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The relationship between aging and sleep quality in Drosophila

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Thesis

THE RELATIONSHIP BETWEEN AGING AND SLEEP QUALITY IN DROSOPHILA

by

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ABSTRACT

Background: Current research regarding the impact aging has on characteristics of sleep suggest that over the course of one's lifespan, the quantity and quality of sleep declines. Sleep quality is a measure of the extent to which sleep is consolidated- the less number of brief awakenings one experiences during a period of sleep, the better. The extent to which degradation of sleep quality may impact overall health and increase susceptibility to age-related diseases is currently unknown, nor is the mechanism that mediates sleep fragmentation and consolidation understood.

Objective: The purpose of this study was to ascertain if the increased sleep fragmentation experienced with age is due to a decreased arousal threshold towards external stimuli. A decreased ability to inhibit sensory processes during sleep could potentially trigger a greater number of brief awakenings and negatively impact sleep quality.

Methods: Various age groups of inbred wild-type genotypes of *Drosophila melanogaster* had arousal threshold tested during the night using mechanical stimuli to see if the older flies were more likely to be woken up. Sleep characteristics at baseline and after the arousal assay were compared to observe any impacts aging has on the ability to recover from a mild sleep deprivation as such.

Results: I observed an increase in arousal threshold with age; older flies were less likely to be aroused by the presentation of the mechanical stimulus. Arousal threshold findings were consistent between sexes but not between genotypes. It was noted that the degree to which aging impacted arousal threshold was affected by the expected lifespan of a genotype. In terms of sleep characteristics measured outside of the arousal assay, I noticed an increase in quantity of sleep and decrease in activity as flies aged, including a greater reliance on day sleep. This was further reflected by a decrease in rebound sleep after the arousal assay was performed. More specific sleep architecture characteristics such as bout number and bout length were greatly impacted by both sex and genotype.

Conclusion: Our results were greatly unexpected in comparison to previous studies, especially in regards to older flies having an increased arousal threshold and an increased quantity of sleep. This is not conclusive, however, as previous studies have shown that the saliency of the stimulus presented may prove important, especially when considering the internal state of the fly. Instead of solely focusing on if older flies are more or less easy to wake up with the application of an external stimulus, it may instead be beneficial to also consider their ability to discriminate between salient stimuli while quiescent.

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LIST OF ABBREVIATIONS

ANOVA …..……Analysis of Variance

ARAS …….…….……..…Ascending Reticular Activating System

ATP ……...…….Adenosine Triphosphate

CDC ……...Center for Disease Control

CS ……...Canton S

DA ……Dopamine

DAM ……...……Drosophila Activity Monitor

EEG ……...Electroencephalogram

GABA ……Gamma-aminobutyric acid

HPC ……...Hypocretin-producing cells

Hsp70 …….Heat shock protein 70

LD ……..Light: Dark

LDT ……...Laterodorsal tegmental nuclei

MCH ……Melanin-Concentrating Hormone

NREM ……..Non-rapid eye movement

PPT ……...Pedunculopontine tegmental nuclei

REM ……Rapid eye movement

ROS ……..Reactive Oxygen Species

TMN ……Tuberomammillary nucleus

VLPO ……Ventrolateral preoptic area

INTRODUCTION

Sleep is defined as a "tightly regulated, reversible state of quiescence" [1], characterized by an inhibition of movement, increased arousal threshold, and changes in electrophysiological cerebral activity.

Sleep is regulated by processes under a "two-process model": the circadian clock, which integrates the 24-hour periodicity of the day with sleep behaviors, and homeostatic mechanisms, the compensation of sleep after a period of some substantial sleep loss [2].

As we age, we become increasingly susceptible to the acquisition of sleep disturbances that leads to daytime fatigue [3].Despite how integral sleep is to our everyday lives, the effects of its dysregulation are often minimized or overlooked. The extent to which accumulation of sleep loss and poor sleep quality over time can damage the body is an unknown researchers are still pursuing.

Why Do We Sleep?

We are generally advised to get at least 8 hours of sleep per night, meaning that it is optimal for us to spend at least a third of our lives in dormancy. In the world of nature, this would leave us severely vulnerable to predators- so our continued reliance on it signifies the evolutionary necessity for adequate sleep [1].

Current leading hypotheses suggest that sleep performs restorative functions in the body, of note:

1. The replenishment of energy stores that were used up whilst awake [4].

2. Aiding the removal of the harmful by-products accumulated from metabolic processes performed whilst awake [5].
3. Supporting the neural plasticity characteristic of memory and learning consolidation [6].
4. Aiding in the maintenance of thermoregulation [7].

All of which have been observed on a correlational basis within various separate experimental studies: Animals that were more physically active during wakefulness were shown to have slept more later in proportion to the amount of activity [4]; in organisms that were deprived of sleep, increased accumulation of harmful metabolic byproducts like reactive oxygen species in the brain and gut have been observed [5,61], as well as a decline in cognitive function and consolidation of memory in both humans and animals; in learning assays, animals that had performed worse were recorded as having poor sleep the night after initially learning the task [6,8] . Additionally, humans that suffered sleep loss were more susceptible to heat loss, where their body's ability to recover warmth after being exposed to cold was impaired [7].

In order to best prevent disruption to sleep and understand its potential harm to our health and well-being, we must first study the physiological changes associated with sleep.

The Electrophysiology of Mammalian Sleep

Although sleep behavior itself is outwardly dormant, sleep itself is anything but stagnant. It proceeds through stages in a cyclical manner that are thought to each indicate

a different function being performed during that time [9]. By observing brain activity measured by an electroencephalogram (EEG), we can reliably indicate what stage of sleep a normal individual is in [9,10].

An EEG works by detecting positive and negative postsynaptic potentials from cortical neurons through nodes affixed to one's scalp, thereby allowing us to observe and quantify the electrical activity of the brain [11].

Sleep initiates with "non-rapid eye movement" (NREM) stage 1, denoted as N1, and progresses cyclically through N2, N3, N4, and then enters rapid eye movement (REM) sleep before restarting. The amount of time spent in each stage changes throughout the course of the night and varies with age [12]; generally as sleep progresses, REM will increase and eventually become the longest stage. - Overall, NREM sleep constitutes 75-80% of total sleep whilst REM sleep makes up the remaining 20-25%. After the onset of sleep and passage of the first cycle, the rest of the cycles that occur will last approximately 90-120 minutes in length.

Each sleep stage has a predominant waveform that allows one to distinguish it from the others- which also suggests that during each the brain is performing distinct actions [13]. As NREM sleep progresses through its substages, the individual will become harder to wake (also known as having an increased arousal threshold), and the waveforms will change from the high frequency and low amplitude characteristic of wake to low frequency, high amplitude rhythmic patterns characteristic of waveforms present in N3 and N4, also known as slow-wave sleep [13,14]. Additionally, the presence of

NREM-specific waveforms will begin to appear, namely sleep spindles and K-complexes.

It Is hypothesized that sleep spindles are important for memory consolidation- individuals that had learned a new task earlier that day had a significantly higher density of sleep spindles present during their sleep compared to those who had not [12,15]. These spindles form from the activity of neurons that discharge rhythmically; on an EEG [16], they appear as a burst of fast activity that will increase and decrease in amplitude similar in appearance to that of an eye.

K-complexes are characteristic of NREM sleep, more specifically stage N2 [17]. They are thought to reflect the sensory processing of external stimuli when one is sleeping- previous studies have observed them as large, distinct waveforms that will often present in response to environmental stimuli [18].

REM sleep is the stage most associated with dreaming. It is generated in the pons and characterized by its namesake- the eyes moving rapidly side-to-side while remaining closed. In this stage, the individual's EEG will more closely reflect that of wakefulness [18], and they are more easily woken up. Likely to prevent "acting out" one's dreams and risk harming themselves, voluntary motor functions in the limbs are inhibited upon the onset of this stage [19]. If this is disrupted, individuals may experience sleep behavior abnormalities such as somnambulance or sleep-talking [20,21].

The Neurobiology of Sleep

Sleep, unlike many other physiological functions, is not largely confined to one region of the brain. Instead, multiple systems will converge onto common effectors-regions within the thalamus and cortex, in order to mediate sleep behaviors [22]. **Table 1** summarizes key areas involved with the regulation of sleep and wake. Using Fos protein expression, studies have been able to identify a number of regions that become maximally active during sleep [22,23], allowing researchers to begin to examine and understand the molecular mechanisms and cellular interactions that take place during different sleep stages.

The neurons of the ventrolateral preoptic nucleus (VLPO) are often considered the switch that initiates the changes in cellular activity associated with sleep and wake. Previous studies were able to use an anterograde tracer to confirm its descending innervations in prominent arousal promoting regions, such as the tuberomammillary nucleus (TMN), the dorsal raphe nucleus, and the locus coeruleus [22]. VLPO neurons have been found to contain both gamma-aminobutyric acid (GABA) and Galanin, both of which have inhibitory effects on the TMN and locus coeruleus, suggesting that VLPO is sleep-promoting in nature via the inhibition of the arousal system [23]. This is further supported by VLPO neurons being maximally active during sleep, especially during recovery sleep.

Interestingly, the projections from the VLPO are reciprocated by its targets: the TMN with histamine, locus coeruleus with norepinephrine, and dorsal raphe nucleus with serotonin all inhibit the VLPO via axonal innervation. Their firing is maximal during

wake, decreases in NREM, and is minimal during REM. Their projections ultimately diffuse across the cerebral cortex and they are considered the hypothalamic branch of the ascending reticular activating system (ARAS) [22].

Studies lesioning the VLPO to observe effects on sleep found that this area may have functions that affect REM and NREM sleep differently based on specialized subregions. Lesioning of its dense cluster of nuclei mainly decreased NREM sleep, whilst lesioning of more diffuse axonal extensions medially and dorsally impacted the quantity of REM sleep.

The extended VLPO also has inhibitory projections in the pedunculopontine tegmental nuclei (PPT) – laterodorsal tegmental nuclei (LDT) in the pons and midbrain, respectively. The PPT-LDT contribute to the ARAS via cholinergic projections towards the thalamic region, and are activated during both wake and REM, due to the release of tonic monoamine inhibition. Similar to how VLPO projections don't actually contact the cholinergic cell bodies directly, it is hypothesized that the PPT-LDT inhibits the VLPO via some downstream target [22].

It is hypothesized that these systems, VLPO and ARAS, work against each other to promote the maintenance of the current state of consciousness. During wake, the ARAS inhibits the VLPO, and the cessation of inhibition that causes further enhances ARAS activity. The reverse would also be true for the VLPO during sleep. It is hypothesized that some other building homeostatic drive would eventually overcome this inhibition and allow the other to become active [24]. Of note, damage to either system

would also damage this self-reinforcement and would likely cause more frequent shifts between sleep and wake rather than prolonging one state over the other.

The orexin/hypocretin neurons in the lateral hypothalamus are also greatly important for sleep to wake regulation, although the exact mechanism by which it does so is relatively unknown. They are maximally active during the day, and the number of active neurons correlates closely with the ability to maintain wakefulness- a major loss of these hypocretin cells (HPCs) produces sleep dysfunction similar to the presentation of narcolepsy, causing inappropriate sleep to wake transitions [24,25,26]. HPCs may therefore have some influence in further promoting the stability of the wake state. Administering hypocretin near the VLPO promotes wake, although VLPO neurons have not been found to have any hypocretin receptors- it is then currently hypothesized that it may act to inhibit VLPO via some presynaptic action as it does have projections to both the monoaminergic and the cholinergic ARAS neurons.

Sleep to wake regulation is not limited to this system alone. Although we know less about its mechanism in sleep processes, dopamine (DA) has clinically been associated with promoting wake [27]. For example, sleepiness and fatigue are common in those with Parkinson's Disease, where there is a loss of DA-producing neurons [21]. Additionally, medication that disrupts DA release or reuptake often produce side effects like sleepiness, such Modafinil. Conversely, many well-known stimulants such as amphetamines work by prolonging DA reuptake via the disruption of the DA transporter [28].

There are also some links to physiological stress promoting sleep via natural somnogens [29]. Melanin-concentrating hormone (MCH), involved with energy regulation and food intake [30], is produced by REM-on neurons in the lateral hypothalamus that innervate similar regions as HPCs but promote inhibition of arousal systems. Adenosine acts as a marker for our cells ability to produce ATP [4]; high concentrations signal some form of metabolic challenge, disinhibiting VLPO neurons and promoting sleep. It increases with sleep deprivation and will decrease with recovery sleep. Lastly, Immune system signaling molecules such as cytokines are also associated with the promoting of sleep, specifically NREM sleep [31]. Studies that administered interleukin-1ß and tumor necrosis factor-α into the preoptic area saw a reduction in REM-off neuron firing and an increase in NREM sleep [23].

Brain Stem	**Dorsolateral Pons**	Controls the generation of REM sleep.
	Ventral Medulla	Initiates sleep-associated atonia.
	Locus Coeruleus	Regulates REM sleep generation in relation to GABA present.
	Laterodorsal Tegmental Nucleus	Related to the maintenance of sleep states. Damage can cause increased fragmentation but also increase REM sleep.
	Periaqueductal Gray	Promotes cortical activity. When damaged, REM duration and number of bouts increase.
Hypothalamus	**Ventrolateral Preoptic Area**	Promotes REM and NREM sleep. Damage causes fragmentation and a decrease in both sleep stages. The amount of damage in the core is correlated with the amount of NREM sleep loss, while the same is true for REM sleep loss in the dorsomedial region.
	Suprachiasmatic Nucleus	Responsible for circadian rhythms. Related to amount of REM in the light sleep stage.

	Median Preoptic Area	Mediates sleep debt, sleep coordination, and thermoregulation.
	Dorsomedial Hypothalamus	Responsible for circadian rhythms involved with sleep/wake.
	Orexin/Hypocretin	Promotes sustained wakefulness. Damage to this area produces narcolepsy, potentially with cataplexy.
Forebrain	**Basal Ganglia**	Involved with sleep consolidation and the transition between sleep and wake pertaining to electrophysiology.
	Substantia Nigra	Promotes sleep induction and maintenance. Damage induces insomnia.

Table 1. Cerebral Regions Responsible for Sleep/Wake Regulation. A large portion of these make up the Ascending Reticular Activating System, , a collection of monoamine cell groups in the pons and midbrain that project to the cerebral cortex, hippocampus, hypothalamus, amygdala, and thalamus in order to regulate sleep and wakefulness [32].

Sleep/Wake Dysfunction with Age

Previous studies have shown that as we age, we experience alterations to our circadian and sleep-related behaviors, mainly:

1. "Phase Advance", the change in the adults' internal clock that promotes a shift towards going to bed and waking up earlier [33,34].
2. Increased sleep fragmentation [35].
3. Decreased NREM stage 3 and 4 sleep [36,37].
4. Decreased ability to recover sleep [7, 38]

Some may even experience "sundowning", the phenomena where elderly patients exhibit periods of uncharacteristic disruptive behavior- most commonly in the evening- such as agitation, aggression, confusion, paranoia, or disorientation [21].

With age, we are also more likely to experience disease or medications that may promote sleep dysfunction. Per a 2015-2016 CDC study, 85% of adults aged 60 years or

older had used prescription drugs within the previous 30 days; in comparison, only 45.8% of the US population had done so [39]. This increased intake in drugs is compounded by the age-related decline in liver function, pronouncing their stimulant effects.

In even healthy humans, aging is reflected externally with wrinkles, hair color, musculoskeletal weakness, etc. These are considered normal, albeit unwelcome, even though they represent failings of the body to maintain itself as it once was able to. This is reflected internally as well, with decreased immune system production, decreased dopamine, dysfunction with insulin, cholesterol, and more that all contribute to increased risk of cardiovascular diseases, stroke, cancers, and overall decrease in organ functioning.

Although sleep dysfunction in these circumstances is often seen as being secondary towards other degeneration as a result of normal aging, the extent to which poor sleep compounds these issues and accelerates other disease is unknown. A previous study by Vaccaro et al., 2020, that observed the relationship between sleep and the gut's ability to clear harmful metabolic byproducts, showed that in flies that were sleep deprived there was an increased accumulation in reactive oxygen species (ROS) in the gut prior to death [5]. ROS and its contribution to cell senescence and aging is a widely considered topic; it is no longer a simple byproduct of cell metabolism but is believed to be important in the signaling and regulation of normal physiological functions [40-43].

Whether or not its imbalance directly or indirectly impacts cell functioning, multiple studies have linked ROS to a cell's susceptibility to stress [44]. In other words, although degeneration and dysfunction is tied with normal aging, rendering older

populations more vulnerable to diseases that affect sleep, the lack of sleep itself can only exacerbate this.

Sleep Disorders that Increase in Incidence with Aging

Insomnia

Insomnia is defined as an insufficient quantity or quality of sleep, characterized by difficulty falling or staying asleep. It can present on its own, or as a symptom from other external factors [45].

Those with insomnia may complain of issues including daytime drowsiness, lethargy, or even cognitive difficulties. It is especially prevalent in adults over the age of 65, with 50% experiencing occasional incidents and 10-15% experiencing chronic insomnia [21].

Although there are genetic influences towards the development of insomnia in adults, other factors such as shifts in work schedule or stress levels contribute largely. Studies have observed a greater prevalence and genetic link within older female populations than males, but this correlation may be related to sleep dysfunction secondary to post-menopausal hormone changes, such as estrogen deficiency [62].

Positron emission tomography (PET) imaging studies have observed altered brain activity in those with insomnia: notably the lack of normal sleep-induced reduction of glucose consumption in regions such as the anterior cingulate cortex, the medial prefrontal cortex, and other limbic or arousal systems [6]. Furthermore, an insomnia model involving a cage-change stressor noted Fos protein expression in both arousal and

sleep-promoting regions, suggesting that a better approach to alleviating insomnia is not to promote sleep, but to inhibit wake [1].

Other Common Disruptive Sleep Disorders

Typically, during REM, the body's voluntary muscles are inhibited in order to prevent the "acting out" of our dreams [19]. During "REM-sleep behavior disorder", however, affected individuals lose this muscle atonia and may engage in movement ranging from thrashing about to walking around or eating [21]. Disorders such as this one can cause those affected to feel tired during the day despite having obtained a good amount of sleep at night.

Other such disorders include obstructive sleep apnea, the relaxing of the tongue and palate during sleep that block the airway, causing dyspnea that can cause one to "jerk" awake in order to catch their breath; Periodic Leg-Movement Disorder a motor disorder where patients will flail their limbs about during sleep; Restless Legs Syndrome, those afflicted experience a sensation in the legs that causes the irresistible urge to move or massage them. All of these will present with variable dysfunction to sleep and are often overlooked when determining causes for poor sleep quality causing daytime fatigue. The presence of disorders like these usually mean that there are defects in other body systems- often in regard to degeneration related to normal aging- that by association produce sleep-disturbing symptoms. [21].

Drosophila Melanogaster Model For Sleep

The usage of an appropriate animal model is essential towards the reliability of research as it is applied toward human systems. In observing other organisms, we use a set of behavioral criteria in order to identify sleep:

1. Evidence of circadian-regulated periods of quiescence [2,38].
2. A temporary increase in arousal threshold [2].
3. Evidence of a homeostatic component where the organism will attempt to regain sleep after deprivation [46].

Drosophila melanogaster is frequently used in research due to their abundance and short lifespan. Despite their appearance, they share many human gene orthologs- since its simple genome is well characterized, it is a useful tool to observe features of specific genetic mutations[1,2].

The arousal threshold of sleep during the day is lower than that of sleep during the night [1]. In flies, there are sex differences in circadian sleep and activity behaviors. When in 12 hour light:dark (LD) conditions, both sexes are highly active at circadian time (CT) 0 and CT 12 (dawn and dusk); females generally sleep less during the day, whilst males sleep about as much during the day as they do at night.

Unfortunately, we cannot observe brain activity in *Drosophila* in the same electrophysiological manner that we can with mammals; however, their sleep can be quantified by using measurements of activity. In the comparison of sleep in mammals and *Drosophila*, both experience recovery sleep after deprivation, observed as periods of sleep longer than baseline with fewer brief interruptions of wake [1].

The sleep of *Drosophila* is also affected by exogenous substances similarly to mammals. Studies that fed flies caffeine, a stimulant that works by inhibiting sleep-promoting systems, saw an increase in wakefulness [63]. Experiments where flies were given antihistamines, which work by inhibiting wake-promoting systems, saw an increase in sleep [64]. This suggests that flies have wake and sleep promoting systems that are at least pharmacologically similar to those in mammals [1,47].

The Present Study

It is of note that many experimental studies suggest that aging is associated with a decrease in total sleep. A 2020 USA national health interview survey reported that individuals above the age of 65 were the population most likely to sleep 8 or more hours each night despite their complaints of fatigue during the day [48,49]. In these cases, if sleep quantity is not the main issue, then perhaps it is decreasing sleep quality that has a greater effect on restfulness.

Sleep quality is measured by the number of times one wakes up during the night, and the amount of time within each bout of sleep- generally it is considered better to have greater bouts of sleep with less interruptions of wake, also known as having a greater sleep consolidation [50]. In this study, we would like to see whether or not the increased sleep fragmentation that occurs with age is due to a decreased arousal threshold- as in, if older populations are more sensitive to external stimuli during sleep causing a greater frequency of brief awakenings.

DESIGN AND METHODOLOGY

Fly Stocks and Rearing Conditions

Multiple genotypes of wild type *Drosophila* at 25°C 12 hr LD were tested: Iso31, white-118 (w118), Canton S (CS), Florida 9, Berlin K, Oregon, and Hikone. Additionally, Iso31, w118, and CS were separated into two different groups based on how they were obtained, which is why they will be referred to as either iso31 or $iso31^{KK}$; CS or CS^{FR}; w118 or $w118^{RJ}$.

Previous studies have noted that results from cross-sectional assays mirror longitudinal assays [8]; due to wanting to see if mating status or rearing group may affect results, a cross-sectional paradigm was selected. Groups of flies from each genotype would be collected and set aside until they reached a specified age. They would be split into groups of approximately 20 to 25: “Virgin” males (as in, separated shortly after eclosure. Previous sexual contact was not considered); Mated Males and Mated Females (collected together but separated approximately 5 days later in order to ensure that females had mated); and females and males that were kept together until they had together reached the age for experimentation.

Virgin females were obtained by crossing wild-type flies with a stock that contains a hsp70 promoter that drives expression of the hid protein, an apoptosis activator, in the Y-chromosome; creating progeny that are denoted as virginators. Virginator stocks were kept at 21°C in order to prevent leaky activation of the hsp70 promoter prior to collection. After parent flies were removed, larvae were exposed to a

45-minute heat shock using a water bath at 37^{o}C in order to eliminate all males, preventing the females from mating prior to collection. After collection, virgin females were aged at 25^{o}C along with the other groups.

Flies were grouped by age in stages of 10: from collection (day 1) to day 10, 10-20, 20-30, and so on until age 80 days. The genotypes used have differing life expectancies; therefore collection had to be halted at a younger age for some.

Sleep Recording

Fly activity was assayed using the Trikinetics "Drosophila Activity Monitor System" (Waltham, MA), specifically the "DAM2 Drosophila Activity Monitor", which measure locomotor activity by recording interruptions in infrared beams that bisect each of the spaces where tubes contain the flies. Using minimal CO_2 anesthetic, flies were loaded individually into 5 mm diameter 65 mm length glass tubes filled with approximately 40 mm of cornmeal-agar food. Cotton plugs were used to prevent escape and to limit the amount of space the flies could move to about 25 mm.. and moved to an incubator at 25 C with a 12-hour LD cycle. In order to let flies recover from the anesthetic, no data was collected for the first 24 hours. Then, fly activity was recorded for at least 24 hours prior to the 10-hour arousal threshold assay, and recorded for another 24 hours afterwards. Sleep was defined as 5 or more consecutive minutes of inactivity [51,52,59,63]. Resulting data was collected using MATLAB2014a software (MathWorks, Natick, MA) via a program that would integrate MATLAB inputs with DAMs locomotion data

(https://github.com/IrisTitos/Atanalysis/blob/master/ReadDamsPullDown, https://github.com/ArousalThreshold/Software). Further analysis was performed by a script that applied the locomotion data over the time period the experiment was performed in order to calculate sleep and activity measurements (https://github.com/CrickmoreRoguljaLabs/SleepAnalysis) as described in the protocol by Titos et al., 2023 [51].

Mechanical Arousal Threshold Assay

After 24 hours collecting baseline sleep, flies would be introduced to intermittent mechanical stimulation using an automatic speaker paradigm programmed in MATLAB as detailed in Titos et al., 2023 under the name Good Morning that delivers vibrations (https://github.com/IrisTitos/Atanalysis/blob/master/Goodmorning.md) during the next 12-hour dark period, 1.5 hours after incubator lights were turned off. The flies would experience a 1.0V mechanical stimulus lasting 2 seconds at a random point every 30-40 minutes. Their activity was recorded in order to identify the arousal effect of the stimulus. Flies that had shown activity 10 minutes prior to the stimulus as well as dead flies were excluded.

Table 2 defines each measure returned from our paradigm that helped us observe arousability in the flies. Within age groups, each sex within each genotype was tested 3 independent times with 6-8 flies used per test,

%Awoken	After the presentation of the stimulus, the percentage of flies that showed activity. Flies that had showed activity within 10 minutes prior were excluded.

#Events Awoken	After the presentation of the stimulus, the sum of number of activity events recorded by the DAMS. Activity from flies that had previously been awake were excluded.
%Spontaneous Arousal	10 minutes prior to the presentation of the stimulus, the percentage of flies that showed activity.
#Events Spontaneous	10 minutes prior to the presentation of the stimulus, the sum of number of activity events recorded by the DAMS.
%Reactivity	The percent change in activity in flies based on the number of activity events from before the stimulus compared to after. Calculated separately for each row of the activity monitor.

Table 2. Arousal Threshold Measurements. An explanation of how measurements used to define arousal threshold were quantified in this study.

Statistical Analysis

Statistical Analysis was performed using GraphPad Prism (version 9.0.0 GraphPad Software, San Diego, California USA). Correlations between variables and age were done using linear regression, which uses a linear model to predict a relationship between variables by estimating a line of best fit and approximating its resemblance to the data. The pearson r value returned is a measure of the association between the variables ranging between +1 and -1; the closer the value is to ±1, the stronger the likelihood that the independent variable has a direct influence on the dependent variable, either in terms of a positive or a negative relationship. The 95% confidence interval (95% CI) is interpreted as a 95% probability that the true linear regression line of the relationship between variables will fall between the confidence interval calculated by the data given. The smaller the confidence interval, the better the relationship between the variables fits the line.

Comparisons between age and gender and/or genotype were measured by two-way analysis of variance (ANOVA). An ANOVA is used to compare the means of two or more groups in order to determine any significant relationship between them. A two-way

ANOVA is used when there are two independent variables that each may impact a dependent variable. It will return the F-statistic, the likelihood that the change in the dependent variable over time was directly related to one or both variables. A two-way ANOVA was used since the observations being made pertained to how some variable was affected in a group over the course of their lifespan. For example, when seeing if genotype was significant to the changes in baseline sleep observed between age groups, age and genotype were used as the independent variables that may impact the baseline sleep quantities obtained. The test then returned if there was a significant relationship in the data that suggested that the changes in baseline sleep seen was due to age and/or genotype or neither. All sleep data was separated between sexes and normalized prior to analysis. For all tests, significance was set at $P<0.05$.

RESULTS

Arousal Threshold Increases with Age

Across genotypes there was a clear and significant negative linear relationship between age and arousal threshold. As flies aged, they were less likely to arouse from the mechanical stimulus presented, as depicted in **Figure 1**. Flies that were woken up were less likely to move around as much, as shown in **Figure 2. Table 3** lists the correlation values between arousal threshold measures and the age group of the flies.

Interestingly, there was a significant positive relationship between increase in age and the percentage of flies that would arouse spontaneously prior to the presentation of any stimulus, as shown in **Figure 3.** When percentage of spontaneous wake was compared to age and sex, there was only a significant variance between sexes; however, increasing age has a significant negative relationship with the number of spontaneous events**, Figure 4.**Therefore, aging may not indicate how many flies will wake up spontaneously, but it will affect the amount of activity the fly exhibits if awoken spontaneously. Additionally, whether flies were previously awake or asleep older flies exhibited less activity in response to the onset of the stimulus, as shown by the negative linear relationship depicted in **Figure 5.**

Other sex comparisons show a significant difference in the number of spontaneous events and the number of waking events in response to a stimulus, where females show a more significant decline in activity after being awoken with increasing age. Other arousability measures that were affected by age showed no significant additional relationship to sex differences. **Table 4** shows the result of a two-way

ANOVA comparing age and gender in relation to their contribution to the variance in the arousability measures.

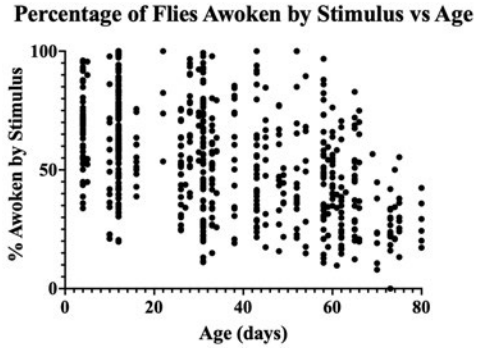

Figure 3. Percentage of Flies Awoken from Stimulus

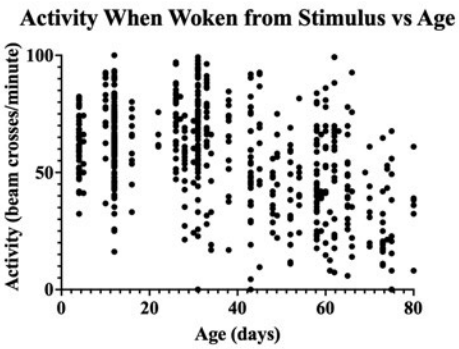

Figure 1. Activity From Stimulus

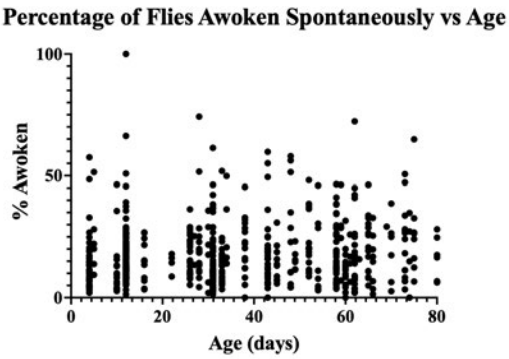

Figure 4. Percentage of Flies Woken Spontaneously

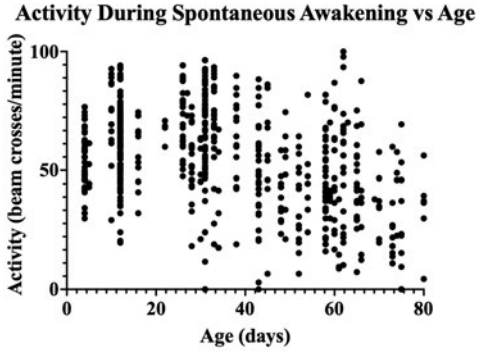

Figure 2. Activity with Spontaneous Wake

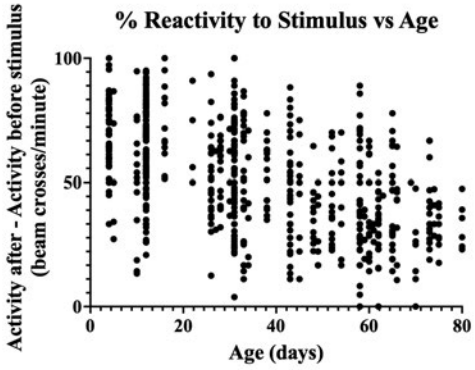

Figure 5. Reactivity to Stimulus

		%Awoken	*#Events Awoken*	*%Spontaneous Arousal*	*#Events Spontaneous*	*%Reactivity*
All WT Flies	**Pearson r**	-0.4861	-0.4637	0.08485	-0.4046	-0.5347
	95% CI	-0.5439 to -0.4238	-0.5228 to -0.4001	0.006465 to 0.1622	-0.4678 to -0.3372	-0.5886 to -0.4762
	P Value	<0.0001 ****	<0.0001 ****	0.0339 *	<0.0001 ****	<0.0001 ****
Female WT Flies	**Pearson r**	-0.6176	-0.5326	0.1004	-0.4741	-0.5827
	95% CI	-0.6844 to -0.5404	-0.6099 to -0.4454	-0.01536 to 0.2135	-0.5583 to -0.3804	-0.6543 to -0.5007
	P Value	<0.0001 ****	<0.0001 ****	0.0890	<0.0001 ****	<0.0001 ****
Male WT Flies	**Pearson r**	-0.3549	-0.3912	0.04110	-0.3325	-0.4945
	95% CI	-0.4449 to -0.2578	-0.4781 to -0.2968	-0.06602 to 0.1473	-0.4243 to -0.2340	-0.5712 to -0.4091
	P Value	<0.0001 ****	<0.0001 ****	0.4520	<0.0001 ****	<0.0001 ****

Table 3. Linear correlations between wild type fly arousability and age in all flies, female flies, and male fly subgroups.

	%Awoken	*#Events Awoken*	*%Spontaneous Arousal*	*#Events Spontaneous*	*%Reactivity*
Gender	F Statistic: F (1, 7) = 0.7805 P Value: P=0.4063	F Statistic: F (1, 7) = 12.15 P Value: P=0.0102 *	F Statistic: F (1, 7) = 63.18 P Value: P<0.0001 ****	F Statistic: F (1, 7) = 14.28 P Value: P=0.0069 **	F Statistic: F (1, 7) = 0.3893 P Value: P=0.5525
Age	F Statistic: F (7, 7) = 14.10 P Value: P=0.0012 **	F Statistic: F (7, 7) = 18.87 P Value: P=0.0005 ***	F Statistic: F (7, 7) = 1.644 P Value: P=0.2639	F Statistic: F (7, 7) = 15.17 P Value: P=0.0010 ***	F Statistic: F (7, 7) = 15.18 P Value: P=0.0010 ***

Table 4. Two-Way Anova comparing variance in arousability measures between aging and gender. The F-statistic from a two-way ANOVA gives a measure of explained vs. unexplained variance in the overall data. The higher the F statistic, the more likely any observable changes are due to the variable presented and are not random.

Additionally, genotype plays a minor role in how arousal threshold is impacted by aging. **Table 5,** a two-way ANOVA comparison between genotype and age show that both incur variance in arousal measures. Similarly to the comparisons with sex, we see that the percentage of flies that spontaneously arouse is dependent on genotype and not age.

Previous studies have shown that sleep changes are dependent on physiological age and not chronological age, i.e., the rate at which one experiences alterations to sleep is not dependent on how old the person is numerically, but the level of development or deterioration one has functionally within their body systems. As flies were aged in order

to conduct experiments, it was clear that some genotypes lived longer than others which may impact how their sleep behaviors change over the same course of time.

	%Awoken	*#Events Awoken*	*%Spontaneous Arousal*	*#Events Spontaneous*	*%Reactivity*
Genotype	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:
	F (9, 52) = 9.281	F (9, 52) = 4.464	F (9, 52) = 4.826	F (9, 52) = 3.972	F (9, 52) = 3.751
	P Value:	P Value:	P Value:	P Value:	P Value:
	P<0.0001 ****	P=0.0002 ***	P=0.0001 ***	P<0.0001 ****	P=0.0011 **
Age	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:
	F (7, 52) = 9.791	F (7, 52) = 23.27	F (7, 52) = 0.4122	F (7, 52) = 19.26	F (7, 52) = 7.693
	P Value:	P Value:	P Value:	P Value:	P Value:
	P<0.0001 ****	P<0.0001 ****	P=0.8904	P<0.0001 ****	P<0.0001 ****

Table 5. Two-way ANOVA comparing variance in arousability measures between aging and genotype.

Table 6 shows the age correlations between two genotypes, $w118^{RJ}$ and CS^{FR}, that live a relatively similar length of time- about 80 days at 25° C. Their arousability measures similarly to the group as a whole, except with the percentage of spontaneous arousal. Although both have significant p-values, $w118^{RJ}$ flies have a negative relationship whilst CS^{FR} flies experience a positive relationship- in other words, as $w118^{RJ}$ flies age, they will be less likely to wake up spontaneously. As CS^{FR} flies age, they will become more likely to wake up spontaneously. The same was not true for genotypes with a shorter lifespan, such as Berlin K, Hikone, CS, w118, etc. (which would

live on average only about 50 days at 25ºC), where any significant relationships seen were not consistent between genotypes. Their correlations are depicted on **Table 7.**

	All w118RJ Flies	***%Awoken***	***#Events Awoken***	***%Spontaneous Arousal***	***#Events Spontaneous***	***%Reactivity***
All $w118^{RJ}$ Flies	**Pearson r**	-0.4679	-0.4426	-0.2552	-0.3902	-0.2658
	95% CI	-0.6253 to -0.2739	-0.6055 to -0.2441	-0.4521 to -0.03466	-0.5638 to -0.1836	-0.4611 to -0.04603
	P Value	<0.0001 ****	<0.0001 ****	0.0241 *	0.0004 ***	0.0187 *
All CS^{FR} Flies	**Pearson r**	-0.6350	-0.5209	0.2837	-0.3934	-0.7045
	95% CI	-0.7387 to -0.5018	-0.6501 to -0.3623	0.09343 to 0.4540	-0.5468 to -0.2145	-0.7910 to -0.5904
	P Value	<0.0001 ****	<0.0001 ****	0.0040 **	<0.0001 ****	<0.0001 ****

Table 6. Linear correlations between aging and arousability measures within two different wild-type genotypes with longer lifespans.

Since sex also plays a role in arousability, we then obtain correlations between male and female CS^{FR} flies, as depicted in **Table 8.** With male CS^{FR} flies, there was no significant relationship between age and percentage of flies that woke up spontaneously, nor the number of spontaneous events. Even previously strong relationships, the percent that woke up and the number of events with waking, albeit still significant, show a much lesser degree of correlation. With females, both spontaneous measures were significant; the number of events decreased with age, but the percent of flies increased. This may indicate that the impact of age on sex differences are dependent on the genotype.

The genotypes with shorter lifespans also had clear gender differences in their arousability as depicted for Hikone in **Table 9.** How each genotype's arousability

changed with age is depicted on **Figures 6-10**. No obvious patterns were noticed between arousability measures and genotypes with longer or shorter lifespans.

There was no significant variance in arousal threshold measures between virgin and mated females.

		%Awoken	*#Events Awoken*	*%Spontaneous Arousal*	*#Events Spontaneous*	*%Reactivity*
All Berlin^{K} Flies	**Pearson r**	-0.3772	-0.1890	-0.05653	-0.07356	-0.3602
	95% CI	-0.5953 to -0.1074	-0.4464 to 0.09741	-0.3324 to 0.2283	-0.3476 to 0.2120	-0.5824 to -0.08796
	P Value	0.0075 **	0.1935	0.6996	0.6154	0.0110 *
All CS^{DR} Flies	**Pearson r**	-0.3820	-0.4796	0.2436	-0.3932	-0.5183
	95% CI	-0.5797 to -0.1419	-0.01101 to 0.4685	-0.5884 to -0.1547	-0.5884 to -0.1547	-0.6824 to -0.3044
	P Value	0.0026 **	0.0001 ***	0.0607	0.0019 **	<0.0001 ****
All Hikone^{AW} Flies	**Pearson r**	0.1962	-0.4973	0.2899	-0.4409	-0.02249
	95% CI	-0.09980 to 0.4603	-0.6882 to -0.2419	-0.0004264 to 0.5352	-0.6482 to -0.1727	-0.3108 to 0.2696
	P Value	0.1913	0.0004 ***	0.0507	0.0022 **	0.8821
All w1118^{DR} Flies	**Pearson r**	-0.1888	-0.2614	-0.2145	-0.2635	-0.3164
	95% CI	-0.4871 to 0.1490	-0.5433 to 0.07340	-0.5073 to 0.1227	-0.5448 to 0.07124	-0.5842 to 0.01360
	P Value	0.2702	0.1235	0.2091	0.1205	0.0601

Table 7. Linear correlations between aging and arousability measures, in wild-type genotypes that have shorter lifespans.

		%Awoken	*#Events Awoken*	*%Spontaneous Arousal*	*#Events Spontaneous*	*%Reactivity*
Male CS^{FR} Flies	**Pearson r**	-0.3353	-0.3090	-0.04162	-0.2725	-0.5847
	95% CI	-0.5656 to -0.05656	-0.5452 to -0.02721	-0.3219 to 0.2454	-0.5166 to 0.01260	-0.7450 to -0.3604
	P Value	0.0198 *	0.0326 *	0.7788	0.0609	<0.0001 ****
Female CS^{FR} Flies	**Pearson r**	-0.7825	-0.5944	0.2973	-0.3740	-0.7950
	95% CI	-0.8690 to -0.6496	-0.7450 to -0.3862	0.02938 to 0.5254	-0.5851 to -0.1154	-0.8768 to -0.6682
	P Value	<0.0001 ****	<0.0001 ****	0.0306 *	0.0058 **	<0.0001 ****

Table 8. Linear correlations between aging and arousability in genders within one longer-lifespan genotype.

		%Awoken	*#Events Awoken*	*%Spontaneous Arousal*	*#Events Spontaneous*	*%Reactivity*
Male Hikone Flies	**Pearson r**	0.6262	-0.6282	0.6411	-0.5963	-0.07063
	95% CI	0.2779 to 0.8289	-0.8300 to -0.2810	0.3009 to 0.8366	-0.8134 to -0.2333	-0.4780 to 0.3618
	P Value	0.0018 **	0.0017 **	0.0013 **	0.0034 **	0.7548
Female Hikone Flies	**Pearson r**	-0.05192	-0.4102	0.04456	-0.3263	0.04786
	95% CI	-0.4460 to 0.3590	-0.6981 to -0.008207	-0.3654 to 0.4400	-0.6448 to 0.08879	-0.3625 to 0.4427
	P Value	0.8096	0.0465 *	0.8362	0.1197	0.8243

Table 9. Linear correlations between aging and arousability in genders within one shorter-lifespan genotype.

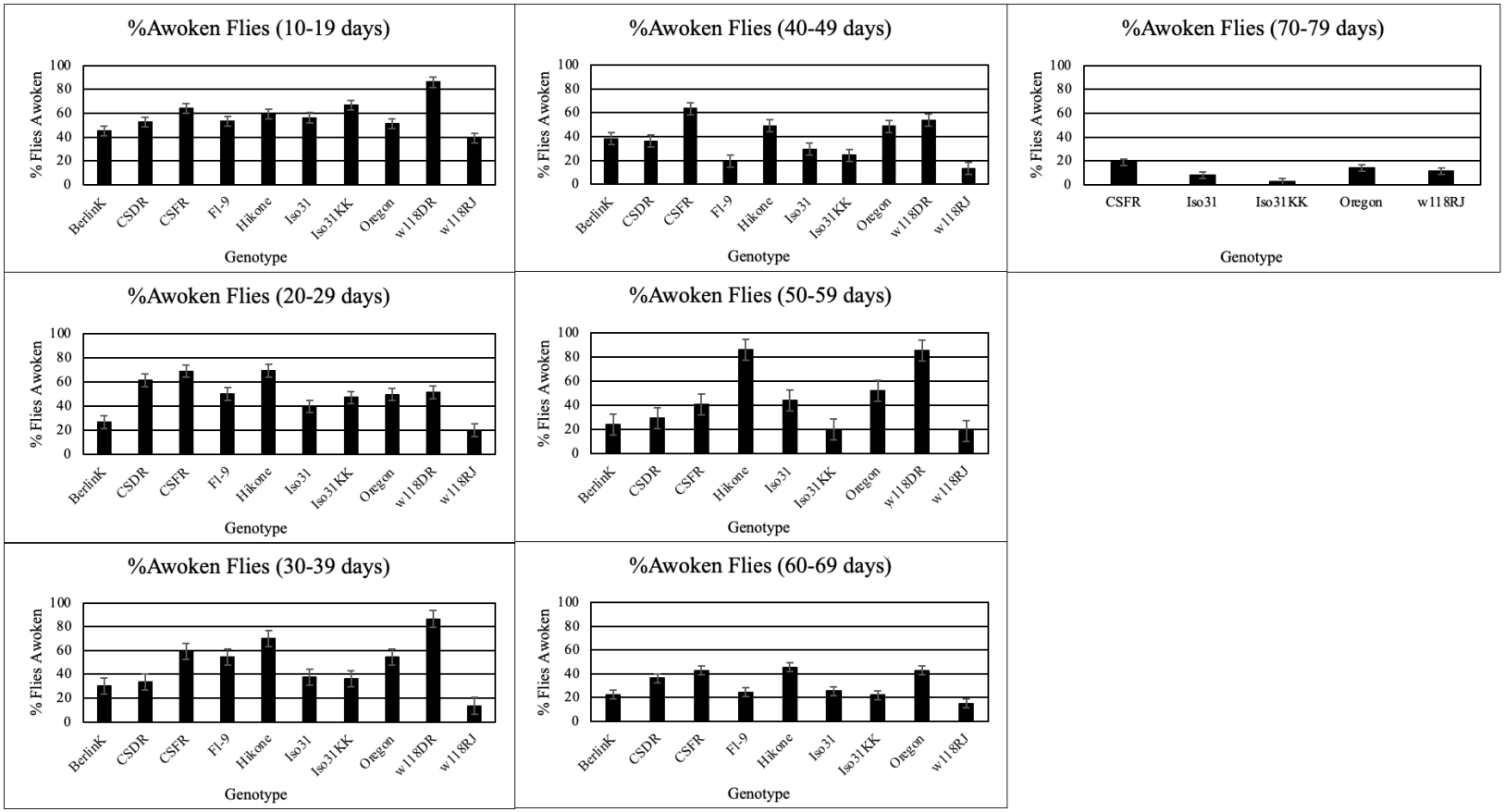

Figure 6. Percentage of flies awoken by a stimulus, comparing genotypes within each age group. Any genotypes not listed in the (70-79 day old) age group indicates that their lifespan did not reach that length.

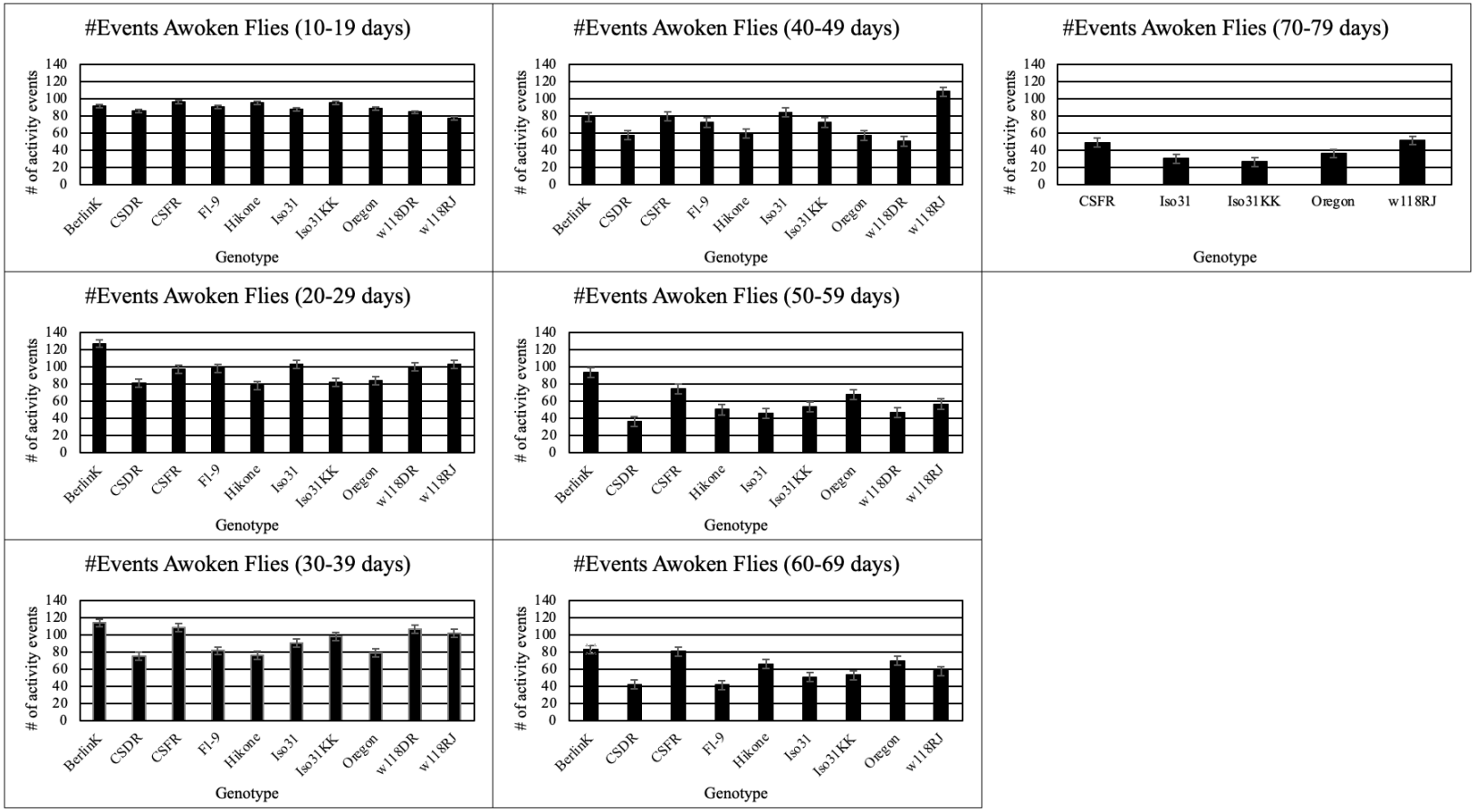

Figure 7. The activity measured from flies awoken by a stimulus, comparing genotypes within each age group. Any genotypes not listed in the (70-79 day old) age group indicates that their lifespan did not reach that length.

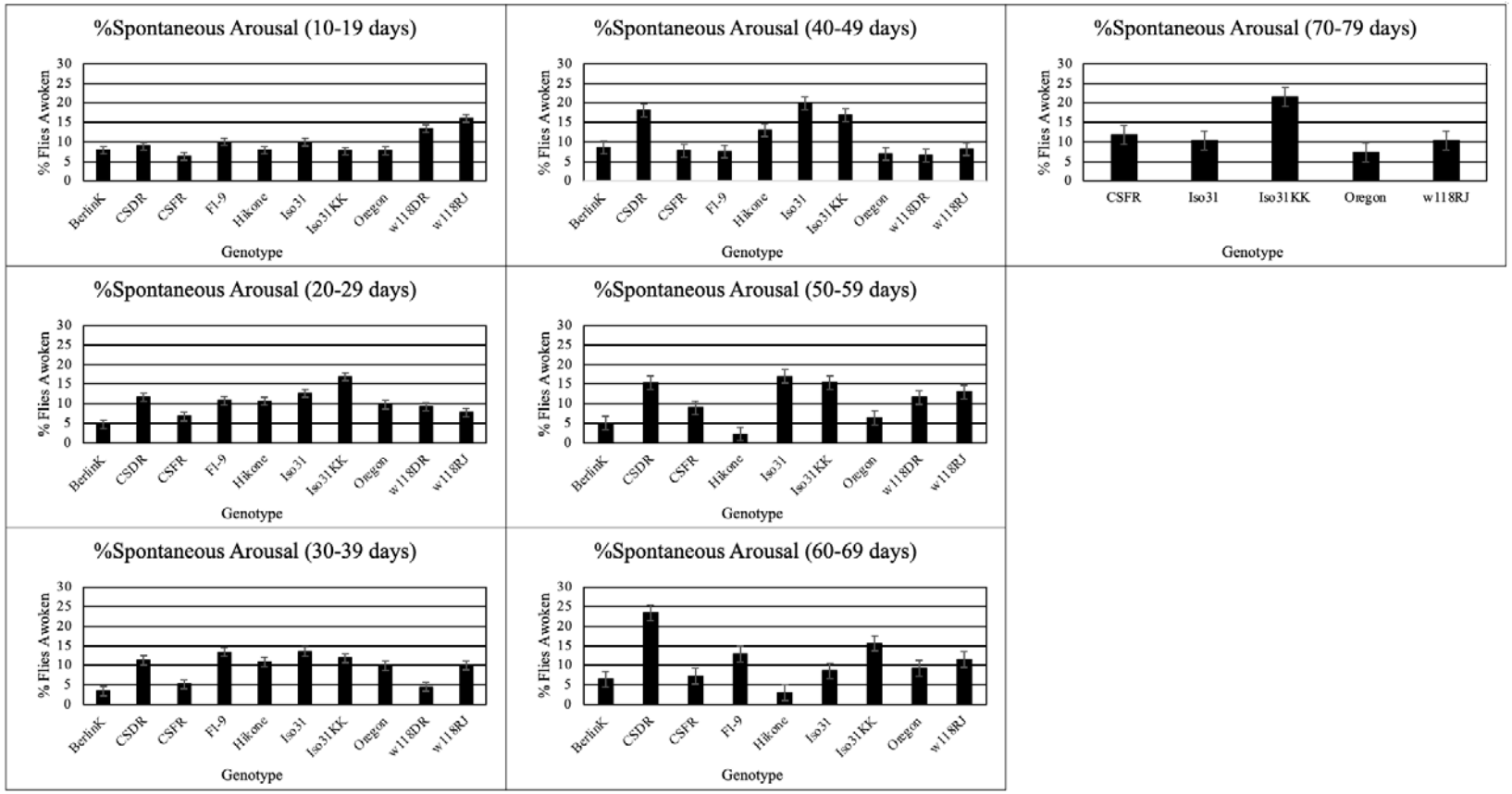

Figure 8. Percentage of flies that woke spontaneously without the presentation of a stimulus, comparing genotypes within each age group. Any genotypes not listed in the (70-79 day old) age group indicates that their lifespan did not reach that length.

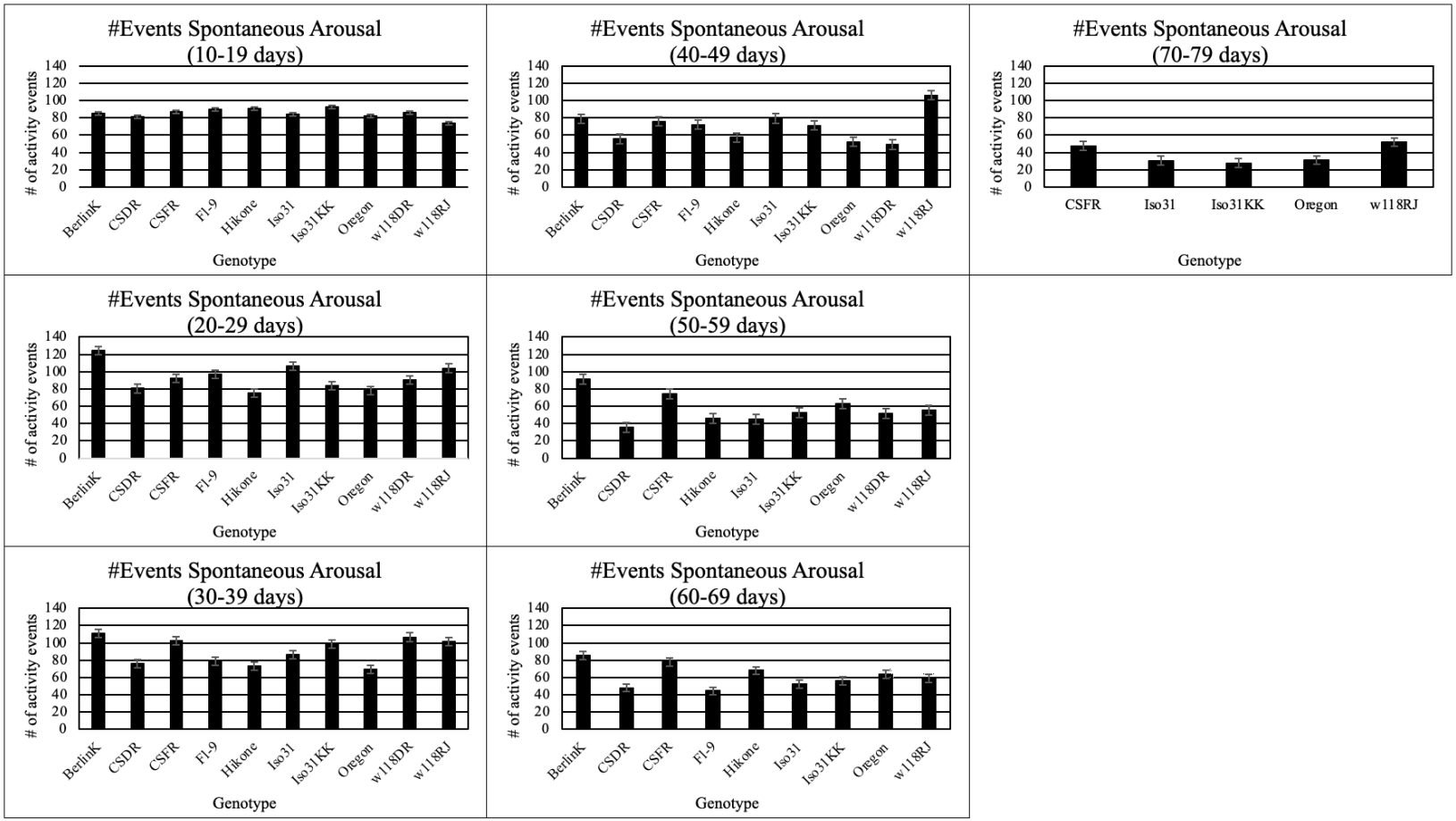

Figure 9. The activity measured from flies that woke up spontaneously, comparing genotypes within each age group. Any genotypes not listed in the (70-79 day old) age group indicates that their lifespan did not reach that length.

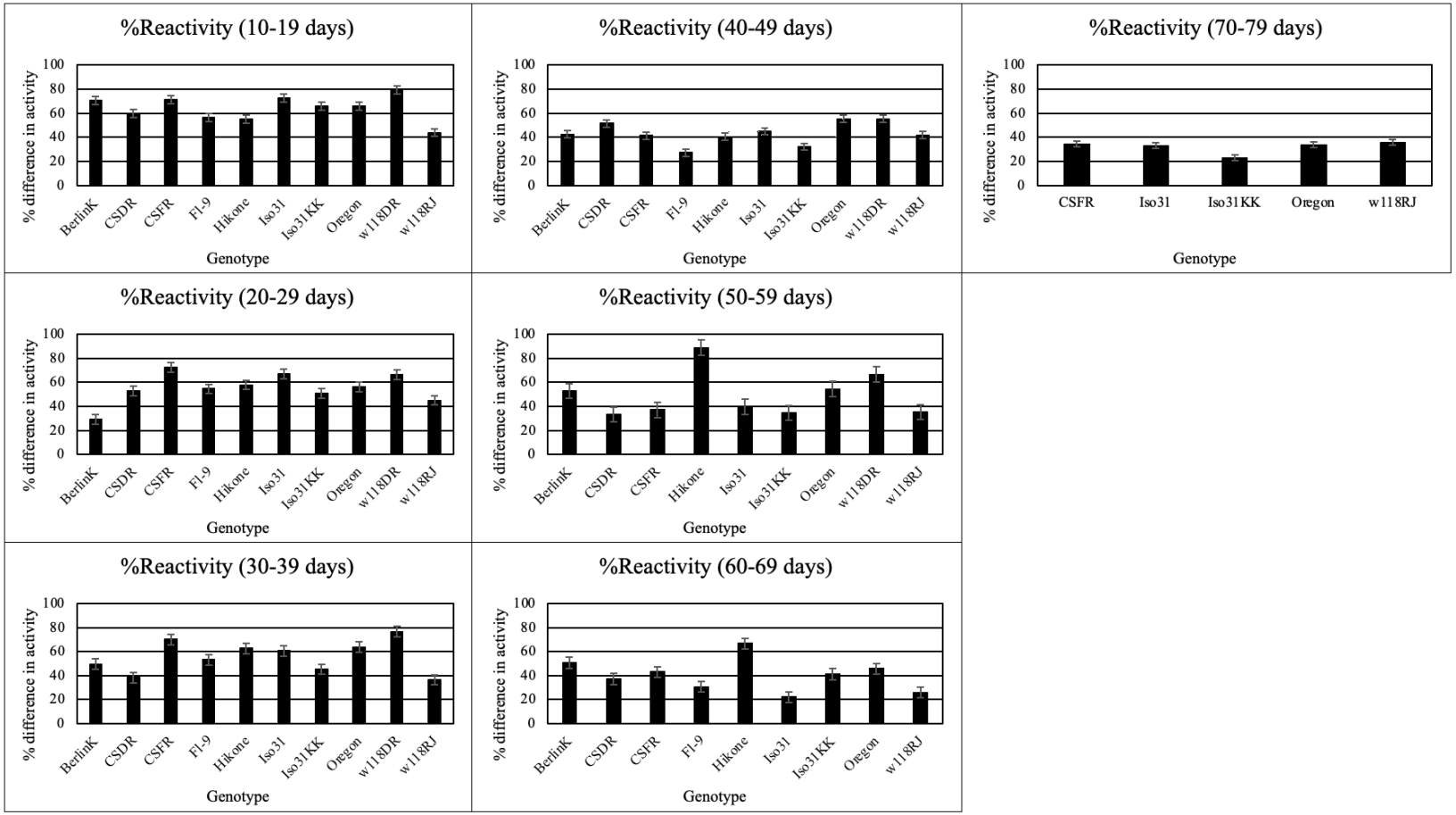

Figure 10. The percent reactivity in flies measured via activity before and after the presentation of a stimulus, comparing genotypes within each age group. Any genotypes not listed in the (70-79 day old) age group indicates that their lifespan did not reach that length.

Sleep Quantity Increases with Age

The measures obtained while studying sleep were separated between males and females prior to analysis due to their differences in baseline sleep patterns. An overview of their relationships regarding aging and aspects of sleep behavior is detailed in **Table 10.**

Sleep was obtained over the course of 3 days: one day before the arousal assay, the day of, and the day after. In order to observe results without the direct interruption of the mechanical stimuli, data from the day of was excluded. Upon further analysis it became likely that the mild deprivation from the arousal assay was enough to cause rebound sleep the day after. Therefore, the total sleep from day one may be referred to as "baseline sleep" and from day three as "recovery sleep".

With baseline sleep, there was a positive relationship between the amount of sleep and the age of the flies tested- showing that generally, with increasing age one will sleep more. This was seen regardless of sex. **Tables 11-12 & 13-14** detail sleep and arousal threshold data averages from the raw data.

Older Flies Compensate with more Day Sleep

Female flies experience prolonged wake during the light period and sleep during the dark period [1]. With age, we see an increasing reliance on day sleep via longer bouts of sleep. Males also experience an increase in sleep during the day, although this is attributed to both the length of the sleep periods as well as an increase in their frequency.

Both genders show an overall decrease in night sleep with age; however, with females there is no significant attribution to either sleep bout length or frequency. In males there is an increase in the frequency of bouts of sleep at night, but a decrease in their length.

Sleep Latency is Sex Dependent

Sleep latency (also known as sleep delay) refers to the length of time it takes for one to fall back asleep after a brief awakening. The sleep latency of females increases with age, possibly relating to why the number of sleep bouts may not change but the length of said bouts do- previous studies have shown that number and length of bouts are typically inverse, with having less but longer bouts being ideal for a better quality of sleep. So, although males have a shorter sleep latency, they are experiencing greater fragmentation. It might be noted, however, that this is consistent with their baseline sleep patterns while young, as supported by **Table 10** and **Figures 12-13 and 14-15-** females experience extended durations of quiescence and activity whilst males have more frequent but shorter periods of activity, clear circadian patterns of which become distorted with age.

Female WT Flies

	Day 1 Sleep	*Day 2 Sleep*	*Day 1 Activity*	*Day 2 Activity*	*Day Sleep*	*Night Sleep*	*Day Bout Length*	*Night Bout Length*	*Day Bout Number*	*Night Bout Number*	*Sleep Delays*
Pearson r	0.09907	-0.07053	-0.09900	0.1316	0.1669	-0.1286	0.1388	-0.05925	0.03994	0.02438	0.2227
95% CI	0.03857 to 0.1588	-0.1307 to -0.009837	-0.1588 to -0.03851	0.07147 to 0.1908	0.1073 to 0.2253	-0.1878 to -0.06836	0.07869 to 0.1979	-0.1196 to 0.001482	-0.02084 to 0.1004	-0.03640 to 0.08499	0.1642 to 0.2797
P Value	0.0014 **	0.0228 *	0.0014 **	<0.0001 ****	<0.0001 ****	<0.0001 ****	<0.0001 ****	0.0559	0.1977	0.4318	<0.0001 ****

Male WT Flies

Pearson r	0.3400	0.2066	-0.3504	-0.2062	0.4211	0.07408	0.1246	-0.02092	0.1380	0.06553	-0.1181
95% CI	0.2893 to 0.3889	0.1521 to 0.2598	-0.3988 to -0.3000	-0.2594 to -0.1516	0.3737 to 0.4664	0.01787 to 0.1298	0.06882 to 0.1796	-0.07711 to 0.03541	0.08230 to 0.1927	0.009283 to 0.1214	-0.1733 to -0.06221
P Value	<0.0001 ****	<0.0001 ****	<0.0001 ****	<0.0001 ****	<0.0001 ****	0.0099 **	<0.0001 ****	0.4667	<0.0001 ****	0.0225 *	<0.0001 ****

Female

	Δ Sleep (Day 2 – Day 1)	*Δ Activity (Day 1 – Day 2)*
Pearson r	-0.2004	-0.2453
95% CI	-0.2580 to -0.1414	-0.3015 to -0.1873
P Value	<0.0001 ****	<0.0001 ****

Male

	Δ Sleep (Day 2 – Day 1)	*Δ Activity (Day 1 – Day 2)*
Pearson r	-0.1040	-0.2785
95% CI	-0.1594 to -0.04803	-0.3296 to -0.2258
P Value	0.0003 ***	<0.0001 ****

Table 10. Linear correlation of sleep characteristics within male and female wild-type flies.

Female WT Flies	Day 1 Sleep (min)	Day 2 Sleep (min)	Day 1 Activity (# beam crosses)	Day 2 Activity (# beam crosses)	Day Sleep (min)	Night Sleep (min)	Day Bout Length (min)	Night Bout Length (min)	Day Bout Number	Night Bout Number	Sleep Delays (min)	Δ Sleep	Δ Activity
Age Groups (days)													
1-9	731.0	844.3	2506.6	1561.3	278.6	482.0	13.4	25.8	20.7	20.7	25.5	113.4	945.3
10-19	810.4	834.3	2142.8	1488.9	302.8	495.1	16.3	27.9	19.8	21.6	23.8	23.9	653.9
20-29	599.0	500.1	3139.6	2503.7	143.6	376.0	9.1	19.2	14.8	20.6	25.1	-98.9	635.9
30-39	877.9	739.0	1510.3	1562.0	221.8	553.4	14.4	26.7	16.2	22.4	17.6	-138.9	-51.8
40-49	823.2	817.5	1475.5	1271.4	317.5	486.0	13.7	23.3	24.0	23.6	33.4	-5.7	204.0
50-59	789.0	739.3	2296.6	1903.6	323.2	435.2	16.3	20.3	20.7	23.5	35.2	-49.6	393.0
60-69	849.4	850.9	1714.7	1486.7	362.7	473.0	17.6	25.8	21.7	21.9	38.1	1.6	228.0
70-79	855.0	789.7	2149.1	2252.8	370.6	450.5	20.2	26.8	20.1	19.2	53.2	-65.3	-103.6

Table 11. Sleep Data Averages between all Female WT Flies

Female WT Flies	%Awoken	#Events Awoken	%Spontaneous Arousal	#Events Spontaneous	%Reactivity
Age					
1-9	65.82	79.86	11.39	69.64	64.18
10-19	66.74	84.12	11.20	79.46	69.73
20-29	50.15	87.80	13.44	85.05	56.29
30-39	47.94	90.54	11.54	86.35	57.61
40-49	41.97	62.46	14.89	59.29	45.39
50-59	31.89	53.81	15.50	53.97	37.46
60-69	29.86	64.13	12.04	63.30	41.05
70-79	14.18	38.50	13.72	37.85	33.90

Table 12. Arousal Threshold Averages between all Female WT Flies

Male WT Flies

Age Groups (days)	***Day 1 Sleep*** *(min)*	***Day 2 Sleep*** *(min)*	***Day 1 Activity*** *(# beam crosses)*	***Day 2 Activity*** *(# beam crosses)*	***Day Sleep*** *(min)*	***Night Sleep*** *(min)*	***Day Bout Length*** *(min)*	***Night Bout Length*** *(min)*	***Day Bout Number***	***Night Bout Number***	***Sleep Delays*** *(min)*	***Δ Sleep***	***Δ Activity***
1-9	954.0	921.9	2126.3	1824.4	391.3	515.1	25.3	42.3	17.3	14.2	44.6	-32.1	301.9
10-19	1002.9	957.8	2274.5	1578.2	420.4	537.4	26.9	38.8	18.0	17.5	31.4	-45.1	696.3
20-29	992.3	939.5	1736.4	1586.0	420.9	530.4	20.4	32.5	22.4	18.7	35.9	-52.8	150.4
30-39	931.9	865.8	2190.3	1833.1	359.4	524.8	15.7	27.4	23.1	20.9	34.7	-66.1	357.2
40-49	1045.6	987.7	1356.8	1262.8	484.2	524.0	24.6	36.7	20.6	16.0	39.4	-57.9	94.0
50-59	1064.5	1003.3	1411.8	1381.0	490.2	533.8	26.7	41.0	20.3	15.9	35.6	-61.2	30.8
60-69	1119.3	1017.0	1293.7	1347.2	529.2	528.0	31.6	37.0	19.5	17.0	30.1	-102.3	-53.6
70-79	1203.7	1150.1	909.7	1002.1	595.2	581.8	37.0	40.2	20.0	19.2	10.1	-53.6	-92.4

Table 13. Sleep Data Averages betwen all Male WT Flies

Male WT Flies

Age	***%Awoken***	***#Events Awoken***	***%Spontaneous Arousal***	***#Events Spontaneous***	***%Reactivity***
1-9	56.87	87.64	7.09	78.32	72.22
10-19	49.00	93.45	8.50	90.22	59.88
20-29	52.76	91.90	9.47	90.58	53.68
30-39	44.47	94.54	8.21	92.11	52.29
40-49	39.35	76.54	8.84	74.80	41.76
50-59	42.84	64.03	8.55	62.03	45.69
60-69	31.21	63.80	9.27	63.76	35.24
70-79	10.97	62.29	8.10	62.82	33.39

Table 14. Arousal Threshold Averages between all Male WT Flies

Recovery Sleep Decreases with Age

When subtracting day 1 sleep from day 3, both genders showed a significant negative relationship. In other words, the difference between baseline sleep and recovery sleep decreases with age, showing a decreased ability in older flies to compensate sleep after a deficiency. This recovery sleep data is also detailed in **Table 10.**

In female flies, there was a negative significant relationship between sleep quantity on day 3 and age; meaning that with age, they experienced so much less sleep after a mild deprivation or disturbance to the point that they showed more activity than younger flies. With males, no such day 3 negative relationship was seen- although the difference in sleep between baseline and recovery decreases with age, the amount of sleep obtained during recovery is still more than that of younger flies.

Total Activity Decreases with Age

Inverse to the relationship between aging and sleep, there is a decrease of baseline activity with an increase in age, as shown in **Figure 11.** The same differences during the recovery period between genders are reflected with activity as well, with females experiencing an increase in activity in the recovery period compared to their baseline in relation to age. With males, no such inverse in relationship with age is present between baseline and recovery period activity, although the total activity on day 1 is less than that of day 3, showing that there is an increase in activity comparable to their baseline but it is still less than the amount of activity younger flies exhibit even during the recovery period.

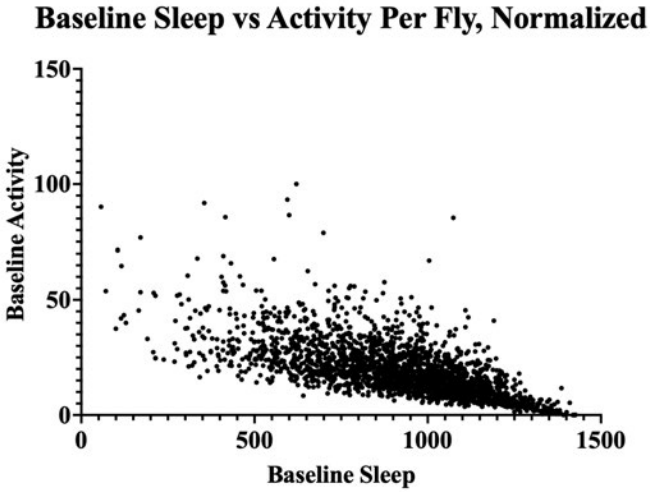

Figure 11. Comparing the amount of sleep to the amount of activity each fly gets.

Sleep and Activity Become Disorganized with Age

As mentioned previously, flies have peaks of activity during the 24-hour period that is dependent on gender. With age, we can see how this changes and becomes less prominent. **Figures 12 and 13** depict actograms, graphical representations of their activity over the three days of iso31^{KK} female and male flies over a range of increasing ages. Although varying genotypes have slight differences, the general change is comparably similar. **Figures 14 and 15** show the average sleep per 30 minutes of male and female flies as they age. The older flies not only spend more time in average sleeping within 30 minutes, but also clearly show a disruption in their sleep/wake patterns.

Sleep Architecture and Gender Differences are Dictated by Genotype

Despite overall changes noted within all male and female wildtype flies, it is important to note that this was highly variable between genotypes- both changes due to

age and changes due to age that are also influenced by gender. Overall, female flies across all genotypes shared only a negative relationship between increasing age and the difference in activity rebound. **Tables 15-17** show examples of sleep architecture between genotypes, and **Tables 18 & 19** depict the variance that exists between genotypes without added gender variability. This would indicate that sleep behaviors not only arise differently between sexes and genotypes, but also the degree to which each genotype experiences gender discrepancies is different. Of note, **Tables 20 & 21** depict mating status differences in females within one genotype. Besides baseline activity, there were no significant variances obtained between virgin and mated female $iso31^{KK}$ flies, and that discrepancy was not consistent in other genotypes examined.

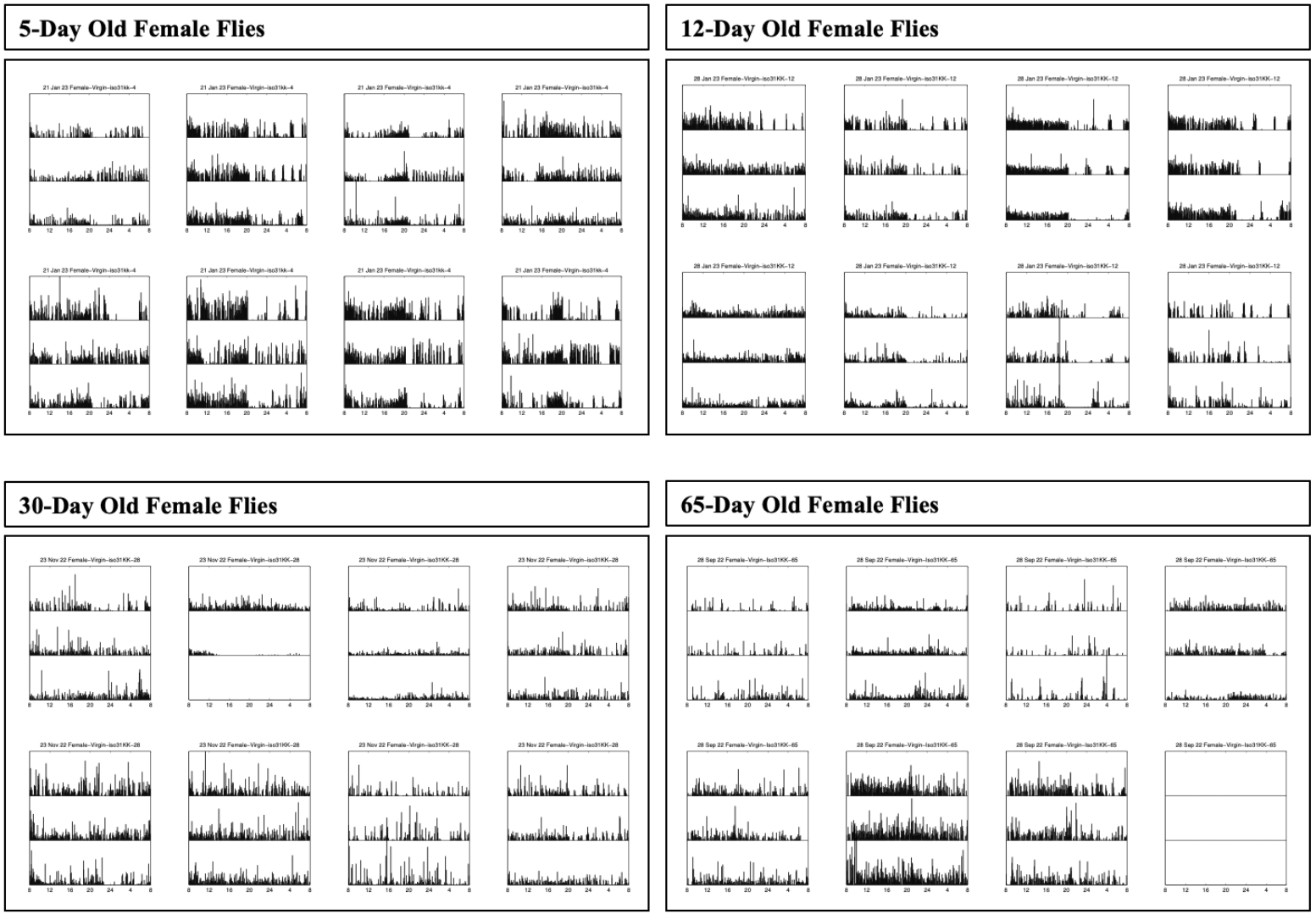

Figure 12. Actograms of female iso31^{KK} flies over the course of 3 days as they age.

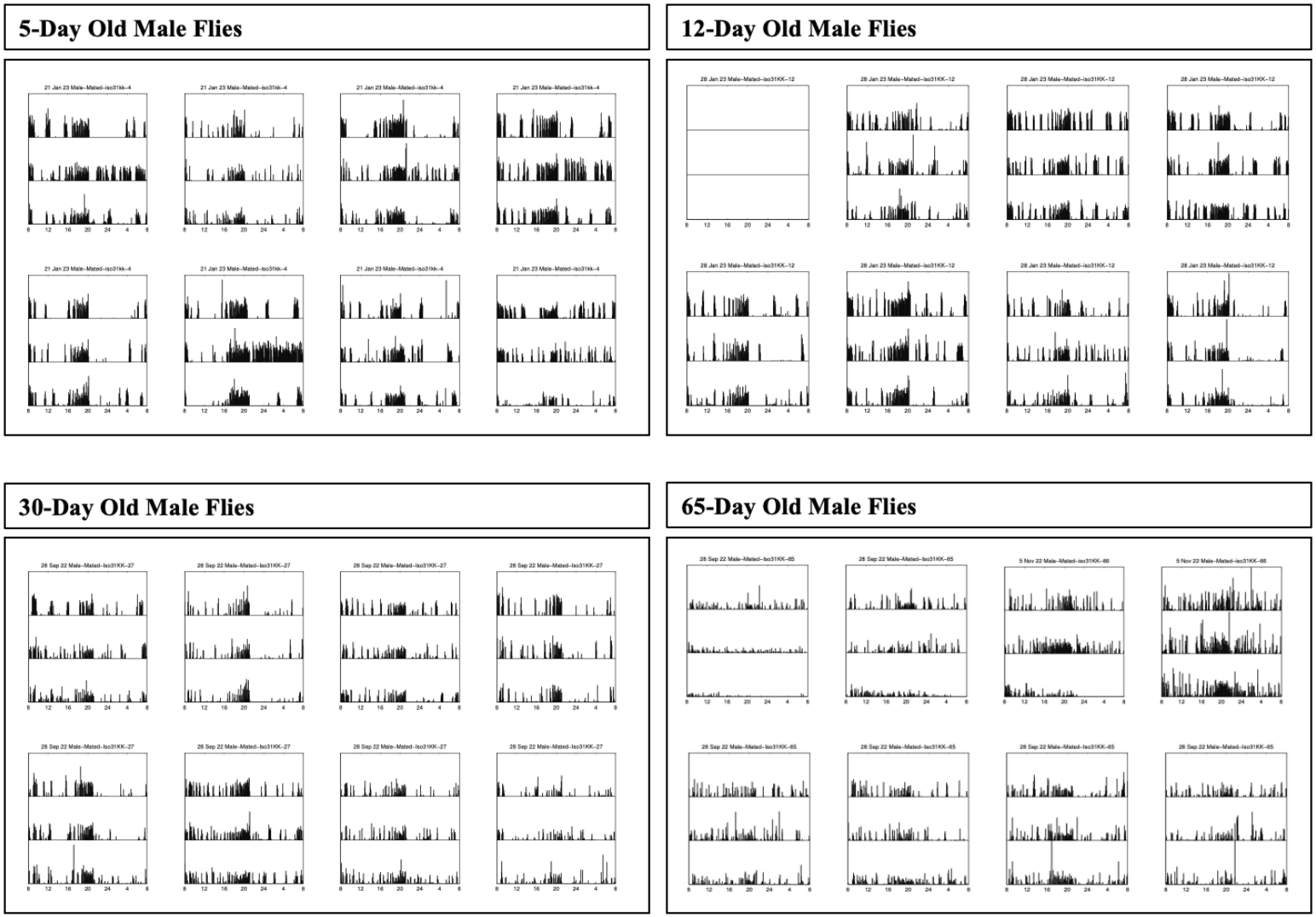

Figure 13. Actograms of male iso31^{KK} flies over the course of 3 days as they age.

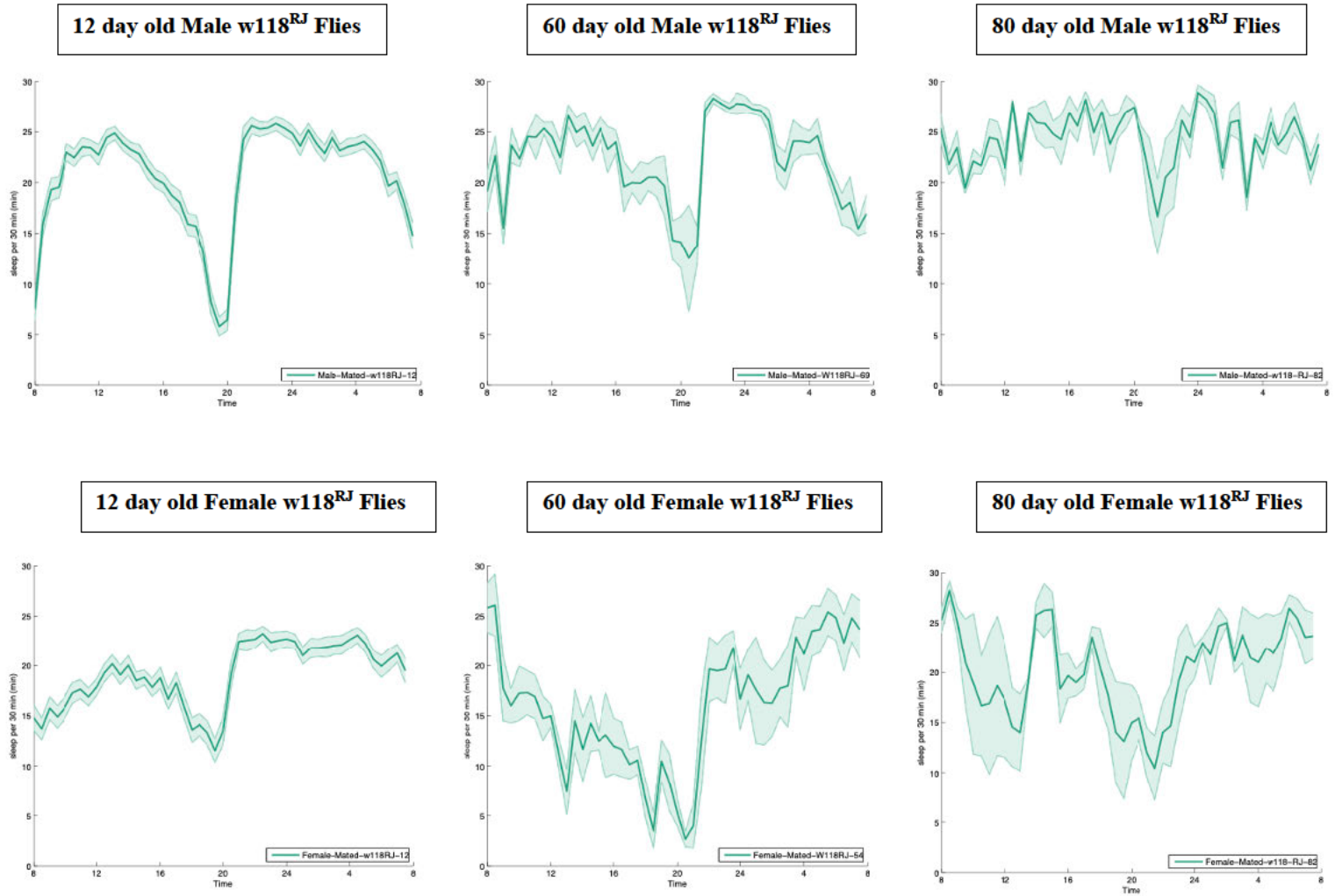

Figure 14. Sleep per 30 minutes of male and female w118^{RJ} flies as they age. The day 12 graph is representative of their healthy baseline circadian patterns. As they age their sleeping behaviors become disrupted.

Male Berlin^{K} Flies

	Day 1 Sleep	*Day 2 Sleep*	*Day 1 Activity*	*Day 2 Activity*	*Day Sleep*	*Night Sleep*	*Day Bout Length*	*Night Bout Length*	*Day Bout Number*	*Night Bout Number*	*Sleep Delays*
Pearson r	-0.0469	0.07875	-0.5581	-0.2276	0.1285	0.02564	-0.4629	0.05543	0.6271	-0.03674	0.6268
95% CI	-0.2639 to 0.1746	-0.1434 to 0.2934	-0.6929 to -0.3857	-0.4260 to -0.008334	-0.09383 to 0.3387	-0.1952 to 0.2440	-0.6196 to -0.2707	-0.1663 to 0.2718	0.4725 to 0.7443	-0.2544 to 0.1845	0.4721 to 0.7441
P Value	0.6794	0.4875	<0.0001 ****	0.0423 *	0.2558	0.8214	<0.0001 ****	0.6253	<0.0001 ****	0.7463	<0.0001 ****

Female Berlin^{K} Flies

	Day 1 Sleep	*Day 2 Sleep*	*Day 1 Activity*	*Day 2 Activity*	*Day Sleep*	*Night Sleep*	*Day Bout Length*	*Night Bout Length*	*Day Bout Number*	*Night Bout Number*	*Sleep Delays*
Pearson r	0.4896	0.4707	-0.6343	-0.4465	0.6279	0.3192	0.1804	0.4046	0.4912	-0.4123	0.2734
95% CI	0.3110 to 0.6348	0.2886 to 0.6199	-0.7453 to -0.4889	-0.6006 to -0.2603	0.4809 to 0.7406	0.1163 to 0.4964	-0.03147 to 0.3767	0.2120 to 0.5669	0.3129 to 0.6360	-0.5732 to -0.2209	0.06664 to 0.4577
P Value	<0.0001 ****	<0.0001 ****	<0.0001 ****	<0.0001 ****	<0.0001 ****	0.0026 **	0.0946	0.0001 ***	<0.0001 ****	<0.0001 ****	0.0104 *

Female

	Δ Sleep (Day 2 – Day 1)	*Δ Activity (Day 1 – Day 2)*
Pearson r	0.1100	-0.5781
95% CI	-0.1030 to 0.3134	-0.7031 to -0.4184
P Value	0.3104	<0.0001 ****

Male

	Δ Sleep (Day 2 – Day 1)	*Δ Activity (Day 1 – Day 2)*
Pearson r	0.1172	-0.5063
95% CI	-0.1052 to 0.3285	-0.6534 to -0.3225
P Value	0.3004	<0.0001 ****

Table 15. Linear correlations between aging and sleep architecture in males and females within a genotype of fly with a shorter lifespan.

Male w118^{RJ} Flies

	Day 1 Sleep	*Day 2 Sleep*	*Day 1 Activity*	*Day 2 Activity*	*Day Sleep*	*Night Sleep*	*Day Bout Length*	*Night Bout Length*	*Day Bout Number*	*Night Bout Number*	*Sleep Delays*
Pearson r	0.5816	0.3757	-0.3925	-0.2394	0.6583	0.3285	0.3120	0.2307	-0.1784	-0.1992	-0.5854
95% CI	0.4581 to 0.6830	0.2214 to 0.5117	-0.5261 to -0.2401	-0.3919 to -0.07401	0.5511 to 0.7442	0.1695 to 0.4708	0.1516 to 0.4564	0.06491 to 0.3841	-0.3367 to -0.01042	-0.3556 to -0.03196	-0.6861 to -0.4627
P Value	<0.0001 ****	<0.0001 ****	<0.0001 ****	0.0050 **	<0.0001 ****	<0.0001 ****	0.0002 ***	0.0069 **	0.0377 *	0.0201 *	<0.0001 ****

Female w118^{RJ} Flies

	Day 1 Sleep	*Day 2 Sleep*	*Day 1 Activity*	*Day 2 Activity*	*Day Sleep*	*Night Sleep*	*Day Bout Length*	*Night Bout Length*	*Day Bout Number*	*Night Bout Number*	*Sleep Delays*
Pearson r	-0.0558	-0.1938	0.09961	0.2532	-0.07858	-0.1315	0.3083	0.1104	-0.2702	-0.2188	0.4076
95% CI	-0.2397 to 0.1320	-0.3669 to -0.00766	-0.08842 to 0.2808	0.07011 to 0.4198	-0.2612 to 0.1094	-0.3103 to 0.05631	0.1294 to 0.4679	-0.07763 to 0.2908	-0.4347 to -0.08827	-0.3894 to -0.03383	0.2393 to 0.5520
P Value	0.5610	0.0416 *	0.2983	0.0073 **	0.4123	0.1690	0.0010 ***	0.2489	0.0041 **	0.0210 *	<0.0001 ****

Female

	Δ Sleep (Day 2 – Day 1)	*Δ Activity (Day 1 – Day 2)*
Pearson r	-0.2061	-0.1960
95% CI	-0.3779 to -0.02045	-0.3689 to -0.009974
P Value	0.0300 *	0.0392 *

Male

	Δ Sleep (Day 2 – Day 1)	*Δ Activity (Day 1 – Day 2)*
Pearson r	-0.1383	-0.1944
95% CI	-0.2997 to 0.03074	-0.3513 to -0.02700
P Value	0.1083	0.0233 *

Table 16. Linear correlations between aging and sleep architecture in males and females within a genotype of fly with a longer lifespan.

Male iso31^{KK} Flies

	Day 1 Sleep	*Day 2 Sleep*	*Day 1 Activity*	*Day 2 Activity*	*Day Sleep*	*Night Sleep*	*Day Bout Length*	*Night Bout Length*	*Day Bout Number*	*Night Bout Number*	*Sleep Delays*
Pearson r	0.3266	0.1428	-0.5917	-0.5161	0.4256	0.01378	-0.4344	-0.2798	0.7247	0.4020	-0.03774
95% CI	0.1722 to 0.4653	-0.0213 to 0.2994	-0.6886 to -0.4740	-0.6268 to -0.3851	0.2817 to 0.5509	-0.1501 to 0.1770	-0.5583 to -0.2916	-0.4240 to -0.1218	0.6366 to 0.7942	0.2553 to 0.5307	-0.2001 to 0.1266
P Value	<0.0001 ****	0.0877	<0.0001 ****	<0.0001 ****	<0.0001 ****	0.8698	<0.0001 ****	0.0007 ***	<0.0001 ****	<0.0001 ****	0.6534

Female iso31^{KK} Flies

Pearson r	0.07775	-0.0657	-0.2426	-0.0762	0.2029	-0.2831	0.01165	-0.3776	0.3001	0.3374	0.2567
95% CI	-0.05728 to 0.2100	-0.1984 to 0.06937	-0.3651 to -0.1118	-0.2085 to 0.05888	0.07041 to 0.3284	-0.4022 to -0.1546	-0.1230 to 0.1458	-0.4873 to -0.2562	0.1726 to 0.4177	0.2126 to 0.4513	0.1266 to 0.3781
P Value	0.2586	0.3402	0.0004 ***	0.2685	0.0029 **	<0.0001 ****	0.8658	<0.0001 ****	<0.0001 ****	<0.0001 ****	0.0002 ***

Female

	Δ Sleep (Day 2 – Day 1)	*Δ Activity (Day 1 – Day 2)*
Pearson r	-0.1459	-0.2164
95% CI	-0.2749 to -0.0117	-0.3409 to -0.08446
P Value	-0.2749 to -0.01167 *	0.0015 **

Male

	Δ Sleep (Day 2 – Day 1)	*Δ Activity (Day 1 – Day 2)*
Pearson r	-0.1463	-0.2656
95% CI	-0.3027 to 0.01765	-0.4113 to -0.1066
P Value	0.0801	0.0013 **

Table 17. Linear correlations between aging and sleep architecture in males and females within the genotype of fly that was previously used in the depiction of actograms.

	Baseline Sleep	*Sleep, Recovery*	*Baseline Activity*	*Activity, Recovery*	*Day Sleep*	*Night Sleep*	*Day Bout Number*	*Night Bout Number*	*Day Bout Length*	*Night Bout Length*	*Sleep Delay*
Genotype	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:
	F (6, 20) = 2.633	F (6, 20) = 11.08	F (6, 20) = 2.706	F (6, 20) = 5.694	F (6, 20) = 1.153	F (6, 20) = 3.079	F (6, 20) = 2.792	F (6, 20) = 7.848	F (6, 20) = 1.575	F (6, 20) = 4.409	F (6, 20) = 0.2594
	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:
	P=0.0478 *	P<0.0001 ****	P=0.0433 *	P=0.0014 **	P=0.3692	P=0.0266 *	P=0.0387 *	P=0.0002 ***	P=0.2060	P=0.0054	P=0.9494
Age, Female	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:
	F (6, 20) = 2.219	F (6, 20) = 6.815	F (6, 20) = 3.259	F (6, 20) = 5.526	F (6, 20) = 2.943	F (6, 20) = 1.728	F (6, 20) = 1.394	F (6, 20) = 2.439	F (6, 20) = 2.924	F (6, 20) = 3.035	F (6, 20) = 2.425
	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:
	P=0.0838	P=0.0005 ***	P=0.0212 *	P=0.0016 **	P=0.0317 *	P=0.1662	P=0.2653	P=0.0620	P=0.0325 *	P=0.0282	P=0.0632

Table 18. A two-way ANOVA comparing the variances in sleep behaviors between genotype and age of female flies.

	Baseline Sleep	*Sleep, Recovery*	*Baseline Activity*	*Activity, Recovery*	*Day Sleep*	*Night Sleep*	*Day Bout Number*	*Night Bout Number*	*Day Bout Length*	*Night Bout Length*	*Sleep Delay*
Genotype	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:
	F (5, 18) = 0.8794	F (5, 18) = 1.286	F (5, 18) = 2.714	F (5, 18) = 1.996	F (5, 18) = 2.786	F (5, 18) = 4.204	F (5, 18) = 12.05	F (5, 18) = 19.86	F (5, 18) = 1.295	F (5, 18) = 11.36	F (5, 18) = 5.488
	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:
	P=0.5146	P=0.3130	P=0.0536	P=0.1281	P=0.0492 *	P=0.0105 *	P<0.0001 ****	P<0.0001 ****	P=0.3096	P<0.0001 ****	P=0.0031 **
Age, Male	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:
	F (6, 18) = 0.7337	F (6, 18) = 1.665	F (6, 18) = 1.945	F (6, 18) = 2.417	F (6, 18) = 1.545	F (6, 18) = 1.166	F (6, 18) = 3.901	F (6, 18) = 1.739	F (6, 18) = 0.7900	F (6, 18) = 1.429	F (6, 18) = 1.686
	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:
	P=0.6290	P=0.1870	P=0.1280	P=0.0685	P=0.2203	P=0.3670	P=0.0114 *	P=0.1692	P=0.5894	P=0.2580	P=0.1819

Table 19. A two-way ANOVA comparing the variances in sleep behaviors between genotype and age of male flies.

		Day 1 Sleep	*Day 2 Sleep*	*Day 1 Activity*	*Day 2 Activity*	*Day Sleep*	*Night Sleep*	*Day Bout Length*	*Night Bout Length*	*Day Bout Number*	*Night Bout Number*	*Sleep Delays*
Female Mated iso31^{KK} Flies	**Pearson r**	0.1397	-0.1165	-0.3631	0.03293	0.1464	-0.3223	-0.00841	-0.3633	0.2629	0.3050	0.4095
	95% CI	-0.0771 to 0.3438	-0.3228 to 0.1004	-0.5358 to -0.1613	-0.1828 to 0.2456	-0.07024 to 0.3498	-0.5020 to -0.1159	-0.2224 to 0.2064	-0.5359 to -0.1614	0.05136 to 0.4518	0.09694 to 0.4875	0.2139 to 0.5735
	P Value	0.2052	0.2913	0.0007 ***	0.7662	0.1840	0.0028 **	0.9395	0.0007 ***	0.0157 *	0.0048 **	0.0001 ***
Female Virgin	**Pearson r**	0.08522	0.1403	-0.1554	-0.2078	0.3666	-0.1811	0.1957	-0.3333	0.3318	0.3588	0.09934
	95% CI	-0.08894 to 0.2543	-0.03335 to 0.3058	-0.3196 to 0.01797	-0.3674 to -0.03624	0.2068 to 0.5073	-0.3432 to -0.008505	0.02360 to 0.3565	-0.4786 to -0.1702	0.1686 to 0.4773	0.1983 to 0.5006	-0.07480 to 0.2676
	P Value	0.3369	0.1127	0.0787	0.0181 *	<0.0001 ****	0.0400 *	0.0263 *	0.0001 ***	0.0001 ***	<0.0001 ****	0.2627

Female Mated		*Δ Sleep (Day 2 – Day 1)*	*Δ Activity (Day 1 – Day 2)*
	Pearson r	-0.2483	-0.4756
	95% CI	-0.4393 to -0.0358	-0.6262 to -0.2909
	P Value	0.0228 *	<0.0001 ****

Female Virgin		*Δ Sleep (Day 2 – Day 1)*	*Δ Activity (Day 1 – Day 2)*
	Pearson r	0.07903	-0.01128
	95% CI	-0.09513 to 0.2485	-0.1838 to 0.1619
	P Value	0.3733	0.8990

Table 20. Linear correlation between age and sleep behaviors of female flies within the genotype previously used in creating actograms. Comparing female iso31^{KK} flies that had previously been mated and those that were virgin.

	Baseline Sleep	*Sleep, Recovery*	*Baseline Activity*	*Activity, Recovery*	*Day Sleep*	*Night Sleep*	*Day Bout Number*	*Night Bout Number*	*Day Bout Length*	*Night Bout Length*	*Sleep Delay*
Mating Status	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:
	F (1, 3) = 6.808	F (1, 3) = 4.182	F (1, 3) = 21.04	F (1, 3) = 0.08120	F (1, 3) = 6.993	F (1, 3) = 5.117	F (1, 3) = 2.476	F (1, 3) = 2.113	F (1, 3) = 5.211	F (1, 3) = 0.008472	F (1, 3) = 0.7975
	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:
	P=0.0797	P=0.1334	P=0.0195 *	P=0.7942	P=0.0774	P=0.1087	P=0.2137	P=0.2420	P=0.1067	P=0.9325	P=0.4377
Age (iso31^{KK})	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:	F Statistic:
	F (3, 3) = 3.142	F (3, 3) = 0.7476	F (3, 3) = 0.3619	F (3, 3) = 1.165	F (3, 3) = 3.543	F (3, 3) = 10.16	F (3, 3) = 4.125	F (3, 3) = 7.379	F (3, 3) = 0.3329	F (3, 3) = 9.017	F (3, 3) = 2.530
	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:	P Value:
	P=0.1861	P=0.5916	P=0.7870	P=0.4516c	P=0.1632	P=0.0443 *	P=0.1374	P=0.0674	P=0.8048	P=0.0519	P=0.2330

Table 21. A two-way ANOVA comparing the variances in sleep behavior that were due to age and/or the mating status of female iso31^{KK} flies (flies that were previously mated or virgin when tested).

DISCUSSION

The disruption of sleep is commonly associated with age, often associated with disease or medication use, yet the implications this may have on one's health are often overlooked and underestimated. Besides common sleep deprivation disorders such as insomnia, even the changes in sleep associated with age in perfectly healthy adults can be disruptive- one such change being a decrease in the quality of sleep, i.e., increased sleep fragmentation. The purpose of this study was to ascertain a potential cause of this fragmentation by seeing if a decreased arousal threshold to external stimuli may cause wake more frequently in older flies.

The Effect Aging Has on Sleep Quality, Arousal Threshold, and Recovery

Based on the results of this study, older populations will generally experience an increased arousal threshold; however, aspects of sleep quality obtained are varied between both genotype and gender. Additionally, the expected lifespan of different genotypes may have some impact upon the amount to which aging affects arousability and reactivity observed from the presentation of a stimulus.

A similar study performed previously by Vienne et al., 2016, obtained results that were largely contrary to ours; they observed a decrease in arousal threshold with age, causing more sleep fragmentation. It is of note that the mechanical stimulus they presented was using a vortexer with an anti-vibration platform instead, therefore the type of stimulus presented may be an important differentiating factor [52]. Additionally, the ages tested in their assay were of 8-, 20-, and 35-day old Canton S and w118 wild type

flies and since no gender discrepancies were tested, flies were reared together, which may also be a factor in the difference in results.

Based on previous study results, arousability in sleep is directly related to the sleep depth (the degree to which sleep is consolidated) and the stimulus presented. In an experiment conducted by French et al., 2021, air puffs were used as a mechanical stimulation to wake male flies, paired with increasing concentrations of acetic acid- a substance that is produced by fermenting fruits that in low concentrations is highly attractive to *drosophila*, but in high concentrations less so. Comparably to a control or repulsive (very high) concentration, the flies that experienced the stimulus with a concentration of acetic acid that was attractive exhibited the strongest reaction [53]. These observations were dependent on time of day- between siesta sleep, early night sleep, and late-night sleep. Arousal threshold was highest during siesta sleep and sleep consolidation was greatest during early night sleep; despite this, flies were more likely to wake up to a salient stimulus during siesta sleep. In other words, while the flies were harder to wake, they were better able to unconsciously discriminate between a regular stimulus and an attractive one. Flies that were previously sedated or sleep deprived were showed less responsivity, and flies that were starved woke up more easily only when presented with food-related odors; showing that not only is the internal state of the fly is greatly related to arousal thresholds, but able to modulate the type of stimuli to become more sensitive to.

Studies that have tested animals' ability to receive sensory signals when asleep in the past have been very conflicting- some show that the thalamus blocks the processing

of all sensory information when asleep, when other studies show that sensory information are more weakly processed than when awake [54-56]. Even discrimination between auditory stimuli has been noted; a person will more likely rouse at the sound of their own name than someone else's. In a study by Issa and Wang, 2008, where auditory stimuli were used, they observed a bidirectional effect from sleep, where when stimulated, auditory neurons could present as more or less reactive than when stimulated during wake [55].

Based on this, it could be likely that the reason our data was different than previous studies of sleep and aging was due to the type of stimulus used, where our stimulus was not salient enough to overcome the internal state. The degree to which the internal state-mediated arousal is impacted by age is unknown.

Sleep Quality Observations to Previous Studies

In just the comparison of how sleep changes with age, our data is contradictory to the generally accepted notion that total sleep is decreased in older populations [52,57]. Our data does agree with previous studies that with age flies begin to rely on day sleep more and their ability to recover sleep is decreased; however specific differences in sleep architecture due to age were highly varied between both genotypes and genders, making it difficult to attribute one change to one aspect over another. Even when just comparing sleep characteristics between the flies with longer and shorter lifespans did not return any significant pattern. Our observations rely on the validity of the measurements recorded by the DAM software, which previous studies have suggested may overestimate the length

of sleep, especially day sleep, and may not accurately describe specific aspects sleep architecture [58,59]. This is especially true for the older populations of flies, which due to their lower baseline activity, it can be unclear as to whether or not they are sleeping or simply not moving.

Future Implications

Generally, for future studies it may be useful to obtain data using video capture software in order to best overcome any differences in general activity that may present with age; older flies showed decreased activity overall, and it could well be that the 5-minute sleep threshold included periods where older flies were awake and just not moving [59]. Additionally, our observation of large genotypical and gender differences in sleep architecture warrants broader exploration into- if effects of aging apply differently between genotypes, it brings into question how their different sleep architectures may contribute to their health and lifespan.

Although previous studies that compare flies raised at 21^{o} C vs 25^{o}C have noted that the effects of aging on sleep is consistent with physiological age [52,60], the variation in sleep changes that presents with genotypes with worse survivability may warrant further exploration on sleep when raised at 21^{o}C, as temperature regulation is directly affected by sleep and the lifespan of *Drosophila* is directly linked to the temperature they are housed at. There is certainly no shortage of factors to consider when applying the methods of one study to others.

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