

High-throughput Recording, Analysis and Manipulation of Sleep in *Drosophila*

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This report is the result of my own work

No part of this dissertation has already been, or is currently being submitted by myself for any other degree, diploma or other qualification.

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Abstract

Sleep is a fascinating mystery that has bemused thinkers since the dawn of civilisations. Scientifically, the formalisation of sleep as an observable behaviour has been a conceptual milestone, which has enabled researchers to address the question of its ubiquity and ultimately led to the discovery of sleep-like states in most animal phyla. The fruit fly *Drosophila melanogaster* has long been at the vanguard of the discovery of many biological processes. In particular, it has been instrumental to explain the genetic determinism of behaviours. In the 2000s two seminal studies reported their discovery of a state of quiescence in *Drosophila* that had all characteristics of sleep. Rapidly, the fruit fly became a significant and widely adopted model of sleep. However, despite the large palette of advanced tools to study various aspects of the genetics, development and neurobiology, the methods and conceptual tools to investigate the behavioural aspect of sleep in *Drosophila* lag behind, which has limited our understanding of its phenomenology and function.

In the thesis herein, I first present the ethoscope platform, a tool to score behaviour in a large number of isolated animals. I explain how its modular design allows for large-scale, real-time, long-lasting experiments. Secondly, I provide **rethomics**, a general framework to analyse the large amount of resulting behavioural data, which has the potential to bridge the gap between experimentalists and data scientists. Thirdly, thanks to these methodological developments, I reconsider the binary definition of activity in the context of sleep, address some ambiguities in the literature regarding the effect of mating and address new questions about the endogenous determinism of sleep. Finally, I employ the ethoscope to perform, to my knowledge, for the first time in *Drosophila*, an automatic real-time sleep deprivation. The specific and parsimonious nature of this new treatment permitted a chronic depletion of sleep on a large population of flies. I show that, in stark contrast with the belief in the field, flies can perhaps survive with no sleep, challenging the notion that sleep is a universal vital need.

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Writing a thesis is a unique experience that I will not reiterate any time soon — at least I hope. In addition to being an excruciatingly long writing process, I was led to think it aims at showing one's *individual* contribution to her or his field. Sadly, this format makes it difficult to convey my personal scientific experience of, instead, a highly collaborative exercise. In truth, all the work I present here results, in one way or another, from a team effort. Therefore, I would like to start by dedicating this thesis to all the members of my group for their direct or indirect scientific contribution but, equally, for the deep friendship that unites us all.

Firstly, to Anne Petzold who, with myself, was the first member of the team. I still remember vividly the first time we met, the many scientific, political and philosophical discussions we had as well as the emotions we have shared. Then, I cannot forget when, later, Alice French joined the lab as a post-doc. She inspired me immensely by her intellectual creativity, but also by her resilience and bravery, both personal and professional. Then, I truly cherished the deep, sometimes spiritual, discussions that I had with Diana Biczak, another PhD student. I also admire Hannah Jones, who joined the team much later, for her determination and her ability to navigate in the most challenging social environments — including the clique we had created — and I can only wish I had more time to get to know her.

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Acronyms

API Application Programming Interface.

D Dark.

DAM Drosophila Activity Monitor.

DSD Dynamic Sleep Deprivation.

EEG Electroencephalography.

FPR False Positive Rate.

FPS Frame Per Second.

IR Infrared.

L Light.

REM Rapid Eye Movement sleep.

ROC Receiver Operating Characteristic.

ROI Region Of Interest.

SWS Slow Wave Sleep.

UPGMA Unweighted Pair Group Method with Arithmetic Mean.

ZT Zeitgeber Time.

1 | Introduction

‘For six days and seven nights,
come, do without slumber.’

— Uta-napishti, in *The Epic Of
Gilgamesh* [Ref. 1, tablet XI 209]

1.1 General introduction

1.1.1 Sleep, the history of a mystery

1.1.1.1 In the oldest tale we know of

From 668 BC to 627 BC, the Neo-Assyrian empire was ruled by King Ashurbanipal. Proud of his literacy and aware of the power of knowledge, he ordered his armies to collect all clay tablets they could find throughout Mesopotamia and he stored them in his capital, Nineveh. After his death, civil wars weakened the state and, in 612 BC, Babylonian invaders eventually burned the city to the ground. Ashurbanipal’s library collapsed, and the ancient tablets were buried and forgotten in its ruins^{2,3}.

Until, in the mid-nineteenth century, Austen Henry Layard discovered the remains of Nineveh⁴. A few years after starting the excavations, his assistant, Hormuzd Rassam, came across a very special set of tablets. They told the story of Gilgamesh, the semi-fictional king of the great city of Uruk². This tale, the ‘Epic of Gilgamesh’, is considered the most ancient piece of literature known to date⁵.

In many respects, the epic reflects how the world was seen and understood by the people who forged the first civilisations. It is quite clear that sleep was already seen as a fascinating, but also mysterious, topic. In particular, several references

show that sleeplessness was thought of as a supernatural trait, comparable to immortality. For instance, Gilgamesh slays Humbaba, the ferocious giant that never sleeps [Ref. 1, I 239]. The epic climaxes when Gilgamesh, who seeks eternal life, is challenged to defeat sleep — the younger brother of death — to prove himself. However, despite his great strength, he fails to remain awake even for a single night [Ref. 1, XI 209]. Altogether, the tale hints that *sleep is an intrinsic limitation which is inherent to the human — and maybe to the mortal — condition.*

1.1.1.2 Sleep and death, a cultural perspective

There are many examples showing a deep interest in sleep in other mythologies and cultures, and the interested reader will find ample material published on the matter^{6,7}. In connection with the present thesis, I was particularly interested in one aspect: the analogy, already found in the Epic of Gilgamesh, between the two mysteries that are sleep and death, and wanted to illustrate their — sometimes implicit — association with a few examples drawn from culture and mythology.

In ancient Egypt, death was seen as an ‘eternal sleep’ and, during sleep, the living could communicate with the world of the dead⁸. In Greek mythology — for instance, in Hesiod’s theogony — Hypnos, the god of sleep, is the half-brother of Thanatos, the personification of death⁹. Homer and Virgil themselves use the euphemism of ‘deep sleep’ to name death¹⁰. Later, in the dedication to his *Natural History*, Pliny the Elder writes that ‘life properly consists in being awake’ [Ref. 11, book I]. In fact, for him, sleeping could, in some cases, rejuvenate. For instance, he surmises that ‘[the] old age of [dormice] is put an end to by their winter’s rest, when they conceal themselves and sleep’ [Ref. 11, book VIII, chap. 82].

The metaphor of sleep is also part of the Judeo-Christian tradition. For instance, for Paul the Apostle, graveyards are large dormitories¹⁰. Later, Milton will also question whether sleep and death are two degrees of the same phenomenon: ‘the lifeless body does not sleep, unless inanimate matter can be said to sleep’ [Ref. 12, p. 285]. In *Sleep And Societies*, Simon Williams points out that the intimate link between sleep and death is present throughout literature, for instance in the work of Shelley, Donne, Bunyon and Shakespeare [Ref. 13, p. 150].

For the modern reader — who, I am sure, knows more about physiology than the ancients did — it may be intuitive to comprehend sleep and death as two different, even dichotomous, processes. In particular, sleep is reversible, whilst death is not. However, in the old times, it seems as though the similarities, rather than the

differences, between these two mysteries were highlighted. This may arise partly from the fact that both states involve closed eyes as well as a lack of movement and responsiveness. Perhaps an even more fundamental resemblance, though, is that both sleep and death appear ineluctable.

1.1.2 Phenomenological definition of sleep

1.1.2.1 Towards an open definition

Often, as I finish a presentation of my work on sleep in fruit flies, an inquisitive listener from the audience takes the opportunity to raise their hand and challenge its very premise: my *definition of sleep*, and whether I should use the word ‘sleep’ at all. Sometimes, I am even charged guilty with the sin of anthropomorphism, as if using the same name for two processes implied a complete identity of functions, origin and implementation.

I find comfort in the realisation that, in behavioural biology, others have faced similar criticisms. A famous example is the use of the word ‘culture’ by primatologists, a term historically reserved for humans, but now accepted — at least, amongst scientists — as a phenomenon with wider scope^{14,15}.

In his prologue to *The Ape And The Sushi Master*, Frans de Waal delivers a powerful argument in favour of ‘open definitions’ in general¹⁶. He argues that we tend to overestimate the risk of anthropomorphism, but dismiss the benefits of open definitions. He anecdotally points out that we use the term ‘bipedal’ to describe both humans and chickens, whilst their implementation is rather different. I would add, to pick an example closer to my field of research, that we speak of insect ‘respiration’, whilst they have neither lungs nor red blood cells^{17,18}, therefore without implying macroscopic identity with the physiological processes that happen in, say, mammals. In fact, calling two processes or structures the same does not even necessarily involve a shared evolutionary origin. For instance ‘wings’ and ‘eyes’ have evolved multiple times^{19,20}, but sharing terminology is important to fully understand the convergence of innovations to similar evolutionary and physical constraints.

Our comprehension of numerous biological processes has resulted from a translation between model organisms. Arguably, using different names reduces visibility across fields and ultimately may prevent collaboration. For instance, if one had restricted the notion of immunity to its adaptive arm, or even to the presence of

antibodies — which are exclusive of vertebrates²¹ —, it could have been difficult to initiate, and eventually translate, the seminal work that was done on the innate immunity of *Drosophila melanogaster*²².

The above processes (*i.e.* immunity and respiration) and structures (*i.e.* bipedal legs, wing and eyes), however, differ fundamentally from sleep insofar as *their function is known*, or at least postulated, whilst the role of sleep is, at best, elusive^{23,24}. In other words, we cannot provide an open definition of sleep that would be based upon its main function, because we simply do not know it. In fact, we often define sleep as a lack of function, for instance a loss of awareness to the environment, a lack of movement and so on (see subsection 1.1.2.1 and²⁵). It therefore seems as though, as long as we have no compelling evidence for a shared role for sleep across all species, we should be very cautious about using this term.

There are, however, widespread phenomena that have been defined and extensively studied, without their function being known *a priori*. For instance, senescence, and eventually death, are broadly accepted as ubiquitous and necessary. The function of ageing — and, in fact, whether it has one at all — has nevertheless long been debated by evolutionary biologists^{26–28}. Instead of postulating a function, and making the risky assumption of a phylogenetic conservation, we use a ‘*phenomenological*’ definition. That is, we study ageing as an observable phenomenon whereby the functions of an organism gradually deteriorates until it eventually dies — even though it manifests itself in various forms in different organisms and has multiple levels (*i.e.* molecular, cellular and physiological)^{29,30}.

The field of ageing, despite a fundamentally function-agnostic framework, has historically drawn from a comparative approach, both describing similarities and differences between organisms^{27,31}. This example shows that phenomenological definitions, too, can be the substrate for a deeper understanding of biological processes. Therefore, if we would like to study sleep with the lens of comparative biology, and without presupposing a function, we ought to provide a phenomenological definition.

1.1.2.2 From behaviour to brainwaves

Historically, sleep was first scientifically investigated in mammals, including humans. It was then defined as an overt *behaviour*³². However, the observation of macroscopic behavioural states was to be superseded by the rise of a new science: electrophysiology.

Indeed, in the late 1930s, Frédéric Bremer noticed that sleep behaviour correlated with changes in the electrical activity of the brain^{33,34}. From then onwards, and with the advance of the Electroencephalography (EEG), the field progressively — and eventually almost entirely — adopted a quantification of sleep based on brainwaves. EEG represented a non-invasive, but also relatively high-throughput and objective alternative to behavioural observation. In addition, it could be used for, and compared between, a variety of model organisms and even reveal cryptic states, such as different sleep stages^{35–38}. Ultimately, EEG became the *de facto* standard to study sleep in vertebrates, to the point that brainwaves became implicitly included in the definition of sleep.

1.1.2.3 From brainwaves to behaviour

In the last few decades, a growing interest in a comparative approach^{25,39} and in ‘simpler’ — non-avian and non-mammalian — models of sleep⁴⁰ arose, which prompted the need for an inclusive definition of sleep.

Although EEG has become the standard for studying sleep in mammals and bird, it cannot be used to study sleep in other organisms such as insects or even fish, as brainwaves originate from structures that are simply not present in most animals — namely, cortical regions.

Since brainwaves are merely a convenient, but specific, read-out of a process that is mostly unapparent, it would have been too restrictive to limit the definition of sleep to the exclusive observation of EEG. To use an intentionally provocative analogy, it would have been comparable to stating that sleep necessarily implies eye closure, *de facto* excluding most animals since eyelids — and, for that matter, eyes — are rather rare in animals.

It, therefore, became necessary to open the scope of the definition of sleep to one that could, in principle, be applicable to a wide range of life forms. This concern led to a return to a *behavioural* definition of sleep, which is based on three fundamental observations^{23,25,32}.

Firstly, it implies a relative *immobility* (*i.e.* quiescence) with a particular posture⁴¹ that can be species-specific. For instance, apes, including humans, lie down and close their eyes²⁵. Importantly, such quiescence must be rapidly reversible, which differentiates sleep from dormant states (*e.g.* torpor, hibernation and aestivation), but also from pathological states such as coma²³. In addition to the posture, sleep

may involve a preferred resting site⁴².

Secondly, during sleep, organisms must have a *reduced awareness* of their surrounding environment. Empirically, this translates in a higher arousal threshold — *e.g.* a stimulus that would cause an awake animal to escape will not startle a sleeping one⁴³.

Thirdly, sleep is defined as a *homeostatic* process. In other words, the loss of sleep will increase the need for sleep, and may eventually lead to a so-called ‘rebound’, during which sleep propensity and depth are increased⁴⁴. Importantly, the recovery of sleep loss is not expected to be — and rarely is — as long²³ as the total sleep deficit.

Another interesting consideration is that sleep is often intimately linked to an animal’s circadian clock, and is almost always studied in this context. The propensity of an individual to sleep is then thought of as the addition of two processes (see subsection 1.1.5 and⁴⁵): the homeostatic drive (process S) and the clock (process C). In other words, sleep is not modulated by its homeostat only, but also, and to a large extent, by the internal clock of an animal. Sleep is therefore generally recurrent (*i.e.* daily), and happens throughout life⁴⁶.

1.1.3 Ubiquity of sleep

The question of the ubiquity of sleep amongst animals is very ancient — and, I would argue, entangled with its definition. For instance, in his monumental ‘Natural History’, Pliny the Elder writes a short chapter entitled ‘the sleep of animals’, where he asserts that all animals ‘with eyelids’, but also fish and insects undoubtedly sleep [Ref. 11, book X, chap. 97], a view that can actually be traced back to Aristotle’s ‘History of Animals’ [Ref. 47, *e.g.* book IV, chap. 10].

Using the permissive behavioural definition I have just presented, it becomes conceivable to investigate sleep in a vast array of organisms, throughout phylogeny. Various authors, who have reviewed the literature on the subject, consider sleep a ubiquitous phenomenon^{24,25,48}. It is also even presented as an evolutionary ‘conserved’ phenomenon^{46,49} — *i.e.* the common ancestors of all Metazoans would have already slept. Such views are, however, equivocal as others maintain that the evidence for ubiquity is not satisfying and that behavioural sleep could simply not be characterised in some models³⁹. In this section, I will attempt to provide an overview of the evidence — or lack thereof — for the existence of sleep in several

taxa from both perspectives.

My purpose is not to make a long bestiary of all species in which sleep has been described—as this has already been done multiple times, for instance in^{24,25,46,49,50}—, but rather, for the purpose of this thesis, to summarise the main properties of sleep by taxum. As a comparative biologist, I will focus on the description of sleep in distant and atypical—and sometimes controversial—models such as insects, molluscs, roundworms and jellyfish, but first, will very briefly summarise the—less contentious—work on vertebrates, starting with mammals.

1.1.3.1 Mammals

As I explained above, sleep in mammals has mostly been, and still is, investigated through the use of EEG. The study of brainwaves has led to the separation of sleep in two main states: Rapid Eye Movement sleep (REM) and Slow Wave Sleep (SWS). The former, also known as paradoxical sleep, is characterised by high-frequency brainwaves that are indistinguishable from wake state, and accompanied with a loss of muscle tone (except for the eye muscles which, in humans at least, are active), whilst the latter involves brain activity with macroscopically lower frequency peaks. Most of the experimental evidence for sleep in mammals comes from human and several domesticated land species^{39,50}, namely rat, mouse, cat, dog, and several species of monkeys—though an increasing number of studies have investigated sleep in wild animals⁵¹. Interestingly, marine mammals have long puzzled sleep researchers—so much so that I thought they deserved their dedicated subsection.

Land mammals The fact terrestrial mammals sleep is widely accepted (reviewed, for instance, in^{25,51–54}). Indeed, multiple experiments have shown that they exhibit all the features of the definition of sleep. For instance, sleep has extensively been studied in rodents, in particular in rats and mice that have long periods of inactivity during the day (since they are nocturnal) that correlate with electrophysiological measurements. When engaged in such states, they have been shown to be less responsive to external stimuli such as vibrations⁵⁵ or sounds⁵⁶—though arousal threshold differs between models⁵⁷. When sleep-deprived, they recover the lost sleep, with more, but also deeper, quiescence^{58,59}.

There is also a vast literature specifically in human, the description of which goes beyond the scope of this general introduction. For completeness purposes, I will,

however, —very superficially, I am afraid— mention the singularity of the work on human sleep. Firstly, several large-scale epidemiological studies have been performed in order to statistically link sleep to other variables such as genetics^{60,61}, and age, sex, lifespan and health conditions^{62–65}. Then, psychology experiments that address the relationship between sleep and high-order cognitive tasks⁶⁶ or emotions⁶⁷ have been carried out. Furthermore, idiosyncratic phenomena such as dreaming⁶⁸ or sleepwaking^{69,70} have been under scrutiny. Lastly, there are interesting considerations on how sleep interacts with culture, for instance with the use of artificial light⁷¹, and society^{72,73}.

In addition to the corpus of studies, with clinical applications, on these conventional, domesticated, models, there are also many research articles on sleep in other mammals (reviewed in^{25,51}). They show a large variability in the amount of sleep. For instance elephants⁷⁴ and giraffes⁷⁵ reportedly sleep as little as 4 h, whilst species of bats would do so almost 20 h each day^{25,76}. Although most authors have worked with animals kept in zoos³⁹, an increasing number of studies have managed to record sleep in the field⁷⁷.

Marine mammals Between 50 and 30 million years ago, three groups of mammals—namely, the Cetaceans, the Pinnipedes (*e.g.* seals and walruses) and the Sirenians (*i.e.* dugongs and manatees)—independently diverged from their respective land ancestors and evolved specific adaptations to the marine environment⁷⁸. Since all marine mammals need to regularly emerge at the surface to breathe, sleep researchers have long wondered how this seemingly active behaviour was compatible with sleep⁷⁹.

Cetacean sleep is particularly well studied (reviewed in⁸⁰) and has several unique features. Firstly, the presence of REM sleep has not been shown conclusively^{80,81}. Secondly, cetaceans seem able to sleep whilst moving⁸². Thirdly, they are capable of ‘unihemispheric’ SWS sleep, a state where only one hemisphere of the brain shows features of sleep, the other being electrophysiologically awake⁸³.

Noticeably, some mother cetaceans have been reported to be constantly active after giving birth⁸⁴, which indicates that sleep can evolve to be optional, at least under some conditions.

1.1.3.2 Birds

Birds (a taxum with approximately 10,000 species) are very interesting for sleep research in, at least, three ways. Firstly, there is a long lasting tradition of studying them in the field, which constitutes an unusual opportunity to investigate sleep in ecologically relevant contexts. Secondly, some migratory birds are known to fly over long distances without ever landing, which has puzzled scientists. Lastly, birds have evolved, independently of mammals, cortical structures, which makes EEG applicable to them, and explains the vast literature on avian sleep⁸⁵.

One seminal study on the barbary dove (*Streptopelia risoria*) showed that exposure to a predator created a sleep debt that was then compensated⁸⁶. Multiple studies following this early work have led to the broad consensus that, like mammals, birds have both REM and SWS⁸⁵.

Like sea mammals, several species of bird, exhibit unihemispheric SWS, including blackbirds (*Turdus merula*)⁸⁷, mallards (*Anas platyrhynchos*)⁸⁸ and others (see table 1 in⁸⁵) In addition, there is some evidence that some gliding species can sleep in flight⁸⁹.

A notable study showed that, during the breeding season, some male pectoral sandpipers (*Calidris melanotos*) mate with multiple females with very little rest, and that this sleep loss is neither compensated nor does it impact fitness⁹⁰, fuelling controversy on the idea that sleep is a fundamental necessity.

1.1.3.3 Fish

Early work There are approximately 25,000 species of Teleost (bony) fish with very diversified ecological niches⁹¹. An early study on a small number of goldfish (*Carassius auratus*) and perches (*Cichlosoma nigrofasciatum*), has shown that both species experienced rest, and that light exposure during the night could increase their activity, with a subsequent rebound⁹². However, no changes in responsiveness were characterised. Behavioural states, including quiescence, were determined by human observation.

Zebra fish The zebrafish (*Danio rerio*) is an important and genetically tractable model that has traditionally been used in developmental biology, genetics, toxicology^{93,94} and, more recently, behavioural genetics⁹⁵.

There is a growing pool of accepted evidence that zebrafish exhibit a sleep behaviour^{96–99}. Some of the studies that investigated sleep in zebrafish also show surprising neurochemical similarities between mammalian and fish sleep. For instance, some hypnotic drugs affect sleep in both models^{96,99}.

It is important to notice that most research is done on very young fish larvae (typically, 7–14 days old) as opposed to adult animals. Since zebrafish larvae are very small and aquatic, electrophysiological measurements are not performed. Instead, sleep is generally assessed by high-throughput automatic behavioural scoring of video recordings.

Blind cavefish More recently, a very interesting model of sleep was suggested: *Astyanax mexicanus*, a species in which several populations have independently adapted to life in the absence of light, and eventually lost their eyes^{100,101}. This model is particularly interesting as the circadian clock of the cave-dwelling populations is altered¹⁰², prompting questions of the evolution of sleep when biological rhythms are dampened. The blind populations were shown to have rapidly evolved towards very low amounts of sleep¹⁰⁰. The same team of researchers further characterised a neuronal pathway that is involved in this reduction of sleep¹⁰¹. This comparative approach shows that the amount of sleep can be a labile and rapidly evolving phenotype.

1.1.3.4 Arthropods

Arthropods are the most diversified phylum with an estimated five million species, including, amongst others, all insects and crustaceans¹⁰³. The diversity of their ecological niches as well as their overall biomass and environmental impact^{104,105} makes them an unavoidable group—at least in the context of comparative biology—, but sleep has only been studied in a few of them.

Early work on cockroaches and scorpions A series of landmark experiments on several species of cockroaches showed that, at least under some conditions, immobile body posture correlated with increased arousal threshold^{106,107}. Furthermore, animals that were forced to remain active exhibited a subsequent inactivity rebound. The same authors were able to replicate their findings in scorpions¹⁰⁸. More recently, an interesting study concluded that sleep deprivation was deleterious to another species of cockroach, even suggesting a lethal effect¹⁰⁹. In the

early studies, behavioural states, including quiescence, were determined by human observation.

Evidence in crayfish The crayfish is a historical model in neurobiology, in particular, due to the extensive study of the encoding of its fast escape behaviour¹¹⁰. An interesting study, which investigated sleep in *Procambarus clarkii*, found that quiescence behaviour was consistent with an elevated response to mechanical stimuli and that keeping individuals awake (using bubbling water) resulted in a rebound¹¹¹.

Furthermore, electrophysiological recordings of resting crayfish indicated a consistent slow rhythm, which was later studied by others^{112,113} and suggested as an analogue of mammalian SWS.

Honey bees The honey bee, *Apis mellifera*, has a remarkable brain plasticity and is notoriously capable of unique cognitive abilities such as various forms of learning, advanced navigation and communication^{114–116}. In addition, circadian clocks are essential for bees as they use it, in conjunction with the position of the sun, to navigate^{117,118}. For these reasons, they constitute a compelling model for the study of sleep and circadian rhythm in the context of learning and plasticity.

It was shown that bees have long bouts of inactivity that correlate with an increased arousal threshold^{119–121}. Furthermore, it was suggested that sleep deprivation led to a subsequent alteration of their behaviour, with bees showing deeper — but not longer — sleep episodes¹²².

Nurse bees and foragers are reported to have different sleep patterns¹²³. Foragers are also capable of dynamically allocating sleep time according to food availability¹²⁴.

Sleep deprivation is thought to impact cognitive functions. Namely, impairing the waggle dance¹²⁵ and the consolidation of odour memory¹²⁶. In addition, the presentation of a context odour during sleep has been shown to enhance memory consolidation of bees¹²⁷ — a phenomenon previously observed in mammals¹²⁸.

Drosophila The fruit fly *Drosophila melanogaster* is one of the most widely used biological models. Historically, it has primarily been instrumental to the field of genetics and developmental biology, but grew as a model for, amongst a long list, ageing, population genetics, immunity and behaviour²².

Indeed, from the late 1970s, Seymour Benzer used it as a tool to demonstrate that behaviours could be determined by genes¹²⁹. Perhaps the most successful achievement of this approach was the uncovering of the molecular mechanisms of the circadian clock.

It is, however, only later, in the early 2000s, that *Drosophila melanogaster* emerged as a model of sleep, with two seminal articles showing that its quiescence fulfilled the three criteria of the behavioural definition of sleep^{130,131}. In order not to interrupt our journey, from branch to branch, into the phylogeny of sleep, I will restrain my urge to immediately elaborate on the literature that followed these seminal studies and refer the impatient reader to section 1.3.

1.1.3.5 Nematodes

In the last decade, the existence of a sleep-like state has also been shown the roundworm, *Caenorhabditis elegans*¹³². This widely used biological model only possesses a simple nervous system with barely more than 300 neurons¹³³, which makes it a compelling candidate to study the genetics and neuronal origins of behaviours¹³⁴.

C. elegans develops by going through discrete moults, and it had been noticed that, during the few hours that precede each ecdysis (*i.e.* the process of moulting), worms exhibit a period of quiescence that was named ‘lethargus’¹³⁵. Later, it was proposed that lethargus is a ‘sleep-like state’¹³⁶. Indeed, the latency of a lethargic worm to respond to a relevant chemical stimulus is increased. In addition, lethargus, seems homeostatically regulated since mechanically or chemically stimulating quiescent animals results in a subsequent decrease of activity^{136,137}. Lethargus is therefore seen as a ‘developmentally-timed’ form of sleep¹³².

Another sleep-like state was identified in worms which, in contrast, is induced by stress. This ‘stress-induced’ sleep occurs after the exposition to a stressor and also involves, at least, an elevated response threshold¹³⁸.

Crucially, both types of sleep described in worms are *not* regulated in a circadian manner. Stress-induced sleep is fundamentally different from sleep described in other animals insofar as it is not constitutive, but induced. Developmentally-timed sleep is also singular in that it is only described in the immature larvae rather than in the adult animal.

1.1.3.6 Molluscs

Molluscs are a major phylum with nearly 100,000 species, most of them aquatic ones, but also some adapted to life on land (*e.g.* snails)¹³⁹. From a neurobiological perspective, they are particularly interesting as they feature a range of cognitive capabilities, from sessile organisms, such as Bivalvia, with a limited nervous system, to Cephalopoda and their astonishing learning capabilities.

Aplysia In the 1960s, it was using a mollusc, the sea slug *Aplysia californica*, that Eric Kandel and his team were able to reveal the cellular basis of simple memories and habituation^{140,141}. Recently, a study investigated sleep in this landmark model, showing that *Aplysia* exhibits prolonged periods of quiescence, which are associated with place preference¹⁴². In addition, its latency to respond to ecologically relevant stimuli, such as the addition of food or an increase in salt concentration, was increased when quiescent. Finally, a 12 h sleep deprivation in the night, led to a large rebound during the following day. Rest state was determined by human observation.

Lymnea An interesting study was carried on the great pond snail, *Lymnaea stagnalis*¹⁴³. The authors videotaped the behaviour of animals for almost three months and described the occurrence of quiescence bouts lasting several minutes. They also reported that behavioural rest correlated with higher response thresholds, to both appetitive and aversive stimuli. Crucially, they failed to characterise an underlying homeostatic control of sleep — *i.e.* no rebound. In addition, the organisation of quiescence bouts was characteristic of a random walk rather than regulated in a circadian fashion. In this study, quiescence was determined by human observation.

Cephalopoda Cephalopods are noticeable amongst molluscs for their outstanding learning ability. A study reported the existence of period quiescence in *Octopus vulgaris* that correlates with a decreased brain activity¹⁴⁴.

Another article, this time in the cuttlefish, *Sepia officinalis*, characterised a resting behaviour that is, to a limited extent, homeostatically regulated. Indeed, compensatory quiescence was observed after a 48 h sleep deprivation — which was performed using a monitor to display constant visual stimuli¹⁴⁵.

Neither studies reported a change in arousal threshold during quiescence and both scored behaviour manually, using explicit criteria.

1.1.3.7 Cnidaria

A recent study has pushed further the boundaries of the field with the discovery of a ‘sleep-like’ state in the jellyfish *Cassiopea spp*¹⁴⁶. The authors found, firstly, that the pulsatile activity of *Cassiopea* was much lower, and had long pauses, at night. Secondly, during their quiescence bouts, animals showed lower responsiveness to a mechanical stimulus. Lastly, recurrent perturbation during the night (*i.e.* a jet of water every 20 min), which increased activity, resulted in a subsequent rebound after the perturbation had stopped.

These findings is particularly interesting as the Cnidaria (jellyfishes, but also sea anemones and corals) branched away from the Bilateria (*e.g.* arthropods, molluscs and vertebrates) 600 million years ago, before the latter evolved a central nervous system, and therefore have only a simple, distributed, network of neurons^{147–149}, hence challenging the notion that sleep evolved as a requirement for the central nervous system.

1.1.4 Functions of sleep

Since, as I just described, sleep appears so widespread throughout phylogeny, it is plausible that it could serve a core set of functions that all animals would need. There has been a broad range of suggestions as to what such roles could be: development of the brain, learning and memory, metabolism, immunity and others. However, there is no universal consensus on one single function of sleep^{23,48}. Before presenting the three main hypotheses in the literature on this subject, I would like to address, perhaps, the most obvious question at this stage: *is sleep a vital need?*

1.1.4.1 Is sleep a vital need?

There is no doubt that the lack of sleep has widespread physiological consequences in some organisms. However, the idea that sleep is vital — to the extent that sleep deprivation eventually causes death — is not consensual. For example, Chiara Cirelli and Giulio Tononi have argued that sleep is essential⁴⁸, whilst Jerome Siegel

pointed out the lack of evidence to support such a view²³. Indeed, the literature on the prolonged effects of sleep restriction is far from comprehensive — considering the central importance of the question — and is partly dated. Experiments addressing this matter have hitherto been reported only in a handful of model organisms: dogs¹⁵⁰, rats^{151,152}, pigeons¹⁵³, cockroaches¹⁰⁹ and fruit flies¹⁵⁴.

In all but one tested models, sleep deprivation led to death. However, the physiological explanations are, to date, unclear. In both rats and dog pups, death was linked to dramatic metabolic changes and obvious signs of distress, altogether suggesting that mortality could result from a generalised and confounding effect of the undergone stress rather than the sleep deprivation itself^{150,152}. The same chronic sleep deprivation paradigm that had been reported to kill rats did not kill pigeons¹⁵³. In the Pacific beetle cockroach (*Diploptera punctata*), sleep deprivation was performed by continuously stimulating the insects¹⁰⁹, but did not account for exhaustion-induced stress — which is known to be lethal to other species of cockroaches¹⁵⁵. Finally, in *Drosophila* the evidence is limited to one study that observed partial lethality in a very small number of wild-type flies (4 of the initial 12 individuals died after 70 h), which the authors sleep deprived manually by tapping the insects' tube with their fingers¹⁵⁴.

An alternative approach to address this question is to study the trade-off between sleep and fitness by asking whether sleep amounts correlate with, for instance, lifespan. Humans affected by fatal familial insomnia, a rare condition, are known to live only a few months after the onset of the disease¹⁵⁶. In fruit flies, some mutants that have reduced sleep incidentally die faster¹⁵⁷, but others do not¹⁵⁸. Such correlations have, however, been more difficult to characterise in non-pathological cases. For instance, in humans, epidemiological studies have shown that low, but also high, sleep amounts are associated with increased mortality (*i.e.* a U-shape relationship)^{64,65}, but there is no consensus on the matter⁶³. In fruit flies, a recent study artificially selected populations according to their amount of activity, and showed that, even though sleep amount is highly heritable, selected low and high sleepers had the same lifespan as the control populations¹⁵⁹.

1.1.4.2 Energy conservation

Perhaps the most obvious general function that sleep could have is simply not to waste energy being active. Indeed, within an ecosystem, organisms tend to adapt to partition their respective niches, one example being temporal division. Namely,

most animals are either nocturnal, diurnal or crepuscular, with various degrees of specialisation. A consequence of temporal partitioning of ecological niches is that the physiology of organisms is tuned to a specific range of abiotic environment that vary over 24 hours (*e.g.* light, humidity and temperature). In addition, their biotic environment may oscillate too. For instance, the parasite and predation pressure, as well as the availability of food, have their own daily dynamics. In this context, it is advantageous to minimise risk and expend the least possible energy whilst waiting for the time of the day with the most favourable conditions¹⁶⁰.

Following this principle, sleep would have evolved as—and, to a large extent, would still be—a daily state of dormancy or torpor²³. In addition to daily oscillations, there are ecologically relevant rhythms along which many animals engage in reversible states of immobility. At low tide, many marine invertebrates become immobile which both protects them and saves their energy^{161–163}. Likewise, some species respond to seasonal variation by entering states such as hibernation or aestivation, which, in many respects, resembles sleep²³. In temperate and dry climates, for instance, land snails are mostly dormant during the summer with a greatly reduced overall metabolism^{164,165}, but can revert to an active state when and if precipitations occur. Over evolutionary times, organisms that developed adaptations to seasonal variations may eventually depend on them, which could mean they have become homeostatically controlled. For instance, many plants absolutely need periods of dormancy to germinate or flower^{166,167}. Similarly, sleep could have evolved from an opportunity to a necessity. In brief, under the energy conservation hypothesis, sleep is primarily and mostly a daily state of torpor that in some, but not all, taxa would have been co-opted to support a variety of other physiological processes.

1.1.4.3 Restoration

Alternatively, it has been suggested that sleep primarily serves a restoration purpose whereby the physiological and metabolic expenditures that happened during the day, can be counterbalanced by a period of rest during the night¹⁶⁸ (for nocturnal animals). There are several examples of such ‘active’ recuperative processes, many of them relating to brain functions, but also several showing the implication of sleep in the replenishment of other physiological systems.

Brain functions Prolonged wakefulness has been shown to result in the accumulation of reactive oxygen species, causing oxidative stress that explains some of the effects of sleep deprivation¹⁶⁹. Similarly, a notable study in rats suggested that sleep was linked with an increased interstitial space between brain cells, which could be crucial in order to clear toxic by-products that accumulate during wakefulness¹⁷⁰.

Distributed functions The immune system has also been linked to sleep in two ways (reviewed in^{171,172}). Firstly, sleep is altered during infections. For instance, in rabbits, the infection by various micro-organisms causes an increase in sleep¹⁷³. This also happens during the response to non-infectious components of the pathogens, such as lipopolysaccharide¹⁷⁴. Secondly, sleep deprivation compromises immune competence. For instance, it was shown that partially sleep-deprived humans immunised against the flu had subsequently fewer antibodies¹⁷⁵. However, the conclusions are not consensual, as some studies show, instead, a protective effect of sleep deprivation on immune functions¹⁷⁶. Suggesting non-linear effects of sleep loss on immunity¹⁷¹.

Beyond immunity, sleep has been suggested to take part in other distributed processes. In rats, it is thought to be involved in efficient wound healing¹⁷⁷. Sleep has also been presented as a mechanism to restore muscles¹⁷⁸. Recently, an interesting study in mice proposed that the skeletal muscles could be implied in its regulation, suggesting a crucial role in muscular recovery¹⁷⁹.

1.1.4.4 Learning and memory

One of the most compelling putative function of sleep is its role in brain plasticity. Indeed, most animals with a central nervous system are capable of some sort of learning, the flip side of which could be the burden of sleeping. The idea that sleep plays a crucial role in the formation of memories is relatively old¹⁸⁰. It has since been supported by a large corpus of experimental work on various models (reviewed in¹⁸¹). For example, spatial memory is impaired in sleep-deprived rats¹⁸². Some forms of learning, such as the visual discrimination task, requires sleep after training¹⁸³. In human subjects, the learning of motor sequences is greatly improved after a night of sleep¹⁸⁴, but not if subjects are kept awake¹⁸⁵.

The mechanism by which sleep affects memory is, however, unclear and two apparently contradictory theoretical frameworks are currently debated: the *active*

system consolidation hypothesis and the *synaptic homeostasis hypothesis* (reviewed in¹⁸⁶ and¹⁸⁷, respectively).

Active system consolidation hypothesis It states that, during wake, events are perceived and encoded in the brain in a shallow fashion. During sleep, the nervous system would ‘replay’ these events and the connections would be strengthened and redistributed^{188,189}. This would allow brains to form new, persistent, representations that are more symbolic and abstract^{186,190}.

In mammals, there is evidence that such process happens during SWS in particular, highlighting the interplay between the hippocampus and the neocortical regions^{128,191}. Behaviourally, it also accounts for the observation that sleep qualitatively changes memory, allowing for new representations^{192,193}.

Synaptic homeostasis hypothesis A relatively recent theoretical framework that associates sleep to brain plasticity is the synaptic homeostasis hypothesis^{194,195}. It accounts for sleep as the ‘price to pay for plasticity’. It is built on the notion that learning generally involves gathering input from the environment, which happens during wake — as the nervous system is connected to the outside world. It postulates that, during wake, synapses between neurons are potentiated (*i.e.* strengthened), hence creating associations and memories. Then, during sleep, the brain is essentially off-line, which allows it to sample comprehensively the links previously drawn. In this process, synapses that are not strong enough may be down-selected (*i.e.* downscaling) in order to preserve only robust associations¹⁸⁷. In addition to reducing the noise generated during wake, downscaling would also promote the encoding of memory in subsequent wake periods.

The synaptic homeostasis hypothesis has been corroborated at several levels — at least in mammals (reviewed in¹⁸⁷). Firstly, molecularly, proteins that are involved in potentiating synapses are more abundant and active during wakefulness^{196,197}. Secondly, several studies have excited upstream neurons during sleep and measured the post-synaptic response, showing that, after sleep, synapses were globally weaker^{196,198,199}. Lastly, structurally, it has been observed that mice neurons have more dendritic connections during wake, and that their number reduce after sleep²⁰⁰.

1.1.5 Regulation of sleep

Sleep is generally postulated to be regulated both by the internal clock (process C) and by the homeostat (process S). This view has been formalised Alexander Borbély as the ‘two-process model of sleep regulation’ (fig. 1.1)^{45,201,202}. In its original form, it states that the propensity to sleep is the sum of the effect of C and S . The first arm, C , is a periodic function of time and independent of the occurrence of sleep. The second one, S , represents the accumulation of sleep pressure in an animal that remains awake and is, therefore, a conditional function of the realisation of past sleep. When sleep happens, S decreases exponentially. On the other hand, when sleep cannot occur, it increases asymptotically. $S + C$ defines the overall sleep pressure that, above a certain threshold H^+ , triggers sleep. Conversely, when $S + C$ becomes lower than H^- , the animal wakes up.

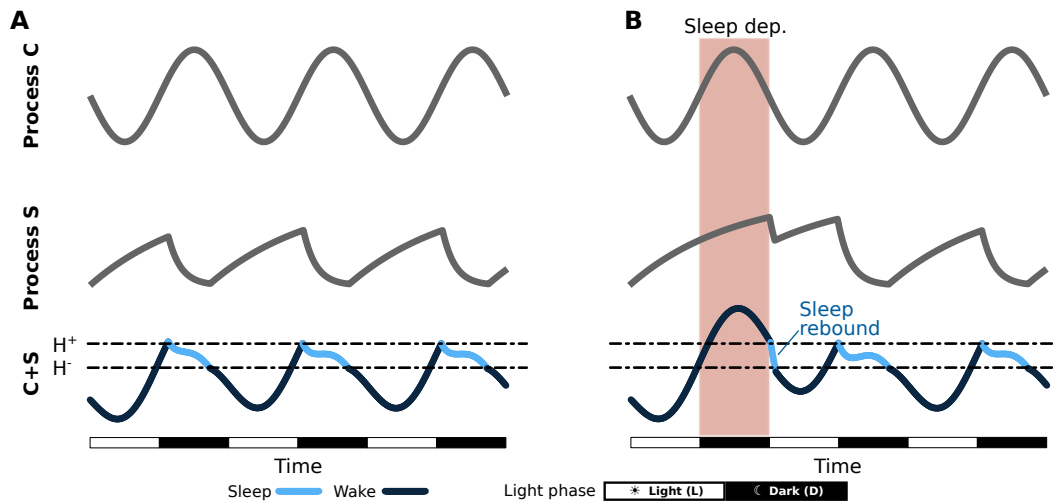


Figure 1.1: *The two-processes model of sleep regulation.* Theoretical example illustrating the model for a diurnal animal, over three days, in two scenarios: either it is allowed to sleep throughout (**A**), or it is prevented to do so during the first night (*i.e.* sleep deprivation, red background, **B**). The propensity to sleep is the sum of two processes: C and S . C , the clock, is a periodic function that does not depend on sleep pressure. S , represents the homeostat. It decreases when sleep occurs (blue), and increases when it does not. Sleep occurs when $C + S > H^+$, and an animal wakes up when $C + S < H^-$. **A**, In normal circumstances, C and S add up to a periodic function that is the sleep propensity, and sleep is the realisation of such propensity. **B**, During sleep deprivation, the clock (C) is unaltered, but the sleep homeostat (S) increases asymptotically. When sleep deprivation is released, the animal may still have an overall sleep pressure ($C + S$) greater than (H^+) and will dissipate S exponentially. In this example, there is such a sleep rebound, during the day, immediately after sleep deprivation.

Under normal conditions, when an animal is allowed to sleep, process S synchronises with C to produce a regular binary sleep pattern (fig. 1.1A). When sleep cannot occur—for instance, because an external factor prevents it—process S continues to increase until it can dissipate (fig. 1.1B). Interestingly, in some cases

the recovery of S (sleep rebound) can be only partial as process C may wake an animal before it has fully recovered. Under the two-process model, this may result in an earlier onset of subsequent sleep. If this model has served as a major conceptual framework since its postulation, in the 1980s, it has also been refined²⁰³ and adapted²⁰².

In conclusion to this general introduction, the open definition of sleep has allowed is discovery and investigation in many species, which has led to the view that sleep its a ubiquitous behaviour. In the next two sections, I will describe why *Drosophila melanogaster* has emerged as a powerful model to unravel the mechanistic aspects of behaviour, and how it became instrumental in studying sleep.

1.2 *Drosophila melanogaster*, a toolbox to study behaviours

In order to fully grasp how and why the fruit fly became a significant model to study sleep, I believe it is necessary to first see how it emerged as a powerful tool to study the genetics of behaviour in general. In this section, I will briefly present, for the reader who may not be familiar with it, general aspects of the biology of *Drosophila melanogaster* — the interested one will easily find much better written historical, practical and fundamental material, for instance^{204–206}. Then, I will explain specifically the context in which it developed as a model for behaviour. Finally, I will describe some of the methodological resources that have emerged in the field.

1.2.1 Life history of *Drosophila melanogaster*

Drosophila melanogaster is a 3 mm long insect belonging to the Diptera order — a taxum with more than 100,000 species of insects, including all flies and mosquitoes²⁰⁷. The genus *Drosophila* itself contains more than 1500 species²⁰⁸.

The original habitat of *D. melanogaster* is thought to be in sub-Saharan Africa from which it would have dispersed to Europe and Asia only 10,000 to 15,000 years ago, and more recently to America and Australia. The expansion of its habitat is thought to be related to human migrations²⁰⁶.

Like other Dipera, fruit flies are holometabolous organisms (*i.e.* with complete metamorphosis) whose life history is characterised by an asexual larval stage that has a different ecological niche and morphology from the adult. Adult females lay up to 100 eggs per day in rotting fruits in which their larvae will hatch and develop. *Drosophila melanogaster* larvae, like other arthropods', grow discontinuously through successive moulting events. In the case of fruit flies, there are three larval stages (*i.e.* instars). It takes about four days (at 25°C, with unlimited food supply) for larvae to reach the end of their third, and last, instar, after which they crawl out of their substrate and initiate pupariation. During pupariation, which lasts approximately four days in *Drosophila melanogaster*, the insect undergoes dramatic anatomical and physiological changes. When the metamorphosis is complete, the insect's puparium splits, and the imago (*i.e.* the final adult form) emerges. Adult flies live up to 90 days at 25°C, in the laboratory, though their

lifespan in the wild is thought to be shorter²⁰⁹.

1.2.2 A century of research on the fruit fly

In the beginning of the 20th century, the principles of heredity, and their link to the theory of natural selection, were a matter of great interest and controversy, but an outlier group, led by Thomas Hunt Morgan, was to revolutionise the field of genetics with the help of a tiny insect: the fruit fly *Drosophila melanogaster*.

Drosophila melanogaster had been described by Johann Meigen in 1830, and was studied by several groups. In particular, Frank Lutz was using it to perform experimental evolution, in a Darwinian framework. It is thought to be Lutz who introduced Morgan to the fruit flies. Morgan eventually noticed heritable changes, such as the white eye mutation, and observed that their transmission was non-Mendelian: instead, some heritable traits were linked to one another, which corroborated a chromosomal theory of heredity²¹⁰.

His seminal work initiated a century of fruitful work using *Drosophila*, which was punctuated by several Nobel prizes, starting by Morgan himself in 1933. Later, Morgan's former student, Herman Muller, developed a technique to generate random mutations using X-rays which was also awarded the prize in 1946. The use of the *Drosophila* model then propagated to other fields than genetics. In 1995, Edward Lewis, Christiane Nüsslein-Volhard and Eric Wieschaus, were credited for their use of the fruit fly to unravel the genetics of embryonic development²¹¹. In 2011 the prize was shared by Jules Hoffman for the work he and his team carried on innate immunity^{212,213}. Altogether, by the mid-twentieth century, it made no doubt that *Drosophila* was an instrumental tool to study the genetic underpinnings of physiological and developmental processes.

The extent to which single genes could determine much more 'complex' phenotypes such as behaviours was, however, vividly debated^{129,214}. In the late 1960s Seymour Benzer reasoned that specific behaviours could be investigated using the same principles as for other traits, and he designed a simple quantitative assay to score light preference in a population of flies. He then carried a genetic screen and discovered mutants with altered phototaxis and isolated the genes responsible for it²¹⁵, hence demonstrating genetics could be used to address the molecular basis of behaviour^{216,217}. In the early 1970s, Benzer and his team used the same approach and created an assay to score eclosion time and locomotion which led to the discovery of the *period* gene, a core gear of the circadian clock. Follow-

ing Benzer’s footsteps, in 2017, Jeffrey Hall, Michael Rosbash and Michael Young were awarded the Nobel prize for their work of the molecular mechanisms of the circadian clock^{218–222}.

By the end of the twentieth century, two lessons had been learnt. Firstly, the research on fruit flies had provided the community with a formidable set of tools to study of how genes can determine even complex phenotypes. Secondly, the molecular determinism of ubiquitous processes, such as development, immunity and circadian clocks could then be generalised and translated to other organisms. In the early 2000s, when the community needed a simple model to study the genetics and neuronal basis of sleep⁴⁰, the fruit fly emerged as a natural candidate^{130,131}.

A large part (the next two chapters) of the work I present in the thesis herein is methodological. Therefore I will use this section, to specifically review the methods developed to study behaviours (in particular sleep behaviour) in *Drosophila*, and will dedicate the next subsection (1.3) to the discoveries resulting from their application to sleep during the last two decades.

1.2.3 Study of behaviour in *Drosophila*

1.2.3.1 What are behaviours

It is complicated to formally define behaviour in the context of biology, and—implicit or explicit—definitions vary between authors^{223–227}. Here, I will define behaviour in a broad and computational sense as a time series of observable states that are organised in a hierarchical fashion. States can be, for instance, postures, sounds, visual displays, chemical signals or any combinations of these. They can vary over time and may be either discrete (within a repertory) or continuous (bounded to a range). Behavioural states are hierarchical insofar as a state may be defined by a time-series of sub-states²²⁷. In this respect, states are contained in states of higher order, much like linguistic entities (*e.g.* syllables make words and words make sentences)^{228–231}.

To illustrate this hierarchical organisation, one can think of the courtship behaviour in fruit flies. Throughout its life, a male may engage in different states such as feeding, resting, flying, walking, grooming or courting a female. These are behaviours of high order, which are observable over a long timescale. However, in our example, the courting behaviour is itself made of a series of lower order behaviours such as chasing, wing extension or wing vibration (see²³² for a review).

Then, at an even shorter timescale, a state such as ‘wing vibration’ can be seen as a series of individual singing bouts, and each bout as a series of pulses — each level having its own ‘grammar’²³³.

In the next two subsections, I will start by describing a few landmark studies to illustrate how the interest in behavioural genetics preceded by far the application of high-throughput methods to it. Then, I will show how, in recent years, ethology has increasingly relied on high-throughput data acquisition and data sciences. Even though the study of behaviour goes far beyond *Drosophila* — with lots of laboratory work on mice, *C. elegans*, zebrafish and many more —, I will narrow the scope of this introduction to the work on the fruit fly.

1.2.3.2 Predigital era

During most of the 20th century, it was not possible, or straightforward, to digitalise data. Benzer, whose early work I just described, was not the only researcher who had to invent ingenious paradigms to score high-level behaviours. Beyond circadian activity and phototaxis, behaviours such as courtship, foraging, egg laying or even, for instance, learning were under scrutiny.

Courtship is a stereotyped behaviour that is generally directed from males to females, and that is central to a male’s fitness²³⁴. As such, it has been extensively studied. It was noticed that this fundamental behaviour could be altered in the *fruitless* mutants^{235,236}. In particular, it was observed that, in this genotype, males courted other males, sometimes forming ‘chains’. Courtship is subdivided in several parts which were — and often still are — scored through subjective observation. Interestingly, in order to increase throughput and decrease subjectivity, assays such as the ‘chaining index’, which scores the proportion of males engaged in courting chains, were proposed^{237,238}.

In 1980, Marla Sokolowski published her discovery of a naturally occurring difference of foraging behaviour in the larva. In the presence of patchy food, she noticed that some animals, the ‘rovers’, were consistently covering a large distance whilst others, the ‘sitters’, had a lower propensity to leave their food patch. This foraging behaviour could be quantified with an elegant assay as larvae leave a visible trail — whose length can be approximated — on their substrate. The molecular determinism of this trait was then identified²³⁹, and is considered ‘the first example of the molecular identification of a naturally occurring behavioural variation’²¹⁴.

1.2.3.3 Ethomics, a high-throughput approach to behaviour

In the last few decades, our capacity to record and process large quantities of scientific data has tremendously increased²⁴⁰, and biology is undergoing a transition towards data sciences. These recent developments have virtually affected all fields from structural biology²⁴¹ and genetics²⁴² to neurosciences²⁴³ and ecology²⁴⁴.

The study of animal behaviour is also undergoing its own computational transition, which has prompted the terms ‘ethomics’^{223,245} and ‘computational ethology’²²⁴. It is now accepted that ethology too can benefit from quantitative sciences such as machine learning, physics and computational linguistics^{225–227,246}. There are two types of interdependent technological breakthroughs that underlie the current transition. Firstly the availability of new high-throughput data acquisition methods. Secondly, the application of computer vision, statistics and the computational techniques to the processing of the resulting data.

In the context of high-throughput biology, data acquisition techniques involve recoding physical phenomena as *digital* time series. There is a variety of approaches ranging from building a specific hardware that records directly variables that are biologically meaningful, to using general purpose recording equipment and algorithmically interpreting the primary data into meaningful secondary data.

One of the most widely used specific hardware is the *Drosophila* Activity Monitor (DAM) system (Trikinetics Inc.) that I will describe in detail in subsection 1.3.2.1 which is used to score walking activity. Two recent methods have been proposed to monitor feeding behaviour using the electrical signal generated when the fly touches its food^{247,248}. Jamey Kain and co-workers described an advanced system designed to track the movement of all six legs on a fly walking on a ball, and analyse behaviour in real time, with the possibility of performing closed loop experiments (*e.g.* deliver stimuli when the animal turns right)²⁴⁹. Karl Gotz designed a system to record the amplitude of wingbeats in a tethered fly²⁵⁰. In a series of experiments, the group of Michael Dickinson then used this system, in addition to the recording of several flight variables (*e.g.* pitch, roll and yaw) in a closed-loop manner to study response to visual signals^{251,252}.

The other approach is to use off-the-shelf tools such as microphones and cameras. For instance, Benjamin Arthur and collaborators have used an array of microphones to record the courtship songs of 32 males at a time, for several consecutive hours²³³. They then used signal processing techniques to extract meaningful acoustic secondary data.

In recent years, cameras and computer power have become less expensive. Therefore, there have been countless applications of video tracking to the study of fly behaviours. From the 1990s already, video analysis had been used, for instance, to study whether the locomotor activity was organised in a fractal fashion²⁵³ and to the dynamic of trajectories²⁵⁴. Various implementations of video monitoring have been used for sleep research to score immobility of many animals over several days^{255–258} (see subsection 1.3.2.1). Interestingly, setups using multiple cameras have been used to reconstruct flight trajectories²⁵⁹.

Rather than focusing on one individual, some paradigms have been designed to automatically score interactions between two animals, for instance aggression²⁶⁰ and courtship²⁶¹. Furthermore, some software tools were designed to track multiple animals in a social context^{245, 262–264}. In the last year, algorithms using deep learning have been proposed to track unmarked animals, whilst minimising identity loss²⁶⁵ and to detect body posture²⁶⁶.

Noticeably, some methods have also been proposed to video track larvae, which are translucent^{267–269}. Recent developments include the use of frustrated total internal reflection to increase optical contrast^{270, 271}.

In general, the low-level representations (*e.g.* pixel and position) that are generated by recording equipment (or by algorithms) need to be processed further in order to make biological sense, and there is also an ongoing effort to develop methodological and conceptual frameworks in this direction^{226, 227}.

For instance, Joshua Vogelstein and colleagues applied multiscale unsupervised structure learning²⁷² to hierarchically cluster larval behavioural time-series, and studied the genetic determinism of these complex phenotypes²⁷³. Another study, by Berman *et al.*, proposed a pipeline in which the repertoire of behaviours is represented as a behavioural space²⁷⁴. The authors first used spectral features to generate a high-dimensional space, and then applied t-SNE²⁷⁵ to embed feature vectors and cluster states. In a follow-up article, they develop the idea of transitions and hierarchy in the behavioural space²³⁰.

In conclusion to this section, *Drosophila* has proved as a powerful tool to study the genetic determinism of behaviour. In recent years, technological and conceptual developments have promoted it at the forefront of neurobiology and ethomics. In the next section, I will describe how, from this substrate, *Drosophila* emerged as a model for sleep.

1.3 Sleep in *Drosophila melanogaster*

1.3.1 Generalities

The ‘droso’ in ‘*Drosophila*’ means ‘dew’, reflecting its preference for dawn and dusk, when dew condensates. Indeed, it has long been known to be crepuscular (*i.e.* active during the twilight). It is consensual that fruit flies have an evening and a morning peak of activity, centred on the light transition time (fig. 1.2). Conversely, sleep is thought to peak in the middle of the day (*i.e.* ‘siesta’) and night.

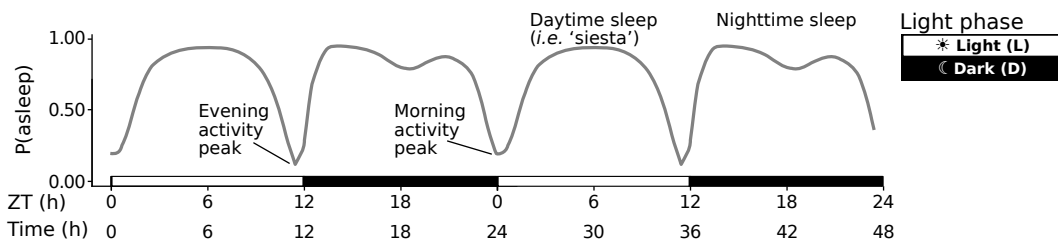


Figure 1.2: *Example of sleep-time visualisation.* Simplified ‘sleep trace’ representing the probability of observing a sleeping fly, $P(\text{asleep})$, over time. This example represents the usual behaviour of a male population. Time is often expressed as Zeitgeber Time (ZT) which is $ZT = \text{Time} \pmod{24 \text{ h}}$, and zero corresponds to the start of the day (phase L). In this example, the light regime is 12 h of light and 12 h of darkness (12:12 h, L:D). Males tend to have a bimodal activity with a morning and an evening peak, and are inactive in between.

The dynamic of sleep is greatly affected by biotic and abiotic factors, but also by how sleep is defined and scored. In this section, I will first detail the methods that have been used to characterise, score and perturb sleep in *Drosophila*, and their recent development. Then, I will describe how internal and external variables impact sleep. Finally, I will present the putative functions of sleep in fruit flies.

1.3.2 Methods and paradigms to study sleep

Methods often frame and drive scientific research, but have also been argued to hold epistemic value and, as such, they impact the nature of scientific conclusions in a given field²⁷⁶. The behavioural definition of sleep I presented in subsection 1.1.2.1 relies on three pillars (*i.e.* quiescence, homeostasis and arousal), which prompts the need for three corresponding methods: the ability to *score immobility*, the possibility to *sleep-deprive* flies and tools to *probe arousal*. In this section, I will describe the tools and paradigms that have been proposed to address each of these three needs.

1.3.2.1 Immobility scoring tool

Early work and manual scoring In 2000, two seminal studies addressed the question of sleep in the fruit fly^{130,131}. Hendricks and colleagues performed an Infrared (IR) beam cross locomotion assay (see fig. 1.3) on a small number of animals ($N = 11$), but crucially also videotaped the flies¹³⁰. They noticed that flies engaged in long bouts of inactivity in proximity with, but not on, the food and during such immobility bouts, animals adopted a specific posture. They then videotaped a population of flies in a Petri dish and scored, manually, their immobility. The authors noticed a satisfactory correspondence between the manual scoring from video data and the IR assay. Therefore, in order to automatise behaviour quantification and, ultimately, increase throughput, they continued with the latter assay.

The second study used an ultrasound system to perform high-resolution measurement of fine movements¹³¹. The authors first ensured their system matched visual observations. Then, in order to work with a larger number of flies, they adopted the same IR assay as¹³⁰. They validated this decision by recording 7 flies for 18 h, only finding 2.35% false negative of motion (animals that were scored as moving by the ultrasound system, but not crossing the IR beam).

The Drosophila Activity Monitor (DAM) The DAM system (TriKinetics Inc.) is a tool to measure the activity of fruit flies which was primarily designed for the study of the circadian rhythm²⁷⁷. A single device (fig. 1.3A) can record the movements of up to 32 animals over long durations. Each fly is contained in a narrow glass tube along which it walks. When an animal crosses the midline of its tube, the device detects it through an IR beam and sensor (fig. 1.3B). Because of its prior availability, robustness and scalability, this system has met large adoption in the field and, to date, remains the most widely used tool to score sleep in fruit flies.

The DAM paradigm cannot exclude the possibility that an animal could be very active without crossing the midline of its tube. For instance, a fly could walk and turn before reaching the tube's midline, or spend a large fraction of time eating or grooming, which would result in falsely scoring immobility and ultimately overestimating sleep¹²⁷. In order to reduce the possibility of such low amplitude movement being unnoticed, TriKinetics also developed a 'multibeam' system (DAM-5), which is an upgraded version with 17 beams. However, it is less cost and space effective

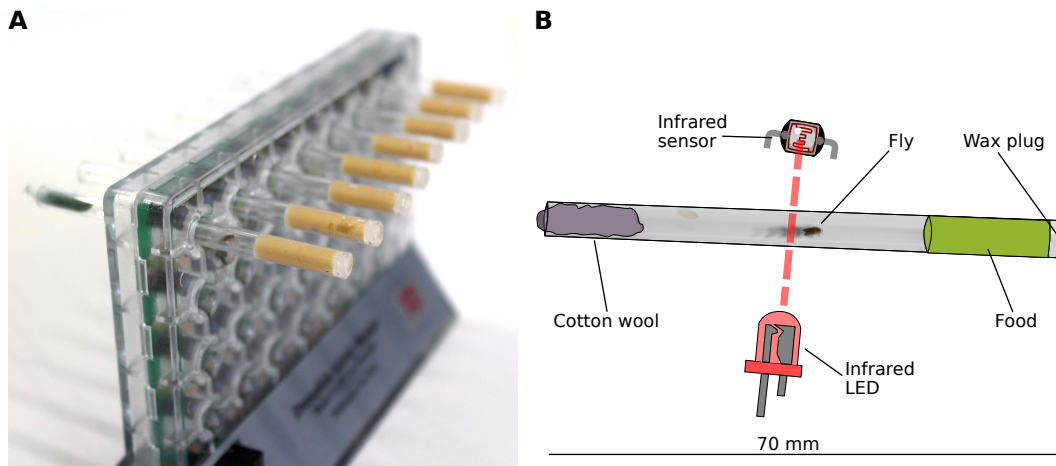


Figure 1.3: *Drosophila Activity Monitor*. **A**: a Drosophila Activity Monitor (DAM), the most widely used system to monitor walking activity and sleep in *Drosophila*, with 8 tubes loaded. Each monitor can hold up to 32 single flies in glass tubes. Multiple monitors can run in parallel, and their data is centralised on an acquisition computer. **B**: Schematic of a single tube (≈ 70 mm long) showing the underlying mechanism of the DAM. Each fly is contained between food on one side and cotton wool on the other. Tubes are sealed with paraffin wax. When the fly walks through the midline of its tube, it crosses an infrared beam which is detected as movement. The numbers of beam crosses are generally aggregated over one minute (*i.e.* the resulting sampling rate being 1 min^{-1}).

and has therefore only been used in a few sleep studies (for instance,^{278,279}).

Video tracking Another solution to address the issue of movement false negatives is to video track flies. Various authors, including myself, have contributed in this direction^{255–258,280,281}, but there seems to be little overall adoption in the field despite the availability of a wide panel of tools.

Zimmerman and colleagues developed a **Matlab & C++** software specifically to score sleep in *Drosophila*²⁵⁵. Using simple background subtraction and binary threshold, they were able to show that, under some circumstances (sex, age, genotype and time of the day), DAM critically overestimated sleep. Donelson *et al.* wrote ‘Tracker’ (a **java** program) also to quantify sleep and confirmed these findings²⁵⁷. A video-based tracking tool was also developed by my supervisor, Giorgio Gilestro, with emphasis on scalability²⁵⁶. Ethovision, a commercial software with a broader scope, has also been used in some studies^{278,282}. ‘DART’, a **Matlab** program was designed as a solution to track and stimulate flies in the context of sleep²⁵⁸. Recently, Murphy and co-workers developed ‘ARC’, a system to score activity and feeding at the same time²⁸⁰.

Electrophysiological attempts There have been several attempts to find electrophysiological correlates of sleep behaviours in *Drosophila* by measuring the local field potential of tethered flies^{283–285}. In addition, calcium imaging²⁸⁶ and single neurone recording²⁸⁷ have been used to compare neuronal activity during sleep or wake. However, the evidence for an unambiguous read-out for sleep remains unclear—and the setup too impractical—to justify the wide adoption of this method.

The ‘five-minutes rule’ The first two studies in the field reported that startled wild-type flies were less likely to respond if they had been quiescent for five minutes or more^{130,131}. Later, another study corroborated this observation⁴³, which has led most authors to generalise this finding to define sleep as, at least, five consecutive minutes of immobility—a rule now widely used.

For instance, in some of the early landmark papers: ‘sleep was measured as bouts of 5 min of inactivity, as described previously’²⁸⁸, ‘a sleep episode was defined as a 5-min bin of uninterrupted quiescence’²⁸⁹, ‘sleep is defined here, as in previous work, as behavioural immobility lasting 5 min or more’¹⁵⁷ and ‘5 min intervals without any locomotor activity’¹⁵⁸.

However other studies have shown that responsiveness is not constant, but follows a temporal dynamic that may vary. In other words, it seems that arousal threshold is not a binary—or even a monotonic—function of the time spent immobile, and that it depends on other variables such as time of the day, sex and genotype^{258,285}.

1.3.2.2 Sleep deprivation tools

Several paradigms have been proposed to keep animals awake for various durations. They generally include the delivery of recurrent mechanical stimuli^{43,122,154,258,290,291}, but also hypnotic and stimulant drugs^{292,293}, exposure to conspecifics^{294,295}, olfactory cues (French *et al.*, in prep.) and thermogenetics^{296,297}.

Manual sleep deprivation In the first study reporting sleep in *Drosophila*, the authors started by sleep depriving flies manually¹³⁰. This allowed them to tune the timing of the stimulus delivery *dynamically*—that is, according to whether flies were immobile. Specifically, ‘mechanical stimuli were applied whenever ≥ 1 fly was immobile for ≥ 1 min’. In addition, they were able to deliver stimuli of appropriate strength: ‘a more intense stimulus was applied after 15 s if needed.

The stimuli were graded as 1 (one tap), 2 (two taps), 3 (move dish 1 mm) and 4 (lift dish and tap forcefully)'.

A similar approach was used by Shaw *et al.* in the second seminal study¹³¹, which allowed the authors to deprive individuals of rest 'by gentle tapping [of their tubes] for 12 hours during the dark period' to perform prolonged sleep deprivation¹⁵⁴.

Mechanical Automatic mechanical sleep deprivation has been adopted by many laboratories, but differ in terms of stimulus type, frequency and intensity between teams. For instance, Hendricks *et al.* spun fly tubes with a series for very short rotations, by short steps, alternating clockwise and counterclockwise. The stimulus lasted less than half a second and repeated each T , according to a uniform random distribution: $T \sim \mathcal{U}(30, 90)$ s⁴⁰. Shaw *et al.* built a system that dropped the glass tubes ten times a minute [Ref. 131, sup. material]. They later also developed a 'Sleep Nullifying Apparatus' (SNAP) that tilted animals, also every 6 s¹⁵⁴. Huber *et al.* implemented a similar solution with a device that would drop DAMs 1 cm (making the subjected fly fall to the bottom of its tube), 2–3 times per minute⁴³. Li *et al.* rotated vials with approximately 100 animals, around their long axis, for 1 min every 2 min²⁹⁰. Faville *et al.* use vibration motors to deliver a train of pulses every $T \sim \mathcal{U}(20, 40)$ s, with variable pulse number and duration²⁵⁸. In addition to devices that are built specifically to perform sleep deprivation, a number of studies have used readily available laboratory equipment such as orbital shakers and vortices^{297, 298}.

Drug Sleep deprivation can be achieved pharmacologically, using drugs such as caffeine²⁹², or methamphetamine²⁹³, which has the obvious limitation that the effects of such drugs are often unspecific and may impair a broad range of neuronal and behavioural functions²⁹⁹.

Thermogenetics Thermogenetics is a set of tools that allows neurobiologists to activate or inhibit a targeted set of neurons at a given time. For instance, using the UAS/Gal4 system, a population of cell expresses a thermosensitive channel. During an experiment, when the temperature is raised, the channels open and activate targeted neurons. This is reversed by lowering the temperature³⁰⁰. Such an approach was used to characterise wake-promoting neurons³⁰¹. Recently, it has also been used as a mean of sleep-depriving animals^{296–298}.

Social Instead of using a mechanical stimulus my team and myself have suggested that it could be more ecologically meaningful to deprive flies of sleep by exposing them to conspecifics. For instance, two males kept in a confined space for several hours are effectively sleep-deprived^{294,295}, and males are kept restless during their interaction with a female²⁹⁵.

Light Light is sometimes used as a mean to keep animals awake³⁰². It, however, also disrupt the circadian clock, which makes it difficult to separate clock from sleep drive.

1.3.2.3 Arousal probing

The paradigms to test the arousability of animals generally involve the delivery of stimuli and the quantification of the propensity of animals to respond to them. Often, it uses the same methods as sleep deprivation, but must also monitor their effect on flies. Instead of delivering binary stimuli and interpreting the response — or lack thereof — as the realisation of an underlying probability, sometimes, stimuli of ramping intensity are given until the animal wakes up^{43,130,258}.

Manual As described above, Hendricks and coworkers manually delivered stimuli of increasing discrete intensity¹³⁰. This way, they were able to categorise sleep depth in a population of flies. Some authors have assessed responsiveness by dropping one or two heavy objects near their experimental animals²⁷⁹.

Mechanical Several studies have used vibrations of increasing intensity to demonstrate that flies that had been immobile responded exclusively to the strongest disturbance^{131,258}. Huber *et al.* designed an automatic system in which the glass tube containing each fly was hit vigorously by a flap [Ref. 43, fig. 1A].

Heat Interestingly, escape response to gradual temperature increase was presented as a way to test sleep depth [Ref. 43,157, fig. 1D]. A faster variant of this approach was later used³⁰³. Surprisingly, although both studies indicate that flies do not habituate to heat — *i.e.* the response probability is consistent in time — over 48 h, neither suggest using heat as an effective mean of sleep-depriving flies.

1.3.3 Determinants of sleep

Like most complex phenotypes, sleep results from the interaction between genes and environmental variables. In a broad sense, environmental variables are either external factors, such as light, temperature and social context, or internal states such as the circadian clock, age and level of satiety. A variety of environmental and endogenous variables have been reported to impact the amount and the structure of sleep. In this subsection, I will describe what is known regarding the main determinants of sleep.

1.3.3.1 Genetic background

Quantitative genetic Quantitative genetic studies have described sleep amount as a very heritable trait. Across several mutant lines the broad-sense heritability — *i.e.* the proportion of the variance explained by genes — was high: $H^2 \approx 0.5$ ³⁰⁴. Similar results were reported by a genome-wide association study starting from a collection of natural variants (the *Drosophila* genetic reference panel)³⁰⁵. Furthermore, a recent study showed a high responsiveness to artificial selection, reporting a narrow-sense heritability of $h^2 \approx 0.2$, and was able to select both for long (≈ 650 min) and short sleepers (≈ 100 min) after less than 10 generations¹⁵⁹. In addition to agreeing on the description of sleep as a highly heritable phenotype, these three studies also recognise that sleep is a complex phenotype not only determined by multiple genes, but also by the interaction between genotype and environment.

Genetic screens Forward genetic — the approach that consists in first finding phenotypical variants to then discover a gene responsible for it — has historically been very fruitful in *Drosophila*. Several landmark studies have identified short-sleeping mutants in this manner^{157,306–308}. Cirelli and coworkers identified a mutation in the *Shaker* gene (encoding for a potassium channel) that reduced sleep, but also suppressed sleep rebound¹⁵⁷. Later, Koh *et al.* identified *sleepless*³⁰⁶. Liu and colleagues identified *wide awake* whose mutation reduces sleep amount. They presented it as an interface between the clock and the homeostat, determining the timing of sleep³⁰⁷.

Other single genes were discovered more serendipitously¹⁵⁸, using direct approaches³⁰³ and reverse genetic screen³⁰⁸. Kume and co-workers described *fumin*

and the role of the neurotransmitter dopamine in the regulation of arousal¹⁵⁸. Bushey *et al.* associated the previously known *hyperkinetic* locus to sleep, but also to memory³⁰³ and Pfeifferberger and Allada characterised *insomniac*³⁰⁸.

1.3.3.2 Sex

Fruit flies are a gonochoric species where the two sexes, males and females, are determined by chromosomes. Beyond the anatomical sexual dimorphism (*e.g.* females being larger), there are noticeable differences in their physiology — for instance in metabolism, immunity³⁰⁹ and ageing³¹⁰. Behaviourally, both sexes are also very dimorphic, with male-specific behaviours such as male-male fighting interactions and an elaborate courtship towards females. Female-specific behaviours include their choice of egg lying site³¹¹ and post-mating male rejection³¹².

It had been noticed by circadian researchers already that males tend to be overall less active than females. This finding was confirmed by the early work in the sleep field. In particular, females are active during the day whilst males are quiescent — a period of inactivity that was wittily named the male *siesta*. This finding was corroborated by many studies, using different genetic backgrounds (*i.e.* reference wild types). A genome-wide association study has, however, showed that there is an interaction between genetics and sex, with several out-bred lines in which females sleep more than males³⁰⁵.

1.3.3.3 Circadian clock

Perhaps, the single most important endogenous determinant of sleep is an animal's internal clock. In fact, the circadian field had long established that the activity of a fly cycles, even in the absence of any circadian clues (*i.e.* in constant darkness and temperature). According to the two-process model of sleep regulation (see section 1.1.5), the propensity of a fly to sleep is, to a large extent, modulated by its clock (process C), which acts independently from the homeostat (process S)³¹³.

These two processes can be dissociated experimentally. For instance, mutants of the core clock genes have been shown to have unaltered sleep amount, but lose circadian regulation of sleep³¹⁴. Conversely, flies with very low sleep still show robust circadian activity rhythms. The clock neurons have been shown to consolidate sleep once night has started and wake flies up in anticipation of the dawn^{307,315}.

1.3.3.4 Age and development

In many organisms, sleep amount reduces with age³¹⁶. When sleep was first characterised in the fruit fly, this trend was also noticed¹³¹. Sleep in aged flies is not only shorter, but also more fragmented^{255,316}. In addition, flies treated with oxidative stressors show a similar loss of sleep consolidation³¹⁶. Such change in sleep architecture (*i.e.* number and length of bouts) is, however, not necessary and could depend on genetic and environmental factors^{317,318}. Age-associated sleep fragmentation also seems related to a high-caloric diet³¹⁹. Young flies have been reported to sleep in a consistent location in their tube, but this preference seems to fade in with age²⁷⁹.

In laboratory conditions, fruit flies larvae develop very rapidly, it has therefore been very difficult to maintain them long enough to study their sleep pattern. However, a recent study has shown that larvae may engage in rest that qualifies as sleep³²⁰.

1.3.3.5 Diet

Flies allocate their time to several competing needs according to their internal states. In some circumstances, both sleep and nutrients may be needed, and the amount and the type of food available may directly impact sleep. Short-term starvation (12 h) has been shown to induce a large reduction of sleep amount³²¹, possibly linked to the lipid metabolism³²². Interestingly, sleep deprivation induced by starvation does not always result in a compensatory sleep rebound. In fact, it was shown that a naturally occurring allelic variant confers resistance to starvation-induced sleep loss³²³.

A complete, yeast and sucrose medium, decreased sleep in males, but increased daytime sleep in —presumably mated— females³²⁴, though this latter result is likely a misinterpretation resulting from the use of the single-beam DAM system³²⁵. It was also shown that fruit flies engaged in postprandial sleep. In other words, sleep is increased after a meal, and more so if it contained protein and salt^{280,326}.

1.3.3.6 Temperature and humidity

Fruit flies face two important biophysical constraints: they are small and endothermic. This implies a very limited thermal inertia and results in high poik-

ilothermy — *i.e.* their body temperature essentially equal to the temperature of their surrounding environment³²⁷. In addition, their surface-area-to-mass ratio is very high, which renders them very vulnerable to the risk of desiccation³²⁸. However, they are very mobile and can change their environment quickly, hence *behaviourally* regulating their humidity and temperature³²⁹.

High temperatures (shift from 25°C to 30°C) have interestingly been found to both increase and reduce sleep in the Light (L) and Dark (D) phases, respectively^{330–332}. In addition, the loss of sleep due to a night at 29°C is compensated by a rebound³³². These results are particularly interesting in the context of thermogenetics as the temperature will affect a wide range of physiological functions, including sleep itself. To my knowledge, there is no study on the direct effect of humidity on sleep in *Drosophila* — perhaps because it is difficult to control and measure humidity in the traditional paradigm.

1.3.3.7 Light and photoperiod

Almost all experiments on sleep in the fruit flies are carried under a 12:12 h, L:D sleep regime (with notable exceptions where flies are in DD^{40,288,333} and LL³³⁴). However, according to the latitude, there are seasonal variations in photoperiods, that were studied in the circadian field^{335,336}, but, to my knowledge, the regulation of sleep under different photo-period has not been investigated (Ko-Fan Chen, personal communication).

In addition, in the wild, there are wide spatial, daily and seasonal variations both in light intensity and quality, but their effect has not been comprehensively studied in the context of *Drosophila* sleep. One study, however, reported that flies prefer to engage in daytime sleep in shaded, darker, locations²⁷⁸.

1.3.3.8 Intra-specific interactions

Besides social interactions between males and females, *Drosophila* has rich social interactions³³⁷. For instance, males are known to fight one another for access to territory, food and mates³³⁸. In addition, emergent gregarious behaviours have been observed in populations as they spatially organise in patches^{339,340} and exhibit cooperative foraging^{341,342}.

Population A recent study reported that the structure of populations affects sleep. Specifically, flies in a group may synchronise their sleep with one another³⁴³. However, this study did not have access to single animal data, making it difficult to untangle the individual from the populational effects³⁴⁴. The density at which larvae were raised has also been shown to impact their sleep as adults³⁴⁵ — though larval density is known to affect many traits such as longevity, starvation resistance and size³⁴⁶.

One-to-one interactions In a study I took part in, males were kept in a confined space for several hours either with a female or a male²⁹⁵. We were able to show that both conditions resulted in effective sleep deprivation for the male²⁹⁵ (see also²⁹⁴ for preliminary work). Whilst males kept with males experienced a compensatory rebound after the removal of their competitor, males that had lost sleep due to their interaction (courtship and mating) with a female did not²⁹⁵. We were able to show that sexual clues raise males' arousal, which changes their internal state and effectively negates sleep rebound. In a separate study, Machado *et al.* characterised a wake-promoting neuronal circuit also involved in the direct regulation of courtship^{347,348}.

Mating After mating, virgin females are known to exhibit profound physiological and behavioural switch that is induced by a peptide present in the males seminal fluid^{349–351}. Namely, they produce more egg, become refractory to mating and less attractive³⁴⁹, their immune system is altered^{352,353} and their food preference also switched towards more salt and a high-protein diet, which they require to produce eggs^{354,355}.

Mating has been reported to affect sleep behaviour of females by several studies^{278,318,325,356}. Isaac *et al.* scored sleep using DAMs and showed that mating reduced sleep, but only during the L phase — night sleep being unaffected³⁵⁶. Garbe and co-workers also applied video tracking and multibeam and could corroborate the observation of a reduction of daytime sleep after mating²⁷⁸. They also noticed a small but very consistent reduction of night sleep. Both studies, however, used food that contained only 5% sucrose and water,

In a follow-up article, Garbe *et al.* used nutritious food (*i.e.* with yeast) instead. In this context, they found that single beam DAM failed to detect any change in daytime sleep. They went further using a multibeam DAM and concluded that daytime sleep was indeed reduced in females after mating, but only when scored

with the spatial resolution of the multibeam system. This latter study suggests that mating induces feeding when nutritious food is available, which the single beam is likely to miss since food is on the extremity of the tube³²⁵. Unfortunately, the authors do not address whether night sleep, post-mating, could be altered in the presence of nutritious food. Interestingly, the effect mating was shown to be dependant on the genetic background used³¹⁸.

1.3.3.9 Inter-specific interactions

The evolution of *Drosophila* has likely been driven partly by its interaction with members of other species such as predators, pathogens, commensals and symbionts. To the best of my knowledge, there is no work on how, for instance, the presence of predators affects sleep in the fruit fly — though it is studied in other models³⁵⁷. There are, however, some studies on the effect of infection, gut microbiota and symbionts.

Infections and immune response During an infection, the behaviour of an animal may be altered for two main reasons. Firstly, there are multiple examples of pathogens manipulating, directly or not, the behaviour of their hosts towards increasing their own fitness³⁵⁸. Secondly, the host may face a new trade-off and alter its behaviour accordingly, for instance by modifying its preferred temperature³⁵⁹ and diet³⁶⁰, which could mitigate the impact of an infection. It has been shown that the immune system interacts with the circadian clock³⁶¹ and sleep has been hypothesised to serve an immune function (see subsection 1.1.4.3).

There are several studies on the interactions between sleep and the immune system in *Drosophila*. Some have suggested that infected flies reduce sleep levels³⁶² and that sleep deprivation activates immune genes³⁶³, possibly strengthening immune response and promoting survival^{364,365}. In contrast, others have concluded that sleep increases immune defences. For instances, infections have been reported to induce additional sleep³⁶⁶, which could increase survival³⁶⁷.

Microbiome The microbiota colonising *Drosophila*'s gut is intimately linked to its immunity, metabolism and food preference³⁶⁸. However, it has not yet been found to play a direct or indirect role in sleep regulation. In fact, a recent pre-print reported no effect of microbiota removal on the host's sleep³⁶⁹.

Wolbachia *Wolbachia* is a genus of endosymbiotic bacteria prevalent in a wide range of arthropods³⁷⁰. It primarily infects their gonads and has extensively been reported to manipulate their reproduction (reviewed in³⁷¹) and immune system (reviewed in³⁷²). *Wolbachia* can also be found in neurons and its presence has been linked to reduced aggressiveness in male fruit flies that host it³⁷³. Interestingly, it has recently been shown to increase arousability and decrease sleep³⁷⁴.

1.3.4 Necessity and function of sleep in *Drosophila*

The question of the role sleep to flies has been of great interest. First and foremost, its vital nature was assessed. Then, its global function was investigated in the framework of the three main hypothesis presented in subsection 1.1.4. Namely, energy conservation, restoration and learning.

1.3.4.1 Lethality of sleep deprivation

To my knowledge, there is only one study reporting a lethal effect of sleep deprivation in the fruit fly¹⁵⁴. In this milestone article, Shaw *et al.* deprived 12 wild-type flies of sleep by tapping on their tube as soon as they appeared immobile, for 70 h. They report that 4 out of 12 animals died due to this treatment. Despite obvious experimental and statistical limitations, the authors conclude that ‘sleep does indeed serve a vital biological role’¹⁵⁴.

The notion that sleep is vital to flies has nevertheless been supported by another line of evidence: the fact that many mutants that have reduced sleep have also shorter lifespan⁴⁸. For instance, *Shaker*¹⁵⁷, *sleepless*³⁰⁶, *Hyperkinetic*³⁷⁵ and *insomniac*³⁷⁶ mutants are all short-lived.

However, *fumin* mutants, which exhibit one of the most severe short-sleeping phenotypes, have the same longevity as their genetic controls¹⁵⁸. In addition, when the expression of *insomniac* is inhibited specifically in the brain of flies, they have a normal lifespan, but reduced sleep³⁷⁶. Finally, the lifespan of artificially selected populations of low or high sleepers does not differ from the one of the control groups¹⁵⁹.

1.3.4.2 Energy conservation and metabolism

In an interesting study, Stahl *et al.* were able to combine the traditional beam crossing assay with a device that can measure CO₂ production³⁷⁷. They could show that sleep was negatively correlated with a metabolic rate, and that this relationship is not only—and trivially—due to the absence of walking, as metabolism reduces proportionally to the time spent being immobile. In addition, a sleep deprivation that leads to a subsequent rebound also causes in a coincident reduction of metabolism, altogether suggesting that sleep could help flies minimise their energy expenditure.

1.3.4.3 Restoration

There is not a lot of evidence regarding of specific processes being restored by sleep in *Drosophila*. As discussed above, sleep is thought to play a role in certain immune processes, and could therefore help recovery after infection^{366,367}. Another possibility is that sleep could be involved in processes such as digestion, which may have a component of active recovery. Indeed, the fact that flies show postprandial sleep²⁸⁰ could suggest—though there are other explanations—that digestion could come at a physiological cost, possibly involving an active compensation mechanism that would be facilitated by sleep. A last, and perhaps more compelling, example is that sleep is associated with widespread synaptic downscaling in fruit fly brain²⁹⁴. Since some affected neurons are not thought to be particularly involved in learning, this process could be seen as a mechanism by which the central nervous system restores its plasticity.

1.3.4.4 Learning and memory

There is a growing literature on the involvement of sleep in learning in *Drosophila*, involving both behavioural and structural evidence.

Behaviourally, some mutants that are impaired in learning have reduced sleep³⁷⁸. In addition, their deficit can be rescued, to some extent, with extra sleep^{379,380}. Conversely, there are documented cases of low-sleepers that also have poorer learning performance^{303,381,382}. Furthermore, learning mutants do not seem to modulate their sleep levels according to their previous experiences³⁸³—which wild-type animals do³⁷⁵.

Molecularly and structurally, it has been shown that, after long periods of wakefulness, for instance due to sleep deprivation, the number of synapses (inferred by protein markers) increased, which supports the synaptic homeostasis hypothesis^{294,375}.

1.4 Summary, aims and scope

1.4.1 State of the art

Sleep is a fascinating mystery that has bemused thinkers since the dawn of civilisations. In fact, the first written tale known to date already illustrates both its importance and elusiveness. The ancient authors, such as Aristotle and Pliny the Elder, who pioneered our comprehension of the living world, already thought that most animals, even distant ones, slept. Today, an increasing amount of empirical evidence has since emerged to support their intuition. Indeed, a wide range of animals has been shown to sleep, including, vertebrates, arthropods, molluscs and even cnidarians.

The conceptual proximity between sleep and death in western culture is particularly fascinating and begs the question of whether sleep is necessary to life, an enigma that is largely unresolved. In fact, despite its ubiquity, the core functions of sleep themselves are still heavily debated.

Drosophila has grown an instrumental model in our understanding of fundamental biological processes such as immunity, senescence. It also proved a crucial asset to understand the genetics of several behaviours including circadian rhythms, foraging and mating.

In the last two decades, the fruit fly emerged as a model to study sleep. Since then, multiple genetic and environmental determinants have been characterised, altogether leading to the notion that sleep is a dynamic process that depends on contexts such as sex, mating status, social clue, interaction with pathogens, temperature and diet. It has been suggested that sleep helps conserving energy, restore the immune and nervous systems, and promotes learning in flies. However, the question of whether fly can live without sleep remains largely open — considering the lack of conclusive evidence.

The field of *Drosophila* research benefits from a vast and constantly evolving palette of scientific tools such as genetic expression systems, themogenetics, confocal imaging, genomics, transcriptomics and many others. In comparison, the paradigms used to score and alter sleep lag behind. Indeed, despite recent advances in hardware and software for video tracking, which can detect movement, immobility — and therefore sleep — has been almost exclusively inferred with the DAM, a device designed to detect large-amplitude walking activity, which cannot account for

low-amplitude movements such as feeding and grooming. Likewise, the sleep deprivation paradigms employed to understand homeostasis have traditionally been unspecific as they consist of untargeted, frequent, mechanical disturbance.

1.4.2 The scope of this thesis

In the next chapter of the thesis herein, I will present the ethoscope platform, a practical solution I developed with my collaborators to address some of the methodological limitations associated with the detection of movement and ultimately sleep. I designed it aiming to deliver a scalable, open and modular tool for the community, not only to score sleep in *Drosophila* but, in principle, to analyse the behaviour of small animals in general.

The development of the ethoscope ultimately resulted in the acquisition of large individual datasets. I wanted to enrich the traditional analysis of movement with a modern approach based on data sciences, which made me realise the absence of a general programmatic framework to, specifically, process and visualise high-throughput behavioural data from several inputs. I, therefore, led the development of **rethomics** as a collection of R packages aiming to fill this niche. In the third chapter, I will describe its scope and originality.

I was very interested in understanding how the use of my novel tracking tool could yield informative variables and ultimately affect the quality of our biological conclusions. I will present, in my fourth chapter, a series of experiments going in this direction. I specifically focussed on acquiring large amounts of data for wild-type animals, comparing them between DAM and ethoscopes, enriching the behavioural space, characterising the consistency of behaviour over time and using positional data in addition to discrete behavioural states.

In my fifth chapter, I will show the results I obtained when employing the ethoscope's modularity to perform dynamic (*i.e.* real-time) sleep deprivation. Such a technique enabled me to prevent animals from being quiescent by startling them only when they were immobile. This approach being more parsimonious, it eventually allowed for a final experiment in which flies were chronically sleep-deprived, ultimately addressing the crucial question of the vital necessity of sleep.

2 | The ethoscope platform

‘All entities move and nothing remains still.’

— Heraclitus, in Plato’s
Cratylus [Ref. 384, 401d]

2.1 Background

The importance of movement and immobility to our understanding of the physical world was already considered a crucial question by the pre-Socratic philosophers. The divergence between Heraclitus and Parmenides as well as Zeno’s paradoxes famously illustrate the intellectual challenges associated with the notion of movement and, ultimately, the difficulty of defining it.

Parmenides argued that movement was ‘impossible’ whilst Heraclitus thought that ‘everything was in motion’. Modern science has since revealed, that we live on continents that are in constant motion, on a planet that endlessly turns around its sun, within a solar system that also circles around the centre of our galaxy at nearly a million kilometre per hour, and that our galaxy itself drifts away from others. It then results that physical objects are never truly immobile and that the definition of motion is a matter of scale.

Interestingly, the inclusive definition of sleep I described in the introductory chapter (see section 1.1.2.1) relies on the observation of a *lack of movement* in an animal. In *Drosophila*, sleep has traditionally been inferred and, in fact, quantified by scoring long bouts of uninterrupted immobility. At the beginning of the twenty-first century, when fruit flies emerged as a model for sleep, the field of circadian biology was already well established and there were obvious conceptual, sociological and methodological overlaps between the two fields. In particular, cir-

cadian researchers had overwhelmingly adopted the *Drosophila* Activity Monitor (DAM) system (see introduction fig. 1.3), an automatic platform to score the activity of hundreds of flies over days and weeks. DAMs implemented a simple idea: when a fly is active, it walks and, restrained in a narrow glass tube, eventually crosses its midline, which is recorded by an Infrared (IR) beam and sensor.

In addition to the momentum the DAM system had, it offered a simple read-out as well as a robust and scalable platform, making it instrumental for wide genetic screens. It was, therefore, *de facto* adopted as the tool to score movement — and thus sleep — in our emerging field. Implicitly, using the break of an IR beam to score movement comes with the assumption that ‘micro-movements’ (*i.e.* activities that do not involve walking), such as eating, grooming and egg-laying, either do not interrupt sleep bouts or that they are sufficiently unimportant to be ignored.

This assumption has been either challenged^{256,258} or corroborated^{131,278} by different studies, but attempts to provide tools based on video tracking have not yet been widely adopted, and most recent studies still use exclusively the DAM system. These video-based alternatives have, at least, two limitations. Firstly, they fail to provide a system that scales ‘horizontally’ — *i.e.* a set of autonomous acquisition devices organised in a platform that centralises data collection. Indeed, at the scale of a laboratory, where multiple users will run simultaneous experiments, a degree of compartmentalisation is needed. Secondly, and more importantly, these methods do not provide an objective or validated algorithms to score movement, nor do they assess their respective sensitivities and specificities.

In the chapter herein, I describe the ethoscope, a platform I developed to address these two issues. With a highly modular design, it offers a general solution to acquire high-throughput behavioural data of small animals over long durations. First, I introduce the ‘ethoscope’, which is a portable autonomous tracking device. Then, I describe two hardware modules that can be programmed to perform closed-loop experiments in order to, for instance, dynamically alter sleep. Afterwards, I illustrate how ethoscopes interoperate within a network and, importantly, how my platform can be operated in practice through its user interface. I conclude by presenting the tracking algorithm that I devised to robustly monitor single animals over long durations. Crucially, I describe and validate, using human-annotated ground-truth data, a simple method for movement classification that can be applied in real-time.

This chapter complements and extends the article that I published, with my team, on the same matter²⁸¹.

2.2 Device

2.2.1 Core

In very close collaboration with Luis Garcia (then working at Polygonal Tree), I developed a novel video tracking device which we named ‘the ethoscope’ (fig. 2.1). Ethoscopes are built around the ‘Raspberry Pi’ (<http://www.raspberrypi.org/>), a single-board micro-computer originally designed for outreach purposes. It has the advantage of being very well documented, inexpensive and offers a range of readily available modules such as several IR cameras.

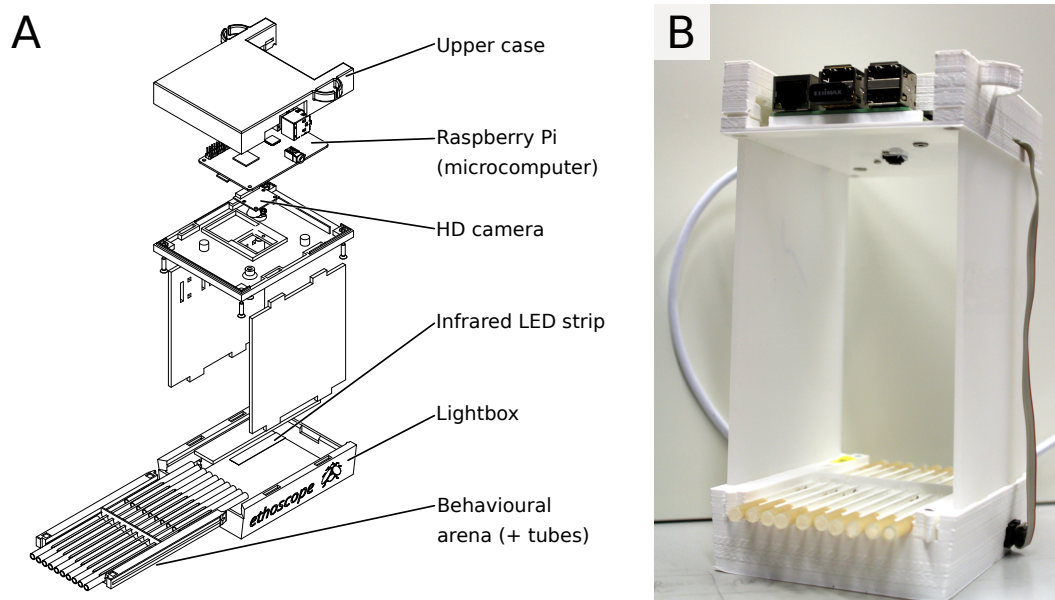


Figure 2.1: *The Ethoscope*. **A**, Exploded drawing of an ethoscope. The upper case encloses a Raspberry Pi microcomputer running its own operating system. It controls a camera that acquires video of the behavioural arena. IR light is transmitted through the arena, to allow recording during the dark phase. **B**, Picture of an actual ethoscope loaded with 20 experimental tubes.

I wanted the ethoscope to be open-hardware, open-source, inexpensive and simple to build. For this reason, all its components were either widely commercially available, or could be created and assembled with basic knowledge of electronics and technology such as 3D printing. Alongside my original peer-reviewed article, I released a full documentation, including building and maintenance instruction at <https://qgeissmann.gitbooks.io/ethoscope-manual>.

Briefly, an upper case contains the micro-computer and a camera facing downwards. It is physically connected, with two laser-cut walls to a lightbox, which

can fit experimental arenas in the field of view. The adjustable-focus camera can acquire standard 1280×960 pixel videos at 25 Frame Per Second (FPS). I chose to use—and customise—the ‘Arch Linux’ distribution as the operating system since it is very well documented and allow for extensive configuration. All software is installed on a micro SD card inserted into the microcomputer.

The device measures less than $190 \times 120 \times 90$ mm. It is only powered through a standard USB port (5 V, 2 A) and has a wireless network card. For prototyping and debugging purposes, it can also be controlled through a keyboard and screen. Altogether, parts of the core cost approximately £70.

2.2.2 Lightbox and arenas

Before they start an experiment, users enclose animals in a so-called behavioural ‘arena’, which is their environment. In this respect, arenas define the experimental paradigm more than any other part. In order to promote modularity and diversity of set-ups, I designed a support that acts as a lightbox and could fit a variety of arenas. It transmits IR light upward, through the arena, so that videos can be acquired during the Dark (D) phase. All the results I present in this thesis were acquired with the ‘sleep arena’, as shown in fig. 2.2A and B.

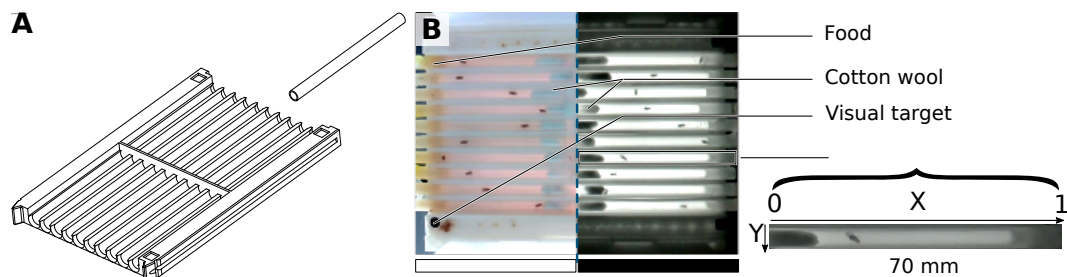


Figure 2.2: *Sleep arena*. The behavioural arena designed to study sleep. It holds twenty fruit flies in individual $70 \times 5 \times 3$ mm ($L \times OD \times ID$) glass tubes. **A**, Drawing of an experimental arena and a single glass tube. **B** Image of the arena taken by the device’s camera (original size 1280×960 pixel). The frame is artificially split into two parts by a dashed line: the left (white bar) and right (black bar) sides represent two portions of the same arena taken either during Light (L) and D phases, respectively. One of the twenty Region Of Interests (ROIs) is shown to illustrate the coordinate system within each ROI.

I also endeavoured to provide a system of templates that my collaborators could derive and adapt quickly in order to implement a specific paradigm. For instance, my team took advantage of this design to develop arenas aimed at studying decision making, response to odour, foraging behaviour, larval sleep and others (see fig. 3 in²⁸¹).

The sleep arena used throughout this thesis holds twenty 70 mm long and 3 mm narrow glass tubes with food on one side and cotton wool on the other side. The tube dimensions being the same as for the DAM system, results can be compared between platforms. It is also very efficient at preventing fast desiccation of the food as it can be sealed to minimise the interface between food and air. Therefore, fruit flies can be kept without external interventions for around ten days at 25°C, which is very convenient in the sleep and circadian fields. In addition, for my purpose, since individuals are hosted in separate compartments it is simpler to startle animals specifically — *i.e.* without disturbing others — in closed-loop experiments.

2.3 Modules

In addition to the core of the ethoscope described above, optional hardware modules can be controlled by ethoscopes in real-time. Here, I present two modules that I used in my thesis: the ‘servo’ module (fig. 2.3) and the ‘optomotor’ (fig. 2.4).

2.3.1 Servo-module

The servo module (fig. 2.3) was built primarily to perform Dynamic Sleep Deprivation (DSD), a paradigm I will describe extensively in chapter 5, by turning the glass tubes that host flies when they have been immobile.

The module fits the bottom of the lightbox and features an array of ten servomotors (five aside) which are individually connected to ten glass tubes through 3D printed pulleys and o-rings. All servos are controlled by a commercial Lynxmotion board, which communicates with the ethoscope through a standard serial interface (USB).

The machine can only rotate half of the tubes in a sleep arena. However, in practice, the neighbouring tubes host non-startled controls, encouraging systematic interspersal — which is a good practice.

2.3.2 Optomotor

I observed that the servo module often failed for various reasons. For instance, o-rings or pulleys would break, servomotor became dysfunctional and loss of electrical power could damage the board irreversibly. In addition, it was time-consuming to set up and complicated to perform quality controls (*i.e.* ways to ensure the

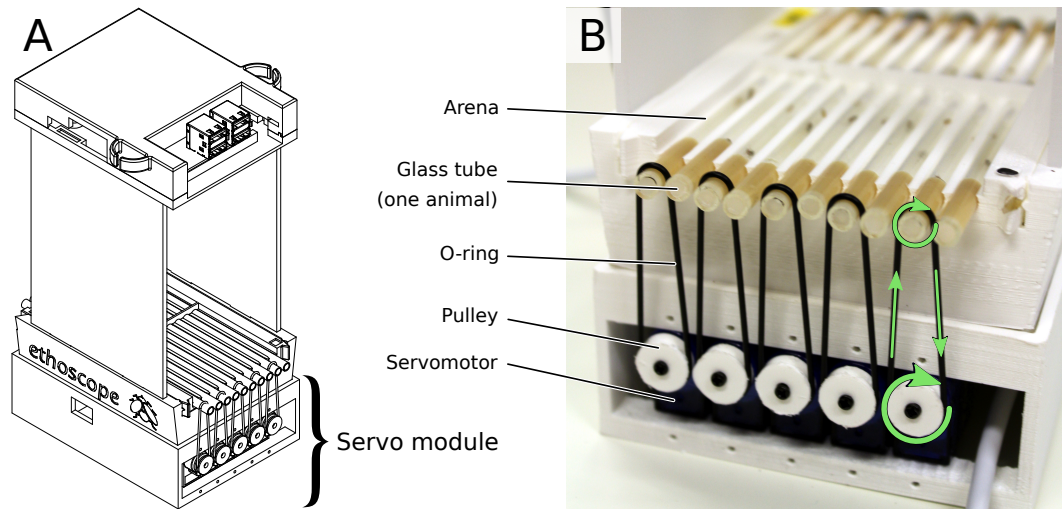


Figure 2.3: *The servo module.* **A**, Drawing of the servo module attached to an ethoscope. A USB cable (not shown) allows the communication between ethoscope and to the module. **B**, Picture of a module loaded with experimental tubes. o-rings transmit rotation from servomotors to individual tubes (green arrows). Ten of the twenty tubes can be rotated to startle flies (the others usually serve as paired experimental controls).

machine functions). For these reasons, the optomotor (fig. 2.4), a new and more robust module, was designed.

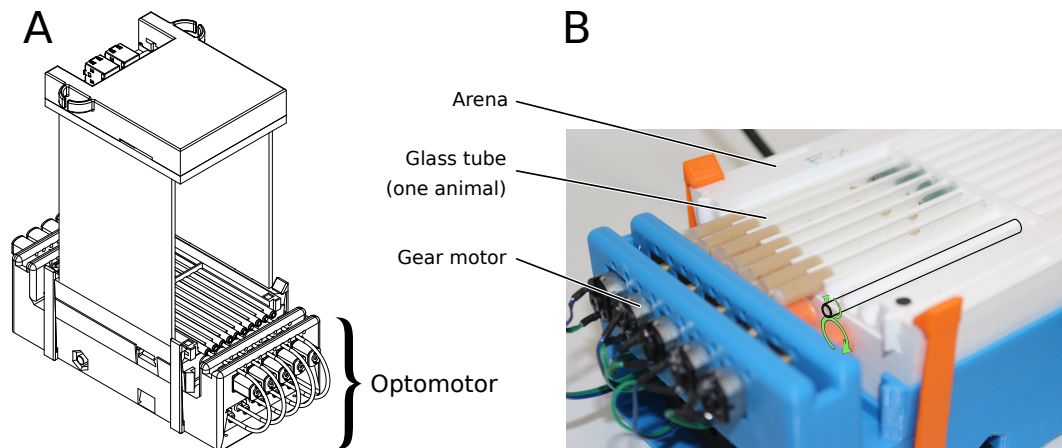


Figure 2.4: *The optomotor.* **A**, Drawing of the optomotor attached to an ethoscope. A USB cable (not shown) allows the communication between ethoscope and to the module. **B**, Picture of a module built without optic fibres (motors only) loaded with experimental tubes. Each other tube rests on top of the shaft of a small motor. Motors can be controlled individually, in real time, to startle specific animals by spinning their tubes (green arrows). The module can also be assembled with LED lights and optical fibres so that light can be guided inside each tube.

It uses gear motors instead of servomotors, and rotate tubes using simple friction, hence abolishing the need for o-rings (which were time time-consuming to install and prone to failure). To improve quality control, a push-button was configured as an interface to test the hardware post-construction, but also before and after experiments.

Arian Jamasb, an undergraduate student I supervised at the time was simultaneously working on a module to perform optogenetics (*i.e.* manipulate populations of neurons using light, reviewed by Riemensperger *et al.*³⁸⁵), and he had the very good idea to combine both functionalities. Therefore, I merged his design to mine in a single machine that can also, using optical fibres, send pulses of light directly inside the tubes. Both functionalities are optional, and devices can be built with either or both.

Light and motors are both controlled by a commercial board (TLC5947 PWM Driver, Adafruit). For robustness and real-time applications, I implemented the controller firmware in an Arduino Micro board, that receives instruction from the ethoscope through a serial connection.

2.4 Platform

2.5 Implementation

Ethoscopes are small and can only track a few individuals at one time, so they only become interesting for high-throughput application when multiple ones are used simultaneously. The ethoscope ‘platform’ is a system designed to handle multiple devices and users. It is made of a central computer, the ‘node’, and multiple tracking devices (*i.e.* *ethoscopes*), all connected wirelessly by a custom wifi access point (fig. 2.5A).

The node is a standard desktop computer that orchestrates the platform, but does not perform any data processing. Indeed, all the video analysis is done, in real time, by the ethoscopes (fig. 2.5B) themselves. Such distribution of computing power renders closed-loops experiments possible and makes the platform extremely scalable — insofar as the addition of new devices does not result in increased workload for the node.

I anticipated that multiple updates of the software would be needed and that it would become challenging to maintain multiple devices. I, therefore, implemented a system that fetches remote updates and makes them available on the local network (through a `git` repository). A website user interface then allows maintainers to selectively update connected devices.

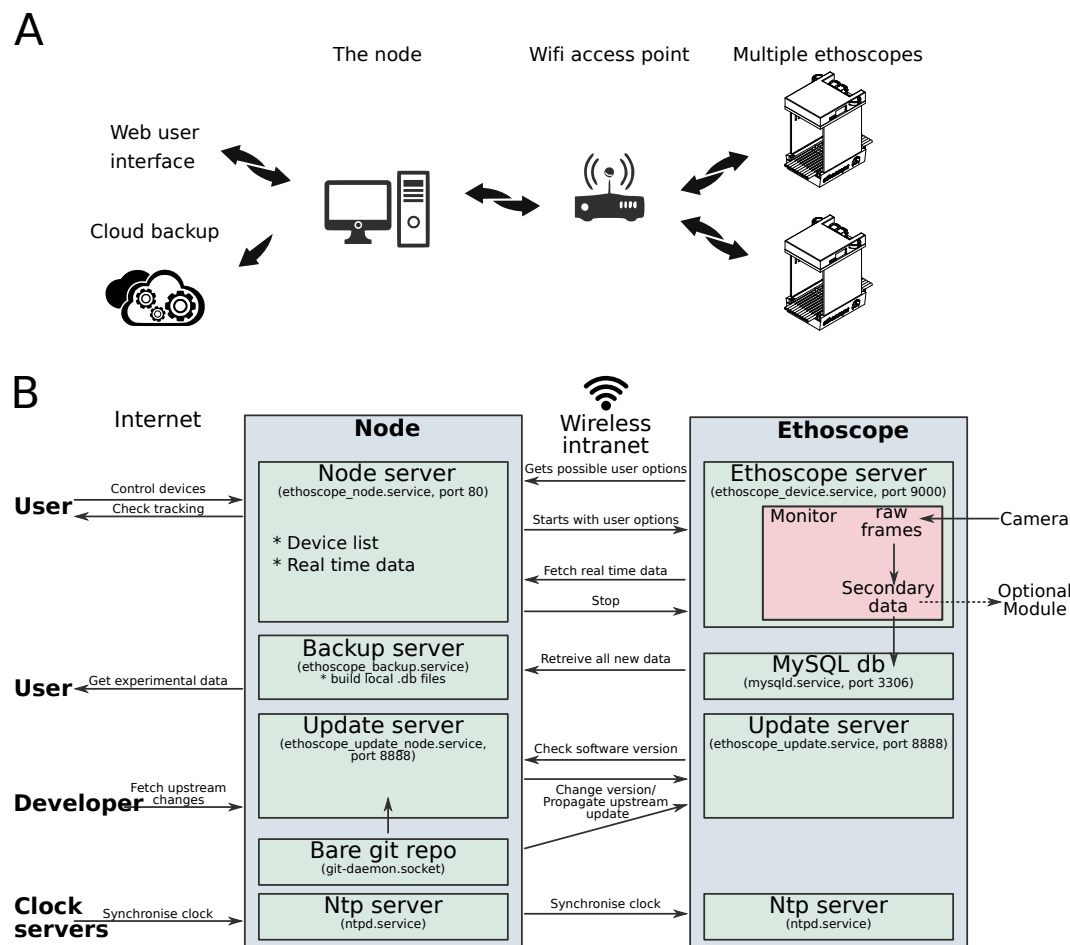


Figure 2.5: *The ethoscope platform*. **A**, Schematic representation of the hardware components of the ethoscope platform. It contains multiple ethoscope and a single ‘node’. Each device is a standalone tracking unit that processes video on-board. In the laboratory, the devices communicate through a custom wireless network. **B**, Software side of ethoscope platform. The node runs three main services: the ‘node server’ to list and control ethoscope through a web interface, the ‘backup server’ to save experimental results and the ‘update server’ to maintain the platform. The ethoscope devices run the ‘ethoscope server’ that performs video tracking and exposes their status, in real time, to allow for quality control. They can also be maintained through their own ‘update server’. Data is saved on a MySQL database by each ethoscope, and mirrored as an SQLite .db file on the node side.

2.6 User interface

I invested considerable effort into making the system user-friendly. Indeed, its complexity is hidden to users as they interact (*i.e.* start and stop experiments, update the system, download data and view arenas in real time) through a website interface (fig. 2.6) which can be accessed using devices such as smartphones, tablets or computers, which renders interaction with the system very straightforward. It features a homepage that gives an overview of the state of the whole platform and lists all devices so they can be individually controlled (fig. 2.6A). Each device can

be accessed through its own web-page (fig. 2.6B), which allows users to start and schedule experiments.

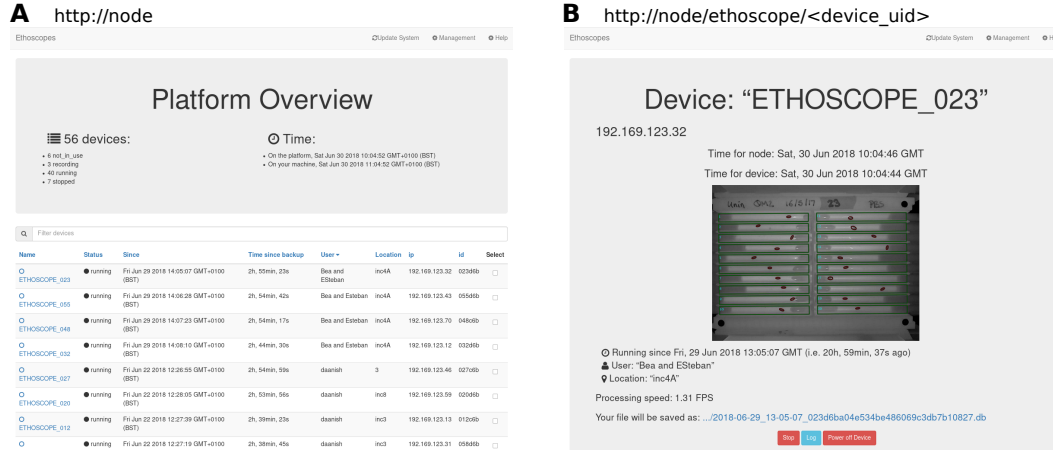


Figure 2.6: *The user interface.* **A**, Homepage of the ethoscope platform. It shows the list of all devices detected on the local network in a table that can be filtered by device status, user and location (only the first seven are shown in this figure). The name of each device links to its control page. **B**, Device control page. The control page of a single running ethoscope. It shows a real-time preview of the tracking result to the user. Buttons allow to stop, start and power-off this specific machine. When the machine is stopped, the start button opens a dialogue window (now shown) that allows users to parametrise their experiment. The top of all pages is a navigation bar containing links to management tools, such as update and file managers.

2.7 Software and tracking algorithm

I also attempted to match the modularity of the hardware described above at the software level. Indeed, by design, it is flexible and general: it makes very few assumptions about the source of the primary data, the nature of the processing, the number of animals tracked and such. This feature allowed some of my collaborators to define their own region of interest, variables or even tracking algorithm. For instance, Diana Bicazan a PhD student in my team has developed a module to track simultaneously multiple animals with the ethoscopes. In the restricted scope of this thesis, I will however only describe the tracking algorithm I designed and implemented to quantify quiescence—and ultimately infer sleep.

I was specifically interested in monitoring the position of animals isolated in individual ROI. Individuals in different ROIs could also be assumed to be visually fairly homogeneous. The challenges where the relatively low resolution of the video, the necessity of a real-time implementation on a micro-computer with limited performance and the long duration of the tracking in a possibly changing (spatially and

temporally) environment. The algorithm also needed to deliver variables tuned for movement detection (*e.g.* sub-pixel-resolved position).

I considered using tracking tools that were already available for fruit flies (described in subsection 1.2.3.3), but most were designed as standalone tools, which limited control and the possibility of integration within my own software. I therefore instead implemented myself a tracking algorithm using open-source Application Programming Interfaces (APIs) that have ports to `python` such as OpenCV³⁸⁶, `numpy`³⁸⁷ and `scipy`³⁸⁸.

2.7.1 General description

A flowchart of the tracking algorithm I designed is shown in fig. 2.7 and each step is detailed below. Briefly, the first few frames are used to automatically detect ROIs (see subsection 2.7.2). Then, each sub-image is preprocessed (see subsection 2.7.3). Dynamic statistical models of the background and the foreground are applied to detect the animal (see subsection 2.7.4). Conditionally on its successful detection, statistical models are updated (see subsection 2.7.4.4). Sub-pixel centres of mass are used to define the position of each animal and compute velocity. It is corrected according to the instantaneous frame rate (see subsection 2.8.3). Finally, corrected velocity is used in a simple classifier to score a behavioural state (see section 2.8).

2.7.2 Automatic ROI detection

It was not possible to guaranty that, across all devices within the platform, the position of the behavioural arena in the video frames would be consistent. Indeed, small variations in the manufacture and assembly of the parts implied that the position and angle of the arena could vary between devices. Therefore, I preferred to automatically define ROIs from the visual ‘targets’ that are part of the arenas (see fig. 2.2) rather than hard-coding their positions. Prior to tracking, the first five frames of the video stream are acquired and stacked in one average image that is used as a reference. Then, the three targets—black dots on the edges of the arena—are located in it using a recursive thresholding algorithm for circular blob detection (similar to the one I had previously implemented for detecting bacterial colonies, see³⁸⁹). Their position is further used to compute and apply an affine transformation, and ultimately to map the image to a predefined grid specifying the relative position of the ROIs.

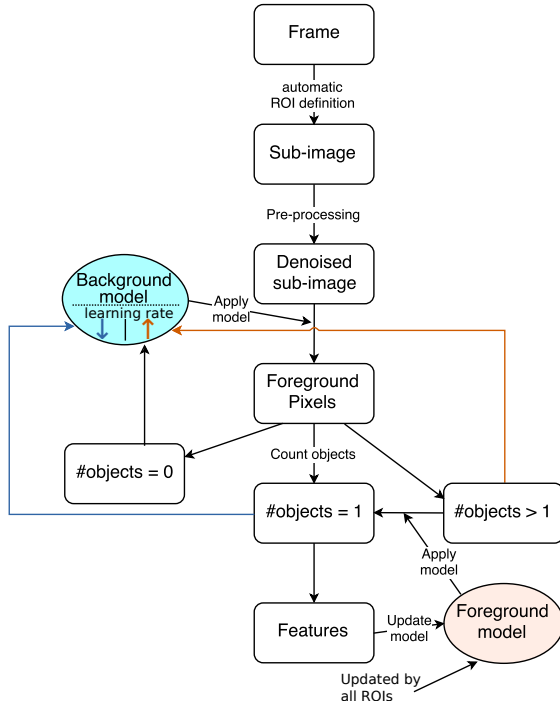


Figure 2.7: *Flowchart of the video tracking algorithm.* ROIs are automatically defined and applied to extract as many sub-images. All sub-images are then processed independently. In a first place, they are denoised. Then, the background statistical model is applied in order to extract foreground pixels. In addition, the preprocessed image updates the current background model. Afterwards, all objects (*i.e.* connected components) are found in the foreground. If none were detected, then tracking is aborted for this frame. When several objects are detected, the foreground model applied to keep only the ‘best’ one (*i.e.* the most likely). Features of the remaining likely object are computed and serve to update the foreground model. The learning rate of the background model is increased either when several or no objects were detected. If only one object was found (*i.e.* unambiguous match), the learning rate is instead decreased. Features, including position, are further used to characterise behaviour.

2.7.3 Preprocessing

Firstly, the sub-image is converted to greyscale. Then, a Gaussian blur with $\sigma = 1.2$ and a kernel size of 11×11 pixels is applied to denoise each image. This convolution reduces the high-frequency noise in each frame, which typically results from the low exposure of the sensor. Insofar as my algorithm aims at locating the centroid of a fruit flies, high-frequency details such as the texture of the animal are not required. The value of σ was defined empirically by visual assessment. The kernel size is sufficient (radius greater than 3σ) so that the contribution of the pixels in its edge is negligible (*i.e.* their coefficients tend to zero).

Finally, the intensity of each pixel in the blurred source image bl is multiplied by a constant so that the resulting preprocessed image pp has its mean intensity $\overline{pp} = 128$ (which is half of the 8-bit range):

$$pp_{x,y} = bl_{x,y} \cdot \frac{128}{\overline{bl}} \quad (2.1)$$

Where,

- x is the index of a pixel in the horizontal dimension,

- y is the index of a pixel in the vertical dimension and
- $\overline{bl}$ is the arithmetic mean of the pixel intensity in the blurred source image.

2.7.4 Background and foreground model

2.7.4.1 Background model

In order to discriminate the animals from their background in a robust fashion, I implemented a background model based on the weighted running average of each pixel. This method creates a persistent representation of the background that is updated by each new frame with a learning rate α . I observed that flies tended to remain in the same location for long durations (sometimes hours), but moved rather quickly when they were active. This feature made it difficult to find a suitable value for α : large values meant the static animals would fade in the background whilst low values would not update the background quickly enough when animals were active and the environment was changing. I, therefore, decided to solve this issue by applying the foreground mask in order to update the background only where the animal was not detected, an approach used by other authors^{390,391}.

$$bg_{t+1,x,y} = \begin{cases} (1 - \alpha) \cdot bg_{t,x,y} + \alpha \cdot pp_{x,y} & , \text{ if } mask_{t-1,x,y} > 0. \\ bg_{t,x,y} & , \text{ otherwise.} \end{cases} \quad (2.2)$$

Where,

- x is the index of a pixel in the horizontal dimension,
- y is the index of a pixel in the vertical dimension,
- bg_{t+1} is the updated background model,
- bg_t is the current background model,
- pp is the current preprocessed image (see eq. 2.1)
- α is the learning rate (see eq.2.3) and
- $mask_{t-1}$ is an image that represents the location of the animal in the previous frame (see eq. 2.4).

I observed that the frame rate was heterogeneous over time, or between devices, depending on system load. Therefore, instead of assuming constant frame rate (*i.e.* α is constant), my background model explicitly accounted for the actual time difference, δt , between two consecutive frames:

$$\alpha = 1 - \exp\left(-\frac{2}{t_{1/2}} \cdot \delta t\right) \quad (2.3)$$

$t_{1/2}$ corresponds to the ‘half-life’ of the information in a pixel. The larger $t_{1/2}$, the longer it takes for the model to learn. Importantly, the learning rate is dynamic $t_{1/2}$ bounded to $[1, 100]s$ (see sub-section 2.7.4.4).

2.7.4.2 Foreground features

At each frame, the preprocessed image (pp) is subtracted from the background model (bg) and intensity-thresholded with the value thr . The resulting image is a foreground mask ($mask$) that has values 1 in the foreground (where the animal is) and 0 everywhere else:

$$mask_{x,y} = \begin{cases} 1 & , \text{ if } bg_{x,y} - pp_{x,y} > thr \\ 0 & , \text{ otherwise.} \end{cases} \quad (2.4)$$

Where,

- x is the index of a pixel in the horizontal dimension,
- y is the index of a pixel in the vertical dimension,
- $mask$ is the resulting foreground mask,
- bg is the background model (see eq. 2.2),
- pp is the current preprocessed image (see eq. 2.1) and
- thr is the value of the intensity threshold which was empirically set to 20. This value was picked as a compromise to reveal the salient foreground features (such as a well-contrasted fruit fly) without detecting low-contrast noise (*e.g.* image flickering).

Then, connected components are detected in order to separate individual objects in $mask$. If none are present, then tracking is aborted. If more than one object is found, then the foreground model (see next subsection) is used to select only

the most likely object. When exactly one object is detected, it is considered an ‘unambiguous’ match and used immediately.

Several primary features such as XY position, orientation, width, height, area and average pixel intensity are computed on the single resulting foreground object. In order to obtain an accurate measurement of velocity, sub-pixel XY position was computed using greyscale image moments as opposed to binary moments.

2.7.4.3 Foreground model

For each frame, a vector of three features — $\log_{10}(\text{area})$, height and average pixel intensity — describing the detected foreground object is stored as a new row in a feature matrix M . The likelihood $\mathcal{L}$ of candidate foreground objects, with a vector of feature X , is then computed based only on marginals, assuming a Gaussian distribution for the three features. For each column j of M , we have,

$$L_j = \frac{1}{\sigma_j \sqrt{2\pi}} e^{-\frac{(X_j - \mu_j)^2}{2\sigma_j^2}} \quad (2.5)$$

Where,

- j is the index of the feature ($j \in [1, 3]$),
- μ_j is the standard deviation of all stored values of a feature j (see below),
- σ_j is the standard deviation of μ_j ,
- L is a resulting vector of likelihood for all three features

The mean value of a feature j over the $N = 1000$ past observed values is the average of the column j in the feature matrix M :

$$\mu_j = \sum_{i=1}^N M_{i,j} / N \quad (2.6)$$

Then, the total likelihood ($\mathcal{L}$) is the product of all marginal likelihoods:

$$\mathcal{L} = \prod_j L_j .$$

Importantly, the foreground model is shared between all ROIs, which assumes

relative feature homogeneity between foreground objects. When tracking visually similar animals (*e.g.* animals of similar size)—an assumption that is expected to hold unless tracking visually very different animals.

2.7.4.4 Updating the background model

The background model is systematically updated for each frame, but its half-life is set dynamically. Namely, it decreases when foreground identification was ambiguous (several objects), or aborted (no objects).

$$t'_{1/2} = \begin{cases} c \cdot t_{1/2} & , \text{ if unambiguous foreground} \\ c^{-1} \cdot t_{1/2} & , \text{ otherwise.} \end{cases} \quad (2.7)$$

Where,

- $t_{1/2}$ is the half-life used to calculate the learning rate α in eq. 2.3,
- $t'_{1/2}$ is the updated half-life to be used for the next frame ($t'_{1/2}$ is bounded in $[1, 100]$ s) and
- c is set empirically to 1.2. This value implies that the learning rate can scale approximately 10 times in 13 frames ($1.2^{13} \approx 10$).

Such a dynamic learning rate allows the algorithm to use prior knowledge to decide when the background information should be kept or discarded. Indeed, the assumption that exactly one animal should be located in each frame can be used to tune the learning rate in real time. Namely, when multiple or no objects are detected, a fast learning rate allows the background model to quickly update to resolve this ambiguity. In this case, a half-life of 1 s, which has the same order of magnitude as the frame rate ($\approx 2 \text{ s}^{-1}$), is appropriate. When only one object is detected (in most cases), a relatively slow learning rate (half-life of 100 s) allows the background to persist in a time scale that is compatible with the duration of immobility bouts of a fly (often minutes). Since ambiguities are often only transient, I opted to change the learning rate smoothly (eq. 2.7). This avoids unnecessarily discarding further background information once the ambiguity has been resolved.

2.8 Validation and behaviour scoring

2.8.1 Ground truth

There were two aspects of the tracking algorithm that I thought were necessary to validate. Firstly, how much error is associated with the detected position of an animal. Secondly, how sensitive and specific to subtle movements the algorithm was. In order to address both points, I acquired a 144 h video at 25 FPS of 19 fruit flies with an ethoscope. Importantly, a visually heterogeneous dataset was generated by using both males and females, which differ in size and aspect. In addition, since images are different between L and D phases (see fig. 2.2B), the video was acquired during both phases (12:12 h, L:D), using only transmitted IR light during D phases.

Then, ten-second videos of each ROI were systematically sampled from the raw video every hour, generating more than 2,500 sub-videos. Sub-videos were then presented one by one, and in a random order, to several independent fruit fly researchers who were asked to:

1. click on the centre of the fly on the first frame, and
2. score one of three behaviours in the whole ten seconds.

Ground-truth XY position was then obtained by computing the median X and Y between human annotations (to make position robust to spurious clicking errors). *A priori* defined behaviours were:

- walking,
- micro-movement, and
- immobile.

If several behaviours were observed within the same ten seconds sub-video, observers were asked to prioritise the behaviour with the largest amount of movement, $\mathcal{M}$, such that

$$\mathcal{M}_{walking} > \mathcal{M}_{micro-mov.} > \mathcal{M}_{immobile} .$$

As a result, 1413 annotations were generated by, at least, three experts each.

Furthermore, a subset of 1297 consensual (*i.e.* majority rule) videos were kept for validation.

2.8.2 Results

Independently to the ground truth generation, the same raw video was re-sampled at 2.1FPS, which is a realistic operational frame rate for the ethoscope, and subsequently processed with the tracking algorithm described in section 2.7. Both position (fig. 2.8A) and behaviour scoring (fig. 2.8B) were compared to ground truth for validation.

The median of distances between ground truth and inferred position was 0.31 mm, corresponding to a tenth of a fly body length (fig. 2.8A). Over the 1297 annotation, all distances were lower than one body length (with a maximum distance of 2.6 mm).

Several scalar variables, such as cumulative distance, mean and maximal velocity, mean proportion of pixels displaced and others were defined for each 10 s sub-video. Random forest variable importance³⁹² was used to empirically rank predictors. Maximal velocity ranked on top as the single ‘best’ predictor, and appeared to provide, on its own, a reliable classifier of movement. In other words, maximal velocity in a 10 s epoch could predict behaviour with a pair of thresholds. In order to assess the performance of such classifier, I computed Receiver Operating Characteristic (ROC) curves for both the movement detection and the walking detection thresholds (fig. 2.8B).

2.8.3 FPS-dependent velocity correction

I then noticed that the computed velocity depended on the frame rate of the processed video. Indeed, when performing the above analysis at different frame rates, the computed velocities appeared unstable and artificially increased with FPS (fig. 2.9A). It is difficult to predict analytically the exact relationship between the measured velocity and FPS since it depends both on the structure of the background noise and the types of foreground movements.

Therefore, I proceed empirically: I down-sampled the original validation video (144 h at 25 FPS) to eight different new ones with a frame rate between 1 and 5 FPS. Then, I used the tracking algorithm described above on each new video and

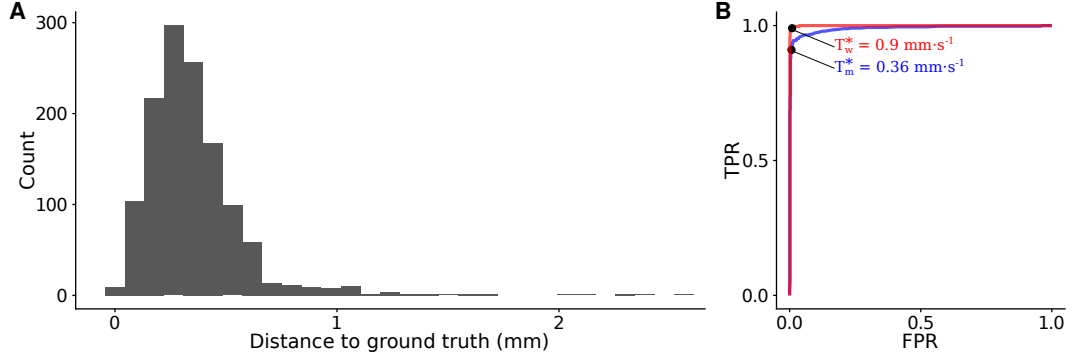


Figure 2.8: *Validation of the algorithm.* Comparison between algorithmic results and a ground truth of 1297 human annotations. **A**, Distribution of the Euclidean distances between tracking and human annotations. **B**, ROC curves for movement and walking classifiers. Relationship between true positive rate (TPR) for false positive rate (FPR) for variable thresholds T . The blue and red curves are for a threshold of the movement (T_m) and the walking (T_w) detectors, respectively. The dots and label show the values of the selected thresholds T^* that correspond to ‘conservative’ FPRs of 0.005.

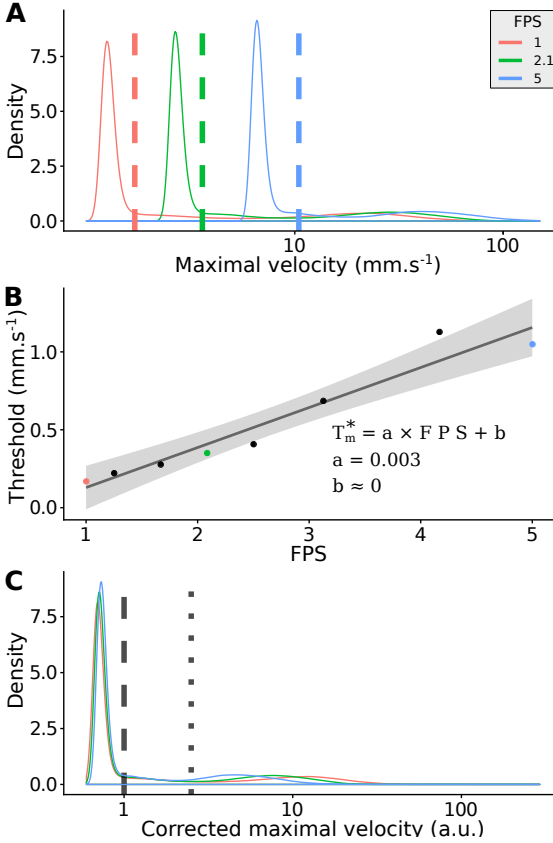


Figure 2.9: *Velocity correction.* **A**, Distribution of the maximal velocity in all animals and 10 s epochs at different FPS. The dashed line indicates the different movement detection threshold obtained. Only three frame rates are shown for the sake of simplicity. **B**, Regression between the obtained threshold and the eight different frame rate tested (all obtained by resampling the reference video). **C**, Distribution of the *corrected* maximal velocity in all animals and 10s epochs the same FPS as in **A**. Correction was performed using equation 2.9.

computed all instantaneous velocities. The maximal velocity in each consecutive 10 s epoch ($\max(V)$) was computed in all cases. Ground truth data was used to perform ROC curves and, for each FPS, define the threshold (T_m^*) that lead to a False Positive Rate (FPR) of movement equals to 0.5% ($T_m^* = T_{m_{FPR=0.005}}$). The relationship between FPS and the threshold on maximal velocity was then

modelled with a linear regression (fig. 2.9B):

$$T_m^*(FPS) = a \times FPS + b \quad (2.8)$$

Where,

- $T_m^*(FPS)$ is the movement threshold that gives $FPR = 0.005$ (see fig. 2.8)
- a is the slope of the linear model and
- b is the intercept

Linear model fitting gave $a = 0.003$ and b was not statistically significantly different from zero, so I used $b = 0$. Therefore, velocity (V_{corr}) calculations were corrected with:

$$V_{corr} = \frac{V}{a \times FPS} \quad (2.9)$$

This way, when greater than 1, the maximal value of the corrected velocity ($\max(V_{corr})$) in a 10 s epoch indicates movement in the foreground with a specificity of 99.5%.

To further ensure the effectiveness of this method, the density of all instantaneous velocity was re-computed after correction (fig. 2.9C). Visual inspection confirmed the effectiveness of my empirical correction.

2.9 Implementation and availability

Both device and node run a version of the GNU/Linux operating system, which is highly customisable and open-source. Their respective APIs were implemented, as part of this project, in `python` programming language. An extensive documentation of both hardware and software, alongside building and maintenance instruction was made available at <http://gilestrolab.github.io/ethoscope/>.

2.10 Summary

- Ethoscopes are open-source and inexpensive video tracking tools for small animals. They can be extended through the addition of hardware and software modules.
- All machines are controlled through a scalable platform that centralises updates and data management.
- The framework can be used remotely through a user interface designed for experimentalists.
- The default tracking algorithm detects small movements in single fruit flies with great accuracy. Its simple design means it can be used in real time on raspberry pis.

3 | Rethomics

‘I suppose it is tempting, if the only tool you have is a hammer, to treat everything as if it were a nail.’

— Abraham Maslow,
*The Psychology of
Science* [Ref. 393, p. 15]

3.1 Background

Tools such as the ethoscope platform, that I described in the previous chapter, and others allow biologists to gather a large amount of behavioural data from multiple animals. Some authors have suggested the use of the term ‘ethomics’, an analogy between the high-throughput acquisition and analysis of behavioural time series and other large-scale phenotyping approaches such as transcriptomics, proteomics and metabolomics²²³. The transition to ethomics era is extremely promising as it paves the way for in-depth quantitative analyses which, in turn, leads to the characterisation of new principles and ultimately a better understanding of biology³⁹⁴.

Most methods that are suffixed with the term ‘omics’—sometimes, I concede, generously so¹—share, at least, two features that, I would argue, are equally crucial. Firstly, they rely on technological breakthroughs that have, over the last few decades, improved throughput and reduced cost of data acquisition by orders of magnitude. Secondly, they adopt advances in data sciences, data management and software engineering to provide communities with sets of maintained analysis

¹see Jonathan Eisen’s ‘Worst New Omics Word Award: Museomics’ post <https://phylogenomics.blogspot.ca/2009/01/worst-new-omics-word-award-museomics.html>

tools and databases which intercommunicate and share standards (*e.g.* file formats, programming languages and software libraries).

In the last decade, several commercial and academic platforms were developed to acquire various behaviours such as activity²⁵⁸, position³⁹⁵ and feeding^{247,248} of single or multiple^{262,269} animals over long durations (days or weeks). However, regarding the subsequent analysis of the results, there is less effort in the development of a high-level, unified, programmatic framework that could be used, for instance, to create pipelines.

For instance, in the field of sleep and circadian rhythm, analysis tools are generally graphical user interfaces built in a ‘top-down’ manner. That is, they are designed to achieve a set of predefined functionalities in the scope anticipated by their developers. In other words, instead of focussing on defining general data structures and concepts to then specify them to their own cases, authors have generally opted for a more rigid approach^{299,396,397}.

In contrast, in the bioinformatics community, many widely adopted tools are instead built in a ‘bottom-up’ fashion and opt for either a command-line or a programming interface. At the foundation level, Application Programming Interfaces (APIs) are designed and provide a well-documented set of tools for developers to build libraries. Then, these libraries are used by individual researchers to write scripts, or can even serve as a back-end to build user interfaces. This approach favours collaboration in favour of replicated work and ultimately promotes maintenance of software (*e.g.* if and when the original authors retire from the field). Programming interfaces are generally more robust. In addition, they are flexible since they can be used in conjunction with a set of other tools to build a pipeline. For this reason, state-of-the-art analysis and visualisation tools are based on programming interfaces³⁹⁸.

The type of data studied in traditional bioinformatics is discrete both in space and state (*i.e.* nucleotide and amino acids) and conveniently ubiquitous, which certainly encourages a shared toolbox. In ethomics, the data gathered could be, at first, assumed to be prohibitively inconsistent to justify sharing tools (*e.g.* what do fish movements and fruit fly feeding behaviours share?) However, I argue that behavioural data is conceptually largely agnostic of the acquisition platform and paradigm. Typically, the behaviour of each experimental individual is described by a long time series (possibly multivariate and irregular). Importantly, individuals are labelled with arbitrary metadata defined by the experimenter (*e.g.* sex, treatment and genotype). At a low level, linking combining and processing meta-

data and data from hundreds of individuals, each recorded for days or weeks, is not a trivial challenge, and the community may benefit from shared solutions.

In the chapter herein, I present **rethomics**, an efficient and flexible tool in R to unify the analysis of behavioural data. Firstly, I will describe a general design and workflow. Secondly, I will detail my computational solution to store and manipulate behavioural data. Thirdly, I will conceptualise a general principle to import ethomics results. Fourthly, I will explain how I extended the concept of the grammar of graphics^{398,399} to the visualisation of behavioural data. Finally, I will briefly present some of the functionalities of **rethomics** to the analysis of sleep and circadian rhythm.

Waiting for peer review, I have made available the manuscript describing **rethomics** as a preprint⁴⁰⁰ (see availability section 3.7).

3.2 Workflow

rethomics is implemented as a collection of complementary R packages (fig. 3.1). This architecture follows the model of modern frameworks such as the **tidyverse**⁴⁰¹, which results in enhanced maintainability and testability, as each step of the analysis workflow (*i.e.* data import, manipulation and visualisation) is handled by a different package.

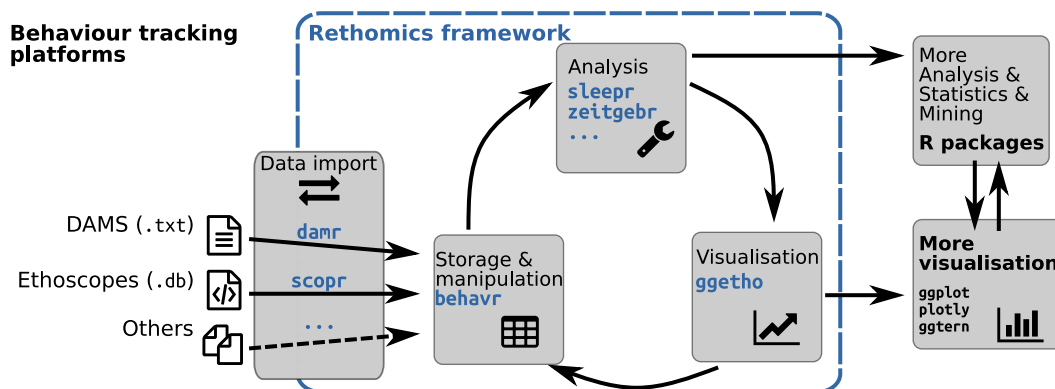


Figure 3.1: *The rethomics workflow.* Diagram representing the interplay between, from left to right, the raw data, the **rethomics** packages (in blue) and the rest of the R ecosystem. This figure is adapted from my own work⁴⁰⁰.

The **behavr** package lies at the centre of **rethomics**. It was designed as a flexible and efficient solution to store large amounts of data (*e.g.* position and activity) as well as metadata (*e.g.* treatment and genotype) in a single **data.table**-derived object⁴⁰². Input packages all import experimental data as a **behavr** table which

can, in turn, be analysed and visualised regardless of the original input platform. Results and plots integrate seamlessly into the R ecosystem, thus providing users with state-of-the-art visualisation and statistical tools.

3.3 Internal data structure

behavr (fig. 3.2), is a new data structure, based on the widely adopted **data.table** object⁴⁰². It is designed to address two challenges that are inherent to handling ethomics results. Firstly, behavioural time series can be very long as one or multiple variables may be sampled—sometimes heterogeneously—several times a second, for days or weeks. Each behavioural series could represent variables that encode the activity, position, orientation, size, colour and so on. Secondly, a large number of individuals are studied (often several hundreds of animals). In order to statistically account for covariates—for instance, in the context of full factorial experiments—it is important that each individual is associated with ‘metadata’. The metadata is a set of ‘metavariables’ that describe all experimental conditions.

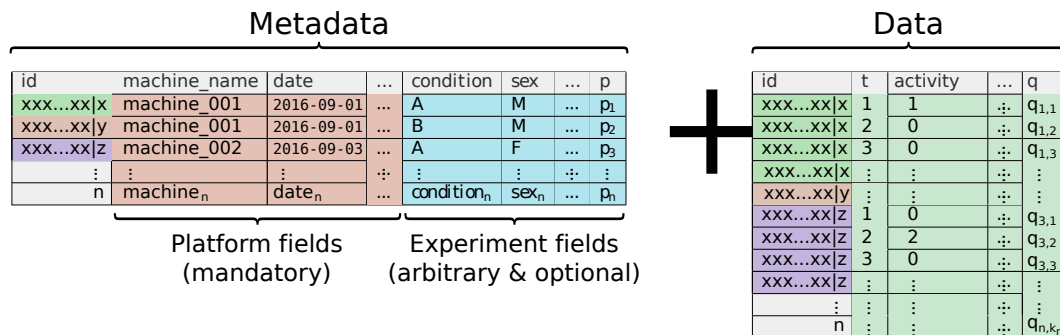


Figure 3.2: *The behavr data structure.* Schematic representation of a **behavr** object, the central data structure in **rethomics**. In the metadata (left), each row refers to one experimental individual. Columns are metavariables that can be either *required* (i.e. defined by the acquisition platform) or *user-defined* (i.e. arbitrary). In the data (right), each row represents a time point, that is the information about one individual at one time-point. Data and metadata are internally joined on the **id** field, the unique identifier of an experimental individual. The column names used in this figure are only examples which will differ in practice. This figure is adapted from my own work⁴⁰⁰.

A **behavr** object contains a pair of tables: metadata and data. The two tables are semantically linked by the extension of the **data.table** syntax, thus rendering the manipulation, the joining and the accession of metadata transparent. In other words, this architecture allows users to map data to its parent metadata without explicit knowledge of databases, in a statistical environment (fig. 3.3). For instance, when data is filtered, only the remaining individuals are left in the meta-

data. It is also important that metadata and data can interoperate. For example, when one wants to update a variable according to the value of a metavariable (say, alter the variable x only for animals with the metavariable $sex = 'male'$).

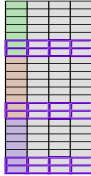
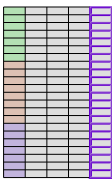
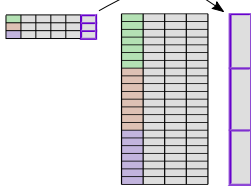
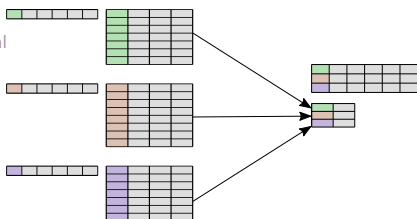
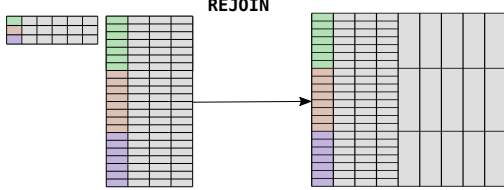
	Metadata	Data
Select	dt[CRITERIA, meta = TRUE]  <pre> # to subset the metadata only for males > male_meta <- dt[sex == "M", meta = TRUE] </pre>	dt[CRITERIA]  <pre> # to keep only data > 5s > late_dt <- dt[t > 5] </pre> <p>Note: metadata is updated when selection removes all data from one id.</p>
Alter, create & delete (meta)variables	dt[, X := value, meta = TRUE]  <pre> # to create a metavariable set to "wt" > dt[, genotype := "wt", meta = TRUE] # delete > dt[, sex := NULL, meta = TRUE] </pre>	dt[, Y := value]  <pre> # to create t_2 (t - 1) > dt[, t_2 := t - 1] # to delete t > dt[, t := NULL] </pre> <p>Note: update data in place. No copy of dt in memory.</p>
Expand metavariables as variables	dt[xmv(X)]  <pre> # to select data with sex > dt <- dt[xmv(sex) == "M"] # to copy a metavariable as a variable > dt[, s := xmv(sex)] </pre>	
Aggregate & summary	dt[, OPERATION, by = id]  <pre> # to compute mean activity, per individual > dt <- dt[,.(mean_act = mean(activity)), by = id] # to count reads per id > dt[, .N, by = id] </pre>	OPERATION
Join data & metadata	rejoin(dt)  <pre> # to reunite data and metadata > full_table <- rejoin(dt) </pre> <p>Note: used mostly after aggregation or preprocessing</p>	REJOIN

Figure 3.3: *Summary of operations in **behavr***. Summary of the functionalities provided by **behavr** data structure. Code examples (prefixed by `>`) are provided and commented. All operations supported by **data.table** are inherited. Additional utilities are specifically implemented to manipulate both metadata and data together. This figure is adapted from my own work⁴⁰⁰.

3.4 Linking and loading data

Metadata encapsulates the information describing exhaustively and unambiguously each experimental individual. As such, it must be provided by the user and constitute a precise record of the experiment. I reasoned that is the if the exper-

imeter was required to provide a metadata table describing each individual, it should also include information that can be parsed to allocate a unique identifier and fetch the data in the file system. Indeed, the process of finding the data for each animal, when multiple devices are used and when experiments are repeated several times, is prohibitively error-prone and hardly reproducible. Although result files are organised in a very different manner across acquisition platform, conceptually the operation of enriching the metadata — which I call ‘*linking*’ — with technical details to, later, fetch the matching data — which I name ‘*loading*’ — is general and can be abstracted (fig. 3.4).

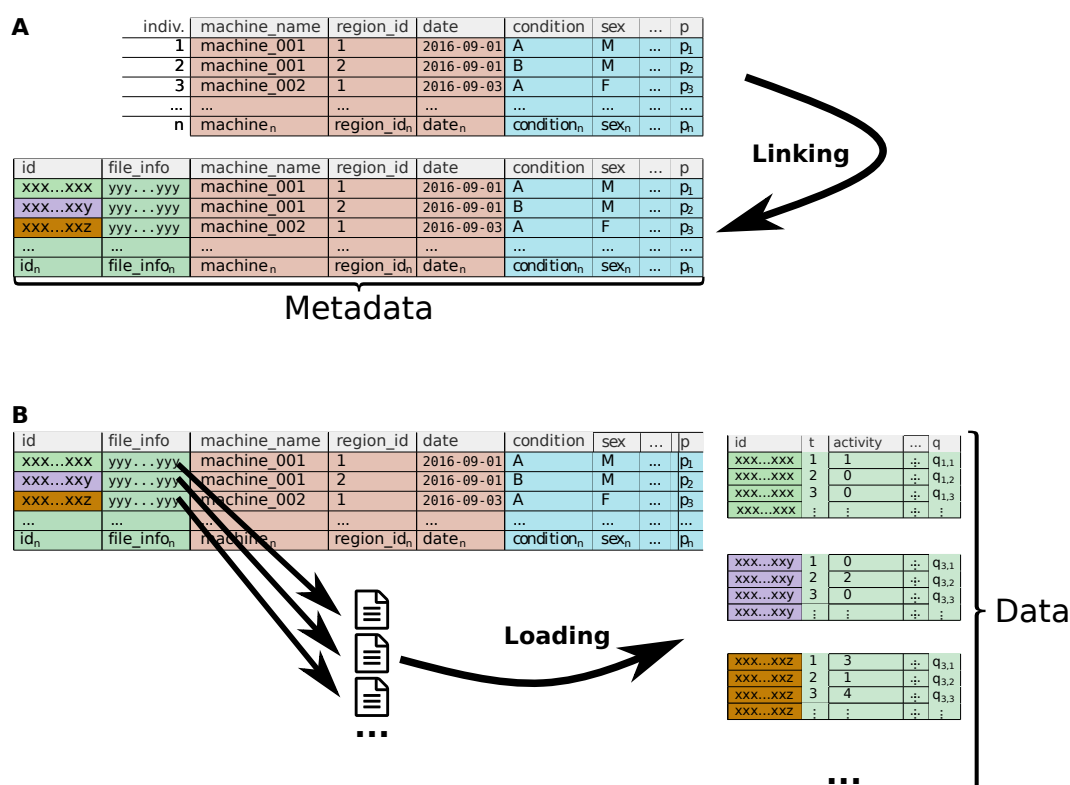


Figure 3.4: *Linking and loading*. **A**, Linking. The mandatory fields in the user-provided metadata are used to automatically locate the corresponding files. Location data is stored as an additional field. This process also checks for existence and integrity of the data. Individuals are also automatically allocated a unique identifier during this process. **B**, Loading. The linked metadata is then used to fetch and optionally pre-process data. All data is grouped and eventually stored, with the metadata in a single **behavior** object.

Naturally, each acquisition platform will have a set of mandatory metavariables that are necessary for linking, which are clearly stated the documentation. Currently, I provide a package to read single or multibeam *Drosophila* Activity Monitor (DAM) data and another one for ethoscope files²⁸¹.

This linking and loading approach has several advantages. Firstly, the metadata file is a standardised structure which allows different users to collaborate

using a shared format. Secondly, since each individual is a row in the metadata, users can — and are encouraged — to intersperse experimental conditions (otherwise, experimenters tend to confound metavariabls — for example, one genotype per machine and multiple machines). Furthermore, this framework facilitates the addition of new replicates insofar as additional individuals will just be represented by new rows — and the date of the replicate can be statistically accounted as a covariate.

3.5 Visualisation

Visualisation is essential for behavioural researchers who use it to control the quality of their data, summarise population trends and generate hypotheses. In R, the widely adopted `ggplot2`³⁹⁸ package implements and extends the concept of the grammar of graphics³⁹⁹, providing users with a very high-level syntax for data visualisation.

Since behavioural time series can be prohibitively large, preprocessing is often needed before visualisation. In addition, `ggplot2` does not have access to metadata, which are often needed since they encapsulate biological questions. In order to interface the `rethomics` framework with `ggplot2` I developed `ggetho`, which is fully compatible with `ggplot`, but also provides new layers and scales to represent behaviours. Figure 3.5 illustrates the use of `ggetho` in a restricted use case — and more examples are available on the `rethomics` website.

3.6 Sleep and circadian analysis

`rethomics` is not exclusively designed for the analysis of sleep and circadian rhythm but, in the context of my research — and in order to attract users and collaborators —, I endeavoured to reproduce and extend some of the visualisation tools and computational methods used by my community. In the manuscript describing `rethomics`, I provide a reproducible code example to illustrate how it can be used to perform ‘canonical’ circadian analysis⁴⁰⁰. In this short section, I will succinctly show some of the main functionalities, and refer the reader to the documentation for a comprehensive description. Specifically, I will show examples of ‘double-plotted actograms’ and periodograms generated in the `rethomics` framework.

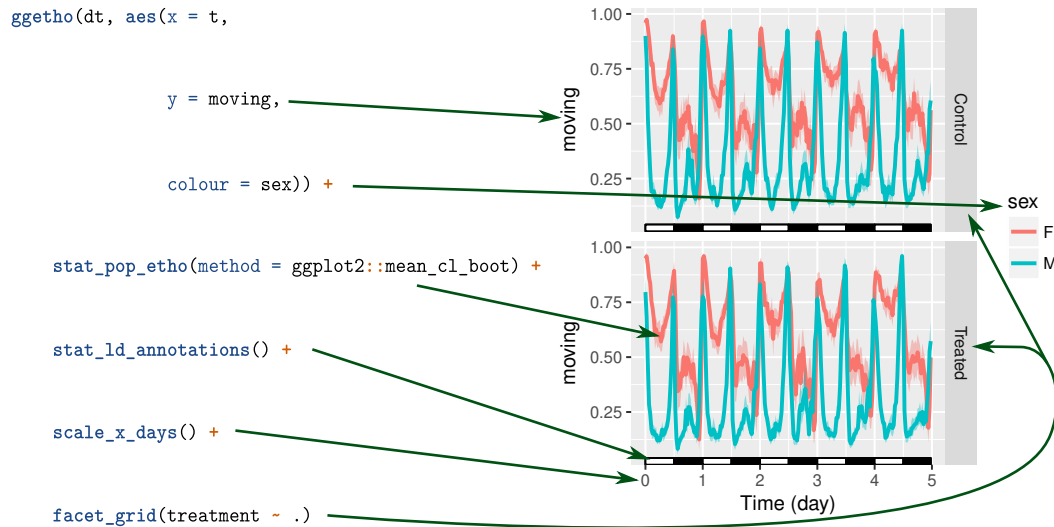


Figure 3.5: *Visualisation with ggetho*. Code snippet (left) and resulting plot (right) for an example visualisation of population trend. The arrows indicate where each code expression is represented on the plot. In this example, the input data, `dt`, is a `behav` table, with variables `t` (continuous) and `moving` (logical), and metavariables `treatment` ('Control' or 'Treated') and `sex` ('M' or 'F'). The `ggetho()` function, the `stat_pop_etho` and `stat_ld_annotations` layers, and the `scale_x_days` scale are part of `ggetho`, but integrate seamlessly with `ggplot`, for instance with the faceting mechanism shown here (*i.e.* `facte_grid`).

Double-plotted actograms are widely used by chronobiologists to visually assess the regularity and period of individual behaviours. `ggetho` provides a generalised implementation to 'multi-plot' time series (*i.e.* double-plotted, triple-plotted and so on). Importantly, the faceting functionality of `ggplot` makes it possible to sort and draw immediately one plot per individual (fig. 3.6A).

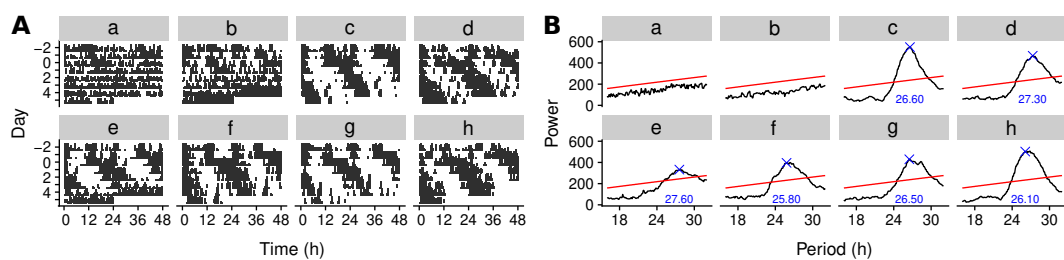


Figure 3.6: *Analysis of the periodicity in the activity in DAMs*. Example of visualisation and analysis of the circadian periodicity in *Drosophila* activity with `rethomics`. Eight selected animals are shown on different facets and labelled alphabetically from a to h. **A**, Double-plotted actograms. The height of the bars represent the relative average activity binned over time (here, 30 min bins, the default). The Y axis shows the time onset, in days, and the X, the time after the onset, in hours. Each value is repeated on the next row to facilitate visualisation (*i.e.* avoid edge effects). **B**, χ^2 periodograms for the activity data visualised in **A**. The blue 'x' symbols represent the most significant peak (if present). The red line shows significance at $\alpha = 0.05$ (the default). Note that a right slant in **A** corresponds a peak period greater than 24 h in **B**. This figure is modified from my own work⁴⁰⁰.

In addition to the double-plotted actograms, which are mostly for quality control and illustrative purposes, circadian analysis often requires the computation

of periodograms, the most widely used being the χ^2 ⁴⁰³ and the Lomb-Scargle⁴⁰⁴ methods. In the `zeitgeber` package, I implemented several periodogram methods. In addition, I endeavoured to formalise their output so that further analysis can be directly compared and reiterated with different functions. I also provided tools to automatically find significant peaks as well as display and summarise periodograms. For instance, figure 3.6B shows the χ^2 periodograms, and their respective peaks, matching the plots in figure 3.6A.

The `sleepr` package, in `rethomics`, provides several routines for the analysis of sleep behaviour in *Drosophila*. In particular, it can be used to apply the five-minute rule (even in heterogeneously sample data), it implements the ethoscope definition of movement and velocity correction presented in the previous chapter and has a method to encode categorical behavioural variables as ‘bouts’ (*i.e.* onset and duration).

3.7 Availability and future directions

I implemented `rethomics` in R⁴⁰⁵ since it is widely taught and adopted by statisticians and computational biologists. The packages developed during my thesis are hosted on CRAN, the official repository. An overall exhaustive tutorial is publicly available at <http://rethomics.github.io>. This whole documentation is automatically compiled and deployed upon upstream software update, which ensures reproducibility of the examples. According to state-of-the-art practices, all packages are also individually documented, continuously integrated and unit tested on several versions of R.

3.8 Summary

- `rethomics` provides a set of packages that integrate with one another and with R.
- The `behavr` object is an efficient and flexible data structure that stores both variables and metavariables.
- The ‘linking and loading’ approach is a consistent and exhaustive method to associate experimental conditions to behavioural data.
- The `ggetho` package extends `ggplot` to visualise ethomics data
- `rethomics` already implements a working set of tools for circadian and sleep quantification, and could develop to include other aspects of behavioural analysis.

4 | Baseline Sleep

‘It is plain also that insects sleep;
for there can be no mistaking their
condition of motionless repose.’

— Aristotle, *History Of
Animals* [Ref. 47, book IV,
chap. 10]

4.1 Background

The idea that, like vertebrates, insects sleep is by no means new. Indeed, Aristotle himself was already convinced of it more than two millennia ago. In fact, Pliny the Elder had also noticed that during their bouts of immobility, insects were less responsive to external stimuli as he wrote, four centuries after: ‘It is quite evident, also, that insects sleep, from the silent stillness which they preserve; and even if a light is put close to them, they will not be awoken thereby.’ [Ref. 11, book X, chap. 97].

It is however only recently that insect sleep became a subject of experimental research. In particular, in the early 2000s, its characterisation in *Drosophila* paved the way for an ever so growing literature. Even though the first studies, and a few others, worked on its phenomenological description as a behaviour, research, since, seems to have mostly focussed on the genetic determinism and neuronal circuitry of sleep behaviour — possibly because of the pre-existing background and interest of the involved scientist, but also, maybe, because the tools were already available to carry out neuro-genetic experiments. Indeed, despite the description of sleep mutants and the characterisation of neuronal circuits, there are many uncertainties regarding the answer to higher level questions — for example, whether

sleep happens during the Light (L) phase, what is the validity of the ‘five-minute rule’, how sleep changes after mating, what happens when flies cannot sleep and so on. These gaps in our knowledge suggest that, perhaps, a phenomenological analysis of sleep behaviour from the ground up would complement the ongoing enquiries on the low-level mechanisms of sleep.

In chapter 2, I presented a novel method to measure the stillness that is characteristic of sleep as an alternative to the traditional motion detection paradigm. I also emphasised that my method could be applied to a large number of animals. Then, in chapter 3, I provided a solution to handle the large amount of data generated in a statistical framework, and suggested it could be instrumental for new types of analysis.

In the chapter herein, I will use these two tools to, firstly, illustrate how the scoring of immobility, and ultimately sleep, differ between ethoscopes and Drosophila Activity Monitor (DAM) and show how such a difference can be critical even when comparing groups of healthy wild-type males and females. Secondly, I will show that it is possible to transcend the binary understanding of activity in the context of sleep by introducing a new state: ‘micro-movements’. Thirdly, I will use this addition, but also positional data, to understand the discrepancies between ethoscope and DAM. Fourthly, I will show how the use of micro-movements sheds light on the change of behaviours that occur after mating, in females. Finally, I will examine the extent to which behaviours are variable between, but consistent within, animal.

Part of the work presented in this chapter is available as a preprint⁴⁰⁶. It results of a collaboration, in equal part, between Esteban Beckwith, without whom this work could not have been carried, and myself.

4.2 Comparison between ethoscope and DAM

I was first interested in comparing activity and sleep in wild-type flies when scored either with ethoscopes or the traditional DAM system. Since the ethoscope tracks position, it is possible to emulate DAM data from ethoscope results by numerically detecting ‘midline’ crosses — a method also known as ‘virtual DAM’²⁵⁶ — and analyse the same data with both approaches.

I, therefore, started by monitoring a large population of socially naive males and females ($N_{male} = 485$ and $N_{female} = 881$) hosted in separate tubes for

five consecutive days (see method subsections 4.6.1 and 4.6.2 for details) and compared both immobility and sleep between both methods (fig. 4.1). Compared to the ethoscope, DAM scoring consistently overestimated both immobility (fig. 4.1A) and sleep (fig. 4.1D)—as scored using the five-minute rule. Overall, immobility was higher with DAM, both for females ($P(\text{immobile}_{DAM}) = 0.747$, $CI_{95\%} = [0.742, 0.753]$, against $P(\text{immobile}_{etho}) = 0.419$, $CI_{95\%} = [0.411, 0.426]$) and, to a lower extent, males ($P(\text{immobile}_{DAM}) = 0.829$, $CI_{95\%} = [0.824, 0.834]$, against $P(\text{immobile}_{etho}) = 0.684$, $CI_{95\%} = [0.678, 0.691]$).

Overall, sleep was also higher with DAM, both for females ($P(\text{asleep}_{DAM}) = 0.607$, $CI_{95\%} = [0.600, 0.614]$, against $P(\text{asleep}_{etho}) = 0.208$, $CI_{95\%} = [0.201, 0.215]$) and males ($P(\text{asleep}_{DAM}) = 0.786$, $CI_{95\%} = [0.779, 0.793]$, against $P(\text{asleep}_{etho}) = 0.430$, $CI_{95\%} = [0.421, 0.438]$).

When comparing males to females with both methods, different qualitative observations were made. For instance, immediately after the onset of the Dark (D) phase (time $\in [12, 14]$ h), midline crossing results suggest that females were less active than males. Instead, my scoring method shows that, during these two hours, females were, in fact, more active than males. It, therefore, appears that the level of consensus between both methods depends on the time of the day and sex.

In order to understand how and when methods critically differed, I computed a rank correlation (Spearman's ρ) on the amount of immobility (fig. 4.1B) and sleep (fig. 4.1E) between both methods for all consecutive 30 min time windows in 24 h. Values close to one denote a strong consensus between methods—since individual ranks are conserved.

Interestingly tools were very consensual with one another for males at the transition time, which corresponds to their active periods. For females, DAM and ethoscopes had the strongest consensus when females were the most inactive, by the end of the D phase (at time $\in [22, 23.5]$). In contrast, ρ was below 0.5 around the L $\rightarrow$ D transition, which indicates low consensus between both methods.

To characterise inactivity and sleep architecture, I also examined the relationship between bout number and average bout length, during both L and D phases (fig. 4.1C and F). There were striking differences between the two scoring methods. Firstly, and unsurprisingly, the overall average duration of inactivity bouts was greatly reduced in ethoscopes ($duration_{etho} = 104.7$ s, $CI_{95\%} = [102.4, 106.8]$) compared to DAM ($duration_{DAM} = 819.4$ s, $CI_{95\%} = [773.7, 874.5]$) (fig. 4.1C). Secondly, and less trivially, the comparison of sleep architecture between males and

females yielded in different conclusions according to the method used (fig. 4.1F). Indeed, during the L phase, males had longer sleep bouts according to the midline approach, whilst ethoscopes scored a mostly higher number of sleep bouts.

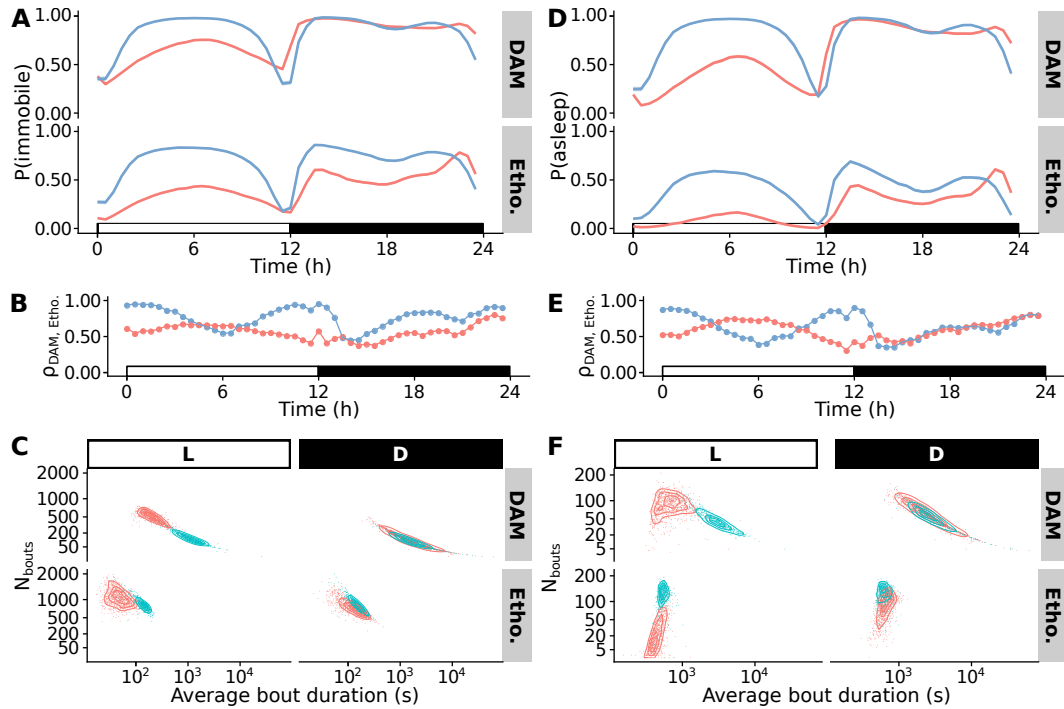


Figure 4.1: *Comparison to DAM*. Scored immobility (A–C) and sleep (D–F) for male (blue) and female (red) flies. **A** and **D**, Population average of activity (A) and sleep (D) over a circadian day (average of each 30 minutes in five consecutive days, modulus 24 hours). **B** and **E**, Spearman's ρ for activity (B) or sleep (E) between DAM and ethoscope scored for each 30 minutes in a day. High values denote a strong correlation between both methods. **C** and **F**, Immobility and sleep fragmentation, respectively. Each point shows data from a single individual, with its total number of bouts in 5 days, on the y-axis, and the average duration of all its bout, on the x-axis. L and D phase bouts were computed separately. Shaded areas in **A** and **D** show a 95% bootstrap resampling confidence interval around the average. Contour lines in **C** and **F** are density levels. $N_{male} = 485$ and $N_{female} = 881$.

4.3 A three-states model of behaviour

My results hinted that DAM and ethoscope scoring accounted for different — and possibly complementary — aspects of behaviours. The former detecting exclusively animals walking along their tube, whilst the latter also included small movements in the activity. In other words, the ethoscope detects ‘micro-movement’, a new class of movement that was traditionally accounted as quiescence. I speculated that micro-movements were qualitatively different from both walking activity and quiescence, and therefore decided to capture behaviour as a discrete variable with three states: walking, micro-movement and quiescence. Using the same raw data

as in fig. 4.1, I scored one of these three states for each consecutive minute of recording (fig. 4.2, see method subsection 4.6.5 for detail).

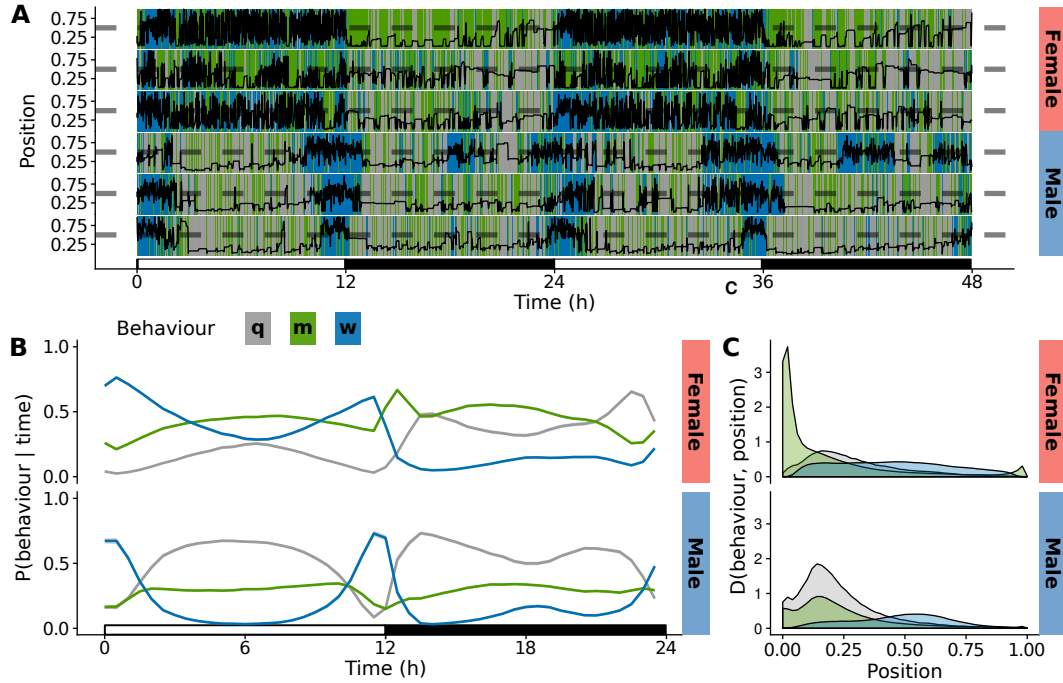


Figure 4.2: *Behavioural state modulation*. **A**, Six, randomly selected, 48-hours, individual behavioural time series. The background colour indicates behaviour state whilst the solid black line shows the average relative position with respect to the food (*i.e.* $Position = 0 \Rightarrow$ the fly is on the food”). Both variables are scored at a frequency of 1 min^{-1} . The three behavioural states are quiescent (q, grey), micro-movement (m, green) and walking (w, blue). The grey dashed line at $Position = 0.5$ shows the location of the ‘virtual beam’, that would emulate DAM data. **B**, Population averages of the proportion of time engaged in one of the three behavioural state over 24 hours. Shaded areas show a 95% bootstrap resampling confidence interval around the mean. **C**, Distribution of the *position* variable computed separately for each behavioural state. Within each group (sex), densities are scaled to reflect the overall occurrence of each state b over the space x (*i.e.* $\int_{x=0}^1 D(b, x) = P(b)$ and $1 = \sum_b P(b)$). In **B** and **C**, $N_{male} = 485$ and $N_{female} = 881$.

To illustrate the nature of this new variable, the data of six individual animals are shown in fig. 4.1A. Interestingly, the top row shows a female that featured a large amount of micro-movements during its D phases, but only a few midline (grey dotted line at $Position = 0.5$) crosses. Indeed, it appears that such micro-movements occurred in close proximity to the food ($Position \rightarrow 0$).

To understand the time dynamic of these three states at the population level, I computed the average occurrence for each behavioural state in each consecutive 30 minutes window of a circadian day (fig. 4.1B). In females, the overall quiescence, micro-movement and walking probabilities were $P(q) = 0.269$, $CI_{95\%} = [0.262, 0.277]$, $P(m) = 0.433$, $CI_{95\%} = [0.427, 0.438]$, and $P(w) = 0.298$,

$$CI_{95\%} = [0.292, 0.304].$$

Quiescence was higher during the D phase compared to the L phase. Furthermore, it was only dominant in the at the end of the D phase. Interestingly, micro-movement highly varied with the time of day, with a peak immediately after $L \rightarrow D$ transition. Walking was higher during the L phase compared to the D phase and dominant after the $D \rightarrow L$ as well as before the $L \rightarrow D$ transitions.

In males, the overall quiescence, micro-movement and walking prevalences were $P(q) = 0.519$, $CI_{95\%} = [0.512, 0.527]$, $P(m) = 0.286$, $CI_{95\%} = [0.281, 0.291]$, and $P(w) = 0.195$, $CI_{95\%} = [0.190, 0.200]$. Quiescence was the dominant behaviour for most of the time, except around phase transitions. Micro-movement did not appear to vary strongly over time and was overall much lower than in females. Walking was overall rare and happened almost exclusively before and after both transitions.

Subjective observation (not shown) of video recording indicated that micro-movements in females were consistent with feeding behaviour (proboscis extension on the food). In order to corroborate the hypotheses that female micro-movements correspond to feeding, I computed the distribution of position for specific behaviours (fig. 4.1C). As expected, females micro-movements happened in close proximity to the food with 51.3%, $CI_{95\%} = [50.3, 52.2]$, of them occurring within only 4 mm of the food end. As previously suggested¹³⁰, overall, flies seemed to engage in quiescence in a preferred position that was close, but not on, the food (approximately at $Position \approx 0.2$). Surprisingly, in males—and in contrast with females—the position distributions of micro-movements and quiescence were extremely similar, and micro-movement did not seem to often occur on the food.

To visually summarise the above results, I developed a representation that could, in principle, be used by experimentalists to create a ‘behavioural fingerprint’ of multiple populations, which would be instrumental for screens. I opted to represent behavioural trajectories in a ternary plot, as a timeline through a 2-simplex (fig. 4.3). Along a day, females (fig. 4.3A) ‘visited’ all dimensions of the behavioural space whilst the variation in males (fig. 4.3B) followed a direction mostly orthogonal to micro-movement.

To consider whether the individual paths through this behavioural space could be instrumental to classify flies, I performed a hierarchical clustering of all animals and assessed how such unsupervised approach could recapitulate known individual labels (*i.e.* male or female). Briefly, a distance measurement, based on Bhattacharyya coefficient⁴⁰⁷, was computed between each pair of animals, and

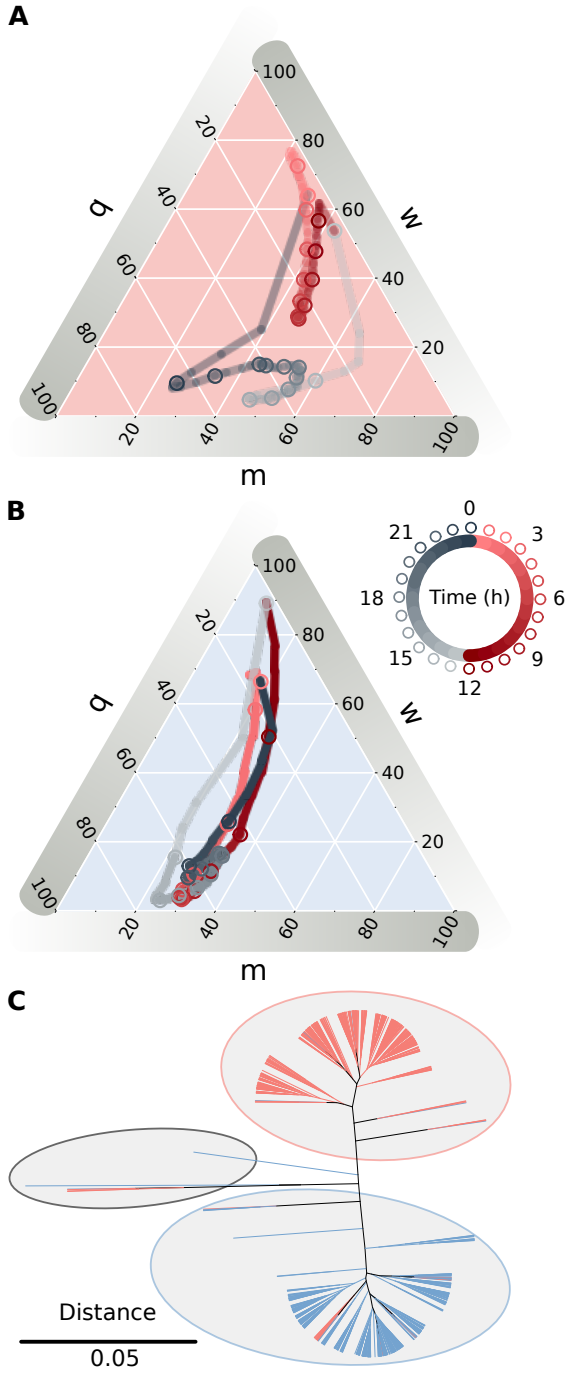


Figure 4.3: *Behavioural state modulation.* **A** and **B**, Ternary representation of behavioural state values along a circadian day, for female and male populations, respectively. Each point represents the average proportion of time spent engaging in quiescence (q), micro-movement (m) and walking (w). The colour of each point and line indicates the time of the day (see the circular colour key). Average were computed for every 15 minutes. Points are circled every hour. $N_{male} = 485$ and $N_{female} = 881$. **C**, Hierarchical clustering of a random sample of 400 males and 400 females (a subset was used for performance considerations only). The average Bhattacharyya distances across all time points were used as a dissimilarity measure. The dendrogram was computed using UPGMA (see method subsection 4.6.6). The three ellipses show partitions of the dendrogram corresponding to males, females, and a heterogeneous outlier group.

Unweighted Pair Group Method with Arithmetic Mean (UPGMA)⁴⁰⁸ was applied (see method subsection 4.6.6) (fig. 4.3C). Both groups clustered very well with a Fowlkes-Mallows index $FM = 0.930$ larger than expected under the null model, $FM_{H_0} = 0.497$, $CI_{95\%} = [0.495, 0.498]$, (see method subsection 4.6.6). Interestingly, a group of 13 females, mostly from different experimental blocks, clustered together within the male group suggesting a cryptic, but consistent, sub-population of females that behave very differently from others.

4.4 Effect of mating on female behaviour

Since, in females, micro-movements occur largely on the food, I had hypothesised that they correspond to food-related behaviours, such as eating and egg laying. I then reasoned that the possibility of scoring micro-movements would reveal crucial to assess activity in animals that modulate their feeding behaviour. It had been shown that, after mating, females *Drosophila melanogaster* dramatically increase their feeding behaviour³⁵⁴, providing a convenient example of a documented and naturally occurring change of feeding behaviour. I, therefore, designed an experiment aiming at comparing mated females to non-mated controls with respect to their behavioural states and position preference (fig. 4.4).

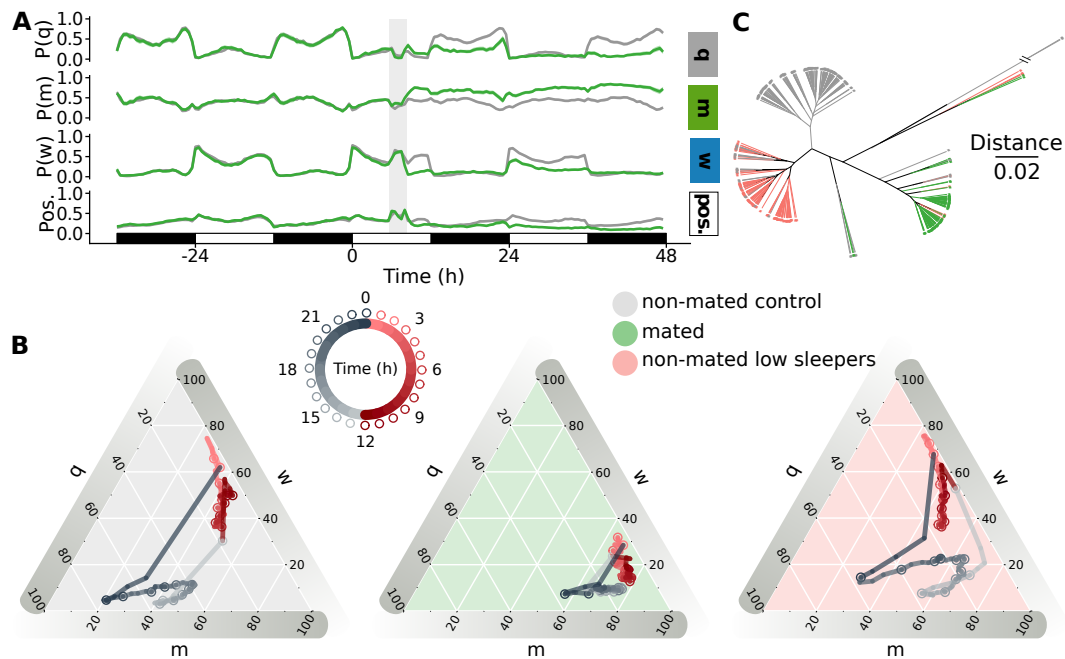


Figure 4.4: *Effect of mating on female behaviour.* **A**, Proportion of time spent in quiescent (q), micro-moving (m) and walking (w), as well as average position, over the course of the experiment. Females were allowed to interact, for an hour, with a male during the first day (grey bar). Females that had mated and non-mated controls are shown as in green and grey bars, respectively. **B**, Ternary representation of behavioural state values along a circadian day, for both mated and non-mated populations. In addition, a third, independent, group of non-mated female that had low quiescence was included for comparison purposes. **C**, Hierarchical clustering of the control and mated female groups as well as an independent, low sleeper, third group. The average Bhattacharyya distances across all time points were used as a dissimilarity measure. The dendrogram was computed using UPGMA (see method subsection 4.6.6). $N_{mated} = 86$, $N_{non-mated} = 152$ and $N_{low-sleeper} = 110$.

To start with, a population of 238 virgin females was monitored for a baseline assessment of 48 h. Then, a male was introduced in all tubes for a short duration which allowed only for partial mating efficiency, resulting in two populations of

females, $N_{mated} = 86$ and $N_{non-mated} = 152$. After removing the males, all females were scored for another two days (see method subsection 4.6.3). Conveniently, the non-mated had likely also experienced the presence of a male and courting, but not mating, and could serve as a faithful control.

Immediately after mating, grey bar in figure 4.4A, mated females widely reduced walking and, concomitantly, increased their micro-movements. The overall elevation of micro-movements was very sustained throughout the rest of the experiment, with consistently higher values in the mated population. On average, mating resulted in an increased probability of micro-moving to 0.712, $CI_{95\%} = [0.694, 0.729]$, against 0.409, $CI_{95\%} = [0.395, 0.425]$, in the controls.

Interestingly, mated females reduced their walking probability during the L, but not in the D, phase. In contrast, quiescence was almost exclusively reduced in the D phase. Furthermore, the position of mated females was on average much lower (*i.e.* closer to the food), which also corroborates the hypothesis of increased feeding (fig. 4.4A, bottom panel).

Because of the probabilistic nature of the mating protocol, the possibility that, for instance, females that had micro-moved more may have had greater mating success could not be *a priori* excluded. However, no statistical effect of average behaviour state values during baseline on mating success could be found (binomial regressions, $p\text{-value} > 0.05 \forall \text{behaviour}$). In other words, there were no obvious and strong link between the behaviour before mating and the probability of mating.

To put in context the amplitude and the nature of such sustained behavioural change after mating, I decided to compare both mated and non-mated populations to an independent percentile group of $N_{low-quiescence} = 110$ virgin females (from fig. 4.1) that had approximately equal overall quiescence, $P(q) = 0.168$, $CI_{95\%} = [0.164, 0.173]$, as the mated group $P(q) = 0.154$, $CI_{95\%} = [0.141, 0.168]$ (fig. 4.4B). Despite quiescence amounts being equivalent, the topology of the behavioural fingerprint appeared radically different between this spontaneously active but non-mated group and the mated females. In fact, former seemed to resemble more the non-mated group.

To further describe the similarity of fingerprints between and within groups, I performed hierarchical clustering using UPGMA (fig. 4.4C). The obtained clusters recapitulated the initial labels accurately $FM = 0.800$, with $FM_{H_0} = 0.328$, $CI_{95\%} = [0.324, 0.334]$ (see method section 4.6.6).

Furthermore, the average pairwise distances between individuals suggested that

the mated female group behaved as an outgroup: $\bar{D}(\text{mated}, \text{non-mated}) = 0.118$, $\text{CI}_{95\%} = [0.117, 0.119]$, $\bar{D}(\text{mated}, \text{low-quiescence}) = 0.093$, $\text{CI}_{95\%} = [0.092, 0.094]$, and $\bar{D}(\text{non-mated}, \text{low-quiescence}) = 0.082$, $\text{CI}_{95\%} = [0.081, 0.083]$, confirming that the consequences of mating on behaviour go beyond a mere scaling of quiescence.

4.5 Endogenous determinism of behaviour

I noticed that most behavioural variables I had recorded so far were surprisingly both very consistent within individuals, over several days, but highly variable between individuals, despite biologically homogeneous population (genetics, age and social interactions). For instance, figure 4.5 shows the sleep amount, as colour intensity, for all individuals used in figure 4.1. Animals are sorted by sleep amount and represented by a single row.

The inter-individual variability in the amount of sleep was high for both males ($P(\text{asleep}) = 0.43$, $\text{sd} = 0.10$) and females ($P(\text{asleep}) = 0.21$, $\text{sd} = 0.11$). In addition, it appeared that individuals had a tendency to retain their sleep pattern over time. In other words, there seemed to be a strong positive correlation between sleep at day d and $d + 1$, for the same animal.

It was however unclear whether this hypothetical correlation resulted from intrinsic biological variables or, rather, from confounding factors (such as the differences in light intensity and food amount across experimental tubes or even from scoring discrepancies between ethoscopes). To gain insight into the source of such variability, I designed a new experiment in which flies (males and females) were monitored for six days. Then, animals were all transferred to a new experimental tube and systematically interspersed into a new region of interest, machine and experimental incubator (see methods subsection 4.6.4). Subsequently, monitoring was carried out for another six days (fig. 4.6).

The three behaviours of interests (quiescence, micro-movement and walking) were scored (fig. 4.6A). For all individuals, the average time engaged in all three states was computed for each day. Since preliminary enquiry had suggested that animals tend to be quiescent in a consistent position along days, I also computed the average location at which animals rest ($\text{Position}|q$).

Then, I studied how all four variables remained correlated to their own value on the first day and, in particular, the extent to which the change of environment

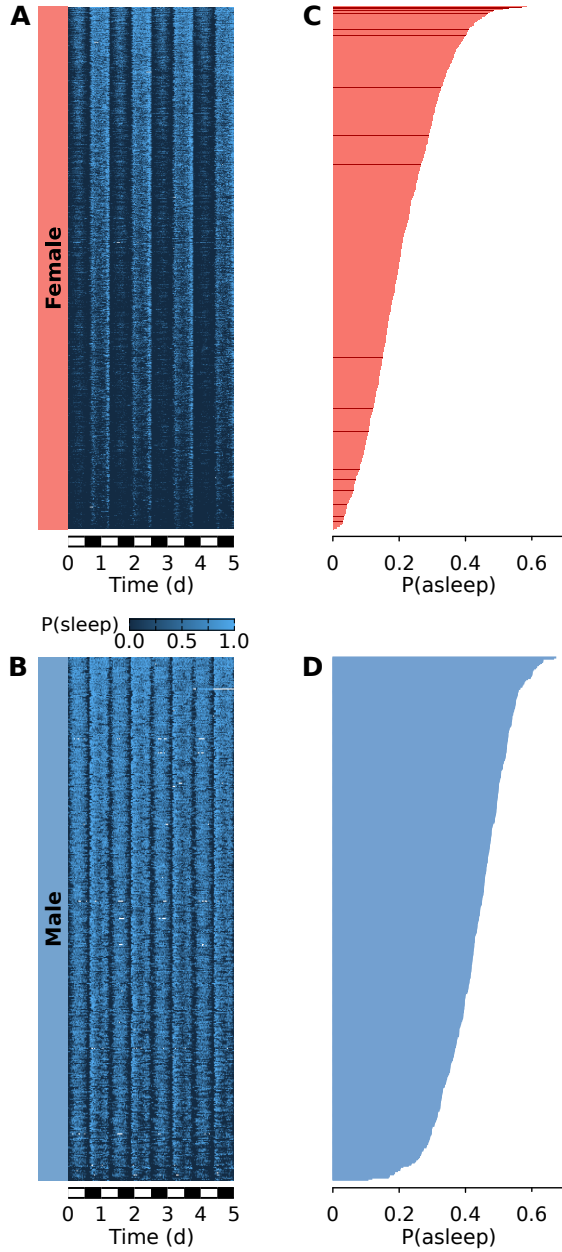


Figure 4.5: *Sleep distribution.* **A** and **B**, Individual sleep profiles. Each row shows data from an individual animal for which sleep amount was scored and represented by a colour intensity every 30 minutes. **C** and **D**, Average sleep over five days for each individual. Flies were sorted in decreasing order, from top to bottom, with respect to their overall sleep amount. Each row in **A** and **B** is aligned to the bar representing the same animal, in **C** and **D**, respectively. The top (**A** and **C**) and bottom (**B** and **D**) sub-figures represent males and females, respectively and $N_{male} = 485$ and $N_{female} = 881$.

disrupted this relationship (fig. 4.6B).

Very interestingly, all three behavioural states had a positive correlation $\rho > 0$ throughout the experiment (before and after shuffling flies in the animals' environment), which indicates that intrinsic factors determine, at least partially, the amount of time engaged in these behaviours. In sharp contrast, the preferred resting position, which was also auto-correlated before tube change, lost all significance $\rho \approx 0$ after the tube transfer, suggesting rather an environmental determinism of resting position.

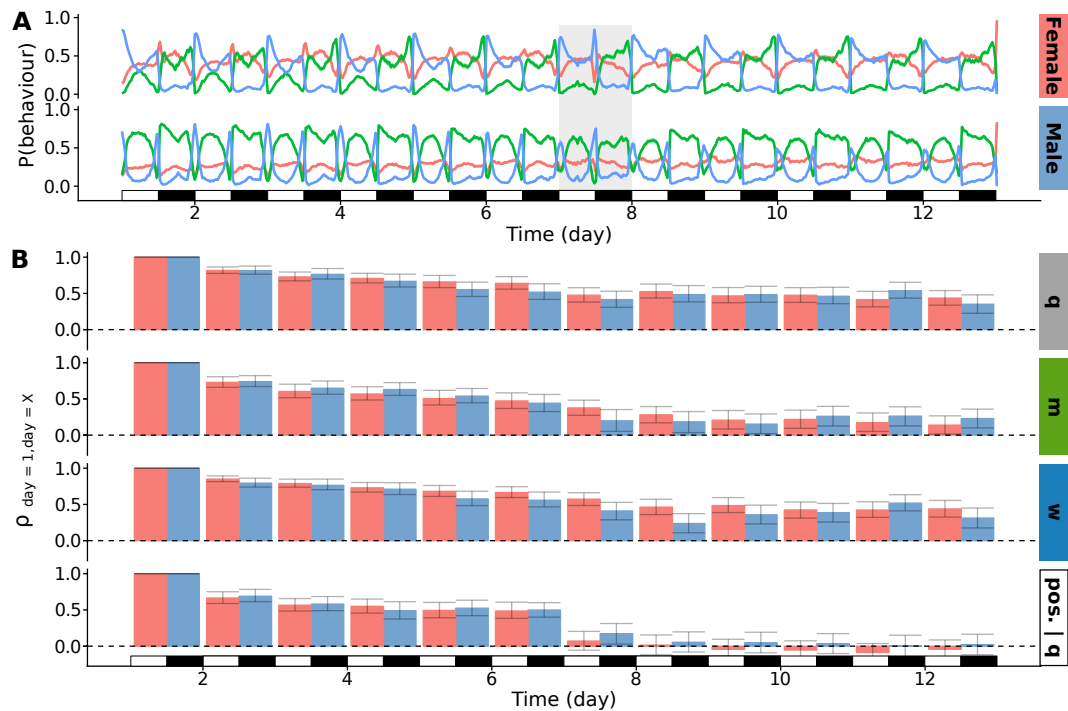


Figure 4.6: *Individual consistency of behavioural states* **A**, Population averages of time spent in quiescence (q), micro-movement (m) and walking (w). On day 7 (grey rectangle) animals were transferred into fresh tubes and systematically interspersed to a new location (within experimental incubators and ethoscopes). **B**, Means of Spearman's ρ showing the correlation of average time spent engaged in a behaviour between day 1 and all subsequent days, by the same animal. In addition to the three behavioural states, the average position when quiescent is also shown. Shaded areas, in **A**, and error bars, in **B**, show 95% bootstrap resampling confidence intervals around the mean. $N_{\text{male}} = 204$ and $N_{\text{female}} = 242$.

4.6 Methods

4.6.1 Fly stocks

CantonS *Drosophila melanogaster* obtained from Ralf Stanewsky's laboratory (University of Münster) were used for all experiments. All flies were raised under a 12 h light:12 h dark (LD) regimen at 25°C on standard corn and yeast media. Unless otherwise stated, all animals were socially naive.

4.6.2 Experimental conditions

For all experiments, 7–8 days old pupae were sorted into glass tubes (70×5×3 mm [length × external diameter × internal diameter]) containing regular food. When animals reached 2–3 days old, tubes were loaded into ethoscopes (20 animals per

machine). All experiments were carried out under constant LD condition, 50–70% humidity, in incubators set at 25°C. Animals always had *ad libitum* access to regular food. Flies that died during the experiment were excluded from the analysis.

4.6.3 Female mating

To evaluate the effect of mating on sleep (fig. 4.4), a naive male was introduced in the tube of each naive female and allowed to interact for 2 hours, $ZT \in [06, 08] h$. Afterwards, males were all discarded and the activity profile of the females was recorded for another 3.5 days. Because of the short duration of the interaction and the restrictive space of the glass tube the probability of mating was reduced, which resulted in two groups: females that were either mated or only courted (non-mated). Effective mating was determined *post hoc* by visually scoring the presence of larvae in the tube four days after the interaction.

4.6.4 Endogenous determinism of behaviour

To study the individual consistency of behavioural states over several days (fig. 4.6), behaviour of both males and females was first recorded for seven days. Then, individual animals were transferred to new tubes. In order to minimise the effect of hidden confounding variables (*e.g.* variation of light, humidity, vibration, ethoscopes and incubators), on behaviour, the new position of all the tubes was systematically interspersed (*sensu*⁴⁰⁹).

4.6.5 Behaviour scoring

‘Immobility’ was scored by thresholding corrected maximal velocity on ten-seconds epochs as described in section 2.8. ‘Sleep’ was computed using the five-minute rule: immobility bouts longer than 300 s were counted as sleep bouts.

Behavioural states ($B \in \textit{quiescence}, \textit{micro-movement}, \textit{walking}$) were defined for each consecutive minute of behaviour according to the following rule:

$$B_j = \begin{cases} \textit{quiescence}, & \text{if } V_{corr_i} < 1 \forall i \\ \textit{micro-movement}, & \text{else if } \sum^i |X_i - X_{i-1}| < T_d \\ \textit{walking}, & \text{otherwise} \end{cases} \quad (4.1)$$

Where,

- i is the index of successive frames (at approximately 2 FPS) in a minute,
- j represents the resulting minute of scored behaviour,
- V_{corr} is the corrected velocity as described in subsection 2.8.3,
- X is the position along the tube and
- T_d is a constant threshold of 15 mm on the distance moved above which *walking* is scored. T_d was defined empirically based on the observation of a bimodal distribution of the total distance moved in a minute (see fig. 4.7).

All instantaneous velocities being lower than 1 in a minute implies the maximal velocity in any ten seconds epoch is also lower than 1.

The space available inside each experimental tube was variable between individual animals due to the different amount of food and cotton wool. In order to compare flies position with respect to the boundary of their respective experimental environments, their position was expressed relative to the food ($Position = 0$) and the cotton wool ($Position = 1$) edges:

$$position = \frac{X - Q_{0.01}(X)}{Q_{0.99}(X) - Q_{0.01}(X)} \quad (4.2)$$

Where, Q_p is the quantile function.

First and last percentiles were used instead of minimum and maximum to avoid the possible effect of spurious artefactual detections beyond the physical limits of the tube.

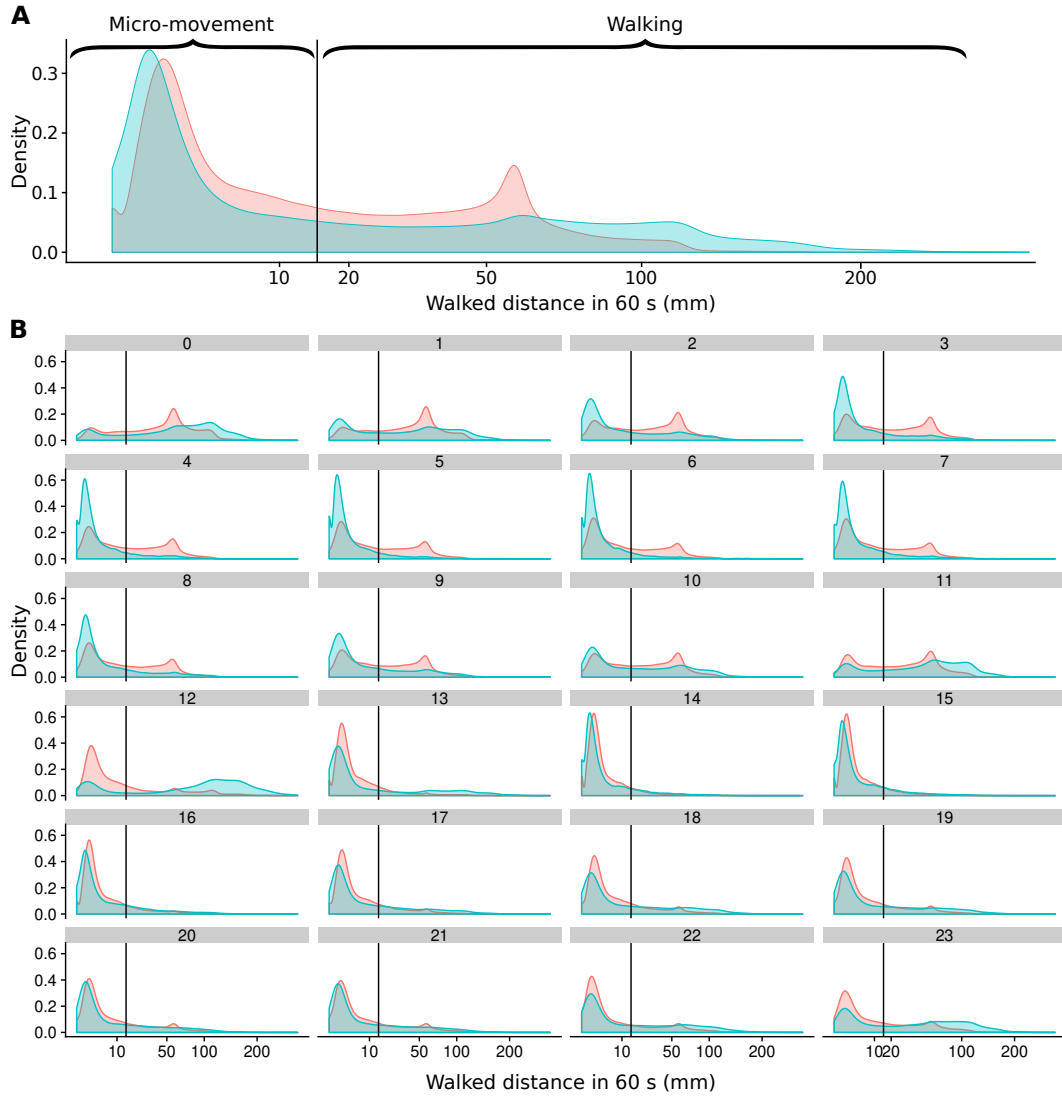


Figure 4.7: *Distribution of walked distance in one minute* **A**, Overall distribution of walked distance for all one-minute bouts that were *not* scored as quiescent, for males (blue) and females (red). **B**, Distribution of walked distance per hour of the day. The black vertical line shows the value of threshold T_d used to score bouts as micromovement or waling (see eq. 4.1) The label of each facet represents the hour of the day from which the data was used (*e.g.* 0 means $ZT \in [0, 1)$). $N_{male} = 485$ and $N_{female} = 881$. The X axis is square-root-transformed.

4.6.6 Hierarchical clustering

The dendrograms in figs. 4.3 and 4.4 are the result of a hierarchical clustering using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method⁴⁰⁸.

During an interval of time, the proportion of time spent by an animal in a behavioural state can be formulated as an empirical discrete probability density function. In this context, the distance between each pair of animals was computed using the average of Bhattacharyya distances⁴⁰⁷ over the entire day:

$$D(p, q) = \frac{\sum_{t \in T} BD_t(p_t, q_t)}{|T|} \quad (4.3)$$

$$BD_t(p_t, q_t) = -\ln(BC(p_t, q_t)) \quad (4.4)$$

$$BC(p_t, q_t) = \sum_{x \in X} \sqrt{p_t(x)q_t(x)} \quad (4.5)$$

Where,

- BD_t is the Bhattacharyya distance at a time interval t ,
- T is the set of consecutive 15 min time intervals within a day:
 $T = \{[0, 0.25), [0.25, 0.5), \dots, [23.75, 24)\}h$,
- BC_t is the Bhattacharyya coefficient at a time interval t ,
- p and q are the observed distributions of behaviour for two different individuals and
- X is a the set of discrete behaviours as defined in eq. 4.1:
 $X = \{quiescent, micro-movement, walking\}$.

To assess the consensus between unsupervised hierarchical clustering and ground-truth labels, Fowlkes-Mallows index⁴¹⁰ (FM) was computed with:

$$FM = \sqrt{\frac{TP}{TP + FP} \cdot \frac{TP}{TP + FN}} \quad (4.6)$$

Where,

- TP is the number of true positives,
- FP is the number of false positives and
- FN is the number of false negatives.

The expected value of FM under H_0 (FM_{H_0}) was obtained by computing the mean and percentiles on 10,000 label permutations.

4.6.7 Statistics

Unless otherwise stated, the error bars and shaded areas around the mean are 95% confidence interval computed using basic bootstrap resampling⁴¹¹, with $N = 1000$.

4.6.8 Software tools

All data analysis was performed in R⁴⁰⁵, using the rethomics framework (see chapter 3 and⁴⁰⁰). Figures were drawn using `ggplot2`³⁹⁸ and ternary representations in were generated with `ggtern`⁴¹².

4.7 Summary

- Traditional activity scoring greatly overestimates immobility and sleep compared to ethoscopes, and bias depends, at least, on time of the day and sex.
- The addition of a new behavioural state, micro-movements, is very informative and can account for such inconsistencies.
- Micro-movements are particularly relevant in females, for which they seem to correspond to feeding instances.
- In females, mating dramatically increases micro-movements and proximity to the food whilst reducing walking and quiescence.
- Although behaviour states are very variable between animals, they remain consistent throughout time and environment within the same individual.

5 | Sleep Deprivation

‘To die: to sleep;
No more; and by a sleep to say we
end’

— Hamlet, in
Hamlet [Ref. 413, Act III, Scene 1]

5.1 Background

In the 17th century, Robert Boyle initiated the first chemical revolution, which transmuted alchemy into chemistry. It culminated, a century later, with Antoine de Lavoisier’s law of conservation of mass. The contribution of Lavoisier — and other chemists such as Joseph Priestley — to chemistry is well known, but less so is his work on the physiology of respiration⁴¹⁴. He applied the same analytic and rigorously quantitative method to understand both combustion and respiration — *i.e.* measuring the heat animals produce and the oxygen they consume. In order to prove oxygen was crucial for respiration, the chemists would deprive an animal of it⁴¹⁵. The idea of *removing a single component from a biological system in order to understand its necessity* was both powerful and modern.

In this ‘analytic’ perspective, the first step to understanding the functions of sleep would be to deplete sleep altogether. As simple as this idea may seem, it has proven very difficult to implement. Indeed, the various ways of forcing an animal to be awake may themselves create confounding stresses. For instance, an animal exposed to recurrent stimuli that are primarily intended to remove sleep may incidentally stop eating, and the observed physiological consequences could result from starvation more than sleep deprivation.

As I have discussed in the introductory chapter, the question of the inevitabil-

ity and necessity of sleep is central (see subsection 1.3.4.1). In order to address it, authors have mostly performed chronic sleep deprivation, sometimes until the subjects died. In this type of experiments, the above consideration — of reducing confounding stresses — becomes crucial. Indeed, various stresses are known to impact lifespan, presumably independently of sleep. Hoping to render sleep deprivation more specific, authors have sometimes used a paradigm that I call Dynamic Sleep Deprivation (DSD), which implies startling an animal only when it is asleep.

Perhaps the most illustrative example of DSD is the ‘disk-over-water’ method developed by Allan Rechtschaffen and Bernard Bergmann^{151,152,416}. It pairs two rats: the ‘totally sleep deprived’ and the ‘control’ animals and records their brainwaves. As soon as the former starts sleeping, a feedback, computer controlled, mechanism rotates the disk on top of which both animals stand. Unless they wake up and walk, this rotation makes them fall into a water container underneath. The control rat is therefore disturbed the same number of times as the deprived one but can sleep when its pair was awake. Since its invention, the disk-over-water paradigm was also used on pigeons¹⁵³. In different model organisms, for instance, in mice, other automatic tools were developed to perform long lasting DSD⁴¹⁷.

However, in insects, the only form of DSD that has been performed, to my knowledge, is *manual*. That is, researchers have startled animals immediately after they had noticed — subjectively — them being immobile. For instance, cockroaches have been kept awake by ‘gentle shaking of the cages whenever the animals were immobile’¹⁰⁷, or to dynamically sleep deprive fruit flies, ‘experimenter[s] would gently tap on [the fly’s] tube’¹⁵⁴.

In addition to the subjective nature of the scoring and stimulus delivery, manual sleep deprivation also severely reduces experimental throughput, especially when performing chronic sleep deprivation, which also explains the low number of individuals used: 12 flies¹⁵⁴ and 10 cockroaches¹⁰⁷.

In chapter 2, I presented the ethoscope as a device capable delivering real-time stimuli and, in chapter 4, I showed how video tracking was instrumental for augmenting our understanding of sleep, at least in undisturbed animals. In the chapter herein, I will, firstly, validate ethoscopes as a tool to perform DSD, study the induced quiescence rebound and explore the effect of important parameters. Secondly, I will show that rebound is conditional on stimuli being given when animals were quiescent. Thirdly, I will investigate how the effectiveness of sleep deprivation depends on when it occurs. Lastly, I will conclude by presenting the results of an unprecedented large-scale chronic sleep deprivation and study its effect on

longevity.

Part of the work presented in this chapter is available as a preprint⁴⁰⁶. It results from a collaboration, in equal part, between Esteban Beckwith—without whom this work could not have been carried—and myself.

5.2 Results

5.2.1 Dynamic Sleep Deprivation (DSD)

Most sleep deprivation experiments in *Drosophila melanogaster* had been performed in a mechanical and *static* fashion. In this paradigm, stimuli are delivered to animals independently of their behavioural states (*i.e.* walking, feeding and immobile individuals are startled regardless). Since the ethoscopes allow both for real-time tracking and can be extended with modules that deliver individually targeted stimuli, it is possible to employ a *dynamic* paradigm instead. Such DSD, implies animals are only startled when they were already sleeping—or at least immobile.

Since, to my knowledge, this was the first implementation of DSD in *Drosophila melanogaster*, I focussed on validating this novel paradigm. Specifically, I wanted to verify that it was effective at depleting quiescence and could indeed elicit a homeostatic rebound. In addition, I wanted to take advantage of the behavioural states I had defined to better characterise sleep deprivation.

I recorded baseline behaviour of both males and females for two days, after which they were either dynamically sleep deprived for 12 h, over their Dark (D) phase, or left undisturbed as controls ($N > 145$ for all four groups). Servo modules were fitted to ethoscopes so that every other experimental tube could be turned with a servomotor, pulley and o-ring system (see subsection 2.3.1). Devices were programmed to startle animals when and only when they had been immobile for 20 s. The trends of each behavioural state, position and number of stimuli given during the experiment as well as the following quiescence rebound were then analysed (fig. 5.1).

During DSD, the propensity of all three behaviours and the average position were altered, both in males and females (fig. 5.1A). Throughout the treatment, animals that received stimuli were more active: quiescence was greatly reduced whilst

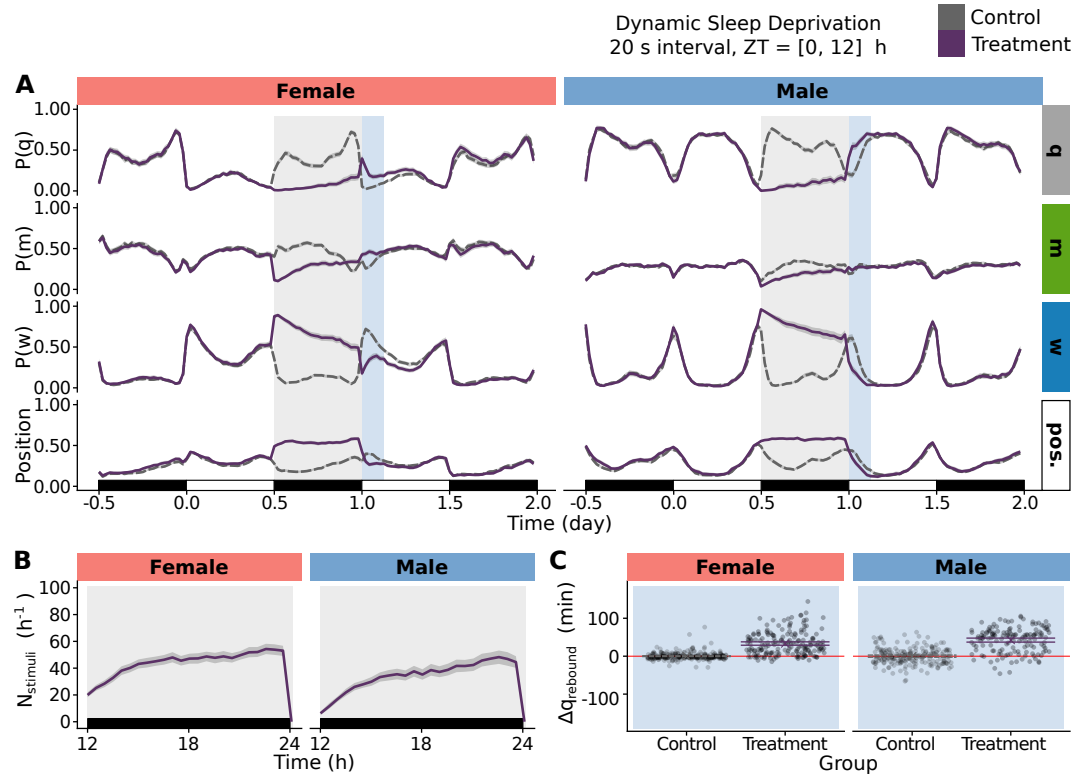


Figure 5.1: *Overnight dynamic sleep deprivation causes a homeostatic rebound.* **A**, Proportion of time engaged in either of the three behavioural states (q: quiescence, m: micro-movement and w: walking) and relative position (from the food, 0, to the cotton wool, 1) are represented as different rows. Females and males are shown on the left and right, respectively. The grey rectangle in the background, between ZT=12 and ZT=24 h, coincides with the permissive time window of stimulus delivery, Stimuli were delivered to animals each time they had been immobile for 20 consecutive seconds. Controls animals (grey) were undisturbed and spatially interspersed (in the neighbouring tube) with the treated individuals (plum). **B**, Average number of stimulus delivered in each consecutive 30 min. **C**, Extra quiescence during the 3 h of rebound (blue rectangle in the background of **A**), expressed in extra minutes compared to the predicted quiescence (see method subsection 5.3.4). The shaded areas around the average lines, in **A** and **B**, and the error bars in **C** are 95% bootstrap resampling confidence intervals on the mean. $N_{sex,treatment} > 145 \forall sex \times treatment$.

walking frequency was increased. To my surprise, the fraction of time spent micro-moving was also reduced for both sexes along the night. Startled animals were also, on average, further away from the food, and closer to the middle of their tube, which is typical in highly walking animals.

Interestingly, the number of stimuli delivered increased monotonically during the night, to reach nearly 1 min^{-1} (fig. 5.1B). In a dynamic context, the delivery of an increasing number of stimuli could indicate a growing sleep pressure insofar as increasingly more startling events were required to keep animals active. In total, females and males experienced 631, $CI_{95\%} = [586, 680]$, and 558, $CI_{95\%} = [499, 617]$, stimuli, respectively.

On the day immediately following the end of the treatment ($t \in [1, 1.5]$ d) the behaviour of the startled population was different from their respective controls (fig. 5.1A). In particular, quiescence, which had been reduced during treatment, was then increased. Such rebound is generally interpreted as partial homeostatic recovery, which is defining of sleep. Conversely, walking was decreased throughout this post-treatment period. Another noticeable difference was that females increased their proportion of micro-movement immediately after the DSD, which coincided with a greater proximity to the food. This observation suggests that this paradigm may also incidentally reduce feeding, and therefore cause a similar homeostatic recovery (*i.e.* a ‘feeding rebound’).

After the end of the treatment, the differences with the controls faded over time, to the extent that both groups had the same levels for all behaviour states after 12 h of recovery. In fact, most of the rebound happened in the first three hours. I was interested in expressing rebound in terms of relative quiescence (*i.e.* compared to predicted values) in this 3 h period. To predict quiescence during the rebound I used a linear model, fitted on the undisturbed control populations (see methods subsection 5.3.4). Both females and males displayed a consistent rebound after treatment, recovering 33.4, $CI_{95\%} = [29.0, 37.9]$, and 42.4, $CI_{95\%} = [37.6, 47.6]$, minutes, respectively (fig. 5.1C).

Since a 12 h DSD, with a 20 s interval, seemed to cause stress that impacted behaviour beyond quiescence — *e.g.* micro-movements were reduced — I suspected that such treatment resulted in confounding stress. I postulated that such off-target effects would increase — and the specificity of the sleep deprivation decrease — with the number of stimuli. I, therefore, became interested in investigating the extent to which the interval could be lengthened. In other words, whether sleep deprivation would be effective, and possibly more parsimonious if animals were allowed to rest for longer than 20 s at a time. To investigate this avenue, I widened the scope of this experiment by testing a range of intervals, from 20 to 1000 s (fig. 5.2).

In this experiment, animals were only allowed to be quiescent for a given interval of time, after which they were startled by a stimulus. Each fly has a constant interval over the 12 h treatment, but intervals varied between animals. From the distribution of bout length in preliminary experiments, I observed that approximately 5% of animals never experienced a quiescence bout longer than 1000 s over 12 h (not shown). Therefore, I used this value as the longest interval. I hypothesised that the difference of effect between two intervals would depend more on their ratio

than on their difference, and opted for a (pseudo-)dynamic, rather than linear, range, with ten intervals between 20 and 1000 s (see, for instance, the x-axis in fig. 5.2A).

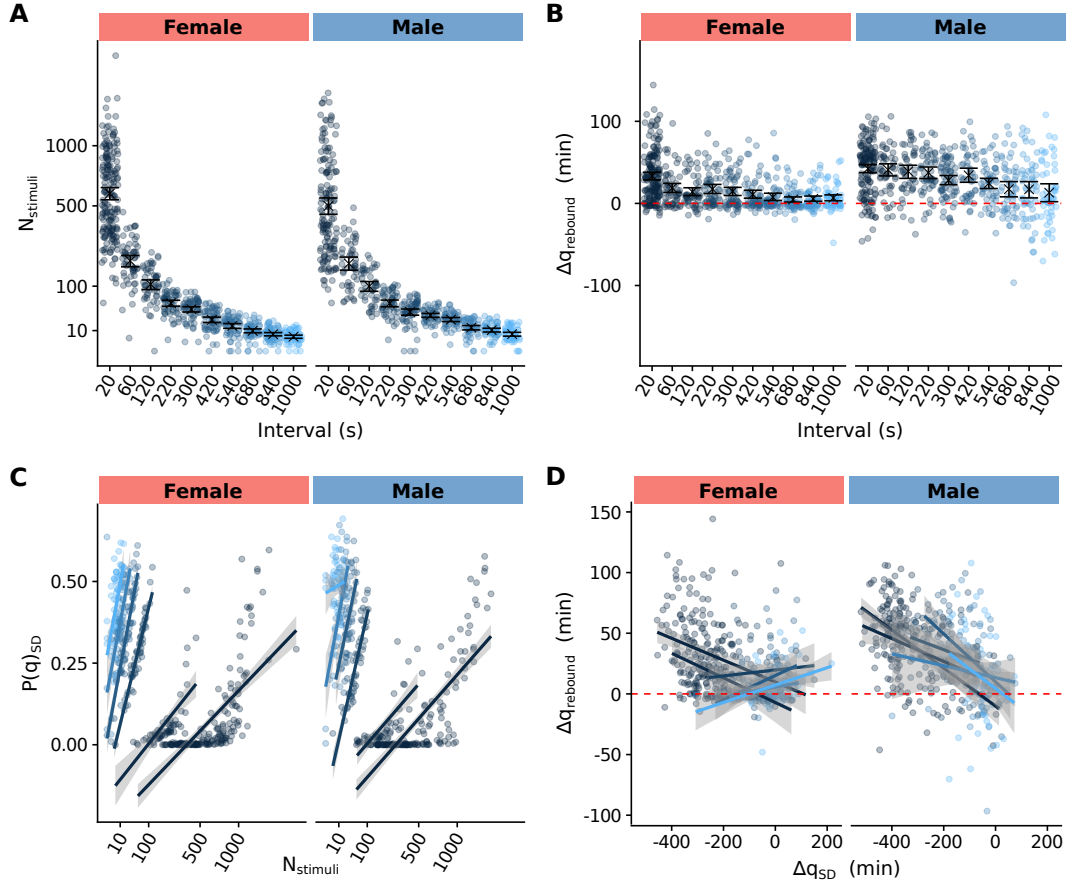


Figure 5.2: *Only a few targeted stimuli causes a homeostatic rebound.* **A**, Number of stimuli delivered over the 12 h of dynamic sleep deprivation. The point colour and x axis represent different intervals (*i.e.* time an animal can remain immobile without being startled). **B**, Extra quiescence during the 3 h of rebound, expressed in extra minutes compared to the predicted quiescence (see method subsection 5.3.4). **C**, Relationship between quiescence during sleep deprivation and number of stimuli delivered in the same period. The error bars, in **A** and **B**, are 95% bootstrap re-sampling confidence intervals on the mean (black crosses). **D**, Relationship between lost quiescence during the sleep deprivation and rebound (*i.e.* regained quiescence) in the subsequent 3 h. The red dashed line at $Y = 0$, in **B** and **D**, shows the value of rebound expected by chance. For the sake of clarity, only five intervals are shown in **C** and **D** ($interval \in [20, 120, 300, 540, 840]$ s). Lines in **C** and **D** show linear model fit with standard errors (shaded areas). $N_{sex, interval} > 45 \forall sex \times interval$.

As expected, the overall number of stimuli delivered decreased with interval duration were very similar between sexes, for the same interval (fig. 5.2A). For the longest interval, 1000 s, the average was only 6.83, $CI_{95\%} = [5.95, 7.77]$, stimuli, overnight. In fact, several individuals never experienced long enough quiescence bouts to be startled ($N_{stimuli} = 0$).

Following the 12 h of treatment, the quiescence rebound was quantified (fig. 5.2B).

In females, the amplitude of the rebound appeared to be a monotonic function of the interval, with the largest rebound for 20 s. In males, however, this relationship resembled more a step function, with rebound of similarly high amplitude for intervals under 500 s, and of similarity low amplitude for longer intervals.

I also wanted to investigate how the number stimuli explained the actual quiescence during the treatment, and whether these two variables could predict the amplitude the rebound itself. Within each interval group, there was a positive relationship between quiescence during the sleep deprivation night, and the number of stimuli delivered (linear model, overall $R^2 = 0.83$, fig. 5.2C). Indeed, the dynamic nature treatment means that animals that had a higher propensity to sleep exhibited more quiescence, but also needed more stimuli to be kept awake.

To some extent, the amount of quiescence loss during sleep deprivation (compared to forecasted quiescence) linearly predicted rebound in males. Overall, 1 minute was recovered for every 2.53, $CI_{95\%} = [2.21, 3.05]$, min of depleted quiescence ($R^2 = 0.199$). Interestingly, the slope was not significantly affected by the value of the interval.

In females, the picture was more complex since there was an interaction between the interval and the slope. Namely, the effect of lost quiescence on rebounded time was only clear for short intervals.

5.2.2 Movement control

It was plausible that rotating tubes multiple times during the night, regardless of the state of the animal inhabiting it, would alter behaviour the next morning. If so, what I had interpreted as a rebound would have been, in fact, a behavioural response to the stimulus rather than to the loss of quiescence. To address this issue, and to assess the specificity of this response, I made the hypotheses that animals which were stimulated when already active, would not lose sleep, and therefore would not show or need a rebound. To verify this prediction, I programmed modules to deliver the same stimulus but, this time, when and only when animals had just crossed the midline of their tubes (fig. 5.3). The other experimental conditions were otherwise identical: males and females received a 12 h treatment after two days of baseline.

In males, no alteration of any behavioural state or position could be characterised during the treatment night (fig. 5.3A). Furthermore, after the end of the treatment,

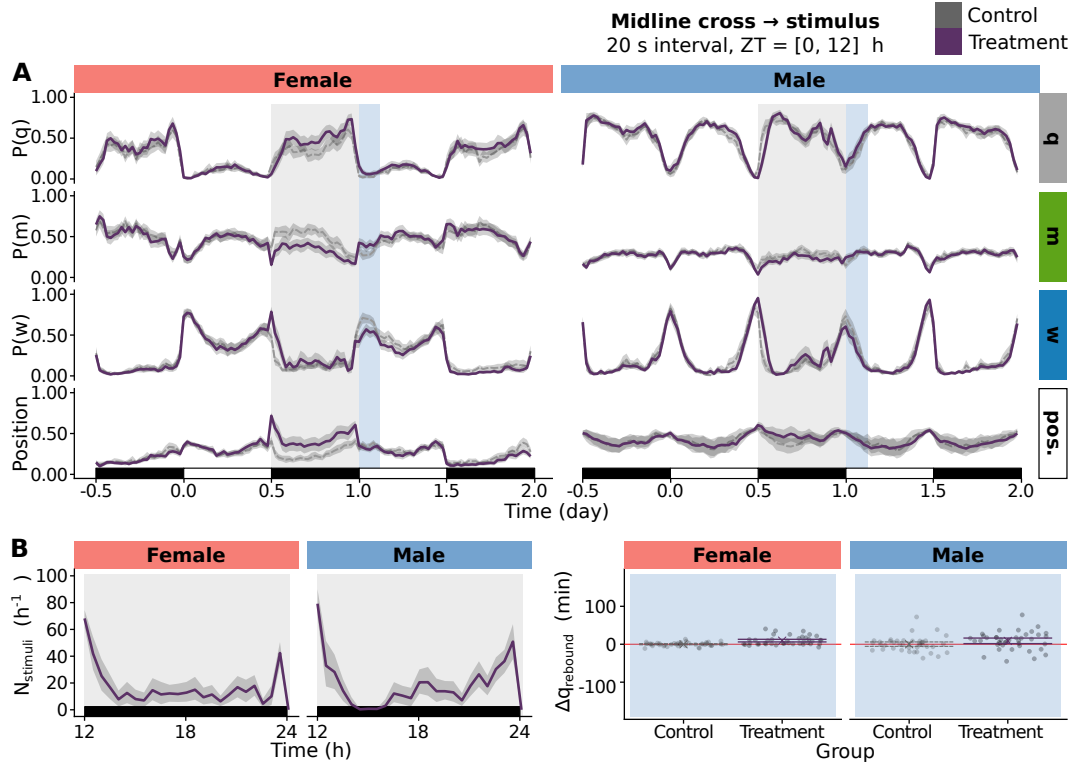


Figure 5.3: *Stimuli do not account for rebounded quiescence.* **A**, Proportion of time engaged in either of the three behavioural states (q: quiescence, m: micro-movement and w: walking) and relative position (from the food, 0, to the cotton wool, 1) are represented as different rows. Females and males are shown on the left and right, respectively. The grey rectangle in the background, between ZT=12 and ZT=24 h, coincides with the permissive time window of stimulus delivery, **Stimuli were delivered to animals each time they actively crossed the midline** of their tubes. Controls animals (grey) were undisturbed and spatially interspersed (in the neighbouring tube) with the treated individuals (plum). **B**, Average number of stimulus delivered in each consecutive 30 min. **C**, Extra quiescence during the 3 h of rebound (blue rectangle in the background of **A**), expressed in extra minutes compared to the predicted quiescence (see method subsection 5.3.4). The shaded areas around the average lines, in **A** and **B**, and the error bars in **C** are 95% bootstrap resampling confidence intervals on the mean. $N_{sex, interval} = 40 \forall sex \times treatment$.

the behaviour of treated animals was not different from the control group.

In contrast, in females, micro-movements were reduced throughout treatment. In the following hours three, there appeared to be less walking, but more micro-movements in startled animals. In addition, quiescence seemed slightly increased in the startled group after treatment.

For both males and females, the number of stimuli delivered followed the expected activity pattern: many stimuli around the phase transition times ($t \in \{12, 24\}$), and fewer in between (fig. 5.3B). In total, females and males experienced 237, $CI_{95\%} = [191, 290]$, and 383, $CI_{95\%} = [329, 438]$, stimuli, respectively—a value comparable to the total number of stimuli delivered during dynamic sleep de-

privation with a 20 s interval.

After treatment, females and males had more quiescence than expected, with mean rebound amplitude of 9.38, $CI_{95\%} = [6.03, 12.97]$, and 8.83, $CI_{95\%} = [0.88, 15.73]$, extra minutes in 3 h, respectively (fig. 5.3C). Despite statistically significant values, the effect size was very moderate compared to the DSD experiment (see fig. 5.1C), suggesting only a partial effect of stimuli-induced stress on rebound.

5.2.3 Timing of Sleep Deprivation

The previous experiment showed that stimuli delivery itself seems to cause a mild rebound. In addition, it appeared that sleep deprivation implied delivering an increasing number of stimuli, which suggested that flies may habituate to the stimulus. For these two reasons, I was interested in increasing specificity of sleep deprivation by further reducing unspecific stress.

The first obvious approach to reduce the absolute number of stimuli was to increase the immobility interval that triggers a stimulus, which I already presented in fig. 5.2. A simple alternative is however to shorten the permissive window during which animals can be startled. As a proof of principle, I decided to pursue this direction, and targeted the DSD, with the original interval of 20 s, to the end of the D phase, in $t \in [20, 24] h$ (fig. 5.4).

Consistently with the results of the 12 h DSD, the treatment reduced quiescence and micro-movement, whilst increasing walking (fig. 5.4A). Conversely, in the three hours following it, there was more quiescence, but less walking.

The number of stimuli delivered also increased monotonically during the 4 h of treatment. By the end of the night it had reached approximately 30 stimuli per hour — whilst the overnight DSD experiment was nearly $60 h^{-1}$ (fig. 5.4B). The average total number of stimuli delivered to females was 72.5, $CI_{95\%} = [58.2, 89.3]$, and males were startled 105, $CI_{95\%} = [80, 132]$, times.

Both sexes significantly increased quiescence after four hours of DSD. Altogether, females and males recovered an average of 26.7, $CI_{95\%} = [20.7, 33.3]$, and 27.1, $CI_{95\%} = [19.7, 34.0]$, minutes of quiescence, respectively (fig. 5.4C). In comparison, depriving the same treatment performed over 12 h had resulted in rebounds of 33 and 42 minutes, for females and males, respectively.

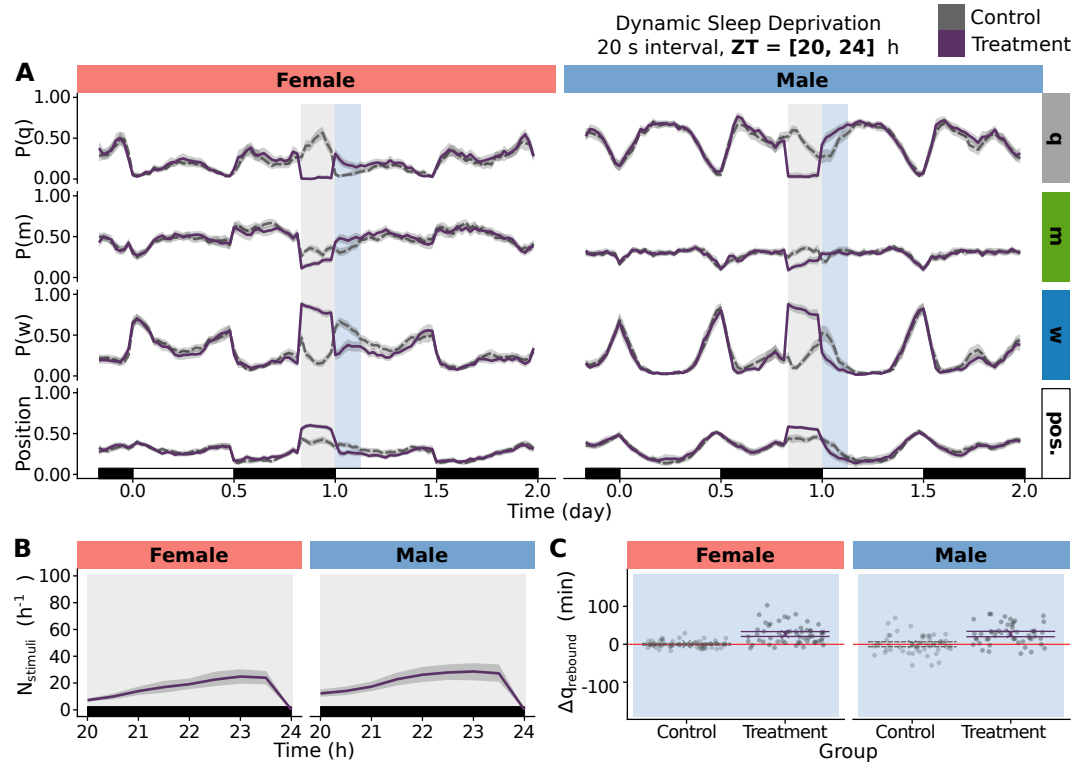


Figure 5.4: *Four hour sleep deprivation causes a homeostatic rebound.* **A**, Proportion of time engaged in either of the three behavioural states (q: quiescence, m: micro-movement and w: walking) and relative position (from the food, 0, to the cotton wool, 1) are represented as different rows. Females and males are shown on the left and right, respectively. The grey rectangle in the background, between ZT=20 and ZT=24 h, coincides with the permissive time window of stimulus delivery, Stimuli were delivered to animals each time they had been immobile for 20 consecutive seconds. Controls animals (grey) were undisturbed and hosted in neighbouring tubes, interspersed with the treated individuals (plum). **B**, Average number of stimulus delivered in each consecutive 30 min. **C**, Extra quiescence during the 3 h of rebound (blue rectangle in the background of **A**), expressed in extra minutes compared to the predicted quiescence (see method subsection 5.3.4). The shaded areas around the average lines, in **A** and **B**, and the error bars in **C** are 95% bootstrap resampling confidence intervals on the mean. $N_{sex,interval} > 51 \forall sex \times treatment$.

5.2.4 Light phase sleep deprivation

Some of the first studies in the field, had shown that no sleep rebound occurred when deprivation was performed during the Light (L) phase^{43,130}. In addition, it had been shown that day and night quiescence have different architecture and that, during the L phase, flies had higher arousability^{43,258}. I was very curious to reassess these findings with my novel, more specific, sleep deprivation paradigm. Therefore, I carried an experiment where animals were startled after an interval of 20 s in Zeitgeber Time (ZT) $\in [0, 12]$ h, and quantified the quiescence rebound (fig. 5.5).

Throughout the 12 h of treatment, quiescence was reduced and walking activ-

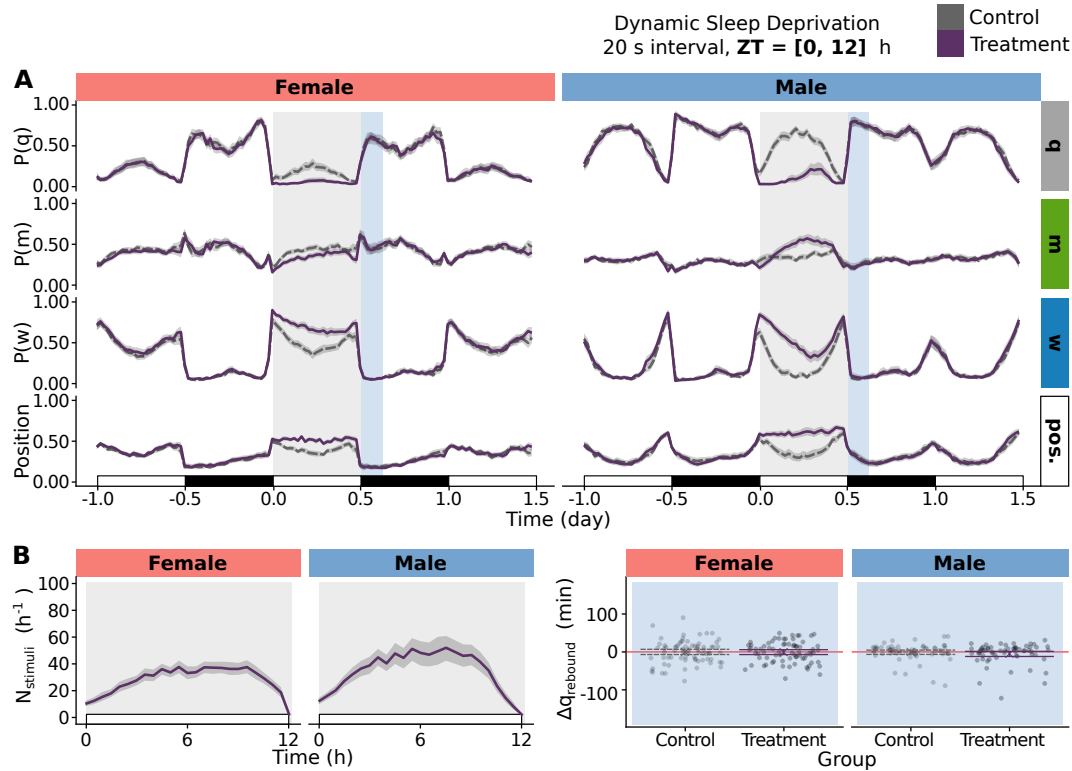


Figure 5.5: *Quiescence depletion during the L phase does not cause rebound.* **A**, Proportion of time engaged in either of the three behavioural states (q: quiescence, m: micro-movement and w: walking) and relative position (from the food, 0, to the cotton wool, 1) are represented as different rows. Females and males are shown on the left and right, respectively. The grey rectangle in the background, **between ZT=0 and ZT=12 h**, coincides with the permissive time window of stimulus delivery, Stimuli were delivered to animals each time they had been immobile for 20 consecutive seconds. Controls animals (grey) were undisturbed and spatially interspersed (in the neighbouring tube) with the treated individuals (plum). **B**, Average number of stimulus delivered in each consecutive 30 min. **C**, Extra quiescence during the 3 h of rebound (blue rectangle in the background of **A**), expressed in extra minutes compared to the predicted quiescence (see method subsection 5.3.4). The shaded areas around the average lines, in **A** and **B**, and the error bars in **C** are 95% bootstrap resampling confidence intervals on the mean. $N_{sex, interval} > 60 \forall sex \times treatment$.

ity increased, both in male and females (fig. 5.5A). In females, micro-movement was slightly reduced in the startled group, whilst treated males had, to my surprise, more micro-movement than their control groups. The increased micro-movement in males did not coincide with an increased proximity to the food. Indeed, the average position of startle animals was very close to the middle of the tube ($position = 0.5$), suggesting no dramatic increase in feeding. Following the end of the treatment, neither position nor behavioural states were different between treated groups and their respective controls.

In contrast with the overnight DSD (fig. 5.1B), the number of stimuli delivered during the L phase did not increase monotonically (fig. 5.5B). Instead, it peaked after

approximately eight hours before decreasing. This suggests either no cumulative effect of sleep pressure or an overriding control of the clock on the sleep homeostat. Over the 12 h of treatment, females and males received 355, $CI_{95\%} = [307, 406]$, and 517, $CI_{95\%} = [444, 586]$, stimuli, respectively.

No rebound (*i.e.* increase in quiescence in the three hours following the treatment) could be characterised—for females and males, $\Delta q_{rebound} = -0.470$, $CI_{95\%} = [-6.61, 5.38]$, and $\Delta q_{rebound} = -4.67$, $CI_{95\%} = [-12.0, 1.85]$, minutes (fig. 5.5C).

Altogether, this experiment suggests that day and night quiescence are indeed very different. In the light of this results and other studies^{43,130,258}, and insofar as homeostasis is a cornerstone of the definition of sleep, it seems difficult to consider quiescence during the L phase as *de facto* sleep.

Another explanation for this result is that animals were indeed sleeping, and that my treatment was effective at depriving them. However, the lack of observable rebound could have been due to the possibility that the circadian clock overrode the homeostat specifically during $ZT \in [12, 15]$ h. In other words, there could be non-permissive time windows during which the clock unilaterally determines activity and therefore masks the otherwise observable sleep rebound.

In order to decide between these two mutually exclusive hypotheses, I decided to perform a similar quiescence restriction experiment that also started at the onset of L phase but, this time, stopped four hours before the transition to the D phase. If a rebound could then be observed, despite a shorter treatment, the ‘clock-masking’ hypothesis would be favoured (fig. 5.6).

As expected from the previous experiment, during the eight hours of treatment both males and females reduced their quiescence level whilst increasing their walking activity (fig. 5.6A). Males also increased their amount of micro-movements. Immediately after the treatment had stopped, at $ZT = 8$ h, treated animals exhibited more quiescence than their respective control groups. However, the effect in females was limited in both amplitude and duration.

The number of stimuli increased monotonically, to peak around 50 h^{-1} , in the end of the treatment (fig. 5.6B). Overall, females and males were startled 208, $CI_{95\%} = [185, 235]$, and 377, $CI_{95\%} = [309, 456]$, times, respectively.

The further quantification of the homeostatic rebound showed an effect of the treatment (fig. 5.6C). Indeed, females and males slept 9.91, $CI_{95\%} = [6.03, 14.4]$ and

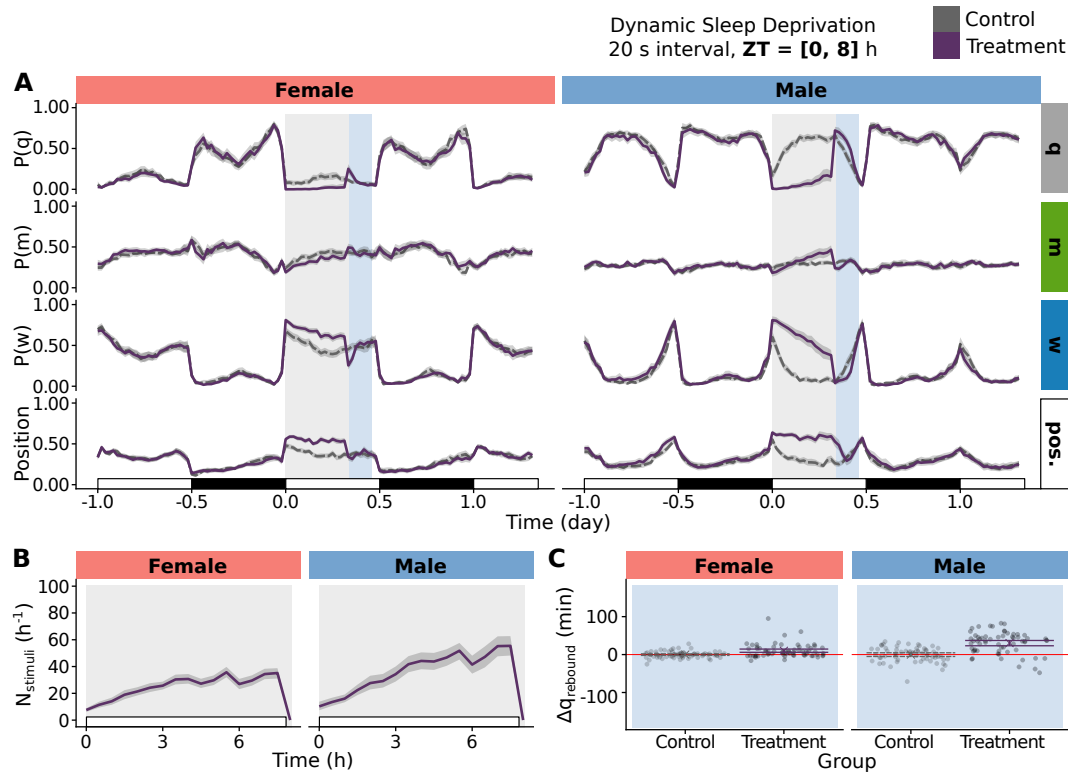


Figure 5.6: *Daytime sleep deprivation causes rebound when it finished before dusk.* **A**, Proportion of time engaged in either of the three behavioural states (q: quiescence, m: micro-movement and w: walking) and relative position (from the food, 0, to the cotton wool, 1) are represented as different rows. Females and males are shown on the left and right, respectively. The grey rectangle in the background, **between ZT=0 and ZT=8 h**, coincides with the permissive time window of stimulus delivery, Stimuli were delivered to animals each time they had been immobile for 20 consecutive seconds. Controls animals (grey) were undisturbed and hosted in neighbouring tubes, interspersed with the treated individuals (plum). **B**, Average number of stimulus delivered in each consecutive 30 min. **C**, Extra quiescence during the 3 h of rebound (blue rectangle in the background of **A**), expressed in extra minutes compared to the predicted quiescence (see method subsection 5.3.4). The shaded areas around the average lines, in **A** and **B**, and the error bars in **C** are 95% bootstrap resampling confidence intervals on the mean. $N_{sex, interval} > 60 \forall sex \times treatment$.

30.4, $CI_{95\%} = [22.7, 37.3]$ additional minutes, compared to prediction, respectively.

The difference of constitutive day quiescence between both sexes makes it difficult to directly compare the amplitude of their respective rebounds. Indeed, females are only rarely quiescent during the L phase, whilst males are known to have a so-called ‘siesta’. Therefore, it is conceivable that L phase sleep deprivation only depletes a modest amount of sleep in females, which could explain the lower amplitude of their rebound.

In males, at least, this result suggests that, like nightly sleep, daily quiescence is, to some extent regulated by a homeostat. However, it is yet unclear whether day and night quiescence are functionally related, if they share the same homeostat

and how they interplay with one another as well as with the internal clock.

5.2.5 Prolonged sleep deprivation

Sleep is often assumed to carry out a vital function⁴⁸, which is empirically supported by the observation that sleep deprivation is lethal¹⁵⁴. In the introduction to this chapter, I have explained that this conclusion is not always supported by conclusive evidence (see subsection 1.1.4). In particular, in *Drosophila melanogaster*, it remains unclear whether—and why—flies die during prolonged sleep deprivation. One landmark study attempted to address this question, but the authors could not perform automatic DSD at the time, which severely limited their objectivity (*e.g.* ‘manually’ delivered stimuli for several days) and throughput ($N = 12$) of their results¹⁵⁴.

I consider this question crucial for the field, and saw an opportunity to address it by using my automatic DSD apparatus over both a long duration and a large number of animals. The original study described a lethal effect after 48 h to 72 h of sleep deprivation¹⁵⁴. Therefore, I decided *a priori* to dynamically deprive flies of sleep (with an interval of 20 s) over 9.5 days (fig. 5.7).

To my great surprise, only 3.3% of the animals died during the treatment (*i.e.* 6 controls and 7 sleep-deprived animals, all sexes). The reduction of quiescence seemed however effective throughout the experiment—though it appeared less so at the end (fig. 5.7A). In the controls, the daily amount of quiescence appeared stationary.

Wrapping measurements, during treatment, over one day shows how quiescence restriction affected all behavioural states with respect to the time of the day (fig. 5.7B). For both treated males and females, quiescence was reduced nearly to zero, whilst walking was consistently increased. In the sleep-deprived population, the walking activity was increased, but remained variable along the day, suggesting the persistence of the circadian drive. The average position was also affected, with values close to 0.5 (the midline of the tube) at any time of the day for treated animals.

In treated females, micro-movements were overall reduced, except in the end of the D phase, where their propensity increased, compared to the undisturbed control. Interestingly, the peak of micro-movement that was present in controls, after the L→D transition, was not visible in the treated group. In contrast, in males, the

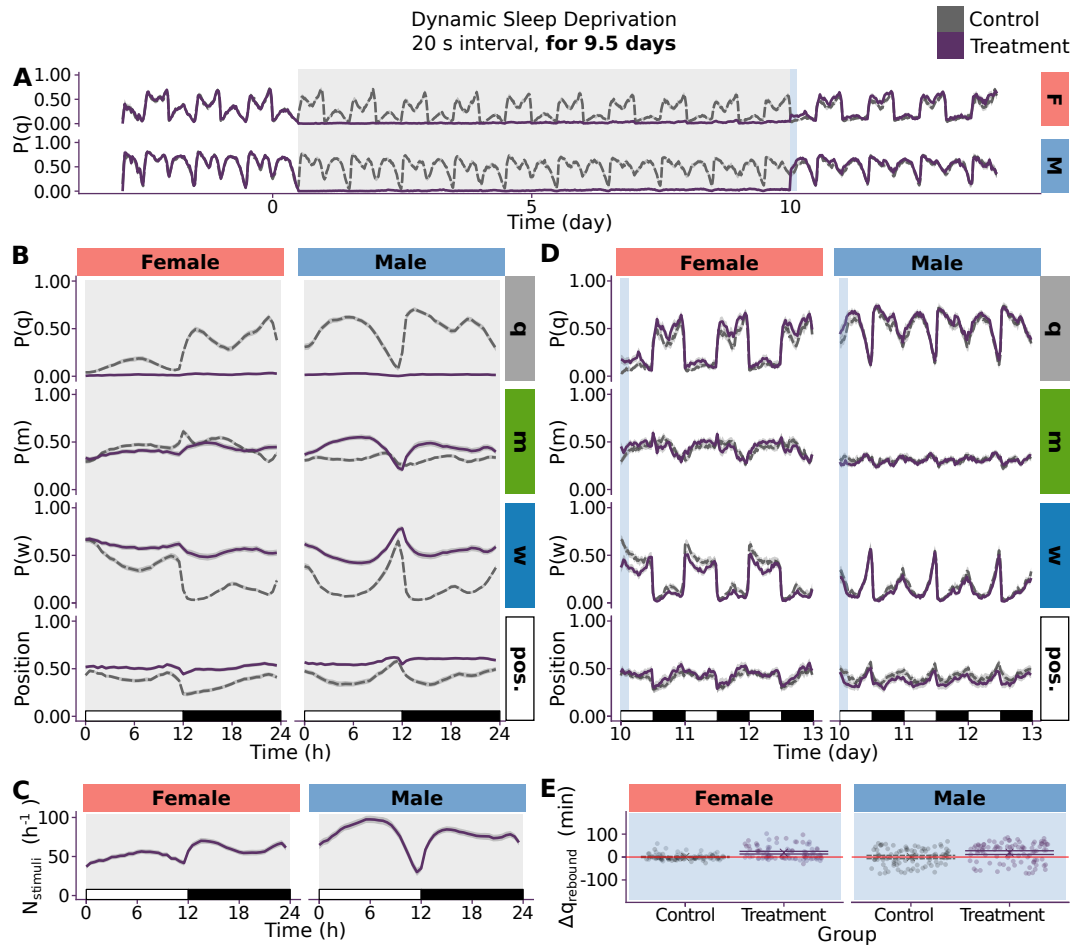


Figure 5.7: *Prolonged sleep deprivation is effective.* **A**, Proportion of time engaged in quiescence. Females and males are shown on the top and bottom panels, respectively. The grey rectangle in the background, between $t=0.5$ d and $t=10$ d, coincides with the permissive time window of stimulus delivery. Stimuli were delivered to animals each time they had been immobile for 20 consecutive seconds with the motors of the ‘optomotor’ device (see method subsection 5.3). Controls animals (grey) were undisturbed and spatially interspersed (in the neighbouring tube) with the treated individuals (plum). **B**, Proportion of time engaged in either of the three behavioural states (q: quiescence, m: micro-movement and w:walking) and relative position (from the food, 0, to the cotton wool, 1) during treatment. Left and right columns show females and males data, respectively. **C**, Average number of stimulus delivered in each consecutive 30 min, during treatment. **D**, Population averages of the three behavioural states, and position, in the three days following the end of the prolonged sleep deprivation. **E**, Extra quiescence during the 3 h of rebound (blue rectangle in the background of **A**), expressed in extra minutes compared to the predicted quiescence (see method subsection 5.3.4). Values between day 1 and 10 are the average over one day in **B** and **C**. The shaded areas around the average lines, in **A-D**, and the error bars in **E** are 95% bootstrap resampling confidence intervals on the mean. $N_{sex,treatment} > 97 \forall sex \times treatment$.

treated group had increased micro-movement throughout the day.

The number of stimuli delivered was highly modulated along the day (fig. 5.7C). Interestingly, males and females experienced more stimuli during the L and D phases, receptively. This results suggests a possibly large effect of the circadian

clock on the sleep pressure, despite a consistent deprivation.

Immediately after the end of the DSD, slightly more quiescence and less walking were observed in both males and females. In females, the effect persisted during the following days (fig. 5.7D). The quantification of the quiescence rebound showed a significant effect in both males and females (fig. 5.7E), though the amplitude was limited to 18.867, $CI_{95\%} = [13.343, 24.756]$, minutes for females and 19.272, $CI_{95\%} = [10.168, 28.175]$, minutes for males. It is however not possible to directly compare such numbers with previous experiments as flies were also older.

Despite animals surviving throughout the treatment, I hypothesised that sleep deprivation could have persistent effects on overall longevity. I, therefore, decided to keep animals individually after the experiment and monitor their survival daily in order to assess their overall lifespan (fig. 5.8).

The effect of 9.5 days of quiescence restriction did not alter lifespan in either males or females (fig. 5.8A). In fact, all groups had statistically the same longevity (l). For females, $median(l_{control}) = 26$, $CI_{95\%} = [25, 28]$, days and $median(l_{SD}) = 26$, $CI_{95\%} = [24, 27]$, days. For males, $median(l_{control}) = 26$, $CI_{95\%} = [25, 27]$, days and $median(l_{SD}) = 26$, $CI_{95\%} = [25, 28]$, days (Kaplan–Meier estimates of the median).

I then wondered whether, within the treated population, mortality could be affected by how much stress animals had undergone. I, therefore, attempted to predict lifespan from the number of stimuli delivered, but found, instead, that it was overall increased by 0.529, $CI_{95\%} = [0.338, 0.710]$, $h \cdot stimulus^{-1}$ ($R^2=0.100$). (fig. 5.8B).

The control population provided me with a collection of animals for which I had both quantified lifespan and behavioural states. I hypothesised that if sleep was crucial for lifespan, then animals that had spontaneously lower quiescence would live shorter. Therefore, I studied the relationship between the overall proportion of time engaged in behavioural states and longevity (fig. 5.8C), for both sleep-deprived and controls. I first tried to explain lifespan with a linear model using treatment, sex and behavioural state as covariates, but could not find a significant effect of any predictor on lifespan ($R^2 = 0.023$). I also attempted using non-linear regression techniques. For instance, random forest regression³⁹² explained only 0.48% of the variance, suggesting no obvious relationship between overall behaviour occurrence and longevity.

The results of this experiment are in stark contrast with the consensus that sleep deprivation is lethal to flies. It also shows that in such experimental conditions,

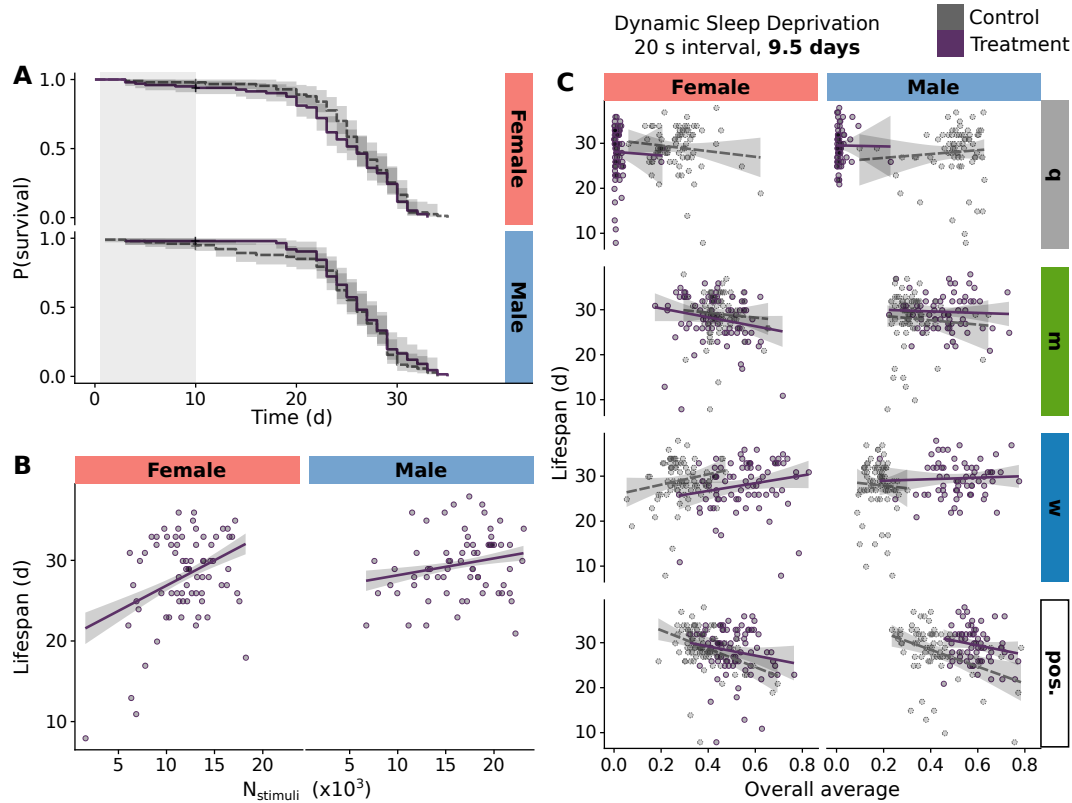


Figure 5.8: *Prolonged sleep deprivation is not lethal.* **A**, Kaplan–Meier plot showing the proportion of animals alive since the onset of the treatment. Females and males are shown on the top and bottom, respectively. The grey rectangle in the background coincides with the permissive time window of stimulus delivery. Stimuli were delivered to animals each time they had been immobile for 20 consecutive seconds. Controls animals (grey) were undisturbed and spatially interspersed (in the neighbouring tube) with the treated individuals (plum). **B**, Effect of number of stimuli on lifespan, for the treatment group. Lines show the result of a linear regression. **C**, Effect of the overall average time spent in a behavioural state (and position), during the experiment, on the subsequent lifespan. Behavioural variables are represented as labelled rows (q: quiescence, m: micro-movement, w: walking, pos.: position — from the food, 0, to the cotton wool, 1). Straight lines in **B** and **C**, are the result of a linear regression. Shaded areas represent 95% confidence intervals. $N_{sex,treatment} > 97 \forall sex \times treatment$.

lifespan is hard to predict from overall quiescence (or, for that matter, other behavioural variables), altogether challenging the notion that there is a necessary trade-off between living and sleeping.

5.3 Methods

5.3.1 Experimental conditions

Unless otherwise stated, experimental conditions were similar to the ones described in the previous chapter’s methods section (see subsection 4.6.2). The rare flies that

died during the experiment were excluded from the analysis — expect, of course for the last experiment, which specifically measures lifespan.

5.3.2 Sleep deprivation

With the exception of the last experiment (fig. 5.8 and 5.7), sleep deprivation was carried by rotating individual tubes with the servo module (see description in subsection 2.3.1).

In all cases, animals were recorded for, at least, two full days of baseline before treatment. Treated animals were systematically interspersed with non treated controls.

5.3.3 Prolonged sleep deprivation

To test the effect of long-lasting DSD on lifespan (fig. 5.8 and 5.7), the motors of the ‘optomotor module’ were used (see description in subsection 2.3.2). An interval of 20 seconds of immobility was set to trigger the rotation of an experimental tube. In order to ensure good quality food (*e.g.* prevent it from drying) throughout the whole experiment, flies were transferred to a fresh tube at day 7 after the onset of the experiment. After 9.5 days of DSD, flies were allowed to recover for 3 days whilst still monitored in ethoscopes at 25°C. After this recovery phase, each individual animal was moved to a new tube (flies remained individually housed) and kept at 29°C. Mortality was scored daily and flies were changed to a fresh tube every ten days.

5.3.4 Rebound calculation

Quiescence rebound was expressed as the difference between the average quiescence during rebound and the expected quiescence. The values of expected quiescence were inferred by a linear regression between the reference baseline quiescence and the value during the rebound period, in the relevant control population. The rebound period was always the first three hours following the end of a given treatment. Formally, the homeostatic rebound h_i of an individual i was expressed as:

$$h_i = r_i - \hat{r}_i \quad (5.1)$$

$$\hat{r}_i = \alpha + \beta b_i \quad (5.2)$$

Where,

- $\hat{r}$ is the *predicted* quiescence *after* treatment ($t \in [x, x + 3]$ h),
- r is the *measured* quiescence *after* treatment ($t \in [x, x + 3]$ h),
- b is the *measured* quiescence *before* treatment ($t \in [x - 24, x - 21]$ h), and
- α and β are the coefficients of the linear regression $r_C = \alpha + \beta b_C$ on the control group C .

$$\alpha = \bar{r}_C - \beta \bar{b}_C \quad (5.3)$$

$$\beta = \frac{\text{Cov}(r_C, b_C)}{\text{Var}(b_C)} \quad (5.4)$$

5.3.5 Statistics

Unless otherwise stated, the error bars and shaded areas around the mean are 95% confidence interval computed using basic bootstrap resampling⁴¹¹ with $N = 1000$. For the survival analysis, the Kaplan–Meier, which contained right-censored data, estimates of the median were computed on bootstrap replicates.

5.3.6 Software tools

All data analysis was performed in $\mathbf{R}$ ⁴⁰⁵, using the rethomics framework (see chapter 3 and⁴⁰⁰). Figures were drawn using `ggplot2`³⁹⁸ and survival plots were generated `GGally`⁴¹⁸.

5.4 Summary

- Dynamic quiescence restriction overnight leads to subsequent rebound.
- It is parsimonious as rebound can be elicited by only a few stimuli.
- It is also specific as quiescence is not (or only slightly) increased after startling walking animals.
- No lethality from prolonged dynamic quiescence restriction could be measured.
- Average quiescence does not predict lifespan.

6 | Discussion

‘Consciously or not, the decision to employ a particular piece of apparatus and to use it in a particular way carries an assumption [...]’

— Thomas Kuhn, *The Structure of Scientific Revolutions* [Ref. 419, VI]

In the introductory chapter, I presented an open and phenomenological definition of sleep that relies on three behavioural observations: immobility, homeostasis, and lowered responsiveness. As I investigated the literature on sleep in *Drosophila*, I identified two methodological limitations. Firstly, the scoring of sleep implies measuring immobility, but the conventional tool can only detect walking, thus omitting behaviours such as grooming and feeding. Secondly, measuring homeostasis involves depleting sleep, and quantifying its subsequent rebound. However, the sleep deprivation paradigms that are generally used disturb animals regardless of their state at the time, which is unspecific.

The consequences of these limitations are twofold. Firstly, they both carry implicit assumptions which could lead to intractable biases. For instance, what has been interpreted as alterations in sleep could, in some cases, have in fact been changes in feeding or grooming. In addition, static—as opposed to dynamic—mechanical sleep deprivation, the most commonly used paradigm, may also incidentally perturb other processes, such as feeding, so the observed sleep rebound could be, in fact, a ‘feeding rebound’.

Secondly, these two limitations have restricted our ability to address certain questions. For example, the trade-off between sleeping and other states remains poorly

understood since other behaviours are not measured at the same time. Critically, unspecific and repetitive stimuli can hardly be applied to deprive animals of sleep for more than a few hours and, therefore, cannot soundly be used to address the question of the viability of sleeplessness.

The focus my work has been, in a first place, to develop the *ethoscope*, a new device that could address these two limitations. In addition, I have provided *rethomics*, an analysis framework for behavioural data, which I hope will serve as a community interface between experimentalists and data scientists, and ultimately promote a high-throughput analysis of sleep. Then, I have employed these two tools to study the baseline behaviour of wild-type flies in the context of sleep experiments. I have shown that these improvements have the potential to resolve some ambiguities in the literature and to ultimately deepen our understanding of sleep. Finally, I used and validated a novel, real-time, sleep deprivation paradigm and managed to perform sleep restriction for nearly ten days but, in stark contrast with the existing literature, noticed neither immediate nor delayed effect on lifespan.

In this final chapter, I will first critically elaborate on the above methodological — but also epistemological — limitations that initially motivated my work and will discuss how the solutions I have developed during my doctorate address them in comparison to alternatives. I will also pinpoint their own shortcomings and examine possible improvements. Secondly, I will discuss how their application to sleep — in particular with the addition of micro-movements — already challenges the established knowledge. Thirdly, I will highlight the originality of the findings that resulted from the application of the Dynamic Sleep Deprivation (DSD) protocol. Finally, I will conclude by examining new directions and general considerations.

6.1 Methodological improvements

In fruit flies, sleep is inferred from movement, and movement is traditionally detected, by the *Drosophila* Activity Monitor (DAM) paradigm. In this section, I will first highlight the limitations of the DAM system, and explain how video-tracking alternatives, in general, address them. Secondly, I will compare the ethoscope platform with other video-tracking methods, and describe its originality. Lastly, I will discuss the validity of the ‘five-minute rule’, the wildly used methodological assumption that animals should be scored as sleeping when they have been immobile for 300 seconds.

6.1.1 The *Drosophila* Activity Monitor

The DAM (see introduction fig. 1.3) is undoubtedly a powerful experimental paradigm. At its core, the principle of keeping flies into small glass tubes has the advantage of summarising their movement to one dimension. Tubes are also standardised, simple to prepare, isolable and durable, which is crucial for experiments on sleep. They are also cost-effective and can be cleaned easily. However, the simplification of movement to the action of walking through the midline of a tube may be somewhat restrictive. Namely, movements related to feeding are undetected, otherwise informative positional data cannot be recorded, subtle movements such as twitches are missed and only isolated individuals can be studied.

6.1.1.1 The importance of detecting feeding

The first and foremost consideration is that experimental tubes are asymmetrical, with food on one side. The DAM, which is traditionally used to score activity, is, by design, incapable of discriminating between long bouts of feeding and sleeping. From the extensive literature on the matter, we know, however, that feeding is regulated by many variables such as genotype, sex, internal states and so on (reviewed in⁴²⁰). For instance, there are known mutants of food intake^{421,422}, and feeding behaviour is regulated by the circadian clock⁴²⁰.

Therefore, there have been concerns as to whether inactive animals could, in fact, be feeding. The first authors in the field were very aware of this limitation, and some of them actually assessed how feeding could render activity estimations erroneous. For example, Shaw and co-workers starved wild-type flies for 12 hours,

during their Dark (D) phase — which should make them subsequently eat more — , but saw no change in beam crossing, and concluded that ‘eating was not miscoded as rest’ [Ref. 131, supplementary material]. However, it seems unreasonable to assume that this finding is *a priori* generalisable to all future experimental contexts. In particular, studies on the genetics of sleep rely on the observation of mutants that are chosen because they widely differ from the wild-type. In other words, the population used to draw this conclusion is not representative of the distribution of possible phenotypes.

A sensitive enough video tracking provides a clear improvement towards solving this issue as feeding is visually identifiable. Indeed, with good enough definition, feeding animals will often move sufficiently to be detected — and video-based validation of feeding assays has been proposed in other contexts^{247,423,424}. In addition, video-tracking generates positional data and the location of feeding animals is necessarily in very close proximity to the food.

The ethoscope and most other video tracking tools, however, do not, and cannot, measure feeding behaviour or food intake as such — as there are other behaviours that involve micro-movement on the food such as egg laying or tasting. Furthermore, the proboscis is often occluded by the rest of the animal when it faces down. In order to be more conclusive regarding trade-offs or transitions between feeding and sleeping, it would be necessary to measure the correlation between micro-moving on the food and food intake, in different contexts. To go further, it would be very interesting to develop a module that could measure more precisely how and when each animal feed. For instance, it should be possible to use a pair of thin electrodes inside of the glass tube to detect each ‘sip’ and infer feeding whilst video tracking flies²⁴⁷ (Pavel Itskov, personal communication).

A very interesting study proposed to measure feeding and sleeping at the same time using a video camera for both^{280,326}. In this setup, the flies feed from capillaries whose level can be visually measured. However, it requires daily human interventions to change the food, and liquid food in capillaries is known to be difficult to reach for flies²⁴⁸.

6.1.1.2 The added value of positional data

The position of a fly in its tube in the context of sleep is an interesting variable^{130,257,278,279}. Hendricks and coworkers already noted that animals rest close to, but not on, the food¹³⁰. Although this type of information could be studied

in the context of sleep, it cannot be recorded with the conventional single beam paradigm.

Experimentally, it is very interesting to study how animals move in an asymmetrical environment and video tracking, as well as multibeam DAM, have both been suggested as tools to study the position preference over time. For instance, Garbe *et al.* have proposed a setup where a fly can move either to a bright or a dark side in their arena, and concluded that animals preferred to sleep in shaded areas²⁷⁸. In such a paradigm, the position can be interpreted as a choice, which makes it possible to address the question of the interaction between sleep and decision making.

Furthermore, restricted in narrow glass tubes, flies cannot display the full extent of their behavioural repertoire. The analysis of trajectories²⁵⁴ or foraging⁴²⁵ of *Drosophila* in an open-field arena have been scrutinised with video tracking, but rarely in the context of sleep. In order to work at the intersection between these questions and sleep, it would be necessary to use another paradigm than the glass tube. In fact, in my laboratory, two doctoral candidates are currently using the ethoscope to undertake this task.

6.1.1.3 Twitches during sleep

In their seminal paper, Hendricks *et al.* interpreted uncoordinated movements such as respiratory contractions of the abdomen or unsolicited proboscis extensions as part of sleep⁴⁰. In other words, these very short micro-movements have been considered compatible with sleep, which is the accepted view in mammals⁴²⁶. Since sleep twitches cannot be detected by the DAM, we have little evidence either against or in favour of this hypothesis, nor do we understand their biological significance in *Drosophila*.

Sleep twitches have, however, been suggested as an interesting variable, and video tracking has the potential to study them²⁵⁵. A recent study used video recording to manually score their prevalence²⁷⁹. The authors reported a larger number of twitches in young, compared to aged, flies. However, to my knowledge, we do not know whether twitches relate to sleep depth or responsiveness. It would be very interesting to address this question by dynamically probing, or attempting to sleep-deprive, animals that had just exhibited short uncoordinated movements.

The algorithm I presented in section 2.8 attempts to detect *any* movement and

considers that sleep is interrupted by twitches. However, in practice, given the standard spatiotemporal resolution ($\approx 30 \times 20$ pixel per fly at ≈ 2 FPS), twitches are often missed. Indeed, as I annotated myself many 10 s video fragments (which were at a higher temporal resolution, 25 FPS), I could often not see well enough to confidently score very small micro-movements. In order to work on, and properly account for, twitches, a better resolution would be needed, and their study goes beyond the scope of my work so far.

6.1.1.4 Multiple animals

There is very little work on how flies sleep inside a group. Liu *et al.* addressed this question by generalising the single beam approach to a population of flies held in a large vial³⁴³. Their set-up could only count, in a given time window, how many flies crossed the midline. Although they attempt to draw conclusions regarding the effect of population density on sleep, such inference requires many assumptions that are very difficult to verify. For instance, one must hypothesise that the spatial distribution of flies is homogeneous and that individuals have the same propensity to be active. Furthermore, the use of a ‘five-minute rule’ for a population adds a level of non-linearity that is difficult to account for.

The solution to address sleep in groups of flies would be to use video-tracking tools that can label each animal. It is notoriously difficult to preserve the identity of animals in large groups for long durations²⁶⁹. Indeed, even if the probability of swapping individual labels is very low, errors do propagate, but promising solutions have been developed^{262, 265, 269}.

In a typical sleep experiment, where animals are held for several days, it seems ambitious to preserve identity throughout. However, a lot of information could already be gathered even if animals become sometimes mislabelled. In addition, using *Drosophila*, it is also possible to visually ‘tag’ animals. For instance, some mutants are darker or have coloured eyes. Therefore, instead of tracking an entire population, perhaps a more reasonable approach is to surround a focal animal by visually different ones.

6.1.2 Alternative video tracking tools

Several video tracking platforms have been presented as alternatives to the DAM system^{255–258, 280}. However, they suffer from two kinds of limitations. Firstly, they

are not always trivial and practical to use in a laboratory context. Secondly, their performance is rarely extensively evaluated and they only detect basic features.

6.1.2.1 Practicality

In order to promote widespread adoption of a method, it is crucial that it is usable in a laboratory context. Indeed, even the best tracking algorithm will meet limited adoption if it is not designed and implemented with real-life practicality in mind. In this respect, the DAM system is a good example of a tool that scales well, is relatively simple to use and has its own, user-friendly, acquisition software.

Scaling The transition from DAM to video tracking comes with additional digital layers, at least two orders of magnitude more data and is often less robust — *i.e.* more prone to failure. Although the proposed video-based alternatives allow for the tracking of dozens of animals in one machine, it is not clear how they offer to accommodate the needs of a laboratory with multiple users, acquiring thousands of animals' position over several days^{255–258}. Namely, how are the data labelled, centralised and archived, and how does the system scale up (*e.g.* more computers, more cameras or more time)?

In order to improve throughput, I proposed a network of microcomputers that all perform their own acquisition and real-time processing, rather than a single, powerful, computer that handles all the functionalities of the platform (fig. 2.5). In other words, the computing load is distributed on multiple devices. This architecture addresses several practical issues related to scaling.

Firstly, it promotes compartmentalisation. For instance, a user can start their own experiment in a different physical location without risking to impact on another's work. Specifically, in the context of sleep and circadian rhythm, behavioural monitoring must be performed for days with a specific temperature, light and humidity regime, and with minimal human intervention (*e.g.* interference between experimenters). Therefore, in practice, all experiments are carried in a separate incubator that remains closed for the entire duration of the experiment. Compartmentalisation also increases robustness as the failure of one device will have a very limited overall effect. At the scale of the laboratory, portable, wireless and self-contained devices such as the ethoscope allow for a dynamic and flexible resource allocation between experimenters that could not be achieved by having a rigid, single-computer, platform. Indeed, users can focus on setting up their

experimental environment (as long as it can be reached by the wireless network) and then plug the number of machines they wish. Ethoscope can run on standard 5 V batteries for several days which also makes them suitable for field studies.

Secondly, it centralises and organises all results in the same file system. The data can then be transparently queried using the `rethomic` workflow (fig. 3.4). Indeed, data management becomes crucial when increasing experimental throughput, and it becomes necessary to implement mechanisms to prevent data loss, mislabelling and error-prone operations (such as manually finding and copying files from multiple experiments).

Lastly, throughput can be increased simply by building more tracking devices, without changing how users interact with the platform. In an alternative scenario, where a large computer would handle all the tracking from multiple cameras, scaling up would eventually require the acquisition of a new machine, which would complicate the logistics of data handling and compromise the possibility to allocate dynamically resources in a laboratory.

In the Gilestro laboratory, the ethoscope has become the only sleep scoring system, with 80 devices used by five to ten users on a daily basis. Over the last three years, more than one terabyte of behavioural data has already been acquired by the platform. At this stage, the throughput of video tracking in the laboratory is no longer limiting. Instead, the time-consuming preparation and cleaning of tubes as well as the availability of environmental chambers (*i.e.* incubators) are now the main bottlenecks. Therefore, a further scale-up of the system may involve automating such routine tasks.

Modularity Most video-tracking software tools that apply in the context of sleep are built in a top-down manner: features are defined *a priori* and centred around a graphical user interface, the functionalities being implemented to serve the interface. This choice of architecture can provide a quick solution to specific problems, but makes software overall less modular which hinders adaptation and partial reuse by other researchers. In contrast, the community could develop thoroughly documented libraries and Application Programming Interfaces (APIs) to standardise shared principles. The bonsai platform is a good example of a tool that emphasises on modularity, allowing users to define their own protocols with a visual programming interface⁴²⁷.

For this reason, I have tried to develop the ethoscope software as an API, in

the form of a `python` package. As such, it can interact with a rich ecosystem of scientific libraries. It features a set of core modules, but also many classes that can be derived for a specific use. For instance, the source of the raw data, tracking algorithm, real-time stimulus and other utilities can all be adapted from templates.

Several of my collaborators and students I have supervised have already taken advantage of software modularity to develop different tracking algorithms, Region Of Interest (ROI) definitions and hardware modules, without having to reimplement other functionalities.

The ethoscope's modularity is also reflected at the hardware level. In my thesis, I have presented two tools that extend the ethoscope: the servo module (fig. 2.3) and the optomotor (fig. 2.4). Interestingly, I had not anticipated the development of the optomotor itself, but the modular architecture of the ethoscope allowed me to support it by only writing one short source file.

As I have experienced, it is difficult to predict future experimental needs and possibilities. Therefore, unless a clear definition of all required features can be provided, it is sensible to develop a modular tool that can accommodate unanticipated functionalities.

One of such directions I am currently investigating is the potential of optogenetics³⁸⁵ in the context of sleep. For instance, it could be possible to use the optomotor to find, or validate the implication of, a set of neurons taking part in sleep or arousal. In short, one could probe arousal or sleep deprive animals by stimulating a population of neurons, using light that is invisible to flies.

Post hoc analysis The acquisition is often only the first step of the analysis. Then, data must be loaded, processed, summarised, visualised and modelled. I would argue, with others, that high-quality, original, reproducible and scalable, data analysis can only be achieved with scripting and programming interfaces — see my introduction to the `rethomics` manuscript⁴⁰⁰.

However, a programming interface does not mean that each user has to implement low-level functions. In fact, there are many specific high-level data analysis libraries and packages available for various aspects of data sciences. As I developed the ethoscope, I realised there was no satisfying programmatic toolbox that was adapted to specifically analyse large behavioural datasets in a platform-agnostic fashion and interacting with other data-science packages.

I, therefore, developed **rethomics**, a framework intended to fill this niche. Rather than focusing on sleep and circadian rhythm, it provides an interface for high-throughput behavioural analysis that is independent of the acquisition tool and field of research (fig. 3.1). Together with ethoscopes, **rethomics** facilitates the transition towards a quantitative approach to behaviour — *i.e.* ethomics.

Maintenance A last practical consideration is the lack of long-term support for video-tracking alternatives to DAM. Indeed, most of them are developed independently by each laboratory, often by one or two researchers. However, modern software, in particular when it interfaces closely with hardware, will require long-term maintenance, which is difficult to provide in the current academic environment⁴²⁸. Altogether, delivering a scalable tracking software is an engineering challenge that would require a team or community of qualified developers with long-term interests in mind to be addressed.

I personally do not provide a definitive solution to this issue and would argue that it relates more to the sociology and politics of academia, which is, sadly, mostly out of my control. However, I will note that the openness of the ethoscope makes it conceivable that other actors could maintain it and continue developing it.

The scalability and practicality of the ethoscope have already attracted external users. For instance, several collaborators have adopted it in their own laboratory and a startup company is working on commercialising it. Since the ethoscope has been built using inexpensive and freely available technology, it is also perfectly suited for outreach and teaching. For instance, my team and I have used it in the Imperial festival and high school students have contacted my laboratory to use the machine as part of their respective projects.

6.1.2.2 Validation and scope

Validation As I discussed in my introduction to chapter 2, defining motion is not trivial, and finding a set of pixels that defines an animal to then decide whether it is ‘moving’ comes with its own set of assumptions. Interestingly, most authors in the field have applied arbitrary criteria to decide whether a fly, in a video, was moving.

For instance, Zimmerman *et al.* defined a fly as immobile if ‘no pixels [had] moved’. Based on a one hour of video of two aged male flies, they reported a sensitivity of 92.6% to movement, but do not mention specificity. In addition, their tracking

algorithm is limited by the fact that amount of pixel moved is an integer, which suggests sensitivity cannot be improved by lowering the threshold.

The ‘Tracker’ method, proposed by Donelson *et al.*, records the position of 10 pixel long flies. The authors define movement by thresholding the distance that animals moved. After trying three values (2, 5 and 10 pixel·frame⁻¹), they decide to use 5 pixel·frame⁻¹, which they see as a trade-off between false positives and false negatives. They did, however, not report specificity and sensitivity at these thresholds.

Faville *et al.* compared three movement thresholds: 1, 3, 20 mm to DAM results. Without explicitly including ground truth, they empirically conclude that 1 mm has too many false positive and 20 mm, too many false negatives.

In contrast, I endeavoured to validate my motion detection algorithm. Firstly, I generated a large amount of ground-truth data (19 animals for 144 h), in conditions that matched future experiments as much as I could. Secondly, I validated the predicted position by showing its very close similarity to the ground truth (fig. 2.8A). Then, I based my decision of movement threshold by scanning multiple values and by drawing Receiver Operating Characteristic (ROC) curves, thus explicitly presenting the trade-off between specificity and sensitivity (fig. 2.8A). Lastly, I accounted for the possibility of different —and heterogeneous— frame rates in the context of real-time analysis on underpowered devices (fig. 2.9).

Scope One serious limitation of video alternatives to DAM, including the ethoscope, is that they only record simple features such as centroid, area, width and orientation of animals. Instead of focusing on acquiring data for a large number of animals, it would be possible to increase the single-animal resolution and quantify subtle changes in pose, but the tools proposed so far are restricted both in terms of hardware and tracking algorithms.

The ethoscope software can function on a desktop computer, and it would be possible to use high-resolution cameras to address the hardware limitation. Then, there are very promising developments of algorithms based on machine learning such as LEAP²⁶⁶ and DeepLabCut⁴²⁹ that, in principle, could be included as software dependencies.

Another limitation that is related to software is the lack of tools to score sleep in multiple interacting animals, in the same ROI. In this area too, methods are advancing rapidly^{262, 265}, and it should be possible to perform experiments to un-

derstand how populational variables (such as sex ratio and density) affect sleep. One could also use such tools to investigate how the immediate experience with conspecifics alter sleep, possibly in real time too.

6.1.3 The five-minute assumption

In addition to the scoring of movement using DAM, there is another widespread layer of assumptions made by the community: the ‘five-minute rule’, or the idea that sleep should be scored from the observation of five consecutive minutes of immobility. This assumption appears in almost all sleep studies, that use DAM (for instance the ones I quoted in subsection 1.3.2.1), but also in later ones that have employed video tracking. The rule originates from the observation, in a limited number of homogeneous wild-type animals, that arousal tends to globally decrease after approximately five minutes^{43, 130, 131}.

In 2000, Hendricks and coworkers defined and quantified ‘rest’ as five minutes of immobility without justifying their decision. In fact, they ethologically observed that flies ‘relax’ after nine minutes. In the second seminal paper, Shaw *et al.* report that, overall, flies that had been awake respond more than the ones that had been immobile for five minutes, but did not show the relationship between time immobile and arousability (*i.e.* they do not describe arousability before and after five minutes)¹³¹.

The most compelling evidence for the five-minute rule came four years later from Huber *et al.*⁴³. They quantified the escape response according to the time engaged in immobility bouts and observed that responsiveness decreased to eventually reach an asymptote after five minutes [Ref. 43, fig. 2] — though the large size of the error bars makes this result rather statistically inconclusive. In this study, the authors used only 28 virgin female Canton-S flies and aggregated response data for the first 8 h of the Light (L) phase. Although they discuss their observation that, during immobility bouts in the D phase, responsiveness decreases to an even lower level than during the L phase, they curiously do not show data for the D phase. Following these three articles, almost all studies have scored sleep as five minutes of immobility²⁵⁸ (see for instance some of the most widely cited ones:^{157, 158, 288, 289}).

More recently van Alphen *et al.* comprehensively scored responsiveness of immobile flies under different condition (genotype, sex and social context)²⁸⁵. They concluded that, firstly, arousability was dynamic. Secondly, in most context, it was the lowest between 15 and 30 min (*i.e.* it continued lowering after 5 min)

Thirdly, the relationship between bout length and behavioural response was modulated by the conditions they tested.

In a complementary study, Faville *et al.* found that, firstly, in the night, arousability continues to decrease after five minutes²⁵⁸. Secondly, the proportion of animals reacting to vibration does not decrease monotonically with bout length in the L phase and globally remains very high — sometimes it even increases during long bouts. However, the authors do not discuss how their results challenge — or even falsify — the established five-minute rule, which they themselves apply throughout their work.

Using calcium imaging, Bushey *et al.* were able to show that after five minutes, Kenyon cells, a population of neurones involved into the coding of olfactory input, were less spontaneously active, and that they responded less readily to odours when the animals had been immobile for five minutes. However, the authors did not investigate the time dynamic of the response²⁸⁶.

Because of its extensive adoption throughout the literature, the five-minute rule can be seen as part of an ‘operational definition’ of sleep — together with the use of DAM. That is, an animal is defined as ‘sleeping’ if and only if ‘it does not cross the mid-line of its tube for at least five minutes’. As I have just presented, recent investigations have shown that variables such as time of the day and sex may compromise this view²⁵⁸. In addition, the architecture of inactivity bouts is very different when using video tracking, implying that five minutes of immobility does not correspond to five minutes without walking.

Furthermore, the observation of lowered responsiveness emphasises the arousal part of the behavioural definition of sleep, without accounting for the homeostasis criterion. Indeed, even if arousal were consistently decreased after exactly five minutes, in all immobility bouts, we would still need to show that all of these events are under homeostatic regulation. For instance, it is plausible that even if animals are still very responsive after one minute, these first 60 seconds already take part in the homeostatic process. Conversely, in some context (*e.g.* during the L phase, discussed in subsection 6.3.2), an animal may stop and becomes less responsive, but no rebound can be observed if it is prevented to do so. In other words, homeostasis and arousal can be expected to mismatch and the five-minute rule should rely on both aspects.

Altogether, it seems very speculative and contradictory to systematically score sleep with this criterion. Indeed, on the one hand, it is accepted that sleep is

regulated by the clock and determined by very complex interactions between genes, internal state and environment (see subsection 1.3.3). In other words, we explain that sleep is highly variable. On the other hand, the five-minute rule is expected to be static and apply to all new conditions we tests, without global validation. I would argue that, unless a very compelling evidence was provided towards the universality of such a rule, it should be seen as a risky inductive inference. It is plausible that the ‘rule’ could be, say, five minutes in the middle of the night, but one minute in the end of the day. Likewise, why should we accept that mutations or other treatments severely compromise the dynamic of quiescence on a large time scale (hours and days), but assume that the five-minute rule, which is the micro-dynamic of sleep (over minutes), is intact?

There are also obvious cases where this assumption is expected to fail. For instance, several studies have been performed at different temperatures (*e.g.* thermogenetics)^{295,297,298}. However, the metabolism and physiology of poikilothermic animals scales drastically with temperature — unless, like the circadian clock, it is temperature-compensated. In fact, the first quantitative validation of the rule was performed at 21°C⁴³, whilst most experiments in the field are conducted at 25°C. There is therefore no *a priori* reasons to think that micro-dynamic of sleep will be preserved.

In conclusion, the evidence behind the idea that, after five minutes of immobility, animals are asleep, regardless of genetic background, age, sex, environment and so on seems as fragile as universally adopted, and its use is, at best, unnecessary. In my thesis, I have tried, as much as possible to make the economy of this assumption.

6.2 Baseline sleep

In this section, I discuss the first aspects of my findings: the difference between my results and DAM data, the inclusion of micro-movements as a new behavioural state, the effect of mating on female sleep and the sources of behavioural idiosyncrasies in flies.

6.2.1 Differences with beam crossing

Video tracking tools had revealed that DAM globally overestimates inactivity — and therefore sleep^{258,299}. However, descriptions of how this bias changes over time and for different biological variables are rare²⁷⁸. If, as proposed by its proponents, video tracking is more sensitive to small movements and if small movements are themselves non-stationary, then this bias cannot be seen as constant.

When I compared both immobility and sleep between DAM and the ethoscope, with my movement detection algorithm, I discovered that the consistency between the two methods depended on the time of the day and sex (fig. 4.1). Even though the proportion of animals scored as immobile was reduced in both sexes and at any time, the amplitude of this reduction greatly varied (fig. 4.1A), which was qualitatively reflected in biological conclusions regarding the differences between males and females. Namely, sensitive movement detection led to the conclusion that females were more active than males at night, whilst beam crossing suggested that they were equally immobile.

To go further in the description of the consensus between methods, I also analysed their rank correlation, including all animals, at different times of the day (fig. 4.1B and E). Since this approach compared the respective ranks of animals between both methods, it accounted for consistency at the individual level. I discovered that the correlation between methods greatly changed over time. In particular, it was rather low (< 0.5) for females around midday (ZT=12 h). I, therefore, concluded that this discrepancy could not be accounted by a constant ‘scaling’ of the activity.

6.2.2 Micro-movements and behaviour space

I reasoned that the immobility, as defined by ethoscopes, included small movements whilst DAM's activity consisted solely of walking bouts. In addition, I noticed that within every minute of ethoscope-scored activity, the distribution of total distance moved was bimodal, suggesting a natural dichotomy between either walking a lot or, instead, moving without walking. I, therefore, decided to explicitly account for these small movements as a third behavioural state, which I named — rather unoriginally — ‘micro-movement’. In other words, instead of seeing behaviour as moving *vs* immobile, I described it as a variable with three states: quiescent, micro-moving and walking. Interestingly, alternative video tracking tools for sleep can, in principle, score micro-movements too²⁵⁵. However, to my knowledge, they had not been studied as an explicit state in the context of sleep, and authors in the field consistently use and underlying binary definition of activity: immobile or moving.

For this reason, I started by characterising the dynamic of these three states (fig. 4.2B). I discovered that micro-movements in females were overall high, and changed over 24 h. In particular, they peaked after the transition to D phase. In addition, micro-moving females were very often in close proximity to the food end of their tube (fig. 4.2C), which suggested micro-movement corresponds to feeding instances.

In males, micro movements were, however, rarer and not as strongly circadianly modulated (fig. 4.2B). Furthermore, the distribution of the animals' location indicated they micro-moved and quiesced at the same place: close to, but not on, the food (fig. 4.2C). Males fruit flies have been reported to eat less than virgin females⁴²², which could explain this variation. It is therefore possible that, in males, most of the detected micro-movement correspond to instances of grooming, twitches or even false positives — which would have a probability of occurrence essentially proportional to the amount of quiescence given the time of the day. Moreover, males may also feed in a more fragmented manner (*i.e.* in shorter bouts) as opposed to the consolidated, long, epochs that female display.

In order to show the informativeness of my ternary definition of behaviour, I visualised population trajectories in a 2-simplex behavioural space (fig. 4.3A and B). I envisage this tool as a mean to capture, summarise and compare the daily behaviour of large populations of flies. This representation could be particularly useful to generate hypothesis in the context of genetic screens or quantitative

genetics. It also demonstrated the added value resulting from the description of behaviour as a series of discrete probability distributions to assess the similarity between individuals (fig. 4.3C). The proof of principle I presented by clustering males and females could be extended to detect cryptic clusters and, for instance, statistically link behavioural proximity to other phenotypical variables.

Altogether, as long as video tracking can be used, the addition of the micro-movement dimension to the behavioural space is experimentally and conceptually costless, and I see no reasons to work exclusively within the restrictive two-states behavioural representation. In particular, in the context of sleep, explicitly defining micro-movements makes it possible to comprehend the difference between video tracking experiments and the existing literature—mostly based on DAM data. Arguably, micro-movements may not be informative in all cases (for instance, in my results, in males), but they have great potential to increase our understanding of sleep and, more generally, of behavioural trade-offs. Indeed, they can be used to address whether animals regulate quiescence by altering their micro-movements or, rather, by changing their walking propensities (or both).

The same argument could be used to extend—perhaps with better resolution—this idea to more than three states. For instance, we could score other behaviours (*e.g.* grooming, twitches), using human knowledge, in a supervised fashion, which has the advantage of being immediately interpretable in a biological context. Likewise, it could be helpful to experimentally validate further the three behavioural states to ensure they are not largely arbitrary constructs.

Another, more sophisticated, approach could be to discover and score behaviours in an unsupervised manner^{274,275}. This would be particularly interesting to study sleep with videos at higher temporal and spatial resolution, and in an open arena, which could reveal sub-states. In this respect, pioneering work has been carried in the roundworm^{430,431} and in *Drosophila*²⁷⁴ and could be adapted to the study of sleep. In fact, it is possible to compare behavioural time-series in an even more agnostic fashion. For instance, Ben Fulcher and Nick Jones developed a framework that computes thousands of features on phenotypical time series and that has the potential to classify individuals, find outliers and generate new hypothesis—*i.e.* suggest candidates variables⁴³². It could prove very interesting to use both the hypothesis-driven and agnostic approaches together.

One aspect of my work that I have not presented in this thesis (because too preliminary) is the analysis of the transitions between behavioural states. Indeed, I have been interested in modelling behavioural time series as (hidden) markov

chains in order to characterise its architecture. For instance, the succession of sleep stages in mammals is often seen as a series of transitions^{433,434}. With such a framework it would also be possible to account for the time-embedding of different behavioural orders. We could, for instance, borrow concepts and tools from computational linguistics and theory of speech generation, which are very advanced. In this perspective, we would aim at formalising a ‘generative grammar’ of behaviour to understanding it as a probabilistic sequence, which could serve to make, for instance, predictions regarding sleep regulation.

6.2.3 Mated females may feed day and night

The widespread metabolic, physiological and behavioural effects of mating on females has been extensively studied^{349–351}. Therefore, unsurprisingly, sleep researchers had asked whether mating impacts sleep^{278,325,356,435}. The consensual view is that mating reduces daytime sleep, but does not affect night sleep^{278,356,435}. These conclusions have, however, been drawn from studying flies that did not have access to nutritious food (proteins). A recent study failed to show such a large reduction in sleep in females that did have access to food containing yeast, employing DAM³²⁵. The authors then increased the spatial resolution of their assay by using multiple Infrared (IR) beams, and could then show a reduction of daytime sleep, but did not present any data for the D phase.

Since females are known to increase feeding in response to mating, I thought that the detection of their micro-movements would be crucial to understanding how they modulate sleep. I anticipated that the previously described phenotypes may have been instrumental artefacts due to the use of the DAM. Therefore, I attempted to resolve the confusion in the literature by scoring the behaviour of mated females (fig. 4.4). Very interestingly, I found that immediately after mating, micro-movements increased and remained consistently high throughout the rest of the experiment. During the L phase, mated females reduced walking in favour of micro-movements whilst, in the night, they reduced quiescence. Since the average position of mated females was very close to the food, my interpretation is that they reallocated their time to feeding and laying eggs.

This example shows that the assumption behind the use of the DAM may fail conditionally on experimental variables such as the type of food used. The observation of elevated activity after mating in the first study is, therefore, most likely a foraging behaviour exhibited by flies questing for laying sites and food, a

behaviour that is restricted to the absence of yeast. The decision to prevent flies from laying eggs by not providing them with the appropriate type of food seems curious, and one could wonder if the same authors had not performed the experiment with yeast, but found no effect — which may have been seen as a negative result, and not reported.

Beyond the differences between experimental paradigms, the study of mated females, which is likely the default state in wild fruit flies⁴³⁶, can be very informative to understand the nature of sleep. Indeed, the observation that quiescence is reduced to an extremely low level after mating requires particular attention, and I provide two interpretations for this result. Firstly, females may ‘sacrifice’ a part of their sleep to perform activities that are likely more relevant to their fitness: feeding and egg laying.

Alternatively, a large part of the quiescence exhibited by non-mated females could be an accessory form of inactivity that is simply opted-out after mating. This latter explanation would imply that the amount of rest we measure had, at least, two cryptic components: *sleep* and *accessory rest* — theorised by Alexander Borbély as ‘recovery sleep’ and ‘inactivity sleep’⁴³⁷. Experimentally, one may suggest that the former, but not the latter, is under homeostatic control and that the study of mated females provide a framework where only actual sleep remains.

In *Drosophila*, genetic tools would allow us to address this question by, for instance, activating a subtest of neurons involved in post-mating behaviours, in a conditional manner. In short, it would be possible to make a female behave as though it had mated, for say 12 h, and then reverse the mating switch⁴³⁸. If no rebound is observed after such quiescence loss, it would suggest that the lost rest was accessory. An interesting preliminary analysis which I have not performed would be to study the distribution of behaviour bout length after mating in order to understand whether quiescence is reduced due to fewer episodes or, rather, shorter ones.

6.2.4 Behavioural states are idiosyncratic

Phenotypical variance is traditionally seen as the interaction between two partitions: genetic variance and environmental variance. However, under certain conditions, biological entities that have the exact same genetic make-up and that experience the same environment may still exhibit different phenotypes. There are, for example, stochastic processes, or so-called ‘developmental noise’, that take

part at the cellular level and account for phenotypical differences⁴³⁹. During the development of a multicellular organism, such small stochastic cellular perturbations of the initial conditions may propagate and eventually generate stable, but different, phenotypes between adult organisms—though mechanisms of ‘developmental stability’ are in place to counteracts extreme variations (see, for instance,⁴⁴⁰ for a review).

Behaviours are often studied as macroscopic phenotypes produced by nervous systems. In addition, brains are examples of highly plastic systems that continue accumulating noise (*e.g.* due to experience) after the end of an organism’s ‘development’ (*i.e.* in adults). Therefore, there has been growing interest in understanding the processes by which animals develop behavioural personalities and idiosyncrasies⁴⁴¹. In a broader evolutionary context, it has also been suggested that developmental stability, or the propensity to minimise adult variance, is itself an inherited trait. In other words, two isogenic populations may have the same average value for a given phenotype, but their variance may differ and is selectable⁴⁴². This process is evolutionary and ecologically important insofar as it takes part in the capacity of a genotype to generate diversity play a role in adaptation to changing environments⁴⁴³—and, in this respect, is conceptually related to ‘second-order selection’⁴⁴⁴.

As I started accumulating several days of behavioural data for hundreds of animals, I was first puzzled by the extent of the variability between animals. In contrast, each animal appeared to behave consistently throughout an experiment (fig. 4.5). Since the laboratory populations of flies I used are highly inbred and that individuals had been isolated from birth, I thought the simplest explanation to such large inter-individual variance was the product of the cryptic environmental noise in lighting, food, temperature, humidity, vibrations and so on. To corroborate this intuition, I recorded flies for a week, shuffled them in a new environment and tracked them for another week (fig. 4.6). To my surprise, the amount of quiescence, micro-movement and walking remained highly auto-correlated despite the change of environment, suggesting an endogenous determinism of behaviour. In contrast, the variation in preferred resting position seemed explained mostly by the environment.

At this stage, it is not possible to decide the extent to which genetics explains phenotypic variance as, even in inbred lines, genetic polymorphisms often remain^{445,446}. I attempted to obtain isogenic fly lines in order to address this concern but, for technical reasons, did not manage to record their behaviour. It would,

however, be important to perform similar experiments in genetically identical animals. A complementary direction could be to start from a genetically heterogeneous population and use a quantitative genetics approach to study the ability of genotypes to generate variable behaviour trajectories.

6.3 Sleep deprivation

Two of the strengths of *Drosophila* as a model are its small size and its short life cycle, which allows for very large numbers of animals to be used. Paradoxically, relatively few individuals have been used in the pioneering experiments (*e.g.*^{40,43,131,154}). In particular, the manual arousal probing and sleep deprivation protocols that have been applied by some authors were understandably very low-throughput^{40,154}. Aware of these limitations, some authors developed automatic assays to keep animals awake, but these methods (which I have presented in subsection 1.3.2.2) deliver numerous unspecific stimuli. Therefore, it is very difficult to isolate the effect of forced activity from the possible impact of repeated stimuli on the animal's physiology.

In order to address both the issue of the limited throughput and the lack of specificity of the treatment, I inspired myself from pieces of apparatus used for other model organisms^{151,152,416} to develop an automatic Dynamic Sleep Deprivation (DSD) module. In this section, I will first elaborate on the parsimonious and specific nature of this novel approach. I will then discuss the validity of the consensus that daytime rest is actually sleep. Finally, I will conclude by examining, perhaps, the most interesting — and controversial — of my results: the observation that flies do survive chronic sleep deprivation.

6.3.1 Dynamic Sleep Deprivation (DSD)

6.3.1.1 DSD leads to a rebound

In order to validate the use of a dynamic approach to sleep deprivation in flies, I programmed the servo module (fig. 2.3) to startle animals every time they had been immobile for an ‘interval’ of 20 s. This treatment considerably reduced quiescence throughout. After sleep deprivation had stopped, animals did show a clear and significant quiescence ‘rebound’ (fig. 5.1). This result comes alongside many others to corroborate the early observations of a homeostatic rebound after rest deprivation^{40,131}.

Despite, a clear effect of the treatment in the early hours of the following day, all three behavioural states returned to normal levels after less than 12 h, for both males and females. In fact, most of the effect was limited to the first three hours of the day. In contrast, Hendricks and coworkers had measured an increase

in rest for as long as three days post-treatment¹³⁰. Shaw *et al.* measured a rebound that seemed to last for only 12 h, but its amplitude appeared much larger compared to the one I could record. In several other studies, rebound seemed, however, limited in time and amplitude^{43,158}. The quantitative difference between studies, including the one herein, could originate from genetic or environmental discrepancies between experiments. It is also possible that conventional, static, sleep deprivation also prevents animals from feeding as they involve very frequent stimuli (sometimes as often as every 6 s^{131,154}). This off-target effect, alongside the use of the DAM, could have led to an overestimation of the rebound in previous literature.

Instead of to measuring quiescence only, I scored the three behaviours I had defined as well as the average position. I predicted that, since animals were startled when and only when they were resting, micro-movements would be either increased or unaffected during treatment. However, I found that the DSD, with an interval of 20 s did not exclusively reduce quiescence. In particular, in females, micro-movements were clearly less frequent over the 12 h of treatment. Furthermore, females had more micro-movements and were closer to the food immediately after the sleep deprivation night. Even though stimuli were given to immobile animals, it is conceivable that they mounted an escape response that changed their internal state, which reduced their propensity to feed during the night.

The idea that females could have been both starved and sleep-deprived in the morning could be seen as an exciting opportunity for future experiments. Indeed, it would be interesting to study the hierarchy between two homeostatically regulated processes: sleeping and feeding. In figure 5.1, I show the population average propensities to micro-move and quiesce, which are both increased post-treatment. However, at the individual level, these two processes are exclusive, and there could be a sequence of action (*e.g.* first feeding then sleeping) that may be an individual trait — and which could be experimentally manipulated.

Another interesting observation I made is that the number of stimuli required to keep animals moving increased during the night. In fact, the effectiveness of the treatment seemed to reduce as, by the end of the 12 h, the average quiescence is greater than zero. The observation that sleep deprivation was decreasingly efficient has been pointed out before, and there are two non-exclusive explanations. Firstly, sleep pressure increases, which results in flies engaging more readily in quiescence bouts, when they can. Secondly, they habituate to the recurrent stimulus and therefore have a lower propensity to respond. For the latter reason, authors

often delivered of stimuli that have some level of randomness in their timing⁴⁰ or intensity²⁵⁸.

DSD is parsimonious Despite the DSD protocol seemingly mildly depriving females of food, it can be assumed to be more parsimonious than static methods for the same effectiveness. In particular, the average number of stimuli received by the animals was approximately one per minute (fig. 5.2). In contrast, many studies startle animals three times more often to observe an effect^{43,131,154}. I was nevertheless interested in investigating the extent to which fewer stimuli could still be effective at eliciting a rebound, which I tested by lengthening the initial interval of 20 s to a range of values up to 1000 s. I found that, in general, higher interval led to fewer stimuli and also lower rebound. However, in males, rebound was not statistically different between intervals lower than 7 min. That is, animals that were allowed to rest for a maximum of 20 s and 7 min per bout had the same rebound, even though more quiescence was ‘lost’ in the first case.

Some authors calculate rebound by comparing quiescence loss during deprivation to quiescence regained afterwards, which seems burdened with the premise that, in general, there is a positive relationship between both variables¹⁵⁴. However, I found that, in males, rebound was only partially ($R^2 = 0.20$) explained by quiescence loss. This relationship only held for low intervals in females. The number of stimuli delivered was also only a poor predictor of rebounded time (not shown).

Interestingly, I could characterise a statistically significant, but only modest, rebound for long intervals (> 10 min), which corresponded to an average number of stimuli lower than ten. In contrast, other studies had delivered, systematically, one stimulus per hour to a population of flies overnight and saw no rebound^{258,285}. In their case, it is possible that the animals that were woken up had the opportunity to immediately recover (as they had one hour before the next event).

Since flies seemed to habituate to stimuli over 12 h (and 20 s interval), I decided to assess the efficiency of a DSD that would be carried over the last four hours of the night. This treatment led to a significant and large quiescence rebound (fig. 5.4). However, even though this four hours window corresponds to the time during which females display the least micro-movements and the most quiescence, the treatment clearly decreased micro-movement. Conversely, females showed a subsequent ‘micro-movement rebound’ and were closer to the food post-treatment. Which prompts the question of whether it is possible to alter sleep without incidentally generating some stress that reduces feeding.

DSD is specific Systematic sleep deprivation experiments make it difficult to decide whether rebound is a consequence of loss of rest, or, rather, a response to, for instance, mechanical stress. With a dynamic protocol, it is, however, possible to provide a control by stimulating animals when and only when they are already moving (fig. 5.3). Using this approach, I was able to show that although there is a mild quiescence rebound, its amplitude is very limited and it appears that the number of stimuli delivered, alone, does not explain rebound.

Interestingly, this treatment also reduced micro-movements in females and seems to have resulted in subsequent feeding redound, with more micro-movement and increase proximity to the food in the morning, which supports the idea that increased micro-movements after DSD resulted from a feeding homeostasis and that startled animals feed less.

The limits of mechanical sleep deprivation Although DSD is certainly more parsimonious and specific than its static counterpart, this series of experiments also reveals that it may have been naive to assume that mechanically stimulating a fly when it rests will only alter its sleep. Indeed, the sort of complex disturbance that it undergoes could change profoundly the internal state of an animal, which likely impacts other behaviours. It has been suggested that there is a hierarchy between feeding and escaping in *Drosophila* and other models, the latter inhibiting the former⁴⁴⁷, and it seems reasonable to think that startled animals will attempt to escape.

In addition, I would argue that it is logically impossible to ‘remove sleep’—or, for that matter, any behavioural state—without incidentally affecting another behaviour (*e.g.* feeding, walking, flying etc). Indeed, if we view behaviours as mutually exclusive and complementary, then the sum of behavioural propensities at any time, over the entire behavioural space, is one. Therefore, ‘depleting’ a behavioural state necessarily results in increasing the occurrence of, at least, another one. Then, it is not possible to know whether the observed phenotype results from the removed sleep, or from the alteration of other behaviours. Therefore, it could be wiser to draw conclusions based on several sleep-deprivation methodologies. For instance by assessing the consistency of results between genetic, mechanical and ‘ecological’ (*i.e.* conspecifics or predator) sleep deprivation protocols.

6.3.2 Daytime rest is maybe not sleep

As I planned my experiments on sleep deprivation, I realised that most previous studies had either altered both daytime and nighttime sleep or nighttime sleep only. The apparent lack of reported rebound in animals that would have been startled only during the day made me question whether L-phase rest qualifies as sleep altogether. Since a large portion of total inactivity happens during the day I believe this point is crucial for the field. In this subsection, I would like to discuss the contrast between the lack of empirical support for daytime sleep on one side, and its—stated or tacit—acceptance on the other side.

Homeostasis—the observation that animals compensate rest loss by subsequently increasing their immobility—is fundamental to the definition of sleep. In the two seminal articles of the field, homeostasis was shown elegantly by depriving animals of rest during their D phase and by observing a rebound from the beginning of the following L phase (*i.e.* in the morning) onwards^{40,131}.

Interestingly, although both articles report a large fraction of the rest happening during the day (almost half of it), they do not thoroughly report daytime rest deprivation. Hendricks *et al.* do not mention a treatment that would have occurred exclusively during the day⁴⁰ whilst Shaw *et al.* do so, but without presenting any data: ‘rest deprivation using the automated system revealed that both nighttime rest and rest during the day are homeostatically regulated (not shown)’¹³¹. In contrast with the latter claim, four years later, Huber *et al.* reported convincingly the *absence* of rebound when sleep deprivation is performed during the L phase⁴³.

To my knowledge, most following landmark studies that have performed sleep deprivation have then—at least partially—altered rest during the D phase, thus solidly corroborating the existence of a rebound after nighttime rest deprivation (for instance,^{157,158,288,289,294,306} and many more). Whilst I cannot exclude the possibility that some authors did observe of a rebound after daytime deprivation, no such work has come to my attention.

In addition to the observation of a homeostatic rebound, sleep is also defined by an increased arousal threshold. There are several studies that have compared responsiveness during the day and night and concluded that flies are a lot more arousable during the former than the latter^{43,258,285}. In fact, Faville *et al.* indicate that flies remain highly arousable throughout daytime immobility bouts²⁵⁸.

From these two independent lines of evidence, I would expect that the most rea-

sonable interpretation is that daytime rest does *not* qualify as sleep, and assume that immobility during the day is phenomenologically and ontologically different from sleep. However, I do not feel this view is shared by many of my peers.

There may be a growing consensus that sleep in L and D phases could be qualitatively different^{258,285}, but it has not been suggested flies do *not* spontaneously sleep during the day. Instead, virtually all studies implicitly assume sleep happens during the day (for instance by plotting population ‘sleep’ amount over 24 h). There are even studies that have observed differences in ‘sleep’ restricted to the L phase and that have provided a mechanistic explanation regarding their regulation^{333,383,435,448}.

To address this crucial question, I performed DSD over the entire L phase (fig. 5.5) and made two important observations. Firstly, despite the reduction of quiescence, no rebound was noticeable at the end of the treatment. Secondly, the number of stimuli that were automatically given to flies did not increase monotonically—in contrast to nighttime sleep deprivation. My interpretation is that there was no build up of a sleep debt and therefore no rebound.

An alternative explanation was, however, that, perhaps, the circadian drive somehow prevented rebound to happen. To address this point, I performed another experiment but, this time, stopped the treatment four hours before the end of the L phase. To my great surprise, despite a shorter treatment, I was able to quantify a significant rebound. This last experiment appears to support the conventional view that daytime rest is indeed homeostatically regulated.

I have to admit that I find these two results difficult to reconcile without making more *ad hoc* assumptions. These two experiments were not performed at the same time, and therefore cannot be genuinely compared. In addition, the overall number of involved animals is still low. Considering the scope of their conclusion, I would suggest treating them as preliminary work and propose to perform a larger scale experiment that compares the ability to rebound according to the timing of sleep deprivation in a full factorial design.

In conclusion, I remain very sceptical about the existence of a daytime sleep in *Drosophila melanogaster*, but hold it would be very important to investigate this avenue. Indeed, it could reveal rather problematic for the credibility of the field if we later discovered that what had been called ‘daytime sleep’ has, in fact, little in common with sleep.

6.3.3 Chronic sleep deprivation

It is often claimed that sleep is essential, the extreme manifestation of this biological need being the fact that chronic sleep deprivation is lethal⁴⁸. In *Drosophila*, the evidence supporting the notion that sleep is vital lies on one cornerstone study in which Shaw *et al.* manually deprived 12 animals of sleep over approximately three days¹⁵⁴. Only four animals have been reported to die before 70 h. Considering the contrast between the importance and of this question and the elusiveness of the answers that had been provided, I decided to employ DSD to address it in a more meticulous and statistically sound fashion.

6.3.3.1 Chronic DSD is efficient

A 9.5 days DSD reduced quiescence throughout the experiment and resulted in a significant quiescence rebound (fig. 5.7). Surprisingly, the amplitude of the following rebound was limited in males, but appeared to last several days in females. It is, however, unclear whether the rebound in these two-weeks-old animals can be compared, in any way, with the one exhibited by animals less than half their age (shown in all the other experiments). It would be necessary to understand the effect of age on sleep homeostasis in order to provide any meaningful conclusion regarding the amplitude of the rebound.

When examining with more attention the effect of DSD on behaviours, during treatment, I noticed micro-movements were profoundly affected. Indeed, treated females had lost their characteristic peak of micro-movement after the transition to D phase, but micro-moved more than the control at the end of the night. In males, micro-movements in sleep-deprived animals were increased throughout and were curiously even higher than control females.

I observed, by inspecting individual stimulus data, that animals that were startled often responded by walking two or three millimetres, perhaps repositioning themselves before engaging in a new quiescence bout. Given that the shape of the micro-movement traces matches very well the average number stimulus delivery, I suggest most of the micro-movement, in chronically sleep-deprived animals, corresponds to responses to stimuli with no walking. If this interpretation is correct, I do not see it as an argument against my paradigm, as I make the hypotheses that an animal must be awake to reposition.

To understand further the behaviour of animals during chronic sleep deprivation,

it would be necessary to decompose micro-movement in more biologically meaningful behaviours. In the results I presented, repositioning, feeding, grooming and twitches are all aggregated into a single variable, which is clearly a technical limitation. I suspect feeding-related micro-movements are masked by the larger amount of repositioning events after a stimulus.

6.3.3.2 Chronic DSD is not lethal

In contrast with Shaw *et al*'s work¹⁵⁴, in my experiment, neither male nor female populations ($N \approx 100$ each) died faster than their respective controls during a 9.5 days of sleep deprivation (fig. 5.8). Furthermore, their lifespan post-treatment, at 29°C, was unaffected.

The first obvious explanation would be that complete loss of sleep is, in fact, viable—rather than a vital need—to *Drosophila melanogaster*. I cannot exclude that some flies managed to sleep during the 20 s that separated two consecutive stimuli. However, there is little doubt that the amount of time that treated animals could rest was severely impacted, whilst their lifespan was absolutely identical to the control's. If sleep was indeed physiologically crucial, even a partial chronic sleep deprivation should have affected lifespan post-treatment, even subtly.

There are, however, several alternative explanations for this result. One possibility is that only the 'accessory rest' would have been depleted. Indeed, the small amount of unconsolidated quiescence, between two stimuli, could have been sufficient for animals to survive. In which case, it prompts the question of whether it is experimentally possible to completely deprive an animal of sleep, whilst remaining specific.

Another explanation could be that sleep was indeed depleted and that is a crucial determinant of fitness, but that lifespan of individual animals in small glass tubes does not capture this effect. Indeed, sleep could serve a function that, in a less 'forgiving' environment (*e.g.* with predation and competition), is vital. In this case, one could argue that sleep is evolutionary, but not physiologically, vital. Using an analogy, a fly that would have no wings can live long in our laboratory, but I doubt it would be fit in the wild, and one may argue that wings are 'virtually vital' to flies in the latter environment. It would be very interesting to assess how sleep-deprived animals perform in competitive mating assays, learning tasks and other 'challenging' contexts.

An alternative possibility is that sleep deprivation actually reduces lifespan. However, as I discussed above, it appears that treated animals feed less, and caloric restriction is known to enhance lifespan in *Drosophila* and other models (reviewed in⁴⁴⁹). Therefore, it is possible that my DSD protocol both increased and decreased longevity, with antagonistic mechanisms.

6.3.3.3 Lifespan is not explained by behaviour

Another argument that is put forward by the defenders of the vital need for sleep is that animals that sleep very little, such as some sleep mutants, tend to have a shorter lifespan^{48,157,306,375,376}. However, most genes are pleiotropic and a phenotype as general as longevity likely results from sleep-independent effects. To consider an almost absurd example, in *Drosophila*, there are mutations that affect both eye colour and lifespan, but we cannot claim that red or white eyes themselves are ‘vital’⁴⁵⁰. Likewise, the argument according to which ‘sleep must be vital since mutants that sleep less also die faster’ is not receivable. Furthermore, the lifespan of *fumin* mutants and brain-specific *insomniac* knock-downs, which have reduced sleep, is intact^{158,376}.

There is often an evolutionary trade-off between longevity and other traits, such as fecundity⁴⁵¹ and immunity^{452,453}. If sleep was indeed a crucial physiological function, one may predict that animals that invest more in resting would also live longer. However, in my experiments, lifespan could not be explained by quiescence—or, for that matter, by other behaviours (fig. 5.8). This finding is also supported by the discovery, from another team, that flies which had been selected over several generations became low-sleepers without either their lifespan or egg-to-adult viability being altered¹⁵⁹.

Altogether, the result of my chronic sleep deprivation experiment challenges the recurrent claim that sleep is vital to *Drosophila* and invite us to rethink what is often presented as an established fact. Whilst I cannot exclude that some animals may die from sleep deprivation, the statement that sleep is a universal need does not appear supported by extensive evidence.

6.4 Conclusion

In the thesis herein, I have first described the ethoscope, an instrument that can be used to monitor simple behaviours of multiple animals over long durations. Although it can clearly be improved, and I hope others will attempt this task, it already provides a competitive alternative to the *Drosophila* Activity Monitor (DAM) and to other video-tracking methods.

In addition to the ethoscope, I delivered **rethomics** a set of R packages intended to streamline and standardise the analysis of behaviour such as sleep and activity, in particular when many animals and covariates are included.

My new tools allowed me to account for daily behaviour with a new state, micro-movement. This addition was very informative in general and particularly instrumental to reconcile the inconsistent results regarding post-mating sleep in females. I also described sleep as an idiosyncratic behaviour and paved the way to experiments that would explain the origin of the large variability in daily behaviour.

The real-time capabilities of the ethoscope enabled me to apply Dynamic Sleep Deprivation (DSD), a paradigm that had not been used previously on fruit flies. Although its specificity can be discussed, it provides a clear improvement over the static alternatives that are commonly used. Using this new method to deplete sleep, I reassess the accepted view that daytime rest is sleep, and recommend a cautious approach to this question. Finally, I was able to perform an unprecedented chronic sleep deprivation experiment, but could not show any effect of sleep loss on longevity, a very controversial finding.

The work I have presented in this thesis emphasise that there are several important claims regarding the phenomenology of what we call sleep that may need to be re-evaluated. Since, for *Drosophila*, the definition of sleep we operate with is behavioural only, it is paramount that the conceptual, but also the technical, tools we use to study behaviour match the quality of the arsenal of techniques we already use to study the biology of the fruit fly.

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