

Stress behavior and biochemical profiling of *Opsarius bendelisis* under different photoperiods

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Received: 29 January 2024

Revised: 09 April 2024

Accepted: 08 May 2024

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Abstract

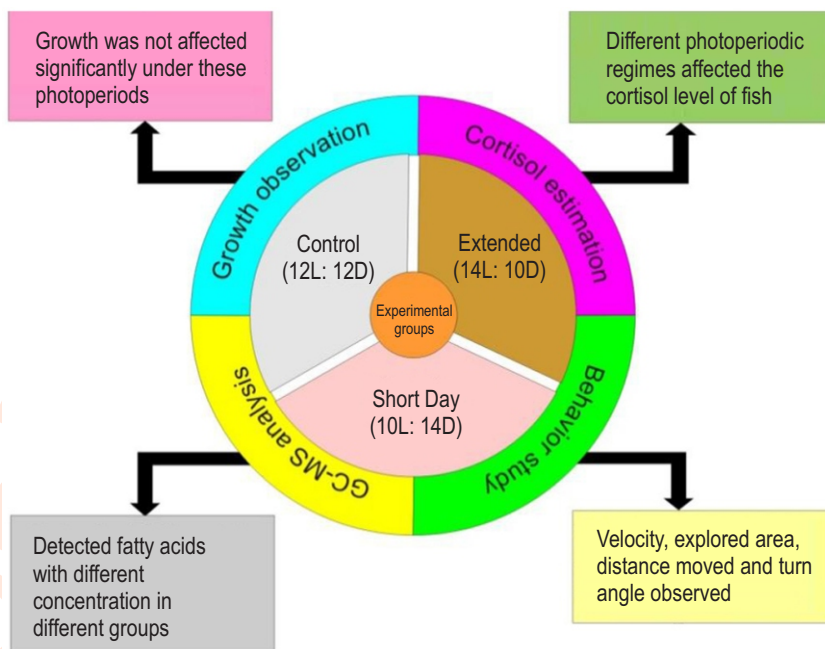
Aim: The investigation evaluated the impact of varying photoperiods on the physiological regulation of stress pathway and the biochemical concentration of metabolites in hillstream trout, *Opsarius bendelisis*.

Methodology: Different light phases were used in the present study to quantify their weight and chronic stress marker cortisol using enzyme-linked immunosorbent assay (ELISA). The behavior of the fish was investigated using the highly sophisticated software Ethovision XT 13 with a frame rate of 25 per sec. The fish's lipid profiles were measured using gas chromatography-mass spectrometry (GC-MS).

Results: The chronic marker of stress cortisol showed a relevant difference among the various regimes of photoperiod at $P \leq 0.0046$. The different observed behavior profiles, such as velocity distance traveled, also showed a significant difference at $P < 0.0001$ in the different light-exposed durations. The 9-octadecenoic acid, methyl ester, (E)- (I), and hexadecanoic acid, methyl ester also showed a significant difference at $P < 0.05$.

Interpretation: Extended photoperiod lowers stress levels and influences fish behavior. The results of this investigation will offer fresh perspectives on cognitive-behavioral tests.

Key words: Cortisol, ELISA, *Opsarius bendelisis*, Photoperiods, Stress behavior



Introduction

The family Cyprinidae includes the genus *Opsarius bendelisis* (Hamilton 1807), one of the widely dispersed species in Asia. Currently, 20 species of *Opsarius* are known from the Himalayan and sub-Himalayan areas. The Indian Hill Trout, *O. bendelisis*, is a widely dispersed demanding ornamental and food fish. Most significantly, they are easy to maintain and monitor in a laboratory setting and are well-suited to environmental conditions (Dewan et al., 2016). *Opsarius bendelisis*, Previously known as *Barilius bendelisis* (Kottelat, 2013), categorized under the least concern in IUCN, has a flattened body, blackish-blue bars on the body and a dorsal fin behind the body's center. These notable morphological features contribute to the unique physical appearance and identification of *Barilius bendelisis* in its natural habitat, which was studied by Panda et al. (2019). Chand et al. (2022) studied different behaviors (swimming speed, exploration, turn angle) and other stress aspects of *Barilius bendelisis* post exposure to vitamin C.

The daily endogenous rhythmicity of fish is also regulated by photoperiod, which influences fish development, locomotion, metabolic rates, body coloration, sexual maturity and reproduction. Photoperiod also functions as an artificial Zeitgeber (trigger or synchronizer) (Elsbaay, 2013). Fish stay active for more extended periods and exhibit more activity as a result of lengthy photoperiods, which can have an indirect effect on the growth. Lengthy photoperiods also impact muscle mass development by increasing locomotor activity and improving nutrient usage efficiency (Boeuf and Le Bail, 1999; Biswas et al., 2006). In addition to having an impact on eating behaviors, photoperiod is essential for the growth, survival, and social behavior. (Almazán-Rueda et al., 2005; Ergün et al., 2003; Abdollahpour et al., 2020). According to Bani et al. (2009), photoperiodic fluctuations affect the levels of steroid and corticosteroid hormones, although stress is not always a noticeable result. A light environment affects the biochemical profiling, including the fish's lipids or fatty acids of the *Scylla paramamosain*, as per a study of Li et al. (2020).

A dim light at night promoted the lipid hepatic triacylglycerol accumulation and upregulated hepatic genes, which elevated the glucose and fatty acid uptake, but in parallel, it suppressed fatty acid synthesis in the adipose tissue in Wistar rats (Okuliarova et al., 2020). The effects of photoperiod (16L:8D) on the growth, stress and behavior *Tor putitora* has been studied by Kalkhundiya et al. (2021). They reported that short-day (8L:16D) photoperiod was favorable for *Tor putitora* in response to behavioral responses. According to Biswas et al. (2006), photoperiod affects the development and stress of *Pagrus major*. In Himalayan hillstream fishes, limited research has been done on photoperiodic modulation of behavior, stress and lipid profiling using sophisticated methods. A significant lacuna exists in the natural behavioral performance of these fishes. studies on the effects of various photoperiods on *Opsarius bendelisis* in relation to their growth, stress, behavioral and lipid profiling are limited.

Therefore, the current study aimed to determine the effects of different photoperiods on the growth, stress behavioral and lipid profiles of *Opsarius bendelisis*.

Materials and Methods

Procurement of fish: Adult *Opsarius bendelisis* were sampled from the Suwalekh stream (29.66°N, 80.22°E) in Pithoragarh, Uttarakhand, transported to the laboratory, and acclimatized in natural habitat there. Artificial food was provided to fish after the onset of natural light. After acclimation, the experiment was conducted according to the Institutional Animal Ethical Committee Guidelines (1449). Fifteen individuals of *O. bendelisis* were selected and equally distributed into three tanks, with five fish in each tank (2.41 -10.79 g) for the photoperiodic stimulation of 28 days.

Experimental design: The fish were grouped into three groups in separate aquariums (30x42x36 cm). Black sheets were placed over each tank to block outside light. Each group was given a different photoperiod, maintained with the timer's quick sense (Qs-T1), and manually monitored the timer's functioning.

Experimental protocol of all the groups is as follows: Control (12L: 12D) 12 hr light and 12 hr dark (L^{on} at 12 am and L^{off} at 12 pm); Short day (10L: 14D) 10 hr light and 14 hr dark (L^{on} at 12 pm and L^{off} at 10 pm) and Extended day (14L: 10D): 14 hr light and 10 hr dark (L^{on} at 12 am and L^{off} at 2 pm).

All tanks were routinely aerated with air pumps and air stones. The excretory matter was removed every 4th days, and 70% of the water was refilled every fourth day. A 5W LED. (Syska) was placed 10 cm above the water to produce artificial light. Light intensity was maintained at 100 lux in the light phase and one lux in the dark phase. The intensity was assessed with a digital lux meter. Fish were fed artificial food ad libitum. The composition of artificial food was 32% crude protein, 4% crude fat, 5% crude fiber, 10% crude ash, 31% nitrogen and 9% moisture. The weight of each fish was recorded using an electronic balance (Roy Electronics India model number CBCAB300) with an accuracy of 0.001 g.

Open square tank test: A digital Basler camera with four pylon cameras was used to record the video of individual fish in an open square tank (50 x 30 x 30 cm) to study their behavior. Ethovision XT 13 Software (Noldus Infotech, Wageningen, the Netherlands) was used to analyze different behavioral scores (an area of exploration, distance traveled, velocity, and turn angle) for 300 sec at 25 frames per sec.

Stress quantification: At the end of the experiment, fish were anesthetized with clove oil. Fish cortisol levels were measured using an enzyme-linked immunosorbent test kit (Cayman Chemical, Cortisol ELISA Kit) as per the protocols of Cachat et al. (2010).

Lipid extraction: Post exposure to three different photoperiods, i.e., Control (12L:12D), Short day (10L:14D) and Long day

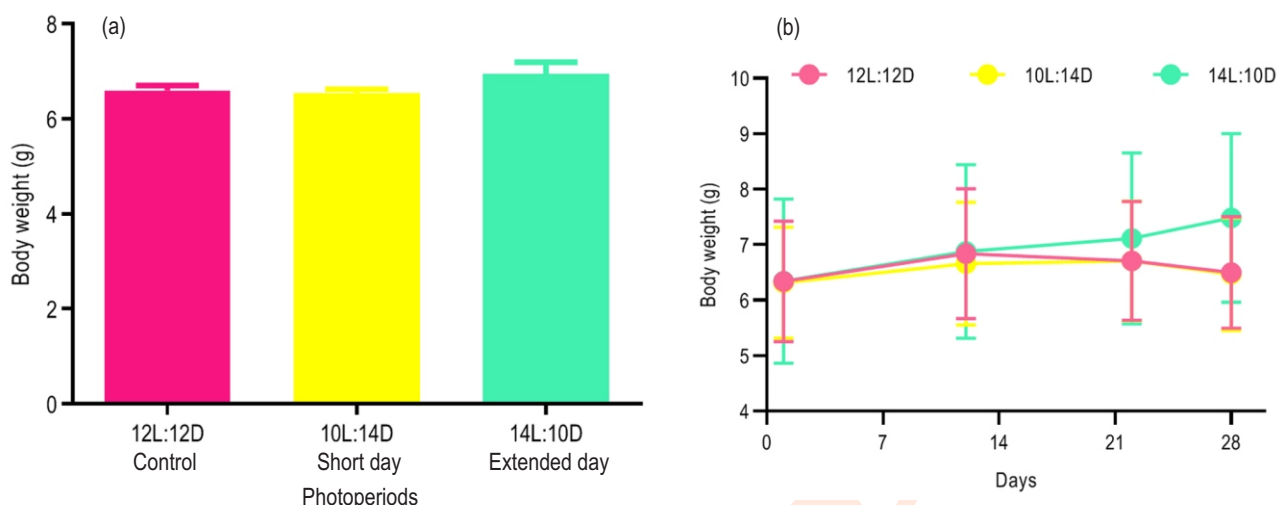


Fig. 1: (a) Average body weight of *O. bendelisis* after photoperiodic exposure; (b) Temporal profiling of body weight of *O. bendelisis*.

(14L:10D), fish were killed using clove oil, and the muscle samples were collected for estimating lipids content. Lipid was extracted as per the protocol of Folch *et al.* (1957). In this method, 50 mg of tissue was homogenized in 2 ml chloroform-methanol mixture 2:1 (v/v) and then diluted with chloroform-methanol mixture to make a volume of 10 ml, there after it was filtered. A 2ml of 0.9% NaCl salt solution was added, mixed well, and centrifuged at 2400 rpm for 20 min. Two phases, i.e., upper and lower phases were separated after centrifugation. The supernatant was removed by pipetting, and the lower phase was used for GC-mass analyses.

Gas Chromatography-Mass Spectrometry (GC-MS): The lipid samples were dissolved in a mixture of methanol: ethyl acetate and injected in a GC-MS- Agilent 7890B G.C. connected to 5977A M.S.D., Column HP_5MS 5% Phenyl Methyl Silox-60°C-325°C, 30m x 250 μm x 0.25μm through Splitless Injection mode. The operating conditions of the G.C.-M.S. set for the analysis were as follows: Oven temperature at 45 °C for 2 min, followed by 140 °C at 5 °C min⁻¹, and finally increased to 280 °C and held isothermally for 10 min. The sample injection was 2 μl, and helium at a flow rate of 1ml min⁻¹ was used as a carrier gas. The ionization of the sample was carried out at 70 eV. The running time of the G.C. was 54.0 min. NIST14.L library (2020) was then searched to compare the structures of the compounds with those of the NIST database. Compounds were then identified based on the retention times and mass spectra with already known compounds in the NIST library (C:\Database\NISTMS2011.L).

Statistical analysis: Graph Pad Prism was used for the statistical analysis by applying One-way ANOVA, followed by a post hoc Tukey's multiple comparison test.

Results and Discussion

The perusal of data showed that the average body weight of fish was 6.594 ± 0.11g in the control group (12L:12D), 6.537 ±

0.09 g in short-day group (10L:14D) and 6.952 ± 0.24 g in the long-day group (14L:10D). Body weight was highest in long days and lowest for short days. One-way ANOVA (F 2, 9 = 1.969 at P=0.1953) followed by post-hoc Tukey's multiple comparison tests showed no significant difference in the body weight of any group. (Fig. 1a, b). In the present study, no significant growth performance was observed in *O. bendelisis* after exposure to photoperiod Fig. 1. However, the growth performance in long day group onwards 21 days increased as compared to the control and short day light exposure groups (Fig. 1 b). The light intensity in the light-phase was 100 lux compared to the dark phase, which was 1 lux. The species-specific growth performance depends on the light-intensity uptake and feeding strategy of fish. Bani *et al.* (2009) examined the effects of photoperiod on young *Huso huso* fish and found no discernible changes in the growth of any group of fish. Different light hours of photoperiod impact the fish performance, which is species-specific.

In another investigation, *Miichthys miiuy* larvae were raised under several photoperiodic regimes, and the results revealed that the photoperiod had no impact on the development of larvae younger than 20 Dph (Shan *et al.*, 2008). Juveniles of *Pleuroctes ferrugineus* showed no significant difference on the growth when exposed to different photoperiods (Purchase *et al.*, 2000). The light intensity and photoperiod have no impact on the growth of juveniles of *Oreochromis niloticus* (Wang *et al.*, 2020). According to Abdollahpour *et al.* (2020), numerous elements like diet, hormones, stocking density, physical conditions and chemical factors affects the growth and development of fish.

At the end of the study, the average cortisol levels was 4.44 ± 0.08 pg ml⁻¹ in the control group, 4.25 ± 0.06 pg ml⁻¹ in the short day group, and 4.07 ± 0.02 pg ml⁻¹ in the extended day group, respectively. The control group had the highest cortisol levels. The log transformed data of cortisol concentration showed a (one way ANOVA, F 2, 11= 8.5 at P ≤0.0046) significant

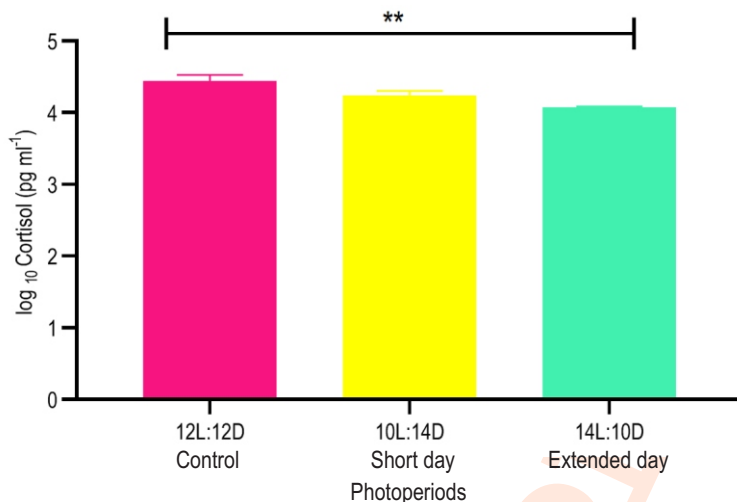


Fig. 2: Average cortisol concentration of fish after photoperiod experiment; ** Indicated the statistical significance of $P < 0.0046$.

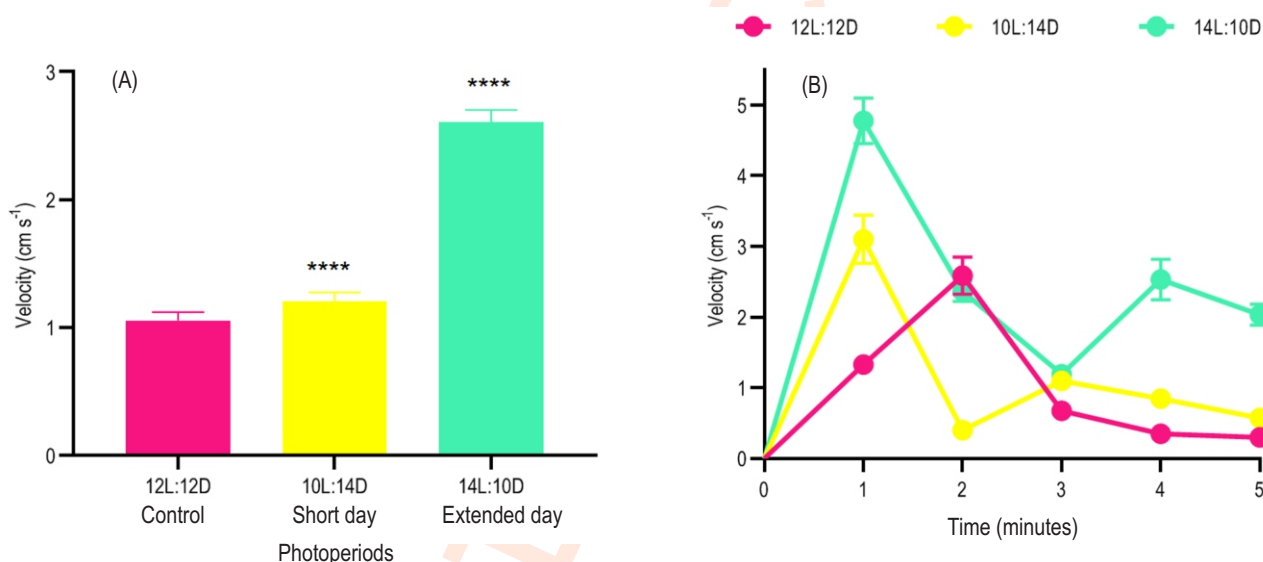


Fig. 3: (A) Average velocity of fish after photoperiodic treatments; **** Indicated the statistical significance of $P < 0.0001$; (B) Temporal observation of velocity (cm s⁻¹) during a 5 min open tank test of fish.

difference according to the post-hoc Tukey's multiple comparison tests (Fig. 2). In the current study, the maximum concentration of cortisol was in the control group. In contrast, the minimum concentration was measured in the extended day group. The minimum concentration of cortisol indicated that these light hours have no impact on the chronic stress of *O. bendelisis* (Fig 2).

According to Tian *et al.* (2019), cortisol in fish plays a role in various biological functions such as growth, improved lipid metabolism, stress response, reproduction, behavior and osmoregulation. Additionally, Almazán-Rueda *et al.* (2005) found that photoperiod impact stress levels in *Clarias gariepinus*. Further, they found that increased photoperiod affected cortisol

levels, oxidative stress and chronic stress. Similarly, Biswas *et al.* (2004) observed a difference in the cortisol levels in Nile tilapia (*Oreochromis niloticus*) with photoperiodic manipulation. These findings highlight the importance of light hours in influencing chronic stress levels.

The behavior study showed that the average velocity in the control group was 1.056 ± 0.064 cm sec⁻¹, short day group was 1.204 ± 0.07 cm sec⁻¹, and extended group was 2.605 ± 0.098 cm sec⁻¹. One-way ANOVA (2, 44882 = 117.5 at $P < 0.0001$) was used to evaluate the velocity data, tracked by as post hoc Tukey's multiple comparison tests, which revealed a significant difference among the distinct photoperiodic regimes (Fig. 3). Fish travelled more

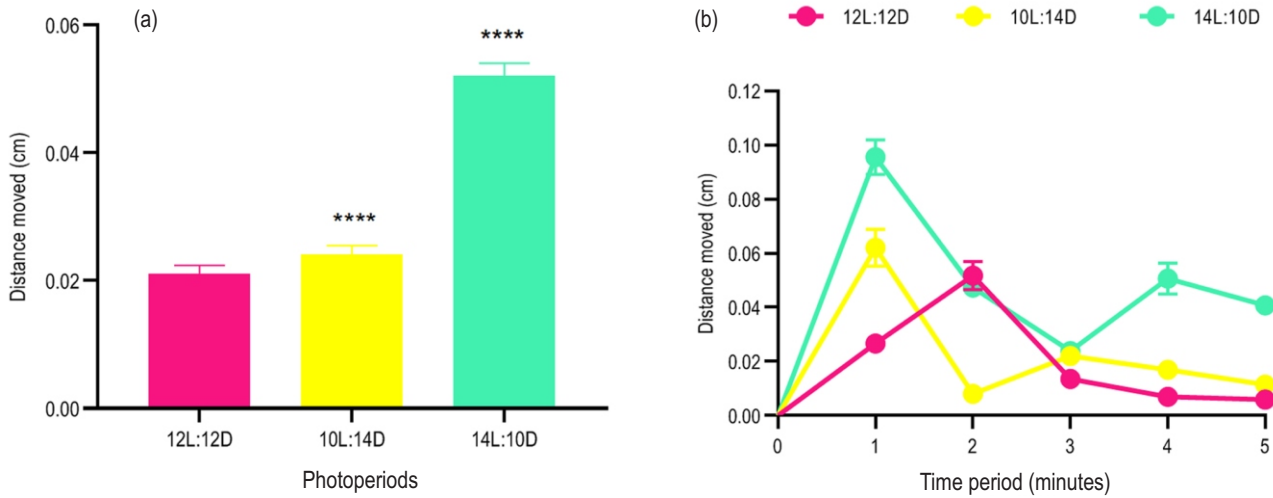


Fig. 4: (A) Distance moved by fish during behavior study after photoperiod experiment, ****Indicated the statistical significance of $P < 0.0001$; (B) Temporal observation of distance moved (cm) during 5 min open tank test of fish.

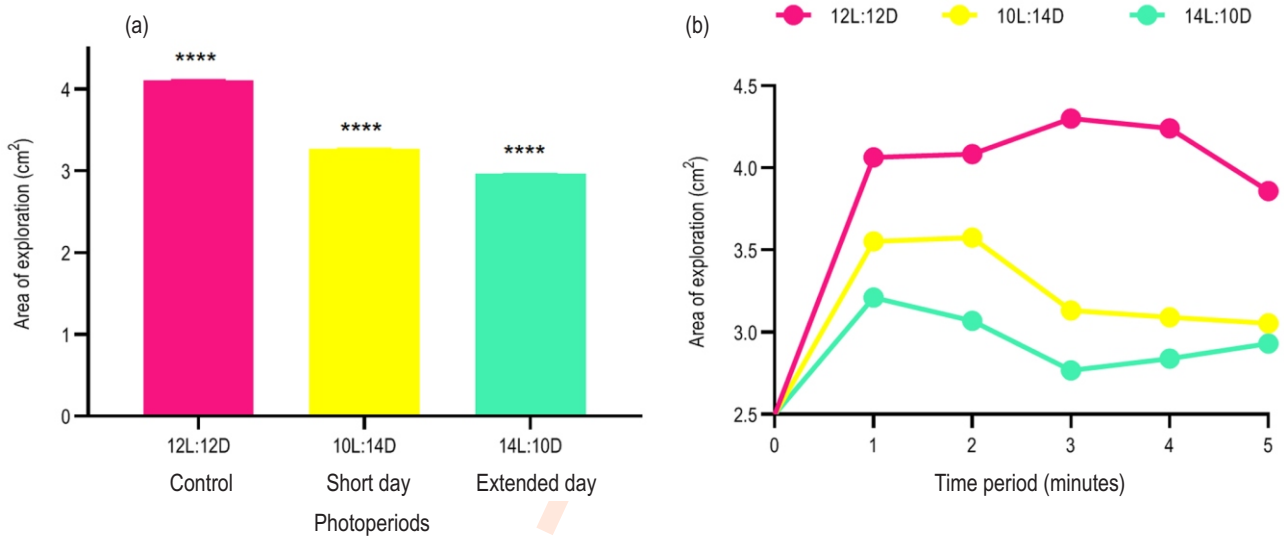


Fig. 5: (a) Area explored by fish after the photoperiod experiment, ****Indicated the statistical significance of $P < 0.0001$; (b) Temporal observation of area of exploration (cm²).

distance in the extended day group (0.052 ± 0.002 cm) as compared to the short day group (0.024 ± 0.001 cm) and control group (0.021 ± 0.001 cm), (2, 44882 = 117.5 at $P < 0.0001$) respectively (Fig. 4).

The current investigations measured all behavioral indicators using highly sophisticated software Ethovision XT 13 (Noldus). The swimming exercises in the extended day group was more as compared to short day group and control group. The minimum concentration of cortisol and maximum swimming speed in the extended day group showed a correlation among the regulation of stress (Fig. 3A and B). In the first minute, the

maximum velocity was observed in the fishes of extended day group as compared to other photoperiodic regimes. The temporal expression of swimming behavior showed a drastic change during the entire duration of observation. Several motions have been documented to express the behavioral profile of fish, including velocity, distance traveled, an area explored, and turn angle. In this study, speed and distance moved by fish were maximum in the extended day group and maximum in the control group. A similar study on *Tor putitora* post exposure to different light spectra (16L:8D and 8L:16D) affected the swimming velocity and distance travelled (Kalkhundiya et al., 2021). The area of fish explored area was maximum in the control group (4.11 ± 0.004

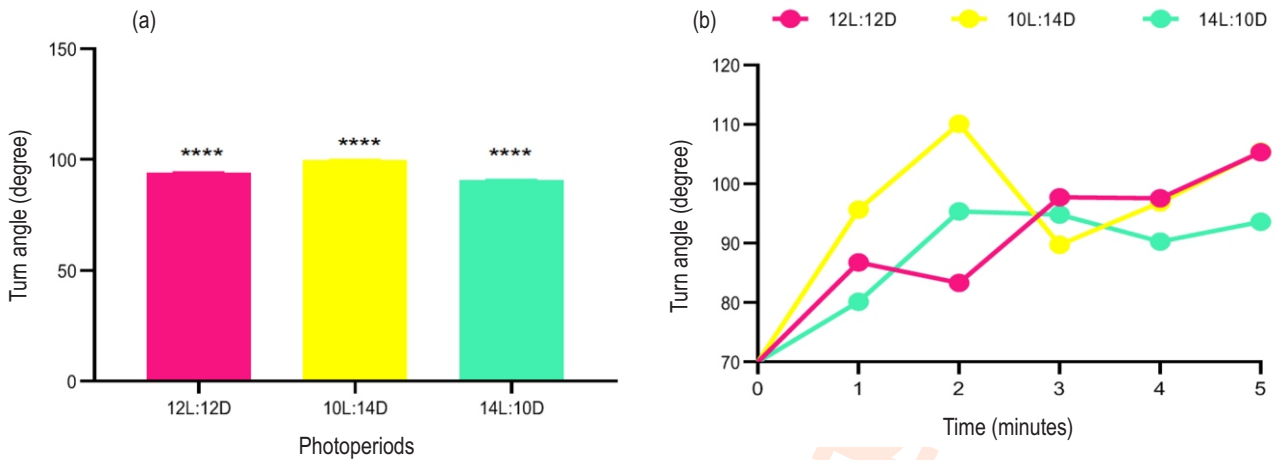


Fig. 6: (a) After photoperiodic exposure, turn angle of fishes; ****Indicated the statistical significance of $P < 0.0001$; (b) Minute-wise observation of Turn angle.

cm²), followed by short day group (3.27 ± 0.004 cm²), and extended day group (2.96 ± 0.003 cm²) respectively. A significant difference between various groups was found by One-way ANOVA ($F_{2, 44882} = 21499$ at $P < 0.0001$) and Post-hoc Tukey's multiple comparison tests (Fig. 5). The temporal expression of the exploratory behavior of fish was maximum in the first-minute duration, and thereafter it was approximately in constant nature. The continuous approach of fish in constant conditions highlight the cognitive abilities of *O. bendelisis*.

The exploratory nature of fish opens many dimensions in the neurobehavioral aspects of the fish. The action of the brain mainly governs each behavioral execution. According to a study by Cachat *et al.* (2010), zebrafish exhibit decreased area exploration, freezing, and increased diving under stressful environmental conditions. As the zebrafish adjusts, it exhibits increased area exploration and decreased freezing (Stewart *et al.*, 2010). A novel tank experiment with zebrafish showed that gradual changes in freezing behavior increased exploratory behavior. Many zebrafish mutant strains show less exploratory behavior, indicating a stronger anxiety reaction to perceived stressors and higher baseline anxiety than the wild-type strain (Egan *et al.*, 2009). The turn angle of the fish in the control group was 94.16 ± 0.27 degree, 99.9 ± 0.27 degree in the short day group and 90.93 ± 0.25 degree in the extended day group, respectively. Tukey's multiple comparison tests after one-way ANOVA ($F_{2, 44882} = 296.9$ at $P < 0.0001$) showed a significant difference in the turn angles of all groups (Fig. 6).

A simple change in the direction of swimming is the turning behavior, and its angle is called the turning angle. Fish mark their territory in search of food. In the current behavioral investigation of *O. bendelisis*, the turn angle was highest in the short day (10L: 14D) group and lowest in the extended day (14L: 10D) group (Fig. 6). Stewart *et al.* (2015) pointed out that treating zebrafish with nicotine (an anxiolytic chemical) increased the rate of swimming and distance covered by fish, while decreased the

turn angle. Anosmia (loss of olfaction) is produced in zebrafish (*Danio rerio*). In adult zebrafish, it modifies behavior that resemble anxiety. Their locomotor activity indicators, such as distance travelled, crossing, absolute turn angle, and entrances into higher and lower zones decrease (Abreu *et al.*, 2016). Our previous study on *Barilius bendelisis* showed that vitamin C treatment affected the explored area, velocity, distance travelled, and turn angle of fish (Chand *et al.*, 2022).

In the present study, GC-MS detected various compounds in the lipid samples of fish. GC-MS analysis detected 49 compounds in the 12L:12D group, 86 compounds in the 10L:14D group, and 58 compounds in the 14L:10D group in the total ion chromatogram. In the present study, the maximum concentration of n-Hexadecanoic acid was expressed in the short day group as compared to other groups. The concentration of Hexadecanoic acid methyl ester showed a significant difference (One-way ANOVA, $F_{(2, 2)} = 32.40$ at $P \leq 0.0299$) in Fig. 7f. The maximum light exposure of fishes also affect their fatty acid composition. The increasing concentration of fatty acid after light exposure opens many dimensions in lipidomics concerning environmental factors. The fatty acid expression also depends on the climatic and natural habitat conditions of fish. In the current investigation, the n-hexadecanoic acid concentration was higher in the extended day 14L:10D cycle. n-hexadecanoic acid also known as palmitic acid is an anti-inflammatory compound.

The light phase of the fish increased the concentration of n-hexadecanoic acid to prevent inflammation in fish. n-Hexadecanoic acid is a competitive inhibitor of phospholipase A(2), which hydrolyses the ester bond of the membrane phospholipids and initiates the inflammation process (Aparna *et al.*, 2012). 9,12-Octadecadienoic acid (Z,Z)-, methyl ester (C₁₉H₃₄O₂) is an unsaturated fatty acid through a double bond at ($\Delta 9,12$) commonly known as methyl linoleate. The maximum concentration of 9,12-Octadecadienoic acid (Z, Z)-, methyl ester (C₁₉H₃₄O₂) was found in the 10L:14D group (Fig 7b). The

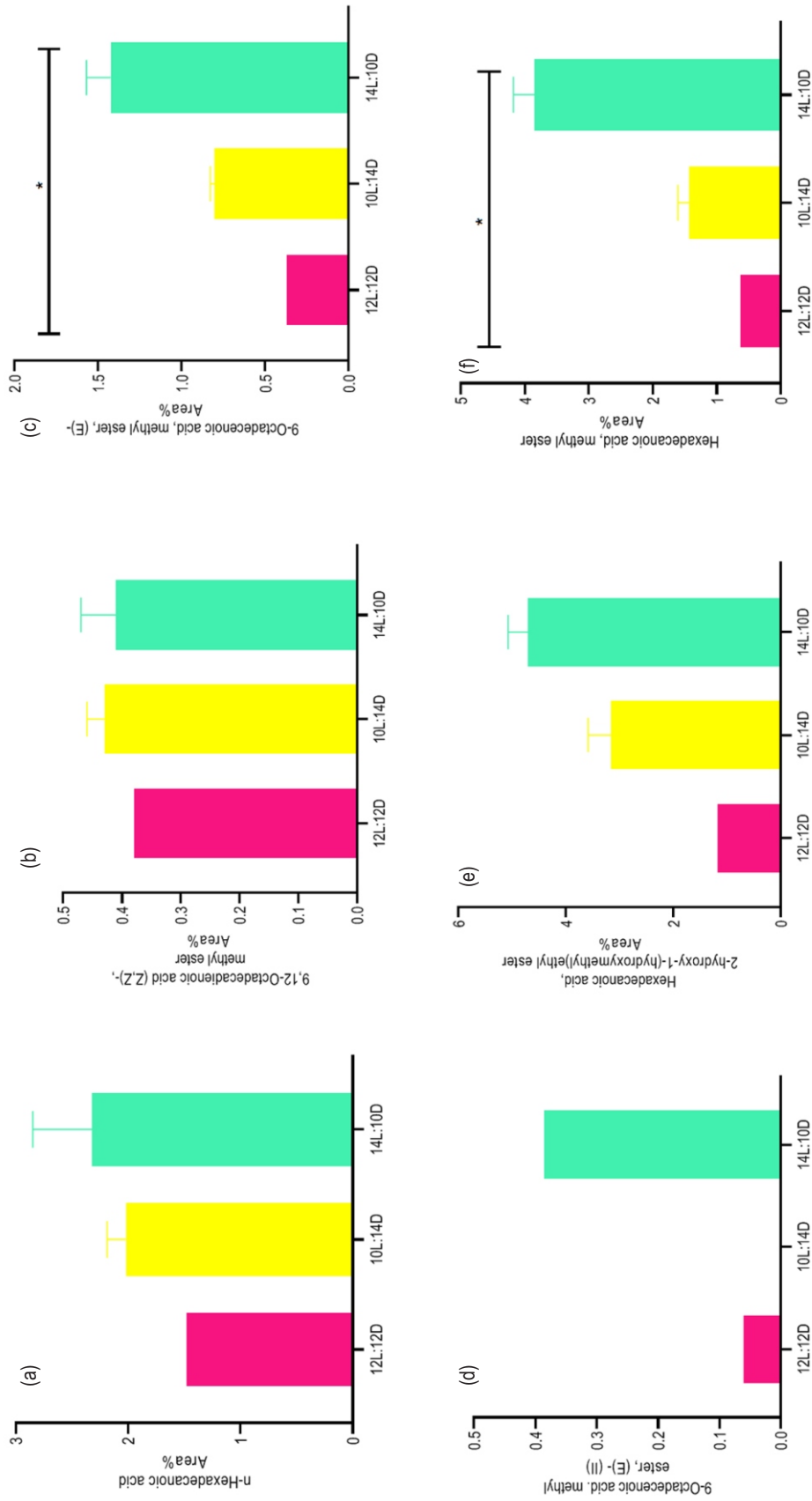


Fig. 7: Average concentration of common fatty acids in the photoperiodic exposure groups; * Indicated the statistical significance of $P < 0.05$.

herbivorous nature of *O. bendelisis* indicated the presence of these compounds in fish. Habitat plays a vital role in the expression of these compounds. The general role of this compound is to transport the cholesterol molecules (Sipes *et al.*, 2013). 9 - Octadecenoic acid, methyl ester, (E)- (I) also showed a remarkable difference after the data exposure of One-way ANOVA analysis ($F(2, 2) = 19.13$ at $P \leq 0.0497$) (Fig. 7c).

In the present study, the maximum concentration of 9 - Octadecenoic acid, methyl ester, (E)- (I) in the 14L:10D phase of photoperiod was compared to that of the control. The long exposure to light intensity (100 lux) affects the expression of these fatty acids in *O. bendelisis*. 9 - Octadecenoic acid, methyl ester, (E)- (I) is a monounsaturated fatty acid. The analgesic, anti-inflammatory and ulcerogenic activities of these compounds have been reported by Hadi *et al.* (2016). 9 - Octadecenoic acid, methyl ester, (E)- (II) was not detected in the short day (10L:14D) phase of photoperiod (Fig. 7d). This indicated that the ten-hour light phase was insufficient in triggering this form of fatty acid; a further downstream study will be required to decipher the pathway of its expression after the threshold light phases. A similar study was conducted to extract these compounds from the seed kernels of *Manilkara bidentata* (Powder-George and Mohammed, 2018). A similar study on Atlantic salmon also showed diversity in monounsaturated and polyunsaturated fatty acid content after exposure to 16L:8D and 24L:0D photoperiodic cycle (Nemova *et al.*, 2020). This study showed the maximum concentration of Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester, in the 14L:10D light phase of the photoperiodic regimes (Fig. 7e). The light phase affects the expression of these fatty acids in the *O. bendelisis*. An in depth study will open the dimensions of the mechanism of action of these chemicals after exposure to environmental factors such as photoperiod. The role of these compounds is to prevent ulcers and improve the proinflammatory cytokines and apoptosis markers (Syed *et al.*, 2022). Light plays an underlying role in the expression of these molecules and opens a broad spectrum about the tracking of pathways and their consequences in the freshwater hill stream fish *O. bendelisis* (Shaaban *et al.*, 2021).

Palmitic methyl ester protects against cardiac arrest as a neuroprotective agent in rats by increasing cerebral blood flow, acting as a vasodilator, and enhancing cognitive abilities such as memory and learning (Lee *et al.*, 2019). The enhanced levels of fatty acids during 14 hours of light at 100 lux intensity opens a new dimension of this compound in the therapeutics of cardiovascular disease protection. A further downstream study will be required on the pathway's role of palmitic methyl ester in protecting cardiac arrest.

Acknowledgments

The authors thank the Principal of Govt. P.G. College Pithoragarh and M.B. Govt. P.G. College Haldwani for their unconditional support during the study. We are also grateful to CytoGene Research & Development Laboratory, Lucknow for providing the GC-MS instrumentation facility.

Authors' contribution: J. Chand: Sampling, experimentation, paper writing and experiment execution; N.S. Karki: Helped in GC-MS study; J. Bhatt: Helped in experiment and writing; S. Bohra: Helped in experimentation; B.C. Pathak: Designed and provided guidance during experimentation, penned the report, performed statistical analysis, proofreading, and other tasks.

Funding: Not applicable.

Research content: The research content of manuscript is original and has not been published elsewhere.

Ethical approval: As per the Institutional Animal Ethical Committee Guidelines (1449).

Conflict of interest: There is no conflict of interest among the authors.

Data availability: Data available with the corresponding author.

Consent to publish: All authors agree to publish the manuscript in *Journal of Environmental Biology*.

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