Universität Regensburg

PHOTOTACTIC BEHAVIOUR SUPPRESSION IN *DROSOPHILA MELANOGASTER*

Bachelor's Thesis

Accomplished at

The Institute of Zoology – Neurogenetics

Scientific Faculty III

Biology and preclinical Medicine

University of Regensburg

Submitted by

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7 April 2015

Declaration

I hereby declare that I have written this thesis independently and that I have used nothing else than the referenced sources of information and implements. I hereby declare that this thesis has not been submitted to any other university for a degree.

Ich habe die Arbeit selbständig verfasst, keine anderen als die angegebenen Quellen und Hilfsmittel benutzt und bisher keiner anderen Prüfungsbehörde vorgelegt.

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CONTENT

ABSTRACT

In this bachelor's thesis, a Phototaxis Suppression Paradigm for one single fly was adapted to an assay for a group of flies. Both positive and negative phototactic behaviour of flies with intact or clipped wings, respectively, could be suppressed by pairing light/dark with an aversive stimulus. Two different aversive stimuli were used to suppress the phototactic behaviour in flies, a gustatory (quinine) and an olfactory (benzaldehyde) stimulus. In single-fly experiments, it has been shown that flies are only able to remember the association between the light and the aversive stimulus during the training phase. To test if on the conditions for the new group assay, flies are able to remember the association after the training, flies were also tested on extinction. However, no completely reversal of their phototactic behaviour could be achieved in absence of the aversive stimulus. On regard to the efficiency of both aversive stimuli, the gustatory stimulus was not an appropriate stimulus to use in this paradigm. Quinine could only evoke a change in phototaxis behaviour in flies with intact wings, but it could not change the behaviour of flies with clipped wings. On the contrary, benzaldehyde was effective in experiments on flies with both intact and clipped wings. Although flies only showed a suppression in phototaxis as long as the aversive stimulus was present, and not on extinction, their behaviour could not be considered as naïve anymore. In conclusion, it was possible to convert the Phototaxis Suppression Paradigm from a single fly assay into an assay in which a group of flies can be tested. The results also showed that light became a strong aversive stimulus for flies without wings, since a stronger stimulus was needed to suppress their phototactic behaviour.

ZUSAMMENFASSUNG

In dieser Bachelor Arbeit wurde ein Paradigma zur Unterdrückung der Phototaxis einer einzelnen Fliege zu einem Testverfahren für eine Gruppe an Fliegen angepasst. Sowohl positives als auch negatives phototaktisches Verhalten von Fliegen mit entsprechend intakten oder geschnittenen Flügeln, konnte durch das Paaren von Licht/Dunkelheit mit einem aversiven Stimulus unterdrückt werden. Zur Unterdrückung des phototaktischen Verhaltens von Fliegen, wurden zwei unterschiedliche aversive Stimuli verwendet, ein gustatorischer (Chinin) und ein olfaktorischer (Benzaldehyd) Stimulus. In Experimenten

anhand einzelner Fliegen hat es sich gezeigt, dass Fliegen nur während der Trainingsphase dazu fähig sind sich an die Assoziation zwischen dem Licht und dem aversiven Stimulus zu erinnern. Um zu überprüfen, ob Fliegen unter den Bedingungen des neuen Gruppentestverfahrens dazu fähig sind, sich nach dem Training an die Assoziation zu erinnern, wurden die Fliegen auch auf Extinktion überprüft. Jedoch konnte in Abwesenheit des aversiven Stimulus keine komplette Umkehrung ihres phototaktischen Verhaltens erzielt werden. Im Hinblick auf die Effektivität beider aversiven Stimuli war der gustatorische Stimulus zur Verwendung in diesem Paradigma nicht geeignet. Chinin konnte nur eine Veränderung im phototaktischen Verhalten von Fliegen mit intakten Flügeln hervorrufen, jedoch konnte es das Verhalten von Fliegen mit abgeschnittenen Flügeln nicht verändern. Im Gegensatz dazu, war Benzaldehyd in Experimenten an Fliegen mit sowohl intakten als auch abgeschnittenen Flügeln wirksam. Obwohl Fliegen eine Unterdrückung der Phototaxis nicht unter Extinktion, sondern nur dann gezeigt haben, solange der aversive Stimulus präsent war, konnte ihr Verhalten nicht mehr als naiv betrachtet werden. Schlussendlich war es möglich, das Phototaxis Unterdrückungsparadigma von einem Testverfahren für eine einzelne Fliege zu einem Testverfahren umzuwandeln, in welchem eine Gruppe von Fliegen getestet werden kann. Die Ergebnisse zeigen ebenfalls, dass das Licht für Fliegen ohne Flügel zu einem starken aversiven Stimulus geworden ist, da ein stärkerer Stimulus notwendig war um ihr phototaktisches Verhalten zu unterdrücken.

1. INTRODUCTION

As other flying insects, *Drosophila melanogaster* is instinctively positive phototactic (Hirsch & Boudreau, 1958) and is even more responsive to light after given them a start impulse (Benzer, 1967). In 1918 McEwen and in 1967 Benzer showed that the removal of the wings reduces a fly's response to light (McEwen, 1918; Benzer, 1967). The wing-clipping effect is even obtained when the response relates to mostly walking rather than flying (Benzer, 1967). This switch in phototactic behaviour depends on the amount of the wings removed while the activity of the fly is not affected (McEwen, 1918). In fact, a flightless fly is actively avoiding the light and become negatively phototactic. Moreover, Gorostiza showed that it is not the injury itself (clipping wings) what causes the change in behaviour, but rather the disruption of flying ability, which is constantly monitored by the fly. This relationship between flying ability and phototaxis prompt the hypothesis that phototaxis is more a decision-making process than a simple response to a stimulus (Gorostiza et al., in preparation).

Phototaxis is a robust behaviour and only an association with a punishment can confound it. In 2002, Le Bourg developed and presented the Phototaxis Suppression Assay (Le Bourg & Buecher, 2002). In 2009, Seugnet further studied this paradigm and explored the learning process beneath, where one single fly learns to avoid a light source that is paired with an aversive stimulus (Seugnet et al., 2009). They used a T-maze where a fly could choose between a lighted and a dark vertical vial over a course of 16 trials. Quinine was placed into the lighted vial and was used as a negative reinforcer in this assay to induce avoidance behaviour in flies (Le Bourg & Buecher, 2002; Seugnet et al., 2009). Le Bourg also tested flies with a humid filter paper in the light while a dry filter paper was on the dark and found that water could also promote an increasing number of photonegative choices in flies. In addition, Le Bourg analysed flies on extinction, i.e., after training flies were tested in the absence of the aversive stimulus to see if the association remained. He found that the flies showed low avoidance of the lighted vial on extinction, but had a significantly greater tendency to choose the dark vial. Therefore, he concluded that flies subjected to the training cannot be considered as naïve flies (Le Bourg & Buecher, 2002).

The main intention of this thesis was to adapt Le Bourg's Phototaxis Suppression Paradigm from a single fly to a group assay, in order to reduce the variability obtained when single

animals are tested, and secondly to test if wing-clipped flies can change their negative phototaxis again to positive by associating the darkness with an aversive stimulus. The T-maze Phototaxis assay, which is used to evaluate light/dark preference in flies, was used as a start point for the new Phototaxis Suppression Assay. The proper conditions were established by testing the positive phototaxis suppression of intact flies in several experiments. Once this was achieved, flies with clipped wings were tested in the new paradigm, placing the aversive stimulus in the dark arm of the T-Maze. Additionally, flies with intact or clipped wings were tested on extinction to determine whether they were able to remember the association between the light/dark and the aversive stimulus using the new paradigm.

2. MATERIALS AND METHODS

2.1 FLY STRAIN AND CARE

The experiments in this thesis were conducted with wild-type *Berlin* (*WTB*) flies. Flies were always tested in groups and irrespective of their gender.

Flies were raised in a controlled density in vials containing standard cornmeal/molasses medium, in a 25°C chamber with 60% humidity and on a 12:12 h light and dark cycle. A dab of fresh and living yeast paste and a filter paper were added in the middle of the food medium. The optimal density, i.e. the amount of flies for breeding in one vial, is achieved at a state in which the food medium can be liquefied during the larval stages and all larvae have pupated before the first flies hatch (Brembs, 2008). After laying eggs for 24 hours in one vial, flies were transferred into a new vial. By performing this procedure every day, an appropriate and controlled growth of the animals could be provided, and newly eclosed flies were available every day. Flies used for egg-laying were not used for experiments and they were replaced every two weeks in order to maintain the optimal density.

2.2 WING CLIPPING

WTB flies from 0-1 day old were briefly anaesthetized with carbon dioxide or cold (0°C), and both wings were cut to 1/3 of their original length. Then, fifty wing-clipped flies were transferred into small vials with food and placed at 25°C for 24 hours in order to recover from the treatment.

2.3 T-MAZE

2.3.1 APPARATUS

To adapt the Phototaxis Suppression Paradigm for *Drosophila melanogaster* from a single fly to a group assay a T-Maze was employed (figure 1). This apparatus, used to assess phototactic choice (see section 2.3.2 below), consists of a mobile part containing an elevator in which flies can be transported to three separate tubes (1.5 cm internal diameter and 0.5 cm wall thickness): An entrance tube (10 cm long), a lighted transparent tube and a dark opaque tube (either 20cm or 10 cm long tubes were used, see section 2.4).

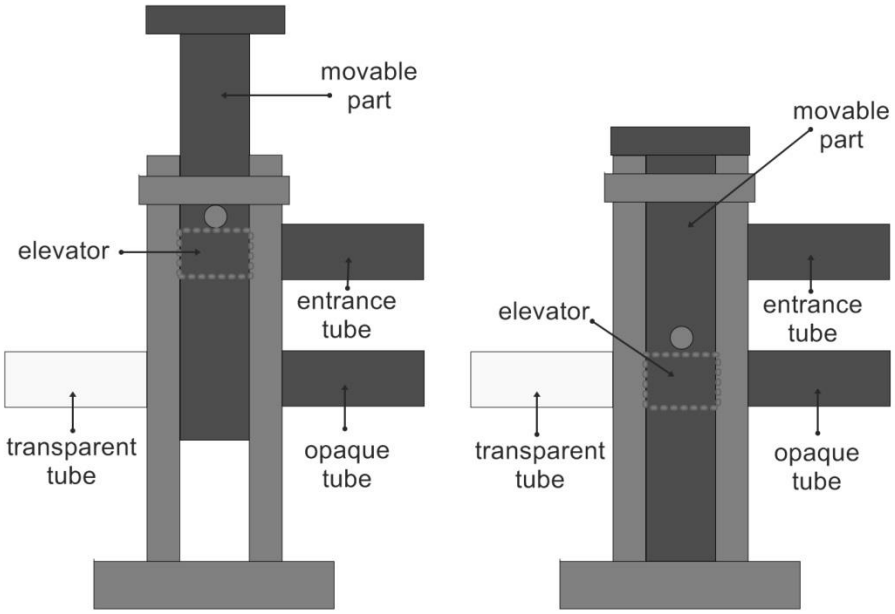


Figure 1: T-Maze. Experimental apparatus to evaluate the phototaxis suppression in *Drosophila melanogaster.*

2.3.2 T-MAZE PHOTOTAXIS PARADIGM

The T-maze phototaxis paradigm was used to assess the light/dark preference in flies. One day before the experiment, wings were clipped under CO_2 (see section 2.2). Then, 30 flies with clipped wings and 30 flies with intact wings were placed into one vial, and let them recover from anaesthesia until the experiment began. In the dark room, where the experiment was conducted, a light source was placed above the T-maze to provide homogenous lighting conditions. To start the experiment, flies were placed into the entrance tube for 10 minutes in order to adapt to the apparatus. The movable part was shifted all the

way up to tap the flies gently into the elevator, and immediately after this it was moved to the middle position of the T-maze, i.e. between the entrance tube and the opaque tube. At this positon flies were kept for 30 seconds. Then the elevator was shifted all the way down, and flies were able to choose between the lighted tube and the dark tube for 30 seconds. After the experiment was conducted, the movable part was shifted up in order to encase the flies in their selected tube. The number of flies in the transparent/lighted tube (#FL), the opaque/dark tube (#FD), the elevator (#FE) and the total number of flies (#FT) were counted by anaesthetizing the flies. A Choice Index (CI) was calculated using the following formula:

$$CI = \frac{(\#FL \cdot 1) + \big(\#FD \cdot (-1)\big) + (\#FE \cdot 0)}{\#FT}$$

The CI values range from 1 to -1. A CI of 1 means, that all flies chose the light, whereas a CI of -1 means, that all flies chose the dark. A value of zero means that the flies showed no preference. A CI was calculated for both, wingless flies and the intact flies in each experiment.

2.4 PHOTOTAXIS SUPPRESSION ASSAY

A preselection step was conducted to ensure that only photopositive or photonegative flies participate in the experiment. For this purpose, flies with intact wings or flies with clipped wings were placed into the entrance tube of the T-maze, and a regular phototaxis experiment was performed. The preselected flies were transferred back to the entrance tube. In regard to the suppression of the phototactic behaviour in flies, a filter paper wetted with an aversive stimulus was placed into the transparent or the opaque tube of the T-maze to provide an aversive association, depending on the conditions of the flies' wings (intact or clipped) and thus their phototaxis (positive or negative, respectively). Flies were allowed to choose between the lighted tube and dark tube for 30 seconds in each trial over a course of multiple trials. After one trial, all the flies were transferred again to the entrance tube for 30 or 15 seconds (see result section), which was considered as the intertrial interval time.

In order to assess if flies were able to suppress their phototaxis preference while avoiding the aversive stimulus, the number of flies in the transparent/lighted tube (#FL) and the opaque/dark tube (#FD) was counted under CO_2 anaesthesia after the last trail with the aversive stimulus in the T-maze. To determine whether flies were able to form a memory

during this Phototaxis Suppression Assay, flies performed one additional trial (test) without the aversive stimulus and were counted afterwards (Extinction experiment). In both cases a Choice Index (CI) could be calculated by using this formula:

$$CI = \frac{(\#\mathrm{FL} - \#\mathrm{FD})}{(\#\mathrm{FL} + \#\mathrm{FD})}$$

Since light reach the elevator in a lower intensity, forming a shadowed area between the lighted and the dark tube, flies which stayed in the elevator were not considered in the calculation, in order to avoid counting flies which have not made an explicit choice for either the light or the dark. The Choice Index values range from 1 to -1. A CI of 1 means, that all flies chose the light, whereas a CI of -1 means, that all flies chose the dark. A value of zero means that the flies did not show a preference.

To find out an appropriate aversive stimulus to use for the Phototaxis Suppression Assay for a group of flies, two different aversive stimuli were tested independently, a quinine/water solution (a gustatory stimulus) and a benzaldehyde/paraffin oil mixture (an olfactory stimulus). For each aversive stimulus, different numbers of trials were conducted and different lengths of the intertrail interval times (ITIT) were tested (see results). During the transfer step between trials, flies were tapped into the tube which did not contain the aversive stimulus in order to prevent an unrequested contact between the flies and the aversive stimulus.

For the gustatory stimulus, different quinine concentrations were tested (see results). The filter paper wetted with quinine covered the walls of a 20 cm long appropriate tube, i.e., the transparent or the opaque tube, such that the flies were able to get in contact with the substance. Le Bourg found that flies prefer to stay on a dry paper rather than on a wet one, and therefore water can potentially act as an aversive stimulus (Le Bourg & Buecher, 2002). To minimize the differences between tubes to only lighting conditions and the presence of quinine, the opposite tube was covered with a filter paper moistened with water. The filter paper was selected to be thin enough to provide sufficient light in the transparent tube, and rigid enough to prevent it to collapse when it was wet. A common chromatography paper served for this purpose. For experiments, the amount of liquid on the filter paper was adjusted to be sufficiently available for the flies to absorb the substance, but low enough to

prevent them to get wet or stuck. After several attempts, 1.5 ml of a quinine/water solution or water was considered to be sufficient. As control for the experiments, groups of flies were tested with filter paper wetted with water placed in both, transparent and opaque, tubes.

For the olfactory stimulus, different concentrations of benzaldehyde in paraffin oil (0.1, 0.01 and 0.001 (v/v)) were compared with the following experiment. The openings of two vials were put together and a filter paper wetted with the mixture was placed only in one of these vials to provide an odour gradient. Ten flies were placed inside and their behaviour was observed for 3 minutes. The 0.1 mixture caused the strongest aversive effect, and therefore it was chosen for further experiments. In a pilot experiment with benzaldehyde in the 20 cm long tube, it became apparent that the odour only range until the half of the tube. To ensure that the odour was distributed in the relevant tube of the T-maze, the long tubes were replaced with shorter tubes (2.5 cm diameter and 10 cm long). A piece of chromatography paper with 60µl of the benzaldehyde/paraffin oil mixture was placed at the end of the tube. The opposite tube was always empty. As control for the olfactory stimulus, flies were tested with empty tubes.

2.5 STATISTICAL ANALYSIS

Statistical analyses were performed using the statistical program *InfoStat* (InfoStat Group, FCA, National University of Córdoba, Argentina). A two sample t-test for paired samples was used to compare experimental groups with control groups, when flies came from one vial and were tested at almost the same time. A two sample t-test for independent samples was used to compare the different quinine/water concentrations in figure 2. In figure 4 a two way ANOVA was performed for multiple comparisons.

3. RESULTS

3.1 LIGHT/DARK PREFERENCE

First of all, it was essential to determine whether the light/dark preference in flies was affected after multiple trials in the T-maze. Therefore, the T-maze Phototaxis assay was conducted for 6 consecutive trials with an ITIT of 30 seconds. Filter paper wetted with 1.5 ml water was placed into both choice tubes to provide the same humid conditions which quinine would implicate in the Phototaxis Suppression Assay. A positive CI was obtained for flies with intact wings suggesting that they still preferred the light, but with a higher variability (figure 2). Flies with clipped wings showed a statistically significant tendency to the dark. This showed that the preference for light or dark remains unaffected by the repetition of trials.

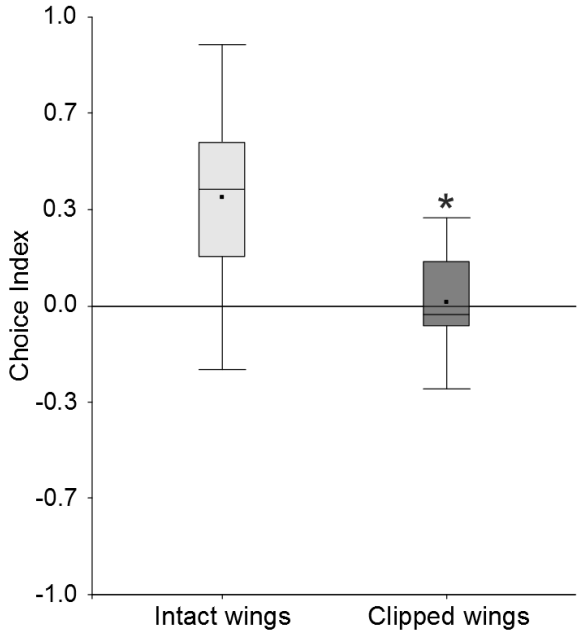


Figure 2: T-maze Phototaxis assay conducted with multiple trials. Flies with intact wings preferred the light (CI = 0.38), while flies with clipped wings preferred the dark (CI = 0.01). T-Test, p- value = 0.0053; N =10. * means significant differences

3.2 QUININE AS THE AVERSIVE STIMULUS

In Le Bourg's Phototaxis Suppression Assay for one single fly, a 0.1 M quinine/water solution was used to suppress the positive phototactic behaviour over a course of 16 trials (Le Bourg & Buecher, 2002). To achieve an aversive effect in flies with intact wings for the group assay,

it was necessary to determine an appropriate quinine concentration. Two concentrations were compared; 0.1 M and 0.15 M. The wetted filter paper was placed into the transparent/lighted tube and the number of trials was reduced from 16 to 9, after considering that 16 trials could be too stressful for the flies given the increased variability observed for the CI after 6 trials (figure 1). The ITIT was kept in 30 seconds. Flies which were exposed to a concentration of 0.1 M displayed a positive choice index and therefore no phototaxis suppression was observed (figure 3). Compared to this, an exposure to a 0.15 M quinine/water solution caused a significant change in flies from a positive into a negative phototactic behaviour. Therefore, a 0.15 M quinine/water solution was selected for further experiments.

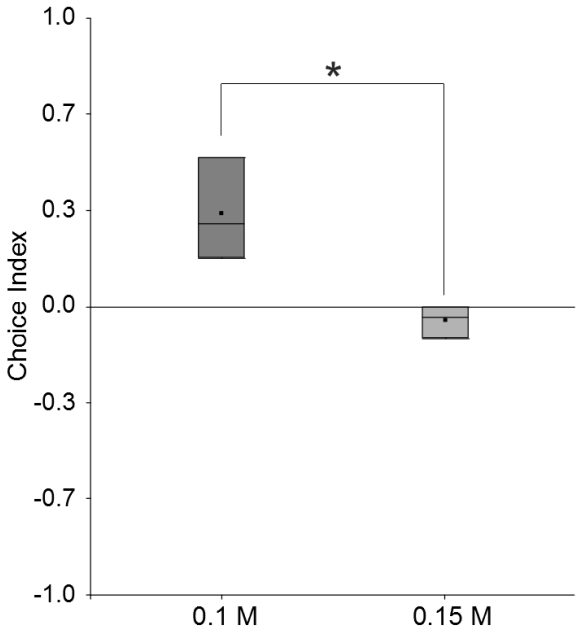


Figure 3: Different quinine/water concentrations. For 0.1 M no phototaxis suppression was obtained in flies with intact wings; CI = 0.32. For 0.15 M an aversive effect was obtained in flies with intact wings, CI = -0.05. The different quinine concentrations were significantly different (*). Paired T-Test, p-value = 0.0551; N =3.

Then, the next step was to adjust the number of trials which were necessary to improve the phototaxis suppression. Therefore, the phototactic behaviour of intact flies after 6 trials and 8 trials was compared. Flies in both control groups (- Quinine, 6 and 8 trials) showed a positive phototactic choice and they were not significantly different (figure 4). Moreover, the different number of trials did not affect the phototactic behaviour in flies in the experimental groups (+ Quinine) and no significant differences were observed between 6 and 8 trials for these groups. Surprisingly, no phototaxis suppression was observed for the

flies after 6 or 8 trials. But, since after 8 trials with quinine/water the CI of the flies was less positive than after 6, even when the outlier was taken into account, the number of trials was fixed in 8 and other parameters of the paradigm were investigated.

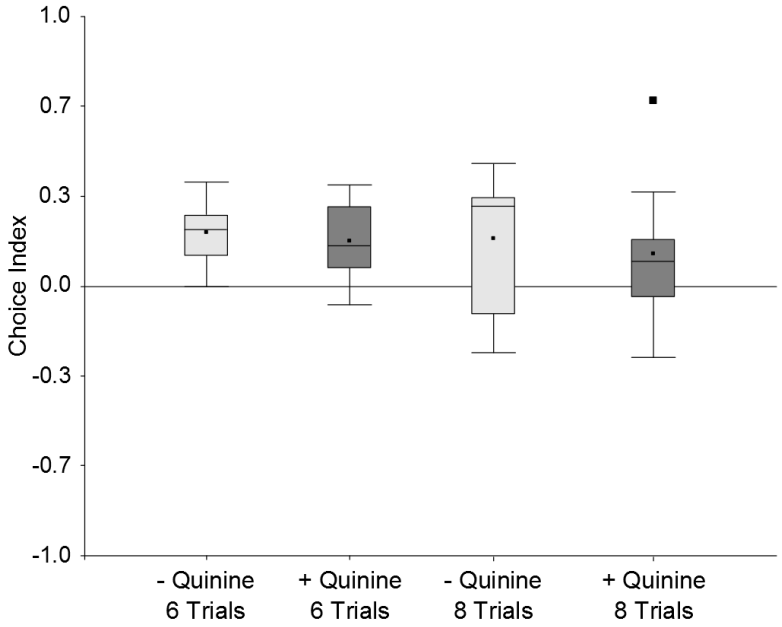


Figure 4: Phototactic behaviour of flies with intact wings in the T-maze for 6 and 8 trials. Both control groups (6 trials, CI = 0.20 and 8 trials, CI = 0.18) and experimental groups (6 trials, CI = 0.17 and 8 trials, CI =0.12) were not significantly different from each other; Two way ANOVA, Interaction p-value =0.8502, Treatment p-value = 0.4710, Trial p-value = 0.6371; N =10.

Seugnet conducted the Phototaxis Suppression Assay for one single fly without delay between trails and therefore no intertrial interval time (ITIT) was specified. This means that after a fly chose between light and dark, it was immediately transferred back to the entrance of the T-maze to start a new trial (Seugnet et al., 2009). However, considering that in learning processes, the ITIT is an important variable during training, and that a complete reduction of the ITIT could be too stressful for the group of flies in the Phototaxis Suppression Assay, a reduction of the ITIT from 30 seconds to 15 seconds was conducted for further experiments. With this conditions, flies in the control group (- Quinine) still showed a positive phototactic behaviour, while flies in the experimental group (+ Quinine) showed a statistically significant phototaxis suppression (figure 5). Thus, a similar result of the single fly assay was achieved: Flies avoided the lighted tube when it was paired with an aversive stimulus. Therefore, an ITIT of 15 seconds and 8 trials were selected to use for further experiments.

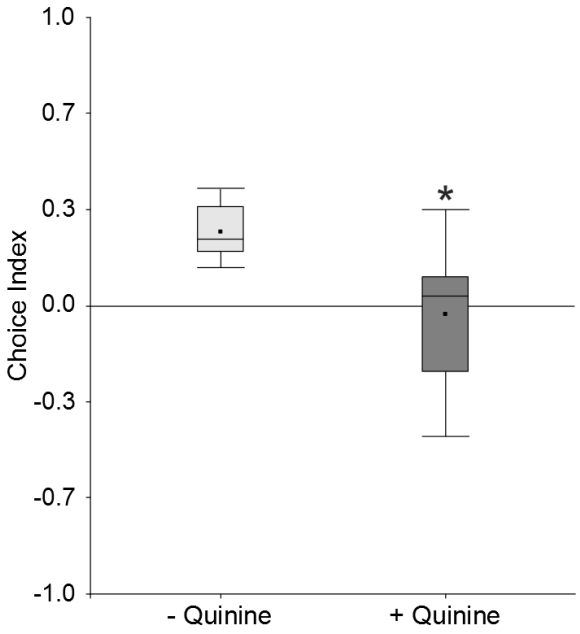


Figure 5: Intertrial interval time reduction in the Phototaxis Suppression Assay. The intertrial interval time was reduced from 30 seconds to 15 seconds. Flies in the control group (-Quinine) showed positive phototactic behaviour (CI = 0.25). Positive phototaxis was suppressed in flies exposed to quinine in the bright tube (+ Quinine) (CI = -0.03). Both groups were significantly (*) different; Paired T-Test, p-value = 0.0065; N =10.

After the proper conditions for the Phototaxis Suppression Assay were established, the next step was to test whether the flies were able to remember the association right after the last trial. Therefore, a 9th trial was added as a test, in which flies faced the choice in the absence of the stimulus, i.e. on extinction (see section 2.4). The filter paper with Quinine was replaced by another one with water. Unfortunately, no memory formation was observed (figure 6). After removing the aversive stimulus, flies in the experimental group (+ Quinine)

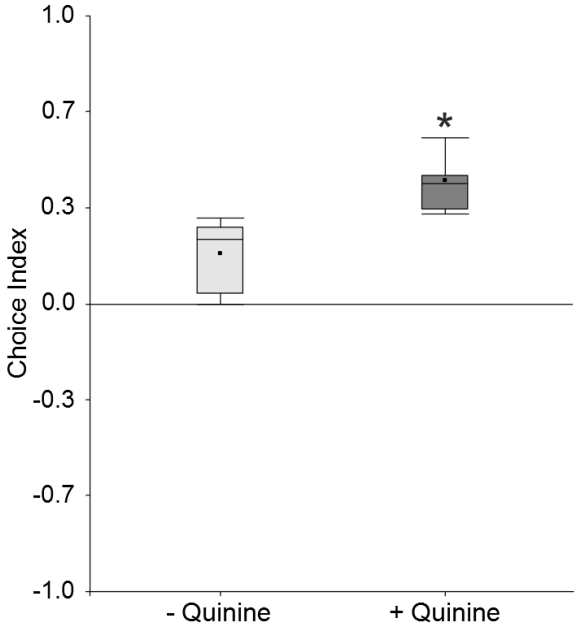


Figure 6: Flies with intact wings tested on extinction. Flies did not show a memory formation after training. Flies in the experimental group displayed a more positive choice (CI = 0.43) than flies in the control group (CI = 0.17); Paired T-Test, significant (*) p-value = 0.0055; N =8.

showed a significantly more positive phototactic behaviour than flies in the control group (- Quinine).

These results were in accordance with Le Bourg's and also Seugnet's findings, which also showed that flies recovered their previous preference after removing the aversive stimulus. Therefore, despite the absence of retention of the association, the Phototaxis Suppression Assay was conducted on flies with clipped wings to test if it was possible to reverse the negative phototactic preference of the flies, caused by clipping their wings, to their prior photopositive behaviour. Wings were clipped under CO_2 (see section 2.2) and a Phototaxis Suppression Assay was performed with the conditions previously established, but placing the aversive stimulus in the dark tube. Surprisingly, no change in phototaxis was observed after the 8 trials for the experimental group. Both groups of flies (+ Quinine and - Quinine) were indistinguishable and displayed a negative phototactic preference (figure 7). Quinine appeared to be not strong enough to induce phototaxis suppression in flies with clipped wings. Taking this into account, the absence of a behavioural change on extinction and the small changes seen in some phototaxis suppression experiments, a different and stronger aversive stimulus was used to repeat the experiments.

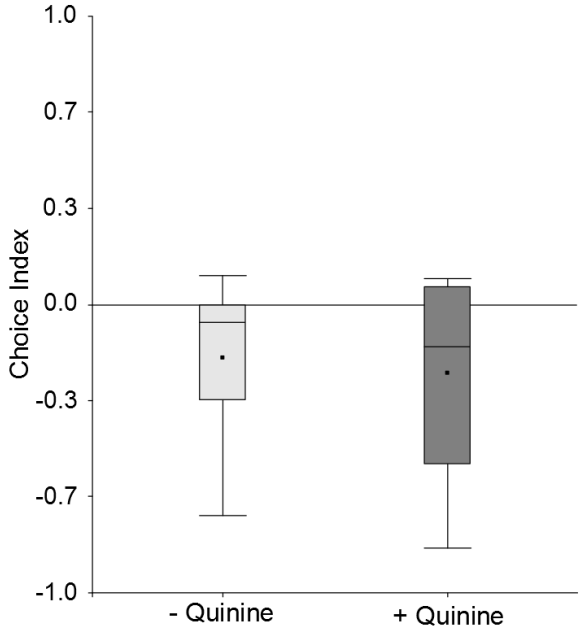


Figure 7: Phototaxis Suppression experiment for flies without wings. No significant differences were obtained between groups (-Quinine, CI = -0.18; +Quinine, CI = -0.24); Paired T-Test, p-value = 0.6329; N =10.

3.3 BENZALDEHYDE AS THE AVERSIVE STIMULUS

In 2012, Knaden screened 110 odours for their attractive and aversive effect on *Drosophila melanogaster* and found benzaldehyde to be the most aversive one (Knaden et al., 2012). Thus, benzaldehyde was a good candidate to be used as a stronger aversive stimulus in the Phototaxis Suppression Assay. To test this hypothesis a phototaxis suppression experiment with intact flies was conducted with a 0.1 mixture (v/v) of benzaldehyde/paraffin oil in the lighted tube (for details on this dilution see section 2.4). On a pilot experiment (data not shown), flies were suspected to habituate to benzaldehyde before reaching the 8th trial in the T-maze. Therefore, the number of trials was reduced to 5. Supporting the hypothesis, flies in the experimental group showed a very strong phototaxis suppression using benzaldehyde as an aversive stimulus (figure 8).

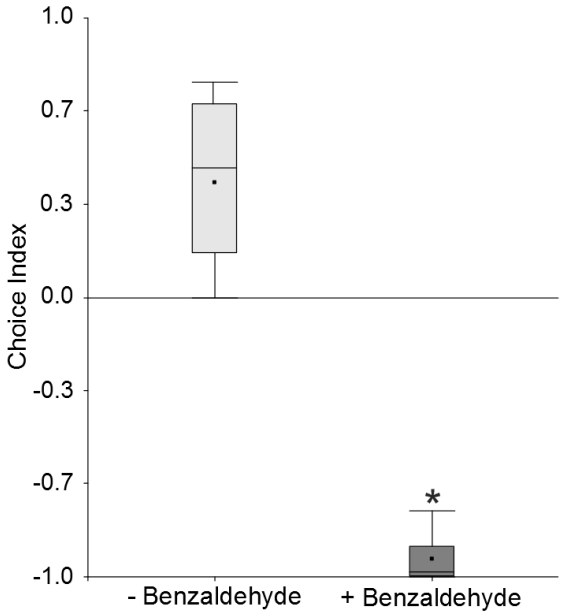


Figure 8: Phototaxis suppression with benzaldehyde in the lighted tube of the T-maze. Flies in the control group displayed a positive phototactic behaviour (CI = 0.41). A significant (*) effect with benzaldehyde was obtained for flies with intact wings (CI =-0.93). Paired T-Test, p-value <0.0001; N =10.

Since a strong phototaxis suppression was obtained with benzaldehyde, flies were also tested on an extinction experiment. Flies were trained for 5 trials with benzaldehyde in the lighted tube and then tested without the aversive stimulus. Both groups, control (- Benzaldehyde) and experimental group (+ Benzaldehyde) displayed positive choice indexes, but benzaldehyde caused a significant reduction in the positive phototactic behaviour (figure 9).

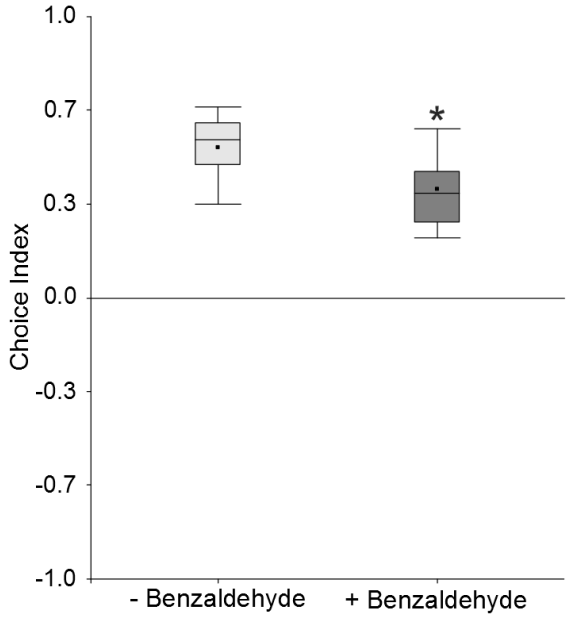


Figure 9: Extinction experiment with benzaldehyde in the lighted tube of the T-maze. Flies with intact wings in both the control group (CI =0.54) and the experimental group (CI =0.39) displayed a positive phototactic behaviour but a significantly (*) statistical difference could be obtained; Paired T-Test, p-value =0.0425; N =10.

The strong results in Phototaxis Suppression Assay obtained with benzaldehyde, encourage the use of benzaldehyde to test the paradigm with wing-clipped flies. Interestingly, flies in the experimental group showed a significant change on their behaviour after being exposed to benzaldehyde in the dark tube (figure 10).

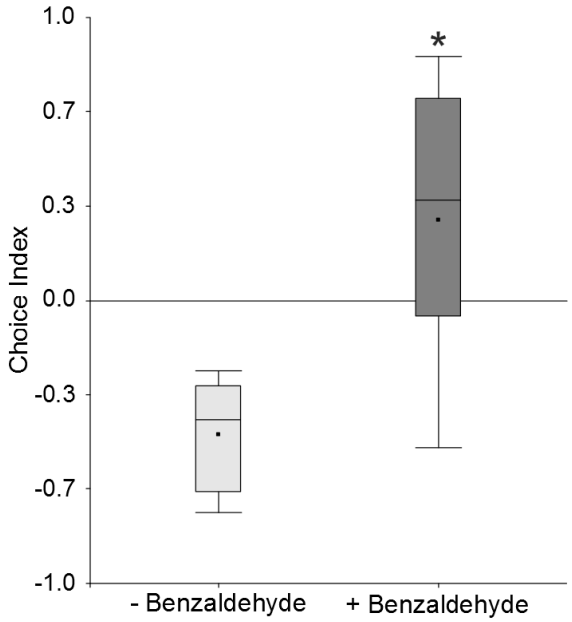


Figure 10: Flies with clipped wings in the Phototaxis Suppression Assay with benzaldehyde in the dark tube of the T-maze. Flies in the control group (CI = -0.47) displayed a negative phototaxis, while flies exposed to benzaldehyde showed a significantly (*) positive phototaxis (CI =0.28); Paired T-Test, p-value =0.0010; N =10.

To know if this aversive effect was strong enough for the flies to form a memory they were tested on extinction. Flies performed 5 trials with benzaldehyde in the dark tube then they were tested without the aversive stimulus. Flies in the experimental group were not significantly different from flies in the control group but the CI was slightly less negative and highly variable (figure 11a). To see whether this result was in fact a real change in the behaviour, another experiment with an N of 10 was conducted. A similar result was obtained and figure 11b shows the 2 experiments combined (N=20). Although the high variability remained, the differences became significant with an N of 20.

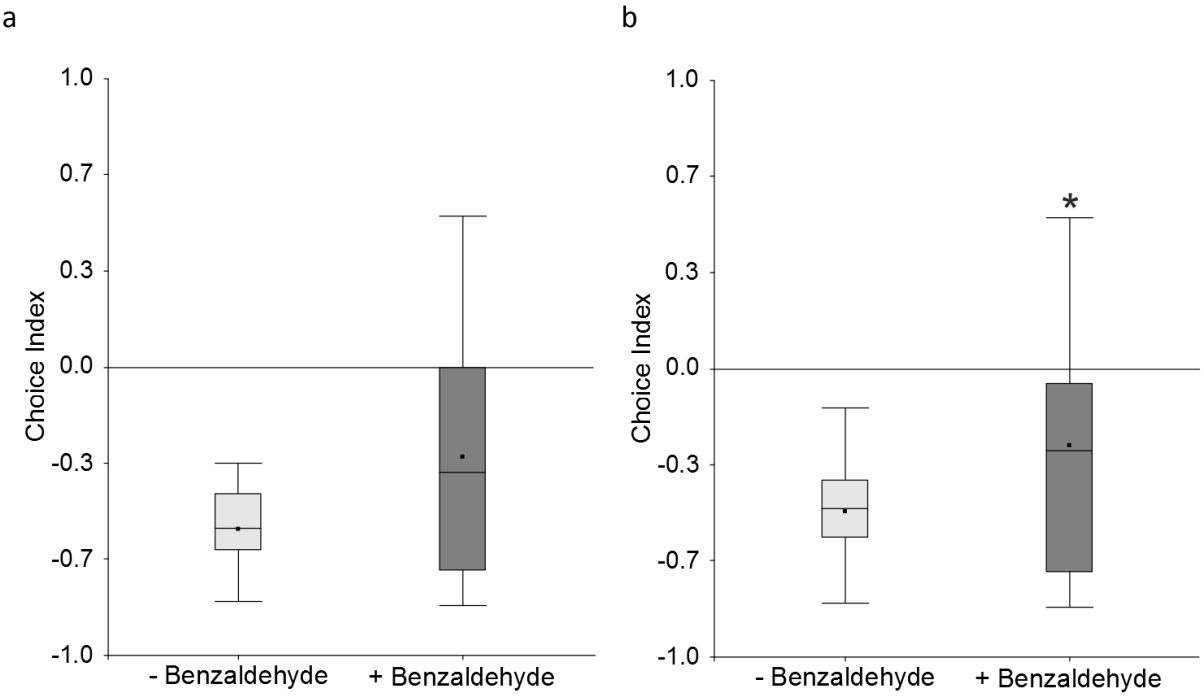
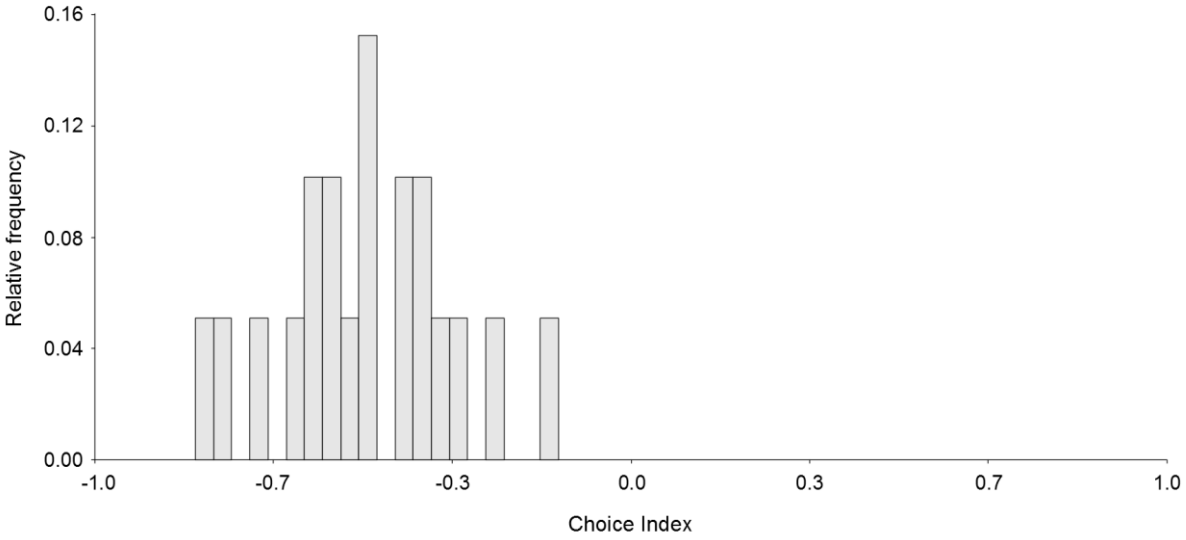


Figure 11: Extinction experiment with benzaldehyde in the dark tube of the T-maze. a. Example of one of the two experiments with 10 replicates. No significant (*) differences were obtained between the control group (CI =-0.56) and the experimental group (CI =-0.31); Paired T-Test, p-value =0.1110. **b.** N =20. A significant (*) difference was obtained between the control group (CI =-0.49) and the experimental group (CI =-0.27); Paired T-Test, p-value =0.0165.

To see if the increased variability was uncovering a change in the distribution due to the fact that only a few experimental groups remembered the association, the histograms of the results were plotted. Figure 12a shows the normally distributed results from the control group. After removing the aversive stimulus, it seems like the choices of the flies in the experimental group can be separated in two groups, one in which flies remember and another one in which flies do not remember the association (figure 12b).

a



b

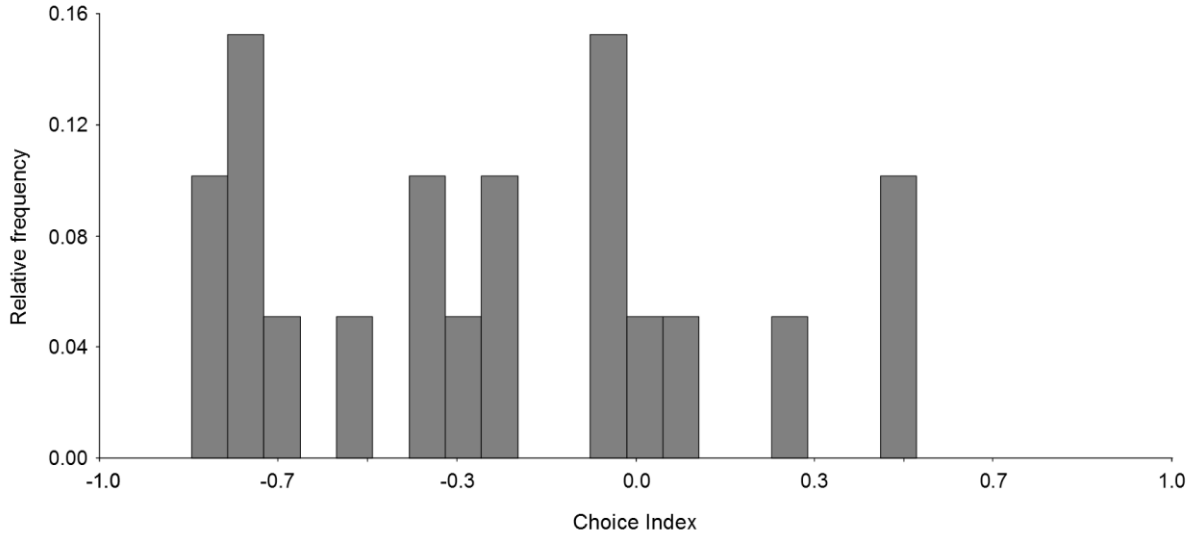


Figure 12: Histograms from extinction experiments of wing-clipped flies trained with benzaldehyde in the dark tube of the T-maze. a. The results of flies in the control group are normal distributed. N =20. **b.** The results of flies in the experimental group seem to be divided in a group in which flies remember and another group in which flies do not remember the association. N =20.

In 1993, Seiger and Kink found that anaesthetising *Drosophila melanogaster* with CO_2 affects their phototactic behaviour (Seiger & Kink, 1993), and it is also known that it could affect other processes such as learning. Thus, it could be possible that the high variability obtained in previous experiments was due to the exposure to CO_2 while clipping the wings which caused that some groups did not learn. To diminish these effects, wings were clipped on a cold station, at 0°C. Then, flies were trained for 5 trials (a normal Phototaxis Suppression

Assay with benzaldehyde) and tested on extinction. As expected, with this pre-treatment under cold temperature, a lower variability could be achieved (figure 13). Unfortunately, although flies in the experimental group displayed significantly higher CIs than flies in the control group, they still remained photonegative. Hence, no reversion to a positive phototactic behaviour was achieved in flies with clipped wings, but a little effect could be observed.

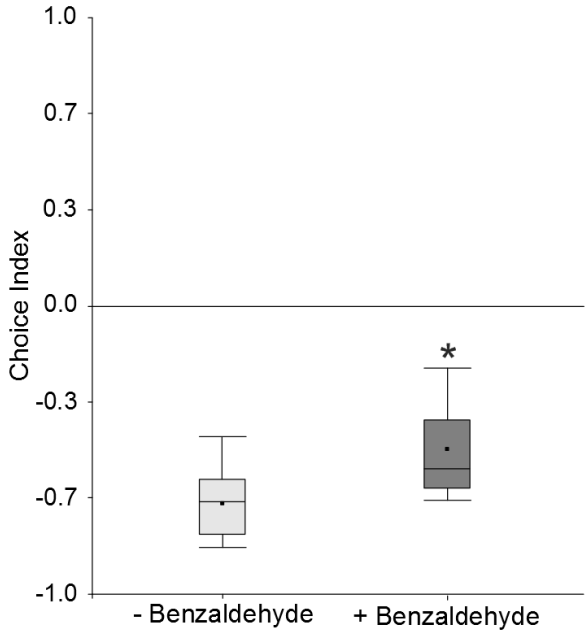


Figure 13: Extinction experiment after clipping flies' wings on cold temperature. Both groups of flies displayed a negative phototactic behaviour. But the CI of the experimental group was significantly less negative than one of the control group (CI =-0.69 and CI = -0.49, respectively). Paired T-Test, p-value =0.0099; N =10.

4. DISCUSSION

The experiments in this thesis have shown that it was possible to convert the Phototaxis Suppression Paradigm from a single fly assay into an assay for a group of flies. The robust phototactic behaviour in flies could be confounded through an association of their light/dark preference and a punishment. Flies with intact wings were able to inhibit their positive phototactic behaviour when the light source was paired with both the gustatory and olfactory aversive stimulus. It is worth to notice, that flies with intact wings showed suppression of the phototactic behaviour for the first time, after the intertrail interval time was reduced. It is possible to hypothesize that the presentations of the aversive stimulus paired to the light need to be executed in quick succession in order to get a significant association between the stimuli. Interestingly, quinine was not strong enough to act as an aversive stimulus for flies with clipped wings, and a stronger aversive stimulus (benzaldehyde) was necessary to suppress their phototactic preference. It is also noticeable, that the avoidance to benzaldehyde was lower in flies with clipped wings than in flies with intact wings. All these indicate that light could become a strong aversive stimulus for flies after losing their flying ability, and therefore an even stronger aversive stimulus is required to suppress the new preference for the dark. This further confirms Gorostiza's finding that the phototactic behaviour is more flexible than thought (Gorostiza et al., in preparation).

Once flies were subjected to extinction by removing the strong olfactory stimulus (benzaldehyde) both flies with intact and clipped wings have shown a reduction in their photopositive/photonegative choice, but no completely switch to the respectively opposite phototactic behaviour could be obtained. In other words, flies were able to form an association between the aversive stimulus and the light (or dark) during training, and it seems that flies maintained that association afterwards, but it was not strong enough to show a behavioural switch in the absence of the aversive stimulus. These results confirm Le Bourg's conclusion that flies cannot be considered as naïve flies after being subjected to a Phototaxis Suppression Assay (Le Bourg & Buecher, 2002). In 1885, Ebbinghaus first identified the phenomenon of the spacing effect, which refers to the fact that information is learned and retained more effectively and led to better memory when presented over spaced intervals rather than repeatedly studied in a short span of time (Ebbinghaus, 1885; Sisti et al., 2007). Therefore, one conceivable extension of the extinction experiment could

be to repeat the procedure of exposing flies to benzaldehyde for 5 trials for more than just one occurrence and with a rest period in between, before subjecting these trained flies to the test on extinction. This experiment could be conducted for different amount of repetitions and different rest period durations. Performing these multiple groups of training phases before testing flies on extinction, may finally improve memory formation in this paradigm. Another possibility to extend the extinction experiment could be to test flies on extinction not only right after presenting them the aversive stimulus but also after a certain time. In other words, different time durations could be set between the last trial with the aversive stimulus and the test. Conducting this procedure may help to identify if flies still perform less positive/negative than the control group after a certain time, which may suggest a learning effect, or if they behave like naïve flies again.

The variability was consistently high across the experiments. One possible reason might be the multiple cycles of tapping and shaking the flies in the T-maze, which could be an excess of mechanical stimulus and causes stress on flies. This could be observed in figure 4, where the variability increase from 6 to 8 trials, but further experiments need to be done to confirm this. Moreover, Gorostiza observed that if the density of flies is too high in the T-maze apparatus, the results become more variable (Gorostiza et al., in preparation). Thus the amount of flies, participating in T-maze experiments, may also be an issue for the Phototaxis Suppression Assay. Furthermore, the results of the extinction experiments after clipping flies' wings on cold temperature (figure 13) supports the hypothesis that this variability may occur due to the previous wing-clipping procedure under CO_2. Flies in the control group as well as the flies in the experimental group were much less variable when pre-treated with cold temperature than with CO_2 (figure 12a/12b). For future experiments it would be better to clip flies' wings on cold temperature. The variability in the first light/dark preference experiment might be because of insufficient tapping to transfer the flies from one tube to the other, and/or because of killing a few flies in the elevator during the transfer-steps, as it is usual for beginners.

In 2014, Ramdya found that *Drosophila melanogaster* shows collective odour avoidance to CO_2 which arises from cascades of appendage touch interactions between pairs of flies (Ramdya et al., 2014). It cannot be excluded that these inter-fly interactions prevail in the Phototaxis Suppression Assay for a group of flies as well. For example, these interactions

could be one reason for the result in figure 6, where flies with intact wings which were subjected to extinction displayed an even more positive phototactic choice than the control flies. It could be possible, that a few flies realised, that there was no quinine, and therefore no punishment in the lighted/dark tube anymore. These flies could have been interacting with the other flies to trigger a cascade of encounters and interactions between flies, which provoked an enhanced collective photopositive choice. This is just one possibility how inter-fly interactions could influenced the flies choices and behaviour. In addition, it can be assumed that these inter-fly interactions may also be affecting the results of the test on extinction. Further research with regard to the interactions between flies during the experiments in this thesis, would help to understand its relevance for the Phototaxis Suppression Assay.

ACKNOWLEDGEMENTS

At this point I would like to take the opportunity and thank all those who have supported and motivated me during my Bachelor's project.

I am deeply grateful to Prof. Dr. Björn Brembs. Thank you for granting me the opportunity to have been part of your lab and to become insights in such an alluring and fascinating field of research. Thank you for giving me constructive comments and suggestions on my work, for teaching me how important and beneficial it is to share your thoughts and that it is worth to be brave enough to investigate new aspects in science.

My heartfelt thanks and appreciation goes to Dr. E. Axel Gorostiza who not only supervised my work but also me. Thank you so much for your time you have given me, even if you had other things to do. Thank you for supporting and encouraging me constantly and for never letting me down, especially when the results of the experiments frustrated me. I want to express my gratitude to you for your friendship.

I also want to thank Christian Rohrsen for always bringing a smile on my face.

Now I would like to express my gratitude to the three of them. Thank you so much for your patience, your confidence in me and for welcoming me with so much kindness, that I felt that I belonged to you. Thanks for showing me how to work on a project as a scientist, for teaching me concepts and methods in Drosophila and also how to cope with failure. It was an honour to work with you and I really appreciated the feedback, support and help you offered me all the time. I will always look back to my Bachelor's project with great memories. The time I spent with you to work on my thesis strengthened me in my decision to go my way in science.

I would like to offer my special thanks to my family. I cannot find words to express how important all of you are to me. Thank you so much for always supporting me in every aspect and situation of my life.

LITERATURE

Benzer, S. (1967) Behavioral mutants of Drosophila isolated by countercurrent distribution. *Proc Natl Acad Sci U S A 58,* 1112-9.

Brembs, B. (2008) Operant learning of Drosophila at the torque meter. *J vis Exp 16,* 731.

Ebbinghaus, H. (1885) *Über das Gedächtnis.* Leibzig, Germany : Verlag von Duncker & Humblot, 1885.

Gorostiza, E.A., Colomb, J. & Brembs, B. (in preparation) A value-based behavioural choice underlies phototaxis in Drosophila.

Hirsch, J. & Boudreau, J.C. (1958) Studies in experimental behavior genetics. I. The heritability of phototaxis in a population of Drosophila melanogaster. *J Comp Physiol Psychol 51,* 647–651.

Knaden, M., Strutz, A., Ahsan, J., Sachse, S. & Hansson, B. S. (2012) Spatial Representation of Odorant Valence. *Cell Rep 1,* 392-399.

Le Bourg, E. & Buecher, C. (2002) Learned suppression of photopositive tendencies. *Animal Learning& Behavior 30,* 330-341.

McEwen, R.S. (1918) The reactions to light and to gravity in Drosophila and its mutants. *Journal of Experimental Zoology 25,* 49 - 106.

Ramdya, P., Lichocki, P., Cruchet, S., Frisch, L., Tse, W., Floreano, D. & Benton, R. (2014) Mechanosensory interactions drive collective behaviour in Drosophila. *Nature 519,* 233-236.

Seugnet, L., Suzuki, Y., Stidd, R. & Shaw, P.J. (2009) Aversive phototaxic suppression: evaluation of a short-term memory assay in Drosophila melanogaster. *Genes Brain Behav 8,* 377-389.

Sisti, H.M., Glass, A.L. & Shors, T.J. (2007) Neurogenesis and the spacing effect: learning over time enhances memory and the survival of new neurons. *Learn Mem 14,* 368-375.

FIGURES