Sleep patterns in Generalists and Specialists

Final Project Report

Submitted by:

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Certificate

This is to certify that this dissertation entitled "**Sleeping patterns in Generalists and Specialists**" towards the partial fulfilment of the BS-MS dual degree program at the Indian Institute of Science Education and Research, Pune represents study/work carried out by "Mr. Utkarsh Shrivastava" at "National Centre for Biological Sciences (NCBS), Bangalore" under the supervision of "Dr. Shannon B. Olsson, Associate Professor, Naturalist-Inspired Chemical Ecology (NICE) Lab, NCBS" during the academic year 2019-2020.

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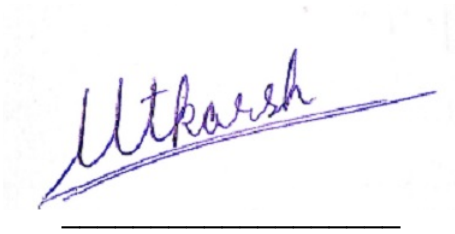
Declaration

I hereby declare that the matter embodied in the report entitled "**Sleeping patterns in Generalists and Specialists**" are the results of the work carried out by me at **Naturalist-Inspired Chemical Ecology (NICE) Lab**, National Centre for Biological Sciences Bangalore, under the supervision of Dr. Shannon B. Olsson and the same has not been submitted elsewhere for any other degree.



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Abstract

Most animals show sleep-like behaviors. The presence of quiescence or a sleep-like state across taxa indicates an early appearance in animal evolution. The amount of sleep required by an animal differs widely between species and can depend on a lot of factors like body size, foraging strategies, diet, as well as external factors. However, for every species, there exist an optimum range of sleep and sleeping pattern, which would be followed by the individuals of the species. In this project, we attempt to provide a correlation between the foraging strategies and sleep, and use a new, robust method for observing sleep in insects. The aim of the project is to study the sleeping patterns of insects, and compare between closely related species that employ generalist and specialist strategies and see if organisms with different lifestyles but similar physiologies sleep differently.

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Acknowledgements

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I would also like to thank Dr. Collins Assisi at IISER Pune for being my guide in the project and helping me with the computational part of the analysis. At last but not the least, I would like to thank my parents for being immensely supportive in all the decisions I have taken, and guiding me whenever I needed, always providing the moral support, and believing in me, even in adverse conditions, when no one else would and I will be forever grateful for that.

Introduction

Sleep is a naturally occurring state of mind and body and exists in most species of animals (2, 3). Why animals sleep is not a completely understood phenomena. However, it is hypothesized to serve many important functions, like consolidation of memory (1, 13), altering brain plasticity (4, 7) and maintaining homeostasis in the body (10). It has also commonly been observed that deprivation of sleep adversely affects learning and consolidation of memory as well as many different activities (6) such as reduced DNA repair (4), disturbed metabolic rates and disruption of synaptic homeostasis (17), and even increased chances of behavioral disorders like ADHD and depression (7).

In terms of ecology, sleep is a costly behavior. It reduces the time available for utilizing resources, searching for mates, and increasing the number of progeny through reproduction. It also makes the animals susceptible to predation during their inactive state, thus potentially lowering their fitness. As a result, it should be an important life strategy for any animal species to maximize the efficiency of sleep while minimizing the time spent sleeping.

Now, given that sleep is important for a proper brain functioning and learning (12), we hypothesize that animals with a lower demand of processing and/or learning and memory should need less sleep compared to similar animals which make much more daily choices during their lifetimes, hence requiring more amounts of learning and memory. According to information processing hypothesis, when making decisions since neural processing has evolved to allow the animal to respond to relevant information in their environment, the amount of processing required by an organism differs with their life histories. For example, for a polyphagous animal, there would be a cost associated with being able to discriminate between and evaluate a large number of host species in terms of requiring more machinery for and a broad sensory capacity combined with an ability to switch attentiveness and an extensive use of learning for choosing among many objects (26). On the other hand, a specialist would require limited amount of information to locate its object of interest, and can have a tradeoff with the neural machinery against efficient decision making.

The extent to which organisms are required to evaluate and retain information about their surroundings should therefore differ with their life histories, including their resource utilization. For example, specialist organisms, such as specialist insects, have innate preferences for specific resources and environments to which they are adapted. Meanwhile, generalists have the ability to survive on different kinds of diets, hosts and varying environments. Hence, compared to specialists, generalists are thought to perform much more decision making over their lifetimes (11). Consequently, they should need to learn and retain a lot more information during their lifetime compared to similar specialist species and therefore require more learning and more memory for these choices. It has also been observed, that this also affects the size of brain and mushroom bodies, and in fact generalist species tend to have bigger mushroom bodies compared to specialist species (12).

Now, since sleep is a costly behavior, we hypothesize that given similar physiological conditions between two animals; if sleep is required for learning, memory and proper decision making, then the amount of sleep required by a specialist should be less than the amount of sleep required by a closely related generalist species. To test this hypothesis, we chose 3 different pairs of closely-related specialist and generalist insects, and compared the periods of inactivity between each pair, as per the definition of sleep in insects (5).

For insects, sleep has been determined as more than 5 minutes of inactivity (15). This 5-minute threshold has been defined based upon observations with regards to the 5 characteristics required by an organism when considering it to be asleep (14). They include:

- Consolidated circadian periods of immobility
- Species specific resting place or posture
- Reversibility to wakefulness
- Increased arousal threshold
- Homeostatic regulatory mechanism

Based on these characteristics, it has been observed, that 5 minutes of inactivity is the minimum amount of time when all 5 criteria of sleep in insects can be met (9, 18).

Materials and Methods

Choice of Animals:

To observe and compare the difference sleep between generalists and specialists, 3 different pairs of insects were chosen. The animal pairs were chosen based upon the following criteria:

- Different degree of specialization regarding their accepted food source
- Relatively close evolutionary relationship
- Similar body size, optimum to be able observe them in the recording setup
- Ease of availability
- Must be able to survive on similar food source and climate

This was done to observe if we see any correlation in sleep and position of insect on the scale of generalists to specialists. The pairings were as follows:

1. *Drosophila melanogaster* vs *Drosophila sechellia*: These species are both part of the *Drosophila* melanogaster clade and are closely related species. Here, *D. melanogaster* is a generalist (19), and can survive and reproduce on nearly all kinds of fruits, and *D. sechellia* is a specialist, and exclusively reproduces on ripe fruit of *Morinda citrifolia* (20).
2. *Bacterocera dorsalis* vs *Rhagoletis pomonella*: These species are both in the Dipteran family Tephritidae. Here, *B. dorsalis* is a relative generalist, with many species of fruits as possible hosts (22). Whereas *R. pomonella* is an extreme specialist and can survive on only a single species of fruit tree (apple or downy hawthorn depending on the race) (21).
3. *Corcyra cephalonica* vs *Galleria mellonella*: These species are both in the Lepidopteran family Pyralidae. Here, *C. cephalonica* is a relative generalist and can survive on dried plant matter and grains (23). On the other hand, *G. mellonella* is a specialist, and only resides inside the walls of hives of honeybees (24), and very specifically feeds on pollen and bee extracts, like honey, wax, and pupal skins.

Insects were maintained in well humidified chambers at 25^{o} C, in 14h/10h day/night cycle and were recorded by keeping in petri plates (Fig.1).

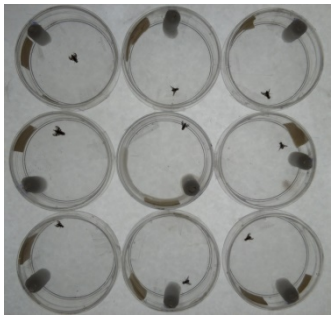


Fig.1: Flies in the Experimental Setup

Choice of monitoring system:

Traditionally, for the study of sleep in flies a Drosophila Activity Monitor (DAM) has been used. However due to limitations in type and amount of data collected, it is not considered an efficient method (25). In recent studies, a camera system was used in coordination with the DAM, which gave the data in the form of position of fly as a percentage of body length. This also felt a little limiting, as the flies were still in the DAM, and had very restricted movement, and it would not have worked for bigger flies without redesigning the setup.

For our experiments, a Raspberry Pi microcomputer with raspberry pi NOIR camera module was used. This was chosen over the Drosophila Activity Monitor (DAM) system due to the higher flexibility, robustness and accuracy of the imaging system over DAM system. Also, the DAM does not detect the minor movements exhibited by the fly and tends to overestimate the amount of sleep in the fly (25). The camera system on the other hand gives a more accurate data about fly sleep and also enables us to dwell deeper into sleep dynamics of the fly and enables us to correlate it with the sleeping pattern of the insect. Also, we kept the insects in the petri plates to enable higher freedom of movement and make it easier to record for flies other than Drosophila as well. The data collected was in the form of a sequence of images, and was analyzed using image analysis software.

Animals were kept in the setup for acclimatization for 1 day and then recorded for 3 days. The data from 3 days was then used to get amount of sleep per day for each insect.

Experimental Setup:

Sleep boxes were made at civil workshop at NCBS to facilitate the easy day/night recording of flies (Fig. 3). A 30cm x 30cm x 30cm box was made out of wood and was painted white on the inside. A transparent acrylic top was made and fixed on the box and covered with a parchment paper to allow diffusion of light. For illumination, electric circuits were made in the electronic workshop at NCBS. 9 white LEDs (1W, 350mA) were connected and fixed to the base of the box. Also, 9 infrared LEDs (100mW, 100mA, 940nm) were connected and fixed on the walls of the box. Both the circuits from both the boxes were then connected to an automated timer, which was responsible for switching between white light and infrared light during day and night at fixed times. The switch happened at 8:00 AM from infrared to white and at 10:00 PM from white to infrared light. The infrared light was used to record during the night without disturbing the day-night cycle of the animals, since it is not visible for these insects.

A metal frame external chamber was covered with black paper to prevent external light from entering the setup (Fig. 2). The sleep boxes were then placed in the chamber and the Raspberry Pi along with the camera were fixed over the setup. The screen, keyboard, mouse and USB drive attached to the Pi, and the camera's focus was adjusted to focus on the petri plates using a small focus adjustment tool. After this was done, the animals were kept in the setup for acclimatization for 24 hours and then recorded for 3 days in the form of time-lapse photography. The frame rate of recordings was kept at 1 frame per 30 seconds.

The animals were kept in petri plates, which were large enough to give the animals a freedom of movement, as well as to be able to record 9 animals per round per setup. Food was provided as a mixture of 50% Brewer's yeast and brown sugar, mixed in distilled water and dried on filter paper. The paper was then cut in smaller pieces, and attached to the walls of the petri plate. In addition, 10% sucrose solution was also given to the flies through cotton plugs (fixed in the petri plate) through holes in the petri plate lids two to three times during the experiment. The petri plates were kept over the illuminated boxes with automated light timings of 14h/10h day/night cycles in congruence with overall rearing regime; i.e. for rearing, a temperature of 25^{o}C, and humidity of 60% to 70% was maintained. For experiments, the

temperature and humidity could not be monitored due to the illumination and water given to the flies, which could potentially change the micro-environments inside the petri plates.

In insects, unlike mammals there are no clear behavioral markers for sleep. Sleep is therefore defined by many criteria, including more than 5 minutes of absolute inactivity (9, 18). Hence for our purpose, lack of any movement (including appendages) for more than 10 frames was counted as sleep.

The experiments were performed in pairs with closely related species being recorded together. This was done to minimize the effect of any other external factors, such as weather or air pressure, that could externally influence sleep. Nine animals per species were recorded each time, and this was replicated 3 times. For the experiments, virgin animals were used of approximately 2-5 days of age at the start of the experiment with approx. equal number of males and females.

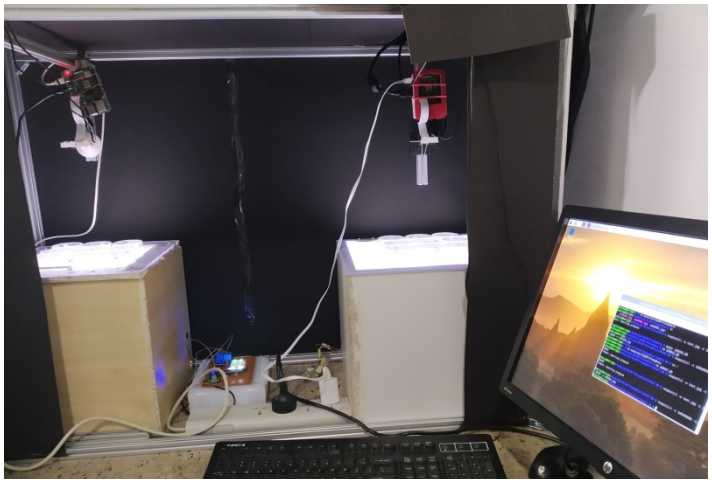


Fig.2: Experimental Setup

Fig.3: Camera Setup

Standardizing the experimental protocol:

For the experiments, multiple food and water trials were performed to optimize the efficient recording of animals. The food choices were kept as such, that the animals' survivability is not affected, can be used for both species of animals being compared, doesn't hinder the recording process, and which not create problems while subtracting background for analysis.

First problem faced was the flies sitting on the walls of the plates, making it harder to detect the animals specially *Drosophila*. This was also problematic, as sometimes, the view of the flies would be obscured due to the walls of the petri dishes. For that we tried giving the flies a rough surface, as flies might have a preference towards them (27). We gave them cotton, tissue paper and filter papers in different shapes to optimize the view for efficient recording of flies (Fig. 4). However, many of these surfaces led to blocking of view of the flies, hampering the data analysis. Moreover, no significant difference was observed due to these surfaces. Hence, not using any such rough surface was decided.

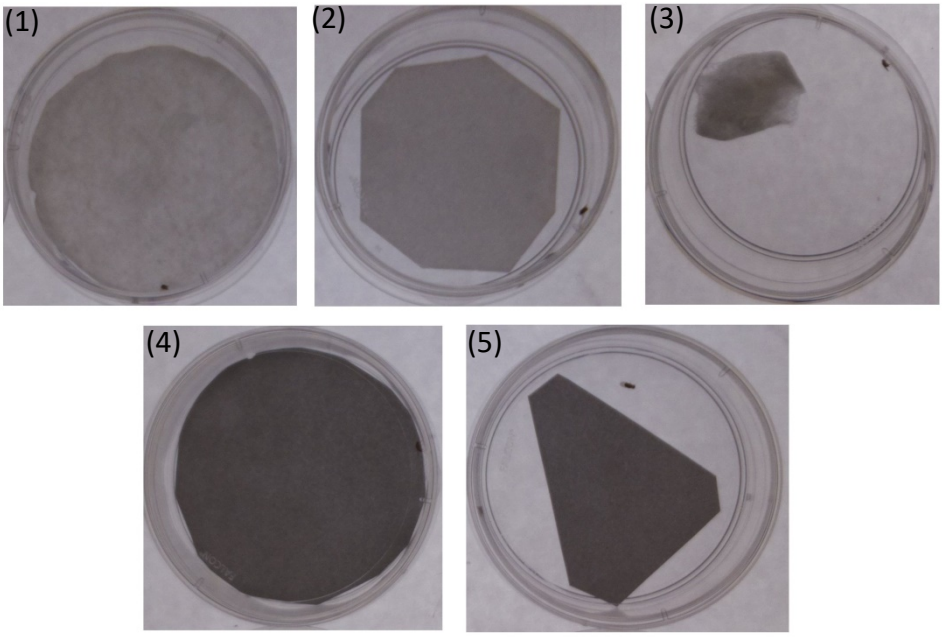


Fig. 4: 1) Tissue paper surface sprayed with water. 2) Dry tissue paper surface. 3) Cotton piece flattened to make a surface. 4) Filter paper sprayed with water. 5) Dry filter paper surface

For the next step, multiple food sources were tried. For that, first, we gave them 10% honey solution soaked on cotton plugs (Fig. 5 (1)). However, the plugs were too big and wet for the flies, and many flies got entangled with the cotton fibers and died. This also was a huge problem for analysis as the software could not detect the flies sitting on the cotton plugs, and the method was abandoned. Next, we gave the flies standard Drosophila fly food (Fig. 5 (2)). But over the course of 4 days, the food dried up and shrank in size, and was problematic for analysis. Also, if the fly sat on the food, it was hard for the software to detect the fly.

Next, a mixture of 50% Brewer's yeast and brown sugar was mixed in distilled water and dried on filter paper, cut in circles and was given to the flies (Fig. 5 (3)). In these experiments, most of the flies were dying before the end of the experiment, supposedly due to lack of water. To overcome this problem, holes were made on the top of petri plates and cotton plugs were cut, soaked in 10% sucrose solution, and placed inside the petri plates. The cotton plugs were cut to the thickness of the petri plates, and fixed under the holes. This way, the sucrose solution could be added many times during the experiment. Also, since the food papers hindered the analysis by making it hard to detect the flies when they stayed over the food, they were glued to the walls of the petri plate for further experiments (Fig. 5 (4)).

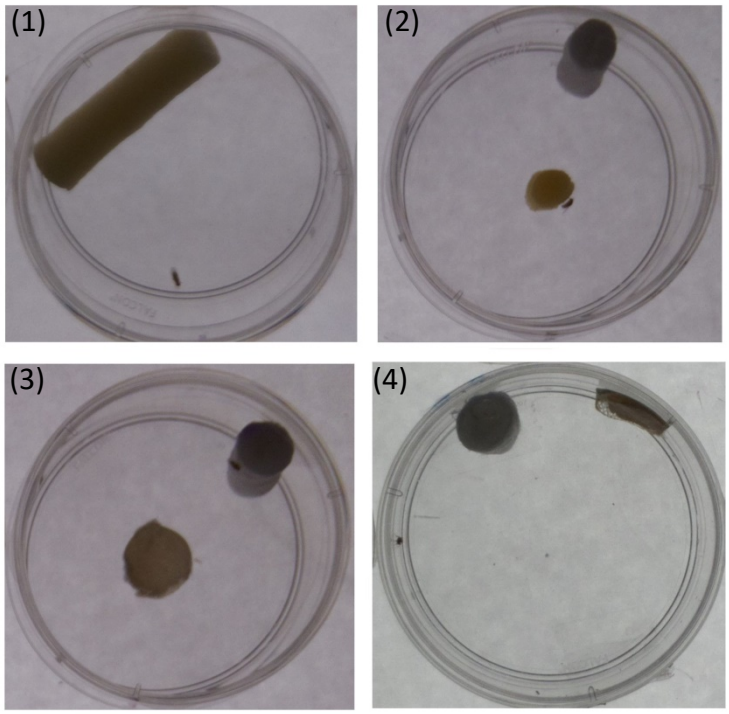


Fig. 5 1) Cotton soaked in 10% honey sol. 2) Drosophila fly food. 3) Dry food on filter paper 4) Final setup with holes on top to refill water

Maintenance of Fly Cultures:

The lab cultures of *Drosophila melanogaster* and *Drosophila sechellia* were obtained from the national fly facility at NCBS, and were maintained in test tube vials along with Drosophila media. The flies were flipped every 4 days into new vials. For the experiment, virgin collections were performed, i.e. flies were separated at the pupal

stage into separate vials. This was to disable egg laying behavior which might affect the recordings, as well as due to the evidence of change in sleeping patterns in female flies post mating (16).

Bacterocera dorsalis fly pupae were obtained as pupae from ICAR – Indian Institute of Horticulture Research, Bangalore. The flies were then maintained in 10h/14h day/night cycle and were provided food in the form of mixture of 50% Brewster's yeast and brown sugar, mixed and dried on filter paper.

Rhagoletis Pomonella fly pupae were imported as pupae from U. Notre Dame, India, where they had been collected from fruit and allowed to pupate in laboratory conditions. The flies were then maintained in 10h/14h day/night cycle and were provided food in the form of mixture of 50% Brewster's yeast and brown sugar, mixed and dried on filter paper.

Corcyra cephalonica and *Galleria mellonella* larvae were obtained as larvae in final instar from National Bureau of Agricultural Insect Resources. The moths were then maintained in 10h/14h day/night cycle and were provided food in the form of 10% honey solution. For the experiment, age of the insects was maintained at 2-5 days.

Choice of analysis software:

Image-J was chosen for analysis, as the data collected was in the form of images, and image-J provides with a good range of tools for data analysis, as well as tracking software (Fig.6). Traditionally, sleep has been measured using motion sensors, where the animals must pass through the light beam to indicate activity. However, this technique is coarse and cannot detect subtle activity such as small appendage movements and grooming. Here, we can visualize the movement in the entire animal, and can pick up subtle appendage movements. This

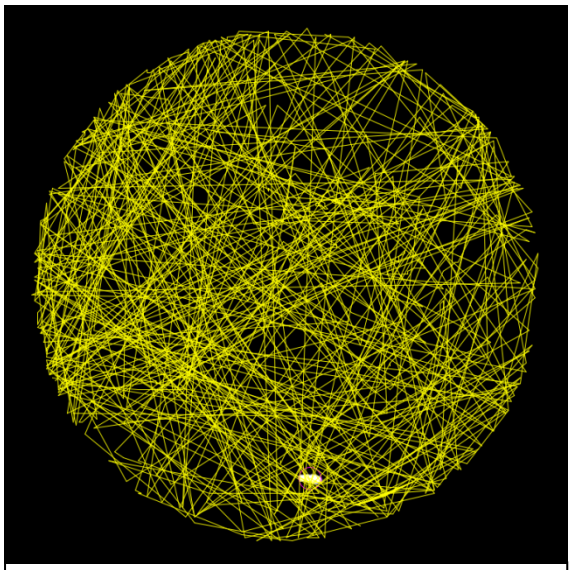


Fig.6: Tracking result of a Drosophila after 8 hr. recording.

is done through getting the mean position of fly in every frame. As such, we can detect short periods of sleep as well. The data can also be exported to an excel file, as well as in the form of XML file, which gives us the flexibility to perform further analysis in python as well.

For the analysis, the data was divided into day and night segments for background subtraction, which were then analyzed using track-mate plugin provided in the FIJI imageJ software.

Data Analysis:

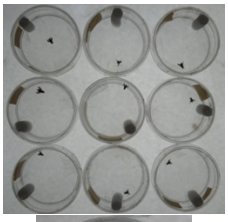
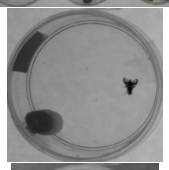
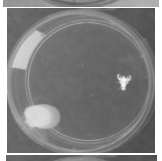
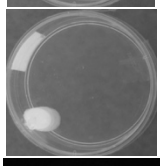
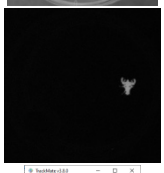
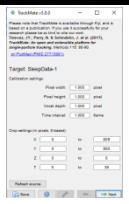
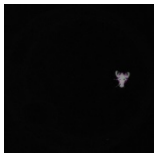
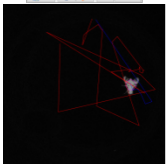
After data collection, position data of flies was extracted using FIJI imageJ software. First, images were divided into smaller stacks for efficient background subtraction. Days and nights were also divided into smaller stacks. Then, the stacks were loaded into FIJI imageJ software, and each petri plate was separated as a stack by cropping from the main stack. Now, background subtraction was done by inverting the images and subtracting the median of the stack (Table 1). Now the fly was visible in the form of a white spot on black background.

Tracking: For tracking of these spots, TrackMate plugin was used. For tracking the position of the flies, based upon the size of the spots, Downsample LOG detector was used, which uses Laplacian of Gaussian method to find the spots based upon their brightness and intensity. Now, the size of spots, intensity threshold, and other parameters, such as position as required by the individual spots, were applied (Table 1), such that only the fly was detected in each image. Then, a Nearest Neighbor Search was done to find the trajectory, and the position data of spots was saved in the form of *x* and *y* positions with respect to time. This was extracted in the form of a table, and saved in the .csv format. Lastly, fly movement was calculated in each file using the formula:

$$d = \sqrt{\left(x_2 - x_1\right)^2 + \left(y_2 - y_1\right)^2}$$

The tables were then collated to make files with position data over the course of three days for each insect.

Tracking protocol:

Step	Action	Supporting Image
1	Load the images as a stack and convert it to 8-bit Greyscale	
2	Crop each petri plate as a stack	
3	Invert image in Edit Tab	
4	In Image Tab, use Z-project to calculate median of the stack	
5	From Process Tab, use Image Calculator to subtract median from the stack	
6	Open Track-mate plugin from Analysis Tab	
7	Select Downsample LOG Detector and set Estimated blob diameter, Threshold and the downsampling factor (=2)	
8	Adjust other filters such that only one spot is selected per frame	
9	Select the nearest neighbor search tracker and set plate size as 800 pixels	

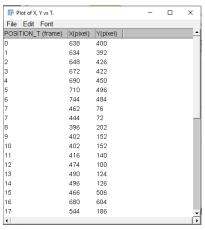
10	Plot X and Y vs T, display data tables, and save the file in .csv format	

Table 1: Analysis Protocol for Tracking of animals through ImageJ

Extraction of data:

A python program was written to import the position data of each fly and plot the movement of animal against time for each insect (For example Fig. 7). “Pandas” library was used for this purpose. For the output, the program gave the total sleep time per day for the flies, number of sleep bouts, length of the bouts, as well as total amount of sleep exhibited by a single insect in 3 days.

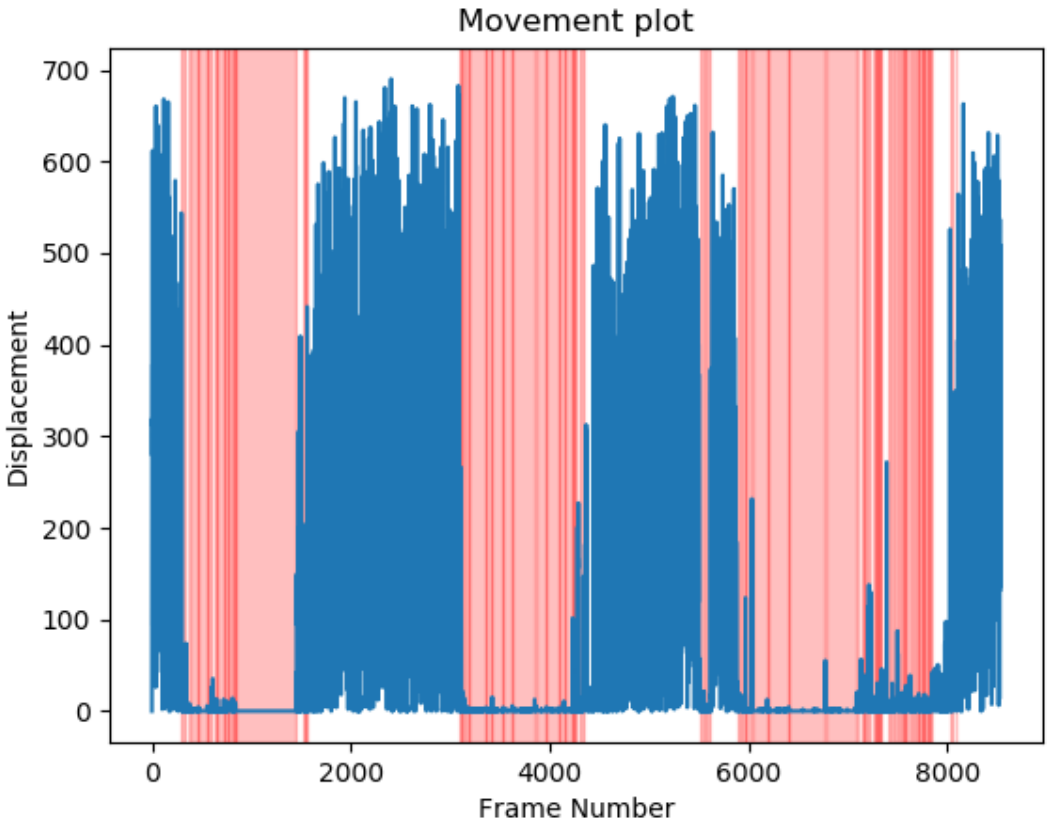


Fig.7: Activity plot of a single *Rhagoletis pomonella* fly. Red regions mark the sleep periods.

The program was written based upon the criteria of sleep, i.e. more than 5 minutes of inactivity was considered as sleep. Another important thing considered while writing the program was the movement threshold. This was important as even in the best recordings, there existed slight anomalies, due to manual errors, inefficient background subtraction, etc. Hence, we needed to optimize the number of pixels to be considered as noise against subtle movements exhibited by the animal which render it not asleep.

After observing and analyzing, a displacement 4-pixel in the mean position of animals was found to be the required threshold for an animal to be considered sleeping. i.e. the movements below 4-pixel threshold are due to errors in data tracking. This is also evident from Fig. 8, the software calculated a difference of 2 pixels between images (1) and (2). As we can see, the position of the fly seems unchanged. However the difference between images (1) and (3) was found to be 4 pixels and the fly has changed its position slightly, marking a break in sleep.

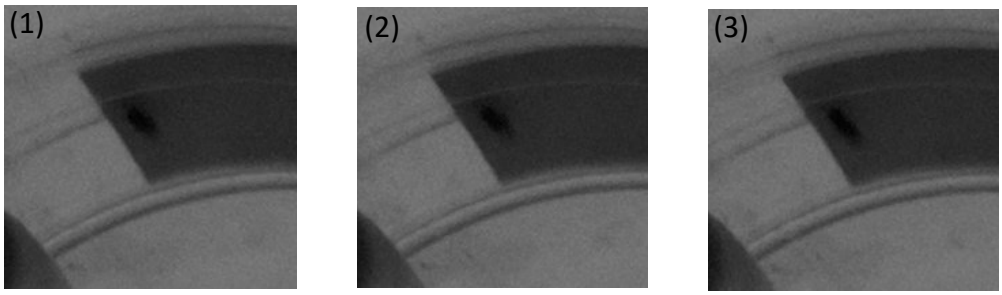


Fig. 8: Threshold visualization in *Drosophila Sechellia*. 1) Reference Fly. 2) Pixel difference detected = 2 pixels. 3) Pixel difference detected = 4 pixels

Similarly in Fig. 9, we can see, that tracking software detected a difference of 4.5 pixels between the mean positions of the fly in images (1) and (2). Upon a closer look, we can clearly see that this is caused due to a Leg extension by the fly in (2), which counts as a break in sleep. Similarly, the difference detected between images (1) and (3) is about 2 pixels. Both these images are from the same sleep bout, and no visible difference can be observed between the images. The difference might have been caused due to slight changes in lighting, or just random error in pixel alignment while clicking the photo.

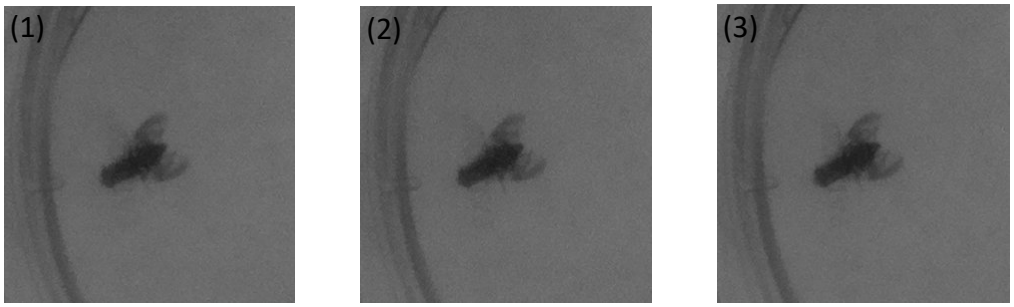


Fig. 9: Threshold visualization in *Rhagoletis Pomonella*. 1) Reference Fly. 2) Pixel difference detected = 4.5 pixels. 3) Pixel difference detected = 2 pixels

The program imports the libraries, using which it reads the data file. From the file, the variables are imported as series. Now a loop runs through each element of the series, and checks if the displacement is less than 4 pixels. If this condition is satisfied, then it checks if it remains consistent for more than 5 minutes. For each time, the conditions are satisfied, the program also counts the number of bouts along with the total sleep time. These results are also put up on a plot, and all the results are displayed at the end of the program. (Fig. B).

Now, after the program was compiled for all the data, results were then loaded on spreadsheets, and analyzed using Microsoft Excel and Graphpad Prism software. A Python program was also written to collate all files containing day and night data into one for each individual insect.

Statistical Analysis:

For the analysis of the data extracted through images, Microsoft Excel and Graph-pad Prism 8 software were used. The datasets for the Tephritidae and Drosophilidae flies were analyzed separately. Kolmogorov-Smirnov test was used to test for normalcy of datasets. Later, student's t-test was used to check the differences between the two species.

Results and Discussion

Due to the excessive time spent in analysis and the experiments, and anomalies in eclosion timings of moths, there was shortage of time, and the moth experiments could not be performed.

To reduce the difference due to environmental conditions, all pairs of insects were recorded simultaneously on two setups, with similar conditions kept in the same room, with cameras focused on the flies.

Tephritidae Flies:

The sleeping pattern of the flies seemed to be neatly arranged with high activity observed during the day, and more periods of inactivity in the night. The sleep data for all the animals of both species was aligned with time and plotted together in a single graph to form consolidated graphs of movement observed with time over the span of three days (Fig. 10, Fig. 11). Our goal was to observe the difference between sleeping patterns of both the species. This included total amount of sleep, number of sleep bouts, average length of sleep bouts, and the timing of bouts to determine if any patterns exist.

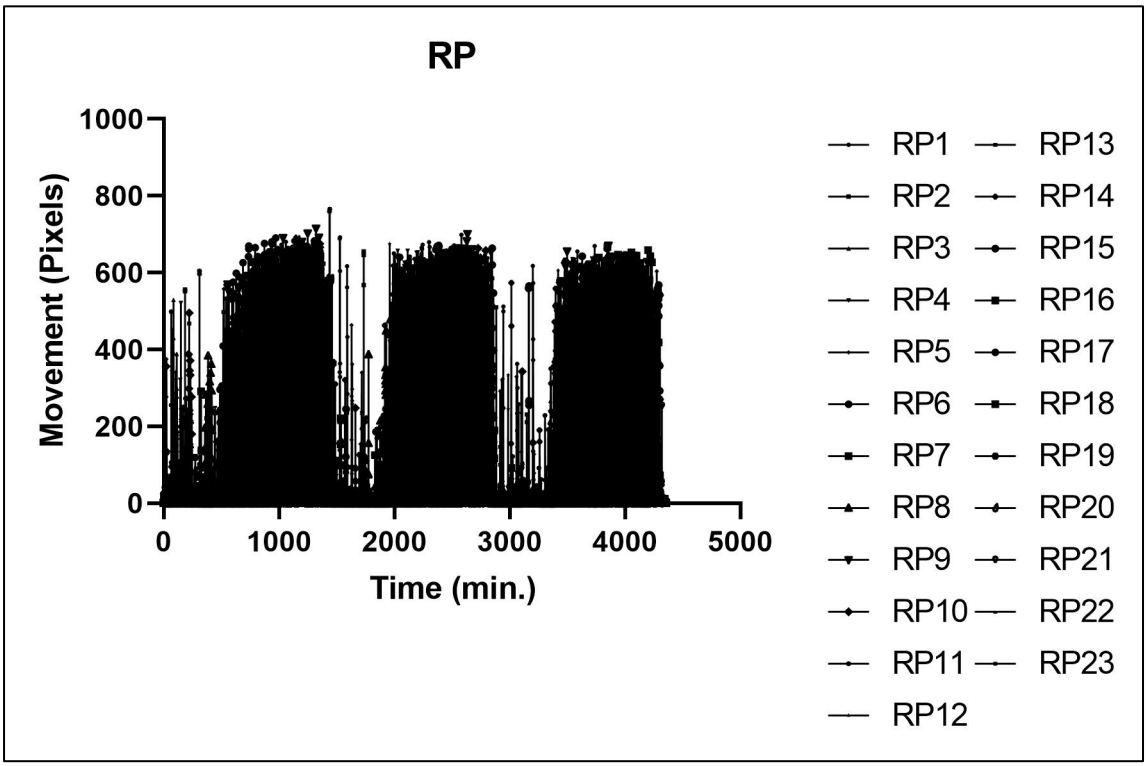


Fig.10: Consolidated activity plot of *Rhagoletis pomonella* recorded every 30 seconds.

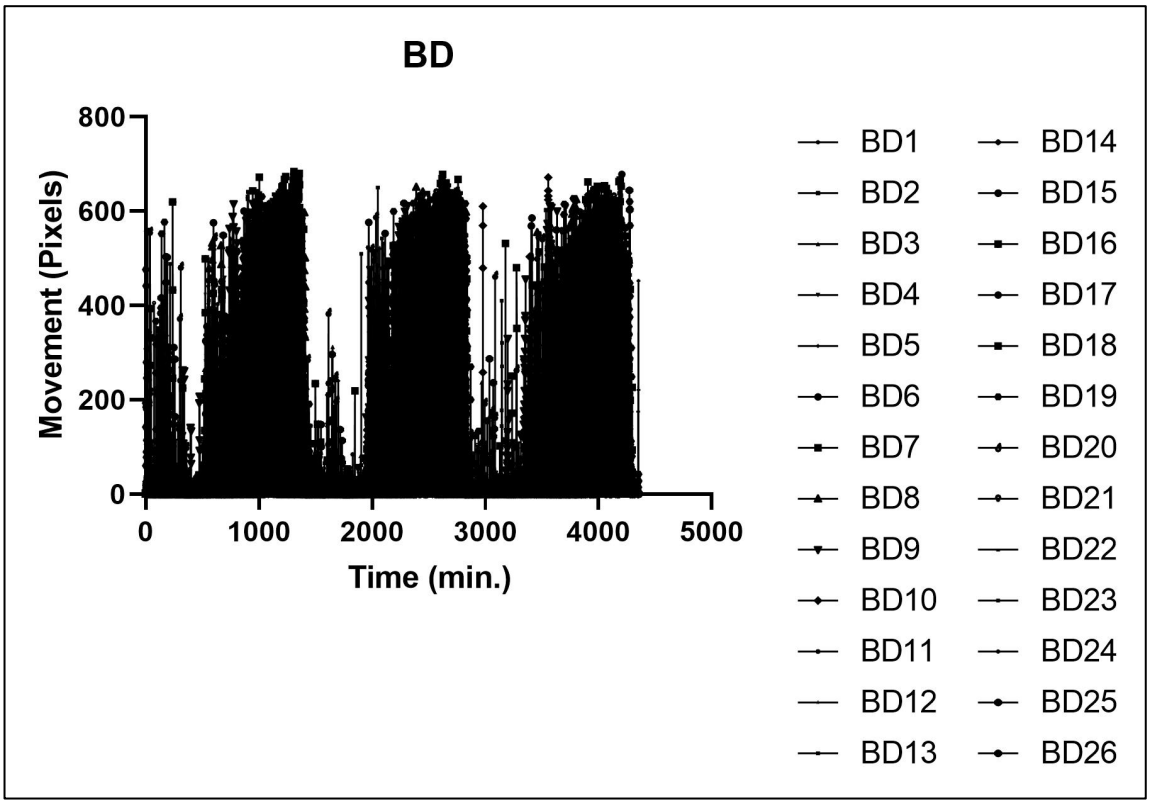


Fig.11: Activity of *Bacterocera dorsalis* recorded every 30 seconds.

Comparing between RP and BD: To check for the Normality of the data, Kolmogorov-Smirnov (KS) test was performed to confirm whether to use parametric or non-parametric tests to analyze the data. The KS test confirmed that the data is in fact normal. Hence, student's t-test was done between the sleep times as listed in the table (Table 2). For the t-test, hypotheses were as follows:

H_0: There is no difference between sleeping times of both the species.

H_1: There is a significant difference between the sleep times of both the species.

S. No.	*Rhagoletis pomonella* (RP)				*Bacterocera dorsalis* (BD)			
	No. of sleep bouts	Total Sleep Time (s)	Sleep per day (hrs.)	Avg. bout Length (min.)	No. of sleep bouts	Total Sleep Time (s)	Sleep per day (hrs.)	Avg. sleep bout Length (min.)
1	67	100590	9.313889	25.02239	98	99390	9.202778	16.90306
2	81	111270	10.30278	22.89506	85	140850	13.04167	27.61765
3	94	112320	10.4	19.91489	138	139980	12.96111	16.9058
4	108	122280	11.32222	18.87037	68	135840	12.57778	33.29412
5	-	-	-	--	97	146460	13.56111	25.16495
6	124	72060	6.672222	9.685484	65	116910	10.825	29.97692
7	65	117540	10.88333	30.13846	105	107250	9.930556	17.02381
8	75	87990	8.147222	19.55333	52	113850	10.54167	36.49038
9	91	115650	10.70833	21.18132	91	110610	10.24167	20.25824
10	97	119160	11.03333	20.47423	84	149250	13.81944	29.6131
11	112	96690	8.952778	14.38839	106	90210	8.352778	14.18396
12	152	128040	11.85556	14.03947	94	145800	13.5	25.85106
13	-	-	-	-	89	138090	12.78611	25.85955
14	-	-	-	-	92	137040	12.68889	24.82609
15	77	36000	3.333333	7.792208	76	135360	12.53333	29.68421
16	-	-	-	-	105	146850	13.59722	23.30952
17	154	102390	9.480556	11.08117	111	153210	14.18611	23.0045
18	117	116100	10.75	16.53846	-	-	-	-
19	93	124470	11.525	22.30645	145	129300	11.97222	14.86207
20	116	109920	10.17778	15.7931	68	93240	8.633333	22.85294
21	130	99210	9.186111	12.71923	129	111720	10.34444	14.43411
22	70	106200	9.833333	25.28571	143	115770	10.71944	13.49301
23	130	116580	10.79444	14.94615	129	128460	11.89444	16.5969
24	107	78180	7.238889	12.17757	106	162960	15.08889	25.62264
25	126	159960	14.81111	21.15873	135	129960	12.03333	16.04444
26	161	110280	10.21111	11.41615	54	108420	10.03889	33.46296
27	110	138420	12.81667	20.97273	149	116520	10.78889	13.03356
Average	106.8261	107882.6	9.98913	17.75439	100.5385	127050	11.76389	22.70652
Standard Deviation	27.90186	24321.1	2.251953	5.657452	28.23222	19342.35	1.790959	6.790513

Table 2: Total sleep time per hour and number of sleep bouts for each individual in RP and BD

The t-test was performed in Microsoft Excel, and the sleep times between RP and BD were found to be significantly different (p = 0.0036). Also, as can be seen from the Table 1, on average *Bacterocera dorsalis* (generalist) seems to be sleeping more than the *Rhagoletis pomonella* (specialist). This can also be seen in Fig. 12 and Fig. 13(A).

Though, no significant differences were found in the number of sleep bouts (p=0.44), the lengths of bouts however were found to be significantly different (p=0.0091). *Baterocera dorsalis* seems to be having longer sleep bouts than *Rhagoletis pomonella* and can be observed in Fig. 13(B).

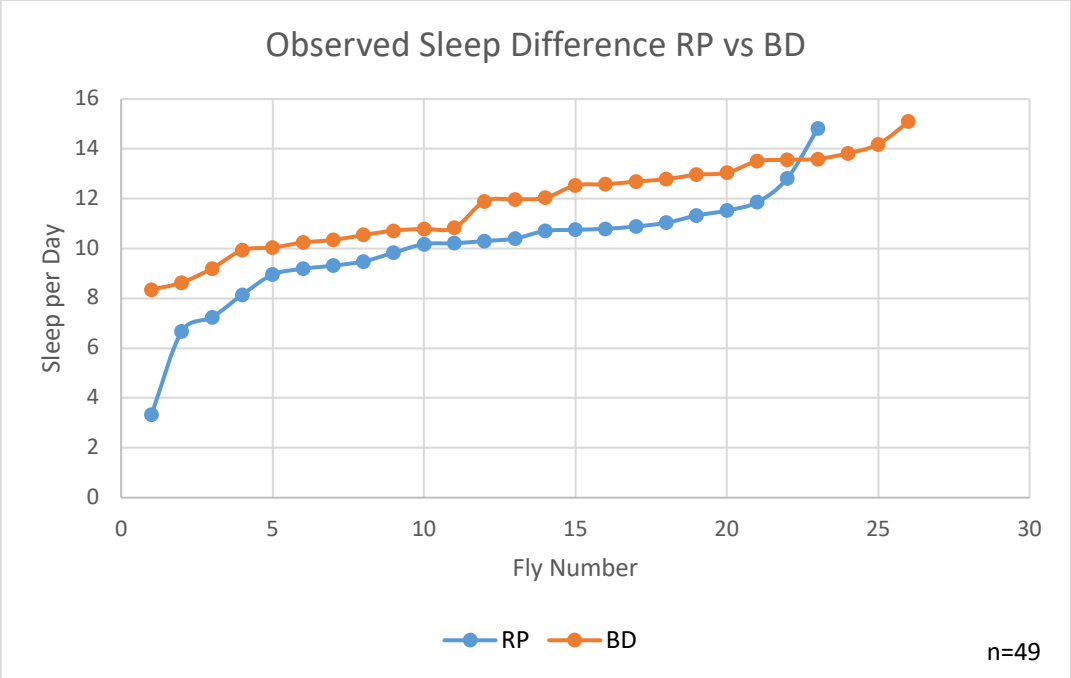


Fig. 12: Sleep times of each individual, which was recorded in the setup. Each point in the graph depicts a single individual's sleep time. All the individuals of each species were arranged in order of increasing sleep times in order to better visualize the difference.

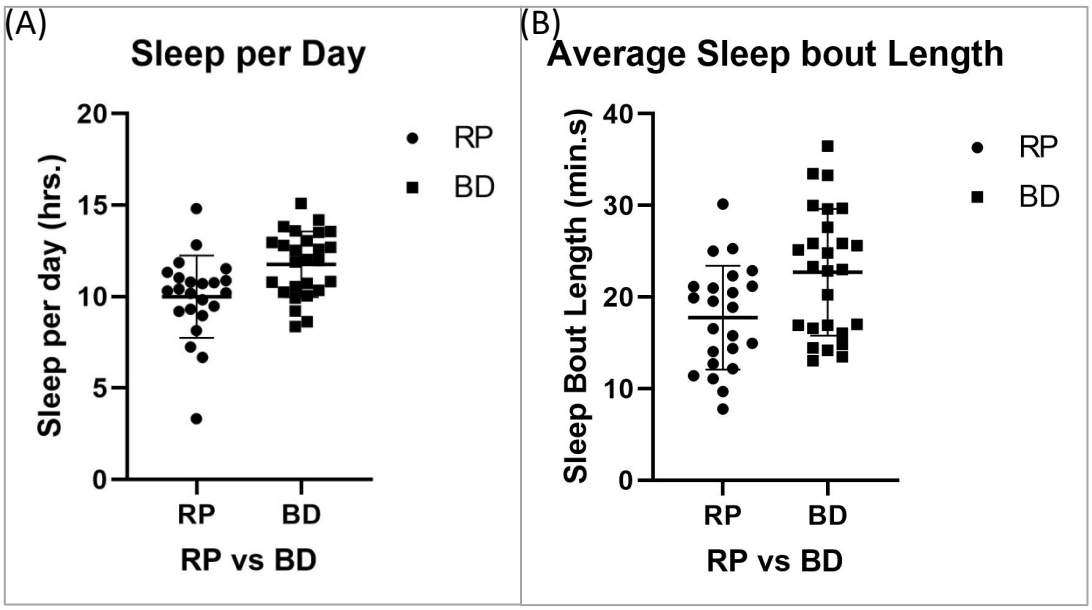


Fig. 13: A) Difference between sleep per day of individuals of both the species.
B) Difference between the lengths of sleep bouts of each species.

Drosophilidae Flies:

Observing sleep in Drosophilidae was much harder than Tephritidae flies. This was due to the smaller size of the flies that produced more noise in the data. The software detected large difference between the total amounts of sleep exhibited by both the species, with *Drosophila sechellia* sleeping more than *Drosophila melanogaster*. However, this could be due to the unrealistically low amount of sleep detected for *Drosophila melanogaster*, which is much lower than what has previously been reported in the literature. This could have been due to numerous reasons, as has been discussed in the discussions section. Also, due to the size of the flies, the data extraction was hindered and during analysis, it was not possible to align the sleeping pattern of the flies in a single graph. Hence, for the Drosophilidae species, we observed differences in amount of sleep and number of sleep bouts, and the average length of sleep bouts.

S. No.	*Drosophila melanogaster* (DM)				*Drosophila sechellia* (DS)			
	No. of sleep bouts	Total Sleep Time (s)	Sleep per day (hrs.)	Avg. bout Length (min.)	No. of sleep bouts	Total Sleep Time (s)	Sleep per day (hrs.)	Avg. sleep bout Length (min.)
1	23	11430	1.058333	8.282609	96	116310	10.76944	20.19271
2	31	34170	3.163889	18.37097	146	134220	12.42778	15.32192
3	81	60060	5.561111	12.35802	94	130410	12.075	23.12234
4	66	66450	6.152778	16.7803	122	104730	9.697222	14.30738
5	51	54510	5.047222	17.81373	106	140940	13.05	22.16038
6	62	45270	4.191667	12.16935	108	162450	15.04167	25.06944
7	50	43500	4.027778	14.5	83	122010	11.29722	24.5
8	32	29430	2.725	15.32813	102	159300	14.75	26.02941
9	47	49860	4.616667	17.68085	101	131910	12.21389	21.76733
10	35	28380	2.627778	13.51429	72	66540	6.161111	15.40278
11	41	36780	3.405556	14.95122	84	119160	11.03333	23.64286
12	35	29580	2.738889	14.08571	66	121740	11.27222	30.74242
13	-	-	-	-	60	88890	8.230556	24.69167
14	3	1680	0.155556	9.333333	83	143640	13.3	28.84337
15	12	8490	0.786111	11.79167	130	96180	8.905556	12.33077
16	12	10050	0.930556	13.95833	78	101520	9.4	21.69231
17	23	35850	3.319444	25.97826	-	-	-	-
18	12	5160	0.477778	7.166667	55	72090	6.675	21.84545
19	75	63780	5.905556	14.17333	113	81960	7.588889	12.0885
20	74	50010	4.630556	11.26351	84	129420	11.98333	25.67857
21	59	56040	5.188889	15.83051	57	74970	6.941667	21.92105
22	18	25020	2.316667	23.16667	75	101640	9.411111	22.58667
23	47	50790	4.702778	18.01064	83	124410	11.51944	24.98193
24	56	60630	5.613889	18.04464	141	151560	14.03333	17.91489
25	-	-	-	-	75	89910	8.325	19.98
26	50	33870	3.136111	11.29	64	61620	5.705556	16.04688
Average	41.45833	37116.25	3.43669	14.82678	91.12	113101.2	10.47233	21.31444
Standard Deviation	22.05325	19540.88	1.80934	4.333568	25.2971	28954.51	2.680974	4.8994

Table 3: Total sleep times per hour and number of sleep bouts for each individual for *Drosophila melanogaster* (DM), and *Drosophila sechellia* (DS).

Comparing between two Drosophilidae species: To check for the Normality of the data, Kolmogorov-Smirnov (KS) test was done. This was done to confirm whether to use parametric or non-parametric tests to analyze the data. The KS test confirmed that the data is in fact normal. Hence, student's t-test was done between the sleep times as listed in the table (Table 3). For the t-test, hypotheses were as follows:

H_0: There is no difference between sleeping times of both the species.

H_1: There is a significant difference between the sleep times of both the species.

The t-test was performed in Microsoft excel, and the sleep times between DM and DS were found to be significantly different ($P<0.05$). However, as can be seen from the Table 3, on an average, the total amount of sleep exhibited by D*rosophila melanogaster* (generalist) seems to be much less than the *Drosophila sechellia* (specialist). This can also be seen in Fig. 14 and Fig. 15(A) along with the differences in other parameters, which can be seen in Fig. 15(B), however due to the anomaly and surprisingly low amount of sleep for DM flies, a presence of error in data collection is suspected and no definitive inferences can be made.

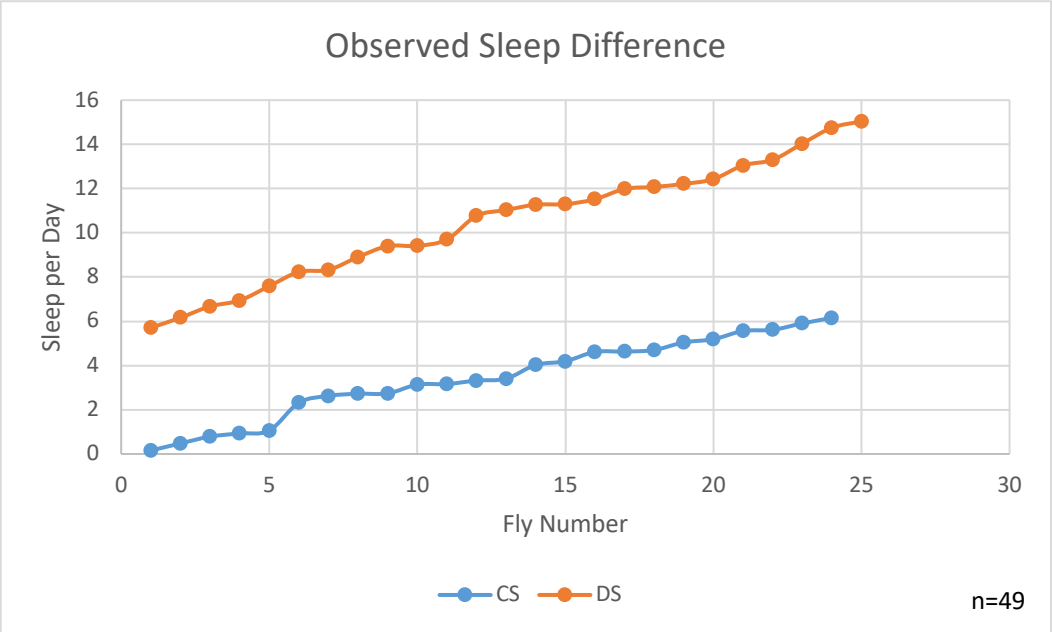


Fig. 14: Sleep times of each individual, which was recorded in the setup. Each point in the graph depicts a single individual's sleep time. All the individuals of each species were arranged in order of increasing sleep times in order to better visualize the difference.

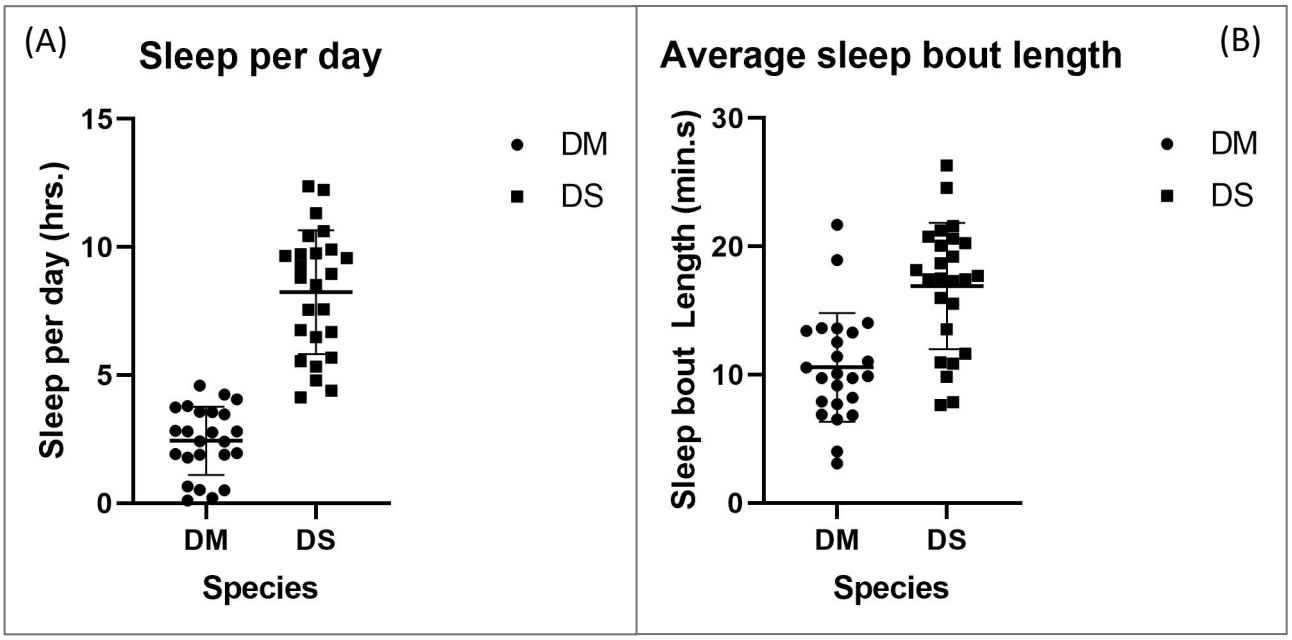


Fig. 15: A) Plot of sleep per day by the individuals of both species.

B) Average length of sleep bout for each individual of both species.

Discussion:

As we can see, Tephritidae flies showed a significant difference (p=0.016) between the amount of sleep needed by both species in accordance with our hypothesis. The *Rhagoletis pomonella* seems to sleeps less compared to the *Bacterocera dorsalis*, and also have shorter sleep bouts. However, the same cannot be said about the *Drosophila* flies. The sleeping pattern of *Drosophila sechellia* was found similar to what has been previously reported (25). However the sleeping pattern of *Drosophila melanogaster* was far from expected. They seemed to be sleeping on an average (3.4 ± 1.8) hours a day which is very different from the known data. This is also very much different from the *Drosophila sechellia*, which is again not expected (p<0.00001).

We suspect that there might be many reasons behind this. We thought that it could be due to the difference between the two setups, since both species were kept on the same setup consistently, and there might be a difference between the lighting systems, especially the infrared, which might cause a temperature difference

between the two setups. However, upon inspection, no difference in illumination or temperature, and humidity of the chambers was found. Also, we considered using only the day-time data to avoid the possible effects of temperature because of possible difference in infrared light intensity. But again similar patterns were observed. The flies were always given the same food, and same 10% sucrose solution at the same times. The other conditions were also kept constant, so the presence of the difference could not have been due to these factors. The pupae were separated at the same times, so the eclosion times were also close. So no difference in external conditions was observed. Since the *Drosophila* experiments were performed multiple times over the span on 4 months, any differences that might have occurred due to possible external weather were rejected.

Next, we thought there might be some problem with the batch of Drosophila that was received. So we changed the flies and did the experiments with a fresh batch. However even this time we got similar results. Then we thought if the flies had received any physical damage, which would be stressful towards them. So instead of using a fly-sucker to pull the flies out, we anesthetized using CO_2 and were transferred carefully into the petri plates. Still, the data we got was consistent, and flies with much lower sleep time i.e. highly active *Drosophila melanogaster* were seen. As of now, we don't know the reason behind the anomaly. Even after giving very similar conditions, food, water, treatment, size of boxes, illumination, and light-control apparatus, still the activity of *Drosophila melanogaster* was much higher than has been previously reported. More experiments need to be done to verify the cause of such difference for example, flies can be mixed on setups to give exactly the same conditions. Experiments can be performed using Drosophila fly food instead of dry diet and sucrose, so that it is closer to what the flies are familiar with, etc.

Multiple sleep bouts were also observed for all the flies during the day. It has been observed that the flies do take an afternoon siesta, sometimes multiple times a day, but no such patterns with regards to siesta timings were seen. The unusually high number of bouts of sleep can be explained evolutionarily, that it would give the flies a much higher alertness and defense against predators compared to a single long bout of deep sleep.

Unfortunately, with the current amount of data, no inferences about the sleeping strategies of generalists and specialists could be made.

Problems Faced:

There were numerous problems faced during the project, which hindered and slowed down the pace substantially.

Eclosion of Flies: The *Rhagoletis* fly pupae were imported from USA, and we had eclosion at onsistent rates. On the other hand, the *Bacterocera* fly pupae were received from ICAR Horticulture institute in Bengaluru. So getting enough eclosion in a limited time, and was quite unpredictable. Consequently, keeping flies at a similar age was a challenge. Since both species of fly do not mature sexually till about 12 days post eclosion, so getting virgin flies was not a hard task.

The *Drosophila* flies were separated in the final pupal stage, so that virgin flies could be used. However, getting sufficient healthy pupae, in enough numbers for both the species was sometimes difficult.

Moth experiments: Moths were obtained from NBAIR institute in the form of larvae in their last instar. However, their eclosion took too much time, and was uneven. The *Galleria* eclosed early, and some moths were of 5 days of age when the *Corcyra* started eclosing. Due to this, we couldn't get enough moths of same age at a time when the experiments needed to be done. Also, there was a huge size difference between the two species, which we had not accounted for previously. The biggest problem however was the petri plated in which the experiments were conducted. The plates were too small for *Galleria*, and were not enough for the moths to exercise free movement. Also, the wings of the moths released the scales, which hindered with proper recording and transparency of the setup.

Ants: Ants were a huge problem for a few rounds of data, as they would be attracted to the 10% honey solution given to the moths, as well as 10% sugar solution given to the flies. Being mobile objects, ants could not be removed from the recordings. Also, they drank the fluids, which are required by the insects in experiment, and might affect their behaviors. Due to this, a few rounds of data had to be discarded and performed again. We used sucrose instead, and being odorless, somewhat solved this problem, but still the data which was already collected with ants needed to be discarded.

Analysis of Data: There were many problems faced while doing the analysis which slowed down the process and made it difficult to analyze the data. The size of the stacks was too large, and loading each dataset would take a lot of time, and many a times lead to crashing of the systems. The background subtraction was not efficient, and it still left a lot of noise, which would be detected by the tracking software. This was especially problematic for the *Drosophila* flies, as the size of noise spots was very much comparable with the flies. Due to this, we had to divide the stacks into smaller stacks, which solved both the problems to a small extent, but in turn led to a huge increase in the time required for analysis.

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