Dozing Off With Drosophila: The Effect of Disrupted Circadian Rhythms and Sleep Disturbance on Mortality, Mood, and Addiction

Neuroscience Insights
Volume 18: 1–6
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DOI: 10.1177/26331055231218698

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ABSTRACT: Many environmental factors can disrupt sleep and circadian rhythms, yet the consequences of such disruptions are poorly understood. The main goals of this project were to study the effects of disrupted circadian rhythms and sleep disturbance on *Drosophila melanogaster's*: (1) lifespan, (2) depression-like behaviors, and (3) propensity to consume caffeine-containing media. Three experimental groups were used: controls, Circadian Dysfunction (CD), and Sleep Disturbance (SD). Circadian disruption (CD): used flies with Tim^{01} mutation, which eliminates circadian behavioral rhythms. Sleep disturbance (SD): used flies subjected to hourly light exposure and manual mechanical disruption, for 48 hours. To assess the effect on lifespan, the percent of flies surviving over time, within each group, was calculated. Impaired geotaxis, or loss of climbing motivation, was assessed as a measure of a depression-like state. Preference for caffeine-containing food was evaluated using a choice chamber where caffeine enriched, and regular media were presented to flies. Group differences were analyzed with survival curves. Chi-square tests were used for the categorical variables. Survival curve analysis showed that Flies with the *timeless* gene mutation (tim^{01}) have a significantly shorter lifespan than controls. Geotaxis was not significantly impaired by sleep disturbance, but it was negatively affected by circadian dysfunction. Both the Circadian Dysfunction and Sleep Disturbance groups showed a preference for caffeine-containing food, after 72 hours of exposure to it, although the Circadian Dysfunction group was much more affected than the Sleep Disturbance group. Sleep and circadian disturbances can negatively influence physical and mental wellbeing and the accompanying molecular mechanisms, as well as disrupted brain physiology, must be studied. It is critical to identify and minimize social and environmental disruptors of such biological rhythms.

KEYWORDS: Drosophila, circadian rhythms, depression, sleep, addiction, behavior, mortality

RECEIVED: July 20, 2023. **ACCEPTED:** November 20, 2023.

TYPE: Original Research

FUNDING: The author(s) received no financial support for the research, authorship, and/or publication of this article.

DECLARATION OF CONFLICTING INTERESTS: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Introduction

Circadian rhythms are daily rhythms in behavior or physiology that reoccur approximately every 24 hours. Biologic clocks produce circadian rhythms and regulate their timing but external environmental cues (*Zeitgebers*), especially daylight, can also affect circadian rhythms. Humans and other mammals have a master clock in the brain called the suprachiasmatic nucleus, or SCN, which is located in the hypothalamus and receives direct input from the retina in our eyes. The SCN then coordinates all the other biologic clocks located in various tissues and organs keeping them in sync. While the sleep-wake cycle is one of the most common and obvious circadian cycles, many others exist such as body temperature, hormone release, eating habits, and digestion. When circadian rhythms are disrupted, it can affect everything from learning and memory to metabolic and cardiovascular disease.[1]

Changes in our body and environmental factors can cause our circadian rhythms and the natural light-dark cycle to be out of sync. For example, mutations or changes in certain genes can affect our biological clocks. Jet lag or shift work causes changes in the light-dark cycle. Light from electronic devices at night can confuse our biological clocks. These changes can cause sleep disorders and research suggests this may lead to other chronic health conditions, such as obesity, diabetes, and mental health disorders such as depression and anxiety.[2] The increasing use of mobile technology and social media, especially among youth, is having a profound impact on their sleep habits and circadian rhythms.[3] According to the Centers for Disease Control, data from the Youth Behavior Risk Surveillance Data from 2007, 2009, 2011, and 2014 (N = 50 370 US students) found that two-thirds of students in grades 9 to 12 reported 7 hours or less sleep on school nights.[4]

Many human studies typically use short perturbations in sleep, and they are unable to directly measure the long-term consequences of chronically disturbed sleep on various aspects of physical and mental functioning. Systematically assessing the impact of altered sleep physiology in mammalian models is difficult due to ethical concerns, high costs, and their relatively long-life spans. However, *Drosophila melanogaster* or the common fruit fly has the potential to reveal the complex neuroanatomy and neurophysiology involved in sleep and circadian cycles. Due to evolutionary conservation, the neurochemistry of Drosophila sleep-wake systems parallels those that exist in mammalian organisms[5] and they share many of the sleep and wake-promoting neurotransmitters and neuropeptides, including dopamine and adenosine, with mammalian species.[5] In

fact, the master gene, called "period," that controls circadian rhythms in all mammals was first isolated in fruit flies.[6] Also, sleep in flies closely resembles sleep in larger organisms and is marked by measurable periods of quiescence.[7]

Circadian behavioral rhythms in Drosophila depend on the appropriate regulation of at least 2 genes *period* (or *per*) and *timeless* (or *tim*).[8] The level of PER protein follows a 24-hour cycle, increasing at night and decreasing during the day. The PER protein is coded for by the *period* gene at night. In the cytoplasm, the PER protein couples with the TIM protein, allowing it to enter the nucleus. When inside the nucleus, PER inhibits its own production. During the day, PER is slowly destroyed. As night approaches, the amount of PER in the cell is so low that the whole cycle begins again, and the production of PER resumes. The whole cycle takes 24 hours. Flies with the tim^{01} mutation, which harbors a deletion of 64 bp causing a frameshift in the coding sequence, have severely impaired circadian behavioral rhythms.[9]

Disturbances in sleep and circadian rhythms are well-documented in people who suffer from mental disorders[10] as well as substance use.[11] However, the exact direction of this relationship is hard to discern. Interestingly enough, insect behavior is also organized and driven by reward. Studies have established *Drosophila melanogaster* as an excellent model to explore drug addiction.[12] Such studies utilized ethanol and assessed voluntary consumption.[13] There has also been extensive literature demonstrating that fruit flies respond to caffeine much like mammals do.[14] Thus, a fly model may provide a unique opportunity to study the risk of drug addiction after disrupted circadian and sleep physiology, using the stimulant caffeine as a potential substance. Similarly novel protocols have established stress-induced depression-like states in *Drosophila melanogaster*[15] and feelings of hopelessness and anhedonia have been proposed as core manifestations of this depression in flies. Thus, behavioral despair tests are popular assays for the assessment of animal models of depression phenotypes.[16]

In this study, we hypothesized that circadian dysfunction (CD) and sleep disturbance (SD) will lead to measurable negative impacts on *Drosophila melanogaster's* lifespan, increased propensity of flies to consume caffeine-containing food, and depression-like behaviors such as an impaired response to gravity or altered geotaxis.

This study used a mutant form of *Drosophila* with a clock mutation, *timeless* (tim^{01}), that produces flies with severely disrupted circadian rhythms.[17]

Materials and Methods

Fly Stocks, wild-type (Samarkand strain; BDSC Ref. no 4270), and Timeless (y[1] w[*]; tim[01]; BDSC Ref. No 80922), were obtained from Bloomington Stock Center (Bloomington, Indiana) and raised at 23°C on standard cornmeal-molasses medium. Flies used for experimentation were 10 to 12 days post eclosion and all flies were subjected to 12 hour:12 hour Light: Dark cycle conditions.

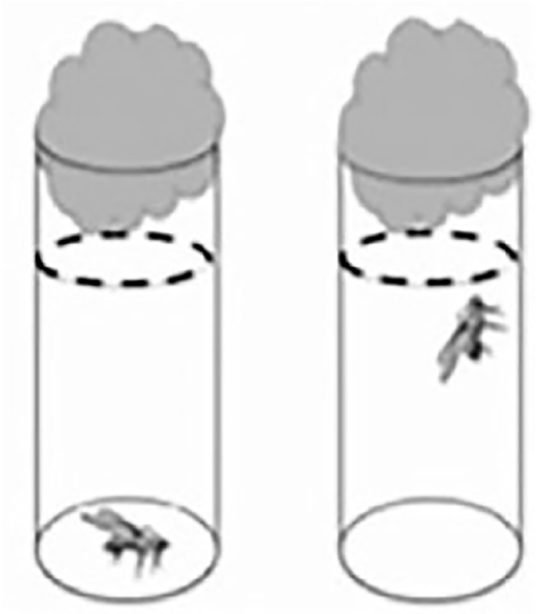

Figure 1. Geotaxis assay[19] to assess lack of motivation or depression like states.

Mechanical sleep deprivation has been a standardized way to induce sleep deprivation in flies and involves the use of the Drosophila Activity Monitors (DAM), which displaces flies 2 seconds randomly within every 20 seconds window.[18] Such automated methods provide reliable sleep deprivation for prolonged periods of time but may not necessarily reflect milder sleep disturbances that occur commonly among humans. In this study, we evaluated a model of sleep disturbance where flies were subjected to manual mechanical disturbance for 20 seconds each hour, for a 48-hour period.

Longevity

Twenty to 30 adult flies were placed in each of 3 vials, per condition (control, CD, and SD). Flies were transferred to new vials every 3 days to avoid including their offspring in the longevity count. Flies were counted daily, and the mean number of dead flies, living flies, and the percentage of surviving flies were recorded.

Negative geotaxis or depression-like state

Negative geotaxis is defined as the motion in response to the force of gravity.[19] Flies placed in a vial were tapped to the bottom and were given 10 seconds to demonstrate negative geotaxis by migrating upward to a line 2″ below the vial lid. The number of flies above the demarcated line, at 10 seconds, was recorded (Figure 1)[19].

Caffeine addiction

Caffeine tablets were crushed and boiled with water at a concentration of 0.5 mg/ml consistent with previously established literature.[20] When individual caffeine particles had limited

visibility, the solution was then cooled while the standard medium (as aforementioned) was prepared for the 3 experimental groups. For each condition, a choice chamber was constructed as shown below (Figure 2).

Each choice chamber had 2 options for the cohort of flies: caffeinated medium or un-caffeinated medium. The caffeinated vial combined the standard *Drosophila* medium with the prepared caffeine solution (ratio 1:1); the un-caffeinated vial combined the standard medium with distilled water (ratio 1:1). After vials were prepared and inserted into the PVC pipe, the appropriate cohort of flies was introduced via the remaining opening.

Flies were given a 20-minute acclimation period before recording the first observation. Each observation noted the number of flies in each section of the chamber (including the middle). In a study researching preferential ethanol consumption in Drosophila, the relative preference for ethanol increased over the course of 3 to 5 days.[12] Therefore, we took 1 observation each day for 3 successive days.

For the longevity outcome, data were tabulated and graphed on survival curves using Microsoft Excel. For the depression and addiction assays, raw data was taken, and a chi-square analysis was conducted for each experimental group. The odds ratio is the relative odds of the occurrence of the outcome of interest given exposure to our variable of interest. The MedCalc software was used for this calculation.[21]

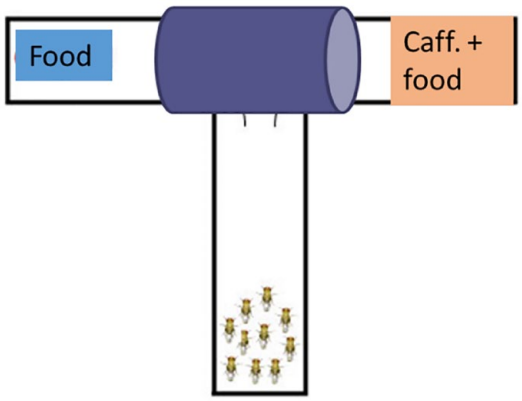

Figure 2. Choice chamber to assess preference for caffeine-containing food.

Results

Longevity

Survival curves were plotted using a Kaplan Meir model and showed that circadian dysfunction significantly decreased *Drosophila* lifespan while the effect was not as pronounced for sleep deprivation (Figure 3). Additionally, the time taken for 50% of the flies to die was 7.2, 13.5, and 15.3 days among the CD, SD, and control groups, respectively.

Geotaxis assay

As shown in Table 1 and Figure 4, 41.7% of flies with circadian dysfunction demonstrated impaired geotaxis compared with 17.9% percent of control flies (OR = 3.26, $P = .024$). Sleep-deprived flies did not appear as much affected when compared to control flies (OR = 1.89, $P = .239$).

Propensity to caffeine

As shown in Table 2 and Figure 5, significantly more flies were observed in the vial containing caffeinated medium, for both the circadian dysfunction and sleep disrupted groups. More specifically, compared to the control where about 13% of flies were on the caffeinated side, the CD group had 74% of flies in the caffeinated medium and they were 26 times as likely to prefer caffeinated media compared to the controls ($P < .001$). The sleep-disrupted group was also more likely to prefer caffeine-containing media compared with the controls, but they were not as affected as the circadian mutants (Odds Ratio = 5.4, $P = .017$). No significant difference was seen between days 1 through 3, post-exposure to caffeine.

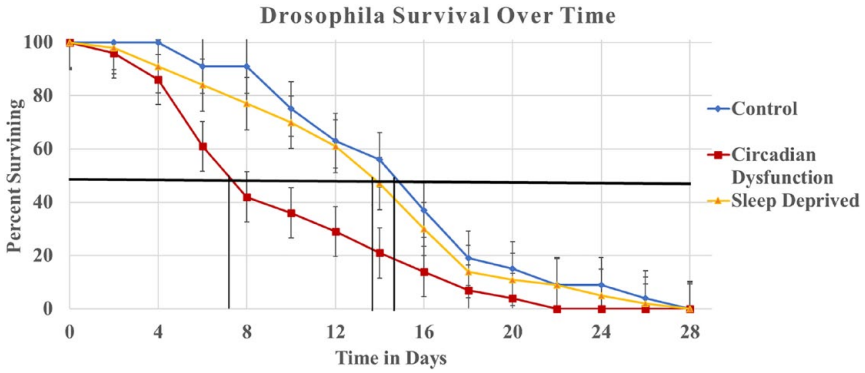

Figure 3. Percent flies surviving over time.

Table 1. Proportion of flies with impaired geotaxis/depression like state, by group.

FLY COHORT	N/TOTAL (%)	ODDS RATIO, RELATIVE TO CONTROL (95% CI)	*P* VALUE
Controls	7/39 (17.9%)	1.00	N/A
Circadian mutants	15/36 (41.7%)	3.26 (1.14-9.36)	.024
Sleep disrupted	13/45 (28.9%)	1.89 (0.65-5.26)	.240

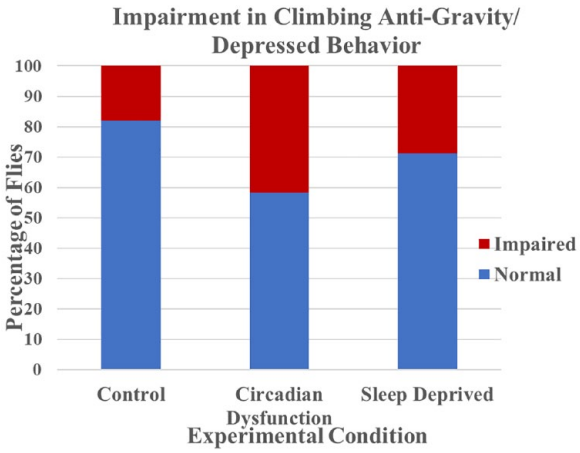

Figure 4. Indicated above is the percentage of flies with impaired geotaxis for the various groups.

Discussion

The purpose of this study was to utilize *Drosophila melanogaster* as a model organism and study the effects of circadian disruption and sleep disturbance on mortality, mood and addiction-like behaviors. Flies with the *timeless* gene mutation (tim^{01}) have a significantly shorter lifespan. Circadian mutants had severely impaired negative geotaxis or increased depression-like behavior. Both sleep-deprived and circadian mutant flies preferred the caffeinated medium after 48 hours, indicating increased risk for substance use among both these groups.

Circadian dysfunction, sleep disturbance, and mortality

Results from this experiment indicate a strong association between circadian dysfunction and mortality, in flies. It is known from human studies that many biological functions are under circadian control, including hormonal activity, blood pressure, and heart rate, as well as sleep-wake cycles.[1,22] Also, altered circadian rhythms such as seen in night shift workers have been associated with a higher risk of breast cancer, cardiovascular disease, and metabolic derangements.[23] Despite multiple studies linking altered circadian physiology with various diseases,[24,25] there is limited evidence for a clear association between disrupted circadian rhythms and mortality. Data from this study establishes a clear link between circadian dysfunction and increased mortality. The association is likely explained by the desynchronization of various internal clocks in the body[26] which can ultimately cause many physiologic alterations and induce pathology.[27-31] The sleep-disturbed group did not show a significant increase in mortality possibly because it involved the disruption of only 1 circadian cycle. Moreover, while tim^{01} is an established model of circadian dysfunction, the hourly manual sleep disruption used in this experiment may not be the ideal way to model sleep disturbance.

Circadian dysfunction, sleep disturbance, and mood

Both circadian mutants and sleep-deprived flies showed impairment in geotaxis but only the circadian group differed significantly from the controls. Negative geotaxis in flies is the innate motivation to climb vertically when startled; impairment in this response has been observed in flies with lower serotonin and octopamine levels,[32] which are associated with a depression-like state in flies.[15,33] While it is possible that impaired climbing behavior in this study was related to decreased endurance, previous studies in *Drosophila* have shown that depression does not cause diminished general activity.[15] Studies have consistently shown that depressed individuals have altered sleep-wake cycles, but it is not clear if depression precedes altered sleep or vice versa. Studies in rodents have shown that experimentally induced circadian dysfunction can lead to mood changes and resynchronization of circadian rhythms can improve mood symptoms.[34] Along with such prior data, the current experiment suggests that while sleep and circadian disturbances may not be the sole cause of depression, they may produce or worsen symptoms in subjects with a predisposition to mental health problems.

Circadian dysfunction, sleep disturbance, and addiction

Like mood disorders, substance use has been linked to disordered sleep. Flies with both circadian dysfunction and sleep disturbance showed an increased propensity towards caffeine-containing food. These results are consistent with prior literature that reports that flies lacking the timeless gene can sensitize to substances such as cocaine.[35] Our sleep disturbance findings correlate with longitudinal human studies that show youth who sleep less go on to consume more cigarettes and alcohol in later years.[36] Research suggests that sleep deprivation can downregulate dopamine D2 receptors that are involved in wakefulness.[37] This in turn could cause behaviors leading to drug use, which produces huge amounts of dopamine, to compensate for this deficit. Caffeine in particular increases the

Table 2. Proportion of flies in the caffeinated chamber (after 72 hours), by group.

FLY COHORT	N/TOTAL (%)	ODDS RATIO, RELATIVE TO CONTROL (95% CI)	*P* VALUE
Controls	5/36 (12.8%)	1.00	N/A
Circadian mutants	17/23 (73.91%)	26.3 (6.23-111.4)	<.001
Sleep disrupted	7/16 (43.75%)	5.43 (1.36-21.70)	.017

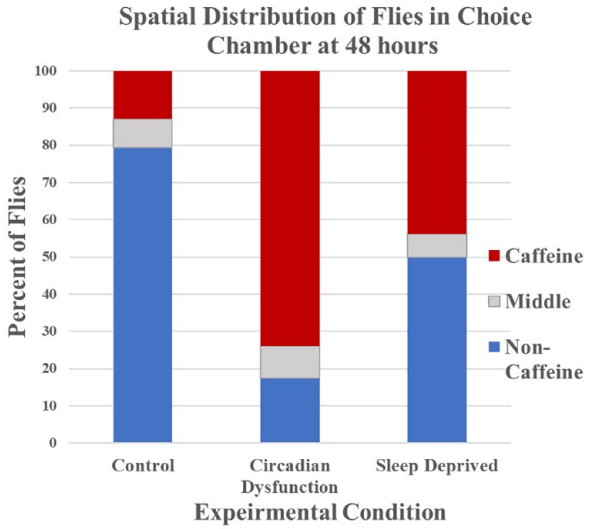

Figure 5. Proportions of observed flies in regions of the choice chamber are shown.

dopamine D2/D3 receptor availability in the brain,[38] which may explain our experimental findings. Also, human research indicates that disturbed sleep can affect all phases of the addiction cycle including initiation, maintenance, and relapse.[39]

We fully recognize the limitations of our model of mechanical sleep disturbance but also suggest that it is more consistent with human life scenarios which do not typically involve a complete lack of sleep for days at a time. But we acknowledge that utilizing systems such as the Drosophila Activity Monitoring device can allow more precise characterization of sleep-wake cycles and activity levels.

Findings from this study have serious implications for various environmental disrupters of circadian dysfunction such as shift work, jet lag, and nighttime exposure to artificial light. About 36% of adults and 34% of children sleep with a light-producing electronic device, such as a television or computer.[40] Exposure to light at night confuses the circadian system because light is the body's cue to distinguish day from night. Mistimed light exposure can severely desynchronize biological and behavioral rhythms causing many negative health consequences.[41]

Our results underscore the importance of intact circadian rhythms and sleep-wake cycles to physical and mental health. Future studies must evaluate genetic and physiologic mechanisms underlying poor outcomes associated with disrupting sleep and circadian rhythms, thereby allowing targeted interventions. It is also a public health imperative to identify and prevent societal causes of circadian and sleep disruption, as it can have deleterious consequences across the lifespan and in multiple biological systems.

Author Contributions

Rania Lateef has contributed to the conception and design of the research study, data collection, data analysis and interpretation, and drafting the article as well as revising it.
Bibhaw Pokharel has contributed towards research design, data analysis and interpretation, and critical revision of the article.
Tasnuva Shafin has contributed towards data interpretation, drafting the article, critical revision of the article.

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