OPTOGENETICS AND BEHAVIOR: THE EFFECT OF RED LIGHT EXPOSURE ON THE COURTSHIP EXPOSURE ON THE COURTSHIP BEHAVIOR IN *DROSOPHILA MELANOGASTER*

By

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ABSTRACT

Optogenetics is a powerful piece of technology that is highly utilized in neuroscience research. It makes use of light and channelrhodopsin proteins to remotely manipulate neuronal circuits and behavior of organisms *in vivo*. Ecdysis Triggering Hormone (ETH) is a peptide hormone that is produced by endocrine Inka cells in arthropods. Previous work showed that ETH injection inhibits courtship behavior in male *Drosophila melanogaster*, indicating a reproductive role in adult *Drosophia melanogaster*. The goal of this study is to use optogenetic manipulation to test the hypothesis that elevation of endogenously released ETH likewise inhibits courtship behavior. That is done using red light exposure at a wavelength of ~600nm and a dose dependent duration. That allows cuticle penetration, which should elevate endogenous ETH levels using light-gated CS-Chrimson channels. Red light entry into the cell causes the channel to open and an influx of Na+ and Ca+, depolarizing the Inka cell and activating vesicular exocytosis of ETH. This exposure will be done on progeny of GAL-4 ETH x UAS CS-CHRIMSON and we will focus on the male behavior in this study. We will have two controls that consist of a female Canton S and male GAL-4 or UAS respectively. Results will be measured by placing the male cross line x female Canton S. They will be allowed to court for 10 minutes and video recorded. These videos will then be scored by recording expressed courtship behavior and calculating the courtship index (CPI). While previous studies in the Adams lab show inhibition of courtship behavior by the injection of ETH, this approach is less invasive and allows physiological levels of ETH. but is expected to provide similar results. This knowledge of optogenetics is important to understand organisms' behavior in a less invasive methodology to genetic manipulation of behavior and its further uses in neuroscience research.

ACKNOWLEDGMENTS

I would like to thank my faculty mentor and primary investigator, Dr. Michael Adams, for his continued support throughout this project, in the lab, as well as in my academic career over the past 3 years. I would also like to thank my lab peers Ramtin Ghafoori and Cameron Zappetta for explaining many concepts to me and keeping me motivated. Finally, I would like to thank my partner throughout this project, Aiden Bryan, for dedicating countless hours with me in the lab for the success of this project, even when I was unable to.

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INTRODUCTION

Drosophila melanogaster, commonly known as the vinegar fly or fruit fly, has been a model for neuroscience for decades. That is due to our great knowledge of its neurobiology and its ability to reproduce quickly, while allowing for genetic manipulation. 60% of *Drosophila* DNA is homologous to humans (Mirzoyan, 2019), making it advantageous as a model organism for understanding human physiology and pathophysiology.

Optogenetics refers to use of light and biological elements for remote manipulation of target neuroendocrine cells. The National Institutes of Health (NIH) defines it as a technology that allows targeted, fast control of precisely defined events in biological systems as complex as freely moving animals (2011). In simpler terms, it is the ability to target and control the functioning of a specific cell, in our case the endocrine Inka cell and source of ETH, using light. It's a revolutionary piece of technology used in neuroscience and neurobiology. It is especially useful for behavioral studies due to its ability of application in-vivo, preserving the animals' life and allowing for the measurement of certain behaviors. This technology has found wide applications in studying various aspects of brain function, including sensory processing, motor control, learning, and memory (Fenno et al., 2011).

In this study, our aim is to combine our knowledge on *Drosophila* and application of optogenetics to understand the effect it has on courtship. Ecdysis Triggering Hormone (ETH), a peptide hormone produced by endocrine Inka cells in arthropods, has been implicated in modulating courtship behavior in male fruit flies. Previous studies have shown that exogenous administration of ETH inhibits courtship behavior, suggesting its role in regulating reproductive activities in adult flies (Meiselman et al., 2022). Based on

that knowledge, we hope to understand the effect of red light on release of endogenous ETH, and its effect on courtship behavior on the fly. We hypothesize that usage of red light in transgenic flies will increase release of endogenous ETH, which may inhibit courtship behavior and will cause our courtship index to decrease rapidly.

The aim behind this study is to understand the possible application of optogenetics in different ways. By manipulating the activity of specific neuronal populations in behaving animals, researchers can elucidate the causal relationships between neural activity patterns and behavior (Yizhar et al., 2011). The understanding of the effect of red light exposure on courtship and the underlying neural mechanisms, can provide invaluable insight into potential applications into human studies in patients that suffer from neurobehavioral, neurological, or psychological disorders.

BACKGROUND

My study is focused on the elevation of endogenous ETH using optogenetic, red light exposure, during the reproductive period of male drosophila. By combining the principles of genetics and optics, researchers can now remotely manipulate neural circuits with unprecedented accuracy and spatiotemporal resolution. (Boyden, 2015)

A crucial aspect of this study is using Channelrhodopsin (ChR1), a light-gated cation channel. This opsin protein is what allows for the targeted control of the cell. Retinal, which binds to the opsin proteins, is naturally produced by the body to promote visual transduction. We used ChR2, which meant that at light exposure, ion channels open, resulting in sudden depolarization and excitation, making neurons more likely to fire action potentials (excitation). ChR1 and ChR2 are the more common variations, however, researchers tend to use ChR2 more frequently as it allows for a more accurate response time, with minimal time delay between the exposure or removal of light and the activation of the opsin. We will be using ChR1 present in CS-Chrimson in this experiment.

In recent years, researchers have been using optogenetic techniques to manipulate *Drosophila* behavior in order to understand the neural basis of many complex behaviors. By expressing light-sensitive proteins in specific neuronal populations, researchers have been able to modulate locomotor activity, feeding behavior, social interactions, and courtship behavior in fruit flies (Inagaki et al., 2014; LeDue et al., 2016). Ecdysis Triggering Hormone (ETH) is the peptide hormone produced by endocrine Inka cells in arthropods, including *Drosophila melanogaster* (Figure 2). The primary role of ETH during juvenile development is regulation of ecdysis, in which the fly sheds old it's cuticle at the end of each larval stage

as it grows and advances to the adult stage, as seen in Figure 1, which demonstrates the full life cycle of *Drosophila*. However, researchers found that ETH production and secretion doesn't stop after eclosion to the adult stage; rather, it persists into adulthood, but the reasons were unknown at first. Meiselman et al. (2022) demonstrated that exogenous administration of ETH inhibits courtship behavior in male fruit flies, indicating its role as a modulator of reproductive activities in adult flies. With that knowledge, we were able to put that to the test by inhibiting courtship behavior by "remotely" elevating endogenous ETH levels on the fly.

A significant difference in this study compared to previous work is our ability to elevate endogenous ETH levels without using pharmacolical approaches such as administering exogenous ETH through injection. These previous approaches provided the basis for my study, however, they were highly invasive and could potentiall manipulate the behavior of the fly due to the injury associated with injection as well as using physiologically excessive amounts. We are using optogenetics, which is less physically invasive on the fly and more chemically dependent mechanism as shown in Figure 3. It works by allowing the red light to infiltrate the cuticle and open the light gated ion-channel (Chr1), which causes Na+ & Ca+ to enter the cell, activating vesicular exocytosis and release of ETH from the Inka cells.

MATERIALS & METHODS

For this experiment, we used fruit flies, *Drosophila melanogaster*. We used multiple lines that were all purchased from Bloomington Drosophila Stock Center. The lines used are: GAL-4 ETH, UAS CS-Chrimson, Canton S. All the lines were maintained in stock and contamination error was prevented. The UAS CS-Chrimson expresses a red shifted channelrhodopsin. GAL-4 ETH expresses GAL-4 under control of regulatory sequence from ETH neuropeptide solely in Inka cells. The Canton-S line is a wild type obtained from the lab of Anupama Dahanukar at the University of California, Riverside.

Making of the Cross and Controls:

In order to successfully express the CS-Chrimson in ETH producing neurons, we created a cross line that consisted of GAL-4 ETH that had the promoter, and the UAS effector line, which included the CS-Crimson transgene (Figure 4). This allowed us to create a fly line that releases ETH from Inka cells when the opsin (channelrhodopsin) is activated using the red light. The promoter we used is unique to the Inka cells, which allowed cell-specific expression of GAL-4 expression solely in these cells. The progeny then created allows for GAL-4 expression in the Inka cells and consequently for UAS-CsChrimson expression.

We created 4 different groups, 3 controls and 1 experimental group. Control groups allowed us to assess behavior that is due to optogenetic manipulation and dismiss non-specific effects that could arise. Two of our controls were simply missing one or the other, ChR1 or ETH promotor. The controls were (1) GAL-4 male x Canton S Female and (2) UAS male x Canton S Female. The purpose of this comparison was to see how expression is

affected on a genetic level. The other control group used the cross made (GAL-4 ETH x UAS CS-Chrimson). However, despite this group containing both gene expression and ETH promotor, it was lacking the activation factor, which is the red light. This line was a direct comparison with the experimental, which was the cross progeny ((GAL-4 ETH x UAS CS-Chrimson) Male x Canton S Female). One of the groups was not exposed to the light set up, and the other was. It is important to note that the flies need to be of similar age for each stage, hence why we collect pupae and cross adults at around the same time at 4-6 days post-eclosion.

Experimental Set-Up:

As for the actual set-up, the purpose was very similar for control and experimental groups. The flies are always collected as pupae, and checked daily in order to log the day of eclosion, since we do these experiments 4-6 days after eclosion to allow for full reproductive maturity. For all 3 control groups, we extracted adult pupae (4-6 days old), and transferred them into courtship chambers (Figure 5). The two control groups missing Chr1 or the promotor, they'd be placed under the red light for 60 minutes, which is at ~600 nm. Red light has sufficient penetration depth to reach Inka cells, allowing for ETH release *in vivo* (Inagaki et al., 2014). The two control groups are then placed under a SONY HDR-XR150 camera set-up and the chambers are then opened and courtship behavior is recorded for 10 minutes. The chambers are then cleaned with ethanol and cotton swabs prior to subsequent experiments..

For the second experiment, the cross groups are used. However, one of the groups (control) will not be placed under the red light set-up (Figure 5). Rather, it's immediately

placed under camera, chambers opened, and courtship is recorded for 10 minutes, then the chamber is cleaned again. Finally, for the experimental group, after placing in the chambers, they were placed under the red light LED set up for 60 minutes. Then, they will be transferred to the recording room, where their chambers will be opened allowing them to court over 10 minutes and be recorded.

Scoring and Data Analysis:

Although behavior can be very variable, there are certain behaviors that these flies will do to court and copulate (Figure 6). After obtaining all the recordings, we used a single blind technique to "score" the videos presented with minimal personal bias. Scoring is a form of behavioral assay in which we were able to calculate the change in the fly's behavior based on the stimulus, light in this case. The scale used was 10 minutes, or 600 seconds. The videos were analyzed by taking note of when and for how long the male cross would perform any courtship behavior (Figure 6). Once all 10 minutes were completed, we'd note whether successful copulation, mating, occured between the pair. The time recorded is then summed and the courtship index is calculated and recorded using the following formula:

CPI (%)= total courtship activity time (seconds)/ total time in chamber (seconds)

RESULTS & DISCUSSION

By having multiple controls, we were able to draw several conclusions. To start, having the control group that doesn't have the cross (Gal-4 ETH x UAS) which expressed the CS-Chrismson gene, allowed us to display a baseline of what a courtship index would be (Table 1). To build on that, we tried another form of control, in which we still used the cross, but one set was exposed to red light for 60 minutes and the other set had the gene, it wasn't activated using red light, which kept it dormant and didn't affect ETH levels (Table 2). What we observed was that in all controls, the courtship index (CPI) was significantly higher and copulation was successful in the 10 minutes alotted time frame. However, this wasn't the case in the experimental group, which was the (Cross (M) * Canton-S (F)) that was in fact exposed to the red light and the CS-Chrimson gene was expressed and activated. That supports our hypothesis, in which light exposure and the activation of the light-gated ion channel elevated the levels of ETH, which resulted in an inhibition of courtship behavior in male flies.

Despite observing some form of CPI, it was very low compared to the control, other observations made include unsuccessful copulation in that group. It is also worth noting that in some cases we also observed the male actively escaping the female when an attempt of courtship was made by the female. This was not the case for the flies that were (1) not a cross with an expression of CS-Chrimson or (2) not exposed to light. The difference in CPI % can be attributed to the fluctuation of ETH and its role in reproductive modulation, which can be visualized in Graph 1.

Despite our best ability to obtain data for this study, it is important to understand that there are some limitations that could be worked on and will later be discussed in

Future Directions. Those limitations include the use of strictly behavioral mechanisms instead of immunohistochemistry and enzyme immunoassays (EIA) to measure the quantitative change in ETH and be able to quantify the effects of the light on the elevations, which could help understand other aspects such as dose dependence based on frequency and length of exposure.

FUTURE DIRECTIONS

This project is one of many ways optogenetics is used in neuroscience and behavioral studies. This research builds on the original study where ETH was injected into the fly, which is relatively more invasive as (1) injections stress the fly which could affect behavior in studies and (2) the injected dosage was higher than the naturally occurring amounts hence not ideal for drawing conclusions. Future research could look at different ways to refine the use of optogenetic tools. Development of an opsin protein that is more light sensitive and cell-type specific could be very beneficial for result isolation in many more precise studies before they can be used in human trial research.

However, another importance of future studies is understanding the direct correlation of ETH on courtship behavior and if the same applies to other species. By understanding how these organisms interact and court, we can conclude their method of reproduction and control to some extent. For instance, if we can understand how to control reproduction of some insects that carry vector-borne diseases, we can maintain population control in small amounts and proactively work on public health issues such as disease breakouts and epidemics. Future research can also transfer to different genetic models such as zebrafish and rodents, which would allow a more intimate understanding of the applicability to humans. Optogenetics, if used to its full extent, holds a very important role in understanding neurological disorders that occur due to problems in neural circuitry. It can allow for a more therapeutic approach to the management and possible cure for diseases that affect behavior and normal brain function such as mood disorders, autism spectrum disorders, trauma…

CONCLUSION

In conclusion, using the results from this data, we were able to demonstrate the importance of ETH in modulating courtship behavior in male *Drosophila melanogaster*. We did so by targeting endocrine cells in the fly responsible for production and release of the hormone resulting in elevation of the hormone concentration in the hemolymph. That resulted in successful inhibition of courtship behavior compared to our controls. This confirms the role of ETH in reproductive activity in *Drosophila*. These findings align with our original hypothesis and allow for an understanding of the neurobiological relationship of light and behavior. This allows for future research in the field of optogenetics as discussed above.

FIGURES

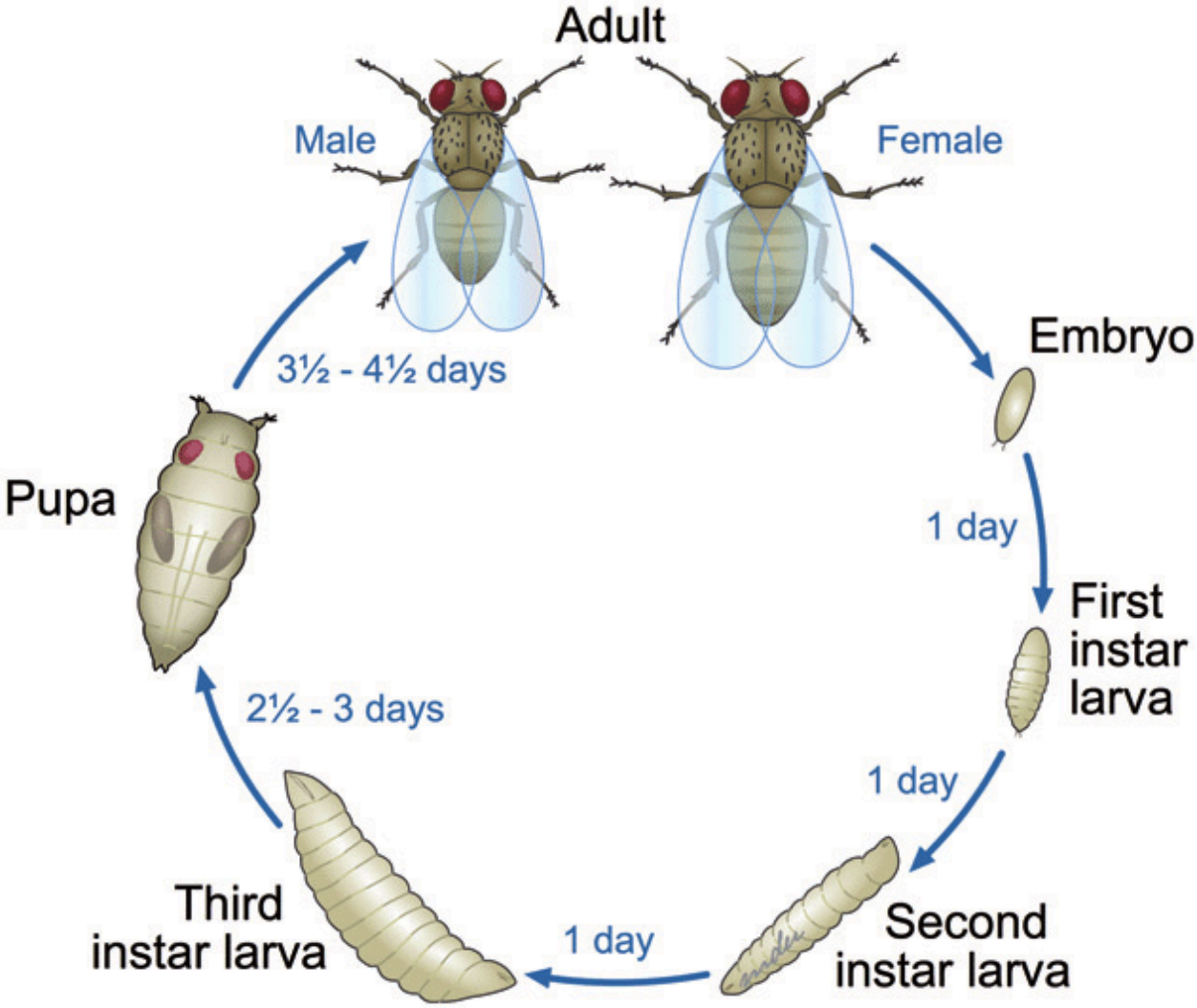


Figure 1. *Drosophila melanogaster* life cycle.

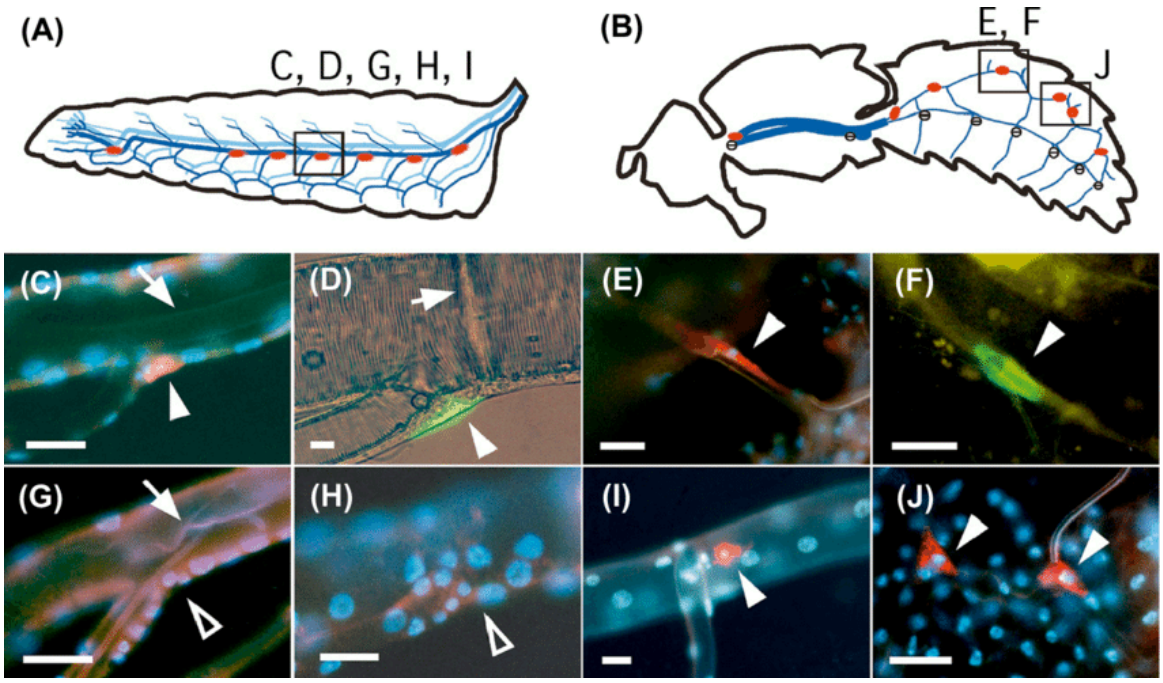


Figure 2. Drosophila Inka Cells.

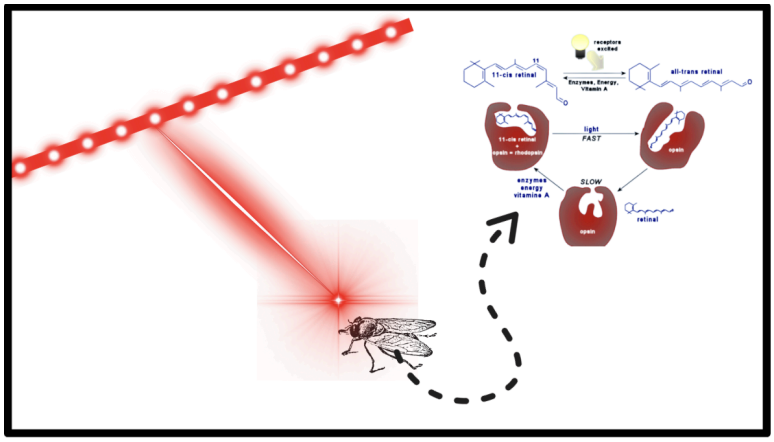


Figure 3. Optogenetics. Demonstrated above is the method in which the light acts on the neuron allowing it to affect ETH production and secretion.

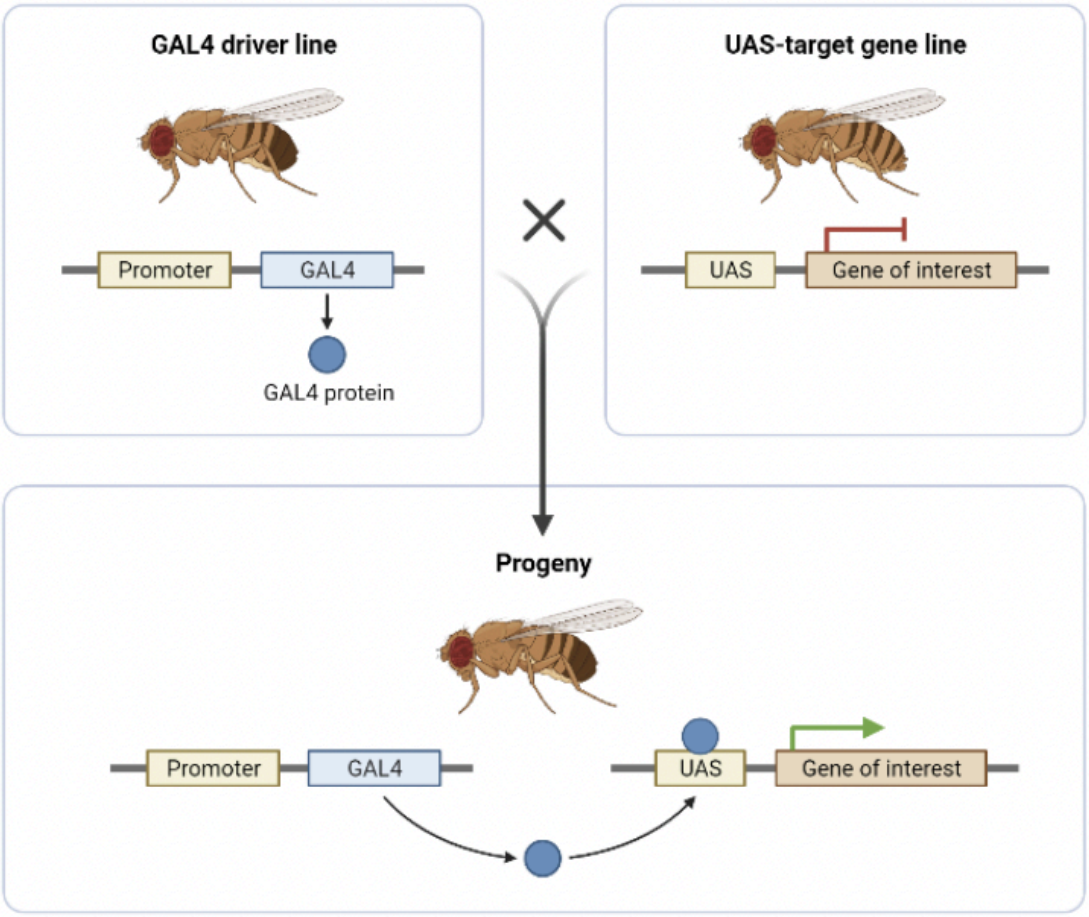


Figure 4. Experimental Cross. In order to make the cross that expresses the CS-Chrimson gene that responds to light, we mated GAL4 driver line and a UAS target gene line and used their progeny in our experimental group.

Figure 5. Physical Set-up: The right figure shows the courtship chambers that were used throughout the experiment and the left figure is the light set-up in which the flies were kept for an hour before recording.

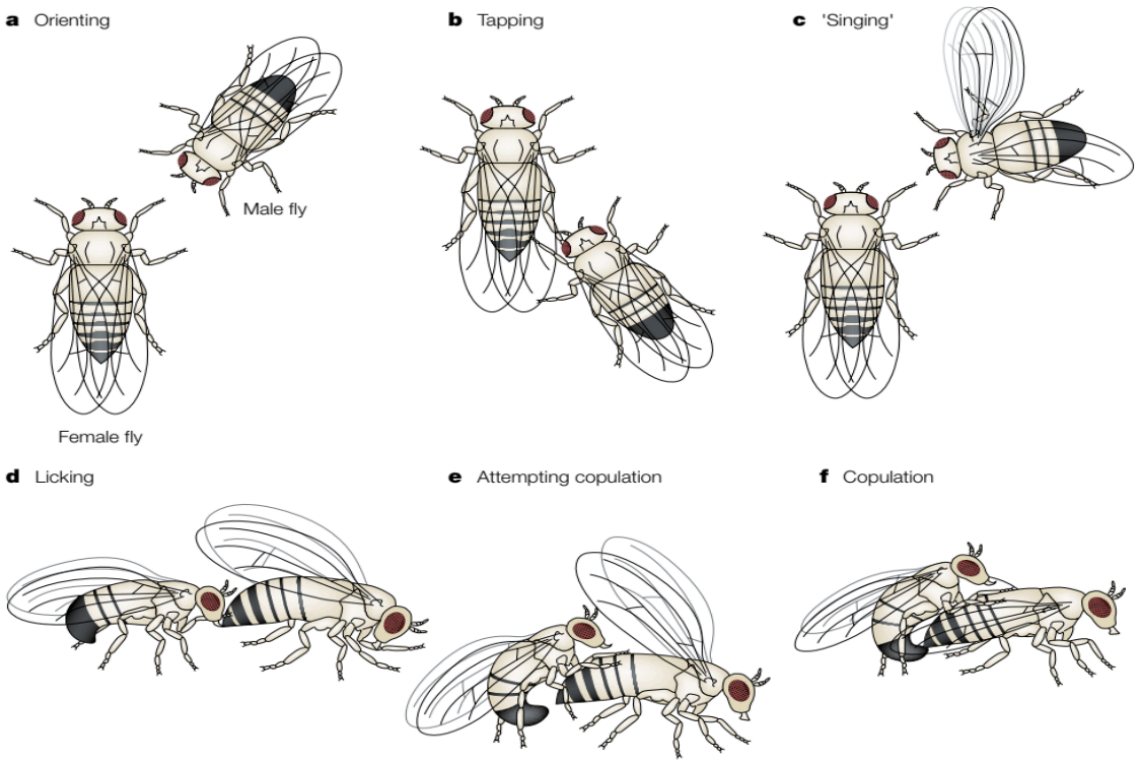


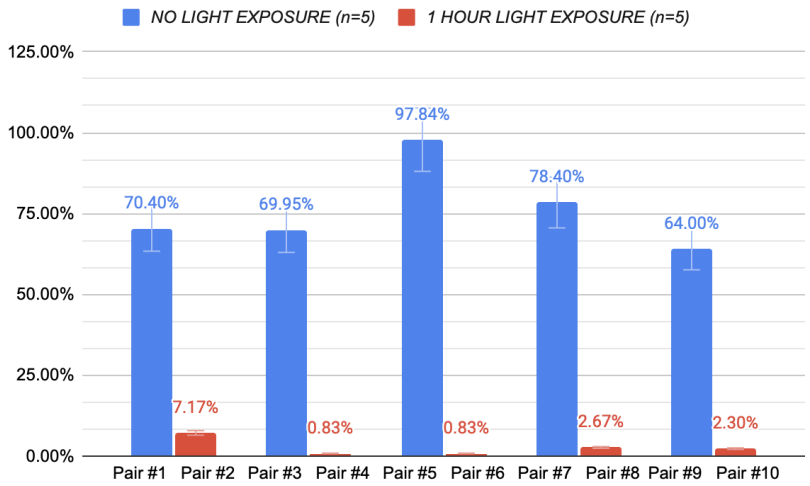
Figure 6. Courtship Behavior. The figures demonstrate the behavior exhibited by the male fly that we "count" as courtship behavior. There are 6 measurable types of this behavior that are later summed and used to calculate the courtship index (CPI).

CONTROL EXPERIMENTS			
UAS (M) * CANTON S (F)		***GAL- 4 (M) * CANTON S (F)***	
Courtship Index (CPI)	**Successful Copulation?**	**Courtship Index (CPI)**	**Successful Copulation?**
50.50%	Yes	50.80%	Yes
50.20%	Yes	56%	Yes
71.40%	Yes	56.20%	Yes
62.80%	Yes	25.57%	Yes
46.50%	Yes	87.30%	Yes

Table 1. Control Experiments. (no CS-Chrimson expression or light exposure).

Light Exposure Experiment *(CROSS(M) * CANTON S (F))*			
NO RED LIGHT EXPOSURE		***1 HOUR RED LIGHT EXPOSURE***	
Courtship Index (CPI)	**Successful Copulation?**	**Courtship Index (CPI)**	**Successful Copulation?**
70.40%	Yes	7.17%	No
69.95%	Yes	0.83%	No
97.84%	Yes	0.83%	No
78.40%	Yes	2.67%	No
64.00%	Yes	2.30%	No

Table 2. Light Exposure Trials.ETH transcription precursor and CS-Chrimson ChR1 present.



Graph 1. Light Exposure Experiment results in a bar graph format that allows a visual of the effect of an increase of ETH on courtship and provides a comparison between males that were exposed to red light and those that were not exposed to red light.

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