Behavioural Adaptations to Light Deprivation

Fast and Furious: Tōhoku Drift

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“You have to be realistic about these things”

-Logen Ninefingers

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Summary

Micro-evolution is a natural process, driven by natural or sexual selection, mutations, genetic drift or genetic flow. While physiological and anatomic adaptations are well studied, behavioural adaptations are rarely observed in a micro-evolutionary context. The *dark-fly* strain, a *Drosophila* strain that has been reared in total darkness conditions for over 1500 generation, presents a great opportunity to study adaptations of visually-guided behaviours in a micro-evolutionary scale. This study focusses on two visually-driven behaviours: the locomotion strategy and courtship behaviour.

The ability to extract 3D-information from the environment is crucial for successful navigation and exploration behaviour in non-sedentary species. However, most insects lack stereoscopic vision and therefore other cues for distance estimation become prevalent. The optic flow, the retinal image shift induced by self-motion, is utilized to gain 3D-information. The saccadic movement strategy, consisting of long phases of translation separated from very short and fast rotations, called saccades, has been shown to facilitate the 3D content in the optic flow. Experiments with canonical mutations of the visual neuropiles suggest a correlation between the saccadic movement strategy and the status of the visual system. We found that the classic saccadic strategy is changed by manipulations of the visual system and is lost due to lack of visual cues. Phases of translations are severely reduced, while rotations and saccades become more abundant.

This change in locomotion strategy is accompanied by a change in the exploration strategy: the *dark-fly* strain shows a significantly higher exploration rate compared to wt flies, which can be accounted to a drifiting movement while curve walking: the *Tōhoku drift*. We conclude that *dark-fly* developed a new strategy that seems to optimize mechanosensation, rather than optic flow.

Previous studies showed a severe influence of vision on courtship success, courtship initiation and timing of specific behaviour. The volume of courtship song has been shown to be distance dependent and vision responsible for distance estimation. In a

competitive mating assay *dark-fly* surpassed wt strains and seemed to be able to identify another *dark-fly*. Hence, the question arises whether *dark-fly* has changed their courtship strategy.

In accordance with other studies we find that courtship is disrupted in dark conditions in both wt and *dark-fly*. Curiously, *dark-fly* performed worse in a single pair courtship assay and did not successfully copulate. Changing the approach to a group courtship assay restores courtship success in *dark-fly* to an even higher level than the wt *OregonR* suggesting a change in strategy from competitive to cooperative.

The courtship song of *dark-fly* is still functional but shows an adaptation to higher volume. While wt females are repelled by loud courtship songs, *dark-fly* shows a sexual dimorphism in hearing ability. Female *dark-fly* are less sensitive compared to males. This is evidence for a sex-specific co-evolution that has been widely observed in the animal kingdom.

Taken together this study provides evidence for adaptation of visual-based behaviours to the absence of visual cues. Both locomotion and courtship are still functional in *dark-fly*, however the strategies have changed to optimize survival in a changed environment.

1. Introduction

1.1 Micro-evolution

One of the fundamental features of life is the adaptation to changing environmental conditions. While macro-evolution is describing these adaptations on a large scale, i.e. the emergence of new species, micro-evolution is characterized by rapid evolutionary adaptations on a smaller scale, i.e. within and among populations (Hendry and Kinnison, 2001). Within a population most characteristics are manifested in different forms based on genetic variation. If evolutionary processes act on this variation, certain traits can become either advantageous or disadvantageous leading to a change in occurrence. Micro-evolution is usually driven by either natural or sexual selection, mutations, genetic drifts or genetic flow. This mechanism can guarantee the survival of a species and has given rise to the great biological diversity observable on different scales (Hendry and Kinnison, 2001; Reznick and Ricklefs, 2009). The emergence of novel molecular techniques that allow to link physiological traits with the genome, increased the interest in studying micro-evolution in the last years.

Classic examples for micro-evolution often include the change of appearance to changing environmental factors. One of the most prominent cases is the directional colour change observed in peppered moths, often found on the trunk of birches. Peppered moths exist in two morphs, a white-bodied form (*Biston betularia f. typica*) and a black-bodied form (*Biston betularia f. carbonaria*). Pre-industrialisation, the white-bodied form was predominantly found within the population. During the industrialisation and associated increase in pollution, the trunks of birches were darkes in colour and correspondingly the frequency of the black-bodied form increased. After pollution was reduced, the white-bodied form was again predominantly found. This process is known as industrial melanism (Kettlewell, 1955; Majerus, 1998). Another well studied example of micro-evolution on an anatomical scale are the changing beak sizes of Galápagos finches (Grant and Grant, 1995). More recent examples gaining increasing

importance are the occurrence of resistances to both pesticides and antibiotics (Tabashnik, 1994; Baquero and Blázquez, 1997). Evolutionary processes can not only act on anatomical and colouration but also on behavioural traits. While behavioural adaptations are well studied on a macro-evolutionary scale they are less often observed in a micro-evolutionary context and are predominantly described in birds (Berthold et al., 1992; Cattau et al., 2018).

Studying evolutionary and micro-evolutionary processes proves difficult since observations have to include different generations and populations might spread over great distances. Hence, *Drosophila* is a convenient model to study micro-evolution due to their short generation cycle, high number of offspring and minimum space requirements. In laboratory conditions the environment of *Drosophila* can be easily modified and therefore different traits like senescence (Rose, 1984), tolerance to alcohol (McKechnie and Geer, 1993), cold (Kellermann et al., 2009) and desiccation (Folk and Bradley, 2005) have already been studied. Additionally, several adaptations in appearance like pigmentation (Rajpurohit and Gibbs, 2012) and wing evolution (Houle et al., 2017) could be shown.

In the mindset of studying *Drosophila's* capability to adapt to various changing environmental conditions, in 1954 Professor Shuiti Mori at the University of Kyoto started a series of experiments which exposed a *Drosophila* wildtype stain to different changes in environmental conditions. One part of the series consisted of generating an isogenetic *Drosophila* strain and raising it in complete darkness (*3.1.1 Generation of dark-fly*(Fuse et al., 2014).

1.2 Dark-fly as a model for genetic adaptation

The isogenetic dark-raised *Drosophila* strain established by Professor Mori, has been maintained for over 1500 generations and is still sustained in different laboratories to this day. Over the decades, several experiments have been performed on dark-fly to understand the extend of the behavioural and genetic adaptations.

Typically, *Drosophila* shows a strong phototactic behaviour which can be measured by illuminating one side of a transparent glass tube and counting the flies that cross the

midline towards the light source in a defined time interval. Phototactic behaviour of *dark-fly* was tested in generation 39, 51, 80, 82, 108, 135, 168, 202, 304, 582 and shows stronger bias towards light than the control group raised in a dark:light cycle (Mori & Imafuku, 1982; Mori & Yanagishima, 1959).

The dark:light cycle is the most important zeitgeber for synchronisation of the internal clock of *Drosophila*. *Drosophila* shows a bimodal activity pattern, characterised by one peak in activity in the morning and one in the evening (Aschoff, 1966; Peschel and Helfrich-Förster, 2011). It has been suggested that the two activity peaks are the result of two coupled circadian oscillators; one that would be accelerated by light and responsible for morning activity and a second one that would be slowed down by light and therefore induce the evening peak in activity (Daan & Pittendrigh, 1976; Picot et al., 2007). This system would allow the circadian rhythm to be more flexible and react to seasonal changes in illumination (Stoleru et al., 2007). Furthermore, various other influences like temperature, social interactions and magnetism can act as *zeitgebers* and harmonize the internal clock to the environmental conditions (Levine et al., 2002; Majercak et al., 1999; Yoshii et al., 2009). These findings imply that the circadian rhythm in *Drosophila* is not a rigid system but can rather be adapted to different environmental factors. Previous research showed that wt *Drosophila* display an arrhythmic activity pattern under constant light conditions but are able to maintain robust oscillation for a prolonged time in constant dark conditions (Dows et al., 1987; Konopka et al., 1989). Despite being reared in DD conditions over many generations *dark-fly* shows no differences in circadian rhythm in LD conditions compared to wt control flies, indicating that the light-driven circadian rhythm is still functional (Imafuku & Haramura, 2011). Furthermore, the developmental rhythm was not influenced by a completely dark environment (Imafuku and Haramura, 2011). The ultrastructure of photoreceptors shows no significant difference comparing wt and *dark-fly* (Fuse et al., 2014a)

.

Interestingly, the tactile bristles covering over the whole body are significantly longer in *dark-fly* compared to wt (Fuse et al., 2014a; Imaizumi, 1979). The bristles are of the external sensory organs of *Drosophila* and react primarily to tactile stimuli. They provide

proprioceptive feedback on limb position and locomotion and if located on the mouthparts, wings and legs can act as a contact chemosensor. This suggests an increase in mechanosensory and chemosensory sensitivity in the *dark-fly* strain. Early experiments using spoiled fly food suggest indeed an increased sensitivity in olfaction in *dark-fly* but no further studies on the olfactory or gustatory system have been performed (Fuse *et al.*, 2014a).

Since the aim of the *dark*-fly project was to gain insight into the genetic mechanisms of adaptation, a recent study performed whole genome sequencing and a subsequent analysis of single nucleotide polymorphisms (SNPs). SNPs are defined as a variation in a single nucleotide occurring at a specific position in the genome that occur at a perceptible degree within the populations. Areas with a high frequency of SNPs can be considered a candidate for adaptations. This study revealed about 220 000 SNPs and furthermore 4700 insertions and deletions (InDels) when comparing the *dark-fly* genome with an *OregonR* control (Izutsu *et al.*, 2012). Inconveniently the light raised control group of *dark-fly* perished in 2002. Consequently, a subsequent study reared mixed populations of *dark-fly* and *OregonR* in both dark and light conditions respectively, to reselect dark-adapted traits (Izutsu et al., 2015). Comparing the SNP and InDel analyses showed condition-depended genetic adaptation in about 6% of the genome and rendered 84 candidate genes for dark-adaption These include genes involved in olfaction, detection of pheromones, metabolism of fatty acids and neural development (Izutsu *et al.*, 2015).

Furthermore, the mating fitness of the *dark-fly* strain was tested in a competitive fecundity assay. *Dark-fly* males and females were paired with different wt strains and the offspring were allocated to their parents strains, utilizing transgenic markers (Izutsu *et al.*, 2015). In dark conditions, the *dark-fly* strain dominated over the wt strains, producing more offspring and seemingly preferring other *dark-fly* as mating partners (Izutsu *et al.*, 2015). Successful copulation in *Drosophila* is highly dependent on a functional visual system (Spieth and Hsu, 1950; Markow, 1987), raising the question whether *dark-fly* has developed a new strategy and method to recognize conspecifics.

Hitherto, no detailed analysis of visually guided behaviours has been performed in *dark-fly*.

1.3 Visually guided behaviours in Drosophila

Visual cues contain a high amount of information about the environment that can be crucial for the survival of a species. Vision has evolved independently several times with many organisms dedicating large amounts of energy and parts of their brain to perceiving and processing visual information (Land and Fernald, 1992).

In *Drosophila* vision is a crucial environmental cue and about half of the brain is utilized to process visual cues (Rein et al., 2002). The primary visual sensors in *Drosophila* are the compound eyes with sensory neurons projection into the visual ganglia of the brain. These form distinct neuropils known as lamina, medulla, lobula and lobula plate. Each compound eye contains about 780 optical units termed ommatidia. Each ommatidium consist of eight circular arranged photoreceptors either involved in motion vision (outer photoreceptors R1 – R6) or colour vision (inner photoreceptors R7 – R8). The both pathways are separated in *Drosophila* and can be fully functional independent of each other (Yamaguchi et al., 2008). Compared to other insects like *Apis mellifera* or *Calliphora*, the visual acuity of *Drosophila* is limited. The inter-ommatidial range is approximately 4.5° and *Drosophila* can therefore optically resolve objects that cover more than 8° of the fly's visual field; *Calliphora* and *Apis* mellifera on the other hand can resolve object of 1° angular extension (Borst, 2009; Geurten, et al., 2014; Gonzalez-Bellido et al., 2011).

Drosophila shows a range of visually guided behaviours, that have been studied extensively (Heisenberg and Götz, 1975; Borst, 2009) including positive phototaxis (Carpenter, 1905), optomotor response (Heisenberg and Götz, 1975), initiation of flight and escape response (Tanouye and Wyman, 1980), initiation of landing (Waldvogel and Fischbach, 1991) and walking as well as flying (Katsov and Clandinin, 2008; Mronz and Lehmann, 2008). In *Drosophila* two main approaches can be used to study visually guided behaviour: the manipulation of the visual system or processing of visual cues using the broad genetic toolkit that *Drosophila* provides, and the external removal of

visual cues by either manual manipulation (i.e. covering the eyes) or exposing the flies to total darkness.

This study is focussed on two behavioural strategies that have been shown to be visually driven in *Drosophila*: the locomotion strategy during walking and courtship behaviour.

1.4 Locomotion strategies

Movement through the environment will generate relative motion of all objects, surfaces and edges between the observer and the scene. This apparent movement is known as optic flow (Gibson, 1950). During forward movements the image shift of objects close to the animal travel with a high velocity, while objects further away travel with ever slower velocities. This allows for the extraction of 3D information from the optic flow. However, during purely rotational movements all objects move with the same speed and therefore extraction of 3D information is not possible (Koenderink and Doorn, 1987). Optimizing optical flow is crucial to efficiently extract 3D information from the environment.

Different locomotion strategies for optic flow optimization have evolved in different animals. Prominent examples are the stabilization of the head against external movement in birds (Frost, 2009; Frost, 1978; Katzir et al., 2001), often shown in herons or chickens, or the saccadic strategy. The saccadic strategy consists of long stretches of translational (forward movement) during which 3D information can be extracted. Rotations are reduced to short phases for reorientation with a high rotational velocity, called saccades (Collett and Land, 1975a, 1975b; Geiger and Poggio, 1977). The saccadic strategy has been shown for flying *Apis melifera*, *Calliphora*, *Eristalis tenax*, *Musca domestica*, *Drosophila* and walking *Calliphora* and *Drosophila* (Geurten et al., 2010; Ribak et al., 2009; Schilstra & Hateren, 1999; Srinivasan et al., 1996; van Hateren & Schilstra, 1999). Further, zebra finches during flight and different aquatic species like zebrafish, cuttlefish and seals apply this strategy during swimming (Eckmeier et al., 2008; Geurten et al., 2017; Helmer, 2017). The widespread use of the saccadic strategy in different species and different forms of locomotion illustrates its fundamental importance.

Most insects lack stereoscopic vision and therefore display a distinct form of saccadic strategy with short and fast head saccades followed by body saccades. However, walking Drosophila diverge from this strategy and only shows body saccades (Geurten et al., 2014). Modelling of ommatidial maps revealed a very low visual acuity of *Drosophila* compound eyes compared to those of *Calliphora* and *Apis melifera* rendering head saccades, as described for these species, obsolete for *Drosophila* (Geurten et al., 2014). However, tethered *Drosophila*, in response to visual stimuli still display head saccades (Fujiwara et al., 2016; Williamson et al., 2018), demonstrating that they are physically able to move their head independent from the body. This divergence from the saccadic strategy due to visual constraints raises the question of the influence of the visual system on the locomotion strategy.

1.5 Exploration strategies

All non-sedentary organisms, like *Drosophila*, need to move to gather the resources crucial for survival: food and mating partners. Both of these resources are needed to produce offspring and therefore guarantee a successful survival of the respective species. Furthermore, predators, obstacles and possible noxious areas have to be avoided during the search for resources. As locomotion is the basis of exploration, a change in locomotion strategies due to the availability of visual information might be indicative of a change in exploration strategy.

Many exploration strategies can be described as defined mathematical models, most prominent amongst them the random walk. Random walk models describe a path consisting of a sequence of steps with a random direction independent of the direction of the previous step. The step-length is determined by a Gaussian probability distribution (Pearson, 1905). A prominent example of a specific random walk model is Brownian motion.

During the last decades, the *Lévy flight* has been a candidate to model optimal foraging and exploration strategies. Like Brownian motion, the Lévy flight is a specialised random walk model. It is characterised by a heavy-tailed probability distribution determining the step-length and giving *Lévy flight* a bias towards longer step-lengths compared to

classical random walk models. A typical *Lévy flight* consists of long stretches of forward movement and short pausing phases deciding a new direction (Mandelbrot, 1982). Due to the longer step-length, *Lévy flight* has an advantage over classical random walk models in finding randomly distributed objects in a defined area and time frame (Cole, 1995). This feature makes *Lévy flight* a candidate for an optimal foraging strategy.

Indeed, Lévy flight has been used to model the foraging and exploration strategies of an array of different organisms: typical examples can be found in T-cells, foraging albatrosses, different marine predators, bees and human hunter-gatherers (Harris et al., 2012; Humphries et al., 2010; Humphries et al., 2012; Korobkova et al., 2004; Raichlen et al., 2014; Reynolds et al., 2007; Sims et al., 2008, 2014; Tu & Grinstein, 2005).

Drosophila has been shown to apply *Lévy flight* during odour tracking while flying and in walking behaviour. While in flight, *Drosophila* shows near optimal *Lévy flight*, during walking is can still be detected but far from optimal (Reynolds, 2015; Reynolds & Frye, 2007).

One of the main characters of *Lévy flight*, the separation into phases of forward movement and reorientation phases is shared by the saccadic strategy: the reorientation phases are corresponding with the saccades found in the saccadic strategy. As elaborated above, the saccadic strategy is utilized to optimise optic flow. Due to its similarities, *Lévy flight* will not interfere with the 3D-information generated by optic flow. In the absence of visual cues the constraints that favour a saccadic strategy are lost. This raises the question if both the saccadic strategy and *Lévy flight* will be subject to change in a light-deprivation context. If behavioural adaptations of this strategy are in fact adapting to the absence of visual cues, the dark-raised *Drosophila* strain *dark-fly*, maintained in darkness for over 1500 generations, would be a sufficient model.

1.6 Drosophila courtship

Courtship in *Drosophila* is characterized by a series of highly stereotyped and genetically hard-wired behaviours (*Figure 1*) performed in a variable sequence before mating is initiated (Sturtevant, 1915; Manning and Bastock, 1955; Hall, 1994; Yamamoto and

Koganezawa, 2013). This intricate courtship ritual involves the mutual exchange of signals in utilizing different sensory modalities serving the purpose of communicating species and sex recognition, the state of receptivity and the display of abilities (Bennet-Clark & Ewing, 1968; Greenspan & Ferveur, 2000; Kyriacou & Hall, 1982; Ritchie et al., 1994).

1.6.1 Courtship behaviour

Unlike in most other flies, especially in the super family *Cyclorrhapha, Drosophila* courtship is done walking, rather than flying.

Upon detecting a female, the male starts orienting its body axis towards the female and starts following her. Commonly, while following the male starts tapping the female abdomen using his forelegs (Hall, 1994). As a mandatory step in courtship the male extends the wing, closest to the female and starts producing the species-specific courtship song (for a more detailed description see *1.6.2 Courtship song*)(Schilcher, 1976).

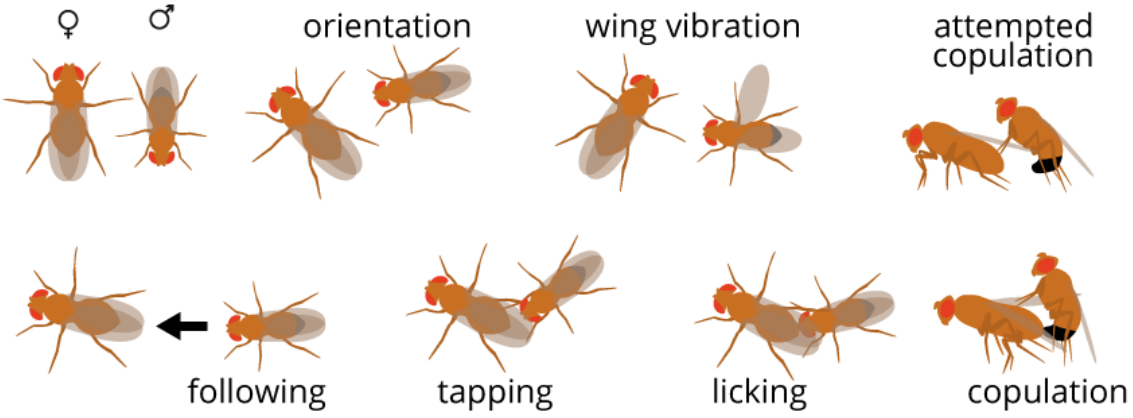


Figure 1 Canonical courtship behaviours of *Drosophila* males. Typical behaviours displayed by males during courtship. Orientation, following, tapping, licking, wing vibration, attempted copulation and successful copulation.

After perceiving the male courtship song and as a reaction to the male courtship behaviour, the female reduces her locomotion speed, signalising her receptivity (Ewing,

1983; Schilcher, 1976). Subsequently, the male is licking the female abdomen and will attempt copulation. Copulation can be only successful, if the female raises her wings and opens her genital plate. If the female rejects copulation, the male either retracts or resumes courting. Furthermore, the females can in turn also actively stimulate male courtship by partial ovipositor extrusion, emission of droplets from the ovipositor tip or abdominal preening (Lasbleiz et al., 2006; van Dijken et al., 1987).

Drosophila courtship behaviour is mediated by the integration of different sensory modalities: vision, gustation, olfaction and audition (Ralph J. Greenspan and Ferveur, 2000; Billeter and Levine, 2013; Auer and Benton, 2016). The decision of the male to initiate courtship is thought to be influenced by both the olfactory and gustatory system (Dweck et al., 2015; Thistle et al., 2012; Toda et al., 2012). If any of these sensory modalities is absent, courtship was consistently shown to be impaired, showing that all of these are needed to guarantee successful copulation. The importance of the different sensory systems varies with the courtship distance: to locate and approach a possible mating partner and subsequently courtship initiation, both the visual and olfactory system are needed (Agrawal et al., 2014; Tompkins & Hall, 1981), the volume of the courtship song is also dependent on distance estimation and reliant on visual cues (Kohatsu & Yamamoto, 2015; Pan et al., 2012). To maintain contact to the female during courtship males need intact vision, since courtship success does rely on the male's ability to follow (Cook, 1979; Krstic et al., 2009; Sakai & Ishida, 2001).

With increasing proximity to the female, other sensory signals become prevalent. Close contact courtship is mostly driven by olfactory and gustatory cues, signalling receptivity but also gender and species of the potential mate (Dweck et al., 2015; Kurtovic et al., 2007; Lu et al., 2012; Thistle et al., 2012; Toda et al., 2012). This information is transmitted *via* both volatile and non-volatile pheromonal cues (Cobb & Jallon, 1990; Ferveur, 2005; Kohl et al., 2015). Female-specific pheromones like 7,11-dienes or methyl laureate have been reported to stimulate male wing extension and copulation attempts (Antony and Jallon, 1982; Dweck *et al.*, 2015). The male-specific volatile pheromone 11-*cis*-veccenyl acetate (cVA) is transferred to the female during copulation and subsequently reduces the attractiveness of recently mated females to other males

(Kurtovic, Widmer and Dickson, 2007). If no visual cues are available, olfactory cues are necessary for the male to position himself behind the female and find the correct location to initiate copulation (Kimura et al., 2015). Contact chemosensation, i.e. gustation, has been reported to stimulate ipsilateral wing extension and following behaviour in males (Kohatsu et al., 2011; Kohatsu & Yamamoto, 2015). Loss of olfaction or one of the gustatory receptors involved in detection of female pheromones (Gr68a and Gr39a) does not prevent male courtship behaviour, but does significantly decrease male courtship success (Bray & Amrein, 2003; Markow, 1987; Watanabe et al., 2011).

The auditory system is mainly needed to mediate and receive courtship songs. Females, upon perceiving the male courtship song show increased arousal and initiate pausing to let the male approach and proceed with close range courtship behaviours (Schilcher, 1976; Ewing, 1983). Males, upon hearing courtship song not produced by themselves still maintain courtship behaviour (Corthals *et al.*, 2017). *Drosophila* males lean their courtship song from con-specifics but can be even trained by speakers playing artificial courtship songs (Li et al., 2018; Riabinina et al., 2011). This suggests that the system of courtship songs itself allows for a certain flexibility. If no visual cues are available, auditory cues can act as long-distance signals to enable the location and direction of the female (Ejima and Griffith, 2008). While a deficiency of auditory functions only shows a minor effect on male courtship success, female seems highly dependent on perception of auditory cues (Markow, 1987).

Contrary to other members of the *Drosophilidae* family, *Drosophila melanogaster* still reproduce in darkness, indicating vision is not a mandatory prerequisite for successful courtship (Spieth and Hsu, 1950). However, several studies show disputed courtship behaviour in the absence of visual cues and visually deprived or blind males are at a disadvantage when competing with wt males (Connolly et al., 1969; Hirsch & Tompkins, 1994).

In a recent study the LC10 visual projection neurons have been implicated in mediating all of these behaviours: orientation, maintaining proximity to the female and ipsilateral wing extension are all impaired in males with silenced LC10 neurons (Ribeiro *et al.*, 2018a). These neurons respond to visual stimuli matching the size and speed a female

Drosophila would normally display (Ribeiro *et al.*, 2018a). Interestingly, the potency of the LC10 controlled wing extension is enhanced in a state of arousal, mediated by the male-specific P1 neurons (Kimura, Hachiya et al., 2008; Ribeiro et al., 2018; von Philipsborn et al., 2011). These studies provide evidence of a LC10 driven pathway transmitting visual information to the neural courtship circuits in males and further indicate the importance of the visual system in guaranteeing successful courtship behaviour.

1.6.2 Courtship song

During courtship *Drosophila* males produce a species-specific courtship song *via* unilateral wing extension (*Figure 2 A*). Acoustic signals in *Drosophila* can only be used as short-range signals; due to the rather small wing size, pressure waves are produced ineffectively and the particle velocity of the produced sound decreased sharply after only a few millimetres (Göpfert and Robert, 2002; Billeter and Levine, 2013).

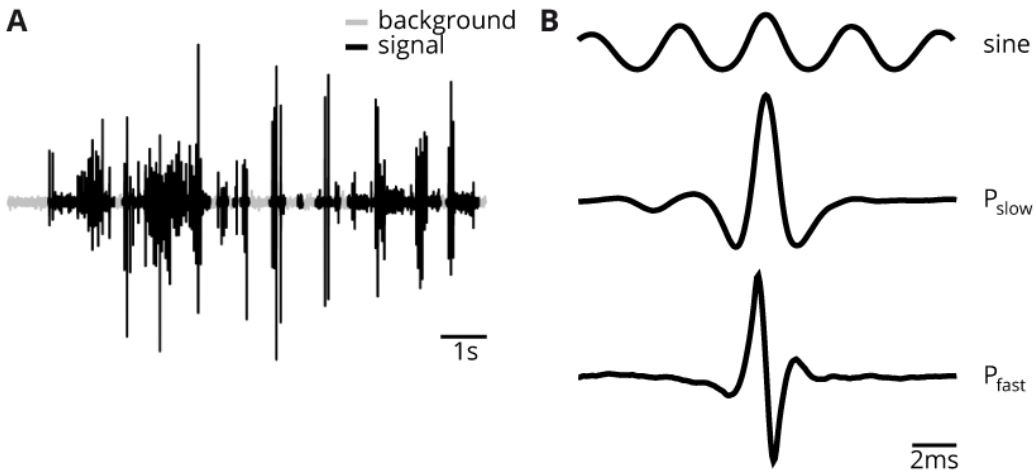


Figure 2 Courtship song of Drosophila melanogaster. (A) Example of song recording. Typical song recoding showing both background noise (grey) and signals that can be further analysed. **(B) Shapes of courtship song.** Drosophila courtship song can be divided in three types: sine song (top), Pslow (middle) and Pfast (bottom). Every song type shows their own, distinct function.

Drosophila courtship song can typically be divided into one type of sine song and two types of pulse song, P_{fast} and P_{slow} (Clemens et al., 2018; Greenspan & Ferveur, 2000; Kyriacou & Hall, 1982; Ritchie et al., 1999) (*Figure 2 B*). While the sine song joint with the interpulse interval communicates the species to possible mating partners, both pulse songs are used to arouse and attract the female (Clemens et al., 2018; Greenspan & Ferveur, 2000). The use of pulse song modes correlates with distance to the female: P_{slow} is used for close range courtship and a rather fainter sound while the loud P_{fast} is used at a larger distance to the female (Clemens et al., 2018). Intra-specific female mate choice was reported to be correlated to the total amount of pulse song per time unit (Talyn and Dowse, 2004). Since production of courtship song by wing vibration is rather energy consuming it is thought to be an honest indicator the male's fitness and health status. Additionally, the wing vibration might serve as a fan-like transfer of pheromones during courtship (Talyn and Dowse, 2004).

1.7 Dark-fly as a model for micro-evolution

So far, behavioural micro-evolution was mainly observed in field studies, having the disadvantage of long generation cycles and an uncontrolled environment. The dark-raised *Drosophila* strain presents the possibility to study behavioural adaptations of visually-guided behaviours in a controlled environment, that can easily be manipulated. This study aims to assess the adaptation of both courtship behaviour and the locomotion strategy to the absence of visual cues as both behaviours have been shown to be heavily dependent on a functional visual system.

To date, neither of these strategies have been extensively studied in dark conditions. Courtship behaviour was shown to be disrupted in wt *Drosophila* when assessed in darkness (Sakai et al., 1997), however, *dark-fly* dominates over wt in dark conditions in a competitive fitness assay, producing more offspring and preferring *dark-fly* as mating partner (Izutsu *et al.*, 2015). This suggests an adaptation of *dark-fly* courtship strategy to long-term light deprivation, allowing them to localize conspecifics more efficiently. This could either involve a divergence from the canonical courtship behaviours, a change in courtship song or a change in exploration and locomotion strategy.

As explained above, *Drosophila* utilizes a saccadic locomotion strategy, optimizing 3D-information generated by optic flow by reducing the time spent with rotations. This strategy is clearly influenced by the visual system, as *Drosophila*, displaying a highly reduced visual acuity compared to *Apis melifera* and *Calliphora*, lacks the head saccades characterizing this strategy in insects. Abolishing visual cues might therefore lead to a relinquishment of the saccadic strategy and the emergence of new strategy, superior in darkness.

In this study, the *dark-fly* strain was tested in different courtship assays while courtship songs were simultaneously recoded. Furthermore, a detailed locomotion analysis was performed. To further understand the progression of possible adaptations a second dark-raised strain *Goe-dark* was established and examined for 15 generations in darkness.

2. Materials

2.1 Media

2.1.1 Standard apple juice Drosophila medium

fresh yeast	500 g
sugar	500 g
flour	250 g
salt	20 g
propionic acid	30 ml
apple juice	1000 ml
agarose	60 g

Water was added to reach a total volume of 7 l, medium was prepared in a Systec mediaprep cooker (Systec GmbH, Lohfelden, Germay) filled in vials and sealed with mite-proof plugs (K-TK e.K., Retzstadt, Germany; #1002). The medium recipe is also described in (Corthals *et al.*, 2017).

2.1.2 Agarose medium for locomotion experiments

agarose	5 g
glucose	5 g
deionized water	500 ml

Ingredients were mixed in a glass bottle and brought to boil using a microwave. Medium was stored at 4°C until further used. Before every experiment medium was heated until liquid, filled into the arena and cooled down to room temperature until reaching a firm state at room temperature.

2.2 List of used materials

Kits

DNeasy Blood&Tissue	Quiagen, Valencia, CA, USA
QuantiTec Reverse Transcription	Quiagen, Valencia, CA, USA
ZR Tissue and Insect RNA MicroPrep	Zymo Research Europe GmbH, Freiburg, Germany

Chemicals

Agarose food grade BioChemica	AppliChem GmbH, Darmstadt, Germany
Anhydrous D-glucose BioChemica	AppliChem GmbH, Darmstadt, Germany
Biozym LE Agarose	Biozym Scientific, Hessisch-Ohlendorf, Germany
Chemosolute® Ethanol absolute	Th. Geyer Ingredients GmbH & Co. KG, Höxter, Germany
iQ™ SYBR® Green Supermix 2x	Bio-Rad Laboratories GmbH, Munich, Germany
Proprionic acid	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sigmacote	Sigma-Aldrich, St. Louis, Missouri, USA

Electronics

AxioCam MRc	Carl Zeiss AG, Oberkochen, Germany
Dual Microphone Supply Type 5935	Brüel & Kjær, Nærum, Denmark
Microphone Type 4165	Brüel & Kjær, Nærum, Denmark
Hercules Optical Glass webcam	Guillemont Cooperation S.A., Carentoire, France
Kayeton KYT-U200-MR01	Kayeton Technology Co., Shenzhen, China
LUXEON SunPlus dim-red LED	Lumileds Holding B.V., Amsterdam, Netherlands
Pollin infrared LED	Pollin Electronic GmbH, Pförring, Germany
xiQ MQ042RG-CM	Ximea GmbH, Münster, Germany

Lab equipment

DAM2	TriKinetics Inc., Waltham, Massachusetts, USA
MyiQ Single color RT PCR Cycler	Bio-Rad Laboratories GmbH, Munich, Germany
SteREO Lumar.V12	Carl Zeiss AG, Oberkochen, Germany
Systec mediaprep cooker	Systec GmbH, Lohfelden, Germay
Ultimaker 3D printer	Ultimaking Ltd., Geldermalsen, Netherlands

Lab utensils

Blu Tack	Borstik GmbH, Borgholzhausen, Germany
Eppendorf Tubes® 3810X 1,5 ml	Eppendorf AG, Hamburg, Germany
Måla, silver	Inter IKEA Systems B.V.. , Delft, NEtherlands
mite-proof plugs	K-TK e.K., Retzstadt, Germany

PW6 titanium white	DF.FR.Schoenfeld GmbH & Co, Düsseldorf, Germany

Software

AxioVision SE64	Carl Zeiss AG, Oberkochen, Germany
DAMSystem308	TriKinetics Inc., Waltham, Massachusetts, USA
Etho-Scorer	Geurten & Kuhlemann
FlySongSegmenter	https://github.com/FlyCourtship/FlySongSegmenter
MATLAB R2102b	The MathWorks Inc., Naticks, Massachusetts, USA
Python 2.11.7	
StreamPix	NorPix Inc., Montreal, Quebec, Canada

2.3 Flystrains

Strain	**Genotype**	**Source**
dark-fly	+/+;+/+/;+/+	provided by Dr. Naoyuki Fuse
dark fly light	+/+;+/+/;+/+	provided by Dr. Naoyuki Fuse
Goe-dark	+/+;+/+/;+/+	generated by me, based on *OR*
OregonR	+/+;+/+/;+/+	Bloomington #5
ora	ort^{1}	Bloomington #1133
sineoculis	so^{D}/Cyo	Bloomington #4287
sol	w[*] P{w[+mC]=EP}sol[G1689]	Bloomington #63253

3. Methods

3.1 Animal handling

3.1.1 Generation of dark-fly

To investigate the genetic adaptation to environmental conditions the group of Prof Dr Mori at Kyoto University started raising a *Drosophila* wildtype strain *OregonR* in dark conditions since 1954 (see introduction). To this end the offspring of a single *OregonR-S* pair was divided into six groups, three were raised in dark conditions and three were raised as control lines in a 12:12 dark:light cycle (Fuse et al., 2014; Izutsu et al., 2012). Since the original control lines all perished by 2002, we reinstated *dark-fly* in light conditions further referred to *dark-fly light* (***Fehler! Verweisquelle konnte nicht gefunden werden.*** *A*). Locomotion analysis is shown for *dark-fly* in both dark and light conditions and for *dark-fly light* after being raised in a 12:12 dark:light cycle for 5 generations (*dark-fly light 05*)(*Figure 3*).

3.1.2 Generation of Goe-dark

To assess whether behavioural adaptation is a slowly progressing or rather instant effect, we started maintaining *OregonR* flies in dark conditions, further referred to as *Goe-dark* (*Figure 3 A*). Numbers after the strain name indicate the generation of being raised in certain conditions.

We recorded locomotion behaviour for every generation between *Goe-dark01* and *Goe-dark10*, followed by intervals of 5. Locomotion analysis is shown for *OregonR* in both dark (*Goe-dark 01)* and light conditions, for generation 5, 10 and 15 (*Goe-dark 05, Goe-dark 10, Goe-15*) in dark conditions. Locomotion data for all progressing generations from 01 to 15 can be found in the supplements. Flies of the generations 5 and 10 were also tested in light conditions (*Goe-dark light 05, Goe-dark light 10*) (*Figure 3 B*).

3.1.3 Fly rearing and basic experimental conditions

Flies were maintained at 18°C and 60% humidity with either a 12h:12h dark:light cycle or a dark:dark cycle on apple juice medium. Dark-flies were handled under dim red light (λ_{min} = 720nm; Lumileds Holding B.V., Amsterdam, Netherlands; #L1SP-FRD00035R0000). *Drosophila's* photoreceptors cannot detect wavelengths over 700nm since the spectral sensitivity of R6, the photoreceptor absorbing in the longest wavelength range (λ_{max} = 510nm), drops to zero at around 650nm (Salcedo et al., 1999; see QUERVERWEIS). For transport of *dark-flies* the vials were wrapped in aluminium foil and put in styrofoam boxes. To generate socially isolated males, flies were removed from the vials 24h before experiments and transferred to 1,5 ml microtubes (Eppendorf Tubes® 3810X, Eppendorf AG, Hamburg, Germany; #0030125150) containing apple juice medium and were sealed with a cotton wool plug.

Unless stated otherwise flies were tested at the age of 5-7days.

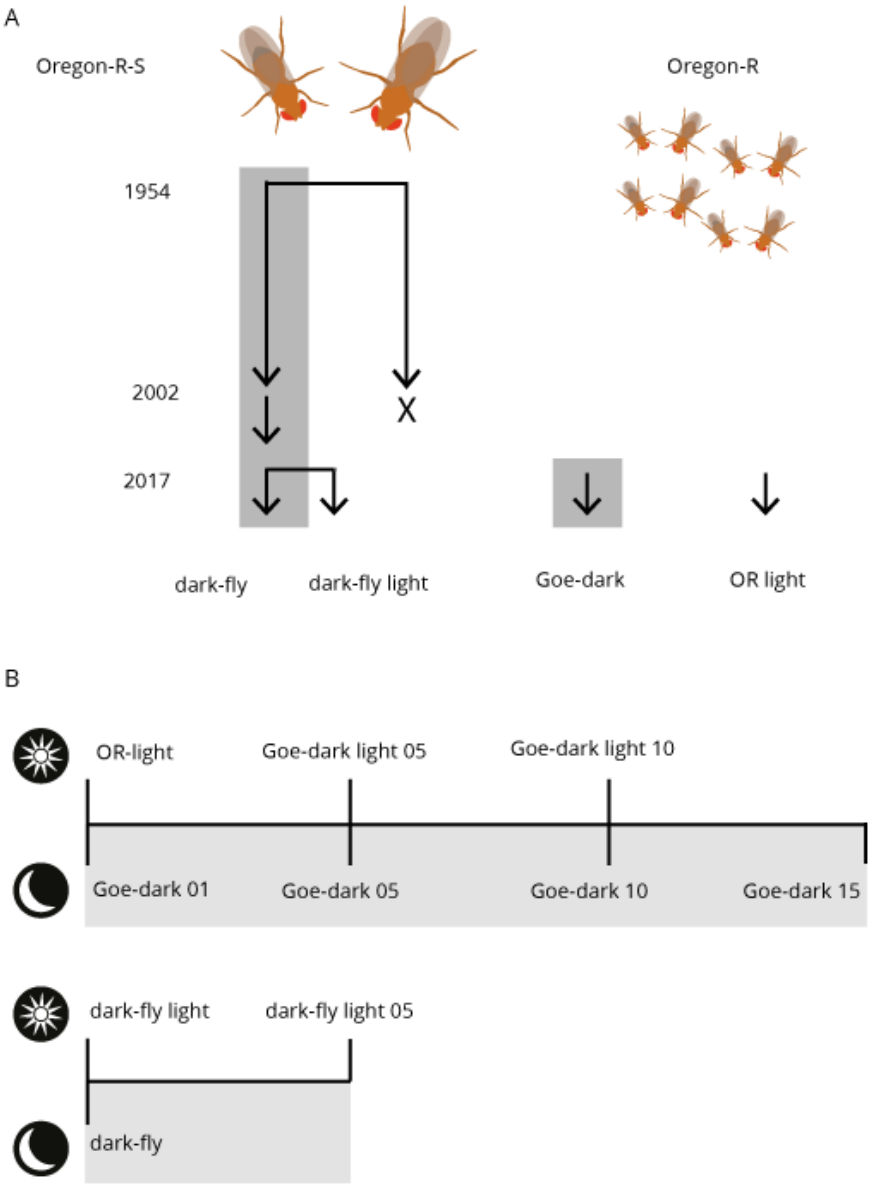


Figure 3 **Generation of dark-adapted *Drosophila* strains and their controls. (A) Dark-fly and Goe-dark strains.** The dark-fly strain was generated in 1954 by separating the offspring of one *Drosophila OregonR-S* pair and rearing them in dark and light conditions. The light control strain was lost in 2002. After acquiring the dark-fly strain in 2017 they were raised in both dark light conditions. The *Goe-dark* strain was established by raising *OregonR* flies in dark conditions. **(B) Experimental design.** Oregon-R flies were tested first in light and subsequently in dark conditions (*Goe-dark 01*) and further maintained in darkness. Locomotion analysis will be shown for generation 5 (*Goe-dark 05*), generation 10 (*Goe-dark 10*) and generation 15 (*Goe-dark 15*). At generations 5 and 10 locomotion analysis was also done in light conditions (*Goe-dark light 05* and *Goe-dark light 10*). *Dark-fly* was both tested in dark and light (*dark-fly light*) condition. After raising *dark-fly light* for 5 generations in light conditions, locomotion was again assessed (*dark-fly light 05*).

3.1.4 Analysis of body pigmentation

Visual comparison of dark-flies and OR flies showed obvious differences in pigmentation. To document pigmentation differences we used a SteREO Lumar.V12 stereomicroscope (Carl Zeiss AG, Oberkochen, Germany) equipped with a camera (AxioCam MRc; Carl Zeiss AG, Oberkochen, Germany). Picture acquisition was done using the AxioVision SE64 software (Carl Zeiss AG, Oberkochen, Germany). The observed flies were 5 days old and raised in mixed-sex group of 10 flies (5 males, 5 females). To ensure that differences in pigmentation are not caused by rearing or light conditions, flies of both strains were either raised under a light:dark cycle or constant darkness. In this case flies were anaesthetized with CO_2 prior to data acquisition.

3.2. Behavioural Analysis - Locomotion

3.2.1 Acquisition of locomotion data

To record the free walking behaviour of *Drosophila* individual flies were transferred into a circular arena with a 40 mm diameter filled with 1% agarose/1% glucose using a suction tube. The arena was closed with an anti-glare acrylic glass pane covered with Sigmacote (Sigma-Aldrich, St. Louis, Missouri, USA; #SL2) to prevent the flies from walking on the ceiling, creating a gap of 2mm between the medium and the pane. This distance allows for the fly to freely walk but not to start flying. Hence, the flies‘ wings are left intact in the setup and thereby possible alterations of free walking behaviour are avoided (as described in Corthals et al., 2017;*Figure 4*).

The arena was produced using an Ultimaker 3D printer (Ultimaking Ltd., Geldermalsen, Netherlands) and data was recorded using StreamPix software and a xiQ camera (MQ042RG-CM, Ximea GmbH, Münster, Germany) at 500 frames per second (fps). The arena was illuminated from below with infrared LEDs (Pollin Electronic GmbH, Pförring, Germany; #351090) (*Figure 4 B*), which allowed us to record in dark conditions. For recordings in light conditions additional LEDs within the spectrum of visible light were placed around the arena.

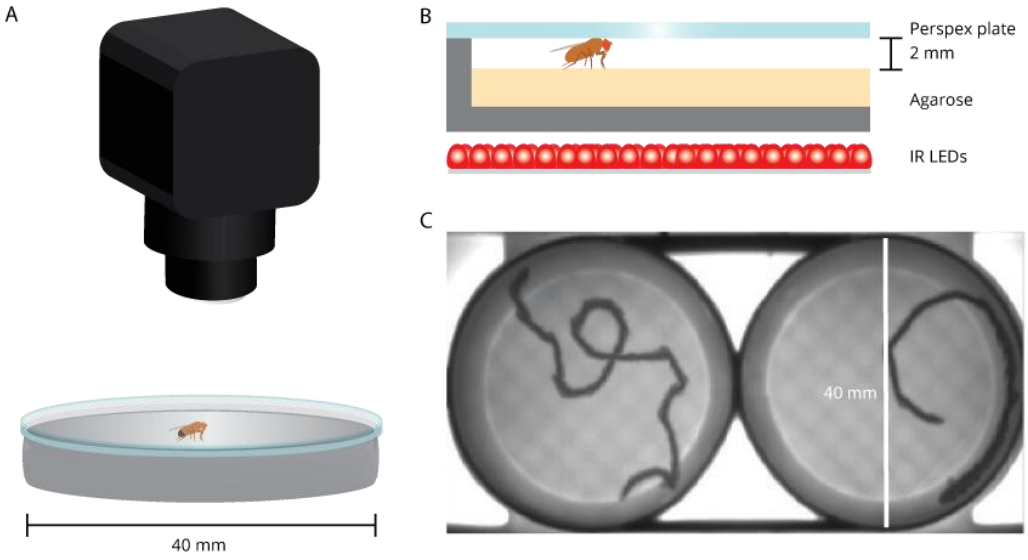


Figure 4 Data acquisition for locomotion analysis. (A) Model of the arena used for locomotion experiments. The arena consists of a circle of 40 mm diameter filled with 1% agarose and covered with an anti-glare Acrylic glass pane, leaving a space of 2 mm for the fly to move [zoom-in of the arena in **(C)**]. Experiments are recorded *via* a highspeed camera placed above the setup. To facilitate tracking, the arena is illuminated from below with an array of infrared LEDs light conditions additional visible light sources are positioned in the proximity. **(B) Zoom-in cross-section of the arena.** The arena is filled with 1 % agarose and covered with an anti-glare acrylic glass pane covered with Sigmacote to prevent the flies from walking on the ceiling. Arrays of infrared LEDs are placed under the arena for illumination during dark conditions and to facilitate tracking. **(C) Example trajectory.** Example trajectories are calculated as a minimum of every pixel in each frame. If this is done for a complete movie over 5001 frames the image of the fly will overlap, rendering a dark line, displaying the trajectory.

3.2.2 Tracking analysis

To acquire walking trajectories that will provide us with location information for every frame of the videos a MATLAB-based tracing software provided by Dr Bart Geurten was used.

First, a region of interest (ROI) is defined by calculating the minimal background over all frames. Since the darkest pixels in a frame are always descendant form the fly, the trajectories of the flies over the whole length of the video could be obtained (*Figure 4 C*). To identify the fly in every frame the maximum background is subtracted, and the image is binarized. If the size of an ellipsoid object lies within a predetermined threshold it was

detected by ellipse detection in the Hough transform (Xie & Ji, 2002; Duda & Hart, 1972). The resulting trajectories provide us with the position and orientation of the fly for every frame in the Cartesian coordinates *x* and *y*.

If the automatic tracing algorithm fails to identify the fly, it interpolates the trajectory. Subsequently, the result is presented to a human observer, who decides whether the interpolation was accurate, or the result not usable.

3.2.3 Analysing 2D velocities

To characterize the different features of locomotion the flies' trajectories are divided into the three 2D velocities thrust (along the caudal-cranial, forward movement), slip (orthogonal to the thrust vector, sideways movement) and yaw (rotation around the norm of the plane defined by slip and thrust) based on a fly-centred coordinate system (*Figure 5*). To transform the moved distances from pixel to mm, the length of the behavioural setup was taken as scale and subsequently measured for every video. Trajectories were smoothed using a Butterworth filter to avoid digitisation noise from the automatic object recognition.

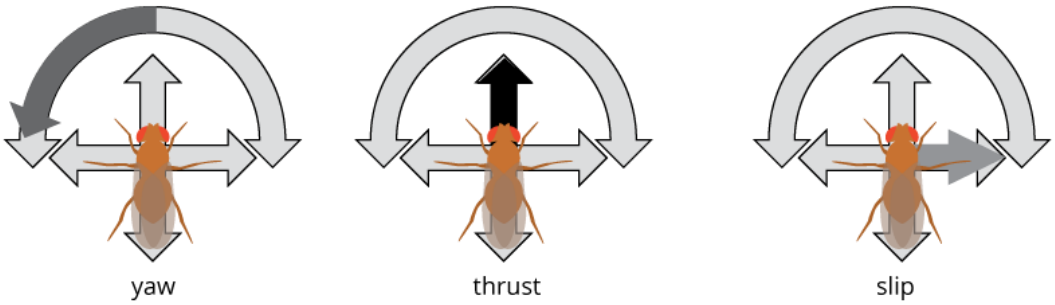


Figure 5 The three movement directions extracted from a 2D walking trajectory. 2D trajectories allow for the extraction of three movement directions in a fly-centred coordinate system: yaw, thrust and slips. Yaw is defined as a rotation around the normal vector of the thrust-slip plane. Thrust is the movement along the caudal-cranial axis. Slip is the movement orthogonal to the thrust axis.

The three velocities were calculated from the difference in position and orientation between two frames. For this the image-centred Cartesian coordinates (top left corner is 0,0) derived from the tracing analysis were transformed into a fly-centred coordinate system in which the *y*-axis represents the thrust and the *x*-axis the slip movement. Differences in position compared to the following frame are calculated by using vector

analysis and render the velocities for thrust (y-axis) and slip (*x*-axis). The angle at which the orientation from one frame to the next is rotated provides the angular velocity of the yaw movement. Using the Fick rotation matrix, the coordinate system is also rotated to be aligned with the orientation of the fly in the next frame.

Rotations were defined as saccades if they reached a yaw velocity threshold of 200deg/sec. Saccades that were not captured completely and either start or end are missing (broken saccades), were excluded from the analysis.

3.2.4 Prototypical Movements

To describe the syntax of locomotion prototypical movements (PMs) for each *Drosophila* strain were computed. Prototypical movements are reoccurring movement patterns, consisting of distinct combinations of movement directions and their respective velocity (Braun et al., 2010). The 2D trajectories obtained in this study allow for the extraction of three movement directions: yaw, thrust and slip (*Figure 5*).

To identify the most common velocity combinations two clustering algorithms, agglomerative hierarchical clustering and k-means clustering were utilized (MacQueen, 1967; Milligan & Cooper, 1987).

To narrow down the number of PMs the agglomerative hierarchical clustering approach was used. This approach is only feasible for smaller data sets; therefore, the data was divided into 200 chunks in a round-robin fashion. This identified less than 20 possible PMs which were then tested with k-means clustering for the whole data set. To find the number of PMs best representing my data set, the quality and stability were used as operational criteria. Stability was tested by omitting 10%, 25% and 50% of the data in a round-robin fashion to test whether the clustering result was persistent. Quality of the clustering was calculated as the distance between the different PMs divided by their individual density.

3.2.5 Exploration rate

For each recorded fly I obtained 10 sec of freely walking and traced the trajectory in the video *post hoc* analysis. To analyse the percentage of the arena area covered in a 10 s time interval, a mechanosensory field overlaying the fly and including mechanosensory organs was calculated. The mechanosensory field allows them to discover possible objects in their environment. In normal conditions *Drosophila* can use its visual field, however, in dark conditions only the mechanosensory field will produce valid information about their surroundings.

3.2.6 Probability density

Through tracing of the flies' trajectories, the Cartesian coordinates *x* and *y* were obtained and subsequently transformed into polar coordinates with the polar angle θ and the radius *r*. For each fly the histogram of r was calculated and then used to produce a median histogram for each strain. Afterwards we normalized the histogram for every bin, then normalized so that the integral of the histogram is 1. This renders a probability density for the circular arena (diameter: 40 mm).

3.2.7 *Tōhoku* drift

The additional area covered by the drifting movement of *dark-fly* was determined in three different ways. **I**) The simplest mode was to calculate the summed trajectory (see *Figure 4 C*) and binarize it, using the contrast threshold (see *3.2.2 Tracking analysis*). The obtained number can be defined as the exploration rate. The body surface area is also directly determined by our automatic tracker. This allows us to calculate a median body surface for each fly individually, as well as the median body long axis. Using these two parameters, an ellipse with the major axis identical to the median long axis of the fly can be defined. The surface of the ellipse is therefore equal to the median body surface of the respective fly. **II**) The tracking analysis extracted the coordinates and orientation of each individual fly for every single frame, allowing us to orient the obtained ellipse accordingly. Thereby, small arena differences, such as appendages (e.g. legs, antennae)

are eliminated, but possible benefits of the orientation of the animal during locomotion can still be observed. **III**) As a null model we used a circle with a surface identical to the ellipse. As a circle has no observable orientation, moving it along the trajectory would render the same amount explored of explored area as the ellipse, except of possible orientation bonuses. The difference of the area covered by the ellipse and covered by the circle amounts to a drifting motion referred to as *Tōhoku* drift.

3.2.8 Circadian rhythm

Circadian rhythm was assessed using the *Drosophila* Activity Monitoring System (DAM2, TriKinetics Inc., Waltham, Massachusetts, USA). Single *Drosophila* males were individually put in glass tubes (diameter: 3 mm; lenght: 70 mm) that were filled with standard fly food medium (see chapter *2.1.1* Standard apple juice Drosophila medium) on one end and sealed with a gas permeable cap. The tubes were inserted in an incubator with a dark:dark cycle that was switched to a 12:12 dark:light cycle after four days of recording. Activity was measured by interruptions of an infrared beam and were automatically counted for 7 days with the DAMSystem308 software (TriKinetics Inc., Waltham, Massachusetts, USA). For analysis the first 24 h of recording were discarded to avoid behavioural changes resulting from relocation of the flies. The data set used for analysis consisted of three days of a dark:dark cycle followed by three days of a dark:light cycle.

Data analysis was done using a customized MatLab script (R2012b, The MathWorks Inc., Naticks, Massachusetts, USA). Sleep was defined by phases of inactivity for at least 5 min, and activity by the number of beam crossings in a 30 min interval.

3.3 Behavioural Analysis – Peripheral Auditory Functions

3.3.1 Laser-Doppler-Vibrometry

Analysis of hearing ability was done by Dr Thomas Effertz (Deparment of Cellular Neurobiology & UMG, Göttingen) utilizing Laser-Doppler-Vibrometry. Both female and male *Drosophila* were fixed to a focus holder (Gras, 2014) using wax, and the hearing

ability was determined by measuring vibrations of the antennal sound receiver (Göpfert and Robert, 2002)

Sound receiver vibrations were measured at the top of the arista, using a PSV-400 Laser-Doppler-Vibrometer (Polytec GmbH, Waldbronn, Germany). Sound stimulation was archived by broadcasting pure tones *via* a loudspeaker positioned behind the animal. Stimulus amplitudes were matched to the individual best frequencies. Best frequencies were determined from the power spectrum of the arista's vibration in the absence of sound (Effertz et al., 2011). To determine compound action potentials (CAP) of auditory receptor neurons electrophysiological recordings were performed using an etched tungsten electrode positioned next to the auditory nerve, between head and antenna (Nadrowski et al., 2008; Kamikouchi *et al.*, 2009).

3.4 Behavioural Analysis – Courtship Behaviour

3.4.1 Sound recordings

Male courtship songs (CS) were recorded in presence of females under both dark and light conditions using a microphone (Brüel & Kjær, Nærum, Denmark; Type 4165) placed under the arena and covered with a fine mesh located in a soundproof chamber. The recorded acoustic signals were amplified (Brüel & Kjær, Nærum, Denmark; Dual Microphone Supply, Type 5935), band-pass filtered (70-5,000 Hz; model 3550 filter, Krohn-Hite) and instantly digitised with a sampling frequency of 44,100 Hz. For every group over 80 min of courtship song was recorded, adding up to a total of over 5.5 h of data to analyse.

3.4.2 Analysis of Courtship Songs

Recorded courtship songs were segmented using the open-source software *FlySongSegmenter* (Arthur et al., 2013). The software automatically detects sine song and both types pulse songs P_{fast} and P_{slow} (Clemens et al. , 2018). Individual pulses are detected by utilizing the continuous wavelet transform (Mallat, 2008). To identify trains of sine song, a multitaper spectral analysis was employed (Walden, 1993). To exclude noise of fly handling, the analysis window was set at 90sec after the start of the recording.

The analysed parameters were latency to first courtship song performance, duration of courtship song, the median interpulse interval (IPI), the fraction of sine song and P_{fast} pulse song and the amplitudes of P_{fast} and P_{slow}, indicating the volume of the produced song. Significances were determined using a Kruskal-Wallis test.

3.4.3 Single Courtship Assay

To assess courtship behaviour in *Drosophila*, a pair of a virgin female and a socially isolated male were put together in an arena (diameter: 10 mm; height: 3 mm) placed over a microphone (Type 4165, Bruel&Kjær) covered with a fine mesh and covered with an anti-glare acrylic glass plate covered with Sigmacote (Sigma-Aldrich, St. Louis, Missouri, USA; #SL2) used to prevent the flies from walking on the ceiling (*Figure 6 A*). Behaviour was recorded at 25 fps using either a Hercules Optical Glass webcam (Guillemont Cooperation S.A., Carentoire, France) or Kayeton KYT-U200-MR01 (Kayeton Technology Co., Shenzhen, China). We recorded in both light and dark conditions (*Figure 6 B*) using indirect illumination with a lightbulb (light conditions; DIAG GU10 1X3W) placed above the arena or infrared LED-arrays arranged around the arena (*Figure 6 A*). Data acquisition was done with a customised Ubuntu bash-script (using *arecord* and *streamer*) and compressed with *avconv*.

Flies were introduced into the arena using a suction pipette and recording was started directly after. Recordings were done for at least 5:30min, for analysis the first 30 sec were discarded to obtain an analysis window of 5min. Analysis was done frame-by-frame using the open-source Python-based tracking software *Etho-Scorer* (by Geurten & Kuhlemann).

We were not able to identify and distinguish all of the previously described canonical courtship behaviours (Hall, 1994; Sakai et al. , 1997) since several of those often occur simultaneously (i.e. orientation, following and wing extension). Due to the video resolution and recording angle, it was not possible to reliably identify both tapping and licking behaviour. Therefore, next to the classical courtship behaviours wing extension, copulation attempt and mating, new categories were defined. Male courtship behaviour was further distinguished into locomotion, wing extension (correct and incorrect),

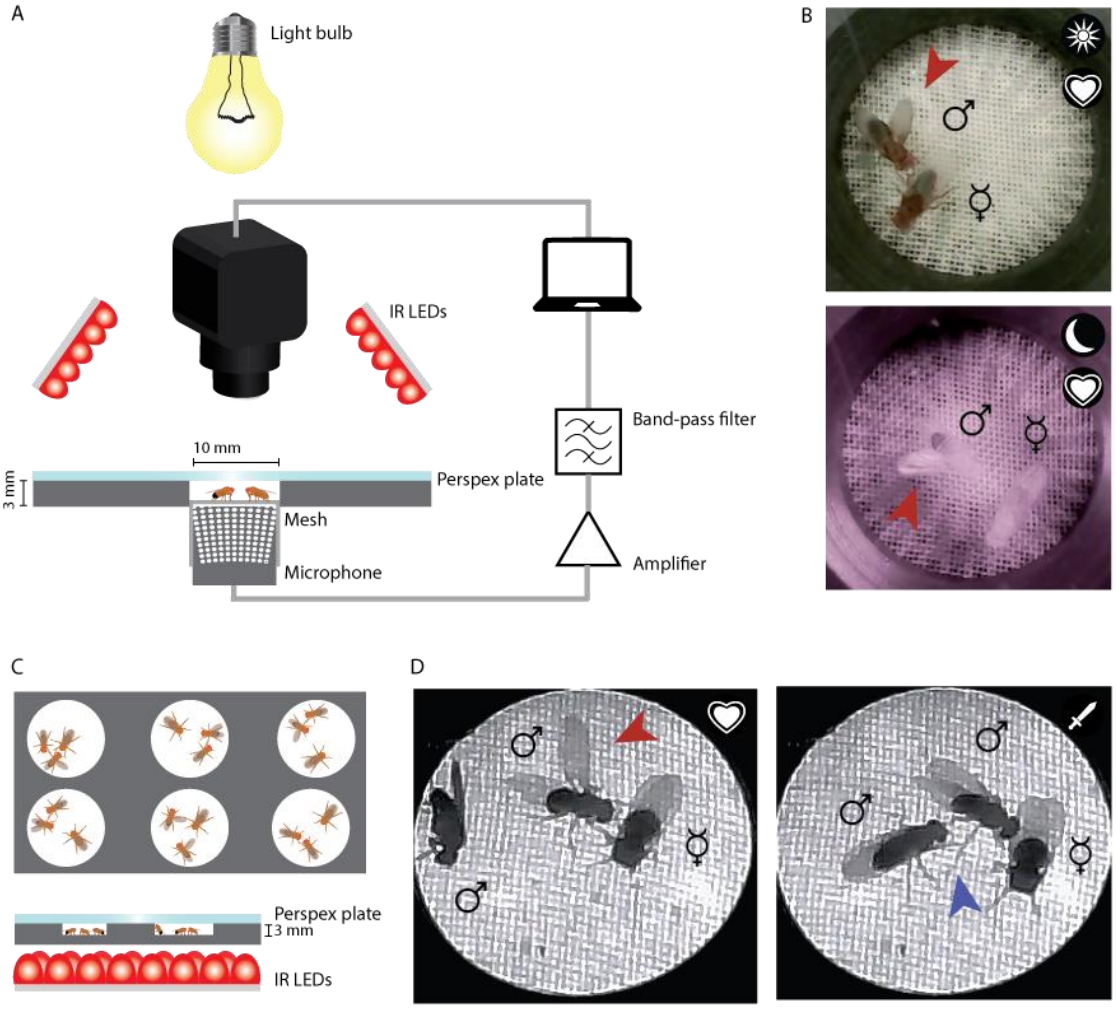


Figure 6 Sketch of setups used for courtship experiments. (A) Model of the single pair courtship assay setup. A circular arena of 10 mm diameter is placed above a microphone covered with mesh and closed with an anti-glare Acrylic glass pane, leaving 3 mm for the flies to move. The microphone allows for recording of courtship song during the assay. The arena is filmed from above, for light conditions a light bulb was placed in the setup, for dark conditions arrays of infrared LEDs are positioned around the arena. The microphone is connected to an amplifier and band-pass filter**. (B) Examples for courtship behaviour in dark and light condition.** Gender symbols mark the male and the virgin female, red arrowheads point to extended wing of the male. Wing extension is generally associated with the production of courtship song and therefore a typical characteristic of a courtship approach. Sun and moon symbols are used to label either light or dark conditions, heart symbol indicates courtship behaviour.

(C) Model of the competitive courtship assay setup. The setup consists of 6 neighbouring arenas with a diameter of 10 mm each, allowing for high-throughput analysis. Arenas are covered with an anti-glare Acrylic glass pane, creating a 3 mm high space for the animals to move. Illumination is provided by infrared LED arrays positioned below the arena. For light conditions, visible light sources are stationed in close proximity. Two socially naïve males and a decapitated virgin female were used for the experiments. **(D) Examples for courtship and aggression behaviour.** Gender symbols indicate the males and decapitated virgin female, red arrowhead marks wing extension. The blue arrowhead points to leg fencing between to males, a typical characteristic of male aggression behaviour. Heart symbol indicates courtship, sword symbol indicates aggression behaviour.

copulation attempt (correct and incorrect) and mating. Following behaviour and tapping/licking if identifiable, are enclosed in the term female-directed behaviour (*Table* **1**).

Female courtship behaviour was divided into locomotion, rejection and mating. Rejection behaviour in females include kicking, jumping, wing fluttering and decamping. Other subtler behaviours of female courtship like ovipositor extrusion or droplet emission from the ovipositor tip could not be reliably identified due to both video resolution and camera angle.

Using the modulator “correct action” or “incorrect action” allows us to distinguish between correctly and incorrectly performed wing extension and copulation attempts. For optimal presentation of the courtship song, the male extends the wing closest to the female (ipsilateral wing extension). Use of the contralateral wing was therefore defined as “incorrect wing extension”. Copulation attempts were classified as “incorrect” if initiated towards the head of the female or the female was no longer present.

3.4.4 Competitive Courtship Assay

Competitive courtship assays were performed by presenting two socially naïve males with a decapitated virgin female. The setup consists of six neighbouring arenas, each with a diameter of 10 mm and 3 mm height, allowing for a high-throughput analysis. Illumination is provided by an array of infrared LEDs (Pollin Electronic GmbH, Pförring,

Germany; #351090) below the arena, for light conditions additional visible light was provided by a lightbulb (DIAG GU10 1X3W) close to the arena (*Figure 6 C*).
Virgin females were anesthetised with CO_2 before decapitation. If assays were performed with mixed genotyped males (*dark-fly* and *OregonR*) they were marked with acrylic paint to enable a distinction. The flies were recorded for 30 min at 30 fps. For analysis 5 min of the recording were selected and frame-by-frame analysis was done using the open-source Python-based tracking software *Etho-Scorer* (by Geurten & Kuhlemann).

After video annotation, the parameters male-male aggression behaviour and male courtship behaviour were evaluated (*Figure 6 D*). Male courtship behaviour was classified by the previously described features wing extension and copulation attempts. Male aggression behaviour is characterized by agonistic interactions including leg fencing, boxing, lunging or hunting. Leg fencing is depicted by shoving a conspecific with one leg; boxing describes a match between two conspecifics using the forelegs; lunging is classified as the shoving of a conspecific using the whole body; hunting is described by a male following a conspecific and attempting to initiate antagonistic interactions.

3.4.5 Group Courtship Assay

To assess the relevance of groups on dark-fly courtship we introduced 10 flies (5 virgin females, 5 socially isolated males) into a circular arena (diameter: 58mm, height: 8.5mm) that was covered with an anti-glare acrylic glass pane. Flies were recorded in both light and dark conditions using indirect illumination with a light bulb (light conditions; DIAG GU10 1X3W) placed above the arena and infrared LED-arrays (dark conditions; Pollin Electronic GmbH, Pförring, Germany; #351090) arranged 5 cm under the arena to avoid an increase in temperature. Before each trial the arena was cleaned with 70 % EtOH to remove possible pheromone traces.
Flies were introduced in the arena through an opening at the side that was subsequently closed with Blu Tack (Borstik GmbH, Borgholzhausen, Germany; #30811745). Courtship behaviour was recorded for 60 min at 10 fps using a Hercules Optical Glass webcam

(Guillemont Cooperation S.A., Carentoire, France). Frame-by-frame analysis was done using the Python-based tracking software *Etho-Scorer* (by Geurten&Kuhlemann). After video annotation the parameters latency to first courtship, courtship success and copulation duration were evaluated. Courtship behaviour was identified by the previously characteristics following, wing extension and copulation attempt.

3.4.6 Video Annotation using the *Etho-Scorer*

Recoded videos were analysed using the open-source Python-based tracking software *Etho-Scorer* (by Geurten & Kuhlemann). The software allows for high-throughput video annotation using a gamepad. Videos are scored frame-by-frame, annotating the observed behaviours for each respective frame and fly.

behaviour	**executed**	**description**	**mode**
unilateral wing extension	male	male extends wing to produce courtship song	courtship
		correct: ipsilateral wing	
		incorrect: contralateral wing	
female-directed behaviour	male	umbrella term for male courtship behaviour,	courtship
		subdivided into	
		following: male follows female after decamping	
		licking: male extends proboscis towards female genitalia	
		tapping: male taps female abdomen using the forelegs	
locomotion	male &	movement to cover a certain distance	courtship
	female	mode of locomotion usually walking, since	
		flying is suppressed due to the arena height	
attempted copulation	male	male bends abdomen under his body and towards a courted object;	courtship
		fails to successfully initiate copulation	
copulation/mating	male &	Successful copulation lasts for > 1 min	courtship

	female	(or initiated directly before the end of the recording)	
leg fencing	male	shoving conspecific with one leg	aggression
boxing	male	match of two conspecifics using forelegs	aggression
lunging	male	shoving conspecific with whole body	aggression
hunting	male	following conspecific attempting to	aggression
		initiate agonistic behaviour	

Table 1 Ethogram of Drosophila courtship and aggression behaviour described and classified in this study.

For efficient video annotation we classified different categories of behaviour. The behavioural categories evaluated for each type of courtship assay are described in detail in the respective sub-chapters and can be extracted from the generated ethogram (*Table 1*).

The generated data was analysed using MATLAB R2012b (The MathWorks Inc., Naticks, Massachusetts, USA). Parameters analysed include latency to first courtship and copulation, latency to wing extension, the courtship success defined as the proportion of successfully copulated pairs per strain, duration and frequency of aggression, courtship, copulation attempts, copulation and wing extension.

For the comparison of the distances of courtship behaviour, the proximity to the female was determined: observations show that the boundaries in which the male could physically interact with the female were similar to the distance between the tip of the female abdomen to the tip of the folded wings. This distance *x* was therefore defined as an approximation to the male reaching distance which would be constant between trials. Courtship behaviour towards the female within the distance *x* are classified as "close interaction".

For comparison of different courtship parameters a Michelson contrast was calculated (Michelson, 1927). To compare the fraction of close vs far courtship behaviour, a proximity (ProxI) index was defined from the duration of male courtship behaviour in close (Dnear) or far (Dfar) interaction range. ProxI = (Dnear – Dfar)/(Dnear + Dfar)

Positive values indicate a higher amount of courtship behaviour in the close interaction range, negative values show a higher amount in the far interaction range. Analogously, an index for correct wing extension (CorrI) was calculated from the duration of ipsilateral ($D_{correct}$) and contralateral ($D_{incorrect}$) wing extension.

$CorrI = (D_{correct} - D_{incorrect})/(D_{correct} + D_{incorrect})$

Positive values show a higher fraction of correct wing extension, negative values denote a higher amount of incorrect behaviour.

3.4.7 Hidden Markov Model of male courtship behaviour

Markov processes in general can be utilized to describe the discrete directly observable states of a system. At distinct times, the system transitions between states according to a set of transition probabilities linked to the respective state. If only the output of the states and not the states itself are observable, a Hidden Markov Model (HMM) can be compiled. The underlying "hidden" states can only be observed through their transition probabilities, generating the sequence of observations (Rabiner, 1989). HMMs can be used to build and optimize a model of the transition probabilities of an observed sequence of behaviours (Geurten et al., 2010; Hofmann et al., 2014). Previous studies have already applied HMMs to model the courtship syntax of *Drosophila* (Lasbleiz et al., 2006; Markow & Hanson, 1981; Sakai et al., 1997). In this study an HMM was compiled to compare the courtship syntax of *OregonR* and *dark-fly* in both light and dark conditions.

Since an HMM can only describe discrete states, but the previously annotated behaviours often occur simultaneously, the categories were redefined. The defined states of male courtship behaviour used in this model are: Locomotion, pausing, wing extension, copulation attempt, successful copulation, other female-directed courtship behaviour in a close interaction range (*other courtship behaviour near*) and other female-directed courtship behaviour in a far interaction range (*other courtship behaviour far*). It was assumed, that behaviourally relevant state-transitions occur significantly more often than random state-transitions. These behaviourally relevant transitions were extracted using Bernoulli statistics and comparing the proportions of

each occurring transition from state x to state y to the a priori distribution of the states. Transition models were subsequently generated from the transitions occurring with significantly higher probability than chance level.

3.5 Software

All calculations were done in MATLAB R2012b (The MathWorks Inc., Naticks, Massachusetts, USA) running on Ubuntu 14.04 LTS (Debian-based Linux distribution) in a Java 1.6.0_17-b04 system (Sun Microsystem Inc., Santa Clara, California, USA). For all other used software, please refer to the list of materials.

3.6 Statistical Analysis

If not indicated otherwise, differences of medians were tested for significance applying Fisher's exact permutation test (Fisher, 1970). The Benjamini-Hochberg false correction rate (Benjamini and Hochberg, 1995) was used to correct the obtained *p*-values. Significances in all figures were indicated as: * = $p < 0.05$; ** = < 0.01; *** = 0.001.
All statistical analyses and graphs were done using MATLAB R2102b (The MathWorks Inc., Naticks, Massachusetts, USA).

4. Results

4.1 Circadian rhythm of dark-fly shows no difference to wt

The *dark-fly Drosophila* strain has been raised in dark conditions for over 1500 generations, which raises the question if their circadian rhythm has changed. To test this, we used *Drosophila* Activity Monitoring System (DAM), a setup in which animals are placed into a horizontal glass cylinder and crossings of the middle are counted via an infrared light beam. Phases of inactivity that lasted longer than five minutes were categorized as sleep. Both *dark-fly* and *OregonR* were expose to 12:12h dark:dark illumination conditions for four days, subsequently followed by five days in a 12:12 dark:light cycle.

In a 12:12h dark:dark cycle both male *dark-fly* and *OregonR* show similar activity patterns with a weakly oscillating locomotion pattern with a phase duration of approximately 25 h (*Figure 7 A).* When presented with a 12:12h dark:light cycle both *OregonR* and *dark-fly* display a diurnal rhythm with elevated activity associated with dark-light or light-dark switches (*Figure 7 B*). Activity is decreasing during midday, which has previously been reported for wt flies in a dark:light cycle (Aschoff, 1966; Corthals *et al.*, 2017; Jarabo and Martin, 2017).

During the dark phases both strains show intermediate activity, with *dark-fly* showing slightly higher activity levels during the first days (*Figure 7 B*). This might be due to a novelty effect for the *dark-fly* strain which is exposed to light for the first time. This effect is slowly decreasing over the course of the five days.

In both *dark-fly* and *OregonR* the observed locomotion pattern is mirrored in the sleep pattern. In 12:12h dark:dark conditions the sleep patter of both strains oscillates weakly with a phase of 25 h (*Figure 7 C*). After switching to a 12:12 h dark:light cycle the two strains show similar sleep patterns (*Figure 7 D*). During dark phases the sleep pattern of *dark-fly* animals shows a slight decrease compared to the *OregonR* strain. However, this effect is fading over the course of the five days.

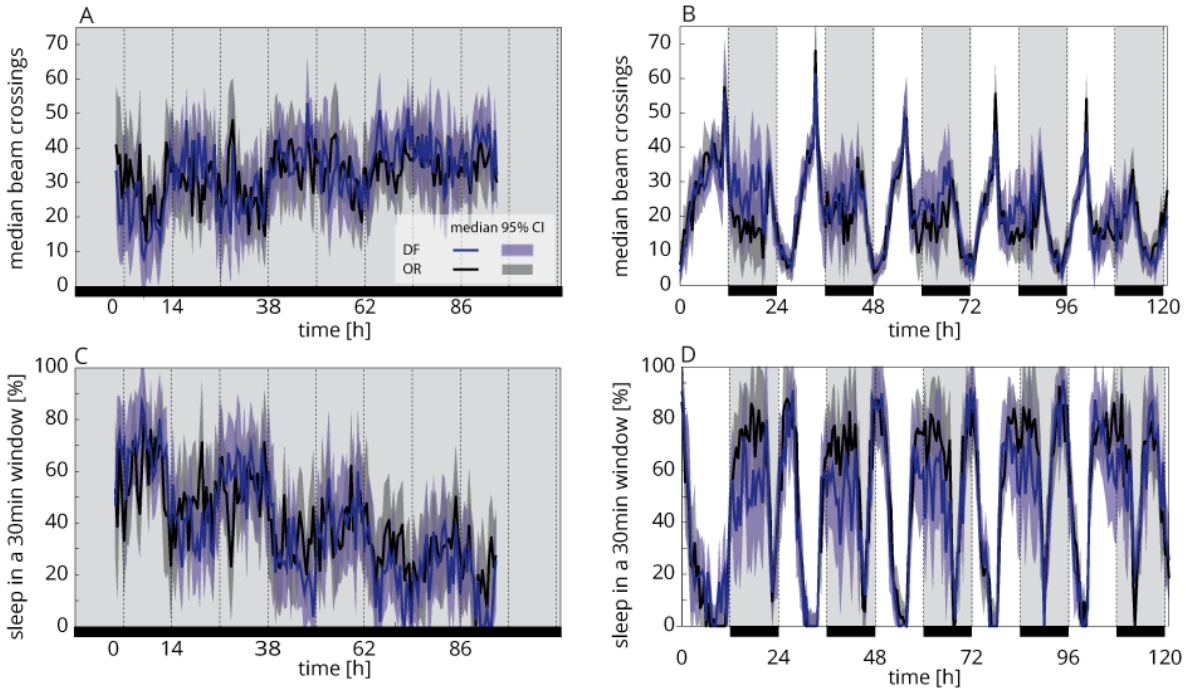


Figure 7 Circadian rhythm of male *dark-fly* and *OregonR*. Solid line marks the median, 95% confidence interval is depicted by the shaded areas. Black bars below graph and grey background indicate dark conditions, white bars and white background indicate light conditions. N*(OR light)* = 32, *N(dark-fly)* = 32 **(A) Median number of beam crossings in a dark:dark cycle.** Comparison of beam crossings in male *dark-fly* and *OregonR* during 4 days in a 12:12h dark:dark cycle shows no difference in activity pattern. **(B) Median number of beam crossings in a dark:light cycle.** Comparison of beam crossings in male *dark-fly* and *OregonR* during 5 days in a 12:12h dark:light cycle shows no difference in activity pattern. **(C) Median duration of sleep in a 30 min window in a dark:dark cycle.** Sleep pattern of male *dark-fly* and *OregonR* during 4 days in all dark conditions show no difference. **(D) Median duration of sleep in a 30 min window in a dark:light cycle.** Sleep pattern of male *dark-fly* and *OregonR* during 5 days in a 12:12h dark:light cycle show no difference.

These findings show, that the circadian rhythm of *dark-fly* is not significantly different from our wt control *OregonR*. *Dark-fly* is still able to entrain to a dark:light cycle and displays the characteristic bimodal activity pattern. It can be concluded, that even after 1500 generations in darkness, the switch in illumination condition still acts as a functional zeitgeber.

4.2 Saccadic strategy requires visual cues

4.2.1 Absence of visual cues decreased the duration of thrust movements

The saccadic locomotion strategy is widely believed to have been developed to facilitate motion vision by reducing rotational optic flow (Collett and Land, 1975a; Geiger and Poggio, 1977; Koenderink and Doorn, 1987). I therefore hypothesised that mutations to the motion vision pathway might reduce the benefit of the saccadic strategy and thereby change the locomotion pattern of these animals. To this end, locomotion was studied using the arena and tracking analysis explained in the method section. The saccadic locomotion strategy shows two types of locomotion: thrust movement and rotational movement, called saccades. I examined the locomotion behaviour of three *Drosophila* mutant strains with various degrees of impairment in the visual system. *ora* shows impaired motion vision but retains an intact colour vision pathway (Yamaguchi et al., 2008). The *sineoculis* strain has an impaired development of compound eyes and is therefore blind if the mutation is homozygously present in the fly genome (Helfrich-Förster et al., 2000; Kenyon et al., 2005; Weasner et al., 2007). The *sineoculis* mutant flies used in this experiment were maintained as a heterozygotic strain and crossed for the experiment to generate first generation blind flies. *sol* displays developmental degeneration of columnar neurons, abolishing the processing of visual cues (Delaney et al., 1991). If behavioural adaptations to the absence of visual cues are present in the locomotion strategy, the homozygous strain *sol* would rather show them then the first-generation blind flies from the used *sineoculis* strain. We therefore hypothesise a progression with severity of the mutation in altered locomotion behaviour: while *ora* flies would show only minor changes in locomotion, *sol* would be expected to be most different from wt flies with the first-generation blind flies of the *sineoculis* strain displaying an intermediate phenotype.

Thus, the velocity in direction of movements is significantly increased comparing the visual mutants *ora* (7.54 mm/s), *sineoculis* (8.48 mm/s) and *sol* (13.06 mm/s) with the wildtype control *OregonR* (6.57 mm/s) (*Figure 8 A*). It can be observed that the velocity is indeed progressing with severity of mutation.

The saccadic strategy is further characterized by long stretches of translational bouts, punctuated by saccadic rotations. The wt control strain *OregonR* shows a thrust bout duration of 37 ms, which is slightly shorter in *ora* (31 ms). Both mutant lines *sineoculis* (25 ms) and *sol* (15 ms) show significantly decreased bout durations of the thrust movement (*Figure 8 B*).

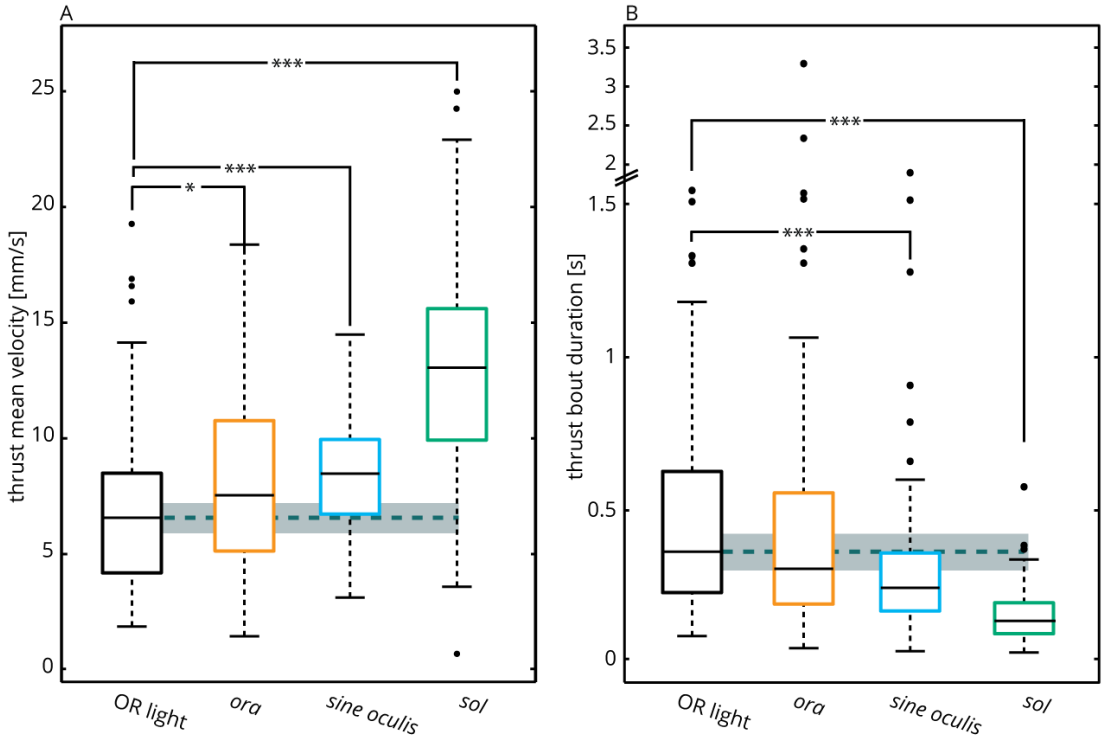


Figure 8 Characteristics of translational movements. Black lines indicate the medians; boxes include 50% of the data set around the medians; whiskers indicate 1.5* interquartile distance; outliers are marked by black circles. Green dashed line indicates the median of the wt control (OR light), the shaded area marks the 95% confidence interval. To test for significance, we used a two-sample Kolmogorov-Smirnov test and corrected *p*-values using the Benjamin-Hochberg false FDR. Significance is indicated as follows: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. N(*OR light*) = 97, N(*ORT*) = 86, N(*sineoculis*) = 96, N(*sol*) = 124 **(A) Boxplots of the mean velocity of thrust movements.** Comparing the mean thrust velocity of the wt strain *OregonR* with the visual mutants *ORT*, *sinceoculis* and *sol* shows an significant increase of velocity consistent with the severity of the mutation. [*p*-values: *ORL* vs *ORT* 46,73 x 10^{-2}; *ORL* vs *sineoculis* 21 x 10^{-5}; *ORL* vs *sol* 12 x 10^{-5}] **(B) Boxplots of duration of thrust bouts.** Duration of thrust bouts is significantly reduced in *sineoculis* and *sol* compared to wt control. [*p*-values: *ORL* vs *ORT* 19,084 x 10^{-2}; *ORL* vs *sineoculis* 16 x 10^{-5}; *ORL* vs *sol* 10 x 10^{-5}]

4.2.2 Absence of visual cues prolongs the time spent with rotations

After analysing the translational component of the *Drosophila* locomotion strategy, a detailed description of saccade characteristics was done. The criterion to classify a rotational movement as a saccade was a peak yaw velocity over 200 deg/sec. For better comparison the saccades were all arranged at the peak velocity which is now visible at 0 ms in the graph (*Figure 9*). In the wt strain *OregonR* a yaw peak velocity of 258.1 deg/sec can be found, which is consistent with the saccadic velocity we previously reported for walking *Drosophila* (Geurten et al., 2014). In comparison, both mutant strains *ora* (349.4 deg/sec) and *sol* (351.1 deg/sec) show a significantly higher velocity for saccadic turns. Furthermore, the corresponding change in angular heading was analysed. Within a 130 ms window *OregonR* flies turn on average by 18.1 deg, whereas *sol* mutants show a significantly larger turning angle of about 20.83 deg the same time window. *ora* flies change their angular heading by about 16.01 deg (*Figure 9*).

In conclusion, we see an increase in saccade velocity and turning angle in the mutant *Drosophila* strain with impaired visual system. To further understand the impact of mutations in the visual system on the saccadic strategy different saccade characteristics like duration, amplitude and frequency were analysed in detail. The saccade duration shows no significant difference if compared between *OregonR* (0.078 s) and *sol* (0.079 s); however, the saccades for both *ora* (0.082 s) and *sineoculis* (0.1 s) show a significantly higher duration in relation to *sol* and the wt control (*Figure 10 A*)The saccade amplitude is significantly rising with increasing severity of the mutation. The wt *OregonR* shows a mean saccade amplitude of 298.8 deg; *ora* (322.8 deg), *sineoculis* (399.29 deg) and *sol* (408.66 deg) all reach significantly higher saccade amplitudes (*Figure 10 B*). Interestingly, a similar effect can be observed in the increase in saccade frequency correlating with the severity of the visual manipulations (*OregonR* 2.7 Hz; *ora* 3.43 Hz; *sineoculis* 3.95 Hz; *sol* 6.41 Hz) (*Figure 10 C*). Accordingly, there is also an increase in the time the flies spent with saccadic movements. *OregonR* spends 21.37% of the recorded dataset with saccades. This is significantly increased in the three visual mutant strains *ora* (27.87%), *sineoculis* (39.2%) and *sol* (51.12%) (*Figure 10 D*).

Additionally, the remaining rotational movements which were not classified as saccades were analysed. The mean residual yaw velocity of the wt *Drosophila* strain *OregonR* was at 61.74 deg/s and a significant derivation of this can be found in *ora* (63.75 deg/s) and *sineoculis* (64.16 deg/s) and *sol* (73.32 deg/s) (*Figure 10 E*). Furthermore, the integral of rotation velocity was calculated. The integral of rotational velocity for the control strain *OregonR* is at 88.23 deg/s and levels are significantly increased for the three mutant lines (*ora* 100.55 deg/s; *sineoculis* 131.23 deg/s; *sol* 160.51 deg/s) (*Figure 10 F*).
To summarize, I observed that severe mutations in the motion vision system correlate with severe changes in the saccadic strategy. Furthermore, with progressing severity of the mutation, the severity of the locomotion change is increasing. This can be observed in a rise of thrust velocity, and a decrease of thrust bout duration.

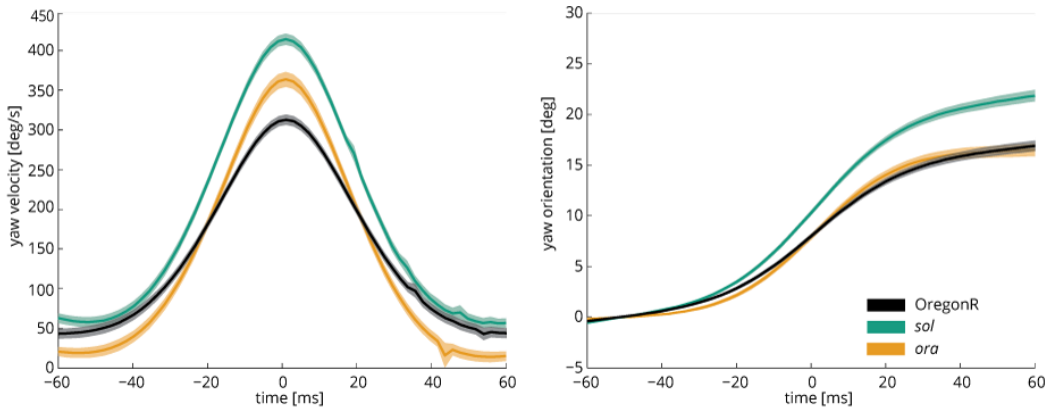
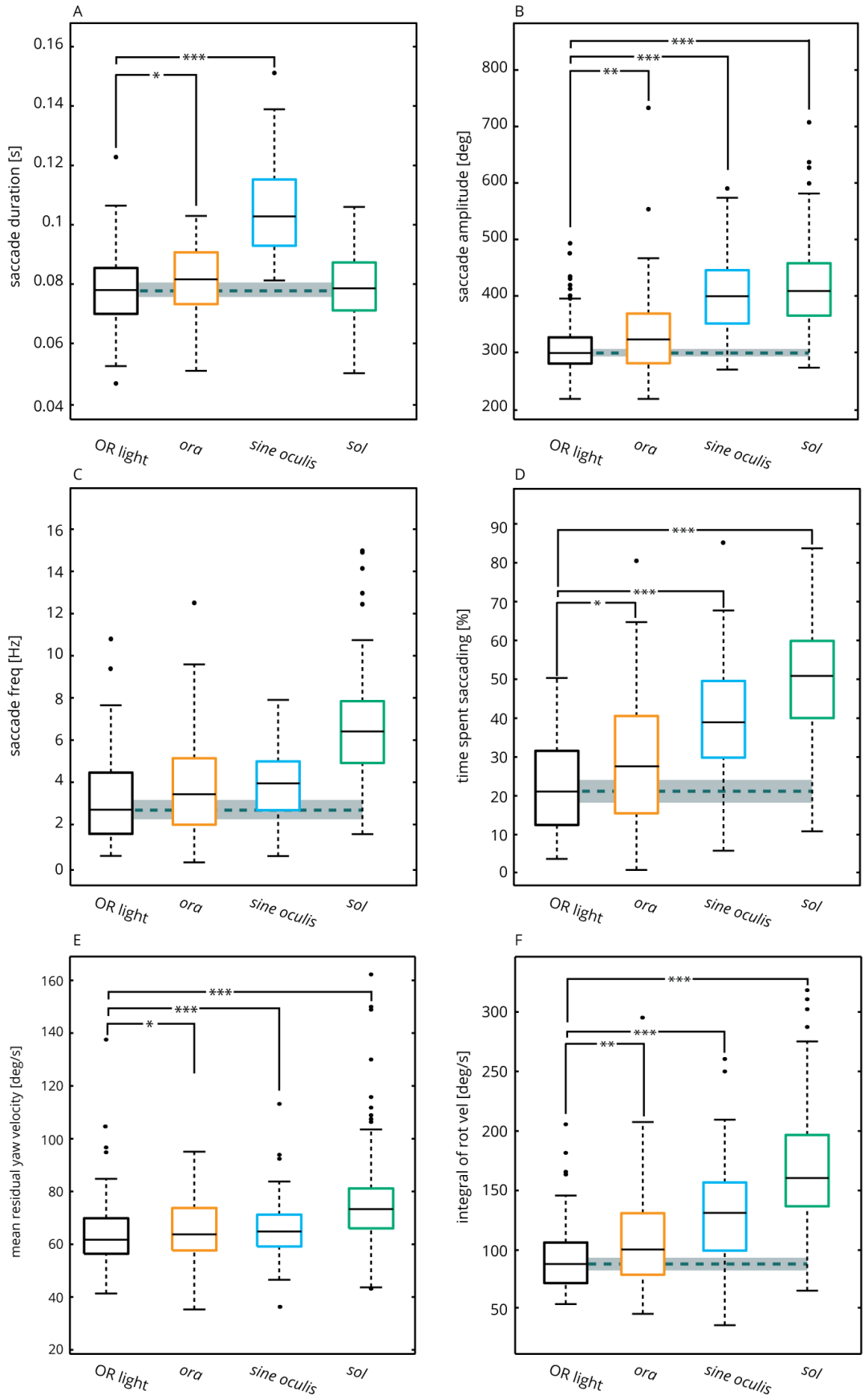


Figure 9 Analysis of saccade velocity and angle depicted as saccade triggered averages. The solid line indicates the median, the shaded area shows the 95% confidence interval (CI) of each group. N(*OR light*) = 98, N(*ORT*) = 99, N(*sineoculis*) = , N(*sol*) = 124 **(A) Mean yaw velocity of the saccade.** Saccades were identified by using a yaw velocity of 200 deg/sec as threshold and were superimposed so that the peak velocity is at 0 ms. Preceding the analysis left and right saccades were separated leading to mirror-symmetric velocity profiles. *OregonR* as a wt control shows a peak saccade velocity of about 200 deg/sec whereas both *ORT* and *sol* show highly significantly faster saccades with a peak velocity of about 370 deg/sec. **(B) Mean corresponding turning angle.** Within a window of 120 ms the wt *Drosophila OregonR* change their angular heading by about 15 deg. The turning angle of *sol* is significantly larger than wt with over 20 deg, whereas *ORT* shows a slightly smaller angle of about 18 deg.


A
saccade duration [s]
0.16
0.14
0.12
0.1
0.08
0.06
0.04
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*
OR light
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800
700
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OR light
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16
14
12
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8
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2
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OR light
ora
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D
time spent saccading [%]
90
80
70
60
50
40
30
20
10
0
***
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*
OR light
ora
sine oculis
sol
E
mean residual yaw velocity [deg/s]
160
140
120
100
80
60
40
20
***
***
*
OR light
ora
sine oculis
sol
F
integral of rot vel [deg/s]
300
250
200
150
100
50
***
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OR light
ora
sine oculis
sol

Figure 10 Characteristics of saccadic and rotational movements. Black lines indicate the medians; boxes include 50% of the data set around the medians; whiskers indicate 1.5* interquartile distance; notches display the 95% confidence interval; outliers are marked by black circles. Green dashed line indicates the median of the wt control (OR light), the shaded area marks the 95% confidence interval. To test for significance, we used a two-sample Kolmogorov-Smirnov test and corrected *p*-values using the Benjamin-Hochberg false FDR. Significance is indicated as follows: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. N(*OR light*) = 97, N(*ORT*) = 86, N(*sineoculis*) = 96, N(*sol*) = 124 **(A) Boxplots of the duration of saccades.** *OregonR* and *sol* show saccade durations that are in a comparable margin. *ORT* and *sineoculis* both show significantly longer saccade durations. [*p*-values: *ORL* vs *ORT* 43,35 x 10^{-2}; *ORL* vs *sineoculis* 8 x 10^{-5}; *ORL* vs *sol* 35,67 x 10^{-2}] **(B) Boxplots of mean saccade amplitude.** The saccade amplitude is significantly increased in *ORT*, *sineoculis* and *sol* compared to wt control. [*p*-values: *ORL* vs *ORT* 20,2 x 10^{-4}; *ORL* vs *sineoculis* 9 x 10^{-5}; *ORL* vs *sol* 9 x 10^{-5}] **(C) Boxplots of saccade frequency.** Comparing the saccade frequency shows a significant increase in *ORT*, *sineoculis* and *sol* to the wt control *OregonR*. [*p*-values: *ORL* vs *ORT* 41,38 x 10^{-3}; *ORL* vs *sineoculis* 77 x 10^{-5}; *ORL* vs *sol* 8 x 10^{-5}] **(D) Boxplots of the time spent saccading.** Comparing the mean time each strain spends with a saccadic movement, shows a significant increase in *ORT*, *sineoculis* and *sol* to the wt control *OregonR*. [*p*-values: *ORL* vs *ORT* 14,02 x 10^{-3}; *ORL* vs *sineoculis* 9 x 10^{-5}; *ORL* vs *sol* 9 x 10^{-5}] (**E) Boxplots of the residual yaw velocity.** Comparing the mean thrust velocity of the wt strain *OregonR* with the visual mutants *ORT*, *sinceoculis* and *sol* shows a significant increase of velocity consistent with the severity of the mutation. [*p*-values: *ORL* vs *ORT* 43 x 10^{-3}; *ORL* vs *sineoculis* 10 x 10^{-5}; *ORL* vs *sol* 10 x 10^{-5}] **(F) Boxplots of mean integral of velocity.** Duration of thrust bouts is significantly reduced in *sineoculis* and *sol* compared to wt control. [*p*-values: *ORL* vs *ORT* 33,5 x 10^{-4}; *ORL* vs *sineoculis* 8 x 10^{-5}; *ORL* vs *sol* 8 x 10^{-5}]

Subsequently, the saccade frequency and time spent saccading is increasing significantly, reducing the time in which 3D information could be extracted from the optic flow generated by moving in the arena. Additionally, the saccade amplitude shows significantly larger angles in the mutants compared to wt. These findings give evidence, that the saccadic strategy is indeed highly influenced by the visual system.

4.3 Light deprivation severely influences the saccadic strategy

4.3.1 Light-deprived flies show altered walking trajectories

After finding that an impairment of the visual system correlates with a severe alteration of the locomotion strategy in *Drosophila*, we wanted to examine possible adaptations

to a total lack of visual cues. A straightforward way to assess this is to expose the flies to darkness and see if the changes to the locomotion strategy found in chapter *4.2* Saccadic strategy requires visual cues are also emerging in flies raised in light-deprived conditions. From the experiments with canonical mutants, we can conclude that mutations instated over many generations lead to a more severe behavioural phenotype. We therefore not only maintained an *OregonR* strain in light-deprived conditions for 15 generations but also obtained specimen of the *dark-fly* strain (N. Fuse; Tōhuku University, Sendai, Japan).

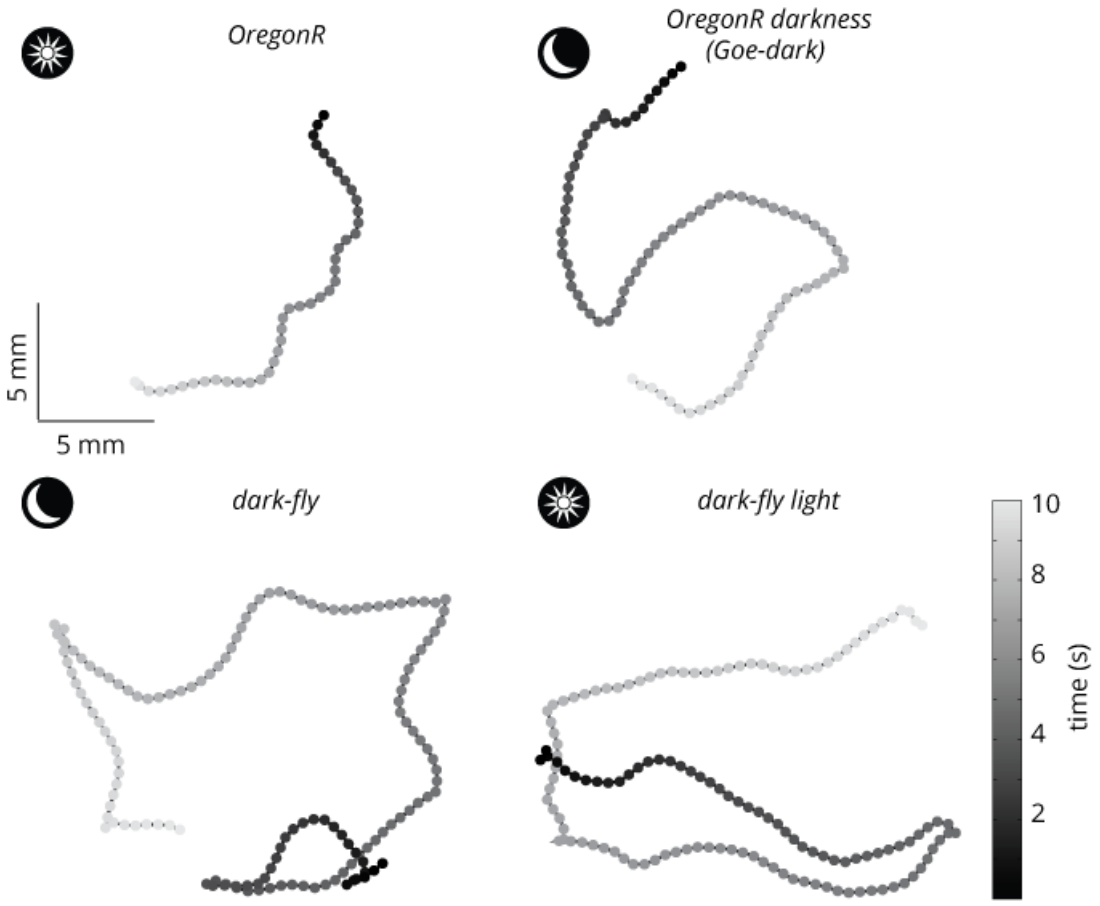


Figure 11 Example traces of individual flies. Traces are examples for the trajectories that were derived from highspeed recordings and display the fly's movement over 10 sec. Sun icon is indicating the recording was performed under light conditions, moon icon indicates dark conditions. Trajectories display locomotion differences between *dark-fly* and *OregonR* strains. The traces were smoothed using the smoothing algorithm after Günther et al., 2016.

Both strains are derived from an *OregonR* strain and therefore closely related. The *dark-fly* strain has been maintained in dark conditions since 1954 and is currently sustained for over 1500 generation. To guarantee dark conditions, we used infrared lights during recordings and far-red lights, outside of *Drosophilas* visual spectrum, for handling (*3.1.3 Fly rearing and basic experimental conditions*). Unfortunately, the light-raised control line of the *dark-fly* strain perished in 2002. To establish a comparable line, we started raising *dark-fly* specimen in light conditions (*dark-fly* light).

Comparing example trajectories of *OregonR*, *OregonR* raised in darkness (*Goe-dark*), *dark-fly* and *dark-fly* raised in light conditions shows direct differences between the light-raised wt *OregonR* and the dark-raised *dark-fly* (*Figure 11*). The walking path of *dark-fly* covers considerably more area than the wt path and shows more points of rotation. Furthermore, *dark-fly* animals cover more area in the same time as *OregonR* flies and show an increase in locomotion speed (*Figure 12 A*).

4.3.2 Light-deprived flies favour faster and shorter thrust movement

With the finding of faster but shorter thrusts in visually impaired flies (*Figure 8*), I wanted to see if this might represent a general adaptation strategy of locomotion in the absence of visual cues.

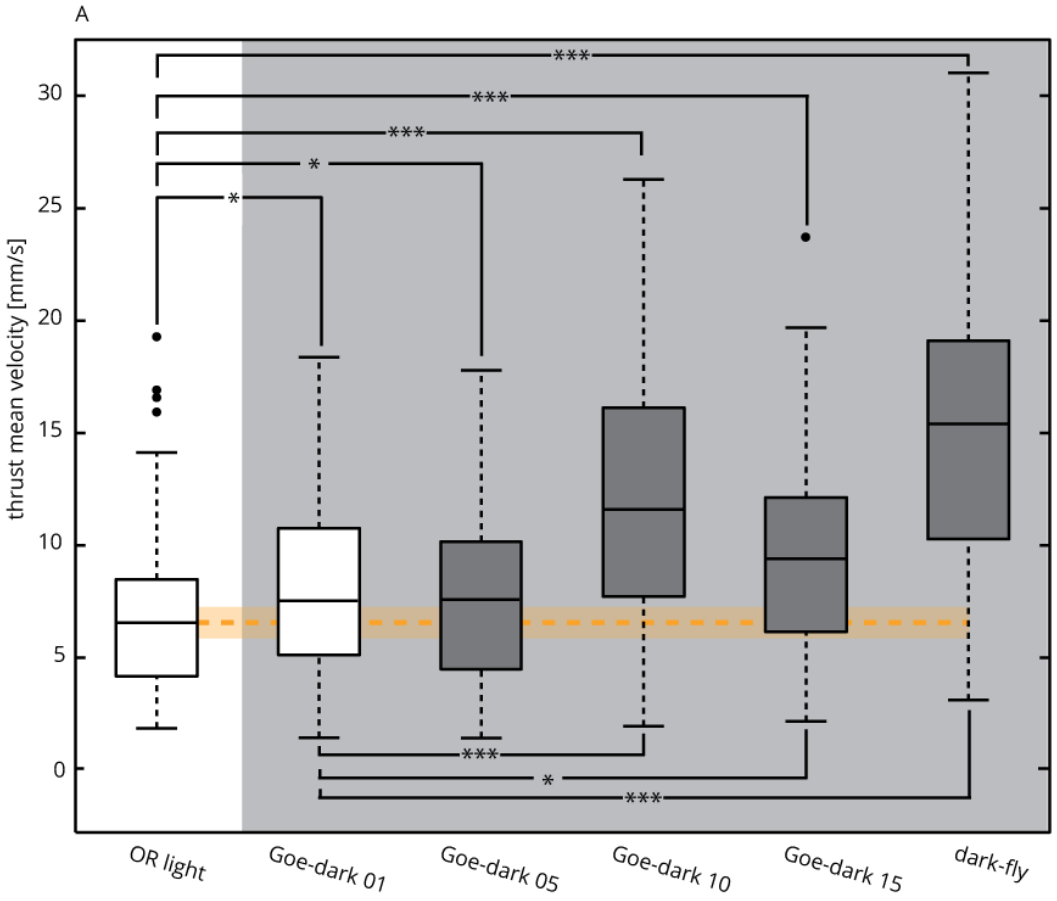
To understand if there is a progression in the adaptation of the locomotion strategy to light-deprivation an *OregonR* strain was raised in complete darkness for 15 generations and subsequently tested; the strain is further referred to as *Goe-dark* (generations are indicated by suffixed numbers). Comparing the thrust velocity of *OregonR* raised in light and tested in light conditions (*OregonR light*; 6.57 mm/s) with the speed of *OregonR* raised in light but tested in darkness (*Goe-dark 01*; 7.54 mm/s) an immediate significant increase can be observed (*Figure 12 A*). This increase can further be seen in the sequential generations (*Goe-dark 05* 7.59 mm/sec; *Goe-dark 10* 11.6 mm/sec; *Goe-dark* 15 9.41 mm/sec) which show a significant increase in thrust velocity compared to both *OregonR light* and *Goe-dark 01*. The *dark-fly* flies show the highest thrust velocity with 15.4 mm/s, which is significantly higher than all other velocities found in this experiment (*Figure 12 A*).

As a next step these flies were reintroduced into light condition, recorded and the thrust velocity was analysed (*Figure 12 B*). Re-establishing *dark-fly* back into light (*dark-fly light*) directly leads to a significant decrease in thrust velocity from 15.4 mm/s in *dark-fly* to 12.76 mm/s in *dark-fly light*; the velocity then stays stable after 5 generations in light:dark conditions (*dark-fly light 05*; 13.09 m/s). To understand how persistent the effect is *Goe-dark 05* and *Goe-dark 10* were recorded under light conditions. In both cases the thrust velocity was decreased compared to dark conditions and reached *OregonR light* levels (*Goe-dark 05 light* 6.19 mm/s; *Goe-dark 10 light* 6.11 mm/s).

Taken together we see an increase in trust velocity, if flies are deprived of light over a prolonged time period, cumulating with *dark-flies* reaching the highest velocity. Reintroducing dark-raised flies back into light conditions leads to a decrease in velocity. Since we found prolonged thrust bout durations in fly strains with visual impairments (see chapter *4.2.1* Absence of visual cues decreased the duration of thrust movements) we were intrigued if a similar change can be observed in light-deprived flies. Recording *OregonR* in darkness leads to a slight, non-significant, decrease in duration of thrust bouts (*OregonR* 0.37 s; *Goe-dark 01* 0.31 s) (*Figure 13 A*). If comparing *OregonR* with the dark-raised flies *Goe-dark 05* (0.24 s), *Goe-dark 10* (0.23 s) and *Goe-dark 15* (0.2 s) a significant and progressing decrease in thrust bout durations can be observed where *Goe-dark 15* shows the shortest durations. However, *dark-fly* (0.15 s) displays even lower thrust bout durations (*Figure 13 A*). which are significantly shorter compared to both wt and *Goe-dark 15* flies.

Reintroducing the *dark-fly* strain back into light directly shows a significant increase in thrust bout duration (*dark-fly light*; 0.24 s) and become indistinguishable from *OregonR* levels after only five generations in light conditions (*dark-fly light 05*; 0.3 s) (*Figure 13 B*). Recording the dark-raised flies of generation 5 and 10 in light conditions shows *Goe-dark 05* (*Goe-dark 05 light*; 0.33 s) returning the thrust bout duration back to wt levels; *Goe-dark 10 light* (0.5 s) shows prolonged durations that are significantly longer compared to *OregonR* (*Figure 13 B*).

Summed up, consistent with the findings of visually impaired flies an increase in velocity and decrease in thrust bout durations can be observed in absence of visual cues.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25
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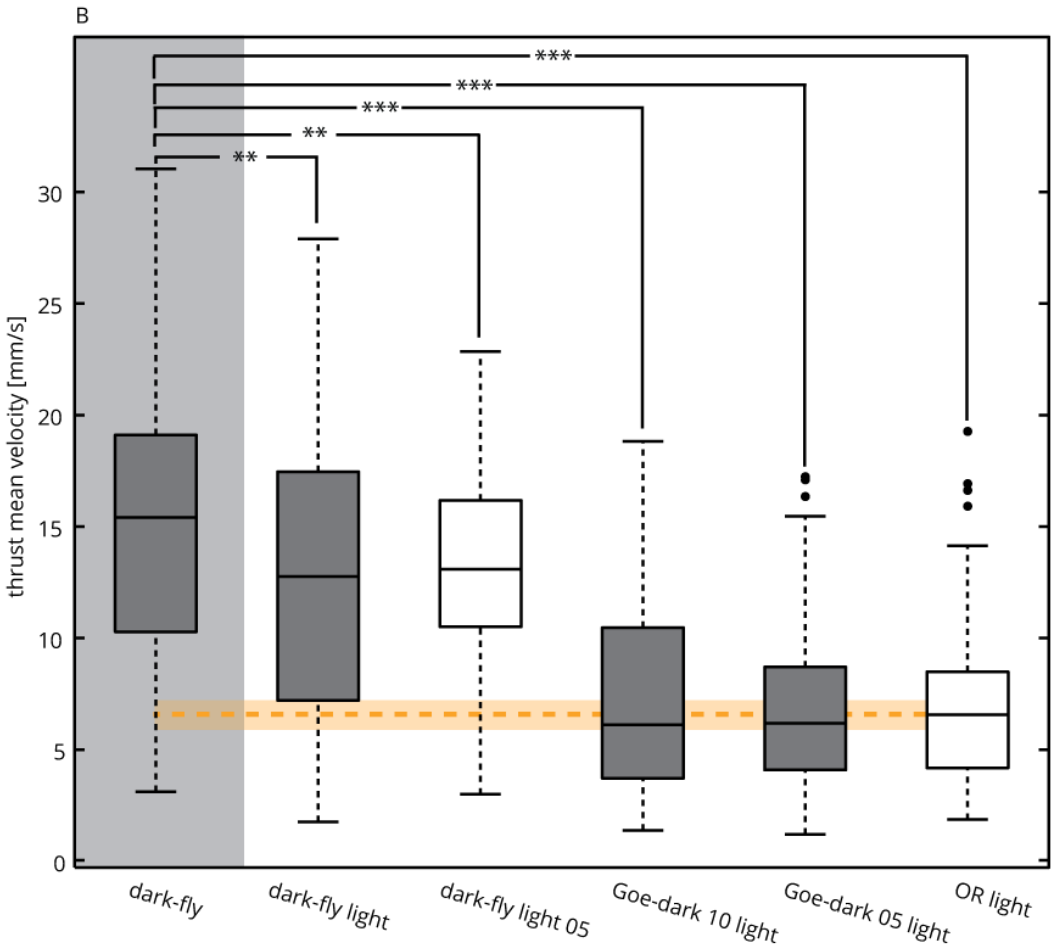
OR light
Goe-dark 01
Goe-dark 05
Goe-dark 10
Goe-dark 15
dark-fly


B
thrust mean velocity [mm/s]
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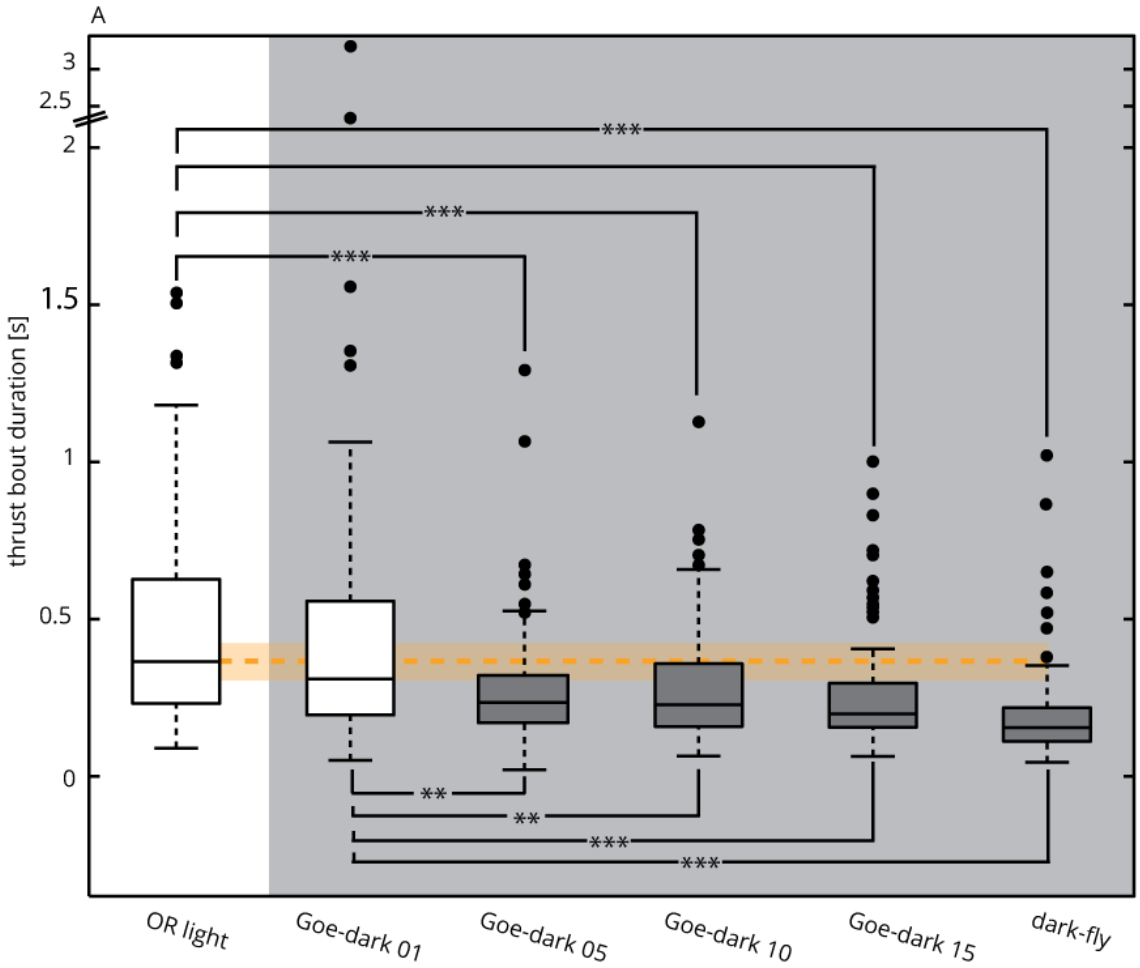
dark-fly
dark-fly light
dark-fly light 05
Goe-dark 10 light
Goe-dark 05 light
OR light

Figure 12 Boxplots of the mean velocity of thrust movements. Black lines indicate the medians; boxes include 50% of the data set around the medians; whiskers indicate 1.5* interquartile distance; notches display the 95% confidence interval; outliers are marked by black circles. Orange dashed line indicates the median of the wt control (OR light), the shaded area marks the 95% confidence interval. Rearing and experimental conditions are indicated by the colour of the boxes and the background: a white box illustrates rearing in a 12:12 dark:light cycle, a grey box rearing in a 24h dark cycle. White background indicates that the recordings were done in light, grey background indicates that the recordings were done in darkness. To test for significance, we used a two-sample Kolmogorov-Smirnov test and corrected *p*-values using the Benjamin-Hochberg false FDR. Significance is indicated as follows: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ (**A) Into darkness** Comparing the mean velocity of the forward movement (thrust) shows a progression to higher velocities with more generations in darkness. *Dark-fly* shows the highest mean thrust velocity with around 15mm/s and *OR light* the slowest with around 7mm/s. We see a significant increase in thrust velocity in *Goe-dark 10* and *Goe-dark 15*. *N(OR light)* = 97, *N(Goe-dark 01)* = 86, *N(Goe-dark 05)* = 83, *N(Goe-dark 10)* = 127, *N(Goe-dark 15)* = 112, *N(dark-fly)* = 124 [*p*-values: *ORL* vs *GD01* 37,222 x 10^{-3} ; *ORL* vs *GD05* 43,51 x 10^{-3}; *ORL* vs *GD10* 9 x 10^{-5} ; *ORL* vs *GD15* 9 x 10^{-5} ; *ORL* vs *DF* 9 x 10^{-5} ; *GD01* vs *GD05* 45,73 x 10^{-2} ; *GD01* vs *GD10* 9 x 10^{-5} ; *GD01* vs *GD15* 27,88 x 10^{-3} ; *GD01* vs *DF* 9 x 10^{-5}] (**B) Back to light** The mean thrust velocity shows a significant decrease for *dark-fly* the longer they are reared in light conditions (DF 15 mm/s, DFL and DFL05 around 13mm/s). Reintroducing GD05 and GD10 back in light conditions also leads to a decrease in velocity which is not significantly different from ORL. *N(OR light)* = 97, *N(Goe-dark light 05)* = 94, *N(Goe-dark light 10)* = 106, *N(dark-fly)* = 124, *N(dark-fly light)* = 101, *N(dark-fly light 05)* = 119, *N(Goe-dark light 05)* = 94, *N(Goe-dark 10)* = 106 [*p*-values: *DF* vs *DFL* 79,8 x 10^{-4} ; *DF* vs *DFL05* 21 x 10^{-4} ; *DF* vs *GDL10* 8 x 10^{-5} ; *DF* vs *GDL05* 8 x 10^{-5} ; *DF* vs *ORL* 8 x 10^{-5}]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1
0.5
0
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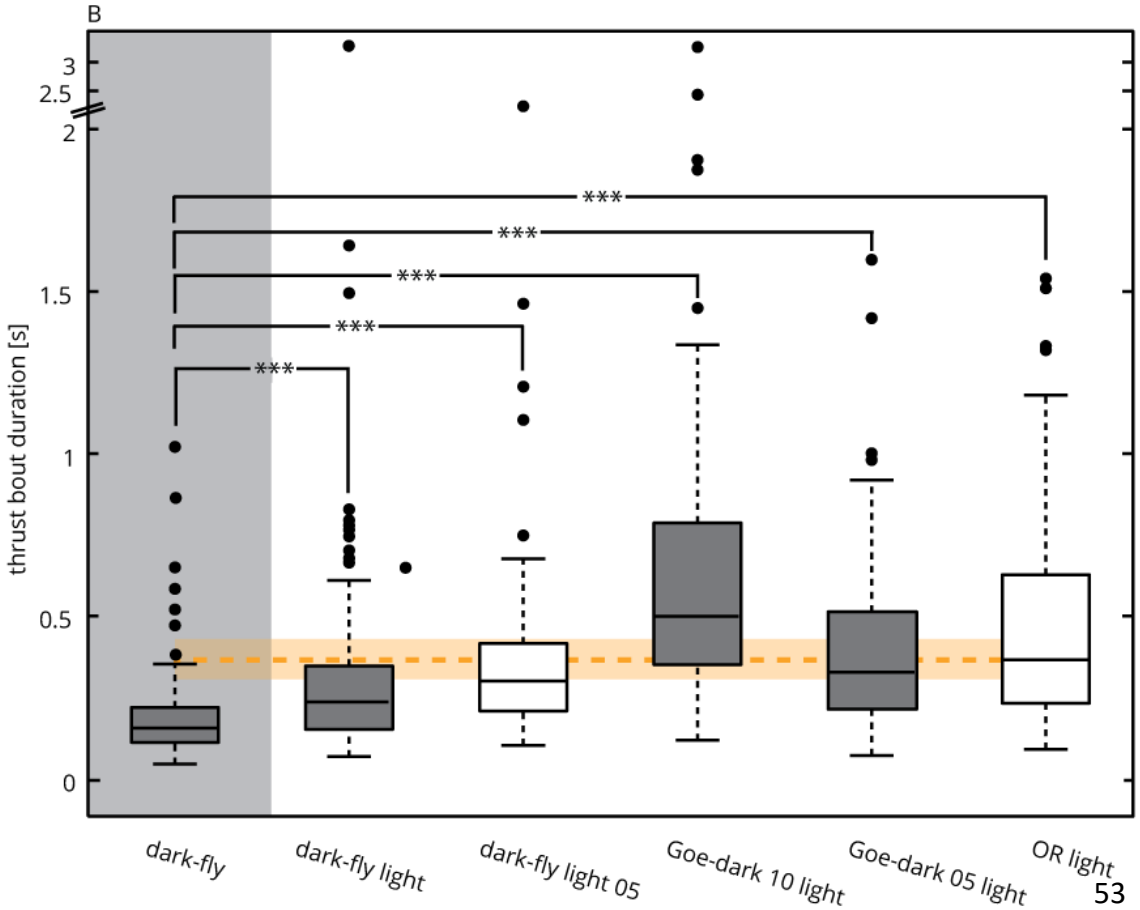
OR light
Goe-dark 01
Goe-dark 05
Goe-dark 10
Goe-dark 15
dark-fly


B
thrust bout duration [s]
3
2.5
2
1.5
1
0.5
0
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dark-fly
dark-fly light
dark-fly light 05
Goe-dark 10 light
Goe-dark 05 light
OR light

Figure 13 Boxplots of the duration of thrust bouts. Black lines indicate the medians; boxes include 50% of the data set around the medians; whiskers indicate 1.5* interquartile distance; notches display the 95% confidence interval; outliers are marked by black circles. Orange dashed line indicates the median of the wt control (OR light), the shaded area marks the 95% confidence interval. Rearing and experimental conditions are indicated by the colour of the boxes and the background: a white box illustrates rearing in a 12:12 dark:light cycle, a grey box rearing in a 24h dark cycle. White background indicates that the recordings were done in light, grey background indicates that the recordings were done in darkness. To test for significance, we used a two-sample Kolmogorov-Smirnov test and corrected *p*-values using the Benjamin-Hochberg false FDR. Significance is indicated as follows: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ (**A) Into darkness.** The longer Drosophila is reared under dark conditions the more a decrease in length of thrust bouts can be observed. *OregonR ligt* shows a mean duration of 0.4s and *Goe-dark 05* to *Goe-dark15* show a significant decrease of mean thrust bout duration compared to the wildtype control. *Dark*-fly shows the lowest values with a mean bout duration of about 0.2s. N*(OR light)* = 97, *N(Goe-dark 01)* = 86, *N(Goe-dark 05)* = 83, *N(Goe-dark 10)* = 127, *N(Goe-dark 15)* = 112, *N(dark-fly)* = 124 [*p*-values: *ORL* vs *GD01* 21,659 x 10^{-2} ; *ORL* vs *GD05* 35 x 10^{-5}; *ORL* vs *GD10* 13 x 10^{-5} ; *ORL* vs *GD15* 13 x 10^{-5} ; *ORL* vs *DF* 13 x 10^{-5} ; *GD01* vs *GD05* 39,8 x 10^{-4} ; *GD01* vs *GD10* 31,3 x 10^{-4} ; *GD01* vs *GD15* 13 x 10^{-5} ; *GD01* vs *DF* 13 x 10^{-5}] **(B) Back to light.** Reintroducing *Drosophila dark*-fly back into light conditions leads to a direct significant increase of thrust bout duration. N*(OR light)* = 97, *N(Goe-dark light 05)* = 94, *N(Goe-dark light 10)* = 106, *N(dark-fly)* = 124, *N(dark-fly light)* = 101, *N(dark-fly light 05)* = 119, *N(Goe-dark light 05)* = 94, *N(Goe-dark 10)* = 106 [*p*-values: *DF* vs *DFL* 9 x 10^{-5} ; *DF* vs *DFL05* 9 x 10^{-5} ; *DF* vs *GDL10* 9 x 10^{-5} ; *DF* vs *GDL05* 9 x 10^{-5} ; *DF* vs *ORL* 9 x 10^{-5}]

4.3.3 Light-deprived flies show higher turning angle

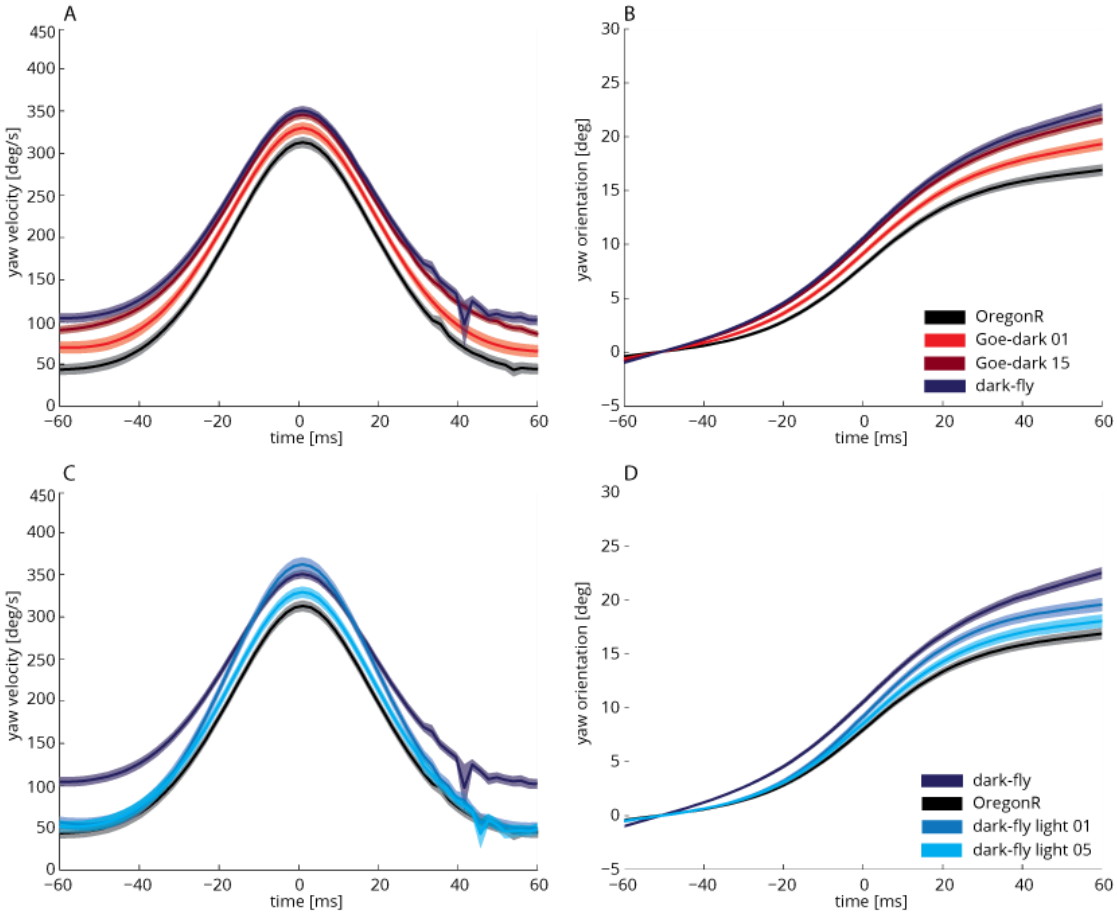
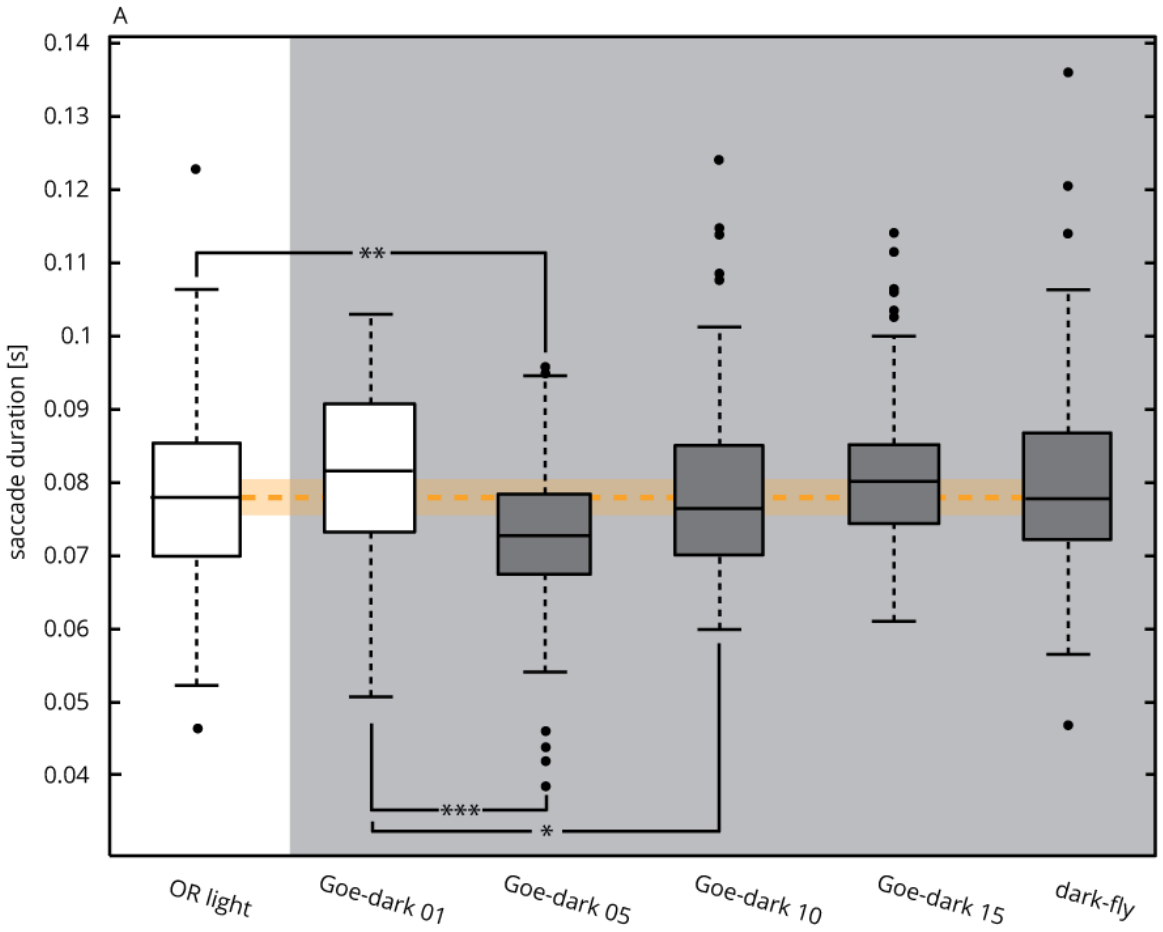


Figure 14 Analysis of saccade velocity and angle depicted as saccade triggered averages. The solid line indicates the median, the shaded area shows the 95% confidence interval (CI) of each group. N(*OR light*) = 98, N(*DF*) = 124, N(*GoeD01*) = 86, N(*GoeD15*) = 112 , N(*DFL*) = 100, N(*DFL05*) = 120 **(A) Mean yaw velocity of the saccade – into darkness.** Saccades were identified by using a yaw velocity of 200 deg/sec as threshold and were superimposed so that the peak velocity is at 0 ms. Preceding the analysis left and right saccades were separated leading to mirror-symmetric velocity profiles. The mean yaw velocity of *dark-fly*, *Goe-dark 01*, *Goe-dark 15* are not significantly different from the wt control *OregonR*. **(B) Mean corresponding turning angle – into darkness.** The angular heading of *dark-fly*, *Goe-dark 01* and *Goe-dark 15* is significantly bigger than of the wt control *OregonR*. **(C) Mean yaw velocity of the saccade – back to light.** Saccades were identified by using a yaw velocity of 200 deg/sec as threshold and were superimposed so that the peak velocity is at 0 ms. Preceding the analysis left and right saccades were separated leading to mirror-symmetric velocity profiles. Yaw velocity of *OregonR*, *dark-fly* and *dark-fly 05* does not significantly differ from each other. *Dark-fly light* shows a significantly higher yaw velocity compared to the other. **(D) Mean corresponding turning angle – back to light.** The change in angular heading of *dark-fly*, *dark-fly light* and *dark-fly light 05* are significantly bigger than the wt control.

All rotations with a yaw velocity not crossing the threshold were omitted for this analysis. The median was calculated as triggered averages, with the saccade peak velocity being the trigger. The mean yaw velocity of the wt control *OregonR* is 258,1 deg/sec. The yaw velocities of *dark-fly* (240.2 deg/sec), *Goe-dark 01* (235.2 deg/sec) and *Goe-dark 15*(246.6 deg/sec) (*Figure 14 A*). The corresponding change of angular heading within a 130 ms window was 18.1 deg for the wt *OregonR*, *dark-fly* (23.6 deg), *Goe-dark 01* (20.37 deg) and *Goe-dark 15* (22.08 deg) show all significant higher yaw angles (*Figure 14 B*).Again, the flies reintroduced back into light were tested; *dark-fly*, *dark-fly light 05* and wt show no significant differences in the yaw velocity. However, *dark-fly light 01* reaches a significantly higher yaw velocity compared to the other groups (*Figure 14 C*). The change of angular yaw heading in *dark-fly light 01* (20.62 deg) and *dark-fly light 05* (17.53 deg) is significantly larger than the wt control (18.1 deg) but does not reach *dark-fly* levels (23.6 deg) (*Figure 14 D*). Interestingly, the light-deprived strains show a significantly higher rotational velocity starting into the saccade than *OregonR* flies suggesting an increased amount of rotations in the light-deprived flies (*Figure 14 A & C*). Already in this rather simple analysis a clear trend towards higher rotational velocities and turning angles is observable in the light-deprived strains. This corresponds to the findings observed in the visual mutant strains. These data suggest that absence of visual cues not only influences thrust movements and but also promotes changes in saccadic and other rotational movements.

4.3.4 Saccades are increased in light-deprived flies

For a detailed characterization of saccade characteristics, the different parameters saccade duration, saccade amplitude, saccade frequency, and the time spent saccading were analysed. The saccade duration is not significantly different comparing the wt control *OregonR* (0.078 s) with *Goe-dark 01* (0.082), *Goe-dark* 10 (0.076 s), Goe-*dark 15* (0.08 s) and *dark-fly* (0.78 s)(*Figure 15 A*). *Goe-dark 05* shows a significantly reduced duration of 0.073s. Reintroducing *dark-fly* back into light conditions leads to a significant increase in saccade duration to 0.084 s. After five generations raised in dark:light conditions, *dark-fly light 05* flies reduce the saccade duration to 0.079 s (*Figure 15 B*).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saccade duration [s]
0.14
0.13
0.12
0.11
0.1
0.09
0.08
0.07
0.06
0.05
0.04
**
***
*
OR light
Goe-dark 01
Goe-dark 05
Goe-dark 10
Goe-dark 15
dark-fly

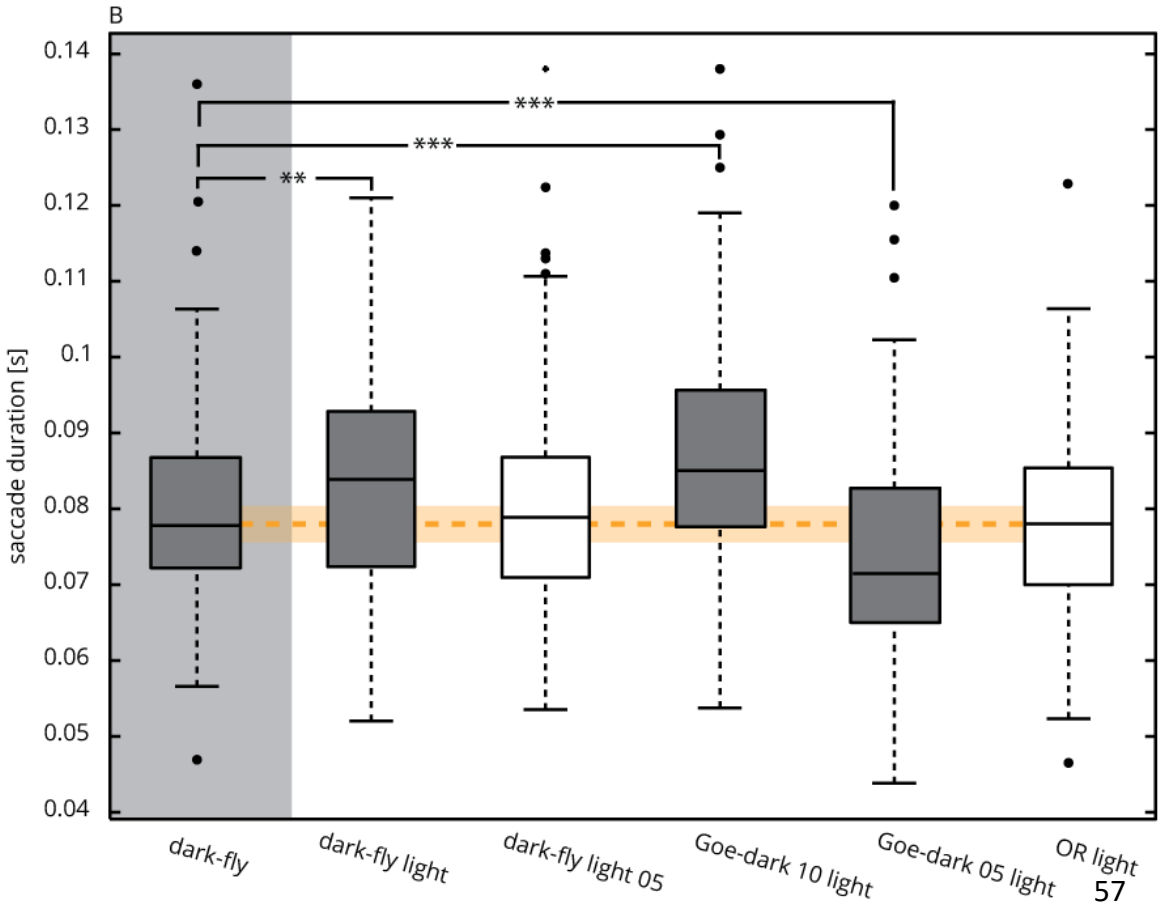
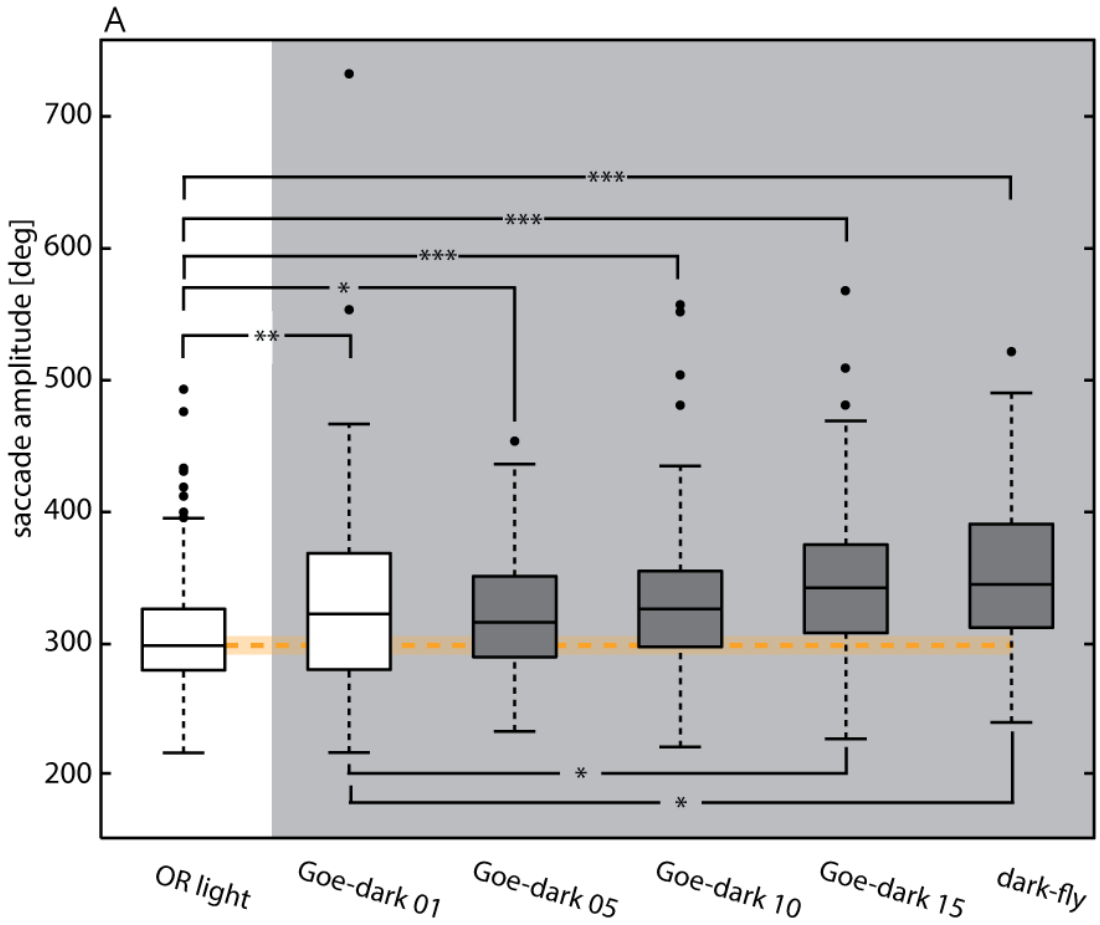

B
saccade duration [s]
0.14
0.13
0.12
0.11
0.1
0.09
0.08
0.07
0.06
0.05
0.04
***
***
**
dark-fly
dark-fly light
dark-fly light 05
Goe-dark 10 light
Goe-dark 05 light
OR light

Figure 15 Boxplots of the duration of saccades. Black lines indicate the medians; boxes include 50% of the data set around the medians; whiskers indicate 1.5* interquartile distance; outliers are marked by black circles. Orange dashed line indicates the median of the wt control (OR light), the shaded area marks the 95% confidence interval. Rearing and experimental conditions are indicated by the colour of the boxes and the background: a white box illustrates rearing in a 12:12 dark:light cycle, a grey box rearing in a 24h dark cycle. White background indicates that the recordings were done in light, grey background indicates that the recordings were done in darkness. To test for significance, we used a two-sample Kolmogorov-Smirnov test and corrected *p*-values using the Benjamin-Hochberg false FDR. Significance is indicated as follows: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ **(A) Into darkness.** The duration of saccades is similar in most of the groups, with an exception in *Goe-dark* 05, which shows significantly shorter saccades compared to OregonR and *Goe-dark* 01. N*(OR light)* = 97, *N(Goe-dark 01)* = 86, *N(Goe-dark 05)* = 83, *N(Goe-dark 10)* = 127, *N(Goe-dark 15)* = 112, *N(dark-fly)* = 124 [*p*-values: *ORL* vs *GD01* 21,659 x 10^{-2} ; *ORL* vs *GD05* 35 x 10^{-5}; *ORL* vs *GD10* 13 x 10^{-5} ; *ORL* vs *GD15* 13 x 10^{-5} ; *ORL* vs *DF* 13 x 10^{-5} ; *GD01* vs *GD05* 39,8 x 10^{-4} ; *GD01* vs *GD10* 31,3 x 10^{-4} ; *GD01* vs *GD15* 13 x 10^{-5} ; *GD01* vs *DF* 13 x 10^{-5}] **(B) Back to light.** Saccade durations of *dark-fly light*, *Goe-dark 05 light* and *Goe-dark 10 light* are significantly different from *dark-fly*. N*(OR light)* = 97, *N(Goe-dark light 05)* = 94, *N(Goe-dark light 10)* = 106, *N(dark-fly)* = 124, *N(dark-fly light)* = 101, *N(dark-fly light 05)* = 119, *N(Goe-dark light 05)* = 94, *N(Goe-dark 10)* = 106 [*p*-values: *DF* vs *DFL* 9 x 10^{-5} ; *DF* vs *DFL05* 9 x 10^{-5} ; *DF* vs *GDL10* 9 x 10^{-5} ; *DF* vs *GDL05* 9 x 10^{-5} ; *DF* vs *ORL* 9 x 10^{-5}]

This is not significantly different from both *dark-fly* or *OregonR*. Recording the dark-raised flies of generation 5 and 10 shows a small, but not significant decrease in duration (*Goe-dark 05 light*; 0.071 s), *Goe-dark 10 light* shows an increased saccade duration of 0.0085 s (*Figure 15 B*).

Analysing the saccade amplitude shows a direct and significant increase from *OregonR* (298.81 deg) to *Goe-dark 01* (322.82 deg) (*Figure 16 A*). The subsequent generation *Goe-dark 05* (316.46 deg) stays at about the same level and both *Goe-dark 10* (326.56 deg) and *Goe-dark 15* (342.51 deg) display significantly larger amplitudes compared to wt. *Dark-fly* reaches the highest saccade amplitude with 345.14 deg (*Figure 16 A*). *Dark-fly* recorded in light conditions shows a small, not significant increase in saccade amplitude to 350.4 deg, which is decreased after sustaining *dark-fly light* in a dark:light cycle (*dark-fly light 05*; 319.16 deg) (*Figure 16 B*). Reintroducing both *Goe-dark 05* and *Goe-dark 10* back into dark:light conditions shows a slight, not significant increase in *Goe-dark 05 light* (337.29 deg) and a small decrease in *Goe-dark 10 light* (322.91 deg) (*Figure 16 B*).


A
saccade amplitude [deg]
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OR light
Goe-dark 01
Goe-dark 05
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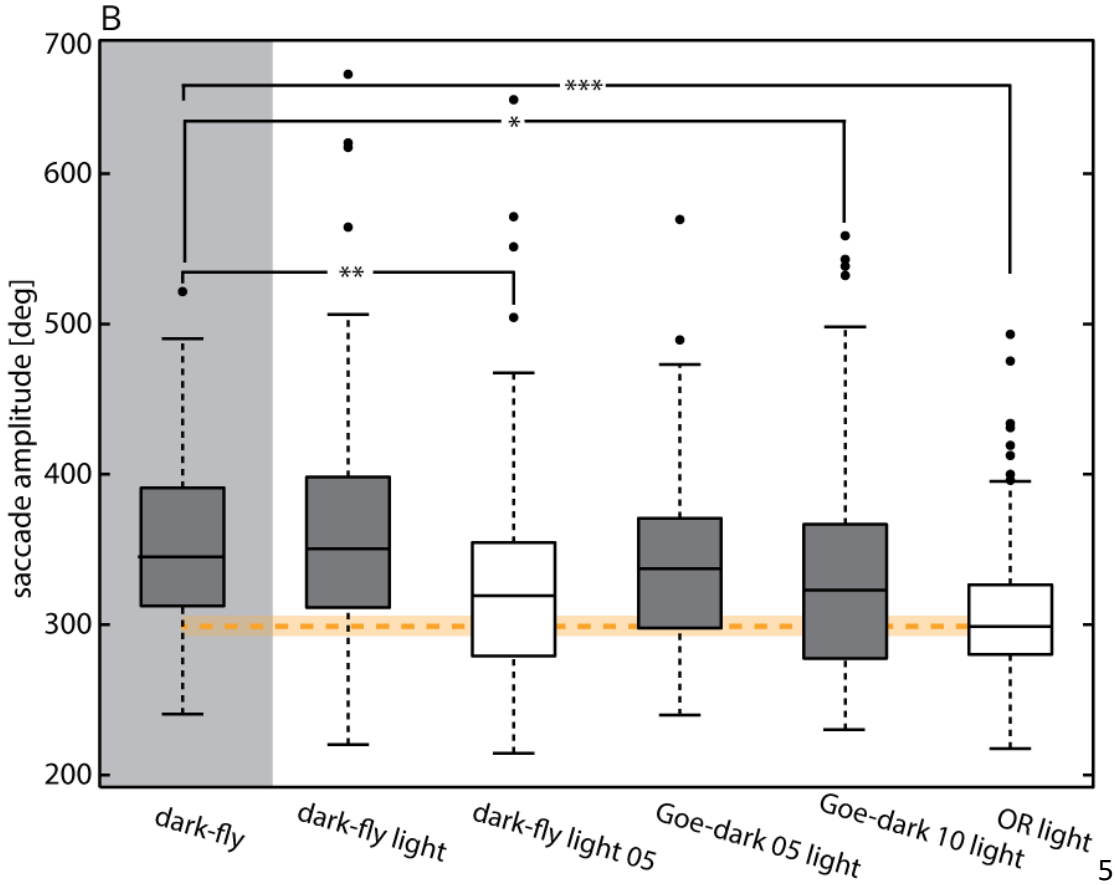
Goe-dark 15
dark-fly


B
saccade amplitude [deg]
700
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400
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*
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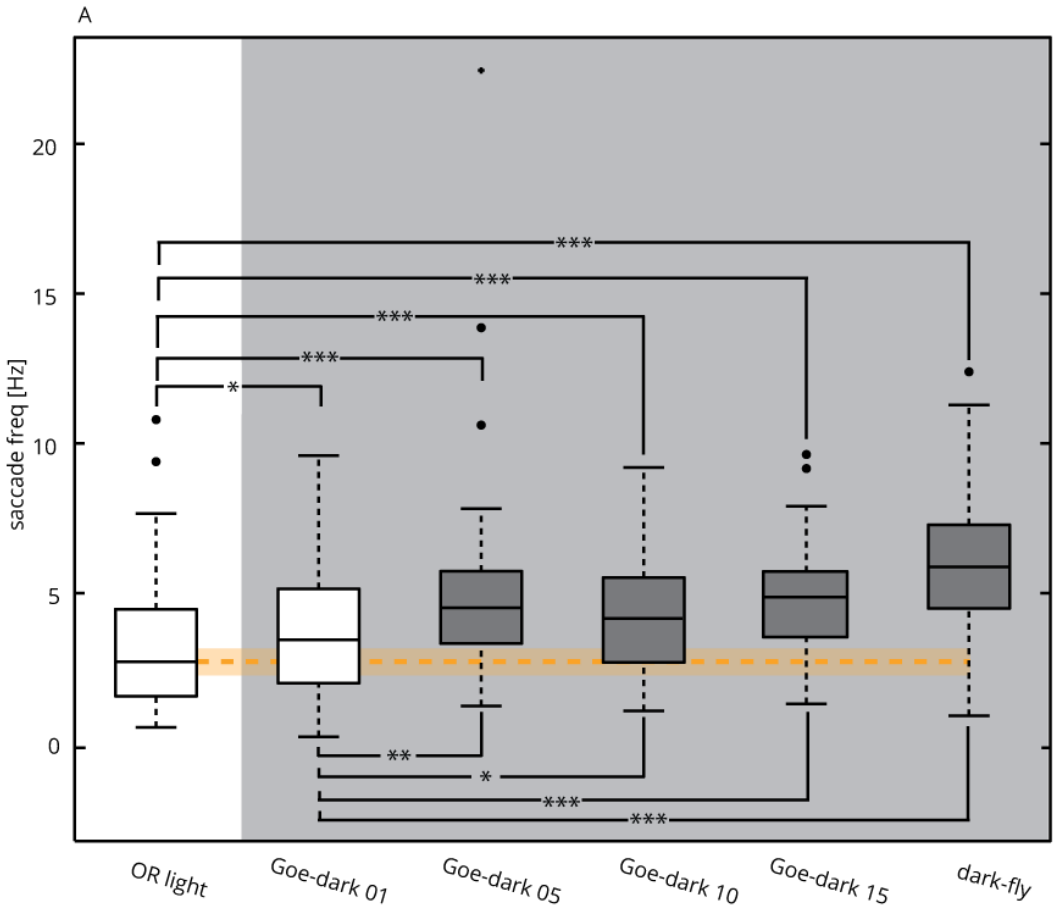
dark-fly
dark-fly light
dark-fly light 05
Goe-dark 05 light
Goe-dark 10 light
OR light

Figure 16 Boxplots of mean saccade amplitude. Black lines indicate the medians; boxes include 50% of the data set around the medians; whiskers indicate 1.5* interquartile distance; outliers are marked by black circles. Orange dashed line indicates the median of the wt control (OR light), the shaded area marks the 95% confidence interval. Rearing and experimental conditions are indicated by the colour of the boxes and the background: a white box illustrates rearing in a 12:12 dark:light cycle, a grey box rearing in a 24h dark cycle. White background indicates that the recordings were done in light, grey background indicates that the recordings were done in darkness. To test for significance, we used a two-sample Kolmogorov-Smirnov test and corrected *p*-values using the Benjamin-Hochberg false FDR. Significance is indicated as follows: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ **(A) Into darkness.** Mean saccade amplitude is progressing to significantly higher amplitudes with more time in darkness. *Dark-fly* shows the highest saccade amplitude. N*(OR light)* = 97, *N(Goe-dark 01)* = 86, *N(Goe-dark 05)* = 83, *N(Goe-dark 10)* = 127, *N(Goe-dark 15)* = 112, *N(dark-fly)* = 124 [*p*-values: *ORL* vs *GD01* 38,9 x 10^{-4} ; *ORL* vs *GD05* 19,13 x 10^{-3}; *ORL* vs *GD10* 35 x 10^{-5} ; *ORL* vs *GD15* 35 x 10^{-5} ; *ORL* vs *DF* 35 x 10^{-5} ; *GD01* vs *GD05* 41,347 x 10^{-2} ; *GD01* vs *GD10* 37,633 x 10^{-2} ; *GD01* vs *GD15* 15,4 x 10^{-3} ; *GD01* vs *DF* 17,54 x 10^{-3}] **(B) Back to light**. Mean saccade amplitude is getting smaller after reintrocuing *dark-fly* into light conditions. N*(OR light)* = 97, *N(Goe-dark light 05)* = 94, *N(Goe-dark light 10)* = 106, *N(dark-fly)* = 124, *N(dark-fly light)* = 101, *N(dark-fly light 05)* = 119, *N(Goe-dark light 05)* = 94, *N(Goe-dark 10)* = 106 [*p*-values: *DF* vs *DFL* 31,757 x 10^{-2} ; *DF* vs *DFL05* 3 x 10^{-3} ; *DF* vs *GDL10* 12,47 x 10^{-3} ; *DF* vs *GDL05* 20,492 x 10^{-2} ; *DF* vs *ORL* 38 x 10^{-5}]

The next characteristic that was analysed is the frequency with which the flies performed saccadic movements (*Figure 17*). Note that the definition of saccades includes all rotational movements with a yaw velocity of over 200 deg/s. Start and end points of a saccade were determined in the yaw velocity by detection of the crossing of the zero velocity or a pivot point. Saccades where either start or end of the rotation were not captured in the recording were excluded from the analysis.

The saccade frequency of wt flies is 2.7 Hz; when exposed to dark conditions we can observe a shift to higher frequencies (*Goe-dark 01*; 3.43 Hz; *Goe-dark 05* 4.51 Hz; *Goe-dark 10* 4.15 Hz, *Goe-dark 15* 4.87 Hz) (*Figure 17 A*). *Dark-fly* shows a 2x fold increase in saccade frequency of compared to *OregonR* (*dark-fly* 5.87 Hz) (*Figure 17 A*).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OR light
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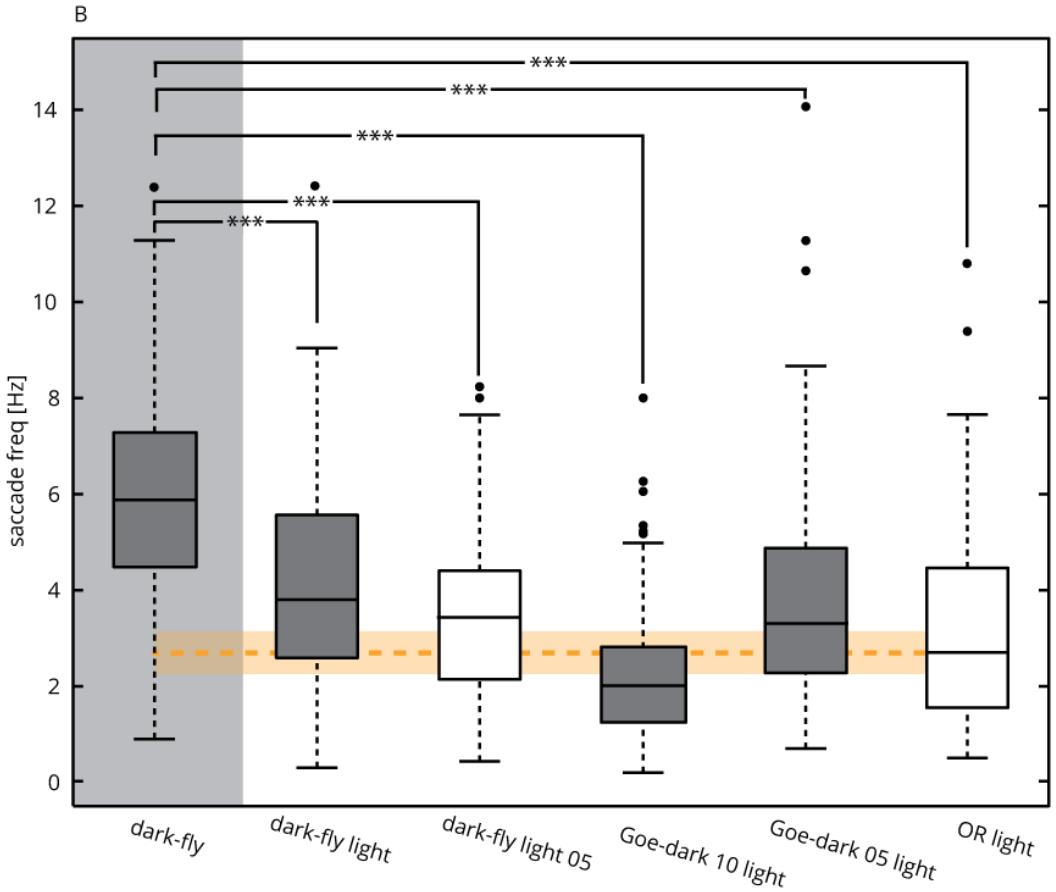
Goe-dark 05
Goe-dark 10
Goe-dark 15
dark-fly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6
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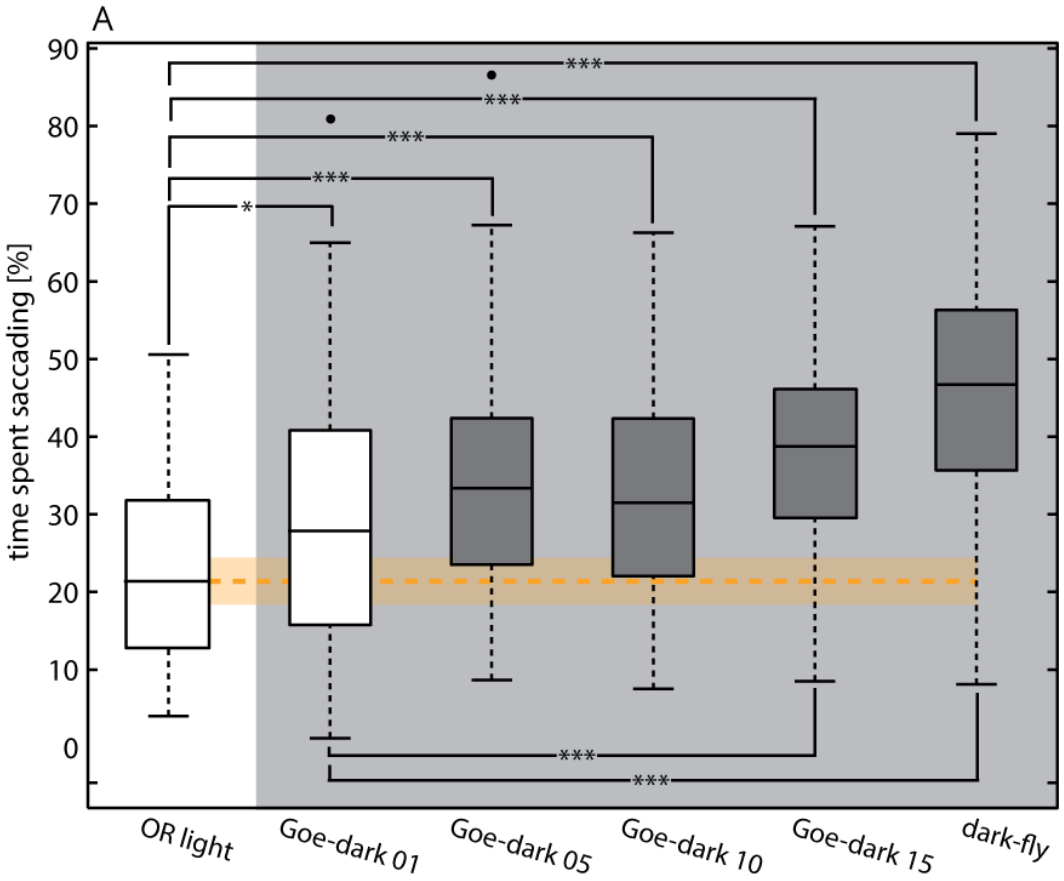
dark-fly
dark-fly light
dark-fly light 05
Goe-dark 10 light
Goe-dark 05 light
OR light

Figure 17 Boxplots of saccade frequency. Black lines indicate the medians; boxes include 50% of the data set around the medians; whiskers indicate 1.5* interquartile distance; outliers are marked by black circles. Orange dashed line indicates the median of the wt control (OR light), the shaded area marks the 95% confidence interval. Rearing and experimental conditions are indicated by the colour of the boxes and the background: a white box illustrates rearing in a 12:12 dark:light cycle, a grey box rearing in a 24h dark cycle. White background indicates that the recordings were done in light, grey background indicates that the recordings were done in darkness. To test for significance, we used a two-sample Kolmogorov-Smirnov test and corrected *p*-values using the Benjamin-Hochberg false FDR. Significance is indicated as follows: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ **(A) Into darkness.** Saccade frequency is significantly increased with time spent in darkness; *dark-fly* shows the highest saccade frequency. N*(OR light)* = 97, *N(Goe-dark 01)* = 86, *N(Goe-dark 05)* = 83, *N(Goe-dark 10)* = 127, *N(Goe-dark 15)* = 112, *N(dark-fly)* = 124 [*p*-values: *ORL* vs *GD01* 38,5 x 10^{-3} ; *ORL* vs *GD05* 13 x 10^{-5}; *ORL* vs *GD10* 13 x 10^{-5} ; *ORL* vs *GD15* 13 x 10^{-5} ; *ORL* vs *DF* 13 x 10^{-5} ; *GD01* vs *GD05* 39,4 x 10^{-4} ; *GD01* vs *GD10* 33,73 x 10^{-3} ; *GD01* vs *GD15* 13 x 10^{-5} ; *GD01* vs *DF* 13 x 10^{-5}] **(B) Back to light.** Reintroducing *dark*-fly and *Goe*-dark back to light shows a direct decrease in saccade frequency. N*(OR light)* = 97, *N(Goe-dark light 05)* = 94, *N(Goe-dark light 10)* = 106, *N(dark-fly)* = 124, *N(dark-fly light)* = 101, *N(dark-fly light 05)* = 119, *N(Goe-dark light 05)* = 94, *N(Goe-dark 10)* = 106 [*p*-values: *DF* vs *DFL* 9 x 10^{-5} ; *DF* vs *DFL05* 9 x 10^{-5}; *DF* vs *GDL10* 9 x 10^{-5}; *DF* vs *GDL05* 9 x 10^{-5} ; *DF* vs *ORL* 9 x 10^{-5}]

Re-establishing the *dark-fly* strain back to light conditions leads to a significant decrease in saccade frequency to 3.8 Hz which is further decreasing after 5 generations in dark:light conditions (*dark-fly light 05* 3.43 Hz) (*Figure 17 B*). *Goe-dark 05* decreases its saccade frequency to 3.31 Hz, *Goe-dark 10 light* to 2.01 Hz (*Figure 17 B*).

As explained before the saccadic strategy is characterized by reduced rotations to minimize the time in which extraction of 3D information is not possible. We already saw a significant increase in the time spent saccading in *Drosophila* with impaired visual system, and subsequently analysed this in the dark-raised strains.

As explained before the saccadic strategy is characterized by reduced rotations to minimize the time in which extraction of 3D information is not possible. We already saw a significant increase in the time spent saccading in *Drosophila* with impaired visual system, and subsequently analysed this in the dark-raised strains. *OregonR* flies show a significant increase in the time spent with saccadic movements when recorded in dark (*OregonR* 21.37%; *Goe-dark 01* 27.87%) (*Figure 18 A*).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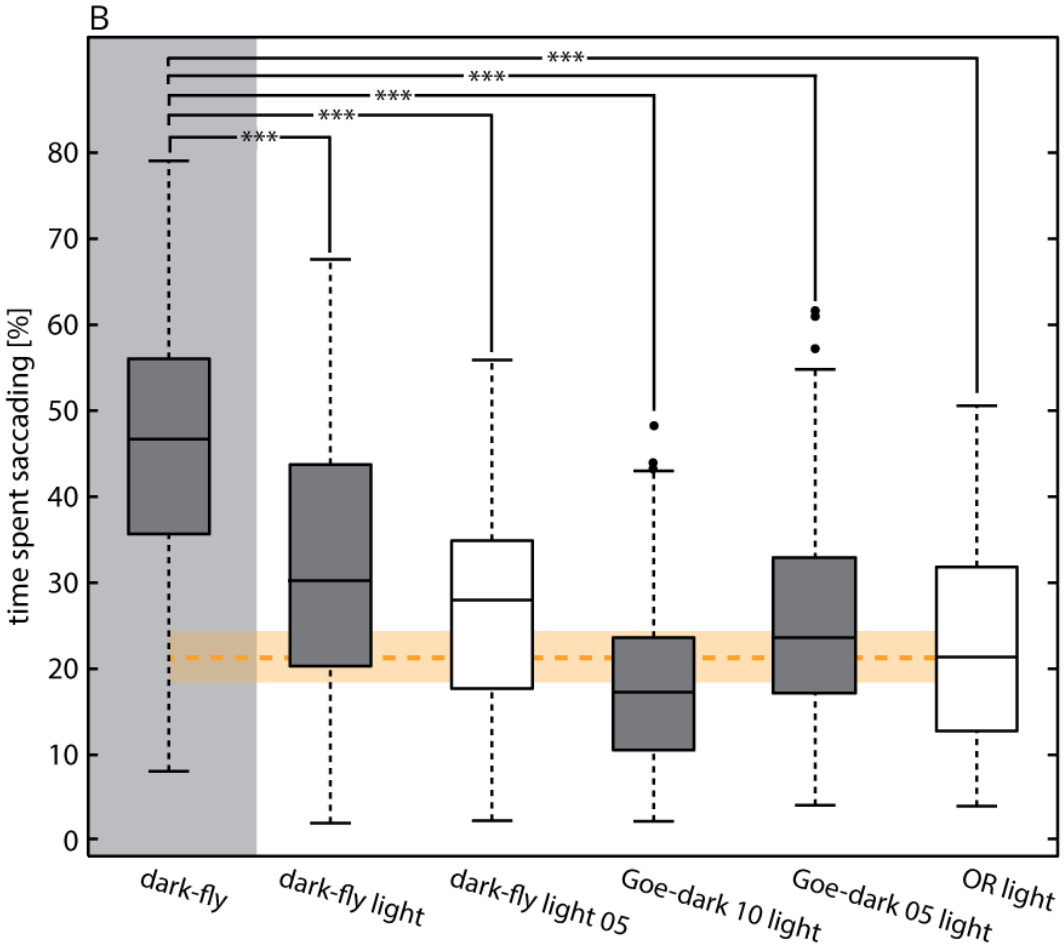
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Goe-dark 10 light
Goe-dark 05 light
OR light

Figure 18 Boxplots of the time that was spent saccading. Black lines indicate the medians; boxes include 50% of the data set around the medians; whiskers indicate 1.5* interquartile distance; notches display the 95% confidence interval; outliers are marked by black circles. Orange dashed line indicates the median of the wt control (OR light), the shaded area marks the 95% confidence interval. Rearing and experimental conditions are indicated by the colour of the boxes and the background: a white box illustrates rearing in a 12:12 dark:light cycle, a grey box rearing in a 24h dark cycle. White background indicates that the recordings were done in light, grey background indicates that the recordings were done in darkness. To test for significance, we used a two-sample Kolmogorov-Smirnov test and corrected *p*-values using the Benjamin-Hochberg false FDR. Significance is indicated as follows: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ **(A) Into darkness.** The time spent saccading is significantly increased with time spent in darkness and shows the highest percentage in *dark-fly*. N*(OR light)* = 97, *N(Goe-dark 01)* = 86, *N(Goe-dark 05)* = 83, *N(Goe-dark 10)* = 127, *N(Goe-dark 15)* = 112, *N(dark-fly)* = 124 [*p*-values: *ORL* vs *GD01* 14,42 x 10^{-3} ; *ORL* vs *GD05* 16 x 10^{-5}; *ORL* vs *GD10* 23 x 10^{-5} ; *ORL* vs *GD15* 16 x 10^{-5} ; *ORL* vs *DF* 16 x 10^{-5} ; *GD01* vs *GD05* 57,93 x 10^{-2} ; *GD01* vs *GD10* 64,85 x 10^{-2} ; *GD01* vs *GD15* 23 x 10^{-5} ; *GD01* vs *DF* 16 x 10^{-5}] **(B) Back to light.** Upon reintroduction to light conditions the fraction of time spent saccading is significantly reduced compared to *dark*-fly. N*(OR light)* = 97, *N(Goe-dark light 05)* = 94, *N(Goe-dark light 10)* = 106, *N(dark-fly)* = 124, *N(dark-fly light)* = 101, *N(dark-fly light 05)* = 119, *N(Goe-dark light 05)* = 94, *N(Goe-dark 10)* = 106 [*p*-values: *DF* vs *DFL* 9 x 10^{-5} ; *DF* vs *DFL05* 9 x 10^{-5} ; *DF* vs *GDL10* 9 x 10^{-5} ; *DF* vs *GDL05* 9 x 10^{-5} ; *DF* vs *ORL* 9 x 10^{-5}]

This trend towards an increased time spent with saccading is progressing with the successive dark-raised generations (*Goe-dark 05* 33.56 %; *Goe-dark 10* 31.48 %; *Goe-dark 15* 38.76 %) (*Figure 18 A*).

Reintroducing *dark-fly* in light decreases the percentage of time spent saccading to 30.25 % and gets even shorter after five generations in dark:light cycle (*dark-fly light 05* 27.97) (*Figure 18 B*). Recording *Goe-dark 05* in light conditions leads to a decrease back to wt levels (*Goe-dark 05 light* 23.6 %) and even lower in *Goe-dark 10 light* to 17.27 % (*Figure 18 B*).

The detailed analysis of saccade characteristics shows a severe impact of light-deprivation, and therefore non-availability of visual cues, on the generation of saccadic movements. With progressing time *Drosophila* is sustained in darkness the frequency of saccades and correspondingly the time spent with saccadic movements is increased, which is immediately reversed by reintroducing these flies back to light. Furthermore,

the saccade amplitude is raising with subsequent generations in darkness and is reduced upon reintroduction to light conditions. This suggests that these changes in locomotion strategy are highly and transiently adaptable to environmental conditions.
These findings provide evidence, that diversion from the saccadic strategy is indeed mediated by the absence of visual cues.

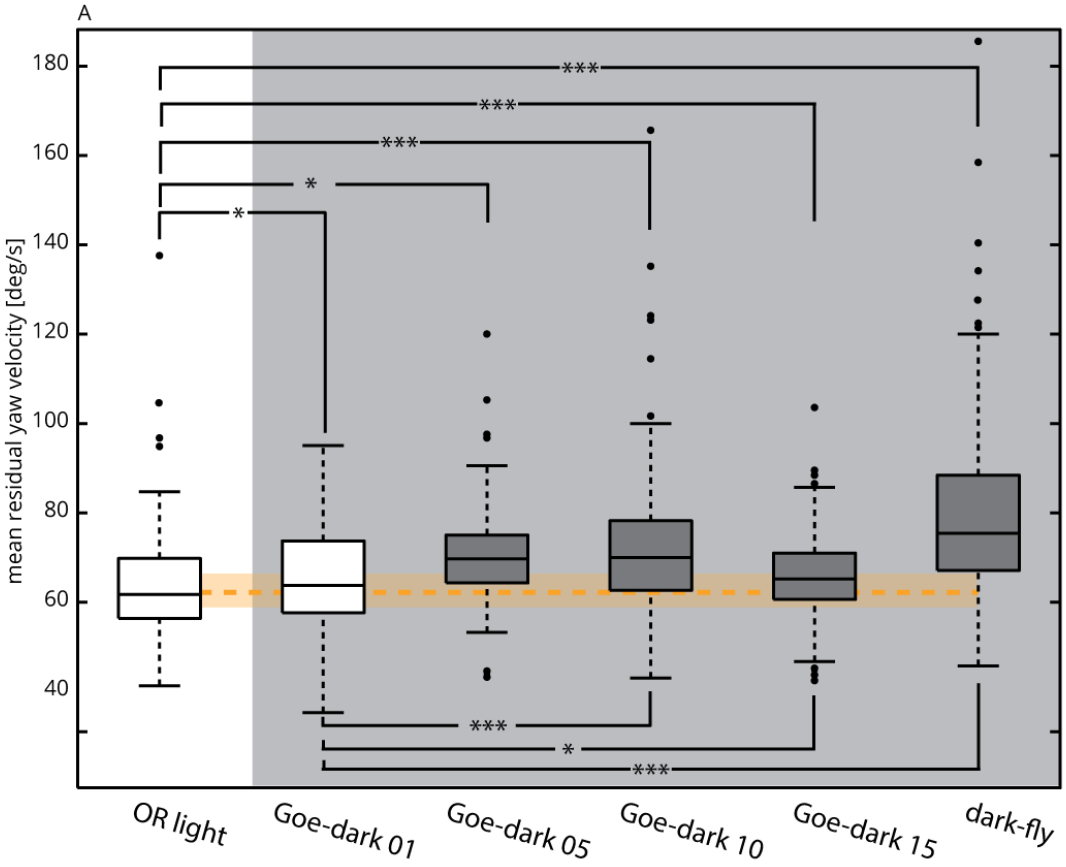
4.2.5 Other rotations are increased in light-deprived flies

The detailed analysis of saccadic movements showed significant changes in light-deprived *Drosophila* (chapter *4.3.4* Saccades are increased in light-deprived flies). While saccades are a stereotypical, hard-wired behaviour, other rotational movements are supposedly more flexible and should therefore be influenced by light-deprivation. *Drosophila* strains with differently impaired visual systems show an increase in rotational velocity (chapter *4.2.2* Absence of visual cues prolongs the time spent with rotations) suggesting that the residual yaw movements would similarly be influenced by light-deprivation.

In *OregonR* a mean residual yaw velocity of 61.74 deg/s can be found which is increased to 63.74 deg/s when recordings are performed in darkness; subsequently, the dark-raised generations showed further increasing velocities (*Goe-dark 05* 69.73 deg/s; *Goe-dark 10* 70.03 deg/s, *Goe-dark 15* 65.19 deg/s) (*Figure 19 A*). In the *dark-fly* strain, the mean residual yaw velocity is further accelerated to 75.44 deg/s. Re-establishing *dark-fly* back in light conditions leads to a direct increase in residual yaw velocity, levels stay stable after these flies were raised in darkness for five generations (*dark-fly light* 66.86 deg/s; *dark-fly light 05* 65.83deg/s) (*Figure 19 B*). In both dark-raised groups *Goe-dark 05* and *Goe-dark 10* a decrease of velocity can be found; in *Goe-dark 05 light* levels (62.78 deg/s) come close to wt, but *Goe-dark 10 light* reaches significantly lower velocities of 51.32 deg/s (*Figure 19 B*).
Calculating the integral of rotational velocities shows a significant increase from *OregonR* (88.26 deg/s) to *Goe-dark 01* (100.54 deg/s) and subsequent generations (*Goe-*

dark 05 114.57 deg/s; *Goe-dark 10* 115.4 deg/s; *Goe-dark 15* 120.69 deg/s). *Dark-fly* displays the highest levels in the current data set with 148.74 deg/s (*Figure 20 A*).
Recording *dark-fly* in light conditions lowers levels to 114.63 in *dark-fly light* and show further decline after *dark-fly* was sustained in light conditions for five generation (*dark-fly ligher 05* 101.11). Consequently, in both *Goe-dark 05 light* (96.65) and *Goe-dark 10 light* (74.49) values abate and are approaching wt levels (*Figure 20 B*)
This shows that dark-raised flies and *dark-fly* not only show more and faster saccades but also the remaining rotational movements, not reaching saccade threshold, are increased in both velocity and abundance. This indicates a reduced gaze stabilisation, as a direct consequence of the loss of vision.

We also tested our observations by segregating locomotion trajectories using unsupervised data driven algorithms to eliminate any observer bias. Therefore, an analysis of prototypical movements, obtained by a cluster algorithm, was performed.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Goe-dark 05
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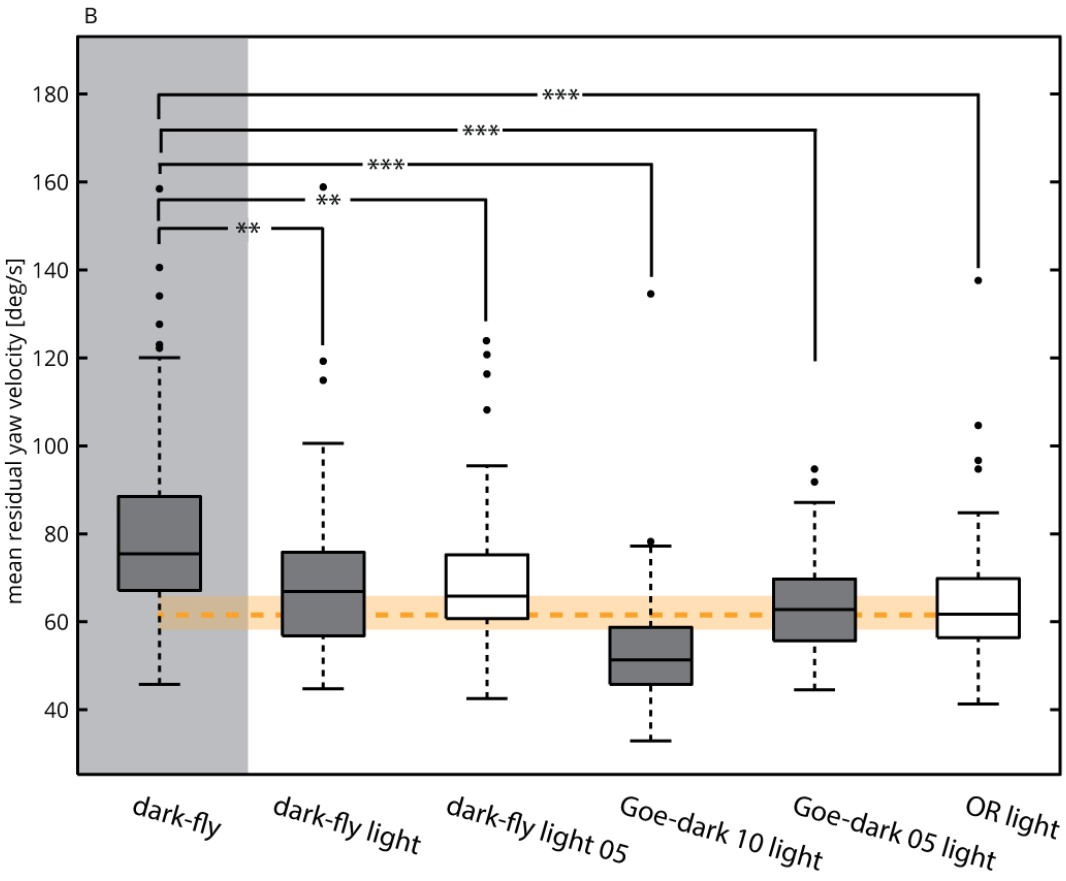
Goe-dark 15
dark-fly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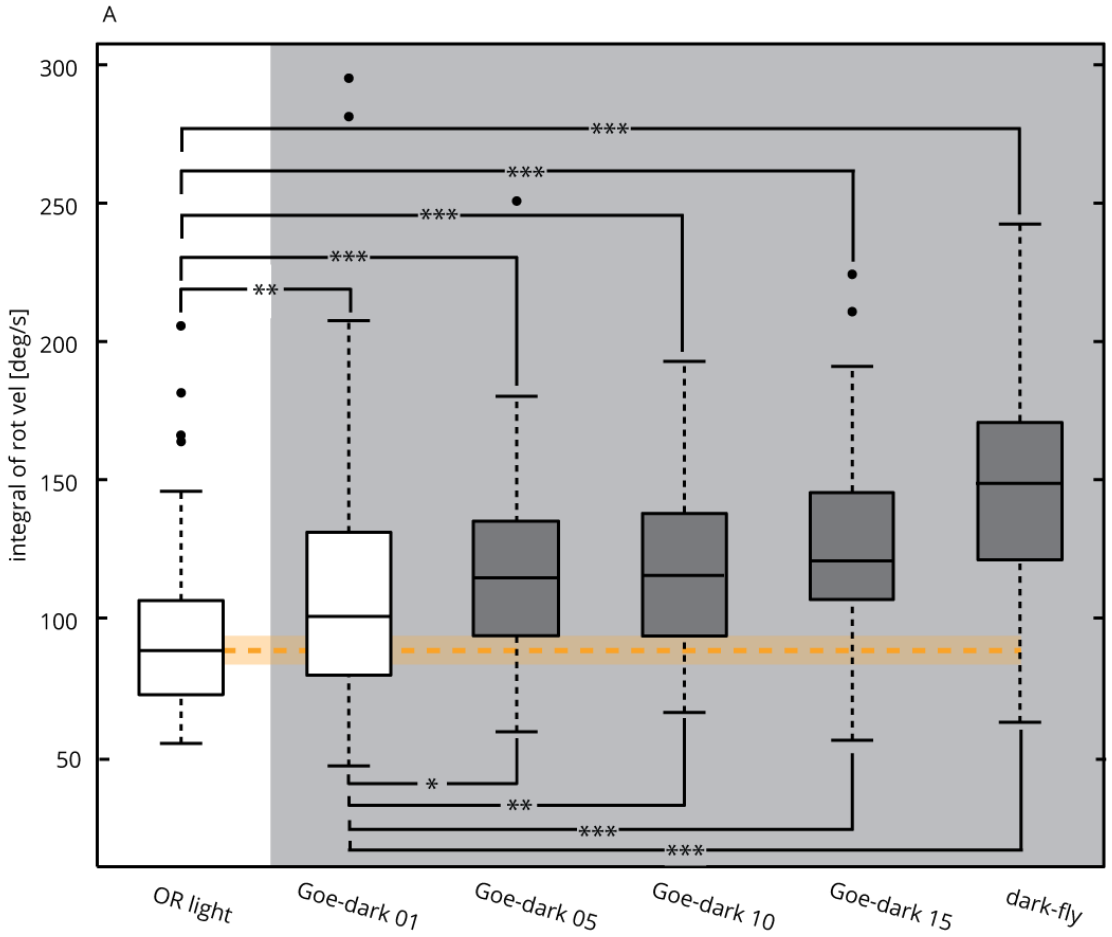
Goe-dark 10 light
Goe-dark 05 light
OR light

Figure 19 Boxplots mean residual yaw velocity. Black lines indicate the medians; boxes include 50% of the data set around the medians; whiskers indicate 1.5* interquartile distance; outliers are marked by black circles. Orange dashed line indicates the median of the wt control (OR light), the shaded area marks the 95% confidence interval. Rearing and experimental conditions are indicated by the colour of the boxes and the background: a white box illustrates rearing in a 12:12 dark:light cycle, a grey box rearing in a 24h dark cycle. White background indicates that the recordings were done in light, grey background indicates that the recordings were done in darkness. To test for significance, we used a two-sample Kolmogorov-Smirnov test and corrected *p*-values using the Benjamin-Hochberg false FDR. Significance is indicated as follows: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ **(A) Into darkness**. The velocity of the residual rotations is increasing with time spent in darkness. N*(OR light)* = 97, *N(Goe-dark 01)* = 86, *N(Goe-dark 05)* = 83, *N(Goe-dark 10)* = 127, *N(Goe-dark 15)* = 112, *N(dark-fly)* = 124 [*p*-values: *ORL* vs *GD01* 44.51×10^{-3} ; *ORL* vs *GD05* 45.18×10^{-3}; *ORL* vs *GD10* 9×10^{-5} ; *ORL* vs *GD15* 9×10^{-5} ; *ORL* vs *DF* 9×10^{-5} ; *GD01* vs *GD05* $45{,}69 \times 10^{-2}$; *GD01* vs *GD10* 9×10^{-5} ; *GD01* vs *GD15* 20.88×10^{-3} ; *GD01* vs *DF* 9×10^{-5}] **(B) Back to light**. Reintroducing dark-raised flies back into light conditions decreases the yaw velocity of rotations. N*(OR light)* = 97, *N(Goe-dark light 05)* = 94, *N(Goe-dark light 10)* = 106, *N(dark-fly)* = 124, *N(dark-fly light)* = 101, *N(dark-fly light 05)* = 119 [*p*-values: *DF* vs *DFL* 78.8×10^{-4} ; *DF* vs *DFL05* 20.2×10^{-4} ; *DF* vs *GDL10* 9×10^{-5} ; *DF* vs *GDL05* 9×10^{-5} ; *DF* vs *ORL* 9×10^{-5}]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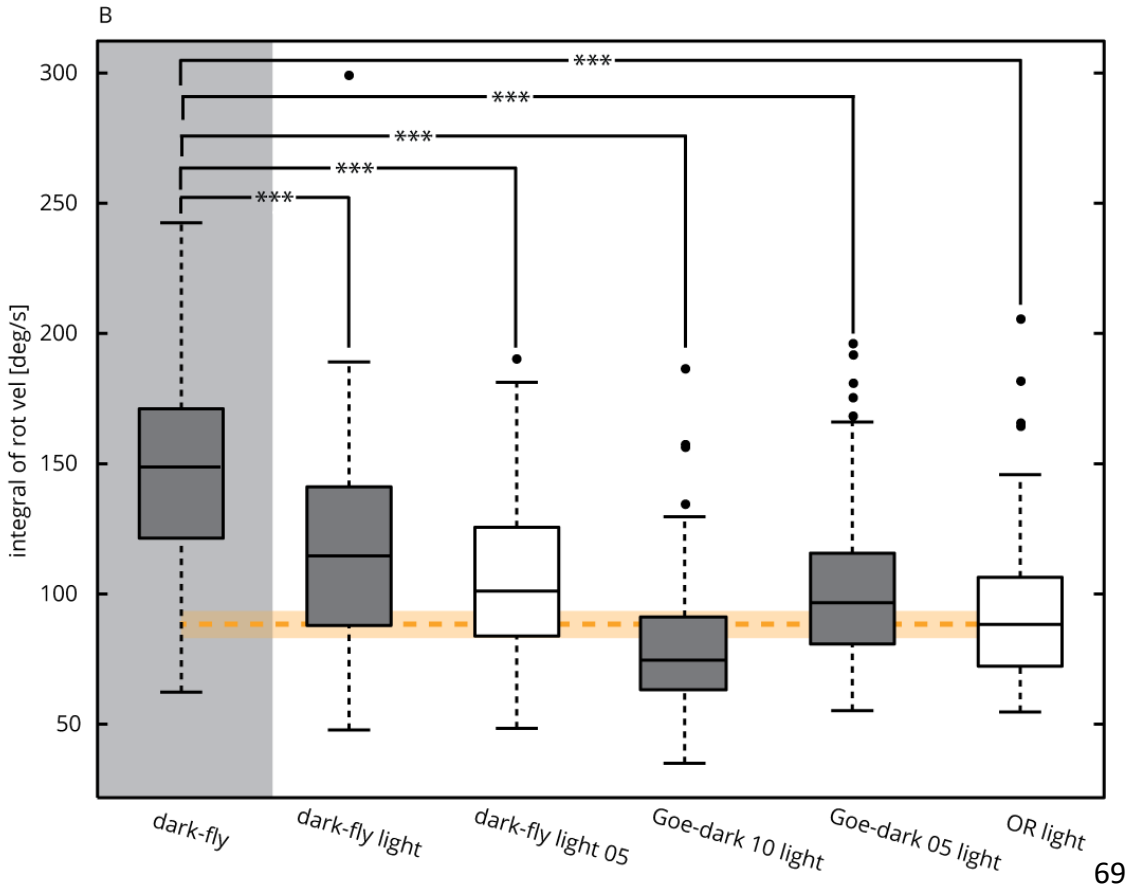
Goe-dark 15
dark-fly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Goe-dark 10 light
Goe-dark 05 light
OR light

Figure 20 Boxplots showing the integral of rotation velocities. Black lines indicate the medians; boxes include 50% of the data set around the medians; whiskers indicate 1.5* interquartile distance; notches display the 95% confidence interval; outliers are marked by black circles. Orange dashed line indicates the median of the wt control (OR light), the shaded area marks the 95% confidence interval. Rearing and experimental conditions are indicated by the colour of the boxes and the background: a white box illustrates rearing in a 12:12 dark:light cycle, a grey box rearing in a 24h dark cycle. White background indicates that the recordings were done in light, grey background indicates that the recordings were done in darkness. To test for significance, we used a two-sample Kolmogorov-Smirnov test and corrected *p*-values using the Benjamin-Hochberg false FDR. Significance is indicated as follows: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ (**A) Into darkness.** The integral of rotation velocity is increasing with time spent in darkness N*(OR light)* = 97, *N(Goe-dark 01)* = 86, *N(Goe-dark 05)* = 83, *N(Goe-dark 10)* = 127, *N(Goe-dark 15)* = 112, *N(dark-fly)* = 124 [*p*-values: *ORL* vs *GD01* $43{,}6 \times 10^{-4}$; *ORL* vs *GD05* 23×10^{-5}; *ORL* vs *GD10* 12×10^{-5} ; *ORL* vs *GD15* 12×10^{-5} ; *ORL* vs *DF* 12×10^{-5} ; *GD01* vs *GD05* $43{,}75 \times 10^{-3}$; *GD01* vs *GD10* $42{,}9 \times 10^{-4}$; *GD01* vs *GD15* 32×10^{-5} ; *GD01* vs *DF* 12×10^{-5}] **(B) Back to light.** Dark-raised flies show a decreased integral of rotation velocity after reintroduction to light conditions. N*(OR light)* = 97, *N(Goe-dark light 05)* = 94, *N(Goe-dark light 10)* = 106, *N(dark-fly)* = 124, *N(dark-fly light)* = 101, *N(dark-fly light 05)* = 119 [*p*-values: *DF* vs *DFL* 8×10^{-5} ; *DF* vs *DFL05* 8×10^{-5} ; *DF* vs *GDL10* 8×10^{-5} ; *DF* vs *GDL05* 8×10^{-5} ; *DF* vs *ORL* $8 \times 10^{-}$

4.3.6 Prototypical movements show increase in rotation in light-deprived flies

A previous study on walking behaviour in wt *Drosophila* revealed a separation of translational and rotational movements by analysing prototypical movements (PM; Geurten et al., 2014). PMs are defined as reoccurring movement patterns, consisting of a combination of the three movement directions slip, thrust and yaw (*Figure 5*). To identify PMs, the respective movement velocities were deduced from the free walking behaviour and the most common combination extracted by utilizing a cluster analysis.

Computation of PMs rendered four classes for all strains: two rotations (left and right), translation and a phase of inactivity (*Figure 21*). However, in *Goe-dark 01* and *Goe-dark 05* the fraction of translational movements is decreased while the fraction of rotations shows an increase for both rotation directions (*Figure 21*). The higher translational velocity during rotational movements and the increase in fraction of rotations indicates

a divergence from the classic saccadic movement strategy in which the amount of rotational movements is decreased to guarantee optimal exploitation of the 3D-information generated during translational movements.

Furthermore, the velocity of the respective movements, shows an increase from *OregonR* to both *Goe-dark 01* and *Goe-dark 05* (*Figure 12*), suggesting an overall increase in movement velocities with ongoing light deprivation.

The fraction of inactivity is decreased from *OregonR* to both *Goe-dark 01* and *Goe-dark 05*, indicating an increase in activity in dark conditions (*Figure 21*).

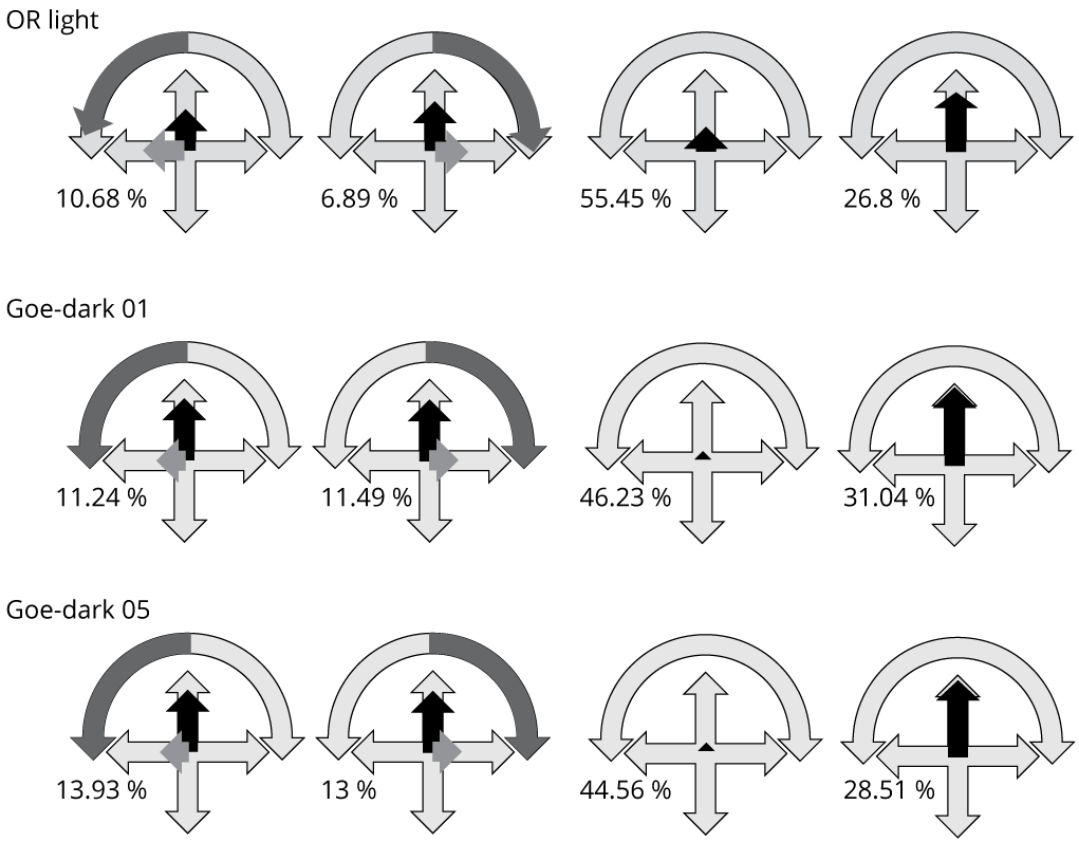


Figure 21 Prototypical movements for *OregonR*, *Goe-dark 01* and *Goe-dark 05*. Prototypical movements (PMs) were computed from the free walking behaviour, recorded at 500 fps. Grey arrows indicate the velocity combination characterizing each PM, size of arrows indicate the velocity of each movement direction. Light grey background shows the fastest movement velocity found in all data sets. The respective abundance in the dataset for each PM is indicated in percent. The combination of PMs does not differ between *OregonR*, *Goe-dark 01* and *Goe-dark 05*. The respective abundance of the different PMs shows an increase in both left and right rotations and a decrease in translation. Pausing behaviour is reduced in *Goe-dark 01* and *Goe-dark 05*, indicating a higher overall activity

4.4 Light-deprived *Drosophila* develop new locomotion strategy

4.4.1 Exploration rate is increased in *dark-fly*

The previous results obtained in this study show a diversion from the classical saccadic strategy. This is not surprising as the saccadic strategy optimises the visual input to extract 3D information during locomotion. In light conditions, *Drosophila* can use their visual field to navigate around the environment, however, in dark conditions they must rely on the mechanosensory field composed by the mechanosensory organs. This raises the question, whether *dark-fly* displays a new locomotion strategy, guaranteeing higher explorative success in dark conditions. Other senses might have become more dominant in darkness and the locomotion behaviour could have adapted to facilitate these senses. A simple hypothesis might be that mechanoreception is emerging as one of the dominant senses as the bristles of *dark-flies* are elongated (Fuse et al., 2014; Imaizumi, 1979). In light conditions, *Drosophila* can use their visual field to navigate around their environment, however, in dark conditions we hypothesis that they rely on the mechanosensory field composed by the mechanosensory organs. In this case, the animal would need touch sensations to gain information about their environment. Consequently, in dark conditions the sensory reception field of *Drosophila* would be reduced from about 2 cm in light conditions (size of the visual field, see: (Geurten et al., 2014; Schneiderr et al., 2018) to roughly its own body surface.

To assess if *dark-fly* utilizes a new and dark-adapted strategy the exploration rate was analysed (*Figure 22*). *OregonR* (5,18 %) shows slightly elevated levels of exploration rate when introduced to dark condition (*Goe-dark 01* 5,86 %), however, it does not reach significance. Maintaining those flies in darkness shows a shift to a significantly increased exploration rate (*Goe-dark 05* 6.33 %; *Goe-dark 10* 7.73 %; *Goe-dark 15* 8.38 %). *Dark-fly* shows an exploration rate over twice as high as *OregonR* (10.94 %) (*Figure 22 A*).

Interestingly, the fast changes in locomotion strategy after only a few generations in persistent darkness are equally fast “recovering”/reversed to wild type levels in a dark:light cycle. Comparing *Goe-dark 05* and *Goe-dark 10* in light and dark conditions shows a significant decrease in exploration rate when recorded in darkness (*Goe-dark* 05_{light} 6.7 %; *Goe-dark* 10_{light} 5.82 %)(*Figure 22 B*). Even though significant, the change

after reintroduction of *dark-fly* in light conditions is less severe as for example the change in rotational and translational velocity, implying at a more persistent adaptation in the exploration behaviour.

4.4.2 *Tōhoku drift* increases exploration rate in light-deprived flies

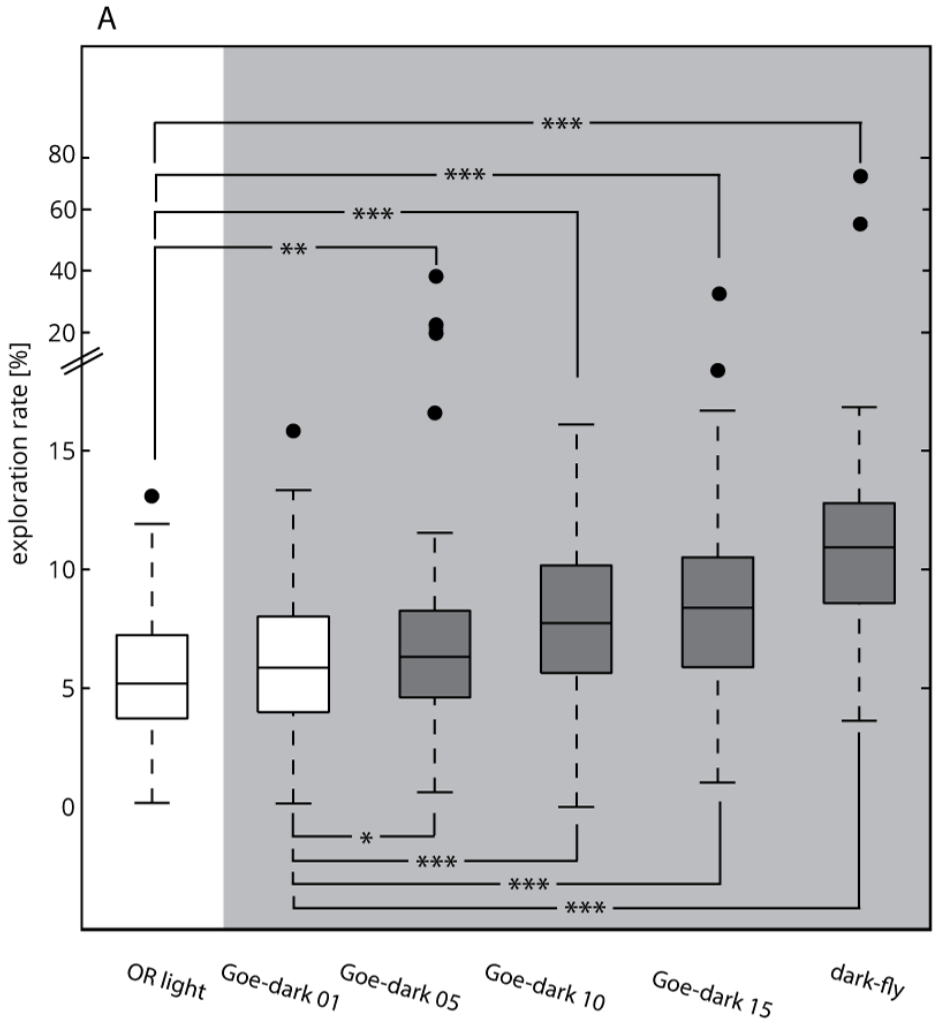
To understand the strategy responsible for the increase in exploration strategy found in long-term light-deprived flies, the curve walking of the different strains was examined. This revealed some severe differences in the aforementioned strains: while *OregonR* flies in light conditions normally manoeuvre around a corner pirouetting. In contrast *dark-fly* shows a drifting movement. This allows *dark-fly* to effectively cover more area with their mechanosensory field, thereby generating an increased exploration rate. I call this drifting motion *Tōhoku drift*.

To quantify the proportion of increased exploration rate that is due to the *Tōhoku drift*, the walking trajectories were again compared. We performed a detailed comparison of the wt and dark-fly trajectories. If the *Tōhoku drift* indeed increases the mechanosensory field during locomotion, the orientation of the fly during rotational movements is critical. While drifting through a rotation would prolong rotation duration, it is also increasing the area covered by the body. By superimposition of the location and orientation of each respective fly for every frame in its trajectory we obtained a so-called summed trajectory which allows us to calculate the surface area covered by the fly. We also calculated an ellipse with the median fly surface and a median fly anterior-posterior axis. This ellipse was superimposed on all trajectory positions in the orientation of the fly, thereby creating an abstract fly. As circles have no orientation, superimposing circle on the fly's trajectory omitted all orientation biases. The difference in area covered by the circle and area covered by the ellipse amounts to the drifting motion *Tōhoku drift*.

This comparison shows no significant difference for *OregonR* in light or dark conditions (*OregonR* 8.55 mm^2; *Goe-dark 01* 13.43 mm^2) (*Figure 23 A*). In subsequent generations, only *Goe-dark 10* displays a significant increase (*Goe-dark 05* 14.15 mm^2; *Goe-dark 10* 37.55 mm^2; *Goe-dark 15* 23.24 mm^2). However, *dark-fly* shows a significantly increased difference of 68.06 mm^2 (*Figure 22 A*).

Even more interesting, while dark-flies rapidly re-establish a classic saccadic locomotion strategy *(*see *4.3 Light deprivation severely influences the saccadic strategy*), when shifted back to a dark:light cycle, *Tōhoku drift* was persistently found under these conditions (*dark-fly light* 71.15 mm^2; *dark-fly light 05* 61.44 mm^2) (*Figure 22 B*). In contrast, besides the mild trend of exhibiting *Tōhoku drift* of Goe-dark flies even the Goe-dark 10 strain rapidly dropped this locomotion strategy.

Additionally, I calculated the gain in exploration rate amounting to the *Tōhoku drift* (*Figure 24*). Correspondingly, a strong contribution of the *Tōhoku drift* to the increased exploration rate during long-term light deprivation can be observed (*OregonR* 11.41 %; *Goe-dark 01* 19.69 %; *Goe-dark 05* 19.41%; *Goe-dark 10* 39.56%; *Goe-dark 15* 20.68 %) (*Figure 24 A*). In *dark-fly* a significant increase can be detected which is not influenced by a change in illumination conditions as is still persistent after 5 generations in light conditions (*dark-fly* 49.83 %; *dark-fly light* 54.01 %; *dark-fly light 05* 50.55 %) (*Figure 24 B*). While *Goe-dark 10* shows no significant difference from *OregonR* in light conditions (*Goe-dark* 10_{light} 21.85 %), *Goe-dark 5* shows an increase in difference when being introduced into light (*Goe-dark* 05_{light} 32.98 %) (*Figure 24 B*).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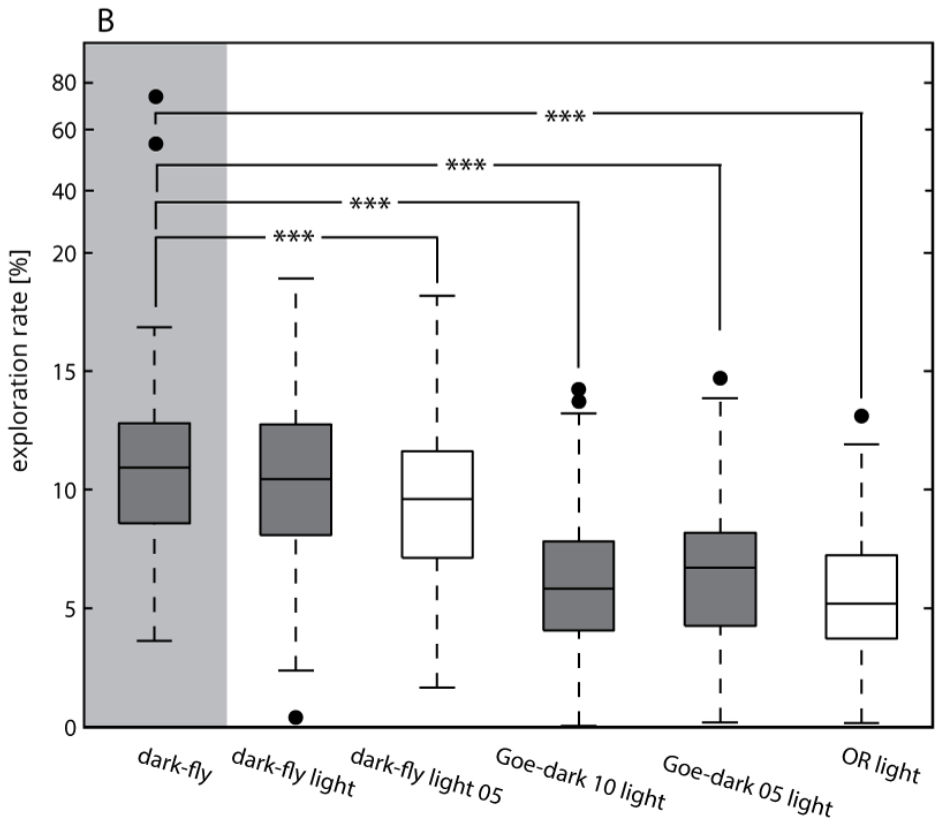
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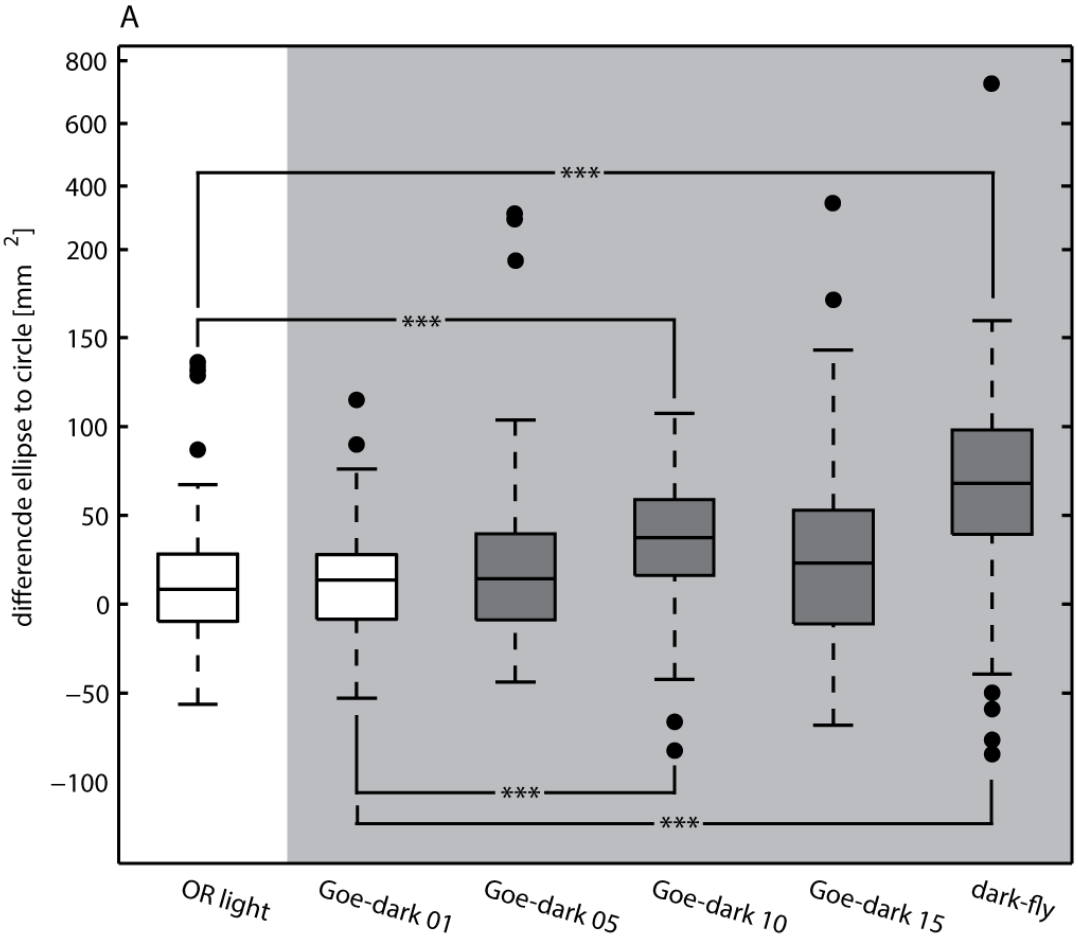
Goe-dark 05 light
OR light

Figure 22 Exploration rate. The exploration rate displays the fraction of arena area covered in the 10 s of experimental recording. Black lines indicate the medians; boxes include 50% of the data set around the medians; whiskers indicate 1.5* interquartile distance; notches display the 95% confidence interval; outliers are marked by black circles. Orange dashed line indicates the median of the wt control (OR light), the shaded area marks the 95% confidence interval. Rearing and experimental conditions are indicated by the colour of the boxes and the background: a white box illustrates rearing in a 12:12 dark:light cycle, a grey box rearing in a 24h dark cycle. White background indicates that the recordings were done in light, grey background indicates that the recordings were done in darkness. To test for significance, we used a two-sample Kolmogorov-Smirnov test and corrected *p*-values using the Benjamin-Hochberg false FDR. Significance is indicated as follows: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ **(A) Into darkness.** The exploration rate is significantly increased with progressing generations in darkness. *Dark-fly* shows the highest exploration rate. N*(OR light)* = 116, *N(Goe-dark 01)* = 142, *N(Goe-dark 05)* = 106, *N(Goe-dark 10)* = 223, *N(Goe-dark 15)* = 137, *N(dark-fly)* = 137 [*p*-values: *ORL* vs *GD01* 65.95×10^{-5} ; *ORL* vs *GD05* 14.3×10^{-5}; *ORL* vs *GD10* 8×10^{-5} ; *ORL* vs *GD15* 8×10^{-5} ; *ORL* vs *DF* 8×10^{-5} ; *GD01* vs *GD05* 31.21×10^{-5} ; *GD01* vs *GD10* 8×10^{-5} ; *GD01* vs *GD15* 8×10^{-5} ; *GD01* vs *DF* 8×10^{-5}] **(B) Back to light.** N*(OR light)* = 116, *N(Goe-dark light 05)* = 141, *N(Goe-dark light 10)* = 177, *N(dark-fly)* = 137, *N(dark-fly light)* = 139, *N(dark-fly light 05)* = 194 [*p*-values: *DF* vs *DFL* 8×10^{-5} ; *DF* vs *DFL05* 8×10^{-5} ; *DF* vs *GDL10* 8×10^{-5} ; *DF* vs *GDL05* 8×10^{-5} ; *DF* vs *ORL* 8×10^{-5}]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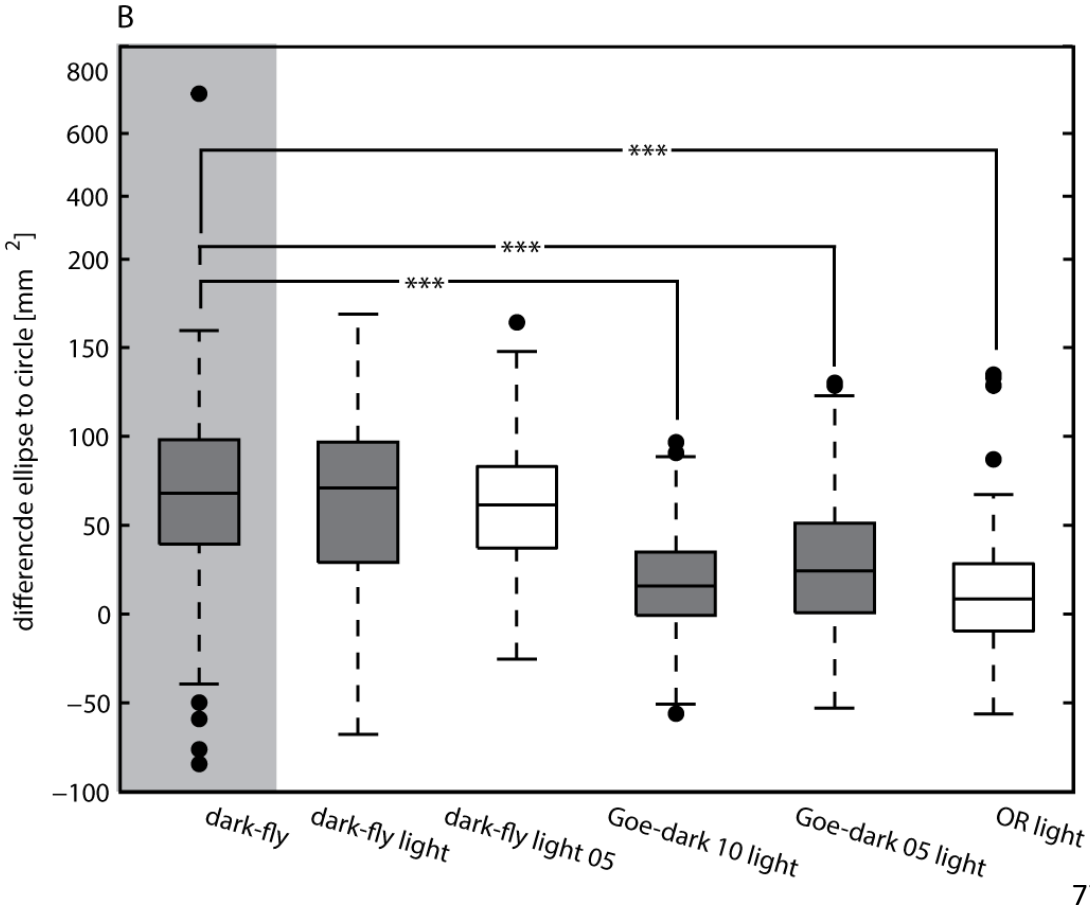
OR light
Goe-dark 01
Goe-dark 05
Goe-dark 10
Goe-dark 15
dark-fly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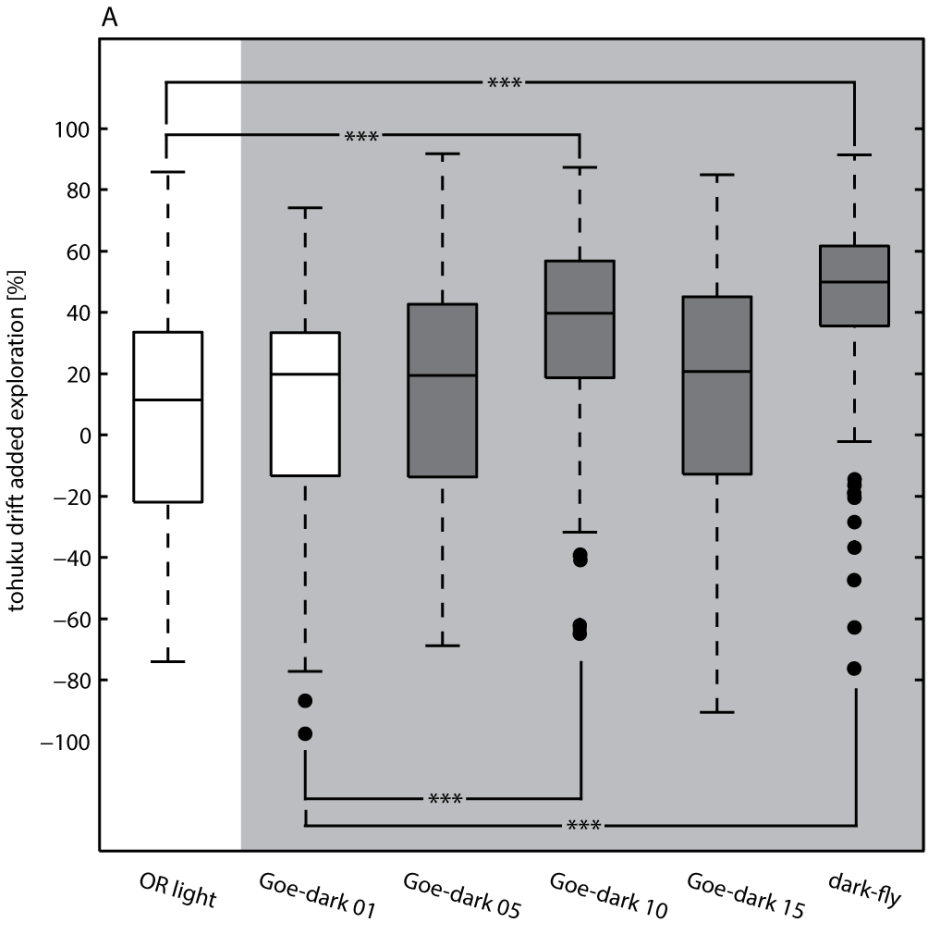
dark-fly
dark-fly light
dark-fly light 05
Goe-dark 10 light
Goe-dark 05 light
OR light

Figure 23 Boxplot showing the Tōhoku drift. Black lines indicate the medians; boxes include 50% of the data set around the medians; whiskers indicate 1.5* interquartile distance; outliers are marked by black circles. Rearing and experimental conditions are indicated by the colour of the boxes and the background: a white box illustrates rearing in a 12:12 dark:light cycle, a grey box rearing in a 24h dark cycle. White background indicates that the recordings were done in light, grey background indicates that the recordings were done in darkness. To test for significance, we used a two-sample Kolmogorov-Smirnov test and corrected p-values using the Benjamin-Hochberg false FDR. Significance is indicated as follows: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

A Into darkness N*(OR light)* = 63, *N(Goe-dark 01)* = 87, *N(Goe-dark 05)* = 85, *N(Goe-dark 10)* = 131, *N(Goe-dark 15)* = 116, *N(dark-fly)* = 124 [*p*-values: *ORL* vs *GD01* 28,495 x 10^{-2} ; *ORL* vs *GD05* 14,306 x 10^{-2}; *ORL* vs *GD10* 9 x 10^{-5} ; *ORL* vs *GD15* 76,57 x 10^{-3} ; *ORL* vs *DF* 9 x 10^{-5} ; *GD01* vs *GD05* 42,155 x 10^{-2} ; *GD01* vs *GD10* 9 x 10^{-5} ; *GD01* vs *GD15* 11,161 x 10^{-2} ; *GD01* vs *DF* 9 x 10^{-5}]

B Back to light. N*(OR light)* = 63, *N(Goe-dark light 05)* = 95, *N(Goe-dark light 10)* = 107, *N(dark-fly)* = 124, *N(dark-fly light)* = 98, *N(dark-fly light 05)* = 119 [*p*-values: *DF* vs *DFL* 30,32 x 10^{-2} ; *DF* vs *DFL05* 14,763 x 10^{-2} ; *DF* vs *GDL10* 8 x 10^{-5} ; *DF* vs *GDL05* 8 x 10^{-5} ; *DF* vs *ORL* 8 x 10^{-5}


A
tohuku drift added exploration [%]
100
80
60
40
20
0
−20
−40
−60
−80
−100
***
***
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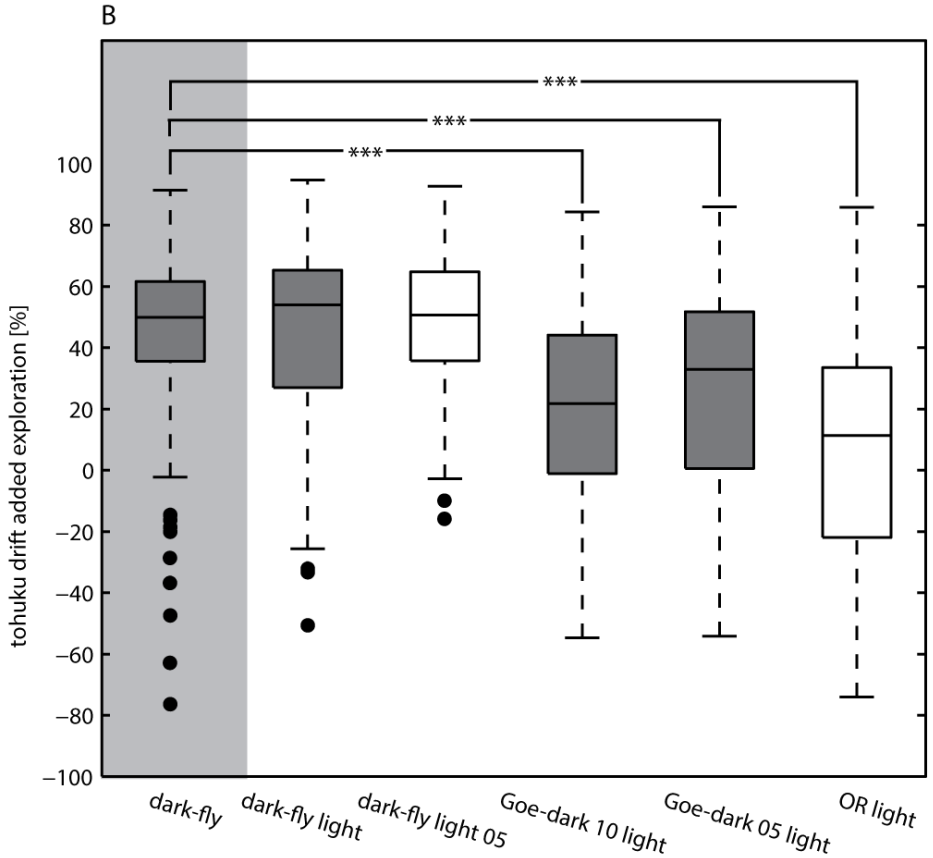
OR light
Goe-dark 01
Goe-dark 05
Goe-dark 10
Goe-dark 15
dark-fly


B
tohuku drift added exploration [%]
100
80
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−20
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−80
−100
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***
***
dark-fly
dark-fly light
dark-fly light 05
Goe-dark 10 light
Goe-dark 05 light
OR light

Figure 24 Boxplot showing the added exploration rate due to the Tōhoku drift. Black lines indicate the medians; boxes include 50% of the data set around the medians; whiskers indicate 1.5* interquartile distance; outliers are marked by black circles. Rearing and experimental conditions are indicated by the colour of the boxes and the background: a white box illustrates rearing in a 12:12 dark:light cycle, a grey box rearing in a 24h dark cycle. White background indicates that the recordings were done in light, grey background indicates that the recordings were done in darkness. To test for significance, we used a two-sample Kolmogorov-Smirnov test and corrected p-values using the Benjamin-Hochberg false FDR. Significance is indicated as follows: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ **(A) Into darkness** N(OR light) = 63, N(Goe-dark 01) = 87, N(Goe-dark 05) = 85, N(Goe-dark 10) = 131, N(Goe-dark 15) = 116, N(dark-fly) = 124 [p-values: ORL vs GD01 20.08 x 10^{-2} ; ORL vs GD05 20,.8 x 10^{-2}; ORL vs GD10 9 x 10^{-5} ; ORL vs GD15 27.537 x 10^{-2} ; ORL vs DF 9 x 10^{-5} ; GD01 vs GD05 48.23 x 10^{-2} ; GD01 vs GD10 9 x 10^{-5} ; GD01 vs GD15 48.23 x 10^{-2} ; GD01 vs DF 9 x 10^{-5}] **(B) Back to light**. N(OR light) = 63, N(Goe-dark light 05) = 95, N(Goe-dark light 10) = 107, N(dark-fly) = 124, N(dark-fly light) = 98, N(dark-fly light 05) = 119 [p-values: DF vs DFL 14.608 x 10^{-2} ; DF vs DFL05 41.88 x 10^{-2} ; DF vs GDL10 8 x 10^{-5} ; DF vs GDL05 8 x 10^{-5} ; DF vs ORL 8 x 10^{-5}]

4.5 Courtship strategy is influenced by light-deprivation

Previous studies of *Drosophila* courtship could show a severe impact on copulation success and courtship behaviour in the absence of visual cues (Sakai et al., 1997) as parts of the courtship rely on a functional visual system (Markow and Manning, 1980; Ribeiro *et al.*, 2018b). With over 1500 generations in constant darkness I was curious if *dark-flies* may adapted courtship behaviour in some manner as I could already show that locomotion strategy is changed in the long-term absence of light.

Courtship behaviour was examined in three different assays: a classical single pair courtship assay, consisting of a virgin female and a socially naïve male observed for a distinct time. A group courtship assay consisting of 10 flies (5 males and 5 females). And a competitive courtship assay, in which two socially naïve males are presented with a decapitated female and show both reciprocal antagonistic interactions and female-directed courtship behaviour. An ethogram of observed behaviours can be found in chapter *3.4.6 Video Annotation using the Etho-Scorer* (*Table 1* **Ethogram of Drosophila courtship and aggression behaviour described and classified in this study.**)

4.5.1 *Dark-fly* performs worse in single pair courtship assay

A single pair courtship assay was used to analyse different components of *Drosophila* courtship like courtship success, courtship latencies, wing extension and the fraction of female pausing while recording courtship song (*Table 1* **Ethogram of Drosophila courtship and aggression behaviour described and classified in this study.**). This assay was performed using both *dark-fly* and *OregonR* flies in two illumination conditions (*OregonR*$_{light}$: *OregonR* in light conditions; *dark-fly*$_{light}$: *dark-fly* in light conditions; *OregonR*$_{dark}$: *OregonR* in dark conditions; *dark-fly*$_{dark}$: *dark-fly* in dark conditions).

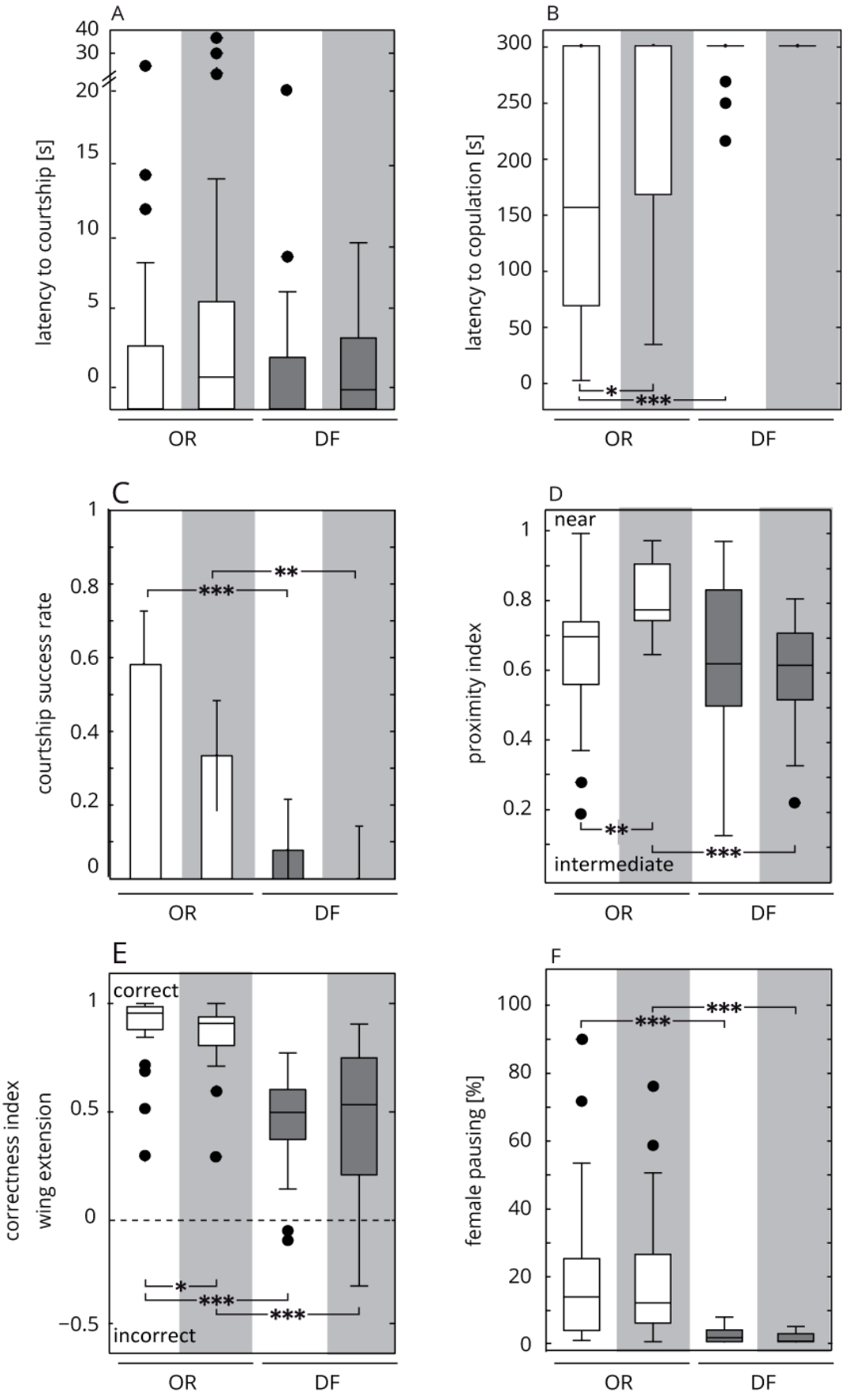
First the courtship latency was tested. To initiate courtship the male needs to detect the female either *via* vision, olfaction or gustation. Within the analysis window (00:30 min – 5:30 min after introduction to the arena) both the wt control *OregonR* and *dark-fly* initiated courtship behaviour similarly fast. A general bias towards slightly delayed courtship initiation could be observed in dark conditions (*OregonR*$_{light}$ 0 s; *dark-fly*$_{light}$ 0 s; *OregonR*$_{dark}$ 2 s; *dark-fly*$_{dark}$ 1.2 s) (*Figure 25 A*).

Subsequently, the latency until copulation was initiated was analysed. However, over 50% of tested *OregonR* pairs in darkness and *dark-fly* pairs in both illumination conditions did not mate successfully within the 5 min analysis window. Therefore, the median copulation latency in these groups showed the same median values (*dark-fly*$_{light}$ = *OregoR* $_{dark}$ = *dark-fly*$_{dark}$ 299.9 s) (*Figure 25 B*). It can be assumed, that selected time window for analysis was too short for flies in dark conditions to successfully copulate.

However, in *OregonR* dark conditions lead to a significant increase in the time until a first copulation attempt was made (*Figure 25 B*). In light conditions the initiation time was also significantly decreased in *dark-fly* compared to *OregonR* . With the loss of visual cues due to darkness, flies are limited to olfactory and gustatory cues. As this could limit their detection range, courtship behaviour might be restricted to close proximity as well. The distance of the male to the female while courting was assessed by calculating a proximity index $(D_{near} – D_{far})/(D_{near} + D_{far})$. In all four groups the males showed a higher proportion of courting in proximity to the female then at larger distances (*OregonR*$_{light}$ 0.7; *dark-fly*$_{light}$ 0.78; *OregonR*$_{dark}$ 0.62; *dark-fly*$_{dark}$ 0.63 s) (*Figure 25 D*). In dark

conditions *OregonR* significantly increases the proportion of courtship in proximity to the female, whereas the *dark-fly* ratio is independent of illumination conditions.

Another important result of this behavioural assay is the courtship success rate. *OregonR* shows a significantly reduced success rate when introduced to dark conditions (*OregonR*$_{light}$ 0.59; *OregonR*$_{dark}$ 0.32) (*Figure 25 C*). *Dark-fly* (*dark-fly*$_{light}$ 0.08) in light conditions already reached a lower success rate than *OregonR* in both illumination conditions and declines even further in dark conditions (*dark-fly*$_{dark}$) (*Figure 25 C*). This finding was highly surprising since the *dark-fly* strain has shown an increased mating fitness under dark conditions compared to wt flies (Izutsu et al., 2016). Furthermore, the *dark-fly* strain was maintained for over 1500 generations. It is therefore highly unlikely, that *dark-fly* flies are generally unable to copulate and motivated us to analyse this behaviour in greater detail. An important aspect of *Drosophila* courtship behaviour is the production of male courtship song, which is generated by unilateral wing extension using the wing closest to the female. For orientation towards the female an intact visual system is needed. It was previously reported, that male *Drosophila* show a higher fraction of incorrect wing extension in the absence of visual cues (Cook, 1979). To classify this behaviour a correctness index $(D_{correct} - D_{incorrect})/(D_{correct} + D_{incorrect})$ was calculated.


A
latency to courtship [s]
40
30
20
15
10
5
0
OR
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B
latency to copulation [s]
300
250
200
150
100
50
0
*
***
OR
DF
C
courtship success rate
1
0.8
0.6
0.4
0.2
0
**
***
OR
DF
D
proximity index
near
intermediate
1
0.8
0.6
0.4
0.2
**
***
OR
DF
E
correctness index
wing extension
correct
incorrect
1
0.5
0
−0.5
*
***
***
OR
DF
F
female pausing [%]
100
80
60
40
20
0
***
***
OR
DF

Figure 25 Single courtship behavioural assay for *dark-fly* and *OregonR*. Black lines indicate the medians; boxes include 50% of the data set around the medians; whiskers indicate 1.5* interquartile distance; outliers are marked by black circles. Rearing and experimental conditions are indicated by the colour of the boxes and the background: a white box illustrates rearing in a 12:12 dark:light cycle, a grey box rearing in a 24h dark cycle. White background indicates that the recordings were done in light, grey background indicates that the recordings were done in darkness. N*(OregonR$_{light}$)* = 21, *N(OregonR$_{dark}$)* = 21, *N(dark-fly$_{light}$)* = 21, *N(dark-fly$_{dark}$)* = 21. To test for significance, Fisher's exact permutations test and Benjamini-Hochberg correction were used. Significance is indicated as follows: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ **(A) Latency to first courtship.** Time until first courtship was initiated is similar in all four groups, with a trend to delaying initiation in dark conditions. [*p*-values: *ORL* vs *DFL* 0.12; *ORD* vs *DFD* 0.16] **(B) Latency to copulation.** *OregonR* shows a delayed onset of copulation in dark conditions compared to light conditions. In *dark-fly* copulation was significantly delayed. [*p*-values: *ORL* vs *DFL* < 0.01; *ORD* vs *DFD* 8×10^{-5}] **(C) Courtship success rate.** Courtship success rate is decreased in *OregonR* in dark conditions. In *dark-fly* courtship success is severely and significantly reduced in both dark and light conditions. **(D) Proximity index for male courtship behaviour.** Index was calculated as (Dnear – Dfar)/(Dnear + Dfar). In light conditions no significant difference in courtship proximity can be found. In darkness *OregonR* shows closer courtship while *dark-fly* does not change the distance significantly. **(E) Correctness index for wing extension.** Index was calculated as $(D_{correct} - D_{incorrect})/(D_{correct} + D_{incorrect})$. Compared to *OregpnR dark-fly* shows a significantly reduced amount of correct wing extensions in both illumination conditions. **(F) Fraction of female pausing.** The amount of female pausing in *dark*-fly in both illumination conditions is significantly reduced compared to *OregonR*.

In light, *OregonR* flies almost always use the ispilateral wing to produce courtship song (95% correct choices) (*Figure 25 E*). However, in the absence of light they show slightly more errors (9% error rate). *Dark-fly* flies on the other hand seem to have dropped this behaviour. They only use the ispilateral wing in roughly half of their courtship singings, which appears more like a random 50:50 strategy than an actual attempt to use the closer wing (*Figure 25 E*). Even more surprising *dark-fly* flies did not change this strategy even when visual cues were available again. This seems contradictory since the *dark-fly* strain was expected to be better adjusted to dark conditions. However, it can be hypothesised that the male courtship song might have changed in fashion that renders correct wing extension obsolete.

To guarantee successful courtship the female pauses, upon recognizing the male courtship song, and lets the male approaching. *Dark-fly* shows an extremely low courtship success in both illumination conditions, raising the question if not only male, but also female courtship behaviour is affected. The fraction of female pausing was analysed and *OregonR* shows no difference when comparing both light conditions (*OregonR*$_{light}$ 13.3%; *OregonR*$_{dark}$ 11.5%) (*Figure 25 F*). *Dark-fly* females on the other hand, nearly abolished pausing in reaction to male courtship song. No changes could be observed for dark-fly in dependence of the lighting condition (*dark-fly*$_{light}$ 1.2%; *dark-fly*$_{dark}$ 0.2%) (*Figure 25 E*). While these results give a possible explanation for the reduced courtship success in the *dark-fly* strain, this still contradicts the finding of increased mating fitness in the *dark-fly* flies (Izutsu *et al.*, 2016).

In the wt flies OregonR a severe influence of illumination conditions on different aspects of courtship behaviour can be observed. Furthermore, dark-fly shows a significantly reduced courtship success in this behavioural assay and females show a low fraction of pausing. To understand if the reduced courtship success in dark-fly is due to less courtship behaviour or a lower number of copulation attempts, the frequency of general male courtship behaviour and wing extension, as well as the frequency of copulation attempts was analysed (*Figure 26*).

Overall, flies exhibit more courtship behaviour in the absence of light (*Figure 26 A)*. Moreover, the courtship frequency of the *dark-fly* strain under light conditions is comparable to elevated levels of *wt* flies in the dark. Nevertheless, dark-fly courtship frequency still more than triples in darkness (*Figure 26 A*). This suggests that the reduced courtship success of the *dark-fly* strain might indeed be due to disrupted female behaviour. In *OregonR*, the frequency of wing extension, a specific feature of male courtship behaviour used for production of courtship song, is largely unaffected by changing illumination. The frequency of wing extension behaviour in *dark-fly* in light conditions is analogous to *OregonR* levels. Interestingly, in *dark-fly* an influence of change in illumination is observable: the wing extension frequency is decreasing in dark conditions (*Figure 26 A*)

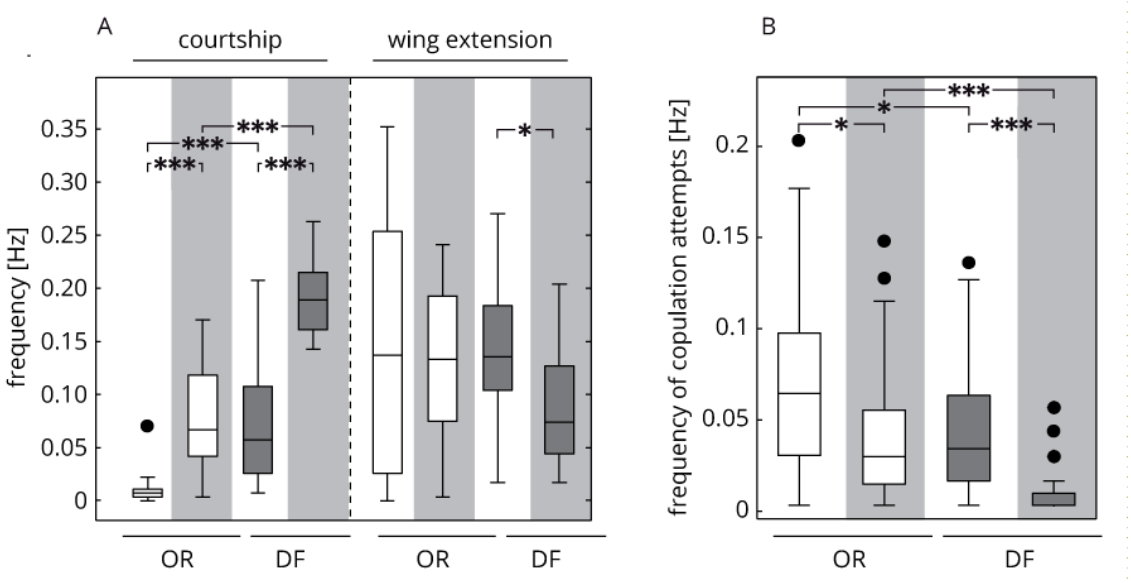

Figure 26 Courtship frequencies of OregonR and dark-fly in single courtship assay. Black lines indicate the medians; boxes include 50% of the data set around the medians; whiskers indicate 1.5* interquartile distance; outliers are marked by black circles. Rearing and experimental conditions are indicated by the colour of the boxes and the background: a white box illustrates rearing in a 12:12 dark:light cycle, a grey box rearing in a 24h dark cycle. White background indicates that the recordings were done in light, grey background indicates that the recordings were done in darkness. N($OregonR_{light}$) = 21, N($OregonR_{dark}$) = 21, N(dark-fly_{light}) = 21, N(dark-fly_{dark}) = 21. To test for significance, Fisher's exact permutations test and Benjamini-Hochberg correction were used. Significance is indicated as follows: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ **(A) Frequency of general male courtship behaviour and wing extension.** The frequency of general courtship behaviour is increasing in darkness. In OregonR the frequency of wing extension is unaffected by illumination conditions; in dark-fly frequency is decreasing in dark conditions. [p-values courtship: ORL vs ORD 6 x $10^{-4;}$ DFL vs DFD 6 x 10^{-4}; ORL vs DFL 6 x 10^{-5}; ORD vs DFD 6 x 10^{-5}; p-values wing extension: : ORL vs ORD 0.47 ; DFL vs DFD 0.02; ORL vs DFL 2 x 10^{-4}; ORD vs DFD 0.08] **(B) Frequency of male copulation attempts.** The frequency of copulation attempts is decreasing with a switch from light to dark conditions. [p-values courtship: ORL vs ORD 0.014; DFL vs DFD 4 x 10^{-3}; ORL vs DFL; DFL vs DFD 6 x 10^{-5}

Furthermore, the frequency of attempted copulations observable in the analysis window was analysed. In both strains the frequency is significantly reduced from light to dark conditions. In both illumination conditions, the frequency of copulation attempts in *dark-fly* males is significantly lower compared to *OregonR* (*Figure 26 B*).

To verify, that the low courtship success of *dark-fly* is at least partly due to the reduced pausing behaviour in *dark-fly* females, the single courtship assay was performed using mixed pairs (*Figure 27*).

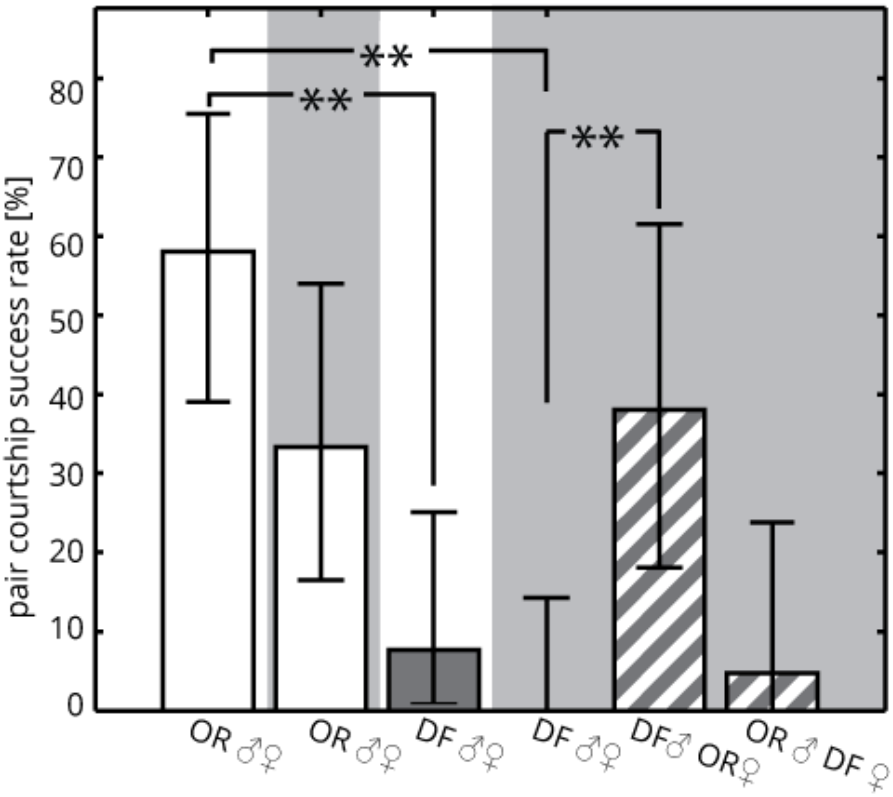

Figure 27 Courtship success in same and mixed pairs. When males and females of the same strain are paired, courtship success is reduced in dark conditions in *OregonR*. *Dark-fly* shows a significantly reduced courtship success rate in both illumination conditions. If a *dark-fly* male is paired with a wt female, courtship success in darkness is restored to similar levels found in *OregonR*.

Pairing a *dark-fly* male with an *OregonR* female indeed restores the courtship success in dark conditions to a level similar as in *OregonR*. However, pairing a *dark-fly* female with an *OregonR* male still shows a significantly reduced courtship success, suggesting a severe influence of female behaviour on male copulation success (*Figure 27*).

To summarize, *dark-fly* shows a severely reduced copulation success in a single pair behavioural assay, likely linked to reduced pausing behaviour in *dark-fly* females.

Furthermore, *dark-fly* males also display a lower number of correct wing extensions in both illumination condition. This suggests that the *dark-fly* strain might have changed their strategy of courtship songs and extension of the wing closest to the female is irrelevant.

4.5.2 The courtship success of the *dark-fly* strain is restored in a group courtship assay

In the classical single courtship assay *dark-fly* shows a significantly worse performance and success rate than *OregonR* in both illumination conditions. Overall, the fecundity of the *dark-fly* strain is not strongly affected, and the strain has been maintained over 1500 generations. Therefore, a group courtship assay was designed to recreate more natural condition. T this end, the courtship behaviour of 5 pairs (5 male, 5 female) was tested in different illumination conditions and different parameters were assessed after 60 min

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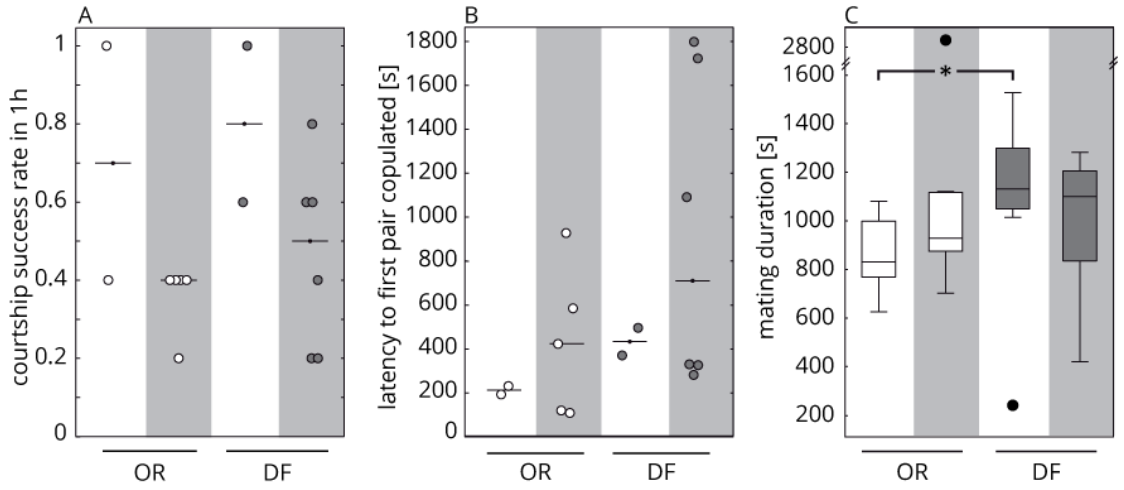


Figure 28 Group courtship assay. Dots represent individual values; black line indicates median. Boxes include 50% of the data set around the medians; whiskers indicate 1.5* interquartile distance; outliers are marked by black circles. **(A) Courtship success.** In both strains the courtship success is lower in dark conditions, however, *dark-fly* shows a higher success rate compared to *OregonR*. **(B) Latency to first pair copulated.** In dark conditions latency to first successful copulation is higher than in light conditions. Latency in general increased in *dark-fly* compared to *OregonR*. **(C) Mating duration.** Change in illumination condition shows no significant effect on mating durations within the strains. In light conditions *dark-fly* displays a significantly longer mating duration than *OregonR*. N*(OregonR$_{light}$)* = 7, *N(OregonR$_{dark}$)* = 9, *N(dark-fly$_{light}$)* = 8, *N(dark-fly$_{dark}$)* = 14. [*p*-values: *ORL* vs *ORD* 0.24; *DFL* vs *DFD* 0.3; *ORL vs DFL* 3 x 10^{-2}]

After the testing period the courtship success rate was analysed. In both strains the courtship success rate was higher in light than in dark conditions; however, *dark-fly*

shows an increased courtship success rate in both illumination conditions compared to *OregonR* (*OregonR*$_{light}$ 0.7; *dark-fly*$_{light}$ 0.8; *OregonR*$_{dark}$ 0.4; *dark-fly*$_{dark}$ 0.5) (*Figure 28 A*). In the classical single pair courtship assay the flies were examined in an analysis window of 5 min and *dark-fly* showed a severely decreased courtship success compared to *OregonR*. To understand if the different time scales of the two assays an influence on the courtship success have the latency to the first pair copulated was analysed (*Figure 27 B*). In light conditions *OregonR* flies took about 212.8 s until the first pair was mated; this time is prolonged in dark conditions to 423.5 s. In *dark-fly* an increase in time to first pair copulated can also be observed with a change in illumination conditions (*dark-fly*$_{light}$ 433.5 s; *dark-fly*$_{dark}$ 710.5 s). In both light conditions *OregonR* flies show a trend to earlier copulation compared to *dark-fly*. This indicates that the selected time window for the single courtship assay was indeed not feasible to analyse copulation latencies in darkness.

Regarding mating duration, there was no significant difference within the strains with changing illumination conditions, however, the mating duration in *dark-fly* seems to be prolonged compared to *OregonR* (*OregonR*$_{light}$ 831.2 s; *dark-fly*$_{light}$ 1188.1 s; *OregonR*$_{dark}$ 992.5 s; *dark-fly*$_{dark}$ 1099.9 s) (*Figure 27 C*).

4.5.3 Competitive courtship assay.

Wt *Drosophila* show a competitive courtship strategy. To test whether this is changed in *dark-fly* a competitive courtship assay was performed. The courtship performance of *dark-fly* is significantly improved in the group courtship assay compared to the single courtship assay. In the latter, over 50% of *dark-fly* were not able to perform successful copulation, while in a group setup they showed an increased courtship success even surpassing *OregonR* levels in both illumination conditions. This suggests a change in courtship strategy in *dark-fly* to tolerate other males courting the same female simultaneously.

The competitive courtship assay pairs two socially naïve males with a decapitated female. The males switch between male-male agonistic interaction and courtship behaviour towards the female.

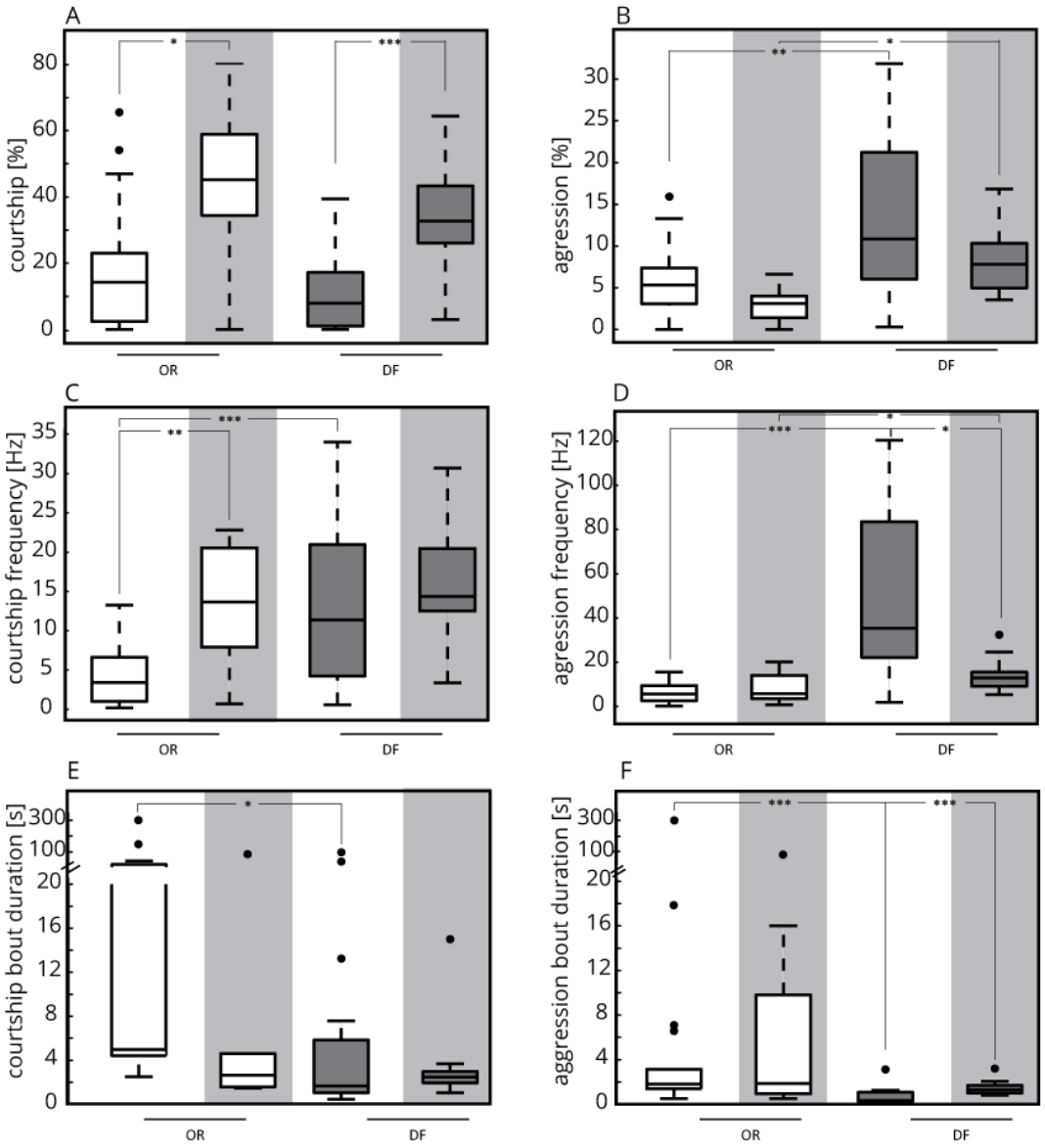


Figure 29 Competitive courtship assay. Black line indicates median. Boxes include 50% of the data set around the medians; whiskers indicate 1.5* interquartile distance; outliers are marked by black circles. N*(OregonR$_{light}$)* = 22, *N(OregonR$_{dark}$)* = 10, *N(dark-fly$_{light}$)* = 22, *N(dark-fly$_{dark}$)* = 16. **(A) Fraction of courtship behaviour.** The fraction of time spent with courtship is increased from dark to light conditions in both strains. **(B) Fraction of aggression behaviour.** The level of aggression is elevated in light conditions compared to dark conditions. *Dark-fly* shows a significantly increased fraction of aggression behaviour in relation to *OregonR*. **(C) Courtship frequency.** The courtship frequency in *OregonR* in increased from light to dark conditions. In *dark-fly* illumination had no impact on the courtship frequency, which did not significantly differ from *OregonR* in dark conditions. **(D) Aggression frequency.** Aggression frequency is not influenced by illumination condition in *OregonR*. In *dark-fly* the aggression frequency is significantly increased in light conditions.

(E) Courtship bout duration. Courtship bout duration is significantly decreased from light to dark conditions in *OregonR*. In *dark-fly* levels in both illumination conditions are similar to *OregonR* in darkness. **(F) Aggression bout duration.** *Dark-fly* shows significantly shorter aggression bout durations compared to *OregonR*.

The duration that each male spent with the respective behaviours was determined. Analysing the fraction of courtship behaviour, both *OregonR* and *dark-flies* spent much more time on courtship in dark conditions. A comparison within the two strains also shows severe differences (*Figure 29 A*). This correlates with more aggression behaviour: in both *OregonR* and *dark-fly* aggression behaviour is reduced by a change into dark conditions. However, *dark-fly* shows significantly increased aggression levels in both illumination conditions compared to *OregonR* (*Figure 29 B*).

The frequency of courtship bouts is significantly increased in *OregonR* with a shift from light to dark conditions (*Figure 29 C*). In *dark-fly* illumination conditions show no significant effect on courtship frequency; levels are similar to *OregonR* in dark conditions. In *OregonR*, frequency of aggression behaviour is not influenced by a change from light to darkness, while in *dark-fly* aggression frequency is significantly increased in light conditions compared to darkness. In both illumination conditions, *dark-fly* shows higher levels than *OregonR (Figure 29 D)*

The courtship bout duration in *OregonR* is significantly decreased from light to dark conditions; in *dark-fly* the courtship bout duration is not influenced by a changing illumination condition and similar to *OregonR* in darkness (*Figure 29 E*). The aggression bout duration in *dark-fly* is significantly shorter compared to *OregonR*. However, *dark-flies overall* exhibit higher aggression levels than *OregonR (Figure 29 F)*.

To better understand the relationship between aggression and courtship behaviour a behavioural contrast was calculated. Both tested strains have a bias towards courtship behaviour in dark conditions. On the contrary, while *wt* flies still prefer courtship over aggression under light, *dark-fly* flies completely flip their behaviour and become predominantly aggressive.

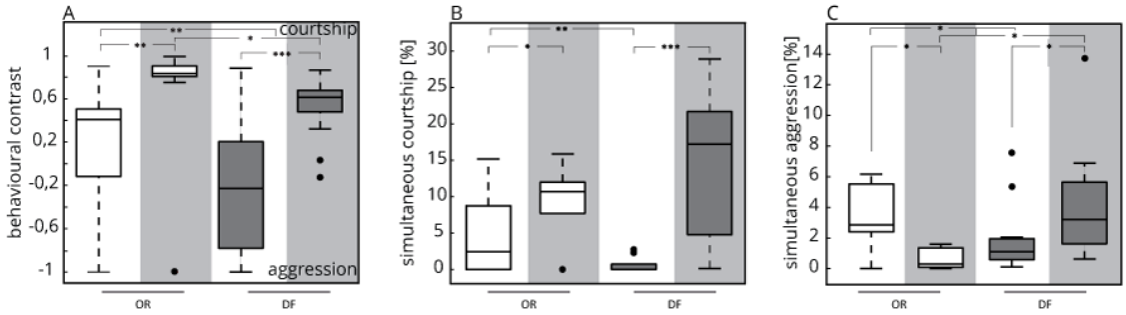


Figure 30 Comparison of courtship and aggression behaviour in the competitive courtship assay. Black line indicates median. Boxes include 50% of the data set around the medians; whiskers indicate 1.5* interquartile distance; outliers are marked by black circles. N*(OregonR$_{light}$)* = 22, *N(OregonR$_{dark}$)* = 10, *N(dark-fly$_{light}$)* = 22, *N(dark-fly$_{dark}$)* = 16. **(A) Behavioural contrast.** The behavioural contrast was calculated as $(D_{courtship} - D_{aggression})/(D_{courtship} + D_{aggression})$. Both *OregonR* and *dark-fly* show a significantly increased inclination towards courtship in darkness. In light conditions *dark-fly* shows a preference towards aggression behaviour. **(B) Synchronicity of courtship behaviour.** The synchronicity of behaviour indicates the fraction in which both males show the same behaviour simultaneously. In both strains the fraction of simultaneous courtship is significantly increased by a switch from light to darkness. Compared to *OregonR* the fraction is increased in darkness but decreased in light conditions in *dark-fly*. **(C) Synchronicity of aggression behaviour.** The synchronicity of behaviour indicates the fraction in which both males show the same behaviour simultaneously. In *OregonR* the fraction of simultaneous aggression is decreased by a switch from light to darkness, while in *dark-fly* the fraction is higher in dark conditions

As hypothesised above, in *dark-fly* a tolerance towards other males courting simultaneously might have evolved. To verify this, the synchronicity of both behaviours was analysed. In both strains the synchronicity of courtship behaviour is significantly increased by a transition from light to dark conditions. In *dark-fly* the level in darkness is higher and in light lower compared to *OregonR*. The fraction of simultaneous aggression behaviour is increased from light conditions to darkness in *dark-fly* and decreased in *OregonR*.

These findings support the hypothesis that *dark-fly* males indeed have evolved a higher tolerance to other males courting simultaneously.

4.6 HMM show changes in courtship syntax in darkness

A previous study could show, that light deprivation has a severe influence on the syntax of male courtship behaviour in wt flies (Sakai *et al.*, 1997).

To understand the impact of generations of light deprivation on the transitions between the distinct male courtship behaviours, a Hidden Markov Model, representing a syntax of male courtship behaviour, was compiled from the data derived by behavioural screening during the single pair courtship assay. Since many of the described behaviours, the categories were redefined before the HMM was compiled. Only transitions that are occurring significantly more often than chance level were classified as behavioural relevant state-transitions. All transitions shown in the HMM are therefore positively significant transition between states (chapter *3.4.7* Hidden Markov Model of male courtship behaviour.

All four groups shared positively significant bidirectional transitions (*Figure 31*, yellow triangles) between pausing and locomotion as well as wing extension and other courtship behaviour close to the female. Furthermore, the unilateral transitions from wing extensions to courtship attempt and from other courtship behaviour in near proximity to farther away were found in all groups (*Figure 32*).

Further analysis of the courtship syntax suggests that the core pattern of *OregonR* courtship in light conditions consists of the behavioural states wing extension and other courtship on both near and far distance (***other courtship behaviour near***; ***other courtship behaviour far***); all these states show positively significant bidirectional transitions.Abortion of courtship behaviour only occurred as a transition from other courtship behaviour in far distance to the inactive state of pausing.

The courtship syntax of *OregonR* in darkness shows several severe changes compared to courting in light conditions. The bilateral transition between wing extension and other courtship behaviour in far distance as well as the transition from ***other courtship behaviour far*** to ***other courtship near*** were lost; there are no transitions from courtship in far distance to any other courtship behaviours, indicating an abortion of courtship. This implies that although detection of the female is possible from a distance (transition locomotion to ***other courtship behaviour far***) the initiation of courtship farther away

from the female is not successful and ultimately leads to abortion of courtship rather than transitioning to more intense courtship like production of courtship song. However, we find a new transition from locomotion to courtship in near proximity, suggesting that detection of the female happens at a closer distance and leads directly to initiation of courtship behaviour.

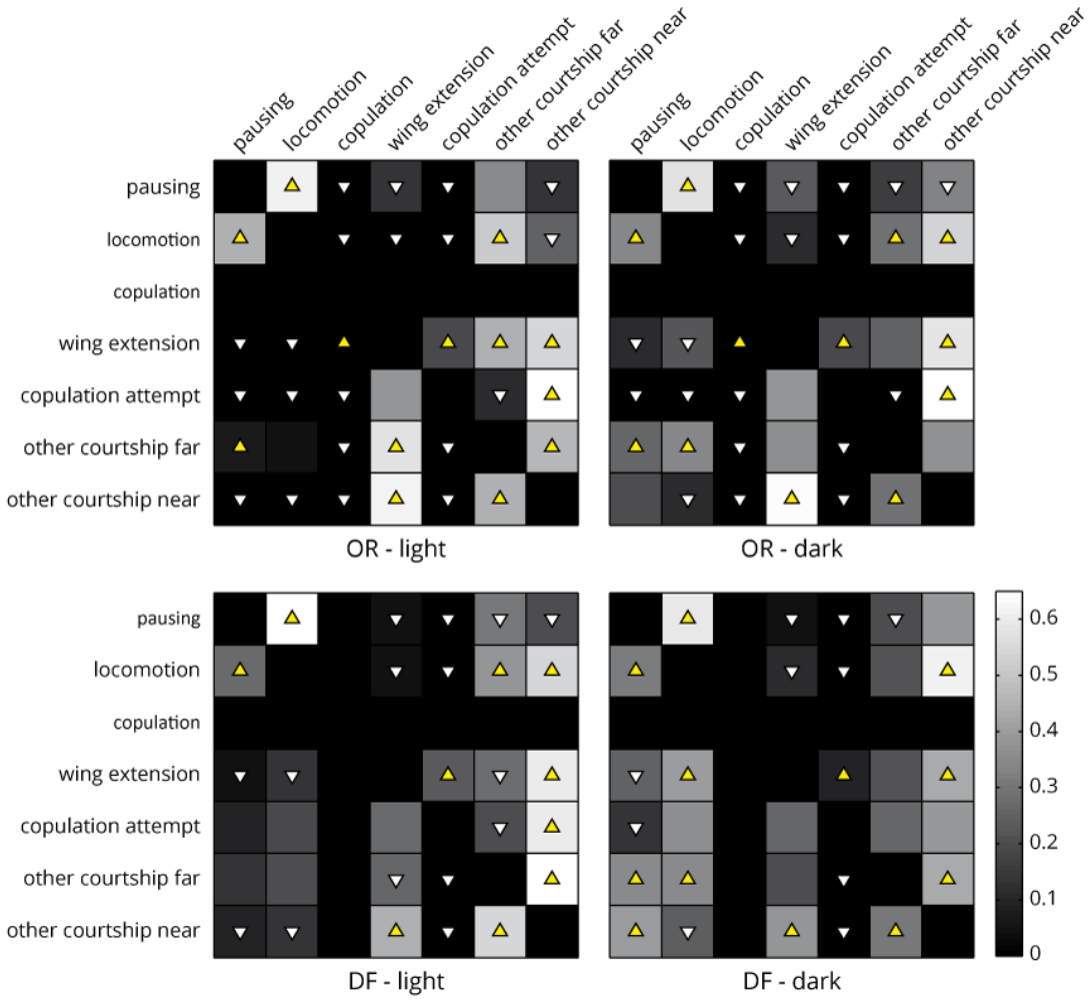


Figure 31 Hidden Markov Model – Transition matrices of male courtship behaviour. Scale bar from black to light grey indicates transition probability. Triangles display transitions that are significantly above or below chance level: yellow triangles denote positive significant transitions that were later used in the transition diagrams. N*(OregonR$_{light}$)* = 31, *N(OregonR$_{dark}$)* = 27, *N(dark-fly$_{light}$)* = 26, *N(dark-fly$_{dark}$)* = 24. Values for transition probabilities and *p*-values can be found in the supplements (*A1 HMM transition probabilities and p-values*).

Comparing the *dark-fly* courtship syntax in both illumination conditions the most apparent feature is the missing transition to successful copulation. The syntax of *dark-fly* in light conditions appears as an intermediate between *OregonR* in light and dark conditions. The bilateral transition between wing extension and ***other courtship far***, as well as the unilateral transition from ***other courtship far*** to pausing are missing, suggesting that only courtship behaviour close to the female leads to the initiation of wing extension. Similar to *OregonR* in dark conditions, *dark-fly* in light conditions shows a direct transition from locomotion to courtship close to the female. The transition from ***other courtship far*** to ***other courtship near*** is again found to be bilateral, suggesting that light conditions indeed facilitate the maintenance of courtship behaviour even if the female is farther away. Comparable with the *OregonR* syntax in both illumination conditions unsuccessful copulation attempts lead to a restart of courtship behaviour close to the female.

Rather surprisingly, the courtship syntax of *dark-fly* in dark shows several transitions from courtship behaviours to abortion of courtship. This can be seen in a newly arisen transition from both wing extension and ***courtship near*** to locomotion. Furthermore, unsuccessful courtship attempts did not lead into a transition to courtship close to the female but to random transition to all other states. The transition diagram implies that *dark-fly* males in darkness were not able to locate the female from a distance. However, the bilateral connection between ***other courtship far*** and ***other courtship near*** suggesting that *dark-fly* is capable of maintaining contact and restoring proximity to the female.

Taken together light deprivation shows a severe effect on courtship syntax in both strains, shown in the increased transitions leading to courtship abortion. *OregonR* in darkness was not able to restore proximity to the female when she was moving to a distance, which seemed to still be functioning in *dark-fly*.

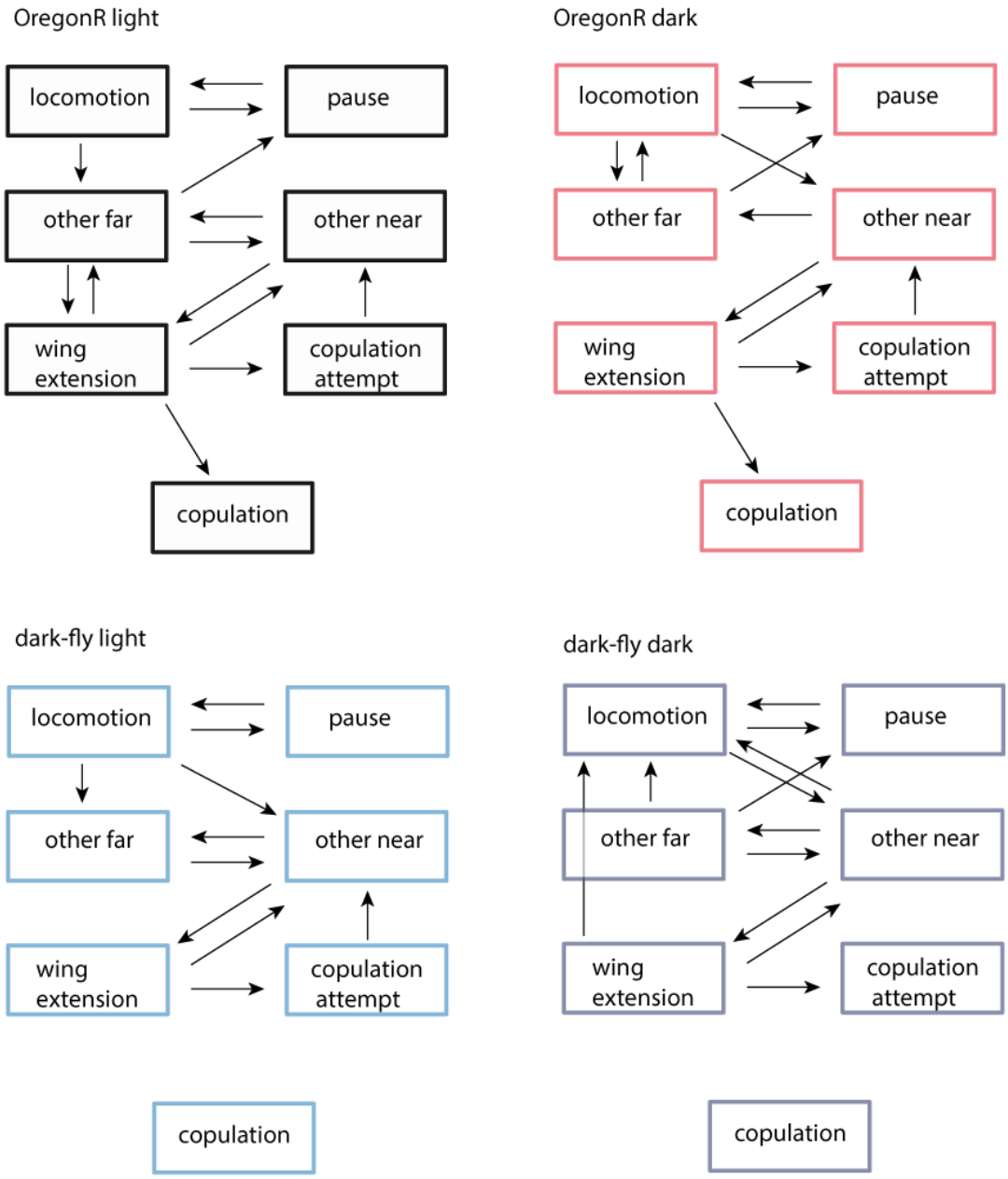


Figure 32 Hidden Markov Model – Transition diagram for male courtship behaviour. Arrows represent positive significant transitions between respective states (see also *Figure 31*). Colours indicate lighting condition and Drosophila strain.

4.7 Courtship songs are influenced by light-deprivation

The analysis of *dark-fly* courtship behaviour showed a rather surprising decrease in courtship success under dark conditions. Restored success in a group courtship assay suggests that this effect can be mended by simultaneous song production of the male flies. The obvious next step was to analyse the male courtship songs, which were recorded during the single couple courtship assay.

In *Drosophila*, the courtship song produced by male wing vibration is one of the critical features of courtship behaviour. Upon perceiving the courtship songs, female *Drosophila* pauses, indicating receptivity (Schilcher, 1976). The song can be divided in three distinct modes: two pulse songs, P_{fast} and P_{slow}, the use of which correlates with the distance to mating partner. P_{fast} is used in longer distance to the female, whereas P_{slow} is used in close proximity (Clemens et al, 2018). Furthermore, one type of sine song can be identified, which together with the interpulse interval (IPI) communicates species identification (R J Greenspan and Ferveur, 2000).

To extract possible differences in the shape of P_{fast} and P_{slow}, the pulses were z-scored scored (normalised to mean = 0 and standard deviation = 1) and superimposed. This rendered no difference between the four groups (*Figure 33 A*). However, a change in amplitude on both pulse forms within the two strains and two illumination conditions can be observed, indicating a change in volume.

Analysing the amplitude of pulse songs shows an increase in both strains when changing the illumination condition from light to dark. In both conditions the *dark-fly* pulse songs have a higher amplitude than *OregonR* (*Figure 33 B*). This demonstrates that the courtship song of *dark*-fly is indeed increased in volume compared to *OregonR.*

Subdividing the amplitudes into the two pulse forms P_{slow} and P_{fast} indicates a trend towards higher amplitudes in darkness in both strains but does not reach significance. *Dark-fly* produced significantly louder courtship song in both illumination conditions compared to *OregonR* flies, corresponding to the observation that the overall pulse amplitude is increased in *dark-fly* (*Figure 33 C & D*).

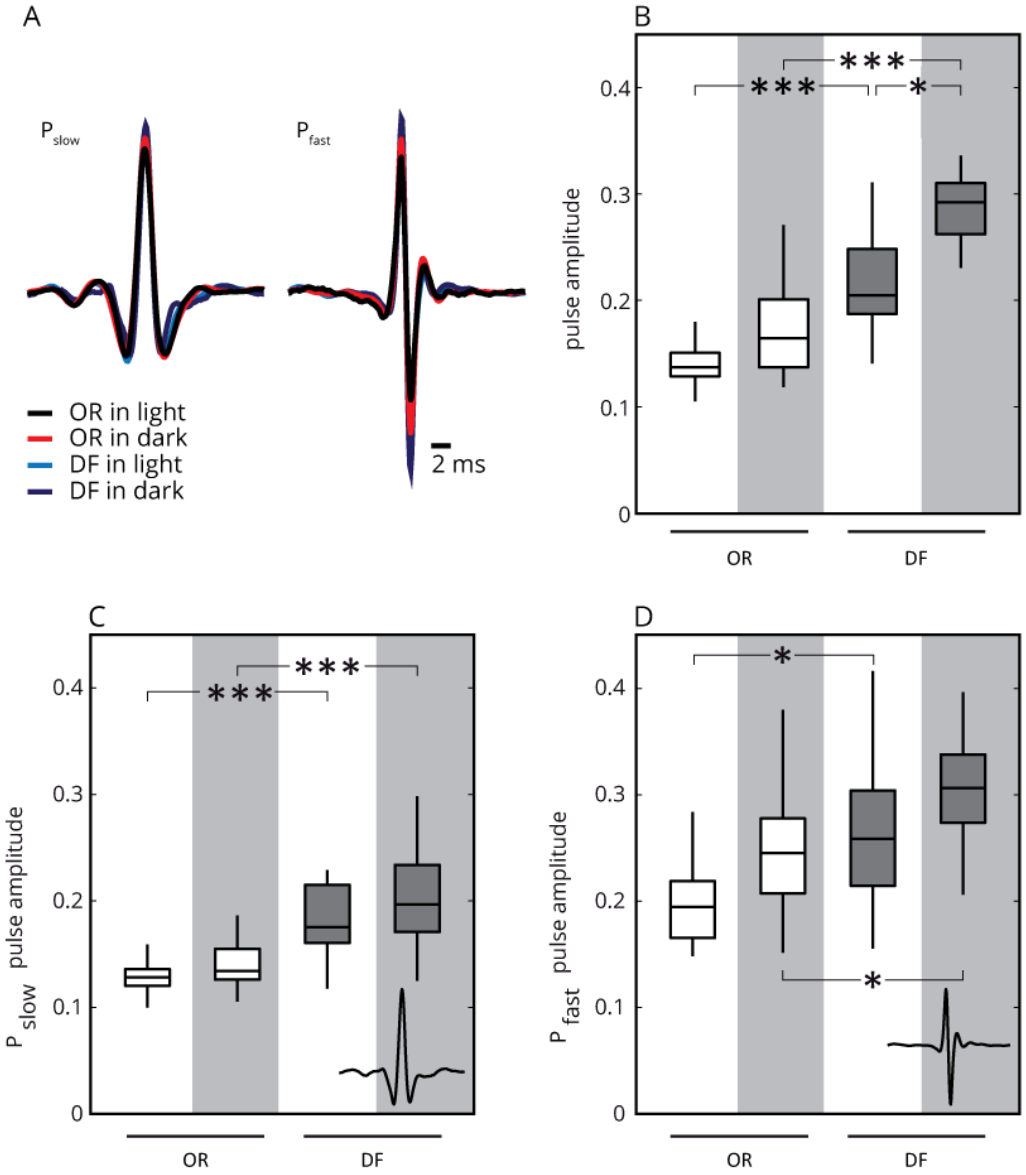


Figure 33 **Pulse songs and courtship songs amplitude.** Dots represent individual values; black line indicates median. Boxes include 50% of the data set around the medians; whiskers indicate 1.5* interquartile distance; outliers are marked by black circles. N*(OregonR$_{light}$)* = 21, *N(OregonR$_{dark}$)* = 21, *N(dark-fly$_{light}$)* = 21, *N(dark-fly$_{dark}$)* = 21. To test for significance, Kruskal-Wallis test was used. Significance is indicated as follows: ** p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001* **(A) Shape of pulse songs.** Z-scored superposition of pulse shapes. The shape of the two forms of pulse songs P_{fast} and P_{slow} does not differ between *dark-fly* and *OregonR* in both illumination conditions. **(B) Overall amplitude of pulse songs.** In *OregonR* and *dark-fly* the amplitude of pulse songs is significantly increased from light to dark conditions. The overall amplitude is significantly increased if comparing *dark-fly* and *OregonR*. [*p*-values: *ORL* vs *ORD* 0.27 ; *DFL* vs *DFD* 0.04 *; ORL vs DFL* 2 x 10^{-3}; *ORD vs DFD* 1 x 10^{-4}]

(C)Amplitude of P_{slow}. P_{slow} shows a trend to higher amplitudes in dark conditions compared to light conditions in both *dark-fly* and *OregonR*. The amplitude in *dark-fly* is significantly increased compared to *OregonR*. [*p*-values: *ORL* vs *ORD* 0.74; *DFL* vs *DFD* 0.72 *; ORL vs DFL* 1 x 10^{-4}; *ORD vs DFD* 1 x 10^{-4}] **(D) Amplitude of P_{fast}.** P_{fast} shows a trend to higher amplitudes in dark conditions compared to light conditions in both *dark-fly* and *OregonR*. The amplitude in *dark-fly* is significantly increased compared to *OregonR*. [*p*-values: *ORL* vs *ORD* 0.14; *DFL* vs *DFD* 0.15; *ORL vs DFL* 0.02; *ORD vs DFD* 0.03]

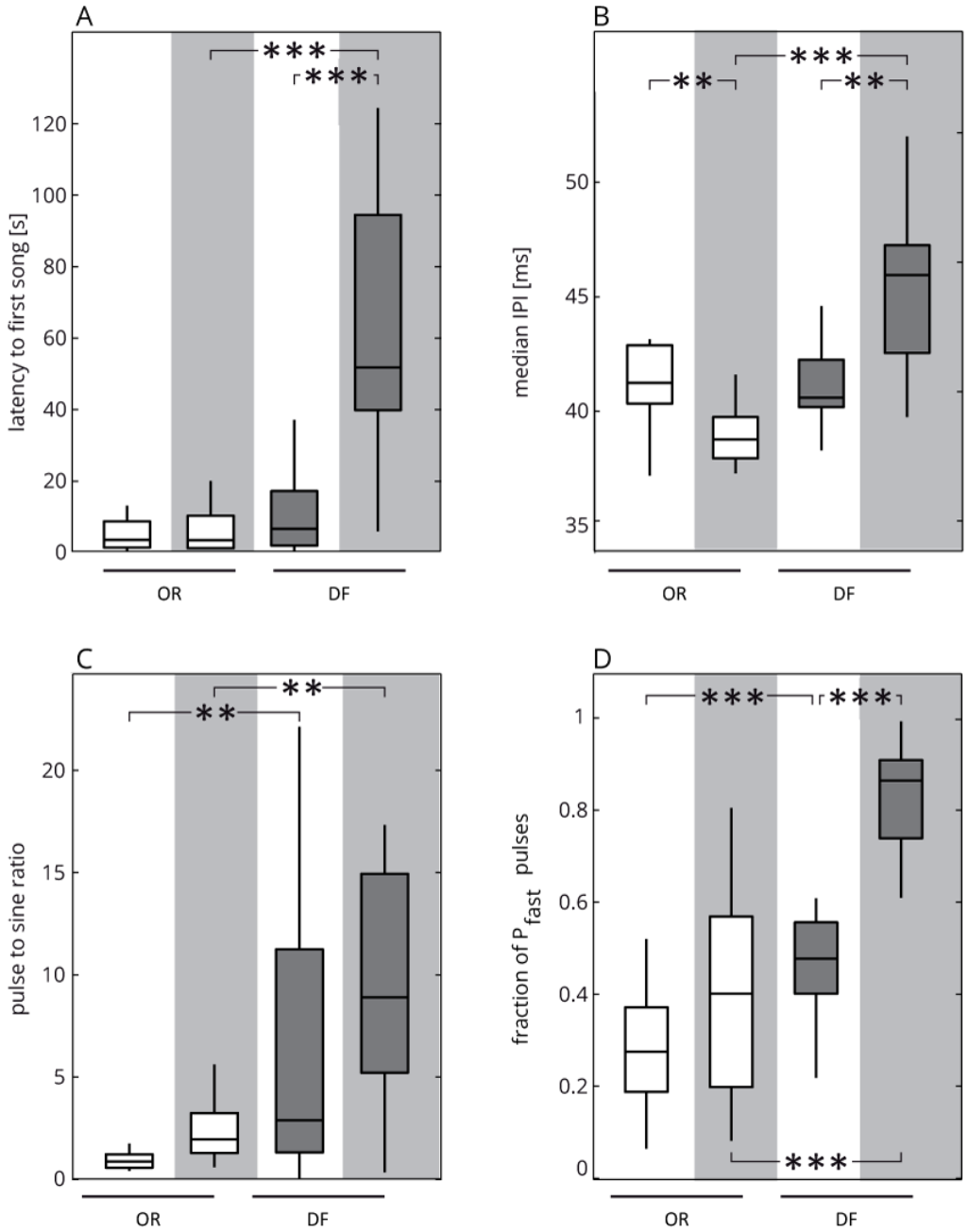


Figure 34 Male courtship song characteristics. Dots represent individual values; black line indicates median. Boxes include 50% of the data set around the medians; whiskers indicate 1.5* interquartile distance; outliers are marked by black circles. N*(OregonR$_{light}$)* = 21, *N(OregonR$_{dark}$)* = 21, *N(dark-fly$_{light}$)* = 21, *N(dark-fly$_{dark}$)* = 21. To test for significance, Kruskal-Wallis test was used. Significance is indicated as follows: ** p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001* **(A) Latency to first courtship song.** Latency to first courtship song is similar for *OregonR* in both illumination conditions and *dark-fly* in light conditions. In dark conditions, *dark-fly* shows a significant increase in latency compared to the other three groups. [*p*-values: *ORL* vs *ORD* 0.97 ; *DFL* vs *DFD* 2 x 10^{-4} *; ORL vs DFL* 0.88; *ORD vs DFD* 1 x 10^{-4}] **(B) Median interpulse interval (IPI).** In *OregongR* the IPI is significantly reduced from light to dark conditions. In *dark-fly* the IPI is significantly increased from light to dark conditions. Median IPI is similar for both strains in light conditions but significantly different in dark conditions. [*p*-values: *ORL* vs *ORD* 43 x 10^{-4} ; *DFL* vs *DFD* 99 x 10^{-4} ; *ORL vs DFL* 0.85; *ORD vs DFD* 1 x 10^{-4}] **(C) Pulse to sine ratio.** Pulse to sine ratio shows a trend to be increased in darkness in both strains. Compared to *OregonR*, the ratio is significantly increased in *dark-fly*. [*p*-values: *ORL* vs *ORD* 0.09; *DFL* vs *DFD* 0.12; *ORL vs DFL* 4 x 10^{-3}; *ORD vs DFD* 7 x 10^{-3}] **(D) Fraction of P_{fast}.** The fraction of P_{fast} is increased in darkness for both strains. Overall, *dark-fly* shows a higher fraction of P_{fast} compared to *OregonR*. [*p*-values: *ORL* vs *ORD* 0.27; *DFL* vs *DFD* 0.04; *ORL vs DFL* 15 x 10^{-3}; *ORD vs DFD* 1 x 10^{-3}]

The latency to performance of first courtship song was not influenced by illumination condition in *OregonR* flies and similar to *dark-fly* in light conditions. Interestingly, in dark conditions the latency was significantly prolonged in *dark-fly* compared to all other groups (*Figure 34 A*).

As mentioned above, the IPI has an important role in species identification and was therefore compared between the four groups. Both *dark-fly* (40.6 ms) and *OregonR* (41.3 ms) show a similar IPI in light conditions. Interestingly, with a change to dark conditions, the interval is significantly reduced in *OregonR* but significantly increased in *dark-fly* (*OregonR$_{dark}$* 39.0 ms; *dark-fly$_{dark}$* 45.9 ms) (*Figure 33 B*).

Comparing the pulse to sine ratio, a significantly higher proportion of pulse song is found in *dark*-fly for both illumination conditions. Within the groups a trend to an increase in proportion of pulse song can be observed but does not reach significance (*Figure 33 C*). Correspondingly, the fraction of P_{fast} is significantly increased in *dark-fly* for both illumination conditions compared to *OregonR* (*Figure 33 D*). In *dark-fly* the proportion is also significantly higher in dark conditions than in light conditions, whereas in *OregonR*

a trend towards an increased fraction of P_{fast} in dark conditions can be observed but does not reach significance (*Figure 33 D*).

In dark conditions, courtship song volume is increased compared to light conditions. Since male are not able to reliably locate the female in the absence of visual cues and distance to the female mediates courtship song volume, this result was to be expected. However, *dark-fly* males increase their courtship song volume significantly compared to *OregonR* males, suggesting an underlying mechanism that favours louder courtship songs.

4.8 *Dark-fly* shows altered pigmentation

Comparing the morphology and pigmentation of both the *dark-fly* and *OregonR* strain rendered colouration differences in the abdomen and scutum of *dark-fly* compared to the *OregonR* strain. To quantify this, males and females of both strains at the age of 5 days and reared under different illumination conditions were compared.

The most prominent difference is found in the pigmentation of the abdomen: the colouration of the dark band at the posterior end of the abdominal tergites in *dark-fly* is considerably lighter *(Figure 35)* The transition from the darker coloured band to the background pigmentation is rather gradual in *dark-fly*, while in *OregonR* the colour change is indicated with a sharp border. This gradual transition of colouration in *dark-fly* also influences the stripe pattern on the female abdomen. While it is distinct in *OregonR*, in *dark-fly* females the stripe pattern is rather blurred. Furthermore, the thorax of *dark-fly* shows a distinct pigmentation change. Indicated by the dashed line, the scutum of *dark-fly* holds a pattern of darker pigmentation in trident form, which is mostly absent in *OregonR* (Figure 35 *B*). If *OregonR* displays a trident shaped pattern on the scutum, it is of a lighter colour compared to *dark-fly*. Raising both groups in different illumination conditions did not show an influence on the pigmentation pattern.

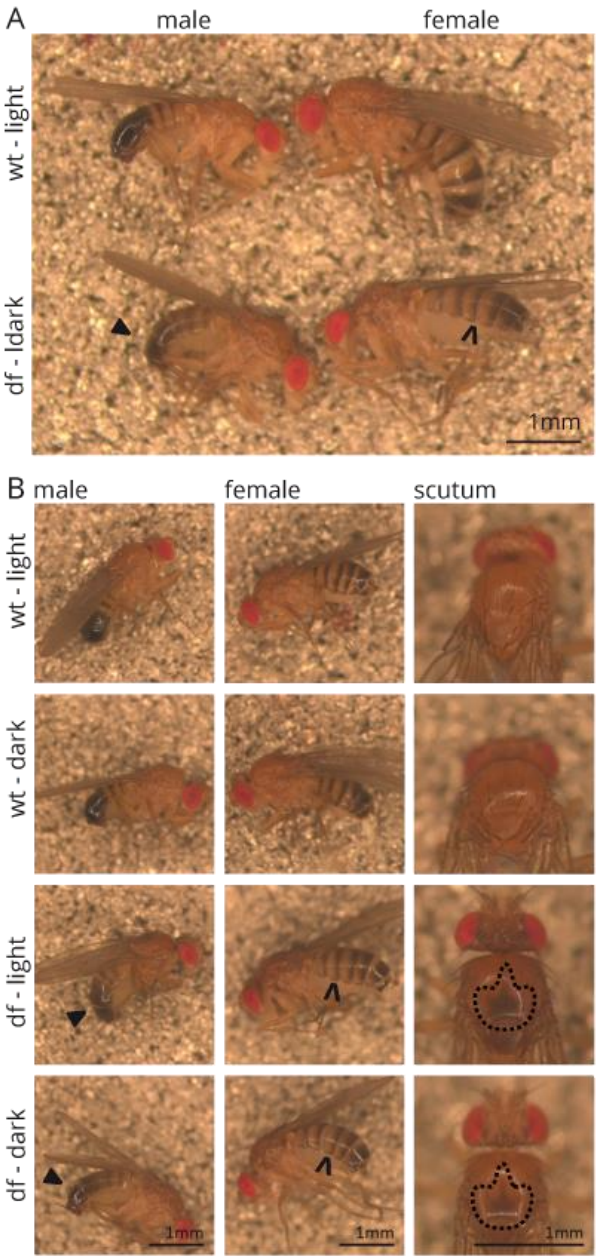


Figure 35 Pigmentation in dark-fly and OregonR. $N(OregonR_{light})$ = 5, $N(OregonR_{dark})$ = 5, $N(dark\text{-}fly_{light})$ = 5, $N(dark\text{-}fly_{dark})$ =5 **(A) Overview of pigmentation.** Filled arrowheads indicate lighter pigmentation of male dark-fly abdominal segments, empty arrowheads indicate lighter pigmentation and blurred stripe pattern in dark-fly females. **(B) Pictures of males and females of both strains raised in different illumination conditions.** Filled arrowheads indicate lighter pigmentation of male dark-fly abdominal segments, empty arrowheads indicate lighter pigmentation and blurred stripe pattern in dark-fly females. The dashed trident shape depicts the darker pigmentation pattern on dark-fly scutum.

5. Discussion

Behavioural adaptations in a micro-evolutionary context have only been sparsely described. These mechanisms are mainly studied in field studies, with the disadvantage of uncontrollable environmental conditions. In this study I examined a dark-raised *Drosophila* strain and its behavioural adaptation to long-term light deprivation, an environmental factor that can be easily controlled in laboratory conditions.
The *dark-fly* stain was established in 1954 and has been sustained for over 1500 generations. While it has been extensively studied on a molecular and anatomical level, to date no detailed behavioural assessment has been done. I concentrated on two visually guided behaviours that are crucial for the survival of *Drosophila*: the locomotion strategy and the courtship behaviour.

Here I present evidence of the emergence of new behavioural strategies in dark-raised *Drosophila*. The saccadic locomotion strategy, optimizing the optic flow, is replaced by a strategy optimizing the mechanosensory field, characterised by the *Tōhoku drift*. This also suggests, that the saccadic strategy is indeed solely mediated by the visual system as it is abolished in the absence of visual cues.
The classically competitive courtship strategy is superseded by a cooperative courtship approach in *dark-fly* males, guaranteeing higher courtship success in dark conditions. Furthermore, this study shows indication for a sex-specific co-evolution in the *dark-fly* strain.

5.1 Circadian rhythm unaffected after 1500 generations of light deprivation

In *Drosophila* several behaviours such as courtship, mating and general locomotion activity are driven by the circadian clock and its regulating clock genes (Allada & Chung, 2010; Fujii et al., 2007; Sakai & Ishida, 2001). The *dark-fly* strain has been raised in darkness for over 1500 generations raising the question if the circadian rhythm shows divergence from the wt pattern. Using the DAM system, the daily activity of both male *OregonR* and *dark-fly* was assessed, presenting them with 4 days of constant darkness

followed by 5 days of a 12:12 h dark:light cycle. Neither the circadian activity nor the sleep pattern rendered a significant difference comparing both strains. In constant darkness, both strains display concurrent weakly oscillating activity patterns with a phase duration of approximately 25 h. Introducing a dark:light cycle elicits a strong diurnal circadian rhythm showing the characteristic bimodal activity peaks associated with switches from either dark to light or light to dark (Aschoff, 1966; Dubowy and Sehgal, 2017). During the first day, *dark-fly* shows a higher activity during dark phases compared to *OregonR*, however, this effect is fading over the concurring days. Overall, the activity patterns of the both strains do not significantly differ from each other.

Even after 1500 generations in darkness, *dark-fly* still entrains to a dark:light cycle and displays a diurnal activity similar to wt flies. This finding shows, that the dark:light cycle is still used as an external zeitgeber to sustain rhythmic activity. These data are in accordance with previous studies on *dark-fly* and wt Drosophila activity pattern (Fuse et al., 2014; Imafuku & Haramura, 2011; Mori, 1986). Even after over 60 years in constant darkness, *dark-fly* animals are maintaining a bimodal circadian rhythm in a dark:light cycle, suggesting that the underlying mechanism is still functional.

It has been shown that *Drosophila* in constant dark conditions maintains a robustly oscillating circadian rhythm. However, the weakly oscillating activity pattern we find for both strains in darkness can also party rely on other external *zeitgebers* present in laboratory conditions that could lead to entrainment of the *dark-fly* strain to a distinct circadian rhythm ahead of the experiment. Possible examples would be vibrations of the used incubators and temperature changes due to opening the incubator in the mornings for fly housekeeping (Busza et al., 2007; Majercak et al., 1999). Furthermore, in laboratory conditions, day-time is associated with an elevated general activity of walking, talking and opening/closing of doors. Although the experimental setup was situated in a closed room placed on a passive stabilized table, and the incubators were nor opened during the course of the experiment, these influences cannot be fully ruled out to act as external *zeitgebers* and therefore might play a role in inducing an oscillating pattern of daily activity.

To conclude, these findings show no difference in activity pattern in *OregonR* and *dark-fly*. We can therefore assume that behavioural differences in *dark-fly* and *OregonR* are not biased by changes in the circadian activity of those strains. Since *dark-fly* still entrains to a dark:light cycle, it can be assumed that the underlying mechanisms responsible for the typical bimodal activity pattern driven by the switch in illumination conditions are still functional in *dark-fly*.

5.2 *Drosophila* locomotion strategy is dependent on the visual system

Drosophila, similar to most other insects, displays a distinct locomotion strategy: since they lack stereoscopic vision (Land, 1999), the optic flow, the retinal image shift generated by self-motion, is used to extract 3D-information from the environment (Gibson et al, 1955). During translational movements, objects generate different velocities across the retia, dependent on the distance: close objects generate higher velocities than objects farther away. This allows for the extraction of distance information. During rotational movement, however, all objects move with the same speed. Therefore, only translational movements can provide distance information (Koenderink and Doorn, 1987). Since 3D-information is crucial for successful navigation, insects overcome this problem by separating translational from rotational movements. Phases of translation are prolonged while rotations are reduced to very short and fast turns, called saccades (Collett and Land, 1975a, 1975b; Geiger and Poggio, 1977). This saccadic strategy was consistently found in different insects (Geurten et al., 2010; Ribak et al., 2009; Schilstra & Hateren, 1999; Srinivasan et al., 1996; van Hateren & Schilstra, 1999), zebra finches (Eckmeier *et al.*, 2008) and different aquatic species (Geurten et al., 2017; Helmer et al., 2017). *Drosophila* has been shown to apply this strategy during both walking and flying (Geurten et al., 2014; Tammero & Dickinson, 2002). Furthermore, this strategy is not only present in behavioural observations but this information is also represented on a neuronal layer, allowing the animal to extract spatial information about their environment in a computationally efficient fashion (Kern *et al.*, 2005; Geurten, Kern and Egelhaaf, 2012).

It can be concluded, that the locomotion strategy is adapted to allow for 3D information extraction by the visual system (Land, 1973). In this study the effect of long-term light

deprivation and possible adaptations to absence of visual cues was examined. Locomotion experiments were performed with genetically impaired visual mutants, the long-term dark-raised strain *dark-fly* and a newly dark-raised strain *Goe-dark* which was tested at every generation.

5.2.1 The absence of visual cues leads to an increase in locomotor velocity

Flies in darkness, as well as fly strains with impaired visual systems, display an increased thrust velocity. In light conditions, we find a median thrust velocity for *OregonR* of 6.57 mm/s, which is comparable to previously reported walking speeds for *Drosophila* (Berendes et al., 2016; Mendes et al., 2013; Robie et al., 2010). The thrust velocity immediately increases when *OregonR* flies are light deprived. The thrust velocity further increased after 15 generations in darkness and the *dark-fly* strain shows the highest thrust velocity with 15 mm/s.

A similar progression is observed in the visual mutants. *ora* shows a mutation in the ionotropic histamine-gated chloride channel ora transientless essential for motion vision and is therefore motion blind. Experiments suggest the colour vision pathway is at least functional (Harris, 1977; Yamaguchi et al., 2008). Sine oculis is a homeobox-containing transcription factor that functions together with eya as a transcriptional co-activator mediating the development of the compound eyes (Helfrich-Förster et al., 2000; Kenyon et al., 2005; Weasner et al., 2007). *sineoculis* mutants therefore have no eyes. The *sineoculis* mutant flies used in this study were maintained as a heterozygotic strain and crossed for the experiment to generate first generation blind flies. The *sol* strain shows a mutation in the gene *small optic lobes*. It is involved in the neurogenesis of the nervous system and mutations in this gene cause neuronal degeneration in columnar neurons, severely impairing the processing of visual stimuli (Delaney et al., 1991). The *sol* stain in this study has been homozygous for this mutation for several years (Bellen *et al.*, 2011), therefore possible behavioural adaptations to the absence of visual cues could be expected in this line. Compared to wt all three strains show an increase in locomotion velocity. *ora* shows the lowest increase while *sol* shows the highest, displaying a progression in locomotor speed corresponding to the severity of impairment of the visual system.

Light-deprivation and therefore loss of visual cues in a behavioural setup will increase the stress level in *Drosophila*. This potentially explains the sudden acceleration of walking speed in wt *Drosophila* exposed to dark conditions. However, when maintained in darkness, *OregonR* further increased their velocity. A comparative study on the Mexican blind cave fish, that has adapted their sensory system to light-less environments, and the closely related surface form (*Astyanax mexicanus*) showed a reduced stress response of the blind form to novel environments (Chin *et al.*, 2018). Stress as the source of increased walking velocity in dark-raised flies seems therefore less likely.

In light conditions, insects use the visual system to navigate and find resources (reviewed in: Heinze, 2017). In Drosophila, their visual field allows them to successfully navigate and discover important resources. However, in dark conditions, the visual field is not available and other sensory cues become prevalent. Aside from olfaction, gustation and audition, *Drosophila* can use their mechanosensory field, consisting of the mechanosensitive organs like antennae, legs, wings and bristles. An accelerated walking velocity can therefore increase the area covered by the mechanosensory field. Indeed, *dark-fly* shows an increased exploration rate that can be in part accounted to a higher walking velocity (for further discussion of the exploration strategy see *5.2.3*).

5.2.2 The absence of visual cues mediates a diversion from the saccadic strategy

While thrust velocities are accelerated and thrust bouts reduced, the time spent with saccades increased, which is observed in *dark-fly*, *Goe-dark* and the vision impaired mutants. Together these results suggest that in the absence of visual cues, and thus optic flow, the flies' locomotion strategy is less optimized for visual based 3D-information gathering.

The average yaw velocity over the course of a saccade displays the characteristic bell-shape reported for eye saccades in mammals and saccadic body turns in insects (Blaj & van Hateren, 2004; Geurten et al., 2014; Kress & Egelhaaf, 2012; Land, 1997; Ribak et al., 2009; Stanford et al., 2010). However, flies with an impaired visual system as well as both dark- dark-raised strains *dark-fly* and *Goe-dark* show an increase in rotational

velocity both at the start and the peak of the saccade. This indicates, that not only the saccade is faster, but contrary to wt *Drosophila*, which enter saccades after translational motion, there is a residual rotational component in the inter-saccadic interval of dark-flies and *Goe-dark*. Congruously, not only the number of saccades but also the amount of other rotations, below the saccade threshold of 200 deg/s, is increased in both dark-raised strains.

During a saccade, the wt strain *OregonR* changed its angular heading 15 deg, consistently with reported angular heading changes in the wt *Drosophila* strain *CantonS* and within the range of walking *Calliphora* (Blaj & van Hateren, 2004; Geurten et al., 2014). Both dark-raised and mutant *Drosophila* showed a significant increase in the angular heading changes. As explained above, in darkness flies can use their mechanosensory field instead of the visual field for orientation in the environment. Higher angular changes lead to an increase in area covered by the mechanosensory field, which would be beneficial for navigation and the discovery of resources.

Taken together, our findings show, that absence of visual cues either by genetic manipulation or light deprivation promotes abolishment of the saccadic strategy. The features allowing for the optimal exploitation of the 3D-information generated by optic flow (long phases of translation and reduced points of rotations) are inverted: phases of translational motion are severely reduced while rotational movements are increased. The higher angular changes and accelerated walking speed suggest the emergence of a new locomotion strategy, that substitutes the saccadic strategy in the absence of visual cues. These results present evidence that the sole sensory modality mediating the saccadic strategy is indeed vision.

5.2.3 *Dark-fly* locomotion strategy optimizes the mechanosensory field

The *dark-fly* strain shows a significantly increased exploration rate in both illumination conditions compared to wt flies. Contrary to the changes that can be observed in the time spent with rotational and translational motion, the increased exploration rate in the *dark-fly* strain is not immediately affected by a change to light conditions. Behavioural adaptation to the environmental conditions could have driven the *dark-fly*

stain to develop a new locomotion strategy generating a persistent increase in exploration rate.

As I discussed previously, this effect can be partly ascribed to an acceleration in walking speed in the absence of visual cues, which allows the flies to cover more area in the same time (see It can be concluded, that the locomotion strategy is adapted to allow for 3D information extraction by the visual system (Land, 1973). In this study the effect of long-term light deprivation and possible adaptations to absence of visual cues was examined. Locomotion experiments were performed with genetically impaired visual mutants, the long-term dark-raised strain *dark-fly* and a newly dark-raised strain *Goe-dark* which was tested at every generation.

5.2.1 The absence of visual cues leads to an increase in locomotor velocity*).* Comparing the walking trajectories of *OregonR* and *dark-fly* animals, one of the biggest differences is curve walking: wt *Drosophilae* pirouette around a corner, while *dark-fly* animals display a drifting movement, comparable to a racing car, termed *Tōhoku drift*. In *dark-fly* nearly 50% of the increased exploration rate can be accounted for by the *Tōhoku drift*. This is a persisting effect even after *dark-fly* animals were raised in light conditions for 5 generations. While the saccadic strategy optimises optic flow, the newly arisen strategy rather optimises the mechanosensory system, allowing flies of the *dark-fly* strain to cover more area and successfully encounter resources like food and mating partners. The importance of the mechanosensory system for the *dark-fly* strain is also reflected in the elongated bristles, external sensory organs of *Drosophila* reacting mostly to tactile stimuli (Fuse et al., 2014a; Imaizumi, 1979).

This holds interesting consequences for the underlying navigational strategy. Over the last decades, the *Lévy flight* was identified as the optimal foraging strategy that utilizes the visual system. The *Lévy flight* is a specialised random walk model, characterised by a heavy-tailed probability distribution for the determination of step-length (Mandelbrot, 1982). Compared to a classical random-walk model, the *Lévy flight* model is more successful at finding randomly distributed objects in the same time (Cole, 1995). Due to the heavy-tailed probability distribution, *Lévy flight* favours longer step-lengths, which

allow to cover more area than a classical random walk[1]. In previous studies, evidence for *Lévy flight* as a foraging strategy was found in T-cells, albatrosses, marine predators, bees and human hunter-gatherers (Korobkova *et al.*, 2004; Tu and Grinstein, 2005; Reynolds *et al.*, 2007; Sims *et al.*, 2008, 2014, Humphries *et al.*, 2010, 2012; Harris *et al.*, 2012; Raichlen *et al.*, 2014).

While flying *Drosophila* display a nearly optimal *Lévy flight* when odour tracking (Reynolds & Frye, 2007), walking *Drosophila* still show characteristics of this strategy but far from optimal (Reynolds et al., 2015).

Considering the evidence for *Lévy flight* in *de facto* blind objects like T-cells and *Bivalvia* (de Jager et al., 2011; Kölzsch et al., 2015) this raises the question whether the *dark-fly* strain still displays *Lévy flight* as an exploration strategy. One of the main characteristics of *Lévy flight* is its segmentation in long stretches of forward movement, favoured by the heavy-tailed probability distribution determining the step-length, and short points to reorient the gaze, often coupled with a rotation to change direction (Mandelbrot, 1982; Cole, 1995). These characteristics match those of the saccadic strategy, which is characterised by long phases of translation and short phases of reorientation and has been shown to be visually driven (Collett and Land, 1975a, 1975b; Geiger and Poggio, 1977). The similarities between *Lévy flight* navigation and saccadic movement strategy indicate that in fact both strategies are relying on the visual system. This gives rise to the question if the *dark-fly* strain not only changed their locomotion strategy but also changed their navigational strategies. An indication of such change in navigational strategy is the appearance of the *Tōhoku drift.* The geometrical analysis presented in this study shows evidence that employing the *Tōhoku drift* during curve walking expands the area swept by mechanosensors. If the environmental conditions favour the use of mechanosensation over other senses, as could be the case in dark conditions, use of the *Tōhoku drift* indeed presents major advantages and surpasses *Lévy flight*. It can be concluded, that the *Tōhoku drift* model developed in this study seems to render a better suited description of the locomotion strategy in persistent darkness than the

[1] Heavy-tailed probability distribution $p(l) \sim l^{-\mu}$ ana $1<\mu<3$. list the step-length, μ the Lévy exponent. An optimal Lévy flight would be reached at $\mu \approx 2$.

conventional *Lévy flight* model. As a next step the *dark-fly* walk can be modelled and compared to *Lévy flight* and a classical Random Walk model similar to the study by Cole, 1995.

In conclusion I present evidence, that the visually-dependent saccadic strategy is abandoned in dark conditions in favour of a newly emerging strategy that is dependent on the optimisation of the mechanosensory field.

5.3 Light-deprived *Drosophila* show changes in courtship behaviour

Drosophila courtship is characterized by a succession of elaborate, male behaviours. The typical courtship actions include following behaviour, tapping the female abdomen, licking the female genitalia, unilateral wing extension and production of courtship song, attempted copulation and successful copulation (Hall, 1994). Before courtship initiation, the male has to assess species, gender and receptivity of the potential mate. Successful courtship therefore relies on the perception and computation of multisensory inputs.

Species identity is communicated by the type of sine song and the interpulse interval (IPI) *via* the male courtship song and through a combination of olfactory and gustatory cues. Also gender and female receptivity is examined by the male though the olfactory and gustatory sensory system (Dweck et al., 2015; Greenspan & Ferveur, 2000; Kurtovic et al., 2007; Lu et al., 2012; Thistle et al., 2012; Toda et al., 2012). Vision was previously shown to have a severe influence of vision on courtship success, courtship initiation and timing of specific actions (Agrawal et al., 2014; Markow, 1987; Markow & Hanson, 1981; Markow & Manning, 1980; Ribeiro et al., 2018).

This raised the question how courtship is affected by long-term light deprivation and if adaptation to these change in environmental conditions have arisen. To gain deeper understanding three types of courtship assay were performed: a single pair courtship assay, a group courtship assay and a competitive courtship assay. Both *dark-fly* and *OregonR* strains were examined in light and dark conditions. Simultaneously courtship songs were recorded, and a detailed analysis was performed.

5.3.1 Dark-*fly* shows reduced courtship performance in single courtship assay

In a dyadic single pair courtship assay we could show a significantly impaired courtship success in the absence of visual cues. Unexpectedly, *dark-fly* performed worse than *OregonR* in both illumination conditions. Since *dark-fly* was sustained in dark conditions for over 1500 generations and has been shown to have reproductive dominance over wt flies (Izutsu et al., 2015) a change in courtship strategy to cope with light deprivation was expected. The latency to courtship initiation is not significantly different in the four groups; *dark-fly* therefore has no advantage in locating females faster in darkness.

For a detailed characterization of the courtship behaviour a Hidden Markow Model (HMM) of courtship syntax was compiled. In dark conditions, *OregonR* looses the bilateral transition ***of other courtship behaviour far*** to ***other courtship behaviour near***; there is a transition from ***other courtship behaviour near to other courtship behaviour far*** but no transition to any other courtship behaviour like wing extension or copulation attempts. This suggests, that *OregonR* in darkness are not able to relocate the female when she leaves the close interaction range. Additionally, females can be detected at larger distances in general, but flies lack positional information and aborted courtship in darkness more likely.

A previous studies could show, that the behaviour most influenced by absence of visual cues is the following behaviour (Sakai et al., 1997). Our definition of the state other courtship behaviour far includes following behaviour. The missing connection from this state to other courtship behaviours, indicating abortion of courtship once the females leaves the close interaction range, corresponds to the finding of Sakai et al.

Dark-fly animals in both dark and light conditions retain the bilateral connection between other courtship behaviour far and other courtship behaviour near. The calculated proximity index showed that the *dark-fly* strain is not limited to courtship near the female in both illumination conditions. However, courtship behaviour is not initated over other courtship behaviour far. This indicates that *dark-fly* flies are capable of maintaining conctact to the female and reestablishing courting in close proximity, but is not able to find the correct position of a female in darkness. Analysis of the exploration

rate has shown a significant increase in the *dark-fly* strain compared to *OregonR* flies; this could be responsible for *dark-fly* males to efficiently relocating the female.

Based on the HMM, a severe influence of the illumination condition on wing extension is suggested. While *OregonR* in light shows a bilateral transition from wing extension to all other courtship behaviour, the connection from other courtship behaviour far to wing extension is lost in the other three groups. The correctness index for wing extension shows reduced levels for *dark-fly* compared to *OregonR*; while in *OregonR* dark conditions lead to a decrease in correctness index, in *dark-fly* no influence of illumination condition can be found. The frequency of wing extension is similar in *OregonR* for both illumination conditions and *dark-fly* in light, *dark-fly* in darkness shows a significantly reduced frequency.

These findings imply that the absence of visual cues impaires functional wing extension; this is corresponding to previous studies showing that *Drosophila* uses vision to locate the female and choose the correct wing for wing extension (Cook, 1980; Kohatsu & Yamamoto, 2015; Pan et al., 2012; Ribeiro et al., 2018; Schneider et al., 2018)(Cook, 1980; Pan, Meissner and Baker, 2012; Kohatsu and Yamamoto, 2015). However, in dark conditions courtship songs show an increased volume (see *5.3.5 Light deprivation might trigger sex-specific co-evolution*), suggesting that the importance of extending the ipsilateral wing is decreasing in darkness.

As outlined above, *dark-fly* shows a significantly reduced courtship success compared to OregonR, which is further shown by the missing transition to sucessful copulation in both *dark-fly* groups displayed in the HMM. To understand which part of the mating behaviour is interrupted in *dark-fly* the latencies to courtship and copulation were analysed. The latency to start of courtship was not significantly different in all four groups; a slight trend towards a later start of courtship behaviour in dark conditions can be observed for both strains. It can be concluded that both strains start courtship behaviour as soon as the female is found. In darkness *Drosophila* can only rely on olfaction, mechnosensation and audition to locate the female, which can account for the slightly higher latency to courtship initiation in dark conditions. The latency to copulation was significantly increased by change in illumination condition in *OregonR*.

This might be due to the higher chance of loosing and failing to reestablish contact to the female in darkness. Correspondingly, *OregonR* displays a higher courtship initation frequency in darkness; since copulation attempts are often unsuccessful, courtship behaviour is resumed until mating can be completed. The latency to copulation is severly affected in *dark-fly* in both illumination conditions: over 50% of tested *dark-fly* pairs were not able to mate within the 5 min of our analysis window. The courtship frequency is significantly increased compared to *OregoclonR* in both illumination conditions.
Taken together *dark*-fly males show a highly disrupted courtship in both dark and light conditions. Altough the results suggest an increased ability in relocating the female after leaving the close interaction range, *dark-fly* is unable to successfully copulate in a single pair courtship assay.

5.3.2 *Abdominal-B* might be involved in disrupted female courtship behaviour

In a natural setting, the ability to successfully couplate in *Drosophila* is not only dependent on male courtship behaviour but also on female receptivity. It was therefore imporant to analyse female behaviour as well.
Repeating the single courthsip assay with mixed pairs in dark conditions increases coursthip success in the *dark-fly* strain to wt levels, if a *dark-fly* male was paired with an *OregonR* female. This indicates that although male courtship behaviour is severly altered from wt courtship, *dark*-fly males are still able to successfully complete mating. However, if a *dark-fly* female is paired with an *OregonR* male courtship success is again significantly reduced.
The female part of courtship is charaterized by pausing, to allow the male to initiate copulation, and opening the cuticular vaginal plate, to reveal their genitalia (Hall, 1994). Pausing is typically initiated after perceiving the male courtship song and signals readiness to mate to the male (Schilcher, 1976). There was no significant influence of the illumination condition onto female pausing. However, *dark-fly* females show significantly lower levels of pausing compared to *OregonR*. *Dark-fly* males do initate succesful copulation with wt females. Therefore an alteration in *dark-fly* courtship song rendering them insufficient to persuade the females can be excluded. This suggest an

"internal" cause within the female. A recent study described neurons expressing the homeobox transcription factor *Abdominal-B* (*Abd-B*) mediating female pausing behaviour. Silencing adult *Abd-B* expressing neurons lead to a significant decrease in female receptivity, characterized by reduced pausing behaviour but did not affect opening of the vaginal plate (Bussell et al., 2014). This suggests that both components are functionally different. Interestingly, *Abd-B* carries a point mutation in *dark-fly* subsituting an Alanine for a Serine (Izutsu *et al.*, 2012). Futhermore, *Abd-B* is overexpressed in *dark-fly* (N. Fuse, personal communication).

Abd-B is further involved in the biosynthesis pathway determining the colour of *Drosophila* by influencing *yellow* (Jeong et al., 2006; Kopp et al., 2000). After closer inspection, colouration differences between in *OregonR* and *dark-fly* become apparent, illustrating a change in *Abd-B* funtcion and further supporting the hypothesis that it could also be responsible for alterations in receptivity of *dark-fly* females. To validate this hypothesis, the courtship assays would have to be repeated using an *Abd-B* overexpression strain. Expression levels of *yellow* and other genes involved in colour determination should be examined in *dark-fly*.

Taken together we see an increase in *dark-fly* courthsip success, even surpassing *OregonR* in dark conditions, when switching from a classical single courtship assay to a group courtship assay. Males show disrupted courtship behaviour in darkness and relocation of the female is impaired. Furthermore, *dark-fly* females show defective pausing behaviour, impeding succesful copulation. This defect in female receptivity seems to be linked to an irregularity in *Abd-B* expressing in the *dark-fly* strain and is countermanded by courtship in groups.

5.3.3 *Dark-fly* males show changes in behaviour towards conspecifics

The reduced courtship success and overall performance of *dark-fly* in the single pair courtship assay conflict with the study of Izutsu et al., finding an increase performance of *dark-fly* compared to wt flies in a competitive mating assay (Izutsu *et al.*, 2015). In laboratory conditions, *Drosophila* is maintained in vials filled with food and about 100 conspecifics. Regarding these rearing conditions, a single pair courtship assay presents a very unnatural setting for *Drosophila*. To recreate a more natural assay, a group

courtship assay was designed, testing 10 flies (5 males, 5 females) over the course of 60 min.

Although the latency until the first pair successfully copulated is prolonged in *dark-fly* in both illumination conditions, the courtship success of *dark-fly* is increased in respect to *OregonR* in both conditions. This increase cannot be accounted to the longer observation period since an analysis assessing the number of mated females every five minutes shows that *dark-fly* were similar or even more successful.

Assuming a decreased receptivity of *dark-fly* females in single pair courtship assays, the addition of multiple males leading to an increased amount of courtship song and other sensory modalities is likely sufficient to overcome that obstacle. Furthermore, in darkness the presence of more males and therefore a higher amount of courtship song might be beneficial: since an exact localization of females is increasingly difficult in the absence of visual cues a male could accidentally enter the close interaction range of a female that was aroused by the courtship song of another male. However, this would require males tolerating other males courting simultaneously.

This would hold interesting implications for the courtship strategy in *dark-fly*. Wt *Drosophila* display a competitive courtship strategy: when presented with a competing male during courtship, reciprocal aggression behaviour towards the competitor is initiated (Dow & van Schilcher, 1975; Sturtevant, 1915; Versteven et al., 2017). We assessed this behaviour by performing a competitive courtship assay: two socially naïve males are presented with a decapitated virgin female (Hahn *et al.*, 2013; Corthals *et al.*, 2017). Copulation can never be successful; therefore, the males will perpetually court the female and display reciprocal aggression behaviour. In both strains the amount of courtship is increased in dark conditions. While in *OregonR* the aggression behaviour is not affected by a change in illumination conditions, in *dark-fly* a significant increase of aggression behaviour in light conditions can be observed.

Courtship behaviour is mediated by pheromones and other chemosensory cues, courtship song and auditory cues by the female (i.e. cleaning behaviour or walking; Ejima & Griffith, 2008) as well as the visual system (ie *via* LC10 neurons; Ribeiro et al., 2018). Although courtship success is impaired in the absence of visual cues (Agrawal et al.,

2014; Markow & Manning, 1980; Sakai et al., 1997) the HMM of courtship syntax compiled in this study shows that the presence of females is still perceived by males and courtship behaviour initiated. The olfactory and auditory cues present in darkness seem to be sufficient for courtship initiation, however the exact localisation of females and therefore copulation is severely impaired. Aggression behaviour is mainly modulated by pheromonal cues. It can be hypothesised that the detection of females and initiation of courtship in darkness is easier than the detection of opponent males, since even in the absence of vision different sensory inputs are available. This would explain the increased levels of courtship behaviour in dark conditions compared to light conditions.

During the competitive courtship assay *dark-fly* males are exposed to light conditions for the first time meaning this would be the first encounter with visual images of their conspecifics. This could explain the elevated levels of aggressiveness in *dark-fly* males in light conditions compared to *OregonR* males.

To understand if *dark-fly* males changed their courtship strategy, the synchronicity of both courtship and aggression behaviour was analysed. Both strains show a higher amount of simultaneous courting in dark conditions. As described above, this could be due to easier recognition of females than of opponents in the absence of visual cues. In darkness, *dark-fly* males show indeed a higher rate of simultaneous courtship compared with *OregonR* males. This could point to an increased tolerance of *dark*-fly males to concurrently courting competitor males. Interestingly, in *dark-fly* males the synchronicity of aggression behaviour is increased in dark conditions while it is reduced in males of the *OregonR* strain. *Dark-fly* males might have evolved a better system to recognize possible opponents and therefore engage in reciprocal aggression behaviour. A previous study suggests that the olfactory system of *dark-flies* is more sensitive compared to *wt* flies (Fuse et al., 2014b). Thus, d*ark-fly* flies might be better equipped to pick up traces of pheromones. Furthermore, the auditory system of *dark-fly* males shows a higher sensitivity and increased mechanical amplification compared to *OregonR* flies (T. Effertz, personal communication). The higher sensitivity of these systems might allow *dark-fly* to easier recognize opponents and therefore account for the increase in reciprocal aggression behaviour. Also, a previous study found increased aggression

behaviour in genetically blinded and socially isolated *Drosophila* (Ramin et al., 2014). The regulation of aggression behaviour might be part of a visually mediated network which for now remains elusive.

In summary, it can be hypothesised that *dark-fly* shows a higher tolerance of other males courting the same female in darkness. This indicates a strategy change in the *dark-fly* strain: the competitive courtship strategy is replaced by a cooperative strategy, allowing multiple males to court simultaneously. This strategy change might act to overcome the reduced receptivity in *dark-fly* females by increasing the amount of available auditory cues.

5.3.4 Light-deprivation influences interpulse interval

As described above, male *Drosophila* need to assess gender, species and female receptivity before successful copulation can be initiated. This recognition is mediated with the olfactory, gustatory and auditory system (Kurtovic, Widmer and Dickson, 2007; Lu *et al.*, 2012). For species communication the interplay of sine song and species-specific interpulse interval (IPI) is important (Bennet-Clark et al., 1969; Bennet-Clark et al., 1968; Kyriacou & Hall, 1982; Ritchie et al., 1999). In *Drosophila melanogaster* the average IPI amounts to around 34 ms; its closely related sister species *Drosophila simulans* shows a longer IPI with 48 ms (Bennet-Clark and Ewing, 1968).

During the single pair courtship assay male courtship songs were recorded simultaneously, and different parameters were analysed. In this study we find that the IPI is not different for *dark-fly* and *OregonR* in light conditions but an inverse change in darkness. While *OregonR* flies significantly decrease their IPI, *dark-flies* actually shift to longer IPIs. As *OregonR* females still mate with *dark-fly* males, this suggests some variance in the recognition of IPI. Indeed, previous studies show IPI differences in geographically separated populations of *Drosophila melanogaster* (ranging from 33 to 36 ms; Ritchie et al., 1994). It was further reported, that playback experiments with different IPIs ranging from 28 ms (*Drosophila mauretania*) to 48 ms (*Drosophila simulans*) showed no effect on mating success (Talyn and Dowse, 2004). This suggests that the variability in IPI between *dark-fly* and *OregonR* are not behaviourally relevant.

Nevertheless, a preference of *dark-fly* females for the altered IPI in *dark-fly* males cannot be excluded.

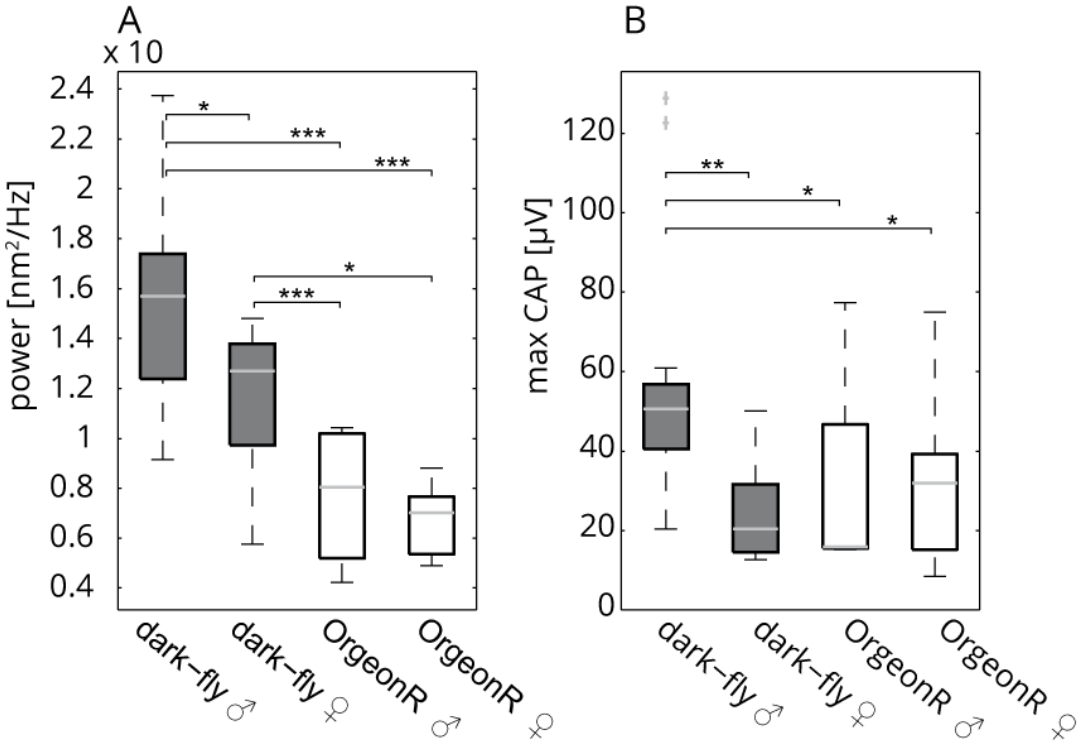
5.3.5 Light deprivation might trigger sex-specific co-evolution

As described in previous studies, the different modes of the male courtship song each have a distinct function. The sine song together with the IPI are species-specific (Greenspan & Ferveur, 2000). The pulse song is divided in P_{fast} and P_{slow}. Both are directed to the female, the use of P_{fast} correlates with a higher distance to the female while P_{slow} is used in a close interaction range (Clemens et al., 2018).

Superimposing the pulse shapes of P_{fast} and P_{slow} for all four groups renders no visible difference, implying the courtship song in *dark-fly* is indeed fully functional. However, the amplitude of courtship song is significantly changed. In both *dark-fly* and *OregonR* the amplitude of courtship song is increased when switching from light to dark conditions, which holds true for both P_{fast} and P_{slow}, with a fractional larger increase of P_{fast}. *Dark-fly* in darkness almost exclusively use P_{fast} to call out to the female. It was previously reported that the fainter pulse song P_{slow}, when perceived by a female, initiates pausing over a wide variety of distances. P_{fast} is only effective when the female is at a larger distance. For an optimal presentation of courtship song, males can modulate the volume in a distance dependent manner, using sensory feedback from the courted female and the visual system to estimate the distance (Coen et al., 2014; Coen et al., 2016). As blind flies also show an increased fraction of P_{fast} compared to wt, it can be assumed, that in the absence of visual cues the distance estimation is impaired (Clemens, Ozeri-Engelhard and Murthy, 2018). This would explain the increase in courtship song volume by increasing both amplitude of songs and fraction of P_{fast} in darkness. The males modulate their courtship song according to the lacking visual feedback towards higher volumes. However, P_{fast} has been shown to repel females rather than attract them, if emitted at close range (Clemens et al., 2018).

Interestingly, the *dark-fly* strain displays a change in hearing ability when compared to *OregonR* (T. Effertz, personal communication). *Drosophila* actively amplifies acoustic signals which can be assessed by measuring the antennal displacement using a laser doppler vibrometer and antennal nerve recordings (Kamikouchi *et al.*, 2009). *Dark-fly*

females show a reduced active amplification compared to *dark-fly* males. This is also visible in the antennal nerve recordings: the compound action potential (CAP) is significantly reduced in *dark-fly* females compared to males. The reduction in CAP might be due to a reduction in the mechanosensory transduction channel NompC localized in sound-sensitive neurons (Effertz et al., 2011).



F**igure 36 Hearing ability of *dark-fly* and *OregonR* recorded by laser doppler vibrometry.** This data is courtesy of Thomas Effetz (Department of Cellular Neurobiology and University Medicine Göttingen). **(A) Gender specific amplification of the *Drosophila* ear.** While *dark-fly* animals show a nearly twice a high amplification of sound stimuli compared to *OregonR* flies, also a sexual dimorphism emerges. Contrary to *OregonR* animals, *dark-fly* females show a significantly reduced amplification compared to *dark-fly* males. **(B) Maximum compound action potential (CAP) responses.** The CAP response of *dark-fly* males is significantly increased compared to *dark-fly* females.

So far, in *Drosophila* no sexual dimorphism in hearing ability was described. The sexual dimorphism in *dark-fly* suggest a micro-evolutionary scenario: *dark-fly* males, as soon as transferred to darkness, produced higher volume courtship song. Recent studies showed that male Drosophila can learn their courtship song from con-specifics and even speakers playing artificial courtship songs (Li et al., 2018; Riabinina et al., 2011). Taking

into account the naïve preference for P_{fast} in blind or dark conditions, it might be a simple learning context that first triggered the adaptation to increased P_{fast} production. Females, possessing a random mutation decreasing their hearing ability, would be less repelled by the loud courtship song and therefore able to mate and produce more offspring. This would lead to an establishment of the two traits in the *dark-fly* population. Co-evolution between males and females driving the evolution of sex-specific traits can widely be found in the animal kingdom. A common example is the ornamental display of feathers in a courtship context in male birds (Lebbin, 2007; Loyau et al., 2005).

5.4 *Dark-fly* as a model for micro-evolution

In nature, micro-evolution is a frequent phenomenon, defining the rapid evolutionary adaptation within and among populations. Micro-evolution is commonly driven by natural and sexual selection, mutations, genetic drift and genetic flow (reviewed in Hendry & Kinnison, 2001; Reznick & Ricklefs, 2009). Due to its very short generation time and usually high population size, *Drosophila* is a convenient model to study micro evolution. Previous studies include traits like pigmentation (Rajpurohit and Gibbs, 2012), senescence (Rose, 1984) and wing evolution (Houle et al., 2017). Furthermore, adaptation to tolerate various environmental factors like desiccation (Folk and Bradley, 2005), cold (Kellermann et al., 2009) and alcohol (McKechnie and Geer, 1993) were reported. Adaptations to absence of visual cues, as presented in this study, were previously studied in the Mexican blind cave fish (*Astyanax mexicanus*) and their closely related surface species These fish show many sensory adaptations in hearing, olfaction, stress response and electro- and magnetoreception (Chin et al., 2018; Soares & Niemiller, 2013; Soares et al., 2016).

The *dark-fly* strain was initially generated to study genetic adaptations to changing environmental conditions (Mori and Yanagishima, 1957). It has now been raised in darkness for over 1500 generations and the genome is fully sequenced which allows the linkage of physiological traits and genes (Izutsu *et al.*, 2012, 2015). This makes the *dark-fly* strain an interesting model to study micro-evolution. Previous studies found

sensitisation of the olfactory, visual and mechanosensory (elongated bristles) systems (Mori and Imafuku, 1982; Fuse *et al.*, 2014a).

In this study evidence for adaptations of behavioural strategies were found for the first time: both courtship and locomotion strategies have changed to guarantee mating success and better navigation in darkness.

Dark-fly abolishes the saccadic locomotion strategy and Lévy flight as a foraging strategy, both of which have been shown to be most successful in light conditions, and favours optimization of the mechanosensory field by incorporating the *Tōhoku drift* into their locomotor behaviour.

In *dark-flies* a sex-specific co-evolution can be observed. Due to lack of visual cues and therefore impossible distance estimation the courtship song of *dark-fly* males is significantly increased in amplitude and volume. Equally, *dark-fly* females developed bad hearing, that allows for better tolerance of the louder courtship songs.

In summary, the *dark-fly* strain represents a powerful tool to study micro-evolution. The establishment of the new light-raised strain *dark-fly light* gives further opportunity to understand how the locomotion and courtship strategies did arise.

This is the very first account of *Drosophila* undergoing a behavioural micro-evolution. This opens the field to analyse the adaptation of behaviour to a changing environment on a strategic, and in future neuronal, level.

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List of abbreviations

CCA	competitive courtship assay
CI	confidence interval
CO_2	carbon dioxide
CS	male courtship song
DAM	*Drosophila* activity monitoring system
deg	degree
DF	*dark-fly*
DFD	*dark-fly* dark conditions
DFL	*dark-fly light*
DNA	deoxyribonucleic acid
EtOH	ethanol
FDR	false detection rate
GD	*Goe-dark*
GDL	*Goe-dark* light conditions
h	hour
HMM	Hidden Markov Model
Hz	hertz
IPI	interpulse interval
IR	infrared
LED	light-emitting diode
min	minute
mm	millimeter
ms	milliseconds
OR	*OregonR*
ORD	*OregonR* dark conditions
ORL	*OregonR* light conditions
ORT	ora transientless
PM	prototypical movement
RNA	ribonucleic acid
ROI	region of interest
sec	second
s	second
SNP	single nucleotide polymorphism
sol	small optic lobes
wt	wild type

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Declaration

I, Kristina Corthals, hereby declare that my thesis entitled “Behavioural Adaptations to Light Deprivation” was written independently. No other sources and aids other than quoted were used.

_____

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Poster, Meeting of the German Neuroscience Society, Göttingen, Germany, 2015

Characterisation of a dnl2-ko in Drosophila melanogaster
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Acknowledgements

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In case anyone was wondering what happens Tuesdays and Thursdays at night in the lab: I am torpedoing Thomas and Phil no matter if we're on the same side or not. Thanks to you two for advice on applications, thesis writing and warships.

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And of course, endless thanks to my parents Johan and Angelika. Thank you for your constant support, advice and love, for encouraging me to pursue whatever I want, whether it's career or travel.

Appendix

A1 HMM transition probabilities and *p*-values

For information about the computation of the HMM transitions diagrams, please refer to *3.4.7 Hidden Markov Model of male courtship behaviour*. A graphical representation can be found in *3.4.7 Hidden Markov Model of male courtship behaviour*. (*Figure 31 and Figure 32)*. For an explanation of the categorization of courtship behaviour, please refer to *Table 1 Ethogram of Drosophila courtship and aggression behaviour described and classified in this study.*

Table 2 HMM Transition probabilities and *p*-values for *OregonR* light conditions

transition probabilities ORL

	pause	locomotion	copulation	wing ext	cop attempt	other far	other near
pause	/	0.5946	0	0.0676	0	0.2703	0.0676
locomotion	0.3793	/	0	0	0	0.4598	0.1609
copulation	0	0	/	0	0	0	0
wing ext	0.0041	0.0049	0.0089	/	0.0984	0.3846	0.4992
cop attempt	0	0	0	0.2960	/	0.0560	0.6480
other far	0.0310	0.0258	0	0.5455	0	/	0.3977
other near	0.0045	0.0045	0	0.6036	0.0045	0.3827	/

p-values ORL

	pause	locomotion	copulation	wing ext	cop attempt	other far	other near
pause	/	0.5705	-0.0031	-0.2752	-0.0350	0.0016	-0.2830
locomotion	0.3585	/	-0.0031	-0.3428	-0.0350	0.1911	-0.1447
copulation	NaN	NaN	/	NaN	NaN	NaN	NaN
wing ext	-0.0168	-0.0193	0.0059	/	0.0634	0.1159	0.1936
cop attempt	-0.0208	-0.0241	-0.0031	-0.0468	/	-0.2127	0.3424
other far	0.0102	0.0017	-0.0031	0.2027	-0.0350	/	0.0921
other near	-0.0163	-0.0196	-0.0031	0.2609	-0.0304	0.1141	/

Table 3 HMM Transition probabilities and *p*-values for *OregonR* dark conditions

transition probabilities ORD

	pause	locomotion	copulation	wing ext	cop attempt	other far	other near
pause	/	0.5387	0	0.1347	0	0.0773	0.2494
locomotion	0.2618	/	0	0.0508	0	0.1959	0.4915
copulation	0	0	/	0	0	0	0
wing ext	0.0563	0.1260	0.0083	/	0.1000	0.1573	0.5521
cop attempt	0	0	0	0.3019	/	0.0094	0.6887
other far	0.1710	0.2604	0	0.2803	0	/	0.2883
other near	0.1056	0.0532	0	0.6372	0.0090	0.1950	/

p-values ORD

	pause	locomotion	copulation	wing ext	cop attempt	other far	other near
pause	/	0.3918	-0.0022	-0.1306	-0.0292	-0.0618	-0.0570
locomotion	0.1508	/	-0.0022	-0.2144	-0.0292	0.0567	0.1851
copulation	NaN	NaN	/	NaN	NaN	NaN	NaN
wing ext	-0.0548	-0.0208	0.0061	/	0.0708	0.0182	0.2458
cop attempt	-0.1110	-0.1468	-0.0022	0.0366	/	-0.1297	0.3823
other far	0.0600	0.1136	-0.0022	0.0150	-0.0292	/	-0.0181
other near	-0.0054	-0.0936	-0.0022	0.3719	-0.0292	0.0558	/

Table 4 HMM Transition probabilities and *p*-values for *dark-fly* light conditions

transition probabilities DFL

	pause	locomotion	copulation	wing ext	cop attempt	other far	other near
pause	/	0.6512	0	0.0249	0	0.2150	0.1090
locomotion	0.1777	/	0	0.0261	0	0.2979	0.4983
copulation	0	0	/	0	0	0	0
wing ext	0.0259	0.0687	0	/	0.1394	0.1912	0.5747
cop attempt	0.0417	0.0972	0	0.1806	/	0.1111	0.5694
other far	0.0688	0.1115	0	0.1687	0.0007	/	0.6503
other near	0.0485	0.0667	0	0.3835	0.0027	0.4987	/

p-values DFL

	pause	locomotion	copulation	wing ext	cop attempt	other far	other near
pause	/	0.5429	0	-0.1642	-0.0275	-0.0455	-0.2450
locomotion	0.1171	/	0	-0.1630	-0.0275	0.0374	0.1442
copulation	NaN	NaN	/	NaN	NaN	NaN	NaN
wing ext	-0.0347	-0.0395	0	/	0.1120	-0.0693	0.2207
cop attempt	-0.0190	-0.0110	0	-0.0086	/	0.1141	0.2154
other far	0.0082	0.0033	0	-0.0204	-0.0268	/	0.2962
other near	-0.0121	-0.0416	0	0.1943	-0.0248	0.2382	/

Table 5 HMM Transition probabilities and *p*-values for *dark-fly* dark conditions

transition probabilities DFD

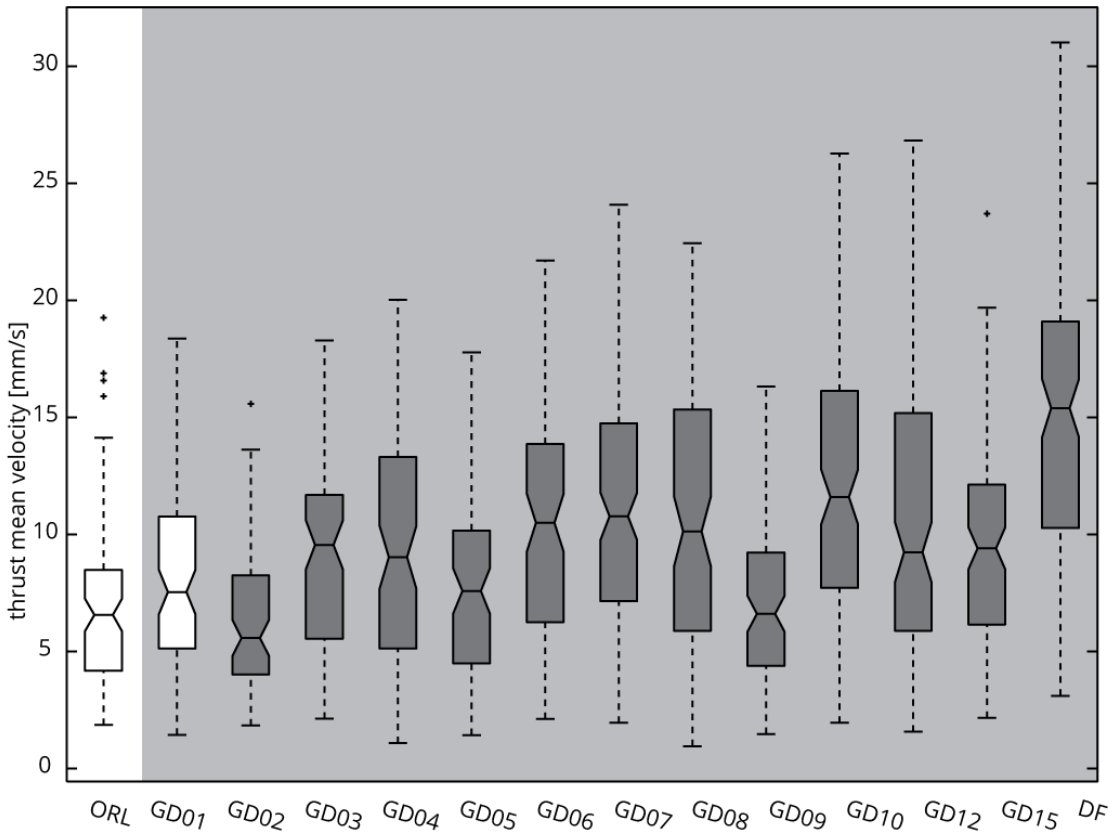
	pause	locomotion	copulation	wing ext	cop attempt	other far	other near
pause	/	0.5656	0	0.0254	0	0.1027	0.3063
locomotion	0.2256	/	0	0.0585	0.0008	0.1178	0.5973
copulation	0	0	/	0	0	0	0
wing ext	0.1534	0.3212	0	/	0.0415	0.1214	0.3626
cop attempt	0.0690	0.2759	0	0.1724	/	0.1724	0.3103
other far	0.2693	0.2663	0	0.1059	0.0015	/	0.3570
other near	0.3198	0.1519	0	0.3045	0.0007	0.2232	/

p-values DFD

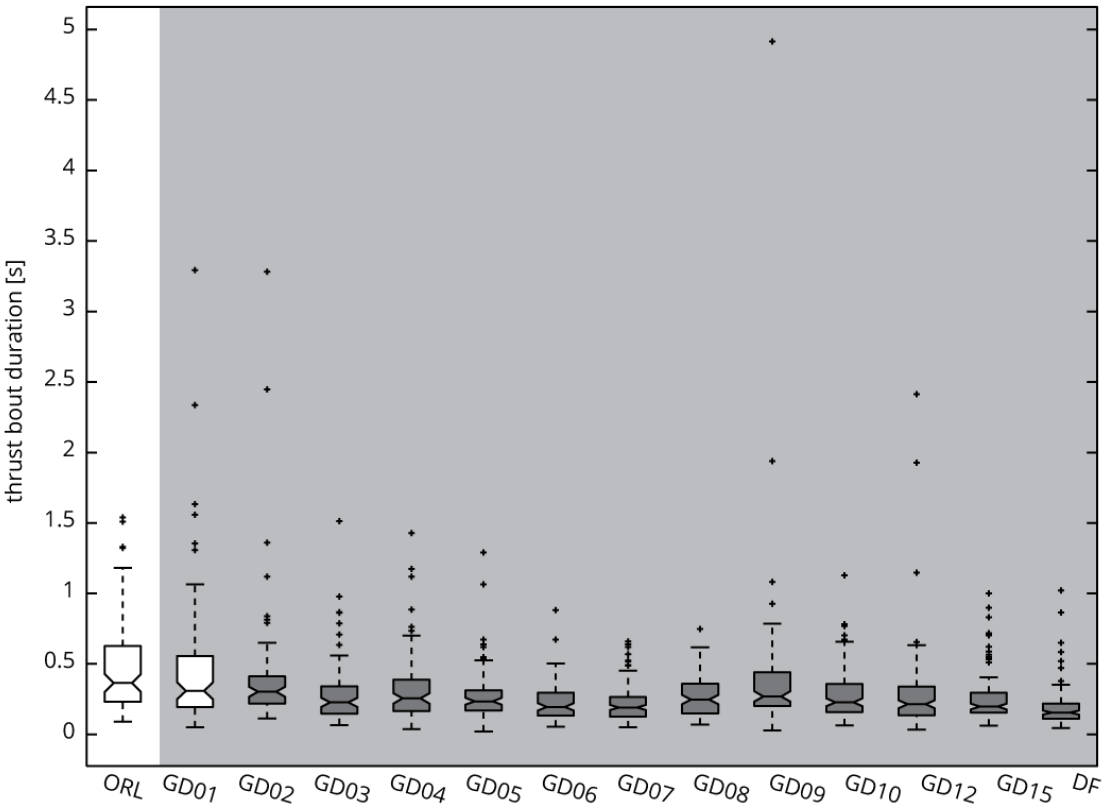
	pause	locomotion	copulation	wing ext	cop attempt	other far	other near
pause	/	0.3281	0	-0.0992	-0.0057	-0.0285	0.0088
locomotion	0.0221	/	0	-0.0662	-0.0049	-0.0134	0.2999
copulation	NaN	NaN	/	NaN	NaN	NaN	NaN
wing ext	-0.0501	0.0836	0	/	0.0358	-0.0098	0.0652
cop attempt	-0.1345	0.0384	0	0.0477	/	0.0412	0.0129
other far	0.0658	0.0288	0	-0.0188	-0.0042	/	0.0596
other near	0.1163	-0.0856	0	0.1798	-0.0051	0.0920	/

A2 Locomotion characteristic of the *Goe-dark* strain

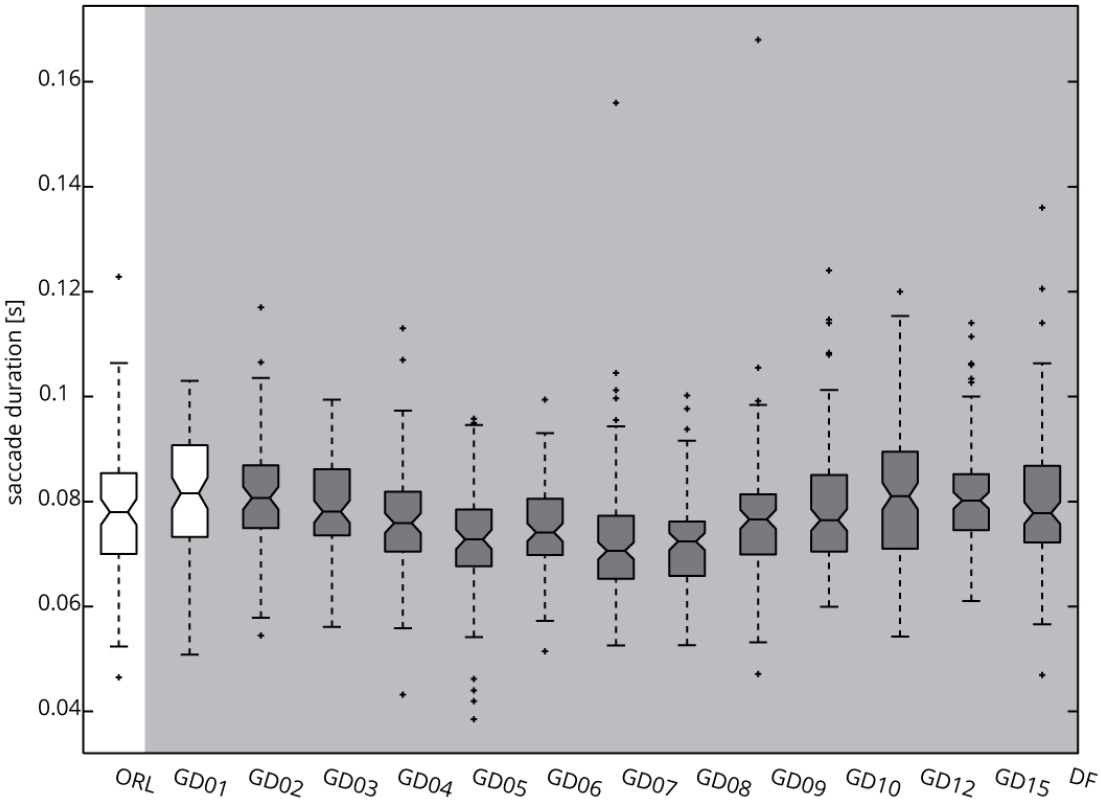
Correspondingly to the data found in chapter *4.3 Light deprivation severely influences the saccadic strategy*, here the data of the concurring *Goe-dark* generations from 01 to 15 can be found. Medians are indicated for every group in the respective figure caption. p-values can be found in *A3 p-values of locomotion characteristics.*



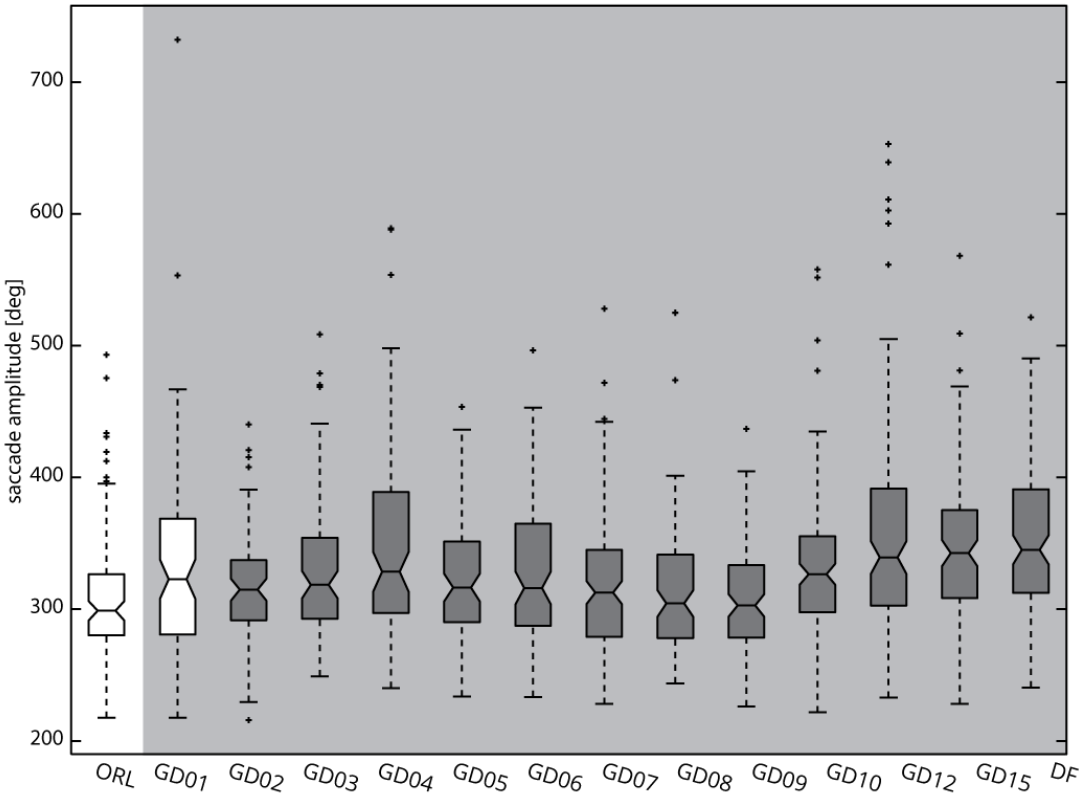
Appendix figure 1 Thrust velocity. Medians: ORL 6.57 mm/s; GD01 7.54 mm/s; GD02 5.58 mm/s; GD03 9.56 mm/s; GD 04 9.031 mm/s; GD05 7.589 mm/s; GD06 10.503 mm/s; GD07 10.78 mm/s; GD0 8 10.14 mm/s; GD09 6.62 mm/s; GD10 11.6 mm/s; s GD12 9.25 mms/; GD15 9.41 mm/s; DF 15.4 mm/sk



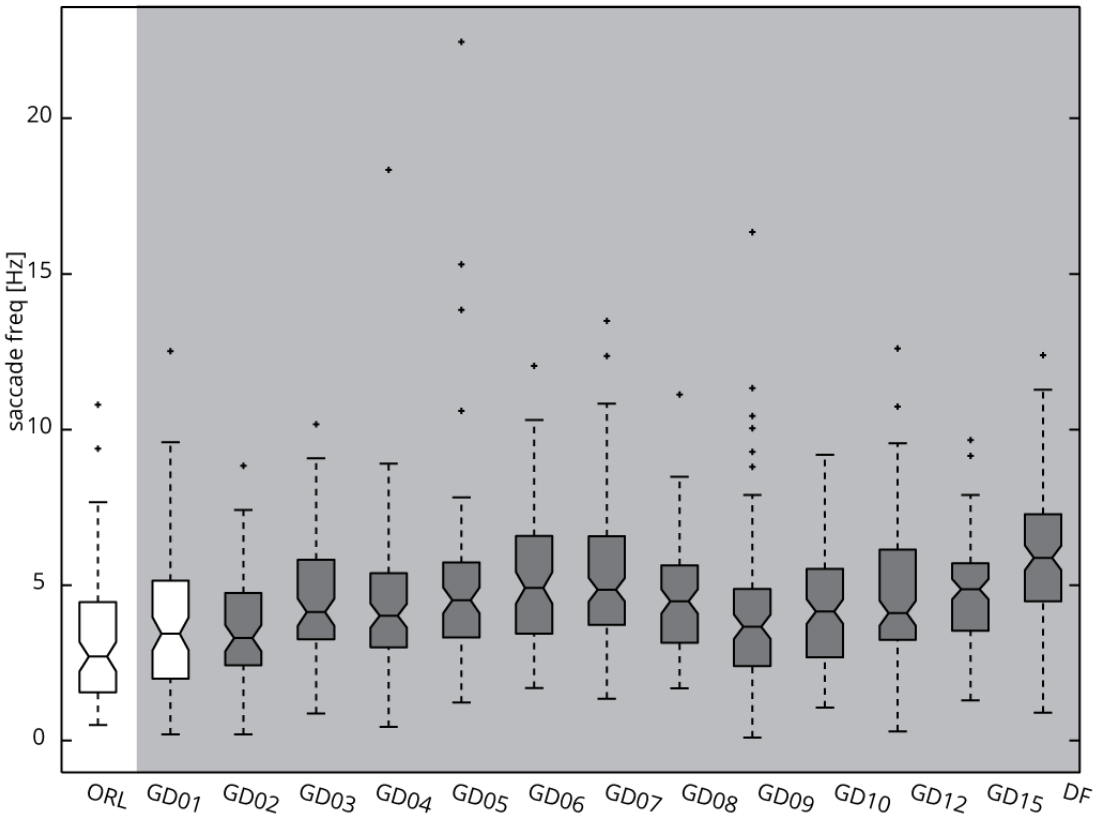
Appendixl figure 2 Thrust bout duration. Medians: ORL 0.365 s; GD01 0.309 s; GD02 0.303 s; GD03 0.227 s; GD 04 0.256 s; GD05 0.2599 s; GD06 0.1952; GD07 0.191 s; GD0 8 0.47 s; GD09 0.269 s; GD10 0.228 s; GD12 0.186 s; GD15 0.198 s; DF 0.1548 s



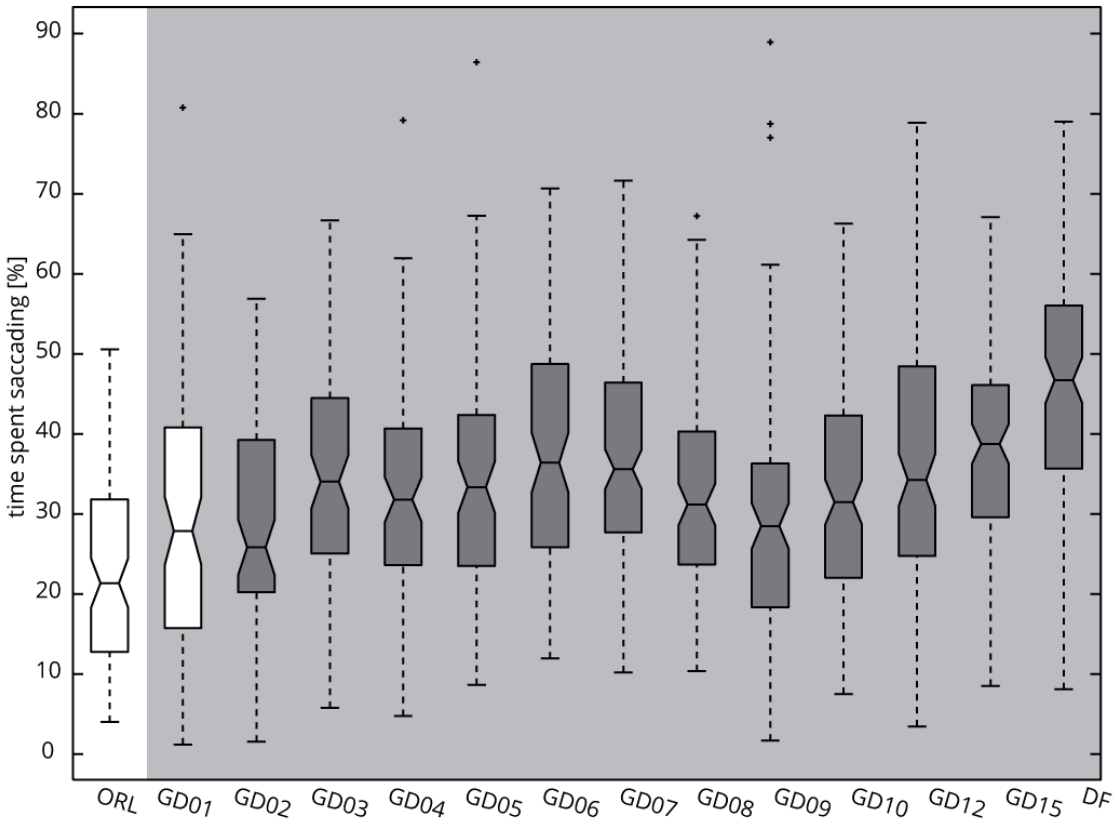
Appendix figure 3 Saccade durations. Medians: ORL 0.078 s; GD01 0.082 s; GD02 0.081 s; GD03 0.078 s; GD04 0.076 s; GD05 0.073 s; GD06 0.074 s; GD07 0.071; GD08 0.072 s; GD09 0.077 s; GD10 0.076 ; GD12 0.081 s; GD15 0.08 s; DF 0.078 s.



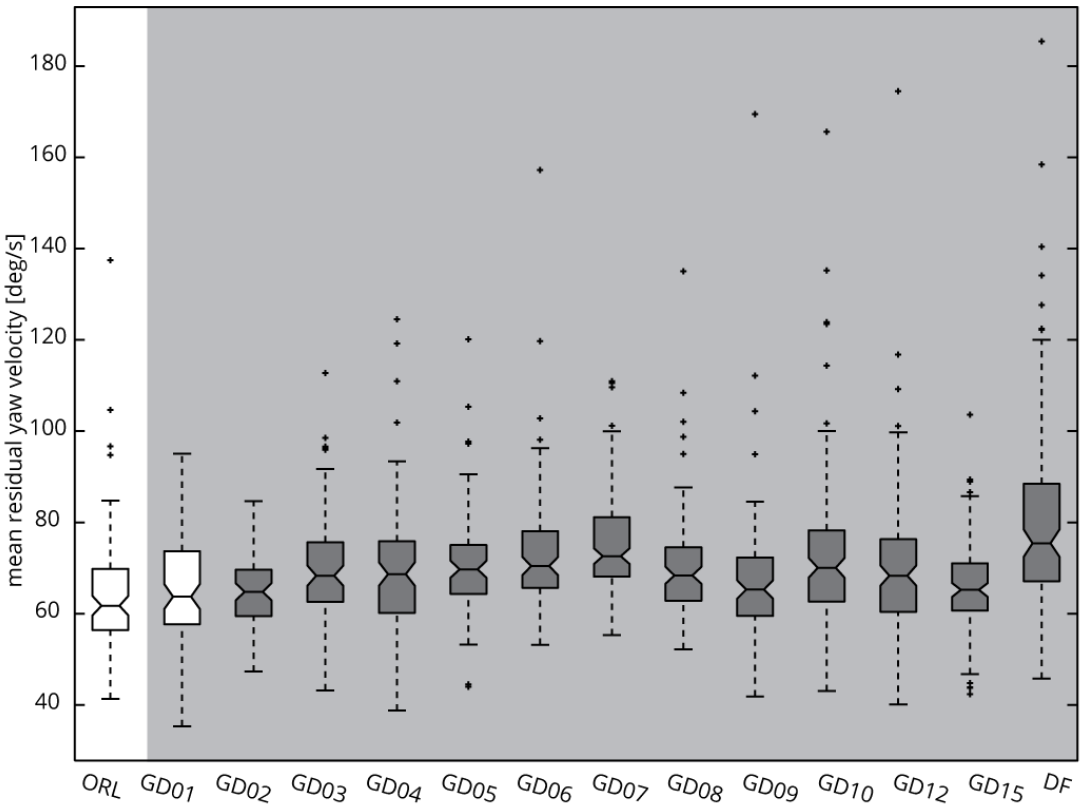
Appendix figure 4 Saccade amplitude. Medians: ORL 298.8 deg/s; GD01 322.82 deg/s; GD02 314.897 deg/s; GD03 318.6 deg/s; GD04 328.39 deg/s; GD05 316.46 deg/s; GD06 316.15 deg/s; GD07 312.581 deg/s; GD08 304.32 deg/s; GD09 302.86 deg/s; GD10 326.56 deg/s; GD12 339.31 deg/S; GD15 342.51 deg/s; DF 345.14 deg/s



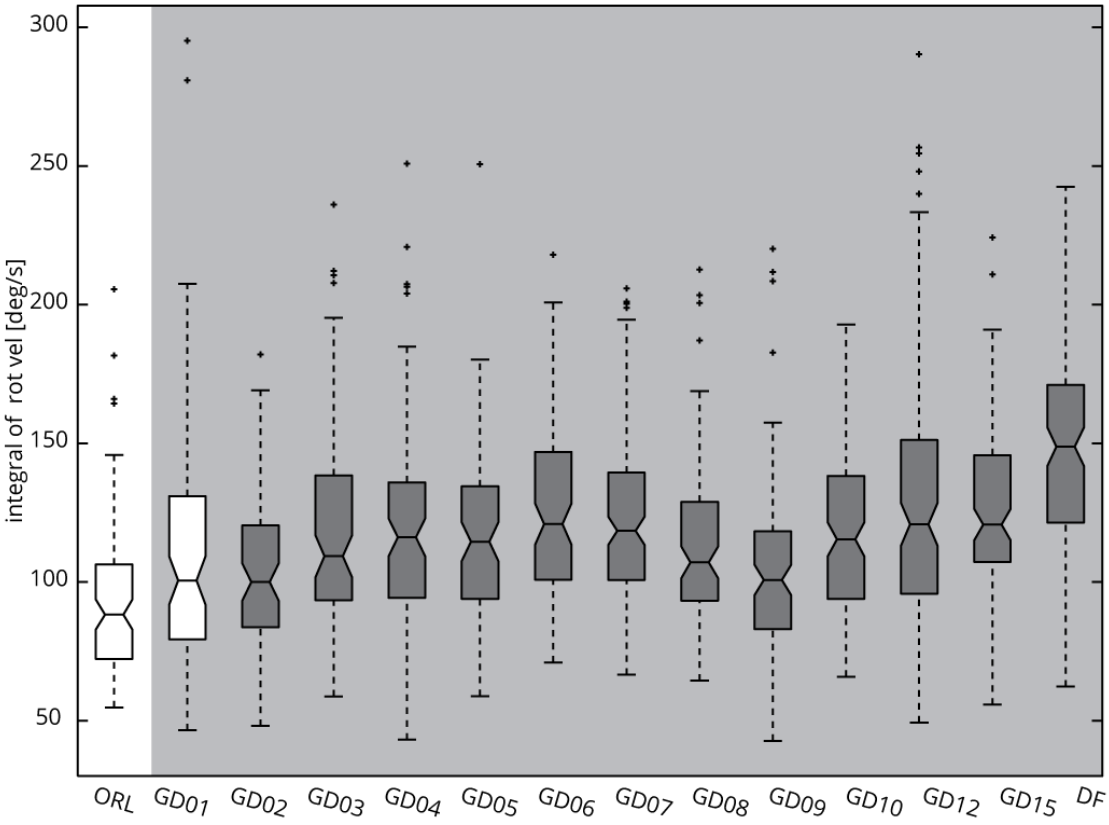
Appendix figure 5 Saccade frequency. Medians: ORL 2.7 hz; GD01 3.43 hz; GD02 3.29 hz; GD03 4.13 hz; GD 04 4.02 hz; GD05 4.53 hz; GD06 4.92 hz; GD07 4.85 hz; GD08 $.48 hz; GD09 3.66 hz; GD10 4.15 hz; GD12 4.09 hz; GD15 4.87 hz; DF 5.87.



Appendix figure 6 Time spent saccading. Medians: ORL 21.37 %; GD01 27.87 %; GD02 25.84%; GD03 34.03 %; GD 04 31.79 % GD05 33.36 %; GD06 36.42 %; GD07 35.58 %; GD08 31.18 %; GD09 28.49 %; GD10 31.48 %; GD12 34.27 %; GD15 38.76 %; DF 46.72 %



Supplemental figure 7 Residual yaw. Medians: ORL 66.7 deg/s; GD01 63.75 deg/s; GD02 64.79 deg/s; GD03 68.31 deg/s; GD 04 68.67 deg/s; GD05 69.73 deg/s s; GD06 0.1952; GD07 70.43 deg/s; GD08 68.39 deg/s; GD09 65.29 deg/s; GD10 70.03 deg/s; GD12 68.34 deg/s; GD15 65-19 deg/s; DF 75.44 deg/s



Supplemental figure 8 Integral of rotational velocity. Medians: ORL 88.26 deg/ss; GD01 100.55 deg/s; GD02 99.97 deg/s; GD03 109.28 deg/s; GD 04 116.09 deg/s; GD05 114.67 deg/s; GD06 120.88 deg/s; GD07 118.48 deg/s; GD0 8 107.08 deg/s; GD09 100.75 deg/s; GD10 115.41 deg/s; GD12 120.78 deg/s; GD15 120.67 deg/s; DF 148.74 deg/s

A3 *p*-values of locomotion characteristics

Table 6 *p*-values for concurring *Goe-dark* strains. Abbreviations: Th vel thrust velocity; Th dur thrust duration; sac du saccade duration; sac amp saccade amplitude; sac freq saccade frequency; T sacc time spent saccading; rot vel mean residual yaw velocity; sum integral of rotation velocity

	Th vel	Th dur	Sac dur	Sac amp	Sac freq	T sacc	rot vel	sum
	ORL	ORL	ORL	ORL	ORL	ORL	ORL	ORL
GD01	0.05164	0.22694	0.06480	0.00490	0.04566	0.02253	0.19906	0.00527
GD02	0.04644	0.08669	0.08357	0.02503	0.03924	0.03739	0.07735	0.01055
GD03	0.00013	0.00023	0.48110	0.00273	0.00021	0.00017	0.00051	0.00017
GD04	0.00013	0.00234	0.09800	0.00041	0.00021	0.00017	0.00158	0.00017
GD05	0.05639	0.00057	0.00179	0.03185	0.00021	0.00017	0.00030	0.00017
GD06	0.00013	0.00023	0.02099	0.01962	0.00021	0.00017	0.00030	0.00017
GD07	0.00013	0.00070	0.00021	0.07234	0.00021	0.00017	0.00030	0.00017
GD08	0.00013	0.01468	0.00038	0.36274	0.00021	0.00017	0.00051	0.00017
GD09	0.45425	0.00023	0.13081	0.0782	0.02012	0.00816	0.02597	0.00569
GD10	0.00013	0.00023	0.22065	0.00041	0.00035	0.00017	0.00030	0.00017
GD12	0.00013	0.00023	0.06651	0.00041	0.00021	0.00017	0.00083	0.00017
GD15	0.00013	0.00023	0.06267	0.00041	0.00021	0.00017	0.01494	0.00017
DF	0.00013	0.00023	0.44317	0.00041	0.00021	0.00017	0.00030	0.00017