

**The quantitative description of heading choices of
flying *Drosophila* under changing angles of
polarized light**

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I declare that this thesis was composed by myself except when explicitly stated otherwise in the text.

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2. Introduction

Within the animal kingdom, the evolution of visual systems has brought forth remarkable mechanisms for collecting and integrating visual information in order to produce meaningful behaviors within environments of varying optical complexity (Land & Fernald, 1992; Heinze, 2017). Amongst other sensory input, visual cues can be used to very reliably orient and even navigate over vast distances (Heinze & Reppert, 2011; Warren et al., 2019; Dickinson, 2013). Classic insect models for studying navigation are central place foragers like honeybees or desert ants, whereas other species like ball-rolling dung beetles have developed into a powerful model system for studying visually guided orientation behaviors. Through catch-and-release experiments in the desert, even a well-researched laboratory model insect - the fruit fly (*Drosophila melanogaster*) – was previously shown to be able to keep a relatively stable heading while traveling over many kilometers, even in an environment without many visual landmarks (Coyne et al., 1987). Importantly, the visual cues used in both orienting and navigating insects appear to be largely the same (Heinze, 2017).

2.1 Skylight polarization

One of the most prominent visual cues that can be used for orientation and navigation is the celestial body (during the day: the sun), since it provides robust spectral and intensity cues throughout the sky. However, for instance even on very cloudy days, when the sun is occluded and no celestial intensity gradients are visible, its position can still be inferred through a property of light that most humans are not naturally sensitive to – skylight polarization. Linear polarization can be described as the confinement of the oscillation of a light wave to a specific plane oriented orthogonally to the light path. Through scattering of sunlight by molecules in the sky, the unpolarized sunlight becomes partially linearly polarized, creating a celestial polarization pattern that is symmetrical around the sun and can therefore be used as a reference system for orientation and navigation even if the sun itself is not directly visible (Wehner, 2001). This natural pattern is characterized by differences in both the degree of polarization (describing the portion of the incoming light that is completely linearly polarized) and the angle of polarization (i.e. the e-vector orientation) which is a measure of the angle of the plane of the vibration.

2.2 Polarization-based orientation and navigation

Past studies have shown that many animals can utilize skylight polarization for a wide variety of behaviors (Cronin et al., 2003; Nilsson & Warrant, 1999). Polarization-based navigation was first described in honeybees which routinely use the position of the sun as a global reference frame to signal food source location and to find back to the hive (von Frisch, 1949). Also other central place foragers like desert ants have been found to use skylight polarization for finding back to their hive (for review, see Wehner, 2003). Even long-range (monarch butterflies), or short-range migratory animals (locusts) have the ability to detect the polarization pattern of the sky to accurately navigate over vast or short distances, respectively (Mappes & Homberg, 2007; Sauman et al., 2005). But even for orientation behavior over comparably small distances, skylight polarization can serve as a robust external cue. For instance, it was shown that dung beetles use the e-vector pattern in the sky to roll dung balls in a straight path to get away from the main pile as quickly as possible, which minimizes the probability of encountering a competitor (Baird et al., 2012). Being able to avoid walking or flying in curves when actually trying to keep a steady heading can be a very beneficial trait, especially in visually scarce or challenging environments that don't allow for landmark-based orientation. Spontaneous orientation responses to linearly polarized light (polarotaxis) were also observed in walking and flying houseflies (von Philipsborn & Labhart, 1990, Warren et al., 2018). Later studies revealed that *Drosophila* performs polarotaxis when walking under laboratory conditions (Wolf et al., 1980; Wernet et al., 2012; Velez et al., 2014a, 2014b), as well as when flying under the natural sky or under an artificially polarized stimulus under closed loop conditions (Weir & Dickinson, 2012; Warren et al., 2018).

2.3 Anatomical adaptations for celestial polarotaxis in *Drosophila melanogaster*

Due to a large variety of genetic tools available, *Drosophila* serves as an excellent model organism for studying visual circuits and how they contribute to the motor output of the animal, in order to generate meaningful behaviors. The *Drosophila* visual system - despite its comparably small size - is a complex neural network with approximately 100,000 neurons in the central brain alone, able to detect motion, perceive objects, and distinguish colors ranging from UV to red. Within each unit eye (or ommatidium) of the adult fly eye, the inner photoreceptors R7 and R8 share the same optical axis (by forming light-gathering rhabdomeres sitting on top of each other), while being surrounded by outer photoreceptors R1-6. In the main part of the eye, R7 and R8 process color information by expressing different rhodopsins in order to compare light of different wavelengths (Wernet & Desplan, 2004). However, previous work has shown that a small subset of ommatidia near the dorsal edge of the eye - the so called dorsal rim

area (DRA) - has morphological and physiological adaptations for detecting skylight polarization (Wada, 1974; Labhart & Meyer, 1999; Wernet et al, 2012). In the main part of the eye, R7 and R8 photoreceptor rhabdomeres are twisted along their optical axis, making them sensitive to incoming light regardless of its e-vector orientation. This twist is necessary to eliminate the inherent polarization sensitivity of rhabdomeric photoreceptors, allowing for unambiguous wavelength comparison. In the DRA however, both inner photoreceptors contain the UV-sensitive rhodopsin 3 (Rh3) and form untwisted rhabdomeres that are oriented orthogonally to each other, making them potent analyzers for skylight polarization (Wernet et al, 2012). Importantly, the analyzer directions of DRA rhabdomeres gradually shift about a total of 180° from the anterior to the posterior edge of the DRA, thereby forming a fan-shaped array for the detection of skylight polarization (Weir et al., 2016). In *Drosophila*, much research is being undertaken to genetically dissect the neuronal circuits forming the skylight polarization pathway. This is aided by recent developments towards generating a diverse toolkit of powerful neuroanatomical and physiological methods. The successful dissection of neuronal circuits involves the characterization of cell types involved, their synaptic interconnections, as well as their physiological properties in the behaving animal. Additionally, it is of great importance to define a quantitative model for polarization-based orientation.

2.4 Quantifying vision-based behavior in flies

Studying vision-based orientation and navigation behaviors under laboratory conditions poses many challenges, yet also provides many possibilities for gaining insights into neuronal network properties, signal processing and circuit hierarchy, by quantifying specific motor output parameters as part of the behavioral output, depending on the tightly controlled visual input. Many different designs exist for quantitative assays studying motor output in response to visual stimuli in flies, especially for *Drosophila melanogaster*. For instance, flies can be allowed to freely fly or walk in groups or after being singled out (Mathis et al., 2018; Branson et al., 2009). However, in many cases the degrees of freedom in which flies are allowed to move must be restricted in order to keep their position relative to the visual stimulus constant. For this, tethering approaches exist for both walking as well as flying flies, where a single fly is usually glued to a metal pin or wire (Moore et al., 2014; von Philipsborn & Labhart, 1990). After being attached to a sturdy wire, flies may be positioned to walk on a spherical treadmill (i.e. an air-suspended ball). For flying flies, differences in wing beat amplitude can be recorded when the animals are placed on top of infrared sensors (Götz, 1987; Tammero et al., 2004) or high-speed-cameras can be used (Suver et al., 2016). Importantly, scenarios where the animal's body axis is fixed allow for closed-loop stimulus

presentation, where the motor output of the animal directly controls stimulus presentation. Alternatively, flying flies might also be allowed to rotate freely around their yaw-axis while being tethered to a small steel pin by placing them between two magnets ('magnetotether') (Bender & Dickinson, 2006; Weir & Dickinson, 2012).

2.5 Designing behavioral assays - challenges and opportunities

Despite being used by many laboratories all over the world interested in vision-based insect orientation and navigation, the necessary behavioral assays are often custom-built setups whose assembly may require a workshop, sophisticated tools, special expertise, or even complete outsourcing of construction to engineering departments or companies. Such restrictions might pose limitations for those laboratories with limited funds, potentially slowing down or even limiting the use of quantitative behavior experiments. The recent development of affordable 3D printers and user-friendly hard- and software environments opened up new possibilities for designing sophisticated behavioral setups that are affordable and fairly easy to replicate. Moreover, 3D data for the construction of functional parts and the necessary software can easily be shared, modified and remixed by the scientific community in order to improve designs and help facilitate the establishment of common standards between laboratories. This is especially useful when working with visual stimuli, since they are prone to intensity, chromatic or polarization artefacts which themselves might influence the behavioral response (Foster et al., 2018). In this case, a standardized choice of materials and parts also allows for better comparability of results across different laboratories. Finally, the ongoing development of new digital image processing methods allows for rapid code development for unbiased and fully automated quantification of behavioral parameters through camera-based animal tracking (Mathis et al., 2018). Such code can then also be shared and improved within the scientific community. Vision-based orientation can rely on intensity, chromatic and polarization cues. Under laboratory conditions these properties of light can also be artificially modulated using modern LED technology and optical filters and - if documented properly – be accurately replicated across different laboratories.

2.6 Polarized reflections as behavioral cues

In addition to polarized skylight being an orientational cue, linear polarization can also serve to signal specific environmental surface properties (Wehner, 2001; Heinloth et al., 2018; Mathejczyk & Wernet, 2017): When unpolarized light gets reflected off a smooth non-metallic surface, it becomes partially or in

some cases even fully linearly polarized. The degree of polarization varies depending on the wavelength and incident angle of reflected light relative to the observer and the refractive index of the surface. Importantly, the angle of polarization is influenced by orientation of the surface. When unpolarized light is reflected off of a water body, it will become partially or fully horizontally polarized. Hence, in nature angle and degree of polarization can serve as powerful indicators for finding water bodies. Finding water is essential for every animal on earth. However, while seeming like an easy task for humans, detecting water bodies based on intensity and chromatic cues alone might pose a big challenge to animals with relatively small visual systems due to the reflective properties of water surfaces. Polarization therefore serves as an attractive modality signaling the presence of water. Although not much is known yet about the neuronal substrates that convey such ventral (i.e. non-celestial) polarization vision, an increasing number of behavior experiments have shown that many insects are either repelled or attracted by heavily polarizing surfaces (Farkas et al., 2016; Bernath et al., 2002, Shashar et al., 2005; Wildermuth, 1998). Interestingly, anatomical specializations in the ventral retinas of some semi-aquatic insects might not only be utilized to find or to avoid water surfaces but in turn they serve to filter out unwanted polarized reflections in order to be able to see deeper into the water (Schneider & Langer, 1969; Trujillo-Cenóz & Bernard, 1972). Finally, in addition to finding or avoiding water bodies, polarized reflected light appears to be utilized for evaluating the surface qualities of shiny leaves (e.g. for oviposition of certain butterfly species) (Kelber et al., 2001), or for finding prey whose body surface consist of shiny fur (i.e. detection of prey by visuall-guided horse flies) (Horváth et al., 2008; Meglič et al., 2019).

Taken together, skylight polarization and polarized reflections both are powerful cues for guiding the visual orientation and navigation of many different insect species, potentially informing a plethora of behavioral strategies in very different ecological niches (desert versus water surface). This thesis is a summary of my efforts towards assembling an overview of current knowledge on polarization vision in insects, providing a novel behavioral assay for studying visual orientation and navigation in both walking and flying insects, and conducting an in-depth experimental assessment of skylight-mediated polarotaxis in *Drosophila melanogaster*.

3. Aims

In the process of working towards my dissertation, my strategy included both initial literature research as well as practical experiments (building quantitative assays and performing behavior experiments).

1. The first aim was to work on an overview of current knowledge on polarization vision in insects. The resulting Manuscript I is a review article intended to illustrate the occurrence of polarized light in nature and its implications as a possible orientational and navigational cue for many insect species. It aims to compare anatomical adaptations and behavioral strategies utilizing polarized skylight and surface reflections across different insect orders.
2. The second aim was to design and build new behavioral assays for the quantitative study of visually guided orientation and navigation in insects. These setups were intended to be versatile and modular, allowing for presentation of intensity-, chromatic- and polarization cues to single insects that could either be walking or flying. The goal was for the setup to be well documented, affordable, easy to replicate and to make accessible all the codes for automated tracking and data analysis. As a result, Manuscript II addresses those efforts.
3. The third aim was to do an in-depth assessment of polarotactic behavior in flying *Drosophila* within the new modular flight arenas described in Manuscript II. Through quantification of fly headings in response to a constantly rotating e-vector presented dorsally, I intended to find out whether flying flies would correct their course by following this rotation. Special attention was given to the chosen heading of each fly and how those preferred e-vectors might be distributed throughout the tested fly population. As a result, Manuscript III addresses those questions.

Taken together, these three aims provide significant progress towards the dissection of neural circuits and possible behavioral strategies underlying skylight polarization vision and visual navigation in *Drosophila melanogaster*.

4. Manuscripts

4.1. Manuscript I

Sensing Polarized Light in Insects.

Thomas F. Mathejczyk and Mathias F. Wernet

Oxford Research Encyclopedia of Neuroscience, published online: 26 September 2017.

Contribution:

I created figure 1, assisted in assembling figures, and wrote the manuscript under the supervision of Prof. Mathias Wernet.

The original article is presented on the following pages and can be downloaded at:

<https://oxfordre.com/neuroscience/view/10.1093/acrefore/9780190264086.001.0001/acrefore-9780190264086-e-109>

<https://doi.org/10.1093/acrefore/9780190264086.013.109>

4.2. Manuscript II

Modular assays for the quantitative study of visually guided navigation in both flying and walking flies.

Thomas F. Mathejczyk and Mathias F. Wernet

Journal of neuroscience methods, 340, 108747. Published July 2020.

Contribution:

I designed the assays, performed all experiments and data analysis and created all figures under the supervision of Prof. Mathias Wernet. I wrote the manuscript together with Prof. Mathias Wernet.

The original article is presented on the following pages and can be downloaded at:

<https://www.sciencedirect.com/science/article/abs/pii/S0165027020301709>

<https://doi.org/10.1016/j.jneumeth.2020.108747>

4.3. Manuscript III

Heading choices of flying *Drosophila* under changing angles of polarized light.

Thomas F. Mathejczyk and Mathias F. Wernet

Scientific reports, 9(1), 16773. Published November 2019.

Contribution:

I performed all experiments and data analysis and created all figures under the supervision of Prof. Mathias Wernet. Together with Prof. Mathias Wernet I designed the experiments, assembled the figures, and wrote the manuscript.

The original article is presented on the following pages and can be downloaded at:

<https://www.nature.com/articles/s41598-019-53330-y>

<https://doi.org/10.1038/s41598-019-53330-y>

5. Discussion

5.1 Flexibility of the behavioral assays

Visually-guided orientation and navigation is a challenging task for any animal. Optical signals within an animal's visual field not only need to be translated into electrical potentials and synaptic signals but ultimately must enable the animal to initiate an appropriate response, i.e. a motor output that probabilistically increases chances for survival and/or successful reproduction. The major challenge when performing visually-related behavioral experiments under laboratory conditions is to carefully present tightly controlled optical stimuli and also precisely quantify the animal's motor output in response to these stimuli. In preparation for this thesis, I have developed a series of highly modular behavioral setups that allow for presentation of stimuli with clearly defined intensity-, chromatic- and polarization properties to both walking and flying insects while providing a fully automated analysis of the resulting motor output. Much care was given to producing clear and easy to understand building instructions with a detailed documentation to support its establishment in other labs. Although many behavioral assays already exist for studying visually-guided behaviors in insects (Bender & Dickinson, 2006; Moore et al., 2014; von Philipsborn & Labhart, 1990; Götz, 1987; Tammero et al., 2004; Suver et al., 2016) the setups described in Manuscript II are unique in their modularity and flexibility. The 3D models for constructing these setups can conveniently be downloaded and printed on most commercially available 3D printers at very low costs. Due to its modular conception, only certain elements or assays can be chosen to be utilized at a given time, depending on the specific experimental demands, further reducing cost. All 3D models can easily be modified and shared throughout the scientific community, aiding further development of the setup and also opening up possible applications for non-visually guided behavior experiments. For instance, by adding small holes to the surrounding semi-hollow cylinder, odor plumes or air currents could be introduced to study the influence of specific odors (Frye & Duistermars, 2009) or wind strength and direction (Okubo et al., 2020; Dacke et al., 2019) on the orientation behavior of walking and flying insects. In future studies, such a stimulus could then even be combined with visual cues to study the integration of these signals and their hierarchy in guiding navigational decisions.

Since these new assays provide the possibility of testing both walking as well as flying flies, they also allow for studying the influence of different behavioral states on orientation behavior. In walking *Drosophila* differences in optomotor responses have previously been described in response to intensity stimuli but not to dorsally presented linear polarization. Would a fly that followed a rotating e-vector

during flight also robustly follow the rotation of a polarized stimulus when walking on an air-suspended ball? Data from houseflies certainly points in that direction (von Philippsborn and Labhart, 1990), yet the behavioral performance appears less strong than expected from flying flies. In addition to the behavioral performance, would a fly also choose the same preferred e-vector when walking? The modular assays presented in Manuscript II now provide the hardware as well as the software to conduct these kinds of experiments, not only in *Drosophila* but also in other insect species (Mathejczyk & Wernet, 2020).

The setups presented here allow not only for landmark- or polarization-based orientation behaviors to be quantified, but may also enable future experiments where both cues can be presented in combination in order to study multimodal integration of these stimuli, as well as their hierarchy when guiding the behavioral response, while keeping in mind that both intensity- and polarization-inputs could be depended on the behavioral state of an animal (Gorostiza et al., 2016).

Temperature and humidity are important environmental factors that might influence the behavioral performance of animals. Precise climate control during behavioral experiments ensures constant environmental conditions but is often achieved using expensive incubators or air-conditioned rooms. Manuscript II also features an optional temperature- and humidity-control that is affordable, easy to assemble and might be of potential use for other applications (Mathejczyk & Wernet, 2020). Additionally to keeping temperature- and humidity levels constant during experiments, the climate control can also be used to quantify fly behavior at specific elevated temperatures. This may not only be useful for scientific examination of the general influence of temperature on behavior, but might also enable dissecting neuronal circuits by using thermogenetic tools like *shibire^{ts}* (Kitamoto, 2001) allowing to silence neural activity in defined groups of cells. Specific neurons could also selectively be activated by expressing the temperature gated ion channel TrpA1 (Rosenzweig et al, 2005) or expression of the red-shifted channelrhodopsin molecule CsChrimson (Inagaki et al., 2013), which could easily be activated utilizing the red channel of the LED matrix used in Manuscript II.

Working with polarized light often requires special adaptations to the design of behavioral assays in order to avoid creating chromatic or intensity artefacts that might be used for orientation rather than polarization itself (for review, see Foster et al., 2018). These Polarization artefacts can be reduced by keeping incident angles of light shining through the polarization filter as low as possible and by choosing matte white surfaces when designing the inside of the apparatus. In addition, Manuscript II contains an additional, novel approach to further reduce intensity artifacts at the arena walls by using a back-lit, white cylinder surrounding the fly (Mathejczyk & Wernet, 2020).

Although all code necessary for tracking and data analysis are freely available for download (<http://flygen.org/modular-assays/>) to further support open science, the recording and data-analysis software (Matlab, Streampix) used in manuscript II is in fact commercial software that is quite expensive. To further reduce costs, future versions of the setups described here could therefore utilize alternative, open-source software like python to acquire videos, track an animal's heading, as well as for data analysis. This would also allow to extract an animal's heading in real-time. The setup as presented in Manuscript II relies on the analysis of pre-recorded videos, not allowing for real-time tracking (Mathejczyk & Wernet, 2020). Tracking an animal in real time, while its responding to the stimulus, would further allow for closed-loop experiments where the motor output of an animal could control certain parameters of stimulus presentation. Apart from preventing a tethered fly from rotating (via gluing to a fixed needle, instead of a magnetotether), no additional modifications would need to be implemented to the setup's hardware, since the original camera position also allows for image-based wingbeat amplitude measurements, which can be used to deduce a fly's intended turning tendency (Götz, 1987; Suver et al., 2016). In such a scenario, speed and direction of a polarization filter's rotation might be coupled to differences in wingbeat amplitude allowing a flying insect to control the e-vector angle of light presented dorsally (see Warren et al., 2018). Closed-loop scenarios for walking flies could also be achieved using the pre-existing software fictrac (Moore et al., 2014). Taken together, the affordable behavioral assays presented here allow for a variety of experiments and in the future can further be modified to hopefully enable even more sophisticated applications by the scientific community. As an example, the data presented in Manuscript II laid the groundwork for the quantitative behavior experiments described in Manuscript III (Mathejczyk & Wernet, 2019).

5.2 Flying *Drosophila* choose and keep arbitrary headings relative to a celestial polarization pattern

In Manuscript II I was able to reproduce previous experiments where flying flies compensated rapid switches in e-vector orientation by adjusting their heading accordingly (Mathejczyk & Wernet, 2020; see Weir & Dickinson, 2011). However, I quickly realized that a finely resolved quantification of polarotactic episodes was not easy using the setups I had built. Whenever a fly flew with a relatively constant heading, it was hard to determine whether this was at all in response to the mostly static e-vector. To resolve polarotactic episodes with finer detail and temporal resolution, I therefore chose to confront flying flies with a constantly rotating e-vector presented dorsally, under open-loop conditions. The rationale was to motivate the flies to constantly adjust their heading while performing polarotaxis.

Indeed this revealed, that flying flies transit in and out of polarotactic episodes under linearly polarized UV (365nm) light, while showing much less to no polarotaxis under a linearly polarized green (532nm) stimulus. This confirmed our assumptions regarding wavelength specificity of skylight polarization vision in *Drosophila*, since DRA inner photoreceptors R7 and R8 both express the UV-sensitive Rh3 (Fortini & Rubin, 1990). Although not statistically different, flies flying under linearly polarized green light showed a slight tendency to follow the rotating e-vector slightly more than under unpolarized UV control conditions. It remains unclear whether this is due to an optical artefact or how else such a response might be mediated. Furthermore, we found that while flies can set an arbitrary heading relative to a rotating e-vector, they can even maintain this heading in a second trial after being interrupted by being forced to fly under unpolarized UV light for 5 minutes. These findings are in accordance with previous studies (that were published while I was working on these experiments) demonstrating polarization-mediated orientation behavior in flying *Drosophila* (Warren et al., 2018). In similar experiments, flies also chose arbitrary headings in response to an unpolarized intensity stimulus mimicking the sun (Giraldo et al, 2018). In nature, a flying fly keeping a constant heading relative to the sun and/or a celestial e-vector pattern over the course of minutes would fly a straight course. But why would a fruit fly need to keep a straight course? One explanation might be related to energy efficiency: If a fly finds itself in a scarce area without vegetation or food sources, the ability to leave such a terrain in a straight path in search for more promising environments will be quicker and more efficient than through saccadic flight patterns which are often observed in flying flies (Schilstra & Hateren, 1999; Bender & Dickinson, 2006). In this context, the saccadic episodes that I observed interrupting polarotactic phases (see Manuscript III: Mathejczyk & Wernet, 2019) might be an interesting topic for future research. In general, these two distinctly different flight patterns (straight heading versus saccadic search) might reflect different behavioral strategies, with polarotactic episodes aiding long-range distance travel and saccadic episodes representing a rather local search mode, where a fly might search for food or mating partners. These behavioral strategies might strongly depend on an animal's internal state which could be modulated by a variety of sensory cues, and/or mediated by different neuromodulators. For instance, a certain smell or high-contrasted object could potentially interrupt a polarotactic episode. An indicator for a putative hierarchy in processing different stimuli might be found in the fine structure of the data presented in Manuscript II (Mathejczyk & Wernet, 2020) and III (Mathejczyk & Wernet, 2019). While many flies did not follow a rotating celestial e-vector, almost all tested flies followed the rotation of a high-contrast vertical stripe (Mathejczyk & Wernet, 2019; Mathejczyk & Wernet, 2020). Such findings might indicate a higher priority of the pathway processing motion on a fly's motor output, as opposed to the pathway processing skylight-polarization. However, such hierarchies would need to be tested within controlled

experiments presenting intensity and polarization cues simultaneously. Those kinds of experiments can now be done using the behavioral assays presented in Manuscript II.

5.3. A polarization-based dispersal strategy?

The findings presented in Manuscript III (Mathejczyk & Wernet, 2019) suggest another possible evolutionary cause for why a fruit fly might need the ability to keep an arbitrary, but straight course. Confirming previous findings of Warren and colleagues (see Warren et al., 2018), my inter-individual examination of the distribution of preferred e-vectors during flight also revealed that flies choose arbitrary headings relative to a celestial e-vector and tend to keep those headings even after a 5-minute interruption. These results suggest that the arbitrary choice of preferred headings could be part of an innate dispersal strategy. Such a dispersal strategy based on skylight polarization vision might pose evolutionary benefits since arbitrary angles would ensure a homogenous distribution of flies, when dispersing from the same place. Although this might lead to some flies flying towards environments with less resources, it would minimize the risk of losing large parts of a fly population, e.g. by all flies displaying the same e-vector preference and setting the same course depending on the position of the sun. Interestingly, the choice of arbitrary headings was also observed when flies were presented with an unpolarized intensity stimulus representing the sun (Giraldo et al, 2018).

It remains unclear how these arbitrary preferred e-vectors originate within the fly brain. The analyzer directions of DRA rhabdomeres forming the fan-shaped array of skylight polarization detectors appear to be stereotypical between flies (Weir et al., 2016). It therefore seems reasonable to assume that an e-vector preference would probably originate downstream of DRA photoreceptors. A preferred e-vector could be determined neuro-developmentally, as idiosyncratic differences have recently been shown to be neurodevelopmental in origin (Linneweber et al., 2020). Alternatively, they could be variable and e-vector preference could shift, potentially via neuromodulatory control. Studies in the central complex have shown, that the absolute representation of the azimuthal position of visual landmarks within the brain varies between flies (Seelig & Jayaraman, 2015; Kim et al, 2017). Since polarization is also represented within in the central complex (Timaues et al., 2020), the mechanisms causing inter-individual differences in the neuronal representation of landmarks and e-vectors might be related, or even have the same origin.

Unfortunately, testing preferred e-vectors of the same flies on consecutive days is extremely difficult, due to the gluing procedure required for both closed-loop and magnetotether experiments.

Nevertheless, closed-loop behavior experiments may reveal important details about how e-vector preference originates. In these experiments, a specific e-vector range could be rewarded or punished (for instance by shining a painful laser beam onto the fly, Colomb & Brembs, 2016) to test whether a preferred e-vector could be shifted to another angle. Alternatively, both reward or punishment could be triggered using optogenetic activation of neurons mediating the respective signals (Lyutova et al., 2019).

5.4. Time compensation

In nature, setting a fixed course relative to the sun or a celestial e-vector pattern while navigating for longer periods of time poses a problem: Over the course a day, the sun's position in the sky and the associated polarization pattern change quite drastically by approximately 15°/hour (for sun movement East to West in 12h). If an animal would not compensate for this movements of the sun-centered stimulus system, it would fly an arc. This is not in agreement with what has been described for *Drosophila* and other insects (Dickinson, 2013). Hence, long-scale sun- or polarization-based orientation and navigation would require a compensation for the time of day to correct for the movement of the sun across the sky. Such a time-compensated polarization sensitivity was previously demonstrated for the foraging behavior of honeybees (Gould, 1980) and electrophysiologically described in specific polarization-sensitive neurons in locusts (Pfeiffer & Homberg, 2007). Although catch-and-release experiments using *Drosophila* revealed that they are able to travel straight paths over hours in a visually scarce landscape (Coyne et al., 1987), time-compensated navigation behavior was never described under laboratory conditions. In fact, one publication demonstrating the fly's ability to use an artificial sun as an orienting cue ('menotaxis') found no evidence for time compensation (Giraldo et al, 2018). The dorsal polarization flight assay described in Manuscript II (Mathejczyk & Wernet, 2020) in combination with the findings presented in Manuscript III (Mathejczyk & Wernet, 2019) are a good starting point for future experiments examining whether an e-vector preference might shift over the course of hours. However, during the experiments I performed for this thesis, tethered flies were often not motivated to fly for elongated periods of time, making it difficult to observe a shift in the preferred e-vector. Additionally, precise quantification of preferred e-vectors is not easy, since many flies don't strictly follow the e-vector rotation during polarotaxis but rather allow for a certain deviation from it until they realign their body-axis, often resulting in what appear to be saccadic movements (Mathejczyk and Wernet, 2019). However, it remains unclear whether this an artefact of the magneto-tether due to flies having difficulties rotating around their yaw axis or an actual property of the polarization-vision pathway. Future

experiments with rigidly tethered flies under closed-loop conditions may answer this question, since this would cancel out rotational difficulties as root cause of saccadic-like movements.

Even experiments spaced apart as little as 2-3 hours may be suited to resolve a time compensation of preferred e-vectors if the flies can be motivated to fly a second time. For instance, time compensation might be achieved neurally by the polarization pathway getting direct or indirect input from clock neurons or by expressing the pigment dispersing factor PDF (see Shafer & Yao, 2014), and evidence for such input indeed exist (Hulse et al, 2020). Further research into the possible existence of time-compensated navigation in flies and the underlying circuit mechanisms might be an interesting field of study for fully understanding the skylight polarization vision pathway in *Drosophila* and other insects.

5.5 Dissecting neuronal circuits through behavioral experiments

Much research is currently being done to neuroanatomically and functionally dissect the neuronal circuits processing skylight polarization in *Drosophila* (Sancer et al., 2019; Sancer et al., 2020; Hardcastle et al., 2021; for review, see Sancer & Wernet, 2021). Recently it was shown that that the polarization-vision pathway contains modality-specific circuit elements in its periphery which differ from their color-vision counterparts in both anatomy and connectivity (Sancer et al., 2019, Sancer et al., 2020). Polarized skylight is perceived by DRA inner photoreceptors, processed in the DRA region of the medulla neuropil, and synaptically transferred to the anterior optic tubercle (AOTU), an optic glomerulus in the central brain (Omoto et al., 2017; Hardcastle et al., 2021). From there, polarized light information is transferred to the bulb neuropil of the central complex (Timaues et al., 2020), where it is most likely integrated with the system computing head direction and landmarks (Seelig & Jayaraman, 2015; Kim et al, 2017; Hulse et al, 2020; Heinze & Homberg, 2007). By inhibiting synaptic transmission in cell types postsynaptic to DRA photoreceptors, their role within the dorsal polarization pathway could be characterized through behavioral experiments performed like in manuscript II (Mathejczyk & Wernet, 2020) and III (Mathejczyk & Wernet, 2019), using the setups that I have developed. For doing so, the temperature-sensitive *shibire^{ts}* (Kitamoto, 2001) could be expressed cell-type specifically, for instance in only Dm-DRA1 or Dm-DRA2 neurons. At an elevated temperature *shibire^{ts}* inactivates synaptic transmission, allowing for a stepwise genetic dissection of modality-specific circuits. The function of Dm-DRA1 or Dm-DRA2 in processing skylight polarization would then be revealed through changes in the quality or quantity of polarotactic responses when compared to wild-type and parental control groups. The active climate control described in Manuscript II allows for such experiments.

Since the visual cues that a fly retrieves from its environment are not limited to polarized skylight, intensity- and chromatic cues also need to be integrated within the fly brain to produce meaningful behaviors. One possible center for this multimodal integration may be the AOTU, since this neuropil consists of different subunits, each collecting different synaptic input, organized in parallel channels (Omoto et al., 2017; Tai et al., 2020; Timaeus et al., 2020). The different perceived modalities might require a hierarchical integration in order for an animal not only to be able to differentiate between behaviorally relevant cues, but also to weight their importance depending on an animal's behavioral state or visual ecology. Experiments examining multimodal integration and signal hierarchy may also be conducted using the modular assays described in Manuscript II.

5.6. Ventral polarization vision

Behavioral experiments under laboratory conditions require setups with optimal experimental design. These designs for behavioral assays intended to study vision-based orientation and navigation need to take into account the properties of the animal's visual system. Manuscript I (Mathejczyk & Wernet, 2017). provides a comprehensive overview of polarization vision, anatomical adaptations and associated behavioral strategies in different insects. While compiling this data, it became clear that until today much research on insect vision and navigation was conducted with a focus on skylight polarization, while much less is known about the neuronal basis of ventral polarization vision, i.e. the detection of polarized reflections. Like for celestial polarization vision, *Drosophila* might also be a very promising candidate to study ventral polarization vision under laboratory conditions. Previous studies in walking *Drosophila* showed responses to linearly polarized light presented ventrally (Velez et al. 2014a and 2014b; Wernet et al., 2012). However, the retinal substrate and underlying circuits for ventral polarization responses remain unclear (Heinloth et al., 2018). Further electron microscopic sections of the ventral retina may reveal ultrastructural adaptations of photoreceptors for ventral polarization vision. Considering how polarization sensitivity is achieved in the DRA, rhabdomere twist would probably be the main indicator of polarization sensitivity to look for ventrally. Indeed, such rhabdomeric specializations exist in other insect species, like the hemipteran *Notonecta glauca* (Schwind, 1983), or horseflies (Meglič et al., 2019). To minimize cost- and labor-intensive electron microscopic sections and ultrastructural reconstructions, a promising starting point might be to identify the retinal area that during flight faces the ground at the Brewster angle. At this incident angle light reflected off water surfaces is maximally horizontally polarized and might be the ideal angle to measure polarized reflections. Although *Drosophila* is not particularly known to interact with large water bodies like many semi-aquatic insects do (Heinloth et al.,

2018; Mathejczyk & Wernet, 2017), fruit flies also may need to seek out water in order to survive if no moist food is available for water supply. In behavioral experiments, this could be tested by confronting freely moving flies with a shiny, non-metallic surface for creating horizontally polarized reflections, to see if they would behave like a control group presented with water. Based on their internal state, flies might either be attracted to large horizontally polarizing areas, e.g. when thirsty, or might want to avoid them in order to prevent them from drowning. The neural circuits underlying any of these potential behaviors remain unknown in flies, as well as in other insects (Heinloth et al, 2018). The variety of genetic tools available *Drosophila* provide a very promising platform when aiming to dissect the neuronal circuits underlying ventral polarization vision. Through EM reconstruction as well as methods like GRASP (Feinberg et al., 2008; Macpherson et al., 2015) or trans-tango (Talay et al., 2017) downstream partners of yet-to-be identified polarization-sensitive photoreceptors in the ventral half of the retina may be identified. Subsequently, these could then be further characterized using cell-type specific expression of genetically encoded calcium-indicators in combination with 2-photon imaging (Chen et al., 2013). This might reveal the physiological properties of circuit elements processing polarized reflections, thereby helping to explain how ventral polarization is processed and integrated in order to produce meaningful behavioral output. Utilizing the Crispr/Cas9 system (Jinek et al., 2012) it would also be possible to use a variety of genetic tools in other insect species, opening up many opportunities for revealing the neuronal basis of ventral polarization vision in more specialized, semi-aquatic species.

Studying ventral polarization vision is also important in the context of understanding the ecological implications of human-made objects placed in nature. For instance, human-made objects like solar panels, cars or streets often have very smooth, non-metallic surfaces, that produce linearly polarized reflections similar to water or shiny leaves (Blaho et al., 2014; Egri. Et al., 2013; Blaho et al., 2012; Robertson et al., 2018; Kriska et al., 2008). These artificial polarization cues might falsely signal water or oviposition sites to insects, thereby forming evolutionary traps. Vice versa, man-made sources of unpolarized light (like lamps placed close to water bodies) are known to destroy important sources of linearly polarized reflections sought out by insects (Szaz et al., 2015). The recent decline in insect biomass is a concerning ecological threat (Hallmann et al., 2017). While factors like the industrialization and destruction of natural habitats, as well as the use of neonicotinoids are known to be factors linked to insect decline, human-made polarization (or the destruction of it) may also have a large ecological impact. Therefore, studying and understanding ventral polarization vision in insects may aid future designs and the development of materials aimed to conserve insect population by reducing human-made linearly polarized surface reflections (Fritz et al., 2020).

Taken together, Manuscripts I-III provide insights into skylight polarization vision in insects, provide methods for studying vision-based orientation and navigation and - through behavioral experiments - investigate behavioral responses to linearly polarized light of different wavelengths. The major goal was to reveal possible behavioral strategies underlying skylight-polarization-based orientation in *Drosophila*, to make this behavior and the underlying circuits accessible to the molecular genetic tools available in this model organism. The tools presented here therefore serve as the groundwork for behaviorally dissecting the modality-specific visual neural circuits underlying skylight navigation and multisensory integration, ranging from characterization of circuit properties in wild-type animals to allowing for future thermo- or optogenetic manipulations.

6. Summary

Many insect visual systems are comprised of dedicated neuronal circuits that allow for perceiving, processing, and integrating different modalities of light in order to orient or even navigate within visually complex environments. In addition to intensity or chromatic cues, many insects can utilize the linear polarization of light as a separate modality (Labhart, 2016) for orientation. For instance, polarized reflections can signal specific surface properties, aiding in the detection of water bodies, finding oviposition sites, or localizing prey. Additionally, due to scattering of sunlight in the atmosphere, a celestial polarization pattern is created that can be used by many insects as a reference for both orientation and navigation. Here in this thesis, Manuscript I provides an overview over polarization vision in insects, by summarizing the current knowledge (as of 2017) on anatomical and physiological adaptations of insect visual systems and their behavioral implications in different species (Mathejczyk & Wernet, 2017). To experimentally study visual orientation and navigation in insects, I designed affordable and highly modular behavioral assays, which are described in Manuscript II (Mathejczyk & Wernet, 2020). These assays provide a quantitative behavioral readout in response to panoramic intensity and chromatic patterns or to polychromatic linearly polarized light presented dorsally, when insects are either walking on a spherical treadmill or flying in a magneto-tether. This publication provides 3D model data and building instructions for those modular assays, including an easy-to-build tethering station and a low-cost temperature and humidity control. All code for tracking and data analysis was also made available online to aid the scientific community in establishing and modifying the presented assays in the spirit of open science. In this publication, I further demonstrate the setup's functionality and versatility by describing opto-motor responses of walking and flying flies to rotating panoramic intensity patterns as well as behavioral responses to rapid e-vector switches of polarized light presented dorsally in flying *Drosophila melanogaster*. Using the setup described in Manuscript II, I assessed polarotactic responses in flying *Drosophila* in response to a constantly rotating e-vector presented dorsally, under open-loop conditions (Mathejczyk & Wernet, 2019). I found that flying flies align their body axis in response to linearly polarized UV, but not under polarized green or depolarized UV light. Every fly chose an arbitrary preferred heading relative to a celestial e-vector and most flies were able to keep those headings at least over the course of several minutes. Taken together, these findings confirm that *Drosophila* can utilize wavelength-specific skylight polarization for orientation and suggest that in *Drosophila* celestial polarization vision might serve an underlying dispersal strategy, optimizing chances of survival and reproduction on a population level.

7 Zusammenfassung

Die visuellen Systeme vieler Insekten bestehen aus spezialisierten neuronalen Schaltkreisen, welche es ihnen ermöglichen, verschiedene Lichtmodalitäten wahrzunehmen, zu prozessieren und zu integrieren, um sich in visuell komplexen Umgebungen zu orientieren, oder gar über weitere Entfernungen zu navigieren. Neben Intensitäts- oder Farbinformationen können viele Insekten die lineare Polarisation des Lichts als potenziell separate Modalität (Labhart, 2016) zur Orientierung nutzen. So können z.B. polarisierte Oberflächenreflektionen bestimmte Oberflächeneigenschaften signalisieren und dabei helfen, Wasseroberflächen, Eiablageorte oder Beutetiere zu detektieren. Außerdem erzeugt atmosphärische Streuung von Sonnenlicht ein Polarisationsmuster am Himmel, welches von vielen Insekten als Referenzsystem zur Orientierung und Navigation genutzt werden kann. In dieser Dissertation beschäftigt sich Manuskript I als Übersichtsartikel mit dem Polarisationssehen der Insekten und bietet einen Überblick über aktuelles Wissen (Stand: 2017) zu anatomischen und physiologischen Anpassungen und deren Auswirkungen auf das Verhalten von verschiedenen Insektenarten (Mathejczyk & Wernet, 2017). Um visuelle Orientierung und Navigation von Insekten experimentell zu studieren, entwickelte ich günstige und modulare Verhaltensapparaturen, welche detailliert in Manuskript II beschrieben sind (Mathejczyk & Wernet, 2020). Diese Apparaturen ermöglichen die Quantifizierung der Verhaltensantworten von Insekten auf panoramische Intensitäts- und Farbmuster oder polychromatisches linear polarisiertes Licht, welches dorsal präsentiert wird, wobei die Versuchstiere stets auf einer sphärischen Tretmühle laufen oder magnetisch fixiert auf der Stelle fliegen. Diese Publikation bietet detaillierte 3D Modelldaten und Bauanleitungen für diese modularen Verhaltensapparaturen, inklusive einer einfach zu bauenden Klebestation und einer günstigen Temperatur- und Luftfeuchtigkeitssteuerung. Code zum Tracken der Tiere und zur Datenanalyse wurden online verfügbar gemacht, um der wissenschaftlichen Gemeinschaft im Sinne von ‚offener Wissenschaft‘ behilflich zu sein, damit die präsentierten Verhaltensapparaturen modifiziert und weiter optimiert werden können. Weiterhin demonstrierte ich die Funktionalität und Vielseitigkeit der Verhaltensapparaturen, indem ich Verhaltensantworten von laufenden und fliegenden Fliegen auf rotierende panoramische Intensitätsmuster, sowie Verhaltensantworten von fliegenden *Drosophila melanogaster* auf schnelle Änderungen des dorsalen e-Vektors quantitativ beschrieb. Daraufhin nutzte ich die Verhaltensapparaturen aus Manuskript II, um polarotaktische Verhaltensantworten von fliegenden *Drosophila* auf einen konstant rotierenden, dorsal präsentierten e-Vektor zu untersuchen (Mathejczyk & Wernet, 2019). Es zeigte sich, dass fliegende Fruchtfliegen ihre Körperachse in Antwort auf linear polarisiertes UV Licht ausrichten, aber nicht unter polarisiertem grünen oder depolarisiertem

UV Licht. Jede Fliege wählte hierbei eine arbiträre bevorzugte Richtung relativ zu einem dorsal präsentierten e-Vektor und konnte diese Richtungen über mehrere Minuten stabil halten.

Zusammengenommen bestätigen diese Ergebnisse, dass *Drosophila* Wellenlängen-spezifische Himmelpolarisation zur Orientierung nutzen kann und suggerieren, dass dorsales Polarisationssehen in *Drosophila* einer Verbreitungsstrategie dienen könnte, welche die Überlebens- und Reproduktionschancen der Fliegen auf der Populationsebene optimiert.

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