Baffling behavior in the tree cricket *Oecanthus henryi*: how is leaf area measured?

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CERTIFICATE

This is to certify that this dissertation entitled "Baffling behaviour in the tree cricket *Oecanthus henryi*: how is leaf area measured?" towards the partial fulfillment of the BS-MS dual degree program at the Indian Institute of Science Education and Research, Pune represents the research carried out by Harsha K Kumar at IISc, Bangalore under the supervision of Professor Rohini Balakrishnan, Centre for Ecological Sciences (CES), IISc and Dr. Deepak Barua, IISER, Pune during the academic year 2015-2016.

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Declaration

I hereby declare that the matter embodied in the report entitled ""Baffling behavior in the tree cricket *Oecanthus henryi*: how is leaf area measured?"" are the results of the investigations carried out by me at the Centre for Ecological Sciences (CES), IISc Bangalore, under the supervision of Prof. Rohini Balakrishnan and the same has not been submitted elsewhere for any other degree.

Harsha K Kumar

Abstract

Acoustic communication in animals generally serves many purposes including mate attraction and aggression. Males of tree crickets use acoustic communication to attract females. It has been observed that males of the tree cricket species *Oecanthus henryi* sing from leaf edges to attract females from a distance. Males have also been observed making holes in leaves and singing through them to amplify their song. Previous work has shown that *O. henryi* can measure leaf sizes and that they always choose relatively large leaves to make holes on, and sing through them to amplify their sound. Song amplitude gain is known to be much higher when a cricket sings from a hole on a bigger leaf compared to that on a smaller one. With females selectively choosing loud males in the environment, there would be immense pressure on such a behavior to evolve. This thesis attempts to elucidate the mechanisms underlying the leaf area-measuring ability of *O. henryi*. Experiments conducted, indicated that the behavior of individuals on different sized leaves are different. Insects were found not to be using petiole features or hypothetical walks which were correlated well with area. The mean free path of individuals on small leaves was significantly lower than that on large leaves suggesting that it could potentially be used for estimation of leaf areas.

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ACKNOWLEDGMENTS

This thesis was a result of the hard work of many others besides me. It is only just to thank them for their contributions to this thesis. I personally have never liked writing. And if not for people nudging me and keeping on my feet, this thesis would have never come together.

First, I would like to thank Rohini Balakrishnan, my advisor for teaching me to ask the relevant questions in science. She taught me to constantly keep looking at the bigger picture during any study. She taught me to logically connect all the dots to see this bigger picture. The enthusiasm and spirit she has for pursuing science is something that I admire about her. Those are things I will take along with me for the rest of my life. During my stay in her lab, I came to the realization that the entire field of animal behavior and cognition like many others has no immediate goal or purpose to aid or support human life. It is simply for the joy of learning how tiny 'insignificant' creatures in today's concrete jungle lead their lives.

I would like to thank Dr.Deepak Barua for taking interest in my project and suggesting experiments and ways to analyze data though he doesn't particularly work on anything closely related to animal cognition.

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Introduction

Oecanthus henryi is a small tree cricket of the subfamily Oecanthinae (Family Gryllidae). Individuals of this species are 12.4 ± 0.73 mm in length (Metrani and Balakrishnan, 2005) and are green-white in color and have transparent large wings. They are usually found on their host plants *Hyptis suaveolens* and *Muntingia calabura* ; both of which are exotic species (Fig 1.1 a,b) (Deb, 2015).

Figure 1.1a: *Hyptis suaveolens*   Figure 1.1b: *Muntingia calabura*

Photo Credits : Figure 1.1a: https://species.wikimedia.org/wiki/Hyptis_suaveolens

Figure 1.1b: http://foodnsport.com/blog/muntingia-jamfruit.html

The acoustic communication system in this species involves males being stationary and calling on different bushes. Females move from bush to bush and are known to use male calls for three different purposes: a) Identification of con-specific males in the environment; b) For mate choice; c) For localization of calling males.

Calling loudly would increase the chance of a female hearing a male and therefore would increase his chances of mating (If sound is louder at source, sound travels further in space when compared to a fainter sound at source). Therefore, there is evolutionary pressure on males to call loudly.

Adult males of most cricket species have forewings with a stridulatory apparatus and are thus equipped to produce sounds (Forrest, 1982). Forewings have a sclerotized plectrum and a file each (a row of fine teeth which are engaged by the plectrum) (Fig 1.2a, b) (Forrest, 1982; Bennet-Clark, 1989, 1998, 1999). Sound is produced when the plectrum of one wing rubs over the file of the other wing (Forrest, 1982). This mechanical activity causes vibrations of the membranous wings and sound is produced (Forrest, 1982).
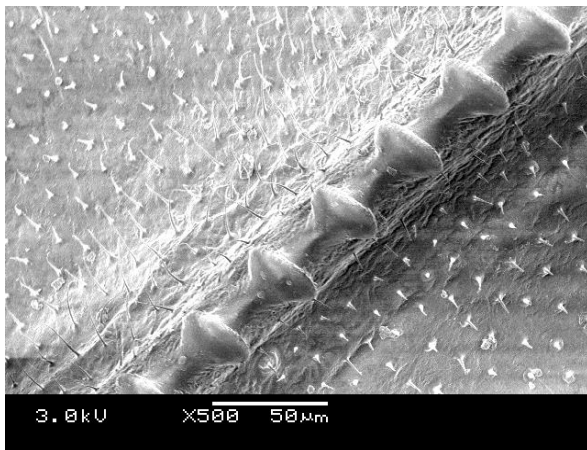

Figure 1.2a. File of *O. henryi*   Figure 1.2b: Plectrum of *O. henryi*
 (Photo Credits- Sapna Metrani)

The vibrating wing membrane (which vibrates to and fro from its natural position) sends out waves of sound which are exactly opposite in phase in opposite directions (away from the head and in the direction of the head) (Forrest, 1991, Bennet-Clark 1970). The vibrating wing membrane compresses the air molecules on one side and relaxes the air on the other side (Fig 1.3). This sets up a small pressure difference across the vibrating wing membrane. The natural tendency of air is to rush back to equalize this pressure difference. The wavelengths of the calls of *Oecanthus henryi* are larger than their wing sizes (*Oecanthus henryi* are 12 mm in length (Metrani and Balakrishnan, 2005) and wavelength of song is 11.5 cm at 25 degrees Celsius) (Forrest, 1991; Bennet-Clark 1970; Deb, 2015). Therefore, there is a destructive interference of sound at the edges of the wings of the insect when the air rushes from one side of the wing to the other to equalize the pressure difference (interfering sound waves are completely out of phase). This causes a loss in acoustic output (Forrest, 1991, Bennet-Clark 1970). This also makes the sound field of the cricket

look dipole like (Fig 1.4). The wings of the cricket insulate the air rushing from one side of the wing to the other but are not large enough in the species to prevent a major loss of sound amplitude.
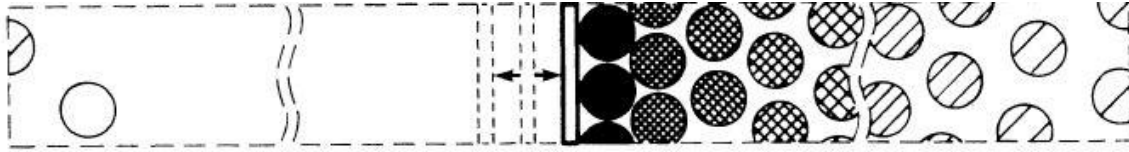
Figure 1.3: This figure shows how a vibrating wing membrane compresses and relaxes air on either sides of it creating a small pressure difference.
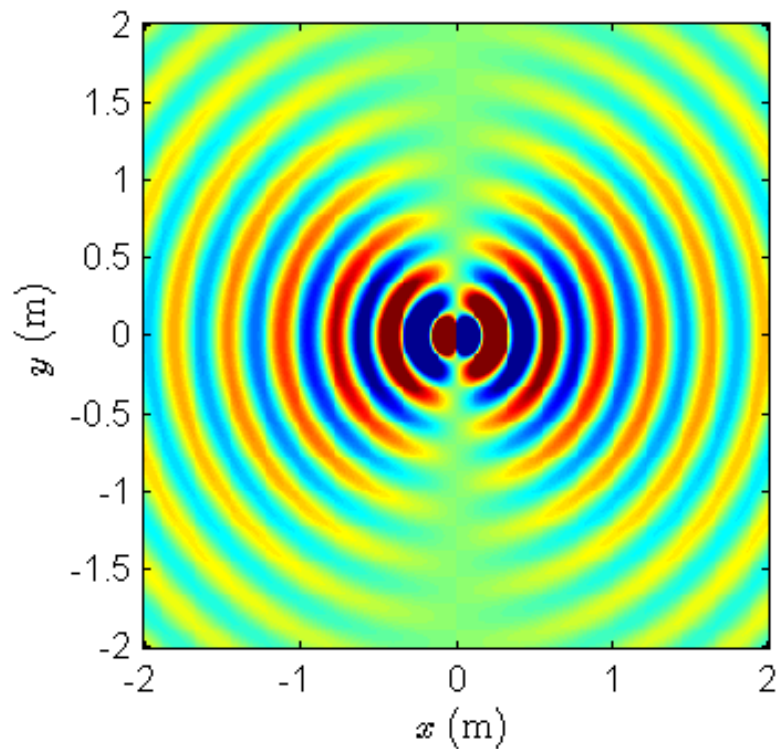
Figure 1.4: An example picture of a dipole source of sound. Shades of red and blue are used to represent the phase of waves; dark red and dark blue being completely opposite in phase. The regions of no red or blue color are where destructive interference has occurred (Credits: http://www.soundfieldsynthesis.org/other-resources/).

As calling loudly increases the probability of females hearing males, there are two strategies that can be employed by male tree crickets to reduce this destructive

interference and make their calls louder: singing at higher frequencies or increasing the area of the sound radiator (use of other objects to extend insulating surface (wing) or a direct increase in wing size) to insulate air from rushing from one side of the wing to the other (Forrest, 1982, Bennet-Clark, 1989, 1998, 1999). Singing at high frequencies reduces the destructive interference as the wavelength of song is reduced. If this wavelength falls below the size of the radiator, there is no destructive interference and thus a louder song results. But high frequencies have the disadvantage that they attenuate quickly over small distances (Forrest, 1982, Bennet-Clark, 1989, 1998, 1999) and therefore reduce the broadcast area of the signal, which will in turn decrease the probability of attracting females.

As this problem of sound loss due to destructive interference is common in crickets; different cricket species have found ways to increase their broadcast area in different ways. The mole crickets and some field crickets use burrows with one or two openings to amplify sound without increasing radiator size (Forrest, 1982). In the scenario where there are two holes, the tunnel simply channels away the two sources of sound in different directions (Forrest, 1982). In the scenario where there is only one hole, the cricket sits exactly at half wavelength distance away from the wall such that the sound waves reflected off the wall and the source of sound away from the wall are in phase (Forrest, 1982). Such a cricket would be amplifying sound in one direction at the cost of broadcasting sound in two directions.

Tree crickets and other crickets that sing on vegetation often sing from the edges of leaves where the edge of one wing is in continuum with the leaf (Forrest, 1982, Prozesky-Schulze et al, 1975). This technically extends the margin of one wing to the extent of the size of the leaf (partial baffle) (Fig 1.5b). *Oecanthus henryi* and *Oecanthus indicus* have been observed to sing from between two leaves by clasping the leaves with their legs (nearly complete baffle; Personal observation; Deb, 2015). *Oecanthus henryi* has also been observed to make a hole (baffle; Fig 1.5a) in the leaf and sing by pressing their wings against it. This amplifies the outputted sound by about 2 to 3 times by making use of a baffle (Prozesky-Schulze et al, 1975; Deb, 2015). The use of a baffle or a partial baffle typically expands the sound field in 3 dimensions and also increases sound amplitude (Forrest, 1982).

Figure 1.5a: Individual singing through a baffle

1.5b: Individual singing from leaf edge

It would be expected that individuals would preferentially choose relatively large leaves to make baffles on as it directly follows from the physics discussed that the sound advantage gained by singing through baffles on big leaves is higher than that obtained by singing through baffles on small leaves. This is because the insulation achieved is much higher when larger leaves are used (Bennet-Clark, 1989, 1998, 1999). Theoretically, sound amplification would be the largest when sung through a hole on an infinite plane.

In *Oecanthus henryi*, the frequency of baffling behavior was observed to increase with increasing leaf size as expected (Deb, 2015: No choice experiment). When given a choice to make baffles from small (4.5 ± 0.2 cm in length and 3.5 ± 0.1 cm in breadth) and large leaves (11 ± 0.4 cm in length and 9 ± 0.2 cm in breadth), all individuals chose to make baffles on large leaves (N=15, Deb, 2015). This suggests that *O. henryi* males are able to distinguish between small and large leaves for the purpose of baffling.

This raises a particularly interesting question on how nocturnal insects such as *Oecanthus henryi* measure leaf areas to make ecologically relevant decisions. There exist only a handful of papers in the field of animal cognition, which go on to

elucidate mechanisms of decision-making in the context of measuring the areas/volumes of ecologically relevant structures such as leaves, burrows and shelters by insects.

All mechanisms elucidated so far for measuring area (Mallon et al, 2015) and volumes (Seeley, 1977) in insects are dependent on measurement of distances.

Hypotheses

The following are a few plausible hypotheses of how the insects could be measuring leaf areas.

A) Petiole features that are well correlated with leaf area (as shown in *Tabebuia aurea and Tabebuia impetiginosa:* Capuzzo *et al*. 2012*)* could in principle be used.

B) Walking along paths that are informative of leaf area (where path length is correlated with leaf area); See materials and methods : Part B (b)).

C) Mean free path hypothesis: The mean free path algorithm calculates area of the leaf as a function of mean distance between collisions with leaf boundaries. The mean free path was shown to be correlated with area in some studies (Wittilinger et al. 2007).

D) Buffon's needle algorithm: The area of the leaf can be best calculated as a function of the number of intersections between a randomly laid trail path and a random survey path (to reduce variance in estimation of leaf area); provided the lengths of both walking paths are remembered (Mallon et al. 2015).

E) Acoustic Feedback – A cricket in theory could estimate leaf area by listening to its own call, which is amplified when sung at a leaf edge (partial baffle). In theory, the amplitude gain must be proportional to leaf area (Bennet-Clark, 1989, 1998, 1999).

This thesis goes on to describe and compare the activity of individuals on big and small leaves to see if certain activities precede the activity of baffling, thus suggesting mechanisms for area measurement. It also tries to test the hypotheses A, B and C.

Materials and Methods

<u>General</u>

<u>Note : Big and large leaves have been used interchangeably in this thesis.</u>

a) Collection of individuals: Individual adult males of *Oecanthus henryi* were collected from Ullodu (Chikballapur district, Karnataka, India) from the open fields of *Hyptis suaveolens .*

b) Animal maintenance: Individuals were housed in small circular plastic boxes of height 4 cm (lid closed) and diameter 6.5 cm. Holes were made in the lid of these boxes for free exchange of air. The crickets were fed *ad libitum* with dog food (Pedigree) and a moistened piece of cotton (Deb, 2015). During the months of September and November, the crickets were given folds of tissue paper to protect themselves from the cold.

c) Experimental setup: The experimental setup included a dried, cut stem of *H. suaveolens* standing on a base made of themocol in an anechoic room. The free end of the stem was split. A leaf was wedged in this split and wrapped around with moist cotton to keep the leaf from wilting during the course of the experiment (Deb, 2015). The insect was released at the base of the stem with the help of a stick. Videos of the entire apparatus were captured for analysis. Sometimes, the insect flew directly onto the leaf without walking on the stem or the petiole. Such trials have also been considered, as these insects are known to use flight over short distances to move between plants.

d) Video capture – All videos were recorded at 25 frames per second (fps) with two tripod mounted cameras (Sony, Model No. HDR-XR500E and Sony, Model No. DCR-SR65E) capturing the upper-side and under-side of the leaf respectively. On 3 days, a Sony Model No DCRA-C152 was used instead of Sony Model No HDR-XR500E. The inbuilt sources of infrared light on the cameras were adjusted such that the distortion of the shadow of the insect on the leaf was as little as possible on the camera recording the upper-side of the leaf. The recordings from the camera facing

the upper-side of the leaf were used to trace the entire path of the insect. The camera facing the underside of the leaf was simply used as another source of infrared light to illuminate the leaf and also served as back-up data if something was not clearly visible on the upper-side of the leaf. By default, Sony Model No HDR-XR500E always captured the upper-side of the leaves.

e) Classifying behaviors performed on the leaf by male individuals of *O. henryi*: The behaviors performed on the leaf were categorized and analyzed as the time spent on the leaf doing that particular activity. The individuals released on the stem usually got onto the leaf eventually and explored it. They did various things on the leaf and finally either made a baffle or not within the observation time of 10 hrs. The possibility of a few individuals making baffles after a span of 10 hours cannot be excluded, as there are no data to suggest otherwise.
The list of behaviors observed included:

Walking and resting on stem (Stem) – This is the time spent on things other than the leaf and its petiole such as the stem, the cotton and the thermocol base.

Walking on Petiole (PW) – This is time spent by the insect walking on the petiole. This has been characterized as a different behavior as the insect could potentially estimate leaf area based on petiole features if they were correlated with area.

Walking on leaf (Walking) – This is the time spent walking on the leaf. There is evidence to suggest that insects such as ants walk to estimate nest sizes. Hence it has been classified as a different behavior.

Singing at leaf edge (SALE) – This is the time spent singing at the leaf edge (partial baffle).

Singing at places other than the leaf edge (SNALE) – This is the time spent on the leaf singing at places other than the leaf edge.

Walking and singing (WS) – This is the time spent on the leaf singing and walking simultaneously. These singing behaviors are classified separately as individuals could be using auditory feedback to measure leaf areas.

Resting and grooming (Resting) – This is the time spent in resting on the leaf and grooming on the leaf. It has been represented as one unit under the name 'Resting' as it is not likely to provide any information about the area of the leaf anyway.

Making baffle (MB) – This comprises the activity of making the baffle.

Singing through the baffle continuously for a period of 20 min has been used as an indicator to suggest the end of making baffle. During the activity of chewing the leaf to make a baffle, individuals have been observed to intermittently 'sing close to the hole (SNH)' and 'sing and make the baffle simultaneously (SMB)'. For all practical purposes, both these behaviors SNH and SMB have been treated as a part of baffle making as it is hypothesized that these behaviors might be necessary for building a 'good' baffle.

Stem to leaf (STOL) – This is the activity of a jump from stem to leaf without walking on the petiole.

Jump – This is the activity of jumping off the setup after having explored and walked on the leaf at least once.

Eating- This activity involves the chewing of the leaf by individuals without singing through the hole.

We believe that the entire time duration just before singing through the self- made baffle is spent in making three decisions; **whether** to make a baffle or not on that particular leaf, **where** to make the baffle on the leaf and **how large** to make the baffle to ensure maximum possible acoustic output.

The time spent on the leaves can be broadly classified into two phases: **'decision-making'** and **'baffle-making'** (if the decision is to make a baffle). 'Decision making' refers to the time taken by the insect till it initiates making a baffle. This could include the time taken to decide if a baffle should be made and also where to make the baffle on the leaf.

All experiments carried out were done so with two clear logical questions in mind:

1) Are behavioral repertoires on large and small leaves different? Do these provide clues as to how leaf area is being measured?
2) Are there ways in which *O.henryi* could potentially measure leaf area? Are they using those methods?

PART A) – Activity Budgets

A) Average Activity budgets: The time spent on different activities by different individuals were pooled and plotted as a proportion of the total time spent to an idea of the average population behavior on small and large leaves.

B) Multivariate overall comparison of behaviors on big and small leaves and supervised clustering:

As the important behavior is baffle making and how individuals make this decision of whether to make the baffle, the end of a trial was decided accordingly. The end of a trial was considered as the completion of making a baffle or jump off the setup (no interest in making baffle). As a result of this end of trial criterion, most individuals on small leaves were observed for a maximum period of 8hrs.
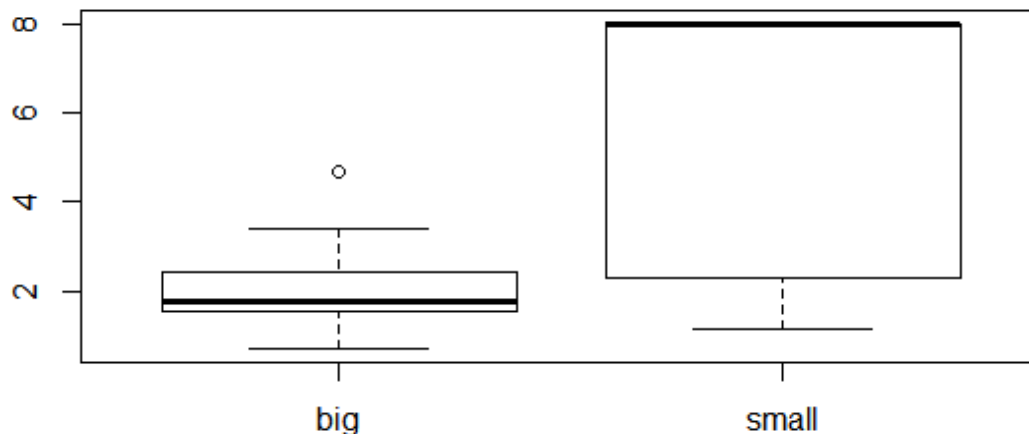

Figure 2.1: Boxplot showing time durations of observations in **hours** on large and small leaves.

As the time of observation of individuals on small and large leaves was different, this had to be normalized for; to be able to perform meaningful comparison between small and large leaves. This was done in two different ways:

a) Absolute time data – This was done to make trial lengths on small and large leaves comparable. End of trial: Completion of making a baffle, jump or 9000s (2.5hrs).

b) Proportion time data – Proportion of time spent on different activities were used for comparisons here.

As the data gathered were multivariate and numerical (Column 1 – Activity, Column 2- Time spent), a multivariate analysis was performed to investigate if behaviors performed on big leaves are overall statistically different from behaviors performed on small leaves.

The MVN (Multivariate normality) package in R (Korkmaz et al , 2015) was used for exploratory data analysis . Three tests of normality; Mardia's test, Henze- Zirkler's test and Royston's test were used.

Absolute time data - All three tests suggested that the dataset of absolute time was not multivariate normal (p value skew = 0.0002, p value kurtosis = 0.4379, P value small <0.001; HZ= 1.1436 , p value =0; H = 190.62, p value < 0.001).
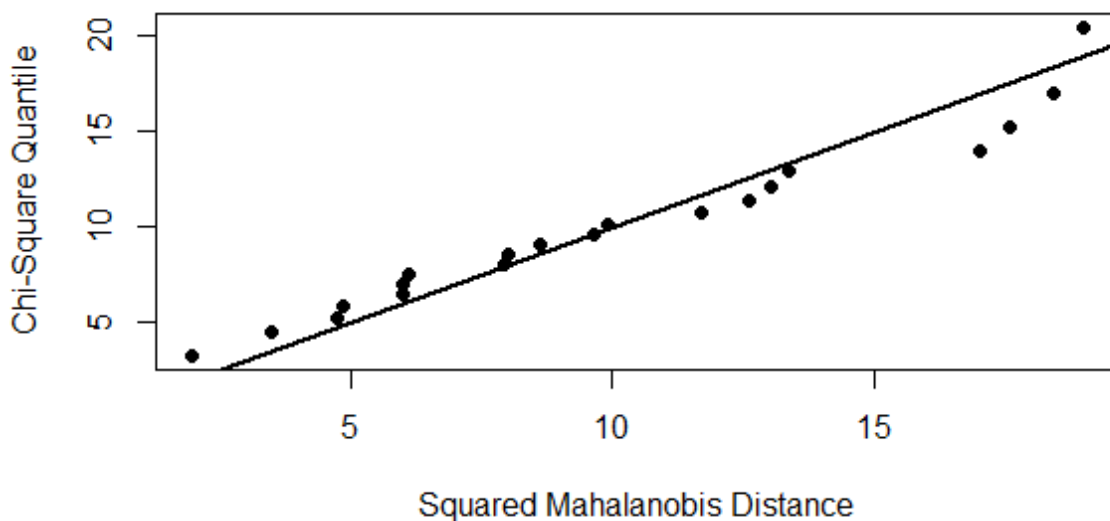The Q-Q plot suggested a deviation from multivariate normality (Fig 2.2).

Figure 2.2: A Q-Q plot showing deviation of data from multi-variate normality.

Uni-plots were created to check the time spent on which behaviors followed normality individually and which ones did not. It was found that time spent on most behaviors had skewed normal distributions.

Proportion time data- Tests for multivariate normality were not carried out, as the tests were sensitive to zeros (singularity) that were found in proportion data.

A **factor analysis** was carried out on the **absolute time data**. PCA is known to be more robust at preserving distances between points whereas factor analysis is known to be robust at preserving correlations (DeCoster, 1998). Since the data collected was well correlated (behaviors transitioned back to each other with high probabilities), a factor analysis was carried out.

A **factor analysis was not** carried out on **proportion time data** as the system was found to be singular (many zeros). Therefore, a **PCA** was carried out on **proportion time data,** as PCA is less sensitive to zeros in the data.

Variables in both cases were not normalized as they were on the same scale of measurement (seconds). Scree plots and Eigen value decomposition of the correlation matrix (Horn's analysis) was used to estimate the number of factors and components needed to explain the data satisfactorily.

A supervised clustering analysis was done on R using the K-means algorithm with a randomly set seed for 1000 iterations. This was done to see if individuals on big leaves clustered separately from individuals on small leaves.

C) Activity of *O. henryi* on large and small leaves: Variation in proportion of time spent on different behaviors and the total duration of time spent on each behavior was analyzed to identify behaviors that might be useful in measuring leaf area.

D) Transition probability charts: The aim of this analysis was to identify behaviors, which transitioned to baffle making with high probabilities. The calculation of these probabilities was done on Libre Office using the 'Sumif' function.

e) Testing sub-hypotheses –
A trial time of 100 minutes was chosen to test our hypotheses as 14 out of 17 individuals on large leaves had already started making the baffle (decision taken) within this time.

a) Do individuals on small leaves sing for longer than individuals on large leaves in the first 100 minutes?

This tests the assumption that the activity of 'decision making' is costly and that time spent in 'decision making', could have been spent singing on leaves.

b) Do individuals on large leaves also walk for longer in the first 100 minutes?

This tests the assumption that individuals on large leaves walk more simply because there is more area. However it is possible that individuals on small leaves walk the same small paths over and over again.

A Wilcoxon unpaired rank sum test was used to test for differences between these behaviors on large and small leaves in the first 100 minutes after release of the insect on the setup.

Part B) – Hypotheses related to walking

a) Are petiole features correlated with leaf area?

Petiole features could be correlated with leaf area and hence might provide clues about the area of the leaf to the insect. To test this, leaves were sampled (N=43) from different *H. suaveolens* plants in IISc, Bangalore. As petiole length is known to be controlled by the shade avoidance response in plants, sampling was done adequately at all heights. The following petiole features were measured in these leaves: petiole length (full length of the petiole), petiole thickness at the node (point of attachment of leaf to the stem; petiole thickness 1) and at the point where the leaf blade begins (identified by viewing the underside of the leaf; petiole thickness 2).

Petiole length and thickness were found to be normally distributed using Shapiro's normality test. The p-values for all three variables were significantly higher than 0.05 indicating normality. Hence, a Pearson's correlation test was performed to investigate the association between the petiole features and leaf area.

b) What hypothetical walks are good indicators of area?

On the same set of leaves that were used for the petiole features experiment described above, (N=43), a measurement experiment was done. The leaf

measurement experiment tries to assess the information obtained about leaf area by crickets when they walk different paths on a leaf. This experiment assumes the measurement of distance walked by some means (see discussion for different ways in which measurement of distances in insects has been known to occur). If a walking strategy is being used to compare leaf sizes, the insect could trace out the same path on different leaves to estimate and compare the computed leaf areas to make decisions. Therefore, all paths considered for this experiment were simple in the sense that the chosen paths could be replicated fairly identically on different sized leaves given the natural variation in leaves.
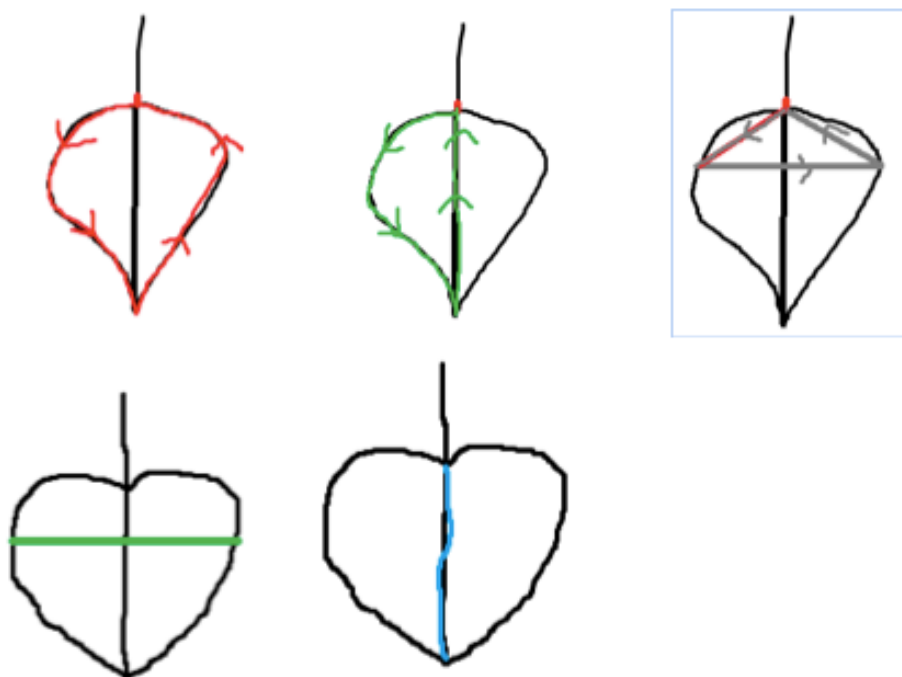

Figure 2.3: Different theoretical walks by insects on leaves. From top left to bottom right; Full perimeter, half perimeter, triangle (One arm of the triangle is the breadth of the leaf), breadth (widest points apart on the leaf) and length walks.

All path lengths (variables) were tested for normality using Shapiro Wilk's test. Leaf length was the only variable found to be not normal. A Pearson correlation test was done on all the normal variables and a Spearman correlation test was done on the single non-normal variable.

c) <u>What do actual paths look like?</u> – The intent of this analysis was to find out if insects were using any hypothetical walks, which were well correlated with area, for area estimation. Tracker (copyright Douglas Adams; Version 4.87) was used to track the movement of individuals on the leaves. Tracker allows the user to set an origin on the video clip and allows for tracking objects as point masses, vector and based on RGB color spaces. Tracker also provides for Auto-tracking objects. The program generates coordinates of the object being tracked as per the specified scale which can copied as a table onto an excel sheet. To generate raw data for Tracker to track objects on, the entire duration of recording was broken down into walking and stationary phases. Only the walking phases were tracked using the software, as tracking insects through the entire duration of experiment was computationally expensive. Walking bouts with less than 5 second intervals were considered as a single walking phase. If the stationary interval was more than 5 seconds, the next walking bout was considered as a separate path. The tip of head of the insect was tracked as a point particle using the auto-track feature. After the auto-tracker had finished tracking the insect, the video was scanned again manually for mistakes if any that might have occurred during the tracking. If a mistake was found, it was corrected manually by using the cursor click. The coordinates obtained from the software were saved as comma delimited files (.csv) and were used to plot the paths in R using the 'plot' function and the 'add' feature. The paths were color coded to indicate the temporal order of occurrence.

d) <u>Can insects use the mean free path algorithm to estimate leaf area?</u> - The mean free path algorithm calculates area as a function of average distance traveled between two collisions with a boundary (leaf boundary). The hypothesis states that leaves with larger area will have longer mean free paths (average distance; adapted from Mallon et al, 2015).

The boundary points (where the insect met the leaf boundary) were manually located on Tracker files (1/5s time resolution). The first head movement away from the boundary was considered to be the start of the path. The first head movement on arrival near the boundary was considered the end of the path. The distance covered by a path was measured as the sum of Euclidean distances between coordinates visited on that particular path. The average of these distances is the mean free path.

The mean free-hypothesis was tested to see if *O. henryi* could use it to measure area of leaves. The aim of this experiment was to experimentally measure mean free-path of different individuals on different leaves to check if mean free path was larger on large leaves when compared to small ones like Mallon et al suggested (Nl = 8, Ns= 8).

Results

Part A) - Activity Budgets

A) Average Activity budgets:

The average time spent on behaviors by individuals on small and large leaves were investigated (Fig 3.1). It was found that the average proportion of time spent walking and resting was comparable between small and large leaves. Singing at leaf edge (SALE) was found to be represented at high proportions on both small and large leaves (78% and 43% respectively). The behavior 'making baffle' consumed an average of 28% of the trial duration on large leaves. This is a behavior that was never observed on small leaves in time course of the experiment. The behaviors STOL, Jump and eating were observed only small leaves.
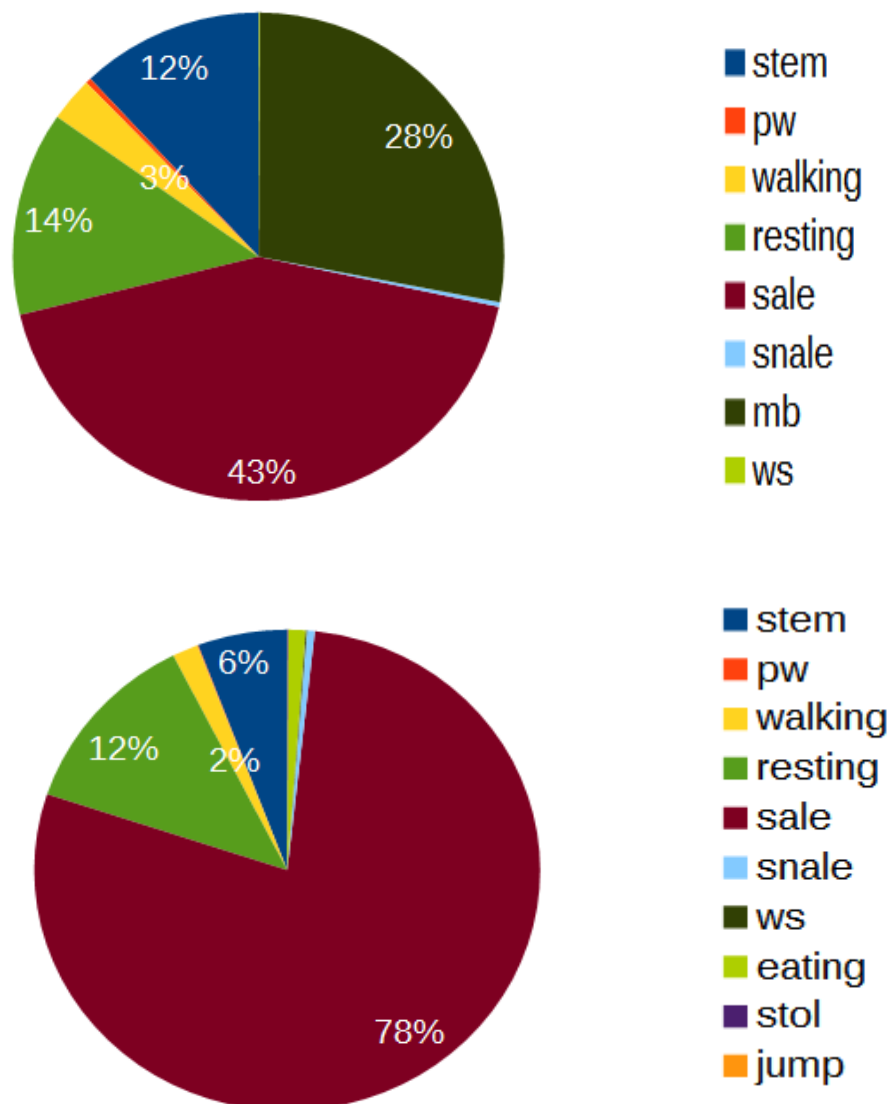

Figure 3.1: Average activity budgets of individuals on large and small leaves in order

As there seemed to be a fairly large difference in averages of the behavior SALE (Singing at leaf edge), the hypothesis that individuals on small leaves sing for a larger proportion of time was tested (Fig 3.2). It was found that the proportion of time spent singing on small leaves was not different from that on large leaves (W = 72, p-value = 0.0514). As the P-value is very close to 0.05, it is likely that this result will change if more individuals are sampled.
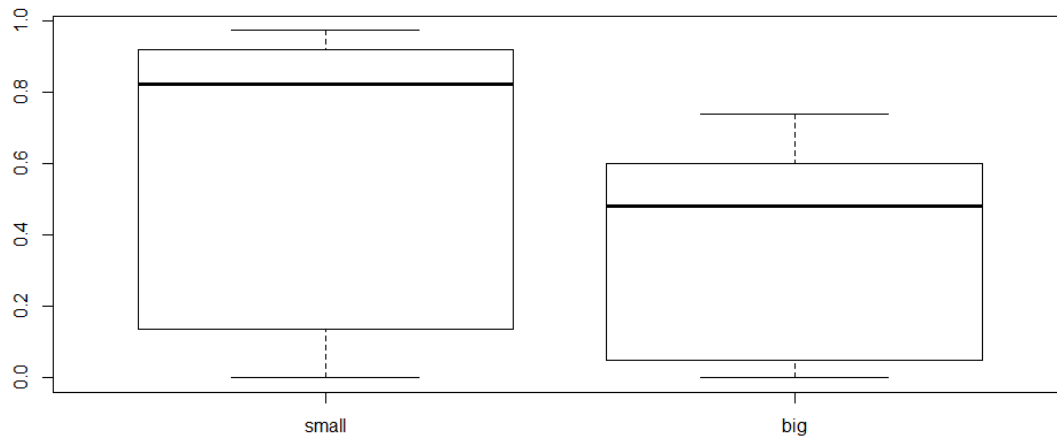

Figure 3.2: Box plots showing proportion of time-spent SALE (Singing at leaf edge) on small and large leaves.

B) Multivariate overall comparison of behaviors on big and small leaves and supervised clustering

To ascertain quantitatively if behavior of individuals on small and large leaves were different, an overall multivariate comparison analysis was carried out. This involved a component analysis for dimensionality reduction coupled with a supervised clustering analysis.

Absolute time data – Before a factor analysis was carried out, the number of factors to use was estimated using Horn's analysis and the scree plot (Fig 3.3a,b). Both methods indicated that 4 factors must be chosen to represent the data set.

As 5 factors explained only 77% of the variance and 6 was the maximum allowed factors (0 degrees of freedom), a decision to NOT reduce dimensionality was taken (Table 1). A clustering analysis was performed on the entire data set [20,10; (individuals, behaviors)]. **5 individuals on small leaves and 8 individuals on large**

leaves formed one cluster. 5 individuals on small leaves and 2 individuals on large leaves formed the other cluster.

This indicates that the behavioral repertoires of individuals on large and small leaves were not different when the absolute time data was looked at.

```
----------------------------------------------------------------
Component    Adjusted      Unadjusted     Estimated
             Eigenvalue    Eigenvalue     Bias
----------------------------------------------------------------
1            1.610552      2.933523       1.322970
2            1.259572      2.099861       0.840289
3            1.035824      1.528453       0.492629
4            1.025621      1.240017       0.214396
5            0.938776      0.910841       -0.02793
6            0.734302      0.494153       -0.24014
7            0.756474      0.330582       -0.42589
8            0.908803      0.318914       -0.58988
9            0.826975      0.095711       -0.73126
10           0.903097      0.047940       -0.85515
----------------------------------------------------------------

Adjusted eigenvalues > 1 indicate dimensions to retain.
(4 components retained)
```
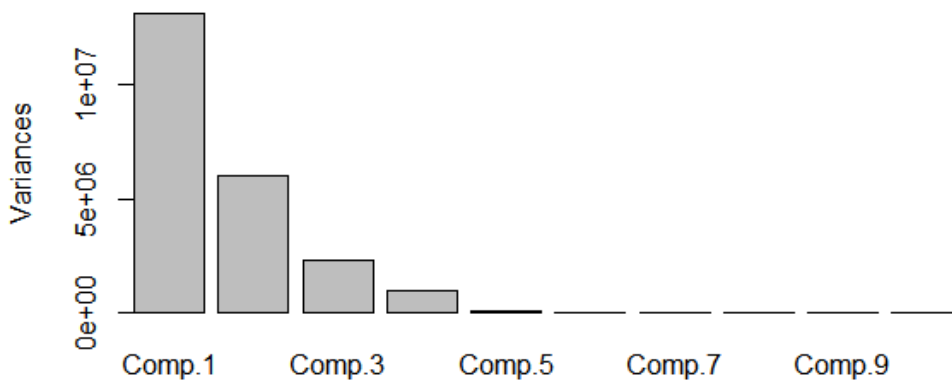

Figure 3.3 a): Horn's analysis indicating that four factors must be chosen (Eigen values>1); b): Scree plot indicating that four factors must be retained.

	Factor1	Factor2	Factor3	Factor4	Factor5
SS loadings	2.094	1.994	1.497	1.239	0.941
Proportion Var	0.209	0.199	0.150	0.124	0.094
Cumulative Var	0.209	0.409	0.558	0.682	0.776

Table 1: This table shows how the proportion of variance explained by factors.

Proportion time data – It was found that 3 components explained more than 99% of the variance (Scree plot). Therefore, a decision to reduce dimensionality was taken. A clustering analysis was performed on the data set with dimensionality reduction [20,3]. **5 individuals on large leaves and 3 individuals on small leaves formed one cluster. 5 individuals on large leaves and 7 individuals on small leaves formed the other cluster (Fig 3.4).** This indicates that the behavioral repertoires of individuals on large and small leaves were not different when the proportion time data was looked at.
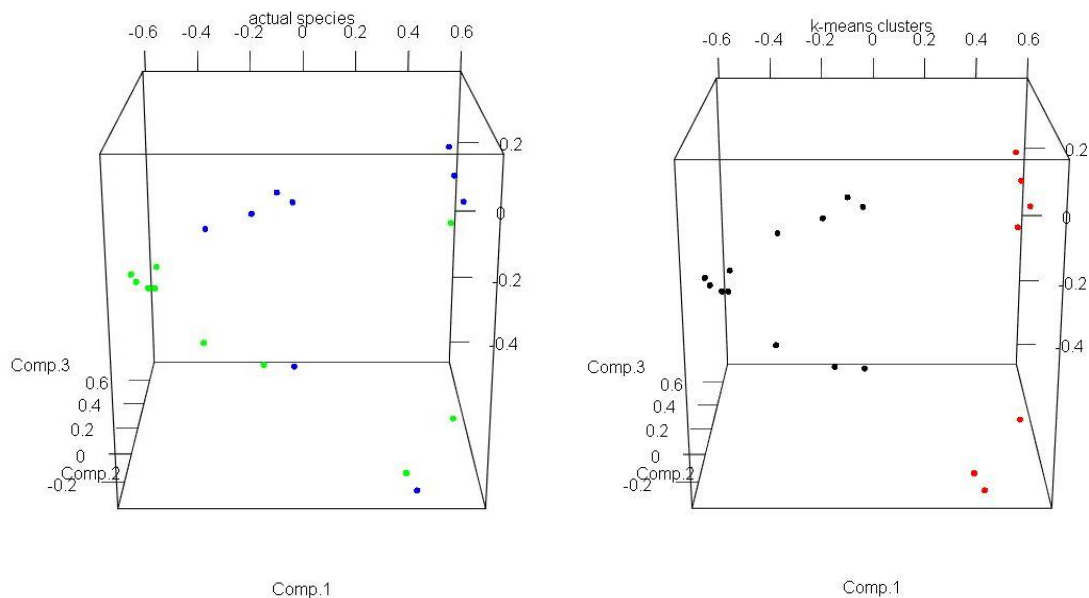
Figure 3.4 a): 3d plot showing the reduced data set with actual identity tags (green points- individuals on small leaves; blue points – individuals on large leaves).
3.4 b): 3d plot showing k-mean clusters of the reduced data set (Red and Black dots form 2 clusters). Observe the differences between the two.

A qualitative estimate of behaviors on small and large leaves indicated that baffles are made only on large leaves. This would indicate that individuals are in fact measuring leaf area. There were also differences found between large and small leaves. (Unique behaviors performed on both small and large leaves). It is therefore likely that component analysis, which looks at the overall data, misses out on these crucial details.

Therefore, the individual proportion of time spent on different activities (Fig 3.5) was estimated to pinpoint behaviors that might be important in estimating leaf areas.

C) Activity of *O. henryi* on large and small leaves: Large leaves- the median of time spent on behaviors; singing at leaf edge (SALE) and making baffle (MB) consumed most of the proportion of time. Resting and stem followed this. The individuals spent a small median of approximately only 10% of their time in walking.

Small leaves- the median of time spent on behaviors singing at leaf edge (SALE) and **resting** constituted a large proportion of the time. A small comparable proportion of time was spent on walking. **This makes SALE (singing at leaf edge) and walking possible candidates for leaf area measurement**.

However, these behaviors were also found to be very variable. It is likely that these behaviors are variable as the observation time for different individuals were different due to the criterion used for the end of a trial.
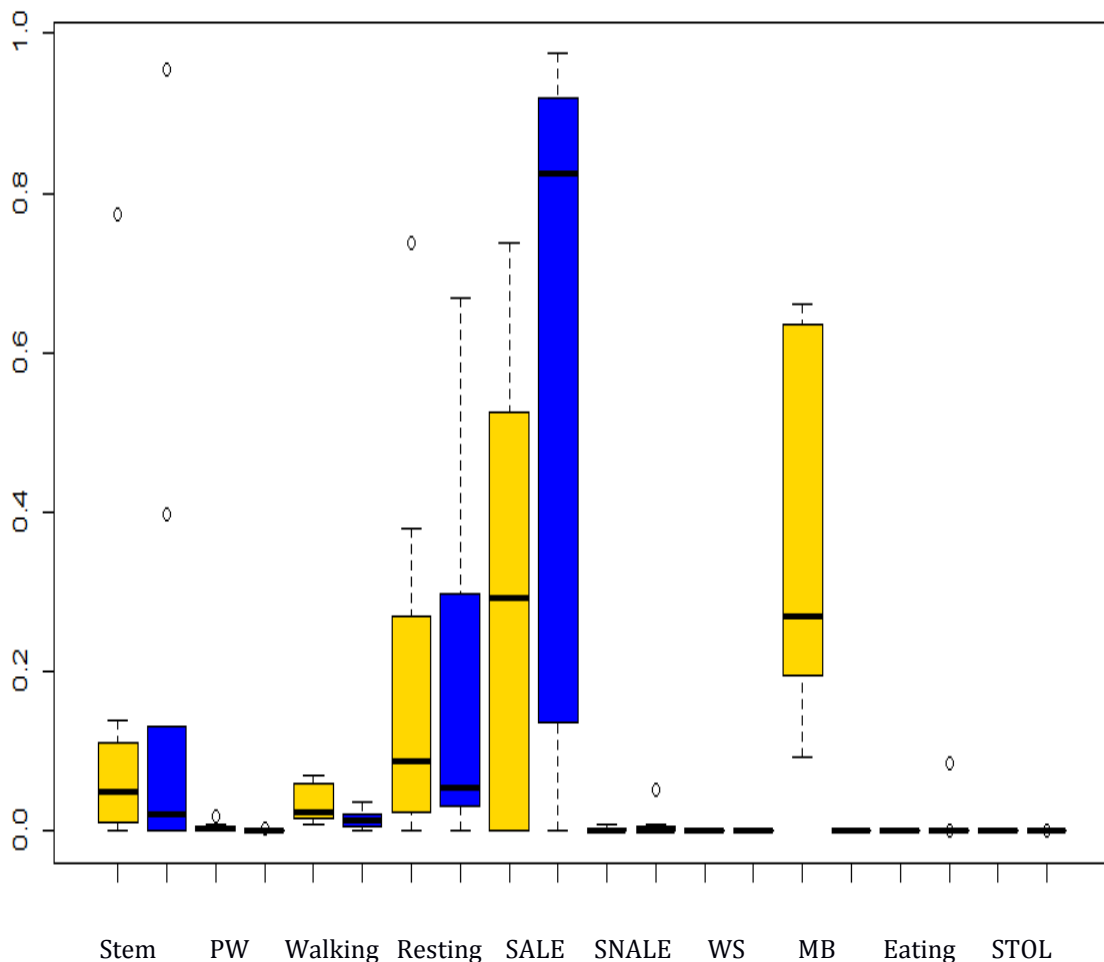
Figure 3.5: Yellow bars – Individuals on Large leaves; Blue bars – Individuals on small leaves; Proportion of time spent on different behaviors.

Therefore, the absolute time spent on different behaviors on **large (Fig 3.6)** and **small leaves (Fig 3.7)** was also looked at. A comparatively lower variance in the

absolute time spent on different behaviors would indicate that the results from the proportion data were an artifact of trial length and would go on to provide actual details of time spent on each behavior.

It was observed that individuals on **large** leaves spent maximum amount of time on four activities: **making baffle** (median time approximately 30 minutes), **resting and grooming** (median time approximately 15 minutes; shown as resting on graph to avoid clutter), **singing at leaf edge** (median time approximately 15 minutes) and **resting on the stem** (median time approximately 10 minutes).

Individuals on **small** leaves were observed to have a median of close to 400 minutes (> 6.5 hours) of **SALE** (Singing at leaf edge). **Resting** behavior had a median of approximately 20 minutes. Walking behavior had a median of approximately 4 minutes.

Resting is unlikely to be a behavior that is informative of leaf area. **SALE might be** informative about leaf area (see discussion). **Walking** is another potential way in which insects could be measuring leaf area.

As the variation of absolute time spent on behaviors observed were qualitatively observed to be smaller than that represented by the proportion data, it is likely that this is a result of variable trial lengths of observations between individuals.
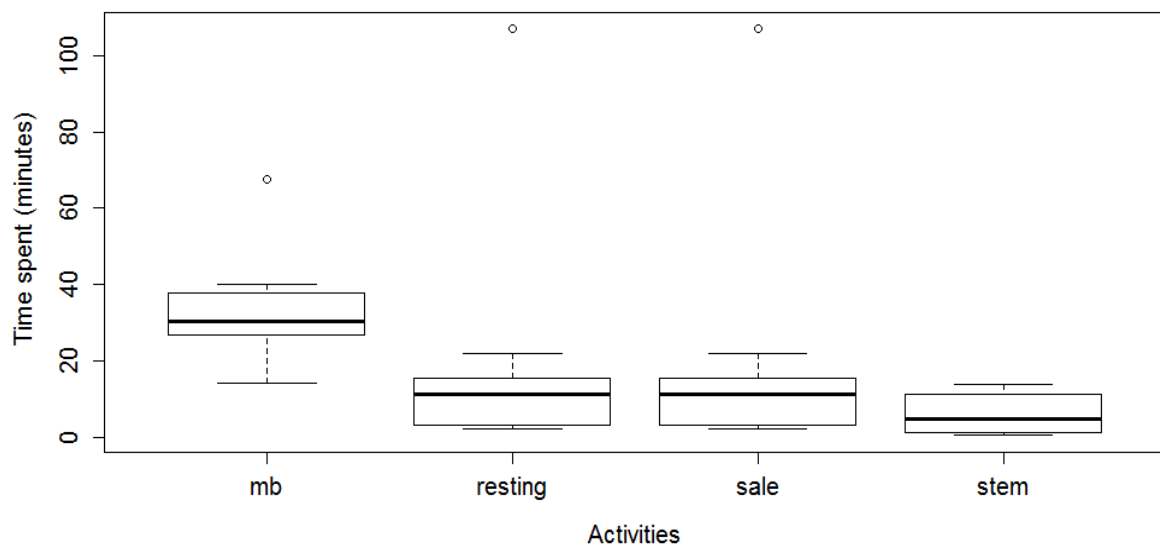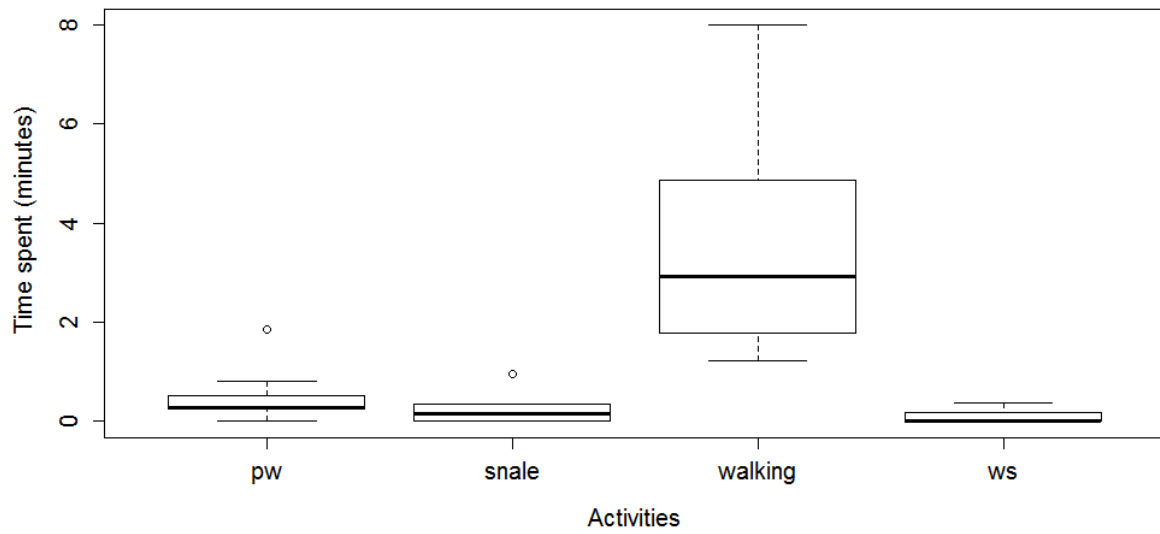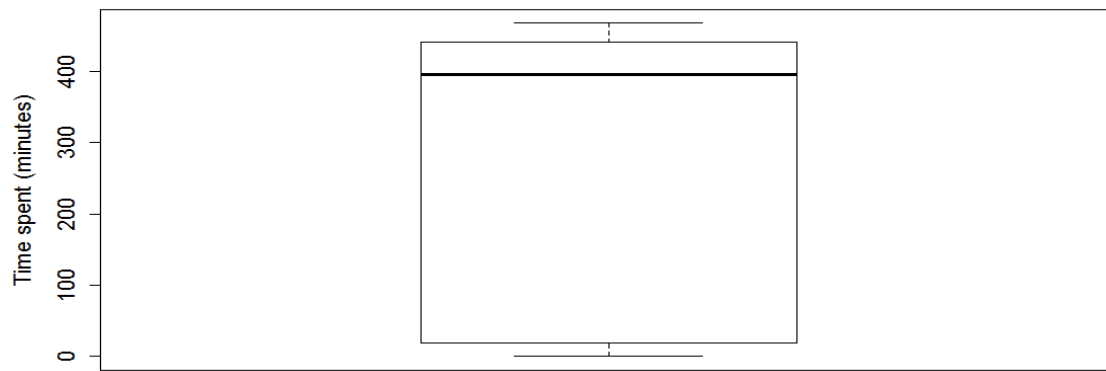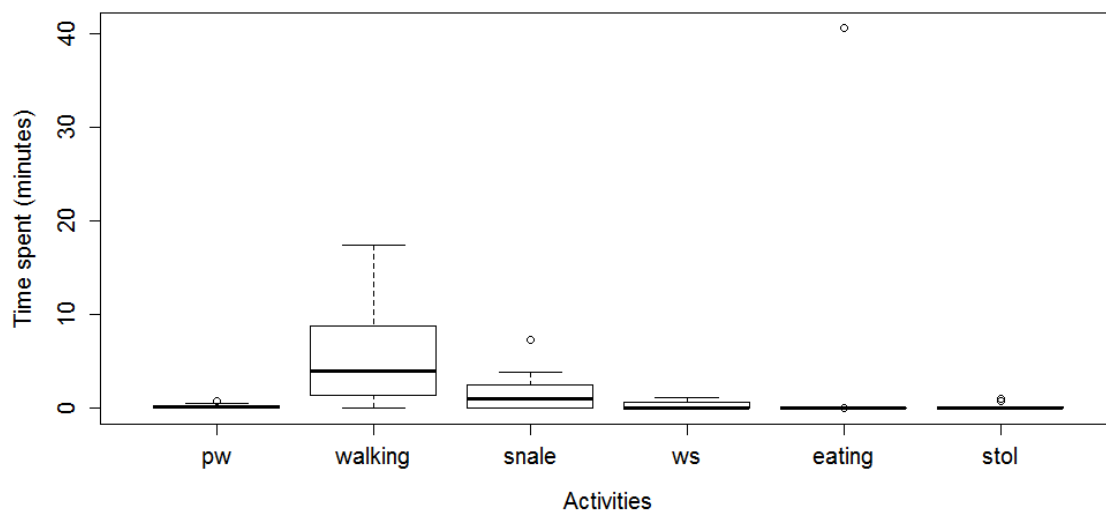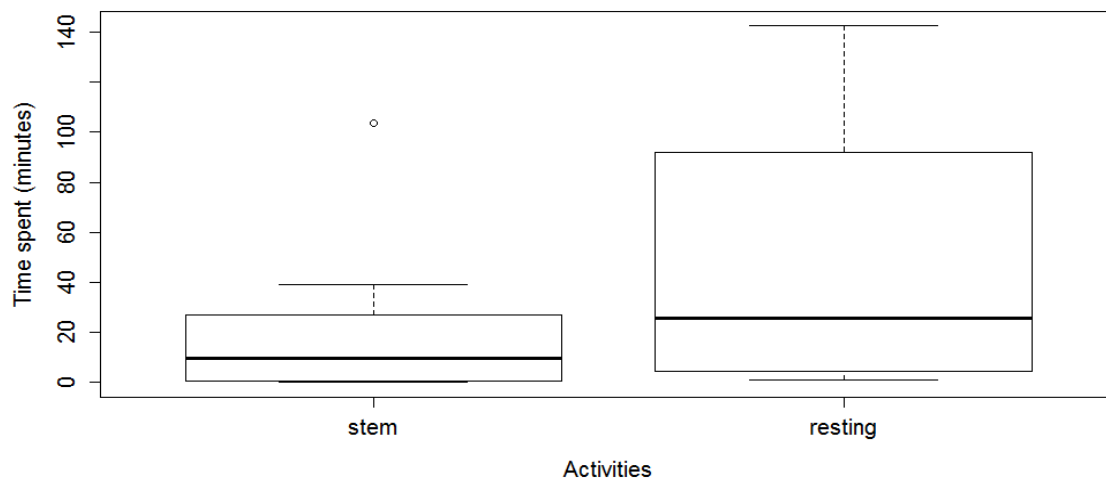
Figure 3.6a,b): Boxplot showing variation in absolute time duration for different activities on big leaves (N=10). Legend: pw- petiole walk; snale – singing not at leaf edge; ws – walking and singing; mb – making baffle; sale – singing at leaf edge.

Figure 3.7 a,b,c : Boxplot showing variation in time duration for different activities on small leaves (N=10). Legend : pw- petiole walk ; sale – singing at leaf edge ; snale – singing not at leaf edge ; ws – walking and singing; stol – stem to leaf.

The next line of inquiry was to identify behaviors that transitioned to baffle making with high probabilities (if any could be identified).

D) Transition probability charts:

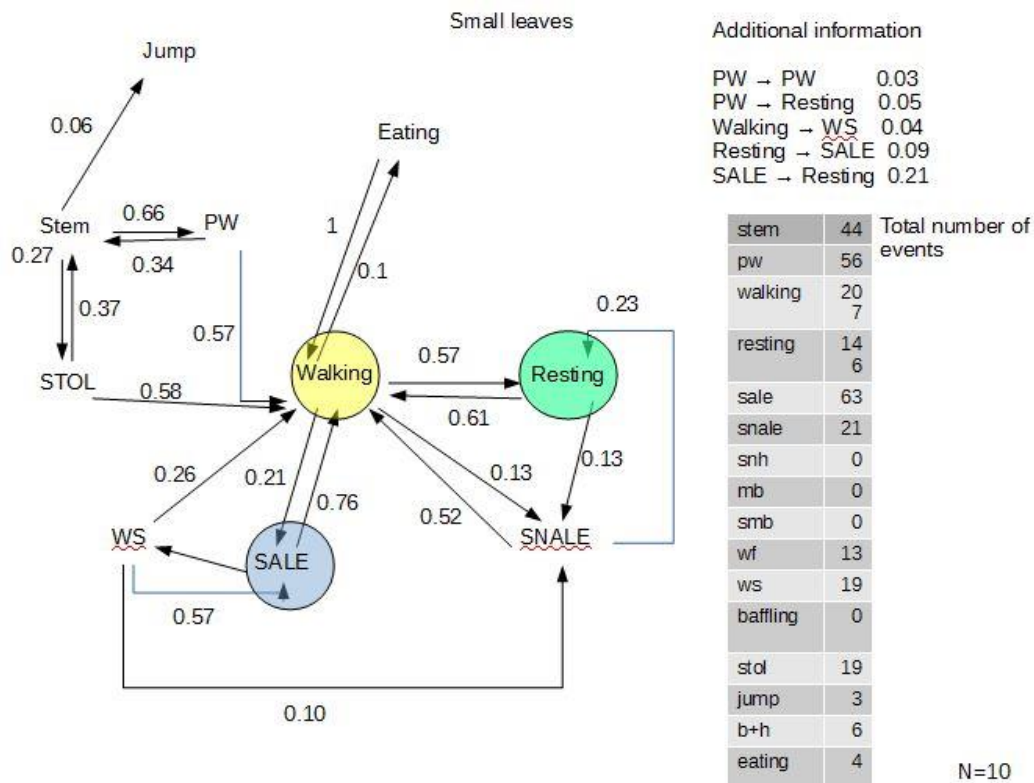**Transition probability chart for individuals on small leaves (Refer to Chart 1) –** Individuals on small leaves clearly did not make baffles. The transition between walking and resting and vice versa occurred with high probabilities (>50%) and the transition from walking and resting to singing occurred at lower probabilities (34%) (Chart 1).

Transition probability chart for individuals on large leaves (Refer to Chart 2) - The transition between walking and resting and vice versa occurred with high probabilities (>50%) and the transition from walking and resting to singing occurred at lower probabilities (24%). Singing often transitioned back to walking or resting with high probabilities. This was much like the scheme that observed on small leaves (Chart 2).

A second circuit of behaviors that were absent on small leaves, linked to baffle making through walking was observed. This involves all activities besides making baffle (MB), singing near hole (SNH) and singing and making baffle (SMB).

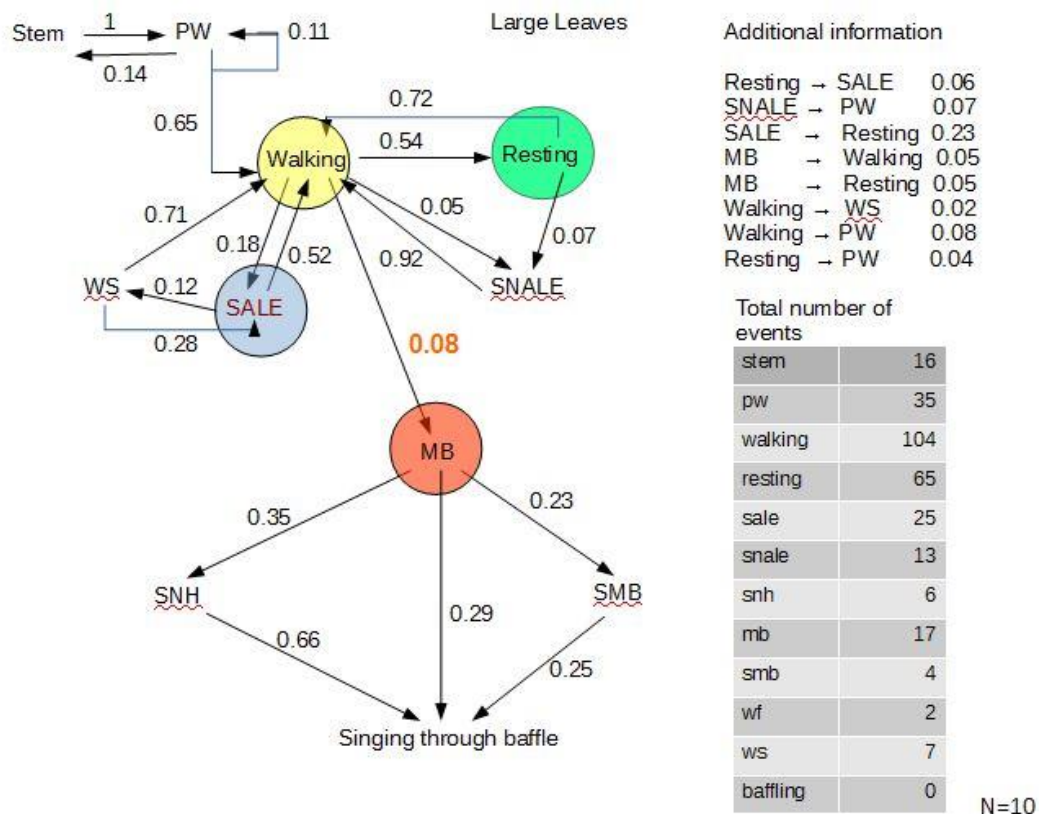It was found that only a **mere 8% of the total walks transitioned to baffle making**.

Small leaves

Additional information

PW → PW   0.03
PW → Resting   0.05
Walking → WS   0.04
Resting → SALE   0.09
SALE → Resting   0.21

		Total number of events
stem	44	
pw	56	
walking	20 7	
resting	14 6	
sale	63	
snale	21	
snh	0	
mb	0	
smb	0	
wf	13	
ws	19	
baffling	0	
stol	19	
jump	3	
b+h	6	
eating	4	N=10

Large Leaves

Stem —1→ PW ←— 0.11
0.14

0.65

0.72
0.54

Walking

Resting

0.71
0.18 / 0.52   0.92
0.05
0.07

WS ←— 0.12
SALE
SNALE

0.28

0.08

MB

0.35
0.23

SNH
SMB
0.29

0.66
0.25

Singing through baffle

Additional information

Resting → SALE   0.06
SNALE → PW   0.07
SALE → Resting 0.23
MB   → Walking 0.05
MB   → Resting 0.05
Walking → WS   0.02
Walking → PW   0.08
Resting → PW   0.04

Total number of events

stem	16
pw	35
walking	104
resting	65
sale	25
snale	13
snh	6
mb	17
smb	4
wf	2
ws	7
baffling	0

N=10

Chart 1, 2: Transition probability chart showing transition between different behaviors on small leaves and large leaves respectively (N=10). Legend: PW- petiole walk ; SALE – singing at leaf edge ; SNALE – singing not at leaf edge ; WS – walking and singing; STOL – Stem to leaf ; Eating – eating the leaf with no intent of singing through the hole; SNH – Singing near hole ; SMB – Singing and making baffle; SNH – Singing near hole; MB – Making baffle.

As it was found that a small proportion of walking transitioned to baffle making, the variation in time taken by different individuals to initiate making a baffle was looked at.

Individuals were observed to spend variable amounts of time on 'decision making' (Fig 3.8). 14 individuals out of 17 had started making baffles by the end of 100 mins. However, individuals took more or less the same duration of time to complete making a baffle (~25 to 35 minutes).
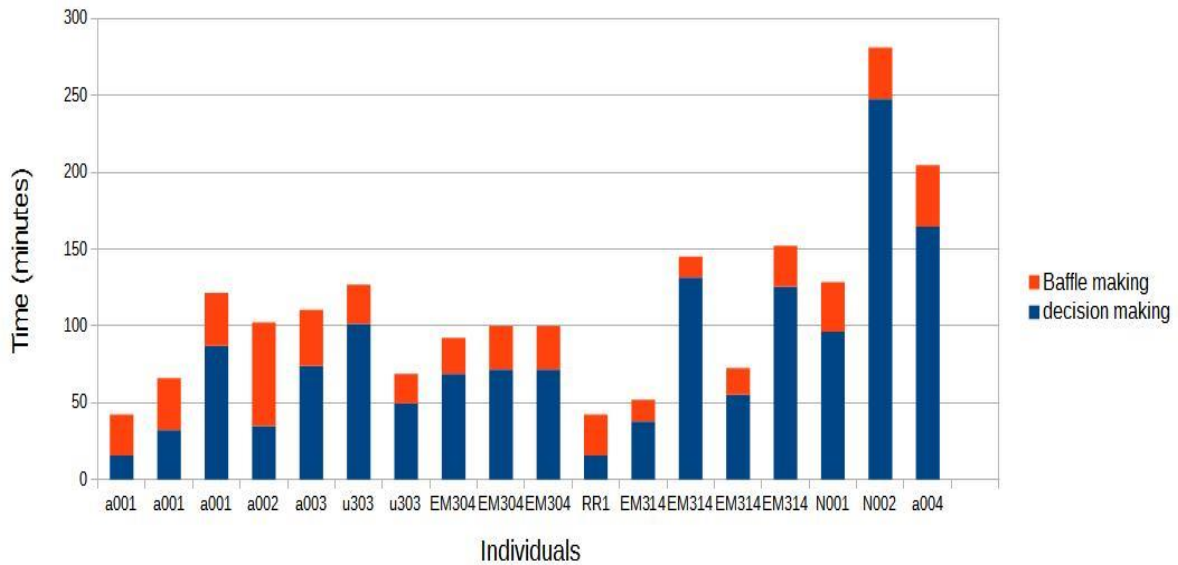
Figure 3.8: The graph above shows individuals only large leaves. Observe that time taken for 'decision-making' is a variable behavior. The time taken to make a baffle remained fairly constant except for an outlier a002, which took an un-usually large time to make, it's baffle.

e) Testing Sub-hypotheses -

a) Do individuals on small leaves sing for longer than individuals on large leaves in the first 100 minutes?
The differences between the groups were not found to be statistically significant
(W=26, P value=0.29) (Fig 3.9).
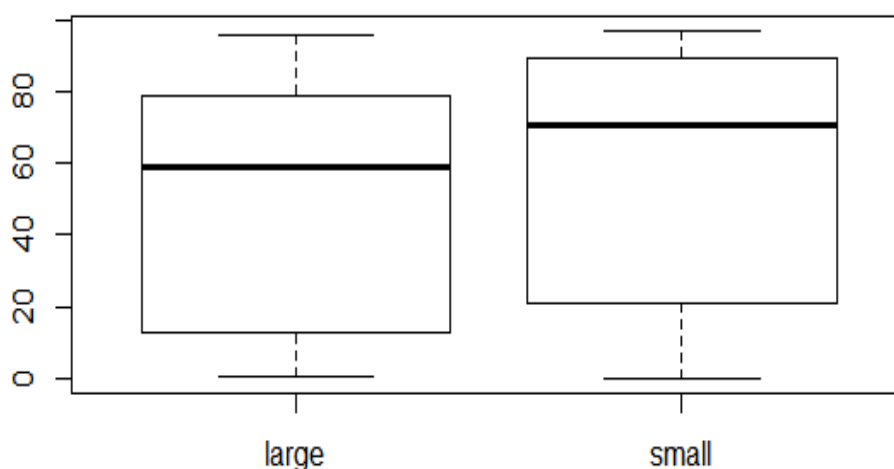
Figure 3.9 a: Box plots showing time spent singing in the first 100 mins.

b) Do individuals on large leaves also walk for longer in the first 100 minutes?

The differences between the groups were not statistically different (W=42, P value=0.16) (Fig 3.9b).
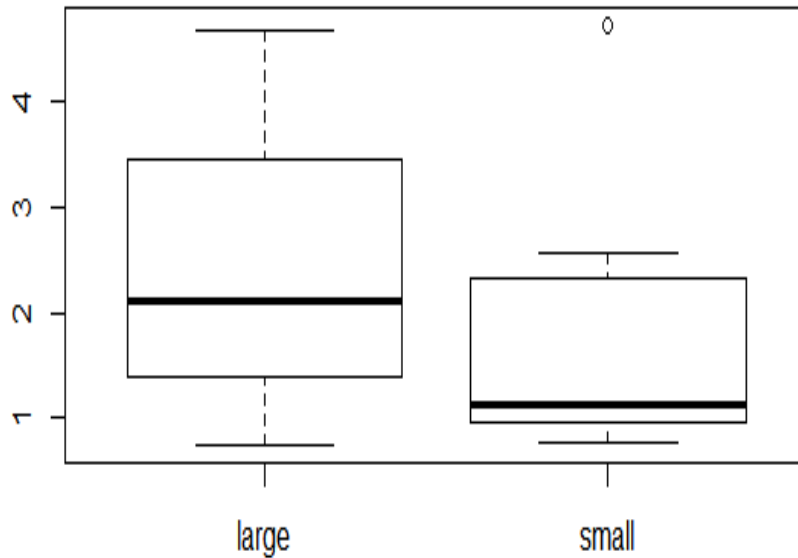
Figure 3.9 b: Box plots showing time spent walking in the first 100 mins.

Results : Part B)

a) Are petiole features correlated with leaf area?

All petiole features were found to be significantly positively correlated with leaf area in *H. suaveolens* suggesting that they could be used in measurement of areas (Petiole length: Pearson's r =0.8199, P value <0.001 ; Petiole thickness 1 : Pearson's r =0.8271, P value < 0.001; Petiole thickness 2: Pearson's r= 0.7684, P value < 0.001) (Fig 3.10a,b,c).
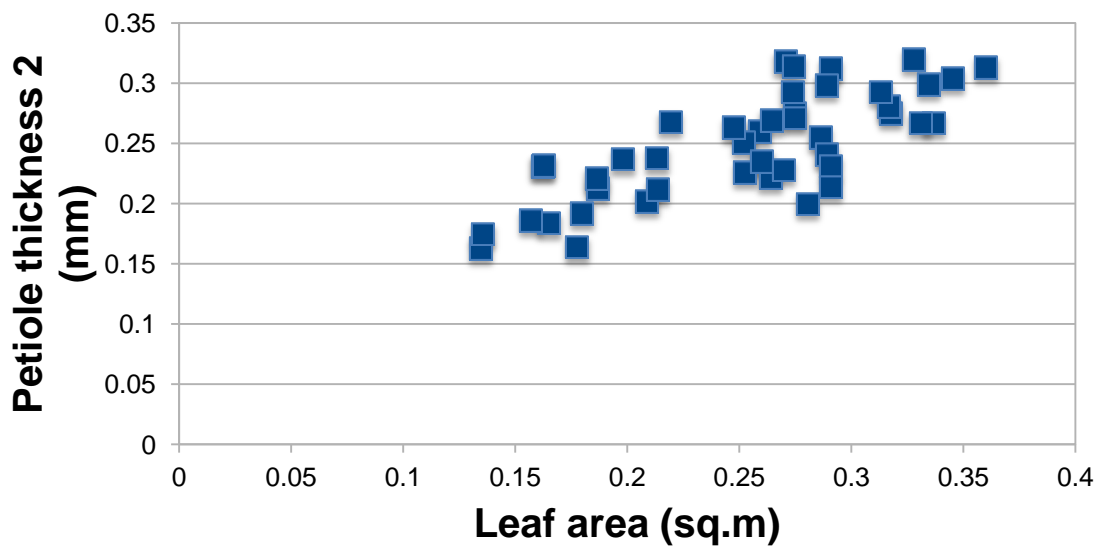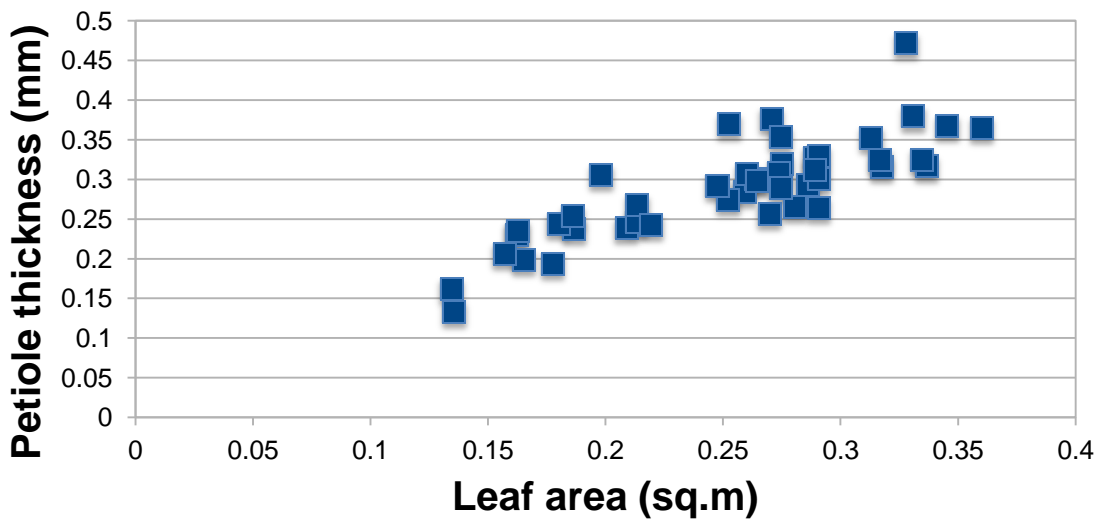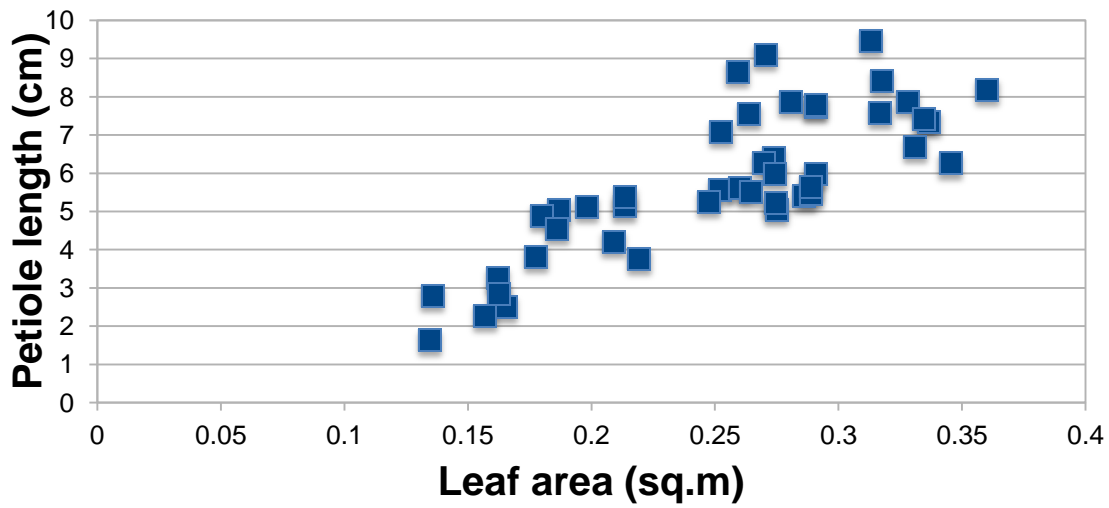
Figure 3.10 a,b,c: Association between petiole features and leaf area.

b) What hypothetical walks are good indicators of area?

All hypothetical walking paths were found to be well correlated with area (Full perimeter: Pearson's r = 0.997, P value < 0.001; Half perimeter: Pearson's r =0.9617, P value < 0.001; Triangle: Pearson's r =0.9165, P value < 0.001; Leaf length: Spearman's r= 0.9463, P value < 0.001 (distribution of leaf length walks were not normal) and Leaf Breadth: Pearson's r= 0.9525, P value < 0.001). Since, all path lengths have high correlation values, they can be used to measure leaf area in theory (Fig 3.11).
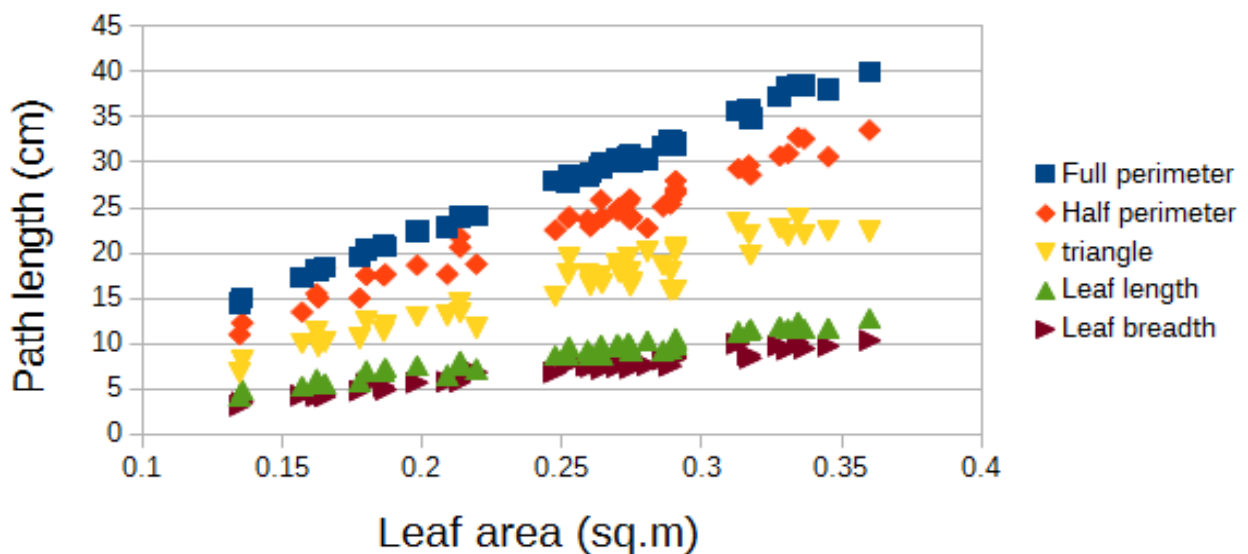

Figure 3.11: Association between lengths of hypothetical paths and leaf area.

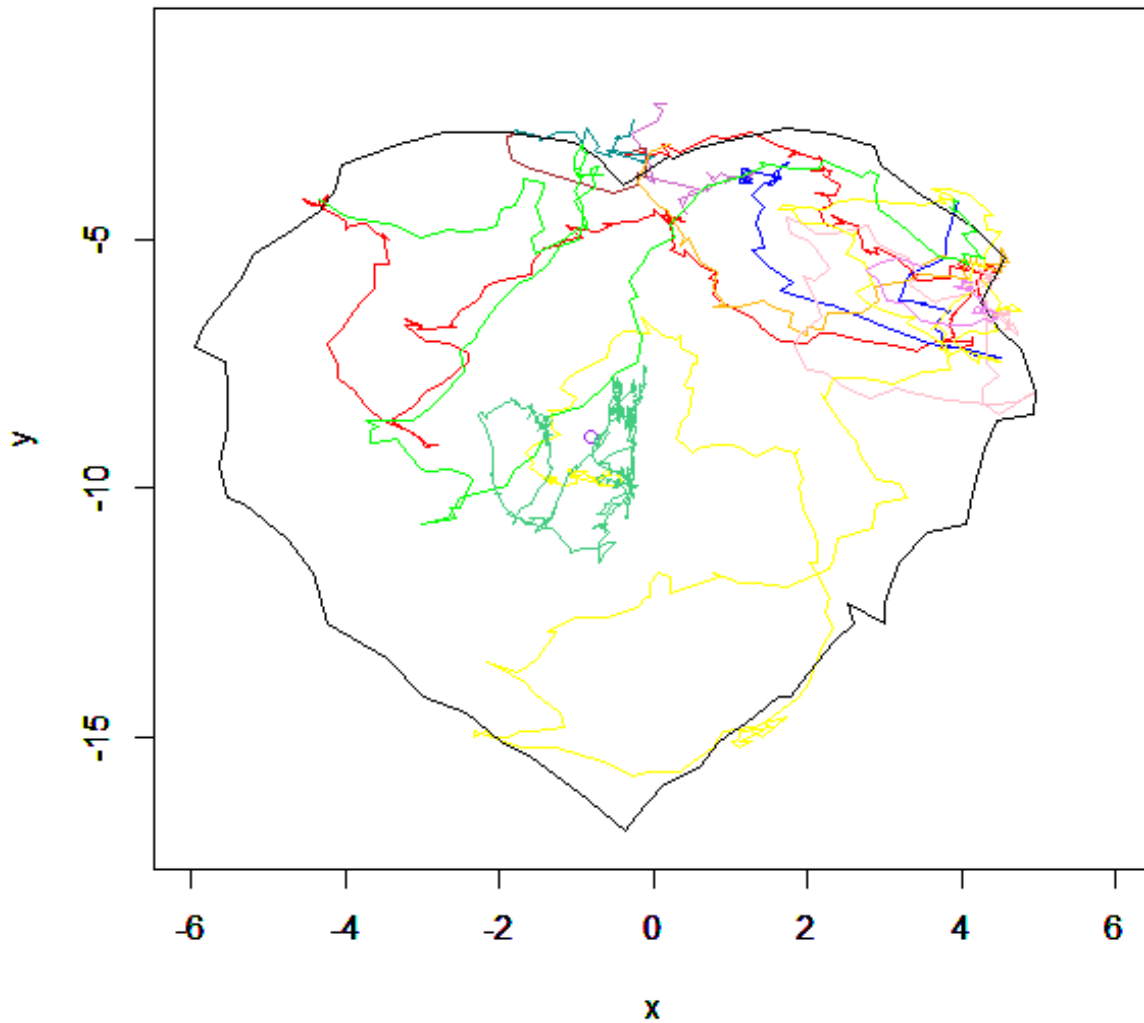C) What do actual paths look like?

Figure 3.12: The plot above shows a representative example of different walking paths of individuals. All walking paths (all paths both on under-side [UN] and upper-side [UP] of the leaf) of an individual on a large leaf have been plotted. The colors denote the order in which the paths were walked from start of experiment to baffling. The order of walks based on time, occurred in the following sequence: 1) Red UN, 2) Green UP, 3) Blue UN, 4) Orchid UN, 5) Brown UN, 6) Cyan UN, 7) Golden rod UN, 8) Violet UP, 9) Pink UN, 10) Yellow UN, 11) Sea green UN; Purple - Baffle, Leaf - Black.

When the experimentally obtained walking paths (like the ones shown above), were examined for the occurrence of the hypothetical leaf-area-measuring paths described previously, resemblance was found only in very few cases (Table 2). The proportion of hypothetical walks recognized was higher on small leaves. However,

the insects mostly walked in paths that did not resemble these hypothetical paths on both small and large leaves.

big leaves	breadth	length	triangle	half perimeter	full perimeter	Total walks	proportion
1	0	0	0	1	0	7	0.14
2	0	0	0	0	0	6	0.00
3	0	0	0	0	0	8	0.00
4	0	0	0	0	0	5	0.00
5	0	0	0	0	0	6	0.00
6	0	1	0	0	0	24	0.04
7	0	1	0	0	0	10	0.10
8	0	0	0	0	0	17	0.00
9	1	0	0	1	0	13	0.15
10	0	0	0	0	0	7	0.00

small leaves	breadth	length	triangle	half perimeter	full perimeter	Total walks	proportion
1	0	3	0	1	1	15	0.33
2	0	0	0	0	0	2	0.00
3	0	5	0	2	1	38	0.21
4	0	4	0	0	1	14	0.36
5	0	2	0	0	0	19	0.11
6	0	3	0	0	0	23	0.13
7	0	9	0	0	0	50	0.18

Table 2: Table showing the number and proportion of hypothetical walks on large and small leaves. Each row is an individual. Total walks = hypothetical walks + other walks. Observe that there are many zeros in the proportion column on large leaves.

d) Can insects use the mean free path algorithm to estimate leaf area?

A categorical pooling of data was done here. As suggested by Mallon et al, it follows that the mean free path must increase with increase in area of the leaf. Therefore a wilcoxon un-paired test was done to check if the mean free path on large leaves was statistically different from that observed on small leaves.

The mean free path on large leaves was found to be significantly larger than the mean free path on small leaves (W=1; p-value = 0.0003) suggesting that the mean free path could be used in theory to measure leaf area (Fig 3.13).
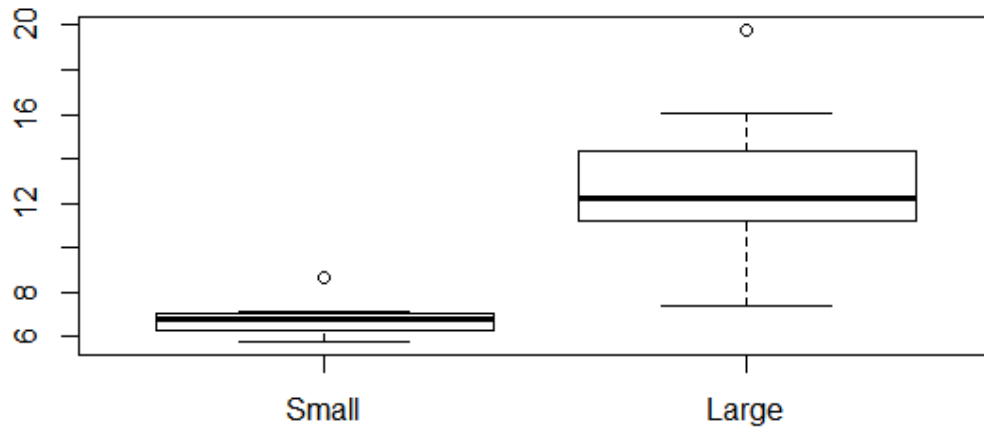
Figure 3.13: Mean free path length of individuals on small and large leaves (Ns = 8, Nl=8).

Label the Y axis.

DISCUSSION

Previous studies on the baffling behaviour of *Oecanthus henryi* have shown that males of this species distinguish between small and large leaves to make baffles. Therefore, the aim of this study was to elucidate mechanisms by which *Oecanthus henryi* measures leaf area in the context of baffling behavior. First, activity budgets of individuals on different leaf sizes were analyzed and compared. The overall behavioral repertoires of individuals on small and large leaves were not different when both the absolute time data and proportion time data was looked at. However, the fact that 7/10 individuals stayed on small leaves without making baffles or jumping off the setup for a period of 8 hrs is an important result already making the behavioral repertoires on small and large leaves different (2/10 individuals on small leaves made baffles after 8 hours and 3/10 of them had jumped off the setup).

The behaviors 'resting and grooming' and 'resting and walking on stem' were found to be well-represented behaviors on both small and large leaves. However they do not seem to be important for area measurement, as they are not likely to provide any information about leaf area (Walking on stem does not include petiole walks).

Singing at leaf edge and Auditory feedback - It was found that 'Singing at leaf edge' occupied a large time window on both small and large leaves. SALE (Singing at leaf edge) could be providing information about leaf area to the insect through auditory feedback. A cricket could estimate leaf area by listening to its own call, which is amplified when, sung at a leaf edge (partial baffle) as the amplitude gain is proportional to leaf area (Bennet-Clark, 1989, 1998, 1999). It could also be picking up asymmetry in it's own sound field to estimate leaf area. However, in the scenario where tiny individuals call with large wavelengths on leaves (Individual –12 mm, wavelength – 11.5 cm at 25 degrees Celsius), it is unlikely that auditory feedback can provide information about leaf area.

It has also been known that cricket auditory neurons are preferentially less sensitive to their own sounds in order to increase sensitivity of auditory neurons to other sounds while calling (Poulet et al, 2003) as a possible adaptation against predation pressure or because of habituation to loud calls of individuals. As it has been shown

before that an immobilization of the tympanum is not enough to stop firing in auditory neurons of *Teleogryllus commodus* and *T. oceanicus* (because of presence of tibial vibrations), a bilateral deafening experiment has to be carried out to rule out this hypothesis (Ball et al, 1978).

Petiole Features - As petiole features correlated well with area, it is possible that individuals estimate leaf area when they walk on petioles. However, individuals (4/10) on small leaves were observed to jump from stem to leaf (STOL) directly without walking on petioles. If walking on PW was the way individuals were measuring area, these individuals on small leaves would be expect to make mistakes and make baffles in the 8-hour recording period. However, this was not the scenario. Therefore, it is unlikely petiole features alone are being used for estimating area.

Walking- The total duration of time spent walking on leaves was very small on both large and small leaves. As all the elucidated mechanisms for area measurement are dependent on walking, this hypothesis was studied carefully though individuals spent very little time walking.

A measurement experiment revealed a set of hypothetical paths that were well correlated with area suggesting that they could be used. However, *O.henryi* was found to be not using those hypothetical paths.

It was found that the mean free-path length was larger on large leaves when compared to small ones; indicating that it can be used for reliable measurement and comparison of areas of leaves in theory. This however needs to be tested using manipulative experiments such as ones described by Mallon et al, 2015 which make use of a partial barrier to experimentally change mean free path without changing the area of a leaf.

It is believed that measurement of areas and volumes depend on the measurement of distances in one of the above ways: 1) Walking path lengths measured are themselves correlated with area or volume; 2) Use of algorithms such as mean free

path or Buffon's needle which computes area or volume based on a set of distances calculated (computationally more expensive).

However, these distances measured on walks or flights, can be measured in any of the following ways:

a) Optical flow or Motion Parallax – The idea revolves around the fact that when an observer is moving in a stationary environment, the objects which are closer to observer seem to move out of the frame of sight faster than farther objects do. (Animation: http://psych.hanover.edu/Krantz/MotionParallax/MotionParallax.html) Optical flow or Motion Parallax has been known to be used by bees (Esch, 1995) and wood crickets (Goulet et al,1981) for estimation of distances. Wood crickets were found to be using Motion parallax by swinging their heads to and fro to estimate distance to other objects around them when they reached the edge of a stem (Goulet et al, 1981). The possibility that *O. henryi* could be measuring distances based on optical flow cannot be ignored. A confirmatory experiment, which involves blinding males of *O. henryi*, is needed to disprove this.

b) Stride integration – The idea revolves around measurement of distances based on feedback generated by motor organs during movement or by time integration (if distance walked is correlated with time spent walking; Wittilinger et al, 2007). If time integration is being used, for easy comparison of distances walked on different leaves, the insect would have to walk at constant velocity to estimate and compare distances. An initial qualitative analysis of velocity plots of walking bouts on leaves did not seem to suggest such a region of constant velocity. However, there is no reason not to believe that a higher order computation using distances measured based on walks of different velocities is possible.

c) Energy hypothesis – This hypothesis proposes that distances are measured as a function of expenditure of energy (Karl Von Frisch, 1967). The strong evidence for the hypothesis comes a study of bees on a mountain. Individuals that went up the mountain indicated larger distances to food resources through their waggle dance when compared to individuals that went downhill to food resources that were equally far away (Heran, 1956). This was not explicitly tested during the study.

Interesting observations

During the activity of baffle making, individuals would have to not only choose the position on the leaf to make the baffle to ensure maximization of sound field in all directions, but also choose what size the baffle has to be. The baffle size has to be exactly as big as the wings. If the baffle size is not approximately the size of the wings, there is a loss in acoustic output. Theoretically, the cricket can make a hole in the leaf in two ways; cut out the circumference of the hole or to chew and enlarge the hole. It was observed that in all instances, a hole was chewed and enlarged. Individuals were seen singing near freshly made baffles and adjusting sizes of baffle while singing. These activities singing near hole (SNH), singing and making baffle (SMB) intermittent with the activity of chewing the leaf, could indicate an acoustic method by which *O. henryi* could control size of baffles it makes.

It must also be noted that; during the course of the study, males in captivity were observed to call for much longer hours than individuals in the wild. The natural calling period of *O. henryi* is between 6 pm and 9 pm. However, the individuals continued to sing from leaf edges or baffles even after 9 pm with little to no inhibition. This is probably an effect of nutrient rich diet (dog food) given to the crickets. The activity of baffle-making in some individuals also started after the calling period (4/20). This may seem disadvantageous as the natural calling period is almost over and this is a waste of effort. However, this in theory would ensure perfect utilization of the next calling period as songs can be broadcast through the baffle the entire duration. The possibility exists that individuals make baffles prior to calling period to ensure optimum use of the calling period.

Individuals in the semi-natural conditions have also been observed to call from the same baffle on consecutive nights (personal communication, Manjunath Reddy and Viraj Torsekar independently). This could support the fact that the probability of finding a baffler is so low in the wild because the probability of finding large leaves is also small (as suggested by Deb, 2015). It could also reflect a cost of the activity of baffle making, which still remains unknown.

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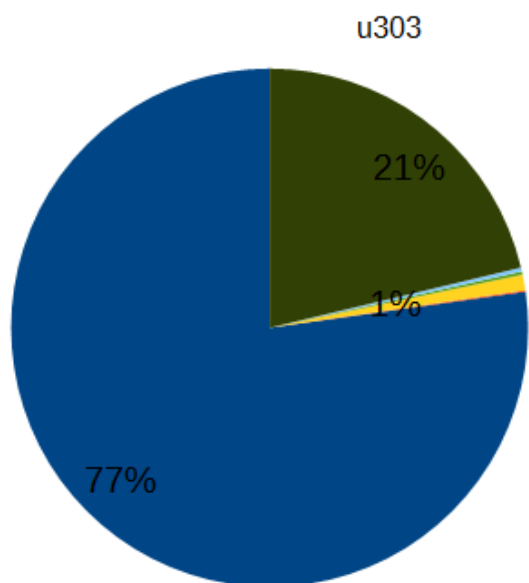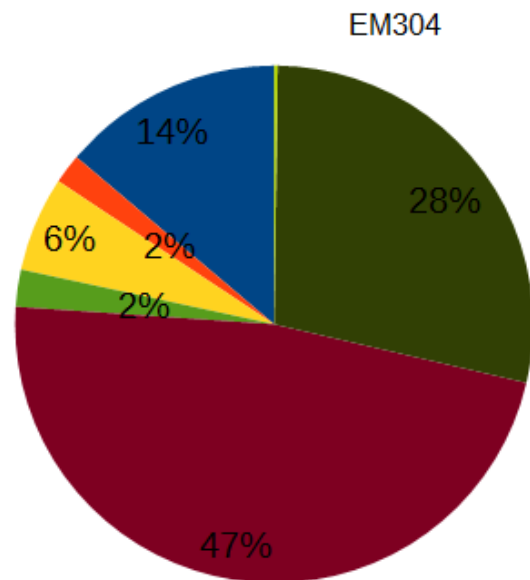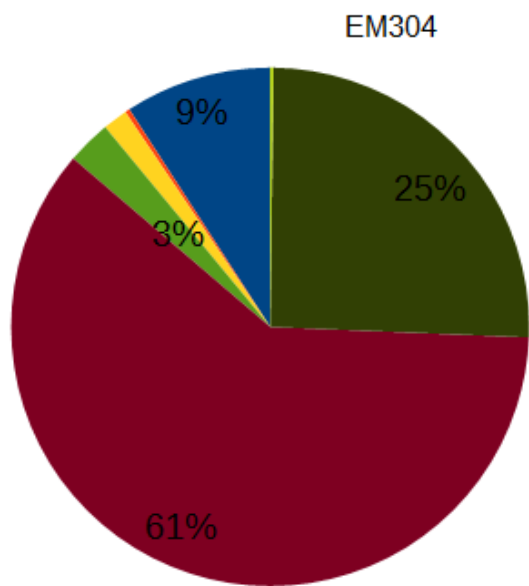
Appendix

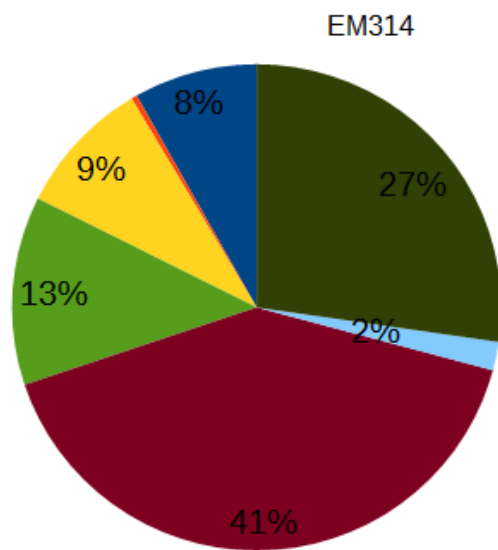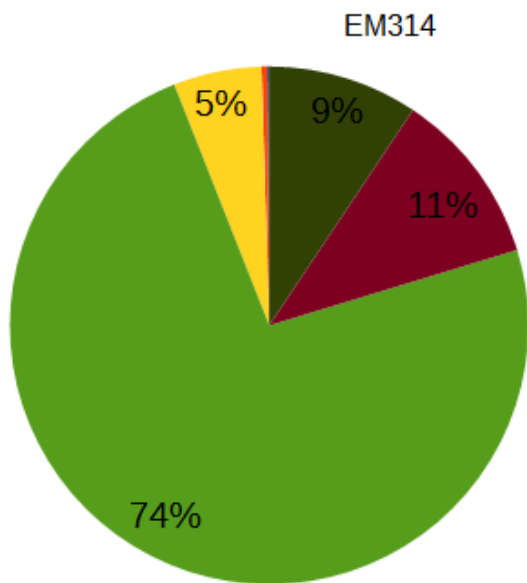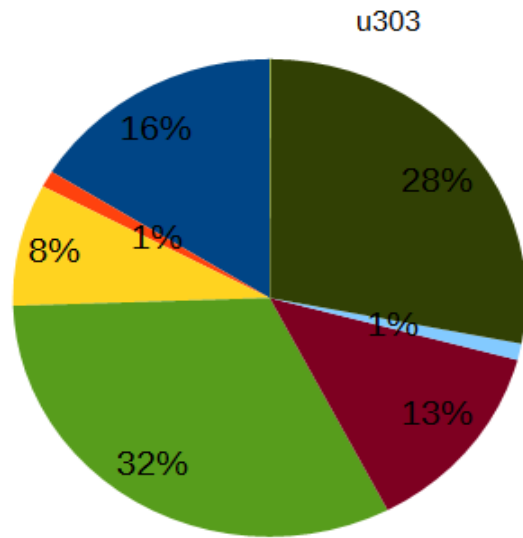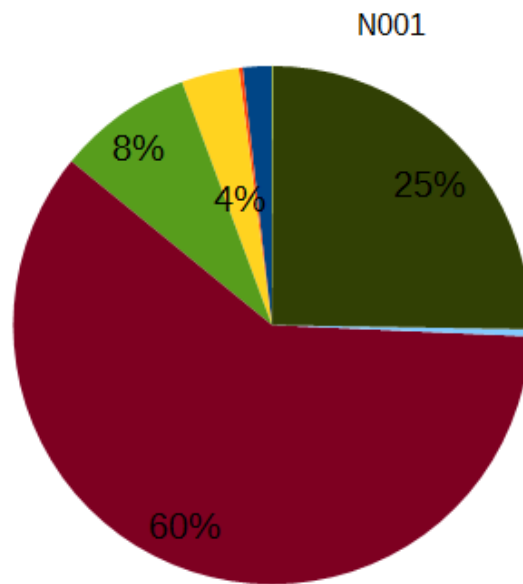**Activity budgets – Large leaves**
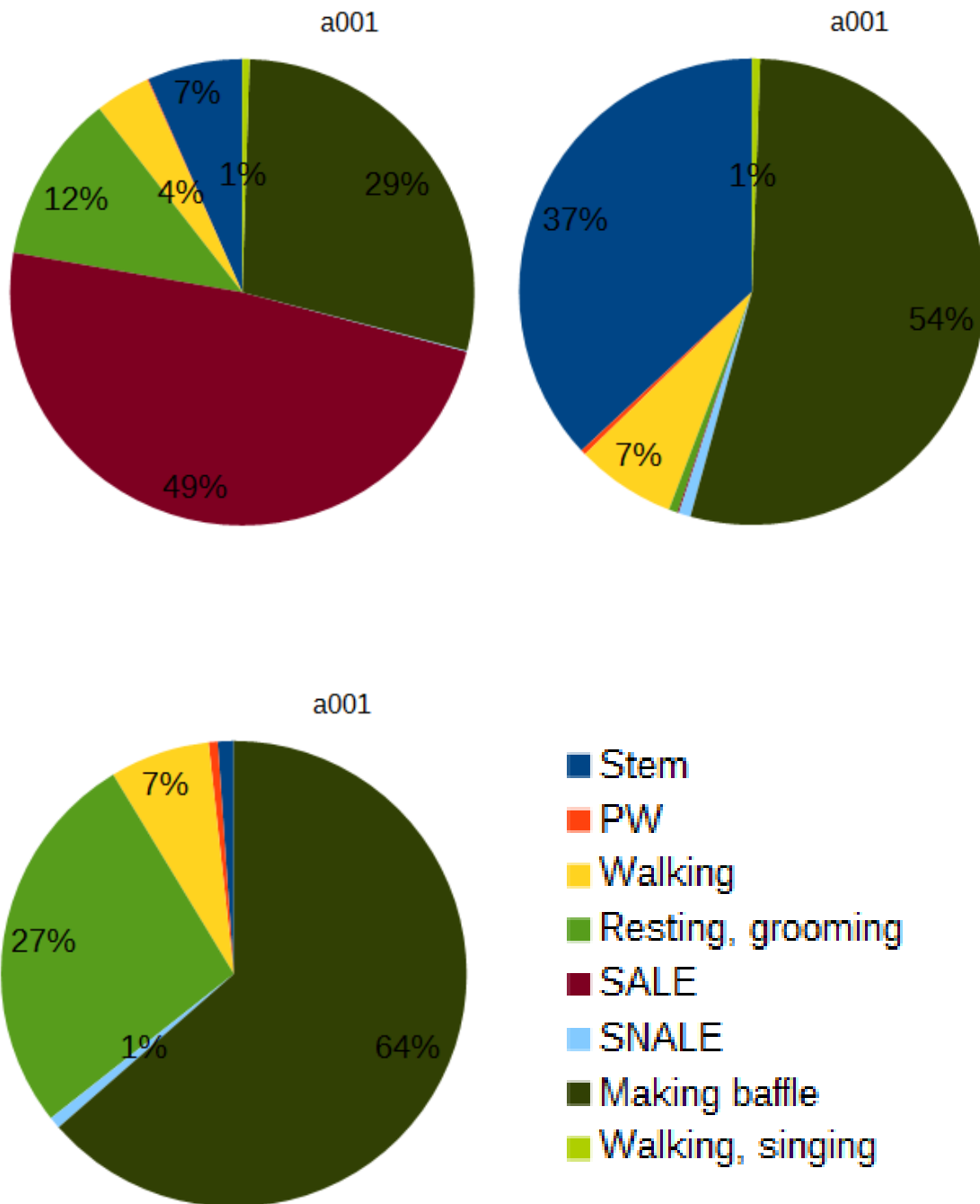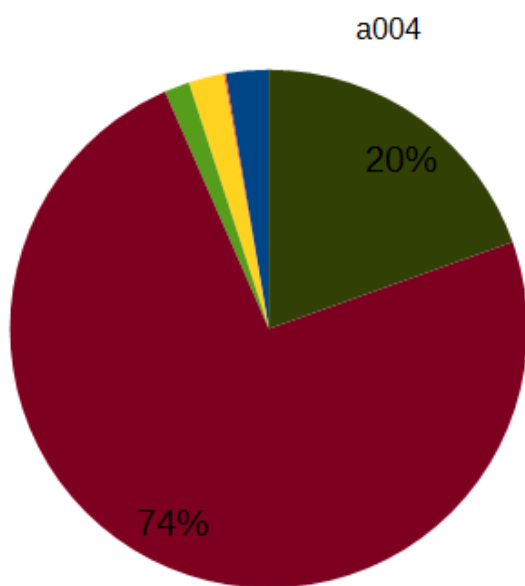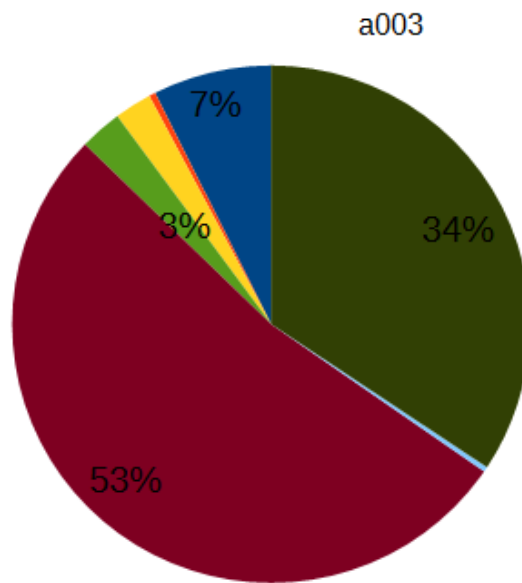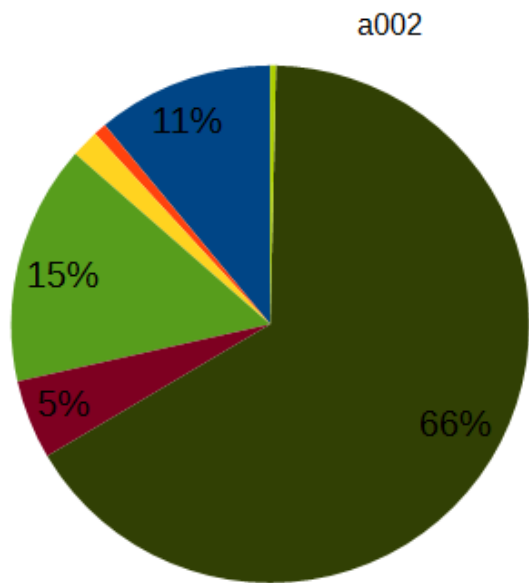
a001

a001

a001

- Stem
- PW
- Walking
- Resting, grooming
- SALE
- SNALE
- Making baffle
- Walking, singing

(Chart 1: 29%, 1%, 7%, 4%, 12%, 49%)

(Chart 2: 37%, 1%, 7%, 54%)

(Chart 3: 7%, 27%, 1%, 64%)

a002

a003

a004

Stem
PW
Walking
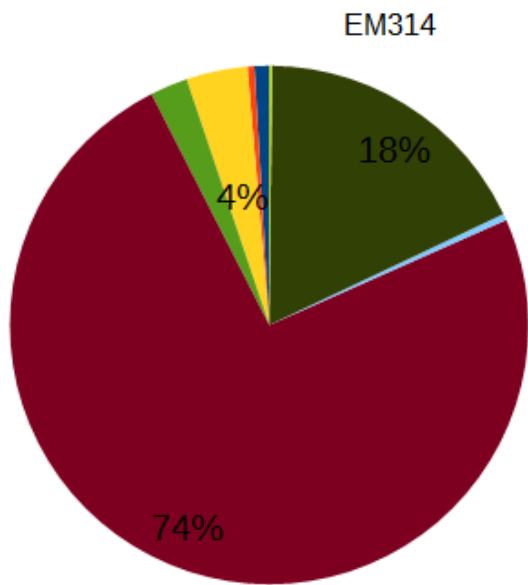Resting, grooming
SALE
SNALE
Making baffle
Walking, singing

EM304

EM304

u303

Stem
PW
Walking
Resting, grooming
SALE
SNALE
Making baffle
Walking, singing

Legend: Stem, PW, Walking, Resting, grooming, SALE, SNALE, Making baffle, Walking, singing

u303: 16% Stem, 1% PW, 8% Walking, 32% Resting grooming, 13% SALE, 1% SNALE, 28% Making baffle

EM314: 5% Walking, 9% Making baffle, 11% SALE, 74% Resting grooming

EM314: 8% Stem, 9% Walking, 13% Resting grooming, 41% SALE, 2% SNALE, 27% Making baffle
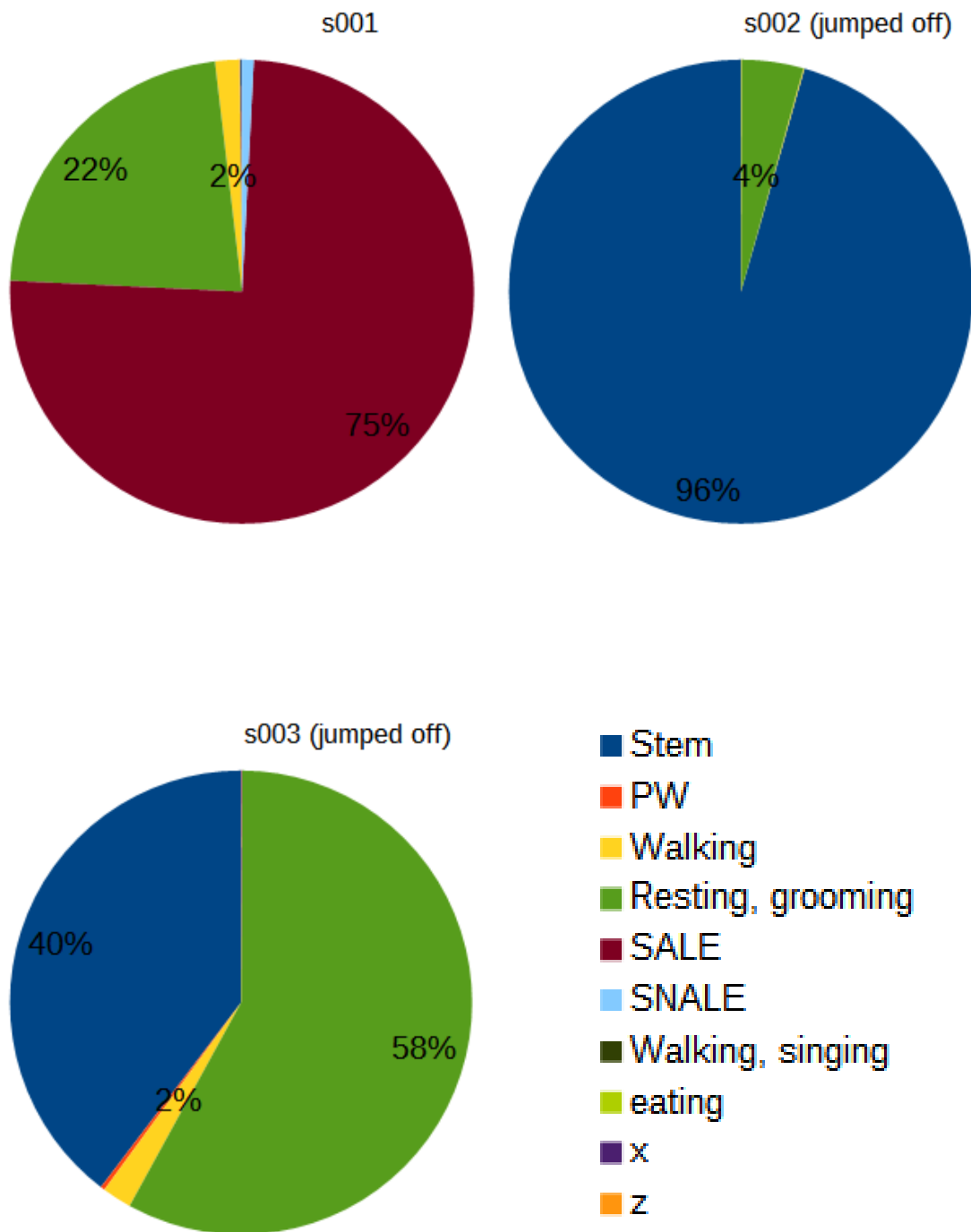
EM314

Legend:
- Stem
- PW
- Walking
- Resting, grooming
- SALE
- SNALE
- Making baffle
- Walking, singing

Stem: 45%
Making baffle: 24%
SNALE: 2%
Resting, grooming: 27%

EM314

Making baffle: 18%
Resting, grooming: 4%
SALE: 74%

N001

Resting, grooming: 8%
Walking: 4%
Making baffle: 25%
SALE: 60%

N002

- Stem
- PW
- Walking
- Resting, grooming
- SALE
- SNALE
- Making baffle
- Walking, singing

12%

2%

38%

48%

RR1

7%

27%

1%

63%
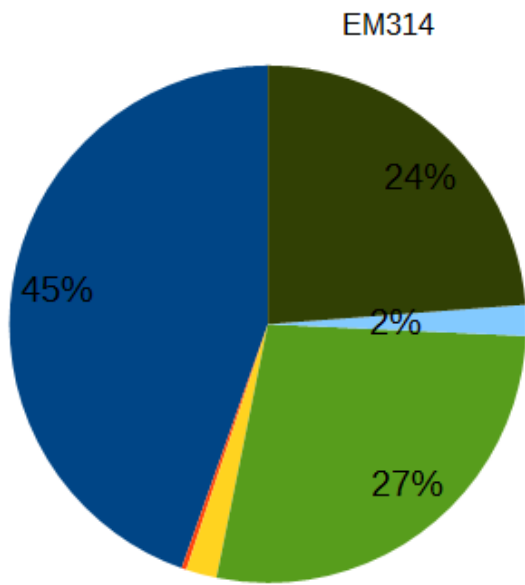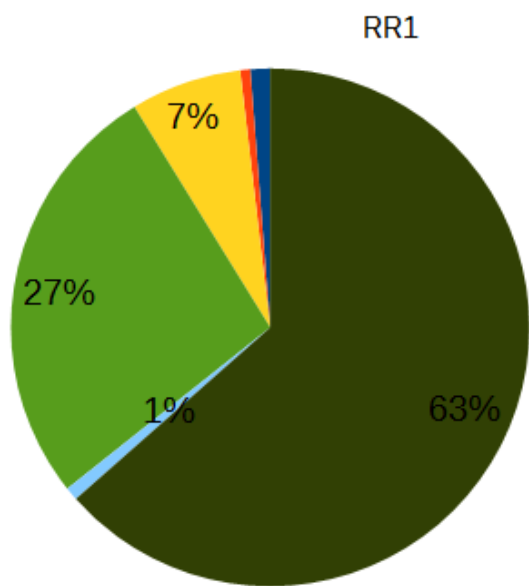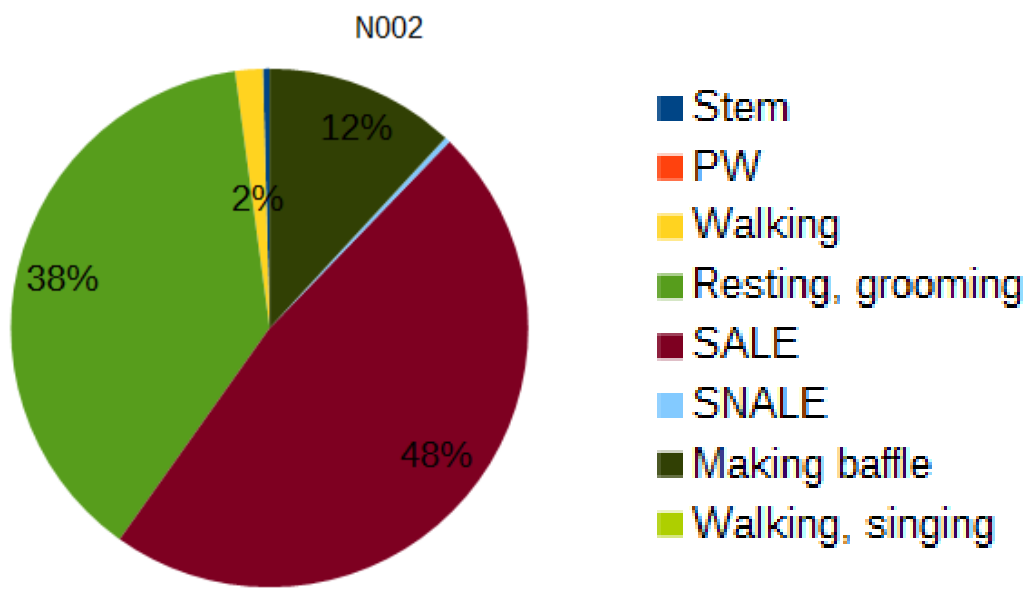
Activity Budgets on Small leaves

s001

22%   2%

75%

s002 (jumped off)

4%

96%

s003 (jumped off)

40%

2%   58%

■ Stem
■ PW
■ Walking
■ Resting, grooming
■ SALE
■ SNALE
■ Walking, singing
■ eating
■ x
■ z

X – STOL ; Z- Jump

s004 (jumped off)

s005

s006

Legend:
- Stem
- PW
- Walking
- Resting, grooming
- SALE
- SNALE
- Walking, singing
- eating
- x
- z

X- Stol ; Z -Jump

s007

s008

s009

Legend:
- Stem
- PW
- Walking
- Resting, grooming
- SALE
- SNALE
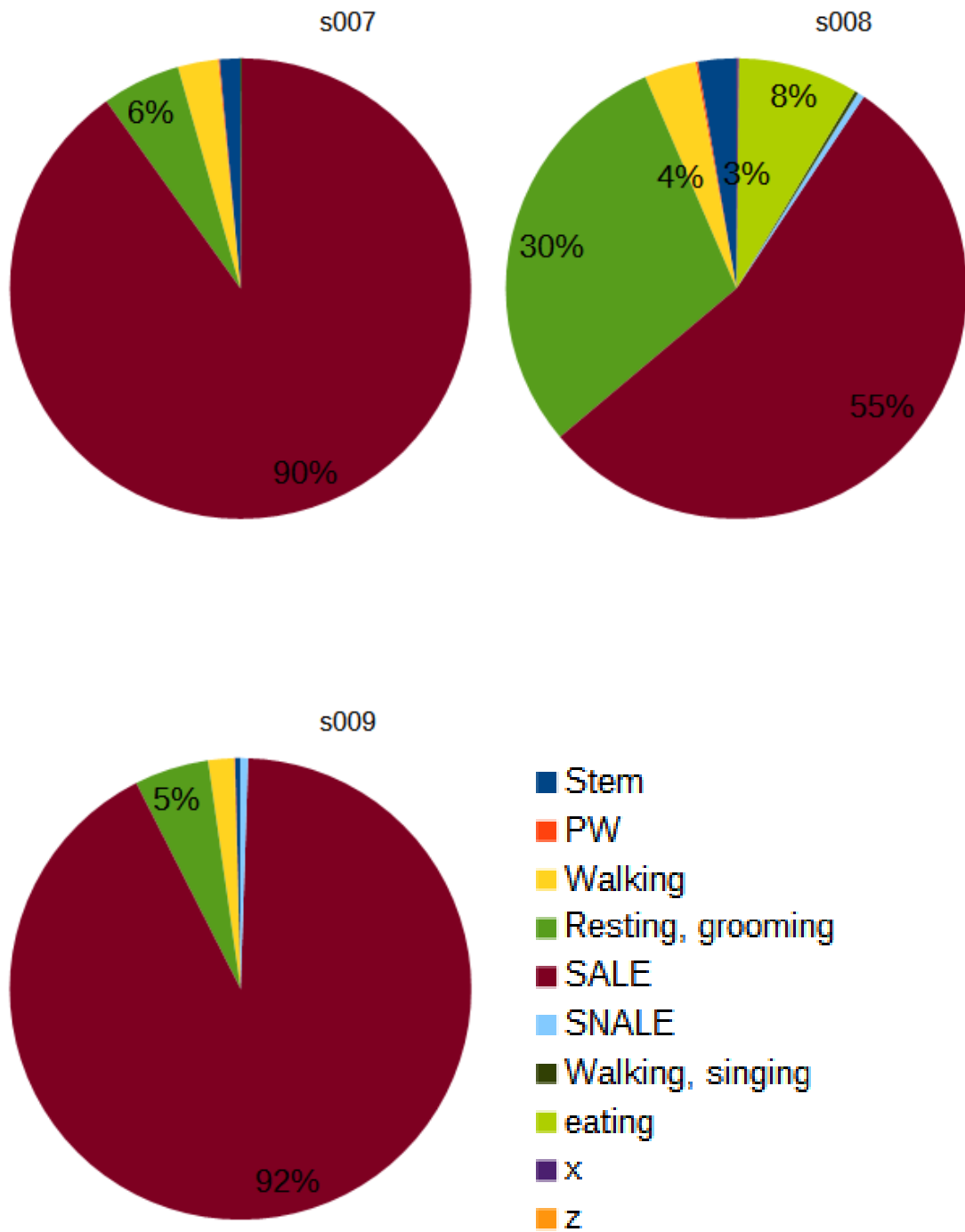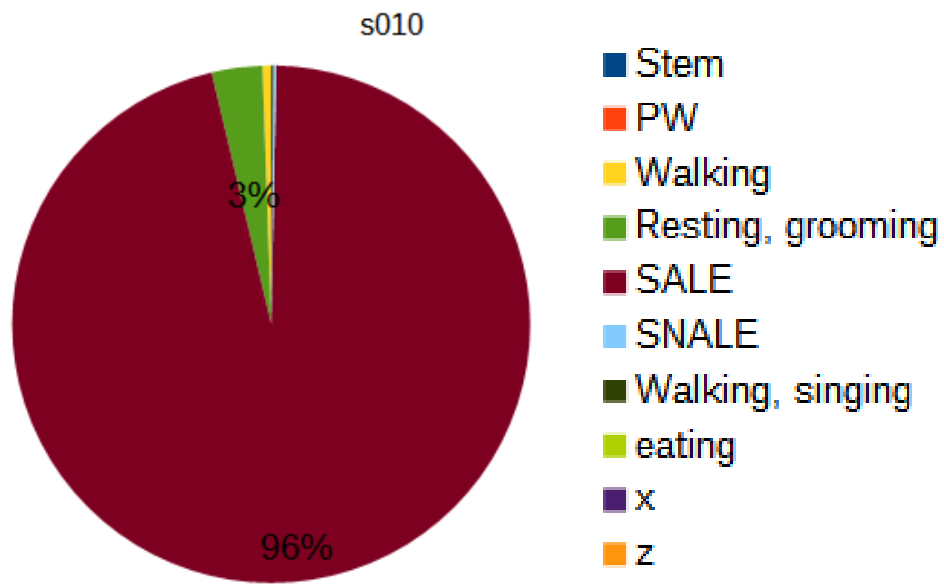- Walking, singing
- eating
- x
- z

X- STOL; Z -Jump

s010

Legend:
- Stem
- PW
- Walking
- Resting, grooming
- SALE
- SNALE
- Walking, singing
- eating
- x
- z

96%

3%

X- STOL; Z - Jump