A Time-Dependent Color Preference in Adult Drosophila

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A TIME-DEPENDENT COLOR PREFERENCE IN ADULT *DROSOPHILA*

By

Stanislav Lazopulo

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A TIME-DEPENDENT COLOR PREFERENCE IN ADULT
DROSOPHILA

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Light is essential for the survival of almost all organisms. Development of biological light detectors, called photoreceptors, allowed bacteria, plants, and animals to sense light. The evolution of the photoreceptor organs led to the appearance of visual systems. Through light perception, organisms started to adapt their behavioral and physiological processes to variations of light intensity and spectrum. Animals developed stereotypical behavioral responses to light. Profound understanding of photoreception in fruit fly *Drosophila melanogaster* allow to study how the light information is processed and responses are generated. In this study, we report previously undescribed color-specific behavioral responses of fruit flies to light and identify the phototransduction pathways, which are required for these responses.

Coupling between daily varying external light and an endogenous time-keeping mechanism known as the circadian clock produces a periodic pattern in the locomotion activity of *Drosophila*. This pattern is commonly characterized by the so-called morning and evening peaks of activity. Here we show that activity patterns are punctuated by even stronger bouts of activity that are reminiscent of bursts. The bursts are more than ten standard deviations larger than basal activity.
and appear in two bands, on average around 2 hours after the morning peak and 2 hours before the evening peak. The bursts are virtually undetectable in population-averaged data due to the variability of their timing and short duration. We further show that the bursts are likely generated as a light response and independently of the circadian clock.

Visual and nonvisual responses to light are often color specific due to the natural spectral sensitivity of photoreceptors. Adult Drosophila senses light mostly through the eyes, the ocelli, Hofbauer-Buchner eyelets and structural changes in the protein cryptochrome. These photoreceptors each have distinct activation spectra. To identify if the color of the light affects activity peaks and bursts, we measured fly locomotion patterns under different wavelengths of light. Data on the activity of flies illuminated with blue light showed an unequal effect of that light on activity peaks. Elevated movement speed of flies in blue color suggests blue light to be the possible driver of the activity bursts. In contrast, flies in green light reduced their activity. Distinct and non-uniform effects of light colors on fly activity hinted at a possible variation of Drosophila color preference with time.

Typically, color preference in Drosophila is studied using phototaxis paradigms, which do not consider time dependence of the behavior. Here we show that when given a choice between blue, green and dim light, fruit flies exhibit an unexpectedly complex pattern of color preference that changes with the time of day. Flies display a strong green color preference in the morning and late afternoon, reduced green preference at midday, and a robust avoidance of blue throughout the day. We further show that the color preference depends on multiple photoreceptor systems.
The peaks in green preference require visual photoreceptors. The midday reduction in green preference depends on the Transient Receptor Potential (TRP) channels dTRPA1 and Pyrexia (PYX) and is timed by the circadian clock. In contrast, blue avoidance is primarily mediated by class IV multidendritic neurons that tile the fly body. The avoidance requires the TRP channel Painless and is independent of the clock. Together, our results reveal the complexity of the \textit{Drosophila} color-specific light responses and identify multiple photoreception and transduction pathways, which mediate those responses.
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<th>Symbol</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>12:12LD</td>
<td>12 hours light/12 hours dark</td>
</tr>
<tr>
<td>5-HTP</td>
<td>5-Hydroxytryptophan</td>
</tr>
<tr>
<td>CLK</td>
<td>clock</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
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<td>CRY</td>
<td>cryptochrome</td>
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<tr>
<td>CYC</td>
<td>cycle</td>
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<tr>
<td>d</td>
<td>dorsal</td>
</tr>
<tr>
<td>DAM</td>
<td><em>Drosophila</em> activity monitor</td>
</tr>
<tr>
<td>DD</td>
<td>constant darkness</td>
</tr>
<tr>
<td>DFT</td>
<td>discreet Furrier transform</td>
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<tr>
<td>DRA</td>
<td>dorsal rim area</td>
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<tr>
<td>E</td>
<td>evening</td>
</tr>
<tr>
<td>gl</td>
<td>glass</td>
</tr>
<tr>
<td>GMR</td>
<td>Glass Multimer Reporter</td>
</tr>
<tr>
<td>H-B</td>
<td>Hofbauer-Buchner</td>
</tr>
<tr>
<td>hdc</td>
<td>histidine decarboxylase</td>
</tr>
<tr>
<td>hid</td>
<td>head involution defective</td>
</tr>
<tr>
<td>IR</td>
<td>infra-red</td>
</tr>
<tr>
<td>I</td>
<td>lateral</td>
</tr>
<tr>
<td>LD</td>
<td>light/dark</td>
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<td>LED</td>
<td>light emitting diode</td>
</tr>
<tr>
<td>LS</td>
<td>Lomb-Scargle</td>
</tr>
<tr>
<td>M</td>
<td>morning</td>
</tr>
<tr>
<td>md</td>
<td>multidendritic</td>
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<tr>
<td>min</td>
<td>minute</td>
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<tr>
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<td>millimeter</td>
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<tr>
<td>mM</td>
<td>milli Molar</td>
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<tr>
<td>ninaE</td>
<td>no inactivation no after-potential E</td>
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<tr>
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<td>nanometer</td>
</tr>
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<td>norpA</td>
<td>no receptor potential A</td>
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<tr>
<td>pdf</td>
<td>pigment dispersing factor</td>
</tr>
<tr>
<td>PER</td>
<td>period</td>
</tr>
<tr>
<td>ppk</td>
<td>pickpocket</td>
</tr>
<tr>
<td>pr</td>
<td>photoreceptor</td>
</tr>
<tr>
<td>R</td>
<td>Receptor</td>
</tr>
<tr>
<td>Rh</td>
<td>Rhodopsin</td>
</tr>
<tr>
<td>RNAi</td>
<td>Ribonucleic acid interference</td>
</tr>
<tr>
<td>TeTxLC</td>
<td>tetanus toxin light chain</td>
</tr>
<tr>
<td>TIM</td>
<td>timeless</td>
</tr>
<tr>
<td>TRP</td>
<td>Transient receptor potential</td>
</tr>
<tr>
<td>TRPL</td>
<td>TRP-like</td>
</tr>
<tr>
<td>UAS</td>
<td>upstream activating sequence</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
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<tr>
<td>v</td>
<td>ventral</td>
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<tr>
<td>W</td>
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<tr>
<td>w</td>
<td>white</td>
</tr>
<tr>
<td>ZT</td>
<td>Zeitgeber time</td>
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</tbody>
</table>
Chapter 1 Introduction.

1.1 Photoreception in *Drosophila*.

1.1.1 Light perception in nature.

Light perception, an ability to sense and interpret light information can provide critical survival advantages. Through evolution, organisms have developed various light-sensitive organs called photoreceptors, to receive light information. This information is used to regulate many behavioral and physiological processes. Simple organisms like cyanobacteria can detect light and respond to it (Montgomery 2007). Cyanobacteria use light for photosynthesis, and to maximize exposure they move towards light of higher intensity. This positive movement towards light is called phototaxis. Due to rotation of Earth, light intensity changes rhythmically with 24-hour period and cyanobacteria adapted this oscillation. Cyanobacteria developed a molecular mechanism called circadian clock, to predict daily variations of light (Golden and Canales 2003; Cohen and Golden 2015). However, the circadian clock also requires adjustments to the seasonal changes of day duration and are entrained by external light. Phototaxis and entrainment of the circadian clock are common behaviors among prokaryotes, plants, and animals. They depend only on the intensity and spectrum of ambient light and do not require complex photoreceptors. The type of responses such as phototaxis and circadian clock entrainment is called nonvisual, and also includes regulation of sleep, metabolism and synthesis of some proteins (Mohawk, Green, and Takahashi 2012; LeGates, Fernandez, and Hattar 2014; Lucas 1999; Nitabach and
Taghert 2008; Ceriani et al. 1999; Mazzoni, Desplan, and Blau 2005; Yamaguchi and Heisenberg 2011). As the name suggests, nonvisual responses do not require visual system.

In contrast, visual responses require eyes, which are complex light-sensitive organs, typically containing many receptors. Eyes use light reflected from surfaces to reconstruct the image of surrounding objects and track them. With many receptors, vision provides high spatial and temporal resolution to help animals orient in the environment, avoid predators or find food and mates. Another advantage of multiple receptor systems is the possibility of color discrimination. The ability to distinguish colors of objects provides a further advantage to animals, by enabling them to determine from a distance if the other animal is a predator or not, or if the food is edible or poisonous. Depending on the importance of the color information, different animal species developed a color vision of varying complexity. Primates and humans can discriminate between more than 300 hues with the highest resolution in the green-red region, which is advantageous for finding ripe fruits and young leaves (Dominy and Lucas 2001). Among insects, butterflies rely heavily on their ability to distinguish flowers and mates by color and developed up to 15 types of photoreceptors (Arikawa 2003; Perry et al. 2016). In contrast, fruit flies only have four and do not see red light (Paulk, Millard, and van Swinderen 2011).

With the development of visual and nonvisual photoreception, animal’s perception of the color of light became more prominent. Animals and plants invented coloration as a way of communicating, to attract a potential mate or avert
predator (Cuthill et al. 2017). As a result of an interplay between all the visual and nonvisual responses, strong innate color specific associations and preferences have been established. In such complex organisms like humans, the innate responses are often masked by social interactions and are hard to track and verify (Palmer and Schloss 2010; McManus, Jones, and Cottrell 1981). In contrast, insects are significantly simpler animals and show more direct responses to light (Giurfa et al. 1995; Fischbach 1979). The progress in studies of Drosophila light perception allows detailed analysis of how light information is processed and responses are generated.

1.1.2 Photosensitive organs in Drosophila.

The photosensitive system of the Drosophila melanogaster consists primarily of three visual photoreceptor organs: compound eyes, ocelli and Hofbauer-Buchner eyelets (Figure 1). In addition to visual photoreceptors, many light sensitive proteins are expressed across the fly body and activate various groups of neurons. A UV-A/blue light sensitive photo-pigment cryptochrome (CRY) is not involved in classical phototransduction but required for light entrainment of the circadian clock. Some light sensitive proteins - rhodopsins - are expressed in peripheral sensory neurons called multidendritic (md). Other rhodopsins are expressed in temperature sensitive neurons, however, there is no data showing light affecting signaling in those neurons.
Figure 1. Visual System of Drosophila. Schematic of the Drosophila head with visual receptors shown with arrows. Visual system includes two compound eyes, three ocelli and two Hofbauer-Buchner (H-B) eyelets (from Buschbeck & Friedrich, 2008).

Compound eyes are complex systems, composed of multiple modules. The retina is an outer layer of the eye, consisting of approximately 800 subunits, called ommatidia, which are arranged in a hexagonal pattern (Fig. 2A, B). Each ommatidium, in turn, consists of 8 photoreceptor cells that absorb light of certain wavelengths and 11 accessory cells (Fig. 2D). The accessory cells include cone cell, bristles and a layer of pigment cells to block off light from the sides and let light only through the cornea. The brown (ommochromes) and red (pteridines) pigments are the sources of the red color of a wild-type fly eye. In the white Drosophila mutant, a fly with a null mutation in the gene white, which is required for synthesis of the pigments, the pigment cells fail to express pigments, resulting in the cells being transparent, and overall eye appearing white. As a result, the photoreceptors in each ommatidium start to receive light from sides, leading to abnormal blurry vision. The eight photoreceptor cells are arranged in structure with
six outer cells (R1-R6), and two central cells positioned one (R7) on top of the other (R8). Each of the cells expresses one of 5 rhodopsins (Rh1,3-6) with distinct absorption spectra. Photoreceptors R1-6 all express Rh1, rhodopsin that responds to a wide range of wavelengths. Those cells are mostly involved in motion detection (Heisenberg and Buchner 1977; Yamaguchi et al. 2008).

Figure 2. Organization of the compound eye. *Drosophila* eye (A) consists of ~800 similar ommatidia (B) forming a hexagonal pattern. (C) Top view of an ommatidium with rhabdomeres of 7 photoreceptors (R1-R7). (D) Schematics of a single ommatidium, side view on the left and cross-section on the right. Each ommatidium includes 8 photoreceptors surrounded by a layer of pigment cells (PC) with cone cell (CC) on top. Photoreceptors are organized in 6 outer receptors (R1-R6) encircling R7 and R8 which are positioned on top of each other (A-C, from Kumar 2012 and D from Wang and Montell 2007).
Downstream of the retina are more complex structures of neurons, called neuropils, which process signals from photoreceptors: medulla, lamina, lobula and lobula plate (Fig. 3). Together those neuropils form optic lobe. Each neuropil consists of multiple layers of interneurons which projections remain inside optic lobe and not only go towards deeper layers but also towards neighboring neurons. The other type is “projection” neurons, which connect optic lobe to the central brain (Nériec and Desplan 2016). Many structures and neurons inside neuropils have been identified, however, the processing of visual information remains hindered.

Figure 3. *Drosophila eye and optic lobe.* The retina of an eye carries ommatidia with photoreceptors (PRs) projecting to the optic lobe. Optic lobe consists of lamina, medulla, lobula and lobula plate. Motion-sensing photoreceptors 1-6 (R1-R6) from all ommatidia innervate lamina and color photoreceptors 7,8 (R7, R8) innervate medulla (modified from Nériec & Desplan, 2016).

In addition to compound eyes, *Drosophila* has three simple eyes on top of the head, called the ocelli, which express Rh2. The role of the ocelli is not well defined, but they can sense small changes in light intensity, the information that can be
used during flight (Taylor and Krapp 2007). The last component of the fly visual system is the Hofbauer-Buchner (H-B) eyelets, the remnants of the larval eye, Bolwig organ. Each H-B eyelet consists of four neurons, expressing Rh5 and Rh6. The H-B eyelets send projections towards circadian pacemaker neurons, contributing to the photo synchronization of the circadian clock.

1.1.3 Rhodopsins.

Rhodopsins (Rhs) form the group of biological pigments expressed primarily in *Drosophila* visual photoreceptors that activate the phototransduction cascade. Upon exposure to light Rhs undergo conformational change into active metarhodopsin, which then activates heterotrimeric $G_q$ protein and realizes the $G_q\alpha$-subunit. $G_q\alpha$ next activates the phospholipase C (PLC) and initiates the phosphoinositol signaling (Fig. 4). This leads to opening of the Transient receptor potential channels (TRP and TRPL) and cation influx, therefore, membrane depolarization. Each rhodopsin is tuned to absorb specific wavelengths enabling color vision. Metarhodopsins have shifted, relatively to rhodopsin, absorption spectra. The proportion between rhodopsins in different states helps to accommodate vision to light conditions and fine-tune color discrimination.
1.1.3.1 Rhodopsins expression in the visual system.

In *Drosophila*, seven different rhodopsins (Rh1-7) have been identified, though the role of Rh7 is not yet well determined. Rh1-6 are all expressed in *Drosophila* visual system and differentiate by the distinct sensitivity spectra (Fig. 5C). Rh1, the first major rhodopsin to be identified, is encoded by no inactivation no after-potential E (*NinaE*) gene. It is expressed in all six outer photoreceptor cells R1-6 of all ommatidia and has a long blue-green spectral range with a peak at 486 nm. The long-range makes Rh1 more suitable for motion detection and shape recognition. In contrast to Rh1, the Rh2 to Rh6 rhodopsins have more narrow ranges of sensitivity spectra peaking at 418, 331, 355, 442 and 515 nm respectively (Table 1). When rhodopsins are in metarhodopsin states, their absorption peaks are shifted towards longer wavelengths, except for Rh6 (Table 1). The two UV sensitive rhodopsins Rh3 and Rh4 can be expressed in photoreceptor R7, and blue sensitive Rh5 and green sensitive Rh6 can be
expressed in photoreceptor R8. Due to different expressions in R7 and R8, four types of ommatidia are formed: “pale”, “yellow”, “dorsal rim area” and a specialized “yellow” (Fig. 5B). In pale ommatidia, R7s express Rh3 and R8s express Rh5. In yellow ommatidia R7s express Rh4 and R8s express Rh6. In small subset of dorsal rim area (DRA) ommatidia both R7s and R8s express Rh3. During development, each ommatidium stochastically self-specifies into pale or yellow type with 30%/70% correspondingly through repression feedback signal from Rh6 (Fig. 5A) (Vasiliauskas et al. 2011). Some specialized yellow ommatidia in a dorsal third of an eye co-express Rh3 and Rh4 in R7s. In addition to R8s, Rh6 is also expressed in H-B eyelets. The violet sensitive Rh2 is expressed only in ocelli.

Table 1. Expression patterns and absorption peaks of rhodopsins.

<table>
<thead>
<tr>
<th>Rhodopsin</th>
<th>Expression</th>
<th>Absorption peak of rhodopsin (nm)</th>
<th>Absorption peak of metarhodopsin (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rh1</td>
<td>Compound eye, R1-6</td>
<td>≈486</td>
<td>≈566</td>
</tr>
<tr>
<td>Rh2</td>
<td>Ocelli</td>
<td>≈418</td>
<td>≈506</td>
</tr>
<tr>
<td>Rh3</td>
<td>Compound eye, R7</td>
<td>≈331</td>
<td>≈468</td>
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<td>Rh4</td>
<td>Compound eye, R7</td>
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<td>Compound eye, R8</td>
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<tr>
<td>Rh6</td>
<td>Compound eye, R8</td>
<td>≈515</td>
<td>≈468</td>
</tr>
<tr>
<td>Rh7</td>
<td>Clock neurons and PRs</td>
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1.1.3.2 Extraocular expression of Rhodopsins.

Rhodopsins are not limited to light capturing role in photoreceptor cells of the fly visual system. In *Drosophila* larvae, Rh1 is required for temperature discrimination in a comfortable range (Shen et al. 2011). This discrimination is light-independent and seems to be coupled with Transient receptor potential A1 (TRPA1) channel, an ion channel already known for modulation temperature responses. Rh1 is co-expressed with TRPA1 for the temperature discrimination role, though the level is extremely low for detection with an antibody or fluorescence staining.

Rh5 and Rh6 are additionally expressed outside the visual system in multidendritic neurons in larvae (Sokabe et al. 2016). Sokabe et al. showed that
larvae, lacking either Rh5 or Rh6 lost a characteristic switch in temperature preference during third instar. Unlike Rh1, the expression of Rh5 and Rh6 was strong enough to label type IV neurons ddaC and v’ada and the external sensory organ neuron vp5. The rhodopsins are co-expressed in those neurons, and their overlapping function suggests the possibility of Rh5 and Rh6 forming heterodimers.

The newest characterized Rh7 has been a mystery since 2000 when it was discovered from full *Drosophila* genome, as it is not expressed in visual photoreceptors. Rh7 is highly conserved across *Drosophila* genus and also found in other arthropods (Senthilan and Helfrich-Förster 2016). The structure of Rh7 has most of the signature components of rhodopsins but lacks G-protein activating motif. When Rh1 was replaced with Rh7, it was able to maintain rhabdomeres but did not activate phototransduction (Grebler et al. 2017). Rh7 does not seem to function in visual photoreceptors, but, may work in other photoreception pathways. Recently two groups have shown that Rh7 is expressed in circadian clock neurons in the brain and plays a role in photoentrainment and light-dependent activity (Ni et al. 2017; Kistenpfennig et al. 2017). The knockdown of Rh7 gene alone or with CRY did not seem to abolish photoentrainment but sometimes led to arrhythmic flies and modified activity pattern. So far, the role of Rh7 remains elusive and requires additional studies.

In addition to Rhodopsins, a blue-sensitive pigment CRY is expressed in circadian clock system so will be discussed later. Before going into issues concerning of color vision in *Drosophila*, let us discuss multidendritic neurons.
These neurons have not been yet reported for phototransduction but are possible candidates to mediate color specific response in our research.

1.1.4 Multidendritic neurons.

Multidendritic (md) sensory neurons, also called dendritic arborization (da) sensory neurons have, as their name suggests, multiple dendrites, which branch into nearly all epidermal cells in *Drosophila* larvae (Fig. 6A). Based on the size of dendritic arbors, md neurons are divided into four classes represented in every hemisegment: three Class I, four Class II, five Class III, and three Class IV md neurons (Fig. 6B, C) (Grueber et al. 2007). Different classes provide information about mechanical stimuli and proprioception, thermal and chemical signals (Geffeney and Goodman 2012). The most complex in terms of dendritic arbors class IV md neurons are polymodal nociceptors. These neurons express multiple ion channels and initiate aversive responses to heat, mechanical loads and UV/blue light (Hwang, Stearns, and Tracey 2012; Tracey et al. 2003; Xiang et al. 2010). During metamorphosis, md neurons undergo extensive dendritic pruning and subsequent regrowth (Shimono et al. 2009). Some of the neurons degenerate at pupal stage, others go through cell death within one week after eclosion, but some immediately reshape and persist throughout the lifespan. Among the neurons which survive through morphogenesis are two class IV neurons ddaC and v’ada, which were required for light sensing in larvae (Xiang et al. 2010) (Fig. 6C). Recently those neurons were also shown to express Rh5 and Rh6 (Sokabe et al. 2016), which makes it highly possible, that these neurons maintain their function in adult fly as well.
Figure 6. Multidendritic (md) sensory neurons in *Drosophila* larvae. (A) md neurons cover almost entire body of the larva (modified from Xiang et al., 2010). (B) Four classes of md neurons are specified, based on the complexity of dendritic arbors (from Grueber et al., 2007). (C) Schematic of positions of md neurons in a hemisegment of the larval peripheral nervous system with nomenclature. Neurons are shown with diamonds, dorsal, lateral, ventral prime, and ventral neuron clusters are shown with d, l, v’ and v respectively (from Shimono et al., 2009).

### 1.2 Phototaxis and color vision in *Drosophila*.

Studies of color vision in *Drosophila* started more than half a century ago (Schumperli 1973; Hernández de Salomon and Spatz 1983; Fischbach 1979). These studies attempted to establish reliable and reproducible paradigms in fly behavioral response to light and colors. One of the earliest experiments were done by Schumperli, with the introduction of Y-mazes in studies of phototaxis (Schumperli 1973). Schumperli used a simple maze with two arms illuminated with different light: one arm was illuminated with white or monochromatic light, and the other arm – with monochromatic lights of various intensities and wavelengths from UV to red (Fig. 7, left). The idea of the experiment was to “demonstrate wavelength specific reactions which are inexplicable with the properties of the receptors alone”
which would indicate not only discrimination between colors but also innate color preference. In the experiment, a total of 50 flies were separately introduced in the maze and collected after choosing an arm. The total number of flies choosing each option was used to show preference. The experiment has clearly shown the attraction of flies to the light of a higher intensity and also led to some conclusions towards the ability to discriminate colors and preference of light of shorter wavelength. The former was mainly driven by the requirement of monochromatic light of a long wavelength to have a higher intensity than the light of a shorter wavelength to achieve similar preference. However, the strong dependency of the response to the intensity of light does not allow firm support of color vision. One of the main requirements of color vision is the independence of intensity.

A group of studies tried a more sophisticated approach of training flies to discriminate between colors (Menne and Spatz 1977; Hernández de Salomon and Spatz 1983). This approach avoids the idea of innate preference and can drive response independently from the intensity. In those studies, ~100 flies were placed in a container with two halves illuminated by distinct colors (Fig. 7, right). A shaker was attached to the container and flies were trained through conditional punishment. First, the intensities of colors were adjusted so that ~50% of flies were in each color. That condition ensured the equal attractiveness of both options. During entrainment, one color was associated with shaking, which evoked an aversive response, while another color was not. Immediately after the entrainment flies were tested and number of flies in each color counted. The method not only showed that flies discriminate between colors, but also allowed to estimate the
resolution of discrimination through spectral discrimination function. However, the experimental design does not allow to find if flies have an innate color preference. Additionally, the experiment requires measurements of a population of flies, introducing a possible social component to a decision.

![Figure 7. Experimental setups for original studies of *Drosophila* phototaxis.](image)

(A) Y- or T-mazes are simple mazes, which provide the animal with a choice between two options. (B) A color choice assay Y-maze used by Schumperli called the double choice apparatus (from Schumperli, 1973). Schumperli used the single light source, divided into two beams and a set of color and neutral density filters to adjust to light conditions. (C) An apparatus used by Spatz to study wavelength discrimination in *Drosophila* (from Hernández de Salomon & Spatz, 1983). Spatz designed conditioning choice assay using a shaker to elicit aversion.

Going back to innate phototaxis, Heisenberg and Buchner (Heisenberg and Buchner 1977), and later Fischbach (Fischbach 1979) had used a “slow” phototaxis paradigm with light versus dark options to show a preference for light colors. The previous experiments were designed to show the immediate response from flies. This “fast” phototaxis response was most likely evoked by escape reaction. In Heisenberg and Buchner “slow” phototaxis paradigm, flies were given time to settle and stop moving. Therefore, that was considered a truly preferred choice and not escape reaction. In the experiment, the two arms of T-maze were
illuminated in turn by green light. Simultaneously, both ends were illuminated by UV light, which was monotonically increased. Both UV and green light showed previously an increased attractiveness with an increase in intensity (Schumperli 1973), however, when UV light reached 1% of the intensity of green light, the fly response changed to aversive. In the Fischbach experiment, the gradual increase in green light intensity alone after certain value led to the decrease of preference and eventual avoidance.

Together these data indicate, that flies show a nonlinear response to the intensity of light. Flies display maximum preference to light at low intensities followed by decrease towards avoidance at higher intensities. This response, however, is dependent on the spectral composition of light with a stronger preference towards light of shorter wavelengths. However, even in “slow” phototaxis experiments, flies are subjected to stressful conditions, which might affect their response. It is not a direct escape response like in “fast” phototaxis, but flies might be startled. Also, the absence of food does not allow long-term measurements and to observe phototactic behaviors in a more comfortable to flies conditions a different approach is required.

1.3 Circadian clock.

Circadian clocks are endogenous molecular mechanisms found in almost all organisms from bacteria to humans. Those mechanisms drive rhythms precisely adjusted to predict periodic changes in the environment, which occur daily, giving the name circadian (from Latin “circa diem” meaning “about a day”). In contrast to the simple response to the periodic cues, circadian clock affects physiological,
molecular and behavioral processes in an anticipatory way. One of the properties of the circadian clock is that it can sustain the oscillations even in the absence of cues. In such cases, the internal free-running rhythm is often slightly different from 24 hours.

The field of circadian clocks was pioneered by Pittendrigh, who studied the behavioral output of circadian rhythms in *Drosophila* and various rodents (Pittendrigh 1960; Pittendrigh and Daan 1976). Pittendrigh used light to entrain animal’s locomotor activity to circadian rhythms, the result of the second property of the circadian clock: ability to synchronize to periodic external cues. Aschoff introduced term “zeitgeber” (from German “time giver”) for external cues which entrain or synchronize the innate circadian clock to the Earth’s 24-hour day cycle (J. Aschoff 1960). Among zeitgebers, light and temperature are the most dominant (LeGates, Fernandez, and Hattar 2014). The requirements for zeitgebers also include the ability to start circadian oscillations in initially arrhythmic animal (the animal that was raised in the conditions without zeitgebers).

Using his experimental setup, Pittendrigh was also able to show the third property of the circadian clock, a tolerance to external temperature. Many molecular mechanisms depend greatly on the temperature, as the protein interaction is generally accelerated by higher temperature. For the clock to be reliable, it should be fairly stable at a wide range of temperatures. Pittendrigh showed that *Drosophila*, entrained to 12:12LD cycles at 16°C, 21°C, and 26°C retained similar rhythm in constant darkness.
1.3.1 The molecular organization of circadian clock in *Drosophila*.

The potential of *Drosophila* to be a model organism for the detailed study of the circadian clock was revealed by Konopka and Benzer by discovering first clock gene *period* (Konopka and Benzer 1971). Since then, the search for other clock genes led to discovering four core proteins: PERIOD (PER), TIMELESS (TIM), CLOCK (CLK) and CYCLE (CYC), expressed by corresponding genes. The main part of the mechanism is the negative feedback loop where PER and TIM essentially repress their own synthesis (Fig. 8) (Tataroglu and Emery 2014). CLK and CYC are transcriptional factors, which form a heterodimeric complex and promote transcription of PER and TIM. During night PER and TIM accumulate in the cytoplasm and in their turn form a heterodimeric complex. That complex enters the nucleus and promotes phosphorylation of the CLK/CYC complex, suppressing its activity. In addition, PER and TIM also gradually degrade, leading to release of CLK/CYC inhibition and start of a new cycle. The interactions result in close to sinusoidal oscillations of PER and TIM (Nitabach and Taghert 2008).
1.3.2 Central clock neurons.

Even though the clock genes are expressed in many cells in the body, the certain set of neurons in the brain is considered to be the core or pacemaker neurons, which synchronize all others. In mammals, the pacemaker function is performed by Suprachiasmatic Nucleus (SCN), which consist of approximately 20,000 neurons, each containing a self-autonomous oscillator (Mohawk, Green, and Takahashi 2012). In Drosophila, the central clock is composed of ~150 neurons bilaterally clustered into multiple groups: small ventral Lateral Neurons (s-LN₉s), large ventral Lateral Neurons (l-LN₉s), the dorsal Lateral Neurons (LNds), the Dorsal Neurons (DN₁, DN₂ and DN₃), and Lateral Posterior Neurons (LPNs) (Fig. 9). These groups of neurons are characterized by distinct functions (Tataroglu and Emery 2014) as well as signaling method (Nitabach and Taghert 2008).
Figure 9. Circadian clock neurons in *Drosophila* brain. Groups of clock neurons in are clustered by size and position, but not function. Total core clock consists of ~150 neurons divided into groups of lateral and dorsal (from Tataroglu and Emery 2014).

As light is one of the strongest zeitgebers, it has multiple ways to affect the circadian clock. One of the most reliable ways to study clock function and to observe the effect of light on the clock is the analysis of locomotor activity.

1.3.3 Photoentrainment of fly locomotion.

1.3.3.1 Bimodal activity.

One of the most obvious behavioral outputs of the circadian clock is the bimodal locomotor activity patterns, occurring in light/dark cycles. A popular way to visualize the time series of activity data is through graphical representation – actograms (Fig.10). *Drosophila*, as well as many other animals, including fishes, birds and some mammals, show two peaks of activity, one in the morning (M peak) and one in the evening (E peak) (Jurgen Aschoff 1966), and reduced activity (“siesta”) in the middle of the day. *Drosophila* is a crepuscular animal, which is most active during dusk and dawn. The name *Drosophila* from Greek “drosos
philos”, meaning “dew-loving”, dew – droplets of water appearing due to condensation in the morning and evening. The bimodal activity pattern is the most robust in standard laboratory conditions where the temperature is kept constant and the light pattern is varied in a rectangular cycle with a period of 24 hours - 12 hours of constant light followed by 12 hours of complete darkness (12:12LD). Population averages of locomotor activity recordings produce two robust peaks at transitions between lights on/off states of illumination (Fig. 10, top). However, these peaks are not a simple response to light turning on or off, but a clock-controlled change in behavior. This control can be seen from the activity starting to increase in anticipation of the light transitions (Fig. 10, top) and also, in continuation of the bimodal activity when LD is followed by constant darkness. Nevertheless, the masking effect of light is still present and can be observed in the activity of arrhythmic flies. Flies which have a non-functional circadian clock, do not show the anticipation of light transitions, but still increase their activity in response to light (Fig. 10, bottom).
Figure 10. Activities of rhythmic and arrhythmic flies under light/dark conditions. Fruit flies display a bimodal activity with morning (M peak) and evening (E peak) peaks of activity. Rhythmic flies (top) show anticipation of light on/off and off/on transitions and start activity earlier. In comparison, arrhythmic flies (bottom) do not show anticipation and increase activity in response to light transitions. Light on and off states shown with step function on top, two days are shown with time in Zeitgeber time (ZT). Both panels show population-average activity (N=18 for each).

1.3.3.2 Dual oscillator model.

Pittendrigh and Daan initially proposed a dual oscillator model of independent clock control of M and E peaks. The two oscillators have opposite responses to light: the first gets accelerated by light exposure, while the second gets decelerated (Pittendrigh and Daan 1976). The model had explained the possibility to adapt the activity to longer summer and shorter winter days (Jurgen Aschoff 1966). However, it did not have a neuroanatomical backing until studies of Grima and Stoleru (Grima et al. 2004; Stoleru et al. 2004). Using genetic tools available in *Drosophila*, Rouyer and Rosbash groups were able to block specific groups of neurons and separately
eliminate each of the activity peaks. The conclusion was that the M oscillator is in four s-LN_v neurons (M cells) expressing neurotransmitter pigment-dispersing factor (PDF) and the E oscillator is in three LN_d, DN_s and 5th s-LN_v (E cells) (Fig. 11) (the 5th s-LN_v was added later by Rieger (Rieger et al. 2006)). As PDF is considered the main intracellular messenger that synchronizes rhythms between PDF-positive and other neurons, the M oscillator is thought to be the main pacemaker. Since all PDF-positive neurons also express CRY, they are also responsible for photoentrainment of the circadian clock.

**Figure 11. Dual oscillator model of clock control.** A group of pigment dispersing factor expressing (PDF+) neurons is responsible for the control of morning peak and PDF non-expressing neurons are responsible for evening peak of activity. The exposure to light advances the morning peak and delays evening peak (modified from Stoleru et al., 2004; Tataroglu & Emery, 2014)

1.3.3.3 CRYPTOCHROME.

*Drosophila* cryptochrome (dCRY) is a blue sensitive photopigment which is mainly detected in visual photoreceptor cells and multiple circadian clock neurons: all LN_v, in the 5th s-LN_v, in three LN_d, and some DN_1. dCRY has a light
sensitivity spectra peaking in UV and blue regions (Fig. 12, left). Cryptochromes belong to a class of flavoproteins and are found in plants and animals. In mammals, two cryptochromes CRY1 and CRY2 are expressed and function in a core molecular circadian feedback loop in a light-independent manner (Mohawk, Green, and Takahashi 2012). The loop consists of transcriptional activators CLOCK and BMAL1, which promote expression of PERIOD and CRY. PERIOD and CRY, in turn, form a heterodimer and repress transcription of CLOCK and BMAL1. In Drosophila, this function of CRY is fulfilled by TIMELESS (TIM), and even though dCRY is a homolog of the CRY1, it has a different role in circadian clock mechanism. Upon activation by light CRY binds to TIM and leads to its degradation (Fig. 12, right). Degradation of TIM destabilizes PER and pauses the oscillation of both PER and TIM. This effectively resets the clock.

Figure 12. Cryptochrome is the circadian photoreceptor. Cryptochrome (CRY) mediates entrainment of the circadian clock to external light. (Left) Absorption spectra of CRY peaks in UV (~330-370 nm) and blue (~430-460 nm) regions of wavelengths. Solid line: CRY held in dark, dashed line: light-exposed CRY (from Ozturk, Selby, Annayev, Zhong, & Sancar, 2011). (Right) A functional diagram of CRY interfering in TIM oscillations. Upon light-activation, CRY binds to TIM and induces its degradation (from Hardin, 2005).
Null mutation of cry gene results in significantly slower clock re-entrainment, whereas wild-type fly requires about one day for synchronization, cry<sup>b</sup> mutant needs approximately seven (Stanewsky et al. 1998; Dolezelova, Dolezel, and Hall 2007). While wild-type flies become arrhythmic in constant light, cry mutants maintain rhythmicity. However, flies lacking CRY still can synchronize their activity and keep the free-running rhythm in constant darkness. Experiments with mutations in cry and visual receptors demonstrated that mainly compound eye, but also Hofbauer-Buchner eyelets play roles in photoentrainment (Helfrich-Förster et al. 2001). Whether the ocelli (Fig. 1) also play a role is not understood.

**1.3.3.4 Natural light entrainment.**

The bimodal activity is robust and clear in the laboratory 12:12LD pattern. In natural light cycle, the intensity of light does not change by step. Instead, it can be roughly characterized by a rectified sinusoid during the day with constant low intensity (≤1) during the night. Effects of the natural light pattern on the activity of flies have been actively studied and revealed complex modifications to the behavior (Yoshii, Rieger, and Helfrich-Förster 2012; Rieger et al. 2009; Y. Zhang et al. 2010; Bachleitner et al. 2007). In addition to the pattern of the light cycle, the spectrum of the sunlight is also unlike that of the standard fluorescent laboratory light. The sun’s spectrum can be understood in terms of black body radiation with the peak at ~450 nm and a gradual decrease towards the infrared region. In contrast, white fluorescent light is produced by a combination of three narrow bands of spectrum in blue, green and red regions. Everywhere else the intensity is close to zero (for details see Chapter 2.3.1). Studies with natural light conditions
have shown an ability of M cells and E cells to take each other functions. If the M cells are abolished, the E cells can control morning activity under low light intensity (Rieger et al. 2009). When E cells are inactive, the M cells can take over the control over the evening activity under high-intensity light or temperature cycles (Y. Zhang et al. 2010). These studies suggest a more complex mechanism of the activity control by the nervous system and lead to a proposal of multiple oscillators orchestrating the locomotion through complex interactions (Yao and Shafer 2014).

As more experiments were performed in natural and semi-natural conditions, a new peak of activity in the middle of the day was discovered, questioning the current approach in studying fly locomotor behavior (Fig. 13) (Vanin et al. 2012; Menegazzi et al. 2013; De et al. 2013; Menegazzi, Yoshii, and Helfrich-Förster 2012). Current methodologies use unnatural conditions and population averaged data to produce robust activity data. However, these methodologies might mask some prominent features of fly behavior in favor of robustness. A more sophisticated experiments and analysis of individual data can provide insight into new unexplained behavioral traits.

**Figure 13. Simulated natural conditions reveal an additional peak of activity.** Activity under varying temperature and non-square light/dark cycles show the presence of an afternoon activity peak (modified from Green et al. 2015).
1.4 Aims of this thesis.

The first part of this thesis focuses on a light-driven stochastic burst of activity in single fly locomotion data. Traditionally, the analysis of fly locomotion under LD conditions is done on population-averaged data. While such averaging is effective in revealing features like the M and E peaks that appear in close synchrony among individuals, it is less effective in identifying other potentially important locomotion features that lack temporal synchrony. However, study such locomotor features of single fly recordings can circumvent this limitation in current methods of parallel analysis. We report on a brief but strong stochastic burst in fly activity that, in contrast to M and E peaks, is detectable only in single fly recordings. In a single fly recording, the burst is likely to appear once randomly within 0.5-5 hours after lights turn on, last for only 2-3 minutes and yet show 5 times greater activity compared to the M peak. Owing to its variable timing and short duration, the burst is virtually undetectable in population-averaged data.

The second and major part of this thesis focuses on an unexpectedly complex pattern of color preference in *Drosophila* when given a choice among blue, green and dim light. The preference changes with the time of day: flies show a strong preference for green in the early morning and late afternoon, a reduced green preference at midday and a robust avoidance of blue throughout the day. Most of the studies of the color preference in *Drosophila* use well-established paradigms of slow and fast phototaxis, which show that flies are attracted to the light over dark and prefer light of shorter wavelengths(Schumperli 1973; Fischbach 1979). In these experiments, flies are released in T-mazes, given two choices and the
number of flies in each option is counted immediately or shortly after the release. However, the procedure most likely involves stress affecting the decision of flies and the response being an escape reaction. As a result, the effect of more slow-paced behaviors, such as sleep and feeding on color preference cannot be observed. Interaction of color preference with the circadian clock also cannot be determined. One of the key experiments in the field of circadian studies is a measurement of fly activity under 12-hour light/12-hour dark ambient light conditions. This long-term interaction between light and the circadian clock results in two peaks of activity (morning and evening) near the transition between light on/off with a period of low activity in the middle of the day (siesta) (Yoshii, Rieger, and Helfrich-Förster 2012; Grima et al. 2004; Stoleru et al. 2004). We wondered if the color preference in flies also changes with the progression of the day, and what photoreceptors and neural networks could be behind it.

To address this problem we, first, designed an experimental setup, which would allow us to measure the color preference throughout 24 hours a day and written custom code for analysis of data. Second, using available genetic and biochemical tools and various visual and clock mutants, we analyzed possible phototransduction pathways, candidates to mediate preference and interaction with circadian clock.
**Chapter 2 Material and Methods.**

**2.1 Fly strains and crosses.**

Table 2. *Fly strains used in this thesis.* # in source column indicate stock number in Bloomington Drosophila Stock Center.

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<th>Source</th>
<th>Reference</th>
<th>Details</th>
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</thead>
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<td>(Lindsley and Grell 1968)</td>
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<tr>
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<td>(Lindsley and Grell 1968)</td>
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**Ion channels**

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**Dmel/Mi{ET1} Gr28b<sub>MB03888</sub>**

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**Clock mutants**

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Flies were reared in plastic vials on a standard *Drosophila* medium at room temperature in laboratory conditions. The medium was prepared with cornmeal, molasses, agar, yeast, propionic acid and antibiotic (Tegosept).

### 2.1.1 The GAL4/UAS system.

The UAS/GAL4 system is a powerful genetic tool developed by Hitoshi Kakidani and Mark Ptashne and later adapted for *Drosophila* by Brand and Perrimon, which
is widely used in genetics, developmental biology and neuroscience (Kakidani and Ptashne 1988; Brand and Perrimon 1993). The system consists of two parts, first is the transcription activator protein GAL4, initially extracted from yeast. GAL4 specifically binds to the second component of the system, upstream activation sequence (UAS), and activates transcription. GAL4 is expressed under control of a native promoter, which makes it tissue-specific and UAS is inserted with the gene of interest to drive the expression of that gene in the tissue. The two components usually are inserted in separate fly lines and are brought together by the simple cross (Fig. 14). The line that carries GAL4 is called GAL4-line and the line with UAS is called a reporter or responder line. For practical application in our research, we used GAL4/UAS system to silence or ablate certain neurons.

Some GAL4/UAS transgenic flies did not survive to adulthood. In such conditions, one of the lines was crossed to a strain containing tub-Gal80ts, a temperature dependent repressor of GAL4, expressing under tubulin promoter, effectively in every cell of the fly. The establishment of stable transgenic lines, insertions were mapped and balanced by crossing to double balancer mutants w; Sp/CyO; D3/TM6B.
Figure 14. GAL4/UAS system. A simple cross of two strains carrying GAL4 and UAS constructs leads to expression of the UAS-target gene X in tissues specified by GAL4 promoter. For details see text (from St Johnston, 2002)

2.2 Behavioral assays.

2.2.1 Illumination system.

A typical environmental chamber for Drosophila studies is an incubator with a regulated light, temperature and humidity conditions. Temperature and humidity can be relatively easily adjusted to simulate natural conditions if needed. However, mimicking the natural light conditions is much more complicated. Not only is the change of the intensity throughout the day is non-trivial, but also the spectrum of the sunlight is hard to imitate. Sunlight has a spectrum close to that of the black body with intensities across a long continuous range of wavelengths from far ultraviolet (~100 nm) to far infrared (above 3000 nm) with a peak around blue (~450 nm) (Fig.15, grey line). In comparison, fluorescent light that is typically used in
laboratories, has three sharp peaks at 440 (blue color), 550 (green color) and 615 (red color) nm, while, in other regions, the spectrum shows smaller peaks or no irradiance at all (Fig.15, dashed line). That makes the light much more different, especially for animals like *Drosophila*, which has different photoreception spectra and therefore experience light differently from humans.

![Figure 15. Power spectra for sunlight, white fluorescent and LED lights.](image)

Typical irradiance spectra for the installed LED lamp (solid line) compared to the previous fluorescent lamp (dashed line) and local (25° 43' 24.26'' N, 80° 16' 45.89'' W) sunlight (grey line, not to scale). LED lamp spectrum is broadly distributed, while fluorescent irradiance is concentrated in three narrow peaks. The LED peak at 450 nm coincides with one of the peaks in the action spectrum of *Drosophila* cryptochrome. Compared to fluorescent lamps, the LED spectrum better approximates light conditions in the natural environment.

The standard fluorescent light in our experimental setup was replaced with white light emitting diodes (LED) for multiple reasons. First, the LED spectrum more closely imitates that of the sun. Fluorescent light has roughly equal intensity in three narrow bands of blue, green and red regions of spectra, while sunlight and
LED light is richer in blue. Since flies do not see the red light, they perceive fluorescent light more weakly. Another important aspect is the shelf-life of the lamp. With time fluorescent lamps lose brightness, specifically in blue, which shifts overall light towards red dominated. The LEDs have significantly more stable radiation and for a longer time. The critical point is that fluorescent lamps produce light from electrical discharge and require certain minimum power. This presents a threshold to how low can one reduce the intensity and sets a step in the adjustment of the light conditions. In comparison, LEDs allow smooth control over an entire range of irradiation. The LED lamps (LED-88020-120V Neptun Light Inc., IL) used in our setup have 576 LEDs each housed in a 4 ft. aluminum (T8 standard) support with a sturdy polycarbonate frosted cover. An important aspect of the LEDs used in our setup is that it has a high peak at 450 nm that coincides with one of the CRY absorbance peaks, a property that makes it strong entraining tool for circadian clock.

To create customizable light patterns, we designed a light control system and a custom code written in MATLAB (MathWorks Inc.). The system consists of described above lamps, light sensors to monitor and adjust intensity, and a light control interface box to communicate with a computer or manual remote. Electronics gathered in the light control box include an interface board (Part#1018_2, PhidgetInterfaceKit, Phidget Inc., Canada), optocouplers, a command decoder, and a dimmer (Fig. 16B). The light control box translates commands from computer or remote to electrical signals and sends them individually to each lamp, allowing separate control over each lamp. The box also
sends data from the light sensors to the computer. The dimmer consists of four digital potentiometers which receive a signal from the interface and changes resistor values at 1% steps. This varies the input voltage to each lamp and allows to change light intensity in 1% steps. The manual remote control can be used in the event communication with the computer is lost (Fig. 16A).

Figure 16. Schematic and diagram of the illumination system, designed for the study. (A) Schematic of the experimental setup and the production and detection of light. Each of the eight LED lamps is controlled from a computer to provide illumination between 0 and 8800 lux. The level of illumination is reported to a computer by three pairs of photodiode-based sensors. (B) Information flow between main components of the light control box and peripheral devices. Interface board receives control signals from the computer and forwards them to the command decoder which interprets the signal to set the desired light intensity.

The system can produce any arbitrary pattern of light intensity with an error of less than ±2.5%. As an example, Figure 17A shows square waves of random intensities and durations. For simulating the shape of the natural light cycle, another example, a half wave rectified sine pattern (Fig. 17B) or a simple
A trapezoidal pattern with a linear change of light can be used to mimic natural light. The light system was tested for stability by maintaining constant intensity for 100 minutes and has kept it at ±2.5% of the target level (Fig. 17C).

**Figure 17. The illumination system can produce light of arbitrary pattern with less than 5% error.** (A) Square patterns of different LED intensities and durations. Error bars at peak values represent 5% variation with respect to each target value. (B) Half-sine light cycles of different maximum intensities. The half-sine shape with a period of 24 hours closely simulates variations in daily sunlight intensity. (C) Stability of the system for three different LED intensities each maintained for 100 minutes. For all tests, intensity variations were limited to 5% error bounds.

The intensity of the light inside the chamber was constantly monitored using two types of light sensors. The first type (Part#1143_0, Phidget Inc., Canada) was used to cover high intensities and has a logarithmic response up to 70 000 lux. However, this makes it unreliable at low intensities, so a different sensor (Part#1142_0, Phidget Inc., Canada) was used to read intensities below 10 lux. The second type has a linear response and reliably measures light from 1 to 1000 lux. The sensors are sensitive to the direction of light and were modified with diffusers to overcome that deficiency. The sensors are analog photoresistors, which means they output measured intensity in arbitrary units and require calibration. Total of three groups, each consisting of two sensors were installed inside the chamber (Fig. 16A) and
all were individually calibrated using a handheld light meter (LM-200LED Amprobe, WA) with 5% uncertainty. For low-intensity sensors, the data points are best fitted with function $I = a \cdot S^b$ (I – light intensity, S – analog signal, a and b – fit parameters) for intensity up to 65 lux (Fig. 18). For high intensity sensors, the data points are best fitted with function $I = e^{\alpha \cdot S + \beta}$ (α and β – fit parameters) deviating from this only below 10 lux (Fig. 18). To obtain maximum accuracy, the system automatically switches from low to high intensity sensors at intensity of 38 lux.

**Figure 18. Calibration data for a low range (left) and a high range (right) sensor.** The low range sensor has a linear response while the high range sensor has a logarithmic response. Lines show fits of the data points shown in symbols. For the sensor shown parameters were $a = 0.29, b = 0.86, \alpha = 2.33 \times 10^{-2}, \beta = 6.71 \times 10^{-2}$. Software controlling the light system automatically switches from low to high range sensor at 38 lux (horizontal line).
2.2.2 Video recording.

For the recording of fly activity and color preference, a home-designed setup was built. The setup consists of a transparent acrylic platform positioned above the array of infrared LEDs and a CCD camera above the platform. The infrared LEDs were attached to the aluminum sheet to disperse the heat. For the experiments, the tubes with flies were placed in transparent acrylic racks on top of the platform. A layer of a plastic diffusor allowed to create a uniform infrared background from LEDs. A long pass filter lens with a transmitting range above 780 nm wavelength was attached to the camera to capture video in infrared light only. That allowed to record flies throughout 24 hours a day in light and dark conditions without alterations in the background intensity.

Figure 19. Experimental setup for measuring color preference using the illumination system. An incubator with an installed illumination system and video recording setup.
2.2.3 Color filters.

For the experiments with light of different colors, the white LED light was filtered through plastic bandpass filters (Roscolux, Rosco Laboratories, Inc.). Based on the sensitivity peaks of Rh5 and Rh6, the rhodopsins, which are thought to play a leading role in the color vision of fruit fly, the bands for two filters were chosen with peaks at 450 nm and 528 nm (Roscolux #58 and #389). A red filter was used as the dark and has transmission above 580 nm (Roscolux #19, non-visible for flies). The intensities of light transmitted through filters were 626 μW/cm² for green, 572 μW/cm² for blue and 571 μW/cm² for red (Fig. 20A). When blue, green and red filters were overlapped, the resulting dark filter was almost completely opaque to white LED light. In addition, multiple neutral density filters were used to decrease overall intensity of white light or certain color or region in a tube. At last, a yellow filter with transmission range above 500 nm was used to see if the light above green range contributes to response form flies (Roscolux #10). For three-color choice assays filters were cut into pieces, glued together with ~1 mm overlap and wrapped around tubes creating ~2 cm long regions of each color. In two color-choice assays, a similar procedure was used but with each color region ~3 cm long. To avoid any effect of food and cotton, the filters were used in all possible combinations (two for two colors and six for three colors, Fig. 20B). For simplicity, tubes were arranged in order as in Fig. 20B,C, however, randomized ordering showed same results; sample sizes were chosen to reliably measure experimental parameters above noise levels.
Figure 20. **Bandpass filters for color experiments.** For experiments with colors, light was transmitted through plastic bandpass filters. (A) Properties of light of different colors. (B) Six possible combinations of color filters for three-color choice assays. (C) Example of typical tubes arrangement in an experiment.

### 2.2.4 Locomotor activity recordings.

Locomotor activity of the flies in the experiments was measured either in *Drosophila* activity monitors (DAM) (TriKinetics Inc., MA) or from video recordings. For both methods, flies were placed in individual glass tubes of length 6.5 cm and diameter 5 mm. One end of a tube had *Drosophila* medium (see Chapter 2.1) and a wax seal and the other end was plugged with cotton for air access. The measurement of locomotion in DAMs was performed with an infra-red (IR) beam bisecting each tube perpendicular to its long axis. Every time a fly intersects the beam, activity is detected by the IR sensor and counted into the time series. The number of beam crossings was aggregated over 20 seconds’ intervals. For the measurement of the locomotion with video recordings, the fly positions were tracked every second. The locomotion was then defined as the displacement of the fly in mm between two consecutive time points (for tracking algorithm see...
Chapter 2.3.1). For both methods, the measurements were done in an environmental chamber (Percival Scientific, IA) maintained at 25°C and 70–80% relative humidity, unless otherwise stated. Light was produced by our control electronics as described in Chapter 2.2.1.

2.3 Data collection and analysis.

2.3.1 Fly tracking.

Videos were processed and analyzed using the custom-written code in MATLAB (MathWorks, Inc.). Flies were recorded at ten frames/second and their locations detected every second using background subtraction (Fig. 21 A). The background frame is created for each video from the first (reference) frame. To create background frame, ten additional frames are chosen throughout the video with approximately same intervals, (Duration of video/11) and are compared to the reference. The flies in the video are represented by pixels, which a darker than the background. So, to extract the flies from the reference frame, the frame is consecutively compared to additional ones and if it has a darker pixel in a place where the additional frame has a brighter one, the pixels are exchanged. As a result, the reference frame without flies is created. The number of ten additional frames is chosen to reliably extract all the flies in case if some flies accidentally appear in the same place in multiple frames. Next, the background frame is used to extract the flies in the tracking frames.

The background subtraction is sensitive to noise and any instabilities in video recording, so multiple steps are taken to eliminate noise and correctly find flies in
frames. First, each analyzed frame undergo video stabilization processing to eliminate effects of vibrations or shifts in setup. A feature point matching method (MATLAB) is used, which automatically finds salient (by contrast) points in video frames and uses them as anchor points to cancel out perturbations.

After stabilization, the background subtraction is applied leaving the only binary difference between background and tracking frames (Fig. 21 A,B), which next amplified linearly (Fig. 21 C). The resulting binary image then is filtered to eliminate noise, leaving only closed objects corresponding to flies (Fig. 21 D). First, all objects with an area smaller than 1/3 of fly size and all object with an area bigger than 2x size of a fly or length more than the 2x length of a fly were deleted. Next binary masks were applied to specify only regions with tubes and limitation of maximum one object per tube is implemented. In the end, only one object, which corresponds to a fly, per tube was left. To validate the method, 50 random frames from every experiment were analyzed by eye and fly positions compared to the ones from computer tracking. Multiple parameters, defined by the user, are used to adjust the software to the video:

1) an estimated size of fly – determined from a single video frame. As the videos for different experiments were taken at various distances from the tubes, the size of flies varied.

2) The tracking frame/background frame difference amplification coefficient – determined by the amount of noise. The bigger coefficient resulted in more definite fly-objects but increases the noise level.

3) Dimming coefficient – determined by fluctuations in background illumination. Dimming coefficient allowed to adjust if the intensity of the background
illumination in the tracking frame was different from the one in the background frame.

4) Stabilization threshold – determined by a number of matching point features. The stabilization threshold defines the contrast level for consideration of matching points and is required for precise aligning of frames. After adjusting the four parameters, the software was able to track all flies and no discrepancy was found between manual and automatic fly tracking.

Figure 21. Example frames of automatic tracking of flies in tubes with filters. (A) First, the background was obtained using multiple frames to extract flies. (B) Tracking frame with flies was stabilized to reduce noise and align tubes with the ones in the background. (C) Background subtraction was applied to tracking frame after stabilization. (D) Flies (green objects) were detected following digital filtering and noise elimination. For more details see text.

2.3.2 Locomotion.

Data for locomotor activity from the DAMs and video recordings were visualized and processed with custom written MATLAB (MathWorks Inc., MA) codes. The
locomotor activity in DAMs is calculated as the number of times the fly crosses IR beam per 20 seconds intervals (for details see Chapter 2.2.4). For the analysis of single fly locomotion, the activity was further binned over 3-minute intervals. For the population-level analysis data was binned over 30-minute intervals and averaged over a total number of flies. The line or bar actograms were plotted as activity in units of a number of beam crosses over time intervals versus time (Fig. 22 left). The data from the actograms were used for measuring activity levels, detecting circadian periodicities using power spectrum analysis and bursts in activity.

2.3.2.1 Power spectrum analysis.

The Lomb-Scargle (LS) method was used to calculate power spectrum of fly locomotion (Lomb 1976; Scargle 1982). The method has several advantages over the more commonly used fast Fourier transform algorithms in that LS can be used with unequally spaced data, produces a higher resolution power spectrum and allows for the calculation of the statistical significance of peaks in power spectrum (Ruf 1999). Traditionally, the power spectrum of time series data calculated using Discrete Fourier Transform (DFT). In this method, the periodicity of the signal is analyzed by trying to fit it with a linear combination of sinusoidal curves. The DFT transforms the signal from time domain time series \( X \equiv \{x(t_i), i = 1, 2, \ldots, N\} \) to frequency domain periodogram \( S(\omega) \). The \( S(\omega) \) is given by:

\[
S(\omega) = \frac{1}{N} \left( \sum_{i=1}^{N} x(t_i)e^{-i\omega t_i} \right)^2
\]
where \( \omega \in \left\{ \frac{2\pi k}{N\Delta t} \mid k = 0, \ldots, \frac{N}{2} \right\} \) are discrete frequencies, \( N \) is the number of samples in data, and \( \Delta t \) is the sampling time interval.

For LS method, Lomb used a similar to DFT approach of fitting signal with sinusoidal functions but used a least-square fitting. Next, Scargle adjusted the sample times \( t_i \) with a time delay \( \tau \) for an unequally separated time series. The resulting LS periodogram (Fig.22) \( S(\omega) \) is calculated using the equation:

\[
S(\omega) = \frac{1}{2} \left\{ \frac{\left[ \sum_i^N x(t_i) \cos \omega(t_i - \tau) \right]^2}{\sum_i^N \cos^2 \omega(t_i - \tau)} + \frac{\left[ \sum_i^N x(t_i) \sin \omega(t_i - \tau) \right]^2}{\sum_i^N \sin^2 \omega(t_i - \tau)} \right\}
\]

where \( \tau \) is defined by

\[
\tan(2\omega \tau) = \frac{\sum_i^N \sin 2\omega t_i}{\sum_i^N \cos 2\omega t_i}
\]

In addition to periodogram, Scargle suggested an easy way to test the statistical significance of periodicities in calculated periodogram. Scargle had shown that for the Gaussian noise, the probability density function of the \( S(\omega) \) is an exponent, therefore, the probability of the \( S(\omega) \) at frequency \( \omega \) to be below threshold \( \Lambda \) is given by the formula:

\[
P[S < \Lambda] = 1 - e^{-\frac{\Lambda}{\sigma^2}}
\]

The periodogram is in a discrete form with \( N \) independent frequencies \( \{\omega_1, \omega_2, \ldots, \omega_N\} \), then the probability that all \( S(\omega): \{S_1, S_2, \ldots, S_N\} \) are all below the threshold \( \Lambda \) is:
\[ P[S_i < \Lambda, i = 1, 2, ..., N] = \left(1 - e^{-\frac{\Lambda}{\sigma^2}}\right)^M \]

Then the false probability, which is the probability that the periodogram for noise will have at least one \( S(\omega) \) above the threshold \( \Lambda \) will be:

\[ P[S_{max} > \Lambda] = 1 - \left(1 - e^{-\frac{\Lambda}{\sigma^2}}\right)^M \]

The variable \( M \) depends on a number of data points and sampling intervals and if the sampling intervals at least two times shorter than the tested periods, \( M \) can be set to be equal to the total number of data points \( N \). At last, in biological studies, the false probabilities used for the test of the significance of data are \( \alpha = 0.05, 0.01, 0.005 \). The thresholds for those probabilities can be calculated using:

\[ \Lambda = -\ln \left(1 - (1 - \alpha)^{\frac{1}{M}}\right) \]

In this work, we utilized a publicly available implementation of LS (http://www.mathworks.com/matlabcentral/fileexchange/22215-lomb-normalized-periodogram).
Figure 22. Example of activity data with power spectra analysis. (Left) The actogram shows a normalized average activity of 16 wild-type flies in 12:12LD cycle. Each row shows two days and for row 2 and 3 first day is the repeat of the second day from the previous row. The white/grey background shows day/night in LD. (Right) Lomb-Scargle periodogram shows a strong peak at 12 hours period, representing 12-hour separation between peaks of activity.

2.3.2.2 Burst detection.

The DAM locomotion recordings (see Chapter 2.2.4) of an individual fly revealed presence of bursts of activity. The activity binned over 3-minute intervals was found to be an optimal duration that avoided oversampling but remained the more detailed features. The bursts were identified as single bins with the largest number of activity counts. Occasionally, multiple bursts appeared within ~10 min window, then an average bin was calculated without significant error to burst count or timing. If the burst appeared within 5 minutes of light transition, it was assumed to be the startle response to the changing light and not counted. The number of counts in one bin to consider it to be a burst was ten standard deviations above mean activity.

2.3.3 Color preference.

For color preference, user-defined binary masks corresponding to each color filter (Fig. 23B) were applied to background-subtracted images to determine fly
position relative to the color zones (Fig. 23C). In the situation, when a fly was positioned between two filters with parts appearing in both, the bigger part of the fly was counted, but only if it was bigger than 1/3 of estimated fly size. The number of flies in each color was divided by the total number of flies to calculate fractions at each time point (Fig. 23E). These data were typically averaged over 1-hour interval to produce the time-dependent patterns in color preference (for example, Fig. 23F).

Figure 23. Color preference calculation procedure. (A-C) User-Defined binary masks were applied to the detected flies in each frame to find which flies were in what color zones. Panel C shows an example of flies detected in blue zones. For details on fly detection see Chapter 2.4.3.1 (D) Example of 12 randomly picked single fly trajectories, moving between green (G), blue (B) and red (R) zones over 24 hours. Fly positions were determined every minute, but data shown are 5 minutes apart for clarity. (E) Population-averaged color preference of the 12 flies. The number of flies in each color was divided by total number of flies and the fraction shown with the line of the corresponding color. As with the individual data, the time interval here is 5 minutes. (F) Same color preference but averaged over 1-hour intervals.
2.3.3.1 Green preference peaks.

The green preference peaks were determined from the color preference patterns when the fraction of flies in green color zones was above 1/2 while fractions in red and blue about or below 1/3.

2.3.3.2 Avoidance and preference calculations.

In the three-color assay, if 1/3 fraction of flies was positioned in a color zone, the preference was concluded to be neutral. For values from 0 to 1/3, the color was assumed to be avoided, for values from 1/3 to 1 color was assumed to be preferred. For blue avoidance in the three-color-choice assay, the fraction of flies in the blue zone was compared to the fraction of flies in the other two colors combined, according to

\[
\text{Avoidance index} = \left( (A - B) - \frac{1}{3} \right) \times \frac{3}{2} = 1 - 3 \times B
\]

where \(A\) is the total fraction of flies in green and red zones and \(B\) is the fraction of flies in the blue zone. The simplified expression with only \(B\) follows from the normalization constraint \(A + B = 1\), at all times for these three-colour experiments. Defined this way, the Avoidance index = 1 if there are no flies in blue, and 0 if 1/3 of the flies are in blue. The avoidance index was calculated for each hour from ZT 2-10, then averaged. When only two colours were involved, the standard Preference index = \(A - B\) was used, where \(A\) and \(B\) are fractions of flies in two colors.
Chapter 3 Results.

3.1 A transient burst of activity in *Drosophila* locomotion data.

Locomotor activity measurements using DAMs provide an excellent way to study the effect of light on fly behavior. Under 12:12LD light cycles, the population-averaged recordings reveal two peaks of activity – morning (M) and evening (E) peaks (Fig. 10). On a large timescale of circadian clock studies, the small details of activity are usually disregarded. The data is typically binned in up to 30 minutes intervals to reflect major changes in timings of activity. Averaging over population of flies also refines the data from any random or non-periodic events. We wondered, however, if there are important features in locomotor data, which are missed due to population-level activity analysis.

Our examination of an individual fly activity showed the presence of unexpected bursts of activity, which appeared regularly, but stochastically and lasted only 2-3 minutes (Fig. 24A). Even though bursts appeared to be a more prominent feature than the M and E peaks, the inconsistency in bursts’ timings led to a weak autocorrelation, in comparison to that of the M peak (Fig. 24B). The activity bursts were detected in three different control strains (*iso31*, *yw*, and *2U*) with roughly same frequency. The frequency of the bursts increased with the light intensity, with up to ~85% of flies (N=32 for each strain) showing the burst at ~8000 lux illumination (Fig. 24C). Consistent with the correlation of frequency of bursts' occurrence and the light intensity, the burst was rarely observed in the dark phase of LD.
Figure 24. Individual flies show a significant burst of activity after the start of morning activity. (A) Examples of activity (3 minutes bins) for two different flies with midday light intensity of 8000 (left) and 4000 (right) lux. Morning/evening peaks denoted as “M” and “E” respectively, the burst of activity as “B”, delay of B peak after the light turns on as $\Delta t$ (activity in black columns and light patterns in black line on top). (B) Autocorrelation functions for the stochastic burst (left) and the periodic M peak (right) for a fly measured in LD for six days. (C) A fraction of iso31 and yw flies (N=32 for each), which displayed the burst at various midday light intensities. Grey dashed curve is a guide to the eye and not a fit to the data.

Further analysis suggested that there are two bands of bursts, one band appears ~2 hours after lights turn on and a second band appears ~10 hours after lights turn on (Fig. 25A). Compared to the first band, the second band consists of 20% fewer bursts which are spread sparsely over a time window that is roughly twice as long (standard deviations of the two bands, 1.3 hours and 2.7 hours, respectively, Fig. 25B). Even though the two bands might be triggered by the same mechanism, they have significant differences in frequency and temporal distributions. For simplicity, we focused mainly on the more frequent and denser first band of bursts. Regardless of light intensity, the activity burst consistently appeared after the start of the day with an average delay $\Delta t \sim 2.3$ hours (Fig. 25C). The magnitude of the bursts also did not depend on the light intensity and on average was ~5 times larger than the M peak (Fig. 25D).
Figure 25. The appearance of bursts during the light-dark cycle. (A) Examples of delay, $\Delta t$, between the burst and the moment light turns on for 32 flies measured for 7 days. The first group of 16 flies is iso31 and the remaining 16 are yw. White and black bar on the right shows light/dark conditions at $\Delta t$. (B) Distribution of $\Delta t$ calculated for iso31 flies (N=159). Bursts are clustered into two groups, one centered ~2 hours and another ~10 hours. The standard deviation for the first group $\sigma_1 = 1.3$ hours, and for the second $\sigma_2 = 2.7$ hours. (C) The onset of the burst after lights on for iso31 and yw for different midday light intensities. $\Delta t \approx 110 - 150$ minutes on average, independent of light intensity and genetic background. (D) Ratio of the burst activity to the average morning activity for iso31 and yw at different midday light levels. Average value of the ratio ~5 for iso31 and ~6 for yw. (C-D) iso31 (N=32) shown with black diamonds and yw (N=32) shown with grey diamonds.
3.1.1 The burst requires light but not the circadian clock.

Since in a 12:12LD light cycle the burst appears periodically, our experiments suggested a possible role of the circadian clock in timing the activity burst. To test this hypothesis, the activities of the \textit{per}^{0}, \textit{dbt}^{AR} and \textit{cry}^{01} mutants were measured. PER is a core clock protein, which levels oscillate with a \(~24\)-hour period (Chapter 1.3.1). DOUBLETIME (DBT) is a kinase, which is involved in circadian clock regulation (Hardin 2005). Ablation of \textit{period} and \textit{doubletime} genes in \textit{per}^{0} and \textit{dbt}^{AR} flies leads to a non-functional clock. CRY is a photopigment, which mediates entrainment of the circadian clock to external light (Chapter 1.3.3.3). A null mutant of \textit{cry} gene \textit{cry}^{01} has a severely reduced ability to entrain activity to light cycles. However, \textit{per}^{0}, \textit{dbt}^{AR} and \textit{cry}^{01} all showed the burst with main features unmodified: the delay $\Delta t\sim0.5$-5 hours after lights turn on (Fig. 26A-C, left panel, arrows), and the magnitude of \sim10-12 times of basal levels (Fig. 26G, arrow and maximum in distribution). The bursts also occurred in data as frequently in circadian mutants as in controls (Fig. 26H).

\textbf{Figure 26.} Light/dark experiments with \textit{Drosophila} mutants suggest light is required for activating burst in locomotion. (A) \textit{per}^{0}, (B) \textit{dbt}^{AR}, (C) \textit{cry}^{01}, (D) \textit{hdc}, (E) \textit{ninaE}, and (F) \textit{norpA} under 12:12 LD conditions. Single fly (left, 3 minutes bins) and population averaged (right, 30 minutes bins). (A-C) Black arrows indicate bursts. (G) The probability distribution of the 3 minutes bins in activity. Grey line – \textit{iso31} (N=25, for 5 days), cyan – \textit{per}^{0} (N=14, for 5 days), green line - \textit{dbt}^{AR} (N=16, for 2 days), orange line – \textit{cry}^{01} (N=32, for 3 days), violet line – \textit{hdc} (N=16, for 5 days), black line – \textit{ninaE} (N=10, for 5 days), and pink line – \textit{norpA} (N=8, for 2 days). Corresponding arrows show the average value of activity burst in each population. (H) Percent of flies per day that showed burst under 12:12LD. Stars denote significant difference compared to \textit{iso31} control flies (p<0.05, Mann-Whitney-Wilcoxon test). For \textit{norpA}, a same number of flies showed bursts each day, providing insufficient statistics for error bars.
The independence of the burst from the circadian clock only increased the possibility that the light is the major driver of the phenomena. To support this hypothesis, the activity of control flies under non-periodic light pulses of different durations was measured. The light pulses were set to random lengths between 1 and 12 hours with the maximum light level of 8000 lux (Fig. 27A, top). The bursts can occur up to 5 hours after the light turns on, so when the light pulses were shorter than that, they were followed by a minimum of 5 hours of darkness. However, only ~40% flies showed the burst after a 1 hour-long pulse (Fig. 27A and B, top). As the duration of the light pulse increased, so did the frequency of bursts, with a maximum of ~85% for ≥8 hours of illumination (Fig. 27B, top). The data also showed that the frequency of burst occurrence did not depend on the duration of the light-off period (Fig. 27B, bottom). These results offered an additional support for the hypothesis of light being an essential driver of the burst.

Since light is required for triggering the burst, disruption of fly phototransduction pathways should lead to decrease in the frequency of bursts. Two visual mutants, partially blind mutant ninaE17 that lacks rhodopsin Rh1 and blind mutant norpA P24 with interrupted phototransduction pathways were tested and showed significantly lower frequencies of the burst (Fig. 26H). In addition, Mi{ET1}HdcMB07212 (called hdc in this thesis) flies, which have impaired light transduction owing to depleted histamine levels, also showed reduced frequency of bursts (Fig. 26D, H) despite showing increased levels of basal activity compared to other strains (Fig. 26D, right-shifted maximum in the violet curve). The suppression of burst in the hdc animals coexisting with increased locomotion suggested the burst has a weak
correlation to activity. Interestingly, hdc flies showed wildtype M and E peaks in LD cycles, implying that sufficient light was available to drive their M and E cells but not those responsible for the burst (Fig. 26D, right panel). These results suggested that light is a major driver of the observed burst in fly activity.

Figure 27. Experiments with variable light pulses show a correlation between duration of “day” and occurrence of the burst. (A) Light pattern with various durations of day and night (top) and examples of activity profiles for iso31 (middle) and yw (bottom). (B) The percentage of flies, which show the burst increases monotonically with increasing length of day (top), but remains roughly constant with changes in the length of night (bottom). iso31 shown in white triangles and yw in black circles, grey dashed curve is a guide to the eye, top and bottom panels.

Using a traditional experimental method but the non-traditional analytical approach, we were able to find a novel behavioral trait – the activity burst – in Drosophila locomotion. Conditional analysis revealed a light-induced nature of the burst and pushed us to further investigate how the light affects the behavior of fruit flies. Particularly, does light of different spectrum promote different response and
if this response changes with time? To address this question, we looked at the activities of flies in LD conditions with light of different colors.

### 3.2 Effect of the color of light on *Drosophila* locomotion.

The activity bursts are dependent on light, and their occurrence correlates with the light intensity. However, properties of light are not limited to intensity but also the spectrum. The frequency of bursts was found to be higher under light produced by white LEDs in comparison to fluorescent light of similar illuminance. *Drosophila* possesses multiple photoreceptors with distinct spectral sensitivity, and it is possible that the mechanism that triggers bursts is attuned to a certain spectral range of light. This notion raised a question if the spectral composition of light affects the locomotor activity of flies. Previously, the role of the color in light-driven behaviors have already been shown in mammals and insects. In mice, light can promote sleep or wakefulness depending on the color: green light increases sleep latency while blue light reduces it and also promotes aversive response (Pilorz et al. 2016). In humans, the color of light can modulate brain activity, alertness, and cognition (Lockley et al. 2006; Vandewalle et al. 2007; Vandewalle et al. 2010). In insects, the phototactic behavior (movement towards or away from light) have been shown to depend on the color of light (Hanai, Hamasaka, and Ishida 2008).

To study the effect of light color on the activity of *Drosophila*, locomotor activity was measured under 12:12LD with white LED light transmitted through color filters (Fig. 20 A). The colors for the transmitted light were chosen based on the maximum sensitivity of the *Drosophila* photoreceptors within the range of the LED spectrum. Blue and green filters transmitted at peak sensitivity of Rh5 (~440 nm) and Rh6
(~515 nm) respectively (Table 1 and Fig. 5C). Additionally, a red filter with transmission above ~590 nm and yellow with transmission above ~500 nm were used to test the effect of longer wavelengths. For comparison, a combination of overlapped blue, green and red filters were used to almost completely block white light (shown as dim in graphs). The locomotor activity data were calculated from the video recording by tracking fly positions and measuring distance traveled by flies over 3-minute intervals. The resulting distances were plotted versus time in a fashion similar to actograms (Fig. 28). The data revealed that blue light affects morning and evening peaks of activity in opposite ways. The M peak was significantly higher and lasted longer than in dim light. However, the E peak was reduced. In contrast, flies under green illumination showed less activity, with both M and E peaks reduced. The activity during “siesta” time was almost completely absent. Interestingly, flies under dim light conditions displayed normal bimodal activity.
Figure 28. Activities of flies in 12:12LD with light of different colors. Two days of activity for wild-type flies (CS-1, N=24 for each) in colors from top to bottom: dim white light, blue (peak at 450 nm), green (peak at 528 nm), yellow (includes both green and red peaks) and red (peak at 620 nm). Morning/evening peaks denoted as “M” and “E” respectively. Black/white bars indicate light and dark phases of LD cycle, the time is shown in Zeitgeber Time (ZT) with light turning “on” at 0 and turning “off” at 12.
Extremely high sensitivity of flies to light has been shown previously, suggesting that some light still passes through overlapped filters (Bachleitner et al. 2007). Flies in red color light showed similar activity to the one under dim light. It is possible that Rh1 and Rh6 have some sensitivity at shorter red wavelengths and are responsible for the activity pattern in dim and red light. However, at such low intensities, the photoreception most likely becomes scotopic and is not sensitive to the color of light. The entrainment of the circadian clock by red light was also suggested previously with Rh1 playing a major role (Hanai, Hamasaka, and Ishida 2008). However, flies in yellow light, which included wavelengths in both green and red region, showed activity similar to flies in green color, indicating that the perception of green had overridden the red sensitivity.

Next, the activity was quantified by calculating the average distance flies traveled per minute in each color during light and dark phases of 12:12LD. As expected from the actogram, flies in the green and yellow colors had reduced activity, but flies in dim, red and blue showed similar levels (Fig. 29, left). The increased morning activity of flies in blue color was compensated by reduced evening activity. As a result, on average activity in blue was similar to that in red and dim. During the night all flies in all colors had similar average activity (Fig. 29, right).
Figure 29. Average activity of wild-type flies in colors during day and night. Bars of corresponding colors show activity in terms of average distance traveled by one fly per minute, black bar corresponds to activity in dim light. Error bars show standard deviation between activities of different flies (N=24).

Since the activity in our assay is measured as average distance moved over unit of time, the difference in activity between colors could have resulted from the change in overall time spent active or change in how much flies moved during the active state. The time was divided into 3-minute bouts and the average speeds (mm/min) during these bouts calculated. If the fly did not move more than half body size, it was considered resting. The analysis showed that the reduced activity in green and yellow colors during the day was primarily the result of slower speed (~2 times less than in dim light) and only secondly due to the amount of time spent resting (Fig. 30A, B). However, during the night all flies rested significantly more (Fig. 30D bottom), which led to lower average activity. Therefore, the changes of average activity during day and night are of different nature.
Figure 30. The discrepancy in average activity between colors is caused by differences in speeds. Flies in green and yellow colors have reduced activity primarily due to lower average speeds. (A, C) Distributions of speed bouts during the day (A) and night (C). Activity data were binned into 3-minutes bouts, and for each bout, average speed was calculated by dividing total distance by bout duration. (B, D) Comparison of median speeds (top) and total active times (bottom) between colors for the day (B) and night (D).

Flies in green and yellow have on average slower speeds than in blue, red and dim. The distributions of bouts over speeds of flies appeared to be a combination of Poisson and multimodal distributions (Fig. 31, left with cartoon). However, the
contribution of the multimodal component was not the same for different colors. Green and yellow distributions showed close to exponential decay which can be expected from random data (Fig. 31, left). Interestingly, the speeds of flies in dim and red colors showed a strong contribution of peaks. This peaks correspond to values of \( \text{length of tube/ min} \times \text{multiplier} \). \( \text{length of tube} \) with food and cotton is \( \sim 43 \) mm, and multipliers were from one to four. Therefore, the peaks were at values 43 mm/min, 86 mm/min, 129 mm/min and 172 mm/min. While the speed distribution of flies in dim and red had all four peaks, flies in blue had a single peak around 86 mm/min (2 tube lengths) (Fig. 31, left). Distributions of speed were significantly modified during night with a major contribution from the peaks at 86 and 129 mm/min (Fig.31, right). Strangely, flies in yellow had the strongest contribution. Difference in speed distributions between colors can potentially hint at the effect of colors on fly behavior. Previously, studies of sleep in mice suggested a sleep-promoting effect of green color light and stressful arousal-promoting effect of blue color (Pilorz et al. 2016). Our results suggest that, the distribution in blue could indicate a stressful or uncomfortable light condition while distributions in green and yellow - calming conditions.
Figure 31. Distributions of average speeds during the day (left) and night (right). The 3-minute activity bouts were clustered by the average speed from 1 to 300 mm/min, binned into 3 mm/min bins. The distribution then is plotted as a number of activity bouts at each speed bin, and for each color is shown in the corresponding color. The schematic cartoon shows a possible explanation of the distributions. Arrows with a number indicate values of 43 mm/min, 86 mm/min, 129 mm/min and 172 mm/min, which corresponds to length of tube/min × multiplier.

Flies in green and yellow colors showed significantly less activity than flies in dim with slower speed bouts, and flies in blue color had most of their activity concentrated in the M peak. This result indicates that the color of the light plays a significant role in shaping the activity profile of Drosophila. The effect of light color on Drosophila locomotion is not surprising since in nature, flies experience distinct colors of light due to the environment and the time of day. However, the only reported color specific behaviors right now are the phototactic paradigms and entrainment of the circadian clock (Fischbach 1979; Schumperli 1973; Hernández de Salomon and Spatz 1983; Menne and Spatz 1977; Ceriani et al. 1999; VanVickle-Chavez and Van Gelder 2007). While the paradigms, were able to help study visual system and color vision in Drosophila, they do not provide information about the response of flies to ambient illumination during the day. The photoentrainment of the circadian clock affects activity only when the external light
cycle and circadian clock are out of phase and does not affect already synchronized activity. While we can speculate about the meaning of the modifications in the activity due to the color of light, an assay where flies have a choice between colors of light for the entire day may give us a better understanding of how light color affects Drosophila behavior. To that end, we have designed a color-choice assay, where flies reside for multiple days in individual tubes with food and can choose to stay in multiple light colors. In the next chapter, we will report the results obtained using that assay.

3.3 Color preference of Drosophila throughout the day.

Most of the studies of the color preference in Drosophila use well-established paradigms of slow and fast phototaxis, which show that flies are attracted to the light over dark and prefer light of shorter wavelengths (Fischbach 1979; Schumperli 1973; Yamaguchi, Desplan, and Heisenberg 2010). In typical phototaxis experiments, flies are released in T-mazes, given two choices, and the number of flies in each option is counted immediately or shortly after the release. Sudden manipulation of flies and introduction them to a new environment with unfamiliar light conditions drives an escape or startled response. As a result, the effect of more slow-paced behaviors, such as sleep and feeding, on color preference cannot be observed. Interaction of color preference with the circadian clock also cannot be determined.

To address spectral preference of Drosophila during the day, the behavioral experiments were performed providing flies with choices of blue, green and red (red can also be associated with low intensity for fruit flies) colors throughout the
day (Fig. 32). In such experiments, flies are housed in individual glass tubes covered with three plastic color filters dividing tubes into three equal color zones. The filter positions are combined in all six possible ways to avoid bias towards food (see Chapter 2.2.3). To quantify the preference for colors, the number of flies in each color was counted every minute, using custom computer code. Next, the number of flies was averaged over 1-hour time intervals, providing the distribution of the flies between colors during the day (see Chapter 2.3.3).

Figure 32. Schematic of the experimental setup. Tubes with flies were wrapped in three color filters and placed in an environmental chamber under controlled conditions. Individual fly positions in tubes were recorded every minute for multiple days using a CCD camera. Broad-band white LED lamps simulate alternating LD conditions while infra-red lights permit CCD to continuously track flies (for details see Chapter 2.3.1).

From previous studies, we expected to see the highest preference for blue color and lowest for red. The analysis of color preference of CS-1 (further called wild-type) flies has shown an unexpectedly complex pattern in the behavior (Fig. 33A, B). Flies strongly avoid blue color during almost all light phase of the light cycle (Fig. 33C). In contrast, their preference between green and red changes with time: ~2 hours after the light turns on flies are attracted to the green color.
(Fig. 33D). Next, during the middle of the day flies shift their preference towards red or split between green and red in almost equal proportion (Fig. 33D). At last, approximately 1 hour before the light turns off flies return to the green color.

Figure 33. The distribution of flies in color zones under 12:12LD conditions. The fraction of flies in green, blue and red zones for wild-type during six days (A) and for four control genotypes during one day (B, w^{1118} flies have mini-white) are shown in the corresponding color line. Shaded band surrounding each line represents standard deviation in data from 3-6 independent experiments. Each experiment used 18 or 24 flies. Black and white bars indicate dark and light part of LD cycle. (C) Comparison of blue avoidance. The avoidance index was calculated (see Chapter 2.3.3.2) for each hour from ZT 2-10, then averaged. (D) Preference between red and green colors near timing of the first green peak ZT2 (Morning) and midday ZT6 (Midday). Preference for green is shown as positive and preference for red as negative.
The experiments were run for up to 2 weeks, and flies showed similar color preference pattern for the whole duration of the experiment with the exception of the first, and sometimes, second days. During first days fruit flies most likely were acclimating to new LD conditions, which can explain the deviation in color preference pattern. To ensure that the choice of colors was not driven by some positional rather than light stimuli, the LD conditions were followed by constant darkness (DD). In DD, however, flies immediately lost color preference and equally spread between colors (Fig.34A), indicating no position preference.

**Figure 34. The color preference pattern is driven by the light and not temperature.** Distribution of flies across color zones in 12:12LD conditions, followed by constant darkness (A) or constant light (B) (N=24, each experiment). The fraction of flies in green, blue and red zones shown in the corresponding color. Black and white bars indicate dark and light part of LD cycle. (C) Average temperature in the green, blue, and red zones, regardless of proximity to food or cotton plug. (D) Average temperature near food, near cotton and in the middle, regardless of filter positions. (C, D) Measurements were taken with lights turned off (grey bars) and after lights have been on for 10 hours (white bars). Error bars are standard deviations from 12 independent measurements.
Another possible driver of the pattern in color preference could be temperature variation. To test this possibility, the temperature was measured along the length of 12 tubes with filters in different combinations. No discrepancies were found between different colors and different positions inside the tubes, which reassures that thermotaxis does not affect fly choices of locations in tubes (Fig. 34C, D).

### 3.3.1 Color preference at various intensities.

The color preference pattern observed in our assay is at variance with previous phototaxis studies. However, the conditions in our experimental assay are very different, and one of the major differences is the intensity of the light. To test whether reduction of the light intensity would change the preference multiple experiments with different light conditions were performed.

First, flies were tested with light of overall lower intensity: light through each color was equally reduced. For each intensity level, a separate experiment was performed for one week with 24 flies. As the intensity was reduced, the height of the green peaks gradually got lower, but the blue avoidance decreased rapidly, and already at 67 μW/cm² was almost absent (Fig. 35). At ~1 μW/cm² the avoidance of blue reversed to preference, which was even stronger than the preference for green. This result is in agreement with the previous works on *Drosophila* phototaxis (Schumperli 1973; Yamaguchi, Desplan, and Heisenberg 2010) and shows that there are no specific alterations of our experimental setup, which would change the conventional response of flies. At extremely low intensities (<1 μW/cm²) flies still preferred light over dark, but lost any preference between green and blue colors, possibly switching to scotopic (twilight) vision.
Figure 35. **Color preference with reduced light intensity.** The intensity was reduced by step logarithmically: the first experiment had intensity 67 μW/cm², second 6.7 μW/cm², third 0.7 μW/cm², and fourth less than 0.1 μW/cm². The fraction of flies in green, blue and red zones for wild-type are shown in the corresponding color line. Shaded band surrounding each line represents standard deviation in data from four consecutive days. Each experiment used 24 flies.

In the second set of experiments, the intensity was reduced only through blue filters, using neutral density filters of different transmission power. The intensity was reduced to 50% (286 μW/cm²), 25% (143 μW/cm²), and 10% (57 μW/cm²) of original 572 μW/cm². It was found, that at 50% intensity flies still showed similar avoidance to that at full power, however, already at 25% the blue avoidance was nearly gone (Fig. 36). This result indicates that the blue avoidance is only driven by the high-intensity blue light, and there is some threshold in the behavior. However, the response to blue never reversed to preference, and the green peaks with midday switch to red preference were always present. This agrees with previous studies, where flies showed a preference for green when its intensity was significantly higher than the intensity of blue/UV light (Gao et al. 2008). Similarly, in our assay flies preferred blue when both green and blue light intensities were low, but still preferred green when only the blue light was low. Interestingly, even when flies were not avoiding blue color, they still showed familiar switch from green
preference to red in the middle of the day. This suggests that the response to green color is independent of the blue avoidance. Next, the role of the intensity in the variation of the green preference was addressed.

Figure 36. Color preference with reduced intensity through a blue filter. (A) Average daily preference between green, red and blue color light with 100%, 50%, 25% or 10% of maximum (572 μW/cm²) intensity through a blue filter. The fraction of flies in green, blue and red zones shown in the corresponding color. The standard deviation between multiple consecutive days is shown in a shaded band around each line. 18 or 24 flies were used for each experiment. Black and white bars indicate dark and light part of LD cycle. (B) Average avoidance of blue light at different intensities. The avoidance index was calculated (see Chapter 2.3.3.2) for each hour from ZT 2-10, then averaged.
In the third set of experiments, flies were measured in the two-choice assay with both options being a green color, but with different intensities. One option had full intensity while the other had intensity reduced via neutral density filters. We hypothesized, that if the green preference peaks and midday switch were due to the intensity of light rather than the color, flies would show the behavior for experiments, where one option is relatively dimmer. However, the pattern was found to be a combination of spectral and intensity preference (Fig. 37A). The two peaks of green preference only appeared when the second option had intensity 10-fold lower than that of the first option (Fig. 37A, B). At all higher intensities, flies did not show any preference during the typical timing of green preference peaks, indicating the behavior driven primarily by color. It is possible that at the lower intensities fly vision becomes scotopic (colors loose significance) and flies simply prefer brighter light. In contrast to green peaks, the midday switch was present in all experiments, indicating its intensity driven nature (Fig. 37A, C). The overall result was that flies prefer to stay in green color in the morning and late afternoon, shift/switch preference towards dimmer environment during the middle of the day and avoid blue light throughout the day.
Figure 37. Color preference in the two-choice assay with green lights of different intensities. (A) Average daily preference between two intensities of green color light with the second option being 100%, 50%, 25%, 10% or 4% of the intensity of the first (626 μW/cm²). The standard deviation between multiple consecutive days is shown in a shaded band around each line. 18 or 24 flies were used for each experiment. Black and white bars indicate dark and light part of LD cycle. (B) Average preference for green light of higher intensity in the first and last 3 hours of the day. Red line with shaded zone shows preference and standard deviation for green in the green vs. red assay. The abscissa shows the ratio of intensities in two choices in logarithmical scale. (C) Change in preference for green of higher intensity during first and last 3 hours of the day (M and E) and at ZT6 (Midday).

3.3.2 Locomotion in color-choice assays.

3.3.2.1 Average locomotion of flies in color experiments.

Flies change their preference for colors as the day progresses. Another commonly studied behavior that changes with time is fly locomotion, and it has some features that are reminiscent of color preference – the two peaks of activity. We were interested how the two behaviors relate and examined the locomotion of flies in the color preference experiments. Flies had shown a typical wild-type
locomotor behavior with morning and evening peaks of activity near “on” and “off” transitions of light and low activity in the middle of the day, known as “siesta” (Fig. 38). When compared to the color preference in time scale, the green peaks of activity occur at the end of the M peak and shortly before the E peak. The switch in green preference happened during the “siesta” time and did not have a strict locomotor feature.

![Figure 38. Correlation between color preference (top) and locomotor activity (bottom, black line) averaged in 3 min intervals.](image)

Locomotion of flies in color experiments reminded of that in experiments with white light. However, the choice of ambient color can produce a more fine effect on fly locomotor behavior. The activity of flies in color experiment was compared to the activity in an experiment with white light, which intensity was adjusted to match the average irradiance of the light through filters. Average irradiance
transmitted through the filters was 590 μW/cm². This value is comparable to the irradiance of white LED light with an illuminance of ~2000 lux. The peaks of activity in two experiments were found to be very close in both height and width (Fig. 39). Interestingly, the activity during the “siesta” was significantly lower (~70% less between ZT4 and ZT8) in color experiments in comparison to experiments with white light, with the morning and evening activities ending/starting more abruptly. As seen from the green preference peak, it is possible that with the option of green color, flies can stop their activity earlier, and rest in green. Similar behavior has been seen in our experiments with light of only one color (Chapter 3.2), where flies with green illumination had significantly less activity.

Figure 39. Average activity of flies in tubes with color filters (black line) and without filters (orange line) under 12:12LD. Light intensity was set at 2000 lux for tubes without filters, which is similar to the intensity of light transmitted through the filters in the color preference experiments (see Chapter 2.2.4). Average activity was calculated as the distance moved by individual flies between consecutive video frames (captured at 1 frame/sec) and binned over 3-minute intervals. Black and white bars indicate dark and light portions of LD cycle.
3.3.2.2 Individual locomotion of flies in color experiments.

The difference in locomotion between experiments with colors and white light suggests light color affecting the behavior of flies. To test this idea, individual flies’ locomotion and positioning in tubes were analyzed. The experimental setup involves six possible combinations of three color filter with food on one end of the tube and cotton plug on the other. Visual observations of videos showed that after the morning period of intense activity flies rapidly shift to almost complete tranquility, stopping primarily in green color zones. Next, flies shift their positions towards closer proximity to food with one exception, the blue color is still avoided. As can be seen in Fig. 40 depending on the position of colors in tubes, the behaviors of flies are different. When the blue zone is in the middle, flies almost exclusively stay in the zone next to food, regardless if it is green or red (Fig. 40A, F). In these situations, flies often spend all the time right next to food. When the green zone is next to food and next to it is red, flies spend time in both colors (Fig. 40E). When the red zone is next to food and next is green, flies mostly stay in red (Fig. 40C). And lastly, when the blue is next to food, flies spend most of the time in the middle zone, often right on edge to the blue (Fig. 40B, D). It seems that flies are trying to be near the food but stay outside the blue zone, and make brief intrusions into blue to feed. The fact that flies prefer to stay away from food to avoid blue light indicates how strong of the aversive stimulus the blue color is. Next, through measurements of various mutants, we addressed the possible sensory systems and pathways that might mediate the color preference behavior.
Figure 40. Flies tend to stay near food in the middle of the day. (A-F), Examples of the fly positions during ZT3.5 to ZT8.5. Flies tend to stay near food when a green or red filter is nearest (A, C, E, F). When a blue filter is nearest to the food (B, D), flies enter the blue zone only briefly to eat. Ordinates represent the coordinate along the tube length. Horizontal orange lines represent edges of the color zones.

3.3.3 Color preference of white-eyed flies.

Wild-type fruit flies have red and brown pigments pteridines and ommochromes in their ommatidia, which prevents light from leaking into the neighboring ommatidia. That screening protects photoreceptors from the excessive light intensity and flies that lack pigmentation in their eyes have a defective vision (Cosens and Briscoe 1972). Vertebrate albino mutants, which lack melanin and do not have pigmentation, experience similar vision defects. In Drosophila, the white gene encodes ATP-binding cassette transporter, which carries red and brown pigments to the eye during development. Knockdown of this gene leads to non-colored (white) eyes, an important phenotypic marker. White mutants are often used as a control background in genetic experiments. To see if the lack of pigmentation affects color preference, two white eyed mutants $w^{118}$ and iso31
were tested. Both mutants displayed an abnormal pattern in color preference. The color preference pattern did not show the first green peak, and instead, flies preferred red from the start of the day (Fig. 41). That preference for red continued through first half of the day but still switched to the green peak in the late afternoon.

**Figure 41. Color preference of white-eyed flies.** The fraction of flies in green, blue and red zones shown in the corresponding color. The standard deviation between multiple consecutive days is shown in a shaded band around each line. 18 or 24 flies were used for each experiment. Black and white bars indicate dark and light part of LD cycle.

The disappearance of the first green peak can be explained by light being too bright. However, the remaining second green peak indicates that either the perception changes with progression of the day or the attractiveness of the green color in the afternoon is higher. Understanding of the primary driver of the change in color preference will require additional measurements and will be discussed later. However, the white-eyed fly behavior further indicated the importance of the visual system. Next, we tested color preference in several blind mutants.

**3.3.4 Color preference of the blind mutants.**

Like most organisms, *Drosophila* perceives light information mainly through the visual system. Extensive genetic studies on *Drosophila* have generated a detailed
understanding of the phototransduction pathways and the development of vision in fruit flies (Paulk, Millard, and van Swinderen 2011; Zhu 2013; Yamaguchi and Heisenberg 2011; Morante and Desplan 2008; Desplan 2001; Behnia and Desplan 2015). A large collection of mutants with knockdown genes involved in vision allows studying roles of visual organs, and even individual rhodopsins in the color preference behavior. To find if the light information for the avoidance is transmitted through the visual system, three blind mutants were tested: \textit{gl60j}, \textit{norpA}^{p24}, and \textit{GMR-hid}. \textit{gl60j} are flies with a null mutation of \textit{glass}, a gene that is necessary for the development of retinal photoreceptors, ocelli and Hofbauer-Buchner (H-B) eyelets (Bernardo-Garcia et al. 2017). In addition to the visual system, \textit{glass} is expressed in a subset of clock neurons DN1p\(_s\), and the mutation eliminates those neurons (Head et al. 2015). \textit{GMR-hid} are flies with proapoptotic gene \textit{head involution defective} (\textit{hid}) expressed under control of \textit{Glass Multimer Reporter} (\textit{GMR}) in all visual photoreceptor cells. Similar to \textit{gl60j} these flies do not develop normal compound eyes, ocelli, and H-B eyelets, but have clock neurons intact. Third mutant \textit{norpA}^{p24} are flies with a null mutation in phospholipase C-\(\beta\) encoding gene \textit{no receptor potential A} that disrupts phototransduction cascade (Randall 1996). In addition to blind mutants, a strain lacking only ocelli (\textit{ctn,oc01}) was tested. Surprisingly, for all blind and ocelli-less mutants, the blue avoidance was near wild-type level (Fig. 42A, B). This result raised a question if there are other than visual photoreceptors, which are primary mediate blue avoidance.
Figure 42. Color preference of blind and ocelliless flies. (A) The fraction of flies in green, blue and red zones shown in the corresponding color. The standard deviation between 3 independent experiments is shown in a shaded band around each line. 18 or 24 flies were used for each experiment. Black and white bars indicate dark and light part of LD cycle. (B) Comparison of blue avoidance. The avoidance index was calculated (see Chapter 2.3.3.2) for each hour from ZT 2-10, then averaged. (C) Preference between red and green colors near timing of the first green peak ZT2 (Morning) and midday ZT6 (Midday). Preference for green is shown as positive and preference for red as negative.

While blue avoidance was not reduced, the peaks of green preference did not appear for blind mutants, and the fractions of flies in green and red colors were always similar (Fig. 42A, C). This indicated that the green color was perceived through the visual system and flies lacking visual organs did not see the difference between red (dark) and green. As expected, the ocelliless fly still showed close to normal green peaks (Fig. 42A, C).
The red color is outside of sensitivity of all *Drosophila* rhodopsins and can be assumed as a dark or dim without color specificity. Then in terms of overall preference, one can assume:

$$Green > Red(Dark) > Blue$$

For the blind flies than one can say that green and red are equal:

$$Green = Red(Dark) > Blue$$

Green and red seem indistinguishable for blind flies in the three-color choice assay. However, the strong aversion from blue might affect preference of flies between green and red

$$? \quad Green = Red(Dark) > Blue$$

Similarly, the green preference peaks in wild-type flies' color preference could be affected or even somehow generated due to blue avoidance. To see if the green preference, or in case of blind mutants, neutrality, was affected by avoidance of blue color, flies were tested in green/red two-choice assay.

The blue avoidance, in turn, can also be affected by the preference for green. During green preference peaks almost all flies go to green, however, if the green color was not available, would flies go to red or blue?

$$? \quad Green = Red(Dark) > Blue$$
To see if the avoidance of the blue color does not require the presence of green option flies were tested in blue/red two-choice assay.

3.3.4.1 Wild-type and blind mutants in green/red two choice assays.

For the two-color choice assays, the same setup was used as for three colors, with the only difference that this time tubes were wrapped in a combination of two colors. First, wild-type flies were measured and found to have similar color preference features: two green preference peaks in the morning and late afternoon, separated by the switch to red preference in the middle of the day (Fig. 43A, B). The midday switch showed a higher preference for red relative to green than in the three-color assay. This difference can be explained by the design of the experimental setup: flies could be reluctant to move from green to red when these colors are separated by blue. Indeed, locomotion of individual flies (Chapter 3.3.2.2) supported this hypothesis. The red color was preferred over green when they were not separated by blue. Whenever red was closer to food than green, flies consistently stayed in red (Fig. 40B, C), but when green was closer, flies still spent considerable time in red (Fig. 40D, E). Flies consistently stayed in green only when it was near the food and next was blue (Fig. 40A). This would lead to an artificially increased fraction of flies in green.
Figure 43. Color preference of blind and ocelliless flies in green/red assay. (A) The fraction of flies in green and red zones shown in the corresponding color. The standard deviation between 3 independent experiments is shown in a shaded band around each line. 14 or 24 flies were used for each experiment. Black and white bars indicate dark and light part of LD cycle. (B) Preference between red and green colors near timing of the first green peak ZT2 (Morning) and midday ZT6 (Midday). Preference for green is shown as positive and preference for red as negative.

Next, blind mutants were tested in the green/red assay. As in three-color assay, $g^{60j}$ showed constantly equal distribution between green and red (Fig. 43A, B). However, $norpA^{P24}$ had shown a preference for red throughout the day. The preference was not very high and probably was masked by blue avoidance in the three color choice assay. However, the result suggests that there are photoreceptors in the fly visual system, which use a norpA-independent pathway but require functioning $glass$ gene. Phospholipase C is encoded by the $norpA$ gene and is a part of canonical phototransduction pathway, a cascade of activations
from rhodopsins to TRPs. The presence of non-canonical phototransduction pathways has been reported by studies in both larvae and adult *Drosophila* (Busto, Iyengar, and Campos 1999; Saint-Charles et al. 2016; Szular et al. 2012). Busto et al. have studied the behavioral responses from larva to on/off and off/on transitions of light. Wild-type flies when exposed to light, shorten their path, perform head swings, and turn their direction. *norpA* mutants did not shorten their path and have not performed head swing, indicating that norpA is essential for the behavioral response. However, the third behavior, the turn of direction, have shown near wild-type levels. This result indicates that larvae were still perceiving the light and that there should be an additional transduction pathway, which does not require norpA. The study of Szular et al. group was focused on entrainment of the circadian clock. Szular used multiple mutants to check the ability of flies to synchronize their clock to external light. Double mutants *norpA*;*cry* quickly adapted their activity to shifted light cycles, but triple mutants *norpA*;*Rh6*;*cry* and *norpA*;*Rh5*;*cry* had severely slowed re-entrainment and the quadruple mutant *norpA*;*Rh5*;*Rh6*;*cry* nearly lost the ability to re-entrain the activity. These data indicate additional light-sensing pathways which do not depend on classical phototransduction cascade. However, it seems that in this pathways, light is still captured by the rhodopsins.

### 3.3.4.2 Wild-type and blind mutants in blue/red two choice assays.

The measurements in three color assays have indicated an active avoidance of the blue color in both wild-type and blind flies. To see if the possibility of a choice of green color can amplify the blue avoidance a series of experiments were
performed with tubes wrapped in combinations of only blue and red color filters. The experiments produced avoidance index similar to that of three-color experiments, indicating that the avoidance is not dependent on the presence of green (Fig. 44).

Figure 44. Color preference of blind and ocelliless flies in blue/red assay. (A) The fraction of flies in blue and red zones shown in the corresponding color. The standard deviation between 3 independent experiments is shown in a shaded band around each line. 14 to 24 flies were used for each experiment. Black and white bars indicate dark and light part of LD cycle. (B) Comparison of blue avoidance. The avoidance index was calculated (see Chapter 2.3.3.2) for each hour from ZT 2-10, then averaged.

3.3.5 Cryptochrome does not mediate color preference.

To find if the blue light sensitive photopigment cryptochrome (VanVickle-Chavez and Van Gelder 2007) is involved in blue avoidance, cry<sup>01</sup> mutants were tested (Dolezelova, Dolezel, and Hall 2007). However, no reduction of blue avoidance was observed for those mutants either (Fig. 45A).
3.3.6 Color preference of rhodopsin mutants.

In the visual system, the photoreceptors respond to light through the activation of rhodopsins and following canonical phototransduction cascade. In addition to visual photoreceptors, various rhodopsins are found in the peripheral sensory neurons: Rh1 is found in TRPA1-positive neurons (Shen et al. 2011), and Rh5 and Rh6 in multidendritic (MD) neurons (Sokabe et al. 2016). Even though the visual system is not required for blue avoidance, rhodopsins in other sensory neurons might be necessary for the behavior, and the green preference peaks might depend on a single rhodopsin. The light mediating role of the rhodopsins in the periphery has not been reported, but it is highly possible that those neurons sense the light. To investigate the role of individual rhodopsins in the color preference flies with null mutations knocking down Rh1 (Nina\textsuperscript{E17}), Rh5 \((rh5^{2})\) or Rh6 \((rh6^{1})\) were tested (Fig. 45).

Among the three mutants only \(Rh5^{2}\) showed significantly reduced blue avoidance (Fig. 45B). Even though, \(Rh5^{2}\) expressed less pronounced green peaks, but qualitatively the behavior was still present (Fig. 45). \(Rh5\) and \(Rh6\) genes are most likely co-expressed in the multidendritic neurons (Sokabe et al. 2016), but in the compound eye, they cannot be expressed in the same ommatidium, and which rhodopsin to express is determined stochastically through the feedback exclusion (Vasiliauskas et al. 2011). The modification of the green preference seen in both \(Rh5^{2}\) and \(Rh6^{1}\) could be the result of this exclusion.

While \(Rh5^{2}\) and \(Rh6^{1}\) had only weakly modified green preference, the \(ninaE^{17}\) flies completely lost it. Instead, they preferred red throughout the day. Rh1 is
primarily responsible for motion detection and is tuned to low intensities, its role in
the color vision remains controversial (Schnaitmann et al. 2013).

Figure 45. Color preference of rhodopsins’ mutant flies. (A) The fraction of flies in green, blue and red zones shown in the corresponding color. The standard deviation between 3 independent experiments is shown in a shaded band around each line. 18 or 24 flies were used for each experiment. Black and white bars indicate dark and light part of LD cycle. (B) Comparison of blue avoidance. The avoidance index was calculated (see Chapter 2.3.3.2) for each hour from ZT 2-10, then averaged. (C) Preference between red and green colors near timing of the first green peak ZT2 (Morning) and midday ZT6 (Midday). Preference for green is shown as positive and preference for red as negative.

Measurements in two-choice assays supported the results of experiments in the three-choice assay, showing the significantly reduced blue avoidance for Rh5 mutants and the modifications in green preference similar to observations seen in three color assays (Fig. 46). Interestingly, the preference of NinaE17 in green/red assay was very similar to that of norpA^P24. While ninaE encodes only one rhodopsin Rh1 and therefore interrupts only some of the phototransduction
pathways in the visual system, *norpA* encodes phospholipase C which is a part of all canonical pathways. It is possible that there is a competitive interaction between visual and peripheral sensory systems, where one drives attractive response and the other – aversive. Therefore, the loss of visual system leads to avoidance of all light.

Figure 46. **Color preference of rhodopsins’ mutant flies in two-color choice assays.** (A, B) The fraction of flies in blue and red (A) or green and red (B) zones shown in the corresponding color. The standard deviation between 3 independent experiments is shown in a shaded band around each line. 14 to 24 flies were used for each experiment. Black and white bars indicate dark and light part of LD cycle. (C) Comparison of blue avoidance. The avoidance index was calculated (see Chapter 2.3.3.2) for each hour from ZT 2-10, then averaged. (D) Preference between red and green colors near timing of the first green peak ZT2 (Morning) and midday ZT6 (Midday). Preference for green is shown as positive and preference for red as negative.

### 3.3.7 Class-VI multidendritic neurons mediate blue avoidance.

None of the flies discussed this far with abnormal vision, lack of rhodopsins or CRY have shown the absence of the blue avoidance, with only Rh5 and Rh6 flies having a minor decrease. The fact that the blue avoidance is so robust with avoidance index often above 0.8 suggests that the blue light might be noxious.
Multiple types of noxious stimuli, including heat, chemical and mechanical, are modulated through the network of multidendritic neurons, which cover nearly whole body in both larva and adult *Drosophila* (Im and Galko 2012). Until recently, however, light was not among the known noxious stimuli sensed by md neurons. The Jan group in their studies identified class-IV multidendritic neurons to respond towards strong blue and UV light mediating avoidance behavior in larvae (Xiang et al. 2010). Xiang et al. had first shown that class IV md neurons are activated by light in isolation. They further showed that ablation of those neurons, in addition to Bolwig organ, leads to loss of the avoidance behavior. For their experiment, they expressed tetanus toxin light chain (TeTxLC) with pickpocket driver (ppk-Gal4) (Sweeney et al. 1995; Grueber et al. 2007; Shimono et al. 2009) in larvae with ablated Bolwig organ (using *GMR-hid*).

In our experiment, the same transgenic construct *UAS-TeTxLC/ppk-GAL4* was used. Blocking the md neurons neurotransmitter resulted in a significantly lower blue avoidance (Fig. 47A, B). Ablation of both external photoreceptors with *GMR-hid* and md neurons with TeTxLC led to an even stronger reduction of blue light avoidance (Fig. 47A, C).

In *Drosophila* larvae class IV md neurons express Rh5 and Rh6 (Sokabe et al. 2016). We hypothesized that depletion of Rh5 using RNAi in flies with abolished external photoreceptors would lead to similar blue avoidance as for *GMR-hid; UAS-TeTxLC/ppk-GAL4*. The *GMR-hid; UAS-Rh5-RNAi/ppk-GAL4* flies produced the same change in blue avoidance supporting our hypothesis (Fig. 47A, C). Together, these data suggest that multidendritic neurons mediate blue
avoidance in adult *Drosophila* as well as in larvae and utilizes Rh5 for capturing light. However, the ppk-GAL4 expression is not limited to md neurons (Lamaze et al. 2017; Seidner et al. 2015). To confirm the role of the md neurons in adult *Drosophila* blue avoidance a more targeted study would be required. One of the solutions for that problem could be a gradual elimination of potential candidates among ppk-expressing neurons by means of conditional restriction of GAL4 expression or the split-GAL4 technique.

![Figure 47. Color preference of flies with ablated md neurons and visual organs.](image)

- **(A)** The fraction of flies in green, blue and red zones shown in the corresponding color. The standard deviation between 2-3 independent experiments is shown in a shaded band around each line. 18 or 24 flies were used for each experiment. Black and white bars indicate dark and light part of LD cycle.
- **(B, C)** Comparison of blue avoidance for flies with ablated md neurons only (B) and with visual photoreceptors (C). The avoidance index was calculated (see Chapter 2.3.3.2) for each hour from ZT 2-10, then averaged.

In addition to color preference, the activity of the *g[60j], GMR-hid, UAS-TeTxLC/ppk-GAL4* and *GMR-hid; UAS-TeTxLC/ppk-GAL4* was measured (Fig. 48 A-D). As was shown in previous studies, *g[60j]* flies loose morning peak of activity
(Fowler and Montell 2013; Venkatachalam and Montell 2007), however, the GMR-
_hid showed normal wild-type activity. UAS-TeTxLC/ppk-GAL4 also did not have altered activity. Interestingly, flies with a combination of GMR-hid and UAS-
TeTxLC/ppk-GAL4 significantly reduced their activity and also did not show typical morning and evening peaks.

Figure 48. Activities of flies with ablated md neurons, visual organs, circadian clock and TRPA1 channel. (A-D) Abolishing visual photoreceptors and md neurons drastically reduce response to external light. However, the activity is still periodic and even though reduced, is comparable to per^0 flies. (E, F) While both per^0 and TrpA1^1 mutants lose midday green/dim switch, TrpA1^1 retain the anticipation of morning and evening. The peaks that can be seen in per^0 activity are not anticipatory M and E peaks but the response to the light on/off switches. (A-F) Average activity was calculated as the distance traveled by individual flies between consecutive video frames (captured at one frame/sec), binned over 10-minute intervals, and then normalized. Black and white bars indicate dark and light portions of LD cycle.
3.3.7.1 TRP channel Painless is required for blue avoidance.

The blue avoidance in larvae is mediated through the Transient receptor potential A1 (TRPA1) channel (Xiang et al. 2010). TRP channels comprise a superfamily of cation channels which are conserved from worms to humans and involved in the sensation of nearly all kinds of stimuli (Fowler and Montell 2013; Venkatachalam and Montell 2007). The first identified TRP channels are TRP and TRPL, which are part of the canonical phototransduction pathway, and therefore mediate light perception. Though the TRPA1 channel is required for the light avoidance in larvae, it belongs to TRPA subfamily and mainly functions in nociceptive temperature response. The TRPA1 and another TRPA channel Painless were identified in larvae as nociceptors of high temperature (>39°C) and strong mechanical stimulation (Tracey et al. 2003; Hwang, Stearns, and Tracey 2012). Both channels are expressed in class IV md neurons. In the adult, at least one more TRPA channel Pyrexia (PYX) was found to contribute to temperature nociception. PYX is directly activated by temperatures above 40°C and helps prevent paralysis at hot temperatures.

To see if the TRPA1 is also required for blue avoidance in adult Drosophila, a strong loss-of-function TRPA1 mutants (TrpA1


) were tested. However, the mutants did not show a reduction of blue avoidance (Fig. 49 A, B). Additional flies were further tested with null mutations of other TRP channels: TRP (Trp0), TRPL (Trpl), Painless (painpc) and Pyx (pyx3). Out of all mutants, only painpc showed a reduction in blue avoidance (Fig. 49 B). In fact, painpc was the first mutant fly that had almost completely lost blue avoidance.
Figure 49. Color preference of TRP channels’ mutant flies in three and two-color choice assays. (A, D, E) The fraction of flies in green, blue and red (A), blue and red (D), or green and red (E) zones shown in the corresponding color. The standard deviation between 3 independent experiments is shown in a shaded band around each line. 14 to 24 flies were used for each experiment. Black and white bars indicate dark and light part of LD cycle. (B, F) Comparison of blue avoidance for three-choice (B) and two-choice (F) assays. The avoidance index was calculated (see Chapter 2.3.3.2) for each hour from ZT 2-10, then averaged. (C, G) Preference between red and green colors near timing of the first green peak ZT2 (Morning) and midday ZT6 (Midday). (C) shows preference in three-choice assays and (G) – two-choice assays. Preference for green is shown as positive and preference for red as negative.
pain<sup>pc</sup> flies lost the blue avoidance, but remained the pattern in the green preference (Fig. 49A, C). Painless had never been reported for involvement in light avoidance, and the current study is the first to show that new modality of Painless functions. pain<sup>pc</sup> also showed almost complete lack of blue avoidance in the blue/red two-choice assay nearly wild type behavior in green/red two-choice assay supporting data from three colors experiments.

### 3.3.8 TRPA1 and PYX mutants loose midday preference switch.

TRPA1 and PYX mutants did not show any reduction of blue light avoidance. However, they did not switch their preference from green in the middle of the day (Fig. 48 A, C, E, G). The temperature maintained in the color preference experiments significantly lower than the ones which directly activate TRPA1 and PYX. However, these channels were also shown to influence other behaviors at temperatures far from their direct activation range. Under natural conditions, TRPA1 is required for the presence of the afternoon activity peak (A-peak) (Green et al. 2015). In addition, TRPA1 affects midday activity under temperature cycles (Roessingh, Wolfgang, and Stanewsky 2015). PYX was shown to mediate entrainment of the circadian clock to low-temperature cycles (Wolfgang et al. 2013). Similarly to how rhodopsins appear to play a dual role in light and temperature sensation, it is possible that TRPA1 and PYX are involved in light responses.

### 3.3.9 The circadian clock in color preference.

The repetitive pattern of the color preference with two green preference peaks and a switch in the middle of the day hint at a possible involvement of the circadian
clock. Furthermore, the second green preference peak seems to appear in anticipation of the evening activity bout. To follow up with these observations, flies with a modified circadian period were tested. Per is the essential component of the molecular mechanism of the circadian clock, and per$^0$ is the first circadian clock mutant with a non-functional clock that helped to establish the field of chronobiology (Konopka and Benzer 1971). Konopka and Benzer had generated three period mutants: arrhythmic per$^0$, per$^S$ with a short circadian rhythm period of ~19 hours, and per$^L$ with a long period of ~28 hours. Later, new mutants were generated, which targeted other core clock components like Tim (tim$^0$) and Cyc (cyc$^0$), and helped to unravel the mechanism of the circadian clock in Drosophila (Vosshall et al. 1994; Rutila et al. 1998).

First, three arrhythmic mutants: per$^0$, tim$^0$, and cyc$^0$ were tested in three-color choice assay under 12:12LD. All three mutants had shown no variation in color preference and kept constant preference for green (Fig. 50 A). The midday switch in preference was not present. Next, per$^S$ and per$^L$ were measured in 19-hour long cycle and 29.5-hour long cycle correspondingly. As expected, both flies showed “normal” behavior with two green preference peaks and a switch in the middle of the day (Fig. 50 B, left). The difference from the wild-type behavior was that the length of the midday switch was shorter for per$^S$ and longer for per$^L$. The green peaks were still ~2 hours after lights turn on and ~1 hour before lights turn off, like in wild-type behavior. The two green peaks seem strongly tied to the activity bouts, the first peak appearing at the end of morning activity and second peak -shortly before the evening activity.
Figure 50. Color preference of wild-type and clock mutant flies in light cycles of different durations. (A) Color preferences for clock mutants \(\text{per}^0\), \(\text{tim}^0\), and \(\text{cyc}^0\) under 12:12LD cycle. (B) Color preferences for wild-type and period mutants in three different LD cycles: 9.5:9.5LD (top), 12:12LD (middle), and 14.75:14.75LD (bottom). (A, B) The fraction of flies in green, blue and red zones shown in the corresponding color. The standard deviation between two independent experiments is shown in a shaded band around each line. 18 or 24 flies were used for each experiment. Black and white bars indicate dark and light part of LD cycle.

When wild-type and period mutants were measured in light cycles whose periods did not correspond with innate circadian rhythm periods, the behaviors were modified. Wild-type, \(\text{per}^0\), \(\text{per}^S\) and \(\text{per}^L\) flies were tested in 19-hour (9.5:9.5LD), 24-hour (12:12LD) and 29.5-hour (14.75:14.75LD) days. Only \(\text{per}^0\) flies had same behavior regardless of different durations, and simply kept constant preference for green (Fig. 50 B, right). This result was consistent with the idea of clock modulating the color preference, and since \(\text{per}^0\) has nonfunctional circadian
clock it should not synchronize with any light cycle. In other mutants and wild-type, both color preference and activity pattern were adjusted.

We already discussed the color preferences of the tested flies in the light cycles of innate circadian periods. So next, we will separate other cases in two groups: first, when the period of the external light cycle is shorter than an innate circadian period, and second when the period of the light cycle is longer than the innate period.

3.3.9.1 Flies in short light cycles.

The first group includes three cases: wild-type in 19-hour days, \textit{per}^{\text{L}} in 19-hour days and \textit{per}^{\text{L}} in 24-hour days. In those cases, the light turns on earlier than anticipated by the circadian clock and the activity does not start before the light transition (Fig. 51 B, C). The change in light conditions accelerates the morning activity, so it starts immediately after lights turn on. The first green peak follows the activity and also appears shortly after lights-on. As a result, the first green peak whenever appears always has the same timing (Fig. 51 D). Next, in the middle of the day, the preference switches from green preference to equal between green and red, though the timing of the switch was not consistent, and for \textit{per}^{\text{L}} in 19-hour switch never occurred. The likely reason for the switch not to occur for \textit{per}^{\text{L}} in 19-hour day is that the period of the light cycle was approximately half of the innate period of fly’s circadian clock, which is too short and can disbalance the clock. A similar conclusion can be drawn from activity data, where the bimodality was completely lost (see next chapter). The light cycle is short, so the light transition to dark phase again happens before the anticipated by the circadian clock. As a
result, the second green peak never occurs, and the evening activity bout takes place during the night.

**Figure 51.** Circadian clock regulates the timing of changes in preference between green and red colors. (A-C) per\textsuperscript{S} (A), wild-type (B) and per\textsuperscript{L} (C) in three different LD cycles (9.5:9.5LD, 12:12LD, 14.75:14.75LD). The grey area shows dark part of LD cycle; black and white arrows indicate the timing of morning and evening bursts in locomotor activities respectively (for color preference with activity see Fig.52). (D), Timings of the first (open symbols) and the second (filled symbols) green peaks in Zeitgeber time. Peaks for per\textsuperscript{S} shown with squares, wild-type with circles and per\textsuperscript{L} with triangles. (A-C). The fraction of flies in green, blue and red zones shown in the corresponding color, the standard deviation between two different experiments are shown in the shaded bands; 18 or 24 flies were used for each experiment. Black and white horizontal bars indicate dark and light parts of LD cycle.

3.3.9.2 Flies in long light cycles.

The second group of experiments includes wild-type in 29.5-hour days, per\textsuperscript{S} in 24-hour days and per\textsuperscript{S} in 29.5-hour days. Here the modulation by circadian clock led to opposite effect from the previous paragraph: the morning activity bout starts early in anticipation of the light transition to light phase, but because of the longer period, the phase remains dark (Fig. 51 A, B). As a result, the first green preference peak does not occur, and when the light finally turns on, the color preference pattern starts with the green/red switch. Interestingly the absence of first green
peak resulted in a higher preference for red in the switch than in normal conditions. The delayed light-on transition did not affect the timing of the evening activity bout and the second green peak as well. Both appeared approximately 1 hour before the anticipated light-off transition. However, as the light cycle is long, the light-off anticipation is not satisfied, and the flies after a short interval of evening activity return to green color. This result and the color preference of the arrhythmic flies indicate that green is the overall more preferable than red, and the midday switch represents the change in a “default” preference.

3.3.9.3 Light cycles and activity bouts.

An interesting observation from the interaction of activity bout timings with the light cycles of different lengths is that the timing of the morning peak is defined by the light-off transition, but can be advanced by earlier light-on transition (Fig. 52). Among the combinations of circadian and external light periods studied here, the morning activity timing was the same if the time counted from the start of the dark phase for each mutant (Fig. 51 D). The exception is when the light turned on before the anticipated time; then the morning activity was accelerated. The evening activity is tied to the morning and always occurs after the same time interval, regardless of light conditions (Fig. 52 B, the distance between black lines is always constant). Since the primary pacemakers neurons, which are responsible for the M peak (M-cells) coordinate the E-cells (Stoleru et al. 2005), The E peak is controlled by M peak. However, it is interesting that the timing of the morning activity bout is set by the start of dark phase with possible acceleration by the early transition to light phase.
3.3.9.4 The role of the circadian clock neurons in the color preference.

The central circadian clock in *Drosophila* includes ~150 neurons which are well described and grouped into multiple subsets (Tataroglu and Emery 2014). The subsets of circadian clock neurons are involved in multiple distinct functions, from
the primary pacemaker function to arousal and temperature synchronization. The advancements of *Drosophila* genetic studies have allowed building an extensive collection of tissue-specific Gal4-drivers. To investigate what neurons are required for the color preference pattern, three Gal4-drivers were used to express a human inward rectifier K+ channel (*UAS-kir2.1*) in order to silence large (l-LNᵥ, with *C929-Gal4*) and small ventral lateral neurons (s-LNᵥ, with *R6-Gal4*) and a group of dorsal neurons (DN₁p, with *Clk4.1-Gal4*). These subsets are important for generating the daily M and E activity peaks (Grima et al. 2004; Stoleru et al. 2004). In addition, the *pdf-Gal4* driver was also used, which is less specific and expressed in both l-LNᵥs and s-LNᵥs but is a better-described driver. However, none of the lines survived to adulthood, therefore, a temperature dependent repressor of the Gal4, *tub-Gal80ts* was used to raise animals under restrictive conditions and silence clock neurons only in the adult stage. Flies with *C929-Gal4* and *Clk4.1-Gal4* driven silencing largely retained wild-type behavior indicating that l-LNᵥ and DN₁p are not required for the control of the color preference (Fig. 53 A). In contrast, both *R6-Gal4* and *pdf-Gal4* lines showed a modified pattern in color preference. *Pdf-Gal4* did not have the first green preference peak and started immediately from the midday switch (Fig. 53 B, top). The second green peak was there, though it started earlier than in wild-type. *R6-Gal4* seemed more similar to the arrhythmic flies and preferred green during all light phase, though their morning green preference was lower. The result suggested s-LNᵥs to be the primary controlling neurons of the color preference behavior.
Figure 53. Color preference of flies with ablated subsets of clock neurons. (A) Silencing s-LNvs leads to loss of green/red switch in preference. Comparison of color preference of flies with silenced I-LNvs (top, C929-GAL4), s-LNvs (middle, R6-GAL4) and DN1ps (bottom, CLK4.1-GAL4) and corresponding control flies. (B) Flies without PDF receptors (pdf\textsuperscript{ran5304}) and with ablated PDF-expressing neurons I-LNvs and s-LNvs (pdf-GAL4) also lost normal color preference pattern. (A, B) The fraction of flies in green, blue and red zones shown in the corresponding color. The standard deviation between 2-3 independent experiments is shown in a shaded band around each line. 18 or 24 flies were used for each experiment. Black and white bars indicate dark and light part of LD cycle.

s-LNvs are thought to be the main pacemaker neurons, which uses a neurotransmitter PDF to synchronize other neurons. The requirements of this neurons for the color preference pattern suggests that disruption of the PDF signaling would lead to the similar effect. Flies with an abolished gene \textit{pdfr}, which encodes PDF receptor (pdf\textsuperscript{ran5304}) was tested and found to have similar to pdf-
Gal4 preference (Fig. 53B, bottom). This confirms the role of the PDF in the color preference, however, a more detailed study of the PDF function would require for the understanding if the clock control of the color preference can be separated from the activity.

3.3.10 Role of temperature in color preference.

The interaction between light and temperature sensory systems of adult Drosophila have been studied mostly through the change in temperature preference (Head et al. 2015). No data on interaction is currently available from larval studies, though multiple works have found rhodopsins being involved in temperature discrimination (Sokabe et al. 2016; Shen et al. 2011). As of now, no studies of temperature affecting color preference have been carried out. We performed two additional color-choice assays with wild-type and white-eyed (iso31) flies in 18°C and 30°C (Fig. 54). At 18°C wild-type flies lost midday preference switch and showed similar to TRPA1 mutants behavior. TRPA1 is a high-temperature sensitive channel, which was shown to modulate activity. The temperature dependent switch of color preference only supports the role of TRPA1 in the behavior. The white-eyed flies at 18°C, instead started to show the pattern, reminiscent to that of wild-type at 25°C. At 30°C, wild-type and iso31 started to behave similarly, losing green peaks and increasing the red preference. This result indicates an important role of temperature on the color preference, especially the preference of green in the middle of the day. The observation that the cold conditions led to behavior similar to the one of TRPA1 mutant suggests the integration of temperature and light processing circuits to form a combined output.
The wild-type-like behavior of more light-sensitive *iso31* at lower temperatures suggests a similar conclusion.

**Figure 54. Color preference of wild-type and white-eyed flies at different temperatures.** Preference of wild-type and *iso31* (white-eyed) flies between three colors in 18°C (left), 25°C (middle) and 30°C (right). The fraction of flies in green, blue and red zones shown in the corresponding color. The standard deviation between 2-3 independent experiments is shown in a shaded band around each line. 18 or 24 flies were used for each experiment. Black and white bars indicate dark and light part of LD cycle.

**3.3.11 Serotonin is responsible for the white-eyed fly color preference.**

*White* gene encodes ATP-binding cassette transporter, which is required for transport of tryptophan, a serotonin precursor. Therefore, flies with *white* mutation have reduced levels of serotonin. Serotonin is the neurotransmitter used for neurons signaling in a variety of animals and widely expressed in *Drosophila* brain.
Previously, serotonin had been linked to the stress, anxiety-like states and aggression (Maier and Watkins 2005; Ries et al. 2017; Gibson et al. 2015). We hypothesized that the high preference for red could be a symptom of stress.

Wild-type and iso31 flies were fed with the serotonin precursor 5-Hydroxytryptophan (5-HTP) to restore wild-type levels of serotonin. After two days of 5-HTP treatment, iso31 flies started to show wild-type behavior (Fig. 55 bottom). In contrast, wild-type flies did not change their behavior and kept the same color preference pattern (Fig. 55 top). This result could indicate that the color preference of white-eyed flies is an attribute of a depression-like state. One of the typical symptoms of the depression-like state is anhedonia (lack of interest in enjoинments). In a study, where the depression-like state in flies was induced by non-controllable stress, flies showed a reduction in a climbing attempt (Ries et al. 2017). In Ries' paradigm, the phenotype was recovered by feeding flies with 5% sucrose. However, neither wild-type nor white-eyed flies have shown any change of color preference after feeding sucrose. The abnormal color preference, shown by white-eyed flies most likely is not caused by the depression-like state, however, it still can represent stressful condition. The ability of flies to chose their light conditions allows them to reduce stress, therefore it does not induce depression. The color preference assays used in our study could potentially be used as a stress paradigm. However, a more detailed study, including the targeting of serotonergic neurons and serotonin receptors is required.
Figure 55. Color preference of wild-type and white-eyed flies with a supplement of serotonin precursor 5-HTP. Wild-type and iso31 flies were fed 100mM of 5-HTP for the duration of the experiment. The fraction of flies in green, blue and red zones shown in the corresponding color. 24 flies were used for each experiment. Black and white bars indicate dark and light part of LD cycle.

3.3.12 Histamine deficiency eliminates color preference.

In *Drosophila*, histamine is the neurotransmitter in mechanosensory and visual transduction (Melzig et al. 1996; Sarthy 1991; Gao et al. 2008). It is used to project signals from receptor cells to interneurons, and complete elimination of histamine synthesis is lethal, while severe depletion of histamine levels leads to loss of visual and mechanical response (Dau et al. 2016; Melzig et al. 1996). Restoring the histamine levels by simple uptake rescues both responses (Melzig et al. 1998). Histamine was also shown to modulate temperature preference in adult flies (Hong et al. 2006).
Two fly strains were tested, which have strong loss of function mutation for gene hdc – a gene that encodes the histamine precursor histidine. The first mutant is $hdc^{JK910}$ with almost complete loss of histamine and the second mutant is $hdc^{P211}$ that lacks histamine in the brain (Melzig et al. 1996). The $hdc^{JK910}$ mutants the most severe color preference phenotype, with nearly no avoidance of blue and preference of green (Fig. 56, left). Interestingly, the $hdc^{P211}$ flies showed close to $ninaE^{17}$ phenotype with normal blue avoidance but lost green preference peaks (Fig. 56, right). This result supports the hypothesis that the blue avoidance is mostly mediated by the peripheral photosensors, while the green preference is mediated by visual phototransduction.

![Figure 56. Color preference of histamine mutant flies. (A)](image)

(A) Histidine decarboxylase produces histamine from histidine. (B) The fraction of flies in green, blue and red zones shown in the corresponding color. The standard deviation between multiple consecutive days is shown in a shaded band around each line. 18 flies were used for each experiment. Black and white bars indicate dark and light part of LD cycle.

In accordance with the rescue of the visual response by histamine uptake (Melzig et al. 1998), flies which were fed histamine decarboxylase almost completely restored the color preference pattern (Fig. 56, top right). In the case with $hdc^{P211}$, flies had white eyes, therefore restored white-eyed phenotype (Fig.
After flies restored color preference, they were not fed histamine anymore, and gradually lost the color preference pattern in ~5 days (Fig. 57, bottom right). This result indicates that the histamine is the primary neurotransmitter for the light information not only in visual photoreceptors but in the peripheral system as well. There are multiple histamine receptors utilized in the nervous system and it would be interesting to study which receptors are required for the behavior, and track projections from peripheral photoreceptors to the brain.

**Figure 57.** Color preference of wild-type and white-eyed flies with a supplement of serotonin precursor 5-HTP. Wild-type (top left), *hdcJK910* (top right) and *hdcP211* (bottom left) flies were fed 100mM of histamine diphosphate for the duration of the experiment. (bottom right) *hdcJK910* flies were fed 100mM of histamine diphosphate for five days prior to the experiment, and then measured with regular food. The fraction of flies in green, blue and red zones shown in the corresponding color. 24 flies were used for each experiment. Black and white bars indicate dark and light part of LD cycle.
Chapter 4 Discussion.

4.1 A transient burst of activity in *Drosophila* locomotion.

Since studies of Pittendrigh and Aschoff, morning and evening peaks of locomotor activity have become some of the most reliable traits of *Drosophila* behavior, especially for studies of the circadian clock. Detailed analysis of the peaks allows for extraction of information about photic and mechanosensory pathways in *Drosophila*, neural circuits and behavioral responses (Yoshii, Rieger, and Helfrich-Förster 2012; Prabhakaran and Sheeba 2014; De et al. 2013; Guo et al. 2014; Helfrich-Förster 2000; Lazopulo and Syed 2016; Simoni et al. 2014; Vanin et al. 2012). In our study, we addressed finer structure of *Drosophila* locomotion and found a prominent feature reminiscent of burst. The feature unlike the peaks of activity occurs stochastically in time and, therefore, does not appear in population-averaged data. The feature is characterized by extremely high activity in a very short time and was called a burst of activity. The burst usually lasts for ~3 minutes and has at least 5 times the activity count of the morning peak (~12 counts per 3 minutes). The short duration and non-precise timing do not allow burst to appear in standard population-averaged actograms. Bursts are common in nature and can be defined as a rapid series of events, preceded and followed by a period of quiescence. In insects and animal behaviors bursts have already been reported and efforts to understand and predict them through behavioral studies and computational modeling are starting to arise (Wiens et al. 1995; Barabási 2005; Chou et al. 1999; Sorribes et al. 2011; Berman et al. 2013; Martin, Ernst, and Heisenberg 1999). To characterize the activity burst, reported in our study, we
analyzed its features. The burst’s frequency of appearance is highly dependent on the illumination level, however, its timing and magnitude are not. Both timing and magnitude of the burst appear consistent across different genetic backgrounds, unlike the peaks of activity, which appear to differ considerably between distinct genotypes. The consistency is surprising, given many fundamental behaviors like sleep and nociception can vary significantly between different genetic backgrounds (Harbison, Mackay, and Anholt 2009; Rode et al. 2007). The burst is independent of the clock, and the absence of a strict time-tracking driver could be the reason for stochasticity. The neuronal pathways are intrinsically noisy, and internal electrical or biochemical fluctuation can cause variation in timing. Stochastic behaviors have already been reported in *Drosophila* including flight and larval thermotaxis (Luo et al. 2010; Palmer and Schloss 2010). Even though the stochasticity can cause a decrease in quality of behavior, it, as often in nature, can add to efficiency and creativity (Tumer and Brainard 2007; Edwards et al. 2007). Why the burst occurs stochastically is currently unknown. However, the understanding of the actual behavioral state during the burst can give the insight into the nature of the burst.

The regular appearance of bursts supports our hypothesis of the burst being part of the important daily activity. It most likely reflects one type of behavior from the variety exhibited by *Drosophila* (Kain et al. 2013). Due to limitations of the experimental procedure, we were not able to identify the activity performed by a fly during the burst. However, we can speculate based on the burst’s characteristics. Given an extremely high activity during the burst, and the way it is
counted from crossings of the IR beam, it is unlikely that flies can travel along the entire length of tube between each beam crossing. Instead, it is more likely some fast repetitive action near the beam, like grooming (Seeds et al. 2014). The rapid body and wing movements can produce a large number of IR beam counts in a very short time. Since the IR beam is essentially is the beam of heat, it is possible that it causes a thermotactic response from flies (Kwon et al. 2008). Visual observations of video recordings of fly locomotion suggest that the presence of the IR beam may increase the frequency of the burst in the presence of light. These conclusions would be consistent with several lines of evidence, including our finding of color preference depending on the temperature, which has reported substantial overlap between light and temperature responses in *Drosophila* (Shen et al. 2011; Kwon et al. 2008).

The timing of the bursts did not depend on the circadian clock but instead was tied to the light switching on. Moreover, the bursts occurred more frequently at higher intensities. *Drosophila* is a diurnal crepuscular animal, which is most active during dusk and dawn. In nature, during these times light intensity is relatively low. In the middle of the day, when light intensity is at peak, fruit flies have the least activity and display so-called “siesta.” Studies with the dim light illumination have shown that flies prefer lower light levels despite positive phototaxis (Rieger et al. 2007). We speculate that light above a certain level of intensity could be a noxious stimulus and inflict stress on flies. Therefore, the burst could indicate a response to stressful conditions. One of the observed behavioral responses to stress is altering self-grooming (Kalueff et al. 2016). It is possible that the activity burst is
an output of self-grooming behavior in response to stress-inducing levels of light intensity.

4.2 Effect of the color of light on *Drosophila* locomotion.

Simple exposure to white light during photic entrainment of circadian clock produces changes on both behavioral and molecular levels. However, the entrainment, as well as other light-dependent processes, are sensitive to distinct light spectra. In human, the visual system displays spectral sensitivity and most sensitive to light of 555 nm wavelength. However, many physiological processes are governed by melanopsin, which has sensitivity peak at ~480 nm (Provencio et al. 2000; Panda 2002; Roecklein et al. 2013; Lockley et al. 2006; Altimus et al. 2008; LeGates, Fernandez, and Hattar 2014). The spectral sensitivity of different physiological processes can lead to such interesting and conflicting behavioral outcomes, as light promoting both sleep and wakefulness (Pilorz et al. 2016).

When tested in LD cycles under illumination of assorted colors, flies displayed distinct activity profiles. In blue light, flies showed an elevated long-lasting morning peak, but reduced evening peak of activity. In *Drosophila*, the circadian photopigment cryptochrome is most sensitive to UV/blue region of wavelengths (Ceriani et al. 1999). Cryptochrome is the primary source of photic synchronization of the circadian clock to light cycles, therefore, blue light is the best for the clock entrainment. However, blue light was also shown to produce a damaging effect on *Drosophila* (Hori et al. 2014) and decrease lifespan. The increase in height and duration of morning peak could indicate stressful or noxious conditions and an active search of escape. In comparison, in green light, the activity during morning
and evening was both lower and shorter. Flies in the dark for them conditions showed very similar behavior to the one in white light. Therefore, the decrease in green was not a product of poor sensitivity to green light. Similar to how green light promote sleep in mice (Pilorz et al. 2016), it can have a calming effect on fruit flies. In nature, green can be associated with protection of leaves, while blue – with the danger of open space.

Further analysis of activity bouts revealed that the reduced activity in green primarily comes from the slower average speeds. Flies still spent similar time resting and acting, but moved slower, which can also hint at more comfortable conditions. Surprisingly, even though, the activity in blue light was significantly different from activity in both white and red, the average activity, speed and time spent active were very similar. It seems that averaging the data removed the difference observed in timeseries. Therefore, we analyzed the distribution of activity bouts by speed. Interestingly, the distributions for red and dim light produced discreet uniformly separated peaks. These peaks correspond to values of multiples of tube lengths per minute. The distribution shows that when active, flies tend to move full tube distances and end up either near food or cotton plug. Whether this is an exploratory behavior or a search for an escape is currently unknown. In comparison, flies in green had an exponentially decreasing distribution of speeds, reminiscent of Poisson distribution. Such distribution is produced when individual events are independent of previous ones. In our case, this most likely means that flies do not express the same trait as in white and red colors and do not always end up near food or cotton plug. Such behavior once
again supports the hypothesis of a calming effect of the green color. In contrast, the distribution of speeds for flies in blue color had one peak around two tube lengths per minute.

The relatively simple central nervous system of *Drosophila* permits observation and analysis of the physiological changes in the fly through behavioral outputs. Clear modulations of *Drosophila* locomotion driven by colors of the light indicate the importance of the spectral composition of light on the daily life of an animal. In humans, the behavior is much more complex, and the color specific modulations are hard to track. However, multiple studies demonstrated the effect of light color on brain activity, and light therapies are widely used to treat psychological disorders (Vandewalle et al. 2010; Terman 2007). *Drosophila* has already proven to be a successful model for the human nervous system and helped to study multiple neuronal disorders (Terman 2007). Studies of color-specific effects on fruit flies can improve our understanding of human interaction with light, which is currently obscured by the complexity of the behaviors. One of the highly debatable topics, which can be addressed through *Drosophila* is the innate color preference and if it exists in humans (Palmer and Schloss 2010).

### 4.3 Time-dependent color preference.

The interaction of LD cycles with circadian clock produces a bimodal pattern in *Drosophila* locomotor activity (Pittendrigh 1960; Jurgen Aschoff 1966). Same interaction also produces a change in temperature preference (Kaneko et al. 2012). However, the possible influence of circadian clock on color preference was not yet addressed. In most phototaxis studies fruit flies are assumed to have a
constant color preference that does not depend on the time of day. This assumption can be justified for “fast” phototaxis paradigm, where the choice of color is made in an escape reaction (Schumperli 1973). However, we found that this assumption does not hold for extended periods of time. Using our color-choice assay, we were able to show a previously undescribed periodic variation in color preference. Flies display two peaks of preference for green light in the morning and late afternoon. In the middle of the day, green preference experiences a deep drop due to increase in preference for dim light. Simultaneously, the blue light is continuously avoided with remarkable robustness. The circadian variation in Drosophila raises the possibility that color preference in humans may also depend on the time of day, in addition to its dependence on seasons (Schloss et al. 2017). Further analysis allowed to split the behavior into two parts: green/dim light preference and blue avoidance. While two parts are not entirely independent, they seem to primarily rely on two separate phototransduction pathways: visual system and peripheral sensory neurons. Such design further pursues similarity to mammalian phototransduction, with rods and cones in the visual system and melanopsin in extraocular phototransduction. However, this concept does not deny other possible contributors, like dermal photosensitivity.

4.3.1 Variation in green preference.

The green preference in the color choice assay required visual system and was eliminated in flies without visual organs. However, ablation of the norpA gene, and therefore, interruption of classical phototransduction pathway did not fully mirror the elimination. In the three-color choice assay, the color preference was similar
to eyeless gi^{60j}, with small red preference in the middle of the day, which at first was disregarded as insignificant. However, in green/red assay the preference for green was reversed to avoidance, similarly to Rh1 mutant. An additional norpA-independent pathway for Rh5 and Rh6 was proposed by several studies (Szular et al. 2012; Saint-Charles et al. 2016). Our finding further supports the possibility that Rh5 and Rh6 have an alternate phototransduction pathway. The pathway still recruits TRP channel for the final output of transduction, since trp ablation also removed preference.

The periodic variation of green preference strongly depends on the circadian clock. One of the earliest reported phenotypic outputs of the circadian clock is the locomotor activity with morning and evening peaks. The pattern in green preference is reminiscent of that in activity with two peaks, but timings are shifted. Where activity peaks occur at light transitions, the green preference peaks occur ~2 hours after light-on and ~1 hour before light-off. Ablation of three core clock genes per, tim, and cyc, all eliminated the variation in preference, and instead produced the uniform pattern. Furthermore, the mutation of gene pdfr, which is required for morning activity peak, also removed the first green peak. Consistent with the role of PDF, the color preference relies on the subset of small lateral ventral neurons, the main pacemaker neurons in the central clock. The tight control and robustness of the green preference suggest the substantial role of the color preference in everyday life of Drosophila.

Results from our study suggested the requirement of TRPA1 and PYX for the midday switch in green preference. Interestingly, neither of the two has a primary
role in color discrimination. TRPA1 and PYX primarily contribute to temperature-dependent behaviors (Fowler and Montell 2013). Both channels are directly activated by hot temperatures and considered nociceptors. However, they also indirectly track lower temperatures and modulate activity in temperature cycles (Lee and Montell 2013; Wolfgang et al. 2013). The experiments with different temperature conditions further supported the effect of temperature on color preference. Temperature and light often drive similar responses suggesting integration of information from temperature and photoreceptors for decision making. Using our assay, we further support such integration. White-eyed flies lack red pigments in their ommatidia, which increases the amount of light received by the photoreceptors. Therefore, these flies are more sensitive to light and require lesser intensity to produce the wild-type behavior. However, the same result could be achieved by decreasing ambient temperature. On the other hand, the increase of temperature leads to wild-type flies behaving like white-eyed. This result suggests that the behaviors, which are modulated by both temperature and light, are regulated by their linear combination. The sensory information from all kinds of stimuli is projected to specific brain centers, primarily mushroom body, and lateral horn, where information is processed. While the receptor and projection neurons are well identified for both temperature and photoreceptions (Frank et al. 2015; Vogt et al. 2016) the possible interaction between two systems is still concealed.
4.3.2 Blue avoidance as a response to noxious stimulus.

The blue light avoidance, observed in our study presented a remarkable contrast to green preference: it did not depend on circadian clock and visual organs. Instead, it rapidly increased at the beginning and persisted until the end of the light phase. Previously, the blue avoidance was reported in larvae with md neurons playing the role of photoreceptors (Xiang et al. 2010). However, the common assumption for adult *Drosophila* was that due to morphological changes during metamorphosis the behavior was lost. That assumption is supported by the phototaxis studies and the changes to md neurons: some experience programmed apoptosis, and others undergo a complete redesign of dendrites (Shimono et al. 2009). However, the intensity of blue light, required for excitation of md neurons was significantly higher than the one typically used in color preference experiments. Secondly, the class IV neurons, which are responsible for the blue avoidance in larvae still survive in the adult. The results of our study support the persistence of the avoidance through metamorphosis and potentially same neuronal pathway. Like in larvae, the behavior requires high-intensity light and significantly decreases at lower intensities. The avoidance is also reduced by ablation of the blue sensitive rhodopsin Rh5. This initially raised a possibility of the visual system being still involved in avoidance, but a recent study discovered expression of Rh5 in md neurons in larvae (Sokabe et al. 2016). Therefore, it is still possible that md neurons are solely responsible for the blue avoidance. Interestingly though, the behavior employs a different than in larvae TRP channel, Painless, which is common in nociceptor responses. However, the involvement of Painless only adds to our
hypothesis of bright blue light being a noxious stimulus. Our experiments with flies in light of different colors proposed a similar conclusion. Previous studies of blue light affecting *Drosophila* lifespan further point at the harmful influence of blue light (Hori et al. 2014). However, the mechanism of deadly influence of blue light is still unknown and requires further research. The experimental assay, used in the current study can provide a reliable phenotypic paradigm for such research.
References.


