

Finding an assay that reveals effects of sleep deprivation on decision making in *Drosophila*

by

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Statement of originality

I hereby declare that everything presented in this thesis is my own work or it was appropriately referenced. I have reported from experiments always designed and planned by me personally. The data was collected either by myself or by students that I supervised during my Phd as specifically acknowledged.

THIS THESIS IS DEDICATED TO MY BROTHER DAN, AND TO MY FRIEND CLEMENT. THE DEPTHS OF THEIR HEARTS HELPED ME TO KNOW MYSELF BETTER.

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Abstract

This study makes use of cutting-edge technology to tackle the intricate links between decision making and sleep in the *Drosophila Melanogaster* fly using various innovative behavioural paradigms.

The assays developed are iteratively improved to be suitable for high-throughput experiments and automatic video tracking of desired features. The final paradigm presents the fly with the simple decision of passing through two holes of different sizes: a large hole through which it can pass easily and a smaller one through which it can pass with some difficulty. Independently of the external conditions such as light, shape of the assay and time of the day or of the internal states such as genetic background and sleep state, all the flies choose to make multiple passages through both holes and unanimously show a preference for the bigger hole. Our paradigm is unique in that it allow us to assess flies decisions independently of the locomotion state by evaluating the proportion of passages through the two holes. Remarkably, this parameter is conserved in the same genetic background. Moreover, our results confirm and strengthen previous studies showing that locomotion is increased by space restriction in males.

When faced with our non-conventional paradigms, after prolonged sleep deprivation, flies suppress their sleep rebound. In addition, they show no behavioural differences to control flies. It appears that the sleep pressure built up through homeostatic regulation is not important enough to counteract the increased arousal state resulted from space restriction in order to trigger sleep. However, when experiments are performed in the afternoon during flies "siesta", they fall asleep, indicating that the sleep pressure arising from circadian regulation is more significant than the sleep pressure coming from homeostatic regulation.

Contents

COPYRIGHT DECLARATION	i
STATEMENT OF ORIGINALITY	ii
DEDICATION	iv
ACKNOWLEDGMENTS	v
ABSTRACT	vi
LIST OF FIGURES	xi
LIST OF TABLES	xii
o INTRODUCTION	1
o.1 Brief history of this study in time	1
o.2 Behaviour	2
o.2.1 What is behaviour?	2
o.2.2 Behavioural variability	2
o.3 Decision making	3
o.4 Sleep	4
o.4.1 Brief history of sleep science	4
o.4.2 Sleep and circadian rhythms	5
o.4.3 Sleep deprivation as a means for studying sleep	5
o.4.4 Two-process model	5
o.5 Sleep, decision making and mental illness	6
o.6 <i>Drosophila</i> : an atom of behaviour	7
o.6.1 <i>Drosophila</i> , frontliner of genetics research	7
o.6.2 <i>Drosophila</i> and behaviour	8
o.6.3 <i>Drosophila</i> and sleep	9
o.6.4 <i>Drosophila</i> and decision making	10
o.6.5 Link between sleep and decision making in <i>Drosophila</i>	12
o.6.6 Advantages of using <i>Drosophila</i> as an animal model	13
o.6.7 <i>Drosophila</i> as a model for psychiatric disorders	13
o.7 Advances in Technology	14
o.7.1 Hardware	14
o.7.2 Software	14
o.7.3 Genetics	15
o.7.4 Platforms used to study sleep and decision making in <i>Drosophila</i>	15
o.7.5 Limitations for studying behaviour in <i>Drosophila</i>	17
o.8 Lack of good behavioural assays	17
o.9 Scope of this thesis	17

I	Y-MAZE PARADIGM	18
1.1	Introduction	18
1.2	Materials and methods	20
1.2.1	Data analysis and statistics	20
1.2.2	Y-maze design	20
1.2.3	3D modelling and 3D printing	21
1.2.4	Sleep deprivation	21
1.2.5	Arena cleaning	21
1.2.6	Video recordings	22
1.2.7	Experimental setup and conditions	22
1.2.8	Experimental protocol	22
1.2.9	Video tracking	24
1.2.10	Workflow overview	25
1.2.11	Ethoscope - a flexible platform for monitoring behaviour	26
1.2.12	Data analysis	26
1.3	Results	26
1.3.1	Exit pattern of flies from maze to field	27
1.3.2	Exploratory behaviour in the maze	29
1.3.3	Fractals in behaviour	35
1.4	Discussion	38
2	CONCENTRIC CIRCLES AND HOLES	41
2.1	Introduction	41
2.2	Material & Methods	42
2.2.1	Fly stocks:	42
2.2.2	Experimental conditions:	43
2.2.3	Sleep deprivation	43
2.2.4	Models manufacture	43
2.2.5	Experimental protocol	43
2.2.6	Video recordings	44
2.2.7	Data collection and analysis	44
2.2.8	Mazes design	45
2.2.9	Technical developments	48
2.3	Results	56
2.3.1	Preliminary mazes examination, results and future directions	56
2.3.2	Preference for the big hole in the concentric rings maze	57
2.3.3	Groups of flies - maze exploration	59
2.3.4	Individual fly - maze exploration	62
2.3.5	Latency to enter the maze	62
2.3.6	Social influence	63
2.3.7	Size of the arena	64
2.4	Discussion	65
2.4.1	Hole preference	66
2.4.2	Effect of light	66
2.4.3	Sleep deprivation	66
2.4.4	Social influence	67
2.4.5	Anxiety	67
2.4.6	Restricted space	68
2.4.7	Software developments	68

3	BIG HOLE, SMALL HOLE	71
3.1	Introduction	71
3.1.1	Outcomes and shortfalls of the concentric rings assay	72
3.2	Materials and methods	73
3.2.1	Fly stocks:	73
3.2.2	Experimental conditions:	74
3.2.3	Mini rings, mini squares and plug stoppers manufacture	74
3.2.4	Sleep deprivation	74
3.2.5	Video recordings	74
3.2.6	Tracking	75
3.2.7	Data analysis and statistics	75
3.3	Refinement, validation and analysis of hole assay	76
3.3.1	Results	77
3.3.2	Discussion	93
3.4	Effect of sleep on hole choice	96
3.4.1	Results	98
3.4.2	Discussion	124
4	FINAL DISCUSSION	127
4.1	Technology	127
4.2	Randomness in behaviour	127
4.3	Development of own tools	127
4.4	Mini square arena - a simple decision	128
4.5	Large number of decisions	128
4.6	Confirmation of Heisenberg's theory	128
4.7	Confirmation that locomotion is increased by space restriction	129
4.8	Suppression of the sleep rebound	129
4.9	Sleep pressure vs space pressure	130
4.10	Reproducible results in different contexts	130
4.11	Drosophila as model for psychiatric disease	130
4.12	Limitation of this study	131
5	CONCLUSION	132
	REFERENCES	148

Listing of figures

1	Diagram of a decision making process	3
2	A two-process model of sleep	6
3	Lifecycle and morphological differences between sexes in <i>Drosophila melanogaster</i>	7
4	Sleep in <i>Drosophila</i> follows a two-process model	9
5	<i>Drosophila</i> Activity Monitor	16
1.1	Galton box	19
1.2	Y-maze arena	21
1.3	Experimental setup y-maze paradigm	23
1.4	Experimental protocol y-maze	24
1.5	Methods used to define regions of interest in the y-maze	25
1.6	Population and individual sleep profiles of female flies before and after the y-maze experiment	27
1.7	Exit distribution of male flies	28
1.8	Total distance walked in the y-maze	29
1.9	Y-maze Arena exploration	31
1.10	Number and duration of the activity and inactivity bouts in the y-maze	32
1.11	Number of intersections crossed	33
1.12	Time spent in intersections	34
1.13	Example of fractals	35
1.14	Self-similarity in the activity of <i>Drosophila</i>	36
1.15	Log-log plot analysis of the temporal pattern of activity in the y-maze	37
2.1	Ethoscope and concentric rings maze setup	43
2.2	Spherical ball design for studying the individual and population passing through holes behaviour	45
2.3	Consecutive discs paradigm to study passages through holes	46
2.4	Concentric rings maze design and dimensions	47
2.5	Mini ring maze	48
2.6	Accurate mapping of each individual region of interest	50
2.7	Flexible way of defining regions of interest of any geometrical shape	50
2.8	Identifying passages using subregions of interest	51
2.9	K-means clustering algorithm strategy	52
2.10	K-means multi-tracking algorithm in practice	53
2.11	Multi-tracking of males and females during copulation	54
2.12	Initiation of passages in population experiments performed in varying external and internal conditions	60
2.13	Big vs small hole preference in population experiments performed in varying external and internal conditions	61
2.14	Distribution of passages between concentric rings	62
2.15	Latency to enter the maze in individual flies	63
2.16	Latency to enter the maze for gradually increasing fly densities	64
2.17	Latency to initiate a crossing depends on the available space	65
3.1	Masks that define the region of interests	75
3.2	Mini rings arena	77
3.3	Longer time to pass through big hole than small hole.	78

3.4	Big vs small hole preference of wild-type flies (CS) in the circle arena	80
3.5	Square rings arena	81
3.6	Longer time to pass through big hole than small hole confirmed in mini square arena.	82
3.7	Big vs small hole preference of wild-type flies (CS) in the square arena	84
3.8	Mini rings versus mini squares arena crossings comparison	87
3.9	Mini rings versus mini squares arena distance walked and velocity comparison	88
3.10	No holes square arena vs ibig_ismall square arena	89
3.11	No_hole vs ibig_ismall number of passages	90
3.12	No_hole vs ibig_ismall distance and velocity	92
3.13	1 night sleep deprived flies vs control number of passages	97
3.14	1 night sleep deprived flies vs control, velocity, sleep and PCA analysis in the ibig_ismall maze type	99
3.15	1 night sleep deprived flies vs control, hole preference and distance walked in the ibig_ismall maze	100
3.16	3 days sleep deprived flies vs control, population and individual sleep profiles	102
3.17	3 days sleep deprived flies vs control, number of passages	103
3.18	3 days sleep deprived flies vs control, hole preference and distance walked in the ibig_ismall maze	104
3.19	3 days sleep deprived flies vs control, sleep architecture and PCA analysis in the ibig_ismall maze	105
3.20	CLK mutants vs CS wild-type in the ibig_ismall mini square maze, number of passages	108
3.21	CLK mutants vs CS wild-type in the ibig_ismall mini square maze, hole preference and distance walked	109
3.22	CLK mutants vs CS wild-type in the afternoon, number of passages	111
3.23	CLK mutants vs CS wild-type in the afternoon, hole preference and distance walked	112
3.24	Morning vs afternoon, number of passages and hole preference	114
3.25	Morning vs afternoon, distance and velocity	115
3.26	Population and individual sleep profiles in the CLK and CS flies during the morning and afternoon experiments	117
3.27	CLK vs CS sleep analysis	118
3.28	Morning vs afternoon sleep analysis	119
3.29	Correlation between distance walked and number of passages	121
3.30	Number of passages and distance walked in individual wild-type (CS) flies	122
3.31	Number of passages and distance walked in individual wild-type (CLK) flies	123

Listing of tables

1.1	Participation in the maze.	28
2.1	Multi-tracking Analysis	55
2.2	Multi-tracking Analysis (Continued)	56

O

Introduction

0.1 BRIEF HISTORY OF THIS STUDY IN TIME

This thesis focuses on the study of animal behaviour using emerging technologies available today on the market. The study of animal behaviour falls in the field of ethology. Even if naturalists have long been interested in the animal behaviour, the scientific world started to pay more attention to it in the late 19th century when Charles Darwin published his theory of evolution. The field became more consolidated in the 1920s thanks to the contributions of Nikolaas Tinbergen, Konrad Lorenz and Karl von Frisch. From its beginnings the field found itself at the intersection of several disciplines which included field work and neurobiology. The arise of technology offered new venues for the study of animal behaviour as it gives the experimenter the opportunity to observe and interact with the animals at an improved resolution. Furthermore, automation of processes enable the experimenter to increase the throughput of animals used, generating more data. This comes with its challenges. If more data means more precision and accuracy of one's results it also implies creating new methods of data analysis for extracting useful information. Technological advances offer a new wing to the study of animal behaviour also in the design of the experiments. Many studies of animal behaviour fields end up using the same experiment assay for decades. This can be quite risky because of the tendency of making general conclusions based on a unique context. Being able to reproduce results in different external contexts, adds validity to a study. Measuring the same variables in slightly different contexts brings up new perspectives and gives a new understanding of the animal's behaviour. In this study, several concepts used in software design have been applied to the design of experimental paradigms. In that sense the assays were changed, tested and adapted continuously. This study is an exploratory quest into how technology can help us shape the entire life-cycle of an experiment to produce more robust and reproducible results. It involves building a flexible workflow, made up of micro-components that can be each one easily adapted to new conditions.

0.2 BEHAVIOUR

0.2.1 WHAT IS BEHAVIOUR?

Behaviour is something that we do. It is the external output of something that is alive. Behaviour happens at the intersection of two worlds: the inside world of the entity performing the behaviour and the outside world in which the entity finds itself when performing that behaviour. The inside world is in itself a very complex environment governed by physiological needs like the need for water, food and sleep. The external environment in which a behaviour takes place feeds the entity performing the behaviour with an extensive range of feelings and sensations like the sensation of being cold or hot, the smell of food, the sight of a possible mate etc. These feelings and sensations are perceived by the entity performing the behaviour through a very complex set of receptors like tactile, olfactory and visual receptors that update its internal world in permanence, re-prioritizing what is the new behaviour to be performed. These receptors project their signals to the brain which decides what are the new behavioural decisions to be taken. Several definitions of behaviour exists some of them contradicting each other or being applicable only to specific taxa. An effort has been made to search for a succinct and widely applicable definition of behaviour which is: *Behaviour is the internally coordinated responses (actions or inactions) of whole living organisms (individuals or groups) to internal and/or external stimuli, excluding responses more easily understood as developmental change.*¹

0.2.2 BEHAVIOURAL VARIABILITY

Behaviour is highly variable. Genetically identical individuals under the most rigorously controlled conditions of pressure, temperature, humidity and other variables still display differences in their output behaviour. Even same individuals display a variability in their output response to the same sensory information.² It is not clear through which mechanisms the inter-variability (between different individuals) and intra-variability (between the same individual) are produced.

Potential sources of intra-variability can arise from the external world where very small differences in conditions can give rise to complex dynamics that get propagated. Another potential source for the variability is the internal state of the individual, that can change quickly through time. Many regulatory mechanisms exists in the organism that are responsible for maintaining internal conditions such as body temperature, fluid balance, blood sugar level or sleep in a state of equilibrium. Therefore, even small changes in the internal world of an individual can be at the source of the behaviour variability.³

Another important source of variability comes from randomness or noise, that is present in the behavioural loop at many stages as external information is not perfectly passed to cells and further to the central nervous behaviour.⁴

Recent studies have also looked at how genetics control the phenotypic variability, suggesting that the phenotypic variability in itself is a trait that can be heritable³. Finally, there a few studies suggesting the existence of a non-linear deterministic endogenous initiator of spontaneity inside each organism responsible in part for the behavioural variability.

Harvard's Law summarize the above by stating:

"Under the most rigorously controlled conditions of pressure, temperature, volume, humidity, and other variables, the organism will do as it damn well pleases"

Behavioural variability is essential to be taken into consideration when planning experiments and it is one of the main reasons for which a high-throughput of animals is desired to validate results.

0.3 DECISION MAKING

Decision making is the process through which an organism selects actions based on the possible values or outcomes that he expects from those actions. An effective decision making is aiming at choosing the most advantageous outcome in a particular context. The decision making process is influenced by all the external environmental cues specific to that context such as temperature, light etc. and by all the internal states specific to that organism such as hunger, lack of sleep, fear, love, anger etc. Every decision is also accompanied by some randomness, which it is believed to be endogenously generated^{5,6}.

Figure gives an overview of the decision making process.

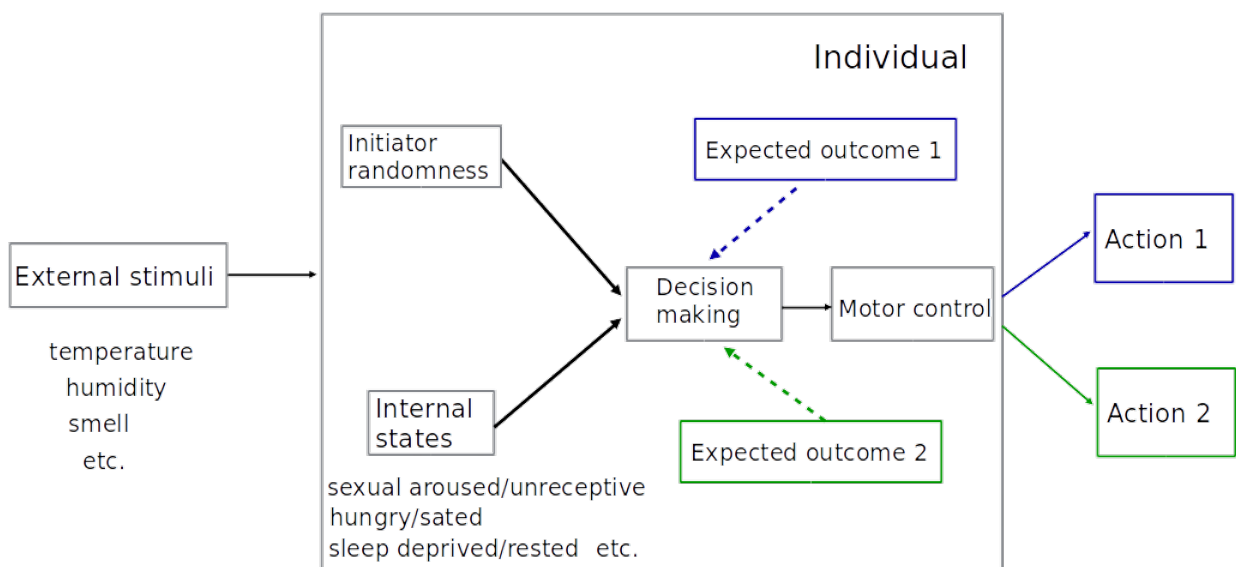


Figure 1: Diagram of a decision making process A decision making process selects a certain action by evaluating expected outcomes for possible actions and going for the most advantageous one. To do that it integrates external factors, internal state and some specific randomness.

In humans, decision making is often studied using a two-alternative forced choice (2AFC) which is a method developed by Gustav Fechner⁷, and used in behavioural experiments to assess an individual's subjective experience. It consists in presenting the subject with two different possibilities from which one has to be chosen, and then analyse the pattern of choices and reaction times. Different 2AFC tasks have been used predominantly in the field of decision making, which lead to the development of several formal models. One example is the drift-diffusion model⁸, generally used for the perceptual decision making tasks. In this model a subject is accumulating noisy evidence at each time step and integrates this evidence until a decision threshold is reached. There are several studies in monkeys that started to shed light on how decisions form and how they are implemented in the brain.³ One such task involves motion discrimination of a set of random dots out of which a percentage move together in one direction (up or down). The subject makes a choice of the direction of movement using a saccade. Recording from the parietal lobe, lateral intraparietal cortex (LIP) shows that neuronal population sensitive to the two possible choices, increase firing at stimulus onset⁹.

0.4 SLEEP

Sleep is a behaviour consisting of an altered state of consciousness that is recurring and is characterised by a decreased ability to react to the stimuli, a particular position, and specific brainwaves. Its function are poorly understood.

0.4.1 BRIEF HISTORY OF SLEEP SCIENCE

Historically, humans were always aware of the essential importance of sleep in their lives. The idea that the main purpose of sleep is to get rest is deeply rooted in the collective consciousness. It is just recently, that a scientific approach is being used in order to examine what is the function of sleep, and what is its importance and interaction with specific physiological processes. This section presents some results that can be considered as pillars to the foundation of sleep as a well defined field. Progress has been done on two fronts that merge into each other: the study of sleep itself and the study of circadian rhythms. William Dement, the pioneer of sleep medicine, humorously called the period before 1952 as 'pre-historic', for its lack of experimental methods and tools to study sleep.¹⁰

0.4.1.1 SLEEP SCIENCE PROGRESS

Often in the history of science, the invention of a new tool precedes the birth of a new field. In the case of sleep it was the invention of electroencephalography (EEG) by German psychiatrist Hans Berger, in 1924 that enabled scientists to measure and record "brain waves" in a non-invasive manner. Not long after, in 1937 Frédéric Bremer¹¹ used EEG to study sleep and understood that just by looking at the frequency of the EEGs, one can know if a person is awake or asleep. Moreover, in 1952, Kleitman and his student Aserinsky noticed that during sleep there were periods in which the eyes were moving rapidly back and forth, to which they coined the name "rapid-eye movement" or REM sleep. REM sleep is characterized by rapid-eye movement together with low muscle tone, giving rise to low-voltage, desynchronized brainwaves, very similar to the recordings during waking state. They also showed that this stage of sleep appears while dreaming.¹²

In 1968, the first EEG manual for scoring sleep was published. Based on the amplitude and frequency of the EEG, the sleep cycle was divided into four non-REM stages of sleep plus REM sleep.¹³ Our deepest sleep happens in stages 3 and 4, also known as deep sleep, which distinguish themselves by the presence of slow-waves. In this period of sleep the activity of the EEG is synchronized giving rise to slow-waves with a frequency range of 5 Hz-10 Hz and an amplitude greater than 75 μ m. On contrast with REM sleep, deep sleep is characterized by slow and absent eye movements accompanied by moderate muscle tone.

0.4.1.2 CIRCADIAN RHYTHMS PROGRESS

In 1729, the beginning of research on circadian rhythms was led by French astronomer Jean-Jacques d'Ortous de Mairan who observed that the *Mimosa pudica* plant will continue to rhythmically open and close its leaves even in constant darkness.¹⁴

Two centuries later, in 1929, Erwin Bunning, put forward the idea that circadian rhythms are not governed entirely by external cues, as previously believed. He proved that there is an internal peacemaker, sen-

sitive to light, in charge of the circadian rhythms. Moreover, he demonstrated the existence of clocks across species including plants, flies and fungi. His experiments also suggested that clocks are heritable.¹⁴

This was essential to understand that there is an endogenous clock governing rhythmic processes like eating, sleeping, mating etc. in living things and dismisses the idea that these rhythms were simply the result of environmental cues. Sleep, is a rhythmic process, controlled by an internal clock in a circadian manner.

0.4.2 SLEEP AND CIRCADIAN RHYTHMS

In humans, the circadian rhythms are controlled by a small region of the hypothalamus called the suprachiasmatic nucleus (SCN)¹⁵, also called the master clock because of its responsibility of coordinating all the other peripheral clocks.¹⁶ When light arrives on the retina, a set of photosensitive ganglion cells from the retina can directly inform the SCN which therefore can use direct light from the external environment to entrain and synchronize an organism's daily rhythms to the 24-hours light cycle. It is light travelling from the outside to the inside, that can be considered as the main factor responsible for regulating an organism's behaviour from the micro level to the macro level. The clock system is adaptable and in the face of varying external conditions it can reset. A good example of clock reset is the jet lag that people experience when changing time zone. It has been shown that repeated shifting of the dark and light cycle results in long term deficits of the learning and memory functions of the brain together with less neurogenesis in the hippocampus¹⁷. Generally, people that experience chronic circadian shifting, like shift workers or airline workers are known for experiencing mental and mood alterations¹⁸.

0.4.3 SLEEP DEPRIVATION AS A MEANS FOR STUDYING SLEEP

The record for the longest recorded sleep deprivation belongs to Randy Gardner, who in 1963, at the age of 17 years old decided with his friend to beat the world's record for staying awake which was just under 11 days at the time. Failing to do this by themselves they contacted one of the only sleep researchers at the time, William Dement from Stanford to help them out, and he stayed awake for 11 days and 25 minutes. During this experiment, Randy's physical and cognitive abilities were continuously monitored. Unexpectedly, Randy demonstrated no physical impairment, no major psychotic episode or emotional change during the entire experiment. He recovered fully after his first sleep following the experiment which was of only 14h and 40 minutes, which was abundant in REM sleep.

0.4.4 TWO-PROCESS MODEL

In the last 30 years, Borbély's two-process model was widely accepted as a good framework for understanding sleep regulation¹⁹. This model considers that sleep has a circadian component (Process C) and a homeostatic component (Process S) each one regulating independently the drive to sleep. The homeostatic drive increases sleep pressure gradually during the day being maximal when we fall asleep. The circadian drive is oscillating in phase with the light cycle and it is responsible for the oscillation of our energy throughout the day. The two process work in opposition with the circadian component being mainly responsible for the sleep during the day when the homeostatic drive is still low. At the beginning the night the reverse happens and the homeostatic drive is at its maximum

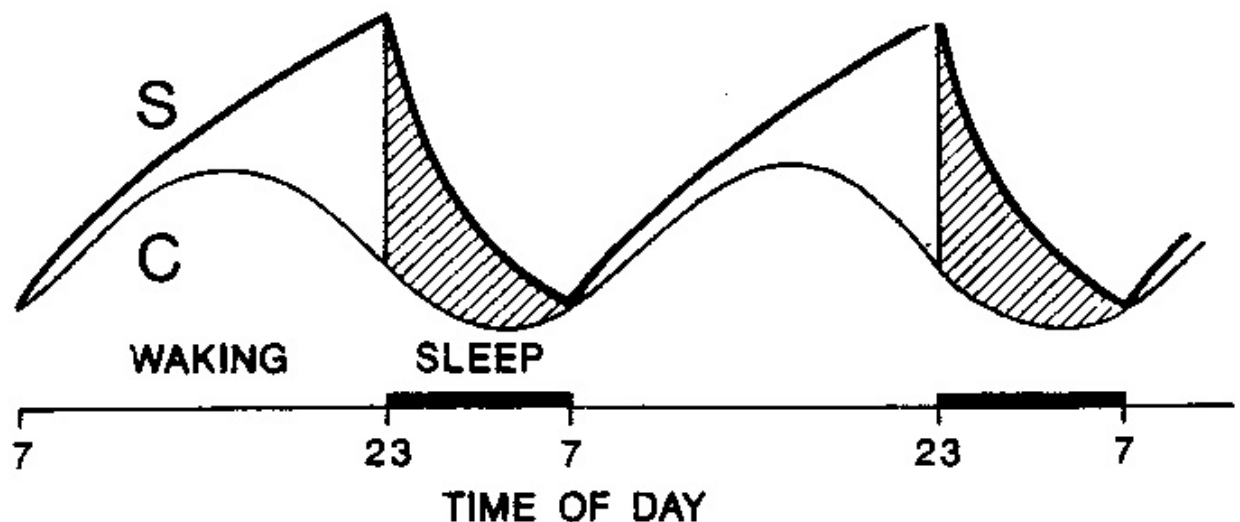


Figure 2: A two-process model of sleep A generally accepted model explaining the drive to sleep by the means of two processes: a circadian drive (process C) and a homeostatic drive (process S). Process S increases the need for sleep the longer we have been awake, and Process C is in phase with the light cycle and it prepares us to sleep at the beginning of the night and to wake up in the morning. Process C is responsible for alertness and tiredness during the day and the combination of these two processes explain why we are not gradually becoming more and more asleep during the day, but we are oscillating between peaks of energy. Adaptation sourced from <https://commons.wikimedia.org/wiki> under the Creative Commons license.

0.5 SLEEP, DECISION MAKING AND MENTAL ILLNESS

There is an intimate link between circadian rhythms disruption and mental illness, in particular depression, mania and schizophrenia²⁰. All these neuropsychiatric disorders show massive disturbances in the sleep patterns^{21,22,23}. It is unclear through which mechanisms chronic circadian disturbance result in such conditions.

These diseases show deranged internal and external characteristics of the persons affected. In humans, speech is the most relevant correlate of the internal state of an individual, and the actions of a person are probably the best external read of its state. In healthy individuals there is a perfect multi-level coordination that takes place both internally and externally. But speech and action can be seen as macro-outputs that can be decomposed in smaller elements. Speech is made out of words that come together in a specific order, or grammar, and there is a very fine coordination that happens for someone to make sense. Similarly, the locomotion of a person can be looked at as a macro-behaviour that can be decomposed in several sub-behaviours: the locomotion of legs, arms, neck, fingers. Everything needs to be perfectly coordinated in order for the whole body movement to be in harmony. In people with schizophrenia, two main symptoms are: disorganised speech and disorganised behaviour, which clearly show a lack of internal and external coordination which might be rooted in their disrupted circadian clocks.

People with psychiatric disorders are also known to take bad decisions²⁴ and often lack motivation for taking action²⁵. This can be caused by an impairment in the initiation of action mechanism²⁶. It has been shown that mental diseases can be seen like a salience dysfunction syndrome²⁷, as the brain of these ill people perceives the salience of stimuli that it receives from the outside world in a deformed way. It is not surprising that if their attention is dispersed on things that should normally be ignored, and if they are trying to make sense of those things, their decision-making ability becomes highly affected. In order to take a

decision, the brain has to operate with the variable on which our attention lie. In people with mental illnesses there must be a struggle to make sense of specific information put forward by their brain.

0.6 *DROSOPHILA*: AN ATOM OF BEHAVIOUR

Drosophila melanogaster, also known as the fruit fly or the vinegar fly is a species of fly in the family Drosophilidae, commonly observed in kitchens around fermented food, especially rotten fruit. It has a body size of 2-3 mm and a brain size of approximately 100,000 neurons. *Drosophila* has been used for over a century in biological research like genetics, behavioural and neuroscience studies. The fly also showed its great potential of producing breakthrough results in Medicine. Since the beginning of the 20th century, research using *Drosophila* received 8 Nobel prizes for outstanding work achievement in Medicine and Physiology²⁸. The lifespan of the wild-type *Drosophila* in laboratory conditions, at 25 °C can go up to almost three months, however it is greatly variable between individuals and under the influence of many different factors including diet, light-dark cycle, temperature, humidity and the presence of conspecifics.^{29,30,31,32,33} The life cycle of *Drosophila* takes on average one to two weeks, at room temperature. It consists of a complete metamorphosis and it goes through four main developmental stages: egg, larva, pupa and adult. (Fig. 3 A) A wild-type female can lay hundreds of eggs daily.

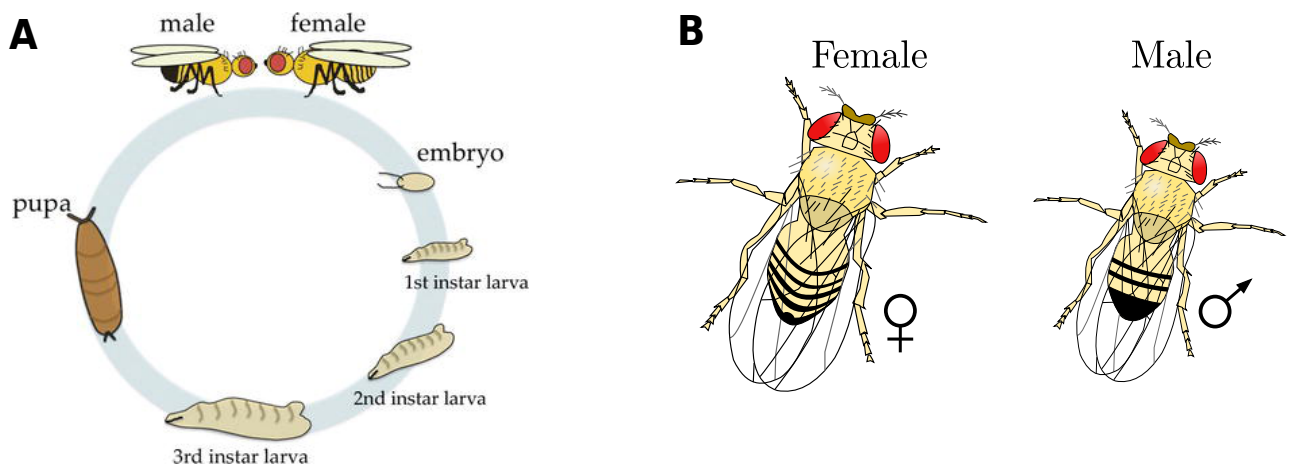


Figure 3: Lifecycle and morphological differences between sexes in *Drosophila melanogaster* **A** Metamorphosis of the fly from embryo to larva, then into pupa and finally emerging as an adult. Image reproduced under the Creative Commons license³⁴. **B** An illustration of morphological differences between male and female flies. Adaptation sourced from <https://commons.wikimedia.org/wiki> under the Creative Commons license.

The sex of the flies is easy to recognize, even with the naked eye as males are smaller in size than females and they have a very distinguishable black and rounded back. (Fig. 3 B)

The genome of *Drosophila* has been completely sequenced and contains only 4 pairs of chromosomes, with the first pair being a sex chromosome.

0.6.1 *DROSOPHILA*, FRONTLINER OF GENETICS RESEARCH

In the late 1800s, the monk called Gregory Mendel made a revolutionary observation regarding the way in which physical traits are passed over from one generation to the other³⁵. He bred the common pea plant and followed how characteristics like plant height, seed shape and colour were inherited. This enabled him to establish the basic principle of heredity of dominant and recessive discrete inheritable units,

that will later be assigned the term of "genes". Thomas Morgan was inspired by the humble fruit fly and used it as an animal model to study genetics. He considered *Drosophila* an excellent model because of its size (the fly is small enough to be able to run large experiments, but big enough to be easy to observe and handle), fast breeding, and the ability of easily distinguishing between the male and the female. He used milk bottles to grow the flies and yeast and banana to feed them. His first major breakthrough consisted in the discovery of the white-eye mutant³⁶, which was a spontaneous mutation on the eye-colour gene situated on the X chromosome. By breeding *Drosophila* white eye mutant with normal wild-type flies he was able to observe an different pattern of inheritance of the white-eye trait that did not match Mendel's law. An analysis of the transmission of the mutation over several generation, led him to the elucidation of the role that chromosomes play in heredity, for which he received a Nobel prize in 1933.³⁷

The interest in *Drosophila* grew considerably with the finding that *Drosophila* shares 65% of its genome with the humans making it a powerful system to investigate humans genetic diseases^{38,39}. *Drosophila* has shown potential in studying neurodegenerative disorders like Alzheimer's⁴⁰ and Parkinson's⁴¹. Moreover, it is believed that almost 75% of the genes responsible for human diseases have an homolog in flies.⁴²

0.6.2 *DROSOPHILA* AND BEHAVIOUR

The advent of genetics prompted scientists to ask what is the interaction between the genes and the behaviour? If genes are able to encode physical traits, and if there are single genes that match with unique characteristics of an individual, does it mean that there are single genes that correspond to specific behaviours? In 1970s, Seymour Benzer, physicist by formation, got attracted by the molecular biology revolution that was taking place at the time, and decided to use *Drosophila* as a model for studying behaviour.

Drosophila exhibits various complex behaviours such as the courtship behaviour in which the male fly goes to the female and sings a song with its wing to impress her. The female decides weather to accept or deny the male, depending on whether she likes the song and his looks. If she accepts him, they will copulate otherwise she runs away⁴³. Also, two males that are courting the same female will show an aggressive behaviour in which they extend their wings and hit each other with their forelegs similarly to a boxing match.^{44,45}. Moreover, *Drosophila* proved to have some sort of cognitive skills, being able to learn specific behaviours and remember them during their life⁴⁶. Females also show a maternal response as they decide very carefully where to lay their eggs, in order to ensure the best nutriments for their progenitors. Most relevant to us, flies show specific rest and activity periods that resemble human sleep⁴⁷, which seem to be in intimate connection with most of the other behaviours^{48,49}.

It is amazing that the small brain of the fly is able to produce such a diverse repertoire of behaviours. For this reason, Benzer saw in the fly an atom of behaviour. The complex behaviours of the fly have close correspondents in the human behaviour, which suggest the possibility of matching homologous genes and molecular mechanism of behaviour between the fly and humans. The method introduced by Benzer in order to understand the molecular basis of behaviour consists in isolating mutants that show a specific behavioural phenotype and use specific genetic tools to understand which genes and what neural network are responsible for that phenotype, and therefore part of the mechanism that performs the normal behaviour. This approach called forward genetics is the most used up until today, but more genetic tools are available, allowing a better resolution and manipulation of the genes and neurons of flies.

Benzer and his students developed many assays that helped them to identify genes involved in those behaviours. These include the countercurrent machine to investigate the phototactic behaviour of the

fly⁵⁰, olfactory-based shock avoidance assay to genetically dissect learning and memory⁵¹ and using infrared light to measure activity in *Drosophila* in order to understand the sleep in *Drosophila*⁵². His assays became standards and many labs still use them today, of course, with some technical improvements but keeping the original core ideas.

So how far did we get after almost 50 years of trying to understand the molecular basis of behaviour using *Drosophila*? First of all, it is clear that single genes do not determine a specific behaviour, but rather they are involved in the development of the parts of the brain that are responsible for producing that specific behaviour⁵³. The complete circuits of some very simple reflex responses⁵⁴ of the fly have been identified and understood, but none of the more complex behaviour exemplified above has been entirely elucidated. Some believe that this is due to the fact that complex behaviour involves many overlapping circuits which makes their identification a very difficult task, while others believe that the approaches used in the neurogenetics might not work because of some deep dogmas that dominate the field but that might not match reality. An example of such a dogma would be looking at organisms as input-output systems instead of focusing on how they select their actions based on their outcomes expectations⁵⁵.

0.6.3 *DROSOPHILA* AND SLEEP

It is believed that *Drosophila* flies sleep because their resting periods share all the characteristics of mammalian sleep: a reduced activity, a circadian and homeostatic component regulating their sleep (Figure 4), a typical posture⁵⁶, a decreased arousal⁵⁶ and their sleep is a state relatively easy to reverse (compared to coma or hibernation) by applying a sufficiently high stimulus to wake them up.

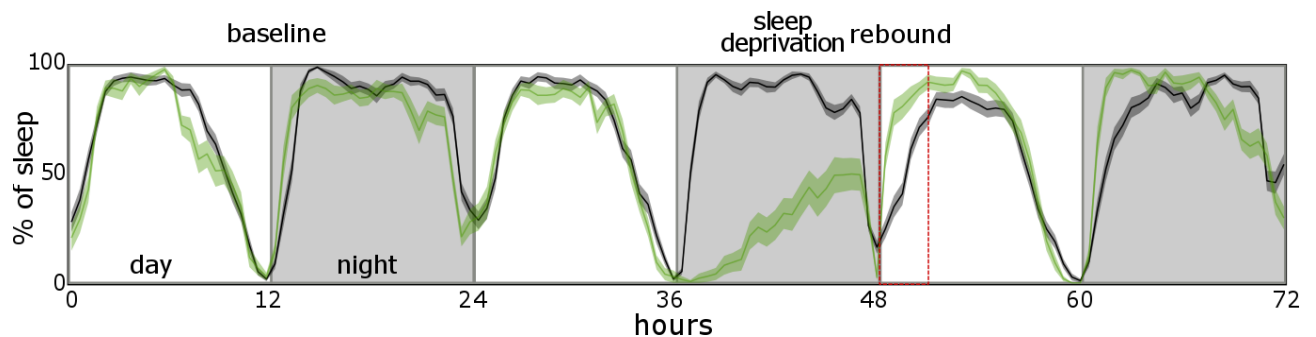


Figure 4: Sleep in *Drosophila* follows a two-process model A sleep ethogram showing the percentage of time flies are asleep during three consecutive days in a 12h light : 12h dark regime. Sleep is defined as an inactivity period longer than 5 minutes. Two groups of flies are shown a control group (grey) and a sleep deprived group (green). The sleep deprived group undergoes mechanical sleep deprivation during the second night and shows a longer sleep on the following day, shown in red. This is an indication of the homeostatic regulation of sleep. The movement pattern follows a circadian pattern, with flies showing an increased sleep during the night and a "siesta" in the middle of the day. Flies activity peaks at the transition between the two light regimes. (Reproduced with the permission of Esteban Beckwith)

Sleep in *Drosophila* follows a two-process model showing a circadian regulation and a homeostatic regulation, that act independently (Figure 4), and is modulated by various internal states and external factors.

From the internal states, the circadian clock of the fly is the major regulator of sleep as flies pattern of sleep presented in (Figure 4) does not change in the absence of light. The circadian clock is involved in the hormonal, physiological and behavioural rhythms of mammals. Circadian oscillators can be found at many different levels in the organism and they are coordinated by a central pacemaker. They tick autonomously and orchestrate behaviours from the micro universe found at the cell level to the macro universe found at the organism level^{57,58}. In *Drosophila*, circadian clock is involved in locomotion by control-

ling the activity and quiescence of flies. Circadian clock is also responsible for the eclosion time, it coordinates the courtship behaviour and the timing of feeding^{59,60}.

Seymour Benzer and Ronald Konopka were the first ones to identify a clock gene, and open the gate to study the mechanisms of circadian clocks at the molecular level. By using a forward mutagenesis screen they were able to identify the *per* gene⁶¹. Further, another student of Seymour Benzer, invented the first device to monitor the activity of the flies using infrared light to assess the passing of the flies to the middle of the tube, the same technique adopted by Trikinetics for the DAM monitors 0.7.4.1. The main advantage of looking at activity instead eclosion consists in the fact that eclosion is a one time event, therefore by using the infra-red device Benzer and Konopka were able to prove that *per* gene was not specific to eclosion, but was also responsible for controlling the activity of the flies.

The chronobiology field uses a special nomenclature to describe the circadian behaviour of the fly called “zeitgeber” (ZT), by which time is defined based on light or other environmental cue with ZT0 corresponding to the onset of light and ZT12 corresponding with the onset of darkness.

A strong sexual dimorphism is observed in the sleep of the flies, with males sleeping for longer periods overall, and females showing an increased activity especially during the day⁵⁶.

Sleep is also modulated by other internal states such as hunger, as starved flies show a decrease in their sleep⁶². Diet in general it is known to have an important impact on flies sleep⁶³. Moreover, the sexual state of a fly modulates sleep with sexual aroused males being able to abolish their sleep rebound normally observed after a night of sleep deprivation⁶⁴. The amount and architecture of sleep are age dependent, with young flies sleeping more than adults which also show a more fragmented sleep⁶⁵.

From the external factors, temperature is an important modulator of sleep. In laboratory conditions flies are usually kept at 25 °C. They can also survive at higher temperatures that go up to 29 °C. High temperatures change flies sleep profile by modifying both the amount and architecture of their sleep: they increase their sleep during the day having longer resting inactivity bouts, while their night sleep is diminish and more fragmented^{66,67}.

0.6.4 DROSOPHILA AND DECISION MAKING

Drosophila flies exhibit behaviours involving rather complex decisions, some of them being sex specific: flies have to decide what to eat and how to find potential food sources, male flies decide who to court and females decide whether to accept a male or not, based on context males can decide to initiate a fight with another male and females are in charge of selecting what is the most appropriate medium to lay their eggs. These natural decisions are modulated by many environmental cues. For example, before accepting or rejecting a male, a female integrates information about his smell, the specific songs that he sings to impress her and his overall looks. The complexity of this decision makes it very difficult to understand what are the natural pathways that govern flies behaviour and an effort is being made to find simple models to study decision making.

From the natural behaviours of the flies, the choice of the oviposition site by females flies has been recently addressed in the context of decision making because of its simplicity. Females assess the nutritional value of available food patches and make sure to lay their eggs on the sites that will ensure the optimal survival of their progeny⁶⁸. While the decision making mechanism involved in this choice is still poorly understood, recent studies revealed that the amesiac mutant shows defects in the egg-laying decisions and was able to identify two neurons that modulate flies choices⁶⁹.

Another natural behaviour investigated in the context of decision making is the escape behaviour, the sequence of movement that a fly performs in the presence of a predator. Several teams at Janelia Research Campus (institution pursuing fundamental science research) collaborate to put together the state of the art genetical and technological tools in the hope of identifying the complete sets of neurons involved in this behaviour. Progress is slow, but they identified a large set of descending neurons responsible for activating and modulating locomotion and generated transgenic lines targeting individual neuron cells⁷⁰.

Finally, olfactory decisions are the natural behaviours that led to the most advanced understanding of the neural mechanism in charge of integrating information and selecting an action^{71,72}.

Olfactory decisions play an essential role in many important behaviours like feeding, reproduction and navigation. The olfactory research has been very attractive given that the sensory periphery of the fly is very well understood, and most of the olfactory receptor neurons have been identified and have complete molecular descriptions⁷³. Moreover, it's starting to be well understood how these receptor neurons project to the next processing area, called the antennal lobe and little is known about how the projection neurons from the antennal lobe send information to higher olfactory centres⁷⁴.

Dasgupta *et al.* showed that flies integrate olfactory information coming from the olfactory receptor neurons in order to make a choice. They came up with a discriminative odour choice assay and trained the flies to be repelled by a particular concentration of the odorant 4-methylcyclohexano by pairing it with electric shocks. By presenting the flies with two odours one that has the same concentration used using training and one that has a lower value, they demonstrated that the flies were able to choose quickly and accurately when the lower concentration was much lower than the trained one. However, if the two odours had similar concentration, the decisions became harder and the flies needed more time to accurately decide which one they should go for⁷¹.

Further, by conducting a genetic screen the FOXP gene was identified as playing a critical role. The flies lacking FOXP gene in a specific set of neurons, called " α " β c Kenyon cells, located in the mushroom body, which is a higher region where the neurons from the antennal lobe project to, took longer to make decisions. This suggests that the FOXP transcription factor in these specific neurons modulate the decisions timing.

Moreover, in a recent study, Groschner *et al.*⁷² looked in greater detail at the Kenyon cells identified in the previous mentioned study. Firstly, they sent electric shocks to the antennal lobe and looked at how the signal was propagated to the " α " β c Kenyon cells, whose response was measured using a genetically expressed calcium indicator. They found that the response of wild type flies increased faster and saturated at bigger amplitudes than the response of FOXP mutants. To better understand why this is happening, they performed single-cell patches and showed that in the FOXP deprived flies, the pulse trains could not accumulate efficiently because of current leakage.

Further, they showed that " α " β c Kenyon cells in the FOXP flies have currents leaking because they expressed more voltage-gated potassium channels Shal. They were able to show that by lowering Shal expression in the FOXP flies, the decisions times of the FOXP flies lowered as well, being similar to decision times of the wild-type. Also, by expressing more Shal in the wild-type " α " β c Kenyon cells, they increased the decision time of these flies making them similar to those of FOXP mutants.

The above mentioned studies approach decisions as the response of flies to a certain stimulus. Heisenberg suggests that this description might be limiting in understanding the means by which brain generates decisions and proposes that closer look should be given to how flies initiate actions based on expected out-

comes⁵⁵.

Probably the most simple decision that one can address when investigating the initiation of action is the stop-go behaviour. Several studies^{75,76,77,78} have examined the specific characteristics of flies activity in an open and unconstrained space. The temporal pattern observed in flies movements shows a complex and non-linear organisations of intermittent activity and inactivity periods, following a structure order. It is believed that there is a neural mechanism, the central pattern generator, responsible for sprinkling with some randomness every behaviour. If this is true, the behavioural variability specific to any behaviour is inherited in each individual and must have a specific biological function.

Another study⁵ confirms and extends these results by the means of a more sophisticated paradigm called a flight simulator. In this assay, the fly is attached with the help of a string to a torque-meter and can fly stationarily performing left and right turns. A mathematical analysis of the sequence and timing of fly's specific turns reveals that random noise is not the only source causing behavioural variability and it suggests the existence of an initiator internal to the fly able to generate behavioural variability.

The stop-go behaviour and the body-movements of the tethered flies are both capturing the initiation of movement made by the flies in the absence of any cues. They are the most raw output of flies behaviour when no stimulus is offered to them. Another paradigm able to capture the initiation of movement based on an external stimulus information is the gap-climbing behaviour⁷⁹. This assay uses high-definition cameras to analyse from close the behaviour of *Drosophila* when faced with gaps of different sizes. Flies estimate the width of the gap before attempting to reach the other side. This study is capturing a simple decision in which *Drosophila* initiates actions based on an external stimuli. A large screen using an improved paradigm modified for high-throughput was able to identify flies that try to climb unsurmountable gaps and used them to identify neurons specific to visual-distance estimation⁸⁰. The gap-climbing decision could be used to further study how flies select their actions based on outcome expectations by presenting the flies with multiple gaps of different widths and investigate their choices.

The stop-go behaviour and the body-saccades of tethered flies also confirmed the internal generated randomness specific to action selection in the absence of any outcomes. The most relevant paradigm able to capture initiation of action in *Drosophila* and to analyse flies selection of actions based on various possible expected outcomes, it is the flight simulator. It is the same device used for studying the body saccades of tethered flies. The fly is attached to a torque meter and surrounded by a cylindrical screen on which the experimenter can project different combinations of visual patterns. Flies respond to those patterns by generating left and right turns and trying to stabilize their flight.

0.6.5 LINK BETWEEN SLEEP AND DECISION MAKING IN *DROSOPHILA*

There are a few studies investigating the interactions between sleep and decisions in *Drosophila*. One such study suggests that following a night of sleep deprivation, flies are less likely to initiate a fight.⁴⁸ Another study is able to demonstrate that sleep deprivation affects visual decisions in *Drosophila*. A modern paradigm is employed to present flies with competing foreground and background visual cues. Sleep deprived flies are able to respond similarly to control if only one visual stimuli is presented, however in the presence of other background cues sleep deprived flies are more distracted⁸¹. This study makes the link between attention, decision and sleep. The attention can be seen as the precision with which one is able to initiate an action. A lack of attention is possibly caused by an inefficient decision mechanism which becomes unstable due to sleep deprivation. While this study captures a true effect of sleep deprivation on the

decision making mechanism it employs a sophisticated apparatus to assess the behaviour of *Drosophila*.

0.6.6 ADVANTAGES OF USING *DROSOPHILA* AS AN ANIMAL MODEL

Drosophila melanogaster offers many advantages: the fly is small, easy to breed and cheap to maintain. It can easily be raised in the laboratory in large numbers, as female flies can lay up to 100 eggs per day⁸². Its life cycle is short, taking approximately 12 days at 25 °C allowing large number of crosses to be followed during several generations in only a few months⁸².

Another important advantage of using *Drosophila melanogaster* is the vast amount of knowledge that we gathered about this organism in the past century along with the genetic tools available with it. 0.7.3

Finally, it a powerful system to investigate humans genetic diseases as it share 65% of its genome with humans^{38,39}.

0.6.7 *DROSOPHILA* AS A MODEL FOR PSYCHIATRIC DISORDERS

Drosophila has been successfully used as a model for studying human diseases such as Parkinson's and Fragile X (a mental retardation system)⁸³. Two main strategies are often employed: the first consists in inserting random mutations and test the resulting mutants for behavioural phenotypes and a second approach looks for homologs of humans candidate disease genes in *Drosophila* and assess their function in the fly's behaviour⁸³. The Fragile X syndrome is a disorder induced by a mutation in one single gene, FMR-1 which is highly expressed in the brain^{84,83}. A homolog gene, dFMR1 has been identified in *Drosophila* and the fly has been successfully used to better understand the morphological and behavioural phenotypes caused by the syndrome^{83,85}.

Unfortunately, the situation is very different when looking for *Drosophila* models of psychiatric disorders like schizophrenia, autism and ADHD⁸³. Schizophrenia is characterized by abnormal behaviour, false beliefs confused thinking and an overall lack of motivation. The mechanisms causing the disease are very poorly understood. A *Drosophila* model has the potential of being used as therapeutic discovery tool. In spite of that, up to present there are only two potential schizophrenia genes that were identified in the fly^{83,86,87} and the situation is very similar for other psychiatric diseases. The psychiatric disease have an extended range of symptoms, the majority being mind related which makes it difficult to translate to animal models, and especially to insects. It is the anthropomorphic view that needs to be countered, as all these psychiatric disease share an attention deficit which could be study by the means of arousal thresholds⁸³. Moreover, many psychiatric diseases share a disrupted sleep, and *Drosophila* is now an established model for studying sleep.

This study⁸³ suggests that the main problem in the finding of good *Drosophila* models for studying psychiatric disorders is the lack of appropriate behavioural assays. The most relevant paradigm able to test arousal thresholds up to date is the flight arena which present the flies with different visual stimuli and is able to capture their attention. However this paradigm is not suitable for large genetic screen.

Hence, finding a behavioural assay able to give us insights on how sleep deprivation affects decision making in a high-throughput manner, can offer potential benefits for the discovery of *Drosophila* models of psychiatric disorders.

0.7 ADVANCES IN TECHNOLOGY

The recent advances in technologies are very diverse and produced a new approach in the study of animal behaviour. They offer a new resolution into the external animal behaviour through the means of new hardware and software, but also into the internal state of the animal which can be manipulated using various genetic tools. Here I am going to describe specific advances in technology employed to study behaviour in *Drosophila*.

0.7.1 HARDWARE

The study of fly behaviour used to be made by a human observer. The recent progress in technology enables the experimenter with cheap acquisition methods like video recording offering a better resolution into the spatial and temporal behavioural space of the fly compared to the human eye. Augmented observations are now possible through the use of high speed cameras which are able to capture micro-movements of the flies giving access to a fantastic temporal resolution. Moreover, infrared illumination offers the possibility of monitoring flies in darkness and is now indispensable to the sleep field.

The recently introduced Arduino microcontrollers brought new possibilities for interacting with the animals during the experiments. The microcontroller, combined with video acquisition can be employed to trigger specific interactions based on the flies actions, in the complete absence of the experimenter.

Another game-changer technology is the Raspberry Pi, a very tiny and cheap computer that facilitates a modular design for easily collecting more data and processing it in real-time.

Finally, 3D printers technology like Formlabs or Ultimaker enable the experimenter to quickly produce behavioural paradigms at a high resolution using a great diversity of materials.

0.7.2 SOFTWARE

On the software side a multitude of open-sources packages exists to assist the experimenter with the analyse of the big data acquired through different devices. Several trackers have been developed to detect the position of flies in videos, for example the ethoscope software⁸⁸ is able to automatically assess individual flies positions in real-time for long periods of time. Ctrax⁸⁹ and idTracker⁹⁰ offer semi-automatic solutions to track offline multiple flies in videos of decent resolution.

An advanced tracking algorithm, DeepLabCut⁹¹, developed to be used with videos recorded by high speed cameras at relatively high-resolution is able to track specific body parts of the fly's body giving access to an enormous amount of information. The extremely good resolution into the fly's behavioural space offers new possibilities for defining micro-behaviours in *Drosophila* inaccessible to the human eye.

With the improved resolution into the animal behaviour, the amount of data collected can quickly become unmanageable and the experimenter might find it hard to extract relevant information from it. Specific software is used to assist behavioural experiments analysis of data. Such a software is JAABA⁹², a machine-learning based interactive system that enables users to annotate specific behaviours in a reduced number of frames and uses these annotations to create classifiers able to recognise the specific behaviours.

Another example is the rethomics framework⁹³ which consists in a set of R packages that facilitates the manipulation and visualisation of large amounts of behavioural data sets.

Besides these tools specifically targeting *Drosophila* there is a wide range of programming language available, like python that come equipped with specific packages specialised in computer vision which can be

used to respond to personal requirements not already available.

Finally, Onshape is a very intuitive design software available online that enables the experimenter to easily create 3D models of potential paradigms.

0.7.3 GENETICS

A huge amount of genetic tools enable researchers to manipulate the genome of the flies and to knock-down, overexpress, mutate and alter the expression of genes in *Drosophila*³⁴. Researchers are able to generate transgenes, which are pieces of foreign DNA introduced into the flies genome. One such technique employs P-element transposons which are pieces of DNA that are able to cut themselves from the genome and reintegrate in another location³⁴. Another technique is the homologous recombination which uses the flies DNA repair mechanism to swap out one piece of DNA for another³⁴. The recently introduced CRISPR/Cas9 also brought a lot of excitement as it enables the editing of the genome in a more efficient and accurate way³⁴.

Another way of studying genes is by generating mutations and identifying behavioural phenotypes in order to understand the function of specific genes in certain behaviours³⁴. A very large number of mutants flies are already available for purchase in stock centres.

Balancer chromosomes are another technique that made *Drosophila* famous as an animal model, because it enables genetic screens for interesting genes involved in specific behaviours. They help with the mating design and the maintaining of stable flies stocks, by suppressing recombination and containing a dominant phenotypic marker visible in the offsprings³⁴.

Probably the most used techniques in *Drosophila* genetics is the UAS/Gal4 system^{34,94}. It is a powerful tool that allows to spatially control the expression of transgenes coding receptor proteins capable of exciting or inhibiting neural activity. Many different Gal4 lines are available with expression patterns ranging from a group of cells to entire brain regions. The very specific ones express Gal4 in a small sets of neurons linking two different brain regions. The presence of Gal4 does not have any effect on the neurons in which it is expressed, but it can bind to a UAS (Upstream Activation Sequence) to activate gene transcription. Therefore the Gal4 lines are used together with reporter lines, which are special strains of flies that have the UAS region next to the desired gene. In the cells in which, Gal4 is expressed, it will bind to UAS and the desired gene will be expressed. One possible use is to express Gal4 in specific neurons of interest and cross the fly with a channelrhodopsin reporter line. Channelrhodopsin will be expressed in those specific cells in the offspring and will allow the triggering of those neurons using light.

Neurons targeted with the UAS/Gal4 system can also be manipulated using thermogenetics⁹⁵, by expressing a thermosensitive channels that activates or inhibits the specific cells by raising the temperature during the experiment.

0.7.4 PLATFORMS USED TO STUDY SLEEP AND DECISION MAKING IN *DROSOPHILA*

0.7.4.1 *DROSOPHILA* ACTIVITY MONITORS (DAMs)

Drosophila Activity Monitor (DAM) is a device produced by Trikinetics that can hold 32 glass tubes of size : (70 x 50 x 3 mm) (length x external diameter x internal diameter)) (see Fig. 5). Tubes are placed in the monitor symmetrically so that a beam of infrared light passes through their middles. Normally, flies walk in the tube from one side to the other, and a movement is detected whenever they cross the beam of light.

The monitors are linked to a computer where recorded data is analysed by a software provided by Trikinetics. The software counts the number of times the beam has been crossed in one minute, therefore the data obtained has a temporal resolution of 1 min.

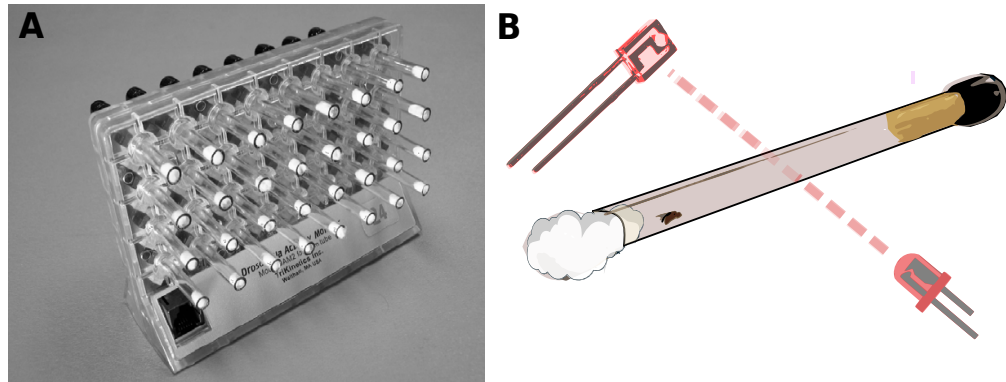


Figure 5: Drosophila Activity Monitor **A** An example of a Drosophila Activity Monitor (DAM) (image reproduced from Trikinetics Users Guide 8, <<http://www.trikinetix.com/>>) used for quantifying the locomotion of *Drosophila* flies. The monitor can hold 32 tubes and can be used to record the activity of the flies for long periods of time. The tubes contain solid food with agar on one side and cotton wool on the other side. Flies walk steadily between the two end of the tube. **B** Illustration of the basic mechanism of DAM. (image reproduced from ⁹⁶) Around each tube there is an infrared LED that sends a beam of light which is captured by an infrared receptor on the other side of the tube. Whenever a fly passes through the middle of the tube, the beam of light is broken and it does not reach the infrared receptor anymore. This is scored as movement of the fly.

0.7.4.2 ETHOSCOPE

The ethoscope is a great example of a device that integrates advances in hardware and software to generate a modular platform offering the ability to track flies in real-time and interpret collected data by the means of machine learning techniques in order to be able to deliver behaviourally triggered stimuli in a feedback-loop. It has a modular design that can be scale up cheaply to provide a robust solution for high-throughput experiments involving individual flies. The device integrates an Raspberry Pi microcomputer together with an infrared camera, an Arduino controller and can be built using 3D printing technology. It has been successfully used for high-throughput long-term sleep studies^{97,64}, but it can be adapted for studying other behaviours.

0.7.4.3 DROSOPHILA AROUSAL TRACKING SYSTEM (DART)

DART platform was developed for the study of sleep and is able not only to track the flies but also to deliver mechanical stimuli of different intensities in order to characterise flies sleep depth. The definition of sleep based on the activity record consists in the five minute rule which considers sleep any immobility period longer than 5 minutes. Such a rigid definition of sleep based on a fixed threshold is likely to deform reality. By also probing arousal-thresholds, the DART system is offering a multi-variate solution able to characterise sleep with a great precision.

0.7.4.4 FLIGHT ARENA

The flight arena consists of a cylindrical arena made out of LEDs that are able to project different combinations of stripes and patterns. A fly sits in the middle of the arena and is attached to a torque meter using a string. The yaw-torque response of the flies in response to visual patterns are continuously recorded. This arena has been used for studying visual attention in flies⁹⁸.

0.7.5 LIMITATIONS FOR STUDYING BEHAVIOUR IN *DROSOPHILA*

The biggest limitation for studying behaviour in *Drosophila* is the inability to record brainwaves while the flies are freely moving, because of unavailable technologies suitable for its small brain. A few attempts^{99,100} of electrophysiological recording from tethered flies suggests that electric activity measured using local field potential correlates with the rest and activity states in *Drosophila*.

0.8 LACK OF GOOD BEHAVIOURAL ASSAYS

In spite of the extensive range of technological advances that enable the experimenter to collect high-resolution data and easily design sophisticated arenas, there is a lack of good behavioural assays for studying decision making in *Drosophila* using simple decisions, which involve the minimum number of external stimuli and that can be used with cheap, unsophisticated setups for high-throughput experiments. The gap-climbing assay is able to capture a simple decision but it requires a sophisticated setup not suitable for high-throughput experiments. The same is true for the flight arena. A good behavioural assay would be able to capture the initiation of the flies decision and to address the randomness specific to each decision. We look for a paradigm that is able to reveal insights of the decision making mechanism and how this is modulated by different factors using behavioural measurements.

0.9 SCOPE OF THIS THESIS

The scope of this thesis is to find a behavioural paradigm that can reveal insights about how sleep modulates decision making in a high-throughput manner using inexpensive tools. Ideally, this paradigm will also be suitable for finding appropriate *Drosophila* models for psychiatric diseases.

In the first chapter, I will present the first paradigm designed to investigate the link between sleep and decisions in *Drosophila*, the y-maze paradigm. The results obtained with this assay are an exploratory quest for behavioural differences between sleep conditions that fail to report any behavioural changes caused by sleep deprivation but are able to reveal the endogenous randomness specific to each fly. This first piece of work resulted in the creation of a flexible workflow transferable to future paradigms.

The second chapter presents my attempts for designing a decision making paradigm (the passage through holes), able to capture with precision the initiation of a decision (initiation of a passage) and its modulation by one external stimuli: the size of the hole. There are many iterations of the design which converge into a setup suitable for video-tracking, that can be used to test flies preference for holes of different sizes. This chapter also presents the software implementation designed for automatically tracking flies with precision in the new behavioural arenas. Specific features are added to enable the tracking of the flies initiation of passages through holes in future experiments. Moreover, this chapter also includes the multi-tracking algorithm developed to be used with the ethoscope and the new implemented features. Unfortunately this algorithm was not used in future experiments as using individual flies was considered to be the good direction to be taken.

The third chapter presents two refined versions of the hole paradigm suitable for high-throughput of individual flies. They are used to investigate how sleep modulates flies decisions to pass through the holes and reveal the preference for the bigger hole in every single fly.

1

Y-maze paradigm

1.1 INTRODUCTION

This chapter presents our first attempts of finding an assay that can capture modification of the behaviour due to sleep deprivation.

The y-maze was inspired by the Galton's box which is a board that holds several interleaved pins. Balls are dropped from the top and they are collected in the bottom in separated bins. The device was invented by Sir Galton Francis to prove the central limit theorem which states that a binomial distribution approximates a normal distribution. Therefore we looked for a paradigm in which flies can walk freely and in which they are faced with several spontaneous decisions of moving right or left.

Hirsh¹⁰¹ designed a "multiple unit classification maze" to make a mass screening for geotaxis and Hadler¹⁰² used similar mazes to study phototaxis. Both of these studies use a paradigm that is close in design with the one I will describe in this chapter. Unfortunately, these studies were undertaken at the beginning of *Drosophila* behaviour research and I was not aware of them at the time of building my maze. They would have been of great help in choosing the right parameters for the maze that fit the fly. Because the size of the fly is so small, the adjustment of dimensions of the maze had to be very precise in order for the fly to walk straight through the intersection instead of wondering around on the walls. This ended it up being very tedious.

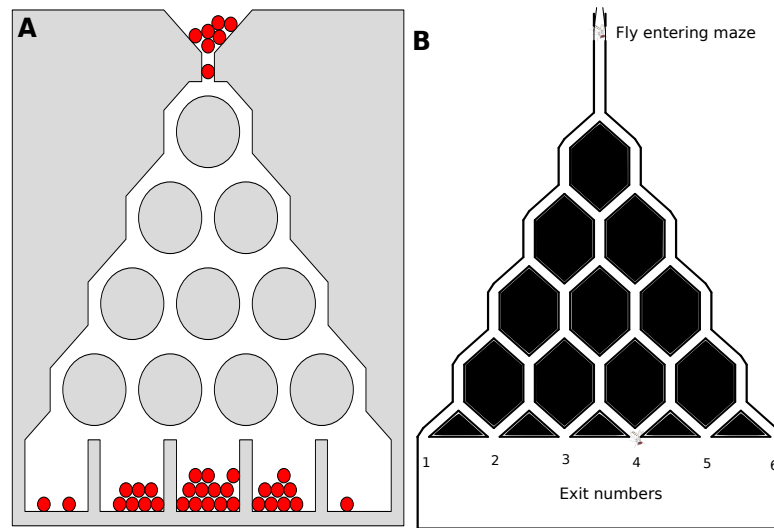


Figure 1.1: Galton box **A** An example of a Galton board. Red balls are dropped from the top and at each intersection they have equal chances of going either left or right. They accumulate at the bottom of the board in equal size bins giving rise to a normal distribution. **B** Idea of our paradigm inspired by the Galton Box. Flies are able to enter the maze from the top. The paradigm consists of two compartments: a maze, in which the flies are faced with intersections where they have to take either a left or a right turn and an open field arena, where they can move freely. At any time there will be only one fly in the maze. For presentation purpose this figure contains one fly entering the maze and one fly exiting the maze through exit number 4.

A part of the motivation for building this maze came from wanting to know more about how similar is a fly to a random ball. Therefore we were curious of how the exit-distribution of the flies from the arena will differ from a normal distribution. Nevertheless, a lot of the motivation for building this y-maze came from our interest in the exploratory behaviour of the flies in a novel environment. Most of the locomotor and sleep research in *Drosophila* has been conducted in glass tubes using TriKinetics activity monitors 0.7.4.1 which are very simple and restrictive environments.^{103,104,105} The Trikinetics system has a very low resolution of the activity of the fly because it only tracks the fly passages through the light beam in the middle of the tube. Recently, more studies started to employ a video tracking system to track the flies in tubes in order to gain better resolution on the movements of the fly^{64,106,88}. A few studies exist in the literature that analyse the locomotion of the fly in a square or circular arena instead of a tube^{77,107}. These studies are able to look in more depth at the temporal organisation of activity and inactivity bouts, and suggest that the overall pattern of activity seems to have a fractal-structure. The question arose of how does the internal state of sleep deprivation affect the temporal and spatial resolution of behaviour?

It has been shown that the reaction times of decisions depend on the quality of the external information. For example, the speed and accuracy of reaction times required to discriminate between two different concentrations of the same odour depends on how strong the difference is between the two concentrations. A big contrast between concentrations is easier for the flies to distinguish than a very subtle difference.⁷¹ How much the reaction times depend on the quality of the internal information? When faced with an intersection, the fly has to choose between going left or right. Is the reaction time of this choice dependent on the sleep that the fly took the previous night?

It is established that after a night or several days of sleep deprivation, there will be a sleep homeostatic response in the flies resulting in more sleep, also called sleep rebound¹⁰⁸. This sleep usually arises in the first few hours straight after sleep deprivation. The homeostatic response of sleep is largely unelucidated, and some organisms are able to cope better with sleep deprivation than others. Here, we would like to look at how the homeostatic system adjusts itself when flies are introduced into a new challenging environment

straight after sleep deprivation.

Our idea of experiment came with the challenge of finding out which are the appropriate tools that should be employed in order to create a workflow that ensures the quantification of the data at a suitable resolution to tackle the questions described above. Suitable tools are needed for designing and building the arena and suitable hardware and software should ensure that several flies can be recorded in the same time. We should also be able to make sure that sleep deprivation is performed at the individual level and that it is efficient. Tracking of the flies can be done post experiment, but suitable software is needed to accurately know the location of the fly at each frame. Own data analysis software is required to explore the behaviour of the flies in the paradigm. Finally, our framework should also guarantee a decent throughput of flies.

Therefore this chapter attempts to build a new paradigm from scratch and integrate it in a workflow that allows the recording, tracking and data analysis of individual flies in parallel in order to undertake an exploratory work into what are the behavioural differences between mock control and sleep deprived flies when faced with a new environment straight after sleep deprivation.

1.2 MATERIALS AND METHODS

1.2.1 DATA ANALYSIS AND STATISTICS

Data analysis was performed in R. Own scripts were used to count the number of passages through the holes using the parameters that define the position of the fly in the maze, provided by the tracking algorithm. Plots were generated using ggplot2 and ggpubr packages. For all the boxplots, the first and third quartiles (Q_1 and Q_3) are the ends of the box and the median is indicated with an horizontal line in the box. The whiskers go from the bottom and top of the box to the minimum and maximum values situated in the $\pm 1.5IQR$ range, where $IQR = Q_3 - Q_1$. The errors bars present in some figures indicate the standard error. Statistical analysis was carried out using the pairwise Wilcoxon rank sum test available in the ggpubr package. Outliers were always kept, and each experiment was repeated at least twice. The ethogram and overview plots were generated using the Rethomics packages⁹³ (<https://rethomics.github.io/intro.html>). The following convention is used to indicate statistical significance: ns: $p > 0.05$, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$.

1.2.2 Y-MAZE DESIGN

The y-maze has been through several versions since its inception as presented in Fig. 1.2. An initial attempt has been made to build the maze out of printed lego tiles.(left and middle images in Fig. 1.2) However the flies were very sensible to the small spaces between the tiles and to small unevenness of the surface. Therefore, a smooth surface was preferred and the y-maze was redesign in one piece. The final version of the y-maze paradigm consists of an acclimatisation chamber, a maze and an open arena 1.2 B). The fly is loaded in the acclimatisation chamber using a set of two doors. The acclimatisation chamber allows the fly to get used to the new conditions of light, temperature and humidity that exist in the incubator before starting the experiment. In doing so we hope to eliminate part of the initial stress of the fly, which can give rise to very noisy and variables responses. The maze has the shape of a 5-level binary tree in which at each intersection the fly can go either left or right. At the end of the maze, the fly exits from the maze through one of the six available exits to the open arena where it can move freely. The dimensions of the y-maze arena (1.2

B) are 120 x 80 mm (length x width) and the Y shape maze path has dimensions of 2 x 7.5 x 1.3 mm (width x length x height).

A



B

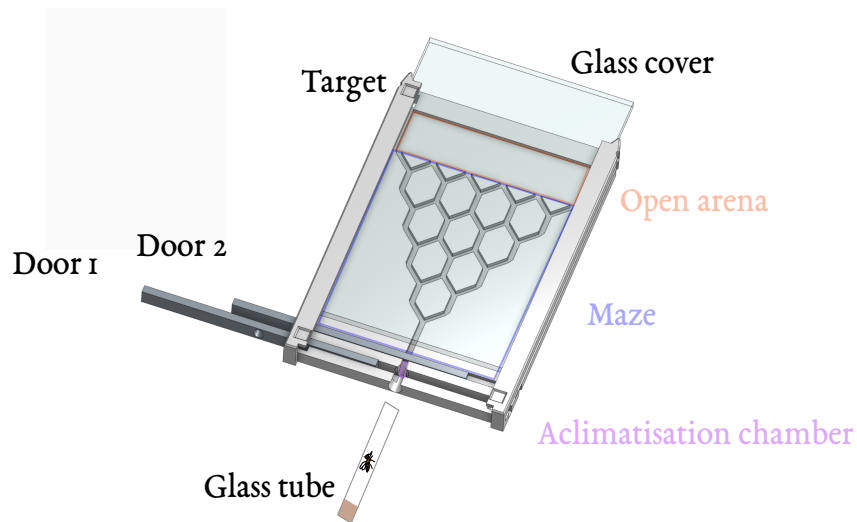


Figure 1.2: Y-maze arena Initial designs together with the final version of the y-maze paradigm presented in this chapter. **A** Initial designs of the y-maze paradigm. The left and middle photos present an attempt of creating the assay using several y shaped tiles, and the right photo presents the version of the y-maze without an open arena or acclimatisation chamber. **B** The final version of the y-maze arena that is formed of an acclimatisation chamber, a maze and an open area where the flies can move freely. The flies are loaded straight from their glass tubes and are kept in the acclimatisation chamber with a set of two doors. The arena is covered by a glass lid

1.2.3 3D MODELLING AND 3D PRINTING

All the assays have been designed using the onshape platform www.onshape.com. The initial designs (Fig. 1.2 A) were printed using the Ultimaker2 3D printer with PLA material. The final version of the maze (Fig. 1.2 B) was printed with a higher resolution printer using a transparent material called VeroClear.

1.2.4 SLEEP DEPRIVATION

The activity of the flies was monitored continuously for the duration of the entire experiment using DAM monitors (Fig. 5). Each fly was sleep deprived during one night (12 hours during the dark cycle, from ZT₁₂ to ZT₀) by fixing the DAM monitor on an PSU-10i Orbital shaker setted up to rotate at a speed of 450 rotations per minute around its vertical axis every 1-7 minutes, for a random time of 5 to 30 seconds.

1.2.5 ARENA CLEANING

In order to remove any odours or impurities left by the flies in the maze, the arenas were washed between each experiment using 100% ethanol. A paintbrush was used to wash between the narrow y-maze tunnels.

The arenas were dried extensively to make sure that all the ethanol had evaporated before loading a new fly. The doors and transparent plastic sheets used to tighten the space between the maze and the glass lid were also washed in 100% ethanol. At the end of a day of experiments all the arenas would also be wash in distilled water.

1.2.6 VIDEO RECORDINGS

In the initial design experiments flies were filmed using a Canon 10D camera that sat on a tripod. The distance between the camera and the arena varied slightly between experiments, making the videos difficult to align. The video recordings in the subsequent phases of the experiments were taken using the ethoscope platform developed in our lab by Quentin Geissmann together with his close collaborator Luis Garcia.⁸⁸. The ethoscope is a device that contains a raspberry pi computer and a high definition camera. It can be controlled through a web interface to acquire videos of 1280×960 pixel resolution at 25 Frame Per Second (FPS). The platform was originally created for doing real-time tracking over extensive periods of times at a resolution of 2 frames/seconds. This resolution was not sufficient for extracting information like the time flies spend in intersections before deciding if they want to go left or right, or even the number of the intersections they crossed. I am very thankful to Quentin for adding the feature of recording videos through the web interface upon my request. This enabled me to record videos at a resolution of 25 frames/second and to analyse them subsequently offline using my own tracker.

1.2.7 EXPERIMENTAL SETUP AND CONDITIONS

Experiments were performed in incubators at 60% humidity and 25 °C. Flies are very sensitive to light therefore any light gradient will bias their choice in the maze. In order to ensure a uniform light environment the arenas were placed on a uniform led lightbox that was able to hold 4 ethoscopes (Fig. 1.3 C). Two lightboxes were used in the same time, and were placed in an incubator that had no other source of light inside. The incubator was placed in a dark room. Flies were loaded from their individual tubes in the acclimatisation chamber of the arena where they will spend 10 minutes, following which the second door of the y-maze will be open allowing them to explore the y-maze for a period of 15 minutes.

1.2.8 EXPERIMENTAL PROTOCOL

Flies are placed in tubes at the pupa level, for them to be socially naive. Post-eclosion, males and females are identified. Experiments were run with only one sex at a time. Flies are randomly separated into two groups, Gr1 and Gr2, and carefully placed in DAM monitors (Section 0.7.4.1) inside the incubators. Following one baseline day, on the second night inside the incubator flies from Gr1 are mechanically sleep deprived for 12 hours from (ZT12 to ZTo). Straight after sleep deprivation each fly is individually placed in the y-maze and kept for 10 minutes in the acclimatisation chamber (Figure 1.2 B), after which the doors are opened and each fly is given 15 minutes to explore the maze. At the end of the experiment they are removed from the y-maze and placed in their original tubes. Four ethoscope are used at a time (Fig. 1.3 C) resulting in a throughput of approximately 25 flies in the 3 hours window. Once all the flies enter the y-maze, their DAM monitors are placed back in the incubators and left to recover for the rest of the day and on the following day. On their fifth night, flies from Gr2 are sleep deprived and straight after the 12h period

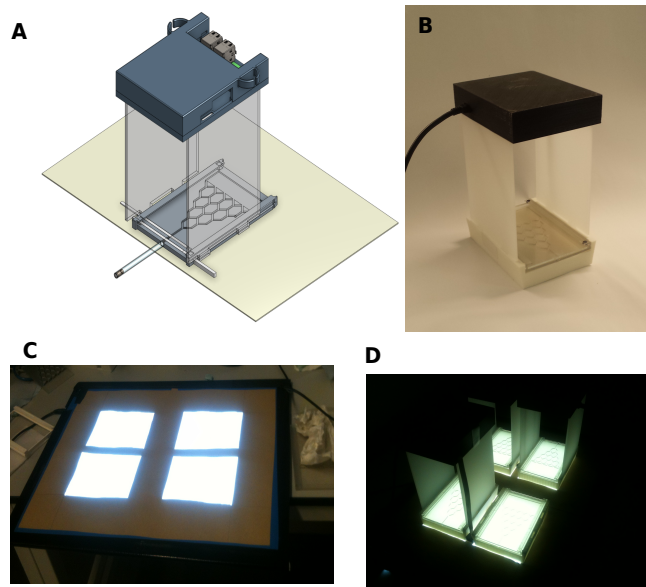


Figure 1.3: *Experimental setup y-maze paradigm* **A** An illustration of a lightbox that holds an ethoscope which records a y-maze arena. **B** A real image of the ethoscope device holding a y-maze arena. **C** A lightbox covered in a symmetrical fashion to ensure uniform light in the 4 ethoscope placed upon it. **D** A lightbox holding 4 ethoscopes.

of sleep deprivation all the flies reenter the maze a second time, but this time in a different sleep condition. Again, experiments happen in a time window of three hours and follow the same routine explained before.

The experimental protocol follows a matched pairs design. This design makes it less probable to have a lurking variable like temperature, humidity or light causing an imbalance, skew the results or give a biased result. It increases our chances of obtaining real and unbiased information and to be able to trust that a certain explanatory variable has a causal effect on a response variable. In our case we want to be able to trust that is sleep deprivation that is responsible for any effects observed in the behavioural responses.

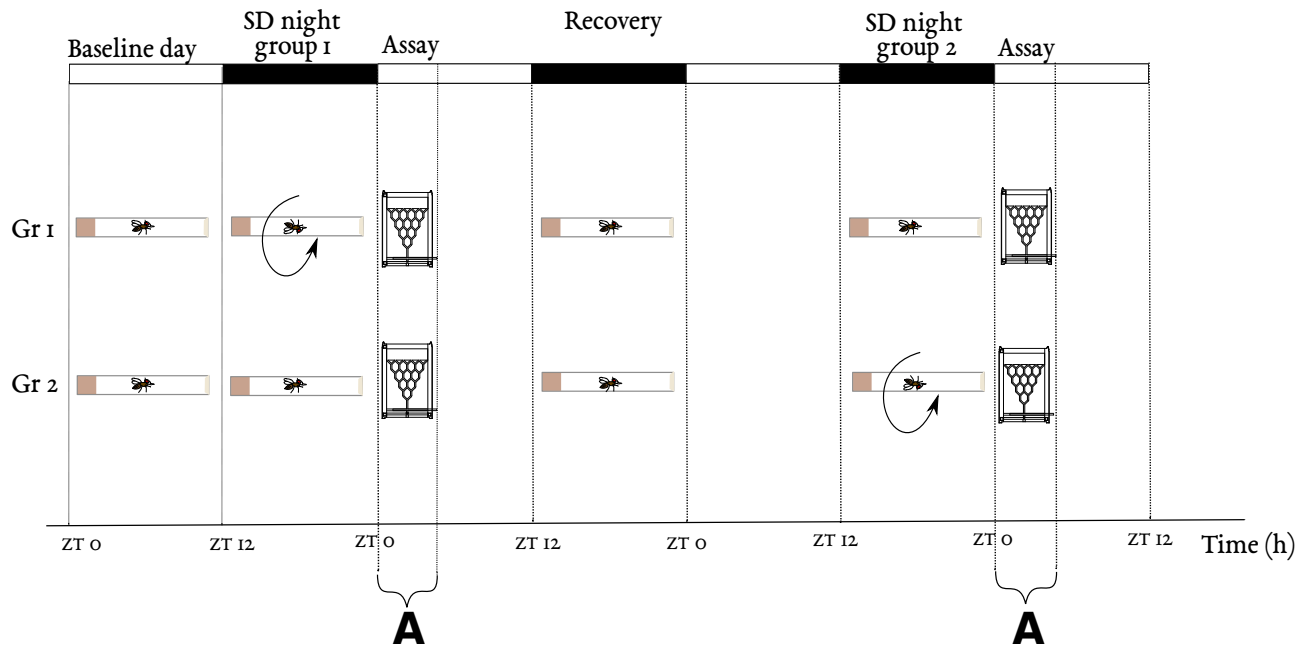


Figure 1.4: Experimental protocol Flies are randomly placed into two groups: Gr1 and Gr2 and placed in DAM monitors, to be recorded for 5 days in total. Both groups have a baseline day, and in the second night Gr1 is mechanically sleep deprived for 12 h. Between ZT0 to ZT3, on the following day, all flies from both groups spent 13 minutes in the y-maze, following which they are placed back in their original positions in the DAM monitors. They are left to recover on the next day, and on the night of the fourth day of experiment, flies from GR2 undergo mechanical sleep deprivation. Again, in the morning of the fifth day, between ZT0 and ZT3 each fly from both groups will individually enter the maze for a period of 13 minutes. The baseline and recovery periods are shortened in the figure for presentation purposes.

1.2.9 VIDEO TRACKING

The tracking information of a fly includes the pixel-accurate coordinates (x, y), the orientation angle and the dimensions of the ellipse (width and height) that fits the best the fly's body. Moreover, at every frame the ellipse is superimposed over the mask to provide the quantification of the number of pixels of different greyscale contained in the fly's body.

Figure 1.5 A shows the standard sleep arena used with the ethoscope to study sleep in wild-type flies. The arena can hold 20 flies at a time and there are 8 parameters that have to be fixed in order to define a grid holding 20 regions of interests. The position of the arena with respect to the camera varies slightly between experiments, therefore three black targets are used to align the regions of interest on the arena by the means of an affine transformation. My initial tracking results were obtained using the region of interest presented in Figure 1.5 B. The y-maze engraving was not precise between videos making it difficult to analyse the behaviour of the flies in the intersections. As I kept the raw videos of all my experiments, I was able to come back to them and apply my improved definition of regions of interest and subregions of interest (Section 2.2.9) to get a clearer understanding of the flies behaviour in the intersections. Figure 1.5 C shows the mask used to define the regions of interest. Each intersection is defined as a subregion of a different greyscale in order to be easily identifiable.

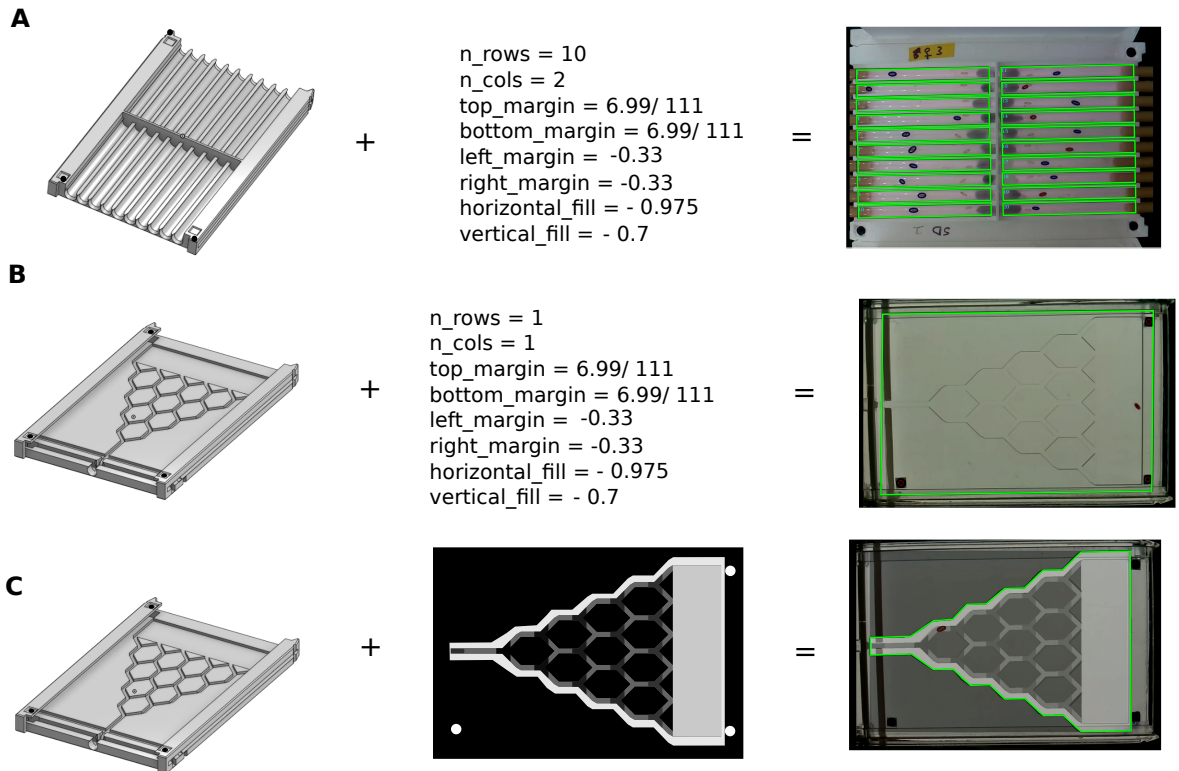


Figure 1.5: Methods used to define regions of interest in the y-maze **A** Standard arena used with the ethoscope to study sleep in flies. The 8 parameters provided in the image are used to define the 20 regions of interest which are highlighted in green on top of the sleep arena. **B** Initial definition of the region of interest in the y-maze using the method delivered with the ethoscope as presented in **A**. **C** Flexible definition of the y-maze region of interest and subregions of interest using a mask. (Section 2.2.9)

1.2.10 WORKFLOW OVERVIEW

The work presented in this chapter led to the development of a workflow that integrates and adapts a set of tools and methods in order to assist the lifecycle of a novel behavioural assay from creation, to data acquisition and data analysis. My workflow consists in the following steps:

1. Design a new behavioural arena using Onshape design software system, available via the Internet as a Software as a Service model and available in any browser. Draw the most minimalist design that can fit the ethoscope to ensure the highest-throughput.
2. 3D print the arena from step 1 in low resolution and cheap material (PLA) using the Ultimaker 2 printer. Assess its functionality using preliminary experiments; if any changes are needed, modify the 3D model and reiterate until satisfied
3. Final version of the arena from step 2 is printed with more accuracy using a high-resolution 3D printer and it is to be used in experiments.
4. Establish a protocol, run the experiments and obtain the 5-minutes chunks of raw video recordings (.h264) from the ethoscopes
5. Unite and convert the .h264 files to a single .mp4 file corresponding to one experiment
6. Use ffmpeg software to cut unnecessary parts of the video and align the experiments in the same time frame
7. Create a mask to define the regions and subregions of interest in the videos: export a drawing of the 3D arena from step 1 and use Inkscape to draw in greyscale the regions and subregions of interest
8. Adapt the tracking algorithm and use it offline, with the mask on the videos obtained in 6.

9. Results from the tracking algorithm are stored in a sqlite3 file, having a .db extension and are loaded together in R using an adapted version of the rethomics package⁹³
10. Write own R scripts to analyse results

1.2.II ETHOSCOPE - A FLEXIBLE PLATFORM FOR MONITORING BEHAVIOUR

The ethoscope platform⁸⁸ was the main tool that enabled me to create a flexible workflow for running experiments. From my perspective, the main advantage of the ethoscope is its modularity on both the hardware and the software sides. On the hardware side it gave me the opportunity to quickly adapt any assay to fit in the standard arena that slides inside the ethoscope. On the other side, the software is written in a way that is easily extensible allowing a straightforward integration of new features such as new regions of interest builders or new trackers. The ethoscope platform was build to provide real-time automatic tracking over long periods of time. Each ethoscope holds a Raspberry pi in charge of the real time processing of the video data. Even if efforts has been done to improve the efficiency of the automatic tracking algorithms as much as possible, the Raspberry Pi does not have enough power to apply the video tracking algorithm to high resolution videos, being able to process on average only 2 frames per second. My interest in the decisions of the flies demanded detailed information about their temporal sequence of movements, as only 2 frames per seconds were insufficient to get a clear understanding of the behaviour of the flies in the intersection of the y-maze. Therefore, I persuaded Quentin into extending the ethoscope software to enable me to obtain videos at the maximum possible resolution offered by the Raspberry Pi Camera: 25 frames per second. Apart from gaining a better resolution into the fly's behaviour it also enabled me to keep the raw videos of all my experiments. With the improved trackers and regions of interest that I implemented throughout this project, I was able to go back to my data and extract from it more detailed information with the help of the new features.

1.2.I2 DATA ANALYSIS

1.3 RESULTS

In this experiment each fly enters the y-maze twice in two different sleep states: once well rested (control) and once after 12 hours of mechanically sleep deprivation (sleep deprived). Figure 1.6 B shows the sleep profile of individual flies, monitored continuously for 5 days and Figure 1.6 A presents the sleep profile at the population level in the two randomly assigned groups: Gr1 and Gr2. (Section 1.2.8) During the baseline and recovery days the sleep of the flies is consistent and follows the regular circadian pattern observed in flies. They sleep less during the day and their day-sleep culminates with a siesta in the course of which they are asleep approximately 50% of their time. Their night sleep is more consolidated The sleep profiles confirm that the sleep deprivation was efficient in both nights, as flies in the sleep deprived groups spent less than 20% of their time sleeping during the course of that night while flies in the control group spent more than 75% of their time sleeping. From Figure 1.6 B we observed that there are differences in the efficiency with which the sleep deprivation works in individual flies. While some flies have no sleep during the sleep deprivation periods, other get to rest for short amounts. Interestingly, after the first night of sleep deprivation and following the experiment in the y-maze, a sleep rebound is observed. During the first recovery day, the sleep deprived flies spend almost 75% of their time sleeping, an increased amount compared to the baseline day. The sleep of control flies in the first recovery day is no different from baseline. Moreover, the

sleep deprived group is indistinguishable from the control group from the first recovery night and thereafter. Figure 1.6 B gives detailed information about the sleep of the flies at the individual level. While great inter-variability is observed in the amount of sleep of different flies, the sleep of the same fly is more robust between consecutive days.

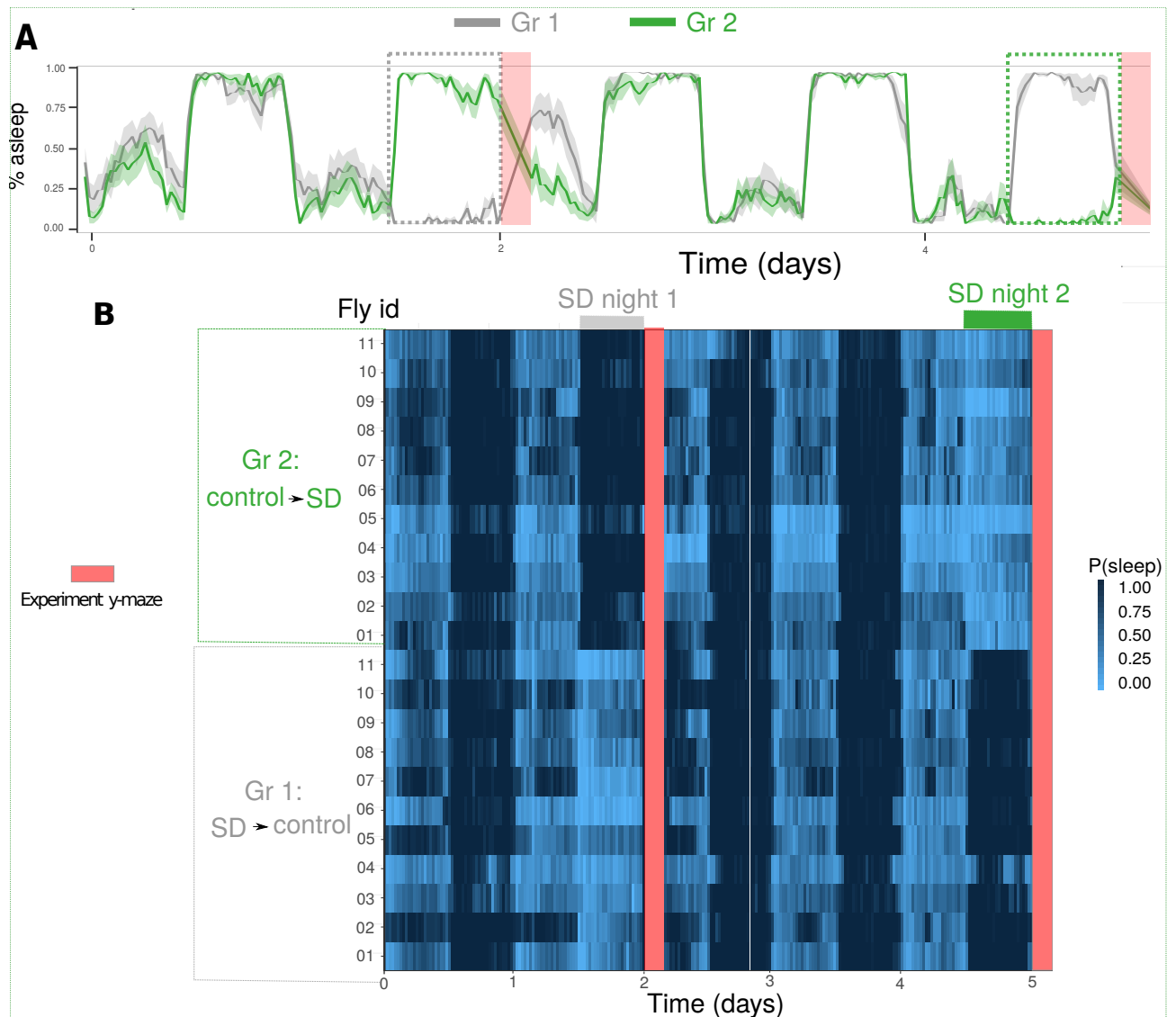


Figure 1.6: Population and individual sleep profiles of female flies before and after the y-maze experiment. Flies are randomly separated in two groups: Gr1(gray) and Gr2(green). Each fly enters the maze twice, once as a control and once as sleep deprived. Flies in Gr1 enter the maze as sleep deprived first, and as a control the second time. Vice-versa for the flies in Gr2. **A** Population sleep profiles for the two groups of flies during the 5 days of experiment. The first night of sleep deprivation is surrounded in a gray dashed rectangle and the second night of sleep deprivation is surrounded in a green dashed rectangle. Straight after the nights of sleep deprivation flies are loaded in the y-maze in the 3 hours time window, shown by the red rectangle. **B** Individual sleep profiles, each row represents on fly. The bottom half shows flies in Gr1 and top half shows flies in Gr2. It allows the visual assessment on the performance of the sleep deprivation in each individual fly. Sleep amount is assessed in bouts of 30 minutes and represented by a color intensity, as specified in the legend.

1.3.1 EXIT PATTERN OF FLIES FROM MAZE TO FIELD

The shape of the y-maze arena was inspired by the Galton box (Figure 1.1) and we wanted to investigate how the exit distribution of the flies from the maze is different from a normal distribution. The y-maze is a binary tree with five levels and six exits identified like in Figure 1.1 B. Females and males in either the control or sleep deprived state were allowed 15 minutes to explore the y-maze, however not all of them

reached the open area (Figure 1.2). Table 1.4 summarizes the participation of flies in the maze. Flies show a pronounced sexual dimorphism in the exploration of the y-maze arena with almost all the males exiting the maze to the open arena compared to only one third of the females. As more flies arrived to the open area compared to females, they are a better candidate for analysing how their exit-distribution resembles a normal distribution. Figures 1.7 A,B present the histogram and density plot of the exit distribution of the control flies. A Shapiro-Wilk normality test was used to show that the exits of the flies are not normally distributed and a Q-Q plot confirmed the result (Figures 1.7 C). Sleep deprived flies have a similar distribution to control flies as presented in Figures 1.7 D, E, F.

Table 1.1: Participation in the maze.

	number	% enter maze	% enter open arena
Males (control)	57	96	87
Males (SD)	57	95	88
Females (control)	101	75	35
Females (SD)	101	81	32

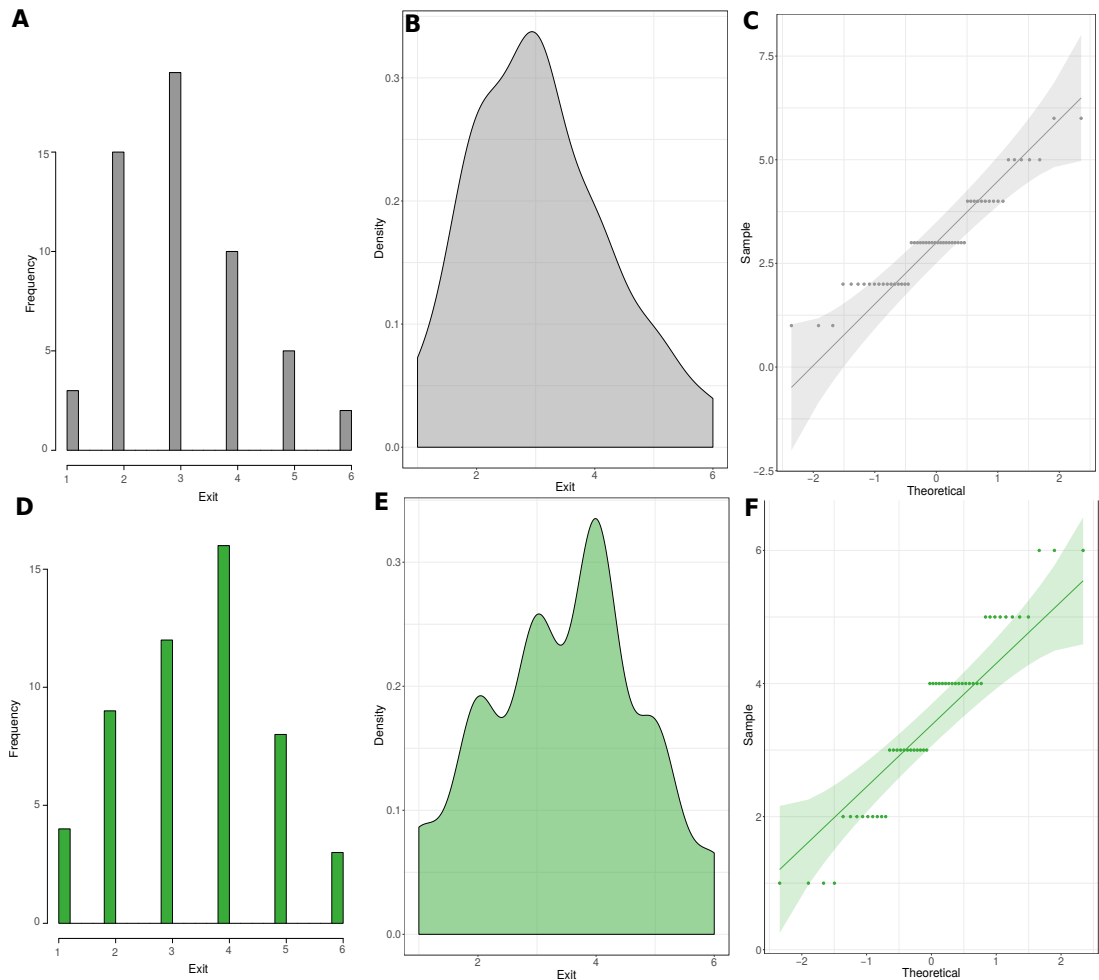


Figure 1.7: Exit distribution of male flies **A** Histogram presenting the exit distribution of control male flies that reach the open area of the y-maze. **B** Density plot showing the exit distribution of control male flies that reach the open area of the y-maze. **C** Normal Q-Q plot with line of best fit. (Shapiro-Wilk normality test $W = 0.92$, $p\text{-value} = 0.001$) **D** Histogram presenting the exit distribution of sleep deprived male flies that reach the open area of the y-maze. **E** Density plot showing the exit distribution of sleep-deprived male flies that reach the open area of the y-maze. **F** Normal Q-Q plot with line of best fit. (Shapiro-Wilk normality test $W = 0.92$, $p\text{-value} = 0.001$) $N_{\text{flies}} \geq 50$

1.3.2 EXPLORATORY BEHAVIOUR IN THE MAZE

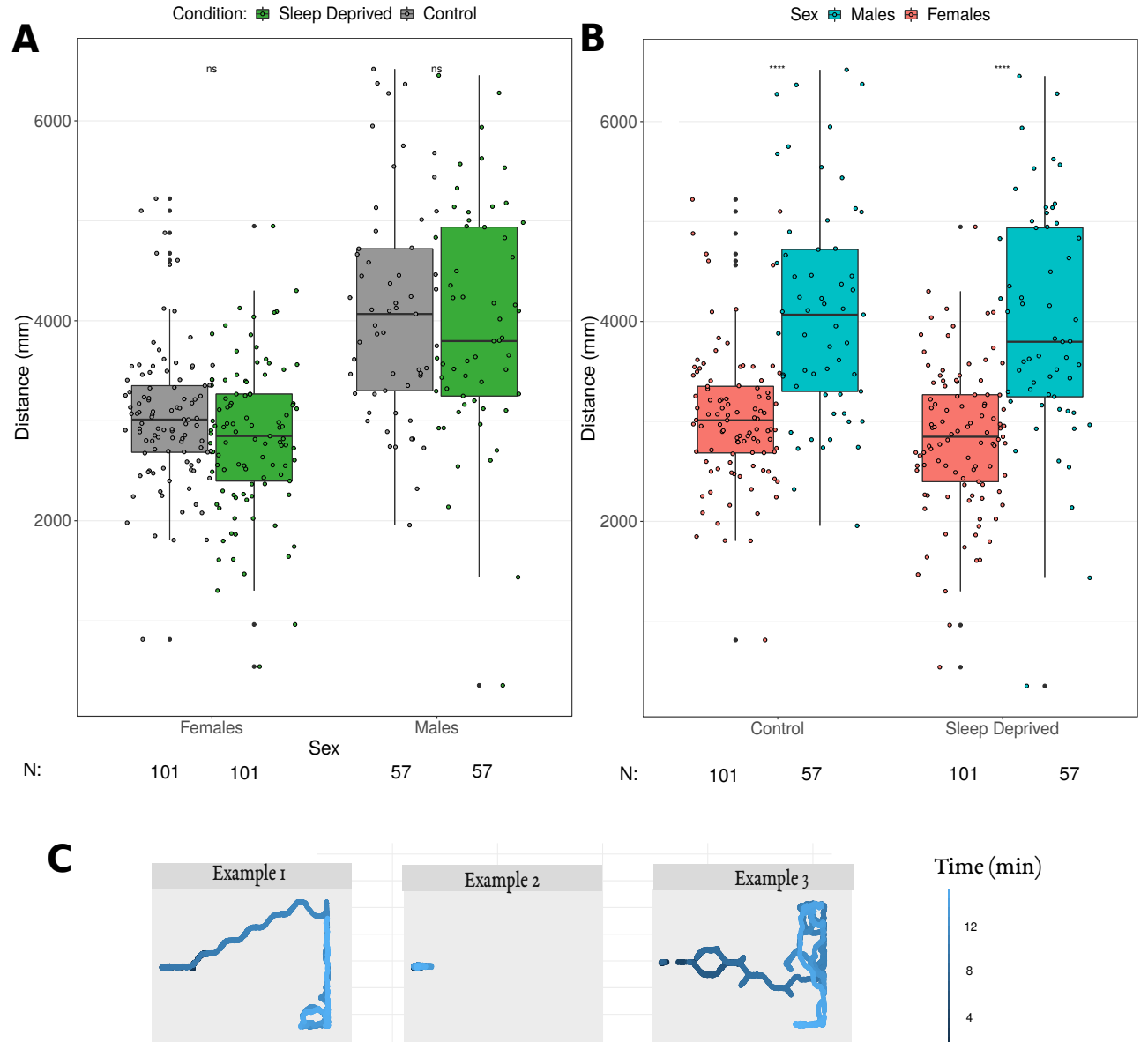


Figure 1.8: Total distance walked in the y-maze. Both graphs present the same data. **A** Comparison between the total distance walked by flies in control and sleep deprived conditions, in animal of the same sex. **B** Comparison between the total distance walked by males vs females in the same sleep condition, either control or sleep deprived. **C** Three examples of the trajectories of the flies in the maze. A code color is used to map each point in the trajectory with a point in time. The beginning of the trajectory is represented with dark shades of blue which gradually decrease in intensity through the 15 minute of experiment.

Locomotion in *Drosophila melanogaster* is modulated by the internal states of the animal and external states like temperature, humidity and light¹⁰⁹. From the internal states, hunger is known to increase flies activity¹¹⁰ as starved flies enhance their food-seeking behaviour¹¹¹. The activity of *Drosophila* is the main indicator of how much *Drosophila* sleeps, and it is established that the internal sleep state also impacts the locomotor activity of the flies. Sleep depriving flies for 12 hours, or even several days⁹⁷ results in a decreased activity during the first hours following sleep deprivation^{56,97}. It is also established that several sleep aspects are sexual dimorphic. For example, females flies sleep less than males overall, with some females flies having little or no sleep⁹⁷. Females are also more sensitive to sleep deprivation, and a continuous sleep depriva-

tion treatment over their entire lifetime reduces more seriously their life expectancy, compared to males⁹⁷. Most studies in *Drosophila* locomotion and sleep research look at the behaviour of *Drosophila* in standard glass tubes of size 70 x 50 x 3 mm, using DAM monitors 0.7.4.1, with flies staying in the same environment following sleep deprivation.

The y-maze is a new environment, and our interest was to better comprehend what are the observable effects of sleep deprivation and how many of the usual behavioural changes observed in the standard tubes are still conserved when flies are faced with a new context.

In *Drosophila* sleep research, sleep is assessed using the five minute rule assumption which scores as sleep any inactivity bout longer than 5 minutes. This rule is rooted in some early studies and initial observations that flies arousal decreases after approximately five minutes^{104,56}. This rule has been generally accepted and is suitable for long experiments, but it is quite limiting for shorter length experiments like our assay (only 15 minutes). Using the five-minutes rule no sleep is observed neither in the sleep deprived group, nor in the control group in the y-maze. To get a more detailed idea of the behavioural changes in flies locomotor activity we looked at their total distance walked in the y-maze, the exploration of the arena and at the architecture of their activity and inactivity bouts. Figure 1.8 A shows that sleep deprivation does not change in a significant way the total distance walked by the flies during their 15 minutes in the maze, neither in females nor in males. However, the total distance walked in the y-maze reflects a strong dichotomy between sexes presented in Figure 1.8 B. By fixing the sleep condition, we observe that in both control and sleep deprived groups males total distance walked is significantly greater than the total distance walked by females flies.

In humans, the lack of sleep causes fatigue which reduces the ability of engagement in activities¹¹². The exploration of the arena is highly variable between flies. To grasp their behaviour in the y-maze we started by first visually assessing their trajectories in the y-maze using a code colour to map their position to each moment in the time-window of 15 minutes of experiment. Three distinctive behaviours were observed: flies that do not enter the maze and spend the entire time on a very restrictive area at the entrance of the y-maze (Figure 1.8, example 2), flies that walk through the maze to the open arena and after some exploration they come back to the maze, often up to the entry of the maze, their point of departure (Figure 1.8 C, example 1) and flies that walk through the maze and once they reached the open arena they stay in there until the end of the experiment ((Figure 1.8 C, example 3)). The flies that go to the open arena and come back to the maze often make several crosses back and forth from the open arena to the maze.

Even though all these trajectories may end up in similar distances walked in the maze, the distance variable is not able to capture the amount of exploration of the arena. To assess the engagement of the flies in the y-maze we quantified the area explored by each individual fly. To do that a very fine grid was superimposed on top of the y-maze, and we counted the number of grids cells covered by each fly during their exploration of the maze. Figure 1.9 C is a zoom on one intersection of the y-maze and shows an example of how the grid cells are filled in as the flies walk through the maze. Sleep deprivation does not have any impact on the flies exploration of the arena, neither in males nor in females Figure 1.9 A. However, a strong sexual dimorphism is observed between flies, as the area covered by males is more than double of the area covered by females. This result is present in both control and sleep deprived conditions.

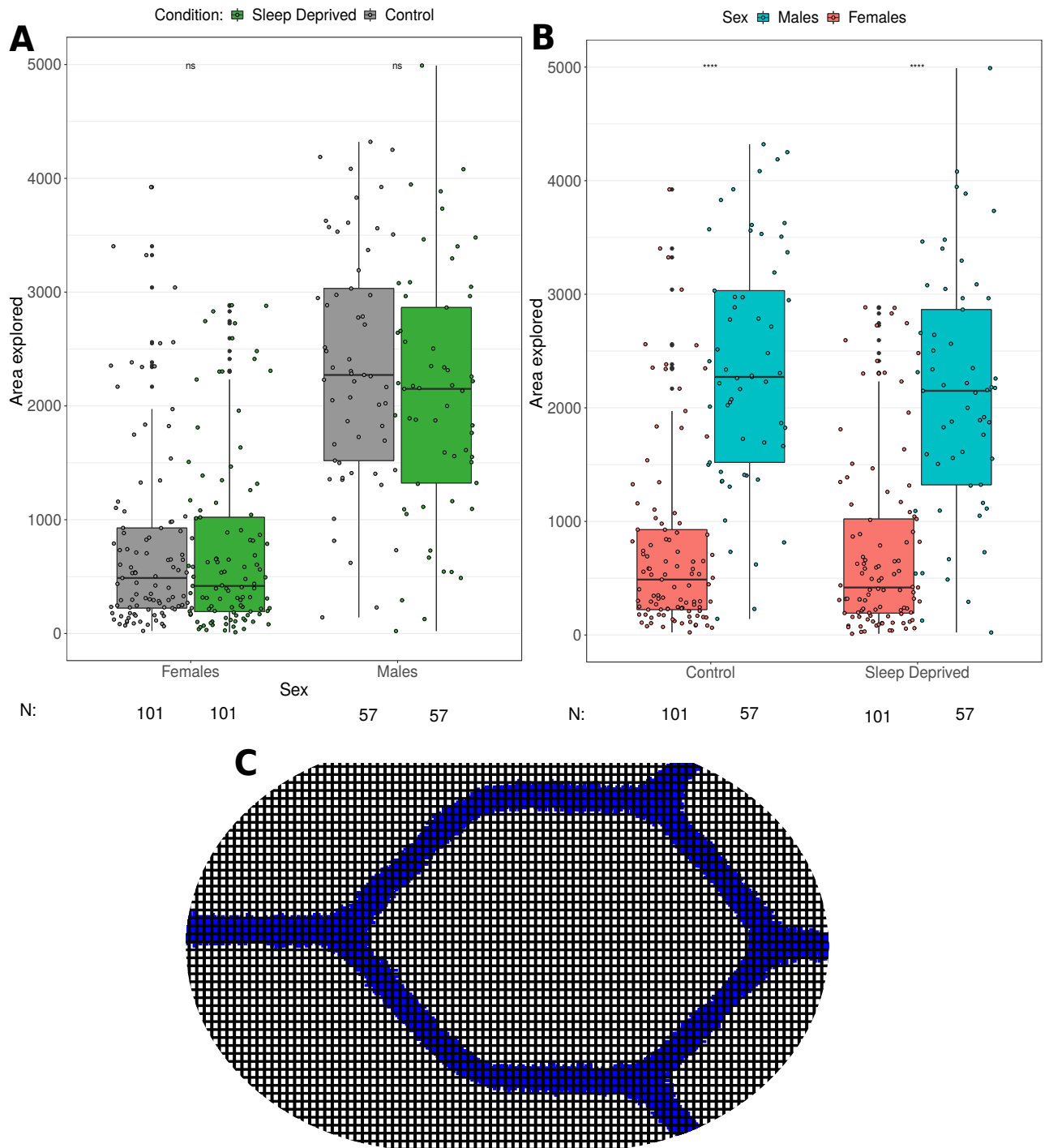


Figure 1.9: Y-maze arena exploration. To quantify the amount of exploration of the arena in each individual fly, a fine grid is superimposed over the y-maze (**C**) and the total number of grid cells touched by the body of the flies are sum together to show an approximate of the area explored. The same data is presented twice: once to compare between sleep conditions and once to compare between sexes. **A** Comparison between the area covered by flies in control and sleep deprived conditions, in animal of the same sex. **B** Comparison between the area covered by males vs females flies, for a fixed sleep state: control or sleep deprived. **C** Zoom on a y-maze cell that has a fine grid on top of it. The blue dots represent the area covered by one fly in that specific cell.

As mention at the beginning of this section, following a night of sleep deprivation, and when in the standard glass tubes flies show a sleep rebound which is represented by a larger number and longer inactivity bouts compared to control. To investigate if there are any behavioural changes into their time organization of movements, we further looked at the architecture of their stop and go behaviour by quantifying the total number and duration of activity and inactivity bouts.

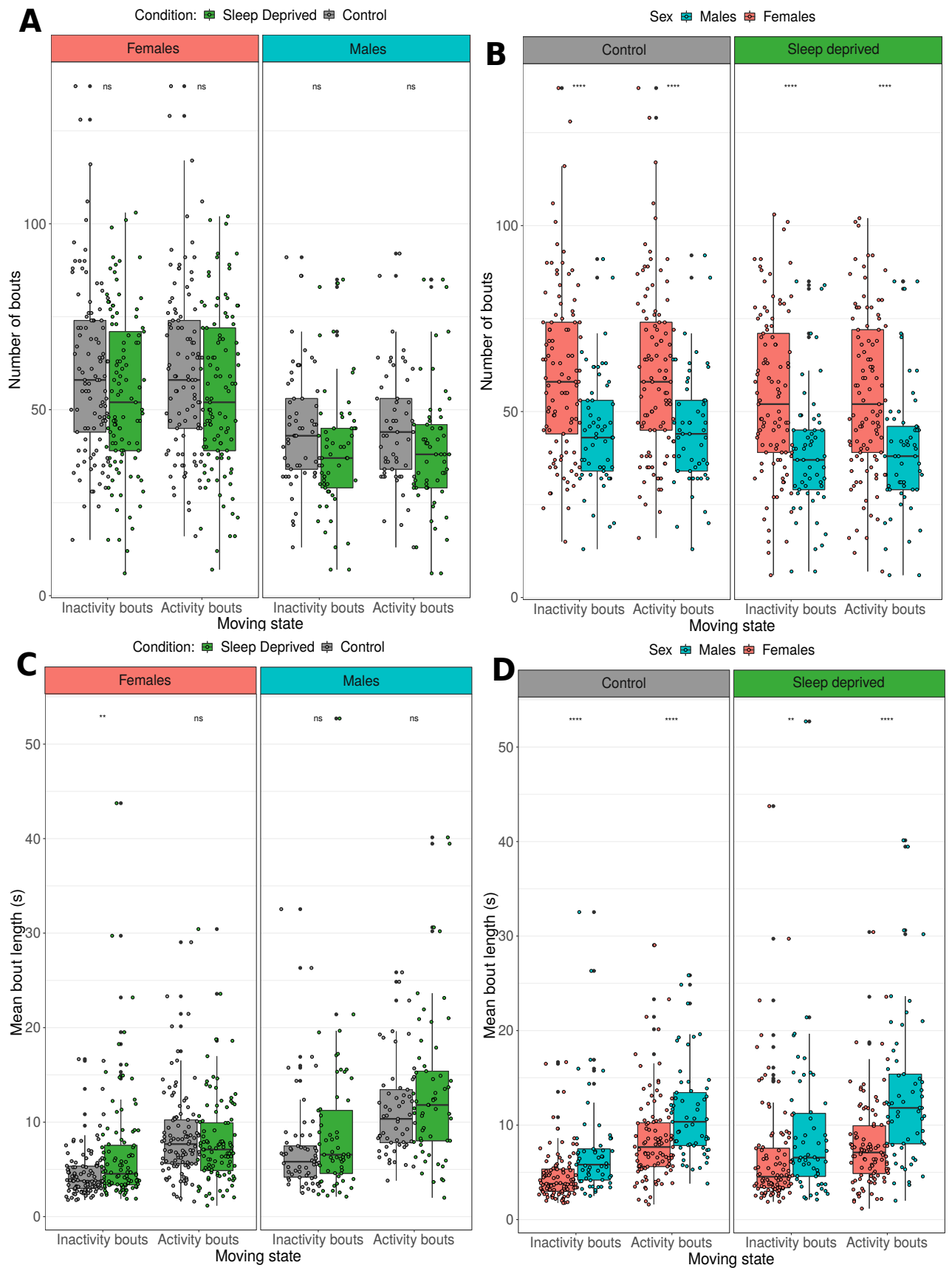


Figure 1.10: Number and duration of the activity and inactivity bouts in the y-maze. Same data is shown twice: once to compare the metrics between sleep conditions and once to compare them between sexes. **A** Number of inactivity and activity bouts for control and sleep deprived conditions. Left panel shows results for females and right panel shows results for males. **B** Number of inactivity and activity bouts for males and females. Left panel shows results for control flies and right panel shows results for sleep deprived flies. **C** Mean bout inactivity length and mean bout activity length for control and sleep deprived flies. Left panel shows results for females and right panel shows results for males. **D** Mean bout inactivity length and mean bout activity length for males and females. Left panel shows results for control flies and right panel shows results for sleep deprived flies.

Female flies show no differences in the amount of activity and inactivity bouts (Figure 1.10 A) but they show an increased mean duration of their time spent inactive (Figure 1.10 A). This suggests that sleep deprived flies rest more during the experiment compared to control, but is difficult to assess if this states are similar to sleep without a way of testing their arousal when inactive. Sleep deprivation does not have any significant impact on the amount and duration of movement organisation in males. Control and sleep deprived males have make a similar number of activity and inactivity bouts (Figure 1.10 A), and there are no important differences in the durations of neither their activity, nor their inactivity bouts. The results in males show that in spite of the lack of sleep, males do not rest more in the maze and their locomotion architecture is undistinguishable so far between the two sleep conditions. By fixing the sleep state and comparing the architecture of sleep between the two sexes we observe again a significant sexual dimorphism. Females activity is more sporadic compared to males, as they show an increased amount of both activity and inactivity intervals (Figure 1.10 C). Males have less bouts of movements but a more continuous activity with longer bouts compared to females (Figure 1.10 A). The sexual dimorphism is conserved in both sleep states of the flies.

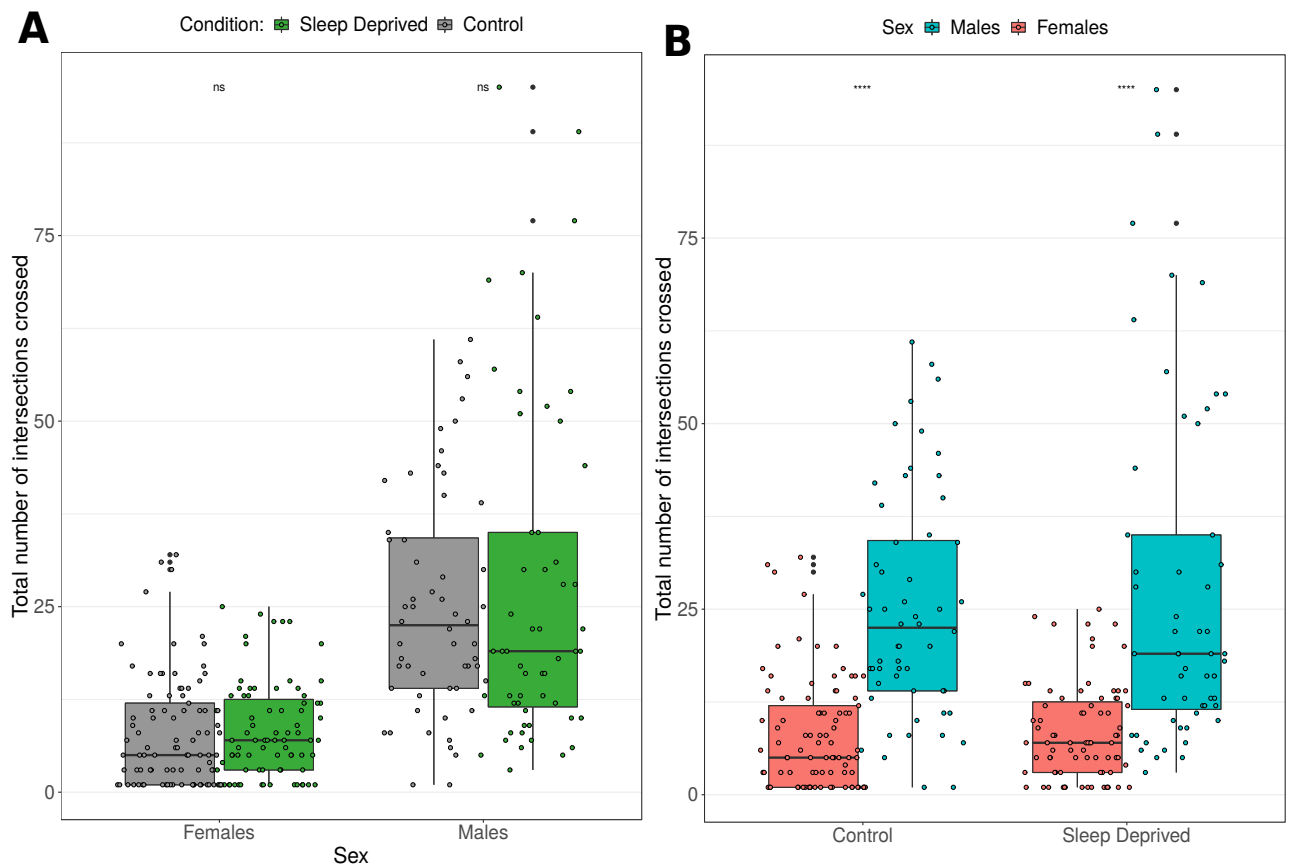


Figure 1.11: *Number of intersections crossed* Same data is shown twice: once to compare the time spent in intersections between sleep conditions and once to compare it between sexes. **A** Total number of intersections crossed by each individual fly. Comparison between control and sleep deprived conditions, in animal of the same sex. **B** Total number of intersections crossed by each individual fly. Comparison between the males and females deprived in the same sleep state. $N_{flies} > 24$

The y-maze design faces the flies with a complex environment to navigate in that it prompts them to choose a specific strategy of navigation inside the maze. They are also offered the possibility of spending their time in either a challenging part, the maze, characterized by a more restrictive space or in the open area where they can freely move. To investigate into flies approach of the intersections of the y-maze we

counted the number of intersection crossed by each fly. Given that the amount of activity between sleep conditions is stable, we further wanted to investigate if the lack of sleep is reflected in preference for a less challenging environment, which will be reflected in a decrease amount of intersections crossed. Our data suggests that this is not the case, as sleep deprived flies show a similar number of crossed intersections to control (Figure 1.11 A). A sexual dimorphism is still observed as male flies cross on average significantly more passages than females flies (Figure 1.11 B). However, it is difficult to know if this is purely due to an increased locomotion or if the increased number of crosses also reflects a sexual difference in the perception of the intersections, which would be less attractive to females compared to males.

In humans, sleep deprivation drastically deteriorates reaction times in activity^{113,114}, suggesting a decreased ability to respond to the environment. As flies navigate through the maze, they are faced with consecutive intersections in which they spend variable times before deciding between going left or right. To investigate if sleep deprivation impacts their reaction times in the intersections, by modulating their decision times, we quantified the amount of time they spent in the small area surrounding each intersection crossed. The areas defining each intersection are shown in the mask presented in Figure 1.5 C.

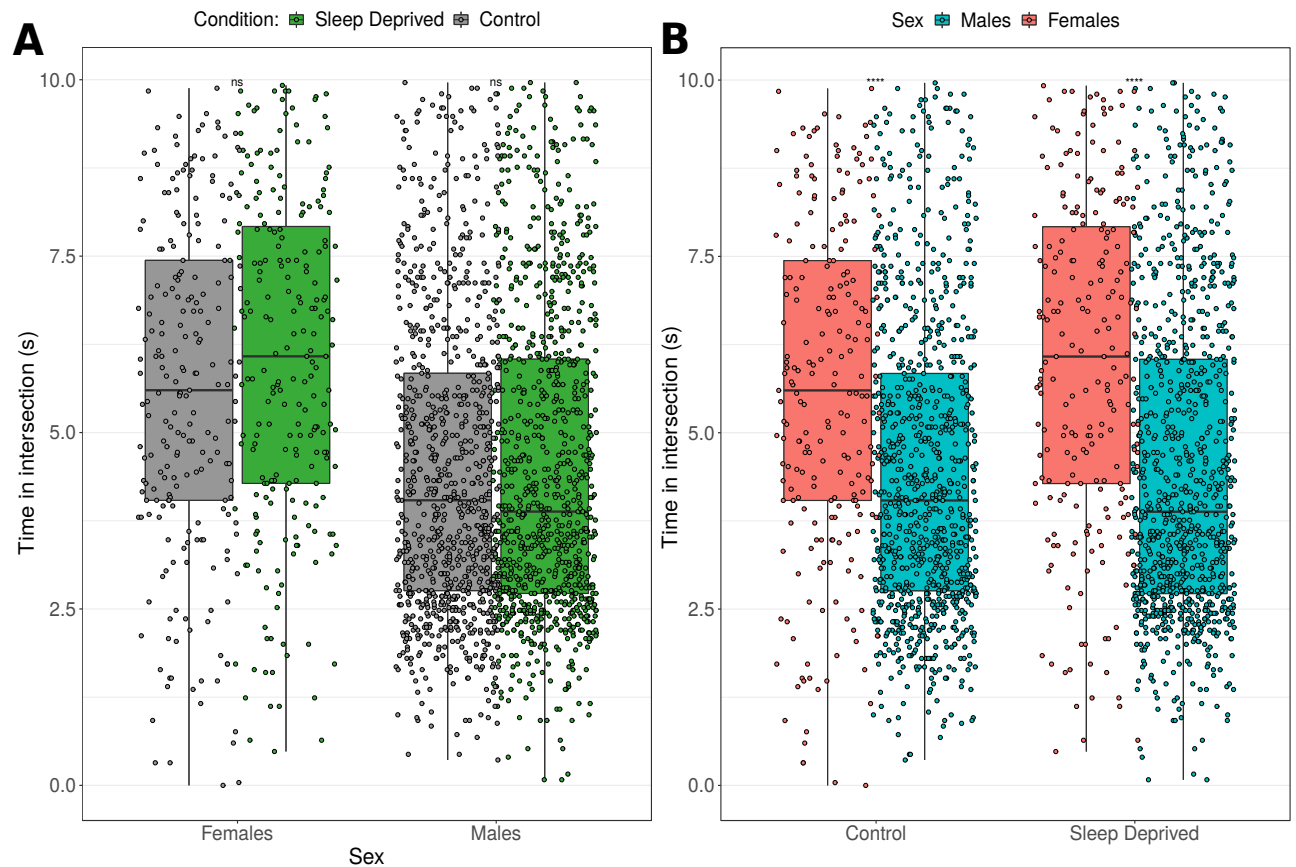


Figure 1.12: Time spent in intersections. The intersections positions in the y-maze are highlighted in the mask presented in Figure 1.5 C with different grayscale shades. Same data is shown twice: once to compare the time spent in intersections between sleep conditions and once to compare it between sexes. In order to exclude the flies that stopped for grooming in the intersections for large amounts of time, and to consider only the passages that reflect the decision of going left or right, the data was truncated and only decisions shorter than 10 seconds were considered. **A** Comparison between the time spent in intersections by flies in the control and sleep deprived conditions, in animal of the same sex. **B** Comparison between the time spent in intersections by males vs females flies, for a fixed sleep state: control or sleep deprived.

Figure 1.12 A shows that sleep deprivation does not impact the time flies spent in intersection, suggesting that their ability to respond to the environment and to decide if they want to go left or right is conserved.

However, the decisions durations are significantly reduced in males, which respond quicker and spend less time in intersections compared to females (1.12 B).

1.3.3 FRACTALS IN BEHAVIOUR

Previous sections describe the exploratory behaviour of *Drosophila* using average quantities of different metrics. These measures are very coarse-grained as they do not take into account the entire temporal pattern of the activity of the flies. The timings at which a fly initiates a moving bout or stops for an inactivity bout are internal to the flies and must be under the control of external environment, internal states and a bit of randomness. How exactly are the inactivity and activity bouts organised in time? Their organisation is rather complex, several studies show that the locomotor behaviour of *Drosophila* follow a fractal structure.

The word "fractal" was coined by Mandelbrot which used it to define a mathematical topology that has the property of self-similarity¹¹⁵. This implies that no matter how much we zoom in we will get the same pattern. An example of a computer generated fractal can be seen in Figure 1.13 A. The fractal geometry gives a better description of the complex shapes found in nature like the shape of blood vessels in humans body¹¹⁶ or the shape of the Romanesco vegetable shown in Figure 1.13 B.

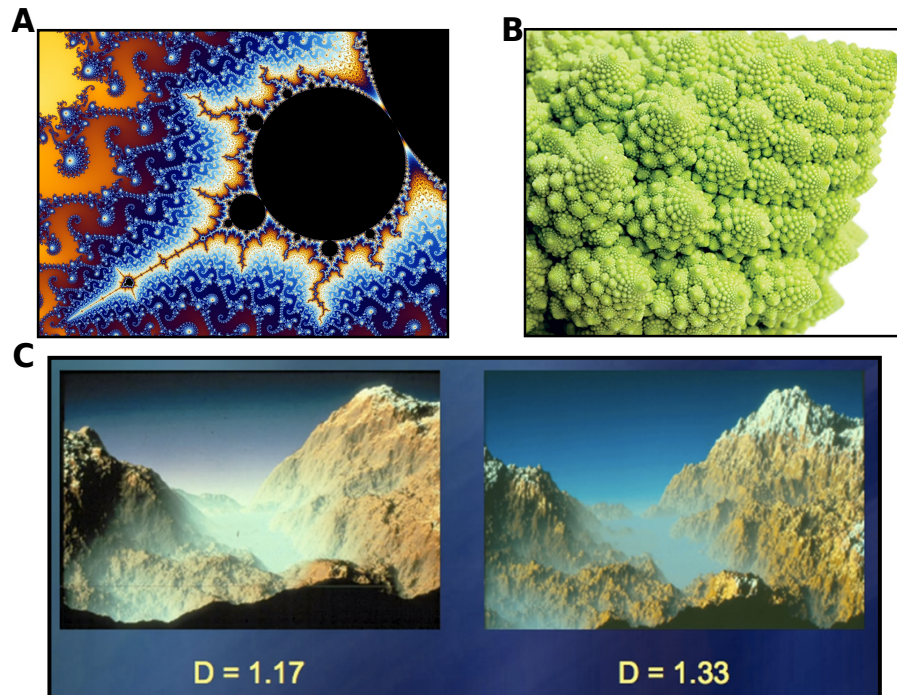


Figure 1.13: Examples of fractals. **A** Computer generated fractal using the recursive function $z_{n+1} = z_n^2 + c$. **B** Romanesco broccoli vegetable whose form is a natural approximation of a fractal. **C** Beautiful computer generated images (by Richard F. Voss in 1983) using only fractal equations. Adaptations sourced from <https://commons.wikimedia.org/wiki> under the Creative Commons license

More interestingly, fractals are better not only at modelling various complex shapes present in the material world but they are also better descriptors of temporal events happening in nature such as the opening and closing of ion-channel¹¹⁷ or behaviour in general¹¹⁸. Using the tracking data generated by mobile phone users it has been shown that their movement follows a fractal pattern⁶. In *Drosophila*, several studies found fractal like temporal patterns in flies behaviour. A few examples are the feeding behaviour¹¹⁹, the stop-go behaviour^{75,76,77}, and the body saccades of tethered flies⁵.

Fractal patterns shows a self-similarity in the temporal pattern. In the *Drosophila* locomotor activity, this can be easily observed by plotting the velocity of flies over time. Figure 1.14 plots the velocity of a single fly has activity and inactivity bouts over the time course of 5 days.

By plotting the velocity of the fly over the course of time we get a pattern of intermittent activity and inactivity bouts. A two-fold expansion of the activity of the flies shows exactly the same pattern, suggesting indeed that there are activity bouts nested inside the inactivity bouts which get revealed by zooming in. However, in practice this has a limit, as we can only zoom in as much as the resolution of our data allows.

The fractal structure of the temporal organization of behaviour can be assessed by generating the log-log plot of the number of inactivity bouts of a certain duration as a function of that specific duration. The linearity of this plot indicates a power-law distribution, which implies a fractal pattern^{75,76,77}. Figure 1.15 A shows the log-log plots of the number of inactivity bouts in the y-maze against their duration in 6 individual flies, 3 in the control state and 3 in the sleep deprived state. The linearity of these plots suggests that in the y-maze the inactivity bouts follow a fractal structure, confirming results of previous studies^{75,76,77}.

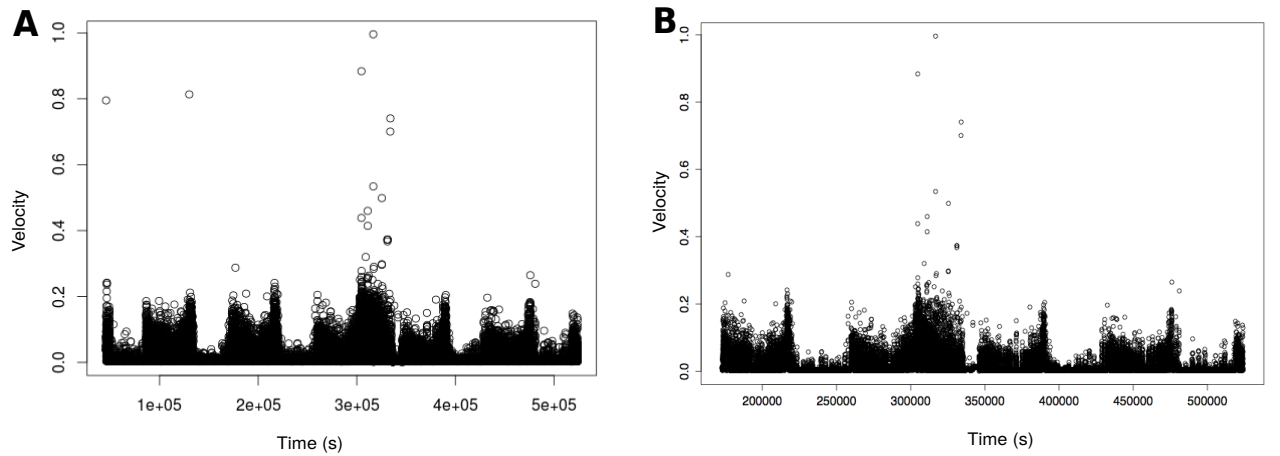


Figure 1.14: Self similarity in the activity of *Drosophila*. Data collected by following individual wild type flies over several days in standard glass-tubes using the ethoscope platform. **A** Velocity of one individual fly over the course of the experiment. **B** Two fold expansion of the time-scale of **A** results in the same pattern.

Fractals are defined by a dimension which reflects the measure of complexity or the granularity of a certain pattern. Figure 1.13 C shows an example of a computer generated image using fractals equations with different dimensions. It is visible from these images that a greater dimension implies more detail. The functions presented in Figure 1.15 A are approximated by the power-law equation $y = 1 - \exp(-(x/a)^{-b})$ with b representing the slope of the log-log plot and a representing the relative position on the abscissa⁷⁶. The dimension of the fractal D corresponding to the power-law distribution is inversely proportional with the slope b of the log-log plot⁷⁶. Therefore the slope of the linear plot describes the complexity of the temporal pattern present in each individual fly. The complexity of the temporal pattern is dependent on the age and on the genotype of the flies. The locomotor activity of older flies remains fractal but the slope decreases suggesting that the complexity of the pattern is reduced with age⁷⁶. It has also been shown that the fractal dimension changes with genotype and WT Berlin flies have a higher fractal dimension compared to CantonS flies suggesting a more complex temporal pattern in their activity⁷⁶. Age and genotype are internal characteristics of the flies therefore I hypothesize that sleep deprivation might also change the com-

plexity of the pattern of movements in *Drosophila*. By using log-log plot analysis in each individual fly I computed plotted and computed the linear equation characteristics to their temporal pattern (Figure 1.15 A). A comparison of the fractal dimension between sleep conditions suggests that control and sleep deprived flies have no differences in the complexity of their activity pattern in the y-maze. Moreover, there was no significant difference in the complexity of the movement organisation of males and females.

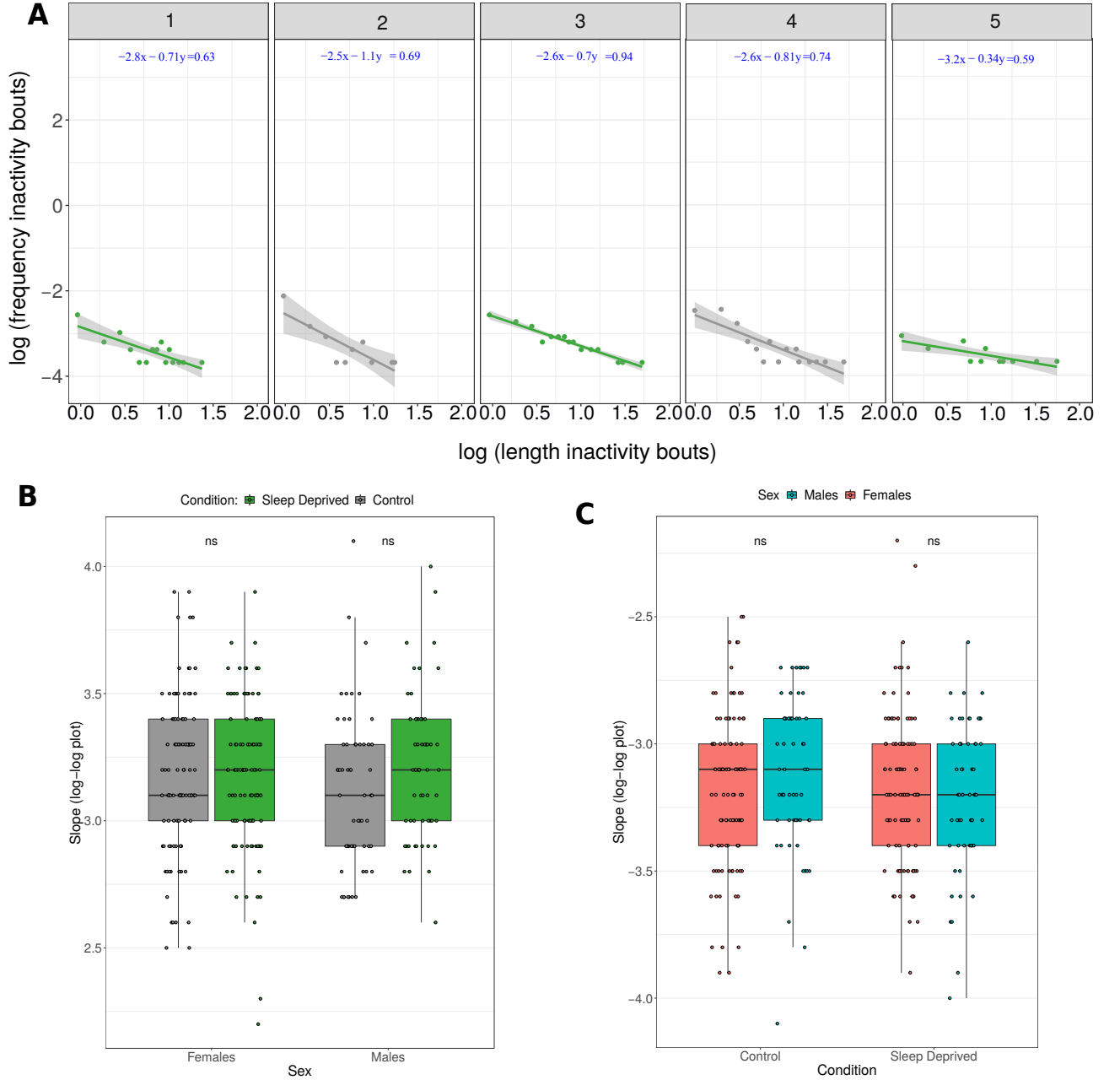


Figure 1.15: Log-log plot analysis of the temporal pattern of activity in the y-maze **A** Examples of log-log plot of the length of inactivity bouts as a function of their frequency. Each panel represents an individual fly. The characteristic linear equation defining the log-log plot is presented in blue. The slope of these lines are inversely proportional with the dimension of the fractal structure characteristic to the complexity of the temporal pattern. **B** Comparison between the fractal dimension of the temporal activity pattern between sleep conditions, for females and males. **C** Comparison between the fractal dimension of the temporal activity pattern of males vs females, for a fixed sleep condition.

1.4 DISCUSSION

The work presented in this chapter is a first attempt to investigate the link between decisions and sleep in *Drosophila*. Strikingly, the behaviour of the sleep deprived flies of both sexes is indistinguishable from the control flies of the same sex in all the metrics used to assess flies behaviour in the y-maze. Sleep deprivation does not change the exploratory drive as the distance and area covered in their 15 minutes in the y-maze are no different than control (Figure 1.8 A, Figure 1.9 A). A closer look into the architecture of their movement bouts reveals that in males, the number and the duration of the activity and inactivity bouts is no different than control. Therefore, male flies do not sleep in the y-maze and there is no fragmentation of their movement caused by sleep deprivation. Female flies have slightly longer inactivity bouts suggesting that they might be resting, however these periods of rest are too short to be scored as sleep using the methods employed in the field^{104,56}. Existing literature shows that following a night of sleep deprivation flies exhibit a longer rest in the first hours straight after sleep deprivation, called a sleep rebound. This is usually observed during the first 3-6 hours of the next day^{56,97}, and flies enter in the y-maze in this time-window. A possible explanation for the lack of a sleep rebound inside the y-maze is the novelty of the environment which might make flies prioritize the exploration of maze and be able to delay coping with the sleep deprivation. Figure 1.6 A reinforce this idea by showing that after the first round of experiments in the y-maze, the sleep deprived group shows an increased sleep during the day following the experiment, and once back into the original tubes used for monitoring them over five consecutive days. The absence of the sleep rebound might also be due to the shape of the new environment rather than its novelty. The y-maze has a rather complex architecture with space ranging from very restricted in the maze engravement to an unconstrained space in the open-arena. Sleep research in *Drosophila* was undertaken by placing flies in individual glass tubes⁵⁶ using DAM monitors (Section 0.7.4.1) and recently using video acquisition⁹⁷ which offers a better resolution into their behaviour, without changing the environment, flies being kept in standard glass tubes. This medium has been established as being very convenient for keeping *Drosophila* in good health for long periods of times. Regardless, I think it is important to be able to reproduce the results on which an entire field rests in slightly different contexts and to investigate the robustness of the results and their dependence to the shape of the external environment. A recent study shows that the locomotion of *Drosophila* is highly increased by reducing the space of the environment in males¹²⁰. Given that we assess sleep in *Drosophila* based on flies locomotion, being able to reproduce results in different contexts is fundamental for trustful results.

Our investigation reveals a strong sexual dimorphism in the exploration of the maze. Male flies engage much more in the y-maze compared to females which in great part do not even attempt to enter the maze and only approximately 30% walk through the maze to the open arena, compared to more than 87% of the males that make it to the open arena. Moreover, the distance walked and area explored by females is highly less than males (Figure 1.8 B, Figure 1.9 B). It is possible that the dimorphism in exploration is caused by differences in size, as females are known to be larger than males¹²¹. The perception of the space might be different for females which can feel more restrictive in their movements and having therefore a diminished exploratory drive. This study¹²⁰ also reveals a strong sexual dimorphism in the locomotion of the flies, with male flies having an increased locomotion induced by space restriction but not females. If this is the case, the more intense activity observed in males might be a response to the restrictive space in the narrow tunnels of the binary tree engravement on the y-maze. A strong dimorphism is also observed in the architec-

ture of movement organisation, as females show an increased number and significantly longer inactivity and activity bouts compared to males, independent of the sleep state (Figure 1.10 B and D).

Even more sophisticated methods able to assess the complexity of the temporal pattern of the flies movement as a whole, were not able to capture any differences between sleep conditions (Figure 1.15 B). Moreover, they suggest that independent of the sleep state or sex, every single fly's organisation of movement in the y-maze is well characterized by a fractal structure. This originates in the power-law distribution linking the duration of inactivity bouts to their frequency, confirmed by the linearity of the log-log plot (Figure 1.15 A). There is one study that shows that age, genotype and sex have an influence on the complexity of the temporal sequence of flies movements. Our results suggest no difference between the fractal dimension characteristic to the locomotion of males and females. The difference in results might be due to the different acquisition methods employed⁷⁶, as they used the DAM system (Section 0.7.4.1) to collect data which has a lower resolution in the movement of flies compared to the improved quality offered by our videos recording at 25 frames/s. Their estimation of the fractal dimension might be imprecise, as the DAM monitors fail to capture the activity of the flies while in the regions separated by the infrared beam. Otherwise, the difference in results might be a reflection of the specific shape of the y-maze, which might give rise to a similar complexity in their movement pattern. Remarkably, in spite of the fact that the environment is not completely open, and flies are faced continuously with intersections and a complex navigation path in the maze, the randomness in their behaviour is conserved, suggesting that the inherited stochastic specific to any behaviour has an important impact on their stop-go behaviour and it is not abolished by the architecture of the maze. The persistent randomness characteristic to flies behaviour in the y-maze occludes any effects of the sleep deprivation, which might be perceivable if more data was available. However, the y-maze paradigm is not suitable for very high-throughput experiments as only 1 fly can be followed by an ethoscope at a time.

More recent studies¹²² bring advanced methods to be employed when assessing a power-law, and they suggest that the linearity of the log-log plots is a required but not sufficient condition to confirm the existence of a power-law distribution in the data. For meaningful outputs, the new methods request high amounts of data, equivalent with several weeks of fly's activity records, not being therefore applicable to our work.

The time that flies spent in the intersections is a good read of how they perceive the choice of going left or right. Observations of their behaviour in the maze, together with the fact that sleep deprivation has no impact on their reaction times in the intersections makes me think that the y-maze paradigm fails at capturing the moment in which the decision is actually taken, making it difficult to assess if an actual decision is taken or if flies behaviour in the y-maze is purely a consequence of their locomotor activity. Certainly, sleep deprived flies do not show any anxiety with regards to the intersections as they choose to pass through a similar number of intersections as control (Figure 1.10 A). A strong sexual dimorphism is again observed in the way in which males and females approach the intersections with the females choosing to pass through significantly less and they also take longer time to decide weather to go left or right. Given that the decision is so tight to the locomotion, without any other reference, and knowing that the locomotion of males and females is distinct with females walking less overall, it is impossible to know if the differences in reaction times are simply a causation of their different activity or if there is also a sexual dimorphism in the mechanism employed for choosing between going left or right.

Flies that arrived in the open arena showed hesitation of going back into the maze, by repeatedly mak-

ing turns around the same exit before attempting to enter. This observed behaviour also trigger the idea of studying flies passages through hole of different sizes, explore in Chapter 2 and Chapter 3.

The non-gaussian exit distribution (Figures 1.7 C), seems to be caused by a high percentage of flies exiting the maze through the lateral exits. This suggests that flies movement in the maze is not purely random, but rather modulated by external and internal forces. Some of the flies choose to go only left or only right. Once study that investigated the handedness in *Drosophila*² also shows the existence of individuals that are strongly left or strongly right, however a close observation of their video data and my experience with the y-maze makes me believe that the choice of the flies of going left or right is highly-modulated by their wall-following behaviour. It seems to me that flies that keep their legs and body very close to one wall side will continue walking on that side with high probability. To stop them to have this behaviour, our paradigm was refactored and tested several time to minimize their ability of holding to one side of the wall and forcing them to face the intersection, in order to ensure relatively equal chances of choosing left or right.

An important achievement of the work undertaken so far is the creation of a flexible workflow that iterates the life cycle of a behavioural assay from creation to data collection and data analysis. I was in charge of every step from building the behavioural arena from scratch, finding the tools that will enable me to collect accurate data and writing the scripts to analyse the data. The ethoscope platform and the rethomics package⁹³ gave me a very strong backbone on which I could build a software that adapts to my needs. Every tool integrated in this workflow needed adaptation to my particular environment and needed continuous testing and integration. Apart from being hard and time consuming to put in place, especially on the software side, this approach gave me a depth knowledge into the different components involved and enabled me to easily transfer it to future behavioural paradigms.

To summarize, this paradigm gave me the opportunity of building a workflow and unravel to me the randomness specific to flies behaviour which needs to be taken into consideration when building a decision making paradigm. The y-maze has a relatively low-throughput, that is not able to average out the randomness of the activity in order to reveal insights about how sleep deprivation impacts decisions. The relatively low-throughput also put a restriction on the length of the experiments, and it is the very brief experiments of only 15 minutes which might be a possible reason for no visible effects between control and sleep deprived flies. Another limitation of the paradigm was the small number of decisions. A larger number of crossings might be needed to understand the consequences of sleep deprivation on flies behaviour. The continuity of their movements, and the resolution of our video acquisition makes it difficult to assess the initiation of the decisions of going left or right. It is hard to understand decision making if the only metrics that we have are linked to the locomotion which is involved in all the behaviours. A paradigm able to assess the initiation of decisions and their modulation by internal and external states based on a different metric than locomotion would therefore be highly desirable. The ability of better disentangling the locomotion from the actual decision might be able to give us better insights into how the decision making mechanism integrates different external and internal variables before taking action. The y-maze was good for an exploratory investigation into flies behaviour, and provided some interesting results into the flies homeostatic regulation mechanisms of sleep, however it fails at capturing the decision making mechanism in an easily quantifiable way. The ability of the flies of going back and forth through the intersections adds extra complexity to the data collected.

The only source of knowledge is experience.

Albert Einstein

2

Concentric circles and holes

LOREM IPSUM DOLOR SIT AMET, Miesenbock

2.1 INTRODUCTION

In humans, the intersection between sleep and decision making seems to be best illustrated in people with psychiatric diseases. Most such disease including schizophrenia, autism and bipolar disorder have common and possible interlinked behavioural symptoms: propensity to make poor decisions²⁴, lack of attention^{123,124,125} and deficient sleep¹²⁶. Sleep is characterized by increased arousal thresholds: deeper the sleep, stronger the arousal needed to get a response. One suggestion is that the link between mental illness and sleep comes from a shared fundamental mechanism in charge of controlling the arousal thresholds and attributing relevant salience to appropriate stimuli⁸³. Deficiency of such a mechanism would lead to poor decisions, sleep problems, attention shortfalls and mental illnesses. For example, hallucination in psychotic patients can be viewed as an effort of making sense of noise signal from the environment that fails to be suppressed by the appropriate mechanism and is leaking into the higher cognitive levels^{127,128}.

All of the above are human specific and it appears difficult to transpose these anthropomorphic concepts proper to the human mind into animal models, especially insects like *Drosophila*. Nevertheless, approaching these psychiatric diseases not by looking at them as human mind problems but rather as a salience dysfunction syndrome^{83,128,127} could open new venues into studying such diseases even in the humble fruit fly. Apart from the benefits brought to the medical world, looking at arousal thresholds can be key into understanding the interplay between decision making and sleep. A close examination into how sleep modulates behavioural responses of flies to slightly variable environmental cues might reveal new insights into the functional role of sleep in our life which in spite of great scientific efforts, remains a mystery. The major obstacle for such a scientific direction resides into a lack of suitable behavioural paradigms. Are there any ways of capturing the attention of a fly? A relevant assay is the flight simulator in which a tethered fly is presented with different visual stimuli^{98,129,130}. Flies visual attention can be measured by examination of their body movements and particular combinations of visual stimuli can reveal insights about their attention-like states. The downside of this particular paradigm is its sophisticated design, difficult to

put in practice and not suitable for high-throughput experiments, as only one fly can be present in a flight arena at a particular time. Observing subtle changes in behaviour requires large numbers in order to compensate for the variability specific to any action.

The work presented in this chapter is an attempt to find a relevant paradigm suitable for the study of the intersection between sleep, decision making and attention using *Drosophila*, in a high-throughput manner. My strategy was to start searching for a simple assay able to capture the behaviour response of flies to increasing levels of an external stimuli and look at how sleep deprivation impacts flies responses. Preferably, the behavioural paradigm would also expose the flies with a decision that separates the initiation of action from the modulatory effects of internal states and external conditions. This separation seemed essential for a straightforward quantification of individual modulatory factors. Ideally, the decision should be binary, and happen fast in order to be easily measurable and repeated over long period of times.

Finding a binary response to external stimuli of different intensity will lead to an easily quantifiable way of assessing the insights of the decision mechanism responsible for choosing actions. To simplify even more, the decision should be under the control of as few external stimuli as possible, preferably one. The gap-climbing assay⁷⁹ was inspiring in that direction as it is a binary decision (attempt to pass or no attempt to pass over a gap) modulated by one main external stimuli: the width of the gap. The behavioural assay that presents the flies with only one gap was already challenging to put in practice, therefore giving flies access to multiple gaps of different widths was not amenable for high-throughput experiments.

The inspiration for the assays presented in this chapter came from the natural feeding behaviour of the flies. Most species of *Drosophila* like feeding on rotten fruit. The foraging behaviour in itself is very complex and has a lot of facets, requiring a multisensory integration in order to make appropriate nutritional and oviposition decisions. Flies usually make small circular chambers in the rotten fruits to gain easy access to the food source or to find the best nutritional place to lay their eggs. In doing so, the fly has to make a correct estimation of the size of the cavity through which it would be able to pass. It is known that *Drosophila* is able to visually approximate distances in quite a refined manner. As mentioned before, when faced with a chasm, flies will spontaneously decide to cross if the gap seems surmountable based on their distance estimation⁷⁹.

Inspired by this natural behaviour, I started to look into the flies ability of distinguishing between holes of different sizes.

This chapter presents a set of preliminary assays which are designed to get a rough idea about flies hole preference and which explore several ways of pushing the flies to pass through holes. The initial paradigms are gradually refined into a final assay, the concentric rings arena, which reveals some potentially interesting results about the flies choices and how they are modulated by internal and external forces. The Material & Methods section includes technical advancements of specific software developed in parallel to enable the future exploration of the hole preference assay in a high-throughput manner.

2.2 MATERIAL & METHODS

2.2.1 FLY STOCKS:

The flies used in this experiment were *Drosophila melanogaster* CantonS (CS) and were obtained from the Bloomington Laboratory. They were raised under a 12 h light: 12 h dark (LD) regimen on standard solid corn and yeast media. Flies are reared in standard 25mm vials from Flystuff. The same number of parent

flies (4 females and 2 males) were kept in the vials for 2 days to ensure similar social conditions and food availability.

2.2.2 EXPERIMENTAL CONDITIONS:

Experiments were conducted on 3-5 days old mated males, in incubators set at 60% humidity and 25 °C, in either dark or light conditions as specified. Preliminary experiments were run at different times of the day at room temperature.

2.2.3 SLEEP DEPRIVATION

Flies are sleep deprived using mechanical sleep deprivation during 12h from ZT₁₂ to ZT₀. The vials containing the flies are placed on an orbital shaker (Grant PSU-10i Orbital Shaker) which rotates continuously at 450 rpm for the entire dark cycle.

2.2.4 MODELS MANUFACTURE

All the mazes were designed using Onshape software available online at www.onshape.com. The preliminary designs were printed using the Ultimaker 2 3D printer, in PLA material. All the other designs were printed with the Form2 printer by Formlabs using clear resin. Prints were washed for 10 minutes in a bucket with isopropanol after which they were removed from the first bucket and placed in a second bucket with clean isopropanol for another 10 minutes. Longer times were used if necessary to remove all the excess resin. Finally, prints were cured in UV light for one hour at 60 °C to achieve their optimal properties and future resistance.

2.2.5 EXPERIMENTAL PROTOCOL

Flies were loaded using a mouth aspirator in the 2D arenas at ZT₀ (9am) and placed inside ethoscopes. Their behaviour was video recorded for 3 to 5 hours.

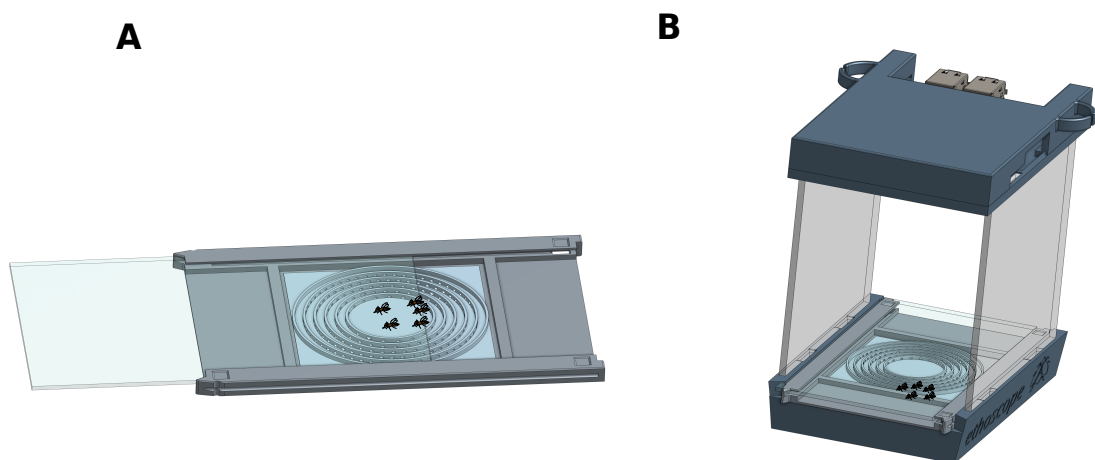


Figure 2.1: Ethoscope and concentric rings maze setup **A** Flies were loaded into the middle circle of the concentric rings maze using a mouth pipette. The glass covers almost entirely the inner circle, leaving only a small aperture for loading the flies using a mouth pipette and preventing them to escape. **B** Ethoscope recording flies in the concentric rings arena.

2.2.6 VIDEO RECORDINGS

Videos of 1280×960 pixel resolution were recorded using the ethoscope platform⁸⁸ at 25 frames/s. The ethoscope platform generates one compressed .h264 file every 5 minutes. Ffmpeg software is used to unite all the .h264 files corresponding to an experiment into one single .mp4 file, on which the tracking algorithm is applied.

2.2.7 DATA COLLECTION AND ANALYSIS

The passing through holes behaviour of the flies is scored manually by dividing the three hours .mp4 video into 30-seconds extracts with 2 minutes interval, producing 90 videos to be scored per experiment. Data analysis was performed in R. Plots were generated using ggplot2 and ggpubr packages. For all the boxplots, the first and third quartiles (Q_1 and Q_3) are the ends of the box and the median is indicated with an horizontal line in the box. The whiskers go from the bottom and top of the box to the minimum and maximum values situated in the $\pm 1.5IQR$ range, where $IQR = Q_3 - Q_1$. The errors bars present in some figures indicate the standard error. Statistical analysis was carried out using the pairwise Wilcoxon rank sum test. Outliers were always kept, and each experiment was repeated at least twice. The following convention is used to indicate statistical significance: ns: $p > 0.05$, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$.

2.2.8 MAZES DESIGN

2.2.8.1 PRELIMINARY DESIGNS

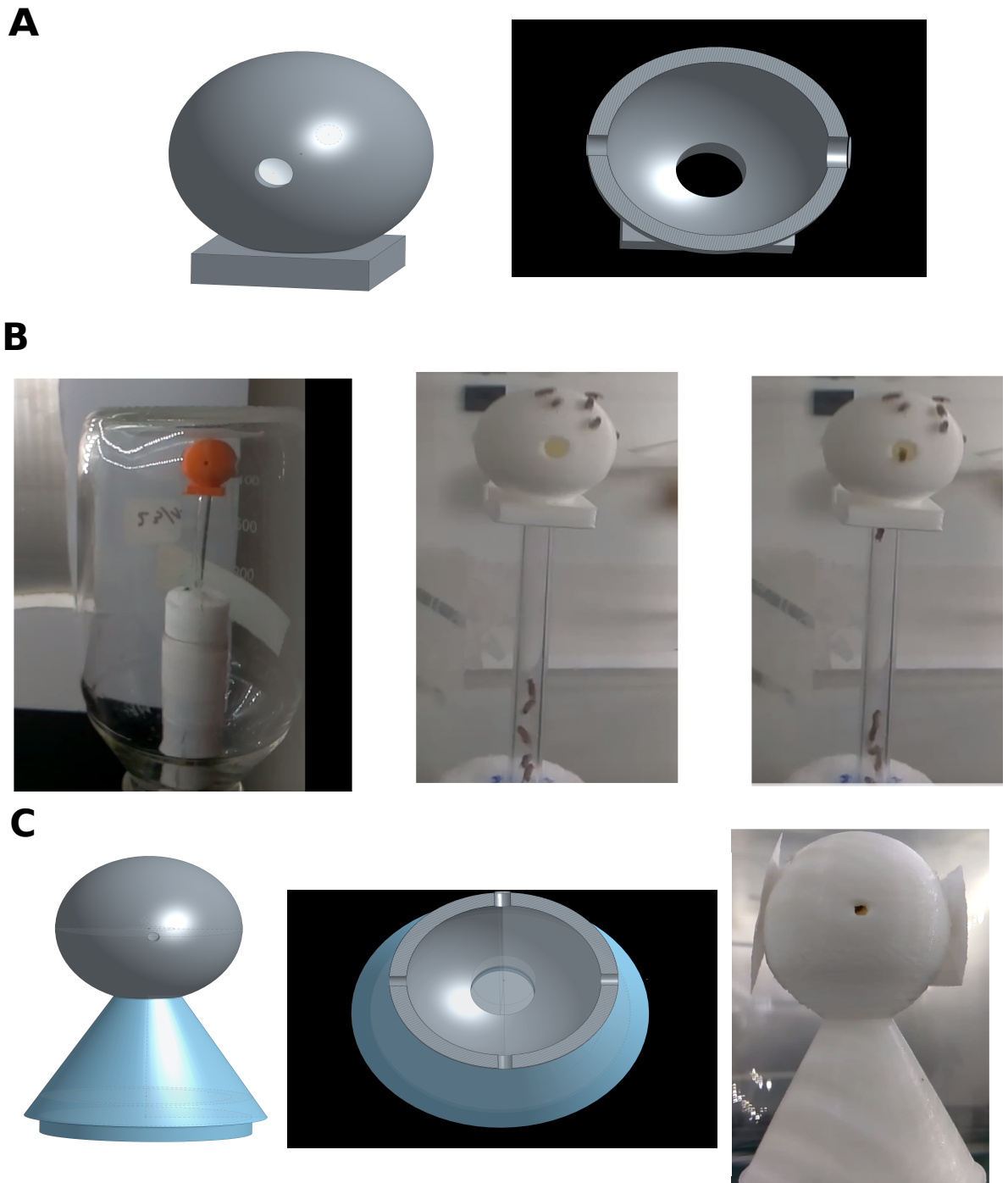


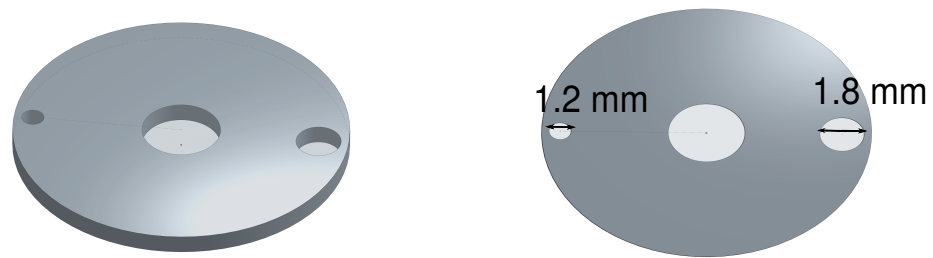
Figure 2.2: Spherical ball design for studying the individual and population passing through holes behaviour **A** Isometric and section view of the initial assay design for studying individual holes behaviour. It consists of a plastic spherical ball of 20 mm diameter which has 2 holes of different sizes (2mm and 1.5mm) along its diameter. The bottom part of the ball its designed to fit a standard glass tube through which flies can be loaded inside the ball. **B** Initial experimental design to study the passage through holes behaviour. Left image shows the experimental design which consists of a standard vial used for rearing flies. A glass tube is inserted into the cotton plug used to close the vial. The plastic ball with holes is placed at the free end of the tube and flies walk through the glass tube from the vial to the spherical ball and then escape through one of the hole of their choice. Middle image presents a close zoom on the big hole of the sphere taken during the experiment. It shows the flies walking up the tube and a few of them that already exited the ball and remained on top of it. Right image shows a fly escaping through the hole. **C** Left image presents the plastic ball designed to fit directly on the top of the vial used for flies rearing, allowing many flies to enter the ball in the same time. The ball holds 4 holes of 1.1, 1.2, 1.3 and 1.4 mm diameter respectively placed symmetrically on its middle section. Middle image shows a section view through the holes. Right image shows a zoom on the 1.1mm hole during an experiment in which this was the only hole available. A fly is trying to exit through the hole without success.

Figure 2.2 A presents the initial design for studying how flies pass through holes. It consists of a spherical ball holding two holes of different sizes (2mm and 1.5 mm respectively). Flies enter the ball through its bottom, advancing individually through a glass tube connecting a vial containing the flies to the plastic ball. Once in the sphere they can escape through one of the holes 2.2. The vials are fully covered in opaque tape to keep the flies in complete darkness. Both the negative geotactic¹³¹ and positive phototactic⁵⁰ behaviours of the flies are used to force them to exit the vial and reach the ball as quickly as possible in order to escape.

Figure 2.2 C presents a version of the ball paradigm that fits directly on the vial allowing many flies to enter the ball directly, therefore improving the throughput of the flies passing through the holes.

Finally, Figure 2.3 presents a paradigm consisting of symmetrically placed discs inside a vial. Each disc contains holes through which flies are able to advance from the bottom to the top of the tube. In this paradigm only the geotactic behaviour is sufficient to drive the flies to advance through the tube. Discs are all kept in the exact same position, having all the big holes on one side, and all the small holes on the other side. Flies are presented with four consecutive decisions of passing through the holes.

A



B

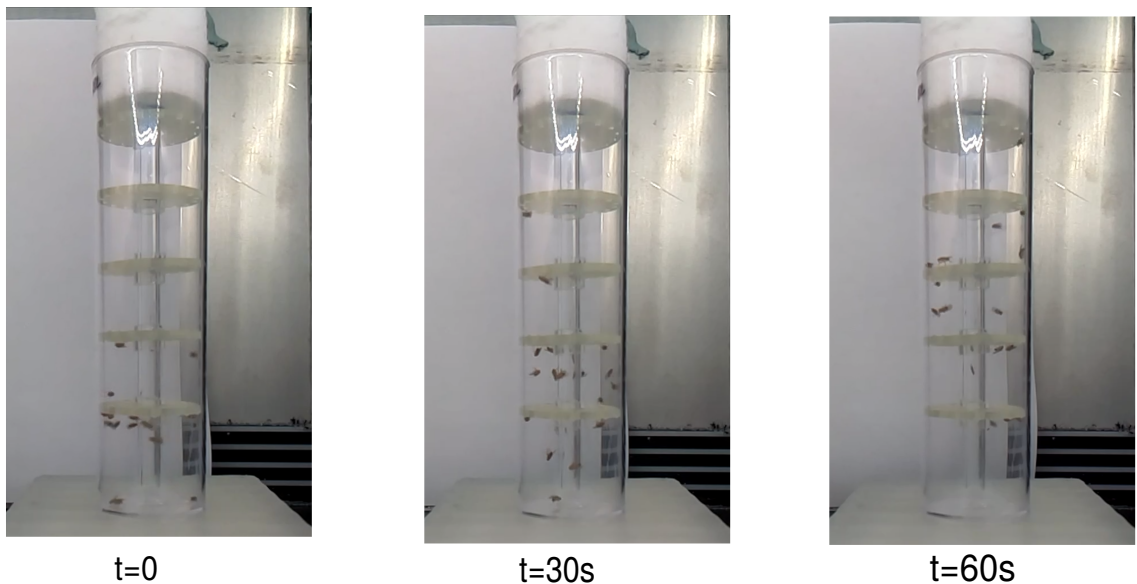


Figure 2.3: Consecutive discs paradigm to study passages through holes. **A** Disc holding two holes of different sizes on its exterior, designed to perfectly fit in a standard vial. The middle hole fits a glass tube through it, used for supporting the disc in the vial as shown in **B**. **B** Experimental design consisting of four consecutive discs placed symmetrically inside a vial. Discs and cotton plug are attached together by a glass tube and they can all be introduced into the vial at once. Flies advance from the bottom to the top of the vial through the holes of their choice. Three different frames are extracted from the experiment showing flies at the beginning of the experiment ($t=0$, left frame), after 30 s (middle frame) and after 1 minute (right frame).

2.2.8.2 CONCENTRIC RINGS

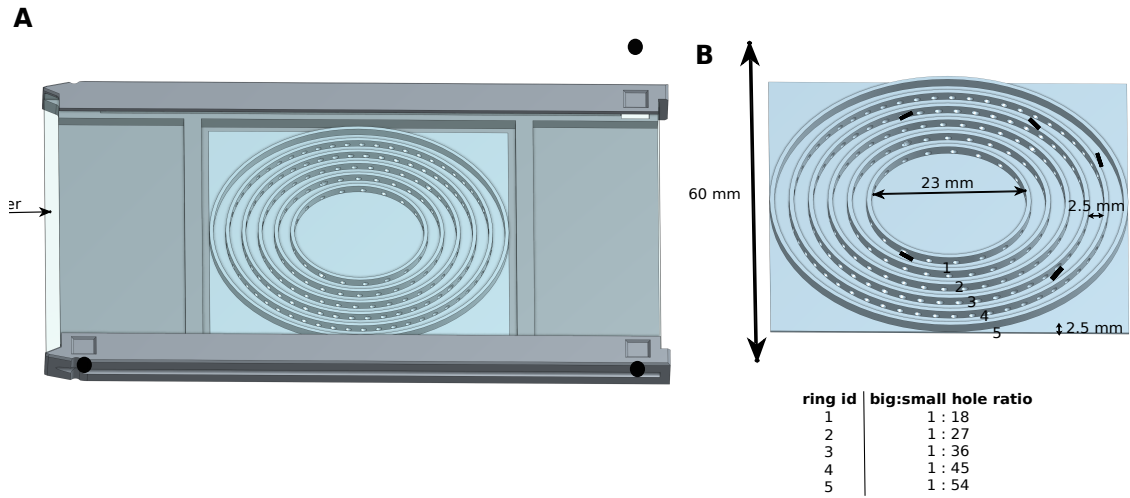


Figure 2.4: Concentric rings maze design and dimensions. It consists of 5 concentric and equidistant circular walls each holding one big hole ($\varnothing = 1.8\text{mm}$) and several small holes ($\varnothing = 1.2\text{mm}$) as specified in **B**. **A** Concentric rings maze with support arena that can slide into the ethoscope for recording. A glass cover is slid on top of the arena to prevent the flies from escaping. **B** Dimensions of the concentric rings arena. The position of the big hole on each ring was randomly assigned and is specified by a small black line.

Given that the preliminary designs capturing the holes passings behaviour offered some interesting results, I wanted to further understand how flies distinguish between holes. All the preliminary experimental setups (Figures 2.2 and 2.3) are in 3D. This makes data collection extremely difficult as it requires either an extremely attentive observer or a multi-camera setup to have access to several angles, but this demands more resources and an extra effort for merging the collected data for efficient scoring. A 2D arena design would offer the advantage of being easily recorded with a single camera. If videos are recorded at a decent frame rate than the passing through holes behaviour can be scored after the experiment by analysing one single video. Moreover, a 2D design will also be fit for potential tracking automation. Apart from the 2D design, I desired an assay in which flies are able to make several passages through the holes. The main blocker when trying to pass from a 3D to a 2D arena design was the driving force that pulls the flies to pass through the holes. If in all the preliminary designs the pulling force was the negative geotactic behaviour specific to *Drosophila*, in 2D, the few initial designs (not shown) failed to get the flies to pass through the holes. The inspiration came from Triphan et al. who uses a gap-climbing disks⁸⁰ to force the flies to make passages over consecutive gaps. Flies placed in the center of an arena are actively moving towards the outside part of the arena, avoiding the centre, behaviour known as centrophobism.¹³² Our concentric rings arena exploits the centrophobism of the flies together with their wall following behaviour¹³² to push them to make several crossings through the holes. From the preliminary results we decided to pick two sizes of holes that have a reasonable difference between them, one hole through which the fly can pass with no effort and one hole that is tighter, therefore demanding some effort to pass through. For the tight hole we chose 1.2 mm, the smaller size through which flies could fit in the preliminary results. Flies seemed able to distinguish a difference of more than 0.5 mm, therefore we chose 1.8 mm for the size of the big hole to make sure that flies perceive it as considerable different from the small hole.

2.2.8.3 MINI RINGS

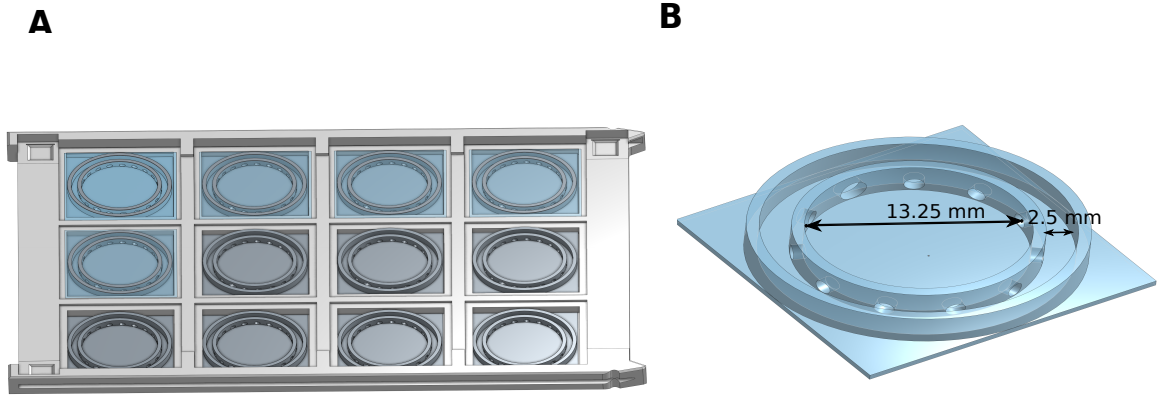


Figure 2.5: Mini ring maze **A** Arena holding 12 mini ring mazes. **B** Mini ring maze with one wall holding 1 big hole ($\varnothing = 1.8mm$) and 10 small holes ($\varnothing = 1.2mm$).

2.2.9 TECHNICAL DEVELOPMENTS

In both experiments undertaken up to this point: the spontaneous exploration of the y-maze (Chapter 1) and the spontaneous initiation of passages under the modulation of diverse internal and external conditions (Chapter 2) I felt the burden of not being able to automatically track the flies in small spaces with a great precision that can be maintained between experiments in spite of small variations in experimental conditions, like small movements of the camera that records, which can result in slightly deformed images. Given the small sizes of the regions of interests in my arenas (the y-maze engraving has a width of only 1.3 mm and the distance between concentric rings is of only 2.5 mm), any distortion of the images as a result of a change in the camera position produced perceivable changes in the amount of pixels and pixels positions representing those region of interests. The ethoscope platform had a rigid way of defining the regions of interest by specifying a set of 8 non-intuitive parameters.

Because small changes in the camera positions during the experiments the rigidly defined region of interest will often be misaligned with the real region of interests resulting in loss of continuity in data and worst data which cannot be aligned together to be analysed. Moreover, the ethoscope software allowed only the definition of a grid, but if the region of interest had another shape, like in the case of the y-maze, there was no way of defining the engraving of the y-maze. In this case a one cell grid was used as a region of interest over the entire arena containing the y-maze engraving. The definition of a new region of interest was very cumbersome as it was not developed to suite a frequent change of arenas, but rather one specific arena that holds flies in tubes.

At this point in my project I hit a wall. Having some interesting results to exploit, it was impossible to continue without a flexible and accurate way of defining the regions of interests and the ability of tracking the flies with precision in the smallest regions of interest. Also the concentric rings arena assay used both individual and group of flies. Therefore a multi-tracking algorithm of the flies which keeps individuals identity was highly desirable. Finally, in the prospect of a flexible and accurate way of defining the regions of interest, being able to automatically track the sequence of passages was necessary for a trustful validation

of the initiation of passages through the holes paradigm. Given that the passage of the holes happened so quickly, a high speed camera would have been appropriate to get a detailed description of the behaviour. However, I enjoyed the modularity of the ethoscope and the ability of running high-throughput experiments and considered it was more valuable to invest time into trying to adapt the ethoscope platform to my needs rather than acquiring expensive resources. Fortunately, around this time Raspberry Pi camera was updated from Module 1 which has a resolution of 5 Megapixels to Module 2 which has a resolution of 8 Megapixels. The improved resolution motivate me to try and reach the above goals by extending the ethoscope platform.s

2.2.9.1 FLEXIBLE DEFINITION OF REGIONS OF INTEREST

In order to be able to extract trustful data from my experiments I decided to first implement a flexible, automatic and fast way of defining accurate regions of interests in my videos. The circular shapes of the walls holding the holes in the concentric rings arena and mini rings will also not be suitable for this definition and giving that we are interested in this region in particular to capture the passage to the holes, a way of defining any geometrical curvature was highly desirable

Defining the regions of interest using a mask seemed to be a decent solution to my problem. The plan was to provide a mask which is an image that contains the targets of the arena together with drawings of the region of interests at the correct proportions. The tracking algorithm will detect the positions of the targets in the mask (example in Figure 2.6 A) and in the first frame of the video (example in Figure 2.6 B) on which is applied. Having access to three pairs of corresponding points in two images is sufficient in order to get the matrix that describes the affine transformation that maps the two images, and it can easily be done using Opencv open-source computer vision library¹³³. Once we know the matrix transformation, we will extract the regions of interest from the original mask, based on their colour and apply the transformation to each individual region so that they align precisely on their real correspondents. Figure 2.6 C shows an example of how the mask looks like after such a coordinate transformation and Figure 2.6 D presents the results of applying the transformed mask to the video.

Finally, Figure 2.7 shows that using a mask we can define regions of interest of any geometrical shape.

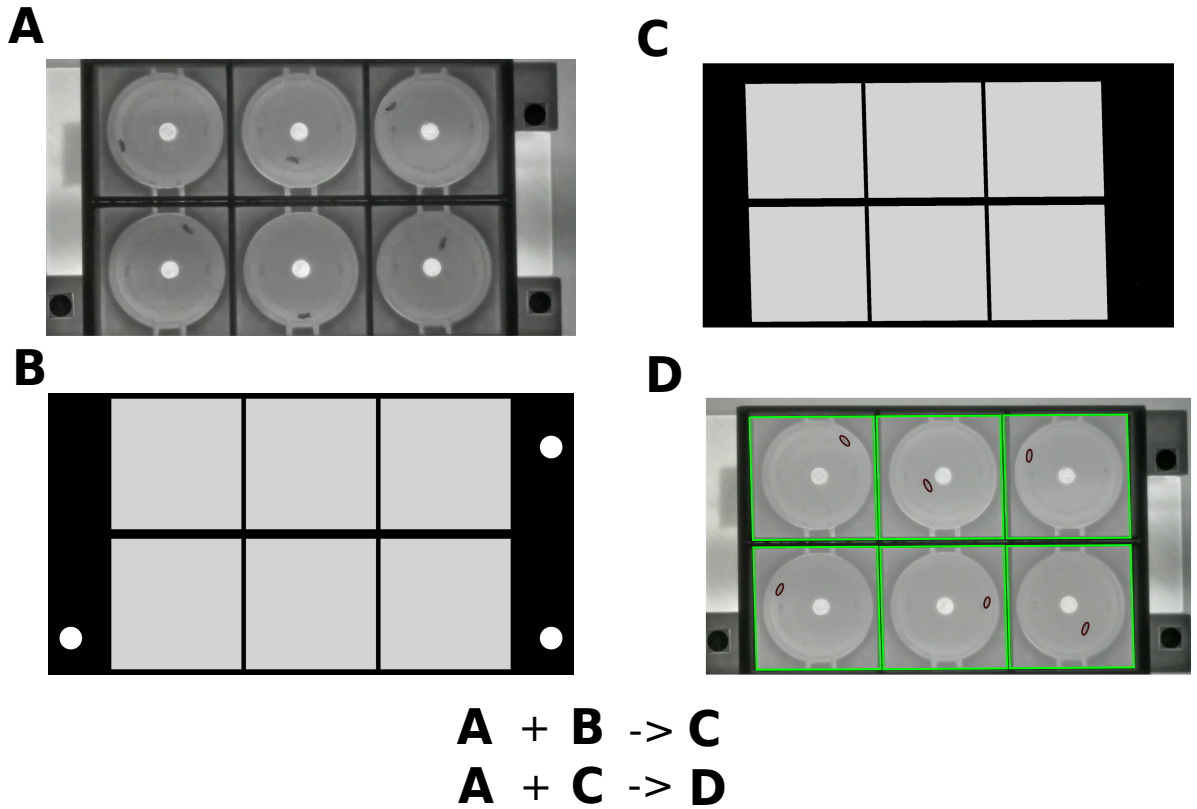


Figure 2.6: Accurate mapping of each individual region of interest Example showing how the regions of interest are mapped through the mask on the raw video via an affine transformation of the mask. **A** Example of a frame from a video holding 6 mini rings. **B** Mask used to define the regions of interest. **C** Transformed mask in which an affine transformation was applied on each individual ROI. The affine transformation was obtained by mapping the three black targets in **A** with the three white targets in **B**. **C** Transformed regions of interest are superimposed on the original frame from **A**.

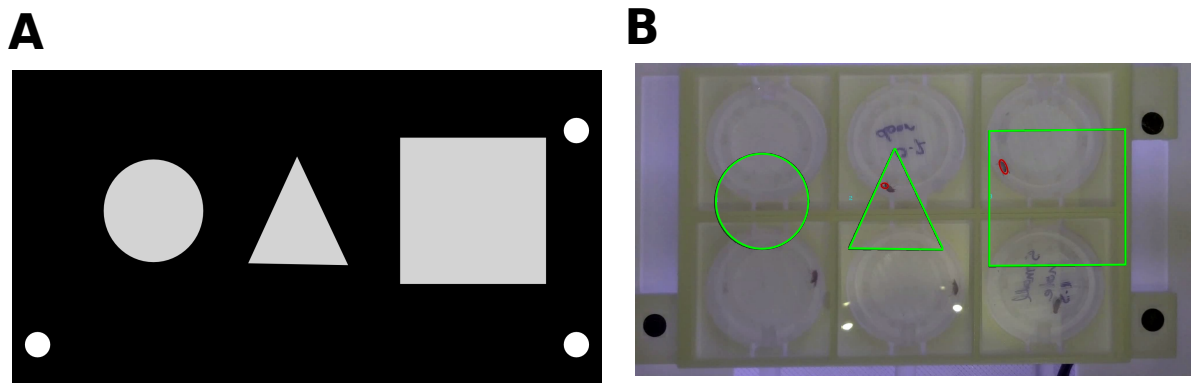


Figure 2.7: Flexible way of defining regions of interest of any geometrical shape **A** Mask that defines region of interest of various geometrical shapes. **B** Frame from video that defines its regions of interest using the mask from **A**, and tracks the flies in those specific regions..

2.2.9.2 DEFINING SUBREGIONS OF INTEREST, IDENTIFYING THE HOLES AND COUNTING THE PASSAGES

The manual counting of flies going through holes was cumbersome, error-prone and employed only to get an estimate of the passages of flies through holes and of their preferences, in the hope of capturing interesting results worth pursuing. Flies preference for holes of larger size was confirmed in this chapter using population experiments in the concentric rings arena. Being able to automatically track the flies passing through the holes became therefore compulsory for a in depth validation of these results and in the prospective of quantifying the effects of sleep deprivation on hole choice.

To do that I extended the ethoscope software to allow the definition of subregions of interest. Each subregion of interest is defined using a different grayscale value therefore each region of interest can contain a maximum of 255 subregions of interest. The outermost gray regions will be interpreted by the algorithm as the main region of interest, and everything that they hold inside is interpreted as subregions of interest.

The flexible definition of regions of interest of any geometrical shape together with the new feature that allows the definition of subregion of interest, enable us to define the passage through a hole like the passage of one subregion to another subregion. By using different subregions around the holes of different sizes we can efficiently identify the type of the holes.

The tracking algorithm was extended to extract the body of the fly at every frame and to superimpose on it the mask defining the regions and subregions of interest. If the fly is not passing through a hole, its entire body will be included in only one region of interest (Figure 2.8). Whenever the fly is passing through a hole, its body will contain pixels of two different greyscales. Figure 2.8 B gives an example of the fly passing through a hole.

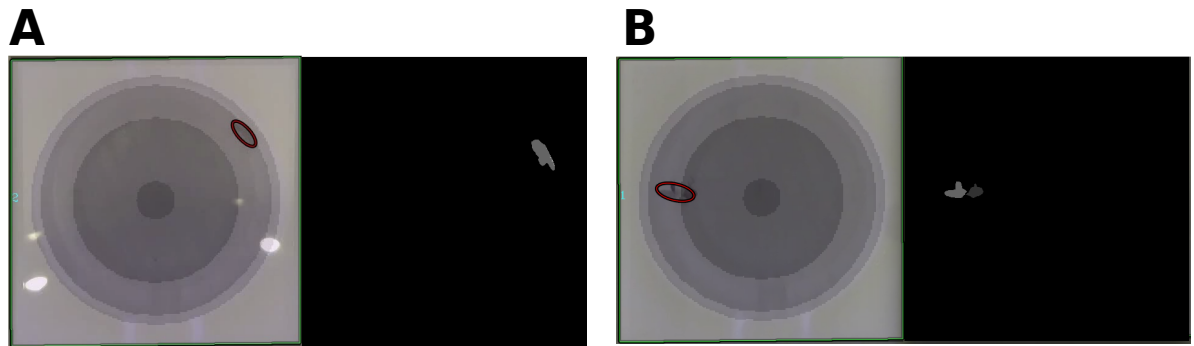


Figure 2.8: Identifying passages using subregions of interest **A** Image showing a fly that does not initiate a passage together with the superimposed mask. Its body contains only pixels of the same greyscale colour. **B** Image showing a fly that initiates a passage through a hole. Its body is between two regions, therefore it contains two different greyscale colours.

2.2.9.3 MULTI-TRACKING OF FLIES USING K-MEANS

I struggled to use existing software for tracking multiple *Drosophila* flies. My attempt in using CTRAX⁸⁹ failed as this software accepts only a particular video format called UFMF format, and my converted files from .mp4 to UFMF were not suitable for some reason. Also, using idtracker⁹⁰ failed because of too few pixels representing the flies. On top of these reasons, none of the mentioned software were easily extensible into using regions of interest and more importantly subregions of interest that will allow tracking the number of passages through the holes together with the hole type. I therefore decided to attempt to implement a new tracker that can track any number of animals for long periods of times, by extending on the current tracking algorithm used by the ethoscope. The algorithm uses the dynamical foreground and background statistical models already implemented in the ethoscope software for detecting an individual fly together with the number of flies required parameter to automatically detect the contours of all the flies in every frame. From the contours of the flies it creates a binary image in which the foreground is drawn in white and the background is in black. The positions of all the foreground pixels are fed into the K-means algorithm, which identifies which pixels corresponds to each animal. The k-means algorithm¹³⁴ is a clustering algorithm which partition any group of n observations $(x_1, x_2, x_3, \dots, x_n)$ into k cluster in a way that minimize the sum of squares within each cluster. Each observation x_i is a vector of fixed dimension. Our

multi-tracking algorithm takes as an input the positions of all the pixels representing all the flies and separates them into a number of clusters equal with the number of flies provided as an input.

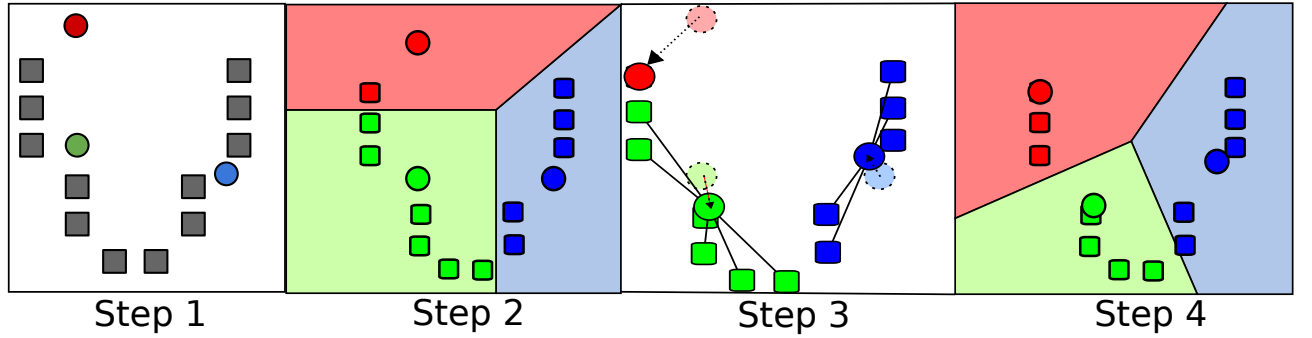


Figure 2.9: *K-means clustering algorithm strategy.* The k-means algorithm partitions a set of n observations into k clusters using the following steps:

Step 1 Randomly generates k means (centres) in the domain of the data. In the figure the data is represented by the gray dots and we want to separate it in three cluster. The 3 randomly generated means are shown in colour. **Step 2** Each data point is assigned to its closest mean. To do that we compute the distance from each data point to every k -mean and choose the mean corresponding to the minimum distance. **Step 3** Now that all the points are assigned to a cluster, we can compute the centroid of each cluster which becomes the new mean. **Step 4** Step 2 and Step 3 are repeated until the algorithm converges.

Figure 2.10 gives an example of tracking 7 flies using the algorithm described above, in a simplified version of a concentric ring arena, having only one ring (Figure 2.10 A and B). It shows one frame from the video (Figure 2.10 C) from which the contours of the flies are extracted and a binary image is created which fills with white pixels the contours of the flies (Figure 2.10 D). The positions of all these white pixels are supplied to the k-means algorithm which divides them in 7 clusters. For the first frame in a video the initial clusters are chosen randomly like explained in Figure 2.9, however for the following frames the initial clusters are set to the k-means of the previous frame in order to keep the ids of the flies. Figure 2.10 E shows a result of the tracking algorithm by drawing a contour around each cluster of points identified using a different colour and superimpose it on the original flies. Moreover, Figure 2.10 F presents the trajectories of the flies in the first 20 minutes of experiment.

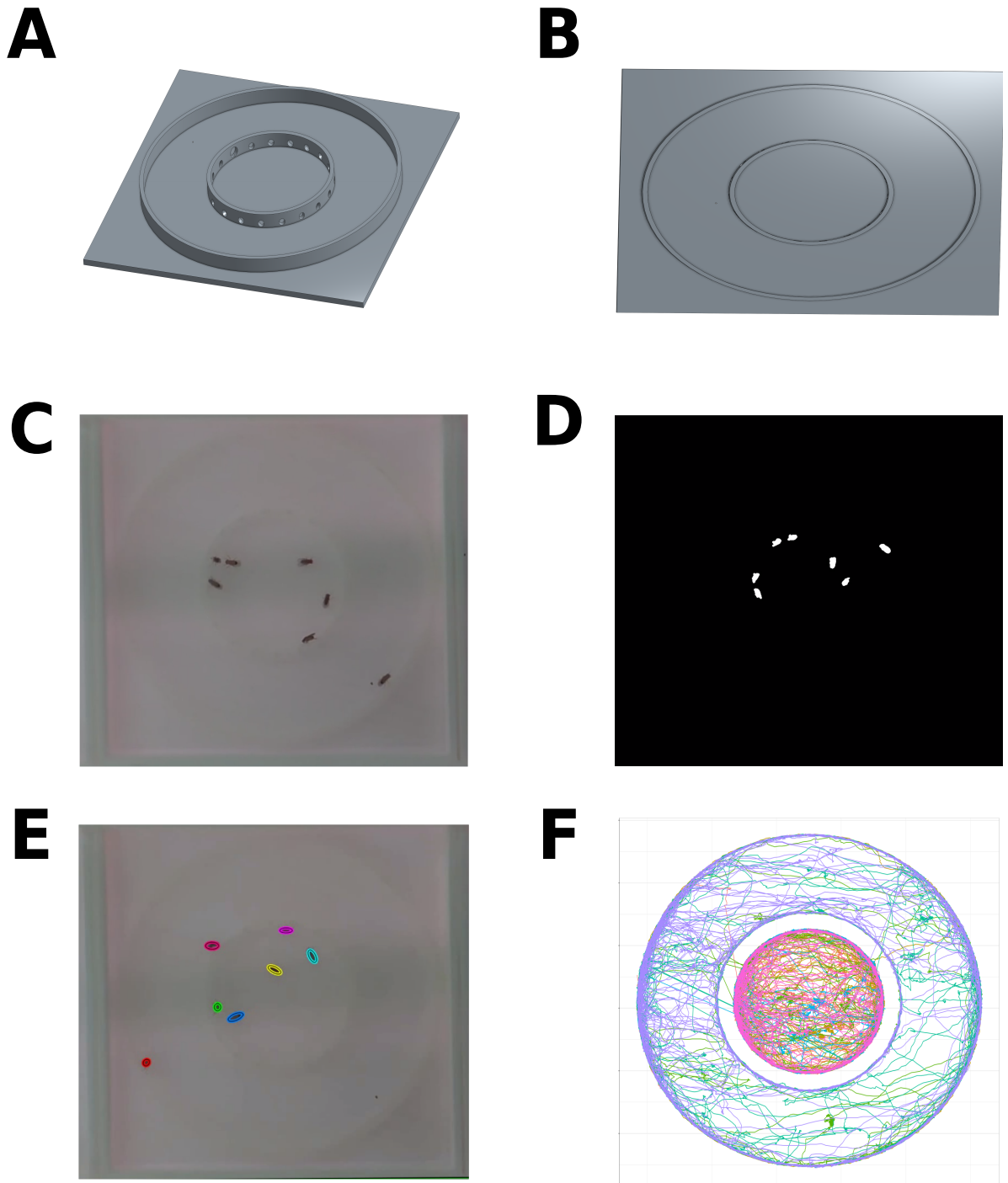


Figure 2.10: *K-means multi-tracking algorithm in practice.* Example showing the multi-tracking of 7 flies in a simplified version of the concentric ring arena. **A** Simplified version of the concentric ring arena holding only the first ring. **B** Top view of **A**. **C** Frame from experiment with 7 flies exploring the arena in **A**. **D** Binary image showing an example of foreground subtraction from the background. **E** Identification of the flies by applying the k-means algorithm on the white pixels from **D** and separate them in 7 means. The ids of individual flies are represented with different colours. **F** Trajectories of the flies in the first 20 minutes in the maze.

The multi-tracking algorithm is very stable if only males flies are used. Even sudden changes in the movement of the flies, like jumping of the males during a fight are correctly tracked without any id swaps or lost ids observed in the videos manually annotated. The limitations of the multi-tracking algorithm consists in the swapping of the ids if strong illumination variations arise in the videos. Another limitation is that it can take long time to start if the flies do not move at the start of the videos, as this delays the identification and subtraction of the foreground from the background. Finally, we applied the algorithm to pairs of male and female flies that are copulating. The copulation between *Drosophila* flies involves the

male mounting behind the female, and attempting the coupling of their genitals. Their interaction lasts between 10-20 minutes¹³⁵ and involves their bodies appear like merging into one. Nonetheless, the algorithm can distinguish between the male and the female and keep their identities impressively at moments when the visual identification of the two flies appears very difficult for a human scoring the behaviour. A few such examples are shown in Figure 2.11 C. Our algorithm was applied on 12 videos of 5 minutes length showing pairs of *Drosophila* copulating in glass tubes. Approximately 2-3 swaps were observed per video as shown in Table 2.2. These happen especially at moments of sudden movements with flies swapping positions between each other which were very difficult to perceive with the naked eye, and could be confirmed only by rewatching the videos in slow motion. Given the lack of tools allowing the automatic scoring of courtship in *Drosophila*, most of the labs turn to manual scoring. Despite of the swaps of ids having access to the trajectories of the flies in different time intervals can be useful for a visual assessment of the amount and quality of their interaction. A visual analyse of the trajectories corresponding to different time windows in the video can be used to efficiently to find the moments of interaction (Figure 2.11).

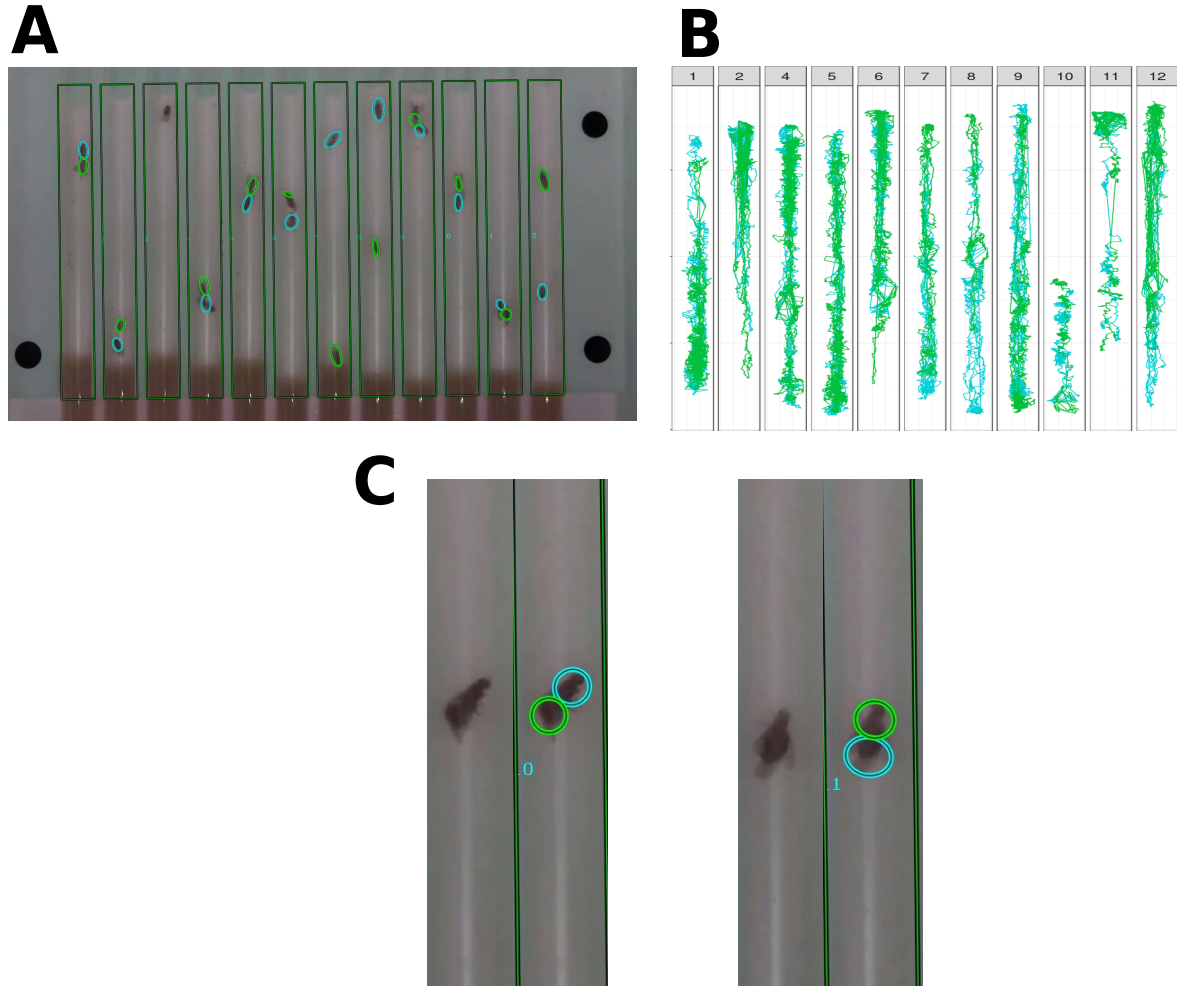


Figure 2.11: Multi-tracking of males and females during copulation **A** Multi tracking using k-means applied to an arena with 12 individual tubes, each holding a male and female fly. **B** Trajectories of the flies from **A** during the first 20 minutes of experiment. **C** Ability of the multi tracking with k-means algorithm to find the contours of the flies in situations in which they are hardly depicted with a naked eye.

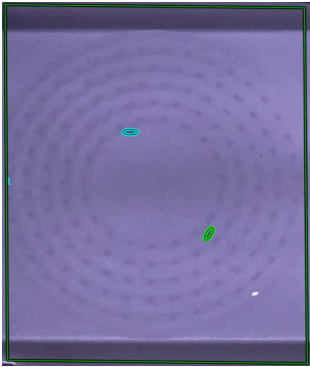
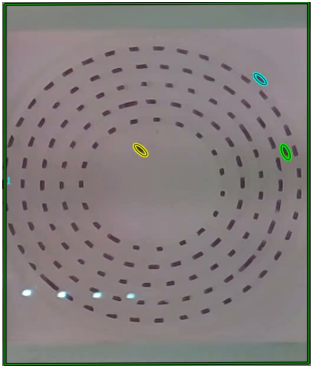
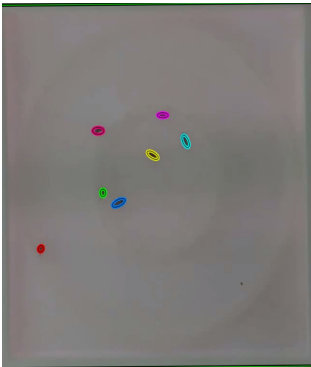
Tracking context	Details videos	Swaps and id loss
	• Number of flies = 2 • Sex of flies = 2 males • Duration of video = 1h • Light condition = dark • Arena type = concentric ring arena (Fig. 2.4) • Number of videos with the above conditions = 2	• Swaps = 0 • Id loss = 0
	• Number of flies = 3 • Sex of flies = 3 males • Duration of video = 2.5h • Light condition = dark • Arena type = concentric ring arena (Fig. 2.4) • Number of videos with the above conditions = 2	• Swaps = 0 • Id loss = 0
	• Number of flies = 7 • Sex of flies = 7 males • Duration of video = 1h • Light condition = light • Arena type = concentric ring arena with one ring (Fig. 2.10 A) • Number of videos with the above conditions = 1	• Swaps = 1 • Id loss = 1

Table 2.1: Multi-tracking Analysis

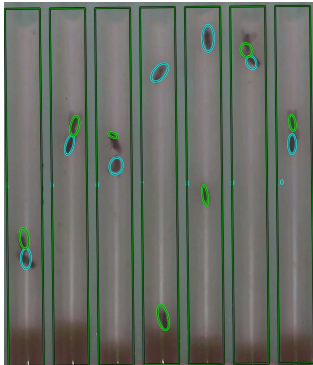
Tracking context	Details videos	Swaps and id loss
	• Number of flies = 14 • Sex of flies = 14 males • Duration of video = 5h • Light condition = light • Arena type = concentric ring arena with one ring (Fig. 2.10) • Number of videos with the above conditions = 1	• Swaps = 3 • Id loss = 3
	• Number of flies = 2 • Sex of flies = 1 male, 1 female • Duration of video = 5 min • Light condition = light • Arena type = glass tubes • Number of videos with the above conditions = 12 • Additional note = Flies were actively copulating for the most part of the video	• Swaps = 2-3/video • Id loss = 0

Table 2.2: Multi-tracking Analysis (Continued)

2.3 RESULTS

2.3.1 PRELIMINARY MAZES EXAMINATION, RESULTS AND FUTURE DIRECTIONS

The initial design (Figure 2.2 A, B) consisting of a plastic sphere holding two holes confirmed that flies choose by themselves when to escape through the holes. The counting of the flies escaping through each of the hole was done by the experimenter. Observing both holes at the same time demanded a lot of visual attention and was prone to errors. An initial experiment counted 17 flies coming out through the big hole and 3 flies coming out through the smaller hole, suggesting for the first time the preference of the flies for the bigger hole. The throughput varied as flies could take from a few seconds to more than 5 minutes to pass from the vial to the plastic ball. Therefore, this setup was not time efficient for the testing of different

hole sizes.

Further, we wanted to get a higher throughput in order to test our hypothesis that flies prefer the big hole to the small hole, and gain more knowledge into how well can they distinguish between holes. The initial ball design was adapted to fit exactly on the vial containing the flies (Figure (Figure 2.2 C)), allowing many flies to enter the plastic sphere directly. In order to test for several sizes of holes, the new ball design hold four different holes: 1.1 mm, 1.2 mm, 1.3 mm, 1.4 mm. Experiments were run with either one, two, three or four holes available to the flies to escape. In the experiment which allowed the escape only through the 1.1 mm door, no flies exited the plastic ball, suggesting that the hole was too small for the flies to fit in it. Also, keeping only the 1.2mm hole, the 1.3 mm and the 1.4 mm show similar flows: 10 flies/1 minute, 9 flies/1 minute and 15 flies/1 minutes. In another experiment only the 1.2 mm and 1.4 mm holes were available for exit. Out of 20 flies, 11 exited through the 1.4 mm hole and 9 through the 1.2 mm hole. This suggested that flies were not able to distinguish such a small difference between the holes (0.2 mm). However, given the difficulty in the manual scoring, these results were also prone to errors, strongly suggesting that an improved design was necessary.

Initial experiment results shown that flies have a preference for the bigger hole, for a considerable difference between the big and the small hole (0.5 mm in our case). However, after reflecting on the results above, it was not clear if flies prefer the big hole because of a tactile preference for it or because more light is coming through, making it more attractive due to the flies phototactic behaviour.

Figure 2.3 is an initial change in design meant to separate the phototactic behaviour of the flies from the actual tactile preference for the holes. The discs holding the holes are made from a transparent material diffusing light in a more uniform way. Results gathered using this paradigm were purely qualitative. We could observe a bigger proportion of the flies advancing on the side of the vial with the big hole than on the other side. Results suggested therefore that flies preference for the big hole was not purely light related but also tactile related. This paradigm also brought up the value of looking at several decisions of each fly instead of one only one passage. The discs containing the holes were also a first attempt into transforming our paradigm from the 3D space to the 2D space, towards something that is more easily tractable and quantifiable.

These preliminary results indicate that the passing through holes behaviour offers potential into better understanding the decision-making processes as it captures the active component of the behaviour of the fly to cross on the other side. The choice of initiating a passage is binary (flies can either initiate a passage or not). Moreover, the hole size, which is an easily controllable stimuli, seemed to modulate the passages through the holes, with flies being able to perceive subtle differences between the holes. A design which allowed a robust quantification of the passages was necessary to confirm the hole preference result.

2.3.2 PREFERENCE FOR THE BIG HOLE IN THE CONCENTRIC RINGS MAZE

In ordered to understand how well can flies distinguish between holes sizes we conducted a first experiment in which we placed 12 male flies in the concentric rings arena and counted the number of big holes and small holes crossed in 3 hours of experiment, scoring 45 passages through the big holes and 17 crosses through the small holes. Therefore, the probability of choosing a small hole given that a passage is initiated is:

$$P(\text{small}|\text{passage}) = \frac{\text{passages}_{\text{small}}}{\text{passages}_{\text{total}}} = \frac{\text{passages}_{\text{small}}}{\text{passages}_{\text{small}} + \text{passages}_{\text{big}}} = \frac{17}{17 + 45} = 0.27 \quad (2.1)$$

and the probability of choosing a big hole given that a passage is initiated:

$$P(\text{big}|\text{passage}) = \frac{\text{passages}_{\text{big}}}{\text{passages}_{\text{total}}} = \frac{\text{passages}_{\text{big}}}{\text{passages}_{\text{small}} + \text{passages}_{\text{big}}} = \frac{45}{17 + 45} = 0.73 \quad (2.2)$$

The small holes outnumbered by many the big holes, making the passages through the small holes more probable. Despite that, experimental results suggest the contrary. Given our current holes arrangement on the concentric rings maze, let's compute what should be the theoretical probability of passing through the big and small holes if flies were not able to distinguish between the holes and compare it to our experimental data.

If flies are not able to distinguish between the holes this means that the probability of making a passage given that they encounter a big hole would be equal with the probability of making a passage given that they encounter a small hole, otherwise said, the probability of a passage would be hole size independent. This can be expressed in the following way:

$$P(\text{passage}|\text{big}) = P(\text{passage}|\text{small}) \quad (2.3)$$

To compute the theoretical probabilities of choosing either a small or a big hole given that a passage is initiated we used Bayes conditional probability theorem which states that:

$$P(A|B) = \frac{P(B|A)P(A)}{P(B)} \quad (2.4)$$

Further let's express the probability of choosing a small hole given that a passage is initiated using Bayes theorem:

$$P(\text{small}|\text{passage}) = \frac{P(\text{passage}|\text{small})P(\text{small})}{P(\text{passage})} \quad (2.5)$$

But the probability of making a passage can be written as:

$$P(\text{passage}) = P(\text{passage}|\text{small})P(\text{small}) + P(\text{passage}|\text{big})P(\text{big}) \quad (2.6)$$

By replacing eq. 2.6 into eq. 2.5 we obtain:

$$P(\text{small}|\text{passage}) = \frac{P(\text{passage}|\text{small})P(\text{small})}{P(\text{passage}|\text{small})P(\text{small}) + P(\text{passage}|\text{big})P(\text{big})} \quad (2.7)$$

In our maze configuration, holes density is the same on each ring and there are 180 small holes in total and 5 big holes. Therefore the probability of passing next to a big hole is:

$$P(\text{big}) = \frac{\text{number of big holes}}{\text{total number of holes}} = \frac{5}{185} = 0.03 \quad (2.8)$$

and the probability of passing next to a small hole is:

$$P(\text{small}) = \frac{\text{number of small holes}}{\text{total number of holes}} = \frac{180}{185} = 0.97 \quad (2.9)$$

By replacing equation eq. 2.3 into eq 2.7 it simplifies as follows:

$$P(\text{small}|\text{passage}) = \frac{P(\text{small})}{P(\text{small}) + P(\text{big})} \quad (2.10)$$

Finally, introducing the values obtained from eq. 2.8 and 2.9 into 2.10, we obtain the theoretical probabilities for choosing either the big or the small holes

$$P(\text{small}|\text{passage}) = \frac{0.97}{0.97 + 0.03} = 0.97 \quad (2.11)$$

$$P(\text{big}|\text{passage}) = \frac{0.03}{0.97 + 0.03} = 0.03 \quad (2.12)$$

Comparing theoretical results from eq. 2.11 and 2.12 with the experimental results expressed by equations 2.2 and 2.1 it becomes clear that flies are able to distinguish between holes and that our assumption was false.

2.3.3 GROUPS OF FLIES - MAZE EXPLORATION

Initial experiments were performed by manually introducing male flies in various conditions and numbers into the concentric ring maze, at ZTO and record them for three hours. The number of the flies varied between experiments from 10 - 20 flies and experiments were run either in light or in dark condition. The groups of sleep deprived flies which performed the experiment were subject to 12 hours of mechanical sleep deprivation.

2.3.3.1 INITIATION OF PASSAGES UNDER VARYING INTERNAL AND EXTERNAL CONDITIONS

Flies most obvious behaviour in the maze is walking around the walls of the concentric rings, being therefore continuously surrounded by holes offering them the possibility to cross at any moment in time. Even though flies are surrounded by many holes, the decision of passing through a hole and the timing at which this happens seems to be actively chosen by each individual fly. Flies can walk around in the same circle, passing next to the same holes many times in a row before choosing to pass. The initiation of the passages is under the control of various internal states and external stimuli together with some randomness. The number of crossings is a good measure of the flies propensity to pass at a particular moment in time. Initial results obtained using the preliminary designs and the concentric rings proposed that the size of the holes is an external factor that plays an important role in their decision of passing through holes, as they seemed more inclined in choosing the big hole to make a passages even though the small holes outnumbered by many the big holes. To better comprehend flies ability of preferring the big hole and by what means they are able to distinguish holes size we decided to run experiments in complete darkness and in light conditions. Sleep deprived flies were also employed to explore if the lack of sleep changes in any way their ability to distinguish between the big and the small holes or their predisposition to initiate passages. Experiments for each combination of condition were repeated at least 5 times.

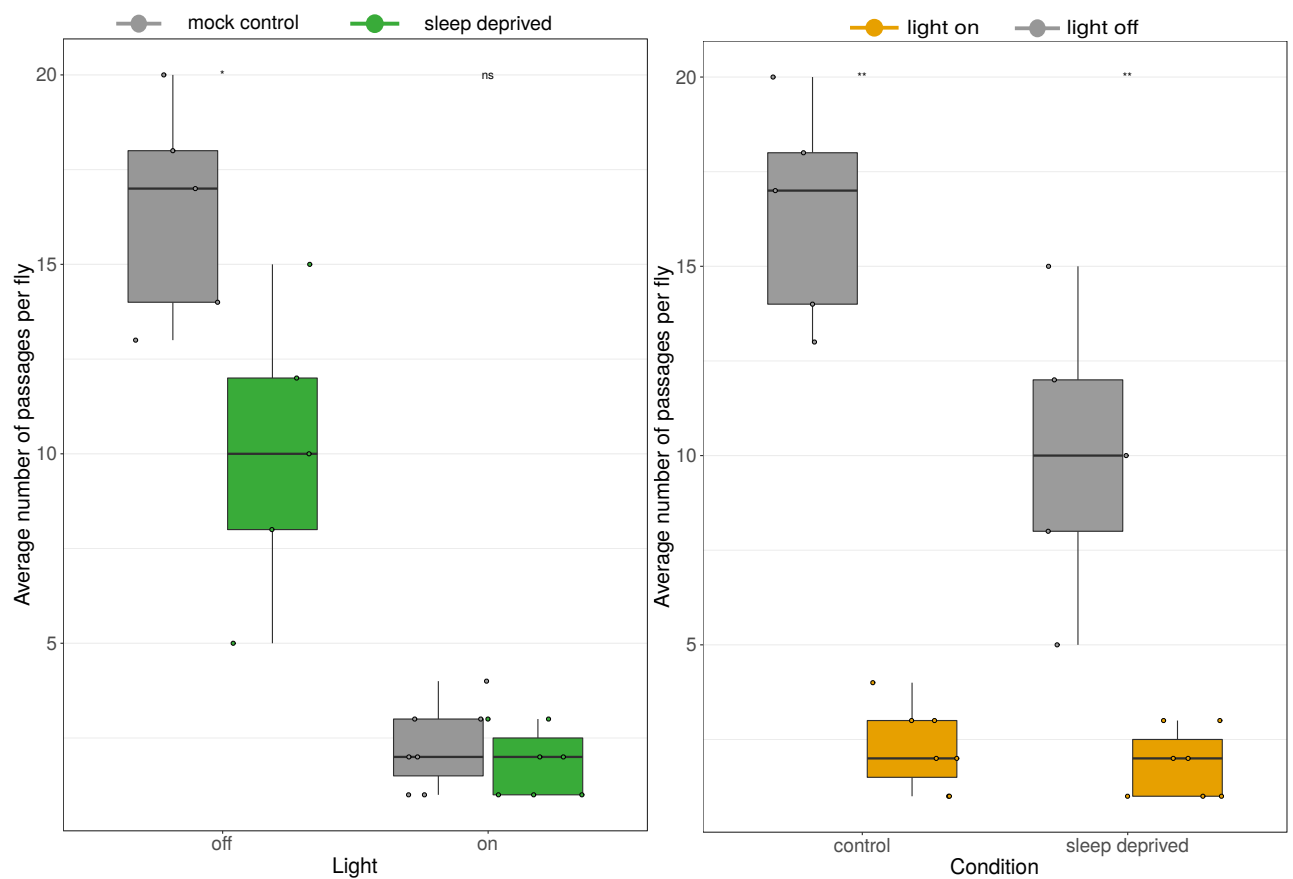


Figure 2.12: Initiation of passages in population experiments performed in varying external and internal conditions. Figures show the average number of passages per fly in different conditions. The average number of passages per fly is computed by dividing the total amount of passages counted during the entire duration of an experiment (3 hours) by the number of flies present in that particular experiment. At least 5 experiments were conducted for each combination of light and sleep condition. Each point represents one experiment. **A** Comparison between the average number of crossings per fly in either control or sleep deprived condition, for a fixed light condition. **B** Comparison between the average number of crossings per fly in either dark or light condition, for a fixed sleep state.

Figure 2.12 A shows that in light condition sleep deprived flies initiate passages similarly to control but in the dark condition they are less driven to cross compared to control. The absence of light proves to be a great means for pushing the flies to make more passages (2.12 B). Regardless of their internal sleep state, they make significantly more passages in the dark compared to the light condition.

2.3.3.2 HOLE PREFERENCE UNDER VARYING EXTERNAL AND INTERNAL CONDITIONS

Given the big variation in the number of passages corresponding to each combination of light and sleep condition, it will be irrelevant to compare the number of passages through the big and small holes. To get a rough idea of how well flies are able to distinguish between the holes we looked at the proportion with which they choose each size of the holes out of the total. The proportions of choices presented in Figure 2.13 represent the concrete proportions of big holes and small holes out of the total passages. These do not take into consideration the design of the concentric arena with the higher density of small holes and more therefore more probable compared to the big holes. Regardless of being eclipsed in number by the small holes, when in light condition, the big holes are preferred by flies despite their internal sleep state (left panels of Figure 2.13). When in dark, the amount of passages through the small holes increased and flies chose the big and small holes in the same proportion. However, by taking into account that small holes surpass the big holes, it is clear that even in dark flies are able to distinguish the big from the small

hole, preferring the bigger one.

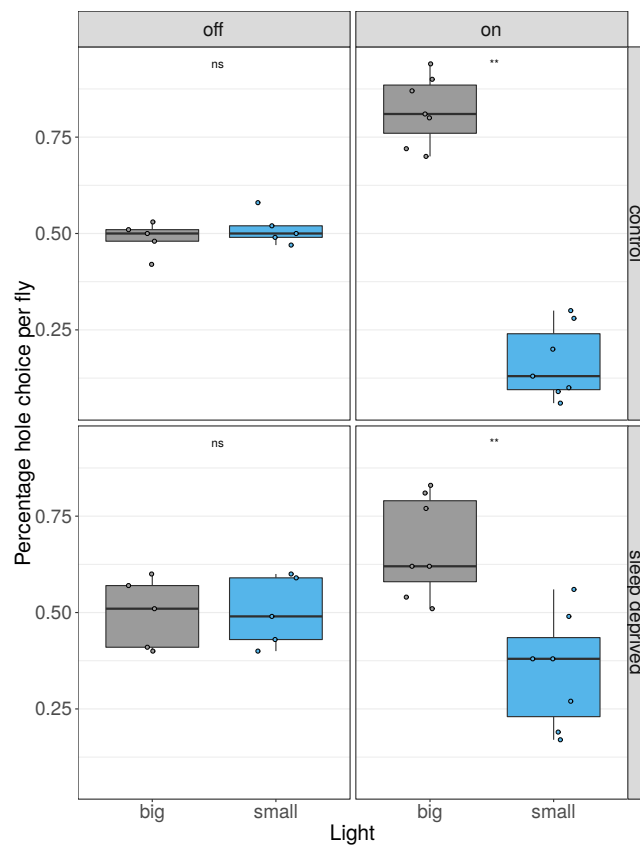


Figure 2.13: *Big vs small hole preference in population experiments performed in varying external and internal conditions.* Each panel presents a comparison between the proportion of passages through the big vs the proportion of passages through the small holes in a particular combination of light and sleep state. (upper right panel: control in dark, upper left panel: control in light, bottom left panel: sleep deprived in dark, bottom right panel: sleep deprived in dark).

2.3.3.3 CENTROPHOBIA

We further wanted to verify our hypothesis that the driving force that pushes the flies to pass from one ring to the other is their centrophobic behaviour. Moreover we were interested to what degree is this behaviour modulated by external and internal factors like the presence or absence of light and the lack of sleep. The centrophobic behaviour of the flies remains consistent in every combination of conditions (Figure 2.14), with flies making much more passages through the outer rings compared to the first ones. The large amount of passages on the fourth and fifth rings suggests that once arrived in the external part of the concentric maze flies do not come back towards the inner circle. No observable effect from the varying conditions on the distribution of passages suggests the robustness of the mechanism in the fly in charge of exploring the space and avoiding the centre.

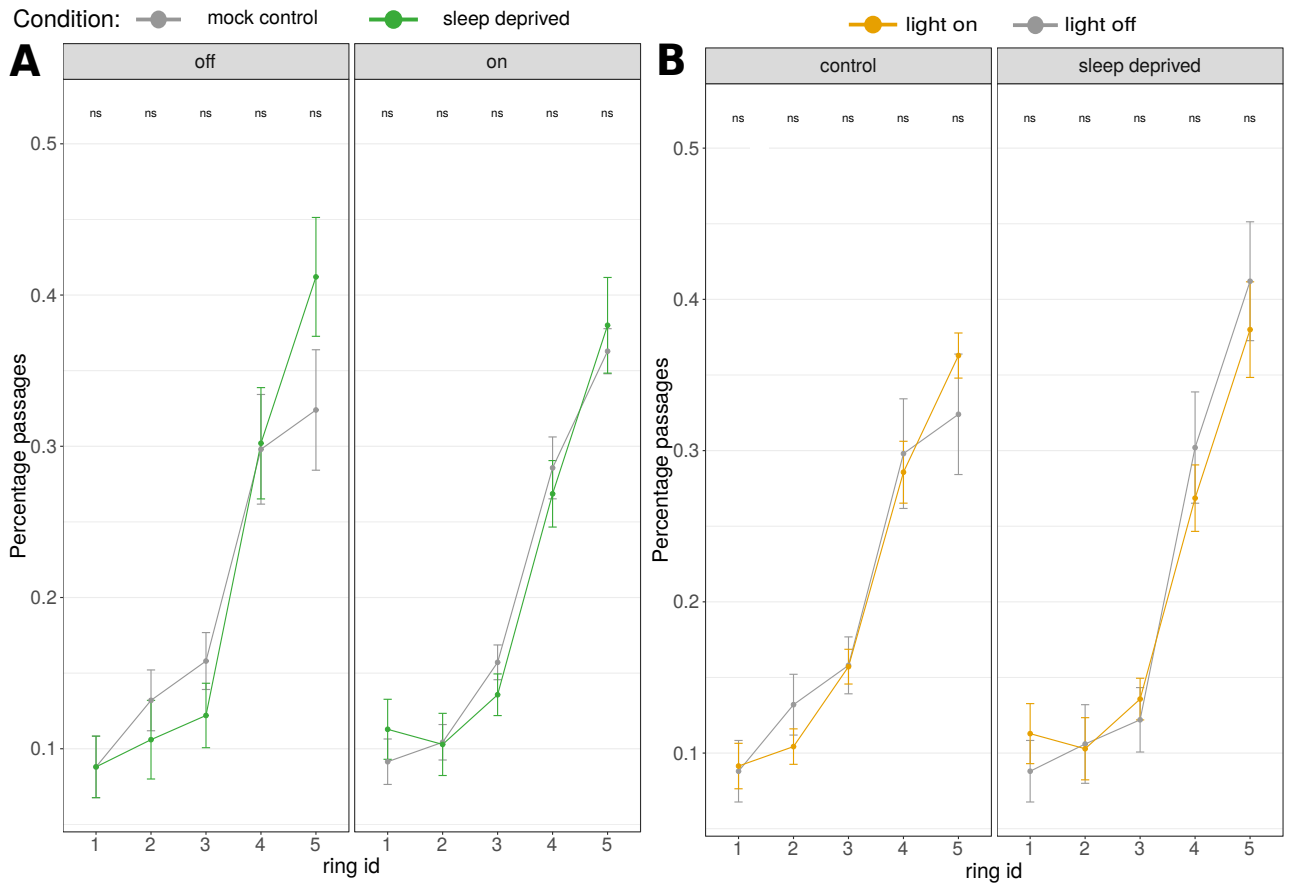


Figure 2.14: Distribution of passages between concentric rings. Each panel presents the percentage of passages corresponding to each ring. **A** Comparison between the distribution of passages in the control vs sleep deprived group of flies, for a fixed light condition: dark (left panel) and light (right panel). **B** Comparison between the distribution of passages in the dark vs light condition, for a fixed sleep state: control (left panel) and sleep deprived (right panel).

2.3.4 INDIVIDUAL FLY - MAZE EXPLORATION

Population experiments were very useful for bringing in our attention the trends of the flies to make passages in the concentric rings maze, by ensuring a large number of passages made collectively by all the flies in a small amount of time. However there was great variability observed in the behaviour of individual flies as some will make many passages while other will make very few or no passages. In the sleep deprived group some of the flies remained in the inner circle being completely immobile for extended periods of time, therefore clearly sleeping. The loading procedure involved some randomness, as some quick flies were able to escape at times. Therefore we were not able to control for the number of flies in each experiment. Variable fly density involves different amount of space in the central ring and might play an important role in the initiation of passages. Apart from the different amount of space, flies were also subject to different social context which might modulate their decision in a way that fits the group. To be able to separate the hole choice behaviour from the social influence we decided to perform experiments in individual flies.

Flies were loaded in arenas at ZT0 and video recorded for 5 hours.

2.3.5 LATENCY TO ENTER THE MAZE

To our great surprise, individual flies in light condition refused to initiate any passages through the holes for very long amounts of time (Figure 2.15 A, left panel), with some taking more than 5 hours before their

first passage. Once they passed the first ring they will quickly advance to the external part of the arena. The refuse of entering the maze was observed in both control and sleep deprived flies, the centrophobic force driving the flies in the population experiments 2.3.3.3 being silent in individual flies in light, independent of their sleep state. Interestingly, the absence of light suppressed the delay before entering the maze in both sleep conditions (Figure 2.15 B).

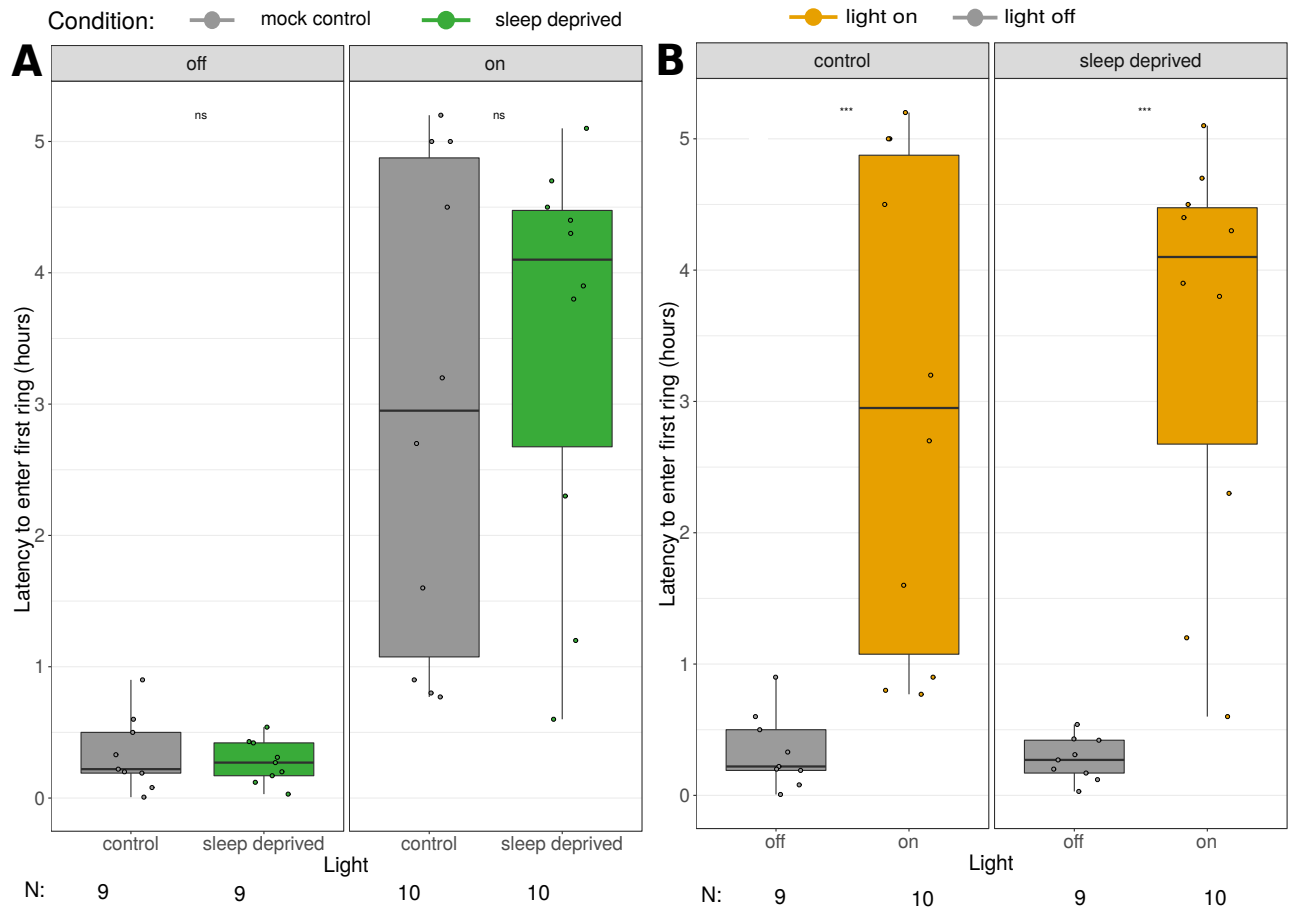


Figure 2.15: Latency to enter the maze in individual flies. The latency to enter the maze represents the amount of time that passes from the beginning of the recording to the first passage initiated by each individual fly. **A** Comparison between control and sleep deprived fly latency to enter the maze in the same light condition: dark (left panel) and light (right panel) **B** Comparison between flies latency to enter the maze in dark and light condition, for a fixed sleep state: control (left panel) and sleep deprived (right panel)

2.3.6 SOCIAL INFLUENCE

In the population experiments some flies entered the maze instantly after loading them into the maze and occasionally before even the recording was started. The striking latency of individual flies in entering the maze in light condition brought the opportunity of exploring what is the social influence that flies have upon each other. To quantitatively investigate how the presence of each extra individual modulates the drive to pass through the holes we decided to run parallel experiments with either 1, 2 or three individuals. Given the small number of flies required for these three situations, it was straightforward to control for it when loading the flies in the maze, without any loss.

Either one, two or three flies were loaded in the mazes at ZTo and video recorded for 5 hours.

The presence of a second fly in the maze triggered an immediate initiation of a passage through the first ring, consistent with the population experiments 2.16. Adding a third fly also accelerated the crossing but not in a significant way than having only two flies.

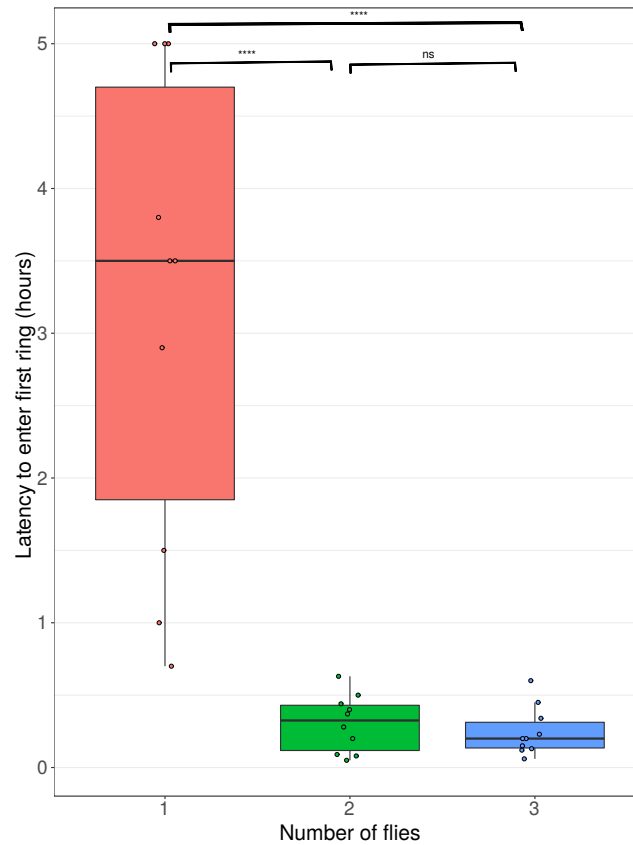


Figure 2.16: Latency to enter the maze for gradually increasing fly densities. Latency to enter the maze when either one, two or three individuals are present. In this case the latency represents the amount of time that passes from the beginning of the recording until the first initiation of a passage by any of the flies.

The presence of another fly implies a different social context but also a different amount of space as *Drosophila* males are known for fighting for territories, making it unclear what is the source for the change in the drive to pass.

2.3.7 SIZE OF THE ARENA

In order to try to separate how space availability influence the latency of entering in the maze from the social influence, we further conducted experiments in individual flies in mazes having the diameter of the inner circle approximately half the size of the one from the concentric rings arena. Restricting the amount of available space pushed the flies to enter the maze immediately. Furthermore, a qualitative observation suggested an increased amount of passages overall in the mini ring compared to the concentric rings arena.

The rapidity of the flies in initiating passages in the mini ring arena resulted in many of them making several crosses before the recording started, making the comparison between individual flies rather difficult.

Last two section shown that fly density and the amount of space available modulate the drive to initiate passages. Increasing fly densities and restricting available space push the fly to a rapid first crossing through the holes.

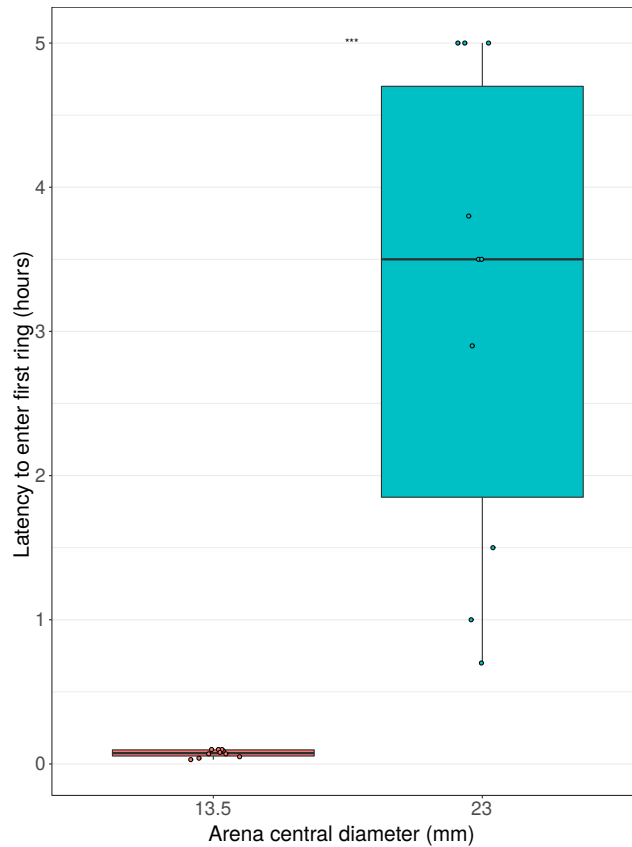


Figure 2.17: *Latency to initiate a crossing depends on the available space* Latency of initiating a first crossing in individual flies placed either in the concentric rings arena 2.2.8.2 which has an inner diameter of 23 mm or in the mini rings arena 3.3.1.1 which has a central ring diameter of 13.5 mm.

2.4 DISCUSSION

The work presented in this chapter was an effort of finding a paradigm able to capture behavioural changes in the flies due to small variations in external and internal conditions, in a high-throughput manner. Our aim was accomplished through a continuous refinement of our designs which culminated with the concentric rings arena (Section 2.2.8.2) and the mini rings (Section 3.3.1.1). Manual scoring was employed for analysing the results and decide on future steps to be taken. If preliminary paradigms were in 3D and required the permanent attention of the experimenter to collect the data, the concentric rings arena and the mini rings have a 2D design. This brought several advantages, first of all the flies behaviour could be easily assess and observed using video recording. The presence of the experimenter was not anymore required during the experiment. Moreover, having access to the videos and not relying only on the attention of the observer made the scoring of the passages through the holes more trustful. The concentric rings arena was also suitable to use with the ethoscope platform¹³⁶ and its 2D design allowed setting up experiments inside an incubator, ensuring controlled temperature and humidity. The concentric rings arena has the advantage of being able to capture multiple passages through the holes instead of a single choice like in the 3D plastic ball design (Section 2.2.8.1). Experiments ran using the concentric rings arena were manually scored to gain an approximate idea of the assay's ability of capturing behavioural differences in flies behaviour when crossing holes of different sizes. The manually scored data was enough for making sure that flies choose more the big holes, and gaining confidence that our paradigm is worth pursuing (Section 2.3.2). However, a detailed understanding of flies choices of holes requires validation using individual flies, to con-

trol for social influences and avoid averaging between flies. This will be done in Chapter 3. In parallel with running experiments in the concentric rings arena, own software was developed to ensure future flexible and automatic tracking of the flies positions and passages through the holes in a high-throughput manner.

2.4.1 HOLE PREFERENCE

This chapter proposes the study of a robust, well defined and easily quantifiable decision in *Drosophila*: to pass or not to pass through a hole. Flies freely walking in different environments are faced with holes and based on a unification of the internal and external cues they decide to either pass or not. The power of this specific decision consists in its ability of revealing the salience that flies give to one particular environmental cue: the size of the hole. Moreover, the paradigms are able to capture how small changes in this external cues are reflected at the behavioural level. The size of the holes modulates the initiation of passages through a specific hole. All the paradigms presented in this chapter prompt flies with the opportunity of passing through holes of different sizes. In spite of their ability to fit through all the available holes, they show a preference for passing through the bigger hole (Figure 2.13).

2.4.2 EFFECT OF LIGHT

One of the most striking results observed in the concentric rings arena is the large increase in the number of passages in the absence of light through all the available holes (Figure 2.12). This can be due to changes in locomotion and increased levels of activity due to positive phototaxis¹³⁷. The experiments in darkness run between ZT₀ to ZT₅, at a time of the day when the internal clock of the flies expects light. To better understand the effects of the light and separate them from the circadian rhythm effects, experiments should be run at several times of the days. In darkness, flies change the proportions with which they choose the big and the small holes, finding the small holes more attractive than before. Nevertheless, their preference for the big hole is conserved in darkness given that 50% of their passages happen through the big hole, in spite of the very low occurrences of the big hole compared to the small holes. The greater attraction for the smaller hole in darkness compared to light suggests a different perception of the holes. It can be that the absence of light increases the salience of the holes, empowering the fly with a more accurate and trustful estimation of whether its body will fit or not. This hypothesis is reinforced by the behaviour of individual flies in darkness and their immediate entry through the first ring compared to individual flies in light condition which have a latency of several hours on average (Figure 2.15 B).

2.4.3 SLEEP DEPRIVATION

Mechanically sleep deprived flies behave very similarly to the control flies both at the group and at individual level. The only exception is the group experiment performed in darkness whose results suggests a slightly decreased average number of passages per fly in the sleep deprived condition compared to control (Figure 2.12 A). To compute the average number of passages per fly, the total number of passages observed is divided by the total number of flies in that experiment. Our method is prone to error, especially because in the sleep deprived group some of the flies remain immobile in the inner circle of the arena showing similar states to sleep. Without considering the sleep in individual flies, computing the number of passages as we do might result in a underestimation of the performance of the sleep deprived flies which stay awake. To do that, it is necessary to be able to assess the sleep of the flies during the experiments. The rethomics

package⁹³, which analyses the sleep of flies in videos recorded at 2 frames/second was adapted to be used for the quantification of sleep in videos recorded at 25 frames/second in future experiments presented in Chapter 3. It is also possible that the results obtained in darkness are more relevant as flies initiate more passages overall. The increased number of passages might be able to capture the fine effects of sleep deprivation on decision making. Experiments in individual flies using mini rings arena will be able to provide more relevant results on how sleep deprivation affects the passage through the holes, as they capture a higher number of choices and larger number are necessary for identifying subtle changes in flies responses.

2.4.4 SOCIAL INFLUENCE

Flies entries in the concentric rings arena happen in a matter of a few seconds in groups of flies. Individual flies show a delayed response in the initiation of their first passage, waiting on average more than three hours before attempting their first cross. A possible reason for the differences in the individual and collective behaviours is the social influence that flies have on each other. A recent study¹³⁸ looks at how social context affects individual flies behaviour faced with an aversive odour. While individual flies avoid the odour only weakly, groups show stronger responses. Using high-resolution behavioural tracking, the study is able to analyse the inter-fly encounters and suggests that they are responsible for the enhanced decision making observed in the collective behaviour. If the odour avoidance paradigm is able to assess the behaviour of the flies mainly by looking at locomotion, we propose that using the concentric rings arena to study decision making in groups of flies provides a easy quantifiable way of assessing the decision making of each individual fly through the counting of their initiation of passages.

The groups of flies in the concentric rings arena also show a stronger response to the maze, initiating passages more quickly and attempting more passages than individual flies. This has been assessed only through visual examination. Individual flies make several crosses, once they initiate their first passage.

Another possible reason for the initiation of more passages in groups of flies can be linked to the reduction of space available to each individual fly with the increase in fly density. If this is the case, slightly increasing the number of flies should result in a gradual decrease in latency. Our results show that introducing another fly 2.16 is enough to speed up the entry into the maze significantly. A third fly does not decrease the latency in a significant way, suggesting that the space available is not responsible for the enhanced response. These results reinforce the strong impact of social context on flies decisions. Even though introducing only one additional fly is enough for restoring the latency of entering the maze to the group level latency, a closer examination of the total initiation of passages by different fly densities would be able to reflect more clearly any gradual changes happening with increase fly densities and how they vary over time. Unfortunately, manually scoring the total initiation of passages was not feasible, and an automatic tracking method was only available later on in the project.

2.4.5 ANXIETY

When placed in an open field *Drosophila* flies avoid the centre of the arena and show a wall following behaviour (Figure 2.10 F), a phenomenon also observed in rodents. A common hypothesis is that this behaviour is an expression of anxiety, flies preferring walls because they are looking for a shelter. It has been shown that diazepam, a medicine used for calming effects, reduces the wall following behaviour in both flies and rodents¹³². The initiation of passages through holes might also trigger a fear response in the flies.

A possible way to investigate the link between the passage through holes and anxiety would be by administering diazepam and check if this increases the latency of the first passage in individual flies in light. An increased number of total passages due to diazepam in individual and group of flies would also suggest a possible anxiety response of the flies to the maze. Examining the latencies of entries to the first hole, second hole, third hole etc. for each individual fly might give indications of how the anxiety levels vary over time, and can be representative for the fear intensities in the flies.

2.4.6 RESTRICTED SPACE

Preliminary arenas use the negative geotactic and phototactic behaviours of *Drosophila* as driving forces that encourage the passages through the holes. Further, the concentric rings arena uses the centrophobic behaviour observed in flies to prompt flies passages through the holes, but it fails at obtaining a quick response in individual flies. Remarkably, restricting the space of the arena from an inner circle of 23 mm diameter to a 13.5 diameter drastically increases both the latency of the first passage and the total number of passages observed overall in individual flies. As validated in chapter 3 and suggested by previous studies¹²⁰, locomotion is drastically increased by space reduction. This observation gave us a mean for studying the passage of holes in individual flies in a high-throughput manner as presented in Chapter 3.

2.4.7 SOFTWARE DEVELOPMENTS

Section 2.2.9 presents the software developments triggered by the paradigms presented in Chapter 1 and Chapter 2. They were mandatory for establishing an automatic high-throughput tracking method of individual and multiple flies in the paradigms involving the passing through holes behaviour.

The first contribution, presented in Section 2.2.9.1, consists in a flexible way of defining regions of interests. My approach employs a mask on which the regions of interest can be drawn using any image editor. The mapping between the mask and the videos is based on the alignment of three targets and is suitable for defining regions of interests on videos of any resolution using the same mask. This approach avoids the setting of rigid parameters, and does not require any knowledge of the underlying implementation, being user friendly. Further, it gives the user the ability of defining regions of interests of any particular shape, and ensures an accurate and consistent size of the region of interests between videos. Softwares like idtracker allow the user the possibility of defining themselves each region of interest by drawing either a rectangle or a circle of a certain size but fail at providing a way to keep consistency between the regions of interest in the same video or between videos. The size of the different regions are not pixel accurate but rather visually approximate. This gives rise to difficulties in aligning and comparing the data between different regions of interests, and is problematic if the pixel accuracy precision is required for trustful results, like in the case of scoring the passages through holes. Moreover, based on the positions of the targets in the video and in the mask, an affine transformation is computed and applied to each region of interest to ensure the closest possible match between the region of interest and the video in the face of small variations in the position of the arenas between videos. This feature was essential for a trustful retrieval of the intersections positions in the y-maze and of the holes positions in the concentric rings arena and it can be helpful to anyone looking for precision and conducting behavioural studies of insects using video-approaches.

The second contribution is the implementation of a simple way of defining subregions of interests of any shape, represented using different grayscale shades in the provided mask (Section 2.2.9.2). As the im-

plementation of the regions of interest guaranteed pixel-accuracy and a close matching between the regions of interests, the subregions of interests come on top of that to enable the definition of specific sections of interests inside the regions of interests. This facilitates the data analysis of the exploration of the environment by the flies. In particular, it is handy when the interest area is represented by shapes which are difficult to define, for example the intersections in the y-maze. Using subregions of interest offered a straightforward solution for scoring the passages through the holes behaviour as it provided a flexible way of defining the position of the holes on walls of any shape.

Finally, the last contribution provides a novel implementation for tracking multiple flies using the k-means algorithm (Section 2.2.9.3). It uses the dynamical foreground statistical model that defines a fly in the ethoscope software⁸⁸ and adapts it to the multiple flies situation before applying the k-means algorithm. The proposed algorithm is a robust solution for accurately tracking male flies in the limited resolution videos provided by the raspberry pi camera v1 (5 Megapixels), which is one of the main advantage compared to existing software which require better resolution of the flies⁹⁰. Moreover, the algorithm is accurately keeping track of flies individuals ids even in the face of interactions in which their identities are difficult to establish without close visual examination in slow motion. Tracking male and females flies during copulation comes with occlusions of the flies for long periods of times, which results in several losses and swaps of ids. To be suitable for long-term accurate tracking of the flies using the multi-tracking algorithm in a semi-automatic fashion seems promising. A future interface might enable the user to manually attribute identities to flies at moments of uncertainty. Such a solution will facilitate the study of sleep in *Drosophila melanogaster* populations. Up to present there are no video-recorded approaches for studying *Drosophila* sleep in population of flies. The only attempt of looking at sleep in population uses nine individual infrared beams crossing through the middle of a standard vial used for breeding *Drosophila*¹³⁹. Given the size of the vial, such an approach has very poor resolution into the behaviour of the flies. A video-recording solution could give great resolution and provide insights into the amount of sleep that flies have in groups and the impact of the social interaction on their sleep. Apart from being more relevant for real life situations, results might also be more valuable for understanding the function of sleep. Our algorithm could be potentially useful for studying aggression in males as it is stable in keeping the identities of males even in the face of sudden interaction between them like jumps.

The modularity of the ethoscope platform allows an easy integration between the different components developed. Therefore the multi tracking algorithm can be used to accurately track the position of the flies in the concentric rings arena and to efficiently score the passage through holes of individual flies, if a suitable mask is provided. The ability to combine the flexible definition of regions and subregions of interest with the multi-tracking has not been described in any available trackers for *Drosophila*. This algorithm was developed in parallel, while running the experiments presented in this chapter. The results obtained led me into focusing on individual flies as it was the more promising direction for simplifying the paradigm and disentangle the passages through holes behaviour from the social interaction.

In conclusion, this chapter achieves its aim of providing a paradigm able to capture changes in the flies behaviour due to small variations in one external stimuli (the size of the holes). The concentric rings arena was useful at quickly assessing the behaviour of many flies through many available holes and gaining an overview picture of their choice behaviour. The presence of so many holes (190 holes), together with the presence of several rings add a lot of complexity and offer enormous numbers of possible combination of choices. A robust validation of the hole preference and a more detailed overview of the proportion of

choices demand for a simplification of the paradigm. The mini rings arena offers a lot of potential in that direction, as it ensures many choices in flies due to the restriction of space and it also increases the high-throughput of flies per experiment. Moreover, using individual flies removes the social influence effect. Finally, the software developments undertaken so far allow automatic tracking of the positions of the flies and automatic scoring of their passages through holes in the mini ring arena.

There are always two choices. Two paths to take. One is easy. And its only reward is that it's easy.

Unknown

3

Big hole, small hole

3.1 INTRODUCTION

This chapter presents the main results of the thesis: a novel assay to study the interactions between sleep and decisions in a high-throughput manner together with new insights about how the decision making process is affected when disturbing one of the two established components of sleep : homoeostatic component and circadian component.

In trying to understand the link between decisions and sleep, my intuition after running the experiments presented in Chapter 1 and Chapter 2 was that I should find the simplest possible decision which involves an obvious and measurable intention of the fly to perform a particular action, and look at how sleep modulates that intention.

I would like to recall a few of the facts presented in the introduction that guided me up to this point. Firstly, I agree with Heisenberg which considers that behaviour is something active that is generated in the organism and the main function of the brain is to select actions depending on the possible outcomes⁵⁵. Therefore looking at behaviour like a stimulus-response process is not appropriate when trying to understand the decision making process or behaviour in general. Behaviour is something active. There is an active component of the organism choosing to do something. When presented with a new environment, an organism initiates an action by chance, and depending on the outcome that it gets, it will either do or suppress the current action on a future encounter of the same stimuli.⁵⁵ The terminology used by Heisenberg to define this activity in behaviour is called "initiating behaviour"¹⁴⁰. In this viewpoint, a decision could be broken into two components: the active initiation of the action that originates in the organism and the modulatory control of that action which involves the entire spectrum of sensory stimuli including tactile, olfactory, visual etc. and all the internal states of the organism such as hunger, sleepiness, fear, fatigue etc.

Heisenberg also considers that if flies need to adapt to unpredictable conditions, the initiation of actions needs to be error-prone, so probabilistic. Therefore, it is the external stimuli together with the internal states and together with some randomness that are all taken in account by an organism when initiating an action. There are two studies that confirm the randomness in the initiation of behaviour using *Drosophila*: one is the stop-go behaviour which looks at the sequence of activity and inactivity bouts of flies walking in tubes⁷⁸ and the other is looking at the sequences of body saccades of tethered flies⁵.

A relevant example of a decision that exposes the initiation of activity part of a behaviour in the fly is the gap-climbing behaviour⁷⁹. In the gap climbing behaviour the fly is presented with gaps of different sizes and it has to decide whether to pass from one side of the gap to the other. It is proven that the fly estimates the width of the gap, and based on its estimation it will decide whether to make an attempt to pass or not. If the size of the gap is greater than a certain threshold no flies will make an attempt. To analyse the gap behaviour of the fly sophisticated and expensive technology like high-speed cameras are needed, and trying to run this experiment for large numbers of flies seems challenging.

My interest was to find a simple experiment in which the initiation of action of the fly is obvious and easily measurable. More importantly, it must be straightforward to study under laboratory conditions, with minimal equipment and in a high-throughput manner. Also, in order to simplify things as much as possible, there should be as few external stimuli as possible influencing the decision. In the case of the gap climbing experiment, the width of the gap is the only main external stimuli influencing the decision to pass or not. It will also be very informative to find an environment in which the fly needs to make a decision several times and be able to monitor how that specific action is incorporated in their behaviour over time, instead of looking at a one time event.

I consider that the passage through holes is an easily quantifiable experiment for studying the initiation of action in *Drosophila*. When presented with a hole, a fly can choose to pass through it or not. The number of times the flies chooses to pass through the hole must depend on the external conditions like the size of the hole, light, temperature, humidity etc. and internal conditions like hunger, sleepiness etc. together with some stochasticity. Chapter 4 suggests that the size of the hole has a big weight in the initiation of action together with the light condition.

Consider that a fly is presented with two holes: a large hole, through which it can pass comfortably and a tight hole, through which it can pass with some effort. Exposed to this environment, the fly will try both holes and depending on the outcome of their actions they will decide whether to pass or not at the next encounter of the holes. In Heisenberg view, the fly should choose more often the big hole, given that it is easier to pass through it. If he is right, the probability of choosing the big hole should always be bigger than the probability of choosing the small hole, even in varying external conditions, just because that it is the advantageous thing to do. Therefore, action selection behaviour should adapt in that direction. However, the number of passages and the proportions with which flies choose the big and the small hole can be under the influence of external stimuli like the size of the holes and internal states like sleep.

Therefore in this chapter, I would like to find the most simplified version of a hole passing paradigm and check if it verifies Heisenberg's theory. If it does, I would like to use it to see how sleep deprivation or a deficient circadian rhythm mechanism can influence flies decisions.

3.1.1 OUTCOMES AND SHORTFALLS OF THE CONCENTRIC RINGS ASSAY

The previous chapter put forward some observations worth being explored further, in a more refined manner. First, the flies were able to distinguish between holes of different sizes (1.3mm and 1.8 mm) showing a preference for the bigger hole. Secondly, when in darkness, the flies boost their passages through the holes, compared to when they are in white light. The number of passages in dark is almost triple compared to the number of passages in light. Therefore, the preference of the big hole fits our hypothesis that flies should go for the most advantageous thing to them but it needs rigorous validation. The strong effect of light on the initiation of a passage, together with the fact that light has a fundamental role in regulating the circa-

dian rhythm opens up questions on whether there is a link between the circadian component of sleep and the initiation of actions, in this case the initiation of a passage.

One shortfall of the concentric ring arena was our lack of automatic tracking software and automatic detection of the passages through the holes software, during the collection of the data. Therefore, the manual scoring of the passages through the holes was cumbersome and prone to errors, and could not be carried out for larger scale experiments. Also, the throughput was very small, as we could only fit a maximum of two arenas in an ethoscope¹³⁶.

Moreover, the concentric rings arena is very complex, in the sense that the large amount of holes on each ring offers a lot of potential navigation paths through the maze. This makes the data analysis more difficult, and the comparison between different flies becomes possible just by introducing some levels of abstraction, ending up comparing things that are not exactly the same. For example, if one fly chooses a big hole on the first ring, then a small hole on the second ring and then a third small hole on the third ring and another fly chooses one big hole on the first ring, a small hole on the second ring and then it came back through another small hole of the second ring, the sequence of passages for both flies will be (big, small, small) so if the flies were compared only based on this sequence of passages, one can say they decided in the same way. The direction component could be added in and then the first fly will be represented by the sequence ((big, outward), (small, outward), (small, outward)) and the second fly by the sequence ((big, outward), (small, outward), (small, inward)), which has a higher degree of complexity but is able to surprise the difference between the two flies more accurately.

On each ring there is just one big hole and several small holes. Therefore, to gain even more precision, the identity of the small holes can also be retained. This could be helpful if we are interested in knowing if individual flies remember particular paths, repeatedly choosing the same holes. In the example before the first fly will be characterised by a sequence like this: ((big, outward), (small, outward, 3), (small, outward, 5)), if the fly chose the third small hole on the second ring and the fifth small hole on the third ring. Similarly, the second fly can be represented by ((big, outward), (small, outward, 7), (small, inward, 7)) if the fly chose the seventh hole on the second ring and came back through exactly the same hole on the second ring.

The example above is meant to illustrate some prospective research offered by the concentric rings arena which could be adjusted to study learning and memory in flies for example, how well they can remember a specific path, consisting of a combination of big and small holes that leads to a reward. Exploratory work could be conducted and new results observed by changing the size of holes, the pattern of holes, the length between rings, the size of the central ring etc.

The concentric ring arena revealed a potentially interesting behaviour for studying decision making in *Drosophila* which is: the passage through holes. Further, we will try to simplify the paradigm as much as possible by removing unnecessary parameters and finding a suitable way of automatically tracking the passages through the hole at the best possible resolution.

3.2 MATERIALS AND METHODS

3.2.1 FLY STOCKS:

The flies used in this experiment were CantonS and clock *Drosophila melanogaster* and were obtained from Ralf Stanewsky's laboratory at the University of Münster, Germany. They were raised under a 12 h light: 12 h dark (LD) regimen on standard solid corn and yeast media.

3.2.2 EXPERIMENTAL CONDITIONS:

Experiments were conducted on 3-5 days old mated males raised under constant LD conditions in incubators set at 60% humidity and 25 °C, in either dark or light conditions as specified. during the experiment flies had no access to food, but they had access to a small quantity of 1% agar or a filter paper soaked in distilled water, in order to avoid dehydration. Specific conditions used for each experiments are mentioned before each results section.

3.2.3 MINI RINGS, MINI SQUARES AND PLUG STOPPERS MANUFACTURE

The mini rings, the mini squares and the plug stoppers were designed using Onshape software available online at www.onshape.com. They were printed with the Form2 printer by Formlabs using clear resin. Prints were washed for 10 minutes in a bucket with isopropanol after which they were removed from the first bucket and placed in a second bucket with clean isopropanol for another 10 minutes. Longer times were used if necessary to remove all the excess resin. Finally, prints were cured in UV light for one hour at 60 °C to achieve their optimal physical proprieties.

3.2.4 SLEEP DEPRIVATION

3.2.4.1 MECHANICAL SLEEP DEPRIVATION

Mechanical sleep deprivation was performed using the PSU-101 Orbital Shaker. Vials containing several flies, males and females, were fixed with tape on top of the shaker's flatbed. The sleep deprivation was performed at night for 12 h from (ZT₁₂ to ZT₀), unless otherwise stated. The shaker was programmed through an Arduino to move in a pseudo-random fashion every 1 to 7 minutes, for a period of 5 to 30 seconds. The sleep deprivation was always performed in an incubator at 60% humidity and 25 °C.

3.2.4.2 DYNAMIC SLEEP DEPRIVATION

Dynamical sleep deprivation was performed for three days, starting at ZT₀ on the first day. The ethoscope platform together with the servo motors module were employed for the sleep deprivation⁸⁸. The platform allows the continuous monitoring of individual flies in separate tubes. Every half a second the position of each fly is recorded and if a fly is still for 60 s its individual tube will rotate in order to awaken it.

3.2.4.3 ARENAS CLEANING

The mini ring and mini square arenas were soaked in water containing liquid detergent for at least two hours between experiment. They were then rinsed using distilled water to remove the salts and dried for a few hours prior a new experiment. New cover slips were used between experiments to prevent the flies from escaping from the arenas.

3.2.5 VIDEO RECORDINGS

Videos at 1280×960 pixel resolution were recorded using the ethoscope platform⁸⁸ at 25 frames/s. Before starting experiments presented in this chapter, the ethoscope's raspberry pi camera was replaced with the module version 2 (still resolution of 8 megapixels instead of 5 megapixels in module version 1) enabling a

higher definition acquisition. The ethoscope platform generates one compressed .h264 file every 5 minutes. A bash script using ffmpeg software iterates all experiments and unites all .h264 files corresponding to an experiment into one single .mp4 file, on which the tracking algorithm is applied.

3.2.6 TRACKING

Tracking was done post-experiment using our own software. A new tracking algorithm was added to the ethoscope platform that allows a flexible definition of the regions of interest using a mask. The masks used for tracking the flies in the mini ring and the mini square arenas are presented in Figure 3.1. The tracking information is contained in a sqlite3 database file with extension .db . A bash script iterates through all existing experiments and applies the tracking algorithm on all the .mp4 files that were added recently, that do not have a correspondent .db file.

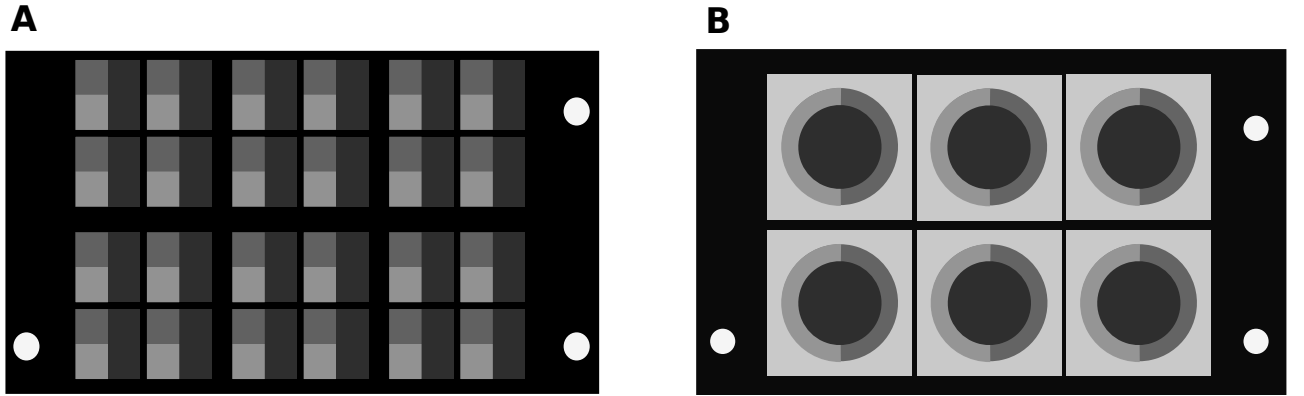


Figure 3.1: Masks that define the region of interests. **A** Mask used to track the flies in the mini square arena. **B** Mask used to track the flies in the mini ring arena.

3.2.7 DATA ANALYSIS AND STATISTICS

Data analysis was performed in R. Own scripts were used to count the number of passages through the holes using the parameters that define the position of the fly in the maze, provided by the tracking algorithm. Plots were generated using ggplot2 and ggpubr packages. For all the boxplots, the first and third quartiles (Q_1 and Q_3) are the ends of the box and the median is indicated with an horizontal line in the box. The whiskers go from the bottom and top of the box to the minimum and maximum values situated in the $\pm 1.5IQR$ range, where $IQR = Q_3 - Q_1$. The errors bars present in some figures indicate the standard error. Statistical analysis was carried out using the pairwise Wilcoxon rank sum test available in the ggpubr package. Outliers were always kept, and each experiment was repeated at least twice. The ethogram and overview plots were generated using the Rethomics packages⁹³ (<https://rethomics.github.io/intro.html>), and the principal component analysis (PCA) was performed using the prcomp function of the stats package. The following convention is used to indicate statistical significance: ns: $p > 0.05$, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$.

It is worth recalling that our concentric rings with holes assay was inspired by the concentric ring-gaps assay⁸⁰ and that it reproduces well the tendency of the flies to move towards the outer rings of the arena. This design was chosen in order to use the shape of the arena to trigger the passages through holes, as the flies will always walk towards the exterior of the arena. However, our experiments using different sizes of arenas and further inspection of the existing literature¹²⁰ unravel to us that space restriction increases locomotion in *Drosophila*. Therefore, a restrictive space guarantees more encounters with the holes, therefore more passages. This was noticeable in the concentric rings arena as well, as flies took longer to pass through the first ring, but they made more passages once arrived in the space between rings which is much more restrictive in size than the centre of the arena.

In order to simplify the arena and to increase the throughput we decided to reduce the size of the central circle and keep only one ring that holds two holes (Figure 3.2 B). Given the smaller size, the new design makes it possible to fit six arenas in one ethoscope (Figure 3.2 A). It also makes the data analysis clearer, by reducing the possible paths through the arena to a few rigid decisions. Having two holes of different sizes is the minimum requirement that can enable us to verify if indeed the flies will choose more the most advantageous action and track how this action gets integrated into their behaviour in time. By simplifying the design, it became more straightforward to tackle the development of a software able to automatically track the fly and extract from the videos information about its position in the maze, the timing of passages through the holes, the total number of passages and the duration of the passages, measurements that might help us further understand how flies prioritize and select their actions.

Along with the arena that has one big hole and one small hole, we built an arena that has only two big holes and another one that has only two small holes (Figure 3.2 B). These arenas were used as controls to make sure that the preference for the big hole is not due to biased external conditions. Therefore, in the arenas that hold same size holes there should be no significant differences between the number of passages through the individual holes. If the two holes are the same size and the shape of the maze is symmetric, than fly's outcome expectations of selecting any of the holes should be the same.

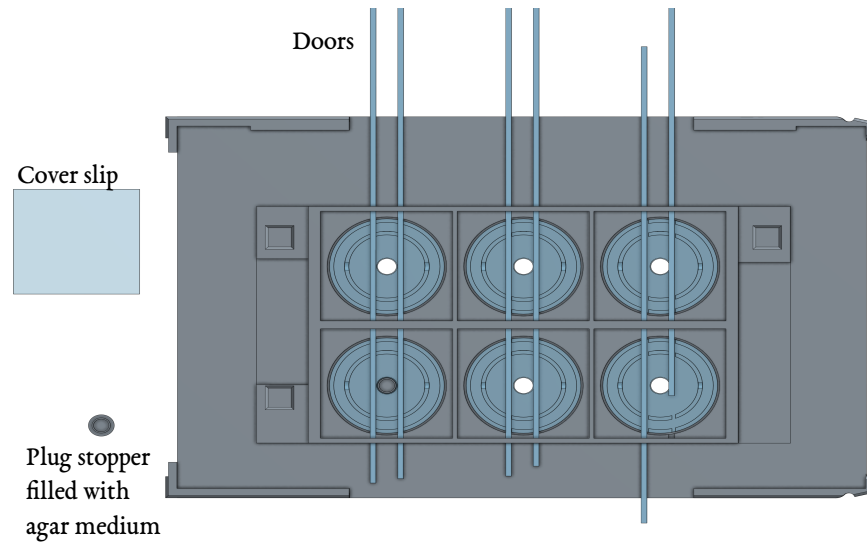
Furthermore, when loading the flies in the concentric rings arena, there was a time difference of a few minutes between loading the first fly and the last fly, time in which the first fly can make several passages that cannot be recorded. Therefore, a door system was added (Figure 3.2 A) that allow the experimenter to load all the flies in the arena, put them in the incubators and start the experiment by releasing all the doors at the same time. In this way, flies were given time to accommodate in the arena and the new environment conditions before starting the experiment.

Finally, as specified in the Methods Section, video recordings were acquired at a higher resolution, using a better camera. The support arena that holds the mini rings and the mini square was adapted so that the high definition mode of the new camera could be used at its maximum potential (Figure 3.2 A). The new resolution allowed a zoom into the fly's finer behaviour and it made possible the observation of their micro-movements during experiments. More importantly, given that each fly was represented by a larger amount of pixels, it enabled us to score the passage through the holes and the time they required to pass through the hole. This would have not been possible with the camera used in previous experiments.

3.3.1 RESULTS

3.3.1.1 MINI RINGS ASSAY - SIMPLIFIED VERSION OF CONCENTRIC RINGS ASSAY

A



B

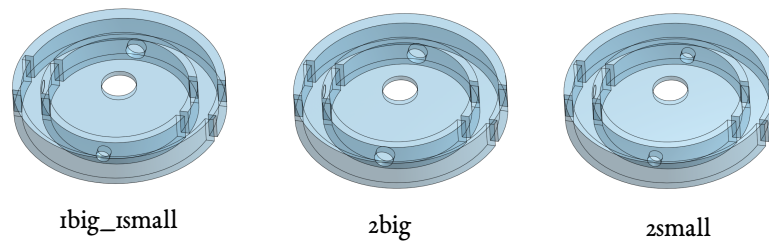


Figure 3.2: Mini rings arena. The behavioural arena designed to study passages through holes. It holds six flies in individual mini rings: inner ring diameter (13.5 mm), outer ring diameter (19.5 mm), wall thickness (0.5 mm). **A** Image of the arena that holds 6 mini rings. Each mini ring is covered with a 22 x 22 mm glass cover slip from above. The fly is loaded through the hole situated in the centre of the mini ring, which is next plugged with a stopper filled with agar, to avoid dehydration of the flies. **B** Image of the three mini arenas used during experiments: **1big_1small** mini ring which holds one big hole of 1.8 mm and one small hole of 1.3 mm; **2big** mini ring which holds two big holes of 1.8 mm each, and **2small** mini ring which holds two small holes of 1.3 mm each.

Flies were loaded in the maze between ZT₀-ZT₁ (9am-10am) and they were given 30 minutes for acclimatization before the doors were open. Video recordings of the flies were taken for three hours after the opening of the doors, either in complete lightness or in complete darkness, using the ethoscope platform⁸⁸. During the experiments flies had no access to food but they had access to 1% agar medium to avoid dehydration. Agar medium has no caloric value.

On the release of the doors the flies started to move back and forth through the holes. Three different types of ring arenas were used, each one holding two holes (Figure 3.2 B). Flies were able to pass through

both size of holes of 1.3 mm and 1.8 mm, and made several passages through all the doors that were available to them. The two sizes of holes were chosen in such a way that the fly would be able to pass easy through the big hole and with some effort through the smaller hole, which would be tighter around the body of the fly. The fact that the smaller hole is more difficult to pass is confirmed by the longer time that the flies take to pass through it compared to the big hole.

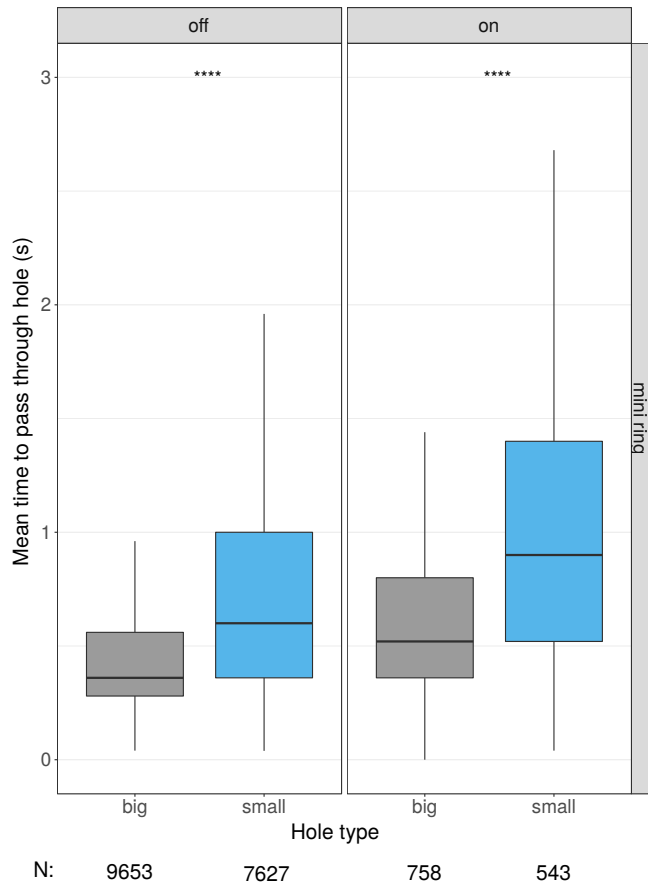


Figure 3.3: Longer time to pass through big hole than small hole Mean time to pass through the big and small hole in either dark (light off) or light condition (light on). N represents the number of passages in each combination of hole type and light condition.

Experiments were run in both light and darkness (light on or light off) (Figure 3.4 A, B, C). In darkness (see Figure 3.4 A, left panel), the total number of passages through all the available doors in the 1big_1small ring is not significantly different from the total number of passages through all the available doors in the 2small ring. Put simply, in dark, having access to one big and one small hole instead of two small holes did not seem to affect the overall passage through holes and the presence of one big hole is not by itself a stimulus for making more passages. However, the total number of passages through both available holes in the 2big ring is slightly above the total number of passages through the two available holes in the 1big_1small arena. Therefore in this case having 2 big holes instead of 1big and 1 small brings an incentive for the fly to pass more. In light (see Figure 3.4 A, right panel), there is a significant difference between the total number of passages through all available doors in the 1big_small mini ring and 2small mini ring, therefore the passage of one big hole forces the flies to initiate more passages. However, no significant difference between the total number of passages in the 1big_1small mini ring and 2big mini ring was found, therefore the presence of another big hole does not imply more passages in this situation, and is unclear at this point how the size of the hole modulates the initiation of passages. The small size of the arena increases locomotion

of the fly and ensures a flux of passages through the holes through the entire duration of the experiment. Even if the arena is symmetric, it is still large enough compared to the body size of the fly to allow it to move freely through it and spend a lot of time far from the holes. This means that the probability of the fly to initiate a passage, either through the small or the big hole is not equal but dependent on the trajectory of the fly in the arena, and the number of times that the fly encounter either of the holes. Regardless of this, it is clear that the fly prefer the big hole, however it would be good to find a way to give the fly equal probabilities of encountering either the small and the big hole, so that the proportion with which the flies select the holes reflects as clearly as possible only the flies preference for the bigger hole.

By comparing the left and right panels in Figure 3.4 A it is obvious that the total number of passages through all available doors is drastically increased by the absence of light. Also, by looking at the number of passages through each individual hole of every mini ring type, it is clear from Figure 3.4 B that both in light and darkness, there is no significant difference between the passages through each of the two available doors in the 2big and 2small rings. This is in accordance to our expectations, if the available doors have the same size the probability of passing through any of them is the same. However, in the 1big_1small ring, there is a significant difference between the number of passages through the big hole and the number of passages through the small hole. The strong preference for the big hole over the small hole is conserved both in light and darkness.

Figures 3.4 A and B indicate, by comparing their left and right panels that the presence of light decreases the number of passages. Therefore, Figure 3.4 C compares the total number of passages through all available doors for each ring type in light or darkness. For each ring type, the absence of light triples the number of crosses through the holes.

To better understand the behaviour of the flies, it is useful to look at how the number of crosses varies in time. Therefore, Figure 3.4 D shows the number of passages through each of the individual holes, every 30 minutes, in darkness. For the 1big_1small ring, the preference for the big hole is constant and conserved in time, the total number of passages through the big hole being significantly above the total number of passages through the small hole at any moment in time. As expected, for the 2big and 2small arenas, whose holes are the same size there is no significant difference between the passages through the two holes at any moment in time, confirming there is no experimental bias.

These results validate the preference of the fly for the big hole in a rigorous manner, showing also that light is a powerful modulator for the initiation of passages. Moreover we looked at the difference between the number of passages through the big hole and the number of passages through the small hole for each individual fly and we found that 100% of the flies made more passages through the big hole than the small hole in the three hours of experiment, confirming the hypothesis that behaviour adapts in time in the most advantageous way for the organism.

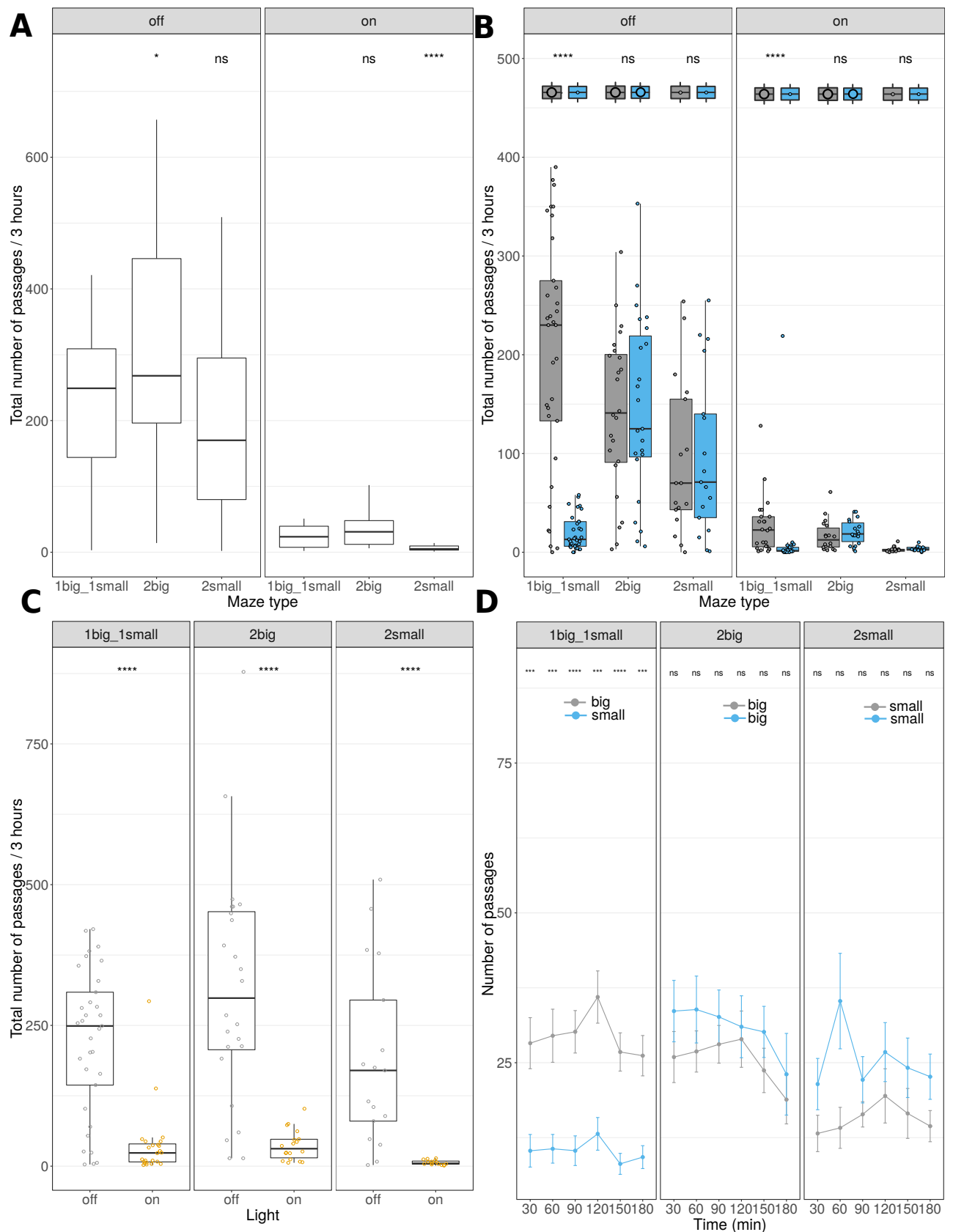
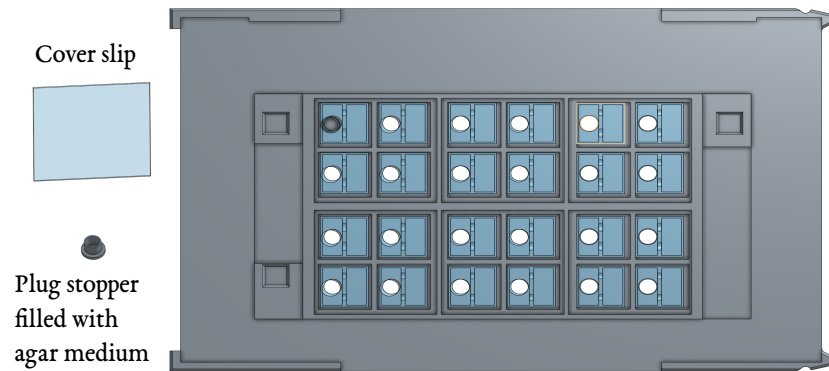


Figure 3.4: Big vs small hole preference of wild-type flies (CS) in the circle arena. **A** Total number of passages in the first three hours through all the possible holes for each of the three version of the circle arena used: *1big_1small*, which holds one big hole and 1 small hole, *2big*, which holds two big holes and *2small*, which holds 2 small holes. The left panel shows results in darkness (light off) and left panel shows results in lightness (light on). **B** Total number of passages through each individual hole contained on each of the three versions of the circle arena, both in darkness (left panel) and lightness (right panel). It is clear that in the versions of the circle arenas that hold holes of same size (*2big* or *2small*), the flies have no preference for any of the holes, having no significant different between their separate total passages, both in darkness (left panel) and lightness (right panel). However, in the *1big_1small* which holds one big and one small hole, the flies choose to pass through the big hole significantly more. (ref give p-value) **C** Total number of passages in darkness versus lightness through all the possible holes for each of the three versions of the circle arena used: *1big_1small*(left panel), *2big* (middle panel) and *2small* (right panel). **D** Quantification of the number of passages in time, in 30 minutes, through each hole, for the first three hours of the experiment. $N_{\text{flies}} > 24$

A



B

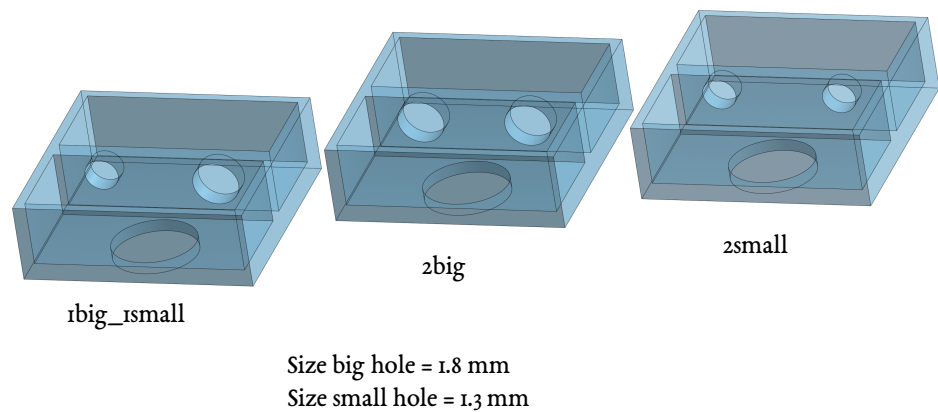


Figure 3.5: Square rings arena. The behavioural arena designed to study passages through holes with an increased high-throughput. It holds 24 flies in individual mini squares of size 9mm x 9mm and wall thickness of 0.75 mm. **A**, Image of the arena that holds 24 mini squares. Each group of four mini squares is covered with a 22 x 22 mm glass cover slip from above. The fly is loaded through the hole situated on the bottom of the mini square, which is next plugged with a stopper filled with agar, to avoid dehydration of the flies. **B** Image of the three mini squares used during experiments: **1big_1small** mini square which holds one big hole of 1.8 mm and one small hole of 1.3 mm; **2big** mini square which holds two big holes of 1.8 mm each, and **2small** mini square which holds two small holes of 1.3 mm each.

Final simplification of our assay consists of an arena that can hold up to 24 flies, each fly being contained in a mini square of size 9 mm x 9 mm. In the middle of each square there is a wall that holds two holes. Again, there are three versions of the arena: **1big_1small** to investigate how the flies choose between different size of holes, and **2big** and **2small** mini square to control that there is no bias from the external conditions.

The fly can make passages between the two sides of the wall (Figure 3.5). With this final version arena, the space in which the fly moves is reduced to a minimum. The new arena has the advantage of increasing the throughput to 24 flies per arena instead of 6. Moreover, by reducing the environment of the fly to a

minimum size, it is expected that the number of passages will increase accordingly¹²⁰. More passages can give us a better resolution into how the flies initiate passages and how these passages are modulated by various factors in time. The arena is so small that the fly is in close contact with both holes, which makes the probabilities of encountering the big and the small hole very similar, compared to the mini rings arena in which the probabilities of encountering either of the holes were dependent on the trajectory of the flies.

Flies were loaded in the maze between ZT₀-ZT₁ (9am-10am). Video recordings of the flies were taken for three hours, either in complete lightness or in complete darkness, using the ethoscope platform⁸⁸. During the experiments flies had no access to food but they had access to a filter paper soaked in distilled water to avoid dehydration.

The square arena has one quarter the surface of the mini ring. By comparing Figure 3.4 A and Figure 3.7 A, we notice that the total number of passages through all the available doors is four times bigger in the mini squares than in the mini rings. Again, the increased locomotion due to space restriction is confirmed, and it seems that the passage through holes behaviour is inversely proportional with the space reduction: by reducing the space to a quarter of the initial space, the number of passages through holes increases 4 times.

Further, we looked at the time that flies take to pass through the hole. We wanted to double check that the flies passage through the small hole is more difficult and this is context independent. This is confirmed again by the fact that flies take longer to pass through the small hole. 3.6

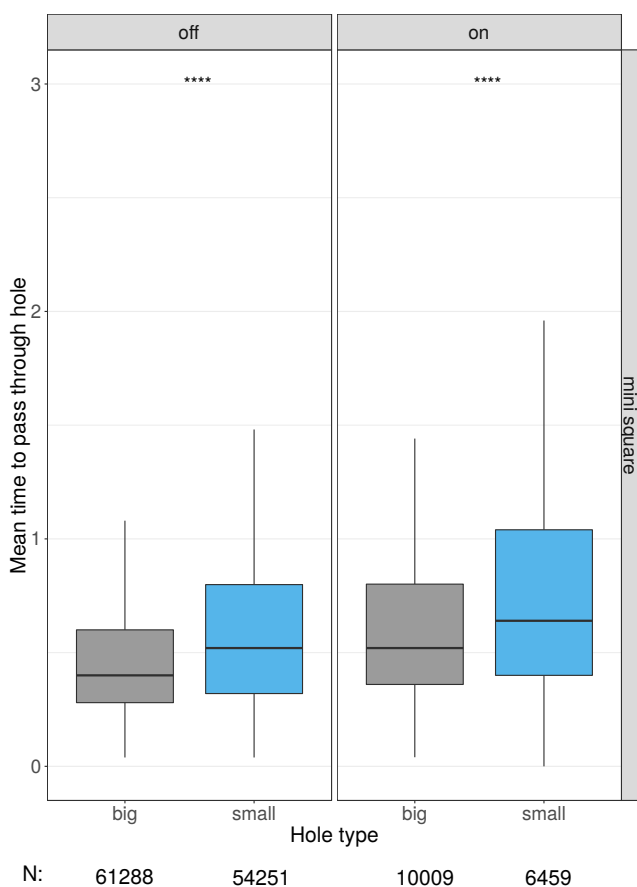


Figure 3.6: Longer time to pass through big hole than small hole confirmed in mini square arena Mean time to pass through the big and small hole in either dark (light off) or light condition (light on). N represents the number of passages in each combination of hole type and light condition.

Experiments are performed again both in lightness and in darkness. However, in the case of the mini

squares it is clear that in darkness there is a strong significant difference between the total number of passages through all the available doors in the different types of maze. The total number of passages through the 1big_1small mini square's holes, when one big hole is present, is bigger than the total number of passages through the 2small mini square's hole, that has no big holes. Also, the number of passages through the 2big mini square's holes, which has two big holes, is above the total number of passages through the 1big_1small mini square's holes, which has only one big hole 3.7 A. Indeed, by reducing the space of the arena and ensuring that the fly encounters each hole type with the same probability it gets clear that each big hole triggers more overall passages. Similarly to the mini rings arena, the number of passages through each individual hole is not significantly different if the holes are of the same size (for the 2big mini square and the 2small mini square). This is the expected result if there is no bias in the experimental conditions and the holes have the same probability of being chosen. This result is conserved both in darkness and in lightness. In the case of the 1big_1small mini square there is a significant difference between the number of passages performed through the big hole and the number of passages performed through the small hole, with light on or off, as expected (Figure 3.7 B left panel).

By comparing the left and right panels in Figure 3.7 A and B it is obvious that the absence of light boosts the passages through the holes. Figure 3.7 C shows that the number of total passages through all the available doors is significantly increased in darkness for each mini square type. Therefore, the absence of light either increases locomotion in flies making them encounter the holes more therefore making more passages, either makes the flies perceive holes in a different way that pushes them to more passages, or a combination of both.

Moreover, Figure 3.7 D shows how the number of passages varies with time, by plotting the number of passages done in chunks of 15 minutes. For the 2big and 2small mini squares, there is no significant difference between the number of passages through each individual hole at all points in time. However, for the 1big_1small hole, the preference for the big hole is conserved for each time point. During the three hours there is a slight decrease in the number of passages, notably for the big hole.

From 76 flies that performed the experiment in the 1big_1small, 100% made more passage through the bigger hole and also 100% made several passages through both available holes, no matter the light condition. This is a very strong result that confirms unanimously that in wild-type flies each individual brain uses the same mechanism in taking a decision and it will select more often the action that gives a more advantageous expected outcome.

The square arena proved to be an excellent environment that creates pressure on the fly to initiate action but also the arrangement of the holes and the size of the arena compared to the size of the fly seem to be great for ensuring equal opportunity of passing through both holes. Therefore, the arena has potential of revealing insights about the initiation of activity in the fly by looking at how flies select holes in time.

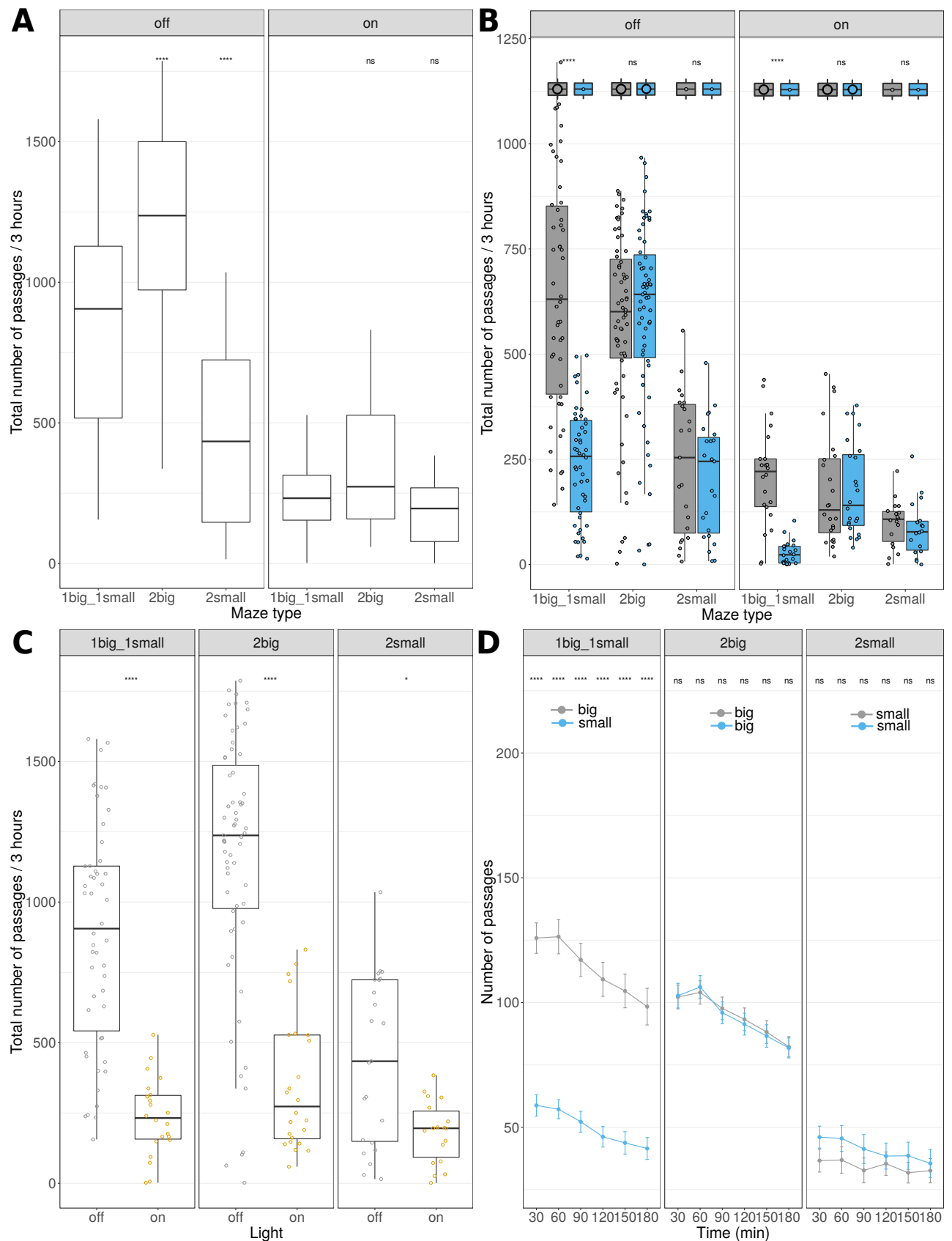


Figure 3.7: Big vs small hole preference of wild-type flies (CS) in the square arena. **A** Total number of passages in the first three hours through all the possible holes for each of the three version of the square arena used: *1big_1small*, which holds one big hole and 1 small hole, *2big*, which holds two big holes and *2small*, which holds 2 small holes. The left panel shows results in darkness (light off) and left panel shows results in lightness (light on). **B** Total number of passages through each individual hole contained on each of the three versions of the mini square arena, both in darkness (left panel) and lightness (right panel). It is clear that in the versions of the circle arenas that hold holes of same size (*2big* or *2small*), the flies have no preference for any of the holes, having no significant different between their separate total passages, both in darkness (left panel) and lightness (right panel). However, in the *1big_1small* which holds one big and one small hole, the flies choose to pass through the big hole significantly more. (ref give p-value) **C** Total number of passages in darkness versus lightness through all the possible holes for each of the three versions of the square arena used: *1big_1small*(left panel), *2big* (middle panel) and *2small* (right panel). **D** Quantification of the number of passages in time, in bouts of 30 minutes, through each hole, for the first three hours of the experiment. $N_{\text{flies}} > 24$

3.3.1.3 MINI RINGS VS MINI SQUARE - TWO DIFFERENT CONTEXTS OF THE SAME TASK

The two hypotheses of Chapter 4 (that the flies prefer the big hole over the small hole and that flies boost their crossings in the absence of light) have now been validated in two different contexts: mini rings assay and mini square assay (see Sections 3.3.1.1 and 3.3.1.2). The two assays differ between them in shape (circle vs square), size(area of the square is $0.25 \times$ area of the mini ring) and positions of the holes.

I want to recall here what we said in the introduction: when faced with a decision (like passing or not passing through a hole), there is an active part of behaviour that performs that action depending on the outcome that it expects from it. However there will be variability in the selection of that action, and this comes from the differences in the external conditions and internal states. However, no matter what the external conditions are, if we have two environments (mini square and mini ring) that present the fly with two expected outcomes (loosely passing through the big hole and tightly passing through the small hole), in the face of varying external and internal states, the flies should always prefer the more advantageous outcome over time. The fact that all the flies that we tested in the *ibig_ismall* mini ring and *ibig_ismall* mini square choose more the big hole than the small hole, confirms that indeed flies go for the most favourable action. One reason might be that they go more for the actions that requires them to consume less energy. Another reason might be that they choose more the less risky option, which in case of danger guarantees a quicker and safer escape. However, we can measure the effects of the external conditions on flies decisions by looking at the number of passages they make and the proportion with which they select the two holes, together with other physiological measurements like distance and velocity.

In this section, the results from Sections 3.3.1.1 and 3.3.1.2 are pulled together and compared. In order to better understand how the environment influences the passages through holes and what are the factors that modulate the initiation of a passing, we look at the differences between the mini ring and the mini square arena.

Figure 3.8 A presents a comparison between the mini ring and the mini square in the same type of assay (*ibig_ismall*, *2big* or *2small*) and same condition of light. Independent of the assay type or light condition, the total number of passages in the mini square assay is always significantly superior to the total number of passages in the mini ring assay, suggesting that the square arena pressures the flies to initiate more passages. The percentage of passages through each of the available doors gives us further information on the link between the external conditions and the initiation of passages. The top panel of Figure 3.8 B presents the results in the absence of light. As expected, for the *2big* version of the arena, flies have no preference for either of the holes, and they choose both of them with 50% probability. Same thing for the *2small* versions of the arena. However, in the *ibig_ismall* mini ring arena type, the percentage of big hole being chosen is greater than in the *ibig_ismall* mini square. This means flies choose with different proportion the big and the small hole in different arenas, in dark. Therefore the degree of preference for the big hole is dependent on the characteristics of the arena. When light is present (bottom panel of Figure 3.8 B) there is no significant difference for the preference of the big hole in the *ibig_ismall* versions of the mini ring and mini square arenas. The results in dark and light taken together together suggest that there might be the combination of both light and characteristics of the arena that modulate the proportion with which flies choose the big and the small holes, in a not so obvious way. However, the smaller number of passages performed in light might be insufficient for portraying the true expected value of the chosen proportions. We should remember that each initiation of action has a degree of randomness involved. A larger amount of

passages is supposed to average out this randomness and give us a closer value to the real percentage value that portrays the way in which flies distinguish between the two outcomes. This is why, results in darkness are expected to reveal less noisy results. Now that we analysed the effects of the characteristics of the arena on the proportion with which flies choose the holes in same light conditions, let's also look at the effect of the light alone on the proportion with which holes are crossed in the same arena. In the mini ring, the presence of light has no influence over the percentage of time the flies choose the big hole over the small hole (Figure 3.8 D top panel). It is not the case for the mini square assay in which the presence of light increases flies preference for the big hole. These results suggest that we cannot uncouple the effect of the light from the effect of the arenas in their modulation of the proportion with which flies choose between the two holes of different sizes. The smaller and similar number of passages in the mini ring in light and dark together with the mini square in light might be insufficient for capturing the effect of light on the proportion with which flies choose the holes.

Figure 3.9 compares the activity of the flies in the mini ring and the mini square arena. Similarly with the number of passages, for the same type of assay (1big_1small, 2big, 2small) and light condition, the flies always walk more in the square arena. Their total distance walked in three hours is always superior in the mini square compared to the mini ring arena (Figure 3.9). Moreover, the three hours of experiments are split in 30 minutes bouts and the distance in each bout is computed, in order to have an idea of how flies walking changes in time. Generally, at every moment in time the distance walked in the mini square is greater than the distance walked in the mini rings. The only exception is the 2small arena type with lights off condition, for which at a few points in time, the distance walked in the mini ring and mini square are not significantly different. This can also be an artifact due to very few passages performed at that point. It is also worth noticing that the distance walked decreases in time both in the mini square and in the mini ring. In the mini square, the slope of the descent is very small, flies walk at an almost constant pace, both in light and in darkness. However, in the mini ring, the distance walked decreases at a much faster rate, especially in the presence of light. This suggest that the increased locomotion due to space restriction might vanish with time, if sufficient time is given to the flies to adapt to the new environment.

The distance walked is a relevant measurement of the general excitement that the flies get from being in the arena. Flies walk the most in the first 30 minutes bout (Figure 3.9 B), and the distance walked in this first bout, in some conditions(1big_1small with light on and 2small with light off) is independent of the context. This might be due to the fact that flies are more aroused at their initial encounter with a new environment. This initial excitement is prioritised in the first 30 minutes following which flies adjust their behaviour to something that is more relevant to the context in which they are.

For the same type of arena and light condition, the flies move always faster in the mini square arena compared to the mini ring. (Figure 3.9 C) The pace of the flies in the square arena is almost constant in time. In the mini rings arena there is a strong decrease in speed during the three hours of experiment. The presence of light increases the rate of decrease in the speed of the flies.

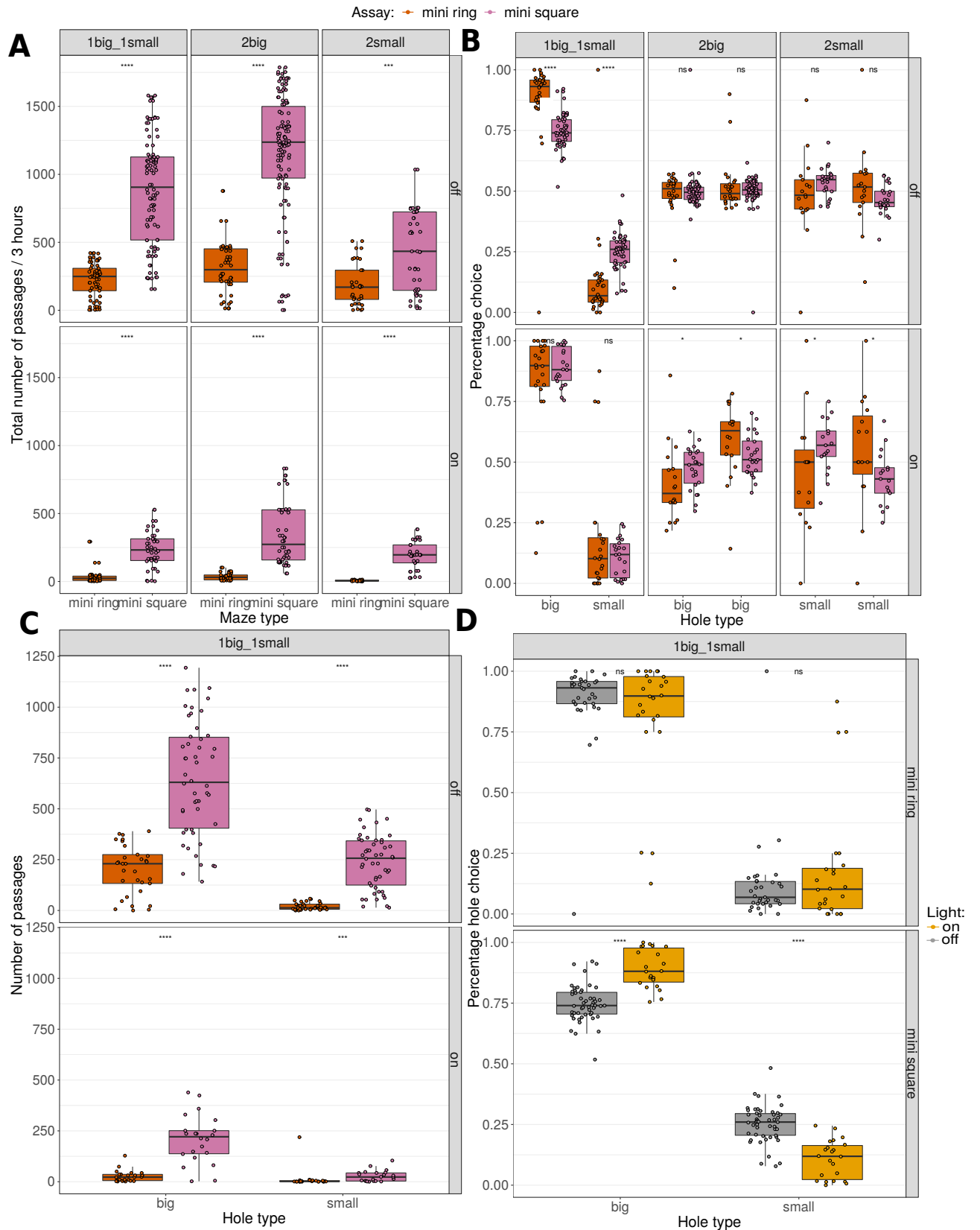


Figure 3.8: Mini rings versus mini squares arena crossings comparison Both assays present the fly with the same task: choosing between two holes. The two assays differ in shape and size, making the context in which the decision between the two holes is taken slightly different. **A** Total number of passages performed in the first three hours of the experiment through all the available doors in the mini rings vs the mini squares assays. Both assays have three different versions, with corresponding same size holes. (See Figures 3.2,3.5) Comparison is done between the corresponding same type of mini rings and mini squares assays with the same light conditions (either light on or light off). **B** Percentage of passages through each of the two possible holes, for the three types of different mazes. Comparison is done between the corresponding same type of mini rings and mini squares assays in either light on or light off condition. **C** Comparison between the number of passages performed through the big hole of the *1big_1small* mini ring assay and the big hole of the *1big_1small* mini square assay. Same for the small hole with light either on or off. **D** Upper panel presents comparison between the number of passages through the big hole in light vs dark condition, in the *1big_1small* mini ring. It also presents the same comparison for the small hole. Bottom panel shows the corresponding results in the *1big_1small* mini square.

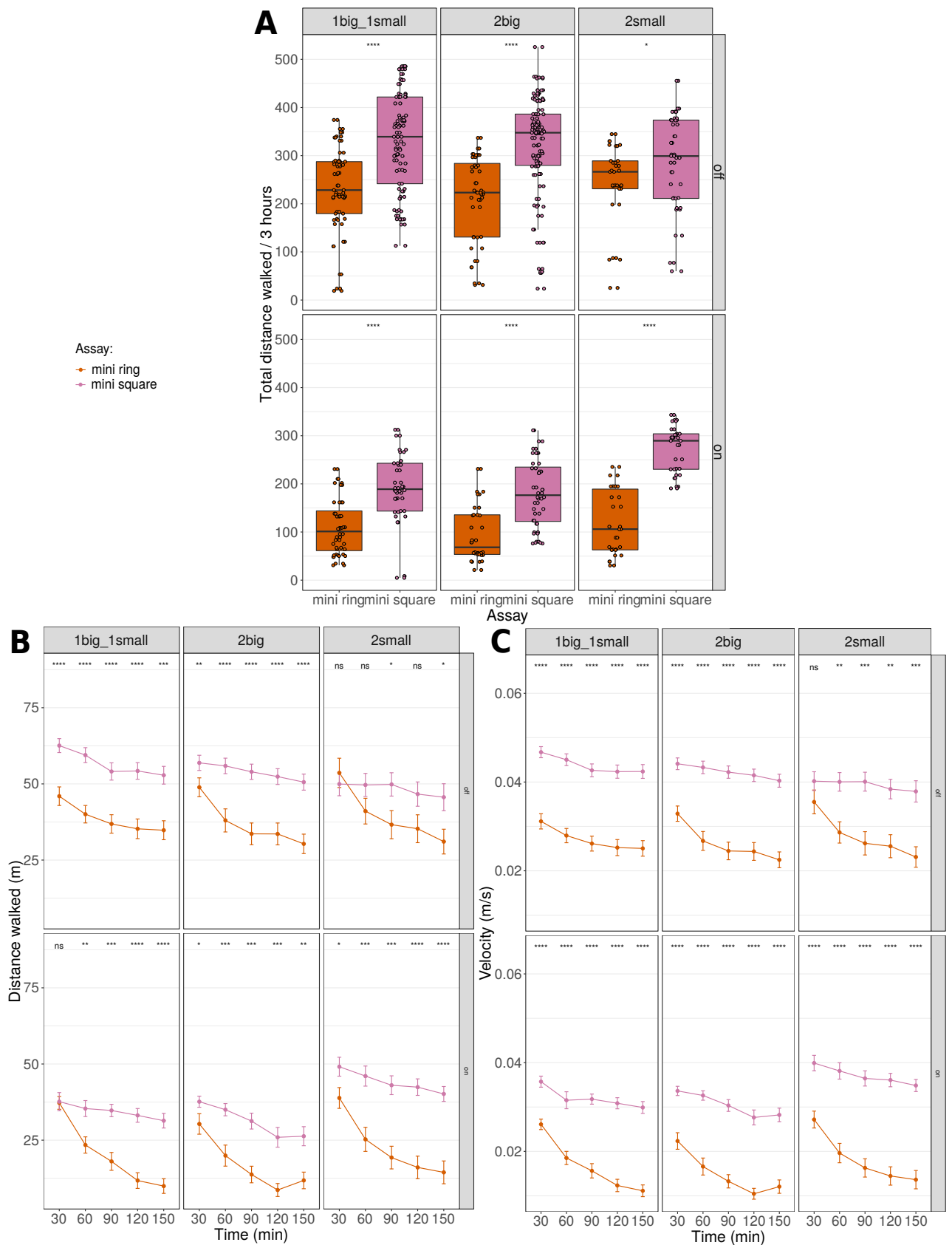


Figure 3.9: Mini rings versus mini squares arena distance walked and velocity comparison Mini rings and mini square assays present the flies with the same task of choosing between two holes. The main difference between the two assay is their size, the circle arena being 4 times the size of the square arena. **A** Comparison between the total distance walked in 3 hours in same type of mini ring and mini square arena. Each column corresponds to one of the three types of arenas (1big_1small, 2big, 2small). Top panels present results in complete darkness and bottom panels present results with light on. **B** Distance walked in bouts of 30 minutes, panels are following same structures as in **A**. **C** Mean velocity in bouts of 30 minutes, panels are following same structure as in **A**.

3.3.1.4 EFFECT OF THE HOLES

In this section we investigate what is the effect of the holes on the flies. In Chapter 4 we observed that individual flies placed in the concentric rings arena in light condition were waiting on average 5 hours before making their first passage through the available doors, suggesting a sort of anxiety to pass. However, in darkness, flies will make their first passage after only a few minutes. In the square arena, similarly to the concentric rings arena, the number of passages in dark surpasses the number of passages in light, and in both conditions flies cross through the holes many times, steadily during the entire length of the experiment. In the square arena, the small size of the area explored increases the locomotion¹²⁰, contributing to the initiation of more passages. It is not clear, however, whether the holes are arousing the flies in order to explore the square arena even more or if they discourage the flies in their search. A commonly used approach when studying any behaviour is to prevent that behaviour from happening in order to understand its functions. One relevant example is the study of sleep in which the organisms are often sleep deprived and their behaviour assessed post sleep deprivation. Similarly, to understand in what way holes influence the behaviour of the flies, they are removed completely from the square arena as presented in Figure 3.10. To assess the exploration of the no_hole in a comparable way to the 1big_1small arena, we imagined a set of virtual holes that sit at the same place as the ones in the 1big_1small arena, and we assess the crossing through them. In the 1big_1small square arena a crossing is counted only if the fly moves its entire body size from one part of the arena through the hole and to the other part of the arena. This applies to the virtual holes as well, therefore a passage is counted only if a fly moves the entire area of its body from one side of the virtual hole to the other side of the virtual hole.

Protocol is the same as the one used previously to validate the square arena results (Section 3.3.1.2) except that the video recordings are taken for 4 hours instead of 3 hours.

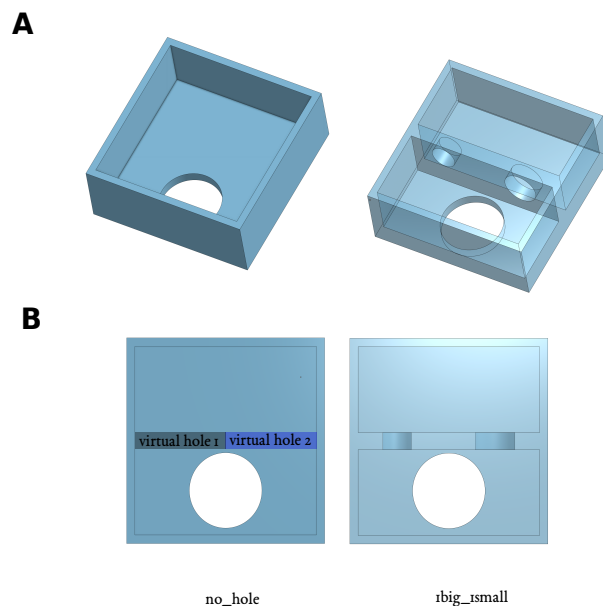


Figure 3.10: *No holes square arena vs 1big_1small square arena* The no_holes square arena has the same size and shape as any of the squares arena, but the middle wall holding the holes is entirely missing. **A** Isometric view of the no_holes (left) and 1big_1small (right) square arenas. **B** Top view of the no_holes and 1big_1small square arenas. Placement of the two virtual holes is indicated in colour and it is accurate in size.

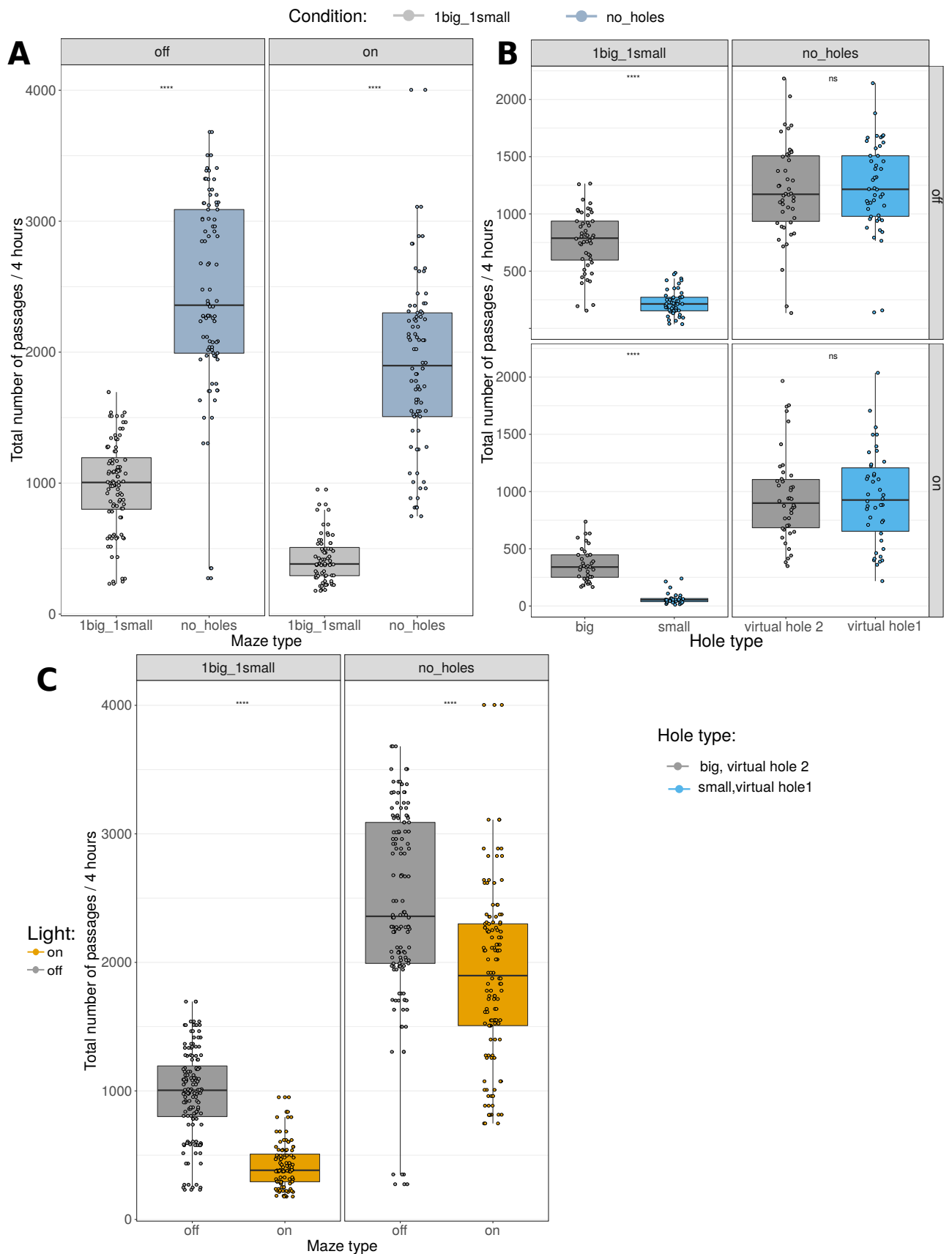


Figure 3.11: No_hole vs 1big_1small number of passages. **A** Total number of passages in 4 hours through the big and small doors of 1big_1small square arena compared to the total number of complete crosses through the two virtual doors in the no_holes arena (see Figure 3.10). A complete passage is when a fly moves their entire body area from one side of the virtual door, to the other side of the virtual door. Left panel shows results in dark (light off) and right panel shows results with light on. **B** Number of passages taken through each specific door: big vs small holes for the 1big_1small maze, and virtual door 1 vs virtual door 2 for the no_holes maze. Upper panel shows results in dark (light off) and bottom panel shows results with the light on. **C** Total number of passages comparison between the light and dark conditions in the 1big_1small maze and no_holes maze.

Figure 3.11 A looks at how the number of passages made by the flies through the big and the small door combined compares with the number of passages made by the flies in the no_hole arena through the virtual hole 1 and virtual door 2. In both light and dark conditions, the number of passages in the no_hole arena is more than the double of the total amount of passages through the ibig_ismall's holes.

It has been shown in Figure 3.7 B that in the ibig_ismall square arena the flies make more passages through the big hole than the small hole. This result is confirmed again in Figure 3.11 B left panel. For the no_hole assay, on which we imagined two virtual doors that are therefore indistinguishable one from the other, the number of passages through each of these doors is relatively the same. (Figure 3.11 B right panel)

The way in which light modulates the activity in the ibig_ismall and no_hole arenas is presented in Figure 3.11 C. As previously shown in Figure 3.7 C, it is verified that the number of passages is boosted by the absence of light. The activity of the flies in the arena with no holes is also increased in darkness, flies making more passages through the virtual doors in this condition than when light is present. The distance walked during the experiment, together with the velocity of the flies are providing valuable information about the excitement of the flies, and how much arousal they receive from the environment. Figure 3.12 A compares the total distance walked in 4 hours of experiment in the ibig_ismall assay and no_holes assay. It shows that in both light and dark conditions, the absence or presence of holes does not have an influence on the total distance walked.

Light modulates the total distance walked in both assays. In the same assay, flies walk more in dark than in light condition regardless of the presence of holes.

If Figure 3.12 A shows that the presence of holes does not influence the total distance walked, Figure 3.12 C reinforces this result proving that the distance walked in the ibig_ismall maze is not significantly different from the distance walked in the no_holes maze at any moment in time. The distance is assessed after every bout of 30 minutes. The only exception is the first bout in the lights off condition for which the distance walked in no_holes condition is slightly superior to the distance walked in the ibig_ismall condition.

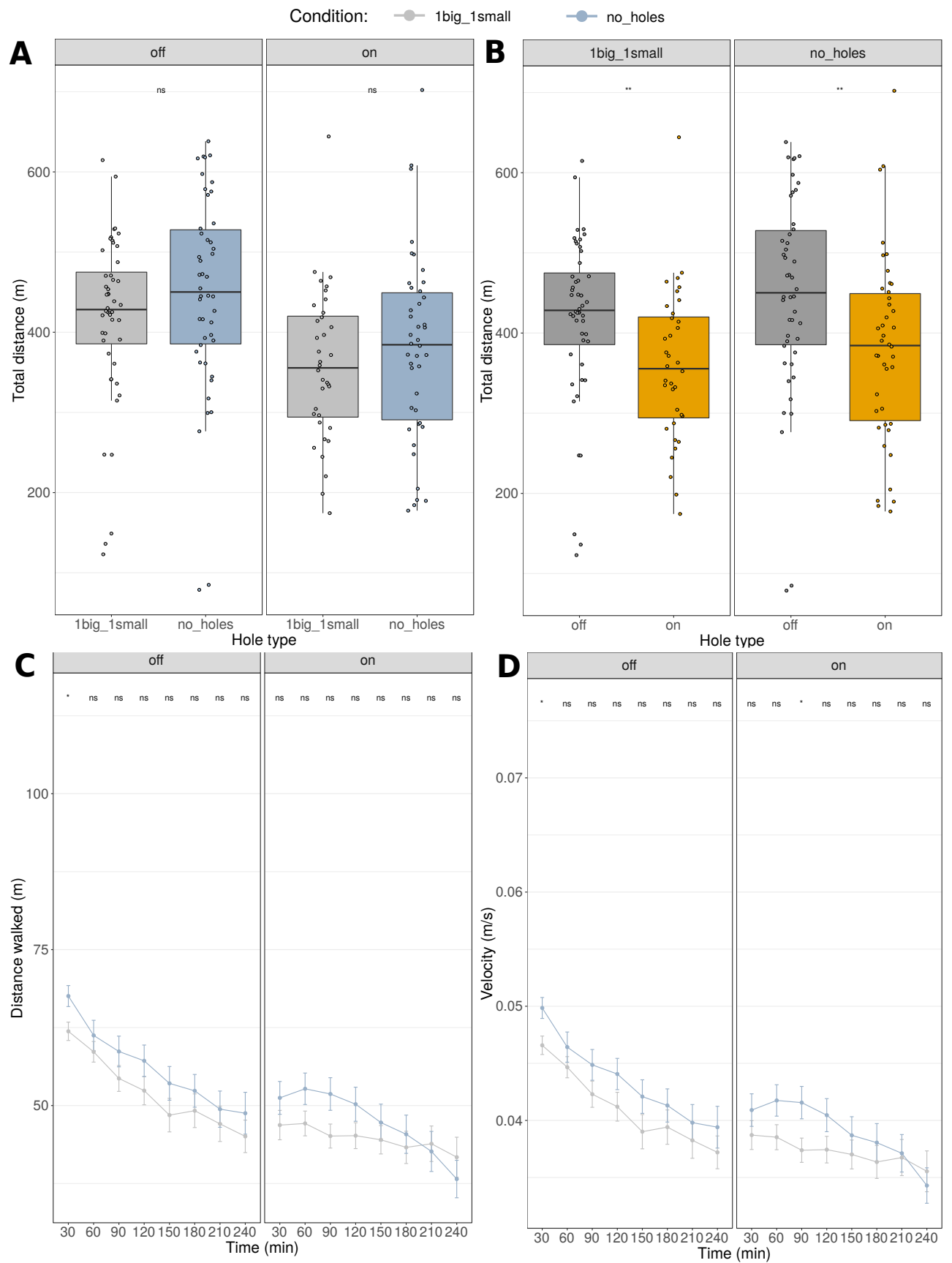


Figure 3.12: No_hole vs 1big_1small distance and velocity. **A** Total distance walked in 4 hours of experiment in 1big_1small arena compared to the no_holes arena, in the absence of light (left panel) and in the presence of light(right panel). **B** Influence of light on the total distance walked in the 1big_1small assay (left panel) and no_holes assay (right panel). **C** Distance walked in bouts of 30 minutes shows how the activity varies in time, in dark (left panel) and light (dark panel). **D** Mean velocity in bouts of 30 minutes in both light conditions. $N_{flies} = 48$ for each condition (1big_1small with light off, 1big_1small with light on, no_holes with light off, no_holes with light on)

3.3.2 DISCUSSION

One of the main findings of this work is the hole assay which consists of a very simple design and is able to capture a robust decision of the flies: the preference for the big hole. Given that all the flies favour the big hole to the small hole fits our hypothesis that flies select their actions based on the expected outcomes, choosing the most advantageous one more often. The unanimous response of the flies advocates for the existing of the same living/active mechanism responsible for generating behaviour. The paradigm of the holes is unique in its ability of uncoupling the initiation of an action part of a decision (initiating a passage) from the modulatory role played by the external conditions (size of the holes, size of the arenas, light) and internal states of the flies. The holes choice paradigm is powerful because it presents the flies with two decisions whose outcomes expectations depends on one main variable: the size of the holes.

This preference for the big hole is context independent because by slightly varying external conditions, flies remain stable in their choices. By keeping the same size of the holes (1.8 mm for the big hole and 1.3 mm for the small hole) we have hypothesised that flies expect the same outcomes for passing through the holes in each of the three contexts. In simplifying our paradigm from the concentric rings up to the square arena, different experimental variables were tried: light (on and off), size of the arena (concentric arena, mini ring, mini square), number of holes (18+ vs 2) and different position of the doors. In all the combinations of these different experimental condition, the preference of all the flies for the big hole was conserved. This confirms on one side that indeed the outcomes expectations from passing through the holes depends only on the size of the holes, but more importantly that the mechanism responsible for generating behaviour and for initiating action is robust.

All the flies made passages through both available doors. The decision of passing through a door is modulated by external conditions like the size of the doors, light, size of the arena and also spiced by some randomness. This is reflected in the variability that exists between the flies in the number of passages they crossed and in the proportion with which they choose the big and the small holes. By video observations, it was clear that they will not initiate a passage every time their legs came in contact with the shape of the holes, but rather whenever they felt like it.

The design of these assays went through different steps and had three main milestones which consisted in the concentric rings arena (Figure 2.4), the mini rings arena (Figure 3.2) and the final square arena (Figure 3.5). Each of the three designs came with their specific improvements, allowing a more detailed and high-throughput acquisition of data. Specific tools for monitoring and tracking the flies were developed in parallel. Even though the passage through holes takes place very fast, recording experiments using a cheap camera proved sufficient for automatically extracting information like the amount of time the flies take to pass through the holes and the number of passages they make. Using a mask for each type of the arena, that defines the interesting areas of the arena using different grayscales colours proved to be an easy solution for creating a flexible feature to assess the passage through holes behaviour in different contexts.

The increased locomotion of the flies due to spatial restriction has been strongly confirmed. Xiao and Robertson¹²⁰ used a restrictive space arena of 12.7 mm diameter which is of similar size with the mini ring arena (13.5 mm inner diameter) and ran their experiment for 1 hour. Therefore we were able to reproduce their results with the mini rings and show that the increased locomotion is sustained for the entire duration of our experiment which was of 3 hours. Moreover we showed that by decreasing the area of the arena four times (mini square) the locomotion increases drastically as indicated by the total distance walked dur-

ing the experiment which is significantly superior to the total distance walked in the mini rings arena, independent of the light condition or size of the holes (Figure 3.9 A). Moreover, by assessing the distance and velocity in bouts of 30 minutes it was obvious that the locomotion in the square arena was increased at every moment in time. Together with the distance and velocity, the total number of passages also boosted in the square arena compared to the circle arena, flies making almost four times more passages (Figure 3.8 A). The locomotion is increased by space restriction and the absence of light but not by the presence of holes, as there is no difference in the distance walked in the square maze in the presence or absence of the holes (Figure 3.12 A).

By completely removing the middle wall that holds the holes flies make many more passages through the middle of the arena (Figure 3.11 A). The presence of the wall with the holes, restricts the width of the space to pass to either the big or the small hole. The number of passages through the middle of the no_holes arena is more than double the number of passages done through the holes of the ibig_small arenas in both light and dark condition. Therefore, reducing the space to pass restricts the movement of the fly, holding the fly back from initiating a passage. This might be similar to an anxiety response from the fly. This result points back to Chapter 4, where it was observed that a single fly placed in light condition in the concentric rings arena which has an inner diameter of 23 mm, took on average more than 3 hours to make a first passage through the holes, again showing an anxiety-like state. *Drosophila* wall following behaviour is thought to be related to anxiety¹⁴¹, and a recent study showed that *Drosophila* has a few genes that influence their defence behaviour similarly to rodent anxiety.¹³² The holes arenas might be useful in that direction.

Together with the reduction in space, the presence or absence of light proved to have a profound effect on the behaviour of the flies. Independent of the presence of holes, we showed that in the square arena flies walk slightly more in darkness compared to light condition (Figure 3.12). However, their number of passages through the holes in darkness is almost four times bigger than the number of passages through the holes in light condition (Figure 3.5 C). The increase in the number of passages is not proportional to the increase in the distance walked and suggests that there must be another component that plays a role in increasing the number of passages. One possible scenario, is that flies perceive the holes differently in darkness. According to some studies^{142,143} tactile acuity is enhanced by visual deprivation, in humans. In a similar way, the flies tactile perception might be enhanced in dark. If this is the case the presence of holes might arouse the flies more in dark, urging them to make more passages. However, little is known about how mechanoreceptors neurons are integrated in the brain in order to evoke a motor response. The body of the *Drosophila melanogaster* fly is covered with bristles which are considered to be main touch receptors¹⁴⁴. They are also involved in environment exploration⁷⁹ and they help flies to elicit very fast and robust escape responses. It is believed that stimuli are represented through parallel computations of signals coming from a combination of mechanosensory receptors¹⁴⁴. The hole assays is therefore also promising in studying tactile perception in *Drosophila*.

Related to the effect of light, it is interesting to notice that in light condition, the flies choose the big and the small hole in the same proportion in the mini rings and mini square arena. Therefore, the shape and placement of the holes do not have an influence of the preference for the big hole. However, in darkness flies will choose the big hole a lot more often compared to the small hole in the mini ring arena the proportion being 90% big hole: 10% small hole. In the square arena the proportion changes to 75% big hole : 25% small hole. Again, the absence of light seems to change the way in which flies perceive the holes, the small

hole becoming more attractive to them in darkness.

The behaviour of the flies in darkness also makes us think of an attention-like process. Attention is the ability of an individual to focus on one group of stimuli and ignoring the other irrelevant stimuli. A very good example of attention is the cocktail party effect, which describes the capacity of an individual to focus his auditory attention on a conversation when at a party, in a noisy environment.¹⁴⁵ If it is easy to understand what is attention in humans, it becomes very difficult to recognise it in the others, especially in invertebrates because of their brain which is completely different to ours. However, visual attention-like states have been studied in *Drosophila* over the last decades^{129,130,98}, using very sophisticated behavioural paradigms. It is established that *Drosophila* is able to fixate its visual attention on specific targets while ignoring background motion⁹⁸.

The way in which flies perceive holes can be of interest in studying tactile attention-like states in flies. The observation that the lack of light seems to enhance their perception of the holes, could be relevant in that sense. The visual attention of flies has been broadly studied, however their tactile perception might have more weight in determining their behaviour, as they are highly sensitive to the movement of their bristles, which allows them to navigate and explore an environment with ease in the absence of light.

A recent paper has shown that sleep deprived flies respond normally to visual stimuli, however they are more distracted by background cues in a visual competition task than flies that had normal sleep⁸¹. In the next part of this chapter, an exploratory investigation is carried out to understand if sleep impacts in any way the behaviour of the fly in the hole assay.

For the rest of our investigation, the square arena was used in dark condition, as this was the context that gave us the highest throughput and the higher number of passages through the holes. According to the law of large numbers, more trials (in our case more passages) will average out the randomness and give us results which will reflect better the mechanism through which flies choose to initiate passages through holes.

So far we achieved the first milestone which consisted of finding an assay that can reflect the mechanisms through which flies take a decision and we have proven that this mechanism is robust in different context. Moreover, we have shown that external conditions like the light and the shape and size of the arena modulate the initiation of passages and changes the average number of passages and the proportions with which the big and the small hole are chosen. Further, we are going to investigate how internal states modulate the initiation of passages, by interacting independently with the homeostatic and circadian components of sleep.

3.4 EFFECT OF SLEEP ON HOLE CHOICE

In humans, sleep deprivation can interfere with a person's capabilities of successfully achieving a motor task¹⁴⁶ and it seems to increase the perception of a task difficulty¹⁴⁷.

This section presents the results of how disturbing the sleep of the flies affected their behaviour in the mini squares arenas, in dark condition. The flies were sleep-deprived with different methods and for different amounts of time from mild sleep deprivation to acute sleep deprivation. Our hypothesis was that disturbing the sleep of the flies will result in a change of their behaviour in the mini squares: a possible drop in the number of total passages or an inability to distinguish with the same precision between the big and the small holes. We have shown in the previous sections that the mechanism used by flies to initiate passages is robust in every fly and in varying external conditions, as they all choose the big hole over the small hole more often. Next, we would like to understand if this mechanism is robust in the face of changes in the internal states, but also what is the weight that sleep deprivation plays in initiating a passage. Moreover, we will use CLK mutants which have a disrupted circadian mechanism and try to understand if this has an effect on the mechanism with which decisions are generated or on the variability present in the sequence of passages that flies make.

In the previous sleep deprivation experiments, done using the y-maze and presented in Chapter 1, we were unable to capture any effects of the sleep deprivation on the performance of the flies in the task. However those experiments were very short, being run for only 15 minutes following sleep deprivation. One of the major understandings up to this point, that came from observations and experimental manipulation is that flies have different modes of functioning and when presented with a new environment they will enter a specific state in order to adjust themselves to the novel conditions. Therefore when starting a new experiment it is necessary to take into consideration this time of habituation which can take from 30 minutes up to an hour, and only afterwards assess the performance in the task. Of course, this does not apply for every experiment, but is particularly required on behavioural experiments studying internal states using deprivation methods to induce sleep deprivation or hunger. The novelty of a new environment might be so arousing that no effects of the internal states appear in the initial excitement of exploring a new space. If this is true, this is possible to be the reason for which no effects of sleep deprivation are recorded in the y-maze. To improve our resolution into the effects due to sleep deprivation on the initiation of passages in the mini squares, we decided to run the experiments for up to 5 hours.

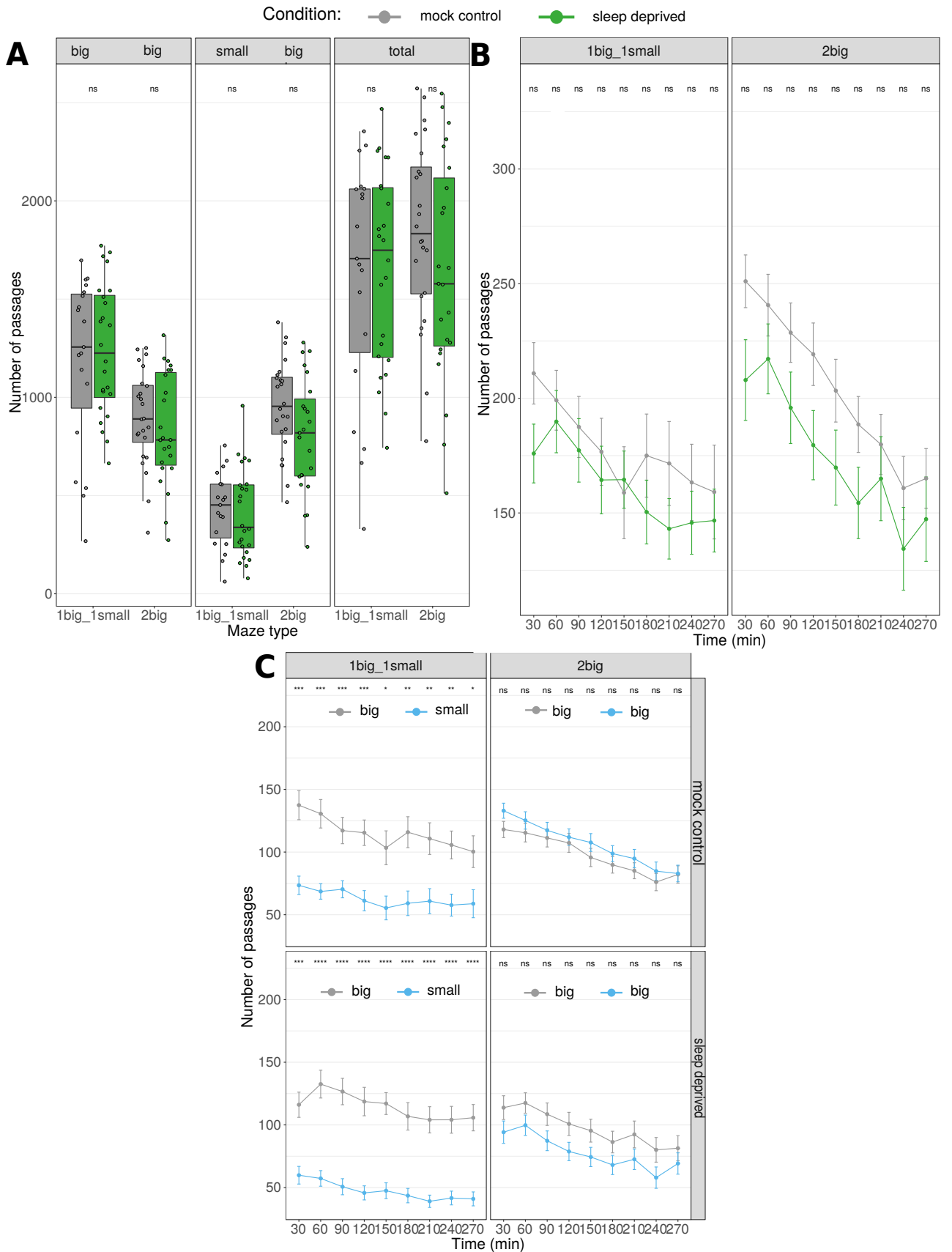


Figure 3.13: 1 night sleep deprived flies vs control number of passages **A** Total number of passages made by sleep deprived and control flies through all available doors and through each individual door in the 1big_1small and 2big square maze types. The left panel presents results through the big hole of the 1big_1small maze and one big hole of the 2big maze, the middle panel presents results through the small hole of the 1big_1small maze and the other big hole of the 2big maze, and the right panel sums up results from the first two panels, giving the total number of passages through all available doors in each maze. **B** Number of passages made every 30 minutes through all the available doors in the 1big_1small mini square maze (left panel) and 2big maze (right panel). **C** Number of passages made every 30 minutes through each individual hole of the 1big_1small maze and 2big maze. $N_{\text{flies}} > 24$

3.4.1 RESULTS

3.4.1.1 INIGHT SLEEP DEPRIVED FLIES

In this experiment, wild-type flies were sleep deprived using mechanical sleep deprivation for one night from ZT12 to ZT0, a commonly used technique in the *Drosophila* sleep field.¹⁴⁸ Immediately following sleep deprivation (ZT0-ZT1) male flies were loaded in the 1big_1small and 2big square arenas and monitored for 4.5 hours.

Figure 3.13 presents a comparison of the number of passages taken by the mock control and sleep deprived flies. There is no significant difference in the mean total number of passages made during the entire length of the experiment (Figure 3.13 A). If we look at the number of passages made every 30 minutes, again there is no difference between the control and sleep deprived groups at any moment in time (Figure 3.13 B). Sleep deprived flies are able to distinguish between the small and the big hole, and they make significantly more passages through the big hole than to the small hole at every moment in time (Figure 3.13 C). Similarly to control, in the 2big maze flies choose the 2 big holes equally.

Figure 3.15 shows a comparison between the preference for the big hole in the control and sleep deprived groups, together with a comparison of the distance walked in the maze in the 1big_1small square assay. Sleep deprived flies choose on average the big and the small hole in the same proportions as the mock control (Figure 3.15). If we look at the proportions of big holes chosen out of the total holes passed every 30 minutes, sleep deprived flies choose the big hole in the same percent as control except at one point in time, in the second hour of experiment when they choose slightly more the big hole. The activity of the sleep deprived flies is not different from control neither. The total distance walked in the maze during the 5 hours of experiment is not significantly different between the two groups (Figure 3.15 C). However, by assessing the distance every 30 minutes, mock control flies walk slightly more than sleep deprived flies at the beginning of the experiment and in the fourth hour of experiment (Figure 3.15).

The mean velocity of sleep deprived is not significantly different from control almost at every moment in time during the length of the experiments. The exception is the first half an hour of the experiment and at one point after 3.5 hours of experiment when control flies move slightly faster than sleep deprived flies (Figure 3.14 A).

Following a sleep deprivation night, flies usually sleep more than control in the three hours following sleep deprivation⁵⁶. Given that the length of our experiment is five hours following sleep deprivation, we assumed that sleep deprived flies will fall asleep in the square maze at one point during the experiment. Figure 3.14 B shows that sleep deprived flies have no sleep in the maze, as they rest for less than 5% of their time in the maze. Their behaviour is not significantly different from control flies which are also very active, resting for only 10% of their time.

Given that one night of sleep deprivation did not change any obvious behaviour of the flies in the square maze, we used the variables measured to make a principal component analysis (PCA) on the control and sleep deprived flies group. Three variables were fed to the PCA: the number of passages through the big hole, the number of passages through the small hole and the total distance walked. The PCA is an algorithm that takes the data and projects it into a new coordinate system such that the greatest variance of some projection lies on the first component and the second greatest variance lies on the second component. Basically, we wanted to know if we can find a space in which the two groups of flies sleep deprived and control are distinguishable. Figure 3.14 C shows the data projected in the new coordinate system, however

even in this new space the two groups of flies overlap, suggesting that there is no difference between them in the way in which they behave during the experiment.

In conclusion, one night of sleep deprivation does not have an effect on the mechanism with which the flies choose to pass through the holes as again, 100% of the flies choose the bigger hole more often and it also does not have a visible effect on the number of passages and proportion with which flies choose the holes, suggesting the small weight that sleep deprivation plays in this decision.

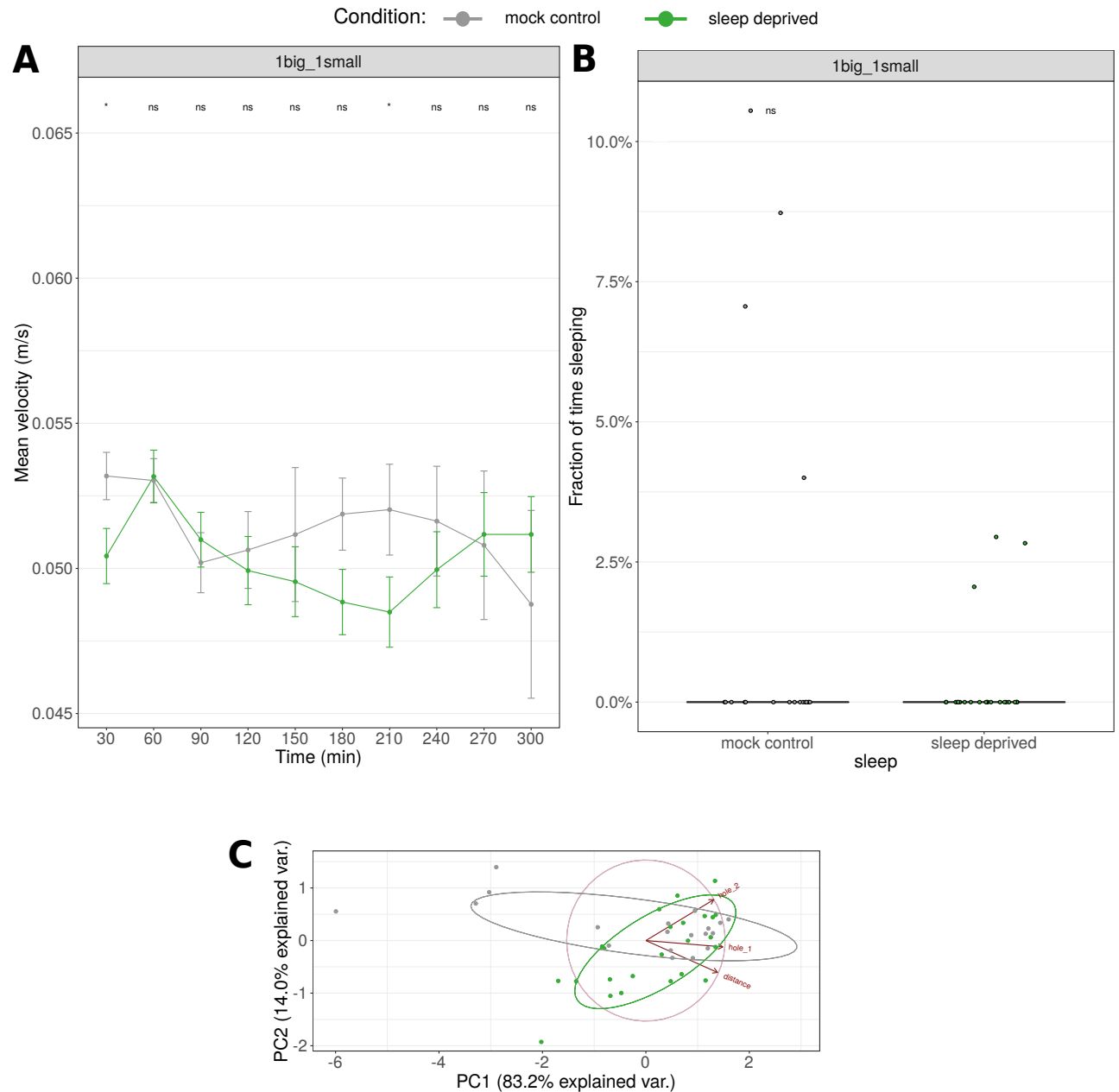


Figure 3.14: **A** Mean velocity every 30 minutes. **B** Fraction of time sleeping during the entire length of the experiment. Sleep is defined as a period of inactivity longer than 5 minutes. **C** Principal component analysis using a singular value decomposition which examines the covariances / correlations between individuals. Each individual fly is represented by 3 variables: the number of passages made through the big hole, the number of passages made through the small hole and the total distance walked. $N_{\text{flies}} > 24$

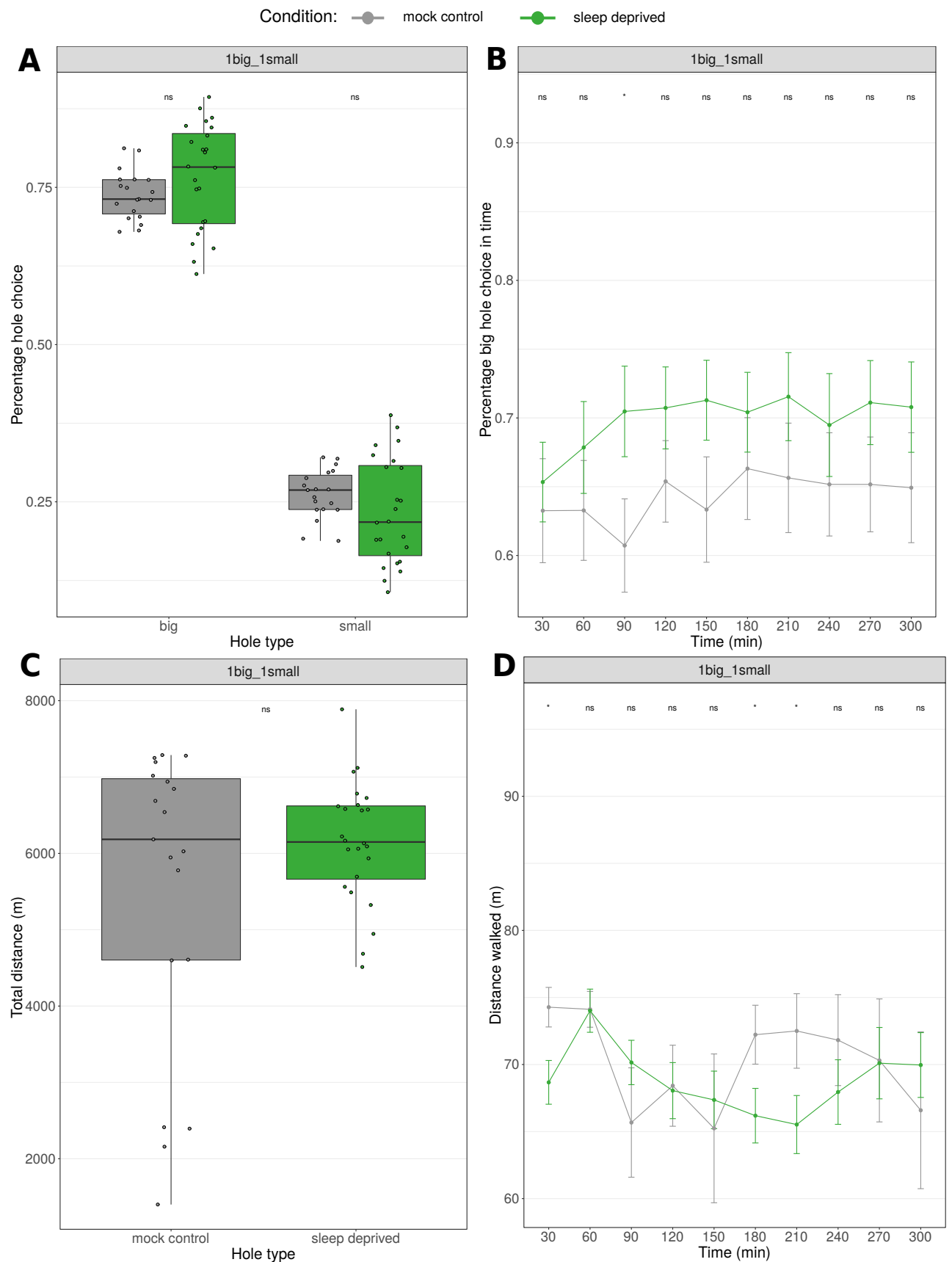


Figure 3.15: 1 night sleep deprived flies vs control, hole preference and distance walked in the 1big_1small maze **A** Percentage of big hole choice in the sleep derived vs control group, and percentage of small hole choice in the sleep deprived vs control group. Percentage big hole + percentage small hole = 1 **B** The percentage of the number of passages through the big hole out of the total number of passages made every 30 minutes. **C** Total distance walked in the 1big_1small maze during the entire experiment. **D** Distance walked after each bout of 30 minutes. $N_{\text{flies}} > 24$

3.4.1.2 3DAYS SLEEP DEPRIVED FLIES

In the previous section, the results show that one night of sleep deprivation does not have any impact on the way in which flies perceive or can distinguish between the small and the big holes. Sleep deprived flies explored the square maze in the same way as the mock control during the entire length of the experiment. Therefore, in the context of the mini square assay flies were able to cope easily with one night of sleep deprivation. Following on anthropomorphic way of thinking, students often end up doing an all-nighter learning session before exams, however they can succeed rather well. Therefore, they are able to cope with the sleep deprivation coming from one night of no sleep during the exam time, when they have to be very focused. However, after several consecutive nights of sleep deprivation, students will not be able to cope with their exams in the same way, as more serious mental effects start to be present like perceptual illusions and hallucinatory episodes, which greatly decreases their focus.^{149,150} In order to test if flies perception of the holes and their behaviour in the 1big_1small mini square assay can be influenced by prolonged sleep deprivation, we decided to sleep deprive them for three consecutive days and nights, so for a total of 72 hours.

For this experiment flies were loaded in individual tubes at the pupa level in order for them to be socially naive. Three days old males were loaded in the ethoscopes and monitored for 2 baseline days (from day -2 to day 0 in Figure 3.16) before starting the 3 days sleep deprivation which started at ZT0 and ended up at ZT0 after 3 days (from day 0 to day 3 in Figure 3.16). Following the sleep deprivation, flies were loaded in the mini square assays and they were able to explore the arena for 5 hours.

Figure 3.16 shows the population (3.16 A) and individual sleep of each fly (3.16 B) before the experiment. Monitoring the individual sleep of each fly allowed us to only keep flies that were completely sleep deprived during the three days and exclude the flies for which the sleep deprivation did not work properly. Poor sleep deprivation arrived in a few cases for which the motor that turns the tube to wake up the fly failed.

Similarly to the previous section, we started by looking at the total number of passages made through all the available doors and through each particular door (big or small hole). Again, there is no significant difference between the control and 3 days sleep deprived group of flies in total amount of crossings (Figure 3.17 A). The prolonged sleep deprivation does not affect the number of passage through the hole at any moment in time, as the average number of passages initiated every 30 minutes by the control and sleep deprived group are not significantly different for the entire length of the experiment (Figure 3.17 B). Also, sleep deprived flies kept their preference for the big hole throughout the whole experiment similarly to control flies (Figure 3.17 C).

Three days of sleep deprivation did not have any impact on the overall average proportion of choice between the big and the small holes (Fig. 3.18 A). The fraction of big holes chosen every 30 minutes by sleep deprived flies was constant during the 5 hours of experiment and not significantly different from control (Fig. 3.18 B). Moreover, their overall activity was similar. The sleep deprived group walked the same average total distance in 5 hours of experiment as the control group and their average distance walked by bouts of 30 minutes was not different from control at any moment in time.

We further looked at the architecture of flies's sleep during the experiment. Both control and sleep deprived flies have very few bouts of sleep, less than 2-3 bouts on average (Fig. 3.19 C). Their average longest bout of sleep in the mini square maze arena is approximately 30 minutes and their mean bout length is less than 10 minutes. Given the 5-minutes rule used to assess sleep, which scores sleep as a period of complete

rest that is longer than 5 minutes, it is clear that flies are active during the experiment.

Again, a principal analysis component was conducted as described in section 3.4.1.1, but no space was found that will separate the control and sleep deprived groups of flies based on the variables measured in the experiment.

These results suggest that the sleep deprived group of flies are able to cope perfectly with the new environment and are able to perceive the size of the holes as well as control flies. This suggests that a three night sleep deprivation has a little weight and does not modulate the initiation of a passage in an easily perceivable way. Given the very subtle effect of sleep deprivation on the hole choice, a larger amount of decisions might be useful in capturing that effect. In contrast with flies's behaviour in DAM tubes, where a sleep rebound is always observed following sleep deprivation, in the mini square mazes, sleep deprived flies do not rebound in the 5 hours of experiment following sleep deprivation. The reasons for the lack of sleep rebound could be either the arousal of the new environment, the smaller size of the space, as the mini square is slightly smaller than the DAM tubes and they have a different shape or the presence of the holes.

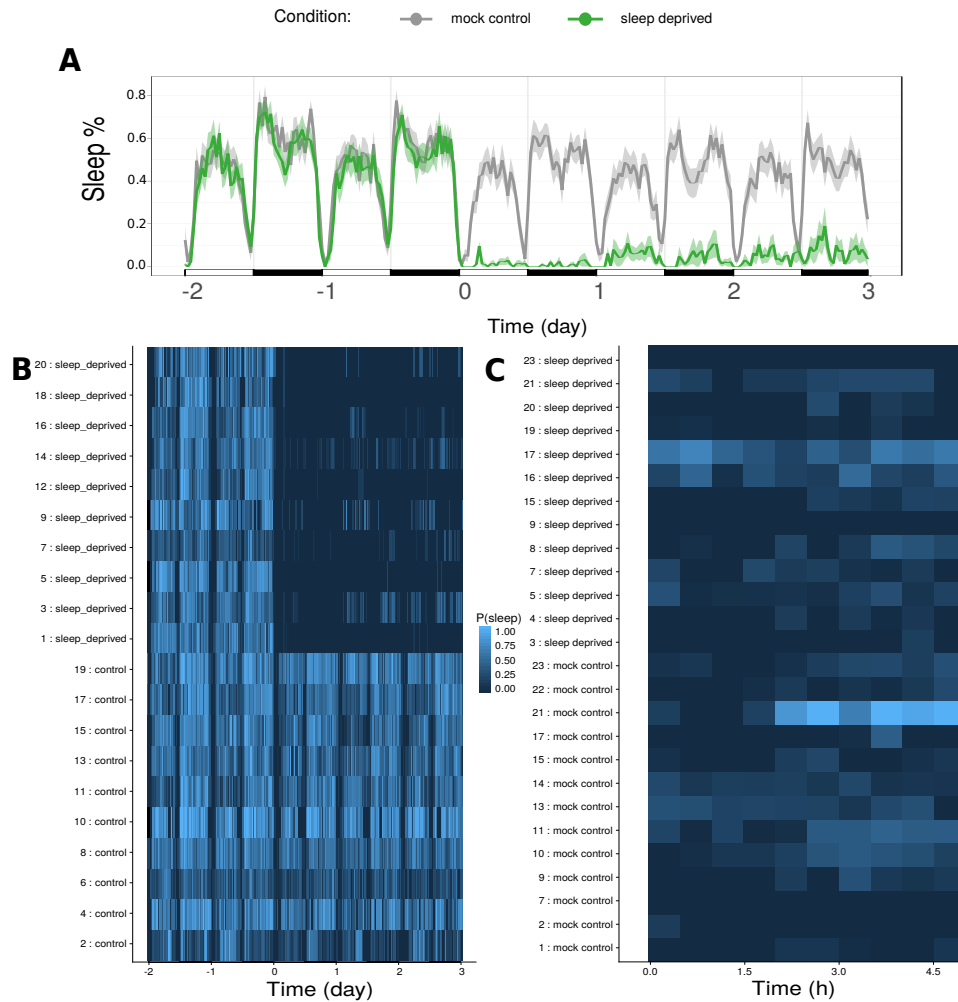


Figure 3.16: 3 days sleep deprived flies vs control, population and individual sleep profiles **A** Population sleep in control and sleep deprived flies before experiment. Sleep deprivation starts at day 0 (ZT0) and finishes at day 3 (ZT0). First two days are baseline days (from day -2 to day 0). **B** Sleep distribution of individual flies before experiment. Each row represents one fly either in the control or sleep deprived groups and their sleep amount every 30 minutes. Sleep amount is represented by a colour intensity, as specified in the legend. **C** Sleep distribution of individual flies during the 5 hours experiment. Each row represents one fly either in the control or sleep deprived groups and their sleep amount every 30 minutes. Sleep amount is represented by a colour intensity, as specified in the legend. $N_{\text{flies}} = 24$

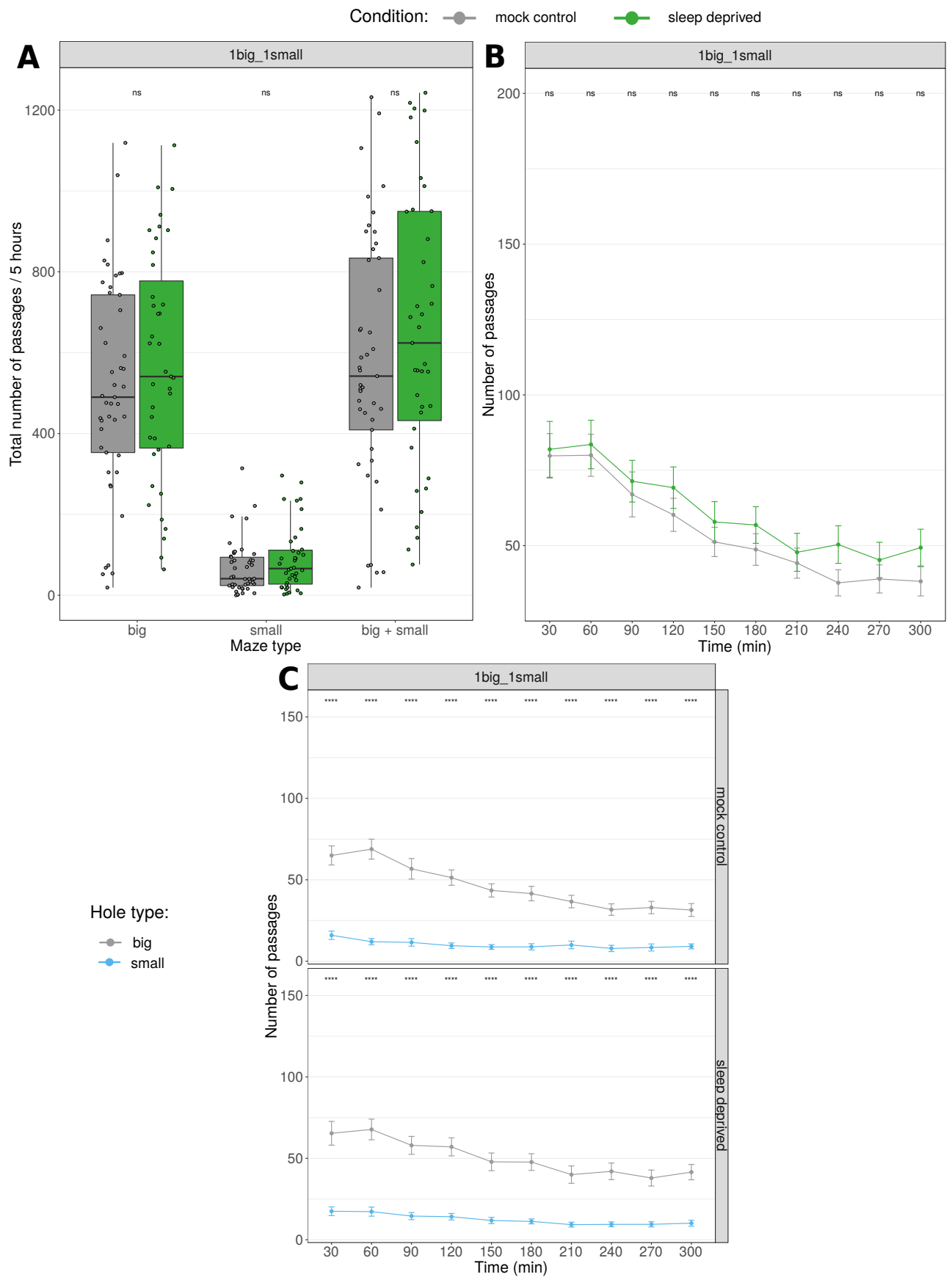


Figure 3.17: 3 days sleep deprived flies vs control, number of passages **A** Number of passages through each hole type (big or small) and total number of passages (big + small) made during the 5 hours of experiment. **B** Number of passages made every 30 minutes through all the available doors in the 1big_1small mini square maze **C** Number of passages made every 30 minutes through each individual hole type of the 1big_1small maze. $N_{flies} > 24$

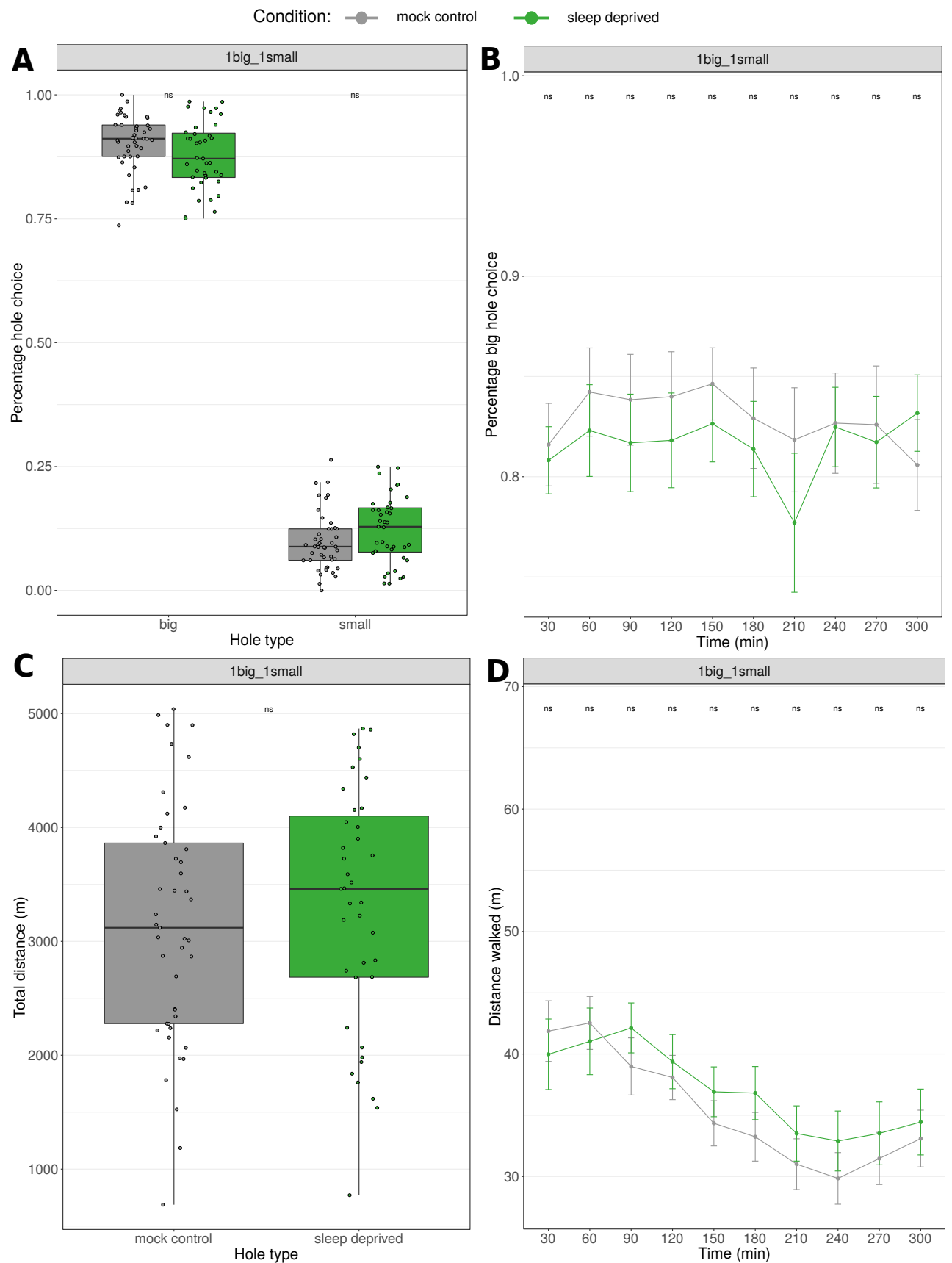


Figure 3.18: 3days sleep deprived flies vs control, hole preference and distance walked in the 1big_1small maze **A** Percentage of big hole choice in the sleep derived vs control group, and percentage of small hole choice in the sleep deprived vs control group. Percentage big hole + percentage small hole = 1 **B** The percentage of the number of passages through the big hole out of the total number of passages made every 30 minutes. **C** Total distance walked in the 1big_1small maze during the entire experiment. **D** Distance walked after each bout of 30 minutes. $N_{\text{flies}} > 24$

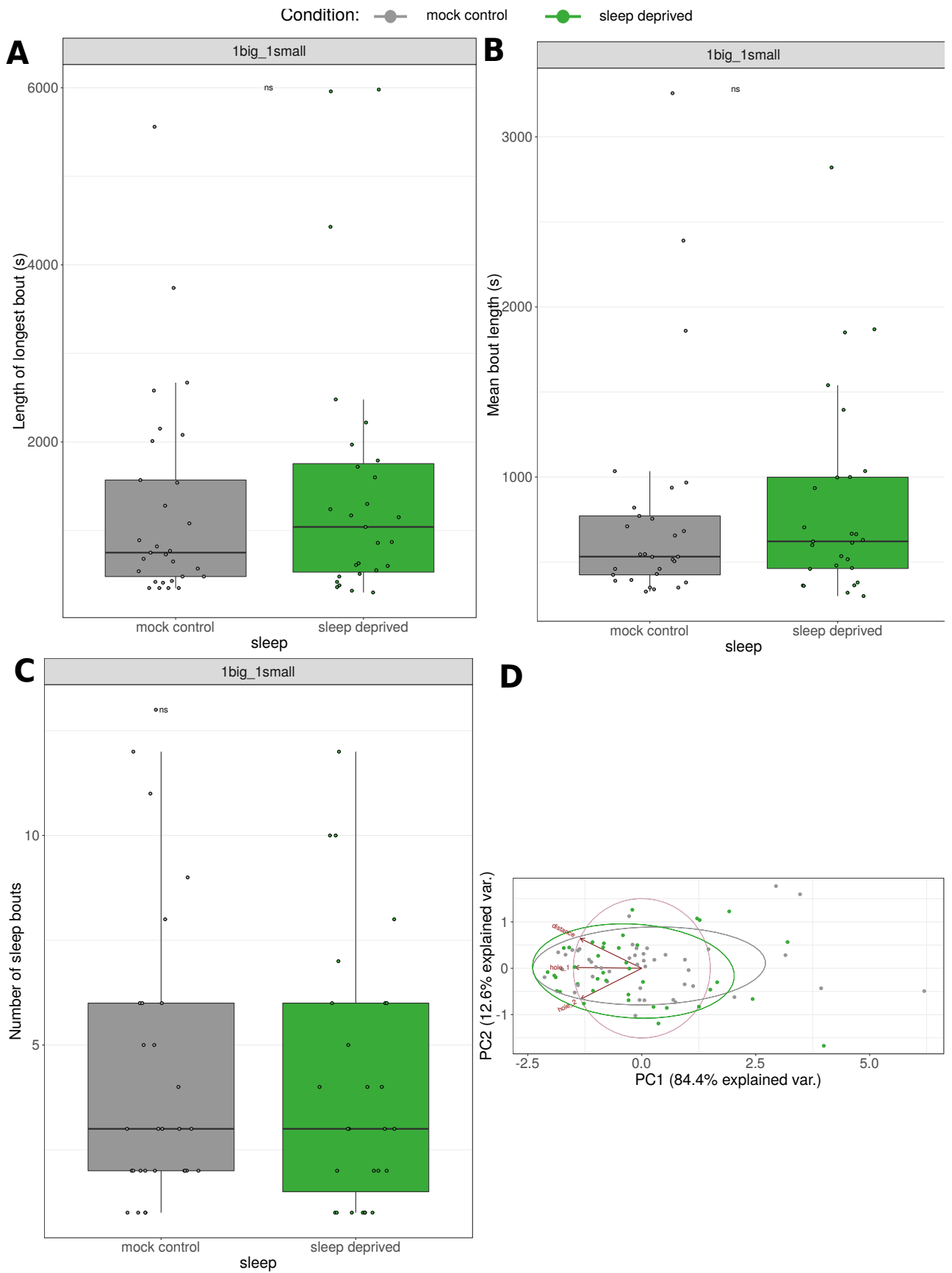


Figure 3.19: 3 days sleep deprived flies vs control, sleep architecture and PCA analysis in the 1big_1small maze **A** Length of the longest sleep bout that was made during the entire length of the experiment by either the control or the sleep deprived group of flies. **B** The mean sleep bout length. **C** Total number of sleep bouts. **D** Principal component analysis using a singular value decomposition which examines the covariances / correlations between individuals. Each individual fly is represented by 3 variables: the number of passages made through the big hole, the number of passages made through the small hole and the total distance walked. $N_{\text{flies}} > 24$

3.4.1.3 CIRCADIAN EFFECT ON HOLE CHOICE

In the previous sections, flies sleep was disturbed using either mechanical or dynamical sleep deprivation for either a short or a long period of time, but no effects were observed on their hole preference choice in the mini square arena. Similar to wild type, 100% of the sleep deprived flies choose to pass through the big hole, more than the small hole, suggesting again the robustness of the mechanisms in charge of generating an action in the face of internal changes. When looking at how external conditions influence the initiation of passages it was obvious that light, the size of the holes and the shape of arenas all had a concrete effect on the number of passages and the proportions with which holes are chosen. Interestingly, sleep deprivation does not have any noticeable effect that can be captured using our current setup. Results show that flies are able to cope very well with sleep deprivation during the experiments, as they are actively exploring the maze, without falling asleep more than control. The homeostatic regulation of sleep mechanism is therefore either delaying or suppressing completely the sleep rebound which normally happens straight after a sleep deprivation period. Given that the homeostatic regulation of sleep was very adaptable to the new environment and sleep deprivation did not have a perceivable effect on the flies behaviour we further decided to investigate whether disturbing the circadian clock component of sleep will have any effect on the flies activity and choices in the mini square maze.

There is a balance in the flies behaviour in the assay, they make passages through both the big and the small hole, and there is robustness in their unanimous preference for the big hole. Any changes in this balance and in this robustness can reveal insights into the mechanism in charge of initiating a passage and its link with the circadian clock. A fly that will choose only the big hole, a fly that will choose equally between the big and the small holes, or even a fly that will choose the small hole more than the big hole are all good examples of imbalances in the choice behaviour, deviates from the "normal" that might be potential models for a depressed or a schizophrenic fly.

In this section, a *Drosophila* behaviourally arrhythmic mutant fly, *CLK^{out}*, was used to investigate the link between the hole preference and the circadian clock. The mutant has a deletion in the CLK gene known to be involved in the circadian rhythms¹⁵¹. The choice of this mutant was motivated by a few studies in mice and humans that show a link between the clock gene and bipolar disorder^{152,153,154}. The bipolar disorder is characterised by episodes of either mania or depression, which show either great excitement in the case of mania or a lack of excitement in the case of depression. At the neurochemical level bipolar disorder is characterised by imbalances in the homeostatic regulation of dopamine. It has been shown that dopamine also regulates the motivation to act in general¹⁵⁵ therefore I hypothesis that there might be a link between the CLK gene and the initiation of passages.

For this experiment CLK mutants and CS flies were loaded in the ibig_small maze at ZTo and placed in the incubators in dark. They were video recorded for 5 hours. Only males flies were used, taken straight from the vials in which they grew using a mouth aspirator (also called pooter) and without being anaesthetized.

Figure 3.20 presents the number of passages done by the CLK and CS flies. CLK mutant flies made significantly less passages than CS flies during the 5 hours of the experiment. Their total number of passages through all available doors is smaller than wild-type, but also the number of passages done through the big hole and the number of passages through the small hole are both significantly lower than CS flies (Fig. 3.20 A). Plotting the number of passages done by both groups of flies every 30 minutes is more informative.

In the first bout of 30 minutes the CLK mutants made more passages through the holes than the CS flies, however their number of passages drops almost linearly in time thereafter. There is no significant difference between CLK and CS in the second bout of 30 minutes of movement, but the number of passages continues to drop and passed that moment CLK mutants make regularly less total number of passages through all available doors throughout the rest of the experiment (Figure 3.20 B).

It is interesting to observe the pronounced difference in the number of passages made by the CLK flies at the beginning of the experiment and at the end of the experiment. If at the beginning of the experiment they made on average 150 passages in 30 minutes, their crossings reduced in an almost linear fashion to less than 20 passages in a 30 minutes bout, by the end of the last hour of experiment. Opposite to them, the CS flies kept a very constant pace during the entire length of experiment making on average between 100 and 120 passages every 30 minutes. (Figure 3.20 B)

CLK flies are able to distinguish between the big and the small holes at the beginning of the experiment, but not towards the end (Figure 3.20 C). If for the first 3 hours of experiment the number of passages through the big holes is significantly higher than the number of passages through the small holes, in the last 2 hours of experiment there are two bouts in which there is no significant difference between the passages through the two hole types. However, as observed in Figure 3.20 B, in the last hours of experiment the total number of crossings in CLK mutants is significantly reduced. The smaller number of passages is probably not able to capture the effect of the size of the hole on the initiation of passages, which will explain why there was no significant difference between the small and big holes in the last two hours of experiment. CS flies make on average 90 crossings through the big hole and 40 crossings through the small hole, every 30 minutes until the end of the 5 hours of experiment (Figure 3.20 C).

CLK mutants prefer the big hole to the small hole more than CS flies as on average the percentage of passages through the big hole out of the total is significantly higher than in CS flies. Complementary to this the percentage of passages through the small hole is significantly less for CLK mutants than for wild-type flies (Figure 3.21 A). Therefore, the genetic background has a noticeable effect on the initiation of a passage, by modulating the proportion with which flies choose the two holes. The weight of the genetic background on the initiation of a passage is large enough to be perceivable. By looking at how the proportion of big holes out of the total varies in time for the two groups of flies, we observe that for the first 1.5 hours of experiment there is no significant difference, therefore CLK flies choose the big hole in the same proportion to the small hole as the CS flies. Thereafter, there are three bouts in which the CLK flies choose significantly more the big hole than the CS flies. The increased preference for the big hole comes in the same time as the drop in the number of crossings. The small number of passages in the last part of the experiment might not be big enough to average out the randomness specific to each initiation of action. If this is true, it's possible that the randomness outweighs the size of the hole input in the initiation of an action, moving the percentage of the big hole away from the true expected value.

Similarly to the number of passages, the total distance walked in 5 hours is also greatly reduced in the CLK mutants compared to CS (Figure 3.21 C). Following the same pattern as the number of passages in time, CLK mutants are more active during the first 30 minutes of experiment than CS flies. Again, an almost linear drop in the distance walked takes place until the last hour of experiment when CLK mutants walk very little on average.

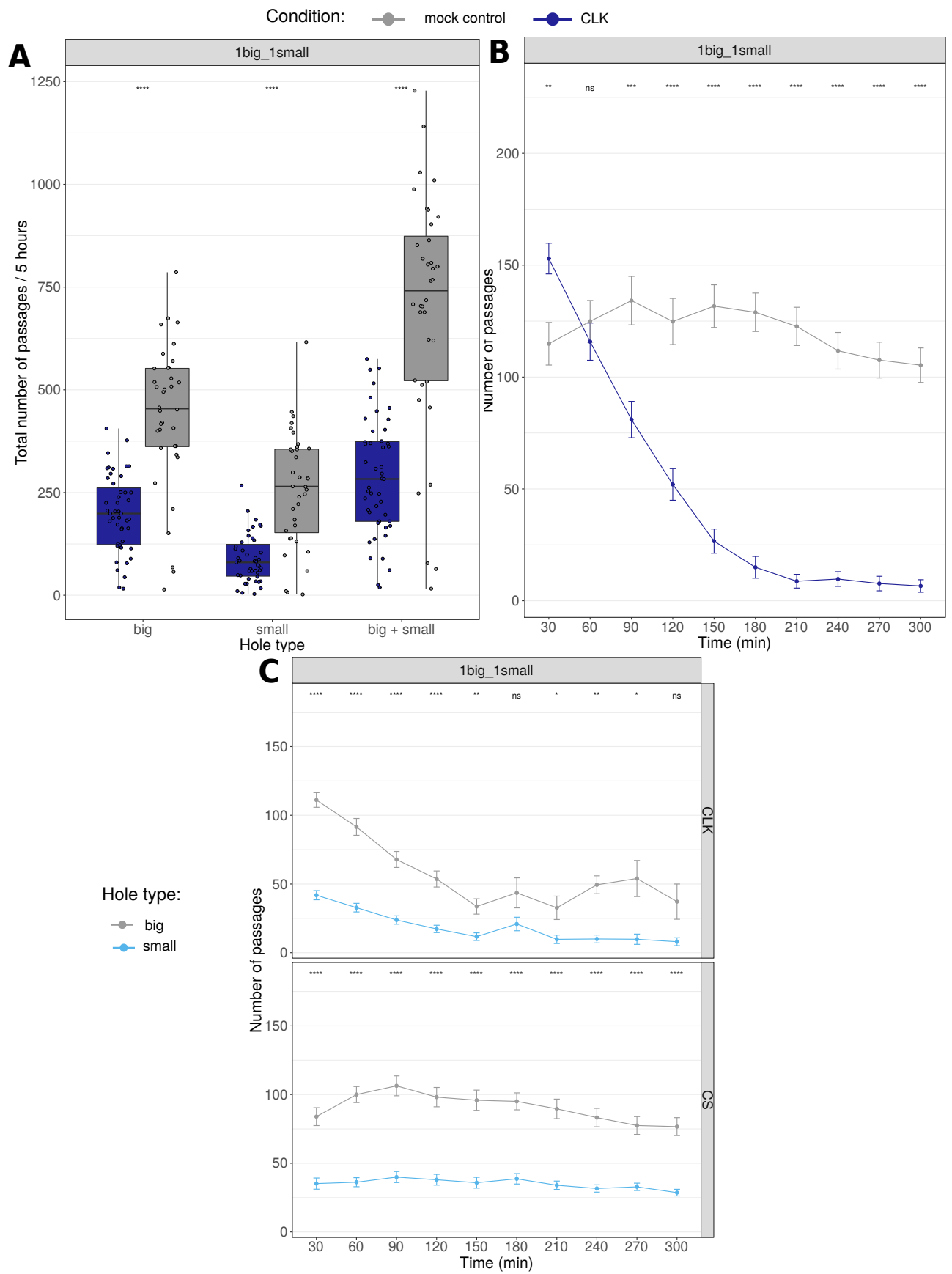


Figure 3.20: CLK mutants vs CS wild-type in the 1big_1small mini square maze, number of passages **A** Number of passages through each hole type (big or small) and total number of passages (big + small) made during the 5 hours of experiment. **B** Number of passages made every 30 minutes through all the available doors in the 1big_1small mini square maze **C** Number of passages made every 30 minutes through each individual hole type of the 1big_1small maze. $N_{flies} > 24$

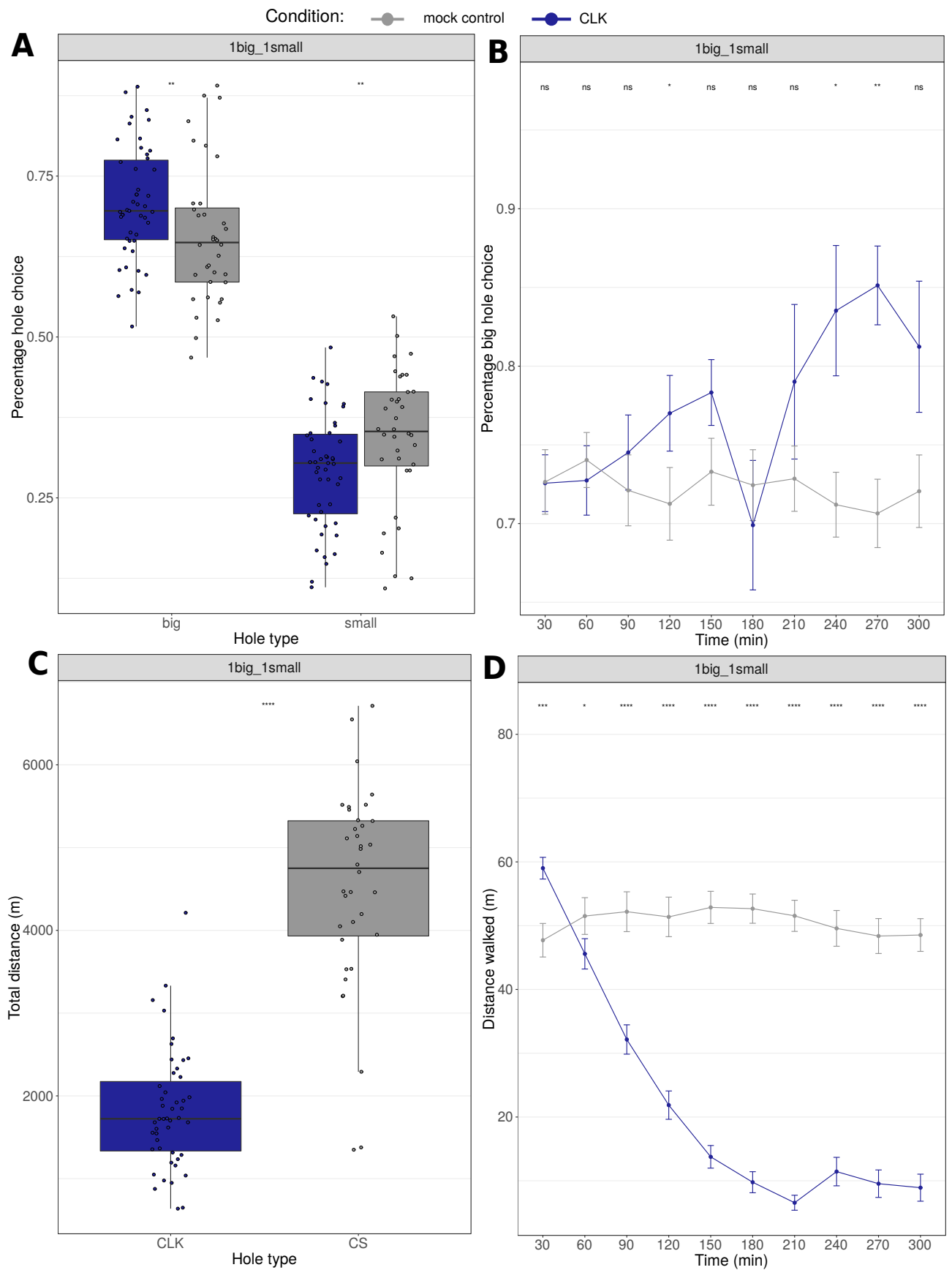


Figure 3.21: CLK mutants vs CS wild-type in the 1big_1small mini square maze, hole preference and distance walked **A** Percentage of big hole choice in the CLK vs CS flies, and percentage of small hole choice in the CLK vs CS flies. **B** The percentage of the number of passages through the big hole out of the total number of passages made every 30 minutes. **C** Total distance walked in the 1big_1small maze during the entire experiment. **D** Distance walked after each bout of 30 minutes. $N_{\text{flies}} > 48$

3.4.1.4 EFFECT OF THE TIME OF THE DAY

The results from the previous section show a different behaviour of the CLK flies compared to wild-type (CS): they are more active at the beginning of the experiment and they experience a drop in their activity, being very quiet in the last hour of experiment. The CS flies are stable and constant in their interaction with the maze during the 5 hours of experiment. The balance of wild-type flies seems to be lost in the CLK mutants whose activity reaches two extremes: a very active episode that slowly progresses into a very quiet episode. CLK mutants are known to have deficit locomotor behaviour as the circadian rhythms are responsible for regulating the walk-rest cycles. To separate the effect of the circadian mutation on the hole choice behaviour from the effect of the circadian rhythm itself, we decided to run an experiment at a different time of the day, in the afternoon.

Experiments were run similarly to section 3.4.1.3 but they started at ZT6 and stopped at ZT11.

In the afternoon the CLK mutants made significantly more passages through the small hole, but the total number of passages through the big and small hole were not significant from the mock control (CS) (Figure 3.22 A). The number of passages made by bouts of 30 minutes is very similar between the CLK and CS groups, being slightly different in the first 30 minutes of experiment and at one point after 3.5 hours when CS flies make slightly more passages than CLK mutants. The total number of passages drops in time, and the shape of the descent is very similar between the two groups (Figure 3.22 B).

CLK mutants are able to distinguish the big hole from the small hole, making more passages through the big hole at all times during the experiment (Figure 3.22 C).

Overall, the number of passages of CLK mutants lean more towards the big hole than for CS flies, as they choose the big hole in a significantly greater percent out of the total number of passages (Figure 3.23). The proportion of big holes chosen every 30 minutes is slightly above for CLK flies compared to CS at several moments in time, however is significantly different only after 1 hour and after 3.5 hours of experiment.

CLK and CS flies seem to respond similarly to the maze in the afternoon, as their activity is very much alike. The total distance walked by the two groups is not significantly different. By assessing the distance walked after 30 minutes we notice that the activity of both CS and CLK mutants decreases quite drastically in time, following the same pattern. CLK mutant flies walk significantly more than CS flies in the first bout of movement, and they also walk significantly less in their last bout of movement.

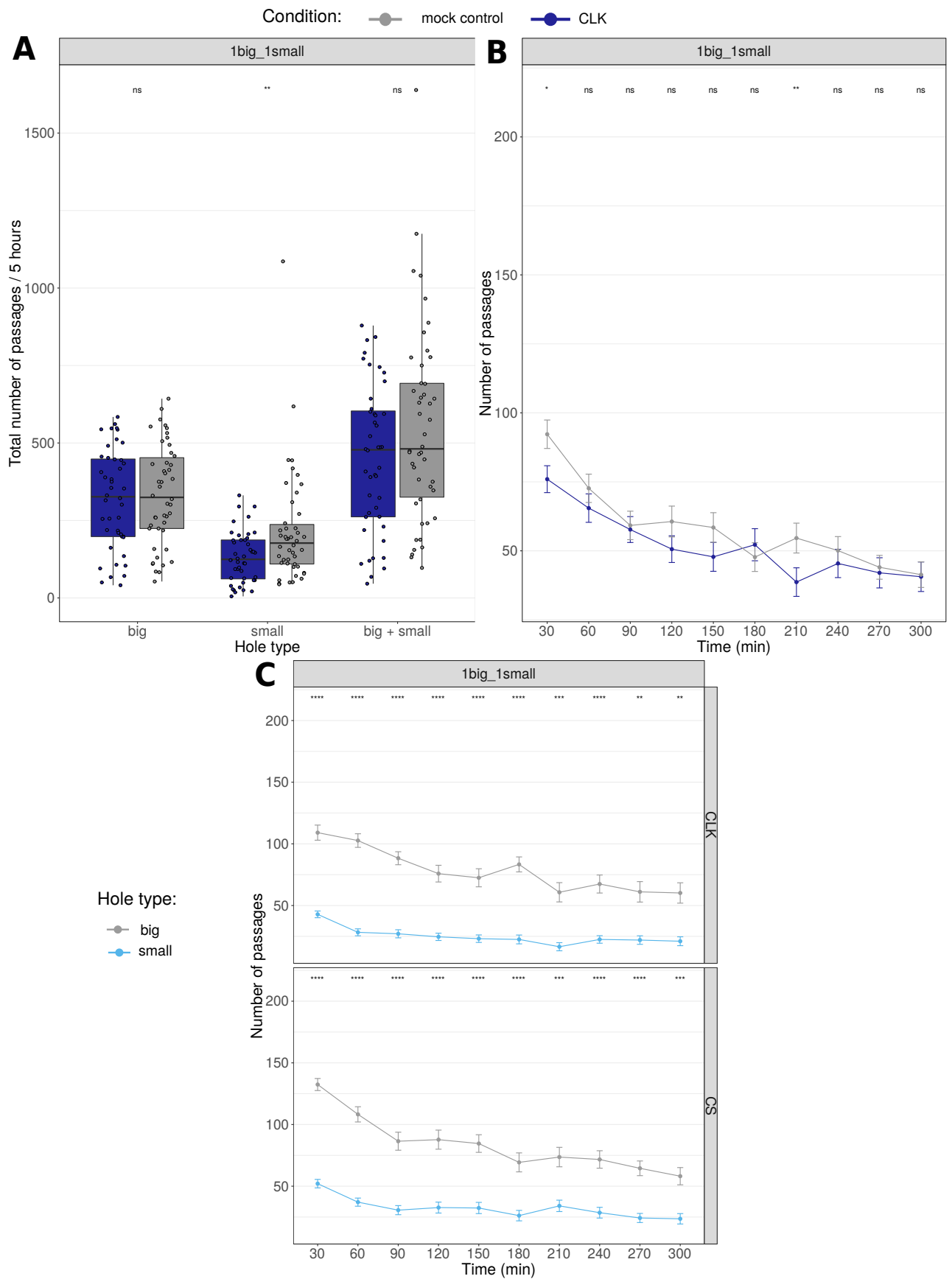


Figure 3.22: CLK mutants vs CS wild-type in the afternoon, number of passages **A** Number of passages through each hole type (big or small) and total number of passages (big + small) made during the 5 hours of experiment. **B** Number of passages made every 30 minutes through all the available doors in the 1big_1small mini square maze **C** Number of passages made every 30 minutes through each individual hole type of the 1big_1small maze. $N_{flies} > 48$

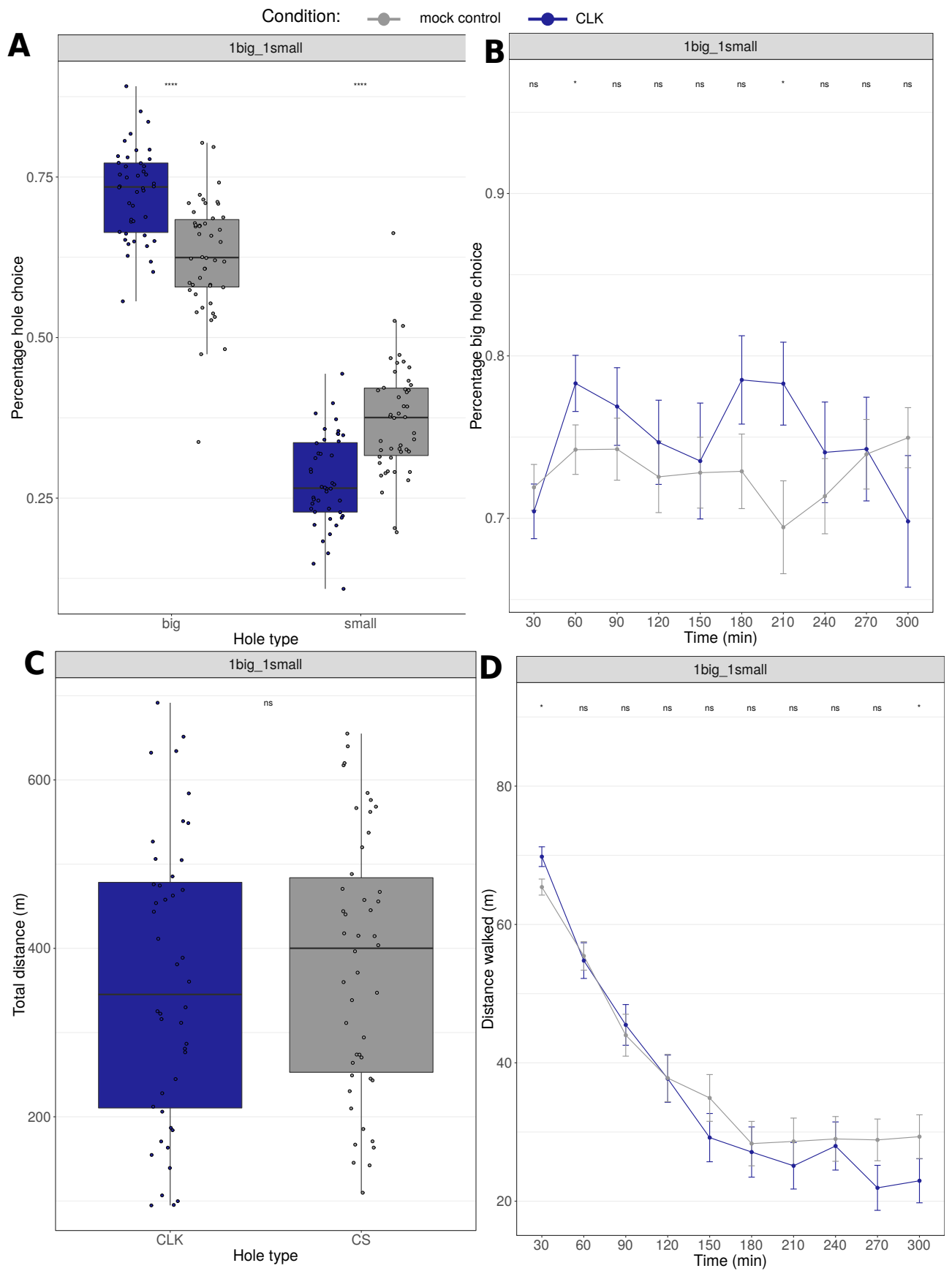


Figure 3.23: CLK mutants vs CS wild-type in the afternoon, hole preference and distance walked **A** Percentage of big hole choice in the CLK vs CS flies, and percentage of small hole choice in the CLK vs CS flies. **B** The percentage of the number of passages through the big hole out of the total number of passages made every 30 minutes. **C** Total distance walked in the 1big_1small maze during the entire experiment. **D** Distance walked after each bout of 30 minutes. $N_{\text{flies}} > 48$

The time of the day impacts the response of both CLK and CS flies to the 1big_1small maze type. The two groups have a very different behaviour in the morning and a much more similar one in the afternoon. To better understand how the time of the day impacts on flies behaviour, the results from this section were merged with the results from section 3.4.1.3 and a comparison between the morning and afternoon activity was made for each genotype.

Both groups show a significant difference in the amount of passages made in the morning vs afternoon. On one side CLK mutants increase their total amount of passages in the afternoon compared to morning, and also the amount of passages through individual holes making more passages through the small hole and more passages to the big hole in the morning than in the afternoon experiment (Figure 3.24 A, top panel). On the other side CS flies decrease their total amount of passages in the afternoon compared to morning, and they also decrease their passages to each individual hole (Figure 3.24 A, bottom panel).

Plotting the number of passages made every 30 minutes reveals that CLK mutants made similar amount of passages in the morning vs afternoon, in the first half of the experiment. In this first 2.5 hours of experiment, the amount of passages dropped in the same way both in the morning and in the afternoon. The second half of experiment (the last 2.5 hours) CLK mutants made significantly more passages in the afternoon than during the morning. The CS flies which were very constant in their number of crossings in the morning experiment show a behaviour similar to CLK flies in the afternoon, making more passages in the first bout of 30 minutes and slowly dropping the number of crossings afterwards, but with a smaller slope than CLK mutants.

Looking at the proportion with which flies choose the big and the small holes reveals that flies of the same genotype are constant in their preference, no matter what the time of day is. In Figures 3.21 A, B and 3.23 A,B, presented previously, we've seen that there is a significant difference between the CS and CLK mutants in their hole preference both in the morning and in the afternoon. Figure 3.24 C, D melt previous results and show that there is no overall significant difference in the percentage of the big hole choices out of the total passages within the same genotype. CLK flies choose the big hole in the same percentage in the morning vs afternoon at every 30 minutes bout. Same for the CS flies.

Distance and velocity are under the influence of the circadian rhythm. For the CS flies the total distance walked during the whole experiment is lower in the afternoon than in the morning, and for the CLK mutants the opposite arise as they walk more in the afternoon than in the morning. By looking at the distance walked every 30 minutes bout we can see that it follows a similar pattern to the number of passages crossed in time. Therefore CS flies walk more in the first 30 minutes bout in the afternoon than in the morning. Their distance walked progressively drops afterwards and flies walk significantly less in the afternoon than in the morning. CLK flies cover a greater distance at any moment in time in the afternoon compared to corresponding bout of movement from the morning.

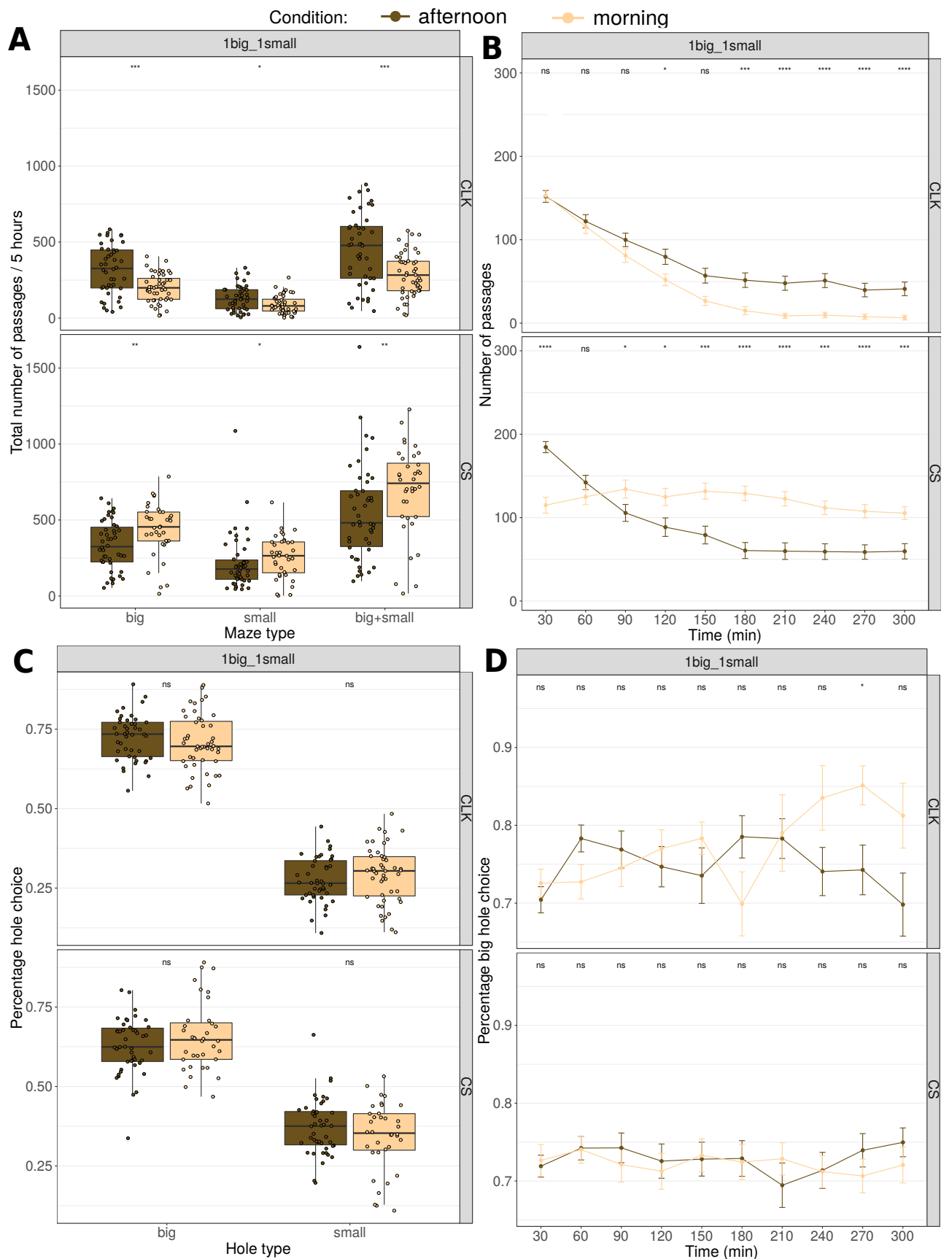


Figure 3.24: Morning vs afternoon, number of passages and hole preference Morning experiments ran from ZT0 to ZT5 and afternoon experiments ran from ZT6 to ZT11. Top panels show results for CLK mutants flies, and bottom panels show results for CS flies. **A** Number of passages through the big hole, small hole and both holes together (big + small) in the morning vs afternoon. **B** Total number of passages through all the available doors made every 30 minutes. **C** Percentage of big hole choice in the morning vs afternoon. Percentage(small) = 1 - percentage(big) is also showed. **D** Percentage of the number of passages through the big hole out of the total number of passages made every 30 minutes. $N_{\text{flies}} > 48$

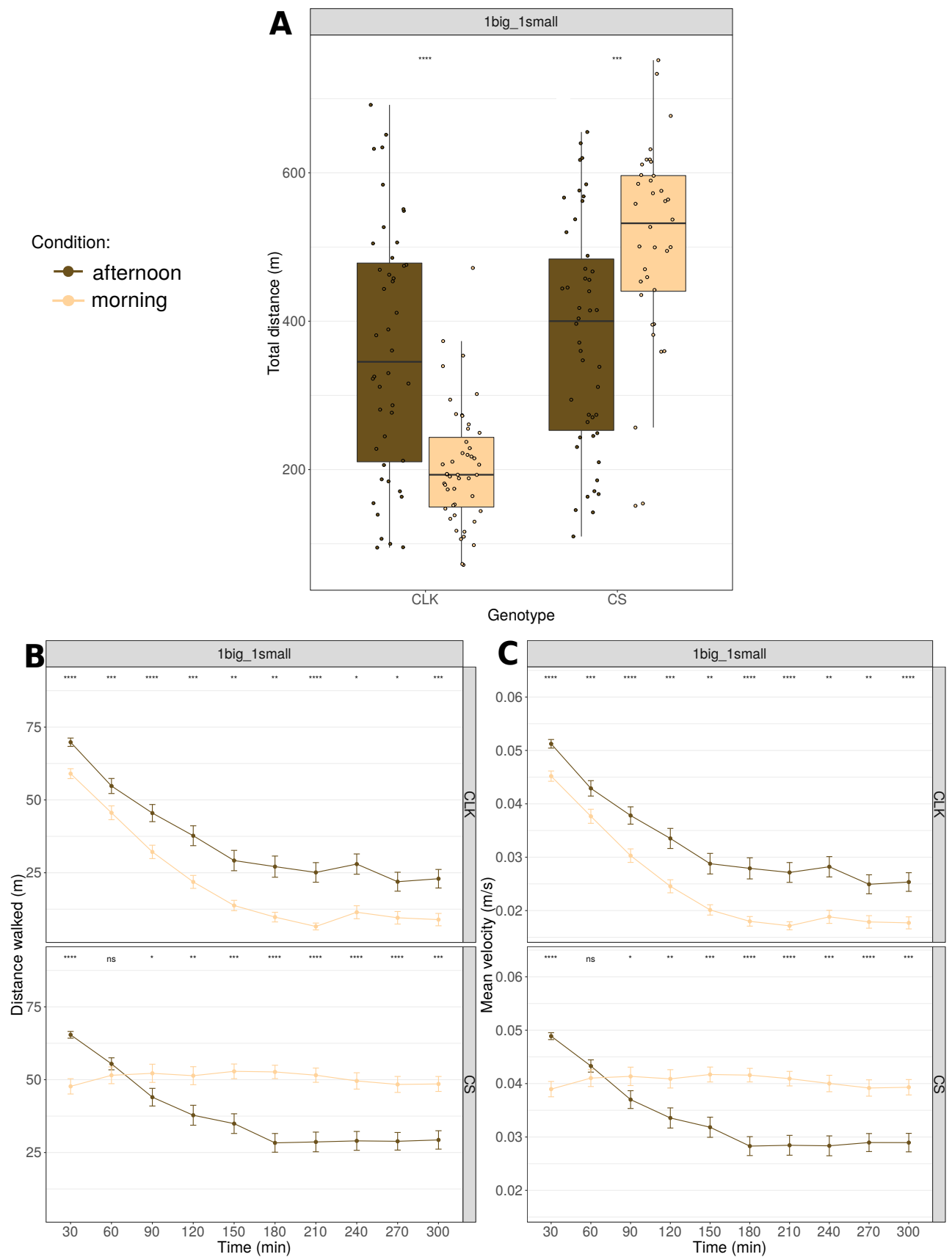


Figure 3.25: Morning vs afternoon, distance and velocity Morning experiments ran from ZT0 to ZT5 and afternoon experiments ran from ZT6 to ZT11. **A** Total distance walked in morning vs afternoon experiments in both CLK and CS flies. **B** Distance walked by bouts of 30 minutes in the morning vs afternoon. Top panel shows results for CLK flies and bottom panel shows results for CS flies. **C** Mean velocity by bouts of 30 minutes in the morning vs afternoon. Top panel shows results for CLK flies and bottom panel shows results for CS flies. $N_{\text{flies}} > 48$

3.4.1.5 HOW MUCH SLEEP DO FLIES GET DURING THE EXPERIMENT?

In the experiments that assessed the behaviour of flies following 1 night of sleep deprivation (Sec. 3.4.1.1) or 3 consecutive days of sleep deprivation (Sec. 3.4.1.2), no sleep was recorded during the 5 hours experiment in the *ibig_small* assay, even if the experiments overlapped with the period in which flies normally recovered their lost sleep after being sleep deprived^{104,65}. Flies were therefore able to suppress their usual homeostatic response for the time of the experiment. The afternoon experiments overlap with the "siesta" of the flies which is usually centred around ZT6¹⁵⁶, and which represents the largest amount of usual daytime sleep in CS flies. Given the significant differences observed in distance, velocity and number of passages in both CS and CLK flies in the morning vs afternoon experiments, the obvious question to ask is how do flies respond to the maze when under the sleep pressure due to the circadian clock in the afternoon. In this section, an analysis of the sleep profile of flies during both morning and afternoon experiments is made in order to better understand the changes observed in their physiological parameters and hole choice behaviours.

Figure 3.26 A shows the sleep profile at the population level during the experiment. In the afternoon, both CLK mutants and CS flies have no sleep at the start of the experiment, but gradually increase their sleep in the first half of the experiment, and they spend half of their time sleeping on the second half of the experiment. In the morning, it is clear from the bottom panel of Figure 3.26 A that CS flies have no sleep at all, but the CLK mutants are active at the start of the experiment following which they linearly increase their sleep, spending more than 75% of their time sleeping in the last 2.5 hours of experiment.

Figure 3.26 B shows a few examples of the sleep profiles at the individual level in the morning experiment. The colour shades represent different amounts of sleep that flies have per 30 minutes bout and indicate that each individual fly of both genotypes is always awake in the first hour of the experiment, with most of the CS flies being awake until the end of the experiment. Exceptions are flies from region 6 and 7 which show a small amount of sleep. Most of the CLK mutants go to sleep in the last 2 hours of experiment, except the individuals from region 5, 10 and 13 who show a similar behaviour to CS flies, being awake the entire duration of the experiment.

In the afternoon, it is not possible to distinguish visually, between the two genotypes by looking at the individual amount of sleep of each fly (Figure 3.26 C). A bit more than half of the flies in the CLK and CS groups will go to sleep in the second half of the experiment. However in both groups there are nearly half of them that choose not to sleep, more precisely 9 out of 22 CS flies with their corresponding ids: 1, 4, 7, 13, 16, 17, 19, 20 24, and 7 out of 20 CLK flies with their corresponding ids: 1, 4, 5, 7, 15, 21, 22.

In order to assess more quantitatively the differences in amount of sleep and sleep architecture we looked at the fraction of time flies spend sleeping, the length of the longest bout of sleep and the mean bout length. As shown in Figure 3.27 A, B, C, in the afternoon, there is no significant difference in the sleep amount and architecture of CS and CLK flies. They sleep on average 30% of their time. In the morning, CLK mutants sleep significantly more than CS flies. They spend on average between 30% and 40% of their time sleeping compared to CS flies which spend on average less than 5% of their time sleeping. The sleep architecture of CLK and CS flies is also similar in the afternoon. Both CLK mutants and CS flies have an average longest sleep bout of around 5000 s (1h 23 min) and an average mean bout length of approximately 2500 s (40 min).

We further investigate what is the effect of the circadian rhythm on each genotype. For the CLK mu-

tants, there is not a significant difference in their sleep and sleep architecture in the morning vs afternoon. They sleep on average between 30% and 40% both in the morning as in the afternoon. The architecture of their sleep is characterised by an average longest bout of sleep of around 5000 s and average mean bout length of around 2500 s. The CS flies have a different sleep and sleep architecture in the morning vs afternoon. If in the morning they have basically no sleep, in the afternoon they sleep on average less than 30% of their time, they have an average longest bout larger than 4000 s and a mean bout length of around 2000s. Therefore, we can observe the effect of the siesta in the *ibig_1small* maze. The circadian rhythm has potentially a stronger weight on the initiation of activity in the fly (locomotion) than the size of the arena.

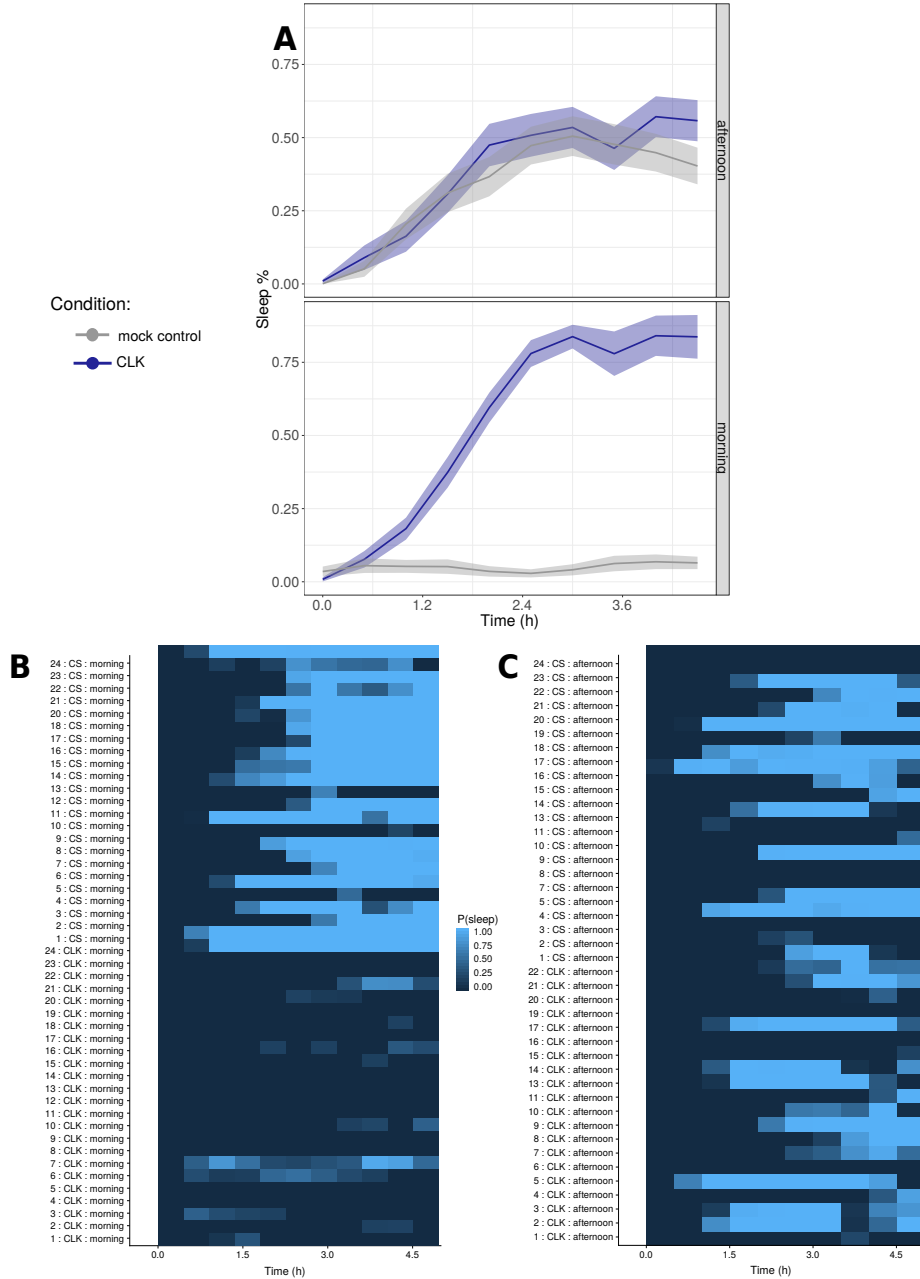


Figure 3.26: Population and individual sleep profiles in the CLK and CS flies during the morning and afternoon experiments **A** Population sleep profile of CLK vs CS flies in the afternoon (top panel) and in the morning (bottom panel) **B** Individual sleep profiles for the morning experiment. Each row represents an individual fly. Y axis indicates the id, genotype and time of the day when the experiment was run. **C** Individual sleep profiles for the afternoon experiment. $N_{\text{flies}} > 48$

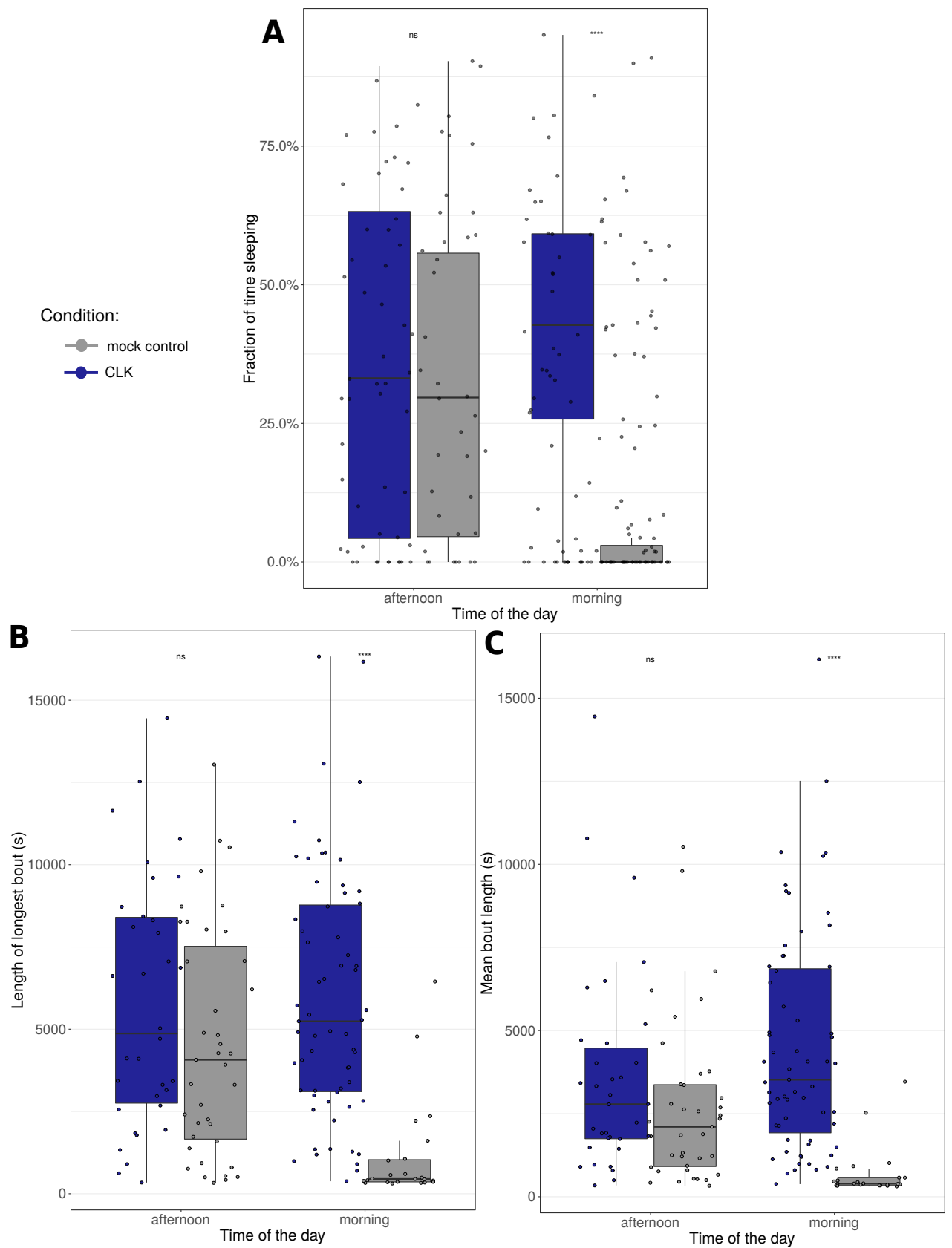


Figure 3.27: CLK vs CS sleep analysis **A** Fraction of time spent asleep during the 5 hours of experiment. **B** Length of the longest sleep bout. **C** Mean bout length.

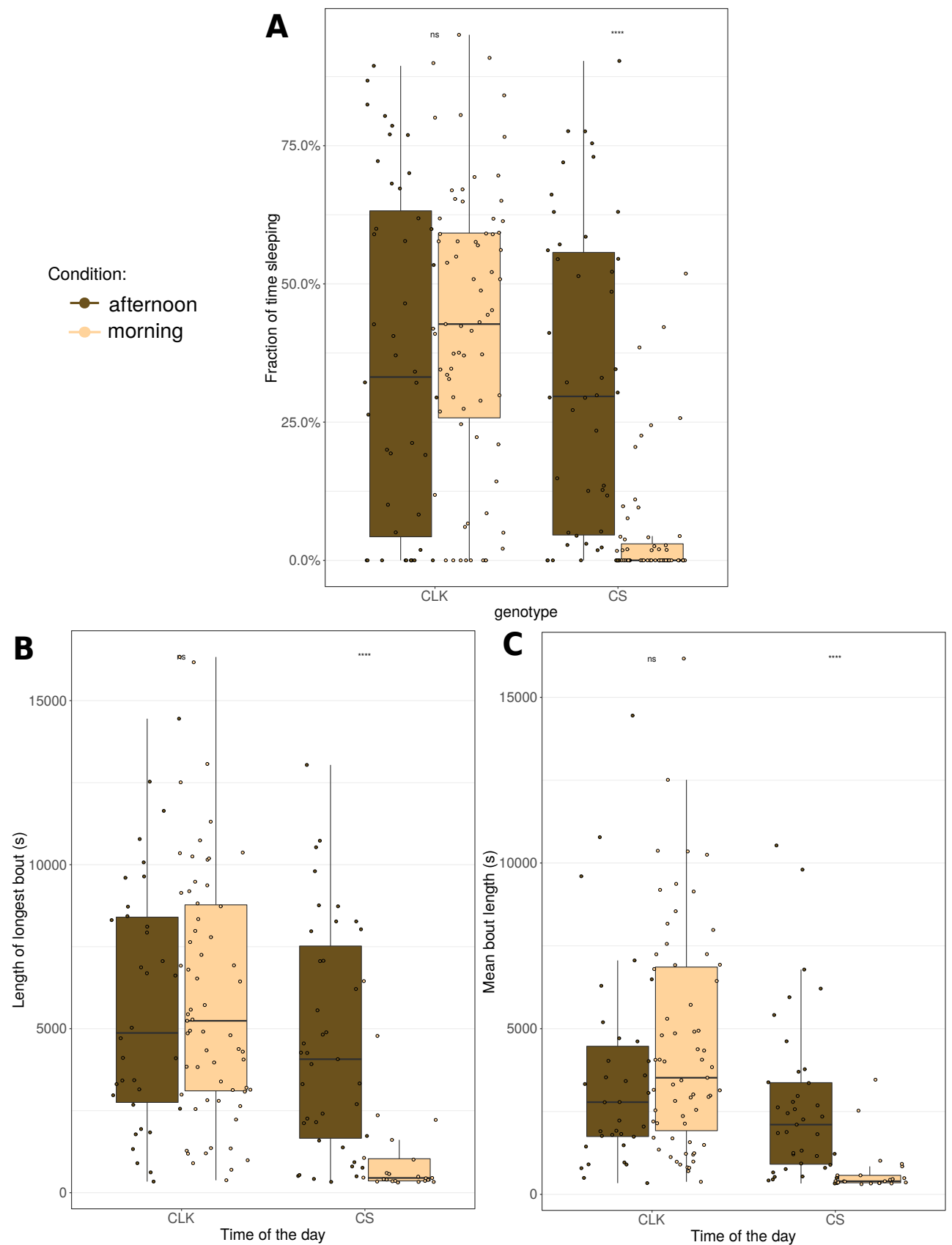


Figure 3.28: Morning vs afternoon sleep analysis **A** Fraction of time spent sleeping during the 5 hours of experiment. **B** Length of the longest sleep bout. **C** Mean bout length.

3.4.1.6 CAN THE NUMBER OF PASSAGES THROUGH THE HOLE BE A MARKER OF SLEEPINESS?

The locomotion of flies consists of a series of activity and inactivity bouts and is modulated by a combination of factors.

From our experiments it was clear that the absence of light increased the locomotion of flies in all the different types of mazes used (Figure 3.12 B). We also confirmed the results saying that locomotion is increased by space reduction¹²⁰ in Figures 3.9 A and B. Also, the time of the day had an impact on wild type flies which in the afternoon walked considerably less than in the morning, but also on the CLK flies (Figure 3.25). This confirms what is already known: that time of the day, size of the arena, genotype, sex, temperature etc. all have an effect on flies locomotion. There is the combination of external and internal variables together with some randomness that decide if the fly should be active or rest. Same must be true for sleep. The weight with which each external and internal variable contributes to put a fly to sleep is definitely context and individual dependent.

From our results we noticed that there is a competition between external pressure and internal states for the activity of the flies. On one hand the small size of the arena together with the absence of light pushes them to be very active, and on the other side there is the sleep pressure that builds up. It is interesting that in the morning, in the sleep deprived flies group, the sleep pressure that presumably built up internally in the flies after the sleep deprivation period, is not strong enough to get them to sleep. It is therefore possible that the arousal that came from being in such a small space outweighs the sleep pressure keeping them awake. However, in the afternoon, the sleep pressure due to circadian rhythms seems to be more powerful as approximately half of the flies fall asleep during the experiments for more than 2.5 hours out of the 5 hours of experiment (Figure 3.26) compared to no sleep at all in the morning experiment in neither the control group nor the 3 days sleep deprived group (Figure 3.16 C).

Is this competition between stimuli reflected in the flies behaviour in an observable way? Can we measure the build up of the sleep pressure? In order to tackle this question I decided to look at the behaviour of individual flies that fall asleep, more precisely at how their activity and number of passages changes in time, especially in the window of time preceding their sleep.

In the initiation of a passage, it makes sense that the activity of a fly has an important weight, as flies that are more active will encounter the holes more often, and therefore they will be faced with the decision of passing or not passing through a hole more often. As the results in the mini square suggest, locomotion and the passages through the holes are highly correlated (Figure 3.29)

I was not able to find in the literature any studies that look at how the activity of animals changes before they fall asleep, in laboratory conditions. In the mini square assay, flies that fall asleep have their sleep mainly organised in one long bout, that takes on average more than one hour, after which they wake up (Figure 3.26 B and C). This is true for both CLK and CS flies. Figures 3.24 B and 3.25 B show the average number of passages and distance walked by flies in time. This average is somehow misleading because it mixes flies that fall asleep with the ones that are awake. In order to better understand the behaviour of flies that fall asleep, I separated the flies that fall asleep from the ones that are awake through the entire length of the experiment and I plotted the distance walked and the number of passages crossed every 30 minutes in each individual fly. Figures 3.30 and 3.31 show a few examples of individual CS and CLK flies both in the morning and in the afternoon, together with their distance walked and number of passages crossed in time.

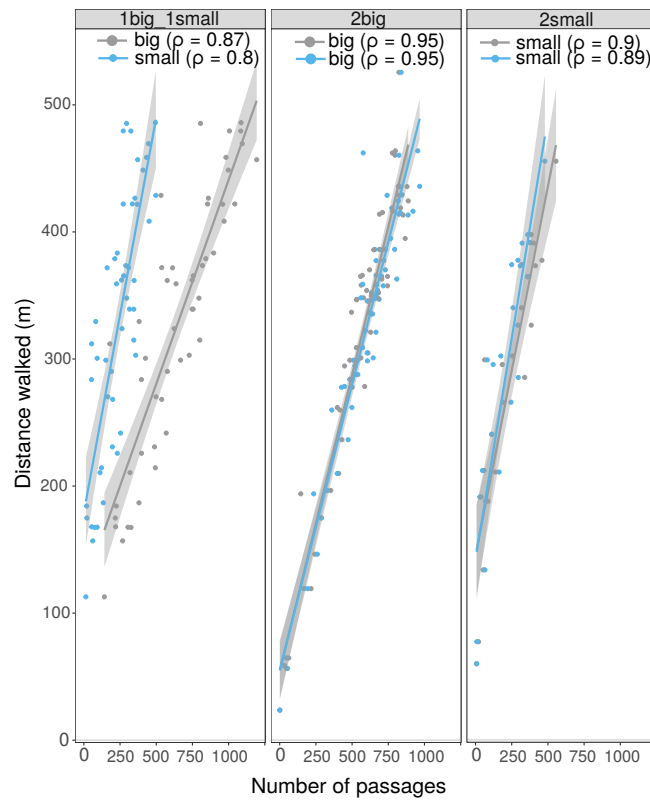


Figure 3.29: Correlation between distance walked and number of passages Correlation between distance walked and number of passages in the three different types of mini square arenas: 1big_1small (left panel), 2big(middle panel) and 2small (right panel). Each panel shows the correlation between the number of passages through each individual hole and distance. The top legend specifies what colour corresponds to what hole type and gives between brackets the Pearson correlation coefficient that corresponds to it.

It is clear from Figure 3.30 A and B that the CS flies which are awake in the morning through the entire length of the experiment move at a constant pace, having relatively little variation in the distance walked and number of passages crossed in the same individual. It seems that there is greater variation in the amount of passages performed between individuals than in the same individual in time. In Figure 3.30 A, fly 1 from ethoscope 1 makes on average 200 passages (between 170 passages and 220 passages) per 30 minutes bout, and fly 3 from ethoscope 1 makes on average 90 (between 60 and 120 passages) passages per 30 minutes bout. In the flies that fall asleep, both CLK mutants and CS wild-type, there is a clear drop both in the distance walked and in the number of passages performed in the few hours preceding sleep (Figures 3.30 C and 3.31 A and C). The observation that both the distance walked and the number of passages decrease in an almost linear way makes us think that they might reflect the sleepiness that gets built in the fly throughout the time.

It is very easy to distinguish visually between the flies that are awake all the time (Figure 3.30 A and B) and flies that fall asleep (Figure 3.30 C, D and 3.31). The passages of the flies that are awake all the time fluctuate by going up and down during the 5 hours of experiment. For the flies that fall asleep, the number of passages in the period preceding sleep is only decreasing. A few flies go to sleep very quickly, like the CLK mutant from ethoscope 4, id 1 in the Figure 3.31 A which goes to sleep during the first hour of experiment. Another observation that the flies that sleep in the first hour of experiment usually stay asleep until the end of the experiment, and from the flies that fall asleep later some of them stay asleep until the end of the experiment and some of them wake up before the end of the 5 hours.

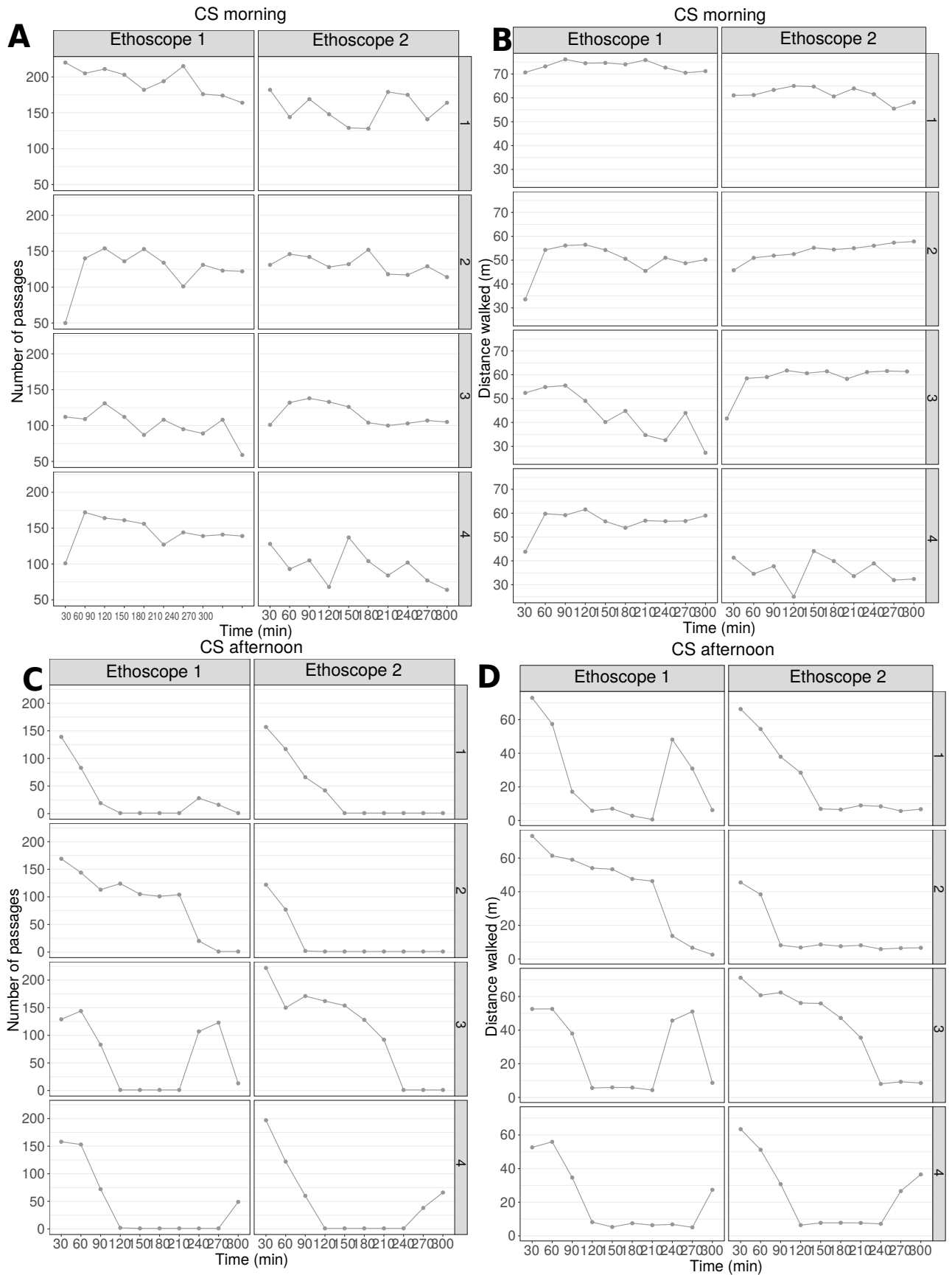


Figure 3.30: Number of passages and distance walked in individual wild-type (CS) flies. Each figure presents 8 individual flies which are identified by the ethoscope number in which they were during the experiment (vertical panels) and by their region of interest id (horizontal panel). **A** Number of passages made every 30 minutes, by 8 individual CS flies during the morning experiment. **B** Distance walked every 30 minutes by the same flies presented in **A**. **C** Number of passages made every 30 minutes, by 8 individual CS flies during the afternoon experiment. **D** Distance walked every 30 minutes by the same flies presented in **C**.

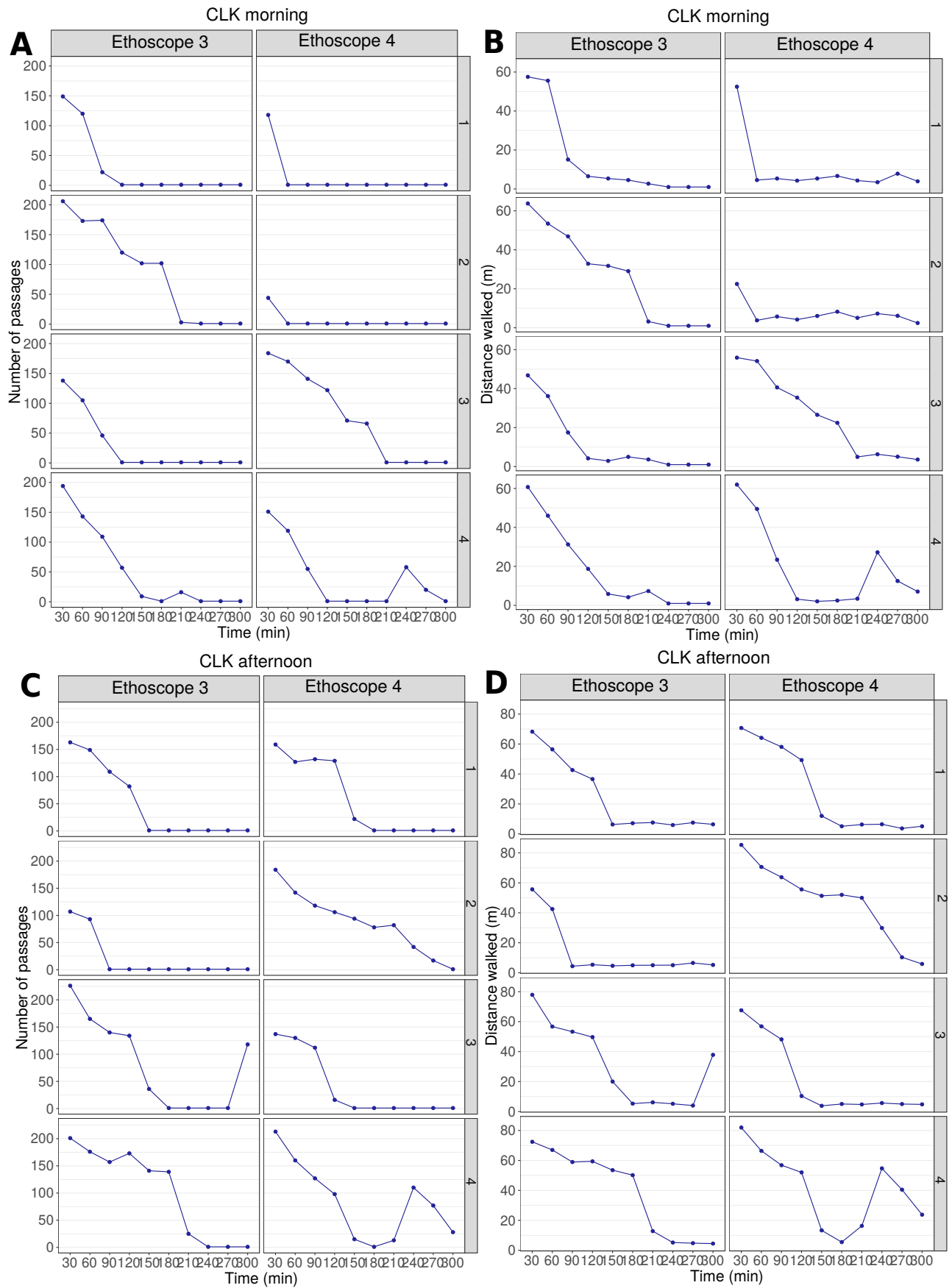


Figure 3.31: Number of passages and distance walked in individual wild-type (CLK) flies Each figure presents 8 individual flies which are identified by the ethroscope number in which they were during the experiment (vertical panels) and by their region of interest id (horizontal panel). **A** Number of passages made every 30 minutes, by 8 individual CLK flies during the morning experiment. **B** Distance walked every 30 minutes by the same flies presented in **A**. **C** Number of passages made every 30 minutes, by CLK individual CS flies during the afternoon experiment. **D** Distance walked every 30 minutes by the same flies presented in **C**.

3.4.2 DISCUSSION

In the first section of this chapter I have described a novel assay that is able to shed a bit of light into how flies assign weight to different external stimuli in order to take a decision. More precisely, we proved that the mechanism that decides if flies should initiate a passage through a hole or not takes into account the size of the hole, making more probable holes of larger size than the ones of small size. In the second part of this chapter I have looked at how internal states influence the passages through the holes, by investigating how sleep modulates the sequence of crossings through the holes.

Overall, I think that by far the most interesting result is that all the flies that performed the experiment, independent of their internal condition and independent of the external context, made more passages through the big hole than through the small hole. This reveals a fantastic robustness of the decision making mechanism which selects actions based on their expected outcome and gives priority to the actions with a more favourable expected outcome. In the face of varying external conditions and different internal states, this mechanism does not fail at assigning different weights to each hole based on their size and at preferring the bigger hole in every single fly. A possible reason for which the big hole is perceived by the fly as being more advantageous are the easiness to pass through it rather than the small hole which might exert too much pressure on the flies body and wings. Another reason might be linked to the flies escaping behaviour, which is perfected for finding the easiest route to escape, therefore possibly preferring the big hole as the quickest and safest option.

On one side, the external conditions like light and size of the arena had an impact on the proportions with which flies choose the big and the small hole, having a noticeable weight into the mechanism deciding if a passage is initiated or not. On the other side, using sleep deprivation as a method for disturbing the internal state of the fly did not have any influence on the flies initiation of passages and preference of the holes. This suggests that the weight of the sleep pressure felt in a fly following a night or 3 days of sleep deprivation is not significant enough to be perceived in the time frame of our experiment and at the resolution provided by our setup into flies behaviour. Using the smallest size arena, the `ibig_small` mini ring arena, and performing experiments in dark, guarantees on average more than 500 passages per fly in 5 hours of experiment. Large numbers are needed to average out the randomness specific to each passage and to have access to the subtle contributions that the external and internal conditions feed into the decision making process. The throughput of decisions was big enough to reveal the effects of the external conditions on the initiation of passages but not big enough to capture the effects of the sleep deprivation.

The function of sleep is unknown, but recent studies⁹⁷ in *Drosophila melanogaster* show that after prolonged periods of sleep deprivation (10 days), flies slept slightly more in the morning following the sleep deprivation period but no other visible effects were observed in their behaviour compared to control. Moreover, males that were sleep deprived for their entire lifetime, had the same life span as control. If this study suggests that sleep is not vital for life in *Drosophila*, my current results extend on them by showing that the sleep pressure that builds up from sleep deprivation is not something that flies need to deal with in priority, and they can either suppress it or delay it. Doing that, does not have any measurable consequences on their behaviour. In humans, hallucinations are observed after approximately 4 days of sleep deprivation¹⁵⁷. For the flies, not dealing with their lack of sleep has no visible effect on their perception of the environment and does not alter in any way their willingness to pass through the holes or ability to distinguish them.

Sleep deprivation has no effect on the choice behaviour of the flies at the timescale of our experiment of a few hours. Some effects might be visible at finer timescales. For example by looking at the first sequence of choices that a fly does, we might be able to gain more knowledge into the way actions are performed over time. It might be that sleep deprived flies need more iterations over the holes to get to distinguish them in the same way as control, and to formulate their expected outcomes in a similar way. We do not know how many iteration flies need to make over the same holes to converge to the stable expected outcomes that we observe. It might be that this happens after the first passage or after several passages. Given the randomness specific to every passage, a much larger throughput of flies would be needed to be able to zoom at that level. Even if there are differences in the decision making at a finer scale, the ability of the sleep deprived flies of coping with them over time is striking.

Without any concrete changes in behaviour following sleep deprivation, we next tried to disrupt flies at a deeper level, in the hope of capturing an effect of the internal states on the mechanism involved in the initiation of a passage. To do that we turned to circadian rhythm mutants. The CLK mutants, known to be behaviourally arrhythmic, initiate passages into the holes with different proportions compared to CS, going through the big hole in a greater percentage than the wild-type flies. The impact of the CLK mutation on the initiation of passages was strong enough to be perceived with our setup. Even though the proportion with which flies initiated passages is dependent on the genotype, it proved to be independent of the activity of the flies. More precisely, both CS flies chose the holes in the same proportion both in the morning as in the afternoon. The stability in the proportion of holes chosen in the same genome, even in the face of significantly different activity, in the same genetic background, suggests a deep link between genetics and the mechanism responsible for assigning values to how the outcome of an action is remembered by the fly and updated after repeating the action. Put simply, it suggests that certain tendencies in behaviour are under precise genetic control. In the initiation of the passages locomotion plays a main role. The locomotion of CLK mutants is different from control, therefore this might explain the dropped willingness of CLK mutants compared to control for initiating passages. However, the mutation in the CLK gene might also contribute to the lack of motivation to pass, and could be investigated by an experiment design able to separate its contribution from the effect of locomotion.

Our paradigm enabled us to explore the effects of internal states on decision making and results suggest that the decision making mechanism responsible for selecting actions based on their expected outcomes and also responsible for assigning outcomes to performed actions, is not intimately linked to the sleep homeostasis mechanism. However, it does seem to be modulated by the genetic background. The mini square assays also unravel some interesting insights about sleep. Firstly, the lack of a sleep rebound during the experiment suggests that the sleep pressure due to the homeostatic regulation of sleep is very small. Oppositely, wild-type flies fall asleep in the afternoon for a large period of time suggesting that the sleep pressure due to circadian regulation is much stronger and it cannot be suppressed or delayed. This confirms recent results stating that sleep pressure is largely under the control of circadian rhythms⁹⁷. Looking at the link between the space of the arena and sleep pressure might lead to more quantitative measures of the sleep pressure itself.

Finally, our paradigm revealed insights into how sleep pressure due to circadian rhythms grows over time suggesting an almost linear build up that is reflected in both the distance walked and the number of passages crossed. It is the linear decrease of these parameters before sleep in every single fly that we looked at, which suggests its possible link with sleep pressure. For some flies, the number of crossings falls with

regularity over more than 3 hours before a sleep episode is triggered. This opens up avenues into quantifying more precisely sleep pressure and understanding the mechanism through which it is generated.

4

Final discussion

4.1 TECHNOLOGY

Available technology for studying the behaviour of *Drosophila* comes with its limitations. Many of the software are implemented for very specific solutions and are not amendable for own needs as often they are not developed with this framework in mind. It takes therefore some effort to put in place a workflow with modular components which can be easily adaptable. Not all the results are worth pursuing further therefore ability to interrupt and change direction based on most recent results is imperative. The ethoscope is a tool that has been created to be extended but its validation has been made using a specific context therefore employing its software in a different context, on a higher resolution temporal data needs to be done with care by adapting specific thresholds to new circumstances.

4.2 RANDOMNESS IN BEHAVIOUR

The y-maze paradigm confirmed previous studies showing an endogenous initiator of randomness in every single fly. The effects of sleep deprivation does not change in any way the complexity of the temporal pattern of flies suggesting its weak effect in this particular context.

4.3 DEVELOPMENT OF OWN TOOLS

The software developed to respond to my specific needs during this project consists in: a pixel-accurate way of defining regions of interest, the ability of defining subregions of interest and use the different subregions of interests to understand specific behaviour of the flies in specific locations in the arena, in my case to trace with a good time resolution the passages through holes. Defining the regions of interest using a mask can facilitate other fields of *Drosophila*. It can be used to easily distinguish between patches of food with different shapes and precisely address the number of visits that a fly does to each one of them. The development of the k-means multi-tracking algorithm, even though unexploited throughout this project offers possibilities into the study of sleep in a population of males. The ability of combining it with the definition of subregions of interest can give increased understanding of the flies spatial distribution and the inter-individual proximity and interactions while sleeping. Up to present, there is no study employing

video-acquisition methods to look at sleep in a population of flies. While the multi-tracking algorithm is stable in males, it cannot be used with a population of males and females as it is not able to keep the ids of the flies throughout the copulation period.

4.4 MINI SQUARE ARENA - A SIMPLE DECISION

The final version of our paradigm is able to capture a simple decision : the passage through a hole, to quantify with precision the moment when the decision is initiated and to observe the modulation of the decision by an external stimuli: the size of the hole. This context offers the possibility of separating the locomotion from the actual decision by the means of two behavioural measurements: the number of passages initiated and the proportion of big and small holes chosen. The presence of two holes of different sizes, two different external stimuli is a necessity that reveals insights of the decision making mechanism by using one hole as a reference. The proportion of flies choices is a measure that characterised the decision mechanism but is independent of the locomotion. This is confirmed by the fact that it does not change between the morning and afternoon experiments (Figure 3.24 C), in the same genotype, even it is obvious that the locomotion is different with flies being more active in the morning. However the proportion with which flies choose the big and the small hole is different between genotypes. Further experiments using several mutants and performed at different time of the day, to ensure different activity can help to better understand how the ability of the proportions of hole choices measure to characterized the decision making mechanism and its link to the genome. The mini square offers many other potential uses for understanding the mechanism governing decision making processes through behavioural responses. The assays can be slightly changed by intruding a wall between the two holes on one half of the square. By placing behind the small hole a food source or a potential mate one can examine how the proportion of choices changes and try to understand how flies attach value to specific stimuli.

4.5 LARGE NUMBER OF DECISIONS

The key to observe and quantify the decision making mechanism and the way in which it integrates various external and internal factors is to be able to look at a large number of decisions. In our paradigm this is achieved by two means: reducing the space to the minimum and running experiments in darkness. It is already known that space restriction increases locomotion¹²⁰ however we do not understand why running experiments in darkness is able to boost so much the number of decisions. Running the experiment at the beginning of the dark cycle can help us understand if the increased number of passages is linked to being in darkness at a moment when they expect light or if flies perceive the holes and the environment in a different way.

4.6 CONFIRMATION OF HEISENBERG'S THEORY

I want to recall a final time Heisenberg's theory⁵⁵ which affirms that behaviour is active and in order to understand it we should look at how actions are selected based on their expected outcomes. In his view, an organism should choose the most advantageous outcome more often. Our paradigm offers us the chance to verify Heisenberg hypothesis as it presents the fly with two decisions: passing through the big hole through which it fits smoothly or passing through the small hole through which it fits tightly. Two pos-

sible actions that have a similar outcome as they both get the fly to the other side, but for the big hole the passage is a bit easier. Each individual fly chooses more often the big hole, even though it makes several passages through both holes. It is impressing that Heisenberg theory is verified in every single fly, independent of genotype.

4.7 CONFIRMATION THAT LOCOMOTION IS INCREASED BY SPACE RESTRICTION

This study¹²⁰ uses large arenas of a similar size to the concentric rings arena (Figure 2.4) and small/restrictive arenas of the size of the mini rings arena 3.2 and run experiments for one hour. Their results suggest that the locomotion of males is highly increased in the small arena during the one hour of experiment. Our results come to confirm and extend their results as we further decrease the space of the flies by using the mini square arena (Figure 3.2), which is approximately one quarter of the mini rings arena. We show that the locomotion is considerably increased in the mini square compares to the mini rings independent of light condition or maze type (Figure 3.9 A). This suggest an interesting relationship between space restriction and locomotion that should be further exploited. It suggests a sort of space pressure that arouses the animal. Our experiments last for either three or five hours and we observe that if in the mini ring the activity of the flies decreases considerably after the first hour (Figure 3.9 B), in the mini square their activity is almost constant for the entire length of the experiment, for the experiments run in the morning.

4.8 SUPPRESSION OF THE SLEEP REBOUND

In both the y-maze and the 24 square experiments using sleep deprived flies we are not able to capture any sleep rebound even though experiments are run straight after the SD period at a time at which the sleep rebound is usually observed. Flies in the y-maze more after the experiment suggesting a possible delayed sleep rebound. Flies in the mini square were not monitored after the experiment therefore we do not know if they rebounded or not. In any case, we can asks what are possible reasons for the suppression of the sleep rebound during the experiment? One hypothesis is the novelty of the arena, however this study⁶⁴ shows that by loading flies into fresh tubes following a night of sleep deprivation they are still showing a sleep rebound. A more probable explanation it is that the space restriction is responsible for the sleep rebound suppression. This is plausible as we have seen before that space restriction enhances locomotion, therefore having an arousing effect on the flies. The previous mentioned paper also founds that sexual arousal suppress the sleep rebound. A similar effect might be therefore triggered by a "restrictive space arousal". To test this hypothesis I suggest an experiment in which flies are sleep deprived during one night and then moved to fresh tubes of shorter size and followed to see when they rebound. If the hypothesis is true, by manipulating the size of the shorter tube we will be able to manipulate the external space pressure as suggested by Section 4.7 and be able to characterised better the internal sleep pressure due to the homeostatic regulation. In any case, the existent internal sleep pressure does not have effect on the computed metrics of the flies in the maze. This can mean either that our paradigm is not able to capture such effects or that the fly is able to cope very well with the sleep deprivation.

4.9 SLEEP PRESSURE VS SPACE PRESSURE

When experiments are run in the afternoon at the moment of their "siesta", day-time sleep strongly regulated by the circadian clock, flies fall asleep in the y-maze. This indicates a strong sleep pressure present in the fly which surpasses the external excitement produced by the restriction of space. The space of the mini square is our reference, and this result indicates that the sleep pressure due to circadian regulation is greater than the space pressure that comes from the mini rings arena. The previous section suggested that the sleep pressure due to homeostatic regulation is smaller than the space pressure of the mini square. Put together these result in the fact that the sleep pressure due to circadian regulation is greater than the sleep pressure due to the homeostatic regulation. This results should also be further investigated using standard tubes to understand if it is reproducible in another context and if it verifies our hypothesis. If this is the case, than space restriction can be used as a ruler able to quantify sleep pressure.

4.10 REPRODUCIBLE RESULTS IN DIFFERENT CONTEXTS

A beautiful result of this work is its reproducibility in different contexts, which reinforces our results and makes them more trustful. Moreover, the diversity of the paradigms used gave us new insights about the regulation of sleep as they brought new perspectives which came purely from integrating the results of different paradigms. Moreover all the paradigms can be used for further studying decision making in flies. The concentric rings arena can be adapted to offer flies with several paths of different lengths to arrive to a certain goal, either some food or the female. These paths would take the form of different combination of holes. It would be interesting to investigate if flies are able to remember the shortest path.

4.11 DROSOPHILA AS MODEL FOR PSYCHIATRIC DISEASE

We mentioned before that in order to be able to translate psychiatric disorder into the animal realm, one suggestion is to stop looking at these disease from a purely anthropomorphic perspective which involves the human mind but rather look at them as an inability of responding to environmental stimuli in an appropriate way. The brain is constantly flooded by stimuli and is the relative salience of these inputs that controls which are more likely to capture attention²⁷. A salience dysfunction syndrome will assign distorted salience to stimuli. The mini square is able to capture the preference for the bigger hole in each individual and it might be that the proportion of passages through the big vs the small hole is linked to the relative salience between the two holes in the flies perception. If this is the case, a genetic screen might found behavioural phenotypes that choose equally between the two holes, or choose more the small hole, or choose only the big hole, or choose only the small hole. All these example can be good phenotypes of a salience dysfunction syndrome as they exhibit behaviour which is in contradiction with the unanimous stable preference for the big hole and unanimous consistent behaviour of passing through the two holes specific to each individual fly tested. Such mutants might be good models of *Drosophila* for the study of psychiatric diseases.

4.12 LIMITATION OF THIS STUDY

A limitation of this study is that flies cannot be observed for longer than 5 hours as their water resources fade and they start to become dehydrated. I deliberately chose to not feed the flies during the experiment in order to keep the external stimuli to a minimum.

Another limitation is that we are not able to capture the first crosses of the flies in the mini square arena, but this does not seem to be problematic as they are excluded for every fly.

Another limitation of this study is the inability to sleep deprive flies directly in the mini square and be able to analyse their passages through holes straight after sleep deprivation without the necessity of loading them into a new arena.

5

Conclusion

This study achieved its aim of finding a paradigm suitable for studying decision making in *Drosophila* using cheap technology and enabling high-throughput experiments. Moreover, the most refined of the paradigms designed, the mini square, is able to quantify characteristics of the decision making mechanism through behavioural responses which are independent from locomotion. Our assay is also capturing with precision the moments when decisions are initiated.

We were not able to capture any effects of sleep deprivation on decision making, but our approach revealed a few interesting perspectives on how sleep pressure might be regulated by space restriction. These results also imply that the sleep pressure due to circadian regulation is more significant than the sleep pressure exerted by the homeostatic regulation. Our work also confirms and extends the results of previous studies¹²⁰ finding that space restriction induces sleep locomotion in *Drosophila* males.

Independent of external conditions and internal states, every single fly passed through both available holes but made more passages through the bigger hole. The mini square assay was able to confirm through its very simple design that flies choose more the actions with a better expected outcome.

The precision in the collected data has been achieved through a continuous development of software able to respond to our specific demands. The software was continuously integrated and tested, and can be used in various behavioural task to facilitate the quality of the collected data. The design of the final paradigm has been achieved through a continuous refactoring and testing of several assays which were gradually refined and culminated with the design of the mini square.

Current advances in technology played a critical role as they allowed a continuous testing of the experimental paradigm and design, rapid modification and rapid integration of the improvements, ensuring a deliverable result.

Finally, the mini square assay offers great potential at being used for the screening of possible models of *Drosophila* suitable for the study of psychiatric disorders.

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THE MAN WHO REALLY KNOWS MUCH, pushing the questions to the limits of science - the consistency and the theoretical proof will take him to sanctuary of religion. Let's suppose we have pushed the research so far that the science could explain all the laws governing the process of the world. Let's assume the theoretical spirit of man celebrated his latter triumph to join in a closed system all the relationship and causes that created the world. And in the eyes of a scientist from the far away future, the universe is known in its ultimate mystery, like a perfect icon, like a closed loop cycle. At this moment, there is a question the science still cannot find the answer. Why universe has the shape it has, and no other limits; and finally, why the world obeys the same laws the science discovered and established, and not other laws?

Here are the questions that science cannot answer, even at the end of its research. But here is a question where religion responds, when is stating fervently the existence of a personal God outside the world but still crossing our world, which created it from nothingness through a sovereign act of choice from an infinity of probabilities, with affection and love for the unique shape it has now.

Without doubt, it seems the spirit of consistency and truthfulness lived in depth comes to confess not only the relativity of the scientific spirit and its insufficiency in front of the last problems of speculation, but more than that. The scientific and religious attitudes convert in ardor, seizing the mystery clouding their area. Scientific investigation is carried out in adoration for the Being beyond the world, the Being who created the world, who leads the world, and supports world with love.

It can be seen - for people who want to see - that the way to solve the fight between science and religion doesn't assume at all a give up to scientific attitude. In the large framework of the world mystery, in the large infinity as well as in the small one, the scientific mystery can continue to develop its own values. But what must be fought against in the light of the analysis so far, is the scientific arrogance and its narrow and short-sighted dogmatism, vices not to be found in real scientist.¹⁵⁸