Modulation of Sleep and Activity in *Drosophila*: a Systems Biology Approach. Genetics, pharmacology and high-throughput analysis of behaviour

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DECLARATION

I declare here that this thesis has been comprised solely by myself and has not been used in any form in a previous application for a degree. Except where stated, the work presented here is wholly my own.

This document does not exceed 100,000 words, including those in the main text, figure legends and those in the bibliography.

Abstract

Drosophila melanogaster is a widely used model organism which for the past 20 years has been employed in a variety of contexts to understand aspects of sleep, activity and more complex forms of behaviour. A challenge within the field of behaviour is how to accurately classify and quantify behaviours that arise from an organism when these behaviours are observed in different contexts. Technological advances have increased the availability of quantitative tools which can be used to examine activity and sleep behaviour. With the use of these tools, we can now answer new questions about the underlying mechanisms of behaviour in different conditions. In the thesis herein, I have examined activity and sleep behaviour in two different contexts, utilising some of these new technological tools, including a novel activity monitoring device and statistical classification techniques. In the first part of this thesis, I use this activity monitoring system to elucidate some of the mechanisms involved in homeostatic sleep behaviour. Specifically, I examine the effect of two different sleep deprivation methods on mated and virgin *Drosophila* females to examine their responses in terms of homeostatic sleep regulation. Using the same methodology and protocol, I then extend this work to examine the role of the neuropeptide, Corazonin, and its receptor, the Corazonin receptor, in these contexts. In the second part of this thesis, I use the same activity monitoring system to record the behavioural responses of flies exposed to different insecticide compounds. I then use both a statistical classification technique and behavioural analysis to attempt to classify these compounds based on their mode of action (MoA) and symptomology. Finally, I apply this methodology to answer other biological questions of interest, classifying both rebound sleep and flies with varying genotypes.

Acknowledgements

Undertaking a PhD is an experience that allows you to put so much of yourself and your personality into a large project, which ultimately feels like something of a reflection of yourself. This may not be a universally shared view, but this is correct in my experience. It has been famously said that a person is the average of the people they are surrounded by. If this is true, and by extension, if this thesis reflects me, then for this work I need to thank the following people who have supported me over the past years.

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scientific discussions with these people, but I have also had many laughs and fun times with them, which I will never forget.

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I would like to finally dedicate this thesis to my sixteen-year-old self, and any other sixteen-year-old who doubts that they can achieve something significant. Never let your

fears be bigger than your ambitions.

Contents

List of Figures

List of Tables

0.1 Acronyms

3IY	3-iodo-tyrosine
AADC	Amino acid decarboxylase
AANAT1	Arylalkylamine N-acetyltransferase
ACh	Acetylcholine
AG	Abdominal ganglion
AGO	Air-Gas-Odour (device)
AKH	Adipokinetic hormone
AL	Antennal lobe
AMPT	α-methyl-p-tyrosine methyl ester
ATP	Adenosine triphosphate
Ca^{2+}	Calcium ion
cAMP	cyclic Adenosine monophosphate
CCAP	Crustacean cardioactive peptide
C.elegans	*Caenorhabditis elegans*
Cl^-	Chloride ion
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system

CRY	Cryptochrome protein
Crz	Corazonin
CrzR	Corazonin receptor
CS	Canton Special (fly line)
CX	Central complex
dCLK	*Drosophila* Clock protein
D2R	Dopamine D2-like receptor
DAM	*Drosophila* Activity Monitor
DAN	Dopaminergic neuron (in the *Drosophila* brain)
DAT	Dopamine transporter
ddc	3,4-dihydroxyphenylalanine decarboxylase
DDT	Dichlorodiphenyltrichloroethane
dFSB	Dorsal Fan-shaped body
dg2	The gene encoding the cyclic guanosine monophosphate dependent protein kinase / protein kinase G
DILP	*Drosophila* insulin-like peptide
DL	Dorso-lateral neurons (a group of sexually dimorphic neurons in the *Drosophila* brain)

DMSO	Dimethylsulfoxide
DopEcR	(non-canonical) Dopamine receptor
DopR	Dopamine receptor, a dopamine D1-like receptor
DopR2	Dopamine receptor 2, a dopamine D1-like receptor
EB	Ellipsoid body
EEG	Electroencephalography
EGFR	Epidermal growth factor receptor
FSB	Fan-shaped body
GABA	γ-aminobutyric acid
$GABA_A$-R	γ-aminobutyric acid receptor type A, a ligand-gated ion channel
GFP	Green fluorescent protein
Gl	Glutamate
GnRH	Gonadotropin-releasing hormone
GRASP	Green fluorescent protein (GFP) reconstitution across synaptic partners
HCTSA	Highly Comparative Time Series Analysis
KC	Kenyon cell
LD	12 hour light : 12 hour dark cycle

L-DOPA	L-3,4-dihydroxyphenylalanine
LNd	Dorsal-lateral ventral neuron
LNv	Lateral ventral neuron
MB	Mushroom body
MBON	Mushroom body output neuron
MoA	Mode of action
Na^+	Sodium ion
nACh-R	Nicotinic acetylcholine receptor
NO	Noduli
NPF	Neuropeptide F
NPY	Neuropeptide Y
OL	Optic lobe
P1 neurons	A group of neurons in the *Drosophila* brain that is considered to be involved in courtship behaviour
PAL	Protocerebral anterior lateral (dopaminergic neurons)
PAM	Protocerebral anterior medial (dopaminergic neurons)
Para	*Paralytic*, a gene encoding a mutated sodium ion channel
PB	Protocerebral bridge

PBST	Phosphate buffered saline with 0.1% Tween-20
PDF	Pigment-Dispersing Factor
PER	Period protein
PKG	Protein kinase G
PPL1	Posterior protocerebrum lateral 1 (dopaminergic neurons)
PPL2	Posterior protocerebrum lateral 2 (dopaminergic neurons)
PPM	Parts per million (a unit of concentration)
PPM1	Posterior protocerebrum medial 1 (dopaminergic neurons)
	PPM2 posterior protocerebrum medial 2 (dopaminergic neurons)
PPM3	Posterior protocerebrum medial 3 (dopaminergic neurons)
PTTH	Prothoracicotropic hormone
Rdl	$Resistance - to - dieldrin$ (a gene conferring resistance to dieldrin)
RNAi	Ribonucleic acid interference, a method to knock down gene expression
ROS	Reactive oxygen species
SD	Sleep deprivation
SEZ	Subesophageal zone

s-LNv	Small-lateral ventral neuron
sNPF	Short neuropeptide F
sNPFR	Short neuropeptide F receptor
SOG	Suboesophageal ganglion
SP	Seminal fluid protein
SPR	Seminal fluid protein receptor
SPSN	Seminal fluid sensory neurons
SSFT	Sperm or seminal fluid transfer
TFNA-AM	4-trifluoromethylnicotinamide (the main metabolite of Flonicamid)
TH	Tyrosine hydroxylase
TIM	Timeless protein
THIP	4,5,6,7-tetrahydroisoxazolopyridin-3-ol (also known as gaboxadol)
TNT	Tetanus neurotoxin
TRPA1	Transient receptor potential ankyrin 1 (a thermosensitive ion channel)
TRP Channel	Transient receptor potential channel
UAS	Upstream Activating Sequence
VMAT	Vesicular monoamine transporter
ZT	Zeitgeber time

Chapter 1

Introduction

1.1 General Introduction

1.1.1 Behaviour and Our Understanding of Behaviour

One of the greatest challenges of modern neuroscience is to understand the way in which behaviours arise: from the molecular level of gene transcription, translation and protein production, to the circuits via which electrical and chemical signals are transmitted, to the phenotype that ultimately results. The fascination of human kind to study movement and behaviour is a curiosity which underlies our desire to understand not only the world around us, but also further understand ourselves. The numerous contexts in which behaviour can be studied provides a great challenge in so far as the number of possibilities of how to do so. Subsequently, the field of neuroscience has developed many tools study behaviour. These tools provide a basis to understand and study this complex topic from the level of proteins to cells and circuits to the whole organism. With the development and increase in power of computational tools and systems, this study has advanced in its ability but also generated further challenges. With an increase

in technical ability and complexity, a great many assumptions must often be made when measuring a certain biological aspect of behaviour. Many different biological measurements act as a proxy for what we perceive the behaviour of an organism to constitute, and with further biological evidence we look to give the necessary support for our definition. Another approach to studying behaviour is to use model organisms. These organisms, for which biological tools and techniques have been designed and established, allow us to not only understand the behaviour of complex animals, but also to advance understanding of possible homologous systems or behaviours in humans.

1.2 Movement and Sleep: Fundamental beginnings and definitions

The typical definition of movement often refers to the change in the position of a whole organism in space, or the change in the position of a singular or number of body parts in space. To the contrary of this, the opposite of movement is a general state of inactivity or quiescence. Sleep, however, is generally thought to imply something more advanced from a simple state of quiescence. The 'prehistoric' definition of sleep, that is the version defined prior to structured scientific study, is linked to the Greek philosopher, Alcmaeon of Croton, who likely lived between 500 and 450 BCE. He defined sleep as a physiological process of withdrawal of blood from the surface of the body to more internal and larger blood vessels (with death being the retreat of blood from the body, entirely) [204]. The prolonged state of inactivity of a living organism is generally defined as sleep. However, the definition of activity may arise at several levels: at a whole organism level, the cellular level or even that within cells. In this work, I am going to assist in defining these behaviours with observations at the level of the whole organism.

I will look at sleep and activity in two different contexts. (1) Understanding sleep in *Drosophila melanogaster*, with particular interest in the homeostatic regulation and recovery or rebound sleep. (2) Activity in the context of behavioural responses to insecticides, with observations of *Drosophila melanogaster* activity in response to compound exposure.

1.2.1 Movement and Sleep: Defining Sleep

The scientific definition and understanding of sleep has developed scientifically far beyond the initial historical and poetic descriptions as outlined above. During the 20th Century, emphasis became focussed on defining sleep biologically in terms of both understanding the brain circuits which regulate sleep and their molecular underpinnings, and in terms of theoretical models.

Mammalian species are the group of organisms in which by far the most research on sleep has taken place. Indeed, granted that many mammals share certain biological traits and mechanisms most closely with humans, this makes perfect sense. However, there are many pitfalls when using mammalian species as models for sleep. The maintenance costs of laboratory animals, their rearing times and the complexity of mammalian models are just a few of the main issues with studying sleep in these species [187].

Mammalian sleep is generally defined in terms of rapid-eye movement (REM) and non-REM sleep. The discovery of REM sleep is credited to Aserinsky and Kleitman, who, in 1953 showed in sleeping adults a type of eye movement which presents as 'rapid, jerky and binocularly symmetrical'. Recordings of these eye movements were quantified using an electroencephalogram (EEG) and correlated with the dreaming sleep stage in the adults [14]. This work was expanded on some years later by Dement and Kleitman [88], who studied EEG recordings of sleeping human subjects throughout the night, estab-

lishing that the brain goes through stages of both REM sleep and non-REM sleep in a cyclical way. Following these studies in humans, REM sleep was shortly after identified in cats [225], [382], rats [445] and rabbits [125]. REM and non-REM correlates of sleep, measured with EEG recordings, have now been observed in many mammalian, reptilian and avian species [58]. However, this is not the only way sleep has been traditionally defined. Four behavioural correlates of sleep were identified very early on by Henri Piéron [201] and have been used in this field ever since. These behavioural correlates are: (1) the adoption of a sleep-specific posture, (2) the state of behavioural quiescence being maintained past a threshold of time, (3) an elevated arousal threshold (whereby an increased stimulus is needed to elicit a movement, compared to that during wake) and (4) the state of sleep being reversible. It was later argued by Hendricks et al. [187] that these criteria should be expanded to include other behavioural correlates (and, as such, I will continue numbering from where I previously finished). (5) State-related changes to the CNS, i.e. a change in the processing of sensory information, (6) a process regulated via circadian and homeostatic mechanisms. It was stated by Campbell and Tobler [58] in their review of sleep in animals that some invertebrate species demonstrate the four original behavioural correlates, and later by Hendricks et al. [185] that these behaviours are also present in *Drosophila*. This opened up a plethora of work using invertebrate species in the field of sleep research. This allowed researchers to use a species which was much simpler, more quickly bred and allowed for higher throughput experiments (as experiments can be conducted on many more individuals) for sleep research.

Specifically, for sleep research in *Drosophila melanogaster*, work so far has looked at probing not only changes in quiescence length, with sleep defined by using the five minute rule, but also the depth of sleep (by measuring arousal threshold) [126], [472],

and changes in electrophysiological signals, measured from single cells [365] or over the whole brain by measuring local field potentials (LFPs) [338], [503].

1.2.2 Movement and Sleep: Defining Activity

Activity can be seen as the opposite of sleep: the animal is awake and producing complex behaviours when not in a state of quiescence. However, due to the many different complex behaviours an organism performs when awake, defining activity is not a trivial task. Activity can either arise from two origins: as a response from the organism to its environment, or as a response to a change in internal state, which then leads the organism to react to the environment [315]. In *Drosophila* specifically, various sensory systems can detect changes in the environment, such as olfaction [480], taste [212], vision [517] and mechanoreception [140]. These senses allow detailed information about the world to be received by the fly, and this in turn may modulate the biology or the internal state of the organism. For example, neuronal circuitry has been shown to develop initially in a predetermined manner and is then modulated by sensory inputs and experiences, both in terms of motor control [141], as well as for sensory processes themselves, such as vision [192] and olfaction [190]. It is also theorised that sleep contributes the reorganisation and consolidation of synapses in response to environmental inputs [57].

Internal state can be defined as the physiological conditions which make up the state of cellular and molecular processes of an organism [318]. This internal state is modulated by sensory inputs and can therefore change the response of the organism to its environment in a process often described as adaptive plasticity [160]. Examples of this include internal-state mediated changes to courtship [93], aggression [199], feeding [366] and food preference [319], and various social behaviours [6].

This constant interplay between environmental variations and changes in internal state lead the organism to dynamically respond to the environment and by producing a wide range of behaviours.

1.2.3 Measuring Behaviour Quantitatively

For many years researchers have been attempting to measure behaviour in a quantitative manner. With the development of computational power and decreasing size of circuit boards, this has enabled researchers to build sophisticated tools to measure behaviour at the whole animal level. Manipulation of an environment and direct observation of responses to changes can now be accurately quantified and analysed. However, much has changed in the field of behavioural research since Aristotle looked at defining the movement of complex organisms in his work *"On the gait of animals"* [389]. This work can perhaps best be described as a more advanced state of ethology, which itself is described as *"the biological study of behaviour"* [462]. This type of work, which combines ethology [52] with high-throughput quantification [47],[82], is now commonly referred to as *"ethomics"* and has expanded and developed over the years and continues to grow with technological advances.

This field relies heavily on big data, and many studies in the past 20 years have looked to use this ethomics approach to investigate questions in *Drosophila* such as feeding [214], [317], social interactions between pairs of flies [82], sleep and activity [150] and 3D behavioural monitoring [9]. All of these ethomics approaches rely on recording devices, but other technologies, such as electronic sensors [399] and GPS systems [350] are other examples of technologies we can now use to quantify behaviour scientifically. Computational visual tracking and analysis has for some time now been regarded as one of the best methods for recording and quantifying animal behaviour, both recording

behaviour of the whole animal and of specific body parts.

1.2.4 Visual Analysis

In the past twenty years, a multitude of video tracking platforms and software analyses methods have been used to discern behaviour, the majority of which look specifically at locomotion and distance travelled [227], [339], [142], [357], [277], [173].

However, many platforms and packages have also looked at specific behaviours and interactions, such as social behaviours [198], [82], [123], [228], [47], [356], or for specific behavioural paradigms, such as animals walking in mazes or undergoing a specific behavioural test [65], [3], [74].

There are a variety of benefits to these systems over techniques which take a single measurement of behaviour as a proxy for whole body movement. Firstly, they can often accurately track many different body parts, even in a dynamic and frequently changing environment. Secondly, they allow for very small changes in behaviour to be detected, in some cases only to a single body part rather than the entire organism. Finally, many of these systems allow tracking of multiple individuals simultaneously, which allows high-throughput data analysis to be achieved quickly and easily.

In recent years, more emphasis has been put on designing flexible software tools readily available to the wider scientific community; open-source, compatible with a variety of hardware set-ups and versatile in their tracking ability. Some prominent examples of these software tools include DeepLabCut [298], LEAP [359], DeepPoseKit [162] and FLIIT [498]. With the exception of FLIIT, which is primarily concerned with predicting the movement of individual appendages [498], these software packages all use image based machine learning techniques predict the movements of animals. In this study, two different recording devices were used to track and collect data from flies: the ethoscope,

which uses custom software to track the whole body of the fly in real-time to provide velocity data, [150] and the superscope, which is a high-resolution recording device used in combination with DeepLabCut [298] pose-estimation software (see section 4.5).

1.3 Circadian and Homeostatic Regulation of Sleep

The most famous model in the field of sleep research describing the regulation of sleep behaviour was coined in 1982 by Borbély [40] in which he postulated a two-process model of sleep regulation by two interconnected, but separately acting, mechanisms: via Circadian regulation (process C) and via Homeostatic regulation (process S). Process C was summarised as a sleep-independent process, which was said to reflect rather than the amount of sleep, the timing of sleep and the demonstration of the rhythmic variation of sleep propensity. Process S is a sleep-dependent process, which is reflected in the level of the homeostat at sleep onset and is defined as the function of time previously awake. The longer you are awake, the greater the pressure to sleep. In this pivotal work, Borbély theorised that the interaction between these two processes is the mechanism by which sleep behaviour is controlled and regulated. This model has been hugely influential in the field of sleep research and sets a conceptual foundation on which most research in the field is built upon. Not only has it set a precedent for research but many discoveries post publication were in line with the models predictions. In terms of electrophysiological descriptors, it was acknowledged that in vertebrates, slow wave activity (SWA) or "delta activity", measured by using EEG recordings, correlates to process S of sleep, based on findings that the EEG power density reflected the effects of sleep deprivation (SD) [41]. The suprachiasmatic nucleus (SCN), a brain region found in mammals, has been correlated to regulating circadian mechanisms in animals.

Animals with lesions in this region show arrhythmic sleep/wake behaviour but have no disruption in reference to their homeostatic sleep drive [116]. These findings, amongst others, demonstrate separate biological mechanisms for the regulation of each of the processes, which ultimately work together to control sleep behaviour.

One of the shortfalls of this model, however, is found in its simplicity. The original authors acknowledged in a review, almost three decades on from the first conception of the model, that many of the original facets of the model were not true. This conclusion was based on research published in the years that followed [42]. In their 2016 review, the authors stated that there were several other complexities and considerations not contemplated in the original framework. These include, but are not limited to: (1) The concept that process S and process C do not just interact at certain times of the day, but are likely constantly interacting (2) Both sleep homeostasis and circadian regulation do not merely have one centre each for regulation of sleep, but several centres, each with differing mechanisms of control (3) The model was limited to defining sleep regulation without postulating on function (4) Other essential drives, such as metabolism and feeding, are also likely to influence circadian and homeostatic processes of sleep.

1.3.1 Circadian regulation of *Drosophila* sleep

The circadian regulation of sleep at the molecular level was first described using *Drosophila melanogaster* as a model organism [35]. The circadian changes in behaviour and physiology, which occur over the course of the day in response to changes in light and temperature, are known to modulate a number of biological processes, including sleep. In laboratory conditions under the constant maintenance of a 12 hour light, 12 hour dark cycle *Drosophila* exhibit changing waves of activity. It is possible to measure these changes in activity with the use of devices called *Drosophila Activity Monitor*

(DAM) [307], [203]. This set-up sees flies placed into individual glass tubes, with a laser beam running through the midpoint of each tube. When a fly crosses the beam, the device records the fly as being awake. Peaks of activity, measured in wild-type flies using DAMs, occur around the times of light transition, in both subjective morning and evening. Sleep is generally greatest during the middle of the day or the middle of the night [171], [184], [384]. *Drosophila* were first used for circadian studies after the discovery that various mutations to a core clock gene, thereafter named *period*, changed the sleep-wake circadian period from 24 hours to 19 hours, 28 hours, or led to a completely arrhythmic phenotype [249]. This work, and the discovery and characterisation of other sleep mutants, allowed researchers to probe the molecular and genetic underpinnings of this process.

The circadian process is based on two transcriptional activators, clock (dCLK) [5] and cycle (CYC) [394] and transcriptional repressors, period (PER) [20], and timeless (TIM) [322]. dCLK and CYC are located in the nucleus, with the levels of dCLK oscillating and levels of CYC remaining the same throughout the 24 hour period. dCLK and CYC heterodimerise and bind to the E-box enhancer elements of the promoter regions of *per* and *tim* [18], which activates their transcription in the light period [394]. Throughout the night, mRNA levels of PER and TIM accumulate in the cytoplasm [172], [413]. The proteins, PER and TIM, form a heterodimeric complex and translocate to the nucleus where they bind to the dCLK-CYC complex. This inhibits the binding of dCLK-CYC to the E-box enhancer elements, preventing the transcription of *per* and *tim* [404], [83]. Although in mammals it is known that light, which is considered one of the main circadian *Zeitgebers*, is responsible for regulating the circadian clock, in *Drosophila* the effect of light on rhythmic behaviour seems to be less important. Experiments have shown that locomotive circadian rhythms persists in mutant flies without a functional circa-

dian clock [487] and that flies kept in constant darkness can still maintain rhythmic behaviour [184]. However, the blue-light sensing protein cryptochrome (CRY) [120], which is expressed in the eyes and in some (but not all) of the clock neurons of the *Drosophila* brain [504] does partially regulate the molecular circadian clock. It has been shown that when CRY is exposed to light throughout the subjective daytime, it mediates the degradation of TIM via the F-box protein, jetlag (JET) [248], which allows the resetting of the circadian clock. Work has shown that decreasing amounts of TIM occur with increasing amounts of light intensity, and mutants for TIM are more sensitive to light [442]. This work and that of others has demonstrated that CRY contributes to circadian oscillations [120], [244] of sleep-wake behaviour in *Drosophila*, but that the circadian cycle does not critically depend on its function.

Another key player which contributes to the regulation of the circadian process is Pigment Dispersing Factor (PDF). This neuropeptide has been shown to be expressed in key circadian regulating cells in the *Drosophila* brain and mutants for PDF show disruptions to rhythmic behaviour [380], [261].Functionally, this neuropeptide is thought to coordinate oscillators throughout the brain [276], [209],[412].

1.4 Circuitry of *Drosophila* homeostatic regulation

In contrast to the wealth of knowledge we have regarding the molecular processes which underlie the circadian regulation of sleep, we know comparatively little as to how the homeostatic process is regulated. In the past 20 years, however, our understanding has grown greatly, and the circuitry which underlies this homeostatic process has begun to be pieced together.

One of the most important structures for sleep regulation in *Drosophila* which has been

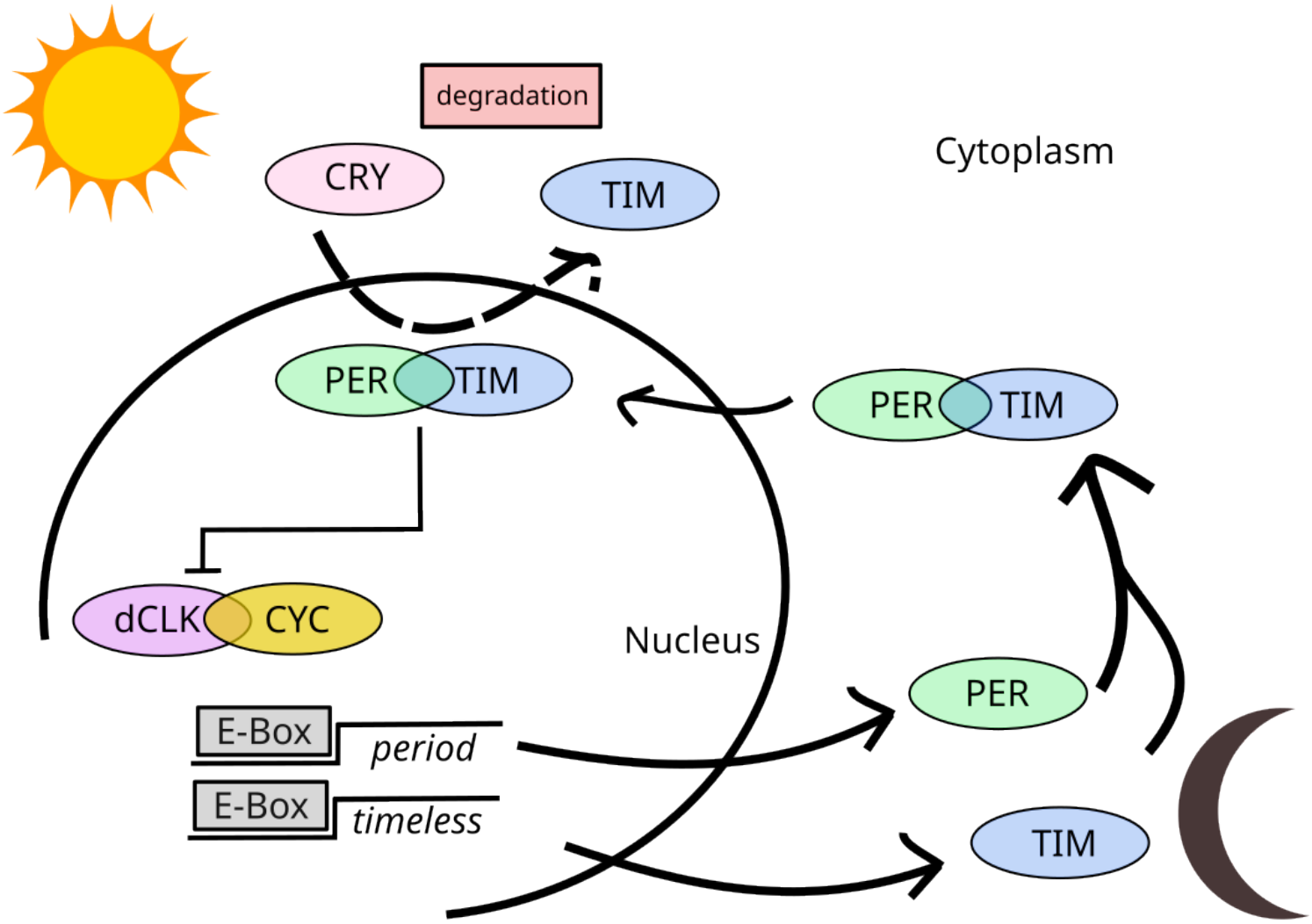


Figure 1.1: *The molecular mechanism of circadian regulation in Drosophila melanogaster* A diagram showing the main components of the molecular transcriptional feedback loop which dictates the circadian regulation of sleep.

identified so far is the mushroom body (MB) [222]. This structure is located centrally in the fly brain, and its connections and sections have been mapped in detail. Aso et al. [16] used split-Gal4 lines to give one of the most detailed accounts of the structure and connectivity of this region. The MB lobes comprise around 2200 neurons, including 7 types of Kenyon cells (about 2000 of which make up the MB itself), 21 types of MB output neurons and 20 types of dopaminergic neurons. Each of the symmetrical structures of the MB is composed of 3 lobes based on the subtypes of Kenyon cells within these structures: α and β lobes, α' and β' lobes and the γ lobe and heel [78]. Its basic structure is composed of a pair of bilateral neuropils. Each of these have an L-like shape which face in on each other on either side of the central complex (CX). They protrude downward through the protocerebrum, with the calyx (a ball-like structure which sits behind each neuropil), connected to the pedunculus, a second ball like shape, which rests behind the tail of the γ lobe [183],[15],[16]. Aso et al. showed that projection neurons from the antennal lobes (AL), which sit just ventrally below the MB, innvervate the calyx. Axons of the KCs run through the pendunculus to the lobes where KCs connect to dendrites of the MB output neurons (MBONs). Dendrites of these MBONs, and terminals of these aforementioned DANs, intersect the lobes and the pendunculus.

Although the MB is an associative region to the CX, it has been shown to connect to structures within the CX which are also important regulators of sleep. The CX is composed of four distinct neuropils in the middle of the protocerebrum; the fan-shaped body (FSB), the ellipsoid body (EB), noduli (NO) and protocerebral bridge (PB) [506]. These regions have been shown to have various different subtypes of neurons, expressing various different neurotransmitters. The EB contains serotoninergic, dopaminergic, GABAergic and glutaminergic neurons. The FSB contains dopaminergic, GABAergic,

glutaminergic and sNPFR neurons. The PB expresses GABAergic and glutaminergic neurons. The NO with only dopaminergic and glutaminergic expression [230]. The diversity of different neurotransmitters in the MB indicates complex neuromodulatory interplay underlying behaviours such as sleep.

One of the most important studies, which linked the CX to the homeostatic regulation of sleep, identified that a subset of EB neurons, R2 neurons, whose activation led to sleep loss and upon the cessation of activation, a sleep rebound occurred [281]. The authors also showed that inhibiting neurotransmitter release in these neurons reduced rebound sleep following mechanical SD.

The dorsal-FSB (dFSB) has also been linked to homeostatic sleep regulation. Early studies showed that activation of both neurons within and projections to the dFSB led to increased sleep but did not affect rebound [469] [108]. The above mentioned R2 neurons were also shown to innervate the dFSB [281]. It was later shown that mutants for the Rho-GTPase-activating protein, crossveinless (cv-c), which show reduced sleep, have reduced sleep rebound, deficits in memory, and decrease the sensitivity of dFSB neurons to their synaptic inputs [105]. The authors went on to postulate, based on their evidence, that an increase in homeostatic sleep pressure increases the activity and sensitivity of the dFSB neurons (measured electrophysiologically).

Building on this work, Pimentel at al. [365], identified the mechanism by which the dFSB switches between the ON state (active and promoting sleep) and OFF state (inactive and not promoting sleep). The authors found that dopamine signalling was the mediator of this switch. Dopamine signalling leads to the expression of specific voltage-gated potassium channels. Silencing these neurons downregulated currents of the potassium channels, Shaker and Shab, and upregulated leak currents through the potassium channel, Sandman.

Interestingly, previous studies, such as that by Liu et al. [279], had already implicated dopamine as a mediator of arousal and wakefulness in *Drosophila*, demonstrating that dopaminergic neurons from the PPL1 cluster increase activity during wakefulness, and that these neurons also project to the dFSB. Liu et al. saw that the sleep reduction they observed in flies upon activation of DA neurons was prevented by the loss of the dopamine receptor, DopR [279].

Later work has build on this homeostatic circuitry and gone further to show the link between dFSB neurons and EB neurons. Helicon cells were shown to be the projection neurons that link the dFSB to the EB and mediate signalling between these regions [106]. Here, it was shown that an increase in wake past a threshold led to the release of Allatostatin-A (AstA) onto helicon cells, resulting in their inhibition and ultimately allowing sleep to occur [106].

Although much of this work has implicated the FSB in homeostatic sleep regulation, microcircuits in the MB have also been shown to have a role in sleep homeostasis. When Sitaraman et al. [422] activated KCs in the α'/β' lobes of the MB, they found that sleep was decreased, whilst the activation of γd KCs led to sleep increase and activation of γm's led to a decrease. Further experiments in this study showed that activating specific MBONs, which connect to different KC clusters can also modulate sleep. Most interestingly, silencing of the MBON $\gamma 2\alpha$'1 outputs prevented rebound following SD, with the same phenomena occurring with the silencing of γd KCs after SD.

As previously stated, KCs in the MB also receive other sensory information such as that for odours [321], [370], [135], visual processing [280], courtship [313], [273] and social interaction [441].

The MB is known to be a hub for regulating learning and memory in flies [354], [467] , [252], with many studies identifying the MB as the centre that links two or more of

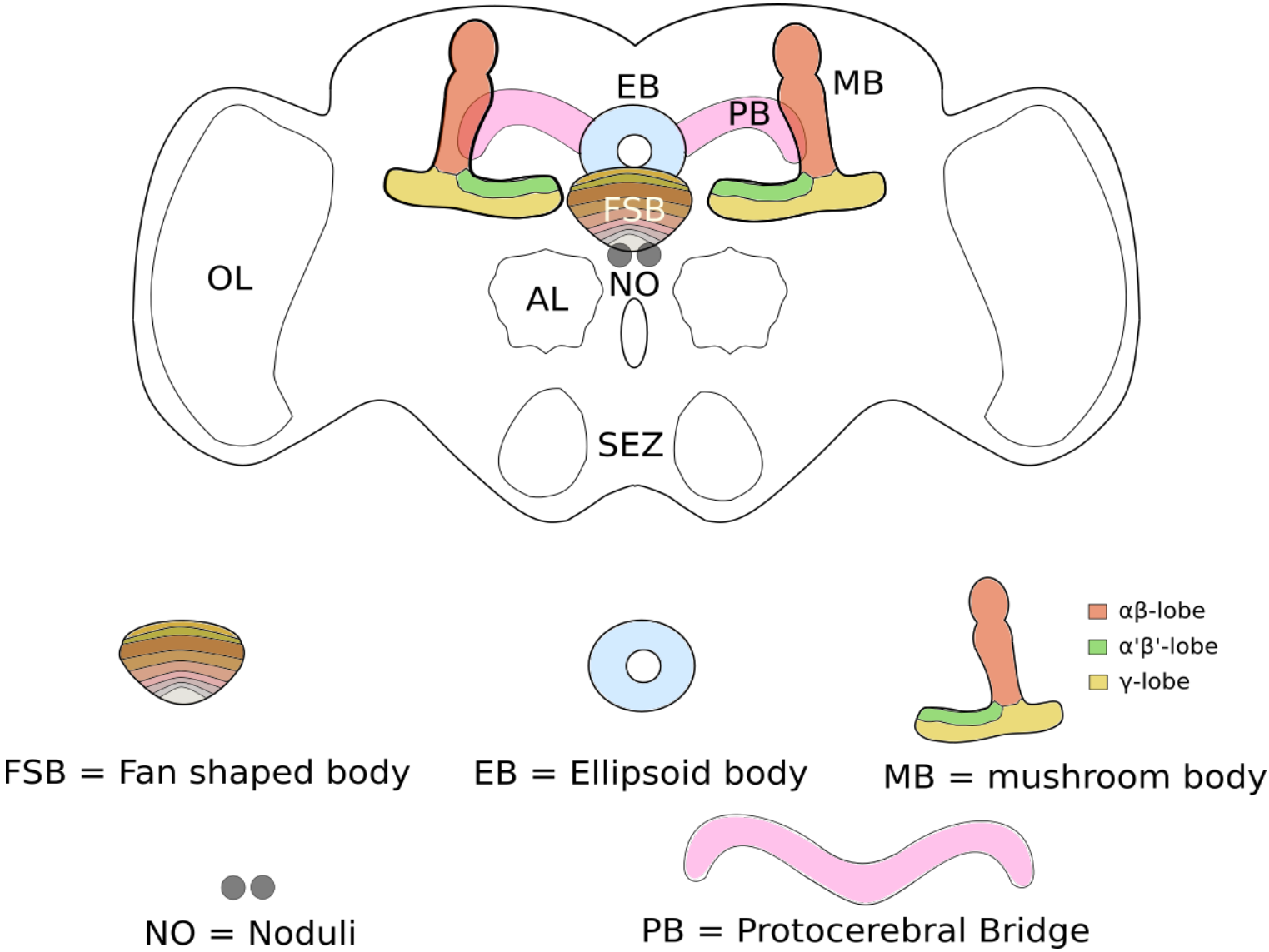


Figure 1.2: *Major components of the Drosophila central complex and the associative structure, the mushroom body, with surrounding brain regions.* Main regions of the fly brain, with emphasis on the central complex components, the PB, FSB, EB and NO and associative structure, the MB. Other regions shown are the antennal lobes (AL), optic lobes (OL) and subesophageal zone (SEZ).

these roles [135], [177], [102].

1.5 The role of Dopamine in sleep and arousal

Many lines of evidence in sleep research have implicated dopamine as one of the main neurotransmitters needed for sleep regulation and the regulation of arousal.

Dopamine is synthesised in *Drosophila* via a two stage process. The first stage involves the conversion of tyrosine into L-DOPA via the enzymatic action of tyrosine hydroxylase (TH), which is encoded by the *pale* gene [336], [193]. The second stage involves the conversion of L-DOPA to dopamine by the aromatic amino acid decarboxylase (AADC), encoded by the *DOPA decarboxylase (ddc)* gene [458], [55], [324], [284].

Drosophila have four dopamine receptors, which can be divided into three types: D1-like receptors, *DopR* and *DopR2* [161], a D2-like receptor *D2R* [127] and a non-canonical receptor, *DopEcR* [434].

Further evidence for its importance in sleep is the large body of work which shows a variety of dopaminergic compounds modulate *Drosophila* sleep, such as caffeine [329], L-DOPA [306], modafinil [186], 3IY and AMPT [364] (discussed in depth in section 1.7.4).

Another factor implicating dopamine in *Drosophila* sleep regulation is that mutations which affect dopamine levels in the fly brain have a significant impact on sleep and arousal.

One of the first sleep mutants, *fumin*, was identified as having an extremely low sleep phenotype which resulted from a mutation in the *Drosophila* dopamine transporter (dDAT) [255]. These flies also have increased locomotor activity, and decreased arousal threshold [126],[255]. *dumb* mutants were first identified as mutants with defects in aversive and appetitive learning [242]. The *dumb* mutation leads to abnormal expression of the *Drosophila* DopR and reduced dopamine signalling, increased sleep and resistant to the wake-promoting effects of caffeine (which is thought to work via DopR

receptors and TH) [329],[7], [242]. Similarly, flies mutant for TH showed decreased locomotor activity, with increased sleep and resistance to the sleep inducing effects of 3IY [385]. Interestingly, in this study, the TH mutants responded normally to the sleep suppressing effects of caffeine (this could be related to the different nature of mutations used by each study [385], [329]). *Insomnia-like* flies, a line which selectively bred for 60 generations for a low sleep phenotype, show a large increase in dopamine levels in the brain and severe learning impairments [409]. The *Insomnia-like* line should not be confused with the *Insomniac* line, which was identified in a forward genetic screen [435] along with its E3-ubiquitin ligase *Cullin-3*, as showing a low sleep phenotype mediated by the dopaminergic pathway [364].

Flies with manipulations for other parts of the dopamine synthesis pathway have also been examined for sleep changes, like that for *ddc*. Interestingly, when neurons expressing *ddc* are silenced with tetanus neurotoxin (TNT), flies did not show changes in baseline sleep [507]. Neurons which express *ddc* are also serotonin producing, which may explain this result, but the same paper showed modifications to serotonin expression can also have an effect on sleep [507].

In the *Drosophila* brain, Mao and Davis [294] identified 13 different clusters of dopaminergic neurons, which are named based on their anatomical location in the brain. 8 of these clusters project to the MB, which include those from the regions of the PAM, the posterior protocerebrum lateral 1 (PPL1) and the posterior protocerebrum lateral 2 (PPL2). Subtypes of posterior protocerebrum medial 3 (PPM3) neurons project to the EB and FSB [294]. Later work has now found 20 types of dopaminergic neurons that connect to the MB [449], [270].

Activating various dopaminergic neurons has an effect on sleep and arousal. As mentioned in section 1.4, two dopaminergic neurons from the PPL1 cluster connect to the

Figure 1.3: Dopamine Synthesis Pathway in *Drosophila*.

dFSB and these neurons promote wakefulness [279]. The neurons are more active during wake and require *DopR* for this effect to occur [279]. Other dopaminergic neurons also connect directly to the FSB [469], [365]. It has been shown that the circadian l-LNvs also respond to dopamine, as they express *DopR*, *DopR2* and *D2R* and show increased activity in response to dopamine application [415]. Direct activation of certain dopaminergic neurons in the PAM and PPL1 clusters also leads to sleep suppression [422].

Together, this shows the considerable effect dopamine, its receptors and neurons expressing them have on sleep and wake activity.

1.6 Types of sleep deprivation

To understand the function and purpose of a molecule, circuit, or process in biology, one of the usual methods to understand it is to remove it. For instance, when one would like to understand what the role of food and feeding is to an organism, the first thought is to remove the food source. This is how the story goes for many lines of scientific exploration. When it comes to sleep, however, the removal of sleep is a little more difficult to enforce. Not only is a constant monitoring system required to ensure that the test organism remains awake, the main difficulty with removing sleep is that most methods of removing sleep may also affect other biological functions which are not the subject of the study. For instance, early SD studies in *Drosophila* concluded that

prolonged SD was fatal [417], a fact which has now been disputed [149]. By examining the specific methodologies of how SD is carried out, it is now seemingly necessary to consider what other functions the type of SD may impact apart from sleep.

1.7 Sleep Deprivation in *Drosophila*

The earliest work investigating sleep behaviour in *Drosophila* aimed to do what many had attempted before in the mammalian field; remove sleep to examine the homeostatic aspects of sleep. The one of the first papers [185] to look at behavioural correlates of sleep in *Drosophila* used two methods of SD, the first method simply being an experimenter tapping the side of the dish each time the fly was determined to be asleep. The second method, used previously and currently, involves a mechanical stimulus which is generated by a stepping motor and configured to perform this method autonomously. Both of these methods were fairly rudimentary in design, but achieved the aim of determining that flies *could* be sleep deprived and that they displayed a sleep rebound following SD [185]. Following this study, and the further development of the *Drosophila* sleep field in general, attempts have been made to expand the number of SD methods available. I will now briefly review the work on each of these methods used in *Drosophila*, summarising these as either natural (ethologically relevant), or artificial (constructed) forms of SD.

1.8 Non-natural methods of sleep deprivation

1.8.1 Mechanical sleep deprivation

By far the most popular method of SD used in flies is that using forms of mechanical stimuli to sleep deprive flies. This method, depending on the specific methodology, usually relies on an apparatus (such as the Sleep Nullifying Apparatus, SNAP). In this paradigm, individual flies are kept in the previously aforementioned DAMS [307], [203] and monitored constantly. The SNAP apparatus works autonomously whilst the flies are in the DAMs [307] and shakes the DAMs, and therefore the flies, from side to side (enforcing negative geotaxis). Almost all studies which use mechanical SD as a method to sleep deprive flies use this apparatus, or some variation of this set-up. Alternatives, such as a vortexer, or a vibrating surface, deliver a mechanical stimulus at either random or evenly spaced intervals (the following citations are examples of such studies, and not an exhaustive list) [447], [417], [72], [257], [459], [485], [218], [238], [53], [477], [132],[126]. However, using a mechanical SD method like this has some flaws.

Firstly, this method does not discriminate between flies which are asleep or awake. The flies stimulated by the vibration or movement are not actively monitored to determine in real time what behavioural state they are in, and so may experience needless stimulation. Secondly, this method could also lead to flies becoming habituated to this stimulus. In fact, one study showed that flies can be "lulled" into a vibration-induced sleep by a continuous mechanical vibration [348]. One methodology which has been developed in the recent past for more targetted mechanical SD is that of dynamic SD. This method relies first on a new kind of tracking device developed in the Gilestro Lab, the ethoscope. The ethoscope is a custom tracking device which can monitor the activity and sleep of flies in real time by imaging the fly whilst it moves in the tube

[150]. Designed to work with the ethoscope tracking device [150], the dynamic SD system uses an add-on device to the ethoscope called an optomotor. The optomotor device consists of a circuit board attached to ten motors. This can be placed below the ethoscope tubes and produces a targetted mechanical stimulus to the specific tube that contains the specific fly intended to be sleep deprived. The optomotor device receives input from the ethoscope based on real time data about the active state of the fly. When the fly is detected to be asleep, the ethoscope triggers the corresponding motor under the tube of the sleeping fly and causes it to rotate, spinning the glass tube and the fly held inside. This method solves both aforementioned problems. As the active state of the fly can always be determined using this method, habituation is less likely to occur (see Fig.2.1 for a diagram of the ethoscope and optomotor).

1.8.2 Genetic sleep deprivation

Another method for SD which has increased in popularity in recent years is that of genetic SD. This is performed either by thermogenetically activating or silencing specific sleep inducing regions in the fly brain [281], or optogenetically activating/silencing [365] for the same aim. This method is popular as it also allows temporal control of the activation or silencing of neurons. Thermogenetic modulation works by using the Gal4/UAS system [304] with temperature sensitive UAS constructs (see Methods for an overview of this system). Thermogenetic activation is achieved by expressing the thermosensitive ion channel, TRPA1, in target neurons [169]. Silencing works by using the same system but with the temperature-sensitive mutation *Shibire* [49], which blocks synaptic transmission. Optogenetic methods achieve this aim via the addition of a light-sensitive genetic construct [373].

This method of SD is highly effective, and leads to behavioural inactivity when key

neurons in wake-promoting regions of the brain are activated [279]. However, there are two problems with this method of SD. Firstly, an increase in temperature has been shown to have a significant effect on sleep and locomotion in flies [156]. Temperature works in combination with light to regulate the circadian variations in activity, as shown by Majercak et al. [290], as the circadian gene, *period*, is differentially spliced in response to low or high temperatures. When the temperature is raised, flies show an increase in daytime sleep and a decrease in night-time sleep [353]. The second possible problem with activation or silencing of neurons directly is that we do not understand the exact effect which is produced from this change. It is difficult to measure what effect the activation or silencing of neurons has on the fly. One study has suggested that chronic activation of the dorsal fan-shaped body (dFSB) sleep promoting neurons in the fly decreases behavioural responsiveness [466]. Many regions in the fly brain are interconnected and complex feedback mechanisms between neurons lead to changes in behaviour. Silencing or activating one of these regions may have complex effects that we currently cannot understand.

1.8.3 Drug-induced sleep deprivation

Many substances can be used to induce sleep loss in *Drosophila*. Many of these compounds which induce sleep loss in *Drosophila* are also known to induce sleep loss in mammals. One such compound is caffeine. Many studies have shown that caffeine reduces sleep in flies in a dose-dependent manner [274], [246], [496], [369].

In mammals it has been well established that caffeine induces its wake-promoting effects via the adenosine receptor [133],[488], specifically the inhibitory A1 and stimulatory A2A variants of the receptor [134]. However, in *Drosophila*, only one type of adenosine receptor has been shown to exist, and surprisingly, caffeine does not have its effect via

this receptor [496]. It was later shown that the effect of caffeine in flies is mediated instead by dopaminergic protocerebral anterior medial (PAM) neurons [329]. Nall et al. also showed that flies deficient for TH did not show a change in sleep in response to caffeine [329].

In a similar vein, feeding flies L-3,4-dihydroxyphenylalanine (L-DOPA), which increases dopamine in flies, can suppress sleep [408], [1], [329]. A reduction in night-time sleep was observed by Melnattur et al. after feeding CS flies L-DOPA (5mg/mL) [306]. Nall et al. observed that the effect of night-time sleep loss was further increased when L-DOPA and caffeine were fed simultaneously [329].

Another compound used to decrease sleep is Modafinil (2-[(Diphenylmethyl) sulfinyl] acetamide). Although the MoA of modafinil is still somewhat unclear, it has been shown to increase extracellular levels of serotonin, dopamine and noradrenaline in rats [86]. Originally, it was thought its mechanism was mediated by dopamine, but studies are somewhat conflicting on this opinion [128], [494],[4]. Despite this, it has been shown to have a significant sleep reducing effect in flies, which occurs in a dose-dependent manner and leads to a significant sleep rebound [186].

Other compounds which affect dopamine signalling are also potent inhibitors of *Drosophila* sleep. These compounds include amphetamine [236], which requires a functional dopamine transporter (DAT) to have a sleep suppressing effect [136]. Similar compounds, methamphetamine [8] and cocaine [263] also suppress sleep. Surprisingly, use of amphetamine can increase sleep in DAT mutants [236], suggesting there may be a secondary mechanism by which an effect can be seen.

In a screen by Nall and Seghal, [328], where over 1200 small molecule compounds were screened to identify those which had a significant effect on *Drosophila* sleep, four compounds, pergolide methanesulfonate, R(-)2,10,11-trihydroxyaporphine, paliperidone and

1,3-dipropyl-7-methylxanthine were identified as having a sleep suppressing effect.

1.8.4 Drugs which induce sleep in *Drosophila*

A variety of different drugs can also be used to induce sleep in flies. As an increase in dopamine has been shown to have the ability to decrease sleep in flies, conversely, feeding substances such as 3-iodo-tyrosine (3IY) or α-methyl-p-tyrosine methyl ester (AMPT), which cause a decrease in dopamine levels, lead to an increase in sleep amount in wild-type flies [364]. In the previously mentioned screen by Nall and Seghal [328], one compound was found to increase sleep. This compound was Reserpine, which inhibits the vesicular monoamine transporter (VMAT). This transporter is responsible for packaging all monoaminergic neurotransmitters into vesicles, and consequently, has a sleep inducing effect on mutants deficient for dopamine, serotonin, octopamine, histamine and GABA. This suggests its MoA occurs via a complex mechanism involving multiple neurotransmitters.

The last sleep inducing compound to be mentioned, which has been studied comprehensively in *Drosophila*, is Gaboxadol (or THIP). Gaboxadol increases sleep [233], [122] in flies and it is reported to have its effect via GABA-A receptors [101]. It has also been shown to reduce activity in *Drosophila* dopaminergic neurons [30].

1.9 Natural forms of sleep deprivation

1.9.1 Starvation-Induced Sleep Deprivation

A method which has been used to understand ethologically relevant affects of SD on *Drosophila* is starvation, a phenomenon which likely effects *Drosophila* in the wild.

Early studies looking at starvation in *Drosophila* found that starvation induces hyperactivity in adult flies [265]. The authors showed that flies lacking the adipokinetic hormone gene (*dAKH*), which mediates the use of energy stores upon starvation, did not show this hyperactive phenotype when starved and also displayed resistance to starvation-induced death. Further to this, it was previously established that sleep is significantly suppressed in both male and female flies in response to starvation [240]. This study also reported some other interesting observations: (1) Flies which were fed sucrose containing agarose medium did not suppress sleep (2) No significant difference in sleep was seen in the first 12 hours of starvation, only during ZT12-ZT24 (3) The circadian genes *cycle* and *Clock* are needed for robust suppression of sleep during starvation. He et al. [181] extended the idea that there is a circadian aspect to starvation-induced sleep loss by showing that in W1118 flies, starvation-induced sleep loss is mediated by the transition of light to darkness. They then showed that the circadian LNvs neurons are required for starvation-induced sleep loss [181]. A further study examined the role of two other genes, *brummer* and *Lipid Storage Droplet 2* as possible regulators of the starvation response in flies [460]. Despite Thigman et al. not seeing a sleep rebound following 12 hour starvation in either W1118, CS or flies mutant for the *Clock* gene, they found that flies with mutations for *cycle* and *brummer* showed a significant rebound response following 12 hours starvation. They also found that this response protected against learning impairments following sleep loss. Donlea et al. [104], implicated the *forgaging* gene as playing a role in starvation-induced SD. In this work, it was found that the levels of Protein Kinase G (PKG) vary in flies with different alleles of the *foraging* gene. The authors postulated that this demonstrated different survival strategies for flies with these different alleles, with flies that were awake during starvation having the opportunity to look for food, while those that slept conserved

energy stores. Brown et al. [53], implicated the insulin peptide, *Dilp-2* as a key regulator of starvation-induced changes to sleep. They found that wild-type starved flies had deeper night-time sleep than those flies with SD due to caffeine or mechanical SD and also exhibited no rebound, contrary to flies exposed to other types of SD. *Dilp-2* was found to be the regulator of this response. Flies mutant for, or with knocked-out, *Dilp-2* showed increased arousal thresholds during starvation and also exhibited a rebound following starvation.

Despite evidence here showing some genetic mutations can lead to resistance or increased susceptibility to starvation, Masek et al. [297] demonstrated that by outbreeding *Drosophila* and selecting for starvation-resistance, flies could be selectively bred to show the ability to survive under starved conditions for up to 18 days. This phenotype was accompanied by an increase in sleep and a decrease in feeding, suggesting that it is possible to genetically select for flies that prioritise conserving energy over foraging.

The role of Dopamine in relation to starvation and sleep

Up to this point, work investigating starvation-induced sleep suppression suggests that increased wake during starvation is a strategy to find a nutritious food source. However, Linford et al. [278] showed that the starvation-induced sleep suppression could be recovered when flies were fed a sweet, but non-nutritious food source. This phenotype was recapitulated when the sweet-sensing receptor, gustatory receptor-64 (Gr64), was knocked out. The authors went on to show that gustatory stimulation led to dopaminergic transmission. Blocking dopaminergic neurons (using tyrosine hydroxylase (TH)-Gal4) led to suppressed sleep and prevented flies from responding to glucose. These results were confirmed by Hasegawa et al. [175], who went further to show that non-nutritive sweeteners, which restored sleep, also decreased arousal threshold and

bout length.

This role for dopamine in starvation and sleep was supported by the work of French et al. [135], who showed that starved and fed flies have different arousal thresholds during sleep. They showed that flies exhibit different arousal responses to aversive, attractive or neutral compounds based on whether they are fed or starved. This phenomenon is regulated by a circuit which connects the olfactory neurons to the antennal lobe via the mushroom body and up to the PAM dopaminergic neurons. The studies by French et al. and Haegawa et al. implicate dopamine as a key mediator of the response to starvation-induced sleep suppression. PAM neurons are also modulated by γ-aminobutyric acid (GABA) signalling, which has been shown to be involved in the regulation of sleep [112]. This work shows that starvation-induced sleep loss may not solely be due to the effects of starvation per se, but a change in the levels of dopamine in the brain as a response to the lack of appetitive signals.

1.9.2 Social methods of sleep deprivation

The waking experience of flies has been shown to significantly change the amount of sleep which subsequently occurs. The first study to identify this change saw that in male flies, socially enriched individuals slept more than socially isolated counterparts [145]. The authors of this study also found that this change, which was dependent on visual and olfactory signals, was somewhat caused by dopamine. Isolated flies had a third less dopamine than social flies and silencing or ablating dopaminergic neurons abolished the increase in sleep in socially enriched flies. Further work confirmed another finding of this study. Flies with mutations in proteins involved in cyclic adenosine monophosphate (cAMP) signalling, with deficiencies in learning and memory, do not exhibit the increased sleep associated with social enrichment [107]. Donlea et al. linked

this process to a circuit in *Drosophila* which also regulates circadian rhythms in flies. The effect of social isolation, which leads to decreased sleep, has been confirmed by other sources [285], [518], who have also determined that genetic background has a significant effect on the social aspects relating to the modulation of sleep. Recently, it was shown that the decreased sleep in socially isolated individuals is accompanied by starvation signalling and increased feeding [271].

Based on this work, which clearly indicates that social experience increases sleep in flies, it is somewhat surprising to note that two SD methods have been developed based on social interactions. However, it should be noted that the effect of social interaction as a prior waking experience was measured in flies which had been isolated following these grouped scenarios. The first of these social SD methods is based on the interactions between two male flies. When two males are kept in a confined environment after an extended period of isolation, the males show aggressive behaviours towards each other, which leads to a significant SD response and subsequent rebound [155],[26], [289]. Sleep is also suppressed when a male is confined with a female fly. Here, rather than aggressive behaviours leading to SD, it is the drive of the male to court with the female which leads to SD [26]. Interestingly, the male-female interaction leads to greater sleep loss than the male-male interaction [289], although interacting males do demonstrate a "freezing" behaviour during confrontations, which is a behaviour easily confused with sleep or quiescence [26].

1.10 Sleep Rebound in *Drosophila*

As has already been discussed, sleep homeostasis is one of the key processes of sleep regulation, as defined by Borbey's model of sleep [40]. A pertinent feature of this

Table 1.1: Type of sleep modulating drugs and mechanisms

Drug	Effect	Mechanism
Caffeine	Wake-promoting [329]	Dopamine signalling via PAM neurons
L-DOPA	Wake-promoting [306]	Dopamine signalling (dopamine precursor)
Modafinil	Wake-promoting [186]	Unknown
Pergolide methanesulfonate	Wake-promoting [328]	Dopamine agonist
R(-)2,10,11-trihydroxyaporphine (TNPA)	Wake-promoting [328]	D2 dopamine receptor agonist
Paliperidone	Wake-promoting [328]	D2 dopamine receptor agonist and serotonin receptor agonist
1,3-dipropyl-7-methylxanthine	Wake-promoting [328]	Adenosine in mammals
3IY	Sleep-promoting [364]	Reduces dopamine signalling
AMPT	Sleep-promoting [364]	Reduces dopamine signalling
Reserpine	Sleep-promoting [328]	Inhibits VMAT
Gaboxadol (THIP)	Sleep-promoting [233]	GABA-A receptor modulator

Table 1.2: Type of SD and resulting sleep rebound: reports from the literature

Sleep Deprivation	Sleep Rebound Observed?	Mechanism
Dynamic SD	Yes [149],[135]	Unknown
Thermogenetic Activation	Yes [405]	Cholinergic neurons
Thermogenetic Activation	Reduced [405]	Dopaminergic neurons
Thermogenetic Activation	No [405]	Octopaminergic neurons
Starvation-induced SD	No [53]	*Dilp-2* signalling pathway
Starvation-induced SD	Yes [240]	
Caffeine-induced SD	Yes [53], [369]	Dopamine signalling
Male-Male-interaction induced SD	Yes [26]	Pheromone signalling (via *ppk*23) and P1 neurons
Male-Female-interaction induced SD	No [26]	Pheromone signalling (via *ppk*23) and P1 neurons
Mechanical SD with axenic flies	Reduced [419]	Gut microbiome
Mechanical SD with aged females	Reduced [477]	Dopamine signalling
Mechanical SD with *Pumillo* mutants (acute - 12h)	Yes [218]	Neuronal homeostasis
Mechanical SD with *Pumillo* mutants (chronic - 84h)	No [218]	Neuronal homeostasis
Mechanical SD with *sleepless* mutants	No/Reduced [247], [255]	Dopamine signalling
Mechanical SD with rhomboid kd	Reduced [132]	EGFR and ERK signalling
Mechanical SD with AANAT1 kd	Increased [84]	Serotonin and dopamine signalling in astrocytes
Mechanical SD with *bubblegum* mutants	Increased [459]	multiple possible pathways
Mechanical SD with *cueball* mutants	Reduced [459]	multiple possible pathways
Mechanical SD with *heimdall* kd	Reduced [459]	multiple possible pathways
Mechanical SD with *hugin* mutants	Increased [403]	*hugin* neurons
Mechanical SD with mir-190 kd	Reduced [158]	Various
Mechanical SD with mir-957,956,313,318,1014,955,281-1,308 kd	Increased [158]	Various

homeostatic process is one that characterises all homeostatic processes: if a homeostatic process is unbalanced, the system will work in order to rebalance that process [31]. This is the case also with sleep homeostasis. If wake persists past a certain threshold, the homeostat will use sleep as a way to correct this imbalance. In this scenario, if an organism experiences more time awake than usual and goes past the usual threshold of wakefulness normally experienced, recovery sleep or sleep rebound, may occur [481]. Early SD experiments in human subjects have shown that recovery sleep is a key feature demonstrated in subjects following prolonged SD [327], [461]. This recovery sleep was shown to have beneficial and restorative effects on cognitive aspects such as memory [39], [176] and mood [343].

In the past 20 years, studies have shown similar phenomena in *Drosophila melanogaster*. As mentioned previously, *Drosophila* show all the behavioural hallmarks of sleep, one of which is the ability to demonstrate sleep recovery in response to a prolonged period of SD [416],[185]. It was initially concluded, in a very early study on SD in flies, that sleep rebound was dependent on prior waking time [203]. Interestingly, most other studies have determined that this is not the case and that the amount of SD does not dictate the amount of recovery sleep needed [416]. Flies which are short sleepers or long sleepers can also exhibit similar sleep rebounds [409]. In fact, one study of chronic SD over 228 hours showed that the resulting rebound was similar to that seen in flies which had only experienced one night of SD [149].

Rebound sleep in *Drosophila* itself is not necessarily a puzzling phenomenon. It is understandable that severe loss of sleep will enforce the homeostatic mechanism to make up the deficit. The most intriguing work which has been done of late is in scenarios where sleep rebound after SD is abolished. Several examples of this exist. It has previously been shown [405] that thermogenetically activating neurons in the

Drosophila brain expressing the neurotransmitter acetylcholine (ACh) leads to a strong SD and robust sleep rebound. However, the authors also found that activation of dopaminergic neurons led to a greater decrease in sleep but much less sleep rebound than seen in flies with activated cholinergic neurons, which experienced a comparable amount of sleep loss. In a previously mentioned study, Brown et al. [53], showed that mated females have no rebound response when sleep deprived by starvation, but a robust rebound response following SD with a mechanical method or with caffeine. This loss of sleep rebound following starvation-induced SD was reversed when the insulin-like peptide, *Dilp-2*, was knocked-down or knocked out.

Other studies have looked specifically at environmental factors which may lead to a reduced or abolished SD. Beckwith et al. [26] demonstrated that males, which are sleep deprived by the presence of another male, had a robust sleep rebound. However, when the focal male was in the presence of a female, it negated sleep in preference of courtship, but did not show sleep rebound. Another study implicated the effects of the microbiome on SD and rebound. Silva et al. [419] studied the effect of SD on axenic flies (flies which are microbiologically sterile) compared to flies with a normal microbiome. They found the axenic flies displayed less sleep rebound compared to controls, regardless of the genetic background. Vienne et al. [477] found that mated females displayed less sleep rebound following mechanical SD at 35-days old compared to flies of the same genotype at 8-days old. The authors observed the same effect of age on rebound when using genetic SD (by activating wake-promoting dopaminergic cells).

Specific genes have also been linked to sleep rebound. One study [218] identified the gene *pumillo*; the protein encoded by this gene is a repressor of mRNA targets and controls neuronal development and neuronal excitability. The authors showed that in flies with knock-down of *pumillo*, sleep rebound was not demonstrated after chronic

(84-hour) mechanical SD. However, after acute SD for only 12 hours, sleep rebound did occur. In both cases, *pumillo* knock-down flies showed SD which was not significantly different to that of controls. Another gene which was identified as being involved in regulating sleep rebound was *sleepless* [247]. The protein product of *sleepless* works partially to upregulate *Shaker* potassium channels, which themselves enable sleep and suppress neuronal excitability [495]. Koh et al. [247] identified that in mutant flies with reduced levels of *sleepless*, sleep rebound was abolished following significant SD. This same phenotype, of abolished sleep rebound following mechanical SD, was also demonstrated in the previously mentioned *fumin* flies [255]. The *Drosophila* mutant, *fumin* (which means sleepless in Japanese) shows high levels of activity throughout the day. Koh et al. showed that the *fumin* activity phenotype arises from the loss of activity of the *Drosophila* dopamine transporter gene, *dDAT*. Another study implicated the EGFR signalling component, rhomboid, as a factor which can change sleep rebound in *Drosophila*. Flies with knockdown of rhomboid showed reduced sleep and reduced sleep rebound following mechanical SD [132]. A role in sleep homeostasis and rebound was also found for the enzyme arylalkylamine N-acetyltransferase 1 (AANAT1) [84], which is specifically expressed in astrocytes and some neurons in adult *Drosophila*, where it acetylates monoamines and inactivates them. The authors found that in flies with mutations in AANAT1, rebound sleep was associated with increases in serotonin and dopamine expression, and specifically, knockdown of AANAT1 in astrocytes limited the amount of rebound experienced [84].

There are also cases in *Drosophila* where flies have shown a significantly larger rebound than normally observed or show a rebound even when SD is not as severe as in other cases. For example, Thigman et al. [459], identified that flies with mutations in the genes *cueball* (*cue*) and *bubblegum* (*bgm*), display significantly more rebound than their

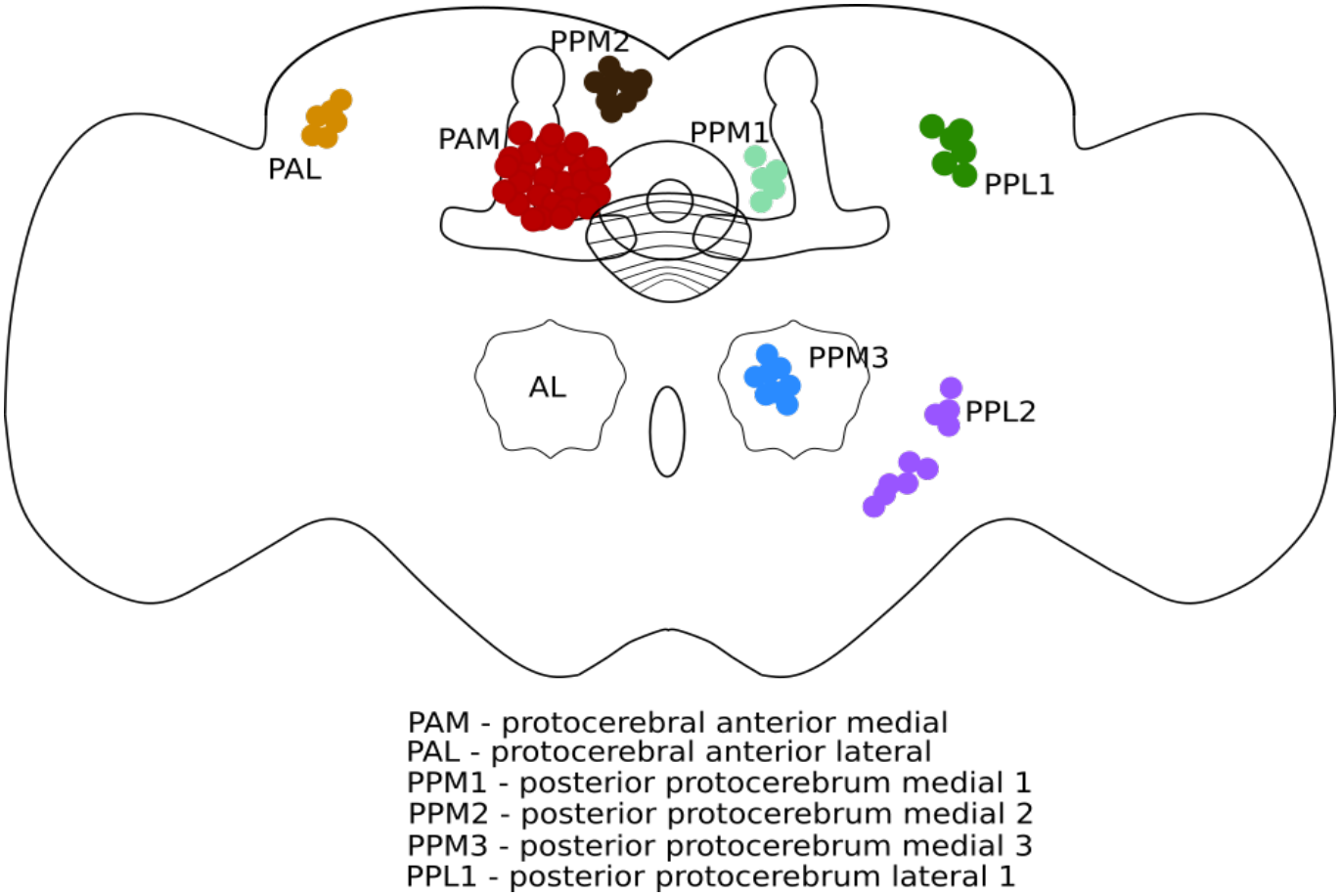


Figure 1.4: Dopaminergic neurons are located in various regions of the fly brain and include PAMs, PALs, PPL1,PPL2,PPM1,PPM2,PPM3 (names of these neurons arise from their locations in the brain).

genetic controls following mechanical SD. Another study showed that mutants lacking the neuropeptide *hugin* had increased rebound sleep following mechanical SD [403]. Finally, one paper [158], identified a role for microRNAs (miRNAs) in sleep rebound and sleep homeostasis. Goodwin et al. performed a genetic screen using a miRNA sponge (which causes a loss of specific miRNAs in transgenic flies) and identified nine miRNAs which affected sleep homeostasis. One of these, miR-190, was identified as being required for rebound and the other eight showed increased rebound compared when compared to the control.

1.11 Drosophila Neuropeptides and sleep

1.11.1 The effect of feeding neuropeptides and their role in sleep

Just as feeding is a behaviour in itself, it can also affect the movement and activity behaviour of the whole organism, as this is how the organism gains access to food. In *Drosophila*, the genetic basis of foraging and how this impacts activity has been widely studied. Early work in this field looked at foraging behaviour and discovered that larvae exhibited one of two behavioural strategies when feeding ("rover" and "sitters"), with these behaviours having a genetic basis to them [431]. It was only many years later that it was understood that the gene controlling foraging in larvae also had the same function in adult *Drosophila*. Behavioural characterisation of the gene, *foraging, (for)*, showed that this gene, found at a single locus, defines "sitter" and "rover" populations [358]. Sitter populations stop moving when they have found food and rovers continue foraging. This is true in both adults and larval populations [358]. It was later found the locus for this gene also contains the cyclic guanosine monophosphate (cGMP)-PKG gene, *dg2*; mutations in the *for* gene mapped in or near *dg2* led to the "rover" phenotype reverting to the "sitter" phenotype and a decrease in PKG levels [346]. This work was pivotal in demonstrating three different things. Firstly, that single genes can underlie and control specific behaviours, secondly that behavioural phenotypes in larvae can persist into adulthood, and thirdly that feeding can alter locomotor activity.

Since these studies examining the *for* gene, many other genes in *Drosophila* have been discovered to underlie feeding-related behaviours. Neuropeptide F (NPF), a homologue to mammalian neuropeptide Y (NPY) [54], which is responsible for vertebrate feeding regulation [168], was initially shown to modulate larval foraging behaviour and food

aversion. High expression of NPF leads to attraction to food, whereas low levels lead to food aversion [497]. Later work in adult flies showed sex-specific expression of NPF and that its expression was under circadian control. The expression of NPF is reliant on the function of the courtship regulating gene, *fruitless (fru)* and is expressed in the dorsal-lateral ventral neurons (LNds) [264]. This was supported by experiments further elucidating the sexually-dimorphic circuitry of NPF cells. These are connected to the P1 neurons, neurons which are regarded as a hub of courtship activity in male flies. The activation of P1 neurons works to increase the activity of NPF male-specific neurons and those of NPFR to suppress courtship drive [282]. However, some of the most interesting work on the diverse functions of NPF revolve around its ability to control feeding and activity. NPF has a role in regulating the internal state of hunger, with one study demonstrating that activation of NPF neurons increased appetitive memory in a food-related learning task, and knockdown of NPFR decreased this memory [250]. This process is modulated by connecting dopaminergic neurons. This study was also supported by later work that found a similar role for NPF and NPFR in feeding dependent memory formation [68].

The first paper to link NPF to sleep showed that an overexpression of NPF, and its receptor, led to an increase in sleep and an impaired homeostatic response, where flies overexpressing NPF showed a reduced rebound following SD [179]. This finding was later contradicted, as a different study showed activation of NPF and NPFR cells in the *Drosophila* brain reduces sleep [70]. NPF has also been implicated in regulating certain circadian processes. NPF was localised to the circadian fifth small-lateral ventral neuron (s-LNv) and the LNds, where it was shown to play a role in regulating the evening activity peak together with the Pigment Dispersing Factor Receptor (PDFR) [401], [262]. Another example of its circadian function is through the micro-RNA, MiR-

276a. It was shown that the loss of this micro-RNA increases sleep and was expressed in key sleep centres in the brain (MB and LNds) where it colocalises with the NPFR [512]. NPFR expression is increased in MiR-276a mutant flies [512]. It was also shown to regulate metabolic components in a circadian fashion in the fat-body [121].

Other more diverse roles of NPF include it's ability, and that of its receptor, to modulate alcohol sensitivity [486] and male aggression [99]. Surprisingly, NPF may also modulate pheromone reception. A paper which examined changes in male flies showed that exposure to male or female pheromones has opposing effects on starvation resistance [152], with fat stores and lifespan also being affected. The silencing of NPF neurons prevented these pheromone-induced changes [152]. This paper also noted that in males exposed to female pheromones, NPF expression was increased [152]. A recent paper may have shed light on how NPF is regulated in relation to feeding. Two crustacean cardioactive peptide (CCAP) neurons in the *Drosophila* brain were shown to act in a NPF suppressing way to regulate feeding and metabolism. Specifically, sugar sensitivity and triglyceride levels, demonstrating how NPF is involved in many feeding mechanisms [492].

One of the reasons for the surprisingly varied roles of NPF may stem from its wide and diverse pattern of expression in the *Drosophila* brain. It is well documented, that NPF is expressed in many areas of the brain such as the FSB (multiple layers), the lobes of the MB, the subsopheagal ganglion (SOG)[232],[250], male-specific neurons (dorso-lateral to the AL) [282] and specific circadian neurons [401],[512].

Short-Neuropeptide F and its receptor

The sequencing of the *Drosophila* genome led to the later discovery of short neuropeptide F (sNPF), which also plays a role in larval feeding and growth [267]. This neu-

ropeptide has been shown to also be important in regulating sleep and activity along with NPF.

sNPF was initially thought to be located in small populations of LNds and s-LNvs [221] and in most parts of the MB [220], with the possibility that they receive input via dopaminergic inputs. Further work has localised sNPF expression to six layers of the FSB (1,2,4,5,6,7) [232],[231] and DL neurons (with sNPFR expressed in IPCs) [235].

Loss of sNPF and its receptor has been shown to lead to an increase in total sleep amount, both during the day, the night and in both sexes [64]. It was also shown to play a role in sleep homeostasis. Sleep deprived flies have increased expression of sNPF following SD [64]. This was supported by evidence showing activation of sNPF neurons increases sleep (release from this activation leads to an immediate negative sleep rebound) and silencing of these neurons leads to a decrease in sleep [414]. The activation-led sleep increase was further shown to act via the circadian s-LNvs and l-LNvs, supporting the previous work showing the co-localisation of SNPF to circadian neurons [414]. Optogenetic activation of these neurons has been shown to also increase sleep amount. Juneau et al. showed that activating these neurons, even for a very short amount of time, leads to an increased sleep amount lasting for much longer than the activation period itself and decreased locomotion [226].

Despite a multitude of work looking into the functions of NPF and sNPF, it is currently not known how they may interact to regulate feeding or how a balance may be struck between whether to feed or whether to sleep.

Drosophila Insulin-Like Peptides

In the *Drosophila* brain, a variety of different centres have been linked to aspects of feeding behaviour. For the process of hunger sensing, insulin-like peptides (DILPs) are

produced in a range of cell types in *Drosophila*, including from the insulin producing cells (IPCs) in the brain. To date, seven DILPs have been identified in *Drosophila melanogaster*, and these are also expressed in 12 other *Drosophila* species, with varying degrees of homology between these peptides [164]. The IPCs are located in the pars intercerebralis, specifically in the median neurosecretary cells (mNSCs) and release only three of the seven DILPs: DILP2, DILP3 and DILP5 [32]. Ablation of these neurons leads to increased lifespan, resistance to oxidative stress and starvation stress, and reduced fecundity [50]. Mutations for DILP2 and DILP5 lead to increased lifespan [368]. These DILPs function through the sole insulin receptor found in *Drosophila*, InR, to regulate glucose and glycogen metabolism [368]. The increased lifespan seen when DILP signalling is reduced or the IPCs are ablated is mimicked when the InR or its substrate, *chico*, is mutated [206]. This is mediated by the forkhead transcription factor, dFOXO [206]. It is no surprise that DILP expression is also necessary for growth regulation in *Drosophila* [511],[12] [424]. Other roles for the DILPs have also been explored in *Drosophila*. For example, it has been shown that mutations for all DILPs in *Drosophila* lead to reduced sleep, and upregulation of DILP2 increases sleep [75]. The authors of this study also found that DILP2 was expressed in the circadian LNvs.

Allatostatins: feeding and sleep

Another neuropeptide which has been implicated in the regulation of feeding and sleep is Allatostatin A (AstA). This neuropeptide belongs to a group of insect neuropeptides called the Allatoregulatory peptides. These can be classified into two categories: the allatostatins (A,B and C), which have inhibitory effects on Juvenile Hormone (JH) synthesis and the allatotropins, which have a stimulatory effect on JH synthe-

sis [17]. In *Drosophila*, only allatostatins and not allatotropins have been identified [331]. The most thoroughly researched of each allatostatin in flies is AstA, and within *Drosophila melanogaster* it has been shown that the AstA precursor produces four peptides, AstA1–4, which are expressed in the CNS [331].

In one of the first studies to examine the role of AstA in *Drosophila* behaviour [189], it was identified that activation of AstA neurons leads to a reduction in feeding in starved flies, although the authors found that there was no observed increase in the energy stores of flies with activated AstA neurons. Work following this showed that AstA regulates the expression of key metabolic genes, such as AKH, Dilp2 and Dilp3, and that mutants for AstA have increased accumulation of lipids in the fat body [188]. The authors also showed that mutants for AstA show increased sucrose intake, and that knockdown of the AstA receptor, *Dar-2*, leads to increased starvation-resistance [188].

Chen et al. [63], were the first to implicate AstA as having a role in sleep regulation. The authors demonstrated that thermogenetic activation of AstA neurons led to decreased locomotion and, furthermore, showed that when starved, flies with activated AstA do not show differences in sleep significantly different to that of fed controls [63]. Further contributions to how AstA regulates sleep has come from Donlea et al. [106]. In their work, it was shown that knockdown or mutant flies for AstA have reduced sleep and reduced sleep rebound following 12 hours of mechanical SD. Ultimately, the sleep regulatory effect was mediated by AstA-R1 expressing helicon cells, which connect to both the FSB and EB, and activation of which can modulate the sleep-wake response [106].

Taken together, this work has contributed to the evidence that AstA neurons regulate feeding responses in *Drosophila* and also implicates these neurons in playing an impor-

tant role in sleep regulation.

More recently, one study has implicated another allatostatin, Allatostatin C (AstC), in the regulation of feeding. Lin et al. [275] showed that in male flies, the Diuretic hormone-31 (Dh31), mediates the switch in behaviour between courtship and feeding (where male flies preferentially choose to feed rather than court, if starved). Knockdown of the receptor for Dh31, Dh31-R, in corazonin expressing neurons promotes courtship and knockdown in AstC expressing neurons promotes feeding, suggesting that AstC may also play an important role in the regulation of feeding in *Drosophila*.

1.11.2 Corazonin and its receptor: A stress neuropeptide

One neuropeptide which so far has not been implicated in sleep, but has been implicated in feeding, is corazonin (Crz). Crz is the invertebrate analogue of human gonadotropin-releasing hormone (GnRH) and it shares a high-degree of sequence similarity to adipokinetic hormone (AKH) [395]. It was initially discovered in the cardiac tissue of cockroaches and named Crz because the Spanish word for heart is "corazon" [476]. Although Crz is known to be present in most arthropods, except for aphids and beetles [37], it has been shown to play diverse roles. It is functionally implicated in processes such as social behaviour and caste identity in ants [159], body pigmentation in locusts [439] and development and ecdysis in various species [243], [499].

In *Drosophila melanogaster*, Crz and the corazonin receptor, (CrzR) have been shown to be involved in many different processes. The first role it was implicated in was the regulation stress responses in flies and several studies have now looked at the effect of stress in association with Crz and CrzR. For example, one of the first of these studies showed that ablating Crz neurons led to extended life-span under starvation conditions, osmotic stress, high salt-diet and oxidative stress [514]. The same authors

reported that silencing these neurons with UAS-ORK led to increased locomotor activity in males with these flies having higher levels of dopamine expression [514]. This work was supported by Kapan et al. [235] who showed that Crz is co-expressed with sNPF in the sexually dimorphic DL neurons in the *Drosophila* brain. Knockdown of Crz in these DL neurons led to increased survival when the flies were starved [235]. Both Zhao et al. and Kapan et al. showed increased glycogen and trehalose levels when Crz was knocked down or silenced [514], [235].

Another study went further to show that flies with knockdown of CrzR in the fat-body, salivary gland and various other areas led to increased survival upon starvation, oxidative and dessication stressors [254].

Two papers have implicated Crz in the process of courtship and mating in males. The first by Tayler et al. [456] documented how silencing Crz or CrzR neurons in the abdominal ganglion (AG) led to the inability of the male fly to undergo sperm or seminal fluid transfer (SSFT). The activation of these neurons did the opposite, and led to males ejaculating within 1 minute of the onset of thermogenetic activation. Tayler et al. also showed that CrzR AG neurons respond to serotonin and serotonin injection could also induce ejaculation [456].

The second of these studies by Zer-Krispil et al. [510] looked at whether the induction of ejaculation was rewarding in males. They expressed a red-light sensitive channelrhodopsin in Crz neurons and found that flies preferentially moved to red-light areas, where activation Crz neurons could occur. This study also found that males with increased activity in Crz neurons, or wild-type flies that were mated, had increased NPF levels.

A role for ethanol regulation was found to involve Crz and CrzR in *Drosophila*. Knockout or knockdown of CrzR or Crz led to a delayed recovery from ethanol sedation and

CrzR mutants show increased alcohol dehydrogenase activity [410].

Similarly to other insects, Crz has a role in ecdysis in *Drosophila*. Crz neurons are connected to both prothoracic gland (PG) cells and prothoracicotropic hormone (PPTH) neurons [210]. PTTH producing neurons innervate PG neurons and regulate the production of ecdysone, which regulates larval and pupal development [302]. Inhibition of Crz neuronal activity causes an increase in pupal size, due to its critical interaction with PTTH at the L3 stage of larval development [210].

Interestingly, Crz is also expressed in neurons in the fly brain which express the sugar-sensing receptor, *Gr43a*, [311]. As it is known that reward pathways that regulate feeding and mating often overlap [66], this could explain some of the diversity in roles implicated in Crz and CrzR function.

1.11.3 Post mating responses and sleep

In female *Drosophila*, seminal fluid protein (SP) detection by seminal fluid protein receptors (SPR) found in seminal fluid sensory neurons (SPSN) are key to inducing the changes in biology which lead the female to be mated and induce behavioural changes which accompany this [253]. A decrease in sleep behaviour is just one of the changes which has been observed following mating [211],[147] [149].

Garbe et al. [148] showed that it is the specific transfer of SP acting via SPRs which leads to this change in sleep. Females mated to males without SP have decreased expression of SPR in SPSN and are, therefore, unable to decrease sleep. However, Garbe et al. showed that silencing the SPSN leads to the exhibition of post mating behavioural changes, including a decrease in sleep [148].

Intriguingly, only one paper has investigated the impact of SD on female flies [369]. The authors showed that SD negatively affects females. Exposure of wild-type flies

to caffeine or mechanical SD reduced fecundity and female *fumin* mutants, which are associated with increased dopamine and show reduced sleep, had reduced egg laying output [369].

1.12 Insecticides and Activity

1.12.1 Phenotypic Screening: The need for classifying behavioural phenotypes

Phenotypic screens using high-throughput methods are a common approach for those working in drug discovery; many compounds can be tested on a cell, organism or even *in silico* to determine whether a compound may have the desired behavioural effect on its target [312]. However, when it comes to understanding sleep and activity, very few phenotypic screens have been developed to assess behaviour for purposes outside traditional pharmacology. Insecticide compounds are developed in a similar fashion to those in pharmacological drug discovery: compounds are synthesised or extracted from organic sources, and tested via various assays to understand whether they elicit desired behaviours. Behavioural assays may be used to look for whether a compound prevents insect feeding, paralyses the target organism or death. These assays are also used to help build a hypothesis as to what the MoA of a compound is, and further investigations using electrophysiological, genetic or proteomic approaches can follow behavioural assays to probe further into the mechanism by which the compound acts. The second part of this thesis contains work relating to an industrial collaboration with the agricultural company, Syngenta. Specifically, the collaboration was with a team investigating behavioural phenotypes resulting from insecticide exposure using

Drosophla melanogaster. Researchers at Syngenta apply compounds to the surface of a food medium distributed in 24-well plates and then expose groups of *Drosophila* males to the compound surface, visually monitoring and recording effects which result over subsequent hours following absorption and ingestion of the compound from the plate. This method yields information about survival length after exposure, allows recording of observable behaviours and informs about the physical state of the fly over time. Despite aforementioned pros, several central questions cannot be addressed using this method, leaving room for methodological improvements.

More specifically, the following shortcomings of this traditional phenotyping method were identified. Firstly, periodical examination of plates by a researcher means data at intermediate time points was lost. Secondly, behaviours which were recorded using this method were assigned meaning by the researchers themselves, that is to say, only behaviours which were sufficiently obvious to the examiner and presented meaning were documented. Hence, some subtle behavioural changes were liable to be missed or assigned a meaning which may be false. Lastly, this method does not allow for quantitative comparisons to be made on a large scale.

1.12.2 Using *Drosophila melanogaster* as a Model Organism for Insecticide Research

Drosophila melanogaster is not widely recognised as being a pest species, but despite this, it has been a powerful model organism for insecticidal research.

Extensive research has allowed the entire *Drosophila* genome to be one of the first to be completely sequenced [62]. Using this information scientists have developed a plethora of genetic tools for use with flies, with the easy manipulation of its genome to be counted

amongst many other benefits, such as a short development time and simplicity in its maintenance and rearing.

Due to these genetic tools and the genetic similarity of *Drosophila* to other insect pests [402], *Drosophila* has been used to map different modes of resistance to insecticides, both genetically and mechanistically [81], [229], [25], [361]. For example, a gene that confers resistance to Dieldrin and other cyclodienes was first discovered in *Drosophila*, and named accordingly *Resistance to dieldrin (Rdl)* [129]. Further work following this found that the same gene confers resistance to these compounds in other insects [379]. *Drosophila* have also been used to identify the modes of action of many different compounds. For example, the MoA of Pymetrozine was uncovered in a study using *Drosophila* which identified TRP receptors in chordotonal neurons as being the targets of its action [337].

Both these and many other examples [363], [109], [81], [229] show that *Drosophila melanogaster* is a powerful model organism for use in the study and discovery of insecticides, their targets and potential resistance genes.

1.12.3 The Use of Insecticides

It is estimated that there are between 2.5 million [316] and 5.5 million [437] insect species present in the world, but it is probable that this is a gross underestimation. Of these species, we can define a subcategory of insect pest species: ones which cause many types of monetary, social, environmental and healthcare costs. These insect pest species can themselves be put into two categories, native pest species and invasive pest species. Native pest species are those which are indigenous to the regions in which they cause harm and invasive pest species are those which are introduced to a region, either by human intervention, or increasingly, due to changes in geographical temperatures

and weather conditions as a result of climate change [196].

Many of the insect species targeted by insecticides are invasive and cause many billions of dollars worth of damage. A study from 2016 conservatively estimated that the cost incurred by invasive insect species to be around US$70.0billion globally per annum, with an extra US$6.9billion in costs associated to health [45]. However, it was noted that this was likely to be a underestimate due to incomplete data and many costs from invasive insect species being indirect. Therefore, it is possible this global cost is much higher [341]. These costs fall into four main categories: (1) economic costs, with damage to agricultural crops and livestock (2) human health, via parasitic insects that transmit or cause disease (3) ecological, where an invasive insect leads to the extinction of a native species (4) environmental, where the pest causes a change in the ecosystem [305],[45]. These costs related to invasive insect species are difficult to determine due the fact that different world regions experience different pests. However, many studies have attempted to quantify the economic costs of invasive pest/ insect species by location based on data from specific geographical regions and considering a variety of different factors [351],[95], [45]. The main economic costs detailed by these studies and the InvaCost database, are defined as primary crop damage and its effects e.g. having to mitigate the loss of crops via pest management, the breeding of new crops and substitutions and imports to cover losses. In a study of all data on invasive species (compiled in the InvaCost database [96]), it was found that invasive invertebrates were the most costly of all pest species, and that low income countries and those which rely most heavily on agriculture are most at risk of economic burden due to invasive invertebrates [96].

Some invasive insect species can cause costs and damage in more than one of these areas outlined above. For example, the red imported fire ant (*Solenopsis invicta*) causes

enormous damage both to crop species and environmentally to indigenous vertebrate and invertebrate species [44].

Insecticides are one method to tackle the costs and problems caused by insect pests, and for the aforementioned reasons, the use of insecticides remains a key strategy of both farmers and governments around the world.

1.13 Insecticide Compounds and Classes

For this study, more 39 insecticide compounds were tested in total. A set of 11 compounds were initially randomly chosen by Syngenta and included compounds which had similar MoAs, such as those that act on the ryanodine receptor, and also included those which had unique MoAs. These compounds were selected for the initial proof-of-concept. Further to this, a larger panel of 28 compounds were randomly chosen. I will give a brief overview of each of the targets of these compounds used, their mechanisms of action (where known), and brief overviews of their usage.

1.14 Overview of Insecticide Targets

Table 1.3: Complete list of insecticide compounds tested and their targets

Insecticide Name	Target	Reference
Abamectin	Glutamate-gated chloride channels	[79] , [11]
Diamide Syn Cpd	Ryanodine receptors	Syngenta in house data.
Chlorantraniliprole	Ryanodine receptors	[76]
Cyantraniliprole	Ryanodine receptors	[76]
Dieldrin	GABA receptors	[386]
Flubendiamide	Ryanodine receptors	[296]
Imidacloprid	nACh-R	[36], [362]
Pymetrozine	TRP channels	[337]
Ryanodine	Ryanodine receptors	[131]
Spiroindoline	Cholinergic transmission	[425]
Tetramethrin	Sodium channels	[500]
DDT	Voltage-Gated sodium channels	[340]
Lufenuron	Chitin synthesis	[493]
Flonicamid	Voltage-gated sodium Channels	[457]
Permethrin	Voltage-gated sodium Channels	[470]
Syngenta Cpd 1	$GABA_A$ receptors	Syngenta in house data.
Juglone	R.O.S	[291]
Diflubenzuron	Chitin synthesis	[73], [308]
Rotenone	Complex I inhibitor	[352]
Methotrexate	Dihydrofolate receptor	[436]
Diafentiuron	Mitochondrial ATPase	[391]
Fenoxycarb	Juvenile hormone	[163]
Diafenthiuron Carbodimide (DCCD)	Mitochondrial FIFO ATP synthase	[390]
Chlorfenapyr	Mitochondrial oxidative phosphorylation	[33]
Thiamethoxam	nACh-R	[490]
Tebufenozide	Ecdysone receptor	[381]
Syngenta Cpd 2	nACh-R	Syngenta in house data.
Quinuclidine hydrochloride	Muscarinic ACh-R	[98]
Indoxacarb	Voltage-gated sodium channels	[259]
Fluazinam	Mitochondrial ATP synthesis	[266]
Syngenta Cpd 3	Mitochondrial ATP synthesis	Syngenta in house data.
Syngenta Cpd 5	Unknown	N/A
Syngenta Cpd 7	Octopamine receptor	Syngenta in house data.
Methoprene	Juvenile hormone	[219]
Tubulin	Binds tubulin	Syngetna in house data.
Metaflumizone	Voltage-gated sodium channels	[396]
Cyenopyrafen	Succinate dehydrogenase	[326], [484]
Syngenta Cpd 10	Octopamine receptor	Syngenta in house data.
Syngenta Cpd 12	Unknown	Syngenta in house data.

1.14.1 Chloride Channel Modulators

A selection of compounds tested in this study act on chloride channels. Chloride channels, like other ion channels, can be of two types, voltage-gated or ligand-gated.

One compound, which targets ligand-gated chloride channels, and that has been studied in detail is Abermectin. Abamectin belongs to the class of insecticides called called avermectins which were first discovered in the late 1970s as compounds naturally produced by the fermentation in the gram-positive bacteria, *Streptomyces avermitilis*, found in Japanese soil [56]. The avermectins consist of four major (A_1a, A_2a, B_1a, B_2a) and four minor (A_1b, A_2b, B_1b, B_2b) (commonly referred to as A_1, A_2, B_1, B_2) components which designate the mixtures of homologous pairs, with the majority of the complex being a major component and a minority being the minor component [260]. Of these components, it is the B_1 which forms Abamectin, initially used as an anti-parasitic agent against nematodes [118]. There are also two other notable compounds in this class (but not tested in this work): Emamectin, which is also used as a pesticide, and Ivermectin, which is used in veterinary science and human health [118].

Initial studies showed that Ivermectin and Abamectin increased GABA-evoked changes of membrane conductance, membrane hyperpolarisation and generally acted as a GABA agonist on somatic cells taken from worm muscle tissue [197], [113]. Therefore, it was first thought that GABA-gated chloride channels were their target site. However, later studies produced evidence to show that mRNA taken from the worm *Caenorhabditis elegans* (*C.elegans*), which was injected into *Xenopus* oocytes, led to the expression of glutamate-gated chloride channels, which responded to avermectins [11]. This discovery was later confirmed by electrophysiological recordings of these channels, which were completely blocked by the application of Ivermectin [10]. *In vivo* studies in *C.elegans* [90] and *Drosophila* [234] showed that mutations in glutamate-gated chloride chan-

nels led to resistance to Ivermectin. This was further supported by *in vivo* data, which showed mutations in the glutamate-gated chloride channel in the diamond-backed moth, *Plutella xylostella*, led to resistance to Abamectin [483]. This evidence points to glutamate-gated chloride channels being the main site of action of avermectins.

Abamectin is commonly used against a variety of pests, including mites, miners and suckers [269] and is specifically documented for effective use against cockroaches, mites, leaf-miners, psyllidae and various lepidoptera [260]. Sub-lethal effects have been reported in the Oriental Fruit Moth, *Grapholita molesta*, following diet incorporation [438], and these effects included shortened adult longevity, reduced fecundity and prolonged duration of growth stages in larval and pupal stages. Sub-lethal activity in spider mites, *Tetranychus urticae*, has also been reported, with one study, [213], reporting reduced female fecundity and death of offspring.

Other compounds with this target are Aldrin and its metabolised product, Dieldrin. These are both organochloride pesticides which were developed for use in the 1950s and used widely worldwide until the 1970s, when they were banned, or in some countries restricted agriculturally due to concerns around public health [345]. Under normal environmental conditions or inside the body, Aldrin decays rapidly into Dieldrin [157].

In a similar mechanism to picrotoxin, Dieldrin has been shown to act on the $GABA_A$ receptors, [207]. Similar to picrotoxin, Dieldrin inhibits $GABA_A$ receptor-mediated chloride influx, leading to increased excitation and facilitated neurotransmitter release, resulting in excitotoxicity [300] [146], [34].

Resistance to Dieldrin was reported as early as 1955, where it was shown that a strain of wild mosquito had developed resistance [378]. This mutation has now been identified in many different insect species[501], [170].

A specific mutation conveying resistance to Dieldrin (Rdl) in field populations was

identified in the early 1990s, which confirmed the target of Dieldrin as being $GABA_A$ receptors [386]. It is thought that this point mutation may account for 60% of the cases of insecticide resistance, as this mutation lead to resistance to many different receptors in the cyclodiene class [501], [386].

1.14.2 Ryanodine Receptor Modulators

The contraction of muscle is regulated primarily via the movement of calcium ions (Ca^{2+}) over the sarcoplasmic reticulum. The ryanodine receptor is the release channel found within this membrane, the mechanism of which was first discovered in 1985 by Fleischer et al. [131]. Since the identification of this mechanism in skeletal muscle, ryanodine receptors have been found in various different cells where they modulate Ca^{2+} signalling [130]. These receptors were first named as such due to their affinity to the natural insecticide and alkaloid compound, Ryanodine. Ryanodine acts on these receptors to prevent Ca^{2+} release, leading to paralysis in target insects [130], [258]. Chlorantraniliprole is in the diamide class of insecticides, which all act on ryanodine receptors. The development of Chlorantraniliprole was spurred on by the need for highly effective synthetic versions of Ryanodine with a similar MoA. Chlorantraniliprole was categorised as belonging to the class of anthranilic diamides, which bind to a chemically different site on ryanodine receptors but lead to similar behaviours upon exposure [76]. However, the MoA of Chlorantraniliprole is significantly different to that of Ryanodine. Ryanodine acts on these receptors to cause Ca^{2+} release, leading to paralysis in target insects. Where Ryanodine prevents the closure of Ca^{2+} channels after they have already been activated, Chlorantraniliprole directly activates ryanodine receptors, opening them fully or partially to deplete Ca^{2+} [406]. As with the natural alkaloid Ryanodine, Chlorantraniliprole leads to excessive release of Ca^{2+} from muscles causing

paralysis, stopping feeding and eventually resulting in death [283]. Specific pests it has been shown to be effective against include European Corn Borers, *Ostrinia nubilalis* [154], cabbage worm, *Pieris rapae* [283], the fall armyworm, *Spodoptera frugiperda* [92], cutworm, *Agrotis ipsilon* [180] and many others.

In a continued effort to produce more anthranilic diamides, there was a later synthesis of the compound Cyantraniliprole, which shares the anthranilic chemical core of Chlorantraniliprole but has a nitrile substitution, leading to a different structure but the same MoA [406]. Cyantraniliprole has been used around the world to control insect pests, with symptoms resulting from exposure correlating to those seen from Chlorantraniliprole exposure: muscle contraction leading to paralysis, followed by death [344].

Flubendiamide is another compound which is known to target ryanodine receptors. Flubendiamide was the first synthetic diamide to be produced, and was classed as a phthalic acid diamide, based on its new chemical structure [464]. This compound binds to a site distinctly different to that of Ryanodine but shows a very similar MoA. Upon binding of Flubendiamide to the ryanodine receptor, the receptor is stabilised to an open state, which causes intracellular Ca^{2+} release [114], [296].

1.14.3 Voltage-Gated Sodium Channel Modulators

Voltage-gated sodium channels are transmembrane proteins, which open following a change in membrane potential across the cell, and in response allow the movement of sodium ions along an electrochemical gradient [85]. Different insecticide compounds can have a variety of different effects on voltage-gated sodium channels.

Tetramethrin is one compound which has been shown to act on sodium channels [330]. Tetramethrin is in the pyrethroid insecticide class of compounds. Pyrethroids are

derivatives of pyrethrins, compounds found in the flowers of *Chrysanthemum cinerariae folium* [332]. Natural pyrethrins are ideal insecticides, as they have low toxicity in mammals and work well as insecticides. However, they are photo-chemically unstable [119]. To tackle this problem, synthetic pyrethroids were developed.

These synthetic pyrethroids can be divided into three classes based on structure and toxicity: Type I (non-cyano) pyrethroids induce repetitive firing in nerve cells after a single sensory stimulus and induce symptoms such as hyperactivity, uncoordinated movement and paralysis [143]. Type II pyrethroids do not induce repetitive firing and cause symptoms such as convulsions [143]. Type III pyrethroids, usually just referred to as non-ester pyrethroids [400], also work on voltage-gated sodium channels but are much less prominent [429]. Tetramethrin is a type I pyrethroid, and pyrethroids in this class have been shown to extend sodium currents by holding the sodium channels in an open state, prolonging the action potential and depleting intracellular energy sources required to extrude Na^+ ions [288], [500]. Type II pyrethroids have a slightly different MoA, causing tonic release of neurotransmitter from ion channels rather than the repetitive release seen with type I pyrethroids [48], [371]. A small amount of work has shown that type II, but not type I pyrethroids, may also have an effect on voltage-gated chloride and $GABA_A$ activated chloride channels [144]. Compounds other than Tetramethrin which are also in the class of type I pyrethroids include Allethrin and Permethrin, and those which are type II pyrethroids include Deltamethrin, Cypermethrin and Fenvalerate [377]. Pyrethroids have been used effectively against beetles, leaf miners and, in particular, lepidoptera [377].

Dichlorodiphenyltrichloroethane (DDT) is another insecticide which works on sodium channels, but is not a pyrethroid. DDT was actually first synthesised in 1874 [509], but its properties as an insecticide were not discovered until many years later. Insecticidal

and public health uses for DDT were discovered just before the outbreak of the Second World War, and it was popularly used as a tool by the military to combat vector-borne diseases such as malaria, typhoid and dengue fever as well as being used as an agricultural insecticide [309]. In animals, toxicological symptoms of DDT include abnormal motor responses and spontaneous movements, a tremor and cessation of feeding [427]. DDT has been shown to have its neurotoxic effect by modifying sodium channel gating, preventing the closing of sodium channels and prolonging the action potential [478], [340].

Although DDT is not particularly toxic to humans or animals, its persistence in the environment has led to a sharp decline in its use [77]. However, in some countries, it is still used against vector borne diseases, such as malaria [473].

1.14.4 Acetylcholinesterase and Acetylcholine Receptor Modulators

ACh receptors come in two forms, nicotinic (nACh) and muscarinic (mACh). The difference between the two being that nicotinic are ligand-gated ion channels, and responsive to the agonist nicotine. Muscarinic receptors work via second messenger cascades and are responsive to muscarine [60]. These two receptors are the targets of several different insecticide classes, along with compounds that target the hydrolysing enzyme of the neurotransmitter ACh, acetylcholinesterase [433].

One famous class of insecticides, which have an effect by acting as agonists of nACh receptors (nAChR), are neonicotinoids [421]. This class includes compounds such as Imidacloprid and Thiamethozam. These compounds were the most widely used insecticides in the world, with low mammalian toxicity and a strong effect against sucking

pests such as aphids, leafhoppers and fleas [465],[217]. Recently, concerns around the use of these compounds have become more widespread in light of research which has shown that they can have an extremely damaging impact on non-target insects and pollinators [94],[295],[451].

Neonicotinoids have faced growing problems of resistance from many of the pests they were designed to target due to their intensive use [237], [24]. Another mechanism by which insects are now becoming immune to the effects of these compounds is via increased metabolism of these compounds, for example, by detoxification enzymes [208], [388].

A compound which works on the nAChR, but with a distinctly different mechanism to neonicotinoids, is Cartap. This compound is a synthetic derivative of the naturally occurring compound Nereistoxin and was first synthesised in the late 1960s [167]. Cartap acts as an open channel blocker at the nAChR [323], acting directly on the receptor [268]. This compound was used to control chewing and sucking pests by preventing feeding and leading to death [268]. Cartap is still used commercially for agriculture in many countries, particularly in Asia, [195], [29].

Spinosyns are another group of compounds which are also known to act on nAChRs, albeit, they have distinctly different binding to the neonicotinoids [398]. Spinosad is one such insecticide in this class and is a mixture of two naturally occurring compounds, Spinosyn A and Spinosyn D, produced by the soil bacteria *Saccharopolyspora spinosa* [397]. Spinosyns are highly effective insecticides and have been credited with having efficacy against a broad range of insect pests, with low mammalian toxicity and low persistence in the environment [111]. Spinosyns have also faced problems with their insect targets becoming resistant, such as the diamond-backed moth, housefly and tobacco budworm [418], [513],[387].

Compounds can also have their effect by disrupting the function of the enzyme, acetylcholinesterase. Aldicarb is one such compound, and is a potent inhibitor of acetylcholinesterase at synapses and neuromuscular junctions, leading to loss of coordination, seizures and death in affected insects [23]. Aldicarb has been used worldwide on a number of insect, nematode and mite pests but also has high-mammalian toxicity [22]. Naturally occurring resistance to Aldicarb has been reported in the Cotton Aphid *Aphis gossypii* [349] and laboratory studies have reported it in *C.elegans* [310].

Compounds which are agonists of the mACh receptors (mAChR) can also be used as insecticides, for example, Quinclidine hydrochloride [98]. This compound was one of several insecticides acting on mAChRs examined by Dick et al. [98], and these authors showed that sucking pests are particularly susceptible to the effects of these compounds. Despite this, these compounds have not been commercialised or used to the same extent as compounds affecting the nAChR.

A relatively new class of insecticide compounds, which have their insecticidal effect via the vesicular ACh transporter, are Spiroindolines [425]. These compounds have a novel MoA, and due to their recent discovery, there is very little literature available on insect responses to their use. However, data from the initial report documenting the use of a compound from this class in *C.elegans* showed that worms responded with uncoordinated locomotion and disrupted feeding when exposed [425].

1.14.5 Other Targets of Insecticides

Growth Regulator Disruptors

Compounds which disrupt the normal cycle of growth in insects can also be used as insecticides. In general, those of interest to this report are of two types: those that

mimic Juvenile hormone and those which inhibit chitin biosynthesis. Benefits of these compounds are that they can be quite specific to certain insect groups. However, they are slower acting than traditional neuro-modulatory compounds [334].

Various types of Juvenile hormones exist in insects, and these hormones act to regulate the timing of larval moulting, metamorphosis and reproduction [219]. Compounds which act via this mechanism include Fenoxycarb and Methoprene. Fenoxycarb mimics the action of Juvenile hormone II, preventing or delaying metamorphosis in insects and, in some insects, interfering with reproductive ability and pheromone production [163]. Methoprene acts in a similar way to Fenoxycarb, and is a synthetic Juvenile hormone mimic with the ability to prevent the progression from metamorphosis to adult stage but can also kill larvae and pupae [444], [411]. Although the effects of these compounds are generally quite specific to insects, they can have toxic effects on crustaceans, which have hormonal analogues to insect Juvenile hormones [202].

Chitin is one of the most important components of insect exoskeletons and is critical for the structure of the cuticle, which is formed and degraded through various stages of the insect developmental process [516]. Insecticide compounds which interfere with the synthesis of chitin work to inhibit growth and disrupt development. These compounds include Cyromazine, Lufenuron and Diflubenzuron. Lufenuron has its main effect during larval stages of development, where it has been seen to kill larvae and pupae, but had little effect on adults (only affecting fecundity) [493], [335]. Cyromazine also shows very similar symptoms, with death occurring more quickly in larvae at higher concentrations [205], with adult fecundity also affected [97]. The symptoms of insect exposure to Diflubenzuron are slightly different, and are reported as an inability to moult, a fragmentation of the chitin exoskeleton and a separation of the cuticle from muscles [314]. Despite small differences between these compounds, they all broadly act on disrupting

the synthesis of chitin during development [110].

Mitochondrial Inhibitors

Due to the growing problem of insecticide resistance, and the fact that resistance generally occurs due to a mutation or insensitivity at a binding site, more effort has been made to find potential insecticidal compounds which have molecular MoAs [342]. One such molecular mechanism that has been investigated for use is that of the mitochondrial electron transport chain. Compounds of interest are those that either target complex I, similar to the classical complex I inhibitor Rotenone [287], or those that target other parts of the electron transfer chain, such as Acequinocyl [28]. Although Rotenone has quite weak insecticidal activity, it has its toxic effect by increasing the production of reactive oxygen species (ROS), and ultimately leading to cell apoptosis [182]. Interestingly, this MoA also gives Rotenone anti-carcinogenic properties, and in recent years this has been the focus of study around the compound [89], [182].

Acequinocyl, like many compounds which disrupt respiration, is an acaricide as well as an insecticide. Differently to the other compounds described in this section, it targets complex III [87].

Diafenthiuron Carbodiimide is another such compound to inhibit mitochondrial ATPase. The precursor compound, Diafenthiuron, does not have insecticidal activity itself, but once it has undergone photodegredation to become Diafenthiuron Carbodiimide it is able to inhibit respiration at complex V in a time and concentration dependent manner [390], [391].

Chlofenapyr also inhibits respiration. However, it does so in a manner which uncouples phosphorylative oxidation in mitochondria [375], and this process causes symptoms such as the prevention of feeding, uncoordinated movements and paralysis in exposed

insects [33]. A compound with a very similar MoA is the fungicide, Fluazinam, which disrupts oxidative phosphorylation and disrupts ATP synthesis [479],[215].
The last mitochondrial inhibitor to mention here is Cyflumetofen, which acts selectively on mites [178]. This compound inhibits the mitochondrial complex II [448].

Feeding Inhibition and Chordotonal Organs

Two compounds which have a striking effect on the feeding of insects, particularly on sucking pests such as the cotton leafhopper (*Amrasca biguttula*), are Pymetrozine and Flonicamid. These compounds both have extremely unique MoAs when compared to the compounds discussed so far.
Pymetrozine exposure to insects was seen to lead to impaired feeding, leading to death by starvation [174]. Despite what would seem to be a simple behavioural effect, the MoA of Pymetrozine was not discovered until many years later. It was ultimately found to target transient receptor potential (TRP) channels found in chordotonal stretch receptor neurons, leading to uncoordinated behaviour and cessation of feeding [337].
Flonicamid to this day has a still unknown MoA, although unpublished data from Syngenta, and one published study, suggests this compound could also work by targetting the chordotonal receptors [457]. Previous work has shown that insect exposure to Flonicamid prevents ingestion in a dose-dependent manner without causing poisoning symptoms in aphids [453], but symptoms of uncoordinated movement and leg-splaying were observed in aphids with the treatment of the main metabolite of Flonicamid, 4-trifluoromethylnicotinamide (TFNA-AM) [457].

Octopamine Receptors

Compounds which target the octopamine receptors can also act as pesticides. Octopamine is an insect analogue of noradrenaline and is a neurotransmitter and neurohormone which is involved in stress responses and key insect behaviours [124]. Compounds which target octopamine receptors are in the formamidine class of insecticides and these compounds generally act to mimic the behaviour of octopamine [165].

1.14.6 Classification Techniques for Biology

Machine learning and statistical learning techniques for various biological classification tasks have gained popularity in recent years due to several factors, which include the increased availability of specialist hardware for the application of parallel data processing (GPUs), advances in computer algorithms, which allow the development of models to process large amounts of data, and the successes which have been seen in using these techniques for biological applications [471], [272]. When it comes to using types of machine learning, these can be broadly divided into two types of learning. (1) *Supervised learning*, where a section of the data is provided with labels or features based on that data. Following this, a model is built based on identifying these pre-determined features, and new data is processed based on its features to allow classification [153]. This is an example of an input:output system, as example inputs and corresponding outputs are given. (2) *Unsupervised learning* uses models which are given data but are not given any information on features inherent to the data; this system will classify or cluster data based on patterns it can identify in the data, which are then unknown to the researcher [452]. This is an input only system. These types of machine learning based classification algorithms have been used for a many different applications in the

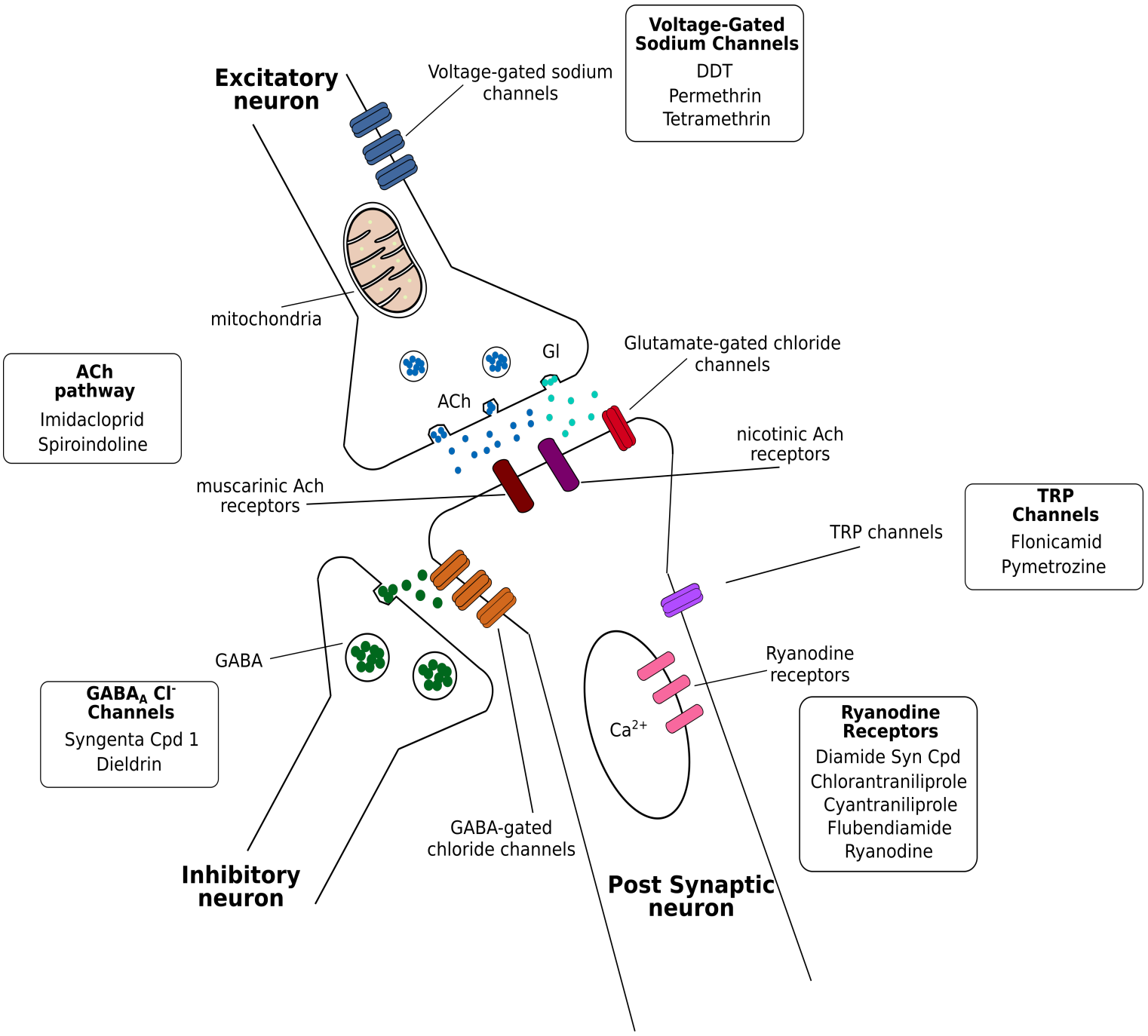


Figure 1.5: Diagram of some of the cellular targets of insecticides

field of biology, such as genetics [27], drug discovery [293], ecology [59], [360] and plant biology [216].

In the field of agriculture, machine learning has been used for many different applications, examples of which include fruit counting [376], estimating soil temperature [325], tracking behaviour changes in pigs [301] and the detection and classification of pests on crops [115].

The work cited so far gives a representation of the variety of applications of many different approaches using techniques that come under the definition of machine learning. This begs the question, what technique is best to use? As might be expected, the exact machine learning technique most suitable depends on the exact nature of the problem to be tackled.

In the case of the work presented in this thesis, the problem can be concisely phrased as *how to classify complex biological time series data*, specifically, behavioural data relating to the exposure of *Drosophila* to insecticide compounds.

Predicting or classifying the behavioural dynamics of organisms is a challenge which can be addressed by different machine learning approaches. In the field of agriculture, some examples of where this has been done are that of Cavendish et al. [61] and that of McDermott-Rouse et al. [303]. Cavendish et al. used an unsupervised machine learning approach to characterise shape changes of the Asian soybean rust crop pathogen, *Phakopsora pachyrhizi*, germination behaviour in control conditions and when exposed to different fungicides [61]. McDermott-Rouse et al. used a high-throughput machine learning approach to classify the behavioural motifs of *C. elegans* extracted from pose-estimation data. This study found that behavioural responses of *C. elegans* to different insecticides could be used to predict the MoA of the compounds with an accuracy of 88% [303].

To be able to address the question of whether behaviours arising from exposure to compounds can be more quantitatively analysed and classified, the focus of this work was turned to the area of behavioural classification.

1.14.7 Statistical Classification

One method of classifying behavioural phenotypes which has been briefly mentioned is that of statistical classification. Statistical classification is a type of feature based classification. This method extracts representative features from the data before the classification procedure and uses these features to group data [443]. This method is particularly good for classifying time series data. One specific method which uses statistical, feature based learning for classification purposes is that of *Highly Comparative Time Series Analysis* (HCTSA), developed by Fulcher et al. [139], [138]. This method is actually something of a composite of different methods: Fulcher et al. made a classifier which first performs massive feature extraction on time series data, using more than 7700 statistical tests, which each give an output (feature) based on the use of the statistical test on each single time series inputted. This means each time series which is inputted will give a number of different features, allowing a feature matrix to be built of all the time series data. Based on this feature matrix, data within this can be classified. This technique was validated by the authors as able to classify subgroups within behavioural data taken from both *Drosophila* and *C.elegans* [138].

Since its development, HCTSA has been used to classify zebra finch songs in different social contexts [355], heartbeats based on electrocardiography data [38] and identify babies with low blood pH from heartbeat recordings [137], amongst other applications. It is this technique which was chosen to be used for classification of behavioural symptoms in this thesis.

1.15 Bridging two fields: How insecticides can modulate sleep

As has been outlined in section 1.2.2, the influence of the external environment of an organism can have a large impact on internal state. The exposure to compounds such as insecticides is one example of how an environmental factor can modulate the internal state of an organism, which includes the sleep:wake state. Although, as it has been discussed, *Drosophila* are not classically seen as a pest species, many studies have used *Drosophila* to show the detrimental effects insecticidal compounds can have on the functioning of insect species in their ability to perform normal processes, such as sleep. For example, it has been shown that compounds in the class of neonicotinoids can disrupt both circadian and sleep behaviour in *Drosophila* [454], [505]. Further to this, it has been shown that other non-pest species in the environment can have their sleep negatively impacted by insecticides. Honeybees have been shown to exhibit negative sleep and circadian rhythm effects as a result of neonicotinoid insecticide exposure [446], [455] and also in response to a commonly used herbicide, glyphosate [475].

Further to the direct impact insecticides can have on sleep and circadian rhythms, mutations which lead to insecticide resistance have also been shown to have an effect on sleep. The best known example of this is that of the gene, *Rdl*. This gene encodes a $GABA_A$ receptor and knock-down of this gene in circadian PDF neurons [69] or in the FSB [241] leads to an increase in wakefulness. It has also been shown that mutants for *Rdl* have increased sleep latency [2]. It has also been reported that a loss of the Dα1 subunit in *Drosophila*, which is a subunit of the nAChR and confers insecticide resistance to neonicotinoids, also demonstrates changes in sleep behaviour [432].

Interestingly, using changes in circadian expression of genes is now being investigated as

a possible pest-management approach. This strategy has been investigated in *Drosophila* owing to the plethora of circadian work done with flies as a model organism. It has been shown that circadian changes in the expression of xenobiotic metabolising genes, which encode detoxifying enzymes, change the susceptibility of *Drosophila* to some insecticides [200]. In other insects, a similar effect has been found and has shown that application of compounds at different times of day can change the susceptibility of houseflies and cockroaches [440], mites [117] and in the pine weevil [372].
Although the interplay between sleep and insecticides is not the main focus of the work herein, this research does give light to the interesting possible cross-overs between these two fields.

1.16 Aims of this work

Based on the background I have laid out in this introduction, I have attempted in my work to tackle two broad aims:

- To understand the effect of social, genetic and pharmacological manipulations on homeostatic sleep regulation.
- To understand and classify the behaviours which result from *Drosophila* exposure to various insecticides.

Chapter 2

Materials and Methods

2.1 Fly Strains and Maintenance

Fly lines were maintained on a 12-hour light: 12-hour dark (LD) cycle and raised on standard corn and yeast media (standard food). A list of all fly strains used and their sources can be found in table 2.1. Canton Special (CS) flies were used as controls for all experiments using mutant flies and were maintained at 25°C. For RNAi and temperature sensitive experiments, flies were reared at 18°C. CS *Drosophila melanogaster* were used as the wild-type line for all experiments.

2.2 Ethoscope tracking

For all sleep experiments in Chapter 3 and insecticide experiments in Chapter 4, the ethoscope was used for recording of fly activity (except when specified in section 4.5). A detailed description of the ethoscope with open source detailed descriptions as to how to make the hardware can be found at http://lab.gilest.ro/ethoscope and these are detailed in the paper by Geissmann et al. [150]. A diagram showing the main features

of the ethoscope is shown in Figure 2.1.

2.3 Behavioural experiments for sleep investigations

For all sleep experiments using the ethoscope, 7- to 8-day-old pupae were sorted into glass tubes [70 mm × 5 mm × 3 mm (length × external diameter × internal diameter)] containing the same standard food which was used to rear the flies. All behavioural experiments (except temperature sensitive-experiments in section 3.6.5) were performed under a 12-hour light: 12-hour dark cycle at a humidity of 50-70% in an incubator maintained at 25°C.

2.4 Fly preparation for social experiments

For sleep experiments, except those which looked at specifically at prior social experience, flies were sorted as two same-sex virgin pupae into glass tubes, or as mixed-sex pairs with a male and a female to allow for mating. Flies were maintained in this tube for 3-4 days before flies of the sex to be tested were transferred into individual tubes.
For experiments where flies were sorted into larger social groups, single pupae were put into individual tubes for a single day to allow for eclosion before being transferred to a vial with up to 10 other flies, the sex of which depended on the specific group. Flies were maintained in these groups for 2-3 days before being transferred to individual tubes and placed in ethoscopes for behavioural monitoring.
To ensure all females tested in the mated group were actually mated, tubes were checked following the experiment for the presence of eggs or larvae.

2.5 Starvation experiments

For starvation experiments, flies were kept in individual tubes and monitored in the ethoscope for 2-3 baseline days before the treatment period. The baseline day immediately prior to treatment was used for analysis. For the treatment day, flies were transferred from their tubes in the ethoscope to tubes with either 2% agarose medium (Sigma, A6236) for the starved flies, or a tube with standard food for the control group. Flies were starved or kept as fed controls for 23 - 24 hours. Following the 24 hour treatment period, flies were transferred back into the same tubes they occupied before the treatment. Flies were tracked for at least one full rebound day following the treatment period.

2.6 Dynamic sleep deprivation experiments

For dynamic SD, flies were kept in individual tubes and monitored in the ethoscope. Optomotor attachments of the ethoscope platform (see Geissmann et al. [150]) were used to perform the 24 hour dynamic SD treatments. A diagram of the main components of the optomotor can be found in Fig.2.1. Flies were given 2-3 baseline sleep days prior to dynamic SD. The baseline day immediately prior to treatment was used for analysis. Flies were tracked for at least one full rebound day following the treatment period.

2.7 Simultaneous sleep deprivation experiments

For the simultaneous (double) SD, flies were kept as described previously, in individual tubes inside ethoscopes with optomotor attachments. For the treatment day, flies were

flipped into either 2% agarose (Sigma, A6236) or standard food tubes before being placed back into the optomotor device to experience 24 hours of dynamic SD. Flies were flipped back into tubes occupied before the dynamic treatment after 23 - 24 hours. Flies were tracked for at least ome full rebound day following the treatment period.

2.8 Immunostaining for *trans*-Tango

CrzR-Gal4/UAS-*trans*-Tango female flies, Crz-Gal4/UAS-*trans*-Tango and sNPFR-Gal4/UA Trans-Tango female flies were raised at 18°C and dissected 30-35 days after eclosion. Flies were cold immobilised on ice and brains were dissected in 0.01 M PBS. Brains were fixed in 4% paraformaldehyde (PFA) for 20 minutes and washed 3x10 minutes in 0.3% PBST (PBS with Triton-X). Brains were then blocked for 1 hour in 5% normal goat serum (NGS, Ab7481, Abcam) and incubated for 48 hours in 1:10 mouse anti-nc82 (Ab 2314866, DSHB), 1:200 rabbit anti-GFP (ab6556, Abcam) and 1:200 rat anti-HA (ROAHAHA, Merck) in 5% NGS in PBST at 4°C. After 48 hours, brains were washed 3x10 minutes in PBST and incubated for 48 hours in 1:200 goat anti-mouse Alexa Fluor 568 (ab175473, Abcam), 1:200 goat anti-rat Alexa Fluor 647 and 1:200 goat anti-rabbit Alexa Fluor 488 (Ab15007, Abcam) at 4°C. Brains were mounted on microscope slides in Vectashield (Vector Laboratories) and imaged using a Leica SPF inverted confocal microscope.

2.9 Immunostaining for GRASP

Flies used for GRASP were of the following genotypes: R58E02-lexA/UAS-post-t-GRASP, LexAop2-pre-t-GRASP;CrzR-Gal4, SNPF-lexA/UAS-post-t-GRASP,LexAop2-pre-t-GRASP;Crz-Gal4,PDF-lexA/UAS-post-t-GRASP,LexAop2-pre-t-GRASP;Crz-Gal4.

Flies were raised at 18°C and dissected 5-8 days after enclosion. Flies were cold immobilised on ice and brains were dissected in 0.01M PBS. Brains were fixed in 4% paraformaldehyde (PFA) for 20 minutes and washed 3x10 minutes in 0.3% PBST (PBS with Triton-X). Brains were then blocked for 1 hour in 5% normal goat serum (NGS, Ab7481, Abcam) and incubated for 48 hours in 1:10 mouse anti-nc82, (Ab 2314866, DSHB), 1:200 rabbit anti-GFP (ab6556, Abcam) in 5% NGS in PBST at 4°C. After 48-hours, brains were washed 3x10 minutes in PBST and incubated for 48 hours in 1:200 goat anti-mouse Alexa Fluor 568 (ab175473, Abcam) and 1:200 goat anti-rabbit Alexa Fluor 488 (Ab15007, Abcam) at 4°C. Brains were mounted on microscope slides in Vectashield (Vector Laboratories) and imaged using a Leica SPF inverted confocal microscope.

2.10 Feeding of sleep-related compounds

Flies were prepared as described previously for starvation, but flipped into tubes with either 5% sucrose (Sigma, S0389) and 2% agarose (Sigma, A6236) for the sucrose treatment group, standard food for food control treatment group, 2% agarose for the starvation treatment. For L-DOPA, 3IY and caffeine treatments, solid L-DOPA (Abcam,ab120573), 3IY (Sigma,I8250) or caffeine (Sigma,58082) was mixed into melted sucrose and agarose food at a concentration of 5 mg/ml and put into glass tubes.

2.11 Temperature sensitive experiments

For experiments where temperature sensitive lines were used, (R58E02-Gal4/TRPA1^{TS}, CrzR-Gal4/TRPA1^{TS}, flies were raised at 18°C until they were 7-8 day old pupae. At this point, they were sorted into same-sex or mixed-sex tubes as described in Fig.3.1 and

maintained at 22°C until the starvation treatment day, during which the temperature was raised to 29°C for the duration of the starvation treatment.

2.12 Arousal Experiments

For arousal experiments, the Air-Gas-Odour (AGO) extension of the ethoscope was used, described by French et al. [135]. A detailed diagram of this is found in Fig.2.1. Female flies were placed into 140mm long glass tubes with standard food or starvation medium, traversed by a 5μL capillary at one end and a 30mm long piece of hollow silicone tubing serving as a plug at the other end. Flies were in these tubes for 18 hours prior to receiving an arousal stimulus. Tubes were placed into AGO customised arenas, which were inserted into ethoscopes and connected to AGO modules. During the arousal period (ZT18-ZT24) 5 second puffs of 5% acetic acid were delivered to flies immediately following 5 minutes of immobility at a flow rate of 0.4L per minute. Fly movement in the 10 seconds following stimulus delivery was recorded. If the fly moved above a pre-determined velocity threshold, it was deemed to have woken. As each fly could receive multiple puffs within the stimulus period, a mean response proportion for the time was calculated for each fly.

2.13 Insecticide compound preparation

All insecticide compounds were supplied by Syngenta Ltd. from their in house stock (see table 1.3 for a full list of compounds used). Compounds were received in solid form and diluted in solvent containing 5% ethanol (VWR, 20821), 5% acetone (Sigma,179124) and 10% dimethylsulfoxide (DMSO) (D2650, Sigma) in distilled water to 1000ppm initially, then further diluted in the solvent mixture to 100ppm and 1ppm.

2.14 24-well plate preparation

Custom 3D-printed plates were designed using the online CAD software Onshape and printed using Ultimaker 2+ 3D printers using PLA plastic. For insecticide assays, 0.5mL of 5% sucrose (Sigma, S0389), 1% agarose (Sigma, A6236) solution was pipetted into each well and allowed to set. Following this, 2 µL of compound solution were placed on the surface and allowed to dry for 30 minutes or more. Male flies were then placed on the surface with a small glass cover slip placed on top (13mm circular cover slip, VWR631-0150). Flies were briefly anaesthetised ($\leq$1 minute) before being placed onto the surface of the plate. Once each well had been filled with a single male fly, arenas were placed into the ethoscope and recorded for a minimum of 2 days. All experiments were started between ZT0 and ZT1 and within 30 minutes of the flies being placed in the wells. For each compound of the 11 original compounds and the 5 blind compounds, 3 repeats were done at different time points. For the data in section 4.2.5, where 30 additional compounds were assessed at 100ppm, 2 repeats were done for each additional compound.

2.15 Survival Analysis

For survival analysis, the same data used for HCTSA classification was used with a custom R script to determine the time points at which flies died over the first 24 hours of recording.

2.16 Design and build of superscope

All superscope parts were designed using the online CAD software Onshape and printed using Ultimaker 2+ 3D printers using PLA plastic. Custom video recording software was written in Python in collaboration with Giorgio Gilestro and burned on a 32GB SD card (Evo Plus microSD Card 32GB). This was placed in a Raspberry Pi (Raspberry Pi 3 Model B+) which was used to run the device. For recording, a 8MP camera (ELP IMX179) camera was used.

2.17 Superscope square preparation

Custom 3D-printed squares were designed using the online CAD software Onshape and printed using Ultimaker 2+ 3D printers using PLA plastic. For insecticide assays, 0.5mL of 5% sucrose (Sigma, S0389), 1% agarose (Sigma, A6236) solution was pipetted into each well and allowed to set. Following this, 2 µL of compound solution were placed on the surface and allowed to dry for 30 minutes or more before male flies were placed on the surface with a small glass cover slip placed on top (13mm circular cover slip, VWR631-0150). Flies were briefly anaesthetised ($\leq$1 minute) before being placed onto the surface of the square. Once each well had been filled with a single male fly, squares were placed into the superscope and a video was recorded for a minimum of 12 hours. All experiments were started between ZT0 and ZT1. At least 14 flies were recorded after being exposed to one of each of the 11 original compounds or the solvent control. Recordings of flies exposed to compounds were done in a randomised manner.

2.18 Superscope video processing

Videos were initially downloaded and divided into smaller video files using a custom bash script. Following this, files were uploaded onto Google Drive and linked to a DeepLabCut Colab project file. Video files were analysed using DeepLabCut and a .csv file for each video for each compound at each time point was derived. This .csv file was processed to give a time series of Euclidean distance for head coordinates of flies in each video.

2.19 DeepLabCut and pose estimation

The use of DeepLabCut (version 2.1) followed the detailed protocol outlined by Nath et al. [333]. Briefly, frames for labelling were extracted from 3 representative videos using a K-means algorithm and frames were labelled with 22 unique body parts (head, left eye, right eye, thorax top, thorax bottom, abdomen top, abdomen middle, abdomen bottom, left wing tip, right wing tip, left foreleg tip, left foreleg middle, right foreleg tip, right foreleg middle, left middle leg tip, left middle leg middle, right middle leg tip, right middle leg middle, left back leg tip, left back leg middle, right back leg tip, right back leg middle). These frames were labelled locally with a DeepLabCut graphical user interface (GUI) before the project file was uploaded to Google Drive for training and video analysis to be done using Google Colab. The data was split into a 9:1 test:train dataset and training was run for more than 150,000 iterations before the average Euclidean error was computed between labels and predictions. The model at the best performing checkpoint was used to predict pose in novel videos.

2.20 Experiments in total darkness

For experiments in section 4.8.1 which investigated the possible circadian phenotype of flies exposed to compounds, flies were raised as described previously in LD conditions except in complete darkness (DD). Flies were briefly anaesthetised under low-light conditions before the start of recording and all recording took place in complete darkness.

2.21 Use of pre-existing datasets

2.21.1 Sleep rebound data

Velocity time series data generated from recording flies exposed to either control conditions (no SD) or incrementally increasing immobility-triggered SD conditions was taken from the dataset generated by Geissmann et al. [149]. Only data for female flies was taken, and where groups consisted of more than 60 individuals, a sample of 60 random individual flies were chosen to take time series data from. Time series data was processed in the same way as described in section 4.2 using the raw ethoscope data.

2.21.2 RNAi and genetic sleep data

Velocity time series data generated from recording flies of different genetic backgrounds was taken from a dataset generated by Michaela Joyce (unpublished). Only 60 flies from each group were used for HCTSA and where groups consisted of more than 60 individuals, a sample of 60 random individual flies were chosen to take time series data from.

2.22 Software and Data Analysis

2.22.1 Data Analysis

Data used for sleep analysis and time series data of maximum velocity was extracted using rethomics [151]. Statistical comparisons between treatment groups for SD data was done by using Wilcoxon rank-sum tests. When comparing groups for baseline sleep and comparing insecticide compounds to the solvent control, Wilcoxon rank-sum tests were performed and followed with False Discovery Rate (FDR) corrections. For ethograms and box-plots, intermediate reference marks indicate the mathematical mean and error estimates indicate the bootstrapped 95% confidence intervals. Where possible, figures explicitly mention the biological *N*, the number of biologically independent individuals for each data point. *P* values shown in figures relate to the result arising from the Wilcoxon rank-sum tests. Summary tables represent full statistical outputs and representative signs of significance.

2.22.2 Insecticide compounds and behaviour compared to solvent control groups

Ethoscope data was initially processed in R[374] using rethomics [151]. For generating insecticide behavioural estimation plots, the dabest package [194] was used to plot all graphs, with a separate plot demonstrating unpaired mean difference as a bootstrap 95% confidence interval. Wilcoxon pair-wise tests, with a FDR adjustment, were used to calculate differences between mean values of each behavioural state for flies exposed to each compound at each concentration, compared to the flies exposed to the solvent control.

2.23 Data Analysis: HCTSA

Ethoscope data was first processed in R: data was extracted from the ethoscope device and processed using rethomics for each fly over a 12 hour period. Once each time series had been extracted, the data was downloaded and processed using a custom bash script to format individual .csv files for each time series correlating to each fly. Each time series was then further processed in Matlab to convert it to .DAT format. A metadata .txt file was made as a reference file of each time series for processing data using HCTSA.

Ethoscope data was initially processed in R[374] using rethomics [151]. For running HCTSA and performing calculations, Matlab was used [299]. The HCTSA packages were used to classify data using a SVM classifier [138], [139].

2.24 Data Analysis: Catch-22

For Catch-22 analysis, Euclidean distance time series data was taken for each fly exposed to each compound and run through the Catch-22 software using Matlab [299], where 22 operations were used to calculate features for each time series file. Catch-22 was used to classify data using a SVM classifier [286]. Figure 4.30 was generated in R[374] using the Tidyverse package [489].

2.25 Data Collection and Generation of Graphs

All data, except for that in the following instances, was collected by the author. The data for knockdown of Crz in the sNPFR neurons was collected by a Masters' student, Martha Gutteridge. The data for the response of *fumin* mutants to starvation SD was

collected by a Masters' student, Alessandra Lodi, and further repeats were conducted in collaboration with PhD student Laurence Blackhurst from the Gilestro Lab.
The Fig.4.28 of pairwise similarity matrices looking at prediction accuracy and body regions was generated by undergraduate student Cleo Zhang.

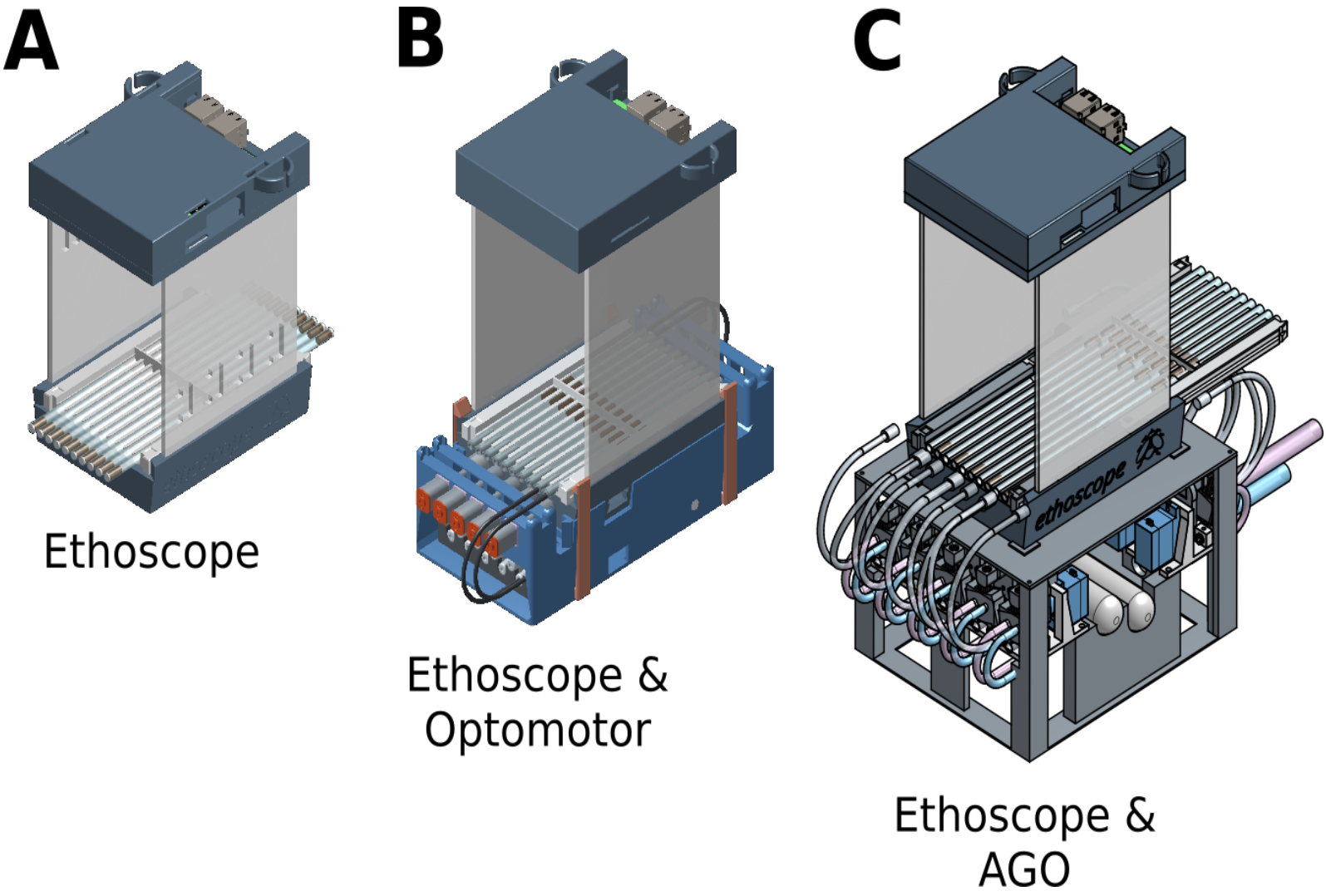


Figure 2.1: *Ethoscope tracking device and its modules* **(A)** The basic design of the ethoscope device use for tracking, where flies are placed in one of 20 individual glass tubes in a 3D printed arena and can be monitored for sleep and activity over time. **(B)** Ethoscope device and its accessory module, the optomotor, which includes 10 motors placed underneath half of the glass tubes and can send targetted mechanical stimulation to the flies, when detected to be asleep. **(C)** Ethoscope device and its accessory module, the AGO, where flies are placed in one of 10 individual glass tubes in a 3D printed arena and receive a targetted puff of odour when detected to be asleep.

2.26 Methods: Explained

2.26.1 The GAL4/UAS System in *Drosophila*

The GAL4/UAS system, used for genetic manipulation in *Drosophila melanogaster* [46], is a transcription activation system taken from yeast and modified for use in *Drosophila*. The fundamental mechanism of this method involves two parts: the yeast transcription factor gene, GAL4 and an upstream activating sequence (UAS). For this system to be functional, a fly must possess both the GAL4 and the UAS. To ensure this is the case, one fly can be genetically manipulated to express the GAL4 gene in a tissue or region of interest and a second fly can be manipulated to express the UAS enhancer sequence, which targets the gene of interest. When both flies are mated together, the progeny will contain both the GAL4 and UAS. The GAL4 gene encodes a transcriptional activator, which binds to UAS in the DNA and recruits transcriptional machinery to induce expression of the gene of interest.

This method can also be used in combination with RNA interference to "knock down" a gene of interest and silence its expression [43]. In this case, the GAL4/UAS will control the expression of a gene fragment that dimerises to give double-stranded RNA hairpins. The production of these have a silencing effect on the single-stranded RNA of the gene of interest and therefore prevent its expression (see figure 2.2 for a graphical representation).

2.26.2 *trans*-Tango

The *trans*-Tango technique is a method used for visualising neurons of interest and their post-synpatic partners. This technique uses a modified version of the Tango system [21], in which a synthetic signalling pathway is introduced to a cell to convert the activation

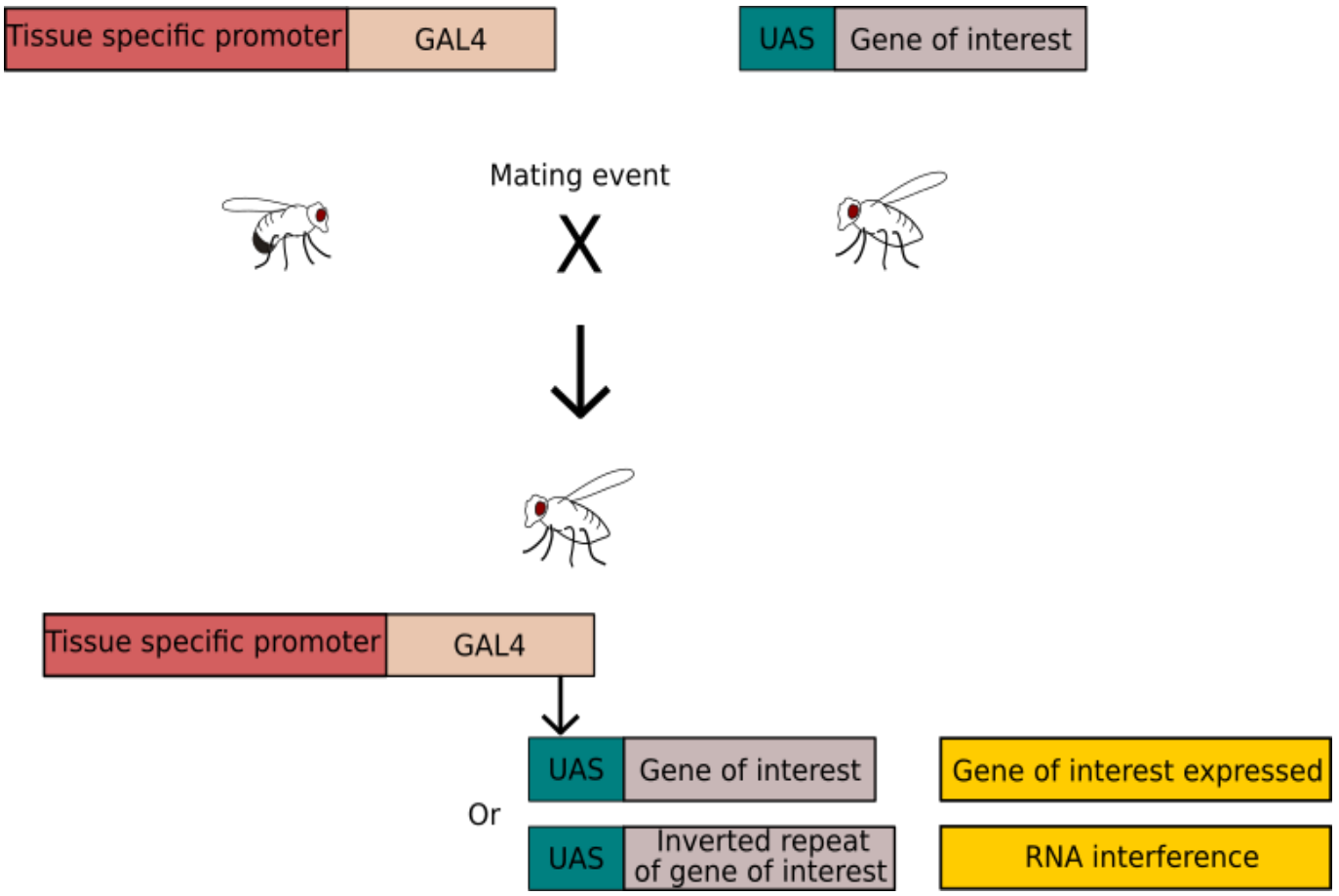


Figure 2.2: GAL4/UAS System in *Drosophila*. Diagram describing how the GAL4/UAS system functions in *Drosophila melanogaster*.

of a receptor on the cell surface to the expression of a reporter via a mechanism of proteolysis. In the *trans*-Tango system, the synthetic signalling pathway is introduced to all neurons and specific neurons of interest are targetted by introducing a tethered ligand in the presynaptic membrane. Contact between the ligand and its receptor at the synaptic membrane leads to the expression of a reporter in post-synaptic cells [450]. Diagram 2.3 represents the key components of this process. The two panneuronally expressed components: hGCGR::TEV::QF, a G-protein coupled receptor (GPCR) bound at the cytoplasmic tail to QF (a transcriptional activator), via a linker which contains the N1a protease cleavage site from the tobacco etch virus (TEV) and hArr::TEV, the human β arrestin 2 protein, which specifically activates GPCRs, fused to TEV. The third component is a reporter, e.g. mdt Tomato, which is under transcriptional

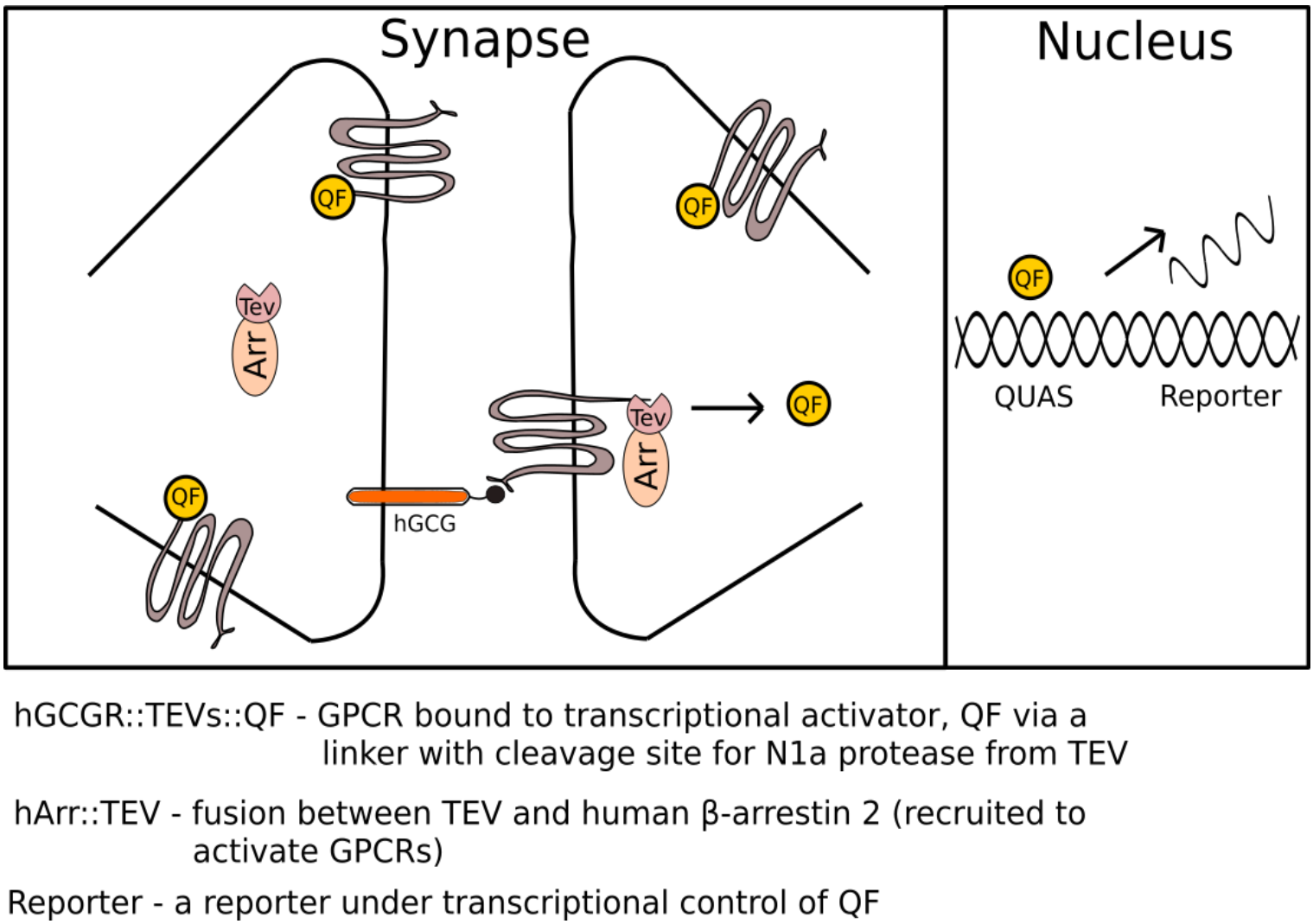


Figure 2.3: *trans*-Tango System in *Drosophila*. Diagram representing how *trans*-Tango signalling functions in *Drosophila*

control of QF. When the membrane tethered ligand is expressed in the presynaptic membrane, trans-synaptic activation of the hGCGR::TEV::QF causes the recruitment of hArr::TEV, which cleaves QF, allowing it to enter the nucleus and induce expression of the reporter.

Table 2.1: Fly stocks used for experiments

Fly stock	Source	Isogenised
Canton Special	Ralph Stanewsky's Lab (UCL,UK)	NA
CrzR Mutant (84488)	Bloomington Stock Centre	No
CrzR-Gal4 (49077)	Bloomington Stock Centre	Yes (with W1118 for 8 generations)
R58E02-Gal4	Alice French (The Francis Crick Institute, UK)	Yes (with W1118 for 8 generations)
CHAT-Gal4 (6793)	Bloomington Stock Centre	Yes (with W1118 for 8 generations)
C564-Gal4	Dionne Lab (Imperial College London, UK)	Yes (before receiving)
nsyb-Gal4	Dionne Lab (Imperial College London, UK)	Yes (before receiving)
GMR89E07-Gal4 (69344)	Bloomington Stock Centre	No
PLE-Gal4(8848)	Bloomington Stock Centre	Yes (with W1118 for 8 generations)
sNPFR-Gal4 (30996)	Bloomington Stock Centre	No
fumin mutant	Jepson Lab (UCL,UK)	No
CrzR-RNAi (43410)	Vienna Stock Centre	Yes (with W1118 for 8 generations)
Crz-RNAi (106876)	Vienna Stock Centre	Yes (with W1118 for 8 generations)
trans-Tango	Jepson Lab (UCL,UK)	No
GRASP (79040)	Bloomington Stock Centre	No
UAS-TRPA1	Jepson Lab (UCL,UK)	No
PDF-LexA (84430)	Bloomington Stock Centre	No
sNPF-LexA (84436)	Bloomington Stock Centre	No
R58E02-LexA (52740)	Bloomington Stock Centre	No
Rdl^{A301S} mutant	Syngenta, Jealott's Hill	No
$Para^{L1029F}$	Syngenta, Jealott's Hill	No

Chapter 3

Investigating Sleep and Homeostatic Rebound

3.1 Introduction

As explained in the introduction, sleep homeostasis has been examined in a variety of contexts. It has been previously shown that a range of environmental conditions, which can change the prior experience of the fly, lead to the fly experiencing SD differently. This can lead to a modulation of sleep rebound exhibited following the SD period.
Sleep can be modulated by social experiences: this includes whether the fly is isolated or group-housed [145], [271], if the fly is paired with a male or a female counterpart [26], and the level of aggression the fly encounters [289].
Flies which experience social types of SD show differing rebound responses depending on whether they are in the presence of a male or female "sleep depriving" fly [26]. This reveals that this experience inherently changes whether rebound need occur or not. One biological factor that has not been investigated in the context of sleep homeostasis

and sleep rebound is mating status. Many biological changes accompany mating in *Drosophila* females, and it has already been shown that mated females have significantly reduced baseline sleep compared to their virgin counterparts [149], [211],[147]. Although some data has shown that mated females have negative biological consequences following mechanical and caffeine induced SD [369], no study has looked at whether this biological change can lead to difference responses in relation to rebound sleep. In this work, I looked to understand whether mated or virgin females display different rebound responses following two different types of SD: dynamic SD and starvation-induced SD. Although several different forms of SD have been outlined in the introduction, these two forms of SD were chosen for the following reasons.

Firstly, as previously discussed, the dynamic form of SD described in the introduction has been shown to be one of the most effective forms of SD. Due to the ability of the ethoscope to conduct real time activity tracking of the flies, it is possible to deliver targeted SD only to the flies which are determined to be immobile past a certain time threshold. This reduces the likelihood of habituation to the dynamic stimulus and ensures that the fly is not disturbed whilst it is awake and carrying out other behaviours such as feeding or egg-laying. This form of SD removes sleep, but from data to this point, does not seem to impact on other forms of physiological processes. Indeed, it has been shown that this form of SD does not impact significantly on lifespan [149].

Although the dynamic SD is efficient at removing sleep, it is an artificial form of SD which would not occur outside a laboratory environment. To counter this and provide a contrasting paradigm, the starvation protocol of SD was chosen as the second method. Starvation, despite leading to in changes in metabolism, provides a more relevant ethological scenario. It is possible that flies have to experience a period of starvation in their natural environment, and this has previously been shown in laboratory studies to

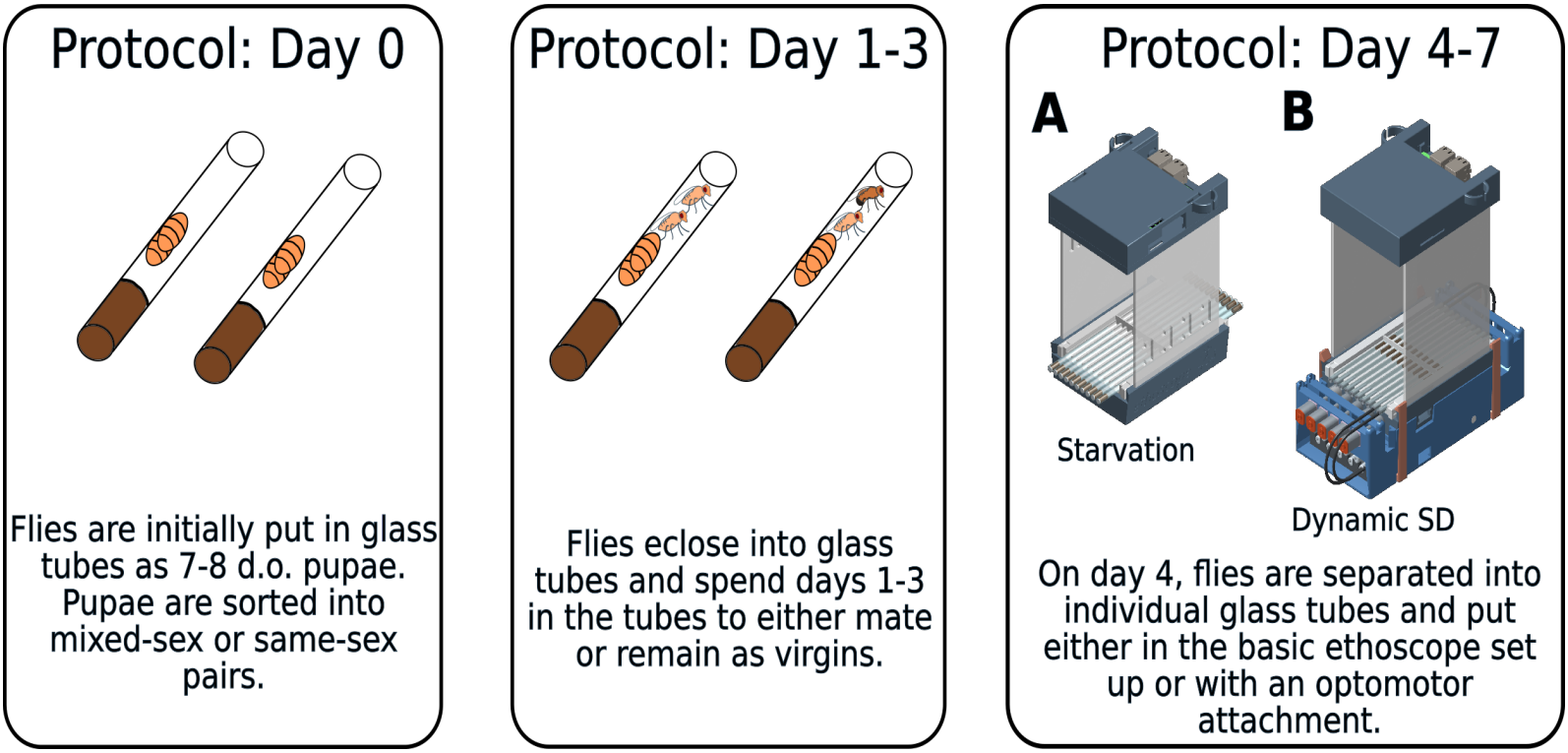


Figure 3.1: *Basic protocol for testing mated or virgin females* Virgin and mated females were initially put into glass tubes as either same-sex or mixed-sex pairs on day 0 and remained in these pairs from eclosion to 4 days old. At 4 days old, flies were separated and put into individual glass tubes in ethoscopes, either in the basic set-up for the starvation SD or in ethoscopes with optomotor attachments for dynamic SD.

have a significant impact on sleep [240]. This form of SD is particularly interesting as it presents a possible behavioural dilemma for the fly: choose to sleep, search for food, or perform a different behaviour. As prolonged periods of starvation lead to death in flies, it also presents a contrast to the dynamic method of SD, where the fly is faced with a severe negative consequence as a result of the SD method.

3.2 Results

3.2.1 Investigating Sleep and Homeostatic Rebound in Wild-Type Females

To understand differences that may arise in sleep homeostasis following mating, I decided to expose females which were either mated (kept with a male from eclosion to 4 days old) or virgin (kept with another female for the same amount of time) to three different forms of SD. These types were dynamic SD, starvation-induced SD and a combination of both methods simultaneously, for a period of 24 hours. Prior to this, I measured the baseline sleep amount exhibited by these females for more than 24 hours before the onset of sleep deprivation treatment, and allowed 24 hours post-treatment to determine whether the sleep was recovered (Fig.3.1).

As presented (in Fig.3.1 and Fig.3.2), both mated and virgin females that were subjected to the dynamic SD paradigm, which leads to total SD during the treatment period, responded with significant rebound sleep in the first 3 hours and 6 hours post-treatment (Fig.3.2 and Fig.3.3).

When subjected to starvation SD, mated and virgin females experienced far less sleep loss when compared to controls, although this sleep loss was significant for daytime sleep. However, only virgin females, but not mated females, showed a significant rebound following this type of SD. It is surprising to see that virgin females actually demonstrate a larger amount of rebound sleep following starvation-induced SD than they do following the dynamic SD, despite losing more sleep with the latter.

To understand what the response would be if both forms of SD were combined, a third group was tested, which underwent simultaneous dynamic and starvation-induced SD. Interestingly, mated females did not show a significant sleep rebound in either the 3

hours or 6 hours following the treatment period, despite losing almost all of their sleep over the 24 hour period. However, virgin females showed a robust rebound, with the response to the "double" SD resulting in a larger rebound than that which was elicited by either method alone. For virgin females, this could demonstrate an additive effect which occurs following both types of SD.

3.2.2 Baseline Sleep of Females with Differing Social Experiences

To understand if this difference in the post-starvation rebound response between mated females and virgin females was based on mating status alone, I decided to next look at different social groups that had differing past experiences, to see how flies in each group responded to SD.

To investigate this, I first devised a methodology to allow flies to only encounter one of seven different past experiences (outlined in Fig.3.4 and Fig.3.5). Flies were isolated in the pupal stage and then, following eclosion, separated into one of the seven group configurations. After 3-4 days in these social groups, the flies were isolated and put individually in glass tubes to be monitored in the ethoscope.

The groups used in this study were as follows (Fig.3.4 and Fig.3.5): isolated females (A), females kept in a same-sex group of 10 (B), females kept in same-sex pairs (C), a single female kept with a single male (D), females kept in a group with an even sex-ration, with 5 females and 5 males (E), females kept in a male biased group, with 8 males and 2 females (F) and females kept in a female biased group, with 8 females and 2 males (G).

My initial baseline sleep recordings from flies kept in aforementioned groups pro-

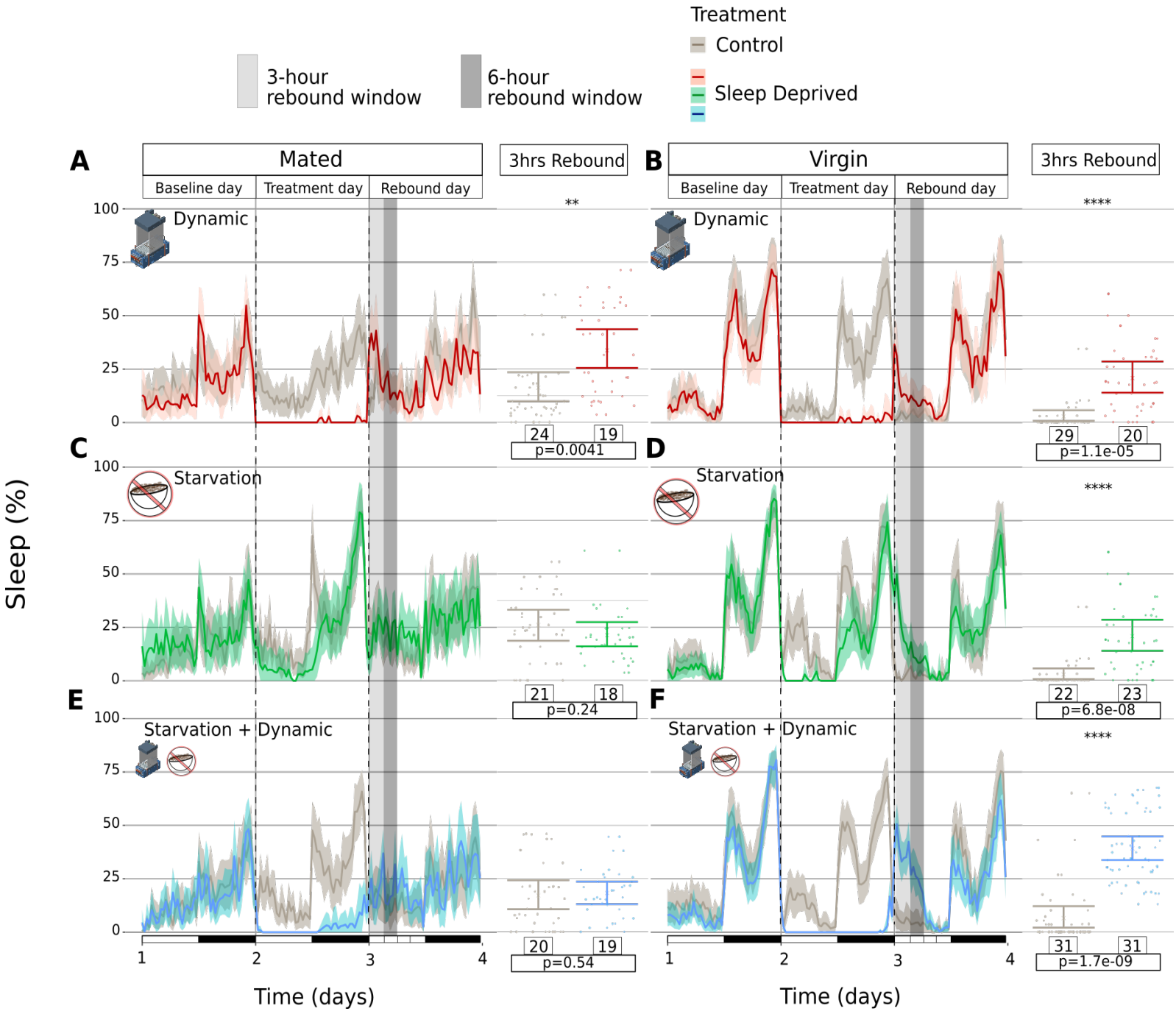


Figure 3.2: *Two sleep deprivation methods and their effect on mated and virgin females* Ethograms for mated and virgin females showing sleep during the baseline day, the SD treatment day and the rebound day with quantification of sleep rebound over the 3 hours following the cessation of treatment. Responses of mated **(A)** and virgin **(B)** females to 24 hours of dynamic SD. Responses of mated **(C)** and virgin **(D)** females to starvation SD. Responses of mated **(E)** and virgin **(F)** females to simultaneous starvation and dynamic SD. Shaded areas show a 95% bootstrap resampling confidence interval around the mean. *N* for each group and significance values (determined using a Wilcoxon rank-sum test comparing control groups to SD groups) for rebound are shown within the figure and data contained is from at least 3 biological repeats per group.

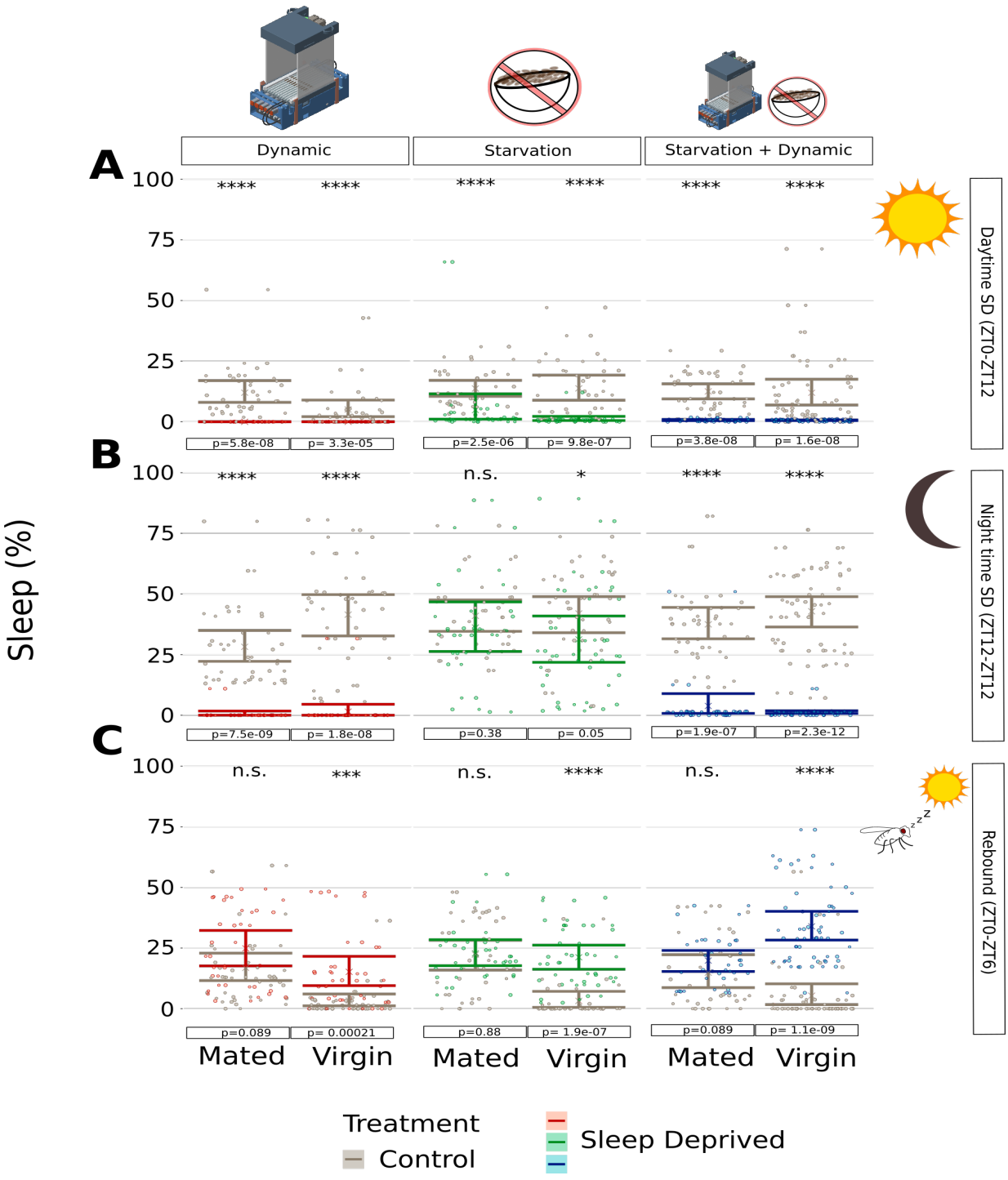


Figure 3.3: *Two sleep deprivation methods and their effect on mated and virgin females* Quantification for mated and virgin females showing sleep during the baseline day, the SD treatment day and the rebound day (over the 6 hours following the cessation of treatment). **(A)** Daytime SD responses of mated and virgin females to dynamic, starvation or simultaneous SD. **(B)** Night-time SD responses of mated and virgin females to dynamic, starvation or simultaneous SD. **(C)** Rebound responses of mated and virgin females within 6 hours following the cessation of dynamic, starvation or simultaneous SD. $N_{Dynamic}$ mated females: control=24, SD=19. $N_{Dynamic}$ virgin females: control=29, SD=20. N_{Starve} mated females: control=21, SD=18. N_{Starve} virgin females: control=22, SD=23. $N_{Simultaneous}$ mated females: control=20, SD=19. $N_{Simultaneous}$ virgin females: control=31, SD=31. Error bars show 95% bootstrap resampling confidence intervals around the mean and significance values are included in the plot (determined using a Wilcoxon rank-sum test comparing control groups to SD groups).

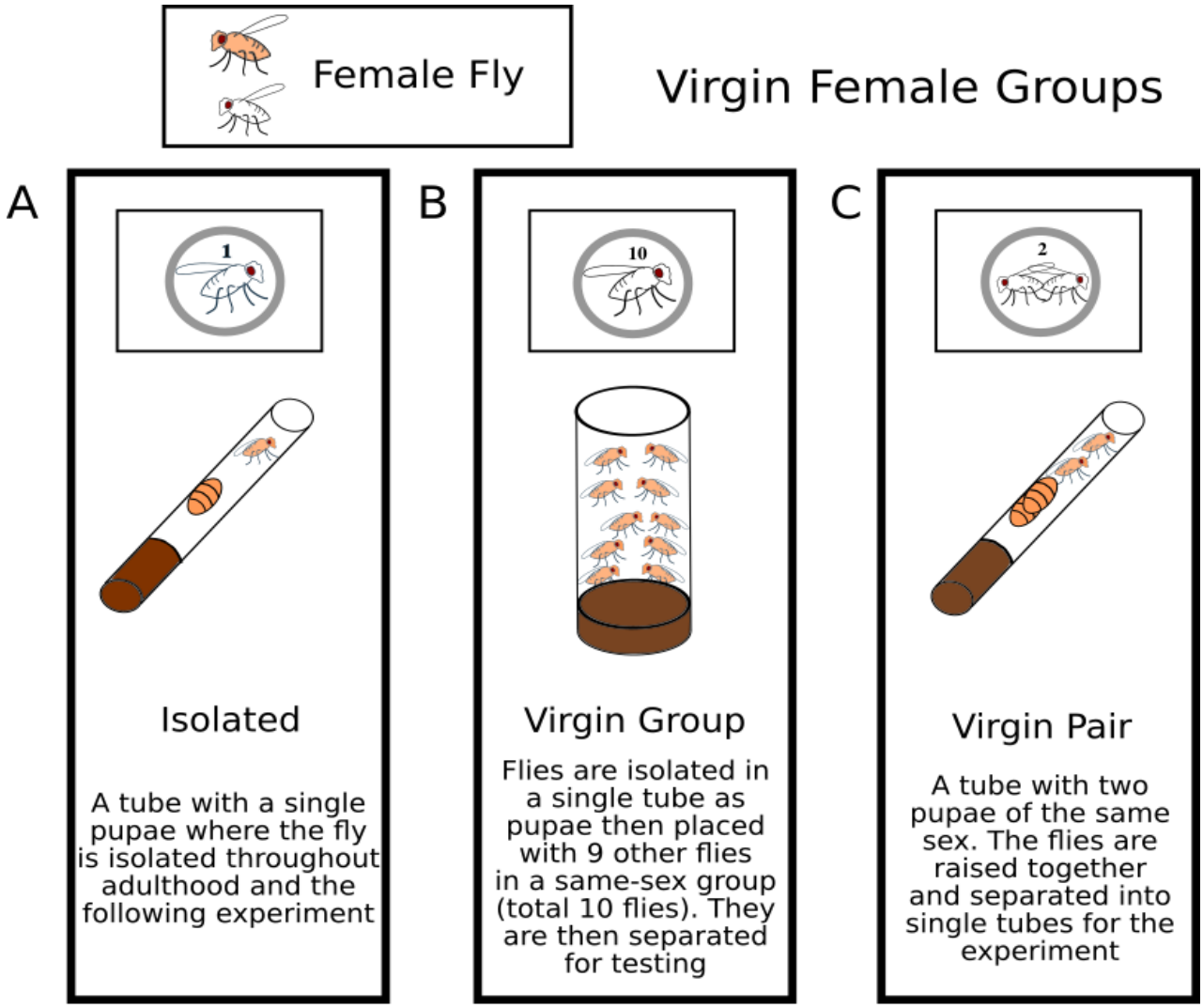


Figure 3.4: *Groups of virgin females* Representative graphic of the different virgin groups used for the modulation of past experiences. **(A)** Isolated females, which were placed as single pupae into glass tubes and maintained as single flies in these tubes throughout adulthood and the experimental protocol. **(B)** Virgin group, consisting of 10 females which were previously isolated as pupae before being transferred to vials with 9 other females before being separated into single tubes for testing. **(C)** Virgin pair, which consisted of two females placed together as pupae in a tube where they were kept throughout adulthood before being separated into single tubes for testing.

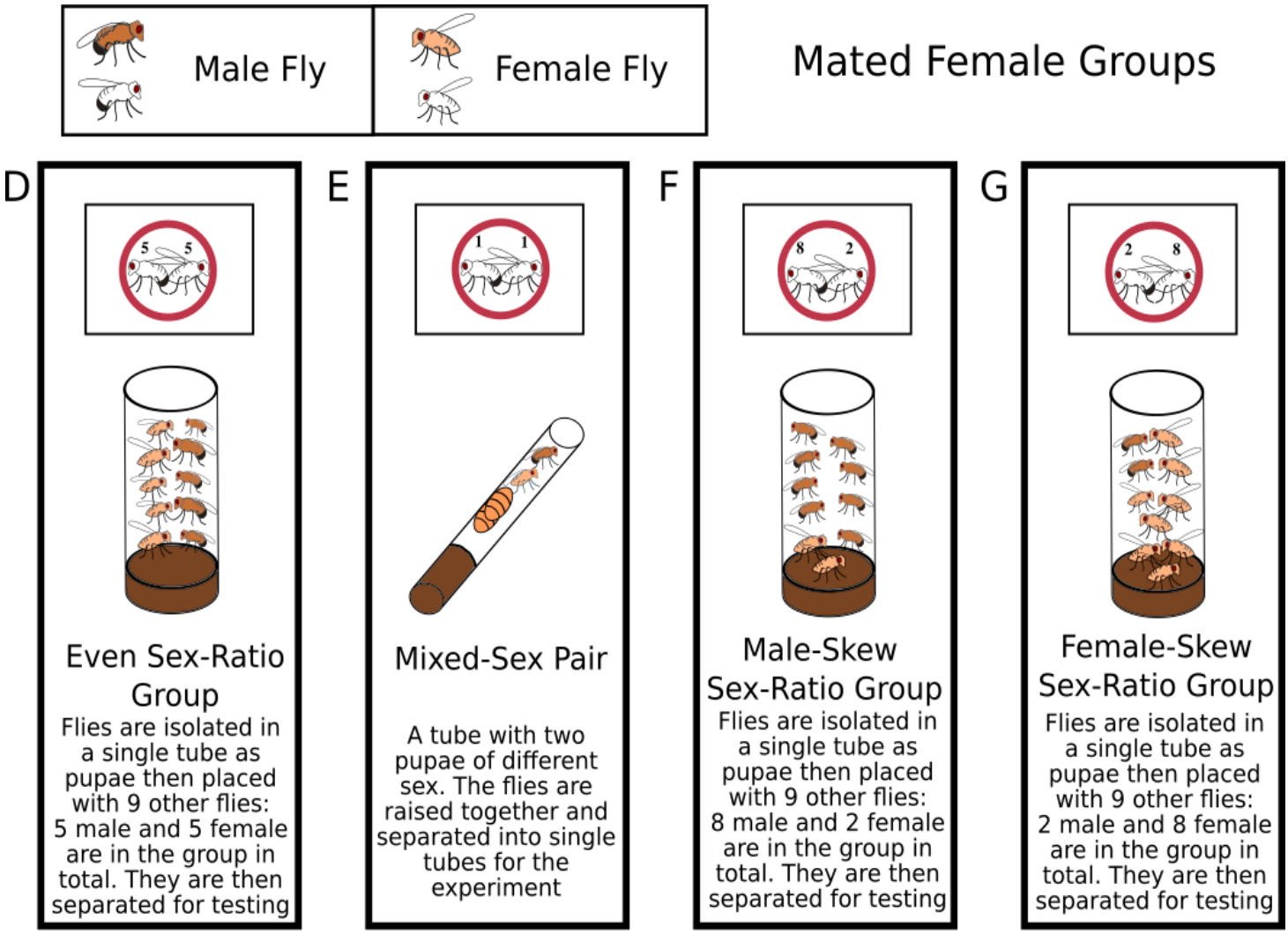


Figure 3.5: *Groups of mated females* Representative graphic of the different mated groups used for the modulation of past experiences. **(D)** Even sex-ratio group, which were initially isolated as pupae before being placed in a group of 10 flies, 5 male and 5 female **(E)** Mixed-sex pair, consisting of a male and a female which were isolated together as pupae and kept together before being separated into single tubes for testing. **(F)** Male-skewed sex-ratio group, consisting of individuals initially isolated as pupae before being placed in a group of 8 males and 2 females. **(G)** Female-skewed sex-ratio group, consisting of individuals initially isolated as pupae before being placed in a group of 8 females and 2 males.

vided data that contradicts published literature. In previous work [271],[149], it has previously been described that mated females sleep less than virgin females, and that isolated flies sleep less than their social counterparts. Fig.3.6 shows a box-plot of sleep over both the daytime (ZT0-ZT12) (Fig.3.6a) and night-time (ZT12-ZT24) (Fig.3.6b) of the baseline day (the day prior to the SD) for females in each of the groups. The quantified differences for sleep between each group can be found in table 3.1.

The virgin female groups (A,B,C), each show significantly different baseline day sleep amounts, when compared to each other. For baseline night sleep, the most interesting groups not to show different sleep amounts between each other were isolated females (A) and females which had experienced the virgin group scenario (B). Despite this, isolated females (A) showed significant differences to all other groups for both baseline day and, except when compared to B, baseline night sleep. Females in the group previously in a same-sex pair slept more during the night-time when compared to all other groups.

The mated female groups (D,E,F,G) had varying levels of differences between each other for sleep.

For the night-time, females which had previously been housed with a single male (E) showed significantly less sleep during the night compared to all other groups.

This demonstrates that past experience has a significant effect on the baseline sleep of flies, both during the day and the night-time, irrespective of mating status.

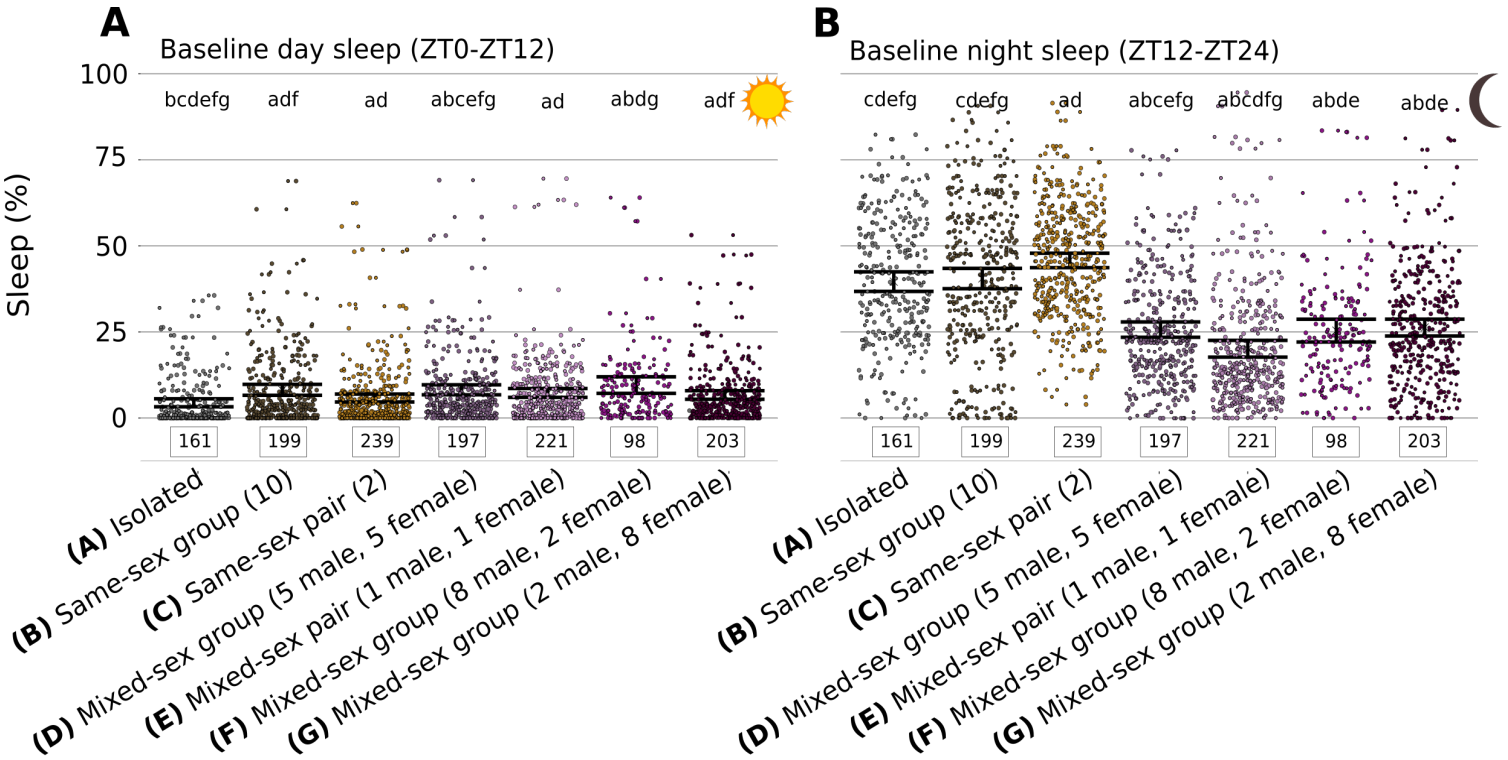


Figure 3.6: *Baseline sleep for females with differing past experiences* Females from the aforementioned groups and their baseline day **(A)** and baseline night **(B)** sleep amounts. Letters above box-plots designate groups against which sleep amount is significantly different (determined using a Wilcoxon rank-sum test comparing all groups against each other and corrected with a FDR adjustment). Error bars show 95% bootstrap resampling confidence intervals around the mean. N for each group is shown within each figure and data is from at least 3 biological repeats per group.

Table 3.1: Differences in mean daytime sleep amounts between CS female groups

Group 1	Group 2	adj. P.value	Significance
A (isolated)	B (virgin group)	2.2e-05	****
A (isolated)	C (virgin pair)	6.3e-10	****
A (isolated)	D (even-SR group)	0.00458	**
A (isolated)	E (ms pair)	6.7e-08	****
A (isolated)	F (male-skewed SR group)	3.9e-09	****
A (isolated)	G (female-skewed SR group)	4.4e-07	****
B (virgin group)	C (virgin pair)	0.10093	n.s.
B (virgin group)	D (even-SR group)	0.03214	*
B (virgin group)	E (ms pair)	0.51438	n.s.
B (virgin group)	F (male-skewed SR group)	0.03395	*
B (virgin group)	G (female-skewed SR group)	0.85300	n.s.
C (virgin pair)	D (even-SR group)	1.4e-05	****
C (virgin pair)	E (ms pair)	0.28047	n.s.
C (virgin pair)	F (male-skewed SR group)	0.30076	n.s.
C (virgin pair)	G (female-skewed SR group)	0.07215	n.s.
D (even-SR group)	E (ms pair)	0.00078	***
D (even-SR group)	F (male-skewed SR group)	8.3e-06	****
D (even-SR group)	G	0.05362	**
E (ms pair)	F (male-skewed SR group)	0.05362	n.s.
E (ms pair)	G (female-skewed SR group)	0.48119	n.s.
F (male-skewed SR group)	G (female-skewed SR group)	0.01292	*

3.2.3 Homeostatic Rebound and Starvation of Females with Differing Social Experiences

Following the results with the initial experiments using the starvation and dynamic methods to sleep deprive the flies, I decided to apply these methods to flies raised in the different social groups described in the previous section. Firstly, I looked at applying the starvation SD method to females from each of the seven groups.

An ethogram of the data for females previously kept in different social groups is shown in Fig.3.7 with a quantification in Fig.3.8 (where the statistical differences were determined using a Wilcoxon rank-sum test comparing control groups to SD groups). Females from

Table 3.2: Differences in mean night-time sleep amounts between CS female groups

Group 1	Group 2	adj. P.value	Significance
A (isolated)	B (virgin group)	0.54650	n.s.
A (isolated)	C (virgin pair)	6.7e-13	****
A (isolated)	D (even-SR group)	0.00121	**
A (isolated)	E (ms pair)	$\leq$ 2e-16	****
A (isolated)	F (male-skewed SR group)	5.5e-10	****
A (isolated)	G (female-skewed SR group)	8.6e-12	****
B (virgin group)	C (virgin pair)	1.4e-12	****
B (virgin group)	D (even-SR group)	0.01831	*
B (virgin group)	E (ms pair)	$\leq$ 2e-16	****
B (virgin group)	F (male-skewed SR group)	2.4e-09	****
B (virgin group)	G (female-skewed SR group)	1.1e-11	****
C (virgin pair)	D (even-SR group)	$\leq$ 2e-16	****
C (virgin pair)	E (ms pair)	5.0e-06	****
C (virgin pair)	F (male-skewed SR group)	0.87792	n.s.
C (virgin pair)	G (female-skewed SR group)	0.75416	n.s.
D (even-SR group)	E (ms pair)	$\leq$ 2e-16	****
D (even-SR group)	F (male-skewed SR group)	$\leq$ 2e-16	****
D (even-SR group)	G	$\leq$ 2e-16	****
E (ms pair)	F (male-skewed SR group)	0.00038	***
E (ms pair)	G (female-skewed SR group)	3.6e-06	****
F (male-skewed SR group)	G (female-skewed SR group)	0.63992	n.s.

all groups showed significant daytime SD induced by starvation, compared to controls from the same group which were not starved, regardless of whether the female was mated or not mated. For night-time SD, no mated female from any group showed significant night-time sleep loss due to starvation. However, apart from those females from the same-sex group scenario, females from both other virgin female groups showed a small but significant SD during the night due to the starvation. Similar to the results shown in Fig.3.2, mated females showed no significant rebound during the day following the starvation period. For all groups, except for females in the male and female pair (E), there was negative rebound demonstrated during the 3 hours of the rebound day, and in the case of females from the male-skew group (F), this negative rebound persisted

to the point that there was significant negative rebound during the 6 hours following SD. This work demonstrates that starvation-induced sleep loss robustly leads to an extended rebound for a period of up to 6 hours for virgin females, regardless of their prior social experience. It also demonstrates that a lack of rebound in mated females is robust and is not affected by prior social experience.

Interestingly, the presence and quantity of sleep lost during the starvation period does not seem to be a predictor of whether a female fly will rebound following starvation induced SD.

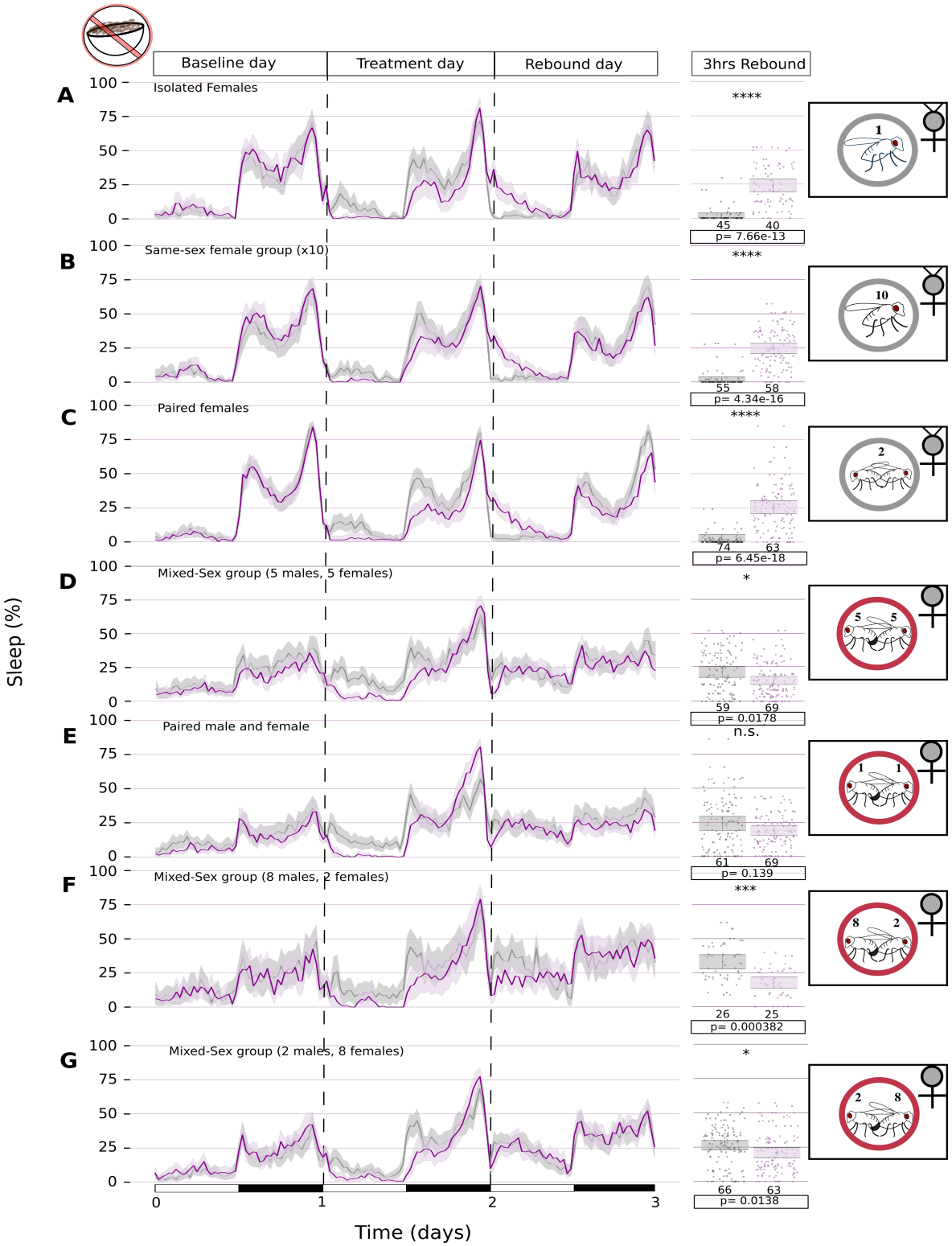


Figure 3.7: *Ethogram plots for females from prior experience groups subject to starvation SD* Ethograms for females from each of the 7 prior experience groups and the rebound sleep amounts of starved females in each group compared to fed controls. **(A)** isolated females **(B)** females from the same-sex female group **(C)** females from the paired female group **(D)** females from the mixed-sex even sex-ratio group **(E)** females from the male and female pair **(F)** females from the male-skewed mixed-sex group **(G)** females from the female-skewed mixed-sex group. Shaded areas show a 95% bootstrap resampling confidence interval around the mean. *N* for each group and significance values for rebound (determined using a Wilcoxon rank-sum test comparing control groups to SD groups) are shown within the figure and data contained is from at least 3 biological repeats per group.

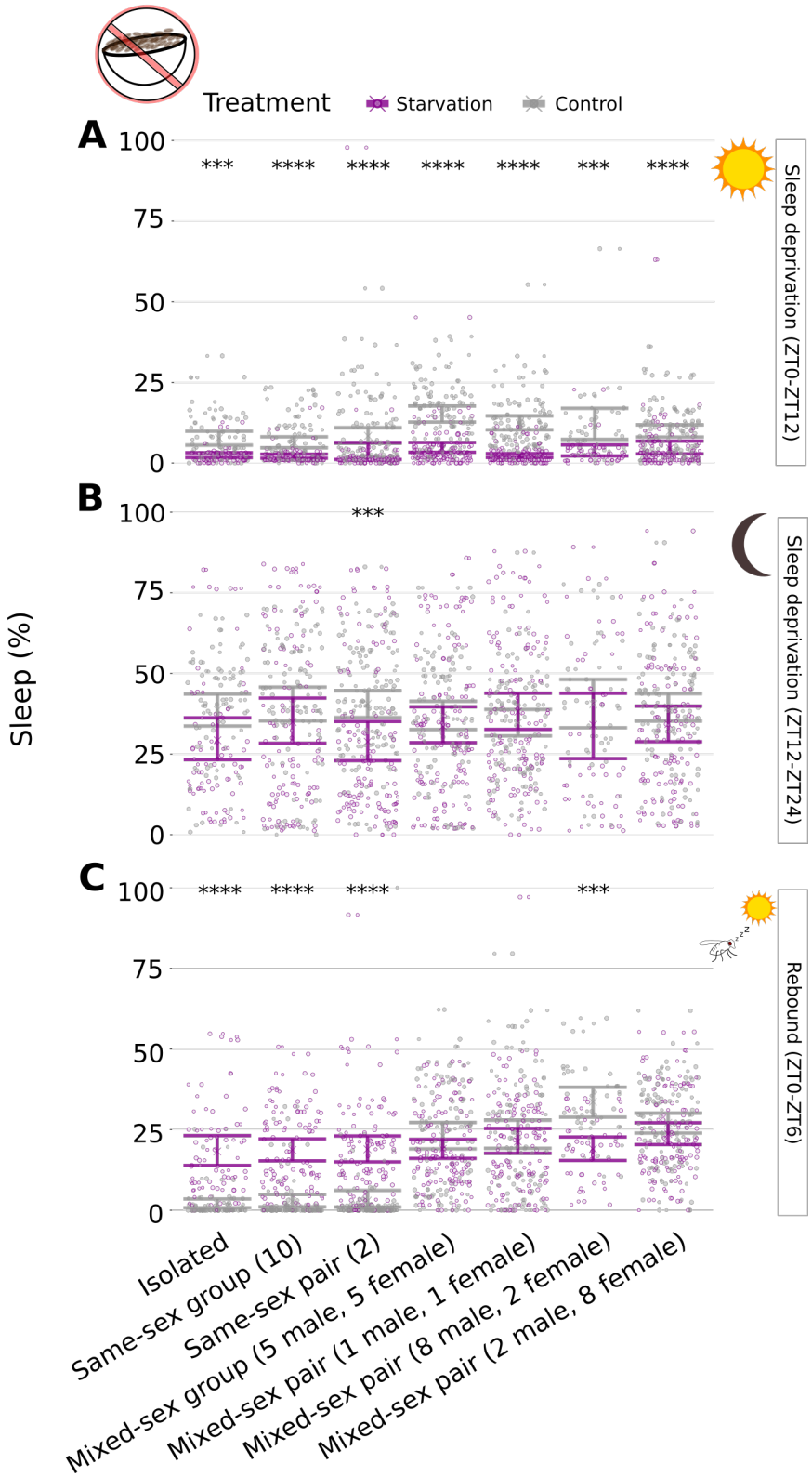


Figure 3.8: *Box-plot quantification for day SD, night SD and the rebound in the 6 hours post starvation treatment* Box-plot quantifications for different time periods for all females in groups with differing past experiences. **(A)** Quantification of day-time (ZT0-ZT12) SD. **(B)** Quantification of night-time (ZT12-ZT24) SD. **(C)** Quantification of rebound in the 6 hours post starvation treatment (ZT0-ZT6). $N_{Isolated}$ control=45, SD=40. $N_{Same\text{-}sex\ group}$ control=55, SD=58. $N_{Paired\ females}$ control=74, SD=63. $N_{Mixed\text{-}sex\ group\ -\ 5\ fem,\ 5\ male}$ control=59, SD=69. $N_{Paired\ male\ and\ female}$ control=61, SD=69. $N_{Mixed\text{-}sex\ group\ -\ 2\ fem,\ 8\ male}$ control=26, SD=25. $N_{Mixed\text{-}sex\ group\ -\ 8\ fem,\ 2\ male}$ control=66, SD=63. Error bars show 95% bootstrap resampling confidence intervals around the mean.

3.2.4 Homeostatic Rebound and Dynamic Sleep Deprivation

Following the work identifying the dichotomy between the response of mated and virgin females to starvation-induced SD, I studied the effect of dynamic SD on mated and virgin females previously kept in one of the seven social groups.

In the case of the dynamic method, no differences were seen between the rebound demonstrated by mated females or virgin females, as females from every group showed significant rebound (compared to slept controls) following the 24 hour period of sleep loss, as well as significant sleep deprivation (as quantified in Fig.3.10, where significance values were determined using a Wilcoxon rank-sum test comparing control groups to SD groups).

3.3 Summary

- Flies with differing prior experiences show differences in baseline sleep.

- Mated and virgin females show significant sleep rebound after dynamic SD, regardless of prior social experience.

- Virgin, but not mated, females show rebound after starvation SD, regardless of prior social experience.

- Simultaneous starvation and dynamic SD leads to rebound in virgin females but not mated females.

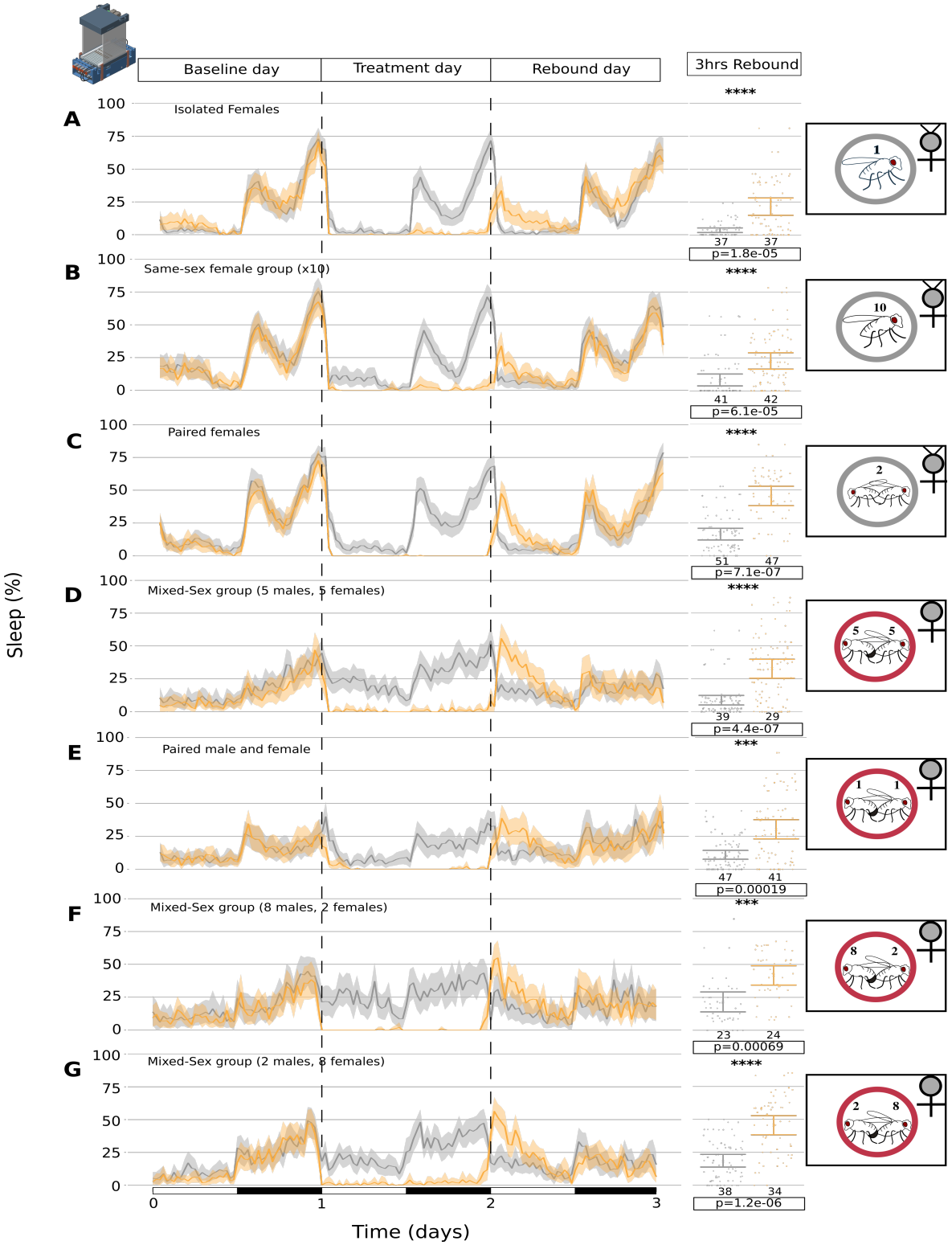


Figure 3.9: *Ethogram plots for females from prior experience groups subject to dynamic SD.* Ethograms for females from each of the 7 prior experience groups and the rebound sleep amounts of dynamically sleep deprived females in each group compared to slept controls. **(A)** isolated females **(B)** females from the same-sex female group **(C)** females from the paired female group **(D)** Females from the mixed-sex even sex-ratio group **(E)** females from the male and female pair **(F)** females from the male-skewed mixed-sex group **(G)** females from the female-skewed mixed-sex group. Shaded areas show a 95% bootstrap resampling confidence interval around the mean. *N* for each group and significance values for rebound (determined using a Wilcoxon rank-sum test comparing control groups to SD groups) are shown within the figure and data contained is from at least 3 biological repeats per group.

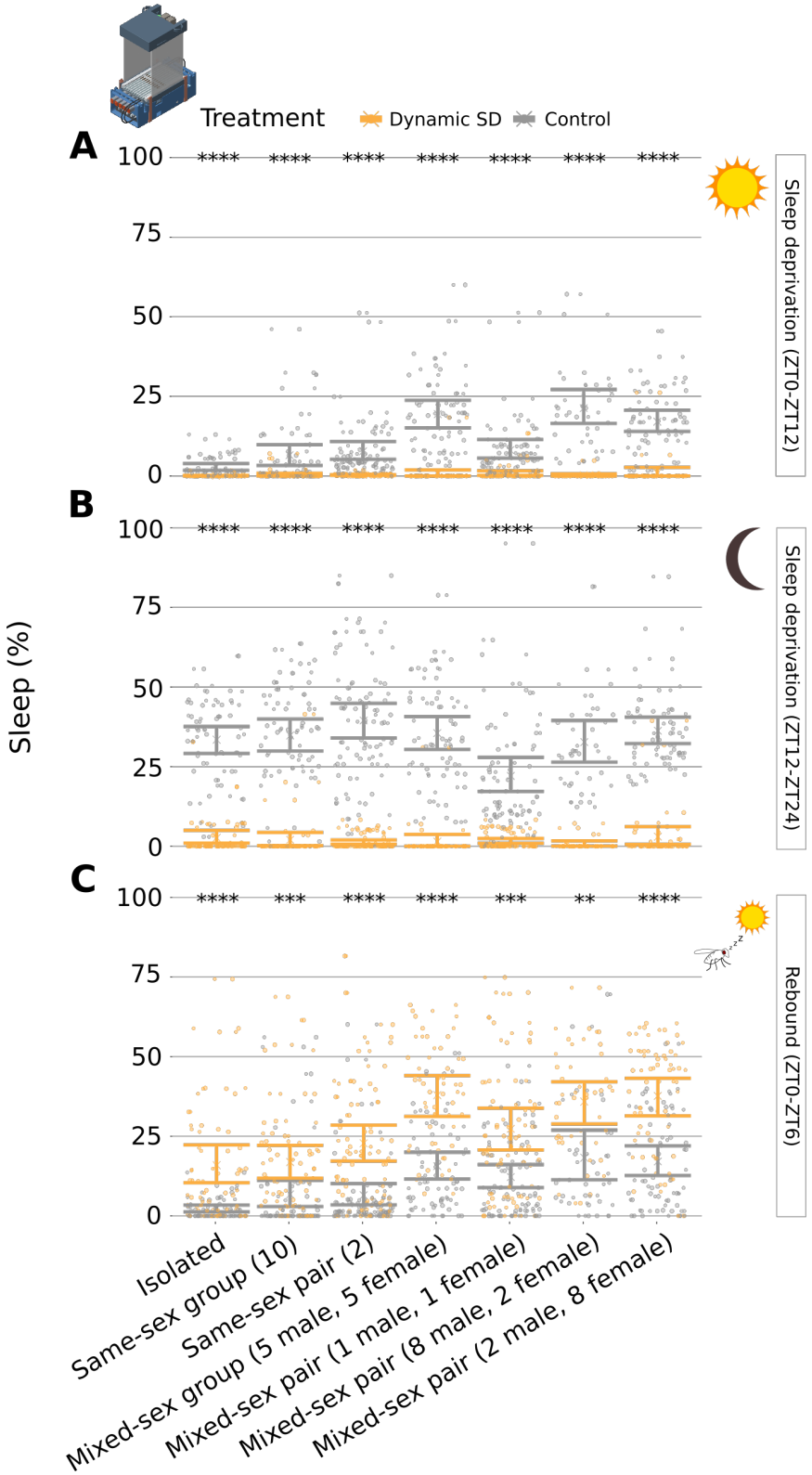


Figure 3.10: *Box-plot quantification for day SD, night SD and the rebound in the 6 hours post dynamic SD treatment* Box-plot quantifications for different time periods for all females in groups with differing past experiences. **(A)** Quantification of day-time (ZT0-ZT12) SD (B) Quantification of night-time (ZT12-ZT24) SD (C) Quantification of rebound in the 6 hours post starvation treatment (ZT0-ZT6). $N_{Isolated}$ control=37, SD=37. $N_{Same\text{-}sex\ group}$ control=41, SD=42. $N_{Paired\ females}$ control=51, SD=47. $N_{Mixed\text{-}sex\ group\ -\ 5\ fem,\ 5\ male}$ control=39, SD=29. $N_{Paired\ male\ and\ female}$ control=47, SD=41. $N_{Mixed\text{-}sex\ group\ -\ 2\ fem,\ 8\ male}$ control=23, SD=24. $N_{Mixed\text{-}sex\ group\ -\ 8\ fem,\ 2\ male}$ control=38, SD=34. Error bars show 95% bootstrap resampling confidence intervals around the mean.

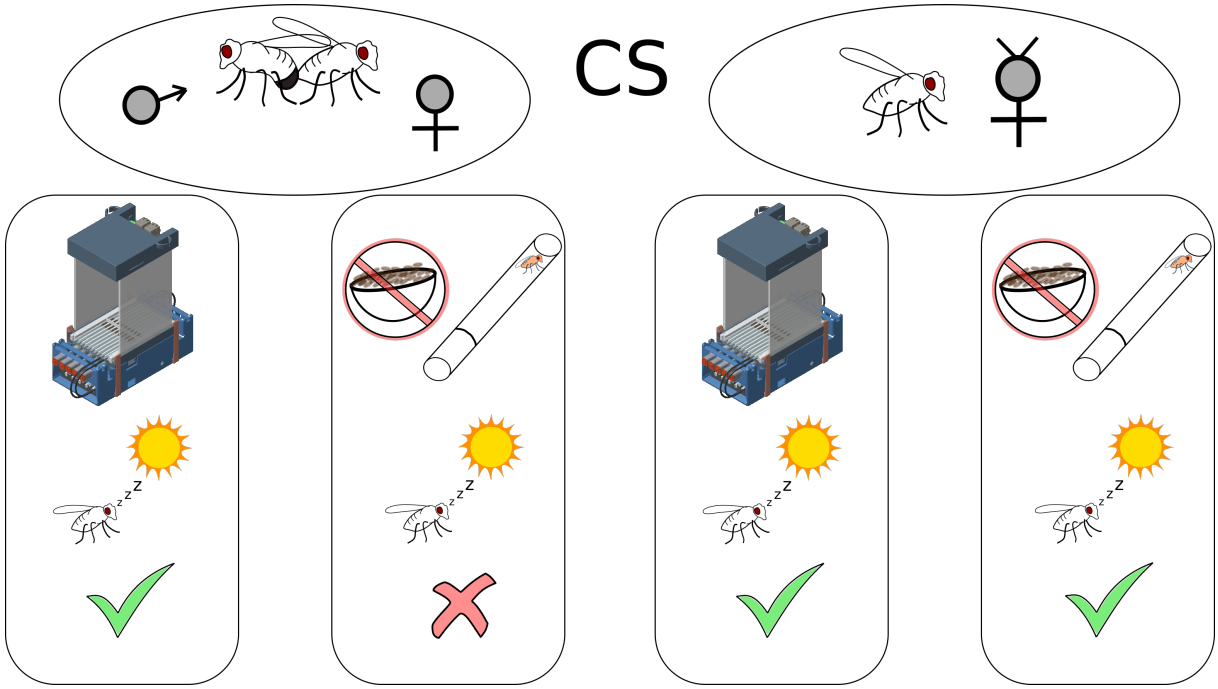


Figure 3.11: *Diagram representing the findings of section 3.2* CS females exhibit different rebound responses to starvation SD but not dynamic SD based on mating status.

3.4 Investigating Sleep Rebound with Two Sleep Deprivation Methods in Stress Mutants

Following my work with wild-type females, I chose to study the impact of stress-related signalling on sleep. As highlighted in the introduction, a large amount of work in the sleep field identifies stress as possible reason for sleep need. In an early paper looking at the modulation of sleep in *Drosophila* stress was implicated as a key component of SD. Shaw et al. [417] found that by activating genes encoding chaperone proteins, which generally protect against the effects of high temperature, the flies could withstand the lethal effects of SD. It has been shown that proteins involved in stress regulation, be that those that regulate temperature, oxidative stress [191], or stress of infection [463] are also involved in the regulation of sleep. I hypothesised that flies showing resistance to different types of stress may also show resistance to various forms of SD.

The neuropeptide corazonin (Crz), which I introduced comprehensively in section 1.9.2, was one such stress candidate. As previously discussed, male and female flies that have a genetic knock-down or knock-out of the neuropeptide Crz or its receptor, CrzR, have been shown to have increased resistance to starvation-induced stress [254], oxidative stress [514] and dessication [508]. Based on this stress resistance, I tested flies with either a knockout or knockdown of Crz or CrzR.

3.4.1 Identification of a neuropeptide and its receptor with expression in key sleep regulating areas in the brain

To understand how Crz and CrzR are expressed in the *Drosophila* brain, I used the *trans*-Tango method [450], a technique used to visualise neurons and their synaptic connections in the brain. This technique is a method of anterograde trans-synaptic tracing, which allows both the target neurons (defined by their Gal4 line) and post-synaptic connections, to be simultaneously visualised (for a detailed description of this technique, see section 2.26.2). Figure 3.12 shows CrzR neurons in the posterior and anterior of the brain. CrzR neurons are visualised in green and the post synaptic connections in cyan, with a structural stain in magenta.

It can be seen that CrzR expression is wide-spread throughout the brain, with expression in key CX region, the FSB and the associative region, the MB. There is also expression in other regions, such as the SEZ, the optic lobes, the protocerebrum. The expression of the post-synaptic connections is more localised; strong fluorescence is seen in the dFSB and some lower layers of the FSB. There are also some connections in the upper regions of the anterior brain.

Crz displayed a different expression throughout the brain. The strongest GFP expres-

sion (indicating the presence of Crz) was in the OL, with a smaller amount of expression in the central brain, seeming to circle the FSB and the lobes of the MB. Post-synaptic connections are much more widespread, with some connections in the OL, and more in the central brain, surrounding the lobes of the MB, with some connections in the dorsal part of the brain in areas known to harbour dopaminergic neurons.

The differing expression of Crz when compared to CrzR is somewhat unusual, as it is usually the case that a neuropeptide is expressed in similar regions to that of its receptor. However, the dissections of fly brains with *trans*-Tango expression were only carried out at one time point during the day (during the morning between ZT0 and ZT3). With this in mind, it is possible that the expression of Crz follows a circadian rhythm and that expression of the peptide changes throughout the day.

When the expression data for the Crz peptide and its receptor, CrzR, were compared, CrzR was deemed the more compelling candidate to study for its ability to modulate sleep. This was due to the expression data which showed CrzR is expressed in several keep sleep regulatory regions in the fly brain: the γ-lobes of the MB [**?**] and some layers of the FSB [105]. Further to this, it was seen that post-synaptic connections also present in the FSB, with possible connections in dorsal dopaminergic neurons. Comparatively, expression of Crz was mainly localised to the optic lobes, with very little expression in the central brain.

Due to the interesting and strong expression pattern of CrzR expression and post-synaptic connections being identified in known sleep regulatory regions of the fly brain, I decided to study sleep in (1) CrzR mutant flies and in (2) CrzR knockdown flies. The latter was done using targeted RNAi knockdown by exploitation of the UAS/Gal4 system (for a detailed explanation of this method, see section 2.26.1).

Some experiments were also done with a knockdown of Crz. However, based on pre-

liminary data, the sleep phenotype produced with knockdown and knockout of CrzR was deemed more interesting.

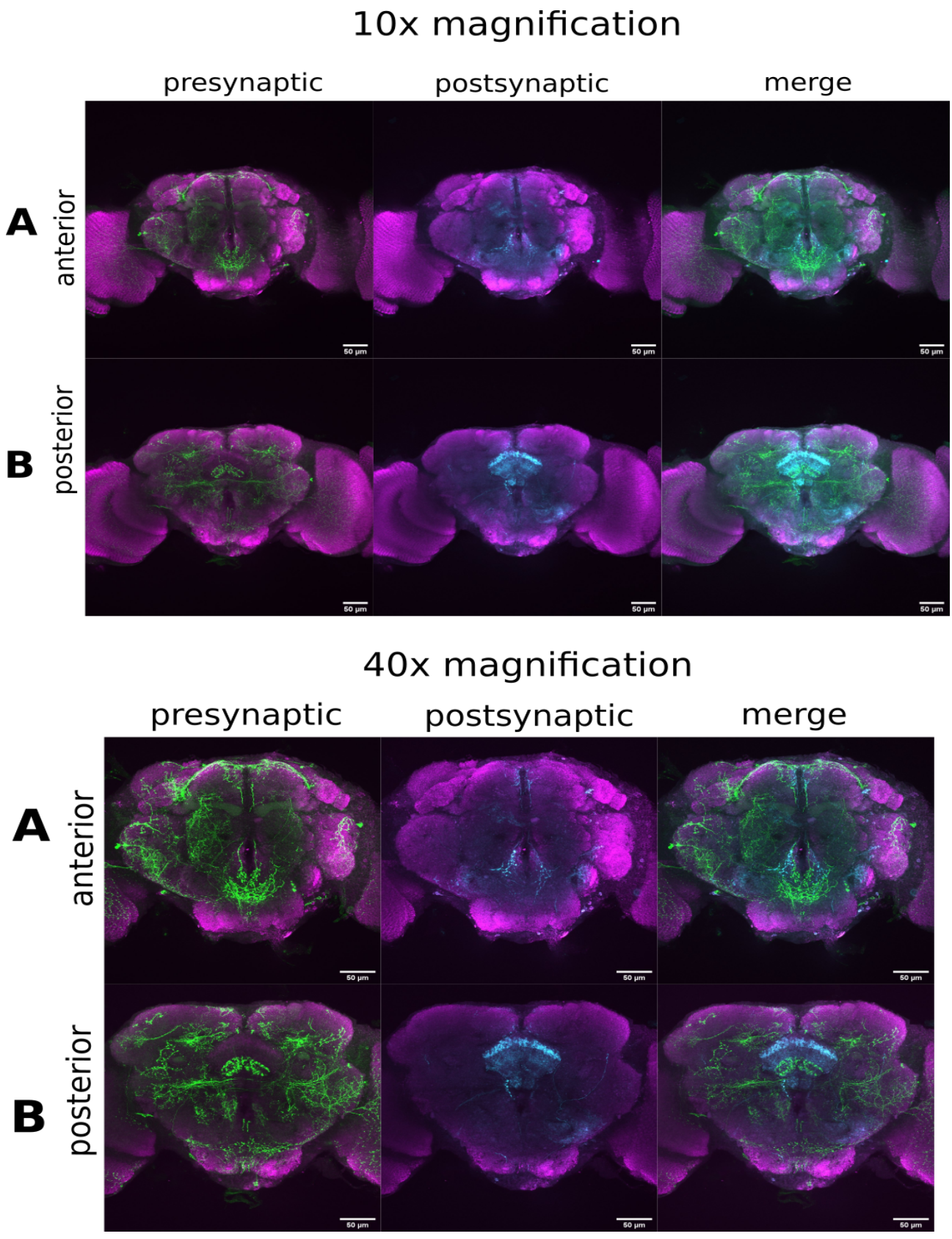


Figure 3.12: *trans-Tango expression of CrzR-Gal4. trans*-Tango assay showing presynaptic connections (green), post-synaptic connections (cyan) and merged images for CrzR-Gal4 in the central *Drosophila* brain at 10x and 40x magnification for the **(A)** anterior part of the brain **(B)** posterior part of the brain. Images shown are representative of what was seen in 8 brains stained with the same protocol. Scale bars, 50μm.

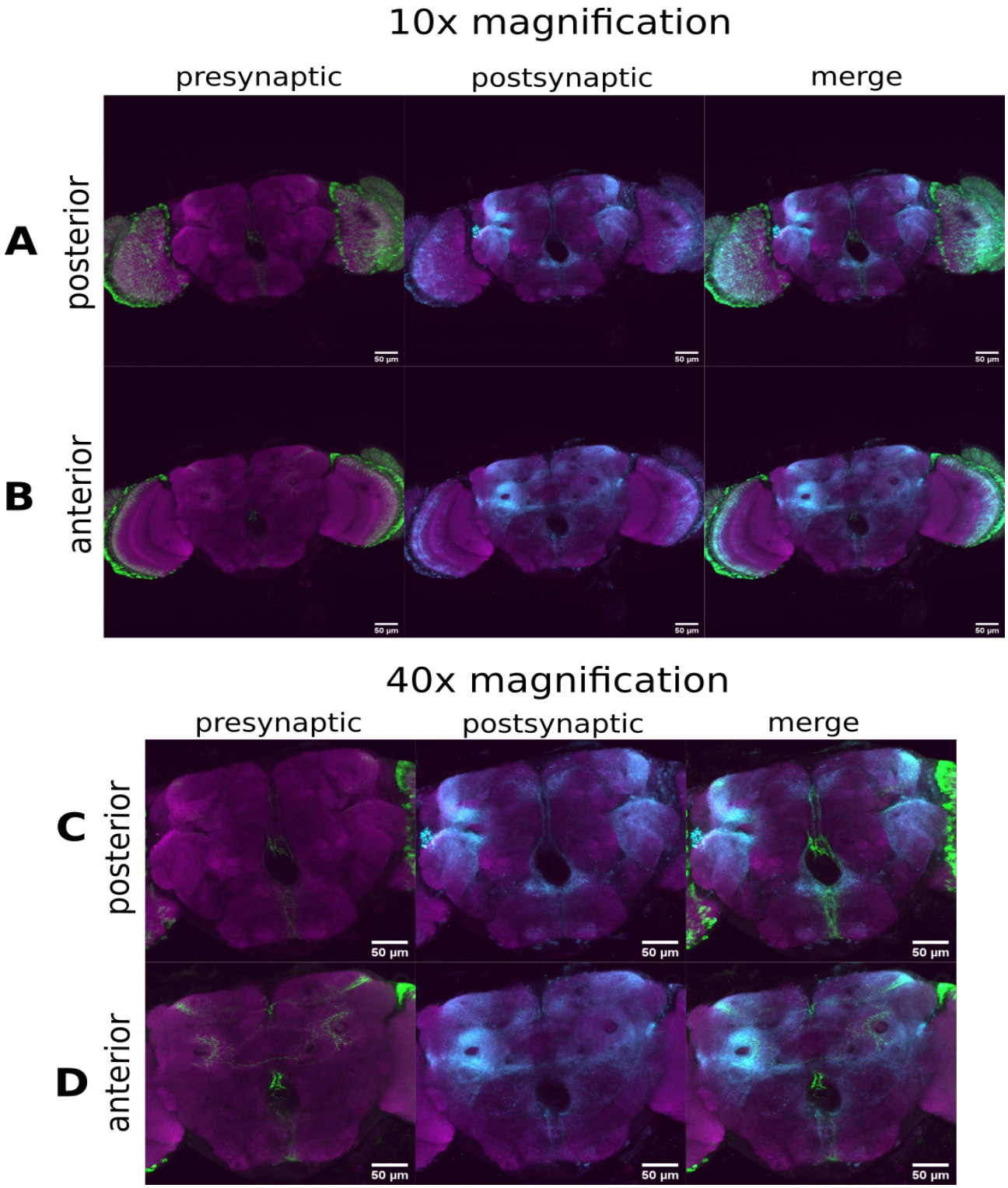


Figure 3.13: *trans-Tango expression of Crz-Gal4.* *trans*-Tango assay showing presynaptic connections (green), post-synaptic connections (cyan) and merged images for Crz-Gal4 in the central *Drosophila* brain at 10x and 40x magnification for the **(A)** anterior part of the brain **(B)** posterior part of the brain. Images shown are representative of what was seen in 6 brains stained with the same protocol. Scale bars, 50μm.

3.4.2 Baseline Sleep of Females with Differing Social Experiences in CrzR Mutants

To understand how baseline sleep in CrzR mutants may be different to that of the wild-type control (CS) and how this might compare depending on previous social experience, I applied the protocol I had applied to wild-type females, where the flies were split into different social groups, resulting in either mated or virgin status.

As can be seen in Fig.3.14, for baseline day sleep, CrzR mutant females from all social groups showed a large increase in sleep when compared to their CS control counterparts, regardless of whether they were mated or virgin. For night-time sleep, CrzR mutant females from all groups, bar one, showed an increase in sleep. The group which did not show altered sleep was the group of females that had been in the male-skewed sex ratio group. Statistical comparisons can be found in tables 3.3 and 3.4 and were determined using a Wilcoxon rank-sum test comparing control groups to SD groups. Interestingly, although I have shown that isolated CS females sleep less during the daytime than females with any other prior group experience, CrzR isolated females did not show the same result. Although the statistical comparisons were not done for CrzR mutant female groups against each other, it is pertinent to observe that these flies do not follow the same pattern of sleep as their wild-type counterparts.

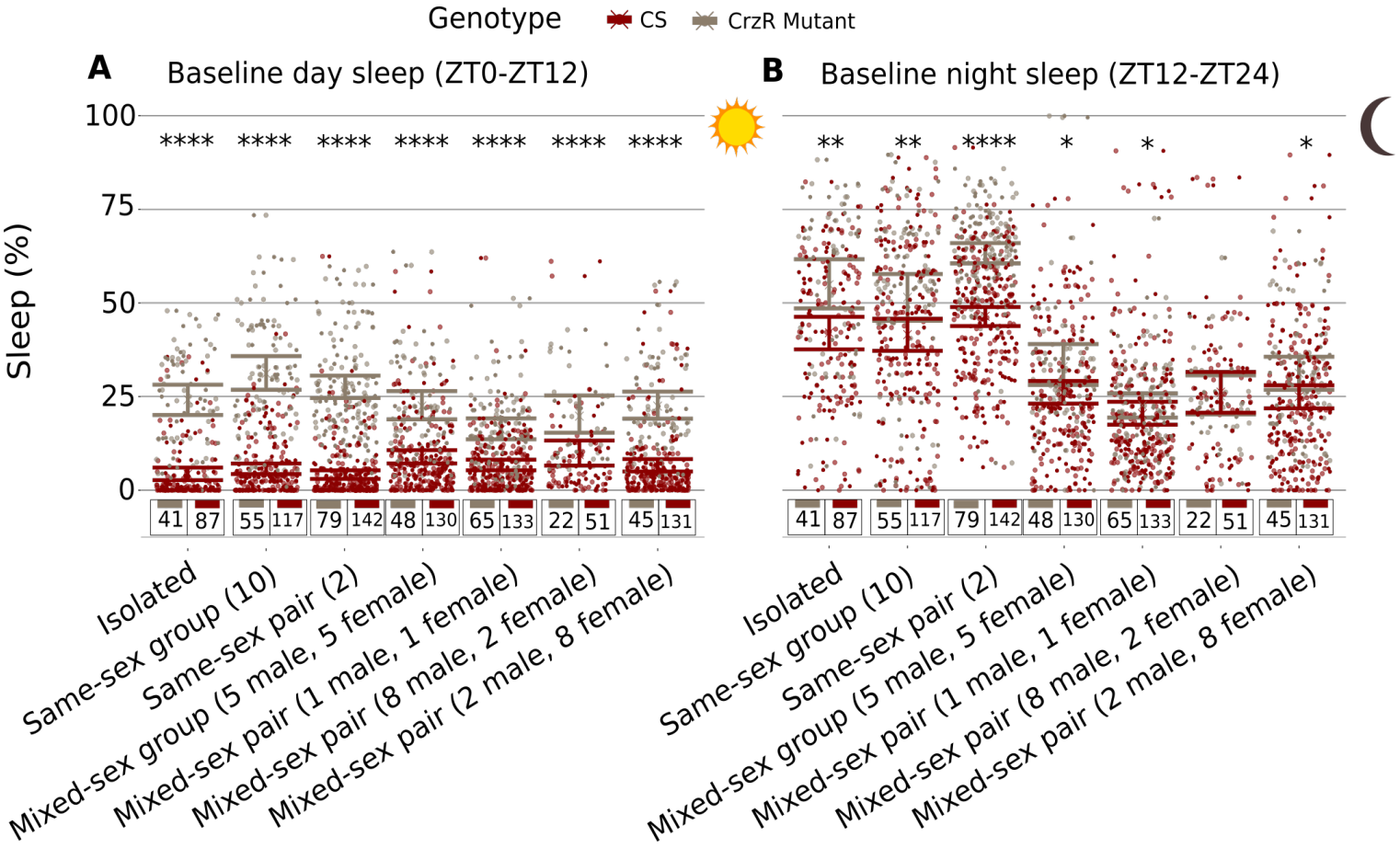


Figure 3.14: *Basline sleep for CrzR Mutant females compared to CS females from differing social groups* Quantification of day-time **(A)** and night-time **(B)** sleep for females from prior experience groups for CrzR mutant females or CS females. Error bars show 95% bootstrap resampling confidence intervals around the mean. *N* for each group is shown within each figure and data is from at least 3 biological repeats per group.

Table 3.3: Differences in mean day-time sleep amount between CS female groups and CrzR mutant female groups

Group 1	Group 2	adj. P.value	Significance
A (isolated) CrzR mutant	A (isolated) CS	4.6e-14	****
B (virgin group) CrzR mutant	B (virgin group) CS	$\leq$ 2e-16	****
C (virgin pair) CrzR mutant	C (virgin pair) CS	$\leq$ 2e-16	****
D (even-SR group) CrzR mutant	D (even-SR group) CS	5e-12	****
E (ms pair) CrzR mutant	E (ms pair) CS	5.4e-11	***
F (male-skewed SR group) CrzR mutant	F (male-skewed SR group) CS	9.6e-5	****
G (female-skewed SR group) CrzR mutant	G (female-skewed SR group) CS	1.5e-14	****

Table 3.4: Differences in mean night-time sleep amount between CS female groups and CrzR mutant female groups

Group 1	Group 2	adj. P.value	Significance
A (isolated) CrzR mutant	A (isolated) CS	0.0014	**
B (virgin group) CrzR mutant	B (virgin group) CS	0.005	**
C (virgin pair) CrzR mutant	C (virgin pair) CS	1.6e-14	****
D (even-SR group) CrzR mutant	D (even-SR group) CS	0.015	*
E (ms pair) CrzR mutant	E (ms pair) CS	0.036	*
F (male-skewed SR group) CrzR mutant	F (male-skewed SR group) CS	0.94	n.s
G (female-skewed SR group) CrzR mutant	G (female-skewed SR group) CS	0.021	*

3.4.3 Investigating Sleep Rebound with Two Sleep Deprivation Methods in Corazonin Receptor Mutant Flies

My first behavioural assays using CrzR mutant flies aimed to assess the effect of the two SD methods, dynamic and starvation, as described in section 3.2. To this end, I performed the experiment analogously to those done on CS flies.

As can be seen from the ethogram and box-plot quantification of the results (Fig.3.15 and Fig.3.16, with significance values determined using a Wilcoxon rank-sum test comparing control groups to SD groups), both mated and virgin CrzR mutants showed significant rebound following the dynamic SD method. Following starvation SD, the mated female group responded in a similar way to that seen in wild-type flies. These mated females did not show significant rebound, and in some cases showed a significant negative rebound, where they slept less than the control groups. A difference is seen when comparing the starved CrzR mutant virgin females to their wild-type counterparts. Whereas wild-type virgin females showed a significant rebound lasting more than 6 hours post starvation-induced SD, the virgin females from the CrzR mutant group did not show a significant rebound in the 6 hours following the SD period. When the two SD methods are combined, it can be seen that the CrzR mated females behaved similar to wild-type females, with no significant rebound following the double SD. However, the CrzR mutant virgin females did not mimic that seen in the wild-type group, with no significant rebound in the 3 or 6 hours following the double SD, despite losing all sleep during the preceding SD period. It can be concluded that although the starvation-induced SD seems to override that caused by dynamic SD when determining whether or not rebound will occur, the CrzR mutant shows mating-dependent differences when compared to wild-type virgin females.

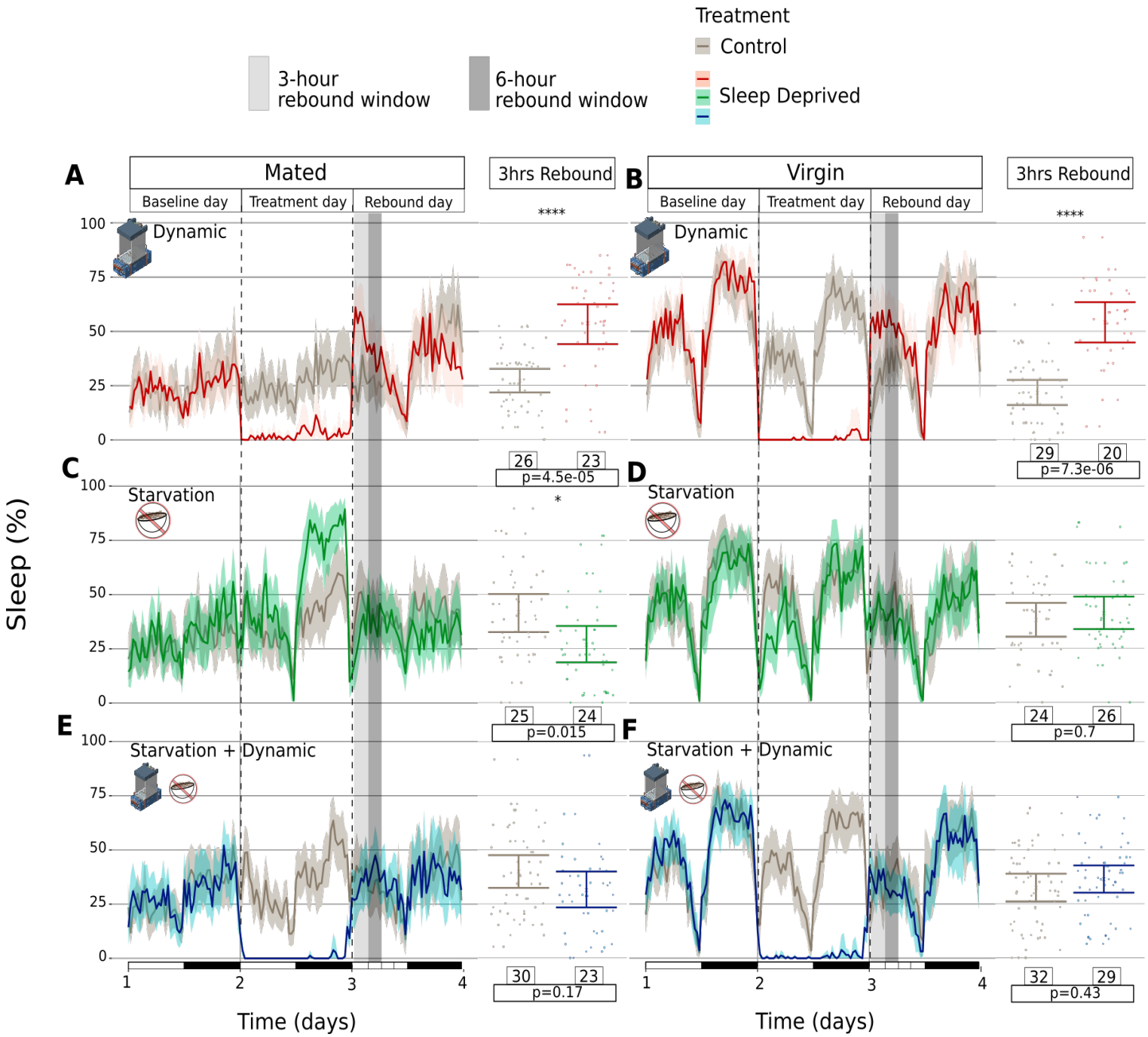


Figure 3.15: *Two sleep deprivation methods and their effect on CrzR mated and virgin females.* Ethograms for mated and virgin females showing sleep during the baseline day, the SD treatment day and the rebound day with quantification of sleep rebound over the 3 hours following the cessation of treatment. Responses of mated **(A)** and virgin **(B)** females to 24 hours of dynamic SD. Responses of mated **(C)** and virgin **(D)** females to starvation SD. Responses of mated **(E)** and virgin **(F)** females to simultaneous starvation and dynamic SD. Shaded areas show a 95% bootstrap resampling confidence interval around the mean. *N* for each group and significance values for rebound are shown within the figure and data contained is from at least 3 biological repeats per group.

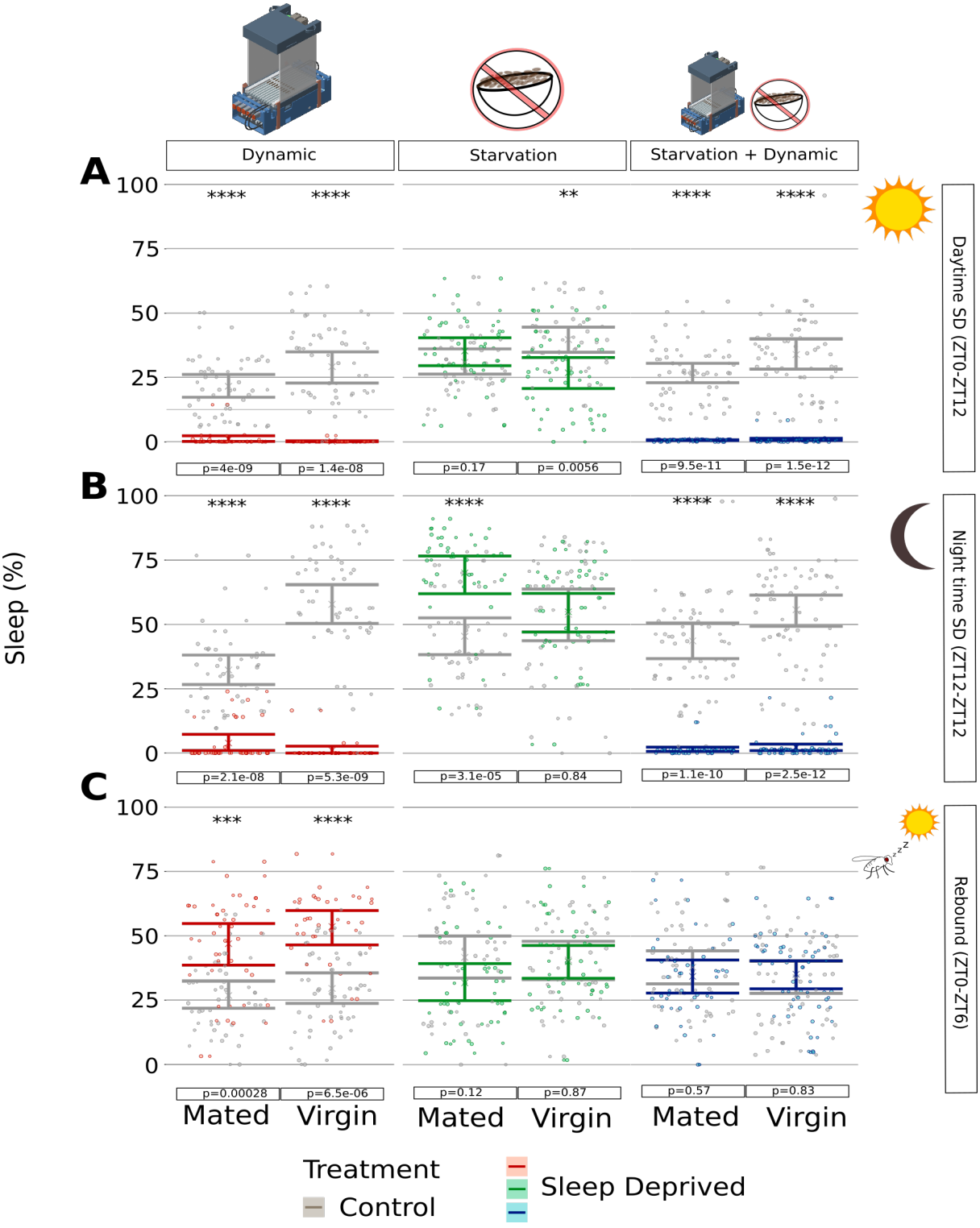


Figure 3.16: *Box-plots of different time periods of sleep deprivation for CrzR mated or virgin females exposed to two different SD methods.* Quantification for CrzR mutant mated and virgin females showing sleep during the baseline day, the SD treatment day and the rebound day (over the 6 hours following the cessation of treatment). **(A)** Daytime SD responses of CrzR mutant mated and virgin females to dynamic, starvation or simultaneous SD. **(B)** Night-time SD responses of CrzR mutant mated and virgin females to dynamic, starvation or simultaneous SD. **(C)** Rebound responses of CrzR mutant mated and virgin females within 6 hours following the cessation of dynamic, starvation or simultaneous SD. $N_{Dynamic}$ mated females: control=26, SD=23. $N_{Dynamic}$ virgin females: control=29, SD=20. N_{Starve} mated females: control=25, SD=24. N_{Starve} virgin females: control=24, SD=26. $N_{Simultaneous}$ mated females: control=30, SD=23. $N_{Simultaneous}$ virgin females: control=32, SD=29. Error bars show 95% bootstrap resampling confidence intervals around the mean.

3.4.4 Homeostatic Rebound and Starvation in CrzR mutant Females with Different Social Experiences

Following the interesting result in the previous section, I went on to follow a similar protocol as with the wild-type females. I arranged CrzR female flies into the social groups previously explained and carried out starvation-induced SD to determine if there were differences between the groups.

As explained in section 3.4.3, I did not see significant rebound in virgin females following starvation (see Fig.3.15). However, when testing the full panel of seven social groups, I found a small yet significant rebound in flies from each of the virgin female groups. Similar to what was seen in CS females, those from groups where the females were mated showed either a non-significant rebound following starvation or a negative rebound, meaning that flies which were starved slept less than the control group.

Fig.3.16 displays the quantification of daytime and night-time SD and rebound in the 6 hours post treatment (where significance values were determined using a Wilcoxon rank-sum test comparing control groups to SD groups). For the SD, only virgin females showed a significant daytime SD, and only one of these groups (females from the same-sex pair) showed significant night-time SD. Interestingly, these virgin female groups did not show rebound in the 6 hours post treatment. The sleep rebound is much shorter and smaller in duration when compared to that of CS females.

Mated females from each of the mated groups showed no change in sleep during the daytime or night-time of the starvation period. Mated females also slept more when starved compared to control flies. Females from only one of the groups, those from the mixed-sex pair, showed a significant negative rebound for the full 6 hours of the rebound day, post-treatment.

This work demonstrates that CrzR mutant females, whether mated or virgin, show significantly different responses to starvation-induced SD than CS females.

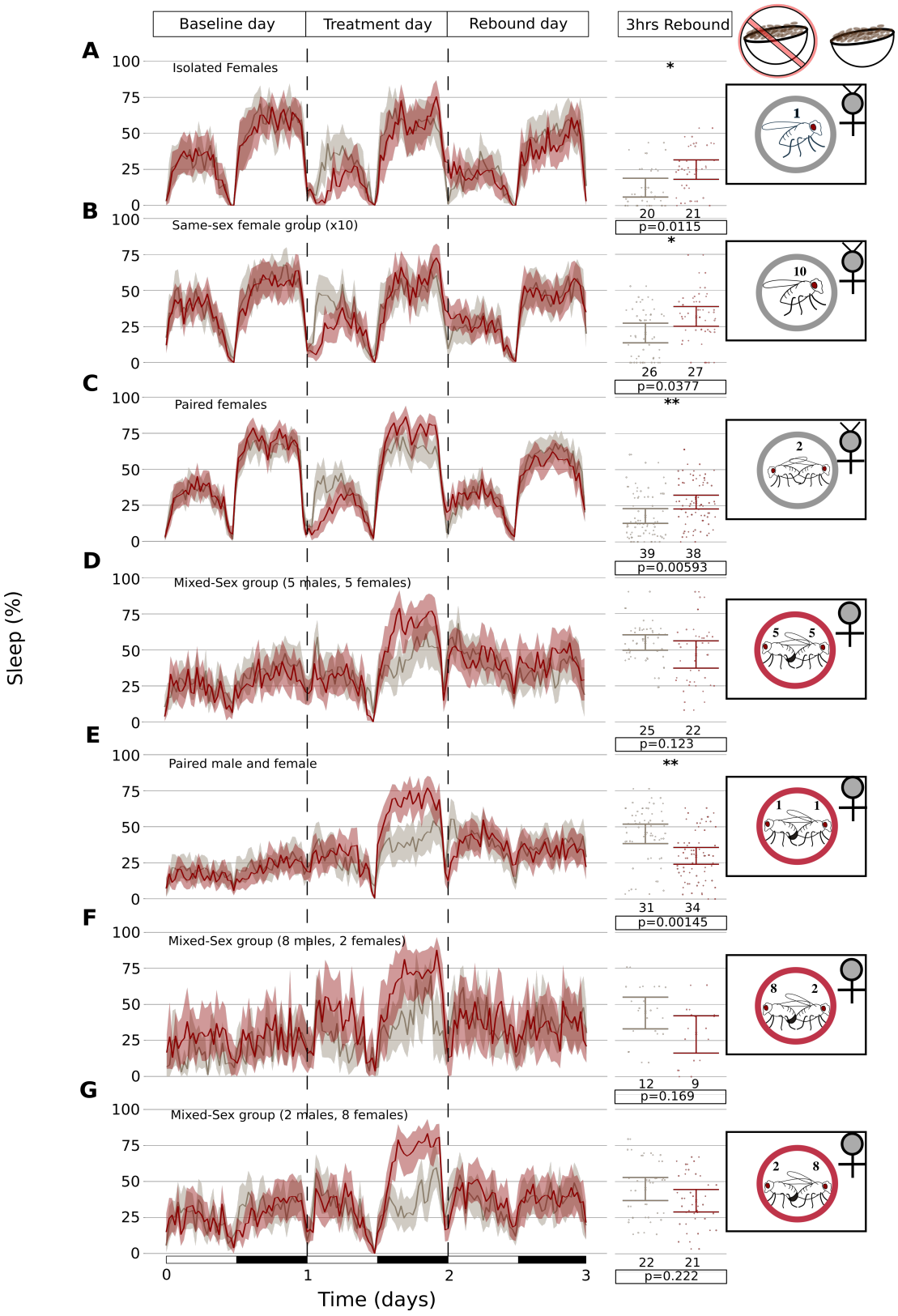


Figure 3.17: *Ethogram plots for CrzR mutant females from prior experience groups subject to starvation SD*Ethograms for females from each of the 7 prior experience groups and the rebound sleep amounts of starved females in each group compared to fed controls. **(A)** isolated females **(B)** same-sex female group **(C)** paired female group **(D)** mixed-sex even sex-ratio group **(E)** male and female pair **(F)** male-skewed mixed-sex group **(G)** female-skewed mixed-sex group. Shaded areas show a 95% bootstrap resampling confidence interval around the mean. *N* for each group and significance values (determined using a Wilcoxon rank-sum test comparing control groups to SD groups) for rebound are shown within the figure and data contained is from at least 3 biological repeats per group.

Table 3.5: Statistical quantification of sleep for starved and control CrzR mutant flies

Group 1	Group 2	P.value	time frame
isolated starved	isolated control	0.00089 (***)	SD day
isolated starved	isolated control	0.99 (n.s.)	SD night
isolated starved	isolated control	0.32 (n.s.)	ZT0-ZT6 rebound
same-sex group starved	same-sex group control	0.0017 (**)	SD day
same-sex group starved	same-sex group control	0.7 (n.s.)	SD night
same-sex group starved	same-sex group control	0.34 (n.s.)	ZT0-ZT6 rebound
same-sex pair starved	same-sex pair control	0.00086 (***)	SD day
same-sex pair starved	same-sex pair control	0.011 (*)	SD night
same-sex pair starved	same-sex pair control	0.25 (n.s.)	ZT0-ZT6 rebound
mixed-sex group (5M,5F) starved	mixed-sex group (5M,5F) control	0.36 (n.s.)	SD day
mixed-sex group (5M,5F) starved	mixed-sex group (5M,5F) control	0.011 (*)	SD night
mixed-sex group (5M,5F) starved	mixed-sex group (5M,5F) control	0.086 (n.s.)	ZT0-ZT6 rebound
mixed-sex pair (1M,1F) starved	mixed-sex pair (1M,1F) control	0.86 (n.s.)	SD day
mixed-sex pair (1M,1F) starved	mixed-sex pair (1M,1F) control	3.7e-06 (****)	SD night
mixed-sex pair (1M,1F) starved	mixed-sex pair (1M,1F) control	0.03 (*)	ZT0-ZT6 rebound
mixed-sex group (8M,2F) starved	mixed-sex group (8M,2F) control	0.069 (n.s.)	SD day
mixed-sex group (8M,2F) starved	mixed-sex group (8M,2F) control	0.0044 (**)	SD night
mixed-sex group (8M,2F) starved	mixed-sex group (8M,2F) control	0.38 (n.s.)	ZT0-ZT6 rebound
mixed-sex group (2M,8F) starved	mixed-sex group (2M,8F) control	0.56 (n.s.)	SD day
mixed-sex group (2M,8F) starved	mixed-sex group (2M,8F) control	1.6e-05 (****)	SD night
mixed-sex group (2M,8F) starved	mixed-sex group (2M,8F) control	0.99 (n.s.)	ZT0-ZT6 rebound

Table 3.6: Summary of findings from CrzR mutant females from prior social groups

Group	Day SD	Night SD	ZT0-ZT3 Rebound	ZT0-ZT6 Rebound
A (isolated) CrzR mutant	Yes (decrease)	No difference	Yes (increase)	No difference
B (virgin group) CrzR mutant	Yes (decrease)	No difference	Yes (increase)	No difference
C (virgin pair) CrzR mutant	Yes (decrease)	No (increase)	Yes (increase)	No difference
D (even-SR group) CrzR mutant	No difference	No (increase)	No difference	No difference
E (ms pair) CrzR mutant	No difference	No (increase)	No (decrease)	No (decrease)
F (male-skewed SR group) CrzR mutant	No difference	No (increase)	No difference	No difference
G (female-skewed SR group) CrzR mutant	No difference	No (increase)	No difference	No difference

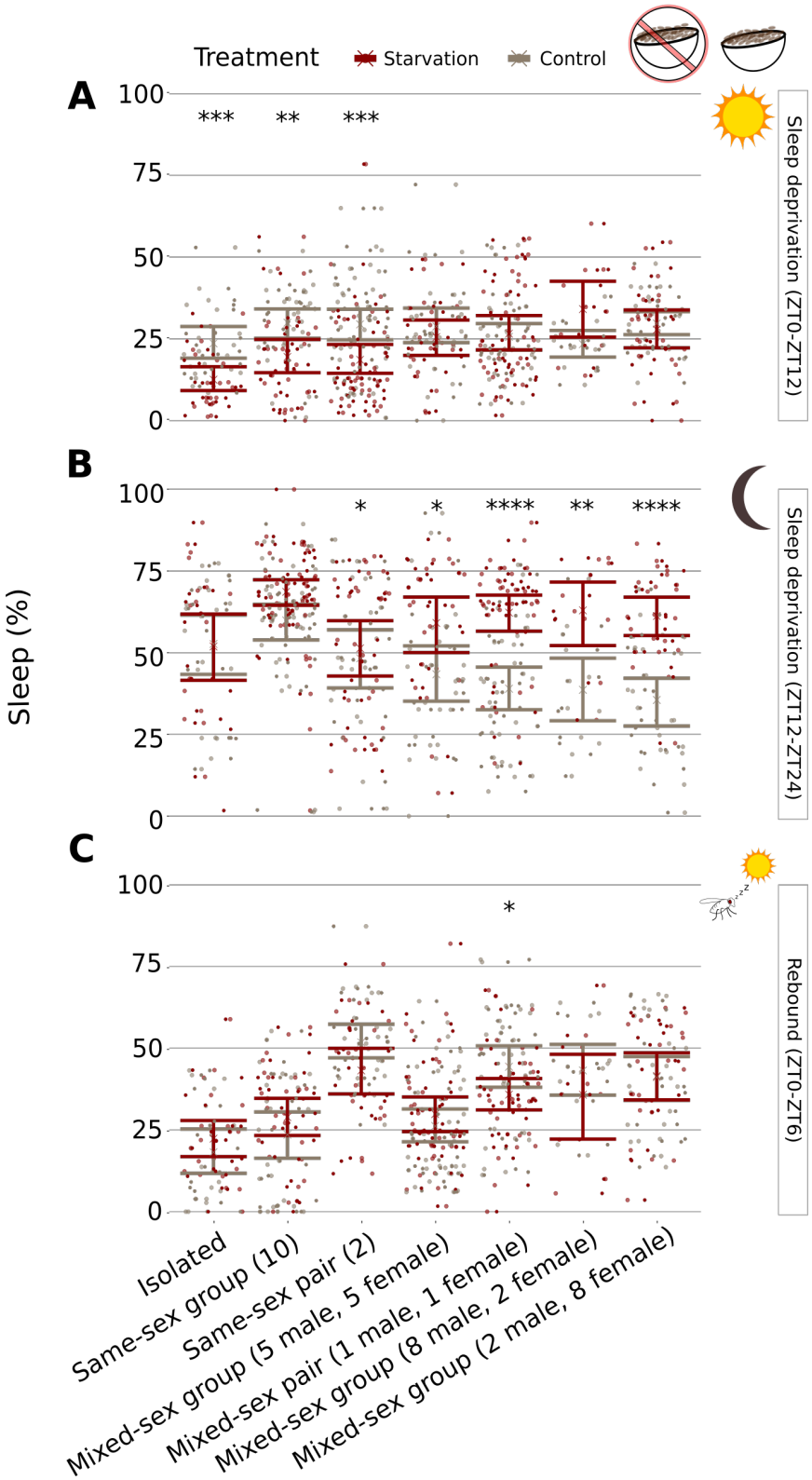


Figure 3.18: *Box-plots for CrzR mutant females showing day SD, night SD and the rebound in the 6 hours post starvation treatment.* Box-plot quantifications for different time periods for all females in groups with differing past experiences. **(A)** Quantification of day-time (ZT0-ZT12) SD **(B)** Quantification of night-time (ZT12-ZT24) SD **(C)** Quantification of rebound in the 6 hours post starvation treatment (ZT0-ZT6). $N_{Isolated}$ control=20, SD=21. $N_{Same\text{-}sex\ group}$ control=26, SD=27. $N_{Paired\ females}$ control=39, SD=38. $N_{Mixed\text{-}sex\ group\ -\ 5\ fem,\ 5\ male}$ control=25, SD=22. $N_{Paired\ male\ and\ female}$ control=31, SD=34. $N_{Mixed\text{-}sex\ group\ -\ 2\ fem,\ 8\ male}$ control=12, SD=9. $N_{Mixed\text{-}sex\ group\ -\ 8\ fem,\ 2\ male}$ control=22, SD=21. Error bars show 95% bootstrap resampling confidence intervals around the mean.

3.4.5 Homeostatic rebound and dynamic sleep deprivation in CrzR mutant females

To confirm the results from section 3.4.3 looking at the two types of SD on CrzR mutants, I separated CrzR mutant females into one of the two previously described groups (Fig.3.1), resulting in either mated or virgin females. I then performed the dynamic form of SD on these flies, whilst also performing the same SD on CS controls. As I have already shown, dynamic SD leads to complete SD for both the day-time and night-time, therefore, I did not quantify the difference between groups which underwent the dynamic SD and sleeping controls. I did, however, quantify the rebound for the 3 hour and 6 hour periods post dynamic SD (seen in fig3.20, where significance values were determined using a Wilcoxon rank-sum test comparing control groups to SD groups). Both CrzR and CS mated and virgin females had significant rebound during both these periods, confirming the results in section 3.4.3.

3.4.6 Summary

- Baseline sleep in both mated and virgin CrzR females was increased, both during the daytime and the night-time when compared to that seen in CS females

- Virgin CrzR mutant females showed a smaller and shorter sleep rebound following starvation SD compared to CS virgin females.

- Mated CrzR mutant females showed similar rebound responses to CS mated females following starvation-induced SD, but showed increased sleep during ZT12-ZT24 of the starvation treatment.

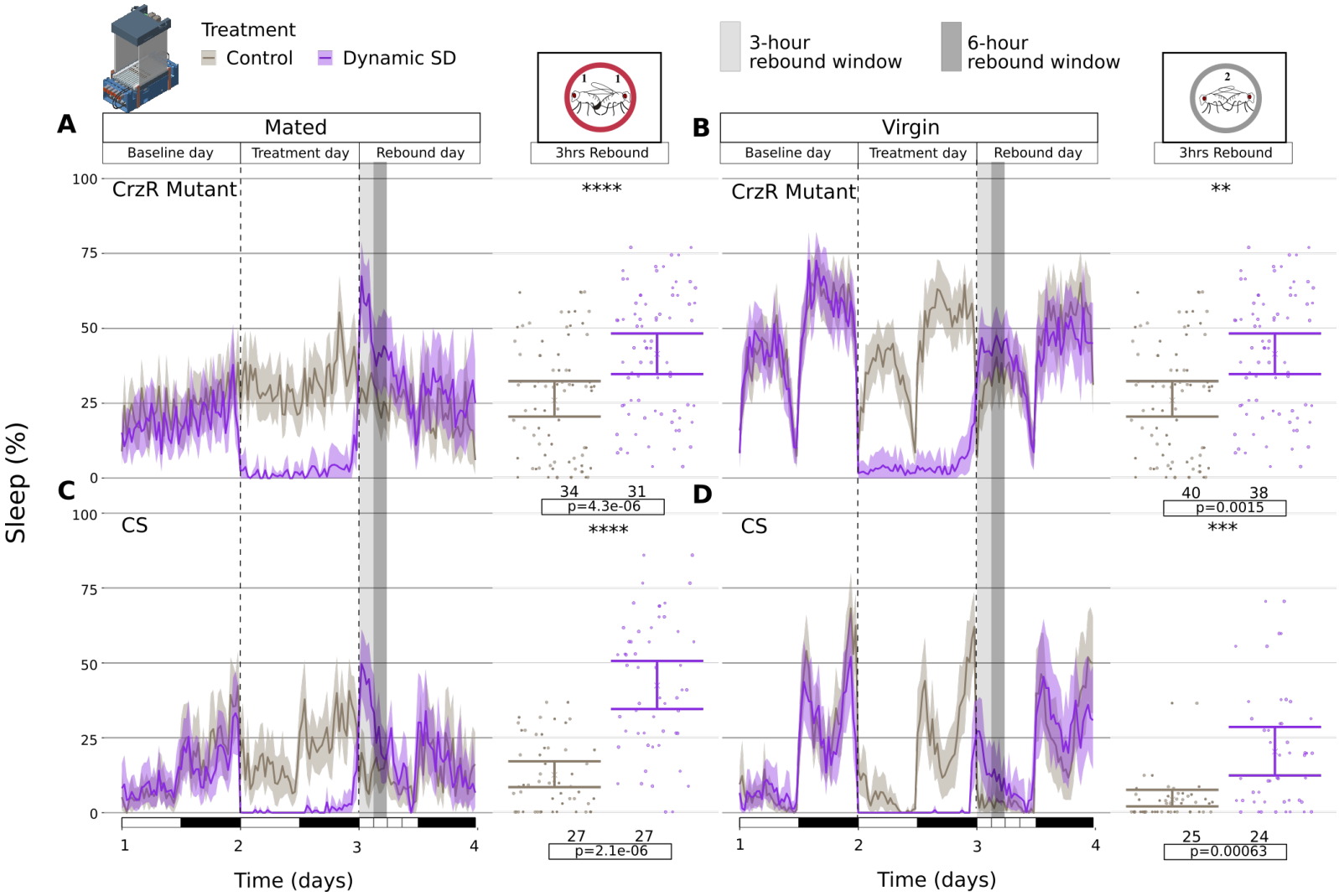


Figure 3.19: *Ethograms of CrzR mutant and CS mated and virgin groups exposed to dynamic SD* Ethograms for **(A)** mated CrzR mutant females **(B)** virgin CrzR mutant females **(C)** mated CS females **(D)** virgin CS females, exposed to 24 hour dynamic SD with the resulting rebound for the 3 hour period post treatment day. Shaded areas show a 95% bootstrap resampling confidence interval around the mean. *N* for each group and significance values for rebound (determined using a Wilcoxon rank-sum test comparing control groups to SD groups) are shown within the figure and data contained is from at least 3 biological repeats per group.

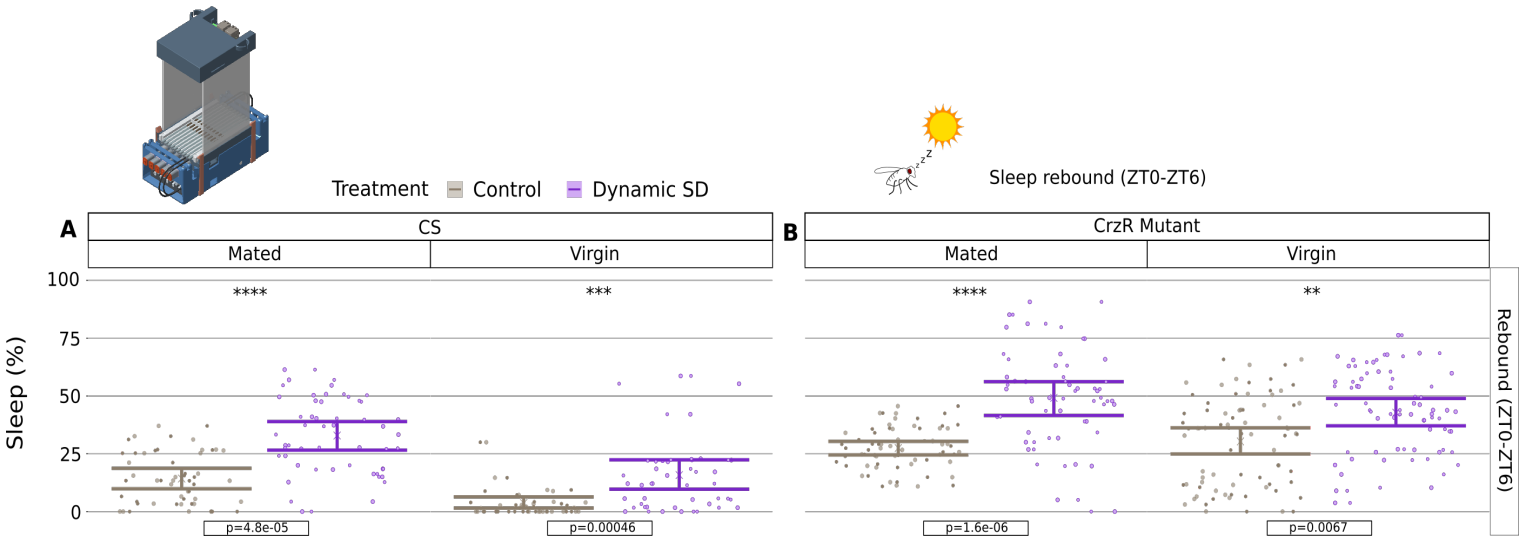


Figure 3.20: *Rebound 6 hours post treatment for CrzR and CS mated and virgin females* Quantification of rebound in the 6 hours post dynamic SD treatment for **(A)** CS mated and virgin females and **(B)** CrzR mutant mated and virgin females. $N_{Dynamic}$ CS mated females: control=27, SD=27. $N_{Dynamic}$ CS virgin females: control=25, SD=24. $N_{Dynamic}$ CrzR mated females: control=34, SD=31. $N_{Dynamic}$ CrzR virgin females: control=40, SD=38.Error bars show 95% bootstrap resampling confidence intervals around the mean.

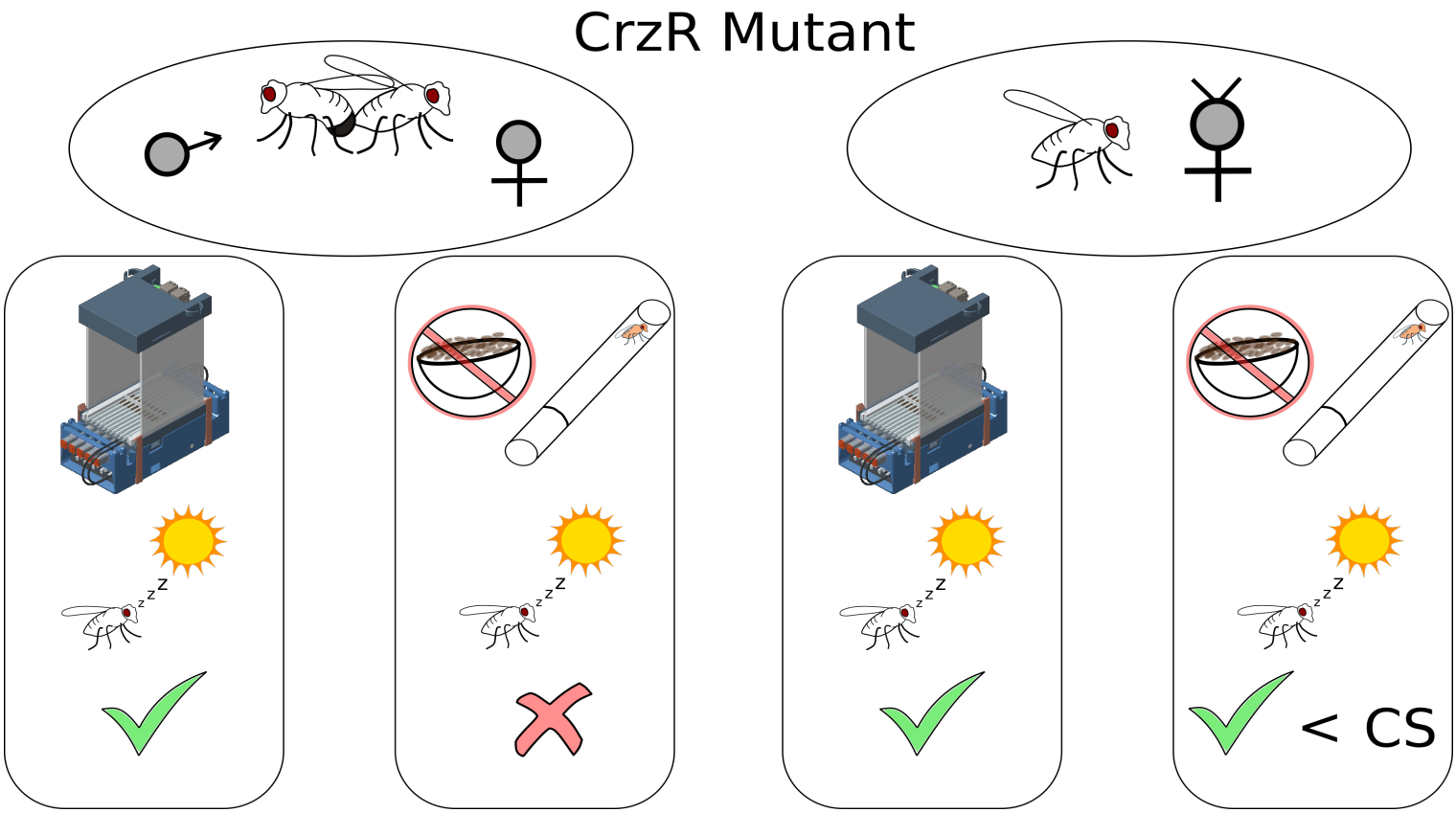


Figure 3.21: *Summary diagram showing rebound responses for CrzR mutant females*

3.5 Knockdown of Crz and CrzR in *Drosophila*

One of the most widely used techniques for genetic manipulation in *Drosophila* is the Gal-4/UAS system for targetting gene expression. This method [223] uses a system from yeast to target specific genes - and thereby their protein products - using RNAi (see section 2.26.1 for a full description of this method). I utilised this technique to target different parts of the *Drosophila* nervous system and specific tissues for CrzR or Crz knockdown. The regions chosen to target were picked based on various reports in the literature of previous survival and stress experiments which yielded changes in behaviours with the knockdown of the Crz peptide and its receptor, CrzR. I also examined the expression of both Crz and CrzR in the brain using the *trans*-Tango data in section 3.4.3. Based on the expression data and results from using the CrzR mutant, I first examined the effects of knockdown of CrzR, in various regions, on baseline sleep in both mated and virgin females.

3.5.1 Baseline sleep and knockdown of CrzR

In total, 6 different Gal4 lines were used (targetting 6 different regions): the nervous system (nsyb-Gal4), layers 2,8 and 9 of the FSB (GMR89E07-Gal4) and the fat-body (C564-Gal4), with two lines specifically looking at dopaminergic neurons (R23E10-Gal4 and PLE-Gal4) and one line specifically targetting cholinergic neurons (CHAT-Gal4). I looked at responses from both mated and virgin females to understand if there were differences in sleep amounts based on these knockdowns. Statistical differences in sleep for the knockdown line were compared to each parental control.

For virgin females, I found that flies with a knockdown of CrzR pan-neuronally (nsyb-Gal4/CrzR-RNAi) increased sleep during the daytime (compared to nsyb-Gal4/CS

(p=3.7e-7) and CrzR-RNAi/CS (p=1.8e-8)) and during the night-time. The only other experimental group to show a change in baseline sleep were flies which had a knockdown of CrzR in the fat-body (C564-Gal4/CrzR-RNAi). These flies showed a decrease in day-time sleep (compared to C56-Gal4/CS (p=0.04831) and CrzR-RNAi/CS (p=0.00157)). For mated females, the sleep profile was quite different. Flies which had a change in day-time sleep were those with a knockdown of CrzR in the (non-PAM) dopaminergic neurons (PLE-Gal4/CrzR-RNAi) (compared to PLE-Gal4/CS (p=0.00086) and CrzR-UAS/CS (p=5.1e-6)). These flies showed an increase in daytime sleep. The two knock-down groups which showed a change in night-time sleep were those with knockdown in the dopaminergic PAM neurons (R58E02-Gal4/CrzR-RNAi) (compared to R58E02-Gal4 (p=0.00845) and CrzR-RNAi/CS (2.0e-13)) and those with CrzR knockdown in layers 2,8 and 9 of the FSB (GMR89E07-Gal4/CrzR-RNAi) (compared to GMR89E02-Gal4/CS (p=0.00064) and CrzR-RNAi/CS (p=0.00049)). Knockdown of CrzR in PAM neurons of mated females led to a decrease in night-time sleep, as did knockdown of CrzR with GMR89E07-Gal4. This demonstrates that CrzR regulates baseline sleep differently in mated and virgin females. (All significance values were determined using a Wilcoxon rank-sum test comparing all groups to each other, and then were adjusted using a FDR correction).

3.5.2 Starvation responses with knockdown of CrzR

My next experiments went on to examine how these lines would respond to starvation SD and how this may change rebound responses. Knockdown of the CrzR receptor was targetted to these areas and responses were compared to the parental controls crossed to a wild-type line (CS).

An ethogram of this data can be seen in Fig.3.23, with quantification of rebound data

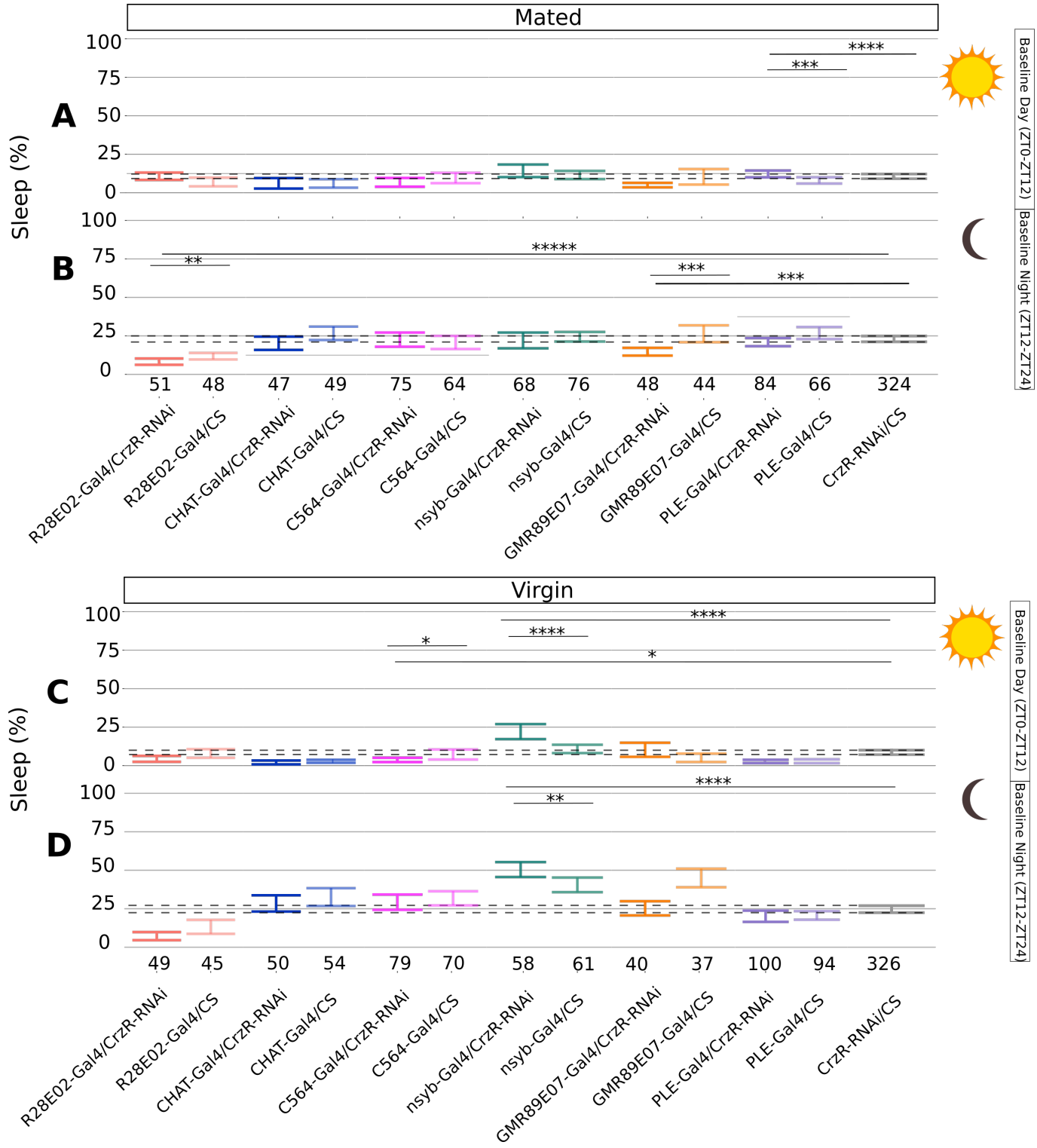


Figure 3.22: *Baseline sleep for knockdown of CrzR in different Drosophila regions* Mean baseline sleep for **(A)** mated females during the day (ZT0-ZT12) **(B)** mated females during the night (ZT12-ZT24).**(C)** virgin females during the day (ZT0-ZT12) **(D)** virgin females during the night (ZT12-ZT24). Fly lines used targetted PAM neurons (R58E02-Gal4), cholinergic neurons (CHAT-Gal4), the fat body (C564-Gal4), all neurons (nsyb-Gal4), layers 2,8 and 9 of the FSB (GMR89E07-Gal4) and non-PAM dopaminergic neurons (PLE-Gal4) with appropriate controls. Error bars show 95% bootstrap resampling confidence intervals around the mean. *N* for each group is shown within each figure and data is from at least 3 biological repeats per group. Grey dotted lines represent the confidence intervals for the control group CrzR-RNAi/CS, which is one of the controls used for every experimental group.

for the first 3 hours of the day post-starvation treatment (where significance values were determined using a Wilcoxon rank-sum test comparing control groups to SD groups). The data shows the robust nature of the phenotype already demonstrated in wild-type females and with the CrzR mutant. Virgin females consistently rebounded following starvation-induced SD and mated females did not. There were two exceptions to this rule; mated females with CrzR knockdown in dopaminergic neurons targetted by PLE-Gal4 did show a small but significant rebound following starvation, which was not seen in either of the parental controls (Fig.3.23Y). The data also demonstrated that mated females are resistant to starvation-induced SD during the night-time, ZT12-ZT24. It also showed that virgin females with a knockdown of CrzR in the PAM dopaminergic neurons had a significant decrease in rebound response following starvation. This implicates two different dopaminergic groups in moderating the starvation SD rebound response in mated and virgin females.

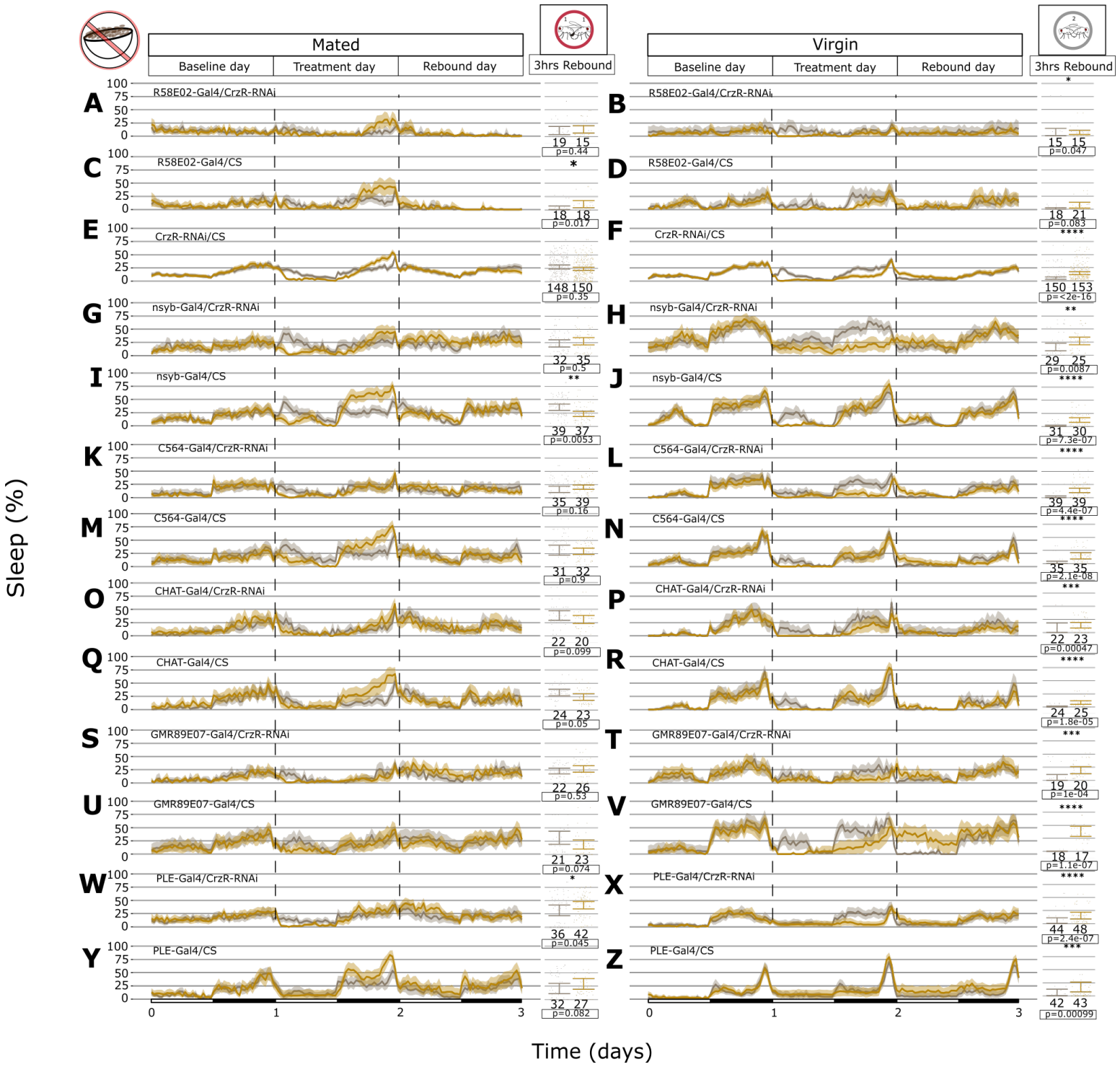


Figure 3.23: *Ethograms showing percentage sleep for knockdown of CrzR in different regions in mated and virgin females with starvation SD.* **(A/B)** Knockdown (kd) of CrzR in PAM neurons (R58E02-Gal4/CrzR-RNAi) **(C/D)** Parental controls for PAM neurons (R58E02-Gal4/CS) **(E/F)** Parental controls for CrzR RNAi line (CrzR-RNAi/CS) **(G/H)** Kd of CrzR neuronally (nsyb-Gal4/CrzR-RNAi) **(I/J)** Parental control for neuronal kd (nsyb-Gal4/CS) **(K/L)** Kd of CrzR in the fat-body (C564-Gal4/CrzR-RNAi) **(M/N)** Parental control for fat-body (C564-Gal4/CS) **(O/P)** Kd of CrzR in cholinergic neurons (CHAT-Gal4/CrzR-RNAi) **(Q/R)** Parental control for cholinergic neurons (CHAT-Gal4/CS) **(S/T)** Kd of CrzR in layers 2,8,9 of the FSB (GMR89E07-Gal4/CrzR-RNAi) **(U/V)** Parental control for layers 2,8,9 FSB kd (GMR89E07-Gal4/CS) **(W/X)** Kd of CrzR in non-PAM dop. neurons (PLE-Gal4/CrzR-RNAi) **(Y/Z)** Parental control for RNAi in non-PAM neurons (PLE-Gal4/CS). Shaded areas show a 95% bootstrap resampling confidence interval around the mean. *N* for each group and significance values for rebound are shown within the figure and data contained is from at least 3 biological repeats per group.

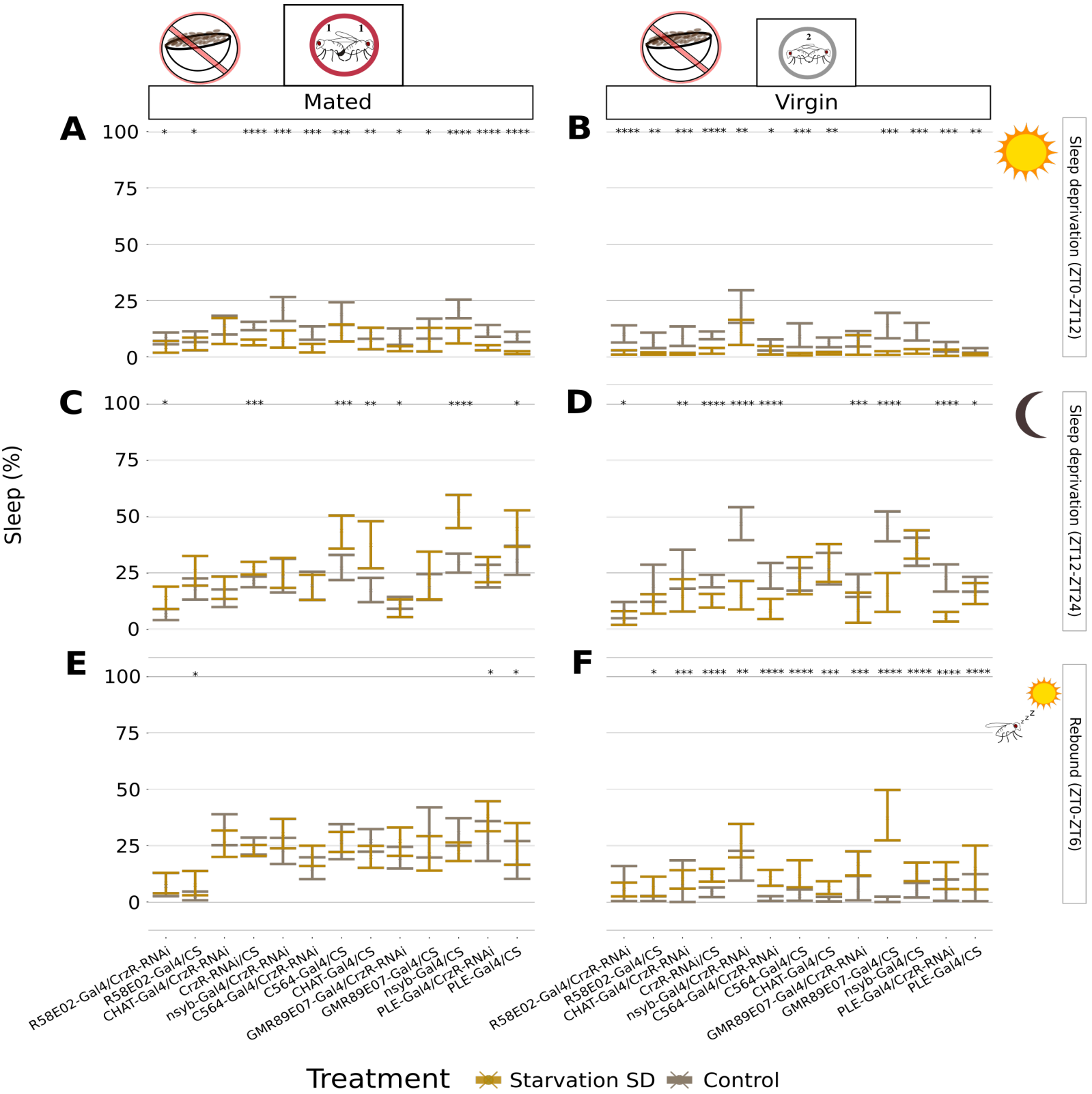


Figure 3.24: *Daytime SD, Night-time SD and 6 hour rebound quantification for CrzR knockdown lines in response to starvation SD.* Quantification of day-time SD for **(A)** mated females with CrzR knockdown in different regions **(B)** virgin females with CrzR knockdown in different regions **(C)** quantification of night-time SD for mated females **(D)** quantification of night-time SD for virgin females **(E)** quantification of rebound in the 6 hours post treatment for mated females **(F)** rebound quantification for the 6 hours post treatment for virgin females. *N* values for each group are shown in the previous figure (3.23). Error bars show 95% bootstrap resampling confidence intervals around the mean and data is from at least 3 biological repeats per group.

Table 3.7: Summary of findings from CrzR RNAi knockdown in various regions: starvation SD with mated females

Gal4 target region of knockdown	Day SD	Night SD	ZT0-ZT3 Rebound	ZT0-ZT6 Rebound
R58E02-Gal4/CrzR-RNAi	Yes (decrease)	No (increase)	No difference	No difference
R58E02-Gal4/CS	Yes (decrease)	No difference	Yes (increase)	Yes (increase)
CHAT-Gal4/CrzR-RNAi	No difference	No difference	No difference	No difference
CrzR-RNAi/CS	Yes (decrease)	No (increase)	No difference	No difference
nsyb-Gal4/CrzR-RNAi	Yes (decrease)	No difference	No difference	No difference
C564-Gal4/CrzR-RNAi	Yes (decrease)	No difference	No difference	No difference
C564-Gal4/CS	Yes (decrease)	No (increase)	No difference	No difference
CHAT-Gal4/CS	Yes (decrease)	No (increase)	No difference	No difference
GMR89E07-Gal4/CrzR-RNAi	Yes (decrease)	Yes (decrease)	No difference	No difference
GMR89E07-Gal4/CS	Yes (decrease)	No difference	No difference	No difference
nsyb-Gal4/CS	Yes (decrease)	No (increase)	No difference	No difference
PLE-Gal4/CrzR-RNAi	Yes (decrease)	No difference	Yes (increase)	Yes (increase)
PLE-Gal4/CS	Yes (decrease)	No (increase)	No difference	Yes (increase)

Table 3.8: Summary of findings from CrzR RNAi knockdown in various regions: starvation SD with virgin females

Gal4 target region of knockdown	Day SD	Night SD	ZT0-ZT3 Rebound	ZT0-ZT6 Rebound
R58E02-Gal4/CrzR-RNAi	Yes (decrease)	Yes (decrease)	No (decrease)	No difference
R58E02-Gal4/CS	Yes (decrease)	No difference	No difference	Yes (increase)
CHAT-Gal4/CrzR-RNAi	Yes (decrease)	Yes (decrease)	Yes (increase)	Yes (increase)
CrzR-RNAi/CS	Yes (decrease)	Yes (decrease)	Yes (increase)	Yes (increase)
nsyb-Gal4/CrzR-RNAi	Yes (decrease)	Yes (decrease)	Yes (increase)	Yes (increase)
C564-Gal4/CrzR-RNAi	Yes (decrease)	Yes (decrease)	Yes (increase)	Yes (increase)
C564-Gal4/CS	Yes (decrease)	No difference	Yes (increase)	Yes (increase)
CHAT-Gal4/CS	Yes (decrease)	No difference	Yes (increase)	Yes (increase)
GMR89E07-Gal4/CrzR-RNAi	No difference	Yes (decrease)	Yes (increase)	Yes (increase)
GMR89E07-Gal4/CS	Yes (decrease)	Yes (decrease)	Yes (increase)	Yes (increase)
nsyb-Gal4/CS	Yes (decrease)	No difference	Yes (increase)	Yes (increase)
PLE-Gal4/CrzR-RNAi	Yes (decrease)	Yes (decrease)	Yes (increase)	Yes (increase)
PLE-Gal4/CS	Yes (decrease)	Yes (decrease)	Yes (increase)	Yes (increase)

3.5.3 Baseline sleep and knockdown of Crz

Following the work performing RNAi knockdown on CrzR, I went on to look at knockdown of Crz in various Gal4 lines, albeit in a smaller number of regions. For virgin females, the data showed that the only group with significant change in sleep is with knockdown of Crz in the dopaminergic neurons of the brain (PLE-Gal4/Crz-

RNAi), which led to a decrease in day-time sleep (compared to parental controls, CrzR-RNAi/CS (p=1.6e-5) and PLE-Gal4/CS (p=0.0015)). For mated females, the only significant change in sleep was a decrease in daytime sleep for knockdown of Crz in sNPFR neurons (sNPFR-Gal4/Crz-RNAi) (compared to the parental controls, Crz-RNAi/CS (p=8.3e-5) and sNPFR-Gal4/CS (p=0.00065)). (All significance values were determined using a Wilcoxon rank-sum test comparing each group to all other groups and then adjusted using a FDR correction).

3.5.4 Starvation responses with knockdown of Crz

I then proceeded to test the rebound and SD responses of *Drosophila* to the knockdown of Crz in the regions described in the previous sections. As seen previously, the data again represents the robust nature of the dichotomy between mated and virgin females following starvation-induced SD. The mated females tested showed either no difference in sleep response when compared to the fed controls for the 3 hours post starvation or showed a negative rebound, with no specific differences between parental and control lines. The only difference seen for virgin females tested was in the experimental line where Crz was knocked-down specifically in neurons which also expressed the sNPFR. In this case, there was no significant rebound compared to the fed control. However, this experiment was performed by another member of our group and the number of flies tested was much lower than in the other groups.

In reference to SD, no group showed significant differences in rebound when compared to parental controls (Fig.3.26. No virgin experimental line tested showed significant sleep rebound in the 6 hours post starvation (Fig.27). (All significance values were determined using a Wilcoxon rank-sum test comparing each group to all other groups and then adjusted using a FDR correction).

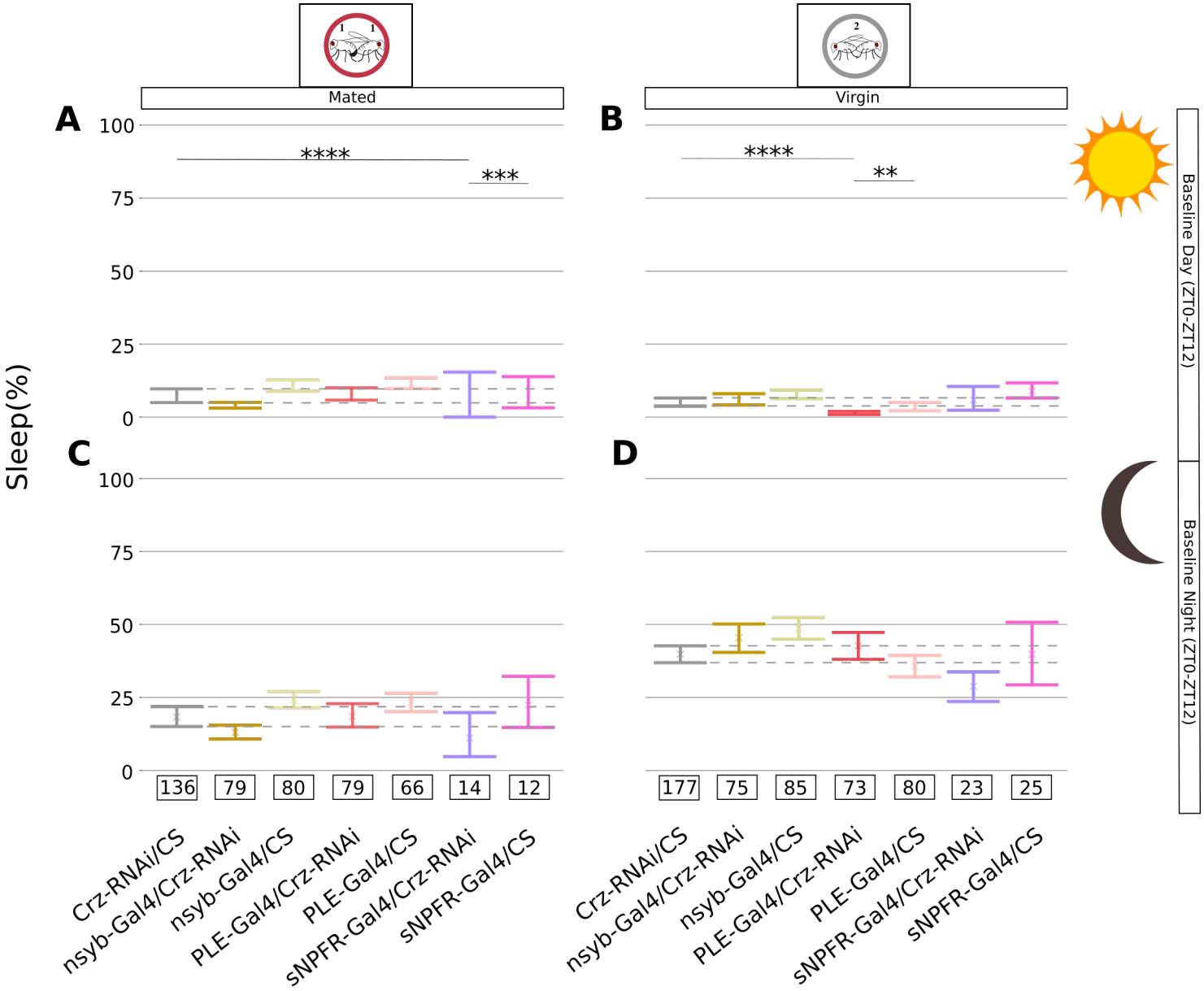


Figure 3.25: *Baseline sleep amounts for mated and virgin females with Crz knockdown in various regions* Baseline sleep amount for **(A)** mated females during the day-time **(B)** virgin females during the day-time **(C)** mated females during the night-time **(D)** virgin females during the night-time. Fly lines used targetted all neurons (nsyb-Gal4), non-PAM dopaminergic neurons (PLE-Gal4) and neurons expressing sNPFR (sNPFR-Gal4), with appropriate controls. Error bars show 95% bootstrap resampling confidence intervals around the mean. *N* for each group is shown within each figure and data is from at least 3 biological repeats per group. Grey dotted lines represent the confidence intervals for the control group Crz-RNAi/CS, which is one of the controls used for every experimental group.

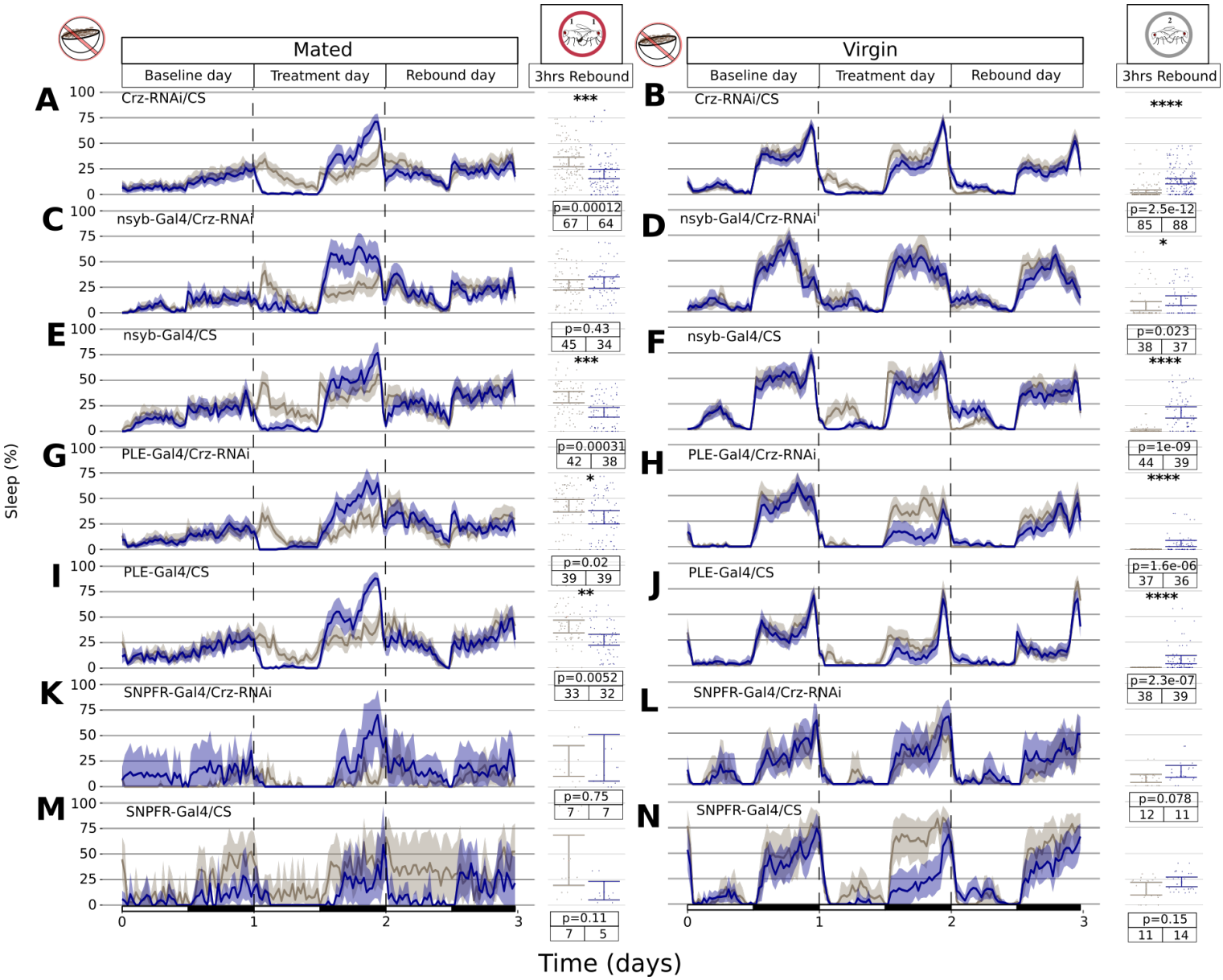


Figure 3.26: *Ethogram plots for Crz knockdown mated or virgin females subject to starvation SD* Ethograms for virgin and female lines with CrzR knockdown. **(A/B)** Parental control line (Crz-RNAi/CS) for mated and virgin females **(C/D)** Knockdown of Crz neuronally in mated and virign females (nsyb-Gal4/Crz-RNAi) **(E/F)** Parental control line for neuronal knockdown of mated and virgin females (nsyb-Gal4/CS) **(G/H)** Knockdown of Crz in the non-PAM dopaminergic neurons for mated and virgin females (PLE-Gal4/Crz-RNAi) **(I/J)** Parental control for knockdown in the non-PAM dopaminergic neurons for mated and virgin females (PLE-Gal4/CS) **(K/L)** Knockdown of Crz in the sNPFR expressing cells for mated and virgin females (sNPFR-Gal4/Crz-RNAi) **(M/N)** Parental control for sNPFR neurons for mated and virgin females (sNPFR-Gal4/CS). Shaded areas show a 95% bootstrap resampling confidence interval around the mean. *N* for each group and significance values for rebound are shown within the figure and data contained is from at least 3 biological repeats per group.

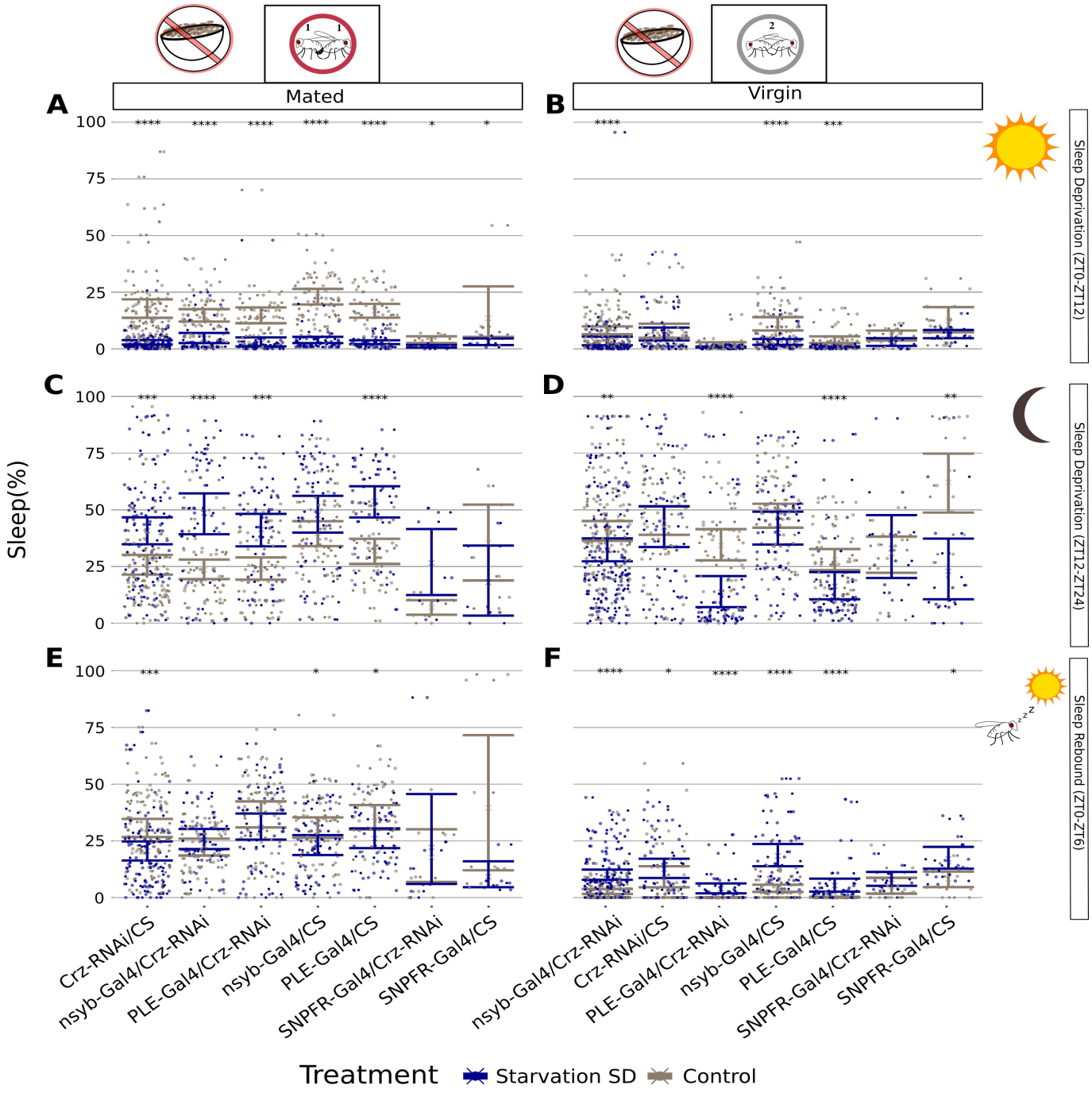


Figure 3.27: *Daytime SD, Night-time SD and 6 hour rebound quantification* Quantification of day-time SD for **(A)** mated females with Crz knockdown in different regions **(B)** virgin females with Crz knockdown in different regions **(C)** quantification of night-time SD for mated females **(D)** quantification of night-time SD for virgin females **(E)** quantification of rebound in the 6 hours post treatment for mated females **(F)** rebound quantification for the 6 hours post treatment for virgin females. N_{Starve} Crz-RNAi/CS mated females: control=67, SD=64. N_{Starve} Crz-RNAi/CS virgin females: control=85, SD=88. N_{Starve} nsyb-Gal4/Crz-RNAi mated females: control=45, SD=34. N_{Starve} nsyb-Gal4/Crz-RNAi virgin females: control=38, SD=37. N_{Starve} nsyb-Gal4/CS mated females: control=42, SD=38. N_{Starve} nsyb-Gal4/CS virgin females: control=44, SD=39. N_{Starve} PLE-Gal4/Crz-RNAi mated females: control=39, SD=39. N_{Starve} PLE-Gal4/Crz-RNAi virgin females: control=37, SD=36. N_{Starve} PLE-Gal4/CS mated females: control=33, SD=32. N_{Starve} PLE-Gal4/CS virgin females: control=38, SD=39. N_{Starve} sNPFR-Gal4/Crz-RNAi mated females: control=7, SD=7. N_{Starve} sNPFR-Gal4/Crz-RNAi virgin females: control=12, SD=11. N_{Starve} sNPFR-Gal4/CS mated females: control=7, SD=5. N_{Starve} sNPFR-Gal4/CS virgin females: control=11, SD=14. Error bars show 95% bootstrap resampling confidence intervals around the mean.

3.5.5 Summary

- Knockdown of CrzR in the nervous system of virgin females, led to an increase in daytime and night-time sleep

- Knockdown of CrzR in the fat body of virgin females led to a decrease in daytime sleep.

- Knockdown of CrzR in non-PAM dopaminergic neurons of mated females showed an increase in daytime sleep.

- Knockdown of CrzR in the PAM neurons and in FSB layers 2,8 and 9 of mated females decreased night-time sleep.

- Knockdown of CrzR in non-PAM dopaminergic neurons of mated females led to a sleep rebound.

- Knockdown of CrzR in PAM dopaminergic neurons of virgin females led to abolition of sleep rebound.

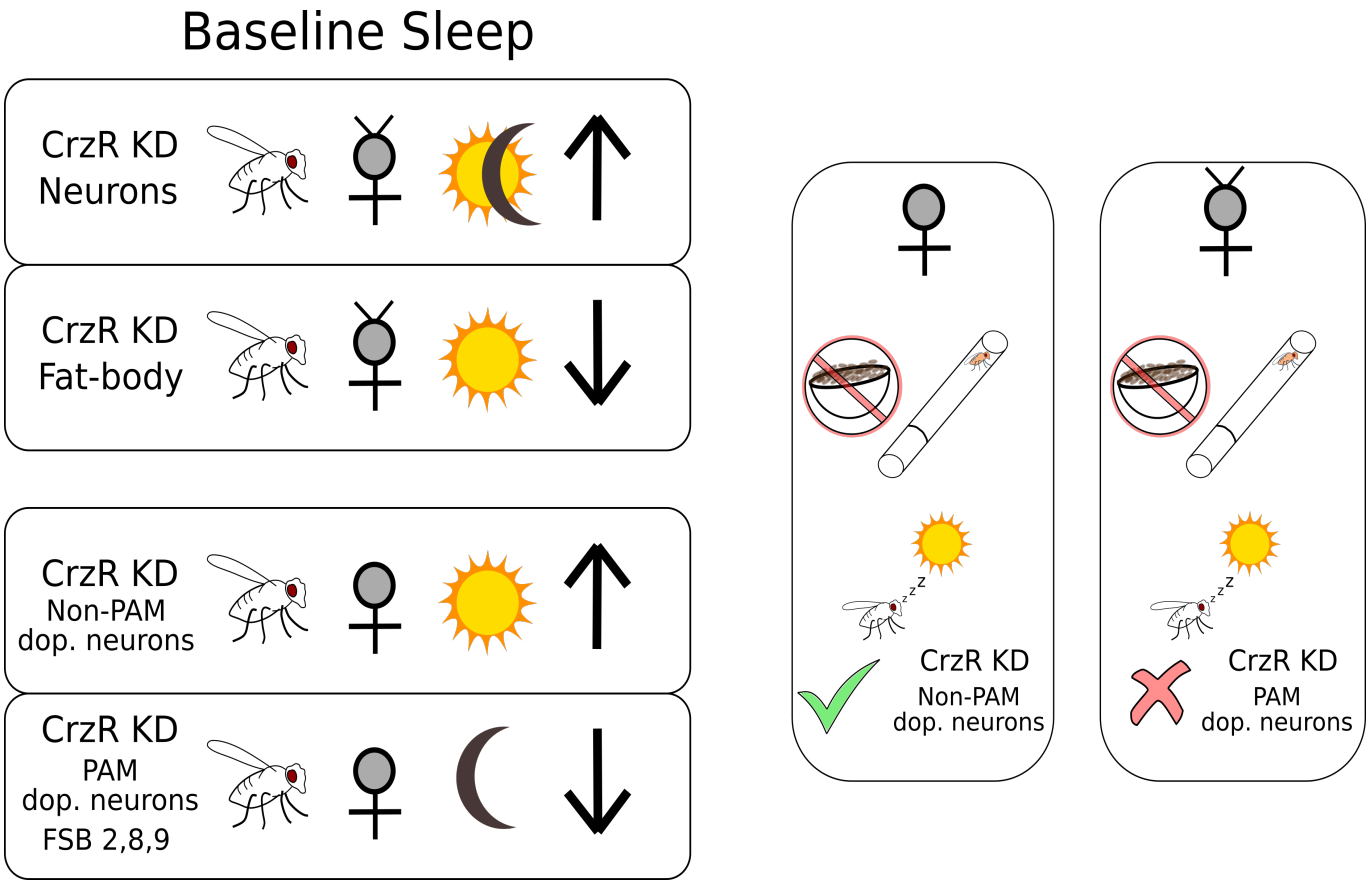


Figure 3.28: *Summary diagram for CrzR and Crz knockdown*

3.6 Investigating the interaction between Dopamine and CrzR

3.6.1 Modulation of sleep resulting from feeding dopamine modulators

As discussed in the introduction, dopamine regulates sleep amount; increases in dopamine lead to decreased sleep and vice versa, with the level of dopamine having an inverse relationship with sleep amount [469]. Several compounds which modulate dopamine levels, through a variety of different mechanisms, can be used to change sleep in *Drosophila*. To understand how changes in dopamine may impact the sleep of CS mated and virgin

females, and that of mated and virgin CrzR mutants, I fed flies one of two different dopamine modulators; L-DOPA (l-3,4-dihydroxyphenylalanine), which has been shown to decrease sleep in wild-type *Drosophila* [329] and 3IY (3-iodo-tyrosine), which has been shown to increase sleep [8]. As I mixed these compounds in a medium of sucrose and agarose, I also tested this as another food condition. Wild-type mated CS females showed a significant decrease in day sleep with all groups apart from L-DOPA compared to when on standard food (Fig.3.29A), whereas for the night time, sleep was decreased with L-DOPA and caffeine but increased by sucrose (Fig.3.29B). Wild-type virgin CS flies showed decreased day sleep in response to L-DOPA, 3IY, agar and caffeine feeding compared to sleep when exposed to standard food (Fig.3.30A). Virgin CS flies showed a decrease in night sleep with agar and caffeine, but increased sleep with 3IY and sucrose, when compared to the standard food control (Fig.3.30B).

The response to 3IY for wild-type virgin females matches that from the literature, as an decrease in day sleep has been reported [8]. However, the increase in night sleep seen with 3IY for virgin females was unexpected.

Comparatively, CrzR mutant mated females, did not show any significant decreases in day sleep with any substance when compared to the standard food control (Fig.3.31A). These flies did show increased night sleep with agar and sucrose, respectively (Fig.3.31B). Caffeine was the only substance which led to a decrease in night sleep for mated CrzR mutant females (Fig.3.31B).

CrzR mutant virgin females had significant decreases in day sleep when exposed to agar and when caffeine was added to agar. This response to caffeine was also significant during the night-time. These flies also showed an increase in sleep when exposed to 3IY, similar to that seen in wild-type flies.

This firstly suggests that mated and virgin females of both genotypes may regulate

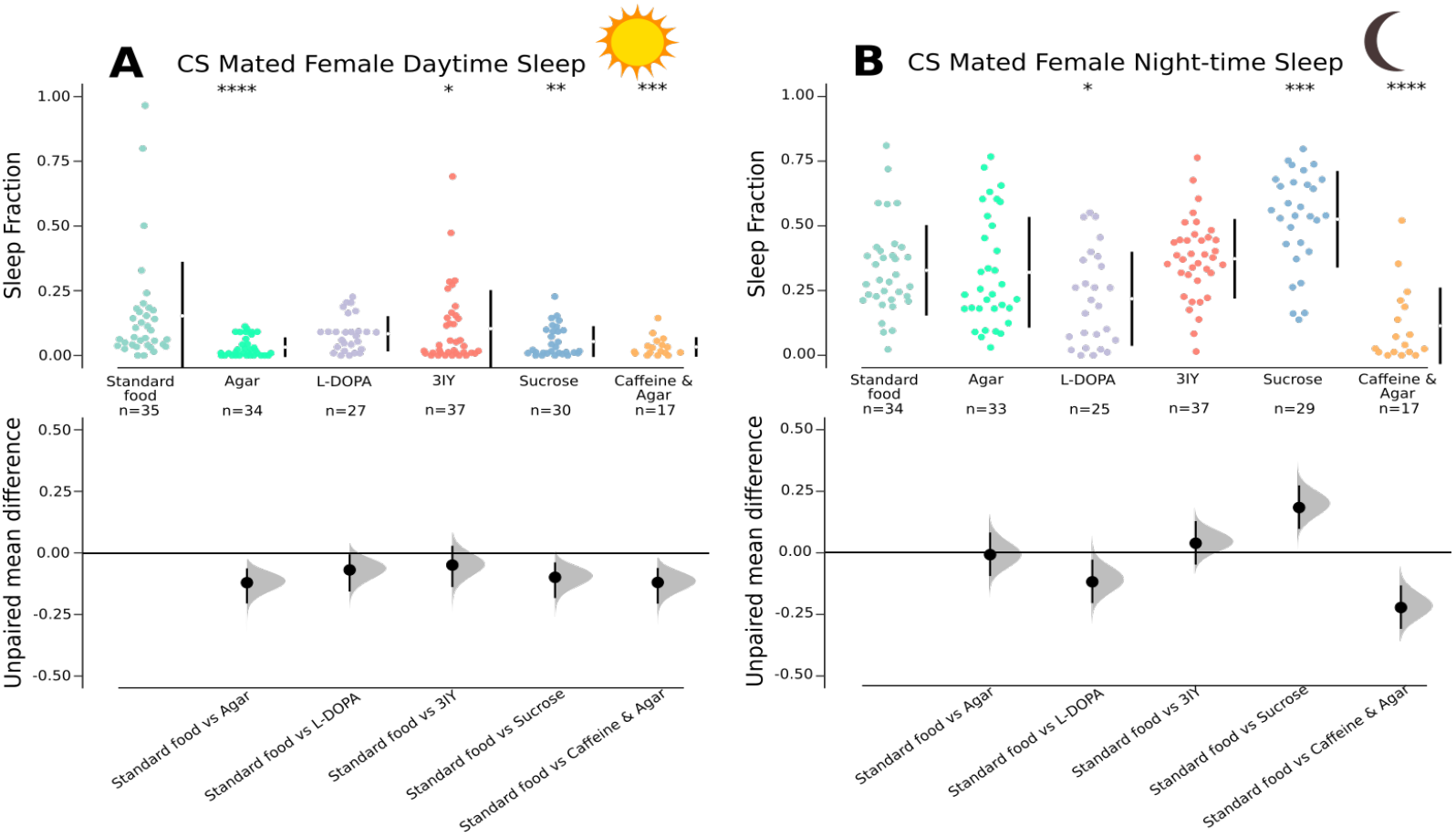


Figure 3.29: *Estimation plots of day-time, night-time SD rebound when feeding various sleep modulators for CS mated females* Quantification of **(A)** Day-time sleep (ZT0-ZT12) for mated CS females **(B)** night-time sleep (ZT12-ZT24) for mated CS females exposed to standard food, agar, L-DOPA, 3IY, sucrose (in agar) and caffeine in agar. The observed effect size between the experimental groups and standard food control group is depicted as a black dot, and the 95% CI is indicated by the ends of the black error bars. *N* for each group and significance values for statistical differences between control and experimental groups are shown within the figure and data contained is from at least 3 biological repeats per group.

dopamine differently, as in both cases they showed different responses to these dopaminergic sleep-modulators. Secondly, as CrzR mutant flies also showed differences in responses to these compounds compared to CS flies, it demonstrates that CrzR may also have a role in dopamine regulation and signalling, or that the mutation leads to a change in dopamine modulation. Statistical quantifications of comparisons of treatments to the standard food group can be found in tables 3.9 and 3.10, where Wilcoxon rank-sum tests were performed between groups and followed with False Discovery Rate (FDR) corrections.

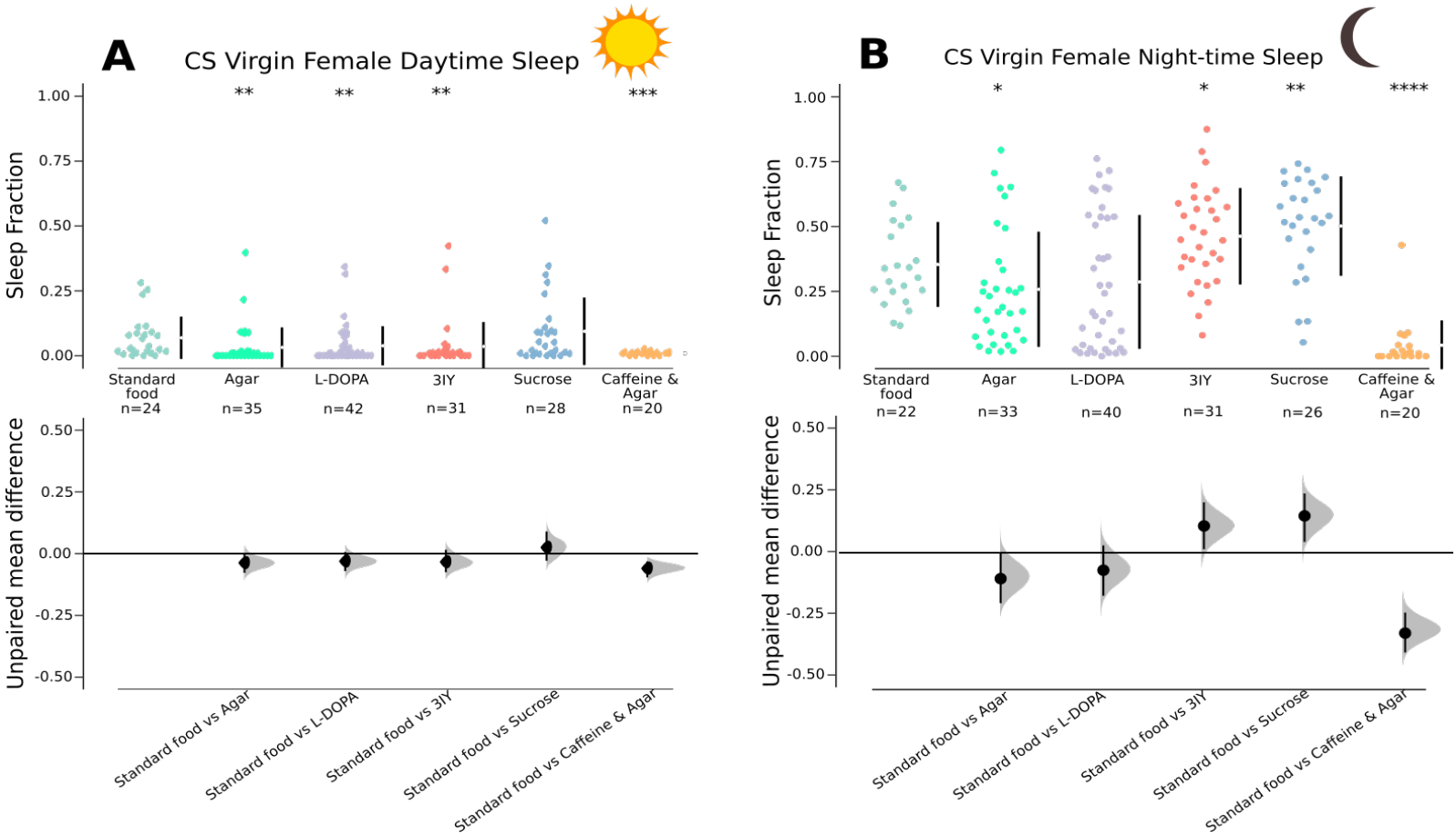


Figure 3.30: *Estimation plots of day-time, night-time SD rebound when feeding various sleep modulators for CS virgin females* Quantification of **(A)** Day-time sleep (ZT0-ZT12) for virgin CS females **(B)** night-time sleep (ZT12-ZT24) for virgin CS females exposed to standard food, agar, L-DOPA, 3IY, sucrose (in agar) and caffeine in agar. The observed effect size between the experimental groups and standard food control group is depicted as a black dot, and the 95% CI is indicated by the ends of the black error bars. *N* for each group and significance values for statistical differences between control and experimental groups are shown within the figure and data contained is from at least 3 biological repeats per group.

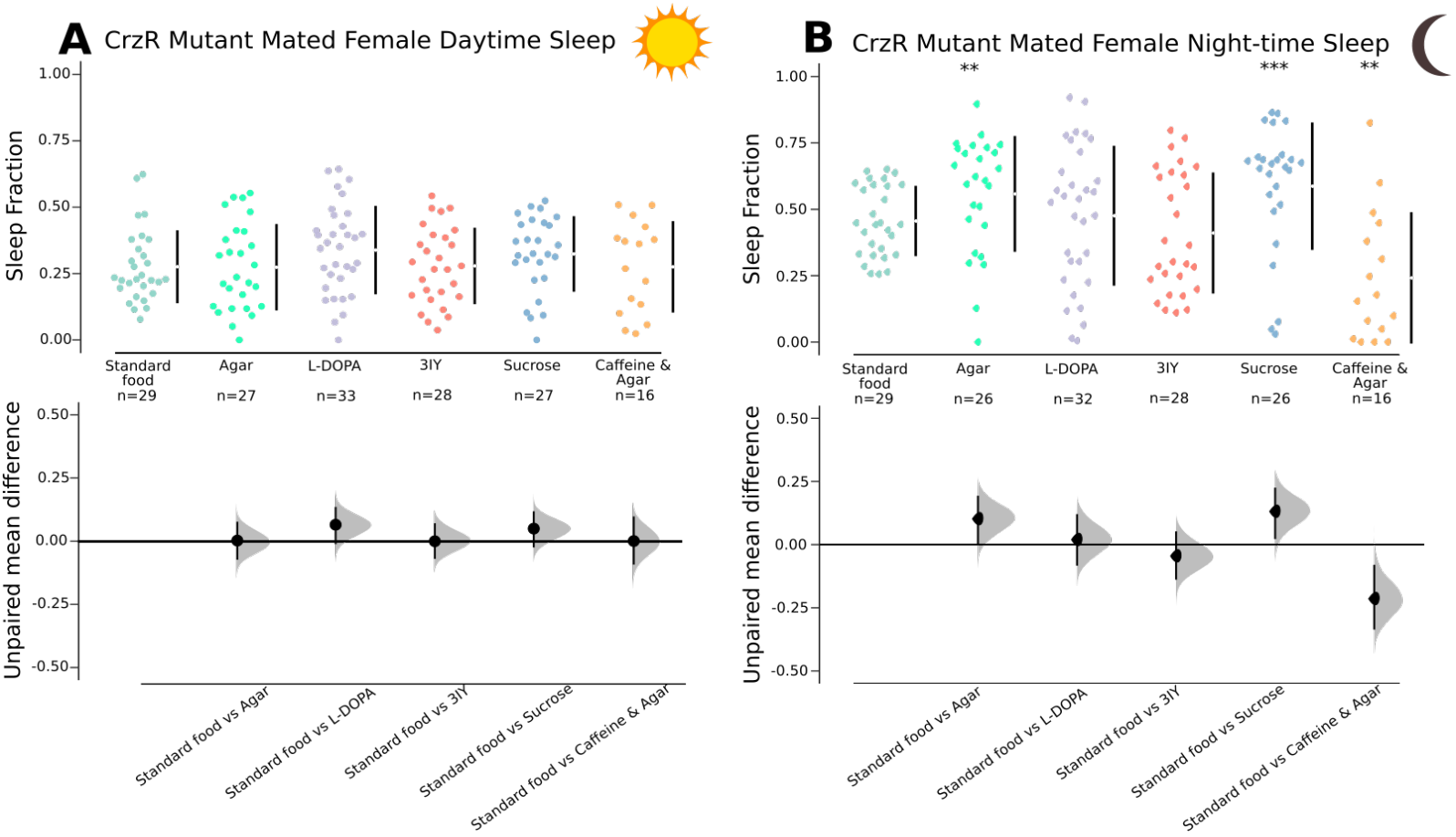


Figure 3.31: *Estimation plots of day-time, night-time SD rebound when feeding various sleep modulators for CrzR mutant mated females* Quantification of **(A)** Day-time sleep (ZT0-ZT12) for mated CrzR mutant females **(B)** night-time sleep (ZT12-ZT24) for mated CrzR mutant females exposed to standard food, agar, L-DOPA, 3IY, sucrose (in agar) and caffeine in agar. The observed effect size between the experimental groups and standard food control group is depicted as a black dot, and the 95% CI is indicated by the ends of the black error bars. *N* for each group and significance values for statistical differences between control and experimental groups are shown within the figure and data contained is from at least 3 biological repeats per group.

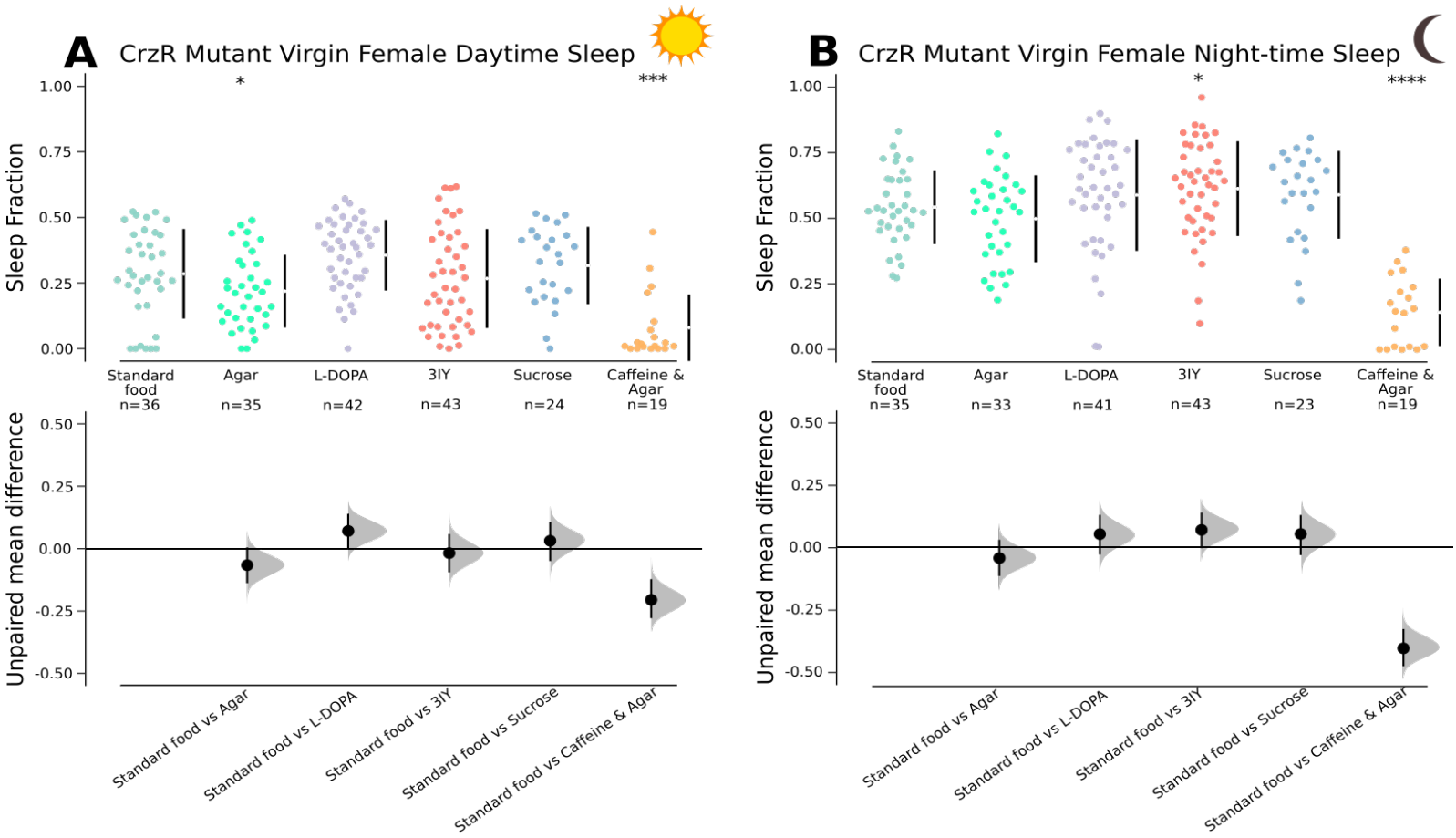


Figure 3.32: *Estimation plots of day-time, night-time SD rebound when feeding various sleep modulators for CrzR mutant virgin females* Quantification of **(A)** Day-time sleep (ZT0-ZT12) for virgin CrzR mutant females **(B)** night-time sleep (ZT12-ZT24) for virgin CrzR mutant females exposed to standard food, agar, L-DOPA, 3IY, sucrose (in agar) and caffeine in agar. The observed effect size between the experimental groups and standard food control group is depicted as a black dot, and the 95% CI is indicated by the ends of the black error bars. *N* for each group and significance values for statistical differences between control and experimental groups are shown within the figure and data contained is from at least 3 biological repeats per group.

Table 3.9: Statistical quantification of sleep for CrzR mutant mated and virgin female flies with different food conditions

Mating status	Food conditions	P.value (vs sucrose only)	time frame
Mated	Sucrose and 3IY	0.231 (n.s.)	SD day
Mated	Sucrose and 3IY	0.00540 (**)	SD night
Mated	Sucrose and 3IY	0.05105 (n.s.)	ZT0-ZT3 rebound
Mated	Agarose	0.280 (n.s.)	SD day
Mated	Agarose	0.66971 (n.s.)	SD night
Mated	Agarose	0.72739 (n.s.)	ZT0-ZT3 rebound
Mated	Agarose and Caffeine	0.478 (n.s.)	SD day
Mated	Agarose and Caffeine	0.00025 (***)	SD night
Mated	Agarose and Caffeine	0.06855 (n.s.)	ZT0-ZT3 rebound
Mated	Standard food	0.085 (n.s.)	SD day
Mated	Standard food	0.00056 (***)	SD night
Mated	Standard food	0.01918 (*)	ZT0-ZT3 rebound
Mated	Sucrose and L-DOPA	0.935 (n.s.)	SD day
Mated	Sucrose and L-DOPA	0.06829 (n.s.)	SD night
Mated	Sucrose and L-DOPA	0.31097 (n.s.)	ZT0-ZT3 rebound
Virgin	Sucrose and 3IY	0.25525 (n.s.)	SD day
Virgin	Sucrose and 3IY	0.6886 (n.s.)	SD night
Virgin	Sucrose and 3IY	0.5996 (n.s.)	ZT0-ZT3 rebound
Virgin	Agarose	0.01384 (*)	SD day
Virgin	Agarose	0.0296 (*)	SD night
Virgin	Agarose	0.4384 (n.s.)	ZT0-ZT3 rebound
Virgin	Agarose and Caffeine	3.5e-05 (****)	SD day
Virgin	Agarose and Caffeine	1.9e-07 (****)	SD night
Virgin	Agarose and Caffeine	0.0982 (n.s.)	ZT0-ZT3 rebound
Virgin	Standard food	0.68356 (n.s.)	SD day
Virgin	Standard food	0.1446 (n.s.)	SD night
Virgin	Standard food	0.7384 (n.s.)	ZT0-ZT3 rebound
Virgin	Sucrose and L-DOPA	0.31743 (n.s.)	SD day
Virgin	Sucrose and L-DOPA	0.8786 (n.s.)	SD night
Virgin	Sucrose and L-DOPA	0.3742 (n.s.)	ZT0-ZT3 rebound

Table 3.10: Statistical quantification of sleep for CS mated and virgin flies with different food conditions

Mating status	Food conditions	P.value (vs sucrose only)	time frame
Mated	Sucrose and 3IY	0.34032 (n.s.)	SD day
Mated	Sucrose and 3IY	0.00049 (***)	SD night
Mated	Sucrose and 3IY	0.191 (n.s.)	ZT0-ZT3 rebound
Mated	Agarose	0.06554 (n.s.)	SD day
Mated	Agarose	0.00064 (***)	SD night
Mated	Agarose	0.646 (n.s.)	ZT0-ZT3 rebound
Mated	Agarose and Caffeine	0.43113 (n.s.)	ZT0-ZT3 rebound
Mated	Agarose and Caffeine	4.9e-07 (****)	SD night
Mated	Agarose and Caffeine	0.139 (n.s.)	ZT0-ZT3 rebound
Mated	Standard food	0.00211 (**)	SD day
Mated	Standard food	0.00014 (***)	SD night
Mated	Standard food	0.745 (n.s.)	ZT0-ZT3 rebound
Mated	Sucrose and L-DOPA	0.12668 (n.s.)	SD day
Mated	Sucrose and L-DOPA	1.5e-06 (****)	SD night
Mated	Sucrose and L-DOPA	0.399 (n.s.)	ZT0-ZT3 rebound
Virgin	Sucrose and 3IY	0.00799 (**)	SD day
Virgin	Sucrose and 3IY	0.26778 (n.s.)	SD night
Virgin	Sucrose and 3IY	0.90971 (n.s.)	ZT0-ZT3 rebound
Virgin	Agarose	0.00353 (**)	SD day
Virgin	Agarose	8.0e-05 (****)	SD night
Virgin	Agarose	0.00197 (**)	ZT0-ZT3 rebound
Virgin	Agarose and Caffeine	0.00948 (**)	SD day
Virgin	Agarose and Caffeine	3.0e-08 (****)	SD night
Virgin	Agarose and Caffeine	0.23854 (n.s.)	ZT0-ZT3 rebound
Virgin	Standard food	0.89758 (n.s.)	SD day
Virgin	Standard food	0.00535 (**)	SD night
Virgin	Standard food	0.00815 (**)	ZT0-ZT3 rebound
Virgin	Sucrose and L-DOPA	0.01001 (*)	SD day
Virgin	Sucrose and L-DOPA	0.00124 (**)	SD night
Virgin	Sucrose and L-DOPA	0.64134 (n.s.)	ZT0-ZT3 rebound

3.6.2 Modulation of Arousal by CrzR and Crz

Dopamine has been shown to be a key regulator of arousal threshold in *Drosophila* [263], [469]. To identify if CrzR also modulated arousal in *Drosophila* females, I used

the AGO attachment for the ethoscope device (see Fig.2.1 for a diagram of this attachment). This "add-on" equipment allows the ethoscope to be modulated in a similar way to how the optomotor device and can be used to modulate sleep and provide a means of dynamic SD. The AGO device allows a puff of odour to be delivered to flies which are registered as asleep (where a threshold of 5 minutes of immobility of any individual fly is determined as being quantified as "sleep").

To understand how arousal may be regulated during both fed and starved conditions, flies were either placed in the device either with agar medium for 18 hours prior to testing, or in a control group with normal food. A recent paper [135] from our group used the AGO device to probe arousal and showed that flies can be aroused from sleep using 5% acetic acid. Acetic acid is the main component of vinegar and an ethologically relevant odour for *Drosophila melanogaster*. This work showed male CS flies, which were starved, had greater arousal responses to acetic acid than fed counterparts. For my work, flies were tested for arousal responses between the times ZT18-ZT24, which correlates to the latter half of the night-period. This was a time period when French et al. [135] found responses to olfactory stimuli led to the most awakenings.

The first groups of flies tested were those with CrzR knockdown in the nervous system, using the nsyb-Gal4 line. The arousal responses of mated and virgin females of the knockdown line (nsyb-Gal4/CrzR-RNAi) were compared to those of the two parental control lines (nsyb-Gal4/CS and CrzR-RNAi/CS). Fig.3.33 displays the results. For virgin females, one of the parental controls showed a significant increase in response in starved flies compared to fed flies (CrzR-RNAi/CS, p=0.000214), but the experimental group (nsyb-Gal4/CrzR-RNAi, p=0.259) and one of the control lines (nsyb-Gal4/CS, p=0.22) did not show changes in response between fed and starved flies. For mated females, flies in the knockdown group did show a significant increase in response be-

tween fed and starved groups (nsyb-Gal4/CS, p=0.0304), as did one of the control groups (nsyb-Gal4/CS, p=0.0213). However, flies in the other parental control group did not show increased responses in starved flies when compared to fed counterparts (CrzR-RNAi/CS, p=0.149). Due to the inconsistencies in responses from the control groups, it is difficult to determine what effect the knockdown of CrzR has on arousal, but the results suggested that the knockdown of CrzR may have different effects on virgin and mated groups. The second group of flies tested were those with knockdown of Crz, with knockdown also specifically targetted to the nervous system (nsyb-Gal4/Crz-RNAi) and compared to parental controls (nsyb-Gal4/CS and Crz-RNAi/CS). In this case, mated females from the experimental line showed no significant differences in arousal response when starved and fed groups were compared (nsyb-Gal4/Crz-RNAi, p=0.531); in mated parental controls, starved flies showed increased arousal compared to fed controls for both parental control lines (Crz-RNAi/CS, p=0.000129 and nsyb-Gal4/CS, p=0.00112). In virgin flies with Crz knockdown, there is also no difference in response between fed and starved groups for the experimental lines (nsyb-Gal4/Crz-RNAi, p=0.887), with only one parental control showing increased response upon starvation (nsyb-Gal4/CS, p=1.46e-05) but the other control group showing no difference (Crz-RNAi/CS, p=0.189).

3.6.3 A dopamine mutant shows different rebound responses

The *Drosophila* mutant, *fumin*, which was previously described in the introduction, has a very low sleep phenotype, which is well documented in the literature [255]. These flies have high activity and low sleep due to the mutated dopamine transporter (DAT), which leads to high levels of dopamine at the synapse. To understand how these flies may respond to starvation, when mated or virgin, and how this may differ to the response

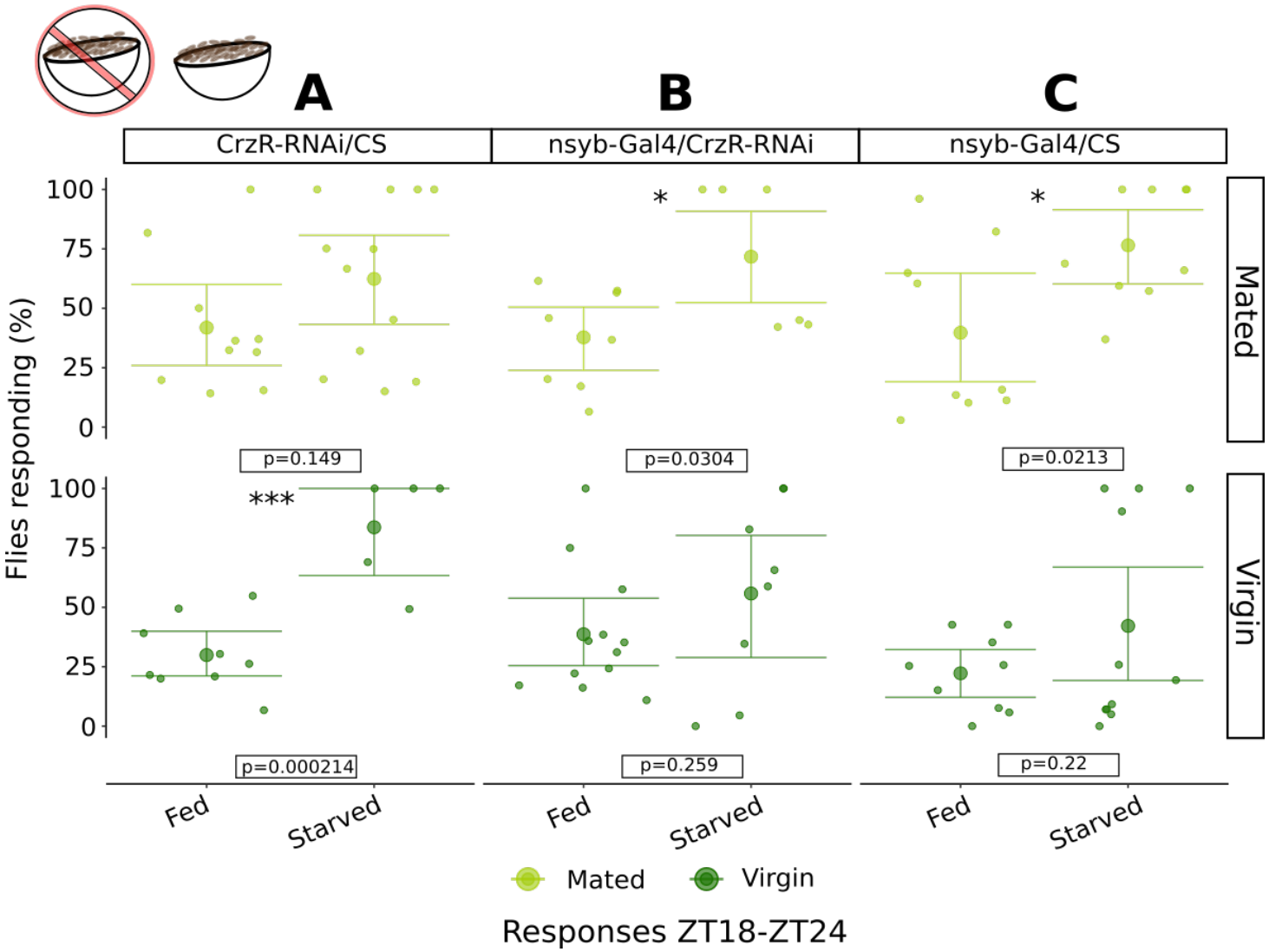


Figure 3.33: *Arousal responses of flies with CrzR neuronal knockdown.* Flies subject to an arousing stimulus of 5% acetic acid whilst asleep, between ZT18-ZT24, when starved or fed. **(A)** Responses of the parental control (CrzR-RNAi/CS) for mated females or virgin females. **(B)** Responses of flies with knockdown of CrzR neuronally for mated females or virgin. **(C)** Responses of the parental control (nsyb-Gal4/CS) for mated females and virgin females subject to an arousing stimulus. $N_{arousal}$ CrzR-RNAi/CS mated females: fed=12, starved=10. $N_{arousal}$ CrzR-RNAi/CS virgin females: fed=5, starved=9. $N_{arousal}$ nsyb-Gal4/CrzR-RNAi mated females: fed=8, starved=6. $N_{arousal}$ nsyb-Gal4/CrzR-RNAi virgin females: fed=8, starved=12. $N_{arousal}$ nsyb-Gal4/CS mated females: fed=9, starved=9. $N_{arousal}$ nsyb-Gal4/CS virgin females: fed=9, starved=9. Significance values for statistical differences between fed and starved groups (calculated with Wilcoxon rank-sum tests) are shown within the figure and data contained is from 2 biological repeats per group.

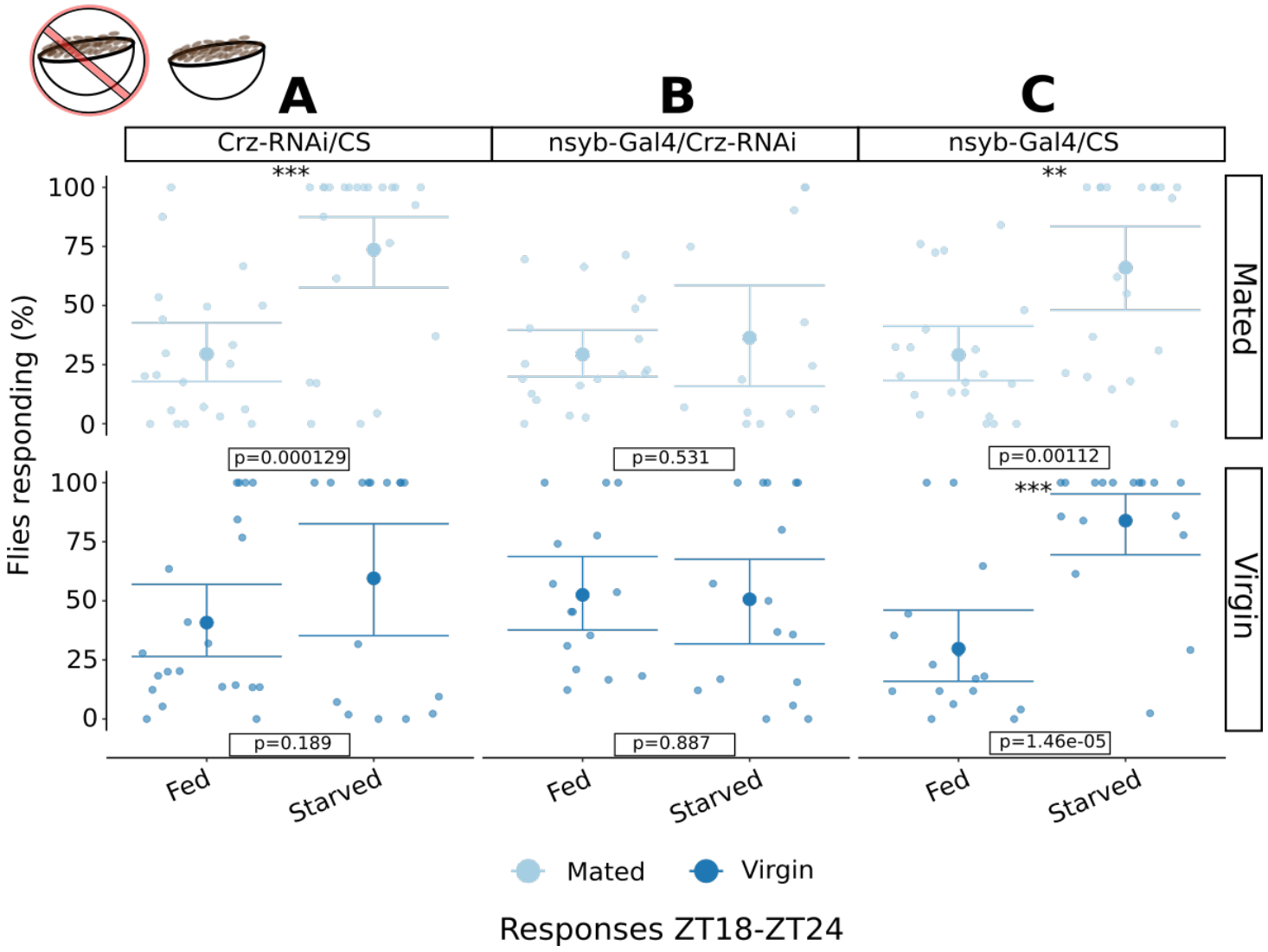


Figure 3.34: *Arousal responses of flies with Crz neuronal knockdown* Flies subject to an arousing stimulus of 5% acetic acid whilst asleep, between ZT18-ZT24, when starved or fed. **(A)** Responses of the parental control (Crz-RNAi/CS) for mated females or virgin females. **(B)** Responses of flies with knockdown of CrzR neuronally (Crz-RNAi/nsyb-CS) for mated females or virgin females. **(C)** Responses of the parental control (nsyb-Gal4/CS) for mated females and virgin females, subject to an arousing stimulus. $N_{arousal}$ Crz-RNAi/CS mated females: fed=23, starved=21. $N_{arousal}$ Crz-RNAi/CS virgin females: fed=16, starved=21. $N_{arousal}$ nsyb-Gal4/Crz-RNAi mated females: fed=13, starved=19. $N_{arousal}$ nsyb-Gal4/Crz-RNAi virgin females: fed=16, starved=21.$N_{arousal}$ nsyb-Gal4/CS mated females: fed=19, starved=21. $N_{arousal}$ nsyb-Gal4/CS virgin females: fed=17, starved=16. Significance values for statistical differences between fed and starved groups (calculated with Wilcoxon rank-sum tests) are shown within the figure and data contained is from at least 3 biological repeats per group.

from CS, I worked with a Master's student to test these flies in the same paradigm as I have described.

Fig.3.32 shows the results when these flies were starved and in the mated or virgin conditions. It can be seen that mated *fumin* females actually showed a significant sleep rebound following starvation-induced SD (p=0.0076), whereas virgin females did not show a significant sleep rebound (p=0.069). Unusually, the CS mated females tested alongside the *fumin* female groups as a control also showed a significant sleep rebound following starvation (p=0.014), an observation which was never seen in any other conditions and casts some doubt on the validity of the of the data. *fumin* virgin females did not show a significant sleep rebound following starvation (p=0.069) and the CS virgin control (p=0.0052), as usual, showed a significant rebound. This suggests that an increase in dopamine in mated and virgin females may lead to changes in starvation-induced rebound responses. However, repeating this experiment would be necessary to understand why the mated control group did not behave as seen in previous data.

Table 3.11: Statistical differences between mated and virgin females, *fumin* or CS

Genotype	mating status	time period	p-value
CS	mated	day SD	0.9 (n.s.)
CS	mated	night SD	0.89 (n.s.)
CS	mated	6hrs rebound	0.066 (n.s.)
CS	virgin	day SD	0.83 (n.s.)
CS	virgin	night SD	0.044 (*)
CS	virgin	6hrs rebound	0.065 (n.s.)
fumin	mated	day SD	0.94 (n.s.)
fumin	mated	night SD	0.1 (n.s.)
fumin	mated	6hrs rebound	0.016 (*)
fumin	virgin	day SD	0.39 (n.s.)
fumin	virgin	night SD	0.3 (n.s.)
fumin	virgin	6hrs rebound	0.34 (n.s.)

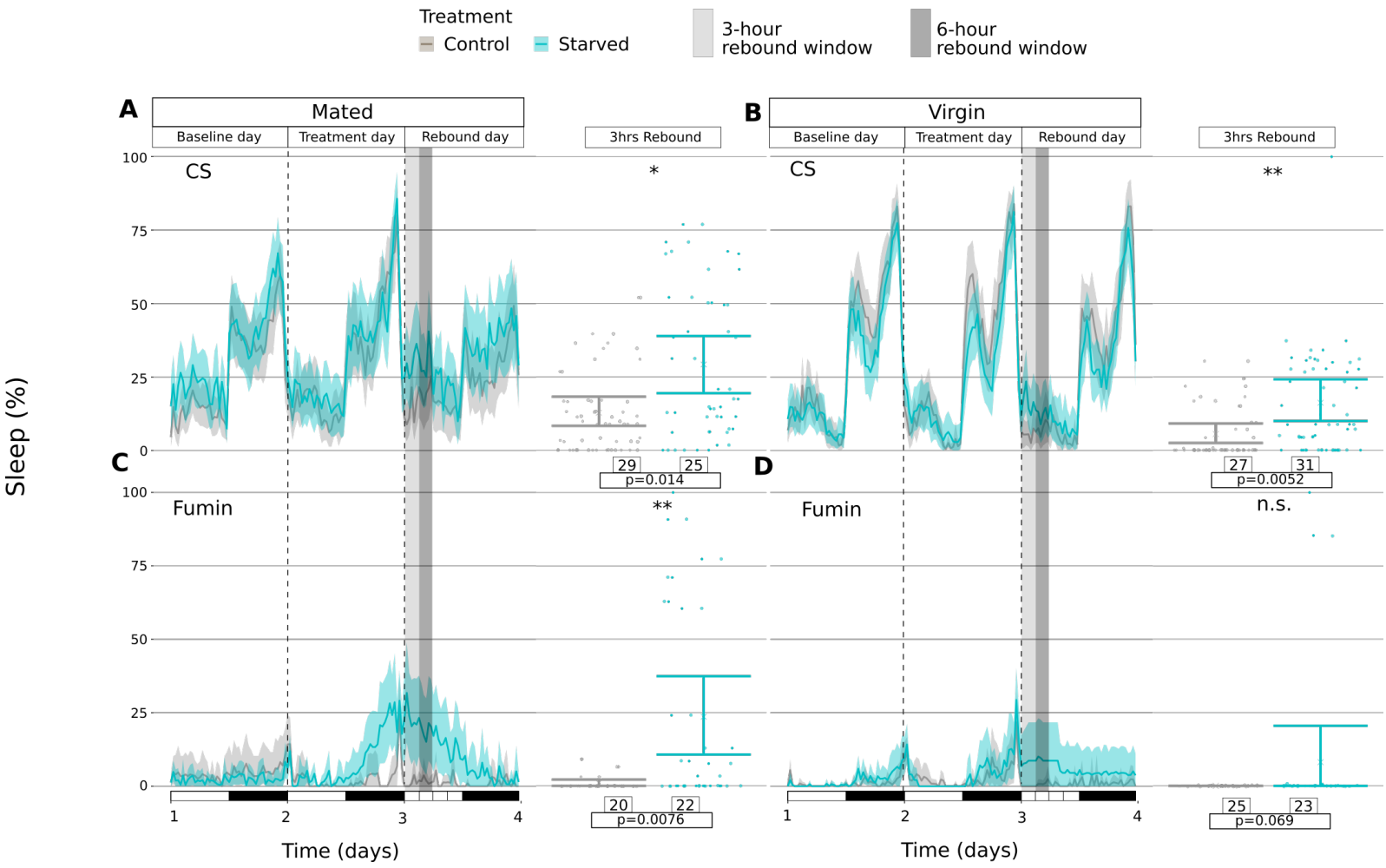


Figure 3.35: *The dopamine mutant, fumin, shows different responses to starvation SD.* Ethograms showing responses of **(A)** mated CS females to starvation SD **(B)** virgin CS females to starvation SD **(C)** *fumin* mated females to starvation SD **(D)** *fumin* virgin females to starvation SD. Box-plots represent rebound quantified in the first 3 hours of the rebound day. Shaded areas show a 95% bootstrap resampling confidence interval around the mean. *N* for each group and significance values for rebound (with differences between control and SD groups calculated with Wilcoxon rank-sum tests) are shown within the figure and data contained is from at least 3 biological repeats per group.

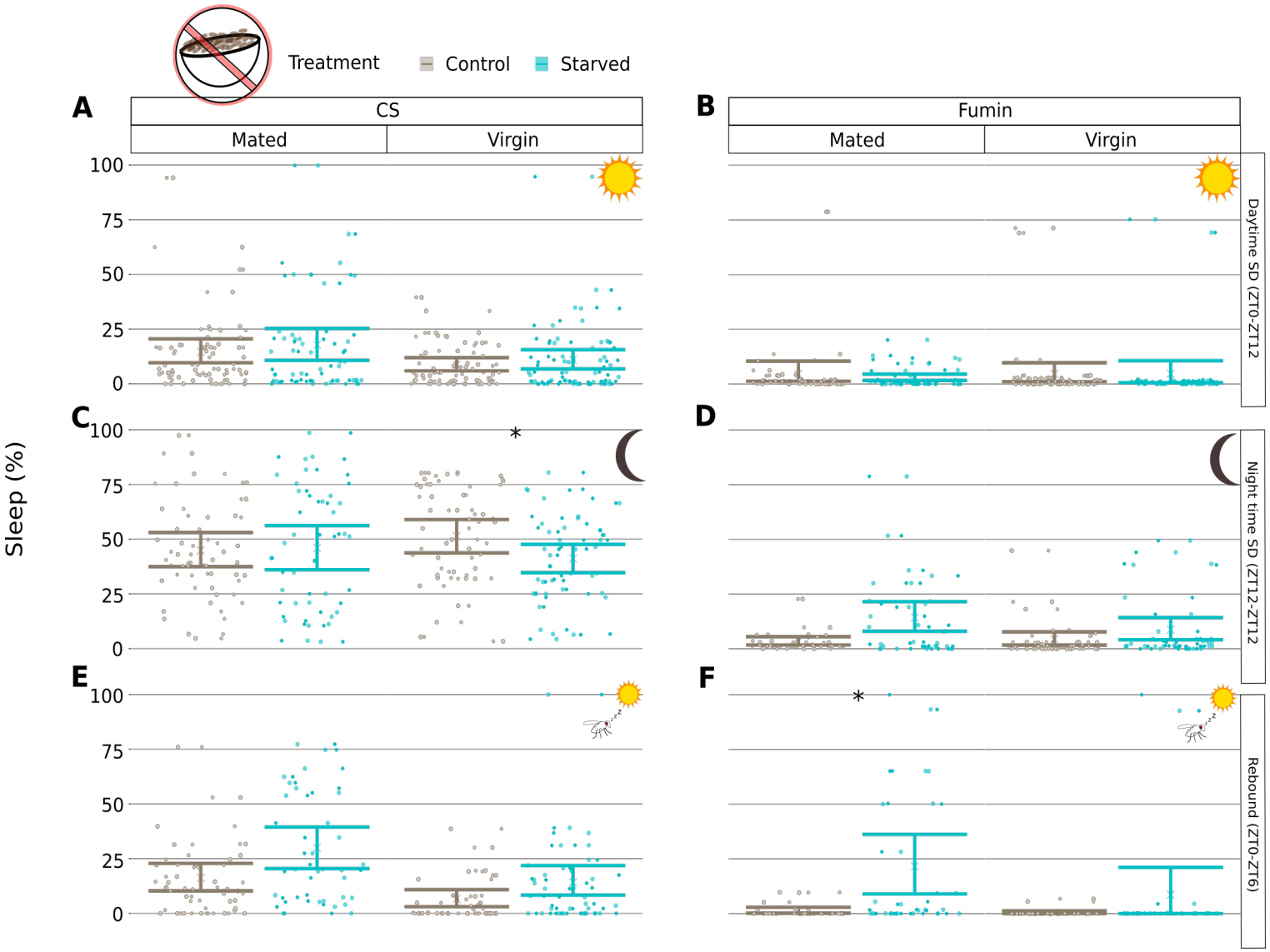


Figure 3.36: *Daytime SD, Night-time SD and 6 hour rebound quantification for CS and fumin flies with starvation SD.* Quantification of day-time starvation SD for **(A)** CS mated and virgin females **(B)** *fumin* mated and virgin females **(C)** quantification of night-time SD for CS mated and virgin females **(D)** quantification of night-time SD for *fumin* mated and virgin females **(E)** quantification of rebound in the 6 hours post treatment for CS mated and virgin females **(F)** rebound quantification for the 6 hours post treatment for *fumin* mated and virgin females. $N_{Starved}$ CS mated females: control=29, starved=25. $N_{Starved}$ CS virgin females: control=27, starved=31. $N_{Starved}$ *fumin* mated females: fed=20, starved=22. $N_{Starved}$ *fumin* virgin females: fed=25, starved=23. Error bars show 95% bootstrap resampling confidence intervals around the mean and data is from at least 3 biological repeats per group.

3.6.4 CrzR connects to a key dopamine regulatory centre in the brain

To understand if the CrzR receptor has a connection to dopaminergic neurons in the brain, I decided to test if there was an anatomical connection between the neurons in which CrzR was expressed (visualised by *trans*-Tango) and the PAM cluster of dopaminergic neurons. Based on previous data highlighting the expression pattern of PAM neurons and the data I obtained highlighting post-synaptic connections of CrzR from the *trans*-Tango assay, I theorised that there could be a possible connection between these groups of neurons. The GRASP technique allows synapses which connect two sets of neurons to emit fluorescence. After crossing the CrzR-Gal4 line with the R58E02-LexA line and then crossing this to the GRASP line, I imaged female flies to determine if there was connectivity.

The GRASP was successful and I saw an anatomical connection between the PAM cluster and the CrzR neurons (shown in Fig.3.34, connecting neurons depicted in yellow). This demonstrates that CrzR neurons receive input from the dopaminergic PAM cluster.

3.6.5 Activation of CrzR neurons and PAM neurons

Activation of dopaminergic neurons targetted by TH-Gal4 (which does not target PAM neurons) has been shown to be caused by gustatory stimulation [278]. Linford et al. also showed that this gustatory stimulation protects against the effects of starvation. If the same is true in PAM neurons, which also express dopamine, we would expect to see activation of these neurons leading to a resistance to starvation-induced sleep suppression. If CrzR neurons are also sensitive to dopaminergic neurons, we would expect

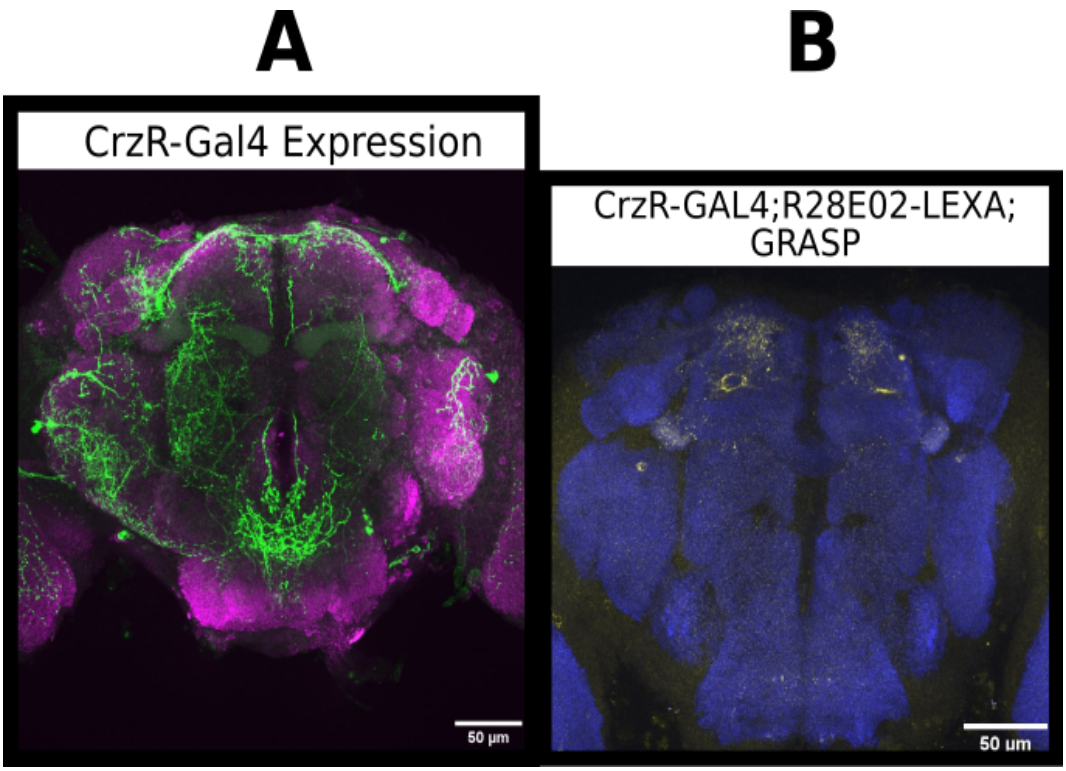


Figure 3.37: *GRASP connection between PAM neurons and CrzR neurons.* **(A)** Expression of CrzR in the fly brain (green). **(B)** Anatomical connection between CrzR neurons and PAM neurons in the dorsal part of the brain (yellow). Figure shown is a representative image of 8 sample brains.

to see a similar phenotype. To investigate if this was true, I activated CrzR neurons and PAM neurons thermogenetically. I initially kept these flies at a lower temperature of 22°C for the baseline day preceding the experiment, before raising the temperature for the 24 hour treatment period of starvation.

Fig.3.35 shows the ethogram of this experiment and Fig.3.36 shows the quantification. I observed that, for mated females, although there was no significant SD for females with activation and starvation of the PAM neurons for either the day or night time (R58E02-Gal4/TRPA1), there was also no significant SD for the control groups either (R58E02-Gal4/TRPA1,CS/TRPA1). However, activation and starvation of CrzR neurons (CrzR-Gal4/TRPA1) also did not lead to significant SD during the day, and an increase in sleep during the night-time and its parental control. (CrzR-Gal4/CS). Total SD, when quantified over the whole 24 hour period gives the same result.

For virgin female flies, activation in PAM neurons (R58E02-Gal4/TRPA1) and CrzR neurons (CrzR-Gal4/TRPA1) with simultaneous starvation led to no SD during the day and led to a significant decrease in sleep in control lines (R58E02-Gal4/CS,CS/UAS-TRPA1,CrzR-Gal4/CS). During the night-time, the only group with no decrease in SD during the starvation was that with CrzR activation (CrzR-Gal4/TRPA1). Total sleep for virgin females showed no significant SD for both CrzR (CrzR-Gal4/TRPA1) and PAM (R58E02-Gal4/TRPA1) activation, although the PAM parental control also did not show significant SD (R58E02-Gal4/CS). Intriguingly, the only flies to show sleep rebound were those which had activation of CrzR neurons (CrzR-Gal4/TRPA1).

This result shows that (1) dopaminergic PAM neurons may partially regulate the starvation response in mated females and (2) dopaminergic PAM neurons and CrzR neurons may regulate the response in virgin females to day sleep, and only CrzR neurons for night sleep.

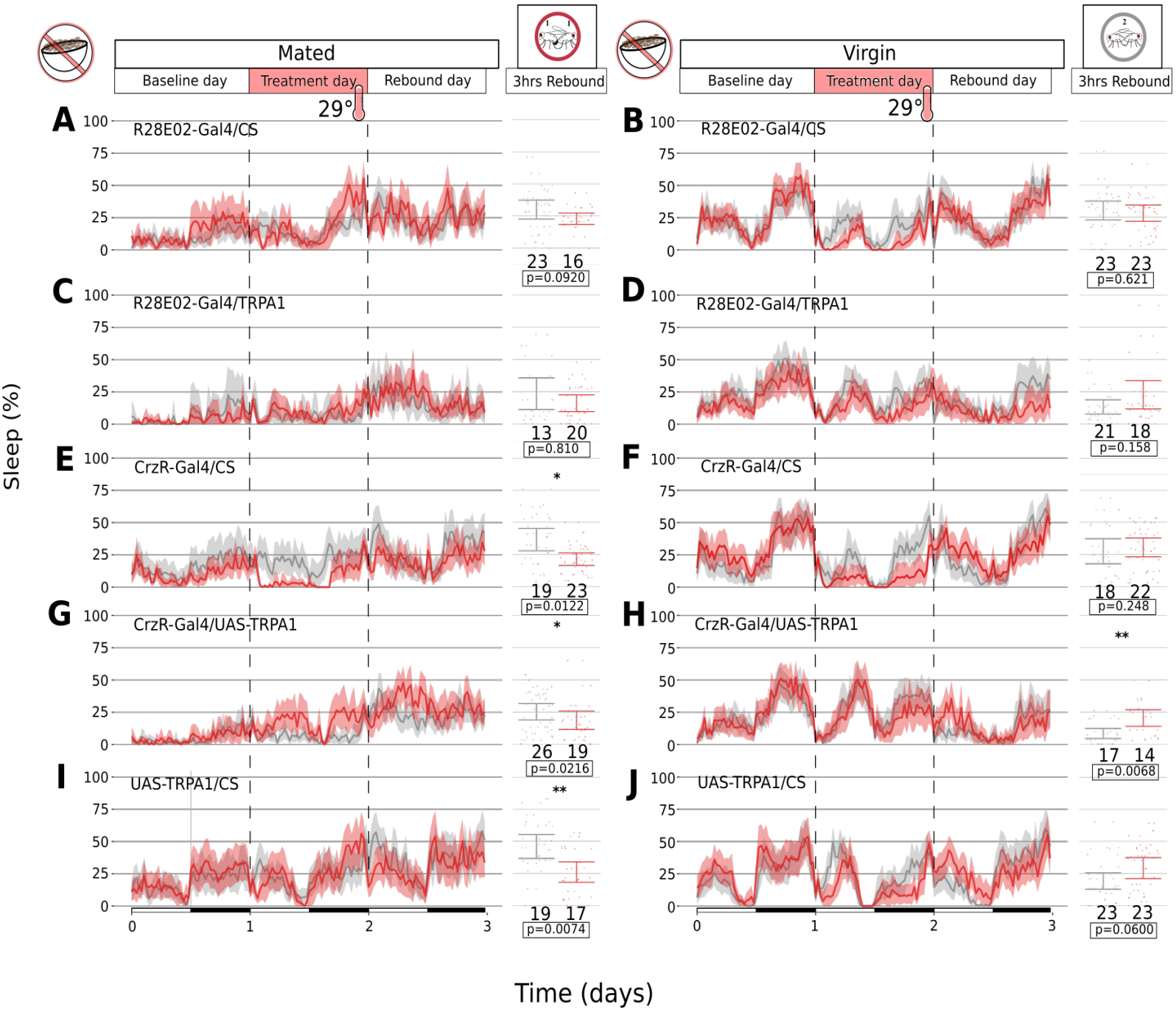


Figure 3.38: *Activation of CrzR neurons and PAM neurons during starvation SD.* Ethograms demonstrating the sleep profile for flies with activation of CrzR or PAM neurons during starvation treatment. **(A)** Parental control line for mated females with PAM-Gal4 targetting. **(B)** Parental control line for virgin females with PAM-Gal4 activation (R58E02-Gal4/CS). **(C)** Activation of PAM neurons (R58E02-Gal4/UAS-TRPA1) in mated females during starvation SD. **(D)** Activation of PAM neurons (R58E02-Gal4/UAS-TRPA1) in virgin females during starvation SD. **(E)** Parental control line for mated females with CrzR-Gal4 activation (CrzR-Gal4/CS). **(F)** Parental control line for virgin females with CrzR-Gal4 activation (CrzR-Gal4/CS). **(G)** Activation of CrzR neurons (CrzR-Gal4/UAS-TRPA1) in mated females during starvation SD. **(H)** Activation of CrzR neurons (CrzR-Gal4/UAS-TRPA1) in virgin females during starvation SD. **(I)** Parental control line for mated females with TRPA1 expression (UAS-TRPA1/CS). **(J)** Parental control line for virgin females with TRPA1 expression (UAS-TRPA1/CS). Shaded areas show a 95% bootstrap resampling confidence interval around the mean. *N* for each group and significance values for rebound (with differences between control and SD groups calculated with Wilcoxon rank-sum tests) are shown within the figure and data contained is from 3 biological repeats per group.

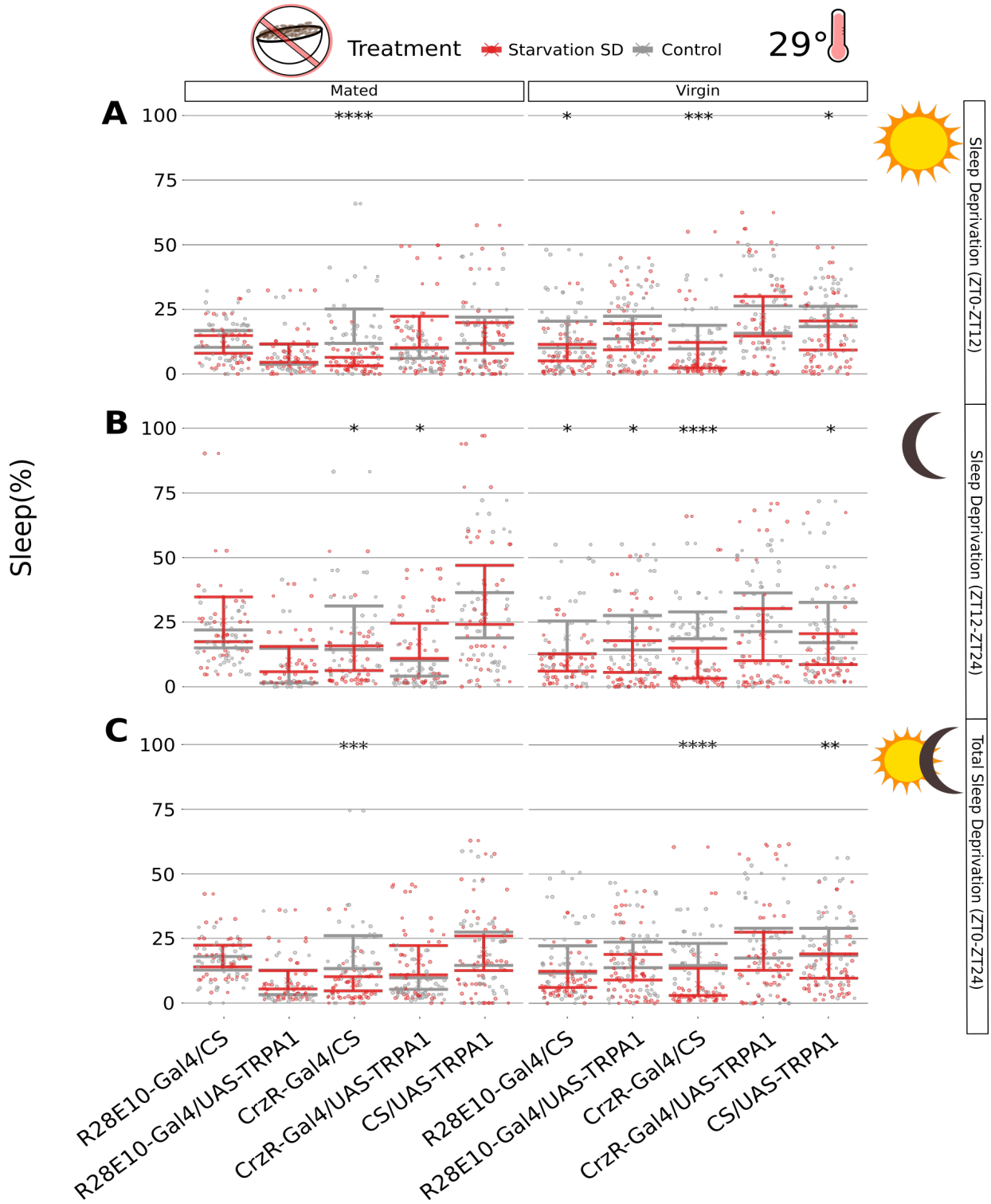


Figure 3.39: *Box-plot quantification for females with activation of CrzR or PAM neurons and their controls for day SD, night SD and the total SD.* **(A)** Day-time SD (ZT0-ZT12) for mated and virgin females. **(B)** Night-time SD (ZT12-ZT24) for mated and virgin females. **(C)** Total SD (ZT0-ZT24) for mated and virgin females. $N_{Starved}$ R58E02-Gal4/CS mated females: control=23, starved=16. $N_{Starved}$ R58E02-Gal4/CS virgin females: control=23, starved=23. $N_{Starved}$ R58E02-Gal4/TRPA1 mated females: fed=13, starved=20. $N_{Starved}$ R58E02-Gal4/TRPA1 virgin females: fed=21, starved=18. $N_{Starved}$ CrzR-Gal4/CS mated females: fed=19, starved=23. $N_{Starved}$ CrzR-Gal4/CS virgin females: fed=18, starved=22. $N_{Starved}$ CrzR-Gal4/TRPA1 mated females: fed=26, starved=19. $N_{Starved}$ CrzR-Gal4/TRPA1 virgin females: fed=17, starved=14. $N_{Starved}$ UAS-TRPA1/CS mated females: fed=19, starved=17. $N_{Starved}$ UAS-TRPA1/CS virgin females: fed=23, starved=23. Error bars show 95% bootstrap resampling confidence intervals around the mean and data is from 3 biological repeats per group.

3.6.6 Summary

- Increasing dopamine with L-DOPA feeding leads to decreased day sleep in CS virgin females.
- Increasing dopamine with L-DOPA feeding leads to decreased night sleep in CS mated females.
- Increasing dopamine with L-DOPA feeding does not change sleep in CrzR mutant or virgin females.
- CrzR neurons are anatomically connected to the PAM dopaminergic neurons in the fly brain.

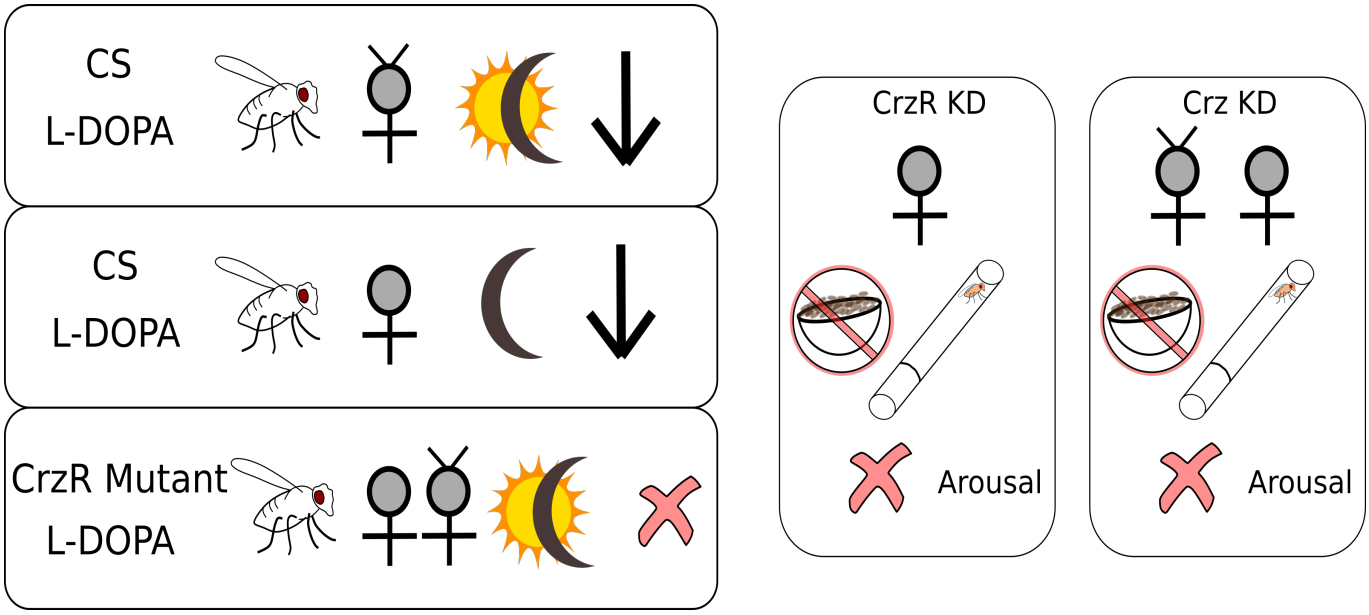


Figure 3.40: *Summary diagram for the investigation of dopamine in sleep responses*

3.7 Other data from sleep investigations

Although males have not been implicated in having differences before and after mating, I was interested to see if there would be differences in sleep when males were (1) put

into different social groups, as was done with females in section 3.4 (2) how males in these groups would respond to starvation-induced SD in these groups.

3.7.1 Baseline sleep in males

To understand how males in different social groups slept during the day and night, I put male flies in the same groups outlined in section 3.4 and monitored baseline sleep. This data confirms that socially isolated males show reduced daytime sleep and not reduced night-time sleep, as was reported by [271]. However, the socially isolated group did not show significantly different sleep to males in the mixed-sex female skewed group. There were no differences to note specifically when examining night-time sleep. Statistical differences between groups are shown in table 3.12 and differences between groups calculated with Wilcoxon rank-sum tests and corrected with FDR adjustments.

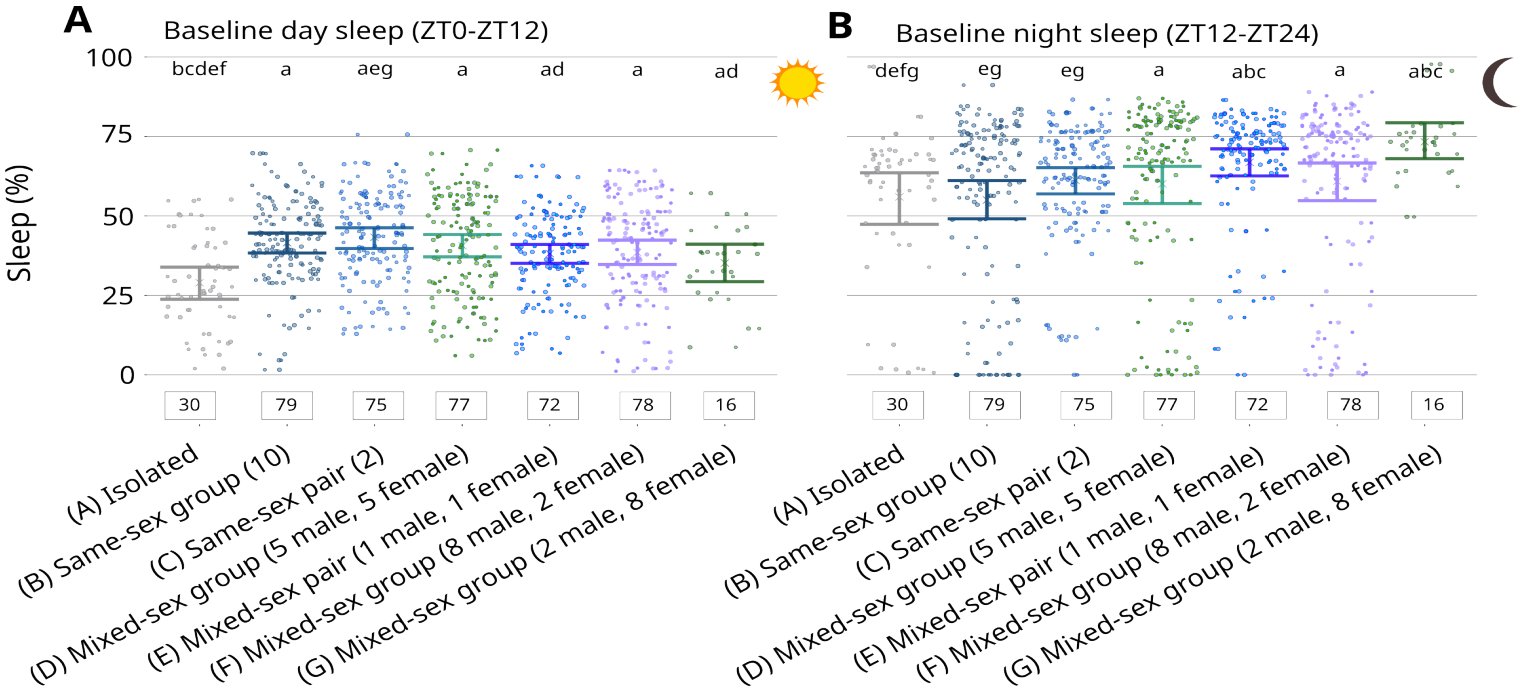


Figure 3.41: *Baseline sleep for males with differing prior experiences.* **(A)** Quantification of male sleep from prior social experience groups during the day-time **(B)** night-time. Error bars show 95% bootstrap resampling confidence intervals around the mean. *N* for each group is shown within each figure and data is from at least 3 biological repeats per group.

Table 3.12: Differences in mean day-time sleep amount between CS male groups

Group 1	Group 2	adj. P.value	Significance
A (isolated)	B (virgin group)	0.00018	***
A (isolated)	C (virgin pair)	5e-05	****
A (isolated)	D (even-SR group)	0.00131	**
A (isolated)	E (ms pair)	0.00480	**
A (isolated)	F (male-skewed SR group)	0.00476	**
A (isolated)	G (female-skewed SR group)	0.16641	n.s.
B (virgin group)	C (virgin pair)	0.46749	n.s.
B (virgin group)	D (even-SR group)	0.83019	n.s.
B (virgin group)	E (ms pair)	0.11419	n.s.
B (virgin group)	F (male-skewed SR group)	0.40340	n.s.
B (virgin group)	G (female-skewed SR group)	0.07588	n.s.
C (virgin pair)	D (even-SR group)	0.39665	n.s.
C (virgin pair)	E (ms pair)	0.02679	*
C (virgin pair)	F (male-skewed SR group)	0.14183	n.s.
C (virgin pair)	G (female-skewed SR group)	0.04049	*
D (even-SR group)	E (ms pair)	0.24200	n.s.
D (even-SR group)	F (male-skewed SR group)	0.50907	n.s.
D (even-SR group)	G	0.19086	n.s.
E (ms pair)	F (male-skewed SR group)	0.62482	n.s.
E (ms pair)	G (female-skewed SR group)	0.38086	n.s.
F (male-skewed SR group)	G (female-skewed SR group)	0.32664	n.s.

3.7.2 Starvation-induced Sleep Deprivation in Males with Different Social Experiences

Following on from looking at baseline sleep in males, I decided to look at how males from these groups would respond to starvation-induced SD.

When tested, all males from all social groups showed a significant decrease in daytime sleep and a significant decrease in night-time sleep in response to starvation. However, only three groups showed a significant rebound following the SD: (A) Isolated males (E) Males which had been paired with another female (G) and males which were in the

Table 3.13: Differences in mean night-time sleep amount between CS male groups

Group 1	Group 2	adj. P.value	Significance
A (isolated)	B (virgin group)	0.44518	n.s.
A (isolated)	C (virgin pair)	0.21058	n.s.
A (isolated)	D (even-SR group)	0.03888	*
A (isolated)	E (ms pair)	0.00061	***
A (isolated)	F (male-skewed SR group)	0.02339	*
A (isolated)	G (female-skewed SR group)	0.00185	**
B (virgin group)	C (virgin pair)	0.69888	n.s.
B (virgin group)	D (even-SR group)	0.07877	n.s.
B (virgin group)	E (ms pair)	0.00576	**
B (virgin group)	F (male-skewed SR group)	0.07677	n.s.
B (virgin group)	G (female-skewed SR group)	0.03624	*
C (virgin pair)	D (even-SR group)	0.13658	n.s.
C (virgin pair)	E (ms pair)	0.00630	**
C (virgin pair)	F (male-skewed SR group)	0.14634	n.s.
C (virgin pair)	G (female-skewed SR group)	0.02745	*
D (even-SR group)	E (ms pair)	0.50320	n.s.
D (even-SR group)	F (male-skewed SR group)	0.84245	n.s.
D (even-SR group)	G	0.41871	n.s.
E (ms pair)	F (male-skewed SR group)	0.39211	n.s.
E (ms pair)	G (female-skewed SR group)	0.71300	n.s.
F (male-skewed SR group)	G (female-skewed SR group)	0.34174	n.s.

female-skewed group. No trend as to why rebound would or would not occur could be established, however. One group, that of males from the same-sex group (B) showed a significant negative rebound for the 6 hours post starvation; this was the only group to do so. As this data allowed no clear conclusions to be drawn as to how mating in males may change sleep and responses to starvation-induced SD, I did not do more experimental investigations into this.

3.7.3 Other GRASP Connections

Further to the GRASP connection which I demonstrated in section 3.6.5 between CrzR neurons and the PAM dopaminergic neurons, I also conducted other GRASP assays,

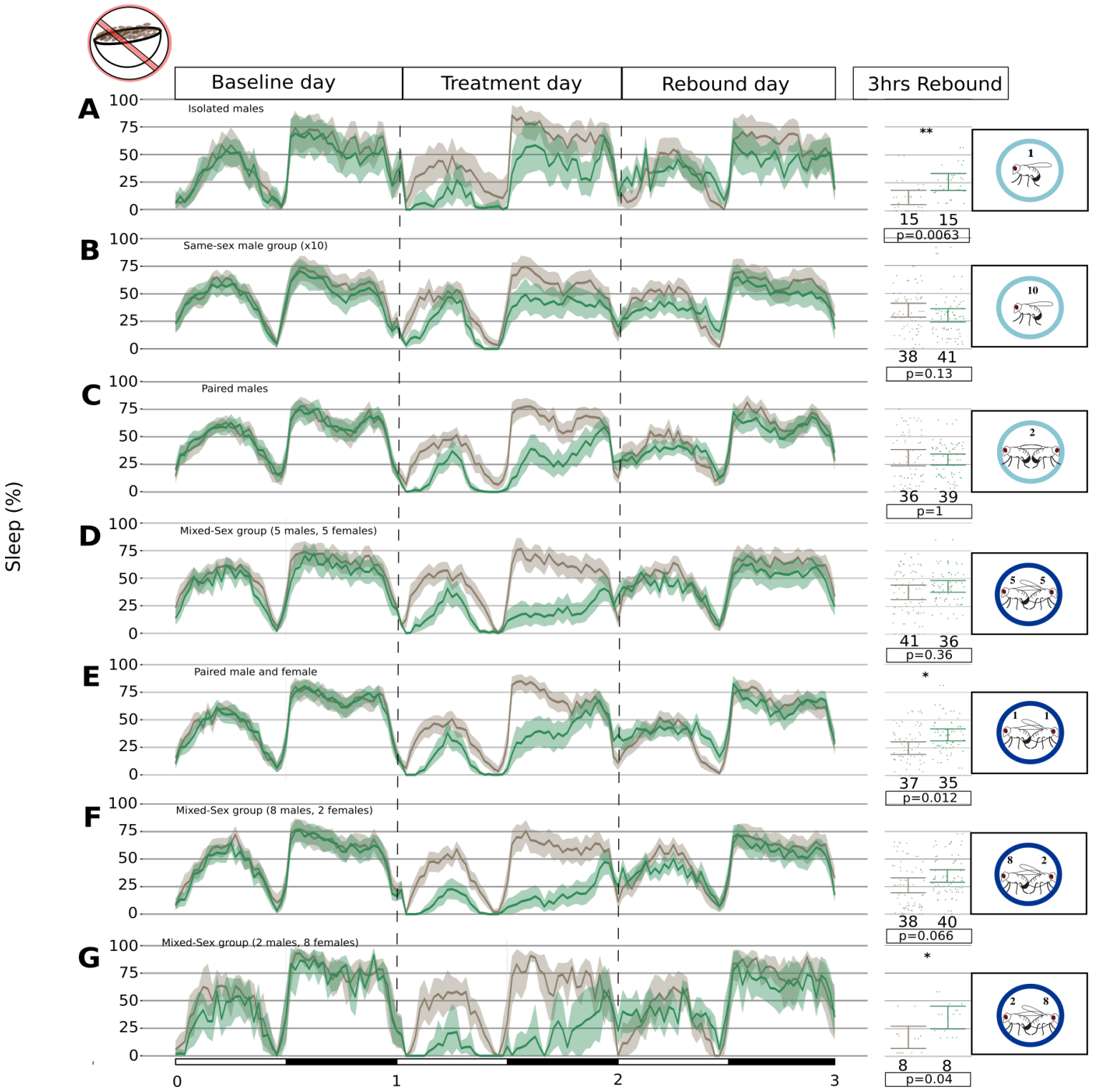


Figure 3.42: *Ethograms for males from prior experience groups exposed to starvation SD.* Ethograms for males from each of the 7 prior experience groups and the rebound sleep amounts of starved males in each group compared to fed controls. **(A)** Isolated males. **(B)** Males from the same-sex male group. **(C)** males from the paired male group. **(D)** Males from the mixed-sex even sex-ratio group. **(E)** Males from the male and female pair. **(F)** Males from the male-skewed mixed-sex group. **(G)** Males from the female-skewed mixed-sex group. Shaded areas show a 95% bootstrap resampling confidence interval around the mean. *N* for each group and significance values for rebound (calculated with Wilcoxon rank-sum tests comparing control groups to SD groups) are shown within the figure and data contained is from at least 3 biological repeats per group.

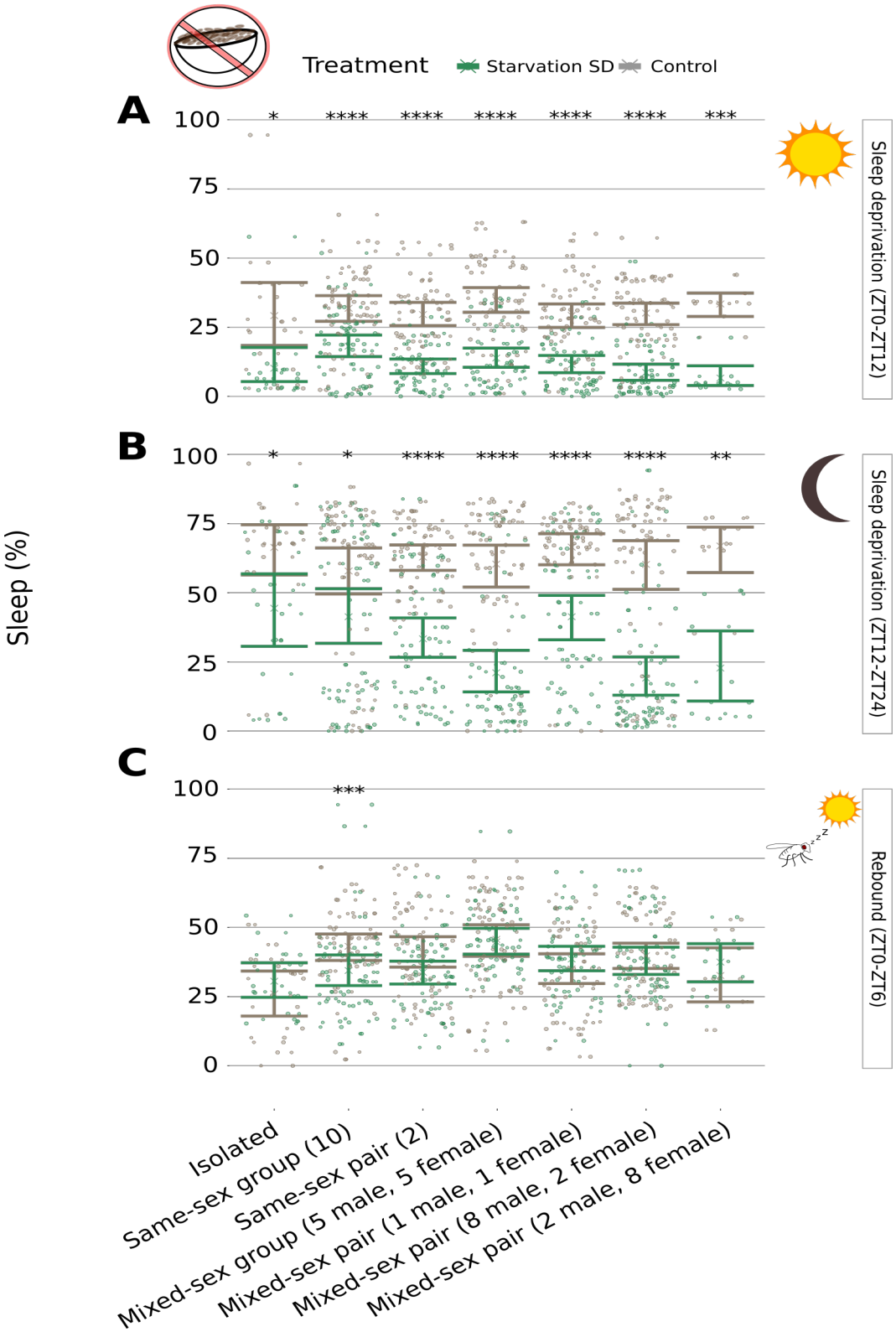


Figure 3.43: *Daytime SD, Night-time SD and 6 hour rebound quantification.* Quantification of various time periods for **(A)** Mated and virgin males for day-time SD. **(B)** Mated and virgin males for night-time SD. **(C)** Mated and virgin males for rebound in the 6 hours post treatment. $N_{Isolated}$ control=15, SD=15. $N_{Same\text{-}sex\ group}$ control=38, SD=41. $N_{Paired\ females}$ control=36, SD=39. $N_{Mixed\text{-}sex\ group\ -\ 5\ fem,\ 5\ male}$ control=41, SD=36. $N_{Paired\ male\ and\ female}$ control=37, SD=35. $N_{Mixed\text{-}sex\ group\ -\ 2\ fem,\ 8\ male}$ control=38, SD=40. $N_{Mixed\text{-}sex\ group\ -\ 8\ fem,\ 2\ male}$ control=8, SD=8. Error bars show 95% bootstrap resampling confidence intervals around the mean.

looking at connections between Crz neurons and two other regions of the brain.

Two other possible connections I looked for were between Crz neurons and neurons expressing the circadian neuropeptide PDF. As discussed in the introduction, PDF is expressed in the main circadian regulating neurons in the brain, including both l-LNvs and sLNVs clock neurons [13]. Based on the *trans*-Tango data from section 3.4.1 which showed that Crz was expressed throughout the eye, a key region where PDF is also expressed, I decided to check to see if neurons expressing PDF and Crz were anatomically connected. Fig.3.41A shows the resulting images from this assay. It was shown that there was an anatomical connection between Crz neurons and those expressing PDF, with points of connectivity highlighted with the white arrows. These points of connection were in several regions: the OL, around the peduncles of the MB, through the midline of the brain and in dorsal areas of the brain.

I also looked for a connection between neurons expressing sNPF and Crz. sNPF is a neuropeptide which is involved in various processes, including sleep [64] and olfactory memory [245]. It has expression in a variety of areas in the brain, including clock neurons and the MB [221]. Based on this data in the literature, I also looked to see if there was a connection between sNPF expressing neurons and Crz neurons. The GRASP assay was successful and showed a strong signal demonstrating connection in the OL (Fig.3.41B).

3.7.4 *trans*-Tango Expression of sNPFR

I have shown that there is an anatomical connection between sNPF and Crz neurons. As I have showed in this work so far, the receptor of Crz is important in sleep regulation. As was stated in the introduction, sNPF has been shown to be linked to sleep regulation, but less is known about its receptor. To understand how the expression of the receptor

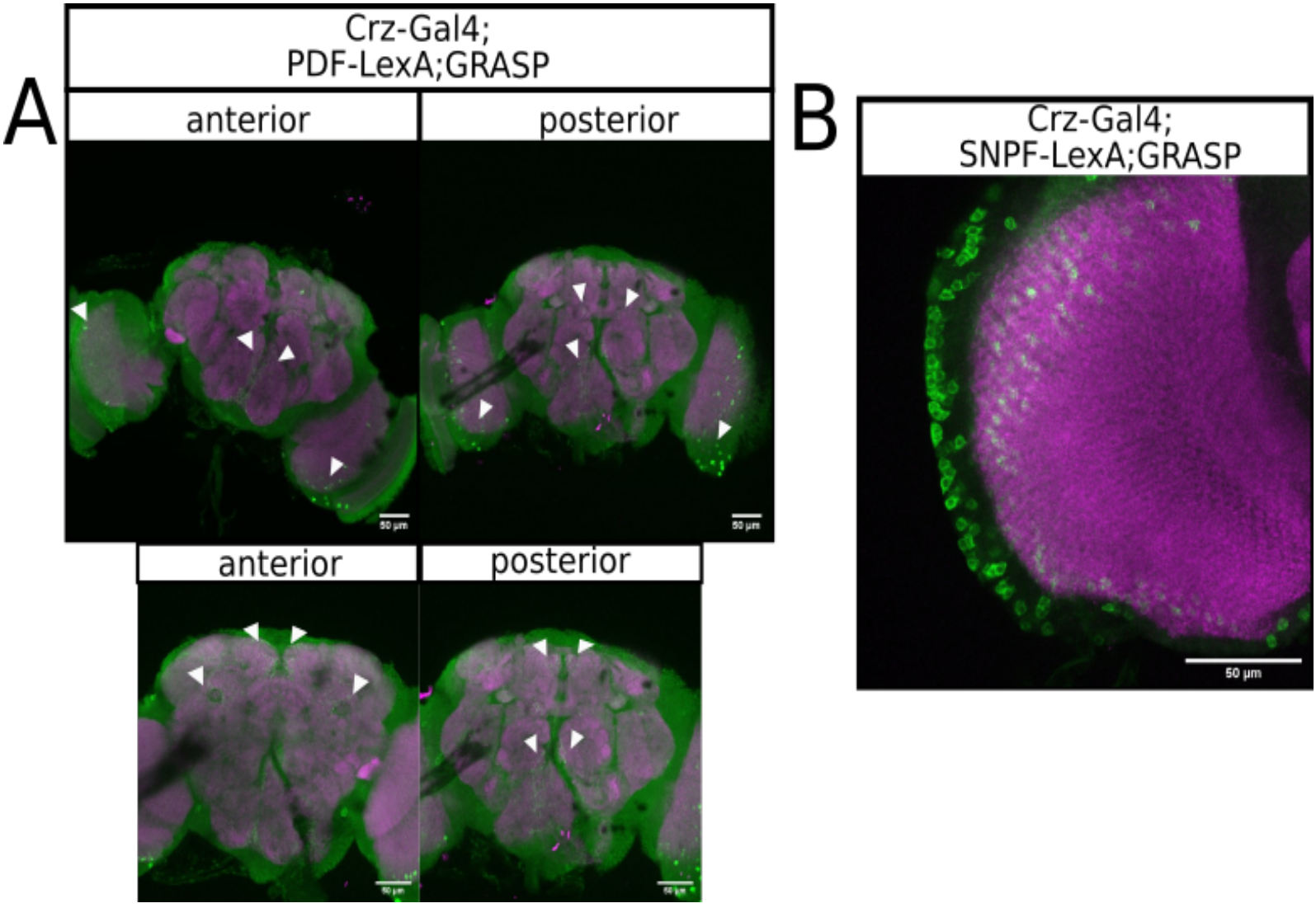


Figure 3.44: *GRASP connections of Crz neurons.* **(A)** Anatomical connection between Crz neurons (CrzR-Gal4) and PDF neurons (PDF-LexA), with connections highlighted by white triangles, for 10x magnification and 20x magnification. **(B)** Anatomical connection between Crz neurons (Crz-Gal4) and sNPF neurons (sNPF-LexA) in the optic lobe. Image shown in A is representative of 6 brains. Image shown in B is representative of 8 brains. Scale bars, 50μm.

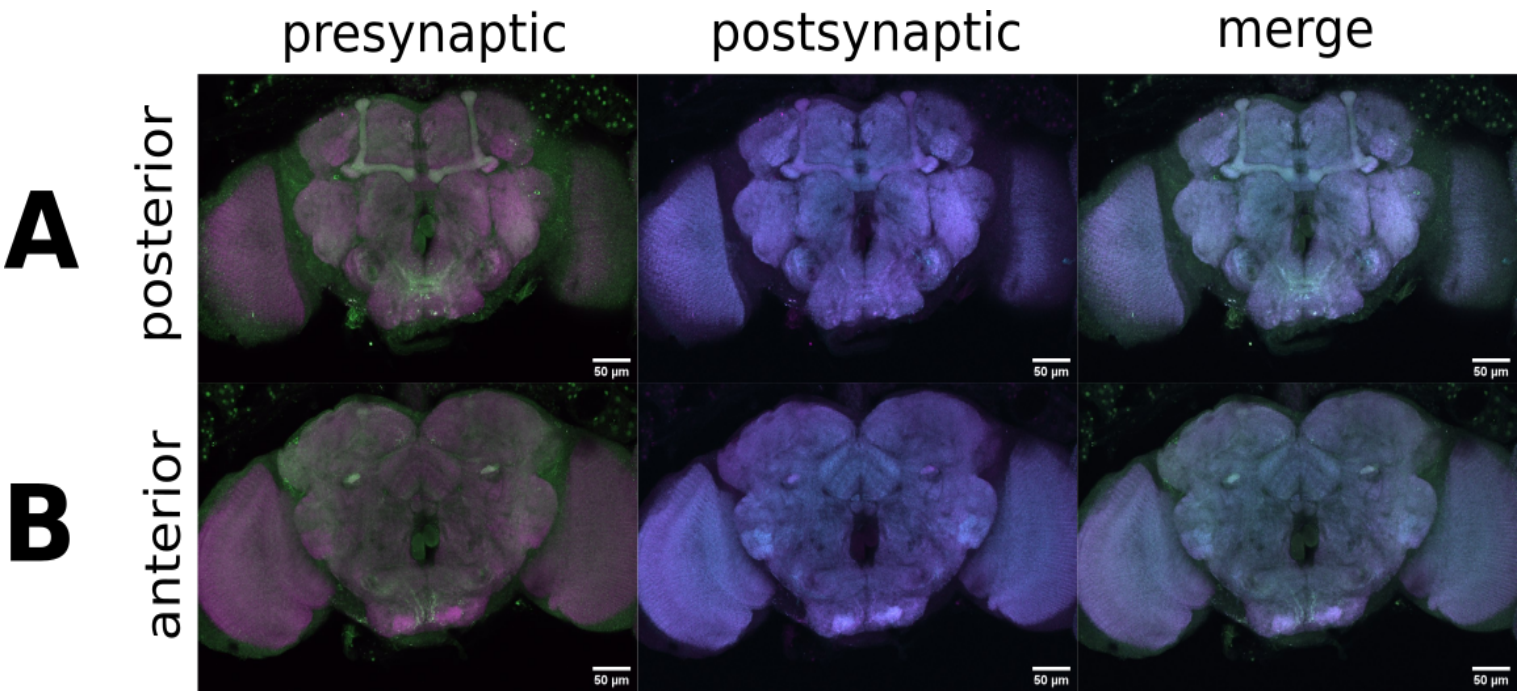


Figure 3.45: *trans-Tango expression of sNPFR-Gal4.* *trans*-Tango assay showing presynaptic connections (green), post-synaptic connections (cyan) and merged images for sNPFR-Gal4 in the central *Drosophila* brain at 10x magnification for the **(A)** anterior part of the brain **(B)** posterior part of the brain. Images shown are representative of 10 brains. Scale bars, 50μm.

of sNPF may be similar to that of CrzR in the *Drosophila* brain, I performed a *trans*-Tango assay.

Expression of sNPFR was seen in various parts of the brain, including the MB, the SEZ and the lowest layer of the FSB. Postsynaptic connections were mainly seen in the SEZ and dFSB.

3.7.5 Summary

- Isolated males show reduced day-time sleep compared to other social groups, as shown in the literature.
- There is no pattern in starvation-induced SD rebound response between mated and virgin males.
- GRASP data shows that there are anatomical connections between Crz expressing

neurons and those expressing PDF and sNPF.

- *trans*-Tango assay of sNPFR shows expression in the MB and the FSB.

3.8 Overall Summary of Sleep Data

In this chapter, I looked uncovering differences in sleep and homeostatic rebound in a number of contexts. I have shown:

- CS mated and virgin females show significant sleep rebound in response to dynamic SD.
- CS mated and virgin females show different rebound responses to starvation SD, with mated females showing no rebound response to starvation and virgin females showing a robust rebound response. This effect was not dependent on prior experience of different group sizes or sex-ratios.
- Baseline day and night sleep differs in CS mated and virgin females based on prior past experience.
- The receptor of the stress neuropeptide, Crz, is expressed in key sleep regulating regions in the *Drosophila* brain.
- CrzR mutant females show increased day and night sleep compared to CS females.
- CrzR mutant females show a significant rebound after dynamic SD, regardless of mating status, however, CrzR mutant virgins show a smaller, shorter rebound following starvation SD.
- Knockdown of CrzR in non-PAM dopaminergic neurons in mated females leads to significant rebound following starvation-induced SD.
- Knockdown of CrzR in PAM dopaminergic neurons in virgin females suppresses sleep rebound in virgin females.

- Dopamine is involved in the changes in sleep homeostasis seen between mated and virgin females, and PAM dopaminergic neurons connect to CrzR neurons in the *Drosophila* brain.

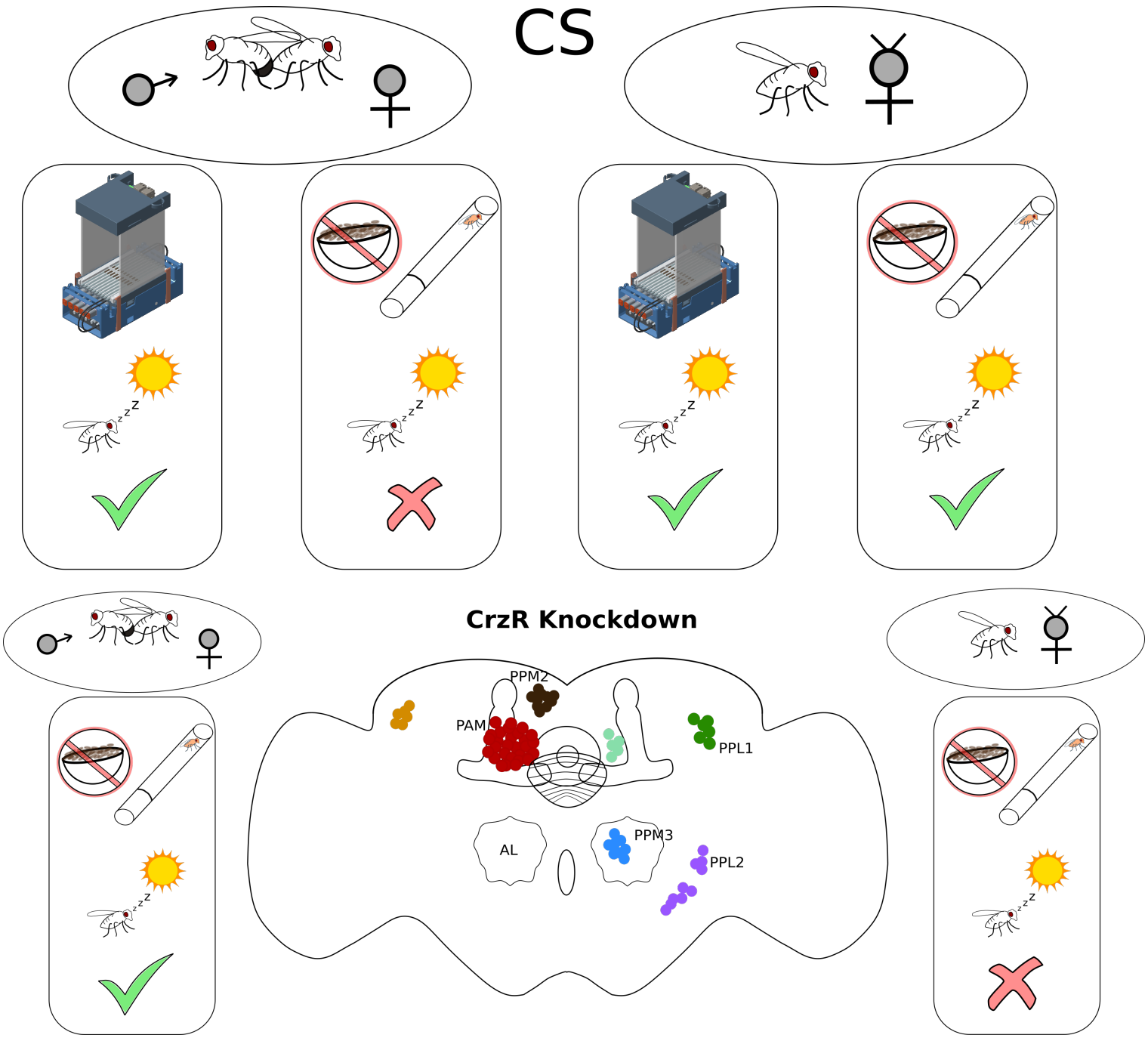


Figure 3.46: *Summary diagram of the work in chapter 3*

Chapter 4

Investigating Activity with Classification: Insecticides, Genes and Sleep

4.1 Introduction

As discussed in the introduction, phenotypic screens are a useful method to process large quantities of data with the aim here of using an *"ethomics"* analysis approach for screening.

This part of my thesis contains work relating to an industrial collaboration with the agricultural company, Syngenta.

These quantitative pipelines which were investigated for use had the aim of giving a greater understanding of whether it was possible to expand behavioural screening to insecticidal compounds. We set out to use a combination of high-throughput and high-resolution methods to quantitatively analyse behavioural phenotypes in *Drosophila*

melanogaster. Further than just simply trying to quantify this process, one of the main aims here was to look at whether behaviours produced by flies to compounds with a particular MoA would also be similar to those produced by flies exposed to a compound with a similar MoA.

4.2 Investigating Phenotypes from Insecticide Compounds using Highly Comparative Time Series Analysis

4.2.1 Development of a high-throughput screening method

To explore the potential of the high-throughput monitoring system developed by the Gilestro Lab, with the design and production of the ethoscope system [150], we firstly decided to conduct a small behavioural screen of various insecticidal compounds to allow us to assess the validity of using activity as a proxy of behaviour. However, to be able to conduct such a screen, various modifications were carried out on the ethoscope system.

In its original form, the ethoscope was designed to monitor sleep and activity behaviour in *Drosophila melanogaster*. Flies are placed in glass tubes with standard fly food at one end (sealed with paraffin wax), to provide nutrition and hydration, and cotton wool at the other end to prevent the fly escaping. In this configuration, flies are able to move away from the food and freely move in the space of the tube, from one end to the other. To measure the activity behaviour resulting from insecticide exposure, the system needed to be modified to allow constant exposure to the compound. It was also determined that the most useful system would be one that replicated that used by our

collaborators. To this end, the behavioural arena was redesigned, with only minor differences to the plate used at Syngeta. The new behavioural arena was designed in the form of a 24-well plate which also fit inside the ethoscope module and each well allowed the placement of a single fly, compared to a group of flies used in Syngenta's manual assay (in order to accommodate the ethoscope's tracking capability a only a single fly per well could be used).

Each well was filled with the same sucrose containing agarose solution and allowed to set before insecticide compound in solvent solution was applied to the surface of the food medium (see Methods). The solution was first allowed to dry before the fly was placed in the well and the arena was then placed in the ethoscope for recording. To capture the effects of the insecticide exposure to the fly, monitoring was done over two days, but only the first 12-hours of behavioural data (from the point of recording onset) was used for subsequent analysis. This was due to death of some flies from longer exposure, data processing restrictions and to negate any circadian effects caused by the onset of darkness. Once the data for the 12 hour period was collected, a single time-series was produced for each individual fly of maximum velocity (see Methods). For the time series analysis and classification, we used a published statistical classification method, HCTSA [138], [139]. As explained in the introduction, this method has been validated on a variety of different applications, including behavioural ethoscope data using *Drosophila melanogaster*, where maximum velocity was used as a proxy for behaviour [138].

HCTSA performs more than 7000 statistical tests (operations) on each time series presented and for the output of each operation returns a value referred to as a feature. Therefore, each individual time-series has the potential to have more than 7000 unique features associated with it. The features, which are produced for each time series, can

be normalised and clustered into a data matrix (see Fig.4.1). Based on this resulting data matrix, information regarding the underlying properties of the time series can be used to classify the time series into groups based on similar properties. This method is beneficial as it does not assume any underlying properties about the data and allows more quantifiable comparisons between the time series data.

4.2.2 Testing the validity of a high-throughput phenotypic screening approach

To test the effectiveness of the methodology, I first attempted classification of a small number of compounds provided by Syngenta tested against a solvent control. The initial compounds which were tested can be found in table 4.1. These initial compounds were chosen as they provided a variety of targets, with many having their effects well documented in the literature and they were each familiar to our collaborators in that they had been noted as producing interesting and obvious behavioural phenotypes. Some were also chosen on the basis that they produced similar phenotypes to each other and therefore had the potential to be easily confused. These compounds were initially only tested at a single high dose, 1000ppm, in order to ensure the behavioural phenotype was as strong as possible.

The confusion matrix classifying these results can be seen in Fig.4.2.

Reassuringly, all the compounds classified very well, with an overall classification accuracy of 73.6% (using a 10-fold SVM linear classification). All compounds showed largely correct predictions with only limited confusion with other compounds.

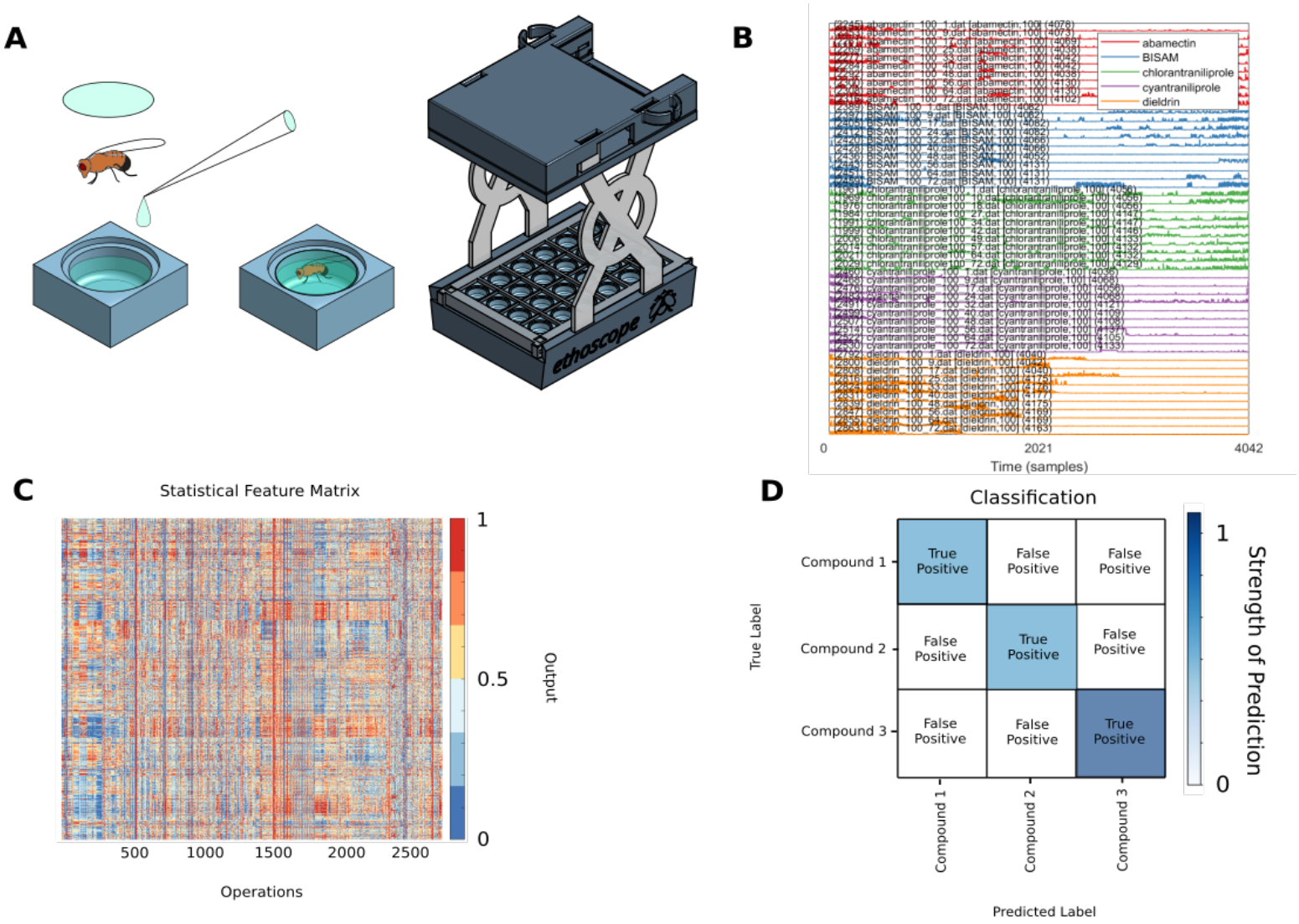
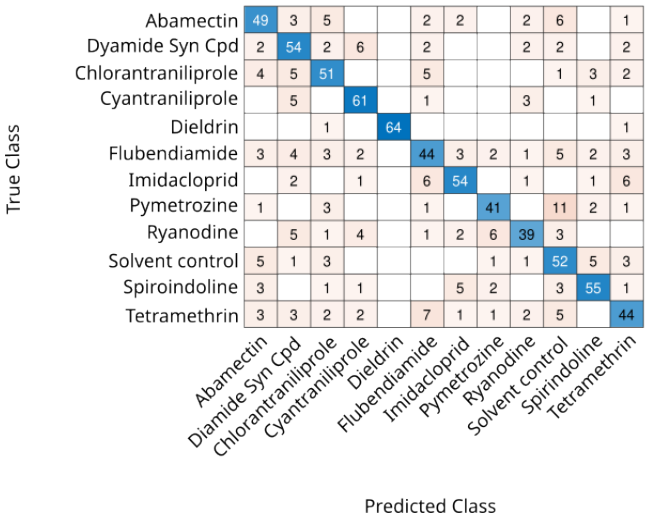


Figure 4.1: *Methodology of insecticide testing with the ethoscope system.* **(A)** Flies were placed in small 3D printed squares with sucrose containing agarose medium and insecticide solution on top before being placed in the ethoscope for behavioural testing. **(B)** Representative time-series traces for maximum velocity of flies exposed to various compounds. **(C)** Representative statistical feature matrix for outputs from time-series data in HCTSA. **(D)** Example classification matrix, describing how "True Positive" values can be identified and how "False Positive" values can be identified.

A



B

Compound	1st Classification	% Predicted	2nd Classification	% Predicted
Abamectin	Abamectin	70	Solvent Control	9
Diamide Syn Cpd	Diamide Syn Cpd	75	Cyantraniliprole*	8
Chlorantraniliprole	Chlorantraniliprole	72	Diamide C.*/Flubend.*	7
Cyantraniliprole	Cyantraniliprole	86	Diamide Syn Cpd*	7
Dieldrin	Dieldrin	97	Chlorantran/Tetram.	1
Flubendiamide	Flubendiamide	61	Solvent Control	7
Imidacloprid	Imidacloprid	76	Flubend./Tetrameth.	7
Pymetrozine	Pymetrozine	68	Solvent Control	18
Ryanodine	Ryanodine	63	Pymetrozine	10
Solvent Control	Solvent Control	73	Abamectin/Spiroindol.	7
Spiroindoline	Spiroindoline	77	Imidacloprid	7
Tetramethrin	Tetramethrin	63	Flubendiamide	10

Figure 4.2: *Confusion matrix for flies exposed to the original 11 compounds and control at 1000ppm.* **(A)** Classification matrix of flies exposed to compounds at 1000ppm **(B)** Table outlining the first classifications of each compound with percentage accuracy of classification and second classification with percentage accuracy of classification. Asterisks represent the classifications which predict a compound in the same class as that of the true class. Overall classification accuracy of the confusion matrix is 73.6%.

Table 4.1: Insecticide compounds tested, concentrations tested and their targets

Insecticide Name	Concentrations Tested (ppm)	Target
Abamectin	1000,100,1	Glutamate-gated chloride channels
Diamide Syn Cpd	1000,100,1	Ryanodine Receptors
Chlorantraniliprole	1000,100,1	Ryanodine Receptors
Cyantraniliprole	1000,100,1	Specific Ryanodine Receptor
Dieldrin	1000,100,1	Ligand-gated chloride channels
Flubendiamide	1000,100,1	Ryanodine Receptors
Imidacloprid	1000,100,1	nicotinic acetylcholine receptor
Pymetrozine	1000,100,1	TRP Channels
Ryanodine	1000,100,1	Ryanodine Receptors
Spiroindoline	1000,100,1	Cholinergic Transmission
Tetramethrin	1000,100,1	Sodium Channels

4.2.3 High-throughput screening: Expanding the dose spectrum

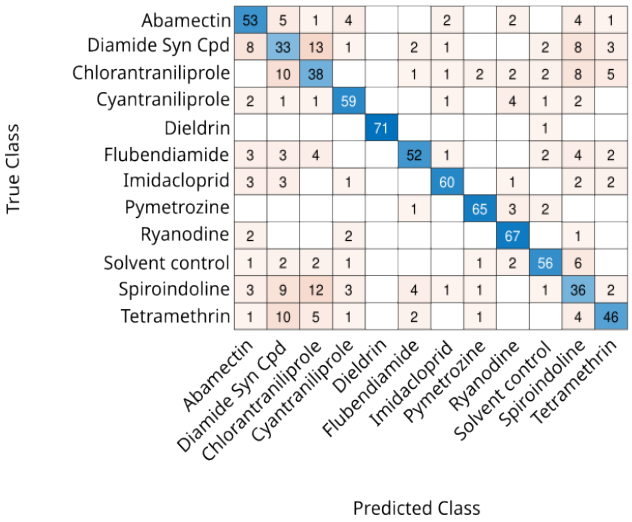
Following on from the above data, where all compounds tested at 1000ppm were correctly classified, I decided to increase the number of doses tested by testing each of the original test compounds at both 100ppm and 1ppm. Whereas the dose of 1000ppm is a very high concentration for most of these compounds, 100ppm and 1ppm are intermediate and low doses, respectively. Using the same methodology, I proceeded to record and classify the original 12 compounds at these two doses. As can be seen from figure 4.3, the compounds at the 100ppm concentration were also all classified correctly, with none being misclassified by HCTSA. Comparatively to 1000ppm, some of the compounds were predicted with less confidence than their when tested at 1000ppm, but this is potentially due to the weaker concentration leading to less obvious phenotypes. In the case of some compounds, such as Ryanodine and Pymetrozine, the classification confidence increased when compared to that at 1000ppm.

At 1ppm, the classification accuracy was still higher for the true class of each of the compounds than it was for the next nearest prediction. All compounds are correctly predicted as their true class, but with less confidence compared to that at the stronger concentrations of 100ppm and 1000ppm.

4.2.4 High-throughput screening: Testing blind compounds

Following on from the successful classification of the first set of compounds and the solvent control at three different concentrations, I expanded the data set by recording and classifying behaviour of five new compounds, all of which were provided by Syngenta without information regarding their mode of action or target. I followed the

A



B

Compound	1st Classification	% Predicted	2nd Classification	% Predicted
Abamectin	Abamectin	74	Diamide Syn Cpd	7
Diamide Syn Cpd	Diamide Syn Cpd	46	Chlorantraniliprole*	18
Chlorantraniliprole	Chlorantraniliprole	55	Diamide Syn Cpd*	14
Cyantraniliprole	Cyantraniliprole	83	Ryanodine*	6
Dieldrin	Dieldrin	99	Solvent Control,	1
Flubendiamide	Flubendiamide	73	Spiroin./Chlorantran.*	6
Imidacloprid	Imidacloprid	83	Abamectin/Diamide C.	4
Pymetrozine	Pymetrozine	92	Ryanodine	4
Ryanodine	Ryanodine	92	Pymetrozine	3
Solvent Control	Solvent Control	79	Spiroindoline	8
Spiroindoline	Spiroindoline	50	Chlorantraniliprole	17
Tetramethrin	Tetramethrin	66	BISAM	14

Figure 4.3: *Confusion matrix for flies exposed to the original 11 compounds and control at 100ppm* **(A)** Classification matrix of flies exposed to compounds at 100ppm **(B)** Table outlining the first classifications of each compound with percentage accuracy of classification and second classification with percentage accuracy of classification. Asterisks represent second classifications which predict a compound in the same class as that of the true class. Overall classification accuracy of the confusion matrix is 74.4%.

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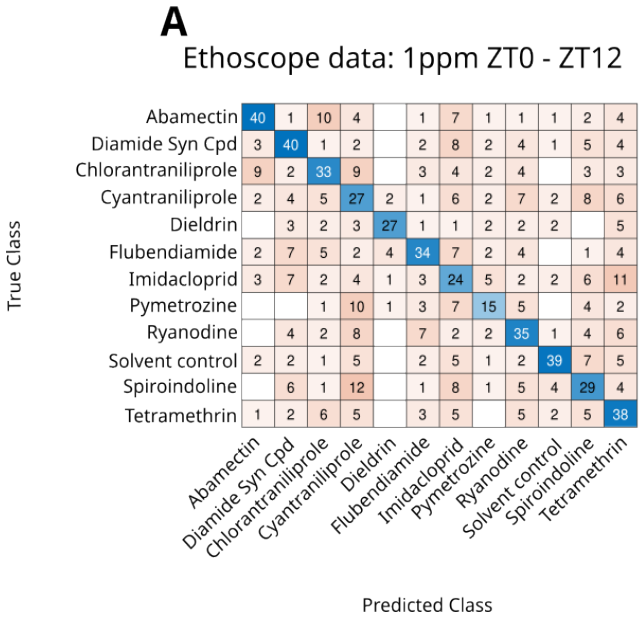


B

Compound	1st Classification	% Predicted	2nd Classification	% Predicted
Abamectin	Abamectin	56	Chlorantraniliprole	14
Diamide Syn Cpd	Diamide Syn Cpd	56	Imidacloprid	11
Chlorantraniliprole	Chlorantraniliprole	46	Abamectin/Cyantran*.	13
Cyantraniliprole	Cyantraniliprole	38	Spiroindoline	11
Dieldrin	Dieldrin	56	Tetramethrin	10
Flubendiamide	Flubendiamide	47	Diamide C.*/Imidaclop.	10
Imidacloprid	Imidacloprid	34	Tetramethrin	16
Pymetrozine	Pymetrozine	31	Cyantraniliprole	21
Ryanodine	Ryanodine	49	Cyantraniliprole*	11
Solvent Control	Solvent Control	55	Spiroindoline	10
Spiroindoline	Spiroindoline	41	Cyantraniliprole	17
Tetramethrin	Tetramethrin	53	Chlorantraniliprole	8

Figure 4.4: *Confusion matrix for flies exposed to the original 11 compounds and control at 1ppm* **(A)** Classification matrix of flies exposed to compounds at 1ppm **(B)** Table outlining the first classifications of each compound with percentage accuracy of classification and second classification with percentage accuracy of classification. Asterisks represent second classifications which predict a compound in the same class as that of the true class. Overall classification accuracy of the confusion matrix is 46.8%.

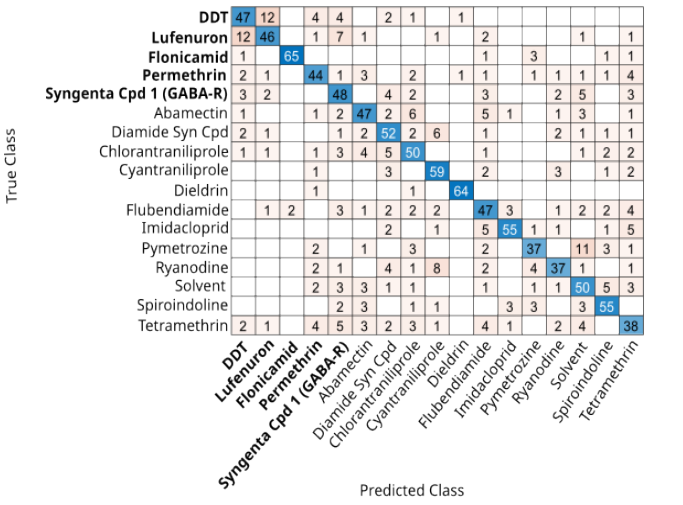
same protocol to test flies exposed to these five new compounds, each tested at the three concentrations previously used and then classified them against the previous 11 compounds and the solvent control. These compounds were tested and processed under their compound codes but were afterwards revealed. These compounds are displayed in table 4.2.

Classification matrices were then made for each of the compounds and they were classified against the original group of compounds and a solvent control. Fig.4.5 shows the classification of all compounds together at 1000ppm with the blinded compounds highlighted in bold. As was seen before when the original compounds were classified alone, all compounds are classified correctly at 1000ppm, with a high-accuracy. This is also true when each compound was tested at 100ppm. However, at 1ppm there were two cases in which misclassification occurred. This was for two of the blind compounds: Permethrin and Syngenta Cpd 1 (an in-house non-commercial compound). Interestingly, these compounds have different MoA, with Permethrin acting on voltage-gated sodium channels [430] and Syngenta Cpd 1 working on GABA receptors.

Table 4.2: Blind insecticide compounds tested, concentrations tested and their targets

Insecticide Name	Concentrations Tested	Target
DDT	1000,100,1	Voltage-Gated Sodium Channels
Lufenuron	1000, 100,1	Chitin Synthesis
Flonicamid	1000,100,1	Voltage-Gated Sodium Channels
Permethrin	1000,100,1	Voltage-Gated Sodium Channels
Syngenta Cpd 1	1000,100,1	$GABA_A$ receptors

A

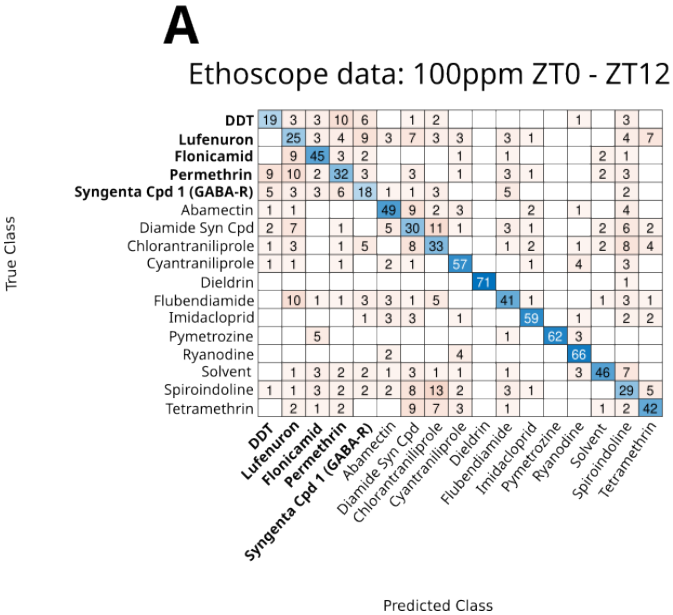


B

Compound	1st Classification	% Predicted	2nd Classification	% Predicted
DDT	DDT	66	Lufenuron	17
Lufenuron	Lufenuron	64	DDT	17
Flonicamid	Flonicamid	90	Pymetrozine*	17
Permethrin	Permethrin	70	Tetramethrin*	4
Syngenta Cpd 1	Syngenta Cpd 1	67	Solvent Control	6
Abamectin	Abamectin	67	Chlorantraniliprole	9
Diamide Syn Cpd	Diamide Syn Cpd	72	Cyantraniliprole*	8
Chlorantraniliprole	Chlorantraniliprole	70	Diamide Syn Cpd*	7
Cyantraniliprole	Cyantraniliprole	83	Diamide C./Ryanodine*	4
Dieldrin	Dieldrin	97	Permethrin/ Chloran.	2
Flubendiamide	Flubendiamide	65	Tetramethrin	6
Imidacloprid	Imidacloprid	77	Flubendiamide/ Tetra.	7
Pymetrozine	Pymetrozine	62	Solvent Control	18
Ryanodine	Ryanodine	61	Cyantraniliprole*	13
Solvent Control	Solvent Control	70	Spiroindoline	7
Spiroindoline	Spiroindoline	77	Abam./Imid./Pymet/Sol	4
Tetramethrin	Tetramethrin	54	Syngenta Cpd 1	7

Figure 4.5: *Confusion matrix for flies exposed to 16 compounds and control at 1000ppm.* **(A)** Classification matrix of flies exposed to compounds at 1000ppm **(B)** Table outlining the first classifications of each compound with percentage accuracy of classification and second classification with percentage accuracy of classification. Asterisks represent second classifications which predict a compound in the same class as that of the true class. Overall classification accuracy of the confusion matrix is 71.6%.

A

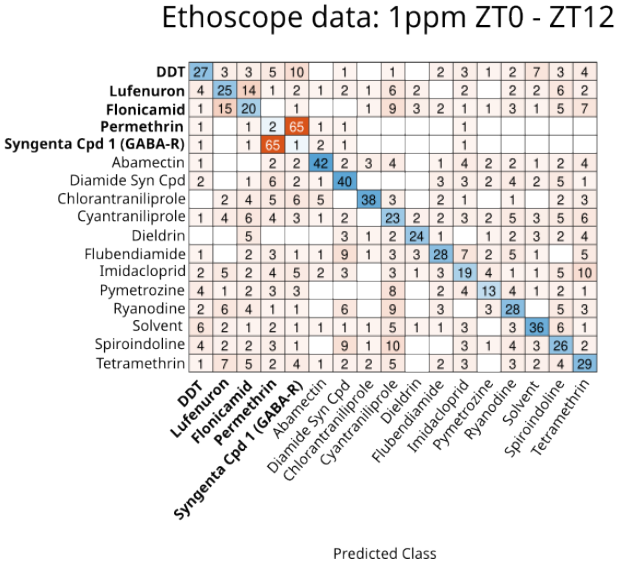


B

Compound	1st Classification	% Predicted	2nd Classification	% Predicted
DDT	DDT	38	Flonicamid	20
Lufenuron	Lufenuron	35	Syngenta Cpd 1	13
Flonicamid	Flonicamid	70	Lufenuron	17
Permethrin	Permethrin	46	Lufenuron	14
Syngenta Cpd 1	Syngenta Cpd 1	38	Permethrin	13
Abamectin	Abamectin	68	Diamide Syn Cpd	13
Diamide Syn Cpd	Diamide Syn Cpd	42	Cyantraniliprole*	15
Chlorantraniliprole	Chlorantraniliprole	48	Ryanodine*	12
Cyantraniliprole	Cyantraniliprole	46	Ryanodine*	6
Dieldrin	Dieldrin	99	Spiroindoline	1
Flubendiamide	Flubendiamide	58	Lufenuron	14
Imidacloprid	Imidacloprid	82	Abamect./Diamide C.*	4
Pymetrozine	Pymetrozine	87	Flonicamid*	7
Ryanodine	Ryanodine	92	Cyantraniliprole*	6
Solvent Control	Solvent Control	65	Spiroindoline	10
Spiroindoline	Spiroindoline	40	Chlorantraniliprole	18
Tetramethrin	Tetramethrin	60	Diamide Syn Cpd	13

Figure 4.6: *Confusion matrix for flies exposed to 16 compounds and control at 100ppm.* **(A)** Classification matrix of flies exposed to compounds at 100ppm **(B)** Table outlining the first classifications of each compound with percentage accuracy of classification and second classification with percentage accuracy of classification. Asterisks represent second classifications which predict a compound in the same class as that of the true class. Overall classification accuracy of the confusion matrix is 62.8%.

A



B

Compound	1st Classification	% Predicted	2nd Classification	% Predicted
DDT	DDT	38	Syngenta Cpd 1	14
Lufernuron	Lufernuron	35	Flonicamid	19
Flonicamid	Flonicamid	29	Lufenuron	21
Permethrin	Syngenta Cpd 1	90	Tetramethrin	3
Syngenta Cpd 1	Permethrin	90	Syngenta Cpd 1	1
Abamectin	Abamectin	58	Cyan./Imid./Tetrameth.	6
Diamide Syn Cpd	Diamide Syn Cpd	56	Permethrin	8
Chlorantraniliprole	Chlorantraniliprole	53	Syngenta Cpd 1	8
Cyantraniliprole	Cyantraniliprole	32	Flonicamid/Tetrameth.	8
Dieldrin	Dieldrin	50	Flonicamid	10
Flubendiamide	Flubendiamide	39	Diamide Syn Cpd*	13
Imidacloprid	Imidacloprid	27	Tetramethrin	14
Pymetrozine	Pymetrozine	27	Cyantraniliprole	18
Ryanodine	Ryanodine	39	Cyantraniliprole*	13
Solvent Control	Solvent Control	51	Spiroindoline/DDT	8
Spiroindoline	Spiroindoline	37	Cyantraniliprole	14
Tetramethrin	Tetramethrin	40	Lufenuron	10

Figure 4.7: *Confusion matrix for flies exposed to 16 compounds and control at 1ppm.* **(A)** Classification matrix of flies exposed to compounds at 1ppm **(B)** Table outlining the first classifications of each compound with percentage accuracy of classification and second classification with percentage accuracy of classification. Asterisks represent second classifications which predict a compound in the same class as that of the true class. Overall classification accuracy of the confusion matrix is 36.1%.

4.2.5 High-throughput screening: Assessing a larger dataset of compounds

Following on from the work done on the 17 compounds tested so far, the original 11 compounds, 5 blind compounds and the solvent control, I decided to expand the panel of compounds tested to add an additional 23 compounds. It was decided that only one concentration of 100ppm would be maintained for the testing of each of these compounds due to reasons of time and availability of equipment. As 100ppm allows for a less severe phenotype to be presented, this was the concentration chosen for the expanded panel of compounds.

As can be seen from the confusion matrix (Fig.4.8A), most of the compounds are again classified with high accuracy. The table in Fig.4.9 sets out the classification accuracy for all of the compounds. Strikingly, only one compound was misclassified, Syngenta

Cpd 5. This compound is misclassified as Chlorantraniliprole. Unfortunately, as this is a new compound developed by Syngenta with a currently unknown MoA, it cannot be said whether this misclassification was based on similar targets between the two.

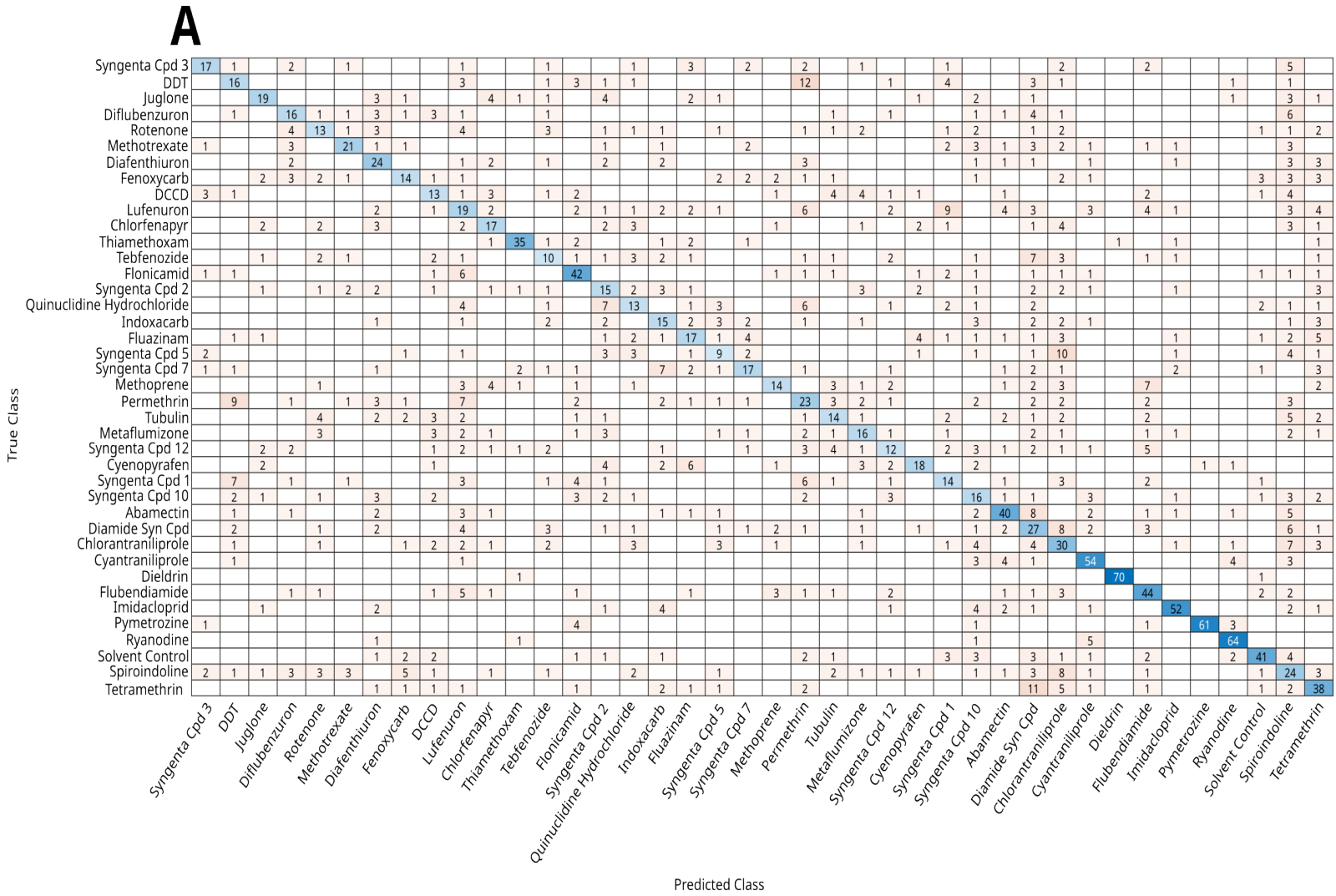


Figure 4.8: Confusion matrix for a larger panel of compounds at 100ppm. Overall classification accuracy of the confusion matrix is 44.5%.

B

Compound	1st Classification	% Predicted	2nd Classification	%
Syngenta Cpd 3	Syngenta Cpd 3	41	Spiroindoline	12
DDT	DDT	33	Permethrin*	25
Juglone	Juglone	42	Chlorfen.Syn. Cpd.2	9
Diflubenzuron	Diflubenzuron	37	Spiroindoline	14
Rotenone	Rotenone	28	Lufenuron	9
Methotrexate	Methotrexate	44	Various	6
Diafenthiuron	Diafenthiuron	50	Permeth/Spiroin/Tetram.	6
Fenoxycarb	Fenoxycarb	31	Various	6
DCCD	DCCD	30	Tubulin/Metafl./Spiroin	9
Lufenuron	Lufenuron	26	Syngenta Cpd 1	13
Chlorfenapyr	Chlorfenapyr	38	Chlorantraniliprole	9
Thiamethoxam	Thiamethoxam	76	Flonicamid/Fluazinam	4
Tebfenozide	Tebfenozide	23	Diamide Syn Cpd	16
Flonicamid	Flonicamid	65	Lufenuron	9
Syngenta Cpd 2	Syngenta Cpd 2	32	Indox./Metaflum./Tetram.	6
Quinuclidine Hycl.	Quinuclidine Hycl.	29	Syngenta Cpd 2	16
Indoxacarb	Indoxacarb	36	SynC.5/SynC.11/Tetra.	7
Fluazinam	Fluazinam	35	Tetramethrin	10
Syngenta Cpd 5	Chlorantraniliprole	24	Syngenta Cpd 5	22
Syngenta Cpd 7	Syngenta Cpd 7	37	Indoxacarb	15
Methoprene	Methoprene	30	Flubendiamide	15
Permethrin	Permethrin	33	DDT	13
Tubulin	Tubulin	30	Spiroindoline	11
Metaflumizone	Metaflumizone	36	Roten./DCCD/Syn Cpd 2.	6
Syngenta Cpd 12	Syngenta Cpd 12	25	Flubendiamide	10
Cyenopyrafen	Cyenopyrafen	42	Fluazinam	14
Syngenta Cpd 1	Syngenta Cpd 1	30	DDT	15
Syngenta Cpd 10	Syngenta Cpd 10	34	Various	6
Abamectin	Abamectin	56	Diamide Syn Cpd	11
Diamide Syn Cpd	Diamide Syn Cpd	39	Cyantraniliprole*	12
Chlorantraniliprole	Chlorantraniliprole	43	Spiroindoline	10
Cyantraniliprole	Cyantraniliprole	76	Abamectin/Ryanodine*	6
Dieldrin	Dieldrin	97	Thiamethoxam/Sol.Cont.	1
Flubendiamide	Flubendiamide	62	Lufenuron	7
Imidacloprid	Imidacloprid	72	Indoxacarb/Syn Cpd.10	5
Pymetrozine	Pymetrozine	87	Flonicamid*	6
Ryanodine	Ryanodine	89	Cyantraniliprole*	7
Solvent Control	Solvent Control	58	Spiroindoline	6
Spiroindoline	Spiroindoline	33	Chlorantraniliprole	11
Tetramethrin	Tetramethrin	54	Diamide Syn Cpd	15

Figure 4.9: Table of extended list compounds tested at 100ppm and their classification accuracies. Asterisks represent second classifications which predict a compound in the same class as that of the true class.

Table 4.3: New Insecticide compounds tested and their targets: 100ppm.

Insecticide Name	Target
Juglone	R.O.S
Diflubenzuron	Chitin synthesis
Rotenone	Complex I inhibitor
Methotrexate	Dihydrofolate receptor
Diafentiuron	Mitochondrial ATPase
Fenoxycarb	Juvenile hormone
Diafenthiuron Carbodimide (DCCD)	Mitochondrial FIFO ATP synthase
Chlorfenapyr	Mitochondrial oxidative phosphorylation
Thiamethoxam	nACh-R
Tebufenozide	Ecdysone receptor
Syngenta Cpd 2	nACh-R
Quinuclidine hydrochloride	Muscarinic ACh-R
Indoxacarb	Voltage-gated sodium channels
Fluazinam	Mitochondrial ATP synthesis
Syngenta Cpd 3	Mitochondrial ATP synthesis
Syngenta Cpd 5	Unknown
Syngenta Cpd 7	Octopamine receptor
Methoprene	Juvenile hormone
Tubulin	Binds tubulin
Metaflumizone	Voltage-gated sodium channels
Cyenopyrafen	Succinate dehydrogenase
Syngenta Cpd 10	Octopamine receptor
Syngenta Cpd 12	Unknown

4.2.6 Summary

- HCTSA can classify compounds correctly in almost all cases for compounds of 1000ppm, 100ppm and 1ppm based on time series data of velocity.

- Classification accuracies generally decrease in a dose dependent manner.

- In some cases, the second predicted classification was for a compound with a similar MoA.

4.3 High-Throughput screening: Investigating sleep and activity in insecticide compounds

Although classification can tell us that behaviours exhibited by flies following exposure to compounds are different, it cannot tell us information about what these differences in behaviour are. To try and elucidate the behavioural changes which accompany exposure to compounds in flies, I looked at quantifying these behavioural changes. To do this, I took the data used for classification and processed this in R using the rethomics package [151], used in the previous chapter to quantify changes in sleep behaviour, and plotted these compared to flies exposed to the solvent control on an estimation plot. For all estimation plots in this section, statistical differences were calculated from comparisons of exposed groups to flies exposed to the solvent control and were calculated with Wilcoxon rank-sum tests followed by corrections with an FDR adjustment.

4.3.1 High-throughput screening: Investigating movement at 1000ppm, 100ppm and 1ppm

The first metric examined for each of the 11 original compounds and the solvent control was movement. The rethomics package can calculate a mean value for movement based on the population of flies tested for each compound, over the 12 hour period for which classification was done.

For flies exposed to the compounds at 1000ppm, the movement data for each population varied greatly compared to flies exposed to the solvent control. Compounds in Fig.4.10 were tested for significant differences compared to the mean movement of flies only exposed to the solvent control. As can be seen, only flies exposed to two com-

pounds, Cyantraniliprole and Tetramethrin, did not show any significant differences in movement compared to the control. Of 9 compounds that showed significant differences to control, 7 decreased movement over the 12 hour period, while the two compounds Dieldrin and Pymetrozine showed the opposite effect.

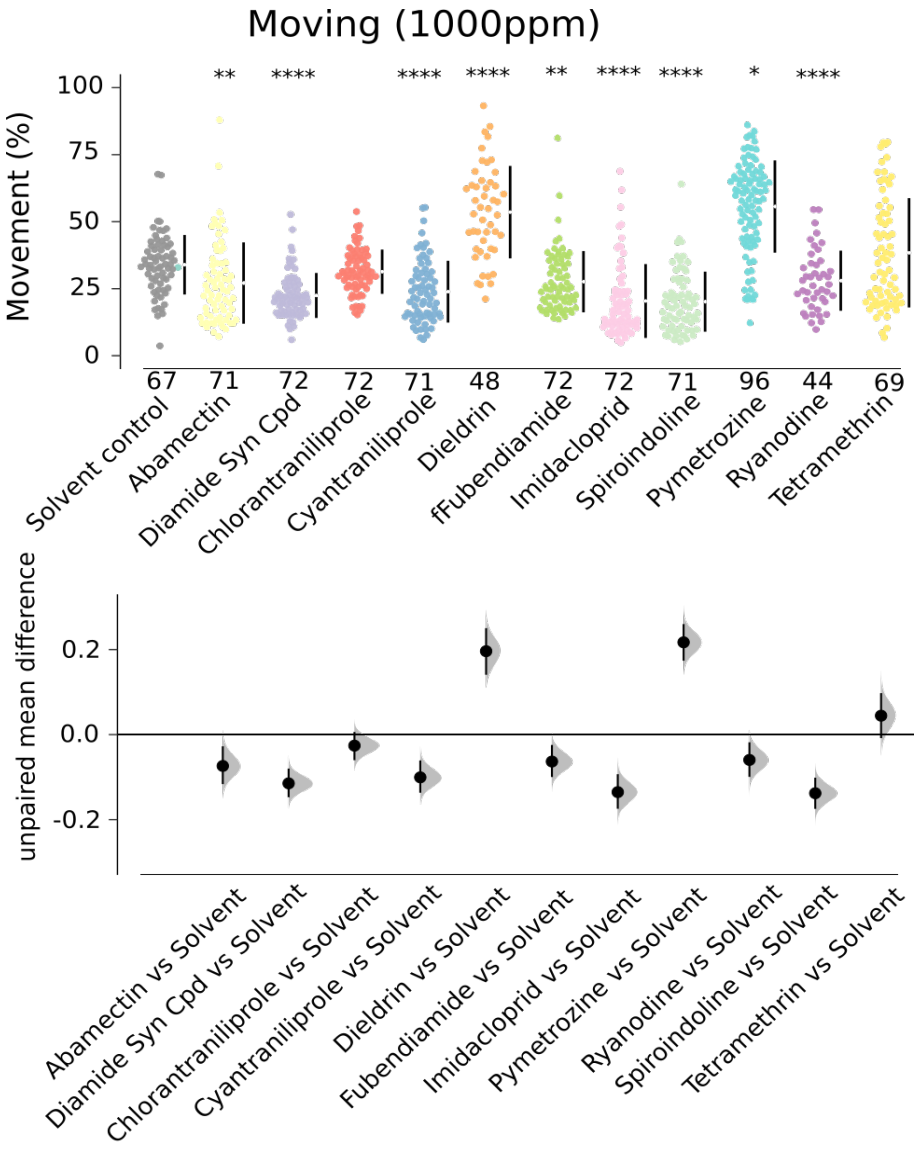


Figure 4.10: *Estimation plot showing the quantification of fly movement for exposure to original 11 compounds compared to solvent control at 1000ppm.* The observed effect size between the experimental groups and solvent control group is depicted as a black dot, and the 95% CI is indicated by the ends of the black error bars. *N* for each group and asterisks' demonstrating statistical differences between control and experimental groups are shown within the figure and data contained is from at least 2 biological repeats per group.

Following the analysis at 1000ppm, a dataset of the 11 compounds at 100ppm against solvent control was obtained. Interestingly, while Abamectin and Tetramethrin

Table 4.4: Quantification of effect of insecticide compounds on movement in *Drosophila* tested at 1000pm.

Insecticide	Adjusted P-Value Significance	Significance
Abamectin	0.0001164066	**
Diamide Syn Cpd	6.996^{-10}	****
Chlorantraniliprole	0.1072078	n.s.
Cyantraniliprole	5.811×10^{-7}	****
Dieldrin	6.983×10^{-9}	****
Flubendiamide	0.0001567628	*
Imidacloprid	7.262×10^{-10}	****
Pymetrozine	1.408×10^{-12}	****
Ryanodine	0.003368	*
Spiroindoline	2.55×10^{-10}	****
Tetramethrin	0.6445273	n.s.

did not affect movement at 1000ppm, both compounds significantly lowered movement levels when tested at the lower concentration of 100ppm. The only compound which did not lead flies to show a change in movement at 100ppm was Chlorantraniliprole. Many of the other compounds tested showed similar behavioural profiles at both concentrations tested. Pymetrozine and Dieldrin both still led to significant increases in movement, although it should be noted that there is a large variability in movement of flies exposed to both compounds, suggesting that there are inter-individual differences in the response of flies to these compounds.

A summary of these results can be found in table 4.13. It can be noted that flies exposed to the lower concentration seem to have a lower degree of variance as compared to those exposed to the higher concentration.

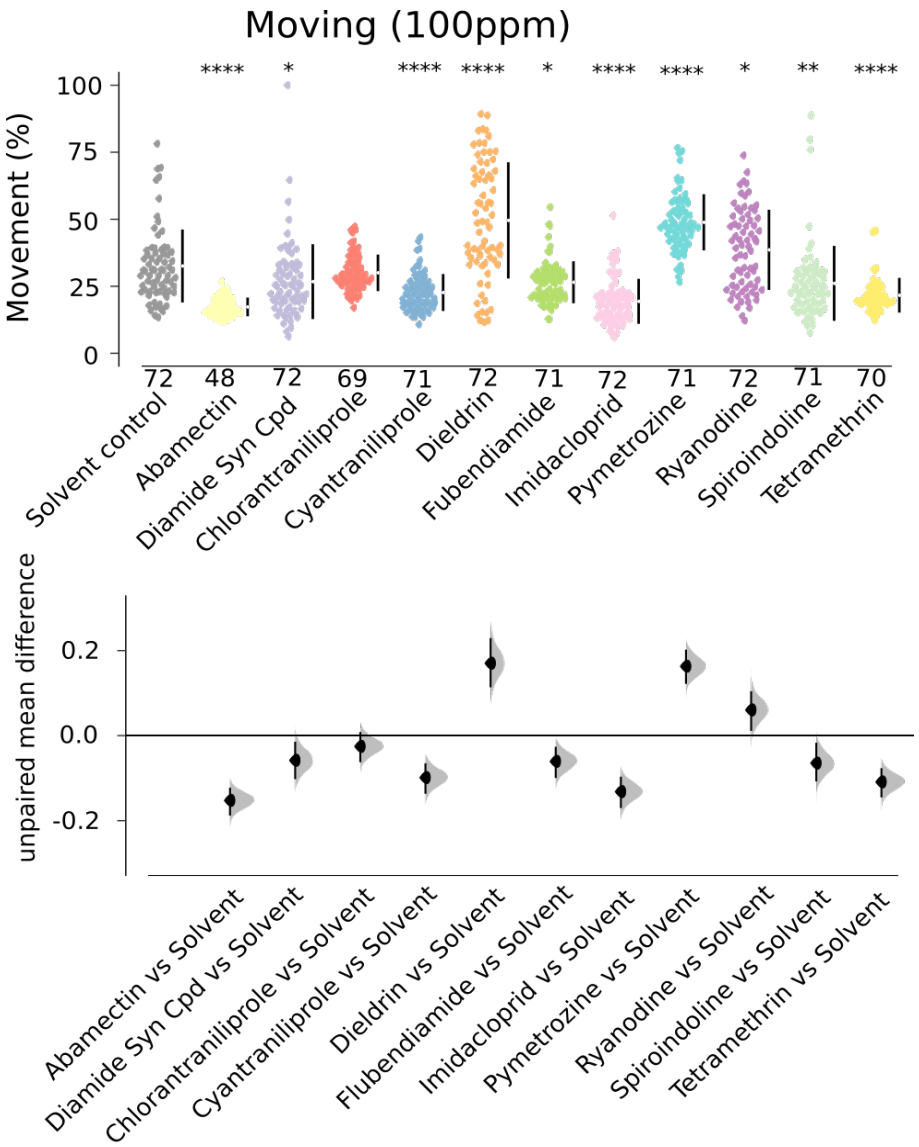


Figure 4.11: *Estimation plot showing the quantification of fly movement for exposure to original 11 compounds compared to solvent control at 100ppm.* The observed effect size between the experimental groups and solvent control group is depicted as a black dot, and the 95% CI is indicated by the ends of the black error bars. *N* for each group and asterisks' demonstrating statistical differences between control and experimental groups are shown within the figure and data contained is from at least 2 biological repeats per group.

Using the data collected, I also analysed the behavioural responses of flies to the compounds at 1ppm. Compared to the flies exposed to compounds at 1000ppm and 100ppm, there is a much lower effect of compounds on flies at this concentration. At 1ppm, only 3 groups exposed to compounds responded with a significant change in movement; Abamectin, Dieldrin and Tetramethrin. All three led to small but significant decreases in movement.

Table 4.5: Quantification of effect of insecticide compounds on movement in Drosophila tested at 100ppm.

Insecticide	Adjusted P-Value	Significance
Abamectin	2.905×10^{-13}	****
Diamide Syn Cpd	0.00314	*
Chlorantraniliprole	0.6575	n.s.
Cyantraniliprole	5.149×10^{-7}	****
Dieldrin	9.454×10^{-7}	****
Flubendiamide	0.00328	*
Imidacloprid	3.106×10^{-11}	****
Pymetrozine	9.402×10^{-13}	****
Ryanodine	0.01195	*
spiroindoline	0.000215	**
Tetramethrin	4.657×10^{-9}	****

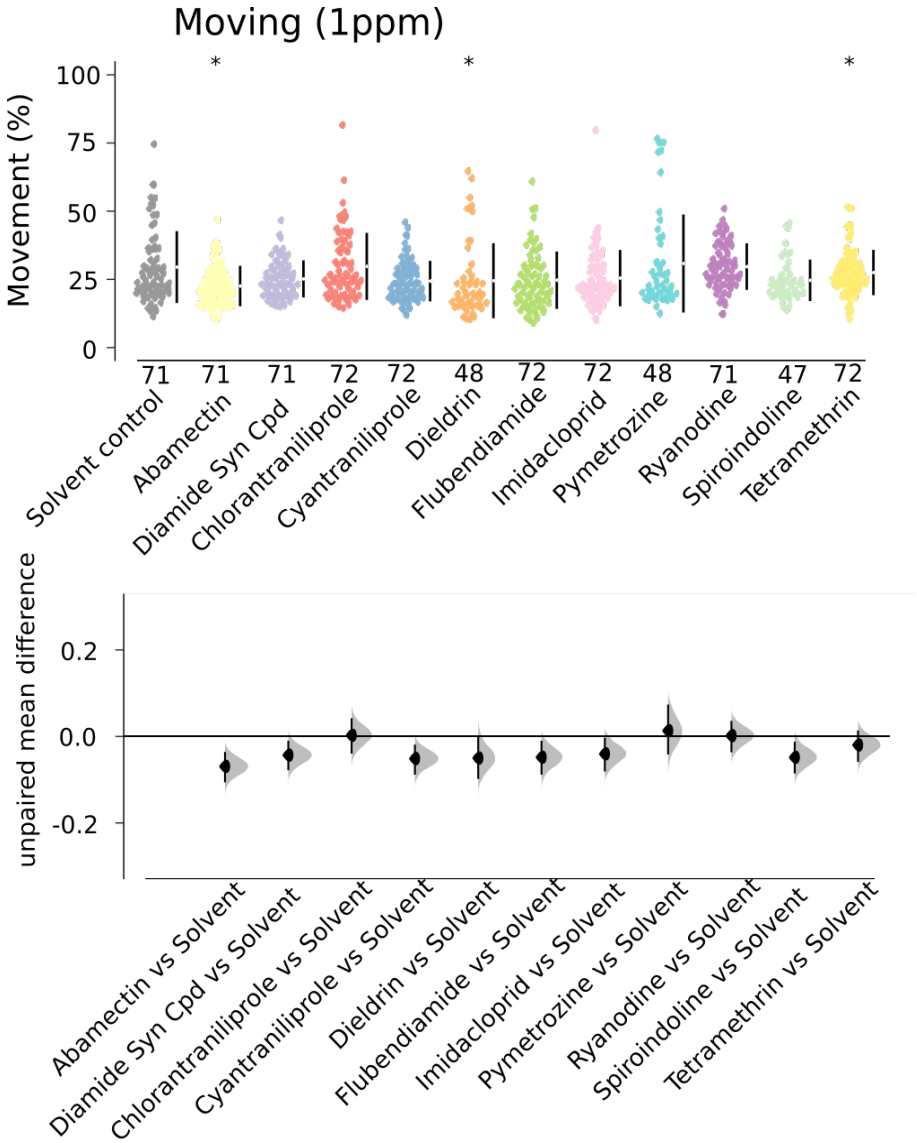


Figure 4.12: *Estimation plot showing the quantification of fly movement for exposure to original 11 compounds compared to solvent control at 1ppm.* The observed effect size between the experimental groups and solvent control group is depicted as a black dot, and the 95% CI is indicated by the ends of the black error bars. *N* for each group and asterisks' demonstrating statistical differences between control

Table 4.6: Quantification of effect of insecticide compounds on movement in Drosophila tested at 1pm

Insecticide	P-Value	Significance
Abamectin	0.0126	*
Diamide Syn Cpd	0.2067	n.s.
Chlorantraniliprole	0.767	n.s.
Cyantraniliprole	0.0738	n.s.
Dieldrin	0.0163	*
Flubendiamide	0.0738	n.s.
Imidacloprid	0.168	n.s.
Pymetrozine	0.5808	n.s.
Ryanodine	0.2067	n.s.
spiroindoline	0.1341	n.s.
Tetramethrin	0.04652	*

4.3.2 High-throughput screening: Investigating micro-movement at 1000ppm, 100ppm and 1ppm

Another metric which can be examined through rethomics is the percentage of time the population of flies spent doing micro-movements. Micro-movements are defined as movements which do not constitute walking activity but are smaller movements such as feeding, grooming or, in the case of females, egg-laying.

Fig.4.11 shows the mean percentage of time spent micro-moving for individuals exposed to each compound at 1000ppm and significant change in micro-movement is calculated compared to the solvent control. As can be seen, all of the groups of flies exposed to the compounds show a significant level of difference in micro-movements when compared to the control. Of these groups, only one group, those flies exposed to Imidacloprid, show a decrease in micro-movements, whilst flies exposed to all other compounds show an increase. The group which showed the greatest increase in micro-movements was Pymetrozine; as shown in Fig.4.11. This group also had a large increase in overall movement, which may in part be due to this large increase in micro-movement. Statis-

tics are quantified in table 4.7. For micro-movements, a more interesting picture

Table 4.7: Quantification of the effect of insecticide compounds on micro-movement in Drosophila tested at 1000pm.

Insecticide	Adjusted P-Value	Significance
Abamectin	0.00763	*
Diamide Syn Cpd	0.000515	**
Chlorantraniliprole	1.86×10^{-8}	**
Cyantraniliprole	9.588×10^{-7}	****
Dieldrin	3.866×10^{-7}	****
Flubendiamide	0.01955	*
Imidacloprid	0.02019	*
Pymetrozine	2.712×10^{-18}	****
Ryanodine	0.000515	**
spiroindoline	0.00298	*
Tetramethrin	0.0241	*

emerges at 100ppm than at 1000ppm. Three groups now did not show significant changes in micro-movements, those exposed to Chlorantraniliprole, Cyantraniliprole and Spiroindoline. For 1000ppm, almost all groups showed significant increases in micro-movements, whereas for the lower concentration of 100ppm, the majority of groups exhibited decreases. Dieldrin and Pymetrozine, and additionally Ryanodine, were the three compounds which led flies to showing increases in micro-movements, at this concentration. When we turn our attention to changes induced in micro-movements at 1ppm, we can see that 5 groups responded with a significant change in micro-movements at this concentration. 3 groups showed a significant increase in micro-movements: Chlorantraniliprole, Pymetrozine and Ryanodine. The remaining 2 groups, those exposed to Spiroindoline and Tetramethrin, showed small but significant decreases in micromovements. Pymetrozine specifically seemed to lead to an increase in micro-movements consistently in flies at all concentrations tested, with Ryanodine acting only on this specific aspect of behaviour at 100ppm and 1ppm. This suggests

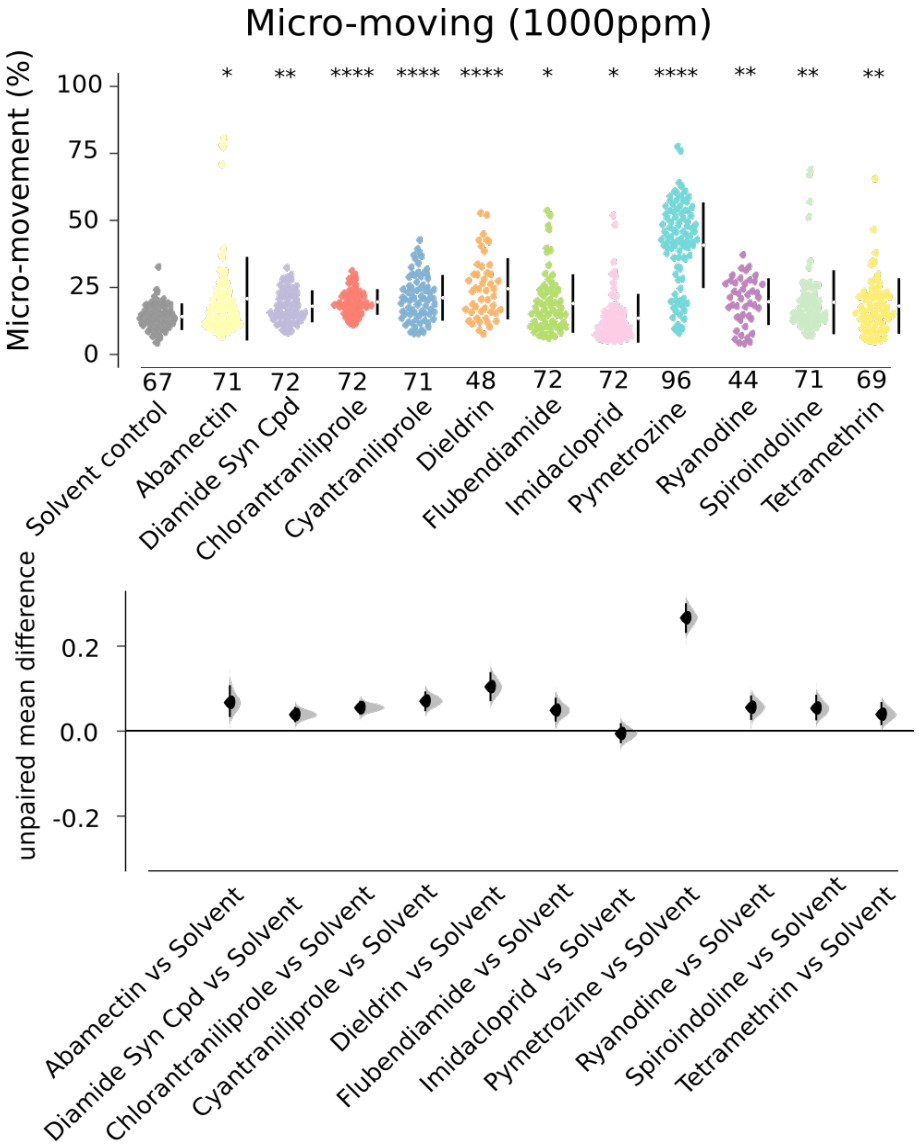


Figure 4.13: *Estimation plot showing the quantification of micro-movements for exposure to original 11 compounds compared to solvent control at 1000ppm.* The observed effect size between the experimental groups and solvent control group is depicted as a black dot, and the 95% CI is indicated by the ends of the black error bars. *N* for each group and asterisks' demonstrating statistical differences between control and experimental groups are shown within the figure and data contained is from at least 2 biological repeats per group.

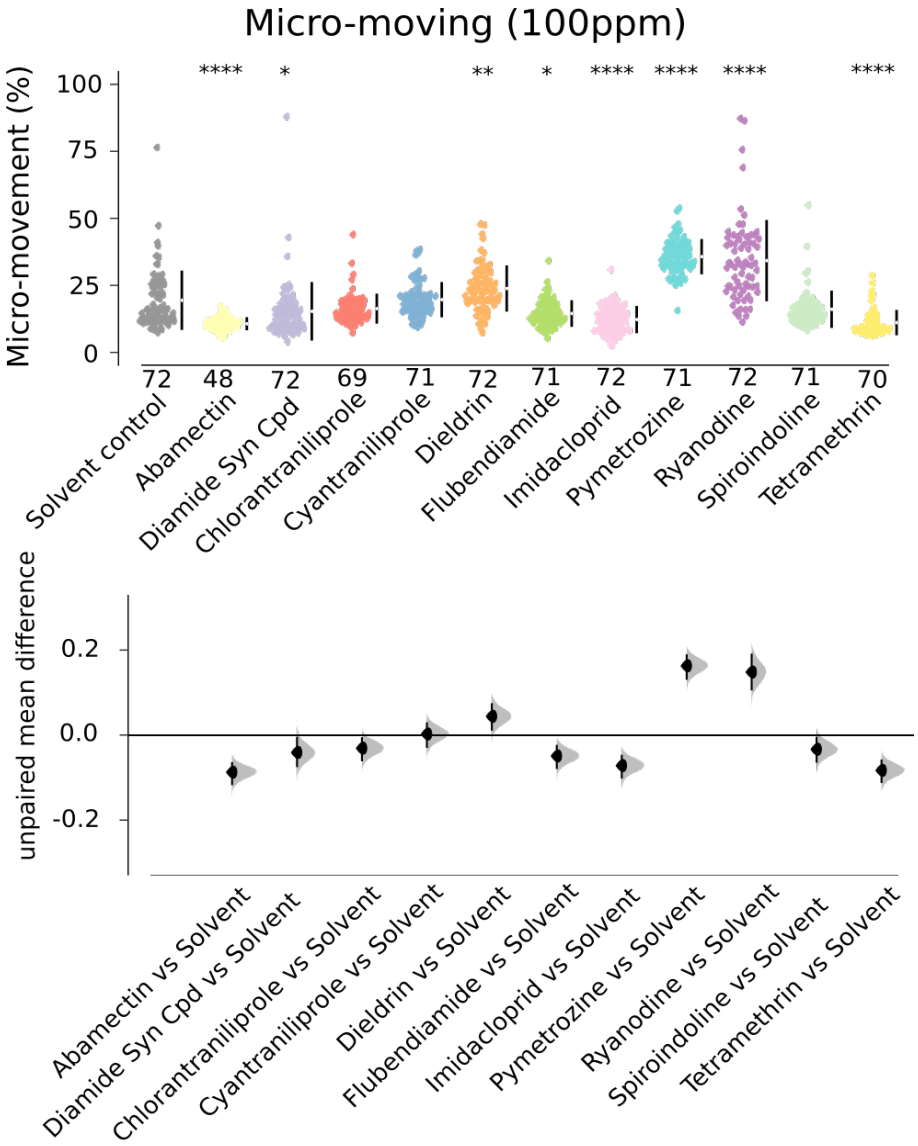


Figure 4.14: *Estimation plot showing the quantification of micro-movements for exposure to original 11 compounds compared to solvent control at 100ppm.* The observed effect size between the experimental groups and solvent control group is depicted as a black dot, and the 95% CI is indicated by the ends of the black error bars. *N* for each group and asterisks' demonstrating statistical differences between control and experimental groups are shown within the figure and data contained is from at least 2 biological repeats per group.

Table 4.8: Quantification of the effect of insecticide compounds on micro-movement in *Drosophila* tested at 100ppm.

Insecticide	Adjusted P-Value	Significance
Abamectin	1.962×10^{-9}	****
Diamide Syn Cpd	0.00286	*
Chlorantraniliprole	0.4320	n.s.
Cyantraniliprole	0.1305	n.s.
Dieldrin	0.000431	**
Flubendiamide	0.01079	*
Imidacloprid	1.079×10^{-5}	****
Pymetrozine	1.799×10^{-16}	****
Ryanodine	8.33×10^{-11}	****
spiroindoline	0.196	n.s.
Tetramethrin	3.323×10^{-10}	****

that at lower concentrations, flies exposed to Ryanodine likely demonstrate more subtle behavioural effects. Flies exposed to Spiroindoline, however, did not show differences in micro-movements at 100ppm but did so at 1000ppm and 1ppm, this also holds true for Chlorantraniliprole.

Table 4.9: Quantification of the effect of insecticide compounds on micro-movement in *Drosophila* tested at 1ppm.

Insecticide	Adjusted P-Value	Significance
Abamectin	0.08191	n.s.
Diamide Syn Cpd	0.0743	n.s.
Chlorantraniliprole	0.000462	**
Cyantraniliprole	0.4328	n.s.
Dieldrin	0.67898	n.s.
Flubendiamide	0.6789	n.s.
Imidacloprid	0.43282	n.s.
Pymetrozine	0.0369	*
Ryanodine	0.000814	**
spiroindoline	0.0398	*
Tetramethrin	0.00387	*

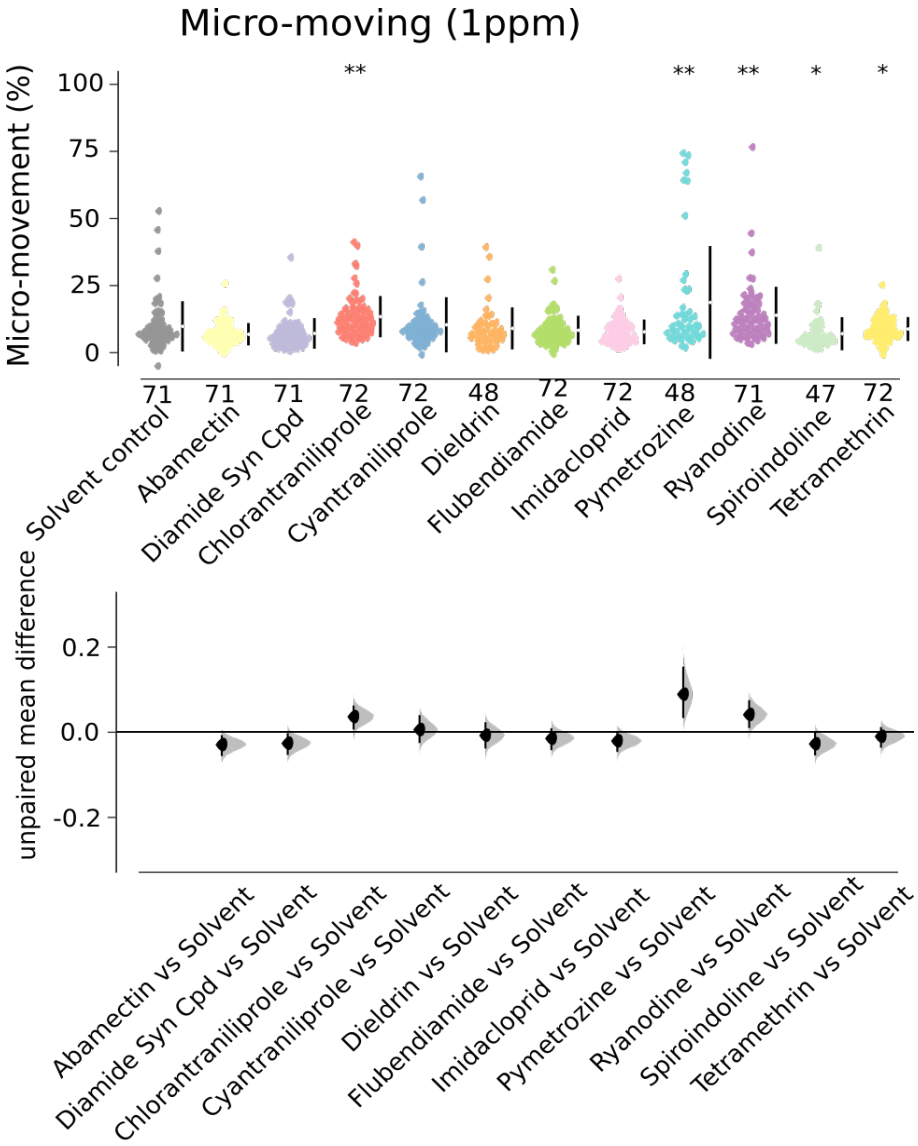


Figure 4.15: *Estimation plot showing the quantification of micro-movements for exposure to original 11 compounds compared to solvent control at 1ppm.* The observed effect size between the experimental groups and solvent control group is depicted as a black dot, and the 95% CI is indicated by the ends of the black error bars. *N* for each group and asterisks' demonstrating statistical differences between control and experimental groups are shown within the figure and data contained is from at least 2 biological repeats per group.

4.3.3 High-throughput screening: Investigating quiescence at 1000ppm, 100ppm and 1ppm

Finally, we can also examine the amount of time spent in quiescence. Quiescence is defined by the ethoscope software as comparative to sleep. That is, when the animal has been immobile for more than 5 minutes, and therefore by behavioural definitions is asleep, this behaviour is noted as quiescence. This measurement is not simply the opposite to movement, as the fly needs to demonstrate prolonged immobility.

Fig.4.15 shows the average amount of time spent in quiescence over the course of 12-hours for the flies exposed to compounds at 1000ppm. Flies exposed to all except three of the compounds showed significant differences in quiescence. The three groups which showed no difference were those flies exposed to Abamectin, Chlorantraniliprole and those exposed to Tetramethrin. Dieldrin and Pymetrozine both showed a decrease in quiescence, which aligns well with the the increase in movement these compounds seem to elicit, as shown by Fig.4.10. The groups exposed to the remaining 7 compounds showed increases in quiescence, which can also be explained by the decreases in movement showed by these groups in Fig.4.10.

As many of these compounds have already been noted in the literature as being neurotoxins, and therefore generally lead to malfunctions in the nervous systems of insects, this could act as an explanation as to why many of these compounds lead to a decrease in movement and an increase in quiescence in exposed flies, when compared to a solvent control.

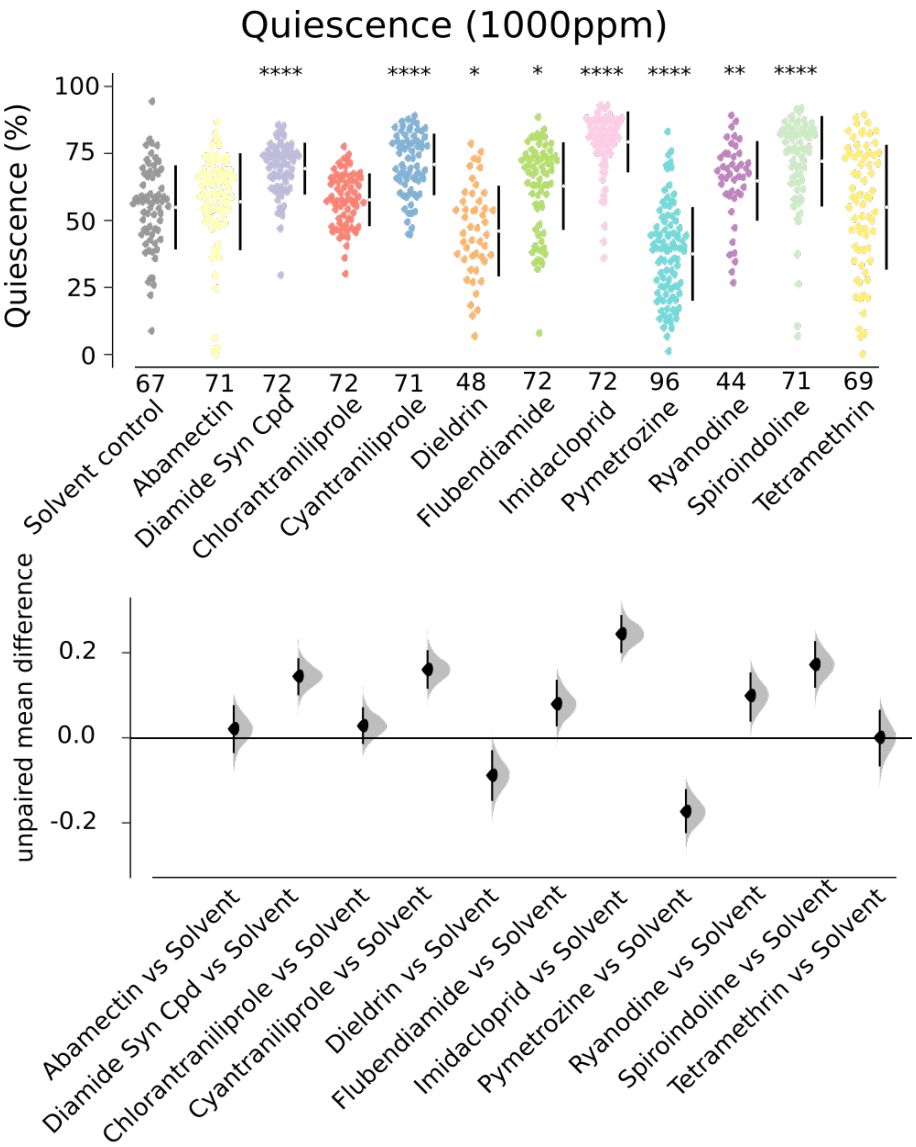


Figure 4.16: *Estimation plot showing the quantification of quiescence for exposure to original 11 compounds compared to solvent control at 1000ppm.* The observed effect size between the experimental groups and solvent control group is depicted as a black dot, and the 95% CI is indicated by the ends of the black error bars. *N* for each group and asterisks' demonstrating statistical differences between control and experimental groups are shown within the figure and data contained is from at least 2 biological repeats per group.

For quiescence, similar to what was seen for movement data, all flies exposed to compounds in all groups, bar one, those exposed to Ryanodine, showed changes in quiescence. Interestingly, flies exposed to Chlorantraniliprole, which showed no difference in movement at 100ppm, showed small but significant increases in quiescence. Ryanodine, which led to a small increase in movement and micro-movements led to no differences in quiescence. As with 1000ppm, Dieldrin and Pymetrozine were the

Table 4.10: Quantification of the effect of insecticide compounds on quiescence in *Drosophila* tested at 1000ppm.

Insecticide	Adjusted P-Value	Significance
Abamectin	0.1745	n.s.
Diamide Syn Cpd	4.959×10^{-9}	****
Chlorantraniliprole	0.337	n.s.
Cyantraniliprole	4.959×10^{-9}	**
Dieldrin	0.00645	*
Flubendiamide	0.00133	*
Imidacloprid	1.663×10^{-15}	****
Pymetrozine	4.959×10^{-9}	****
Ryanodine	0.000592	**
Spiroindoline	1.344×10^{-9}	****
Tetramethrin	0.5480	n.s.

only compounds which led to decreased quiescence, which is to be expected given the increased movement at this concentration.

Table 4.11: Quantification of the effect of insecticide compounds on quiescence in *Drosophila* tested at 100ppm.

Insecticide	Adjusted P-Value	Significance
Abamectin	1.067×10^{-6}	****
Diamide Syn Cpd	0.00069554	**
Chlorantraniliprole	0.04038	*
Cyantraniliprole	9.573×10^{-5}	****
Dieldrin	1.273^{-5}	***
Flubendiamide	0.0274	*
Imidacloprid	1.197×10^{-12}	****
Pymetrozine	1.35×10^{-10}	****
Ryanodine	0.534	n.s.
Spiroindoline	0.00726	*
Tetramethrin	0.01122	*

For quiescence, in flies exposed to compounds at 1ppm, 8 of the 11 groups showed significant changes in quiescence. This is particularly interesting in the light of results in section 4.3.1, which showed that at this concentration, only 3 groups had significant changes in movement. All groups that showed altered movement demonstrated an

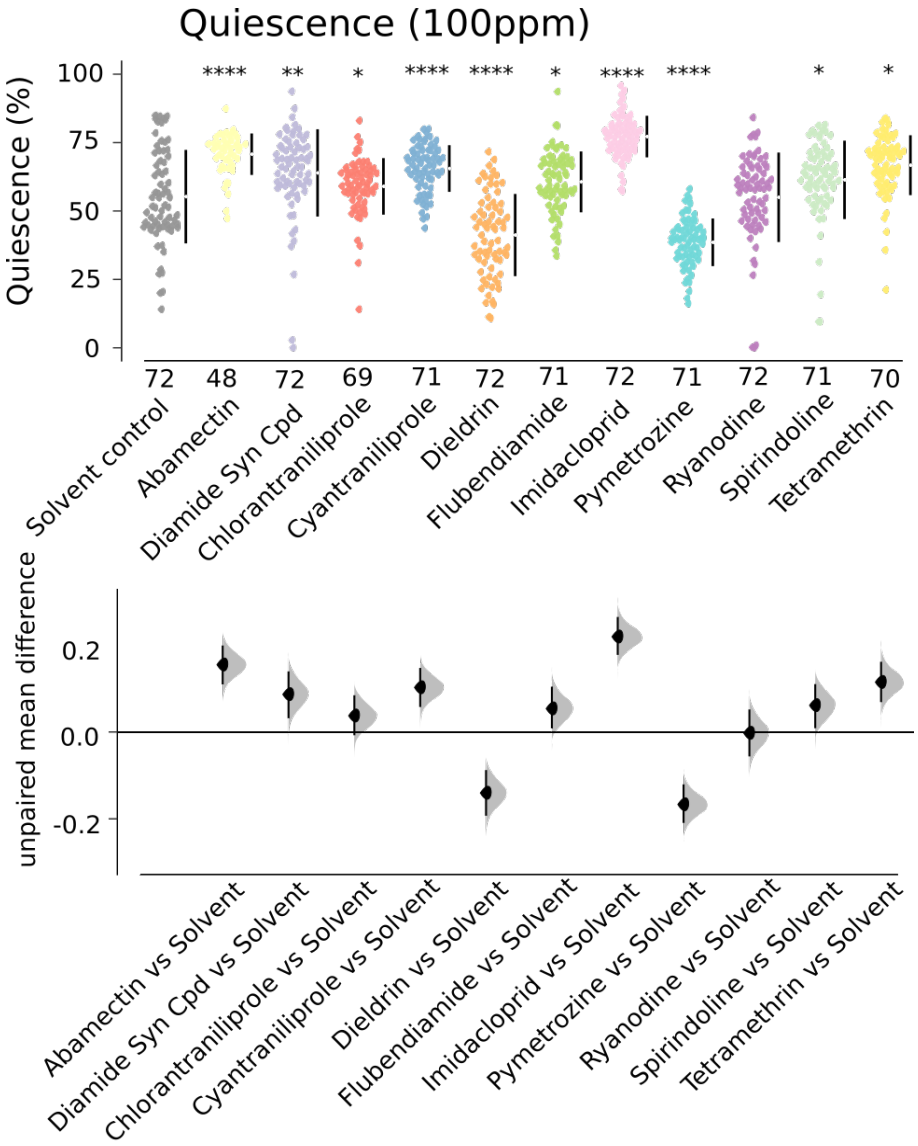


Figure 4.17: *Estimation plot showing the quantification of quiescence for exposure to original 11 compounds compared to solvent control at 100ppm.* The observed effect size between the experimental groups and solvent control group is depicted as a black dot, and the 95% CI is indicated by the ends of the black error bars. *N* for each group and asterisks' demonstrating statistical differences between control and experimental groups are shown within the figure and data contained is from at least 2 biological repeats per group.

increase in quiescence compared to the flies exposed to the solvent control. At this lower concentration, this seems to suggest that the compounds are having less of a hyperactive effect on the behaviour of the flies and are leading the flies to demonstrate more restful behaviour. For almost all of the compounds, flies showed a wide variation in the amount of quiescence, suggesting that the effects of the compounds may be more subject to other influences at this low concentration. This can indicate inter-individual differences in susceptibility to a compound.

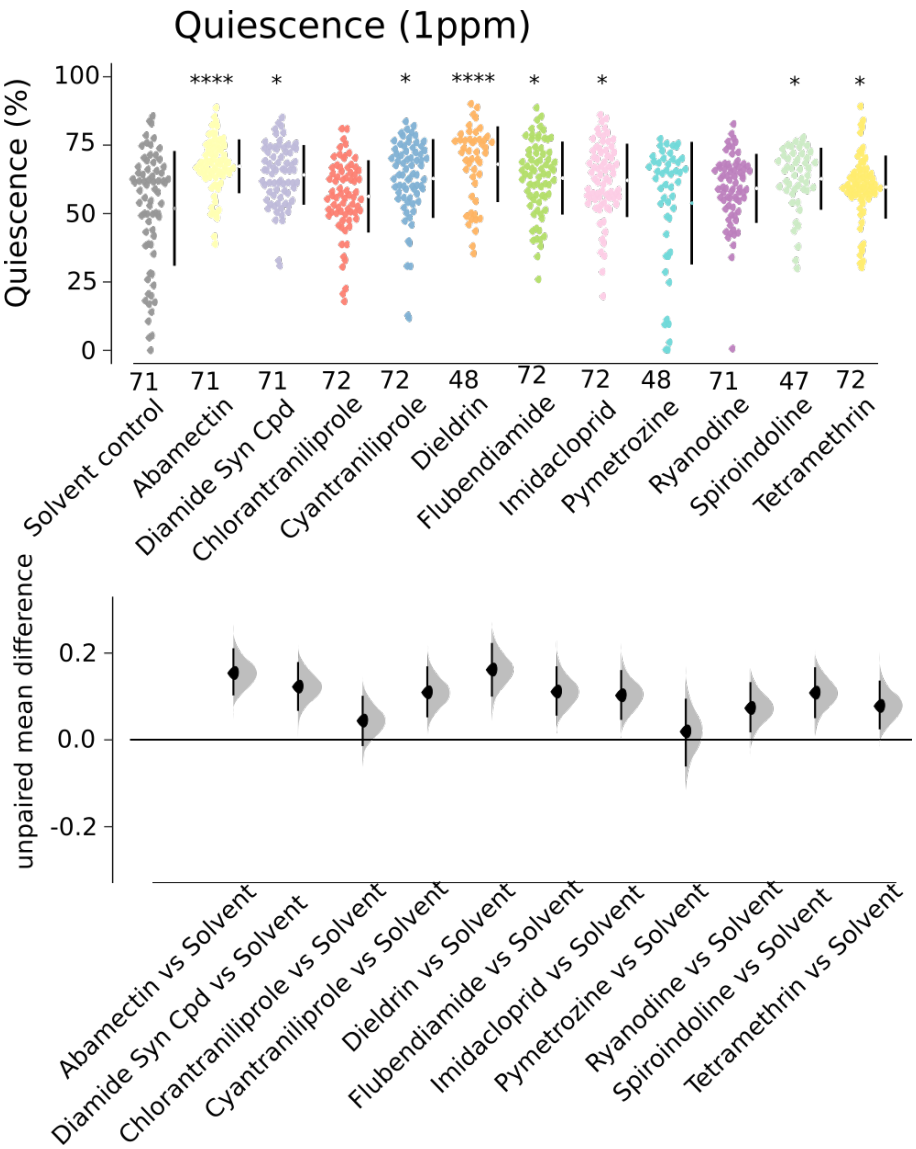


Figure 4.18: *Estimation plot showing the quantification of quiescence for exposure to original 11 compounds compared to solvent control at 1ppm.* The observed effect size between the experimental groups and solvent control group is depicted as a black dot, and the 95% CI is indicated by the ends of the black error bars. *N* for each group and asterisks' demonstrating statistical differences between control and experimental groups are shown within the figure and data contained is from at least 2 biological repeats per group.

Table 4.12: Quantification of the effect of insecticide compounds on quiescence in *Drosophila* tested at 1ppm.

Insecticide	Adjusted P-Value	Significance
Abamectin	$8.9x10^{-6}$	****
Diamide Syn Cpd	0.00209	**
Chlorantraniliprole	0.479	*
Cyantraniliprole	0.00242	**
Dieldrin	0.0000396	****
Flubendiamide	0.00405	**
Imidacloprid	0.00773	**
Pymetrozine	0.335	n.s.
Ryanodine	0.102	n.s.
Spiroindoline	0.00773	**
Tetramethrin	0.0378	*

4.3.4 Summary

The following tables (4.13,4.14 and 4.15) summarise the changes in behaviour seen for each of the compounds at each concentration.

Table 4.13: Changes in behaviours for flies exposed to compounds at 3 different concentrations

Abamectin	1000ppm	Moving	Decrease
		Micromoving	Increase
		Quiescence	No change
Abamectin	100ppm	Moving	Decrease
		Micromoving	Decrease
		Quiescence	Increase
Abamectin	1ppm	Moving	Decrease
		Micromoving	Decrease
		Quiescence	Increase
Diamide Syngenta Cpd	1000ppm	Moving	Decrease
		Micro-Moving	Increase
		Quiescence	Increase
Diamide Syngenta Cpd	100ppm	Moving	Decrease
		Micro-Moving	Decrease
		Quiescence	Increase
Diamide Syngenta Cpd	1ppm	Moving	No change
		Micro-Moving	No change
		Quiescence	Increase
Chlorantraniliprole	1000ppm	Moving	No change
		Micro-Moving	Increase
		Quiescence	No change
Chlorantraniliprole	100ppm	Moving	No change
		Micro-Moving	No change
		Quiescence	Increase
Chlorantraniliprole	1ppm	Moving	No change
		Micro-Moving	Increase
		Quiescence	No change
Cyantraniliprole	1000ppm	Moving	Decrease
		Micro-Moving	Increase
		Quiescence	Increase
Cyantraniliprole	100ppm	Moving	Decrease
		Micro-Moving	No change
		Quiescence	Increase
Cyantraniliprole	1ppm	Moving	No change
		Micro-Moving	No change
		Quiescence	Increase

Table 4.14: Changes in behaviours for flies exposed to compounds at 3 different concentrations: continued

Dieldrin	1000ppm	Moving	Increase
		Micro-Moving	Increase
		Quiescence	Decrease
Dieldrin	100ppm	Moving	Increase
		Micro-Moving	Increase
		Quiescence	Decrease
Dieldrin	1ppm	Moving	Decrease
		Micro-Moving	No change
		Quiescence	Increase
Flubendiamide	1000ppm	Moving	Decrease
		Micro-Moving	Increase
		Quiescence	Increase
Flubendiamide	100ppm	Moving	Decrease
		Micro-Moving	Increase
		Quiescence	Increase
Flubendiamide	1ppm	Moving	No change
		Micro-Moving	No change
		Quiescence	Increase
Imidacloprid	1000ppm	Moving	Decrease
		Micro-Moving	Decrease
		Quiescence	Increase
Imidacloprid	100ppm	Moving	Decrease
		Micro-Moving	Decrease
		Quiescence	Increase
Imidacloprid	1ppm	Moving	No change
		Micro-Moving	No change
		Quiescence	Increase
Pymetrozine	1000ppm	Moving	Increase
		Micro-Moving	Increase
		Quiescence	Decrease
Pymetrozine	100ppm	Moving	Increase
		Micro-Moving	Increase
		Quiescence	Decrease
Pymetrozine	1ppm	Moving	No change
		Micro-Moving	Increase
		Quiescence	No change

Table 4.15: Changes in behaviours for flies exposed to compounds at 3 different concentrations: continued

Ryanodine	1000ppm	Moving	Decrease
		Micro-Moving	Increase
		Quiescence	Increase
Ryanodine	100ppm	Moving	Increase
		Micro-Moving	Increase
		Quiescence	No change
Ryanodine	1ppm	Moving	No change
		Micro-Moving	Increase
		Quiescence	No change
Spiroindoline	1000ppm	Moving	Decrease
		Micro-Moving	Increase
		Quiescence	Increase
Spiroindoline	100ppm	Moving	Decrease
		Micro-Moving	No change
		Quiescence	Increase
Spiroindoline	1ppm	Moving	No change
		Micro-Moving	Decrease
		Quiescence	Increase
Tetramethrin	1000ppm	Moving	No change
		Micro-Moving	Increase
		Quiescence	No change
Tetramethrin	100ppm	Moving	Decrease
		Micro-Moving	Decrease
		Quiescence	Increase
Tetramethrin	1ppm	Moving	Decrease
		Micro-Moving	Decrease
		Quiescence	Increase

4.3.5 Comparing changes in movement to mode of action

When the panel of compounds tested is increased to 16, as it was when the "blind" compounds were added, we can begin to group compounds by MoA. 14 of the 16 compounds tested can be grouped to act on five different targets: ryanodine receptors, sodium channels, $GABA_A$ receptors, chordotonal neurons and on mechanisms involving

acetylcholine. Two compounds each acted individually on other targets and so were not used in this analysis. To see if compounds with similar MoAs would lead to similar types of movement changes in exposed flies, I grouped flies exposed to these compounds at each concentration and analysed changes in movement when compared to flies exposed to the solvent control. For all estimation plots in this section, statistical differences arise from comparisons of exposed groups to flies exposed to the solvent control and were calculated with Wilcoxon rank-sum tests followed by corrections with an FDR adjustment.

Ryanodine Receptor targets and their effect on movement

I firstly looked at the compounds which acted on ryanodine receptors, of which there were five compounds: Diamide Syn Cpd, Chlorantraniliprole, Cyantraniliprole, Flubendiamide and Ryanodine. I plotted the change in movements of flies exposed to these compounds compared to the flies exposed to the solvent control. For 1000ppm, it can be seen that flies exposed to each of these compounds, aside from one, showed a significant decrease in movement (Fig.4.18). Flies exposed to the one compound which did not show a decrease in movement were those exposed to Chlorantraniliprole. These flies showed no significant difference in movement compared to the control.

At 100ppm, four of the five compounds led exposed flies to showed a decrease in movement. However, at this concentration, Chlorantraniliprole did lead to a small but significant decrease in movement, whereas Ryanodine did not.

At 1ppm, only three of the compounds led to a significant decrease in movement to exposed flies. These compounds were Diamide Syn Cpd, Cyantraniliprole and Flubendiamide.

This allows us to conclude that these compounds seem to act in a similar way to de-

crease movement in exposed flies. Flies exposed to Diamide Syn Cpd, Cyantraniliprole and Flubendiamide showed a decrease in movement when exposed to these compounds at all three concentrations, but Chlorantraniliprole only led to a decrease in movement at 100ppm and Ryanodine only at 1000ppm.

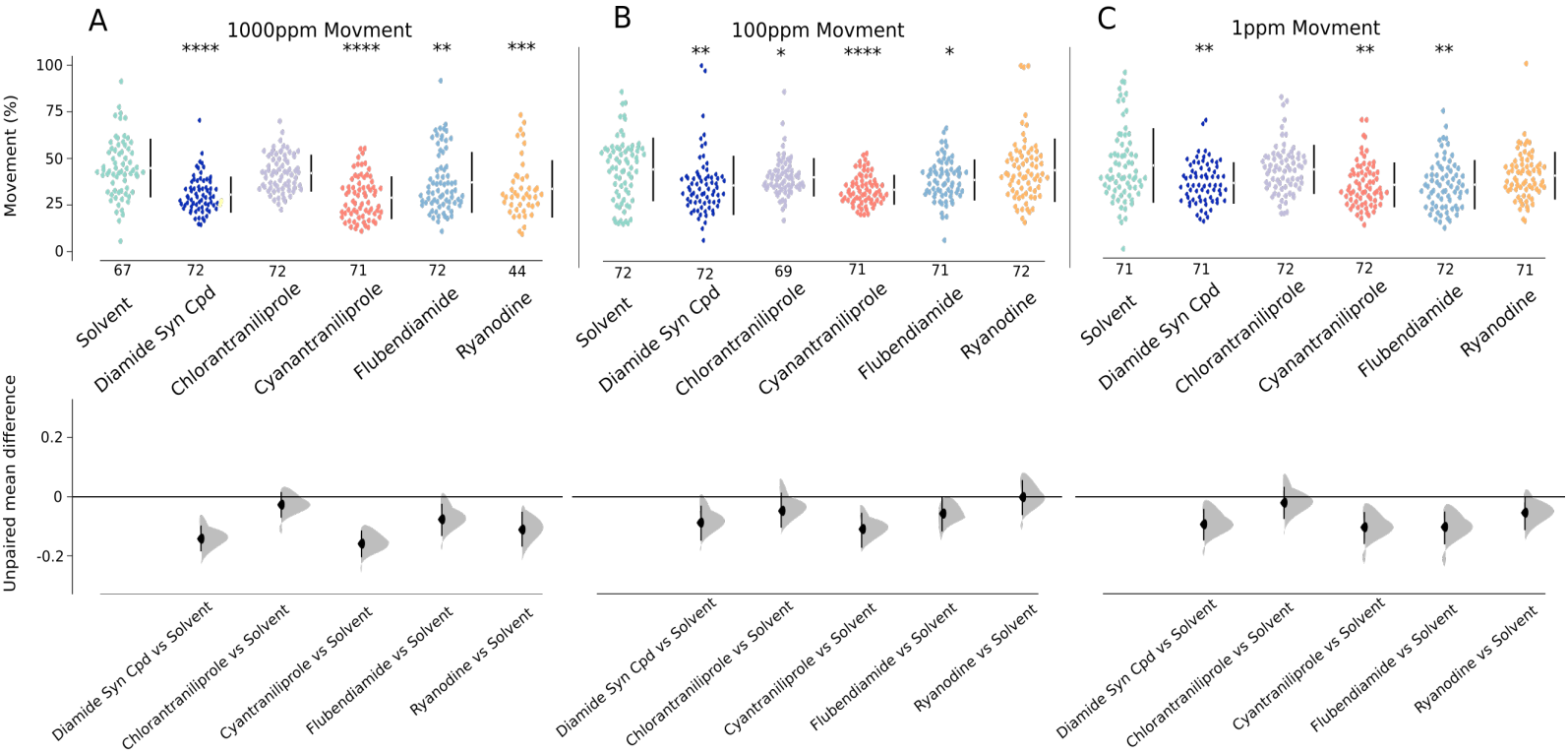


Figure 4.19: *Quantification of movement changes for compounds acting on ryanodine receptors.* Plots showing the change in movement of flies exposed to five compounds - Diamide Syn Cpd, Chlorantraniliprole, Cyantraniliprole, Flubendiamide and Ryanodine - at **(A)** 1000ppm, **(B)** 100ppm and **(C)** 1ppm. The observed effect size between the experimental groups and solvent control group is depicted as a black dot, and the 95% CI is indicated by the ends of the black error bars. *N* for each group and asterisks' demonstrating statistical differences between control and experimental groups are shown within the figure and data contained is from at least 2 biological repeats per group.

Table 4.16: Significant changes in movement for flies exposed to compounds acting on ryanodine receptors at 1000ppm, 100ppm and 1ppm

Concentration	Insecticide	Adjusted P-Value	Significance
1000ppm	Diamide Syn Cpd	$7.75x10^{-9}$	****
100ppm	Diamide Syn Cpd	0.00105	**
1ppm	Diamide Syn Cpd	0.0091	**
1000ppm	Chlorantraniliprole	0.304	n.s.
100ppm	Chlorantraniliprole	0.0385	*
1ppm	Chlorantraniliprole	0.899	n.s.
1000ppm	Cyantraniliprole	$7.75x10^{-9}$	****
100ppm	Cyantraniliprole	$1.5x10^{-5}$	****
1ppm	Cyantraniliprole	0.00325	**
1000ppm	Flubendiamide	$1.45x10^{-3}$	**
100ppm	Flubendiamide	0.0304	*
1ppm	Flubendiamide	0.00348	**
1000ppm	Ryanodine	$1.16x10^{-4}$	***
100ppm	Ryanodine	0.425	n.s.
1ppm	Ryanodine	0.2	n.s.

Table 4.17: Changes in movement for compounds acting on ryanodine receptors

Concentration	Compound	Change in Movement
1000ppm	Diamide Syn Cpd	Decrease
	Chlorantraniliprole	No change
	Cyantraniliprole	Decrease
	Flubendiamide	Decrease
	Ryanodine	Decrease
100ppm	Diamide Syn Cpd	Decrease
	Chlorantraniliprole	Decrease
	Cyantraniliprole	Decrease
	Flubendiamide	Decrease
	Ryanodine	No change
1ppm	Diamide Syn Cpd	Decrease
	Chlorantraniliprole	No change
	Cyantraniliprole	Decrease
	Flubendiamide	Decrease
	Ryanodine	No change

Sodium channel modulators and their effect on movement

I then went on to look at how movement was changed when flies were exposed to compounds acting on sodium channels. Three compounds tested had this MoA: DDT, Tetramethrin and Permethrin. Comparatively to compounds which acted on ryanodine receptors, these compounds did not all lead to flies showing the same changes in movement.

At 1000ppm, both DDT and Permethrin led to flies having a significant increase in movement, whilst flies exposed to Tetramethrin showed no change.

Interestingly, at 100ppm, Tetramethrin led to flies showing a large decrease in movement, whereas flies exposed to DDT had a smaller but significant increase in movement comparatively to those flies exposed to the solvent control. Permethrin had no effect on movement at 100ppm.

At 1ppm, none of the flies exposed to compounds showed a significant change in movement.

From this, we can conclude that exposure to DDT led to increases in movement at 1000ppm and 100ppm but not at 1ppm. Flies exposed to Permethrin had a small but significant increase in movement at 1000ppm only. Flies exposed to Tetramethrin had a decrease in movement at 100ppm, but did not show any changes in movement at either of the other concentrations.

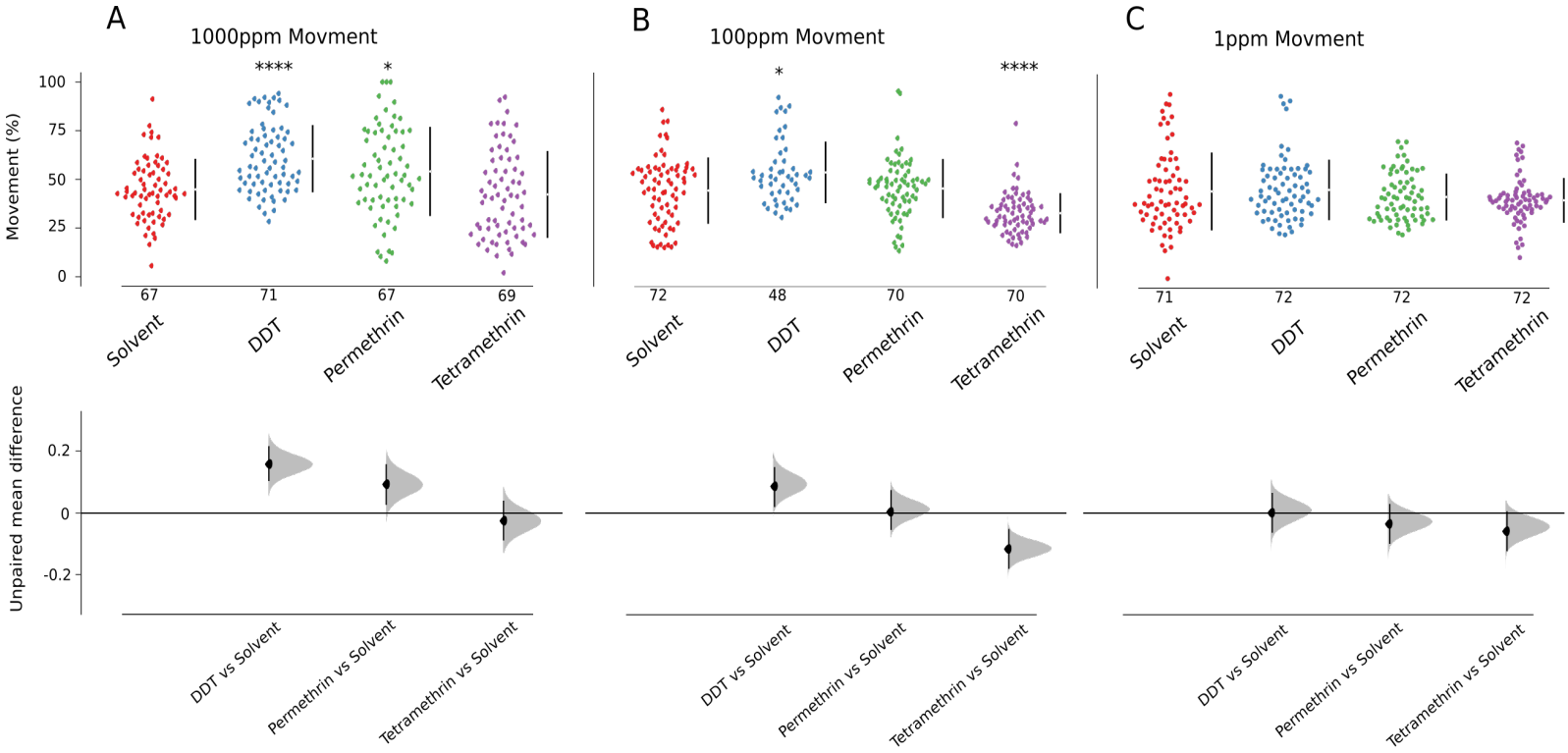


Figure 4.20: *Quantification of movement changes for compounds acting on sodium channels.*. Plots showing the change in movement of flies exposed to three compounds, DDT, Tetramethrin and Permethrin - at **(A)** 1000ppm, **(B)** 100ppm and **(C)** 1ppm. The observed effect size between the experimental groups and solvent control group is depicted as a black dot, and the 95% CI is indicated by the ends of the black error bars. *N* for each group and asterisks' demonstrating statistical differences between control and experimental groups are shown within the figure and data contained is from at least 2 biological repeats per group.

Table 4.18: Significant changes in movement for flies exposed to compounds acting on sodium channels at 1000ppm, 100ppm and 1ppm.

Concentration	Insecticide	Adjusted P-Value	Significance
1000ppm	DDT	$1.9x10^{-6}$	****
100ppm	DDT	0.0354	*
1ppm	DDT	0.41129	n.s.
1000ppm	Permethrin	0.021	*
100ppm	Permethrin	0.9139	n.s.
1ppm	Permethrin	0.661	n.s.
1000ppm	Tetramethrin	0.231	n.s.
100ppm	Tetramethrin	$2.9x10^{-5}$	****
1ppm	Tetramethrin	0.53276	n.s.

Table 4.19: Changes in movement for compounds acting on sodium channels

Concentration	Compound	Change in Movement
1000ppm	DDT	Increase
	Permethrin	Increase
	Tetramethrin	No change
100ppm	DDT	Increase
	Permethrin	No change
	Tetramethrin	Decrease
1ppm	DDT	No change
	Permethrin	No change
	Tetramethrin	No change

Compounds targetting the $GABA_A$ receptor and their effect on movement

The next group I looked at were compounds which target $GABA_A$ receptors and what effect these had on fly movement.

Two compounds tested act on $GABA_A$ receptors. These were Syngenta Cpd 1 and Dieldrin.

Interestingly, these compounds had opposing effects on movement at 1000ppm. At this concentration, flies exposed to the Syngenta Cpd 1 showed a significant decrease in

movement and those exposed to Dieldrin had a significant increase in movement. However, at 100ppm, flies exposed to the Syngenta Cpd 1 had no significant change in movement compared to flies exposed to the solvent control. At 100ppm, Dieldrin again led flies to show an significant increase in movement.

Surprisingly, at 1ppm, flies exposed to each compound had significant decreases in movement. This was more significant for Dieldrin than for the Syngenta Cpd 1.

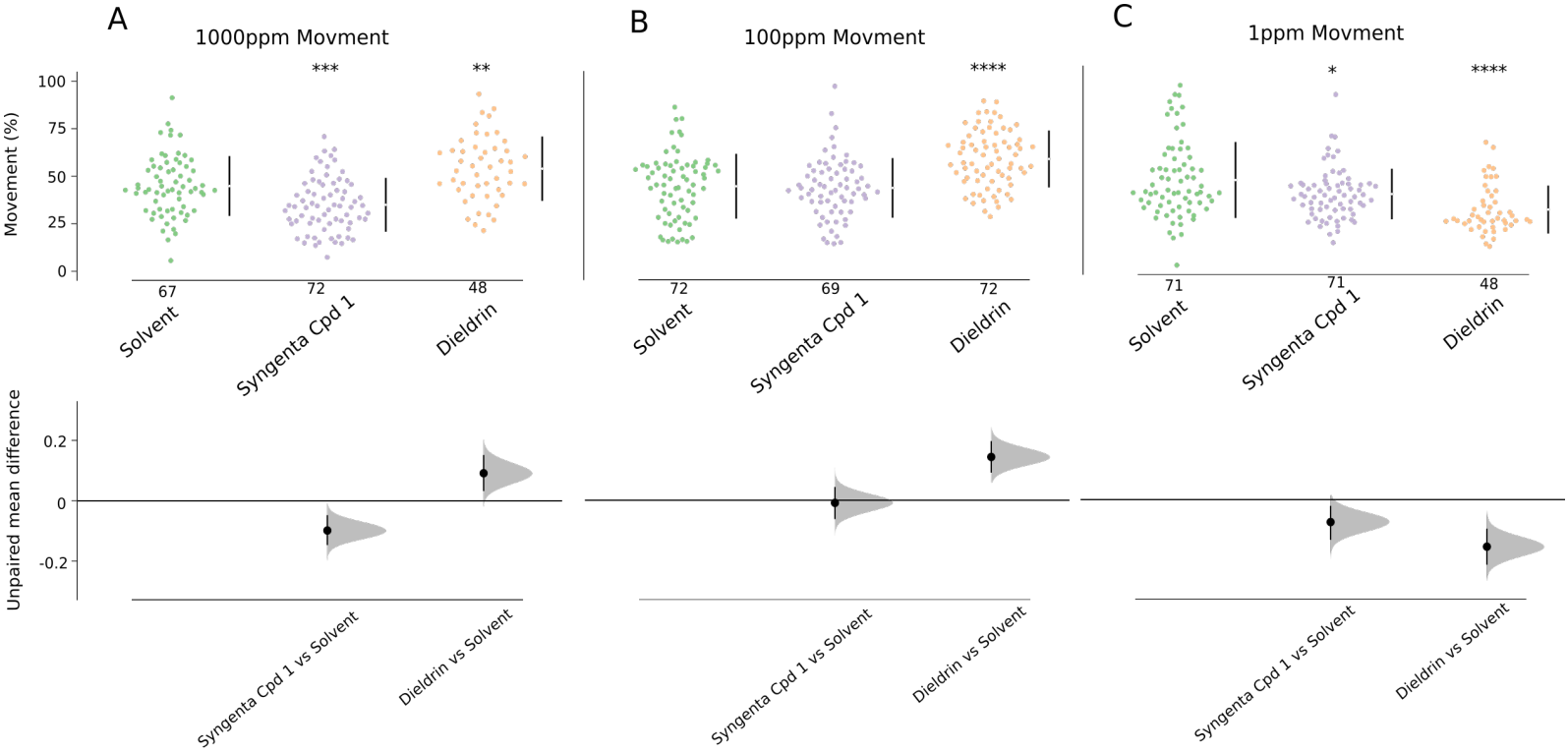


Figure 4.21: *Quantification of movement changes for compounds acting on $GABA_A$ receptors* Plots showing the change in movement of flies exposed to two compounds, Syngenta Cpd 1 and Dieldrin - at **(A)** 1000ppm, **(B)** 100ppm and **(C)** 1ppm. The observed effect size between the experimental groups and solvent control group is depicted as a black dot, and the 95% CI is indicated by the ends of the black error bars. *N* for each group and asterisks' demonstrating statistical differences between control and experimental groups are shown within the figure and data contained is from at least 2 biological repeats per group.

Table 4.20: Significant changes in movement for flies exposed to compounds acting on $GABA_A$ receptors at 1000ppm, 100ppm and 1ppm

Concentration	Insecticide	Adjusted P-Value	Significance
1000ppm	Syngenta Cpd 1	0.00042	***
100ppm	Syngenta Cpd 1	0.540	n.s.
1ppm	Syngenta Cpd 1	0.03	*
1000ppm	Dieldrin	0.00365	**
100ppm	Dieldrin	4.8^{-5}	****
1ppm	Dieldrin	3.0^{-5}	****

Table 4.21: Changes in movement for compounds acting on $GABA_A$ receptors

Concentration	Compound	Change in Movement
1000ppm	Syngenta Cpd 1	Decrease
	Dieldrin	Increase
100ppm	Syngenta Cpd 1	No change
	Dieldrin	Increase
1ppm	Syngenta Cpd 1	Decrease
	Dieldrin	Decrease

Compounds targetting the acetylcholine pathway and their effect on movement

Although Imidacloprid acts on nicotinic acetylcholine receptors [465] and Spiroindoline acts via the vesicular acetylcholine transporter [425], these compounds were most closely related in terms of MoA, hence flies exposed to these compounds were compared.

Although these compounds have different targets within this pathway, flies exposed to each compound showed broadly the same changes to movement.

At 1000ppm, flies exposed to both of these compounds had a significant decrease in movement. This was replicated at 100ppm, albeit with less significance for flies exposed to Spiroindoline. At 1ppm, flies exposed to either compound had smaller but still significant decreases in movement compared to flies exposed to the solvent control.

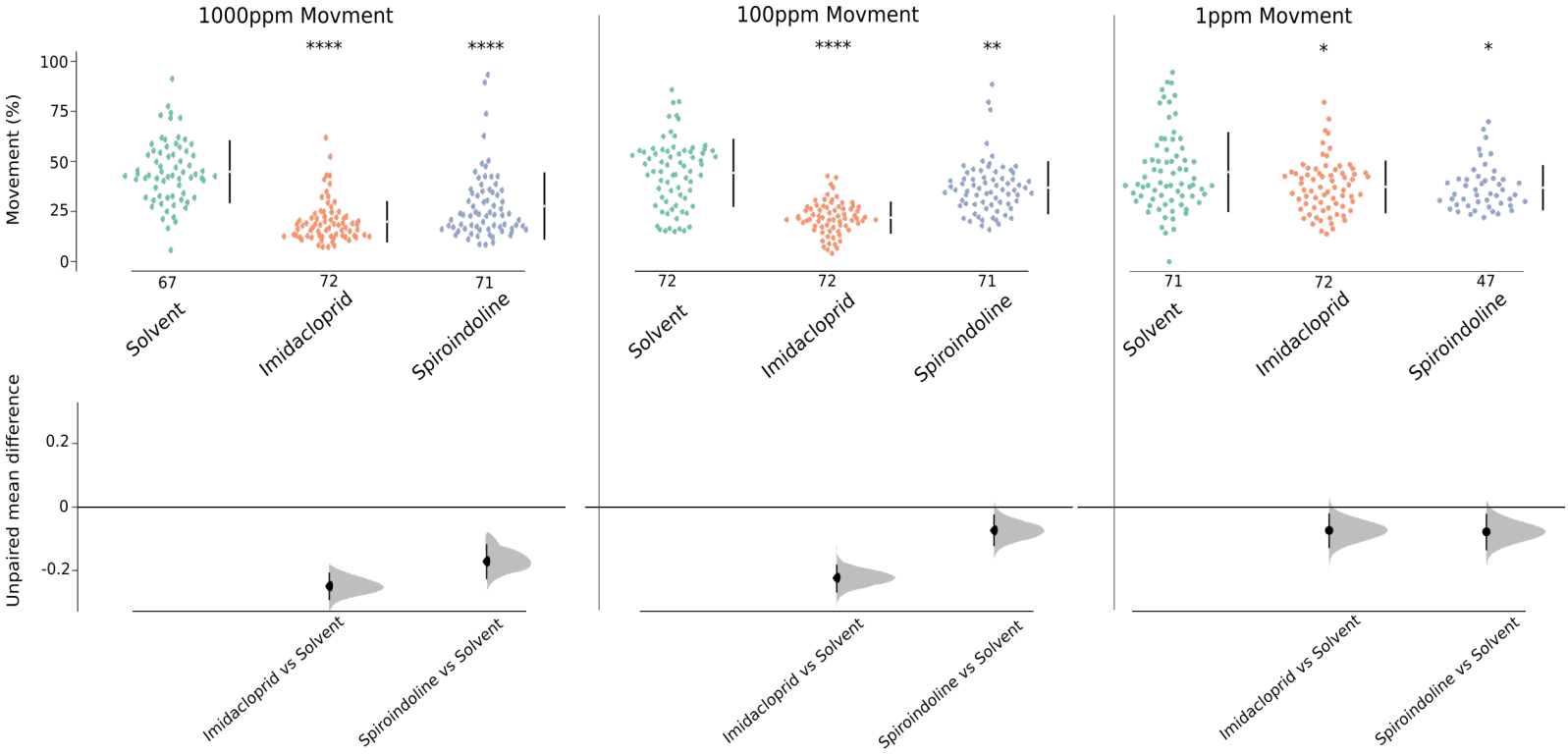


Figure 4.22: *Quantification of movement changes for compounds acting via the acetylcholine pathway.* Plots showing the change in movement of flies exposed to two compounds, Imidacloprid and Spiroindoline - at **(A)** 1000ppm, **(B)** 100ppm and **(C)** 1ppm. The observed effect size between the experimental groups and solvent control group is depicted as a black dot, and the 95% CI is indicated by the ends of the black error bars. *N* for each group and asterisks' demonstrating statistical differences between control and experimental groups are shown within the figure and data contained is from at least 2 biological repeats per group.

Table 4.22: Significant changes in movement for flies exposed to compounds targetting the acetylcholine pathway at 1000ppm, 100ppm and 1ppm

Concentration	Insecticide	Adjusted P-Value	Significance
1000ppm	Imidacloprid	$4.0x10^{-16}$	****
100ppm	Imidacloprid	$1.04.0x10^{-13}$	****
1ppm	Imidacloprid	0.0349	*
1000ppm	Spiroindoline	$2.9x10^{-10}$	****
100ppm	Spiroindoline	0.00148	**
1ppm	Spiroindoline	0.0349	*

Table 4.23: Changes in movement for compounds acting on acetylcholine receptors

Concentration	Compound	Change in Movement
1000ppm	Imidacloprid	Decrease
	Spiroindoline	Decrease
100ppm	Imidacloprid	Decrease
	Spiroindoline	Decrease
1ppm	Imidacloprid	Decrease
	Spiroindoline	Decrease

Compounds targetting the chordotonal organs and their effect on movement

Finally, I looked at two compounds, one of which is known to act on chordotonal organs, Pymetrozine [174], and one which is thought to do so as well, Flonicamid, [457]. Flies exposed to these compounds at both 1000ppm and 100ppm showed highly significant increases in movement compared to controls. However, at the lowest concentration, exposure to neither compound led to a significant change in movement.

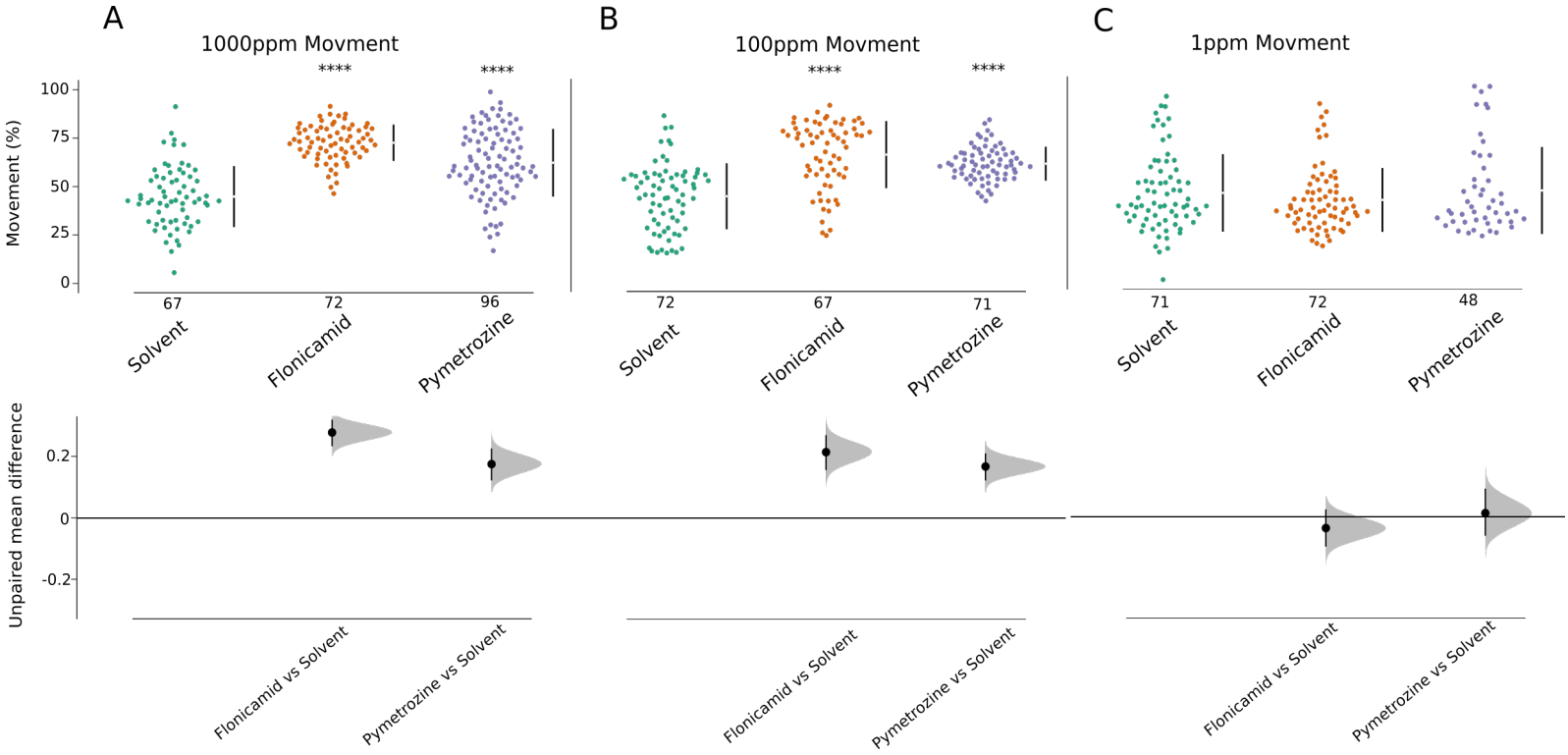


Figure 4.23: *Quantification of movement changes for compounds acting on chordotonal organs.* Plots showing the change in movement of flies exposed to two compounds, Flonicamid and Pymetrozine - at **(A)** 1000ppm, **(B)** 100ppm and **(C)** 1ppm. The observed effect size between the experimental groups and solvent control group is depicted as a black dot, and the 95% CI is indicated by the ends of the black error bars. *N* for each group and asterisks' demonstrating statistical differences between control and experimental groups are shown within the figure and data contained is from at least 2 biological repeats per group.

Table 4.24: Significant changes in movement for flies exposed to compounds targetting the acetylcholine pathway at 1000ppm, 100ppm and 1ppm

Concentration	Insecticide	Adjusted P-Value	Significance
1000ppm	Flonicamid	$4.0x10^{-16}$	****
100ppm	Flonicamid	$2.8x10^{-10}$	****
1ppm	Flonicamid	0.482	n.s.
1000ppm	Pymetrozine	$1.8x10^{-9}$	****
100ppm	Pymetrozine	$7.2x10^{-11}$	**
1ppm	Pymetrozine	0.748	n.s.

Table 4.25: Changes in movement for compounds acting on chordotonal organs

Concentration	Compound	Change in Movement
1000ppm	Flonicamid	Increase
	Pymetrozine	Increase
100ppm	Flonicamid	Increase
	Pymetrozine	Increase
1ppm	Flonicamid	No change
	Pymetrozine	No change

4.4 Measuring lifespan over 24 hours in flies exposed to insecticidal compounds

To increase the understanding of how exposure to these compounds affects flies at different concentrations, I looked to quantify lifespan in flies exposed to each of the 16 compounds. As I only used 12 hours worth of data in my classification and behavioural analysis of flies exposed to these compounds, I restricted the measurement of lifespan to a 24 hour period. I hypothesised this would allow me to understand if any significant changes in lifespan arose during the time I had classified but to also understand if any significant changes occurred afterwards which may impact results.

4.4.1 Survival of flies exposed to compounds at 1000ppm

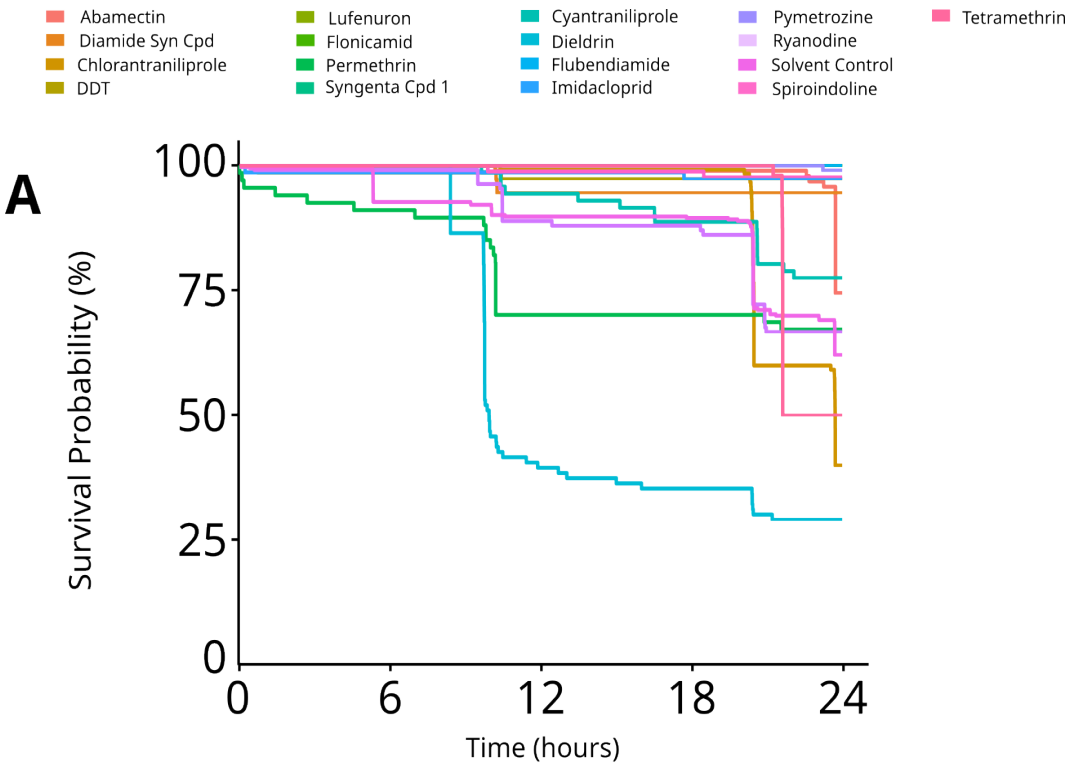
Fig.4.23 shows survival for flies exposed to each of the 16 compounds and the solvent control over a 24 hour period. Exposure to several of these compounds at this concentration led to a reduction in survival. The compound which had the greatest impact on survival was Dieldrin. This compound led to almost total death within the 24 hour period, with only 17% of flies surviving at the end of a 20 hour period.
Other compounds which significantly impacted survival were Ryanodine and Permethrin.

4.4.2 Survival of flies exposed to compounds at 100ppm

Fig.4.24 shows survival for flies exposed to each of the 16 compounds and the solvent control over a 24 hour period in response to the lower dose of 100ppm. At this concentration, survival was significantly improved for many of the compounds.
Although flies exposed to Dieldrin were still the most affected in terms of lifespan, mortality was reduced at 100ppm when compared to that at 1000ppm (68% at 100ppm compared to 17% at 1000ppm).

4.4.3 Survival of flies exposed to compounds at 1ppm

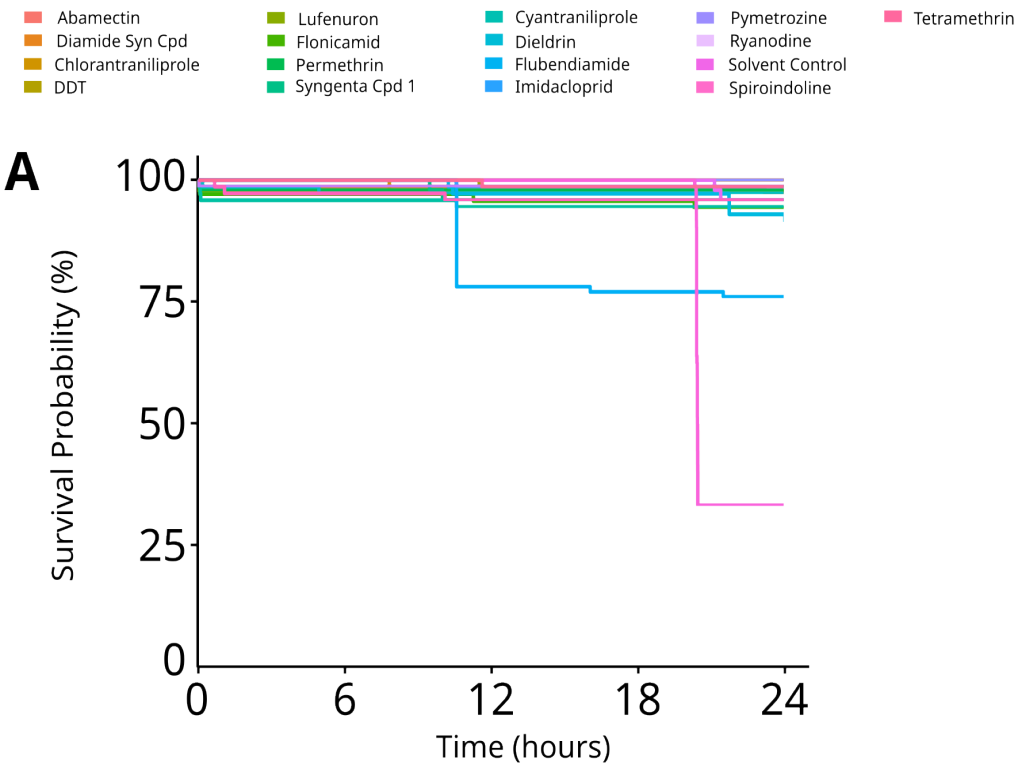
Fig.4.25 shows survival for flies exposed to each of the 16 compounds and the solvent control over a 24 hour period. Interestingly, exposure of flies to Dieldrin at this lowest dose led to a greater decrease in survival than that seen at 100ppm (50% at 1ppm compared to 68% at 100ppm). However, flies exposed to all other compounds were less affected in terms of mortality at this concentration than they had been at higher concentrations.



B

Concentration (1000ppm)	0 hours	5 hours	10 hours	15 hours	20 hours
Compound	n (%)	n (%)	n (%)	n (%)	n (%)
DDT	71 (100)	70 (99)	70 (99)	69 (97)	69 (97)
Lufenuron	72 (100)	72 (100)	72 (100)	72 (100)	72 (100)
Flonicamid	72 (100)	72 (100)	72 (100)	72 (100)	72 (100)
Permethrin	67 (100)	61 (91)	60 (90)	47 (70)	47 (70)
Syngenta Cpd 1	72 (100)	72 (100)	72 (100)	72 (100)	72 (100)
Abamectin	71 (100)	71 (100)	71 (100)	70 (99)	70 (99)
Diamide Syn Cpd	72 (100)	72 (100)	72 (100)	66 (92)	66 (92)
Chlorantraniliprole	72 (100)	72 (100)	72 (100)	71 (99)	71 (99)
Cyantraniliprole	71 (100)	71 (100)	71 (100)	66 (93)	63 (89)
Dieldrin	48 (100)	48 (100)	35 (73)	10 (21)	8 (17)
Flubendiamide	72 (100)	72 (100)	72 (100)	72 (100)	72 (100)
Imidacloprid	72 (100)	71 (99)	71 (99)	71 (99)	70 (97)
Pymetrozine	96 (100)	96 (100)	96 (100)	96 (100)	96 (100)
Ryanodine	44 (100)	44 (100)	44 (100)	40 (91)	20 (45)
Solvent Control	67 (100)	67 (100)	67 (100)	67 (100)	48 (72)
Spirindoline	71 (100)	71 (100)	71 (100)	70 (99)	70 (99)
Tetramethrin	70 (100)	70 (100)	70 (100)	68 (97)	68(97)

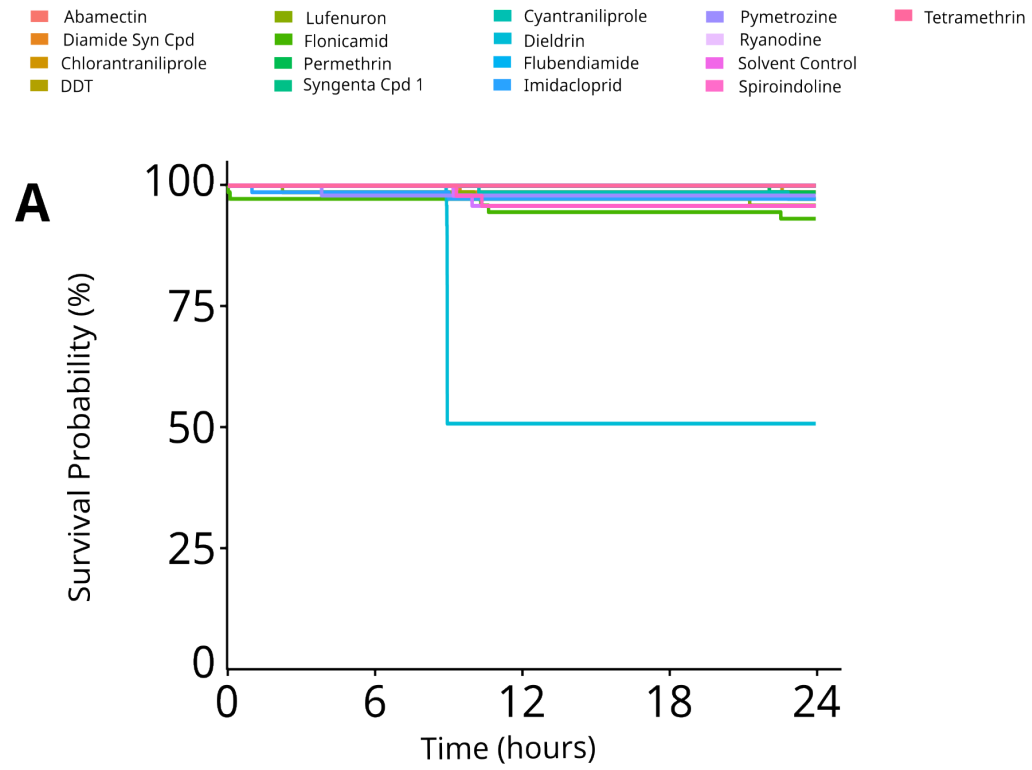
Figure 4.24: *Quantification of survival for flies exposed to compounds at 1000ppm.* Plots showing the change in movement of flies exposed to 16 compounds. **(A)** Survival plot showing a survival trace for each of the 17 groups of flies **(B)** Table quantifying survival number and percentage at increasing time points over the 24 hour period.



B

Concentration (100ppm)	0 hours	5 hours	10 hours	15 hours	20 hours
Compound	n (%)	n (%)	n (%)	n (%)	n (%)
DDT	48 (100)	48 (100)	48 (100)	48 (100)	48 (100)
Lufenuron	72 (100)	71 (99)	71 (99)	70 (97)	70 (97)
Flonicamid	68 (100)	65 (96)	65 (96)	63 (93)	63 (93)
Permethrin	72 (100)	69 (97)	69 (97)	68 (96)	68 (96)
Syngenta Cpd 1	71 (100)	70 (97)	69 (96)	69 (96)	69 (96)
Abamectin	48 (100)	48 (100)	48 (100)	48 (100)	48 (100)
Diamide Syn Cpd	72 (100)	71 (99)	71 (99)	71 (99)	71 (99)
Chlorantraniliprole	69 (100)	69 (100)	68 (99)	68 (99)	68 (99)
Cyantraniliprole	72 (100)	72 (100)	72 (100)	69 (97)	69 (97)
Dieldrin	72 (100)	72 (100)	72 (100)	50 (69)	49 (68)
Flubendiamide	71 (100)	71 (100)	71 (100)	70 (99)	70 (99)
Imidacloprid	72 (100)	72 (100)	72 (100)	72 (100)	72 (100)
Pymetrozine	72 (100)	71 (99)	71 (99)	71 (99)	71 (99)
Ryanodine	72 (100)	72 (100)	72 (100)	72 (100)	72 (100)
Solvent Control	72 (100)	72 (100)	72 (100)	72 (100)	72 (100)
Spirindoline	72 (100)	70 (97)	70 (97)	68 (94)	68 (94)
Tetramethrin	70 (100)	70 (100)	70 (100)	68 (99)	68(99)

Figure 4.25: *Quantification of survival for flies exposed to compounds at 100ppm.* Plots showing the change in movement of flies exposed to 16 compounds. **(A)** Survival plot showing a survival trace for each of the 17 groups of flies **(B)** Table quantifying survival number and percentage at increasing time points over the 24 hour period.



B

Concentration (1ppm)	0 hours	5 hours	10 hours	15 hours	20 hours
Compound	n (%)	n (%)	n (%)	n (%)	n (%)
DDT	72 (100)	72 (100)	72 (100)	72 (100)	72 (100)
Lufenuron	72 (100)	71 (99)	71 (99)	72 (100)	72 (100)
Flonicamid	72 (100)	70 (97)	70 (97)	68 (94)	68 (94)
Permethrin	72 (100)	72 (100)	72 (100)	72 (100)	72 (100)
Syngenta Cpd 1	71 (100)	71 (100)	71 (100)	71 (100)	71 (100)
Abamectin	72 (100)	72 (100)	72 (100)	72 (100)	72 (100)
Diamide Syn Cpd	72 (100)	72 (100)	72 (100)	71 (99)	71 (99)
Chlorantraniliprole	72 (100)	72 (100)	72 (100)	72 (100)	72 (100)
Cyantraniliprole	72 (100)	72 (100)	72 (100)	71 (99)	71 (99)
Dieldrin	48 (100)	48 (100)	24 (50)	24 (50)	24 (50)
Flubendiamide	72 (100)	72 (100)	72 (100)	72 (100)	72 (100)
Imidacloprid	72 (100)	71 (99)	70 (97)	70 (97)	69 (96)
Pymetrozine	48 (100)	47 (98)	47 (98)	47 (98)	47 (98)
Ryanodine	71 (100)	71 (100)	71 (100)	69 (97)	69 (97)
Solvent Control	71 (100)	71 (100)	71 (100)	71 (100)	71 (100)
Spirindoline	47 (100)	47 (100)	47 (100)	45 (96)	45 (96)
Tetramethrin	72(100)	72(100)	72(100)	72(100)	72(100)

Figure 4.26: *Quantification of survival for flies exposed to compounds at 1ppm.* Plots showing the change in movement of flies exposed to 16 compounds. **(A)** Survival plot showing a survival trace for each of the 17 groups of flies. **(B)** Table quantifying survival number and percentage at increasing time points over the 24 hour period.

4.4.4 Summary

- Compounds tested have significant effects on movement, micro-movement, and quiescence and in general these effects decrease with decreasing concentration.
- Compounds acting on chordotonal organs, the ACh pathway and ryanodine receptors led to similar changes in movement, but those acting on sodium channels and $GABA_A$ receptors did not.
- Dieldrin showed the most significant effect on lifespan of all the compounds tested.

4.5 High-Resolution screening: Classification of Insecticide Compounds

4.5.1 Background

As we have seen in section 4.2 , classification of various insecticide compounds can be done based on a high-throughput approach, using ethoscope data and HCTSA as a statistical classification tool. This data showed us that classification is accurate for a number of different doses when the number of flies in each group is very high. However, the other, alternate approach to high-throughput phenotypic classification is by conducting a high-resolution screen.

This approach can be very effective in certain circumstances when looking at behaviours which are smaller. This approach leads to less samples being collected per compound but does provide a benefit of giving more detail for each sample. To evaluate how this approach would compare to the high-throughput approach outlined in the previous sections of this chapter, I determined that a high-resolution screen should be conducted to

understand whether this could be an alternate or complimentary method for screening compounds.

4.5.2 Designing a High-Resolution Pipeline for Insecticide Testing

It has previously been shown that the ethoscope hardware and software was designed for high-throughput screening of sleep and activity in *Drosophila melanogaster*. Based on this, the video tracking software has poor visual resolution and cannot extract individual body-parts of flies; the software draws a circle around each individual fly, the size of which is determined by the width and length of each fly [150]. To develop a high-resolution screening technique, a pipeline using different hardware and software needed to be developed.

As an alternative, a high-resolution hardware device which would allow an increased visual resolution to the ethoscope, was designed with similar principles to the ethoscope: using 3D printed components and a modular design with interchangeable squares in which flies could be placed. This device, however, had a high-resolution camera and a new recording system. To reduce the scale and time needed to build custom software to analyse data collected from the device, it was determined that pre-existing post-estimation software should be used.

Therefore, from this specification, the superscope recording device was born. The device works primarily as a custom video recording device which uses custom software to remotely record and save high-resolution videos which can be then further processed to extract individual body part positions for each video frame. The software used for pose-estimation was DeepLabCut [298], a open-source software tool which uses a pre-

trained neural network to allow the user to label key body parts on a small number of frames, allowing the building of a training data set. The training dataset is then used to train a neural network model which can be used to estimate the position of specified body parts in novel videos. Novel videos trained using this network also produce of a .csv file, which gives time series data on positional coordinates of each body part over time. Using positional body part coordinates, time series data produced by mapping the changes in movement over time of tracked points can be extracted for each individual body part. This data, like the metric for maximum velocity, can be used in combination with statistical classification software to classify flies responding to insecticide compounds. Fig.4.26 represents the steps in this pipeline.

To ensure that flies were imaged at the same dimension, I ensured that the 3D printed square, inside which flies were placed to be recorded, were designed in a specific way to facilitate for this. I designed an arena which had sides with stepped increments of 11°angle based on a design by [420]. This design is shown in Fig.4.27.

4.5.3 Determining body part time series data

As the DeepLabCut software produces data for every labelled body part defined in the labelling phase of the pipeline, and the statistical classification tool used for analysis can only process univariate time series data, I first needed to determine which body part would give the most accurate and informative metric for the movement of the fly. Working in collaboration with an undergraduate student, Cleo Zhang, we used time series data generated from a DeepLabCut pose-estimation model corresponding to different body parts to do a pair-wise similarity score. Using this matrix, we could determine which body parts showed the most similarity. Fig.4.28 shows this data. It can be seen that core body parts, which correspond to the head, thorax and abdomen

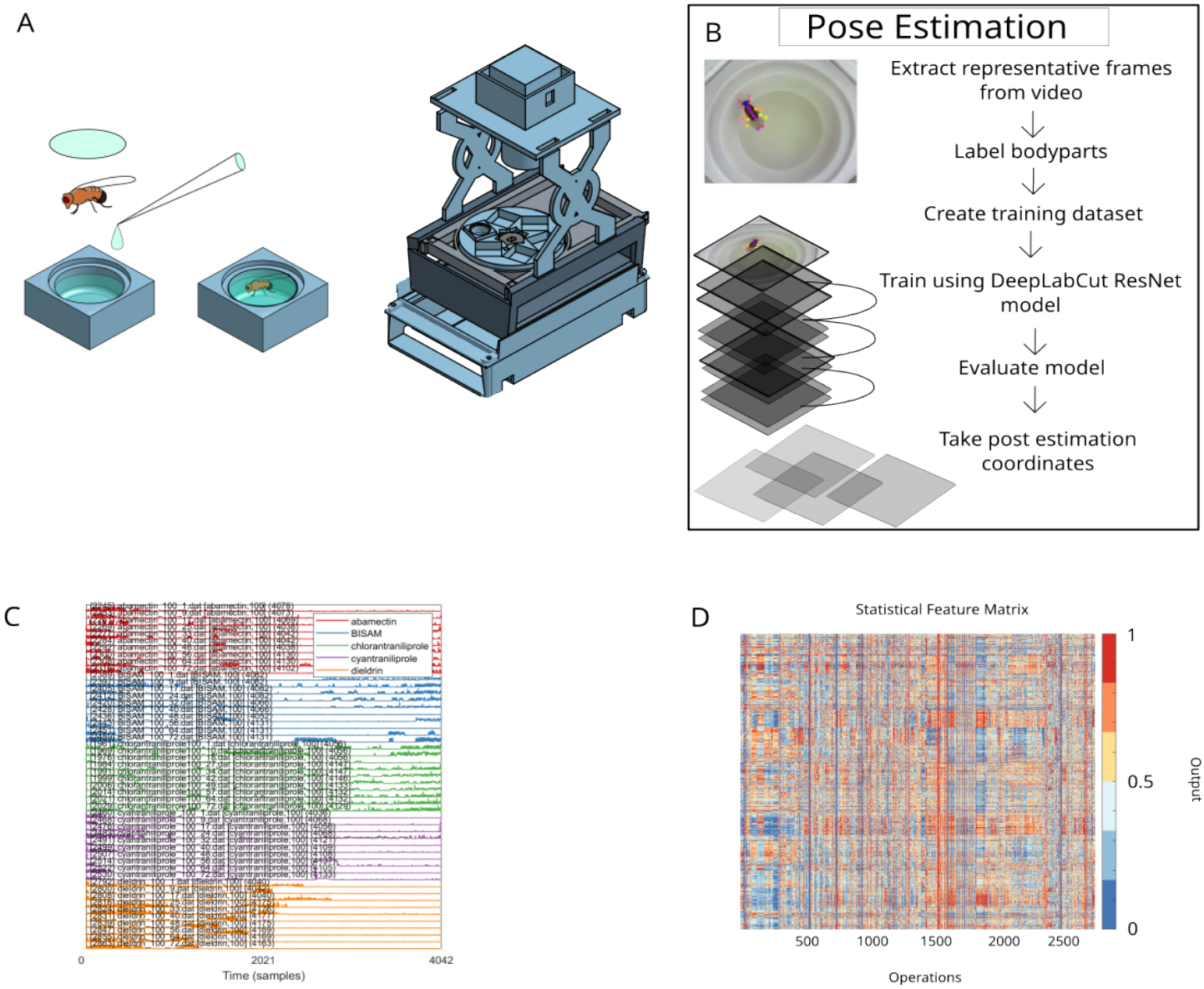


Figure 4.27: *Methodology of high-throughput screening.* **(A)** Flies were placed in small 3D printed squares with sucrose containing agarose medium and insecticide solution on top before being placed in the superscope for video recording. **(B)** Videos were processed using the pose estimation software, DeepLabCut, which allows representative frames to be extracted, labelled and used for training a predictive model which can predict body parts in novel videos. **(C)** Representative time series can be taken from pose estimation of body parts. **(D)** Example of a feature matrix which can be generated based on time series data features generated in HCTSA.

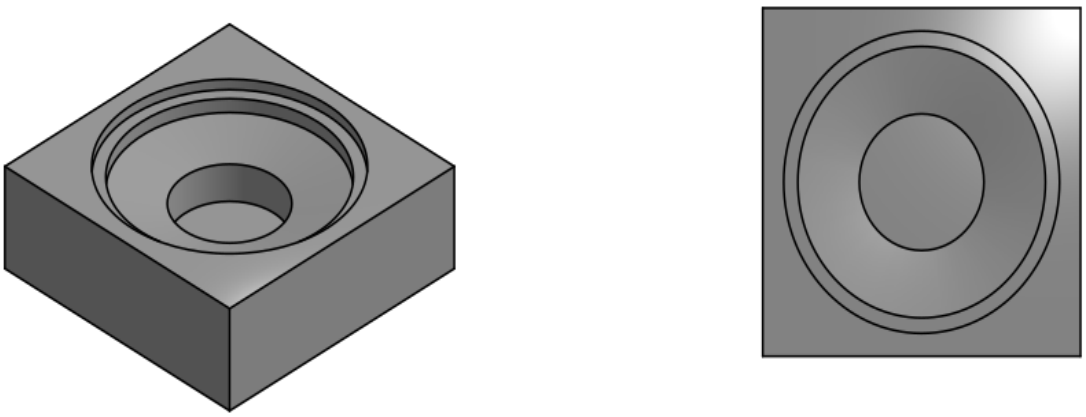


Figure 4.28: *3D printed square design for the superscope.* The design prevented flies from climbing the walls or on the cover slip of the square.

of the fly, conferred the highest similarity. This data also showed that body parts which are the most difficult to consistently label and move the most between frames, the legs and wings, showed the least correlation between compounds. Based on this data, I determined that taking a time series metric for the head would be the best proxy for movement for flies exposed to various compounds. Moving forward, I calculated all of the time series classifications based on the movement of the head over time.

4.5.4 High-resolution Screening: Classification of original compounds

After building a pipeline which would meet the requirements for high-resolution data collection, I proceeded to record and analyse videos of flies exposed to the initial 11 compounds at a single concentration (1000ppm). To ensure that the superscope captured a similar progression of symptoms as that of the ethoscope, videos of each fly responding to each compound were recorded for up to 12 hours. Due to the size of the videos and processing time of videos and pose-estimation analysis, videos were broken down into smaller sections of 30 to 15 minutes for the classification process. Due to

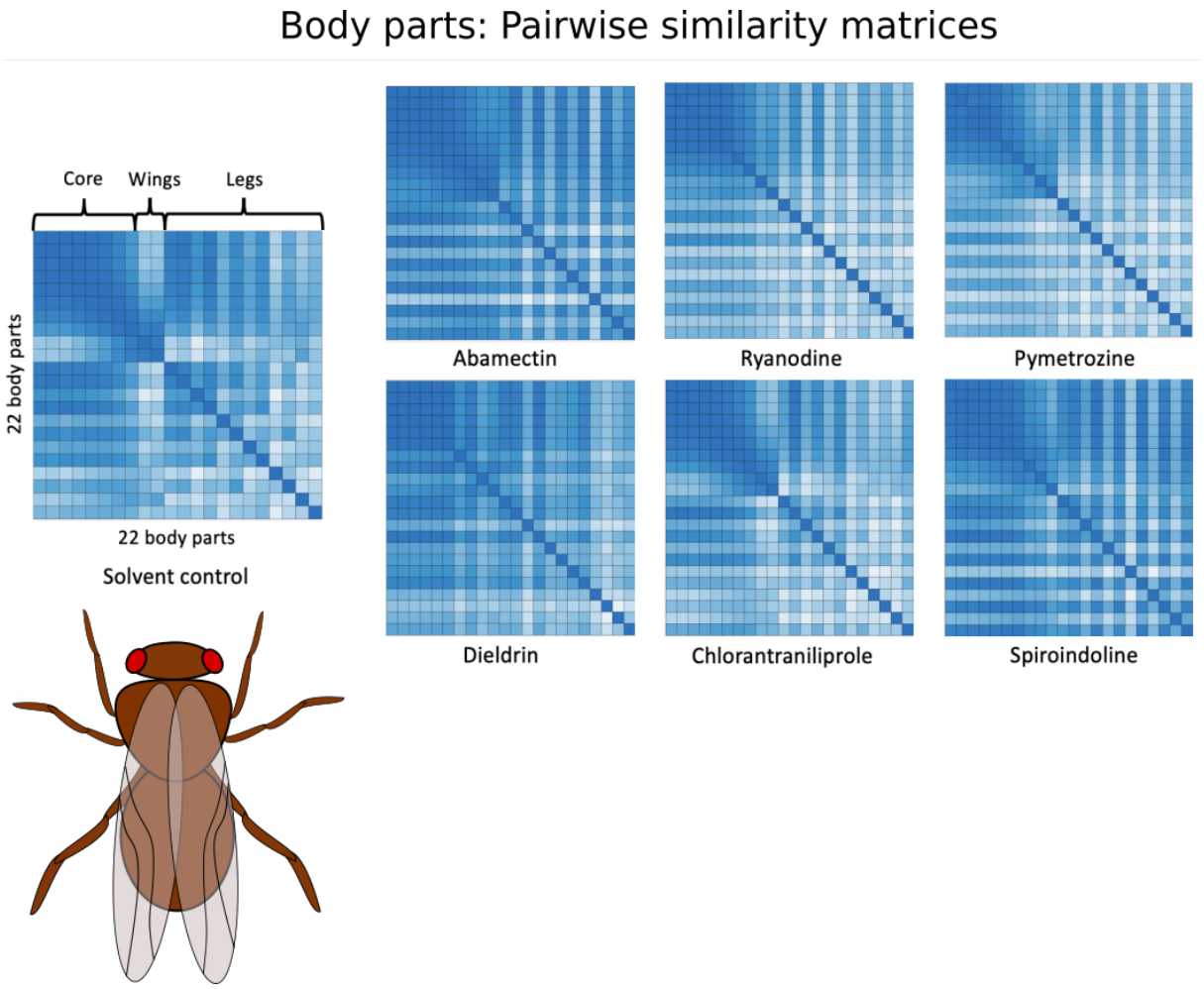
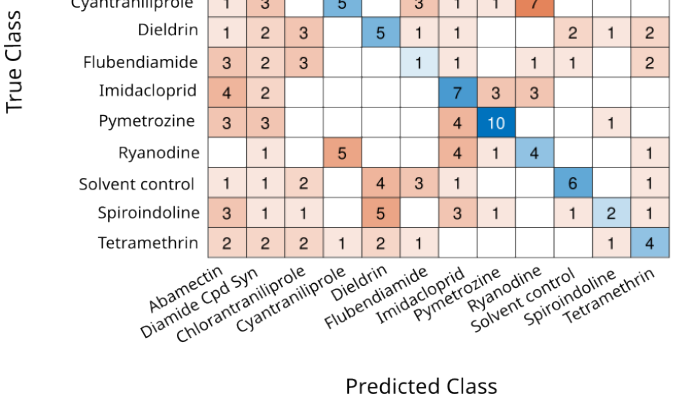


Figure 4.29: *Pairwise similarity matrices of body parts.* Pairwise similarity matrices of body parts demonstrating the correlation between prediction likelihood and body part regions.

the data size of the time series produced by the videos (with a time series data point calculated for every frame, which leads to 44998 frames per 30 minute video, therefore, 44998 data points per video time series) I determined that for validation purposes, the HCTSA sister programme, Catch-22 [286], should be used for statistical classification. Catch-22 works on the same principles as HCTSA, however, rather than using the full feature-set of almost 8000 operations, it uses a smaller feature-set of 22 operations to generate features for each time series. Despite the reduced number of operations performed, it is published as having only a 7% loss in classification accuracy [286]. Based on the reduced number of operations, processing speed is much faster and therefore can give an indication of classification accuracy much more quickly for data files of a very large size.

Fig.4.29 shows the classification matrix produced for video data for flies exposed to the first 11 compounds for the first 30 minutes of video time compared to those exposed to a solvent control. Owing to the time taken to collect and process videos, only 20 videos per group could be collected (20 flies per group). The matrix shows that classification accuracy with this method for the first 30 minutes is comparatively poor when compared to the accuracy seen from the classifications of the ethoscope data. Four compounds are misclassified (Cyantraniliprole, Flubendiamide, Ryanodine and Spiroindoline) and those which are correctly predicted have, in many cases, a 2nd classification prediction which is only a few percentage points behind. Some exceptions to this do exists; Pymetrozine has the highest classification accuracy of 45% and has a second classified compound (Imidacloprid) at only 18%. Tetramethrin has a prediction of 27%, with no clear predicted second classification.

A



B

Compound	1st Classification	% Predicted	2nd Classification	% Predicted
Abamectin	Abamectin	21	Chlorantraniliprole	16
Diamide Syn Cpd	Diamide Syn Cpd	20	Abamectin	20
Chlorantraniliprole	Chlorantraniliprole	22	Solvent/Tetramethrin	17
Cyantraniliprole	Ryanodine	33	Cyantraniliprole	24
Dieldrin	Dieldrin	28	Permethrin/ Chloran.	17
Flubendiamide	Abamectin	21	Chlorantraniliprole	21
Imidacloprid	Imidacloprid	35	Abamectin	20
Pymetrozine	Pymetrozine	45	Imidacloprid	18
Ryanodine	Cyantraniliprole	29	Ryanodine/Imidaclop.	24
Solvent Control	Solvent Control	32	Dieldrin	21
Spiroindoline	Dieldrin	26	Abamectin/Imidaclop.	16
Tetramethrin	Tetramethrin	27	Various	13

Figure 4.30: *Confusion matrix of compounds tested using the high-resolution protocol at 1000ppm.* **(A)** Confusion matrix of flies exposed to compounds and measured using the superscope. **(B)** Table quantifying the first predicted classification of flies exposed to compounds and second predicted classification.

4.5.5 High-resolution Screening: Classification of original compounds over time

As only 30 minutes worth of video data may not be long enough to allow for phenotypes to become apparent in the video data, I then took and processed 15 minute segments worth of data for each of the videos for each compound at increasing time points over the video length. Due to video compression, 12 hours worth of video data was compressed to 6 hours in most cases, so time points ranged from hour 1 to 5 of the compressed video.

As can be seen from Fig.4.30, the classification accuracies for each compound varied, in some cases quite drastically, over time. Some compounds, such as Pymetrozine, Ryanodine and Imidacloprid, had classification accuracies which started with a relatively high accuracy and then decreased at further time points. Ryanodine in particular seemed to show a trend of decreasing accuracy over time, whilst Dieldrin has an initial low accuracy which increased and then remained high. Overall, this data shows that

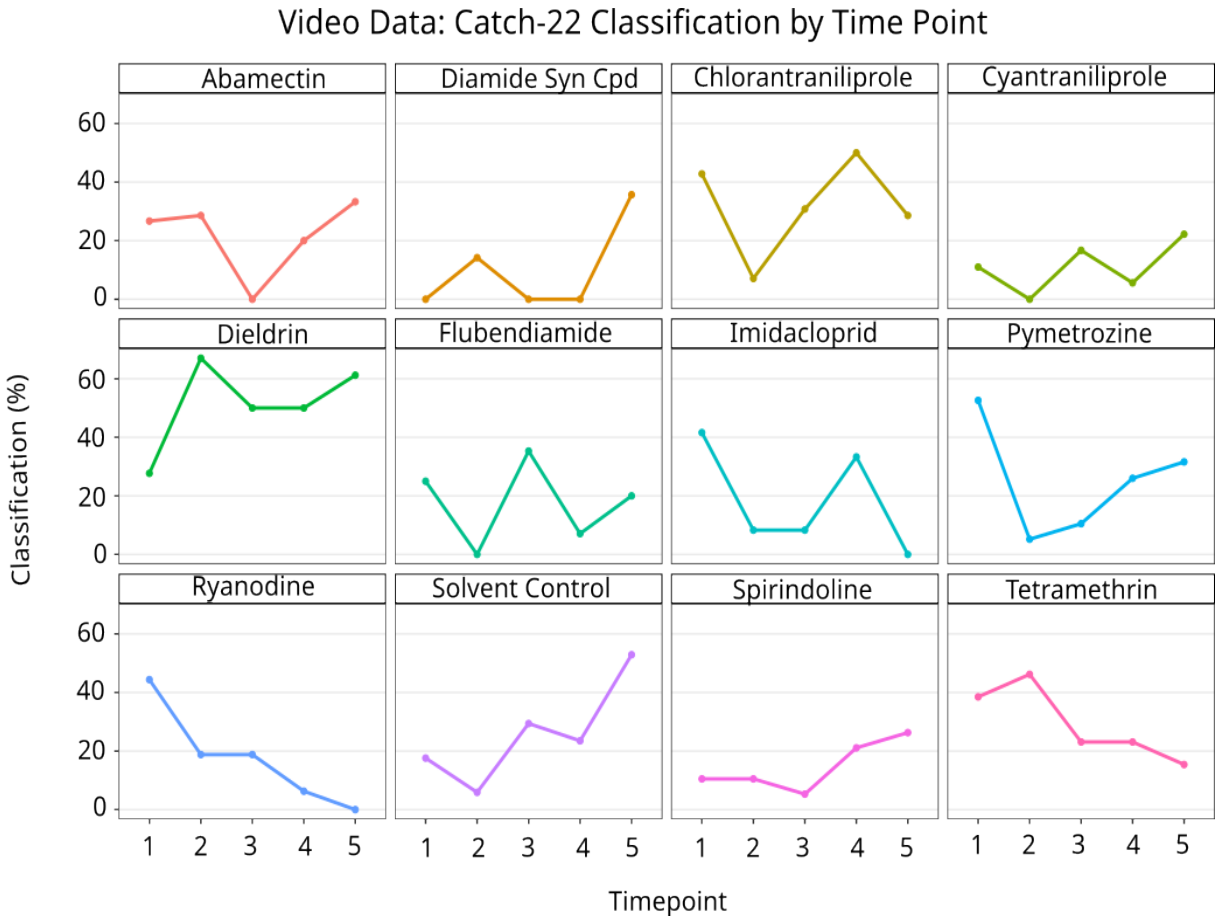


Figure 4.31: *Classification of compounds over time when processed through Catch-22.* Classification accuracy of data collected and analysed with the high-resolution pipeline as outlined above. Video data from five different time points was collected and pose-estimation software was used to track limbs in videos. Vector time series data from the head was then analysed with Catch-22 to classify compounds at different time points.

with a smaller sample size, even with increased resolution, it is much more difficult for the software to classify the compounds correctly. Due to this, it was concluded that further work would only use the high-throughput pipeline for data analysis and further exploratory analysis.

4.5.6 Summary

- The superscope is a custom video recording device which can record high resolution videos of flies.

- Videos can be processed using DeepLabCut to extract pose-estimation informa-

tion of body parts per frame.

- Classification accuracy for compounds at 1000ppm processed with Catch-22 is poor comparatively to that seen with the high-throughput methodology.

4.6 Investigating Interesting Biological Features from Insecticide Exposure

4.7 Background

Based on the data attained from the classification of data in section 4.2 and the interesting aspects of behavioural states in section 4.3, I wanted to look in more detail at some compounds which seemed to lead to unusual behavioural phenotypes in flies exposed to them.

4.8 Results

4.8.1 Investigating Potential Circadian Phenotypes

When investigating the different behavioural states of *Drosophila melanogaster* and how these change with exposure to various insecticide compounds, I noted was that the activity profile of two compounds in particular were quite different in behavioural profile when compared to wild-type flies exposed to only the solvent control. These two compounds were Dieldrin and Pymetrozine. Fig.4.31 displays the movement profiles of flies exposed to these compounds at the three concentrations tested over a 24 hour period in normal light:dark conditions, when compared to a solvent control. The profile

of flies exposed to Dieldrin at the higher doses, 1000ppm and 100ppm, shows that flies initially have a very high level of activity, with the activity gradually decreasing over time. Flies exposed to Dieldrin at 100ppm show very little activity at the transition between the light and dark, period, which suggested that exposure to this compound could change the response of the fly to circadian cues.

Exposure to Pymetrozine at 100ppm and 1000ppm also led to an interesting behavioural profile. Flies exposed to this compound at 1000ppm and 100ppm maintained a high level of activity throughout a 24 hour period.

The activity of flies exposed to these compounds at a lower dose of 1ppm showed a normal pattern of activity, similar to that of flies exposed to the solvent control.

To address whether either of these compounds led to changes in circadian rhythms, I maintained a wild-type (CS) population of flies in total darkness throughout the period of rearing and then exposed these flies to the three different concentrations of Dieldrin and Pymetrozine. Fig.4.32 shows the data from this experiment.

Male flies generally show peaks of activity during the onset of light, with this activity decreasing throughout the daytime and rising again towards the onset of the dark period. A similar pattern then repeats during the 12 hour dark period. When in constant darkness, *Drosophila* begin to lose the normal rhythmicity which is generated from a 12 hour light: 12 hour dark cycle and this rise and fall of activity around changes in light or darkness are not so pronounced (as seen in the flies exposed to the solvent and compounds at 1ppm). When flies are exposed to Dieldrin and Pymetrozine at 100ppm, flies exposed to Dieldrin do seem to show a small change in the pronounced activity peak at the beginning of the onset of the day, which also correlates to the onset of exposure. Flies exposed to Pymetrozine lose the peak of rhythmicity, as with the flies exposed to the solvent control, but still maintain the high level of activity seen in

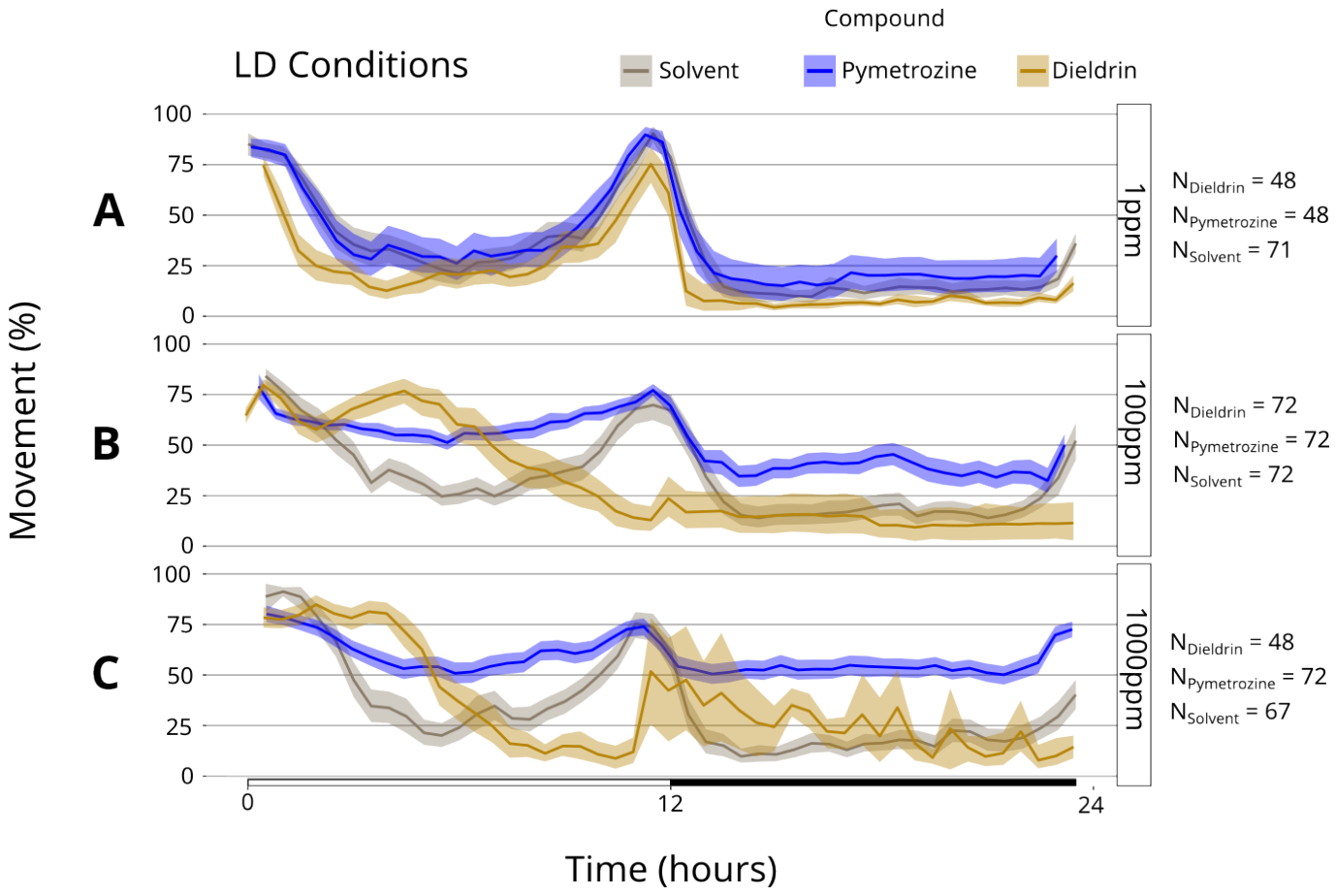


Figure 4.32: *Flies exposed to compounds under LD conditions.* Ethogram of flies exposed to **(A)** Pymetrozine and Dielrdrin at 1ppm compared to flies exposed to the solvent. **(B)** Same as in **A** but for flies exposed to compounds at 100ppm. **(C)** Same as in **A** but for flies exposed to compounds at 1000ppm. Shaded areas show a 95% bootstrap resampling confidence interval around the mean. *N* for each group is shown within the figure and data contained is from at least 2 biological repeats per group.

light:dark conditions.

For flies exposed to Dieldrin at 1000ppm in constant darkness, the flies still demonstrate the same pattern of high to low activity, suggesting that this is a biological phenotype resulting from flies initially showing high activity after compound exposure, likely followed by paralysis and an inability to move rhythmically, rather than a circadian effect.

Interestingly, flies exposed to Pymetrozine at 1000ppm also show this constant high level of activity with no peaks or troughs in movement as seen with the control. This suggests this constant level of activity of flies when exposed to Pymetrozine at this concentration is also a phenomenon arising from the compound leading to differences in behaviour not related to a change in the circadian regulation.

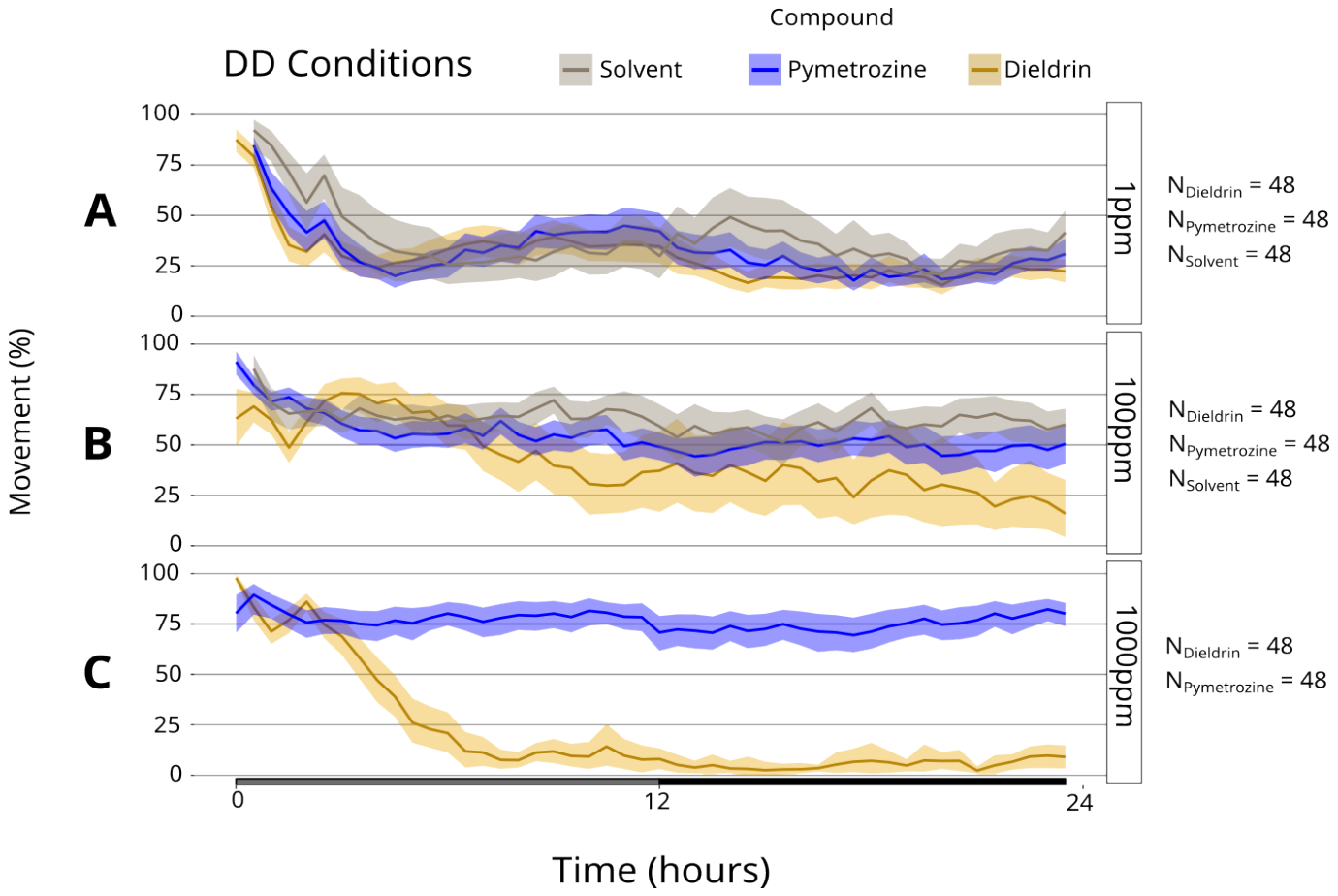


Figure 4.33: *Flies exposed to compounds under DD conditions* Ethogram of flies exposed to **(A)** Pymetrozine and Dielrdrin at 1ppm compared to flies exposed to the solvent. **(B)** Same as in **A** but for flies exposed to compounds at 100ppm. **(C)** Same as in **A** but for flies exposed to compounds at 1000ppm. Shaded areas show a 95% bootstrap resampling confidence interval around the mean. *N* for each group is shown within the figure and data contained is from at least 2 biological repeats per group.

4.8.2 Investigating Responses of Compound Resistant Flies

Insecticide resistance is a prevalent and growing issue throughout the agricultural sector. As explained in the introduction, many target insect pests have developed resistance to some of the most commonly used compounds. Using *Drosophila melanogaster* as a model organism has many benefits, one of which being the ability to generate genetic mutations quickly and easily. To understand insecticide resistance, *Drosophila* has frequently been used as a model, with many *Drosophila* mutants being generated to understand specific insecticide resistant genes. Two prevalent genes which have been

examined are *Resistance to Dieldrin* (*Rdl*) and *Paralytic* (*Para*). *Rdl*, as discussed in the introduction, encodes a GABA-gate chloride channel [379] and the presence of the gene leads to resistance against Dieldrin and fiprioles. *Para* encodes a sodium channel [251],[103]. The research team at Syngenta generated a CRISPR mutant line targetting the *Para* gene ($Para^{L1029F}$) and isogenised the same *Rdl* line used previously in work by ffrench-Constant et al. [386] which encodes an *Rdl* allele from a natural population. Using these genes, I set out to examine three different things: how these compound-resistant flies respond behaviourally to the compounds they are resistant against, how their response compares to that of wild-type flies exposed to these compounds, and whether HCTSA could classify the responses of these flies to the compounds, compared to the behaviour when exposed to the solvent control.

I firstly looked at the movement profiles of flies which were either (A) wild-type (CS) and exposed to either DDT or Dieldrin at 100ppm concentration or the solvent control (B) $Para^{L1029F}$ flies exposed to DDT at 100ppm or the solvent control (C) flies with the Rdl^{A301S} gene and exposed to Dieldrin at 100ppm or solvent control. Fig.4.34 shows that there are some small but recognisable differences in overall movement pattern and profile between CS flies exposed to either DDT, Diledrin or the control. However, the movement profiles for $Para^{L1029F}$ flies exposed to either DDT or solvent and Rdl^{A301S} flies exposed to either Dieldrin or solvent looked remarkably similar. When I quantified movement over the course of the day (ZT0-ZT12) (fig4.35), I found that, although the wild-type flies show significant differences between flies exposed to the solvent and those exposed to Dieldrin and DDT, $Para^{L1029F}$ flies and Rdl^{A301S} did not show significant differences in movement when exposed to either DDT or the solvent or Dieldrin or the solvent. I then processed the time series data for these flies using the HCTSA pipeline I described in section 4.2.

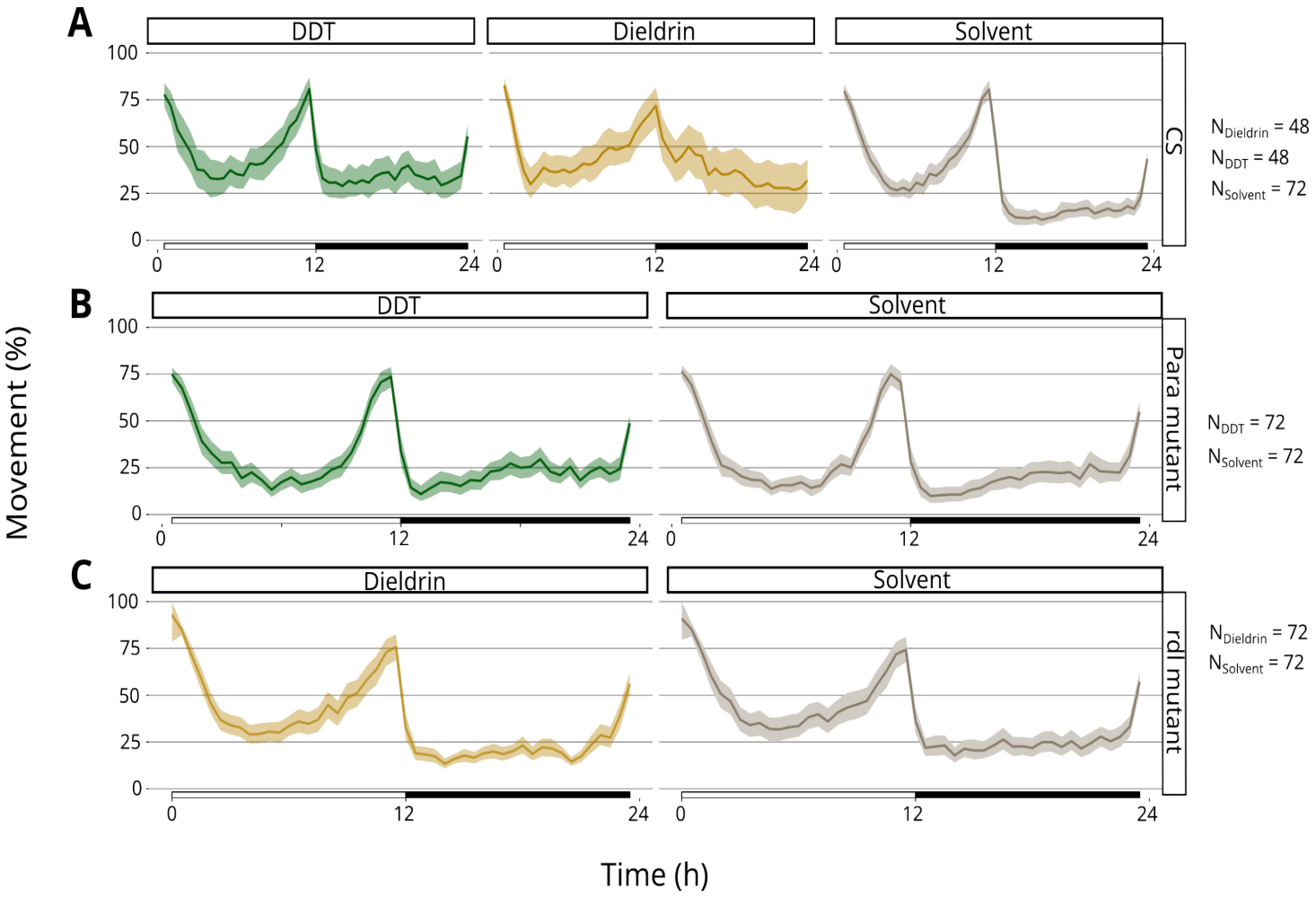


Figure 4.34: Ethogram of CS, $Para^{L1029F}$ or Rdl^{A301S} flies exposed to DDT, Dieldrin or solvent control. Ethograms of movement of **(A)** CS flies exposed to DDT, Dieldrin or solvent control. **(B)** $Para^{L1029F}$ flies exposed to DDT or solvent. **(C)** Rdl^{A301S} flies exposed to either Dieldrin or solvent. Shaded areas show a 95% bootstrap resampling confidence interval around the mean. Data contained in the figure is from at least 2 biological repeats per group.

Fig.4.36 shows the classification matrix for Rdl^{A301S} flies, $Para^{L1029F}$ flies and the CS control groups exposed to either solvent, DDT or Dieldrin. In this case, HCTSA could classify well CS flies exposed to the three different treatments. For $Para^{L1029F}$ flies, HCTSA performed worse here, and although the two treatment groups were classified correctly, there was significant confusion between the DDT exposed flies and solvent exposed flies. For Rdl^{A301S} flies, the system performed quite well, with overall classification being high between the two treatments and only minor confusion.

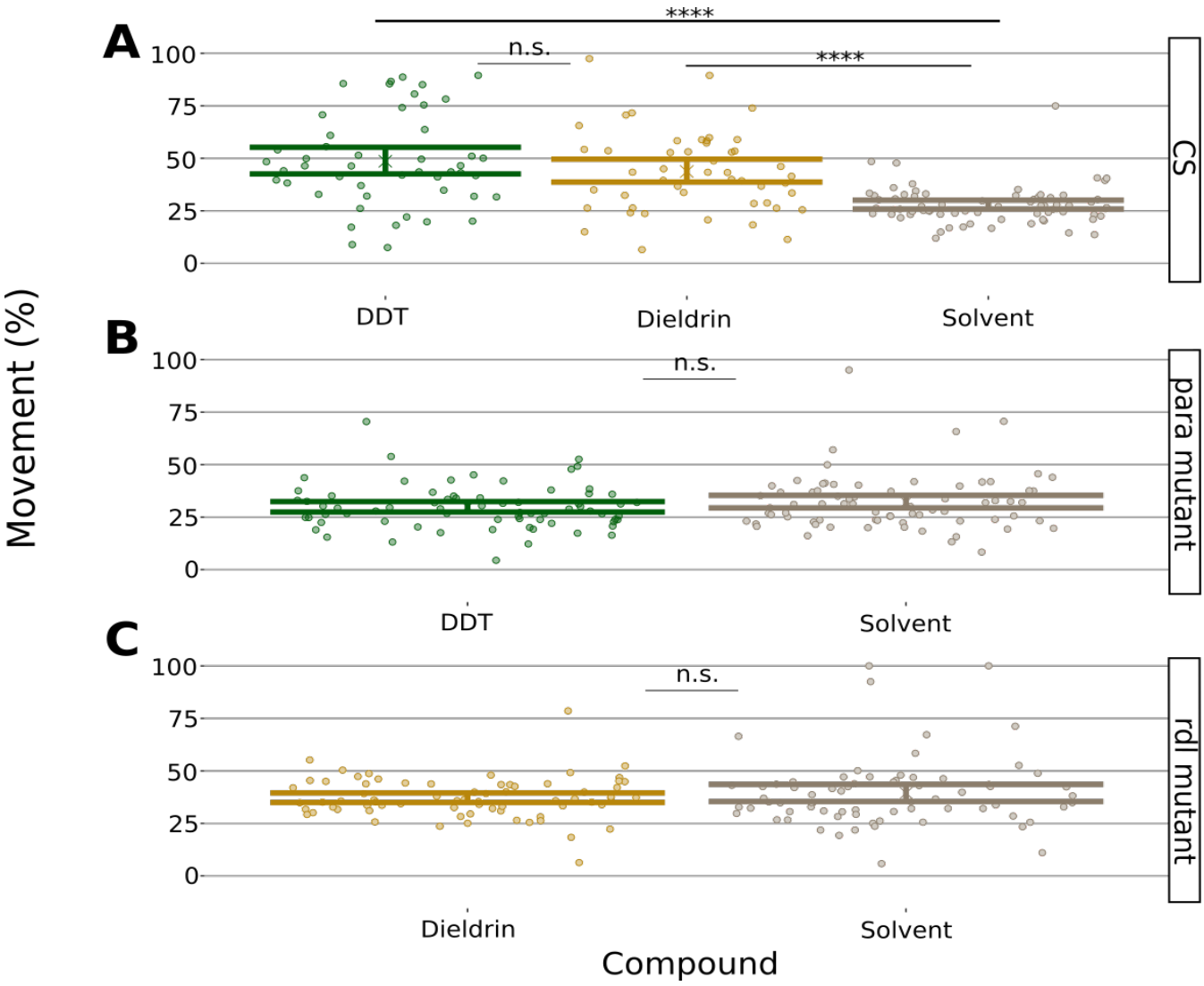


Figure 4.35: Quantification of CS, $Para^{L1029F}$ or Rdl^{A301S} flies exposed to DDT, Dieldrin or solvent control. Ethograms of movement of **(A)** CS flies exposed to DDT, Dieldrin or solvent control. **(B)** $Para^{L1029F}$ flies exposed to DDT or solvent. **(C)** Rdl^{A301S} flies exposed to either Dieldrin or solvent. Data contained in the figure is from at least 2 biological repeats per group. Biological *Ns* are shown in the corresponding ethogram.

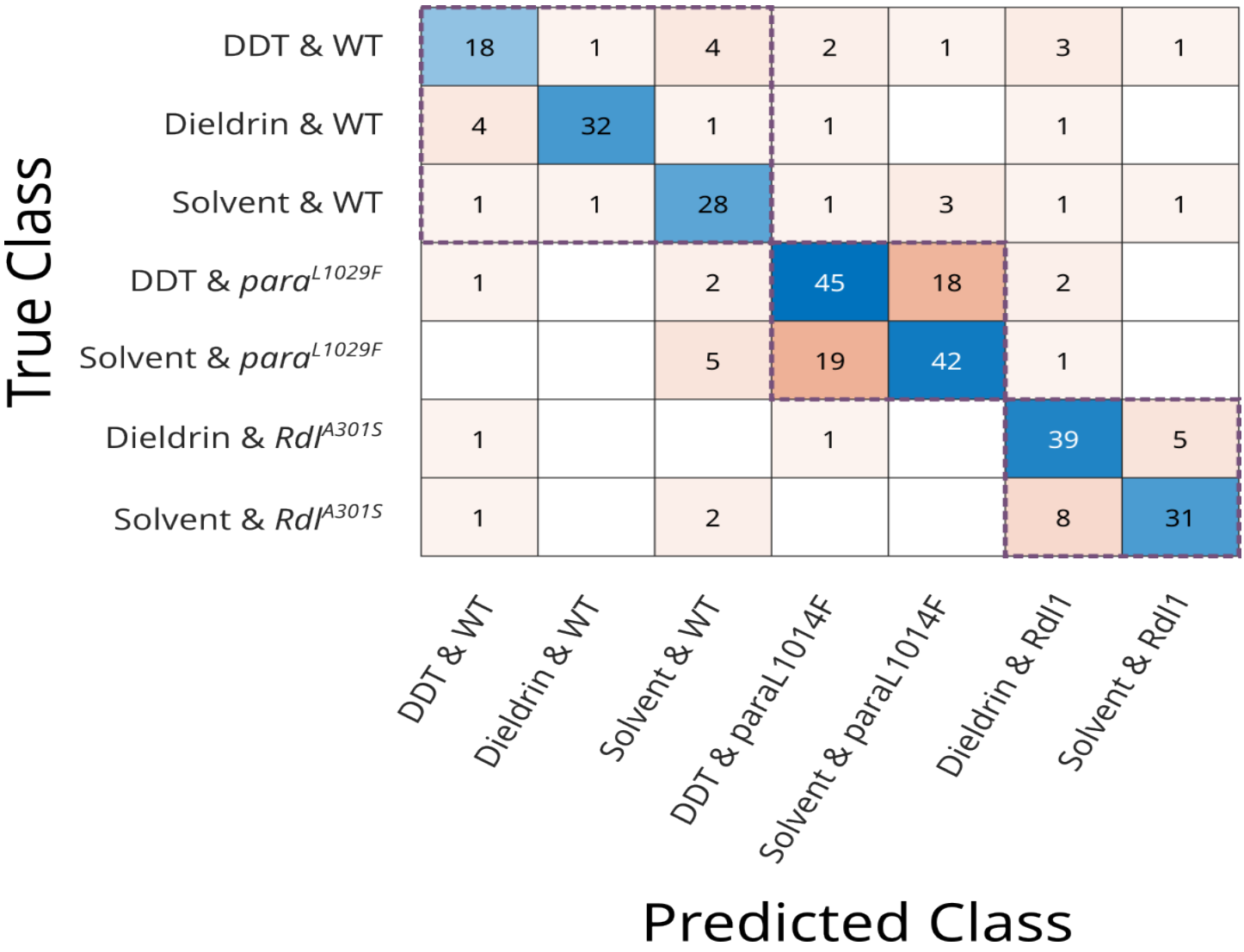


Figure 4.36: *CS or resistance mutant flies exposed to compounds* Classification matrix of movement of CS flies exposed to DDT, Dieldrin or solvent control, $Para^{L1029F}$ mutant flies exposed to DDT or solvent and Rdl^{A301S} flies exposed to either Dieldrin or solvent. Dotted lines around the boxes represent confusion within the genotype and between different compounds. Overall classification accuracy of the confusion matrix is 72.5%.

4.8.3 Summary

- Flies exposed to Pymetrozine and Dieldrin have behavioural phenotypes which are not influenced by constant darkness.
- *Rdl*^{A301S} and *Para*^{L1029F} flies, which have been reported to show resistance to Dieldrin and DDT, do not show differences in movement over the day (ZT0-ZT12).
- Time series data for Para$^{\mathrm{L1029F}}$ and Rdl$^{\mathrm{A301S}}$ exposed to solvent and DDT or Dieldrin (compounds they are resistant against), can still be classified as different by HCTSA, but accuracy was higher for the Rdl$^{\mathrm{A301S}}$ flies than for Para$^{\mathrm{L1029F}}$ flies.

4.9 Other Applications of Statistical Classification

4.9.1 Background

Through the previous chapters, I have shown how HCTSA and its sister software, Catch-22, can be used as a tool to classify time series data produced from the exposure of *Drosophila melanogaster* to different insecticidal compounds. This has been largely successful and has led to interesting discoveries in section 4.2 and 4.8. To understand if HCTSA classification can be applied to other biological questions, I utilised two different datasets of *Drosophila* time series data to explore two different questions unrelated to the previous chapter.

The first dataset was collected by a previous PhD student, Quentin Geissmann and a post-doctoral researcher, Esteban Beckwith, and used in their publication [149]. This dataset contains data on several thousand flies which were tested in ethoscopes for their sleep rebound responses based on the dynamic SD method introduced in chapter 3. In

this study, flies in different groups experienced a dynamic motor spin (over a period of 12 hours) from an optomotor after different periods of immobility, ranging from 1000s to 20s, compared to a control group which experienced no spins (slept control). The resulting rebound in the 3 hours post SD was quantified to probe differences. Using this data, I was curious to know whether the classification software was able to differentiate between flies in which had experienced different levels of SD only based on the 3 hour rebound data of velocity. Very few studies have examined the idea of how quantitative differences in SD lead to quantitative differences in rebound sleep and whether even small disruptions to sleep, like that caused by the 1000s immobility trigger (which only results in very few tube spins per night) lead to significant disruption. The ultimate question here is: does any amount of SD cause a significant change in sleep different to control.

The second dataset was collected by a fellow PhD student, Michaela Joyce. I used this unpublished data which was based on an small RNAi screen, conducted using the ethoscopes. The screen involved knocking-down different genes implicated in the regulation of learning and memory in *Drosophila melanogaster*. The screen used RNAi lines to target 6 different genes, *appl*, *dunce*, *fmr1*, *orb2*, *rutabega* and *stan*, crossed with an nsyb-Gal4 driver to knock-down these genes throughout the nervous system of the fly. The experimental lines (flies in which the genes were knocked-down) were tested to examine their sleep profiles compared to two controls: the RNAi line crossed to a wild-type line and the nsyb-Gal4 driver line crossed to a wild-type line. The data I have taken from this study is only that of the daytime baseline sleep of all the experimental lines and all the relevant controls. I was interested in using this data to determine if it was possible to classify flies with different genetic backgrounds based on their time series velocity data.

4.9.2 Classifying rebound sleep using HCTSA

The sleep data from Geissmann et al [149] is shown in the ethogram in Fig.4.37. SD was restricted to the 12 hours of the night on the second day, with the rebound period quantified for the first 3 hours of the subsequent day. A box-plot seen in Fig.38 quantifies the differences in sleep during the rebound period. This quantification shows that there is very little difference in sleep between both the experimental groups and the control group, particularly between the control group and the experimental groups from 1000s to 420s. Only after 420s does a significant increase in sleep seem to occur, but the latter 5 experimental groups (from 300s to 20s) have very similar amounts of sleep.

To understand if HCTSA could differentiate between the sleep resulting from the different SD immobility periods, I followed the same protocol described in section 4.2. The resulting data is represented in the confusion matrix (figure 4.39). The confusion matrix shows that the two groups showing the strongest classification accuracy are the control group (59%) and the group which had the most frequent immobility trigger of 20s. Aside from the group which had an immobility trigger of 300s, the other groups are either misclassified (680s, 540s,420s,220s,60s) or have a very low classification accuracy. This demonstrates two things. Firstly, that any amount of SD seems to cause a difference to the sleep that allows the time series to be classified as different to the control group. Secondly, it suggests that there is very little difference in the changes caused to recovery or rebound sleep if SD does occur.

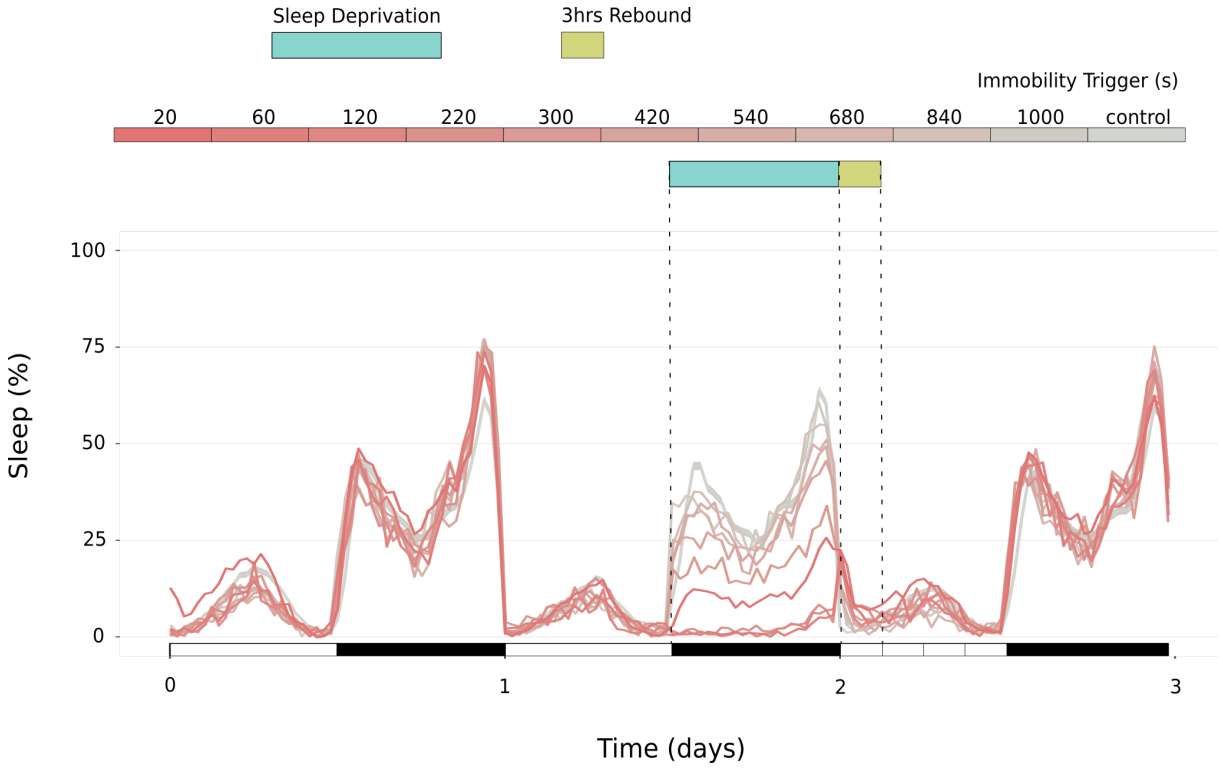


Figure 4.37: *Ethogram representing sleep for female flies exposed to different immobility triggers.* Ethograms showing sleep pattern and SD when exposed to different immobility triggers of SD. 12 hour SD period is designated by dotted lines and blue panel, and 3 hour rebound period is designated by dotted line and green panel.

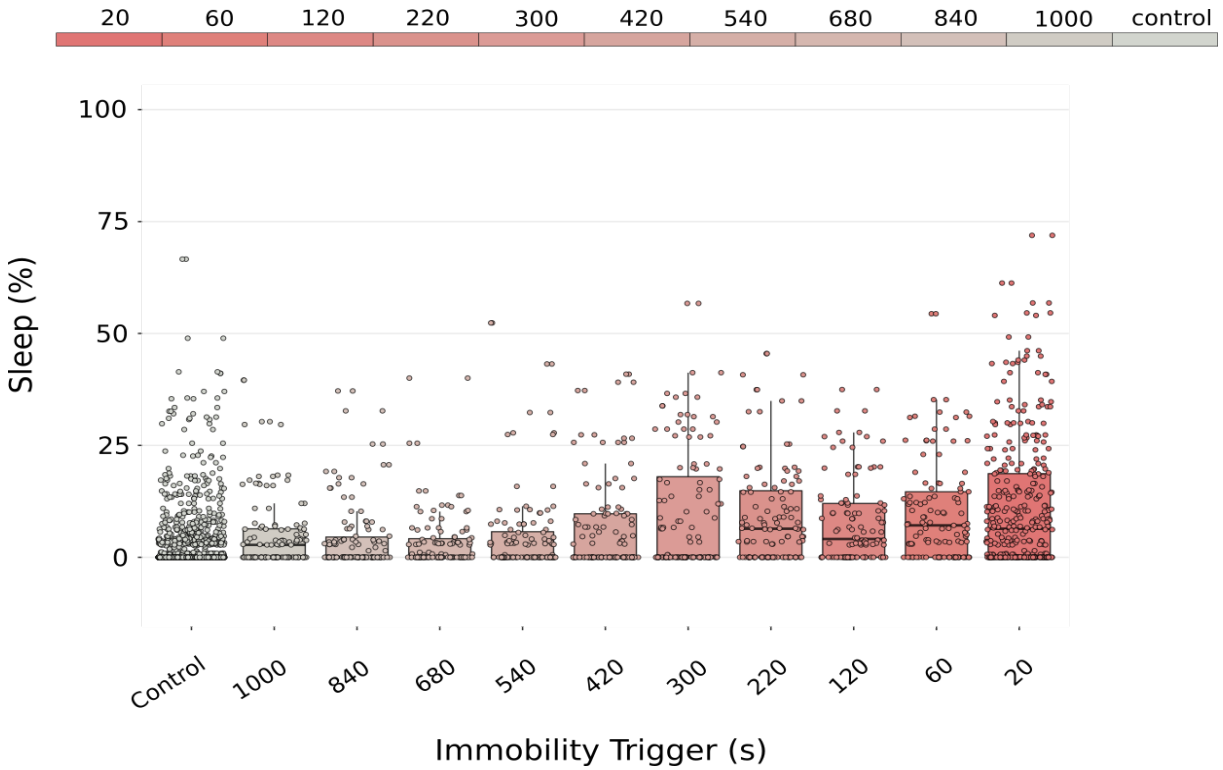
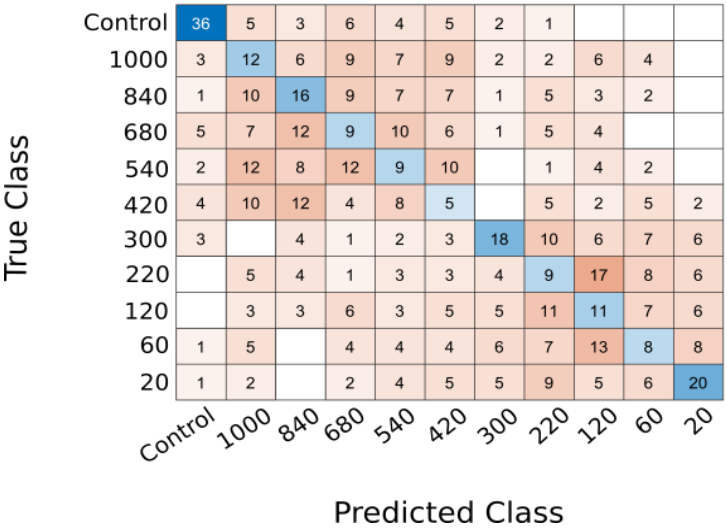


Figure 4.38: *Quantification of rebound sleep of females with different immobility triggers* Quantification of rebound sleep in the 3 hour period following dynamic SD for female flies exposed to different immobility triggers.

A



B

True Class	1st Classification	% Predicted
Control	Control	59
1000	1000	20
840	840	26
680	840	20
540	1000/680	20
420	840	21
300	300	30
220	120	28
120	120	18
60	120	22
20	20	34

Figure 4.39: *Classification of rebound sleep* **(A)** Confusion matrix showing the HCTSA classification for rebound of flies exposed to different immobility triggered dynamic SD stimuli. **(B)** Table quantifying the classification accuracy of HCTSA for different rebound periods.

4.9.3 Classifying differences in sleep based on genotype

Using the data from the RNAi screen, I first generated box plots of the average sleep of flies of each genotype over the daytime, which are shown in Fig.4.40. The data shows that the groups show varying levels of sleep. Many of the groups showed differences between each other, and many of the experimental lines showed significant differences to their parental controls (see appendix for full statistics). However, we know from previous data shown in section 4.2 and 4.3 that differences in sleep or activity data do not necessarily correlate to classification accuracy. Using the time series data for maximum velocity, I proceeded to follow the protocol explained in section 4.2 to calculate a confusion matrix for flies of the genotypes presented in Fig.4.41.

As shown in the confusion matrix in Fig.4.41, it can be seen that all compounds are classified correctly with relatively high accuracy and no confusion. What is interesting here is that for most of the parental control lines, apart from fmr1-RNAi/CS, the second classification is that of another RNAi line, rather than the experimental line.

This is also true for the experimental lines, where the second predicted classification is that of another experimental line rather than the parental control. Fmr1 here is the exception, the only line where the second classification is that of the parental controls. This possibly suggests that genotypes of flies used for knockdown experiments are more similar to other lines with knockdown than their parental controls.

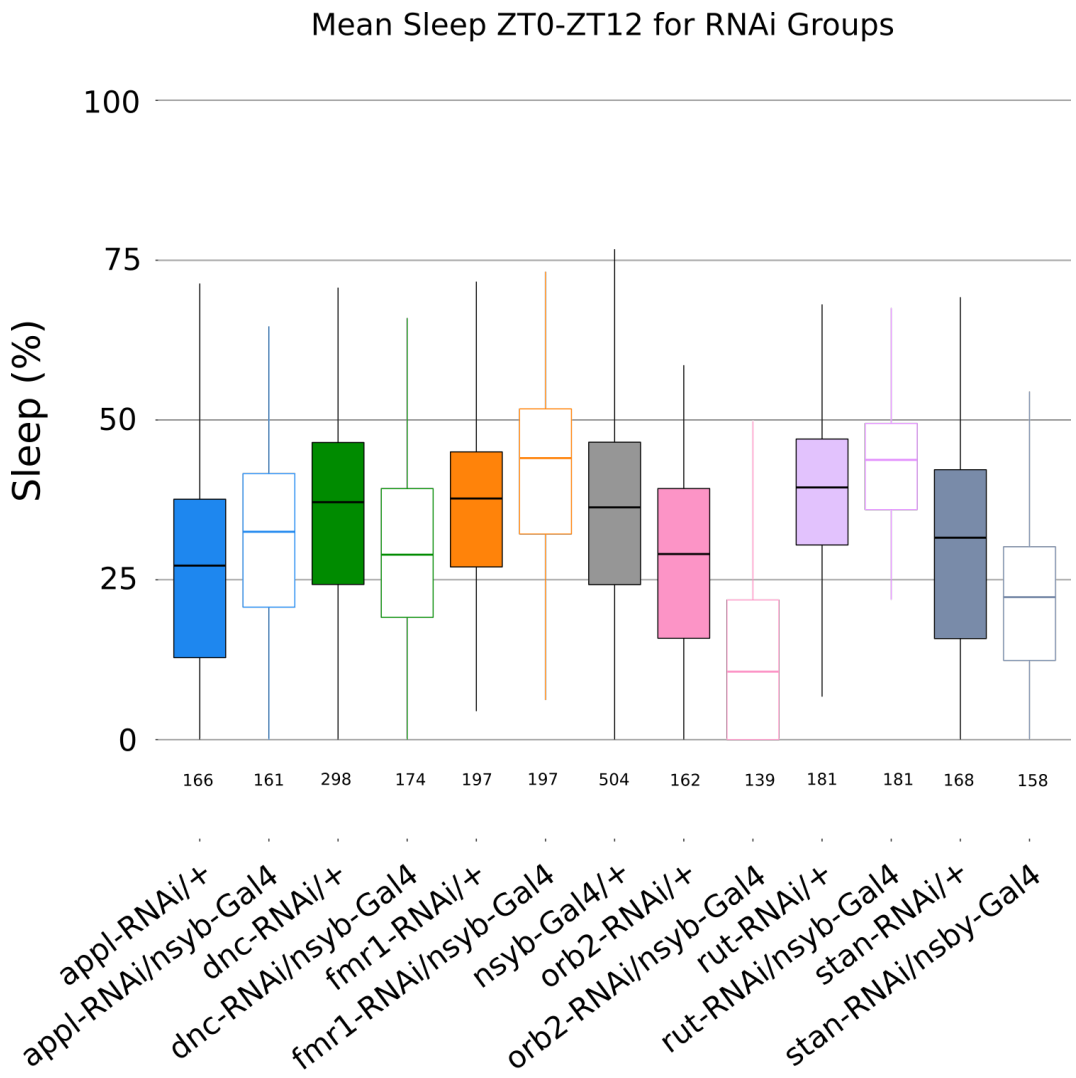
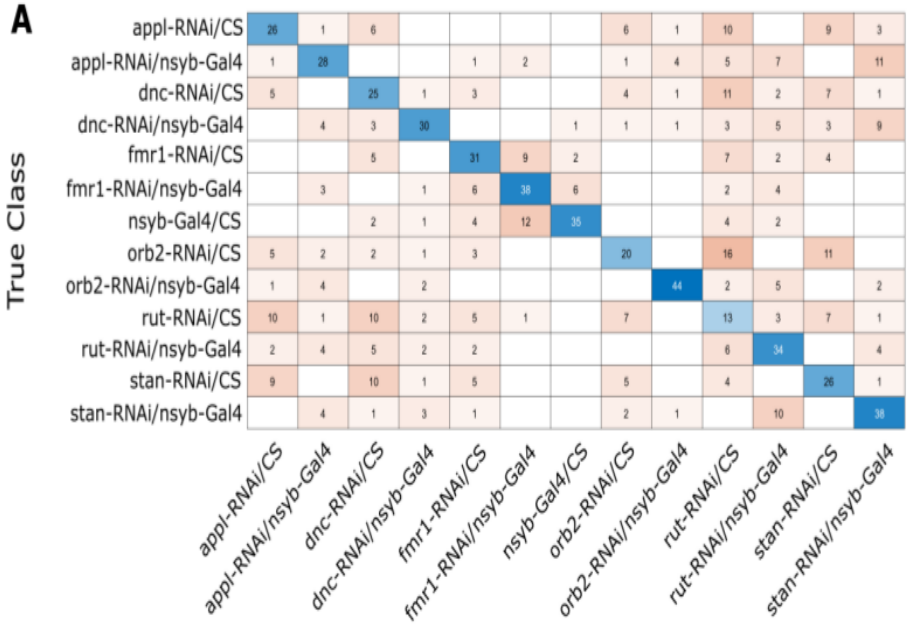


Figure 4.40: *Quantification of sleep for flies with various RNAi knockdowns and their parental controls.* Quantification of sleep amounts for average day-time sleep for flies with knockdown of various synaptic plasticity genes and their parental controls where groups were compared using a Wilcoxon rank-sum test followed by corrections with an FDR adjustment.

A



B

Predicted Class

True Class	1st Classification	% Predicted	2nd Classification	% Predicted
appl-RNAi/CS	appl-RNAi/CS	42	rut-RNAi/CS	16
appl-RNAi/nsyb-Gal4	appl-RNAi/nsyb-Gal4	47	stan-RNAi/nsyb-Gal4	18
dnc-RNAi/CS	dnc-RNAi/CS	42	rut-RNAi/CS	18
dnc-RNAi/nsyb-Gal4	dnc-RNAi/nsyb-Gal4	50	stan-RNAi/nsyb-Gal4	15
fmr1-RNAi/CS	fmr1-RNAi/CS	52	fmr1-RNAi/nsyb-Gal4	15
fmr1-RNAi/nsyb-Gal4	fmr1-RNAi/nsyb-Gal4	63	fmr1-RNAi or nsyb-Gal4	10
nsyb-Gal4/CS	nsyb-Gal4/CS	58	fmr1-RNAi/nsyb-Gal4	20
orb2-RNAi/CS	orb2-RNAi/CS	33	rut-RNAi/CS	27
orb2-RNAi/nsyb-Gal4	orb2-RNAi/nsyb-Gal4	73	rut-RNAi/nsyb-Gal4	8
stan-RNAi/CS	stan-RNAi/CS	43	dnc-RNAi/CS	17
stan-RNAi/nsyb-Gal4	stan-RNAi/nsyb-Gal4	63	rut-RNAi/nsyb-Gal4	17

Figure 4.41: *Classification of flies with RNAi knockdowns and their parental controls* **(A)** Confusion matrix of flies with knockdown of various synaptic plasticity genes and their parental controls **(B)** Table quantifying results from the confusion matrix.

4.9.4 Summary

- HCTSA classification suggests any amount of SD will elicit a response that can be classified as different to the sleep rebound of a control group of flies which have no SD.

- RNAi lines of parental control flies and those with knockdown neuronally are classified accurately by HCTSA.

- Second classification of RNAi lines showed second classification with another parental control line (for most controls) or another experimental line (for most knockdowns).

4.10 Overall Summary of Insecticide and Classification

In this chapter, I looked how behavioural time series data from exposure of flies to insecticides, from rebound data and of flies with different genotypes can be classified . I have shown:

- HCTSA can be used to classify behavioural symptoms of flies exposed to insecticide compounds at various concentrations with high accuracy, using a high-throughput methodology.
- Exposure of flies to these compounds causes a wide range of behavioural changes, which vary based on dose and are often similar between compounds which work via the same MoA.
- High-resolution time series classification is less effective than the high-throughput approach.
- Flies with resistance to DDT and Dieldrin show no changes in movement when exposed to compounds but can be classified as different when behaviour is quantified in HCTSA.
- HCTSA can be used to answer questions about sleep rebound, and help understanding as to how SD can impact recovery sleep.
- HCTSA can be used to look at differences in genetic background and help understanding in how genotypes may be similar or dissimilar.

Chapter 5

Discussion

5.1 Sleep and Rebound

5.1.1 CS females have different rebound responses to different types of sleep deprivation

As covered in the introduction, many different SD methods have been used in the literature to investigate the effects of SD in *Drosophila*, including mechanical methods, genetic methods, and those using various compounds which induce or suppress sleep. In this study, I used two different methods which have been shown to cause sleep loss. The first method, the dynamic SD method [149], uses motors in combination with the ethoscope tracking device to send a targetted stimulus based on when the fly is determined to be asleep. The second method, the starvation method, uses the loss of a nutrient source to sleep deprive the flies, and they are generally seen to preferentially remain active rather than sleeping [104], [240].

I have shown here that both mated and virgin CS females have a significant sleep rebound response following the dynamic SD method but show different responses to

the starvation SD method. When starved, all virgin females, regardless of prior social experience, show a significant rebound response which persists over the 6 hours post 24 hour starvation treatment. However, I have also shown that mated females, regardless of prior social experience, do not show a significant sleep rebound following 24 hour starvation treatment, and in some cases show a negative sleep rebound. When both SD methods are combined, despite flies losing almost their entire sleep over the 24 hour period (as they do with the dynamic method in isolation), their rebound response matches that shown following starvation SD.

This dichotomy in rebound response following the starvation SD method has not been reported before in the literature. Studies using starvation as a SD method have reported mixed results in reference to rebound. For example, some studies have reported a significant sleep rebound in flies following starvation-induced sleep loss [240], [104] and some have reported an absence of rebound [459],[53]. Donlea et al. [104] showed that this rebound response after starvation was different depending on the exact *foraging* allele present: flies with the for_{rover} allele had higher levels of PKA and as a result suppressed sleep rebound following starvation. Flies with one of the two other alleles did not. Although I did not test PKA levels in the mated and virgin groups, it is unlikely the change in rebound response is due to allelic differences, as allelic variations exist randomly throughout in the population, but it may explain the variance seen in the populations tested.

Other previous work looking at starvation and sleep showed that flies have postprandial sleep after a feeding period [320]. Flies eat more following starvation, but it is unlikely that the sleep rebound I observed in virgin females following starvation is due only to a postprandial mechanism, as it is prolonged over 6 hours and Murphy et al. showed postprandial sleep lasts around 20-40 minutes. This would also not explain why mated

females do not show a significant rebound response, as they would then be expected to show a post-starvation rebound.

Previous work has shown that the differences seen in baseline sleep amount between virgin and mated females result from the biological changes which occur following the transfer of the Sex Peptide (SP) and its receptor (SPR) to females from males during a successful copulation event: female flies mated to SP or SPR mutants fail to show a decrease in sleep following copulation [148],[211]. If these proteins are in a large part responsible for the changes in baseline sleep, it is possible these proteins, and their corresponding circuitry, could also be partially responsible for changes in starvation-induced sleep rebound. In flies, Sex Peptide Sensory Neurons (SPSNs), labelled by the *pickpocket-Gal4* and *fruitless-Gal4* lines, can sense the SP and trigger post mating behaviours [502]. However, previous work [67] [456] suggests that Crz is not expressed in the abdomen of adult female *Drosophila*. This suggests that if Crz or CrzR are involved in this change or mechanism, it is modulated via a different set of neurons, or that Crz and CrzR are involved further down in a pathway which may begin with changes in SPSNs and SP and lead to effects which signal to Crz and CrzR in the brain. Another possibility is that Crz and CrzR is involved in dietary sensing and modulation and has its effect via this pathway. Walker et al, [482], showed that the post mating response also incorporates changes in dietary and taste preference: upon mating, the nutrient requirements of the female fly change in such a way that the mated female shows a strong preference for sodium compared to her virgin counterpart. If Crz and CrzR are involved in dietary preference or metabolic changes post mating changes, this could be another possible route via which a change is induced. Further work mating females with SP and SPR mutants or looking at taste responses in mated and virgin flies (both CS and CrzR mutants) could possibly help to understand which mechanism

is at work here.

Another likely reason as to why mated females do not show a rebound response and virgin females do is likely due to starvation impacting on energy levels as well as sleep, negatively affecting egg-laying. As mated females show significantly more egg-laying than virgin females, which is negatively affected by SD [369], it is highly likely that mated females forgo a sleep rebound to feed and/or lay eggs instead. I hypothesise that dynamic SD does not affect egg-laying in the same way that starvation does. This is likely due to (1) the dynamic SD targetting only individuals which are asleep, and not disrupting behaviours during wake (2) and not impacting on energy storage levels. These reasons would also explain why simultaneous starvation and dynamic SD leads to the same rebound responses as seen with starvation.

As discussed in the introduction, DILPs (*Drosophila* insulin-like peptides) have been implicated in sleep regulation [75] and are also implicated in the regulation of appetite [407], metabolism and growth [424]. DILPs have also been shown to be involved in the receptivity of females to re-mating [491], mediate fly lifespan in response to nutrition [51] and have also been implicated as being involved in the mated female starvation-induced rebound response [53]. Although no DILP lines were tested in this work in relation to the differences in mated and virgin female sleep responses, it is conceivable that they may play a significant role in regulating this response. Mating leads to changes in feeding [474], [383], but it has also been shown that mated females have a greater resistance to starvation than virgin females due to their higher fat stores [393]. It has been shown that mutating DILPs leads to a decrease in egg-laying [511] and this may explain why Brown et al. [53] could rescue starvation-induced rebound with a knockout of DILP2. If egg-laying is one of the reasons that mated females forsake rebound following starvation-induced SD, reducing egg-laying by knocking-out DILP2

would possibly lead to females prioritising sleep over what would be a decreased egg-laying ability. To further explore this possibility, and hypothesise if it is true, using both virgin and mated mutant females for DILPs and subjecting them with the starvation-induced rebound protocol would allow explanation of this hypothesis.

5.1.2 Baseline sleep differs between CS groups with prior social experience

It has previously been shown in the literature that mated females show lower sleep amounts that virgin females [149], that socially isolated flies sleep less during the day than their socially grouped counterparts [271], [145]. Although Ganguly-Fitzgerald et al. showed that increasing the size of groups leads to increasingly decreased sleep (as a linear relationship), it has not been reported in the literature as to whether changes in baseline sleep also occur with changing the sex-ratios of different groups. I have shown in this work that female flies which had previously been paired with only one other male (Fig.3.6) show the lowest amount of sleep of all mated female groups. It is possible that this effect could be due to the reduced amount of space these flies experience, as other groups are kept in a vial prior to sleep recording rather than the glass tube. However, females which were previously in the same-sex pair show the highest level of night-sleep compared to there virgin female counterparts, and these flies had only experienced a tube environment, rather than a larger vial like the socially grouped females.

An observation which is somewhat contrary to the literature when describing baseline sleep seen from Fig.3.6 is how there are more differences during day-time sleep between flies of different social groups than between those which are of different mating statuses (i.e. between the virgin groups and mated groups). As seen from the data collected with

wild-type females, mated and virgin females show the greatest differences in sleep at night-time, while virgin females sleep a comparable amount to males and mated females generally sleep a much lower amount. Daytime sleep amounts for virgin and mated females are much more comparable and therefore other changes to sleep behaviour have greater effects and lead to changes which are more easily compared regardless of the mating status. Behaviours such as egg-laying and feeding have been observed to occur preferentially during the night phase in mated females [515], [468], which may explain why mated females reduce their sleep amount in the dark phase. Interestingly, new but data from a pre-print suggests that the timing of egg-laying may vary in mated females depending on their group size [19], with mated females in larger social groups preferentially laying in the light phase and laying more eggs more quickly, and isolated females laying in the dark phase and more slowly. The authors of this work put the change down to increased competition between females in groups. If this is true, it is possible that this group dependent change leads to a reduced sleep amount in groups where competition between females is greater, where females are preferentially egg-laying rather than sleeping. When comparing groups in Fig.3.6., we see this is somewhat true but that the group with the most females does not sleep the lowest amount. This suggests that other conflicting behaviours may be at play here. To understand in greater detail why these changes in sleep occur between the social groups, more work looking at what specific behaviours occur during the light phase for females from each group (e.g. egg-laying and feeding) would provide crucial information as to what may be prioritised over sleep.

5.1.3 Manipulation of Crz and CrzR in females leads to changes in baseline sleep

Previous work looking at knockdown or knockout of the stress neuropeptide, Crz, or its receptor, CrzR, has shown that these flies have increased survival [235],[254] and males but not females show increased locomotor activity [514]. Here, I have shown that compared to a CS control, CrzR mutants, both virgin and mated, have significantly increased daytime sleep and (for social groups apart from those with a male-skew sex-ratio) increased night-time sleep. This is a novel finding and suggests a role for CrzR in normal sleep regulation.

I also showed that knock-out of CrzR in various regions led to mating-dependent changes in baseline sleep. For virgin females, I found that flies with a knockdown of CrzR neuronally increased day and night sleep and knockdown of CrzR in the fat body led to a decrease in day sleep. For mated females, knockdown in the dopaminergic (non-PAM) neurons led to an increase in day sleep and knockdown in PAM neurons and layers 2,8 and 9 of the FSB had a decrease in night-sleep.

Interestingly, the sleep profiles for the mutant flies and CrzR knockdown lines differed in terms of baseline sleep. Knockout of CrzR led to an increased day and night sleep seen in both mated and virgin females, when compared to CS mated and virgin control groups. However, as described above, changes in baseline sleep were variable in the CrzR RNAi knock-down lines, and knock-down groups which showed changes for baseline sleep in virgin females did not show changes in mated females. This result possibly arises for several reasons. Firstly, the knock-down lines target only very specific subsets of tissue, whereas the mutants have a global loss of CrzR. This suggests that tissue-specific differences of CrzR on sleep occur depending on where CrzR may be more necessary for

sleep function. Secondly, the CrzR mutant group was compared to a CS control group when baseline sleep was quantified. As these lines are genetically more dissimilar than the controls used for each knock-out group, it is possible the genetic differences between the mutant and CS line are more pronounced than what is seen when comparing RNAi groups and their parental control lines, and, therefore, greater differences in baseline sleep are seen. Unfortunately, it was not possible to isogenise every RNAi fly line used, therefore possible differences between these groups may occur due to different genetic backgrounds.

I showed in this work that Crz knock-down in (non-PAM) dopaminergic neurons leads to a decrease in day sleep in virgin females and a decrease in day sleep in mated females with knockdown in sNPFR neurons. The *trans*-Tango data of expression of Crz and CrzR showed that CrzR is expressed in the dFSB and the MB, whilst Crz has post-synaptic connections surrounding the penduncles of the MB, suggesting neuronal connections with and expression in sleep regulatory centres. No work has previously examined the expression of CrzR or the postsynaptic connections of Crz or CrzR.

Previous work with NPF, which has also been implicated in sleep, have shown that mated males show increased levels of NPF following mating [179]. Recent data from a pre-print also shows that NPF may be responsible for the switch in feeding behaviour following mating [292]. It has been previously reported that increased activity in Crz neurons is associated with increased levels of NPF in males so it is possible that CrzR and Crz either interact with NPF or perform a similar role in post-mating changes in behaviour of females.

5.1.4 CrzR Mutants show changes in starvation rebound response, but not dynamic

When the CrzR mutant females were tested with the two types of SD, it was shown that both mated and virgin females responded in the same way to dynamic SD, both with significant sleep rebound. Similarly to CS, CrzR mutant mated females also showed no rebound following starvation-induced SD. Interestingly, CrzR mutant mated females showed increased sleep during ZT12-ZT24 of starvation SD.

Although initially (in section 3.4.3) data showed that CrzR mutant virgin females do not have significant rebound after starvation SD, further work (Fig.3.17) showed that with a larger group size the rebound response of virgins was significant, albeit smaller and shorter. This, as with other groups, was not affected by group size or sex-ratio. Several studies I mentioned in my introduction looked at how rebound is shortened or reduced with the manipulation of genes, for example, the mutants *sleepless* [247] and *fumin* [255] show no rebound after mechanical SD. One study has looked at rebound following starvation SD specifically.

5.1.5 Dopamine may be responsible for changes in sleep and rebound responses

The neurotransmitter dopamine has been implicated in the regulation of both arousal and sleep [255] in *Drosophila*. In this work, I have shown a number of pieces of evidence to support the hypothesis that dopamine may be involved in the mating-dependent changes in sleep seen between wild-type females, CrzR mutants and CrzR knockdown flies.

Firstly, I have showed that CS females respond differently depending on mating status

to dopamine modulators. CS mated females show significantly decreased day-sleep in response to 3IY, agarose & caffeine, agarose alone and sucrose when compared to a standard food control, but do not respond to L-DOPA. For night sleep, CS mated females show decreased sleep in response to L-DOPA and the agarose & caffeine group, but increased sleep in response to sucrose. CS virgin females have decreased day sleep with agar, 3IY, agarose & caffeine and L-DOPA and reduced night sleep in response to agarose & caffeine and agar but increased sleep with 3IY and sucrose.

For flies which were mutants for CrzR, mated females showed no changes in day-time sleep to any of the compounds when compared to flies on a standard food control, but did show reduced night-time sleep with 3IY and agarose & caffeine and increased night-sleep with agar or sucrose. CrzR virgin mutants showed reduced day sleep with agarose & caffeine and agar alone, decreased night-sleep with agarose & caffeine but an increased night sleep with 3IY.

In the literature, it has been shown that L-DOPA leads to an increase in dopamine which reportedly leads to a decrease in sleep over a 24 hour period [306] and 3IY leads to an increase in sleep during the daytime, specifically [8].

The CS virgin and mated groups show results which somewhat align with the literature. The CS mated group did show reduced night sleep with L-DOPA and virgin females showed decreased night sleep with L-DOPA feeding. The fact that this compound had different effects on mated and virgin females suggests that day and night sleep may be differently regulated in mated and virgin females, or it could suggest that day or night sleep, depending on mating status, is more susceptible to modulations in dopamine. This data, combined with the data from the responses of mated and virgin females to 3IY (where mated females have decreased night sleep with 3IY and virgins have increased night-sleep with the same treatment), could allow for the hypothesis that

mating status causes changes in dopamine regulation. No mention in the literature has been made to changes in dopamine responses based on the mating-status of females, but it has been shown that mated and virgin females show different nutrient preferences [383], and this circuit is modulated by dopamine [297]. The fact that CrzR mutants (both mated and virgin) did not respond to L-DOPA with reduced sleep and that only virgin CrzR mutants had increased night sleep with this treatment also suggests that these flies modulate dopamine differently. This has been previously implicated by work from Zhao et al. [514].

Knockdown of CrzR in different dopaminergic neurons led to starvation-induced rebound changes which were different in mated and virgin females. I showed that knockdown of CrzR in the PAM dopaminergic neurons led to negative sleep rebound in virgin females after starvation SD and knockdown of CrzR in non-PAM dopaminergic neurons led to a significant rebound in mated females after starvation SD. This suggests that different regions of dopaminergic neurons may separately regulate the rebound responses of females depending on their mating status'. However, the GRASP connection I showed between the CrzR neurons and PAM neurons was from a mated female brain and therefore suggests that even if the PAM neurons are not necessarily regulating the rebound response in mated females, they may still be anatomically connected. The idea that CrzR neurons can both be connected to and knocked-down in PAM neurons (leading to a change in rebound) seems counter-intuitive, as the connection would not necessarily make sense if CrzR is also expressed in these neurons. However, the PAM neurons are widely connected in the *Drosophila* brain to sleep regulating centres. For instance, it has been shown that dopamine signalling from a subset of PAM neurons to the MB contributes to wakefulness of the fly [112], that PAMs signal sugar-mediated reward to MBs [279] and that PAMs signal to the MBs to mediate the arousal threshold

of sleep [135]. As the PAM neurons clearly have several connections to and from the MB, it is plausible that several circuits may be in operation in this area, or that subsets of these regions interact in different ways to CrzR, or its knock-down.

I have shown here that knock-down of CrzR and Crz leads to changes in arousal between flies which are starved and those which are fed. Previous work in male flies has shown that *Drosophila* males which are starved show increased responses to 5% acetic acid during sleep compared to fed and sleeping counterparts [135]. In my work, I used this same protocol to test flies with Crz or CrzR knockdown. I observed that neither mated or virgin flies with Crz neuronal knock-down showed changed arousal when starved (compared to parental controls) and mated CrzR neuronal knock-down females had unchanged responses between fed and starved groups. As dopamine has been shown to be involved in arousal [135], [126], it is possible that dopamine is also involved in the mechanism for this change.

This work here provides a strong evidence based foundation implicating the role of dopamine in both mated and virgin responses to SD, and also its role in regulating sleep and starvation SD rebound responses in CrzR mutant and knockdown females.

5.2 Insecticides, Activity and Classification

5.2.1 Methodology for identifying behavioural symptoms from insecticide exposure

Understanding sub-lethal symptoms arising from exposure to insecticidal compounds has been a challenge for the agricultural industry for some time. Although many studies have tried to quantify the sub-lethal symptoms of exposure [423], [166], [91], [423]. The

pitfalls are generally that each study uses only a small number of compounds, will look at different insect or nematode species and will generally look at very specific symptoms, which are classified by a variety of different assays. In this work, I have tried to do a more comprehensive and quantitative analysis of the behavioural effects of insecticides on a single model organism, *Drosophila melanogaster*. In chapter 4, I have tried to quantify the behavioural affects of flies exposed to different compounds at three different doses. I have used two different approaches for this task. The first of which was initially established to quantify differences in sleep and wake behaviour in *Drosophila* for a different purpose, albeit one which is looking at understanding similar principles [150]. The benefits of this system were the following: (1) Flies could be tested in a very high throughput manner. As 24 flies could be tested in each ethoscope, and many ethoscopes could be run simultaneously, this approach allowed many flies to be tested with different compounds more quickly than other possible approaches. (2) An analysis platform for quantification of behaviours had already been established. The ethoscope system has already been verified as effective at classifying *Drosophila* behaviours by previous members of my lab, and has a partnered analysis system implemented in R [151] had already been designed alongside it. This made it a methodology which can be used quickly, easily and give appropriate results.

Despite the benefits of this system, it also had disadvantages, those of which I will attempt to outline in relation to those appropriate to this work. (1) The quality of visual tracking is of low resolution. Although the key regions of the fly morphology could be made out when observing flies through the ethoscope tracking system, the software is designed to only track the whole body of the fly, rather than individual body parts. (2) Only a specific predefined set of behaviours could be quantified. As seen from the work in section 4.3, only three different behaviours could be quantified

by rethomics: overall movement, micro-movements and quiescence. Some of the key symptoms of insecticide exposure are in the category of micro-movements. Despite the analysis software being able to quantify micro-movements as a whole, this is done based on the overall velocity of the fly being within a specific boundary (e.g. above a certain threshold of immobility but below that characterised as walking), rather than based on these specific movements being observed. This drawback unfortunately restricts specific symptoms, which may arise from insecticide exposure, from being identified. (3) The system requires individual flies in each well being visible to the camera at all times. Although this drawback is not unique to the ethoscope system and can be thought of as being a drawback to visual tracking as a whole, it does prevent the system from being used with other insects, such as aphids. Aphids generally live on top of or below the surface of leaves and are able to reproduce without a partner [426], leading the ethoscope system to be inappropriate for analysis with these insects.

Based on the pitfalls of the ethoscope system, I showed in Chapter 4 how I designed an alternative approach to look at behaviours in higher resolution. The superscope module, which I outlined in section 4.5 was used to record videos of individual flies exposed to compounds, with the video data then being processed through DeepLabCut [333] to track individual body parts of flies and time series data taken from the resulting individual body parts. The benefits of this system were as follows. (1) Flies were tracked in much greater detail and body parts with much greater accuracy. Having the opportunity to specify and label individual body parts of interest was extremely useful for the purposes of increasing the resolution of the tracking ability, and it gave the option for classification to be performed on a number of different body parts. (2) Each time series was much longer and more detailed. Due to the fact that the DeepLabCut software can produce coordinates for each body part for each frame, the length of

time series data was much longer and gave better resolution on the activity of the fly for time points in the millisecond range. However, despite these benefits, there were certain disadvantages of this methodology. (1) Tracking of body parts is not always as accurate as tracking the whole fly. When individual body parts are labelled and tracked per frame, the body parts sometimes move rapidly, meaning that some frames have a blurred image or one limb may be occluded by another. This often means that the only body parts which are always tracked accurately are the largest ones, including the head, abdomen and thorax. Based on this, it does not make tracking in individual frames significantly more informative than the information we get from the ethoscope tracking. (2) Time series data of the head does not classify well. Although I hypothesised that the high resolution data may be easier to classify than lower resolution, this was not the case. This could possibly be due the fact that a longer and more detailed time series actually shows up more of the individual variations in behaviour than a lower resolution time series does, or due to the fact that Catch-22 was used rather than HCTSA. Although Catch-22 has been seen to only provide a small decrease in accuracy when compared to HCTSA [286], due to the smaller set of features used, it is possible that a larger data set is needed to classify these behavioural responses accurately. (3) No custom analysis software has been standardised for DeepLabCut time series data. The authors of DeepLabCut have spent a large amount of time perfecting the post estimation software to accurately track animals in a variety of different contexts and advertise the use of the software for a number of different applications. However, due to the number of different applications the software has been used on, there is (as of the time of writing) no standardised approach to quantify behavioural outputs from the software. As designing such analysis software was outside the scope of this project, it meant that behavioural outputs could not be quantified in the same way as was the

case with ethoscope data.

5.2.2 Suggested improvements to the superscope

Although this approach did not work well, I hypothesise that various improvements could be made to the superscope system to increase the likelihood that it could be used for this type of classification. Firstly, to make the number of frames more comparable to the number that was used with time series data from the ethoscope system, a selection of frames taken from the superscope could be averaged so that a single data point could be given for a 1 or 10 second interval rather than having many frames per second. This would firstly reduce the amount of time and processing power needed to run each time series through HCTSA, whilst still giving many more data points than that collected with the ethoscope. Secondly, a more advanced strategy could be taken, where time series data from multiple body parts could be used together to generate a single time series, based on data from a number of annotated points, over time. This could help give a better picture of the movement of the fly overall, rather than just looking at the head in isolation. This approach could potentially be combined with a change in the set up of the superscope device. DeepLabCut has now been developed for 3D pose estimation (when the software is used with data taken from multiple cameras) [224]. The superscope could be modified to include a second or third camera at a different angle, giving a larger and more accurate overview of the movement of the fly, and therefore potentially improving the classification accuracy of the time series data generated.

The data used from the superscope was that of changes in positional coordinates rather than velocity data, as was used with the data from the ethoscope. Converting the positional data from the superscope to velocity data may help improve accuracy as it

may be more informative of what is happening behaviourally in terms of movements, rather than taking into account only the position of the head in space over time.
Finally, the superscope time series data could be run using the full HCTSA software rather than Catch-22, which would allow a more complete comparison of the data.

5.2.3 Symptomology of exposure to insectides: Understanding behaviours

I briefly mentioned in my introduction that one of the motivations behind this project was to try and make the process of behavioural symptomology more quantitative. This is a process which Syngenta have so far been undertaking manually when working with *Drosophila*. One of the main disadvantages of this is that the behaviours of large groups of flies cannot be readily compared to each other.
As described, I used the data from the ethoscope to classify three behavioural traits in flies exposed to each insecticide compound, compared to those exposed to the solvent control. What I observed were a number of differences between flies exposed to compounds, which varied based on dose and often between compounds which are known to have similar MoAs. One of the most striking observations was the large amount of heterogeneity in behaviours displayed by individuals exposed to compounds. Many of the flies, even those exposed to the same compound at the same dose, showed variation in their responses to the compounds. It is possible that this is one of the reasons why the classification accuracy for many of the compounds is so varied; even though the main classification accuracy is for that compound, many other compounds may also have false positive predictions. When examining changes in overall movement, micro-movements and quiescence in response to insecticide exposure, it was somewhat expected that I

would see such changes in these aspects of behaviour with these compounds. Broadly, it was shown that the effects of decreasing insecticide doses had less of an effect on behaviour than stronger doses do, and this was to be expected. This finding supports that classification of compounds at higher doses shows greater accuracy than classifying compounds at lower doses.

Albeit, there were several unexpected findings from this data. It was unexpected to find that some compounds produce changes in behaviour at certain doses, but not at other doses. For example, changes which accompany exposure at 1000ppm or 1ppm may not lead to a change at 100ppm, which is surprising. This may be due to the higher dose leading to an unspecific effect and the lower dose leading to a more specific effect. However, it is also possible that the intermediate dose may lead to a behavioural change, it may just be that the methods I have used here cannot accurately quantify what this change is.

It is also interesting to note that compounds may have more of an effect on quiescence at 1ppm than at 1000ppm or 100ppm. Quiescence is a subtle behaviour correlating to immobility and not traditionally looked at when examining changes of flies exposed to insecticides. However, as a number of these compounds used here are neurotoxins, many have been shown in the literature to produce symptoms such as paralysis. Quiescence here may be the closest behavioural proxy we have in this analysis to the symptom of paralysis, hence why this behaviour may be the next most appropriate classification.

5.2.4 Statistical Classification Approach

The work outlined in section 4.2 was classified using the aforementioned HCTSA feature based method [138], described in detail in the introduction. When compared to other machine learning approaches described, this method was chosen for the following

reasons. (1) Practicality: due to its ability to classify time series data. Time series data is the primary output of data collected by the ethoscope (see methods) and is a primary output of the pose-estimation software DeepLabCut [298]. (2) As an unbiased approach: if I had based my classification on observed and human characterised descriptions of behaviours, this would have likely resulted in attempting to define the behaviours observed by flies in a way which would have been meaningful to a human, but may not have been an accurate description of what was actually occurring. By using time series data, I could refrain from assigning meaning to observed behaviours based on pre-defined attributes of what humans can define as behaviour. (3) HCTSA combines various statistical classification approaches: HCTSA combines almost 7700 different statistical methods to provide features based on the outputs of these tests. Due to the fact that HCTSA performs many of the statistical tests which are often used in isolation, such as Fourier transformations [256], this saves time and processing power. (4) Pre-made and verified analysis approach: as one of the main objectives of the project was to collect data of flies exposed to different insecticide compounds, process this data and then analyse it, using a technique which had already been developed and verified allowed for more emphasis to be placed on the data collection itself, rather than the project time being wholly consumed by developing an analysis method.

Despite these reasons for my methodology, I do recognise that other approaches could be used for the classification analysis which would also allow for time series data to be processed and classified. Alternatives which have been previously used for other time series classification tasks are Discrete Wavelet Transformation (DWT) [367] (also a feature based approach), Hidden Markov Modelling [428] or a distance based approach such as a k-Nearest Neighbour classifier [428].

5.2.5 Classification of compounds with HCTSA

One of the pitfalls of the manual testing undertaken by Syngenta currently is that by their method, it is impossible to predict the MoA of a new compound. If a fly shows similar symptoms when exposed to compound A as a fly exposed to compound B, it may suggest there is a similar MoA, but it does not allow any form of quantitative classification.

I have shown in this work that the statistical classification software, *HCTSA*, can be used to successfully classify compounds at 1000ppm, 100ppm and (in most cases) 1ppm. From this data, we can make the following conclusions:

(1) The HCTSA software can identify flies exposed to most compounds as behaviourally distinct, when using time series data based on maximum velocity.

The HCTSA software is extremely good here at classifying compounds. However, the accuracy of the software may be due to a number of reasons. The doses of compound flies here are exposed to are likely to be much higher than those used in the field or in other studies. Each individual fly here has acute exposure to the compound both via ingestion and touch, with no ability to escape exposure due to the confined space of the individual squares.

It is difficult to find comparable reports in the literature describing the effects of insecticide exposure on *Drosophila melanogaster*. Compounds which cause changes in sleep behaviour, like those aforementioned in this work, have their effect at concentrations of 5mg/mL (where 1 mg/mL = 1000 ppm), and these are delivered via ingestion only. It is then understandable that more acute exposure at high concentrations would cause more dramatic changes in behaviour. The fact that I observed quite significant changes in behaviour when using behavioural analysis with rethomics suggests that classification with HCTSA at these high concentrations is easy for a software that is designed

to find more subtle changes in time series data.

(2) The accuracy of classification decreases as compound concentration decreases, but in some cases it increases. I showed here that for many of the compounds, classification accuracy decreases when the dose decreases (such as for Flonicamid, Spiroindoline and Cyantraniliprole). However, in some cases the accuracy of classification increased at 100ppm compared to 1000ppm. This increase in accuracy is likely due to the effects at lower doses being less unspecific and lethal, allowing more interesting and less severe symptoms to be measured. However, the accuracy of classification again decreased when comparing flies exposed to 100ppm to those exposed to 1ppm. Although it was not in the scope of this study, measuring responses to more doses would likely allow greater understanding of how symptoms can change with changing concentration.

(3) The classification accuracy depends on the number of compounds to be classified. At each point in this study when the number of compounds classified increased, classification accuracy generally decreases. This is likely due to the number of different groups between which confusion can occur.

5.2.6 Misclassification does not always occur between classes

One of the initial aims of the project was to understand whether compounds which had similar MoAs would be misclassified as each other, when misclassification occurs. Interestingly, this was not seen to be the case. For the initial 16 compounds tested, misclassification only occurred in one case. At 1ppm, Permethrin was misclassified with the Syngenta Cpd 1. These two compounds have different modes of action, with Permethrin acting on voltage-gated sodium channels [144] and Syngenta Cpd 1 acting on $GABA_A$ receptors. It is unknown why these compounds were misclassified as each other.

Interestingly, for many of the compounds, the second classification with the highest predicted class was for a compound which also had the same or a similar MoA, although this was mostly not true for the larger panel of compounds examined.

Very few studies have attempted to classify the behavioural symptoms resulting from pesticide exposure in this way, and none which have done so in *Drosophila*. One study, which used *C.elegans*, used an unsupervised machine learning model to classify behavioural symptoms resulting from exposure [303]. The authors of this study found that the model could classify nematodes exposed to compounds of the same class with a very high accuracy (88%). This model had several key differences to the model used here. Firstly, the model used in this work is an example of statistical feature based classification. HCTSA uses outputs of statistical tests performed on each time series to get an output (feature) which it uses to classify compounds. The model used by McDermott-Rouse et al. used morphological features extracted from behavioural data and then used a reduced feature set from this to perform hierarchical clustering. There are therefore many inherent differences in the two approaches, with one of the most apparent being that the features used for classification in HCTSA do not relate directly to behavioural observations. Further work in this field could look to apply a different method of classification on the data to see if the classification accuracy would be different or, if not, if it could help explain why some time series are classified so well.

5.2.7 Flies with compound resistance can be classified based on exposure

I have shown here that flies with genes resistant to Dieldrin and DDT, Rdl_{A301S} and $Para_{L1029F}$, do not show changes in movement behaviour when exposed to these in-

secticide compounds compared to solvent, but can be classified as different when run through HCTSA. There are two possible reasons for this. The first reason could be that these compounds have a second possible MoA, other than through the targets they are specifically thought to act on.

The second possible explanation is that only quantifying behavioural changes in terms of movement is not enough to quantify the overall impact that these compounds have on behaviour. Quantifying other aspects of behaviour could elucidate further effects these compounds have on overall behaviour. However, it may not be possible to fully understand the more subtle changes to behaviour that these compounds can have on resistance mutants. It may be that although these compounds do not negatively affect resistance mutants in terms of overall survival, that these compounds still produce significant changes to aspects of behaviour.

To go further with this work, different techniques, such as other behavioural assays, could be better suited to identify other symptoms arising from exposure of these mutants to the compounds. It could also be informative to use other classification techniques to see if they identify changes in symptoms and behaviours, or if there is something that the methods used by HCTSA can specifically identify.

5.2.8 Using HCTSA Classification for other purposes

In the final section of my thesis, I have shown that HCTSA can be used to answer other biological questions of interest. The work using sleep rebound data of females, which showed that only flies in the control group or those experiencing the greatest level of SD led to high classification accuracy, tells us two things. It shows that flies which undergo any level of SD, even at a level which only leads to a small disruption of sleep (as with tube spins after 1000s of immobility), have a rebound that can be classified as

significantly different to flies which have not been sleep deprived.

This supports the idea that any amount of SD can be inherently disruptive to fly sleep behaviour, even if it is only very subtle. Stronger types of SD led to a sleep rebound that is not classified as significantly different to types which are less persistent. This shows that there is possibly little difference to the ultimate behaviour of the fly due to the frequency of the stimulus. If any amount of sleep is disrupted, the rebound response will be the same.

The work which looked at classifying genotypes which either have RNAi knockdown, or are parental control lines, also yielded interesting results. This work showed that flies were more likely to be classified as similar if they have a genetic knock-down, or if they are genetic controls. Whether this classification is based on the genetic similarity of the experimental lines, which is genetically similar in sharing a neuronal promoter (with nsyb-Gal4), or due to a different reason, is unknown. However, it does raise questions as to what best genetic control lines are to use, when comparing flies with different genetic backgrounds in behavioural testing. Testing more lines with genetic knock-downs in different regions of the fly may allow greater understanding of what causes this classification.

I have shown in my work that the statistical classification method, HCTSA, has a number of different applications when used in conjunction with behavioural velocity data from the ethoscope. Although the work here covered three possible uses for its statistical classification ability, there is the potential for it to be used in a number of other ways which would extend and deepen the work presented thus far.

In relation to using HCTSA to classify behavioural data relating to insecticide exposure, many more compounds could be tested with *Drosophila melanogaster*. Although a broad range of compounds were tested, classifying compounds at the scale of hundreds

or even thousands would give far more insight as to how insecticide compounds can change behaviour and regarding potential similarities in MoA. The ethoscope device measures a number of different aspects of movement of the fly, but in this study only velocity was taken and used to classify flies exposed to different insecticides. A further study could look at using the distance moved, the angle of change of the direction between frames, or could use velocity values specific for micro-movements, walking or quiescence to classify using HCTSA. Testing flies with a larger number of compounds, at a range of concentrations and using a variety of aspects of movement could give a much better picture of how compounds produce similar or more different responses. If this pipeline was used in this way to test compounds on a much larger scale, this could possibly give insights into how flies respond to new compounds with similar structural or biological properties.

I have shown here that this software can classify resistance mutants as behaviourally different whether they are exposed to the solvent control or a compound they have been shown to be resistant to in the literature. This is somewhat surprising, as I showed that when the behaviour was analysed using the Rethomics [151] software the behaviour of these flies was not statistically different when they were exposed to the control substance compared to the insecticidal substance. If it is possible to classify these behavioural responses as different using HCTSA, it would be very interesting to see if this is possible with other flies which have been shown to be resistant to the effects of various insecticides, and prompts the question as to what 'resistance' means in this context. Although the flies may be resistant to the lethal effects of a compound, it suggests that they may not be behaviourally resistant. Future work looking at identifying these behavioural changes and how it may impact other aspects of the insects behaviour would be insightful in the understanding of what these resistant

genes change in the organism and if there is a possible trade-off to avoiding lethality. Research has shown that as flies age, their behaviour, including locomotor activity, metabolism [347], reproductive ability [392], [71] and plasticity of neurons, changes [80]. Conversely, younger flies have been shown to sleep longer [239] and have reduced locomotion [100]. As the flies I tested in this study were quite young (2-5 days old), repeating the work in older flies could help to understand if classification accuracy for flies is maintained over their lifespan or if this gets more difficult as flies age and change their behavioural habits.

5.3 Conclusion

In this thesis, I have presented work showing how two topics relating to dichotomous behaviours, activity and sleep, in *Drosophila melanogaster* can be examined using similar methods to answer different biological questions.

I first presented my work investigating at how sleep homeostasis can be modulated in various contexts. Here, I examined how differences in two types of SD (starvation and dynamic) can lead to different sleep responses, and how this is further modulated by changes in past experience, including those of mating and social grouping. I then went on to look at how genetics may have an impact on these responses. For this, I examined the neuropeptide Crz, and its receptor, CrzR, which I then implicated in having a role in both regulating the baseline level of sleep and in the response to the two types of SD I chose to use. From this, I attempted to elucidate the mechanism through which CrzR has its effect on sleep and discovered that this mechanism likely occurs through the modulation of dopamine. Future work will further clarify the specifics of this mechanism and the circuitry through which these effects are produced.

In the second part of my work, I went on to present a pipeline for the high-throughput statistical classification of *Drosophila* behavioural time series and compared this to a pipeline using pose-estimation tools for a high-resolution approach. This first method by far outperformed the latter in the ability to classify phenotypes of flies exposed to a range of different insecticidal compounds. These behavioural responses could also be analysed using more basic comparisons of movement, micro-movements and quiescence in an attempt to more deeply understand the phenotypes resulting from exposure. Further to this, I demonstrated how the classification of behavioural time series using my high-throughput pipeline can also lead to the ability to quantify data from other biological areas, including sleep research and genetics. I have presented here an approach which is highly adaptable and has the potential to be used for a number of different applications where there are questions around biological classification.

Overall, this work has emphasised the ability of current quantitative tools which are available to allow for more accurate descriptions of behaviour.

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