

**Homeostasis in Drosophilids: A cross-species
investigation of *Drosophila* sleep**

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Declaration

I declare that this thesis was created by myself, that the work is my own except where explicitly reported in the text, and no part has been submitted for another degree or qualification.

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Abstract

Sleep is a puzzling behaviour that is ubiquitous throughout the animal kingdom, and while it is implicated in many biological processes, its core function remains elusive. Although the core advantage sleep provides for an animal is still debated, it is widely accepted to be regulated by a two-step process that describes the circadian control of sleep timing, ensuring an organism's sleep-wake cycle occurs with the passing day and night and a second homeostatic system which guarantees that animals fulfil their daily amount and quality of sleep. The circadian aspects of sleep regulation are primarily understood, but homeostasis is still a relatively mysterious process. In the last 20 years of studying sleep homeostasis in *Drosophila melanogaster*, a picture of the sleep circuitry and theories of the underlying molecular mechanisms that produce sleep need have emerged. However, the homeostatic control of sleep is complex, with growing evidence of multiple circuits that may differentially influence the amount and quality of sleep determined by the waking environment. Understanding sleep regulation in other species of the *Drosophila* genus may elucidate the emergence of sleep homeostasis in the animal kingdom, utilising their evolutionary distance to compare the shared fundamental aspects of sleep. In Chapter 3 of this thesis, I employed a comparative approach in order to establish the circadian and homeostatic elements of sleep behaviour in a selection of *Drosophila* species, including *D.melanogaster*, *D.simulans*, *D.sechellia*, *D.erecta*, *D.yakuba*, *D.willistoni*, and *D.virilis*. I found a striking disparity in their neuromodulatory sleep signalling and homeostatic response to a mechanical method of sleep deprivation, while the circadian timing of sleep was broadly conserved. In Chapter 4, I investigated the underlying mechanisms of the homeostatic response using the tools available in the *D.melanogaster* model and elucidated that alterations in their synaptic strength dynamics may underscore the divergence in homeostasis observed.

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Acronyms

3IY	3-iodo-tyrosine
AstaA	Allatostatin A
BDSC	Bloomington <i>Drosophila</i> Stock Center
BRP	Bruchpilot
cAMP	Cyclic adenosine monophosphate
CDM	Chlordimeform
CNS	Central nervous system
CREB	cAMP-responsive element binding protein
DAM	<i>Drosophila</i> Activity Monitor
DAN	Dopaminergic
DAT	Dopamine transporter
dFB	Dorsal fan-shaped body
Dop1R	Dopamine-d1-like receptor
DH31	Diuretic Hormone 31
DN1	Dorsal neurons 1
DN2	Dorsal neurons 2
DN3	Dorsal neurons 3
dsRNA	Double-stranded RNA
EB	Ellipsoid body
EEG	Electroencephalogram
FB	Fan-shaped body
FMRP	Fragile X messenger ribonucleoprotein
GABA	γ -aminobutyric acid
GFP	Green fluorescent protein

GluR1-AMPA	GluR1 containing glutamatergic AMPA receptors
GOI	Gene of interest
KCs	Kenyon cells
KD	Knockdown
L-dopa	L-3,4-dihydroxyphenylalanine
LNDs	Dorsal lateral neurons
LFP	Local field potential
LLNvs	Large ventrolateral neurons
LPNs	Lateral posterior neurons
MB	Mushroom body
MBON	Mushroom body output neurons
NREM	Non-rapid eye movement
ORNs	Olfactory receptor neurons
PBS	Phosphate-buffered saline
PDF	Pigment-dispersing factor
PI	Pars intercerebralis
PKA	Protein kinase A
RDL	Resistant to dieldrin
REM	Rapid eye movement
RNAi	RNA interference
SD	Sleep deprivation
SHY	Synaptic homeostasis hypothesis
sLNvs	Small ventrolateral neurons
SWA	Slow-wave activity
SWS	Slow-wave sleep

THIP	4,5,6,7-tetrahydroisoxazolo(5,4-c)pyridin-3-ol
TNT	Tetanus toxin
TrpA1	Transient receptor potential cation channel
UAS	Upstream activating sequence
VDRC	Vienna <i>Drosophila</i> Research Center
ZT	Zeitgeber (time-giver)

Chapter 1

Introduction

1.1 Sleep and its evolution

1.1.1 Sleep generalities

Sleep is a period of reversible quiescence where you are disconnected from the external world but aroused by salient stimuli. For example, while a mother is unlikely to be disturbed by the sound of a car alarm, they would quickly be awoken by the sounds of a crying infant. During such rest periods, an organism cannot satisfy its essential needs. One cannot forage for food, search for an appropriate mate, or remain vigilant to potential threats from predators. Regardless of the apparent inefficiency of such rest periods, every animal meticulously tested appears to partake in this behavioural phenomenon^[1], suggesting that sleep must encode a useful function in order to justify the apparent deficits it presents to an organism and its persistence throughout evolution.

The enigma of sleep has engaged minds since records began, and in 500 BC, a Greek philosopher, Alcmaeon of Croton, postulated that sleep occurred due to the withdrawal of blood from the surface of the body to the interior larger blood vessels ^[2]. However, the scientific sleep field is still a relatively new area due to the previous inaccessibility of sleep for study and the initial thinking that sleep was a unitary 'off-period'^[3]. In the 1950s, the electroencephalogram (EEG) was employed to investigate brain activity during sleep, and it was identified that the behaviour was composed of distinct stages, with one such stage exhibiting the activity associated with an awake brain, greatly challenging the previous conceptions of sleep. The EEG wake resembling sleep stage was found to correlate with bouts of rapid ocular motion amongst bouts of long periods of sleep without eye movement^{[4][5]}. The former sleep stage was indicatively called rapid

eye movement sleep (REM) and the latter non-REM (NREM) sleep. A key aspect of the NREM stage is the delta (0.5-4Hz) slow wave activity (SWA), often referred to as slow wave sleep (SWS). Since this finding, much work has been conducted to understand sleep's function and it has been implicated in many biological processes, including development, immunity^[6], waste clearance^[7], learning^[8], and synaptic homeostasis^[9]. However, the essential function that sleep serves is still debated across the scientific community, and no consensus has been reached on why animals require sleep^[10].

1.1.2 Homeostatic and circadian regulation

Sleep is a complex and puzzling behaviour, and there are ongoing lines of study trying to decode the origin and function of sleep. While there is debate about the unified function of sleep, the conceptual framework for its regulation is widely accepted. In the 1980s, Borbely *et al.*^[11] proposed a model of sleep regulation whereby two processes interact to determine sleep control. This two-process model describes a homeostatic mechanism (Process S) that accounts for the amount and quality of sleep and a circadian mechanism (Process C) that accounts for the timing of sleep with the passing of day and night under the influence of the circadian clock. While the circadian impact on sleep is rhythmic and has a constant cycle, sleep homeostasis is determined by the length and quality of wakefulness, ensuring that an organism achieves its daily amount of sleep. Process S represents sleep need, which builds through the waking period until reaching an upper threshold, initiating sleep, and decreasing sleep need until it reaches a lower threshold, triggering wake. The model attributes the upper and lower thresholds to Process C. The framework established by Borbely and colleagues has been extensively utilised in the sleep field to interrogate the underlying mechanism controlling sleep behaviour. For example, the conceptualisation of a homeostatic process with building sleep need provided a simplistic structure for investigating the biological underpinning of sleep need and Process S. Sleep deprivation (SD) protocols confirm the existence of Process S, as prolonging the waking period past the upper threshold and obstructing the dissipation of sleep need results in a sleep episode of greater length or intensity, in order to dissipate the sleep need signal. This period of compensatory sleep is often referred to as rebound.

The model has undergone several revisions over the years^{[12][13]}, incorporating new research findings, but it prevails as the widely accepted model of sleep regulation.

1.1.3 Ubiquity of sleep behaviour

Sleep is ubiquitous throughout the animal kingdom and has been found to exist in both vertebrate and invertebrate phylum.^{[1][14][15]} The maintenance of sleep across evolution and pervasive selective pressures attests that sleep encodes a significant, beneficial role to an organism. All mammals and birds are found to exhibit the REM and NREM EEG electrophysiological recordings^[16] initially identified in humans; however, across phylogeny sleep is primarily described by its behavioural aspects, and quiescence in animal species must satisfy the following criteria to be considered sleep (1) A circadian-controlled state of quiescence - whether that be diurnal, nocturnal, crepuscular, or arrhythmic. (2) A preferred position and posture, (3) with increased arousal thresholds, distinguishing sleep from periods of immobility that occur during wake, (4) is rapidly reversible when subjected to a more substantial stimulus, discriminating sleep from torpor and (5) is controlled by homeostatic regulation. Such behaviours have been described in vast animal phyla^[17], and this section herein will summarise the sleep in a selection of Chordata, Arthropoda, Nematoda, and Cnidaria animal species.

1.1.3.1 Chordata

Mammals

Humans (*Homo sapiens*) - experience monophasic sleep^[18], sleeping in one extended, largely uninterrupted, bout of immobility. Humans are in the company of other primates, as most other mammals sleep in shorter bouts of polyphasic sleep. In humans, the NREM stage comprises 4 phases which together make up for 75-80% of total sleep^[19]. The phases have a distinguishable EEG pattern and varying arousal thresholds, as described in Fig 1.1. The NREM Stage 1 sleep is the transition from waking before running through the sequential NREM Stages 2,3, and 4 before entering REM sleep, after-which an individual re-enters stage 1. NREM and REM sleep stages cycle 4-6 times throughout the night with a periodicity of 90-100 min^[19].

The final NREM sleep stages are often referred to as SWS due to their large-amplitude, low-frequency SWA. SWS is widely considered to correspond to a deeper stage of sleep due to various findings. Firstly, it is highest at the onset of sleep, mostly occurring in the first 1/3 of the night, with decreasing intensity throughout the sleep period^[19]. Secondly, the SWS stages are associated with increased arousal thresholds, indicative of elevated sleep intensity^[21]. Thirdly, the level of SWA is primarily determined by the length of prior wakefulness^[22] and increases in intensity during SD^{[23][24]}. Finally, compensatory recovery sleep is preferentially composed of this NREM SWA^[25]. For the reasons outlined here, NREM SWA is widely accepted as an electrophysiological

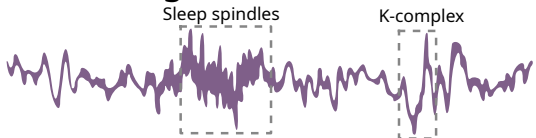
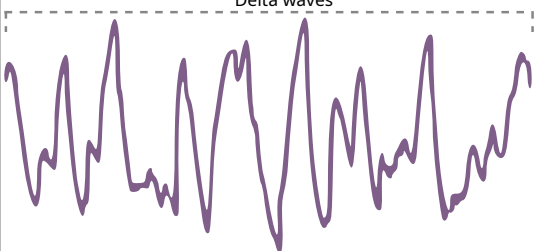
 Human sleep stage	EEG activity, duration and arousal thresholds
NREM Stage 1 	Transition from wakefulness Low amplitude, mixed frequency activity Contain theta waves (4-8 Hz) Low arousal thresholds
NREM Stage 2 	Lengthens with each progressive cycle Low amplitude, mixed frequency activity Contain sleep spindles (10-12 Hz) and K-complexes Higher arousal thresholds than stage 1
NREM Stage 3-4 	Mostly occurs in the first third of the night Synchronised large amplitude, low frequency SWA delta waves (0.5-2 Hz) Stage 3 : lasts a few minutes Stage 4 : makes up 10-15 % of total sleep first cycle lasts approx 20-40 min Highest arousal thresholds
REM 	Similar EEG pattern to wake Desynchronised low amplitude, mixed frequency activity Short bout in the first sleep cycle Lengthens with each progressive cycle Arousal thresholds similar to stage 2

Figure 1.1: The stages and cycles of human NREM and REM sleep
Sleep cycles through NREM and REM stages through the sleep period. NREM stage 1 is the transition from wakefulness, and the EEG pattern contains low amplitude and mixed frequency activity. NREM stage 2 is found to increase in duration with each cycle, and the EEG trace is comparable to stage 1 but is associated with the occurrence of K-complexes. NREM stages 3 and 4 comprise the SWA delta waves (0.5-2 Hz) that correspond to deeper stages of sleep and correlate with the highest level of arousal thresholds. The final stage in the sleep cycle is REM sleep with desynchronised low-amplitude & mixed frequency EEG activity comparable to wakefulness.^[19]. Adapted from Pan *et al.*^[20].

correlate of deep sleep and a primary marker of Process S during sleep^[11].

The impact of sleep loss on REM sleep is less clear than on NREM. Total and selective REM SD has been shown to increase REM sleep intensity; however, the results are inconsistent and are usually less profound than the striking increases seen in the NREM sleep^{[26][27][28]}. Suggesting that while REM sleep is likely homeostatically regulated, it is largely overshadowed by the need for NREM recovery.

Rats (*Rattus*) - are nocturnal mammals that have polyphasic bouts of sleep. It is generally thought polyphasic sleep occurs as it allows for reoccurring vigilance and reduced risk of predation^[18]. Rat sleep predominately occurs during the light phase, but they are found to rapidly cycle through bouts of wake, NREM, and REM sleep over the full day^[29]. The electrophysiological aspects of NREM and REM sleep are primarily comparable between humans and rodents, with large amplitude low-frequency oscillations during NREM sleep and desynchronised low amplitude high-frequency patterns similar to wake during REM^[30]. Rat sleep, however, has yet to be shown to have the distinct stages of NREM described in humans, and their periods of such sleep are much shorter^[31]. Nevertheless, NREM SWS is associated with sleep drive, revealed by the significantly increased levels of NREM SWS after 24 hr enforced waking^[32].

Monotremes (*Monotremata*) - are primitive egg-laying mammals that diverged from other mammalian species approximately 130 million years ago^[33] and today comprise just 5 extant species. Their early evolutionary divergence from other mammalian species and egg-laying physiology make them an exciting study for the evolution of sleep. An initial study of monotreme sleep in the echidna^[34] identified an NREM sleep stage but concluded that there was no evidence of REM. During quiescence, no period of desynchronised EEG activity was accompanied by rapid eye movements, as widely reported in other mammals. The study attempted to identify whether periods of desynchronised activity during quiescence correlated with any other behavioural or physiological characteristics of mammalian REM sleep, for example, increased arousal thresholds and cardiac irregularity. No such correlates were found. However, the study of sleep in another monotreme species, the platypus^[35], found that they spend up to 8 hours a day in their detectable REM sleep, more than any other mammal tested. Conversely to the echidna study, the investigators identified a REM sleep stage based on the behavioural characteristics and, following this, observed the EEG and electrocardiogram (ECG) activity within the defined behavioural bouts of quiescence. Phasic head, bell, and eye movements were associated with moderate to high amplitude EEG measurements, unlike the hallmark low amplitude activity usually found with REM. This described platypus REM stage had elevated arousal thresholds and decreased cardiac rhythmicity, which is common to REM in other mammals^[36]—suggesting that a REM

sleep stage may well exist in the monotreme species but that the cortical desynchronisation evolved later and is unrelated to its function. However, it is unclear whether the REM sleep that is reported in these small number of studies is indeed a true reflection of REM sleep and the monotreme data is often excluded from comparative studies^[37]. It is important to note that the platypus study was only carried out in 4 individuals and that the animals are stated to not acclimatise well to captivity. Therefore the results observed are unlikely to match wild sleeping patterns.

Birds

Birds are the only non-mammalian class to demonstrate REM and NREM sleep unequivocally. Their NREM SWS of high-amplitude, low-frequency activity and REM sleep of low-amplitude, high-frequency comparable to wakefulness^[38] determines the homology with mammalian sleep stages. Birds exhibit unihemispheric sleep, a phenomenon rarely found in mammals and appears restricted to marine animals^[39]. In unihemispheric sleep, an animal engages in sleep in one cerebral hemisphere at a time while the other is awake, and animals often keep one eye open to remain connected to the external environment^[40].

Pigeons (*Columba livia*) - have been found to sleep on average 44% of the day, and their rest periods have elevated arousal thresholds, determined by application of an electric shock^[41]. Birds have a stereotypical sleeping position of immobility with a lowered head, and eye movements accompany REM sleep. Pigeon sleep appears to be homeostatically regulated, and one study found increases in total duration of REM, but not NREM sleep after long-term SD^[42]. Another study, however, showed that after 12 hr SD with gentle handling, there was a global increase in SWA, as an increase in spectral power density, during subsequent sleep, suggesting that comparably to mammalian sleep, NREM is recovered in the form of increased SWA intensity^[43].

White-crowned sparrow (*Zonotrichia leucophrys*) - are migratory birds that show a diurnal sleeping pattern with EEG correlates of REM and NREM sleep^[44]. While no changes in the duration of REM and NREM sleep were found after 6 hr SD, increases in the spectral power density were found during NREM in the first 2 hours following SD, which declined in the subsequent hours as the animals slept^[45]. Again, mimicking what is observed in mammals, where the SWA activity correlates with increased sleep pressure, and suggesting that the white-crowned sparrow sleep is under the control of homeostatic regulation.

Reptiles

It is important to understand non-avian reptilian sleep, as it can illuminate how sleep manifests in the brain of non-endothermic vertebrates with a rudimentary neocortex.

While comprehensive studies on non-avian reptilian sleep are scarce, a picture of the behavioural aspects of their sleep has emerged. However, there are conflicting results on the electrophysiological sleep patterns and whether they share homology with mammalian and bird REM and NREM sleep^[46].

Iguanas (*Iguana iguana* and *Ctenosaura pectinatas*) - were found to have 4 vigilance states, described as active awake, quiet awake, quiet sleep, and active sleep. During the sleep states, Iguanas have a specific sleep posture, where they lie motionless on their torso, with their limbs relaxed in a backward position, and their head resting on a surface with closed eyelids^{[47][48][49]}. Crude interrogation of the sleep states with manual tapping in the *Ctenosaura pectinata* produced a response indicative of decreased arousability^[48]. A methodical electric shock arousal threshold paradigm in the *Iguana iguana* supported this result^[49]; however, these studies were carried out in two different species of lizard. The *Iguana iguana* sleep appears to be influenced by the homeostat since 48 hr enforced waking with manual handling results in approximately 16 hr period of continuous sleep^[49], primarily comprising quiet rather than active sleep. For comparison, the iguanid was found to sleep an average of 12 hours under baseline conditions. Notably, the investigators remark that the *Iguana iguana* animals responded to the enforced waking interventions with threat, attack, and escape behaviours. This raises the issue found in many SD paradigms, but none more plainly evident than here, that researchers may be capturing sleep-independent, stress-induced effects of the SD method. EEG recordings in *Ctenosaura pectinata* and *Iguana iguana* quiet and active sleep states showed distinct patterns, with active sleep sharing commonalities with active wake, yet did not show the epochs of REM sleep. The quiet sleep EEG recordings, while distinct from the wake and active sleep lizard activity, showed no likeness to NREM sleep^{[47][48][49]}. This demonstrates that while distinct sleep stages likely exist within lizards, their homology to endothermic vertebrates is uncertain.

Bearded dragons (*Pogona vitticeps*) - while many early electrophysiological studies of sleep states in non-avian reptiles failed to find correlates of REM and NREM sleep, a more recent study of local field potential (LFP) recordings in the bearded dragon, *Pogona vitticeps* demonstrated homology. Shein-Idelson *et al.*^[50] observed behavioural sleep with high-amplitude sharp waves that show delta SWA, typical of NREM delta SWS. The sharp wave sleep was interspersed between periods of desynchronised activity and sporadic eye movements, a possible homologue of REM^[50]. These results were confirmed within a second study^[51]; however, their investigations of another lizard species, *Salvator merianae*, found two disparate sleep stages with no electrophysiological relevance to mammalian and bird REM and NREM sleep.

Fish

Zebrafish (*Danio rerio*) - exhibit a diurnal sleep state, where periods of larval inactivity lasting 10 min occur predominately at night, associated with specific postures - either floating with their head in a downward position or horizontally near the bottom of the chamber^[52]. Similar results are found in adults, but the bouts of activity are much shorter, lasting up to 4 min ^{[52][53]} where the fish float horizontally with their heads facing upward. The quiescence is accompanied by increased arousal thresholds when presented with a mechanical ^[52] or light^[54] stimulus in larvae and a mechanical ^[52] or electrical ^[53] stimulus in adults. The characterised zebrafish sleep displays evidence of homeostatic regulation, with periods of 6 hr enforced wakefulness with mild vibration leading to increased sleep with elevated arousal thresholds^[52]. However, Yokogawa *et al.*^[53] found that in adult zebrafish, rebound only occurred in constant dark conditions, as terminating of the SD at the beginning of the morning and onset of the light had no rebound. A significant advantage of zebrafish neurobiological research is the optical transparency of larval zebrafish, allowing for in vivo measurement of neuronal activity. A 2018 study^[55] assayed the neuronal signal in sleeping larval zebrafish measuring the fluorescent Ca^{2+} signal in their neocortex homologue, the dorsal pallium. They identified two-distinct activity traces of sleep – short-term bursting and propagating wave sleep, which indicate commonalities with NREM SWS and REM sleep, respectively, supporting the existence of distinct sleep stages in lower vertebrates.

1.1.3.2 Arthropoda

Insects

Cockroaches (*Leucophea maderae* and *Blaberus giganteus*) - were one of the first invertebrate animals identified to present with sleep behaviour. It was well studied for its circadian waking activity (i.e., locomotion)^[56], and in 1983, the focus shifted to its periods of quiescence to establish if it was representative of sleep behaviour. The quiescence was found to be controlled by homeostatic regulation, with enforced waking protocols in the *Leucophea maderae* cockroach resulting in a subsequent elevation in rest^[57]. A following study in the *Blaberus giganteus* cockroach identified that the bouts of inactivity coincided with increased latency of response to a vibrational stimulus, displaying the elevated arousal thresholds that are constitutive of sleep behaviour^[58].

Honey Bees (*Apis mellifera*) - were also among the first invertebrate species to be investigated in the context of sleep. As a diurnal species, they exhibited periods of rest in the nest at night with a specific position within the combs of the nest and characteristic antennae movements, which were also observed under lab conditions^[59]. Crucially, the response to stimuli was reduced while in the characteristic posture. Fur-

ther studies since the description of honey bee sleep found that there are three discrete sleep phases, discerned by the sleep bout duration and classification of their antennae movements^[60]. Arousal threshold analyses established that the deepest stage of sleep occurred at the midway point of a sleep bout and correlated with high levels of antennae immobility; thus, it is commonly used as a metric for sleep depth in honey bees. Finally, SD protocols employing a tilting device that produced rolling movements to isolated honey bees found that 12hr night phase SD resulted in increased levels of antennae immobility during the following night, rather than immediately after the SD had ceased, supporting that honey bee sleep is likely controlled by the homeostat^[61].

Fruit flies (*Drosophila melanogaster*) - at the turn of the millennia, this powerful invertebrate model emerged that had the behavioural hallmarks of sleep and has since been utilised for the mechanistic probing of the enigmatic behaviour. *D.melanogaster* have a diurnal rest-activity pattern, with males having an additional period of rest during the day, known as the siesta^[62]. Hendricks *et al.*^[63] demonstrated with visual observation that flies were found to have a specific rest position and posture, preferring to rest close to, but facing away from the food with a hankered down position (in experimental tubes, see Materials and methods 2.2.2). Fly sleep has been monitored by automated infrared and automated video tracking. Both methods revealed bouts of immobility that preferentially occur during the night phase^{[63][64][65]}. *D.melanogaster* rest has been shown to have increased arousal thresholds using mechanical^[64], visual^[66], auditory^[67] and olfactory stimuli^[68], which is rapidly reversible with a stronger stimulus. Finally, SD with a mechanical stimulus results in a consequential bout of increased quiescent duration; therefore, the humble fruit fly satisfies all the behavioural criteria of sleep. *D.melanogaster* is the focus of this thesis and will be discussed in more detail in the next chapter.

1.1.3.3 Nematoda

Chromadorea

Nematode roundworm (*Caenorhabditis elegans*) - after the substantiation of sleep behaviour in *D.melanogaster*, attention was directed to the nematode roundworm, *C.elegans*, to elucidate whether sleep behaviour was exhibited in lower invertebrates. *C.elegans* possess a simple nervous system comprised of only 302 neurons. The worms demonstrate quiescent behaviour during the transitional periods of their four larval stages, known as lethargus and often described as developmental sleep^[69]. Compared to the larval and adult stages, mechanical and olfactory interrogation of lethargus revealed an altered response to arousing stimuli. Outside of lethargus, approximately

40% of the responses to a mechanical stimulus are composed of complex behaviours, for example, reorientating, acceleration, and shrinking. However, during lethargus, the complex behaviours are restricted to less than 10% of the responses, and the worms predominately perform brief backward movements. This demonstrates that to study the evolution of sleep, the behavioural criteria of sleep behaviour, framed within the mammalian lens, often requires expansion to capture distantly related species' sleep phenotypes. Homeostatic response to enforced wakefulness with a mechanical stimulus is observed in the nematode worm, with a short and sharp increase in quiescence in response to a weak stimulus or a subdued, yet longer lasting overall increase in quiescence after a stronger stimulus^[70]. Finally, this sleep-like state described has since been found to have an identifiable posture which has been described as hockey stick-like^{[71][72]} opposed to their usual sinusoidal shape.

1.1.3.4 Cnidaria

The sleep states described so far have been found in animals with a central nervous system (CNS). However, sleep states have been identified in organisms that lack a CNS and, therefore, do not have a brain. Cnidarians, one of the first metazoans to have evolved tissue-level organisation, have a nerve net that innervates most parts of their body^[73]. The nerve net shares aspects with higher invertebrate and vertebrate nervous systems, including synaptic transmission and neurochemicals^[74]. More recently, sleep-like-states have been described within the cnidarian phylum^{[75][76]}.

Scyphozoans

Upside-down jellyfish (*Cassiopea spp.*)- while the *Cassiopea* upside-down jellyfish rarely swims, they continuously pulse by relaxing and contracting their bell. By measuring the pulse rate, Nath *et al.*^[75] identified that these jellyfish have diurnal activity rhythms, with a reduction in pulsations during the night phase, and are most inactive in the last 6 hours of the night. In order to test whether this rest was accompanied by decreased arousability, the investigators designed an arousal paradigm that utilised their natural movement behaviours. The jellyfish prefer settling on a solid surface, and on placement into the experimental tank, they travel to the bottom. The researchers built a cylindrical arena with a filter screen bottom that they placed inside the tank. As the jellyfish characteristically positioned themselves on the filter screen, the cylindrical arena was lifted and then lowered, leaving the animal floating in the arena. By measuring the time to the first pulse and time to the bottom, they established that the animals had increased arousal thresholds during the night phase, with more extended periods of both metrics in the night compared to the day. Finally, forced movement with a water pulse every 20 min during the night phase led to a reduction in

pulsations in the subsequent light phase, which was, once again, matched with increased arousal thresholds.

Hydrozoans

Hydra (*Hydra vulgaris*) - are freshwater cnidarians found in temperate and tropical regions. They are elementary organisms with only two cell layers and a tubular body with a mouth and tentacles on one end and a basal disk on the other^[77]. With the identification of sleep states in other cnidarians, a key study from 2020^[76] set out to investigate whether this simple organism also had sleep states. Firstly, the *Hydra* were found to show diurnal behaviours, quiescent more in total in the night phase, but also the rest architecture was made up of more prolonged bouts. This quiescence was reversible when interrupted by a light pulse, with reduced latency to arousal with increased light intensity. The rest was associated with increased arousal thresholds, with elevated latency to arousal during extended rest bouts. The *Hydra* seemingly does not have endogenous circadian rhythms, as the rhythmic behaviours ceased in artificial 24 hr light and 24hr dark conditions. Remarkably, their rest is subject to homeostatic regulation, as both mechanical and temperature-induced sleep deprivation during the night phase led to sleep rebound on the subsequent day, indicating that these simple, CNS-less organisms exhibit a sleep-like state.

1.1.4 Proposed functions of sleep

There are many different theories of sleep function, and no single concept fully satisfies all the physiological, molecular, and cellular evidence of sleep across the animal kingdom. It is generally considered that sleep likely encompasses a combination of many aspects of the proposed theories surrounding its function^[10]. Nevertheless, the theories can be broadly characterised as those concerning adaptive, restorative, and cognitive processes.

1.1.4.1 Adaptive theories

The adaptive sleep theories broadly cover the energy conservation^[78] and inactive^[79] origins of sleep. The circadian influence on the sleep-wake cycle determines the timing of sleep. For example, humans are diurnal and restrict their activity to the day and sleep to the night. The energy conservation theory^[78] proposes that sleep reduces an organism's energy consumption during a period it is less suited for. Therefore, restricting energy expenditure to the time of day when it can more effectively execute its essential needs (i.e., mating, hunting, and feeding). However, this passive process

of overall energy conservation is challenged by the finding that sleep itself is relatively energy-consuming, with a minimal reduction compared to wake^[80].

The inactive theory of sleep proposes that it initially emerged as a state of adaptive inactivity comparable to the dormancy found in unicellular organisms and plants. Dormancy allows organisms to survive in hostile environments and sleep may have originated as a daily torpor to shield animals from the danger of exploring an environment they are less suited to, therefore improving survival^[79]. It may well be that sleep initially emerged as a period of daily torpor to limit risk exposure and acquired additional physiological functions through exaptation.

1.1.4.2 Restorative theories

A prevailing concept of sleep is that it serves an essential function of restoration and repair from the physiological and metabolic constraints of wake. In support of this theory, protein synthesis is commonly found to be upregulated during sleep, including those involved in growth and repair^[81]. Another facet of the restorative theories of sleep is the depletion of harmful byproducts accumulated during activity^[82].

Metabolic processes - Multiple genes have altered expression between sleep and wake, including a preferential upregulation in transcripts involved in biosynthesis pathways^[83]. This upregulation is thought to account for replenishing molecular and cellular components that were diminished during wake. Besides restoring depleted stores, sleep is evidenced to allow clearance of harmful byproducts of waking metabolic activity. For example, activity correlates with increased reactive oxygen species (ROS) production during aerobic metabolism and the elevated energy demands associated with high neuronal activity^[84]. In the mammalian brain, waste removal is mediated by cerebrospinal fluid circulation with the interstitial fluid. Indeed, spontaneous and induced sleep drives this exchange and glymphatic clearance, implicating sleep in waste removal^[7].

1.1.4.3 Cognitive function

While many of the functions of sleep remain undetermined, it has an unequivocal association with cognitive processes. Firstly, SD results in various detrimental cognitive effects, including defects in decision-making, attention, learning, and memory^[85]. In the last century, a picture has emerged of the role of sleep in learning and memory. In human studies, SD following training protocols impairs behavioural performance and brain activity during retesting^[86]. Furthermore, brain regions that are activated during training tasks have been found to exhibit substantially elevated activity in the following sleep period^[87].

The ability to form dynamic memories allows organisms to respond to changes in the environment and increase the likelihood of success in a task (hunting, mating, social interactions) based on the retrieval of information from past experiences. This memory process relies upon three categories: encoding, consolidation, and retrieval^[88]. The initial formation of a memory trace during encoding is weak and susceptible to removal; during consolidation, the labile memory is strengthened and transformed into stable long-term memories^[88]. Memory consolidation arises due to the ability of our brains to remain plastic; that is, neurons can dynamically alter their structure and activity in response to neural signals, in some cases with long-lasting changes that underpin memory formation^[88]. The largest share of synaptic strength is accumulated during wake, where an organism continually collects and responds to sensory information from the environment to carry out complex behaviour^[89]. A recent area of thinking is that sleep downscales weaker synapses and strengthens stronger ones to maintain substantial memories and remove the likely unimportant connections^[90].

1.1.5 Evolution of REM and NREM sleep

While sleep or sleep-like states have been found across phylogeny in organisms with a nervous system; it is still unclear whether they have typical sleep stages driven by shared mechanisms^[16].

The activity patterns derived from the EEG are mainly generated from synaptic connections within the cortex^{[91][92]}. The neocortex origin of this activity is likely the cause of the changes seen in the NREM and REM EEG activity profile in reptiles^[93] due to their primitive pallium homologues^[40]. More recently, neuronal signatures comparable to REM and NREM sleep have been described in the zebrafish^[55] when investigating the neuronal activity in their neocortex homologue. With the same line of reasoning, the lack of characteristic EEG-derived waveforms in invertebrates may be due to the lack of a neocortex, and their sleep may have distinct stages with mechanistic and functional similarities. For example, while characteristic SWA delta waves are rarely found in invertebrates, changes in electrical activity indeed exist^{[94][95]}. It is essential to understand what is underlying and causing the EEG activity to investigate whether the mechanisms are conserved. NREM high amplitude, low activity EEG patterns in the delta range arise due to synchronicity in local neuronal circuits^[96]. A recent investigation of *D.melanogaster* network-specific neuronal activity identified delta oscillations within a known sleep regulatory and sensory processing circuit^[97]. The existence of NREM sleep characteristics in *D.melanogaster* is supported by the evidence that a gene identified in mice to regulate NREM sleep, *SIK3*, has conservation in *D.melanogaster*, and mutations in the *SIK3* gene leads to sleep defects^{[16][98]}. Suggesting that SWS

may well be an evolutionarily conserved strategy for regulating sleep across neuronal circuits.

For many years, it was thought that REM sleep only occurred in mammals and birds due to the lack of consensus in the studies of non-avian reptiles^[46]. It was thus considered that REM sleep evolved convergently from a common ancestor^{[99][100]}, which likely exhibited a primitive precursor to REM sleep^[101]. However, this has been challenged with the identification of both NREM and REM sleep states in the *Pogona vitticeps* lizard^{[50][51]}. More investigation is needed in other lizards and reptilian species as the same study that identified REM and NREM signatures in the *Pogona vitticeps* lizard found no such pattern in the *Salvator merianae* lizard^[51]. Nevertheless, this evidence suggests that the emergence of REM sleep in the animal kingdom may have occurred much earlier than initially thought. REM sleep has only been described in vertebrates, with the exception of the cephalopod cuttlefish. The cuttlefish were found to have homeostatically regulated periods of quiescence, with evidence of a REM-like sleep stage associated with rapid eye movements and muscle twitching^[102]. The lack of evidence of REM sleep in other invertebrate creatures may suggest that NREM sleep evolved earlier and is a more ancestral form of sleep. However, further research is required as REM sleep is more challenging to investigate in lower invertebrate organisms that do not have ocular movement or easily identifiable muscle twitches. The lack of such behaviours should not dismiss the possibility that lower invertebrates may have a functionally comparable sleep stage. *D.melanogaster* will likely serve as a valuable model for determining the presence of REM-like sleep states by measuring the synchronicity of neuronal populations to identify distinct stages^[16].

1.1.6 Variability of sleep

Across the animal kingdom, there is large variability in sleep duration, even just within land-dwelling mammals. The African elephant sleeps a reported 2 hours^[103], yet the big brown bat sleeps up to 20 hours^[79]. A close relative to this bat species, the fruit bat, requires 15 hours of daily sleep^[104]. One explanation for this variability is that the big brown bat requires a longer period of sleep to fulfil an essential function, and the fruit bat can simply achieve this in a restricted time frame, potentially through sleep of greater depth. This scenario is unlikely due to the lack of evidence that shorter sleeping mammals have elevated levels of the electrophysiological marker of sleep depth, NREM SWS, duration or intensity^[79]. The adaptive inactive hypothesis of sleep provides another reason for this phenomenon. The big brown bat best navigates its environment with echolocation and preys on mosquitoes that are active from dusk until dawn. There-

fore the animal is best suited to expend energy during this time frame when it can most efficiently interact with its environment and carry out essential behaviours. While if it were to be active in the light phase, it would be at higher risk of predation by predatory birds^[79]. However, one should be cautious about such comparisons about sleep duration, with different experimental paradigms and conditions between studies. The variation in sleep across closely related species requires further study within a controlled comparative investigation to elucidate what has driven the observed changes.

Intraspecies sleep variation also occurs and is well-demonstrated within several marine mammals^[105]. Dolphins and orcas exhibit severe postpartum sleep suppression, often forgoing sleep over periods of weeks^[106]. Moreover, fur seals alternate from the land and the water, migrating to the latter during winter months with an extensive reduction in their sleep time when compared to their land sleep patterns^{[107][105]}. Extended sleepless periods are also found in migratory birds. The white-crowned sparrow migrates 4,300 km between Alaska and the west coast of North America during the spring and fall. Captivity studies found that the birds sleep 63% less during the migratory period compared to non-migratory seasons^[44]. These examples, taken together, demonstrate the adaptability of sleep in an organism depending on its behavioural drive and ecological requirements.

Notably, the investigators did not detect any increase in the spectral power of the EEG activity during the migratory bird's sleep periods, suggesting that the sleep loss was not recovered with bouts of greater depth, despite the drastic changes in their sleep^[44]. Indeed, all the above examples of sleep adaptability were devoid of detectable recovery. Reminiscent results were seen in *D.melanogaster*, where SD with a male and female pair led to considerable sleep loss without any recovery rebound. In contrast, pairs of male flies recovered sleep lost from the social male-male interactions. It was postulated that the sexual arousal evoked from the male-female interaction modulated sleep^[108]. Therefore, sleep can be postponed without recovery in scenarios where other more pressing behavioural drives take precedence. However, whether the behavioural drive modulations of sleep are shared across species and the underlying mechanisms responsible remain to be seen.

1.1.7 Conserved neurochemical regulators of sleep

At first glance, there are many seemingly conserved neurochemical regulators of sleep. The central wake-promoting neurochemicals of mammalian arousal include acetylcholine, dopamine, glutamate, norepinephrine, and hypocretin. The main sleep-promoting neurochemicals are adenosine, serotonin, GABA (γ -aminobutyric acid), and melatonin^[109].

Strikingly, these key neuromodulators of mammalian sleep have been found to have similar effects in even distantly related animals like the zebrafish^[110] and fruit fly^[111].

Mammalian neuromodulatory brain regions are primarily conserved in the zebrafish^[112], and there is significant conservation between zebrafish and mammalian neurotransmitter sleep effects^[100]. The most studied is hypocretin, of which neurons project to many of the same arousal cells as within mammals. Functionally, over-expression of hypocretin results in increased locomotion in larval zebrafish, supporting a conserved wake-promoting effect^[54]. However, a 2007 study^[53] found that hypocretin exhibited sleep-promoting action, opening up the direction of hypocretins influence on the sleep-wake cycle to debate.

The invertebrate *D.melanogaster* also shares many of the above neurochemical regulators with comparable sleep results^[100]. A well-described shared neuromodulator is the arousal-promoting impacts of dopamine. Alongside the findings that dopamine feeding causes arousal, interrogation of the neuronal mechanisms identified that dopaminergic (DAN) clusters of neurons project to, and inhibit, sleep-promoting neurons in the fly brain to initiate wake^{[113][114]}. The arthropod norepinephrine homologue, octopamine, similarly shows conservation and has wake-promoting effects in *D.melanogaster*^[115] and further homology is evidenced with GABA^[116]. Whereas acetylcholine^{[117][118]} and glutamate^{[119][120]} are implicated in both sleep and wake circuits.

The recent *Hydra* cnidarian study found that dopamine was, in fact, sleep-promoting in this nerve net organism, suggesting that the dopamine arousal promotion may have occurred with the development of a CNS^[76]. Studies often concentrate on the neuromodulators that are shared with mammalian sleep regulation. While this is a practical approach to understanding the aspects of sleep that are potentially shared in a common ancestor, studying the differences may elucidate the neurochemical and neuronal circuitry alterations that may underpin the variability of sleep across animal species.

1.1.8 Ecological determinants of sleep

While a unified function of sleep across the animal kingdom is unknown, data from many different animals can inform us about the intrinsic and ecological factors that influence sleep, and provide explanation for the inter and intra species variations that are observed. Sleep and wakefulness has been found to have extensive and widespread impacts on gene expression^{[121][122][123]} and is thought that sleep amount and architecture is determined by the interaction between the genetic factors^{[124][125]} and an animals environment. Levels of predation, mating & food availability, and temperature (among other factors) are suggested to impact the manifestations of sleep within a species^[100].

As sleep is a period of immobility where an organism is largely shut off from the external environment, it is logical to consider that sleep poses an increased risk from predation. In many cases, however, it has been found that sleep largely reduces the risk of predation. Animals often seek safe sleeping settlements to avoid interaction with predators during a time when they are less suited to the environment, and therefore would be at higher risk of predation^[79]. The risk of predation greatly varies from phylum to phylum and species to species, and sleep behaviour may well in part be shaped by an animals risk^[18]. A comparative study of the impact of ecological factors on mammalian sleep identified that overall predatory risk was the greatest predictor of REM sleep, and the second of NREM, with animals associated with a higher risk of predation during sleep in the wild found to perform less REM and NREM under laboratory conditions^[126]. Indeed, there is evidence across the animal kingdom of sleep behaviour which can be explained by a motivation for predatory surveillance. For example, wild ducks have elevated levels of unihemispherical sleep when sleeping at the edge of a group, and respond with escape behaviours when presented with threatening visual stimuli in their open eye^[127]. While the ducks in the centre of the group largely slept bihemispherically with both eyes closed. This demonstrates the adaptability of sleep not only to remain vigilant to predation risk but also within social groups.

Mating and food availability both impact sleep behaviour. Sandpiper birds greatly reduce sleep during the mating season, and this drastic reduction in sleep does not impinge on their activity or performance. In fact, sandpipers which had a more severe sleep suppression were found to produce more offspring^[128]. Highlighting that sleep is a trade-off where other behaviours are neglected. Starvation leads to a reduction in sleep in mammals^[129] and flies^[130], in the latter the sleep reduction is related to hyperactivity. The sleep suppression and hyperactivity presumably act to increase foraging and the likelihood of encountering a food source. *D.melanogaster* research has found evidence that the control of feeding and sleep share neuronal pathways^{[131][132]}. Contrastingly, birds, reduce activity in response to food deprivation, likely as a method of energy preservation^[133]. More studies across the animal kingdom are needed to understand the reason for the disparate results, and understanding the mechanistic alterations will shed light on how environmental perturbations modulate sleep.

Temperature acts as a circadian zeitgeber (ZT - timekeeper) that entrains and synchronises circadian rhythms with daily fluctuations in temperature. In mammals, there is a close relationship between thermoregulation and sleep, and many animals sleep preparatory behaviour includes actions that will provide them with a warmer environment - for example, nest building, curling up, and huddling in groups^[134]. Indeed, in mammals, the ambient temperature is an important factor that influences the transition from wake

to sleep, and alterations in brain temperature are associated with the REM and NREM sleep states, with brain cooling found at the transition of wake to NREM. In contrast, transitions to REM or wake correlate with increases in brain temperature^[134].

Ambient temperature also influences sleep in insects, likely via different mechanisms due to their poikilothermic nature, whereby they rely on the environment to maintain a stable temperature. In *D.melanogaster*, the distribution of their daily sleep and wake cycles is modified by the external temperature. At around 25°C the animals sleep preferentially during the nighttime, with an overall shorter siesta sleep period during the day. However, at lower temperatures, they exhibit shorter siesta periods and extended nighttime sleep, while in warmer conditions, the preference for sleep switches to the daytime, with increased nighttime activity. Parisky *et al.*^[135] reported that elevated daytime sleep exhibited at higher temperatures was due to a combination of circadian and homeostatic influences on sleep. Components of the circadian clock were required for initial temperature-induced daytime sleep elevations. However, they were not necessary for increased day sleep following a temperature-induced night period of reduced rest. This key paper demonstrated how external cues, homeostatic regulation, and circadian sleep control interact to determine sleep behaviour.

While there is evidence of the ecological factors that have shaped sleep across the animal kingdom in individual species, a comparative approach across species and ecology in a single investigation will help understand the alterations in the sleep and arousal circuits that have driven the observed changes.

From mammals with complex nervous systems with an extraordinary number of neurons to elementary cnidarians with a nervous system consisting of a simple nerve net, sleep or sleep-like states appear to persist. In many examples from the animal kingdom, there is likely striking homology in the neural circuits, highlighted by the conserved action of neurochemicals. At the same time, sleep manifests in varying ways (i.e., duration, adaptability & stages), likely depending on complex interactions of ecological, environmental, and intrinsic drives. Investigation of such complexities in higher-order vertebrates is often challenging, with the enormous number of intricate synaptic connections (in humans, a single neuron is thought to connect to tens of thousands of other neurons). The study of sleep in simpler invertebrate models presents the opportunity to interrogate fundamental aspects of sleep in less extensive neural circuits that are likely shared across animal life.

1.2 *D.melanogaster* as a model

1.2.1 A powerful model

1.2.1.1 History of *D.melanogaster* in research

D.melanogaster, commonly called the fruit or vinegar fly, is one of the most advanced and valuable models for studying biological processes. While the first laboratory use of *D.melanogaster* occurred in 1901 under the lead of William Castle^[136], the origin of *D.melanogaster* research is credited mainly to Thomas Hunt Morgan. His team pioneered the use of *D.melanogaster* within research after a groundbreaking discovery, revealing unknown concepts about genetic inheritance^[137]. While housing a population of fruit flies for a year, Morgan identified a white-eyed male fly in a population of the wild-type brilliant red-eyed flies. Subsequent breeding schemes carried out by Morgan identified that the inheritance of the white-eyed trait closely resembled that of the postulated sex determination by a sex factor or what we now refer to as the X chromosome. Additional investigation by crossing this white-eyed mutant male with red-eyed female flies resulted in an all-red-eyed progeny. Further crosses with the F1 progeny produced male and female red-eyed flies and only male white-eyed flies. Examination of the inheritance of the trait with a cross of the red-eyed females to white-eyed males found an equal ratio of red to white eyes in both sexes. Providing the first definitive evidence of the inheritance of traits within chromosomes and fine-tuning the framework provided by Gregor Mendel of Mendelian genetics^[138]. This seminal work and what followed led to the award for the Nobel Prize in Physiology or Medicine in 1933. Fast forward to today, and the widespread use of the *D.melanogaster* model has furthered our understanding of genetics and inheritance, development^[139], immunity^[140], learning^[141], circadian rhythms^[142], and ageing^[143], serving as one of the most effective tools for dissection of the genetic and molecular underpinnings of biological processes.

The flies' relatively short lifespan and low associated maintenance costs make them very appealing models, with the ability to rapidly generate large numbers of genetically identical individuals under controlled rearing conditions. However, their advantages go much further than these practical benefits. The fruit fly is a relatively simple organism with approx 12-15K genes compared to the estimated human 20-25K. Its genome was fully sequenced in the year 2000^[144] and has since been found to have sequence homology with 60-70% of human disease genes^[145]. The power of *D.melanogaster* as a model organism primarily comes from their genetic tractability and elegant genetic tools developed specifically in *melanogaster*, that allow for the spatial and temporal regulation of gene expression to study gene function. Alongside this, there are numerous

databases available for use, including FlyBase^[146] with genetic and genomic information on Drosophilidae based on large-scale genomic studies and primary research, and stock centers (Bloomington *Drosophila* Stock Center -BDSC - <https://bdsc.indiana.edu/> & Vienna *Drosophila* Research Center VDRC - <https://stockcenter.vdrc.at>) for ordering an array of fly stocks for study.

1.2.1.2 Elegant genetic toolbox

Over the last 100 years of research, sophisticated genetic tools have been slowly built upon, leading to the powerful model we have today for interrogating biological processes' genetic, cellular, and molecular underpinnings. Both chemical and ionising mutagens were identified in *D.melanogaster* with ethyl methanesulfonate (EMS) feeding^[147] and X-rays^[148] leading to mutagenesis. Forward genetic screens carried out on mutant specimens to identify corresponding phenotypes led to the functional annotation of many genes. For example, in 1976, Dudai and colleagues tested 500 potential X-chromosome mutant lines after mutagenesis with EMS for learning impairments with an aversive olfactory paradigm^[149]. From this approach, the investigators identified a learning and memory mutant, which they coined *dunce*, and which has since been characterised as a phosphodiesterase involved in cyclic adenosine monophosphate (cAMP) regulation^[150].

Later, the identification and development of P-element transposition, based on an endogenous transposable P-element in the fly genome^[151], was invaluable in developing gene disruption and novel transgenic technologies^[152]. The transposable P-element encoded a transposase enzyme that binds to the P-element's inverted terminal repeats, facilitating its movement to a new genomic location. Researchers developed a system based on this P-element mechanism whereby the transposase gene is replaced with an exogenous gene of interest (GOI) for integration into the fly genome. Injection of the engineered P-element with the GOI alongside an independent source of the necessary transposase would allow for the insertion of the P-element into the developing germline^[153]. The GOI is incorporated alongside a dominant marker for confirmation of the integration of the P-element into the genome. Many tools have since been developed based on this transformation method, including enhancer traps to track the spatiotemporal expression of genes throughout development^{[154][155]}.

The use of P-element insertion led to the design of one of the most important tools for spatially restricted gene expression in a *D.melanogaster* geneticists toolbox: the binary GAL4-UAS expression system (Fig 1.2). GAL4 is a transcriptional activator protein that was identified in the yeast genome^[156]. In 1998, Brand and Perrimon took

advantage of this yeast endogenous transcriptional activator, transforming the GAL4 into the fly genome either downstream of a defined promoter or under the control of a tissue-specific native enhancer^[157]. An upstream activation sequence (UAS) that the GAL4 binds was integrated into another fly genome upstream to a reporter gene. With this binary design, crossing a parent with a GAL4 construct to one with the UAS transgene results in progeny that contains both the GAL4 under the control of a tissue-specific promoter/enhancer and the UAS – reporter gene sequence. Thus, promoter/enhancer activity will lead to transcription of the GAL4, which will bind to the UAS resulting in the reporter gene transcription in a tissue-specific manner. Since its advent, tissue-specific GAL4s have been generated for almost every tissue, with the resolution of small subsets of cells and thousands of lines readily available in stock centres. This has permitted unprecedented specificity in genetic, molecular, and cellular mechanism investigations. One major disadvantage to P-element transformation is that there is no control over the location of the integrated vector into the genome^[158]. Thus a new transformation system has become the preferred method, whereby a serine recombinase from the PhiC31 bacteriophage is utilised for its specific recombination of the *attB* and *attP* binding sites^[158]. Generation of transgenic flies with designated *attP* binding sites allows for the transformation of vectors with the corresponding *attB* binding sites for specific locational integration of transgenes into the host genome.

Common UAS lines that are used include exogenous fluorescent proteins (for example, green fluorescent protein - GFP) to track the spatiotemporal expression of the GAL4, overexpression of genes to elucidate function, and the rescue of genes from a mutant background. The development of RNA interference (RNAi) systems incorporated into the UAS has allowed for the discrete dissection of the impact of a knockdown (KD) of a target gene. RNAi technology takes advantage of an endogenous mechanism for controlling protein expression. In 1998, a pivotal study in *C.elegans* identified that double-stranded RNA (dsRNA) caused sequence-specific inhibition of protein expression^[159]. Over the past decades, *D.melanogaster* transgenic genome-scale libraries have been generated, where the sequence-specific dsRNA is combined with a UAS, allowing for tissue-specific KD of target genes for the study of their function^[160]. RNAi is an advantageous method for gene KD because:

1. It is fast and relatively straightforward
2. It allows for not only sequence-specific but tissue-specific KD of target genes, mediated by a GAL4
3. Generation of genome-scale libraries allows for large-scale screening of lines generated in the same host strain, limiting non-specific phenotypes due to genetic background

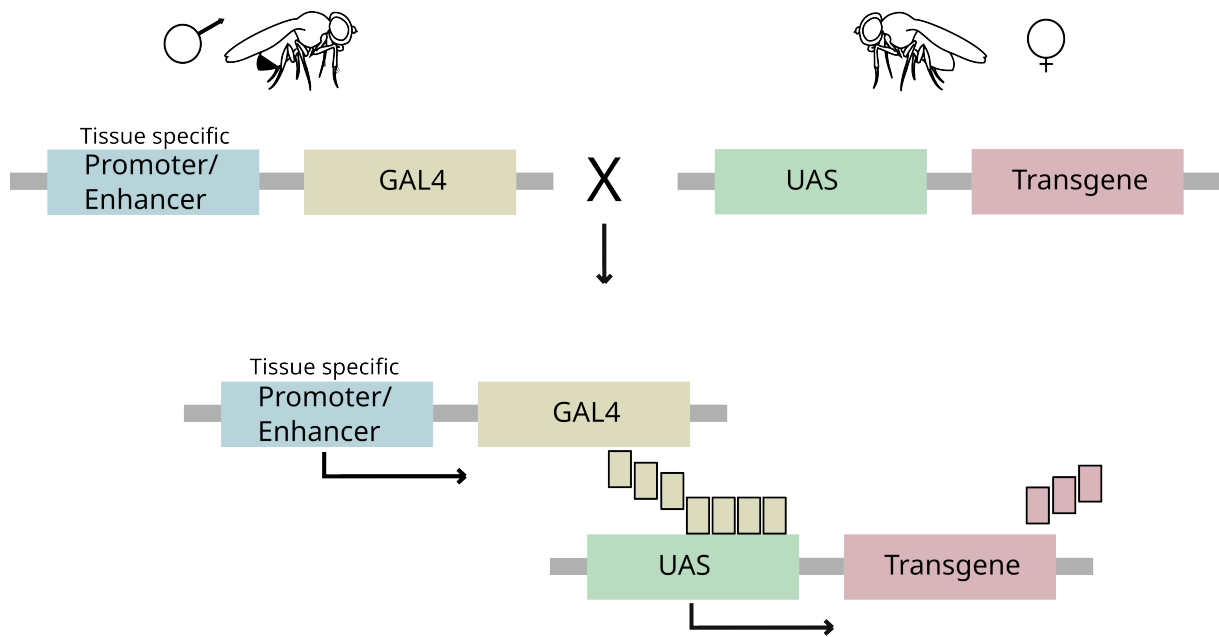


Figure 1.2: The GAL4-UAS system

The GAL4 is a yeast transcriptional activator that is expressed in a tissue-specific manner under the control of a native enhancer or promoter. When expressed, the GAL4 binds to the UAS resulting in the transcription of a downstream transgene. This binary expression system allows for the spatiotemporal control of gene expression.

alterations.

Further temporal controls have been integrated into the system with the development of the TARGET^[161], GeneSwitch^[162] and AGES^[160] approaches. The TARGET system implements a yeast-derived GAL4 repressor, the GAL80 protein^[163]. A temperature-sensitive variant of the GAL80 protein was cloned into the fly genome under the control of a ubiquitous tubulin promoter, repressing GAL4 activity at lower temperatures and allowing expression at temperatures >30C. While this has added significant value to the research area, allowing for the spatiotemporal investigation of transgenes, the higher temperatures necessary for the activation of the GAL4 can have independent impacts on the organism, as *D.melanogaster* are ectotherms and heavily rely on the ambient temperature. Implementing drug-inducible systems produces spatiotemporal control that can be achieved without the need for temperature changes. A hormonal inducible GAL4 system, commonly referred to as GeneSwitch, utilises the RU486-dependent GAL4 progesterone-receptor fusion protein. Only in the presence of the hormone anti-progesterin (RU486) does the RU486-dependent GAL4 bind to the UAS and activate the downstream targets. However, this system is incompatible with previous GAL4 lines, and the drug has been found to cause independent phenotypes^[164]. A recently developed non-toxic drug inducible system uses the auxin-degron system identified in

plants, engineered to a GAL80. The Gal80 suppresses GAL4 activity in the absence of the auxin drug, and thus auxin feeding leads to GAL4 expression.

Over the last century of *D.melanogaster* research (alongside the leaps made in the intricate knockout, KD, and over-expression of transgenes), transgenic constructs have been developed that aid in their genetic tractability and the setting up of genetic crosses. In order to carry out controlled genetic experiments, it is essential to have stable inbred stocks in which transgenic manipulations are easily identified. Many visible markers are employed for the X (*white/ yellow*), 2nd (*Curly*), and 3rd (*stubbly*) chromosomes, which allow researchers to track their desired construct in a genetic cross^[165]. Since the progeny will inherit one allele from each parent, the presence or absence of the visible marker will inform the inheritance of the desired construct. In addition to this, the development of balancer chromosomes which have multiple inversions and thus inhibit viable offspring that have inherited recombinant alleles, prevents unwanted recombination^[166]. One obstacle to stable mutant stocks is that mutations are often homozygous lethal and, thus, over time, are lost from a population. Balance chromosomes address this as they are recessive lethal; therefore, the only viable progeny are the heterozygous mutant and balancer chromosome flies.

1.2.1.3 *Drosophila* neurobiology

D.melanogaster is an attractive model for the study of neurobiology – with a relatively simple CNS made up of a brain and ventral nerve chord. They have around 250,000 neurons compared to 86 billion in humans. Despite their small size, *D.melanogaster* orchestrate an array of complex behaviours, including courtship^[167] and aggression^[168], that share homology with mammals. In executing behaviour, animals must integrate sensory cues through neuronal circuits to determine output. Courtship in *D.melanogaster* is an intricate and robust sequence carried out in male flies that even occurs in flies raised in complete isolation. The courtship behaviours in *D.melanogaster* include the initial orientation, followed by tapping of the prospective female mate, wing vibration and production of the courtship song, licking of the female genitalia, and attempted copulation by thrusting the male genitalia toward the female. Many of these behavioural series will be carried out before female receptivity and successful copulation^[167]. This complex series of actions relies on several genes^[169] and the integration of auditory, olfactory, gustatory, and visual sensory systems^[170]. Aggression is well described in males with motor execution, including charging, wing threat, tussling, fencing, and lunging^[168]. They have been found to establish hierarchies based on the fights winner and loser status, which impacts future aggressive behaviour^[171]. In recent years females have also been shown to have robust aggressive actions. Some are shared with males

(i.e. charging & fencing), while they also carry out sex-specific behaviours (headbutting & shoving)^{[172][173]}. Several genes, hormones, neurotransmitters, neuropeptides, and pheromones influence aggression^[174]. The complex behaviours exhibited by fruit flies make them a suitable model for the dissection of the circuits that control behaviour.

The combination of genetic screens and refined behavioural assays has aided in identifying genes involved in behavioural outputs. For example, the core components of the circadian clock were established with the testing of mutants for disruption of rhythms in eclosion and locomotion^[175]. As discussed earlier, utilisation of the GAL4-UAS system led to the development of refined GAL4s, and lines have been generated that induce gene expression either pan-neuronally (nSyb-gal5, elav-gal4), within glia cells (repo-GAL4) and in subsets of *D.melanogaster* neurons. A recent project at the Janelia Research Campus generated a collection of GAL4 lines in which a short fragment of DNA controls the expression of GAL4, allowing for GAL4 expression in much smaller subsets of neurons integrated into the genome within a known *attP* using the PhiC31 system^[176]. This collection covers almost all of the neurons in the fly brain. The array of GAL4 lines are publicly available at the BDSC, alongside confocal images of the expression of each line which can be found at the FlyLight project (<https://flyweb.janelia.org/cgi-bin/flew.cgi>)^[176]. Such tools allow for the detailed interrogation of the spatial role of genes and neuronal populations in behaviour. As well as interrogating genes, transgene systems have been developed which allow for manipulating neuronal circuits via silencing and activating neurons.

Silencing Silencing of neuronal cells can be achieved by blocking either the synaptic transmission or excitability of the neuron membrane. This is often accomplished by incorporating a target transgene that impacts transmission or excitability under the control of a UAS sequence, allowing for targeted manipulation of global or local neurons^[177]. Synaptic transmission can be inhibited by expressing a neurotoxin that blocks neurotransmitter release, the tetanus toxin (TNT), thus impeding neuronal signalling^[178]. TNT-mediated silencing is irreversible, and the preferred method is the expression of a reversible temperature-sensitive dynamin orthologue, *Shibire*. *Shibire* is involved in synaptic vesicle endocytosis, responsible for the cleaving of synaptic vesicles for the membrane and, therefore, essential for synaptic transmission^[179]. The temperature-sensitive protein functions normally at the permissive temperatures below 20°C but undergoes conformational changes at the restrictive temperatures above 30°C, resulting in a dysfunctional protein and impeding vesicle release, causing a synaptic blockade^[177]. The ability to control neuronal signalling in a reversible temporal manner has allowed the study of specific neurons in a circuit at different stages of a process or behaviour. For example, *shibire*^{TS}-driven silencing of the *D.melanogaster* learning

and memory centre, the mushroom body (MB), at different stages of memory processing identified a specific MB circuit involved in olfactory memory retrieval but not in formation or consolidation^[180]. Blocking excitability of the neuronal membrane can be achieved by manipulating ion channels that determine the excitability of a neuron—for example, the inwardly rectifying potassium channel, Kir2.1^[181]. Overexpression causes membrane hyperpolarization and therefore inhibits action potential firing^[182].

Activating The manipulation of ion channels can also produce the activation of neurons. Overexpression of the bacteria-derived sodium channel, NaChBac, causes membrane depolarization and increased excitability^[183]. A standard neuronal activation tool is the expression of another temperature-sensitive gene, encoding the transient receptor potential cation channel, *TrpA1*, which depolarizes membranes at temperatures $> 27^{\circ}\text{C}$ ^[184]. Using this tool, Chen *et al.*^[185] characterised the role of Allatostatin A (AstaA) expressing neurons in feeding and sleep with *TrpA1* activation. Another way of examining neuronal function is to activate neurons temporally with light-sensitive channelrhodopsins channels, for example, Channelrhodopsin-2 (blue light) or Chrimson (red light). This light-induced neuronal activity is termed optogenetics^[185].

Downstream targets of subsets of neurons can be probed due to the development of other binary expression systems, which work independently of the GAL4/UAS method. The LexA/LexAop system works comparably to the GAL4/UAS system; LexA is a bacterial-derived transcriptional activator that can be expressed in a tissue-specific manner. Upon expression of LexA, it interacts with the LexAop promoter and drives the expression of a downstream transgene^[186]. A combination of non-overlapping binary expression systems enables researchers to investigate neuronal connections within a circuit. For example, one could transiently activate a population of neurons by driving the temperature-sensitive *TrpA1* under the control of a GAL4 and measure its impact on a hypothesised downstream region with a Ca^{2+} indicator under the control of the LexA/LexAop system. An essential aspect of neurobiology is understanding the functional connectivity between neurons that underlie the neuronal circuitry of behaviour. Connectivity can be elucidated by investigating the co-localisation of neurons with layered imaging or using split fluorescent tagging like the GRASP system^[187]. The GRASP system labels synapses fluorescently based on the proximity of the pre and postsynaptic membranes. This is achieved by expressing two complementary, non-fluorescent GFP fragments in individual neurons that are reconstituted in close range, leading to fluorescence and elucidating synaptic contact. However, while these methods illuminate locational proximity, this does not irrefutably deduce functional connectivity. A recent development in *D.melanogaster* neurobiology allows for mapping pre and postsynaptic partners, coined *Trans-tango*. Based on the Tango system^[188], the *Trans-tango*

method employs a ligand in the presynaptic membrane that binds to its receptor in the postsynaptic membrane, causing the cleavage of a transcription factor that will go on to express a target gene, labelling the postsynaptic neurons and elucidating their functional connectivity^[189].

Investigation of the discrete neuronal activity in *D.melanogaster* has been made possible with recent developments in the tools and techniques for electrophysiology and imaging. Extracellular recordings and whole-cell patch clamp recordings facilitate the study of neural activity with the resolution of single neurons^[190]. Genetically encoded reporters are routinely used to measure neuronal activity in combination with multi-photon imaging, including indicators of Ca^{2+} (action potential), voltage (membrane potential), or synaptic transmission (reviewed in^[191]). Presently, multiple methods for measuring the activity of neurons can be performed in an intact fly, allowing for direct interrogation of neural signals and the corresponding behaviour. For example, a method growing in prevalence employs an air-supported ball that the fly can simulate walking behaviour upon, while the head is fixed for two-photon imaging of a genetically encoded activity sensor^[192].

1.2.2 Sleep in *D.melanogaster*

Considering *D.melanogaster* is such an attractive model for the study of neurobiology and behaviour, it is surprising how its use as a model for sleep only came about at the turn of the millennia. In 2000, two independent studies found that *D.melanogaster* shared all of the behavioural hallmarks of sleep and even showed key molecular and genetic correlates of mammalian rest^{[63][64]}. Since these initial papers, *D.melanogaster* has served as an invaluable model for studying the genes and molecular and neural mechanisms of sleep-wake cycles.

1.2.2.1 Measuring sleep

In the initial studies, sleep states were determined by visual observation^[63], ultrasound activity monitoring systems^[64], and infrared activity monitoring systems^{[63][64]}. Sleep is still primarily measured in *D.melanogaster* using the infrared beam activity monitors, the *Drosophila* Activity Monitor (DAM) systems (TriKinetics, Waltham, MA) that were previously widely used to assess the flies' circadian activity. While the DAM system is very effective for studying circadian cycles, it has its limitations in the study of sleep. The DAM devices track activity with the presence of an infrared beam through the midline of the experimental tubes which house individual flies; therefore, fly activity

can be determined by the number of infrared beam breaks. Early models of such systems relied on just one infrared beam. Thus, sleep levels were greatly overestimated as the individual could still execute waking behaviours at either end of the experimental tube without breaking the midline beam^{[193][194]}. In recent years, the system has been updated with models having up to 17 beams for more accurate measurement of the flies' sleep, but between 1 and 4-beam systems are still the most popular tool for sleep measurement.

In recent years, many groups have developed systems that use automated video tracking software^{[193][195][196][197][194]} for behaviour measurement. Video tracking is advantageous due to the high spatial resolution with the ability to track fly behaviour at any point in the tube, even discriminating smaller movements and classifying the waking behaviour^[194]. In 2017, our lab published the manuscript^[194] and accompanying tutorial documentation^[198] for implementing our automated video tracking device, the ethoscope (Materials and methods 2.2.1), which has since been adopted in other sleep investigations^{[108][65][68][199]}.

Sleep deprivation

A substantial aspect of the study of sleep is understanding what happens when sleep is removed to investigate its homeostatic regulation and the consequences of sleep loss. In flies, a period of rebound sleep is found after various methods of enforced waking. There are multiple different SD methods that have been used for the study of *D.melanogaster* sleep. These include mechanical^{[200][65]}, social^[108], starvation-induced^[201], pharmacological, and thermo/optogenetic activation of wake-promoting neurons^{[202][203]}.

Mechanical sleep deprivation The most popular approach is mechanical SD, and initial sleep studies confirmed the homeostatic aspects of sleep using both manual tapping and inversion alongside automatic rotation^{[63][64]}. To date, most approaches use either use a timer-controlled orbital shaker^[200] which provides mechanical stimuli at random intervals through the SD period to disrupt sleep, a rotational device^[204], or a vibrational platform^[197]. All of the above methods are unspecific, providing all flies a stimulus regardless of whether the individual flies are asleep or awake, leading to variability in the amount of sleep loss between individuals. Moreover, the methods likely lead to habituation and the shaking stimuli often cause injury to the flies with damage to their wings and legs^[203]. Therefore, the above mechanical methods have variable success rates in keeping individuals awake, and there are likely sleep-independent artifacts of the response to SD due to stress and injury. A significant advantage to the ethoscope system is that it is modular and can be adapted for use with accessory systems, including an optomotor device that provides a specific, responsive mechanical stimulus to individual flies based on their inactivity duration^[65] (Materials and methods 2.4.1).

Social sleep deprivation A more ecologically relevant waking stimulus is provided by introducing another male intruder into the experimental tube housing a focal male. The interactions of the flies over a 12 or 24 hr period lead to a reduction in sleep^[205], increased activity in sleep centres of the fly brain^[108], and subsequent rebound. This method is favourable for investigating the ecological and social influence on sleep. However, there are a plethora of physiological changes that are likely influencing fly behaviour independent of sleep loss.

Starvation-induced sleep deprivation In many animals across the animal kingdom, starvation leads to sleep loss, and the homeostatic control of feeding and sleep involves overlapping neural circuitry^{[206][207]}. In *D.melanogaster*, starvation causes strong sleep suppression, likely in order to increase activity and forage for food^[208]. However, there are conflicting results on whether starvation-induced sleep suppression results in a recovery period of rebound sleep. Thimgan *et al.*^[201] observed that while starvation caused significant reductions in total sleep amount, the consequences associated with SD were absent. No homeostatic rebound or learning impairments occurred post-starvation-induced sleep loss. The authors identified that starvation response might depend on lipid metabolism, with mutants with increased triglycerides, *brummer*, increasing sleep after SD. In contrast, mutants with reduced triglycerides, *Lsd2*, do not compensate for their lost sleep with rebound, mimicking the result with starvation. A paper published in the same year reported that rebound after starvation did, in fact, evoke an increase in sleep upon cessation of the starvation period^[207]. However, the data is not shown. A 2020 study corroborated the finding that starvation-induced sleep loss is without a compensatory rebound period and proposed that sleep during starvation is composed of bouts of greater depth. In other words, while there is an overall reduction in sleep, the sleep loss is recovered with bouts of increased sleep intensity, and therefore, the starved flies do not require a compensatory rebound. *Dilp2* was required for increased nighttime sleep depth and resilience to starvation sleep loss^[209].

Pharmacologically-induced sleep deprivation Caffeine is a prominent wake-promoting compound known in mammals to action its arousal via adenosine receptors. In *D.melanogaster*, caffeine elicits equivalent wake effects, with reduced nighttime sleep, but the identified adenosine receptor is not involved with the arousal response^[210]. Nall *et al.*^[211] established that a cluster of *D.melanogaster* DAN neurons was required for nighttime sleep reduction, implicating dopamine signalling as the mechanism of caffeine-induced arousal. When fed caffeine for 24 hours, removal from the drug food produced a delayed rebound period of sleep^[209]. Dopamine is implicated in arousal throughout the animal kingdom, and drugs that manipulate dopamine signalling modulate sleep in a predictable nature. That is to say that drugs that increase dopamine levels,

such as feeding the dopamine precursor, l-3,4-dihydroxyphenylalanine (L-dopa), reduce sleep^[212]. While administration of 3-iodo-tyrosine (3IY), an inhibitor of tyrosine hydroxylase - a key enzyme involved in the dopamine biosynthesis pathway, increases sleep^[213]. Feeding with modafinil also promotes wake in *D.melanogaster*, followed by an attenuated recovery period, compared to mechanically perturbed sleep^[214]. While the exact mechanism is unknown, modafinil is found to increase extracellular dopamine levels in rodents^[215]. However, the impact of the drug on dopamine transport in *D.melanogaster* is unknown. Multiple drugs that influence *D.melanogaster* sleep involve alterations to dopamine signalling, but other monoamines drug-induced manipulation also produce wake. Indeed, feeding with octopamine results in sleep suppression^[216].

Nevertheless, pharmacologically induced SD will have widespread sleep-independent effects on an organism and is rarely utilised for specific investigation of sleep loss. However, it can provide broad insight into the potential molecular mechanisms underscoring sleep.

Transient activation of wake-promoting neurons In recent years, with the identification of neuronal circuits involved in sleep-wake cycles and the development of sophisticated genetic tools for the manipulation of neuronal cells, methods of SD have been employed that transiently activate wake-promoting centres in the fly brain^{[202][203][217][218]}.

1.2.2.2 Unitary vs distinct sleep stages

Investigations of *D.melanogaster* arousal thresholds during rest identified that the response to a low-level stimulus decreased during the first 5 min of the bout, and sleep bouts >5 min in length corresponded to the highest arousal thresholds and therefore qualifying as sleep^{[67][64]}. For this reason, 5 min of inactivity is used to investigate sleep throughout the field. Sleep is quantified mainly as a unitary state in *D.melanogaster*; however, it has distinct stages in mammals^{[219][30]} and other animals^[38], usually distinguished by the EEG activity. Heightened arousal thresholds are often found to correspond to the EEG-defined sleep states. Thus, researchers aimed to see if there were changes in the arousal thresholds and altered neuronal activity to investigate the existence of distinct sleep stages in *D.melanogaster*.

A 2002^[66] study demonstrated that *D.melanogaster* brain activity could be measured using LFP recordings, and this study and many others have found that wake is associated with spike-activity that is reduced during sleep^{[220][94][221]}. Further studies revealed that the sleep LFP activity was significantly different between the day and the night, with a more considerable reduction in LFP recordings associated with night sleep^[94]. This differentiation in results corresponds to the flies' arousal thresholds, with a lower

response rate during night sleep. Probing the intensity of sleep with a vibrational stimulus throughout single sleep bouts identified varying arousal levels, suggesting that *D.melanogaster* sleep may indeed cycle through various stages^[94]. Oscillatory LFP activity in the 7-10Hz range was found in a subset of *D.melanogaster* sleep-promoting neurons, the dorsal layer of the fan-shaped body (dFB), confined to the early sleep stages after initiation and during the later sleep stages before awakening^[221]. The differential oscillatory results during a sleep bout suggest that *D.melanogaster* sleep may contain a transitional stage. This reporting suggests that there are indeed neuronal changes associated with sleep, with some evidence of distinct stages. However, LFP recordings did not reveal the electrophysiological correlates of the distinct stages associated with vertebrate sleep (SWA that corresponds to NREM SWS and activity resembling wake of paradoxical REM).

The lack of such distinct stages with LFP recordings may be due to the limitations of such recordings in the vertebrate brain rather than the absence of functionally conserved sleep stages. Studies utilising the technological advancements of *D.melanogaster* neurobiology have used genetically encoded indicators of neuronal activity to investigate local^{[97][222]} and global^[222] network activity during spontaneous and induced sleep. Such methods allow for detailed analysis of the activity of subsets of neurons across the brain. In a 2019^[97] study, network-specific delta oscillations were found within a known *D.melanogaster* sleep centre, the R5 neurons of the ellipsoid body. The researchers identified compound delta oscillations that rise in power throughout the day, falling in subsequent sleep. While disrupting the emergence of the described SWA resulted in altered sleep patterns. The delta activity in this local circuit provides evidence of a potential invertebrate homologue of the SWA observed in higher organisms. Tainton-Heap *et al.*^[222] used a pan-neuronal Ca^{2+} indicator and two-photon live imaging to assess the activity of different neuronal cells during sleep. The global neuronal activity during segments of a sleep bout varied, with an initial period resembling the wake activity, after which the activity decreased, often reaching its lowest levels > 10 min of sleep. Optogenetic activation of the dFB induced sleep, but surprisingly the neuronal signal was comparable to wake, apart from the lack of response in neurons to visual stimulation. The dFB-induced sleep may represent a form of REM sleep in *D.melanogaster*, where brain activity is comparable to waking while disconnected from the external environment. However, artificial activation of neurons is unlikely to resemble their natural physiological activity, and continued stimulation may impact other downstream neurons^[223]. Thus the finding that activation of the dFB causes a sleep state where neuronal activity resembles the elevated signal associated with wake would need further investigation. In order to confirm that it is an intrinsic characteristic of the neurons

during sleep rather than an independent consequence of the artificial stimulation.

1.2.2.3 Circadian and homeostatic control of sleep

Circadian rhythms

Our behaviour and physiology occur cyclically with the turning of our planet and the passing of day and night as organisms have adapted to life on Earth. Circadian rhythms have become entrained to environmental cues, called zeitgebers (ZT), including light^[224], temperature^[225], feeding times^[226], and social interactions^[227]. However, endogenous free-running rhythms persist even without such cues with a period of around 24 hr^[228]. The genetic and molecular underpinnings of the molecular clock have largely been established through the use of *D.melanogaster* as a model. Early studies of eclosion, and later locomotion, timing found the central genes underlying the cellular clock – *period* (*per*)^[229], *timeless* (*tim*)^[230], *Clock* (*Clk*)^[231], *cycle* (*cyc*)^[232] as well as other vital contributors to the clock (reviewed in^[228]).

An essential finding of the underlying mechanisms of the circadian control of behaviour and physiology came from the identification of cyclic levels of the PER and TIM proteins, where increasing protein levels were associated with declining mRNA levels^[233], and so the hypothesis of a negative regulatory feedback loop was born^[228]. The mRNA levels of both proteins rise during the day until peaking in the early evening. Subsequently, protein levels rise in the cytoplasm until the TIM protein facilitates the transport of the PER protein into the nucleus, and levels of the two proteins peak in the nucleus around midnight^[234]. In the nucleus, the *mRNA* levels of both *per* and *tim* decline due to the inhibitory action of the proteins on the *per* and *tim* transcriptional activators: *Clk* and *cyc*, whose protein products form a CLK-CYC complex. A second loop was later identified whereby the CLK-CYC complex rhythmically controls the expression of the *Clk* transcriptional activators and suppressors. Post-translational mechanisms control the timing of the negative feedback loops, mainly in the form of phosphorylation^[228].

Approximately 150 neurons containing the clock components are dispersed throughout the fly brain- broadly split into lateral and dorsal subsets. The lateral neurons include the small and large ventrolateral neurons (sLNvs & lLNvs, respectively), dorsal lateral neurons (LNDs), and lateral posterior neurons (LPNs). There are three subsets of dorsal neurons, DN1, DN2, and DN3^[223]. The ventrolateral neurons, excluding the 5th sLNv, express the neuropeptide pigment-dispersing factor (PDF). At the same time, most clock cells contain the PDF receptor, and evidence suggests that PDF signalling

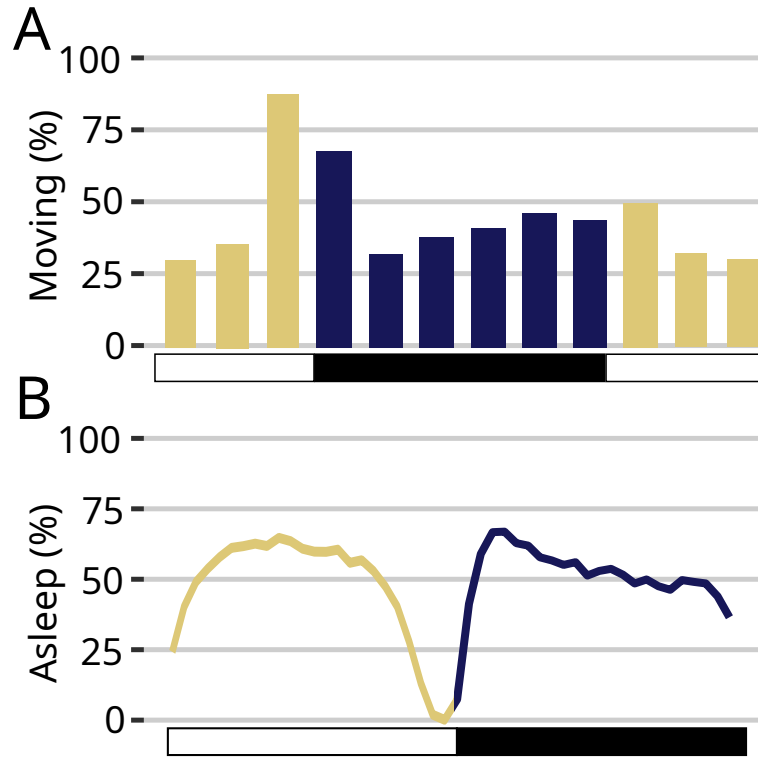


Figure 1.3: Circadian anticipatory activity peaks and sleep timing
(A) Representation of *D.melanogaster* daily activity in 2-hour bins with the evening and morning anticipatory peaks **(B)** Sleep profile of male *D.melanogaster* over the 24hr day with the midday siesta and nighttime sleep periods. *D.melanogaster* are diurnal animals that preferentially sleep more during the night than the day.

synchronises the dispersed clock cells to control behavioural rhythmicity^{[228][235]}. These circadian cells are distinct, with different neuromodulators, receptors, and functions^[236]. The various signalling properties of the relatively small number of circadian cells likely transmit output signals to neuronal populations that lack intrinsic clocks to tightly control behavioural rhythms. For example, DN1 neurons act downstream to the sLNvs and project to non-clock cells in the pars intercerebralis (PI), required for the maintenance of activity rhythms in constant darkness, therefore providing evidence of the DN1's role in mediating the output of circadian rhythms to other circuits^{[237][228]}.

Circadian control of sleep

Sleep timing is considered an output of the circadian clock, restricting the timing of sleep and wake during the environmentally advantageous period. For example, sleep primarily occurs during the night in diurnal animals and the day in nocturnal species. *D.melanogaster* sleep shows a robust diurnal circadian pattern in 12:12 hr light:dark cycles, with strong activity peaks at the onset of dawn and dusk, with activity starting to increase in anticipation of the light transitions. These activity peaks bookmark the day

'siesta' and nighttime sleep periods, with the night containing longer consolidated periods with higher arousal thresholds than the day^[94] (Fig 1.3). Mutants in the circadian clock components show sleep arrhythmicity in constant dark conditions^[204]. However, with the light cue incorporated in the usual LD condition, the mutants maintain some level of a daily rhythm, albeit with altered sleep phenotypes. For example, in LD conditions, many clock mutants display daytime sleep episodes, but the amount of sleep is less than what is reported in the wild-type^[238]. In the case of flies lacking *per* or *tim*, the daytime sleep is later followed by a nighttime sleep period. However, night sleep is abolished in *Clk* or *cyc* mutants^{[238][207]}. The maintenance of sleep-wake rhythmicity in clock component mutants during LD conditions highlights environmental cues' impact on sleep, even in some cases independent of the circadian clock.

Of the 150 circadian neurons, many have been found to have sleep and wake-promoting roles. The sLN_{vs} and lLN_{ds} are considered wake-promoting^[239], while the DN3 neurons have been found to be sleep-promoting^[240]. Evidence suggests that the DN1 neurons may have both sleep^[241] and wake-promoting effects^[242]. The wake-promoting lLN_{vs} fire throughout the day, with a reduction in firing frequency at the transition from light to dark. CLK-CYC mediated transcription of the WIDE AWAKE protein was found to up-regulate the expression of the GABA_A receptor, *Resistant to dieldrin* (RDL), leading to increased inhibitory GABA signalling in the lLN_{vs}, reducing their activity and subsequently leading to the initiation of sleep^[243].

Evidence implicates the DN1s in the transition from sleep to wake at the end of the night via expression of the *Diuretic Hormone 31* (*DH31*) and PDF receptor activity. Specifically knocking down *DH31* in the DN1 neurons leads to increased sleep at the end of the night, while the rescue of *DH31* reduces sleep, as does PDF expression^[244]. However, the DN1 neurons have also been found to have sleep-promoting effects via inhibitory action on neurons involved in the morning (sLN_{vs}) and evening (5th sLN_v & LN_{ds}) anticipatory activity peaks^[245].

A recent study has found that a subset of DN3 neurons acts downstream to DN1 neurons to influence newly identified 'claw' cells. The cells show daily rhythmic changes in activity that correlate with the DN1 and DN3 cycles. The claw neurons were then found to feedback to the DN1 neurons in order to maintain sleep^[240].

Homeostatic control of sleep

The sleep homeostat ensures that an organism achieves its daily level of sleep, controlling the amount and quality of sleep and ensuring lost sleep is recovered with a period of rebound. Conceptually, homeostasis is driven by an increase in the need to sleep,

which builds during the day and reaches a maximum threshold set by the circadian control of sleep, initiating sleep where the sleep need is dissipated^[13].

In order to study the homeostatic arm of sleep control, researchers implement assays of SD to observe the impact of sleep loss on an organism, thus elucidating the mechanisms regulating sleep and its function. Initially, it was reasoned that the genes and circuits involved with rebound periods after SD would also be implicated in the homeostatic control of sleep under baseline conditions^[228]. However, many studies have identified genes^{[246][204]} and circuits^{[247][248]} that impact homeostatic rebound but have no detectable or an opposing effect on baseline sleep. Alongside this, some environments of SD have been found which cause strong sleep suppression without any recovery. For example, sleep suppression induced by male-female interactions^[108] and, in some cases, starvation^[249] has no impact on their subsequent sleep. Thus, there may be distinct context-dependent homeostatic regulation of sleep, depending on the waking environment. A critical study observed that activation of wake-promoting neurons had differential impacts on sleep need and rebound^[247]. Thermogenetic stimulation of cholinergic, DAN, and octopaminergic neurons all resulted in significant sleep loss. However, only cholinergic activity resulted in a rebound response comparable to controls. Contrastingly, DAN activation suppressed the rebound, and octopaminergic stimulation had no impact on subsequent *D.melanogaster* sleep. Further testing combining mechanical SD with neuronal stimulation revealed that octopaminergic, and not DAN, induced waking suppresses sleep need. The behavioural and neuronal SD sleep rebound results demonstrate that in some scenarios, wake may occur without driving changes in sleep need. Nevertheless, a picture of candidate circuitry involved in a sleep homeostatic mechanism has emerged.

Central complex

The central complex describes a collection of neuropils in the midbrain found in many insect species, containing the fan-shaped body (FB), ellipsoid body (EB), protocerebral bridge, and noduli (Fig 1.4). The central complex is evidenced to be involved in the integration and processing of information from environmental stimuli^[250] and the control of motor output^[251] and behaviour^[252]. In recent years it has been implicated in sleep, with many of the neuronal populations found to play integral roles in sleep and homeostasis^{[253][254][255]}. A role of the central complex in the control of wake and sleep may well be conserved with other inveterate species, with evidence of central complex activity with arousal and motor control in the cockroach^{[256][257]}.

The dorsal layer of the FB became of interest for the study of sleep when it was found that *TrpA1* activation of the ExFl2 neurons that project to the dFB induces sleep

and improves the facilitation of long-term memory^[253]. While silencing the dFB has the opposite effect, with sleep suppression^[258]. Since this finding and the research that followed, the dFB has been considered the output arm of the homeostatic mechanism, whose activity is regulated in response to sleep need. The electrical activity, concerning the input resistance and membrane time constraints, of the dFB neurons is suggested to confer increasing sleep pressure into sleep, as the neurons increase in electrical excitability during SD, returning to control levels after rebound sleep^[259]. The Rho-GTPase, from the family of molecular switches that modulates downstream biochemical pathways^[260], is implicated in regulating the innate electrical properties of the dFB neurons. Mutations and other genetic manipulations in the *crossveinless-c* gene that encodes Rho-GTPase were found to abolish rebound and remove alteration in the electrical excitability of the dFB neurons upon SD^[259]. Thus the homeostatic control of sleep may be conducted by altering the activity of this sleep-promoting centre. Pimentel *et al.*^[261] found that dopamine signalling initiated dFB cell hyperpolarization, switching the cells from an electrical excitability ON state to a quiescent OFF state and initiating wake. The expression levels of multiple ion channels modulate neuronal activity within the dFB^[223]. The Sandman leak channel is responsible for the silencing of neurons during waking, and the A-type current Shaker causes neuronal firing during sleep. The Shaker potassium channel has previously been implicated in evoking sleep, as mutants in the genes *Shaker*^[262], *Hyperkinetic*^[263] and *sleepless*^[264] cause reductions in sleep, and thus, these results may well be due to A-type current alterations within the dFB. Hyperkinetic and sleepless are activators of Shaker. A recent study demonstrated that the accumulation of ROS during wake may underlay the build-up of sleep need during wake, regulating dFB activity through oxidation of Hyperkinetic bound NADPH to NADP+. Thus, altering the Shaker A-type current and promoting sleep^[265]citeme.

Another neuronal population in the central complex implicated in sleep homeostasis is the R5 neurons of the EB. Elevating sleep need with SD causes a rise in cytosolic Ca^{2+} levels, a proxy for neuronal activity, alongside increased synaptic plasticity and strength with elevated active zone size and number^[254]. NMDA receptors were required in the R5 neurons for the observed plastic changes in the R5 circuit. R5 neuron inactivation and KD of NMDA receptors in the R5 neurons markedly reduced rebound in response to SD. Therefore, presenting an NMDA-dependent plasticity mechanism within the fly brain that is at least partly involved in homeostatic sleep drive and recovery. The identified EB R5 circuit acts upstream to the established dFB, as the activation of R5 neurons causes increased activity in the dFB projecting ExFl2 neurons, which were also required for full rebound sleep upon SD via thermogenetic activation of the R5 neurons^[254]. Recurrent R5-dFB circuitry was recently described via inhibitory AstaA

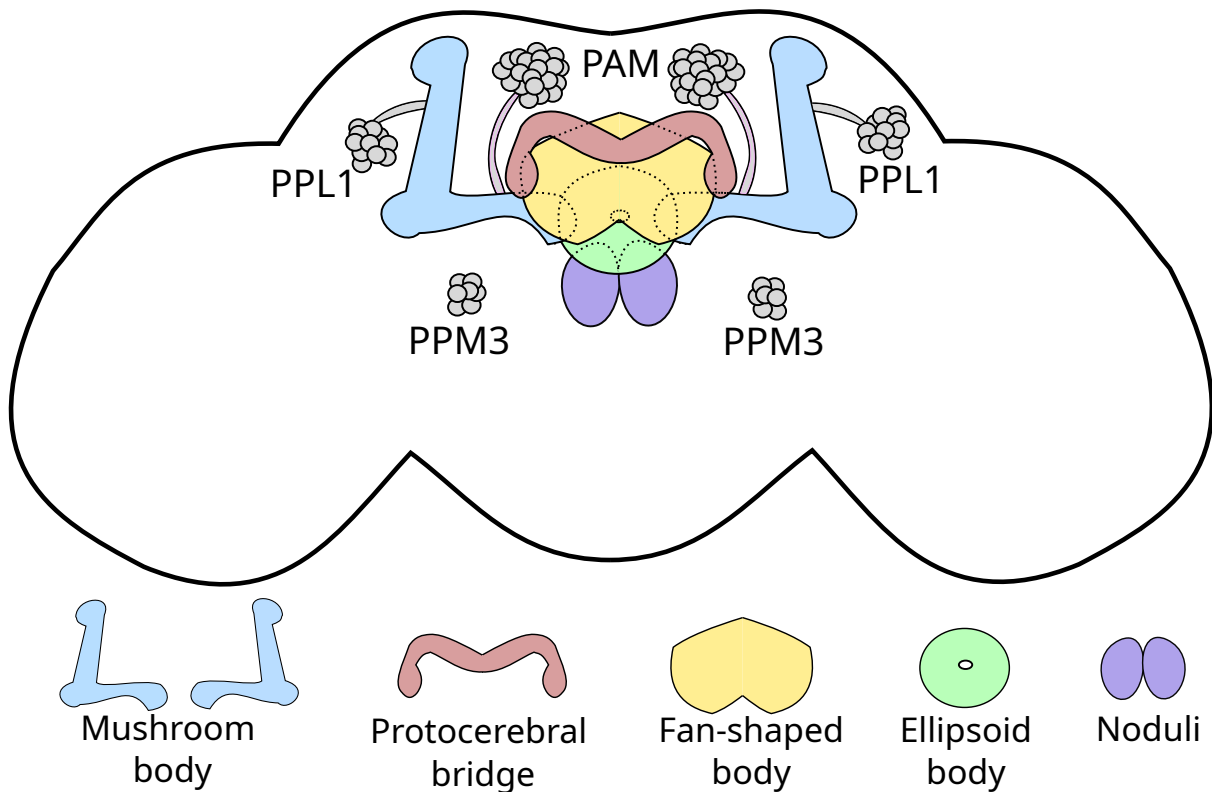


Figure 1.4: Schematic of the central complex, mushroom body and a selection of the dopaminergic neurons in the fly brain

The central complex comprises the fan-shaped body, ellipsoid body, protocerebral bridge and the noduli. The fan-shaped and ellipsoid body are key players in the *D.melanogaster* homeostatic sleep mechanism. The dorsal layer of the fan-shaped body is involved in sleep-promotion and single dopaminergic neurons from the PPL1 and PPM3 cluster promote wake via inhibitory input into the dorsal fan-shaped body. The mushroom body is also implicated in sleep control, with both sleep and wake-promoting compartments. Projections from the PAM and PPL1 dopaminergic neurons innervate distinct mushroom body lobes. This figure was produced with information from Sitaraman *et al.*^[114], Nall *et al.*^[267] & the Virtual Fly Brain (www.virtualflybrain.org).

producing interneurons termed helicon cells. The cells were responsive to visual stimuli and likely to make presynaptic contact with the EB, transmitting visual stimuli information and controlling locomotive output. The dFB ExFl2 sleep-promoting neurons were found to induce sleep via many neurotransmitters, including AstaA, which targets the helicon cells, inhibiting their signal to the R5 neurons, blocking locomotion, and possibly reducing the sleep need that had built through the day^[266]. How the R5 neurons signal increasing sleep need to the dFB and how the sleep need is cleared still needs to be studied.

Mushroom Body

The mushroom body (MB) is one of the most studied areas of the insect brain (Fig 1.4).

Renowned as the associative learning and memory centre^[268], with input from the olfactory circuitry^[269]. The MB structure is located in the midbrain and composed of three main parts: the calyx, pedunculus, and lobes, with the latter classified into ventral (α' and α) and medial (γ , β' , and β) classes^[270]. Around 2000 intrinsic Kenyon cells (KCs) make up each MB, as well as distinct MB output neurons (MBON). The organisation and connectivity of the MB components are well described, thanks to the array of genetic tools available and many years of olfaction and learning investigations^[271]. The MB was the first neuronal population to be implicated in sleep^{[272][273]}. The early studies both identified that the MB played a role in regulating sleep, with ablation causing a reduction in overall sleep amount. Joiner *et al.*^[272] found that cAMP - protein kinase A (PKA) activity alters sleep in the MB in either direction depending on the GAL4 driver used to target the MB cells and the anatomical class of neurons. Since then, distinct wake and sleep-promoting MB circuits have been classified, with five glutamatergic MBON drivers that promote wake and seven GABAergic or cholinergic MBON drivers that promote sleep^[274]. Two of these MBON projections were of particular interest: the cholinergic sleep-promoting MBON- $\gamma 2\alpha'1$ and wake-promoting glutamatergic MBON- $\gamma 5\beta'2a/\beta'2mp/\beta'2mp_bilateral$ share a downstream target and influence sleep in opposing directions.

Further investigation of the connectivity between the KCs and sleep-promoting MBONs identified distinct KCs which promoted sleep or wake via the corresponding wake or sleep-promoting MBONs. Interestingly, the wake-promoting KC-MBON circuits decreased in activity upon mechanical SD. In contrast, the activity of sleep-promoting KC-MBONs increased and was found to be essential for typical rebound^[275].

The MB undergoes structural plastic changes^[276] and elevation in synaptic strength upon SD^[277] that are restored after a period of recovery sleep. Hence, demonstrating the plastic neuronal changes that occur in centres implicated in regulating sleep.

Many modulatory inputs innervate the MB lobes, including DANs and octopaminergic, with the DAN input being the most common. Dopamine is a vital signalling neurotransmitter in many brain processes across neuronal populations and is shown to impact MB functions^[278]. DAN inputs from the DAN PPL1 cluster innervate the MB and are involved in aversive memory^[279], while another cluster of PAM DANs is required for labile memory^[278]. There is a long-standing relationship between learning, memory, and sleep across the animal kingdom^[280], also found in *D.melanogaster*^[281]. Induced sleep in learning and memory mutant flies restores learning levels to wild-type levels^[282], while SD impairs flies' learning and memory scores^[283] and inducing sleep improves memory consolidation^[253]. Dopamine receptors in the MB are required for learning in various

cognitive paradigms^{[284][285]}, and targeted expression of the *dopamine-d1-like receptor* (*Dop1R*) has been found to rescue SD induced cognitive impairments^[284].

1.2.2.4 Neurotransmitters of *Drosophila* sleep

Neurotransmitters are essential in coordinating neural systems in an ever-changing environment for successful behavioural output. Evidence across the animal kingdom and detailed interrogation of model organisms has demonstrated the adaptability of sleep based on the environment and an organism's intrinsic needs^[286]. Neurotransmitters and other neuromodulators likely play a vital role in coordinating sleep circuits in response to external and internal needs. In *D.melanogaster*, wake-promoting (dopamine, octopamine & histamine), sleep-promoting (serotonin & GABA), and dual-function (acetylcholine & glutamate) neurotransmitters have been described^[111]. In many cases, the presynaptic cells and postsynaptic targets of the neurotransmitter's effect on sleep control have been identified. This next section will describe the sleep effect, and proposed mechanism for dopamine, octopamine, and GABA signalling. The details of the other neurotransmitters and neuromodulators involved sleep regulation are reviewed elsewhere^{[267][111]}.

Dopamine As mentioned when discussing pharmacologically-induced sleep, manipulating the dopamine biosynthetic pathway neatly demonstrates the wake-promoting role of dopamine. Several short-sleeping mutants have been linked to impaired dopamine signalling. For example, mutations in the gene which encodes a dopamine transporter (DAT), *fumin*, sleeps two to threefold less than wild-type flies^[287]. DATs are involved with the termination of dopamine signalling with the reuptake of dopamine, and *fumin* mutants are found to have elevated dopamine levels at the synaptic cleft^[288]. A forward genetic screen isolated a short-sleeping mutant, *insomniac* (*inc*), that likely functions as a *Cullin-3* (*Cul3*) ubiquitin ligase complex adaptor, with KD of *Cul3* mimicking the short-sleeping *inc* phenotype^[289]. Further investigation of the *inc* and *Cul3* sleep found that flies lacking either functional protein were resistant to L-dopa-induced sleep elevation, and inhibiting dopamine synthesis with 3IY increased their sleep^[290]. This suggests that the reduced sleep associated with the genetic manipulations of *inc* and *Cul3* are likely due to elevated dopamine levels.

Alongside the aforementioned pharmacological and broad short sleeping affiliation of dopamine with arousal, detailed interrogation of the presynaptic DAN and postsynaptic cells involved with the promotion of wake has been dissected (Fig 1.4). DAN neurons constitute a relatively small number of *D.melanogaster* neurons (130-140) and

are found in clusters throughout the brain, with projections that include MB and structures within the central complex as targets^[291]. Single DANs from each PPL1^[292] and PPM3^[293] cluster were found to promote wake with inhibitory signalling in the dFB via the Dop1R1 dopamine receptor. DANs also exert arousal via projections to MB from the PAM and PPL1 clusters, which innervate distinct MB compartments^[114], and the Dop1R1 receptor is required in the MB for the arousal effects of caffeine^[267]. Dopamine signalling is also implicated in the clock control of sleep. PPL2 clusters of DANs project to the wake-promoting ILNvs and dopamine application increases cAMP levels, depolarizing the cells^[294]; however, the study in question found that dopamine was not responsible for the wake-promoting effects of the ILNvs.

Octopamine Octopamine is a prominent invertebrate neurotransmitter and an orthologue of the vertebrate norepinephrine^[295]. The biosynthesis of octopamine is achieved via the conversion of tyrosine to tyramine catalysed by tyrosine decarboxylase (Tdc) and then the conversion of tyramine to octopamine with tyramine β hydroxylase ($T\beta H$)^[296]. It is found to be involved in regulating multiple physiological and behavioural processes, with an established role in *D.melanogaster* aggression^[297], and more recently, it has been studied in the context of sleep. A picture has emerged of wake-promoting octopamine signalling. Firstly, mutants for the key enzymes involved in octopamine synthesis, *Tdc2*, and *T β H*, exhibit elevated sleep, arousal threshold levels, and reduced latency to sleep onset^[298]. This finding was confirmed by altering the electrical excitability of octopamine (and tyramine) producing neurons, with complementary impacts on sleep - NaChBac mediated activation lowered rest and Kir2.1 silencing increased sleep. Secondly, administering octopamine via food decreases sleep in wild-type flies, implicating octopamine in the wake-promoting effect rather than its precursor tyramine. Feeding of octopamine in the *Tdc2*, and *T β H* mutants supported the specific role of octopamine in the sleep phenotype, rescuing the associated elevated sleep to wild-type levels.

Octopamine is evidenced to exert its wake-promoting effect via cAMP-dependent PKA signalling. Inhibition of PKA with the expression of a regulatory subunit abolished the octopamine feeding-induced sleep decrease found by Crocker *et al.*^[299] Further characterisation of the octopamine wake effect identified that octopaminergic ASM cells project to PI neurons, labelled with the *Dilp2*- GAL4, promoting wake^[115]. Once again, cAMP-dependent PKA signalling was required for the phenotype, as blocking PKA in the PI neurons removed the sleep sensitivity caused by octopamine feeding.

GABA GABA is a prevalent inhibitory neurotransmitter in both vertebrate and invertebrate animals. It exerts its inhibitory effects on postsynaptic GABA receptors,

split into the ionotropic GABA_A and metabotropic GABA_B receptors. The binding of GABA to ionotropic receptors activates the Cl⁻ permeable channel, leading to an influx of Cl⁻ into the postsynaptic neuron, hyperpolarizing the cell and reducing the likelihood of an action potential. GABA activates K⁺ or blocks Ca²⁺ channels in metabotropic receptors, again hyperpolarizing the postsynaptic cell and inhibiting its activity^[300].

The THIP (4,5,6,7-tetrahydroisoxazolo(5,4-c)pyridin-3-ol) compound is an agonist of the ionotropic GABA receptor and has been shown to increase sleep in *D.melanogaster* in a dose-dependent manner^[282]. The periods of sleep associated with the drug feeding shared the hallmarks of characterised sleep behaviour- as it was readily reversible with a strong stimulus and associated with increased arousal thresholds. Alongside this, genetic perturbations that cause elevated GABA levels are found to increase sleep in flies. For example, Chen *et al.* ^[301] reported that a mitochondrial GABA transaminase is implicated in the sleep suppression associated with the *sleepless* mutant. The mutant flies were found to have reduced GABA levels, while loss of the GABA transaminase, and repletion of GABA, restored the sleepless mutants' sleep. As discussed previously, inhibitory GABAergic input in the circadian ILNvs via expression of the RDL GABA_A is involved with suppressing their wake-promoting signal and is necessary for the initiation of sleep^[116].

1.2.2.5 Glial sleep modulation

For many years, investigation of *D.melanogaster* sleep has been restricted to neurons. However, more recently, studies have emerged on the involvement of other non-neuronal brain cells in sleep homeostasis. In particular, sensing the accumulation and dissipation of sleep need during wake and sleep. Glial cells are found in greater numbers than neurons in the nervous system and are implicated in a multitude of functions, including the reuptake of neurotransmitters^[302] and the recovery from neural injury^[303]. A subset of glial cells called astrocytes have processes surrounding neurons, and their morphology indicates they are in a privileged position to sense neuronal activity. While these cells were initially thought of as inactive support cells, work at the turn of the millennia demonstrated that they reciprocally communicate with neurons. Astrocytes express G-coupled protein receptors, which mobilise Ca²⁺ from internal stores in response to presynaptic neurotransmitters. While elevated Ca²⁺ has also been shown to mediate the release of gliotransmitters that bind to neuronal synaptic receptors, impacting synaptic transmission^[304].

A 2021 study^[305] hypothesised that astrocytes likely sense the accumulation of sleep need and signal to relevant downstream homeostatic circuitry to modulate sleep. In

support of this theory, they found that Ca^{2+} levels were enhanced during SD; thus, the astrocyte activity correlates with increasing sleep need. Additionally, reduced Ca^{2+} signalling attenuated the rebound response following SD. Transiently activating astrocytes increased both day and night sleep, suggesting that their glial signals likely communicate with a sleep center to provoke rest. A TyrRII receptor was found to be required in astrocytes for the arousal phenotype and the *tyrRII* expression correlated to increasing sleep need. Thermogenetic elevation of Ca^{2+} in astrocytes impacted two sleep-wake circuits: reducing the membrane potential of the circadian ILNvs, and overnight astrocyte activity resulted in elevated Ca^{2+} levels in the R5 EB neurons the following morning. In support of the latter finding, the R5 neurons and their proposed downstream targets, *ExFl2*, were required for the sleep phenotype associated with astrocyte activation^[305]. The above study comprehensively presents astrocytes as a potential candidate for sensing sleep need (likely through neurotransmitter binding to the TyrRII receptors), computing the sleep need information, and signalling to downstream neuronal sleep circuits.

1.2.2.6 Consequences of sleep loss

In studying the impact of SD on diverse animal species, including *D.melanogaster*, many physiological impairments have been documented, including cognitive, immune, and metabolic effects, to name a few. Exploring the physiological impact of SD provides insight into the potential function/s of sleep and identifies the probable molecular and cellular underpinnings. However, a significant obstacle in the field remains in disentangling consequences of sleep loss that are a direct cause of lost sleep and those due to stress or other sleep-independent effects that correspond to the method of SD. Generally, extended sleep loss is considered fatal, with studies documenting reduced lifespan with chronic sleep paradigms^{[306][307][203]}. These results highlight the essential need for sleep for the survival of an organism. Interpretation of these results should be interrogated similarly to the findings described above, as the fatality found to correspond with sleep loss may well be a consequence of the unnatural and stressful SD paradigms. A 2019 publication in our lab described the striking results that lifelong SD with a responsive mechanical method (Materials and methods 2.4.1) resulted in strong sleep suppression throughout the lives of flies without any impact on their lifespan^[65]. Such a finding is not restricted to flies, in 2008, a group found that pigeons were resistant to the chronic Disk-Over-Water method^[42] that commonly led to death in mice studies^[308]. Suggesting that lack of sleep itself may not directly have deleterious effects on survival. However, the impact of chronic sleep loss on cognitive function and fitness was not explored.

ROS accumulation There is some evidence of sleep causing oxidative stress in mice^[309] and flies^{[310][203]}. Furthermore, mitochondrial ROS activity was found responsible for altering potassium currents and modulation of neuronal activity in the dFB to promote sleep^[265]. However, there are also contradictory findings^{[311][312]}, with some studies unable to correlate the accumulation of oxidative stress markers with sleep loss. The conflicting findings may result from localised rather than widespread accumulation of ROS, as demonstrated by Vaccaro *et al.*^[203] with elevated ROS in the gut of mice and flies after mechanical and thermogenetic SD.

Immune response stimulation SD has been found to increase the expression of immune genes^{[122][313][314]}, and infection or injury promotes sleep in *D.melanogaster*, at least partly due to the inflammatory response associated with the *Relish* gene, as it is required for infection/injury related sleep elevation^[315]. The impact of SD on the immune response is impeded by the subsequent rebound sleep, which increases the resistance to infection and improves survival^[316]. Nevertheless, the results implicate a relationship between sleep and immunity. This relationship was recently mechanistically evidenced with the identification of an antimicrobial peptide encoding gene, *nemuri*. *Nemuri* was required for homeostatic rebound post-SD and infection-induced sleep^[317]. However, the mechanisms by which such immune gene products control sleep are still unknown.

Synaptic strength elevation One emerging consequence of sleep loss and wake is the elevation in various markers of synaptic strength, which disrupts synaptic homeostasis, that is subsequently restored during sleep. It is proposed that interaction with the environment and learning during wake corresponds with increased synaptic potentiation that disrupts neuronal homeostasis. The disruption causes various negative results, including elevated energy consumption, reduced neuronal space, and obstruction of further potentiation and learning. According to the synaptic homeostasis hypothesis (SHY), sleep may serve as a time of reduced net neuronal activity to downscale synaptic strength and restore homeostasis^[318]. Various lines of evidence in mice and *D.melanogaster* support Tononi and Cirelli's SHY proposal. The evidence provided in the latter model will be discussed here, but information on mice can be found in this^[90] review. SD is accompanied by a widespread elevation in synaptic markers, which are reduced after a rebound period^[319]. Alongside this, overexpression of a presynaptic protein elevates sleep while reducing protein levels has the opposite effect^[320]. Mechanical SD increases the volume and number of presynapses in this circuit and the MB^[276]. Therefore, wake corresponds to a net increase in synaptic strength, with elevation in pre and postsynaptic markers observed within the *D.melanogaster* brain during spontaneous wake and upon SD. However, evidence has emerged that the increase may

not be homogenous, as demonstrated by the diverse synaptic scaling within the MB circuits. Weiss *et al.*^[277] found that while overall presynaptic proteins across the MB were increased, further interrogation of the cell types responsible for the elevated signal revealed that while some KCs show increased levels of a presynaptic protein upon SD, other sub population's protein levels are comparable to controls. This demonstrates that the accumulation may be localised to specific circuits, but nevertheless wake still drives a net increase in synaptic strength.

1.3 Other species of the *Drosophila* genus

D.melanogaster is arguably one of the most powerful model organisms for studying biological processes, including behaviour, and is often referred to by its genus moniker, *Drosophila*. However, the other species in the *Drosophila* genus offer opportunities to carry out horizontal comparative studies to understand the emergence of behaviour across ecology and evolution.

Alongside the origin of *D.melanogaster* research in the early 1900s, several labs shifted focus to the study of other species in the Drosophilid family, leading to a comprehensive understanding of the ecology and phylogenetic relationship of Drosophilidae. The Drosophilids were officially catalogued in the 1920s by the pioneering geneticist Sturtevant, who placed 242 species in the *Drosophila* genus^[321], later arranging the genus species into subgenera, including the *Drosophila* and *Sophophora* categories^[322]. Following these foundations of the taxonomic classification, there are now between 1200 and 1500 identified species in the *Drosophila* genus. The phylogenetic tree has been revised over the decades as researchers accumulated insight into their evolution with genetic and morphological data^[323]. In 2007, the genomes of 12 *Drosophila* species (including *D.melanogaster*) were fully sequenced, improving the utility of comparative studies of their differential gene expression, development, ecology and behaviour^[324].

The *Drosophila* genus spans widespread global regions and exhibits considerable variation in morphology, ecology, and behaviour^[325]. Nevertheless, the species within the genus are found to have significant conservation in terms of their cellular, molecular and developmental biology. Therefore, placing the species in a privileged position for the comparative study of the relationship between the environmental cues and underlying mechanisms that drive behavioural variability.

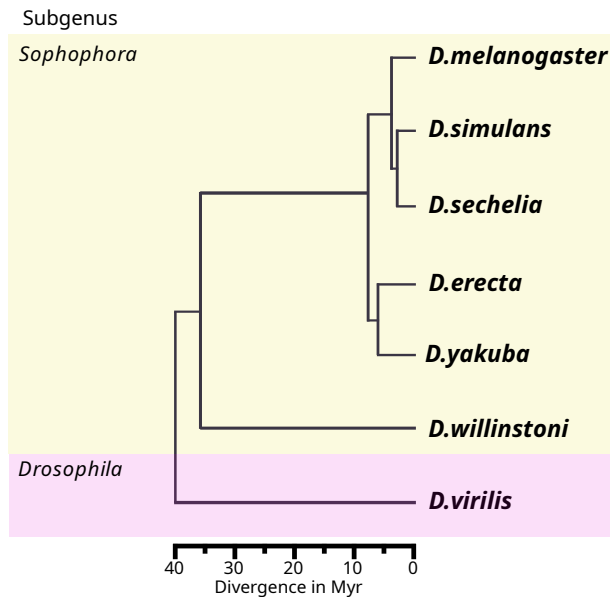


Figure 1.5: A partial phylogenetic tree of a selection of *Drosophila* from the *Sophophora* and *Drosophila* subgenera

1.3.1 Ecology and speciation

The *Drosophila* genus is currently subdivided into ten subgenera, with the *Sophophora* and *Drosophila* subgenera accounting for a vast majority of the genus (approximately 90%)^[326]. Each subgenus is further characterised into groups and subgroups. This next section will briefly overview the ecology and speciation of a selection of species from the *Sophophora* subgenus, including ones from the melanogaster and willinstoni subgroups and a single species in the *Drosophila* subgenus from the virilis subgroup (Fig 1.5). The species described in this next section will be the focus of the investigations in Chapter 3.

1.3.1.1 *Sophophora* subgenus

Melanogaster group *D.melanogaster* are considered a strict human commensal species, originating in the Afrotropical region and now inhabiting all areas of the globe where humans have settled. Their natural populations show significant genetic variation depending on geographic location due to selective pressures and local adaptation^[327].

A close relative to *D.melanogaster*, *D.simulans*, diverged between 2-3 million years ago and is generally considered a commensal species. However, they are found to thrive in unpopulated areas (Eastern Africa, Madagascar, Seychelles & Mascarene islands), and there are regions that they do not inhabit (areas of Western Africa, Southeast Asia & West pacific regions^[327]).

Another Seychelles dwelling *Drosophila* has been of particular interest due to its specialisation to a usually toxic fruit to other Drosophilids. *D.sechellia* is endemic to the Seychelles and selects the *Morinda citrifolia* fruit as its sole host^[327]. A comparative study observed dichotomy in two Seychelles dwelling species, with *D.simulans* demonstrating aversion and a reduction in oogenesis, while the *D.sechellia* species were attracted to the fruit and, in fact, increased oogenesis^[328]. Interestingly, *D.sechellia*'s L-dopa levels are supplemented by the *Morinda* fruit, with the flies having reduced L-dopa levels on non-*Morinda* diets^[329] and contradictory elevated dopamine levels when compared to *D.melanogaster* flies. Some evidence suggests that the higher levels of dopamine shield the flies from the toxic effects of the fruit, with dopamine feeding in other *Drosophila* improving longevity on *Morinda* diets^[329]. However, a satisfactory explanation for the contradictory levels of L-dopa and dopamine is yet to arise, as there are elevated levels of the L-dopa precursor and biosynthesis enzyme, tyrosine and tyrosine hydroxylase respectively, and L-dopa product, dopamine, but yet reduced levels of the L-dopa protein. Studies should investigate dopa decarboxylase levels, responsible for converting L-dopa to dopamine.

Another intriguing species is *D.erecta*, restricted along the Gulf of Guinea coast and specialised to fruit from many species of *Pandanus*^[327]. The *D.erecta* geographical habitat overlaps with *Pandanus* distribution, and due to the seasonality of the fruit, the flies switch to other feeding and breeding sites in the absence of their preferred host^[330].

While *D.erecta* is a specialist to *Pandanus* fruit in terms of oviposition, other *Drosophila* species are known to feed on the fruit, including *D.yakuba*. *D.yakuba* populations are numerous and widespread throughout Sub-Saharan Africa. They are generalists, with many fruit hosts; however, an island Moyotte population has recently been identified that exhibits speciation. The Moyotte *D.yakuba* flies feed and breed on the *Morinda* fruit, with similar genetic differentiation as documented with *D.sechellia*, providing relatively recent evidence of recurrent evolution based on shared ecological drives^[331].

Willistoni group The *D.willistoni* species of the willistoni subgroup are subtropical and inhabit southern Florida, southern South America, and the West Indies^[332].

1.3.1.2 *Drosophila* subgenus

Virilis group The virilis group is thought to have been established in East Asia^[333]. The *D.virilis* species inhabits temperate and usually forest ecosystems, naturally breeding in the slime flux of willows and other decaying parts of trees^[334]. They are almost

cosmopolitan in their distribution, but unlike *D.melanogaster* and *D.simulans*, *D.virilis* is a specialist species. However, they have been identified to use fruits as hosts in urban settings. They have served as a genetic model in evolutionary studies and are found to have elevated temperature^[335] and ethanol tolerance^[336] compared to *D.melanogaster*, likely aiding in their adaptation to vast environments.

In addition to differences in their ecology, the species mentioned above also exhibit variations in body size, with *D.virilis* being notably larger, and smaller differences existing among the other species. These differences in body size may be accompanied by variations in their metabolic rates, as metabolic rate is closely linked to body weight. It is a general trend across the animal kingdom that larger animals tend to have lower metabolic rates, while smaller animals tend to have higher metabolic rates.

1.3.2 Interspecific changes in behaviour: a mechanistic perspective

While the other species of the *Drosophila* genus are generally understudied, exceedingly so compared to the *D.melanogaster* species, a movement towards comparative analysis across the genus has appeared in recent years. A handful of studies have investigated the conservation of circadian activity and the cellular and molecular components of the clock in other species. While *D.sechellia* is emerging as a model for the study of olfactory circuit evolution. The comparative study of the *Drosophila* genus has demonstrated intriguing variability in such closely related species.

1.3.2.1 Circadian activity

D.melanogaster circadian activity shows a robust bimodal pattern of morning and evening activity peaks, with a midday decrease corresponding to their siesta sleep and exhibiting the lowest activity levels during the night phase. Comparable bimodal activity is found in other Drosophilid species, including *D.simulans* and *D.yakuba*^[337] of the melanogaster subgroup and *D.virilis*^[338] of the virilis subgroup. In *D.melanogaster*, constant dark conditions evoke persistent free-running rhythms, and circadian activity is maintained, albeit with a reduction in the morning peak^[228]. The s-LNvs are required for the maintenance of the circadian activity rhythm in the absence of light, primarily due to their expression of the circadian cell coordinator, neuropeptide PDF. Strikingly, Bahn *et al.*^[338] found that under constant dark conditions, *D.virilis* became either weakly rhythmic or arrhythmic, closely resembling the arrhythmic activity of *pdf* mutants in the absence of light. The researchers presented a causal relationship between

the arrhythmicity in the *D.virilis* species with altered PDF expression in a subset of clock neurons, established with immunostaining of PDF expression in the brain. The PDF signal localised to the *D.virilis* equivalent of the l-LNVs but was absent from the s-LNVs, corroborated in another study that found comparable divergence in the PDF expression in the latter clock cells^[339]. This altered PDF expression was common to the *Drosophila* subgenus, while the expression was equivalent to *D.melanogaster* across the *Sophophora* subgenus. Therefore, the loss of rhythmic circadian behaviour in constant conditions may be due to modifications in the PDF transcriptional regulatory mechanisms at the s-LNVs, and the other species of the *Drosophila* subgenus could possibly share the behaviour alteration.

Generally, diurnal animals modulate their activity based on seasonal changes, with increased daytime activity at colder temperatures and increased night-time arousal during the warmer months. To ensure that an animal carries out its essential tasks at an optimal period^[340]. In *D.melanogaster*, thermal-sensitive splicing of a 3' -terminal *per* intron, *dmpi8*, has been identified to modulate the timing of activity based on the external temperature. As a core component of the circadian clock, *period mRNA* and protein levels cycle over the day, and thermal-sensitive splicing of the *dmpi8* is demonstrated to increase *per mRNA* levels and modulate daily activity. For example, splicing is enhanced at colder temperatures, driving elevated *per mRNA* and premature evening activity^{[341][337]}. This splicing mechanism allows *D.melanogaster* to adapt to seasonal changes in temperature. Further investigation of *D.yakuba* revealed that the species was resistant to temperature-induced changes in activity. In *D.melanogaster*, warmer temperatures advance the morning activity, increase the siesta length, and delay evening activity. *D.yakuba*, however, were resistant to the temperature-aligned modulation in the morning and evening activity peaks and the length of the siesta remained stable. *D.yakuba* were found to have a 3'-terminal intron, but the splicing levels remained consistent over the day and at varying temperatures. In turn, the cycling of *per* over the day was also unchanged from 18-29°C. *D.yakuba* inhabit Sub-Saharan Africa and the ancestral regions of the *Drosophila* genus, with a relatively constant temperature over the year. *D.simulans* are close relatives to the *D.melanogaster* species and another human commensal group that have colonised many global regions. *D.simulans* showed comparable activity and siesta modulation at different temperatures to *D.melanogaster*, with cycling in their equivalent 3'-terminal intron splicing and *per* levels across the varying temperatures. This evidence suggests that the modulation in the splicing efficiency of the 3'-terminal intron of the *per* gene likely played an essential role in the Drosophilid commensal species adaptation to a large variety of environmental temperatures. Therefore, likely underscoring the mechanism responsible for the *D.melanogaster* colonisation

of the world. Local equatorial species like *D.yakuba*, where there is minimal fluctuation in the LD cycles and temperature, do not undergo modulation in the splicing efficiency and have fixed activity characteristics at varying temperatures^[337].

1.3.2.2 Olfactory circuitry

Olfaction in *Drosophila* is important for navigating and interpreting their environment and is essential for locating mates, food sources, and appropriate sites for egg laying^[342]. Chemosensory systems have been found to evolve rapidly across insects, and *Drosophila* species and are the driving force behind speciation - as the connection between an organism and its chemical environment^[343].

The *Morinda* fruit preference observed in *D.sechellia* motivated the study of the olfactory circuitry, with many studies revealing the variations that likely elicited the behavioural adaptation of *D.sechellia* to the fruit. One study aimed at investigating the evolution of olfactory circuits across the melanogaster subgroup by studying morphology and electrophysiological recordings in response to odourants of the olfactory receptor neurons (ORNs). Stensmyr *et al.*^[344] studied the ORNs response in three classes of sensillum located in the large basiconic sensilla with distinct functionality - the ab1, ab2, and ab3 sensillum. They found striking conservation in the response of the ORNs in the distinct sensillum across the melanogaster subgroup species included in the study, except the simulans clade, with remarkable alterations identified in the *D.sechellia* flies. The ab2 sensillum was absent in *D.sechellia* and replaced by a higher proportion of the ab3 sensillum. In *D.melanogaster*, ab3A type ORNs primarily respond to ethyl hexanoate, but in *D.simulans* and *D.sechellia*, the ORNs predominately responded to methyl hexanoate, a volatile component of the odour represented in the *Morinda* fruit^[344]. Ibba *et al.*^[345] later identified a second volatile present in the *Morinda* fruit odour bouquet, 2-heptone, which elicited a response in the ab3B type ORNs in *D.sechellia* and the glomeruli receiving input from both the ab3A and ab3B ORNs were enlarged^[345]. Therefore, the ORNs located in the overrepresented ab3 sensillum in *D.sechellia* uniquely respond to the volatiles in the *Morinda* fruit and may underlay the switch in preference of the widely Drosophilid toxic *Morinda* fruit. Comparable results have been observed with another specialised member of the melanogaster group. The specialisation of *D.erecta*, which feeds and oviposits on *Pandanus* fruit, is reflected in its olfactory system with an increased number of the ab3 olfactory sensory neurons, which in turn results in enlarged glomeruli in the antennal lobes^[330].

ORNs usually express a single receptor, characterised as either odorant or ionotropic. ORNs with the same ligand-specific receptor then converge onto a shared glomerulus

in the antennal lobe^[104]. The ab3A ORN expresses the Odorant receptor 22a (OR22a), and the ab3B ORN is associated with the OR85b. Introduction of the *D.melanogaster* *Or22a* in *D.sechellia* flies attenuated the long-range *Morinda* fruit attraction^[346]. This strongly implicates variations in the OR22A receptor in the preferential response of the ab3A ORNs to methyl hexanoate. However, the altered tuning of the ab3A ORNs is present in both *D.simulans* and *D.sechellia*, suggesting an evolutionary variation in a common ancestor of the two species, preceding the host specialisation of *D.sechellia* to *Morinda* fruit. Changes in the number and tuning of other olfactory receptors were also recently described^[347]. Alterations in the number and tuning of many ORNs, alongside the elevated volume of the corresponding glomeruli, are found in *D.sechellia*, and potentially *D.erecta*. However, a direct impact of such alterations on host-specific behaviour is lacking.

1.3.2.3 Sleep and homeostasis

Detailed sleep investigations in isolated species have elicited many biological processes influenced by sleep^{[6][8][9]}. However, there is still no consensus on the specific function of sleep and sleep need^[10]. Observing sleep through the lens of evolution may elucidate its fundamental aspects. Interspecific sleep studies provide a framework for identifying animals that sleep differentially and then investigating the underlying factors contributing to those differences. While a small number of studies have investigated the circadian activity of other species from the *Drosophila* genus, characterisation of their sleep is largely understudied.

A 2013 investigation of the circadian clock gene's involvement in starvation-induced sleep loss recorded the conservation in three melanogaster subgroup species in response to starvation^[348]. A supplementary figure showed that *D.erecta*, *D.yakuba*, and *D.simulans* all exhibited a reduction in sleep when starved. However, this is the only data they included on the species; no comparison of their sleep was calculated, and they did not report any data regarding their rebound response to such sleep loss.

A recently published 2022 study comparatively analysed the effect of temperature on daily activity and sleep in 11 *Drosophila* species^[349]. They reported a large range of interspecific variations in daily sleep levels alongside intraspecific changes in sleep amount over colder and warmer temperatures. Interestingly, while *D.melanogaster* total daily sleep remains constant over 24 hr at varying temperatures (17-29°C), the distribution of their sleep over the day and night switched at warmer environments. At 25°C *D.melanogaster* flies sleep longer during the night, however, studies of the activity

at higher temperatures revealed a nighttime increase in activity, likely as an adaptive response to avoid moving during the deleterious higher temperatures of the day^[340]. The other species tested exhibited alterations in their total sleep at varying temperatures, and *D.simulans* and *D.yakuba* daytime sleep increased with the temperature, but with no change in their night-time sleep appearance. However, they did not provide statistical analysis of the day or night bouts across the temperatures.

Nevertheless, it highlights an interesting aspect of *D.melanogaster* sleep where the daily amount remains constant, ensuring the organism achieves its daily amount of sleep in the different temperature environments but adapting its timing of sleep across the 24 hr day. An aspect that is seemingly absent in the other *Drosophila* species tested. The stable yet, adaptable *D.melanogaster* sleep at different temperatures is reminiscent of the homeostatic sleep mechanism ensuring an organism achieves its daily amount and quality of sleep. Indeed, Parisky *et al.* demonstrated the increased daily sleep amount at higher temperatures in *D.melanogaster* appeared to be a homeostatic sleep response to the reduced sleep levels during the night in warmer conditions^[135]. However, an investigation of the homeostatic mechanism beyond the *D.melanogaster* species has yet to be undertaken.

Comparative studies of the variation of mammalian sleep initially raised some interesting correlations between REM and NREM duration and physiological (mass-specific basal metabolic rate^[350]) or ecological (predatory risk^[126]) factors. However, the studies are limited by their reliance on multiple data sets collected under different conditions with varied sample sizes. In addition, comparing species from vast phylogeny with considerable physiological disparity presents challenges in identifying the mechanism that underlies the observed changes. Furthermore, these studies only concern the sleep stages' duration, which may not constitute changes in the animal's sleep need but rather their sleep availability. An investigation of sleep homeostatic regulation across species in the form of response to SD could provide insight into the evolutionary mechanisms that control sleep.

A comparative study of closely related species, for example, across the *Drosophila* genus, within the bounds of a single controlled study, may advance our understanding of the genetic, molecular, and circuitry alterations that have driven sleep variability and homeostasis - due to their phylogenetic proximity and impressive variability in behaviour.

1.4 Summary and aims of the project

We spend 1/3 of our life sleeping, and it is looking increasingly clear that every animal needs sleep^[1]. The question of why we sleep has intrigued minds since records began, but to this day, there is no unified theory of sleep that can address its ubiquity and provide an adequate explanation for the vast physiological processes in which sleep is implicated^[10]. While sleep can be considered ubiquitous, there is great inter and intraspecies variation in sleep duration. Firstly, the big brown bat requires 20 hr of daily sleep, while the African elephant is reported to sleep for only 2 hr, and the reason for this large discrepancy across animal species remains unanswered^[79]. Secondly, sleep can be modified within a single individual based on the interaction between an organism's external environment and intrinsic needs, for example, in the case of the male sandpipers, which forgo sleep during mating season, highlighting how sleep can be downgraded in the face of more pressing needs^[128].

In the past 20 years, *D.melanogaster* has proven to be a valuable model for studying sleep and its manifestation across neuronal circuits. An in-depth investigation of sleep in *D.melanogaster* has led to substantial evidence of potential sleep need signals in the brain. The recent SHY hypothesis conceptualises the building evidence of the relationship between synaptic plasticity, learning, and sleep, postulating that sleep is the consequence of synaptic plasticity and learning^[351].

However, while the vertical investigation in a single model organism provides the meticulous study of underlying mechanisms and advances biological knowledge, it fails to contextualise biological processes across evolution. Evolutionary biologist Theodosius Dobzhansky famously said, 'Nothing in Biology Makes Sense Except in the Light of Evolution'; thus, a horizontal comparative study between species could facilitate our understanding of sleep and its emergence in the animal kingdom. It is implausible to study the variation across the animal kingdom in such distantly related and morphologically and physiologically disparate species as the bat and the elephant. An investigation of more closely related species may offer a framework for the controlled and systematic examination of sleep behaviour and the genetic, molecular, and cellular underpinnings.

The study of non-*melanogaster* species sleep is limited, and in this thesis, I will present, to my knowledge, the first horizontal investigation of Drosophilid sleep homeostasis, in order to compare aspects of sleep that are shared across the genus and identify what has diverged.

1.4.1 Aims and objectives

In this thesis, I will address the following aims to gain insight of the emergence of sleep and the underlying mechanisms of homeostasis:

1. Study sleep variability across the Drosophilid species

- Characterise sleep in a panel of Drosophilid species
- Investigate the Drosophilid homeostatic response to two methods of SD.
- Determine the conservation of response to neuromodulators

2. Investigate the role of synaptic strength dynamics in Drosophilid sleep homeostasis

- Investigate the impact of sleep deprivation on synaptic strength markers
- Examine the role of synaptic plasticity-associated genes in *D.melanogaster* sleep

Chapter 2

Materials and methods

2.1 Animals

2.1.1 Species stocks

The *Drosophila* species used in this study were mainly sourced from The National *Drosophila* Species Stock Center (NDSSC, Cornell University, USA) but were gifted to us by Lucia Prieto-Godino (Francis Crick Institute, UK). The *D.melanogaster* lab strains used in this study were Canton-S (originally from Ralf Stanewsky - Münster University, Germany) and Oregon-R (#2376, BDSC). The *D.melanogaster* wildcaught strains were gifted to the Gilestro laboratory from Darren Obbard (University of Edinburgh, UK). The non-*melanogaster* fly strains used were as follows: *D.simulans* (14021- 0251.254 #60, 14021-0251.196 #61), *D.sechellia* (14021-0248.25 #3, 14021-0248.28 #53), *D.erecta* (14021-0224.01 #11), *D.yakuba* (14021-0261.01 #5, 14021-0261.48 #51), *D.willistoni* (14030-0811.24 #1, 14030-0811.13 #55) and *D.virilis* (15010-1051.87 #9, 15010-1051.118 #54). White-eyed species strains used in the social SD paradigm can also be found at the NDSSC: *D.simulans w-* (14021-0251.133), *D.sechellia w-* (14021- 02048.30), *D.erecta w-* (14021-0248.30), *D.yakuba w-* (14021-0261.04), *D.willistoni w-* (14030-10811- 33) and *D.virilis w-* (15010-1051.45). The wild-type flies and their original region of collection can be found in Table 2.1.

2.1.2 Transgenic stocks

The GAL4-UAS binary expression system is a powerful tool for the spatial control of gene expression. A detailed explanation of the method can be found in Section 1.2.1.2, but it is covered briefly here. The GAL4 driver is expressed in a tissue-specific manner and binds to the UAS, causing the expression of an upstream reporter gene (Fig 1.2).

Table 2.1: The *Drosophila* species and their collection sites

Species	Strain #	Code	Source	Collection region
<i>D.melanogaster</i>	1	Canton-S	Ralf Stanewsky	Canton, USA
lab strain	2	Oregon-R, 2376	BDSC	Roseburg, USA
<i>D.melanogaster</i>	1	Wildcaught#4	Darren Obbard	Edinburgh, UK
wild caught	2	Wildcaught#3	Darren Obbard	Edinburgh, UK
<i>D.simulans</i>	1	14021- 0251.254 #60	NDSSC	Cape Town, South Africa
	2	14021-0251.196 #61	NDSSC	Antsirabe, Madagascar
<i>D.sechellia</i>	1	14021-0248.25 #3	NDSSC	Cousin island, Seychelles
	2	14021-0248.28 #53	NDSSC	Cousin island, Seychelles
<i>D.erecta</i>	1	14021-0224.01 #11	NDSSC	Unknown
<i>D.yakuba</i>	1	14021-0261.01 #5	NDSSC	Liberia
	2	14021-0261.48 #51	NDSSC	Nairobi, Kenya
<i>D.willistoni</i>	1	14030-0811.24 #1	NDSSC	Guadalupe Island, Caribbean
	2	14030-0811.13 #55	NDSSC	St.Vincent and Grenadines, Caribbean
<i>D.virilis</i>	1	15010-1051.87 #9	NDSSC	Unknown
	2	15010-1051.118 #54	NDSSC	Gikongoro, Rwanda

The UAS can, for example, be found upstream of a target gene for GAL4-mediated overexpression. It can also be combined with a gene-specific RNAi that degrades the target *mRNA*, thus, preventing the translation of a functional protein^[157]. This study herein, applied a variety of neuronal drivers and synaptic plasticity-associated UAS-GOI^{RNAi} reporters for investigations of the spatial requirement of the gene products in sleep and homeostasis. Genes of particular interest were then further studied with investigation of mutant stocks.

Various genes and interactions are implicated in sleep, and background genetic variability can have a profound effect on the sleep phenotypes measured in the fruit fly. Therefore, in studying the genetic underpinnings of sleep, it is crucial to control for any genetic variability other than that of the intended manipulation. Before testing, all GAL4 drivers and UAS-GOI^{RNAi} lines were outcrossed for six generations to the W¹¹¹⁸ background to control for background variability.

2.1.2.1 Drivers

Pan-neuronal KD was achieved with the *synaptobrevin* (nSyb)-GAL4 driver (gifted to us by Crystal Vincent, Imperial College London, UK). *nSyb* encodes a SNAP receptor associated with synaptic vesicle exocytosis, and expression is therefore restricted to neurons. SNAP receptors are required in all neuronal cells, driving widespread neuronal UAS-GOI^{RNAi} expression. To target KD to the MB neurons, a MB010B split-GAL4 construct composed of the R13F02-p65.AD combined with the R52H09-GAL4.DBD was used, which labels all the KCs of the MB (gifted to us by Andrew Lin, The University of Sheffield, UK). All other driver lines used in this thesis were sourced from the BDSC (Indiana, USA). KD of UAS-GOI^{RNAi} was directed to the R5 EB neurons with R30G03-GAL4^[254] (#49646), the PAM DAN neurons with R58E02-GAL4^[277] (#41347) and the ExFl2 FB neurons with R72G06-GAL4^[254] (#39792).

2.1.2.2 UAS-RNAi lines

Synaptic plasticity GOIs were selected from the literature for a small, focused sleep homeostasis RNAi screen. The GOIs, alongside their proposed function in synaptic plasticity and sleep, can be found in Table 4.1. The UAS-GOI^{RNAi} flies were acquired from the KK and GG libraries at the VDRC (Table 2.2).

Table 2.2: UAS-GOI^{RNAi} lines from the VDRC KK and GD libraries

RNAi line UAS -	VDRC Code	Landing site*		Off- targets	Validation**	
		40D	30B		qPCR	Phenotype
<i>dunce</i> ^{RNAi}	#107967 KK	✗	✓	0	A***	[352] [353]
<i>synapsin</i> ^{RNAi}	#109587 KK	✗	✓	1	A	A
<i>appl</i> ^{RNAi}	#108312 KK	✗	✓	2	[354]	[354]
<i>orb2</i> ^{RNAi}	#11753 GD	NA		1	A	A
<i>fmr1</i> ^{RNAi}	#110800 KK	✗	✓	0	A	[355]
<i>rutabaga</i> ^{RNAi}	#101759 KK	✗	✓	0	A	[352]
<i>starrynight</i> ^{RNAi}	#107993 KK	✗	✓	0	A	[356]

*Genotyped using diagnostic PCR^[357].

** Validation of knockdown based on available literature information. The knockdown efficiency of the different lines was established through PCR or confirmed by comparison with a second independent RNAi/mutant phenotype.

***A = Absent from the literature based on a 2023 literature search.

The GD and KK libraries are two resources of RNAi transgene lines with long hairpins that are available from the VDRC and have been prepared with alternative approaches. The older GD library is based on P-element insertion and the UAS-RNAi transgenes are therefore inserted at random sites over the *Drosophila* chromosomes^[358]. The KK library was developed utilising the PhiC31 integrase system which recombines with a specific landing site, spatially controlling the integration of the UAS-RNAi transgene into the genome. Initially, it was thought that the KK transgenes were solely located at the annotated landing site (40D) of the 5'UTR region of the *tio* gene. However, it was later documented that in 75% of the tested cases, the transgenes were integrated into a second, non-annotated region (30B). In the other 25% of the lines, insertions were found in both the 40D and 30B sites and had associated off-target effects^[359] and they have been found to lead to false-positive phenotypes^[360]. The reported off-target effects are likely due to the overexpression of the *tio* gene downstream to the annotated site^[359]. The vast majority of lines, where the UAS-RNAi construct was inserted in the

non-annotated region, behave as expected and have no reported non-specific effects. The UAS-GOI^{RNAi} KK lines used in this study were found to have the insertion only in the 30B landing site with diagnostic PCR genotyping^[357] (Table 4.1). However, each PCR in the Green *et al.* report was conducted only once. Therefore, it is recommended to validate KK library stocks by confirming the insertion site(s) of the UAS-RNAi transgene and corroborating observed phenotypes using a second independent RNAi stock constructed differently and with an alternative insertion site.

2.1.2.3 Mutants

Mutant stocks used in this study were sourced from the BDSC. The *dunce*¹ (#6020) mutant used in this study was generated with chemical mutagenesis with EMS feeding, causing a hypomorphic mutation and reducing *dunce* protein levels by 51%^[149]. The *synapsin*⁹⁷ (#29031) mutation was achieved with P-element activity, leading to an approximate 1400 bp deletion and an amorphic mutation^[361].

2.1.3 Fly maintenance

Flies were reared in vials on a standard yeast-based diet (In H₂O: 0.8% agar - Whole Foods Market, 2% polenta - Whole Foods Market, 8% fructose - Whole Foods Market, 10% brewer's yeast - Whole Foods Market, 0.5% of 15% nipagin in EtOH - Sigma Aldrich & 0.75% propionic acid - Sigma Aldrich) referred to as the standard food diet. In some cases, the *Drosophila* species food was supplemented with a layer of potato starch (1:5 potato starch in H₂O - 66-117, Nutri-Fly® Instant) with filter paper to provide additional surface area for pupation, named the potato starch diet. The selected rearing diet is stated in the figure legends. All flies were kept in a 25°C incubator (LMS Cooled Incubator – Model 400NP) on a light:dark (LD) cycle of 12 hours for each light phase.

2.2 Behaviour

2.2.1 Behavioural tracking

Individual flies were tracked for measuring sleep and activity using the ethoscope automated video tracking system designed in the Gilestro Laboratory (Imperial College London). The ethoscope tracks the fly movement in real-time and is constructed of 3D-printed upper and lower compartments. The upper compartment comprises a Raspberry Pi computer and infrared camera, which overlooks the lower section. The lower compartment contains an infrared light for tracking during the dark phase and can be

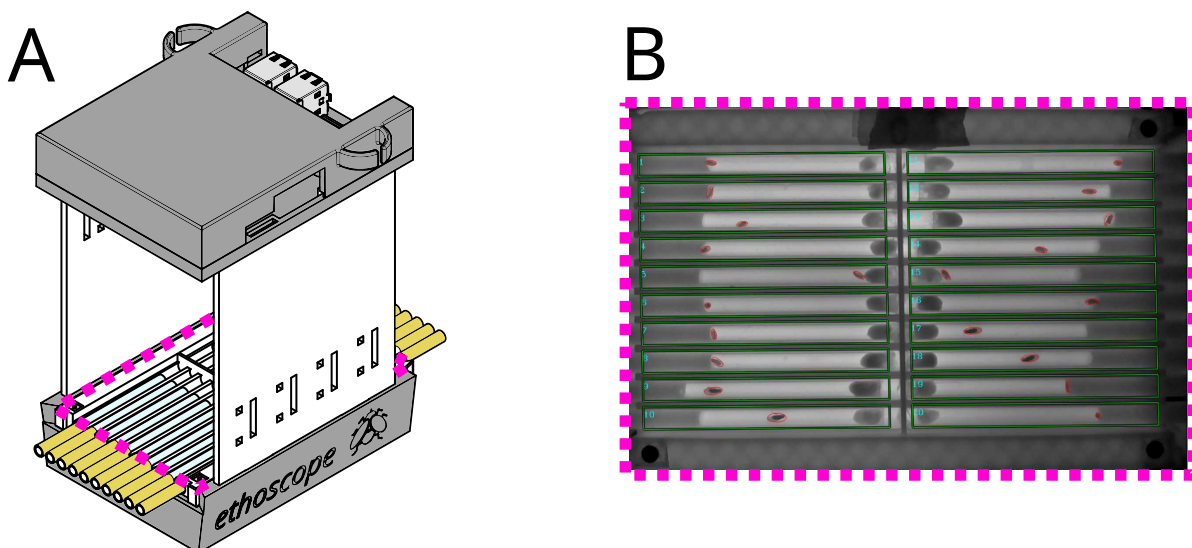


Figure 2.1: The automated video tracking ethoscope system

(A) The ethoscope system used to track the fly behaviour in real-time (B) A view from above of the arena housing 20 tubes of flies with the region of interest applied around the area of the tube (green) and the oval around the fly (red).

customised with arenas for various experimental designs. The two parts of the ethoscope are separated by laser-cut acrylic walls approximately 10 cm in height (Fig 2.1). The ethoscope software and hardware are open source, and additional information can be found in the original publication^[194] and the regularly updated user manual (<http://lab.gilest.ro/ethoscope>).

2.2.2 Experimental setup

For all sleep experiments, non-virgin 0-3 day old individual male flies were mildly anaesthetised on CO₂ and placed into glass tubes of 7 x 5 mm (length x external diameter, internal diameter = 3mm). Mated males were selected for the investigations due to the reported mortality and reduced fecundity with CO₂ exposure during the first three hours following eclosion^[362]. In each tube, standard food (unless stated otherwise) sealed with paraffin was inserted at one end with cotton wool on the opposite side. On the experimental setup day, flies were sorted on a CO₂ pad and positioned with a fine paintbrush into the tube. Each sleep arena holds up to 20 tubes for insertion into the lower ethoscope compartment. The ethoscopes housing the sleep arenas were kept in a 25°C experimental incubator (LMS Cooled Incubator – Model 210) with 50%-70% humidity on a 12:12 LD cycle (unless stated otherwise). The first day of tracking was eliminated from the data to exclude periods affected by CO₂ administration during setup, and at least one full day of baseline sleep was recorded before any manipulation.

2.2.3 Baseline sleep and activity

Detailed information on the ethoscope tracking algorithm can be found in the original publication^[194]. In summary, the flies move freely across the horizontal plane of the tube, and the ethoscope tracks their position in real-time and computes whether the individual is moving or stationary based on its horizontal position over time. Any bouts of immobility >5 min duration are classified as sleep. For the species baseline sleep and activity calculations in Fig 3.2, the sleep was tracked for 6 days and averaged over 24 hr, excluding the wild caught data, which was tracked over 1 day. All other baseline sleep in this thesis was representative of 1 day of tracking, unless stated otherwise. The initial baseline species experiments were run over the extended period of 6 days to generate a robust measure of their sleep amount and architecture throughout the day and night. The SD experiments did not require multiple days of baseline sleep tracking as the objective was to measure the sleep reduction during SD and corresponding rebound response.

The relationship between species size and sleep amount was examined by estimating individual sizes from the ethoscope tracking data and comparing these values to sleep amount. The ethoscope automated video tracking algorithm determines the fly's position in the tube by applying an ellipse shaped mask. Throughout the experiment, the width and height parameters of the ellipse are continuously tracked. To estimate fly size, the data was processed to remove outliers in the width and height that fell outside of 2 standard deviations from the individual. This outlier removal was necessary because an individual's size should remain relatively constant during tracking, and significant changes are likely due to tracking artefacts, such as when the fly is turning in the tube. Following processing, the ellipse area was calculated ($\pi * 0.5\text{width} * 0.5\text{height}$) and then plotted against sleep amount in each fly. Linear regression modelling was used to identify any trends, and Pearson's correlation coefficient was calculated to indicate the strength and direction of the relationship.

2.2.4 Waking activity

During the ethoscope computation, the maximal velocity of each animal in epochs of 10s was calculated based on the fly's horizontal position, as described in^[194]. Threshold values of the maximal velocity have been found to predict the individual's waking behaviour, validated by ground truth examination with researcher annotations. Alongside predicting immobility and mobility (for sleep and activity calculations), the maximal velocity level delimits micromoving and walking behaviour, with maximal velocity values >1 and <2.5 equating micromoving and values >2.5 walking.

2.2.5 Free-running rhythms

Baseline sleep experiments were performed in constant darkness to investigate the *Drosophila* species' endogenous circadian rhythms in the absence of light. The experiments were performed similarly to the baseline tests described above. However, rather than the standard 12:12 hr LD cycle, the flies were raised and tested in DD conditions.

2.3 Drug feeding

The species response to known *D.melanogaster* neuromodulators of sleep was investigated. The average daily sleep was calculated for each individual over 48 hr tracking on drug+vehicle or vehicle-only food. Three drugs were used in the pharmacological experiments: 5mg/ml L-dopa (Ab-cam,ab120573) in sucrose food (1% agar - Whole Foods Market and 5% sucrose - Sigma Aldrich), 0.05mg/ml CDM and 0.1mg/ml or 0.5mg/ml THIP with the latter two drugs administered in the standard food diet.

L-dopa was administered in a sucrose medium because preliminary data collected with the standard food diet revealed an attenuated response, restricted to a minimal increase in activity at the transition from light to dark. The bioavailability of a drug can be influenced by many factors, including diet and feeding conditions^[363]. The standard food diet contained yeast and polenta and it was possible that certain ingredients were impacting the absorption and distribution of the drug. L-dopa is an amino acid and therefore it is likely that the protein content in the standard diet competitively inhibited absorption of the drug, limiting its bioavailability and generating the attenuated sleep response. For this reason, I administered the L-dopa in a solely sucrose diet, comparably to other studies examining the compound.

It is possible that altering the diet in the case of the L-dopa condition in order to improve the bioavailability of the drug may have introduced another factor that can impact the physiology of the fly - protein deprivation. Sucrose only diets generate an environment of malnutrition and are known to reduce lifespan^[364]. However, the flies were only exposed to the diet for a short time frame (48 hours) and protein deprivation diets are reported to have a limited impact on total sleep. Furthermore, inconsistent results have been found, with a slight increase^[208] and decrease^[365] upon yeast deprivation observed. In any case, alterations in sleep were compared to the same sucrose only diet and therefore any changes in sleep can be attributed to the drug rather than the administered diet.

2.4 Sleep deprivation

2.4.1 Mechanical

Mechanical SD was applied with the optomotor device^[194], programmed with an immobility trigger. The optomotor is a 10-motor device and an accessory module that fits beneath the ethoscope. It is designed so that half of the tubes in the ethoscope arena experience mechanical SD while the other half are undisturbed. When a period of immobility lasting 30 sec is measured with the ethoscope automated tracking, the motor spins rapidly (approx. 400 rpm), turning the tube housing the fly and obstructing the individual's transition into sleep. The SD experiments started at ZT0 and predominantly lasted 24 hr, with the exception of the chronic SD which lasted 168 hr.

In most cases, a 1 sec stimulus has been used to prevent sleep; however, the non-melanogaster species had elevated arousal thresholds. Thus, a more prolonged stimulation lasting 3 sec was necessary. Therefore, in the mechanical SD experiments in Chapter 3 & Fig 4.2, the optomotor was programmed to rotate for 3 sec, and in the rest of Chapter 4, 1 sec. At the end of the SD period, the average sleep was calculated between ZT0-3 and compared to that of the undisturbed slept control in order to establish their rebound sleep level.

2.4.1.1 Constant darkness

24 hr mechanical SD was performed as described above but the flies were raised and the experiment was performed in constant darkness. Baseline data was recorded for 6 days before the onset of mechanical SD and the subsequent measurement of rebound.

2.4.1.2 Drug feeding

The response to mechanical SD was measured on the above drug diets. Flies were placed into tubes with the drug+vehicle food, baseline data was recorded for 4 days before the onset of mechanical SD and the subsequent measurement of rebound.

2.4.2 Social

Social SD was achieved in this study with the interactions of two, one focal and one intruder, isolated male flies over 24 hr period. On the experimental setup day, individual focal male flies were placed into tubes and loaded into ethoscopes, where 4 days of isolation occurred, tracking the day 3-4 for baseline sleep measurements. Alongside the setup of the focal male, white-eyed counterparts (i.e. the same species) were prepared;

however, the flies were placed into the same incubator as the focal males and were not loaded into the tracking device. At ZT0 of day 4, the white-eyed intruder was tipped into the focal male tube, where the two males interacted over a 24 period before careful removal of the intruder between ZT23-ZT23.75 in darkness. At each addition/removal juncture, the control tubes were inverted, tipping the fly up and down the tube to generate a mock manipulation. After intruder removal, the average sleep was calculated between ZT0-3 and compared to that of the undisturbed slept control in order to establish their rebound sleep level.

For manual scoring of the interactions between the focal and intruder male, videos were recorded at 15 min intervals of the 24 hr period at ZT0, ZT6, ZT11.75, ZT12, ZT18 and ZT23.75. Three researchers carefully scored the videos in 1 min bins based on a hierarchy of selected behaviours, chosen in accordance with the likely action of socially isolated males - fighting > moving > and immobile.

To establish the correlation between aggression during the Social SD method and rebound sleep, the fighting levels were compared to the change in sleep during the ZT0-3 rebound phase. The change in sleep was determined by calculating the difference between the mean rebound sleep value of sleep-deprived flies of each species and the mean sleep value of control flies. Linear regression modelling was used to identify any trends, and Pearson's correlation coefficient was calculated to indicate the strength and direction of the relationship.

2.5 Immunohistochemistry

Brains were dissected and stained as described in^[366]. *Drosophila* species were exposed to 24 hr mechanical SD or 24 hr social SD. In the former experiment, the flies were aged to 10 days in the 25°C stock incubator. On the morning following SD, at ZT0, flies were immobilised on ice for brain dissection in 0.1M phosphate-buffered saline (PBS). Immediately after the dissection of all brains, fixation was achieved with a 20 min incubation in 4% paraformaldehyde at room temperature before washing the brains in PBST (0.3% Triton X in PBS) for 3 x 10 min. The brains were then blocked for 1 hr in 5% normal goat serum (NGS) in PBST at room temperature before a 48 hr primary body incubation with 1:10 mouse anti-nc82 (AB_2314866, DSHB) at 4°C. The brains were rewashed in PBST for 3 x 10 min and incubated with 1:200 goat anti-mouse IgG (ab150115, abcam) in 5% NGS for another 48 hours at 4°C. A final 3 x 10 min PBST washes were completed, and the brains were mounted on a microscope slide with vectashield (Vector Laboratories) for imaging. Brains were imaged with a Leica SP8 - STELLARIS 5 Inverted Light Sheet Confocal Microscope at x40 magnification to cap-

ture z-stack images of the dorsal and ventral brain regions. For comparative analysis of the BRP expression levels, images from within the same species group were taken in one confocal session using identical laser and confocal settings. Data analysis was performed using Fiji/Image J^[367](NIH, Bethesda). The FB region was manually delimited stack by stack, generating a maximum intensity projection for analysis. Background measurements were taken from the area outside of the brain and subtracted from the region of interest. To normalise the data, an average intensity value of the mean intensity for each condition was calculated within individual microscope sessions. The plotted intensity values were then presented relative to the aforementioned average intensity value.

2.6 Statistics, data collection and visualisation

All of the ethoscope data was analysed using rethomics^[198] or python. Statistical comparisons were achieved with the Wilcoxon rank sum test with correction for the false discovery rate (FDR) and carried out in R or python. Rethomics packages used for the data analysis were as follows: behavr: 0.3.2; sleep: 0.3.0; zeitgebr: 0.3.3; ggetho: 0.3.4; scopr: 0.3.3.

The number of individuals included in each experiment is indicated in the figures. Each individual repeat typically contained 10 individuals for each species and experiments were composed of no less than two independent repeats.

Linear regression analysis was modelled by computing the line-of-best fit that minimises the sum of the squared differences between observed values and the line. The strength and direction of the linear relationship was determined with the Pearson’s correlation coefficient.

The significance of statistical comparisons is indicated in the figures with * = p-value < 0.05, ** = p-value < 0.01 , and *** = p-value < 0.001.

The sleep and activity profiles show the proportion of time spent sleeping or moving averaged for each animal within 30 min. The solid lines represent the mean, and the shaded boundaries indicate the 95% bootstrapped confidence intervals. In all boxplots, the midline indicates the median, the upper and lower limits of the box (hinges) represent the first and third quartiles, and the whiskers extend to the largest and lowest values within a 1.5 interquartile range from the hinges.

All data presented in this work was performed by the author, excluding the species baseline data in Fig 3.2 which was collected by Dr Alice French. The L-dopa drug feeding baseline experiments (Fig 3.16B) and the recording of videos during the social

SD for quantification of the fighting levels (Fig 3.8) was undertaken in collaboration with Dr Alice French. The analysis of the data was undertaken by the author.

Chapter 3

Sleep and homeostasis in *Drosophilids*

3.1 Background

Observations of sleep across disparate species, from terrestrial mammals to CNS-less cnidarians, have raised two critical findings. Firstly, sleep in the animal kingdom is seemingly universal, having persisted under many selective pressures throughout evolution, suggesting a common and fundamental function of sleep. Secondly, sleep is highly variable- some animals need a reported 2 hours of sleep a night (African elephants, giraffes), and others spend most of the day sleeping (big brown bat)^[79]. There is still no consensus on the specific function of sleep^[10], and any such explanation must address the variability across the animal kingdom.

While sleep's function remains ambiguous, the regulation of sleep is widely accepted to be governed by a two-step process of circadian and homeostatic control that together determines the timing, amount, and quality of sleep^[11]. The circadian aspects of sleep control are well-established in both mammals and *D.melanogaster*. The clock cells and molecular mechanisms that determine circadian timekeeping^[368] and their output to control sleep are well established^[369]. Contrastingly, the homeostatic process that controls sleep is still largely mysterious. Conceptually, the homeostatic mechanism is partly driven by a build-up of sleep need during wake that, at peak levels, results in the initiation of sleep where the accumulated sleep need is then dissipated. It is often considered that the essential biological process that sleep provides is encoded in the homeostat. If we understand what is underlying the increased sleep need and why it must be dissipated, we can elucidate its essential function.

Work in *Drosophila* has uncovered many of the mechanistic underpinnings of sleep homeostasis, more so than any other animal^[370]. However, it is becoming increasingly clear that there are likely differential context-dependent homeostatic mechanisms. Different SD methods elicit contrasting results regarding their homeostatic rebound response and the accumulation of sleep need^{[201][108]}. For example, the activation of differential wake-promoting circuits in the fly brain has a varied response on sleep need and the execution of rebound^[371]. This highlights the variability seen within a single species regarding sleep homeostasis. The *Drosophila* genus represents a potential playground for studying sleep from an evolutionary perspective and may shed light on the control of sleep and, ultimately, its enigmatic function.

In this chapter, I incorporated a comparative approach to the study of sleep variability and homeostasis of 7 *Drosophila* species that represent closely and distantly related species from different ecological niches (Fig1.1). Two cosmopolitan (*D.melanogaster* & *D.simulans*), three tropical (*D.sechellia*, *D.erecta* & *D.yakuba*), one subtropical (*D.willistoni*) and one temperate (*D.virilis*) species were used in this study. The panel contained a selection of generalists and specialists, with the *D.secheillia* species known to oviposit and feed on highly toxic *Morinda* fruit, *D.erecta* on *Pandanus* fruit^[327], and *D.virilis* on the slime flux of decaying trees^[334]. The other species can breed in a wide range of rotting fruit^[327]. Firstly, I compared the baseline and circadian aspects of sleep and activity. Secondly, the panel's response to agonist drugs of octopamine and GABA and a dopamine precursor was interrogated to establish if the neurochemical underpinnings of sleep are conserved. Finally, I probed the flies' homeostatic response with mechanical and social SD methods in order to assess whether there was a divergence in the sleep homeostatic mechanism.

3.2 Results

3.2.1 Baseline sleep and activity

In animals with the circadian control of sleep, the circadian clock determines the daily synchronisation and timing of sleep and activity^[228]. I first interrogated their baseline sleep aspects using our automated video-tracking device, the ethoscope (Fig 2.1). Observing their sleep, activity, waking behaviours, and response to drug feeding.

3.2.1.1 Sleep and activity

Wild-type *D.melanogaster* Canton-S male sleep and activity followed a robust pattern of a mid-afternoon siesta sleep, crepuscular peaks in activity, and consolidated night

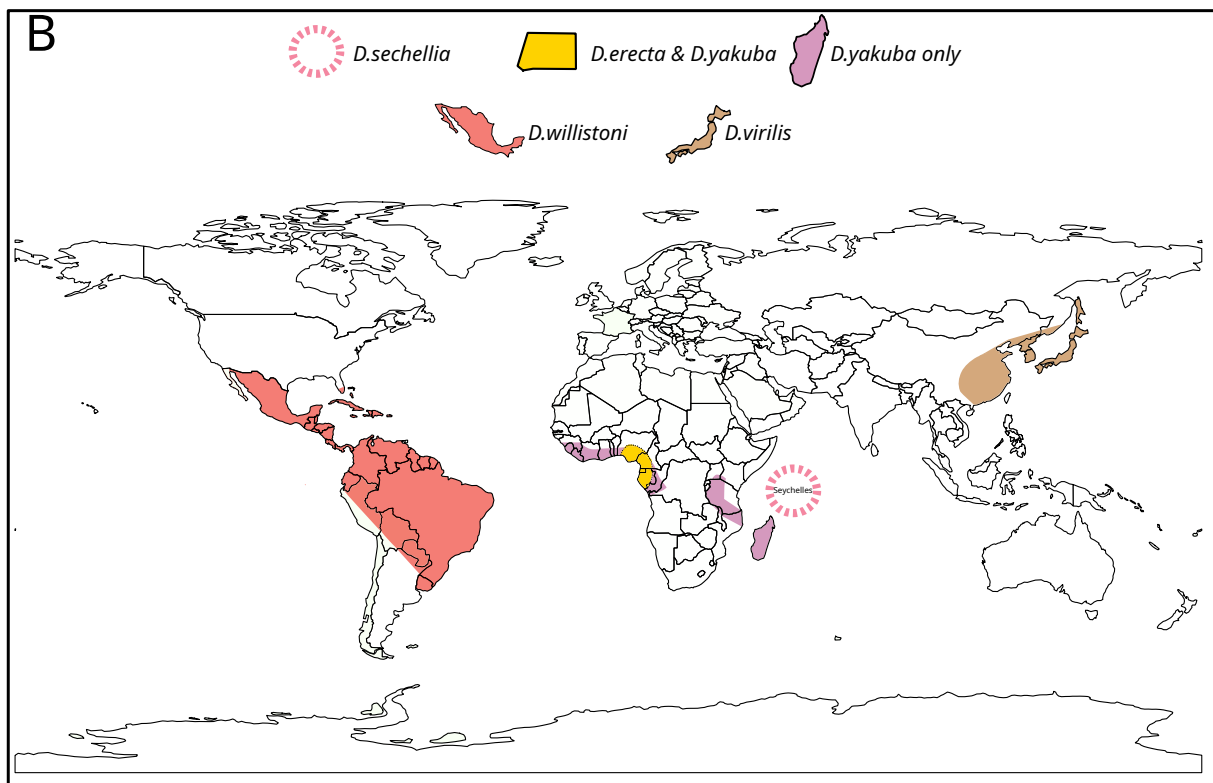
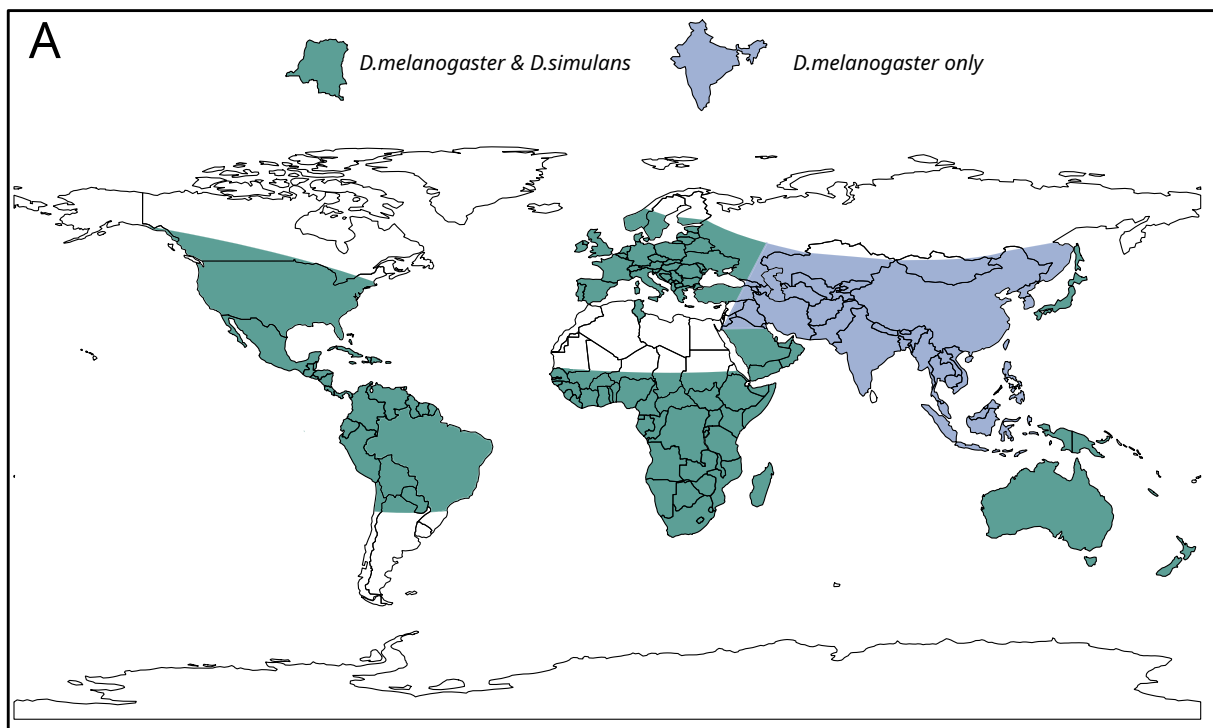


Figure 3.1: The panel of 7 *Drosophila* species approximate global distribution
 (A) Global distribution of two closely related cosmopolitan species of the *Drosophila* genus - *D.melanogaster* & *D.simulans* (B) Global distribution of *D.sechellia*, *D.erecta*, *D.yakuba*, *D.willistoni* & *D.virilis*

sleep (Fig 3.2A,B dark blue solid line). They slept modestly more during the night phase ($\text{asleep}_{\text{DAY}} = 342$ min, $\text{CI}_{95\%} = [329,355]$ against $\text{asleep}_{\text{NIGHT}} = 362$ min, $\text{CI}_{95\%} = [334,391]$, $p = 0.0024$) (Fig 3.4C, dark blue), but the principal distinction in terms of sleep behaviour was found in their sleep bout duration with significantly longer bouts in the evening ($\text{duration}_{\text{DAY}} = 9.2$ min, $\text{CI}_{95\%} = [8.9,9.4]$ against $\text{duration}_{\text{NIGHT}} = 11$ min, $\text{CI}_{95\%} = [10.4,11.3]$, $p\text{-value} = 1.2 \times 10^{-10}$) (Fig 3.6A, dark blue). These aspects of sleep behaviour were echoed in a second *D.melanogaster* wild-type strain, Oregon-R, which had comparable levels of day and night sleep to Canton-S ($\text{asleep}_{\text{DAY}} = 373$ min, $\text{CI}_{95\%} = [349,397]$, $p\text{-value} = 0.055$ & $\text{asleep}_{\text{NIGHT}} = 342$ min, $\text{CI}_{95\%} = [292,392]$, $p\text{-value} = 0.50$) (Fig 3.2A, dark blue dashed line). Alongside the wild-type lab strains, I introduced a true wild *D.melanogaster* species which I termed wild caught, collected from Edinburgh in 2021 by Darren Obbard. Overall, the wild caught flies shared the described behavioural aspects of wild-type sleep in terms of their siesta, crepuscular peaks (Fig 3.2A,B light blue), and night sleep (Fig 3.4C, light blue). In order to determine whether the baseline characteristics are shared in the other *Drosophila* flies, I first tested two strains of each species for their daily sleep and activity pattern to establish that the sleep behaviour was species, rather than strain, specific (Fig 3.4A,B).

The midday siesta prevailed in all the species and was common to both strains (Fig 3.4A). In *D.yakuba* and *D.willistoni*, however, the proportion of sleep during the day was significantly higher in the second strain (dashed line). The *D.yakuba* strains had an average of 176 min difference in daytime sleep amount ($\text{asleep}_{\text{STRAIN1}} = 195$ min, $\text{CI}_{95\%} = [173,218]$ against $\text{asleep}_{\text{STRAIN2}} = 372$ min, $\text{CI}_{95\%} = [325,419]$, $p\text{-value} = 4.2 \times 10^{-8}$), while the *D.willistoni* strains had a 104 min difference ($\text{asleep}_{\text{STRAIN1}} = 204$ min, $\text{CI}_{95\%} = [182,226]$ against $\text{asleep}_{\text{STRAIN2}} = 308$ min, $\text{CI}_{95\%} = [271,345]$, $p\text{-value} = 5.4 \times 10^{-6}$). All of the strains had a distinctive crepuscular activity elevation in anticipation of lights off between ZT9-12 prior to a steep decline in activity as the species entered their first bout of nighttime sleep (Fig 3.2B). After the preliminary examinations of the *Drosophila* sleep confirmed that two strains of each species (excluding *D.erecta*, of which I could only source one strain) displayed the basic qualities of *D.melanogaster* sleep, I conducted further analyses with the first strain (solid line). The Canton-S wild-type was selected for further study due to its common use in the Gilestro lab. The other strains were either selected arbitrarily or because of their enhanced viability under the laboratory conditions employed in the investigations.

To further evaluate the activity over the day, I calculated the time spent moving into 2 hr segments over 24 hr (Fig 3.2C). In all the species, the activity in the initial morning ZT0-2 bout was significantly higher than during the subsequent ZT2-4 time frame, and

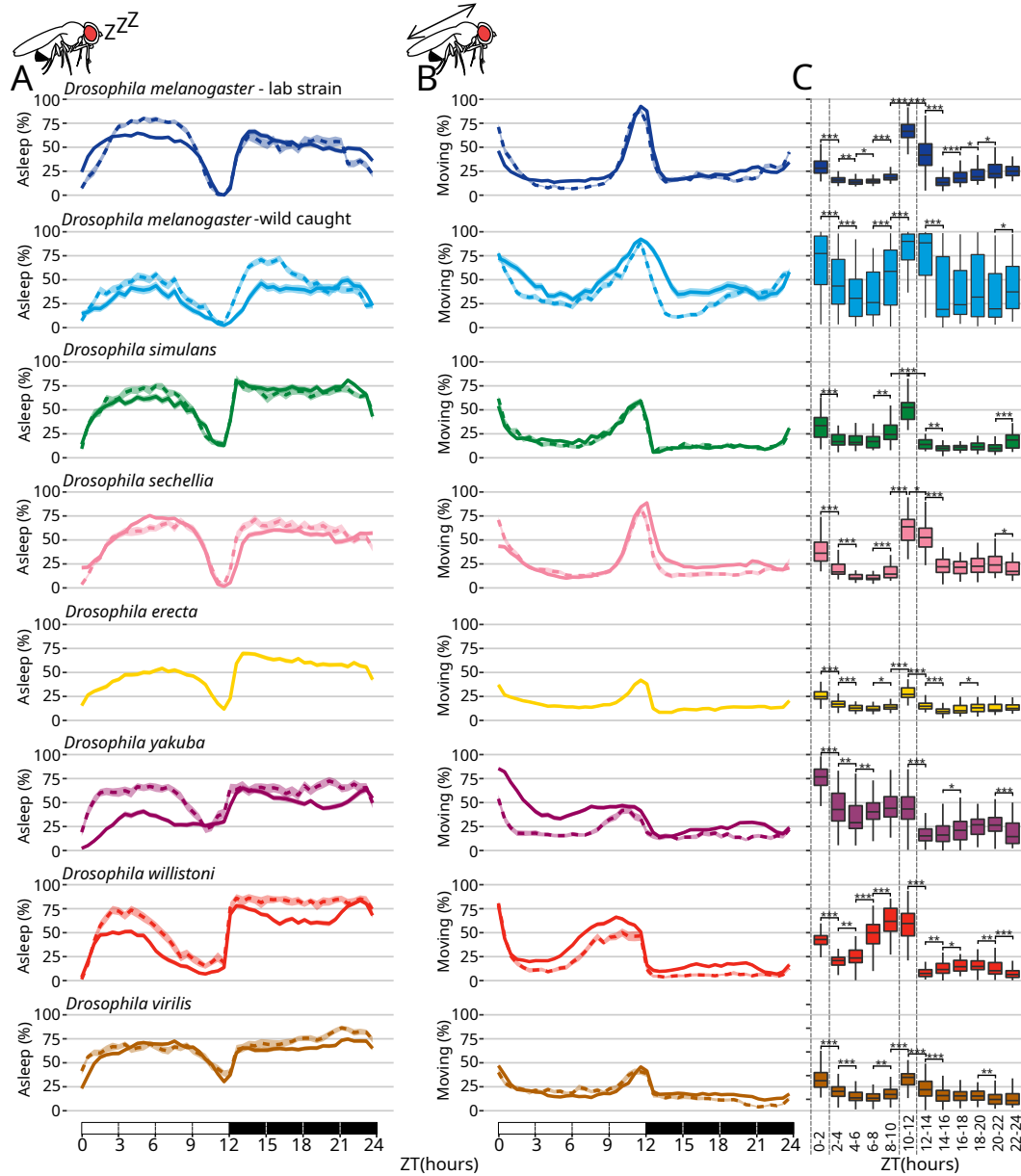


Figure 3.2: Daily baseline sleep and activity in 7 *Drosophila* species

(A) 24 hr sleep profile of 2 strains of *D. melanogaster* lab strain (dark blue: solid line = Canton-S, n = 80 & dashed line = Oregon-R, n = 26), *D. melanogaster* wild caught (light blue: solid line n = 69 & dashed line n = 68), *D. simulans* (green: solid line n = 31 & dashed line n = 20), *D. sechellia* (pink: solid line n = 55 & dashed line n = 22), *D. erecta* (yellow: solid line n = 59), *D. yakuba* (purple: solid line n = 73 & dashed line = 24), *D. willistoni* (red: solid line n = 76 & dashed line = 21) & *D. virilis* (brown: solid line n = 80 & dashed line = 26) (B) 24 hr activity profile (C) Average activity in 2 hour bins. All flies were raised on the potato starch diet and tested on standard food. * = < 0.05, ** = < 0.01 & *** = < 0.001. The duration of the sleep and activity tracking was 6 days in all species apart from *D. melanogaster* wild caught, where the tracking lasted 24 hours. The plots are representative of a minimum of 2 repeats. Wilcoxon Rank Sum Test was used for the statistical comparisons in C.

the highest activity level occurred in the ZT10-12 period. This confirmed that the panel exhibited the crepuscular activity commonly found in *Drosophila*. In *D. willistoni* and *D. yakuba*, however, the anticipatory activity began to reach its maximum in the preceding ZT8-10 period. However, it remained elevated until lights off at ZT12 (*D. yakuba*: activity_{ZT8-10} = 54 min, CI_{95%} = [50,59] against activity_{ZT10-12} = 54 min, CI_{95%} = [49,59], p-value = 0.77 & *D. willistoni*: activity_{ZT8-10} = 74 min, CI_{95%} = [69,79] against activity_{ZT10-12} = 70 min, CI_{95%} = [66,74], p-value = 0.10) (Fig 3.2C).

I had demonstrated that the activity peaks associated with circadian sleep and activity cycles were primarily shared across the panel in LD conditions, however, free-running activity oscillations in the absence of light are a core component of circadian rhythms^[372]. In order to investigate the endogenous circadian activity, I probed the period length in constant darkness (Fig 3.3A-B). In *D. melanogaster* wild caught, there was a strong peak in activity that occurred with a periodicity of 24.2 hr (CI_{95%} = [23.9,24.4]), and therefore, the characteristic circadian rhythm was maintained. An equivalent period was found in *D. erecta* and *D. willistoni*, with a periodicity of 24.1 hr (CI_{95%} = [23.7,24.5], p-value = 0.41) and 23.9 (CI_{95%} = [23.6,24.1], p-value = 0.18), respectively. *D. simulans* period of 24.3 hr (CI_{95%} = [24.1,24.5], p-value = 0.050) was modestly elevated when compared to *D. melanogaster* wild caught, and *D. yakuba* exhibited a longer free-running period of 25.3 hr (CI_{95%} = [25.0,24.7], p-value = 1.3×10^{-9}). Conversely, *D. sechellia* featured a shorter period of 23.3 hr (CI_{95%} = [23.2,23.4], p-value = 9.7×10^{-11}). In DD conditions, most of the *D. virilis* flies were arrhythmic (89.9%), and only three individuals exhibited rhythmic activity. Moreover, the power of the amplitude of their peak above the significance threshold was 9 (CI_{95%} = [0,27]). For comparison, the *D. melanogaster* wild caught peak had a power of 116 (CI_{95%} = [103,129]) (Fig 3.4C).

Total daily sleep duration exhibited variability across the panel, and only the *D. erecta* and *D. willistoni* species had comparable daily levels of sleep to the *D. melanogaster* lab strain (Fig 3.4A,B). Sleeping 712 min (CI_{95%} = [681,743], p-value = 0.54), 693 min (CI_{95%} = [622,723], p-value = 0.86) and 704 min (CI_{95%} = [671,736]) respectively. *D. simulans* and *D. virilis* required more sleep, sleeping 844 min (CI_{95%} = [798,890], p-value = 0.00092) and 889 min (CI_{95%} = [859,920], p-value = 1.8×10^{-13}) respectively. The *D. melanogaster* wild caught flies needed the least sleep, of 454 min (CI_{95%} = [393,515], p-value = $< 2.0 \times 10^{-16}$) (Fig 3.4A,B). Interrogation of the distribution of sleep over the day provided more detail on Drosophilid sleep (Fig 3.4C). Firstly, significantly increased sleep was observed during the night compared to the day in all species, apart from *D. sechellia* (asleep_{DAY} = 357 min, CI_{95%} = [338,376] against asleep_{NIGHT} = 375 min, CI_{95%} = [349,402], p-value = 0.11). *D. sechellia* sleep distribution most closely resembled the *D. melanogaster* lab strain, with no significant difference in the two species

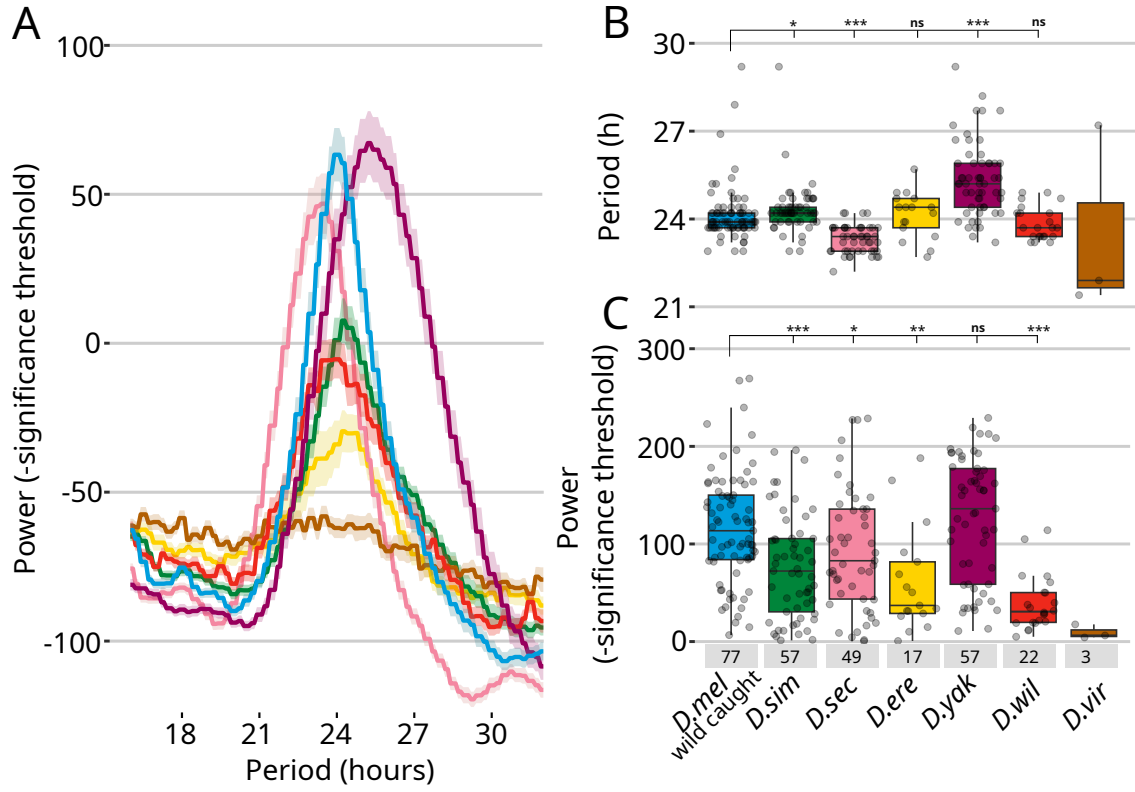


Figure 3.3: Daily period of 7 *Drosophila* species in constant darkness
(A) chi-square x^2 periodogram averaged over 5 days of *D.melanogaster* wild caught (light blue), *D.simulans* (green), *D.sechellia* (pink), *D.erecta* (yellow), *D.yakuba* (purple), *D.willistoni* (red) & *D.virilis* (brown) activity in DD **(B)** Period of the first peak **(C)** Amplitude of the peak above the significance threshold. All flies were raised on the potato starch diet and tested on standard food. * = < 0.05, ** = < 0.01 & *** = < 0.001. The duration of tracking lasted 6 days. The plots are representative of a minimum of 2 repeats. Wilcoxon Rank Sum Test was used for the statistical comparisons in B & C.

day (asleep_{MELs} = 342 min, CI_{95%} = [329,355] against asleep_{SEC} = 357 min, CI_{95%} = [338,376], p-value = 0.10) and night sleep (asleep_{MELs} = 363 min, CI_{95%} = [334,391] against asleep_{SEC} = 375 min, CI_{95%} = [349,402], p-value = 0.89). While *D.erecta* and *D.willistoni* had comparable total sleep to *D.melanogaster* lab strain, the distribution over the light phases was distinct. *D.erecta* slept almost 1 hr less during the day compared to *D.melanogaster* lab strain (asleep_{MELs} = 342 min, CI_{95%} = [329,355] against asleep_{ERE} = 288 min, CI_{95%} = [269,306], p-value = 9.4×10^{-6}) and regained this during the night (asleep_{MELs} = 363 min, CI_{95%} = [334,391] against asleep_{ERE} = 426 min, CI_{95%} = [408,445], p-value = 0.0038). There was a striking disparity with *D.willistoni*, which had one of the lowest levels of daytime sleep and the highest amounts of nighttime sleep (asleep_{DAY} = 204 min, CI_{95%} = [182,226] against asleep_{NIGHT} = 495 min, CI_{95%} = [468,519], p-value = 9.3×10^{-19}). *D.willistoni* daytime sleep was comparable to *D.yakuba* (asleep_{WIL} = 204 min, CI_{95%} = [182,226] against asleep_{YAK} = 195 min, CI_{95%} = [173,218], p-value = 0.80) and *D.melanogaster* wildcaught (asleep_{WIL} = 204 min, CI_{95%} = [182,226] against asleep_{MELwc} = 185 min, CI_{95%} = [151,219], p-value = 0.32). Their nighttime sleep was comparable to the longer sleeping *D.virilis* (asleep_{WIL} = 495 min, CI_{95%} = [468,519] against asleep_{VIR} = 472 min, CI_{95%} = [451,494], p-value = 0.092) and *D.simulans* (asleep_{WIL} = 495 min, CI_{95%} = [468,519] against asleep_{SIM} = 506 min, CI_{95%} = [481,531], p-value = 0.95) flies (Fig 3.4C). These results demonstrated the considerable diversity in *D.willistoni* sleep over the day and night.

Metabolic rate is closely related to body weight and scales with body size, with larger animals having lower metabolic rates than that of smaller organisms^[373]. Metabolic rate is found to be reduced during sleep as measured by oxygen consumption in both mammals and *Drosophila*^[374]. To conduct a preliminary investigation to assess the relationship between sleep amount and metabolic rate across the *Drosophila* species, I compared individual size to total sleep amount (Fig 3.5). The ethoscopes automated tracking algorithm applies an ellipse shape over the fly and measures its width and height, which can be processed to calculate the area of the ellipse and act as a proxy for measuring differences in species size. Initially, there appeared to be a weakly positive correlation between sleep amount and mean ellipse area ($r = 0.39$, p-value = 3.66×10^{-29}) (Fig 3.5A). However, upon further examination, this relationship was non-existent on removal of the *D.virilis* group from the analysis. Despite variation in size among the other species investigated linear regression analysis did not uncover any significant correlation between size and sleep patterns ($r = 0.06$, p-value = 0.085) (Fig 3.5B). Therefore, it appears that the differences in species size alone is insufficient to account for the range of daily sleep duration among the studied species. The larger size of *D.virilis* may restrict their movement within the experimental tube, potentially leading

to the observed correlation between size and sleep within this species as an artefact of limited mobility. However, the relationship between metabolic rate and body size is not linear; this is a general rule, and variations in metabolic rate have been identified even in species of comparable size^[375]. The differences observed in size among the *Drosophila* species do not explicitly determine changes in metabolic rate between these species. Therefore, the data presented here serve as a preliminary assessment, indicating that variations in size across the majority of the panel do not account for the observed sleep differences.

Investigating the *Drosophila* genus' total daily sleep and distribution over the light phases revealed that the panel reflects the variability of sleep duration seen commonly within the animal kingdom, with signs of diverse sleep drives.

D.melanogaster are diurnal animals that sleep significantly more during the night than the day^[376]. This was echoed in the current study, with almost all species sleeping significantly more during the dark phase (Fig 3.4C). Night sleep often has a different architecture associated with more consolidated bouts of increased length and, in some cases, decreased number^[377]. While there was variation in total sleep amount across the panel, the nighttime elevation in sleep bout length compared to daytime was maintained (Fig 3.6A,C). *D.melanogaster*, *D.simulans*, *D.sechellia*, *D.erecta*, *D.yakuba*, *D.willistoni*, and *D.virilis* all exhibited bouts of longer length during the night, while variable results were seen in terms of the bout frequency. Decreased bout number was only found in the *D.melanogaster* lab strain (number_{DAY} = 37, CI_{95%} = [36,38] against number_{NIGHT} = 31, CI_{95%} = [29,34], p-value = 9.3×10^{-5}) and *D.sechellia* (number_{DAY} = 29, CI_{95%} = [27,31] against number_{NIGHT} = 26, CI_{95%} = [24,27], p-value = 0.0066) (Fig 3.6B).

3.2.1.2 Waking behaviours

In addition to examining activity, I delineated their waking behaviour into walking and micromoving to establish whether there were changes in the Drosophilid activity profile. Micromovements captured smaller behaviours when a fly was stationary in the tube, for example, grooming or feeding. Generally examining the panels waking behaviour in this way revealed that micromovements only accounted for a small amount of the species' daily behaviour, with the majority of the panel performing greater levels of walking. *D.erecta* and *D.virilis* were the exceptions to this rule, and both spent more time carrying out smaller micromovements over the 24 hr day (Fig 3.7).

In the *D.melanogaster* lab strain, micromoving levels remained relatively constant over the day and only accounted for 157 min (CI_{95%} = [142,171]) of their daily activity (Fig 3.7A,B). The walking behaviour was significantly higher than micromovements

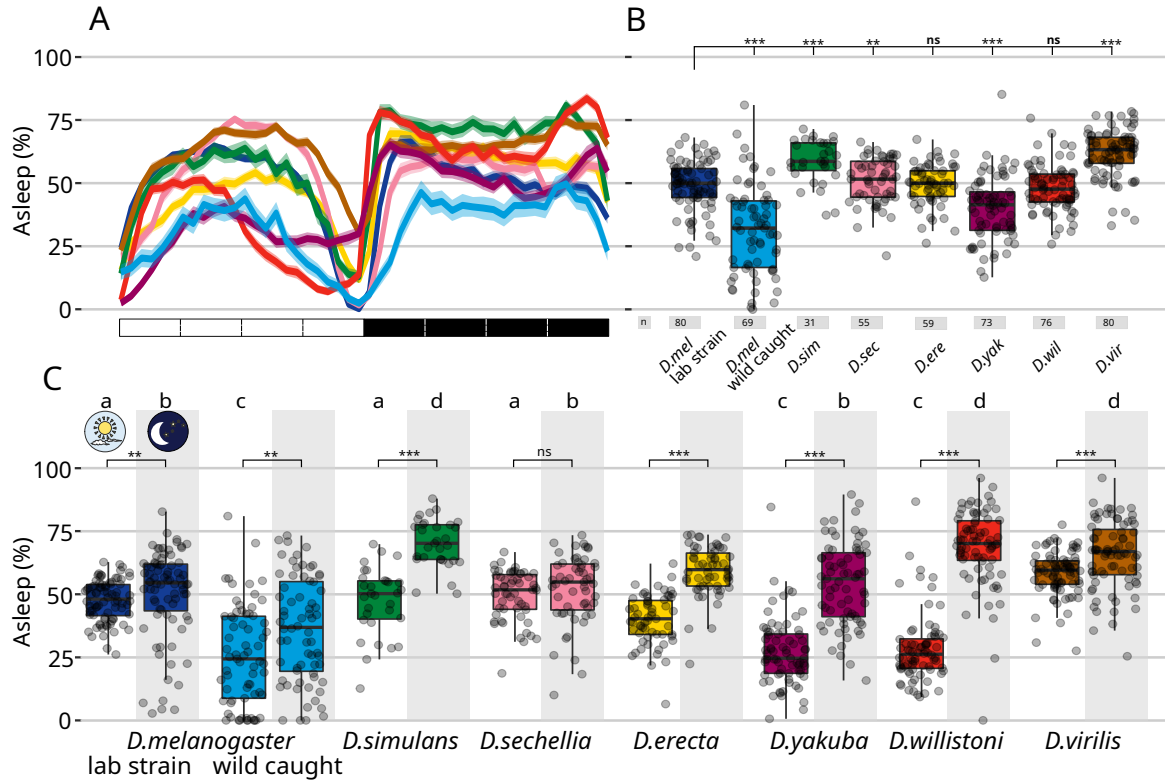


Figure 3.4: Comparison of sleep amount over the entire day and night (A) Strain 1 sleep profile of *D. melanogaster* lab strain (dark blue), *D. melanogaster* wild caught (light blue), *D. simulans* (green), *D. sechellia* (pink), *D. erecta* (yellow), *D. yakuba* (purple), *D. willistoni* (red) & *D. virilis* (brown) (B) Quantification of average total sleep over the 24 hr day (C) Quantification of average sleep over the day and night. All flies were raised on the potato starch diet and tested on standard food. * = < 0.05, ** = < 0.01 & *** = < 0.001. The letters at the top of (C) signify statistically insignificant comparisons and therefore equal sleep levels. The duration of the tracking was 6 days in all species apart from *D. melanogaster* wild caught, where the tracking lasted 24 hours. The plots are representative of a minimum of 2 repeats. Wilcoxon Rank Sum Test was used for the statistical comparisons in B & C.

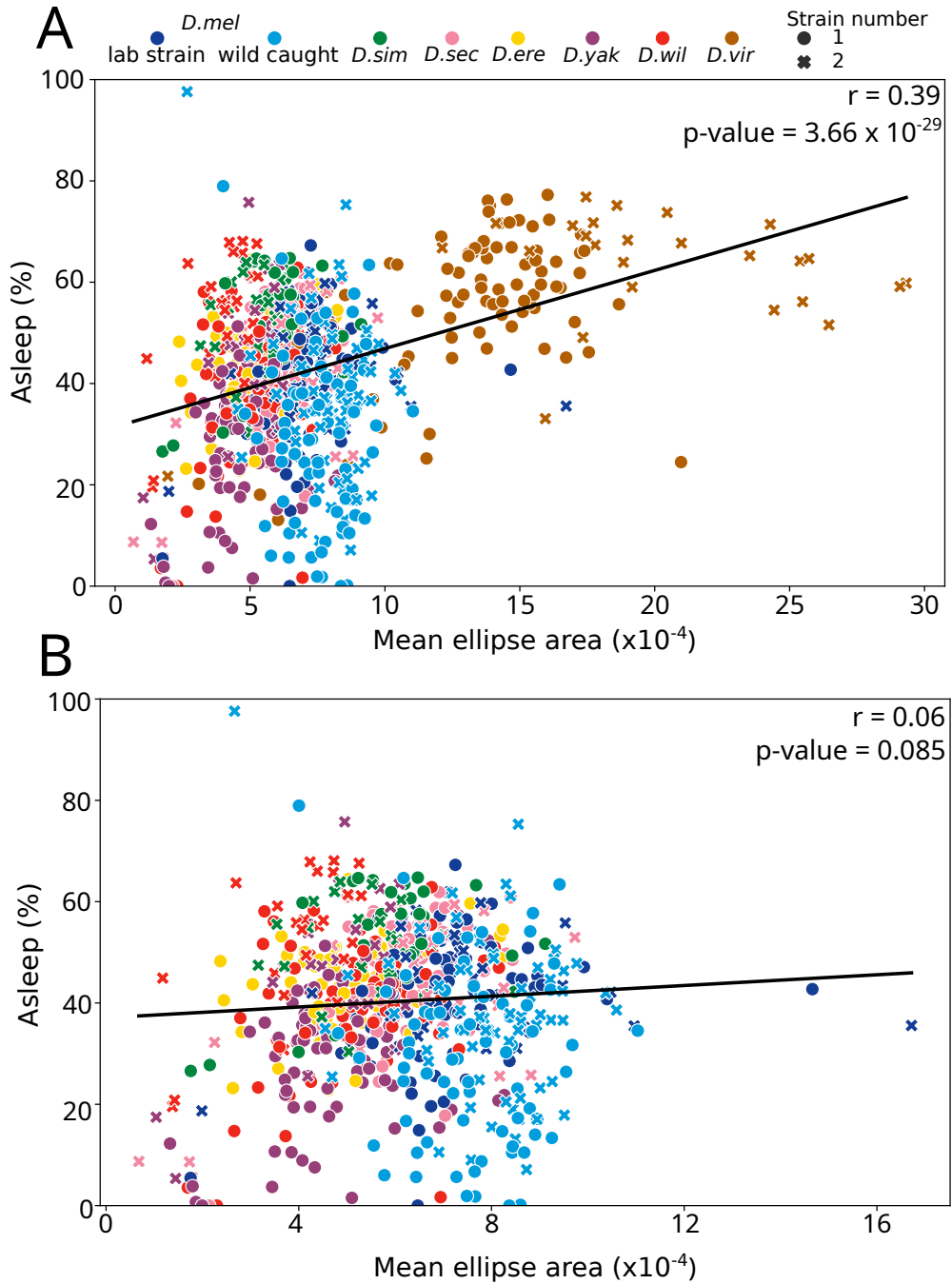


Figure 3.5: The relationship between species size and sleep amount

(A) Linear regression analysis of the relationship between species size as estimated from the mean ellipse size generated from the ethoscope tracking data and sleep for each individual from strain 1 (circle) and strain 2 (cross) of the *D. melanogaster* lab strain, *D. melanogaster* wild caught (light blue), *D. simulans* (green), *D. sechellia* (pink), *D. erecta* (yellow), *D. yakuba* (purple), *D. willistoni* (red) & *D. virilis* (brown) species (B) Linear regression analysis with *D. virilis* removed. The strength and direction of the relationship was quantified using Pearson's correlation coefficient (r). All flies were raised on the potato starch diet and tested on standard food. The duration of the tracking was 6 days in all species apart from *D. melanogaster* wild caught, where the tracking lasted 24 hours. The plots are representative of a minimum of 2 repeats.

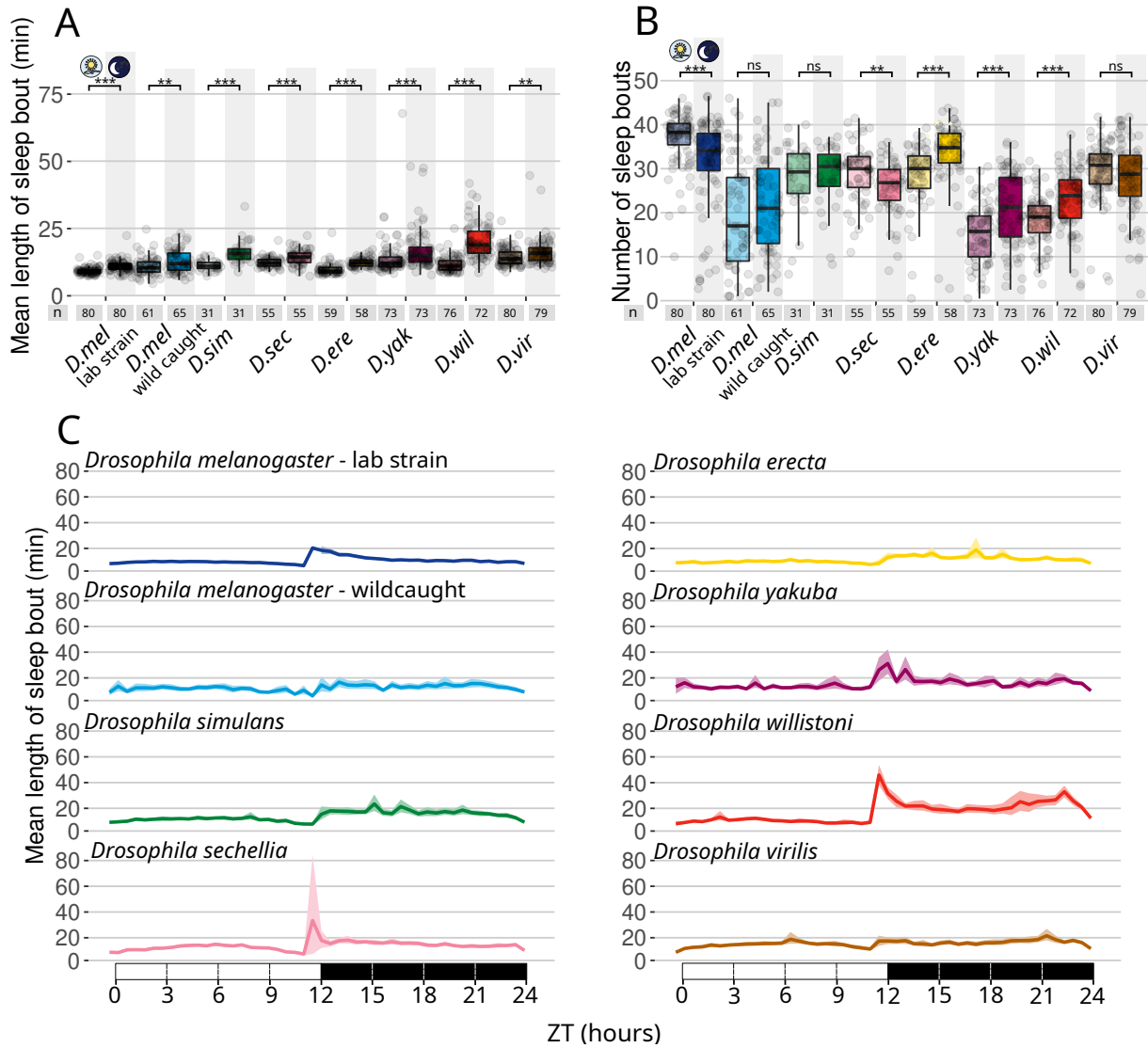


Figure 3.6: Baseline sleep architecture in the 7 *Drosophila* species

(A) Mean duration of sleep bouts during the day (no shading) and night (grey shading) in *D.melanogaster* lab strain, *D.melanogaster* wild caught (light blue), *D.simulans* (green), *D.sechellia* (pink), *D.erecta* (yellow), *D.yakuba* (purple), *D.willistoni* (red) & *D.virilis* (brown) (B) The number of sleep bouts during the day and the night (C) Sleep bout duration profile over the 24hr day. All flies were raised on the potato starch diet and tested on standard food. * = < 0.05 , ** = < 0.01 & *** = < 0.001 . The duration of tracking was 6 days in all species apart from *D.melanogaster* wild caught, where the tracking lasted 24 hours. The plots are representative of a minimum of 2 repeats. Wilcoxon Rank Sum Test was used for the statistical comparisons in A & B.

when quantified over 24 hr with a walking total of 242 min ($CI_{95\%} = [221,263]$, p-value = 6.9×10^{-16}); however, this was mainly due to the large peak in walking at the light to dark transition (Fig 3.7A-C). During the ZT hours 11-13, *D.melanogaster* lab strain spent 82 min walking ($CI_{95\%} = [79,85]$) and 11 min micromoving ($CI_{95\%} = [10,11]$, p-value = $< 2.0 \times 10^{-16}$) demonstrating that elevated walking was responsible for the evening crepuscular activity peak. A similar crepuscular appearance was observed with the *D.melanogaster* wild caught flies, where walking was responsible for the elevated activity (walking_{MELwc} = 98 min, $CI_{95\%} = [8,11]$ against micromoving_{MELwc} = 7 min, $CI_{95\%} = [5,8]$, p-value = $< 2.0 \times 10^{-16}$) (Fig 3.7A,C). However, compared to the lab strain, wild caught flies devoted more time to walking over the entire day (walking_{MELls} = 242 min, $CI_{95\%} = [221,263]$ against walking_{MELwc} = 589 min, $CI_{95\%} = [537,642]$ p-value = $< 2.0 \times 10^{-16}$) and also during ZT11-13 (walking_{MELls} = 82 min, $CI_{95\%} = [79,85]$ against walking_{MELwc} = 98 min, $CI_{95\%} = [91,104]$ p-value = 3.0×10^{-8}) (Fig 3.7D,E). At the same time, fewer minutes were devoted to micromoving over the day (micromoving_{MELls} = 242 min, $CI_{95\%} = [221,263]$ against micromoving_{MELwc} = 589 min, $CI_{95\%} = [537,642]$ p-value = 2.4×10^{-10}) and ZT 11-13 (micromoving_{MELls} = 242 min, $CI_{95\%} = [221,263]$ against micromoving_{MELwc} = 589 min, $CI_{95\%} = [537,642]$ p-value = 7.1×10^{-12}) (Fig 3.7D,E). This demonstrates that the elevated activity in the wild caught flies was due to increased locomotion.

The *D.sechellia* activity profile most resembled that of *D.melanogaster* lab strain, with elevated total walking compared to micromoving (walking_{SEC} = 276 min, $CI_{95\%} = [252,301]$ against micromoving_{SEC} = 130 min, $CI_{95\%} = [116,145]$, p-value = 7.2×10^{-15}) that was primarily due to the crepuscular activity peak, where walking predominates (walking_{SEC} = 83 min, $CI_{95\%} = [79,87]$ against micromoving_{SEC} = 9 min, $CI_{95\%} = [8,10]$, p-value = $< 2.0 \times 10^{-16}$) (Fig 3.7A-C). During ZT11-13, the *D.sechellia* flies walking (walking_{SEC} = 83 min, $CI_{95\%} = [79,87]$ against walking_{MELls} = 82 min, $CI_{95\%} = [79,85]$, p-value = 0.55) and micromoving (micromoving_{SEC} = 9 min, $CI_{95\%} = [8,10]$ against micromoving_{MELls} = 11 min, $CI_{95\%} = [10,11]$, p-value = 0.057) levels were indistinguishable from that of the *D.melanogaster* lab strain (Fig 3.7D,E).

D.simulans spent comparable amounts of time walking and micromoving over the 24 hr day (walking_{SIM} = 177 min, $CI_{95\%} = [139,215]$ against micromoving_{SIM} = 167 min, $CI_{95\%} = [135,198]$, p-value = 0.77) (Fig 3.7A,B). The total micromovement levels were similar to the *D.melanogaster* lab strain (micromoving_{SIM} = 167 min, $CI_{95\%} = [135,198]$ against micromoving_{MELls} = 157 min, $CI_{95\%} = [142,171]$, p-value = 0.89) while walking levels were significantly lower (walking_{SIM} = 177 min, $CI_{95\%} = [139,215]$ against walking_{MELls} = 242 min, $CI_{95\%} = [221,263]$, p-value = 4.7×10^{-5}) (Fig 3.7D). When looking at the population walking behaviour over the 24 hr day and quantified during the ZT11-

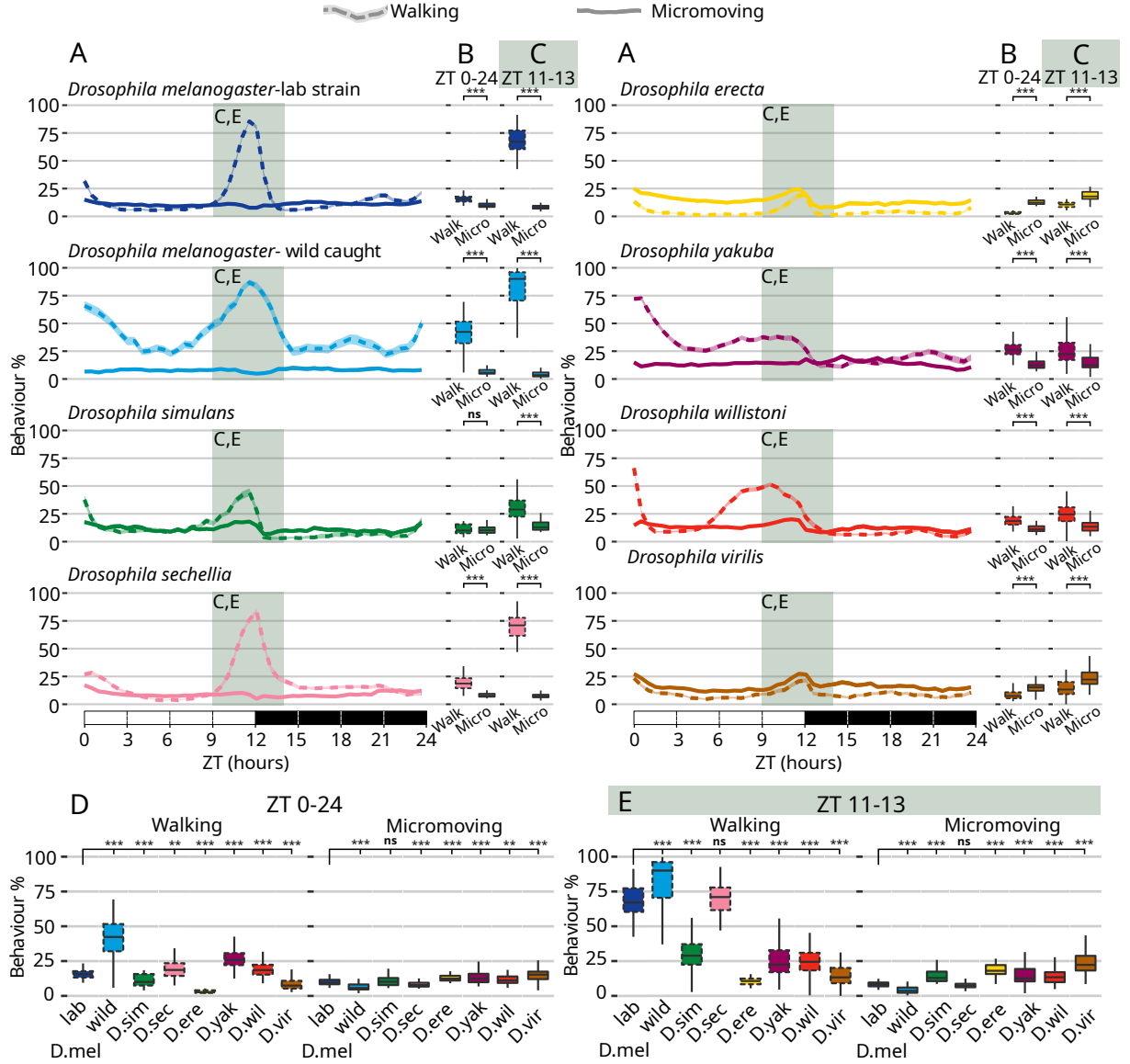


Figure 3.7: The time allocated to walking and micromoving during activity (A) Walking (dashed line) and micromoving (solid line) profile over the 24hr day in *D. melanogaster* lab strain (dark blue), *D. melanogaster* wild caught (light blue), *D. simulans* (green), *D. sechellia* (pink), *D. erecta* (yellow), *D. yakuba* (purple), *D. willistoni* (red) & *D. virilis* (brown) (B) Quantification of the walking and micromoving levels over the entire day and (C) the light to dark transition period ZT11-13 (green shading) (D) The species walking and micromoving levels compared to the *D. melanogaster* lab strain over the entire day or (E) ZT11-13. All flies were raised on the potato starch diet and tested on standard food. * = < 0.05, ** = < 0.01 & *** = < 0.001. The duration of the tracking was 6 days in all species apart from *D. melanogaster* wild caught, where the tracking lasted 24 hours. The plots are representative of a minimum of 2 repeats. Wilcoxon Rank Sum Test was used for the statistical comparisons in B, C, D & E.

13 period (Fig 3.7A,C), it became clear that the reduction in walking was due to a diminished walking peak compared to *D.melanogaster* (walking_{SIM} = 35 min, CI_{95%} = [30,31] against walking_{MELs} = 82 min, CI_{95%} = [79,85], p-value = 2.1×10^{-15}).

D.simulans flies spent significantly more time walking than micromoving during ZT11-13 (walking_{SIM} = 35 min, CI_{95%} = [30,41] against micromoving_{SIM} = 17 min, CI_{95%} = [15,19], p-value = 6.3×10^{-8}); however, they also devoted more time to micromoving compared to *D.melanogaster* (micromoving_{SIM} = 17 min, CI_{95%} = [15,19] against micromoving_{MELs} = 11 min, CI_{95%} = [10,11], p-value = 2×10^{-9}) (Fig 3.7C,E). This demonstrated that the *D.simulans* crepuscular peak was due to elevations in both walking and micromoving activity.

D.yakuba and *D.willistoni* both carried out significantly more walking than micromoving over the 24hr day (walking_{YAK} = 410 min, CI_{95%} = [370,449] against micromoving_{YAK} = 201 min, CI_{95%} = [179,222], p-value = $< 2.0 \times 10^{-16}$ & walking_{WIL} = 275 min, CI_{95%} = [256,294] against micromoving_{WIL} = 186 min, CI_{95%} = [162,211], p-value = 1.4×10^{-3}) and ZT11-13 period (walking_{YAK} = 34 min, CI_{95%} = [29,39] against micromoving_{YAK} = 18 min, CI_{95%} = [16,20], p-value = 2.4×10^{-8} & walking_{WIL} = 34 min, CI_{95%} = [28,38] against micromoving_{WIL} = 18 min, CI_{95%} = [16,20], p-value = 7.8×10^{-10}) (Fig 3.7A-C). They also exhibited significantly increased total levels of walking (walking_{YAK} = 410 min, CI_{95%} = [370,449], walking_{WIL} = 275 min, CI_{95%} = [256,294] against walking_{MELs} = 242 min, CI_{95%} = [221,263], p-value = $< 2.0 \times 10^{-16}$, 0.00025 respectively) and micromoving (micromoving_{YAK} = 201 min, CI_{95%} = [179,222], micromoving_{WIL} = 186 min, CI_{95%} = [162,211] against micromoving_{MELs} = 157 min, CI_{95%} = [142,171], p-value = 5.4×10^{-5} , 0.0050 respectively) over the 24hr day compared to the *D.melanogaster* lab strain (Fig 3.7D). However, investigating the ZT11-13 period revealed that the levels of walking were subdued (walking_{YAK} = 34 min, CI_{95%} = [29,39], walking_{WIL} = 34 min, CI_{95%} = [28,38] against walking_{MELs} = 82 min, CI_{95%} = [79,85], p-value = $< 2.0 \times 10^{-16}$, $< 2.0 \times 10^{-16}$ respectively) and micromoving levels were elevated (micromoving_{YAK} = 18 min, CI_{95%} = [16,20], micromoving_{WIL} = 18 min, CI_{95%} = [16,20] against micromoving_{MELs} = 11 min, CI_{95%} = [10,11], p-value = 4.3×10^{-11} , 1.7×10^{-11} respectively) compared to *D.melanogaster* lab strain (Fig 3.7E). Similarly to the *D.yakuba* and *D.willistoni* overall activity profile, the proportion of time spent walking started to incline around ZT6 and remained elevated until lights off.

Contrastingly to the results observed with the species discussed thus far, *D.erecta* and *D.virilis* executed more micromovements than walking behaviour over the day (walking_{ERE} = 70 min, CI_{95%} = [46,95] against micromoving_{ERE} = 203 min, CI_{95%} = [181,225], p-value = 1.1×10^{-14} & walking_{VIR} = 137 min, CI_{95%} = [116,159] against micromoving_{VIR} = 231 min, CI_{95%} = [211,252], p-value = 2.0×10^{-11}) and during the

evening crepuscular peak (walking_{ERE} = 17 min, CI_{95%} = [12,22] against micromoving_{ERE} = 23 min, CI_{95%} = [21,26], p-value = 5.0×10^{-13} & walking_{VIR} = 21 min, CI_{95%} = [17,25] against micromoving_{VIR} = 29 min, CI_{95%} = [26,31], p-value = 2.7×10^{-8}) (Fig 3.7A,B). *D. erecta* performed the lowest amount of walking of all the panel, closely followed by the *D. virilis* group. Compared to the *D. melanogaster* lab strain, both species walking levels over the day were significantly lower (walking_{ERE} = 70 min, CI_{95%} = [46,95], walking_{VIR} = 137 min, CI_{95%} = [116,159] against walking_{MELs} = 242 min, CI_{95%} = [221,263], p-value = $< 2.0 \times 10^{-16}$, 3.9×10^{-14} respectively) while their micromoving levels were elevated (micromoving_{ERE} = 203 min, CI_{95%} = [181,225], micromoving_{VIR} = 231 min, CI_{95%} = [211,252] against micromoving_{MELs} = 157 min, CI_{95%} = [142,171], p-value = 1.1×10^{-8} , 2.4×10^{-10} respectively) (Fig 3.7D). Equivalent results were found when investigating the walking and micromoving levels during the evening crepuscular peak (Fig 3.7E). Since that both *D. yakuba* and *D. erecta* had diminished walking (walking_{ERE} = 17 min, CI_{95%} = [12,22], walking_{VIR} = 21 min, CI_{95%} = [17,25] against walking_{MELs} = 82 min, CI_{95%} = [79,85], p-value = $< 2.0 \times 10^{-16}$, $< 2.0 \times 10^{-16}$ respectively) and increased micromoving (micromoving_{ERE} = 23 min, CI_{95%} = [21,26], micromoving_{VIR} = 29 min, CI_{95%} = [26,31] against micromoving_{MELs} = 11 min, CI_{95%} = [10,11], p-value = $< 2.0 \times 10^{-16}$, $< 2.0 \times 10^{-16}$ respectively) when compared to the *D. melanogaster* lab strain (Fig 3.7E).

3.2.1.3 Response to neurochemical regulators

Over the decades of *D. melanogaster* sleep research, neurochemical regulators of sleep have been identified as covered in Section 1.2.2.4. There is striking conservation in neuromodulator action between vertebrate and invertebrate species, including *D. melanogaster*^[378]. The common aspects of the role of the neurotransmitter in sleep across evolution support a shared development in molecular pathways that control sleep behaviour. I took a pharmacological approach to investigate the action of three neuromodulators that promote wake and sleep in *D. melanogaster*, to explore the conservation of their action across Drosophilids.

Octopamine and dopamine are established to promote arousal in *D. melanogaster*, while GABA is sleep-promoting^[111]. In each case, I tested the species response to drugs that result in elevated signalling: The octopamine receptor agonist Chlordimeform (CDM), the dopamine precursor L-Dopa, and the GABA_A agonist THIP.

I observed the reported reduction in sleep with CDM (asleep_{CDM-} = 639 min, CI_{95%} = [551,727] against asleep_{CDM+} = 250 min, CI_{95%} = [191,309], p-value = 1.9×10^{-8}) and L-dopa (asleep_{L-DOPA-} = 760 min, CI_{95%} = [715,805] against asleep_{L-DOPA+} = 505 min, CI_{95%} = [461,548], p-value = 3.8×10^{-13}) feeding in our *D. melanogaster* lab strain

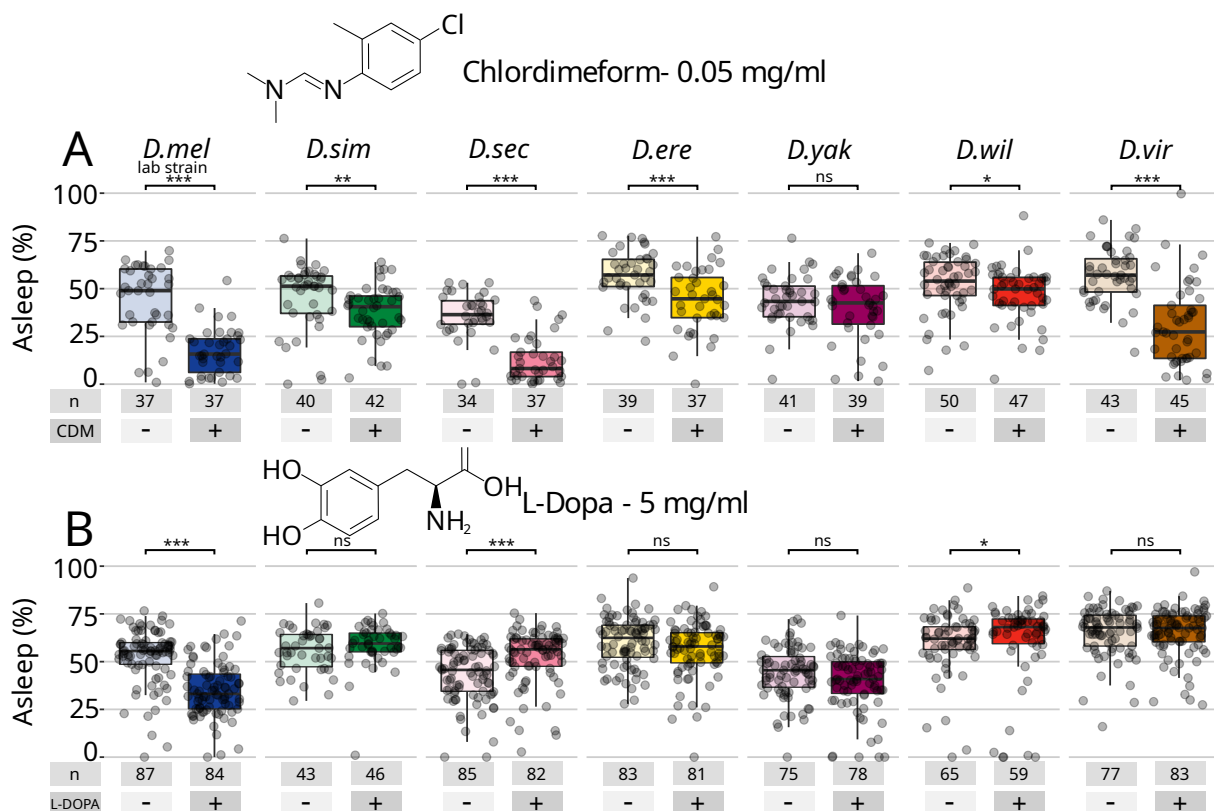


Figure 3.8: The panel of 7 *Drosophila* species response to neurochemical regulators of *D.melanogaster* sleep

(A) Quantification of daily sleep with 0.05mg/ml CDM and (B) 0.5mg/ml L-dopa feeding in *D.melanogaster* lab strain, *D.simulans* (green), *D.sechellia* (pink), *D.erecta* (yellow), *D.yakuba* (purple), *D.willistoni* (red) & *D.virilis* (brown) compared to vehicle controls. All flies were raised on the potato starch diet and tested on drug or vehicle food. * = < 0.05, ** = < 0.01 & *** = < 0.001. The duration of the tracking was 24 hours in all species and the plots are representative of a minimum of 3 repeats. Wilcoxon Rank Sum Test was used for the statistical comparisons.

(Fig 3.8A,B). The wake promoting phenotype associated with CDM in *D.melanogaster* was mimicked in almost all the species: *D.simulans* (asleep_{CDM-} = 659 min, CI_{95%} = [578,739] against asleep_{CDM+} = 544 min, CI_{95%} = [481,608], p-value = 0.00042), *D.sechellia* (asleep_{CDM-} = 514 min, CI_{95%} = [452,577] against asleep_{CDM+} = 184 min, CI_{95%} = [126,241], p-value = 4.6x10⁻⁸), *D.erecta* (asleep_{CDM-} = 816 min, CI_{95%} = [755,878] against asleep_{CDM+} = 635 min, CI_{95%} = [555,715], p-value = 0.00069), *D.willistoni* (asleep_{CDM-} = 751 min, CI_{95%} = [689,813] against asleep_{CDM+} = 680 min, CI_{95%} = [618,742], p-value = 0.035) and *D.virilis* (asleep_{CDM-} = 823 min, CI_{95%} = [760,885] against asleep_{CDM+} = 447 min, CI_{95%} = [355,539], p-value = 8.4x10⁻⁹) all displayed decreased sleep on the drug food, while *D.yakuba* (asleep_{CDM-} = 631 min, CI_{95%} = [572,690] against asleep_{CDM+} = 569 min, CI_{95%} = [490,648], p-value = 0.51) had no change in sleep when administered the drug (Fig 3.8A).

Strikingly, the response to the dopamine precursor, L-Dopa, was diverse across the panel of Drosophilids. *D.simulans* (asleep_{L-DOPA-} = 806 min, CI_{95%} = [755,856] against asleep_{L-DOPA+} = 843 min, CI_{95%} = [793,893], p-value = 0.15), *D.erecta* (asleep_{L-DOPA-} = 865 min, CI_{95%} = [825,905] against asleep_{L-DOPA+} = 809 min, CI_{95%} = [762,856], p-value = 0.093), *D.yakuba* (asleep_{L-DOPA-} = 638 min, CI_{95%} = [593,683] against asleep_{L-DOPA+} = 569 min, CI_{95%} = [518,620], p-value = 0.10) and *D.virilis* (asleep_{L-DOPA-} = 944 min, CI_{95%} = [902,986] against asleep_{L-DOPA+} = 940 min, CI_{95%} = [899,980], p-value = 0.77) had no change in sleep on the L-Dopa diet. Opposing results were found in *D.sechellia* (asleep_{L-DOPA-} = 630 min, CI_{95%} = [585,675] against asleep_{L-DOPA+} = 755 min, CI_{95%} = [709,801], p-value = 9.8x10⁻⁶) and *D.willistoni* (asleep_{L-DOPA-} = 859 min, CI_{95%} = [800,917] against asleep_{L-DOPA+} = 878 min, CI_{95%} = [796,960], p-value = 0.039), with a small yet significant sleep elevation compared to their food controls (Fig 3.8B).

THIP has been shown to cause significant increases in *D.melanogaster* daytime sleep, and such sleep adjustments have been found using concentrations as low as 0.025 mg/ml^[282]. Contrastingly, I found no change in sleep with 0.1 mg/ml or 0.5 mg/ml during the day (asleep_{THIP+0.01} = 379 min, CI_{95%} = [332,425], asleep_{THIP+0.5} = 318 min, CI_{95%} = [270,366] against asleep_{THIP-} = 331 min, CI_{95%} = [288,374], p-value = 0.24, 0.50 respectively) or night (asleep_{THIP+0.01} = 489 min, CI_{95%} = [436,542], asleep_{THIP+0.5} = 447 min, CI_{95%} = [398,497] against asleep_{THIP-} = 487 min, CI_{95%} = [443,531], p-value = 0.85, 0.80 respectively) (Fig 3.9A,B). With only a measurable increase at the end of the light phase between ZT10-12 (asleep_{THIP+0.01} = 51 min, CI_{95%} = [42,61], asleep_{THIP+0.5} = 52 min, CI_{95%} = [42,62] against asleep_{THIP-} = 30 min, CI_{95%} = [18,34], p-value = 0.00086, 0.00096 respectively). Both concentrations led to a significant increase in sleep compared to the normal food controls during the 2 hours prior to the transition from light to dark (Fig 3.9C). However, due to the unexpectedly restricted

changes in sleep observed with THIP feeding in *D.melanogaster*, I excluded the drug from further investigations.

Generally, the circadian aspects of *Drosophila* sleep are seemingly conserved in most species included in the panel. For example, each group exhibited a siesta sleep during the day, and activity peaks at the light transitions in LD conditions. For the most part, the species had endogenous free-running rhythms in constant conditions with a maintained light-to-dark activity peak, excluding the *D.virilis* group, which was arrhythmic under DD and suggesting an alteration in their circadian control. Surprisingly, there was evidence that the molecular mechanisms underlying sleep may have diverged, as the wake-promoting dopamine neurotransmitter elicited either no response or, in fact, elevated total sleep. In order to further characterise their sleep, I investigated the homeostatic control of sleep.

3.2.2 Sleep homeostasis and response to sleep deprivation

While the circadian aspect of sleep control ensures the timing of sleep at an ecologically beneficial period. The homeostatic arm guarantees that an organism achieves its species-specific amount and quality of sleep potentially required to fulfil the essential function/s^[379]. Like many approaches in biology, a common way to study the function of a gene or biological process is to remove or disrupt said process and observe the effects, elucidating the role it may play under baseline conditions. A similar approach is taken for studying sleep and the homeostat, extending the waking period beyond what is natural with a method of SD and measuring the impact of such removal. Through such studies, it has been observed that after SD, a period of rebound sleep occurs in either the shape of increased sleep duration or depth to recover the lost sleep and possibly to counteract the physiological impact of wake^[67].

In order to examine the homeostatic mechanism in other Drosophilids, I performed a mechanical and social method of SD and interrogated the consequential rebound response.

3.2.2.1 Response to mechanical sleep deprivation

Mechanical methods are commonly utilised to study sleep homeostasis and response to SD across the animal kingdom. I employed an accessory module to the ethoscope with a dynamic approach to mechanically enforced waking. The system engages a feedback loop to provide mechanical stimuli in direct response to a bout of 30-sec inactivity, spinning the individual tube housing the fly and obstructing their transition into sleep

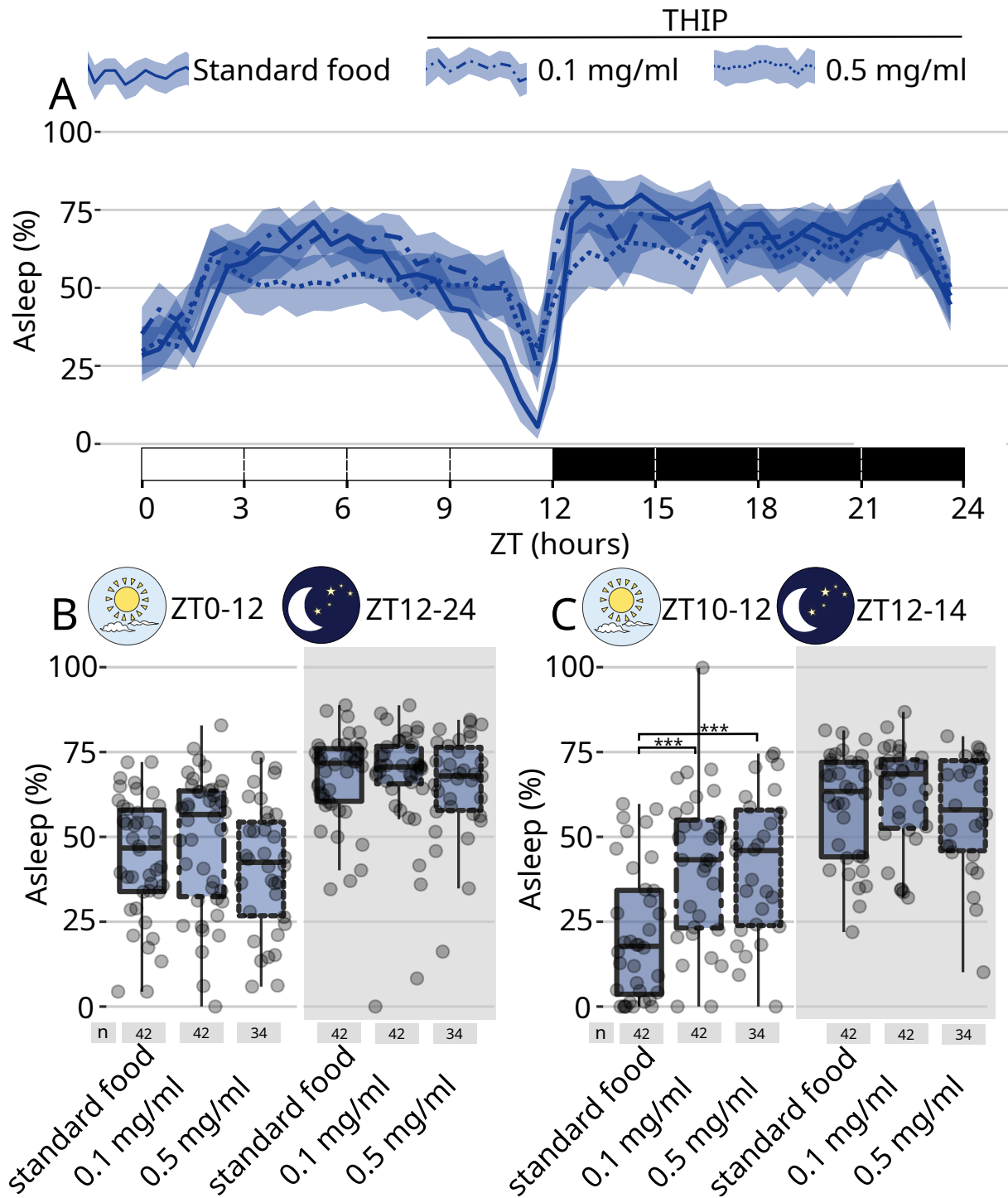


Figure 3.9: *D.melanogaster* lab strain response to THIP feeding

(A) Sleep profile over 24 hr of feeding with standard food (solid line), 0.1 mg/ml & 0.5 mg/ml THIP (B) Quantification of average sleep over the day and night (B) Quantification of average sleep during ZT10-12 and ZT12-14. All flies were raised on the potato starch diet and tested on drug or vehicle food. * = < 0.05, ** = < 0.01 & *** = < 0.001. The duration of the tracking was 24 hours in all species and the plots are representative of 2 repeats. Wilcoxon Rank Sum Test was used for the statistical comparisons in B & C.

(Materials and methods 2.4.1). Any changes in sleep were measured during the 24 hr SD, and the resultant rebound was assessed during the first three hours of the light phase (ZT0-3) immediately following the ceasing of the mechanical SD. While the mechanical method was sufficient at depriving sleep throughout the panel, disparate results were observed regarding the non-*melanogaster* rebound response (Fig 3.10).

In the *D.melanogaster* lab strain, sleep was reduced to 3 min ($CI_{95\%} = [0,7]$) during the 24 hr SD, a 759 min reduction of the flies usual sleep behaviour ($asleep_{CTRL} = 762$ min, $CI_{95\%} = [714,809]$, $p\text{-value} = < 2.0 \times 10^{-16}$). In response to the drastic sleep loss, a strong rebound response was measured during ZT0-3 ($asleep_{CTRL} = 83$ min, $CI_{95\%} = [74,92]$ against $asleep_{SD} = 117$ min, $CI_{95\%} = [105,130]$, $p\text{-value} = 1.5 \times 10^{-6}$) before the sleep returned to the levels of baseline. Comparable results were found in the *D.melanogaster* wild caught flies, sleeping only 56 min during the SD day, which was recovered with 28 min extra sleep during the rebound phase ($asleep_{CTRL} = 20$ min, $CI_{95\%} = [10,31]$ against $asleep_{SD} = 48$ min, $CI_{95\%} = [31,66]$, $p\text{-value} = 0.016$) (Fig 3.10A,B).

Remarkably, while the remainder of the panel had a severe reduction in their sleep over the 24 hr mechanical SD, no detectable changes in sleep were measured to indicate that the sleep loss was recovered with a period of rebound. The non-*melanogaster* panel slept within the range of an average of 22 to 84 min during the mechanical SD ($asleep_{SIM} = 28$ min, $CI_{95\%} = [0,58]$, $asleep_{SEC} = 22$ min, $CI_{95\%} = [7,37]$, $asleep_{ERE} = 79$ min, $CI_{95\%} = [36,119]$, $asleep_{YAK} = 84$ min, $CI_{95\%} = [35,133]$, $asleep_{WIL} = 33$ min, $CI_{95\%} = [4,63]$, $asleep_{VIR} = 30$ min, $CI_{95\%} = [14,46]$) (Fig 3.10A). Nevertheless, in all the SD groups, the sleep during ZT0-3 was indistinguishable from their slept counterparts. *D.simulans* ($asleep_{CTRL} = 69$ min, $CI_{95\%} [20,42]$ against $asleep_{SD} 67$ min, $CI_{95\%} [52,81]$, $p\text{-value} = 0.84$), *D.sechellia* ($asleep_{CTRL} = 31$ min, $CI_{95\%} = [20,42]$ against $asleep_{SD} = 31$ min, $CI_{95\%} = [20, 42]$, $p\text{-value} = 0.84$), *D.erecta* ($asleep_{CTRL} = 71$ min, $CI_{95\%} = [56,85]$ against $asleep_{SD} 68$ min, $CI_{95\%} = [51,84]$, $p\text{-value} = 0.80$), *D.yakuba* ($asleep_{CTRL} = 3$ min, $CI_{95\%} = [1,5]$ against $asleep_{SD} 3$ min, $CI_{95\%} = [0,6]$, $p\text{-value} = 0.28$), *D.willistoni* ($asleep_{CTRL} = 74$ min, $CI_{95\%} = [66,82]$ against $asleep_{SD} 74$ min, $CI_{95\%} = [60,88]$, $p\text{-value} = 1.0$) and *D.virilis* ($asleep_{CTRL} = 121$ min, $CI_{95\%} = [107,136]$ against $asleep_{SD} 107$ min, $CI_{95\%} = [91,124]$, $p\text{-value} = 0.16$) all performed a level of sleep during the rebound phase as if they had not experienced any sleep loss at all (Fig 3.10B).

While changes in sleep amount indicate rebound recovery, it was possible that their sleep was recovered with bouts of greater depth. I tentatively investigated the panel's sleep depth during ZT0-3 by looking at the sleep architecture during the rebound period (Fig 3.10C). The *D.melanogaster* lab strain rebound sleep was composed of bouts of increased duration ($duration_{CTRL} = 10$ min, $CI_{95\%} = [10,11]$ against $duration_{SD} =$

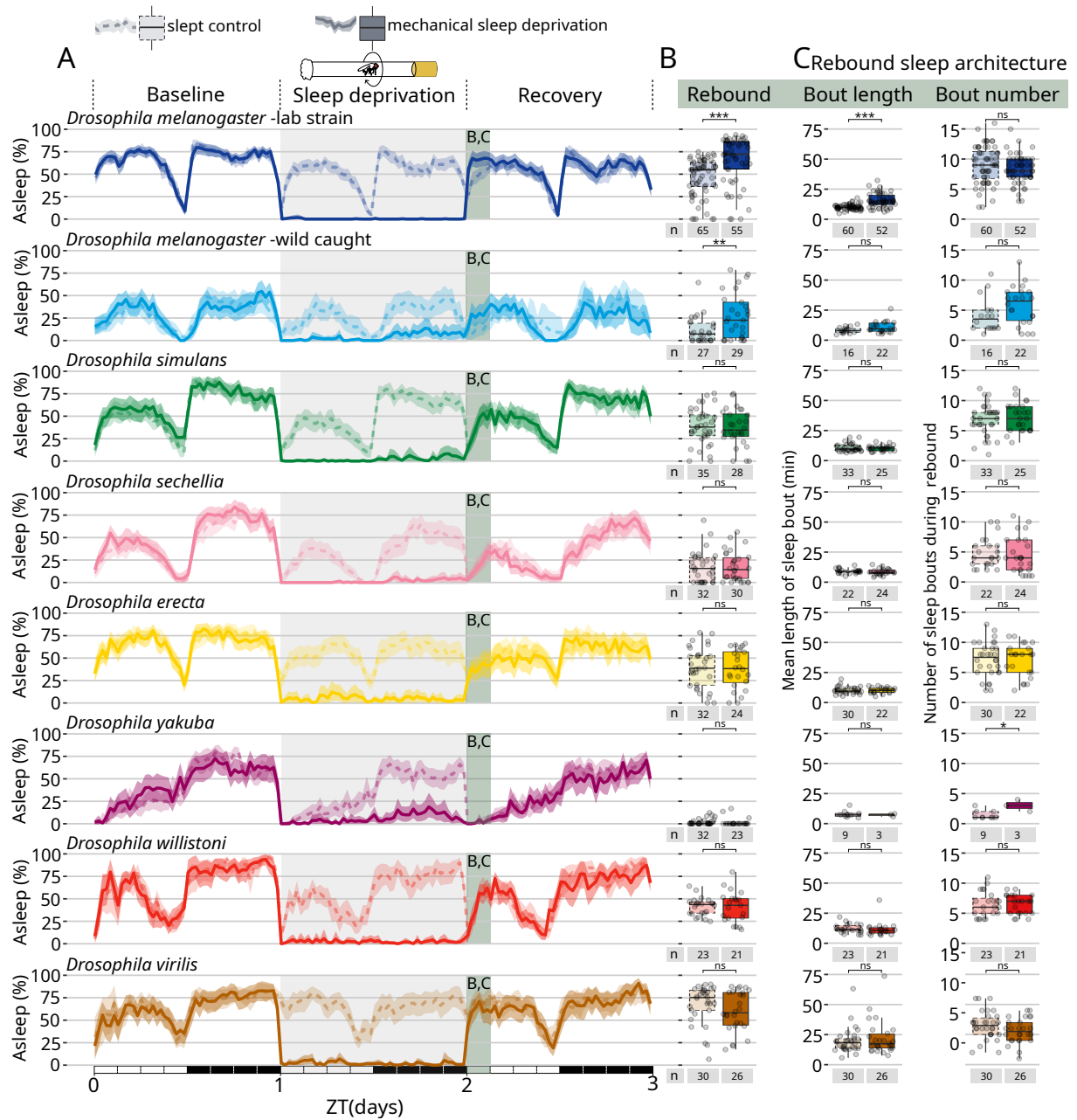


Figure 3.10: The panel of 7 *Drosophila* species response to mechanical SD (A) Sleep profile of *D.melanogaster* lab strain, *D.melanogaster* wild caught (light blue), *D.simulans* (green), *D.sechellia* (pink), *D.erecta* (yellow), *D.yakuba* (purple), *D.willistoni* (red) & *D.virilis* (brown) exposed to 24 hr mechanical SD (solid line) and undisturbed slept controls (dashed line) (B) Quantification of average sleep (C) duration of sleep bouts and sleep bout number during the ZT0-3 rebound period (green shading) following mechanical SD (solid line) or in the undisturbed slept controls (dashed line). All flies were raised and tested on standard food. * = < 0.05, ** = < 0.01 & *** = < 0.001. The duration of the tracking was 3 days and rebound sleep characteristics were measured over ZT0-3 of the recovery day. The plots are representative of a minimum of 3 repeats. Wilcoxon Rank Sum Test was used for the statistical comparisons in B, C & D.

16 min, $CI_{95\%} = [14,17]$, $p\text{-value} = 1.7 \times 10^{-8}$) with change in the number of bouts (number_{CTRL} = 9, $CI_{95\%} = [8,10]$ against number_{SD} = 8, $CI_{95\%} = [8,9]$, $p\text{-value} = 0.15$). In all other species, the mean bout duration and number of sleep bouts were the same in the slept control and SD groups (Fig 3.10C).

3.2.2.2 Waking behaviour during mechanical sleep deprivation

In 3.2.1 of this chapter, I described the variation in waking behaviour across the panel, with different levels of walking and micromoving. Due to such disparity, I postulated that their waking behaviour during SD would also be varied. Thus, I investigated the activity profile during SD compared to slept counterparts and as a fraction of their baseline behaviour to determine the degree to which the mechanical sleep interruptions altered their waking activity.

Firstly, I investigated their walking levels and found that all species executed more walking behaviour during SD (Fig 3.11). *D.melanogaster* lab strain (walking_{CTRL} = 218 min, $CI_{95\%} = [191,245]$ against walking_{SD} = 928 min, $CI_{95\%} = [877,979]$, $p\text{-value} = < 2.0 \times 10^{-16}$), *D.melanogaster* wild caught (walking_{CTRL} = 595 min, $CI_{95\%} = [507,682]$ against walking_{SD} = 1084 min, $CI_{95\%} = [981,1187]$, $p\text{-value} = 1.1 \times 10^{-8}$), *D.simulans* (walking_{CTRL} = 193 min, $CI_{95\%} = [162,224]$ against walking_{SD} = 510 min, $CI_{95\%} = [446,574]$, $p\text{-value} = 2.7 \times 10^{-11}$), *D.sechellia* (walking_{CTRL} = 398 min, $CI_{95\%} = [353,442]$ against walking_{SD} = 768 min, $CI_{95\%} = [702,835]$, $p\text{-value} = 2.4 \times 10^{-11}$), *D.erecta* (walking_{CTRL} = 91 min, $CI_{95\%} = [44,138]$ against walking_{SD} = 503 min, $CI_{95\%} = [428,577]$, $p\text{-value} = 2.8 \times 10^{-11}$), *D.yakuba* (walking_{CTRL} = 448 min, $CI_{95\%} = [393,504]$ against walking_{SD} = 752 min, $CI_{95\%} = [678,825]$, $p\text{-value} = 1.3 \times 10^{-11}$), *D.willistoni* (walking_{CTRL} = 146 min, $CI_{95\%} = [116,177]$ against walking_{SD} = 557 min, $CI_{95\%} = [476,638]$, $p\text{-value} = 1.2 \times 10^{-10}$) and *D.virilis* (walking_{CTRL} = 52 min, $CI_{95\%} = [36,68]$ against walking_{SD} = 335 min, $CI_{95\%} = [283,388]$, $p\text{-value} = 8.4 \times 10^{-10}$) all performed significantly more walking during the 24 hr mechanical SD compared to their slept controls (Fig 3.11A,B). However, the extent of walking behaviour during the mechanical SD differed across the panel. *D.melanogaster* wild caught spent the highest proportion of time walking (walking_{SD} = 928 min, $CI_{95\%} = [877,979]$), followed by the *D.melanogaster* lab strain (walking_{SD} = 928 min, $CI_{95\%} = [877,979]$), and thus the two *D.melanogaster* species carried out the most walking during SD. *D.sechellia* and *D.yakuba* walking levels were significantly equivalent, spending 752 min ($CI_{95\%} = [678,825]$) and 768 min min ($CI_{95\%} = [702,835]$) walking respectively ($p\text{-value} = 0.68$). The second lowest walking levels were executed by *D.simulans* (walking_{SD} = 510 min, $CI_{95\%} = [446,574]$), *D.erecta* (walking_{SD} = 503 min, $CI_{95\%} = [428,577]$) and *D.willistoni* (walking_{SD} = 557 min, $CI_{95\%} = [476,638]$), which carried out the same level of walking as each other ($p\text{-value}$

against *D.simulans* = 0.90, 0.43 respectively). Finally, *D.virilis* performed the lowest proportion of walking over the 24hr mechanical SD, walking for only 335 min ($CI_{95\%} = [283,388]$) (Fig 3.11B). Despite the contrast in walking amount within the panel, all species had a positive change in walking behaviour compared to baseline (Fig 3.11C). *D.erecta* had the largest change in behaviour with a 13 fold increase ($CI_{95\%} = [10,16]$) and the alteration in *D.virilis* walking as a fraction of baseline was the same as that of *D.melanogaster* lab strain (walking $_{\Delta MELIS}$ = 6 fold, $CI_{95\%} = [5,7]$ against walking $_{\Delta VIR}$ = 7 fold, $CI_{95\%} = [4,10]$, p-value = 0.056).

Secondly, the levels of micromovement were mostly increased during SD across the panel, with *D.melanogaster* lab strain (micromoving $_{CTRL}$ = 167 min, $CI_{95\%} = [142,191]$ against micromoving $_{SD}$ = 308 min, $CI_{95\%} = [270,345]$, p-value = 8.1×10^{-11}), *D.simulans* (micromoving $_{CTRL}$ = 171 min, $CI_{95\%} = [151,190]$ against micromoving $_{SD}$ = 615 min, $CI_{95\%} = [542,688]$, p-value = 1.9×10^{-19}), *D.sechellia* (micromoving $_{CTRL}$ = 211 min, $CI_{95\%} = [167,254]$ against micromoving $_{SD}$ = 363 min, $CI_{95\%} = [312,414]$, p-value = 6.6×10^{-6}), *D.erecta* (micromoving $_{CTRL}$ = 214 min, $CI_{95\%} = [175,252]$ against micromoving $_{SD}$ = 587 min, $CI_{95\%} = [517,657]$, p-value = 3.5×10^{-11}), *D.yakuba* (micromoving $_{CTRL}$ = 178 min, $CI_{95\%} = [151,206]$ against micromoving $_{SD}$ = 410 min, $CI_{95\%} = [354,466]$, p-value = 1.4×10^{-8}), *D.willistoni* (micromoving $_{CTRL}$ = 219 min, $CI_{95\%} = [109,328]$ against micromoving $_{SD}$ = 555 min, $CI_{95\%} = [483,627]$, p-value = 7.3×10^{-9}) and *D.virilis* (micromoving $_{CTRL}$ = 263 min, $CI_{95\%} = [190,337]$ against micromoving $_{SD}$ = 780 min, $CI_{95\%} = [729,830]$, p-value = 6.1×10^{-11}) all experiencing elevated micromovements during SD compared to their slept counterparts (Fig 3.12A,B). While the *D.melanogaster* wild caught flies performed the same amount of micromovements during 24hr mechanical SD as control (micromoving $_{CTRL}$ = 131 min, $CI_{95\%} = [110,151]$ against micromoving $_{SD}$ = 143 min, $CI_{95\%} = [104,181]$, p-value = 0.71) (Fig 3.12A,B). Unsurprisingly, the species which carried out the lower levels of walking during SD were found to carry out the largest amount of micromovements , and the ranking of the levels of micromovement was the inversion of their walking amounts. Here the *D.melanogaster* flies spent the lowest time in micromovements (micromoving $_{SD}$ = 308 min, $CI_{95\%} = [270,345]$), followed by *D.sechellia* (micromoving $_{SD}$ = 363 min, $CI_{95\%} = [312,414]$) and *D.yakuba* (micromoving $_{SD}$ = 410 min, $CI_{95\%} = [354,466]$), who executed equivalent levels (p-value = 0.10). *D.simulans* (micromoving $_{SD}$ = 615 min, $CI_{95\%} = [542,688]$), *D.erecta* (micromoving $_{SD}$ = 587 min, $CI_{95\%} = [517,657]$) and *D.willistoni* (micromoving $_{SD}$ = 555 min, $CI_{95\%} = [483,627]$) all spent equivalent time micromoving (p-value against *D.simulans* = 0.89, 0.31 respectively) and performed the second highest levels of the panel. While *D.virilis*, spent the highest proportion of time carrying out micromovements (micromoving $_{SD}$ = 780 min, $CI_{95\%} = [729,830]$) (Fig 3.12B). The change in

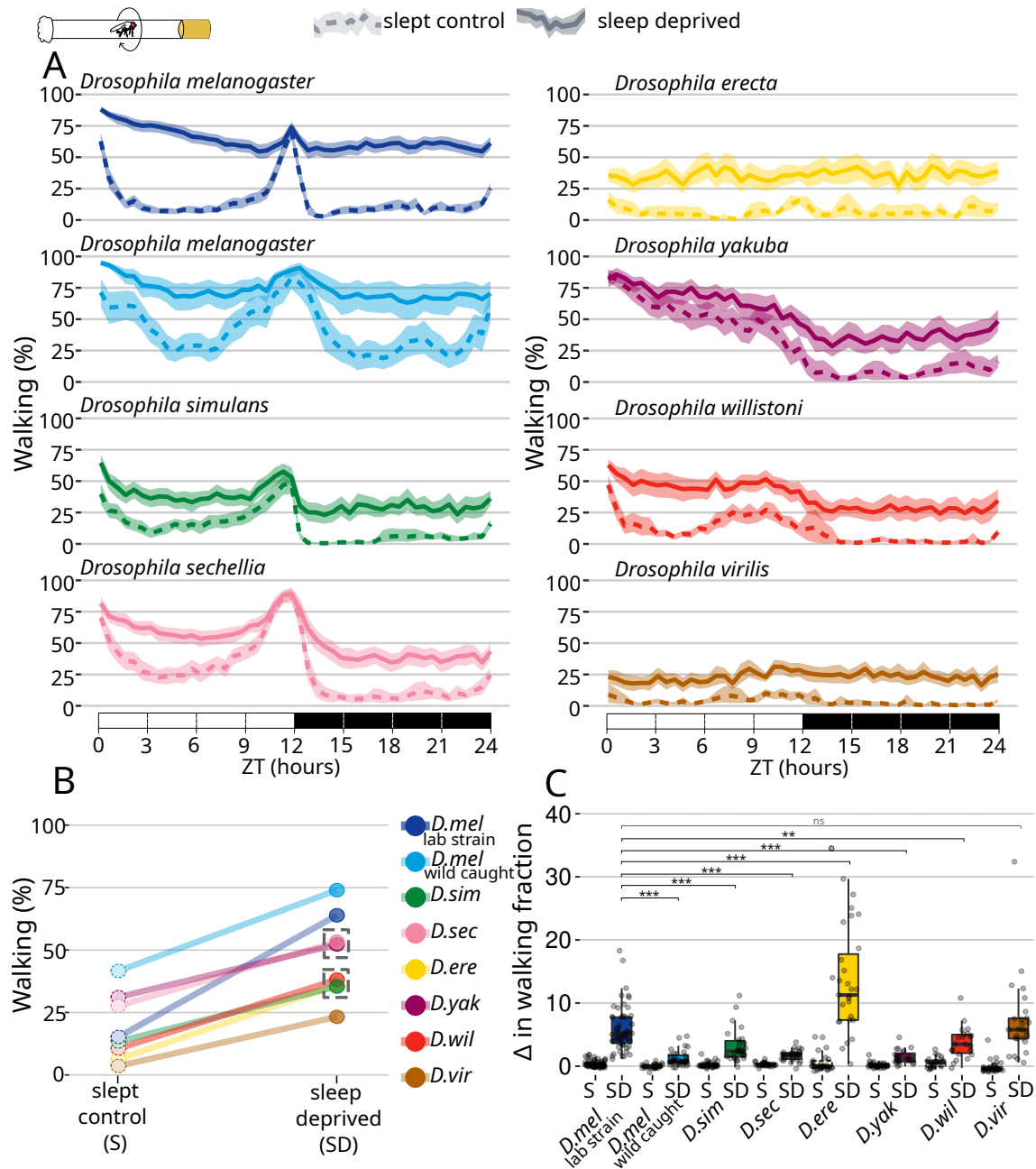


Figure 3.11: The panel of 7 *Drosophila* species walking levels during mechanical SD (A) Walking profile during mechanical SD (solid line) or of the undisturbed sleep controls (dashed line) in *D.melanogaster* lab strain, *D.melanogaster* wild caught (light blue), *D.simulans* (green), *D.sechellia* (pink), *D.erecta* (yellow), *D.yakuba* (purple), *D.willistoni* (red) & *D.virilis* (brown) (B) Quantification of the average time spent walking in the mechanical SD (solid circle) and the undisturbed sleep control (dashed circle), grey dashed boxes signify statistically insignificant comparisons in the mechanical SD groups (C) Quantification of the change in walking from baseline, mechanical SD groups were statistically compared to the change in walking in *D.melanogaster* lab strain. All flies were raised and tested on standard food. * = < 0.05, ** = < 0.01 & *** = < 0.001. The plots are representative of a minimum of 3 repeats and Wilcoxon Rank Sum Test was used for the statistical comparisons in B & C.

micromovement in *D.sechellia* and *D.yakuba* was comparable to the change seen in *D.melanogaster* (micromoving $_{\Delta SEC}$ = 2 fold, CI $_{95\%}$ = [2,3], micromoving $_{\Delta YAK}$ = 2 fold, CI $_{95\%}$ = [1,2] against micromoving $_{\Delta MELs}$ = 2 fold, CI $_{95\%}$ = [1,2], p-value = 0.083, 0.73 respectively), while the other non-melanogaster species micromoving level was significantly higher when compared to the *D.melanogaster* lab strain (Fig 3.12C).

3.2.2.3 Chronic mechanical sleep deprivation

It was conceivable that the different levels of waking behaviour performed across the panel played a part in the abolished rebound of non-*melanogaster* flies. That is, the different magnitudes of waking behaviour during the SD may have elicited inconsistent levels of physical exhaustion, and therefore 24 hr SD would not generate a significant need for sleep. In order to address this, I conducted a chronic mechanical SD experiment to ensure that each species were rigorously sleep-deprived and sufficiently exhausted. If the different rebound responses were due to inadequate sleep loss or physical exertion, an extended 7 day period of mechanical SD should generate high sleep pressure and account for the differential magnitude of waking behaviour.

During the 7 day chronic mechanical SD, all species had a strong suppression in their sleep over the 168 hour enforced waking (Fig 3.13A). *D.melanogaster* lab strain slept for only 3 hr 35 min (CI $_{95\%}$ = [0.99,6.21]) compared to 72 hour 33 min (CI $_{95\%}$ = [67.20,77.91]) in the slept controls. This extensive sleep loss resulted in a strong rebound response in the ZT0-3 period following the SD, with the SD group sleeping 44 min extra compared to their rested counterparts (asleep $_{CTRL}$ = 27 min, CI $_{95\%}$ = [18,35] against asleep $_{SD}$ = 71 min, CI $_{95\%}$ = [53,88], p-value = 0.00013) (Fig 3.13B). Two species from the panel slept less during chronic SD than the *D.melanogaster* lab strain, *D.virilis* sleeping for only 30 min (CI $_{95\%}$ = [7,52]) and *D.erecta* for 2 hour 53 min (CI $_{95\%}$ = [1.72,4.04]). Nevertheless, all of the SD groups had a substantial sleep reduction, with no species sleeping more than an average of 8 hours over the 168 hour SD protocol (asleep $_{SIM}$ = 7 hr 44 min CI $_{95\%}$ = [4.01,11.40], asleep $_{SEC}$ = 5 hr 32 min CI $_{95\%}$ = [2.10,8.97], asleep $_{YAK}$ = 4 hr 9 min CI $_{95\%}$ = [1.52,6.77] & asleep $_{WILL}$ = 4 hr 10 min CI $_{95\%}$ = [0.52,7.77]) (Fig 3.13A). However, despite the extensive sleep loss incurred, there was no detectable change in the non-*melanogaster* sleep in response to the chronic SD. *D.simulans* (asleep $_{CTRL}$ = 13 min, CI $_{95\%}$ = [6,19] against asleep $_{SD}$ = 16 min, CI $_{95\%}$ = [6,27], p-value = 0.99), *D.sechellia* (asleep $_{CTRL}$ = 17 min, CI $_{95\%}$ = [0,33] against asleep $_{SD}$ = 16 min, CI $_{95\%}$ = [0,37], p-value = 0.70), *D.erecta* (asleep $_{CTRL}$ = 38 min, CI $_{95\%}$ = [26,50] against asleep $_{SD}$ = 32 min, CI $_{95\%}$ = [15,49], p-value = 0.21), *D.yakuba* (asleep $_{CTRL}$ = 20 min, CI $_{95\%}$ = [0,41] against asleep $_{SD}$ = 20 min, CI $_{95\%}$ = [0,40], p-value = 0.66), *D.willistoni* (asleep $_{CTRL}$ = 43 min, CI $_{95\%}$ = [32,54] against asleep $_{SD}$ = 44 min,

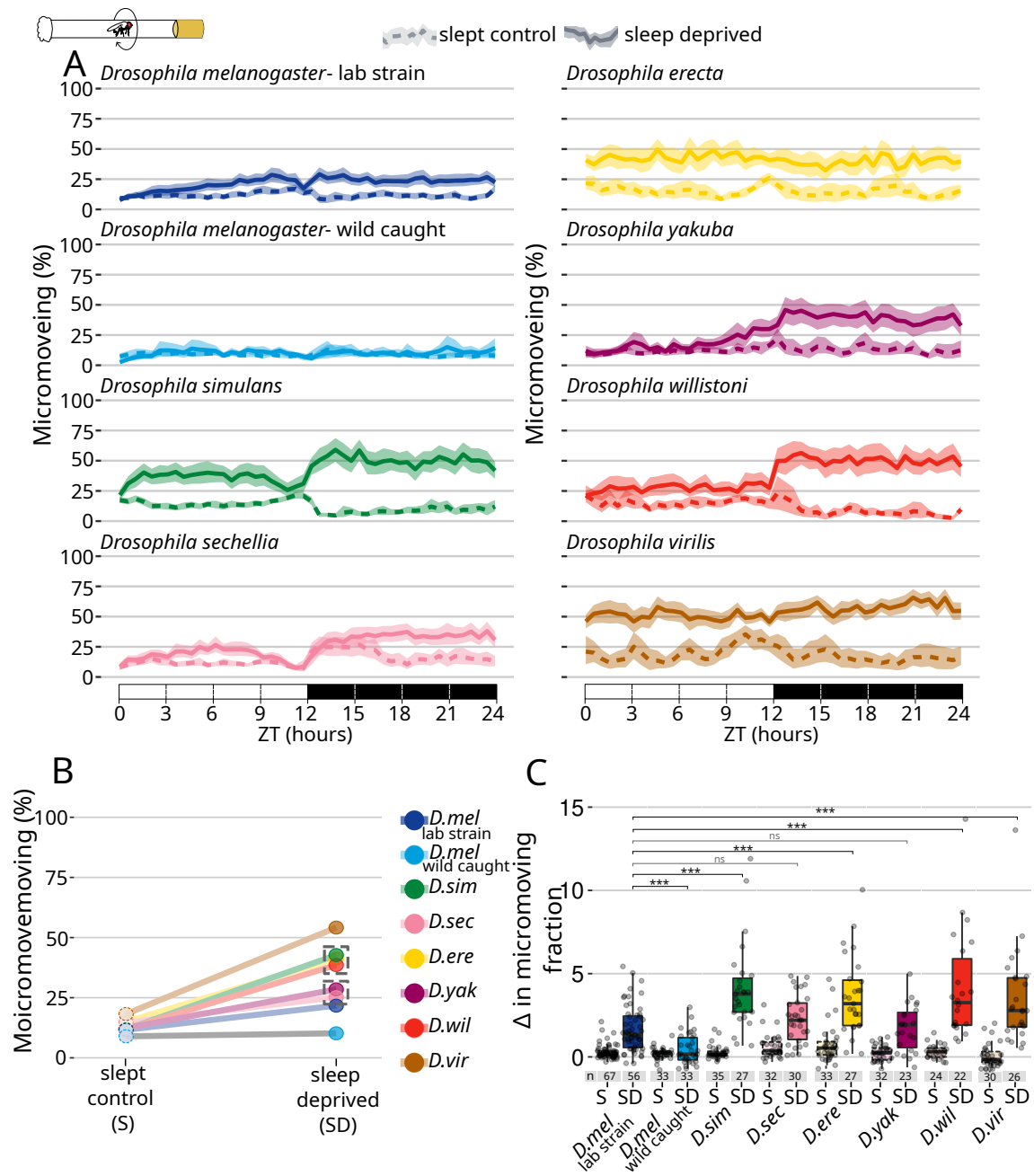


Figure 3.12: The panel of 7 *Drosophila* species micromoving levels during mechanical SD

(A) Micromoving profile during mechanical SD (solid line) or of the undisturbed sleep controls (dashed line) in *D.melanogaster* lab strain (dark blue), *D.melanogaster* wild caught (light blue), *D.simulans* (green), *D.sechellia* (pink), *D.erecta* (yellow), *D.yakuba* (purple), *D.willistoni* (red) & *D.virilis* (brown) (B) Quantification of the average time spent micromoving in the mechanical SD (solid circle) and the undisturbed sleep control (dashed circle), grey dashed boxes signify statistically insignificant comparisons in the mechanical SD groups (C) Quantification of the change in micromoving from baseline, mechanical SD groups were statistically compared to the change in micromoving in *D.melanogaster* lab strain. All flies were raised and tested on standard food.* = < 0.05, ** = < 0.01 & *** = < 0.001. The plots are representative of a minimum of 3 repeats and Wilcoxon Rank Sum Test was used for the statistical comparisons in B & C.

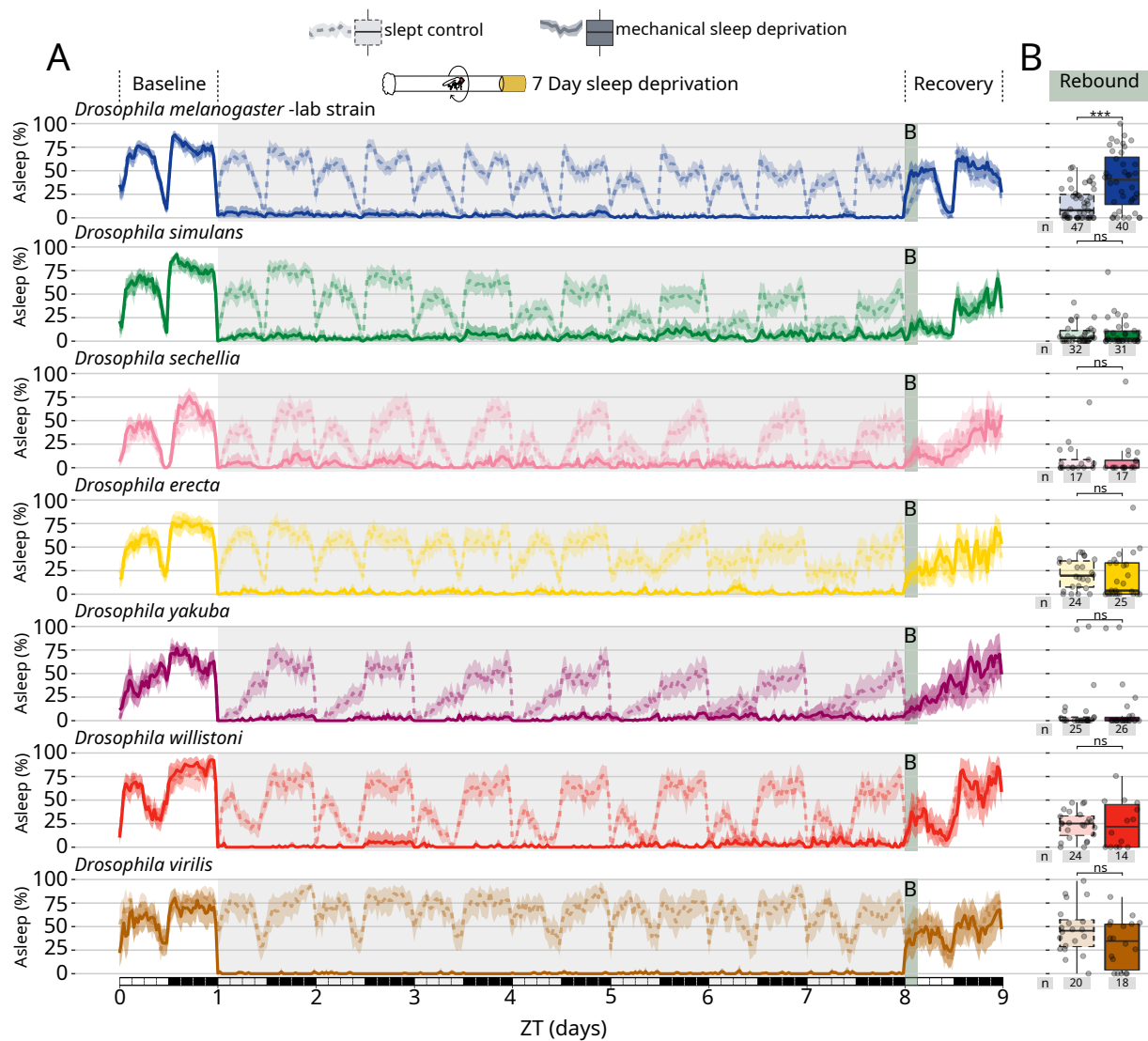


Figure 3.13: The panel of 7 *Drosophila* species response to chronic 7 day mechanical SD

(A) Sleep profile of *D. melanogaster* lab strain, *D. simulans* (green), *D. sechellia* (pink), *D. erecta* (yellow), *D. yakuba* (purple), *D. willistoni* (red) & *D. virilis* (brown) exposed to 168 hr mechanical SD (solid line) and the undisturbed slept controls (dashed line) (B) Quantification of average sleep during the ZT0-3 rebound period (green shading) following mechanical SD (solid line) or in the undisturbed slept controls (dashed line). All flies were raised on the potato starch diet and tested on standard food. * = < 0.05, ** = < 0.01 & *** = < 0.001. The duration of the tracking was 9 days and rebound sleep characteristics were measured over ZT0-3 of the recovery day. The plots are representative of a minimum of 3 repeats and Wilcoxon Rank Sum Test was used for the statistical comparisons in B.

CI_{95%} = [18,70], p-value = 0.89) and *D.virilis* (asleep_{CTRL} = 84 min, CI_{95%} = [62,106] against asleep_{SD} = 57 min, CI_{95%} = [34,80], p-value = 0.14) slept equally during the ZT0-3 rebound phase as the rested controls (Fig 3.13B).

3.2.2.4 Mechanical sleep deprivation in constant conditions

It was plausible that the lack of response was due to a strong arousal drive elicited by the onset of the lights inside the incubator during the ZT0-3 period in which rebound sleep was measured. Therefore, the results I had collected thus far may have been due to a masking effect of the light-driven arousal on the homeostatic response. To address this, I performed the mechanical SD experiments in the same manner as before but in constant darkness removing the light zeitgeber. Once again, mechanical SD only elicited a rebound response in the *D.melanogaster* species, sleeping in excess of 45 min during the ZT0-3 period following SD compared to their undisturbed counterparts (asleep_{CTRL} = 30 min, CI_{95%} = [20,40] against asleep_{SD} = 72 min, CI_{95%} = [56,89], p-value = 0.00018) (Fig 3.14A,B). The remaining species did not incur elevated sleep after the mechanical sleep loss in DD conditions, and each SD group's sleep was indistinguishable from the rested flies. Unexpectedly, *D.virilis* effectively had a negative rebound, sleeping significantly less during this period (asleep_{CTRL} = 134 min, CI_{95%} = [107,161] against asleep_{SD} = 84 min, CI_{95%} = [61,108], p-value = 0.0025) (Fig 3.14A,B).

3.2.2.5 Social sleep deprivation

In both acute and chronic mechanical SD paradigms, the lab strain and wild caught *D.melanogaster* were the only species to exhibit a robust sleep increase. A pattern that persisted even in constant darkness, eliminating the possibility of light-driven masking of the rebound phenotype. The results I had collected thus far suggested that the species had an altered homeostatic mechanism. In the introduction, I explained that the response to sleep loss varies widely depending on the chosen method and waking environment^{[201][108]}. Therefore, the absent rebound in the non-*melanogaster* group may have arisen due to divergent homeostatic mechanisms rather than the lack of homeostatic control of sleep. If this were true, I would expect that another method of SD would elicit rebound.

The mechanical SD method was efficient at reducing sleep to almost zero across the SD period; however, one could argue that the approach was ecologically irrelevant and that while no homeostatic rebound is observed after mechanical sleep loss, a more natural approach would generate different results. In order to probe the existence of homeostatic sleep regulation in the non-*melanogaster* flies, I exposed the panel to a second method of sleep loss termed social SD. The social SD paradigm incorporates

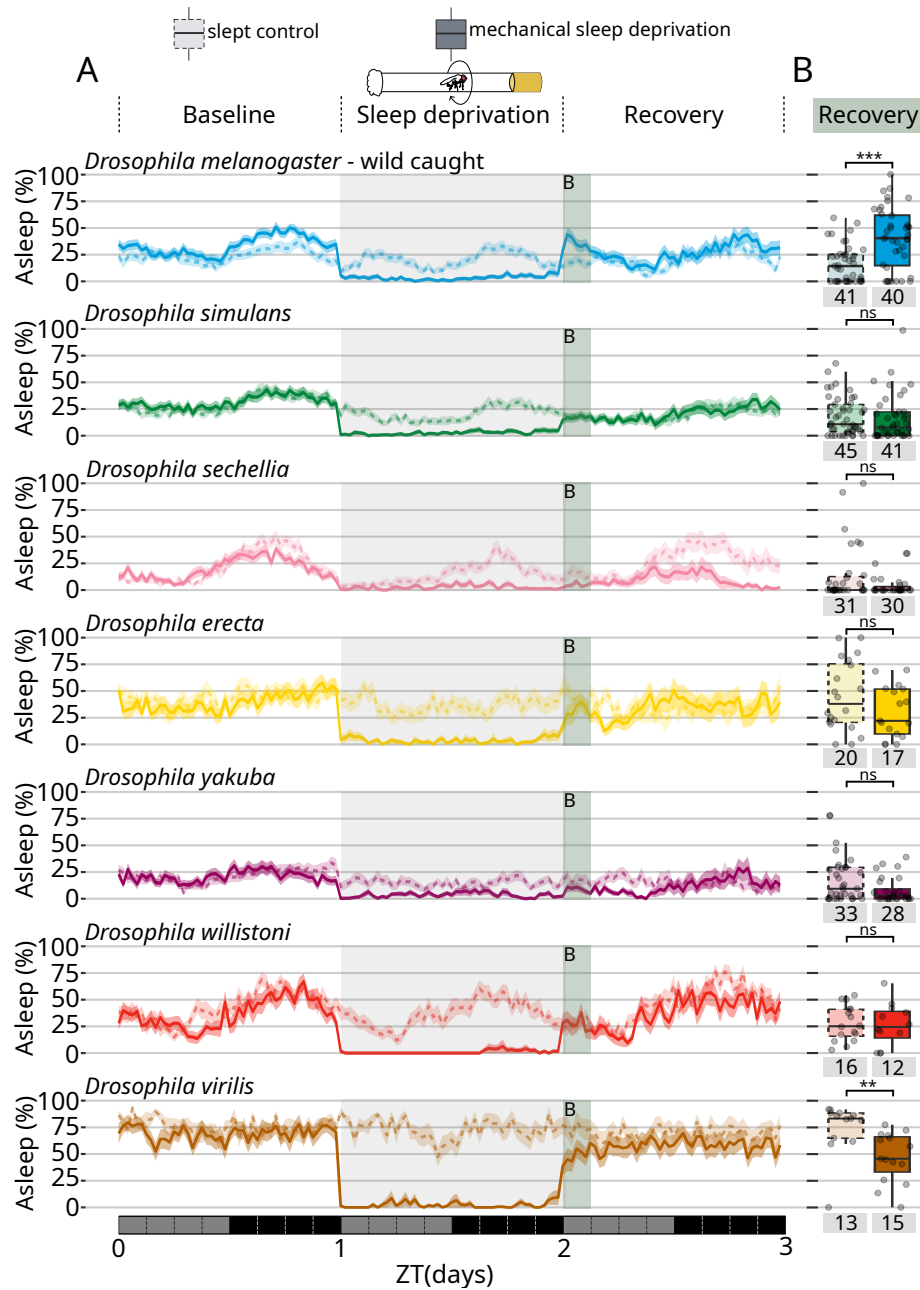


Figure 3.14: The panel of 7 *Drosophila* species response to mechanical SD in constant conditions

(A) Sleep profile of *D.melanogaster* wild caught (light blue), *D.simulans* (green), *D.sechellia* (pink), *D.erecta* (yellow), *D.yakuba* (purple), *D.willistoni* (red) & *D.virilis* (brown) exposed to 24 hr mechanical SD (solid line) and undisturbed slept controls (dashed line) in DD (B) Quantification of average sleep during the ZT0-3 rebound period (green shading) following mechanical SD (solid line) or in the undisturbed slept controls (dashed line). All flies were raised on the potato starch diet and tested on standard food.* = < 0.05, ** = < 0.01 & *** = < 0.001. The duration of the tracking was 3 days and rebound sleep characteristics were measured over ZT0-3 of the recovery day. The plots are representative of a minimum of 2 repeats and Wilcoxon Rank Sum Test was used for the statistical comparisons in B.

the interactions of two previously isolated males over a 24 hr period and has been previously shown to induce sleep loss and subsequent rebound^[108]. After social SD, the lab strain and wild caught *D.melanogaster* flies increased sleep during the ZT0-3 period post-SD, sleeping 22 min and 51 min extra, respectively (*D.melanogaster* lab strain = asleep_{CTRL} = 63 min, CI_{95%} = [55,72] against asleep_{SD} = 85 min, CI_{95%} = [76,93], p-value = 0.00049 & *D.melanogaster* wild caught = asleep_{CTRL} = 9 min, CI_{95%} = [1,17] against asleep_{SD} = 60 min, CI_{95%} = [42,78], p-value = 1.7×10^{-5}) (Fig 3.15A,B). With this approach, a selection of the panel that was previously devoid of a rebound response exhibited a significant elevation in their sleep to the social SD. *D.simulans* (asleep_{CTRL} = 26 min, CI_{95%} = [15,37] against asleep_{SD} = 58 min, CI_{95%} = [45,71], p-value = 0.00016), *D.sechellia* (asleep_{CTRL} = 24 min, CI_{95%} = [16,32] against asleep_{SD} = 43 min, CI_{95%} = [29,56], p-value = 0.052), and *D.yakuba* (asleep_{CTRL} = 4 min, CI_{95%} = [0,10] against asleep_{SD} = 10 min, CI_{95%} = [4,16], p-value = 0.054) all had rebound following the male-male interactions compared to the mock controls (Fig 3.15A,B). However, *D.erecta* (asleep_{CTRL} = 74 min, CI_{95%} = [51,97] against asleep_{SD} = 58 min, CI_{95%} = [43,73], p-value = 0.25), *D.willistoni* (asleep_{CTRL} = 40 min, CI_{95%} = [33,47] against asleep_{SD} = 45 min, CI_{95%} = [35,55], p-value = 0.8), and *D.virilis* (asleep_{CTRL} = 116 min, CI_{95%} = [105,127] against asleep_{SD} = 108 min, CI_{95%} = [93,124], p-value = 0.42) yet again displayed no change in sleep or rebound architecture (Fig 3.15A,B).

Therefore, the species appear to have an intact rebound response, at least in the case of *D.simulans*, *D.sechellia*, and *D.yakuba*. In order to establish what may have caused the elevated sleep need with the social SD approach, I set out to understand how the two males were interacting within the experimental tubes throughout the 24 hr SD. To achieve this, I recorded 15 min videos at intervals over the 24 hr period capturing their behaviour over the day and manually scoring them in 1 min bins based on a hierarchy of fighting > moving > and immobile. In other words, if an individual was identified to carry out all three behaviours during a 1 min bout, it was scored as fighting; otherwise, all other movement was classed as moving, and therefore, only immobility lasting 1 min was scored.

Interestingly, the species that were found to perform higher levels of aggressive behaviour corresponded to their social SD rebound (Fig 3.16). That is, the groups that exhibited significant increases in sleep post-social SD were scored to have allocated more time to fighting. *D.melanogaster* were found to interact with the intruder fly aggressively 52% (CI_{95%} = [41,64]) of the scored bins, *D.sechellia* 39% (CI_{95%} = [29,48]), *D.yakuba* 33% (CI_{95%} = [27,40]), *D.simulans* 35% (CI_{95%} = [26,44]), *D.willistoni* 21% (CI_{95%} = [14,28]), *D.erecta* 10% (CI_{95%} = [6,14]), and *D.virilis* 6% (CI_{95%} = [0,12]) (Fig 3.16A). The *D.melanogaster* group engaged in a higher level of fighting behaviour

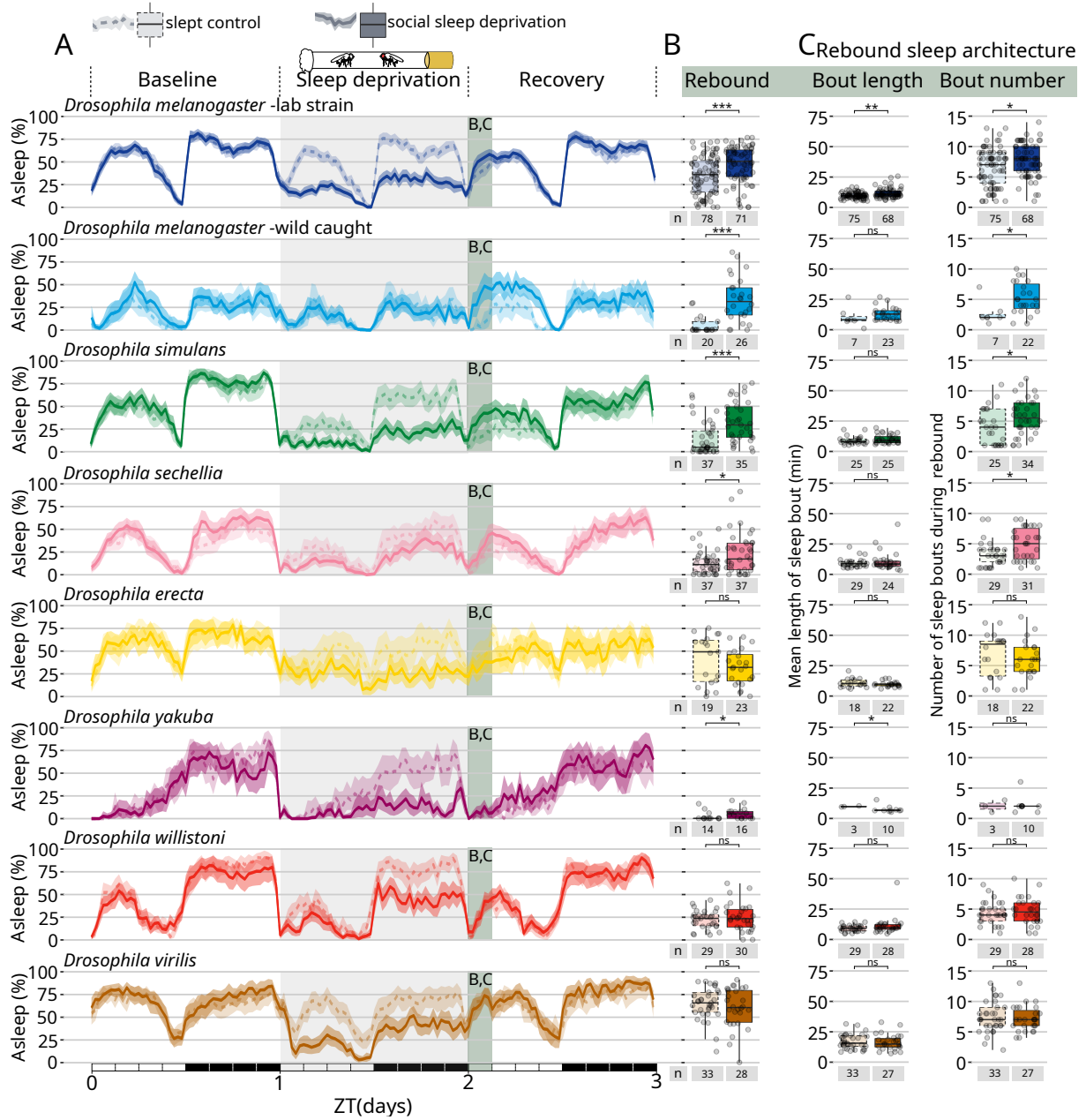


Figure 3.15: The panel of 7 *Drosophila* species response to social SD (A) Sleep profile of socially isolated male *D.melanogaster* lab strain (dark blue), *D.melanogaster* wild caught (light blue), *D.simulans* (green), *D.sechellia* (pink), *D.erecta* (yellow), *D.yakuba* (purple), *D.willistoni* (red) & *D.virilis* (brown) exposed to 24 hr social SD (solid line) and mock slept controls (dashed line) (B) Quantification of average sleep (C) duration of sleep bouts and sleep bout number during the ZT0-3 rebound period (green shading) following social SD (solid line) or in the mock slept controls (dashed line). All flies were raised and tested on standard food.* = < 0.05, ** = < 0.01 & *** = < 0.001. The duration of the tracking was 3 days and rebound sleep characteristics were measured over ZT0-3 of the recovery day. The plots are representative of a minimum of 3 repeats and Wilcoxon Rank Sum Test was used for the statistical comparisons in B, C & D.

than most of the other *Drosophila* species and only the *D.sechellia* aggression scores were statistically comparable (p-value = 0.067). *D.sechellia*, *D.yakuba* and *D.simulans* displayed equivalent fighting behaviour, while *D.willistoni* were significantly less aggressive (p-value = 0.014 compared to *D.simulans*). *D.erecta* and *D.virilis* exhibited the least number of fighting instances, with *D.erecta* scoring 11% below *D.willistoni* (p-value = 0.025). To explore the relationship between aggression and homeostatic sleep rebound in greater detail, I performed linear regression analysis on the mean fighting and sleep change % values for each species (Fig 3.16B). Sleep change was calculated as the increase or decrease in sleep over ZT0-3 following social SD when compared to the sleep amount in the control group during the same period. The analysis revealed a positive correlation between fighting levels during the social SD period and the change in sleep during the rebound period, with a Pearson's correlation coefficient value of 0.81. The linear regression model provided only a partial representation of the data, as demonstrated by the calculated fit of the raw data to the modelled linear trend (adjusted R-squared value = 0.59). Although the relationship between sleep change and fighting level is not strictly proportional, it is noteworthy that species identified as more aggressive tend to exhibit increased sleep following the social SD encounter.

3.2.2.6 Sleep homeostasis with CDM and L-dopa feeding

In order to understand if elevating neuromodulatory signalling in the *Drosophila* genus could restore the abolished rebound to mechanical SD displayed in the non-*melanogaster* species, I exposed the species to the octopamine agonist, CDM, and dopamine precursor, L-dopa, throughout the mechanical SD experiment and measured their rebound response. In both scenarios, the drug feeding did not elicit a change in sleep following the cessation of SD in *D.simulans* (p-value_{CDM} = 0.89 & p-value_{L-DOPA} = 0.35), *D.sechellia* (p-value_{CDM} = 0.09 & p-value_{L-DOPA} = 0.75), *D.erecta* (p-value_{CDM} = 0.33 & p-value_{L-DOPA} = 0.43), *D.yakuba* (p-value_{CDM} = 0.93 & p-value_{L-DOPA} = 0.072), *D.willistoni* (p-value_{CDM} = 0.31 & p-value_{L-DOPA} = 0.74) or *D.virilis* (p-value_{CDM} = 0.4 & p-value_{L-DOPA} = 0.49), with all groups sleeping comparably to their undisturbed controls. However, contrastingly to the result found with 24hr and 168hr mechanical SD, CDM, and L-dopa feeding in *D.melanogaster* wild caught attenuated the elevated sleep during ZT0-3 associated with the mechanical method (p-value_{CDM} = 0.71 & p-value_{L-DOPA} = 0.17) (Fig 3.17).

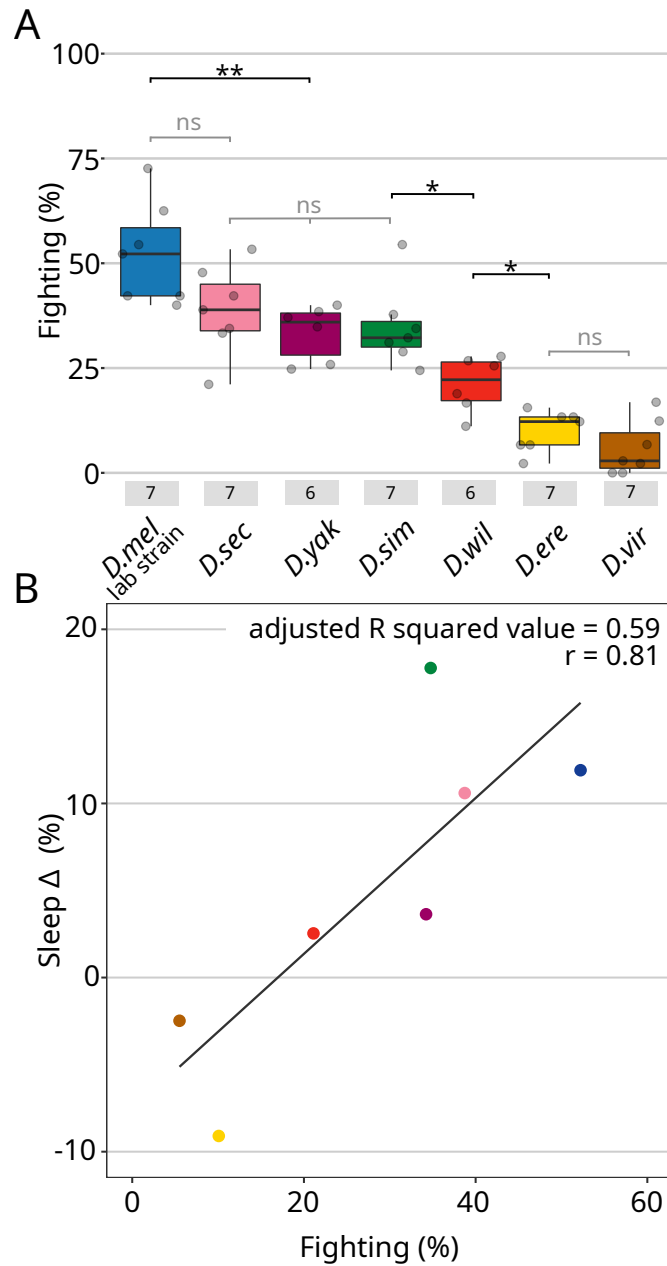


Figure 3.16: Fighting levels and rebound sleep with 24 hr social SD male-male interactions

(A) Quantification of the proportion of 1 min bins that were scored as containing fighting behaviour in *D.melanogaster* lab strain (dark blue), *D.simulans* (green), *D.sechellia* (pink), *D.erecta* (yellow), *D.yakuba* (purple), *D.willistoni* (red) & *D.virilis* (brown). 15 min videos were recorded at 6 time-points (ZT0, ZT6, ZT11.75, ZT12, ZT18, ZT23.5) over the course of the interaction and were manually scored as described in the Materials and methods 2.4.2. (B) Linear regression analysis of the relationship between the fighting levels and the change in sleep during ZT0-3 following social SD compared to the control. Each point represents the group average fighting and sleep change values for each species. The strength and direction of the relationship was quantified using Pearson's correlation coefficient (r). * = < 0.05, ** = < 0.01 & *** = < 0.001. A is representative of 2 repeats and the sleep change in B was from an experiment with a minimum of 3 repeats. Wilcoxon Rank Sum Test was used for the statistical comparisons in A.

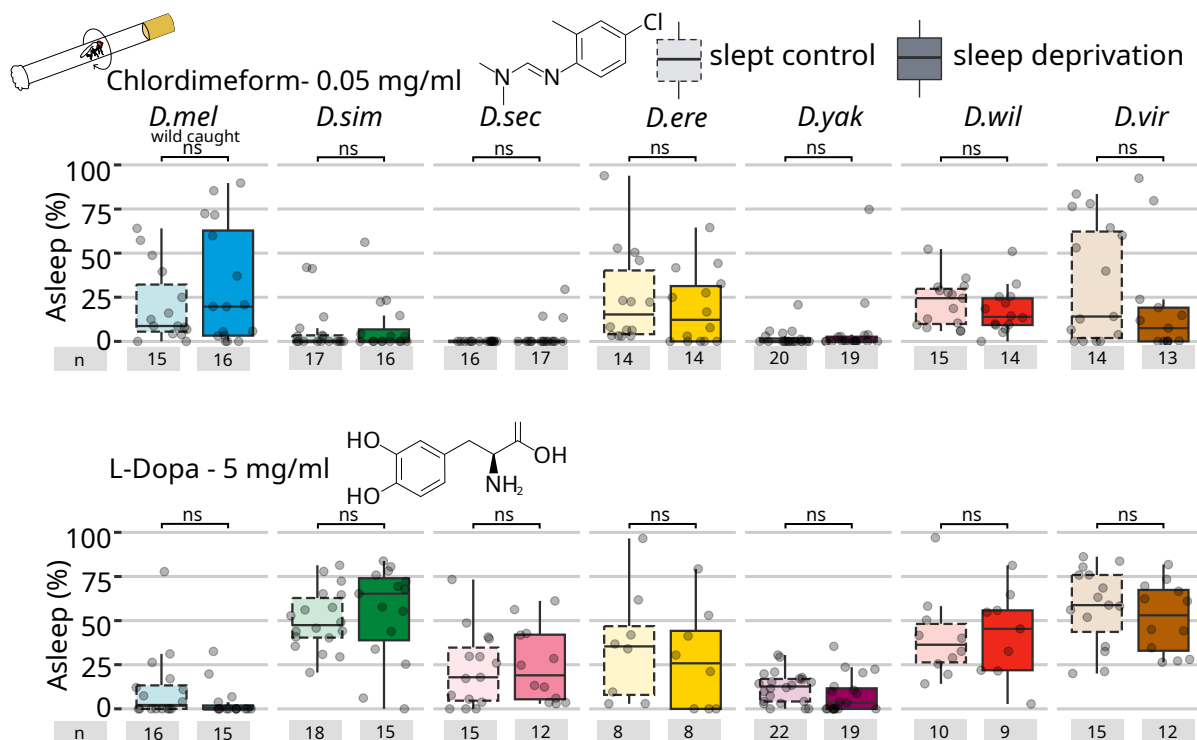


Figure 3.17: Response to mechanical SD with CDM and L-dopa feeding
(A) Quantification of average sleep during the ZT0-3 rebound period following mechanical SD (solid box) or in the undisturbed slept control (dashed box) in *D.melanogaster* wild caught (light blue), *D.simulans* (green), *D.sechellia* (pink), *D.erecta* (yellow), *D.yakuba* (purple), *D.willistoni* (red) & *D.virilis* (brown) exposed to CDM **(B)** or L-dopa feeding for the duration of the experiment. All flies were raised on the potato starch diet and tested on drug food. The plots are representative of a minimum of 4 repeats and Wilcoxon Rank Sum Test was used for the statistical comparisons

3.2.3 Summary

- The circadian aspects of *Drosophila* sleep are mostly conserved in *D.melanogaster*, *D.simulans*, *D.sechellia*, *D.erecta*, *D.yakuba*, and *D.willistoni*. *D.virilis*, however, are arrhythmic in constant darkness.
- There is potential divergence in the molecular mechanisms underpinning sleep, with alterations in response to dopamine feeding.
- *D.melanogaster* are the only group to exhibit homeostatic rebound following 24 and 168 hr mechanical SD.
- A selection of the panel (*D.melanogaster*, *D.simulans*, *D.sechellia* & *D.yakuba*) display rebound following a potentially more ecologically relevant social SD method.
- A casual link between aggression and sleep is revealed with manual scoring of the male-male interactions during social SD.

Chapter 4

Synaptic plasticity and sleep homeostasis

4.1 Background

Conceptually, if a homeostatic mechanism regulates sleep, it must have all the features of a true homeostat. This would require a sensor that measures the change in sleep state, an integrator to compute any change in the system, and an effector, or output arm, to restore the variable to baseline. The underlying cellular and molecular mechanism of this accumulation of sleep need that is sensed within the sleep homeostat likely encodes an essential function of sleep. Currently, there are two branches of thinking which investigate the underlying mechanistic correlates of sleep need - cellular metabolic maintenance (reviewed in ^[380]) and synaptic homeostasis^[90].

Over the past decades, evidence has started to build that demonstrates the relationship between synaptic plasticity, learning, and sleep need. In 2003, the SHY hypothesis^[381] of sleep need was proposed and described that sleep was the 'price of plasticity'. Synaptic potentiation occurs during learning and wake, as neurons selectively distinguish potentially meaningful neuronal inputs from electrical coincidences, firing and thus increasing synaptic strength. As the number and strength of synaptic connections build throughout wake, neuronal selectivity is impaired as the signal-to-noise decreases, and the brain becomes saturated. This negatively impacts the brain and organism due to the increases in energy consumption and cellular stress, ultimately impacting the brain's ability to continue learning ^[90]. The SHY hypothesis postulates that during sleep, when an animal is shut off from the external environment, synaptic strength is downscaled, allowing for less meaningful synaptic connections to be pruned and for consolidating

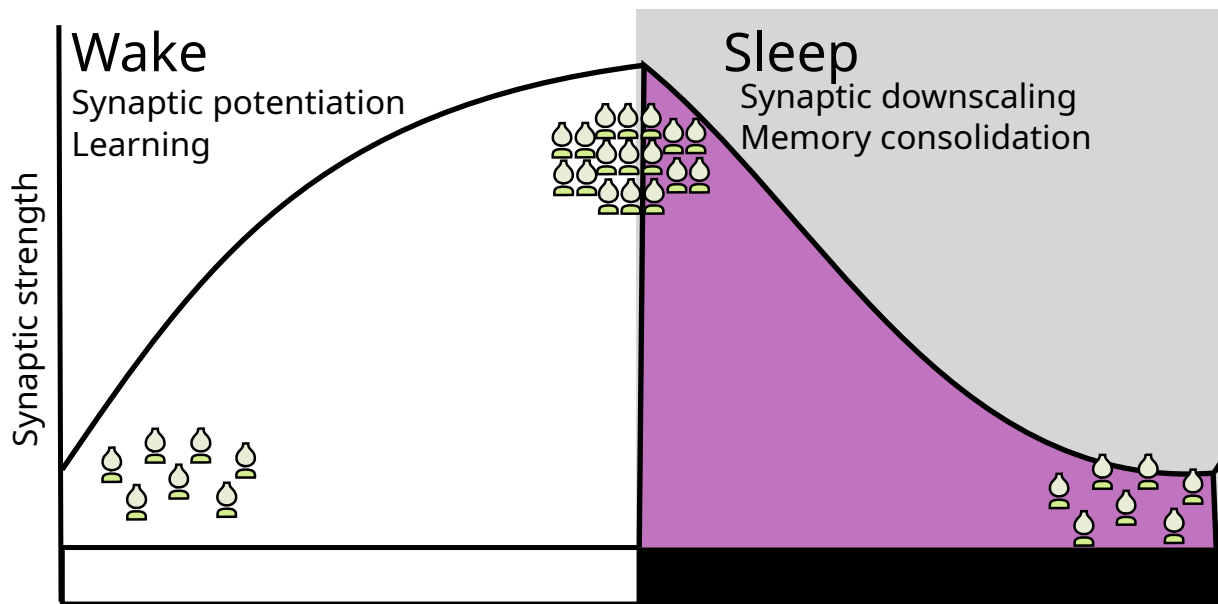


Figure 4.1: Synaptic homeostasis hypothesis of sleep need.

The SHY hypothesis postulates that sleep serves as a period of downscaling the synaptic connections accumulated during wake. While an organism is interacting with its environment and learning, there is a net increase in synaptic strength due to the increasing number and size of synaptic connections. On account of energy and space restrictions in the brain, these connections must be removed and sleep may act as a period in which an organism is disconnected from the external world, and synaptic strength can be downscaled.

relevant information into long-term memory (Fig 4.1). Thus, synaptic plasticity allows us to learn and could be an essential function of sleep.

Mechanisms of activity-dependent synaptic plasticity

Synaptic plasticity can be broadly categorised as morphological and functional changes that modulate presynaptic response to activity-dependent alterations and postsynaptic response to the neurotransmission of presynaptic neurons^[382]. Morphological changes include synaptic number, size and density, branching of dendrites, and alterations in the synaptic contacts. While functional changes broadly incorporate the alteration of neurotransmission via mechanisms involved with the release of neurotransmitters from the presynaptic neuron and the organisation of receptors at the post-synapse.

Many molecules, proteins, and pathways are involved with mechanisms of synaptic plasticity^[382]. A well-studied mechanism is that of the secondary messenger cAMP, found to mediate pre and postsynaptic plasticity in vertebrates and invertebrates, primarily via activation of its downstream target PKA^[383]. The presynaptic mechanisms which evoke alterations in plasticity include PKA phosphorylation of proteins implicated in the mechanisms of neurotransmitter release; for example, activation of ion

channels or synaptic proteins involved in synaptic vesicle processes^[384]. In contrast, the PKA phosphorylation of transcriptional activators causes long-term effects by mediating protein synthesis. For example, the cAMP-responsive element binding protein (CREB) is activated by phosphorylation, stimulating *mRNA* transcription, and plays a prominent role in forming long-term memory^[385].

Early canonical learning and memory mutants for the genes *dunce* and *rutabaga* were later found to have altered cAMP signalling in opposing directions - with *dunce* encoding a phosphodiesterase responsible for cAMP degradation^[386] and *rutabaga* encoding a membrane-bound adenylyl cyclase essential for cAMP synthesis^[387]. A 1990 study of the synaptic plasticity mechanisms in the muscle fibres of the larval neuromuscular junction identified that while *dunce*⁻ and *rutabaga*⁻ mutations impact cAMP levels in opposing directions, they incur several similar impairments in synaptic plasticity, albeit likely via different mechanisms^[388]. The principal difference between the *dunce*⁻ and *rutabaga*⁻ mutations was the elevation in neurotransmitter release in *dunce*⁻ fibres. In comparison, *rutabaga*⁻ neurotransmitter release was unchanged. Despite this difference, both mutations resulted in impaired synaptic facilitation and long-term potentiation, likely underlying the common phenotypes that both mutants exhibit in short-term memory^[388]. A 2011 study observed varied synaptic morphology at the neuromuscular junction, with *rutabaga*⁻ exhibiting decreases in synaptic bouton number, branching, and innervation length, while *dunce*⁻ had increased bouton number^[389]. This evidence, taken together, demonstrates the impact of cAMP homeostasis on structural and functional synaptic plasticity.

Neurotransmitters are stored and transported in synaptic vesicles, which undergo exocytosis from the synapse mediated by Ca²⁺ signalling. Following exocytosis, the vesicles are recycled for reuse. However, at higher activity levels, synaptic vesicles are mobilised from a reserve pool to meet the neurotransmitter demand and maintain synaptic strength under persistent firing^[390]. The synaptic proteins involved with the synaptic vesicle machinery are essential for the function of synapses and synaptic plasticity. For example, mutations in the synapsin phosphoprotein involved in the maintenance and mobilisation of the reserve pool result in multiple functional synaptic plasticity and behavioural impairments. *Synapsin*⁻ flies exhibit a diminished reserve pool^[391], reduced neurotransmitter release upon maintained depolarization^[392], substantial depression, and impaired learning and memory^[393].

Synaptic plasticity and sleep need

Evidence of sleep needs association with synaptic plasticity has been found in humans and multiple model organisms. A small, non-invasive human study demonstrated that

cortical excitability in wake is higher than that of sleep ^[394]. Vyazovskiy *et al.*^[395] comprehensively demonstrated comparable results in rodents, finding molecular and electrophysiological evidence of long-term potentiation and long-term depression during wake and sleep respectively. Levels of GluR1 containing glutamatergic AMPA receptors (GluR1-AMPA) serve as a measure for synaptic strength, as stronger synapses have a higher proportion of the receptors. GluR1-AMPA levels at the cortical and hippocampal synaptoneuroosomes were found to be 50% higher in spontaneously waking rats compared to sleeping animals. Elevated molecular synaptic strength markers were accompanied by increased synaptic efficacy, uncovered by the increased long-term potentiation during wake and decrease during sleep. In *Drosophila*, social enrichment, and therefore the waking environment, increases synaptic density^{[396][397]} with accompanied elevations in sleep ^{[398][399]}. The two sleep studies established that genes involved in learning and memory were essential for social enrichment-induced increases in sleep need, reinforcing the relationship between learning and sleep. Spontaneous wake ^[319] and social enrichment ^[399] led to increased levels of pre and postsynaptic proteins in the fly brain, that was reduced after a period of sleep. Importantly, in all of these studies, SD maintained or heightened the described molecular and electrophysiological changes during wake, which were only reduced after a period of rebound sleep. This evidence supports that the downscaling of synapses that are strengthened during wake is specific to mechanisms that occur during sleep. Gilestro *et al.*^[319] showed that increasing lengths of mechanical and social SD led to accumulation in the levels of the presynaptic protein Bruchpilot (BRP) and postsynaptic protein Discs large, that were all reduced after rebound. Therefore, the pre and postsynaptic protein levels correlate with the wake length. All of this evidence across the animal kingdom supports the hypothesis that sleep downscals the synaptic plasticity-mediated synaptic strength elevations that occur during wake.

In this chapter, I investigated the synaptic strength dynamics underlying homeostatic sleep. Firstly, I measured the levels of BRP across the panel of *Drosophila* species in response to SD to uncover the influence of synaptic strength on their rebound response, or lack thereof. Secondly, I utilised the elegant genetic toolbox available in the *D.melanogaster* model and examined the impact of alterations in synaptic plasticity on both mechanical and social SD. In order to elucidate the possible underlying mechanism responsible for the divergence in response to mechanical SD.

4.2 Results

4.2.1 BRP expression after sleep deprivation

In Chapter 3, I demonstrated that only the canonical *D.melanogaster* line rebounds after mechanical SD, even though a selection of the other *Drosophila* species appear to have a functioning homeostat – illustrated by the rebound sleep after social SD seen with *D.simulans*, *D.sechellia* and *D.yakuba*. It was plausible that the altered rebound following mechanical SD was due to modifications in the accumulation of sleep need. In recent years, evidence has emerged that suggests not all sleep loss is the same. Accumulation of sleep need in *Drosophila* depends on the wake environment; starvation^[201], male-female interactions^[108], and male-male interactions^[319] all result in sleep loss in males. However, only the latter sleep deficit is recovered. I reasoned that the environment of wake within the mechanical SD method was qualitatively different in the non-*melanogaster* species, failing to drive sleep need despite the drastic loss in total sleep. In order to address this, and on account of the strong relationship between homeostatic sleep need and synaptic strength, I wanted to measure changes in synaptic dynamics in response to SD in the panel of species.

Sleep need is correlated with changes in synaptic strength markers, including the presynaptic scaffolding protein, BRP. It is an essential component of the presynaptic active zone, and higher synaptic density will be associated with increased levels of BRP. It is often utilised as a proxy for measuring changes in synaptic strength upon SD, found to be elevated throughout a sleep-deprived fly’s brain. I measured the levels of the BRP protein in the Drosophilid brains upon mechanical SD, in the same manner as the foundational Gilestro *et al.*^[319] study. The flies were exposed to mechanical SD for 24 hr, dissected at ZT0, and immunostained for confocal measurement of the BRP signal in their brains. I investigated the BRP signal specifically in the FB due to its established output role in the sleep homeostat. In both *D.melanogaster* lab and wild species, mechanical SD was accompanied by increased intensity of the BRP signal ($\text{intensity}_{\text{SD}_{\text{lab}}} = 1.17 \text{ a.u.}$, $\text{CI}_{95\%} = [0.97, 1.37]$ & $\text{intensity}_{\text{SD}_{\text{wild}}} = 1.35 \text{ a.u.}$, $\text{CI}_{95\%} = [1.10, 1.60]$) compared to the slept controls ($\text{intensity}_{\text{CTRL}_{\text{lab}}} = 0.91 \text{ a.u.}$, $\text{CI}_{95\%} = [0.79, 1.03]$ & ($\text{intensity}_{\text{CTRL}_{\text{wild}}} = 0.65$, $\text{CI}_{95\%} = [0.53, 0.77]$, $p\text{-value} = 0.032$ & 0.00016 respectively) (Fig 4.2). Interestingly, *D.simulans* and *D.sechellia* both exhibited elevated BRP intensity in response to mechanical SD ($p\text{-value}_{\text{SIM}} = 0.0014$ & $p\text{-value}_{\text{SEC}} = 0.00021$)(Fig 4.2). *D.erecta*, *D.yakuba*, *D.willistoni* and *D.virilis* exhibited equivalent FB BRP intensity after mechanical SD to the undisturbed controls (Fig 4.3) (Fig 4.4) (Fig 4.5) (Fig 4.6) (Fig 4.7) (Fig 4.8) (Fig 4.9). In the *D.melanogaster* flies, the abun-

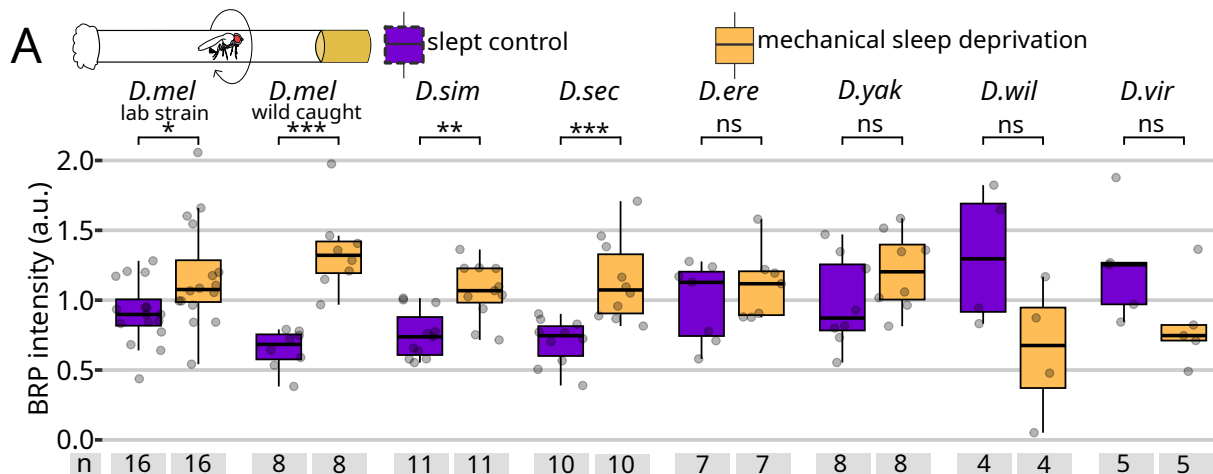


Figure 4.2: BRP intensity in the FB of the 7 *Drosophila* species following mechanical SD

(A) Quantification of the BRP signal intensity in the FB following mechanical SD (purple) and in the undisturbed slept controls (orange). All flies were raised on the potato starch diet and tested on standard food. * = < 0.05, ** = < 0.01 & *** = < 0.001. The plots are representative of a minimum of 2 repeats and Wilcoxon Rank Sum Test was used for the statistical comparisons

dance of the synaptic scaffolding protein complemented the rebound exhibited following mechanical SD. However, I identified two candidates, *D.simulans* and *D.sechellia*, where mechanically induced wakefulness resulted in an elevation in the molecular marker of sleep need without a compensatory sleep response. In contrast, with respect to *D.erecta*, *D.yakuba*, *D.willistoni* and *D.virilis* the lack of rebound correlates with the absence of the stereotypical BRP upregulation usually exhibited upon sleep loss.

4.2.2 Pan-neuronal knockdown of synaptic plasticity genes

Although the research of other *Drosophila* species is currently limited in terms of the genetic tools that are available for the study of the underpinnings that drive changes in sleep need and response to SD; I employed the elegant toolbox available in *D.melanogaster* to further investigate the relationship between synaptic plasticity and sleep. In harnessing the GAL4-UAS system combined with RNAi, I performed a small screen of GOIs that have an established role in synaptic plasticity. More information on the full list of genes involved in the small screen, including their proposed roles in synaptic plasticity are summarised in Table 4.1.

I used an nSyb-GAL4 line that labels all neurons in the fly brain to drive the RNAi-mediated KD of the GOIs and measured changes in their sleep behaviour during baseline

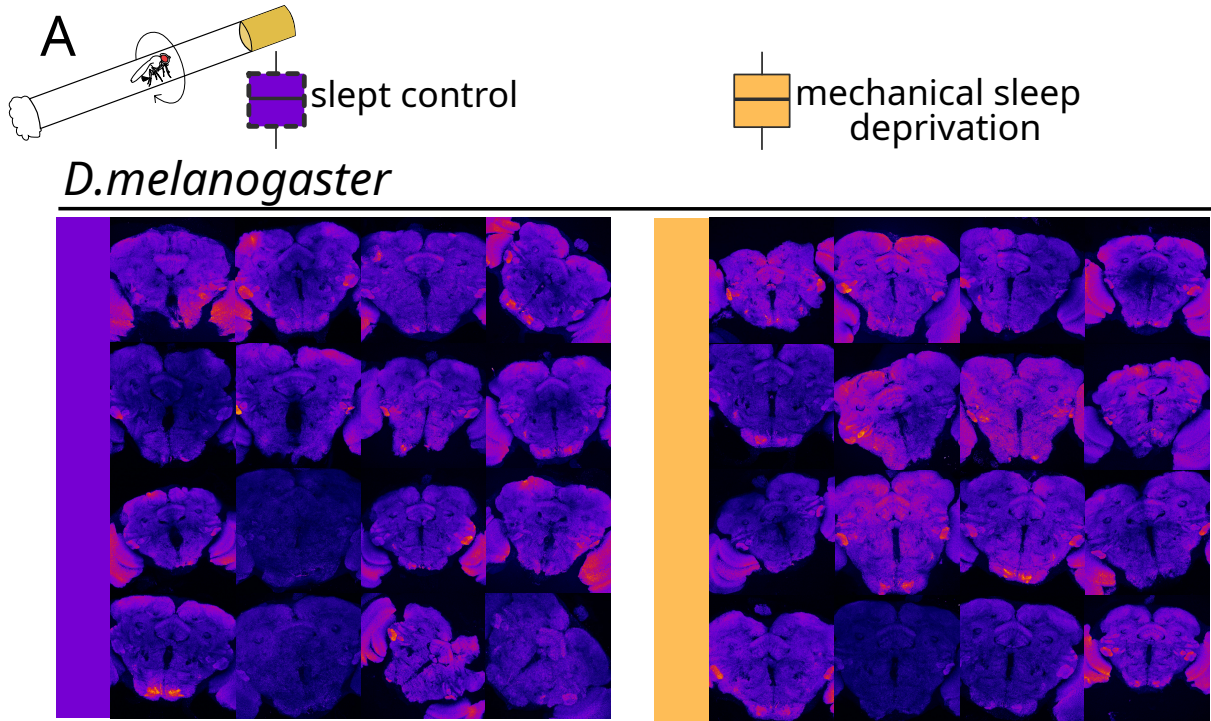


Figure 4.3: *D.melanogaster* brain images used for the quantification of BRP following mechanical SD

(A) *D.melanogaster* brain images used for the quantification of BRP following mechanical SD in Fig 4.2

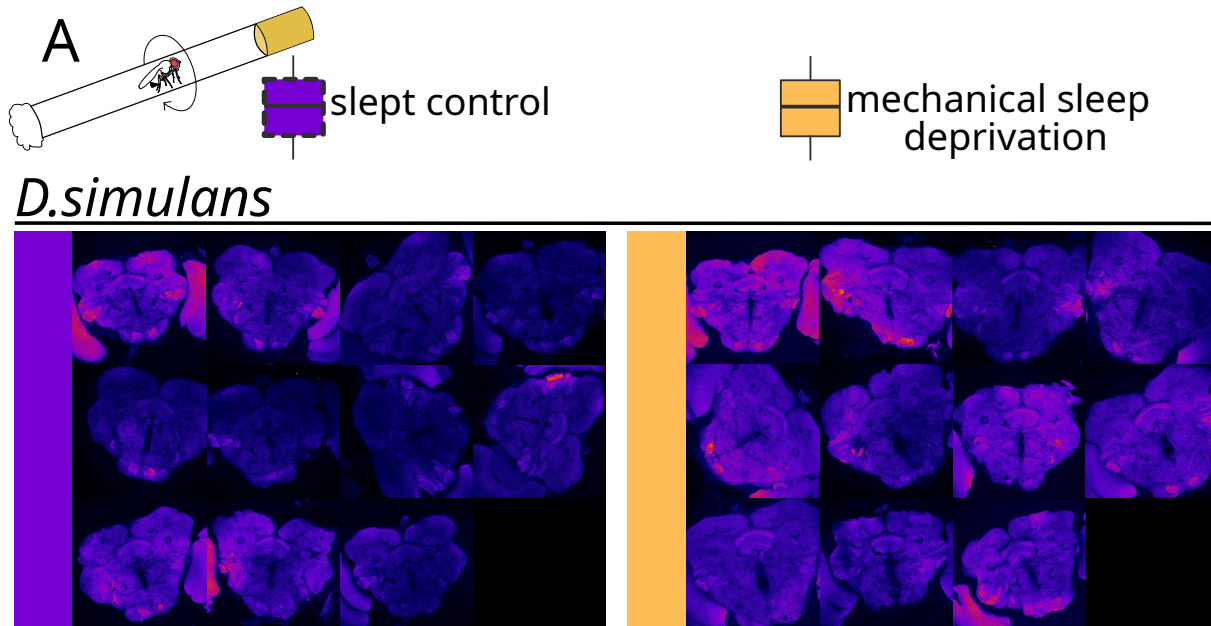


Figure 4.4: *D.simulans* brain images used for the quantification of BRP following mechanical SD in Fig 4.2

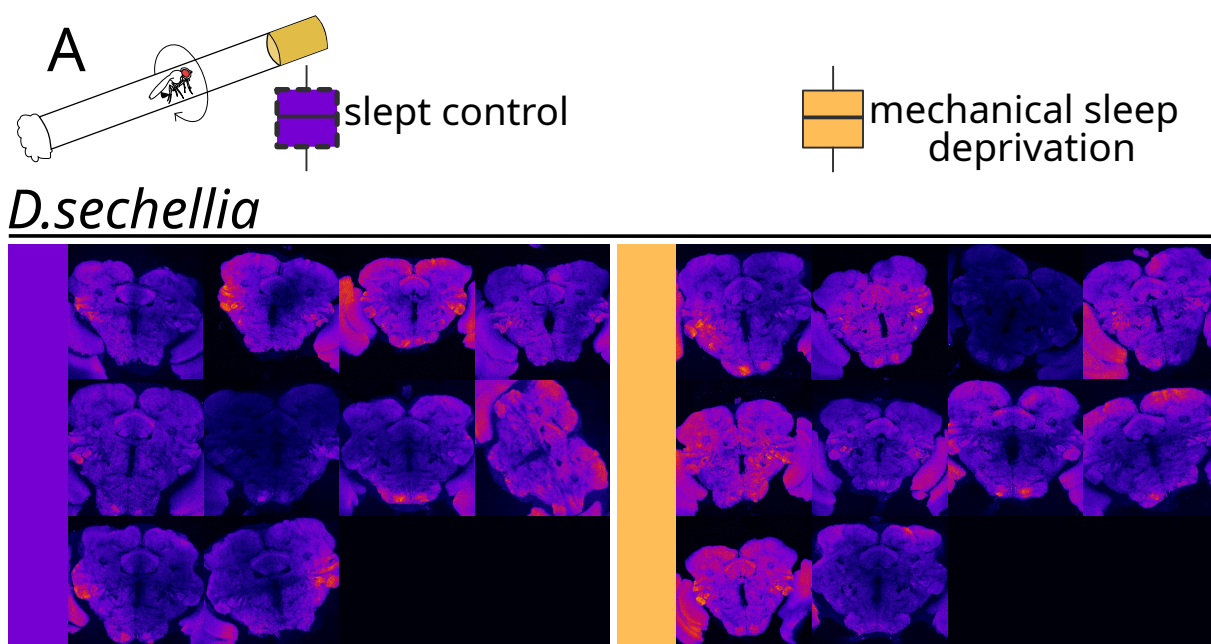


Figure 4.5: *D.sechellia* brain images used for the quantification of BRP following mechanical SD in Fig 4.2

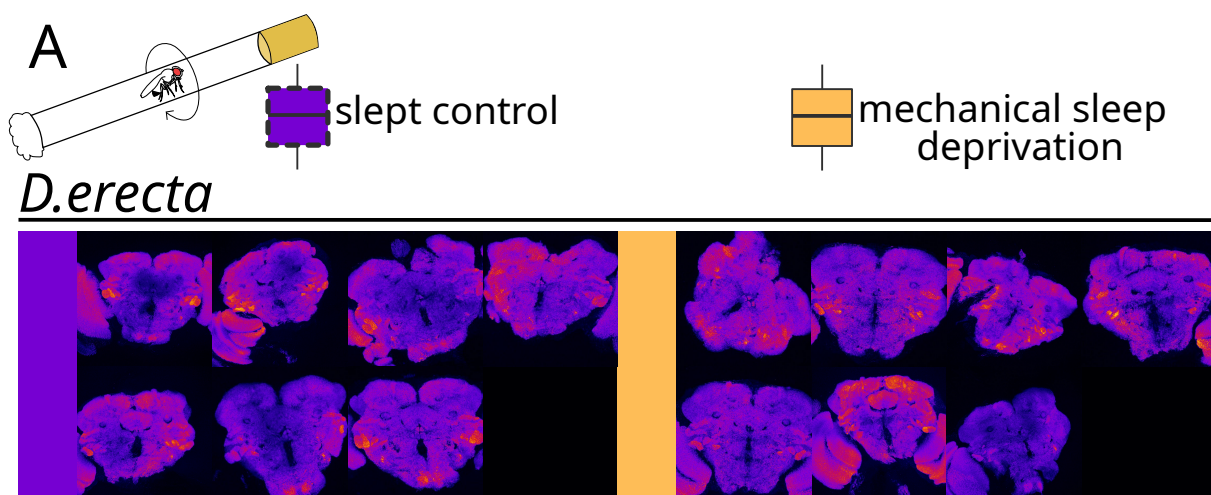


Figure 4.6: *D.erecta* brain images used for the quantification of BRP following mechanical SD in Fig 4.2

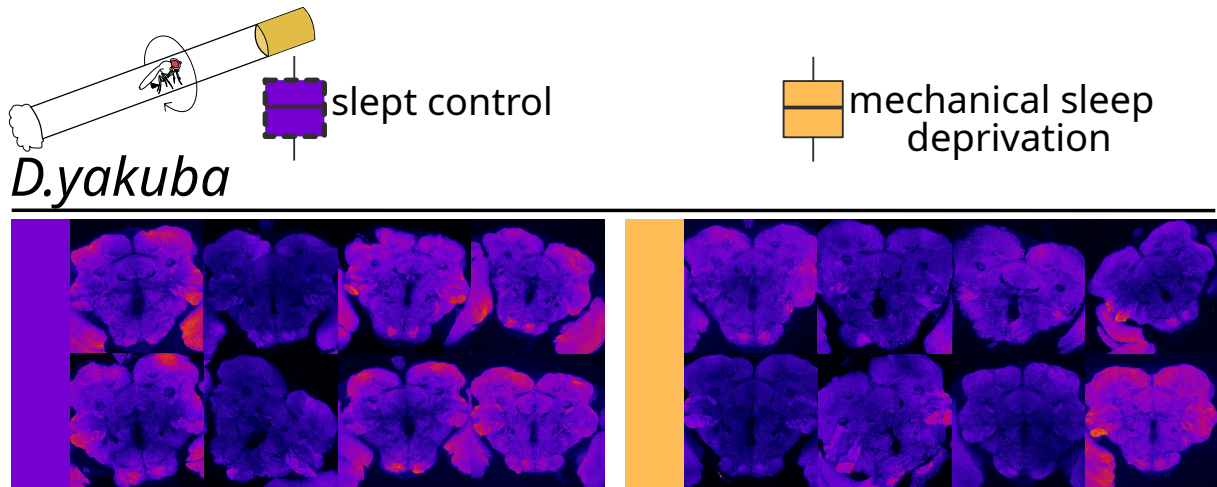


Figure 4.7: *D.yakuba* brain images used for the quantification of BRP following mechanical SD in Fig 4.2

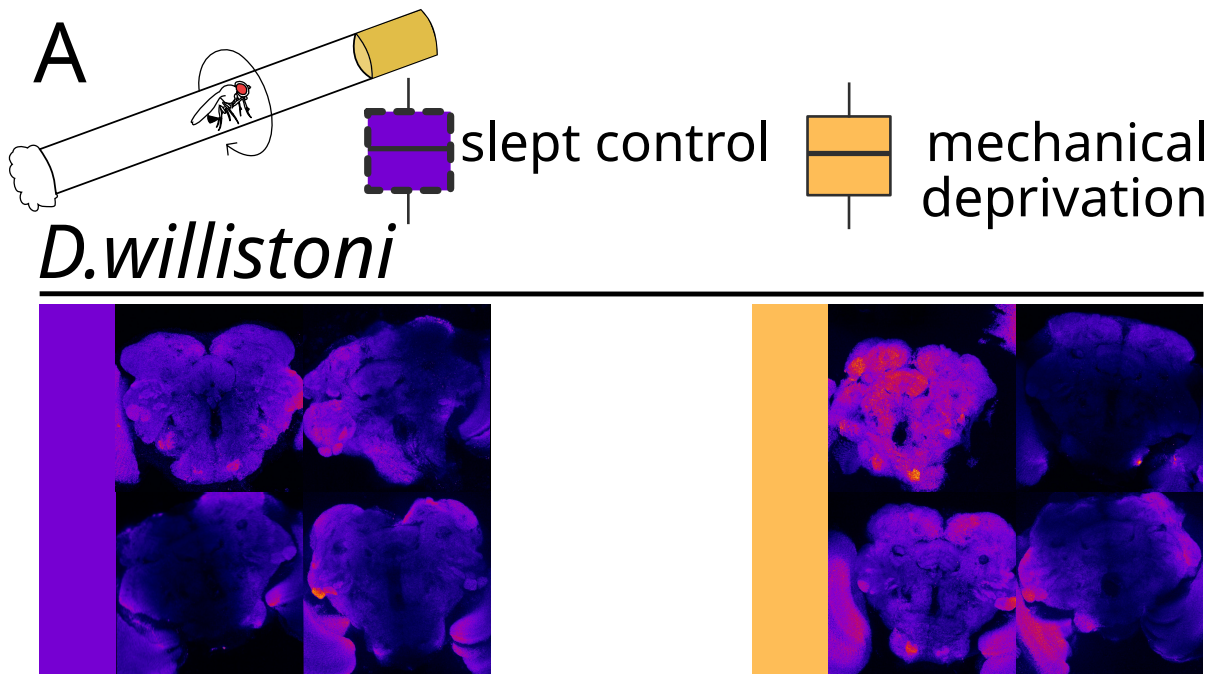


Figure 4.8: *D.willistoni* brain images used for the quantification of BRP following mechanical SD in Fig 4.2

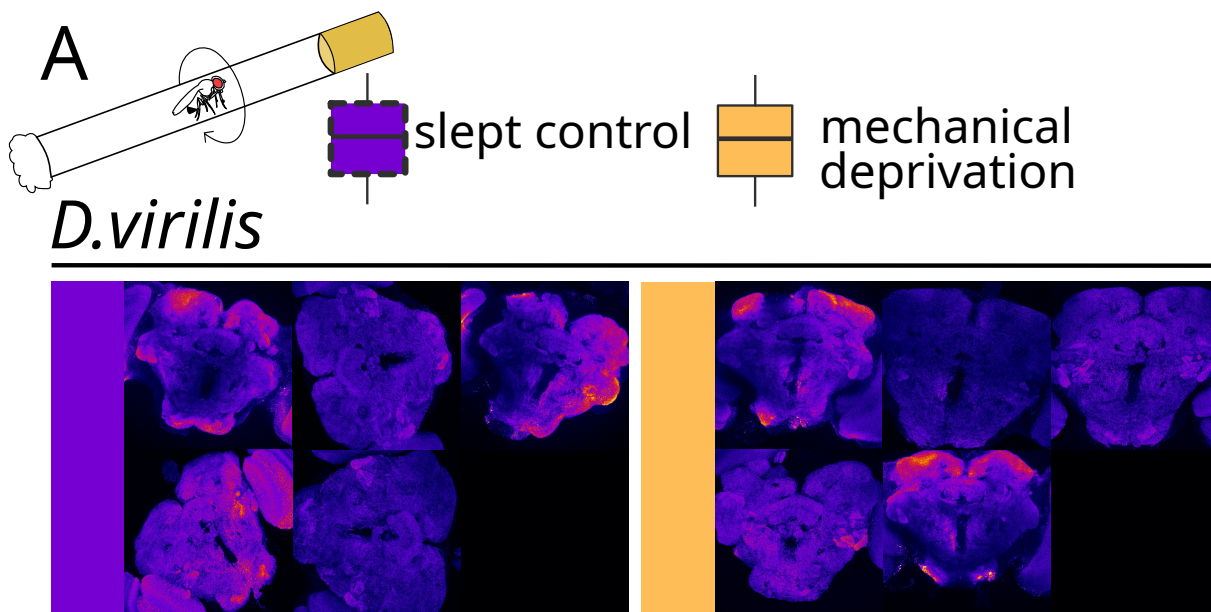


Figure 4.9: *D. virilis* brain images used for the quantification of BRP following mechanical SD in Fig 4.2

and after SD. For each experiment, nSyb-GAL4/+ and UAS-GOI^{RNAi} /+, referred to as the parental controls, were tested alongside the KD groups.

4.2.2.1 Baseline sleep

I first tested whether the GOI KD impacts baseline sleep characteristics. Almost all KD manipulations led to a significant change in the total sleep amount (Fig 4.10). While the sleep of *synapsin* and *starrynight* KD flies over the 24-hour day were equivalent to the UAS-GOI^{RNAi} controls, significant sleep alterations occurred in animals lacking *dunce*, *orb2*, *appl*, *fmr1* and *rutabaga*. Highlighting the role of synaptic plasticity mechanisms in controlling sleep duration.

Pan-neuronal KD of *dunce* resulted in a reduction of total sleep, sleeping 561 min ($CI_{95\%} = [531, 590]$) of the day compared to the +/- UAS-*dunce*^{RNAi} sleep of 633 min ($CI_{95\%} = [605, 660]$, p-value = 0.00030) and nSyb-GAL4/+ sleep of 652 min ($CI_{95\%} = [633, 673]$, p-value = 9.1×10^{-7}). A strong sleep suppression was found in *orb2* KD flies, which slept only 332 min ($CI_{95\%} = [295, 369]$) over the 24-hour day, while the +/-UAS-*orb2*^{RNAi} flies slept for 444 min ($CI_{95\%} = [417, 471]$, p-value = $< 2.0 \times 10^{-16}$) (Fig 4.10).

Conversely, nSyb-GAL4 driven KD of *appl*, *fmr1* and *rutabaga* enhanced daily sleep compared to +/-UAS-GOI^{RNAi} controls. The flies lacking the *appl* gene sustained an approximate 3 hour rise in sleep (asleep_{KD} = 650 min, $CI_{95\%} = [610, 689]$ against asleep_{RNAi} = 464 min, $CI_{95\%} = [432, 496]$, p-value = 3.8×10^{-11}), *fmr1* KD animals had

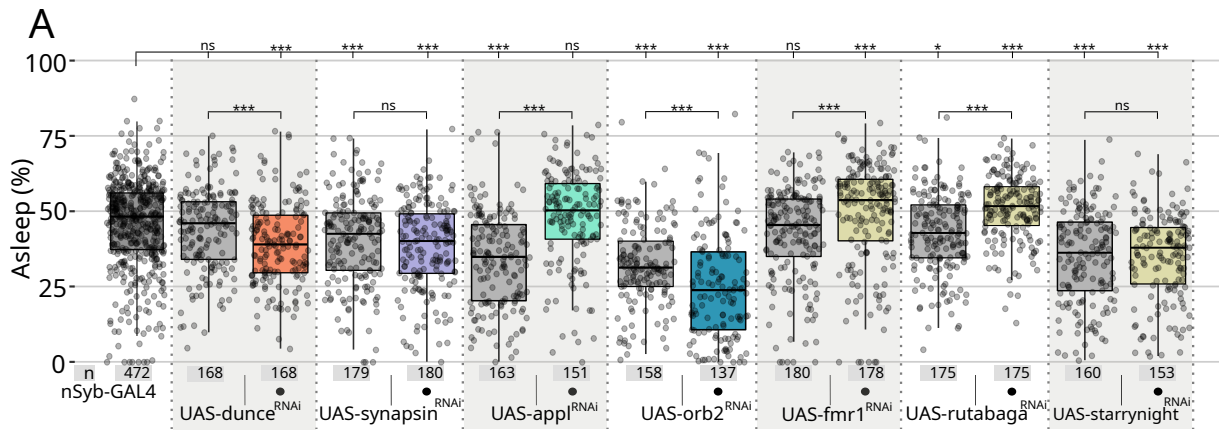


Figure 4.10: Total daily sleep with pan-neuronal KD of synaptic plasticity associated genes

(A) Quantification of the total daily sleep levels with nSyb-GAL4 mediated KD of *UAS-dunce*^{RNAi} (orange), *UAS-synapsin*^{RNAi} (purple), *UAS-appl*^{RNAi} (green), *UAS-orb2*^{RNAi} (blue), *UAS-fmr1*^{RNAi} (yellow), *UAS-rutabaga*^{RNAi} (yellow) & *UAS-starrynight*^{RNAi} (yellow) and their parental controls (grey). All flies were raised and tested on standard food. * = < 0.05, ** = < 0.01 & *** = < 0.001. The duration of the tracking was 24 hours and the plots are representative of a minimum of 4 repeats. Wilcoxon Rank Sum Test was used for the statistical comparisons

a modest 1.5 hour sleep extension ($\text{asleep}_{\text{KD}} = 722 \text{ min}$, $\text{CI}_{95\%} = [691, 754]$ against $\text{asleep}_{\text{RNAi}} = 627 \text{ min}$, $\text{CI}_{95\%} = [598, 656]$, $p\text{-value} = 6.6 \times 10^{-6}$) and *rutabaga* KD flies an approximate 2 hour increase ($\text{asleep}_{\text{KD}} = 733 \text{ min}$, $\text{CI}_{95\%} = [708, 758]$ against $\text{asleep}_{\text{RNAi}} = 612 \text{ min}$, $\text{CI}_{95\%} = [587, 638]$, $p\text{-value} = 2.6 \times 10^{-9}$) (Fig 4.10).

However, the *appl* KD sleep was statistically indistinguishable from the nSyb-GAL4/+ parental control ($\text{asleep}_{\text{KD}} = 650 \text{ min}$, $\text{CI}_{95\%} = [610, 689]$ against $\text{asleep}_{\text{GAL4}} = 652 \text{ min}$, $\text{CI}_{95\%} = [633, 673]$, $p\text{-value} = 0.99$). Therefore I am unable to conclude that the sleep elevation seen is directly due to the absence of the *appl* gene product or simply a result of the nSyb-GAL4 background. Substantial differences were found in the +/UAS-GOI^{RNAi} and nSyb-GAL4/+ sleep across the screen, and only +/UAS-*dunce*^{RNAi} and +/UAS-*fmr1*^{RNAi} flies generated equivalent sleep values to the nSyb-GAL4/+ control ($p\text{-value} = 0.23177$ & 0.25 respectively). Due to the differences observed in the parental controls, I disregarded results where a change is only seen when compared to one of the parental controls (Fig 4.10).

While changes were seen in the total sleep amount, all of the KD flies maintained the relationship between day and night sleep, with all lines sleeping significantly more during the night phase than the dark phase (Fig 4.11). This indicates that the circadian drives for arousal during the light phase and sleep during the night phase are intact across the genetic manipulations.

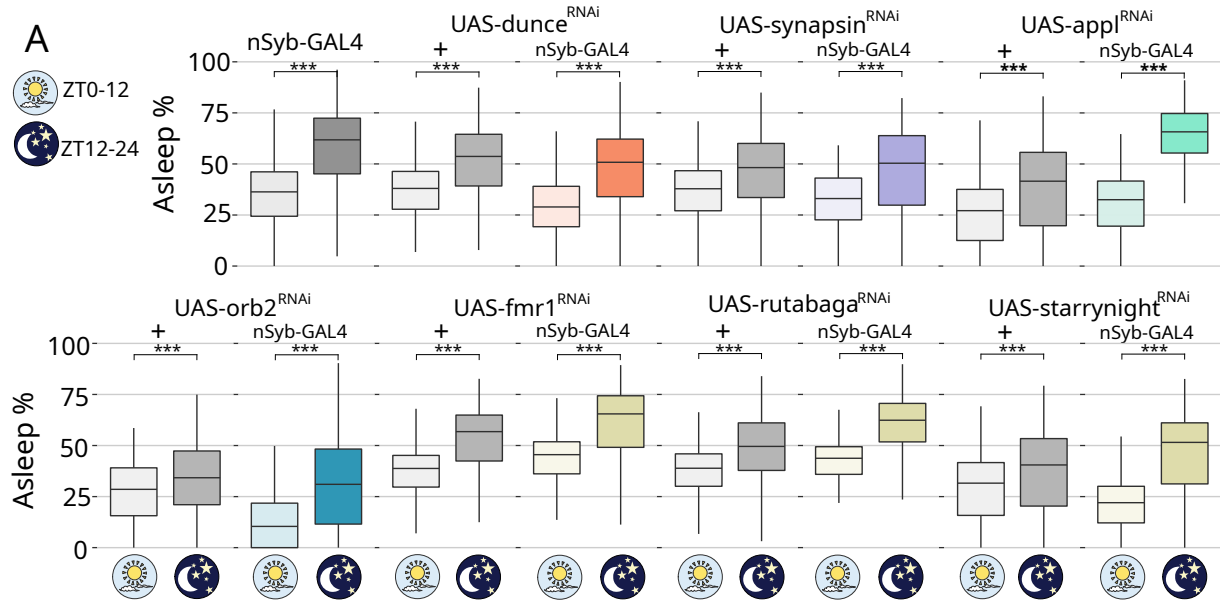


Figure 4.11: Day and night sleep with pan-neuronal KD of synaptic plasticity associated genes

(A) Quantification of the day and night sleep levels with nSyb-GAL4 mediated KD of *UAS-dunce*^{RNAi} (orange), *UAS-synapsin*^{RNAi} (purple), *UAS-appl*^{RNAi} (green), *UAS-orb2*^{RNAi} (blue), *UAS-fmr1*^{RNAi} (yellow), *UAS-rutabaga*^{RNAi} (yellow) & *UAS-starrynight*^{RNAi} (yellow) and their parental controls (grey). All flies were raised and tested on standard food. The ns are equivalent to their total sleep quantification in (Fig 4.10). * = < 0.05, ** = < 0.01 & *** = < 0.001. The duration of the tracking was 24 hours and the plots are representative of a minimum of 4 repeats. Wilcoxon Rank Sum Test was used for the statistical comparisons.

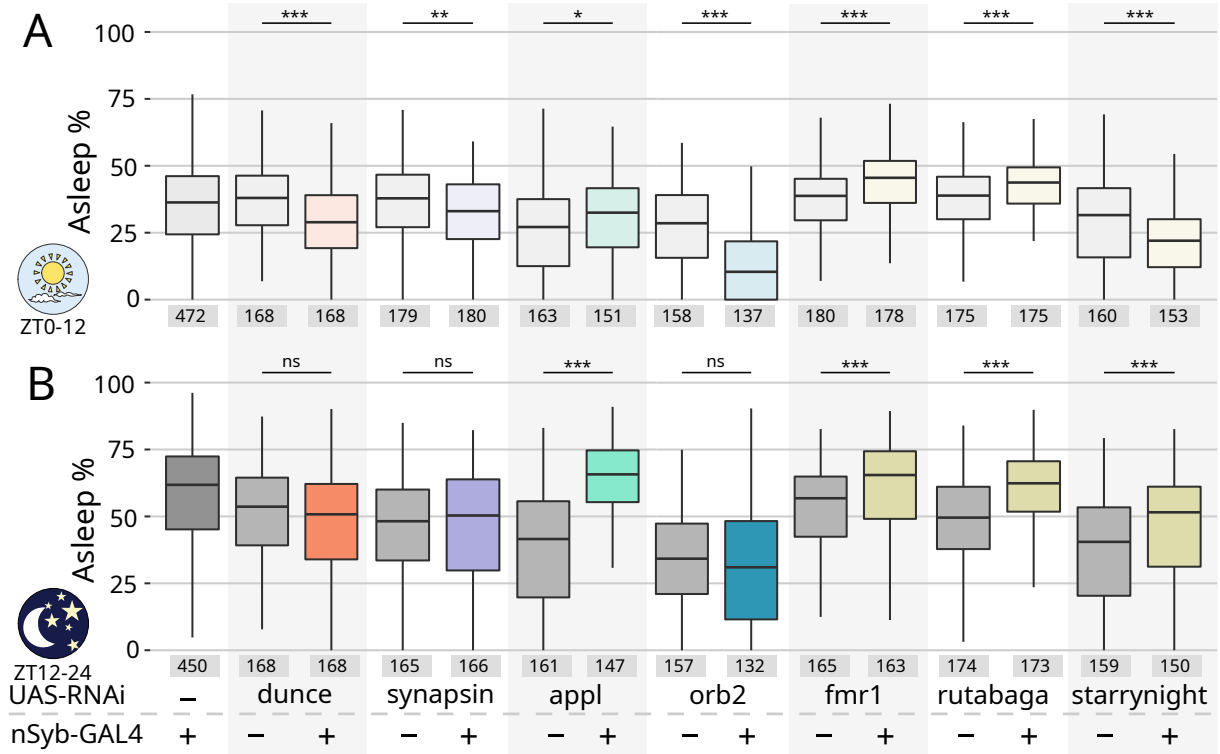


Figure 4.12: The alterations to day and night sleep with pan-neuronal KD of synaptic plasticity associated genes

(A) Quantification of the day sleep levels with nSyb-GAL4 mediated KD of UAS-*dunce*^{RNAi} (orange), UAS-*synapsin*^{RNAi} (purple), UAS-*appl*^{RNAi} (green), UAS-*orb2*^{RNAi} (blue), UAS-*fmr1*^{RNAi} (yellow), UAS-*rutabaga*^{RNAi} (yellow) & UAS-*starrynight*^{RNAi} (yellow) compared to their parental controls (grey) (B) Quantification of the night sleep levels. All flies were raised and tested on standard food. * = < 0.05, ** = < 0.01 & *** = < 0.001. The duration of the tracking was 24 hours and the plots are representative of a minimum of 4 repeats. Wilcoxon Rank Sum Test was used for the statistical comparisons.

To obtain a more comprehensive view of the impact of the global neuronal KD of the genes included in the screen, I explored more extensively the sleep alteration over the light and dark phase. I specifically interrogated the change in sleep time, bout number and bout duration between the KD and their +/UAS-GOI^{RNAi} controls over the day and night. The *dunce* (p-value = 5.3×10^{-6}), *synapsin* (p-value = 0.0035), *orb2* (p-value = 1.9×10^{-13}) and *starrynight* (p-value = 7.6×10^{-6}) pan-neuronal manipulations all displayed a reduction in daytime sleep when compared to their UAS-GOI^{RNAi} parental controls (Fig 4.12A). Conversely, *appl*, *fmr1* and *rutabaga* KD presented with elevated day (p-value = 1.3×10^{-2} , 1.3×10^{-5} & 4.1×10^{-4} respectively) and night sleep (p-value = $< 2.0 \times 10^{-16}$, 8.3×10^{-6} & 4.8×10^{-10} respectively) (Fig 4.12A,B). With respect to the *dunce* KD, the observed reduction in sleep during the light phase resulted from fewer sleep bout occurrences (p-value = 2.4×10^{-10}) rather than a decrease in the duration of each bout, as the average bout length resembled controls (p-value = 0.25) (Fig 4.13A,B). The attenuated daytime sleep found in *synapsin*, *orb2* and *starrynight* KDs manifested from a fall in the number of bouts executed (p-value = 2.4×10^{-10} , 8.8×10^{-10} & 1.5×10^{-11} respectively). However, the mean duration of the bouts were notably longer (p-value = 0.0074, 1.1×10^{-6} & 0.035 respectively), suggesting that the nSyb-GAL4 mediated KD of *synapsin*, *orb2* & *starrynight* may impact sleep and wake transitions. In *orb2* flies, the elevated length (p-value = 3.7×10^{-6}) and reduced number of sleep bouts (p-value = 2.7×10^{-10}) were also exhibited in the dark period, while it was restricted to only the light phase in the case of *synapsin* (Fig 4.13A,B).

In summary, pan-neuronal *dunce* KD resulted in a significant reduction in total baseline sleep, with no change in the organisation of their sleep over the light and dark phases. *Synapsin* and *orb2* KD flies were able to maintain prolonged sleep bouts during the day, demonstrated by the increased length of the day sleep episodes. *Orb2* removal caused the strongest sleep suppression. Both *fmr1* and *rutabaga* KD resulted in a significant sleep elevation. The synaptic plasticity genes appeared to be involved in many aspects of baseline sleep control. While their initial assessment of homeostasis appeared intact, further testing employing SD was required to interrogate their role in the homeostatic control of sleep.

4.2.2.2 Response to mechanical and social sleep deprivation

Analogously to the species experiments executed in Chapter 3, I exposed the KD and parental control flies to 24hr mechanical or social SD and measured the rebound response.

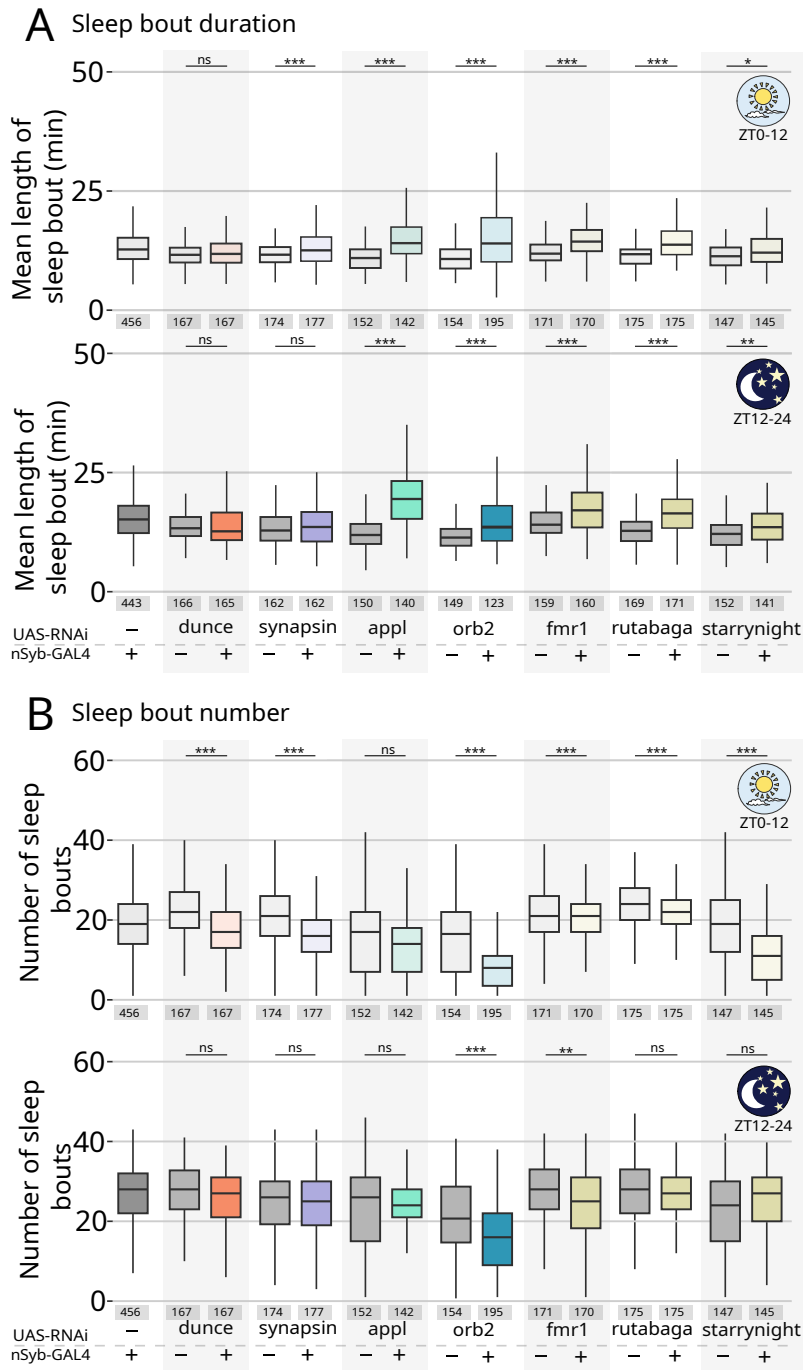


Figure 4.13: The alterations to day and night sleep bout architecture with pan-neuronal KD of synaptic plasticity associated genes **(A)** Quantification of the day and night sleep bout number with nSyb-GAL4 mediated KD of UAS-*dunce*^{RNAi} (orange), UAS-*synapsin*^{RNAi} (purple), UAS-*appl*^{RNAi} (green), UAS-*orb2*^{RNAi} (blue), UAS-*fmr1*^{RNAi} (yellow), UAS-*rutabaga*^{RNAi} (yellow) & UAS-*starrynight*^{RNAi} (yellow) compared to their parental controls (grey) **(B)** Quantification of the day and night sleep bout duration. All flies were raised and tested on standard food. * = < 0.05, ** = < 0.01 & *** = < 0.001. The duration of the tracking was 24 hours and the plots are representative of a minimum of 4 repeats. Wilcoxon Rank Sum Test was used for the statistical comparisons.

All parental controls (nSyb-GAL4/+, UAS-*dunce*^{RNAi}/+, UAS-*synapsin*^{RNAi}/+, UAS-*appl*^{RNAi}/+, UAS-*orb2*^{RNAi}/+, UAS-*fmr1*^{RNAi}/+, UAS-*rutabaga*^{RNAi}/+ and UAS-*starrynight*^{RNAi}/+) showed increased sleep in the ZT0-3 rebound period after both mechanical and social SD, and therefore an intact rebound response (Fig 4.15) (Fig 4.16). Comparable results were found with KD of *fmr1*, *rutabaga*, and *starrynight*, which had significantly elevated sleep compared to the undisturbed flies. I identified four potentially interesting candidates - *dunce*, *synapsin*, *appl*, and *orb2* KD all exhibited impaired rebound after mechanical SD (Fig 4.15). Interestingly, I consistently discovered rebound after social SD in all parental control and KD flies (Fig 4.16). The differential response revealed in the *dunce*, *synapsin*, *appl*, and *orb2* KD to mechanical and social SD casually mimicked the rebound responses revealed in Chapter 3, where *D.simulans*, *D.sechellia*, and *D.yakuba* showed rebound in response to social SD while exhibiting no response to mechanical.

Dunce and *synapsin* knockdown restricts mechanical rebound

Pan-neuronal *dunce* KD flies showed a significant rebound sleep after mechanical SD (asleep_{CTRL} = 93 min, CI_{95%} = [84,102] against asleep_{SD} = 129 min, CI_{95%} = [118,141], p-value = 1.5x10⁻⁸) (Fig 4.15). However, the response was minimal, provisionally demonstrated by the sleep trace over the duration of the SD experiment (Fig 4.17). In the parental controls, there was a clear demarcation between the SD group and slept control during rebound (indicated by the green shaded area)(Fig 4.17A). The *dunce* KD plot was distinct from the parental controls, with little separation between the two traces. Suggesting that the *dunce* KD restricts rebound sleep after mechanical SD. I further empirically probed this finding by calculating the sleep change in rebound compared to baseline, with the following simple calculation:

$$\text{rebound}_{\Delta} = \text{sleep ZT0-3 rebound day} - \text{sleep ZT0-3 baseline day}$$

The baseline day measurements were taken from the morning preceding the 24hr SD period. The above quantification exposed whether any change in their sleep occurred when comparing the sleep amount in the same individual before and after SD. Most rebound change measurements predicatively mimicked the results observed when comparing SD samples to their slept controls (Fig 4.18). For example, the SD parental control groups all had a positive change in their sleep during rebound compared to baseline, which was significantly higher than that of the change in sleep in the slept controls. However, in the case of the mechanical SD *dunce* KD group, there was no change in their sleep during rebound, sleeping 2 min less (rebound_Δ = - 2 min, CI_{95%} = [-9,5], p-value = 0.424) during the rebound period than during baseline (Fig 4.18). For comparison, the SD +/UAS-*dunce*^{RNAi} parental control flies were sleeping 11 mins more

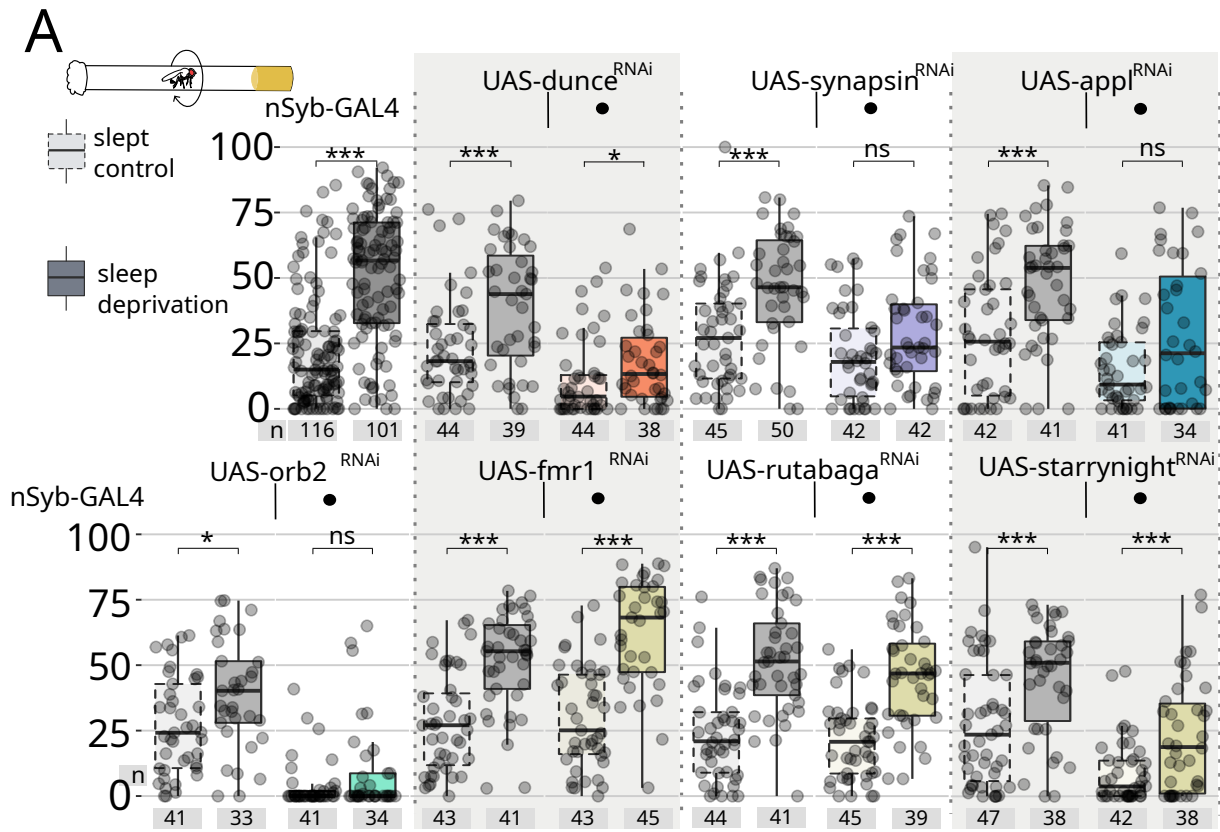


Figure 4.15: Sleep rebound following mechanical SD with pan-neuronal KD of synaptic plasticity associated genes

(A) Quantification of the sleep during the ZT0-3 rebound period with nSyb-GAL4 mediated KD of *UAS-dunce*^{RNAi} (orange), *UAS-synapsin*^{RNAi} (purple), *UAS-appl*^{RNAi} (green), *UAS-orb2*^{RNAi} (blue), *UAS-fmr1*^{RNAi} (yellow), *UAS-rutabaga*^{RNAi} (yellow) & *UAS-starrynight*^{RNAi} (yellow) and their parental controls (grey) following mechanical SD (solid box) or in the undisturbed controls (dashed box). All flies were raised and tested on standard food. * = < 0.05, ** = < 0.01 & *** = < 0.001. The plots are representative of a minimum of 4 repeats and Wilcoxon Rank Sum Test was used for the statistical comparisons.

during rebound ($CI_{95\%} = [1,20]$, p-value = 0.056), and the nSyb-GAL4/+ individuals slept an extra 39 min ($CI_{95\%} = [28,49]$, p-value = 4.55×10^{-9}) (Fig 4.18,A).

Synapsin KD flies' rebound response was impaired, sleeping comparably during the ZT0-3 period in the SD and slept controls (asleep_{CTRL} = 37 min, $CI_{95\%} = [27,46]$ against asleep_{SD} = 52 min, $CI_{95\%} = [40,64]$, p-value = 0.046) (Fig 4.15). The +/UAS-*synapsin*^{RNAi} flies slept 82 min ($CI_{95\%} = [70,95]$) compared to 50 min ($CI_{95\%} = [39,62]$, p-value = 0.00017) in their undisturbed counterparts. However, there was a significant positive change in sleep, with SD flies sleeping 13 min more during the rebound period than in the same time frame during baseline ($CI_{95\%} = [0,25]$, p-value = 0.035) (Fig 4.18A).

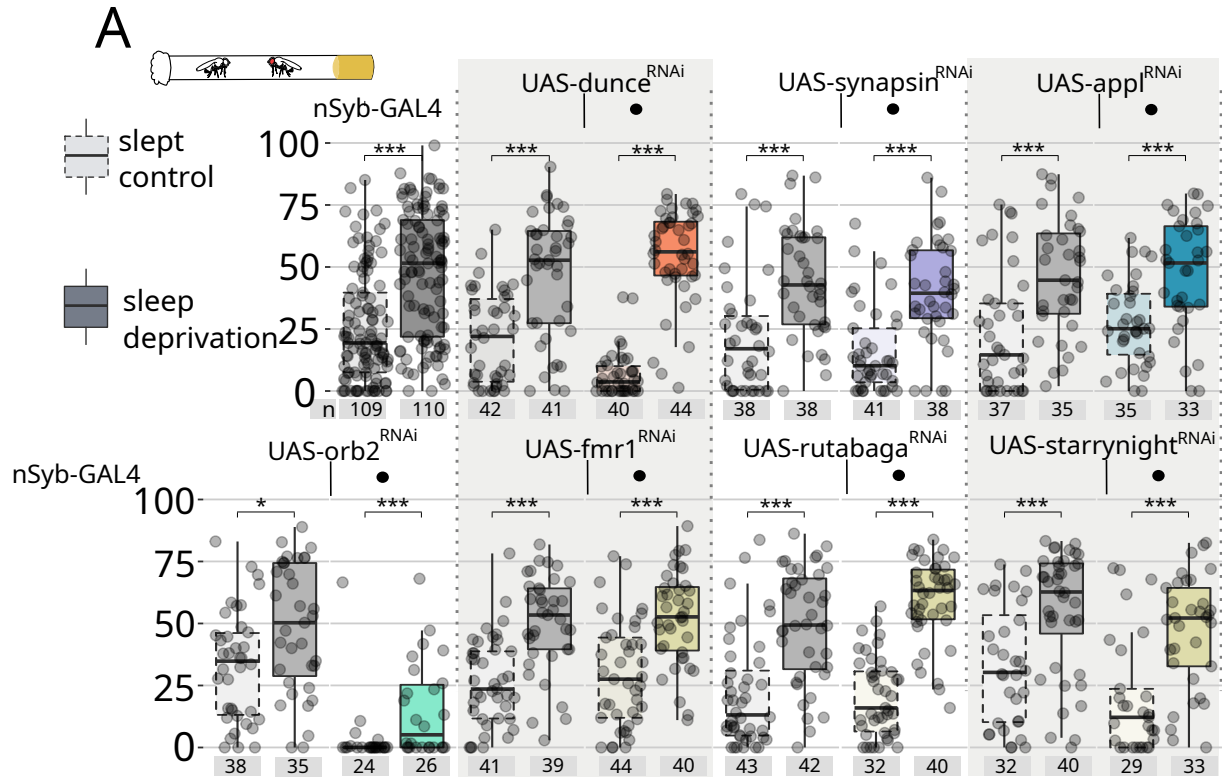


Figure 4.16: Sleep rebound following social SD with pan-neuronal KD of synaptic plasticity associated genes

(A) Quantification of the sleep during the ZT0-3 rebound period with nSyb-GAL4 mediated KD of UAS-*dunce*^{RNAi} (orange), UAS-*synapsin*^{RNAi} (purple), UAS-*appl*^{RNAi} (green), UAS-*orb2*^{RNAi} (blue), UAS-*fmr1*^{RNAi} (yellow), UAS-*rutabaga*^{RNAi} (yellow) & UAS-*starrynight*^{RNAi} (yellow) and their parental controls (grey) following social SD (solid box) or in the mock controls (dashed box). All flies were raised and tested on standard food. * = < 0.05, ** = < 0.01 & *** = < 0.001. The plots are representative of a minimum of 4 repeats and Wilcoxon Rank Sum Test was used for the statistical comparisons.

In both *dunce* and *synapsin* KD flies, there was a strong rebound response to social SD. Socially sleep deprived flies lacking *dunce* slept 86 min more than their slept counterparts (asleep_{CTRL} = 12 min, CI_{95%} = [7,17] against asleep_{SD} = 98 min, CI_{95%} = [87,108], p-value = 1.7 x-10-13) and an extra 58 (CI_{95%} = [46,70], p-value = 2.31x10-10) min than during ZT0-3 at baseline (Fig 4.16) (Fig 4.18B). *Synapsin* KD flies also exhibited elevated sleep compared to slept controls (asleep_{CTRL} = 29 min, CI_{95%} = [19,39] against asleep_{SD} = 72 min, CI_{95%} = [59,84], p-value = 4.6 x-10⁻⁶) (Fig 4.15) and baseline (rebound_Δ = 36 min, CI_{95%} = [23,49], p-value = 4.55x10⁻⁶) (Fig 4.16) (Fig 4.18B).

Appl and *orb2* knockdown abolishes mechanical rebound

Rebound after mechanical SD was abolished in flies lacking *appl* (asleep_{CTRL} = 27 min, CI_{95%} = [18,35] against asleep_{SD} = 50 min, CI_{95%} = [33,66], p-value = 0.13) and *orb2* (asleep_{CTRL} = 7 min, CI_{95%} = [2,12] against asleep_{SD} = 17 min, CI_{95%} = [6,29], p-value = 0.23), with no significant change in sleep between the SD and slept groups (Fig 4.15). Once again, their rebound response was intact after social SD, and *appl* (asleep_{CTRL} = 49 min, CI_{95%} = [39,59] against asleep_{SD} = 84, CI_{95%} = [70,99], p-value = 0.00036) and *orb2* KD flies (asleep_{CTRL} = 7 min, CI_{95%} = [4,17] against asleep_{SD} = 27 min, CI_{95%} = [13,40], p-value = 0.0066) displayed higher values in the SD group (Fig 4.16). The lack of mechanical rebound was recapitulated when looking at the change in rebound compared to baseline. KD of *appl* and *orb2* flies had no significant change in rebound (rebound_Δ = 7 min, CI_{95%} = [-5,18], p-value = 0.59 and rebound_Δ = -6 min, CI_{95%} = [-27,16], p-value = 0.29, respectively) (Fig 4.18A). However, the KD of *orb2* also led to marked reductions in their baseline sleep, with the flies sleeping only 332 min (CI_{95%} = [295,369]) of the day, and 7 min (CI_{95%} = [4,17]) during the ZT0-3 rebound phase, while the +/UAS-*orb2*^{RNAi} controls slept 48 min (CI_{95%} = [38,59]) (Fig 4.15). Therefore, it is plausible that changes found in the rebound response may be due to alterations in the baseline sleep appearance rather than an impaired homeostatic response to mechanical SD.

Taken together, I postulate that the synaptic plasticity genes, *dunce*, *synapsin* and *appl* are involved in the synaptic strength dynamics responsible for increasing sleep pressure or for the initiation of sleep in response to high sleep need.

4.2.3 *Dunce* and *synapsin* mutant sleep

Within *Drosophila* genetic manipulations, it is important to validate any phenotypes observed with a secondary approach to ensure results are due to the specific genetic manipulation and not any off-targets. I controlled for this to some degree in the previous

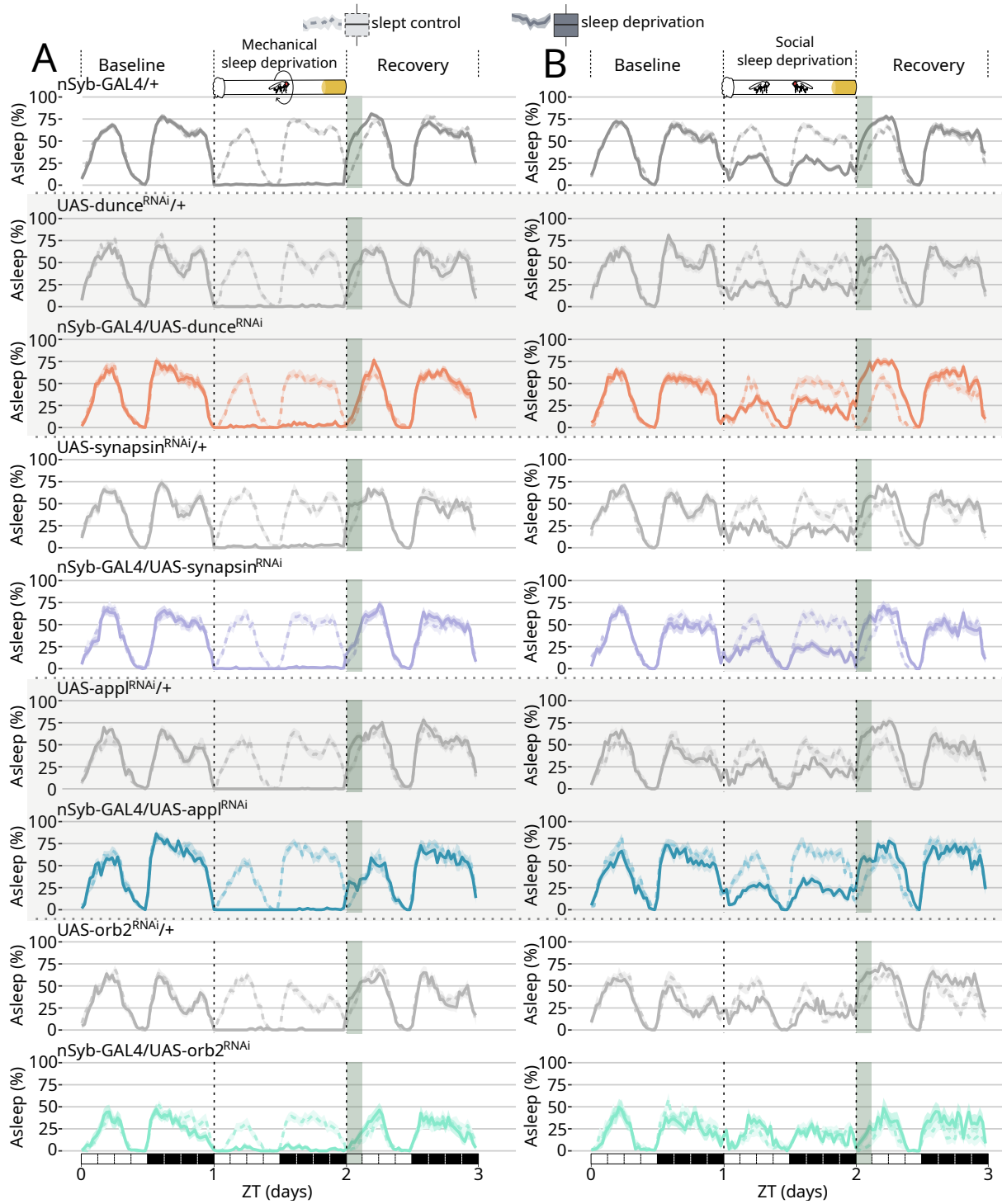


Figure 4.17: Sleep profile of pan-neuronal KD of *dunce*, *synapsin*, *appl* & *orb2* over the baseline, SD and recovery days with mechanical and social SD

(A) Sleep profile of nSyb-GAL4 mediated KD of UAS-*dunce*^{RNAi} (orange), UAS-*synapsin*^{RNAi} (purple), UAS-*appl*^{RNAi} (green) & UAS-*orb2*^{RNAi} (blue) and their parental controls (grey) over the baseline, SD and recovery days with mechanical (B) and social SD. The ZT0-3 rebound period is indicated by the green shaded area. All flies were raised and tested on standard food. The duration of the tracking was 3 days and the plots are representative of a minimum of 4 repeats.

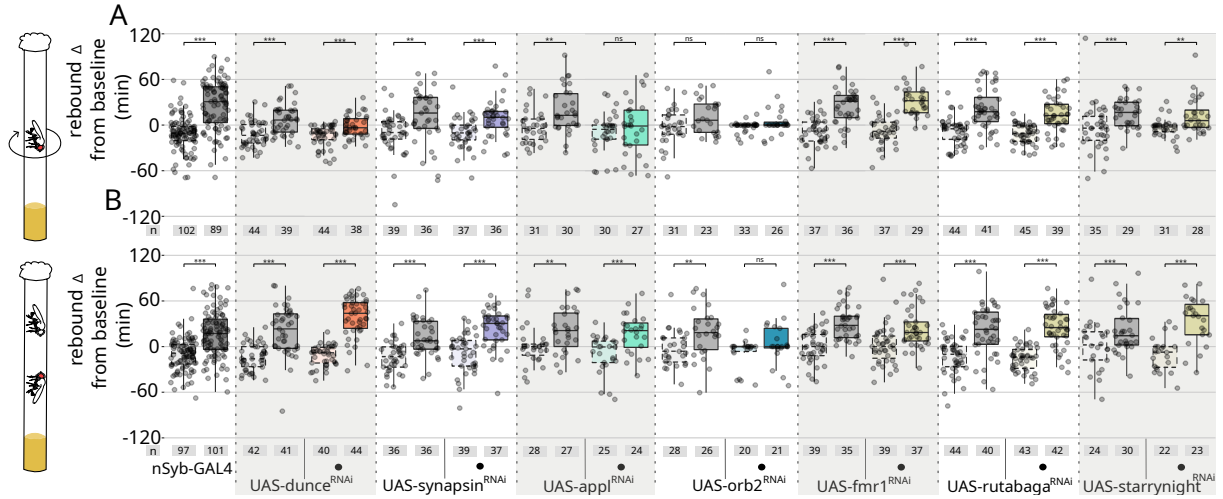


Figure 4.18: Sleep rebound Δ following mechanical and social SD with pan-neuronal KD of synaptic plasticity associated genes

(A) Quantification of the sleep rebound Δ compared to the ZT0-3 baseline period with nSyb-GAL4 mediated KD of UAS-*dunce*^{RNAi} (orange), UAS-*synapsin*^{RNAi} (purple), UAS-*appl*^{RNAi} (green), UAS-*orb2*^{RNAi} (blue), UAS-*fmr1*^{RNAi} (yellow), UAS-*rutabaga*^{RNAi} (yellow) & UAS-*starrynight*^{RNAi} (yellow) and their parental controls (grey) following mechanical (A) and social SD. All flies were raised and tested on standard food. * = < 0.05, ** = < 0.01 & *** = < 0.001. The plots are representative of a minimum of 4 repeats and Wilcoxon Rank Sum Test was used for the statistical comparisons.

nSyb-GAL4 KD experiments by outcrossing lines to a common wild-type and running both nSyb-GAL4/+ and +/UAS-GOI^{RNAi}. With this approach, changes due specifically to other alterations in the fly genome, other than the removal of the target genes *mRNA* product, would be captured in the individual parental controls sleep pattern. In order to address this further and validate the phenotype observed with pan-neuronal KD, I tested the *dunce*¹ and *synapsin*⁹⁷ mutants in the same conditions as the previous assays (Fig 4.19). The Canton-S sleep during rebound was elevated after both mechanical (asleep_{CTRL} = 93 min, CI_{95%} = [84,102] against asleep_{SD} = 129 min, CI_{95%} = [118,141], p-value = 1.5x10⁻⁸) and social SD (asleep_{CTRL} = 77 min, CI_{95%} = [77,85] against asleep_{SD} = 88 min, CI_{95%} = [80,], p-value = 0.034) (Fig 4.19). *Synapsin*⁹⁷ flies mimicked the KD response, with no rebound after 24hr mechanical SD (asleep_{CTRL} = 78 min, CI_{95%} = [62,95] against asleep_{SD} = 96 min, CI_{95%} = [76,115], p-value = 0.33) (Fig 4.19A). In comparison, the rebound post social SD was maintained (asleep_{CTRL} = 46 min, CI_{95%} = [28,65] against asleep_{SD} = 96 min, CI_{95%} = [71,120], p-value = 0.33) (Fig 4.19B). Surprisingly, the results with the *dunce*¹ animals generated conflicting results to what was observed with pan-neuronal KD, as they had a strong rebound after mechanical SD (asleep_{CTRL} = 56 min, CI_{95%} = [44,69] against asleep_{SD} = 89 min, CI_{95%} = [73,105], p-value = 0.00064) (Fig 4.19A). Social SD results were comparable to the KD with sleep elevated during the ZT0-3 rebound period (asleep_{CTRL} = 27 min, CI_{95%} = [17,38] against asleep_{SD} = 80 min, CI_{95%} = [68,93], p-value = 8.2 x-10⁻⁸) (Fig 4.19B).

4.2.4 *Dunce* and *synapsin* targeted knockdown

It seems that *dunce* and *synapsin* are required for normal rebound sleep after mechanical SD, as pan-neuronal KD of *dunce* or *synapsin* abolished rebound, and similar results were collected with *synapsin*⁹⁷. Following the initial assessment of pan-neuronal KD, I wanted to investigate the specific neuronal populations where *dunce* and *synapsin* were required. In order to achieve this, I again utilised the GAL4-UAS system to perform a systematic KD of the *dunce* or *synapsin* genes using GAL4 drivers that label subsets of neurons in the fly sleep centres. The dFB (R72G06-GAL4), R5 EB neurons (R30G03-GAL4), MB (MB010B-GAL4), and the PAM cluster of DAN neurons (R58E010-GAL4)(Fig 4.20A).

Firstly, *dunce* and *synapsin* were required in the PAM DANs for normal recovery sleep, with KD eliminating the rebound response (p-value compared to slept controls = 0.06 & 0.11, respectively) (Fig 4.20B). Interestingly, KD of the genes in the MB (a downstream target of the PAM DANs) had no impact on their homeostatic recovery, with MB010B-GAL4 mediated KD of both *dunce* (p-value = 0.00072) and *synapsin* (p-value

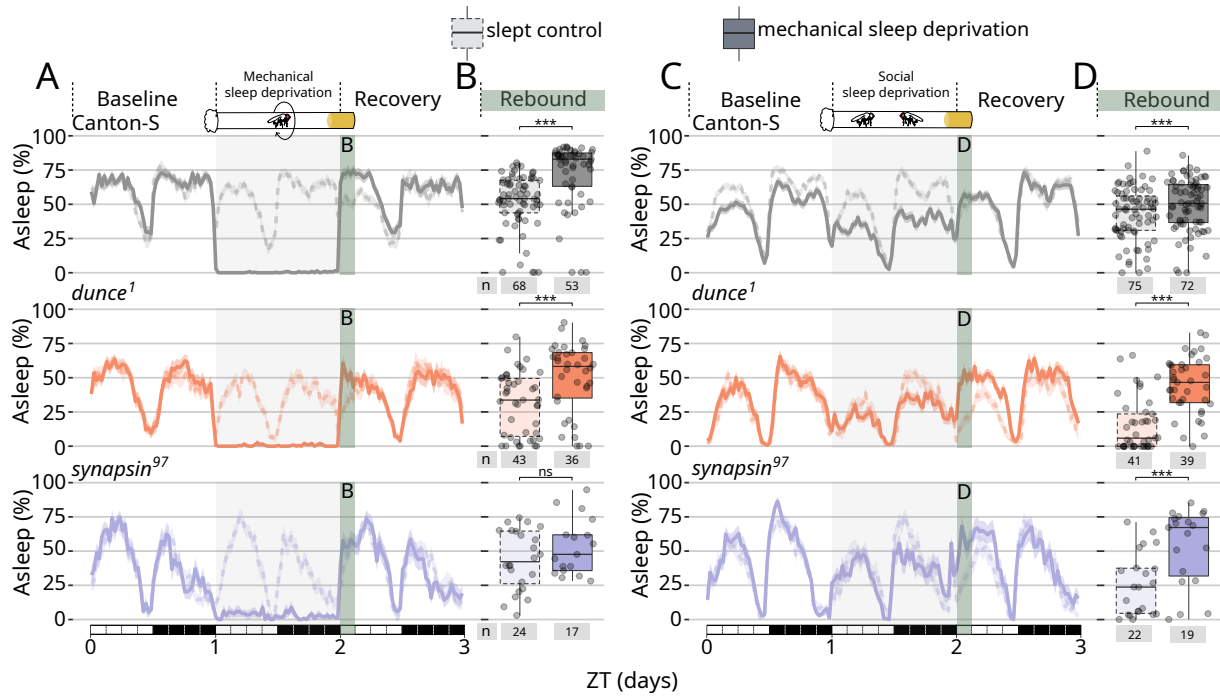


Figure 4.19: Response to mechanical and social SD of *dunce*¹ & *synapsin*⁹⁷

(A) Sleep profile of *dunce*¹ (orange), *synapsin*⁹⁷ (purple) & Canton-S (grey) over baseline, SD and recovery with mechanical SD (solid line) and with the undisturbed controls (dashed line) (B) Quantification of the sleep during the ZT0-3 rebound period in *dunce*¹ (orange), *synapsin*⁹⁷ (purple) & Canton-S (grey) following mechanical SD (C) Sleep profile of *dunce*¹ (orange), *synapsin*⁹⁷ (purple) & Canton-S (grey) over baseline, SD and recovery with social SD (solid line) and with the undisturbed controls (dashed line) (D) Quantification of the sleep during the ZT0-3 rebound period in *dunce*¹ (orange), *synapsin*⁹⁷ (purple) & Canton-S (grey) following social SD. All flies were raised and tested on standard food. * = < 0.05, ** = < 0.01 & *** = < 0.001. The duration of the tracking was 3 days and the plots are representative of a minimum of 4 repeats. Wilcoxon Rank Sum Test was used for the statistical comparisons in B & C.

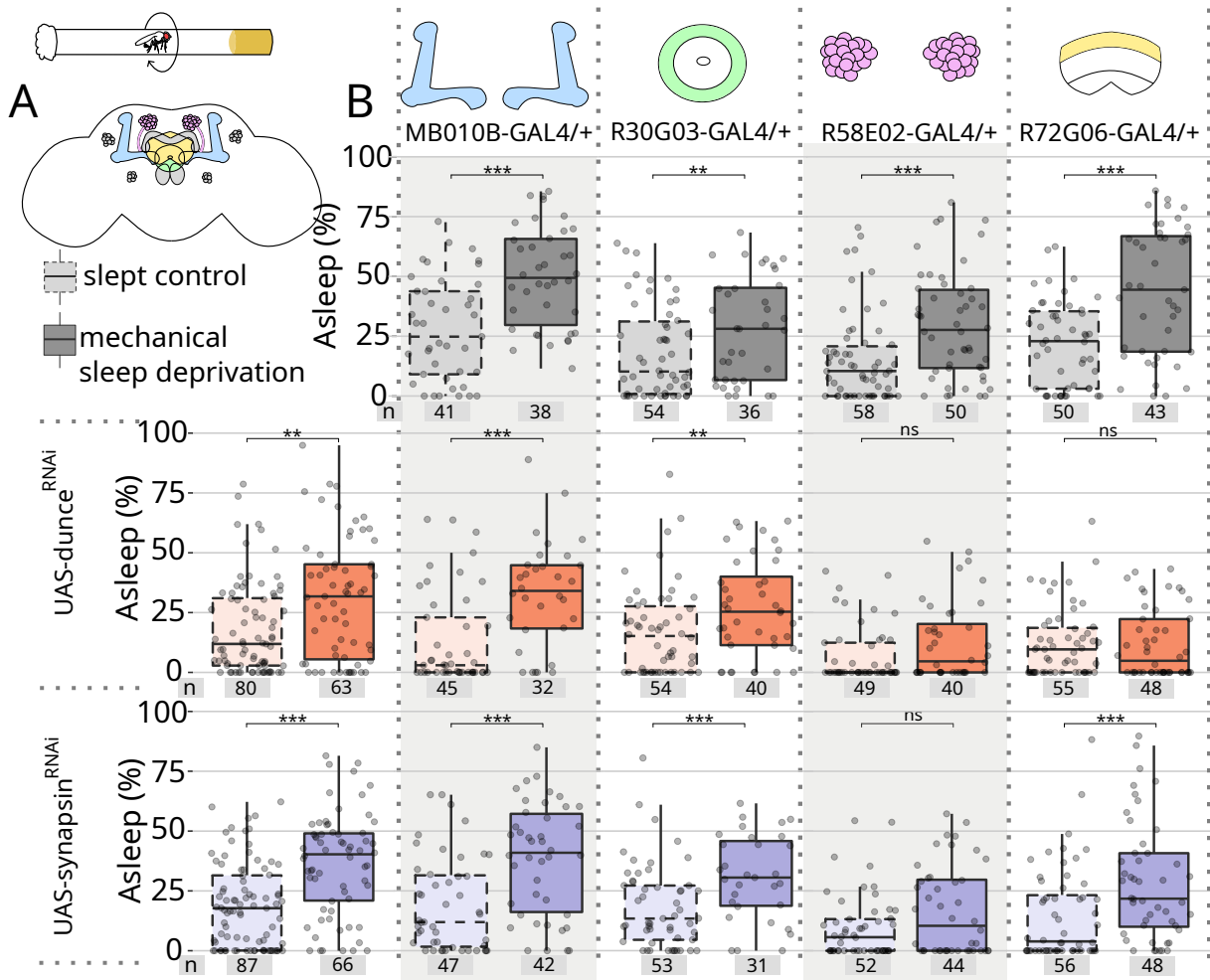


Figure 4.20: Response to mechanical SD following targeted KD of *dunce* and *synapsin* (A) Simplified schematic of the central complex and MB structures of the fly brain, alongside the DANs that have targets in the MB, EB & dFB. The neuronal regions interrogated in this experiment are highlighted – MB (MB010B-GAL4, blue), EB (R30G03-GAL4, green), PAM DANs (R58E02-GAL4, pink) & FB (R72G06-GAL4, yellow). The EB driver labels the R5 ring neurons and the FB driver the ExFl2 dFB neurons (B) Quantification of the sleep during the ZT0-3 rebound period in UAS-*dunce*^{RNAi} (orange) & UAS-*synapsin*^{RNAi} (purple) with MB010B-GAL4, R30G03-GAL4, R58E02-GAL4 & R72G06-GAL4 mediated KD following mechanical SD (solid boxplot) or in the undisturbed controls (dashed boxplot). All flies were raised and tested on standard food. * = < 0.05, ** = < 0.01 & *** = < 0.001. The plots are representative of a minimum of 3 repeats and Wilcoxon Rank Sum Test was used for the statistical comparisons.

= 0.00016) exhibiting a robust rebound response. Similar results were found in the EB R5 circuit, where *dunce* and *synapsin* were not required in the R30G03-GAL4 labelled neurons for rebound sleep (p-value compared to slept controls = 0.0015 & 3.9×10^{-6} respectively). Finally, *dunce* KD in the dFB abolished rebound in the ZT0-3 period following SD, exhibiting sleep levels comparable to the undisturbed controls (p-value = 0.59). However, the KD of *synapsin* in the same region did not influence the homeostatic response to mechanical SD (p-value = 9×10^{-4}) (Fig 4.20B). I conclude that broadly impacting the mechanisms of synaptic plasticity in the PAM neurons leads to sleep rebound impairments, as individual KD of *dunce* and *synapsin* resulted in abolished rebound post mechanical SD. While specific mechanisms of cAMP regulation are required in the ExF12 projection neurons of the dFB, where only KD of *dunce*, and not *synapsin*, remove rebound.

Information of the expression pattern of GAL4s are readily available using resources such as the Virtual Fly Brain^[176] and FlyLight^[414]. Utilising these resources, I examined the expression of the target GAL4 lines used in this study. The MB010B and R58E02 GAL4 lines both exhibit specificity to their intended target neurons, with expression strictly confined to the MB KCs and the PAM DANs, respectively. However, while the R72G06 and R58E010 drivers have been utilised in previous studies to target UAS expression to the dFB and R5 EB respectively, both have expression patterns outside of their target regions. For instance, the R72G06-GAL4 expression is not only present in the dFB but also extends to the superior intermediate protocerebrum neuropil. Similarly, R30G03-GAL4, despite its utility in targeting the EB, exhibits expression in various regions of the fly brain, including the prow, flange, bulb and FB. In both the R72G06 and R30G03 GAL4s, expression is also detected outside of the brain in the ventral nerve chord. Therefore, it is important to note that the phenotypes observed with R72G06 and R30G03 GAL4 mediated UAS-RNAi KD may have potentially arisen as a result of KD in areas outside of the intended region.

4.2.5 RNAi caveats

RNAi-mediated KD exhibits variable efficiency in terms of *mRNA* reduction levels. The KD for specific UAS-GOI^{RNAi} lines targeting the *dunce*, *appl*, *fmr1*, *rutabaga*, and *starrynight* genes have been validated in prior literature, with phenotypic confirmation through use of independent RNAi lines or mutants, as outlined in the Materials and methods Section 2.1.2.2 (Table 4.1). For example, *dunce* and *rutabaga* mutations are well established to result in learning and memory defects^{[149][412]} and Walkinshaw *et al.*, performed pan-neuronal KD of both genes with the same UAS-RNAi lines used in this

study and found poor olfactory memory performance^[352]. While there is no literature available utilising the particular UAS-*synapsin*^{RNAi} and UAS-*orb2*^{RNAi} lines, within this study, the *synapsin* KD phenocopied the *synapsin*⁹⁵ mutant, notably abolishing rebound response following mechanical SD (Fig 4.19).

Although some evidence in the literature validates the RNAi lines based on expected phenotypes, the phenotypes reported in this thesis have not been confirmed, and the efficiency of KD is rarely quantified using methods such as qPCR. Furthermore, the VDRC website reports off-target effects for the *synapsin*, *appl*, and *orb2* UAS-GOI^{RNAi} lines.

Therefore, the findings presented in this chapter could be strengthened by validating observed phenotypes using an independent RNAi line, ideally one without known off-targets, to affirm the specificity of target gene KD. Furthermore, qPCR could be employed to quantify the GOI *mRNA* levels relative to controls, thereby establishing the efficiency of the KD process.

4.2.6 Summary

- The lack of rebound found after mechanical SD correlated with unchanged levels of the synaptic scaffolding protein BRP, in *D.melanogaster*, *D.erecta*, *D.yakuba*, *D.willistoni* and *D.virilis*.
- Pan-neuronal KD of *fmr1* and *rutabaga* increased sleep, while KD of *dunce* and *orb2* suppressed levels.
- The synaptic-plasticity associated *dunce*, *synapsin*, *orb2*, and *appl* are required for rebound in *D.melanogaster* after mechanical SD.
- Rebound following social SD was resilient to synaptic-plasticity associated gene KD.
- General mechanisms of synaptic plasticity were required in the PAM DANs for homeostatic rebound following mechanical SD. While specific downregulation of cAMP was necessary in the dFB.

Table 4.1: Synaptic plasticity associated GOI proposed role in synaptic plasticity

Gene	Encoded product	Proposed role in synaptic plasticity	Mutant phenotypes
<i>dunce</i>	cAMP specific phosphodiesterase responsible for cAMP degradation	Synaptic excitability, long-term potentiation, neurotransmission ^[400]	Increased action-potential independent transmission ^[401] Impaired long-term potentiation and facilitation ^[388] Increased synaptic bouton number and axonal branching ^[389] Impaired short-term memory ^[149]
<i>synapsin</i>	Synaptic vesicle phosphoprotein	Maintenance and release of the synaptic vesicles from the reserve pool under intense stimulation	Diminished reserve pool ^[391] Reduced neurotransmitter release upon maintained depolarization ^[392] , Increased depression ^[392] Impaired short-term memory ^[402]
<i>appl</i>	beta Amyloid Protein Precursor-like	Axon growth and synapse formation ^[403]	Reduced synaptic bouton number ^[404] cite me Impaired long-term memory ^[405]
<i>orb2</i>	Cytoplasmic polyadenylation element binding protein (CPEB) RNA-binding protein that can activate or repress the translation of target mRNA ^[406]	Controls neuronal growth, synapse formation and proteolysis - stabilising activity dependent changes in synaptic efficacy ^[407]	Impaired long-term memory ^[407] ^[408]
<i>fmr1</i>	Fragile X messenger ribonucleoprotein (FMRP) RNA binding translational regulator which mediates activity-dependent control of synaptic structure and function ^[409]	Negatively regulating branching of dendrites and axons and synapse formation. Stabilising neurotransmission ^[410]	Enlarged synaptic boutons ^[410] Increased synaptic vesicle accumulation ^[410] Impaired neurotransmission ^[410] Impaired long-term memory ^[411]
<i>rutabaga</i>	Membrane bound adenylyl cyclase responsible for cAMP synthesis	synaptic excitability, long-term potentiation, neurotransmission	Impaired long-term potentiation and facilitation ^[388] Decreased synaptic bouton number, axonal branching, and innervation length ^[389] Impaired short-term memory ^[412]
<i>starrynight</i>	A transmembrane cadherin protein involved in planar cell polarity that is postulated to regulate the structure of dendrites and synapses	Synaptic target selection and synapse formation ^[413]	Displaced synapse formation along axons ^[413] Degradation of axons ^[413]

Chapter 5

Discussion

5.1 Characterising *Drosophila* baseline sleep

5.1.1 Timing of sleep is largely conserved in LD conditions

D.melanogaster is shown to exhibit two periods of sleep over the 24 hr day. A siesta daytime sleep period and a more consolidated period of nighttime sleep. These baseline sleep period characteristics are repeatedly observed in male flies within the literature across various methodologies and are considered a robust element of *D.melanogaster* sleep^[376]. I have shown that the panel of species tested in this study exhibit equivalent features in LD conditions. *D.melanogaster* lab strain, *D.melanogaster* wild caught, *D.simulans*, *D.sechellia*, *D.erecta*, *D.yakuba*, *D.willistoni* and *D.virilis* all have day and night sleep intervals, with the latter associated with bouts of longer length.

5.1.2 Anticipatory peaks are conserved, with varying activity composition

D.melanogaster shows circadian-driven crepuscular activity peaks that anticipate the changing light phase in between the two sleep times. In addition to the equivalent siesta and night sleep organisation, the crepuscular activity increases in anticipation of the light transition appear to be conserved across the panel. Each species is found to have elevated activity in the 2 hours leading up to lights off. While the light to dark activity peak prevails, I found inconsistent magnitudes in the species' waking activity throughout the transition period (ZT 11 - 13). The panel can be seen as bifurcated into two groups, the *melanogaster*-like activity peak, where they perform larger walking movements, and a second group which executes smaller micromovements. This

division is also represented in their total activity across the 24 hr day, as the higher ranking activity magnitude at the transition from light to dark reflects the waking behaviour that predominates over the 24 hr day. *D.simulans*, *D.sechellia*, *D.yakuba*, and *D.willistoni* could be considered *melanogaster*-like, spending more time walking than micromoving during ZT11-13. However, both *D.melanogaster* species (lab strain & wildcaught) carry out low micromovements during this period, while all other species, excluding *D.sechellia*, micromoving more than *D.melanogaster*. Based on these findings, I will crudely describe the species' transitional waking activity into three classes: almost exclusively walking (*D.melanogaster* lab strain, *D.melanogaster* wild caught & *D.sechellia*), predominately walking (*D.simulans*, *D.yakuba* & *D.willistoni*) and predominately micromoving (*D.erecta* & *D.virilis*). Discriminating waking behaviour is an advantageous feature of video tracking systems. However, data on *Drosophila* activity composition is lacking due to the principal use of infrared beam activity monitors in the field. Therefore, activity is primarily viewed as a binary state where a fly is either mobile or immobile. A previous study using the ethoscope tracking system supports my findings, with males preferentially executing walking at the transition from light to dark. Interestingly, increased micromovements were observed only in female flies^[65].

The increase in activity separating the sleep periods is controlled by the circadian clock, with clock neurons responsible for the increase in movement. The sLNvs^[415] are necessary for anticipatory activity at the onset of the light phase, whilst the 5th sLNv and LNds are implicated in dark phase anticipation^{[415][416]}. Although this is often considered a simplistic and imperfect overview of the clock neuron's anticipatory influence, due to the evidence that numerous clock cells influence both the light and dark crepuscular activity^{[417][418]}, it is clear that circadian neurons are partly responsible for the increased movement. However, it has been shown in the literature that even circadian clock component mutants exhibit some level of rhythmic sleep behaviour in the presence of light. Light is a strong zeitgeber that has been shown to control sleep-wake cycles in the absence of a functioning clock^{[419][420]}. For this reason, I tested the species' activity under constant dark conditions, removing light as a zeitgeber to investigate whether the sleep-wake cycle's circadian control is conserved.

The endogenous circadian rhythms in the absence of a light zeitgeber are primarily maintained across the panel, with *D.melanogaster* wild caught, *D.simulans*, *D.sechellia*, *D.erecta*, *D.yakuba*, and *D.willistoni* all displaying a period of strength above the significance threshold, suggesting rhythmic cycles in their sleep and activity. However, while the *D.simulans*, *D.erecta*, and *D.willistoni* period was equivalent to *D.melanogaster* (24hr), *D.sechellia* had a shorter period (23.3), and *D.yakuba* exhibited an extended

period (25.3). *D.virilis* presented with arrhythmic behaviour, with only three individuals' peak meeting the significance threshold for the period calculation. A previous study observed corresponding results, where the *D.virilis* population exhibited largely arrhythmic behaviour, and the small number of flies that demonstrated rhythmicity did so weakly. The researchers found that the lack of free-running circadian cycles likely manifested from the absence of PDF expression at the s-LNVs^[339].

5.1.3 Variation across the animal kingdom is captured in our panel

As detailed in Section 1.1.6, there is considerable variability in sleep across the animal kingdom, in terms of its total amount, among other elements. I observed a similar sleep disparity in the Drosophilid species over the entire day and the light and dark phases. Such an outcome is not surprising since a combination of ecological factors is speculated to shape the daily sleep amount^[100]. The diverse selective pressures each species would have been exposed to in shaping the evolution of their sleep behaviour may account for the differences I have observed.

The *D.willistoni* night sleep duration is an interesting case, displaying the greatest total amount and bout duration of nighttime sleep. The longer intervals of uninterrupted night sleep may indicate that the dark phase sleep is of greater depth. Thus, this result may suggest that *D.willistoni* individuals experience greater sleep need than the other species, requiring extra rest after a bout of wakefulness. However, van Alphen and colleagues^[94] demonstrated that not all longer-length episodes equated to higher-intensity sleep. Thus, one can only accurately assess sleep intensity by probing animals' arousal thresholds during the sleep bout. A 2015 study of *D.melanogaster* sleep in populations collected from different latitudinal locations across North and Central America found a latitudinal cline associated with nighttime sleep duration, with equatorial populations sleeping almost twice the length in the dark phase^[421]. *D.willistoni* flies were originally collected from Guadalupe Island, located in the West Indies (sub-tropical region), and correspondingly, a latitudinal influence of sleep may account for the nighttime elevation. However, the aforementioned study only included one tropical population, and the investigations were restricted to the North American continent; thus, additional equatorial samples are necessary to confirm the finding of a latitudinal cline in sleep duration. Furthermore, while I have not investigated the impact of latitude on Drosophilid sleep, I think the value in studying such correlations lies within the same species to first determine if intraspecific variation exists. *D.melanogaster* is an excellent candidate for such investigations due to its widespread colonisation of the

continents. Further characterisation of the latitudinal influence in other Drosophilid species will address whether this is a common trend in the *Drosophila* genus and potentially the animal kingdom. However, such investigations are outside the scope of this thesis. Irrespective of such findings, latitudinal and geographical influences on sleep would not, by itself, account for the enigma that is sleep and its variability. For it cannot account for developmental changes in sleep or resolve why one animal can carry out the essential function(s) of sleep in a shorter time than another.

Nevertheless, the variability in sleep across the panel ascertains the *Drosophila* genus as a potential framework for the study of sleep and its variability across the animal kingdom.

5.1.4 *D.sechellia* baseline sleep resembles *D.melanogaster*

Considering their sleep and activity behaviour, I propose that *D.sechellia*'s sleep most closely resembles that of *D.melanogaster*. The first line of evidence in support of this is the total sleep over the light and dark phases, where *D.sechellia* sleep in each period is statistically insignificant when compared to *D.melanogaster* lab strain. Secondly, they are the only species that mimic the *D.melanogaster* activity transitional composition, falling into the 'almost exclusively walking' group. *D.sechellia* is a relatively close relation to the *D.melanogaster* model, a member of the melanogaster subgroup that diverged approximately 2-3 million years ago^[327]. Even so, I found this result surprising on two accounts. Firstly, the two species occupy varying ecological niches, with *D.sechellia* a specialist species that inhabits a restricted geographical region, found solely on Seychelles island. In contrast, *D.melanogaster* is a generalist, human commensal species that has colonised almost every continent, minus Antarctica. Alongside this, the *D.melanogaster* are historically absent from the Seychelles and have only recently been found to inhabit the island, likely due to an involuntary introduction^[422]. The *D.melanogaster* Canton-S lab strain used in this study was collected in Canton, Ohio, in the 1920s. Therefore, finding such comparative sleep characteristics from species from vast ecological niches was unusual, particularly considering the variability in the other Drosophilid species. This leads to the second reason that I find the result that *D.sechellia* best resembles *D.melanogaster* sleep unexpected. *D.simulans* is also closely related to *D.melanogaster*, and has a recent common ancestor with *D.sechellia*. *D.simulans* shares several ecological factors with *D.melanogaster*: both are generalist species inhabiting many of the same geographical regions, mainly due to their status as human commensal animals. I predicted that due to their similar ecological niches, *D.simulans*' sleep would closely resemble *D.melanogaster*. However, this was not the

case as *D.simulans* were found to sleep significantly more during the dark phase and the activity transitional composition varied, with elevated micromovement in addition to walking.

However, I found disparate sleep results between the *Drosophila* strains, while the *D.melanogaster* wild caught flies sleep considerably less than the lab strains. Such results have been identified previously in the literature, with wild-type strains exhibiting vast variation in total sleep amount^[423]. Therefore it is unlikely that the similarities in sleep between *D.melanogaster* Canton-S and *D.sechellia*, strain 1, are species specific. Nevertheless, within the bounds of this study, the resemblance of their sleep establishes that I have observed a group that presents baseline sleep characteristics comparable to *D.melanogaster*.

5.1.5 Response to octopamine is conserved, while dopamine has potentially diverged

A combination of environmental cues and intrinsic mechanisms shapes behaviour, including sleep. Neuromodulators (neurotransmitters, neuropeptides & hormones) coordinate these inputs to drive the appropriate behavioural output. Octopamine, dopamine, and GABA are neurotransmitters implicated in modulating *D.melanogaster* sleep, along with other neurotransmitters and neuromodulators, reviewed in^[424], with the former two bioamines promoting wake and the latter amino acid considered to promote sleep. Many neuromodulator sleep effects, including octopamine, dopamine, and GABA, appear to be conserved throughout the animal kingdom. This conservation is often considered evidence of the shared sleep circuitry within animals and evidence of an ancestral sleep state that has persisted throughout evolution.

While the baseline characteristics of sleep are primarily shared across the panel, further examination is needed to clarify whether the underlying mechanisms which regulate and coordinate sleep are conserved. In order to answer this crucial question, I administered drug agonists of the above neuromodulators in the fly food and examined their sleep response.

Firstly, the action of octopamine on *Drosophila* sleep is generally common across the panel. Previous *D.melanogaster* studies demonstrated that octopamine drives arousal via PKA signalling in the PI region of the fly brain, as the wake-promoting effects of octopamine feeding were abolished via inhibiting PKA in PI neurons^[216]. In this study, *D.melanogaster*, *D.simulans*, *D.sechellia*, *D.erecta*, *D.willistoni*, and *D.virilis* all

experienced a decline in sleep with feeding of the octopamine agonist, CDM, suggesting that the arousal results established in the *D.melanogaster* model are maintained in both close and distantly related Drosophilid species. These results agree with studies of other invertebrates, as a wake-promoting role of octopamine signalling is found in other insects, and octopamine agonists are commonly implemented as pesticides due to the increased activity induced by such drugs^[425]. In contrast to the other species in this study, *D.yakuba*'s sleep was unaffected by the drug treatment, sleeping comparably to the controls. The seemingly pervasive action of octopamine emphasises the unexpected result I obtained concerning *D.yakuba*'s response to CDM feeding.

Secondly, the impact of dopamine manipulation on Drosophilid sleep is disparate, contradicting the general consideration of dopamine as a conserved arousal-promoting neurotransmitter^[426]. Multiple methods of manipulating dopamine signalling, for example, mutations in the dopamine transporter^[287] or dopamine receptors^[427], validate the action of dopamine in arousal. The results I collected on *D.melanogaster* sleep upon feeding with the dopamine precursor, L-dopa, support the previous evidence in the literature on the wake promotion of dopamine since I found L-dopa treatment to reduce daily sleep by 255 min. Remarkably, no other species included in this study were found to suppress sleep on the L-dopa diet, with *D.sechellia* and *D.willistoni* modestly increasing sleep, while *D.simulans*, *D.erecta*, *D.yakuba*, and *D.virilis* sleep was unchanged. Considering the consensus of dopamine in arousal from evidence across the animal kingdom, I found the results obtained on L-dopa rather unusual.

The altered response could be attributed to a variety of factors. For example, the non-*melanogaster* species may have elevated dopamine levels at baseline and therefore are unresponsive to dietary increases. Moreover, several factors could contribute to reduced bioavailability of the L-dopa drug in the other species, including metabolism and excretion. It is possible that the species metabolise L-dopa differently, breaking it down into inactive metabolites, thus showing no influence on sleep. Alterations in the rate of excretion may also play a role; increased excretion would lower the drug's circulation level, potentially causing a highly variable physiological dose across the panel. To explore these possibilities, future studies should investigate the endogenous and response to L-dopa levels of dopamine together with the enzymes and amino acids involved in the dopamine biosynthesis pathway. For instance, ultra performance liquid chromatography-mass spectrometry as performed in^[329] could be employed to aid this investigation.

Alternatively, the divergence may rest in the postsynaptic targets of dopamine signalling. In *D.melanogaster*, the Dop1R receptor is the principal sleep-relevant receptor in the brain, and the explanation for the differential dopamine response could be based

on the expression of Dop1R in the non-*melanogaster* circuits. Further research could conceivably introduce the *D.melanogaster* Dop1R receptor transgenically into the species to test whether it rescues the canonical L-dopa wake-promoting response.

It was plausible that the other species were not eating the L-dopa food, as a reasonable explanation could be that L-dopa was not wake-promoting because they were not consuming the drug. However, I considered this unlikely due to the sleep suppression found in *D.melanogaster* and other Drosophilidae to starvation after 12 hours^[207]. Nevertheless, I have performed a preliminary experiment to validate species feeding with a simple blue dye assay. As anticipated, each species engaged in eating the L-dopa food (data not shown), and therefore a lack of consumption does not justify the diverse findings of wake promotion, sleep promotion, and no effect across the panel.

5.1.6 THIP feeding has a limited effect on baseline sleep

The ionotropic GABA_A agonist THIP had a limited influence on sleep amount in *D.melanogaster*. GABA is a prevalent inhibitory neurotransmitter in vertebrate and invertebrate species alike. Due to its sleep-promoting qualities, GABA circuits are a popular target for pharmacological intervention on sleep impairments and human diseases associated with altered sleep. A previous study demonstrated that THIP feeding at concentrations as low as 0.025mg/ml increased daytime sleep in a dose-dependent manner^[428]. However, my results display only a modest increase in the transition from light to dark. The aforementioned study presented the initial findings on THIP-induced sleep elevation in females, and the pharmacological results within this thesis were undertaken in males. This detail is significant because male flies exhibit a strong siesta sleep during the day, whereas females are more active during this period. Therefore, the lack of sleep promotion generated by THIP feeding may be due to a ceiling effect of the daytime sleep level exhibited in males.

5.2 Investigating *Drosophila* homeostatic response

From cnidarians to mammals, a sleep-like state that encompasses the mammalian behavioural aspects of sleep has been found in almost every animal tested^{[1][14][15][76]}. The *Drosophila* genus presented in this thesis thus far encompasses many of the sleep criteria. They exhibit circadian-driven preferentially rest in prolonged bouts during the night phase. Another aspect of sleep behaviour that is necessary for animal quiescence to be determined as a period of sleep is the existence of homeostatic regulation. Thus, after

characterising the baseline characteristic of quiescence across the panel, I endeavoured to examine their response to enforced waking with a mechanical SD paradigm.

5.2.1 *D.melanogaster* is the only species to exhibit homeostatic rebound in response to mechanical sleep deprivation

The *D.melanogaster* lab strain and wild caught flies exhibited a robust period of rebound following 24 hr mechanical SD, in accordance with previous observations in the literature of mechanical interrogation of *D.melanogaster* sleep^{[63][429][204][65]}. Remarkably, each non-*melanogaster* species encountered a severe loss of sleep without detectable rebound following the suppression. Analysis of the sleep architecture during the ZT0-3 rebound phase revealed that the species did not perform any bouts of longer duration or increased number to their undisturbed counterparts. The unchanged structure suggests that the non-*melanogaster* species are not recovering their sleep in more consolidated bouts, as observed with the increased length of sleep episodes in the *D.melanogaster* lab strain.

In consideration of the differential baseline activity displayed across the species, I examined their waking activity during the 24 hr mechanical SD. Although there was considerable contrast in the time allocated to walking and micromoving, the walking levels of each species were elevated compared to baseline. However, it could be argued that the absence of rebound in response to mechanical SD is due to the level of physical exhaustion generated across the 24 hr period, with species that are predominantly micromoving less fatigued than the predominantly walking groups. In order to address this, I conducted a long-form SD experiment whereby the flies were exposed to 7 days of SD to ensure that the flies were adequately exhausted, referred to as chronic SD. In the first 3 hours following chronic SD, the *D.melanogaster* lab strain slept 44 min more than their slept controls. Once again, the *D.melanogaster* group is the only species to display a compensatory period of rebound. Despite the extensive course of sleep suppression *D.simulans*, *D.sechellia*, *D.erecta*, *D.willistoni*, *D.yakuba*, and *D.virilis* each displayed comparable sleep to their undisturbed controls. Moreover, the absence of a homeostatic sleep response to the mechanical SD persisted in constant darkness, suggesting that the rebound phenotype was not due to the light-promoted arousal at the onset of the morning. The divergent manifestations across the *Drosophila* genus of sleep need in response to SD suggest that the common aspects of their sleep, and likely the ancestral component shared across the animal kingdom, are derived from the circadian shaping of sleep-wake cycles. This would support the theories of sleep surrounding its

adaptive function^[79], that is, that sleep emerged as a period of adaptive inactivity. In this scenario, the homeostatic control may have been later acquired via exaptation and could plausibly be the reason for the plethora of physiological functions in which sleep is implicated.

5.2.2 Several species exhibit rebound in response to social sleep deprivation

Homeostatic control is a widely regarded criterion for sleep behaviour and is required for a state of rest to qualify as sleep^[14]. Considering the ubiquity of sleep across the animal kingdom, I thought it improbable that the entire panel other than *D.melanogaster* were devoid of homeostatic sleep regulation. There are many instances where resistance to sleep loss (i.e., no rebound) has been reported, for example, with the activation of the wake-promoting octopaminergic neurons^[247] or starvation-induced SD^[249]. Hence I monitored modifications in the panel's sleep behaviour in response to an alternative SD approach, an ecologically relevant method of social SD. The social SD paradigm harnesses the sleep suppression that occurs when two previously isolated males are confined to a small space within the experimental glass tubes over 12 and 24 hr. The interactions between the focal and intruder two males have been demonstrated to generate an accumulation of sleep need. In respect of brain-wide elevated synaptic strength^[319], increases in Ca^{2+} activity in the R5 neurons of the EB, comparable to the levels sustained with mechanical SD^[108], and a period of rebound on separation of the male pairs^{[319][108]}. In this thesis, I executed the social SD technique over 24 hr to establish whether the non-*melanogaster* species possessed a sleep homeostat. Comparable to the results obtained by other researchers using the described method, the *D.melanogaster* lab strain and wild caught produced a heightened sleep level on removing the intruder male from the tube. With this ecologically relevant method, *D.simulans* now exhibited a substantial rebound in the ZT0-3 time post-SD, while *D.sechellia* and *D.yakuba* had a significant, albeit modest, elevation in their sleep compared to the slept controls. Thus, providing evidence that *D.simulans*, and potentially *D.sechellia* and *D.yakuba* possess a homeostatic mechanism. I have found three species, *D.erecta*, *D.willistoni* and *D.virilis* that do not display a rebound response within the limits of this study. However, rather than suggesting the absence of sleep in the non-rebounding species, I hypothesise that within the appropriate environmental context, the species will accumulate sleep need and present with the anticipated homeostatic rebound.

The results I have presented in this thesis suggest that the elevated sleep need following social SD may correlate with their aggressive behaviour. Upon recording and manually

scoring the interactions during social SD, a tentative relationship emerged between the proportion of aggressive encounters and the rebound response. In that, the species with a homeostatic recovery period performed higher levels of aggressive behaviour, while the non-rebounding species were scarcely found to exhibit fighting tendencies. Therefore, the waking period over the social SD is qualitatively different across the *Drosophilid* species. While linear regression analysis identified a positive correlation between fighting levels and rebound following social SD, it's important to note that the strength of this correlation is constrained due to the model's limited fit to the data. Future research could focus on assessing fighting levels during the interaction and measuring the subsequent increase in sleep during the rebound phase within the same individuals. This approach would provide a more comprehensive understanding of the direct influence of aggression on rebound sleep and also generate a larger dataset for robust correlative analyses. In support of the connection between aggression and rebound, preliminary experiments suggested that social enrichment in *D.melanogaster* prior to interaction abolished recovery sleep (data not shown). It is widely reported in the literature that social isolation increases aggression and social enrichment suppresses aggressive behaviour^{[430][431][432]}. A key detail in the social SD approach is that both the focal and intruder male are isolated for four days before the interactions. The previous social experience of the fly influences how it responds to the social SD, likely due to alterations in their aggression levels. In future studies, alterations in the method to elicit an aggressive response, for example, extending the period of isolation prior to the interactions, will inform whether the increased sleep need generated by social SD is shared across the *Drosophilid* genus.

A 2015 study found that sleep and aggression are coupled, as SD resulted in suppressed aggression, suggesting that the signals generated by increased sleep need may impact aggression circuits^[218]. However, the study of the influence of aggression on sleep in *Drosophila* is largely understudied. Octopamine is a prominent wake^[433] and aggression-promoting^[297] neuromodulator, and the intersection of sleep and aggression likely lies within the octopaminergic system. Indeed, in the Kayser *et al.*^[218] study, feeding with an octopamine agonist replenished fighting levels after SD. In Section 5.1.5, I established conservation in the wake-promoting action of octopamine, suggesting potentially shared octopaminergic circuits in the brain that impact wake and sleep. Therefore, elevated octopamine signalling may drive wake during the interaction and result in a subsequent rebound.

In order to test this, I conducted experiments on the influence of octopamine agonist feeding on their response to mechanical SD. If elevated octopamine levels are responsible for the accumulation during social SD, then modulating octopamine circuits with

feeding of CDM should result in rebound. This was not the case; contrastingly, CDM feeding abolished rebound in *D.melanogaster* flies. This result may be due to limitations in the experimental design, where the flies were kept on CDM food for the entire experiment, including the rebound period. Therefore the wake-promoting influence of CDM could have masked the rebound response, supported by the abolished recovery in *D.melanogaster*. However, conflicting results are found in the literature on the influence of octopamine on rebound, as one study reported increased sleep following feeding^[433] and another identified that transient activation of octopaminergic neurons suppressed sleep rebound^[371]. Most octopaminergic neurons also release the glutamate neurotransmitter^[434], and thus glutamate transmission with octopaminergic activation may play a role in the rebound suppression observed by Seidner *et al.*^[371]. Therefore, the role of octopamine in regulating sleep-wake cycles is complex and requires further study. Furthermore, as a preliminary experiment, the number of individuals in each group during the drug feeding mechanical SD were relatively low and thus the observed results should be validated. Nevertheless, the species of the *Drosophila* genus offer a potential playground for studying the influence of aggression and octopamine signalling on sleep.

5.2.3 Summary

The circadian aspects of the species sleep-wake cycle are generally conserved, suggesting that the alterations observed in their sleep likely arise from a divergent homeostatic response. However, the *D.virilis* species were arrhythmic in constant conditions, a finding that has also been found in another study^[339]. Therefore the *D.virilis* alterations that I observed may stem from their circadian control, likely due to altered PDF expression in the s-LNvs. Indeed across the panel, widespread impaired homeostatic response to mechanical SD was observed with the non-*melanogaster* species. This was true even in the closely related *D.sechellia* species, whose baseline sleep patterns were primarily reminiscent of *D.melanogaster*. The lack of rebound is unlikely due to an inherent absence of homeostatic sleep control mechanisms due to the intact rebound found with the second method of social SD. Mechanistically, the non-existent homeostatic response to mechanical SD may manifest as a result of modifications in the neurochemical regulators responsible for coordinating sleep-wake circuits. Indeed, the consequence of L-dopa feeding was diverse across the panel, with no other species exhibiting the wake-promoting effects associated with elevated dopamine signalling.

The results I have found across the *Drosophila* genus indicate that the shared aspects of sleep, across the genus and potentially the animal kingdom, primarily rest in their

circadian aspects. This finding supports the notion that sleep initially emerged from circadian-driven periods of inactivity to ensure an animal is active during the period it is best suited for to carry out the essential tasks and inactive during the period where they are at higher risk of predation. It could be that the homeostatic aspects of sleep then were acquired through evolution, shaped by the environment and ecology, resulting in the varying manifestations of sleep that are observed across the animal kingdom - including the duration and physiological functions.

5.3 Examining synaptic strength dynamics

According to the SHY hypothesis, sleep may function as a period where the synaptic connections accumulated through wake with long-term potentiation are downscaled to reduce the strain of high synaptic strength on the neurons and organism (energy consumption, cellular stress, learning)^[90]. In order to elucidate the underlying cellular mechanisms responsible for the contrasting rebound results following mechanical and social SD between *D.melanogaster* and the non-*melanogaster* species investigated in this study, I interrogated the synaptic strength dynamics following SD across the panel. I conducted further analysis strictly in *D.melanogaster*, incorporating the elegant toolbox available in the model organism.

5.3.1 Mechanical BRP results

Many lines of evidence support the notion that sleep need correlates with synaptic strength in vertebrates and invertebrates. Thus, it is possible to elucidate the build-up of sleep need by investigating the level of synaptic strength markers in the brain following SD. Considering the absence of rebound following mechanical SD in the non-*melanogaster* species, I measured the intensity of the synaptic scaffolding protein, BRP, in the dFB following 24hr of sleep loss. *D.melanogaster* exhibited elevated levels of BRP in response to the mechanical method, in accordance with previous findings reported in the literature^{[319] [320]}. Interestingly, although mechanically induced rebound was absent for all the non-*melanogaster* species, their sleep need signal varied. *D.erecta*, *D.yakuba*, *D.willistoni* and *D.virilis* showed no measurable change from the slept controls, while *D.simulans* and *D.sechellia* both exhibited increased BRP signal. My initial postulation was that the lack of rebound observed could be due to the absence of sleep need, which indeed appears to be the case for the *D.erecta*, *D.yakuba*, *D.willistoni* and *D.virilis* flies. These results support the growing evidence of the close connection between sleep need and synaptic plasticity, as the synaptic strength marker levels post SD correlated

with the occurrence of rebound. However, contrary to my expectations, the *D.simulans* and *D.sechellia* species exhibited elevated sleep need despite the absence of rebound. There are various possibilities that would explain these results. Firstly, BRP is a proxy for elevated synaptic strength due to its role at the presynaptic active zone, but is not necessarily directly involved with the rebound response. It could be that while the BRP dynamics are intact in *D.simulans* and *D.sechellia*, alterations exist in other synaptic plasticity markers usually elevated during SD, for example, postsynaptic changes or alterations in the secretory machinery. This could be investigated further by measuring changes in the postsynaptic protein Discs large and in the synaptic vesicle associated proteins synapsin and syntaxin following mechanical SD across the panel. Another explanation could be that while there may be the associated changes in sleep need signals in *D.simulans* and *D.sechellia*, the downstream mechanisms for sensing these changes have diverged, and therefore do not respond to the unexpected changes in sleep need with elevated sleep.

In order to further investigate the influence of changes in synaptic strength on the homeostatic response, I manipulated the plasticity mechanics in *D.melanogaster* neurons and measured the impact on sleep and homeostasis.

5.3.2 Pan-neuronal knockdown of synaptic plasticity candidates influences baseline sleep

Pan-neuronal KD of the candidate synaptic plasticity GOI produced various sleep phenotypes, with *fmr1* and *rutabaga* removal increasing sleep and *dunce* and *orb2* KD reducing sleep, compared to the nSyb-GAL4 and UAS-GOI^{RNAi} parental controls. Mutations in these genes are also associated with numerous learning and memory defects, restating the relationship between synaptic plasticity, learning, and sleep.

***Fmr1* knockdown**

The fragile X messenger ribonucleoprotein 1 gene *fmr1* encodes the RNA binding FMRP protein, and a mutation in humans is related to a neurodevelopmental disorder known as fragile X syndrome^[435]. In *Drosophila*, *fmr1* mutations cause enhanced neurotransmission, overgrown dendrites and synaptic boutons, and its expression is activity-dependent^[410]. In this study, I identified that pan-neuronal KD of *fmr1* resulted in elevated total sleep, a finding that is corroborated in the literature. Bushey *et al.*^[436] identified that the amorphic *fmr1* mutant had elevated baseline sleep, while overexpression of *fmr1* had the reverse effect, reducing daily sleep. The elevated sleep associated with *fmr1* KD and mutant phenotypes supports the notion that sleep plays a role in the downscaling of the synaptic potentiation that occurs during wakefulness.

That is, the heightened functional synaptic plasticity mechanisms reported with *fmr1* mutations requires more sleep in order to reduce the increased synaptic connections acquired during wake.

Contrastingly to what was found in the KD rebound screen in this thesis, the aforementioned study reported that *fmr1* amorphic knockout and overexpression eliminated rebound, whereas I observed intact rebound with pan-neuronal KD. However, Bushey *et al.*^[436] employed the DAM device for sleep measurements, where the crossing of infrared beams indicates movement. In contrast, the sleep measurements in this study were undertaken with the ethoscope video tracking software, which can capture lower-magnitude movements that may be missed with infrared beam crossing. The researchers reported a high level of sleep during the rebound period in the *fmr1* mutants during baseline conditions, and therefore it is plausible that the rebound phenotype observed was due to increased smaller movements, for example, feeding, that would not result in an infrared beam break.

***Dunce and rutabaga* KD**

Dunce and *rutabaga* are the canonical genes identified to impair learning and memory in forward genetic screens^{[149][412]}. Investigations of their function revealed that they encode enzymes responsible for the reciprocal regulation of cAMP. *Dunce* encodes a phosphodiesterase responsible for cAMP degradation^[386], and *rutabaga*, an adenylyl cyclase involved in its synthesis^[412]. Therefore, mutants or KD of the genes has opposing influences on cAMP levels, but both have learning and memory impairments. In this study, the impact of pan-neuronal KD of *dunce* and *rutabaga* generated opposite sleep phenotype results, with cAMP elevations reducing sleep, in the case of *dunce* KD, and cAMP reductions, in the case of *rutabaga* KD, increasing sleep. This finding is echoed in a 2001 study, where Hendricks *et al.* demonstrated that manipulations that increase cAMP signalling or the downstream targets of cAMP signalling, for example, PKA and CREB2 activity, reduced sleep and vice versa, including the *dunce* and *rutabaga* mutants. Thus, sleep timing is inversely related to cAMP signalling in the brain.

Elevated cAMP levels produce wake, likely due to the downstream effects of elevated cAMP-PKA signalling. For example, widespread overexpression of PKA in adult neurons reduces sleep, mimicked with PKA overexpression in distinct MB cells; however, PKA overexpression in another MB GAL4 driver increased sleep^[272]. Since this finding in 2006, the distinct MB circuits responsible for sleep and wake promotion have been identified. The influence of cAMP-PKA signalling on sleep is not restricted to the MB. PKA mediates wake-promotion in the PI in response to octopamine signalling, and cAMP activity downstream of the Dop1R receptor in the dFB inhibits dFB activity,

inducing wake^[437]. Dopamine is a prominent wake-promoting molecule, which exerts its effect by binding to G-protein coupled receptors that modify cAMP signalling ^[438]. Indeed, inhibition of *dunce* in a pair of serotonergic projection neurons was found to stimulate the cAMP-PKA pathway, activating downstream DAN neurons and facilitating the formation of long-term memory in the MB^[439]. Therefore, it is possible that the KD of *dunce* and *rutabaga* is influencing wake-promoting dopamine (or other neurotransmission) signalling in the brain.

Other baseline sleep alterations

The baseline sleep phenotypes I discussed here are based on the significant change compared to both parental controls. There are some instances where the GOI KD produced significantly elevated sleep compared to the UAS-GOI^{RNAi} control, but the levels were indistinguishable from the nSyb-GAL4. Differences in baseline sleep level of parental controls are often found in the literature, primarily as a result of background variability^{[246][440][441]}. The RNAi lines are all sourced from the same VDRC KK library and therefore generated in the same host strain, while the nSyb-GAL4 construct was likely inserted in an alternate background and resulting in the observed changes in the GAL4 and UAS-GOI^{RNAi} controls.

5.3.3 Pan-neuronal knockdown of synaptic plasticity candidates influences sleep homeostasis

Pan-neuronal KD of *dunce*, *synapsin*, *orb2*, and *appl* resulted in impaired rebound. The lack of rebound generated with genes that have associated roles in synaptic plasticity strengthen the building picture found in this study and others on the relationship between synaptic strength and sleep need. Further investigation of the phenotypes generated with *dunce* and *synapsin* with targeted GAL4 KD in established sleep and wake centres in the *Drosophila* brain revealed the requirement of the genes in shared (PAM DANs) and distinct (dFB) circuits for the homeostatic response to mechanical SD. While demonstrating the role of cAMP signalling and neurotransmission in sleep homeostasis throughout the brain and in the PAM DANs and dFB circuits, further study is needed in order to interpret the mechanisms underlying these findings. Herein, I will discuss the results of *dunce* and *synapsin* and provide examples of potential lines of investigation based on evidence from the literature on the gene function and downstream targets on the neuronal cells. However, these lines of reasoning are highly speculative.

Pan-neuronal *dunce* knockdown attenuated mechanical rebound

The rebound found in wild-type flies after mechanical SD was attenuated with pan-neuronal KD of *dunce*, while the homeostatic response persisted in *rutabaga* KD flies. Hendricks *et al.* reported that *dunce*^{ML} mutants exhibited a rebound reduction while *rutabaga* mutants abolished rebound; however, the data was not shown. I tested the response of another *dunce* mutant, *dunce*¹, to mechanical SD and found a strong rebound. While unexpected, the mutant results are likely due to the type of mutation in the *dunce*¹ flies. *Dunce* is seemingly expressed in all tissues within the fly^[442], and many amorphic mutations cause female sterility in flies^[443]. The *dunce*¹ mutant used in this study is hypomorphic, where levels of the *dunce* cAMP phosphodiesterase product are reduced to 51% of the Canton-S wild-type levels^[444]. This perhaps offers an explanation for the disparity in results we see here with the KD and mutant response, as the remaining levels of cAMP phosphodiesterase in the brain may mask the impact of the removal of the *dunce* gene. However, the efficiency of the RNAi mediated KD of *dunce* has not been established in order to confirm whether this is the case.

While few studies directly measure the rebound response of *dunce* and *rutabaga* manipulations, comparisons in the effect of cAMP signalling on rebound sleep in the literature can be explored due to the inverse impacts on cAMP signalling in the *dunce* and *rutabaga* flies. For example, another learning and memory gene with a suspected role in sleep regulation is *amnesiac*, which likely encodes a human neuropeptide orthologue of PACAP and mediates L-type Ca²⁺ channels via cAMP signalling. PKA activity is enhanced in *amnesiac* mutants, with a short sleeping and lack of rebound phenotype, providing another example of where elevated cAMP-dependent PKA activity impacts rebound^[445]. However, *amnesiac* expression is restricted to a small number of neurons, the dorsal paired medial cells, and thus does not resemble the widespread elevation in PKA activity likely associated with pan-neuronal *dunce* KD. Huntington disease models in flies have associated sleep phenotypes, including an impaired rebound in response to mechanical SD, which has been shown to be restored by genetically decreasing PKA levels^[446].

Pan-neuronal *synapsin* knockdown abolished mechanical rebound

In this study, pan-neuronal *synapsin* KD abolished rebound following mechanical SD, with no effect on baseline sleep amount. *Synapsin* encodes a phosphoprotein that forms a complex with the endocytic scaffolding protein Dap160, sustaining the reserve synaptic vesicle pool, and mobilising the reserve vesicles with increased action potential firing^[447]. *Synapsin* mutants show defects in complex behavior, including degraded learning and memory^[393], likely due to impaired functional synaptic plasticity in the absence of the synapsin phosphoprotein^{[391][392]}. Furthermore, synapsin levels in the brain have been found to increase upon SD^[319], alongside the active zone

scaffolding protein BRP. Therefore, the lack of rebound may be due to reduced neurotransmitter release and synaptic strength during SD, attenuating the accumulation of sleep need and initiation of rebound.

Pan-neuronal KD diminished daytime sleep

The *dunce*, *synapsin* and *orb2* nSyb-GAL4 driven KD resulted in a significant reduction of daytime sleep, primarily manifested through bouts of comparable or increased length, alongside a reduction in bout number. Consequently, it could be speculated that the abolished rebound identified in the aforementioned genes may be attributed to a stronger arousal drive during the rebound phase, as indicated by the diminished sleep time. However, a reduction in daytime sleep does not reliably predetermine the loss of recovery sleep following SD, as evidenced by the case of *starrynight* KD. In this instance, the flies exhibited a pronounced daytime sleep suppression, one more severe than *dunce* and *synapsin* KD, yet maintained an intact rebound response to mechanical SD. Furthermore, in all KD groups, socially induced sleep loss elicited a rebound response regardless of the baseline sleep level. While these observations provide support that the abolished rebound in *dunce* and *synapsin* is unlikely to be solely due to the overall reduction in sleep during that period, further studies should investigate their role in the control of early morning sleep and arousal. Such studies will hopefully generate insight into whether the observed rebound phenotypes are due to a key role in homeostasis or circadian driven arousal.

***Dunce* and *synapsin* are required in the PAM DANs for mechanical rebound**

Further interrogation of the *dunce* and *synapsin* KD rebound phenotype revealed that the genes are required in the MB innervating DAN PAM neurons for wild-type levels of rebound sleep, as KD with the R58E02-GAL4 resulted in impaired recovery. This result may arise due to an impaired ability to initiate or maintain sleep need or the disruption in the build-up of sleep need. Synapsin has a specified role in synaptic vesicle release and KD results in impaired mobilisation of the synaptic vesicle reserve pool, limiting neurotransmitter release upon intense stimulation. However, the elevated cAMP with the *dunce* KD and subsequent PKA signalling likely has myriad downstream implications due to the wide-reaching effects of PKA phosphorylation mechanisms.

In this section, I will speculate about possible mechanisms that may cause the observed rebound phenotype. Firstly in the event of increased neurotransmission from the PAM DANs with elevated cAMP in *dunce* KD and secondly with reduced synaptic efficacy in the case of both *dunce* and *synapsin* KD.

Sitaraman *et al.*^[114] established that thermogenetic activation of multiple classes of

PAM neurons results in decreased sleep, and R58E02 labelled neurons were required for the wake-promoting effect. Activation of PAM DANs with R58E02-LexA elevates the activity of the wake-promoting MBON- $\gamma 5\beta'2a/\beta'2mp/\beta'2mp_bilateral$, mediated by the Dop1R receptor, and thus DAN input from the PAM DANs likely activates the MBON- $\gamma 5\beta'2a/\beta'2mp/\beta'2mp_bilateral$ circuit^[114]. Interestingly, the same group identified that this wake-promoting MBON converges on the same downstream target as a sleep-promoting MBON- $\gamma 2\alpha'1$, with the synaptic weight of either circuit likely determining whether the downstream target promotes sleep or wake^[275]. It is reported that the activity of the sleep-promoting MBON- $\gamma 2\alpha'1$ increases with sleep need and is essential for normal rebound sleep^[275]. It is possible that the elevation in cAMP in the PAM DANs upon *dunce* KD results in increased synaptic transmission, with elevated neurotransmitter release at the neuromuscular junction reported in *dunce* mutants^[448]. The impaired homeostatic response elicited with KD of *dunce* may arise due to downstream enhanced activity in the wake-promoting MBON- $\gamma 5\beta'2a/\beta'2mp/\beta'2mp_bilateral$ circuit, overriding the MBON- $\gamma 2\alpha'1$ sleep-promoting signal and thus inhibiting rebound. Indeed, *TrpA1* activation of the MBON- $\gamma 5\beta'2a/\beta'2mp/\beta'2mp_bilateral$ circuit has been reported to suppress homeostatic rebound sleep^[275].

However, this is highly speculative and relies on the assumption that *dunce* KD in the PAM DANs elevates neurotransmitter release. While this is supported by evidence in the literature of *dunce* mutants^[448], I have not confirmed this in this study with *dunce* knockdown. The elevated PKA signalling likely induced with cAMP elevation has a multitude of downstream targets, including the phosphorylation of transcriptional activators that affects the synthesis of many proteins. Evidence from the mouse hippocampus suggests that PKA signalling can result in the synthesis of phosphatases, which have competing actions on protein function to PKA, catalysing the removal of phosphate groups from proteins and potentially reducing the effects of cAMP PKA signalling^{[449][400]}. In addition, while neurotransmitter release is elevated at the neuromuscular junction, the *dunce* mutants were found to have impaired long-term potentiation and failed to recruit synaptic vesicles from the reserve pool upon intense stimulation^{[448][450]}.

An alternative interpretation of the PAM DAN KD results is that a reduction in synaptic efficacy in the MB innervating neurons disrupts the build-up of sleep need. This speculation is supported by the finding that *synapsin* KD exhibits similar rebound deficits.

A 2021 study established that the MB lobes undergo synaptic scaling during mechanical SD, which is diminished after induced rest with dFB activation^[277]. Interestingly, starvation-induced SD did not incur the changes in synaptic strength, suggesting that

the scaling correlated with high sleep need and not simply sleep loss, as starvation-induced sleep suppression is thought to occur without increasing sleep pressure^[201]. The synaptic strength elevation was not found throughout the MB, and subpopulations of MB KCs were responsible for the overall elevation^[277]. Interrogation of the PAM & PPL1 DANs that innervate the MB revealed comparable synaptic scaling upon mechanical SD, suggesting that the MB DANs alongside the MB increase in synaptic strength with growing sleep pressure and may signal to downstream targets to induce homeostatic rebound sleep. Therefore, the result I report here of *synapsin* KD in the PAM DANs highlights the importance of prolonged neurotransmission in this circuit during SD for rebound. One explanation could be that the continued signalling to the MB neurons from the PAM DANs is required for the synaptic scaling in the subpopulations in the MB and necessary for the accumulation of sleep need. KD of *dunce* in this circuit may elicit comparable impairments in the synaptic efficacy of the PAM DAN signalling. Indeed, synapsin is a known target of PKA^[384] and *synapsin* and *dunce* mutations are both reported to have impaired synaptic vesicle release^{[450][391]}. In future studies, the requirement of the PAM DANs in the build-up of sleep need should be investigated in terms of the impact of silencing the neurons on rebound and the synaptic scaling in the MB circuit. Additionally, investigating the effect of *dunce* and *synapsin* KD on the synaptic efficacy of neurons, and the influence on the activity of downstream targets, for example, with genetically encoded fluorescent Ca²⁺ reporters, will provide insight into the mechanisms underlying the rebound phenotype.

Interestingly KD of *synapsin* and *dunce* in the entire MB lobes had no impact on rebound, suggesting that mobilisation of the reserve pool is important in the PAN DANs for effective communication of sleep need, but not in the MB. However, the MBONs are found to have discrete sleep and wake-promoting roles, and the synaptic weight of the inputs likely coordinates the behavioural output. Thus, specific investigations of the distinct circuits may yield more informative results. It is peculiar that impaired synaptic vesicle release from the other known homeostatic centres, the EB and dFB, had no impact on rebound sleep. The firing of the R5 neurons of the EB correlates with sleep drive^[254], and thus one might expect that impaired neurotransmitter release at higher rates of action potentials, as observed with *synapsin* mutants^[392], would abolish rebound. The investigation of the effect of *synapsin* KD on neurotransmitter release in the EB and the resulting influence on the activity of the downstream dFB may provide information on the communication between the centres.

cAMP homeostasis is required in the dFB for mechanical rebound

While the impact of *dunce* and *synapsin* KD elicited similar phenotypes in the PAM DANs, the result was diverse when driven in the dFB. *Dunce*, and not *synapsin*, was

required in the ExF12 neurons for a conventional rebound response.

The dFB is often considered the output arm of the sleep homeostat and is a sleep-promoting centre required for the building sleep need in the EB to affect sleep. *TrpA1* activation of ExF12 neurons labelled with the R72G06-GAL4 elevated sleep, and silencing with another dFB driver, the 104y-GAL4, via expression of a leak K⁺ channel reduced sleep^[258]. The ExF12 neurons were found to be required for sleep promotion following activation of the R5 EB neurons. The R5 EB neurons have been found to increase in activity with extended wakefulness, measured by levels of Ca²⁺, and thus synaptic properties of the R5 circuit are correlated with growing sleep pressure. The increasing sleep need is found to be translated into sleep via activation of the ExF12 dFB neurons^[254].

DAN input into the dFB has been demonstrated to inhibit the dFB via the Dop1R1^[258] and Dop1R2^[451] receptors. D1-like DAN receptors are known to increase cAMP levels, and transgenically increasing PKA activity with the 104y-GAL4 dFB driver has been shown to reduce sleep time^[258]. Further investigations by Pimentel *et al.*^[451] discovered that dopamine hyperpolarizes the dFB neurons via the Dop1R2 receptor mediated by the regulation of potassium conductances. Therefore, the *dunce* KD and likely cAMP elevation in the ExF12 may inhibit the neurons via increased PKA signalling, reducing their activity and abolishing rebound. There is also evidence of recurrent circuitry in the R5 EB and Exf12 homeostatic mechanism, mediated by helicon cells which respond to visual input and play a permissive role in locomotion via activation of the R5 neurons of the EB^[266]. The helicon cells are inhibited by AstA transmission from the ExF12 neurons during sleep, inhibiting the helicon cells influence on R5 neurons and blocking locomotion. Therefore, the lack of inhibitory AstA signalling from potentially reduced electrical excitability in the dFB with *dunce* KD could elevate locomotion and block homeostatic sleep. Once again, these are highly speculative, and further investigation of the mechanisms by which cAMP elevation impacts synaptic plasticity and sleep in specific circuits is essential. Nevertheless, these results reiterate the impact of disrupted cAMP homeostasis on baseline and rebound.

5.3.4 Rebound after social sleep deprivation is resistant to the synaptic plasticity manipulations

While candidate genes were found to impair rebound post-mechanical SD, all KD genotypes exhibited a normal response to social SD. This result highlights the disparity between the types of SD and supports the growing picture that there are likely multiple circuits that mediate homeostatic control in response to different environmental cues.

Previous results in the literature have demonstrated that the social SD method results in synaptic scaling, with elevated BRP at the end of the deprivation period^[319]. Therefore, increases in synaptic strength are associated with social sleep loss, but the manipulations of synaptic plasticity included in my screen were unnecessary for rebound. The above small RNAi screen revealed that alterations in synaptic plasticity likely have different implications depending on the circuit, with both *dunce* and *synapsin* impairments removing rebound in the PAM DANs. However, only *dunce* KD generated a phenotype in the dFB. Further investigations of the social SD method should investigate the circuit by which sleep need is sensed and output. One method by which this can be achieved is by transiently silencing candidate neuronal populations during the sleep loss and recovery in order to identify the circuits required for the social SD rebound response.

Social enrichment increases sleep need, resulting in elevated markers of synaptic strength and overall elevated sleep levels after enrichment. One may elucidate that the rebound observed after social SD may result from accumulated sleep need via social enrichment. However, mutants for the genes *dunce*^[452] and *rutabaga*^[399] have both been shown to be resistant to social enrichment-induced alterations in sleep, sleeping comparably to their isolated controls. However, I have demonstrated here that in the case of pan-neuronal KD of these genes, the increases in sleep post-social SD remain. Suggesting social SD-induced rebound is not due to the increased sleep previously reported with social enhancement. As mentioned in Section 5.2.2, the social SD rebound may occur due to an influence of aggression sleep.

5.3.5 Summary

The absence of homeostatic rebound in the non-*melanogaster Drosophila* species included in this study appears to be due to an alteration in the generation of, or response to, the sleep need signal usually accumulated during mechanically interrupted sleep loss. This is evidenced by the lack of rebound across the panel and varied changes in the synaptic strength marker, BRP. Further interrogation of the synaptic strength dynamics in the *D.melanogaster* species revealed that cAMP homeostasis was necessary for the mechanically induced rebound, as KD of *dunce* pan-neuronally along with in the PAM DAN and dFB neurons resulted in absent rebound response. *Dunce* is responsible for the degradation of cAMP, and the mutant has been found to exhibit elevated overall cAMP levels. The impaired rebound, at the pan-neuronal level, appears specific to elevated cAMP levels, as pan-neuronal KD of *rutabaga*, responsible for cAMP synthesis and associated with overall reduced levels, had an intact rebound

response. Widespread KD of *synapsin* and targeted KD in the PAM DANs resulted in comparable results of the *dunce* manipulations, with no detectable rebound. *Synapsin* is required for synaptic vesicle release from the reserve pool upon intense stimulation. Moreover, *dunce* mutants are found to have impaired synaptic vesicle release, and the *synapsin* protein is a downstream target of cAMP-PKA mechanisms. Therefore, the equivalent results obtained with *synapsin* and *dunce* KD in the PAM DANs may be due to presynaptic cAMP-mediated synaptic plasticity alterations (neurotransmitter release). While the impaired rebound found with KD of *dunce* in the dFB could be a result of cAMP-mediated reduction in the activity of the dFB at the pre-synapse.

5.4 Limitations

5 min rule of sleep

In *D.melanogaster*, sleep is defined as a 5 min bout of immobility established from repeated findings that arousal thresholds were significantly elevated after this time^{[67][94]}. In this study, the 5 min criterion was applied to the other non-melanogaster species, and therefore the 5 min rule may not be appropriate for the measurement of *D.simulans*, *D.sechellia*, *D.erecta*, *D.yakuba*, *D.willistoni*, and *D.virilis* sleep. However, the fact that I can measure increases in sleep in a selection of non-*melanogaster* after social SD rebound partly supports that the 5 min rule is adequate in capturing sleep across the panel. Future studies could investigate the arousal thresholds during the length of an immobility bout to confirm that the non-*melanogaster* exhibit a comparable decrease in response to stimuli.

Arousal

In this study, I have measured the response to SD in terms of the increased level of sleep following sleep loss, a widely utilised parameter to establish rebound. However, sleep duration is only one aspect of rebound, and it is known, particularly in mammals, that individuals can recover from SD with sleep episodes of greater depth^{[453][67][423]}. I accounted for the depth of sleep episodes to some degree by calculating the length and number of sleep bouts during the rebound phase. With this approach, bouts of longer length would provisionally indicate greater sleep depth. Across my investigations, the sleep architecture following SD was unchanged to undisturbed controls, and therefore, within the scope of this thesis, there were no detectable changes in sleep intensity. In the future, researchers should incorporate arousal measurement to stimuli during the rebound phase to further investigate the Drosophilid rebound.

Environmental conditions

It is essential in the study of behaviour and sleep that the environmental conditions are strictly controlled to restrict the variables that could influence the measured phenotypes, particularly considering the impact of external factors on sleep. I tightly managed the environmental settings of the experiments presented in this study, controlling for light, diet, temperature, and humidity. The behavioural experiments were performed in a temperature-controlled incubator, with a 12:12 hr LD cycle (or DD) and humidity of 50-70%. In each incubator, the ethoscopes contained individuals from each *Drosophila* species and were organised irregularly between the arenas to remove any (unlikely) influence of the position in the arena on the sleep and activity measurements. The raised and experimental diets were equal within every experiment, consisting of either the standard food or a potato starch supplemented diet. However, considering the differential ecological niches that the *Drosophila* panel occupies, the environmental conditions considered optimum for *D.melanogaster* may only apply to some of the species. For example, *D.sechellia*, *D.erecta*, and *D.virilis* are specialist species and feed on the *Morinda* fruit, *Pandanus* fruit, and slime flux of decaying trees, respectively. Therefore, there may be more suitable diets; however, within the bounds of this study, I thought it essential to control the diet due to the considerable impact of yeast and sucrose levels on *D.melanogaster* sleep^[454]. Future explorations should interrogate the sleep and activity across the *Drosophila* genus on various diets. A similar argument could be made for temperature, light cycles, and humidity as the species inhabit vast climates. A 2022^[349] study demonstrated that *D.simulans*, *D.erecta*, *D.yakuba*, and *D.willistoni* showed a locational preference for lower temperatures (18°C-23°C). However, *D.melanogaster*, *D.virilis*, and *D.sechellia* all preferred the temperature used in this study, 25°C. Therefore, it is unlikely that the controlled temperature settings in this experiment are related to the striking alterations in the rebound that was observed.

Body size and relative movement in experimental tubes

The *Drosophila* species included in this study exhibit variations in their overall body size. Notably, the *D.virilis* group are considerably larger and this could influence the sleep and activity measured in this specific species. This issue is highlighted in Figure 3.5 where an increase in estimated size corresponds to an increase in sleep, and therefore a reduction in movement. There is a possibility that their size limits their mobility, leading to the observed elevated sleep levels. However, reduced activity can be found with direct visual observation of the *D.virilis* flies in their stock vials. It is important to determine whether the elevated sleep found in *D.virilis* is physiologically related to their size, potentially tied to metabolic rate, or if can be attributed to a confounding factor related to the size of the experimental tube. Future studies involv-

ing *D.virilis* and other larger *Drosophila* species should ensure the experimental arena provides adequate space and is appropriately controlled relative to the mean species size.

Aggression scoring

The aggression scoring was carried out manually by three researchers and included monitoring the incidence of fighting behaviour during 15 min in 1 min bins at ZT0, ZT6, ZT11.75, ZT12, ZT18, and ZT23.75. This approach was reasonable for estimating the amount of fighting across the panel. Still, it is plausible that the timing rather than the fighting levels were altered, as the videos only captured a subset of the 24hr period. On top of this, discrimination between the discrete aggressive behaviours usually performed in a *D.melanogaster* aggressive encounter were not scored. The automated monitoring and analysis CADABRA software, commonly used for the interrogation of aggression and courtship behaviour^{[455][456]}, could be utilised in future research to investigate not only aggression during social SD but to understand how aggression manifests differently across Drosophilids.

5.5 Impact and outlook

This study introduces the *Drosophila* genus as a potential framework for interrogating sleep regulatory mechanisms from an evolutionary perspective, characterising sleep and response to SD across a panel of 7 *Drosophila* species. Alongside the well-established *D.melanogaster* model, I presented sleep's circadian and homeostatic aspects in closely and distantly related species - *D.simulans*, *D.sechellia*, *D.erecta*, *D.yakuba*, *D.willistoni*, and *D.virilis*. Studying the neuromodulatory underpinnings and homeostatic rebound in the *Drosophila* genus revealed striking disparities. Firstly, while the wake-promoting effect of octopamine was generally conserved across the panel, dopamine did not appear to drive arousal in the non-*melanogaster* species, suggesting alterations in the dopaminergic signalling in the Drosophilid brain. Secondly, the accumulation of sleep need and the corresponding rebound in response to substantial sleep suppression with mechanical SD was solely exhibited in the *D.melanogaster* species. However, implementing an ecologically relevant method of social SD demonstrated that a selection of the panel possesses a homeostatic sleep mechanism, presenting with a rebound response (*D.simulans*, *D.sechellia*, and *D.yakuba*). These results indicate that divergent homeostatic mechanisms could be responsible for regulating sleep in the Drosophilids.

Furthermore, utilising the *D.melanogaster* model, I corroborated the literature findings that activity-dependent synaptic plasticity mechanisms are required for rebound following mechanical SD: with alterations in cAMP degradation and the maintenance

and mobilisation of the synaptic vesicle reserve pool resulting in impaired rebound response. Interestingly, the mechanisms of synaptic plasticity implicated in response to mechanical SD were not necessary for social SD-induced rebound. This finding mimicked the results in the *Drosophila* genus, where *D.simulans*, *D.sechellia*, and *D.yakuba* were resistant to mechanical SD but appeared to have a strong (*D.simulans*) or modest (*D.sechellia* and *D.yakuba*) rebound following social SD. These results present a causal relationship between alterations in the dynamics of synaptic strength in the observed changes in the homeostatic sleep mechanism. Although, a further detailed investigation is required in the *Drosophila* species to confirm if such alterations are indeed responsible for the divergence. For example, the generation of CRISPR mutants of the synaptic plasticity-associated genes included in this study or other key players in the *D.melanogaster* homeostatic response. Moreover, mutants in their dopamine receptors could enlighten the role of dopamine in the sleep-wake cycle in the non-*melanogaster* species.

The horizontal comparative analysis, which I have presented here, establishes that the *Drosophila* genus offers a novel approach to the study of sleep. Future investigation will benefit from the development of equivalent tools available in the *D.melanogaster* species for an in-depth systematic interrogation of the underlying genetic, molecular, and cellular mechanisms that have given rise to sleep variability across the *Drosophila* genus and potentially the animal kingdom.

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