Optogenetic replacement of Odor stimuli in Drosophila group conditioning experiments

Max Planck Institute for Neurobiologie and
Ludwig-Maximilians-Universität München

MAX-PLANCK-GESELLSCHAFT

Master Thesis for physics by Marc Eppler

6th of December 2016

Optogenetic als Ersatz für Duft bei langzeit-stimulations-konditionierungs-Experimente in Drosophila

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6ter Dezember 2016

Optogenetic replacement of Odor stimuli in Drosophila group conditioning experiments

Developed in the Lab of and supervised by
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Chemosensory coding and decision-making

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7. November 1988

Handed over at the 6th of December by Marc Eppler

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Abstract

Optogenetics is one of the most powerful recenty discovered tools for the field of Neuroscience and does represent very well the interdisciplinary way modern research is more and more conducted. While replacing traditional way of doing experiments and bringing further possibilities to it, there are also difficulties occurring by the artificial nature of inducing signals into a living model organism. Specially when trying to reconstruct behavior to explore the underlining complexity of the brain it is not trivial to get the the complex circuits within the brain to recognize the stimulus in a way that makes contextual sense and then perform tasks like learning and forming conditioned memories. This work explores a way to have groups of the model organism *Drosophila melanogaster* being conditioned by imitating an odor stimulus with optogenetics over longer periods of time, which always has been a problem with real odor due to its invisible and hard to control dynamic nature. Therefor a chamber is built in which the stimulus is equally illuminating and can be precisely controlled in pulse durations and rate. As read out and calibration tool a complex optogentics arena has been used and it was found that for different kind of receptor neurons, different specific stimulus dynamics are required to have an behavioral relevance. In the larger context of a resulting long-therm conditioning experiment, this advantages has been used to combined it with a also long-therm stimuli of induced sickness which also has been developed. When reaching full functionality the experiment could answer a gang of interesting questions about the nature of integrating long lasting bad states as memories and developing behavior from that, in contrast to the way more explored short pain induced stimulus.

Erklärung:

Hiermit erkläre ich, die vorliegende Arbeit selbsändig verfasst zu haben und keine anderen als die in der Arbeit angegebenen Quellen und Hilfsmittel benutzt zu haben. München, 6. November 2016

Contents

1. Introduction

> *"'You', your joys and your sorrows, your memories and your ambitions, your sense of personal identity and free will, are in fact no more than the behavior of a vast assembly of nerve cells..."* —Crick (1994, p. 3)

In an overall effort of understanding the vast assembly of nerve cells that we call brain, many techniques have been developed and progress is accelerating. The goal of the present master project was to use a recently developed technique, optogenetics, and to develop an experimental setup to ask very precise questions about learning and integration of external and internal sensory stimuli. Specifically, the genetic model organism *Drosophila melanogaster* was employed to dissect the neural mechanisms underlying the memory of long lasting negative internal states. To the end, I developed a method that uses optogenetics and stimulation with light to replicate odor stimuli over long periods of time for large groups of flies. These large and precise stimulation periods were important to allow the induction of a state of malaise via bacterial infection, which ideally causes the brain to couple these stimuli and form a memory. This can then be used to test for consistence while knocking out several genes or use imaging techniques to trace the underpinning mechanisms in the brain and compare them to for example the learning circuits required during more classical electroshock conditioning experiments in follow up projects. In olfactory behavioral experiments regardless of the test subject (this includes humans), there always is a struggle of handling the invisible odor, their different molecular weights and the resulting complication and ever changing streaming dynamics. This is why it is critical to develop other means of odor delivery and/or stimulation in a long-term conditioning set up. Ultimately, the motivation of using optogenetics is to produce more stable test conditions and more reliable behavioral results.

1.1. Optogenetics

1979 the Nobel laureate Francis Crick wrote his famous article in Scientific American, "Thinking about the Brain,". One of his three described needed methods to fully interrogate the complexity of the brain was: a method by which

> *"all neurons of just one type could be inactivated, leaving the others more or less unaltered"*
>
> Francis Crick [8]

While calcium sensitive dyes have opened an avenue for noninvasive imaging of neural activity explored 1990 with modern imagine techniques such as the two-photon microscopy [10], it took some 20 more years until, with Karl Deisseroth [9] optogenetics was a established method for noninvasive stimulation and inhibition of neuronal activity

> *"Optogenetics is the combination of optical and genetic methods to achieve gain or loss in function of well-defined events in specific cells of living tissue"*
>
> Karl Deisseroth [9]

.

Since then this method has widely spread and was further developed; and as Crick predicted, revolutionized the field of neuroscience. Up to this time it was only possible to manipulate the neuronal activity with electrical stimulation, pharmacological compounds and imprecise genetic techniques. Where electrical stimulation is able to have precise kinetic resolution, it was difficult to select only one neuron type because of the bad spacial resolution. Pharmacological compounds and genetic approaches where able to target specific neurons but do not provide no kinetic control of neural spiking. Optogenetics where able to combine the advantages of both approaches. Furthermore it is able to keep the influence on a free moving Drosophila as used in this project to only the interested optogenetic stimuli without other disturbing side effects.

Function

Genetic methods where able to change the nature of neurons in making them sensitive to light stimuli, by extracting genes of light activated proteins from the animal kingdom. While a majority of higher developed animals benefit from light sensitive cells in their complex eye structures for sight, microbes have also developed light-activated proteins for several purposes. It does serve as a mechanism of homeostasis to remain at a certain depth under water [7] or helps to balance osmotic pressure in a highly saline environment [28]. This and similar tasks are often realized by a family of seven-trans-membrane, light-responsive proteins encoded by opsin genes.

Opsins are retinylidene proteins and use retinal as a cofactor which is vitamin A related covalently bound to a conserved lysine residue of helix 7 by forming a protonated retinal Schiff base (RSBH+)and starts the photon induced reaction by isomerization. The spectral and kinetic characteristics of the protein are dictated by their ionic environment of the RSB, which is defined by the residues of the binding pocket. They are divided into two super families: opsins from microbial called type I and opsins from animals called type II depending on their origin. Type I is found mainly in prokaryotes, algae and fungi where they function as ion pumps or contribute to phototaxis. Type II are found in higher eukaryotes photoreceptors such as eyes [27]. Even though both opsin families encode seven-transmembrane structures, phylogenetically the two families are not related [25]. Type II opsins are G protein–coupled receptors, initiate a signaling cascade upon activation, and consequently produce slower changes in neural activity than Type I opsins. The type I opsins makes use of the all trans-retinal change to 13-cis retinal upon photon interaction. The rhodopsin complex then initiates its photocycle which ends with the thermal re-isomerization of the retinal back to its ground state [14]. These are the ones used in the first optogenetics experiments bacteriorhodopsin (BR) in 1971 [23] and now halorhodopsin, channelrhodopsin-1 (ChR1) [21] and channelrhodopsin-2 (ChR2) [22] to control neuronal function, both because of the ease of genetic engineering using a single component protein and because of their faster kinetics, and remain the primary (but not exclusive) source for new natural and engineered opsins.

Upon today there have been several opsins discovered and successfully implemented as optogenetic tools. This gives researches the opportunity to choose from different activation light wavelengths and kinetics. Most of them function as light activated ion channels or pumps that either hyper-polarize or depolarize the cell membrane, but recently G-protein coupled functionality has been developed as well where green (500 nm) light activates the biological functions dictated by the intra-cellular loops (OptoXR) [5].

The most common used one is channelrhodopsin-2 (ChR2) which is a blue light sensitive nonspecific cation Ion channel, conducting H+, Na+, K+, and Ca2+ ions, with the optimal excitation wavelength of $\sim$ 470 nm and an on- and off-kinetics at frequencies around 10Hz in wild type and up to 200 Hz for some mutations. [13] [20]

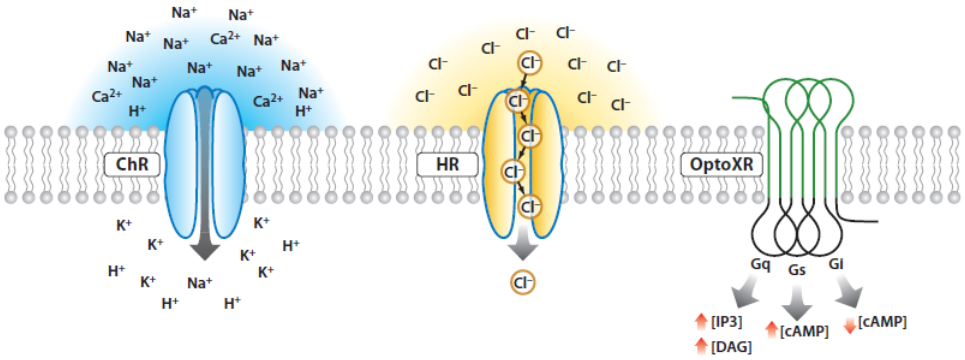

FIGURE 1.1.: Different Optogenetic channels. Channelrhodopsins conduct cations and depolarize neurons upon illumination (left). Halorhodopsins conduct chloride ions into the cytoplasm upon yellow light illumination (center). OptoXRs are rhodopsin-GPCR (G protein–coupled receptor) chimeras that respond to green (500 nm) light with activation of the biological functions dictated by the intracellular loops (right). [11]

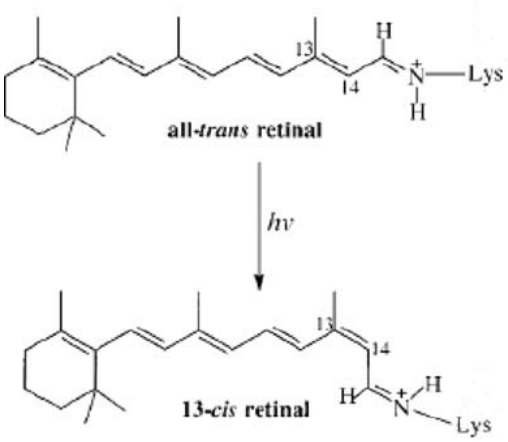

FIGURE 1.2.: The light induced isomerization of all-trans retinal to 13-cis retinal. [24]

CsChrimson is a new red-light red shifted version of the Channelrhodopsin with spectra 45 nm more red-shifted than any previous Channelrhodopsin. Having its optimal excitation wavelength around 620 nm, it enables deeper tissue penetration due to the physical properties

of longer wavelengths. Most importantly for experiments on living Drosophila is that 620nm is outside of their visual range, so that there is no visual bias in behavioral experiment. Precisely for that reason, we chose CsChrimson as the optogenetic method in this project. [18]

1.2. Conditioning

The classical model of conditioning is associated with Ivan Pavlov and has the name Pavlovian conditioning. Ivan Pavlov discovered in the 1890's the phenomena of conditioning, conditioned learning which later granted him the recognition with the Nobel Prize (1904). He developed the terms which are still valid today. So pairing an unconditioned stimulus (UCS) which is the object or event that originally produces the reflexive / natural response that is called the unconditioned response (UCR). The neutral stimulus (NS) is a new stimulus that does not produce a response. Once the neutral stimulus has become associated with the unconditioned stimulus, it becomes a conditioned stimulus (CS). The conditioned response (CR) is the response to the conditioned stimulus.

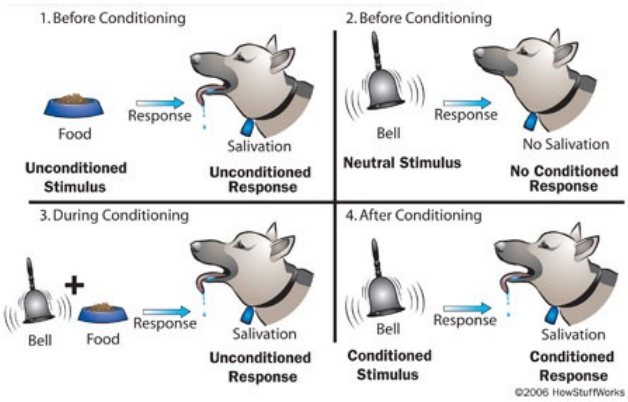

FIGURE 1.3.: The Pavlovian dog showing the conditioning procedure of the famous Pavlovian experiment. Where presenting food is the US, the salivation because of this is the UR, ringing the belle without food is a NS and the conditions salivation response after ringing the belle without having food present is the CR.

[3]

This mechanism is widely used in the field of learning and has been translated to all kinds of animals and also humans and greatly facilitating our understanding of earning and memory. In Drosophila this is usually done by using sugar as a positive US, where the UR simply is an attraction towards the food source, or small electroshocks as a negative reinforcing US. It has been shown that this US can be presented in combination with specific odors NS to achieve a conditioning that leads the flies to be attracted or repelled to that specific odor after conditioning, even when presented without the US.
Rescorla Wagner did define a more precise way to calculate the conditioning strength throughout more complex experiments :

$$\Delta V = \alpha\beta(\lambda - \Sigma V) \tag{1.1}$$

Therefore ΔV is the changed associative strength of the CS that happens on a given trial. λ is the maximum associative strength that a given US will support usually 1 on trials when the US is present, and 0 when the US is absent, ΣV is the sum of the strengths of all stimuli present in the situation. α and β are constants related to the salience of the CS and the speed of learning for a given US. [2]

The newly induced aversion towards a taste or a smell when using a negative US is called conditioned taste aversion or conditioned odor aversion and is a technique that is widely used in animals to sort out bad food sources and learn how not to deal with their environment. This is specifically the mechanism we are interested in this project.

2. Materials and Methods

2.1. Experimental Flies for Optogenetics

All flies (*Drosophila melanogaster*) were stored and reared at 25°C and at 60% relative humidity in a 12h/12h light dark cycle as groups of 100-300 flies per 180 ml bottle holding 45ml standard cornmeal fly food medium. The optogenetic flies are stored under blue light 428nm and were fed with food that has an additional 0.4mM all trans-Retinal (cat# R2500-1G Sigma) while they're parent generation and all other flies are stored and reared under white light.

2.1.1. Gal-4/UAS system

Experimental flies for the optogenetic stimulation where created by using the Gal-4/UAS system, crossing 15-20 UAS (Upstream Activating Sequence) CsChrimson virgin flies created with the virginator stock with 50-60 male flies having the specific Gal-4 drivers on normal food. These crosses had been flipped two times, each after 72h into new bottles. The F1-generation that will be the experimental flies where then collected after 10-11 Days three times where the second and third collection stage where pooled together with the earlier ones. These collected experimental flies where then stored on the retinal food for two-four days so that the, for the optogenetics necessary retinal level, can build up until they been used for the experiment.

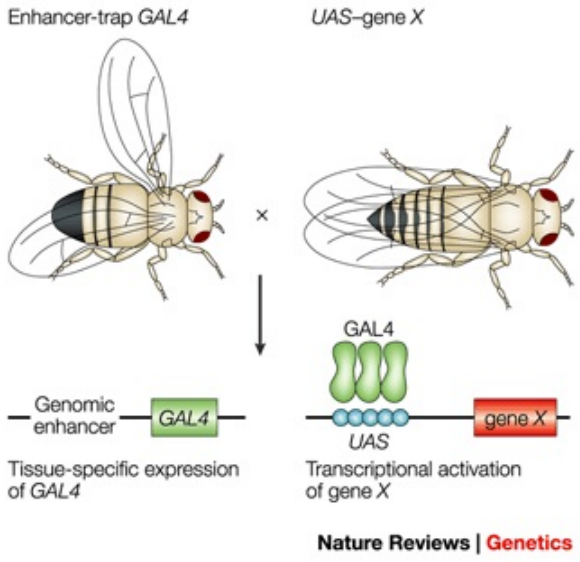

FIGURE 2.1.: Gal-4/UAS system [17]

The flies used as UAS CsChrimson in this thesis are the 20xUAS CsChrimson virginator

flies created by Yoshi Aso into a w+ genetic background. These CsChrimson line has the UAS-trans-gene CsChrimson for the red shifted Channelrhodopsin on the X-chromosome and an additional transgene on the Y-chromosome for the virginator stock.

2.1.2. Virginator Stock

This transgene on "Y" causes lethality on larvae and pupae when expressed under the control of an inducible promoter. In this case, the virginator stock is characterized by the presence of a heat-shock "hid" construct on the Y chromosome. When larvae are subjected to heat shock (37°C. 2-4 hours), the "hid" is overexpressed in males, resulting in lethality leading to easy to collect female virgins only.

2.1.3. Gal4 driver Lines

All Gal-4 driver line transgenes used in the following experiments were inserted into a w- genetic background.

Orco Orco: formerly known as Or83b encodes an odorant co-receptor, it is broadly expressed throughout almost all olfactory neurons.

Gr5a Gr5a neurons encodes for a gustatory receptor that required for the responses to attractive tastants like sucrose, glucose, and maltose. It is mainly expressed in the gustatory receptor neuron in subsets of gustatory neurons on the labellum, legs and wings of the adult animal.

Gr66a Gr66a neurons encodes for a gustatory receptor that is responsible for bitter tastants detection. It is expressed in GR5a negative subsets of gustatory neurons on the labellum, legs and wings of the adult animal.

Or42b Or42b neurons encodes for a olfactory receptor neuron (ORN) and is associated with a flowery smell detection.

Empty Couples with the gal-4 driver but is expressed nowhere in the fly body, and there for a good genetic control.

2.2. Optogenetics Arena

To test the attraction or aversion of the experimental flies to the optogenetic stimuli we used an automated optogenetics arena assay.Figure 2.2 The assay was built by Dr. Laurence Lewis [19] in our Institute, based on the initial design of Dr. Yoshinori Aso at Janelia Research Campus [26], which was based on the original olfactory behavior olfactormeter from 1983 [29] .

The arena was located in the dark, and the experiments where done in a climate chamber with fixed 25°C and 60% humidity. 20-40 of the experimental flies where pulled out of the bottle with a foot pump and inserted in the round 10 cm diameter 3 mm high arena of the assay and been closed with a glass lidFigure 2.3. The system then runs a 30/30/30/30 protocol Figure 2.2, where 30 seconds been there for getting used to the arena, the next 30 seconds the to diagonal quadrants been illuminated from below with "Power SMD LED PLCC-2 Plus" led's at a wavelength of 620-630 that activates the depolarization in the genetically manipulated CsChrimson neurons [18]. The next 30 seconds are to redistribute around the arena with no stimuli and the last 30 seconds has a diagonal quadrants illumination but on the other direction to exclude any spacial bias. One of these runs is then called 1 n. The brightness, power and PWM frequency of the stimulus LED's can be controlled via a "arduino-mega" micro controller that can generate different frequencies and a puls-with-modulation variation in the Matlab code that goes from 0 to 255, where 255 means steady illumination.

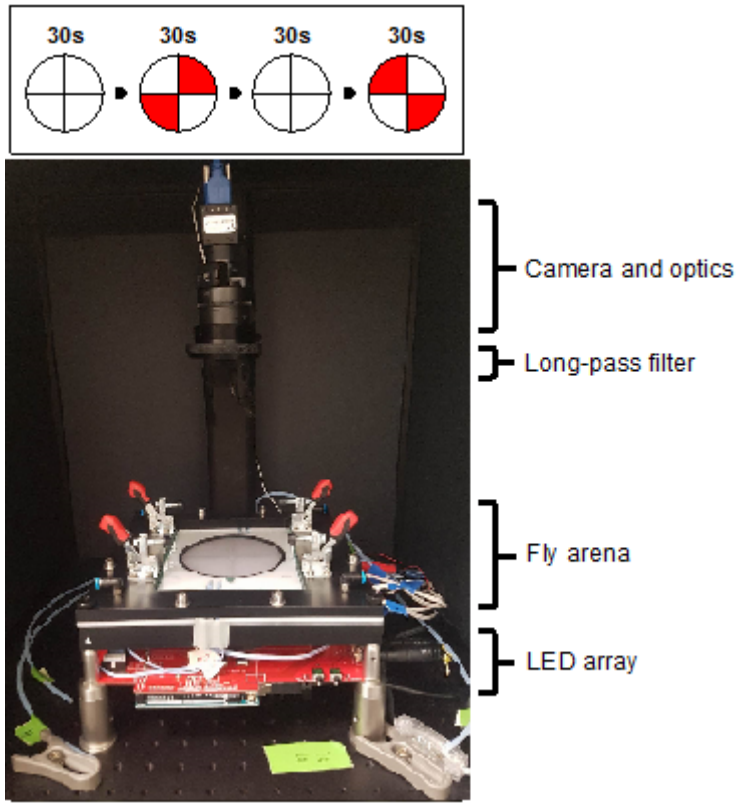

FIGURE 2.2.: Optogenetics arena behavioral assay, The operational protocol used during optogenetics arena testing is represented in the top panel. The bottom panel depicts the arena, filter, and camera arrangement.

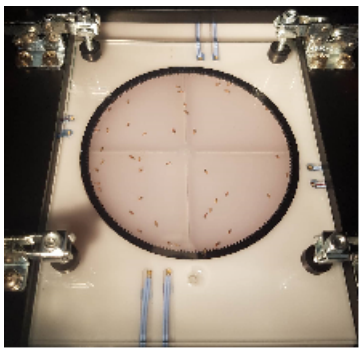

FIGURE 2.3.: Optogenetics arena

2.3. Box and Whiskers plot

To compare the resulting preference indexes from the optogenetics arena Box and Whiskers plots are used displaying 10-90 percentile as lines and out-liners as additional symbols. The middle box shows it's standard deviation and the mean inside of it. Here we are using the average of the last 5 seconds of each stimuli where the flies have orientated already to they're preferred quadrants of the arena to get a good indicator of their behavior. The Box and Whiskers plot is then showing the statistics across the different runs (n). As test for statistical relevant differences between three or more Gaussian experimental groups within one experiment, ANOVA (Bonferroni) is used. Comparison of two Gaussian experimental groups is done with unpaired t-test. These statistical tests are provided by GraphPad Prism 7.02.

2.4. Inducing sickness into groups of Flies

To create the negative internal state of "sickness" within the Drosophila system, some bacteria have been tested for reliability and the potential to make these flies sick to an extend where 10 % to 20 % are dying within the first 24h, to get a large number of assumingly very sick and medium sick flies when considering a Gaussian distribution. The Bacteria that worked best in this context were the Erwinia carotovora carotovora (new name "Pectobacterium carotovorum"), which is gram-negative and of the family Enterobacteriaceae. It has been used to trigger the immune response of *Drosophila melanogaster* before, and will be referred here as Ecc15 from now on [6].

Bacteria

Ecc15 was provided by the Institut de génomique fonctionnelle de Lyon, François Leulier Lab. in two forms:
-Ecc15-pOM1-evf (grow @29°C in LB+ 100μg/mL Spect.) which has an over expression of the viral component of the bacteria and is used to generate the immune response. This bacteria strain will be referred as Pom1 in this thesis.
-Ecc15-Mutant-evf (grow @29°C in LB+ 100μg/mL Rifamp.) where the virulent factors have

been removed so it will function as a control. This bacteria strain will be referred as Mutant in this thesis.

Culturing

The Bacterial stocks were preserved in a 25% glycerol solution at -80°C. Striking out a bit of the bacterial stock on petri dishes with the correlated antibiotics and letting them grow at 29°C for 24 h leads to distinguishable colonies. Adding these colony to 400 ml LB in 4L flasks and shaking them at 200 rpm 29 °C over night. Spun down and washed with PBS by 4000 rpm then spun down again to an OD_{600} of 200 gives the pallet for the feeding solution.

Freezing

For convenience the bacteria pallet was frozen in small handy portions. There for the pallet has been added glycerol instead of PBS until it consists of 15% glycerol and an OD_{600} of 200 to prevent the bacteria from damage. To make the freezing process as fast as possible, the pallet was portioned into screw-able low temperature freezing vials and dropped into liquid nitrogen. The frozen vials are then stored at -80 °C until needed.

Feeding

The OD_{600} 200 pallet mixed 1:1 with 10 % sucrose water gives the solution fed to the flies. 650 μl of the feeding solution getting dropped on a round cut Whatman filter paper that was placed on top of a humidity providing 1 % agarose layer. This feeding assay is designed for 120 flies which been dry starved without water or food in empty standard sized bottles for 6 hours and been selected for females only. Figure 2.4

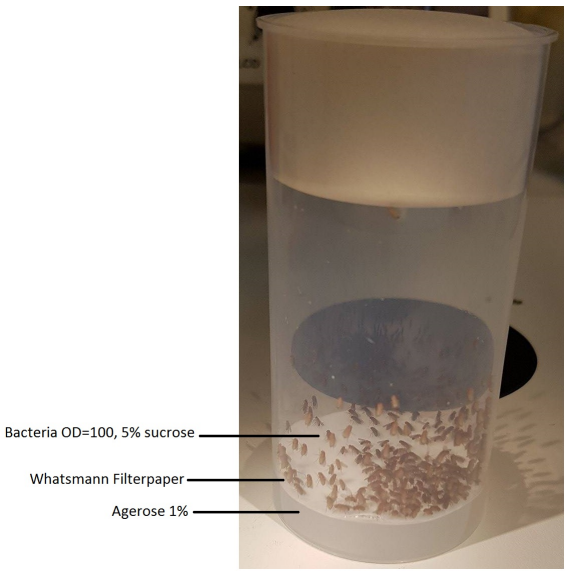

FIGURE 2.4.: Feeding Assay

2.5. Inducing bitter quinine taste

As a different source of negative stimuli to test the optogenetics conditioning chamber a quinine essay is established. The idea is to create a container where the flies are forced to sit on quinine so that even when they are done eating or testing it, they have to stand on the quinine that also induces the bitter taste. This is due the fact that there are gustatory receptor neurons on the feet of Drosophila. To do this, 60mm x 15mm petri dishes (Falcon REF 353004) are filled up with 16 ml of 1% agarose to minimize the surface on the side walls where the flies can hide the quinine. To prevent the flies from avoiding the quinine, the top cover of the petri dishes is coded with Fluon GP1GP1, a fluoropolymer dispersion. The fluoropolymer coated surface then prevents the flies mechanisms to stuck upside down. 0.02g quinine($\geq$ 98.0% 22620-5g Sigma), then is place on top of a Whatman filter paper cut out in a way that it fully covers the agerose. 650 μl filtered water is pulled on top of the quinine and the paste is streaked out over the whole Whatman paper. Figure 2.5 A small hole in the side of the lid is used to insert 50-100 flies via a foot pump and extract them after the conditioning through that hole into a empty fly handling bottle.

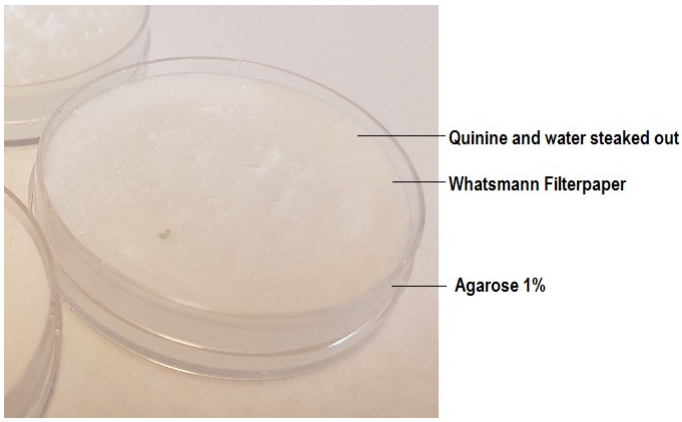

FIGURE 2.5.: Quinine conditioning assay

2.6. Survival test

An easy way to test the fitness of infected flies is to put them into starvation and document how long they can survive. These are done by flipping the infected flies into starvation bottles, which have the same size but instead of food, they contain tissue paper that is soaked with 7ml of filtered water.

2.7. DAM Drosophila Activity Monitor

To get a quantified read out of the induced sickness in the experimental Flies, one tool we used is a DAM (Drosophila Activity Monitor. Specifically the MB5 Multi Beam Activity Monitor bye Trikinetics [4]. The Monitor consists of 16 slots with 17 independent IR beams per slot, detecting activity along 51mm of tube length in 3mm zones.Figure 2.6 Each slot is loaded with a 5mm diameter 75mm long glass tube. Each tube is filled 10mm with the normal fly food and plugged with a black rubber plug to keep the food from drying out. The test fly is placed in the tube while the other end of the tube is closed with a sponge. During the activity monitoring the whole setup is in a temperature and humidity controlled chamber at 29°C and 60% humidity. A white light is installed in the Chamber with the same 12h/12h day night cycle as in the big Chamber where the flies are raised. The IR beams are detecting every crossing of them and counting an overall activity of that tube translating to the activity of that specific fly.

FIGURE 2.6.: Drosophila Activity Monitor. 16 Slots for 5mm diameter glass tubes. Drosophila inside a 5mm diameter glass tubes at the bottom left [4]

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2.8. Dipt-mcherry flies

Another way to evaluate the degree of the induced sickness to the experimental flies is to use the line dipt-mcherry-C1 which expresses the red mcherry fluorescence protein in the gut when the immune response kicks in. This can be made visual under the fluorescent microscope where we used 587nm excitation light to excite the fluorophores and a filter so that the emission light with the wavelength of 610nm will be visible in the captured microscope image.

2.9. Box and Whiskers Plot

To compare the resulting preference indexes from the optogenetics arena Box and Whiskers plots are used displaying 10-90 percentile as lines and out-liners as additional symbols. The

middle box shows 50 % of the data and the mean is a line inside of this box. For the Box and Whiskers plots I use the average of the last 5 seconds of each stimuli for all the runs as the optogenetics arena data. Thereby the flies have orientated already to their preferred quadrant and one gets a very good read out for the preference index. [1]

3. Experiments and Data

3.1. Optogenetics conditioning chamber

To achieve a group conditioning of transgenic Drosophila with optogenetic properties,a conditioning set up was constructed and built, the so called conditioning chamber should be able to illuminate a large enough volume quit equally with high intensity red shifted light to fit the standard bottles used for fly handling in and provide all the flies within the feeding assay with an similar and strong optogenetic activation. The activation of peripheral CsChrimson neurons like the ORN's Olfactory receptor neuron starts at 0.015mW/mm^2 [18]. There fore each of the 4 optogenetics conditioning chambers where designed with 4 powerful wide angle, red LED's LZC-83MC00 by Ledengin with the CsChrimson corresponding wavelength 623nm [18] and a luminous flux of 430lm from. The 90 °radiation angle of the 4 LED's and a all 6 site inside mirroring provides an all over illumination from $>$ 0.06mW/mm^2. This was measured with the power-meter PM100D from Thorlabs.

For powering the conditioning chambers we used a tune able direct current power source (BaseTech Bt-305) put on 4 A and 11 V linked to a arduino uno micro controller and a Matlab program controlling the set up. All 16 LED's are parallel connected which leads to a current of 0.25 and 11 V for each. The Matlab code [1] is designed in a way that enables a easy to use GUI where one can control the intensity via the same 0-255 256-bit pulse-width modulation that is used in the optogenetics arena. Additionally there is an integrated option to operate the set up in an cycling mode where you can define on and off periods which is thought out for long therm stimulus. The idea is to not overwhelm the neurons, imitate a kind of natural bluming effect and also helps preventing the chamber from heating up when doing a conditioning over many hours and high intensity.

A further property of the chambers aims to provide stable temperature is a good heat transport away from the LED's themselves that the inside is not getting heated up by them which would influence the conditioning and later behavior of the experimental flies. There for two powerful aluminum heat sinks 100mm x 80mm x 40mm(Pada Engineering 8214/100/N)have been installed to each two of the LED's with a forced convection thermal resistance of 0.29 °C/W and a natural convection thermal resistance of 1.1°C/W.
To also balance out the heat emitted through the light radiation the setup is operated in a large 19 °C climate chamber with consistent humidity of 60 %, this keeps the temperature inside the fly bottles constant between 22 and 24 °C even when operating on long-therm conditioning.

[1] written with the help of Christian Schmid

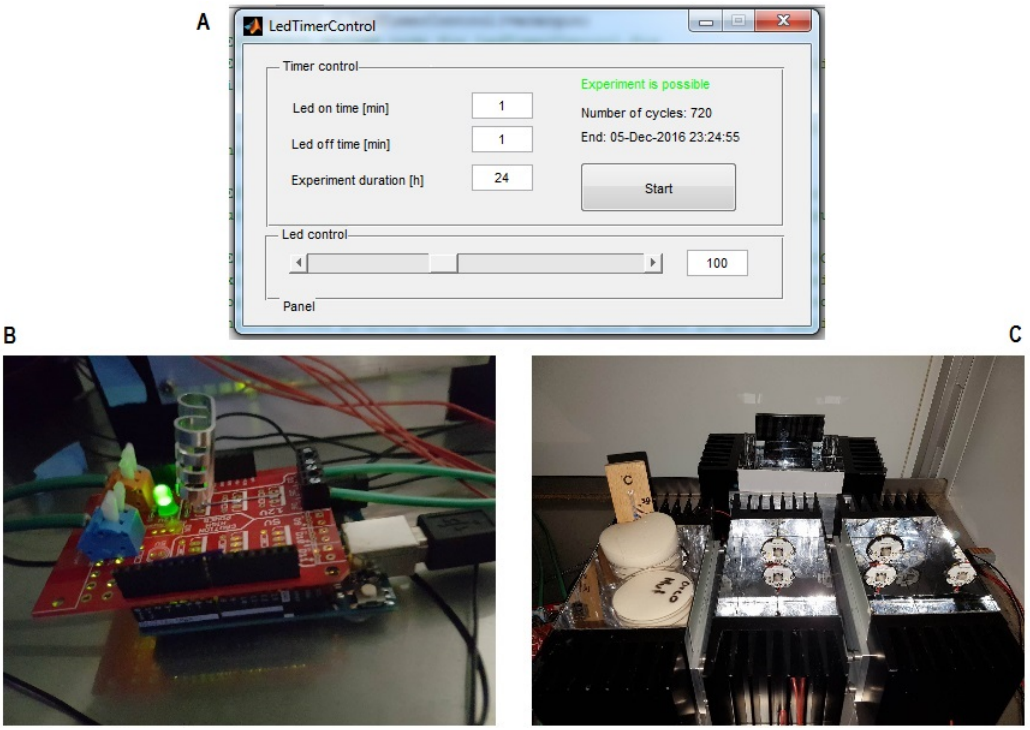

FIGURE 3.1.: A) Matlab GUI Control panel for the optogenetics conditioning chamber, where LED control slider adjusts die PWM 0-255;B)Arduino uno micro controler modified with a Aluminium heat sink to deal with the high current throughput of 4 A; C) 4 optogenetics conditioning chambers.

3.2. Pulse width modulated light stimuli

The LED stimuli in the optogenetics conditioning chamber and also in the optogenetics arena are pulsed. Using a Arduino micro controller to set a frequency and programming a pulse width modulation on top with the controlling software (Matlab 2015) allows to control the pulsing in intensity and pulse durations. The program will divide a cycle that is given by the frequency the Arduino is put on, into 256 bits (0-255) and the program sets how many of these will be on set and the rest will be the of set of the square wave.Figure 3.2 The measured power from the optogenetics arena with the set intensity to 255 uses 256 out of 256 bits onset and translates in full illumination, is 8.8mW/cm^2. So this are the up pulses in a pulse width modulation stimuli. Calculating the Duty Cycle of the PWM:

$$D = used\ bits(set\ intensity + 1)/256(allbits) \tag{3.1}$$

from that we can derive the average Power output:

$$\bar{P} = D * P_{pulse} \tag{3.2}$$

taking also the frequency into account, we can calculate the actual pulse durations:

$$D * (1/frequency) \tag{3.3}$$

. Figure 3.3

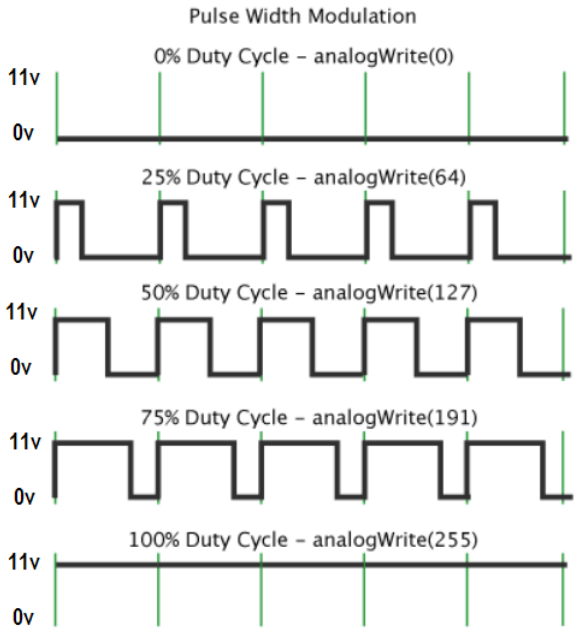

FIGURE 3.2.: pulse width modulation in a 256 bit 0-255 way with different Duty Cycles where 0 of 255 is no stimulus = 0% Duty Cycle and 255 of 255 is a constant illumination with no frequency and a Duty Cycle = 100%. [15]

	pwm=100	pwm=200	pwm=250	
Duty Cycle	39.45%	78.52%	98.05%	
øPower (W/cm^2)	3.47	6.91	8.63	
Freq (Hz)	Cycle duration (ms)	pwm=100 pulse duration	pwm=200 pulse duration	pwm=250 pulse duration
60	16.66666667	6.575520833	13.0859375	16.34114583
244	4.098360656	1.616931352	3.217853484	4.018314549

FIGURE 3.3.: Display of the 6 used settings in this thesis, their average power output and the actual pulse durations.

3.3. Sickness quantification

Having a handle on quantifying the sickness that is induced via the bacteria feeding is crucial for developing a reliable working protocol of growing and feeding the bacteria, as well as handling the flies, as we did successfully here (see materials and methods). Also it is crucial to use the internal state of sickness or malaise as a reliable source for the stimuli in conditioning experiments.

To quantify the sickness that is induced, I used the three different techniques from the materials and methods section.

3.3.1. Survival test

What we see with the survival test is a significant difference in death rate between the virulent *Pom1* and the harmless control *Mutant* strain of the Ecc15 bacteria. This strongly indicates that the thorough trial and error established protocol to induce sickness into groups of flies does work.

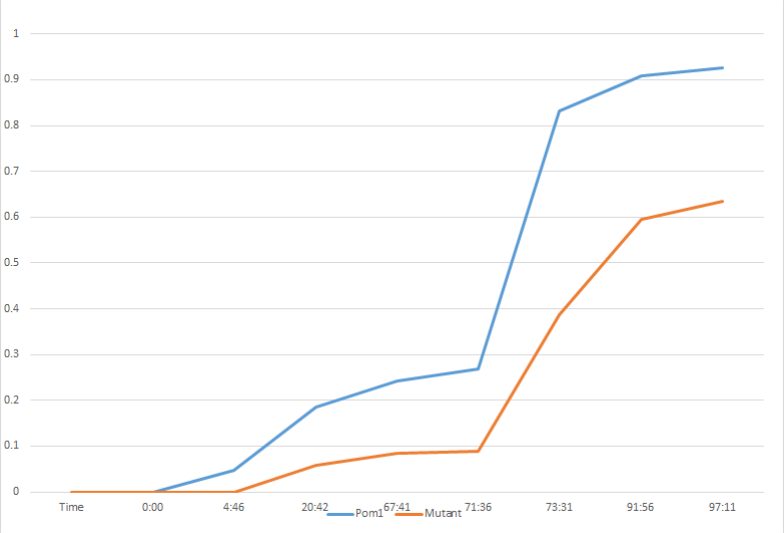

FIGURE 3.4.: Normalized death rate in starvation bottles at 25 °C and 60% humidity n=6 bottles with 50 flies each.

3.3.2. Dipt-mcherry flies

A further test to ensure the liability of the infection protocol and localize the effect in the gut, is the infection of dipt-mcherry transgene flies and then inspect the dissected guts under the fluorescence microscope. There is also a good prove that the freezing didn't took away the virulence of the bacteria, there could also an argument be made, that the additional glycerol helps to infect the guts by ether keeping the bacteria more viral on the filter paper or protecting them pass the defense mechanisms in the fly gut. This observation needs to be further scientifically explored.

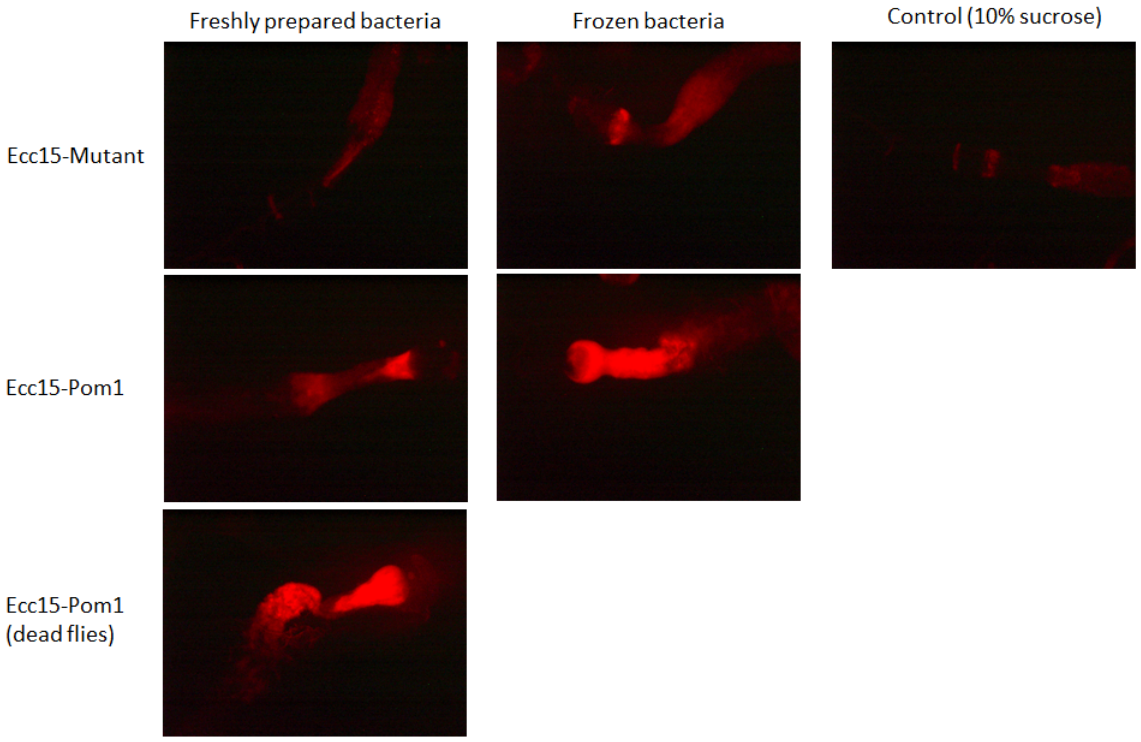

FIGURE 3.5.: Dissected drosophila guts under the fluorescence microscope 48 hours after infection

3.3.3. Drosophila Activity Monitor DAM

Sickness affects the motility and motivation to move in some animals species. Whether this is also the case for Drosophila has not been conclusively determined previously. To more specifically quantify the sickness based on activity differences of the infected as compared to the non-infected animals tested their activity and circadian rhythm in the DAM. None of the results did show any significant differences in the activity recorded by these two groups. From these results, we expect that flies do not show significant altered activity at least not in an artificial setting like the one provided with the DAM where the flies are in a tube and the movement is reduced to almost one spacial-dimension.

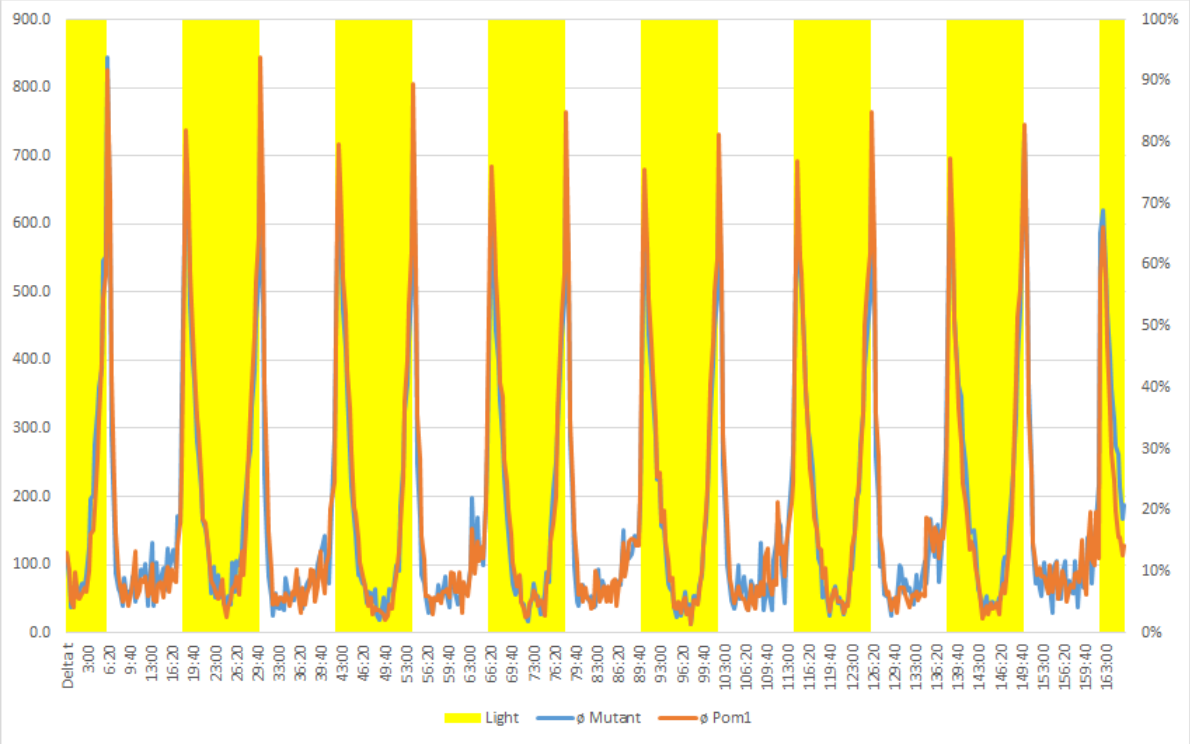

FIGURE 3.6.: The average activity over the 17 measurement beams and over 16 experimental flies per group, with a 9am to 9pm light cycle, similar to the normal circadian condition.

3.4. Conditioning with bacteria

Having established a working protocol for induced sickness in fly groups, we can use this as an US (unconditioned stimulus) and combine it with a NS from the olfactory system (via optogenetics) to get a CS and a CR in form of a COA (conditioned odor aversion). For the olfactory NS we are looking for a fitting Gal4 driver line that expresses the CsChrimson in some olfactory receptor neurons to imitate an odor. Because we are expecting the conditioning to lead to an aversion of that odor replicating optogenetic stimuli, the ideal Gal4 driver for the conditioning experiment has a slight attraction in an optogenetics arena test when no conditioning pre-dated these flies, so that the difference manifests more clear in the behavior. Testing different lines the Orco-Gal4 did show a stable, medium strong attraction in the optogenetics arena and also is probably not a very strongly hard wired stimulus by the nature of co-receptor that it is which is broadly expressed among almost all ORN's. Figure 3.7
That is why we chose the Orco-Gal4xCsChrimson genotype as the experimental flies in the conditioning experiment. The optogenetics arena is ran with a pulse-width modulation of 250 out of 255 and 244hz, which is calibrated by testing different settings on the genotype Gr66a-Gal4xCsChrimson by my colleague Laurence Lewis that shows a very strong aversion in the optogenetics arena and the strongest at these specific settings. This matches with the findings when testing different intensities and frequencies for Orco-Gal4xCsChrimson transgene flies who show also the strongest attraction at these settings. Figure 3.7B

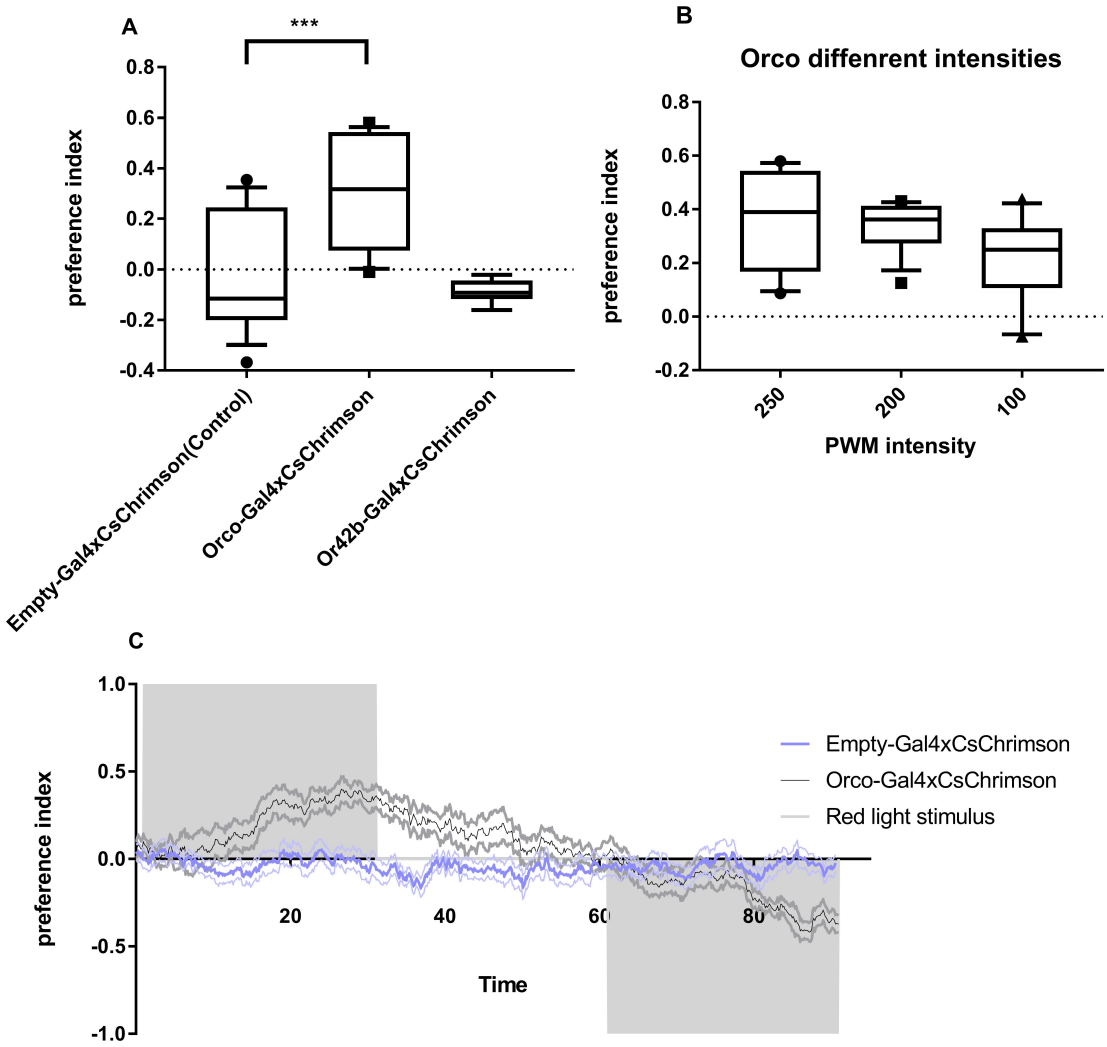

FIGURE 3.7.: A)Box and Whiskers Plot of the tested Gal4-drivers against an Empty-Gal4xCsChrimson control at 250 intensity and 244hz where Orco-Gal4xCsChrimson shows a statistic significant attraction towards the stimulus ***=P$\leq$ 0.001 ANOVA-test B)Box and Whiskers Plot of the same genotype: Orco-Gal4xCsChrimson running the optogenetics arena with 100, 200, and 250 PWM'ed intensity and 244hz. C)Behavior of Orco-Gal4xCsChrimson transgene flies n=11 compared to the control Empty-Gal4xCsChrimson transgene flies n=16 in the optogenetics arena throughout a 90 second protocol. The Y-axes displays the mean preference with the SEM in lighter color.

For conditioning, this stimuli settings (int=250 out of 255 PWM and 244hz) are chosen in the arena as well as in the conditioning chamber. The bacterial feeding assays is then placed inside the conditioning chamber, and for 24 hours a two minutes on two minutes of cycle program was running. Here we used the same settings as used in the optogenetic arena for the illumination of the optogenetic conditioning chamber : 250 out of 255 PWM and 244hz. After one hour of recovery the flies are placed inside the optogenetics arena and tested for preference Figure 3.8

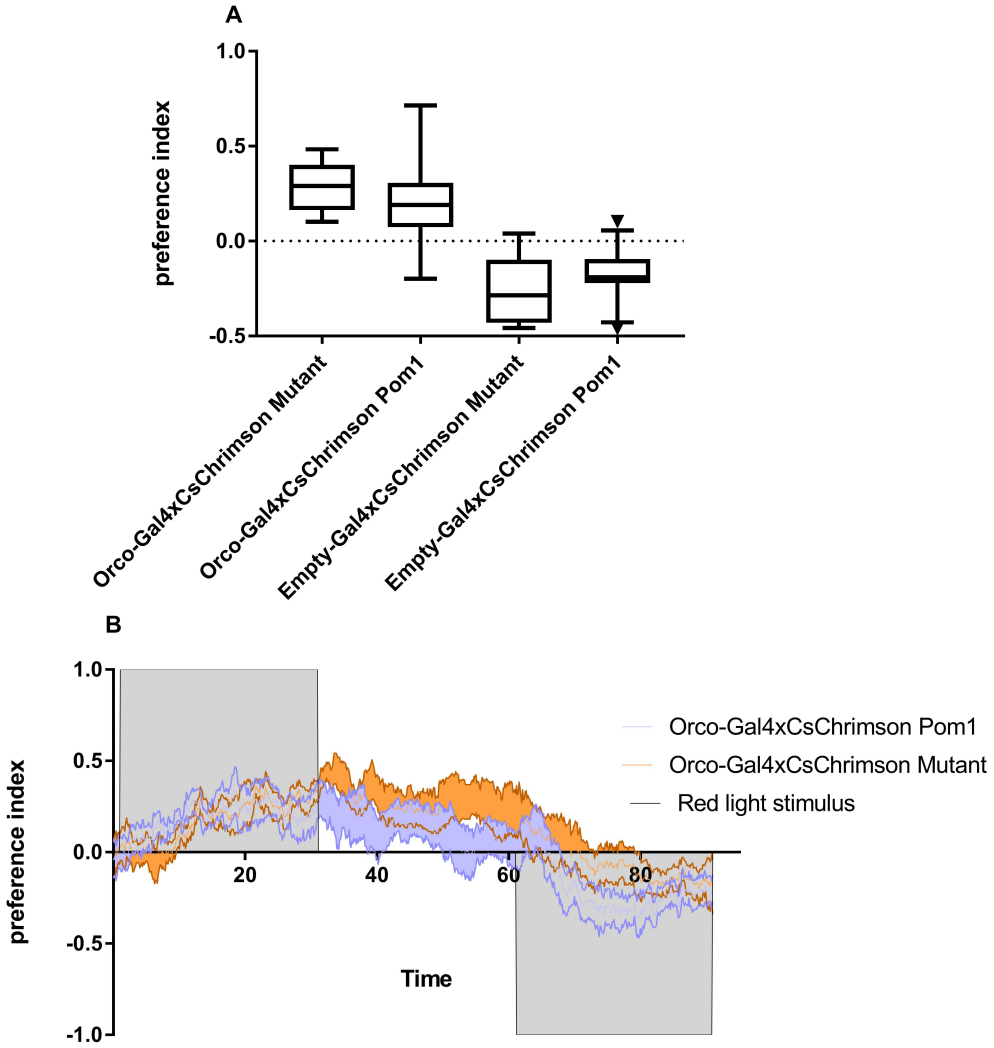

FIGURE 3.8.: A) Orco-Gal4xCsChrimson (Pom1 n=8; Mutant n=6) and Empty-Gal4xCsChrimson (Pom1 n=12; Mutant n=6) flies infected via the sickness inducing feeding protocol with virulent Pom1 and harmless Mutant strains of the Ecc15 bacteria tested in the optogenetics arena running under 250 intensity and 244hz.B) Behavior of Orco-Gal4xCsChrimson fed with virulent Pom1 and harmless Mutant strains of the Ecc15 bacteria in comparison through the whole 90 second testing protocol in the optogenetics arena

3.5. Conditioning with quinine

As proof of principle for the optogenetics conditioning chamber and its ability to condition groups of flies simultaneously the chamber is tested in new experiment where quinine that has a strong bitter taste is the new stimulus instead of the bacteria infected malaise state. There for a gustatory receptor has been chosen to be targeted with the optogenetic CsChrimson channel transgenic expression. Gr5a, which is expressed in the gustatory neurons and act as a primary marker for tastants, especially sugar, in Drosophila. Testing these Gr5a-Gal4xCsChrimson flies in the optogenetics arena without any conditioning the results show no significant distinct behavior response and seem to freeze as soon the light stimulus kicks in. By lowering the PWM intensity to 100 out of 255 and the frequency to 60hz which translates to a power of 3.47W/cm^2 and a way longer pulse duration of 6.57ms Figure 3.3 shows a stable and statistical significant (ANOVA test) attraction as expected from a "sugar receptor". Figure 3.9.

A conditioning experiment using these newly established LED settings for the chamber and arena, specified for the Gr5a receptor is ran. Therefor the in materials and methods described quinine assays were filled with experimental flies and put into the conditioning chambers for 30 min with no on of set in the macro level, only using PWM (100). As a control the same assay has been used with only water instead of quinine and water with the same flies. The flies then been recovered in the dark for 5 minutes and tested in the optogenetics arena.Figure 3.10

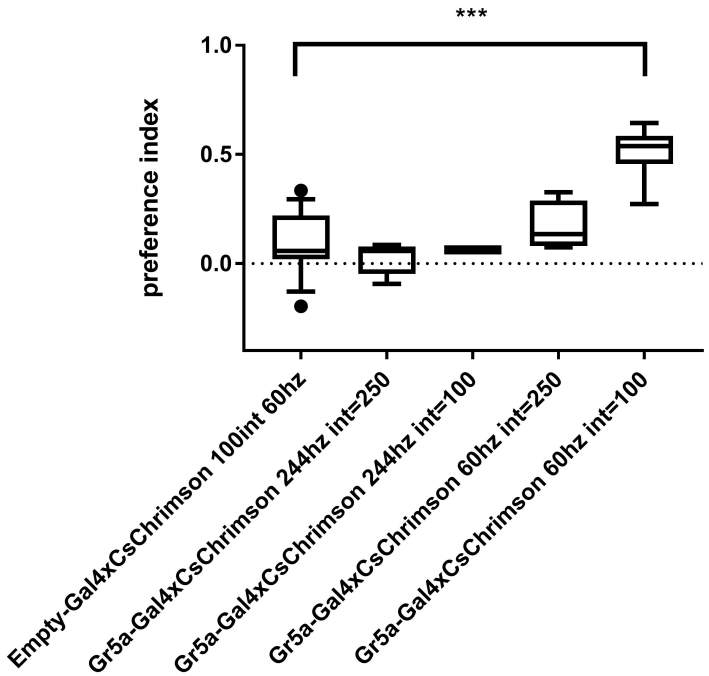

Figure 3.9.: Testing Gr5a-Gal4xCs Chrimson flies with different PWM intensities and frequencies (244hz int=250 n=5 ;244hz int=100 n=2 ;60hz int=250 n=4 ;60hz int=100 n=6)against the Empty-Gal4xCsChrimson 60hz int=100 n=16 ***=p$\leq$ 0.001 ANOVA-test

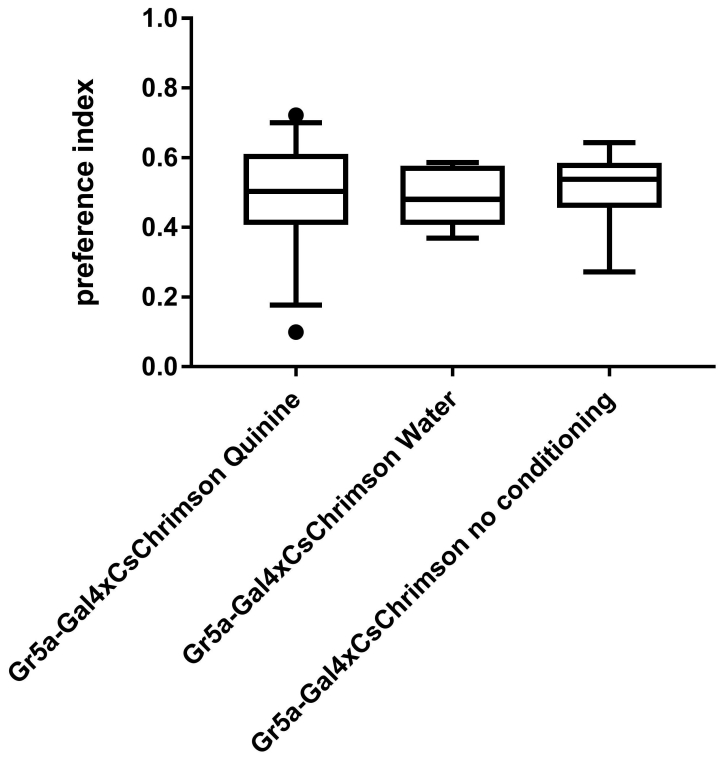

FIGURE 3.10.: Testing Gr5a-Gal4xCsChrimson put on Quinine (n=15) and water (n=9) in the Conditioning Chamber together with unconditioned Gr5a-Gal4xCsChrimson flies n=6. operated at 60hz and int=100

4. Results, discussion and outlook

In trying to establish a complex and novel experimental setup that allows to ask deep neurological questions about the neuronal circuits around learning and memory with the focus on replacing the odor stimulus with precisely timed optogenetical activation over time, not all goals have been accomplished. The, with trail and error developed method of inducing sickness to groups of flies has shown promising results. However the DAM did not pick up a difference in motility, assumingly due to the way *Drosophila melanogaster* deals with bacterial infection without showing significant altered activity. This has also been observed in mice, while rats do show a significant different activity when sick. [16] This part unfortunately took due to a large number of trial and errors a unexpected huge amount of time to the cost of the planed SSR (single sensillum recording) experiments. Nevertheless, the preparatory work present in this thesis now proves very useful. In recent promising results of my college Johanna Kobler using the developed protocol of conditioning with the bacteria and their odor are showing the value of this protocol and the ability to induce learning through negative physiological states in groups of flies.
In the case of conditioning the bacterial induced sickness with a optogenetic stimuli, Orco data analyses shows a trend but no statistically significant difference between the experimental and the control group. This suggests that for some reason the conditioning did not have a significant effect in the protocol used. Whether this effect becomes statistically significant in some further trial and error fine-tuning of the parameters in the protocol will be addressed in future experiments. Reasons for the lack of conditioning effect, could be the unnatural stimulus of activating all of the broadly expressed co-receptors that does not cope as a recognizable stimulus for conditioning. So this experiment will also be tried out in future with an different olfactory receptor.

When testing out, a seemingly more stable conditioning paradigm than bacterial infection, a very bitter unavoidable taste in form of quinine that has produced good learning effects in honeybees [30] but in contrast to our group conditioning approach, they took the single feeding approach where the researcher made sure that the bees had eaten the Quinine. In our case the paring of the quinine assay together with the activation of the gustatory sugar receptors did not show any conditioning effect Figure 3.10. Possible reasons could be the hard wired attraction towards sugar which may be very hard to change by conditioning and/or a to small passive effect of the negative taste experience when not in-taking but simply standing on the quinine solution.

But another interesting observation has been made when calibrating the LED stimuli for the different optogenetic receptor neurons. Here, the naive behavior of flies in the absence of prior

conditioning experience was assessed dependent on the strength of the optogenetic stimulus. My data suggests that the strongest behavioral results for different receptor neurons do differ quit a bit. This correlates with the nonlinear dynamic found for input output correlation in differenet sensory receptor neurons and different kinetics *frequency time constans* by A.S.French [12]2014. While Orco and Gr66a do show the strongest phenotype at a power of 8.63 W/cm^2 and short pulse durations of $\sim$ 4ms, Gr5a with the same optogenetical channel CsChrimson seems to be overactivated leading to no response in the behavioral paradigm. By contrast, a more natural attraction was observed with a lower average power of 3.47 W/cm^2 and a longer pulse duration of $\sim$ 6.6 ms. Figure 3.3 Figure 3.9 Figure 3.7B

So this leads to the conclusion that different receptor neurons, or at least different receptor neuron types have different kinetic patterns and excitation power thresholds on which the optogenetic stimuli can simulate natural signaling and evoke the appropriate behavior. This should and will be further explored for ORN's with single sensillum recordings and correlated with the behavioral data, but unfortunately did exceed the capacity of this master thesis.

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Abbreviations

ANOVA Analysis Of Variance

LED Light-Emitting Diode

ORN Olfactory Receptor Neuron

PBS Phosphate-Buffered Saline

PWM Pulse Width Modulation

SSR Single Sensillum Recording

Nomenclature

Ecc15 Erwinia carotovora carotovora (Pectobacterium carotovorum)

F1-generation The F1 generation is the generation resulting immediately from a cross of the first set of parents (parental generation)

Pom1 A version of ECC15 where the virulent factor is over expressed

A. Matlab code for running the optogenetics chamber

breaklines

```
function varargout = LedTimerControl(varargin)
% LEDTIMERCONTROL MATLAB code for LedTimerControl.fig
%      LEDTIMERCONTROL, by itself, creates a new LEDTIMERCONTROL or raises the existing
%      singleton*.
%
%      H = LEDTIMERCONTROL returns the handle to a new LEDTIMERCONTROL or the handle to
%      the existing singleton*.
%
%      LEDTIMERCONTROL('CALLBACK',hObject,eventData,handles,...) calls the local
%      function named CALLBACK in LEDTIMERCONTROL.M with the given input arguments.
%
%      LEDTIMERCONTROL('Property','Value',...) creates a new LEDTIMERCONTROL or raises the
%      existing singleton*.  Starting from the left, property value pairs are
%      applied to the GUI before LedTimerControl_OpeningFcn gets called.  An
%      unrecognized property name or invalid value makes property application
%      stop.  All inputs are passed to LedTimerControl_OpeningFcn via varargin.
%
%      *See GUI Options on GUIDE's Tools menu.  Choose "GUI allows only one
%      instance to run (singleton)".
%
% See also: GUIDE, GUIDATA, GUIHANDLES

% Edit the above text to modify the response to help LedTimerControl

% Last Modified by GUIDE v2.5 11-Mar-2016 12:37:11

% Begin initialization code - DO NOT EDIT
gui_Singleton = 1;
gui_State = struct('gui_Name',       mfilename, ...
                   'gui_Singleton',  gui_Singleton, ...
                   'gui_OpeningFcn', @LedTimerControl_OpeningFcn, ...
                   'gui_OutputFcn',  @LedTimerControl_OutputFcn, ...
                   'gui_LayoutFcn',  [] , ...
                   'gui_Callback',   []);
if nargin && ischar(varargin{1})
    gui_State.gui_Callback = str2func(varargin{1});
end

if nargout
    [varargout{1:nargout}] = gui_mainfcn(gui_State, varargin{:});
else
    gui_mainfcn(gui_State, varargin{:});
end
% End initialization code - DO NOT EDIT


% --- Executes just before LedTimerControl is made visible.
function LedTimerControl_OpeningFcn(hObject, eventdata, handles, varargin)
% This function has no output args, see OutputFcn.
% hObject    handle to figure
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)
% varargin   command line arguments to LedTimerControl (see VARARGIN)

% Choose default command line output for LedTimerControl
handles.output = hObject;

x = instrfind;
for i = 1:length(x)
    fclose(x(i));
    delete(x(i));
end

handles.arduino = serial('COM6','Baudrate',57600);
```

```
65  fopen(handles.arduino);
66
67  % Update handles structure
68  guidata(hObject, handles);
69
70  % UIWAIT makes LedTimerControl wait for user response (see UIRESUME)
71  % uiwait(handles.figure1);
72
73
74  %--- Outputs from this function are returned to the command line.
75  function varargout = LedTimerControl_OutputFcn(hObject, eventdata, handles)
76  % varargout  cell array for returning output args (see VARARGOUT);
77  % hObject    handle to figure
78  % eventdata  reserved - to be defined in a future version of MATLAB
79  % handles    structure with handles and user data (see GUIDATA)
80
81  % Get default command line output from handles structure
82  varargout{1} = handles.output;
83
84
85
86  function editLed1_Callback(hObject, eventdata, handles)
87  % hObject    handle to editLed1 (see GCBO)
88  % eventdata  reserved - to be defined in a future version of MATLAB
89  % handles    structure with handles and user data (see GUIDATA)
90
91  % Hints: get(hObject,'String') returns contents of editLed1 as text
92  %        str2double(get(hObject,'String')) returns contents of editLed1 as a double
93  val = round(str2double(get(hObject,'String')));
94  if val > 255, val = 255; elseif val < 0, val = 0; end
95  set(handles.sliderLed1,'value',val);
96  set(handles.editLed1,'string',num2str(val));
97
98  pin = 9;
99  analogWrite(handles,pin,val);
100
101 %--- Executes during object creation, after setting all properties.
102 function editLed1_CreateFcn(hObject, eventdata, handles)
103 % hObject    handle to editLed1 (see GCBO)
104 % eventdata  reserved - to be defined in a future version of MATLAB
105 % handles    empty - handles not created until after all CreateFcns called
106
107 % Hint: edit controls usually have a white background on Windows.
108 %       See ISPC and COMPUTER.
109 if ispc && isequal(get(hObject,'BackgroundColor'), get(0,'defaultUicontrolBackgroundColor'))
110     set(hObject,'BackgroundColor','white');
111 end
112
113
114 %--- Executes on slider movement.
115 function sliderLed1_Callback(hObject, eventdata, handles)
116 % hObject    handle to sliderLed1 (see GCBO)
117 % eventdata  reserved - to be defined in a future version of MATLAB
118 % handles    structure with handles and user data (see GUIDATA)
119
120 % Hints: get(hObject,'Value') returns position of slider
121 %        get(hObject,'Min') and get(hObject,'Max') to determine range of slider
122 val = round(get(hObject,'Value'));
123 set(handles.editLed1,'string',num2str(val));
124
125 pin = 9;
126 analogWrite(handles,pin,val);
127
128 %--- Executes during object creation, after setting all properties.
129 function sliderLed1_CreateFcn(hObject, eventdata, handles)
130 % hObject    handle to sliderLed1 (see GCBO)
131 % eventdata  reserved - to be defined in a future version of MATLAB
132 % handles    empty - handles not created until after all CreateFcns called
133
134 % Hint: slider controls usually have a light gray background.
135 if isequal(get(hObject,'BackgroundColor'), get(0,'defaultUicontrolBackgroundColor'))
136     set(hObject,'BackgroundColor',[.9 .9 .9]);
137 end
138
139
140 %--- Executes when user attempts to close figure1.
141 function figure1_CloseRequestFcn(hObject, eventdata, handles)
142 % hObject    handle to figure1 (see GCBO)
143 % eventdata  reserved - to be defined in a future version of MATLAB
144 % handles    structure with handles and user data (see GUIDATA)
145 disp('Try to shut down');
146 pin = 9;
147 value = 0;
```

```
148 try
149 analogWrite(handles,pin,value);
150 catch end
151 x = instrfind;
152 for i = 1:length(x)
153     fclose(x(i));
154     delete(x(i));
155 end
156 try
157     stop(handles.timer);
158 catch end
159 % Hint: delete(hObject) closes the figure
160 delete(hObject);
161
162
163
164 function editLed1On_Callback(hObject, eventdata, handles)
165 % hObject    handle to editLed1On (see GCBO)
166 % eventdata  reserved - to be defined in a future version of MATLAB
167 % handles    structure with handles and user data (see GUIDATA)
168
169 % Hints: get(hObject,'String') returns contents of editLed1On as text
170 %        str2double(get(hObject,'String')) returns contents of editLed1On as a double
171 on_Time  = str2double(get(handles.editLed1On,'String'));
172 off_Time = str2double(get(handles.editLed1Off,'string'));
173 duration = str2double(get(handles.editDuration,'string'));
174
175 period = on_Time + off_Time;
176 cycles = (duration * 60) / period;
177 expGo = mod(duration*60,period);
178
179 if expGo == 0
180 set(handles.textExperiment,'string','Experiment is possible','ForegroundColor','green');
181 set(handles.pbStart,'Enable','on');
182 set(handles.textNumberOfCycles,'string',['Number of cycles: ',num2str(cycles)]);
183 set(handles.textEnd,'string',['End: ',datestr(now+duration/24)]);
184 else
185 set(handles.textExperiment,'string','Experiment is not possible','ForegroundColor','red');
186 set(handles.pbStart,'Enable','off');
187 set(handles.textNumberOfCycles,'string',['Number of cycles: ',num2str(cycles)]);
188 set(handles.textEnd,'string',['End: ',datestr(now+duration/24)]);
189 end
190
191 %--- Executes during object creation, after setting all properties.
192 function editLed1On_CreateFcn(hObject, eventdata, handles)
193 % hObject    handle to editLed1On (see GCBO)
194 % eventdata  reserved - to be defined in a future version of MATLAB
195 % handles    empty - handles not created until after all CreateFcns called
196
197 % Hint: edit controls usually have a white background on Windows.
198 %       See ISPC and COMPUTER.
199 if ispc && isequal(get(hObject,'BackgroundColor'), get(0,'defaultUicontrolBackgroundColor'))
200     set(hObject,'BackgroundColor','white');
201 end
202
203
204
205 function editLed1Off_Callback(hObject, eventdata, handles)
206 % hObject    handle to editLed1Off (see GCBO)
207 % eventdata  reserved - to be defined in a future version of MATLAB
208 % handles    structure with handles and user data (see GUIDATA)
209
210 % Hints: get(hObject,'String') returns contents of editLed1Off as text
211 %        str2double(get(hObject,'String')) returns contents of editLed1Off as a double
212 on_Time  = str2double(get(handles.editLed1On,'String'));
213 off_Time = str2double(get(handles.editLed1Off,'string'));
214 duration = str2double(get(handles.editDuration,'string'));
215
216 period = on_Time + off_Time;
217 cycles = (duration * 60) / period;
218 expGo = mod(duration*60,period);
219
220 if expGo == 0
221 set(handles.textExperiment,'string','Experiment is possible','ForegroundColor','green');
222 set(handles.pbStart,'Enable','on');
223 set(handles.textNumberOfCycles,'string',['Number of cycles: ',num2str(cycles)]);
224 set(handles.textEnd,'string',['End: ',datestr(now+duration/24)]);
225 else
226 set(handles.textExperiment,'string','Experiment is not possible','ForegroundColor','red');
227 set(handles.pbStart,'Enable','off');
228 set(handles.textNumberOfCycles,'string',['Number of cycles: ',num2str(cycles)]);
229 set(handles.textEnd,'string',['End: ',datestr(now+duration/24)]);
230 end
```

```
231
232 % --- Executes during object creation, after setting all properties.
233 function editLed1Off_CreateFcn(hObject, eventdata, handles)
234 % hObject    handle to editLed1Off (see GCBO)
235 % eventdata  reserved - to be defined in a future version of MATLAB
236 % handles    empty - handles not created until after all CreateFcns called
237
238 % Hint: edit controls usually have a white background on Windows.
239 %       See ISPC and COMPUTER.
240 if ispc && isequal(get(hObject,'BackgroundColor'), get(0,'defaultUicontrolBackgroundColor'))
241     set(hObject,'BackgroundColor','white');
242 end
243
244
245
246 function editDuration_Callback(hObject, eventdata, handles)
247 % hObject    handle to editDuration (see GCBO)
248 % eventdata  reserved - to be defined in a future version of MATLAB
249 % handles    structure with handles and user data (see GUIDATA)
250
251 % Hints: get(hObject,'String') returns contents of editDuration as text
252 %        str2double(get(hObject,'String')) returns contents of editDuration as a double
253 on_Time  = str2double(get(handles.editLed1On,'String'));
254 off_Time = str2double(get(handles.editLed1Off,'string'));
255 duration = str2double(get(handles.editDuration,'string'));
256
257 period = on_Time + off_Time;
258 cycles = (duration * 60) / period;
259 expGo = mod(duration*60,period);
260
261 if expGo == 0
262 set(handles.textExperiment,'string','Experiment is possible','ForegroundColor','green');
263 set(handles.pbStart,'Enable','on');
264 set(handles.textNumberOfCycles,'string',['Number of cycles: ',num2str(cycles)]);
265 set(handles.textEnd,'string',['End: ',datestr(now+duration/24)]);
266 else
267 set(handles.textExperiment,'string','Experiment is not possible','ForegroundColor','red');
268 set(handles.pbStart,'Enable','off');
269 set(handles.textNumberOfCycles,'string',['Number of cycles: ',num2str(cycles)]);
270 set(handles.textEnd,'string',['End: ',datestr(now+duration/24)]);
271 end
272
273 % --- Executes during object creation, after setting all properties.
274 function editDuration_CreateFcn(hObject, eventdata, handles)
275 % hObject    handle to editDuration (see GCBO)
276 % eventdata  reserved - to be defined in a future version of MATLAB
277 % handles    empty - handles not created until after all CreateFcns called
278
279 % Hint: edit controls usually have a white background on Windows.
280 %       See ISPC and COMPUTER.
281 if ispc && isequal(get(hObject,'BackgroundColor'), get(0,'defaultUicontrolBackgroundColor'))
282     set(hObject,'BackgroundColor','white');
283 end
284
285
286 % --- Executes on button press in pbStart.
287 function pbStart_Callback(hObject, eventdata, handles)
288 % hObject    handle to pbStart (see GCBO)
289 % eventdata  reserved - to be defined in a future version of MATLAB
290 % handles    structure with handles and user data (see GUIDATA)
291 str = get(hObject,'String');
292 duration = str2double(get(handles.editDuration,'string'));
293 set(handles.textEnd,'string',['End: ',datestr(now+duration/24)]);
294
295 try
296     stop(handles.timer);
297 catch end
298
299 on_Time  = str2double(get(handles.editLed1On,'String'));
300 off_Time = str2double(get(handles.editLed1Off,'string'));
301 duration = str2double(get(handles.editDuration,'string'));
302
303 period = on_Time + off_Time;
304 cycles = (duration * 60) / period;
305 expGo = mod(duration*60,period);
306
307 handles.timer = timer;
308 handles.timer.ExecutionMode = 'fixedRate';
309 handles.timer.Period = (on_Time + off_Time) * 60;
310 handles.timer.Period;
311 handles.timer.TasksToExecute = cycles;
312 handles.timer.StartFcn = {@Led_Timer_Start,handles};
313 handles.timer.TimerFcn = {@Led_Timer_Fcn,handles};
```

```
% handles.timer.StopFcn  = {@Led_Timer_Stop,handles};

guidata(hObject, handles);

if strcmp(str,'Start') == 1
    set(handles.pbStart,'String','Stop');
    disp('Start timer');
    start(handles.timer);
else
    set(handles.pbStart,'String','Start');
    disp('Stop timer');
end

function Led_Timer_Start(obj,event,handles)
disp('Start')

function Led_Timer_Fcn(obj,event,handles)
disp([datestr(now),' - Timer Fcn - Led on']);
pin = 9;
value = get(handles.sliderLed1,'value');
for i = 1:75
    try
        analogWrite(handles,pin,value);
    catch end
end

on_Time  = str2double(get(handles.editLed1On,'String')) * 60;
t = timer;
t.StartDelay = on_Time;
t.Timerfcn = {@Led_turn_off,handles};
start(t);

function Led_turn_off(obj,event,handles)
disp([datestr(now),' - Turn off - Led off']);
pin = 9;
value = 0;
% try
for i = 1:75
    try
        analogWrite(handles,pin,value);
    catch end
end

function Led_Timer_Stop(obj,event,handles)
disp('Stop')
pin = 9;
value = 0;
% try
analogWrite(handles,pin,value);
% catch end
set(handles.pbStart,'String','Start');
```