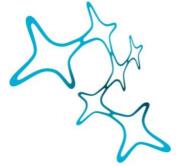
## Navigation in freely moving Drosophila melanogaster

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## Summary

The optomotor response is a behavior displayed by insects like *Drosophila melanogaster* as a response to whole-field motion. When scientists first observed this behavior, they were faced with what we call "a black box system," meaning they could control the visual input (i.e. the velocity of a high-contrast grating) and record the behavioral output (i.e. rotation of the fly), but they did not have any means of understanding the mechanism that processed the visual information and triggered the motor program they observed. Today, the brain of the fly is no longer a black box: the various advanced technologies for genetic targeting and manipulating individual nerve cells have helped us understand the neural network responsible for motion vision.

In Manuscript 1, my colleagues and I aimed to discover the relevance of motion vision for one of the most important but complex tasks that a fly can perform: flight control. During a flight bout, a fly executes a series of long, rather straight flight segments, interrupted by sudden changes in flight direction called saccades. By blocking the primary motion-sensitive neurons of the fly brain, i.e. T4/T5 cells, we could investigate flight performance in motion-blind flies. When inducing an aerodynamic imbalance by clipping the tip of one wing, motion-blind flies show reduced straightness of the intersaccadic segments compared to the wild-type controls.

In **Manuscript 2**, we show that once a fly has discovered a food source, it can remember its location with the help of path integration. Path integration is a process first observed in nesting animals, which refers to the ability of an animal to return in a straight-line route back to the nest after a long, sinuous, foraging trip. The path integrator performs a vector summation of the direction and length of each walking bout, updating itself continuously. We show that flies remember the distance between two reward zones using path integration. In future experiments, we plan to use this experimental approach to investigate the neural substrate of the path integration process.

Taken together, the two manuscripts demonstrate the power of behavioral observations in freely moving animals. Based on technological advancements, animals no longer need to be restrained, thus opening the door to study complex behaviors such as flight control and path integration.

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## Chapter 1: THE ORIGINS OF VISION

## 1.1 How the sun influenced evolution

Life is governed by sunrise and sunset. This recurrent permanence is most likely the leading selective factor in the evolution of nearly all living creatures on Earth (Fernald, 2000). If the Earth would have been a bit closer or a bit further away from the sun, nothing would have evolved the way we know it today (Hart, 1978). The Sun governs everything on our planet: plants use sunlight for nourishment, while animals evolved organs that use light as a means of perceiving their surroundings, helping them locate food sources and mates and avoid predators.

Cyanobacteria evolved a method to use the wavelengths from the sunlight, converting them into energy to upkeep cellular metabolism. This gave way to the evolution of betacarotene, the precursor of retinal. The development of the visual pigment, which was formed by covalently bonding an opsin molecule to retinal, was a critical moment in the evolution of the eye (Schwab, 2018). Sunlight plays a key role in the isomerization process, transforming the visual pigment from cis to trans configuration using the energy of photons. This conformational change triggers a signaling cascade called phototransduction (Kefalov et al., 2005). Using this process, photoreceptors could signal the presence or absence of light, and the first circadian rhythms were established.

The aggregation of multiple such cells with the capacity to measure light intensities from different neighboring points in space has led to the formation of the eye spot (Schwab, 2018). This gave unicellular organisms the capacity to locate the light, which they then used for nourishment. More complex, pluricellular organisms such as the early metazoans (Arendt et al., 2009), not only needed to monitor the amount of ambient light but also to discern the direction from which the light is shining to avoid exposure to extreme intensities. They evolved a screening pigment which partially restricted the field of sensitivity of the photoreceptors, allowing them to discern direction from which the light shines (Land and Nilsson, 2012).

## 1.2 The parallel evolution of the vertebrate and invertebrate eye

Progression to more complex visual tasks, such as detection of self-motion, landmark orientation, and collision avoidance has expanded the list of requirements for the rudimentary eye spot (Land and Nilsson, 2012). The need for monitoring different directions simultaneously has pushed for the distribution of the photo-sensitive cells in a

pit (precursor of ommatidium) or cup (precursor of vertebrate and cephalopod eyes) structure, allowing for a more exhaustive sampling of the environment.

Higher spatial resolution, required for predator evasion and mate detection, derived the necessity of light focusing lenses. Moreover, both compound and camera-type eyes were pushed by evolution to improve mechanisms for contrast and angular sensitivity, to cope with the higher resolution demands (Land and Nilsson, 2012). In the following, I will describe the principles of insect vision by focusing on key moments in the evolution of the insect eye. I will then present some principles of vision and navigation based on the brain of the model organism *Drosophila melanogaster*.

## Chapter 2: INSECT VISION

#### 2.1 Phototransduction

By following the simple timeline provided by evolution, all visual systems can be described. Let's start with the phototransduction mechanism which made vision possible.

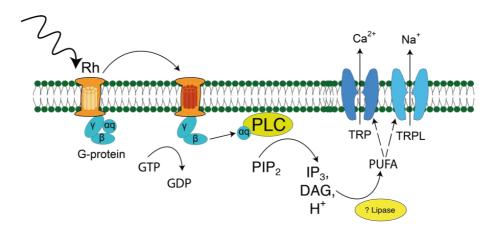


Figure 1: The phototransduction mechanism

The absorption of a photon causes a conformational change of the photoreceptor's chromophore, causing a biochemical cascade: A  $G_q$ -protein-coupled receptor is spliced by a GTPase enzyme. The alpha subunit activates Phospholipase C (PLC). PLC facilitates the hydrolyzation of PIP $_2$  into IP $_3$ , DAG and the free proton. DAG interacts with a lipase to produce PUFAs which, in turn, open  $Ca^{2+}$  and  $Na^+$  ion channels. Figure reproduced and modified with the permission of Tabea Schilling.

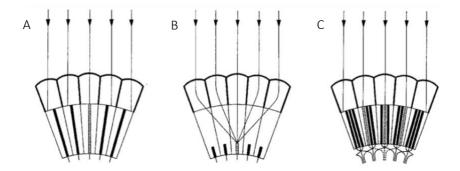
The invertebrate visual pigment is comprised of an opsin which is bonded to a chromophore via a Schiff base. The absorption of a photon is changing the 11-cis 3-hydroxy-retinal chromophore to an all-trans configuration. This conformational change causes a biochemical cascade that ends in an excitation of the photoreceptor cell (Yarfitz

and Hurley, 1994, Schwemer, 1989). A G<sub>q</sub>-protein-coupled receptor is spliced by a GTPase enzyme, and the resulting alpha subunit activates the Phospholipase C (PLC). While for vertebrates, the rest of the phototransduction cascade is well understood, in invertebrates, there are still some questions to be answered. In contrast to vertebrates, in the insect phototransduction mechanism, an increase in photon levels elevates the level of free calcium ions in the cytosol (Yarfitz and Hurley, 1994) via the opening of the transient receptor potential (TRP) and transient receptor potential-like (TRPL) channels (Yau and Hardie, 2009). However, it is still not clear how that happens. It is known that PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) resulting in three byproducts: inositol 1,4,5-trisphosphate (IP<sub>3</sub>), diacylglycerol (DAG) and a free proton (Hardie and Raghu, 2001). One hypothesis states that polysaturated fatty acids (PUFAs) produced by the interaction of DAG with a lipase activate the TRP and TRPL channels (Chyb et al., 1999; Delgado and Bacigalupo, 2009, Hardie and Jusola, 2015). Another hypothesis proposes that these channels open as a result of PIP2 level reduction and simultaneous cytosolic acidification resulted from the hydrolyzation process (Huang et al., 2010). Nonetheless, the opening of TRP and TRPL channels causes an increase in cytosolic Ca<sup>2+</sup> and Na<sup>+</sup> which results in a depolarization of the photoreceptor cells (Stavenga, 1995; Yarfitz and Hurley, 1994).

This phototransduction mechanism has fascinating capabilities in *Drosophila melanogaster*. Fruit flies can sense light levels as low as a single photon as well as variations of illumination in bright environments, simply by adapting to the background level of the environment via the modulation of cytosolic Ca<sup>2+</sup> levels (Yarfitz and Hurley, 1994; Gu et al., 2005).

## 2.2 The insect retina

Nearly every principle known in optics has been employed by evolution during the process of perfecting visual resolution. Of great interest are compound eyes, which treat image formation differently than classic camera-type eyes. Compound eyes, common in insects and some aquatic animals are an accumulation of multiple single-faceted eyes called ommatidia. There are 3 types of compound eyes in nature, characterized by different levels of light sensitivity and spatial resolution: apposition eyes, neural superposition eyes, and superposition eyes. Evolution has attempted with each of these models to design a system that would produce an image of sufficient brightness and clarity within the constraints of optical properties of biological materials (Laughlin, 1989).



**Figure 2: Schematic representation of compound eyes types (A)** Apposition eyes **(B)** Superposition eyes **(C)** Neuronal superposition eyes. Modified from Kirschfield, 1967.

## 2.2.1 Apposition eyes

Appositions eyes are common among arthropods. They are characterized by ommatidia which function as independent, isolated optical units. An ommatidium has several components: a cornea, a cone, a rhabdom, and a pigment screen. Each ommatidium has a group of eight photoreceptors containing rhabdoms which represent the photosensitive structures. From a spatial resolution perspective, they are the least economical, since the lens diameter has to be limited to ensure the acuity of an image, which causes fewer photons to be absorbed by each unit (Nilsson, 1989). To improve the spatial resolution of such eyes, both the number of ommatidia and the lens size would have to be increased (Kirschfield, 1976), which would be ecologically impossible.

## 2.2.2 Superposition eyes

Compared to apposition eyes, superposition eyes do not have optically isolated ommatidia. In the absence of the screening pigment, a clear zone is available for the photons to cross between ommatidia. Thus, one rhabdom receives light from many facets, increasing the system's sensitivity to light (Nilsson, 1989).

#### 2.2.3. Neuronal superposition eyes

In the apposition eyes, rhabdomeres are fused. On the contrary, in Dipterans the rhabdomeres are isolated from one another and distributed in such a manner that the axes of eight rhabdomeres (two from the central ommatidium and one from each of the six surrounding ommatidia) are aligned, allowing seven facets to sample the light in the same space in the visual field. This orientation decreases photon noise by capturing more photons from the same space, thus increasing sensitivity without degrading spatial resolution. These features make it the most successful compound eye of the three types (Nilsson, 1989). Axons from these units reach the same column in the lamina. This

particular arrangement of the axons was coined by Kirschfeld (1967) as "neuronal superposition".

## Chapter 3: DROSOPHILA AS A MODEL ORGANISM

## 3.1 The benefits of using *Drosophila* as a model organism

A common strategy for understanding complex neural processes is to investigate simpler systems, which have a higher probability of being ascertained, given the currently available methods (Hardie, 1986). Thus, the fly motion vision system is a great intermediate level in the journey of understanding how the human brain works. Not only are flies highly genetically amenable, small in size, and cheap to rear, but their visual system has a similar organization to that of vertebrates at only a fraction of the number of neurons (Hardie, 1986; Pak and Pinto, 1976). Moreover, the fruit fly presents a vast repertoire of complex behaviors which have been extensively characterized. Thus, *Drosophila* is a great model organism for analyzing the mechanisms of vision (Franceschini et al., 1989).

## 3.2 The importance of mutations

Before methods for targeted mutation appeared, scientists relied on natural or chemical-induced mutations to study the role of specific genes. For example, flies with a severe mutation in the *norpA* gene present a deficiency in Phospholipase C – an essential component in the phototransduction mechanism (Yarfitz and Hurley, 1994). Such a mutation causes the cell to remain electrically salient due to its inability to increase intracellular Ca<sup>+</sup> levels (Stavenga, 1995). Thus, flies carrying this mutation are blind, becoming useful control flies for experiments regarding vision.

## 3.3 Selective genetic manipulation

## 3.3.1 Gal4-UAS

Expressing a gene of interest in a spatially restrictive manner is of great importance in research. The ease to do so in *Drosophila melanogaster* has made it a favorite in the realm of model organisms (Jenett et al., 2012). A useful tool for targeting multiple, genetically similar cell populations simultaneously is the Gal4-UAS system. Gal4 is a potent yeast transcriptional activator, which can be used with any promoters bearing the Gal4 binding sites. By combining it with genes containing optimized Gal4 binding sites in an Upstream Activation Sequence (UAS) construct, we can drive the expression of any gene of interest in any cell population we desire. Great collections of Gal4 lines controlled by different

transcriptional enhancer fragments have now been fully imaged and documented, thus highlighting the benefits of using *Drosophila melanogaster* as a model organism (Jenett et al., 2012).

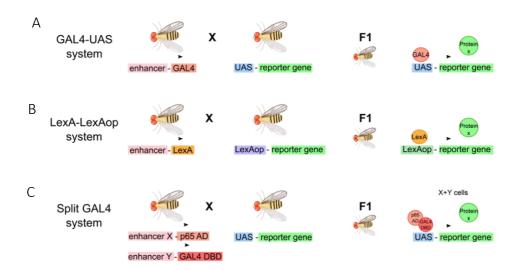


Figure 3: Methods for driving genetic targeted expression

**(A)** Gal4-UAS system: the reporter gene is attached to an Upstream Activation Sequence and is expressed under the presence of the Gal4 transcription activator. **(B)** LexA-LexAop: reporter gene attached to LexA DNA-binding motifs is expressed in the presence of LexA. **(C)** Split Gal4: by splitting the DNA binding domain and the transcription activation domain, the reporter gene is expressed only when both the AD and DBD domains are present in the same cell.

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#### 3.3.2 LexA-LexAop

A similar binary method for driving genetic expression is the LexA – LexAop system. LexA is found in the bacterium *Escherichia coli* and consists of a DNA-binding domain and a dimerization domain. It binds to gene-specific LexA DNA-binding motifs (LexAop) found upstream of the target gene (Pfeifer et al., 2010). By combining LexA-LexAop and Gal4-UAS systems in the same transgenic fly, one can independently express two reporters in two different cell populations. This is tremendously useful in experiments relating to neuronal connectivity (Jenett et al., 2012).

#### 3.3.3 Split-Gal4

Because the binary Gal4-UAS system has some disadvantages in the limited specificity of the available gene promoters, the system has also since been improved. Gal4 can be separated into two elements: a DNA-binding domain (DBD) and a transcription-activation domain (AD). Thus, a system combining an activation and the binding domain via a leucine zipper fragment enables them to become transcriptionally active (Luan et al.,

2006) and generates a higher specificity expression pattern. While individually, they do not drive expression, the heterodimerization of the two domains results in a transcriptionally competent complex, driving expression only at the intersection of the expression pattern of both (Luan et al., 2006, Luan et al., 2020).

#### 3.4 Fffectors

#### 3.4.1 Green Fluorescent Protein

A lot of information about a neural circuit can be inferred from the shape of the neurons, their synaptic partners, and their positioning in the brain. This can be achieved by visualizing neurons under the microscope. One approach is to tag the neurons of interest with green fluorescence protein (GFP) by driving its expression via the Gal4 – UAS system (Casso et al., 1999). GFP is a fluorescent protein found in nature in the bioluminescent jellyfish *Aequorea victoria*. By exciting the cells with blue light (395 nm), the purified GFP emits green light in return (540 nm), making it possible to visualize these cells under the microscope (Chalfie et al., 1994).

#### 3.4.2 Tetanus Toxin

One approach to determine the role of a subset of neurons is to block the neural activity of these neurons and then observe the behavioral output of the animal. In many cases, such an experiment determines the key function the neurons play in the targeted circuit. A great tool for blocking neural transmission is Tetanus Toxin (TNT). This toxin cleaves synaptobrevin, a key protein in the vesicle release process. This results in a complete loss of neurotransmitter release function due to the inability of the vesicle to perform exocytosis, thus blocking neuronal activity. Hence, if selectively expressing TNT in a targeted neuron results in a loss of phenotypical behaviors, it can be inferred that the neuron represents an essential component in the respective neural circuit (Rister and Heisenberg, 2006; Sweeney et al., 1995)

## 3.4.3 Kir2.1

Another approach for blocking neuronal activity is by driving the neuron's membrane potential towards hyperpolarization. This can be achieved by expressing potassium inward rectifying channels (i.e. Kir2.1) which produce a persistent outward K<sup>+</sup> current (Paradis et al., 2001), thereby reducing the excitability of the cell (Johns et al., 1999).

#### 3.4.4 Shibire

Both TNT and Kir2.1 expression cause irreversible neuronal blocking. Additional useful information could be inferred from the ability to switch the neurons on and off. A tool

that enables scientists to reversibly block neuronal activity is a mutated version of the gene *shibire*. This mutation was first discovered as a cause for temperature dependent paralysis. Adult flies carrying this genetic anomaly which were heated up to 29°C were completely paralyzed. However, when cooled down to 22°C, they would recover mobility in a matter of minutes (Grigliatti et al, 1973). This phenotype is explained by the failure of synaptic vesicles to reform, due to the dysfunctionality of Drosophila's dynamin equivalent, a protein important for the endocytosis process (Chen et al., 1991). By expressing the temperature sensitive *shibire* (*shibire*<sup>ts</sup>) protein using the Gal4-UAS system in a subset of neurons, one can observe behavioral changes caused by temperature manipulations which can reveal the function of the investigated neurons (Kitamoto, 2001).

## 3.5 Optogenetics

Sometimes, blocking neuronal activity is too severe a manipulation to understand intricate properties of neuronal circuits. Luckily, additional non-invasive approaches that allow a modulation of the neuronal activity have been developed. Optogenetics refers to the technique of inserting specific light-sensitive proteins in neuronal membranes, intending to manipulate membrane potential via light exposure. By targeting the expression of such genes via the binary expression systems presented above, one can manipulate the activity of a subpopulation of cells with similar genetic background. Lightsensitive channels, which open when excited with the appropriate wavelength, change the composition of ions in the cytosol of the cell, influencing its membrane potential (Fiala et al., 2010). Thus, neurons can be depolarized or hyperpolarized with millisecond precision (AzimiHashemi et al., 2014). One depolarizing optogenetic tool is Chrimson, a red-shifted channelorhodopsin activated by light between 470 and 617 nm. The red shift makes it convenient for deep-tissue stimulation and penetration through the fruit fly's cuticle. Moreover, flies are unable to perceive light in the red and infra-red spectrum (McEwen, 1918), making Chrimson a perfect tool for experiments on freely behaving animals (Klapoetke et al., 2014).

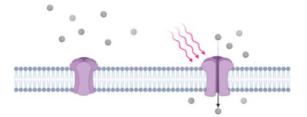


Figure 4: Optogenetic activation of Chrimson

Red light opens the Chrimson channel letting ions into the cytosol, resulting in a depolarization of the membrane potential of the neuron. Illustration created using BioRender.

# Chapter 4: PROCESSING OF VISUAL MOTION SIGNALS IN THE FLY BRAIN

During locomotion, the images on the fly's retina change in a continuous manner, with closer objects "moving" at a faster rate across the fly's retina than those further away (Tammero and Dickinson, 2002). The information provided by the optic flow is indispensable for the fly, producing an external reference system that yields valuable information about certain flight aspects such as altitude and speed (Fry et al., 2009). Thus, optic flow needs to be processed at an extremely fast pace and in a continuous manner.

### 4.1 Local motion detection

Each compound eye of the fruit fly has 750-800 almost identical ommatidia, which span approximately 330° in azimuth and 180° in elevation (Montell, 2012, Ryu et al., 2022). Photons cross the corneal facet lens of each ommatidium to be absorbed by eight photoreceptor cells (Stavenga, 1995). The first six (R1-6) are larger and have three-fold faster dynamics than the R7 and R8 (Cosens and Spatz, 1978). R7 and R8 have long been proposed as the spectral wavelength discrimination cells responsible for color vision (Heisenberg and Buchner, 1977), whereas R1-6 have a key importance in motion vision. Axons from R1-6 group themselves according to the point in space they observe, sending terminals to specific cartridges of the lamina following the retinotopy principles. In other words, two photoreceptors sampling neighboring points in space will synapse in two adjacent cartridges. Thus, the lamina is the first structure of the brain which holds the representation of the visual space as an ordered map (Shaw, 1989).

The luminance information from the light-sensitive cells is then divided into two streams: the ON and the OFF pathway (Jösch et al., 2010). This critical step is a great benefit: by having two parallel pathways in the visual system, the dichotomy between light and dark in natural scenes is better represented (Yang et al., 2016). The ON pathway signals increases in signal density (from dark to light), while the OFF pathway is responsible for processing signals of decreasing light intensity (from light to dark).

### 4.1.1 The ON pathway

Out of all the monopolar lamina cells, L5 presents the strongest increase in activity for increasing light intensities (Arenz et al., 2017, Drews et al., 2020). However, when blocking L1, flies fail to respond to moving ON edges, indicating that they are also important for the ON pathway (Jösch et al., 2010). Experiments blocking L3 activity do

not show a direct involvement in motion detection, but rather a role in contrast sensitivity for both ON and OFF edges (Rister et al., 2007, Tuthill et al., 2013, Drews et al., 2020).

Following again the principles of retinotopy, information from the lamina arrives in the medulla. Of great interest for the ON pathway are three subclasses of medulla intrinsic neurons (Mi1, Mi4, and Mi9), one class of transmedullary neurons (Tm3), and one class of centrifugal neurons (C3) (Arenz et al., 2017; Takemura et al., 2017, Groschner et al., 2022). Interestingly, most of these neurons have center-surround receptive fields: Mi1, Mi4, and Tm3 have an ON center and OFF surround, while Mi9 has an OFF center and an ON surround (Drews et al., 2020).

Electron microscopy data (Shinomiya et al., 2019; Takemura et al., 2017) have resolved the arrangement of these cells onto their postsynaptic partners, the T4 cells. Combining this knowledge with results from cell recordings (Groschner et al., 2022), we have a full picture of the inputs to T4 and the computations that make it the first motion-selective cell in the visual system. T4 dendrites are oriented in the opposite orientation to their preferred direction and span about 7 retinotopic columns, accordingly sampling the environment of 7 adjacent points in space (Shinomiya et al., 2019). The distal end of the dendrite receives glutamatergic input via the Mi9 cell, the middle section of the dendrites forms cholinergic synapses with Tm3 and Mi1, while the proximal end of the dendritic tree receives GABAergic input from C3 and Mi4. The spatial arrangement of inputs, along with the neurotransmitters involved, results in a signal synchronization in the preferred direction. The change in luminance is first received by the distal ommatidium and lastly by the proximal ommatidium column, thus requiring temporal modulation in signal transmission to arrive at the T4 neuron at the same time (Groschner et al., 2022). The same temporal modulation results in a release from glutamatergic inhibition in the preferred direction (Borst, 2018; Groschner et al., 2022) and an activation of inhibitory inputs in the null direction (Arenz et al., 2017; Gruntman et al., 2018). Four subtypes of T4 cells have been identified, each tuned to one of the four cardinal directions. Axon terminals from the cells with the same preferred direction are grouped together in one layer of the lobula plate. Thus, T4a cells tuned to front-to-back motion are located in layer 1, T4b cells responding to back-to-front motion are in layer 2, with T4c (upwards motion) and T4d (downwards motion) being found in layers 3 and 4, respectively (Maisak et al., 2013).

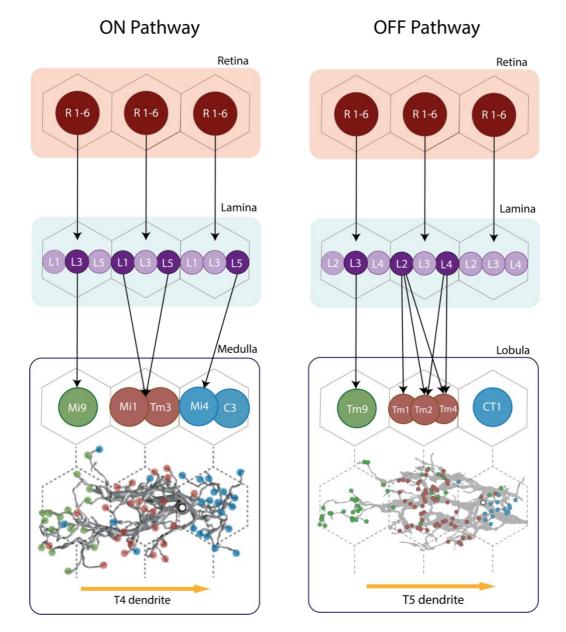


Figure 5: Retinotopic inputs to the ON and OFF motion circuits

(A) Schematic representation of the direct columnar inputs for the ON Pathway: photoreceptors R1-6 from 3 adjacent ommatidia send their inputs into the lamina. In the first column (left), information is relayed via the L3 neuron to the Mi9 in the corresponding column in the medulla. L1 and L5 in the middle column send synapses to Mi and Tm3 in the central medullar column. Inputs coming from the last (right) column are sent into the medulla to the Mi4 neurons via the L5 laminar neurons. Mi9 synapses onto the distal tip of a T4 dendrite. In the median part, synapses from Mi1 and Tm3 cells can be found. At the apex of the dendritic tree, Mi4 and C3 axon terminals can be observed. This arrangement constitutes the basis of the direction selectivity property of the neuron (preferred direction represented with the yellow arrow). (B) Similarly, information in the first column is sent from the ommatidium's R 1-6 photoreceptors via L3 in the lamina, to the corresponding Tm9 neurons in the medulla. Inputs from the central L2 and L4 laminar neurons relay information onto the central Tm1, Tm2 and Tm4. Both C3 (ON pathway) and CT1 (ON and OFF pathway) receive indirect feedback and input from other cells in the optic lobe (feedback loops and indirect input not displayed). Neuronal reconstructions taken with permission from Amalia Braun and Lukas Groschner.

## 4.1.2 The OFF pathway

The principles of the OFF pathway are mostly the same: laminar monopolar cells L2 and L4, along with inputs from L3 synapse in a retinotopic manner in the medulla. Surprisingly both Tm9 which innervates the distal section of a T5 neuron, and Tm1, Tm2, and Tm4 which send their axon terminals to the central part of the dendritic tree, form cholinergic synapses. To achieve null-direction suppression, the multicolumnar CT1 inhibits T5 on the proximal side via GABAergic inputs (Braun et al., 2023). Similar to T4 cells, four subtypes of T5 cells have been identified, presenting the same direction of motion preference and localization of terminals in the lobula plate (Maisak et al., 2013).

## 4.2 Global flow-field analysis

For the fly to see the world, all the information from the local motion detectors must be pooled together to extract global motion information relevant to initiating a behavioral response (Barnhart et al., 2018). This happens at the level of the lobula plate, where axon terminals of T4 and T5 neurons arrange themselves in 4 distinct layers according to their preferred direction (Maisak et al., 2013). Inputs from these neurons are integrated by the so-called lobula plate tangential cells (LPTCs) which have large dendritic trees with preferred directions as well. The horizontal system (HS) cells react to movement in the horizontal plane and receive inputs from the first two layers of T4/T5 neurons, which signal front-to-back and back-to-front movement. The vertical system (VS) cells encode movement in the vertical plane, spanning their dendrites in layers 3 and 4 of the lobula plate, encoding information about upward and downward movement respectively (Scott et al., 2002).

When the fly is moving, LPTC neurons are required to process faster-moving stimuli than when it is stationary. Therefore, the temporal frequency tuning for these cells needs to change (Chiappe et al., 2010). This is achieved via the octopaminergic neurons, which, depending on the state of the fly (walking, flying, or stationary) modulate the temporal tuning of LPTCs (Jung et al., 2011). It was later discovered that octopaminergic input is not only present in LPTCS, but is also responsible for tuning T4 and T5 cells to shift their tuning to higher frequencies (Arenz et al., 2017).

Axon terminals of LPTCs go on to innervate descending neurons, which, in part synapse onto motor control neurons which trigger direct behavioral responses (Ryu, 2022, Busch et al., 2018). The LPTCs are responsible for integrating optic flow information and initiating the appropriate optomotor response, one of the most studied insect behaviors (Haikala et al., 2013).

# Chapter 5: METHODS FOR STUDYING THE OPTOMOTOR RESPONSE

We have seen in the previous chapters how information from a few light quanta is absorbed by photoreceptors, translated into electrical signals, and then processed by higher-order neurons to lead to a complementary behavioral response (Schwemer, 1989). As previously mentioned, one of the most robust and well-studied behaviors is the optomotor response. By genetically manipulating different cells in the circuit linking the visual input to the behavioral output, we have gained tremendous amounts of knowledge about how the brain processes local motion (Zhu, 2013). When the visual scene rotates, the visual input is registered by the LPTCs, which signal to the motor neurons to initiate an equivalent rotational movement in the same direction. This compensates for the involuntary rotation and keep a straight course (Götz et al., 1979). There are several experimental methods useful for assessing the optomotor response, each presenting certain benefits and drawbacks.

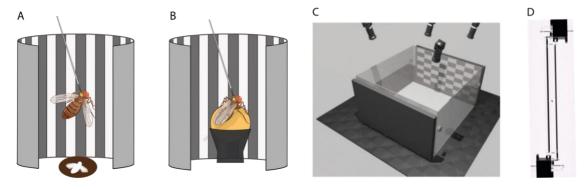


Figure 6: Schematic representation of behavioral setups for *Drosophila* studies

**(A)** Tethered flying setup of a fixed fly. The wing movements are recorded via a wing-beat analyzer. Picture modified with permission from Tabea Schilling. **(B)** Tethered walking ball setup. Picture reproduced courtesy of Tabea Schilling. **(C)** Free flying setup. Flies' movements are monitored with the help of 5 video cameras. **(D)** Free walking chamber. Picture reproduced courtesy of Stefan Prech.

## 5.1 Tethered flies

## 5.1.1 Tethered flying flies

The first characterizations of the optomotor response in *Drosophila melanogaster* were performed by mounting a fly (fixating its head and thorax) to a pin that can rotate (Götz, 1975). The pin was connected to two coils which reported the torque of the fly via a magnetic field. Both the magnitude and the direction of the rotation were successfully

recorded via this elegant yet simple system. When flies flying in this setup were presented with gratings moving in a given direction, they were observed to perform a rotation of the same magnitude in the same direction in a behavior coined as the "optomotor response".

Several other iterations of this setup are available. More modern approaches give the fly complete freedom of rotation by suspending the pin via a magnet and measuring the yaw direction (e.g. Salem et al., 2020). Due to the bilateral changes in stroke amplitude during rotations, the pin can be fixed and the behavioral output can also be read via a wing beat analyzer (e.g. Schilling and Borst, 2015; Götz et al., 1979).

## 5.1.2 Tethered walking flies

Another approach for quantifying the optomotor response was developed soon after the tethered flying setup by Buchner (1976) and Götz (1973). Both setups require the fly to walk on a ball, being kept stationary in orientation via tethering from above. The rotation and translation responses of the fly to visual stimuli presented are recorded via the rotation of the ball in 4 different directions. Götz and Wenking (1973) recorded this rotation via a servo system while Buchner 1976 changed the material and size of the ball so that it can be suspended by airflow, a method used to this day to monitor the intended trajectory of the walking fly (e.g. Bahl et al., 2013).

## 5.2 Unrestrained flies

#### *5.2.1 Freely flying flies*

Simply releasing flies in a transparent acrylic enclosure and monitoring their behavior via a multicamera system is a great experimental method for many studies, including the observation of the optomotor response. Displaying rotating stimuli on the vertical walls of a cylindrical arena evokes circular flight paths consistent with optomotor response. The ability of the fly to move unrestrained in all 3 dimensions reveals new properties of the optomotor response: the radius of the circular flight path increases with increasing velocity of the stimulus, and the flight path is consistently concentric to the center of the arena (Mronz and Lehmann, 2008).

#### 5.2.2 Freely walking flies

Optomotor responses in freely walking *Drosophila* are not a novelty. As early as 1943, flies were released in transparent spherical enclosures and their response to stripe patterns was observed (Kalmus, 1943). Surprisingly, Götz (1975) reported that freely

walking fruit flies move in the opposite direction of a "floating" (continuous translatory) visual stimulation.

## Chapter 6: VISUALLY GUIDED BEHAVIORS

Animals equipped with image-forming eyes process visual features from their surrounding environment and use this information to execute appropriate behavioral responses (Ryu et al., 2022). Light has several properties that need to be extracted by the visual circuit of the fly: intensity, propagation direction, wavelength, and polarization. These characteristics are inhomogeneous and change continuously across the visual field, being influenced by objects and the surrounding environment. Each ommatidium reports the properties of light found at a particular point in space. The brain processes and compares these streams of information in parallel neuronal circuits, leading to ecologically beneficial behaviors for the fly. Here I will present some of these visually guided behaviors.

## 6.1 Phototaxis

One of the most robust behaviors of the fly is phototaxis. In a visual preference task, flies are observed to be attracted by light. The strength of the response (i.e. walking velocity and number of flies moving towards the light source) is modulated by the intensity of the light (Hu and Stark, 1980). However, the spectral composition also plays a role (Ryu et al., 2022). When the fly is faced with multiple light sources of different spectral values, flies favor ultraviolet wavelengths (Hu and Stark, 1980). Surprisingly, this behavior, while present in all individuals, is subject to great variability in response strength across subjects (Kain et al., 2012).

## 6.2 Object fixation

Drosophila melanogaster shows a preference for long vertical bars during locomotion, always keeping them in the center of their visual field (Reichardt and Wenking, 1969; Götz, 1987; Maimon et al., 2008). Ecologically, this is explained by the similarity of this stimulus with a tree-trunk, a common feeding site for flies (Ryu et al., 2022). An intriguing observation is that bar fixation is not completely dependent on motion vision (Bahl et al., 2013). A response asymmetry to the direction of motion of the bar is observed, with control flies responding twice as strongly to front-to-back motion compared to back-to-front when the stimulus was located in the frontal section of the visual field. On the other hand, motion-blind flies are able to fixate a bar to the front of their visual field, but do not show an optomotor response to the motion of a bar presented in the same field of

view (Bahl et al., 2013). This indicates that landmark orientation (i.e. object fixation) is used as a parallel strategy from optic flow processing for navigational purposes.

#### 6.3 Menotaxis

Contrary to expectations, *Drosophila* can execute fly bouts of many kilometers long. Mark-and-recapture experiments revealed the amazing ability of fruit flies to fly almost 15 km in a single afternoon (Coyne et al., 1982). For that to happen, they need to keep the course relatively straight. Menotaxis is the ability of a fly to pick and maintain an arbitrary heading direction relative to an external cue (Fischer, 2022). Flies can use the position of the sun to maintain their straight course. This is different to object fixation, as flies do not keep the sun "in front" of their visual field, but rather choose a random direction relative to the sun's position and maintain this orientation (Giraldo et al., 2018).

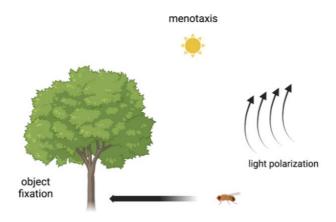


Figure 7: Visually guided orientation

Flies use different elements of the visual field to orient their locomotion trajectories. They can perform object fixation, moving towards a trunk of a tree or orient using celestial cues such as the Sun or the light polarization pattern.

Illustration created using BioRender.

## 6.4 Orientation via polarized light

Celestial cues such as the sun, the moon, or the milky way are useful for navigation. However, these are not always readily available. For example, the Sun's location may be obstructed by clouds or local features, making it impossible for an animal on the ground to orient using its location (Wier and Dickinson, 2012). Evolution has again found an elegant solution to this problem: insect and arthropod eyes are capable of detecting a special property of light undetectable by the human eye: polarization. Light is, in essence, an electromagnetic field (with the electric and the magnetic components being perpendicular to each other) that rotates based on the sun's position.

Von Frisch (1949) first observed that bees use the polarization of the skylight as a compass. Such observations were also made in dragonflies, which use the polarization of the water surface to stabilize flight (Laughlin, 1976). Such a capability has recently been discovered in *Drosophila* as well. Flies can use light polarization patterns as a reference as they remain stable during translation but change when the animal performs a rotation (Heinze et al., 2018). In a portable tethered flight setup, wild-type flies were observed compensating for the rotation of the arena when sky polarization information was available. This phenomenon is similar to the optomotor response and is explained by the intention of the fly to maintain a consistent heading (Wier and Dickinson, 2012). Locomotion modality seems to be of little consequence to the fly: walking flies also align to the direction of the vectors of polarized light coming from below (Wernet et al., 2012).

## 6.5 Landing and escape response

When a predator, such as a dragonfly, is approaching, the fly experiences an expanding dark circle on the retina. This looming stimulus elicits an escape response such as freezing, take-off, or backward walking, a choice which is dependent on the state of locomotion. For example, if the fly is stationary, a fast-looming stimulus will trigger a take-off response. This stereotypical motor program takes less than 300 ms from the onset of stimulus detection (Card and Dickinson, 2008). On the contrary, if this stimulus is encountered mid-flight, it will trigger either landing or escape flight maneuvers (Ryu et al., 2022).

In tethered conditions, responses to looming stimuli are present either in the form of avoidance turns (Schilling and Borst, 2015) or legs extension away from the body in preparation for landing (Ache et al., 2019).

## **Chapter 7: NAVIGATION**

## 7.1 Multi-sensory integration for course control

Visual information is crucial for the fly. However, it is not the only sensory system used for navigation. Flies can move around the environment even in complete darkness. Thus, it is important to understand how information from the other sensory modalities is pooled and processed in the fly's brain. In the following section, I will present some other sensory modalities employed by the fruit fly during the course of a moving bout.

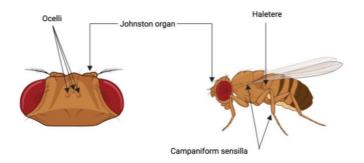


Figure 8: Sensory modalities in flies

Flies integrate and compare inputs from other sensory modalities as well. Ocelli modulate light sensitivity, the Johnston organ provides information about wind direction and velocity, halteres act as gyroscopes for flight maneuvers, and the campaniform sensilla provide proprioceptive information.

Illustration created using BioRender

#### 7.1.1 *Ocelli*

For many insects, especially predatory species like dragonflies, ocelli are important for reporting the position of the head mid-flight. By comparing the 3 luminance values reported by the 3 simple eyes located on top of the head in a triangular distribution, one can infer the tilt of the head (Krapp, 2009). However, the role of ocelli in simpler insects such as *Drosophila melanogaster* is not completely understood. It is thought to be a modulator rather than a direct player in visually guided behaviors, meaning that flies can still perform those behaviors even if ocellar input is missing. One theory states that the role of ocelli is to adjust the light sensitivity of the compound eyes (Hu and Stark, 1980; Kerfoot, 1967). Occlusion of the ocelli causes a slower phototaxis response (Médioni, 1962) and alters color preference and locomotor activity (Hu and Stark, 1980). Interestingly, axon terminals from the ocelli bypass the lamina and medulla, sending projections directly into the lobula and lobula plate (Jean-Guillaume and Kumar, 2022). This indicates a direct influence of the ocelli on visual processing in the optic lobe.

#### 7.1.2 Halteres

Halteres are club-shaped organs which act as gyroscopes for the fly. Their role is to encode self-movement (Dickinson, 1999) used to stabilize the body during flight. Halteres oscillate with the same frequency as the wings but due to their shape, they resist inertia to body rotations which results in Coriolis forces arising at the base of the haltere (Nalbach and Henstenberg, 1994; Yarger and Fox, 2016). Their importance for flight control was revealed by ablation experiments which rendered flies unable to take off (Mureli and Fox, 2015). Moreover, studies modifying the halteres by weighing them down report changes in the dynamics of the saccades by prematurely triggering the motor command for saccade termination (Bender and Dickinson, 2006). Like all other

sensory modalities, halteres do not work in isolation, but rather receive continuous feedback from both the visual system and the wings (Dickerson et al., 2019).

## 7.1.3 Campaniform sensilla

Campaniform sensilla are mechanosensory organs, which detect external perturbation caused by a change in pressure (Dinges et al., 2021). They are located in the edges of the wings and report aerodynamic and inertial forces. The ones found at the base of the halteres are responsible for sensing rotation-induced Coriolis forces (Dickerson et al., 2019). This information is integrated in fast feedback loops, terminals from the sensilla, directly synapsing onto the wing motor neurons (Dickinson and Muijres, 2016).

#### 7.1.4 Antennae

Antennae are very complex organs which have several sensory roles, most importantly olfactory detection. Additionally, the Johnston's organ in the antennae is considered the auditory organ of the fly. A special class of mechanosensory receptors in the Johnston's organ is used for wind direction detection (Yorozu et al., 2009). By comparing the displacement of the two units, the fly can ascertain the direction of the wind (Okubo et al., 2020). Moreover, by actively moving the antennae in response to the optic flow stimulation, the fly can detect the air displacements caused by the movement of the wings and feeds that information back to the steering system (Mamiya et al., 2011).

For a fly to stay airborne, information from all these sensory modalities is enough to maintain an appropriate flight position. However, in nature, damage to the sensory modalities is unavoidable and causes faulty proprioceptive information. In **Manuscript 1**, we analyzed the importance of the optic flow information for maintaining course control in case of wing damage.

## 7.2 Path integration

One of the things that a human, a honey bee, and an ant have in common is the ability to return home. The capability to return to one's dwelling on the shortest route is virtue of path integration. Being able to keep track of the direction and distance traveled and using that information to calculate the shortest path to a significant location is a great advantage in the animal kingdom. Not only does it reduce the amount of energy used for the inbound journey, it also reduces the predatory risk. Therefore, it is a behavior found across all locomotion modalities, with diverse species such as rats (Whishaw, 1998), mantis shrimps (Patel and Cronin, 2020), and dogs (Séguinot et al., 1998) being known for their path integration capabilities. Path integration is also easily observed in nesting insects like honey bees. Not only are they able to return to their nest with the resources

collected during the foraging trip, they are also able to communicate the origin of those resources to other members of their colony. This behavior was extensively characterized by scientists more than 30 years ago and was attributed to path integration (Kirchner and Braun, 1994). However, evidence that *Drosophila melanogaster*, has the capacity for path integration has begun to emerge only recently.

Fruit flies are nestless animals and lack the hierarchical social organization of nesting foragers like bees. To create a significant location to which they can return, one can employ a food source. Fruit flies display a local search behavior when encountering a droplet of food. The fly repeatedly strays from and returns to the food source location, even in the absence of olfactory, visual, and pheromonal cues, or even the food drop itself. This indicates that the fly is employing idiothetic cues to revisit this significant location, suggesting that flies are capable of path integration (Kim and Dickinson, 2017).

Path integration is a navigational strategy that continuously registers and updates the position of an animal in relation to an origin (i.e. nest), enabling a straight-line journey back (Stone et al., 2017). Path integration can be imagined as an accumulator, which registers an initial set of coordinates at the start of the journey as a point of origin (Collett and Collett, 2000). Then, based on egocentric information (Hartmann and Wehner, 1995), the accumulator stores the current location of the animal relative to the point of origin. Thus, a vector encoding distance and direction of the straight-line journey back to the nest is available at all times. It is hypothesized that the neural substrate for path integration is located in the central complex of the fly's brain (Fisher, 2022).

To monitor their traveling direction, flies combine proprioceptive inputs with other sensory information in a continuous manner (Heinze et al., 2018). This pooling happens in the compass neurons, which are organized in an ordered array and are located in the central complex of the brain (Seelig and Jayaraman, 2015). The result is an egocentric heading direction representation, which is then transformed into an allocentric representation (Lu et al., 2022).

However, little is known about how the fly updates the distance from the point of origin. Other animals employ an odometer as a distance indicator. For example, experiments in desert ants *Cataglyphis* elegantly show that ants encode distance using a step counter (pedometer). By modifying step length, ants with shorter legs would trigger a local search behavior faster than necessary, while ants walking on stilts overestimate the distance to the nest (Wittlinger et al., 2006). In contrast, flying bees employ optic flow information to estimate traveled distance (Esch and Burns, 1996; Srinivasan et al., 2000; Kirchner

and Braun, 1994). The question of distance encoding in *Drosophila* is addressed in **Manuscript 2**.

## Chapter 8: CONCLUDING WORDS

Both studies presented in this cumulative thesis address fundamental questions in the field. Firstly, they both present free behavior as an alternative method for discovering the functions of certain neural circuits. Manuscript 1 confirms a long-proposed hypothesis that course control is governed by the optic flow information available. Manuscript 2 presents a new behavior which was for a long time attributed to only nesting animals. Such a complex behavior like path integration would have been impossible to discover in a tethered system, underscoring again the importance of moving away from hypercontrolled experimental setups. Secondly, the experimental setups proposed in these studies are easily scalable and adjustable, enabling scientists to modify them according to the research question and paving the way to deciphering the complex neural circuits responsible for course control and navigation. By combining classical tethered preparations with monitoring freely behaving animals and adding the insights from the *Drosophila* connectome we increase our chances to fully understand the complex computational powers of the fruit fly's brain.

## **Manuscripts**

Manuscript 1: Aerial course stabilization is impaired in motion-blind flies

## Abstract

Visual motion detection is among the best understood neuronal computations. As extensively investigated in tethered flies, visual motion signals are assumed to be crucial to detect and counteract involuntary course deviations. During free flight, however, course changes are also signaled by other sensory systems. Therefore, it is as yet unclear to what extent motion vision contributes to course control. To address this question, we genetically rendered flies motion-blind by blocking their primary motion-sensitive neurons and quantified their free-flight performance. We found that such flies have difficulty maintaining a straight flight trajectory, much like unimpaired flies in the dark. By unilateral wing clipping, we generated an asymmetry in propulsive force and tested the ability of flies to compensate for this perturbation. While wild-type flies showed a remarkable level of compensation, motion-blind animals exhibited pronounced circling behavior. Our results therefore directly confirm that motion vision is necessary to fly straight under realistic conditions.

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## Contributions

Conceptualization: A.L., A.B., A.S.M.; Methodology: A.L., A.S.M.; Software: A.L., A.S.M.; Validation: M.-B.L., A.S.M.; Formal analysis: M.-B.L., A.S.M.; Investigation: M.-B.L., A.S.M.; Resources: A.L., A.S.M.; Data curation: A.S.M.; Writing - original draft: A.S.M.; Writing - review & editing: M.-B.L., A.L., A.B., A.S.M.; Visualization: M.-B.L., A.S.M.; Supervision: A.B., A.S.M.; Project administration: A.B., A.S.M.; Funding acquisition: A.B., A.S.M.



#### **SHORT COMMUNICATION**

## Aerial course stabilization is impaired in motion-blind flies

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#### **ABSTRACT**

Visual motion detection is among the best understood neuronal computations. As extensively investigated in tethered flies, visual motion signals are assumed to be crucial to detect and counteract involuntary course deviations. During free flight, however, course changes are also signalled by other sensory systems. Therefore, it is as yet unclear to what extent motion vision contributes to course control. To address this question, we genetically rendered flies motion-blind by blocking their primary motion-sensitive neurons and quantified their free-flight performance. We found that such flies have difficulty maintaining a straight flight trajectory, much like unimpaired flies in the dark. By unilateral wing clipping, we generated an asymmetry in propulsive force and tested the ability of flies to compensate for this perturbation. While wild-type flies showed a remarkable level of compensation, motion-blind animals exhibited pronounced circling behaviour. Our results therefore directly confirm that motion vision is necessary to fly straight under realistic conditions.

KEY WORDS: Motion vision, Optic flow, Course control, Free flight, Drosophila

#### INTRODUCTION

The execution of coordinated muscle contractions underlying locomotion is inherently imprecise and requires continuous adjustments based on sensory feedback (Taylor and Krapp, 2007; Rossignol et al., 2006; Dickinson, 2014; Tuthill and Azim, 2018). Vision is well suited to keep animals on track as any self-motion evokes characteristic image movements across the eye, termed optic flow (Gibson, 1950; Koenderink and van Doorn, 1987). In principle, optic flow allows moving animals to detect and counteract involuntary course deviations. This is perhaps most relevant during flight, where locomotor trajectories need to be controlled fast and in three spatial dimensions to avoid detrimental collisions (Egelhaaf, 2013).

Flies are among the most agile flying animals, performing minute coordinated changes in various aspects of wing motion to control course (Muijres et al., 2017). A great demand on stabilizing sensory feedback is therefore expected (Egelhaaf, 2013; Dickinson and Muijres, 2016). To investigate the influence of optic flow signals on

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course control, flight behaviour has been probed with visual motion stimuli (Collett and Land, 1975; Mronz and Lehmann, 2008; Stowers et al., 2017). Presented with wide-field image motion, animals display a following response, indicative of intended image stabilization on the eye and interpreted as a strategy to maintain a stable bearing. Further support for this idea is provided by closed loop paradigms in restrained animals, where flight behaviour is read out and fed back to visual stimulation, enabling precise experimental control over behaviour-stimulus coupling (Goetz, 1975; Dickinson and Muijres, 2016; Frye and Dickinson, 2001). However, here, the dynamics underlying sensory-motor integration are altered and both spatial and temporal aspects of visual feedback can only resemble natural reafference to some extent. Furthermore, feedback from other sensory organs is disconnected or entirely missing and behavioural output is severely restricted. Last, visual stimuli usually contain multiple features, and course stabilization also seems feasible without the explicit representation of motion by keeping conspicuous patterns stable on the eye (Bahl et al., 2013). Therefore, is it difficult to draw firm conclusions about the natural role of motion vision from visual stimulation and behavioural analysis.

In flies, the neuronal basis of visual motion processing is understood in great detail, providing further opportunities to investigate visually guided behaviour (Borst et al., 2020). Briefly, the arrival of photons at the level of the photoreceptor cells (mainly R1-6, but also R7 and R8) triggers a cascade of chemical events in a process known as visual transduction (Montell, 2012). Neuronal signals are then passed on to sequential processing stages (represented by laminar and medulla cells), which act as temporal and spatial filters, allowing local motion detection at the level of T4/ T5 cell dendrites (Borst et al., 2020). Axons of T4/T5 neurons project to the lobula plate, where they segregate according to directional preference into four layers: T4/T5 cells tuned to front-toback and back-to-front motion target layer 1 and 2, respectively. T4/ T5 neurons preferring upward and downward motion occupy layer 3 and 4, respectively (Maisak et al., 2013). In the lobula plate, large dendrites of so-called lobula plate tangential cells spatially integrate T4/T5 signals layer specifically (Mauss et al., 2014) and thereby become selective to specific optic flow patterns (Krapp and Hengstenberg, 1996). Tangential cells are prime candidates to detect course deviations and convey signals to downstream motor centres to be used for corrective steering manoeuvres. This notion is supported by various experimental strategies to perturb their activity in restrained walking and flight behaviour (Geiger and Näessel, 1981; Hausen and Wehrhahn, 1983; Heisenberg et al., 1978; Haikala et al., 2013; Busch et al., 2018; Kim et al., 2017; Fujiwara et al., 2017). However, the consequences of manipulating the activity of motion-sensing neurons for course control have not yet been determined in unrestrained flight.

Recently, it has become possible to render *Drosophila melanogaster* motion-blind by selective genetic manipulation of the first stage of visual motion detection, namely T4 and T5 cells (Maisak et al., 2013; Bahl et al., 2013). Importantly, the visual

position system (Bahl et al., 2013) and all other sensory modalities remain functional. Here, we analysed free-flight trajectories of such motion-blind flies. We found that they are well able to fly but exhibit clear deficits in maintaining straight flight trajectories. We therefore provide direct evidence for the conjecture that motion vision is necessary for stable flight performance.

#### **MATERIALS AND METHODS**

#### Flies

Flies were raised on standard fly food (cornmeal-agar) at 25°C and 60% humidity and on a 12 h light:12 h dark cycle. Wild-caught flies ('Luminy') were provided by Frank Schnorrer and collected by Benjamin Prud'homme. NorpA7 flies were obtained from Bloomington Stock Center (no. 5685). The split-Gal4 driver line used to drive expression of effector genes to manipulate neuronal function in free-flight experiments (w-/w-; R42F06.AD/Cyo; VT043070.DBD/Tm6b) was derived in our lab from AD and DBD domains found in Bloomington Stock Center flies (no. 70685 and no. 72763). This line was crossed to w+ cs; UAS-TNT-E/UAS-TNT-E; +/+ (from Roland Strauss, University of Mainz) to create a fly line which blocks activity in all T4/T5 cells through expression of tetanus toxin: w+/w-; R42F06.AD/UAS-TNT-E; VT043070.DBD/+. For parental controls, both the UAS and the split-Gal4 driver line were crossed with CantonS flies, resulting in: w+/w-; R42F06.AD/+; VT043070.DBD/+ and w+ cs; UAS-

Female flies younger than  $48\,h$  were selected for free-flight experiments under  $CO_2$  anaesthesia. Wing ablation was performed using standard micro-scissors (Fine Science Tools, art. no. 15001-08). The cut was executed chordwise to the wing using the spot where longitudinal veins 1 and 2 connect at the distal part of the wing as a landmark. The flies were moved to a new vial with standard food and placed back in the incubator for 24 h to recover from  $CO_2$  exposure. They were then flipped into empty vials to induce starvation for 4 h before being transferred to the experimental arena. All experiments were performed overnight and were started at the same time of the day.

#### **Tethered walking**

We assessed the baseline optomotor response using a locomotion recorder previously described in Bahl et al. (2013). Briefly, a fly whose head, thorax and wings were fixed to a needle using near-ultraviolet bonding glue (Sinfony Opaque Dentin) and strong blue LED light (440 nm, dental curing light, New Woodpecker) was placed on an air-suspended sphere. The movements of the sphere were recorded via two optical tracking sensors. The experiments were performed at 34°C, with the temperature being controlled by a self-designed Peltier controlling system.

The visual stimuli were presented on three LCD screens (120 Hz Samsung 2233 RZ) arranged in a U-shape surrounding the fly. The displays were controlled via NVIDIA 3D Vision Surround Technology on Windows 7 (64 bit) and stimuli were displayed using Panda3D and Python 2.7. The stimuli were presented at 120 frames  $\rm s^{-1}$ .

For each fly, the experiment consisted of 50 trials with stimuli in each trial being presented randomly. Full-field square-wave gratings had a spatial wavelength of  $\lambda$ =20 deg, a mean luminance of 11 cd m<sup>-2</sup> and either high (49%) or low (1.4%) contrast. They moved at a velocity of 20 deg s<sup>-1</sup> either to the left or to the right and were presented for a short (0.5 s) or a long period of time (6 s). Flies that walked continuously for at least 10 trials were selected and only the trials that had an average walking speed of higher than 0.25 cm s<sup>-1</sup>

were included in the analysis. Turning speed traces were determined by taking the average over trials and low-pass filtering the resulting trace ( $\tau$ =0.1 s in all experiments). We performed all data analysis in Python 3.7 using NumPy 1.15.1 and SciPy 1.1.0.

#### Free-flight arena

The arena was a rectangular, transparent enclosure (50×50×30 cm) of acrylic plastic (Evonik, Plexiglas XT). A custom-made array of high-intensity infrared LEDs (Roithner Laser Technik GmbH, H2A1-H830, peak at 830 nm far outside the activation spectrum of fly photoreceptors; Yamaguchi et al., 2010) and a diffuser were positioned below the arena to provide strong background light to facilitate optical tracking by cameras with short exposure time (9 ms) and at high frequency (100 Hz, Point Grey Research, CM3-U3-13S2C-CS).

To encourage flight, small 'buzzing devices' were placed in the four corners of the ceiling of the arena. Each device was based on a small Petri dish with yeast-supplemented fly food, i.e. emanating an attractive odour. Contact of flies with the food was prevented by a grid cover, to which a vibration motor (Pololu, #2265) was attached. Vibration went off every minute and evoked take-off and flight in flies sitting on the grid. This way, sufficient flight data were obtained even from flies in the dark, blind flies and motion-blind flies, all of which are otherwise reluctant to fly.

Static or moving visual patterns were displayed on four monitors (144 Hz, ASUS, VG248QE) placed on the sides of the enclosure. Average light intensity at the level of the monitors was  $0.02 \,\mu \text{W mm}^{-2}$ . A regular checkerboard pattern (dark squares 2 cd m<sup>-2</sup>, light squares 5 cd m<sup>-2</sup>) of 5×5 cm in checker size was displayed as a static visual stimulus for the majority of the experiments. For a fly located in the centre of the arena, the closest square element of the checkerboard pattern subtends an angle of 11.4 deg of visual space. Average luminance of the monitors was 3.5 cd m<sup>-2</sup>. For the experiment shown in Fig. 2B,C, the same pattern moved at 40 cm s<sup>-1</sup> to the left for 10 s, remained static for another 10 s and then moved to the right for 10 s, followed by another 10 s of static display. The displays were controlled via NVIDIA 3D Vision Surround Technology on Windows 7 (64 bit) and stimuli were displayed using Panda3D and Python 2.7. The stimuli were presented at 144 frames  $s^{-1}$ . The temperature inside the arena was approximately 28°C.

#### Free-flight tracking

#### Cameras

Our multi-camera set-up consisted of five mounted units (FLIR Inc., CM3-U3-13Y3M-CS) observing overlapping volumes of the arena (see illustration in Fig. 1A). We used standard machine vision lenses with a focal length of approximately 6 mm (Thorlabs Inc., MVL6WA). Cameras were connected to a single tracking computer via USB-3. To guarantee accurate synchronization across frame captures, image acquisition for all units was triggered by a single external TTL pulse generator that ran at 100 Hz. To prevent leakage of the visual stimulus into tracking images, we equipped all cameras with near-infrared longpass filters (Thorlabs Inc., FGL780M) that separated the displays' spectrum from near-infrared background lighting.

#### Camera calibration

We calibrated intrinsic and extrinsic camera parameters with a singlestep method (Li et al., 2013) that estimates the relevant matrices of all units in a multi-camera set-up from overlapping presentations of a printed random calibration pattern. The underlying camera model was

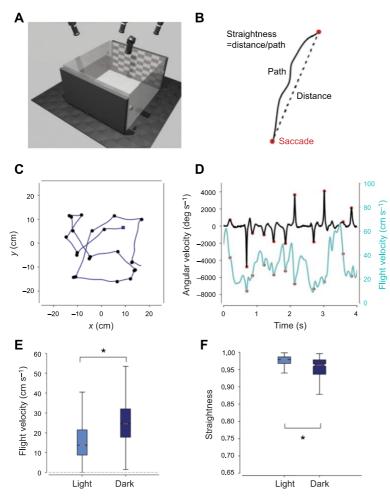


Fig. 1. Free-flight behaviour of wild-type Drosophila in the light and dark. (A) Schematic representation of the free-flight arena, which consisted of a Plexiglas box (50×50×30 cm) surrounded by four monitors to display the visual stimuli. The arena was illuminated from underneath by infrared lights. Five cameras (four shown) fitted with infrared filters recorded the flies from different angles. (B) The straightness index was calculated by dividing the distance between two consecutive saccades by the covered path length. A perfectly straight segment would yield a value of 1.0, while for a trajectory with the shape of a semicircle, the index would be 0.63. (C) Example of a single flight trajectory in the light of a wild-type fly projected in the horizontal plane. The blue square represents the start of the trajectory and the black dots represent individual saccades. (D) Horizontal angular velocity and flight velocity values over time for the wild-type fly in the light. Saccades (red) were identified based on angular velocity (peak values above 300 deg s<sup>-1</sup>). (E) Flight velocity of wild-type (WT) flies in the light and in the dark (n=1988 trajectories in the light and n=310 trajectories in the dark). Significant difference is based on a Kolmogorov-Smirnov test (\*P<0.0001). (F) Straightness of long intersaccadic segments (250-2000 ms) of wild-type flies flying in the light or in the dark. Significant difference is based on a Kolmogorov-Smirnov test (\*P<0.0001, n=4609 segments in the light and n=302 segments in the dark).

a standard pinhole camera (with radial distortion). On average, we were able to achieve a reprojection error below 1 pixel based on 100–200 synchronized multi-camera snapshots of the calibration pattern. We used the algorithm as implemented in the provided MATLAB toolbox (https://sites.google.com/site/prclibo/toolbox). Calibration was performed periodically throughout the experimental phase to safeguard against shifts in camera position that could affect triangulation.

#### Detection

We processed incoming images from 5 cameras running at 100~Hz. Images were acquired as  $640\times512$  pixel single-channel matrices. We estimated the static background by accumulating incoming images with a weight of 0.001 on each time step and subtracted this background estimate from new frames to isolate moving targets. Finally, we applied a threshold on the resulting image at a minimum value of 5 (out of 255) to further suppress photon noise. With each camera image, we applied a standard blob detection algorithm from OpenCV ('cv2.findCountours') to detect contiguous 2D targets. The position of a target was then defined as the weighted centre of the blob. Coordinates of these targets were combined in the triangulation process described below.

#### Reconstruction

Unlike previous studies (Straw et al., 2011), we separated the tracking step into per-frame triangulation and subsequent association to generate trajectories for identified individuals. Triangulation was accomplished using a standard method, the Hungarian algorithm, which can efficiently solve assignment problems in polynomial time (Kuhn, 1955; Ardekani et al., 2013).

Briefly, for each frame our 2D tracking algorithm yields multiple x–y detections which need to be associated across cameras to allow correct reconstruction of 3D positions. If only a single fly moves inside the arena, this operation is trivial; we found a single 2D detection per camera and all detections emanated from the same target. However, when multiple flies moved simultaneously, we needed to correctly assign 2D detections to 3D targets. We always used standard singular value decomposition to estimate the optimal 3D position from a set of 2D noisy observations (Hartley and Zisserman, 2003). The Hungarian algorithm then efficiently calculates a minimum–cost assignment where cost is defined as the reprojection error after assigning particular 2D points to particular 3D targets. We implemented the algorithm in Python 2.7 and Numba, relying on OpenCV or PyMVG (https://github.com/strawlab/pymvg) for various projection operations.

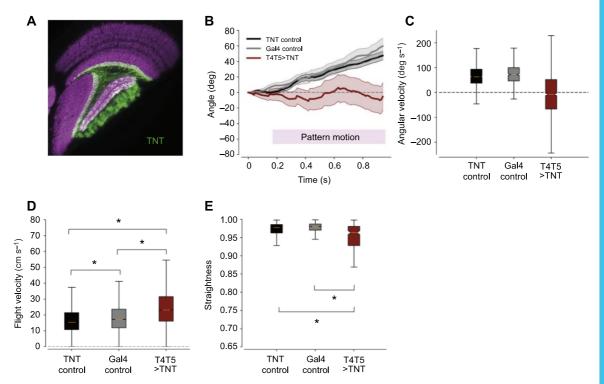


Fig. 2. Response of T4/T5-blocked flies to pattern motion. (A) Confocal image of the split-Gal4 driver line (R42F06.AD; VTO43070.DBD) shown in horizontal cross-section. Neurons (green) were labelled using an antibody against tetanus toxin (TNT); the neuropile (purple) was labelled with an antibody against the postsynaptic protein Dlg. Upon inspection, no off-target expression of TNT was observed in the central brain or ventral nerve chord. (B) Turning response to pattern motion (mean±s.e.m.) of parental controls (TNT control, n=419 trajectories; Gal4 control, n=131 trajectories) and motion-blind flies (T4/T5>TNT, n=67 trajectories) in the light. The stimulus consisted of a regular checkerboard pattern (dark squares 2 cd m<sup>-2</sup>, light squares 5 cd m<sup>-2</sup>) of 5×5 cm in checker size, which would move at 40 cm s<sup>-1</sup> to the left for 10 s, remain static for another 10 s and then move to the right for 10 s, followed by another 10 s of static display. Trajectories selected had a minimum of 150 ms of flight before pattern motion started and at least 800 ms flight during pattern motion. (C) Average angular velocity of parental controls and motion-blind flies in the light during pattern motion (TNT control, n=1622 trajectories; Gal4 control, n=659 trajectories; T4T5>TNT, n=133 trajectories). (D) Flight velocity of parental controls and motion-blind flies in the light (TNT control, n=2107 trajectories; Gal4 control, n=2457 trajectories; T4T5>TNT, n=512 trajectories). (E) Straightness of long intersaccadic flight segments (250–2000 ms) of parental controls and motion-blind flies in the light. Significance in D and E was calculated via a Kolmogorov—Smirnov test: "P<0.0001 (TNT control, n=5842 segments; Gal4 control, n=6612 segments; T4T5>TNT, n=242 segments). Data in D and E were from flies that were presented with a static version of the stimulus described in B.

#### Filtering and association

The method outlined above provides a number of 3D targets per frame. Per-individual analysis requires an association step where these points are aggregated into defined trajectories of single flies. The tracking algorithm treats targets as a collection of linear Kalman filters. Observations are 3D positions. The underlying state consists of six parameters: instantaneous 3D position as well as three velocities in all three directions. All filters are based on a constant-velocity process where manoeuvring is modelled as noisy deviations from this constant velocity. The process matrix simply advances the current position by estimated velocity times the frame length (10 ms). We assumed the following standard deviations for the different components: 2 cm for the measurement noise as well as 1 cm and 50 cm s<sup>-1</sup> for the position and velocity components of the process noise, respectively. Standard deviations for the state covariance matrix were initialized as 10 cm for x-v-z position and 100 cm s<sup>-1</sup> for all velocities. We did not tune these parameters extensively as they had little effect on tracking quality.

On each time step, we predicted the position of each target and used the Hungarian algorithm to assign novel 3D observations to the set of existing filters (based on aggregated distance cost of the assignment). Observations can only be assigned to a filter if the distance is below 1 cm. Any observation that cannot be matched to an existing filter spawns a new target. If a filter does not receive a fresh observation for 20 time steps, the instance is terminated. A trajectory is then simply the filtered position estimate of a single Kalman instance from spawning to termination.

No additional post-processing was applied to disambiguate crossing paths of flies as we found these events to be rare in practice. Reconstruction of 3D points and tracking were computed offline. We used Python 2.7 and the filterpy package (https://filterpy.readthedocs.io/en/latest/) to implement these routines.

Code is available upon request.

#### Free-flight data analysis

#### Trajectory selection and feature extraction

All analysis was carried out in Python 3.7, using the following libraries (among others): NumPy 1.17.2, Pandas 0.25.1 and SciPy 1.3.1. Movement trajectories (walking and flight) were loaded and those below a minimal length (1 s) discarded. Values for x, y and z

were smoothed by convolving them separately with a block filter of size 9. To obtain an initial selection of flight trajectories, only segments within a certain z range (1.5 cm above the floor and 1.0 cm below the ceiling) were included and labelled with a new identifier. From positions over time, the following features were extracted: x-y angle (deg), x-y angular velocity (deg  $s^{-1}$ ), x-y-z flight velocity (cm  $s^{-1}$ ) and x-y (horizontal) flight velocity (cm  $s^{-1}$ ). At this point, manual inspection still revealed a fraction of walking trajectories based on low movement velocity and little x-y displacement over time. Hence, trajectories with a mean flight velocity below 3.0 cm  $s^{-1}$  or the sum of x and y standard deviation below 2 cm were discarded.

#### Saccade detection

Saccade detection was carried out based on angular velocity, obtained by differentiating the angle of consecutive x/y positions relative to the arena coordinates. For each flight trajectory, angular velocity was convolved with a Gaussian kernel of the approximate shape of a saccade ( $\sigma$ =40 ms). Saccade time points were then identified by peak values above a threshold of 300 deg s<sup>-1</sup>. This procedure was done separately for leftward and rightward saccades, taking the respective sign into account.

#### Straightness

For each intersaccadic segment, distance and path length between the two endpoints were computed. To obtain the straightness index, distance was divided by path length. Statistically significant differences between genotypes were established by computing the Kolmogorov–Smirnov statistic on two samples using scipy.stats.ks\_2samp in Python v.3.5.4.

#### Turning

For each trajectory, from the angle value in each frame we subtracted the angle value from the first frame, so that each trajectory angle started at 0. Then, the last angle value from each trajectory was divided by the duration of the respective trajectory to obtain turning in deg s<sup>-1</sup>. To test for statistical significance, we performed a *t*-test using scipy.stats.ttest\_ind in Python v.3.5.4 (significance level adjusted by a Bonferroni correction in case of multiple comparisons). Code is available upon request.

#### **Immunohistochemistry**

Primary antibodies used were: mouse anti-Bruchpilot (1:20, Developmental Studies Hybridoma Bank, AB2314866), rabbit anti-Tetanus Toxin (1:5000, SSI Antibodies, 65873 POL 016). Secondary antibodies used were: ATTO 647N goat anti-mouse (1:400, Rockland, 610-156-040), Alexa Fluor 568-conjugated goat anti-rabbit (1:400, Life Technologies, A-11011).

Brains were dissected in cold PBS and fixed in 4% paraformaldehyde (0.1% Triton X-100) for 25 min at room temperature. They were then washed 3 times with PBST (PBS containing 0.3% Triton X-100) and blocked with normal goat serum (10% NGS in PBST) for 1 h. Brains were incubated at  $4^{\circ}\text{C}$  for 48 h with primary antibodies diluted in NGS solution. They were washed 3 times (for 1–2 h each) with PBST and then incubated at  $4^{\circ}\text{C}$  for 48 h with secondary antibody diluted in NGS solution. Brains were then washed 3 times in PBST before mounting in SlowFade Gold Antifade Mountant (Thermo Fisher Scientific).

#### **RESULTS AND DISCUSSION**

#### **Probing free flight**

We released flies in a transparent enclosure (Collett and Land, 1975; Straw et al., 2011; Tammero and Dickinson, 2002) (Fig. 1A) and

tracked their positions in 3D at 100 frames s<sup>-1</sup> using a calibrated camera system. We calculated features frame-by-frame, such as flight velocity and turning angle. Static or moving visual patterns were displayed on four monitors surrounding the enclosure. We used wild-caught flies for an initial characterization. In agreement with previous accounts, recorded trajectories consisted of straight segments interspersed by sharp turns, so-called body saccades, which have been observed during free flight of different fly species (Collett and Land, 1975; Mronz and Lehmann, 2008; Schilstra and van Hateren, 1999; Tammero and Dickinson, 2002; Egelhaaf et al., 2012) (Fig. 1B–D). We detected saccades on the basis of turning velocity. Another characteristic feature of a saccade is a brief drop in flight velocity (Mronz and Lehmann, 2008; Schilstra and van Hateren, 1999; Tammero and Dickinson, 2002) (Fig. 1D; Fig. S1).

#### Flight with and without vision

Appropriate detection of various self-evoked optic flow components by neural circuits is instrumental in monocular depth perception (Srinivasan, 2011; Ravi et al., 2019; Egelhaaf et al., 2012) as well as estimating and regulating locomotor speed (Baird et al., 2005; Srinivasan et al., 1996; Pfeffer and Wittlinger, 2016). Self-induced image motion may also provide sensory cues to detect and counteract involuntary course deviations (Gibson, 1950; Goetz, 1975; Collett and Land, 1975; Egelhaaf, 2013).

We first asked how missing visual feedback affected flight structure by comparing trajectories of the same wild-type strain under two conditions: with visual patterns surrounding the arena (i.e. with intact visual feedback) and in darkness (i.e. without any visual feedback). Trajectories obtained in the dark were usually shorter in duration. In addition, the average flight velocity in darkness was increased (Fig. 1E), in line with the idea that flight velocity is reflexively modulated by the received optic flow (Mauss and Borst, 2020; Baird et al., 2005; Srinivasan et al., 1996). All saccade metrics, however, were highly similar (Fig. S1). This finding further supports the notion that, although visual signals can trigger saccades, the execution of the underlying motor programme is not reflexively modulated by visual motion feedback (Bender and Dickinson, 2006; Tammero and Dickinson, 2002; Karmeier et al., 2006).

Closer inspection of flight trajectories revealed that vision is more important to maintain a stable bearing during intersaccadic flight. To quantify this, we calculated a straightness index by dividing the distance between two consecutive saccades by the covered path length (Fig. 1B). For each experimental condition, we further divided intersaccadic segments into two groups: short (50–250 ms) and long (250–2000 ms). Comparing straightness indices between bright and dark condition revealed a significant reduction for long segments recorded in the dark (Fig. 1F). We further obtained data from a completely blind fly strain, NorpA, with a mutation in the essential phototransduction enzyme phospholipase C (Hotta and Benzer, 1970). NorpA flies flew even faster and less straight than wild-type flies in the dark (Fig. S2).

#### Free-flight behaviour of motion-blind flies

From the results above, we can conclude that vision is important for course stabilization. However, the question remains whether the stabilizing influence is exerted by visual motion signals. Course stabilization can also be achieved by keeping conspicuous visual features at a constant position on the retina (Bahl et al., 2013; Bar et al., 2015).

In the fly optic lobe, lobula plate neurons integrate the signals from specific sub-samples of local motion detectors T4 and T5.

Thus, they become selective to flow fields such as rotation, translation (Karmeier et al., 2006; Krapp and Hengstenberg, 1996) or expansion (Klapoetke et al., 2017). Any of these self-induced flow components may influence movement trajectories (Collett and Land, 1975; Rock and Smith, 1986; Warren et al., 2001; Mronz and Lehmann, 2008).

To test for the involvement of motion vision, we rendered flies motion-blind by cell-specific expression of tetanus toxin in the primary motion-sensing neurons ('T4T5>TNT'; Fig. 2A). We confirmed the absence of motion vision in these flies in two different ways. First, we found that the optomotor turning response of tethered walking flies was completely abolished (Fig. S3). Second, we measured turning of freely flying flies in response to horizontal pattern rotation around the arena. In contrast to controls, which showed the expected following reaction (Mronz and Lehmann, 2008), responses of T4T5>TNT flies to moving patterns (clockwise and counter-clockwise) did not reveal any average turning response (Fig. 2B,C).

We next analysed flight trajectories of control and T4T5>TNT flies in the presence of static patterns. Flight structure of T4T5>TNT flies appeared normal, albeit an increased flight velocity was observed (Fig. 2D). Furthermore, intersaccadic segment straightness of T4T5>TNT flies was reduced compared with controls. These results are similar to those of wild-type flies in the dark (compare Fig. 2D,E with Fig. 1E,F), demonstrating a role of motion vision for keeping flight trajectories straight.

#### **Compensation of aerodynamic asymmetry**

The above results suggest that the contribution of motion vision to keeping intersaccadic flight straight is significant but subtle. However, inherent left or right turning bias at the level of individuals (Souman et al., 2009) might in part be concealed in the population response that we measured. Furthermore, course control of individuals in nature may be acutely challenged by air turbulence or chronically by wing damage.

In order to test the flies' ability to compensate for a consistent turning bias, we clipped ~25% of the tip of either the right or the left wing. Insects that have undergone wing damage change the dynamics of their wing movements to compensate for the loss of propulsion (Bender and Dickinson, 2006; Mujires et al., 2017; Kihlström et al., 2021). We processed data of left wing-clipped flies as if they were clipped on the right side, allowing us to combine data from both manipulations. We quantified trajectories from the following experimental groups: wild-type flies in the light and dark (Fig. 3A-D), as well as TNT control flies, Gal4 control flies and T4T5>TNT flies in the light (Fig. 3E-H). Visual inspection of individual trajectories (x/y coordinates) from wing-clipped wild-type flies in the light revealed a flight structure similar to that of intact controls (Fig. 3A). However, wing-clipped wild-type flies in the dark behaved differently in that many flight trajectories exhibited a clockwise or counter-clockwise circular structure (Fig. 3B). The same was true when comparing TNT controls (normal flight structure) with T4T5>TNT (curved trajectories) (Fig. 3E,F).

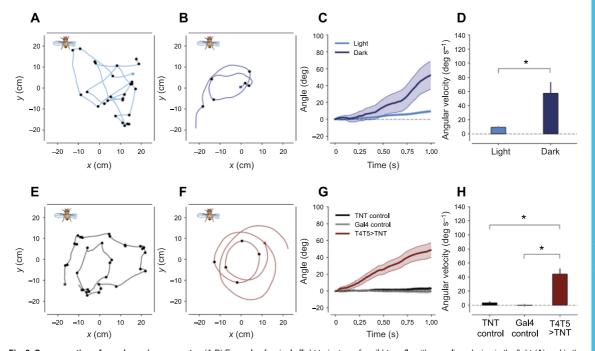


Fig. 3. Compensation of aerodynamic asymmetry. (A,B) Example of a single flight trajectory of a wild-type fly with one clipped wing in the light (A) and in the dark (B). (C) Mean flight angles (±s.e.m.) over time of wild-type flies with one clipped wing (n=6773 segments in the light and n=205 segments in the dark). (D) Turning (integrated angular velocity over time – see Materials and Methods for details) is statistically different between wild-type flies with one clipped wing in the light and in the dark (t-test, \*P<0.0001). (E,F) Example of a single flight trajectory of a parental TNT control (E) and a motion-blind T4T5>TNT fly (F) with one clipped wing in the light. (G) Mean flight angles of parental controls and motion-blind flies with one clipped wing (TNT control, n= 4983; Gal4 control, n= 3893; T4T5>TNT, n=734). (H) Turning is statistically different between parental control flies with one clipped wing and T4T5>TNT flies with one clipped wing (t-test, \*P<0.0001). After Bonferroni correction, no statistical significance was observed between the two groups of parental controls.

Calculating straightness indices revealed a strong reduction for wing-clipped wild-type dark and T4T5>TNT flies, compared with their respective controls (Fig. S4). However, as saccade detection might be compromised by circling flight, we analysed trajectories independent of saccade detection. First, for each trajectory, we took the orientation over time, obtained from the angle of the vector defined by consecutive x-y positions relative to the arena coordinates. We then subtracted the initial angle so that the orientation of each trajectory commenced at zero and computed the average across the first second of recording (Fig. 3C,G). The average orientation of wild-type, TNT control and Gal4 control flies in the light revealed an almost perfect compensation, i.e. a small change in orientation over time. Both wild-type flies in the dark and T4T5>TNT flies in the light, however, exhibited a pronounced average drift in the direction opposite to the wing-clipped side. Furthermore, for each trajectory we calculated a single turning value in  $\deg s^{-1}$  by dividing the total change in orientation from beginning to end by the duration. While the average turning velocity for wildtype, TNT control and Gal4 control flies in the light was close to zero, this parameter was much higher for wild-type flies in the dark and T4T5>TNT flies in the light, at  $\sim$ 45–60 deg s<sup>-1</sup> (Fig. 3D,H).

To summarize, eliminating motion vision had the same effect as removing all visual input: flies lost their ability to compensate for an experimentally introduced turning bias. Hence, these results directly demonstrate a stabilizing influence of motion vision on course control.

#### Sensory cues complementary to optic flow

Animals have various additional sensory cues at their disposal to control heading. For instance, stable bearing can be aided by keeping conspicuous visual features stationary on the retina without the requirement for explicit visual motion representation (Bahl et al., 2013). This involves the computation of an error angle to be minimized by appropriate turning reactions. However, in nature, appropriate visual landmarks may not always be present as for instance in densely cluttered surrounds. Furthermore, using error angle for proportional control of heading is noise sensitive and prone to overshoot, as shown in bats (Bar et al., 2015). Optic flow in turn provides a signal akin to the derivative of an error angle (Bar et al., 2015). Under natural conditions, both position and motion vision system are probably used in a redundant fashion for robust steering.

In addition to vision, mechanosensory feedback from body appendages plays an important role in preventing accidental heading changes. In Diptera, for instance, the halteres – clubshaped appendages modified from hind wings – act as gyroscopes sensing body rotations (Nalbach, 1993; Dickinson, 1999). Because they are tightly coupled to the wing motor system via afferent and efferent connections (Dickerson et al., 2019), they provide ultrafast feedback critical for stable flight. Visual motion in turn signals slower rotations (Sherman and Dickinson, 2004), complementing haltere feedback in a different angular velocity regime.

#### Conclusion

It has long been recognized that motion vision is suitable to subserve various ethological functions. However, the significance of self-evoked visual motion signals for course control has been difficult to address. Here, by combining free-flight tracking with the specific removal of direction-selective neurons in flies, we directly demonstrate an important contribution of the motion vision system to course stabilization. As phenotypes of motion-blind flies are not different to unimpaired flies in the dark, non-motion visual cues do

not seem to contribute substantially, at least in our experimental setup. Our work establishes a basis from which other contributing sensory cues and their integration with motion vision can be further explored under naturalistic conditions.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: A.L., A.B., A.S.M.; Methodology: A.L., A.S.M.; Software: A.L., A.S.M.; Validation: M.-B.L., A.S.M.; Formal analysis: M.-B.L., A.S.M.; Investigation: M.-B.L., A.S.M.; Resources: A.L., A.S.M.; Data curation: A.S.M.; Writing - original draft: A.S.M.; Writing - review & editing: M.-B.L., A.L., A.B., A.S.M.; Visualization: M.-B.L., A.S.M.; Supervision: A.B., A.S.M.; Project administration: A.B., A.S.M.; Funding acquisition: A.B., A.S.M.;

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#### References

Ardekani, R., Biyani, A., Dalton, J. E., Saltz, J. B., Arbeitman, M. N., Tower, J., Nuzhdin, S. and Tavare, S. (2013). Three-dimensional tracking and behaviour monitoring of multiple fruit flies. J. R. Soc. Interface 10, 20120547. doi:10.1098/rsif.2012.0547

Bahl, A., Ammer, G., Schilling, T. and Borst, A. (2013). Object tracking in motionblind flies. *Nat. Neurosci.* 16, 730-738, doi:10.1038/nn.3386

Baird, E., Srinivasan, M. V., Zhang, S. and Cowling, A. (2005). Visual control of flight speed in honeybees. *J. Exp. Biol.* 208, 3895-3905. doi:10.1242/jeb.01818 Bar, N. S., Skogestad, S., Marcal, J. M., Ulanovsky, N. and Yovel, Y. (2015). A sensory-motor control model of animal flight explains why bats fly differently in light versus dark. *PLoS Biol.* 13, e1002046. doi:10.1371/journal.pbio.1002046

Bender, J. A. and Dickinson, M. H. (2006). A comparison of visual and halter-mediated feedback in the control of body saccades in *Drosophila melanogaster*. *J. Exp. Biol.* **209**, 4597-4606. doi:10.1242/jeb.02583

Borst, A., Haag, J. and Mauss, A. S. (2020). How fly neurons compute the direction of visual motion. J. Comp. Physiol. A 206, 109-124. doi:10.1007/s00359-019-01375-9

Busch, C., Borst, A. and Mauss, A. S. (2018). Bi-directional control of walking behavior by horizontal optic flow sensors. *Curr. Biol.* 28, 4037-4045. doi:10.1016/j.cub.2018.11.010

Collett, T. S. and Land, M. F. (1975). Visual control of flight behaviour in the hoverfly, Syritta pipiens L. J. Comp. Physiol. 99, 1-66, doi:10.1007/BF01464710

Dickerson, B. H., De Souza, A. M., Huda, A. and Dickinson, M. H. (2019). Flies regulate wing motion via active control of a dual-function gyroscope. *Curr. Biol.* 29, 3517-3524. doi:10.1016/j.cub.2019.08.065

Dickinson, M. H. (1999). Haltere-mediated equilibrium reflexes of the fruit fly, Drosophila melanogaster. Philos Trans. R. Soc. Lond. B. Biol. Sci. 354, 903-916. doi:10.1098/rstb.1999.0442

Dickinson, M. H. (2014). Death valley, *Drosophila*, and the devonian toolkit. *Annu. Rev. Entomol.* **59**, 51-72. doi:10.1146/annurev-ento-011613-162041

Dickinson, M. H. and Muijres, F. T. (2016). The aerodynamics and control of free flight manoeuvres in *Drosophila*. *Philos Trans. R. Soc. Lond. B.* 371, 20150388. doi:10.1098/rstb.2015.0388

Egelhaaf, M. (2013). Visual processing in free flight. In Encyclopedia of Computational Neuroscience (ed. D. Jaeger and R. Jung), pp. 1-21. Springer.

Egelhaaf, M., Boeddeker, N., Kern, R., Kurtz, R. and Lindemann, J. P. (2012). Spatial vision in insects is facilitated by shaping the dynamics of visual input through behavioral action. *Front. Neural Circuits* **6**, 108. doi:10.3389/fncir.2012.00108

Frye, M. A. and Dickinson, M. H. (2001). Fly flight: a model for the neural control of complex behavior. *Neuron* 32, 385-388. doi:10.1016/S0896-6273(01)00490-1
Fujiwara, T., Cruz, T. L., Bohnslav, J. P. and Chiappe, M. E. (2017). A faithful

Fujiwara, T., Cruz, T. L., Bohnslav, J. P. and Chiappe, M. E. (2017). A faithful internal representation of walking movements in the *Drosophila* visual system. *Nat. Neurosci.* 20, 72-81. doi:10.1038/nn.4435

- Geiger, G. and Nässel, D. R. (1981). Visual orientation behaviour of flies after selective laser beam ablation of interneurones. *Nature* 293, 398-399. doi:10.1038/ 293398a0
- Gibson, J. J. (1950). The Perception of the Visual World. Houghton Mifflin.
- Goetz, K. G. (1975). The optomotor equilibrium of the *Drosophila* navigation system. *J. Comp. Physiol.* **99**, 187-210. doi:10.1007/BF00613835
- Haikala, V., Joesch, M., Borst, A. and Mauss, A. S. (2013). Optogenetic control of fly optomotor responses. J. Neurosci. 33, 13927-13934. doi:10.1523/ JNEUROSCI.0340-13.2013
- Hartley, R. and Zisserman, A. (2003). *Multiple View Geometry in Computer Vision*, 2nd edn. Cambridge University Press.
- Hausen, K. and Wehrhahn, C. (1983). Microsurgical lesion of horizontal cells changes optomotor yaw responses in the blowfly Calliphora erythrocephala. *Proc. R. Soc. Lond. B Biol. Sci.* 219, 211-216. doi:10.1098/rspb.1983.0068
- Heisenberg, M., Wonneberger, R. and Wolf, R. (1978). Optomotor-blind(H31) a Drosophila mutant of the lobula plate giant neurons. J. Comp. Physiol. A124, 287-296. doi:10.1007/BF00661379
- Hotta, Y. and Benzer, S. (1970). Genetic dissection of the *Drosophila* nervous system by means of mosaics. *Proc. Natl. Acad. Sci. USA* 67, 1156-1163. doi:10. 1073/pnas.67.3.1156
- Karmeier, K., Van Hateren, J. H., Kern, R. and Egelhaaf, M. (2006). Encoding of naturalistic optic flow by a population of blowfly motion-sensitive neurons. J. Neurophysiol. 96, 1602-1614. doi:10.1152/jn.00023.2006
- Kihlström, K., Aiello, B., Warrant, E., Sponberg, S. and Stöckl, A. (2021). Wing damage affects flight kinematics but not flower tracking performance in hummingbird hawkmoths. J. Exp. Biol. 224, jeb236240. doi:10.1242/jeb.236240
- Kim, A. J., Fenk, L. M., Lyu, C. and Maimon, G. (2017). Quantitative predictions orchestrate visual signaling in *Drosophila*. Cell 168, 280-294. doi:10.1016/j.cell. 2016.12.005
- Klapoetke, N. C., Nern, A., Peek, M. Y., Rogers, E. M., Breads, P., Rubin, G. M., Reiser, M. B. and Card, G. M. (2017). Ultra-selective looming detection from radial motion opponency. *Nature* 551, 237-241. doi:10.1038/nature24626
- radial motion opponency. *Nature* **551**, 237-241. doi:10.1038/nature24626 **Koenderink**, **J. J. and van Doorn**, **A. J.** (1987). Facts on optic flow. *Biol. Cybernetics* **56**, 247-254. doi:10.1007/BF00365219
- Cybernetics 56, 247-254. doi:10.1007/BF00365219

  Krapp, H. G. and Hengstenberg, R. (1996). Estimation of self-motion by optic flow processing in single visual interneurons. *Nature* 384, 463-466. doi:10.1038/384463a0
- Kuhn, H. W. (1955). The Hungarian method for the assignment problem. Nav. Res. Logist. Q. 2, 83-97. doi:10.1002/nav.3800020109
   Li, B., Heng, L., Koser, K. and Pollefeys, M. (2013). A multiple-camera system
- Li, B., Heng, L., Koser, K. and Pollefeys, M. (2013). A multiple-camera system calibration toolbox using a feature descriptor-based calibration pattern. 2013 IEEE/RSJ Internatl. Conf. Intell. Robots and Systems, Tokyo, 1301-1307.
- Maisak, M. S., Haag, J., Ammer, G., Serbe, E., Meier, M., Leonhardt, A., Schilling, T., Bahl, A., Rubin, G. M., Nern, A. et al. (2013). A directional tuning map of *Drosophila* elementary motion detectors. *Nature* 500, 212-216. doi:10. 1038/nature12320
- Mauss, A. S., Borst, A. (2020). Optic flow-based course control in insects. Curr. Opin. Neurobiol. 60, 21-27. doi:10.1016/j.conb.2019.10.007
- Mauss, A. S., Meier, M., Serbe, E. and Borst, A. (2014). Optogenetic and pharmacologic dissection of feedforward inhibition in Drosophila motion vision. J. Neurosci. 34, 2254-2263. doi:10.1523/JNEUROSCI.3938-13.2014
- Montell, C. (2012). Drosophila visual transduction. *Trends Neurosci.* **35**, 356-363. doi:10.1016/j.tins.2012.03.004

- Mronz, M. and Lehmann, F. O. (2008). The free-flight response of *Drosophila* to motion of the visual environment. *J. Exp. Biol.* **211**, 2026-2045. doi:10.1242/jeb.008368
- Muijres, F. T., Iwasaki, N. A., Elzinga, M. J., Melis, J. M. and Dickinson, M. H. (2017). Files compensate for unilateral wing damage through modular adjustments of wing and body kinematics. *Interface Focus* 7, 20160103. doi:10. 1098/rsfs.2016.0103
- Nalbach, G. (1993). The halteres of the blowfly Calliphora I. Kinematics and dynamics. J. Comp. Physiol. A. 173, 293-300. doi:10.1007/BF00212693
- Pfeffer, S. E. and Wittlinger, M. (2016). Optic flow odometry operates independently of stride integration in carried ants. Science 353, 1155-1157. doi:10.1126/science.aaf9754
- Ravi, S., Bertrand, O., Siesenop, T., Manz, L.-S., Doussot, C., Fisher, A. and Egelhaaf, M. (2019). Gap perception in bumblebees. *J. Exp. Biol.* 222, ieb184135. doi:10.1242/ieb.184135
- Rock, I. and Smith, D. (1986). The optomotor response and induced motion of the self. *Perception* 15, 497-502. doi:10.1068/p150497
- Rossignol, S., Dubuc, R. and Gossard, J. P. (2006). Dynamic sensorimotor interactions in locomotion. *Physiol. Rev.* 86, 89-154. doi:10.1152/physrev.00028. 2005
- Schilstra, C. and Van Hateren, J. H. (1999). Blowfly flight and optic flow. I. Thorax kinematics and flight dynamics. *J. Exp. Biol.* 202, 1481-1490. doi:10.1242/jeb. 202 11 1481
- Sherman, A. and Dickinson, M. H. (2004). Summation of visual and mechanosensory feedback in *Drosophila* flight control. *J. Exp. Biol.* **207**, 133-142. doi:10.1242/ieb.00731
- Souman, J. L., Frissen, I., Sreenivasa, M. N. and Ernst, M. O. (2009). Walking straight into circles. Curr. Biol. 19, 1538-1542. doi:10.1016/j.cub.2009.07.053
- Srinivasan, M. V. (2011). Visual control of navigation in insects and its relevance for robotics. Curr. Opin. Neurobiol. 21, 535-543. doi:10.1016/j.conb.2011.05.020
- Srinivasan, M. V., Zhang, S. W., Lehrer, M. and Collett, T. S. (1996). Honeybee navigation en route to the goal: visual flight control and odometry. J. Exp. Biol. 199, 237-244. doi:10.1242/jeb.199.1.237
- Stowers, J. R., Hofbauer, M., Bastien, R., Griessner, J., Higgins, P., Farooqui, S., Fischer, R. M., Nowikovsky, K., Haubensak, W., Couzin, I. D. et al. (2017). Virtual reality for freely moving animals. *Nat. Methods* 14, 995-1002. doi:10.1038/nmeth 4399
- Straw, A. D., Branson, K., Neumann, T. R. and Dickinson, M. H. (2011). Multicamera real-time three-dimensional tracking of multiple flying animals. *J. R. Soc.* Interface 8, 395-409. doi:10.1098/rsif.2010.0230
- Tammero, L. F. and Dickinson, M. H. (2002). The influence of visual landscape on the free flight behavior of the fruit fly *Drosophila melanogaster*. J. Exp. Biol. 205, 327-343. doi:10.1242/jeb.205.3.327
- Taylor, G. K. and Krapp, H. G. (2007). Sensory systems and flight stability: what do insects measure and why? *Adv. Insect Physiol.* **34**, 232-316. doi:10.1016/S0065-2806/07)34005-8
- Tuthill, J. C. and Azim, E. (2018). Proprioception. *Curr. Biol.* **28**, R194-R203. doi:10.1016/j.cub.2018.01.064
- Warren, W. H., Kay, B. A., Zosh, W. D., Duchon, A. P. and Sahuc, S. (2001). Optic flow is used to control human walking. *Nat. Neurosci.* 4, 213-216. doi:10.1038/84054
- Yamaguchi, S., Desplan, C. and Heisenberg, M. (2010). Contribution of photoreceptor subtypes to spectral wavelength preference in Drosophila. *Proc. Natl. Acad. Sci. USA* 107, 5634-5639. doi:10.1073/pnas.0809398107

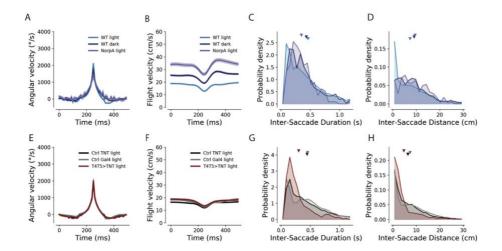


Fig. S1. Saccade characteristics across genotypes and stimulus conditions.

- (A) Angular velocity profile (mean ± sem) during a saccade executed in free flight for wild type flies in light (light blue), in dark (dark blue) and NorpA (blind) flies (magenta).
- (B) Mean flight velocity (± sem) for same saccades as in (A).
- (C) Probability density of inter-saccade duration for all intersaccadic segments in each genotype (same flight trajectories as in (A)). Triangles represent the mean inter-saccade duration for the respective genotype.
- (D) Probability density of inter-saccade distance for all intersaccadic segments in each genotype (same flight trajectories as in (A)). Triangles represent the mean inter-saccade distance of all segments for the respective genotype.
- (E) Same measurements as in (A) for motion-blind flies (T4T5>TNT depicted in dark red), UAS parental control (black) and Gal4 control (grey).
- (F, G and H) Same measurements as in (B), (C) and (D) respectively, for motion blind and parental control flies.

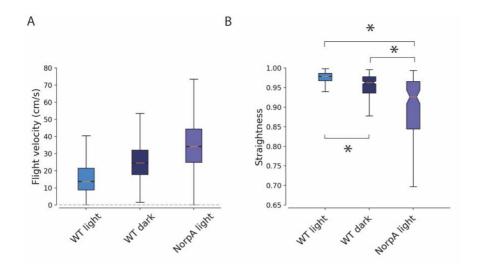


Fig. S2. Free-flight behavior of wildtype flies in the light and in the dark as well as of blind flies.

- (A) Flight velocity of wildtype flies in the light (light blue), in the dark (dark blue) and blind flies (magenta). n=1988 trajectories light, n=310 trajectories dark, n=100 trajectories blind flies.
- (B) Straightness of long inter-saccade flight segments (250-2000 ms). Significant differences based on a Kolgomorov/Smirnov test (p<0.0001, n=4609 segments light, n= 302 segments dark, n=100 segments blind).

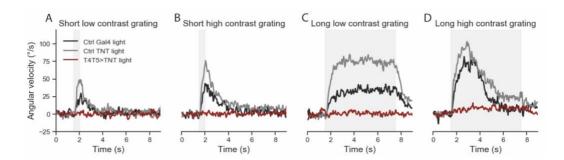


Fig. S3. Motion-blind flies show no optomotor response in tethered walking Angular velocity of tethered-walking flies during presentation of full-field square wave gratings had a spatial wavelength of  $\lambda$  = 20°, a mean luminance of 11 cd m<sup>-2</sup> and either high (49%) or low (1.4%) contrast. They moved at a velocity of 20 ° s<sup>-1</sup> either to the left or to the right and were presented for a short (0.5 s) or a long period of time (6 s). Flies which walked continuously for at least 10 trials were selected and only the trials which had an average walking speed of higher than 0.25 cm·s<sup>-1</sup> were included in the analysis. Turning speed traces were determined by taking the average over trials and low-pass filtering the resulting trace ( $\tau$  = 0.1 s in all experiments).

- (A) Angular velocity response to short low contrast grating.
- (B) Angular velocity response to short high contrast grating.
- (C) Angular velocity response to long low contrast grating.
- (D) Angular velocity response to long high contrast grating. n=7 motion blind flies (dark red), n=9 for TNT Control (grey), n=4 Gal4 Control flies (black)

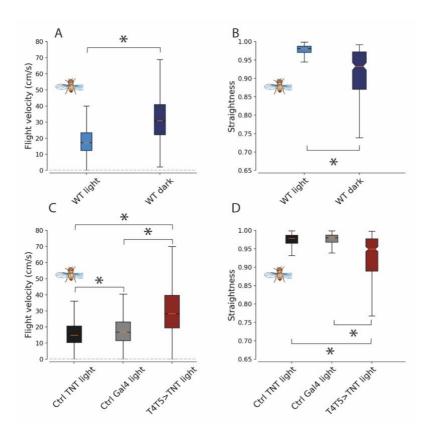


Fig. S4. Free-flight behavior of wing-clipped flies.

- (A) Flight velocity of flies in the light and in the dark (n=6773 trajectories light and n= 205 trajectories dark).
- (B) Straightness of inter-saccade flight segments (250 2000 ms) of wildtype flies. Significant differences based on Kolgomorov/Smirnov test (n=7607 inter-saccade flight segments light and n=241 segments dark p<0.0001).
- (C) Flight velocity of motion-blind flies and parental controls (TNT Control n=4983 trajectories, Gal4 Control n=3893 trajectories, T4T5>TNT n=734 trajectories).
- (D) Straightness of inter-saccade flight segments (250 2000 ms) of parental control and T4T5 > TNT flies. Significant differences are based on Kolgomorov/Smirnov test (p<0.0001, TNT Control n= 6818 inter-saccade flight segments, Gal4 Control n= 7162 segments, T4T5>TNT n=418 segments).

Manuscript 2: A new experimental approach to studying path integration in Drosophila Melanogaster

#### Abstract

Many species rely on path integration to compute and maintain a stable representation of the shortest route home. So far, the search for neural substrates of this type of vector-based navigation has been restricted to species which use their nest as a stable point of reference, such as honeybees or desert ants. In the present study, we develop a behavioral paradigm in the genetically amenable, but a priori nest-less, fruit fly *Drosophila melanogaster*, which allows to study path integration in this species. By optogenetically activating sugar-sensing gustatory neurons whenever the fly occupies a defined place within a chamber, we train it to revisit this position. Flies keep returning to the reinforced location over a few minutes, even after the optogenetic reward is discontinued. When a second reward is introduced at another location, flies successfully oscillate between the two locations, even when reward is withheld at one of them. Future experiments will determine the influence of proprioceptive feedback on the homing behavior and the neural substrate of the path integrator.

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#### Contributions

<u>Conceptualization</u>: L.N.G., A.B.; <u>Methodology</u>: L.N.G; <u>Setup</u>: S.P.; <u>Software</u>: L.N.G, S.P., M.-B.L.; <u>Validation</u>: M.-B.L.; <u>Formal analysis</u>: M.-B.L.; <u>Investigation</u>: M.-B.L., T.R., L.N.G; <u>Data curation</u>: M.-B.L.; <u>Writing - original draft</u>: M.-B.L.; <u>Writing - review & editing</u>: M.-B.L., L.N.G, A.B.; <u>Visualization</u>: M.-B.L.; <u>Supervision</u>: A.B., L.N.G; <u>Funding acquisition</u>: A.B., L.N.G

#### Introduction

Animals spend a great deal of time foraging for food. To find a viable food source, foraging insects have to explore the environment, which may occur in long convoluted trips. Rather than taking the same path on the way back to their home, many animals prefer the most direct route back, in spite of the unfamiliarity with the terrain. This is crucial for the survival of the individual, not only reducing the energy consumption on the return trip, but also minimizing the probability of encountering predators (Heinze et al., 2018).

There are several strategies animals use to return to significant locations such as a nest or a food source. One example is following the gradient of chemical cues such as odors or pheromones coming from the desired location (Steck et al., 2010; Titova et al., 2023). A more suitable strategy for long-distance foraging is path integration, which is prevalent in nesting insects (Fisher, 2022). To keep track of the journey, these insects use a combination of idiothetic and allothetic cues. For example, the desert ant *Cataglyphis* uses celestial cues to determine the walking direction (Wehner and Lanfranconi, 1981) and a pedometer (step counter) as a method of assessing distance (Wittlinger et al., 2006, 2007). Similar to the desert ants, honeybees *Apis melifera* use some celestial cues for navigation (Rossel and Wehner, 1984) but rely on information provided by optic flow to estimate distance (Esch and Burns, 1996; Srinivasan et al., 2000; Kirchner and Braun, 1994).

Central to the path integration process is an accumulator which encodes the location of the nest as an initial state (Collett and Collett, 2000). As the insect is moving about the environment, the accumulator is storing the current position relative to the origin of the journey. This enables the insect to compute a so-called "homing vector" which represents the most direct path back to the nest. The homing vector defines two key attributes of the route back to the colony: the direction and the length of the path (Stone et al., 2017; Collett and Collett, 200; Fisher, 2022).

In recent years, it was proposed that the central complex, a region, highly conserved throughout insects (Heinze et al., 2018), is the neural substrate for path integration (Stone et al., 2017). While clear path integration behaviors have only been observed in homing insects, new studies indicate the possibility that all insects with a central complex might have the capacity of calculating a "homing vector", even in the absence of a nest (Heinze et al., 2018). A great model to study this hypothesis is *Drosophila melanogaster*: a nest-less insect with a well characterized central complex (Scheffer et al., 2020; Hulse

et al., 2020) and a plethora of genetic tools available to manipulate neuronal function (Franconville et al., 2018).

There is already some evidence indicating that fruit flies are capable of path integration. However, most of the current studies focus on the neural encoding of heading direction. Flies combine proprioceptive inputs with other sensory information in a continuous manner (Heinze et al., 2018). This process takes place in the compass neurons of the central complex of the brain (Seelig and Jayaraman, 2015). The result is an egocentric heading direction representation which is then transformed into an allocentric representation (Lu et al., 2022). This representation is then used by the fly to navigate through the environment. When olfactory and visual cues are not available, flies use idiothetic cues to return to a previously discovered food source (Kim and Dickinson, 2017; Corfas et al., 2019).

In this study, we address the ability of the fruit fly to determine the length component of the homing vector. By collapsing a walking chamber to a narrow linear track, we limit the directional component of the homing vector to only two viable options. We further restrict the information available to the fly by eliminating visual and olfactory cues, forcing it to rely on proprioceptive feedback to walk about the track. Using appetitive reinforcement as an incentive to revisit a certain specific location, we demonstrate that fruit flies are capable of path integration. We further test the ability of these insects to estimate distance in a cue-free environment, showing that flies correctly calculate the travelled length towards a secondary reward zone. Our work establishes a novel behavioral paradigm with high throughput that enables use of existing genetic tools to uncover the neural circuit mechanisms underlying path integration in *Drosophila melanogaster*.

#### Materials and Methods

Fly husbandry and genotypes

The following genotypes of *Drosophila melanogaster* were used: w [1118]; LexAop-CsChrimson; + (Bloomington Drosophila Stock Center BL#55138), w [1118]; +; + (stock curtesy of Gertrude Heimbeck), w [1118]; +; Gr43a-LexA (Bloomington Drosophila Stock Centre BL#93446).

All flies were reared on standard cornmeal food in a controlled environment (12h light/12h dark cycle, 60% ambiental humidity).

#### All-trans retinal (ATR) preparation

100 mg of ATR (R2500, P Code 1003530621, Source SLCJ4760, Sigma Aldrich Germany) was dissolved in EtOH 99.8% purity (32221-M, CAS: 64-17-5 Sigma Aldrich Germany) resulting in a solution with a concentration of 100mM.

#### Fly treatment for experiments

To improve the performance of optogenetically activated ion channels, flies were provided with an ATR-supplemented diet, which acts as a photosensitizer for the channels. One-day-old male and female flies were switched to a diet consisting of standard cornmeal mixed with 100 microliters ATR solution (100 mM). After 48 hours, the flies were transferred to containers with no food. To avoid dehydration, the containers had a piece of filter paper saturated with an ATR solution (100 microliters ATR in 5 ml distilled water). Flies were starved for a minimum of 48 hours before performing the experiments. The flies were kept in the dark for the entire duration of the treatment (approximately 4 days). To avoid CO<sub>2</sub> exposure prior to experiment, the flies were transferred to the walking chamber using gentle sucking with a mouth pipette. After each experiment, the walking chamber was cleaned with a mild odor-free detergent solution.

#### Behavioral setup

The experimental setup consisted of a walking chamber made of transparent plexiglass (15.5cm x 0.5cm) (Figure 1A, B). The chamber is illuminated from the sides via infrared lights (850, VSMY1850ITX01, Vishay Semiconductors, USA). Optogenetic stimulation was delivered via an inhouse-built light guide mounted on a 6x6 grid of 590nm LED lights (997-LXZ1-PL03, Lumileds Holding B.V.). Images were acquired at a rate of 10 frames per second via a Blackfly S USB3 camera (BFS-U3-51S5M, Teledyne FLIR USA) mounted with a  $\Phi$ 30.5 objective (Model 25FM50SP, Tamron Japan), covered with an infrared filter (UV/VIS Cut M34.0x0.5 Filter, Edmund Optics, USA). Images were sent to a Dell Precision 5820 Tower computer.

#### Live tracking and optogenetic stimulation

Frames were acquired via a Python 3 script and were immediately processed using the opency 4.5.5 package. After a background subtraction, the centroid coordinates of the mask were extracted and saved in a csv file. If the coordinates corresponded to a designated reward area, a trigger signal was sent to the optogenetic lamp via a Pico Robots Board (v 1.1, Kitronik UK).

#### Results

#### Studying path integration in nest-less insects

For nesting animals, the homing vector represents the direction and distance that an animal has to travel in order to arrive at a significant location, for example, the nest. To focus on the distance component of the homing vector, we limit the directional alternatives by placing individual flies in a linear walking track (Figure 1A). For nest-less insects such as *Drosophila*, a significant location is not readily available. To create such a location, we designed an experiment which uses appetitive reinforcement to build the association of an arbitrary-chosen point in space with the presence of food. The optogenetic stimulation enabled us to induce an appetitive reinforcement without dealing with the "messy" physical rewards. By offering a reward in the form of an illusion of tasting food to the fly each time it visits that point, we create the desired conditioning. We establish a reward zone at approximately 9.5 body lengths (2.27 cm) from the border of the walking chamber. The reward zone is approximately 2.7 body lengths in size (0.65 cm x 0.5 cm).

The experimental setup (Figure 1B) consists of a camera acquiring images at 10 frames per second, which are processed in real time, with the current position of the fly being compared to the location of the reward zone. If the fly is located within the boundaries of the reward zone, a lamp delivers 10 consecutive pulses of light at 590 nm of 20 ms each. This light opens the red-light drivable channelrhodopsin (Chrimson), so that the fructose gustatory receptors Gr43a are activated, thus offering the fly a fictive fructose reward. We observe that flies quickly recognize the requirement of vacating the reward zone to unlock a new reward (for an example see Figure 1 C and D). We train the flies using this paradigm for 30 minutes. Once the training period is over and the optogenetic activation is discontinued, flies continue revisiting the reward zone for a few minutes before changing back to an exploratory walk along the entire length of the arena.

#### Path length is successfully encoded by fruit flies

To determine whether flies are able to encode distance, we provide the flies with a second identical reward zone (4.22 cm from the border), with both zones being activated only if the fly has previously visited the opposite zone (Figure 2A). When calculating the occupancy rate in the chamber, we observe that flies whose Gr43a neurons are optogenetically activated are more likely to inhabit the reward zones rather than the rest of the arena, once the optogenetic stimulation is started (Figure 2 C, D and E, Supplemental Figure 2).

To test the premise that flies do not encode distance but rather walk until they receive a reward and then change direction, we first train the flies to oscillate between the two interest zones by rewarding them for every visit. Later on, we reward the second zone located at 4.22 cm from the edge in only 50% of the visits (Figure 3B). We observe similar results in occupancy rate as in the previous condition, even when the reward is withheld in half of the trials (Figure 3 C, D and E).

#### Learning increases with exposure to reward stimulus

There is a clear variability among individuals, with some flies perceiving the reward rule quickly, and others not (Supplemental Figure 1). To test the assumption that flies which visit reward zones more often perform better during testing, we compare the percentage of time flies spend between the exterior borders of the reward zones (marked as reward associated area in Figure 4A) during the training and testing periods. There is a clear correlation (r = 0.586), indicating that flies which spend more time oscillating between reward zones during the training period, perform better during the testing period (Figure 4B).

#### Flies revisit previously rewarded locations

To further challenge the flies, we doubled the distance between reward zones, and proceeded in repeating the experiments. No significant differences in performance were observed (Supplemental Figure 3). For the analysis in Figure 5, we selected only the flies with a minimum of 10 oscillations between zone 1 and zone 2 during the training period (n=12 flies). Firstly, we selected walking trajectories from the accommodation period, which pass first through zone 1 and then through zone 2. The walking segments displayed in Figure 5A contain data from 100 frames before the fly entered zone 2, traced until the fly changes direction of walking, which in most cases happens when the fly reaches the boundary of the walking chamber. We then plotted in Figure 5B the same type of walking segments produced during the training period (we limited the length of the segments to 300 frames after entering zone 2).

When it comes to the testing period, we split the data into two separate plots: one depicting walking segments when reward in zone 2 is withheld (Figure 5C) and one containing walking segments when the fly is rewarded in zone 2 (Figure 5D). We observe that walking segments are very similar in the case of reward for both training and testing condition. When examining the individual traces in the no reward condition (Figure 5C), we observe that, in most of these trials, flies favor returning to zone 1 when reward is not encountered, rather than continuing their path towards the opposite side of the arena.

The fact that a return to zone 1 is the more frequent behavior shows that flies keep a memory of the distance between the two zones.

#### Discussion

In this study, we confirm that appetitive reinforcement is a powerful conditional stimulus for memory formation of a reward location. This memory is created independent of visual or olfactory cues. Our setup explores the capacity of the fly to encode the distance between two reward zones. We prove that finding the rewards is not accidental, but rather is based on an idiothetic memory of the position of the rewards, relative to each other and relative to the current position of the fly. This indicates that flies are indeed relying on path integration to revisit previously-visited significant locations.

Despite the lack of a nest as observed in ants and bees, fruit flies are a suitable model organism for studying path integration considering the anatomical similarities in brain organization (Strausfeld, 2009) and genetic tools available. There are several behavioral studies, including the results presented above, that demonstrate that fruit flies are indeed capable of path integration (Kim and Dickinson, 2017; Titova et al., 2023; Corfas et al., 2019). For example, in the absence of visual and olfactory cues, flies presented with a food source perform a local search behavior around it, periodically revisiting the location of the food source, even in its absence, to re-zero the accumulator (Kim and Dickinson, 2017). Even clearer indications of path integration are observed when switching from a 2-dimensional walking arena to a narrow circular walking track where flies are optogenetically rewarded every time they visit an arbitrary location (Behbahani et al., 2021, Lu et al., 2022). It was reported that flies walked back and forth around the reward location for a short time after reward was disabled (Behbahani et al., 2021), showing that flies remember the location of the reward.

Our focus on the distance component of the homing vector is warranted by the extensive research on odometers in expert path integrators such as *Cataglyphis* and *Apis melifera*. Desert ants keep track of the distance travelled from the nest to the food source by counting their steps. In a famous experiment performed by Wittlinger and colleagues (2006), it is proven that the ants expect to walk a certain number of steps until finding the nest. By modifying the stride length via amputation or extension of the legs, ants misappraise the distance they travel on the way back to their nest. In comparison to walking ants, when flying, honey bees employ a different odometer for assessing traveled distance, namely, the optic flow generated by their self-motion. When forager bees

travelled through a tunnel (which caused increased optic flow) towards a food source, they reported overestimated distances towards that location to the other worker bees (Srinivasan et al., 2000).

To focus only on the distance component of the homing vector, we transformed the circular walking track used by Behbahani and colleagues into a linear track, collapsing the directionality component to only two options. Both data from Behbahani et al. and the current study indicate that flies remember the location of the reward zone, performing a search around it. Moreover, we observe the same switch from a search to an exploratory behavior after a few minutes from discontinuation of the reward (Figure 1D). We show that fruit flies correctly encode distance, turning back to reward zone 1 when they fail to receive the expected reward in zone 2.

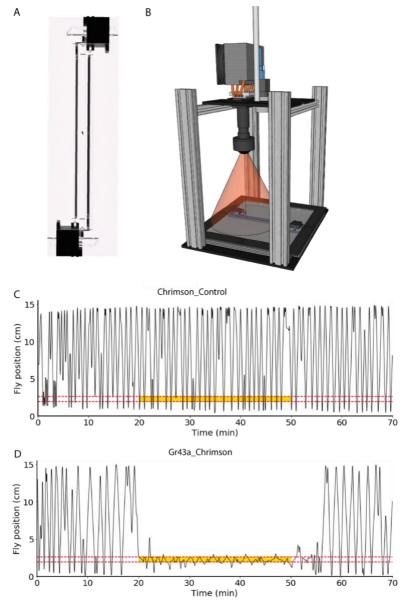
Since there is limited sensory information inside the experimental arena, we theorize that flies use a pedometer similar to that of desert ants to estimate distance travelled. We propose a slight modification to the current walking track set up to test this hypothesis. By lowering the ceiling of the chamber, we could constrain the flies, determining a change in stride due to the modification of the leg geometry. We propose an experiment which starts by training the flies to encode distance using natural step length, then we lower the ceiling to modify stride in the testing period. We expect flies to underestimate the distance to the reward in the testing phase due to shorter step size. Experiments for testing this hypothesis are underway. Such a result would however not deny the possibility that, during flight, fruit flies use an optic flow-based odometer to gauge distances, potentially alternating between the two depending on the adopted locomotion method.

The other component of the homing vector is direction. The neural mechanism for encoding travelling direction has been extensively studied and attributed to the central complex of the fly brain (Pfeiffer, 2022; Seelig and Jayaraman, 2015; Lu et al., 2022). We speculate that the neurons responsible for path integration encoding both the orientation and magnitude of the homing vector can be identified among the downstream post-synaptic partners of the h $\Delta B$  neurons, which are responsible for the egocentric to allocentric position transformation. Our setup provides an opportunity to determine the identity of these neurons: a simple activity block of these neurons would cause the respective flies' inability to locate the reward zone, once reward is discontinued.

There are however certain limitations to the current setup. Recent studies (Titova et al., 2023) raise the concern of a pheromone-based behavior as a confounding variable during path integration assessment. They show that naïve flies concentrate their exploration around droplets of pheromones deposited by previous inhabitants which received optogenetic sugar rewards. While our walking chamber is cleaned after each individual, we cannot control for pheromone deposits excreted around the reward zone during the training period, to which the flies could return to during testing. Further experiments are required to address this concern: by genetically blocking the olfactory system of the fly, one could eliminate the pheromones as confounding variable.

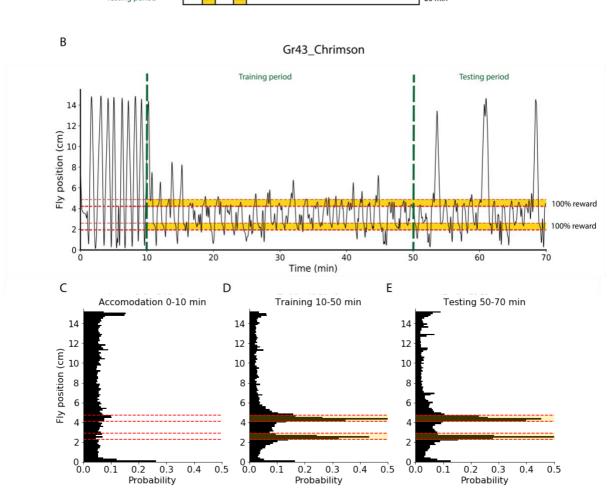
In conclusion, we present a reliable experimental paradigm to study path integration in *Drosophila melanogaster*. We demonstrate that fruit flies can store the path length travelled, being able to predict they are at the location of a food source previously encountered. In future studies, this experimental design can be used to investigate the neural underpinnings of path integration in general and the encoding of path length in particular.

Figure 1

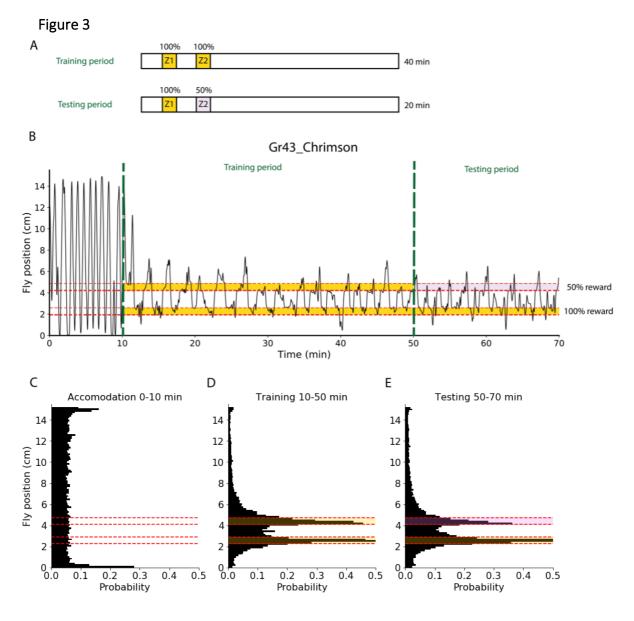


(A) Digital rendering of walking chamber. The track is 15.5 cm long and 0.5 cm wide. (B) Schematic representation of the experimental setup (light shield not included). (C) Example walking trajectory of a single control fly (LexAop-Chrmson Control). Dashed red lines indicate reward position, yellow area indicates the 30 minutes interval when optogenetic activation is enabled. Every time the fly passes through the reward area, it receives 10 consecutive light (590 nm) pulses of 20ms each. (D) Example walking trajectory of a single fly whose sugar-sensing neurons are optogenetically tagged. Every time the fly passes through the reward area, it receives a fictive sugar reward through activation of sugar sensing neurons via 10 consecutive light (590 nm) pulses of 20ms each. Fly has to vacate the reward area to unlock a new reward. During the reward period, the fly walks mainly in the reward area, and it continues to revisit reward area for a few minutes after the reward is no longer offered.



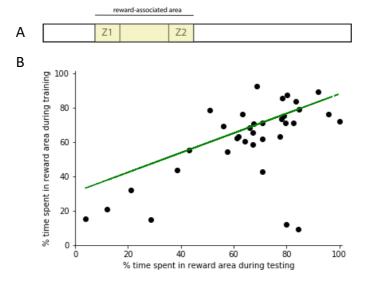


(A) Schematic representation (not drawn to scale) of the experimental protocol. Rectangles labelled Z1 and Z2 represent the designated reward zones. (B) Example walking trajectory of a single fly whose sugar-sensing neurons are optogenetically activated (yellow) when crossing the arbitrary reward zones (2.27 and 4.22 cm from edge). The fly is motivated to alternate between reward zones by offering a fictive sugar reward when entering the zone only if the fly has previously visited the opposite zone. Thus, if fly exits and re-enters the same zone, the sugar sensing neurons will not be stimulated. (C) Pooled (n=11 flies) probability distribution of walking chamber occupancy during the accommodation period, (D) training period and (E) testing period of flies whose sugar sensing neurons are optogenetically activated via the protocol explained above. In this case, training and testing periods have the same conditions.



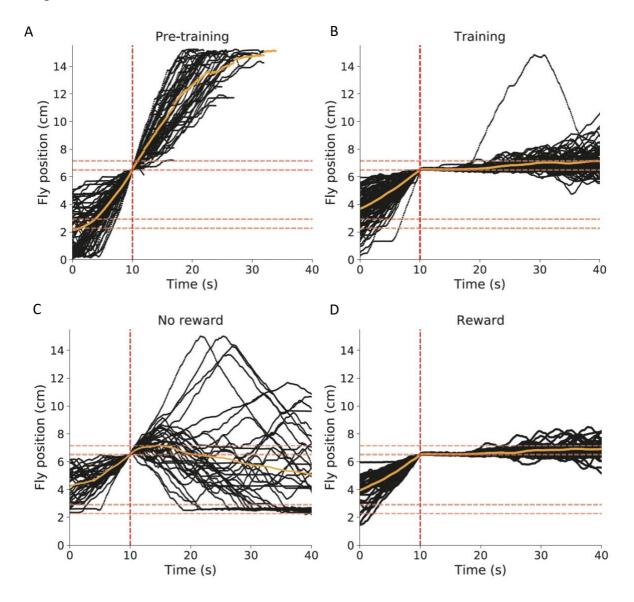
(A) Schematic representation (not drawn to scale) of the experimental protocol. Rectangles labelled Z1 and Z2 represent the designated reward zones. (B) Example walking trajectory of an experimental fly with the same genetic background as in Figure 2. During training (30 min) the fly receives the same stimulation treatment as described before. During testing, however, the fly is only rewarded every second time it visits the second reward zone. (C) Pooled (n=24 flies) probability distribution of walking chamber occupancy during accommodation, (D) training and (E) testing period.

Figure 4



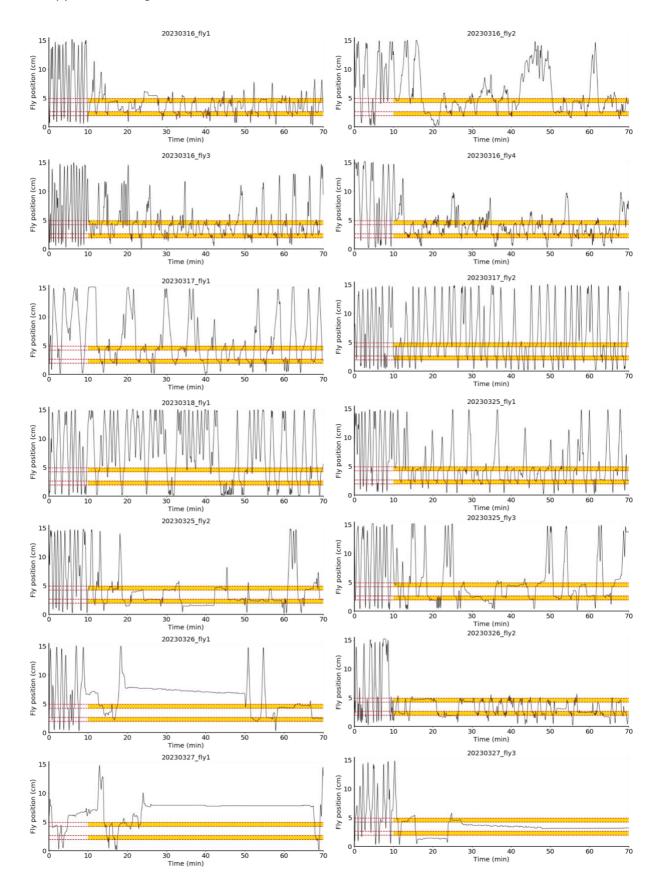
(A) Schematic depiction of walking chamber (not drawn to scale). Rectangles labelled Z1 and Z2 represent the designated reward zones. The yellow shaded area represents the chamber interval where flies spend time when they oscillate between reward zones. We consider that when flies are in this area, they correctly remember the reward zones. When flies exit this area, we consider the fly as not correctly recalling the locations of the rewards. (B) Correlation between the time of Gr43\_Chrimson flies (n=35) spent in the reward-associated zone during training period and the time spent in the same space of same flies during testing period. The Pearson correlation coefficient is 0.586.

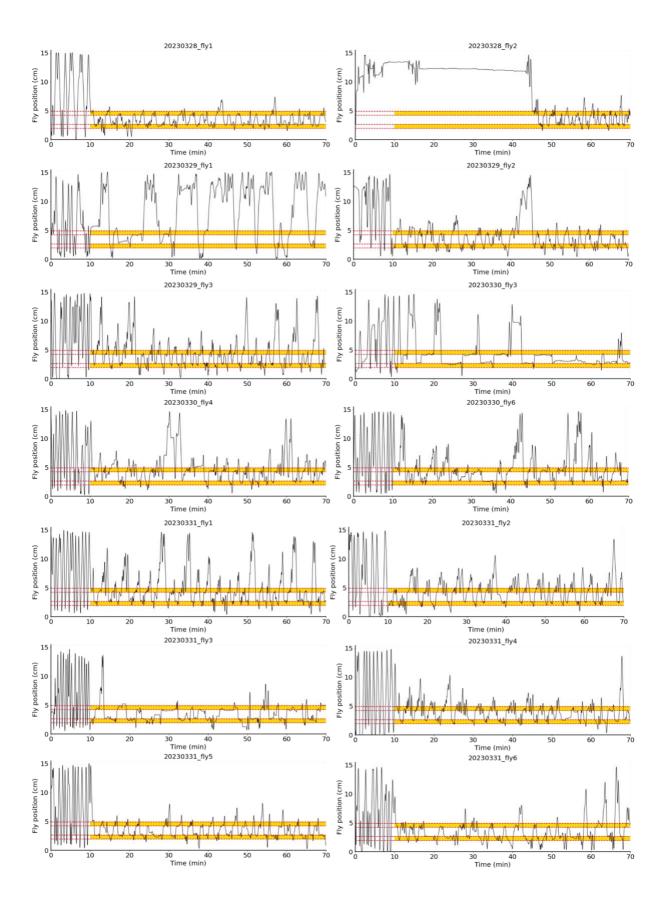
Figure 5

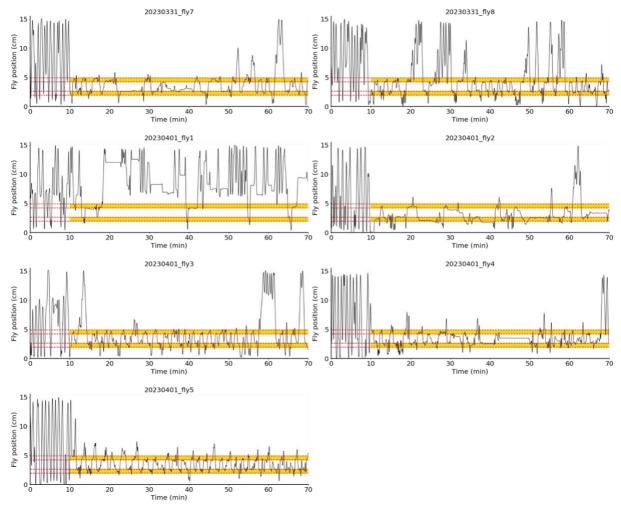


(A) Fragments of walking trajectories of Gr43\_Chrmson flies (n=12 flies). The segments are aligned to the moment the flies enter second reward zone after visiting first reward zone (indicated by red vertical line). The trajectories displayed start 10 seconds before event and stop when fly starts turning around. Average walking segment displayed in orange. (B) Walking segments of same flies during training period. Segments are aligned by the same method, with 10 seconds before entering second reward zone, and 30 seconds after reward is received. (C) During the testing period, the flies get rewarded when entering zone 2 only 50% of the time. Here we display the trials where flies do not get rewarded. (D) Rewarded trials of flies in (C).

## Supplemental Figure 1

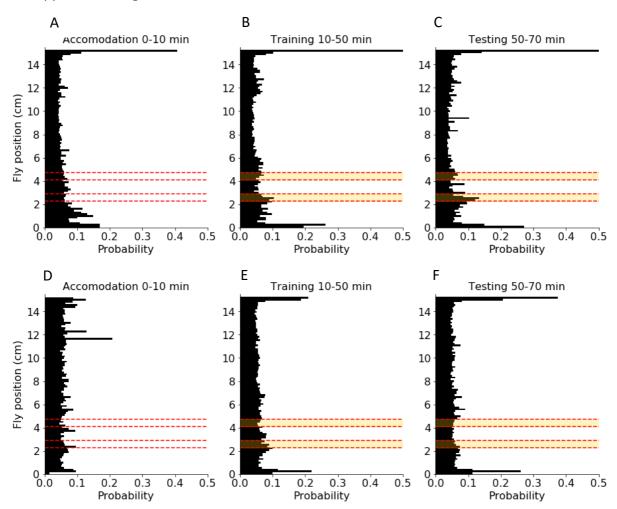






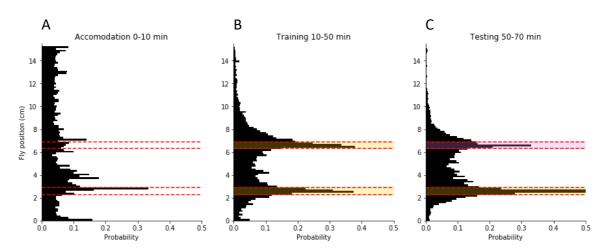
Individual fly walking trajectories of 35 out of the 39 flies of the genotype Gr43-LexA/Chrmson-LexAop. 4 flies were eliminated due to the fact that they did not walk at all or walked very sparsely during the experiment. Flies were left to accommodate to the chamber for 10 minutes. They were then rewarded every time they oscillated between the 2 reward zones by receiving a fictive sugar reward via optogenetic stimulation. This training lasted 30 min. For the remainder of the experiment, they were offered a reward in zone 2 only every other time they visited it.

### Supplemental Figure 2



Probability distribution of the walking chamber occupancy of LexAop-Chrmson controls (n=7) during **(A)** accommodation, **(B)** training and **(C)** testing. Probability distribution of the walking chamber occupancy of Gr43a-LexA controls (n=9) during **(D)** accommodation, **(E)** training and **(F)** testing.

## Supplemental Figure 3



Probability distribution of the walking chamber occupancy of Gr43\_Chrmson controls (n=7) during **(A)** accommodation, **(B)** training, and **(C)** testing for double distance between reward zones (To be compared to Figure 3 C, D, E).

#### References

- Behbahani, A. H., Palmer, E. H., Corfas, R. A., & Dickinson, M. H. (2021). *Drosophila* rezero their path integrator at the center of a fictive food patch. *Current Biology*, *31*(20), 4534-4546.
- Collett, T. S., & Collett, M. (2000). Path integration in insects. *Current opinion in neurobiology*, 10(6), 757-762.
- Corfas, R. A., Sharma, T., & Dickinson, M. H. (2019). Diverse food-sensing neurons trigger idiothetic local search in *Drosophila*. *Current Biology*, *29*(10), 1660-1668.
- Esch, H. E., & Burns, J. E. (1996). Distance estimation by foraging honeybees. *Journal of experimental biology*, 199(1), 155-162.
- Fisher, Y. E. (2022). Flexible navigational computations in the *Drosophila* central complex *Current opinion in neurobiology*, 73, 102514.
- Franconville, R., Beron, C., & Jayaraman, V. (2018). Building a functional connectome of the *Drosophila* central complex. *Elife*, 7, e37017.
- Heinze, S., Narendra, A., & Cheung, A. (2018). Principles of insect path integration. *Current Biology*, *28*(17), R1043-R1058.
- Hulse, B. K., Haberkern, H., Franconville, R., Turner-Evans, D. B., Takemura, S., Wolff, T., ... & Jayaraman, V. (2020). A connectome of the *Drosophila* central complex reveals network motifs suitable for flexible navigation and context-dependent action selection. *bioRxiv*, 2020-12.
- Kim, I. S., & Dickinson, M. H. (2017). Idiothetic path integration in the fruit fly *Drosophila melanogaster*. *Current Biology*, *27*(15), 2227-2238.
- Kirchner, W. H., & Braun, U. (1994). Dancing honey bees indicate the location of food sources using path integration rather than cognitive maps. *Animal Behaviour*, 48(6), 1437-1441.

- Lu, J., Behbahani, A. H., Hamburg, L., Westeinde, E. A., Dawson, P. M., Lyu, C., ... & Wilson, R. I. (2022). Transforming representations of movement from body-to world-centric space. *Nature*, *601*(7891), 98-104.
- Lyu, C., Abbott, L. F., & Maimon, G. (2022). Building an allocentric travelling direction signal via vector computation. *Nature*, *601*(7891), 92-97.
- Pfeiffer, K. (2022). The neuronal building blocks of the navigational toolkit in the central complex of insects. *Current Opinion in Insect Science*, 100972.
- Rossel, S., & Wehner, R. (1984). Celestial orientation in bees: the use of spectral cues. *Journal of Comparative Physiology*, *155*, 605-613.
- Scheffer, L. K., Xu, C. S., Januszewski, M., Lu, Z., Takemura, S. Y., Hayworth, K. J., ... & Plaza, S. M. (2020). A connectome and analysis of the adult *Drosophila* central brain. *Elife*, *9*, e57443.
- Seelig, J. D., & Jayaraman, V. (2015). Neural dynamics for landmark orientation and angular path integration. *Nature*, *521*(7551), 186-191.
- Srinivasan, M. V., Zhang, S., Altwein, M., & Tautz, J. (2000). Honeybee navigation: nature and calibration of the" odometer". *Science*, *287*(5454), 851-853.
- Steck, K., Knaden, M., & Hansson, B. S. (2010). Do desert ants smell the scenery in stereo?. *Animal Behaviour*, 79(4), 939-945.
- Stone, T., Webb, B., Adden, A., Weddig, N. B., Honkanen, A., Templin, R., ... & Heinze, S. (2017). An anatomically constrained model for path integration in the bee brain. *Current Biology*, *27*(20), 3069-3085.
- Strausfeld, N. J. (2009). Brain organization and the origin of insects: an assessment. *Proceedings of the Royal Society B: Biological Sciences*, *276*(1664), 1929-1937.
- Titova, A. V., Kau, B. E., Tibor, S., Mach, J., Vo-Doan, T. T., Wittlinger, M., & Straw, A. D. (2023). Displacement experiments provide evidence for path integration in *Drosophila*. *Journal of Experimental Biology*, jeb-245289.

- Wehner, R., & Lanfranconi, B. (1981). What do the ants know about the rotation of the sky?. *Nature*, *293*(5835), 731-733.
- Wittlinger, M., Wehner, R., & Wolf, H. (2006). The ant odometer: stepping on stilts and stumps. *Science*, *312*(5782), 1965-1967.
- Wittlinger, M., Wehner, R., & Wolf, H. (2007). The desert ant odometer: a stride integrator that accounts for stride length and walking speed. *Journal of experimental Biology*, *210*(2), 198-207.

#### Discussion

## Chapter 1: RESTRAINED VS FREE BEHAVIOR

At first glance, *Drosophila melanogaster* could be perceived as a simple organism with a relatively low number of neurons. However, the vast collection of visually-based behaviors disproves this view. Considering the significant amount of research and the current interest in the field, it is very likely that this will be the first multi-cellular organism, from which we can achieve a complete understanding of the neuronal substrates of visual behaviors (Zhu, 2013). Thus, a plethora of methods to study the link between neuronal processing and behavior were developed. While in the past, most of these experiments were performed on tethered flies, current technological advancements allow studying visual behavior in freely moving flies. In the following, I will highlight some of the studies and contrast them with classic tethered behavioral assays.

The acrobatic abilities of the fly are far superior to any human-made aeronautic advancements (Fry et al., 2009). However, to measure certain aspects of flight control, scientists are forced to tether the animal to be able to perform the experiments necessary (Taylor et al., 2008). This is especially useful if behavioral experiments are coupled with 2-photon calcium imaging or electrophysiological techniques (i.e. Kim et al., 2023; Fenk et al., 2021). However, by tethering the flies to a pin, we dramatically reduce the range of motions the fly can execute, breaking the natural dynamics of the mechanical system of flight (Taylor et al., 2008). Such complex in-flight maneuvers are only possible if all sensory organs provide continuous information about the position of the body in the air. The tethered state destabilizes the feedback loops normally available, causing significant behavioral artifacts (Fry et al., 2009). For example, one of the crucial inputs comes from the fly gyroscopes, the so-called halteres, where haltere-less flies being unable to take off into free flight. Surprisingly, such flies are capable of flying when tethered to a pin (Mureli and Fox, 2015). It has been found that haltere input has a significant influence on certain characteristics of flight trajectories, even in tethered flight: flies able to perform rotations in the yaw axis performed faster saccades compared to rigidly tethered flies (Frye and Dickinson, 2004).

Another example of feedback destabilization can be observed in studies on behavioral responses to looming stimuli. Expanding stimuli are known to elicit avoidance responses

in fruit flies. However, during forward flight, the fly experiences visual expansion as well, indicating the existence of a complementary system that overrides the avoidance response (Budik et al., 2007). Sometimes, due to the overzealousness of the scientist to eliminate as many confounding variables as possible, flight experiments are performed in still air, depriving the flies of the usual air currents caused by their forward motion. In magnetically tethered flies, a dampening in the avoidance response is observed when the Johnston's organ (which reports wind direction) is stimulated (Budik et al., 2007). Such stimulation is generally missing in rigidly tethered preparations, resulting in misleading data.

An interesting dataset from Tuthill et al. (2013) shows the importance of comparing tethered with freely-behaving responses. When unilateral back-to-front motion is displayed, tethered flies show a significantly weaker optomotor response compared to freely-behaving animals. Additionally, comparing tethered flight saccadic escape responses to saccades in free flight shows significant differences, which are accounted for by the lack of haltere input (Tammero and Dickinson, 2002).

Another great limitation of the tethering system is the low throughput: only one fly can be tethered in one setup at a time, forcing labs to either increase the number of setups available or spend many hours performing the same experiments. By contrast, with the help of newer tracking technologies, free behavior setups can house many individuals at once (even up to 100, see Zhu and Frye, 2009), significantly increasing the throughput of these experiments. However, the multifariousness of the behavioral outputs and the significant amount of data generated by such technologies makes this type of research non-trivial (Wang et al., 2022).

Technological advancements such as computer-controllable displays, high-resolution and high-speed video-acquiring equipment expanded the development of experimental paradigms to discover novel behaviors (Zhu, 2013). In parallel, software developments including artificial intelligence video analysis allow the tracking of multiple individuals who are free to move in an experimental enclosure (Stowers et al., 2017). For example, Ctrax is an offline open-source software that provides the ability to track multiple subjects without loss of identity and is capable of detecting behavioral patterns (Branson et al., 2009). Further progress is also made in the live-tracking software. For example, Grover et al. (2008) proposed a system that not only tracks a freely moving fly, but also reconstructs the visual field that the fly experiences. Manuscript 1 describes a free flight enclosure which is monitored via a system on 5 high-speed cameras, surrounded by high-resolution displays that present a static random checkered pattern to the enclosed flies.

It proposes a simple offline tracking and 3D flight trajectory reconstruction software for multiple flies. While this software does not acquire fly identity, it is sufficient for the analysis and characterization of flight trajectories.

Genetic tools have evolved into sophisticated and precise targeting methods for neuronal populations of cells with similar genetic backgrounds. Additionally, the field of optogenetics has brought a revolution to many scientific fields (AzimiHashemi et al., 2014; Rein and Deussing, 2012). Optogenetic manipulations give scientists the ability to modulate neuronal activity and observe their causal effect on behavior (Fiala et al., 2010, Riemensperger et al., 2016). This state-of-the-art technique enables a high temporal resolution, while the binary expression systems enable precise spatial targeting (Fenno et al., 2011, Luan et al., 2006). By far, the greatest benefit of this technique is that light penetrates the cuticle housing the fly's brain, making it ideal for free behavior experiments. Manuscript 2 takes advantage of these techniques, using highly selective LexA lines targeting the cells containing the gustatory receptor Gr43a to drive the expression of the optogenetic channel Chrimson (depolarizing the neurons). By activating the gustatory receptors repeatedly, we created an association between a sweet reward and a certain location in space, without having to deal with difficult-to-manage actual sugar rewards that leave odor traces behind. Moreover, optogenetic activation enables millisecond-resolution stimulus delivery and termination.

No matter how sophisticated the tethering systems become, nothing will compare to monitoring free behavior and natural movements (Fry et al., 2009). As we have seen in the previous section, technological progress facilitates a more detailed analysis of free behavior, while genetic tools advancements enable cell-resolution network manipulation. Object preference (Maimon et al., 2008), multisensory integration (van Breguel and Dickinson, 2014), and course control are just a few of the topics best researched in freely behaving animals. The current studies (Manuscript 1 and Manuscript 2) are examples of observations that would have been missed in a tethered type of setup. For many decades, the importance of perceiving optic flow for course control has been theorized and hypothesized. However, nothing short of observing motion-blind flies in a free flight setup could provide irrefutable proof of the matter (Manuscript 1). Furthermore, to observe the behavioral readout of a complex behavior integrating a plethora of sensory inputs such as path integration is difficult to achieve in a virtual reality setup. Thus, Manuscript 2 finds a compromise between scientific rigor, which minimizes confounding variables, and observing animals performing unrestrained.

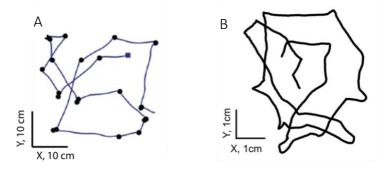
It should be noted, however, that once these strategies are discovered in controlled laboratory conditions, these behaviors must be validated in natural, visually complex, conditions to confirm robustness (Zeil, 2012). Manuscript 1 indeed provides the freedom for the fly to move completely unrestrained, but it still misses many sensory inputs generally found in a natural environment (i.e. olfactory gradients, natural light sources, wind, etc.). Manuscript 2 considerably reduces this freedom: flies are unable to fly, deprived of visual and olfactory inputs. However, only by doing so we can attribute the observed behavior to the path integration system.

Restraining the animal is useful when trying to correlate neural activity with a certain behavior. Fundamental questions regarding neural processing of visual information have been answered by pairing electrophysiological recordings and 2-photon imaging techniques with synchronous observations of tethered behavior (e.g., Kim et al., 2023; Fenk et al., 2021). Moreover, by tethering an animal, scientists limit the confounding variables and are able to better control and manipulate the stimuli delivered, thus characterizing highly robust and reliable behaviors which can then be generalized to more complex environments. With this structured approach, one can quantify the amplitude of behavioral responses to finely controlled stimulation, thus gaining a better understanding of the system.

# **Chapter 2: COURSE CONTROL**

A generic flight pattern of a fruit fly (and many other Dipterans) is characterized by long, relatively straight flight segments, separated by rapid changes in direction, commonly known as saccades (Tammero and Dickinson, 2002; Frye et al., 2003; Manuscript 1). The fixate-and-saccade strategy is very similar to the movement of human eyes (Cellini and Mongeau, 2020; Theobald, 2017), which alternate between smooth pursuits and rapid movements that change the point of fixation (which are also called saccades). The exact mechanism triggering saccades in flies is not yet known. Image expansion might prompt a fly's decision to change course to avoid collision (Tammero and Dickinson, 2002). However, not all saccades are explained by this phenomenon, with some seeming completely random. It has been proposed that the non-visual induced saccades are a result of a searching strategy with "Levy-flight" characteristics. Such a searching pattern is optimal for finding food in an environment where no source is known *a priori*. This locomotor program maximizes the searcher's perceptual range, which in turn maximizes the probability of encountering an attractive odor (Reynolds and Frye, 2007). The ecological efficiency of this search pattern is so useful, that the fly also uses this strategy

in walking (Reynolds and Frye, 2007; Tao et al., 2020). By blocking synapses in the ellipsoid body, this search behavior is abolished in walking (Martin et al., 2001). This indicates that the central complex might be an important brain area related to foraging.



**Figure 9: Comparison of locomotion trajectories in** *Drosophila melanogaster* (A) 2D projection of a free flight trajectory (Reproduced from Leonte et al., 2020). (B) Walking trajectory of a free fruit fly (Reproduced from Cruz et al., 2021).

The flight saccade motor sequence is well understood. Body posture and wing stroke pattern change minutely and precisely to achieve the desired directional change. Once the saccade is initiated, no visual input can change its kinematics (Tammero and Dickinson, 2002). Comparing saccade properties of motion-blind flies and wild-type controls shows no significant differences (Manuscript 1).

Optic flow provides information about the rotation and the translation of the fly in relation to the environment. What this information stream lacks is a distinction between the optic flow caused by self-movement and optic flow caused by external factors such as wind. Unintended changes of course caused by external determinants have to be immediately corrected. This is very challenging for the fly, given the complexity and ambiguity of the optic flow information. One approach to reduce the computational time needed for amending course is keeping inter-saccadic flight segments as straight as possible, thus minimizing the rotational component of the optic flow (Collet et al., 1993; Tammero and Dickinson, 2002; von Holst and Mittelstaedt, 1950). In Manuscript 1 we could demonstrate that this tactic is innate and independent of vision, as flies perform such flight patterns even in complete darkness.

We further show that saccades are independent of visual input. Saccades of motion-blind flies have been compared to those performed by visually apt flies, and no significant differences were observed. To perform a saccade, the optomotor response of a fly needs to be suppressed. It was indeed shown that HS cells are silenced using signals from motor

efference copies when executing an intentional change of flight direction (Kim et al., 2017).

LPTCs play a crucial role in course control during locomotion. Already in the 1960s, electrophysiological recordings performed in *Musca domestica* and *Caliphora phaenicia* revealed that their receptive field resembled an optic flow as elicited by rotation around different body axes (Bishop et al., 1968). Two main classes of LPTCs have been identified: a group of cells (HS) tuned to wide-field motion in the horizontal plane, and a group of cells (VS) tuned to wide-field motion in the vertical plane (Krapp et al., 1998). Due to their functional properties, LPTCs were expected to control optomotor response turns in flies. This was proved by optogenetic activation of HS cells, which evoked turning behavior in flight (Haikala et al., 2013). Directionally-sensitive T4 and T5 cells (Maisak et al., 2013) provide retinotopic visual information to the HS and VS cells, which integrate this information to determine wide-field motion (Schnell et al., 2012). Blocking these inputs causes an absence of the optomotor response. It has long been theorized that the optomotor response plays a key role in course control. In Manuscript 1, we could confirm this hypothesis by introducing an asymmetry in wing surface area, which resulted in a loss of course control ability in motion-blind flies.

Such a complex behavior like aerial maneuvering requires extensive multi-sensory computations and feedback loops from the visual system, the halteres, the ocelli, the antennae, the campaniform sensilla, and the muscles of the wings (Mronz and Lehmann, 2008; Frye and Dickinson, 2004). It is not yet fully understood how the fly can integrate and prioritize such complex multi-sensory information (Gepner et al., 2015).

Interestingly, the fly's reaction time for responding to visual stimulation is about 30 milliseconds (Maimon et al., 2008). By contrast, haltere information processing takes only half of that time (Dickinson and Mujires, 2016). Mechanosensory information from halteres and wings is sufficient to maintain straightness to a certain degree, as seen in the trajectories of flying flies in darkness. However, to keep the intersaccadic segments straight when physiological asymmetries arise, flies seem to rely on optic flow. In cases of dissimilar feedback from other sensory modalities compared to visual information (such as in the case of wing damage), flies favor motion vision information for altering wing stroke patterns, thus maintaining a straight course. Both motion-blind flies and flies which lack optic flow information (i.e. flying in the dark) are unable to maintain straightness during inter-saccadic flight when having asymmetric wing damage (Manuscript 1).

By observing both the predator and prey during free-flight maneuvers, we can learn a lot about the minute differences in flight dynamics, wing kinematics, and body stabilizing approaches of different species. Moreover, we can observe the interplay of different sensory modalities to alter course.

When fruit flies encounter a predator (such as a dragonfly) while flying, they adopt certain changes in flight patterns to avoid danger. As a reaction to a looming visual input midflight flies execute "directed banked turns" which consist of a rapid body rotation followed by a stabilization via a counter-rotation (Muijres et al., 2014). These changes must be performed very rapidly, thus requiring a dedicated circuit for escape maneuvers. Thus, rapid and precise detection of the looming stimulus, followed by immediate initiation of evasive motor programs can help a fly avoid perishing. The sudden change in flight heading is achieved via a subtle change in wing stroke amplitude and takes no longer than two complete wingbeats (Muijres et al., 2014). These evasive flight patterns require a multilevel analysis and pooling of input from the optic flow, halteres, and wings to ensure stabilization after the sudden change in direction.

If we look at the predator flight pattern, taking dragonflies as examples, we observe a wonderful capability to use sensorimotor processing to follow prey in visually cluttered environments (Huston and Jayaraman, 2011). Interestingly, dragonflies aim their flight trajectory towards a predicted point of interception. This point is continuously updated based on the movements of the prey (Olberg et al., 2020). Similar to *Drosophila*, they present key changes in wing kinematics, which result in a subtle asymmetry in beating pattern, thus determining course changes (Fry et al., 2003; Wang et al., 2022). Visual and ocellar information is used to control their course and body posture mid-flight. Compared to the fruit fly, ocellar input is essential, especially during prey pursuit flights, to alter inflight body posture (Krapp, 2009).

# **Chapter 3: PATH INTEGRATION**

We have seen in the previous chapter that flies employ an innate meandering flight pattern when foraging to maximize the probability of finding nourishment. A similar search behavior is observed in walking flies (Reynolds and Frye, 2007). However, this is not the only locomotor program observed in flies, when it comes to food. Fruit flies display a local search behavior when encountering a droplet of food (Corfas et al., 2019). A similar search pattern is observed in desert ants that have been displaced from their homebound path. When the ant cannot locate the nest at the expected destination, it

executes a search behavior around the expected location, which is similar to that of flies. Both behaviors seem to belong to the "Devonian toolkit" and probably stem from an ancient form of path integration that has evolved to serve different purposes in different species. (Kim and Dickinson, 2017; Corfas et al., 2019; Müller and Wehner 1994). Compared to random searches, these behaviors have in common a search point of origin: for the fly, the droplet of food, and for the ant, the expected location of the nest. Such locations are not available for a random search behavior.

An animal can update an estimate of its position in reference to a point of origin in real-time. While the return journey to the origin is not encoded by the path integrator, it is often the best behavioral readout of the path integration process, reporting both the direction and the distance from the point of origin in the form of a vector (Heinze et al, 2018). Nesting animals are known for their exquisite ability to return to their colony. However, evidence that non-nesting animals are also able to integrate their path has emerged recently. For example, fruit flies can return to a location associated with food that was previously encountered without relying on external cues (Kim and Dickinson, 2017; Corfas et al., 2019; Manuscript 2). Path integration is especially helpful when an animal is faced with an unknown environment or deprived of any visual landmarks (Collet et al., 2013).

When it comes to determining the length of the homing vector, different strategies are employed, depending on the animal and locomotion mode. While flying insects (e.g. honey bees) rely exclusively upon optic flow information, walking ants require access to light polarization patterns to extract distance information (Sommer and Wehner, 2005). They combine this information with the pedometer input to reliably asses the traveled distance. Previous literature in the field of *Drosophila* path integration failed to address this question directly. Manuscript 2 offers a behavioral experimental setup perfect for such an undertaking. By linearizing the track, we limit the available number of directions, thus observing exclusively the distance encoding process. We show that flies deprived of any visual input are able to predict the location of a reward and, in the case of discontinued optogenetic stimulation, they perform a search around the area that was previously rewarded. Furthermore, in the double reward zone task during trials where the reward is withheld, flies immediately turned around to the opposite reward zone. This shows that flies can encode the distance between the two. It is hypothesized that flies update their odometer either by using proprioceptive information or efferent copies of motor commands (Corfas et al., 2019). Manuscript 2 proposes a variation of the setup that can modify step size to determine whether walking flies also encode distance using a pedometer.

# Chapter 4: INSECT NAVIGATION – COMBINING NAVIGATIONAL STRATEGIES

Behavioral strategies like path integration are prone to internal noise, especially for longer journeys (Fisher, 2022). Noise comes from many sources, such as quantum uncertainty, stochastic neural spikes, and storing and updating the internal accumulator. (Heinze et al., 2018). Therefore, to navigate, animals are forced to use a combination of external (e.g. sun position) and internal cues (e.g. leg proprioceptive signals) to reliably reach the desired destination (Kim and Dickinson, 2017).

To find their nest, animals use path integration as a general indication of the nest location, relying on visual memory of the scene around the nest to correctly arrive at the destination (Australian desert ant: Narendra, 2007). Other modalities include approaching a nest from downwind to rely on the odor plume coming from the nest (desert ants: Steck et al., 2010; beetle: Dacke et al., 2019; fruit flies: Okubo et al., 2020), learning the color and texture of the nest (honey bees: Cheng et al, 1986; Dittmar et al., 2011) and even analyzing the skylight panorama (ants: Graham and Cheng, 2009).

Celestial cues are extremely useful reference points for determining traveling direction due to the (almost) infinite distance from the animal, which minimizes azimuth variation during translation changes. The dung beetle *Scarabaeus viettei* uses a combination of celestial cues including the sun and the milky way (Dacke et al., 2021), the monarch butterfly *Danaus plexippus* uses a sun compass for establishing the migration pattern (Heinze and Reppert, 2011), and the fruit fly combines the sky polarization patterns with the sun position information (Warren et al., 2018; Giraldo et al., 2018) to keep a straight course on long journeys.

Drosophila can integrate all these distinct cues into a space map that guides navigation. In theory, the neuroarchitecture of the central complex is opportune for the center of path integration (Stone et al, 2017). Of great interest is the ellipsoid body, a torus-shaped brain structure organized in vertical columns. Several neuron types are notable: ellipsoid body-protocerebral bridge-gall neurons (E-PG), the ring neurons which generally provide visual information, and the protocerebral bridge-ellipsoid body- noduli (PEN). These neurons relay inputs from the following different sensory modalities onto the ellipsoid body:

- (1) **Self-motion information** is relayed to the ellipsoid body via PEN neurons (Fisher, 2022).
- (2) Flies detect the **light polarization** direction via the photoreceptors R7 and R8 in the ommatidia facing the sky in the dorsal rim area (DRA). The information is then transmitted via interneurons in the distal medulla to the anterior optic tubercle, passes through the bulb, and eventually arrives in the ellipsoid body of the central complex (Hardcastle et al., 2021).
- (3) For longer journeys, it was found that *Drosophila melanogaster* uses **the Sun as a landmark** for orientation and course control. When E-PG compass neurons are silenced, flies orient directly to the Sun's position (object fixation) rather than adopting an arbitrary heading relative to it (Giraldo et al., 2018).
- (4) In the case of **wind**, the magnitude of displacement of the Johnston's organ in the antennae is relayed to the antennal mechanosensory and motor center (AMMC). The information is then sent to the lateral accessory lobe and then to E-PG neurons in the central complex (Okubo et al., 2020).
- (5) Flies use **visual cues** (place learning) to remember the position of a reward (Ofstad et al., 2011). Ring neurons in the central complex encode these visual memories as demonstrated in silencing experiments (Stern et al., 2019; Kim and Dickinson, 2017).

Information from these modalities is combined in the ellipsoid body torus, which represents an organized map of the visual environment of the fly (El Jundi and Dacke, 2021). It acts as a compass, conveying a bump of  $Ca^{2+}$  activity representing the heading direction of the fly. This direction information is collected by the  $\Delta 7$  neurons and passed on to all columnar cell types in the protocerebral bridge (PB). PfNd neurons (tuned to forward ipsilateral movements) and PfNv neurons (preferring backward contralateral movement) synapse onto  $h\Delta B$  neurons, transforming the direction of movement representation from an egocentric coordinate system to an allocentric set of coordinates (Pfeiffer, 2022). It is believed that distance and direction information conveyed by the path integration system is not used independently, but rather synthesized in an accumulator along with all other navigational cues (Heinze et al., 2018). Therefore, we believe that the homing vector representation is located in this brain region as well. The experimental approach described in Manuscript 2 is ideal for the genetic screening of all candidates for path integration in the central complex.

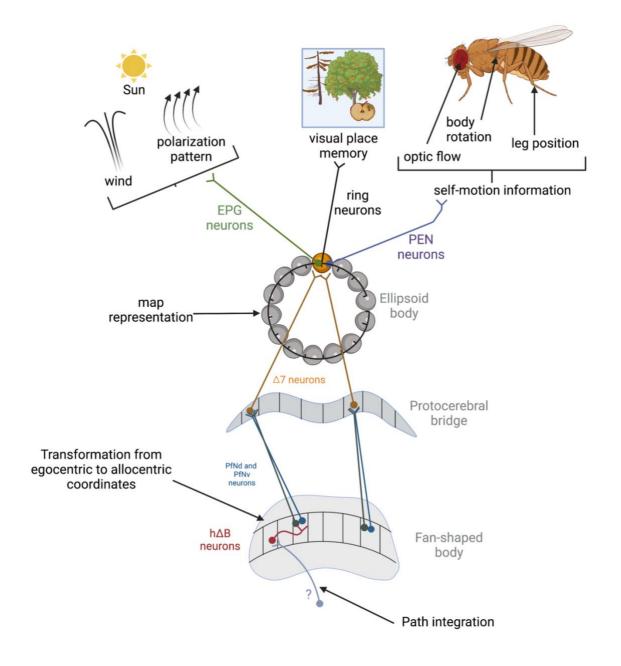


Figure 10: Multisensory path integration in the central complex

The ellipsoid body in the central complex receives information about the fly's position in space from various sources (proprioception, Sun position, polarization patterns, wind direction, etc). It then consolidates this information into a map representation of the position of the fly relative to an egocentric set of coordinates. This position is relayed to the protocerebral bridge, where PfNd and PfNv neurons transform this representation into an allocentric set of coordinates and pass it on to  $h\Delta B$  neurons in the fan-shaped body. Manuscript 2 hypothesizes that cells directly downstream are responsible for representing the homing vector. Illustration created using BioRender

We theorize that cells downstream of the  $h\Delta B$  neurons are responsible for the path integration process. We moreover speculate that blocking these neurons would hinder the path integration process of the fly.

## Chapter 5: MULTIMODAL INTEGRATION

Generally, to study the causal relationship between genes, neuronal circuits and behaviors, scientists prefer well-controlled experiments involving stimulation of one single sensory modality (Sanchez-Alcãniz and Benton, 2017). However, focusing on one sense limits our understanding of the integration of inputs from different modalities. Both Manuscript 1 and Manuscript 2 deal (to some degree) with multisensory inputs and give us an insight into how the fly combines and consolidates different categories of neuronal inputs.

Two approaches can be employed in studying multimodal integration: complete silencing of a sensory modality or distorting the sensory information available to the fly. **Manuscript 1** provides examples for both modalities. By clipping part of the wing, we distort the normally expected propulsive symmetry, forcing the fly to adapt the wing beat frequency to compensate for the imbalance. By depriving flies of visual stimulation (in the dark) or blocking the motion vision pathway, we directly reduce the number of available inputs. These approaches enable us to get a better understanding of how the brain integrates information from different circuits, by observing how these modifications affect the behavioral output.

Manuscript 1 proves the importance of motion-vision information for course control. The study shows that flies rely on visual information to compensate for the bias produced by wing surface asymmetry. However, another important point highlighted in this study is that when the bias is not present, flies are able to fly normally, even in complete darkness. This indicates that completely missing visual input is not consequential to the fly in laboratory conditions. The other inputs are sufficient to maintain normal course if correctly integrated. Similarly, by ablating the wing and leaving the visual system intact, the fly compensates for the asymmetry.

No sensory modality is perfect, and all systems are prone to noise. Therefore, by integrating multiple sensory inputs, the noise is reduced to a statistical minimum. When an input is missing, the animal is forced to recalibrate the weights of every other input to achieve a new noise minimum. By completely silencing a sensory modality, we can

observe how the relative weights of the other inputs are modified to compensate for the missing input. This also implies that the animal is expecting a certain level of congruency between the information streams. When an inconsistency is detected (e.g. when one of the sensory streams is disrupted), the weight of the input is severely reduced. Therefore, both silencing and disturbing the sensory modalities are valuable experimental approaches to understanding the multisensory integration process.

# Chapter 6: CONCLUSION AND OUTLOOK

The availability of genetic tools with precise targeting, the advancements of technology and software enabling observation of freely-moving animals, and the full connectome data available for the fruit fly have resulted in an unprecedented understanding of the neural computations underlying behavioral programs in *Drosophila melanogaster* (Ryu et al., 2022).

There is clear evidence that flies rely on multiple sensory information for performing certain behaviors. A visual stimulus translating or approaching the fly can induce various responses depending on the speed of translation, the position in relation to the fly, and the behavioral context at that moment (Fisher, 2022; Ryu et al., 2022). As an example, object expansion detected in the context of landing will be given a different value than if detected as a possible danger during a foraging flight. For landing, the fly will employ one motor command (extension of legs and change in body orientation to gracefully land), while in the case of predator avoidance, the fly would execute a banked turn to avoid being caught.

The question remaining is how this weight is distributed across sensory modalities in the case of conflicting information. We briefly address this in **Manuscript 1**, where optic flow information is given a higher importance when aerodynamic imbalances are detected. It would be interesting to further study the multisensory integration in the central complex to understand the neural mechanisms that filter and establish a hierarchy of the different sensory inputs.

# **Bibliography**

- Ache, J. M., Namiki, S., Lee, A., Branson, K., & Card, G. M. (2019). State-dependent decoupling of sensory and motor circuits underlies behavioral flexibility in Drosophila. *Nature neuroscience*, 22(7), 1132-1139.
- Arendt, D., Hausen, H., & Purschke, G. (2009). The 'division of labour' model of eye evolution. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *364*(1531), 2809-2817.
- Arenz, A., Drews, M. S., Richter, F. G., Ammer, G., & Borst, A. (2017). The temporal tuning of the *Drosophila* motion detectors is determined by the dynamics of their input elements. *Current Biology*, *27*(7), 929-944.
- AzimiHashemi, N., Erbguth, K., Vogt, A., Riemensperger, T., Rauch, E., Woodmansee, D., ... & Liewald, J. F. (2014). Synthetic retinal analogues modify the spectral and kinetic characteristics of microbial rhodopsin optogenetic tools. *Nature communications*, *5*(1), 5810.
- Bahl, A., Ammer, G., Schilling, T., & Borst, A. (2013). Object tracking in motion-blind flies. *Nature neuroscience*, *16*(6), 730-738.
- Bahl, A., Serbe, E., Meier, M., Ammer, G., & Borst, A. (2015). Neural mechanisms for *Drosophila* contrast vision. *Neuron*, *88*(6), 1240-1252.
- Barnhart, E. L., Wang, I. E., Wei, H., Desplan, C., & Clandinin, T. R. (2018). Sequential nonlinear filtering of local motion cues by global motion circuits. *Neuron*, *100*(1), 229-243.
- Bender, J. A., & Dickinson, M. H. (2006). A comparison of visual and haltere-mediated feedback in the control of body saccades in *Drosophila melanogaster*. *Journal of Experimental Biology*, 209(23), 4597-4606.
- Bishop, L. G., Keehn, D. G., & McCann, G. D. (1968). Motion detection by interneurons of optic lobes and brain of the flies *Calliphora phaenicia* and *Musca domestica*. *Journal of Neurophysiology*, *31*(4), 509-525.
- Borst, A. (2018). A biophysical mechanism for preferred direction enhancement in fly motion vision. *PLoS computational biology*, *14*(6), e1006240.
- Branson, K., Robie, A. A., Bender, J., Perona, P., & Dickinson, M. H. (2009). High-throughput ethomics in large groups of *Drosophila*. *Nature methods*, *6*(6), 451-457.
- Braun, A., Borst, A., & Meier, M. (2023). Disynaptic inhibition shapes tuning of OFF motion detectors in *Drosophila*. *Current Biology*, 33(11), 2260–2269.

- Buchner, E. (1976). Elementary movement detectors in an insect visual system. *Biological Cybernetics*, 24(2), 85-101.
- Budick, S. A., Reiser, M. B., & Dickinson, M. H. (2007). The role of visual and mechanosensory cues in structuring forward flight in *Drosophila melanogaster*. *Journal of Experimental Biology*, *210*(23), 4092-4103.
- Busch, C., Borst, A., & Mauss, A. S. (2018). Bi-directional control of walking behavior by horizontal optic flow sensors. *Current Biology*, *28*(24), 4037-4045.
- Card, G., & Dickinson, M. H. (2008). Visually mediated motor planning in the escape response of *Drosophila*. *Current Biology*, *18*(17), 1300-1307.
- Casso, D., Ramírez-Weber, F. A., & Kornberg, T. B. (1999). GFP-tagged balancer chromosomes for *Drosophila melanogaster*. *Mechanisms of development*, 88(2), 229-232.
- Cellini, B., & Mongeau, J. M. (2020). Hybrid visual control in fly flight: insights into gaze shift via saccades. *Current Opinion in Insect Science*, 42, 23-31.
- Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., & Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science*, *263*(5148), 802-805.
- Chen, M. S., Obar, R. A., Schroeder, C. C., Austin, T. W., Poodry, C. A., Wadsworth, S. C., & Vallee, R. B. (1991). Multiple forms of dynamin are encoded by shibire, a Drosophila gene involved in endocytosis. *Nature*, *351*(6327), 583-586.
- Cheng, K., Collett, T. S., & Wehner, R. (1986). Honeybees learn the colours of landmarks. Journal of Comparative Physiology A, 159, 69-73.
- Chiappe, M. E., Seelig, J. D., Reiser, M. B., & Jayaraman, V. (2010). Walking modulates speed sensitivity in Drosophila motion vision. *Current Biology*, *20*(16), 1470-1475.
- Chyb, S., Raghu, P., & Hardie, R. C. (1999). Polyunsaturated fatty acids activate the *Drosophila* light-sensitive channels TRP and TRPL. *Nature*, *397*(6716), 255-259.
- Collett, M., Chittka, L., & Collett, T. S. (2013). Spatial memory in insect navigation. *Current Biology*, *23*(17), R789-R800.
- Collett, T. S., & Collett, M. (2000). Path integration in insects. *Current opinion in neurobiology*, 10(6), 757-762.
- Collett, T. S., Nalbach, H.O., Andwanger, H. (1993). Visual stabilization in arthropods. In: *Visual Motion and its Role in the Stabilization of Gaze. Elsevier*, 239 263.
- Corfas, R. A., Sharma, T., & Dickinson, M. H. (2019). Diverse food-sensing neurons trigger idiothetic local search in *Drosophila*. *Current Biology*, *29*(10), 1660-1668.

- Cosens, D., & Spatz, H. C. (1978). Flicker fusion studies in the lamina and receptor region of the *Drosophila* eye. *Journal of Insect Physiology*, 24(8-9), 587-594.
- Coyne, J. A., Boussy, I. A., Prout, T., Bryant, S. H., Jones, J. S., & Moore, J. A. (1982). Long-distance migration of *Drosophila*. *The American Naturalist*, *119*(4), 589-595.
- Cruz, T. L., Pérez, S. M., & Chiappe, M. E. (2021). Fast tuning of posture control by visual feedback underlies gaze stabilization in walking *Drosophila*. *Current Biology*, *31*(20), 4596-4607.
- Dacke, M., Baird, E., El Jundi, B., Warrant, E. J., & Byrne, M. (2021). How dung beetles steer straight. *Annual Review of Entomology*, 66, 243-256.
- Dacke, M., Bell, A. T., Foster, J. J., Baird, E. J., Strube-Bloss, M. F., Byrne, M. J., & El Jundi, B. (2019). Multimodal cue integration in the dung beetle compass. *Proceedings of the National Academy of Sciences*, 116(28), 14248-14253.
- Delgado, R., & Bacigalupo, J. (2009). Unitary recordings of TRP and TRPL channels from isolated *Drosophila* retinal photoreceptor rhabdomeres: activation by light and lipids. *Journal of Neurophysiology*, 101(5), 2372-2379.
- Dickerson, B. H., de Souza, A. M., Huda, A., & Dickinson, M. H. (2019). Flies regulate wing motion via active control of a dual-function gyroscope. *Current Biology*, *29*(20), 3517-3524.
- Dickinson, M. H. (1999). Haltere—mediated equilibrium reflexes of the fruit fly, Drosophila melanogaster. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences, 354(1385), 903-916.
- Dickinson, M. H., & Muijres, F. T. (2016). The aerodynamics and control of free flight manoeuvres in *Drosophila*. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *371*(1704), 20150388.
- Dinges, G. F., Chockley, A. S., Bockemühl, T., Ito, K., Blanke, A., & Büschges, A. (2021). Location and arrangement of campaniform sensilla in *Drosophila melanogaster*. *Journal of Comparative Neurology*, *529*(4), 905-925.
- Dittmar, L., Egelhaaf, M., Stürzl, W., & Boeddeker, N. (2011). The behavioral relevance of landmark texture for honeybee homing. *Frontiers in behavioral neuroscience*, *5*, 20.
- Drews, M. S., Leonhardt, A., Pirogova, N., Richter, F. G., Schuetzenberger, A., Braun, L., ... & Borst, A. (2020). Dynamic signal compression for robust motion vision in flies. *Current Biology*, *30*(2), 209-221.

- El Jundi, B., & Dacke, M. (2021). Insect orientation: The Drosophila wind compass pathway. *Current Biology*, *31*(2), R83-R85.
- Esch, H. E., & Burns, J. E. (1996). Distance estimation by foraging honeybees. *Journal of Experimental Biology*, 199(1), 155-162.
- Fenk, L. M., Kim, A. J., & Maimon, G. (2021). Suppression of motion vision during course-changing, but not course-stabilizing, navigational turns. *Current Biology*, 31(20), 4608-4619.
- Fenno, L., Yizhar, O., & Deisseroth, K. (2011). The development and application of optogenetics. *Annual review of neuroscience*, *34*, 389-412.
- Fernald, R. D. (2000). Evolution of eyes. Current opinion in neurobiology, 10(4), 444-450.
- Fiala, A., Suska, A., & Schlüter, O. M. (2010). Optogenetic approaches in neuroscience. *Current Biology*, *20*(20), R897-R903.
- Fisher, Y. E. (2022). Flexible navigational computations in the *Drosophila* central complex *Current opinion in neurobiology*, *73*, 102514.
- Franceschini, N., Riehle, A., & Le Nestour, A. (1989). Directionally selective motion detection by insect neurons. In *Facets of vision* (pp. 360-390). Springer Berlin Heidelberg.
- Fry, S. N., Rohrseitz, N., Straw, A. D., & Dickinson, M. H. (2009). Visual control of flight speed in *Drosophila melanogaster*. *Journal of Experimental Biology*, *212*(8), 1120-1130.
- Fry, S. N., Sayaman, R., & Dickinson, M. H. (2003). The aerodynamics of free-flight maneuvers in *Drosophila*. *Science*, *300*(5618), 495-498.
- Frye, M. A., & Dickinson, M. H. (2004). Closing the loop between neurobiology and flight behavior in *Drosophila*. *Current opinion in neurobiology*, *14*(6), 729-736.
- Frye, M. A., Tarsitano, M., & Dickinson, M. H. (2003). Odor localization requires visual feedback during free flight in *Drosophila melanogaster*. *Journal of Experimental Biology*, 206(5), 843-855.
- Gepner, R., Mihovilovic Skanata, M., Bernat, N. M., Kaplow, M., & Gershow, M. (2015). Computations underlying *Drosophila* photo-taxis, odor-taxis, and multi-sensory integration. *eLife*, *4*, e06229.
- Giraldo, Y. M., Leitch, K. J., Ros, I. G., Warren, T. L., Weir, P. T., & Dickinson, M. H. (2018). Sun navigation requires compass neurons in *Drosophila*. *Current Biology*, *28*(17), 2845-2852.

- Graham, P., & Cheng, K. (2009). Ants use the panoramic skyline as a visual cue during navigation. *Current Biology*, *19*(20), R935-R937.
- Grigliatti, T. A., Hall, L., Rosenbluth, R., & Suzuki, D. T. (1973). Temperature-sensitive mutations in Drosophila melanogaster: XIV. A selection of immobile adults. *Molecular and General Genetics MGG*, 120(2), 107-114.
- Groschner, L. N., Malis, J. G., Zuidinga, B., & Borst, A. (2022). A biophysical account of multiplication by a single neuron. *Nature*, *603*(7899), 119-123.
- Grover, D., Tower, J., & Tavaré, S. (2008). O fly, where art thou?. *Journal of the Royal Society Interface*, *5*(27), 1181-1191.
- Gruntman, E., Romani, S., & Reiser, M. B. (2018). Simple integration of fast excitation and offset, delayed inhibition computes directional selectivity in *Drosophila*. *Nature neuroscience*, *21*(2), 250-257.
- Götz, K. G. (1975). Hirnforschung am Navigationssystem der Fliegen. *Naturwissenschaften*, 62, 468-475.
- Götz, K. G. (1987). Course-control, metabolism and wing interference during ultralong tethered flight in Drosophila melanogaster. *Journal of experimental biology*, 128(1), 35-46.
- Götz, K. G., Hengstenberg, B., & Biesinger, R. (1979). Optomotor control of wing beat and body posture in *Drosophila*. *Biological Cybernetics*, *35*, 101-112.
- Götz, K. G., & Wenking, H. (1973). Visual control of locomotion in the walking fruitfly *Drosophila*. *Journal of comparative physiology*, *85*, 235-266.
- Gu, Y., Oberwinkler, J., Postma, M., & Hardie, R. C. (2005). Mechanisms of light adaptation in *Drosophila* photoreceptors. *Current Biology*, *15*(13), 1228-1234.
- Haikala, V., Joesch, M., Borst, A., & Mauss, A. S. (2013). Optogenetic control of fly optomotor responses. *Journal of Neuroscience*, *33*(34), 13927-13934.
- Hardcastle, B. J., Omoto, J. J., Kandimalla, P., Nguyen, B. C. M., Keleş, M. F., Boyd, N. K., ... & Frye, M. A. (2021). A visual pathway for skylight polarization processing in *Drosophila*. eLife, 10, e63225.
- Hardie, R. C. (1986). The photoreceptor array of the dipteran retina. *Trends in Neurosciences*, *9*, 419-423.
- Hardie, R. C., & Juusola, M. (2015). Phototransduction in *Drosophila*. *Current opinion in neurobiology*, *34*, 37-45.

- Hardie, R. C., & Raghu, P. (2001). Visual transduction in *Drosophila*. *Nature*, *413*(6852), 186-193.
- Hart, M. H. (1978). The evolution of the atmosphere of the Earth. *Icarus*, 33(1), 23-39.
- Hartmann, G., & Wehner, R. (1995). The ant's path integration system: a neural architecture. *Biological Cybernetics*, 73(6), 483-497.
- Heinze, S., Narendra, A., & Cheung, A. (2018). Principles of insect path integration. *Current Biology*, *28*(17), R1043-R1058.
- Heinze, S., & Reppert, S. M. (2011). Sun compass integration of skylight cues in migratory monarch butterflies. *Neuron*, *69*(2), 345-358.
- Heisenberg, M., & Buchner, E. (1977). The role of retinula cell types in visual behavior of *Drosophila melanogaster*. *Journal of comparative physiology*, *117*(2), 127-162.
- Hu, K. G., & Stark, W. S. (1980). The roles of *Drosophila* ocelli and compound eyes in phototaxis. *Journal of comparative physiology*, 135, 85-95.
- Huang, J., Liu, C. H., Hughes, S. A., Postma, M., Schwiening, C. J., & Hardie, R. C. (2010). Activation of TRP channels by protons and phosphoinositide depletion in Drosophila photoreceptors. *Current Biology*, 20(3), 189-197.
- Huston, S. J., & Jayaraman, V. (2011). Studying sensorimotor integration in insects. *Current opinion in neurobiology*, *21*(4), 527-534.
- Jean-Guillaume, C. B., & Kumar, J. P. (2022). Development of the ocellar visual system in *Drosophila melanogaster*. *The FEBS journal*, *289*(23), 7411-7427.
- Jenett, A., Rubin, G. M., Ngo, T. T., Shepherd, D., Murphy, C., Dionne, H., ... & Zugates, C. T. (2012). A GAL4-driver line resource for *Drosophila* neurobiology. *Cell reports*, 2(4), 991-1001.
- Johns, D. C., Marx, R., Mains, R. E., O'Rourke, B., & Marbán, E. (1999). Inducible genetic suppression of neuronal excitability. *Journal of Neuroscience*, *19*(5), 1691-1697.
- Jösch, M., Schnell, B., Raghu, S. V., Reiff, D. F., & Borst, A. (2010). ON and OFF pathways in *Drosophila* motion vision. *Nature*, *468*(7321), 300-304.
- Jung, S. N., Borst, A., & Haag, J. (2011). Flight activity alters velocity tuning of fly motion-sensitive neurons. *Journal of Neuroscience*, *31*(25), 9231-9237.
- Kain, J. S., Stokes, C., & de Bivort, B. L. (2012). Phototactic personality in fruit flies and its suppression by serotonin and white. *Proceedings of the National Academy of Sciences*, 109(48), 19834-19839.

- Kalmus, H. (1943). The optomotor responses of some eye mutants of *Drosophila*. *Journal of Genetics*, 45(2), 206-213.
- Kefalov, V. J., Estevez, M. E., Kono, M., Goletz, P. W., Crouch, R. K., Cornwall, M. C., & Yau, K. W. (2005). Breaking the covalent bond—a pigment property that contributes to desensitization in cones. *Neuron*, *46*(6), 879-890.
- Kerfoot, W. B. (1967). Dorsal light receptors. Nature, 215(5098), 305-307.
- Kim, A. J., Fenk, L. M., Lyu, C., & Maimon, G. (2017). Quantitative predictions orchestrate visual signaling in *Drosophila*. *Cell*, *168*(1-2), 280-294.
- Kim, H., Park, H., Lee, J., & Kim, A. J. (2023). A visuomotor circuit for evasive flight turns in *Drosophila*. *Current Biology*, *33*(2), 321-335.
- Kim, I. S., & Dickinson, M. H. (2017). Idiothetic path integration in the fruit fly *Drosophila melanogaster*. *Current Biology*, *27*(15), 2227-2238.
- Kirchner, W. H., & Braun, U. (1994). Dancing honey bees indicate the location of food sources using path integration rather than cognitive maps. *Animal Behaviour*, 48(6), 1437-1441.
- Kirschfeld, K. (1967). Die Projektion der optischen Umwelt auf das Raster der Rhabdomere im Komplexauge von *Musca. Experimental Brain Research*, *3*, 248-270.
- Kirschfeld, K. (1976). The resolution of lens and compound eyes. In *Neural principles in vision* (pp. 354-370). Springer Berlin Heidelberg.
- Kitamoto, T. (2001). Conditional modification of behavior in Drosophila by targeted expression of a temperature-sensitive shibire allele in defined neurons. *Journal of neurobiology*, 47(2), 81-92.
- Klapoetke, N. C., Murata, Y., Kim, S. S., Pulver, S. R., Birdsey-Benson, A., Cho, Y. K., ... & Boyden, E. S. (2014). Independent optical excitation of distinct neural populations. *Nature methods*, *11*(3), 338-346.
- Krapp, H. G. (2009). Ocelli. Current Biology, 19(11), R435-R437.
- Krapp, H. G., Hengstenberg, B., & Hengstenberg, R. (1998). Dendritic structure and receptive-field organization of optic flow processing interneurons in the fly. *Journal of Neurophysiology*, 79(4), 1902-1917.
- Land, M. F., & Nilsson, D. E. (2012). Animal eyes. OUP Oxford.
- Laughlin, S. B. (1976). The sensitivities of dragonfly photoreceptors and the voltage gain of transduction. *Journal of comparative physiology*, *111*, 221-247.

- Laughlin, S. B. (1989). Coding efficiency and design in visual processing. In *Facets of vision* (pp. 213-234). Springer Berlin Heidelberg.
- Lu, J., Behbahani, A. H., Hamburg, L., Westeinde, E. A., Dawson, P. M., Lyu, C., ... & Wilson, R. I. (2022). Transforming representations of movement from body-to world-centric space. *Nature*, *601*(7891), 98-104.
- Luan, H., Diao, F., Scott, R. L., & White, B. H. (2020). The *Drosophila* split Gal4 system for neural circuit mapping. *Frontiers in neural circuits*, *14*, 603397.
- Luan, H., Peabody, N. C., Vinson, C. R., & White, B. H. (2006). Refined spatial manipulation of neuronal function by combinatorial restriction of transgene expression. *Neuron*, *52*(3), 425-436.
- Maimon, G., Straw, A. D., & Dickinson, M. H. (2008). A simple vision-based algorithm for decision making in flying *Drosophila*. *Current Biology*, *18*(6), 464-470.
- Maisak, M. S., Haag, J., Ammer, G., Serbe, E., Meier, M., Leonhardt, A., ... & Borst, A. (2013). A directional tuning map of Drosophila elementary motion detectors. *Nature*, *500*(7461), 212-216.
- Mamiya, A., Straw, A. D., Tómasson, E., & Dickinson, M. H. (2011). Active and passive antennal movements during visually guided steering in flying *Drosophila*. *Journal of Neuroscience*, *31*(18), 6900-6914.
- Martin, J. R., Faure, P., & Ernst, R. (2001). The power law distribution for walking-time intervals correlates with the ellipsoid-body in *Drosophila*. *Journal of neurogenetics*, 15(3-4), 205-219.
- McEwen, R. S. (1918) The reactions to light and to gravity in *Drosophila* and its mutants. *Journal of Experimental Zoology*, 25,49–106.
- Médioni, J. (1962). Contribution à l'étude psycho-physiologique et génétique du phototropisme d'un insecte: *Drosophila melanogaster meigen*. *Bulletin de psychologie*, *16*(212), 86-93.
- Montell, C. (2012). *Drosophila* visual transduction. *Trends in neurosciences*, *35*(6), 356-363.
- Mronz, M., & Lehmann, F. O. (2008). The free-flight response of *Drosophila* to motion of the visual environment. *Journal of Experimental Biology*, *211*(13), 2026-2045.
- Muijres, F. T., Elzinga, M. J., Melis, J. M., & Dickinson, M. H. (2014). Flies evade looming targets by executing rapid visually directed banked turns. *Science*, *344*(6180), 172-177.

- Mureli, S., & Fox, J. L. (2015). Haltere mechanosensory influence on tethered flight behavior in *Drosophila*. *The Journal of Experimental Biology*, *218*(16), 2528-2537.
- Müller, M., & Wehner, R. (1994). The hidden spiral: systematic search and path integration in desert ants, *Cataglyphis fortis*. *Journal of Comparative Physiology A*, *175*, 525-530.
- Nalbach, G., & Hengstenberg, R. (1994). The halteres of the blowfly Calliphora: II. Three-dimensional organization of compensatory reactions to real and simulated rotations. *Journal of Comparative Physiology A, 175,* 695-708.
- Narendra, A. (2007). Homing strategies of the Australian desert ant *Melophorus bagoti* II. Interaction of the path integrator with visual cue information. *Journal of Experimental Biology*, 210(10), 1804-1812.
- Nilsson, D. E. (1989). Optics and evolution of the compound eye. In *Facets of vision* (pp. 30-73). Springer Berlin Heidelberg.
- Ofstad, T. A., Zuker, C. S., & Reiser, M. B. (2011). Visual place learning in *Drosophila melanogaster*. *Nature*, *474*(7350), 204-207.
- Okubo, T. S., Patella, P., D'Alessandro, I., & Wilson, R. I. (2020). A neural network for wind-guided compass navigation. *Neuron*, *107*(5), 924-940.
- Olberg, R. M., Worthington, A. H., & Venator, K. R. (2000). Prey pursuit and interception in dragonflies. *Journal of Comparative Physiology A*, 186, 155-162.
- Pak, W. L., & Pinto, L. H. (1976). Genetic approach to the study of the nervous system. *Annual Review of Biophysics and Bioengineering*, *5*(1), 397-448.
- Paradis, S., Sweeney, S. T., & Davis, G. W. (2001). Homeostatic control of presynaptic release is triggered by postsynaptic membrane depolarization. *Neuron*, *30*(3), 737-749.
- Patel, R. N., & Cronin, T. W. (2020). Mantis shrimp navigate home using celestial and idiothetic path integration. *Current Biology*, *30*(11), 1981-1987.
- Pfeiffer, K. (2022). The neuronal building blocks of the navigational toolkit in the central complex of insects. *Current Opinion in Insect Science*, *55*, 100972.
- Pfeiffer, B. D., Ngo, T. T. B., Hibbard, K. L., Murphy, C., Jenett, A., Truman, J. W., & Rubin, G. M. (2010). Refinement of tools for targeted gene expression in *Drosophila*. *Genetics*, *186*(2), 735-755.
- Reichardt, W., & Wenking, H. (1969). Optical detection and fixation of objects by fixed flying flies. *Naturwissenschaften*, *56*, 424-424.

- Rein, M. L., & Deussing, J. M. (2012). The optogenetic (r)evolution. *Molecular Genetics and Genomics*, 287, 95-109.
- Reynolds, A. M., & Frye, M. A. (2007). Free-flight odor tracking in *Drosophila* is consistent with an optimal intermittent scale-free search. *PloS one*, *2*(4), e354.
- Riemensperger, T., Kittel, R. J., & Fiala, A. (2016). Optogenetics in *Drosophila* neuroscience. *Optogenetics: Methods and Protocols, 1408*,167-175.
- Rister, J., & Heisenberg, M. (2006). Distinct functions of neuronal synaptobrevin in developing and mature fly photoreceptors. *Journal of neurobiology*, 66(12), 1271-1284.
- Rister, J., Pauls, D., Schnell, B., Ting, C. Y., Lee, C. H., Sinakevitch, I., ... & Heisenberg, M. (2007). Dissection of the peripheral motion channel in the visual system of *Drosophila melanogaster*. *Neuron*, *56*(1), 155-170.
- Ryu, L., Kim, S. Y., & Kim, A. J. (2022). From photons to behaviors: neural implementations of visual behaviors in Drosophila. *Frontiers in Neuroscience*, *16*, 883640.
- Salem, W., Cellini, B., Frye, M. A., & Mongeau, J. M. (2020). Fly eyes are not still: a motion illusion in *Drosophila* flight supports parallel visual processing. *Journal of Experimental Biology*, 223(10), jeb212316.
- Sánchez-Alcañiz, J. A., & Benton, R. (2017). Multisensory neural integration of chemical and mechanical signals. *Bioessays*, *39*(8), 1700060.
- Schnell, B., Raghu, S. V., Nern, A., & Borst, A. (2012). Columnar cells necessary for motion responses of wide-field visual interneurons in *Drosophila*. *Journal of Comparative Physiology A*, 198, 389-395.
- Schilling, T., & Borst, A. (2015). Local motion detectors are required for the computation of expansion flow-fields. *Biology open*, 4(9), 1105-1108.
- Schwab, I. R. (2018). The evolution of eyes: major steps. The Keeler lecture 2017: centenary of Keeler Ltd. *Eye*, *32*(2), 302-313.
- Schwemer, J. (1989). Visual pigments of compound eyes—structure, photochemistry, and regeneration. In *Facets of vision* (pp. 112-133). Springer Berlin Heidelberg.
- Scott, E. K., Raabe, T., & Luo, L. (2002). Structure of the vertical and horizontal system neurons of the lobula plate in *Drosophila*. *Journal of Comparative Neurology*, 454(4), 470-481.
- Seelig, J. D., & Jayaraman, V. (2015). Neural dynamics for landmark orientation and angular path integration. *Nature*, *521*(7551), 186-191.

- Séguinot, V., Cattet, J., & Benhamou, S. (1998). Path integration in dogs. *Animal behaviour*, 55(4), 787-797.
- Shaw, S. R. (1989). The retina-lamina pathway in insects, particularly *Diptera*, viewed from an evolutionary perspective. In *Facets of vision* (pp. 186-212). Springer Berlin Heidelberg.
- Shinomiya, K., Huang, G., Lu, Z., Parag, T., Xu, C. S., Aniceto, R., ... & Meinertzhagen, I. A. (2019). Comparisons between the ON-and OFF-edge motion pathways in the Drosophila brain. *eLife*, *8*, e40025.
- Sommer, S., & Wehner, R. (2005). Vector navigation in desert ants, *Cataglyphis fortis*: celestial compass cues are essential for the proper use of distance information. *Naturwissenschaften*, *92*, 468-471.
- Srinivasan, M. V., Zhang, S., Altwein, M., & Tautz, J. (2000). Honeybee navigation: nature and calibration of the" odometer". *Science*, *287*(5454), 851-853.
- Stavenga, D. G. (1995). Insect retinal pigments: spectral characteristics and physiological functions. *Progress in retinal and eye research*, *15*(1), 231-259.
- Steck, K., Knaden, M., & Hansson, B. S. (2010). Do desert ants smell the scenery in stereo?. *Animal Behaviour*, 79(4), 939-945.
- Stern, U., Srivastava, H., Chen, H. L., Mohammad, F., Claridge-Chang, A., & Yang, C. H. (2019). Learning a spatial task by trial and error in *Drosophila*. *Current Biology*, *29*(15), 2517-2525.
- Stone, T., Webb, B., Adden, A., Weddig, N. B., Honkanen, A., Templin, R., ... & Heinze, S. (2017). An anatomically constrained model for path integration in the bee brain. *Current Biology*, *27*(20), 3069-3085.
- Stowers, J. R., Hofbauer, M., Bastien, R., Griessner, J., Higgins, P., Farooqui, S., ... & Straw, A. D. (2017). Virtual reality for freely moving animals. *Nature methods*, *14*(10), 995-1002.
- Sweeney, S. T., Broadie, K., Keane, J., Niemann, H., & O'Kane, C. J. (1995). Targeted expression of tetanus toxin light chain in *Drosophila* specifically eliminates synaptic transmission and causes behavioral defects. *Neuron*, *14*(2), 341-351.
- Takemura, S. Y., Nern, A., Chklovskii, D. B., Scheffer, L. K., Rubin, G. M., & Meinertzhagen, I. A. (2017). The comprehensive connectome of a neural substrate for 'ON' motion detection in *Drosophila*. *eLife*, *6*, e24394.

- Tammero, L. F., & Dickinson, M. H. (2002). The influence of visual landscape on the free flight behavior of the fruit fly *Drosophila melanogaster*. *Journal of Experimental Biology*, 205(3), 327-343.
- Tao, L., Ozarkar, S., & Bhandawat, V. (2020). Mechanisms underlying attraction to odors in walking *Drosophila*. *PLoS computational biology*, *16*(3), e1007718.
- Taylor, G. K., Bacic, M., Bomphrey, R. J., Carruthers, A. C., Gillies, J., Walker, S. M., & Thomas, A. L. (2008). New experimental approaches to the biology of flight control systems. *Journal of Experimental Biology*, *211*(2), 258-266.
- Theobald, J. (2017). Insect Flight: Navigating with Smooth Turns and Quick Saccades. *Current Biology*, *27*(20), R1125-R1127.
- Tuthill, J. C., Nern, A., Holtz, S. L., Rubin, G. M., & Reiser, M. B. (2013). Contributions of the 12 neuron classes in the fly lamina to motion vision. *Neuron*, 79(1), 128-140.
- van Breugel, F., & Dickinson, M. H. (2014). Plume-tracking behavior of flying *Drosophila* emerges from a set of distinct sensory-motor reflexes. *Current Biology*, *24*(3), 274-286.
- von Frisch, K. (1949). Die Polarisation des Himmelslichtes als orientierender Facktor bei den Tanzen der Bienen. *Experientia (Basel)*, *5*, 142-148.
- von Holst, E., & Mittelstaedt, H. (1950). Das Reafferenzprinzip: Wechselwirkungen zwischen Zentralnervensystem und Peripherie. *Naturwissenschaften*, *37*(20), 464-476.
- Wang, Z. J., Melfi Jr, J., & Leonardo, A. (2022). Recovery mechanisms in the dragonfly righting reflex. *Science*, *376*(6594), 754-758.
- Warren, T. L., Weir, P. T., & Dickinson, M. H. (2018). Flying *Drosophila melanogaster* maintain arbitrary but stable headings relative to the angle of polarized light. *Journal of Experimental Biology*, 221(9), jeb177550.
- Weir, P. T., & Dickinson, M. H. (2012). Flying *Drosophila* orient to sky polarization. *Current Biology*, 22(1), 21-27.
- Wernet, M. F., Velez, M. M., Clark, D. A., Baumann-Klausener, F., Brown, J. R., Klovstad, M., ... & Clandinin, T. R. (2012). Genetic dissection reveals two separate retinal substrates for polarization vision in *Drosophila*. *Current Biology*, *22*(1), 12-20.
- Whishaw, I. Q. (1998). Place learning in hippocampal rats and the path integration hypothesis. *Neuroscience & Biobehavioral Reviews*, 22(2), 209-220.
- Wittlinger, M., Wehner, R., & Wolf, H. (2006). The ant odometer: stepping on stilts and stumps. *Science*, *312*(5782), 1965-1967.

- Yang, H. H., St-Pierre, F., Sun, X., Ding, X., Lin, M. Z., & Clandinin, T. R. (2016). Subcellular imaging of voltage and calcium signals reveals neural processing in vivo. *Cell*, *166*(1), 245-257.
- Yarfitz, S., & Hurley, J. B. (1994). Transduction mechanisms of vertebrate and invertebrate photoreceptors. *Journal of Biological Chemistry*, *269*(20), 14329-14332.
- Yarger, A. M., & Fox, J. L. (2016). Dipteran halteres: perspectives on function and integration for a unique sensory organ. *Integrative and Comparative Biology, 56*(5), 865-876.
- Yau, K. W., & Hardie, R. C. (2009). Phototransduction motifs and variations. *Cell*, 139(2), 246-264.
- Yorozu, S., Wong, A., Fischer, B. J., Dankert, H., Kernan, M. J., Kamikouchi, A., ... & Anderson, D. J. (2009). Distinct sensory representations of wind and near-field sound in the Drosophila brain. *Nature*, *458*(7235), 201-205.
- Zeil, J. (2012). Visual homing: an insect perspective. *Current opinion in neurobiology*, 22(2), 285-293.
- Zhu, Y. (2013). The *Drosophila* visual system: From neural circuits to behavior. *Cell adhesion & migration*, 7(4), 333-344.
- Zhu, Y., & Frye, M. A. (2009). Neurogenetics and the fly-stampede: dissecting neural circuits involved in visual behaviors. *Fly*, *3*(3), 209-211.

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# List of manuscripts and author contributions

### Manuscript 1:

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#### Contributions

<u>Conceptualization</u>: A.L., A.B., A.S.M.; <u>Methodology</u>: A.L., A.S.M.; <u>Software</u>: A.L., A.S.M.; <u>Validation</u>: **M.-B.L.**, A.S.M.; <u>Formal analysis</u>: **M.-B.L.**, A.S.M.; <u>Investigation</u>: **M.-B.L.**, A.S.M.; <u>Resources</u>: A.L., A.S.M.; <u>Data curation</u>: A.S.M.; <u>Writing - original draft</u>: A.S.M.; <u>Writing - review & editing</u>: **M.-B.L.**, A.L., A.B., A.S.M.; <u>Visualization</u>: **M.-B.L.**, A.S.M.; <u>Supervision</u>: A.B., A.S.M.; <u>Project administration</u>: A.B., A.S.M.; <u>Funding acquisition</u>: A.B., A.S.M.

### Manuscript 2:

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### Contributions

<u>Conceptualization</u>: L.N.G., A.B.; <u>Methodology</u>: L.N.G; <u>Setup</u>: S.P.; <u>Software</u>: L.N.G, S.P., M.-B.L.; <u>Validation</u>: M.-B.L.; <u>Formal analysis</u>: M.-B.L.; <u>Investigation</u>: M.-B.L., T.R., L.N.G; <u>Data curation</u>: M.-B.L.; <u>Writing - original draft</u>: M.-B.L.; <u>Writing - review & editing</u>: M.-B.L., L.N.G, A.B.; <u>Visualization</u>: M.-B.L.; <u>Supervision</u>: A.B., L.N.G; <u>Funding acquisition</u>: A.B., L.N.G

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