# Analyzing the use of Humidity Cue in Navigation of *Drosophila melanogaster*

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# Abstract

Humidity is one of the main abiotic factor of ecosystems and affects animals behaviour, fitness and distribution. Due to their small size and inability to thermoregulate using metabolism, insects, such as the fruit fly *Drosophila Melanogaster*, are especially reliant on humidity. They are able to use humidity as a mean of navigation around their environment by using specialised hygroreceptor neurons. While these sensory neurons have been identified, hygrosensation and its transduction mechanism is poorly understood. We have built an experimental setup with adjustable temperature that delivers humidity stimuli, either humid or dry, to flies occupying different lanes in an arena. The idea is to test the effect of temperature on flies reaction to humidity to test hypothesis of transduction mechanism in hygrosensation, the ability to detect humidity. Here we show a clear response from humidity stimuli of *D. melanogaster* in a laboratory environment. The setup gives generally good results and has provided us with data supporting earlier evidence of fly behaviour. The data has been analysed using different features, but no non-trivial pattern of behaviour has been identified outside of a general rise of activity during dry stimuli. With this result we show that our experimental setup can be used for further experiments using more temperatures and control flies to find the means of transduction. We believe that the response of the flies is based on their preferred humidity level of 70 %, and that they are more active in the dry humidity in an effort to locate a more preferred level of humidity.

# Acknowledgements

The work for this thesis was done in close collaboration with Jonathan Lind, who in his master thesis focused more on modelling, especially the Hidden Markov Model[1]. This thesis main interest is the engineering of the experimental setup and trying to iterate on it to reach a result. This thesis also continues where Jonathan's ended, by collecting and analyzing a new dataset with the improved experimental setup, to see if a more clear response could be found. I would of course offer a huge thank to Jonathan, without his help and support this project would not have been done. A big thanks to my always patient and supportive supervisor Anders Enjin as well. For the editing and proofreading of the report my wonderful girlfriend Kajsa Ekström have been very helpful. And of course all the people that has been working in and around the laboratory who's input has helped tremendously.

# Contents

# 1 Introduction

Animal navigation is important for organisms on all scales, from humpback whales migrating thousands of kilometers in water [2], monarch butterflies navigating between southern Canada and the United States to central Mexico [3], to bacteria being able to traverse a food gradient by sampling the chemical concentration and using time comparison [4]. There are a lot of different senses used in navigation, often in combination with each other. These can range from sight, hearing and scent to senses that are more abstract from a human perspective, such as following magnetic or electric fields [5], [6]. Specific navigation senses and methods have evolved over time across different animal species. For example, *Spalax* mole rats are completely blind due to a layer of skin covering their eyes and their brain lacking a visual cortex. Since their life under ground provide little light for vision, they have had to adapt by evolving other means of navigation [7]. One of all these possible senses to use for navigation is humidity. Humidity is a measure of the concentration of water vapour in air. It is a vital variable to describe and predict weather conditions, and one of the fundamental abiotic components that form an ecological system [8]. Humidity is an important factor for many organisms. For example, humans are able to live in different extremes of humidity conditions, but it requires a specialized way of living. The Tuareg and San people are examples of nomadic and semi-nomadic people that inhabit vast, scorching dry deserts. They have developed a way of life based around the harsh conditions in the dry areas, which includes tactics for reserving water, clothing that shields from the sun, and camel herding [9]. Other animals that are highly reliant on humidity in their ecology are amphibians, such as the Colombian terrestrial salamander *Bolitoglossa Ramosi*. The salamander is ectothermic, meaning it does not regulate its inner temperature with metabolism, and is therefore sensitive to the ambient temperature. To keep its water reserve from evaporating from its skin, it is highly reliant on the surrounding humidity conditions. Experimental results suggest that hygroregulation could be more important for the Colombian terrestrial salamander than thermoregulation is. In speculating why, it has been theorized that it could come from the fact that there is more variation in humidity than temperature in the salamander's natural habitat. Bromeliads and other plants in the tropical forests can provide a high-humidity microclimate, suitable for foraging and nesting [10].

Insects are reliant on their environment and microclimates for their storage of water. Their low volume only makes room for small reservoirs, and

with their relatively large surface that makes them prone to drying out, the combination makes water availability and humidity of the climate essential for their survival [11]. Using hygrosensation, they can scout for a preferable level of humidity. The preference varies depending on how dry the fly is. A water deprived fly will have a stronger preference for a higher humidity level. The humidity preference of drosophilids alters radically from the low end of 20% for the north American desert dwelling *Drosophila mojavensis*, to 85% for the *Drosophila teissieri* in the west African rainforest [11]. In this project, we will do experiments on the common fruit fly *Drosophila melanogaster*, a very common model organism. The *D. melanogaster* uses humidity as a cue when navigating its environment. It can use it for finding spaces in a suitable humidity level for maintaining its water balance. Other uses, such as for finding food and laying eggs, have also been hypothesized.

Hygrosensory receptor neurons are specific sensory neurons that detect the difference in humidity. They have been observed in insects such as the honeybee *Apis mellifera*, cockroach *Periplaneta americana*, and of course in the subject of this thesis, the *D. melanogaster* [12]. While other animals, for example humans, are able to roughly sense a difference in ambient humidity, such as when telling apart the dry climate of a desert and the humid air of coastal areas, they do not have sensory neurons dedicated for that purpose. Homeothermic endothermic animals, like humans and most mammals, strive to keep their body temperature at a fixed value using internal body functions, like metabolism. They are in most non-extreme conditions not affected by varying levels of humidity. Many insects are more reliant on ambient heat for controlling their inner temperature and have been observed to be more reliant on surrounding humidity. So far, hygrosensory receptor neurons have only been identified in insects and arachnids. It has been speculated that some amphibians could have a well developed hygrosense, though the evidence is weak and the research dormant. It might also be based on olfactory sensation [13].

The main use of hygrosensation is to regulate water balance, however, from studying behaviour patterns researchers have observed some insects using their hygrosensation for navigating the environment. One example is a biting insect, for instance a mosquito, sensing the humidity from a host's breath and using it as a cue together with other factors such as $CO_2$, heat and visual cues [14].

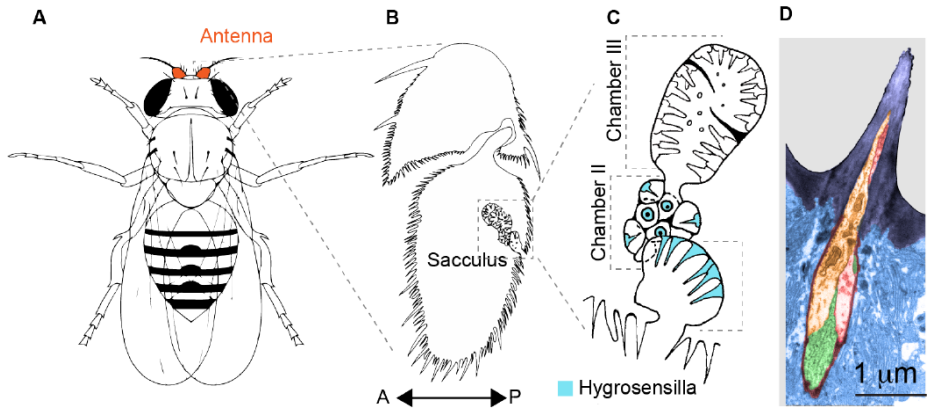


Figure 1: Anatomical illustration of the *D. melanogaster* (used with permission of Anders Enjin), it's sacculus including the triad of hygrosensory neurons. A. The antennas highlighted on a *D. melanogaster*. B. The antenna with the sacculus marked. C. Cross section of the saculus highlighting the hygrosensilla D. Microscopic image of hygrosensory neurons.

Hygrosensory receptor neurons are found on sensilla, antennal sensory hairs. This is analogue to olfactory sensilla which also are located on the antenna. The sensilla are placed posterior on the antenna, on the saculus, see Figure 1.B [11].

The hygroreceptors consist of a triad of neurons housed in the sensilla: dry, moist, and hygrocool.

The mechanism behind hygrosensation has yet to be determined exactly. There are three leading hypotheses on its nature and all of them are based around the three nerve cells found in the sensilla's hygroreceptors: a combination of a dry and a moist cell in an antagonistic pair, and a thermoreceptive cold cell. The hypotheses differ in how these cells in conjunction are used for sensing humidity levels [15].

- The mechanical hygrometer model is based around mechanical deformation of the cuticular wall of the sensilum, in the form of shrinking or swelling from humidity. This deformation is then believed to alter the dendritic membranes and cause a change in voltage. This is similar to how a mechanical hair hygrometer works [16].

- The psychrometer model is also analogous to a common hygrometer. A psychrometer consists of two thermometers. The bulb of one of the thermometers is kept wet by a wick, while the other one is placed in the air. The air humidity can then be calculated from the difference in temperature between the two thermometers [17]. In the psychrometer model, an assumption is made that the dry and moist cells activity correspond to the wet bulb, with the thermoreceptive cold cell acting as the dry bulb.

- The evaporation model uses the rate of evaporation in the sensillum lymph, which is proportional to the surrounding humidity, to measure the humidity. It suggests that there will be a response in the dry cell for increasing evaporation rates and for decreasing in moist, because of their antagonistic relationship.

These hypotheses are quite different from each other, and the driving mechanisms could involve mechanical or chemical processes. They are not without similarities, however. For example, in order for the rate of evaporation during exposure to the ambient air to be measured, both the hygrometer model and the evaporation model require the sensillum lymph to move toward the outside.

An issue for the evidence of the mechanical hygrosensory model lies in that, if given a constant absolute humidity, the relative humidity level will drop if the temperature rises. In this scenario the expectation is that the activity in the hygroreceptors would go down with the rise of temperature. Experiments have shown that this is in fact not the case, and that activity actually measures higher with a corresponding rising temperature. An alternative hypothesis for the underlying mechanisms of hygrosensation has been suggested by the Sensory Neurophysiology group at Lund University. It builds upon the mechanical hygrometer model, and in this augmented theory the antagonistic pair of hygroreceptors works the same way and with the same purpose. The difference lies in the function of the thermoreceptive neuron, which the researchers propose alters the response of the hygroreceptors, and specifically the strength of it. The theory put forth is that the signal transduction is driven by the hygroreceptors and mechanical stress, while lower temperatures inhibit the activity of these neurons via the thermoreceptive neuron. Simply, it expands upon the mechanical hygrosensory model by taking the temperature neuron into consideration. All three neurons are connected via ephaptic coupling, a kind of connection that, unlike chemical or electrical

synapses, is not direct. In this case they are dependant on each other because of the extracellular fluid surrounding them, which the neurons' activity controls the ion concentration of. While researching the thermoreceptive neuron, it has been suggested the neuron is silenced in temperatures above 25 °C, in essence creating a threshold for the temperature dependence at 25 °C.

Initially, we planned to perform a series of behavioral experiments in temperatures around 25 °C, such as 23, 24, 25, 26 and 27 °C, the reason for this being that the threshold is equal to 25 °C. According to our hypothesis, we would have expected the experiments performed in 25, 26 and 27 °C, as well as the control experiments, to have the same humidity responses, while the responses in the experiments at 23 and 24 °C were expected to decline with the decreasing temperature. Furthermore, we wanted to perform the same experiments again, using flies whose temperature neuron were silenced (using genetic modification) as a control group. However, due to lack of time, we settled for one temperature on each side of the threshold: 24 and 30 °C, and we omitted the use of control experiments, with the hope that we might still discover trends that warrant performing several more experiments using different temperatures in future research.

The aim of this report is to answer two questions:

- How do *D. melanogaster* react to humidity stimulus?
- What effect does temperature have on that reaction?

Our hypothesis is that a higher temperature will give rise to a higher humidity response, until the 25 °C threshold has been reached.

# 2 Methodology

## 2.1 Animals

The strain of fly used in the experiments was $w^{1118CS}$, a standard control fly line in experiments with D. melanogaster. Since the experiments were conducted three times a day, it was important that the flies were split up into three separate incubators set to different time schedules, at least for some three days before the experiment. The start of day in the day/night-cycle was set to 8.00, 12.00 and 16.00, respectively. For the 8.00 incubator, the regular laboratory incubator was used, keeping a precise air humidity and temperature, 25 °C and 75 % humidity. The other two incubators were a makeshift compromise consisting of polystyrene boxes with timer controlled lamps inside them. To increase likelihood of a response to the stimuli and increase reproducibility of the results, only males were used in the experiments. To put the flies to sleep the tubes containing the flies were lowered down into ice and kept there for a few minutes. Then the flies were spread out, one in each lane, after which the arena was assembled. The arena is connected to the tubing system and placed into the cabinet. Using this experiment protocol and a total of 81 flies, 27 experiments were carried out: 14 at 30 °C and 13 at 23 °C.

## 2.2 Experimental Setup

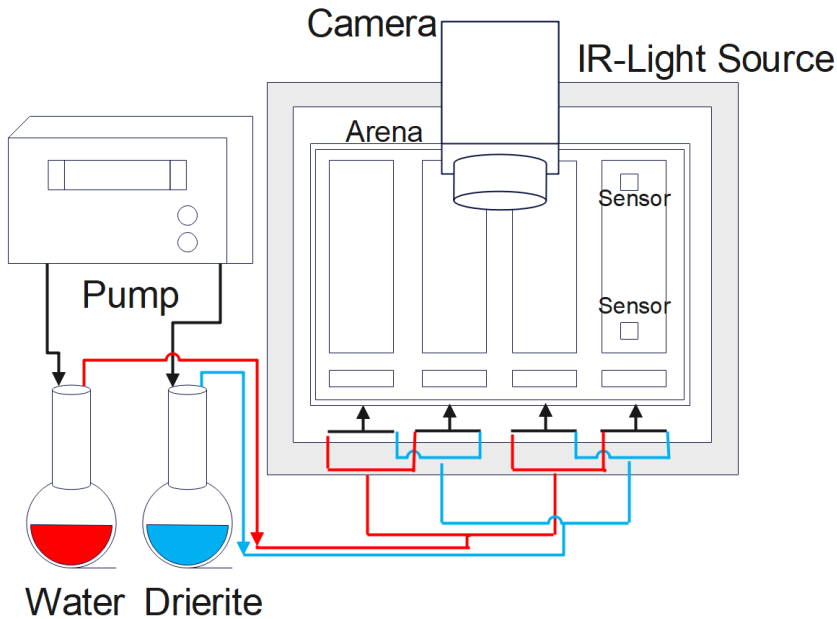


Figure 2: Schematic illustration of the experimental setup. The pump can switch between through two different outputs which then connect to a humidity altering medium each, water or drierite, via tubing. The tubing then continues and splits up and joins together for the different lanes in the arena. All equipment except the pump in the schematic is placed inside the insulated box. The IR-light source is placed on the floor, with the arena then just above it. The camera is placed above the arena filming straight down.

The experimental setup consists of an air pump delivering flow of air through a system of tubes that leads it towards two parallel coupled glass jars, each filled with a humidity controlling medium. It is illustrated in the schematic in figure 2. From the jars, the tubes are split into four, and each is routed into a connection to a chamber in the arena. With a valve it is possible to control the air flow into the humidity medium one at a time. The Arena is placed in an experimental cupboard above an infrared light source, with a infrared camera above the arena.

### 2.2.1 Flow

A Syntech Stimulus Controller CS 55 was used for pumping air into the humidity system[18]. It contains its own air pump and the user can switch between outlets using simple TTL-signals. The CS 55 is designed for being controlled with a foot pedal switch, but was modified in these experiments to be controlled directly via a DAQ-board, the USB-6343 model, and LabVIEW for increased accuracy and consistency between experiments [19].

Two lengths of tubing, one from each output, is lead down to two pieces of glassware, one for each humidity control agent. In the first series of experiments the glassware was Erlenmeyer flask, and in the latter media storage bottles. In total, three different setups of humidity control agents have been employed for the experiments. The reason for the evolution and further development of the methods can be summarized as poor performance and low reliability and consistency of the first versions.

### 2.2.2 Humidity Control

Salts have traditionally been used to control the humidity in a laboratory setup. Some salts are hygroscopic. That is, they hold the ability to hold water from the environment via absorption or adsorption, either in their crystal form or in a saturated water solution. The hygroscopic properties have shown to be highly precise, and are in some cases employed to calibrate humidity sensors[20].

Drierite is a proprietary drying agent made from calcium sulfate (naturally occurring in gypsum), and in some variants also from a color indicator for moisture. [21] Drierite is more efficient than lithium chloride when drying out air humidity. Furthermore, it does not clump together in the same way that lithium chloride does, and thanks to the color indicator, it is easy to see when the agent is losing its potency. All in all, these characteristics made Drierite a more convenient choice of agent for this experiment, compared to lithium chloride.

The first configuration relied on two saturated salt solutions, lithium chloride for the low humidity, and sodium chloride for the high. The resulting difference in humidity between the two mediums were deemed too low, and was

considered one of the primary reasons for the minuscule response of the flies in the experiments where it was used. In addition to the low difference, all the values of humidity given by this system were outside the interval preferred by this type of fly, potentially resulting in the flies not going into different states of navigation. This prompted the change from lithium chloride to Drierite.

### 2.2.3 Control

A LabVIEW-script was used to control the pump. The accuracy of the timing was important for the analysis, and the automation made it easier to carry out the long experiments. A 5V-TTL signal is sent to the stimuli pump via a DAQ-board. The script allows the user to set the pump to a schedule where it automatically switches between the two stimuli based on a millisecond timer. The script then repeats the schedule until the user exits the program.

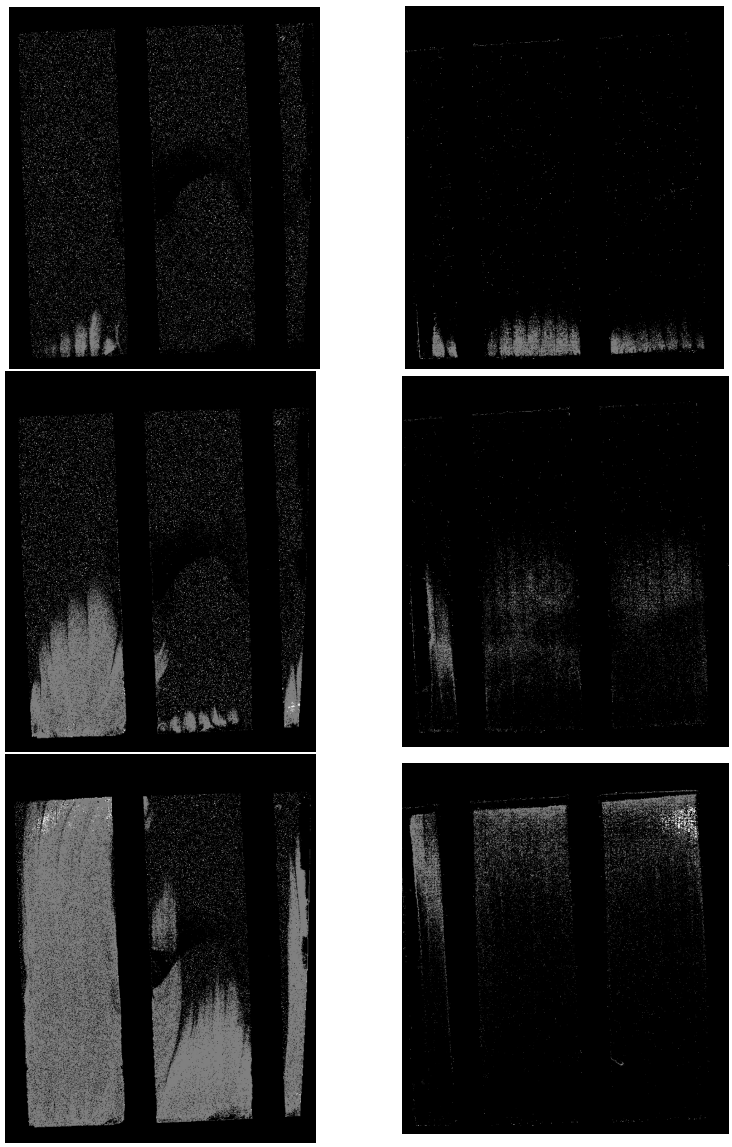

Figure 3: Sequential stills from the smoke tests of the first arena and then the replacement arena. The left column is the first arena and the right the replacement. The leakage is apparent in the first arena. The flow of smoke is significantly delayed in the lanes to the right, and especially the middle one. It then leaks from the left lane to the middle one and the flow profile for the middle one is visibly altered. For the replacement arena the flow is a clear laminar one, where each lane is isolated from each other and there is no clear delay in any lane.

### 2.2.4 Troubleshooting the Flow

To make sure that the flow of air was equally distributed, and in order to measure the speed of the flow, the system was put through a filmed trial run with burning incense in the glass jars instead of Drierite and water. The smoke of the incense was visible in the recorded video, so the flow could be traced. A smoke pen was originally going to be used for this purpose, but incense turned out to be easier to use in the testing tube and was therefore the better choice. Because of the thin chambers, and since the camera was not well-suited for this application, the smoke was very faint in the video. To amplify the traces of smoke in the recordings, an image analysis post processing script was written. A simple KNN background subtractor from the OpenCV library was used to separate the moving smoke from the stationary arena. The result can be seen in figure 3 as some stills from the video. Blockage was found in one of the chambers during the trial run. The arena was switched out and new tests were carried out, and since no blockage was found in the new arena, the experiments could proceed.

## 2.3 Experimental Protocol

In studying the earlier experiments of the thesis it was apparent that the short stimuli did not have a clear effect on fly behaviour. However, in experiments where the duration of the stimuli was longer, a trend could be spotted. The change in behaviour seemed to happen some time after the stimuli started. The first hypothesis was that there was some kind of delay in the fly's reaction, but this did not entirely explain why there was no response to the short stimuli. A new hypothesis was formed, based around the idea that the fly needs to be subjected to the new climate for a while before changing its behaviour, and that it therefore will not react to a short stimuli. The experiment design was modified to test this hypothesis, using significantly longer stimuli than before. So long in fact, that it should be regarded as a change of state in the fly's environment.

The timetable of the experiment is based on blocks of 60 minutes divided as follows:

- 15 minutes of low humidity
- 5 minutes of high humidity
- 10 minutes of low humidity

- 15 minutes of high humidity
- 5 minutes of low humidity
- 10 minutes of high humidity

This was repeated four times, giving the experiments a total running time of four hours.

## 2.4 Data Processing

### 2.4.1 Calibrating the Camera

The focal length of the camera had given rise to a significant amount of distortion in the image, so a calibration of the camera was required. A checkboard calibration pattern was printed on paper and then photographed by the camera from different angles, after which the fisheye camera parameters could be calculated and the image rectified.

### 2.4.2 Collecting the Data

For the tracking of the flies in the experiments, the software Margo (Massively Automated Real-time GUI for Object-tracking) was used, slightly modified to give timestamps based on the computer's system clock, to help when syncing the tracking data with the humidity sensors and LabVIEW pump controller, and also to minimize camera distortion. Margo was devoloped by the de Bivort Lab at Harvard University for tracking individual organisms in real time. Margo provides the data from the session in the form of a struct containing everything recorded as well as the settings of the experiment. The data used for analysis was the centroid, a matrix of the fly's position in x and y coordinates for every recorded frame (the values are set to NaN for a frame if the fly is not tracked on it), a vector of timestamps for the system clock for every time frame (this was added as a modification of Margo by ourselves, for making it easier to sync with the humidity measurement), and a sample image from the recording, used for transforming the coordinates of the centroid.

The data from the Sensirion ControlCenter application comes in the form of an EDF file containing the measured relative humidity level and a system

clock timestamp for each sample. A MATLAB script written for the thesis reads the files, parses and extracts the data from them and rearranges it into a struct with one field for each fly in the experiments.

### 2.4.3 Transforming and Normalizing the Coordinates

The coordinates of the centroid are based on the pixels of the video recording. This becomes an issue for two reasons, firstly it means there is no info on the fly's position in reference to the lane, which we need for comparing the different flies and extracting their features. Secondly, for ease of access and quick set up of the experiments, the positioning of the camera towards the arena was inconsistent. This resulted in an often skewed and poorly scaled recording, making the resulting centroids, and therefore all features, highly camera sensitive. To solve these problems, we implemented an image analysis script. The script takes a sample image from the experiment and finds lines in it using Hough transforms. From the intersections of these lines, it also finds the corners of the lanes. The coordinates of the centroid is then transformed using the corners, and the result is a coordinate system with x ranging from 0 to 400 and y from 0 to 1400.

### 2.4.4 Interpolation and Filtering of Data

Since the centroid only has coordinates in the frames where the fly was tracked, the resulting matrix contains large blocks of NaN-values. In order to complete the trajectories, the centroid is run through a script written by Jonathan Lind, which replaces the NaN-entries with real values using linear interpolation between the adjecent coordinates. To smooth the trajectories, the centroid is put through another script by Lind, which applies a lowpass butterworth filter that removes high frequent noise of the signal. The completed centroids make up matrices that are used to calculate the features that are being analyzed for behavioural traits.

### 2.4.5 Sorting out Data

At this point, sub-par experiments are sorted out and removed based on temperature and humidity. The temperature must be between 29.5 and 30.5 °C during the entire experiment for the higher temperature experiment and 23.5 and 34.5 °C for the lower, and for the high temperature experiment the relative humidity must always reach down to at least 45% for the dry stimuli, and up to at least 60% for the humid one. Because of problems with the drierite for the 24 °C experiments we raised the lower limit of 45% to 50% for the dry stimuli, to get enough data to analyze, see discussion. Experiments in which the fly did not move at all, either because it was dead or because of a tracking error, were also removed.

### 2.4.6 Syncing the Data

To study correlation between the fly's behavior and the stimuli, the matrix for the centroids needs to be synced with the humidity level measurement samples. The measurements of these have been taken with different sampling rates (10 Hz for the tracking of the fly, and 2 Hz for the humidity measurement), and therefore the humidity timestamp vector is expanded and interpolated to be compatible with the tracking data.

The main format of the data analysis is based around the switching point of the valve, when the humidity in the chamber starts to change. To find this change point, the series of humidity values is normalized so that the lowest value is 0 and the highest is 1, and then the humidity signal is differentiated. The signal for the humidity sample tends to become noisy when it reaches saturation, making it challenging to analyze. To counteract this, the differentiated signal is filtered with a Savitzky-Golay filter. The change point is set to be the point where the differentiated signal switches from negative to positive, or vice versa. Data of the different features is extracted from 60 seconds before to 300 seconds after each change point, making each trial 360 seconds long in total. The extracted trials are added as seperate entries into two structs, one for high-to-low humidity, and one for low-to-high.

In order to choose an appropriate threshold for the experiments, a visualization of the distribution of activity in the trials was created using a color map plot. The idea for the plot was adapted from van Brugel et al. [22], who

based the sorting of the trials on average speed during a period before the trial. Since we needed the threshold to be based on total distance moved during the trial in order to compare our result to the results by Alvarez et al. [23], we had to modify the sorting accordingly. Therefore the data is sorted by distance moved in the period after the stimuli. It was decided that flies that moved less than 100 mm in total during the entire trial were discarded.

## 2.5 Analysis and statistics

### 2.5.1 Features

To see if and how the humidity change had an effect on the behaviour of the flies, several specific features from the data were studied. Since the only data collected from the tracking were the x and y position of the centroid in each sample, all features were calculated based on that information. Below follows a list of the studied features.

- Ground speed, defined as the speed of the flies measured in mm/s.
- Upwind speed, defined as the speed of the flies against the air flow, where the positive direction is set to be towards the humidity source, and the negative is set to be against it.
- Angular velocity, defined as the absolute value of the difference in orientation for flies in time. The orientation was not tracked as its own variable during the experiments, but was instead calculated as the direction of the velocity of the centroid. It is measured in degrees/s.
- Curvature, which gives a measure of the sharpness of the fly's turns. It is calculated by dividing the angular velocity with the ground speed, and is measured in degrees/mm.
- Turn probability, which comes from the binarization of the curvature with values higher than 20 degrees being set to 1, and lower values to 0. It is a dimensionless quantity.
- Y-position. That is, the y-coordinate of the fly's position in the arena at a given point in time. Since the length of the arena is 1400 mm, the y-coordinate must be between 0 and 1400 mm, with 0 mm corresponding to the point of inflow of air.

- Percentage of flies that are active, calculated using a binarization of absolute speed. If the absolute speed of the fly is higher than a certain threshold, it is considered active and is set to 1. Otherwise, it is said to 0. The measurement is given as a percentage of the total amount of flies in the trial.
- Y position of only moving flies. This is the same as Y position, but only taking active flies into consideration.

### 2.5.2 Wilcoxon signed rank test

To assess if the flies' behavioral response is statistically significant a statistical hypothesis test was applied. It was decided to use the Wilcoxon signed rank test. Wilcoxon takes two samples and tests if they share a common distribution. It does not assume that the data is normally distributed, like for instance the paired Student's t-test does.[24] The main reason for this choice is that Jonathan Lind used the same test for his experiments, and by using the same it makes it easier to compare the difference in response between the theses. [1] To implement the test, the *signrank* command was used in MATLAB.

# 3 Results

For finding, and justifying, a threshold to use in further analysis of features, a color map of all the trials sorted by total movement over the trial was plotted, as seen in figure 4 and figure 5. It is clear that the distribution is quite varied, and a large amount of trials contain little to no movement. With the distribution of fly movement visualized, choosing a threshold became easier.

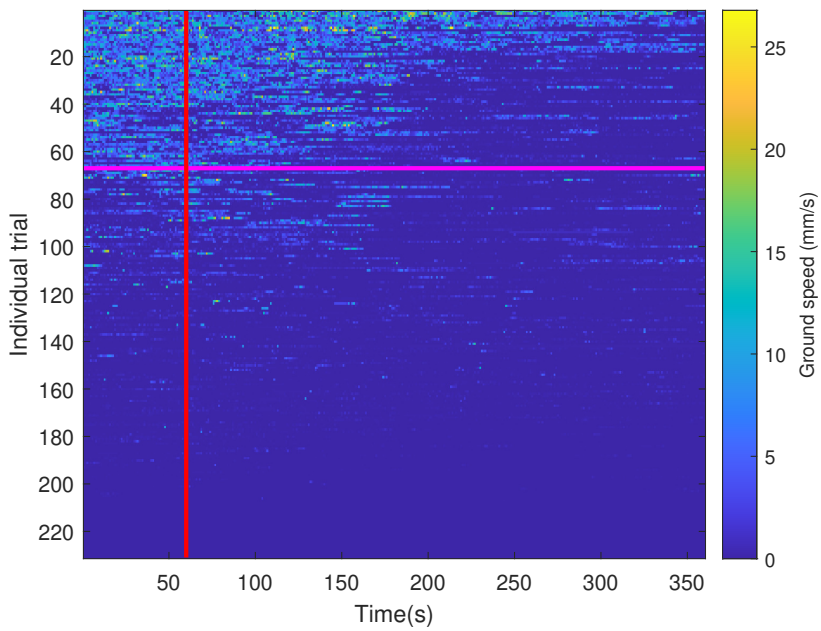


Figure 4: The ground speed of all low to high humidity trials, sorted by total movement over the trial. The red line is start of the trial and the magenta the threshold for trials.

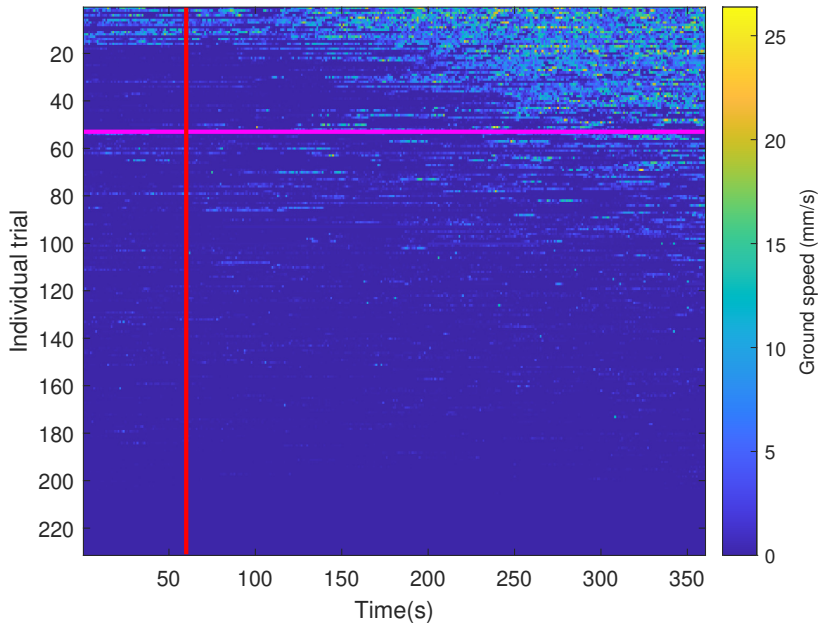


Figure 5: The ground speed of all high to low humidity trials, sorted by total movement over the trial. The red line is start of the trial and the magenta the threshold for trials.

With the threshold decided and the data sorted away, the different features were plotted for the two types of trials in figure 6 and figure 7. In most of the features a clear trend can be seen during both types of stimuli. Curvature, Y-position and turn probability are not statistically significant in the confidence level we set for the experiments. In upwind speed it is hard to spot a trend overall, let alone a significant one.

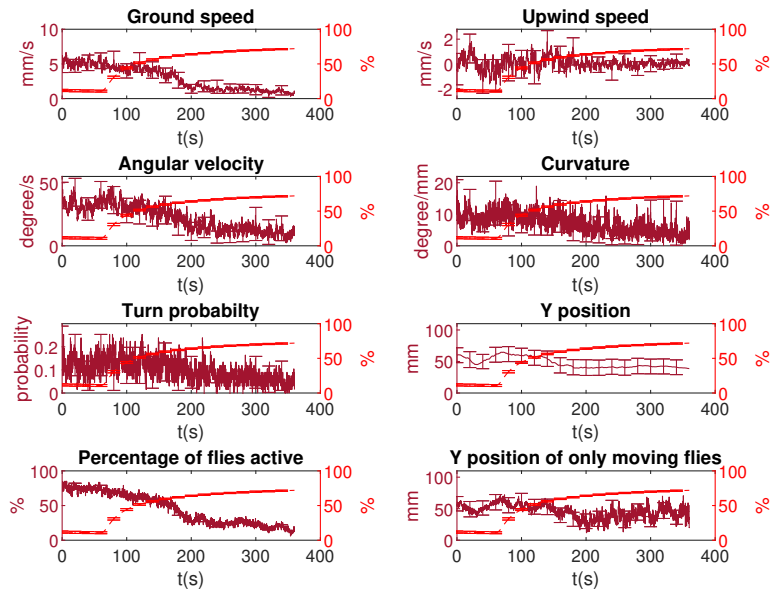


Figure 6: Mean value for the features in the trials going from low to high humidity in 30 °C. The brown line indicates value of the feature and the dashed red line. The error bars show confidence interval with confidence level of 95%. The data is collected from 68 trials spread over 16 flies and 9 experiments.

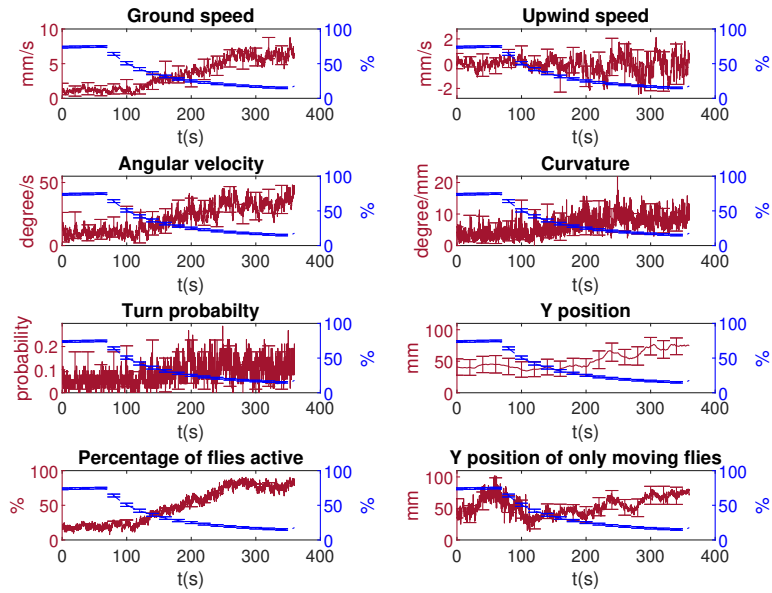


Figure 7: Mean value for the features in the trials going from high to low humidity in 30 °C. The brown line indicates value of the feature and the dashed red line. The error bars show confidence interval with confidence level of 95%. The data is collected from 52 trials spread over 14 flies and 9 experiments.

The same analysis was repeated with flies in 24 °C, as seen in figure 8 and figure 9. Similar trends can be spotted, but much weaker. It is difficult to say how much of it is because of the hypothesized effect of temperature in hygrosensation, and how much is because of the poor quality of the dataset.

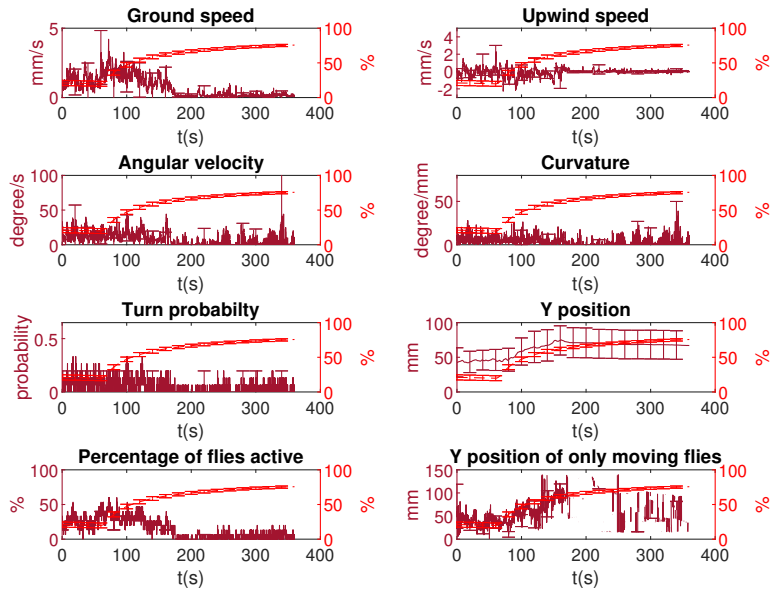


Figure 8: Mean value for the features in the trials going from low to high humidity in 24 °C. The brown line indicates value of the feature and the dashed red line. The error bars show confidence interval with confidence level of 95%. The data is collected from 21 trials spread over 14 flies and 12 experiments.

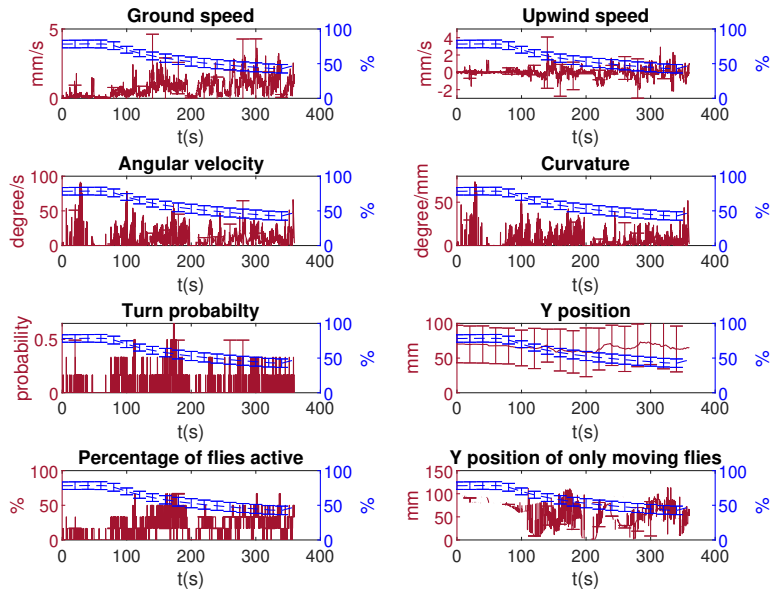


Figure 9: Mean value for the features in the trials going from high to low humidity in 24 °C. The brown line indicates value of the feature and the dashed red line. The error bars show confidence interval with confidence level of 95%. The data is collected from 10 trials spread over 7 flies and 6 experiments.

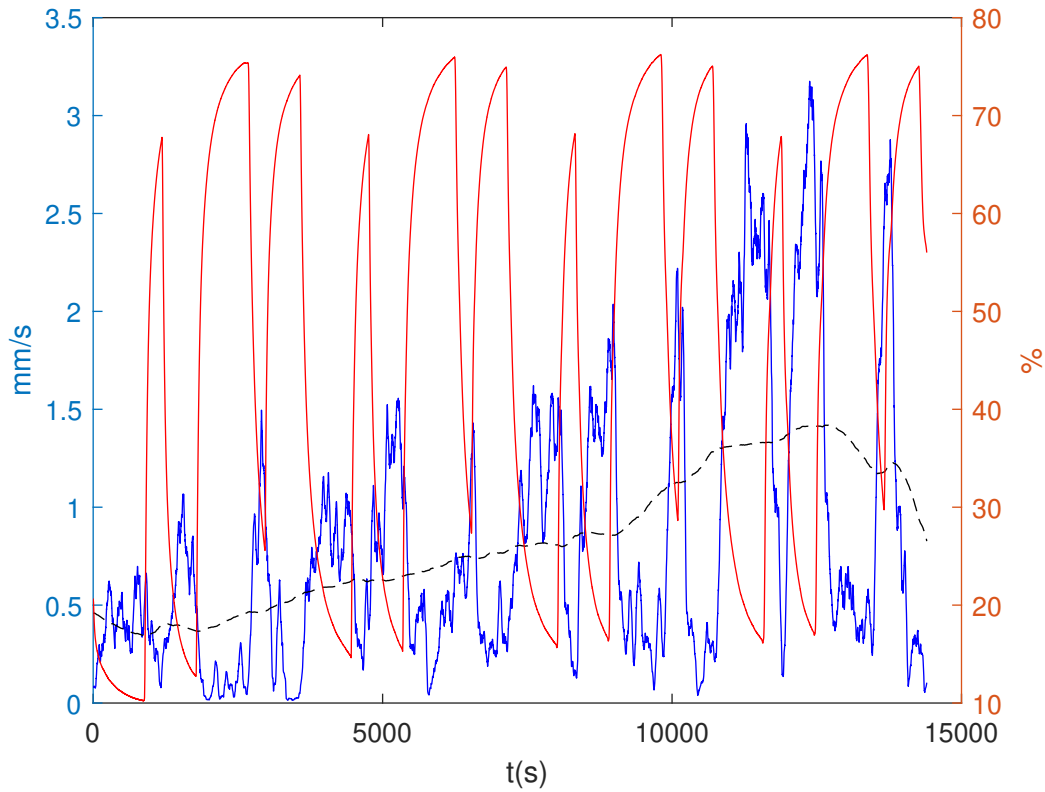


Figure 10: Moving mean of average ground speed for entire four hour experiments. Blue line a 60 s rolling average, and dashed line one hour (3600 s). Red line is the humidity level at a given time point. This plot clearly illustrates how the total activity of the flies grows with time, even though a variation following reaction to the stimuli is present.

Early on when performing the experiments we could see that the average fly became increasingly active during their entire 4 hour run. To be sure that the trends spotted during the average smaller trial were also somewhat present in the much longer experiment sessions, we looked at the average ground speed of the full experiments, as in figure 10. We wanted to study whether or not a reaction to the stimuli could still be observed, as well as the effect time had on the activity of the flies.

# 4 Discussion and conclusions

We can clearly see a shift of behaviour in several behaviour features in both types of trials, high-to-low and low-to-high humidity, in the 30 °C experiments. With a confidence interval of 95%, the change in behaviour after the stimuli seems significant in most features. One of the exceptions is the upwind speed which seems to carry no useful information about a change in behaviour of the flies during stimuli. The hypothesis behind the clear difference in response of this variable compared to the others is based on the movement patterns of the flies. While it can be difficult to see trends in the flies' movement and discern determinism in their path, one of the most visible behaviours is when they go from standing still to moving around the arena for a while, up and down. Most of the time they move back and forth rather than to another end of the arena. This means that when averaging the upwind speed, the flies that move in different directions will cancel each other out. If the setup had allowed for the timing of the experiments to be more precise and the stimuli more immediate, it might have been possible to extract some pattern of interest from the upwind speed, but the variation in the flies' reaction time could make it somewhat challenging. The average y-position for the flies during the trials does not show significant response, however, one can discern a trend when looking at the data. It is possible that this feature contains a high variance and that a response would become significant with more experiments. We can clearly see a improvement in finding a response the stimuli from the earlier experiments we performed that was published in Jonathan Lind's thesis[1]. Comparing results of the hypothesis tests we can see that the reaction to stimuli was only statistically significant in a few features: y position, ground speed and angular velocity. Also they are consistent between types of trials.

| Feature | Low-to-high | High-to-low |
|---|---|---|
| Ground speed | 1.2824e-12* | 5.9141e-10* |
| Upwind speed | 0.7312 | 0.4889 |
| Angular velocity | 8.9311e-11* | 1.2569e-08* |
| Curvature | 4.0011e-08* | 7.3275e-06* |
| Turn probability | 3.3836e-07* | 1.2862e-05* |
| Y position | 0.0327* | 8.0370e-05* |
| Percentage of flies active | 1.4345e-12* | 1.1760e-09* |
| Y position of only moving flies | 0.1894 | 0.0366* |

Table 1: The resulting p-values of the Wilcoxon signed rank tests for the 30 °C experiments. To indicate a significance on a 95 % confidence level, a * is used. The samples being tested are the average of the feature during 60 s before the start of the stimuli (0-60s in the trial) and the last 60 s of the trial, ending 300 s after the start of the stimuli (300-360s in the trial). This was done for the two types of trials separately

| Feature | Low-to-high | High-to-low |
|---|---|---|
| Ground speed | 6.1035e-04* | 0.0313* |
| Upwind speed | 0.2293 | 0.6875 |
| Angular velocity | 0.0040* | 0.0938 |
| Curvature | 0.1763 | 0.4375 |
| Turn probability | 0.0040* | 0.0313* |
| Y position | 0.2078 | 0.8438 |
| Percentage of flies active | 0.0012* | 0.0313* |
| Y position of only moving flies | 0.6250 | 0.2500 |

Table 2: The resulting p-values of the Wilcoxon signed rank tests for the 24 °C experiments. To indicate a significance on a 95 % confidence level, a * is used. The samples being tested are the average of the feature during 60 s before the start of the stimuli (0-60s in the trial) and the last 60 s of the trial, ending 300 s after the start of the stimuli (300-360s in the trial). This was done for the two types of trials separately

As we can see in table 1 for our 30 °C trials, a clear majority of the features chosen have statistically significant response that is consistent between types of trial. For our 24 °C experiments, however, the results are not as stellar, as can be seen in table 2. The expected hypothesized results of the experiments was a lesser response for the lower temperature, in this case, though, it seems

more likely that issues with the experimental setup has a more prominent effect.

The resulting behaviour in the experiments should not be interpreted as a water search behaviour. In fact, it is difficult to categorize as any specific behaviour, as the results are not very precise. Our hypothesis for the interpretation of this behaviour is simply that the *D. melanogaster* have a preference for 70% humidity [11], and when the it gets significantly lower than that, they become more active in an effort to find more humid conditions.

We had some issues with the equipment during the 24 °C experiments, especially concerning the absorption of moisture in Drierite. This resulted in a high amount of subpar experiments that had to be discarded, and for that reason, we have a low sample size for the cold temperature. While the data in the 30 °C experiments are good enough for observing patterns and trends, the vastly different sample sizes makes the comparisons between the temperatures unreliable.

While you can see a clear result with this experimental setup, the result runs a risk of becoming trivial. It is certainly valuable to see how different behavioural features are affected by a stimuli, but the response in behaviour have a tendency to take on an ”On-Off”-characteristic. Meaning, it is hard to describe the behaviour in other terms than simply, ”moving more”, ”moving less”, ”prefer to be closer”, or ”prefer to further away”. The recorded data contains a wealth of information about an advanced and dynamic pattern of behaviour, but also a severe amount of noise. To sift the characteristic features of behaviour through long time series can be difficult. The inexactness of the data is the most obvious issue when trying to find more specific patterns, and the role of choice of feature to parameter should also be taken into consideration. The features for this thesis are very basic. Based on the results of these experiments more specific features could be chosen, by studying the trajectories of the individual flies, observing patterns and finding ways to parametrize them.

More advanced methods for analyzing the data could also be beneficial, especially for finding patterns that are more subtle, of longer duration or more sporadic in nature, like the work Jonathan Lind did with Hidden Markov Models in his thesis [1]. Another method being explored is one using differ-

ent measurements for time series similarity. Dynamic Time Wrapping and Fréchet distance have shown promising results [25]. Recently progress have been made when using deep learning assisted analysis on large sets of movement data in the field of animal trajectories. In regular ”naive” feature design, the researcher studies the trajectory trying to find matters of interest to focus on, like absolute speed, orientation, binarisation of movement, etc. Using the Deep Neural Network approach, labeled (eg. male, female) trajectory data is fed into a neural network. The network extracts segments of interests, which, because the system is a black box, researchers will label as different features [26].

A major issue to take away from this project is the nature of the experimental setup. To which extent can the faults be blamed on the level of optimisation of the current configuration? Will continued iteration of the setup lead towards well-functioning protocol and apparatus, or will they not converge? The principal design of the experiment contains many points of failure, making it far too likely that something will disturb the data collected or that the experiment will be interrupted. These points consist of the communication of the pump breaking up, the humidity control medium varying in efficiency, leakage in the tubing, blockage in the arena, and humidity sensors breaking. Furthermore, the small size of the space experiment cupboard made it difficult to work efficiently. While a great deal of these problems can be phased out by further development, at least to some extent, some of them are in ways inherent to the design. The largest issue is the design of the experiments, the strictly time-dependant nature of them, the extended time it takes to prepare the experiments and the small bottlenecks that can cause delay in the setup. Fruit flies are living organisms and have a circadian rhythm. Their activity vary during the day, and since the experiments are tracking their activity, the flies in the experiment need to be at the same point in their circadian cycle.

It takes a long time to assemble and disassemble the arena. Another design for an arena, that does not need to be disassembled after every experiment, or involved another way of inserting the flies, would be beneficial. This could be performed by using electromagnets to seal the arena, some kind of vise, clamp, or by letting flies into the arena through permanent holes in the lid that can be plugged afterwards. These alternative designs could save copious amounts of time for the researcher, not only in reducing the long preparation time, but also in not having to repeat botched experiments. With the large amount of experiments that is needed to see an effect, this is a relatively small

investment.

An aspect making it difficult to analyze different features of behaviour, especially the mean y-position, is that the level of humidity is not uniform at any given moment. The actual humidity in the arena is formed as a gradient, which mean the humidity a fly experiences is not only dependant on the point of time in the experiment, but also on where in the arena it is located. The precise timing of the signal sent to the pump and meticulous syncing of humidity and behavioural data is very important, but one needs to take into consideration that the difference of a few centimeters in the arena can give an even larger variation in humidity than a few seconds of time. This leads to some amount of inexactness, specifically when trying to analyze immediate behavioural response to a stimuli.

The experimental setup and arena is not without merit. It is easy to set up, and the way the different parts work together is straight forward and simple to understand. Even people with little experience working with this type of equipment should be able to use it, and even modify it for their purpose. It gives a result in which a clear effect of the stimuli can be seen. However, most of the advantages of the setup also give rise to its shortcomings. For this type of research, analyzing large amounts of data for subtle changes in behaviour, precision and replicability are above all the most important factors.

Much of this research has been inspired by the experiments by Alvarez et al. [23], trying to adapt their protocol with humidity instead of olfactory cues. This has given us mixed results, and there are obvious differences in our experiments, like our very laminar flow of humidity in contrast to the wild turbulence of their odour plume. It has however made us speculate in how similar olfactory and hygrosensation actually are, and how similarly they should be tested. The flies' sense of smell is based around sensing a very small amount of particles in the air, that could be as low as a few ppm. Humidity on the other hand is always around us to a varying degree. It is not a question of if it is present or not but rather to which amount. And those amounts are quite high compared to smell, being able to go up to several percent of volume of air. This also means there will be physical differences in how humidity and smell spreads and flows around different environments. It could even be worth considering if another sense, such as temperature for instance, could be a better comparison for hygrosensation.

Even though no clear answer to the question poised in this report has been acquired, and not all the planned experiments could be carried out, it is a clear step forward. The results in this thesis have shown that further research is warranted and with all the lessons we have gotten in how to best perform these further experiments, the future looks bright. The next step is to perform the originally planned experiments with all temperatures and controls. The laboratory is working on building a new and improved experimental setup based around what we learned during this project, and will be using it for further research.

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# 6 Appendices

## 6.1 Equipment

| Equipment | Model name |
|---|---|
| Air pump | Syntech Stimulus Controller CS-55 V2 |
| DAQ Multifunction I/O Device | NI USB-6343 |
| Temperature controller | Ascon Technologic TLK 38*S* |
| Sensor connector | SEK-SensorBridge |
| Humidity/Temperature sensor | Sensirion SHT35-DIS-B |
| IR Light source | Advanced Illumination BXXXYY Backlight |
| Arena | Designed by Alvarez et al. [23] |
| Sensor program | Sensirion ControlCenter |
| Tracking software | MARGO |
| Fly food | Water, sugar syrup, corn flour, yeast, agar, soya flour, propionic acid. |
| Smoke source | Aroma Valley Cedarwood Incense Sticks |

Table 3: Table of all the equipment used in this thesis.

## 6.2 Supplementary figures

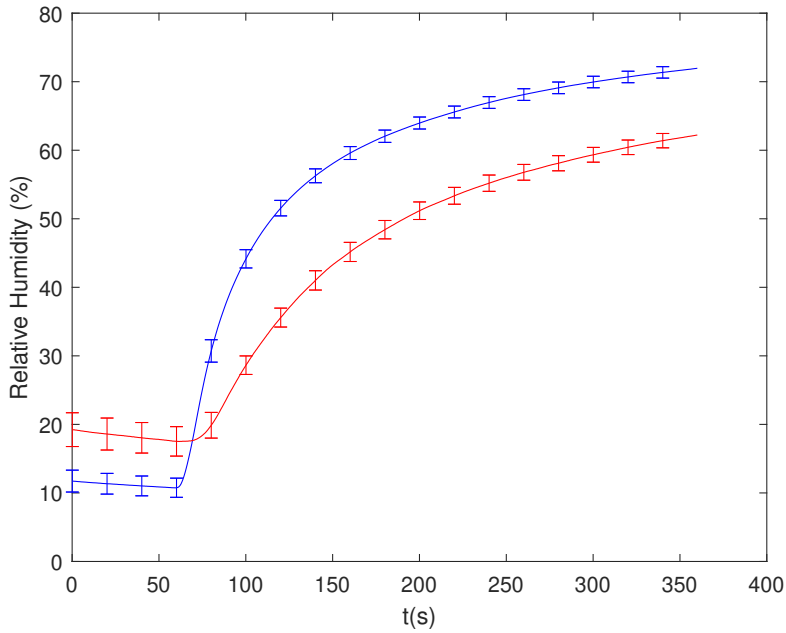


Figure 11: Average humidity level of the low-to-high trial at two points in the arena. Blue line is the sensor at the front of the arena, closest to the inflow. Red is the one at the back, farther from the inflow. The error bars show confidence interval with confidence level of 95%.

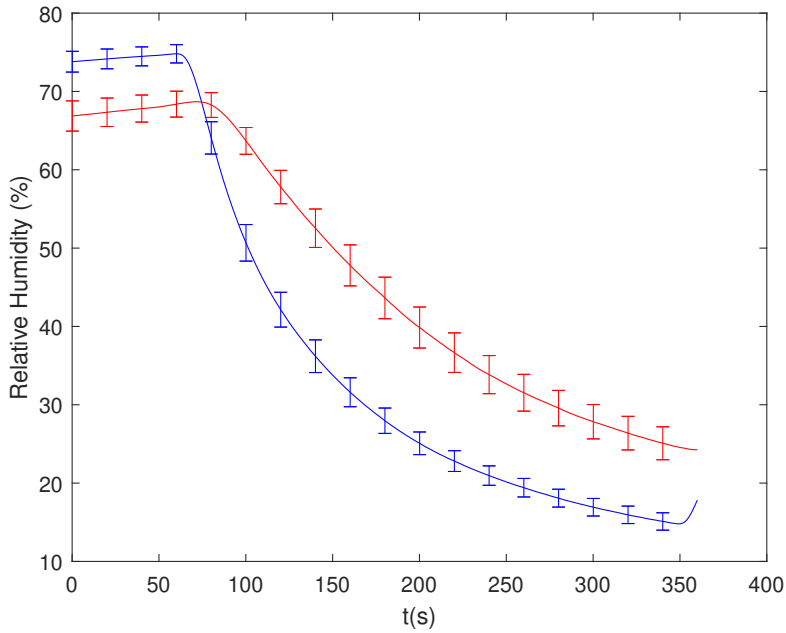


Figure 12: Average humidity level of the high-to-low trial at two points in the arena. Blue line is the sensor at the front of the arena, closest to the inflow. Red is the one at the back, farther from the inflow. The error bars show confidence interval with confidence level of 95%.

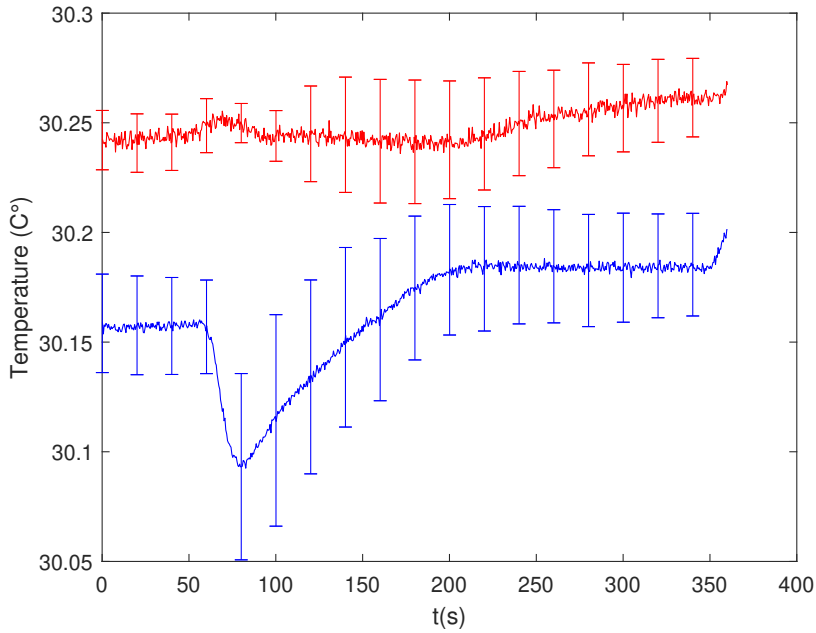


Figure 13: Average temperature level the trials, both low-to-high and high-to-low, at two points in the arena. Blue line is the sensor at the front of the arena, closest to the inflow. Red is the one at the back, farther from the inflow. The error bars show confidence interval with confidence level of 95%.