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Anthropogenic night light affects the muscle histology of redheaded bunting (*Emberiza bruniceps*)

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Abstract

Artificial light at night (LAN) often disrupts the nocturnal behaviors and physiological responses of migratory birds. En route to their migratory journeys, birds come across adversities pertaining to weather, food scarcity, temperature, and several anthropogenic factors. Among these factors, exposure to LAN has garnered much attention in the past decade. Artificial manipulation of daylength is known to cause differential effects on the behavior and physiology of photoperiodic organisms. To test the effect of exposure to different colors of light at night on the histomorphometry of the pectoral and cardiac muscle of birds, we subjected photosensitive redheaded buntings to all LAN (Light at Night) or No LAN treatments under 10L:14D photoperiod for 10 days. All LAN (~5 Lux) treatments comprised two groups having white and blue light at night, namely WLAN and BLAN, respectively. While the group having dark night i.e., 0 Lux at night was named the NLAN group. The daytime illumination with white light was set at 200 Lux. Food and water were *ad-libitum* in all the groups. We found that the pectoral muscle fiber and cardiac muscle fiber width increased under the LAN exposure, but the white and blue color of night light equally affected the muscle morphometry. However, the lipid accumulation in the cardiac muscle fiber showed the differential effect of colored light at night in which the WLAN group showed the highest lipid accumulation. We did not find any significant difference in the density of muscle fiber comprising the fascicle. So, these results suggest that the redheaded bunting showed a differential response to different colored LANs with respect to muscle fiber thickness and lipid accumulation in muscle fibers.

Keywords: Fascicle; LAN; Lipid; Muscle Fiber; Redheaded Bunting

1. Introduction

In urban areas, anthropogenic light sources like streetlamps, searchlights, outdoor advertising boards, etc., turn night into day-like conditions affecting human health and wildlife. Photoperiod is a potent *Zeitgeber* that entrains the biological clock and gives the animal a cue to initiate or terminate reproductive behaviors in photoperiodic species. Researchers have been focusing on studying the effect of exposure to night light on different behavioral and physiological aspects of birds [4, 13,19,57] and claimed that the night phase is getting disturbed due to anthropogenic Artificial Light at Night (ALAN), which is leading to many physiological and metabolic complications and altered immune response [57, 5]. ALAN is a rapidly growing phenomenon with worldwide ramifications on the natural environment, linked to altered biological rhythms as light plays a crucial role in the entrainment of the internal clock/ biological clock. ALAN is directly associated with changes in the daily behavior, reproductive strategies, and physiology of wildlife from plants to animals, unicellular to multicellular, or invertebrates to vertebrates. In the 20th century, the ecological consequences of the LAN came into consideration and now it has imposed a challenge for scientists, environmentalists, conservationists, and industrialists to improve or find alternatives to the bright light sources that reduce the ill effect of light pollution not only on the mankind but also on the wild animals. Birds are sensitive to all three properties of light; photoperiod, intensity, and wavelength, which can be tested individually to determine the effects of different light properties on a bird's physiology and behavior [47,46,27,32,33]. ALAN affects both circadian and seasonal oscillators of

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the organisms, for instance, it affects the sleep/ mood, reproductive cycle, and migratory behavior of birds [2,13,28]. Exposure to ALAN forces the animals to modify/ update their reproductive strategies to perpetuate their species.

de Jong et al. in their research showed that the Great Tits (*Parus major*) laid their eggs earlier in the white light polluted area than in the darker areas [10]. Similar results were also found under the street light in the Blue Tits (*Cyanistes careuleus*) [22]. Whereas, in several taxa, a delay in reproductive response to ALAN, which may be due to the short-day breeding strategy, has been observed; for example, Tammar Wallabies (*Macropus eugenii*) showed a delay in average birth timing [48] and moth (*Mamestra brassicae*) showed the disrupted mating and delayed pupation timing [52,53].

The effect of ALAN exposure is not limited to the reproductive and breeding strategies of birds. A recent study has shown changes in the width of muscle fibers due to light pollution. The diameter of broiler chicken's breast and thigh muscle fiber increased in response to colored light exposure at night measured in the slurry samples of muscles [15]. The poultry producers have thoroughly researched and effectively employed the effect of photoperiod, light intensity, and wavelength on poultry production. Previous studies on broilers have demonstrated that different colored ALAN causes significant changes in pectoral musculature at the histomorphometric level [9,30].

Interestingly, a characteristic feature of several birds is their ability to migrate to and fro, to combat harsh climatic conditions and breed successfully. Bird migration involves considerable changes in physiology and organ remodeling, including modifications to oxidative capacity, hormone levels, fat storage, and individual organ sizes (12,29,34,35). For migratory flights, the songbirds mainly rely on the pectoralis major muscle which is believed to be composed of FOG (fast oxidative glycolytic) fibers [50, 56]. Further research also suggests that the size of the flight muscle may increase in response to or in anticipation of the increased exercise and muscular stress that comes with migration [43]. Moreover, an increment in the size of the pectoralis muscle is seen during the pre-migratory period even in captive conditions, of course, less than in free migratory birds [3,12,54].

Apart from the pectoral muscles, changes in the functioning of cardiac muscles have also been reported during migration. For instance, the cardiac muscles strengthen themselves with the increasing heart rate compared to the resting state to fuel the required oxygen needed by the pectoral muscle during the tedious migratory flight [36]. An increase in the size of the heart during migration is seen to improve the circulatory transport capacity for exogenous fatty acid transport and probably increase the cardiac output [21,40,41]. The main metabolic fuel used by birds during migration is fat. Indeed, migratory birds are unique among vertebrate animals in that, they can power extremely intense exercise using free fatty acids (FFA), which are transported by the circulatory system from the adipose tissue to the working muscle. It has been observed that muscular capillarity increases with migratory distance in passerine bird species, most likely due to the migrants' lower red muscle fiber diameters [31]. However, only about one-third of the necessary increase in exogenous FFA absorption can be accounted for shorter diffusion distances, even in the highly capillary-dense hummingbird flying muscle [49,20].

A recent study has demonstrated that ALAN exposure alters the perception of daylength in birds [24]. As ALAN is known to alter the perception of daylength, therefore, in the present study, we aimed to investigate the impact of colored light at night (CLAN) at histological levels on the cardiac muscles and pectoral muscles of a night migratory songbird, the redheaded bunting. Since these birds show photostimulated changes under longdays [26,51] and these changes have been investigated in the same species under the influence of ALAN [25], we consider getting similar changes in our histological examination. We believe that CLAN will cause photostimulated changes in both pectoral and cardiac muscles in the form of altered muscle fiber characteristics.

2. Materials and Methods

2.1. Animal and maintenance

This work has been performed on redheaded bunting (*Emberiza bruniceps*), a photoperiodic, Palaearctic-Indian migratory bird. It is commonly known as 'laal sar ka gandam' [1]. Since the redheaded bunting is a small bird, it is very easy to handle and needs less maintenance under captive conditions in the laboratory. Besides this, redheaded bunting is photoperiodic in nature; remains photosensitive in shortday conditions, and shows a photostimulated response under longdays. Male redheaded buntings were captured from an overwintering flock using a mist net in the wild. After being taken to the laboratory, the birds were acclimatized for two weeks in an outdoor aviary (3.0 x 2.5 x 2.5m) under natural daylength and temperature conditions. Fresh grasses and green leaves were provided and replaced regularly to offer the birds a semi-natural habitat. Tetracycline hydrochloride solution (Intervet India Pvt. Ltd.) was given as an antibiotic for the first five days of acclimatization. The food mainly included foxtail millets (*Setaria italica*), commonly known as kakuni. An additional/ special meal was also provided to them once a week, made by combining bread crumbs,

boiled eggs, cottage cheese, and Vimeral (vitamins A, D3, E, and B12, manufactured by Virbac Animal Health India Pvt. Ltd.) to ensure the good health of the birds. After acclimatization, the birds were transferred to an indoor chronocubicle for acclimatization to controlled laboratory conditions, before using in the experiment. Food and fresh water were *ad libitum.*

2.2. Experiment

The experiment was conducted according to the Institutional Animal Ethics Committee (IAEC) guidelines at the Department of Zoology, University of Lucknow, Lucknow, India (Protocol #: LU/ZOOL/IAEC/01/21/03). For the experiment, birds (n = 15) were housed in individual cages measuring 45 X 40 X 25 cm having two perches at different heights. Buntings were kept on a daily schedule of 10 hours of light and 14 hours of darkness (10L:14D). All the birds were exposed to 200 Lux of white light during the daytime, while the night was dark. The birds were divided into three groups (n = 5/ group). Group 1, which was not exposed to any light at night was considered an NLAN (No LAN) group, while Group 2 and Group 3 exposed to 5Lux of blue and white light throughout the night, were named BLAN (Blue light at night) and WLAN (White light at night) respectively. Blue colored light and white colored light were provided by LED bulbs (Havells Rojo LED 7W; Havells India Ltd.). When the birds got acclimated to the caged condition, the LAN schedule was started and it lasted for 10 cycles, after which the birds were sacrificed during the midnight of the 11th cycle, and the pectoral muscle and heart were excised from each bird for this study (Fig.1).

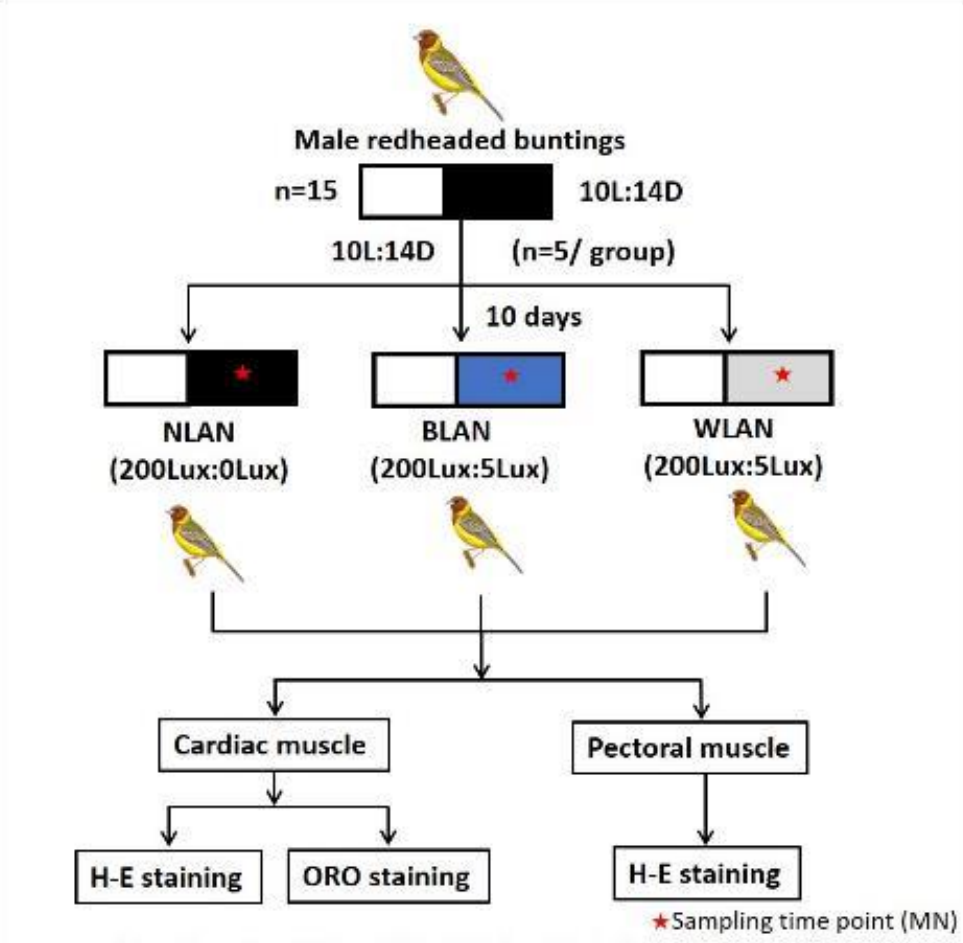

Figure 1 Experimental Protocol: Three groups (n = 5 birds /group) of photosensitive birds were made based on the night light they were exposed to and named NLAN (No light at night), BLAN (Blue light at night), and WLAN (White light at night). LAN exposure was given for up to 10 cycles. After 10 nights of LAN exposure, birds were sacrificed at midnight of the 11th cycle and the pectoral muscle, and cardiac muscle were excised for histomorphometric study

2.3. Tissue preparation and histology

From the harvested tissues, a chunk of tissue (5 x 5 mm) was excised from the heart and pectoral muscle for histology, while the remaining tissue was stored at -80°C. The excised muscle samples were fixed for an entire night at 4°C in

fixative (4% paraformaldehyde in 0.1M phosphate buffer (PB; pH=7.4) solution). After fixation, tissues were transferred to ascending grades of sucrose i.e. 10%, 20%, and 30%, gradually until the tissue sank in the solution.

2.4. Tissue sectioning

The cryostat (CM 1850 Leica, Germany) was used for cryosectioning the tissues. The muscles were sectioned at 14 microns at -25°C. Sections were taken directly on the subbed slides (Blue star micro slides PIC-2, Polar Industrial Corporation, Mumbai, India.). For subbing the slides, they were initially washed with acetone and let dry. These dried slides were then dipped into a subbing solution (prepared by distilled water, gelatin, and chromium potassium sulphate dodecahydrate ($CrK(SO_4)_2.12H_2O$)) and were again left to air dry overnight. Now the slides were ready to take the sections.

2.5. Hematoxylin-Eosin Staining

To ensure that the sections were properly stuck to the coated slides, the slide containing sections was left to air dry for a night before staining. After drying, the slides were cleared in two xylene changes for two minutes each, and then they were rehydrated in descending alcohol grades (100%, 90%, 70%, and 50%) for two minutes each. Sections were cleaned with distilled water for one minute. Following distilled water treatment, the sections were stained for 50 seconds in Hematoxylin (Q38803, Qualigens, Thermo Fisher Scientific India Pvt. Ltd.), and then if excess stained, the excess stain from the sections was removed by dipping them in acid water for 5 seconds. The slides were left for two minutes in tap water after the excess stain was removed. The sections were then dehydrated by passing through ascending grades of alcohol (50%, 70%, and 90%) for two minutes each. Sections were then stained for 50 seconds using Eosin (Q38613, Qualigens, Thermo Fisher Scientific India Pvt. Ltd.), and then they were dipped in acid alcohol once to remove the excess stain, followed by dehydration in 90% and 100% alcohol for an additional two minutes each. The dehydrated sections were cleaned for two and three minutes respectively, in two changes of Xylene before mounting in DPX. Slides were left to dry for at least 72 hours before imaging them.

2.6. Image capturing and analysis

The Leica DM 3000 microscope was used to capture images of the stained slides using a Leica DFC450 C digital camera with the help of the Leica Application Suite (LAS V4.12). We captured photographs of 5 fields per bird in each group. For the measurement, the image of LS and TS of muscles were taken at 400X (10X ocular and 40X objective) and 40X (10X ocular and 4X objective) magnification respectively. The ImageJ program was used to analyze the images of the stained slides [11].

2.7. Measurement of pectoral and cardiac muscle width

We took measurements of the pectoral and cardiac muscle fiber widths by using the straight-line tool to draw a line along the width and then measuring the length of the line drawn in μm, the data transferred to the spreadsheet for calculation and later were represented in the form of graph by using GraphPad Prism Software 8.0.2.

2.8. Pectoral muscle fiber density analysis

To measure the pectoral muscle fiber density, the image was captured at 40X magnification. In ImageJ software, after setting the scale to 0.826 pixels/ μm, we measured the single fascicle area by selecting it with the polygon selections tool. For measuring the total muscle fiber area present in the single fascicle, the rest of the fascicles lying in the image were cropped out by the clear outside option present in the edit drop-down menu and then color thresholding was adjusted to the muscle fiber level, and measured the area of the total muscle fiber in fascicle of our interest. The accuracy of the thresholding was checked with the original image. After that, the ratio of the total muscle fiber area to the fascicle area was taken to calculate the density of the pectoral muscle fibers in the fascicle.

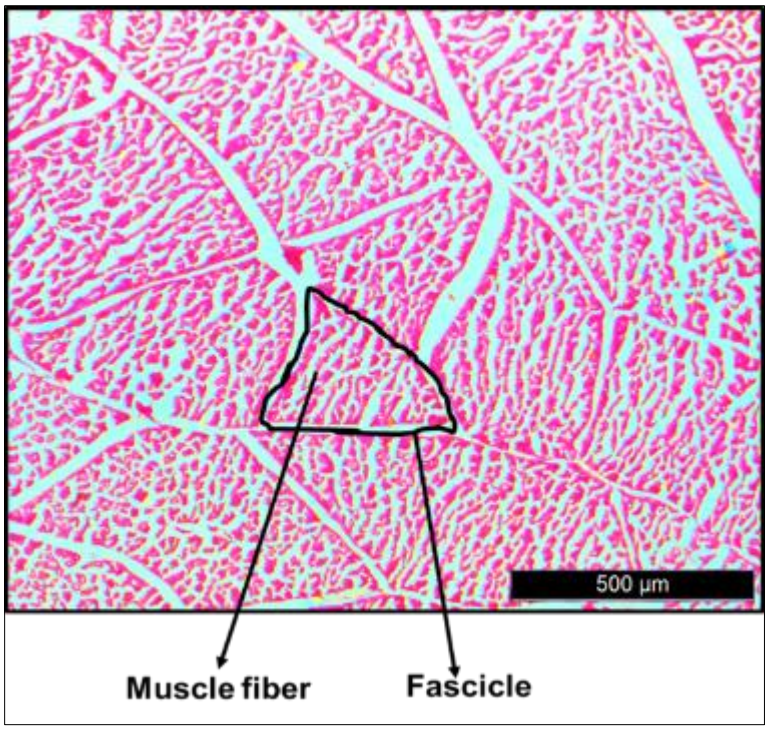

Figure 2 H-E-stained transverse section of pectoral muscle showing fascicle and muscle fibers

2.9. Oil Red O (ORO) staining of cardiac muscle fibers

ORO is a lysochrome (fat-soluble), hydrophobic diazo dye used for staining neutral triglycerides, lipids, cholesteryl esters, and lipoproteins. It has been widely used for intracellular lipid staining and tissue staining due to its intracellular permeability. We analyzed the deposition of lipids in the cardiac muscle by following the ORO staining protocol [37]. The slide preparation and sectioning process was the same as we used for the H-E staining. But for ORO staining the slides having sections placed at -20°C overnight. We prepared an ORO working solution by diluting ORO stock solution (0.5% ORO in isopropanol, 01391, Sigma-Aldrich, Darmstadt, Germany) with distilled water in a 1.5:1 ratio. The solution was kept at 4°C for 10 minutes and then filtered to remove precipitate just before the staining. Then the slides having sections were brought at room temperature, and stained with ORO working solution (~1ml) for 5 minutes without disturbing. Then the sections were washed under running tap water for 45 minutes, mounted in glycerol, sealed, and examined under the microscope (Leica DM 3000) at 400X magnification (ocular=10X; objective= 40X). The sections were photo-micrographed by a Leica DM3000 microscope within 24 hours and ImageJ application was used for further analysis.

2.10. Lipid density measurement in cardiac muscle

We use image analysis software ImageJ for the analysis. Images were opened with the software and RGB images were converted into 8-bit, the scale was set to 8.26 pixels/µm. The threshold was adjusted to the cardiac muscle fiber level and measured the total area occupied by the muscle fiber. Again, the thresholding was done to the lipid content level and measured the total area occupied by the lipid content in a muscle fiber. During thresholding, the original image was opened adjacent to the image opened in ImageJ software to ensure an accurate thresholding. After getting the total area of the cardiac muscle fiber (CMF) and lipid content (L), the ratio of L/CMF was calculated to get the density of lipids in cardiac muscle fiber per μm^2.

Statistics: Data was tested for normal distribution through the Shapiro-Wilk normality test and found that our data was normally distributed then we performed the ordinary one-way ANOVA followed by Tukey's multiple comparison test. For statistical significance, alpha was set at $P < 0.05$.

3. Results

Results are shown as bar graphs in Figures 3-6 along with the representative photomicrographs. The detailed results are as follows:

3.1. Pectoral muscle fiber and cardiac muscle fiber width

LAN treatment significantly affected the pectoral muscle fiber width ($F_{(2,12)}$ = 14.69, *P* = 0.0006*;* ordinary one-way ANOVA; Fig. 3d) and cardiac muscle fiber width ($F_{(2,12)}$ = 15.27, *P* = 0.0005; ordinary one-way ANOVA; Fig. 4d). The width of pectoral muscle fiber was significantly increased in BLAN and WLAN groups relative to NLAN group (Tukey's multiple comparisons post-hoc test; Fig. 3d). Similarly, the cardiac muscle fiber width increased significantly in BLAN and WLAN groups as compared to NLAN group (Tukey's multiple comparisons post-hoc test; Fig. 4d). For both the tissues, there was no significant difference between the effect of blue light and white light at night on the thickness of pectoral muscle fibers.

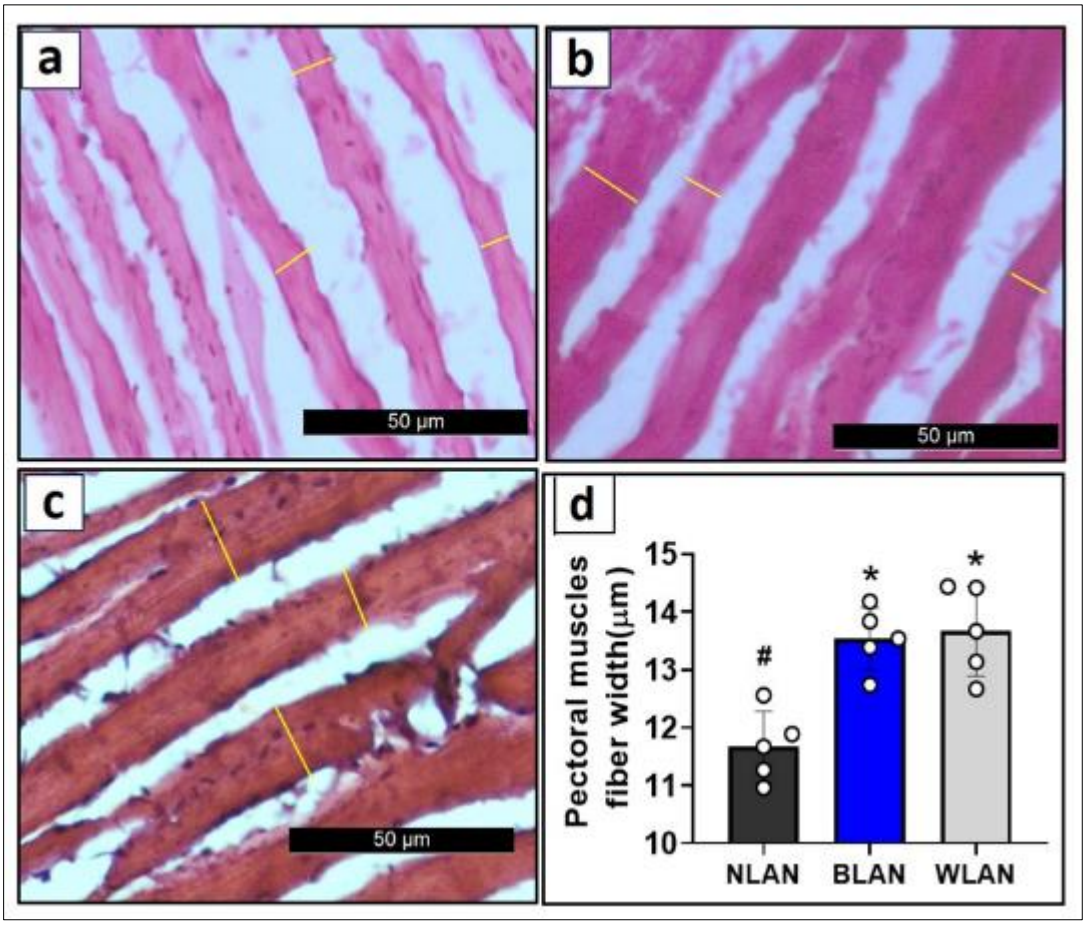

Figure 3 H-E-stained longitudinal sections (LS) of pectoral muscle at 400X magnification of a) NLAN (No Light at Night) b) BLAN (Blue Light at Night) c) WLAN (White Light at Night) group. The yellow line represents the width of the muscle fiber. The Mean (± SEM) (n = 5/ group) width of muscle fiber under different groups is shown in the form of a bar graph (d). The same or different symbols over bars represent the insignificant and significant variations among the groups, respectively. For statistical significance, alpha was set at P < 0.05

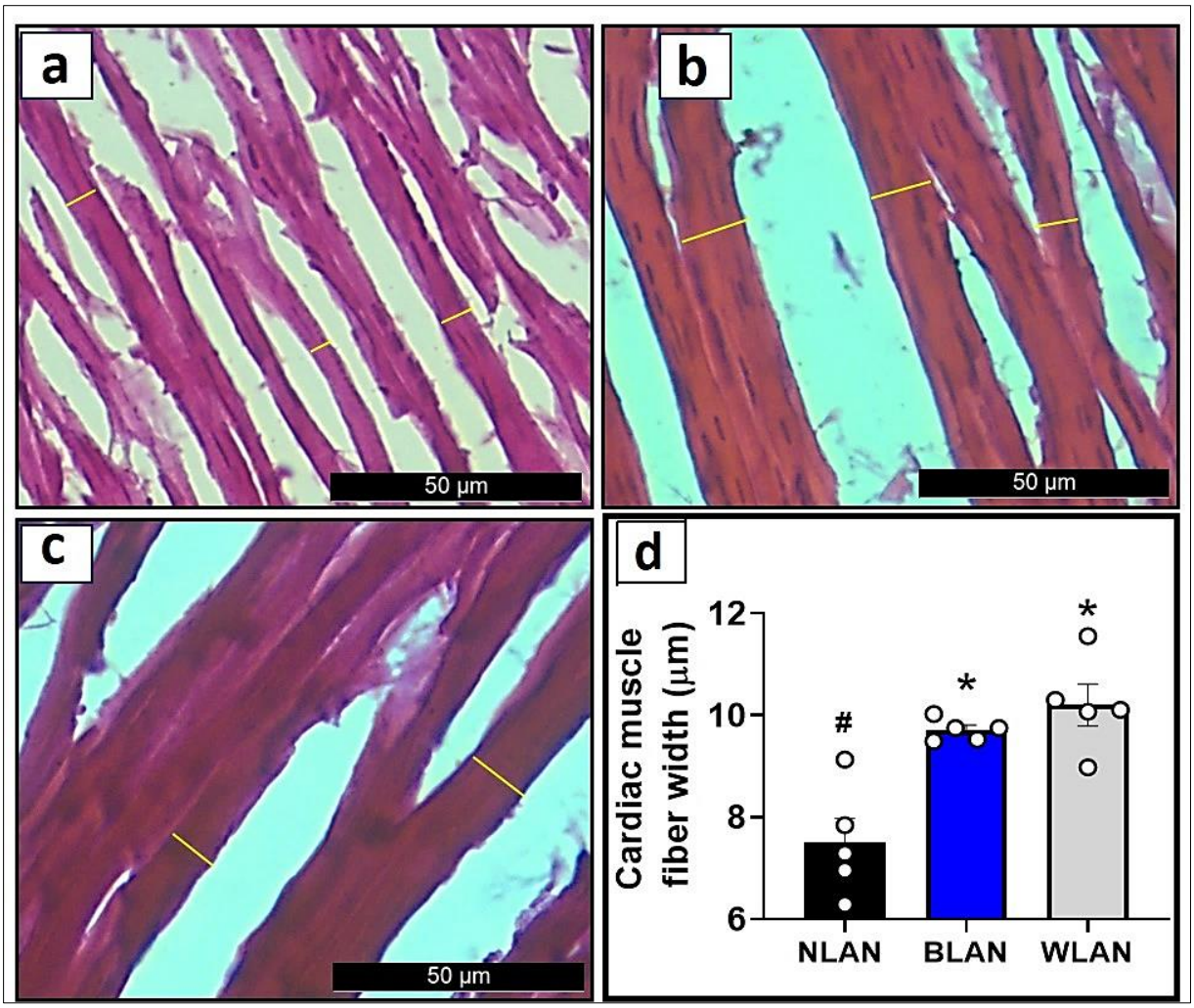

Figure 4 H-E stained longitudinal sections (LS) of cardiac muscle at 400X magnification of a) NLAN (No Light at Night) b) BLAN (Blue Light at Night), and c) WLAN (White Light at Night) group. The yellow line represents the width of the muscle fiber. The Mean (± SEM) (n = 5/ group) width of muscle fiber under different groups is shown in the form of a bar graph (d). Same or different symbols over bars represent the insignificant and significant variations among the groups, respectively. For statistical significance, alpha was set at $P < 0.05$

3.1.1. Lipid density in cardiac muscle fiber

The lipid deposition in the muscle fibers was greatly affected by the artificial light at night ($F_{(2,12)} = 40.14, P < 0.0001$; ordinary one-way ANOVA; Fig. 5d.). Maximum lipid density was observed in the white color light at night (WLAN) group followed by the blue color light at night (BLAN) group and minimum lipid density in the No LAN (NLAN) group (Tukey's multiple comparisons post-hoc test; Fig. 5d).

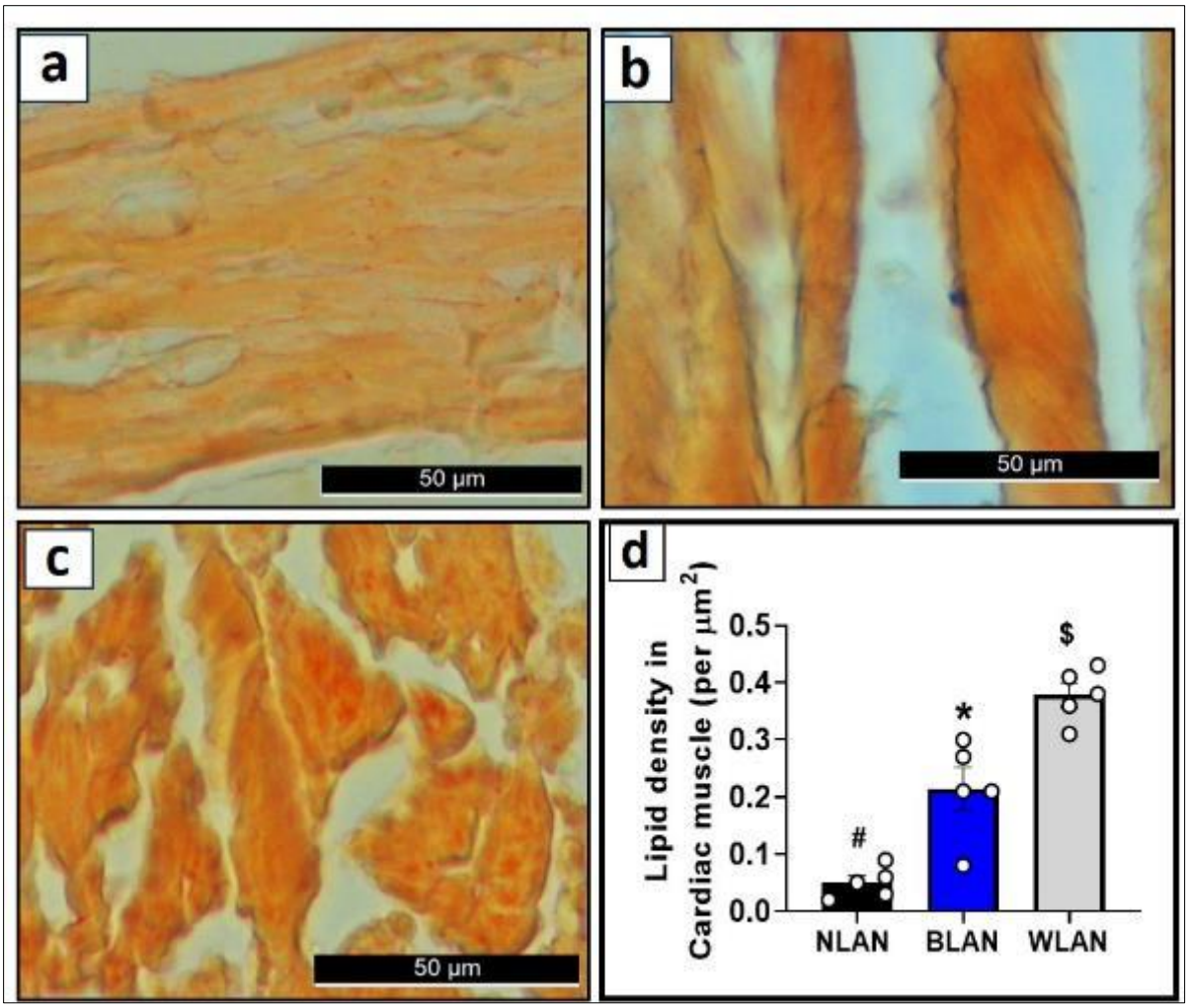

Figure 5 Oil red O (ORO) Stained longitudinal sections (LS) of cardiac muscle at 400X magnification of a) NLAN (No Light at Night), b) BLAN (Blue Light at Night), and c) WLAN (White Light at night) group. The dark stained area represents the lipid content against the light-stained muscle fiber. The Mean (± SEM) (n = 5/ group) lipid density in cardiac muscle fiber under different groups is shown in the form of a bar graph (d). The same and different symbols over bars represent the significant variations among the groups. For statistical significance, alpha was set at $P < 0.05$

3.1.2. Muscle fiber density in pectoral muscle

We checked if the means of different groups were significantly different from each other by ordinary one-way ANOVA. The data was found not significantly different i.e. the artificial light at night had not affected the density of muscle fibers in the pectoral muscle ($F_{(2,12)} = 1.577$, $P = 0.2466$; ordinary one-way ANOVA, Fig.6d).

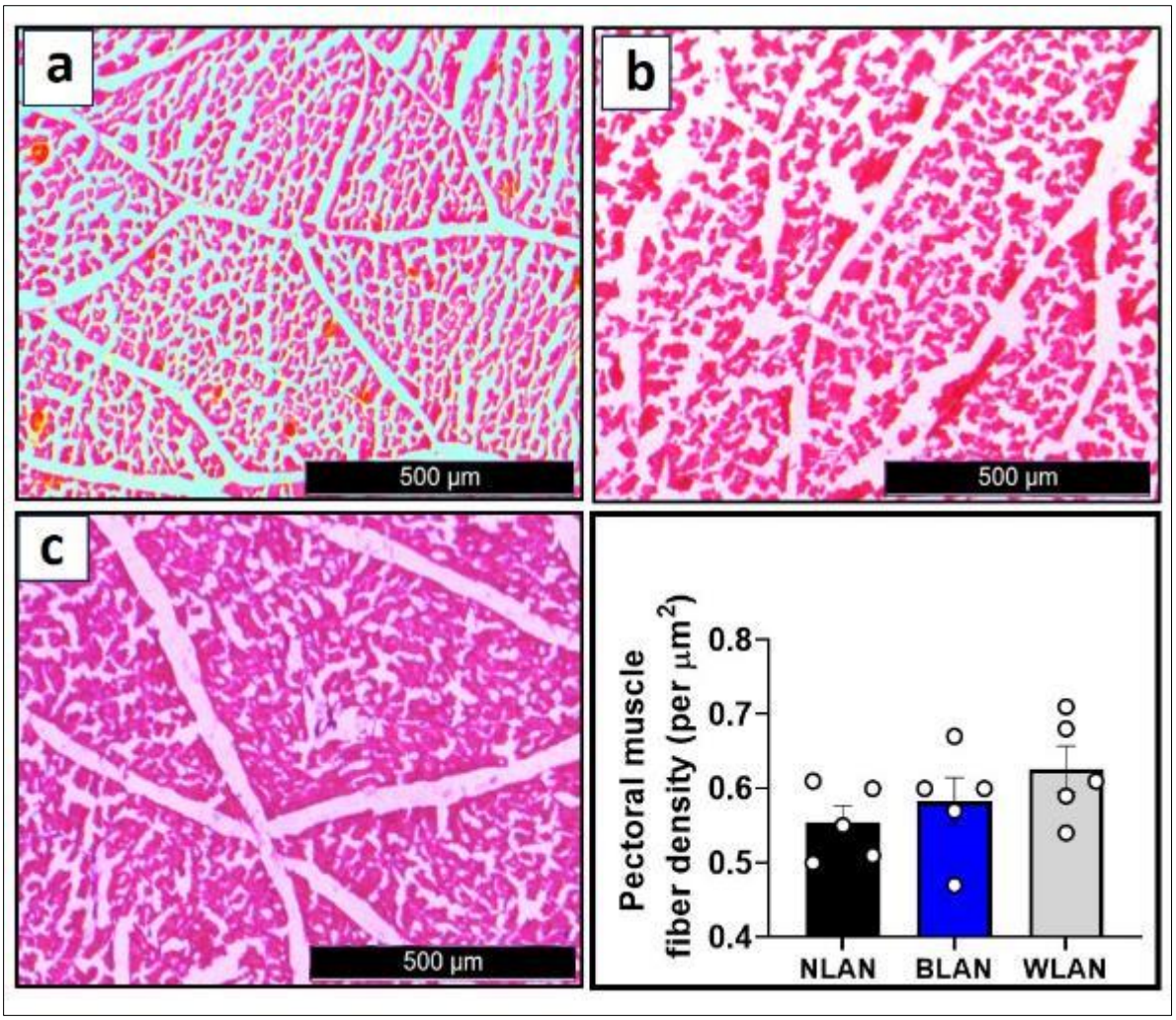

Figure 6 H-E-stained transverse sections (TS) of pectoral muscle at 40X magnification of a) NLAN (No Light at Night), b) BLAN (Blue Light at Night), and (c) WLAN (White Light at Night) group. The Mean (± SEM) (n = 5/ group) pectoral muscle fiber density under different groups is shown in the form of a bar graph (d). The same and different symbols over bars represent the significant variations among the groups. For statistical significance, alpha was set at $P < 0.05$

4. Discussion

Migration in birds involves extensive changes in form and physiology, including hormone levels, fat storage, individual organ sizes, and oxidative capacity [34,12,29,35]. During migration, muscles play a crucial role by providing the necessary force for movement and allowing them to travel long distances [8]. Changes in the morphology of muscles are essential for executing long-distance flights. Flight muscle hypertrophy in birds is linked with an increase in muscle fiber thickness, enhancement of mitochondria density, thermoregulatory function, and an increase in intracellular lipid accumulation within the myocytes [16,14,54]. Studies on migratory passerines like yellow warblers (*Setophaga petechia*), warbling vireos (*Vireo gilvus*), and yellow-rumped warblers (*Setophaga* coronate) show that myostatin plays a major role in facilitating muscle hypertrophy during migration [23]. In our study, we have demonstrated that the pectoral muscle fiber width and cardiac muscle fiber width of the redheaded bunting were increased under the influence of ALAN treatment, probably owing to the misperception of a short day as a long day, resulting in the development of migratory phenology. Such change in perception of day length in the photostimulated behavioral and physiological response to ALAN treatment under experimental conditions has also been shown by Kumar et. al. [24] in a study on resident Indian weaver birds. The role of testosterone, androgen receptors, and insulin growth factors has also been associated with muscle hypertrophy in birds. The gene expression levels of androgen receptors, 5α-reductase (the enzyme that changes testosterone into 5α-dihydrotestosterone), and insulin-like growth factor-1 (an androgen-dependent gene linked to muscle remodeling) all rise in the pectoralis muscle of white-crowned sparrows (*Zonotrichia leucophrys gambelii*) before they migrate [42]. Interestingly, we did not find any change in the fiber density of pectoral muscles probably owing to the short duration of exposure in terms of the number of days. Change in both, pectoral

muscle mass and fiber density has been reported in a recent study on Snow bunting (*Plectrophenax nivalis*) where Vézina et al. [55] demonstrated that during migratory phase, Snow buntings showed pectoral muscle hypertrophy, where both muscle mass and fiber diameter increased by ~17 and 35%, respectively.

Phenological changes associated with bird migration such as an increase in flight muscle score, subcutaneous lipid deposition over the musculature, and lipid infiltration in the muscle fibers have been previously found in several studies [21,35,43,45,44]. In our study, we found that lipid density increased in both the LAN treated groups due to photostimulation. Moreover, the response was higher in WLAN group as compared to the BLAN group. White light probably played a more dynamic role in regulating lipid density in the cardiac muscles of buntings. The heart is a very crucial organ during migration and birds depend on them to sustain high and sustained levels of mass-specific power production required for long-distance flights [7]. Recent research on migratory shorebirds and passerine finches like the western sandpiper and the white-throated sparrow showed that cardiac muscle adaptions play a crucial role in long-distance flights by increasing the uptake of fatty acids through heart-type fatty acid binding protein (H-FABP). Expression of metabolic enzymes like citrate synthase, 3-hydroxyacyl-CoA-dehydrogenase, and carnitine palmitoyl transferase II increases in the cardiac muscle of birds during migration, supporting the hypothesis that the physiological changes necessary for the successful migration are protein-mediated transport of fatty acids dependent [6]

To meet immense energy requirements during migration, long-distance migrants store fat during the pre-migratory phase and utilize it as a primary fuel during migratory flights [5]. During migration in birds, transport and oxidation of lipids occurs ten times faster compared to any other mammal [36]. Migratory flights are copiously fueled by the oxidation of fatty acids which get transported to the mitochondria of the active muscle via the circulatory system. In the muscle's cytoplasm, an important fatty acid transport facilitator is a fatty acid binding protein (FABP) which serves as an intracellular carrier of long-chain fatty acids [39]. It has been shown that the heart rate and the cardiac muscle's lipase activity are directly correlated [17,18]. Fat is a primary source of fuel for cardiac metabolism, and neutral fats may be digested by hydrolytic enzymes to produce fatty acids, which the heart muscles can oxidize [38]. An increase in heart rate should result in an increase in energy expenditure and consequently, in the consumption of fat. In the circumstances, an increase in lipase activity may also be anticipated to produce more fatty acids to meet the heart's higher needs.

5. Conclusion

The present study provides evidence for the disruptive effect of colored light at night under shortdays which misguided the birds as a longday, and ultimately the species started to show migratory phenology which resulted in increased muscle width (pectoral and cardiac muscle) and lipid infiltration in the muscle fibers. Although BLAN and WLAN showed a similar effect on birds, blue light showed comparatively less effect on bird's muscle morphometry. The present conclusions hold relevance since the study was performed at 5Lux light intensity which is often found in our vicinity. This study enhances our understanding of the ill effects of LAN in wild birds and suggests that the effects could be mitigated by the changes in the characteristics of outdoor lighting (such as change in color) and creates possibilities for conservationists and policymakers to use alternative lighting in their daily practice. In the future, intensity-dependent effects of colored light at night could be studied and downstream events involved in regulating the perception of shortday as longday in migratory birds could be studied. It would also be interesting to study if the metabolic costs of exposure to LAN in birds could be mitigated or not under exposure to different colors.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

Authors declare that they have no conflict of interest.

Statement of ethical approval

The experiment was conducted according to the Institutional Animal Ethics Committee (IAEC) guidelines at the Department of Zoology, University of Lucknow, Lucknow, India (Protocol #: LU/ZOOL/IAEC/01/21/03).

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