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Correlation Between Sleep Deprivation and Increased Anxiety In Larval Zebrafish

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Abstract: (1) Background: Current evidence suggests an indirect link between sleep deprivation and anxiety. To explore this relationship more thoroughly, zebrafish provide a valuable model for behavioral research, including anxiety studies, because of their small size and genetic similarity to humans. In this study, we employed an experimental model to compare light/dark preference—a common indicator of anxiety—between sleep-deprived and control larval zebrafish; (2) Methods: We compared two groups of larval (6 days post fertilization) zebrafish that have either been sleep deprived or kept under controlled conditions. Sleep deprivation was induced using constant light. In the morning, we tested both groups' light/dark preference behavior; (3) Results: We found that inducing a sleep deficit was correlated with increased dark avoidance the morning after 10 hours of constant light. This indicates that the larval zebrafish felt higher levels of anxiety; (4) Conclusion: This work demonstrates that sleep deprivation is correlated with increased anxiety-like behavior.

Keywords: sleep deprivation; anxiety; larval zebrafish; light-dark preference; anxiety-like behavior

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1. Background

It is important to understand anxiety in humans, as excessive anxiety can significantly reduce a person's well-being. Anxiety disorders are a type of mental health disorder characterized by 'feelings of intense fear and distress [that] are overwhelming and prevent us from doing everyday things' [1]. Currently, anxiety disorders have been found to be closely related to the brain structures of the hippocampus and amygdala due to their involvement in fear and emotional memory [8,9].

Due to the difficulty in studying anxiety directly in humans, several animal models have been used to study anxiety in recent years. Behavioral tests such as light / dark choice, mazes, and open field tests are used as proxy indications of anxiety in these animal models. Recently, zebrafish have emerged as an attractive model to use in behavioral studies such as these given their rapid development, small size, and genetic similarity to humans. They also have fundamental circuitry in place, as they know how to escape predators and hunt for food as early as 5 days post fertilization (dpf). Larval zebrafish from 5dpf to several

weeks of age are uniquely suited for neurobiological studies given their transparent brain, helping researchers gain a deeper understanding of neural circuitry [4].

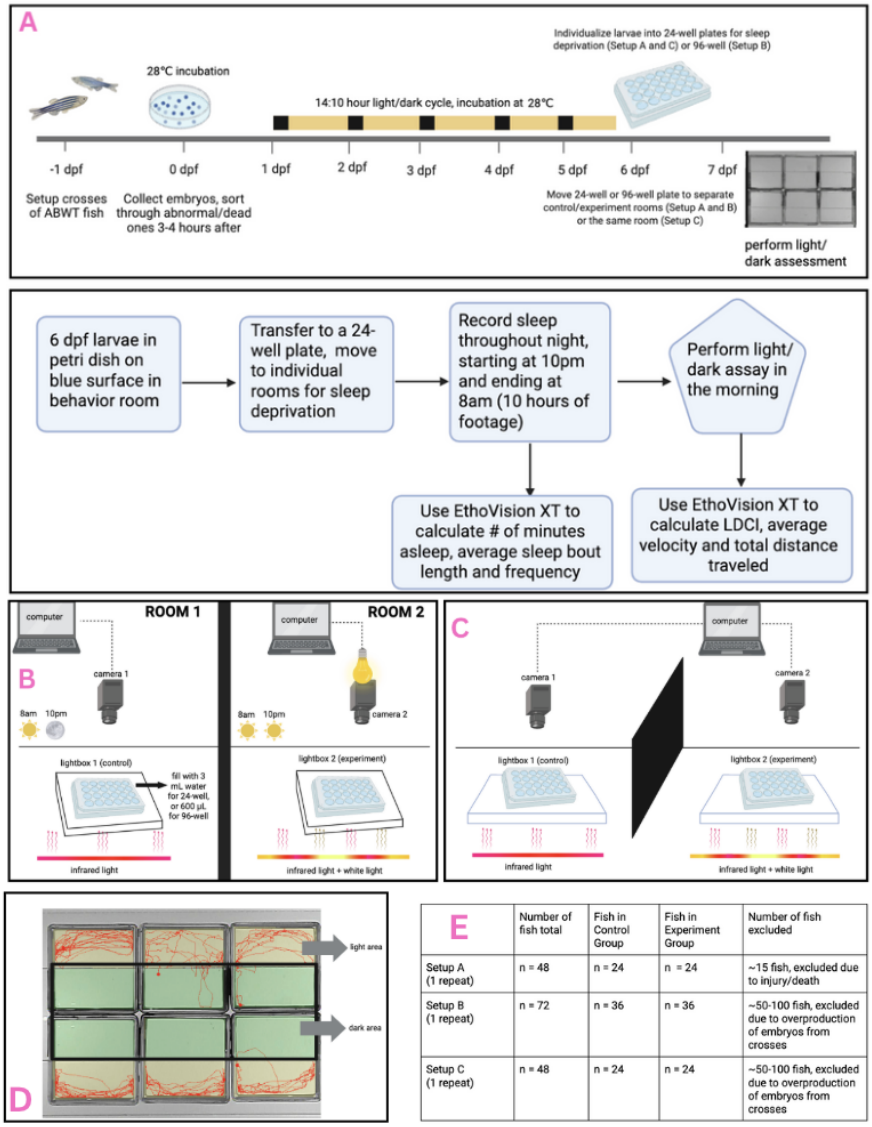
It is known that larval zebrafish naturally prefer light. This preference decreases when larval zebrafish are given antianxiety drugs and increases when larval zebrafish experience stressors such as heat, indicating that their dark avoidance is driven by anxiety [4]. Thus, a light/dark choice assay presents an attractive method to measure a change in anxiety levels after sleep deprivation, where excessive dark avoidance indicates high anxiety.

Currently, there is indirect evidence that sleep deprivation is a significant contributor to anxiety and other mental health disorders such as depression. Studies in rats have shown a direct relationship between sleep deprivation and neuroinflammation [5]. For example, in a comparison between chronically sleep-deprived rats versus control rats, sleep-deprived rats had more inflammatory cytokines, leading to an anxiogenic response and memory loss [13]. Human studies also show an increase in self-reported anxiety after sleep deprivation [12]. However, it is currently unknown whether sleep deprivation induces a similar effect on anxiety in larval zebrafish.

This raises the question: Is there a direct effect of sleep deprivation on anxiety-like behavior in larval zebrafish? If so, the larval zebrafish system, with its amenability to genetics and brain-wide neural activity imaging at cellular resolution, can help decipher the underlying cellular and molecular mechanisms. Larval zebrafish have become a well-established model to study sleep, as they share many sleep/wake dynamics with mammals since they are diurnal and easily trackable in small wells [22]. In addition, their sleep is controlled by the circadian rhythm and melatonin is produced in the pineal gland [16], which are properties that are also shared with humans. Constant light is widely used as a method to invoke a sleep deficit in larval zebrafish, as previous research has found that it can suppress sleep without evidence of a rebound later [11,18,22]. Although there is a wealth of research on the specific genes that increase and decrease zebrafish sleep, there is little knowledge about whether or not sleep deprivation would affect their anxiety on a behavioral level. In this study, we used an experimental model to compare light / dark preference, an established indicator of anxiety level, between sleep-deprived and non-sleep-deprived larval zebrafish.

2. Materials and Methods

2.1. Animals and Housing



	Number of fish total	Fish in Control Group	Fish in Experiment Group	Number of fish excluded
Setup A (1 repeat)	n = 48	n = 24	n = 24	~15 fish, excluded due to injury/death
Setup B (1 repeat)	n = 72	n = 36	n = 36	~50-100 fish, excluded due to overproduction of embryos from crosses
Setup C (1 repeat)	n = 48	n = 24	n = 24	~50-100 fish, excluded due to overproduction of embryos from crosses

Figure 1. Experimental flow diagram from beginning-set up crosses to finish (light dark assay), with three different setups. **(A)** Schematic showing larvae raising and preparation for sleep deprivation and light/dark assessment, using 24-well plates for Setup A and 96-well for Setup B. **(B)** Room setup for Setup A and B, separating control and experiment group into two rooms. **(C)** Room setup for Setup C, using a black barricade between control and experiment group. **(D)** Light/dark assessment setup in a 6-well plate with sample fish tracking. **(E)** Number of fish used total, per control group, and excluded for each setup

Our study used Larval zebrafish from the AB strain that are bred in our facility at the University of California, San Francisco, CA and treated in accordance with IACUC regulations. AB wild-type fish were crossed in the late afternoon and embryos were collected in the late morning the next day; the age was established as 0dpf (day post fertilization) on the day of collection. The embryos were then sorted into separate 100 mm Petri dishes filled with adequate egg water at 40-50 embryos per dish. These embryos were raised at 28 °C ± 0.1 °C starting from day 0 and introduced to a natural circadian rhythm in a 14:10 light / dark cycle. The temperature is maintained using a central air conditioning system in the laboratory, which is in constant use day and night and maintains the same

temperature in all the behavior rooms used. Although small temperature differences may affect the behavior of zebrafish, in our lab the control and experiment groups experience the same temperatures throughout the experiments. The light intensity pointing at the dish during the day period was 350 lux.

On the day of sleep deprivation (6 day post fertilization), 48-72 larvae (24-36 larvae per group for each trial; Figure 1e) were gently pipetted from the 100mm Petri dish they previously lived in either 1) two 24-well plates (6x4) that were filled with 3 ml of blue egg water each to prevent excessive evaporation or 2) two 96-well plates (8x12) filled with 0.6 ml of blue egg water, depending on the setup of the experiment. The larvae were randomly assigned to each well with no consideration of size, movement pattern, or any other factors during the individualization process. The well plates were identical between the two groups, controlling for any positioning that may affect fish behavior. The experimenters were not blinded to the experimental conditions. One plate was assigned as the control group and the other was assigned as the experiment group. At 6:00pm on the day of an experiment, both plates were moved to their respective rooms, kept at 28 ° C ± 0.1 °C (Figure 1A). Each fish went through one night of sleep deprivation and then was discarded following the IACUC protocol after undergoing behavioral tests.

We used three different experiment paradigms:

- Setup A used 24-well plates during sleeping hours and two separate rooms for the control and experiment groups (Figure 1B).
- Setup B used 96-well plates during sleeping hours and two separate rooms for the control and experiment groups (Figure 1B).
- Setup C used 24-well plates during sleeping hours and the same room for the control and experiment groups (Figure 1C).

2.2. Control

The plate holding the control group was placed on top of a light box with only infrared light on so that any movement of the fish could be adequately recorded. After putting the plate in the room, the lights stayed turned off until 8am the next day, when we removed the plate from the light box.

2.3. Experiment

The plate holding the experiment group was placed on top of a light box with both LED lights and infrared lights on at 5000-5500 lux. Above the plate, we also put an LED light to surround all sides of the fish's retina with light, which hit the plate at 500-600 lux. We ensured that no dark objects such as a black curtain or black board were surrounding the fish (to prevent habituation to the light/dark contrast overnight). In addition, we covered anything that may emit light, such as a computer, with a black curtain. We left the room light on for the entire night and removed the fish from the light box at 8 am the next day. For trials that used one room, we used a black barricade between the control and experiment groups and turned the light off in the room from 10 p.m. to 8 a.m.

2.4. Behavioral Tests

The zebrafish were taken off their respective lightboxes and set on a table in a room with lights on at 8am. A collection of 5cm wide black and clear stripes, made of infrared-transmitting acrylic, were set on the top of the lightbox. Then we set a 6-well plate on top of these acrylic strips. Each well was divided into a black and white compartment of equal size, one white and one black. To eliminate transparency on the side of the wells, each well was surrounded by white and black tape. We put 10mL of blue egg water in each well (5mm). Behavioral tests began at 9am, allowing the fish to habituate in a standard room

environment for an hour. After gently pipetting one fish into each well, their behavior was recorded for 8 minutes ((Figure 1D).

2.5. Video Tracking and Data Analysis

Throughout the night, the fish were recorded using cameras set above the apparatus. Videos for both sleep tracking and the light / dark preference behavior test were recorded at 30 frames per second.

In each video, the movement of the zebrafish was tracked using EthoVision XT 13.0. For sleep tracking, the output data points include the mobility state, sorted in a minute-by-minute data table (Figure 2A). To determine the thresholds for the mobility state, several minutes (10-20) were manually watched to ensure that the software was tracking the fish correctly. We used 'Dynamic Subtraction' to detect movement. Thresholds were established per trial as they may vary depending on the size of the well. Setup A and Setup C used 8.00% as the threshold, while Setup B used 9.00% as the threshold. Sleep was defined as >1 minute of immobility, as previous researchers found that 1 minute of immobility was associated with increased arousal after the immobility period [19,20]. Approximately 8.6% minutes were excluded due to failed tracking, as the software sometimes did not track the fish for the entire minute. For light / dark preference behavior, the output data points include time in the light zone, time in the dark zone, average velocity, and total distance traveled (Figure 2B).

To quantify the light/dark preference of larval zebrafish, we used a choice index (CI) to test their anxiety-like behavior, defined as:

$$(DurationInDark - DurationInLight)/(DurationInDark + DurationInLight) \quad (1)$$

Using this, CI = 0 meant that there was no light / dark preference, CI = -1 meant that the larvae had the strongest light preference, and Cl = 1 indicated the strongest dark preference. Statistical analysis and graph generation were performed using Graphpad Prism 5.0. Every graph is presented as scattered plots showing individual sample values and error bars representing mean and 95% CI. Student t-tests (two-tailed, unpaired) were performed to analyze the differences between the CI of the experiment and the control group, minutes of sleep and locomotor activity (distance traveled and average speed). Our data is available at https://github.com/taliaboneh/zebrafishdata/tree/main, and more rigorous statistical analysis is part of our future work.

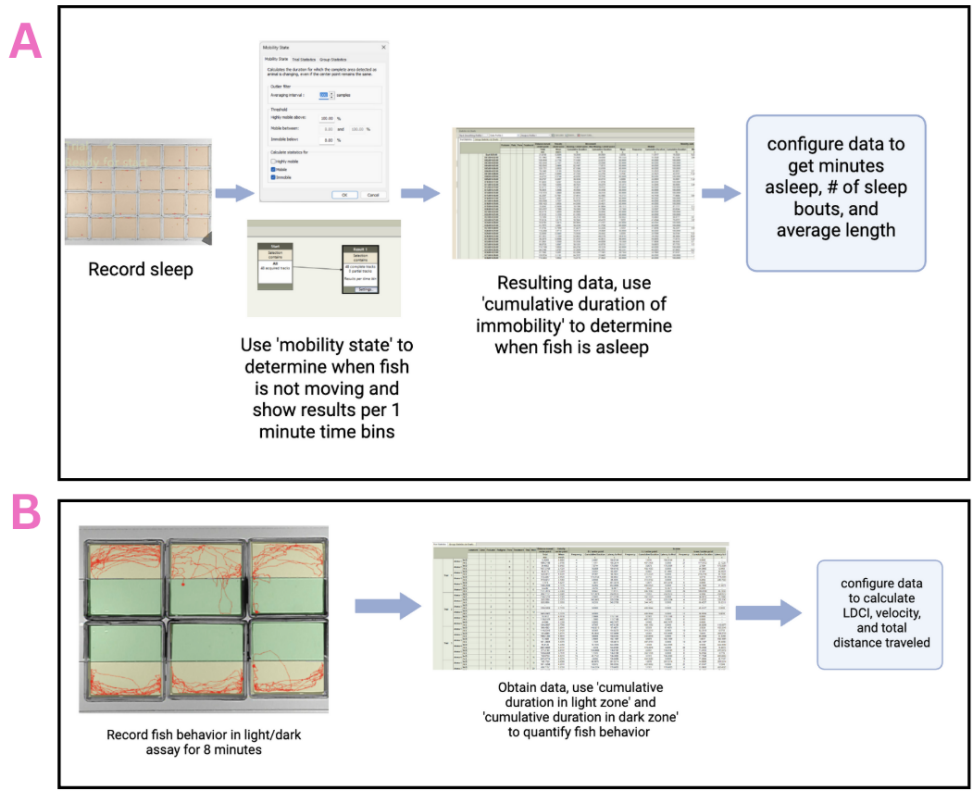


Figure 2. Data analysis pipeline **(A)** Data treatment using EthoVision XT to obtain number of minutes asleep, average sleep bout length, and total number of sleep bouts per fish **(B)** Data treatment using EthoVision XT to obtain LDCI and locomotor activity (total distance traveled and average velocity).

3. Results

3.1. Constant light decreases the total number of minutes asleep

Across the experiments, we observed a significant difference in the total number of minutes asleep and sleep bout length.

Among the trials using Setup B, we observed a mean number of minutes of sleep of 25.94 minutes for the experiment group (Figure 3A). This was a significantly lower number of minutes of sleep (t = 13.500, p = <0.0001, r^2 = 0.8864), given that the control group had a mean of 486.5 minutes of sleep. The minutes of sleep were calculated over the course of the 10 hours of sleep footage obtained the night before (recorded from 10pm to 8am), as mentioned above. This indicates that constant light conditions indeed disrupt the natural circadian rhythm of larval zebrafish. In addition to this, Student t tests showed a significant decrease in sleep bout length (Figure 3B) in the experimental group compared to the control(t = 5.796, p = < 0.0001, r^2 = 0.6043), indicating that the experimental group slept in much shorter episodes.

Due to technical issues, we did not obtain a recording of the zebrafish's sleep overnight for the experiment under Setup A. However, we compared the sleep time of the experiment group in Setup A and the experiment group in Setup B. The experiment group in Setup A has a mean number of minutes asleep of 4.00 minutes, compared to a mean of 25.94 minutes in Setup B, leading us to speculate that not only the constant lights reduce the sleep duration, but larger plate size also reduces sleep duration. The unusually low mean number of minutes of sleep in Setup A indicates that this group is still sleep-deprived.

Though constant light reduces the total number of sleep minutes of the experiment group, its effect on the number of sleep bouts is not significant. In Setup B, there were no

significant differences between the two groups (t = 0.0735, p = 0.1257, r^2 = 0.0001) which suggests a similar number of periods asleep (Figure 3C).

Due to technical issues, we were unable to track the sleep of the control and experiment groups in Setup C. However, we kept the lighting conditions the same as in Setup A and B, so we believe that a similar effect on sleep deprivation would also have occurred in Setup C.

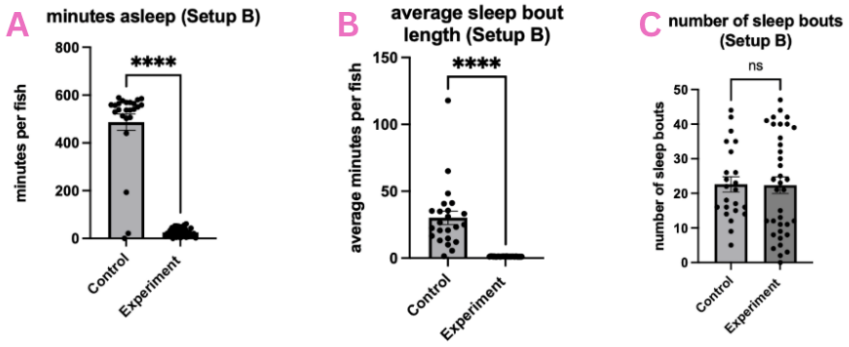


Figure 3. Data from overnight sleep tracking using Setup B (n = 72). **(A)** Asleep minutes between control and experiment groups **(B)** Sleep bout length between experiment and control group **(C)** Average number of sleep bouts per fish between experiment and control group. Data are presented as scatter plots showing individual sample values on top of a box-and-whiskers plot representing mean and 1 SEM; ns p > 0.05; **p < 0.01; ****p < 0.0001; student t tests compared to the control (unstressed) group.

3.2. Sleep deprivation correlated with an increase in dark avoidance in the light/dark preference assay

Among all trials using Setup A, we observed a significant effect in CI for the sleep-deprived and control group. Student t tests (t = 3.3856, p = 0.0015, r^2 = 0.1995) showed that the sleep-deprived group had a much more negative CI than the control group (Figure 4A). This indicates that there was a significantly higher percentage of time spent in the light zone in the sleep deprived group than in the control group and a lower percentage of time spent in the dark zone. Ultimately, these results show that sleep-deprived fish had significantly more dark avoidance, which implies that they were more anxious.

However, the behavior of sleep-deprived fish appeared to be affected by the size of the well that they were sleeping in. In setup B, we used a 96-well plate instead of a 24-well plate to house the larval zebrafish while they slept, making the well size 4 times smaller. In this case, the experiment group had a more positive CI (t = 2.675, p = 0.0120, r^2 = 0.1066; Figure 5A), indicating less dark avoidance. We speculate that the large increase in well size from sleep time to day time introduced another factor that affected fish behavior, namely their exploratory habits of their surroundings, regardless of light/dark preferences.

In setup C (Figure 6A), where the two groups were kept in the same room as each other with a black board between the two groups to prevent light leakage from the experimental group, we observed no significant differences in the CI (t = 0.03053, p = 0.9758, r^2 = <0.0001; Figure 6A). This may have been caused by the black board, which may have created a light/dark contrast around the fish due to light from the lightbox hitting it, allowing the fish to habituate to the contrast overnight. Given that the mean CI for the Setup C control group was -0.1478, which is considerably higher than the control group for Setup A and B (-0.344 and -0.322, respectively), this is a highly probable cause.

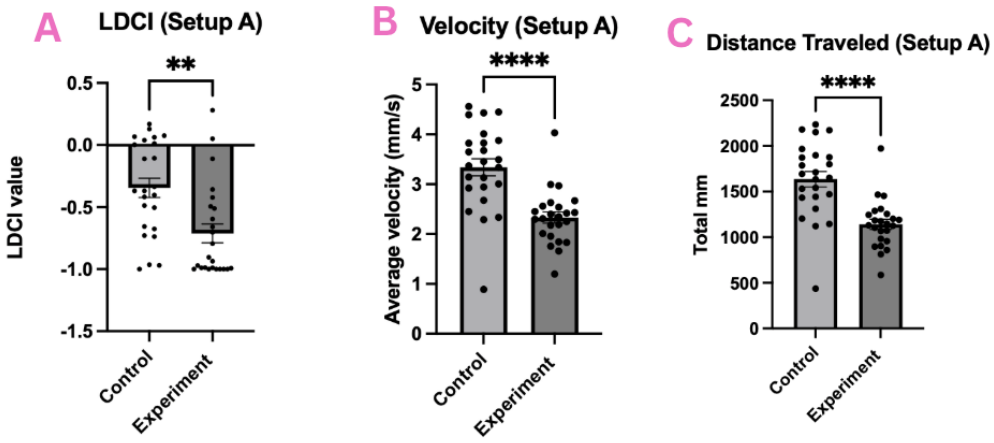


Figure 4. Data from light/dark assessment using Setup A (n = 48). **(A)** Choice index between experiment and control group **(B)** Average velocity per fish (mm/s) between experiment and control group **(C)** Total distance traveled (mm) between experiment and control group. Data are presented as scatter plots showing individual sample values on top of a box-and-whiskers plot representing mean and 1 SEM; ns $p > 0.05$; **$p < 0.01$; ****$p < 0.0001$; student t tests compared to the control (unstressed) group.

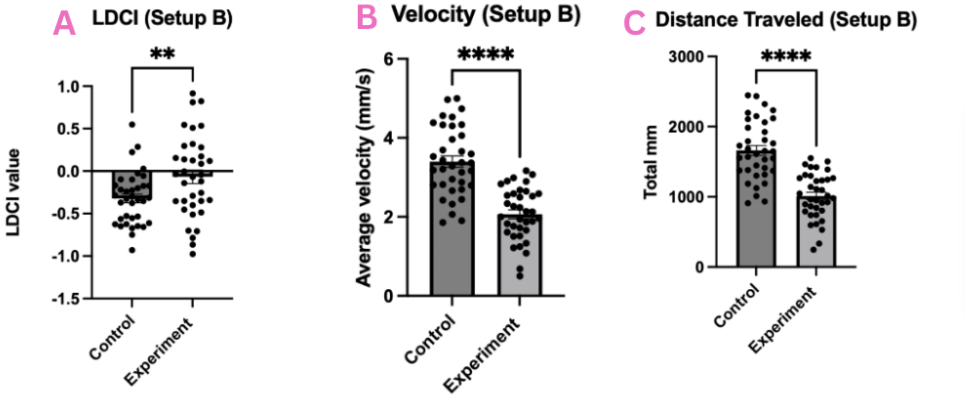


Figure 5. Data from light/dark assessment using Setup B (n = 72). **(A)** Choice index between experiment and control group **(B)** Average velocity per fish (mm/s) between experiment and control group **(C)** Total distance traveled (mm) between experiment and control group. Data are presented as scatter plots showing individual sample values on top of a box-and-whiskers plot representing mean and 1 SEM; ns $p > 0.05$; **$p < 0.01$; ****$p < 0.0001$; student t tests compared to the control (unstressed) group.

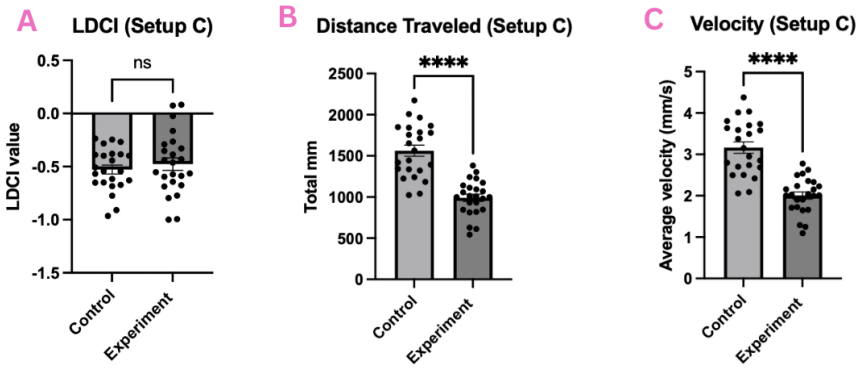


Figure 6. Data from overnight sleep tracking and light/dark assessment using Setup C. **(A)** Choice index between experiment and control group **(B)** Average velocity per fish (mm/s) between experiment and control group **(C)** Total distance traveled (mm) between experiment and control group. Data are presented as scatter plots showing individual sample values on top of a box-and-whiskers plot representing mean and 1 SEM; ns $p > 0.05$; ****$p < 0.0001$; student t tests compared to the control (unstressed) group.

3.3. Sleep deprivation correlated with a decrease of locomotor activity in light/dark preference assay

In all trials, there was a significant difference in locomotor activity (Figure 4B-C, Figure 5B-C, Figure 6B-C). For example, in Setup A, in terms of both velocity (mm / s) and distance traveled (mm), there were differences between the control and experimental groups. The velocity decreased significantly ($t = 4.290$, $p = <0.0001$) in the experiment group (Figure 4B), and the distance traveled also decreased significantly ($t = 4.919$, $p = <0.0001$) in the experiment group (Figure 4C).

4. Discussion

The key finding of this study is that keeping larval zebrafish under constant light conditions, thereby disrupting their circadian rhythm and causing sleep deprivation [1], is correlated with a significant increase in their avoidance of darkness absent other factors.

Our experiments confirm that constant light invokes a sleep deficit in zebrafish. The average number of minutes of asleep in the control group was 486.5 minutes, which falls in the range of values between 219-578 minutes that has previously been seen in wild type larval zebrafish [21,23]. Constant light is a well-established method to deprive fish of sleep with no evidence of a rebound period of sleep, and has been found to reduce sleep in adult zebrafish down to an average of 23.4 minutes of sleep in a 6 hour time period of constant light [24]. Although there is no concrete value for the number of minutes of sleep for larval zebrafish in constant light, we demonstrated a significant decrease in sleep after observing an average number of 4.0-25.9 minutes of sleep over 10 hours of darkness.

Our experiments showed that in zebrafish, sleep deprivation affected both light/dark preference and locomotive activities. In all trials, sleep deprivation led to slower movements and shorter distance traveled, possibly due to fatigue. In Setup B, larva zebrafish experienced an order of magnitude increase in well sizes (such as a 96-well plate used during overnight sleep versus a 6-well plate used during the light/dark assay). This prompted the fish to explore more – both control and experiment groups in Setup B moved faster and traveled longer distances than groups in Setup A and Setup C. Since our light/dark assay included a half-dark, half-light well design, more exploration caused less dark avoidance in our experiments. In Setup C, the fish were kept in the same room and black barricades were used to prevent light leakage. The light from the lightbox hitting the black barricade

formed a light/dark contrast in the room and allowed the larvae to habituate to the contrast, since zebrafish are known to easily adapt to certain object presentations [7]. Both control and experiment groups in Setup C have higher CI than groups in Setup A and Setup B, leading us to suspect that habituation to light/dark contrast also reduces dark avoidance.

Absent the exploration factor and the habituation factor, sleep-deprived larval fish showed a significant increase in dark avoidance, as shown in Setup A.

These results provide supporting evidence for the conjecture that sleep deprivation is correlated with increased anxiety. This builds on existing research regarding the effects of sleep deprivation on fish behavior, which has established that total sleep deprivation in zebrafish led to impaired cognitive responses [18].

Our study is limited by a number of confounding factors that affect larvae dark avoidance in our experiments, including reduced locomotive activities and habituation to light/dark contrast, as discussed above. Another factor may be the increased light sensitivity caused by the experiment groups being in constant light for 10 hours prior to the experiment. Although we did allow the fish to habituate to standard room lighting for an hour prior to the behavioral assay, it is unclear how light sensitivity influences, if at all, dark avoidance. More experiments are needed to understand the effect of these confounding factors, and those experiments remain part of our future work.

5. Conclusions

In this study, using three different experimental setups, we showed that sleep deprivation, invoked by constant light, is correlated with increased avoidance of the dark and increased anxiety in larval zebrafish. This finding adds to the body of research that determines how larval zebrafish react to specific environmental stressors. The experiments showed that the effect of sleep deprivation on zebrafish is similar to that on rats and humans. Thus, our results give validity to future research efforts that use zebrafish to examine the neurological underpinnings of anxiety and to examine which genes or neurons cause circumstances such as sleep deprivation to increase anxiety.

A limitation of this study is that we have not yet determined a casual relationship between sleep deprivation and increased anxiety due to many confounding factors. More experiments can address the issues here. Experiments using drugs such as caffeine (sleep inhibitor) to keep larvae awake for 24 hours in a normal 14:10 light/dark cycle, then finding a significant difference in their light/dark preference, can remove any influence that light sensitivity and light/dark habituation have on light/dark avoidance. Experiments with different well-sizes without sleep deprivation can shed light on how locomotive activities and explorations affect ligh/dark avoidnace. Experiments using melatonin (sleep inducer) to make fish sleep in constant light, then testing their light/dark avoidance, would confirm a causal relationship. These experiments are part of our future work.

Author Contributions: S.G. conceived the idea. T.B., J.X., and S.G. designed the experiment protocol. T.B., J.X., and M.W. managed cameras, lightboxes EthoVision XT, and data treatment. T.B. and J.X. carried out the experiments. T.B. and M.W. provided AB wild-type fish. T.B. wrote the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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