

# Behavior under uncontrollable stress in *Drosophila melanogaster* – Learned Helplessness revisited

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Verhalten unter nicht kontrollierbarem Stress – Neubetrachtung der  
Erlernenen Hilflosigkeit bei *Drosophila melanogaster*



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## Für meine Familie

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„And those who were seen  
dancing  
were thought to be insane  
by those  
who could not hear the  
music.“  
(Friedrich Nietzsche)

## Content

|          |  |           |
|----------|--|-----------|
| <b>1</b> | <b>Introduction .....</b>  | <b>1</b>  |
| 1.1      | Learned helplessness .....   | 1         |
| 1.2      | Studying the response and behavioral changes due to inescapable stress – the shock box ..... | 3         |
| 1.3      | Perception and avoidance of electric shock .....   | 4         |
| 1.4      | Neuronal bases .....   | 4         |
| 1.5      | Aims of this work .....  | 6         |
| <b>2</b> | <b>Materials and Methods .....</b>   | <b>7</b>  |
| 2.1      | Flies .....  | 7         |
| 2.2      | Setup.....   | 7         |
| 2.2.1    | Shock box.....   | 7         |
| 2.2.2    | Free walk arena .....  | 7         |
| 2.2.3    | Mating wheel and courtship behavior .....  | 9         |
| 2.3      | No-idleness experiment.....  | 9         |
| 2.4      | Handling.....  | 9         |
| 2.5      | Place learning .....   | 10        |
| 2.6      | High-performance liquid chromatography (HPLC).....   | 10        |
| 2.7      | Statistical analysis.....  | 10        |
| <b>3</b> | <b>Results .....</b>   | <b>11</b> |
| 3.1      | The shock box.....   | 11        |
| 3.2      | Implementation of suitable test conditions .....   | 11        |
| 3.2.1    | Varying the aversive stimulus .....  | 11        |
| 3.2.2    | Varying the conditioning duration .....  | 14        |
| 3.2.3    | Adjusting shock intensity in place learning.....   | 16        |
| 3.3      | No-idleness experiment.....  | 17        |
| 3.3.1    | Analysis of walking activity.....  | 18        |
| 3.3.2    | Distribution of duration of activity bouts .....   | 19        |
| 3.3.3    | Impact of inescapable shocks on number and duration of pauses .....                          | 21        |
| 3.3.4    | Analysis of short pauses .....   | 22        |
| 3.3.5    | Analysis of walking speed .....  | 24        |
| 3.3.6    | Escape latency.....  | 25        |

---

|          |  |           |
|----------|--|-----------|
| 3.3.7    | Sexual dimorphism .....  | 26        |
| 3.4      | Reversal learning .....  | 28        |
| 3.5      | Comparison between two aversive stimuli.....   | 30        |
| 3.5.1    | Heat versus electric shock .....   | 30        |
| 3.5.2    | Influence of humidity on walking behavior .....  | 34        |
| 3.6      | Impact of inescapable electric shocks on other behavior .....  | 35        |
| 3.6.1    | Impact of stress on place learning.....  | 35        |
| 3.6.2    | Chamber exit latency – Positive phototaxis.....  | 37        |
| 3.6.3    | Courtship behavior subsequent to stress in male flies .....  | 38        |
| 3.6.4    | Impact of inescapable shock on free walk .....   | 39        |
| 3.6.5    | Does handling abolish the after effect observed in the boxes? .....  | 41        |
| 3.6.6    | Impact of inescapable shocks on monoamine levels in the brain .....  | 43        |
| 3.7      | How prior experience influences response to uncontrollability .....  | 44        |
| 3.7.1    | Social isolation as a stressor .....   | 45        |
| 3.7.2    | Comparison of single-reared and group-reared flies in walking behavior<br>without shocks.....                        | 45        |
| 3.7.3    | Comparison of single-reared and group-reared flies during conditioning<br>and test .....                             | 46        |
| 3.7.3.1  | Single-reared female flies react differently to uncontrollable stress<br>compared to group-reared female flies ..... | 46        |
| 3.7.3.2  | Single-reared flies are less affected by uncontrollability .....   | 49        |
| 3.7.3.3  | The reaction to stress does hardly differ between single-reared and<br>group-reared male flies .....                 | 49        |
| 3.7.3.4  | No sustainable after-effect in single-reared male flies can be<br>measured.....                                      | 52        |
| 3.7.4    | Comparison of walking activity between virgin- and non-virgin female<br>flies .....                                  | 52        |
| 3.8      | Genetic intervention .....   | 53        |
| 3.8.1    | Learned helplessness in <i>Drosophila</i> involves dopamine .....  | 53        |
| 3.8.2    | Role of <i>rutabaga</i> in learned helplessness .....  | 55        |
| <b>4</b> | <b>Discussion .....</b>  | <b>61</b> |
| 4.1      | Is uncontrollability the stressor? .....   | 61        |
| 4.2      | Heat versus electric shock .....   | 62        |
| 4.3      | Trans-situationality .....   | 63        |
| 4.4      | Social isolation – influence on learned helplessness.....  | 65        |

|          |   |           |
|----------|---|-----------|
| 4.5      | Dopamine is involved in learned helplessness .....      | 66        |
| 4.6      | Role of adenylate cyclase in learned helplessness ..... | 67        |
| 4.7      | Revisiting Learned helplessness .....                   | 68        |
| 4.8      | Conclusion .....  | 70        |
| 4.9      | Outlook.....  | 70        |
| <b>5</b> | <b>Summary .....</b>                                    | <b>71</b> |
| <b>6</b> | <b>Zusammenfassung.....</b>                             | <b>73</b> |
| <b>7</b> | <b>Literature.....</b>                                  | <b>76</b> |
| <b>8</b> | <b>Appendix .....</b>                                   | <b>84</b> |
| 8.1      | Affidavit .....   | 84        |
| 8.2      | Curriculum Vitae.....                                   | 85        |
| 8.3      | Publication and Conference contributions .....          | 87        |
| 8.4      | Danksagung.....   | 88        |

# 1 Introduction

Animals have to deal with their environment. They need the ability to adapt to certain environmental circumstances in order to save energy, feed, reproduce or escape life-threatening situations. How animals choose the optimal behavior out of all possible behaviors, has been little understood (Heisenberg, 2015). An essential role of the brain is to organize behavior thus in order to understand behavioral control one has to study behavior and the brain. Within the huge field of behavioral control, I study behavior under stressful conditions. Being exposed to uncontrollable stimuli can induce a phenomenon called learned helplessness (Seligman and Maier, 1967). This can in turn impede or change patterns of behavior that do not occur, if stress is still controllable (Seligman and Maier, 1967; Seward and Humphrey, 1967; Braud et al., 1969).

## 1.1 Learned helplessness

For the term learned helplessness the Encyclopedia Britannica provides the following definition: “Learned helplessness, in psychology, a mental state in which an organism forced to bear aversive stimuli, or stimuli that are painful or otherwise unpleasant, becomes unable or unwilling to avoid subsequent encounters with those stimuli, even if they are ‘escapable’, presumably because it has learned that it cannot control the situation.” (Nolen, 2015). The term was introduced in the late 1960’s by two psychologists, Seligman and Maier (1967), who discovered the phenomenon incidentally during an experiment with dogs. First exposed to inescapable stress the dogs later failed to learn to escape in a ‘shuttle box’ experiment. The authors assigned the dogs to three groups (triadic design) which they called master, yoked and control. The master dogs could end the electric shock by pressing a lever, while the yoked dogs were randomly exposed to it, dependent on what the master dog did. Control dogs did not receive any punishment. In the second part of the experiment, both groups could escape the unpleasant shocks by jumping over a low partition (‘shuttle box’). The yoked dogs failed more often to escape the shocks, while the master dogs managed to jump over the low partition. The authors stated that the yoked dogs have learned that they could not escape the shock and therefore failed in the shuttle box. This phenomenon was termed learned helplessness (Seligman and Maier, 1967). Now, 50 years later they revisited their work and reviewed it regarding the discoveries in that research field since the initial finding in 1967. They state, that the failure in the shuttle box was due to passivity, not necessarily due to associative learning (Maier and Seligman, 2016).

Since the introduction of the term learned helplessness, the phenomenon has been investigated as well in other animals than dogs, for instance in cats (Seward and Humphrey, 1967), goldfish (*Carassius auratus*) (Behrend and Bitterman, 1963), but most intensively in rats and mice (Vollmayr and Henn, 2001). Besides vertebrates, learned helplessness has been described for invertebrates such as cockroaches (*Periplaneta americana*) (Brown and Stroup, 1988; Brown et al., 1994a) and slugs (*Limax maximus*) (Brown et al., 1994b). Using the same experimental principles as Seligman and Maier, Brown and Stroup (1988) exposed cockroaches to inescapable shocks (on three consecutive days) and tested them 24 hours later in a shuttle box paradigm. The 'inescapable' group failed to escape while the control and the 'escapable' group performed equally well.

Brown and his colleagues were the first who described learned helplessness for *Drosophila*. Mechanical shaking was used to induce the state of helplessness in flies (Brown et al., 1996). The fruit fly (*Drosophila melanogaster*), as a classical model organism, with its comparatively low number of neurons (~135.000 neurons, (Alivisatos et al., 2012)) is used for studies in behavior and developmental biology. It also plays a crucial role in the research of human diseases, because more than 60% of human genes have orthologues in *Drosophila* (Adams et al., 2000; Wangler et al., 2015). Based on Browns (1996) discovery, Ries (2015) used a similar method. She induced helplessness by shaking the flies for eight hours on three consecutive days. Later, the motivation to climb, walk and to mate was lowered compared to flies which had not been exposed to shaking. Based on the triadic design with a master group that could control the stimulus, a yoked group that could not and a group that did not receive any stressor at all (control), Bertolucci (2008) found that flies which experienced uncontrollable heat (also see chapter 1.2) later failed in a place learning experiment. Yang et al. (2013) characterized the walking behavior under uncontrollable heat in more detail and pointed out that learned helplessness can be divided into a cognitive and a motivational part. Flies exposed to uncontrollable heat showed lower walking activity, walked more slowly (which represents the motivational part) and needed more time to resume walking after the onset of the heat (which represents the cognitive part) than flies that were able to turn the heat off. This may have resulted from a perceived absence of control, because the inherent behavior did not lead to the expected outcome of the situation. Thus the inborn behavioral patterns were suppressed in order to adapt to the new situation. The outcome expectation is what changes, if external conditions change for an animal in such a way, that innate behavior for controlling the situation does not help and therefore can result in learning to suppress the behavior (Heisenberg, 2015). It has been shown that flies also use this mechanism to choose a certain behavior. Yang



et al. (2013) for example also stated that the suppression of inborn behavior, e.g. escape response, due to futile attempts has developed early in evolution, before the separation of vertebrates and arthropods.

It is known already from vertebrates that the state of learned helplessness can persist for some time and has an influence on other behaviors, namely poor learning ability, reduced courtship behavior as well as reduced sleep and immune status (Seligman and Maier, 1967; Willner, 1986; Eisenstein and Carlson, 1997). The behavior, the animals showed during and subsequent to stress, was compared to depressive-like behavior and thus as well studied in the context of depression research (Krishnan and Nestler, 2011). According to the World Health Organization (WHO, 2016), 350 million people are suffering from this disease. Women are twice as often affected as men (Lopez et al., 2006). Therefore, animal models of similar but simpler phenomena are highly welcome. However, little is known about the neuronal underpinnings and the transfer to depression research implies some limitations. In order to gain new knowledge about the disease one can compare and highlight equivalent symptoms in animal models during and subsequent to the exposure to stress and interpret them as homologies to the symptoms of depression as a psychiatric disorder (Nestler and Hyman, 2010).

## **1.2 Studying the response and behavioral changes due to inescapable stress – the shock box**

To understand how behavior is controlled in the brain, one way of analyzing this is to first characterize the behavior in question in detail. An apparatus in which the observation of single fly behavior is possible, was designed by Wustmann et al. (1996). In the so-called heat box, the fly walks along a narrow chamber and can be observed by the experimenter throughout the entire experiment. Contrary to the well-established Tully-machine, which has been used to study fly behavior for over 40 years (Quinn et al., 1974), the heat box provides the opportunity to not only observe the animal's decision post hoc, but also observe the animal during the decision-making process. It was rebuilt and used in many studies (Putz and Heisenberg, 2002; Sitaraman et al., 2008). Since place learning, where a fly has to learn to avoid one side of the chamber (compare Materials and Methods, chapter 2.5), as well as 'no-idleness' learning (avoiding punishment by being active, for more details see Materials and Methods, chapter 2.3) were well characterized in the heat box (Putz and Heisenberg, 2002; Bertolucci, 2008; Yang et al., 2013), the question came up, whether using an aversive stimulus different from heat to condition the flies, would lead to similar results. In the course of this idea, the shock box was designed (Kapustjansky,

2011). Especially because electric shock functions as an immediate stimulus, while heat develops gradually over time. The heat- and the shock box (compare chapter 3.1) were built as multi-chamber setups, which allow to monitor many flies simultaneously. In this study the shock box was used to systematically investigate the behavioral changes due to learned helplessness (Seligman and Maier, 1967) or learned uncontrollability.

### 1.3 Perception and avoidance of electric shock

The sensation of harmful stimuli causes in most cases an immediate avoidance behavior, which is important for survival (Sneddon, 2004). A one-cell organism such as *Paramecium* can already avoid damaging chemicals (Giuffre et al., 2011). In *Drosophila*, the nociception of heat has been studied extensively (Im and Galko, 2012). Because of its importance for survival, nociception was developed early in evolution. In this study, electric shock is used to condition flies. The perception of heat and electric shock is being mediated by different peripheral receptors and neural pathways (Galili et al., 2014) and it is especially interesting to test whether inescapable electric shock leads to similar results as inescapable heat. If that would be the case, it could confirm the theory that the strategy of how an animal copes with uncontrollable situations is evolutionarily highly conserved and independent of the stimulus itself.

### 1.4 Neuronal bases

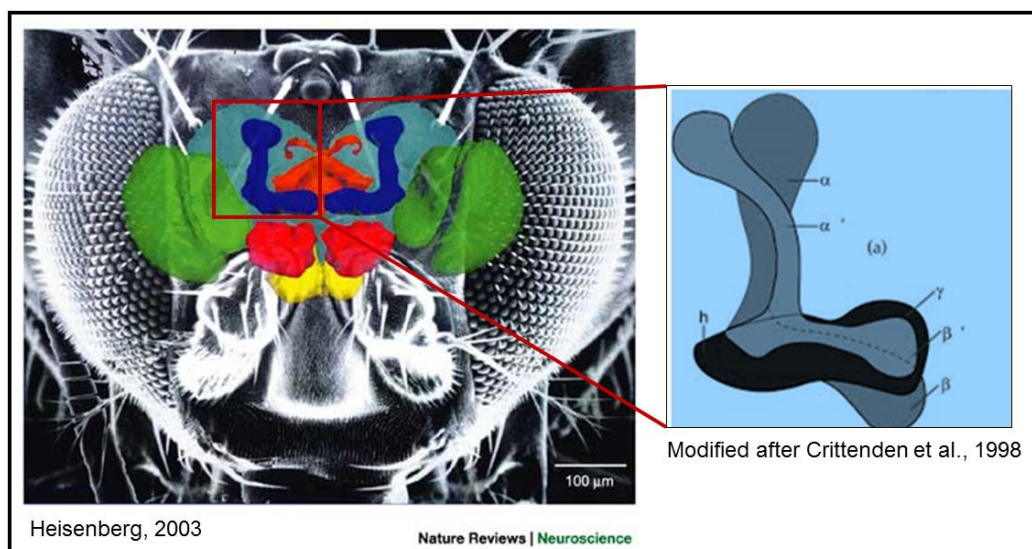
As mentioned earlier, the neuronal underpinnings of the phenomenon learned helplessness are sparsely understood. Serving as an animal model of depression, the biogenic amines are of interest to be studied in the context of learned helplessness. Dopamine for example is involved in behavioral control in vertebrates and invertebrates (Egerton et al., 2009; Van Swinderen and Andretic, 2011). Argue and Neckameyer (2013) found it to be involved in the response to stress in *Drosophila*. The fact that dopamine plays a role in behavioral control under stressful situations suggests dopamine as an interesting subject to be investigated in the context of uncontrollability induced by electric shock.

Electric shock is signaled through dopaminergic neurons in the mushroom bodies (MBs) (Schwaerzel et al., 2003; Aso et al., 2010). The signal releases dopamine which binds to G-Protein coupled receptors which in turn elicits a cAMP cascade. Learning and memory genes, like *dunce (dnc)* and *rutabaga (rut)* as well as the protein kinase A (PKA) are known to play a role in this process (Dudai et al., 1976; Duerr and Quinn, 1982). Since *rut*, which encodes a calcium sensitive adenylylase, first discovered to be associated with

impaired olfactory memory (Livingstone et al., 1984), is highly expressed in the MBs, it seems a promising candidate to test within the learned helplessness paradigm.

The MBs of *Drosophila* hold a major role in several higher brain functions, particularly in olfactory associative learning (Heisenberg et al., 1985; Heisenberg, 2003; Dubnau and Chiang, 2013). Based on electrophysiological experiments, Crittenden et al. (1998) described the neurons of the MBs as a center of sensory integration. Flies without MBs show several deficits. They show difficulties in decision-making, when exposed to two opposing stimuli (Tang and Guo, 2001) and they are impaired in context generalization in the flight simulator (Liu et al., 1999). Additionally it is known that the MBs are also needed for sleep regulation (Yi et al., 2013).

It was shown that several extrinsic MB cells form synapses in the calyx, a substructure of the MBs lying on the posterior-dorsal side of the brain, amongst them neurons releasing biogenic amines such as dopamine and octopamine (Aso et al., 2012; Butcher et al., 2012; Pech et al., 2013). The approximately 2500 Kenyon cells (KCs) receive input in the calyx and form axons building the peduncle and the lobe system. The KCs are subdivided into three categories, the  $\alpha\beta$ - neurons,  $\alpha'\beta'$ - neurons and the  $\gamma$ - neurons, while the  $\alpha/\beta$  compartment can be further subdivided into  $\alpha/\beta_s$  (surface),  $\alpha/\beta_c$  (core) and  $\alpha/\beta_p$  (posterior) (compare Fig. 1). Aso et al. (2014) defined 15 distinct compartments within the MB-lobes. They showed that the lobes are highly specified and play different roles at different time points in distinct tasks.



**Fig. 1:** The mushroom body of *Drosophila melanogaster* (Heisenberg, 2003).

## 1.5 Aims of this work

The present study investigates how the fly *Drosophila melanogaster* copes with uncontrollable stress induced by electric shock and the changes in behavior which occur due to the fact that the animal cannot influence an aversive stimulus.

The study investigates whether stress, induced by electric shocks, leads to similar behavior as already observed in the heat box before (Bertolucci, 2008; Yang, 2015). The first goal is to set up the new multi-chamber device to monitor single fly behavior and to implement the no-idleness- and the place learning paradigms in order to investigate learned helplessness. The present work also deals with the question whether the observations made in the boxes transfer to free walk, to courtship, to phototaxis or to learning behavior and whether a sexual dimorphism exists in the context of this paradigm.

Not only after-effects are studied but also the influence of previous experience on the response to stress in the small chambers is examined. For that purpose, flies – either single- or group-reared – are compared in the presence and absence of uncontrollability.

In the last part of this work, the first steps towards unraveling the neuronal basis underlying learned helplessness are taken, in the course of which the role of dopamine is analyzed by testing a mutant called *fumin* which has a lesion in the dopamine re-uptake transporter gene. Additionally, the role of the adenylate cyclase is examined as well as its role in the mushroom bodies during the exposure to stress.

## 2 Materials and Methods

### 2.1 Flies

All flies used in this study were kept at 25°C on standard food on a 12h light/dark cycle with 60% relative humidity in mixed groups. Flies were tested when they were 3-5 days old. Immediately before the test, flies were transferred to a fresh food vial to avoid grooming behavior during the experiments. *Drosophila melanogaster* (wild type CantonS) and *rutabaga*<sup>2080</sup> flies were obtained from the Biocenter Würzburg. The RNAi stocks were from Vienna Drosophila Resource Center (VDRC) (#106961 and #12082) and the *fumin* line from Kazuhiko Kume (Nagoya University, Nagoya, Japan).

For the courtship experiment, male flies were collected one day after eclosion and kept in small groups until testing. For social isolation, flies were transferred to small food vials, one fly per vial, at 1-2 hours after eclosion. For the experiments using virgins, flies were collected immediately after hatching and kept in female groups, to prevent mating.

### 2.2 Setup

#### 2.2.1 Shock box

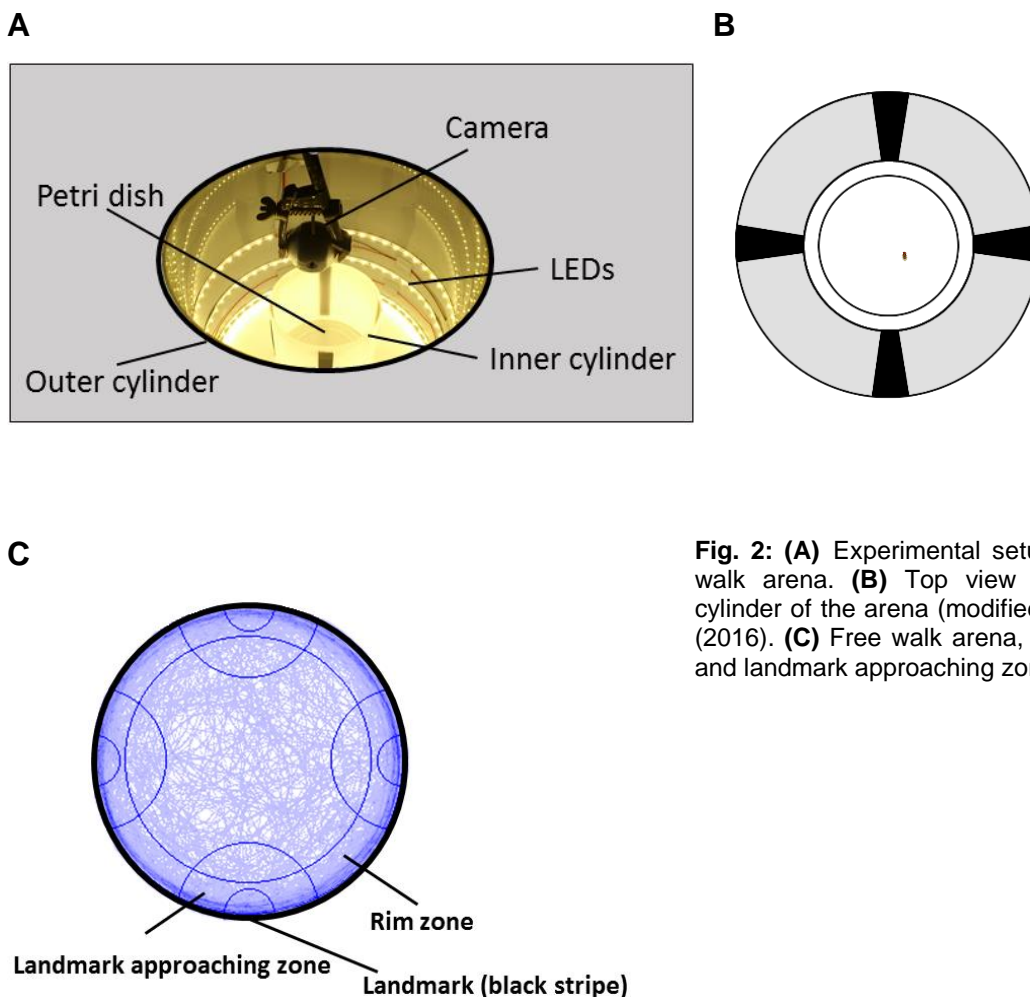
The setup built in the workshop of the Biocenter, Würzburg consisted of 16 shock boxes. Each box contained a chamber (29 x 4 x 2mm<sup>3</sup>) to house a single fly (Fig. 4). Floor and ceiling of the chamber were covered by electric grids to deliver electric shocks. The side walls were made of glass. The chamber was illuminated from one side by an infrared light source and a barcode reader was positioned on the other side to track the fly's shadow. The position of a fly was resolved in 0.2mm units and recorded at 10 Hertz (Hz). The boxes were connected to a computer and operated by a custom made software (shockgui-0.07) written by Andreas Eckert (Biocenter, Würzburg). The temperature in the chamber was kept at 24°C. Humidified air at approximately 90% relative humidity (RH) was provided before and after the experiments using a wash bottle filled with distilled water (Hartenstein, Laboratory equipment, Würzburg, Germany). The chambers were cleaned using an interdental brush (~4mm thick, DONTODENT) after every experiment.

#### 2.2.2 Free walk arena

To record walking trajectories in an open field I built a free walk arena (Fig. 2A, B). It consisted of a modified Petri dish (Ø=86mm, h=4mm) covered by a glass lid and placed on white paper. The arena was surrounded by an opaque acrylic glass cylinder

( $\varnothing=125\text{mm}$ ,  $h=130\text{mm}$ ) with four vertical black stripes ( $h=130\text{mm}$ ,  $w=15\text{mm}$ ) spaced at  $90^\circ$  on its inner surface, serving as landmarks. The cylinder was again surrounded by another translucent milk-glass cylinder ( $\varnothing=295\text{mm}$ ,  $h=240\text{mm}$ ). The inner surface of the outer cylinder carried three horizontally running rows of LEDs (Flex Strip, Synergy 21, Germering, Germany) to illuminate the arena. Trajectories were tracked for 10 minutes at 40Hz using a standard USB webcam (Logitech, C500), stored on a computer and later evaluated using a custom made software (VB.NET). Walking activity in the free walk arena was defined as walking  $>4\text{mm/s}$  and was scored as the percentage of the overall experimental time. The rim zone was defined as the outer 20% of the radius (Batsching et al. 2016).

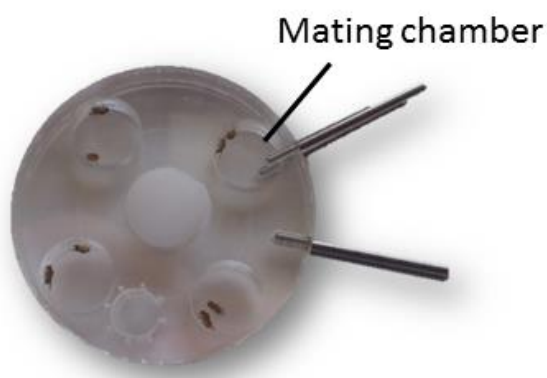
In Fig. 2C, a top view of the arena is displayed, indicating the rim zone and the landmark approaching zone. The inner circle defines the rim zone, while the four areas, represented by the semi-circles, indicate the landmark approaching zones. This threshold area (two semi-circles, indicating the approaching zone) was defined in order to prevent measuring every small movement of the fly. Only flies which crossed both semi-circles were counted as approaching the landmark.



**Fig. 2:** (A) Experimental setup of the free walk arena. (B) Top view on the inner cylinder of the arena (modified after Koenig (2016)). (C) Free walk arena, with rim zone and landmark approaching zone.

### 2.2.3 Mating wheel and courtship behavior

Courtship was recorded in daylight at 24°C room temperature using a "mating wheel" with four chambers ( $\varnothing=8\text{mm}$ ,  $h=6\text{mm}$ ;  $v=0.3\text{cm}^3$ ), modified after Siegel and Hall (1979) in the workshop of the Biocenter, Wuerzburg and a USB webcam (Logitech, C500) (see Fig. 3). Videos were later analyzed by inspection. Courtship and wing vibration indices were calculated as % of time flies showed the respective behavior. Courtship latency is the time the flies started to show any courtship behavior, wing vibration latency is calculated the same way (Batsching et al., 2016).



**Fig. 3:** Mating Wheel

### 2.3 No-idleness experiment

The master fly in the shock box received shocks in case it rested for more than 0.9 seconds. The punishment was immediately switched off if the fly resumed walking. Activity was defined as walking  $>0.6\text{mm/s}$ . Whenever the master fly got shocked the yoked fly in a separate chamber got shocked as well, independent of its locomotor activity. A third group (control flies) did not receive any shocks at all, while walking in the chamber. The experiment consisted of 1 minute pretest, 20 minutes conditioning, followed by 10 minutes test, unless otherwise specified. Only flies showing locomotion during the first minute of the experiment were included in the data evaluation.

### 2.4 Handling

For transfer to the shock box, flies were sucked up into an aspirator and blown into the chamber. For the free walk experiments, flies had to leave the shock box after the 20 minute conditioning phase and walk into a plastic vial, from where they were transferred to the arena. For the courtship experiments, the same procedure was used to transfer a

male fly to the courtship chamber. For transfer to the heat box flies were transported in a vial covered by black foil to keep them in constant darkness.

## **2.5 Place learning**

Whenever the fly entered one side of the chamber of the shock box it received an electric shock (100V peak to peak, 4 Hz, alternating current (AC)). The 'punished' side was alternated from fly to fly to avoid any side biases in the averages. Only flies which crossed the midline during the 1<sup>st</sup> minute pretest and received at least one shock during the first training were included in the evaluation. The performance index was calculated subtracting the time the fly spent on the punished side from the time spent on the unpunished side divided by the total time. The experiment consisted of 1 minute pretest, 1 minute training 1, 1 minute test 1, 1 minute training 2, 1 minute test 2, 1 minute training 3 and 2 minutes post-test (Batsching et al., 2016).

## **2.6 High-performance liquid chromatography (HPLC)**

Three days old female flies were tested in the shock box and afterwards transferred to liquid nitrogen. Frozen flies were vortexed to separate the heads from their bodies and were stored at -80°C. Fly heads were then transferred to the Department of Botany I of the University of Wuerzburg, where a HPLC was performed.

## **2.7 Statistical analysis**

Normal distribution of the data was tested using a Kolmogorov-Smirnov test. Bonferroni corrections were used for multiple comparisons. If no normal distribution could be assumed, two groups were compared against each other using the Mann-Whitney-U-test. Comparison of more than two groups was conducted by a one-way ANOVA with Holm-Sidak's multiple comparisons test, for normally distributed data and otherwise with a Kruskal-Wallis test and a Dunn's test for multiple comparisons. Graphs and statistics were conducted using GraphPad Prism 6. Error bars were represented as the standard error of the mean (SEM), unless otherwise specified. The significance level was set to 5% and asterisks were used to indicate significant differences, unless otherwise stated (n.s.= $p>0.05$ , \* = $p<0.05$ , \*\* = $p<0.01$ , or \*\*\* = $p<0.001$ ).



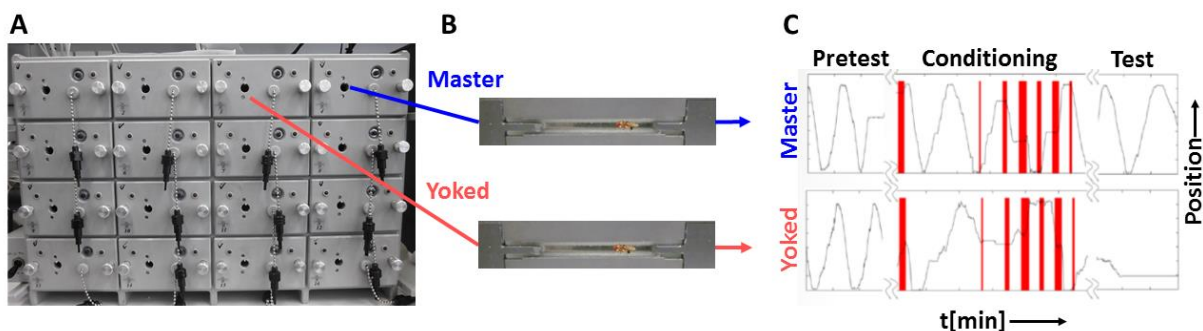
### 3 Results

#### 3.1 The shock box

To investigate how flies cope with uncontrollable, potentially harmful stimuli, a relatively novel apparatus (the shock box) enabling us to study behavioral control under restrained conditions in *Drosophila*, was used (Fig. 4). To address this question, the no-idleness paradigm (compare Material and Methods, chapter 2.3) was utilized. This operant conditioning paradigm has intensely been described within the research context of the heat box before (Bertolucci, 2008; Yang, 2015).

The shock box was built following the design of the heat box (Wustmann et al., 1996) and a prototype of Kapustjansky (2011). I modified the prototype according to my experiences with it (Batsching, 2012). The shock box provides the opportunity to observe a single fly's walking behavior throughout the entire experiment under different conditions.

The setup was used to expose single flies to a random sequence of shock pulses (yoked) and to compare the measured locomotor behavior on the one hand to flies which had control over the shocks (master) and on the other hand to flies which were in the chamber without receiving shocks (control) (triadic design, for more details compare chapter 1.1). Position and shock information were stored and later processed using a self-written software (VB.NET).



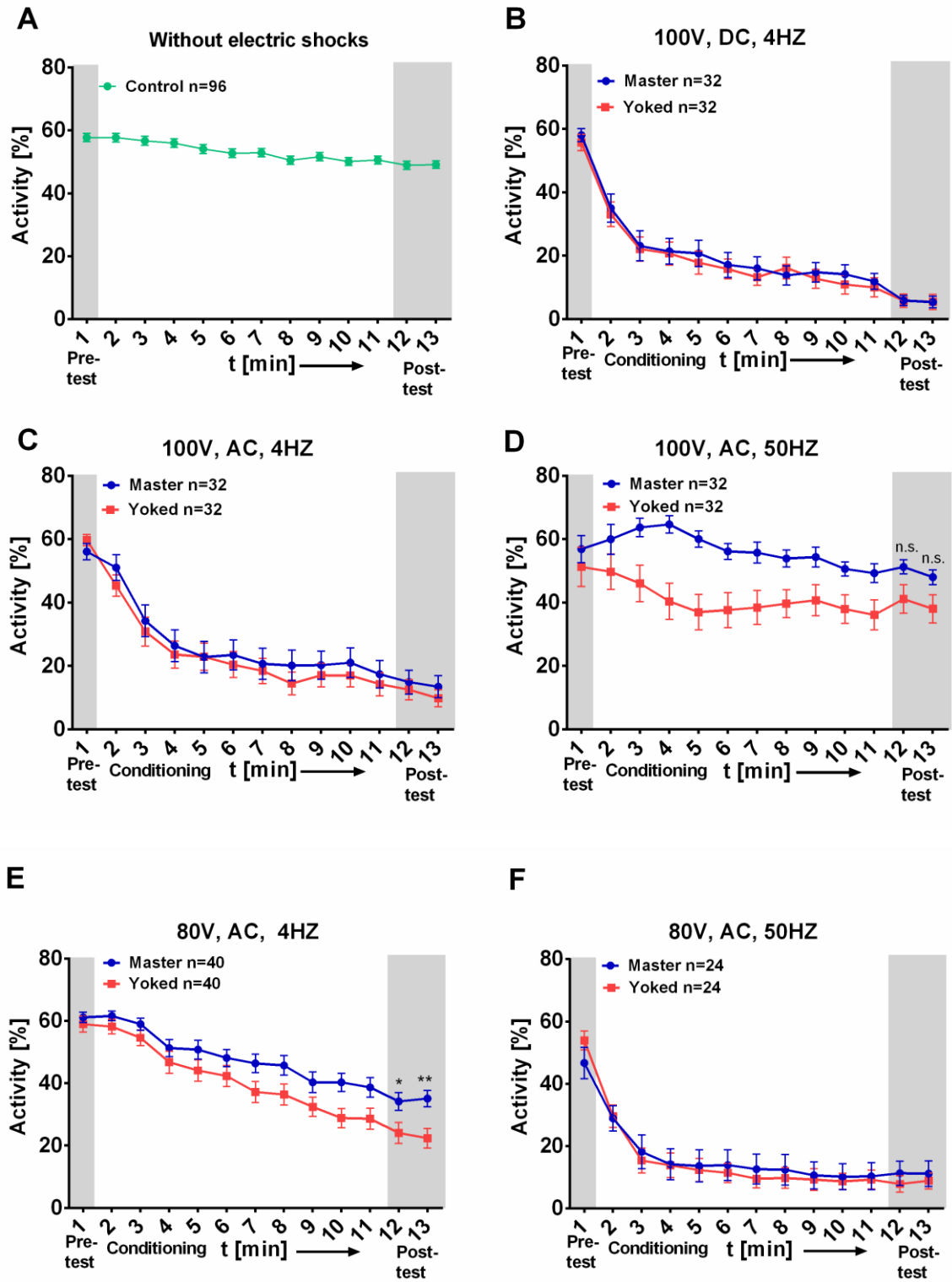
**Fig. 4: The shock box.** (A) Front panel of the 16-boxes setup. (B) Two chambers ( $29 \times 4 \times 2 \text{ mm}^3$ ), each containing a fly inside. (C) In each chamber, the position of the fly is tracked. Rest periods of the master fly cause electric shocks (time periods in red) in both chambers (master and yoked fly).

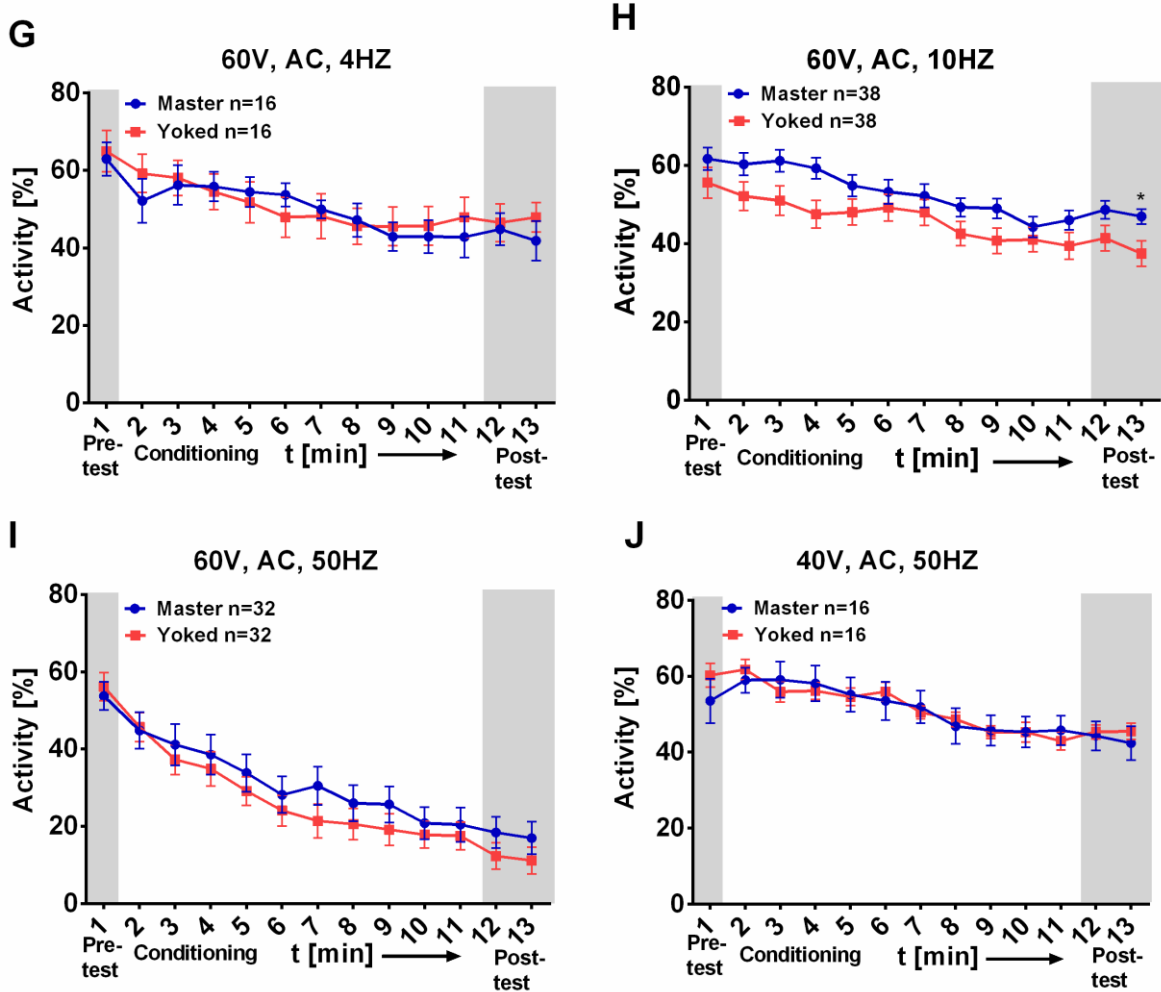
#### 3.2 Implementation of suitable test conditions

##### 3.2.1 Varying the aversive stimulus

To study learned helplessness in the new shock box, I had to find the appropriate shock intensity to condition the flies in an effective but not harmful way. I chose a conditioning

phase of 10 minutes with 1 minute pre-test and 2 minutes post-test. If the flies stayed in the boxes without receiving shocks, their activity dropped only slightly by about 6% (Fig. 5A). In experiments conducted earlier in the prototype 100 Volt (V) direct current (DC), with 2 square pulses per second (each 0,25s long) had been used (Batsching, 2012). In the first conditioning experiment 4 square pulses (DC) per second (each 0.125s) at 100 V were applied, but master and yoked flies showed no difference in walking activity under these conditions (Fig. 5B). It is known that applying electric shock sinusoidally is less damaging for an organism because it lacks the extremely high frequencies which occur when the shock is abruptly turned on. Hence the stimulus was changed to alternating current applied sinusoidally. I started with 100V (50V peak to peak) and 4Hz. Activity levels still dropped to a low level under these conditions in both tested groups and no significant master/yoked difference was observed (Fig. 5C). Increasing the frequency to 50Hz kept the activities of both groups at a high level and led to different activity levels between master and yoked flies during conditioning. Nevertheless, no significant difference in the post-test could be measured (Fig. 5D). Why the high frequency at the same level of shock intensity (100V) keeps the activity high while a frequency of 4Hz lowers it, remains unclear. Next the voltage was set to 80V and the flies were tested at high and low frequencies (50Hz and 4Hz). At 80V and 4Hz the master/yoked flies varied during conditioning and during post-test significantly from each other (Fig. 5E). Conditions of 80V at 50Hz resulted in a major drop in walking activity in both groups (Fig. 5F). Lowering the intensity to 60 or 40V improved the activity levels but led to no or very weak differences between master and yoked flies (Fig. 5G-J). Since 80V at 4Hz (Fig. 5E) was the optimal setting, it was used for the subsequent experiments in this study.



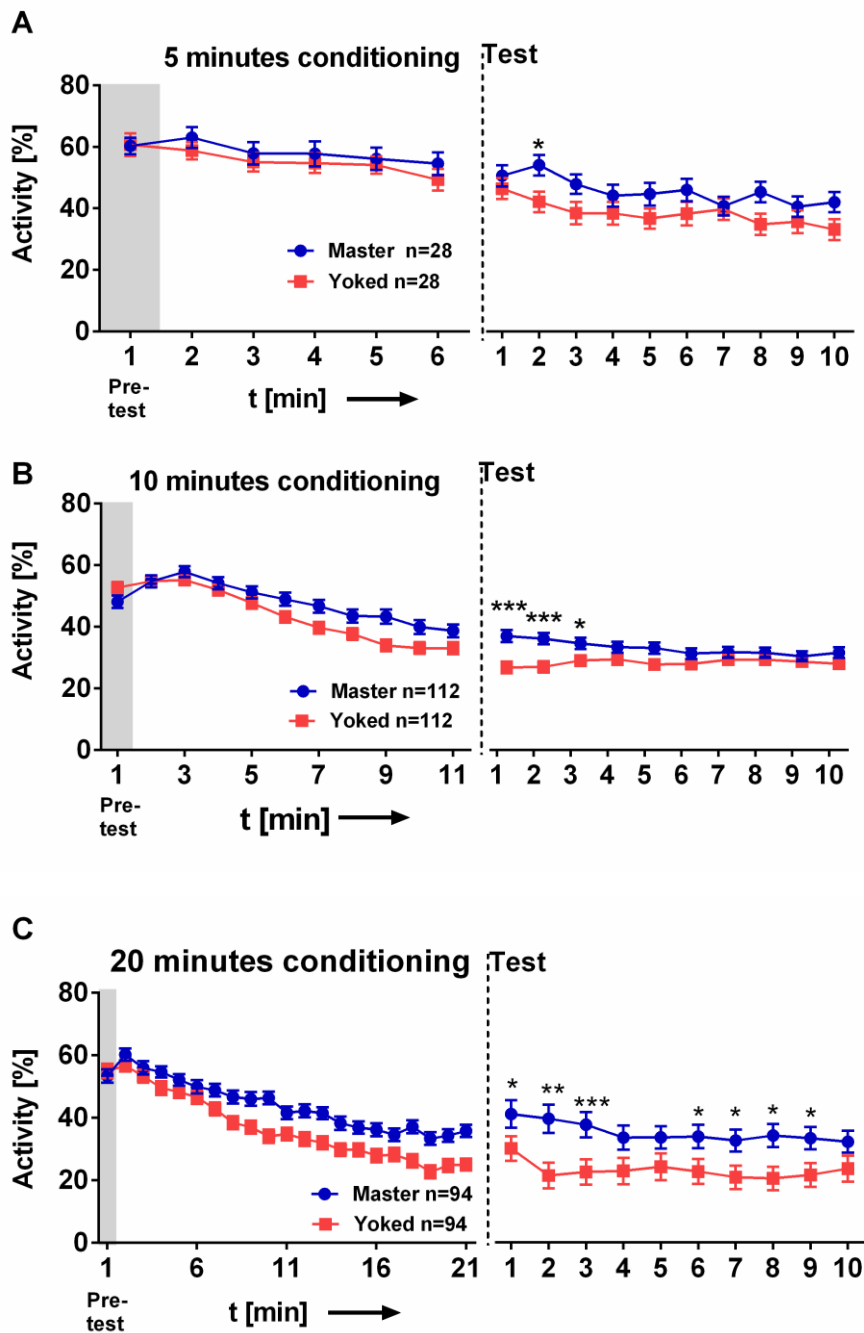


**Fig. 5: (A-J) Walking activity in the shock box during 1min pretest, 10min conditioning and 2min post-test.** Walking is defined as moving more than 0.6mm/s. Master/yoked pairs receive shock pulses if the master fly stops walking. No shock pulses during pretest and test. Only shock intensity is varied between the experiments displayed here. Asterisks are only displayed in the post-test. **(A)** Walking activity without electric shock. **(B)** 100 V, DC, 4Hz: No difference regarding walking activity between master (blue) and yoked (red). **(C)** 100 V, AC, 4Hz: No difference between master and yoked. **(D)** 100V, AC, 50Hz: Master flies are more active during conditioning, but not significantly different from yoked flies during post-test. **(E)** 80V, AC, 4Hz: Gradually, master flies walk more than yoked flies in conditioning and post-test. **(F)** 80V, AC, 50Hz: Activity levels drop to 10% in both groups. **(G)** 60V, AC, 4Hz: Both groups stay at a high activity level but show no difference in activity. **(H)** 60V, AC, 10Hz: Master flies appear to be more active, in particular in the 2<sup>nd</sup> min of the test. **(I)** 60V, AC, 50Hz: Again with higher frequency, the flies' activity drops drastically. **(J)** At 40V, AC, 50Hz activity stays high and flies show no master/yoked differences. \*\*\* $p < 0.001$ ; \*\* $p < 0.01$ ; \* $p < 0.05$ ; n.s  $p > 0.05$ .

### 3.2.2 Varying the conditioning duration

With the aversive stimulus adjusted to 80V at 4Hz, now the optimal conditioning schedule was worked out to obtain a sustained difference regarding the walking behavior between master and yoked flies. For this experiment the post-test was extended to 10 minutes. A five minutes conditioning phase was not sufficient to get a stable master/yoked difference in the post-test (Fig. 6A). Extending the conditioning phase to 10 minutes already led to a

different walking activity during conditioning as shown in Fig. 6B. Additionally, the difference between master and yoked flies was stable for three minutes after the shock was turned off (Fig. 6B). When the conditioning phase was doubled to 20 minutes, an almost constant after-effect in the 10 minutes test phase was found (Fig. 6C). The latter two options were used in this study to investigate the walking behavior of flies under the restrained conditions of the shock box.

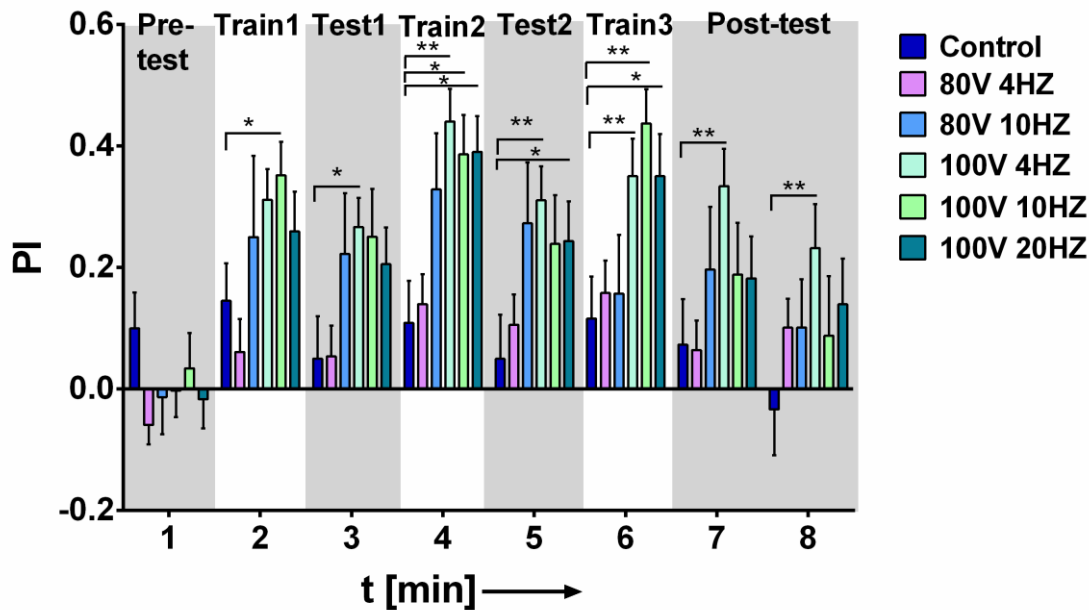


**Fig. 6: Walking activity varying the duration of the conditioning period.** Asterisks are only displayed in the post-test. **(A)** 5min conditioning leads only to a very small difference between master and yoked flies in the 10min post-test. **(B)** 10min conditioning: Yoked flies become less active than master flies. A 3min after effect can be observed. **(C)** 20min conditioning leads to a sustained effect during and after the conditioning which is stable throughout the 10min test period. Also compare Fig. 8. \*\*\* $p < 0.001$ ; \*\* $p < 0.01$ ; \* $p < 0.05$ ; n.s  $p > 0.05$

### 3.2.3 Adjusting shock intensity in place learning

Following up on the idea of Seligman and Maier, to test the dogs' learning ability in a place learning experiment subsequent to stress (Seligman and Maier, 1967), parametrical tests had to be conducted in the new setup in order to test flies in this paradigm after 20 minutes of stress. Earlier work has provided evidence that *Drosophila* was able to avoid one side of the chamber (Wustmann et al., 1996; Kapustjansky, 2011). Back in 2012, I already observed place learning in the prototype of the shock box (Batsching, 2012). For place learning with heat it had been shown that memory levels are influenced by the intensity of the reinforcement (Diegelmann et al., 2006; Zars and Zars, 2006). In the following, a standard place learning experiment was conducted with different settings of the shock intensity (for place learning, more details compare Material and Methods, chapter 2.5). Memory scores were statistically compared to control flies, which received no shocks during their time in the chambers.

In the new version of the shock box a conditioning intensity of 80V at 4Hz (used in the no-idleness experiment) was not sufficient to result in a stable memory effect in place learning. Only with 100V at 4Hz reliable avoidance and memory scores were obtained (Fig. 7). Therefore 100V at 4Hz was chosen for the place learning experiment conducted in chapter 3.6.1. (see Fig. 19).



**Fig. 7: Place learning under various reinforcement conditions:** 1min measuring periods are shown. In pretest, tests and post-test, no shocks are applied: Flies trained under conditions with 100V (AC) at 4Hz show learning starting from test phase 1 until the end of the post-test in every phase, exclusively. Control n=45, 80V, 4 Hz n=119, 80 V 10 Hz n=19, 100 V 4 Hz n=58, 100 V 10 Hz n=31, 100 V 20 Hz n=61. \*\*\*p < 0.001; \*\*p < 0.01; n.s., p > 0.05.

### 3.3 No-idleness experiment

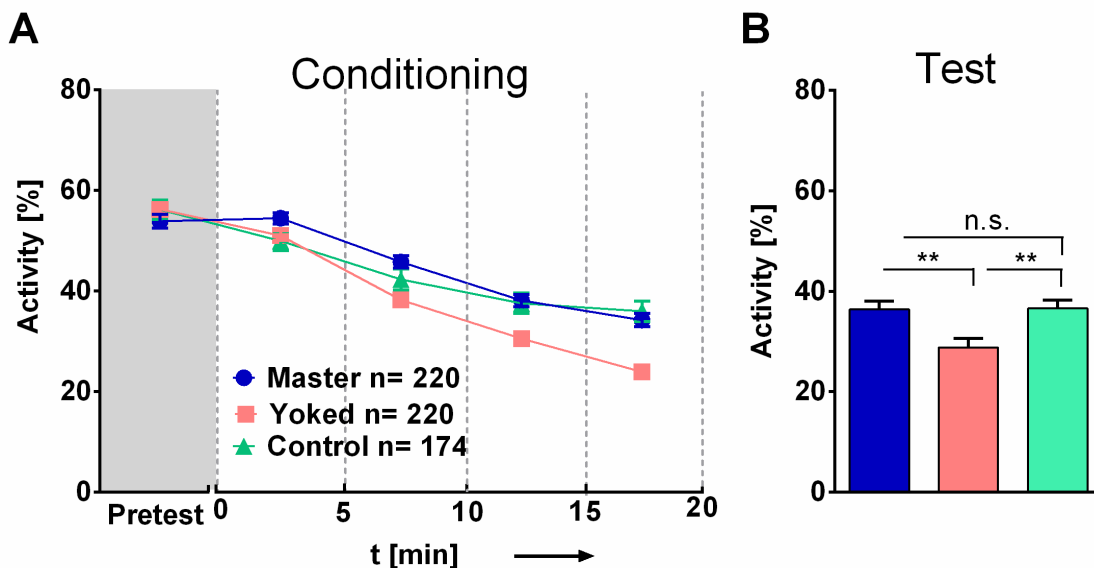
To investigate learned helplessness in flies the so-called no-idleness experiment was conducted. Based on the triadic design (Seligman and Maier, 1967), flies were either in control of the shocks by being active (master group), could not control the shocks (yoked group) or did not receive any shocks at all (control group) (also compare Material and Methods, chapter 2.3).

With shock intensity and duration of the conditioning phase adjusted (chapter 3.2), flies which were not in control of the shocks (yoked group) in contrast to the flies of the master and control groups, modified their walking behavior. These changes were stable throughout the subsequent 10 minute test phase. Surprisingly, there were no differences between master and control flies for the test phase (see Fig. 8B, Fig. 10B, D and Fig. 12B). This phenomenon had already been described in cockroaches (Brown and Stroup, 1988). I consider the stable after-effect in the yoked flies a symptom of learned helplessness and will study whether this behavioral state will transfer to other behaviors in the shock box and elsewhere (see chapter 3.6).

### 3.3.1 Analysis of walking activity

Before proceeding to that step, the walking behavior of *Drosophila* during learned helplessness is described in detail in order to learn more about behavioral control under a stressful situation. Once again, being exposed to inescapable stress has a major impact on the time spent active.

All flies introduced into the chamber started at a comparable activity level in the pretest. Master flies started at 54% and dropped significantly during the following 20 minutes to 34% (Fig. 8A). Yoked flies began at the same level but dropped more drastically down to 24% walking activity. The activity levels of yoked flies diverged significantly from their respective master flies between the 5<sup>th</sup> and the 20<sup>th</sup> conditioning minute. Control flies (which did not receive shocks) started at the same level as the other two groups and did differ from master flies only in the first 10 minutes. For the remaining conditioning and during the post-test, no difference showed between master and control flies. Control flies ended up being significantly more active than yoked flies for the second half of the conditioning (Fig. 8A). In the subsequent test phase, in which the shocks had been turned off for all groups, yoked flies spent significantly less time walking than master and control flies (Fig. 8B).



**Fig. 8: Modulation of walking activity in the shock box.** The pretest lasts 1min. During the 20min conditioning phase, data are pooled in 5min periods. No shock pulses during pretest and 10min test. Control flies (turquoise) receive no shock pulses throughout the experiment. For clarity reasons the asterisks were omitted in the conditioning phase. **(A)** Activity during pretest and conditioning. After 5min conditioning the yoked flies become significantly less active than master and control flies. **(B)** During the 10min test, yoked flies remain significantly less active than master or control flies. No significant difference between the latter two. \*\*\* $p < 0.001$ ; \*\* $p < 0.01$ ; \* $p < 0.05$ ; n.s.  $p > 0.05$ .

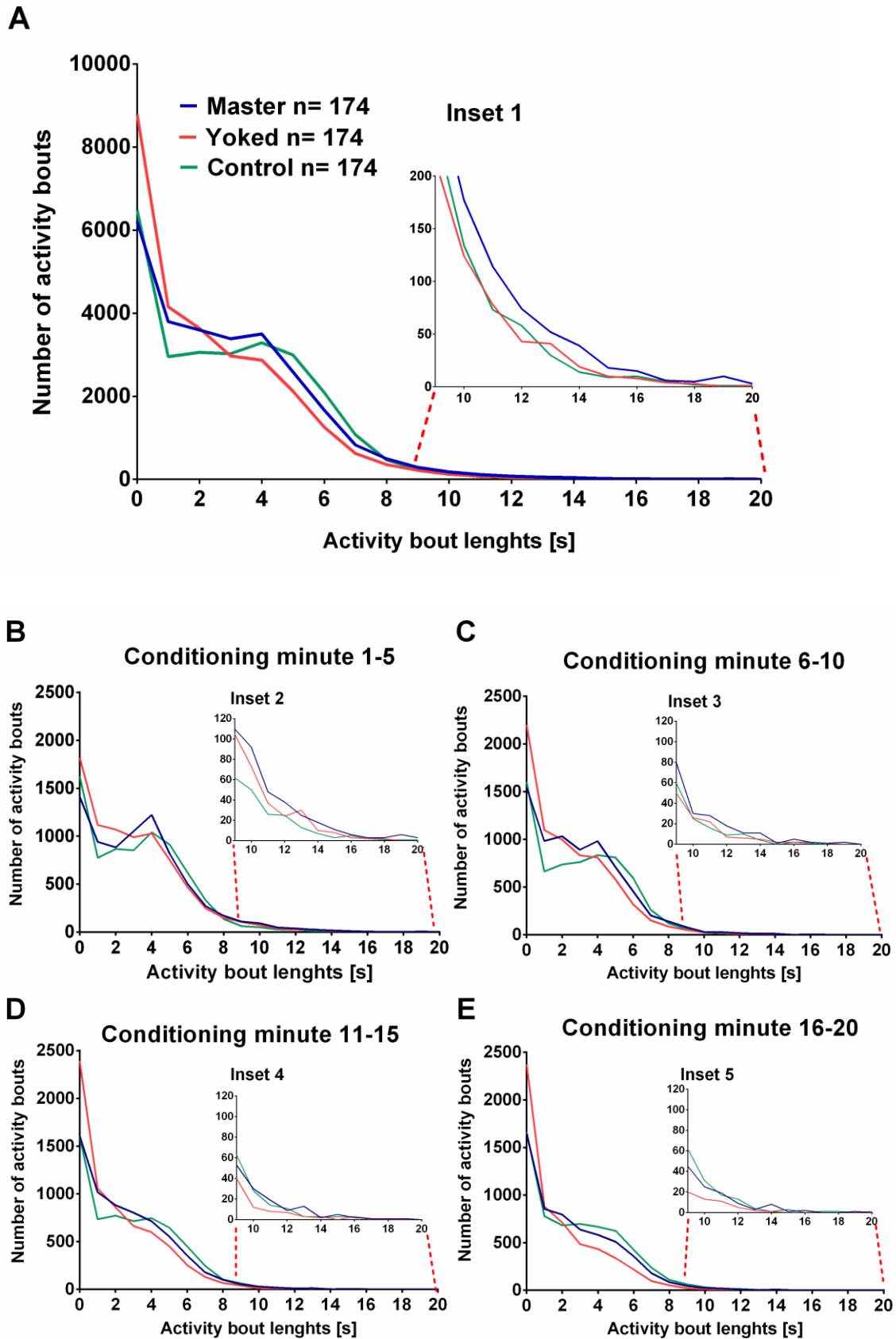


### 3.3.2 Distribution of duration of activity bouts

Averaging the overall activity (Fig. 8) provides an overview of the walking behavior in the box, but does not provide detailed information about the modulation of the walking bouts. In the following I examine the activity bout length of the three tested groups to get a better understanding of how the walking behavior develops during conditioning. Therefore the mean number of activity bouts of all flies during the conditioning phase are summed up and ordered by bout lengths (displayed in Fig. 9A). Yoked flies showed the highest frequency of short bouts (0.1-2s), while master and control flies made more intermediate bouts (3.5-9s) than yoked flies. Master flies showed more long bouts (9-20s, Inset 1) than control and yoked flies. Control flies showed the highest frequency of intermediate bouts (Fig. 9A). A more detailed picture provides the temporal resolution where data are resolved in five minute bins (Fig. 9B-E).

In the first five minutes of conditioning, master flies showed a peak at 4s bout length. In yoked flies shorter bouts at 0.1-2s duration were the most frequent. Control flies made the most frequent bouts of 5-7s bout length, while master flies showed the most frequent long bouts (Fig. 9B, Inset 2). In conditioning minute 6-10, yoked still showed the highest frequency of short bouts, while master flies again showed a peak at 4s and happened to make the most frequent long bouts (9-14s) (Fig. 9C, Inset 3). In conditioning minute 11-15, yoked flies again showed the highest amount of short bouts when compared to master and controls. Master flies made about the same number of long bouts, when compared to control flies (Fig. 9D, Inset 4). In the last five minutes of the conditioning phase, the control group made more intermediate activity bouts than master and yoked flies, while yoked still showed the highest number of short walking bouts. Control flies made more long bouts than master flies (Fig. 9E, Inset 5).

Walking activity of yoked flies consisted mainly of short bouts while master flies displayed a different walking pattern consisting of more intermediate and long bouts. Taken together, master flies made more long bouts in the first half of the conditioning, while yoked flies showed constantly the most frequent short bouts.



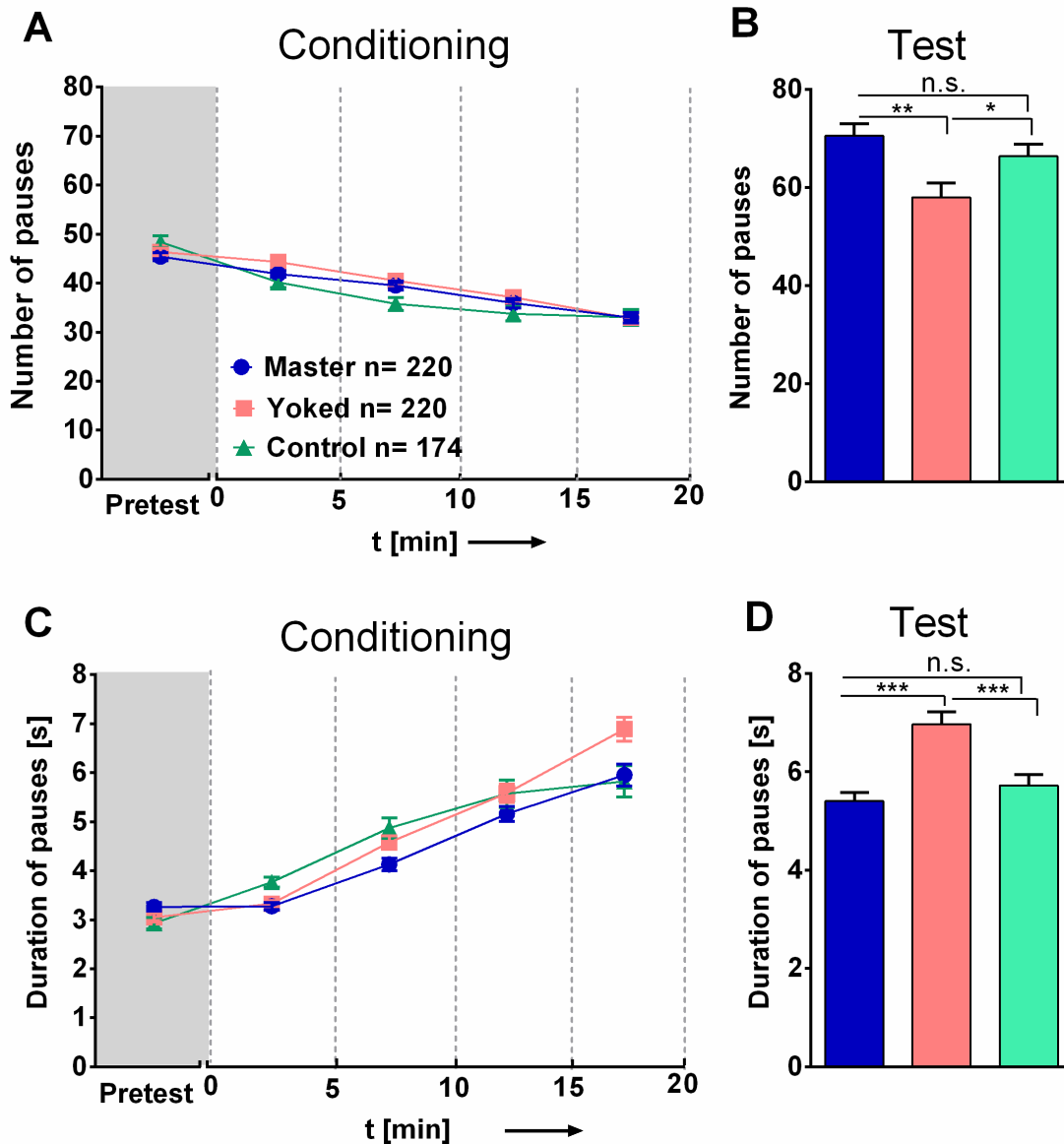
**Fig. 9** Distribution of activity bouts during the conditioning phase ordered by bout length. **(A)** Total number activity bouts. Yoked (red) flies make the most 0.1-2s bouts, while master (blue) and control (turquoise) flies show more 3.5-7s bouts. **Inset 1:** Master flies show more activity bouts which are 10-20s long when compared to control and yoked flies. **(B)** Activity bout distribution during the first five min of

conditioning: Master flies show a peak at 4s. Yoked and controls show the most short walking bouts. **Inset 2:** Master show the most frequent long bouts. **(C)** Activity bout distribution during conditioning min 6-10: Master flies show again a peak at 4s. **Inset 3:** Master still display the most frequent long bouts. **(D)** Activity bout distribution within min 11-15 of conditioning: Master and control make more intermediate bouts than yoked. **Inset 4:** Master and Control flies align concerning the frequency of the long bouts. **(E)** Activity bout distribution within min 16-20 of conditioning: Control flies make more intermediate bouts than master flies, while the latter one make more intermediate bouts than yoked flies. **Inset 5:** Master and control flies do not differ strongly concerning the long bouts. The n was set to an equal value to provide better comparison. Master/yoked, n= 174, control, n= 174.

### 3.3.3 Impact of inescapable shocks on number and duration of pauses

To describe and characterize the behavior in the chambers in more detail, the number and the duration of pauses were calculated. This evaluation provides a detailed picture of how the walking behavior is modulated.

Surprisingly, some of the flies made respectively long pauses, 3% of the master flies and 7% of the yoked flies made pauses which were longer than 20s. These quite rare, but long pauses led to big variations when calculating the mean duration of pauses over the number of flies (data not shown). To accentuate the difference regarding the length of pauses between master and yoked flies in the shock box, I first calculated the mean pause lengths per phase for all flies and divided these values by the number of pauses made by all the flies in this phase. Using this computation, the long pauses impact the mean less and therefore differences could be showed easier (Fig. 10C). Yoked flies made significantly more pauses when compared to control flies between the 5<sup>th</sup> and the 15<sup>th</sup> minute of the conditioning phase (Fig. 10A). During test yoked flies showed less pauses than the other two groups (Fig. 10B). Interestingly, flies did only differ to a small extend during conditioning, but differed strongly during post-test regarding the number of pauses done. When comparing master and control flies I found that master flies made significantly shorter pauses from the 1<sup>st</sup>- 15<sup>th</sup> minute than control flies (Fig. 10C). During the 10 minute test the yoked flies still showed significantly longer pauses than the master and control flies (Fig. 10D).



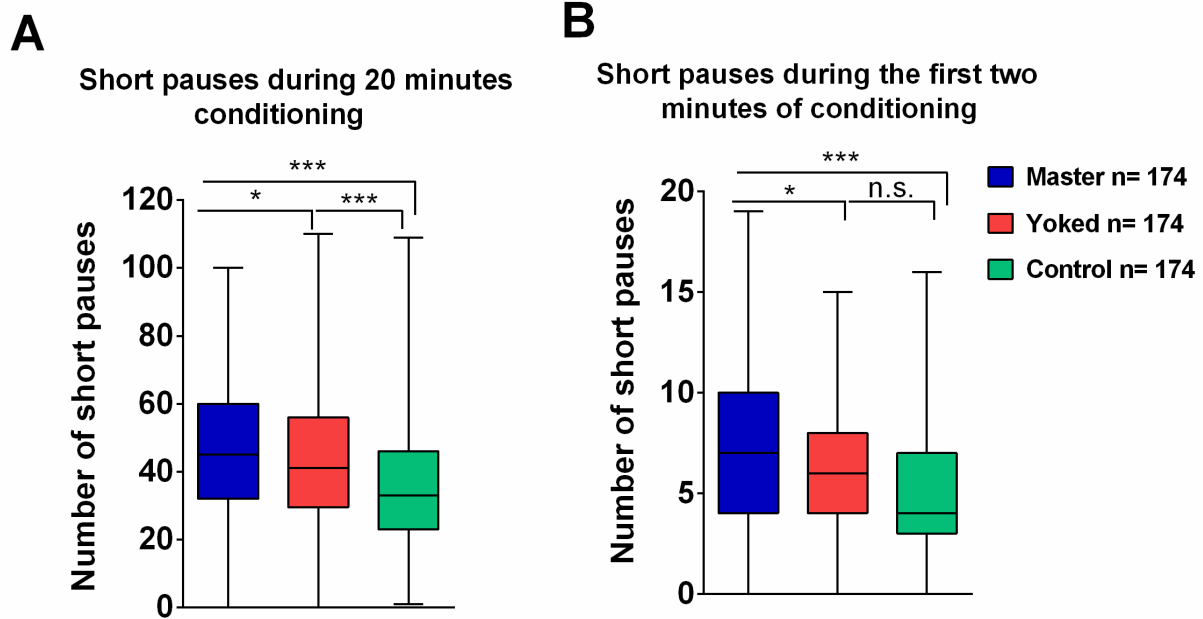
**Fig. 10: Number and duration of pauses.** For clarity reasons the asterisks were omitted in the conditioning phase. **(A)** Number of pauses during pretest and conditioning. Yoked flies make significantly more pauses than control flies between the 5<sup>th</sup> and then 15<sup>th</sup> min. **(B)** During test: Yoked flies make fewer pauses than master or control flies. **(C)** Durations of pauses during pretest and conditioning: Master flies make significantly shorter pauses than controls starting from the beginning of the conditioning until the 15<sup>th</sup> min. Yoked flies sit significantly longer than master flies starting from the 5<sup>th</sup> min until the end of the conditioning phase. Yoked flies sit shorter in the first five min of conditioning and longer in the last five min, when compared to control flies. **(D)** Yoked flies sit significantly longer than master or control flies throughout the 10min test phase. Master/yoked, n= 220, control, n= 174. \*\*\*p<0.001; \*\*p<0.01; \*p<0.05; n.s p>0.05.

### 3.3.4 Analysis of short pauses

A motivational state of uncontrollability would imply, that flies have an innate escape response to electric shock which yoked flies learn to suppress during the conditioning phase. Evidently, electric shock is an aversive stimulus as shown, for instance, in olfactory

learning (Quinn et al., 1974) or in place learning in the shock box, where flies showed avoidance of the shock-associated side (compare chapter 3.2.3).

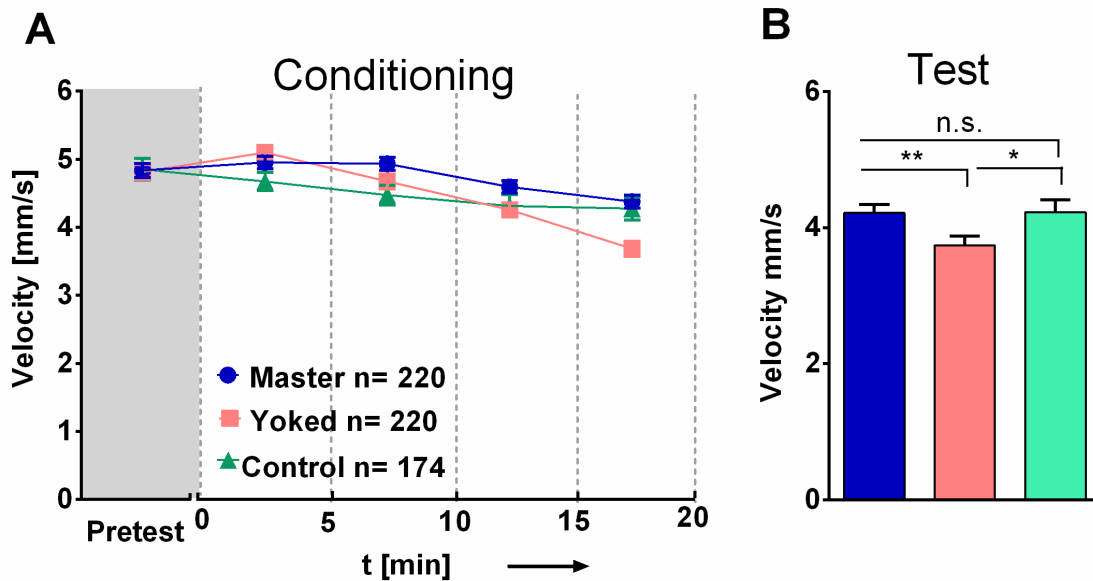
This leads to the question whether in the present experiments a fly at rest tries to jump or start walking in response to a shock. As electric shock for the master flies started 1s after locomotion stopped, I asked whether short pauses during the initial phase of the conditioning – lasting between 1s and 1.5s – were more frequent in master flies than in controls. This would count as indirect evidence for an immediate reaction to shock. Indeed, the short pauses appeared to be most frequent in master, less frequent in yoked and even less in control flies (Fig. 11A). Calculating the short pauses for the first two minutes of the conditioning phase lead to the result that master flies made more short pauses, while yoked and control did not differ significantly anymore (Fig. 11B). The difference between master and yoked flies supports the idea that yoked flies suppressed the escape response, at least to some extent. The fact that the master flies showed more short pauses throughout the entire experiment (Fig. 11A) suggest that they learned to start walking quickly after the onset of an electric shock and this clearly separates them from the control flies. For the different parameters measured in Fig. 8B, Fig. 10B and Fig. 12B master flies ended up at the same level as the controls. This might reflect that master flies found a balance between spending energy on walking and bearing a certain amount of shock. They level off on the same level as the control flies. The shock box enables me to observe the flies' behavior already during conditioning. With that opportunity I found that the way master flies established that certain level was differing from the control flies. Taking activity as an example, master and control flies did not align until the 11<sup>th</sup> minute of the conditioning (Fig. 8A). This effect was even stronger pronounced in Fig. 16A. In short, master yoked and control flies adapted to the external condition individually.



**Fig. 11: Response to electric shock.** All master flies (blue) are hit by a shock at 1s of sitting. Yoked flies have a low probability to be hit by the shock at an arbitrary time during the pause. Non-parametrical presentation of the data. Data in the box represent the 25% and 75% quartiles, while the error bars represent the distribution down to the minimum and up to the maximum values of the dataset. **(A)** Number of short pauses (1-1.5s) during the conditioning phase: Master flies (blue) make more short pauses than yoked flies (red) or controls (turquoise). Yoked make more than control flies. **(B)** Number of short pauses in the first 2min of conditioning. Master flies take more short pauses than yoked and control flies. Yoked show slightly more short pauses than controls, but the difference is not statistically significant. \*\*\* $p < 0.001$ ; \*\* $p < 0.01$ ; \* $p < 0.05$ ; n.s.  $p > 0.05$ .

### 3.3.5 Analysis of walking speed

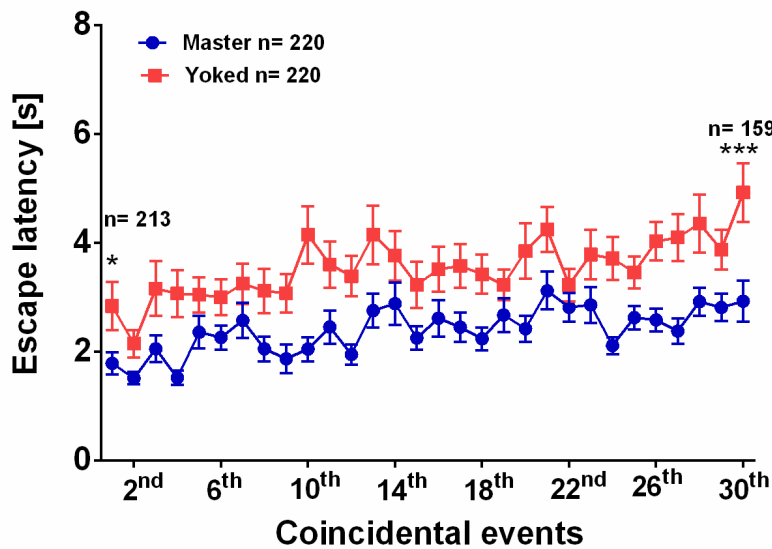
After characterizing the activity pattern and the modulation of pauses in flies (compare chapter 3.3.1-3.3.4), the velocity was measured. Yang et al. (2013) found a reduced walking speed in the post-test for the yoked flies. With electric shock conditioning I also found reduced walking velocities for yoked flies. From minute 10 onwards until the end of the conditioning phase, walking speed differed between master and yoked flies with the yoked flies walking slower than master and control flies. Yoked flies were slower than control flies during the last five minutes of conditioning (Fig. 12A). This effect lasted throughout the whole test phase (Fig. 12B).



**Fig. 12: Velocity.** For clarity reasons the asterisks were omitted in the conditioning phase. **(A)** Velocity of walking during pretest and conditioning: Yoked flies become slower in the second half of the conditioning, when compared to master flies. Starting in the 15<sup>th</sup> min yoked flies also differ from control flies regarding velocity. In the first half, master walk slightly faster than control flies. **(B)** Velocity during test: Yoked flies walk slower than the other two groups. \*\*\* $p < 0.001$ ; \*\* $p < 0.01$ ; \* $p < 0.05$ ; n.s.  $p > 0.05$ .

### 3.3.6 Escape latency

In addition to the analysis of the walking behavior I developed a way to calculate how flies reacted to electric shock when they were hit while sitting. Due to the fact that yoked flies receive electric shock in a random manner one major difference between master and yoked flies is that yoked flies receive shocks during walking as well. Nothing is known about whether shock is sensed differently during sitting or walking. The evaluation software was written such that it detects events in which both flies are sitting already for 0.9s or longer (coincidental events). In such cases the time the flies needed to resume walking was measured. This was named the escape latency. For each master/yoked pair the sequence of coincidental events with the respective escape latencies was recorded. The first pairs of coincidental events occurred already within the first conditioning minute. In Fig. 13 the averaged escape latencies for a sequence of 30 coincidental events are displayed. Coincidental events were included only when the yoked flies had terminated their rest period before a second electric shock occurred. With this computation I measured flies under very similar circumstances: Master and yoked flies were sitting when electric shock arrived. Striking differences could be observed. Yoked flies took longer time to resume walking after the onset of an electric shock in comparison to the master flies. This suggests that the master flies made use of their control.



**Fig. 13: Escape latency.** Time the fly takes to start walking after the onset of an electric shock. Only those events are averaged, in which the master (blue) fly as well as the yoked fly (red) sit during the onset of the shock and the yoked fly is hit only once during that rest period. Yoked flies hit by electric shock during rest period take longer to resume walking than master flies. Master/yoked pairs for first coincidental event,  $n = 213$  and for the 30<sup>th</sup> event  $n = 159$ . \*\*\* $p < 0.001$ ; \*\* $p < 0.01$ ; \* $p < 0.05$ ; n.s  $p > 0.05$ .

### 3.3.7 Sexual dimorphism

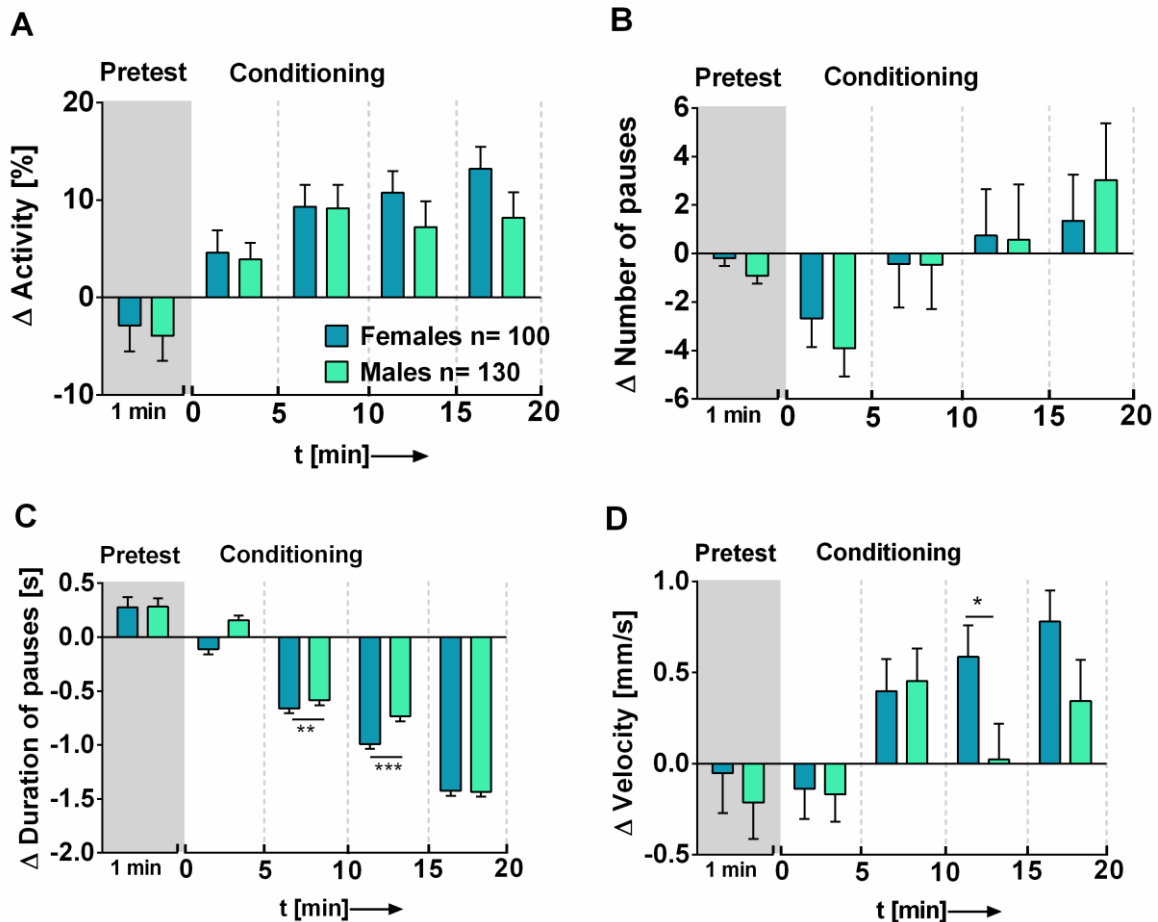
In the previously described experiment only female flies were used (see chapter 3.2-3.3.6). It is known however that in humans the reaction to stress and occurrence of depression has a sexually specific component (Oldehinkel and Bouma, 2011). After the exposure to chronic mild stress (CMS) female rats spend more time immobile in the forced swim test than male rats do (Shors et al., 2007). Yang (2015) and Bertolucci (2008) described already a sexual dimorphism for learned helplessness in *Drosophila* in the heat box. While female flies showed a strong effect concerning the activity levels and the velocity during conditioning, male flies showed a weaker effect (Yang, 2015).

To follow up on these previous findings I compared males and females in the shock box. The same protocol as already shown in chapter 3.3 was used. In this experiment the focus was set to the question, whether master and yoked flies of the respective group would differ in the different parameters measured (activity, number- and duration of pauses and velocity). Thus, for an easier comparison between male and female flies, data are displayed as difference between master and yoked flies for both sexes respectively. Positive values indicate for example that the master flies were more active than yoked flies (master-yoked =  $\Delta$ ). Asterisks therefore indicate differences between males and females. For clarity reasons the asterisk indicating significances between master and yoked are omitted for the conditioning phase, but mentioned in the text.

During conditioning male flies showed a similar although less reliable effect as female flies: Male yoked flies were significantly less active, made the same number of pauses, sat

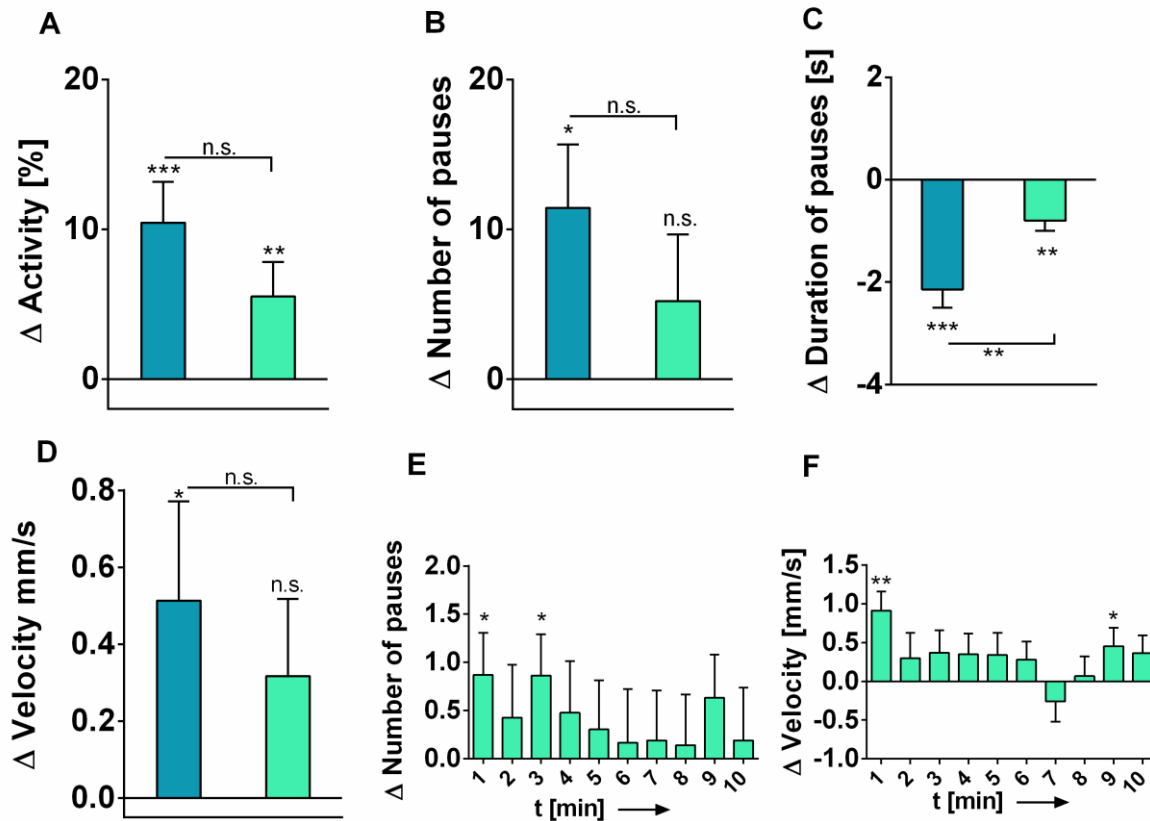


significantly longer and walked significantly slower than their respective master flies (Fig. 14A-D).



**Fig. 14: Comparison between male (turquoise) and female (blue) flies during 20min conditioning.** All graphs display the difference between master and yoked (master-yoked=Δ). Modulation of walking behavior in the shock box. **(A)** Activity during pretest and conditioning: No difference between males and females. **(B)** Number of pauses: Difference between master and yoked is more pronounced in males in the last five min of conditioning, but does not differ significantly from females. **(C)** Duration of pauses: Female master and yoked flies differ significantly stronger regarding the pause length between 5<sup>th</sup> and 15<sup>th</sup> min of conditioning when compared to male flies. **(D)** Velocity: Female flies differ from male flies during the conditioning min 10-15. \*\*\* $p < 0.001$ ; \*\* $p < 0.01$ ; \* $p < 0.05$ ; n.s  $p > 0.05$ .

Interestingly, the after-effect was less stable in male than in female flies, when averaging over the 10 minute test periods. In males, no sustained differences throughout the 10 minute test period with respect to the number of pauses and the velocity were found. However, these values happened not to be significantly different from females (Fig. 15B, D). In Fig. 15E, F the test phase for the male flies is displayed again at a different time scale to present the after effect in more detail. The fact that almost all values appeared to be positive, suggests that the male flies have a tendency to show the same effect as females, but weaker.

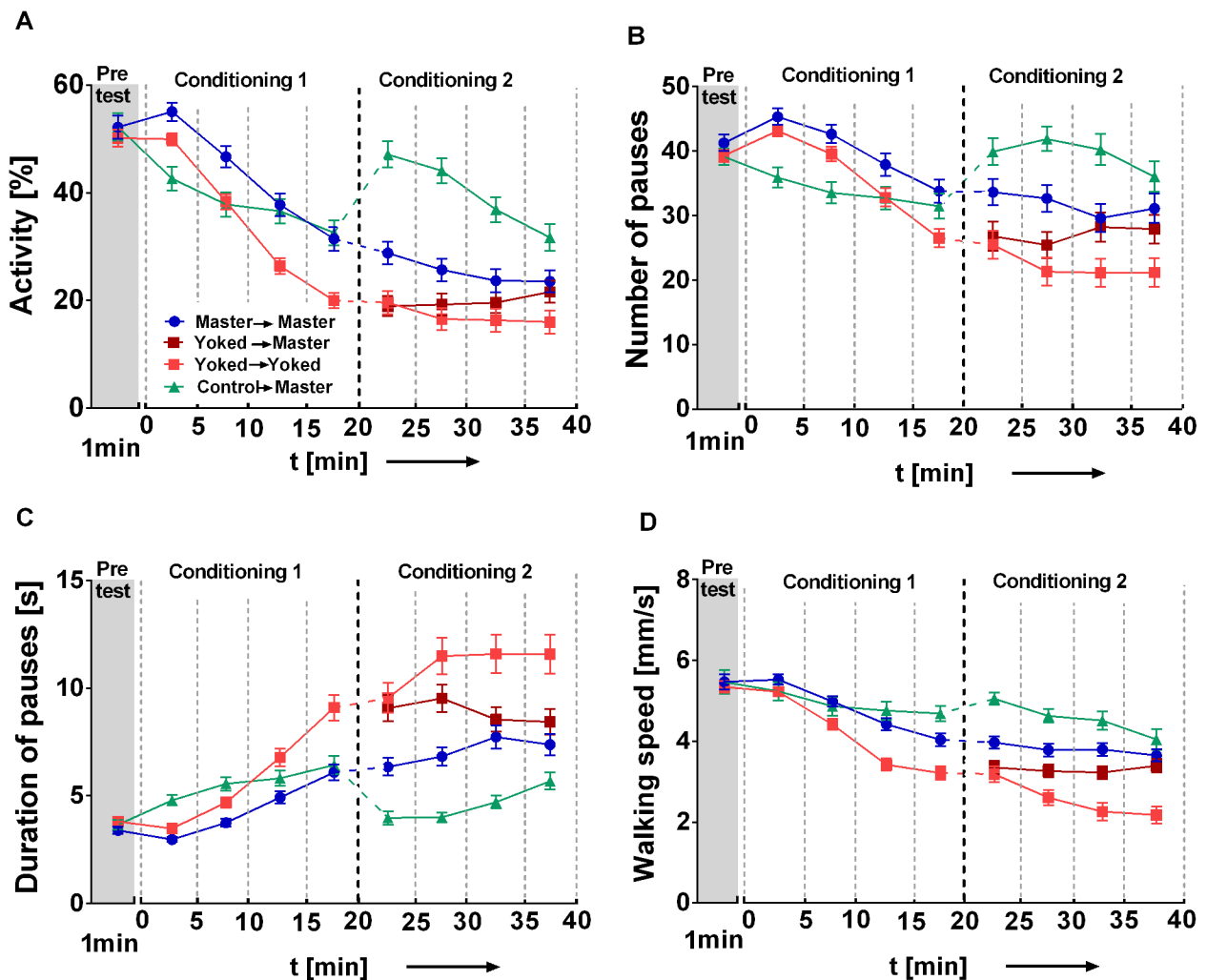


**Fig. 15: Comparison between male (turquoise) and female (blue) flies during 10min test.** All graphs display the difference between master and yoked (master-yoked= $\Delta$ ). Modulation of walking behavior in the shock box. **(A)** Activity during test: No difference between males and females. **(B)** Number of pauses: Female master flies sit significantly more often than their respective yoked flies, while male master flies sit not significantly more often than male yoked flies. **(C)** Durations of pauses during test: Both, male and female yoked flies sit longer than their respective master flies. This effect is more pronounced in females. **(D)** Velocity: Female master flies walk faster than the yoked flies. This differences is not significant in male flies. **(E)** Number of pauses during test in 1min bins: Male master flies make more pauses than yoked flies for about 3min. **(F)** Velocity during test in 1min bins: Male master flies are slightly faster than male yoked flies. Master/yoked females,  $n = 100$ , Master/yoked males,  $n = 130$ . \*\*\* $p < 0.001$ ; \*\* $p < 0.01$ ; \* $p < 0.05$ ; n.s.  $p > 0.05$ .

### 3.4 Reversal learning

Stress before learning can impair or reduce memory retrieval in humans and animals (Shors and Thompson, 1992; de Quervain et al., 1998; Kuhlmann et al., 2005). In order to investigate whether the yoked flies are able to gain back control over the electric shock, a new experiment was designed in which the 20 minutes conditioning period was followed by another 20 minutes period in which the flies of all three groups were conditioned as master flies (Fig. 16A-D). In a fourth group the flies that had started as yoked, stayed yoked in the second part (light red). Flies increased walking activity after the switch from control to master condition by 47% and reduced the mean duration of pauses from 6.4 to 3.9s in the first five minutes of being masters (Fig. 16A, C, green curve). During that period (minute 20-25) yoked-to-master flies were not distinguishable from the yoked-to-yoked flies.

However, gradually they changed and reached the level of the master-to-master flies in all four parameters (activity, speed, duration and number of pauses). Characteristically, the flies that stayed in the yoked mode for the second period walked significantly more slowly, kept significantly longer pauses and rested significantly less frequently than the yoked-to-master flies. Strikingly, yoked-to-master flies took about 7 minutes to notice the change of rules in the second part of the conditioning phase. This shows that the state of learned helplessness is reversible (Batsching et al., 2016).



**Fig. 16: Reversal learning.** Control-to-master (turquoise), master-to-master (blue), yoked-to-master (light red in conditioning 1, dark red in conditioning 2), and yoked-to-yoked (light red) flies. Pretest and conditioning. Pretest lasts 1min. During conditioning data are pooled in 5min periods. For clarity reasons the asterisks were omitted. **(A-D)** Yoked-to-master and yoked-to-yoked flies are pooled. Control flies receive no shock pulses at all. Conditioning 2: Control over the shocks is given to master-to-master, yoked-to-master and control-to-master flies. Yoked-to-yoked flies stayed yoked. I split yoked flies from conditioning 1 into yoked-to-master and yoked-to-yoked flies. **(A)** Walking activity during 1min pretest and 40min conditioning phase: During the first five min all three groups differ in activity. Master-to-master and control-to-master flies remain more active at the end of the first 20min conditioning. Starting from min 20, yoked-to-master flies need several min to align to master-to-master flies. Control-to-master flies show an increasing activity level with the onset of electric shock (min 15-25). **(B)** Number of pauses. Master-to-master and control-to-master flies make more pauses between min 15-20 when compared to yoked. Between min 30-40, yoked-to-master flies align to master-to-master flies. **(C)** Duration of pauses. During min 5-20, yoked flies make longer pauses than the compared groups. During min 20-30, yoked-to-master make longer pauses than master-to-master flies. In the

subsequent 10min, yoked-to-master flies differ from control-to-master and yoked-to-yoked, but not from master-to-master flies. **(D)** Walking speed. Starting from the 5<sup>th</sup> min, yoked flies walk more slowly than the compared groups. In min 35-40, yoked-to-master flies align their walking speed to master-to-master flies. Master-to-master, n= 102; yoked-to-master, n= 102; control-to-master, n= 88 and yoked-to-yoked (light red) flies, n= 103.

### 3.5 Comparison between two aversive stimuli

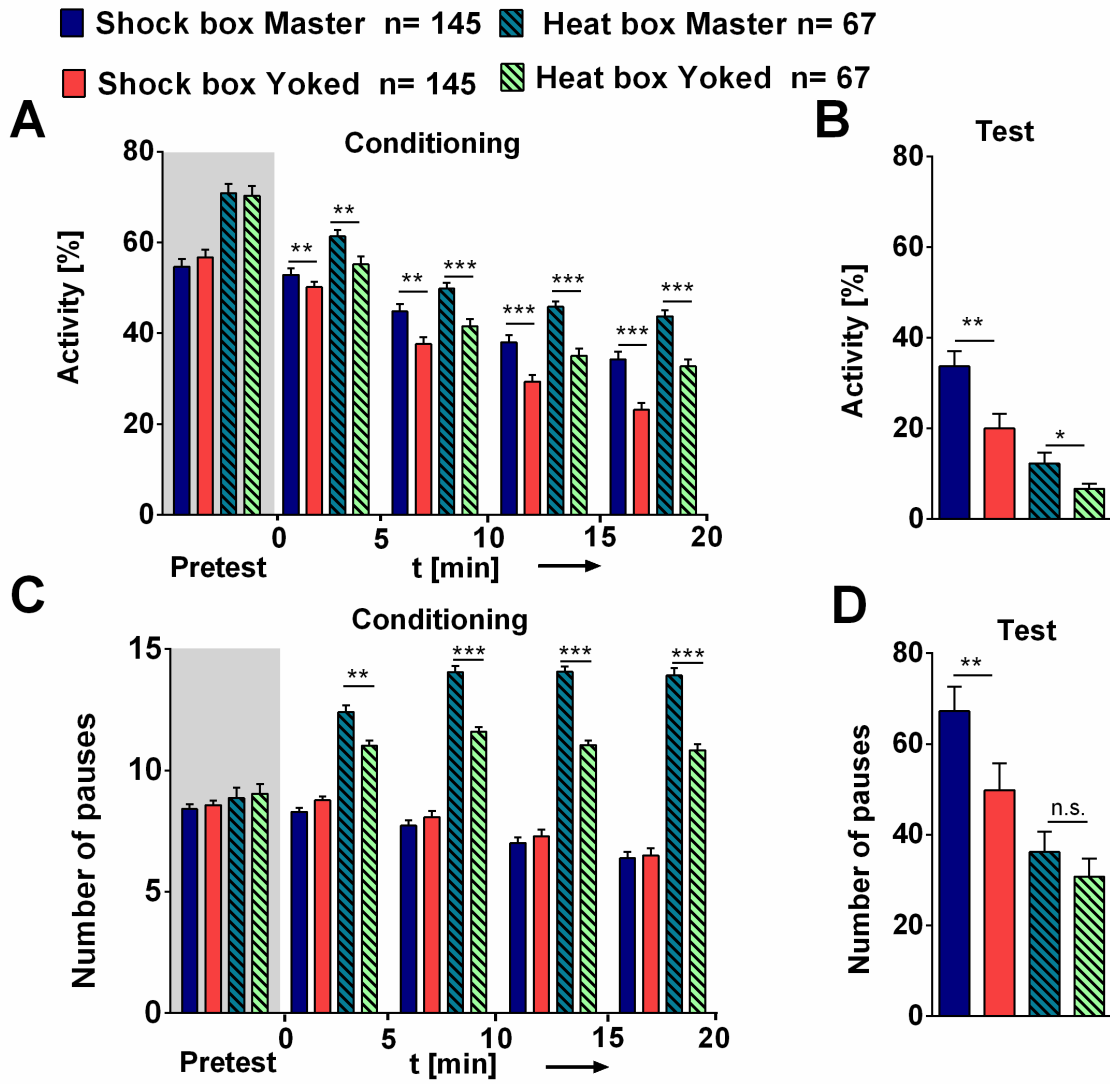
In order to investigate differences between the heat- and the shock box and to test whether changes in walking behavior due to inescapable stress are stimulus dependent, flies were tested side-by-side in the two devices. Comparing two different aversive stimuli in similar devices may provide a deeper insight into the phenomenon of learned controllability/uncontrollability and it may help to answer the question whether it is induced by a specific stressor or by uncontrollability.

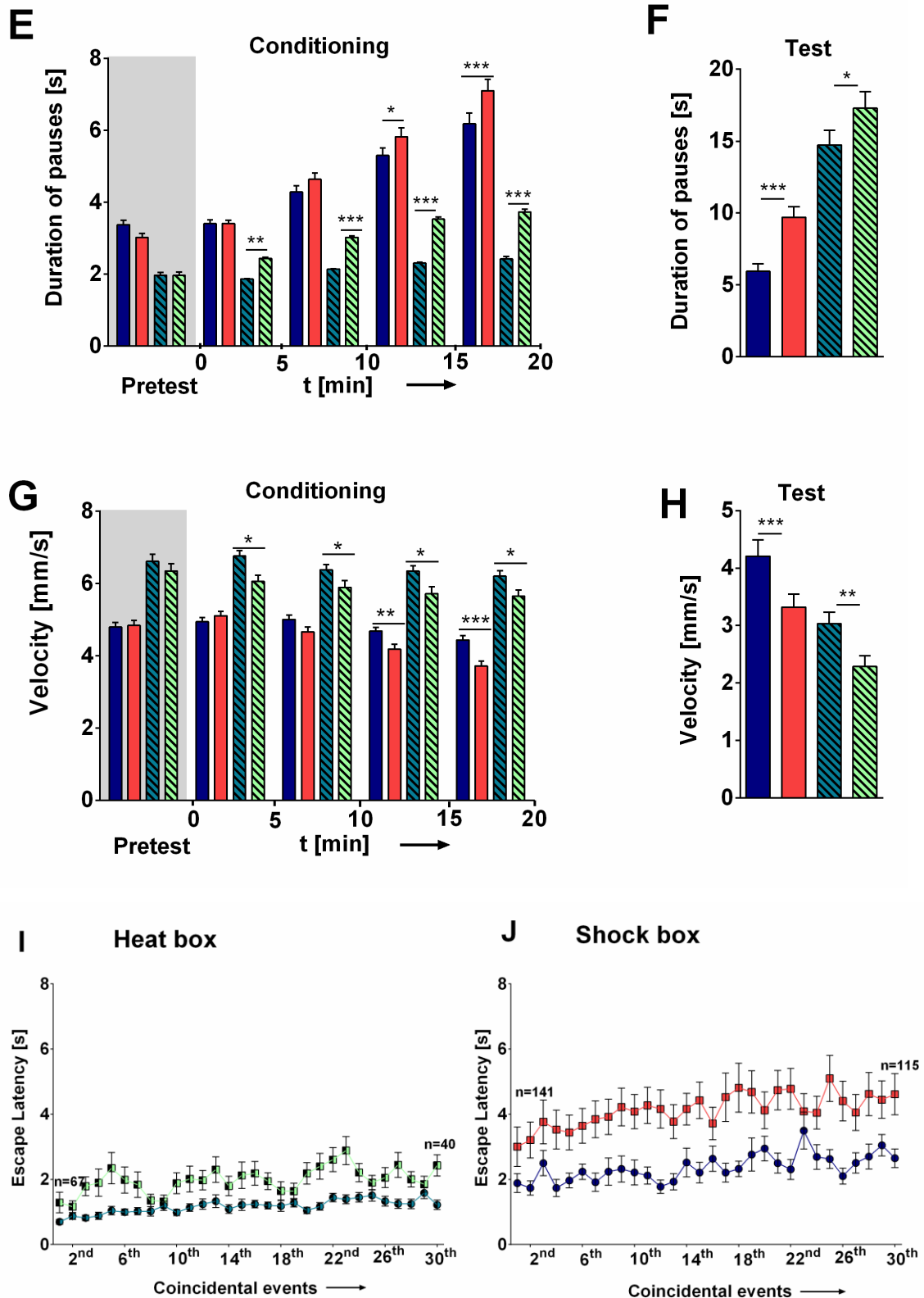
#### 3.5.1 Heat versus electric shock

Yang et al. (2013) could show that flies, which were exposed to uncontrollable heat shocks, displayed a major change regarding their walking behavior. They conducted the experiment with a different protocol. Flies were trained for 10 minutes and tested for 30 seconds. To compare the findings between the devices, collecting a new data set in the heat box which matches the protocol used mostly in the shock box, seemed advisable. Therefore the experiment was conducted the following: 1 minute pretest, 20 minutes conditioning, followed by 10 minutes post-test.

Despite the fact that even with the same protocol it was possible to induce learned helplessness by uncontrollable electric shock and heat, some differences appeared when I directly compared the walking modulation between flies in the two devices. For instance, flies tested in the heat box showed a higher walking activity already from the first minute on, in which no heat pulses were applied (Fig. 17A). In the subsequent test phase the yoked flies remained significantly less active than their respective master flies. This effect could be observed for both devices. Note that the activity level dropped drastically in the heat box down to 12% (master) and 7% (yoked), while the flies in the shock box lowered their walking activity only to 34% (master) and 20% (yoked) (Fig. 17B). Significant variations occurred when comparing the number of pauses between both devices. Master flies in the heat box made more pauses than yoked flies during conditioning. In the shock box, flies showed only small differences concerning the number of pauses between master and yoked (Fig. 17C). Surprisingly, no after effect could be measured between the flies in the heat box during the 10 minutes post-test, while in the shock box master flies sat

significantly more often than yoked flies (Fig. 17D). As mentioned before, flies sat relatively long in the shock box (also compare chapter 3.3.3) when compared to the flies in the heat box (Fig. 17E). In both devices, the differences regarding the duration of pauses which already occurred during conditioning between the tested flies (master/yoked), were still measurable during the subsequent test (Fig. 17F). Flies conditioned in the shock box walked slower when compared to flies in the heat box. However, master flies walked faster than yoked flies in both devices and showed a sustained after effect (Fig. 17G, H). Yoked flies needed longer than master flies to resume walking after the onset of the electric shock in both devices. This phenomenon was more pronounced in the shock box, where yoked flies showed longer escape latencies throughout the 30 coincidental events (Fig. 17I, J). Interestingly, the escape latency of master and yoked flies turned out to be less variable in the heat box (small SEMs). Despite some variations which could be observed between the devices, it is striking that I found similar results. In sum, this finding shows that learned helplessness does not depend on the stressor.





**Fig. 17: Comparison between shock box (no pattern): Master (blue)/yoked (red) and heat box (striped pattern): Master (petrol blue)/yoked (light green). (A)-(I):** Pretest lasts 1min, the test phase lasts 10min, data are averaged during this period. During pretest and test no shock/heat is applied. During conditioning, data are pooled in 5min bins. **(A)** Walking activity during pretest and conditioning: In the heat box as well as in the shock box a difference in activity during conditioning between master and yoked can be observed. **(B)** Activity test: In both devices the difference lasts for the whole 10min test. Note that flies in the heat box end up being less active, than in the shock box. **(C)** Number of pauses during pretest and conditioning: In the heat box, master flies make significantly more pauses than the yoked flies. This is not the case in the shock box.

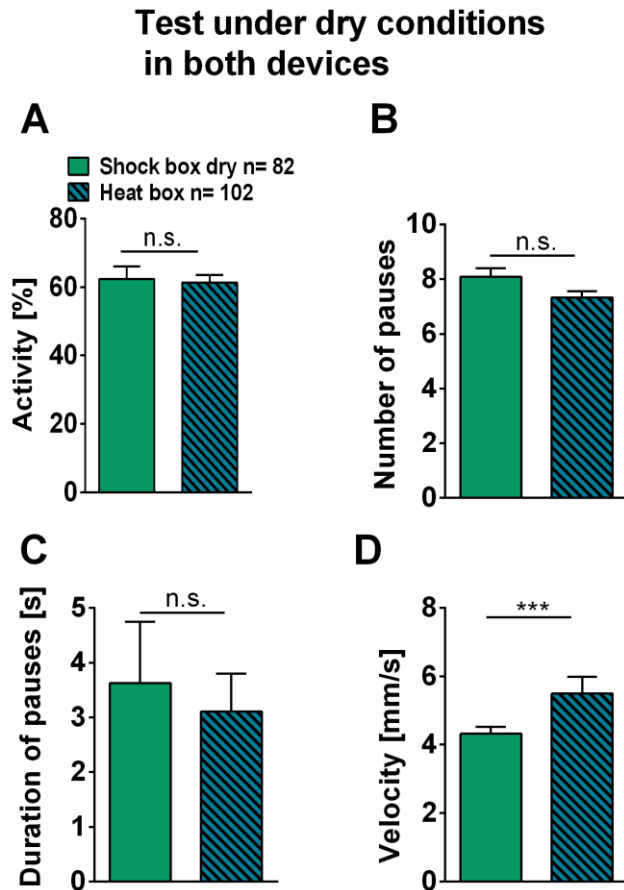
(D) Number of pauses within the 10min test: No difference between master and yoked in the heat box anymore, while in the shock box the master sit more often than the yoked flies. (E) Duration of pauses during pretest and conditioning: Yoked flies make longer pauses than master flies in both devices. (F) Duration of pauses within 10min test: Yoked flies make longer pauses than master flies. This applies for both devices. (G) Walking speed during pretest and conditioning: Master flies are faster in the heat box and the shock box. Altogether, flies walk faster in the heat box than in the shock box. (H) Walking speed within 10min test: Master flies walk faster than yoked flies in both devices. In this phase, flies in the shock box walk surprisingly faster than in the heat box. (I) Escape latency during conditioning in the heat box: Yoked flies need longer time to resume walking after the onset of the heat, when compared to master flies. (J) Escape latency during conditioning in the shock box: Yoked flies need longer time to resume walking after the onset of an electric shock than master flies. \*\*\* $p < 0.001$ ; \*\* $p < 0.01$ ; \* $p < 0.05$ ; n.s  $p > 0.05$ .

### 3.5.2 Influence of humidity on walking behavior

One major difference between the two devices is that humidified air is needed to conduct experiments successfully in the shock box, exclusively. Humidity could influence the walking behavior within the chambers and therefore contribute to the differences observed between the shock box and the heat box. Therefore, flies were tested for 10 minutes in both devices und dry air conditions side-by-side without applying shock/heat. To ensure that the shock box was dry, the air was turned off for two days in advance.

Humidifying the air within the boxes influenced the walking behavior of the flies negatively. As soon as the flies were placed in the shock box under dry conditions flies appeared to be more active and did not differ significantly from flies tested in the heat box (Fig. 18A). Flies in the shock box also showed approximately the same duration of pauses when compared to the flies in the heat box, but with more variations. They also differed slightly regarding the number of pauses and very strongly in velocity (Fig. 18B, D). This result showed that differences between the devices might depend – in parts – on humidity. Interestingly, velocity was not affected by humidity.





**Fig. 18: Walking behavior in the shock and the heat box under the same humidity conditions.** Control flies in shock (green) and heat box (blue, striped) without humidity. No shocks or heat are applied during the 10min test. Phases are displayed as 10min bins. **(A)** Walking activity: No difference between the walking activities of the flies in the two boxes is observed. **(B)** Number of pauses: Flies in the heat box make slightly less pauses. **(C)** Duration of pauses: No difference in pause length can be measured. Note that the variability of the duration of pauses is bigger in the shock box (see SEM). **(D)** Velocity: Flies walk significantly faster in the heat box. \*\*\* $p < 0.001$ ; \*\* $p < 0.01$ ; \* $p < 0.05$ ; n.s.  $p > 0.05$ .

### 3.6 Impact of inescapable electric shocks on other behavior

It has been reported in mammals that in learned helplessness the respective motivational states persisted for a long time and affected other behavioral activities such as sexual behavior and food intake (Maier and Watkins, 2005). In 1967, Seligman and Maier already showed that dogs were unable to learn avoiding the punished side in a shuttle box experiment which was subsequently conducted after exposing the yoked dogs to uncontrollable electric foot shocks (also see chapter 1.1). The shuttle box experiment was repeated with mice and goldfish (Behrend and Bitterman, 1963; Braud et al., 1969). I raised the question whether learned helplessness in the shock box would be context specific or if it would also affect other behavioral activities. For such experiments, the flies might have to be transferred from the shock box to some other environment.

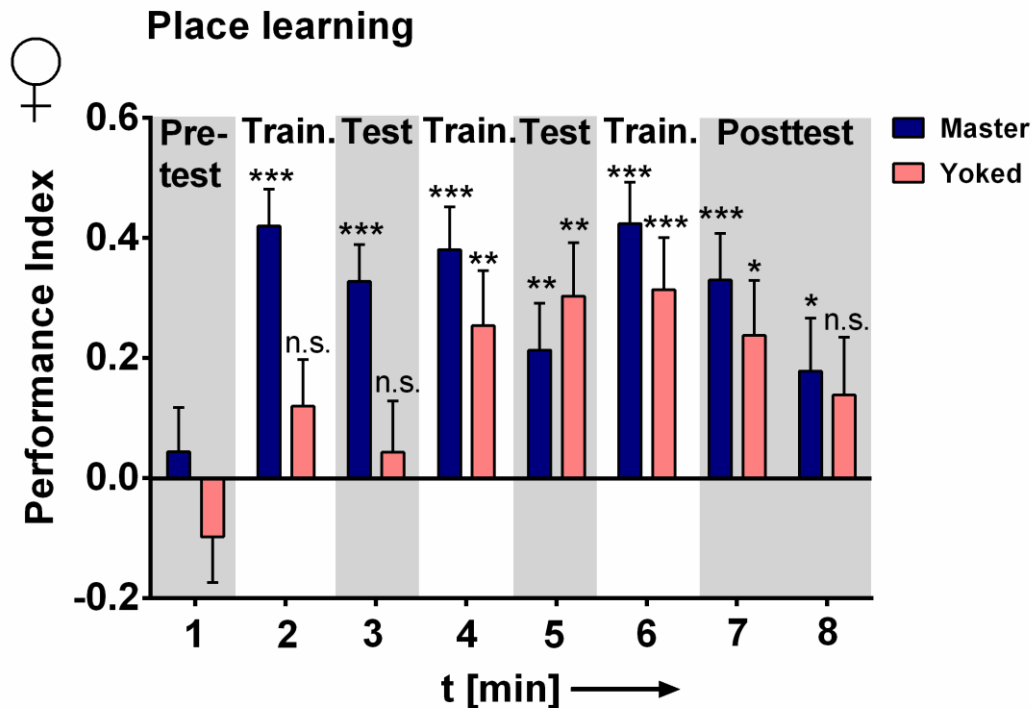
#### 3.6.1 Impact of stress on place learning

Bertolucci (2008) showed that flies which were exposed to uncontrollable heat pulses, were also impaired in subsequent place learning. In order to test whether the animals show a similar behavior in the shock box, the following experiment was conducted: After a 20

minute conditioning phase flies were tested in a 8 minute place learning experiment which consisted of a 1 minute pretest followed by alternating 1 minute training and test phases. Finally a 2 minute post-test was conducted to test how stable their memory was (for more details compare Material and Methods, chapter 2.5).

Seligman et al. (1980) could not fully exclude that the dogs learned to be inactive and were thus not showing an escape behavior. Here only yoked flies which were still active, after being exposed to the uncontrollable conditioning, were used for the place learning task. I used the following criteria: I only included flies in the place learning experiment, if they (a) showed any walking during the 1 minute pretest and (b) received at least one electric shock during the first training. Ten pairs had to be discarded due to these criteria. The remaining master and yoked flies, 58 in each group, differed considerably in the first few minutes (Fig. 19). After one minute pretest master flies immediately avoided the punished side. This did not change during the rest of the experiment. The yoked group required more time to avoid the punished side (two trainings). Comparing the PI's in the post-test, I found only significant memory scores in master flies for both test minutes. For yoked flies the memory, however, seemed to be less stable (Fig. 19, post-test).

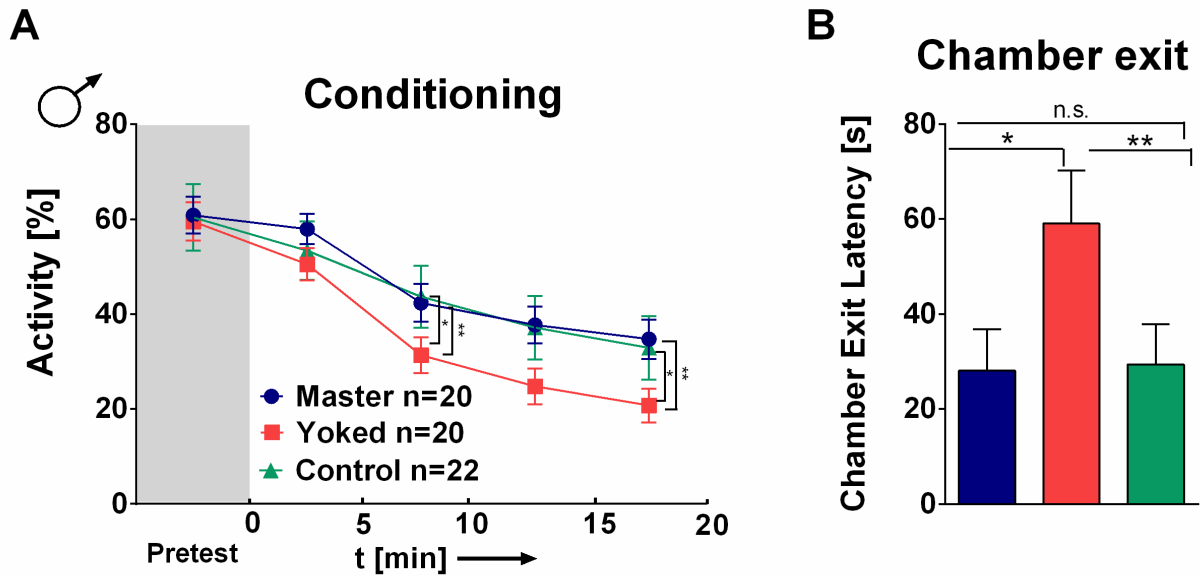
The fact that the master flies showed good memory scores subsequent to the no-idleness experiment, where they were 'pushed' to be more active, is not necessarily self-evident. This is particularly interesting, when it is taken into account that higher activity levels are known to be negatively correlated with successful avoidance behavior in place learning (Putz, 2002).



**Fig. 19: Impact of inescapable shocks on place learning.** Place learning experiment subsequent to 20min no-idleness conditioning in the chambers. 1min measuring periods are shown. Positive performance indices indicate avoidance of the punished side (compare Material and Methods, chapter 2.5): In the first training and test the yoked flies (red) did not avoid the punished side while the master flies (blue) performed normally. During post-test avoidance of the punished side was less stable in yoked than in master flies. Master/yoked pairs,  $n = 58$ . \*\*\* $p < 0.001$ ; \*\* $p < 0.01$ ; \* $p < 0.05$ ; n.s.  $p > 0.05$ .

### 3.6.2 Chamber exit latency – Positive phototaxis

From mice and flies it is known that exposure to stress leads to anxiety-like symptoms as shown, for instance, in the so-called shelter paradigm where the animals avoid exposure to light (Kercmar et al., 2011; Mohammad et al., 2016). In the present experiment I measured the time the flies needed to leave the shock box after the conditioning. Male flies were used to later compare their courtship behavior in a subsequent test. During the 20 minutes conditioning, male yoked flies reduced their walking activity compared to the master flies (Fig. 20A). Directly after the conditioning chambers were opened and flies were free to crawl into a translucent transfer vial to be transported to the courtship chamber. The vial was translucent to elicit positive phototaxis (Benzer, 1967) (a computer screen was positioned in front of the chambers to ensure uniform lighting conditions). Striking differences between master, yoked and control flies were found. Yoked flies took twice as long as master or control flies to walk out into the light (Fig. 20B).



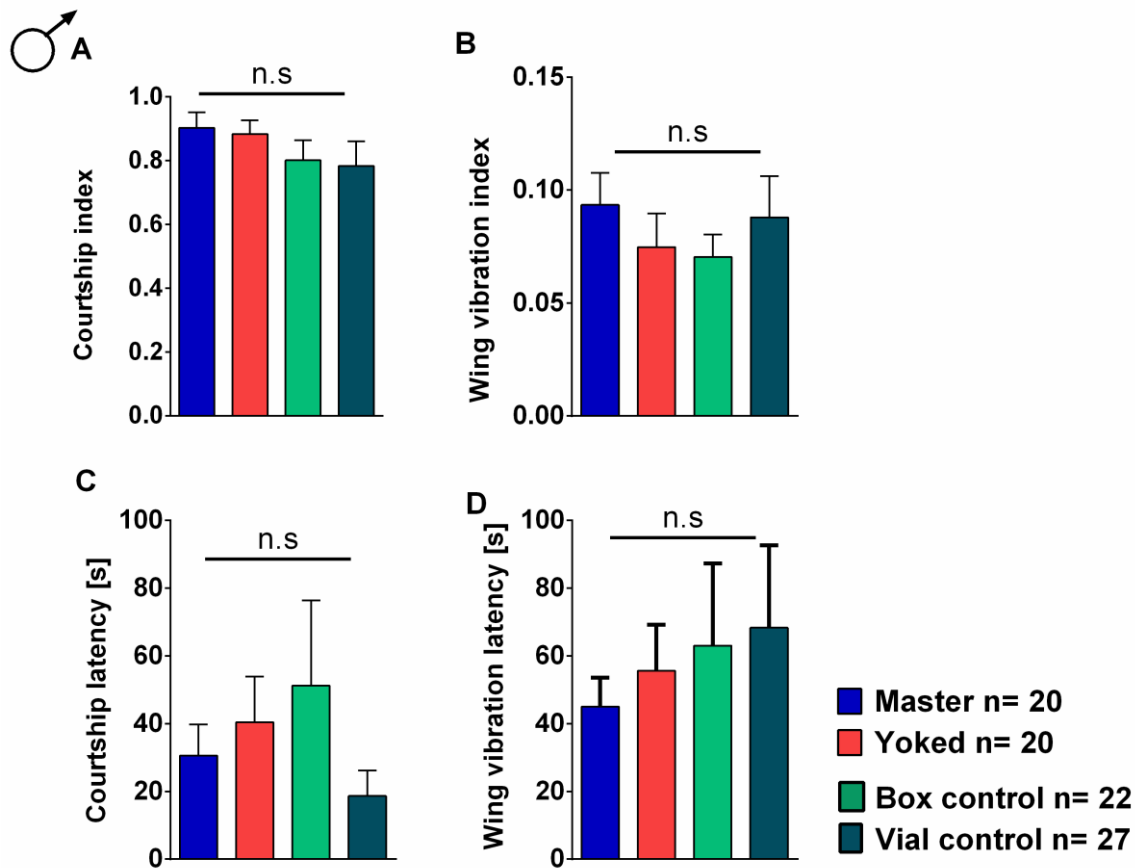
**Fig. 20: Male conditioning and leaving the shock box. (A)** Activity: Male pretest and conditioning: Starting from 5<sup>th</sup> min, yoked (red) flies show a significantly lower activity level than master (blue) or control (turquoise) flies. **(B)** Chamber exit latency: Time the flies needed to crawl out of the chamber into a transport vial. Yoked flies take longer time than master and control flies. Master, n= 38; Yoked, n= 44; Control, n= 41. \*\*\*p<0.001; \*\*p<0.01; \*p<0.05; n.s p>0.05.

### 3.6.3 Courtship behavior subsequent to stress in male flies

Earlier work has provided evidence that exposure to stress negatively impacts the sexual behavior of male rats (D'Aquila et al., 1994). Furthermore, sexual dysfunction is a well-known symptom in depression in humans (Michael and O'Keane, 2000).

Subsequent to the conditioning phase, a single master or a yoked fly was transferred to a small chamber where its courtship behavior was recorded in the presence of a virgin female fly. The following parameters were measured: Courtship index, the wing vibration index, wing vibration latency and the courtship latency (for details compare Materials and Methods, chapter 2.2.3). As an additional control I compared males taken directly from food vials that had not experienced the 20 minutes period in the shock box (vial control) in order to exclude the highly restrained situation in the chamber to be the cause for changes in subsequent behavior. Courtship indices, which included the time the male fly spent with courtship behavior divided by the recording time, lay between 0.9-0.7 (arbitrary units) indicating that the flies spent between 70-90% of the time courting. It seemed that the vial control flies tended to start earlier with the courtship behavior than the compared groups (however, just not significant) (Fig. 21C). Flies needed approximately one minute to start with the wing song (Fig. 21D). In summary, none of the parameters showed any statistical significant differences concerning the courtship behavior between master, yoked and both

control groups. The differences measured in walking behavior in the shock box did not result in different courtship behavior afterwards (Fig. 21A-D).



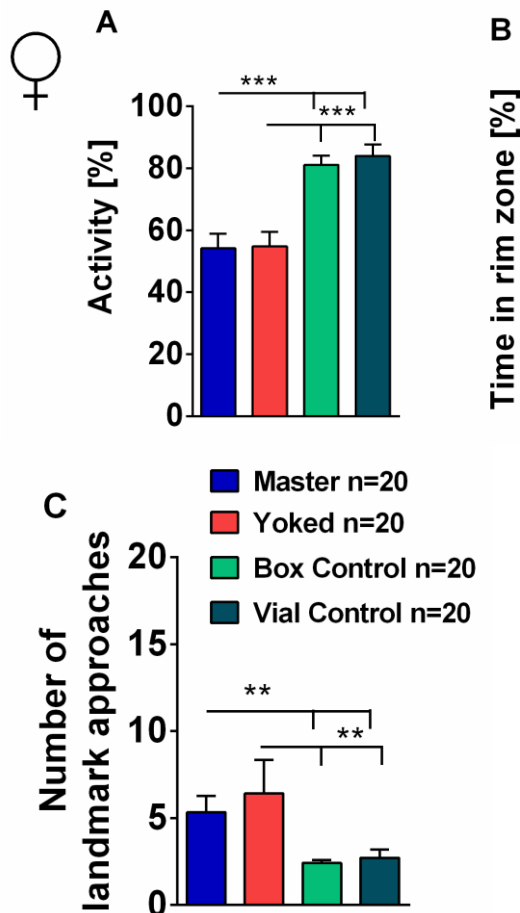
**Fig. 21: Courtship behavior after 20min conditioning.** Male flies were conditioned for 20min in the shock box (see Fig. 20A) and subsequently transferred to a courtship chamber where their courtship behavior was recorded for 10min. Courtship behavior was manually evaluated (compare Material and Methods, chapter 2.2.3) **(A)** Courtship index during 10min recording time: No differences can be observed between master (blue), yoked (red), box control (turquoise) and vial control (petrol blue) flies. **(B)** Wing vibration index: No statistically significant difference regarding the wing vibration indices between the tested groups. **(C)** Courtship latency: Flies of all groups need about the same time to start with their courtship behavior. **(D)** Wing vibration latency: No difference can be observed between the groups. \*\*\* $p < 0.001$ ; \*\* $p < 0.01$ ; \* $p < 0.05$ ; n.s  $p > 0.05$ .

### 3.6.4 Impact of inescapable shock on free walk

In the shock box the flies are highly restrained. A common procedure of testing walking behavior in animals is the open-field test. Hall (1934) introduced it to study anxiety-like behavior in vertebrates. Receiving 20 minutes of uncontrollable shocks had a lasting effect on walking behavior in flies which was represented by the differences observed between the tested groups in the box (Fig. 8, Fig. 10 and Fig. 12). Here, after the 20 minutes conditioning, female flies were free to walk out into a translucent vial, from which they were then aspirated and transferred into the free walk arena (diameter: 86mm, for more details

compare Material and Methods, chapter 2.2.2), where their trajectories were recorded for 10 minutes.

Master and yoked flies spent about 55% of the time active (in this experiment a fly is considered active when walking more than 4mm/s), while control flies that had been in the chamber without shocks (box control) and vial controls (collected from a fresh food vial), spent significantly more time walking (80-85%) (Fig. 22A). Master and yoked flies spent less time in the rim zone when compared to controls (Fig. 22B). Besides the other two parameters, the number of landmark approaches were also measured. Master and yoked flies approached the stripes more often than both control groups did (Fig. 22C). No difference could be found between master and yoked flies in any of the measured parameters. To make sure not to miss an effect, I evaluated the data according to the activity definition used in the shock boxes (0.6mm/s). No differences between master and yoked could be found with these criteria either (results not shown). Uncontrollability seemed not to influence walking behavior in the new environment (open-field, light, different humidity conditions), but a changed and modified walking behavior due to electric shock was observed.



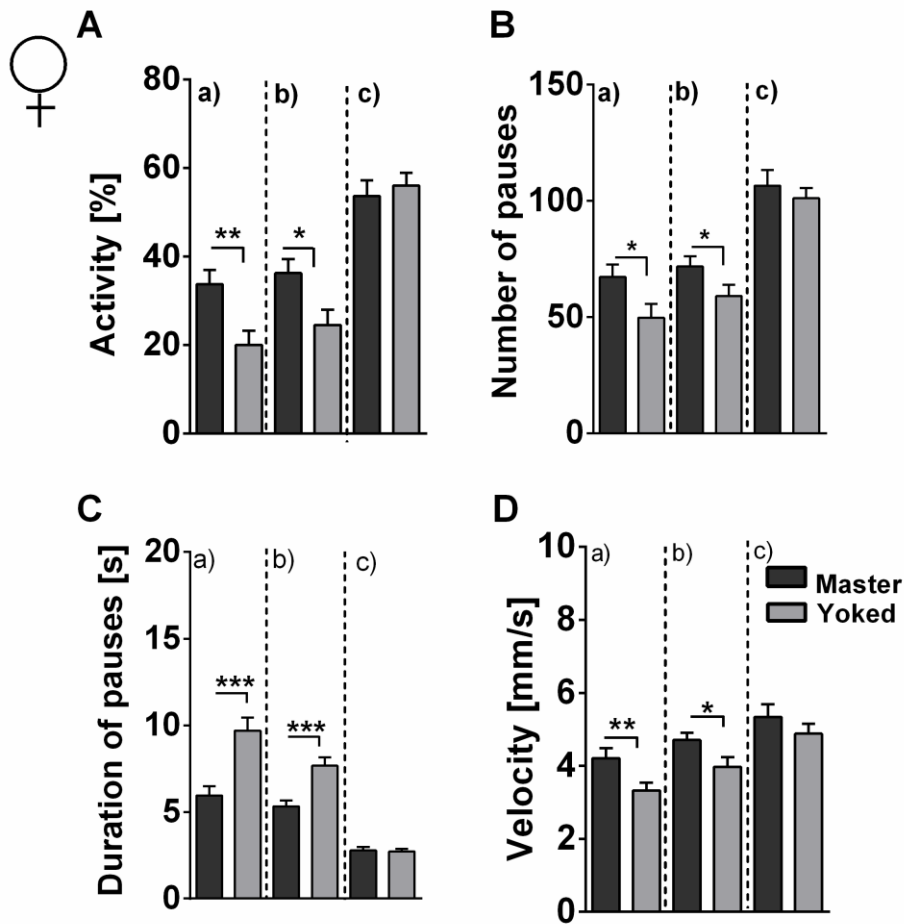
**Fig. 22: Walking behavior in open field in female flies.** Walking activity in the arena is defined as walking more than 4mm/s. Rim zone width is defined as 20% of the arena radius. Data are pooled in 10min periods. **(A)** Walking activity during free walk during 10min subsequent to 20min conditioning in the shock box: Master (blue) and yoked (red) flies walk less than the compared box controls (turquoise) and vial controls (petrol blue). **(B)** Time flies spend in the rime zone. Both control groups spend more time in the rime zone than master or yoked flies. **(C)** Number of landmark approaches: Master and yoked flies approach the landmarks more often than the controls. \*\*\* $p < 0.001$ ; \*\* $p < 0.01$ ; \* $p < 0.05$ ; n.s  $p > 0.05$ .

### 3.6.5 Does handling abolish the after effect observed in the boxes?

The experiments conducted in chapter 3.6 required handling procedures. From aggression studies in flies it is known that aspirating the flies and transferring them to a different environment can interrupt the learning process (Trannoy et al., 2015). To find out whether the handling itself has an impact on the master/yoked differences observed here, flies were transferred back to either the shock- or heat box, respectively after the 20 minutes conditioning phase. Flies were allowed to crawl into a non- translucent transport vial, from where they were taken up into an aspirator and blown back into a different chamber of the same device or transferred to a chamber of the box of the other type. This was conducted under dim light.

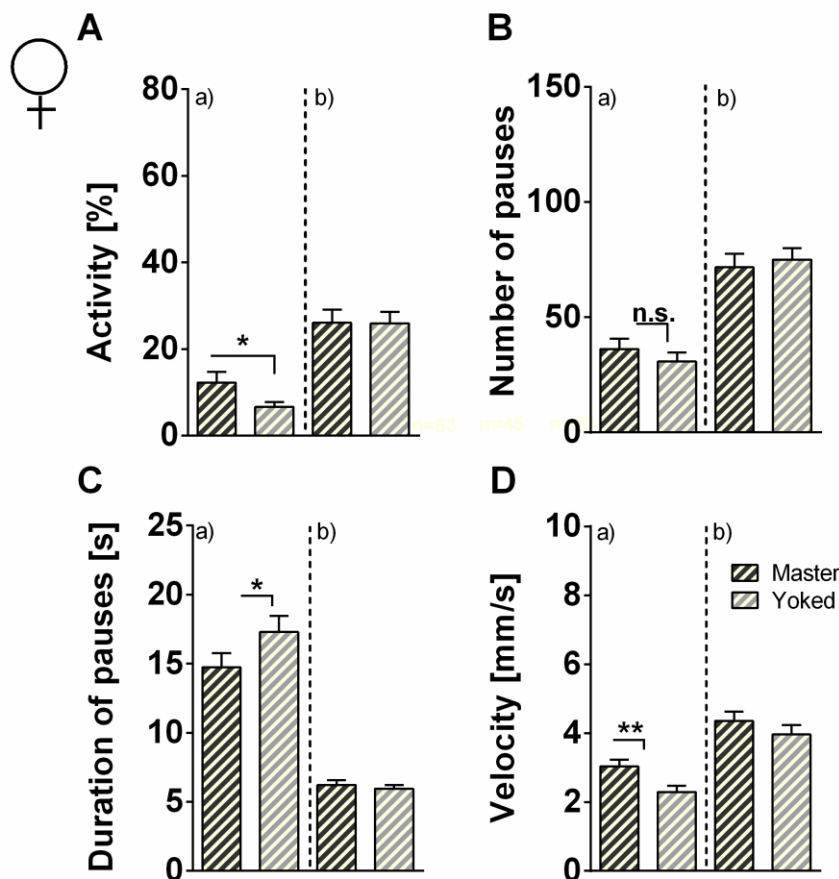
As already seen before, 20 minutes of conditioning lead to a stable after-effect in both devices (Fig. 23A-D (a) and Fig. 24A-D (a)). A 20 minutes conditioning phase followed by handling led to a lower activity level, less, but longer pauses and slower walking of the yoked flies in the shock box but not the heat box (Fig. 23A-D (b) and Fig. 24A-D (b)). After

conditioning the flies in the shock box I also transferred some of them to the heat box. It is to be noted that the humidity was much lower in the heat box. No difference during the test phase was found after the transfer (Fig. 23A-D (c)). Differences between master and yoked remained stable after the handling procedure only in the shock box but not in the heat box (Fig. 23A-D (b) and Fig. 24A-D (b)). In both devices handling led to an increase in activity. This effect was most pronounced in yoked flies in the heat box.



**Fig. 23: Effect of handling on female flies in the shock box.** Master (black), yoked (grey). Walking behavior during 10min test phase (no shocks) subsequent to 20min of conditioning in the shock box. For **(A)** Activity **(B)** Number of pauses, **(C)** Durations of pauses and **(D)** Velocity: **(a)** No handling: Master and yoked flies differ significantly (master/yoked pairs: n= 38). **(b)** After handling: Master and yoked flies differ significantly (master/yoked pairs: n= 34) **(c)** Transfer to the heat box abolishes the uncontrollability effect (master/yoked pairs: n= 29). \*\*\*p<0.001; \*\*p<0.01; \*p<0.05; n.s p>0.05.





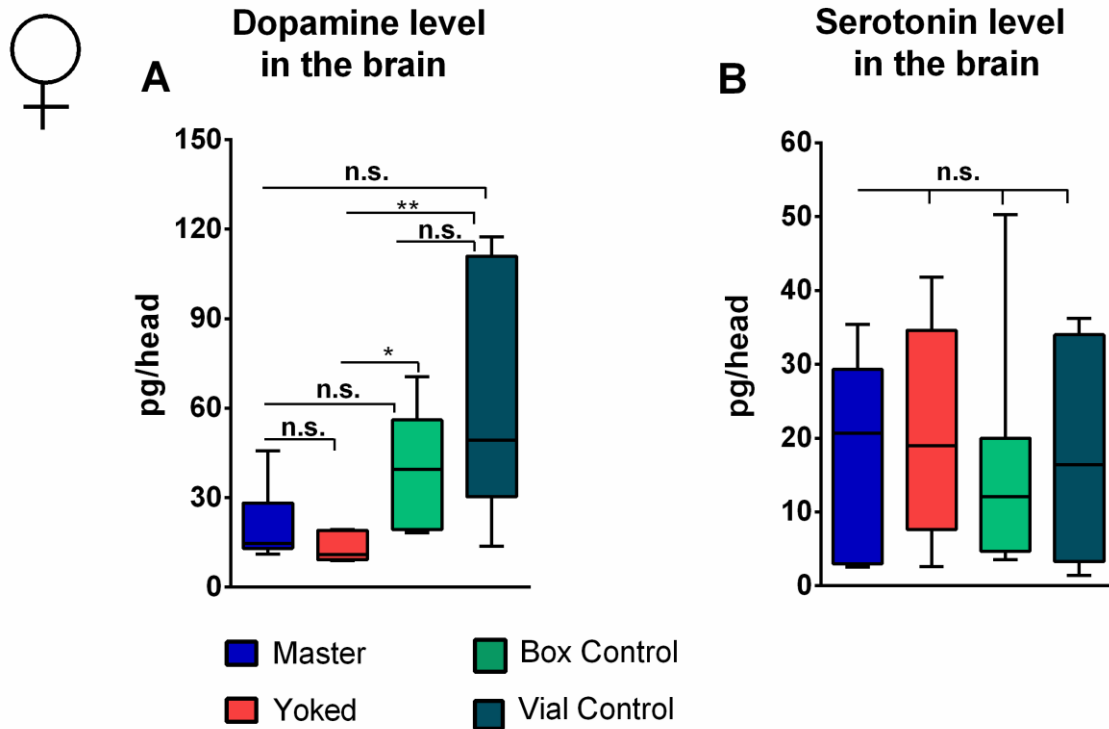
**Fig. 24: Effect of handling on female flies in the heat box.** Master (black-striped), yoked (grey-striped). Walking behavior during 10min test phase (no heat) subsequent to 20min of conditioning in the heat box. For **(A)** Activity **(B)** Number of pauses **(C)** Durations of pauses and **(D)** Velocity: **(a)** No handling: Master and yoked flies differ significantly. Except for the number of pauses (master/yoked pairs:  $n=58$ ). **(b)** After handling: The uncontrollability effect is abolished (master/yoked pairs:  $n=63$ ) \*\*\* $p<0.001$ ; \*\* $p<0.01$ ; \* $p<0.05$ ; n.s  $p>0.05$ .

### 3.6.6 Impact of inescapable shocks on monoamine levels in the brain

Parallel mechanisms have been observed between vertebrate depression studies and the pathophysiology of learned helplessness (Abelaira et al., 2013). Different studies suggest evidence that serotonin plays a role in mediating learned helplessness (Maier and Watkins, 2005). Since learned helplessness also serves as an animal model for depression research, dopamine is also an interesting candidate to focus on. Argue and Neckameyer (2013) found that different dopaminergic neurons are involved in responses to stress in *Drosophila*. I decided to take a look at monoamine levels in the brain after the exposure to stress. Bertolucci (2008) and Ries (2015) showed that feeding a serotonin re-uptake inhibitor or a precursor of serotonin, reversed the effects according to learned helplessness. I conditioned the flies in the chambers for 20 minutes and then transferred them to liquid nitrogen and decapitated them by using a vortexer. Fly heads were analyzed using a High-performance liquid chromatography (HPLC) (compare Material and Methods, chapter 2.6).

I measured a significantly lower dopamine level in the heads of yoked flies but not in master flies, when comparing the levels statistically to the control group. No difference could be

measured between master and yoked flies (Fig. 25A). Serotonin levels did not differ between the three groups (Fig. 25B). Dopamine, but not serotonin levels were reduced due to the inescapable shocks in our experiment.



**Fig. 25: Concentration of dopamine and serotonin in the heads are measured after 20min conditioning in the shock box.** Concentration is measured as picogram/head.  $n=1$  equates 20 fly heads. Non parametrical presentation of the data. Data in the box represent the 25% and 75% quartiles, while the error bars represent the distribution down to the minimum and up to the maximum values of the dataset. **(A)** Concentration of dopamine in the heads: Dopamine is reduced in yoked flies (red) when compared to box controls (turquoise) and vial control (petrol blue), but do not differ significantly from master (blue) flies. Master  $n=6$ ; Yoked  $n=6$ ; Control  $n=9$ ; Vial-Control  $n=9$ . **(B)** Concentration of serotonin in the heads: Flies do not differ concerning the serotonin concentration in the heads. Master  $n=7$ ; yoked  $n=7$ ; box control  $n=11$ ; vial control  $n=11$ . \*\*\* $p < 0.001$ ; \*\* $p < 0.01$ ; \* $p < 0.05$ ; n.s  $p > 0.05$ .

### 3.7 How prior experience influences response to uncontrollability

The loss of control over a stimulus like electric shock influences the locomotor behavior in females and males. My interest was drawn to the question whether flies would show any differences due to social isolation. In the basic experiment shown in chapter 3.3 the flies were transferred from a group-rearing situation to isolation for the experiments. Now, group-reared flies were compared to flies that had lived in isolation already for all their adult life. The purpose of this investigation was to find out whether keeping flies in isolation for three days may influence their walking behavior due to uncontrollability differently in comparison to group-reared flies during the 20 minutes conditioning and the 10 minutes post-test.

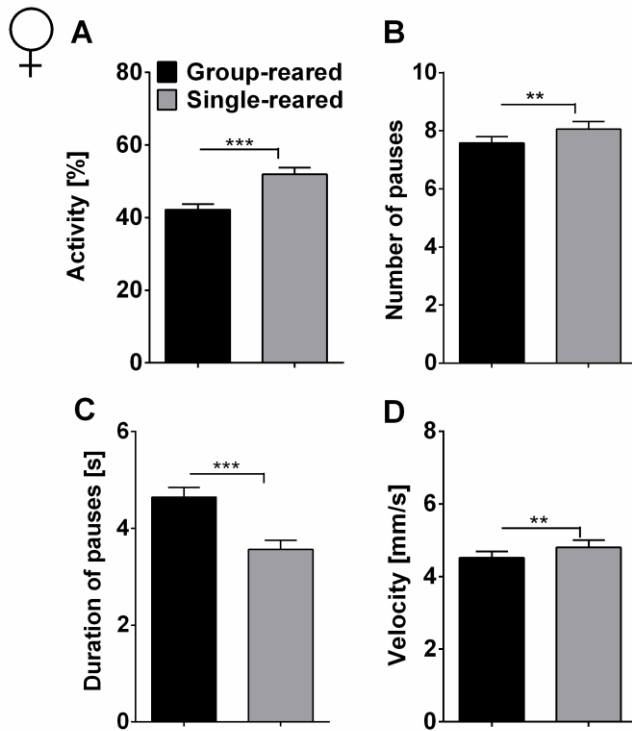
### **3.7.1 Social isolation as a stressor**

Social behavior is defined as interaction between members of the same species. Mice, which are kept in isolation for five days, appeared to be more active (Hilakivi and Lister, 1989; Bakshi and Geyer, 1999; Heidbreder et al., 2000) and more aggressive (Garipey et al., 1995; Matsuda et al., 2001) when compared to group-reared mice. After 20 days they showed more fear-like behavior (Molina-Hernandez et al., 2001) and a higher excitability (Guo et al., 2004) than group-reared animals. Kerckmar (2011) found that social isolation leads to an increased anxiety-like behavior in the light-dark-field experiment in mice (Kerckmar et al., 2011). Such rearing procedures are the reason for a reduction of volume in the medial prefrontal cortex (Schubert et al., 2009), reduction of dopamine and serotonin levels in different parts of the brain as well as changes in the expression of the transcription factor CREB 1 (cAMP responsive element binding protein1) which plays a major role in learning processes (Heidbreder et al., 2000; Wallace et al., 2009). It is known that the rearing situation also has an impact on behavior in flies. Previous studies have shown that social isolation leads to a higher activity level in male flies (Panova et al., 2013) and increases their aggressiveness level (Wang et al., 2008) when compared to group-reared flies.

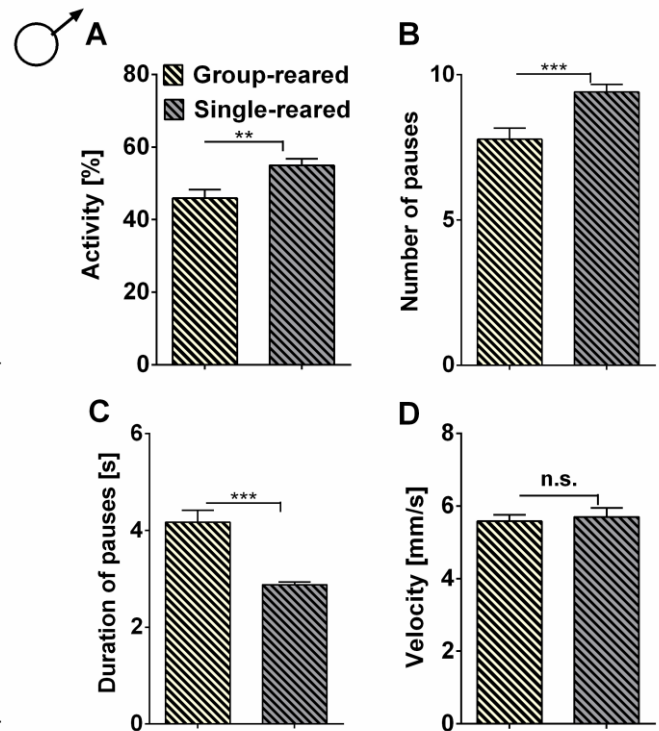
In the following, firstly the walking behavior of single- and group-reared males and females in the chambers without exposing them to electric shock was observed (Fig. 26 and Fig. 27). In a second experiment single-reared and group-reared flies during conditioning and the subsequent test were compared (Fig. 28-Fig. 31).

### **3.7.2 Comparison of single-reared and group-reared flies in walking behavior without shocks**

Single-reared male and female flies showed a higher activity level throughout the 20 minutes in the chamber compared to the group-reared flies (Fig. 26A and Fig. 27A). Group-reared female flies made less but longer pauses and walked slower than the single-reared flies (Fig. 26B-D). Looking at male flies, single-reared flies made more and shorter pauses but did not walk significantly faster than group-reared males (Fig. 27B-D).



**Fig. 26: Impact of rearing conditions on walking behavior in the shock box without punishment in female flies.** Group-reared (black), single-reared (grey). Walking behavior of female group-reared and single-reared flies during 20min test phase. Flies do not receive shocks. **(A)** Walking activity: Single-reared flies are more active than group-reared flies. **(B)** Number of pauses during 20min: Single-reared flies make more pauses than group-reared flies. **(C)** Duration of pauses per min: Single-reared flies make shorter pauses when compared to group-reared flies. **(D)** Walking speed: Single-reared flies walk faster. Group-reared, n= 174; single-reared, n= 113 \*\*\*p<0.001; \*\*p<0.01; \*p<0.05; n.s p>0.05.



**Fig. 27: Impact of rearing conditions on walking behavior in the shock box without punishment in male flies.** Group-reared (white-striped), single-reared (grey-striped). Walking behavior of male group-reared and single-reared flies during 20min test phase. Flies do not receive shocks. **(A)** Walking activity: Single-reared flies are more active than group-reared flies. **(B)** Number of pauses during 20min: Single-reared flies make more pauses than group-reared flies. **(C)** Duration of pauses per min: Single-reared flies make shorter pauses when compared to group-reared flies. **(D)** Walking speed: No difference between single- and group-reared flies. Group-reared, n= 46; single-reared, n= 89. \*\*\*p<0.001; \*\*p<0.01; \*p<0.05; n.s p>0.05.

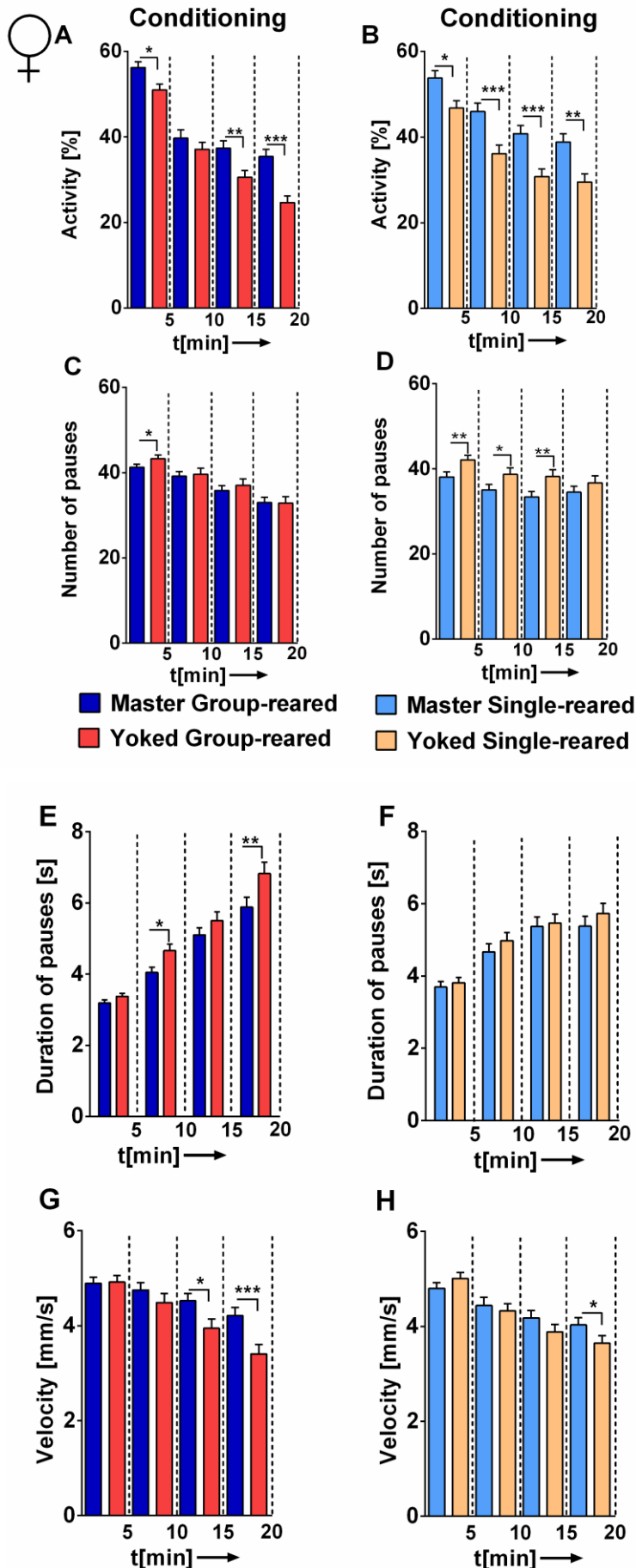
### 3.7.3 Comparison of single-reared and group-reared flies during conditioning and test

After finding a difference in single-reared and group-reared flies without electric shock, another set of single- and group-reared flies was conditioned and tested in the shock box to investigate the impact of inescapable shocks on their behavior.

#### 3.7.3.1 Single-reared female flies react differently to uncontrollable stress compared to group-reared female flies

Once the conditioning started these marked differences in walking activity were not present anymore. Single-reared and group-reared yoked flies showed a lower activity level when compared to their corresponding master fly (Fig. 28A, B). Group-reared yoked flies made more pauses in the first five minutes of the conditioning phase, whereas in the next 15

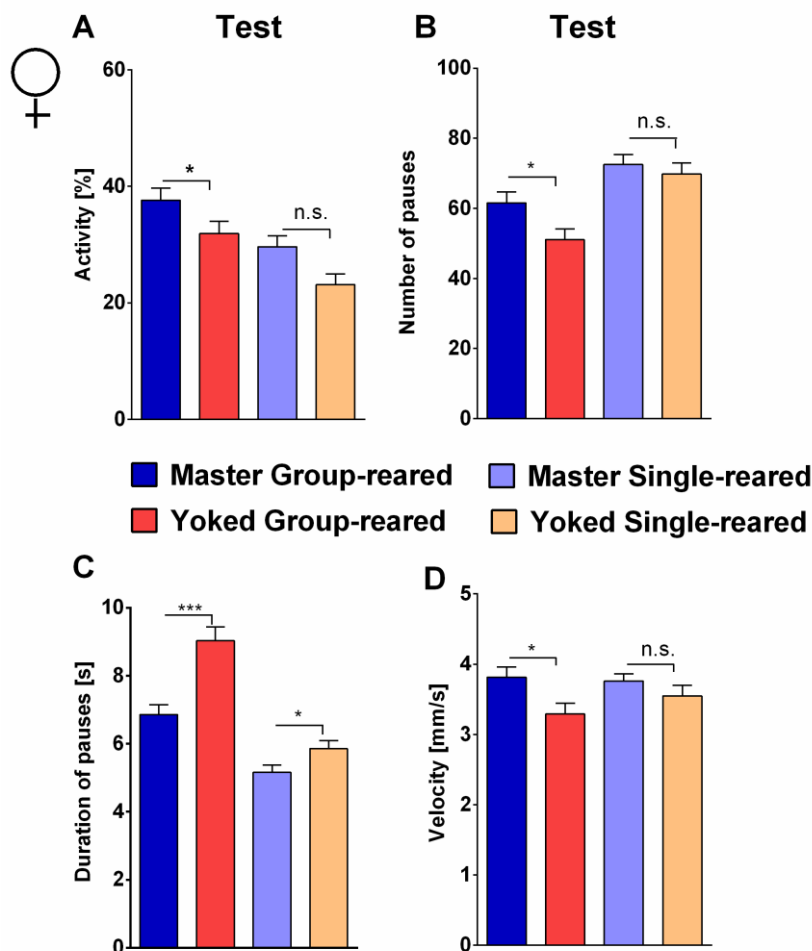
minutes the number of pauses aligned between master and yoked flies (Fig. 28C). In contrast to that, the single-reared yoked flies made more pauses than the master flies (Fig. 28D). Group-reared master and yoked flies differed regarding the length of pauses and concerning their walking speed (starting in the 10<sup>th</sup> minute of the conditioning phase) (Fig. 28E, G), while the single-reared flies did not differ concerning the length of the pauses they made and diverged in walking speed later in the conditioning phase (Fig. 28F, H).



**Fig. 28: Impact of rearing condition on modulation of walking behavior in female flies in the shock box.** During conditioning data are pooled in 5min periods. **(A)+(B)** In group-reared and single-reared flies, master and yoked differ in walking activity. **(C)+(D)** In single-reared flies, the yoked make more pauses than master flies starting from the 1<sup>st</sup> -15<sup>th</sup> min. This only occurs in group-reared flies in the first five min. **(E)+(F)** Yoked group-reared flies make longer pauses, starting after five min of conditioning, while this effect is not observed in single-reared flies. **(G)+(H)** Group-reared master flies walk faster than yoked flies in the second half of the conditioning, this effect is only found in the last five min of conditioning in single-reared flies. Group-reared master/yoked pairs, n= 118; single-reared master/yoked pairs, n= 103. \*\*\*p<0.001; \*\*p<0.01; \*p<0.05; n.s p>0.05.

### 3.7.3.2 Single-reared flies are less affected by uncontrollability

The after-effect turned out to be sustainable only in group-reared flies. With the exception of the pause length during the test, no significant difference between the master and yoked flies under single-rearing conditions was found (Fig. 29A-D).



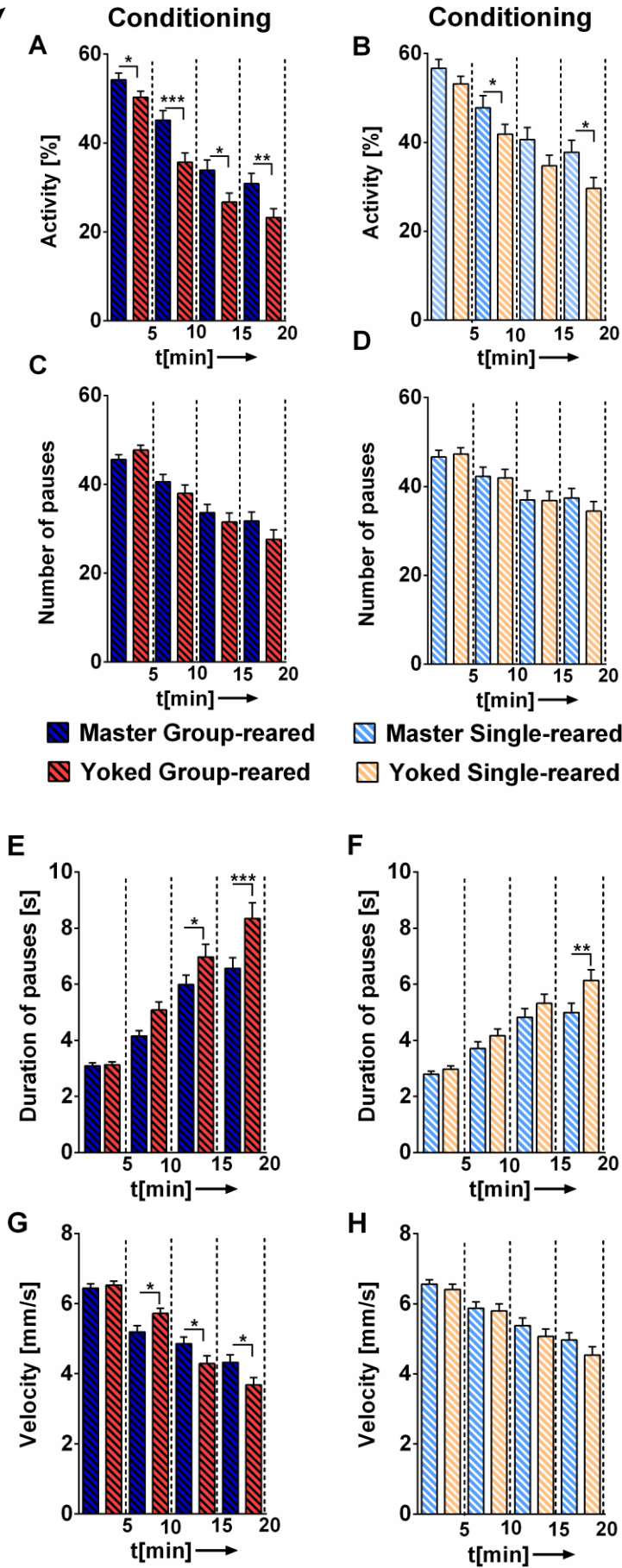
**Fig. 29: Impact of rearing condition on 10min post-test in female flies. (A)** During the test, group-reared yoked flies remain less active than group-reared master flies. No significant difference between single-reared flies. **(B)** Number of pauses during test: The difference is stable in group-reared flies, exclusively. **(C)** Duration of pauses during test: Yoked group-reared flies make significantly longer pauses during the test, in single-reared flies a weak after effect can be observed. **(D)** Walking speed during test: Only in group-reared flies the after effect is stable throughout the 10min test. Group-reared master/yoked pairs,  $n = 118$ ; single-reared master/yoked pairs,  $n = 103$ . \*\*\* $p < 0.001$ ; \*\* $p < 0.01$ ; \* $p < 0.05$ ; n.s  $p > 0.05$ .

### 3.7.3.3 The reaction to stress does hardly differ between single-reared and group-reared male flies

In male and female flies a similar effect was found (for comparison see chapter 3.7.3.1). As soon as the shocks were switched on, the two groups (single-reared and group-reared flies) seemed to deal with the shocks in a similar way. Yoked flies showed lower walking activity and longer pauses in single-reared and group-reared flies (Fig. 30A, B, E and F). No significant difference in walking speed was found in single-reared flies, while group-reared master flies walked significantly faster than yoked did (Fig. 30G, H). A surprising result occurred regarding the velocity in group-reared flies. Between the 5<sup>th</sup> and the 10<sup>th</sup> minutes, yoked flies walked significantly faster than the master flies (Fig. 30G). This effect

did not show, when I conducted the same experiment in male flies earlier (compare Fig. 14D).

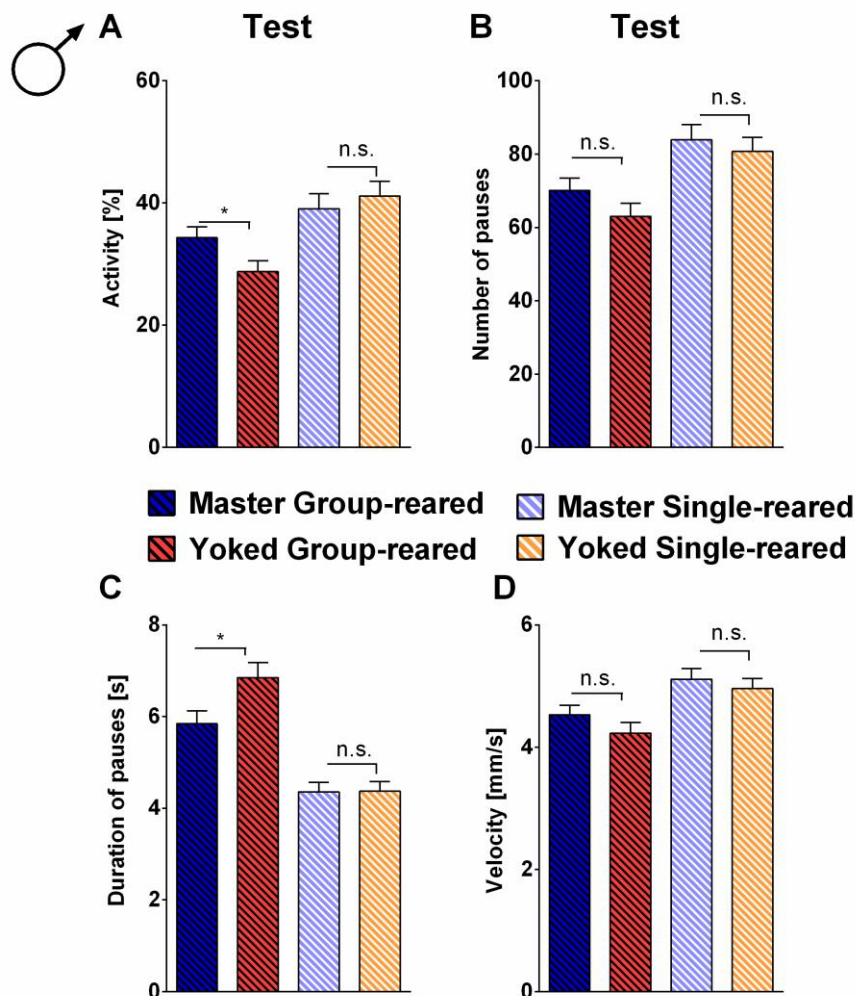




**Fig. 30: Comparison between single- and group-reared male flies during 20min conditioning.** During conditioning data are pooled in 5min periods. **(A)+(B)** In group-reared and single-reared flies, master are more active than yoked flies. **(C)+(D)** In both groups master and yoked do not differ in number of pauses. **(E)+(F)** In single- and group-reared flies, yoked make longer pauses. Stronger pronounced in group-reared flies. **(G)+(H)** Group-reared master flies walk faster than yoked flies, this effect does not apply for single-reared flies. Group-reared master/yoked-pairs, n= 127; single-reared master/ yoked pairs, n= 69. \*\*\*\*p<0.001; \*\*p<0.01; \*p<0.05; n.s p>0.05

### 3.7.3.4 No sustainable after-effect in single-reared male flies can be measured

During the test phase only group-reared flies showed a sustained after-effect in walking activity and pause length (Fig. 31A, C). This confirms the results already measured for group-reared flies (compare Fig. 15A, C). In single-reared male as well as in single-reared female flies, no sustained after-effect was observed (Fig. 31A-D). Apparently single-reared flies of both sexes were less affected by uncontrollability.

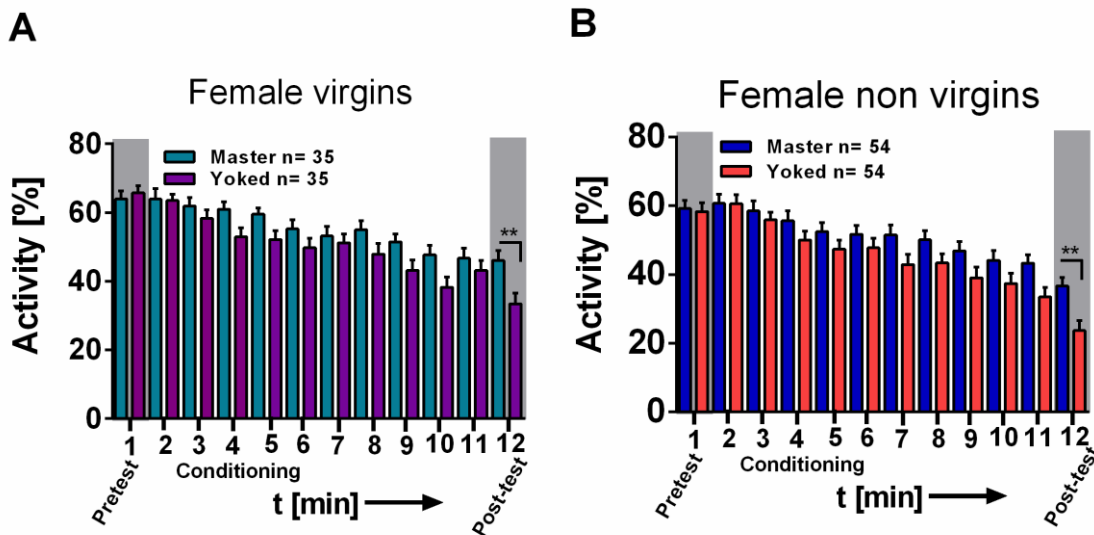


**Fig. 31: Impact of rearing condition on 10min post-test in male flies.** (A) During the 10min test, group-reared yoked flies remain less active than group-reared master. No significant difference between the single-reared flies. (B) Number of pauses during test: In both groups, no after-effect can be measured between master and yoked flies. (C) Duration of pauses during 10min test: Yoked group-reared flies make significantly longer pauses during the test, in single-reared flies no after-effect can be observed. (D) Walking speed during test: In both groups, no after-effect can be measured between master and yoked flies. Group-reared master/yoked pairs,  $n=127$ , single-reared master/yoked pairs,  $n=69$ . \*\*\* $p<0.001$ ; \*\* $p<0.01$ ; \* $p<0.05$ ; n.s.  $p>0.05$ .

### 3.7.4 Comparison of walking activity between virgin- and non-virgin female flies

Hormonal fluctuations in female flies may contribute to their differences in activity. In order to single-house the flies, they were collected after hatching and transferred singly to a vial. In mammals hormonal fluctuations are called activational effects and occur by definition at later stages in adulthood: "Activational effects serve to alter the behavioral state of the animal in response to a variety of changes in environmental or social conditions" (Elekovich and Robinson, 2000). In insects it is also known that mated flies produce for

example the juvenile hormone, which might influence their behavior (Argue et al., 2013). In order to exclude this, I conducted an experiment comparing mated with non-mated females (Fig. 32A, B). The reaction to uncontrollable aversive stimuli did not differ whether flies were mated or not.



**Fig. 32: Comparison of walking behavior between virgin and non-virgin flies:** During pretest and post-test no shocks are applied. Data are presented in 1min bins. Asterisks are omitted in the conditioning phase. **(A)+(B)** Virgin and non-virgin yoked flies develop a significantly lower activity level during the conditioning phase when compared to master flies, which lasts throughout the post-test. \*\*\* $p < 0.001$ ; \*\* $p < 0.01$ ; \* $p < 0.05$ ; n.s  $p > 0.05$ .

### 3.8 Genetic intervention

So far learned helplessness in *Drosophila* has been characterized mainly by its symptoms via behavioral analysis. The choice of the fly as a study case, however, is mostly motivated by the power of its genetics for neural, molecular and circuit analysis. In the following I present two examples of the neurogenetic approach as a first glimpse of this potential.

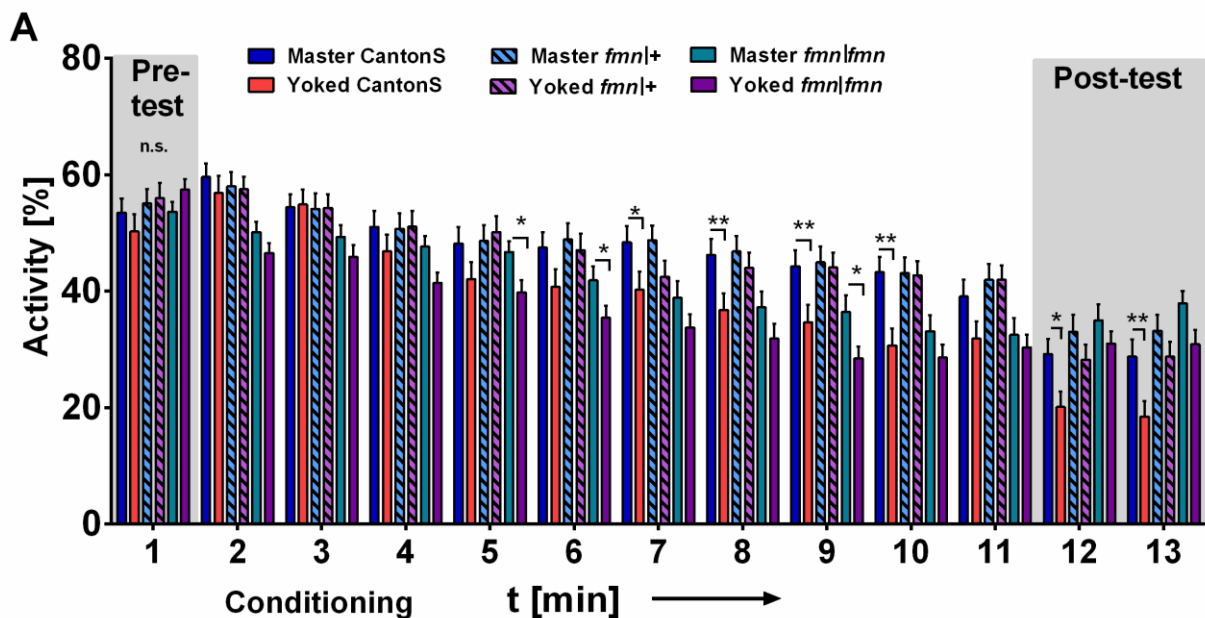
#### 3.8.1 Learned helplessness in *Drosophila* involves dopamine

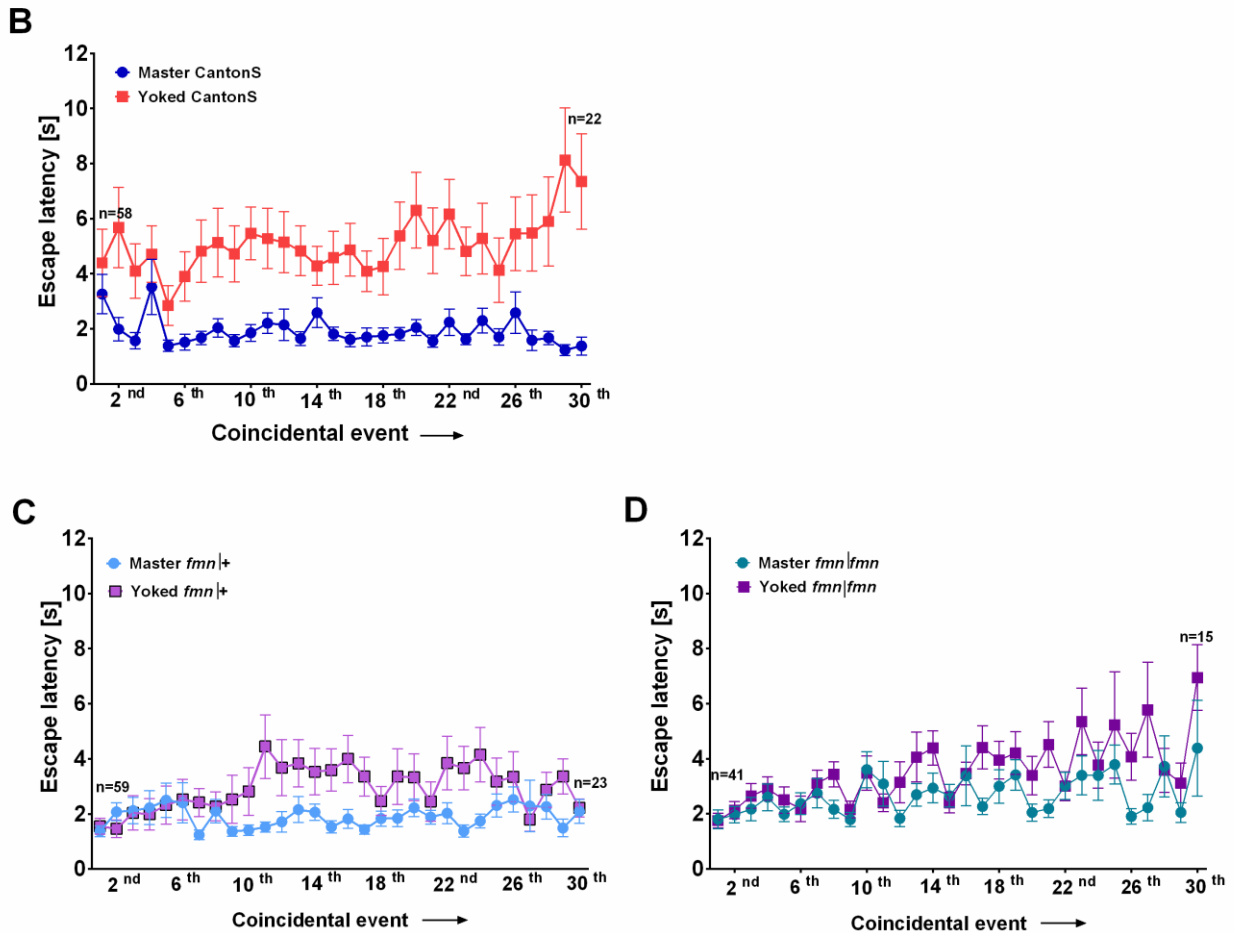
It is known that dopamine plays a role in learned helplessness and depression (D'Haenen and Bossuyt, 1994; Laasonen-Balk et al., 1999; Lambert et al., 2000; McLean, 2004). Moreover, dopamine signaling has been related to the reaction to stress in *Drosophila* (Argue and Neckameyer, 2013). With the triadic design and the study of walking behavior in the heat- and shock box an interesting mutant – *fumin* – came into focus. *Fumin* flies ( $dDAT^{fmn}$  or *fmn*) have an insertion in the *Drosophila* dopamine transporter (dDAT) gene that leads to a loss of function (Porzgen et al., 2001). Mutant flies appear to have a different activity/rest pattern in open field and are affected in arousal (Kume et al., 2005). Here I

used the short conditioning schedule (10 minutes) followed by a 2 minutes post-test to test the role of dopamine in learned helplessness. Homozygous (*fmn/fmn*) and heterozygous (*fmn/+*) mutant flies were compared to their genetic background CantonS.

Flies of all three genotypes started at the same activity levels when introduced into the chambers (Fig. 33A, first minute). One mutant allele (*fmn/+*) was enough to affect behavior in this paradigm. The yoked flies of the *fmn/+* genotype did not drop to the same level of walking activity as wildtype yoked flies ((Fig. 33A, master (blue-striped bars) and yoked (purple-striped bars)). Master/yoked pairs with a homozygous mutation (*fmn/fmn*) (blue and purple bars) differed scarcely in activity (only in minute five, six and nine) and showed no significant differences in the post-test. The *fmn/+* and the *fmn/fmn* flies did not show master/yoked differences in number and duration of pauses as well as in velocity (data not shown).

The escape latency (compare chapter, 3.3.6) in *fmn/+* flies was not as strongly pronounced as in wildtype flies (Fig. 33B, C). In *fmn/fmn* no differences between master and yoked were measured (Fig. 33D). As a result of the mutation in the dopamine re-uptake transporter, dopamine stays longer in the synaptic cleft and therefore may block the outcome learning or increase their resistance to stress in the mutant flies.





**Fig. 33: Comparison of CantonS and *fumin* flies.** (A) Walking activity of CantonS flies compared to *fumin* mutant flies during the no idleness paradigm. 1min pretest followed by 10min conditioning and 2min post-test. In CantonS flies a difference in walking activity between master/yoked emerges starting in the 4<sup>th</sup>min of the experiment and becomes significant from the 7<sup>th</sup> min on and sustains during the 2min post-test. In haploid *fmn*/+ flies a slight difference can be observed in the 7<sup>th</sup>min of the experiment and the post-test, but neither of them are significant. In *fmn*/*fmn* flies a slight difference between master/yoked can be observed starting from the 5<sup>th</sup> min of the experiment, but does not lead to a statistically significant difference in the post-test. Master/yoked pairs: CantonS n=62; *fmn*/+ n=60; *fmn*/*fmn* n= 43. \*\*\*p<0.001; \*\*p<0.01; \*p<0.05; n.s p>0.05. (B-D) Escape latencies compared between master and yoked flies. (B) Escape latencies of CantonS flies (also see Fig. 13): Yoked flies need longer time to resume walking after the onset of an electric shock when directly compared to master flies. Master/yoked pairs for first coincidental event, n=58 and for the 30<sup>th</sup> event n=22 (C) *fmn*/+ yoked flies show a smaller effect in escape latency than CantonS flies, but still need longer time to resume walking after the onset of a shock, when compared to master flies. Master/yoked pairs for first coincidental event, n=59 and for the 30<sup>th</sup> event n=23 (D) *fmn*/*fmn* flies show no difference in their escape response between master and yoked flies. Master/yoked pairs for first coincidental event, n=41 and for the 30<sup>th</sup> event n=15, \*\*\*p<0.001; \*\*p<0.01; \*p<0.05; n.s p>0.05.


### 3.8.2 Role of *rutabaga* in learned helplessness

The gene *rut* encodes an adenylate cyclase which has been shown to be involved in the MBs in olfactory learning in *Drosophila* (Tully and Quinn, 1985; Zars et al., 2000; Akalal et al., 2006; Krashes et al., 2007; Blum et al., 2009). A mutant of this gene – *rutabaga*<sup>2080</sup> (*rut*<sup>2080</sup>) – was tested in the shock box. Therefore flies were again tested in the short version of the standard experiment (as in chapter 3.8.1) and showed an effect of cAMP-signaling in the conditioning phase as well as in the post-test (Fig. 34B). The rescue

experiment for *rut*<sup>2080</sup> did not work due to the usage of a weak Gal4 driver (Gal4-elav). Instead of rescuing the expression of *rut*<sup>2080</sup>, I utilized RNA-interference (RNAi) to understand the neuronal underpinnings of learned uncontrollability. RNAi is a natural mechanism in a cell which controls the gene expression of individual genes, by destroying messenger RNA (mRNA) (Buckingham et al., 2004; Meister and Tuschl, 2004).

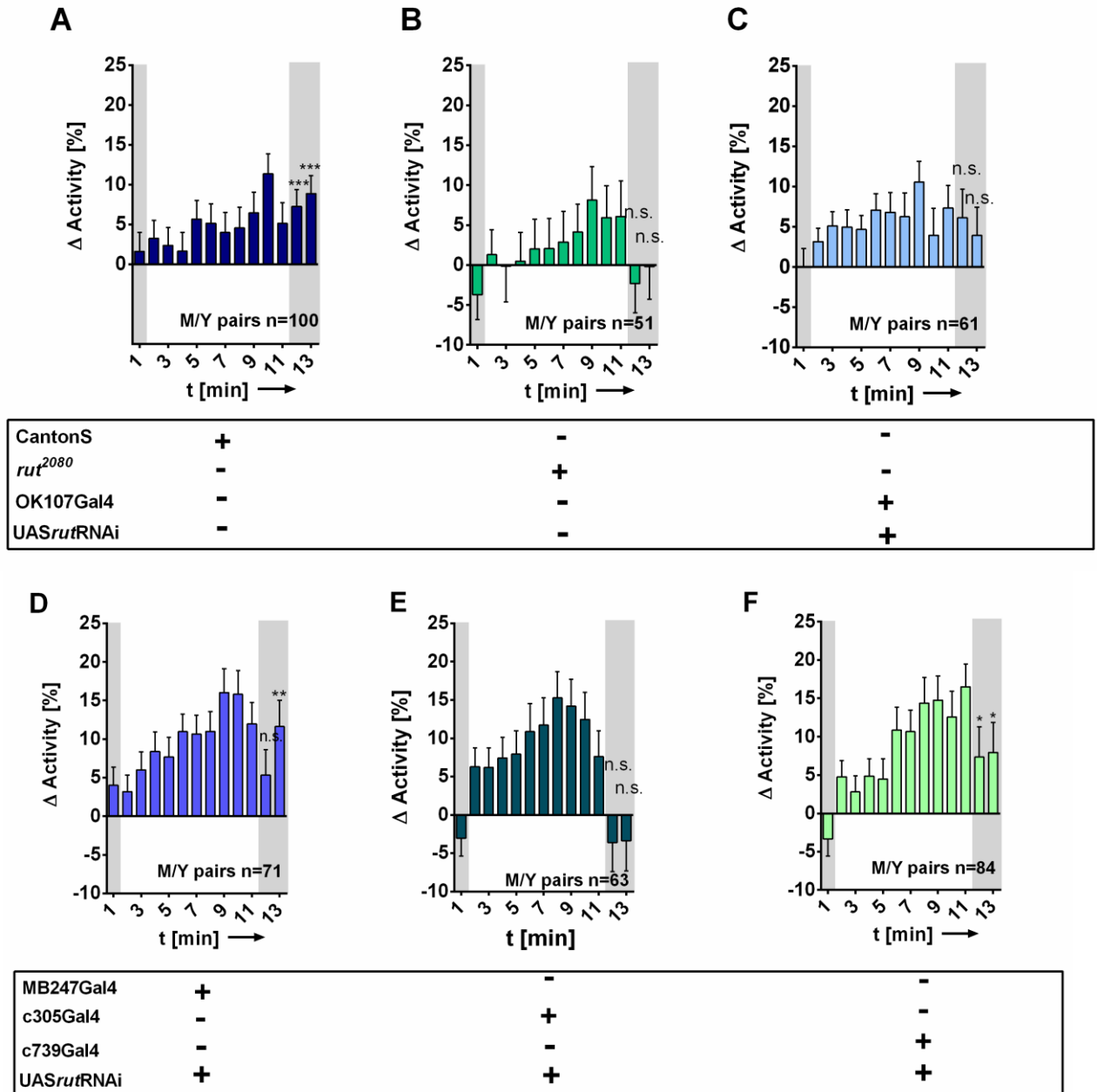
Expressing an UAS*rut*RNAi-line in the entire MB, using the OK107Gal4 driver line (compare Table1) and thus downregulating the *rut* gene in this tissue, gave evidence that it is needed in the MBs for the change in walking behavior due to the exposure to uncontrollable electric shocks (Fig. 34C). However, flies which had a complete downregulation of *rut* in the MBs, showed a higher walking activity in the 2 minute post-test than *rut*<sup>2080</sup> mutants (data not shown).

| GAL4 lines | MB α/βc | MB α/βs | MB α/βp | MB α'/β'a | MB α'/β'm | MB α'/β'p | MB γ main | MB γd | AL | CC fb | CC eb | CC no | CC pb | OL me | OL lo | OL lop | spr | ipr | LH | optu | vlpr | vmpr | plpr | psl | pars in | AN | DE | TR | SOG | Glia |
|------------|---------|---------|---------|-----------|-----------|-----------|-----------|-------|----|-------|-------|-------|-------|-------|-------|--------|-----|-----|----|------|------|------|------|-----|---------|----|----|----|-----|------|
| c739       | ■       | ■       | ■       | ■         | ■         | ■         | ■         | ■     | ■  | ■     | ■     | ■     | ■     | ■     | ■     | ■      | ■   | ■   | ■  | ■    | ■    | ■    | ■    | ■   | ■       | ■  | ■  | ■  | ■   | ■    |
| c305a      | ■       | ■       | ■       | ■         | ■         | ■         | ■         | ■     | ■  | ■     | ■     | ■     | ■     | ■     | ■     | ■      | ■   | ■   | ■  | ■    | ■    | ■    | ■    | ■   | ■       | ■  | ■  | ■  | ■   | ■    |
| OK107      | ■       | ■       | ■       | ■         | ■         | ■         | ■         | ■     | ■  | ■     | ■     | ■     | ■     | ■     | ■     | ■      | ■   | ■   | ■  | ■    | ■    | ■    | ■    | ■   | ■       | ■  | ■  | ■  | ■   | ■    |
| MB247      | ■       | ■       | ■       | ■         | ■         | ■         | ■         | ■     | ■  | ■     | ■     | ■     | ■     | ■     | ■     | ■      | ■   | ■   | ■  | ■    | ■    | ■    | ■    | ■   | ■       | ■  | ■  | ■  | ■   | ■    |

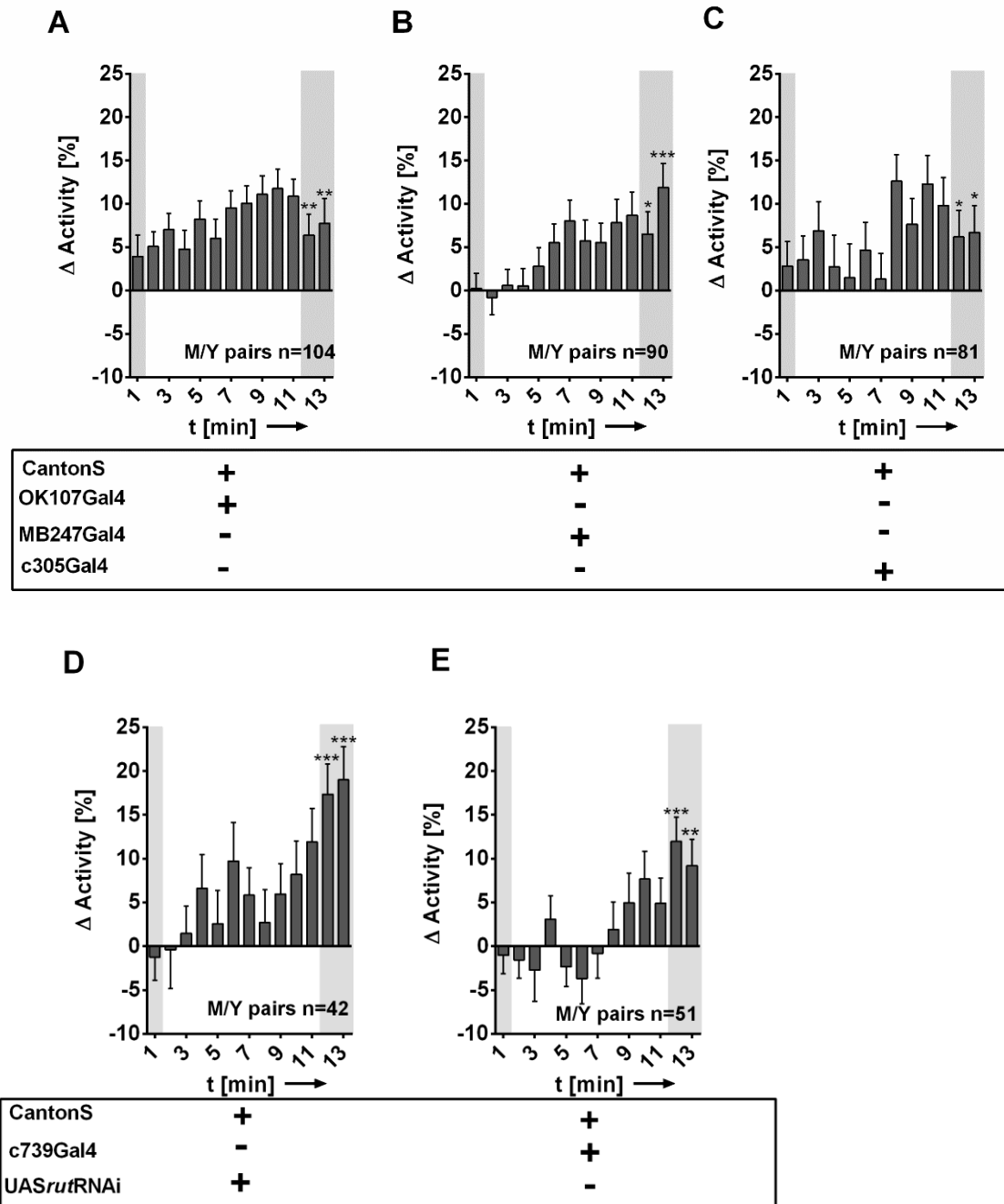
  
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**Table1: Expression pattern of specific Gal4-lines in the brain of *Drosophila melanogaster***, modified after Aso et al., 2009.

Suppressing the expression of *rut* in the αβ'-lobes by driving c305Gal4 together with an UAS*rut*RNAi-line showed no effect in the conditioning phase, but in the test phase (Fig. 34E). *Rut* seems not to be required in the αβ-lobes (c739Gal4/UAS*rut*RNAi, Fig. 34F), but may play a minor role in the γ-lobes (MB247/UAS*rut*RNAi, Fig. 34D). In Fig. 35A-E the control experiments are displayed. All control lines showed the expected difference regarding the walking behavior between master and yoked flies, thus alterations induced by the Gal4 or UAS-RNAi-lines used here can be excluded.



**Fig. 34: Genetically modified flies tested in the standard experiment: 1min pretest, 10min conditioning followed by 2min post-test.** All graphs display the difference between master and yoked (master-yoked= $\Delta$ ). **(A)** CantonS: Master and yoked flies differ during conditioning and post-test from each other. **(B)** *rut*<sup>2080</sup>: Master and yoked flies do not differ in their walking activity in conditioning and post-test. **(C)** OK107/UAS*rut*RNAi: When *rut* is downregulated in the entire MB, flies do not differ significantly concerning their walking activity during post-test. **(D)** MB247/UAS*rut*RNAi: When *rut* is down regulated in  $\alpha\beta$ -lobes and  $\gamma$ -lobes, flies differ during conditioning, but not during post-test. **(E)** c305/UAS*rut*RNAi: *rut* expression seems to be needed in  $\alpha'\beta'$ -lobes for mediating the after-effect in activity between master and yoked, but not during the conditioning phase **(F)** c739/UAS*rut*RNAi: *rut* expression in the  $\alpha\beta$ -lobes is not needed for mediating the difference in master and yoked flies. \*\*\* $p < 0.001$ ; \*\* $p < 0.01$ ; \* $p < 0.05$ ; n.s.  $p > 0.05$ .

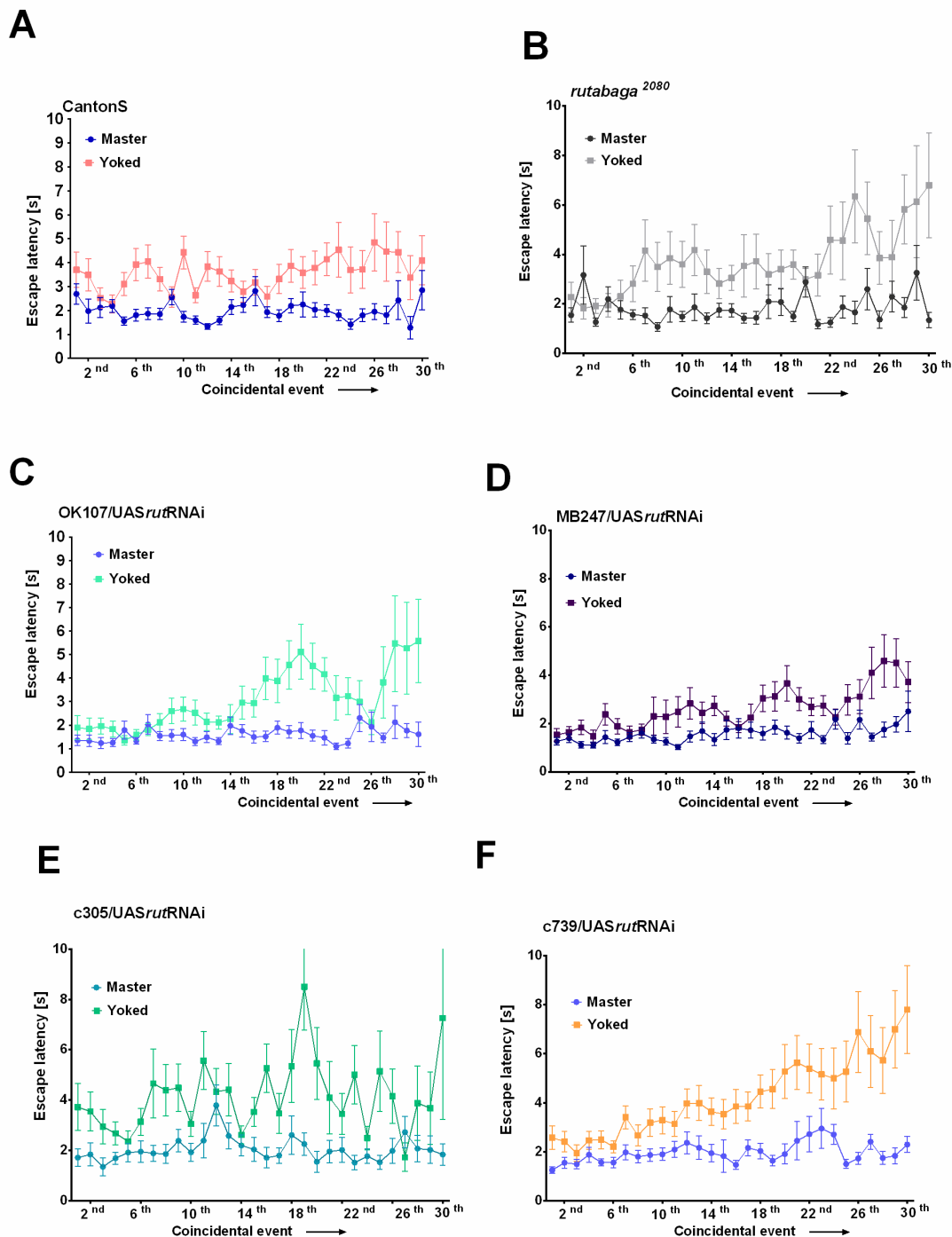


**Fig. 35: Control experiments.** All graphs display the difference between master and yoked (master-yoked= $\Delta$ ). **(A)-(F)** All control flies show the expected differences between master and yoked in such, that the yoked flies become less active during the conditioning. This effect is solid in the 2min post-test. \*\*\* $p < 0.001$ ; \*\* $p < 0.01$ ; \* $p < 0.05$ ; n.s  $p > 0.05$ .

I also evaluated the escape latencies. It serves as a valid test to identify the role of *rut* in the operant part of this experiment (for more details compare chapter 3.3.6). Brembs and Plendl (2008) had shown that operant learning in the flight simulator was not impaired in the *rut*<sup>2080</sup> mutant. In CantonS, yoked flies needed more time to resume walking after the onset of the shock, when directly compared to master flies (Fig. 36A and Fig. 13). This effect was also observed in the homozygous *rut*<sup>2080</sup> mutant (Fig. 36B). In all tested lines



driving *rutRNAi* in the MBs, master/yoked pairs showed a slight but significant difference of the escape response (Fig. 36C-F) showing that outcome learning is still intact.



**Fig. 36: Escape latency.** Time the fly needs to resume walking after the onset of an electric shock. **(A)** CantonS: Yoked flies need more time to resume walking than master flies. Master/yoked pairs,  $n=100$ . **(B)** *rut<sup>2080</sup>*: The same effect can be observed in mutant flies. Master/yoked pairs,  $n=51$ . **(C)** OK107/*UASrutRNAi*: Yoked and master flies show a slightly weaker effect in escape latency, when compared to CantonS flies. Master/yoked pairs,  $n=61$ . **(D)** MB247/*UASrutRNAi*: Again the difference between master and yoked is less pronounced. Master/yoked pairs,  $n=71$ . **(E)** c305/*UASrutRNAi*: Yoked flies show high variations in their escape response and therefore differ not as strong as wildtype flies.

## Results

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Master/yoked pairs, n= 63. **(F)** *c739/UASrutRNAi*: Yoked/master difference is similar to CantonS flies.  
Master/yoked pairs, n= 81.

## 4 Discussion

### 4.1 Is uncontrollability the stressor?

Learned helplessness is one of the most commonly used animal models in depression research, established not only in vertebrates but even in invertebrates (Brown et al., 1996; Yang et al., 2013; Ries, 2015). I studied learned helplessness in flies to arrive at a broader understanding of this behavioral disposition and to find out whether indeed uncontrollability is its main cause. Exposing the flies to an aversive stimulus within a range of minutes already influences their behavior if the stimulus is inescapable. They make significantly shorter walking bouts, walk more slowly, sit less but longer, show delayed responses to the onset of the aversive stimuli and are impaired in learning and phototaxis (Fig. 8, Fig. 10, Fig. 12, Fig. 13, Fig. 19 and Fig. 20B). What turns out with short stress treatments (10 and 20 minutes conditioning), however, is that the behavioral modulations are context dependent (compare chapter 3.6).

Yang et al (2013) trained the flies for 10 minutes to observe an effect during conditioning and a subsequent short test phase. Likewise, 10 minutes of inescapable electric shocks are sufficient to influence the walking behavior. In the shock box 20 minutes are sufficient to observe a 10 minute after-effect (Fig. 6C). In a recent study of learned helplessness (Ries, 2015) the stimulus was applied for much longer time intervals. Flies were exposed for 3 days (3x8-10 hours) to an unpleasant vibrating stimulus. Afterwards changes in climbing and courtship behavior were observed in a different context. With other behaviors even short conditioning phases may have modulatory after-effects. In aggression studies (Trannoy et al., 2015) 20 minutes of conditioning were sufficient to establish the 'loser' and 'winner' effect in the second fight with a new rival.

The close similarity of the effects in the heat- and shock box strongly suggests that the effects are due to the uncontrollability rather than directly to the respective stressor. When an animal can control an aversive stimulus by its behavior, the animal or its ancestors in evolution must have acquired this ability. The heat- and shock boxes provide the opportunity to observe the process in which the fly learns that a controllable stimulus suddenly is uncontrollable or vice versa. Giving back control to the fly after 20 minutes of inescapable stress, is not immediately recognized by the fly. It takes the animal about 7 minutes to reestablish the control (Fig. 16A-D).

For a definite proof of (subjective (compare chapter 4.7)) uncontrollability the triadic design in the shock- and heat boxes still has one limitation. One cannot provide the exact same aversive stimuli to yoked and master animals. Yoked flies receive shock/heat during

walking and pauses, masters only during pauses (Fig. 8A). One cannot fully exclude that the different effects of the conditioning in master and yoked flies might be due to these differences. What makes the latter interpretation most unlikely, however, is the similarity of the behavioral dispositions obtained with heat and electric shocks.

## 4.2 Heat versus electric shock

In general, heat and electric shocks differ in many of their effects on fly locomotion (Fig. 17). For instance, at the onset of the conditioning phase, with electric shocks there is an arousal effect that is not observed with heat pulses. It is most apparent in control flies that are switched to being masters after 20 minute (Fig. 16A; control-to-master, turquoise line). Nevertheless, regarding yoked versus master and control flies electric shocks and heat pulses have very similar effects.

Interestingly, comparing the 20 minute conditioning phase with the 10 minute post-test one finds for all walking parameters differing during the conditioning phase between heat and electric shock, that this difference is inverted in the subsequent test phase. The differences are due to the different stressors and during the subsequent test without the stressor the system compensates for these effects. This inversion between conditioning and test phase is not observed for the differences between master and yoked flies. Most of the differences persisted, an exception being the number of pauses (Fig. 17C, D).

As mentioned earlier, both stressors elicit innate escape responses which have distinctly shorter latencies if the fly is in control of the stressor than if it is not (Fig. 17I, J). The finding that escape responses in the heat box are generally shorter than with electric shock may indicate that the fly notices the gradual increase of temperature and tries to keep it from fully rising to its maximum.

With either stressor yoked but not master flies are transiently impaired in place learning (Fig. 19 and Bertolucci, 2008). Moreover, with both stressors the effects are more pronounced in females than in males (see female/male comparison for electric shocks: Fig. 14A-D and Fig. 15A-F and compare Yang, 2015). These parallels in the behavioral symptoms of objective uncontrollability despite the profoundly different receptive and physiological mechanisms involved with heat and electric shocks (Galili et al., 2014) strongly support the assumption that it is not directly the stressor which causes the effects but indeed its subjective uncontrollability, as is implied with the term 'learned helplessness' (subjective learned helplessness, also compare chapter 4.7).

One striking difference between walking in the two boxes is the duration of pauses. In the shock box during conditioning flies have longer pauses (both groups) and walk more slowly

(Fig. 17E, G). One possible reason for the difference is the surface on which the flies walk. While in the heat box flies walk on Peltier elements which are used to apply high temperatures, in the shock box flies walk on a gold layer which covers the electric grid. Whether the surface is the reason for behavioral differences remains elusive. A second difference between the two boxes is the number of pauses the flies made. Numbers of pauses do hardly differ during conditioning in the shock box, while in the test yoked flies show less pauses than master flies (Fig. 17C, D). This result is different in the heat box. As already found by Yang et al. (2013), master flies made more pauses during conditioning, while the groups did not differ any more during post-test (Fig. 17C, D). A third difference concerns the humidity in the chambers. For sensing the shock pulses the air stream flooding the boxes needs to be humidified (Batsching, 2012). In dry air electric shock cannot be properly transmitted to the tarsi and might therefore not be sensed. Brown and Stroup (1988) for example used a contact cream to conduct experiments successfully with cockroaches. Activity levels of flies under dry conditions in both devices were compared simultaneously and showed that flies walk less under humid conditions. Humidity did not have an impact on velocity (Fig. 18A-D).

### **4.3 Trans-situationality**

The original definition for learned helplessness introduced by Seligman and Maier (1967) involved also the property that it has to show in environments other than where the conditioning took place. When flies in the present study were tested in a new environment, a difference between master and yoked flies was not observed anymore. In free walk both groups walked distinctly less than the control flies, spent less time in the rim zone and showed more frequent landmark approaches (Fig. 22A-C). Following the wall in free walk has been interpreted as anxiety-like behavior (Götz and Biesinger, 1985) but as proposed by Soibam et al. (2012) it might also reflect object exploration. Robie et al. (2010) assumed that landmark approaches could be interpreted as seeking shelter or an exit. As a result of being stressed, the flies might spend less time exploring and more time looking for shelter. In any case, all these effects can be attributed to the electric shock rather than uncontrollability (Fig. 22A-C). Differences between master and yoked male flies that were conditioned in the shock box did also disappear when they were transferred to a chamber with a virgin female to observe their courtship behavior (Fig. 21A-D). In short, the effects of 20 minutes of inescapable stress do not generalize to new environments.

Testing flies in different environments requires handling. In a study, where the influence of pretest handling on subsequent walking behavior was investigated, it was found that

already gentle aspiration impacts walking activity in a negative way. Surprisingly this effect was not detectable in the presence of a second fly (Trannoy et al., 2015). In the present study I excluded handling to be the cause of the loss of the after-effect in free walk and courtship. I tested flies in the shock box and after letting them crawl into a vial they were aspirated and blown back into a different chamber where a 10 minutes post-test was conducted. The activity levels of both groups were slightly increased, but master and yoked flies still showed a significant difference in walking behavior (Fig. 23A (b)). Therefore, an impact through handling could be excluded for the shock box.

In the heat box no after-effect subsequent to handling was found. Flies showed also an increased activity level after handling, particularly yoked flies (Fig. 24A (b)). In the heat box, flies already showed a remarkably reduced activity level after 20 minutes of conditioning without handling (Fig. 24A (a)). This effect was even more pronounced in the study of Bertolucci (2008), who described that over 60% of the flies sat constantly subsequent to the 20 minutes conditioning. The reason for the decrease in activity after a 20 minutes conditioning phase might be that the flies lower their walking activity in order to save energy, especially when they are desiccated under these dry and warm conditions (Kim and Wang, 2016). Further experiments will have to decide whether learned helplessness in the heat box is cured by handling.

I would like to come back to the loss of the after-effect after transferring the flies to a different environment. In the shock box where learned helplessness stands handling, it is abolished by a transfer of the flies to a different environment. There are two explanations one can come up with: First, it might be the case that the after-effect depends on the importance of the subsequent situation. If it is a question of survival it might be covered by behavior which is 'more important' in this situation. This explanation can be applied for the results observed in courtship. Courting is a stress resistant behavior across the animal kingdom (Trannoy, 2015). Reproduction might be so important that 20 minutes of stress would not be enough to have a negative impact.

A second possibility and a more likely one would be, that the differences observed under the conditions in the shock box are context dependent. Abramson and Seligman (1978) published a paper reformulating their definitions given 10 years earlier. They distinguish between *global* and *specific* helplessness. When the deficits were observed in a broad range of situations, they named it *global* and imply it to be very likely to occur across situations. When the deficits appear only in a narrow range of situations they termed it *specific* helplessness. The attribution implies that the animal is helpless only in this special situation.

Becoming *globally* or *specifically* helpless depends furthermore on whether the subjects attribute their helplessness to *global* and *stable* factors or to *specific* and *unstable* factors. The latter would induce no transfer of helplessness to other situations. An example for *global* and *stable* helplessness in humans would be that for example failing in a math test an explanation like: “I am not intelligent“ would be a *global* and *stable* cause and could lead to failure in other situations. The explanation: “I am failing because I am not good at this topic“ would make a transfer to other situations very unlikely and thus serve as an example for *specific* and *unstable* helplessness. “In general, the properties of the attribution predict in what new situations and across what span of time the expectation of helplessness will be likely to recur.” (Abramson et al., 1978). When testing the flies after 20 minutes of conditioning in a place learning task, yoked flies were impaired in avoiding the punished side of the chamber in comparison to master flies (Fig. 19). Furthermore, yoked flies took twice as long to leave the chamber after the conditioning as control or master flies. The reason for this was that their inborn positive reaction to light had changed (Fig. 20). Because the differences between master and yoked occurred only in the shock box, it is likely that the flies developed a *specific*/context dependent helplessness.

The fact that exposing flies to inescapable shaking on three consecutive days had an impact on their courtship and climbing behavior (Ries, 2015), suggests that 20 minutes of conditioning might be too short to elicit *global* helplessness.

#### **4.4 Social isolation – influence on learned helplessness**

Socially isolating an animal for several days after hatching has pronounced effects on various behaviors (Ueda and Kidokoro, 2002). As mentioned in chapter 3.7.1, for male mice and flies, social isolation is known to cause higher walking activity (Guo et al., 2004; Panova et al., 2013) and has also been linked to anxiety in rats (Jankowska et al., 1991; Morinan et al., 1992; Maissonnette et al., 1993), while other scientists found no effect, for example, in the elevated plus-maze (Lapiz et al., 2001).

In the shock box social isolation interferes with learned helplessness in female and male flies (see Fig. 28-Fig. 31). It is possible, that for single-reared flies the transfer to isolation in the shock box is less stressful than for group-reared flies, because they might have already adjusted to being isolated. For group-reared flies, two parameters change at the same time. They are pulled out of their group-rearing situations and at the same time introduced into a dark environment, where only tactile cues can be used for orientation. In the first minute after transfer, the activity level of single-reared flies was comparable to that of group-reared flies (data not shown). During 20 minutes without electric shocks, however,

activity and walking speed declined substantially less in single-reared than in group-reared flies. This can be observed for both sexes (Fig. 26A-D, Fig. 27A-C). The impact of social isolation has also been investigated in insects before. Cockroaches (*Blattella germanica*) for instance, reduced their foraging activity, lost their ability to find a mating partner and lowered their exploration activity (Lihoreau et al., 2009). Other research with *Drosophila* in a light-dark paradigm showed, that socially isolated female flies spent less time in the light than socially isolated male flies did (Mohammad et al. 2016). Concerning the higher activity level of socially isolated flies, Panova et al. (2013) found a similar trend and hypothesized that flies reared in groups might learn to be less active in order to prevent collision and subsequently reduce aggression. Wang et al. (2008) demonstrated that the aggressiveness level of flies reared in isolation was increased in comparison to group-reared flies (Wang et al., 2008). In *Drosophila paulistorum* it was found that socially isolated flies produce greater quantities of cuticular hydrocarbons than group-reared flies (Kim et al., 2004). Cuticular hydrocarbon is a pheromone for inter-individual communication and is also used to protect the animal against environmental factors (Blomquist et al., 1987; Gibbs et al., 2003; Howard and Blomquist, 1982).

In this study single-reared flies show hardly any after-effect during the 10 minutes post-test. The duration of pauses in female and male flies is the only parameter that differed significantly in the post-test (Fig. 29 and Fig. 31). This suggests that after three days of social isolation male and female flies are less affected by uncontrollability than group-reared flies. This finding is also in line with that of Neckameyer and Nieto-Romero (2015), who showed that in the forced swim test socially isolated flies display longer latencies until immobility than control flies. According to their interpretation, flies with longer latencies show less reaction to stress than control flies.

#### **4.5 Dopamine is involved in learned helplessness**

As mentioned earlier in this thesis (chapter 3.6.6) biogenic amines are known to play a role in learned helplessness. In this study, dopamine was studied. Master and yoked flies, which have more dopamine accumulated in the synaptic cleft (*fumin* flies), due to a mutation of the dopamine transporter, showed only a weak difference regarding their walking behavior (Fig. 33A). This finding is in line with the function of antidepressants. Some of them block the re-uptake of dopamine in the synaptic cleft for longer impact of dopamine (Carboni, 1990; Pozzi, 1994). Interestingly, after conditioning a reduced dopamine concentration was measured in the heads of the flies which received inescapable shocks (yoked) in comparison to flies which did not receive electric shocks



(box-control and vial control). No differences could be found between master and yoked flies (Fig. 25A). Since dopamine is involved in many other behaviors in the fly this is not particularly surprising. Yang (2015) found no effect on master and yoked differences when feeding a dopamine inhibitor ( $\alpha$ -methyl tyrosine). The observed effects in the present study, that blocking dopamine re-uptake and thus upregulating dopamine in the synaptic cleft, influences the response to stress positively (Fig. 33A, C, D), seems contradictory to what Yang (2015) found. These findings though would be in line with the inverted-U relationship between dopamine levels and function (Cools and Robbins, 2004).

In this study, no influence of stress on the serotonin level was found (Fig. 25B). Ries (2015) discovered recently, that extensive stress lowers the serotonin concentration in the  $\alpha/\beta$ -lobes, exclusively.

#### 4.6 Role of adenylate cyclase in learned helplessness

Flies carrying a mutation in the *rut* gene which encodes an adenylate cyclase, are known to be deficient in classical odor avoidance conditioning (Dudai et al., 1976; Quinn et al., 1979; Aceves-Pina et al., 1983) as well as in conditioned courtship suppression (Gailey et al., 1984). In this study I found an effect on learned helplessness in homozygous *rut*<sup>2080</sup> mutant flies. During 10 minutes of conditioning only a weak difference between master and yoked flies evolved and no significant difference was observed during the post-test (Fig. 34B). This experiment suggests that *Drosophila* needs the *rut* gene for the expression of the normal learned helplessness behavior.

Many studies provide evidence that the MBs are centrally involved in the organization and modulation of behavior (Aso et al., 2014). Martin et al. (1998) as well as Serway et al. (2009) showed for example that the MBs play an important role in walking behavior. Taking the first small step into the direction of understanding the neuronal underpinnings of learned helplessness, I tested flies in which the expression of *rut* was downregulated in distinct parts of the mushroom bodies (for details compare Table1). At first, *rut* was suppressed in the complete mushroom bodies using OK107Gal4 driving UAS*rut*RNAi (Fig. 34C). This experiment confirmed the hypothesis that *rut* is required in the MBs for its contribution to the downregulation of walking activity due to inescapable stress. Interestingly, *rut* seems not to be required in the  $\alpha/\beta$ -lobes but its expression is needed in the  $\alpha'/\beta'$ -lobes (Fig. 34E, F). These results prove a role for the cAMP signaling cascade in the formation and/or maintenance of the uncontrollability condition in the  $\alpha'/\beta'$ -lobes. Earlier studies in olfactory learning provided evidence that  $\alpha'/\beta'$ -lobes play a role in memory retrieval, shortly after the conditioning while in contrast,  $\alpha/\beta$ -lobes play a role in memory

retrieval after longer breaks between conditioning and test (Sejourne et al., 2011; Cervantes-Sandoval et al., 2013). To investigate the role of the  $\alpha'\beta'$ -lobes in the expression of learned helplessness precisely, one would have to do more experiments.

Master and yoked flies carrying the *rut*<sup>2080</sup> mutation still showed the difference in escape latency (Fig. 36B). The escape latency is considered a purely operant task and *rut* seems not to be involved. Brembs and Plendl (2008) found *rut* to be necessary for solving a classical but not an operant task in the flight simulator, where flies had to associate heat punishment with their own turning behavior. In agreement with this escape latencies seem still to be normal in all the lines with locally suppressed *rut* expression (Fig. 36B-F). Wustmann et al. (1996) found *rut*<sup>2080</sup> mutant flies to be deficient in place learning in the heat box paradigm. Back then, this experiment was considered as purely operant, which has been discussed controversially since then. The fact, that flies may have developed lower walking activity ('stay where you are-effect', (Putz, 2002)) or associate a distinct spot within the chamber with the offset of the stimulus, led to the term 'semi-operant' paradigm (Wustmann et al., 1996; Kapustjansky, 2011; Batsching, 2012). Thus, it is not contradictory that *rut*<sup>2080</sup> mutant flies were not able to perform well in place learning, because it implies also a classical learning component (Wustmann et al., 1996). My focus was on the learned helplessness experiment, thus place learning was not tested.

#### **4.7 Revisiting Learned helplessness**

The general goal was to further characterize learned helplessness in our highly artificial experimental environment, the shock box and learn more about behavioral control under restrictive conditions. The behavior described here is not a disease, but a natural behavior, which occurs when the animal is exposed to changes in environment for which the inborn behavior does not help (Eisenstein and Carlson, 1997). Being exposed to a stressful and inescapable stimulus might influence other behavior and also decrease activity. Following the definition of learned helplessness given in the introduction by the Encyclopedia Britannica (Nolen, 2015), I would like to discuss this definition in the context of my results. In the present study I associate the changes in walking behavior (slower walking and lower activity level) of the yoked flies in comparison to the master and control flies as symptoms of learned helplessness.

It turns out, for instance, that already after 20 minutes of uncontrollability this state persists for at least 10 minutes (Fig. 8, Fig. 10 and Fig. 12). If after 20 minutes of uncontrollability experimental conditions return to controllability (yoked-to-master) it takes the flies several

minutes to adjust to the new condition (Fig. 16). Recently Maier and Seligman (2016) coined the term 'subjectively helpless' implying that the animal measures the outcome of its action, derives an outcome expectation for the future and compares it to the respective outcome expectation derived from similar events in the past. By this the animal learns that its action is ineffective – that it is helpless. This definition is in line with what is observed in the course of this study regarding escape latencies and number of short pauses (Fig. 13, Fig. 11A, B). Yoked flies take longer time to resume walking after the onset of an electric shock. This might show that they learn that their escape attempts are futile and have no effect. In the present study both, master and yoked flies make use of what is offered by the experimental design, control and uncontrollability, respectively. Both results require outcome learning (Heisenberg, 2015). The flies measure the outcome and modulate the response frequency, if the outcome deviates from the outcome expectation. In other words, the yoked fly may be subjectively helpless. Whether all the other behavioral effects such as slower walking, fewer and shorter walking bouts, impaired learning and slower escape to light are adaptive behavioral changes due to subjective uncontrollability, still remains unclear.

Uncontrollability is further characterized by the flies reared in social isolation. Transferred to the shock box, in the first minute their locomotion seems normal. They are as active as group-reared flies. Over 20 minutes without electric shocks, however, activity and walking speed decline substantially less in single-reared than in group-reared flies. Possibly for single-reared flies the transfer to isolation in the shock box is less stressful than for group-reared flies. This interpretation is supported by the finding that the conditioning of uncontrollability is less pronounced in single-reared flies. They show only small 10-minutes after-effects, of which only duration of pauses is significantly different (females) (Fig. 26- Fig. 31).

The *Drosophila* and the mammalian nervous system arise from a common origin. Thus neurobiological pathways may be conserved (Hirth and Reichert, 1999; O'Kane, 2011). It is reasonable to search for similar symptoms which are described in the respective phenomena (e.g. learned helplessness, attention deficit hyperactivity disorder (ADHD) or schizophrenia) to reveal similarities. It is still important to keep in mind that one only compares observed behavior, which in turn proves homology to a limited extend.

The main finding of this study is that not the stressor itself causes learned helplessness but the fact that the stressor is not controllable. In other words, the animals are stressed because of uncontrollability. Whether the fly 'feels' helpless remains elusive, but I found evidence that we observed 'subjective' helplessness.

## 4.8 Conclusion

The shock box is a convenient device to observe and thus characterize walking behavior in *Drosophila*. Several aspects of the learned helplessness phenomenon were studied here, not only in the shock box, but also in other devices subsequent to the conditioning in the chambers. Having the possibility to observe flies during an experiment, contributes to the understanding of how behavior is selected under highly restrictive conditions. The results found in this study contribute to earlier findings on learned helplessness in *Drosophila* and adds new findings, such that learned helplessness has a time component and is independent of the stressor. The fact that the reaction to inescapable stress in *Drosophila* has been described in different studies concluding similar results, makes it likely that what is observed here is indeed learned helplessness. It shows that uncontrollability functions as the stressor, not the stimulus itself. The reaction to stress varied, dependent on sex and the rearing situation as well as dopamine and *rut* happened to be involved during the formation of learned helplessness. The uncontrollability effect was found to be strongly context dependent and flies which were not in control of the shocks, were impaired in place and reversal learning and showed a strongly reduced reaction to light (positive phototaxis).

## 4.9 Outlook

It would be interesting to unravel whether extending the duration of stress (chronic stress) for several days of conditioning, might influence subsequent behavior and what role the  $\alpha/\beta$  lobes would play in that context. Would we also find an inverted U-shaped relationship between dopamine and its function in the MBs?

Especially in understanding the neuronal underpinnings of learned helplessness only the first steps were taken and further investigations of the different mechanisms underlying this phenomenon might considerably advance our understanding of how brains work in order to choose the right behavior in certain situations or even contribute to our understanding of psychiatric disorders.

## 5 Summary

In order to select the appropriate behavior, it is important to choose the right behavior at the right time out of many options. It still remains unclear nowadays how exactly this is managed. To address this question, I expose flies (*Drosophila melanogaster*) to uncontrollable stress to study their behavior under restrictive circumstances by using the so-called shock box. Exposing animals to uncontrollable stress may have an impact on subsequent behavior and can last for some time. The animal learns that whatever it does, it cannot change the situation and therefore can develop something called learned helplessness. The term was first conceptualized by two American psychologists Maier and Seligman (1967), who discovered this phenomenon while doing experiments with dogs. They found out that dogs which are exposed to inescapable stress, later fail in a learning task ('shuttle box').

In this work the walking patterns of three different types of experimental flies, walking in a small dark chamber, were evaluated. Using the triadic design (Seligman and Maier, 1967), flies were either exposed to electric shock randomly (yoked), could turn it off by being active (master) or did not receive punishment at all (control). Master flies were shocked whenever they sat for more than 0.9 seconds. At the same time yoked flies received a shock as well independent of what they were doing, to ensure the same amount of shocks received and to create random punishment pattern for the yoked group. With this so-called no-idleness paradigm flies were conditioned either 10 minutes, which resulted in a short (3 minutes) after-effect, or 20 minutes that turned out to be more stable (10 minutes).

In a second part, the behavior during the 20 minute conditioning and a 10 minutes post-test was described in detail. Female flies of the yoked group developed lower activity levels, longer pauses and walked more slowly than master and control flies during conditioning. In the time after the shocks while still in the box, the yoked flies also reduced the frequency and duration of walking bouts as well as their walking speed. Additionally, they took more time to resume walking after the onset of an electric shock than master flies (escape latency) and turned out to make less pauses lasting between 1-1.5 seconds which supports the finding concerning the escape latency.

Male flies, tested under the same conditions, showed a slightly weaker after-effect regarding the difference between master and yoked during conditioning and post-test when compared to female flies.

When comparing the 20 minutes conditioning with subsequent 10 minutes test in the heat and the shock box in parallel, one finds the same effect: Flies which do not have control over the shocks, lower their activity, make less but longer pauses and walk more slowly

than their respective master flies. Despite the similar effect of heat and shock on the flies, some differences between the devices occurred, which can partly be explained by different humidity conditions as well as by different surfaces within the chambers.

When the control over the shocks is given back to the yoked flies, it takes them about seven minutes to realize it. One could also show that dopamine levels in the brain were reduced in comparison to flies which did not receive shocks. Yoked flies also were impaired in a place learning task (place learning) and their reaction to light (exit from the box towards the light) directly after conditioning.

After characterizing the walking behavior in the chambers, the study deals with the question whether the effects observed in the chambers transfer to different environments. In free walk they only differed from flies which did not receive electric shocks and no effect of uncontrollability was transferred to courtship behavior. Handling as the cause could be excluded. Since handling could be excluded to be the cause of losing the effect, I assumed that the behavior shown in the boxes are context depend.

Not only were the after-effects of inescapable shock subject of the current research also the impact of the rearing situation on the response to electric shock was investigated in the present study. Flies which grew up in a single-reared situation turned out to be less affected by inescapable stress in both sexes.

In the next part, the first steps to unravel the neuronal underpinning were taken. A mutant – *fumin* – which is defective in the dopamine re-uptake transporter showed less reaction to inescapable foot shocks, while a mutant for the gene which encodes an adenylate cyclase (*rutabaga*<sup>2080</sup>) resulted in a good score during conditioning, but showed no stable after-effect. Downregulating the expression of the adenylate cyclase gene (*rutabaga*) in different parts of the mushroom bodies showed, that *rutabaga* is necessary in the  $\alpha'\beta'$ -lobes for expressing the differences between master and yoked flies in the no-idleness paradigm. The study further confirmed previous findings, that *rutabaga* is needed in operant but not in classical conditioning.

As a result, the study could show that not the stimulus itself causes the state of uncontrollability but the fact that the fly learned that it was not in control of the stimulus. This state turned out to be context and time dependent.

## 6 Zusammenfassung

Eine wichtige Aufgabe für ein Tier ist es, das passende Verhalten zur richtigen Zeit zu wählen. Heutzutage ist immer noch unklar, wie dieser Prozess exakt abläuft. Zur Untersuchung dieser Frage werden Fliegen (*Drosophila melanogaster*) in der so genannten Schockbox unkontrollierbarem Stress ausgesetzt um auf diesem Weg Verhaltenskontrolle unter stressigen und stark restriktiven Umständen untersuchen zu können. Wenn Tiere unkontrollierbarem Stress ausgesetzt sind, kann dieser Zustand sowohl langanhaltend sein als auch Einfluss auf das Folgeverhalten haben. Das Tier lernt, dass alle Aktivitäten, die es in dieser Situation unternimmt keinen Einfluss auf die Situation haben. Dadurch kann das Tier einen Zustand der sogenannten Erlernten Hilflosigkeit entwickeln. Dieser Begriff wurde von zwei amerikanischen Psychologen, Maier und Seligman (1976), geprägt, die dieses Phänomen während Experimenten mit Hunden entdeckten und konzipierten. Sie fanden heraus, dass Hunde, die unkontrollierbarem Stress ausgesetzt waren, an einer anschließend gestellten Lernaufgabe scheiterten (,shuttle-box').

Gegenstand der vorliegenden Arbeit ist es, das Laufmuster in einer schmalen und kleinen Kammer an drei verschiedenen Versuchsgruppen von Fliegen zu analysieren. Unter Verwendung des sogenannten triadischen Konzepts (Seligman and Maier, 1967) wurden die Fliegen drei unterschiedlichen Situationen ausgesetzt: Zufällige Elektroschocks (Yoked-Gruppe), durch Laufen abschaltbare Elektroschocks (Master-Gruppe) oder keine Bestrafung (Kontroll-Gruppe). Master-Fliegen wurden immer dann geschockt, wenn sie für länger als 0,9 Sekunden saßen. Unabhängig ihres Verhaltens erhielten die Yoked-Fliegen zeitgleich einen Schock um einen zufälligen Bestrafungsreiz zu generieren. Mit diesem so genannten ,no-idleness' (nicht ruhen dürfen) Paradigma wurden die Fliegen entweder zehn Minuten oder 20 Minuten konditioniert. Während eine zehnminütige Konditionierung zu einem kurzen Nacheffekt führte (Nacheffekt von drei Minuten), stellte sich die zwanzigminütige Konditionierung als nachhaltiger heraus (Nacheffekt von zehn Minuten).

In einem zweiten Teil der Arbeit wurde das Verhalten der Fliegen sowohl während der zwanzig Minuten andauernden Konditionierung also auch im nachfolgenden zehnminütigen Test im Detail beschrieben. Während der zwanzigminütigen Konditionierung zeigten weibliche Yoked-Fliegen eine geringere Aktivität, saßen länger und liefen langsamer als Master- oder Kontroll-Fliegen. In der Zeit nach den Schocks, zeigten sie immer noch eine verminderte Lauffrequenz sowie kürzere und langsamere Laufphasen. Zusätzlich benötigten sie länger um nach dem Einsetzen eines Elektroschocks loszulaufen (Flucht-Latenzzeit) und machten weniger Kurzpausen die

zwischen 1 bis 1,5 Sekunden lang waren. Dies unterstützt das Ergebnis der verlängerten Flucht-Latenzzeit.

Männchen, die unter gleichen Bedingungen getestet wurden, wiesen im Vergleich zu weiblichen Fliegen eine leicht abgeschwächte Reaktion bezüglich des Master-Yoked-Unterschieds auf.

Wenn die Konditionierung mit dem anschließenden Test in der Schock- und der Hitzekammer gleichzeitig durchgeführt wurde, resultierte dies in vergleichbaren Ergebnissen: Fliegen, die keine Kontrolle über den Reiz haben, vermindern ihr Aktivitätslevel, sitzen seltener aber länger und laufen langsamer als die dazugehörigen Master-Fliegen. Neben der Tatsache, dass ein ähnlicher Effekt auftritt, weisen die Apparaturen dennoch kleine Unterschiede auf. Diese können zu Teilen mit den unterschiedlichen Luftfeuchtigkeitsniveaus als auch durch die Verschiedenheit der Lafoberfläche der jeweiligen Kammern erklärt werden.

Wird den Fliegen die Kontrolle über die Schocks zurückgegeben, benötigen sie etwa sieben Minuten um dies zu erkennen. Zudem konnte gezeigt werden, dass die Dopaminkonzentration in den Köpfen, im Vergleich zu Tieren die keine Schocks erhalten haben, vermindert war. Yoked-Fliegen wiesen außerdem unmittelbar nach der Konditionierung Defekte im Ortslernen und in ihrer positiven Reaktion auf Licht auf.

Nachdem das Laufverhalten innerhalb der Kammern ausführlich charakterisiert wurde, geht diese Studie darauf ein, ob die Effekte, die in den Kammern gemessen wurden, auch in anderen Umgebungen zu beobachten sind.

Im freien Lauf unterschieden sie sich lediglich von Fliegen, die keine Schocks erhalten hatten und es sind keine Auswirkungen durch Kontrollverlust im Paarungsverhalten festzustellen. Da die Handhabung der Tiere als Grund für den Verlust des Nacheffektes ausgeschlossen werden konnte, lässt sich schlussfolgern, dass das Verhalten das in den Kammern gemessen wurde, kontextabhängig ist.

Zusätzlich zur Untersuchung der Auswirkungen unausweichlichen Stresses, wurde der Einfluss, der Aufzuchtbedingungen auf die Stress-Antwort in der vorliegenden Studie untersucht. Fliegen, die einzeln aufgezogen wurden, weisen bei beiden Geschlechtern eine verminderte Antwort auf Stress auf.

Im darauffolgenden Abschnitt wurden erste Schritte unternommen, um die neuronalen Grundlagen der Erlernten Hilflosigkeit zu untersuchen. Eine Mutante – *fumin* – die ein defektes Wiederaufnahmetransporter-Gen für Dopamin besitzt, wies eine verminderte Stressantwort auf. Während eine Mutante des Adenylatzyklasegens (*rutabaga*<sup>2080</sup>) normale Ergebnisse während der Konditionierung aufzeigten, war im Post-test kein



signifikanter Nacheffekt messbar. Das Herunterregulieren des Adenylatcyclasengens (*rutabaga*), in verschiedenen Teilen der Pilzkörper, zeigte dass die Expression von *rutabaga* in den  $\alpha'\beta'$ -Loben für die Entwicklung der Erlernten Hilflosigkeit im no-idleness Paradigma benötigt wird. Zudem konnten vorangegangene Studien bestätigt werden, die *rutabaga* eine Rolle im operanten Lernen jedoch nicht im klassischen Lernen zuordnen.

Als Fazit zeigt die Studie, dass nicht der Stressor selbst, sondern die Unkontrollierbarkeit des Stressors der Grund für die Entwicklung der Erlernten Hilflosigkeit darstellt und das Phänomen, innerhalb der hier gewählten Zeitspanne (20 Minuten Stress), kontextabhängig zu sein scheint.

## 7 Literature

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## **8 Appendix**

### **8.1 Affidavit**

I hereby declare that my thesis entitled: „Behavior under uncontrollable stress in *Drosophila melanogaster* – Learned Helplessness revisited” is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore I verify that the thesis has not been submitted as part of another examination process neither in identical nor in similar form.

#### **Eidesstattliche Erklärung**

Hiermit erkläre ich an Eides statt, die Dissertation: „Verhalten unter nicht kontrollierbarem Stress – Neubetrachtung der Erlernten Hilflosigkeit bei *Drosophila melanogaster*”, eigenständig, d. h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen, als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg, den 12. Dezember 2016

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## 8.2 Curriculum Vitae



### 8.3 Publication and Conference contributions

#### Publication:

Batsching S, Wolf R, Heisenberg M. 2016. Inescapable Stress Changes Walking Behavior in Flies - Learned Helplessness Revisited. PloS one 11: e0167066.

#### Conference contributions:

Batsching S, Wolf R, Heisenberg M (2012) Operant conditioning in the shock box, Poster presented at the European Drosophila Neurobiology Conference, Padua, Italy.

Batsching S, Wolf R, Heisenberg M (2013) Learned helplessness in *Drosophila melanogaster*, Poster presented at the Neurobiology of *Drosophila* Conference, Cold Spring Harbor Laboratory, NY, USA.

Batsching S, Wolf R, Heisenberg M (2013) Learned helplessness in *Drosophila melanogaster*, Poster presented at the 10th Meeting of the German Neuroscience Society, Göttingen, Germany.

Batsching S, Weiglein A, Wolf R, Heisenberg M (2014) Learned helplessness in *Drosophila melanogaster*-does it transfer to other behavior? Poster presented at the 79th CSHL Symposium: Cognition, Cold Spring Harbor Laboratory, NY, USA.

Batsching S, Weiglein A, Wolf R, Heisenberg M (2014) Learned helplessness in *Drosophila melanogaster*-does it transfer to other behavior? Poster presented at the European Drosophila Neurobiology Conference, Hersonissos, Greece.

Batsching S, Weiglein A, Wolf R, Heisenberg M (2015) Learned helplessness in *Drosophila melanogaster* -does it transfer to other behavior? Talk presented at the 11th Meeting of the German Neuroscience Society, Göttingen, Germany.

Batsching S, Haberberger A, Wolf R, Heisenberg M (2015) Action selection under restrained conditions. Poster presented at the conference 'Building the brain: from genes to circuits and cognition', Royal Society, London UK.

Batsching S, Haberberger A, Wolf R, Heisenberg M (2016) Talk presented at the Kolloquium der Entwicklungs- und Neurobiology, University of Mainz, Germany.

## 8.4 Danksagung

