CSV) file. The experimenter runs FicTrac simultaneously in order to measure the movements of the animal while FlyFlix presents the stimulus. FlyFlix and FicTrac communicate via a network socket in order to synchronise the movement data of the animal while presenting the stimulus.

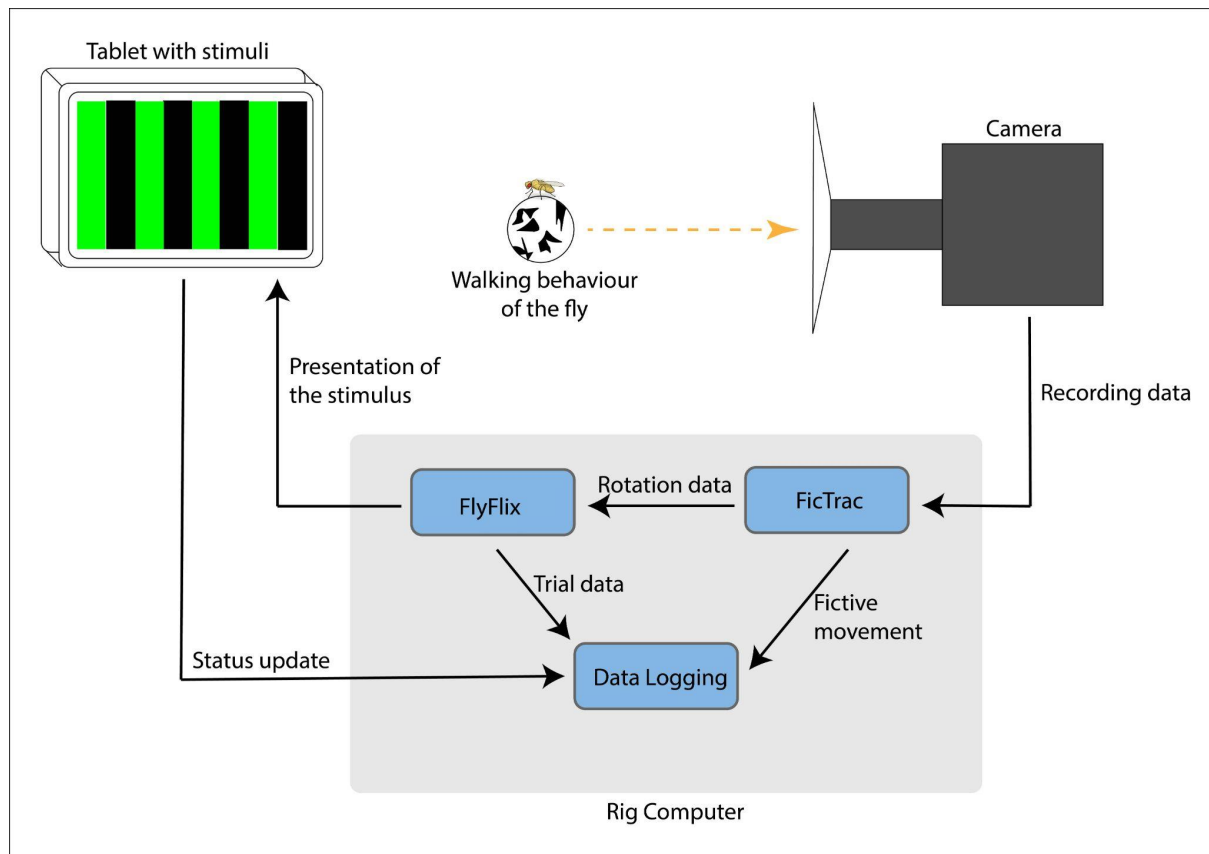


Figure 11. Schematic representation of the working setup.

Communication between FicTrac and FlyFlix is shown.

Definitions used within the protocols

Protocol

A protocol is a system of rules used to generate visual stimuli shown to the fly. Protocols use the terms listed below through their definition.

Spatial period

The angle of the dark and the light gratings as seen from the perspective of the fly. For example, the angle of the light gratings is 30 degrees, and the angle of the dark gratings is 30 degrees.

Total spatial period

The total spatial period is the sum of the angles of the dark and light gratings. In all the experiments, I set it to be 60 degrees.

Temporal frequency

The ratio of the velocity of the stimulus and the spatial period of the pattern being shown. This is measured in Hertz. It usually represents the speed of stimulus movement. For example, the temporal frequency can be 4 Hz. This means that 4 cycles of gratings moves across the screen in one second.

Brightness/Intensity

The intended brightness of the stimulus is a number between 0 and 255 for a given colour, 0 being the dimmest and 255 being the brightest.

Condition

The specific set of stimuli shown to the fly. It defines a set of temporal frequency, spatial period, direction, intensity and colour of the stimulus. FlyFlix usually repeats the same condition many times in an experiment. For example, “Colour: Green, Brightness: 255, Temporal frequency: 4 Hz, Spatial period: 30 deg, clockwise (CW)” would define a condition.

Trial

A single repetition of a single condition. A trial is a part of an experiment, and lasts say, for 8 seconds.

Pre-trial

Stimulus shown before the trial. The stimulus is static in this case, and lasts for say, 1 second.

Post-trial

Stimulus shown after the trial. The stimulus is static in this case, and lasts for say, 1 second.

Experiment

An experiment is a series of trials run for different repetitions. FlyFlix uses a block system of trials, where it stores each condition as a block, and then randomises this to produce the output and show visual stimuli to the fly.

A condition is the particular set of temporal frequency, spatial period, direction and colour of the stimulus. When it is executed by FlyFlix for a single repetition, it is called a “trial”. The static visual stimuli shown right before and after a single trial is the pre-trial and post-trial stimulus respectively. Their duration is encoded in the software and is called the “pre-trial duration” and “post-trial duration” respectively. Different trials fall into different conditions, based on their temporal frequency, spatial period, direction and colour. An experiment runs trials over multiple repetitions.

Protocol 2: Visual stimuli shown to the fly

I considered specific properties of gratings while building the protocol. The colour of the gratings was either green or white, on a black background. The brightness of the gratings was either 50 or 255 for both the colours. The temporal frequency was 5 Hz for all brightness conditions. I used an additional temporal frequency of 0 Hz for the green coloured gratings of brightness 50. The spatial period of the gratings was 30°. The gratings moved either in a clockwise (CW) or counterclockwise (CCW) direction. The Michelson contrast of all the stimuli was equal to 1.

$$\text{Michelson contrast} = (I_{\max} - I_{\min}) / (I_{\max} + I_{\min})$$

Where I is the intended brightness of the stimulus.

I used a spatial period of 30 degrees in all the conditions - 30 degrees of coloured stripes and 30 degrees of black stripes.

Protocol 2 gives rise to a list of 10 stimulus conditions, (2 directions, 2 brightness values, 2 colours = 8 conditions + 2 static conditions = 10 conditions) (Table 2). FlyFlix repeated each stimulus condition six times per fly. This gives rise to a total of 60 conditions per trial. FlyFlix showed each condition to the fly for 10s, with a pre-trial and post-trial duration of 1s each. This equals a total of 10 minutes of the

protocol. I set the brightness of the screen to 67% during the experiments (brightness here refers to brightness of the screen as in a smartphone).

Condition	Colour	Brightness	Temporal frequency	Spatial period	Direction
1	White	50	5 Hz	30°	CW
2					CCW
3		255	5 Hz	30°	CW
4					CCW
5	Green	50	5 Hz	30°	CW
6					CCW
7		255	5 Hz	30°	CW
8					CCW
9		50	0 Hz	30°	CW
10					CCW

Table 2. List of conditions in Protocol 2.

Protocol 3: Intensity protocol of visual stimuli shown to the fly

I considered specific properties of gratings while building the protocol. The colour of the gratings was either green or red, on a black background. The brightness of the gratings was either 1, 2, 4, 8, 16, 32, 64, 128 or 255 for the green coloured gratings, while it was 255 for the red coloured grating. The temporal frequency of all the gratings was 4 Hz, and the spatial period was 30°. The direction of the gratings was either clockwise (CW) or counterclockwise (CCW).

The contrast of all the gratings was 1. The pre-trial duration for each condition was 2s and the moving stimulus was shown for 4s. This gave rise to a total of 6s per condition. 10 such conditions (Table 3) were shown for each direction for six repetitions, giving rise to a total of 120 conditions. This gave rise to a protocol of 12 minutes. The brightness of the screen was 67% during the experiment. I conducted experiments with this protocol in a dark room with the flies in a closed box.

Condition	Colour	Brightness	Temporal frequency	Spatial period	Direction
1	Green	1	4 Hz	30°	CW
2					CCW
3		2	4 Hz	30°	CW
4					CCW
5		4	4 Hz	30°	CW
6					CCW
7		8	4 Hz	30°	CW
8					CCW
9		16	4 Hz	30°	CW
10					CCW
11		32	4 Hz	30°	CW
12					CCW
13		64	4 Hz	30°	CW
14					CCW
15		128	4 Hz	30°	CW
16					CCW
17		255	4 Hz	30°	CW
18					CCW
19	Red	255	4 Hz	30°	CW
20					CCW

Table 3. List of conditions in Protocol 3.

Measurement of optomotor responses

FlyFlix logs the data generated by it onto CSV files. These are then combined with the FicTrac data files using a software called flyhavior (Loesche & Reiser, 2021) in order to combine the data and store them as SQLite tables. These SQLite tables are then loaded as pandas tables, after connecting to the SQLite database using the

sqlite3 library in Python. I separate the data for each condition and plot it to observe behaviour.

Protocol 4: Data analysis of optomotor behaviours

The software separates the data for each of the 10 conditions according to direction, foreground colour, spatial period and speed of the stimulus. It then calculates the rotational velocity using the yaw rotations using the “animal lab heading” column of the dataframe.

$$\text{Rotational velocity (rad/s)} = \Delta \text{Heading direction} / \Delta t$$

(Loesche & Reiser, 2021) used a sliding window of 5 frames in order to smooth their 3s rotational velocity data. In the case of 10 second long experiments (Protocol 2), which were at least 3 times the duration of their experiments, I used a sliding window of 15 frames in order to smooth the rotational velocity data. In the case of 6 second long experiments (Protocol 3, 5, 6), which were 2 times the duration of their experiments, I used a sliding window of 10 frames in order to smooth the rotational velocity data. I thus calculate the turning response and plot them across different stimulus conditions for an individual fly.

The software calculates the walking speed of the fly using the x and y coordinate data, using the formula:

$$\text{Speed} = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2} / \Delta t$$

Where (x_1, y_1) and (x_2, y_2) are consecutive coordinates of the fly (in mm) and Δt is the time difference between two consecutive frames. A 15 frame sliding window smoothes the time series data of walking speed.

The software calculates Δt for both rotational velocity and walking speed using the “Client based timestamp (in ms)” column of the dataframe. It stores the timepoints of the experiment. It calculates the combined responses by multiplying the rotational velocity for counterclockwise conditions by -1 and then averaging the responses with the rotational velocity for clockwise conditions.

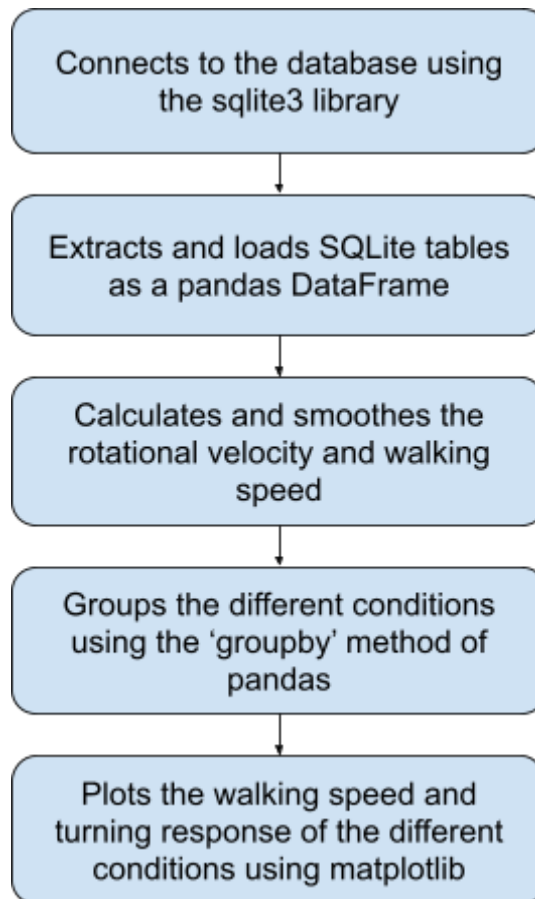


Figure 12. Flowchart for analysing the optomotor behaviour data.

Data plotting conventions

Single fly analysis

I plot the single fly turning responses in Figure 16. The thin lines represent responses over trials, and the thick lines represent the mean over all the repetitions separated by the direction of movement of the stimulus. The first row (Figure 16) is the walking speed of the fly over time in mm/s. The second row is the rotational velocity of the fly in rad/s. The third row is the combined responses of the rotational velocity of the fly over both clockwise (CW) and counterclockwise (CCW) conditions. A total of 6 trials per direction are used. The blue vertical lines represent the start and stop of the trials, respectively.

Multiple flies' analysis

I plotted Mean $\pm$ SEM (Standard error of the mean). I did not plot the last 200 ms of data due to errors arising in this time frame due to the nature of my data analysis in the case of the single tablet experiments. In the two tablet experiments, I did not plot the last 300 ms of data.

Statistical analyses of results of optomotor experiments

I analysed the summary of the optomotor responses to see if they were statistically significantly different from one another using the Kruskal Wallis test, a non-parametric test for multiple comparisons. The Kruskal-Wallis test assumes the data to have a similar scale. If the test revealed that at least one of the means was significantly different from the other, I used a post hoc Dunn's test to determine which of the means were significantly different. I applied a Bonferroni correction during the post hoc test in order to reduce the chances of false positive results (type I errors). I used the `scipy.stats` library to run the Kruskal Wallis test and the `scikit-posthocs` library (Terpilowski, 2019) to run Dunn's test.

Experimental setup of a single tablet in two different positions

In order to test whether there was a difference in optomotor responses to stripes shown to the fly when the tablet was right in front of the fly, versus tilted to an angle of 45°, I used the setup schematised in Figure 13. I rotated the tablet by 45° in the two positions while keeping the distance between the fly and the centre of the tablet in both positions the same.

I then recorded the optomotor responses of each fly after placing the tablet in both positions and then plotted the results (see Results). I did not plot the last 200 ms of the analysis due to errors arising in this time frame due to the nature of my data analysis.

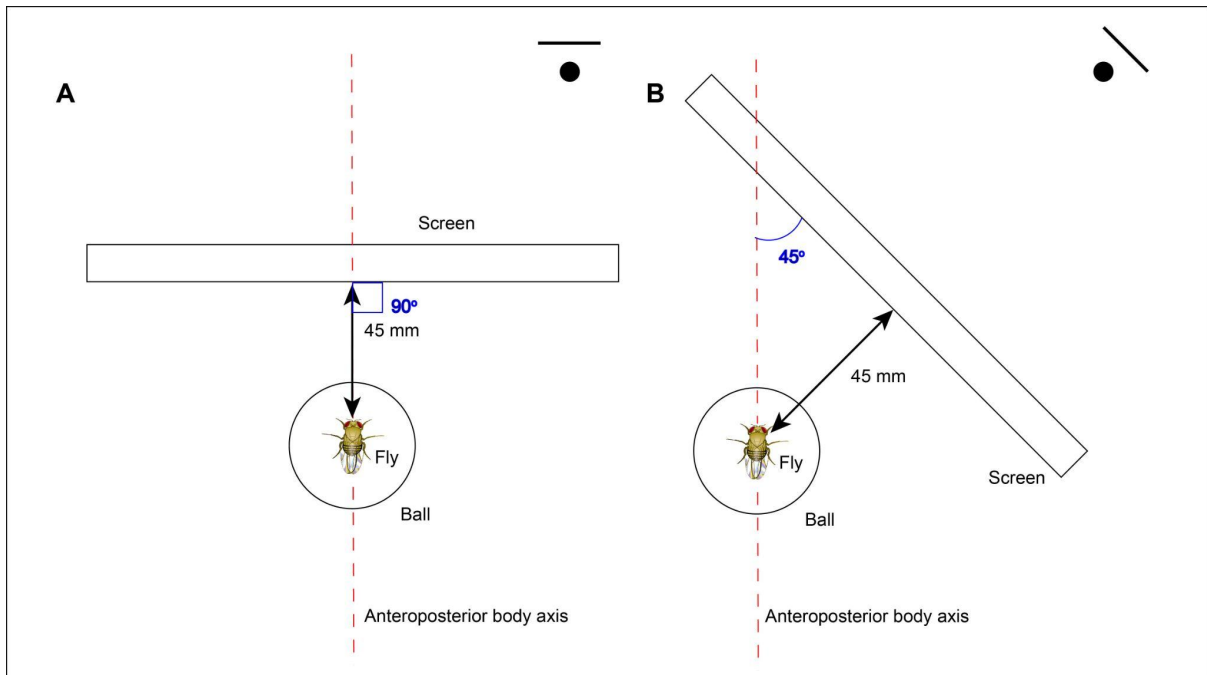


Figure 13. Single tablet in two position design.

The same fly is shown a visual protocol after placing the tablet in the front and at the side of the fly. The distance from the fly to the centre of the tablet in both the positions is 45 mm. I will use the silhouette shown on the upper right of each of the figures when representing results.

(A) Screen at the front of the fly: *Display perpendicular to the anteroposterior body axis of the animal.*

(B) Screen at the side of the fly: *Display at an angle of 45° from the anteroposterior body axis of the animal.*

Figure not drawn to scale.

Protocol 5: Stimulus shown to flies while optimising the virtual reality setup using a single tablet

I considered specific properties of gratings while building the protocol. The colour of the gratings was either green, red or blue, against a black background. The brightness of the gratings was 64 and 255 for the green coloured gratings, while it was 255 for both the red and blue coloured gratings. The temporal frequency of the gratings was either 4 Hz or 10 Hz for the green coloured grating, while it was only 4 Hz for the red and blue coloured gratings. The direction of the stimulus was either

clockwise (CW) or counterclockwise (CCW). The spatial period of the stimulus was either 10° or 30° for the green coloured gratings, while it was only 30° for the red and blue coloured gratings.

This gave rise to a total of 10 conditions for each direction, i.e., to a total of 20 conditions. FlyFlix repeated each condition 6 times, thus giving rise to a total of 120 conditions per trial. Since each condition was 6s long, the total duration of the experiment was 12 minutes. The pre-trial duration of a single condition was 2s, the trial duration 4s, and the post-trial duration 0s.

The above properties gave rise to the conditions shown in Table 4.

The same fly is shown the visual protocol after placing the tablet in the front and at the side of the fly.

Condition	Colour	Brightness	Spatial period	Temporal frequency	Direction
1	Green	50	30°	4 Hz	CW
2					CCW
3				10 Hz	CW
4					CCW
5			10°	4 Hz	CW
6					CCW
7				10 Hz	CW
8					CCW
9		255	30°	4 Hz	CW
10					CCW
11				10 Hz	CW
12					CCW
13			10°	4 Hz	CW
14					CCW
15				10 Hz	CW
16					CCW
17	Red	255	30°	4 Hz	CW
18					CCW
19	Blue				CW
20					CCW

Table 4. List of conditions in Protocol 5.

Experimental setup of two tablets

In order to increase the field of view of the virtual reality setup of the fly, I used a two tablet setup to display visual stimuli. I placed the two tablets perpendicular to one

another, and the centres of both the tablets were equidistant from the fly, as shown in Figure 14.

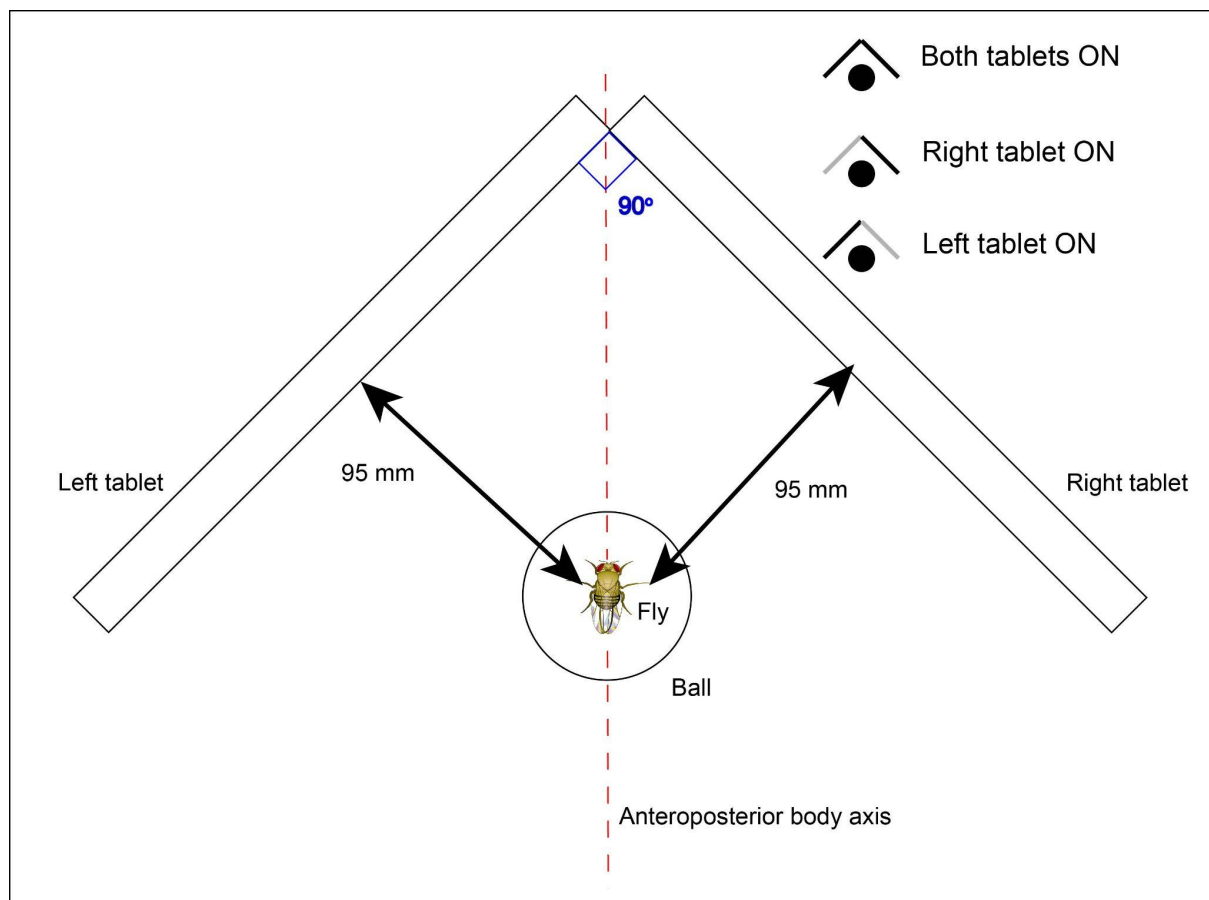


Figure 14. Two-tablet display.

The rods in black represent the tablets that are placed perpendicular to one another. They are also placed equidistant from the fly (95 mm in this case). I used the silhouettes shown on the upper right side of the figure while representing the results. There are three types of experiments, each for which a silhouette has been drawn - one, when both the tablets are ON, second, when only the right tablet is ON, and third, when only the left tablet is ON. Figure not drawn to scale.

Protocol 6: Stimulus shown to flies while optimising the virtual reality setup using two tablets

I next used two tablets in order to optimise virtual reality for the inexpensive fly-on-ball setup as shown in Figure 14. I wanted to check if the optomotor responses of the fly would be according to my expectations and whether reflections from one

tablet to another would affect the responses. In order to tease apart these various factors, I designed a three-factor protocol: factor 1 where both the tablets were on and displaying stimulus, factor 2 where FlyFlix masked the stimulus of the left tablet, and factor 3 where FlyFlix masked the stimulus of the right tablet. The stimulus conditions were the same in all the three factors and the same as Protocol 5 (Table 4), which included 3 different colours, 2 brightness levels, 2 spatial periods, 2 temporal frequencies and 2 directions.

Before going to the Results section, I would like to summarise the list of Figures associated with each Protocol.

Protocol Number	List of Figures
1	15
2	16, 17, 18, 19
3	20, 21
5	22, 23, 24, 25, 26, 27, 28, 29
6	30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41

Table 5. List of protocols and figures associated with each protocol.

Results

Measurement of walking behaviour

I built a binary classifier in order to distinguish between walking and not walking behaviours of the fly using a data driven approach (see Methods). Figure 15 shows the output of the classifier.

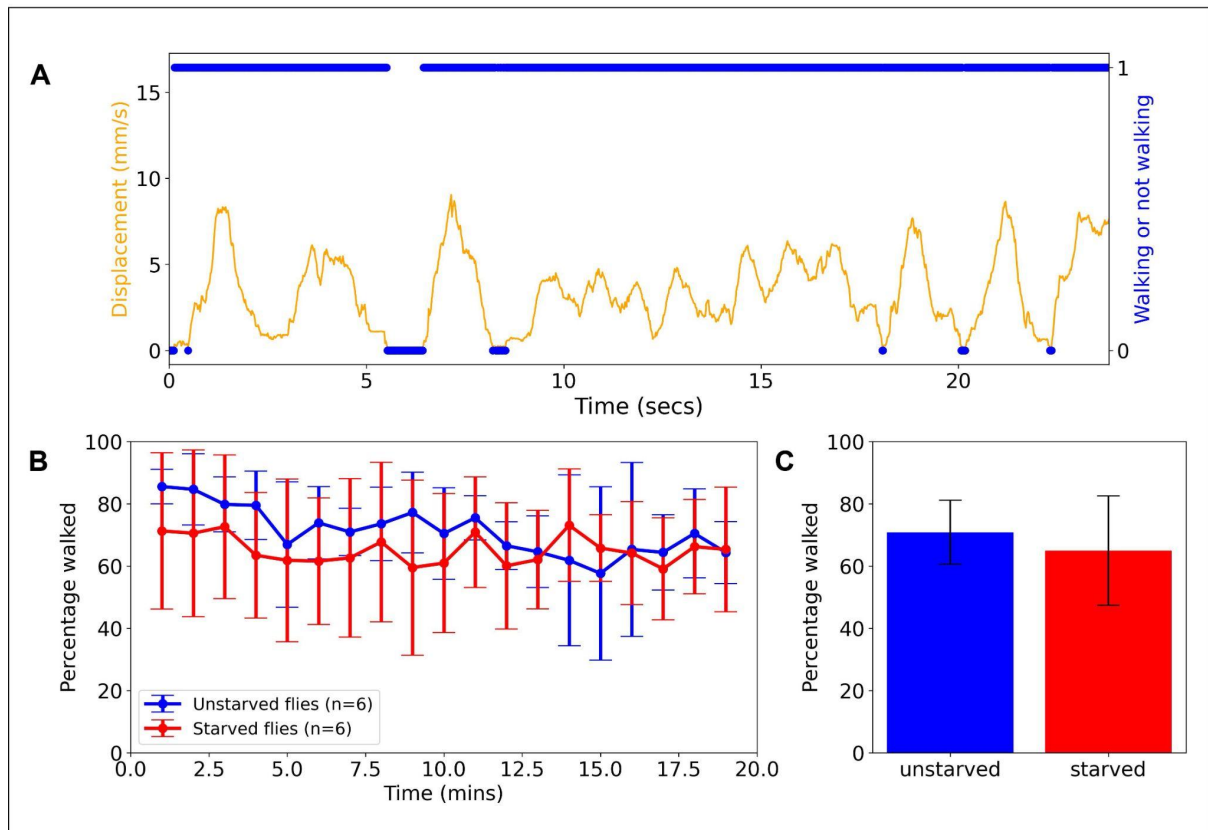


Figure 15. Output of the binary classifier to measure walking behaviours.

(A) Single fly walking analysis. The plot of the output of the binary classifier using a threshold of 30% of the median overlaid over the raw data for a single fly walking on the ball. The blue dots represent the classification of the walking and not walking behaviours at each time point, while the yellow line represents the raw data.

(B) Walking behaviour comparison. Temporal analysis of walking behaviours using the binary classifier in starved and fed flies ($n = 6$ of each group).

(C) Percentage walked by flies kept under different conditions of starvation - fed (unstarved) and starved flies. The differences in percentages of walking behaviour among the two groups are non significant ($p = 0.5$; Wilcoxon signed rank test).

According to the binary classifier, starved and unstarved flies do not walk significantly differently from one another (Figure 15 (C)).

Optomotor responses in *Drosophila melanogaster*

The optomotor responses to Protocol 2 (see Methods) for a single fly can be seen in Figure 16. The responses averaged over multiple flies can be seen in Figure 17. As can be seen in Figure 17, flies show consistent walking while responding to stimuli in both directions.

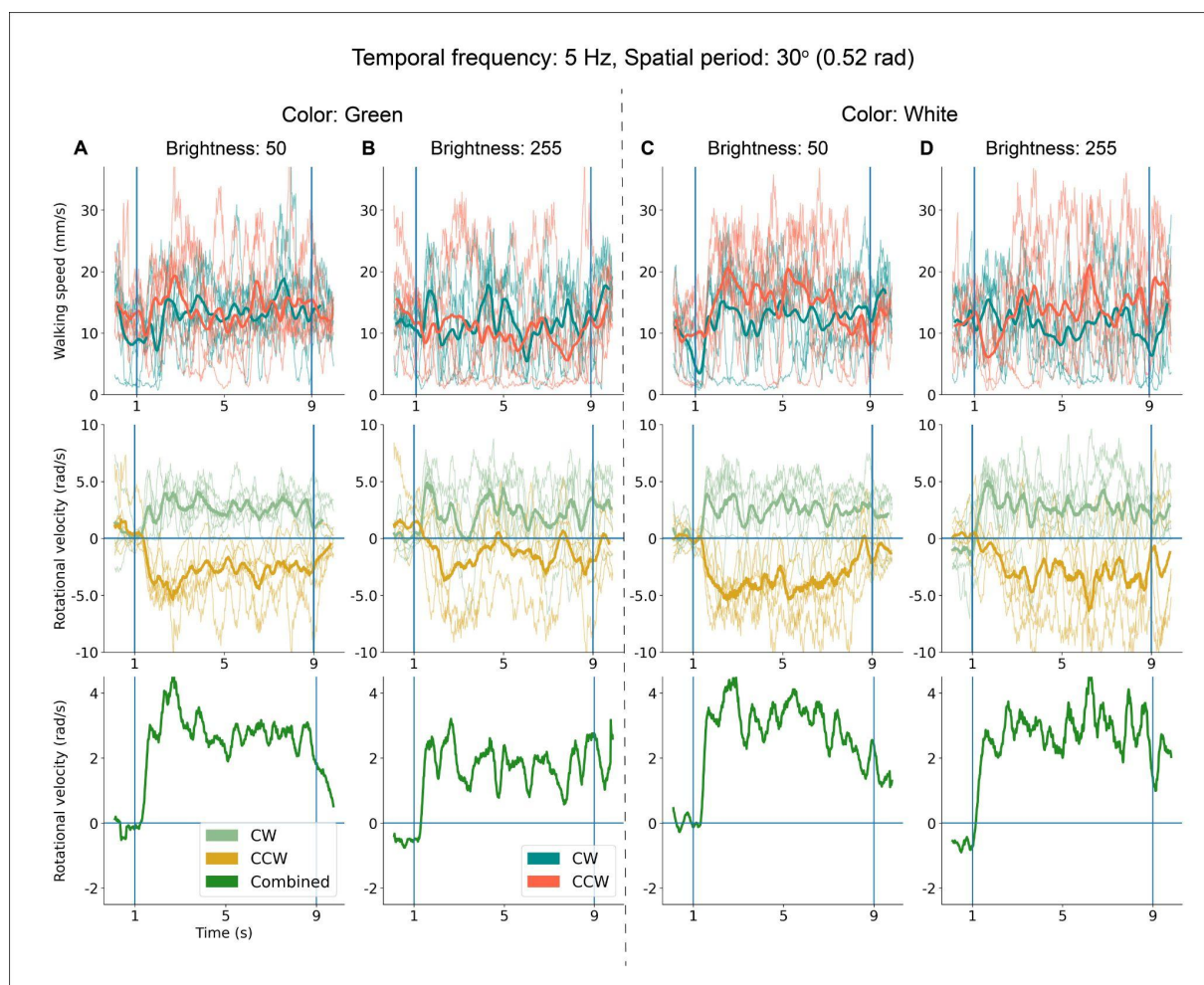


Figure 16. Optomotor response of a single fly.

(Top) Walking speed of the fly. (Middle) Turning responses in both directions. (Bottom) Combined response of the clockwise and counterclockwise directions. (A) - (D) represent conditions as mentioned in the figure.

Figure 16 describes the optomotor responses of a single fly across stimulus conditions. I next wanted to look at the time series plots of the responses across flies (Figure 17).

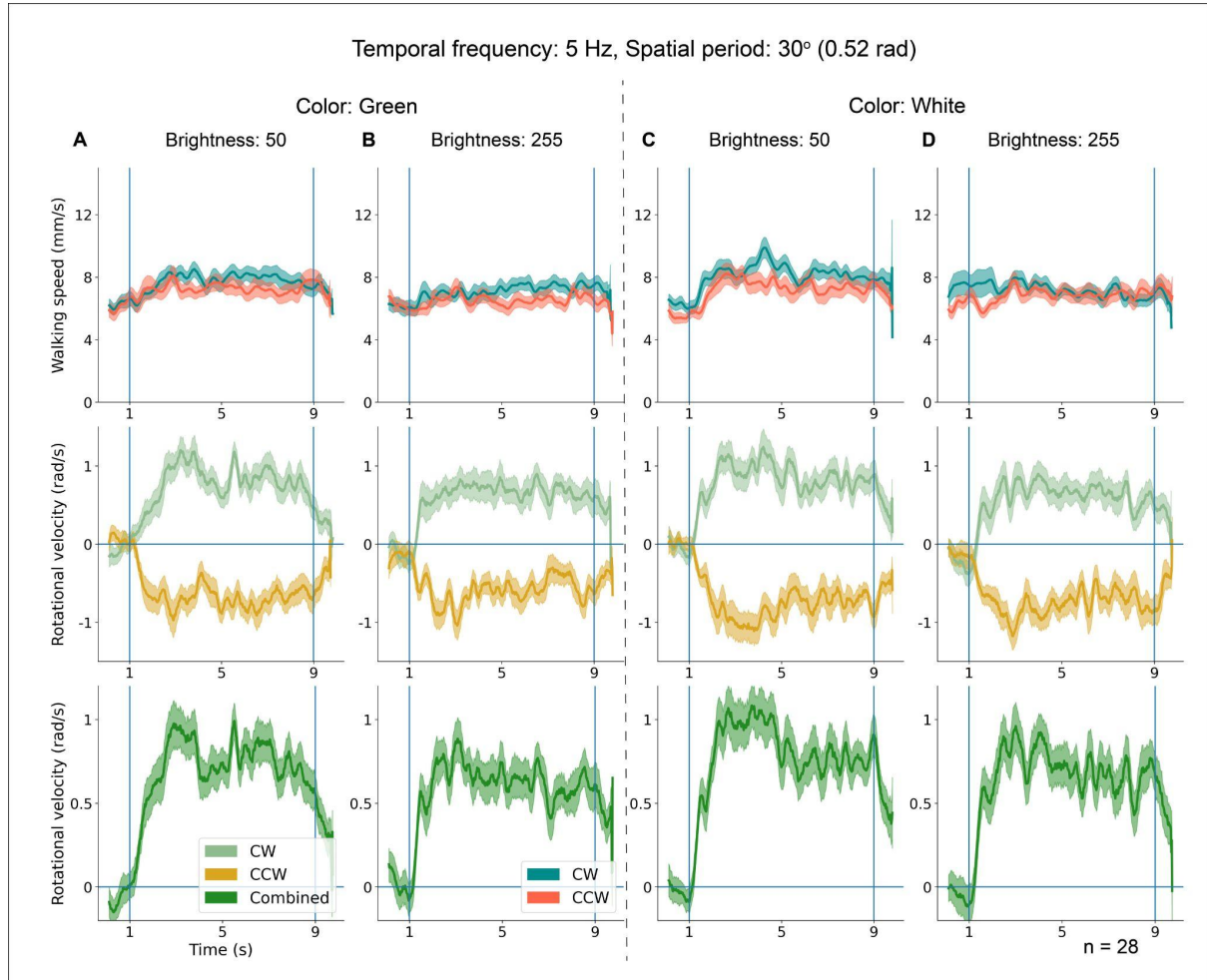


Figure 17. Optomotor responses of multiple flies.

(Top) Walking speed of the flies. (Middle) Turning responses in both directions. (Bottom) Combined response of the clockwise and counterclockwise directions. (A) - (D) represent conditions as mentioned in the figure.

I next asked whether there was a difference in turning response between the different conditions, especially between the bright and dim conditions, and between colours of gratings. From Figures 17 and 18, it can be seen that there is no significant difference between the walking speed of flies between the clockwise and counterclockwise conditions. Moreover, the flies walk consistently, which additionally validates my interpretation of the two figures. Upon observing Figure 17, one can

see that there is no significant difference between the responses to the two different colours. I plotted this summary in Figure 18. As can be seen in Figure 18, I did not find any difference between the responses of different brightness conditions. This later led me to perform an intensity analysis to observe the threshold of intensity of the stimulus at which flies show optomotor responses.

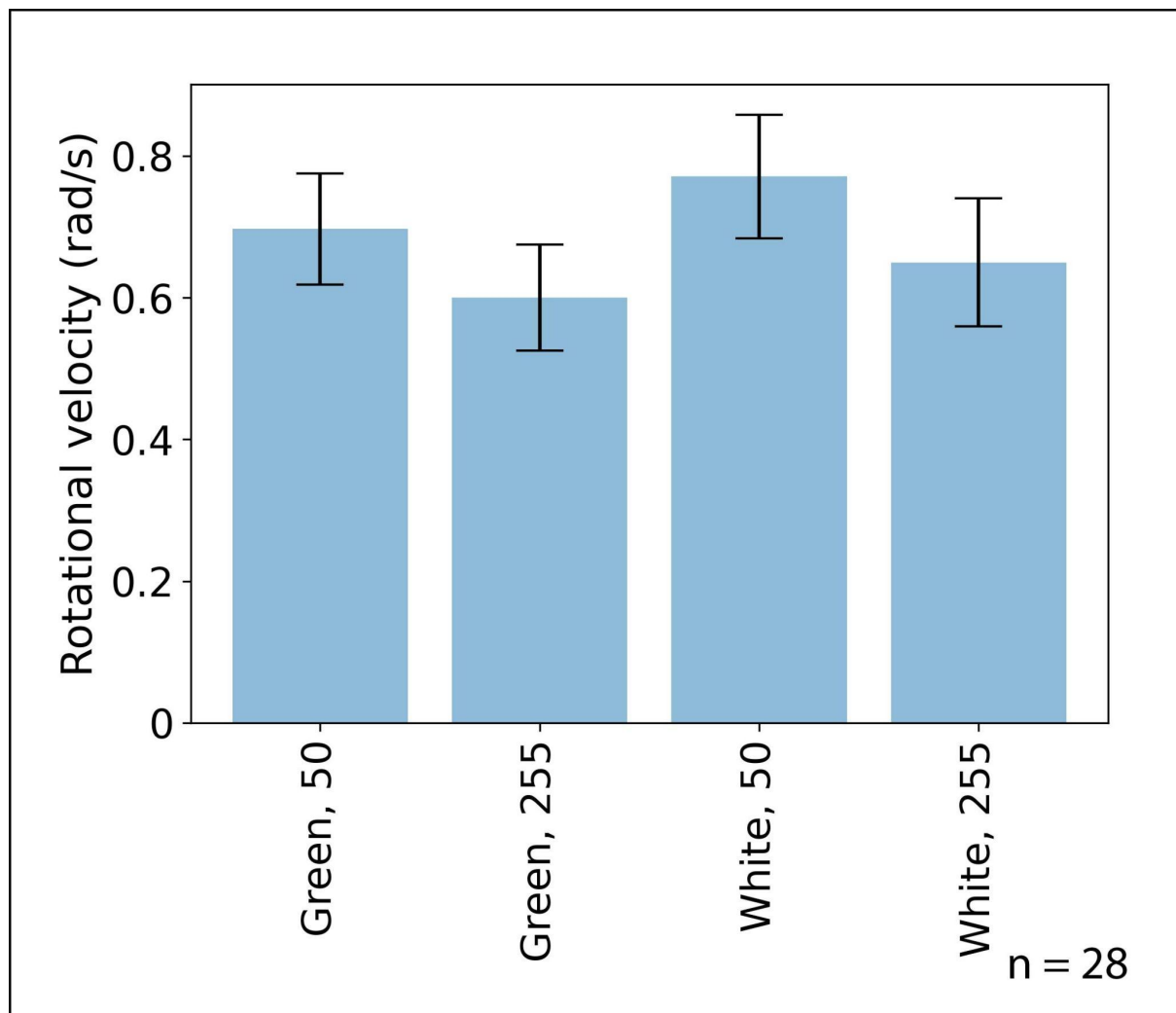


Figure 18. Summary of the optomotor responses across different conditions of colour and brightness.

The means are not significantly different from one another ($p = 0.60$; Kruskal Wallis test).

Figure 18 shows that there is no significant difference between the turning responses between the four conditions. I next asked whether there was a trend in responses

between the early and late trials. The results of this analysis are discussed in the next section.

Optomotor responses to early versus late trials

Early stimuli are defined as stimuli that were given during repetitions 1-3 of the protocol. Late stimuli are defined as stimuli that were given during repetitions 4-6 of the protocol. I hypothesised that since the fly would get acclimated to the ball over time, it would walk and respond more in the late repetitions as compared to the early repetitions. Figure 19 represents the comparison of optomotor responses to early and late stimuli as shown according to Protocol 3 (see Methods). The means of the early and late stimuli of conditions (Colour: Green, Brightness: 50), (Colour: Green, Brightness: 255) and (Colour: White, Brightness: 255) are significantly different from one another. In the (Colour: Green, Brightness: 50) condition, there is a significant *decrease* in the optomotor response, while in the (Colour: Green, Brightness: 255) and (Colour: White, Brightness: 255) conditions, there is a significant *increase* in the optomotor response. This suggests that although the means of the early and late stimuli are significantly different from each other during these conditions, there is no clear trend of increase or decrease across early versus late stimuli.

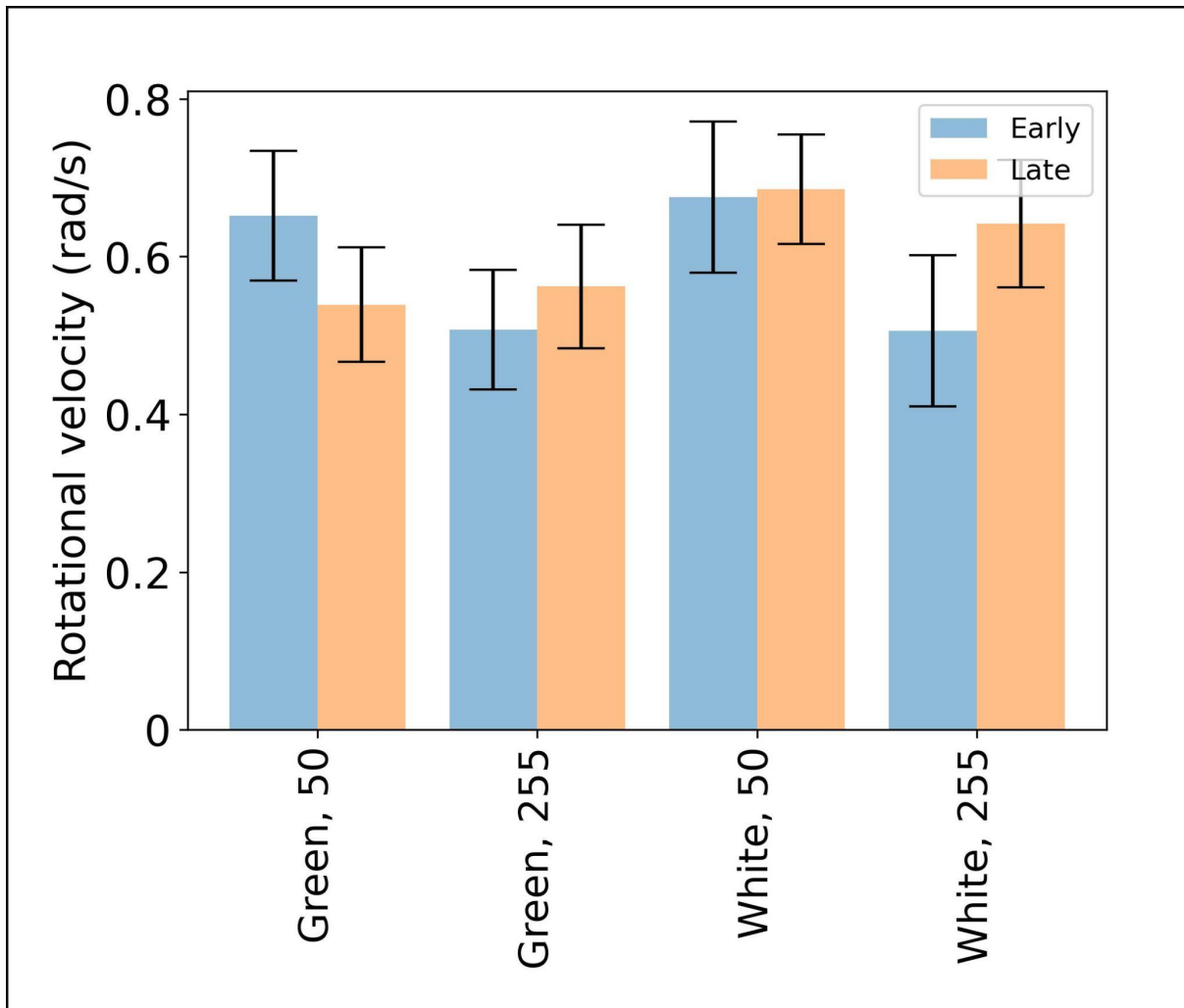


Figure 19. Comparison of optomotor responses to early and late stimuli.

The figure represents the average turning response across flies ($n = 28$) and compares two conditions - the early stimuli and the late stimuli. The means of the early and the late stimuli of conditions (Green, 50), (Green, 255) and (White, 255) are significantly different from one other ($p < 0.001$; Kruskal Wallis test followed by a post hoc Dunn's test). The brightness values vary from 0 to 255, 0 being the most dim and 255 being the brightest.

Intensity analysis of visual stimuli

I next conducted an “intensity analysis” of the visual stimuli being shown to the fly by increasing the intensity of the green gratings in order to measure the threshold at which flies respond to the movement of the gratings. The result of the experiment can be seen in Figure 20.

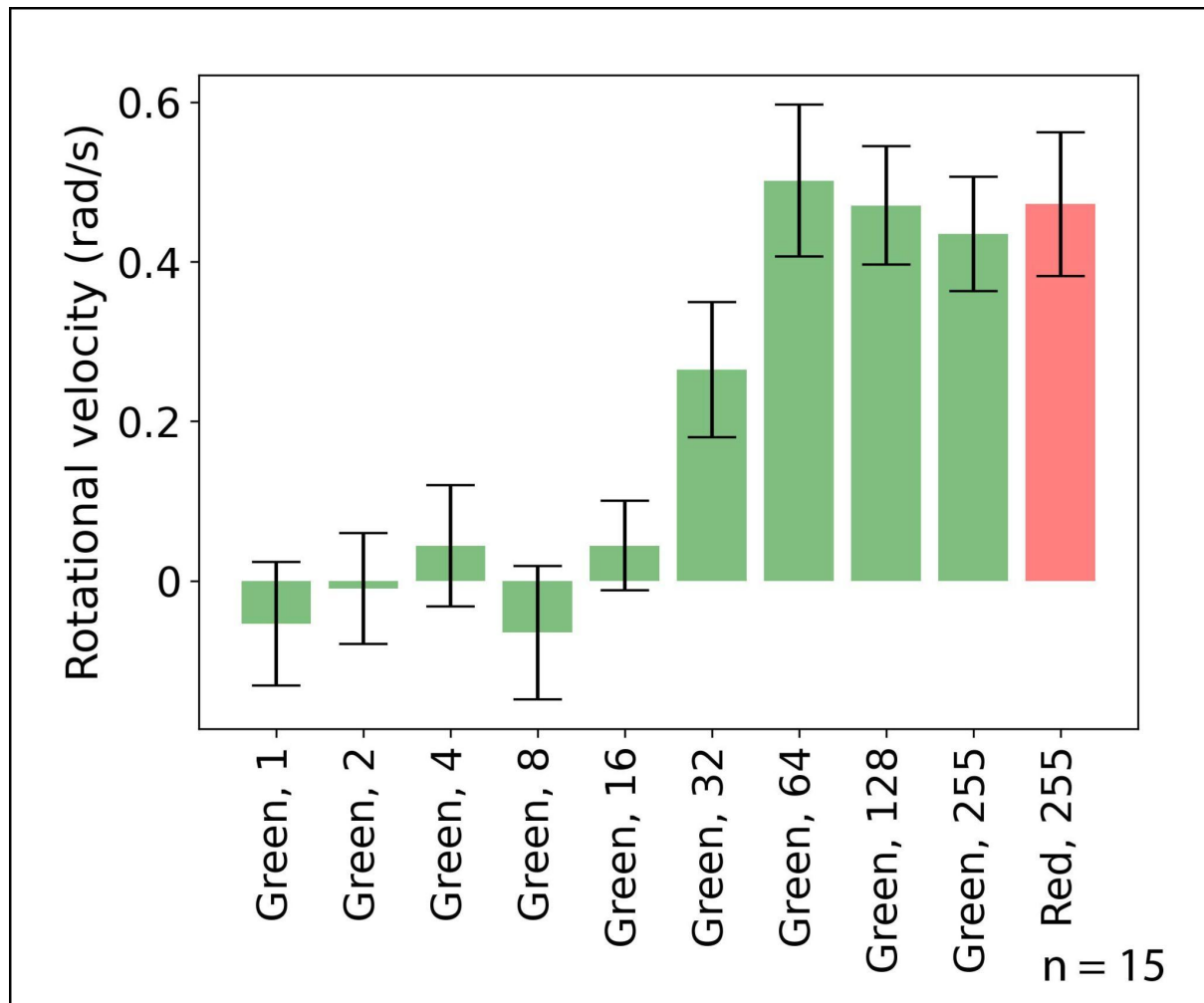


Figure 20. Summary of intensity analysis of the rotational response of the fly.

Change in rotational response of flies (n = 15) across different intensity levels of the green colour in the gratings. I used intensities of 1, 2, 4, 8, 16, 32, 64, 128 and 255. (0 being the dimmest and 255 being the brightest) (see Protocol 3 for details). I also used one condition of red gratings with an intensity of 255. The colours of the bars represent the colour of the stimulus used.

I next asked whether averaging the turning responses for lower intensities masked optomotor responses at lower intensities. In order to do so, I looked at the time series data of the walking speed and the turning responses of flies over all the intensities. The results can be seen in Figure 21. It shows that information is not lost due to averaging the rotational response across time at lower intensities. Flies also show significant optomotor responses only after stimulation to an intensity of 32, as can be seen in Figure 21. This additionally validated my interpretation of Figure 20. Due to the nature of my analysis protocol, and differences in the number of frames captured across conditions and between flies, I clipped the last 200 ms of data in the figures to improve interpretability and generalisation of the results across all flies.

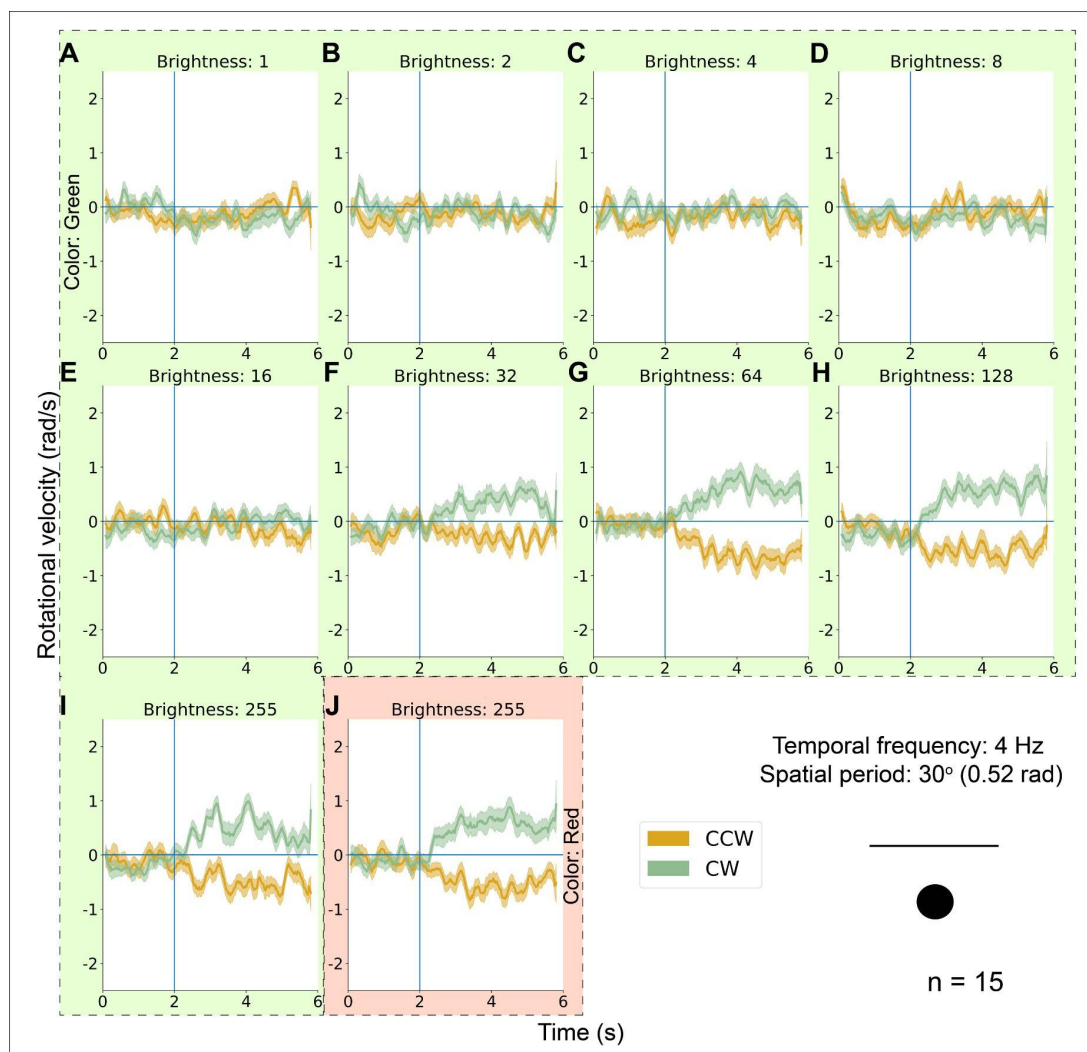


Figure 21. Time series responses of the intensity analysis.

(A) - (J) represent the conditions as mentioned in the figure.

Optomotor responses to a single tablet in two different positions

I wanted to expand the field of view of the virtual reality environment from the perspective of the fly. This required optimisation of the inexpensive fly-on-ball setup with a two-tablet display system (see Methods). As a first step of optimisation of the virtual reality display, I positioned a single tablet to the front of the fly, followed by a rotation of 45 degrees to the right side. I still kept the centre of the tablet in both the positions perpendicular to the position of the fly, and at the same distance from the fly (see Methods). I showed visual stimulus conditions defined by Protocol 5 (see Methods). The results of the turning responses during the front-side experiments are shown in Figures 22 and 24. The walking analysis of the front-side experiments is shown in Figures 23 and 25.

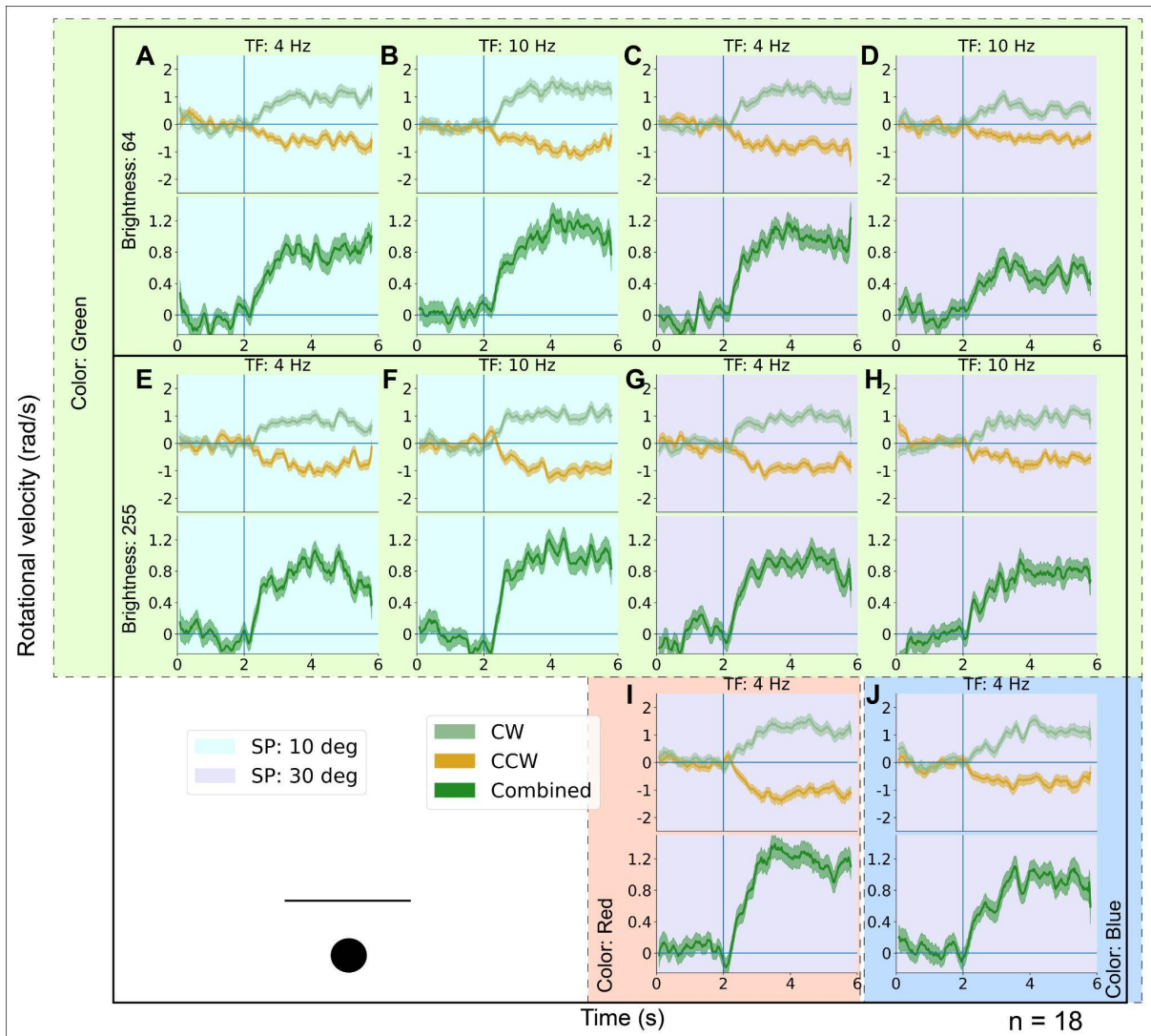


Figure 22. Optomotor responses after placing the tablet in the front.

Turning responses (directional and combined) to stimuli shown after placing the tablet in the front of the fly. The responses for the different combinations of colour, brightness, temporal frequency (TF) and spatial period (SP) as per Protocol 5 are shown. (A) - (J) represent the conditions as mentioned in the figure.

Figure 22 shows the turning responses of the fly after shown stimuli according to Protocol 5, when the tablet is placed in the front of the fly. It shows that the responses to clockwise and counterclockwise directions are symmetric about the line ($y = 0$). Also, there is no significant difference between the walking speeds of the clockwise and counterclockwise conditions in any of the conditions.

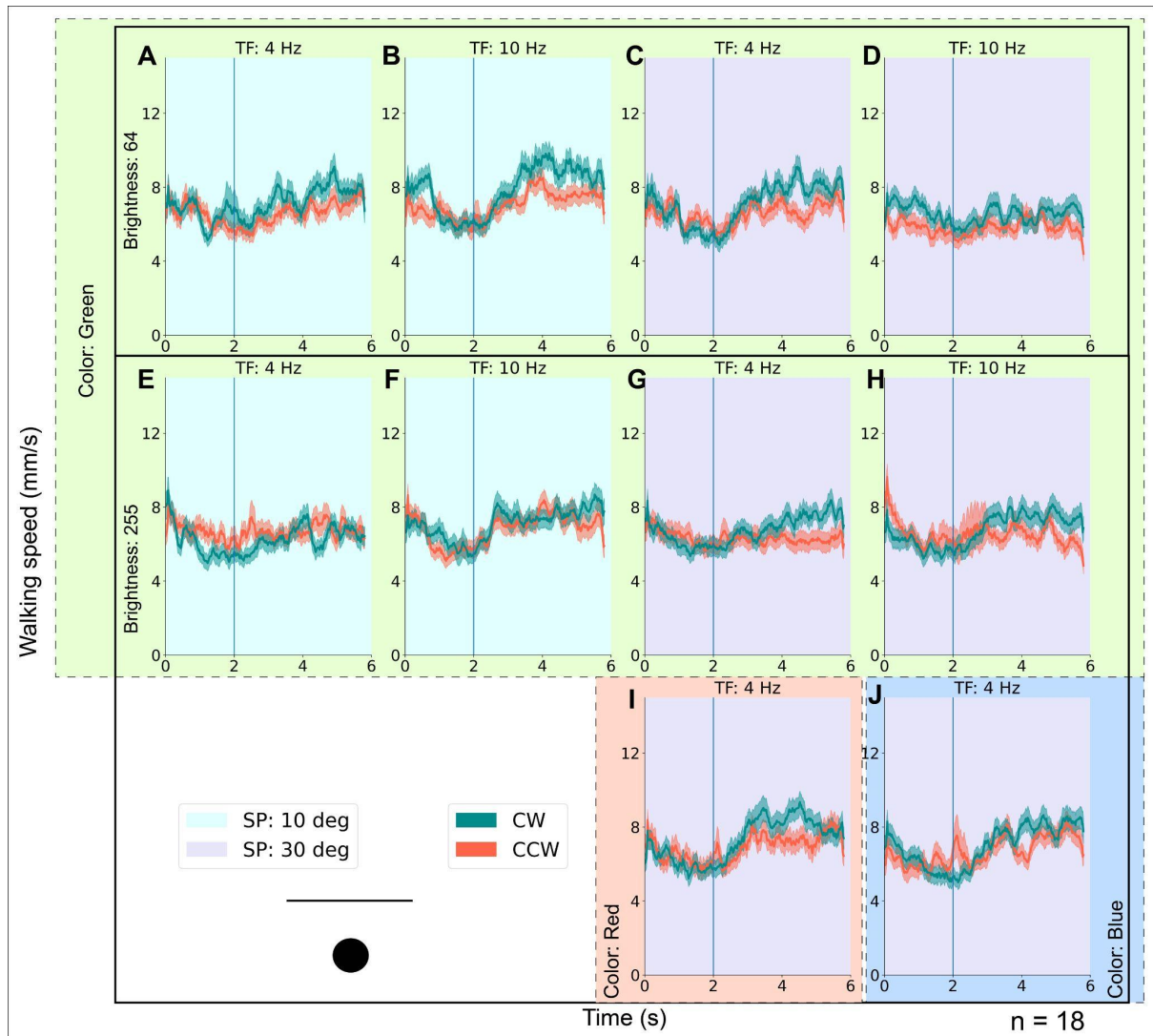


Figure 23. Walking speed after placing the tablet in the front.

The responses for the different combinations of temporal frequency (TF) and spatial period (SP) as given in Protocol 5 are shown. (A) - (J) represent the conditions as mentioned in the figure.

Figure 23 shows the time series responses of the flies when shown stimulus conditions according to Protocol 5, when I placed the tablet in front of the fly. There is no significant difference between the walking responses in the clockwise and counterclockwise directions in this case.

Next, I looked at the responses to the stimuli after placing when the tablet at the side. This serves as a baseline for behavioural responses obtained in the two tablet setup when only one of the tablets is ON.

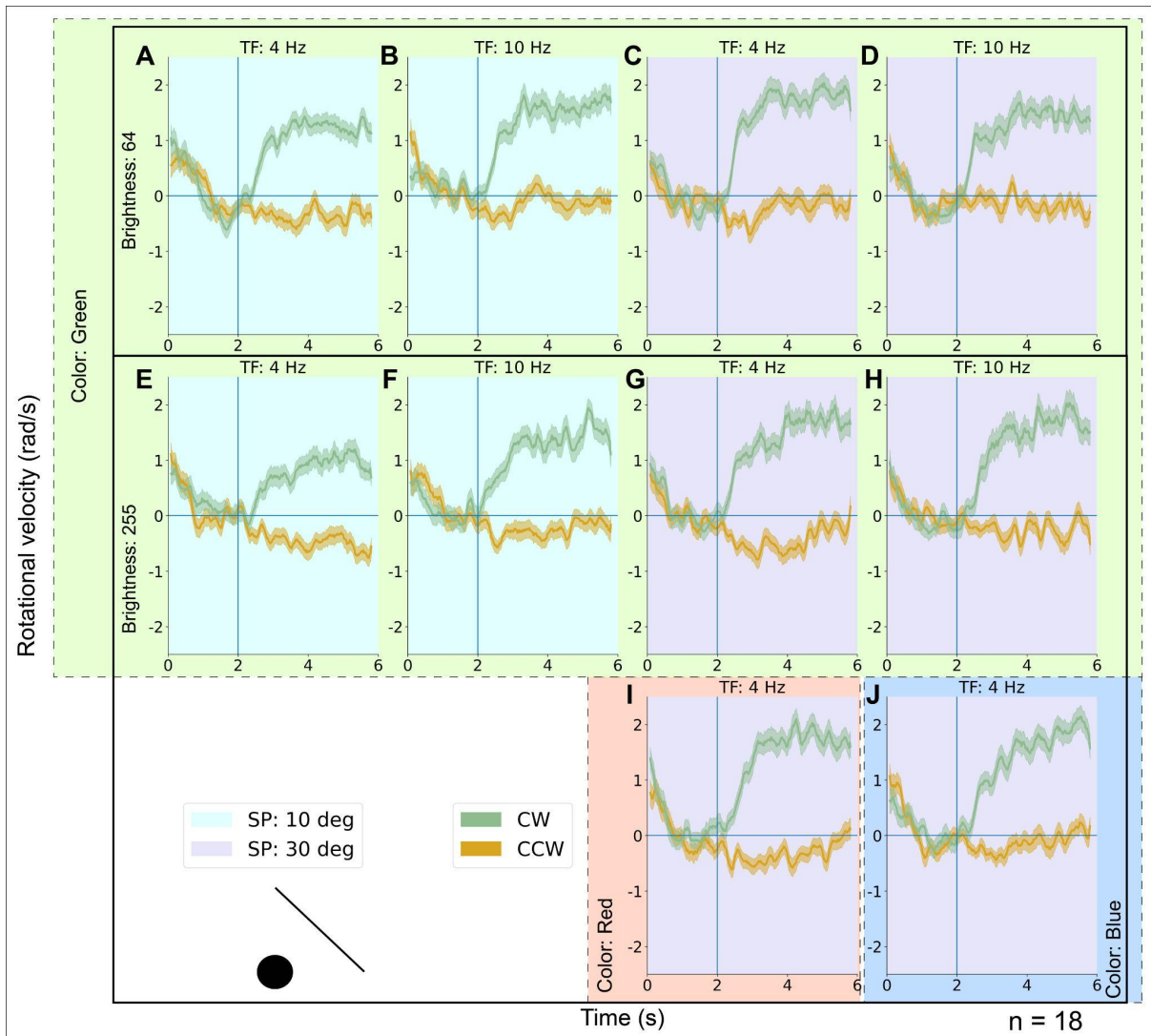


Figure 24. Optomotor responses after placing the tablet at the side.

The responses are for the different combinations of brightness, colour, temporal frequency (TF) and spatial period (SP), as given in Protocol 5. (A) - (J) represent the conditions as mentioned in the figure.

Figure 24 shows the turning responses of the flies after showing the stimulus conditions according to Protocol 5, when I placed the tablet at the side of the fly. The responses are heavily biased to the stimuli moving in the clockwise direction as compared to the responses to stimuli moving in the counterclockwise direction.

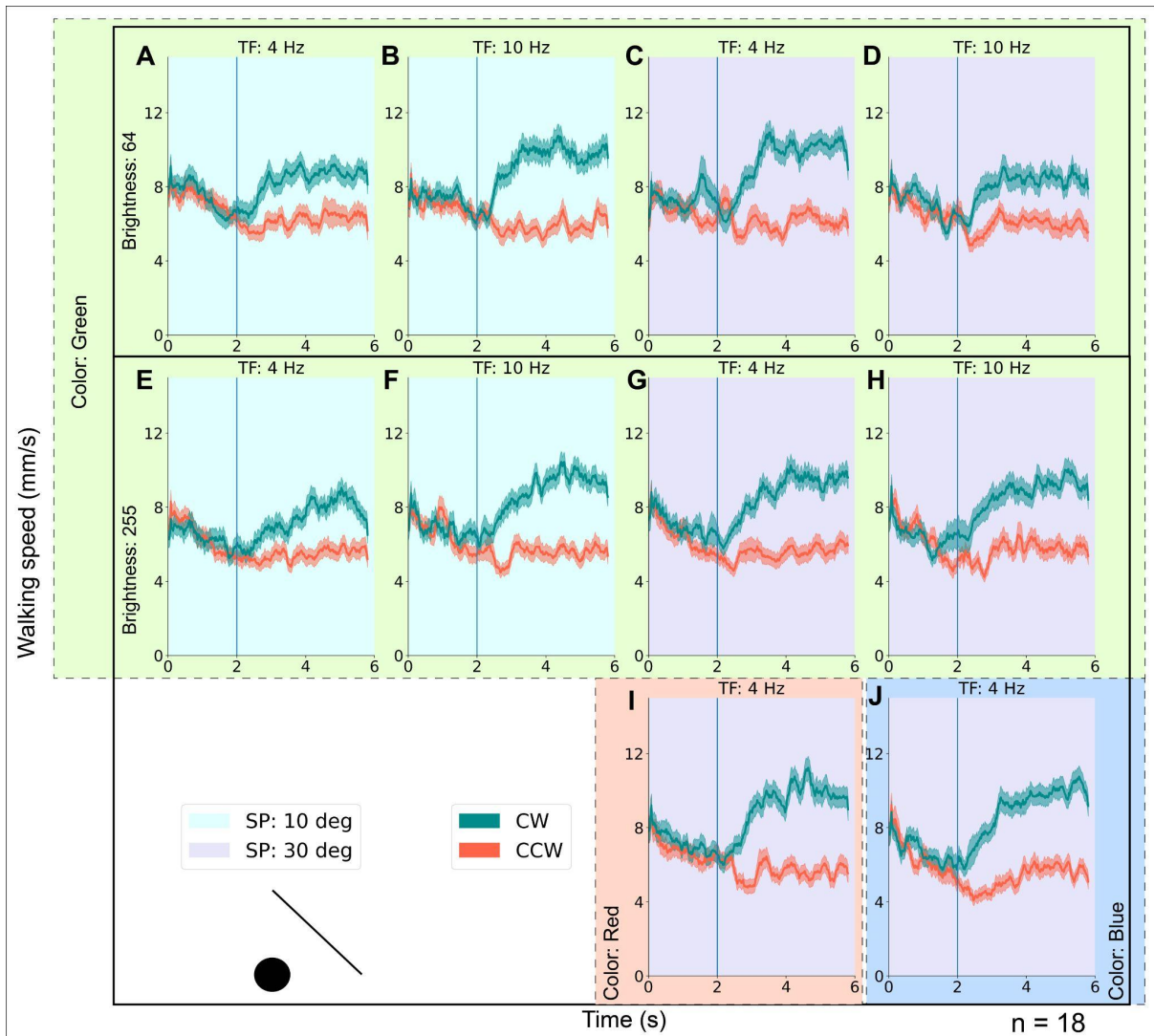


Figure 25. Walking speed after placing the tablet at the side.

The responses are for the different combinations of brightness, colour, temporal frequency (TF) and spatial period (SP) as per Protocol 5. (A) - (J) represent the conditions as mentioned in the figure.

Figure 25 shows the time series of the walking speed of the flies when they are shown stimulus conditions as per Protocol 5, when I placed the tablet at the side of the fly. The walking speed parallels the turning responses. The flies walk significantly more in response to stimulus conditions in the clockwise direction than to the stimulus conditions in the counterclockwise direction. More walking behaviours of the fly imply larger turning responses, and lower walking behaviours imply lesser turning responses.

Summary of the responses to the single tablet placed in two positions experiment

The summary responses of the turning and walking speeds of the above experiments have been plotted in Figures 26, 27, 28 and 29.

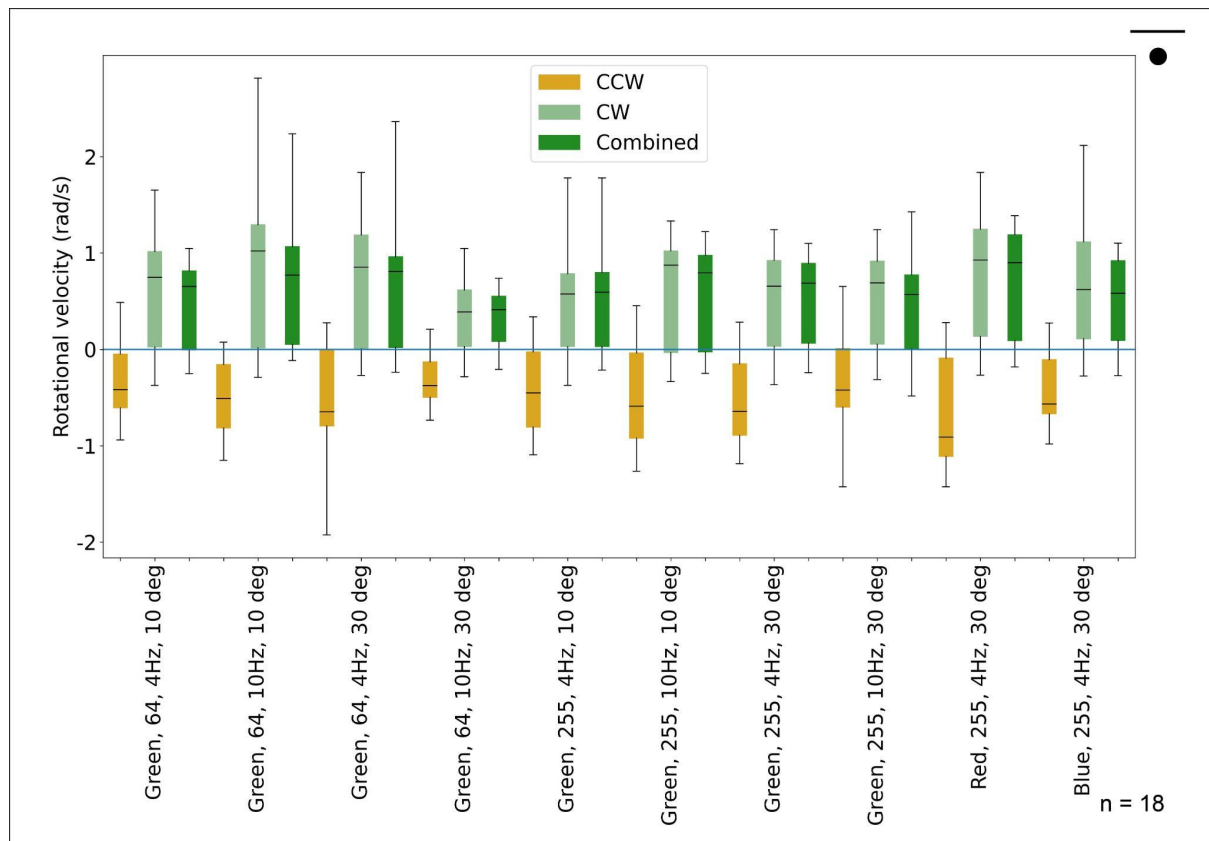


Figure 26. Turning response summary after placing the tablet in the front.

The magnitude of the responses to the clockwise and counterclockwise directions are almost equal. The different conditions are represented in the following order: (direction, colour, brightness, temporal frequency, spatial period).

Figure 26 summarises the turning response of the flies when they are shown visual stimuli according to Protocol 5, when I placed the tablet in front of the fly. The responses to clockwise direction (shown in the lightgreen plot in Figure 26), and the responses to the counterclockwise direction (shown in the yellow plot in Figure 26), are symmetric.

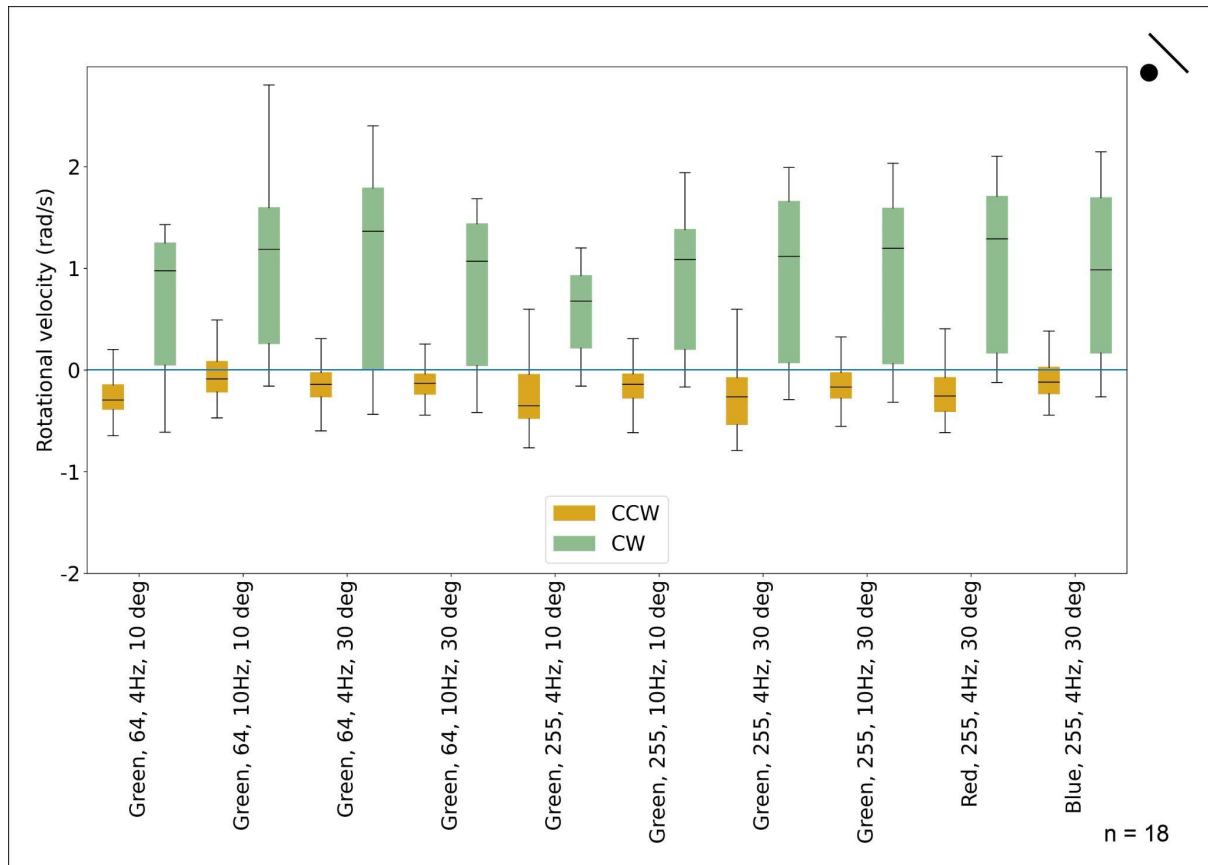


Figure 27. Turning response summary after placing the tablet at the side.

The magnitude of the responses to stimuli moving in the counterclockwise and clockwise directions is not equal. The magnitude of responses to stimuli moving in the clockwise direction is greater than that to stimuli moving in the counterclockwise direction. The different conditions are represented in the following order: (direction, colour, brightness, temporal frequency, spatial period).

Figure 27 summarises the turning responses of the flies when they are shown stimulus conditions as per Protocol 5, and the tablet is placed at the side of the fly. The turning responses are asymmetric, and the magnitude of the responses in the clockwise direction is greater than that in the counterclockwise direction.

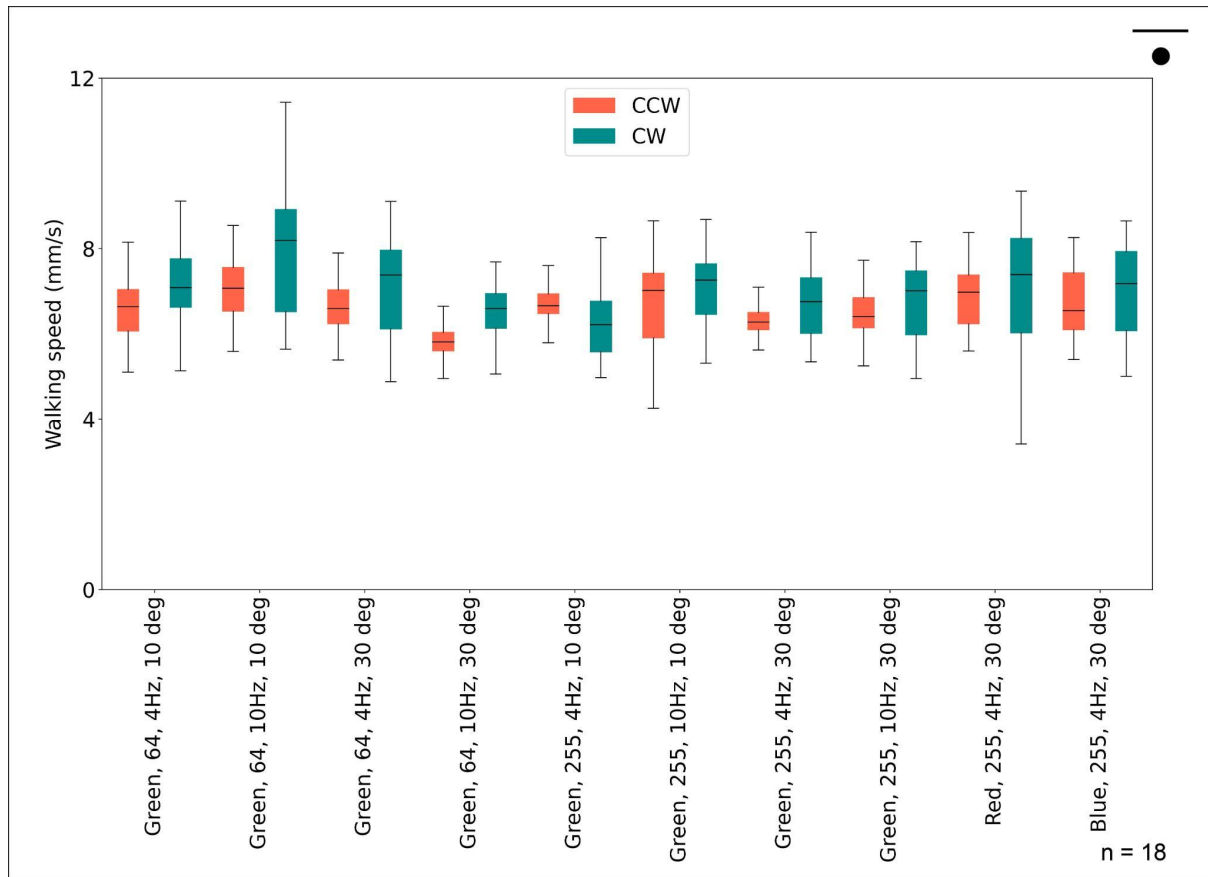


Figure 28. Walking speed summary after placing the tablet in the front.

The magnitude of the responses to the clockwise and counterclockwise directions are not very different from one another, similar to the trend observed in Figure 26. The different conditions are represented in the following order: (direction, colour, brightness, temporal frequency, spatial period).

Figure 28 summarises the walking speed of the flies in the stimulus conditions shown as per Protocol 5, when the tablet is placed in front of the fly. There is no significant difference between the walking speed in the clockwise and counterclockwise directions, implying that the flies are not biased towards turning in one particular direction (Figure 26).

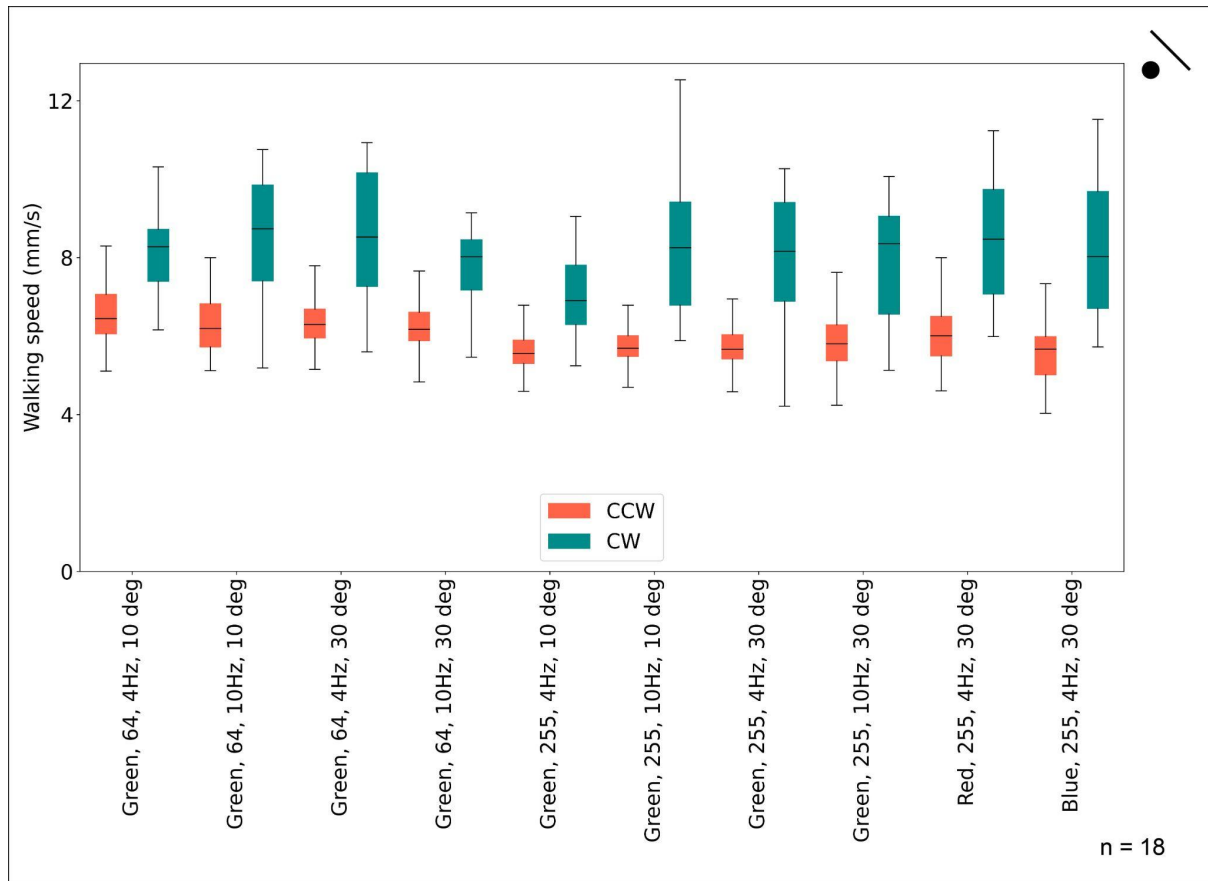


Figure 29. Walking speed summary after placing the tablet at the side.

The magnitude of the responses to stimuli moving in the counterclockwise and clockwise directions is not equal. The magnitude of responses to stimuli moving in the clockwise direction is greater than that to stimuli moving in the counterclockwise direction, similar to the trend observed in Figure 27. The different conditions are represented in the following order: (direction, colour, brightness, temporal frequency, spatial period).

Figure 29 summarises the walking speed of the flies to the stimulus conditions shown as per Protocol 5, when the tablet is placed at the side of the fly. The walking speed of the flies in the clockwise direction is greater than the walking speed in the counterclockwise direction. This is in parallel with the turning responses (Figure 27), where the turning responses in the clockwise direction are greater than those in the counterclockwise direction.

In summary, the turning responses to both directions when the tablet is placed in front of the fly is symmetric about the line ($y = 0$), while it is asymmetric when the tablet is placed to the side of the fly.

Optomotor responses to two tablets

Next, I placed two tablets perpendicular to one another, such that the centre of both the tablets was equidistant from the fly (see Methods). I ran Protocol 6 on the tablets and recorded the responses of the fly. The turning responses can be seen in Figure 30 when both the tablets are ON. In this case, I expect a symmetric optomotor response to the stimuli.

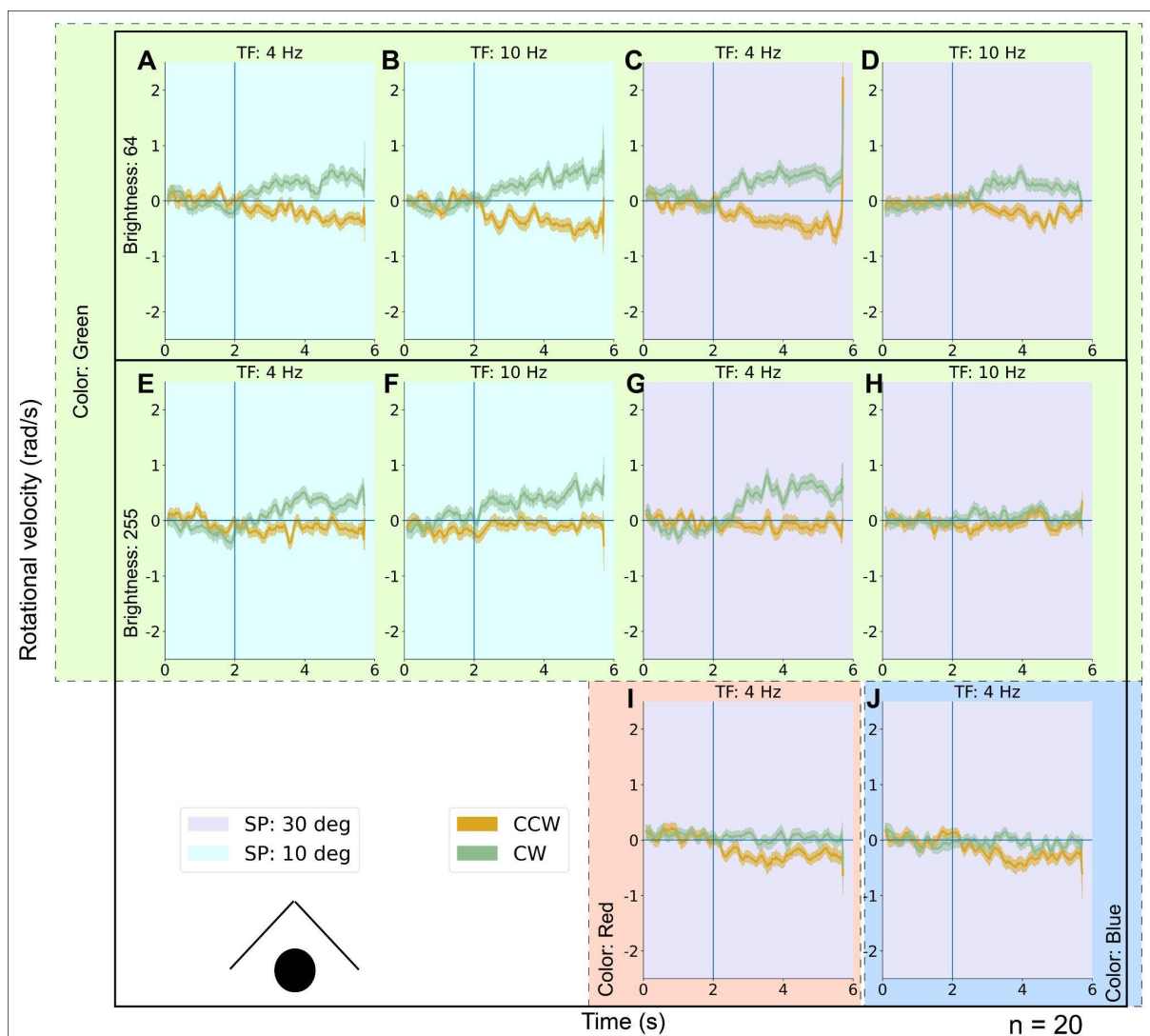


Figure 30. Two-tablet analysis with both tablets ON.

Optomotor responses of the flies ($n = 20$) when both the tablets are ON. TF: Temporal frequency, SP: Spatial period. (A) - (J) represent the conditions as mentioned in the figure.

Figure 30 shows that the turning responses to the two tablet conditions are not symmetric in all the cases. They are symmetric in the cases (Colour: Green, Brightness: 64, Temporal frequency: 4 Hz, Spatial period: 10 deg), (Colour: Green, Brightness: 64, Temporal frequency: 10 Hz, Spatial period: 10 deg), (Colour: Green, Brightness: 64, Temporal frequency: 4 Hz, Spatial period: 30 deg) and (Colour: Green, Brightness: 64, Temporal frequency: 10 Hz, Spatial period: 30 deg). In the case (Colour: Green, Brightness: 255, Temporal frequency: 10 Hz, Spatial period: 30 deg), they are not significantly different from zero in either stimulus direction. In the rest of the cases, the responses are asymmetrically biased either towards the clockwise or counterclockwise direction. In the cases (Colour: Green, Brightness: 255, Temporal frequency: 4 Hz, Spatial period: 10 deg), (Colour: Green, Brightness: 255, Temporal frequency: 10 Hz, Spatial period: 10 deg), (Colour: Green, Brightness: 255, Temporal frequency: 4 Hz, Spatial period: 30 deg) and (Colour: Green, Brightness: 255, Temporal frequency: Spatial period: 4 Hz, 30 deg), the responses are biased towards the clockwise direction, while in the cases (Colour: Red, Brightness: 255, Temporal frequency: 4 Hz, Spatial period: 30 deg) and (Colour: Blue, Brightness: 255, Temporal frequency: 4 Hz, Spatial period: 30 deg), the responses are biased towards the counterclockwise direction.

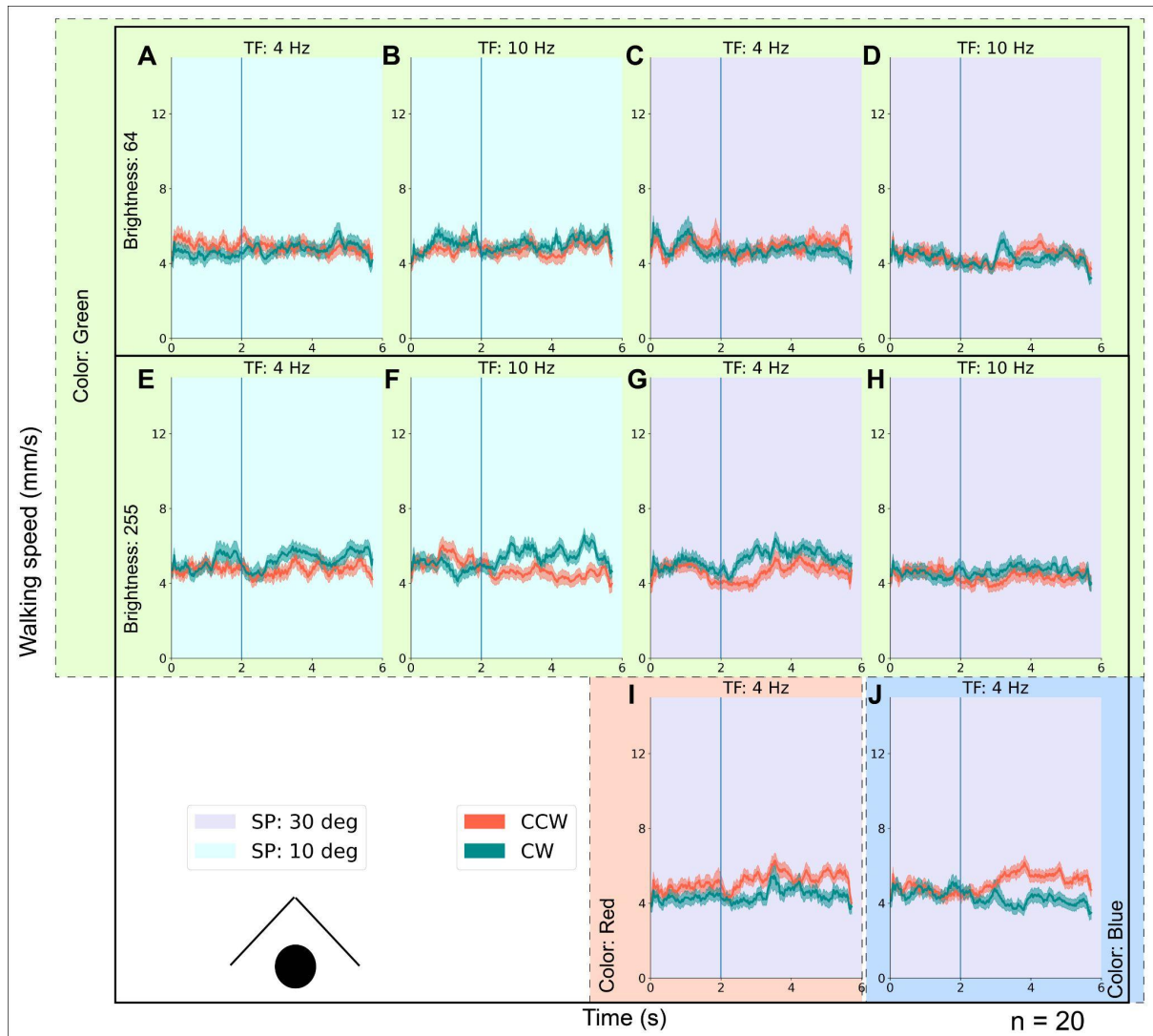


Figure 31. Walking speed with both tablets ON.

(A) - (J) represent the conditions as mentioned in the figure.

Figure 31 represents the time series of the walking speed of the flies (n = 20) across conditions with two temporal frequencies, two spatial periods, and two levels of brightness for the green bars, as well as two conditions with different colours (Protocol 6; see Methods). The figure shows that the differences in walking speed in the clockwise and counterclockwise directions strongly parallel the differences in the magnitude of the turning response across the two conditions. Specifically, if the flies walk less during a certain condition, they also turn less in that condition. This can clearly be seen in the (Colour: Red, Brightness: 255, Temporal frequency: 4 Hz, Spatial period: 30 degrees) condition, where the magnitude of the walking speed in the counterclockwise direction is significantly greater than the magnitude in the

clockwise direction. This parallels the greater magnitude of turning response in the counterclockwise direction as compared to the clockwise direction in Figure 30.

Next, I ran Protocol 6 when only the Right tablet was ON. The results are shown in Figure 32.

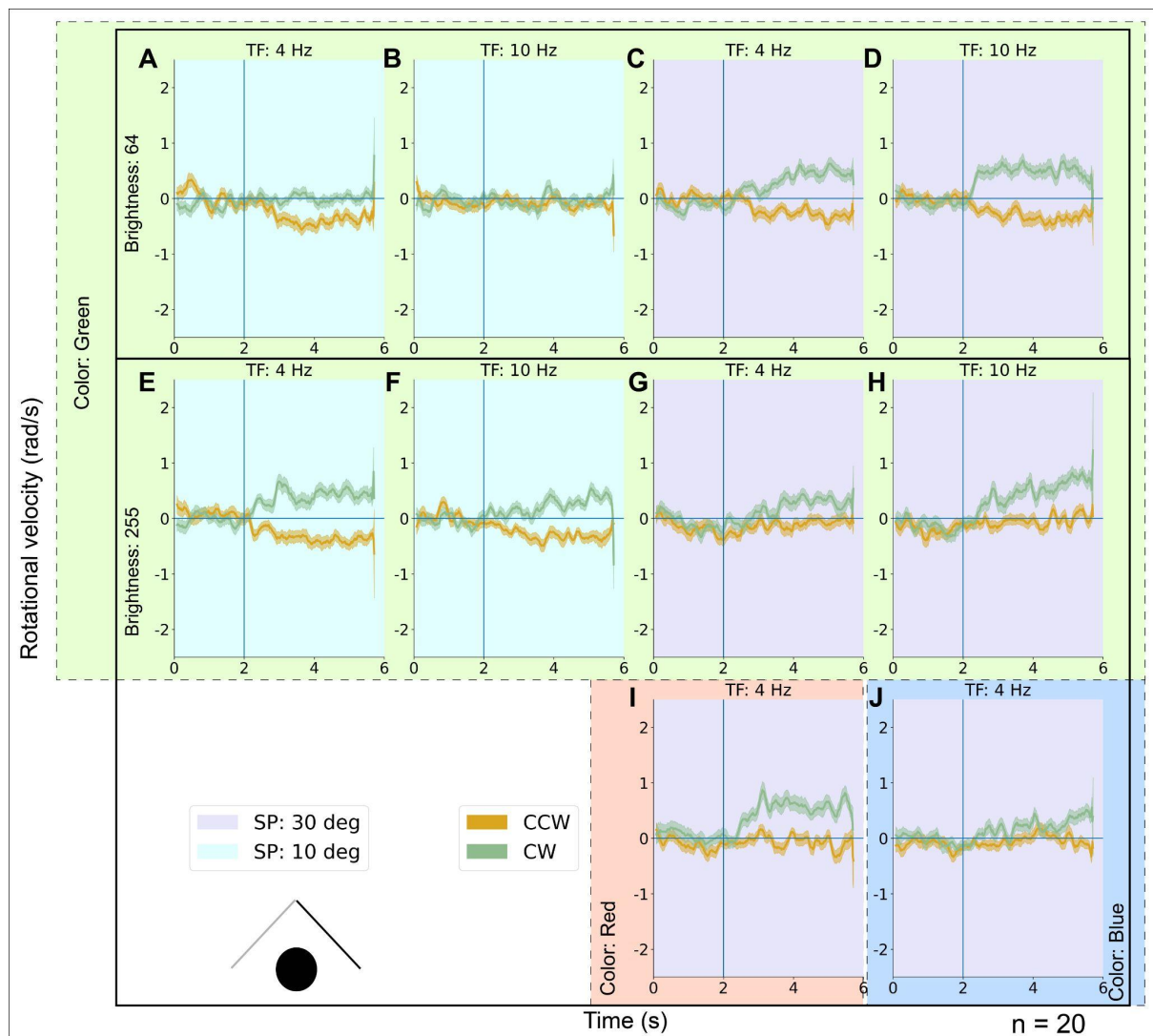


Figure 32. Two tablet analysis with the right tablet ON.

Optomotor responses ($n = 20$) to the two tablet analyses with the right tablet ON. The left tablet stimuli have been masked, and thus, it shows a black colour. TF: Temporal frequency, SP: Spatial period. (A) - (J) represent the conditions as mentioned in the figure.

Figure 32 shows the turning response to the conditions shown as per Protocol 6. At least one of the responses to conditions in one direction is significantly different from zero. An exception is the case of (Colour: Green, Brightness: 64, Temporal frequency: 10 Hz, Spatial period: 10 deg), where the responses in both directions are not significantly different from zero. There is a mix of symmetric and asymmetric responses across the different conditions.

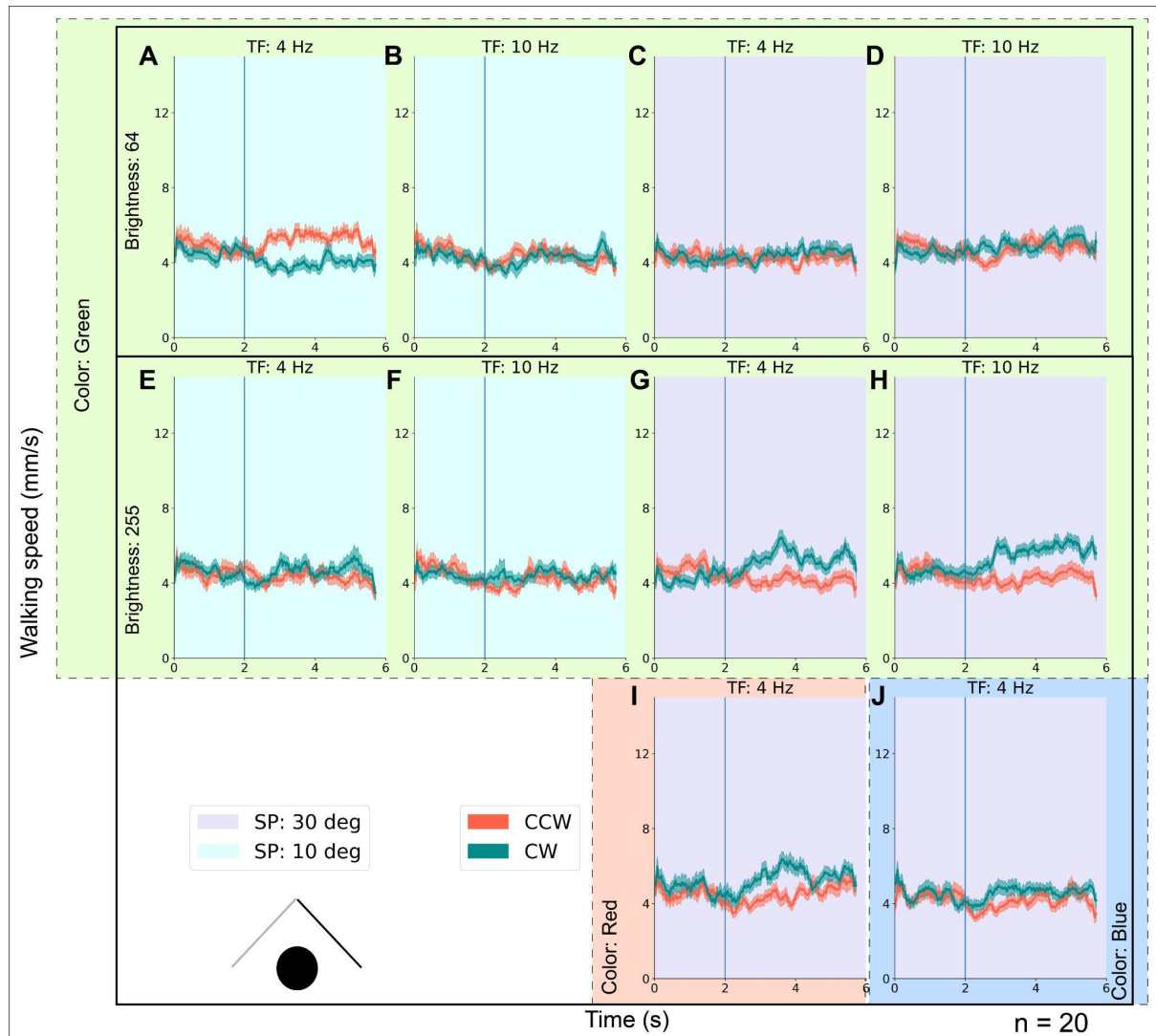


Figure 33. Walking speed with the right tablet ON.

(A) - (J) represent the conditions as mentioned in the figure.

Figure 33 shows the time series of the walking speed of the flies when the right tablet is ON, and the stimulus conditions follow Protocol 6. Similar to Figure 31, the differences in the magnitude of the walking speed between the clockwise and

counterclockwise conditions in Figure 33 parallel the differences in the magnitude of the turning response between the clockwise and counterclockwise conditions in Figure 32. Lower walking responses result in lower turning responses.

Next, I ran Protocol 6 when only the left tablet was ON. Figure 34 shows the results of the experiment.

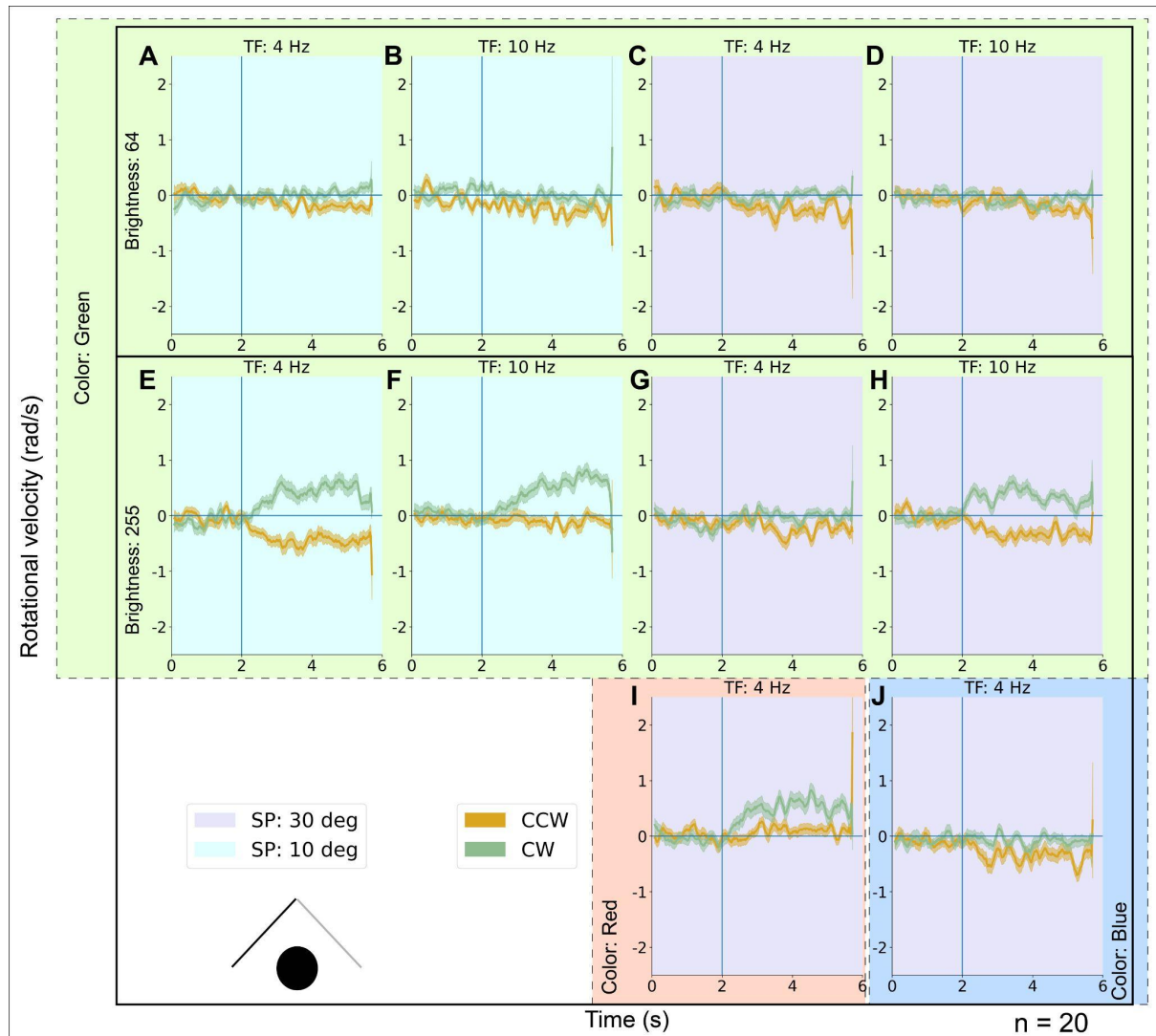


Figure 34. Two tablet analysis with the left tablet ON.

Optomotor responses ($n = 20$) to the two tablet analyses with the left tablet ON. TF: Temporal frequency, SP: Spatial period. (A) - (J) represent the conditions as mentioned in the figure.

Figure 34 shows the responses to the conditions when the left tablet is ON shown as per Protocol 6. The flies turn at least in one direction in all of the conditions, except for the following condition: (Colour: Green, Brightness: 64, Temporal frequency: 10 Hz, Spatial period: 30 deg). In the cases highlighted above, the flies show no significant turning response in either of the directions.

In the case of (Colour: Green, Brightness: 255, Temporal frequency: 4 Hz, Spatial period: 10 deg) and (Colour: Green, Brightness: 255, Temporal frequency: 10 Hz, Spatial period: 30 deg), the turning response is symmetric in both the directions. In the other cases, it is asymmetric and biased either towards the clockwise or the counterclockwise direction. In the cases of (Colour: Green, Brightness: 255, Temporal frequency: 10 Hz, Spatial period: 10 deg) and (Colour: Red, Brightness: 255, Temporal frequency: 4 Hz, Spatial period: 30 deg), the turning response is biased towards the clockwise direction, while in the other cases, it is biased towards the counterclockwise direction.

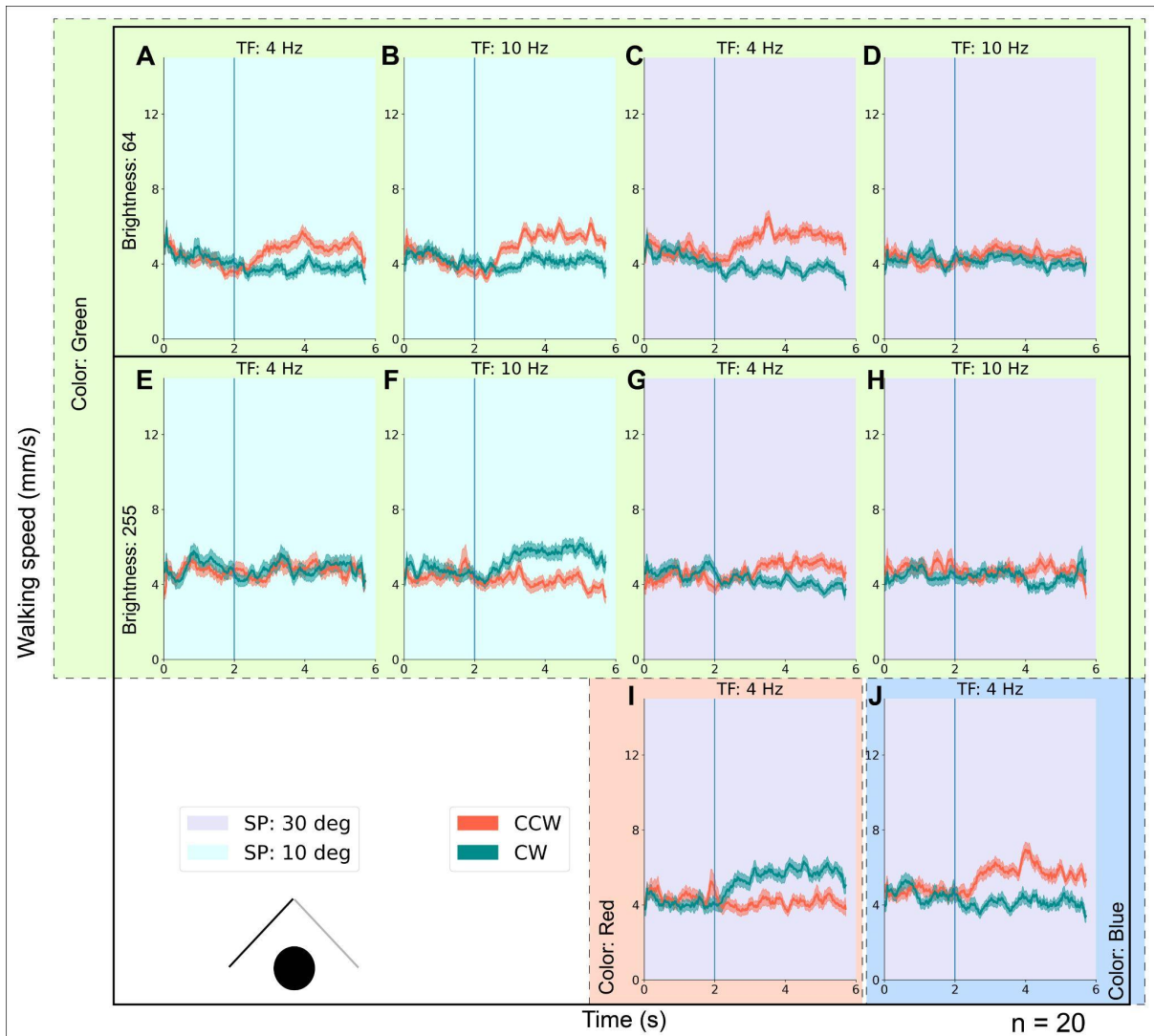


Figure 35. Walking speed with the left tablet ON.

(A) - (J) represent the conditions as mentioned in the figure.

Figure 35 shows the time series analysis of the walking speed of the flies when the left tablet is ON in response to conditions shown as per Protocol 6. Upon comparing Figures 34 and 35, one can see that the turning response magnitude difference between the clockwise and counterclockwise directions stems from the difference in walking speed. This again follows from my interpretation of previous figures, where lower walking speed gives rise to lower turning responses. Additionally, a higher walking speed also gives rise to higher turning responses.

Summary of the responses to the two tablet experiment

The summary of the data from the experiments obtained from the two tablet analyses is shown in Figures 36, 37, 38, 39, 40 and 41.

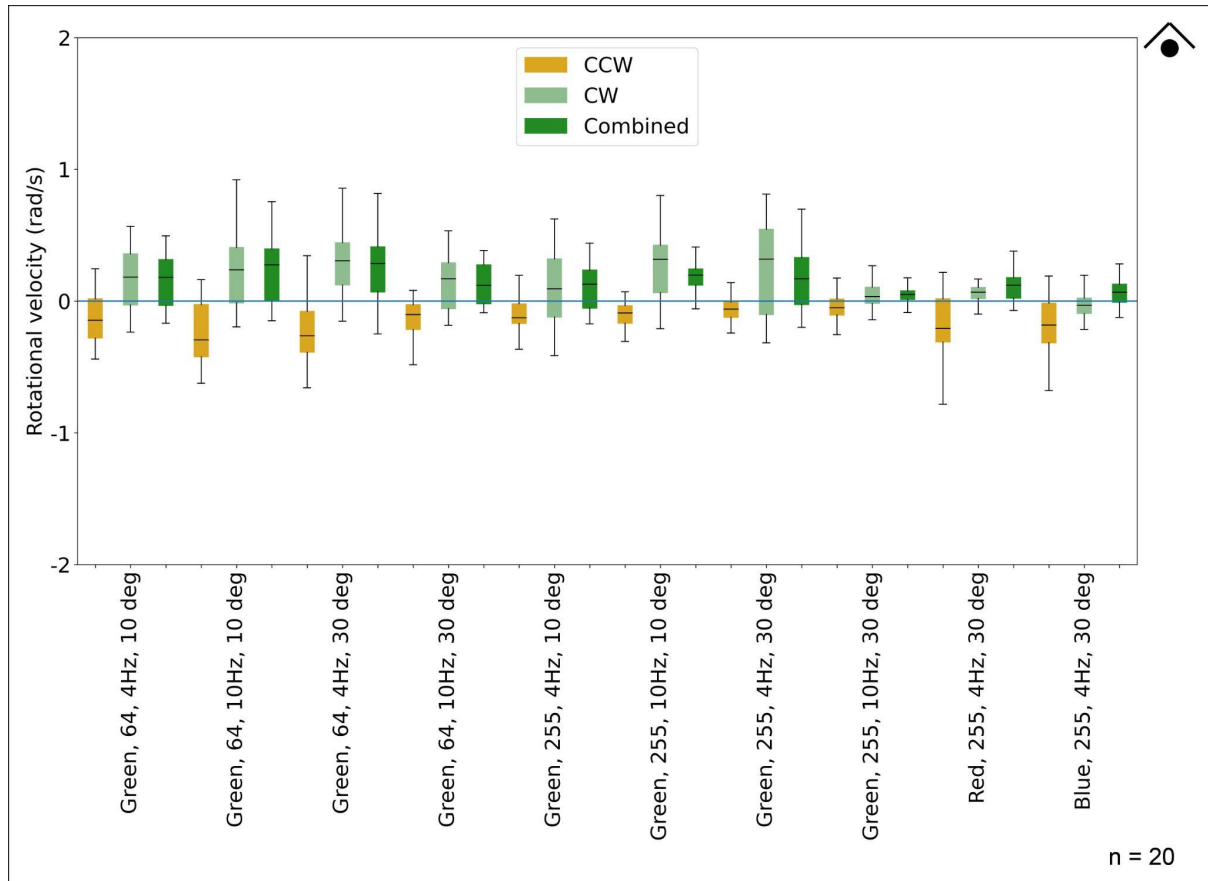


Figure 36. Summary of turning responses when both the tablets are ON.

Figure 36 shows that the clockwise and counterclockwise responses are not as strong as those in the case where the tablet is placed in front of the fly, and the stimulus is run as per Protocol 5. In this case, the responses go up to 2 rad/sec in either direction. In Figure 36, the maximum response is 1 rad/sec. Some of the responses are not even significantly different from zero, like the (Colour: Blue, Brightness: 255, Temporal frequency: 4 Hz, Spatial period: 30 deg), (Colour: Green, Brightness: 64, Temporal frequency: 10 Hz, Spatial period: 30 deg) and (Colour: Green, Brightness: 64, Temporal frequency: 10 Hz, Spatial period: 10 deg) cases.

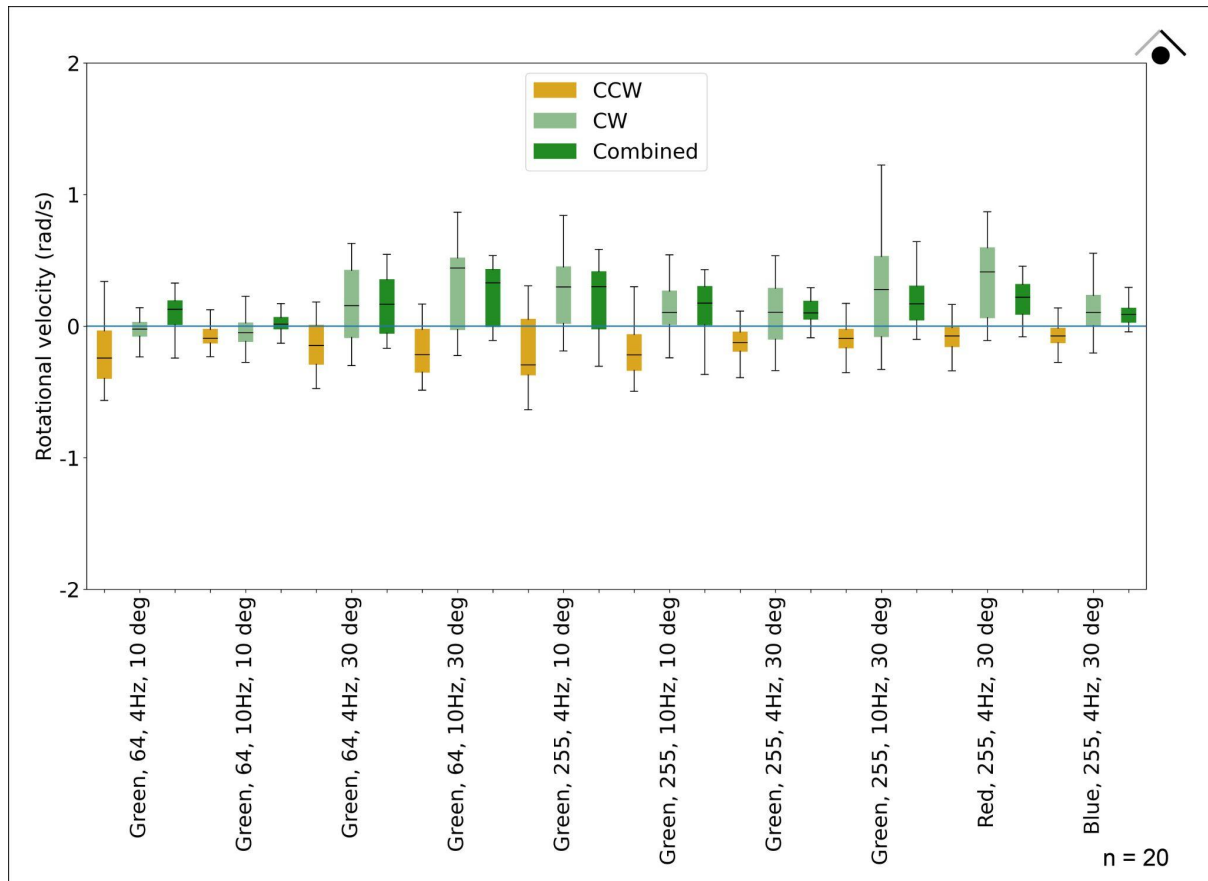


Figure 37. Summary of turning responses when the right tablet is ON.

Figure 37 summarises the turning responses to the stimulus conditions shown when the right tablet is ON, as per Protocol 6. It shows that the responses to the clockwise stimulus are not stronger in magnitude as compared to the counterclockwise responses. In some cases, the optomotor response is not significantly different from zero in the clockwise direction, as in (Colour: Green, Brightness: 64, Temporal frequency: 4 Hz, Spatial period: 10 deg), (Colour: Green, Brightness: 64, Temporal frequency: 10 Hz, Spatial period: 10 deg) and (Colour: Green, Brightness: 255, Temporal frequency: 10 Hz, Spatial period: 30 deg).

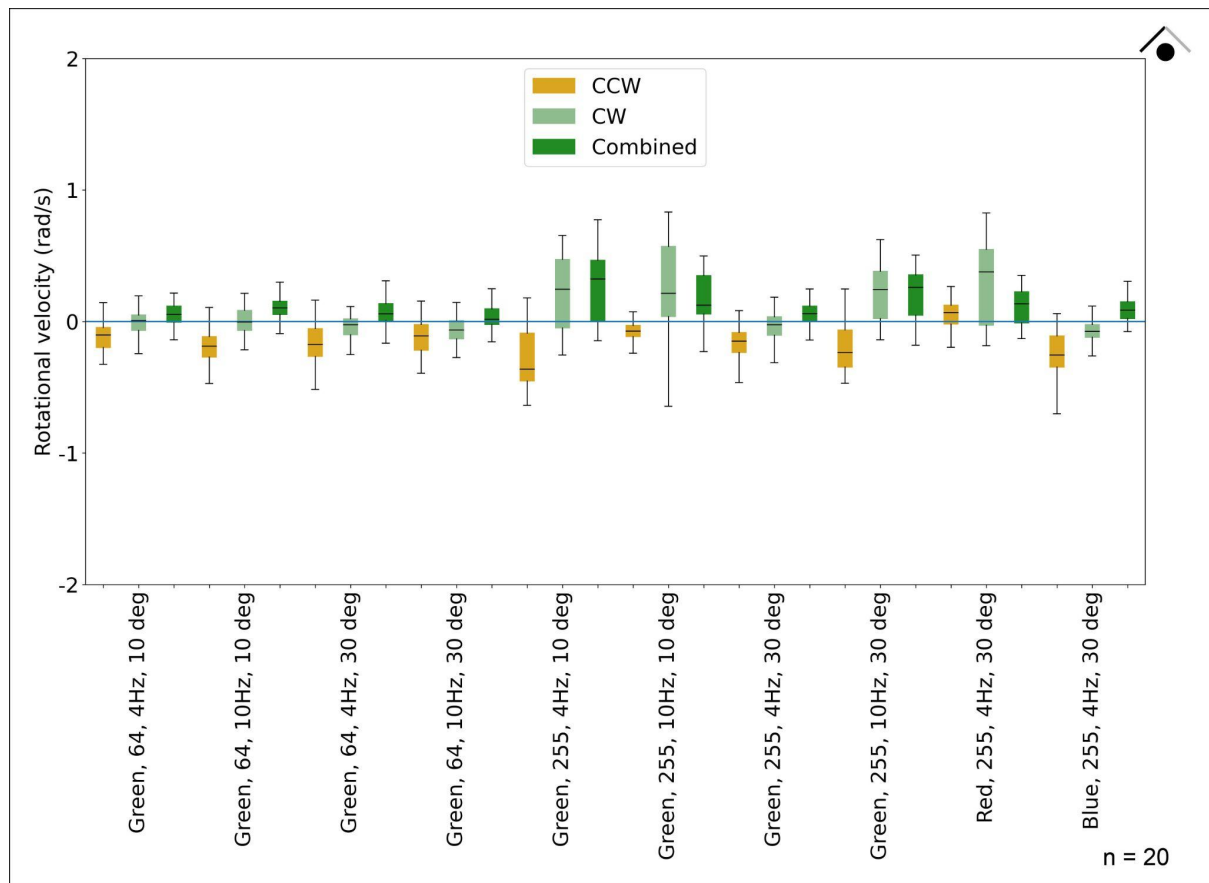


Figure 38. Summary of turning responses when the left tablet is ON.

Figure 37 summarises the turning responses to the stimulus conditions shown when the left tablet is ON as per Protocol 6. It shows that the responses to the counterclockwise stimulus are not stronger in magnitude compared to the clockwise responses. In some cases, the optomotor response is not significantly different from zero in the counterclockwise direction, as in (Colour: Red, Brightness: 255, Temporal frequency: 4 Hz, Spatial period: 30 deg).

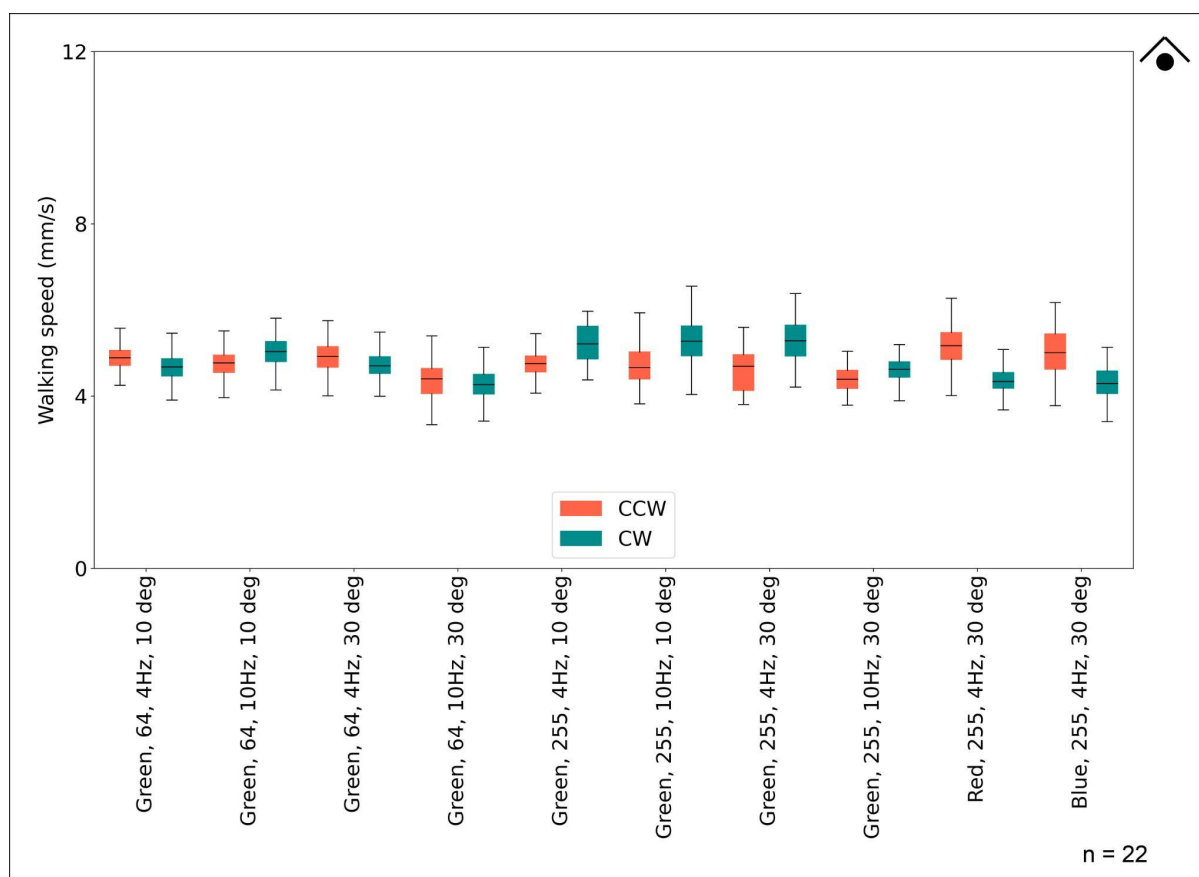


Figure 39. Summary of walking responses when both the tablets are ON.

Figure 39 summarises the walking speed of the flies when both the tablets are ON, and the stimulus conditions are presented as per Protocol 6. The overall walking speed of the flies is lower than the case when the tablet is placed in the front, and the flies are shown stimulus conditions as per Protocol 5 (Figure 28).

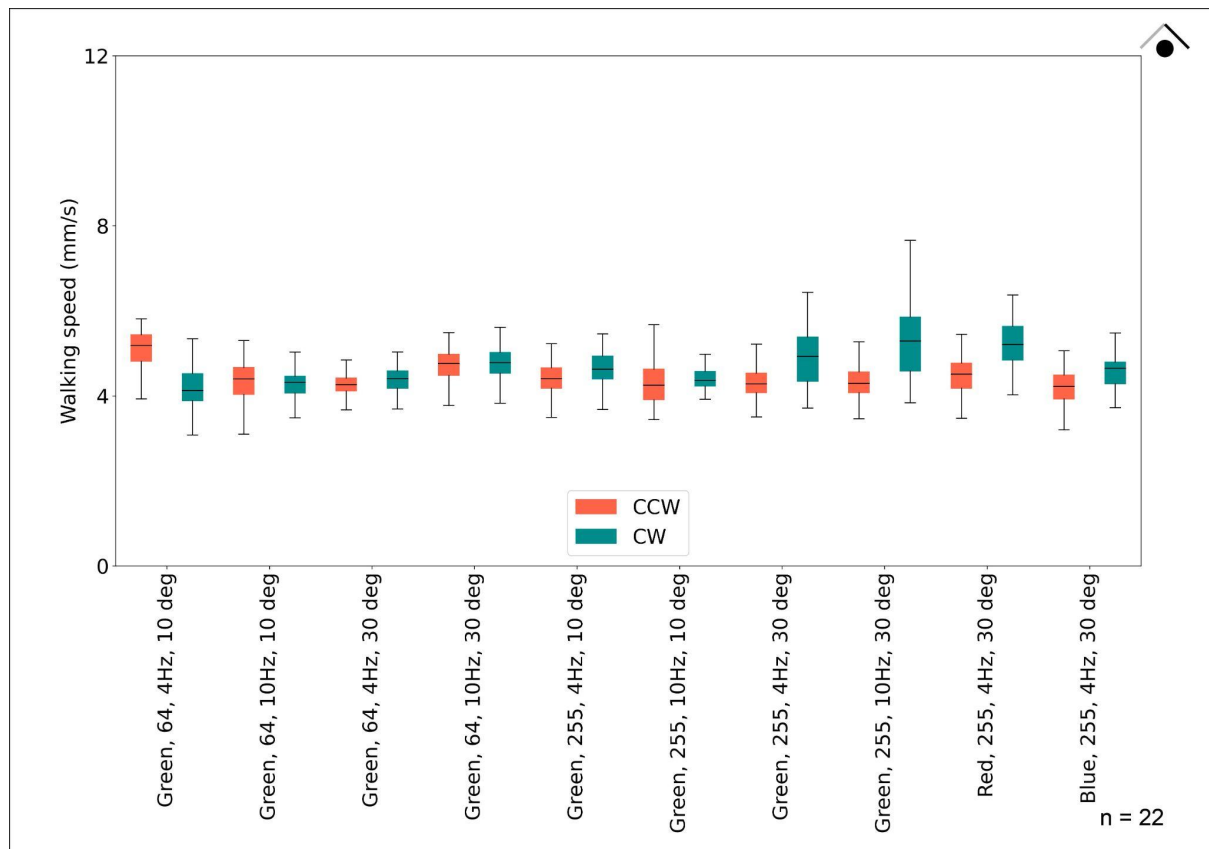


Figure 40. Summary of walking responses when the right tablet is ON.

Figure 40 summarises the walking speed of the flies when the right tablet is ON, and the stimulus conditions are presented to the flies as per Protocol 6. The difference between the turning responses (Figure 37) between the clockwise and counterclockwise directions parallel the differences in walking speed between the two stimulus directions.

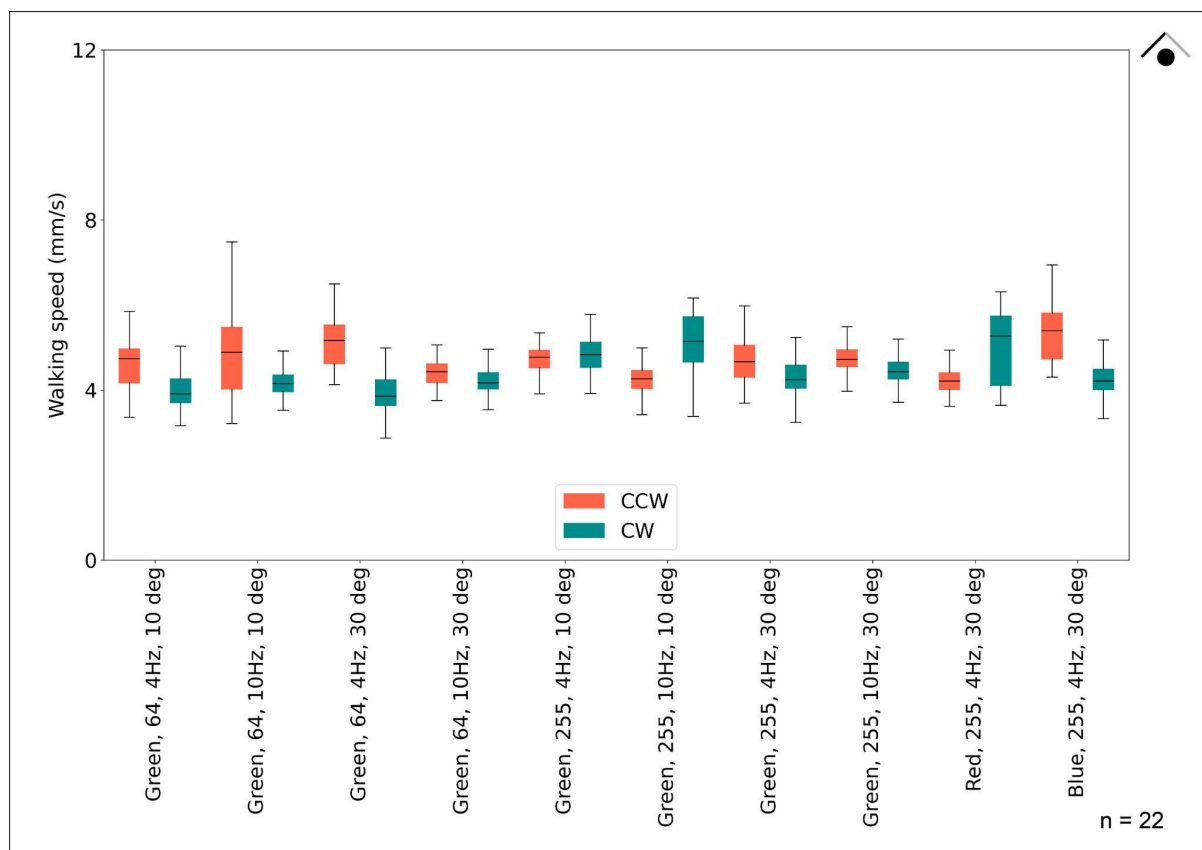


Figure 41. Summary of walking responses when the left tablet is ON.

Figure 41 summarises the walking speed of the flies when the left tablet is ON, and the stimulus conditions are presented to the flies as per Protocol 6. The difference between the turning responses (Figure 38) between the clockwise and counterclockwise directions parallel the differences in walking speed between the two stimulus directions.

Figures 39, 40 and 41 show that the flies walk less as compared to responses seen when stimuli are shown via the single tablet setup. One explanation for the lower turning behaviour as compared to the single tablet setup is the lower walking behaviour of flies. Additionally, a higher walking speed also results in higher turning responses of the flies. However, even while comparing the different setup conditions in the two tablet setup, the flies do not turn as expected, implying that the setup still needs to be optimised further, and this result is not just a fallout of the lower walking behaviour.

Discussion

A binary classifier to distinguish between walking and not walking behaviours

In this thesis, I have presented a binary classifier to distinguish between walking and not walking behaviours using a data-driven approach. Figure 15 shows the result of this classifier. However, this classifier does not replicate previous results suggesting that starved flies walk more than non-starved flies due to increased priorities towards foraging and feeding (Dethier, 1976; Hughson et al., 2018). Flies also walk more consistently in heated environments at temperatures of 32°C compared to room temperature environments (Huey et al., 1992; Soto-Padilla et al., 2018). A plausible explanation for this result is that the rig was already heated up to 32°C in order to ensure that flies walk on it. This could compensate for the increase in walking behaviour when seen in food-deprived flies compared to fed flies.

Optomotor responses in *Drosophila melanogaster*

The optomotor responses shown by flies on the inexpensive setup are similar to the ones shown by flies on setups that use LED displays of stimuli as have been seen before (Mathejczyk & Wernet, 2020) (Figure 42). The turning responses are also similar to those observed in previous experiments on the inexpensive setup (Loesche & Reiser, 2021). Figure 42 shows that flies on setups with an LED display turn up to 40 degrees/sec, while on the setups with the tablet display, they turn up to 60 degrees/sec. This indicates that the optomotor response shown by flies on the inexpensive setup, which uses tablets as the display, is comparable to the expensive setups that use LED arrays as displays.

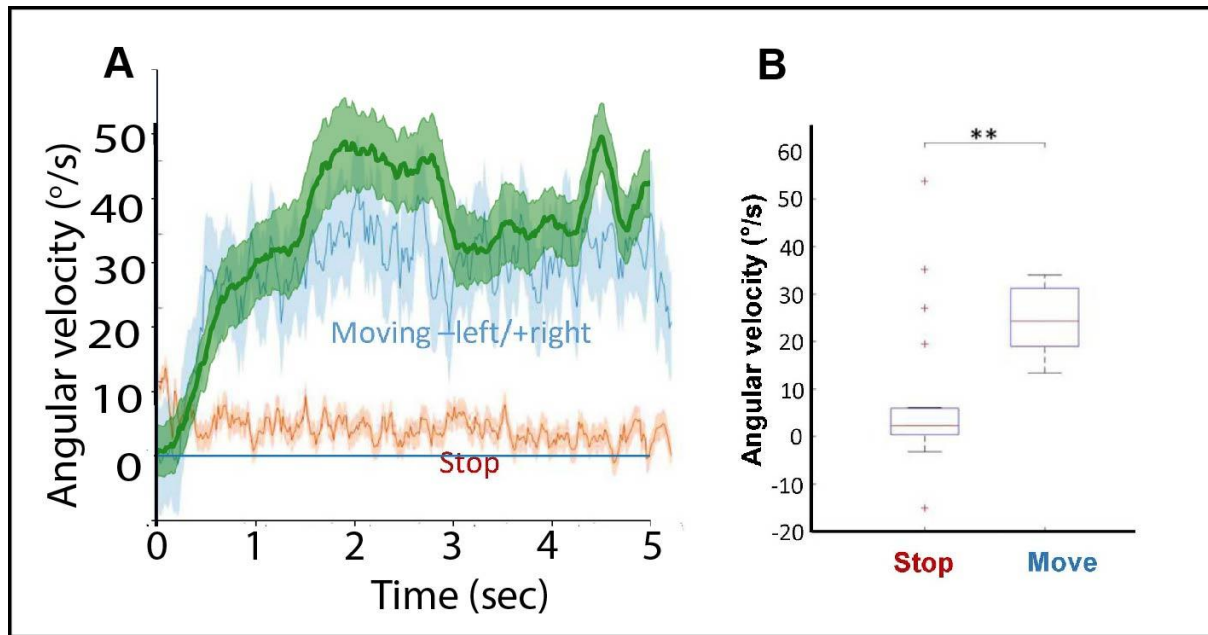


Figure 42. Comparison of optomotor responses in my setup to earlier experiments.

(A) - (B) Rotational velocity response measured in flies in response to stimuli displayed on an LED display. The LED display used here has a refresh rate of 400 Hz and covers 360° azimuth and 45° altitude from the fly's perspective. The stripes shown here are green coloured on a red background. "Moving" represents moving stimuli, while "Stop" represent stationary stimuli. The green curve represents combined data (CW/-CCW) collected from protocol 2 in response to a green coloured stimulus of intensity 50. Figure reproduced with permission from Elsevier (Mathejczyk & Wernet, 2020).

Optomotor responses to early versus late trials

The results of the optomotor experiment comparing early versus late stimuli suggest that although the means of the early and late stimuli are significantly different from each other during these conditions, there is no clear trend of increase or decrease across early versus late stimuli.

Intensity analysis of visual stimuli

I have presented an analysis of the responses to visual stimuli at varying intensities here. The average rotational response of the fly follows a sigmoid-shaped

distribution (Figure 43), typical of such psychometric functions (Wallis et al., 2013). The response is nearly zero at low intensities, and sharply increases at an intensity of 32. It then saturates at higher intensities.

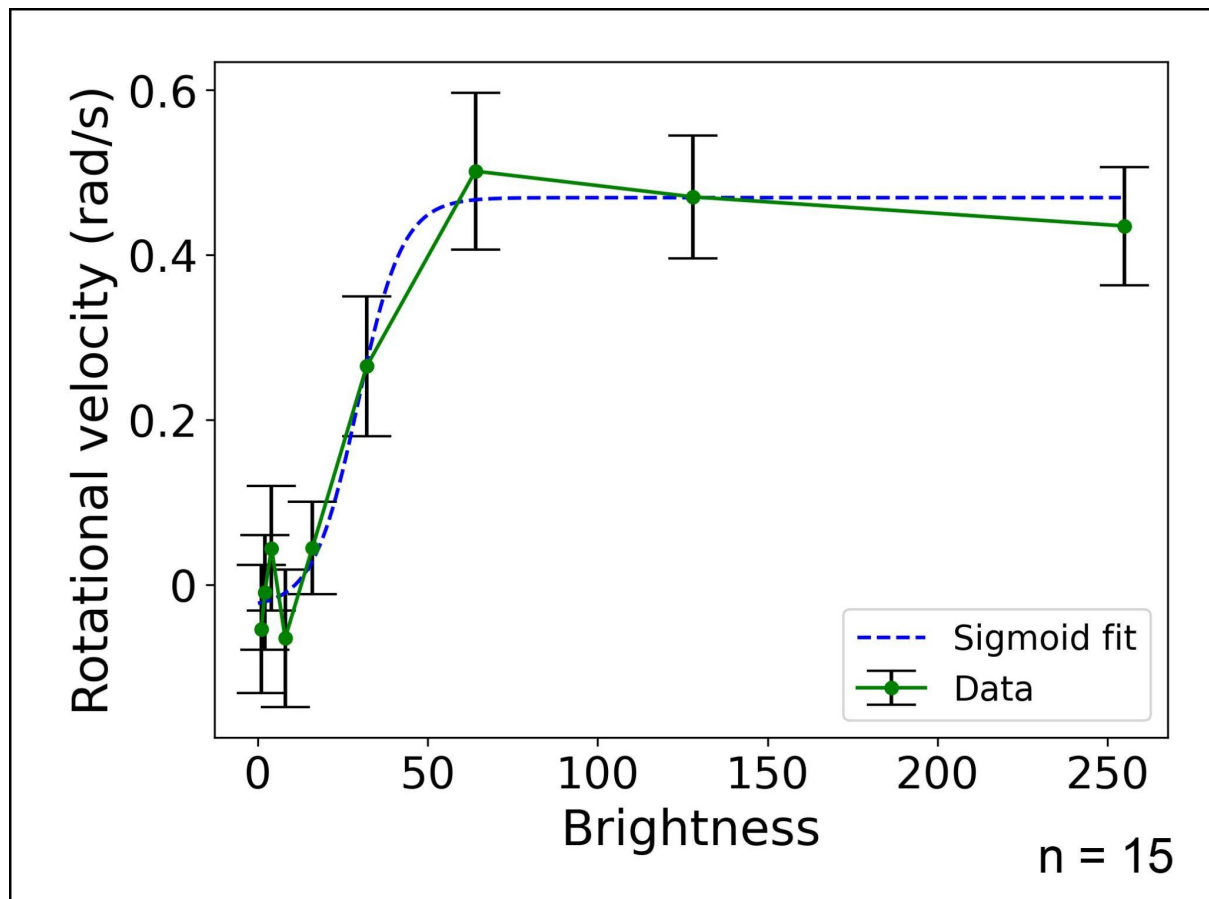


Figure 43. Sigmoid function of optomotor responses across intensities.

Plot of the rotational velocity against intensity (intended brightness) for the green coloured gratings. I fit the sigmoid function to the data using the `scipy.optimize` library.

Interestingly, the flies also have a strong turning response to the red gratings, although flies are known to be insensitive to red light (Hanai et al., 2008). The fact that I am using a tablet to display stimuli can explain this result. The tablet may contain a broad spectrum of wavelengths used to represent “red”, including wavelengths to which a fly is sensitive.

Optomotor responses to a single tablet in two different positions

As seen in Figures 23 and 25, the responses after placing the tablet in front of the fly are symmetric in both stimulus directions. This contrasts with the responses seen after placing the tablet at the side of the fly, where the responses are asymmetric.

One can explain this asymmetry in optomotor responses by the fact that in the case of the tablet placed on the right side of the fly, a clockwise stimulus represents a front-to-back or progressive stimulus on the eye of the fly. In contrast, a counterclockwise stimulus represents a back-to-front or regressive stimulus on the eye of the fly. Earlier measurements have shown that the magnitude of turning responses to front-to-back stimuli is greater than the responses to back-to-front stimuli (Creamer et al., 2018; M. B. Reiser & Dickinson, 2010; Tang & Juusola, 2010). Additionally, researchers have shown that flies walk less in response to a back-to-front stimulus than to a front-to-back stimulus (Creamer et al., 2018). The neural pathways leading to this visual modulation of walking speed are also known (Creamer et al., 2018).

Optomotor responses to two tablets

Case I: Both tablets ON

In this case, I expect a symmetrical turning response in both the clockwise and counterclockwise directions. However, Figure 30 shows that this is not necessarily the case. In some cases, I do observe a symmetric response (Figure 30 A, B, C and D). However, in other cases, I obtain a response which is biased towards the clockwise direction (Figure 30 E and G) or a response biased towards the counterclockwise direction (Figure 30 I) or no significant response (Figure 30 F, H, J).

Case II: Right tablet ON

In this case, I expect the turning responses of the fly when the right tablet is ON to be asymmetric and similar to those obtained in Figure 24, where the flies were shown stimulus conditions according to Protocol 5, and the tablet was placed at the

side. I expect that the responses to the clockwise stimulus are greater in magnitude as compared to the counterclockwise stimulus. However, Figure 32 shows that this is clearly not the case. I observed symmetric responses for a lot of conditions and asymmetric responses, sometimes biased in the opposite direction of expectation. Figure 37 additionally shows that the responses to the clockwise stimulus are not stronger in magnitude as compared to the counterclockwise responses.

Case III: Left tablet ON

In this case, I expect the turning responses of the fly to be asymmetric but biased towards the opposite direction as compared to those obtained in Figure 24, where the flies were shown stimulus conditions according to Protocol 5, and the tablet was placed at the side. In other words, the responses to the counterclockwise conditions should be greater in magnitude than those for the clockwise direction. While this is true for some cases (Figure 34 A and C), it is not for most other cases. In these cases, I observe either a symmetric response in both directions or an asymmetric response in the opposite direction. Figure 37 additionally shows that the responses to the counterclockwise stimulus are not stronger in magnitude as compared to the clockwise responses.

Reasons for unexpected responses

The asymmetric responses obtained when both the tablets are ON can result from reflections between the stimuli of the two tablets leading to one dominant response over another. In some cases, stimuli and responses from both tablets can cancel each other out, giving rise to no significant optomotor response on average across flies. Another factor that could be responsible for giving rise to asymmetric responses is the differences in refreshes of the screen of the two tablets. The two tablets function as a “spatially extended” screen for the stimuli, whereby two or more screens function to display a continuous stimulus. This is different from a single “normal” screen, where a single screen is used to display the stimulus. Though the two tablets have the same refresh rates, the exact time of refreshing the screen is not in sync for the two tablets. This could also account for the unexpected responses. The refresh rate of the tablets is 60 Hz, which is not greater than the flicker fusion frequency of the animal (Autrum, 1958; Cosens & Spatz, 1978;

Haberkern et al., 2019; Miall, 1978), making it plausible for them to sense a difference in image movement between the two displays.

Future work

Exploring the cause of the unexpected symmetries and asymmetries of the optomotor response in the two-tablet setup is the immediate next step in the project. Exploring whether a change in the distance of the tablet from the fly can account for any of these differences can also be tested while optimising virtual reality for the setup.

This setup, coupled with the robotic arm, will enable one to automate experiments and study more complex behaviours involving the circadian rhythms of the fly.

One can do a larger repertoire of optomotor experiments with the two-tablet setup. Experiments where experimenters do not see the optomotor responses when the stimulus is present right in front of the fly, but are present when you increase the visual field of view of the fly, can be done using the multiple tablet setup.

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