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LIGHT EXPOSURE'S EFFECTS ON INACTIVE STATE DURATION AND SLEEP LATENCY IN ZEBRAFISH (DANIO RERIO) LARVAE INSOMNIA MODEL

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ABSTRACT

Background: Insomnia is defined as difficulty falling or staying asleep or a sleep state that cannot restore the body's condition. The zebrafish (Danio rerio) is a vertebrate model that has been extensively studied to study sleep and neurological disorders. One of the most widely used methods to examine the effect of the light-dark cycle on the circadian system is by exposing animals and humans to pulse wave light.
Objective: To see the effect of light exposure on zebrafish larvae by looking at inactive state duration and sleep latency in zebrafish *(Danio rerio)* larvae insomnia model.
Methods: This study used four groups of zebrafish larvae i.e : (1) normal group (2) minutes of light exposure and 2 minutes off (2/2)), (3) Four minutes of light exposure and 1 minute off (4/1), and (4) 24 hours on (24/0). Observation of larval movement was carried out on 5, 6, and 7 dpf (day post fertilization). Observation time was 30 minutes before and after turned off of light exposure.
Results: There were significant differences on days 5, 6, and 7 between the normal group and the three light treatment groups on inactive state duration and sleep latency in dark and light conditions with p-values ($p<0.05$) and ($p< 0.031$), but there was no significant difference among groups of light exposure 2 minutes on 2 minutes off, 4 minutes on 1 minute off, and 24 hours on. The 24-hour on treatment showed the most inactive state duration among the light treatments, while the sleep latency was found in the 24-hour treatment.
Conclusion: Light treatment of 2 minutes on 2 minutes off, 4 minutes on 1 minute off, and 24 hours on can cause insomnia, but the most substantial insomnia effect is obtained from the 24-hour treatment.

Keywords: Insomnia, zebrafish, inactive state duration and sleep latency

Introduction

The zebrafish (*Danio rerio*) is a vertebrate model that has been extensively studied to study sleep and neurological disorders. The zebrafish is a diurnal vertebrate with a simple nervous system that represents an overview of the organization and function of the brain. Zebrafish larvae brain is transparent, and it can draw from single cells and synapses. Over the past two decades, the advantages of this diurnal animal model have attracted the attention of sleep researchers. Research studies of sleep-in zebrafish show advantages over mammals, particularly the presence of voluntary movement, circadian rhythm, reversibility, and homeostasis control.[1-4]

The neuroanatomical and neurochemical systems that regulate sleep and arousal in mammals are primarily found in zebrafish. One crucial difference is that zebrafish do not possess midbrain dopaminergic neurons analogous to the mammalian Ventral Periaqueductal (vPAG) and Ventral Tegmental Area (VTA), which are known to play a role in the sensorimotor function and sleep disturbances in humans. Zebrafish are known to have brain areas that are thought to have areas homologous to dopamine clusters in the ventral diencephalic of zebrafish. Zebrafish lack a layered cortex, which is the main target of the arousal system in mammals. However, homology between mammalian cortical regions and zones in the zebrafish dorsal telencephalon is well known. The cholinergic system in the forebrain and brainstem has not been clearly delineated in zebrafish larvae.[5]

Light is an external timepiece. It will reach the Suprachiasmatic Nucleus (SCN) via afferent projections from the retina through the retinohypothalamic tract. Recent evidence suggests that the primary circadian photoreceptors are retinal ganglion cells containing melanopsin, which transmit light information by projection to the SCN. While circadian rhythms can be synchronized with light-dark cycles that are not precisely 24 hours in duration, synchrony is limited to cycles with periods that are "close to" 24 hours in duration. The range of alignment may vary from one species to another. It depends on experimental conditions (e.g., the intensity of the light-dark cycle, whether its period changes gradually or rapidly), but animals generally cannot combine light-dark cycles of several hours shorter or longer than the endogenous circadian rhythm period. If the cycle period is too short or too long for a union to occur, the circadian rhythm is free flowing, with periods of endogenous pacemaker interspersed.[6]

One of the most widely used methods to examine the effect of the light-dark cycle on the circadian system is by exposing animals and humans to light pulses. The effect of light pulses on the reference point phase of the circadian rhythm (e.g., melatonin onset, minimum body temperature) is determined in the next cycle. The direction and magnitude of the phase shift are highly dependent on the circadian rhythm in which the light pulses occur. Recent findings in humans, both light-induced phase and melatonin suppression, are both susceptible to 460 nm light waves, which can be used as the basis for developing light therapy in the treatment of circadian rhythm sleep disorders.[2,3,6]

In this study, zebrafish will be exposed to light. Like humans, zebrafish sleep at night as opposed to mice that sleep during the day. The characteristics of zebrafish activity during sleep are that the adult ones stop swimming for 6 seconds and the larvae stop for 1 minute—they do not move at the bottom or surface and are less sensitive to external stimuli (such as electric currents). As we know, zebrafish have sleep patterns similar to mammals and humans. When zebrafish experience sleep disturbances, a rebound effect can occur, such as when zebrafish experience sleep disturbances for some time, the fish will oversleep. Exposure to light can cause zebrafish not to sleep at all because zebrafish are sensitive to light. [5-7]

The development of zebrafish larvae within a few days begins with swimming in brief locomotor phasic episodes. The high-speed infrared video capture combined with computational image analysis has been used to quantitatively describe specific locomotor behavior in zebrafish larvae, for example, by measuring the values of variable indicators, such as those characterizing animal posture (tail location and amplitude, turning angle, turning motion) and time (tail-beat frequency, swimming speed).[5,9,10]

Insomnia can be classified as one a sleep disorder. It comes in the form of sleeping difficulty that meets diagnostic standards. According to the fifth edition of The Diagnostic and Statistical Manual of Mental Disorders (DSM-5), insomnia is defined as difficulty in initiating sleep, maintaining sleep, or a state of sleep that is unable to adequately restore physical and mental states associated with disturbances during the day that lasts at least 4 weeks. Insomnia can be a primary disorder or comorbid to other physical and mental illnesses.[11] One of the criteria for insomnia is measured by sleep latency time, the time it takes to fall asleep.[12]

The prevalence of insomnia varies widely from 2% to 48% in adults and from 7% to 40% in young adults. This wide range of differences is influenced by conceptual differences and how insomnia is diagnosed.[13] A survey of the Australian population showed that 13-33% of the adult population had difficulty initiating or maintaining sleep.[11]

An ideal insomnia model should describe the characteristics of insomnia in humans. Animal models of insomnia reduce the amount or quality of sleep. In zebrafish, the quiescent state is regulated by a circadian rhythm that begins about 4 days post-fertilization (dpf). It is reared at 24 hours with alternating light, 14 hours dark, and 10 hours light, indicating daily fluctuations in locomotor activity. An essential difference between zebrafish and mammals is that the zebrafish's peripheral circadian clock is directly controlled by light, whereas mammals require the SCN to regulate circadian rhythms. The study stated that the test to see the regulation of sleep homeostasis by observing the existence of sleep compensation that occurs if the zebrafish lacks sleep is a vibration stimulus given for 6 hours produces sleep compensation the next day. Both larval and adult zebrafish show sleep rebound.[5, 14-16]

A study had tried to validate creating a model for sleep deprivation. Two models were made, first (1) extending the light phase and (2) exposing the zebrafish to a bright flash of light during the dark phase. The research showed that light was a strong stimulus for disrupting sleep. Light can be used as a continuous stimulus for 24 hours without a dark phase or with 18 hours of light exposure and 6 hours of flashes of light during the dark phase with 2 x 2 minutes (light x dark) or 4 x 1 minute (light x dark) to disturb the fish's sleep.[5, 14-16]

Methods

This was a laboratory true experimental, randomized control group post-test only using 8 wild types of zebrafish (standard short-fin phenotype) aged 0 - 7 dpf. Zebra fish was obtained from Fakultas Perikanan Universitas Brawijaya. Divided into 4 groups, i.e., group 1 control given normal light exposure 12 hours on 12 hours off, group 2 given light exposure for 24 hours (24/0), group 3 given light exposure 2 minutes off and 2 minutes on for 6 hours and 18 hours on (2/2). Group 4 light exposure 4 minutes on and 1 minute off for 6 hours and 18 hours on (4/1). This study has been approved by the Health Research Ethics Committee of the Faculty of Medicine, University of Brawijaya, Ethics Permit No: 147/EC/KEPK-S3/06/2021.

Light exposure preparation

A lamp of 200 lux was used for light exposure on insomnia model of zebrafish larvae[17]. The light exposure device consisted of an on and off switch, an adapter, a setting button for dark and light, and a light intensity control button. The lamp setting button featured 3 automatic light-on time modes. In mode 1, the light would be on for 24 hours; in mode 2, the light would be on for 18 hours, then it would be on for 6 hours with 2 minutes on and 2 minutes off; in mode 3, the light would be on for 18 hours then 6 hours with 4 minutes on and 1 minute off.

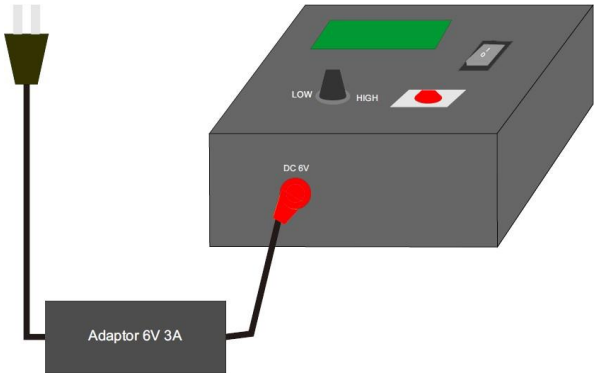
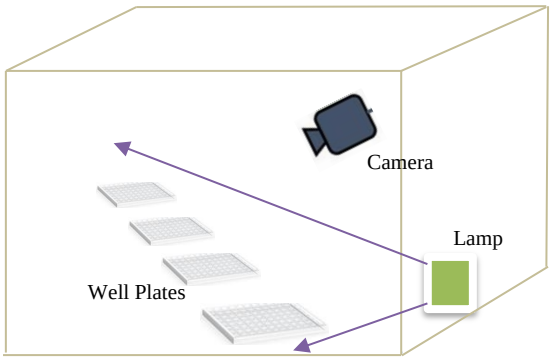


Figure 1. Light exposure lamp design by author



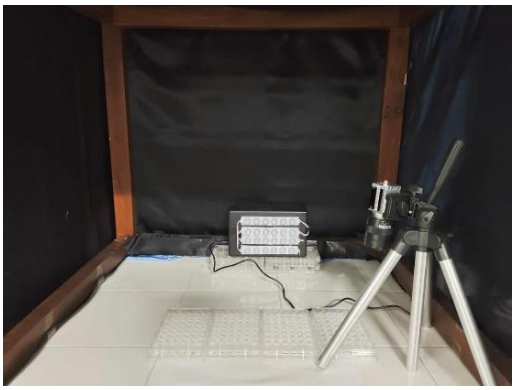


Figure 2. Illustration of a black box cover so that other lights do not interfere with the camera on the top and the light on the bottom design by author.

Figure 3. 48 well plates containing 600 ul of water.

To ensure that outside light does not affect the light-emitting lattice, we made a box 80 cm long, 70 cm wide, and 60 cm high. It is, then, covered with black cloth so that air circulation can still enter. Light exists only from the lamp.

Zebrafish behavior analysis

Sleep latency and inactive state duration were measured on 48 well plates, each containing 600 ul of water. Zebrafish larvae were allowed to move actively. They were observed and recorded using a camera for 60 minutes, divided into 30 minutes before the lights were turned off and 30 minutes after. Fish larvae movements were analyzed from video recordings using Ethovision XT software.

Zebrafish larvae experience insomnia when the rest (sleep) latency is more than 20 minutes. the zebrafish larvae will remain motionless and not move still for at least one minute over a 20-minute period. when the zebrafish is immobile for 1 minute or more for more than 20 minutes the zebrafish larvae have insomnia. Rest (sleep) latency is when the lights are turned off and the first *rest bout* period appears. *Rest (sleep) bout/latency to first/sleep latency* is the time of inactivity or no movement for at least 1 minute.[12] Inactive state duration is the total length of time the fish is asleep or in an inactive condition.

Processing and Analysis of Data

All data were analyzed using SPSS 25, a non-parametric test. Data that were not normally distributed and not homogeneous were analyzed using Kruskal Wallis statistical analysis to determine the difference between the treatment groups provided. If the p-value < 0.05, it was considered statistically significant.

Results

Movement of fish larvae was calculated from video recordings of zebrafish larvae's activity and sleep position. The activity of zebrafish larvae during sleep is characterized by cessation of swimming for at least 1 minute and immobility on the bottom or surface of the water.

The movement inacitivity and total of duration inactive analysis of zebrafish larvae on days 5, 6, and 7 from several groups, namely normal, light exposure of 2 minutes on and 2 minutes off (2/2), light exposure of 4 minutes on and 1 minute off (4/1), and 24 hours on (24/0) results in a table of Activity State Inactive Inactive State Duration as follows.

Based on Table 1, the 24-hour treatment in light and dark has a lower value of Activity State Inactive State Duration than the control group. However, its value is slightly different compared to all light treatment groups, namely 1) 2 minutes on and 2 minutes off (2/2), and 2) 4 minutes on and 1 minute off (4/1). The average 24-hour group (light and dark) possesses values of 430.6047 ± 235.35 per second (for the light cycle) and 655.068 ± 372.903 per second (for the dark cycle) on days 5, 6, and 7. Meanwhile, the 2m on 2m off (light and dark) treatment has values of 649.1679958 ± 351.3376 per second (for light cycle) and 404.9068708 ± 325.4842 per second (for dark cycle). 4m on 1m off (light and dark) treatment has values of 431.3954833 ± 267.4725 per second (for light cycle) and 460.74955 ± 321.75 per second (for dark cycle). These results indicate that light exposure treatment might lead to insomnia with different Activity State Inactive State Durations on zebrafish larvae. Moreover, all light treatment groups have distinguished optimum qualities in triggering insomnia symptoms, and the 24-hour light group appears as the most effective treatment to quicken the symptoms (Figure 1-2).

Table 1. Average of Activity State Inactive State Duration Using Kruskal-Wallis Statistical Analysis on Zebrafish Larvae

Treatment	Total of Sample	Mode	Average of Activity Inactive State Duration ± SD	p-value
normal	8	light	549.5863583 ± 411.2932[a]	**0.001**
normal	8	dark	1115.966042 ± 481.9892[b]	
24 hours	8	light	430.6047 ± 235.35[a]	
24 hours	8	dark	655.068 ± 372.903[a]	
2m on 2 m off	8	light	649.1679958 ± 351.3376[a]	
2m on 2 m off	8	dark	404.9068708 ± 325.4842[a]	
4m on 1 m off	8	light	431.3954833 ± 267.4725[a]	
4m on 1 m off	8	dark	460.74955 ± 321.75[a]	

Description: On the average ± sd, the appearance of different letters implies a significant difference ($p < 0.05$). Meanwhile, the same letters indicate no significant difference ($p > 0.05$).

Table 2. Average of Sleep Latency Using Kruskal-Wallis Statistical Analysis on Zebrafish Larvae

Treatment	Total of Sample	Mode	Average of Activity state	Treatment
normal	8	light	716.159017 ± 520.71a	**0.031**
normal	8	dark	287.5468788 ± 362.10a	
24 hours	8	light	1090.128216 ± 304.27a	
24 hours	8	dark	845.1732331 ± 313.22a	
2m on 2 m off	8	light	708.820765 ± 529.99a	
2m on 2 m off	8	dark	741.8499096 ± 477.96a	
4m on 1 m off	8	light	742.848536 ± 571.06a	
4m on 1 m off	8	dark	550.5247291 ± 511.55a	

Description: On the average ± sd, the appearance of different letters implies a significant difference (p < 0.05). Meanwhile, the same letters indicate no significant difference (p > 0.05).

Figure 3 and 4 demonstrates that the normal group has constant values; the difference in the Inactive State Duration on days 5, 6, and 7 is not far apart. If it is compared to light treatment groups, namely 24-hour (light and dark), 2m on 2m off (light and dark), 4m on 1m off (light and dark), each group has varied values with high standar deviation/standard errors on light and dark cycles.

Sleep Latency

The movement analysis of zebrafish larvae on days 5, 6, and 7 from several groups, namely normal (light and dark), 24-hour (light and dark), 2m on 2m off (light and dark), and 4m on 1 off (light and dark) results in a table of Sleep Latency as follows.

Table 2 shows that the 24-hour treatment in light and dark has a higher value of Sleep Latency than the control group and all light treatment groups—namely 2m on 2m off (light and dark) and 4m on 1m off (light and dark). The average values of the 24-hour group (light and dark) are 1090.128216 ± 304.27 per second (for the light cycle) and 845.1732331 ± 313.22 per second (for the dark cycle) on days 5, 6, and 7. Meanwhile, the values of 2m on 2m off group (light and dark) are 708.820765 ± 529.99 per second (for light cycle) and 741.8499096 ± 477.96 per second (for dark cycle). The last, 4m on 1m off (light and dark) values are 742.848536 ± 571.06 per second (for light cycle) and 550.5247291 ± 511.55 per second (for dark cycle).

These results indicate that 24-hour (light and dark) treatment effectively leads to insomnia symptoms such as difficulty maintaining sleep condition and massive movement or activities at night. Meanwhile, 2m on 2m off (light and dark) and 4m on 1m off (light and dark) treatments can also trigger insomnia symptoms with different Sleep Latency on zebrafish larvae. It can be concluded that all light treatment groups have distinguished optimum qualities in triggering insomnia symptoms, and the 24-hour light group appears as the most effective treatment to faster the symptoms (Figure 5-6).

Figures 7 and 8 exemplify that the normal group has constant values; the difference of the Sleep Latency values on days 5, 6, and 7 is not far apart. If it is compared to light treatment groups, namely 24-hour (light and dark), 2m on 2m off (light and dark), 4m on 1m off (light and dark), each group has varied values with high standar deviation/standard errors on light and dark cycles. However, it is proved that 24-hour (light and dark) treatment has a higher value than other groups in this study. Therefore, this treatment effectively generates insomnia symptoms such as difficulty in sleep maintenance and active movement or activity at night.

Discussion

Zebrafish is an ideal Vertebrata to dissect and analyze sleep disorders and neurological conditions. It is diurnal with relatively simple nervous systems, represents good brain function, and is fitted for genetic manipulation. The Zebrafish brain is transparent so that it attractively shows single cells and synapses during day and night, optokinetic experiments, and markers[3]. This study demonstrates that light exposure treatment can cause insomnia—confirming studies by Yokogawa[18]. Light exposure can be utilized as an intense insomnia stimulus to promote sleep disorders[12].

Inactive State Duration

Inactive State duration is the total duration of sleep (inactive). When sleeping, zebrafish shows several characteristics: stop swimming for 6 seconds (adult) or 1 minute (larvae), are immobile on the bottom or surface and are less sensitive to external stimuli. Zebrafish larvae activities are minimal at night[19]. This study results point out that zebrafish with several light treatments, namely 2m on 2m off, 4m on 1m off, and 24-hour light, experience insomnia more than other groups or treatments. This is because zebrafish have responsive cells to light.[18] Light exposure can reduce melatonin levels, which results in insomnia.

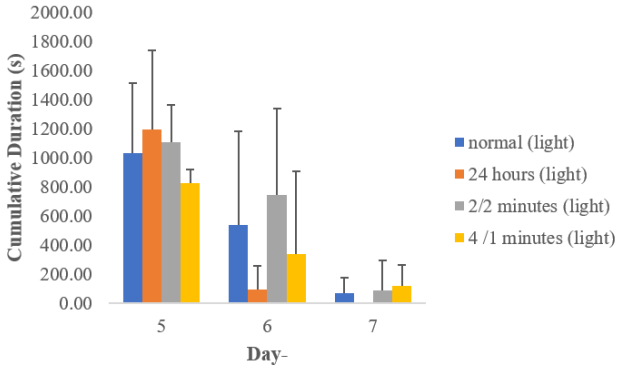


Figure 1. Inactive State Duration of Light Cycle

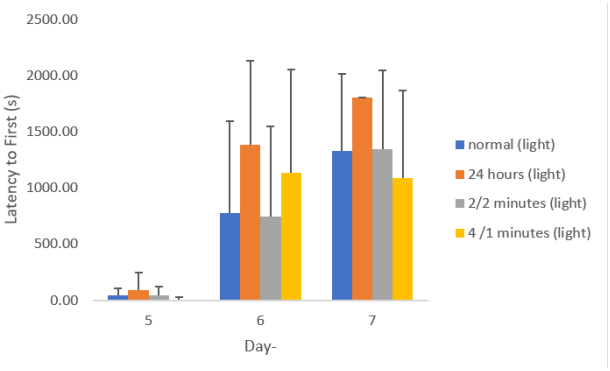


Figure 5. Sleep Latency of Light Cycle

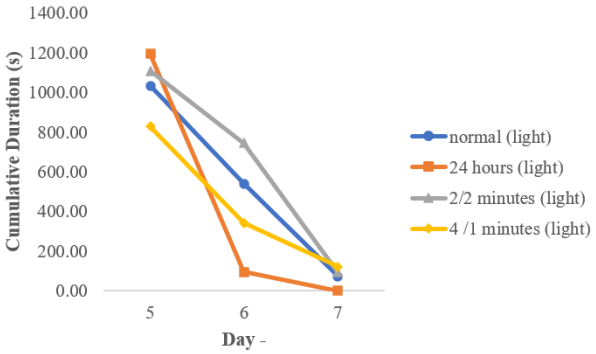


Figure 2. The Zebrafish Locomotoric Activity in Light Cycle on day 5, 6, and 7

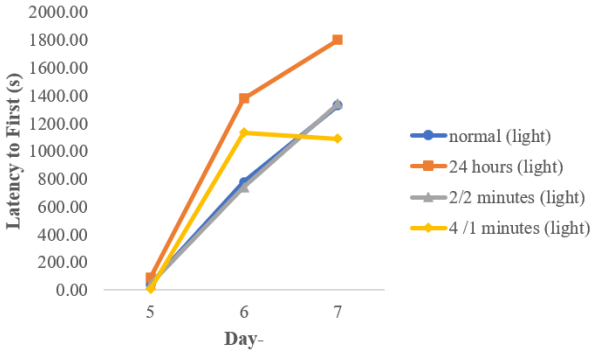


Figure 6. The Zebrafish Sleep Latency in Light Cycle on day 5, 6, and 7

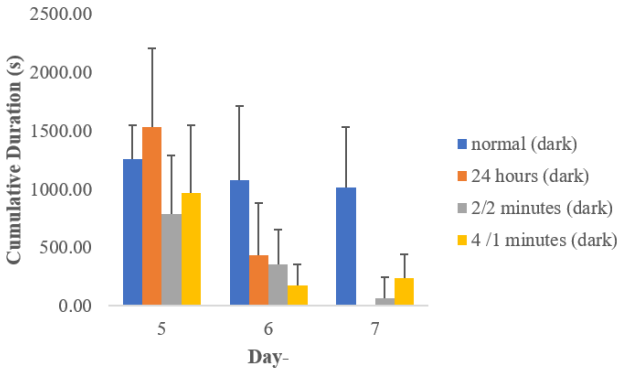


Figure 3. Inactive State Duration of Dark Cycle

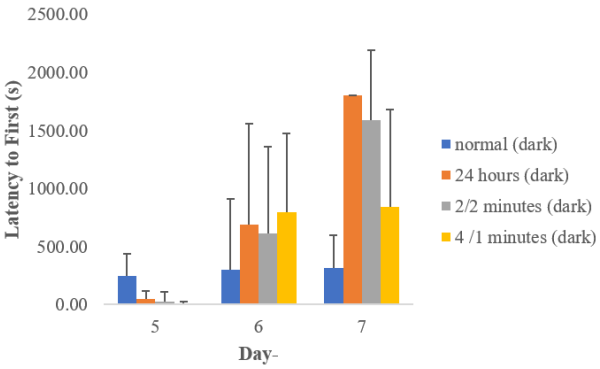


Figure 7. Sleep Latency of Dark Cycle

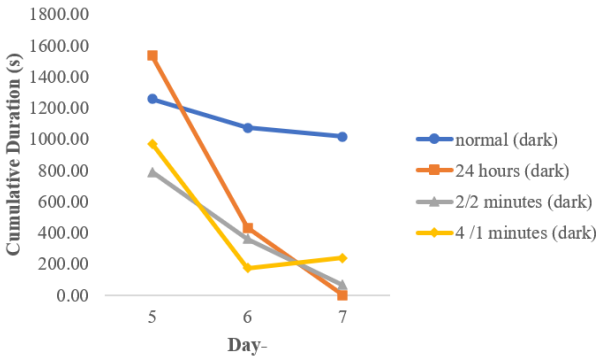


Figure 4. The Zebrafish Cumulative Duration in Dark Cycle on day 5, 6, and 7

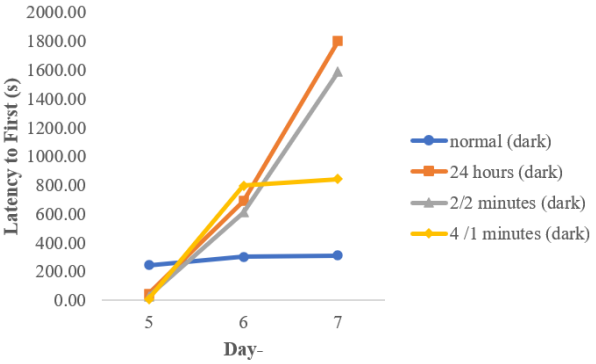


Figure 8. The Zebrafish Sleep Latency in Dark Cycle on day 5, 6, and 7

The length of light exposure duration obtained by them decreases melatonin production, leading to several inputs, promoting excessive wakefulness in the brain.[18]

Sleep Latency

Sleep Latency is defined as a moment or time (both during the day and at night) required to lead to the first sleep.[12] This study shows the prolongation of Sleep Latency in zebrafish larvae—exposed to 24 hours of light. Continuous light exposure can cause this condition, in line with a theory mentioning that zebrafish have endogenously controlled circadian rhythm behavior that light can influence. Another study applied a treatment of 14 hours on (light) and 10 hours off (dark), resulting in data showing that the maximum activities of zebrafish larvae occur when the lighting is turned on. Meanwhile, the average latency to first (light) in that study is 24.8-29.2 minutes o2017 (pp. 414-423). n days 6 and 7 with light exposure during the day.[12] The lighting setting on the sleep-wake-up cycle is related to melatonin production and the hypocretin/orexin (Hcrtr) system. 60% of latency conditions decrease at night, indicating the fragmentation of hcrtr.[18]

Conclusion

Light exposure can disrupt zebrafish sleep and become an intense insomnia stimulus. 24-hour light treatment on zebrafish can reduce the total duration of sleep and increase sleep latency.

Acknowledgement

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