The role of haltere feedback during territorial chases in the house fly, *Musca domestica*

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Thesis submitted in partial fulfilment of the requirements of Five Year BS-MS Dual Degree Program

Under the guidance of:

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Certificate

This is to certify that this dissertation entitled 'The role of haltere feedback during territorial chases in the house fly, *Musca domestica*' towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research (IISER), Pune represents original research carried out by Alisha at NCBS-TIFR under the supervision of Dr. Sanjay P. Sane, Associate Professor, NCBS-TIFR, during the academic year 2013-2014.

Dr Sanjay P Sane

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Declaration

I hereby declare that the matter presented in the thesis entitled ‘The role of haltere feedback during territorial chases in the house fly, *Musca domestica’* are the results of the investigations carried out by me at NCBS-TIFR under the supervision of Dr. Sanjay P. Sane, Assistant Professor, NCBS-TIFR, and the same has not been submitted elsewhere for any other degree.

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BS-MS Dual Degree programme

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Abstract

Of key importance among natural behaviours are territorial chases in which insects guard and defend home territories by chasing away aerial interlopers. Such territorial behaviours allow insects to secure mates and are hence important for both the survival and evolution of insects. To carry out these complex aerobatic manoeuvres requires the nervous system of the chasing fly to rapidly sense and respond to an interloper. Assays for territorial chases in a laboratory environment hence offer an exciting means to experimentally study various questions about sensorimotor integration in flying insects. In this project, we have designed assays for territorial chases between house flies (*Musca domestica*), and filmed them using high-speed cameras. With this assay, we tried to understand the role of mechanosensory input from the halteres. Using multiple, synchronized cameras, we obtain 3D coordinates of the head and abdomen of the territorial defender and the interloper. We compared ethograms to gain qualitative insight in the flight defects of flies with their halteres intact and either haltere ablated. Though, we have their flight trajectories, but we are yet to quantify the defects in flight trajectories when they move in three dimensions.

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Acknowledgements

I would like to thank my mentor, Dr Sanjay Sane, who guided me during this project with his advice, technical and otherwise. I would also like to thank Tanvi for teaching and helping me with experiment and design. She has pushed me to learn and to pay attention to detail by exploration.

This project was a continuation of a summer study by Pranav and Sam with Tanvi, of whom Pranav and Tanvi made time to help set up and understand the details of chase behaviour of house fly which helped me get a head start.

These experiments would have not been possible had the flies were not caught from the wild, for which I would like to thank Mr Kemparaju.

Settling and working in a new lab would have not been easy but for the lovely lab mates of the flight lab at NCBS-TIFR, who were there to share jokes, knowledge, experience, and good times.

Then there were the people of NCBS, warm and bright, inspiring everyday to learn more. They have filled time between work and sleep with memories and laughter.

Discussions with faculty and friends back from campus, who gave me different perspective to the problem at hand, and helped me analyse and understand better. I would like to thank Prof Sanjeev Galande and Dr Raghav Rajan for their valuable inputs. I would especially like to thank Sachit for his patience while I rattled about tangential ideas.

Importantly, my family for helping me get through days good and bad with equal vigour.

1. Introduction:

Insects have inhabited earth for about 400 million years, and since its primitive form has continuously adapted to win the battle of survival. They were the first organisms to evolve flight, and have dominated the aerial territory with marvellous flight manoeuvres (Dudley, 2002). When flying, they exhibit diverse flight behaviours such as hovering, rapid saccades and object tracking which requires them to flap their wings at high frequencies, acquire and process sensory inputs and respond to environmental changes at a high rate (Land 1993; Dudley, 2002).

Insects need to defend their territory, alone or in groups (as a colony), for them to secure mates and forage. Many flying insects chase and catch aerial interlopers to defend territory and secure mates. Such chases also help them determine the gender of the interloper (Baker, 1983). Territorial chases have caught the fancy of biologists and physicists as the flies perform fantastic aerial manoeuvres, track their target and control speed and direction in accordance to the target (Land and Collett,1974; Wehrhahn, 1979; Wagner, 1980). Chases are primarily guided by visual inputs (Land and Collett, 1975), and processed by their nervous system which generates complex motor responses to allow the flies to catch mates and ward off interlopers. In many Dipteran flies, males are usually more territorial than females, and tend to find favourable posts/locations from where they ward off other males and pursue females.

An insect's body is primarily divided in three segments: head, thorax and abdomen. The wings and legs originate from the thorax. In case of Dipteran flies, the hindwings have been modified into dumbbell shaped appendages called halteres. The halteres are believed to function as vibrational gyroscopes (Pflugstaedt, 1912; Fraenkel, 1938; Pringle, 1948). Arranged around halteres base is fields of mechanosensory organs called campaniform sensillae.

Haltere function:

Haltere moves anti phase to the wings, and tends to preserve its angular momentum. While turning, the haltere experiences a Coriolis force perpendicular to the plane of vibration of the halteres. This force acts mainly on the knob, which makes up for the major mass of the haltere. The force's amplitude is proportional to the vector product of the haltere's linear velocity and the fly's angular velocity. Thus haltere can sense angular velocity. The oblique positioning of the haltere helps it encode the forces in all three axis of rotation.

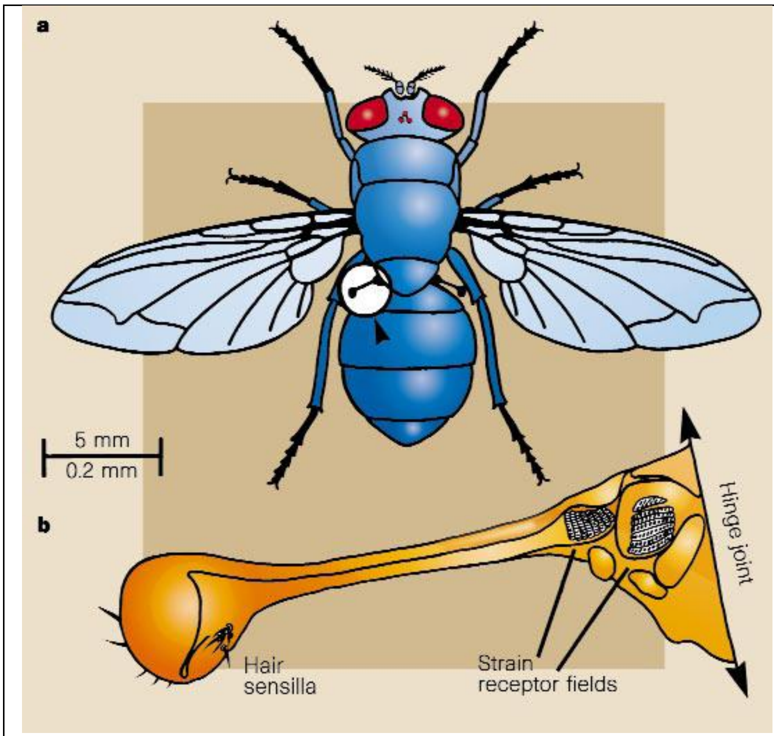


Fig 1) Dipteran Caliphora (Blow fly), and below it is the left haltere. Marked are the sensory fields.

The force experienced by the knob of the haltere, which can be assumed that they have the center of mass, passes the strain along the stock which is later experienced by the strain sensors at the base of haltere called companiform sensillae. The neural pathway of how the signal from the mechanosensors is encoded is poorly understood.

In this project, we have tried to understand the role and functioning of the haltere in free flight though a fly's chasing behaviour. Ethograms were generated to note the differences between normal flies and flies with ablated halters in performance of the aerial manoeuvres (Hengstenberg, R., 1999; Nalbach, G., 1993; Nalbach, G., 1994).

This study is divided in two major parts:

1. First, we standardise the technique for recording the chases between two or more *Musca domestica* in a lab environment, and their 3D reconstruction. This work draws on results and techniques of studies involving the 2D reconstruction of chases (Land and Collett,1974) and 3D free flight tracking (Hedrick, 2008).
2. Second, we use the above setup to study the role of haltere in free flight, in this case, territorial chase. For this we ablated the bulbous end of either left of right haltere, and studied its effect on flight.

The Dipteran house fly (*Musca domestica*) occurs in many parts of the world, and typically prefers hot and humid conditions (Hewitt, 1914). It is easily available and displays robust territorial behaviour. These territorial behaviours are confined to small volumes and hence can be reconstructed in the laboratory. This makes it a good model system to study chases, and further, the role of halteres in free flight.

2. Material and Methods

2.1 Preparation and treatment:

Several variables like the temperature, time of day, luminosity, chase arena, presence of females, food, anesthetisation process etc were explored for their effect on chases in a controlled environment. We standardized a set of these variables (as discussed later in the experimental protocol standardisation in Appendix 1).

Final protocol:

The study was performed on a batch of 40 male *Musca domestica* which were introduced in a 45 cm X 45 cm X 48 cm box (Wehrhahn, 1982), placed in a green house. The flies were caught from the wild, and cold anesthetised (6°C for 4 minutes). They were then placed on a cold metal slab (to maintain anaesthesia) under a microscope (10X zoom) for gender and species identification. Haltere ablation was also carried out during this time for the treatment group. The same batch of flies was filmed for chases 2 days after introduction in the box. The chases were filmed between 10 AM and 3 PM.

The box was illuminated by the diffused natural light of the greenhouse (luminosity ranging from 10000 – 24000 Lux; depending on the weather) on NCBS campus. The ambient temperature ranged between 24 to 30 °C, and humidity about 70 percent. The box was supplied with 2 food plates (35 mm petridish with sugar) and water (60 mm petridish with tissue paper soaked in water).

The box was made of Plexiglas, with two opposite sides of the box covered with random chequer-board pattern to provide an optically rich environment. One of the 4 sides and the roof of the box were left transparent to allow filming, and the sides opposite to the transparent ones were covered with white paper to ensure a good contrast in the recorded videos. Cylindrical posts of height 8 cm and varying diameter (1 cm to 5 cm) were placed inside to provide landmarks to defend.

The chases were filmed using two Phantom V7.3 cameras (at 1000 frames per second and exposure varying from 100-200 µs depending on light conditions) fitted with 18-55mm Nikon lens. The cameras were controlled *via* a software “Phantom Camera controller” for camera control and capture. The cameras were placed approximately orthogonal to each other, to give appropriate depth for 3D reconstruction of the chase. The camera setup and its respective views are shown in the Figure 2.

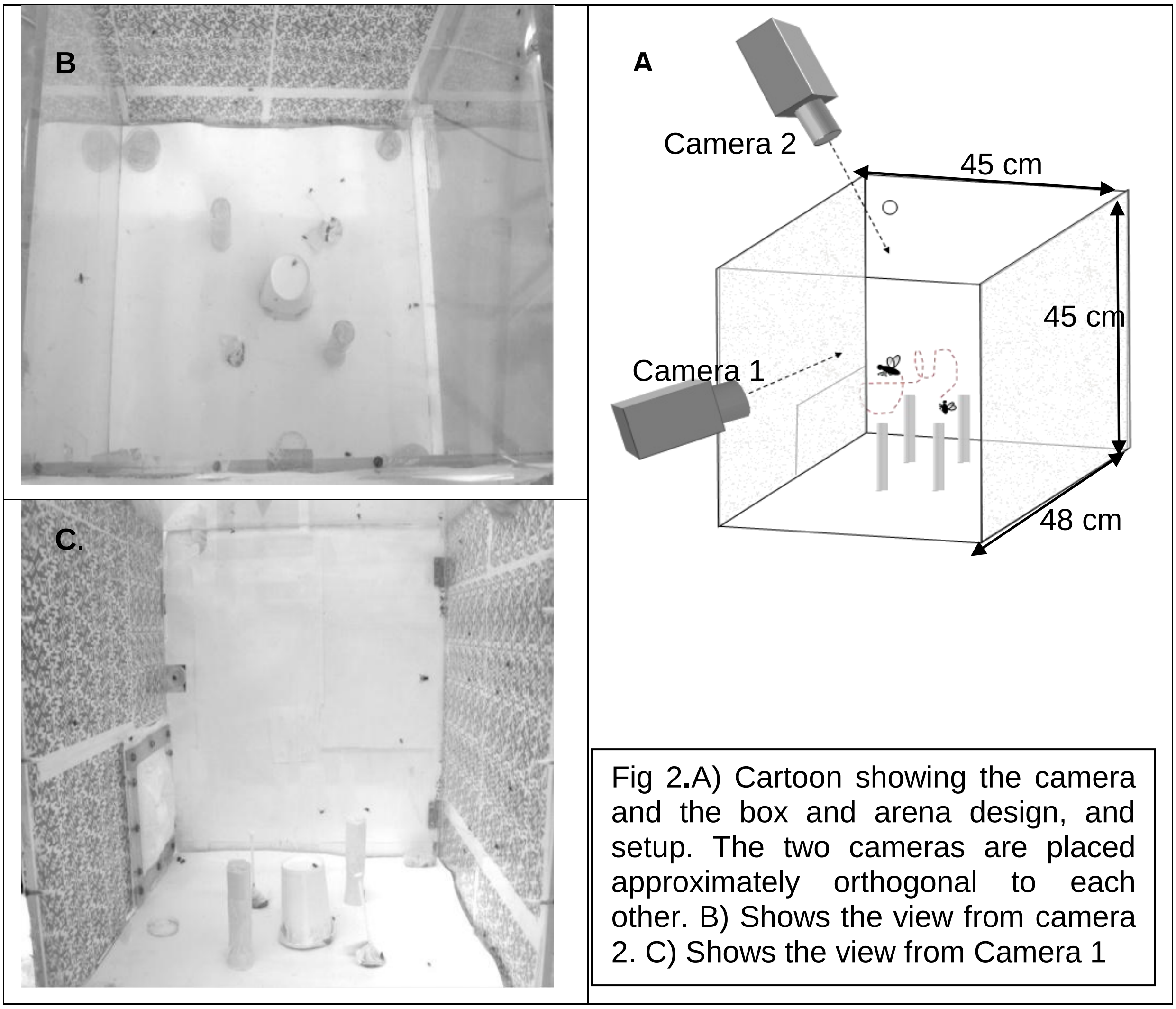


Fig 2**.**A) Cartoon showing the camera and the box and arena design, and setup. The two cameras are placed approximately orthogonal to each other. B) Shows the view from camera 2. C) Shows the view from Camera 1

2.2 Digitization:

The videos were analysed by a specialised MATLAB code developed by Dr. Tyson Hedrick lab for 3D/2D points for each frames (Hedrick, 2008). This code does a 3D mapping using direct linear transformation. The code requires a calibration object (Figure 3), calibration specification file, calibration image file (for each video), and synchronized video from the two cameras. The calibration objecthad16 iron rods (8 cm long and 4 mm diameter) embedded at a gap of 2 cm (center to center distance) in a plexiglas plate. These rods were covered with a sheet of paper marked with black and white bands printed at a 2 cm distance for contrast for marking points. The calibration object chosen should be in accordance to size and detail of the space to be calibrated.

For camera calibration, at the end of each experiment, the calibration object was placed in the field of view of the cameras and synchronised images of the calibration object were taken from each view. These images were fed into the code, and minimum of six corresponding non coplanar points were marked on the calibration object in each camera view which gave the DLTcal file. The DLTcal and videos were fed in the DLTdv code which gave the X, Y, Z coordinates of the point of interest, in this case head and tail of both leading and chasing fly.

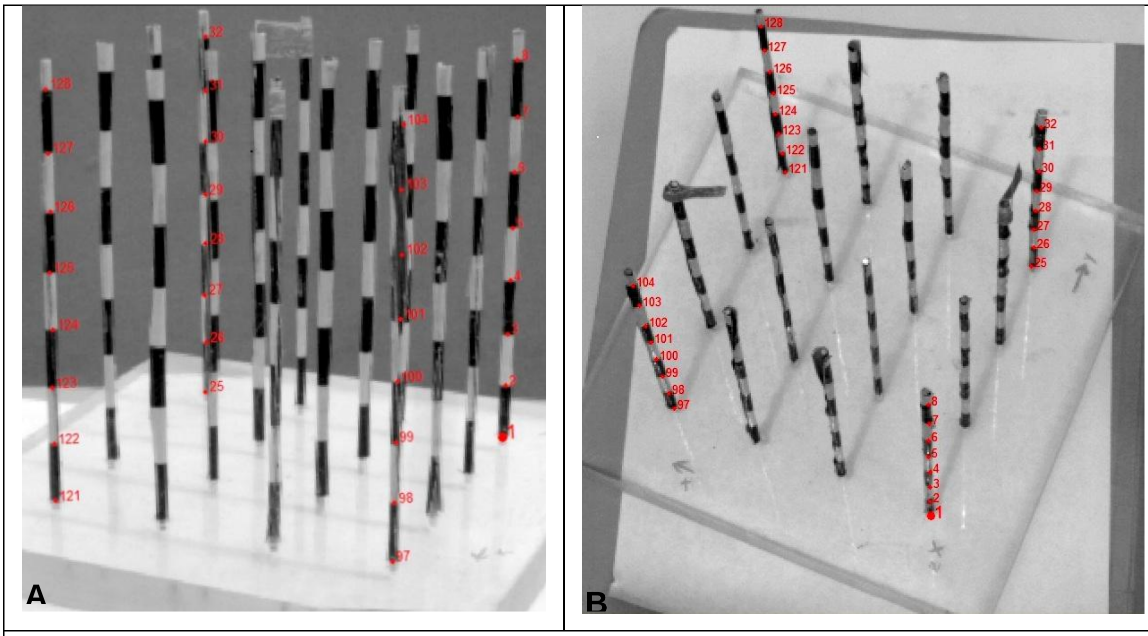
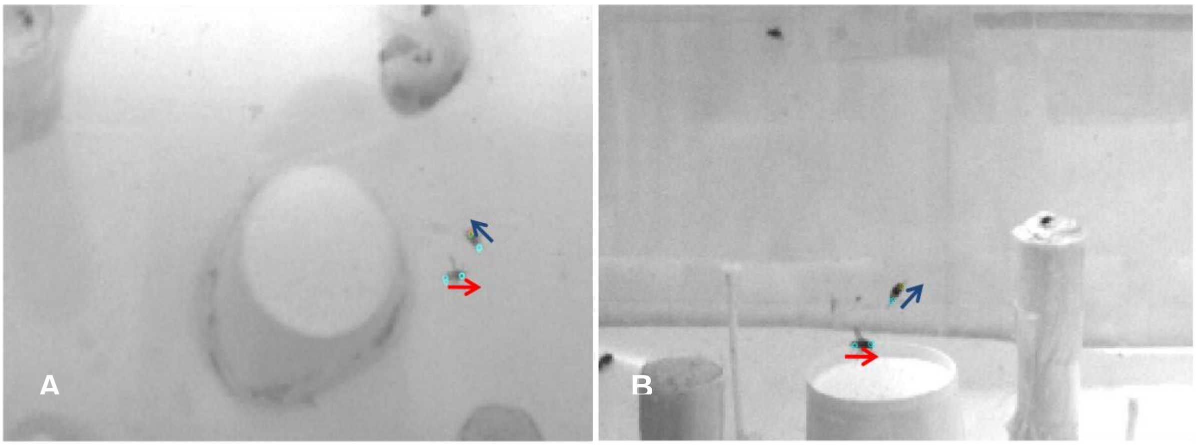


Fig 3.A) The image of the calibration object from the front camera view (camera 1 in Fig 2.1.A), and the top view (B) (Camera 2 in Fig 2.1.A). The figure also shows the corresponding points for calibration.



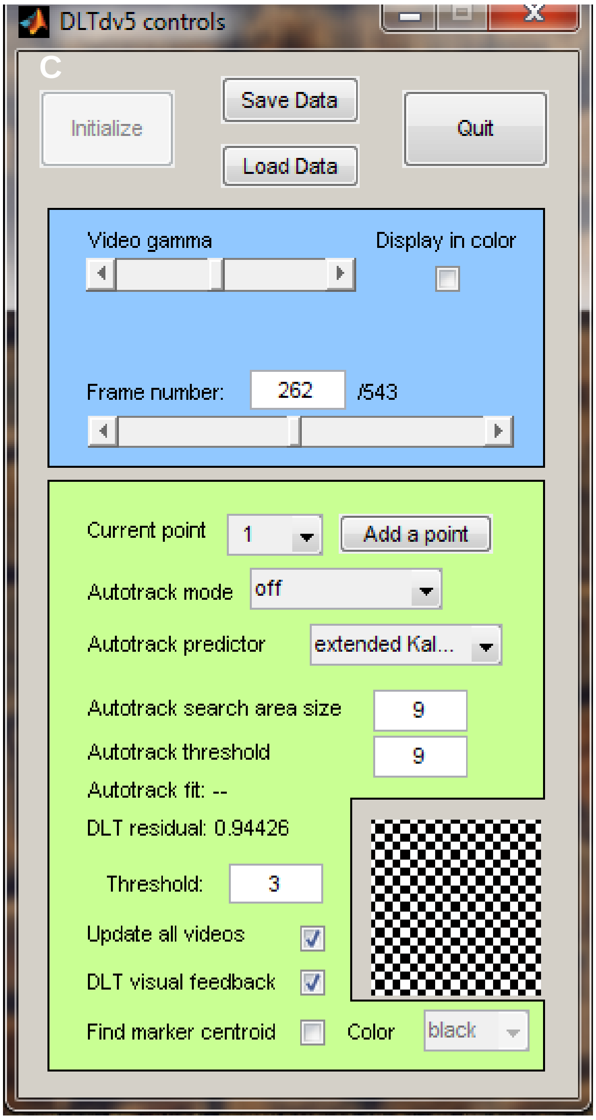


Fig 4) A) View from the over head camera digitised, with head and tail points. B) View from the front camera digitised, head and tail points. Blue arrow is for the leading fly and red for the chasing fly C) DLTdv5, GUI of the MATLAB based software, used for digitising.

2.3 Treatment:

Dipteran hind wings are modified into a club shaped appendage called halteres, which are known to be gyroscopic mechanosensory organs that provide feedback for flight stability. In the experimental group of flies, either left or right haltere knob, for all flies, were cut using surgical scissors. (Figure 5)

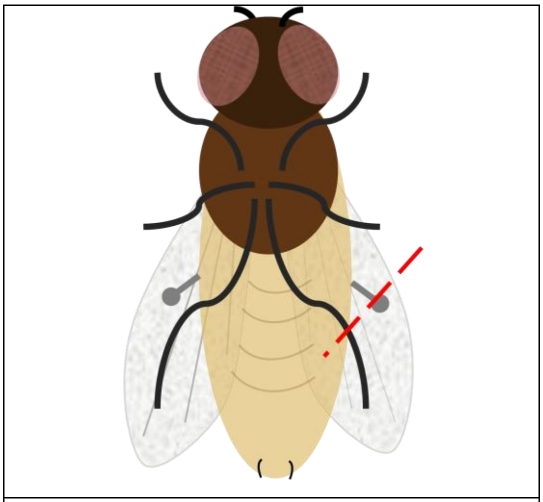


Fig 5) Diagram showing the treatment procedure; cutting the bulbous end of the haltere.

2.4 Analysis:

We analysed the data using MATLAB (R2009a) codes. The head and tail points of the leading and chasing flies were tracked for the entire chase. In the typical case, the video included 30 frames before the chasing fly takes off and ended when the chase was completed. Though, the end of the chase is not clear.

Parameters extracted from the head and tail points:

1. Body vector: Vector pointing from tail towards the head of the fly, calculated from the head and tail points.
2. Body axis angular velocity: The angle between consecutive body vectors divided by the time interval (in this case 1 ms).
3. Trajectory vector: The midpoint of the fly is the midpoint of the head and tail points. If we join the midpoint of the fly for each frame, we get the trajectory of the fly. We use this trajectory to compare the body axis motion when in flight, and the forward velocity of the fly. The rate of change of angle between consecutive trajectory vectors (vector joining two consecutive midpoints) gives us the angular velocity of the trajectory of the fly.
4. Target vector: This is the vector joining the midpoint of the leading and the chasing fly, with the vector pointing in the direction of the leading fly. The magnitude of the vector is the measure of the distance between the flies.
5. Body axis angle, β: Angle between body axis of consecutive frames of F1/F2.

6. Error angle, Ø: Angle between the body axis of F and the vector joining midpoint of F1 and F2
7. Body axis angle, α: Angle between heading vector (body axis) and path vector (trajectory) of either fly
8. Trajectory error angle, ʌ: The angle between the heading axis (the trajectory) and the line joining the midpoints

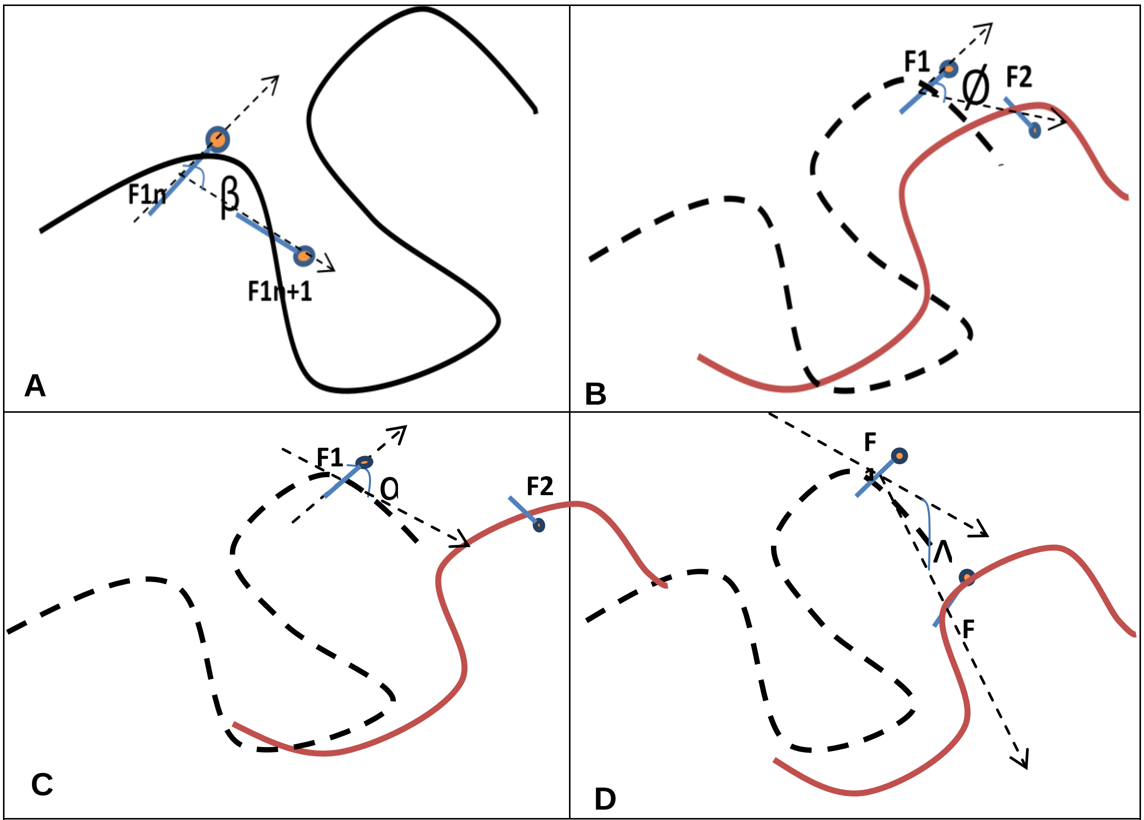


Fig 6) F1 is the chasing fly, dotted red line is the trajectory of the chasing fly. F2 is the leading fly; the dotted blue line is the trajectory of the leading fly. All the angles calculated were in the 2D plane containing the vectors. A) Body axis angle, β, angle between body axes of consecutive frames of F1/F2. B) Error angle, Ø, between the body axis of F and the vector joining midpoint of F1 and F2. C) Body axis angle, α, between heading vector (body axis) and path vector (trajectory) of either fly.D) Trajectory error angle, ʌ, is the angle between the heading axis (the trajectory) and the line joining the midpoints.

Distance plots:

We measure the distance between the flies and plot them (Fig 6). From the plot we can see the distances between the flies decreases, and then the distance between them is almost constant as the leading fly tries to escape and the chasing fly is trying to close in on the leading fly, finally the leading fly is able to escape and that is visible as the sudden increase in the slope of curve in the plot. From this, for each chase recorded, there are three major segments: the approach, follow and escape. We plotted the relative distance between the flies over time for the control and the haltere ablated group, and find how the minimum distance between the chasing and the leading flies varies in the two groups.

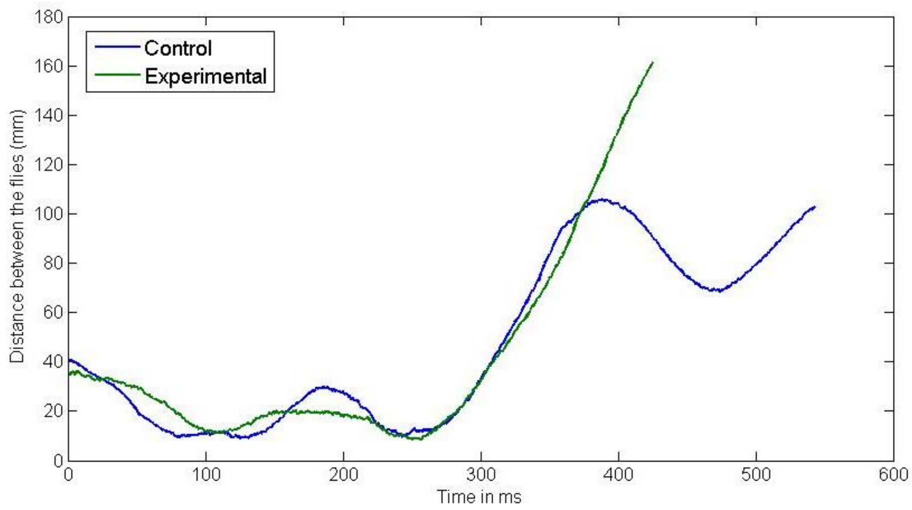


Fig 7) This figure maps the distance between the head of the flies over time for one of the chases.

Ethogram:

Ethograms were generated for quantifying flight defects. We measured the frequency of a) tumbling b) crashing, and c) turns. Tumbling is defined as the vertical *drops* in straight flying paths'; crashing is defined as the *crash* against any of the walls/objects. These parameters capture defects in flight. Number of turns is defined as the *turns* taken while chasing, which may be active or uncontrolled. . The parameter of number of turns in the Ethogram was chosen because the accuracy of a chase can be measured by how the chasing fly is responding to the manoeuvres of the leading fly. If for every turn of the leading fly, the chasing fly can change its direction then the chase is good. This also reflects on the motor control of flight and the motivation of chase.

Statistics:

Unpaired t-tests were done to compare the treatment and the control group of flies on the minimum distance between the flies, the length of chase and the drops, turns and crashes from the ethogram. MATLAB (R2009a) was used for this analysis

3. Results:

3.1 Trajectories:

Using the head, tail and midpoint of the fly, the trajectory and body orientation of the fly was plotted. An example for control group trajectory is as figure 8 and for treatment group of flies is as figure 9.

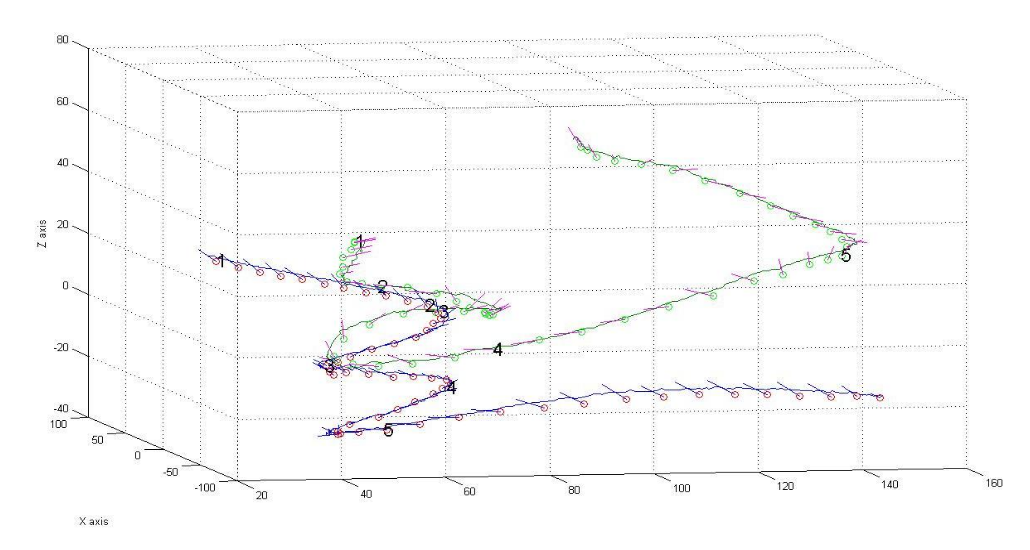


Fig 8) The trajectory of the chase in case of the control group, where the red circle is for the leading fly and the green circle for the chasing fly. The numbers, n, show the corresponding location of the fly at the nX100th ms.

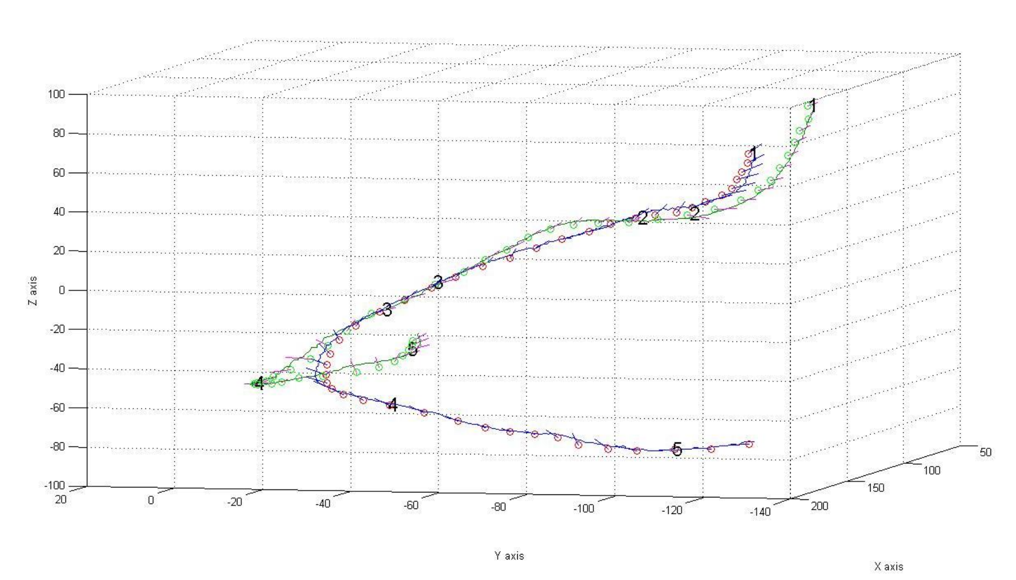


Fig 9) The trajectory of the chase in case of the experimental group, where the red circle is for the leading fly and the green circle for the chasing fly. The numbers, n, show the corresponding location of the fly at the nX100th ms.

3.2 Ethogram:

In case of haltere-ablated flies, both chasing and leading flies had one of their halteres ablated (if right, the right for all the flies in the box). The ethograms shown here contain data for both chasing and the leading fly.

	SN		**Control (mean (SD))**	**Haltere-ablated (mean (SD))**	**P**	**Significance**
Chasing	1	Turns	4.1 (1.9)	1.2 (0.6)	2.46E-04	**
	2	Drops	0.1 (0.31)	1.6 (1.07)	4.1E-04	**
	3	Crashes	0 (0)	0.2 (0.42)	0.1510	
Leading	4	Turns	5.4 (2.06)	1.2 (0.63)	8.33E-06	**
	5	Drops	0(0)	1.6 (1.17)	4.21E-04	**
	6	Crashes	0(0)	0.5 (0.70)	.0382	*

Table 1) This table shows the means, standard deviations and P value obtained from T test comparing the turns, drops and crashes in the control and treatment flies. (P<0.05)

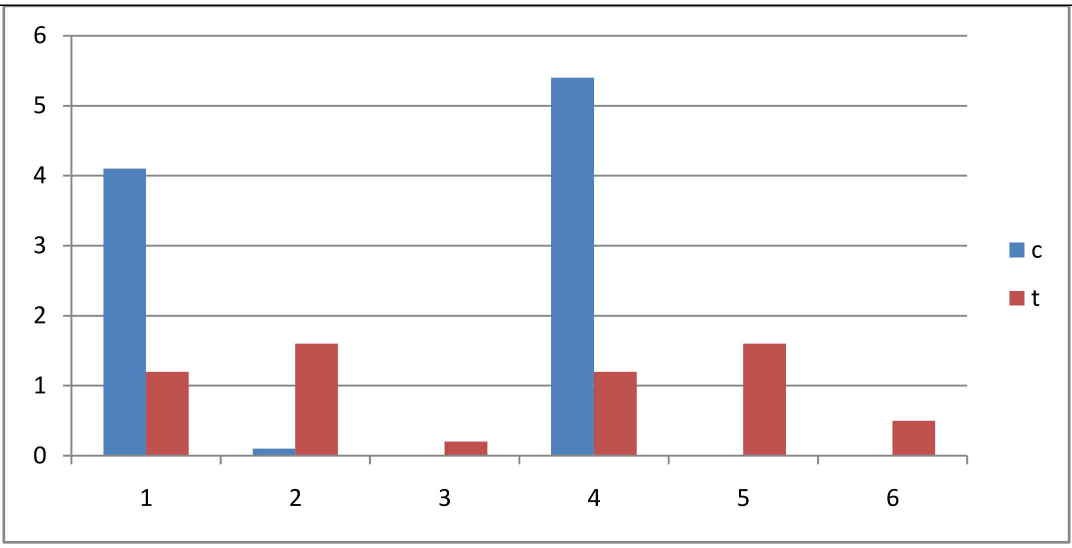


Fig 10) Bar graph showing the differences in control (blue) and haltere ablated (red) group. The numbers on X axis are the corresponding parameters to the numbers in SN (serial number) of Table 1.

3.3 The distance plots:

Minimum distance between the flies:

Minimum distance between the flies			
C1	3.7917	L4	13.3305
c11	11.4471	L5	60.6508
c18	5.9263	L6	35.5162
c23	9.0033	R4	64.3629
c8	5.8455	R5	8.4504
Mean (SD)	7.20 (3.01)		36.46 (25.90)

Table 2) Minimum distance between the flies (distance in mm). (P = .0364).

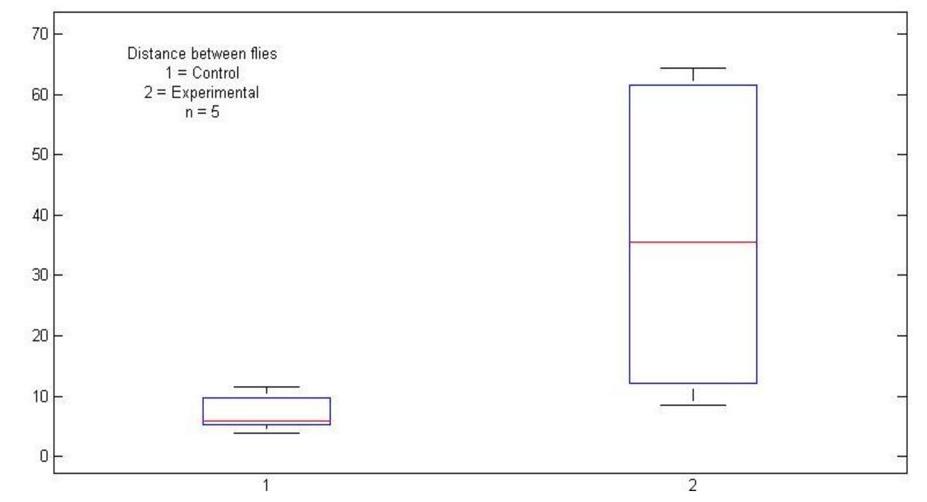


Fig11) Box plot comparing the relative distance between the control and the haltere ablated group of flies. The distance between leading and chasing flies is highly variable in the group of flies with the haltere ablated.

4. Discussion:

4.1 General characteristics of the chase:

Control

A territorial chase involves a fly (typically on a post) taking off and chasing away an interloper from its territory. They engage in various manoeuvres at close range, and attempt to evade or catch the interloper.

Treatment:

In the experimental flies, the halteres (either left or right) are unilaterally ablated. Flies with both halteres ablated are unable to fly. The unilateral haltere-ablated flies, from observation we can say that both chasing and leading typically show lower activity in the box. The chases, and even cruising flights, are fewer than in the control case, and with lesser speed of flight. The chases are short, and the flies seldom take turns. The flight trajectories are often, but not always, erratic and appear uncontrolled. The body axis is changing rapidly with respect to trajectory.

4.2 Trajectory and body axis plot:

Land and Collett in their 1974 study showed that that the trajectory error angle and the angular velocity of the trajectory of the fly are continuously related. Their study was performed on 2D projections of the chase instead of 3D in which the chase happens. Also they recorded the chase at 50 fps where as we did at 1000 fps. We procured the various angles between the leading and the chasing flies, as discussed in section 2.4. We are still analysing the correlations between these angles.

Though, we have a definite start of the chase (30 ms before the chasing flies take off), but the ending of the chase is not very clear. For this reason we cannot make a clear demarcation for the set of values to use as the values beyond the chase end may add to noise and outliers.

The control flies exhibit sharper turns, and the body axis of the fly changes rapidly with respect to the main trajectory. The fly performs complex manoeuvres and with precision while chasing. In the case of treatment flies, as the fly loses stability in flight, the body axis spins about the trajectory. We could identify the tumbling of flies, for they happen not only when fly is turning only but also when fly is flying in a straight path. Hence, if we do analysis of only how the trajectory and the body axis change, we do not infer anything about how the fly speed and direction is chasing. Therefore, we can't differentiate between aerial manoeuvrability and flight instability, which we are measuring as change in body axis with respect to its path.

4.3 Ethograms:

From the ethograms for the number of turns, drops and crashes, the P value shows significant difference in the flight stability between control and treatment flies. Previous studies have stated that the flies with their halteres ablated have unstable flight, especially along yaw axis. With the current 3D coordinates of flies and the body axis, quantifying the instability in flight and the angles can give us better insight into how haltere is a mechanosensory organ.

4.4 Distances between flies:

The distances between flies show significant difference between the control and treatment groups. Though in case of the treatment group, both the leading and chasing flies had their haltere ablated, hence both had impaired flight. Also, the minimum distance between the flies also depends on what was the initial distance between the chasing and the leading fly why the chase started. Thus, the minimum distance between the flies can be biased by sampling alone. Whereas, the number of turns made by the chasing fly in accordance to every turn made by the leading fly shows how close the chase is.

5. Conclusion:

Through the multiple trials for reproducing chases in lab environment, we have established a protocol for studying chase behaviour in lab environment.

With the current analysis, we can say that flies with one ablated haltere are able to fly, but unable to maintain trajectories in tight behavioural chases. This loss of flight ability is not recovered with time.

6. Limitations:

6.1 Variability in the system

1. This is a behaviour experiment of flies, and the insect flight is highly dependent on weather conditions. Well lit environment and high humidity is favoured by them. So the activity of flies varies daily in accordance with weather conditions. Also, as the control and treatment group of flies are filmed on different days, the data might not be comparable.
2. Digitisation is error prone – both due to human and image analysis errors.
3. Because the flies are wild-caught, with different batches being trapped for each trial, we had very little control over the age and prior environmental exposure of the flies. This introduces uncertainty while comparing control and treatment flies. We tried breeding flies in the lab, but the survival rate was very low and the size of the flies was much smaller. Though, as we were interested in flight behaviour during chases, primarily with what happens in natural environment, the flies were caught from the wild.
4. The current protocol for treatment group of flies has all flies with the halteres ablated, which gives no baseline correction/estimation for how the chase should be. Especially, when considering error angles, as both flies have their halteres ablated, the relative error angles and distances fluctuate.

6.2 Sampling Bias:

Not all chases performed between 10 AM to 3 PM are recorded (as some may go out of frame or miss our observation), and out of the ones recorded only the chases with larger number of turns is selected for. Through this experiment we want to compare flight manoeuvrability in flight and how halteres help in that. This can be corrected by digitising all types of chases we get instead of sampling some. As chase trajectories are not consistent across chases, increase in sample size and random sampling can rectify bias.

6.3 Small Sample Size:

Once the final protocol was set, only a single trial was conducted for control and treatment. Though, it is noteworthy that the flight behaviour seemed approximately the same for all protocols. The solution to this is to increase sample size.

7. Future directions:

1. The number of trials should be increased.
2. For better quantification of instability in flight, we can calculate the angles between the chasing and the leading fly, the trajectory, and the forward and angular speeds. Also, to find the similarities and differences in 2D and 3D chase reconstructions, comparing the parameters tested by Land and Collett in 2D chase plots, we need these angles. These angles should be tested only the section where the chases happen.
3. The next step in the experiment should be to use a dummy fly (a comparable sized metal pendulum) which we control and see how the control and experimental fly chases differ, as this way we can control the motion of the chasing fly.

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APPENDIX 1:

Standardisation of protocol:

Initially the chases were observed for both males and females in the box, when we settled on the male:female ratio of 3:1. The animals were cold anesthetised for collection from the container in which they were caught from the wild, and then again cold anesthetised before the flies were introduced in the main box.

We chose to put forty flies in the box as at this number the number of chases were adequate enough, and the clarity in the view though the camera was not lost.

Though, the frequency of chases is remarkably higher when both male and female flies are there in the box, we could not identify the female flies in high speed videos even when the female flies were marked with white acrylic paint. Hence, only all-male chases have been analysed. This was to avoid any behavioural differences in flight behaviour, which may arise due to male-female interactions and which might not be distinguishable in high speed videos.

The chases were recorded 2 days after the treatment and introduction of flies in the box. For coming down to this conclusion, we observed the activity of flies in the box for days after the introduction in box. The day the flies were introduced, the activity was very low and they groomed for most of the time. The activity increased the next day and the day after that. Though no notable differences in flight behaviour was observed in the two days of observation.

The time of recording of chase was chosen after we had observed the distribution of the number of chases over a day. The number of chases was recorded after every half an hour for five minutes. Also, the *Musca domestica* being a diurnal fly, the chases were recorded in the later morning and afternoon.

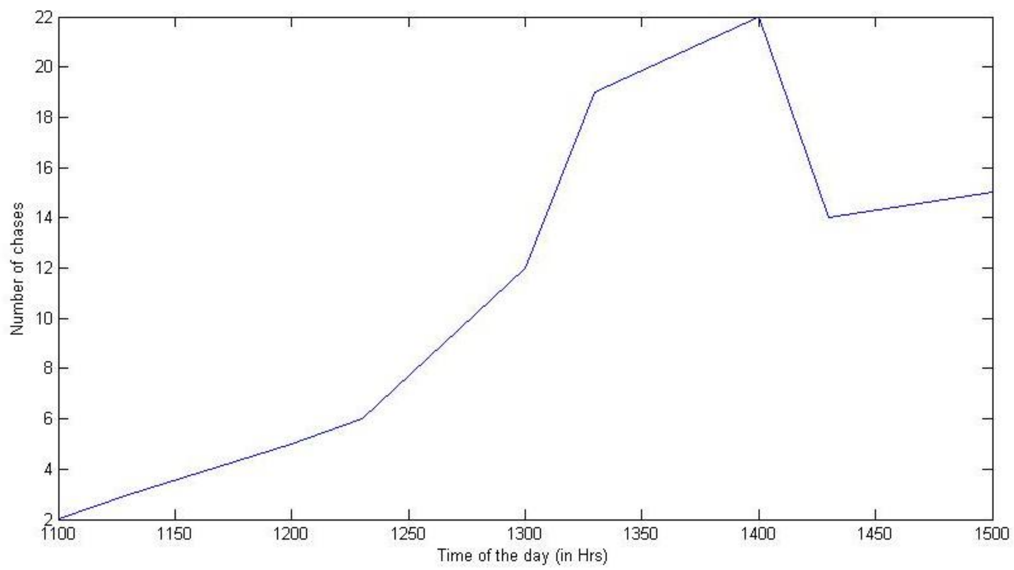

Fig 12) The number of chases per five minutes over the day. The peak of activity in the box is at 2 pm.

After it was established that the chases were highest around 2 PM, we moved on to see how the chases were different for males and females. The females were first marked using nail polish, but the mark was not visible hence we shifted to acrylic white paint which too was not visible in zoomed out high speed videos. Hence we settled on using only all male chases.

Though a major problem remained for the treatment group, in which the mortality rate was really high (50%). The flies were dying after being introduced in observation box and before that as well. To decrease the mortality rate we first increased the gap between the first and second cold anesthetisation, hence delaying the introduction in the observation box. This decreased mortality but not remarkably. Then we skipped the second anesthetisation by potting a side cloth door to introduce flies using smaller containers. This decreased mortality rate to about 10 percent.