

Searching for transgenerational inheritance of acquired behavior in *Drosophila*



Bachelor Thesis

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November 2023

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Abstract

The transgenerational inheritance of acquired behavior in invertebrates is still unexplored. Especially for the model organism *Drosophila melanogaster*, the available literature and evidence is sparse. This study attempts to establish a protocol for investigating possible transgenerational effects of operant conditioning in the flight simulator. A parental generation of animals is conditioned to a stimulus by reinforcement, and their offspring later tested for behavioral abnormalities when exposed to the same stimulus. However, conducting the experiment neither the experimental nor the control groups of either generation showed learning behavior after training. More data are needed to make a definitive statement about transgenerational effects of operant conditioning.

Zusammenfassung

Die transgenerationale Vererbung erworbener Verhaltensweisen bei Wirbellosen ist noch weitgehend unerforscht. Insbesondere für den Modellorganismus *Drosophila melanogaster* ist kaum Literatur verfügbar und die Datenlage dünn. Diese Arbeit versucht, ein Protokoll zu etablieren, welches mögliche transgenerationalen Effekte operanter Konditionierung im Flugsimulator untersucht. Eine Elterngeneration von Tieren wird durch Reinforcement auf einen Reiz konditioniert, und ihre Nachkommen später auf Verhaltensauffälligkeiten getestet, indem sie dem gleichen Reiz ausgesetzt werden. Bei der Durchführung des Experiments zeigten jedoch weder die Experimental- noch die Kontrollgruppen beider Generationen Lernverhalten nach dem Training. Um eine endgültige Aussage über transgenerationale Effekte operanter Konditionierung treffen zu können, müssen weitere Daten erhoben werden.

1 Introduction

The idea that acquired traits can be inherited was first proposed by Lamarck and Darwin (Gowri and Monteiro, 2021) and inspired many researchers in the 20th century to perform rather straightforward experiments trying to prove this new concept (Liu, 2011). Today we know that the inheritance of acquired characters is not as simple as it may have seemed to scientists at the time. At least since Weismann put forward his theory that somatic changes cannot simply enter the germline (Weismann, 1893), we know that cutting off the tails of mice does not result in offspring that lack this important body part. Nevertheless, evidence has accumulated over the years supporting transgenerational inheritance of acquired traits. The best known examples are *Daphnia cucullate* (Tollrian, 1990) and the agouti mice (Wolff et al., 1998). Over the years the explanation arose that transgenerational inheritance is mediated primarily by epigenetic mechanisms. However, the exact pathways underlying this type of inheritance remain largely unknown (Gowri and Monteiro, 2021). Modern research in this field is therefore exciting and challenges scientists to come up with new study design, as the methods are not yet well established.

This bachelor thesis investigates the transgenerational inheritance of acquired behavior in *Drosophila*. The literature on this topic is sparse for two reasons. First, current studies on epigenetic inheritance tend to focus on mammals and not so much on invertebrates (Arzate-Mejía and Mansuy, 2022). Within this field *Caenorhabditis elegans* is the model organism on which most research has been carried out. Recent work on the roundworm discusses RNA-based pathways as possible mechanisms of transgenerational inheritance (Hourri-Zeevi et al., 2020; Toker et al., 2022). RNA-based epigenetic inheritance has already been demonstrated in *Drosophila*, but not in the context of inherited behavior (de Vanssay et al., 2012). Second the inheritance of behavior itself is controversial because memory is stored in a synaptic code that is distinct from the code mediating inheritance through the germline. Thus, inheritance of acquired behavior should not be possible. However, mechanisms that can translate and transfer information between these two planes have been found in *Caenorhabditis elegans* (Miska and Rechavi, 2021). Whether these pathways also exist in *Drosophila* remains to be investigated.

The experiment in the present work sought to establish a basic protocol for studying transgenerational inheritance of acquired behavior in *Drosophila*. A recent behavioral experiment in *Drosophila* (Williams, 2015) was analyzed, and thereupon a new experimental design was set up. Operant conditioning in the flight simulator was used to induce possible

transgenerational inheritance of learning behavior. The trained parental animals were crossed, and the resulting offspring were tested for any behavioral characteristics compared to a control group with untrained parents. Unfortunately, due to time constraints the experiment was not adequately performed, and the results are therefore not fully reliable.

2 Material and Methods

Note: The entire experiment was performed twice with a one-week interval. The results of the two emerging fly populations were later pooled together. This approach allowed more data to be collected over time, as the success of each individual round was limited by the lack of experience with the flight simulator. In addition, the performance of the flies could be checked for season-specific learning impairments or improvements.

2.1 PART ONE: Training of parental generation

Fly stocks and maintenance

In the experiment only Wild type Berlin flies were used. The animals were reared in plastic vials at 25°C and 60% humidity under a 12h/12h light/dark cycle. The fly food of the parental flies consisted of standard *Drosophila* medium and fresh yeast. Filter paper added to the vials protected the medium from drying out.

Preparation

In preparation for the actual experiment virgins were collected to ensure that fertilization takes place after the exposure to the investigated stimulus and that the embryos remain naive to the stimulus. Animals were divided into two groups.

The first group included the parental flies that were later trained in the flight simulator (hereafter referred to as *p(trained)-flies*) and used to produce the offspring of the experimental group. 24 male and female virgins, enough to be sufficient for later preparation, were collected under anesthesia and transferred separately into small glass vials containing instant yeast. It was found that the reproductive activity of the male flies is much higher in two to three day old animals than immediately after hatching. Therefore, the collected virgins were kept at 18 degrees for one day before being prepared for the actual experiment.

The second group contained parental flies that were not trained (hereafter referred to as *p(untrained)-flies*) and used only to produce the control offspring. 12 female and male virgins were collected under anesthesia and transferred separately into small glass vials containing instant yeast. They were also kept at 18 degrees for two days.

Training at the flight simulator

Preparation

Prior to the experiment, the p(trained)-flies were glued to a copper hook between the head and thorax to fix both body parts together and stored overnight at 25°C for recovery (Brembs, 2000).

Flight simulator set up

The flight simulator used for training consists of a “Kopp” torque-meter device (combining the elements of the “Shiming” (Tang and Juusola, 2010) and the “Götz” (Götz, 1964) devices) for measuring the angular momentum of the flying animal and an arena that provides stimuli for the fly (e.g. patterns, colors) if necessary. The arena is cylindrical and uniformly illuminated by a projector (DLP® LightCrafter™ 4500, Texas Instruments). The prepared flies were attached to the torquemeter with a small metal clamp and placed in the middle of the arena. The output data were recorded using LabVIEW (3V19). Punishment could be applied with a laser (Streamline laser, Osela Inc.) that was set at 4.1 V and 50% pulsing intensity. Via a camera (USB-Digital microscope 40x – 1000x magnification, Bysameyee) the behavior of the animal was observed during the experiment for control.

Training

For the training of the parental flies the self-learning paradigm was chosen. No pattern is presented during the whole experiment so the fly can only use its own yaw torque for learning. The total duration of the experiment is 22 minutes (**Table 1**) (Brembs and Plendl, 2008). At the beginning of the training the optomotor response of the fly to a left and right rotating strip pattern (four periods, 30 seconds each) was used to adjust the magnitude of the torque signal. The actual experiment consisted of nine periods of two minutes each. A pretest allowed the fly to become familiar with the environment as the laser remained turned off. Throughout the training periods the laser then was turned on each time the fly’s yaw torque reached the penalized side (Brembs, 2008; Brembs and Heisenberg, 2000; Wolf and Heisenberg, 1991). Test periods were set up to record the learning effort of the fly after two and four training sessions. Finally, the optomotor response of the animal was checked again to detect any shift in the trace indicating a malfunction in the measuring device.

PERIOD	PERIOD	PERIOD	PERIOD	PERIOD	PERIOD	PERIOD	PERIOD	PERIOD
1	2	3	4	5	6	7	8	9
Pretest	Pretest	Training	Training	Test	Training	Training	Test	Test
no Heat	no Heat	Heat	Heat	no Heat	Heat	Heat	no Heat	no Heat

Table 1: Self-learning paradigm for flight-simulator training

To minimize external visual cues the execution of the experiment took place in darkness. Punishment of the right side and left side were randomly alternated for each fly to avoid side preference bias. It was ensured that the naive flies were exposed to the laser at least once during training, otherwise the animals were discarded. Animals showing poor flight performance, e.g., constant stopping, were also discarded. Each fly was trained only once.

Training of the p(trained)-flies took place for five consecutive days, and it was aimed to train at least three flies per day to allow for the later crossing.

Evaluation

The performance index (PI) describes the learning achievement of the flies after the training:

$$PI = (t_p - t_{up}) / (t_p + t_{up})$$

with t_p standing for time spent on punished side and t_{up} for time spent on unpunished side. The PI equals 1 if the animal constantly averts the penalized side and -1 if it constantly resides on the penalized side (Dill et al., 1993). Evaluation of the data was done with the Drosophila Time Series Data Model (“GitHub - bremslab/DTSevaluations: R-Code to evaluate Drosophila Time Series data,” n.d.). The script includes statistical tests of single groups against zero that are based on a Wilcoxon test (significance level set to p-value < 0.005).

Crossing

The p(trained)- and p(untrained)-flies were crossed to produce offspring that would later be used to detect any inherited behavioral traits. Therefore, the flies were pooled over the course of the training experiment. It was aimed at pooling not less than three flies per day (two females plus one male) to obtain a two-to-one male/female ratio in the crossing, to avoid a bottleneck effect, and allow for a high enough larval density in the offspring population.

After the training in the flight simulator, the attached copper hooks were removed from the p(trained)-flies by grabbing the flies by the hook and gently stroking the animal with a brush until both the hook and the glue came off. It was observed that with the copper hook still attached the animals tended to stick to the fly food and became immobilized. The flies were then shaken together in a small vial with fresh yeast and filter paper.

The p(untrained)-flies were pooled in a similar manner using the preserved animals that hatched on the same day as the p(trained)-flies leaving the current experiment.

After five days of experiments, the parental flies remained in the vials for two more days and were discarded on the third day. This allowed the flies added on the last day of the experiment were allowed to mate for two days. Fresh yeast was added to the vials after the parent flies were removed to ensure adequate nutrition for the offspring (**Figure 1**).

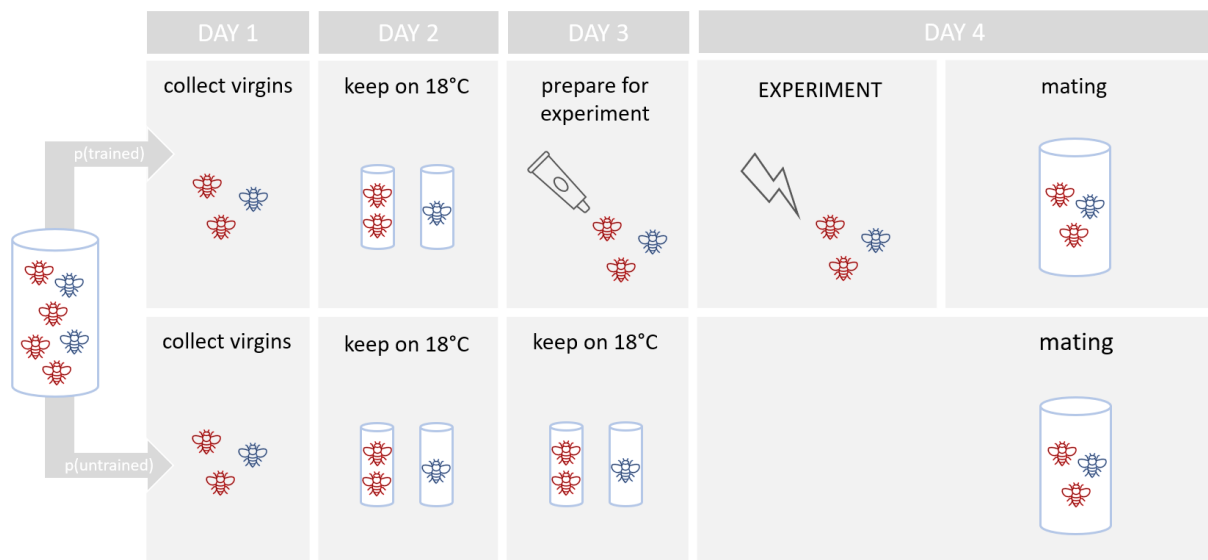


Figure 1: Flowchart of the crossing method; *Virgins are collected and separated in two groups. The p(trained)-flies are later trained in the flight simulator before mating. The p(untrained)-flies are used as untrained control group.*

Note: Due to loss of flies because of natural and random mortality, the age composition within the parent group of flies in each vial could not be fully controlled. To roughly ensure the same conditions for the growing of both populations it was attempted to keep at least the number of flies in both vials adjusted.

2.2 PART TWO: Testing of the offspring

Raising

The larvae were allowed to grow and hatch. The filial flies hatching in the p(trained)-vial will be referred to as $f_{p(\text{trained})}$ -flies, and the filial flies hatching in the p(untrained)-vial as $f_{p(\text{untrained})}$ -flies. Newly hatched $f_{p(\text{trained})}$ and $f_{p(\text{untrained})}$ -flies were separately transferred to small glass vials each day. The flies were kept at 18°C for one day before being prepared for the experiment. This ensured that the offspring flies received the same treatment as the parents.

Testing at the flight simulator

The offspring flies were prepared and trained using the same set up as the parental flies (*Material and Methods, 2.1*). Depending on the hatching time of each population, the testing of the flies was conducted via two schemes:

- 1) Testing of $f_{p(\text{trained})}$ and $f_{p(\text{untrained})}$ -flies on alternate days
- 2) Testing of $f_{p(\text{trained})}$ and $f_{p(\text{untrained})}$ -flies on the same day

Both male and female flies were used in the experiment to allow the results to be examined for sex-specific behavioral differences later.

The training protocol differed from the parent flies and used one-minute periods instead of two-minute periods for the main experiment. The flies are now trained for a total of only four minutes. Wild type berlin flies that are undertrained that way are usually not able to learn the yaw-torque-heat association (Ehweiner, 2022). If previous parental training has a heritable effect on the offspring by improving the animal's learning performance, positive PIs should occur in the $f_{p(\text{trained})}$ -group after training in the flight simulator.

Evaluation of the offspring's behavior was performed similarly to the parental generation (*Material and Methods, 2.1*).

3 Results

3.1 Control experiment

Prior to the actual experiment, it was ensured that the chosen settings for flight simulator set up and laser (*Material and Methods*, **2.1**) could induce learning in the flies (**Figure 2**). For this purpose, the standard learning protocol was implemented (*Material and Methods*, **Table 1**). The test group (N=8) showed an avoidance of the penalized side with PIs between 0.5 and 0.8. A slightly negative PI of -0,1 was observed after the first two training periods. However, the flies preferred the unpunished side during the final test periods (PIs between 0.2 and 0.4). The data showed a large scatter. It is important to note that due to lack of experience with the flight simulator at the beginning of the internship, only left torque was tested. The resulting issues are discussed below (*Discussion*, **4.1**).

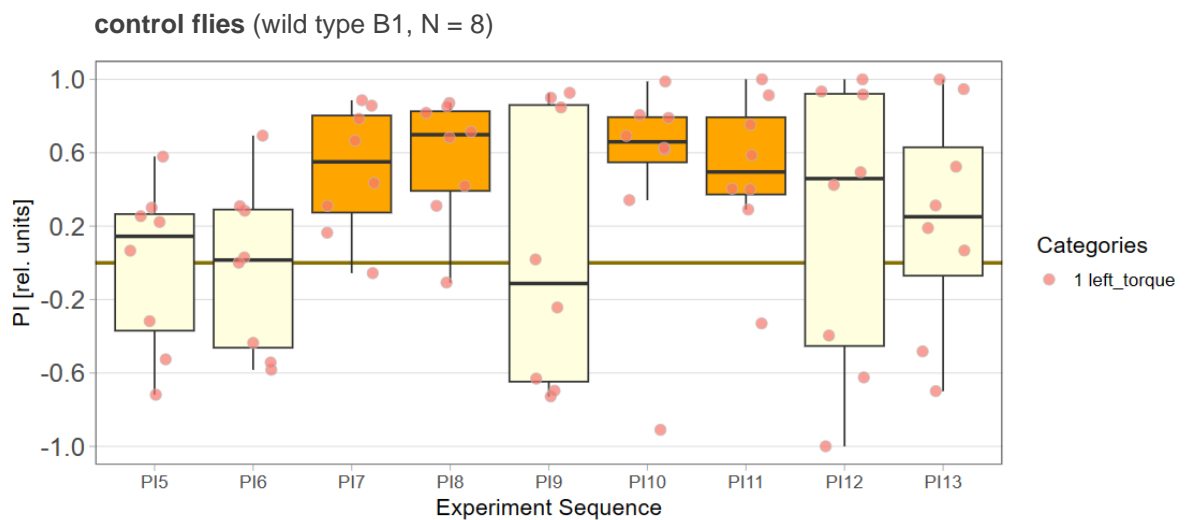


Figure 2: Graphic representation of flight behavior of control group flies in the flight simulator; Performance Index box & dotplot without notches. Y-axis: PI as relative units, X-axis: experimental sequence (punishment applied during sequence 7, 8, 10, 11). Each point represents one fly. Wilcoxon test against 0 with bayesian statistics.

3.2 Training of parents

Flight simulator

The parent flies were trained in the flight simulator using the standard learning protocol (*Material and Methods*, **2.1; Table 1**). Evaluation of the p(trained)-data revealed no memory formation. During the pretests, no fly showed an extreme preference for one side, the observed PIs were between -0.7 and 0.8. The overall naive preference was slightly towards the unpunished side. Avoidance of the penalized side occurred throughout the first two training periods although not very efficiently (average PI of 0.6). Slight learning performance was

observed during the first test period indicated by a PI of 0.3. The second training period showed a similar avoidance as the first although some flies were observed to continuously stay on the punished side (*Results, 3.1*). No memory formation was observed during the final test periods, in fact performance even tended to be negative, as indicated by a PI of -0.4 during the second period. Overall, the average PIs shifted from a slight preference for the unpunished side to a preference for the penalized side. No difference was observed between the PIs of left- and right-punished flies (**Figure 3**).

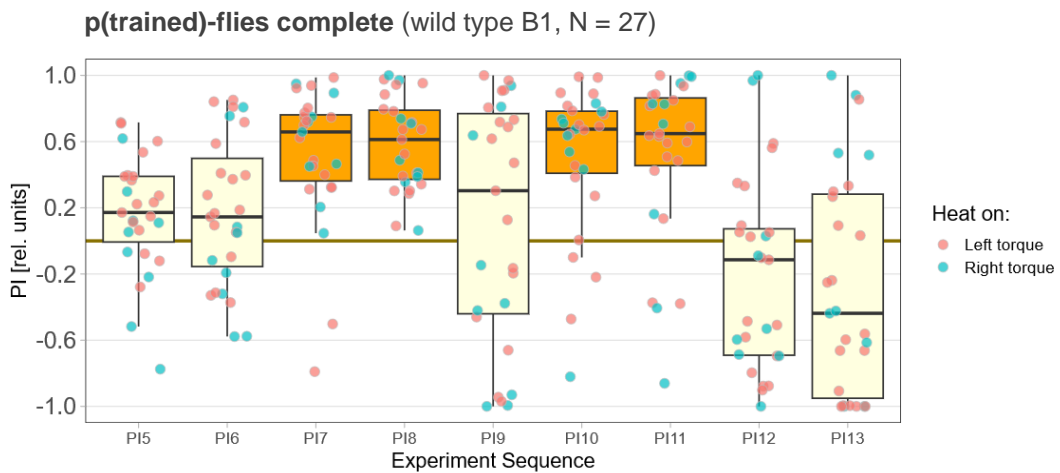


Figure 3: Graphic representation of flight behavior of p(trained- flies); Performance Index box & dotplot without notches. Y-axis: PI as relative units, X-axis: experimental sequence (punishment applied during sequence 7, 8, 10, 11). Each point represents one fly. Wilcoxon test against 0 with bayesian statistics.

Further evaluation

Since it was unexpected that the flies showed no learning performance in the flight simulator set up the behavior of the flies was further evaluated (results compared to data collected by Prof. Björn Brembs with a similar set up (Brembs, 2023a)).

Each individual was examined to verify that the animal was exposed to the stimulus intensely enough. Insufficient exposure leads to poorer learning in the flies and may allow behavior that contaminates the data. Flies were excluded from the evaluation if they fell into one of the following categories (see *Attachments*):

- 1) The average preference during the first two periods was as high or higher than avoidance during the following two training periods. In this situation, it cannot be completely ruled out that the fly avoided the punished side solely because of an innate preference for the unpunished side.
- 2) The flies showed no avoidance during two consecutive training periods. If the fly survives more than 15 seconds of direct exposure to the laser, it is likely that the laser is not properly adjusted to the animal's neck (Ehweiner, 2022).

Excluding these flies from the dataset reduced some of the scatter in the second two training periods, that was mentioned above (*Results, 3.1*). However, it did not seriously affect the results. The flies still showed negative learning on average (**Figure 4**).

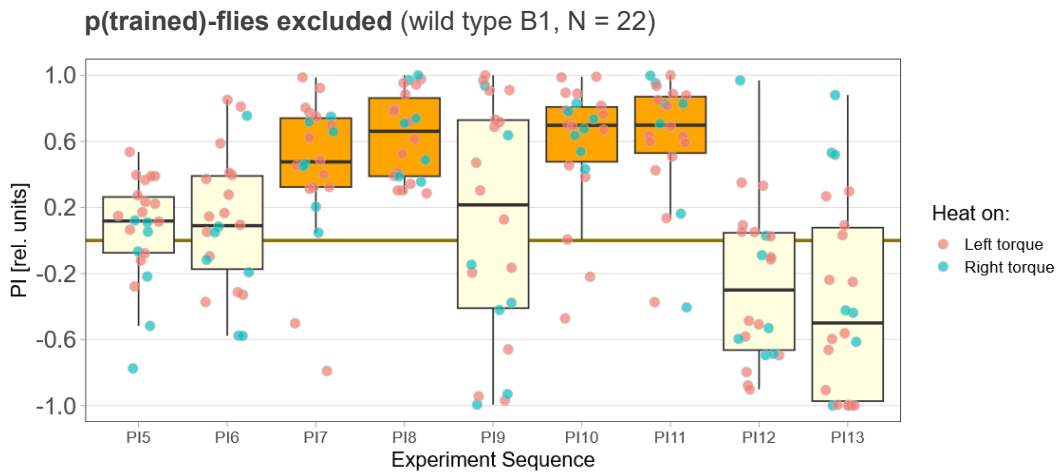


Figure 4: Graphic representation of p(trained)-flies without data from the excluded flies; Performance Index box & dotplot without notches. Y-axis: PI as relative units, X-axis: experimental sequence (punishment applied during sequence 7, 8, 10, 11). Each point represents one fly. Wilcoxon test against 0 with bayesian statistics.

During the evaluation of the individual flies an interesting trend in the behavior of the animals became apparent. The flies could be divided into two groups according to the following criteria:

- 1) Group A: Flies that showed avoidance in all or three of the four training periods and no or negative learning during both final test periods.
- 2) Group B: Flies that showed avoidance in all or three of the four training periods and learning in at least one of the final test periods.

Both groups showed similar trends except for the performance during the first test period. Group A displayed a slight approach of the penalized side (PI = -0,23) whereas Group B showed avoidance (PI = 0,63) (**Figure 5**).

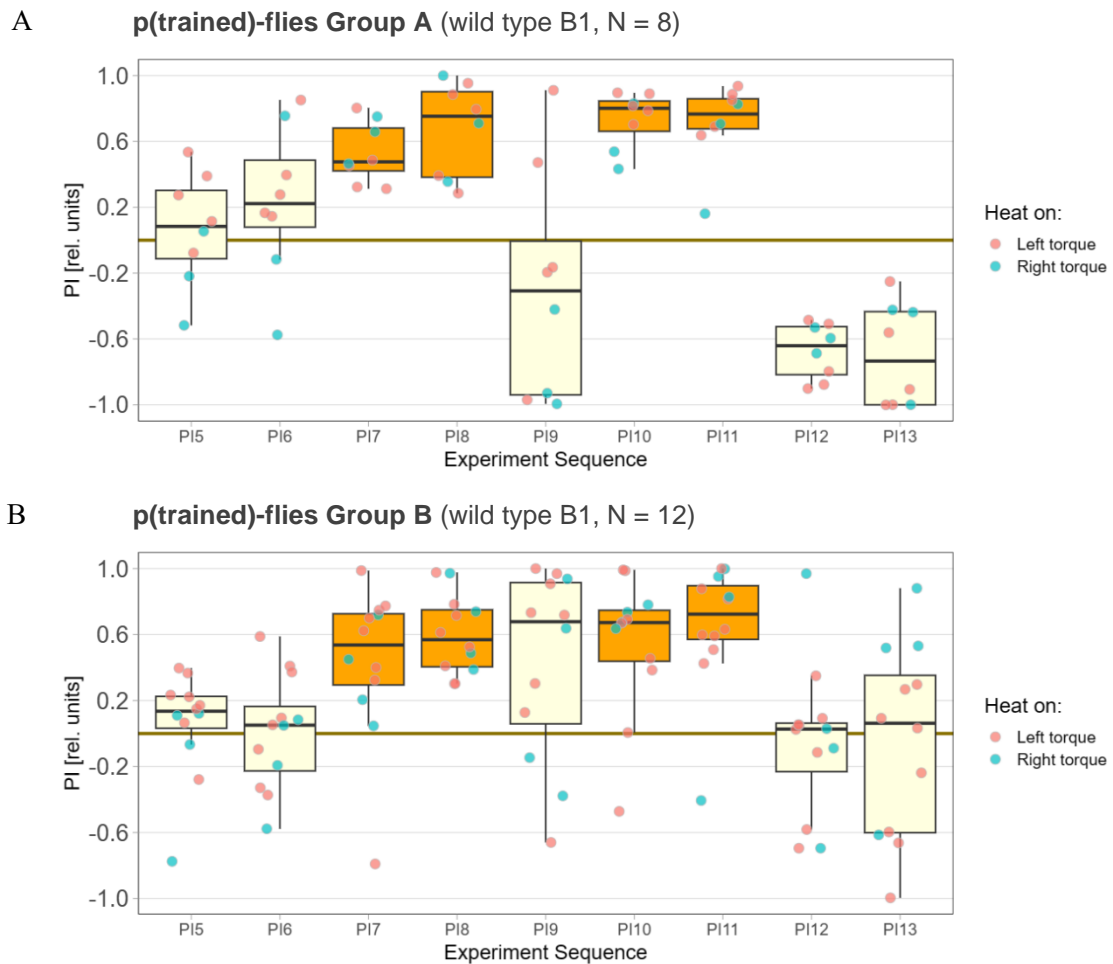


Figure 5: Graphic representation of flight behavior of different subpopulations within the p(trained)-flies; A: Performance Index box & dotplot without notches of Group A containing flies that show avoidant behavior but no learning; **B:** Performance Index box & dotplot without notches of Group B containing the remaining flies of the dataset that show avoidant behavior and learning.

Performance was also evaluated and plotted separately for male and female flies. This was done in order to find any sex specific differences in the behavior of the flies, which could be discussed later. Both sexes showed the same avoidance, however the male flies displayed a slightly worse average learning performance than the female flies. (**Figure 6**).

Avoidance and learning of both sexes were quantified for later comparison (**Table 2**). To do this performance for both categories was averaged using the standard arithmetic mean formula:

$$\bar{x} = \frac{1}{n} \left(\sum_{i=1}^n x_i \right)$$

For average avoidance the mean of all training periods was calculated (period 7, 8, 10, 11). For average learning the mean of the final two test periods was calculated (period 12, 13).

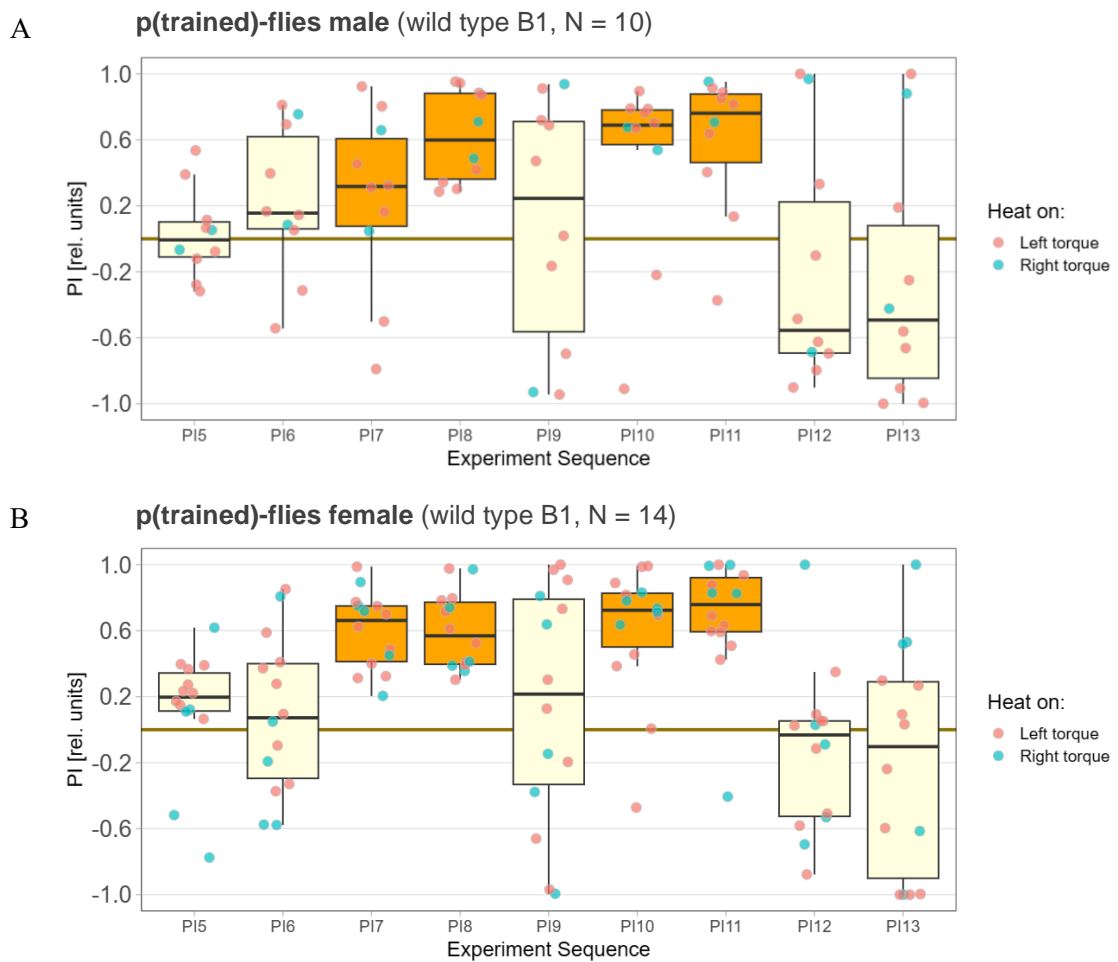


Figure 6: Graphic representation of sex specific behavior of p(trained)-flies; A: Performance Index box & dotplot without notches of male flies; B: Performance Index box & dotplot without notches of female flies.

	MALE FLIES	FEMALE FLIES
average avoidance-PI: $\left(\frac{\sum(PI[training\ periods])}{4}\right)$	-> 0,6	-> 0,65
average learning-PI: $\left(\frac{\sum(PI[final\ test\ periods])}{2}\right)$	-> -0,53	-> -0,05

Table 2: Quantitative comparison of behavioral parameters of male and female p(trained)-flies

3.3 Crossing

To the best of my knowledge, crossing of flies trained in the flight simulator has not been reported before and was therefore carried out for the first time in the context of this bachelor thesis.

It was found that laser exposure did not affect the reproductive behavior of the flies. No obvious difference in the number of pupae was observed between the vial of the p(trained)-flies and the vial of the $f_{p(\text{untrained})}$ -control-group.

The p(trained)-offspring showed no signs of physical impairment under the microscope and remained fully viable during the second part of the experiment.

3.4 Training of offspring

The offspring flies were trained in a similar manner as the parental flies using the adapted one-minute protocol. Evaluation of the offspring flies showed no significant memory formation for the modified protocol (*Material and Methods*, **2.2**). Flies that fell into either of the two categories mentioned above (*Material and Methods*, **3.1**) were excluded. During the first two periods, some flies in both groups showed an extreme preference for one side or the other. Avoidance with a PI of around 0.6 was observed throughout all training periods. Memory formation was not detected in either group during the first test period or during the final test. In fact, learning performance even tended to be slightly negative. No difference was observed between the PIs of left- and right-punished flies (**Figure 7**).

Avoidance and learning of both groups were quantified for later comparison (**Table 3**). To do this performance for both categories was averaged using the standard arithmetic mean formula:

$$\bar{x} = \frac{1}{n} \left(\sum_{i=1}^n x_i \right)$$

For average avoidance the mean of all training periods was calculated (period 7, 8, 10, 11). For average learning the mean of the final two test periods was calculated (period 12, 13).

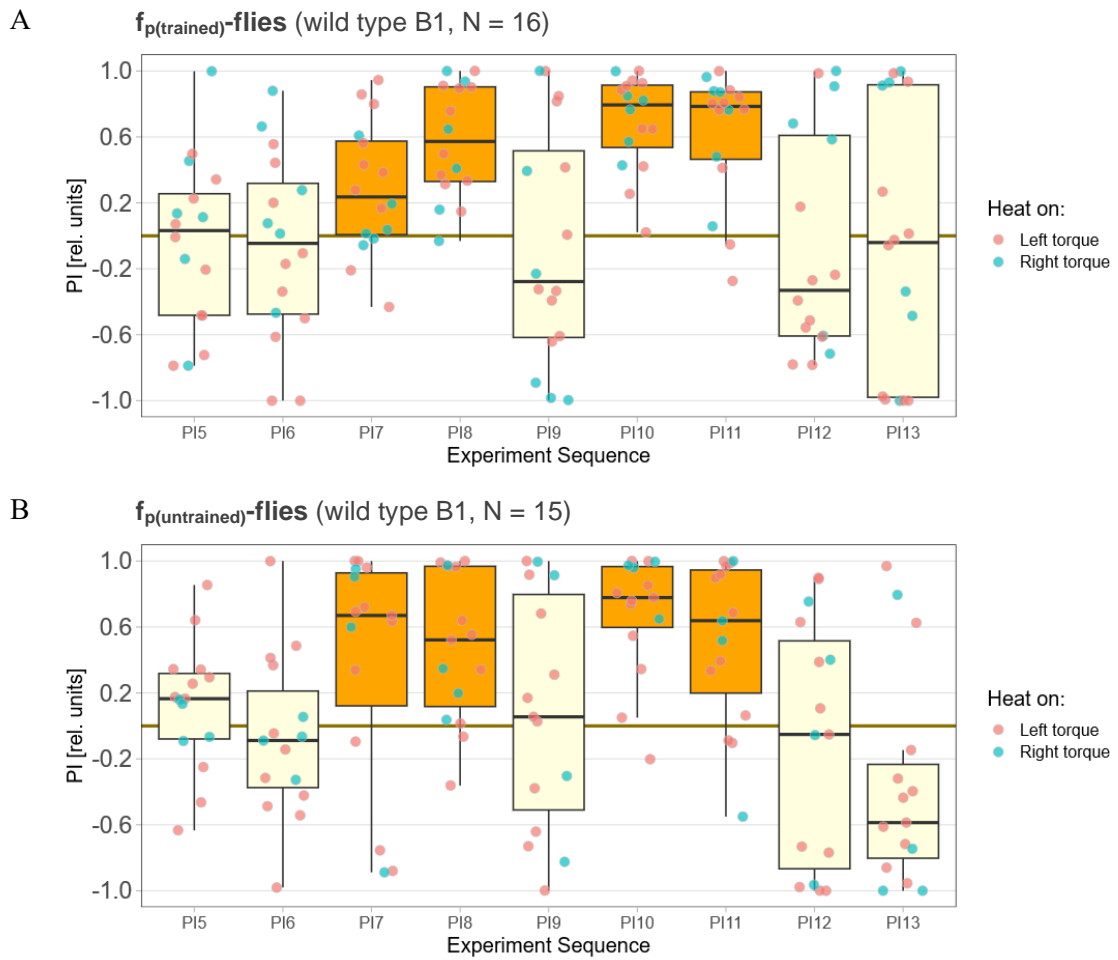


Figure 7: Graphic representation of flight behavior in offspring flies; A: Performance Index box & dotplot without notches of $f_{p(\text{trained})}$ -flies; B: Performance Index box & dotplot without notches of $f_{p(\text{untrained})}$ -flies.

	$f_{p(\text{trained})}$	$f_{p(\text{untrained})}$
average avoidance-PI: $\left(\frac{\sum(PI[\text{training periods}])}{4}\right)$	-> 0,6	-> 0,625
average learning-PI: $\left(\frac{\sum(PI[\text{final test periods}])}{2}\right)$	-> -0,15	-> -0,3

Table 3: Quantitative comparison of behavioral parameters of $f_{p(\text{trained})}$ and $f_{p(\text{untrained})}$ -flies

3.5 Summary

Overall, both parental and offspring flies showed avoidance during the experiment. However, no learning was observed in the animals.

4 Discussion

4.1 Control experiment

To ensure the chosen settings for the experimental set up could induce learning in the flies a control experiment was conducted (*Results*, **3.1**). Avoidance and memory formation were observed, although with lower PIs compared to other lab members (results compared to data collected by Prof. Björn Brembs with a similar set up (Brembs, 2023a)). However, later during the actual experiment the pretest proved to be performed insufficiently. It was observed that memory formation could not be consistently ensured with the chosen settings as previously assumed. This misconception resulted from evaluating only a small sample size (N=8). In addition, only left torque was penalized in the pretest, so it was not checked for any side preferential bias. However, no bias was observed during subsequent testing.

Future studies require careful and well conducted control measurements to provide for the success of the actual experiment.

4.2 PART ONE: Training of parents

In the present work using the torque meter to study operant self-learning, it was not possible to detect learning behavior in the flies as expected (results compared to data collected by Prof. Björn Brembs with a similar set up (Brembs, 2023a)). The experiment required virgin flies (*Material and Methods*, **2.1**) and it proved difficult to compare the collected data because to the best of my knowledge it is not reported that animals have been treated in a similar way before. Nevertheless, experimental data from adult flies trained with a similar set up by other members of the lab was used to point out similarities and differences that are now discussed.

Avoidance

During the training periods higher avoidance was expected in the animals (results compared to data collected by Prof. Björn Brembs with a similar set up (Brembs, 2023a)).

The main reason for these low PIs is considered to be the laser setting. It takes a lot of experience to find a setting that causes avoidance in the flies without killing them too quickly.

Success of the punishment depends on two parameters:

- 1) The heat of the laser must be high enough to be life threatening for the animal (Brembs, 2000). The voltage chosen for the experiment was lower than the settings used by other members of the laboratory (Prof. Björn Brembs, personal communication). Nevertheless,

avoidance was observed during the control experiment (*Material and Methods*, 4.1). Due to limited time resources, the chosen laser setting was not further optimized. However, raising the voltage would result in a higher avoidance.

- 2) The alignment of the laser beam must be exactly on the head of the fly to ensure the heat is perceived by the animal. In retrospect, the head of the fly was sometimes shielded by the clamp during the experiment. The adjustment of the laser to the head of the fly was therefore disabled.

It is possible that the punishment resulting from the chosen laser setting did not present a threat high enough for the animals to exhibit maximum avoiding behavior. In future experiments the laser setting should be chosen more carefully to make sure the punishment is as effective as possible. Pretests with larger sample sizes can validate the success of the punishment (*Discussion*, 4.1).

Learning

Note: This part of the discussion refers to the dataset from which the flies categorized above were excluded (*Results*, 3.2). Earlier it was assessed that the excluded animals did not experience the stimulus intensely enough and learning cannot reliably be presumed. Throughout the final test positive PIs for learning were expected (results compared to data collected by Prof. Björn Brembs with a similar set up (Brembs, 2023a)). However, learning was absent or even negative. Several reasons are possible:

Laser setting

As previously discussed (*Discussion*, 4.2), the chosen laser setting could be the origin for the lack of learning. Learning cannot take place if the punishing stimulus is not great enough (Brembs and Heisenberg, 2001).

Individual behavioral differences

In some flies learning was observed to be absent even though avoidance was taking place. It was pointed out earlier that the flies can be divided into two subgroups, one of which shows normal avoidance but no positive learning PIs (*Results*, 3.1; Group A). It is important to note that this group represents around 40% of all the flies in the dataset. The grouping of flies according to their behavior has been shown before and can be explained by the adaptation of different survival strategies. Dill et al. introduced the two behavioral strategies "Get out of the heat!" and "Stay if it is not hot!", which are parallel to the behavior of the two observed subgroups A and B (Dill et al., 1995).

Future experiments should further investigate the origins of the described strategies to assess their impact on the performance of the whole group.

Sexual maturity

As virgin animals were used for the experiment, it should be considered that the sexual maturity of the flies could have an impact on learning and flight performance. There is no clear evidence that post-mating responses in *Drosophila* females include changes in learning and flight behavior (Chapman et al., 2003). However, there is evidence that the male *Drosophila* sex peptide has an influence on long-term olfactory memory formation by exerting a neuromodulatory influence on serotonergic neurons in the female brain. Virgin females show a deficit in aversive LTM formation while memory formation ability increases after mating (Scheunemann et al., 2019).

Whether the male sex peptide also affects operant visual learning in female *Drosophila* remains to be investigated. Comparing the avoidance and learning behaviors of male and female flies did not find a poorer performance in the female flies. On the contrary, female flies even showed a slightly higher PI for learning (PI = 0,05) than the male flies (PI = -0,53) (*Results*, **3.2**; **Table 2**). However, this could be due to the smaller size of male *Drosophila* individuals compared to female *Drosophila*, which makes it more difficult to properly align the laser beam. That can cause issues already discussed above (*Discussion* **4.2**). In addition, the two subgroups both have a small sample size that is not representative, due to the division into male and female.

A suggested experiment to further investigate the influence of the sex peptide on the learning performance of female flies would be to compare the learning performance of virgin- and non-virgin flies at the same age.

4.3 PART TWO: Testing of offspring

Testing the offspring flies in the flight simulator using the adapted one-minute protocol did not reveal a visible difference in performance between experimental and control group (*Results*, **3.4**). Comparison shows that average avoidance was similar between the two groups (PI \approx 0,6). Average learning was negative in both groups, although slightly higher in the $f_{p(\text{trained})}$ -flies (PI = -0,15) than in the $f_{p(\text{untrained})}$ -flies (PI = -0,3) (*Results* **3.4**; **Table 3**). A possible effect of epigenetic inheritance of the behavior acquired from the parental generation was not detected in the experimental group. Overall, both groups performed similarly to animals tested at the same time in the lab using the same set up (results compared to data collected by Prof. Björn Brembs with a similar set up (Brembs, 2023b)).

Possible epigenetic effect

The present work could not detect a possible epigenetic effect of transmission of behavior acquired in the parental generation to the offspring. The obvious explanation, of course, would be that no such effect exists. However, if an effect does exist, the reasons why it was not detected are discussed below.

Lack of learning in parents

No learning in the parental generation means no acquired behavior that could be passed on to the offspring generation. Repeating the experiment with improved settings that induce a learning effect in the parental flies is key to making a well-founded statement.

Epigenetic reset

During embryogenesis, the gametes of a variety of species including *Drosophila* undergo a heavy reprogramming of epigenetic markers (Iovino, 2014). It is possible that changes in the epigenome resulting from the training are erased by this mechanism. However, since it is not clear if, how and where the corresponding information is stored, it is difficult to draw further assumptions.

Wrong nutrition of offspring

Learning is strongly influenced by environmental factors such as larval density and nutrition. To achieve a good performance in the flight simulator larval crowding should be avoided (Guo et al., 1996). Withholding of fresh yeast during larval development has also been shown to decrease the learning ability of the flies in the flight simulator (Prof. Björn Brembs, personal communication). Although it was tried to provide proper care negative effects caused by inexperienced animal handling cannot be completely excluded.

Inbreeding

Inbreeding can affect epigenetic transgenerational inheritance. Experiment in mice showed that inbreeding can lead to the loss of epigenetically inherited phenotypes (Guerrero-Bosagna et al., 2012). The laboratory flies used in this experiment are inbred over many generations due to long storage periods. However, whether inbreeding also affects transgenerational inheritance in *Drosophila* remains to be investigated.

4.4 Further experiments

Reproduction

Reproducing the experiment with improved settings addressing the above shortcomings is necessary to obtain reliable results. If learning is demonstrated in the parental generation and behavioral variation is found in the offspring, further experiments can be considered.

Follow-up experiments

Several parameters can be further examined:

- 1) To test if the inheritance mechanism of the acquired behavior is sex-specific the performance of $f_{p(\text{trained})}$ -flies with only one trained parent should be compared to the performance of $f_{p(\text{trained})}$ -flies with two trained parents.
- 2) To evaluate if there is any variability in the inherited behavior depending on the conditioning mode, an operant conditioning protocol using reward instead of punishment should be tried out. Other learning protocols, such as pattern learning or classical conditioning, can also be used.

4.5 Transgenerational inheritance of acquired behavior - a general discussion

It remains the greatest challenge in studies of transgenerational inheritance of acquired traits to determine the phenotype that is inherited from the affected parental generation to the filial generation. As discussed earlier, there are several reasons why the experiment described above did not detect a behavioral feature in the $f_{p(\text{trained})}$ -test group. However, it is possible that the trait tested does not represent the trait that is inherited by the $p(\text{trained})$ -test group after operant conditioning in the flight simulator. Two other possible features that may be inherited are discussed below.

Inheritance of preference

As previously shown in mice parental conditioning of a stimulus can induce a selective response to the conditioned stimulus in the offspring generation (Dias and Ressler, 2014). It is therefore possible that after training all the $p(\text{trained})$ -flies by punishing them only on one side the $f_{p(\text{trained})}$ -flies later show a selective preference for the unpunished side. However, it remains a technical problem to measure a selective preference in the flight simulator since it is difficult to determine the “straight-ahead” direction of the fly which is used as reference for the right and left amplitude.

Inheritance of non-neural phenotype

It should be taken into consideration that the inherited phenotype does not necessarily have to manifest as a behavior but as some other adaptation that directly responds to the heat experienced by the $p(\text{trained})$ -flies. Experiments have shown transgenerational effects following heat stress (Folk et al., 2006; Seong et al., 2011). Whether these effects are already

visible after the short training in the flight simulator remains to be investigated. The expression of other non-neural phenotypes cannot be excluded, but they may be difficult to detect.

4.6 Summary

The present work did not detect inheritance of acquired behavior in *Drosophila*. The $f_{p(\text{trained})}$ -test group displayed no behavioral differences compared to the $f_{p(\text{untrained})}$ -control group. However, the results are not fully reliable since the implementation of the experiment was insufficient. Therefore, the data should be reproduced with improved settings and more experience so that a definite statement can be made.

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Attachments

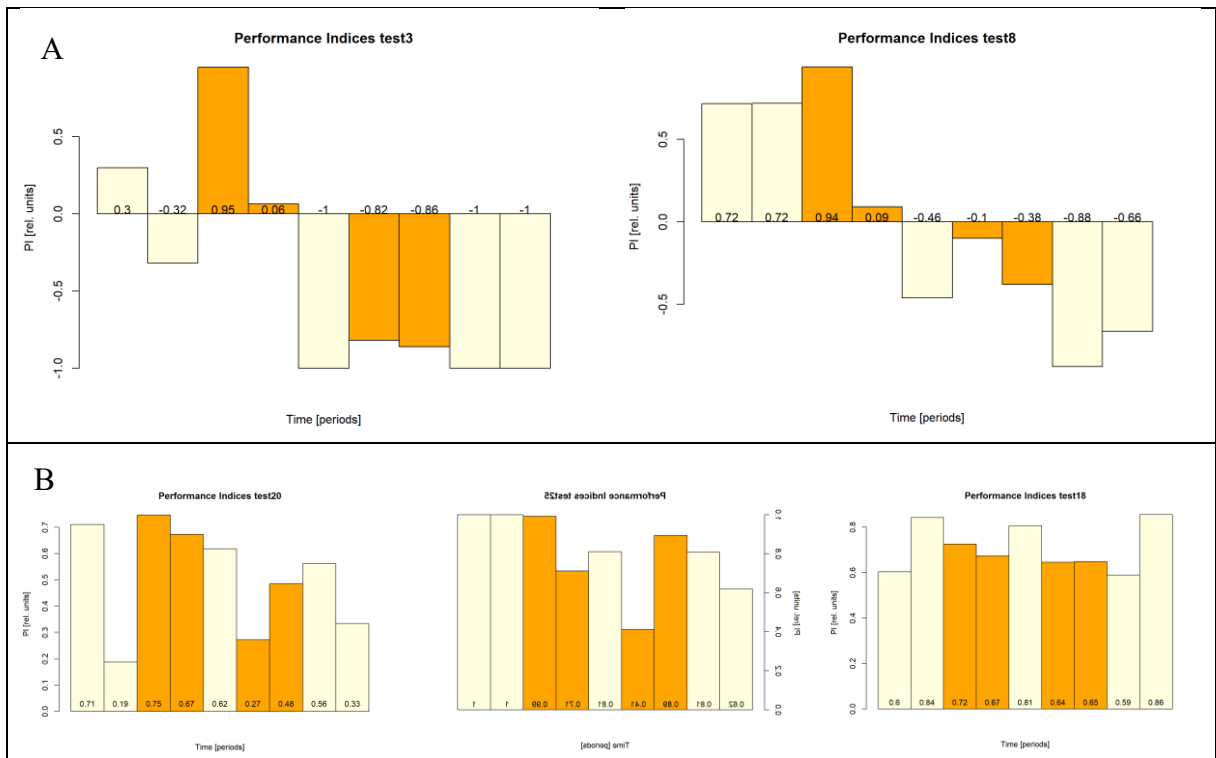


Figure 8: Graphic representation of flight behavior of excluded flies; A: Performance Index bar plots of flies that showed no avoidance during two training periods in a row; B: Performance Index bar plots of flies that showed an average preference during the first two periods was equally high or higher than avoidance during the following two training periods.

Declaration of authorship

Die vorgelegten Druckexemplare und die vorgelegte elektronische Version der Arbeit sind identisch. Ich habe die Arbeit selbstständig verfasst, keine anderen als die angegebenen Quellen und Hilfsmittel benutzt und die Arbeit nicht bereits an einer anderen Hochschule zur Erlangung eines akademischen Grades eingereicht. Weiterhin bestätige ich, dass ich die von dem in § 27 Abs. 5 der geltenden Prüfungsordnung vorgesehenen Rechtsfolgen Kenntnis habe.

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