JOURNAL OF DYNAMICS AND CONTROL

VOLUME 8 ISSUE 9

IMPACT OF CIRCADIAN RHYTHM DISRUPTION ON RED BLOOD CELL COUNT IN *Danio rerio*

Pooja M. Dyapanagoudar, Majesh Tomson

Department of Life Sciences, Christ University, Bangalore, India

IMPACT OF CIRCADIAN RHYTHM DISRUPTION ON RED BLOOD CELL COUNT IN *Danio rerio*

Pooja M Dyapanagoudar[1], Majesh Tomson[2*],
Department of Life Sciences, Christ University, Bangalore, India
majesh[2].tomson@christuniversity.in, pooja[1].dyapanagoudar@res.christuniversity.in
***Corresponding author**

ABSTRACT: The study focused on using Danio rerio as a model organism to study circadian rhythm. Circadian rhythm is a 24-hour sleep wake cycle that has a significant impact on an organism's mental, physical, and behavioural traits. Disruptions in circadian rhythm can lead to imbalance in human health. This study helps in future investigations on the complications related to blood pressure, breathlessness, and body immunity due to circadian rhythm disturbance. To disturb the circadian rhythm in Danio rerio, surface waves in the aquarium was created and exposed the fish to continuous artificial light for 96 hours. Every 24 hours, and estimated the melatonin levels to confirm the disturbance in the circadian rhythm and then estimated RBC count in the blood using a neubauer counting chamber. As the blood of zebrafish is less than 3% of its body weight, this was challenging and made an attempt to estimate RBCs in the model organism. Comparing the RBC count of the experimental fish with the control group (kept under ideal conditions), the researchers found a significant difference. Additionally, the RBC count was compared every 24 hours to monitor changes. The results showed a decrease in RBC count in the experimental group, along with stress and decreased breathing rate in Danio rerio. However, this study contributes to understanding the respiratory physiology of Danio rerio and provides valuable insights for future pharmacological research.

KEY WORDS: Circadian Rhythm, Red Blood Cells, Dissolved Oxygen, Danio rerio, Lipids.

1. Introduction

Blood is the major source for circulation. Red blood cells carry out the exchange of oxygen and carbon dioxide in the cells [1]. In piscine's the red blood cells are nucleated and contains cell organelles which responds to the stimuli [2]. *Danio rerio* blood cells bear resemblance to mammalian blood cells. Even though in contrast to mammals the fish blood cells have retained their nuclei. Zebrafish peripheral blood cells closely resemble mammalian blood cells, while in fish, unlike the mammals, red blood cells retain their nuclei [3]. Circadian rhythm 24 hours cycle completely dependent on the light, also called sleep wake cycle has the control over the biological system and most of the behavioural changes. If circadian rhythm is disturbed or altered, it leads to physiological changes [4]. "Disruption of circadian regulation is associated with a wide variety of adverse health consequences, including increased risk for premature death, cancer, metabolic syndrome, cardiovascular dysfunction, immune dysregulation, reproductive problems, mood disorders, and learning deficits" [5]. According to the study done by the University of Surrey in 2017 by changing the amount of potassium the cell receives, the researchers were able to increase and decrease its levels in the cell and observe the effects on their circadian rhythms. The researchers found that higher levels of potassium negatively impacted the circadian rhythm of the cell, as the day was prolonged for many hours to the fishes the potassium levels were decreased.

Modification in the sleep wake cycle in shift workers leads to change in the WBC count while the red blood cells count is unclear [6]. However the studies show differences in the count of RBCs in the zebrafish embryo when chronodistruption is caused [7]. In this study we have focused on the study of red blood cells in contrast to changes in the circadian rhythm.

The red blood cells in fishes are similar to that of all vertebrates as they also carry the four binding sites, two alpha and two gammas. However, this is changed due to pH and stress in the fishes. This condition is explained by “Roof effect” which says that this functional property of teleost, fish haemoglobin consists of drastic reduction in the affinity towards oxygen molecules in haemoglobin, resulting in decreased transportation of oxygen capability when pH decreases and oxygen concentration is high. This condition in fishes is useful to fill their swim bladder by diffusion of oxygen molecules and also supply oxygen to their retina as they do not possess any separate blood supply to it [8].

Hyperoxia increases the oxidative stress in the fishes [9] [10]. Oxidative stress had a severe effect on the organs like liver, Brain, muscle and kidney of the goldfishes [10]. In all animals the red blood cells produced in the bone marrow but in fishes the Head cells of the kidney is the region where the erythropoiesis begins in fishes. Low levels of red blood cells stimulate the production of erythropoietin which in turn increases the production of RBCs [11].

2. Methods

Zebrafish were acclimatized in accordance with the guidelines given by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The experimental setup was designed to disrupt the resting periods of the zebrafish, thereby altering their circadian rhythms. The levels of melatonin were estimated to confirm the circadian rhythm disturbance and then the red blood cell (RBC) levels were estimated.
This proceeds to provide insights into the broader implications of circadian rhythm disruptions on zebrafish physiology, especially on hematological parameters such as RBC levels.

2.1 Acclimatization of the fishes

Two aquarium setups were made in the aqua lab. Adult *Danio rerio* were introduced (10 in each tanks) into the aquarium each containing bio filter (Waterfall style, RS-4000), thermostat (fully submerged automatic aquarium heater) to regulate the constant temperature, automatic food dispenser (AF012 LCD Auto Feeder) to have an accuracy in the time and the amount of food. Amount of food was determined according to ad libitum. Light and dark 12:12 was maintained. This condition was continued for about 30days for acclimatization.

2.2 Experimental setup

After acclimatization one of the aquariums was considered as control and the other was treated as experimental. In the experimental setup the circadian rhythm of *Danio rerio* was disturbed using white light and surface wave maker. As the white light travels deeper inside while the red light increases the stress [12]. Surface wave maker creates disturbance in the Zebrafishes as they are freshwater organism changes in the flow of water disturbs them [13]. This setup was retained for 120 hours after each 24 hours the required number of the fishes were taken from both control and experimental tanks for haematological studies estimation of melatonin levels.

2.3 Analysis of Dissolved Oxygen in water

Dissolved oxygen was estimated by Winkler’s method. The chemical determination of oxygen concentrations in water is based on the method first proposed by Winkler (1888) and modified by Strickland and Parsons (1968).

2.3 Quantification of Melatonin using fish melatonin ELISA kit (AFG Bioscience)

The fishes were anesthetized using clove oil, ensuring they were placed on an ice bath to minimise pain sensation during the dissection process. Triad fish brains and eyes were pooled in Eppendorf tubes containing phosphate buffer (pH 7.2-7.4) and subjected to sudden freezing using liquid nitrogen, effectively terminating all the brain functions. After allowing the samples to defrost, they were homogenized and centrifuged at 2000 rpm for 20 minutes, the supernatant was collected and utilized for further assay procedures (ELISA KIT). This methodology ensured the preservation of brain samples for further analysis and maintaining the integrity of the biological material during the experimental process.

2.4 Estimation of RBC levels in the zebrafish

Haematological studies were very challenging as the amount of the blood in the zebrafish is less than 3% of its body weight for about 5-10µL [14]. The fish was anaesthetist using clove oil before the collection of blood [15]. The blood collection was done by Novel blood collection method and the blood was collected in the eppendorf containing EDTA 8pH [16]. RBC count was done using a haemocytometer and the red blood cells were counted every 24 hours once and the same was continued for 120 hours.

2.5 Estimation of lipids by Folch Method [17]

Tissue was homogenized with methanol and chloroform (1:2 ratio) then mixture was agitated for 15-20mins and then passed through the filter paper liquid phase was washed with NaCl (0.9%) then centrifuged at 2000rpm for 15mins lower phase was transferred to a weighed crucible, solution is to be evaporated at 40ºC by placing in a hot air oven for 24-48hours.

2.6 Statistical analysis

Statistical analysis was done using ANOVA using the IBM SPSS Statistics 21 software. The sample showed significant difference and the p value was less than 0.05.

3 Results

3.3 Analysis of water parameters

Table 1 Analysis of water parameters

Water Parameters	Optimum Range [18]	Estimated Range (every 24 hours once)
pH	6.8 – 8.5	8.0 ± 0.4
Alkalinity	50 – 150 mg/ L	120±20 mg/L
Hardness	50-100 mg/L	95 ± 2 mg/L
Nitrite	< 0.1 mg/L	0.05 ± 0.01mg/L
Nitrate	< 50 mg/L	30 ± 15 mg/L
Ammonia	< 0.02 mg/L	0.005 ± 0.005 mg/L
Temperature (ºC)	26 – 28.5	26 ± 1.3 (^{o}C)

3.4 Melatonin levels during circadian rhythm disruption.

The melatonin levels in the brains of the experimental fish and control zebrafish were found to be gradually decreasing. This was most noticeable on day two, which occurred after a 48 hour disruption, and then increasing on day three, which occurred after a 72 hour disturbance. The body strives to regain hormonal balance which appears to be the cause of this variation in melatonin release, which was a reaction to stress brought on by the circadian disruption.

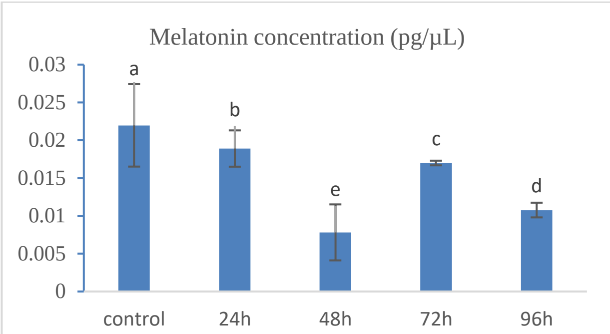

Fig.1. Melatonin concentration in the Brain of zebrafish

2.3 The circadian rhythm disruption on haematological parameters in Zebrafish

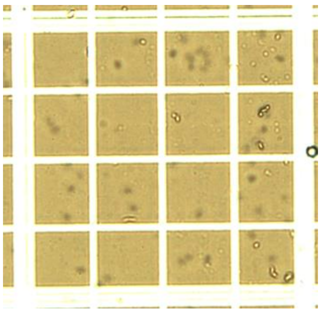

Figure 1: Red Blood Cells

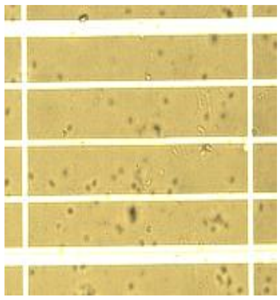

Figure 2: White Blood Cells after 72 hours Circadian rhythm disturbance

Figure 1: Cell count in one of the RBC chamber, Figure 2 shows the white blood cells in the WBC chamber in Haemocytometer

The control fishes showed an average range of (2.8-3.2) × 10^6 cells/µl [19]. Circadian rhythm of the adult zebrafish was disturbed for consecutively for 120 hours. Every 24 hours once the fishes were tested for RBC count. Stress changes the blood composition and decreases the volume of the blood [20] this statement supports our observations after the experiments as shown in the Figure 3.

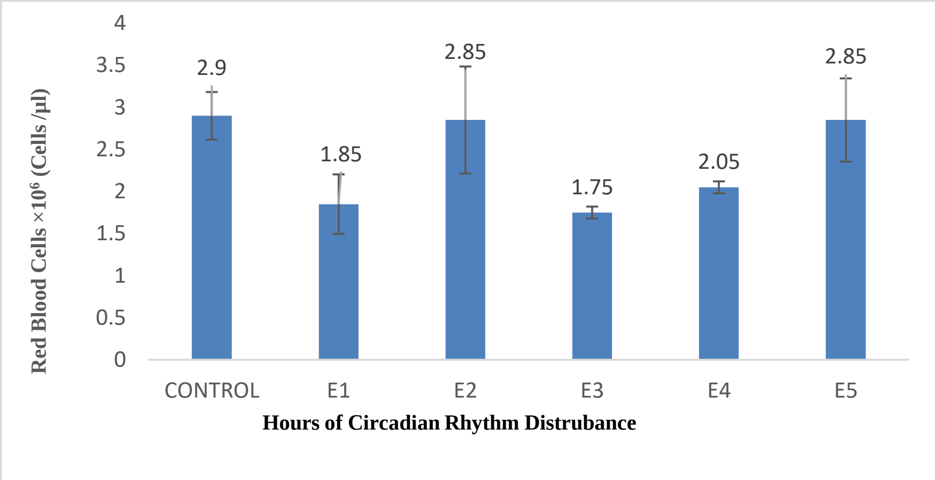

Figure 3: Number of Red Blood Cells in Control and Experimental Condition
(E1 – experimental fishes after 24 hours of disturbance, E2- experimental fishes after 48 hours of disturbance, E3- experimental fishes after 72 hours of disturbance, E4- experimental fishes after 96 hours of disturbance, E5- experimental fishes after 120 hours of disturbance, C1 – Average count of RBC in Control Fishes)

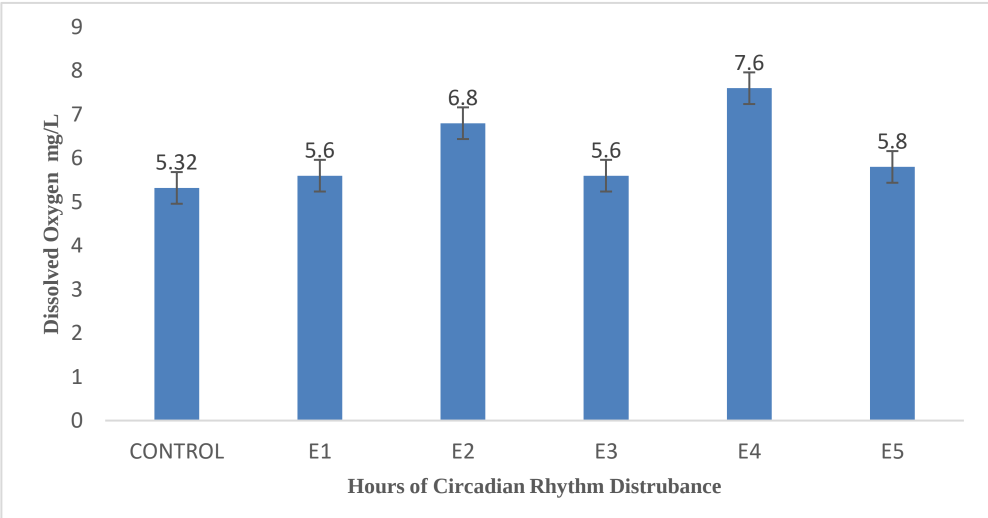

Figure 4: Dissolved Oxygen in control and experimental tanks [21]

In this study it was observed that in the control fishes the Red Blood Cells (RBC's) was found to be 2.9×10^6 cells/µl. after the circadian rhythm was disturbed for 24 hours, 48 hours, 72 hours, 96 hours and 120 hours it was found to be $1.85 \pm 0.35 \times 10^6$ cells/µl, $2.85 \pm 0.63 \times 10^6$ cells/µl, $1.75 \pm 0.07 \times 10^6$ cells/µl, $2.05 \pm 0.07 \times 10^6$ cells/µl, $2.85 \pm 0.49 \times 10^6$ cells/µl respectively. There was a drop in the levels of RBC's after 24 hours of circadian rhythm disturbance, and again upon observation after 48 hours there was an increase in the RBCs levels, as the circadian rhythm disturbance was continued for another 24 hours there was an again drop in the RBCs and then there was an increase in the levels of the RBCs after 96 hours of disturbance and then there was an still more increase in the RBCs in the fishes, which shows that they are trying to acclimatize for the environment. The optimum range of dissolved oxygen for Zebrafishes is not less than 4 mg/L [22]. In our study we have also maintained an average of 5.32mg/L in both control and experimental tanks and was estimated using Winkler's method.

Wave maker was used to disturb the circadian rhythm along with the artificial light [13], the wave maker creates the waves on the surface of the water. Wave maker continuously circulates water and maximum oxygen dissolves in the water which creates Hyperoxia. Hyperoxia changes the levels of oxygen consumption in zebrafish. As the wave maker increases the oxygen dissolving capacity in the water approximately 8 mg/L at 25ºC. As we can observe in figure 4 the breathing rate of the fishes in the experimental tank reduces, as the circadian rhythm disturbance has created stress in fishes which is line with the levels of melatonin. Resulting in clear indication that dissolved oxygen increases as the stress in fish increases. Additionally, Disrupted circadian rhythm is the cause of the variations in the RBC levels is proved by stating that the lipids get damaged due to disturbance in the rhythm [23] these results are also true with our study as observed in the Figure 5.

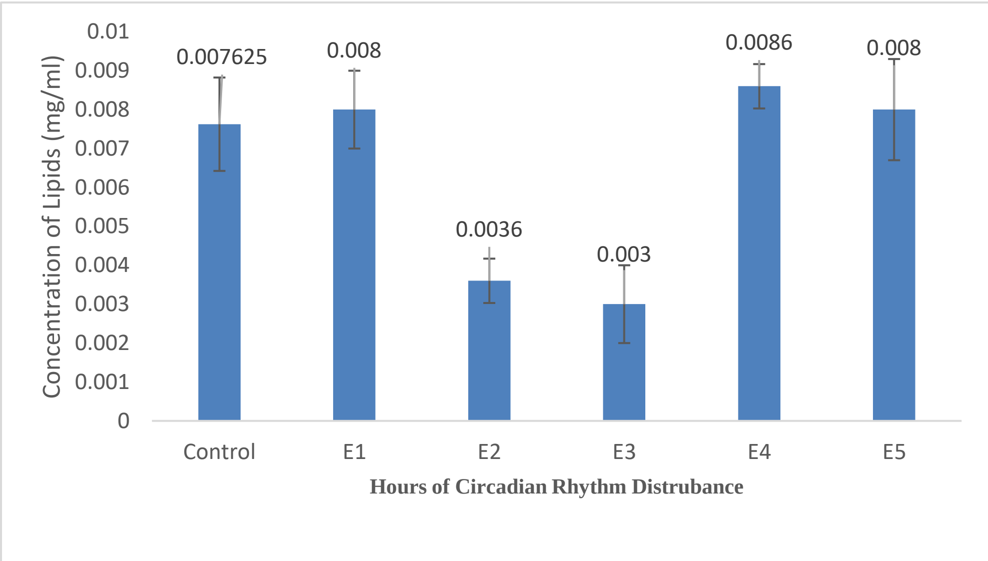

Figure 5: Concentration of Lipids in the zebrafish muscle after the Circadian Rhythm Disturbance
(E1 – experimental fishes after 24 hours of disturbance, E2- experimental fishes after 48 hours of disturbance, E3- experimental fishes after 72 hours of disturbance, E4- experimental fishes after 96 hours of disturbance, E5- experimental fishes after 120 hours of disturbance, C1 – Average concentration of lipids in Control Fishes)

4 Discussion

In this study, the researchers examined the red blood cells in zebrafish over a period of five days while continuously disrupting their circadian rhythm. The average total red blood cell count during this period was 3.02 × 106 cells/µl. The control group, which was not subjected to circadian disturbance, had an estimated total red blood cell count of 2.9 × 106 cells/µl according to a previous study by [19].

Previous studies have shown that the count of red blood cells can vary with changes in the circadian rhythm, as demonstrated by [7]. Our own study also found variations in red blood cell counts after disturbing the circadian rhythm. After 24 hours of disturbance with light and surface waves, the red blood cell count dropped to 1.85 × 106 cells/µl. After 48 hours, the count started to increase and reached 2.85 × 106 cells/µl. However, it decreased again after 72 hours to 1.75 × 106 cells/µl. surprisingly, after 96 hours, there was a continuous increase in the red blood cell count, with a value of 2.05 × 106 cells/µl. The experiment was continued for a total of five days, which revealed that the red blood cell count tried to reach the normal level of 2.85 × 106 cells/µl again. This observation suggests that fish, like humans, try to

restore their red blood cell count to normal levels when their circadian rhythm is disturbed [24].
In zebrafish, erythropoiesis occurs in the anterior and posterior kidneys, which are also referred to as kidney marrow. This process differs from that in mammals, where erythropoiesis primarily takes place in the bone marrow. The transformation of megakaryocytes into erythroblasts and their subsequent maturation into erythrocytes occurs in zebrafish. Low oxygen levels in the blood stimulate erythropoiesis in the kidney, similar to what occurs in mammals. In zebrafish, low oxygen levels increase the expression of epo mRNA in the heart, leading to an increase in the production of red blood cells [25].

Melatonin is a neuro hormone which helps in the regulation of circadian rhythms in humans. Variations in circadian rhythm can significantly affect melatonin levels. Studies have shown that disruptions in circadian rhythm due to environmental factors, physiological changes can lead to the changes in the levels of melatonin secretion patterns. Melatonin levels in humans are intrinsically linked to circadian rhythm, disruptions in the circadian rhythm leading to altered melatonin secretion. However these variations are in line with our studied results. The findings underline the potential therapeutic implications of targeting melatonin and circadian rhythms in clinical settings to improve patient outcomes [26]–[29].
In our study, we observed an increase in dissolved oxygen in the water and a decrease in red blood cells after 48 hours of circadian disturbance, creating a stress condition. To recover from this condition, the red blood cell count in zebrafish increased on the third day (72 hours) of circadian rhythm disturbance. Along with the increase in red blood cells, we also observed numerous white blood cells (WBCs) in the counting chamber of a haemocytometer, indicating the activation of the fish's defense mechanism. However, we found that the increased oxygen levels in the water persisted even after 72 hours of circadian disturbance, and the increase in red blood cells in zebrafish continued even after 96 hours and up to 120 hours. To further support the notion that circadian rhythm only causes variations in red blood cell levels, we also measured the concentration of lipids in the muscle of the fish. We found that there was damage to the lipids after 24 hours of circadian rhythm disturbance, which continued to decrease until 72 hours, and then increased as the fish attempted to adapt to the environment. The similarities between zebrafish and mammals provide a better understanding of haematological changes caused by changes in the circadian rhythm. Further studies using zebrafish as a model organism could contribute to the development of treatments and prevention strategies for haematological disorders that are influenced by changes in sleep patterns in humans.

5 Conclusion

The results of this study show that disturbance in the circadian rhythm of *Danio rerio* leads to changes in red blood cell (RBC) levels. After 24 hours of circadian rhythm disturbance, there was a significant drop in RBC levels compared to the control group. This may be due to the stress caused by the disturbed circadian rhythm, which can have an effect on haematopoiesis and result in a decrease in RBC production.
After 48 hours of circadian rhythm disturbance, there was a significant increase in RBC levels compared to the previous time point. This increase may be a compensatory mechanism of the fish to acclimatize to the altered environment. It is known that stress can stimulate the production of erythropoietin, which in turn increases the production of RBCs. This increase in RBC levels after 48 hours may be a response to the initial decrease in RBC levels observed after 24 hours of disturbance.
The fluctuations in RBC levels observed throughout the 120-hour period of circadian rhythm disturbance suggest that the fish is attempting to adapt and regulate its haematopoiesis in

response to the altered environment. Further studies are needed to investigate the underlying mechanisms and factors involved in this adaptive response.

The presence of hyperoxia in the experimental tank due to the use of a surface wave maker may also contribute to the observed changes in RBC levels. Hyperoxia can increase oxidative stress in fishes, which can have detrimental effects on various organs including the liver, brain, muscle, and kidney. It is possible that the stress caused by hyperoxia and disturbed circadian rhythm may affect erythropoiesis and lead to changes in RBC levels.

Overall, this study provides evidence that disturbance in the circadian rhythm of *Danio rerio* can have an impact on red blood cell levels. Further research is needed to explore the mechanisms underlying these changes and their implications for fish health and physiology. Understanding the effects of circadian rhythm disturbance on haematopoiesis and RBC levels can have implications for both fish health and human health, as disrupted circadian rhythms have been associated with various health problems in humans.

ACKNOWLEDGEMENTS

Authors have made substantial, direct, and intellectual contributions to the article; their efforts in bringing the relevant content were at greater heights. We authors are very grateful to Christ Deemed to be University for providing us the permission, lab facilities and opportunity.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interest or personal relationships that could inappropriately influence the work presented in this manuscript.

STATEMENT OF ETHICS

The Ethical clearance certificate was not required for this research article.

References

[1] S. P. Klinken, "Cells in focus Red blood cells," *Int. J. Biochem. Cell Biol.*, vol. 34, pp. 1513–1518, 2002.

[2] S. Puente-Marin, R. Thwaite, L. Mercado, J. Coll, N. Roher, and M. Del Mar Ortega-Villaizan, "Fish red blood cells modulate immune genes in response to bacterial inclusion bodies made of TNFα and a g-VHSV fragment," *Front. Immunol.*, vol. 10, no. MAY, pp. 1–11, 2019, doi: 10.3389/fimmu.2019.01055.

[3] A. Brownlie and L. Zon, "The for a Model System the Study of Hematopo," vol. 49, no. 5, 1995.

[4] A. Charrier, B. Olliac, P. Roubertoux, and S. Tordjman, "Clock genes and altered sleep–wake rhythms: Their role in the development of psychiatric disorders," *Int. J. Mol. Sci.*, vol. 18, no. 5, pp. 1–22, 2017, doi: 10.3390/ijms18050938.

[5] J. A. Evans and A. J. Davidson, *Health Consequences of Circadian Disruption in Humans and Animal Models*, 1st ed., vol. 119. Elsevier Inc., 2013.

[6] J. Feriel and D. Tchipeva, "Effects of circadian variation , lifestyle and environment on hematological parameters : A narrative review," no. April, pp. 917–926, 2021, doi: 10.1111/ijlh.13590.

[7] M. Egg *et al.*, "reduced clearance of senescent erythrocytes," vol. 31, no. 5, pp. 680–689, 2014, doi: 10.3109/07420528.2014.889703.

[8] P. C. de Souza and G. O. Bonilla-Rodriguez, "Fish hemoglobins," *Brazilian J. Med. Biol. Res.*, vol. 40, no. 6, pp. 769–778, 2007, doi: 10.1590/s0100-879x2007000600004.

[9] S. Chowdhury, S. K. Saikia, and W. Bengal, "Oxidative Stress in Fish : A Review," 2020.

[10] V. I. Lushchak, T. V Bagnyukova, V. V Husak, L. I. Luzhna, O. V Lushchak, and K. B. Storey, "Hyperoxia results in transient oxidative stress and an adaptive response by antioxidant enzymes in goldfish tissues," vol. 37, pp. 1670–1680, 2005, doi: 10.1016/j.biocel.2005.02.024.

[11] Y. Chen, S. Fang, and S. Jeng, "Comparative Biochemistry and Physiology , Part A Zinc transferrin stimulates red blood cell formation in the head kidney of common carp (Cyprinus carpio)," *Comp. Biochem. Physiol. Part A*, vol. 166, no. 1, pp. 1–7, 2013, doi: 10.1016/j.cbpa.2013.05.001.

[12] L. Marı, N. S. Foulkes, and F. J. Sa, "Effect of Lighting Conditions on Zebrafish Growth and Development," vol. 11, no. 2, 2014, doi: 10.1089/zeb.2013.0926.

[13] D. Gronquist and J. A. Berges, "Effects of aquarium-related stressors on the zebrafish: A comparison of behavioral, physiological, and biochemical indicators," *J. Aquat. Anim. Health*, vol. 25, no. 1, pp. 53–65, 2013, doi: 10.1080/08997659.2012.747450.

[14] L. Zang, Y. Shimada, Y. Nishimura, T. Tanaka, and N. Nishimura, "Repeated blood collection for blood tests in adult zebrafish," *J. Vis. Exp.*, vol. 2015, no. 102, pp. 1–10, 2015, doi: 10.3791/53272.

[15] O. Ehrlich, A. Karamalakis, A. J. Krylov, S. Dudczig, K. L. Hassell, and P. R. Jusuf, "Clove Oil and AQUI-S Efficacy for Zebrafish Embryo, Larva, and Adult Anesthesia," *Zebrafish*, vol. 16, no. 5, pp. 451–459, 2019, doi: 10.1089/zeb.2019.1737.

[16] F. Babaei *et al.*, "Novel blood collection method allows plasma proteome analysis from single zebrafish," *J. Proteome Res.*, vol. 12, no. 4, pp. 1580–1590, 2013, doi: 10.1021/pr3009226.

[17] C. Breil, M. Abert Vian, T. Zemb, W. Kunz, and F. Chemat, "'Bligh and Dyer' and Folch methods for solid–liquid–liquid extraction of lipids from microorganisms. Comprehension of solvatation mechanisms and towards substitution with alternative solvents," *Int. J. Mol. Sci.*, vol. 18, no. 4, pp. 1–21, 2017, doi: 10.3390/ijms18040708.

[18] A. Avdesh *et al.*, “Regular care and maintenance of a Zebrafish (Danio rerio) laboratory: An introduction,” *J. Vis. Exp.*, no. 69, 2012, doi: 10.3791/4196.

[19] J. M. Murtha, W. Qi, and E. T. Keller, “Hematologic and serum biochemical values for zebrafish (Danio rerio),” *Comp. Med.*, vol. 53, no. 1, pp. 37–41, 2003.

[20] C. E. Koch, B. Leinweber, B. C. Drengberg, C. Blaum, and H. Oster, “Interaction between circadian rhythms and stress,” *Neurobiol. Stress*, vol. 6, pp. 57–67, 2017, doi: 10.1016/j.ynstr.2016.09.001.

[21] B. Robert and E. B. Brown, “Dissolved Oxygen,” no. 1, pp. 1–14, 2004.

[22] C. Lawrence and T. Mason, “Zebrafish Housing Systems: A Review of Basic Operating Principles and Considerations for Design and Functionality MS [Aquatic Resources Program Manager] and Boston Children’s Hospital,” *Ilar*, vol. 53, no. 2, pp. 179–191, 2015, doi: 10.1093/ilar.53.2.179.Zebrafish.

[23] M. Schieber and N. S. Chandel, “ROS function in redox signaling,” *Curr. Biol.*, vol. 24, no. 10, pp. 453–462, 2014, doi: 10.1016/j.cub.2014.03.034.ROS.

[24] J. S. O-Neill and A. B. Reddy, “Circadian clocks in human red blood cells,” *Nature*, vol. 469, no. 7331, pp. 498–504, 2011, doi: 10.1038/nature09702.

[25] K. Kulkeaw and D. Sugiyama, “Zebrafish erythropoiesis and the utility of fish as models of anemia,” *Stem Cell Res. Ther.*, vol. 3, no. 6, pp. 1–11, 2012, doi: 10.1186/scrt146.

[26] M. Nagane, R. Suge, and S. I. Watanabe, “Relationship between psychosomatic complaints and circadian rhythm irregularity assessed by salivary levels of melatonin and growth hormone,” *J. Circadian Rhythms*, vol. 9, pp. 2–7, 2011, doi: 10.1186/1740-3391-9-9.

[27] T. Paparrigopoulos *et al.*, “Melatonin secretion after head injury: A pilot study,” *Brain Inj.*, vol. 20, no. 8, pp. 873–878, 2006, doi: 10.1080/02699050600832114.

[28] B. H. Kim, S. P. Hur, S. W. Hur, Y. Takeuchi, A. Takemura, and Y. D. Lee, “Circadian rhythm of melatonin secretion and growth-related gene expression in the tiger puffer Takifugu rubripes,” *Fish. Aquat. Sci.*, vol. 20, no. 1, pp. 1–8, 2017, doi: 10.1186/s41240-017-0061-0.

[29] E. Tasali, K. Wroblewski, E. Kahn, J. Kilkus, and D. A. Schoeller, “Effect of Sleep Extension on Objectively Assessed Energy Intake among Adults with Overweight in Real-life Settings: A Randomized Clinical Trial,” *JAMA Intern. Med.*, vol. 60637, no. 4, pp. 365–374, 2022, doi: 10.1001/jamainternmed.2021.8098.