SEASONAL RESPONSES IN REPRODUCTION AND AFFILIATED BEHAVIOR OF TREE SPARROW (*PASSER MONTANUS*) AT AIZAWL, MIZORAM

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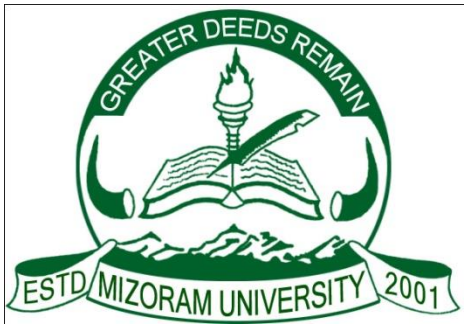

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SEASONAL RESPONSES IN REPRODUCTION AND AFFILIATED BEHAVIOR OF TREE SPARROW (*PASSER MONTANUS*) AT AIZAWL, MIZORAM

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Submitted
In partial fulfillment of the requirements for the degree of Doctor of Philosophy in Zoology of Mizoram University, Aizawl.

Certificate

I certify that the thesis entitled "**Seasonal responses in reproduction and affiliated behavior of tree sparrow (*Passer montanus*) at Aizawl, Mizoram**" submitted to the Mizoram University for the award of the degree of Doctor of Philosophy in Zoology by **Zothanmawii Renthlei** is a record of research work carried out during the period of 2015 - 2020 under my guidance and supervision, and that this work has not formed the basis for the award of any degree, diploma, associateship, fellowship or other titles in this University or any other University or institution of higher learning.

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Declaration

I, **Zothanmawii Renthlei**, hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form the basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University or Institute.

This is being submitted to Mizoram University for the degree of **Doctor of Philosophy** in the Department of Zoology.

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Zothanmawii Renthlei

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GENERAL INTRODUCTION

Seasonality is defined as the initiation-termination-reinitiation of physiological processes. It is an adaptation for survival in most of the species in the fluctuating environment. Birds are seasonal and exhibit seasonality in several physiological functions, for example, change in body weight, deposition in body fat, season dependent parasitic load, hormone levels, change in the bill and plumage color, molt in feathers, immune system, migration, and gonadal growth and development cycle. Seasonality in reproduction and affiliated behaviors is species-specific. Reproduction is timed in such a way that when actual breeding happens, plenty of food resources should be available for both parents and offspring so that chances of survival are maximum.

Various environmental factors help in tracking the time, which includes seasonal variations in day length, temperature, rainfall, humidity, and vegetation (Immelmann, 1971). Baker (1938), for the first time, classified these factors into two types: the proximate and ultimate factors. Proximate factors "gate" a temporal window during the year for any seasonal processes, but the ultimate factors fine-tunes that the actual processes occur during that gated window. For most of the avian species, day length acts as the proximate factor, while food/temperature may act as an ultimate factor. These proximate and ultimate factors do not work as in isolation but in conjunction. Seasonal breeding is tightly coupled with the natural light-dark cycle. To track daily and seasonal timing, almost all organisms have evolved with the endogenous time tracking mechanism known as biological clock(s). These clock(s) are innate (endogenous), inheritable, and genetic in origin (Aschoff, 1981; Kumar, 2001, 2002; Kumar *et al*., 2004). In nature, these time tracking system (oscillatory systems) are in synchrony with the daily cycle of sunrise and sunset, and thus, are represented as daily overt rhythms. When the organisms are exposed to an artificial light-dark cycle, they follow this artificial light-dark environment. The most predictable environmental cue is the daily cycle of the light-dark cycle as in the form of changes in illumination at earth's surface emanating from earth's rotation on its axis and around the sun. Length of the light

phase (day length/photoperiod) changes with the season (at least away from the equator) and intensity of any one time of day changes with the time of year and local weather conditions.

To govern daily and seasonal cycles in their physiology and behavior, most vertebrates exhibit circadian (*circa* = about, *dian* = day) and circannual (*circa* = about, *annum* = year) clocks (Kumar *et al*., 2010), respectively. In mammals, the suprachiasmatic nucleus (SCN) of anterior hypothalamus functions as a circadian pacemaker and involved in the regulation of many behavioral and physiological functions (Rusak and Zucker, 1979; Welsh *et al*., 1995). In non-mammalian vertebrates, these circadian pacemakers are distributed at more than one place within the nervous tissues. In birds, at least three separate circadian oscillators have been attributed residing within the hypothalamus, pineal, and retina of the nervous tissue (Gwinner and Brandstatter, 2001; Kumar *et al*., 2004). A circadian clock exhibits a transcriptional-translational feedback loop (TTFL). The genes referred as clock genes are arranged in an interacting positive (circadian locomotor output cycles kaput, *Clock*/ neuronal PAS

domain protein 2, *Npas2* and Brain and muscle arnt like protein 1, *Bmal1*) and negative (period, *Per* and cryptochrome, *Cry*) loops (Reppert and Weaver, 2002). Several other genes are also involved in stabilizing TTFL (Yin *et al*., 2010). These circadian genes are present in almost all body cells and exhibit differences in phase and amplitude with the central tissues suggesting tissue-level time-keeping in vertebrates (Yamazaki *et al*., 2000; Abraham *et al*., 2002; Yasuo *et al*., 2002; Karaganis *et al*., 2008; Singh *et al*., 2013, 2015; Trivedi *et al*., 2016).

Light at night (LAN) is an ever-growing phenomenon and has been associated with alterations in temporal activity patterns and physiology in vertebrates (Kempenaers *et al*., 2010; Dominoni *et al*., 2013a). The effects of LAN on ecological aspects have received great interest (Rich and Longcore, 2006). The effect of city lights at night has been illustrated using compositional differences on the foraging behavior of shorebirds (Santos *et al*., 2010), survival rates and stress responses in juvenile Pacific bluefin tuna (*Thunnus Orientalis*; Honryo *et al*., 2012)

and the commuting strategies of bats (Stone *et al*., 2009). Ecological research has emphasized the impact of urbanization on animal populations and community dynamics (McKinney, 2006). New interest has emerged in understanding the mechanisms of individual responses to urbanization (Shochat *et al*., 2010) and scattered information is available on the different aspects of animal physiology e.g., stress and reproductive physiology (Partecke *et al*., 2004, 2006; Dominoni *et al*., 2013a; Zhang *et al*., 2019), temporal and spatial activity patterns (Riley *et al*., 2013; Spoelstra *et al*., 2018), metabolism (Liker *et al*., 2008), behavior (Rees *et al*., 2009), social cues (Dominoni *et al*., 2014), sleep (Raapa *et al*., 2017) and on the endocrine system (Dominoni *et al*., 2013b; Zhang *et al*., 2014).

The present thesis

The thesis includes investigations on the population of tree sparrow (*Passer montanus*) that lives in and around Mizoram University campus (23ºN and 92º E). In particular, the emphasis is placed on studying seasonal responses of tree sparrows in the natural environment, the effect of light quality and temperature on gonadal recrudescence, and the regression cycle under laboratory conditions. We also studied the molecular clockwork in central and peripheral tissues of tree sparrows and compared the daily and seasonal responses of tree sparrows from rural and urban habitat. Various studies conducted are summarized in the following sections.

Section 1. Seasonal patterns in breeding and associated behavior

This section deals with changes in body mass, bill color, gonadal status, and molt in birds collected every month from their natural habitat.

Section 2. Seasonal cycles under laboratory conditions

This section includes results from the following experiments performed to investigate responses under a variety of photoperiodic conditions.

Experiment 1: Role of day length in the regulation of the seasonal gonadal cycle

This experiment examined if there was a differential effect of photoperiodic conditions on the responsiveness of the photoperiodic response system of tree sparrow to short and long day lengths.

Experiment 2: Role of food in the regulation of seasonal reproduction

Here, we investigated whether food plays a critical role in photoperiodic responses of tree sparrow.

Section 3. Role of light spectrum on reproduction

In this study, we studied the response of tree sparrow to a different light spectrum to understand the dynamics of their photoperiodic response system.

Section 4. Effect of temperature on reproduction

This section covers the study of the involvement of temperature on the reproductive cycle of tree sparrow. We measured phenotypic responses under varying temperature conditions and studied the candidate genes known to be involved in the regulation of seasonal reproduction.

Section 5. Study of daily rhythms in clock genes

Here we studied the daily expression of clock genes in central and peripheral tissues of tree sparrows collected at six times of the day.

Section 6: Effect of the urban environment on the circadian clock

Here we compared the circadian responses of birds procured from rural and urban habitat.

Section 7: Season dependent effects of the urban environment on the circadian clock

In this section, we studied the effects of season on the molecular machinery of the circadian clock in the birds of rural and urban habitats.

Section 8: Season dependent effects of the urban environment on the seasonal clock

In this section, we studied molecular regulation of reproduction in tree sparrows of rural and urban habitat at a different time of the year.

GENERAL MATERIALS AND METHODS

1. Tree sparrow (*Passer montanus*): the model species

Experiments were carried out on both male and female adult Eurasian tree sparrows (*Passer montanus*), commonly known as tree sparrows. In Mizoram, it is locally known as Chawngzawng. This species belongs to the Passeriformes. In the Indian subcontinent, this species is distributed in Baluchistan, Northeast India, Eastern Ghats, and Bangladesh (Grimmett *et al*., 2015). There is no sexual dimorphism in this species. An adult has a dull chestnut crown, black spot on whitish ear-coverts, small black throat patch not extending onto the breast, and white color separating chestnut nape from the brown-streaked mantle.

2. Procurement and maintenance

Adult sparrows of both sexes were procured locally by mist net within or nearby Mizoram University campus. Mizoram University campus is located on the western side at a distance of about 15 km away from the state capital Aizawl below Tanhril Village. The area of Mizoram University campus lies between 23.7^0 and 92.6^0 longitudes. University campus is spread in 395.86 ha with lush greenery and hills. The University campus contains regenerating tropical wet evergreen and semi-evergreen forests, including a protected forested water catchment reserve in the north. Immediately after procurement, birds were transported to the lab and kept in a room (unless otherwise mentioned) of NDL conditions (L × W × H = 427 × 579 × 304 cm) having two windows (L x W x H = 2 x 5 x 5 feet) facing northeast direction. Birds (N=4) were kept in the cages (L x W x H = 2 x 2 x 2 feet) in the room. Food (seeds of kakuni, *Setaria italica*, and paddy, *Oryza sativa*), mealworm; *Tenebrio molitor*, cultured in the laboratory), and water was available ad libitum and replenished twice daily during the daytime. Cages were cleaned every day. Birds maintained good health under captive conditions. All experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) of Mizoram University.

3. Lighting conditions

In laboratory conditions, birds were kept in the lightproof wooden chambers. The light was available from compact fluorescent light (14-watt CFL, Phillips) at the desired intensity and desired wavelength, as indicated in the respective experiments. No light was available during the night hours. Automatic time switches (Frontier digital timer) controlled the periods of light and dark. In caged condition, four sparrows were kept per cage (size 3 × 2.5 × 3 fits) for experiments. The aeration of photoperiodic chambers was maintained through air circulators.

4. Experimental design and data collection

Different sections of the thesis consist of several experiments, and each of these experiments had specific experimental designs. Detailed experimental protocols and designs are explained in respective experiments. The various measurements for determining the effects of an experiment were used. Data from these measurements were collected at the beginning and at the end of the experiment, and at appropriate intervals during the experiment and mentioned in respective sections.

4.1. Body mass: Body mass of an individual bird was recorded on a top pan balance with an accuracy of 0.1g. A bird was placed in a very thin cotton bag that was initially weighed and tared.

4.2. Gonadal growth and regression (Gonadal status): For the assessment of gonadal growth and regression, birds were anaesthetized (Ketamine and xylazine) and laparotomized with a small incision in between last two ribs on the left flank (Kumar *et al*., 2001). Internal organs were moved aside with the help of a spatula, and the length and width of the left testis or diameter of the largest ovarian follicle were measured. The testicular volume was calculated using the formula $4/3\pi ab^2$, where a and b denote half of the long (length) and short (width) axes, respectively.

4.3 Morphological parameters: We measured various morphological characteristics of a tree sparrow. Following parameters were measured:

4.3.1 Molt: Molt was studied by scores of feathers of primary flight (wing primaries) and body feathers. As explained in Trivedi et al., 2006, we scored primaries in a score of 0–5: 0 – worn or old feather, 1 – missing feather (just dropped), 2 – from a new feather papilla emerging up to the attainment of one-third growth, 3 – new

feather that has attained two-third growth, 4 – new feather grown, but still growth is incomplete, 5 – new feather fully grown. For recording body molt, the whole bird's body was divided into 12 different regions: 1 – head, 2 – neck, 3 –shoulder, 4 – back, 5 – pelvic, 6 – throat, 7 – chest, 8 –abdomen, 9 – flank, 10 – shank, 11 – sub-caudal and 12 – caudal. Any region could have a score of either 0 (old feathers) or 1 (new feathers emerged).

4.3.2 Bill color: Bill color was scored as described by Trivedi *et al*., 2006. The bill color was assessed by scoring in a scale of 0–5, 0: bill straw in color (S); 1: bill straw in color with a little tinge of blackness (ratio = SSS: B); 2: bill slightly blackish in color (ratio = SS: B); 3: bill straw and black with approximately 50: 50 patches (ratio = S: B); 4; bill black with very little straw patch left (ratio = S: BB); and 5 = bill completely black (B)

4.4 Sperm count: We measured the number of sperms by counting the sperms taken from cloacal protuberance; the coiled distal end of the vas deferens where sperms were stored before ejaculation using a hemocytometer.

4.5 General body movement recording: General body movement was recorded as a measure of the response of the circadian system. For this bird was individually caged in a specially designed activity cage (60 x 60 x 60 cm) that was furnished with two perches and mounted with a passive infrared Motion (digital PIR detector) sensor (DSC, Israel) to detect movement of the bird within its cage. Sensors were connected to a separate computer channel of a computer, and the recording was done using a software program of Stanford Software Systems, Stanford, USA.

4.6 RNA Isolation, cDNA Synthesis

Tri reagent (Ambion AM9738; USA) was used to extract total RNA from the hypothalamus of tree sparrow as per the manufacturer's protocol. The quality of extracted RNA was checked by Nanodrop One C (Thermo scientific; US, WI 53711) at 260 nm and 280 nm absorbance, and 260/280 ratio close to 1.9 was considered as 'pure' RNA. To remove the genomic contamination RQ1 DNase (Promega M6101; Wisconsin, USA) kit was used. cDNA was synthesized by the first-strand cDNA synthesis kit (Thermo Scientific, K1622; Lithuania, Europe).

4.7 qPCR for gene expressions

Primer 3 plus online primer design was used to design gene-specific primers. qPCR was performed using Quant-Studio 5 (Applied Biosystem by Thermo Fisher Scientific; USA) with a reaction volume of 7 µL for each gene comprising 1 µL each of cDNA, gene-specific forward and reverse primers, 3 µL Power Up^{TM} SYBR Green MasterMix (Applied Biosystem by Thermo Fisher Scientific; US, A25742) and 1µl of nuclease-free water (Ambion, AM9938). The qPCR cycling conditions used to measure expressions of the target genes were 1 cycle at 95^{0}C (20 sec), 35 cycles at 95^{0}C (01 s), 60 ^{0}C (20 s), 95^{0}C (01 s), additional melt curve plot step included 1 cycle of 60^{0}C (20 s) and one cycle of 95 ^{0}C (01 s). We used the 18S gene as a reference gene for determining the relative expression levels of specific target genes. We ran each sample in duplicate along with non-template and negative RT controls. The relative expressions of genes were determined by the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001).

4.8 Hormone assay

Levels of the target hormones were measured using chicken specific immune assay enzyme kits from KINESISDx with an automated spectrophotometer (SpectramMax M2e microplate reader, Molecular Devices, USA) following the manufacturer's instructions. Briefly, before the beginning of the assay, plasma samples were allowed to thaw on ice. Standards were diluted using the provided standard concentration and standard diluents as per the protocol provided. After that, 50 µL of standards and 40 µL Samples were transferred into respective wells of 96 well plate followed by 10 µL of Biotin conjugate into each sample well. 50 µL of HRP conjugate was pipetted into each sample and standards well and incubated for 1hr at 37 ^{0}C. Then, washed 4 times with 1X Wash Buffer, and the residual buffer was blotted by firmly tapping the plate upside down on absorbent paper. Substrate A 50 µL and Substrate B 50 µL were added to each well, including blank and incubated for 10 min at 37 ^{0}C in the dark. 50 µL of Stop Solution was added, and wells turned from blue to yellow. The absorbance was taken at 450 nm within 15 minutes after adding the Stop Solution.

5. Statistical analysis

Data obtained from different experiments were further analyzed statistically. The student's t-test was used to compare values at two-time points. One-way analysis of variance (1-way ANOVA), followed by Newman-Keuls post hoc tests if ANOVA indicated the significance of difference, was employed to examine the effect of various treatment over a period of time. Similarly, two-way ANOVA was also employed to analyze the effects when two factors (e.g., treatment and duration of the treatment) were taken into consideration, and the Bonferroni test was used as a post hoc test for group comparisons. Significance was always taken at $P < 0.05$. All statistical analysis was done using graph pad prism version 6.

SECTION I: SEASONAL PATTERNS IN BREEDING AND ASSOCIATED BEHAVIOR

1. ABSTRACT

Seasonality is represented as an initiation-termination-reinitiation of physiological processes and is the fundamental property of many organisms. Most of these seasonal processes are centered on and around reproduction. The majorities of birds are seasonal breeders and live in a seasonally varying environment. Here we studied the annual change in body mass, bill color, gonadal cycle, and molt in feathers of tree sparrows living in and around Mizoram University campus and compared their seasonal responses with the conspecifics live in the more urbanized areas of the city. The effect of habitat was observed on body mass, bill color, gonads (testes and ovaries), and timing of the molt in birds. Altogether, our study suggests that there is a slight delay in attaining the gonadal recrudescence in urban birds in comparison to rural birds. These findings are also supported by delays in the molt timings in the urban birds. The difference in the responses of birds of two habitats could be because of urban environmental conditions.

2. INTRODUCTION

It has been estimated that Class Aves contains approximately 10000 living species, while in India, there are 1346 species (Lepage, 2016). Northeast India is rich in biodiversity, and around 900 species have been reported from this region (Chatterjee, 2008). Still, the breeding biology of most of the species is not known. Most of the avian species that have been studied thus far exhibit seasonality in many physiological functions, including body mass, food intake, change in plumage color, variations in bill colors, gonadal growth regression cycle, photorefractoriness, molt in feathers, various hormonal levels, production of the song, immune functions, migration, etc. (for details see Wingfield and Farner, 1993; Kumar, 1997; Renthlei and Trivedi, 2017). In general, the annual change in body mass is exhibited by both migratory and resident bird species. However, in comparison to resident species,

migratory birds show many dramatic changes in the body mass as these species deposit heavy fat deposition, which serves as "fuel" for migration. Further, nonmigratory birds reflect some changes in body mass during the sexual phase than in post-reproductive phase of the annual cycle (Thapliyal, 1968; Saxena and Saxena, 1975), and during the sexual period, males weigh higher than females (Thapliyal and Pandha, 1965; Thapliyal and Biur, 1992).

Reproduction is a fundamental characteristic of living organisms. An essential attribute of any surviving species or population is the ability to reproduce successfully and produce offspring. Although a large number of avian species are known, seasonality in reproduction and linked phenomenon have been studied in only a limited number of species. The list includes European starling, *Sturnus vulgaris* (Bissonnette, 1931; Burger, 1947; Dawson *et al*., 1986); morning dove, *Zenaidure macroura carolinensis* (Cole, 1933); blue jay, *Cyanocilta cristata* (Bissonnette, 1936); house sparrow, *Passer domesticus* (Davis and Davis, 1954); white-crowned sparrow, *Zonotrichia leucophrys* (Blanchard, 1941; Farner, 1961); green finch *Chloris chloris* (Damste, 1947); weaver finch, *Quelea quelea* (Disney and Marshall, 1956); ring dove, *Streptopelia risoria* (Silver *et al*., 1980); emperor penguins, *Aptenodytes forsteri* and adelie penguins, *Pygoscelis adelia* (Groscolas *et al*., 1986); high arctic svalbard ptarmigan, *Lagopus mutus hyperboreus* (Stokkan *et al*., 1986); canary, *Serinus canarius* (Nottebohm *et al*., 1987); white stork, *Ciconia ciconia* (Hall *et al*., 1987); great tits, *Parus major* and willow tits, *Parus montanus* (Silverin *et al*., 1989); macroni penguins, *Eudyptes chrysolophus* and gentoo penguins, *Pygoscelis papua* (Williams, 1992); European quail, *Coturnix coturnix* (Boswell, 1991); Japanese quail, *Coturnix coturnix japonica* (Wada *et al*., 1992); red winged blackbird, *Agelaius phoenicus* (Beletsky *et al*., 1992).

In temperate (high-latitude) regions, most avian species breeding time is restricted to a narrow window of the year during the late spring and summertime. However, breeding timing among mid- and low-latitude species can be more scattered. For example, in tropics, breeding timing can be spread out over the entire year, although individual species or different populations of the same species are essentially seasonal (Chandola *et al*., 1983). Like temperate species, many tropical

birds also breed during spring and summer having cyclicity in reproduction, (Thapliyal and Tewary, 1964; Epple *et al.*, 1972; Lewis *et al.*, 1974; Gwinner and Dittami, 1984; Dittami and Gwinner, 1985; Tewary and Tripathi, 1985; Tewary and Dixit, 1986). Examples of Indian seasonal breeders including those migratory species that overwinter in India are: Indian owls, *Anthena brama*, *Bubo bubo, Ketupa zeylonesis* and *Tyto alba* (Thapliyal, 1954); doves, *Streptopelia tranquebarica, Streptopelia senegalensis* (Singh, 1958); crows, *Corvus macrorhynchos, Corvus splendens* (Prasad, 1965); pigeon, *Columba livia* (Dominic, 1960); Indian weaver bird, *Ploceus philippinus* (Saxena, 1964); mynas: blackheaded myna, *Temenchus pagodarum*, bank myna, *Acridotheres ginginianus*, common Indian myna, *Acridotheres tristis*, pied myna, *Sturnopastor contra* (Tewary, 1967); spotted munia, *Lonchura punctulata* (Chandola *et al*., 1983; Bhatt and Chandola, 1985); blackheaded bunting, *Emberiza melanocephala* (Kumar and Tewary, 1982a); common Indian rosefinch, *Carpodacus erythrinus* (Kumar and Tewary, 1985); redheaded bunting, *Emberiza bruniceps* (Tripathi, 1985); yellow-throated sparrow, *Gymnorhis xanthocollis* (Tewary and Tripathi, 1985); brahminy myna, *Sturnus pagodarum* (Kumar and Kumar, 1991); rose-ringed parakeet, *Psittacula krameri* (Maitra and Dey, 1992); blossom headed parakeet, *Psittacula cynocephala* (Maitra, 1986).

A large number of birds are known for their colored and attractive plumages. These birds do not maintain their plumages all round the year rather than shed off from time to time. The processes of shedding off old feathers and the generation of new feathers are known as molting. Molt is a critical stage in the annual cycle of birds. To maintain the feathers is an energetically demanding process since feathers commonly account for more than 20% of total body dry weight. Therefore, they undergo a series of molt during their life span. At least four different molts occur from hatching to the end of the first year, beginning from natal down, juvenile, alternate to the appearance of basic plumage (Lucas and Stettenheim, 1972). During the rest of life, a large number of birds exhibit two molts every year. One of these is the prenuptial molt that prepares birds to the approaching breeding and mostly acting as a secondary sexual characteristic for male birds. The other is the

postnuptial or prebasic molt that signals the end of the breeding season. In the European starling (*Sturnus vulgaris*), molt starts in June and completes by August (Dawson, 2003) while in house sparrows (*Passer domesticus*) molt begins in May and ends by September (Trivedi *et al*., 2006). The timing of molt is important, and the annual cycle is organized in such a way that molt-breeding and molt-migration overlaps are avoided or minimized (Kjellén, 1994). In a large number of species, the prenuptial molt is not distinct as compared to the postnuptial molt, but the postnuptial molt is more common in the avian species. This postnuptial molt is indicative of several significant physiological changes. For example, postnuptial molting individuals show increased vascularization of the dermis underneath feather follicles and papillae (Stettenheim, 1972), decrease in fat (Kuenzel and Helms, 1974), increased metabolic rate (Perek and Sulman, 1945; Lindström *et al*., 1993), osteoporosis (Meister, 1951; Murphy, 1996), and changes in blood cell profiles (Davis *et al*., 2000). In the white-crowned sparrow (*Zonotrichia leucophrys gambelii*), phospholipid, glyceride, and cholesterol levels, which are high in the premigratory and migratory periods and low in the breeding period, are further reduced during the period of body molt (de Graw *et al*., 1979). There is often a close relationship between the timing of breeding activity and the start of molt (e.g., Hinsley et al., 2003; Morton and Morton, 1990; Morton, 1992; Nilsson and Svensson, 1996; Svensson and Nilsson, 1997).

Several significant physiological changes coincide with the period of postnuptial molt. In a study that induced a forced molt in hens by restricting food and water found thyroxin (T_4) to be an important hormone responsible for initiating the molting process (Brake *et al*., 1979). Experimentally, a single intramuscular injection of progesterone at a dose of 20 mg terminates egg production and subsequently induces complete molt in hens (Shaffner, 1954; Adams, 1955; Smith *et al*., 1957; Tanabe *et al*., 1957; Harris and Shaffner, 1957). Juhn and Harris (1956) showed prolactin-induced molt in hens. In general, high prolactin levels during the late breeding season are implicated in the development of reproductive refractoriness and postnuptial molt in birds (Dawson and Goldsmith, 1982; Blache and Sharp, 2003).

The specific aim of this study was to describe changes in body mass, bill colors, gonadal growth and development, and molt in tree sparrows collected every month over a period of one year from the populations living in and around Mizoram University campus (rural habitat; 23.7^0N, 92.6^0E) and compared these responses with observations recorded at similar intervals on birds from the core area of the city of Aizawl (urban habitat; 23.7^0N, 92.7^0E) to see the effects of the urban environment on the annual reproductive cycle and affiliated responses.

3. MATERIAL AND METHODS

The study was performed on adult birds of both sexes procured locally (within the city Aizawl) during the middle of each month, beginning from January 2017 till December 2017. The study has two components. In the first component, we studied the annual changes in various morphological and physiological characters in birds procured in and around the core area of the city of Aizawl (urban site; 23.7^0N, 92.7^0E). In the second part, we compared all these characteristics with the conspecific procured at the same time but from rural habitat (23.4^0N, 92.6^0E). We measured change in body mass and bill color in both male and female birds (n = 10). Corresponding changes in day-length, temperature, and humidity were also recorded during each month. In order to know the seasonal variations in gonadal status, the size of the testis and ovarian follicles were measured, as mentioned in the general materials and method section. Sperm count was done (only urban birds), as mentioned in the general materials and method section.

3.1 Statistical analysis

Data are presented as mean ± SE. They were analyzed using a 1-way analysis of variance (1-way ANOVA) followed by Newman-Keuls post hoc tests if ANOVA indicated a significance of the difference. 2-way analysis of variance was employed to analyze the effects when two factors (e.g., habitat and time) were taken into consideration, and the Bonferroni test was used as a post hoc test for group comparisons. Significance was always taken at $P < 0.05$.

4. RESULTS

Annual change in temperature, rainfall, and humidity at this latitude is represented in figure 1.

4.1 Urban birds

The results are shown in figures 2 and 3. In male tree sparrows there was an annual change in body mass ($F_{11,84}$ = 3.154, P = 0.0015; 1-way ANOVA; Fig. 2a). Maximum body mass was recorded in April (mean BW19.8 ± 0.3 gm; Fig. 2a), while minimum body mass was observed in January (mean BW8.2 ± 0.3 gm; Fig. 2a). Bill colour also had annual variations ($F_{11,84}$ = 45.87, P= < 0.0001; 1-way ANOVA; Fig. 2b). The maximum score of bill color was observed in April (mean score 4.7 ± 0.1; Fig. 2b), while the minimum bill color score was observed in December (mean score 1.8 ± 0.1; Fig. 2b). Bill color score was high from March till July, while September till February bill color score was low (P < 0.05; Newman-Keuls; Fig. 2b). Testes underwent significant growth-regression cycle ($F_{11,84}$ = 24.09, P < 0.0001; 1-way ANOVA; Fig. 2c). They were found stimulated in March (mean TV= 26.5 ± 3.3 mm^3; Fig. 2c) and attained fully grown size in all birds in April (mean TV= 55.1 ± 7.2 mm^3; Fig. 2c). High testicular volume was observed till July, and By August, testes were regressed (mean TV= 1.9 ± 0.8 mm^3; Fig. 2c). We also accessed testicular activity by counting spermatozoa. The testicular activity was observed from April till July (Fig. 2f). Sperm count was supported by testes size. Within the reproductive phase we observed difference in number of sperm count ($F_{3,14}$ = 9.190, P = 0.0032; 1-way ANOVA). Mature spermatozoa were observed from April to July (Fig. 2f).

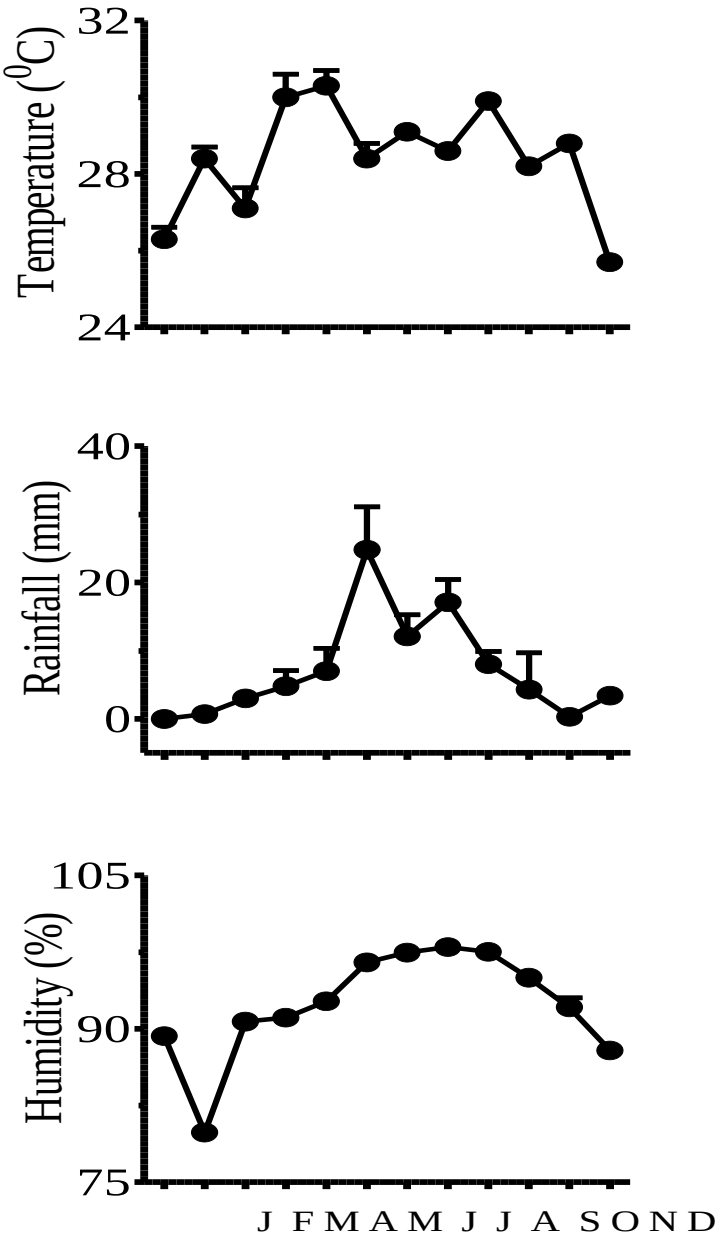

Figure1: Variations in temperature, rainfall and humidity conditions at 23°N, 92°E over the 12 month period, from January 2017 to December 2017.

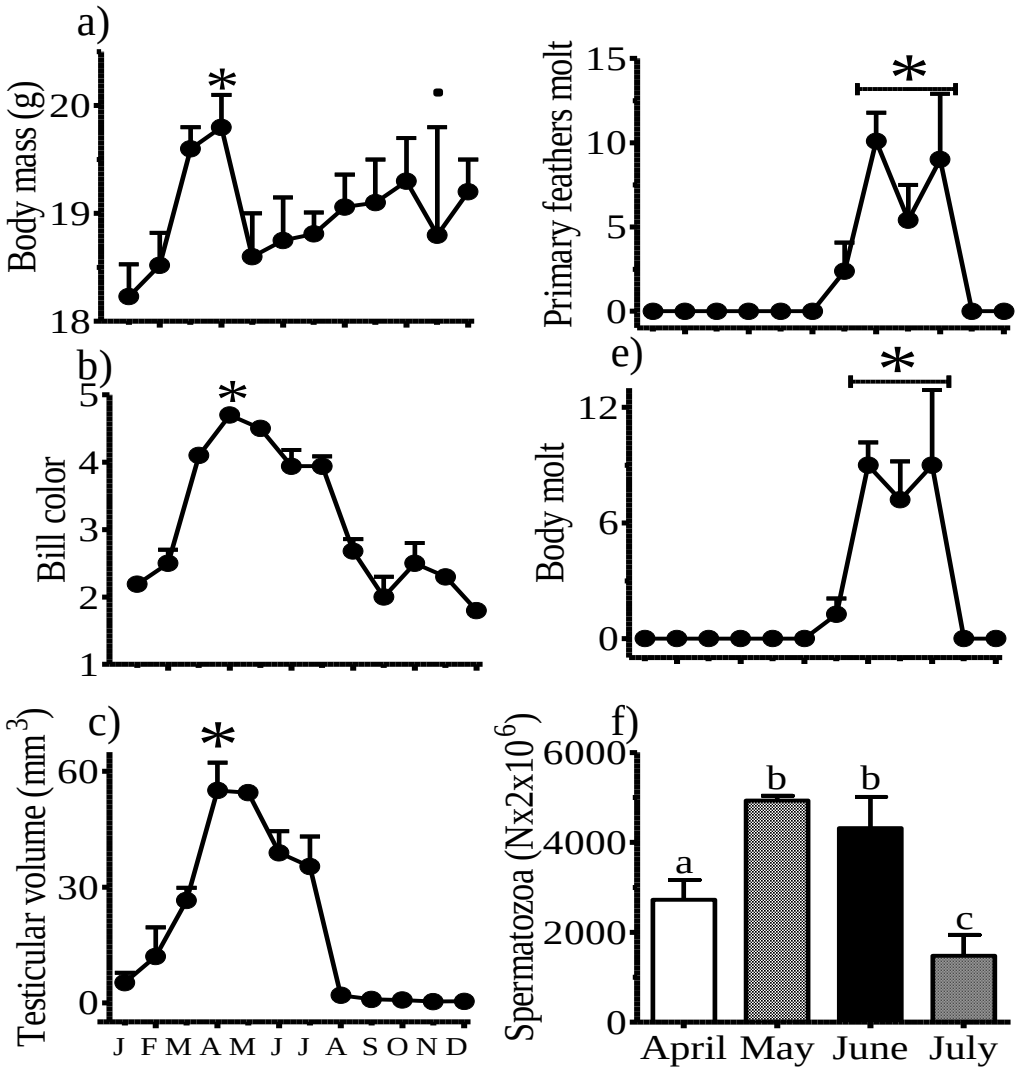

Figure 2: Mean (±SE) body mass (a), bill color (b), testicular volume (c), molt in primary flight feathers (d) molt in body feathers (e) and sperm count (f) of male tree sparrows procured from urban habitat (23.70 N 92.7^0 E) every month from the wild from January till December 2017. Body weight, gonadal weight and gonadosomatic index showed seasonal variations. Asterisks show significant difference ($P < 0.05$).

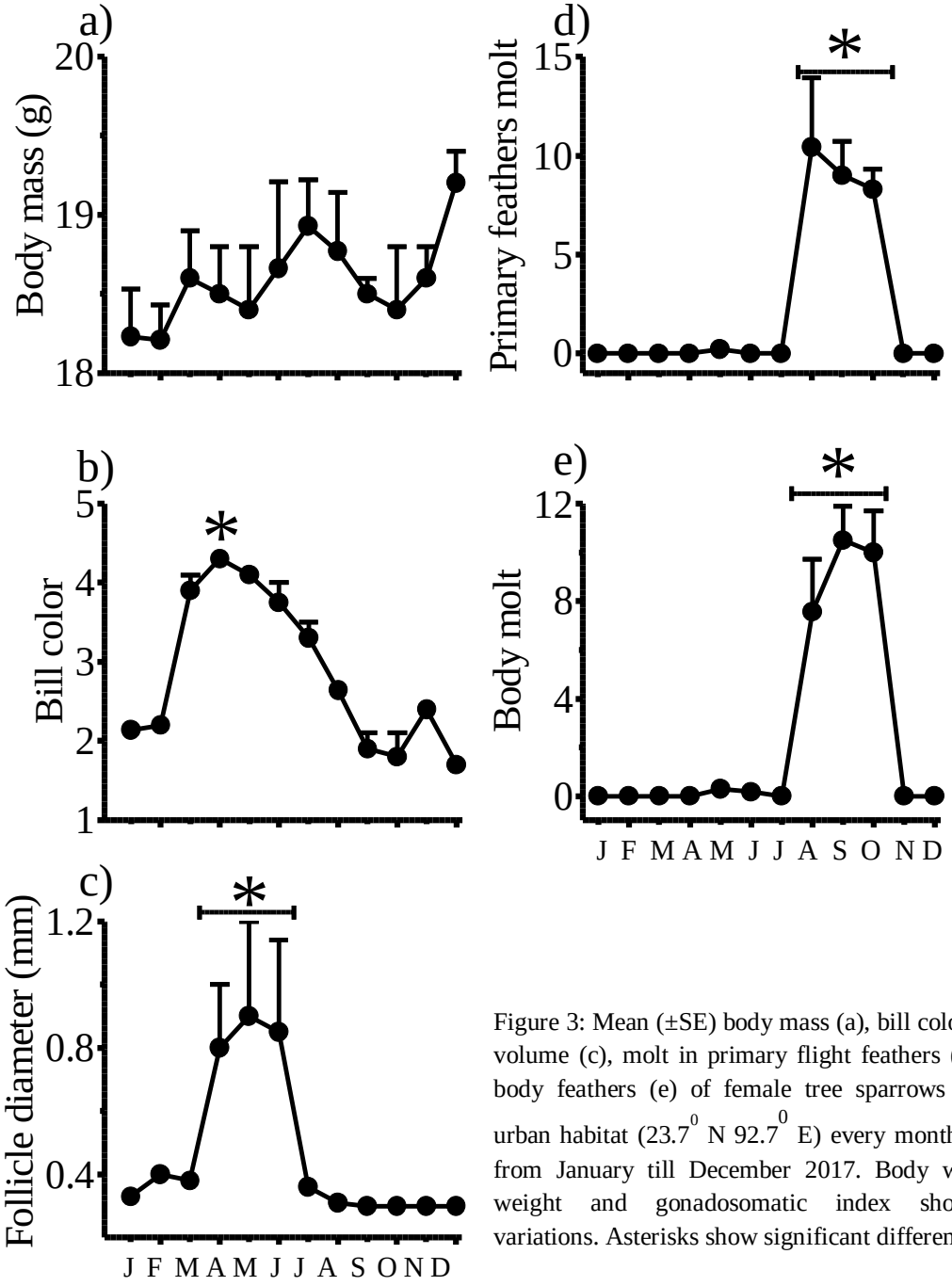

Figure 3: Mean (±SE) body mass (a), bill color (b), testicular volume (c), molt in primary flight feathers (d) and molt in body feathers (e) of female tree sparrows procured from urban habitat (23.7^0 N 92.7^0 E) every month from the wild from January till December 2017. Body weight, gonadal weight and gonadosomatic index showed seasonal variations. Asterisks show significant difference ($P < 0.05$).

Maximum testes activity as reflected by sperm count was during May, while by July, sperm count went significantly low ($P < 0.05$; Newman-Keuls Multiple comparison tests). Molt in body feathers and primary flight feathers followed the annual testicular cycle, and high molt was observed during the post-reproductive phase. There was an annual change in primary flight feathers ($F_{11,84} = 7.190$, $P < 0.0001$; 1-way ANOVA; Fig. 2d) and body molt ($F_{11,84} = 11.58$, $P < 0.0001$; 1-way ANOVA; Fig. 2e). No molt in body and primary flight feathers was recorded till June, slow molting started by July while maximum molt in both body feathers and primary flight feathers were observed during August (Fig. 2d,e). By November, molt was completed (Fig. 2d,e).

In female, we did not see annual changes in body weight ($F_{11,83} = 0.3444$, $P = 0.9723$; 1-way ANOVA; Fig. 3a). However, annual change in bill color was recorded ($F_{11,84} = 45.94$, $P < 0.0001$; 1-way ANOVA; Fig. 3b). Bill color started becoming dark by March, and like male, maximum bill score (darkest bill color) was observed during April (Fig. 3b). The high bill score was maintained until June and started declining by July (Fig. 3b). By September, bill color was minimum, and it was then maintained (Fig. 3b). Like testes, FD also changed seasonally ($F_{11,84} = 73.026$, $P = 0.0020$; 1-way ANOVA; Fig. 3c). No ovarian activity was observed from January till March (Fig. 3c), while maximum ovarian activity was observed during April, May, and June (Fig. 3c). By July, ovarian activity was ceased, and no activity observed until December (Fig. 3c). Molt in feathers followed ovarian cycle (Fig. 3d,e) and showed annual variations both in primary flight feathers ($F_{11,84} = 9.345$, $P < 0.0001$; 1-way ANOVA; Fig. 3d) and in body feathers ($F_{11,84} = 21.22$, $P < 0.0001$; 1-way ANOVA; Fig. 3e). No molting was observed till July while high molting in body feathers and primary flight feathers were observed during August, September, and October while no molting in November and December months (Fig. 3d,e).

4.2 Rural birds

There was an annual variation in body weight in male birds ($F_{11,110} = 1.884$, $P = 0.0490$; 1-way ANOVA; Fig. 4a). Maximum body weight was observed during June

(mean BW19.0 ± 0.2 gm; Fig. 4a), while minimum body weight was observed during August (mean BW17.8 ± 0.2 gm; Fig. 4a). Bill colour changed as per time of the year ($F_{11,110}$ = 15.11, P < 0.0001; 1-way ANOVA; Fig. 4b). Bill color score was low during January and February; high bill color was observed from March till June while July onwards again (Fig. 4b). Bill's color score was minimum-till December. Season dependent change in testes size was observed ($F_{11,110}$ = 32.73, P < 0.0001; 1-way ANOVA; Fig. 4c). The testicular size was small during January and February, but was high during March till July with maximum size during May (mean TV = 49.3 ± 9.0 mm^3; Fig. 4c) while minimum testes volume was observed from August till December (mean TV= 0.3 ± 0.06 mm^3; Fig. 4c). Molt cycle followed annual reproductive cycle and showed seasonal changes in both primary flight feathers ($F_{11,110}$ = 8.406, P < 0.0001; 1-way ANOVA; Fig. 4d) and in body feathers ($F_{11,110}$ = 13.80, P < 0.0001; 1-way ANOVA; Fig. 4e). Both body feathers and primary flight feathers started molt once testes started regressing, and hence maximum molt was observed during August and September months while by November molt was complete (Fig. 4d,e).

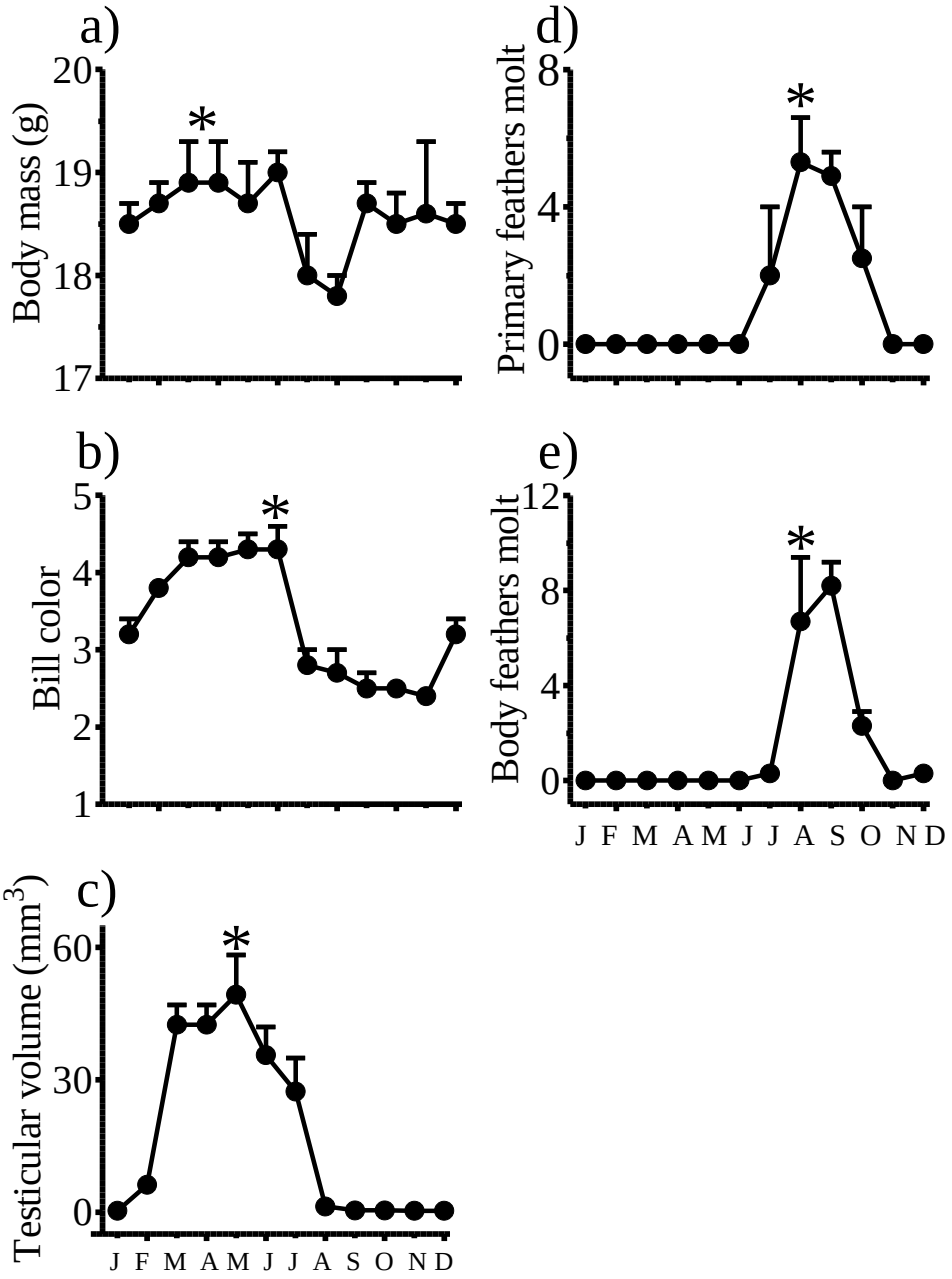

Figure 4: Mean (±SE) body mass (a), bill color (b), testicular volume (c), molt in primary flight feathers (d) and molt in body feathers (e) of male tree sparrows procured from rural habitat (23.7^0 N 92.4^0 E) every month from the wild from January till December 2017. Body weight, gonadal weight and gonadosomatic index showed seasonal variations. Asterisks show significant difference ($P < 0.05$).

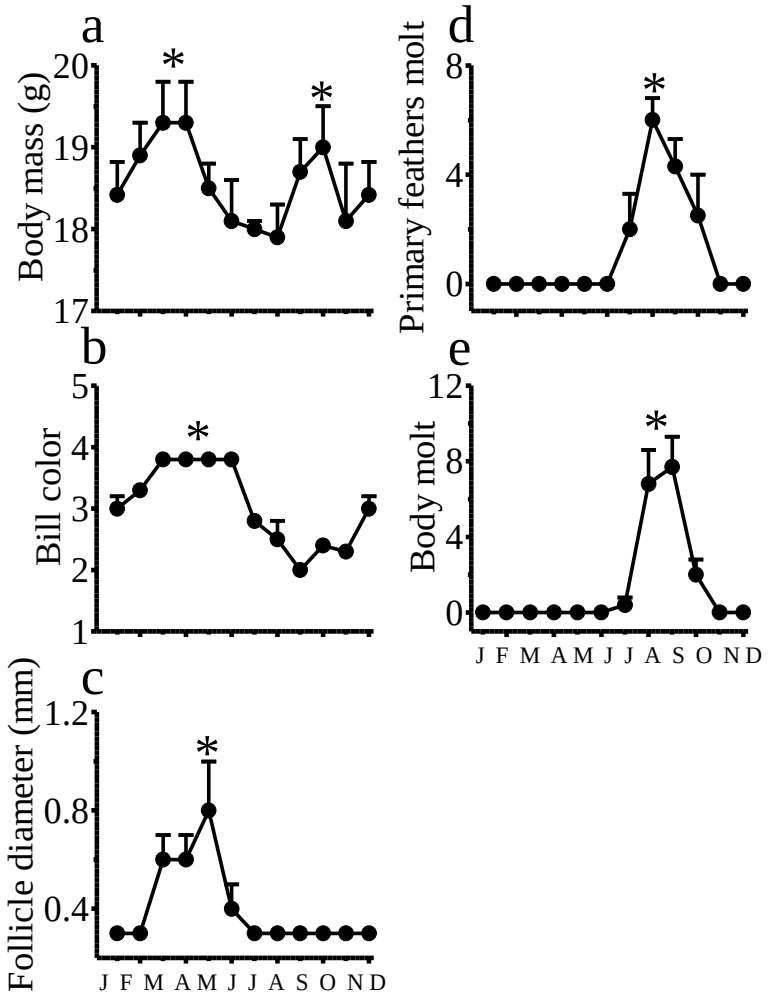

Figure 5: Mean (±SE) body mass (a), bill color (b), testicular volume (c), molt in primary flight feathers (d) and molt in body feathers (e) of female tree sparrows procured from rural habitat (23.7^0 N 92.4^0 E) every month from the wild from January till December 2017. Body weight, gonadal weight and gonadosomatic index showed seasonal variations. Asterisks show significant difference ($P < 0.05$).

Annual change in body mass was also reflected in female birds ($F_{11,108}$ = 1.912, P = 0.0454; 1-way ANOVA; Fig. 5a). Maximum body weight was observed before breeding time, i.e., during March and April (Fig. 5a), while minimum body weight was observed during July, post-reproductive phase (Fig. 5a). There was seasonal variation in the bill colour ($F_{11,110}$ = 20.20, P < 0.0001; 1-way ANOVA; Fig. 5b). High bill color was observed during March till June, while the minimum bill score was observed during September (Fig. 5b). Ovary activity was changing as per time of the year ($F_{11,110}$ = 3.246, P = 0.0010; 1-way ANOVA; Fig. 5c). No follicles were observed during January and February, follicular activity started during March, and maximum ovarian activity was observed during the May, while by July again, no differentiation of ovarian follicle was found (Fig. 5c). Like male birds, molt in feathers were in phase with reproductive cyclicity and maximum molt was observed during post-reproductive phase both in primary flight feathers ($F_{11,110}$ = 8.058, P < 0.0001; 1-way ANOVA; Fig. 5d) and in molt in body feathers ($F_{11,110}$ = 9.457, P < 0.0001; 1-way ANOVA; Fig. 5e) and molt. In body feathers, maximum molt observed during August and September, while in primary flight feathers, high molting was observed during July till October (Fig. 5d,e).

4.3 Rural Vs. Urban Birds

We compared seasonal response of birds from the two habitats. In male birds we observed effect of habitat ($F_{1, 213}$ = 4.703, P = 0.0312; 2-way ANOVA; Fig.6), time ($F_{11,213}$ = 2.076, P = 0.0232, 2-way ANOVA; Fig. 6) and interaction of habitat and time ($F_{11,213}$ = 4.370, P < 0.0001; 2-way ANOVA; Fig. 6) on body mass. The body mass differed in the birds of two habitat at two time of the year i.e., during March and August (Fig. 6a) when the urban birds had significantly higher (P < 0.05; Bonferroni's multiple comparison test; Fig. 6a) body weight in urban birds than rural birds. Effect of habitat, time and interaction of two factors was observed on bill colour (habitat: $F_{1, 213}$ = 8.746, P < 0.0001; time: $F_{11,213}$ = 46.02, P < 0.0001 and interaction of habitat and time: $F_{11,213}$ = 6.591, P < 0.0001; 2-way ANOVA; Fig. 6b). Significant difference between bill colors in the birds of two habitats was observed during January, February and December (P < 0.05; Bonferroni's multiple comparison tests; Fig. 6b) when bill colour was darker in rural birds (Fig. 6b). Effect

of habitat, time and interaction of two factors were also observed on testes size (habitat: $F_{1, 213} = 4.207$, $P = 0.0415$; time: $F_{11,213} = 33.26$, $P < 0.0001$ and interaction of habitat and time: $F_{11,213} = 12.44$, $P < 0.0001$; 2-way ANOVA; Fig. 6c). There was a significant diffference in testicular volume in the birds of two habitat ($P < 0.05$; Bonferroni's multiple comparison test; Fig. 6c) during March. There was a slight delay in the testicular growth in urban birds (Fig. 6c). Both in body feathers and primary flight feathers showed effect of time (body feathers: $F_{11,213} = 18.01$, $P < 0.0001$; primary flight feathers: $F_{11,213} = 10.69$, $P < 0.0001$; 2-way ANOVA; Fig. 6d,e). but not of habitat (body feathers: $F_{11,213} = 2.178$, $P = 0.1428$; primary flight feathers: $F_{11,213} = 2.889$, $P = 0.0920$; 2-way ANOVA; Fig. 6d,e) and interaction of habitat and time (body feathers: $F_{11,213} = 0.9010$, $P = 0.5420$; primary flight feathers: $F_{11,213} = 1.562$, $P = 0.1200$; 2-way ANOVA; Fig. 6d,e).

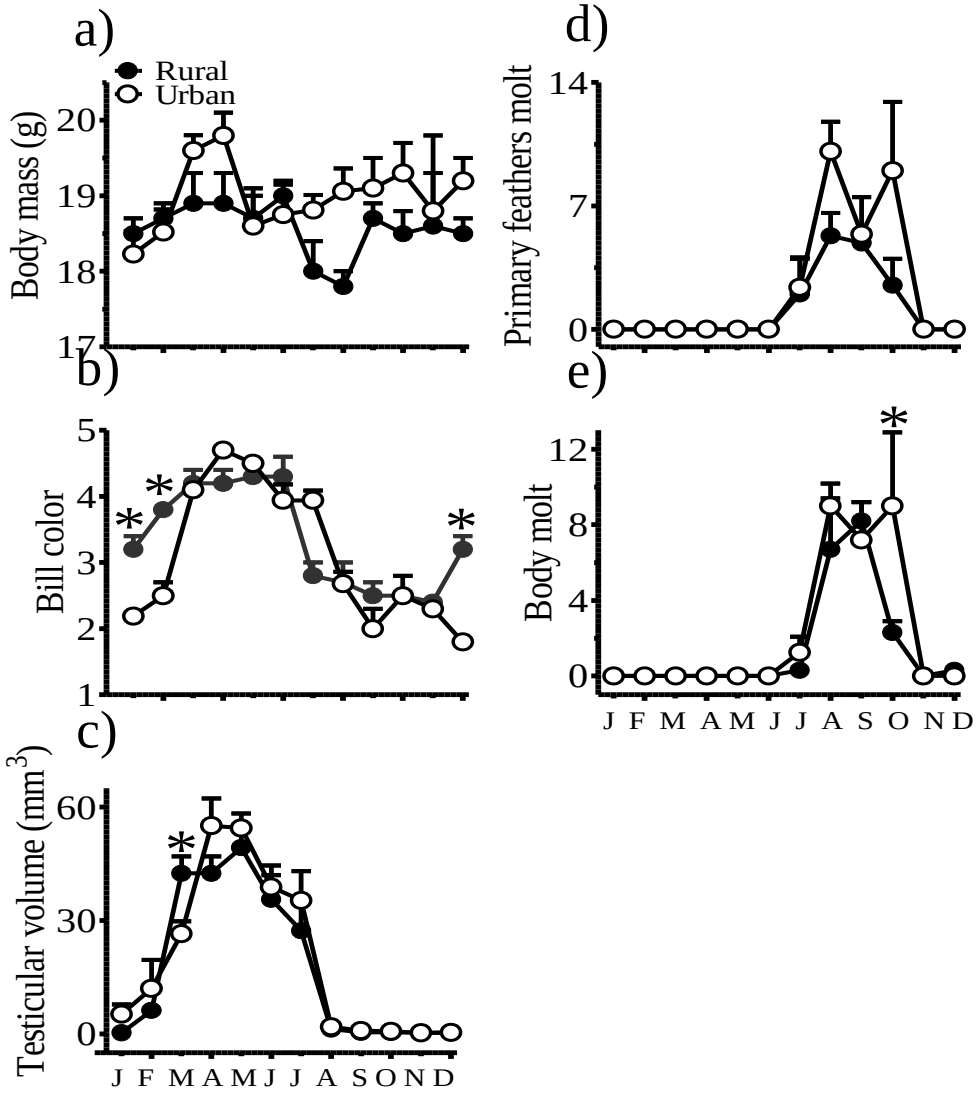

Figure 6: Mean (±SE) body mass (a), bill color (b), testicular volume (c), molt in primary flight feathers (d) and molt in body feathers of male tree sparrows procured from rural (23.7^0 N 92.4^0 E) and urban habitat (23.7^0 N 92.7_0 E) every month from the wild from January till December 2017. Asterisks show significant difference ($P < 0.05$) between two groups at that point of time.

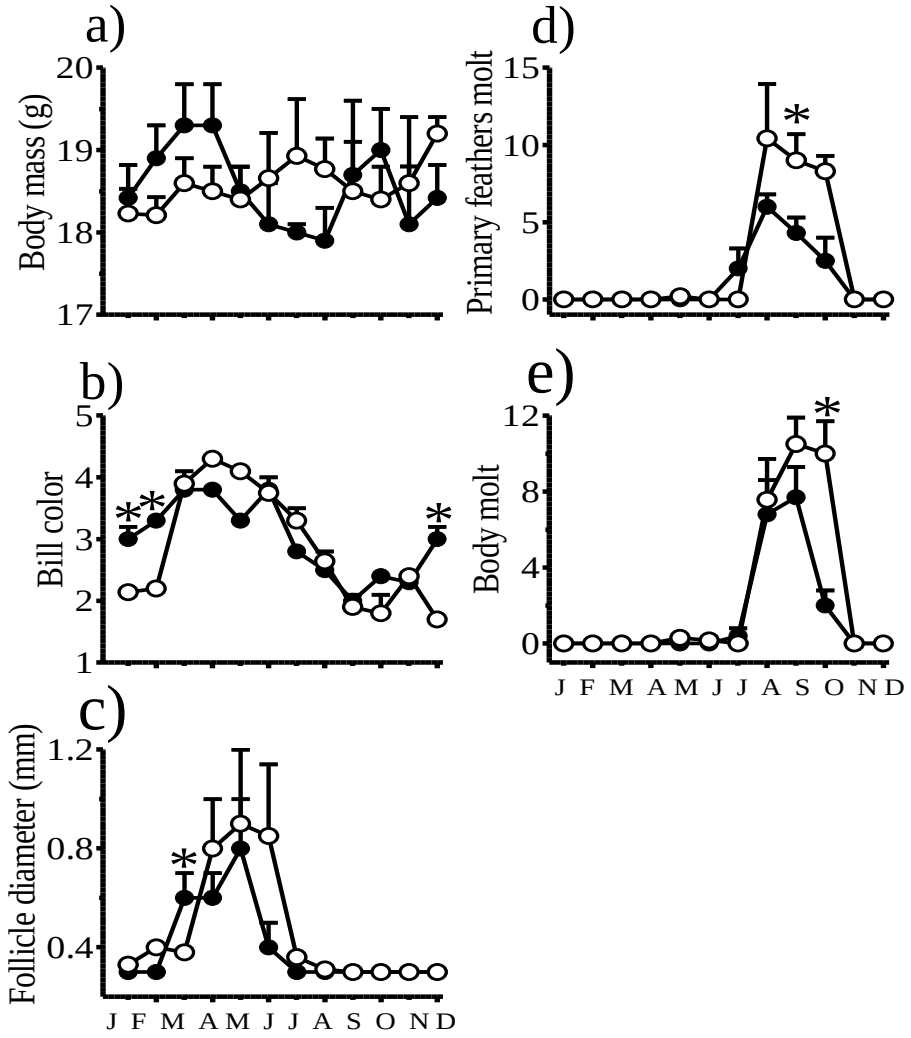

Figure 7: Mean (±SE) body mass (a), bill color (b), testicular volume (c), molt in primary flight feathers (d) and molt in body feathers of female tree sparrows procured from rural (23.7^0 N 92.4^0 E) and urban habitat (23.7^0 N 92.7_0 E) every month from the wild from January till December 2017. Asterisks show significant difference ($P < 0.05$) between two groups at that point of time.

Female birds did not reflect effect of habitat on body weight (habitat: $F_{1, 213}$ = 0.9834, P = 0.3225; time: $F_{11,213}$ = 0.8244, P = 0.6157 and interaction of habitat and time: $F_{11,213}$ = 1.743, P = 0.0658; 2-way ANOVA; Fig. 7a). We observed effect of habitat on bill colour (habitat: $F_{1, 213}$ = 4.117, P = 0.0442; time: $F_{11,213}$ = 56.60, P < 0.0001 and interaction of habitat and time: $F_{11,213}$ = 11.14, P > 0.0001; 2-way ANOVA; Fig. 7a). In rural birds bill colour was significantly darker (P < 0.05; Bonferroni's multiple comnparison test; Fig. 7b) during January, Februrary and December (Fig. 7b) while in urban birds bill colour score was higher during April and May (Fig. 7b). Like male effect of habitat was also observed on follicle diameter (habitat: $F_{1, 213}$ = 6.241, P = 0.0132; time: $F_{11,213}$ = 8.293, P < 0.0001 and interaction of habitat and time: $F_{11,213}$ = 4.705, P < 0.0001; 2-way ANOVA; Fig. 7c). In urban birds there was slight delay in advance (P < 0.05; Bonferroni's multiple comnparison test; Fig. 7c) of FD development and regression was also slightly slower (P < 0.05; Bonferroni's multiple comparison test; Fig. 7c). Effect of time, habitat and interaction was observed on molt in primary flight feathers (habitat: $F_{1, 213}$ = 5.901 P = 0.0166; time: $F_{11,213}$ = 16.22, P < 0.0001 and interaction of habitat and time: $F_{11,213}$ = 2.534.22, P = 0.082; 2-way ANOVA; Fig. 7d) and in molt in body feathers (habitat: $F_{1, 213}$ = 6.3051, P = 0.0134; time: $F_{11,213}$ = 35.32, P < 0.0001 and interaction of habitat and time: $F_{11,213}$ = 2.406, P = 0.0099; 2-way ANOVA; Fig. 7e). There was slight delay in completion of molt in urban birds in comparison to rural birds (Fig. 7d,e).

5. DISCUSSION

Our results demonstrate that both male and female tree sparrows essentially have a similar pattern in annual cycles of body mass, bill color, gonads, and molt. In seasonal breeders, ultimate factors decide the precise timing of reproduction. Annual rainfall is generally predictable at this latitude, and usually, monsoon starts from the second or third weeks of April. Monsoon increases abundance of food availability, and hence many avian species breed in such a way that offsprings should be hatched out during this time of the year (Baker, 1938; Lack, 1950; Marshall, 1961; Immelmann, 1971; Thapliyal, 1978). Therefore, the preparation of reproduction must be started at least 6-8 weeks in advance of the actual breeding, rainfall cannot be the primary factor, although it can facilitate breeding by enhancing the chances of pairing between the opposite sex, and rearing the brood with increased green vegetation and attractive sights of ponds and pools (Misra, 1960). In our study, we observed small changes in body mass throughout the year and are consistent with previous reports of other resident species (Trivedi *et al.*, 2006; Anushi and Bhardwaj, 2006).

In comparison to migratory species, resident species do not accumulate significant fat in the adipose tissues in their body. On the other hand, migratory species before they begin actual migration, they collect a considerable amount of reserve food in their body, which serves as fuel during their spring migration (Trivedi *et al.*, 2014). No such necessity emerges for resident species, and therefore there was a less precise cyclicity in body mass. However, a small gain in body mass before the peak of gonadal development (cf. Figs. 2-5) was observed. For example, birds had gained in body mass in March and April, whereas their peak gonadal development occurred in April-May (though the peak was less evident in females). The peak in body mass before the reproductive phase has been reported in several previous studies (Thapliyal, 1968; Thapliyal, 1969; Thapliyal and Gupta, 1989; Thapliyal and Biur, 1992; Trivedi *et al.*, 2006). The peak in body mass before reproduction suggests the requirement of the energy for the breeding.

Testes began to recrudescence in March, and by April achieved maxima and then start regressing, and fully regressed testes were observed by August. However,

in females, follicles start growing in April, and high ovarian activity was observed from April till June, and by July, fully regressed ovary was found, although day lengths were still long. However, functional spermatozoa were maximum during May, and it is corresponding with the highest activity of ovary in females during May. As reproduction is largely energetically incompatible with other critical life-history stages, such as migration and molt, appropriately timed termination of reproduction is critical. In general, breeding ends, leaving enough time to complete other life-history stages before conditions become unfavorable, thus enhancing adult survival (and thereby future reproductive opportunities) without excessively compromising current reproductive opportunities. Therefore, the neuroendocrine mechanisms evolved to respond to the environment will be key determinants of fitness in variable environments. However, testicular activity starts diminishing gonadal activity after May, although daylight was still increasing. Initially, this type of testicular growth-regression was considered to discount the role of day length as a "driver" in control of the annual testicular cycle. However, early investigations on the annual reproductive cycle (Bissonnette, 1931; Burger, 1949; Farner, 1959; Murton and Westwood, 1977) have established that such gonadal regression when birds are still experiencing increasing or long day lengths are due to the development of refractoriness to stimulatory effects of day lengths; this is called photorefractoriness (Farner *et al*., 1983; Nicholls *et al*., 1988).

Photorefractoriness is an adaptive phase of the annual cycle and has been documented in the annual cycles of several birds (Hamner, 1968; Farner, 1962; Farner and Lewis, 1971; Lofts and Murton, 1968; Murton and Westwood, 1977; Kumar, 1997). The annual cycle of body mass gain, breeding, change in the bill and plumage color, molt, and migration of birds are regulated fundamentally by seasonally changing photoperiod along with internal changes in responsiveness to it (i.e., sensitivity and refractoriness). In many avian species, the onset of an annual plumage molt coincides with the setting/development of refractoriness to the photoperiod for reproductive responses. Photorefractoriness can be either absolute or relative (Robinson and Follett, 1982; Nicholls *et al*., 1988). This absolute photorefractoriness has been characterized in two categories: Refractoriness

Criterion 1: Gonads regress and prebasic molt proceeds without any decline in photoperiod; and Refractoriness Criterion 2: Gonadotropin levels and gonads are unaffected by longer days once gonads have regressed and molt is advanced (see Hamner, 1968; Nicholls *et al*., 1988). Generally, absolute refractoriness is referred to in respect to photoperiod; it appears to eliminate responsiveness to all reproductive cues (Ball, 1993; Goodson *et al*., 2005). However, relative refractoriness requires for its expression a decline in photoperiod, or multiple cues (e.g., photoperiod and temperature; Wada, 1993). Although, gonads regress under relative refractoriness the neuroendocrine system is still capable of an immediate and sustained reproductive response, leading to regrowth of the gonads if the birds experience sufficiently long photoperiod (Robinson and Follett, 1982; Nicholls *et al*., 1988; Hahn *et al*., 1997). The birds are considered refractory as it is not responding to that photoperiod only.

The collapse of the gonads as refractoriness develops appears to coincide with a decline in the release of gonadotropin-releasing hormone (GnRH) at the median eminence (Dawson *et al*., 2001). GnRH is the key regulator of pituitary gonadotropin production and secretion in all vertebrates (Gore, 2002). Therefore, a decline in GnRH leads elimination of gonadotropic stimulation of the gonads. As refractoriness progresses, production and release of GnRH also decline as a result of hypothalamic immunoreactivity for this peptide essentially disappears, and it does not reappear until refractoriness dissipates during the autumn (Ball and Hahn, 1997; Dawson et *al*., 2001). This down-regulation of the GnRH system during absolute refractoriness has now been found in around a dozen of songbirds (MacDougall-Shackleton *et al*., 2005). Photoperiod dependent seasonal cycles and the development of photorefractoriness in annual reproductive cycles have been documented in many species. In another resident species, like tree sparrows in brahminy myna *(Sturnus pagodarum)*, testes began to develop in March/April, attained their peak in May, and remained developed until July, when regression sat in. Fully regressed testes were observed in August/ September (Kumar and Kumar, 1991). Common Indian rosefinch and yellow-throated sparrow also exhibit

seasonality in gonads (Kumar and Tewary, 1985; Tewary and Dixit, 1986; Tewary and Tripathi, 1985).

When we compared the reproductive responses of the birds procured from two sites (rural and urban), we observed differences in their reproductive responses (Fig. 6, 7). Both in male and female bill color scores were high in rural birds during January, February, and December. Similarly, preparation of gonads for reproduction was initiated in advance in rural birds as reflected by higher testicular volume, and larger follicles in March were observed. Delay in the completion of molt in urban birds also suggests that the reproductive cycle was slightly delayed in the urban birds. Habitat dependent variations in the reproductive cycles have been reported for many avian species, including tree sparrows (Zhang *et al*., 2014). However, our results are different with the findings of Zhang *et al*. When they compared the luteinizing hormone (LH), testosterone (T), and estradiol (E_2) levels of tree sparrow from the birds sampled at urban and rural sites, increased levels of LH were observed in urban birds than those of rural ones, but rural populations had higher LH peaks. The difference in the two studies could be because of differences in the local environmental conditions. Further, how the molecules involved in the seasonal reproduction are getting affected in the two habitats will be discussed in the later section.

In conclusion, tree sparrows inhabiting at 23.7^0N, 92.6^0E show distinct seasonality in the gonadal development, molt, and other secondary sexual characters. There is a small change in body mass over the year, which is less prominent in females and less dramatic in comparison to migratory species.

SECTION II: SEASONAL CYCLES UNDER LABORATORY CONDITIONS

1. ABSTRACT

Most of the birds exhibit seasonality in their reproductive function in order to coincide with the timing of the birth with the optimal resource availability and suitable environmental conditions to enhance the survival of offsprings. The control of temporal reproduction is accomplished by complex physiological mechanisms and the use of environmental cues to time and regulate the reproductive state appropriately. Day length is the most consistent and reliable environmental cue used by the organisms for signaling the upcoming favorable and unfavorable seasons to do the necessary preparations accordingly. Other environmental factors like food availability and temperature may play a supplementary but essential role in the regulation of seasonal breeding. Here, we investigated whether photoperiodic induction of seasonal responses in tree sparrow shares the same basic features as shown by its temperate population, which has been investigated. Two experiments were performed with observations recorded on changes in body mass, bill, and gonadal size at the beginning and the end of the experiment, and at intervals during the experiment. Experiment 1 examined if there was a change in responsiveness of the photoperiodic response system of tree sparrows to long day lengths. Birds were transferred to long day lengths (16L:8D) and short day lengths (8L:16D) during March and September. Birds were responsive to long days only during March. Experiment 2 investigated whether food restriction can affect the gonadal response. One group was restricted food availability for the first 7h while the control group had food ad libitum. Both groups were exposed to stimulatory photoperiod of 14L:10D. After 30 days, there was significantly less growth in follicle development in the food-restricted group. Altogether our results suggest that a long day stimulates gonadal responses, but only in photosensitive birds and limited food supply can compromise photoperiod induced gonadal responses.

2. INTRODUCTION

Seasonal breeders are highly sensitive to light duration. In most of these organisms, the annual solar cycle has been found to influence various physiological functions (Murton and Westwood, 1977; Hoffman, 1981; Kumar 1997; Renthlei and Trivedi, 2017). Among seasonal breeders, birds' exhibit pronounced seasonal cycles in various behavioral and physiological functions, and the majority of these processes are influenced by annual changes in day length (Dawson *et al*., 2001). William Rowan (1925), for the first time, demonstrated the involvement of photoperiod in gonadal development of slate-colored junco (*Junco hyemalis*). He established that the vernal migration and gonadal recrudescence could be induced out of season by exposure of birds in the laboratory to manipulating day lengths (Rowan, 1926, 1928, 1929, 1932). Since then, a vast number of avian species have been studied in which day length plays a critical role in seasonal physiology in general and reproduction in particular.

Birds use an endogenous time-keeping mechanism (circadian/circannual clock) to track daily changes in the timing of sunrise and to be in sync with the environment. Seasonal cycles of various physiological and behavioral functions in birds seem to be regulated by two mechanisms viz. photoperiodism and circannual rhythm generation (Kumar *et al*., 2010). In seasonal breeders, the photoperiod regulates seasonal rhythms by stimulating and terminating the physiological processes like gonadal growth and development, feathers molt, and change in bill color and migration, in most photoperiodic birds, cycle between periods of photosensitivity and photorefractoriness. In long-day breeders, initiation of gonadal growth happens during long photoperiod, and then birds became photorefractory under this long stimulatory photoperiod. This photorefractoriness gets terminated, and birds subsequently recovered their photosensitivity under short days (Kumar, 1997). Both long and short days are critical for seasonal reproductive physiology in these birds, although they are used to control different physiologies. Therefore, these species must have some mechanism for the measurement of day length in order to have a successful interaction with day length at appropriate phases of the endogenous clocks (Renthlei and Trivedi, 2017).

The photoperiodic control of reproductive functions in birds involves a circadian rhythm of photoperiodic photosensitivity (CRPP; Rani and Kumar, 1999; Dawson *et al*., 2001). The phase relationships between CRPP and environmental factors change with the season, leading to seasonal responses. The photoperiodic response occurs when the photoperiod coincides with the photoinducible phase of an entrained endogenous circadian rhythm. In circannual rhythm generation, a self-sustained endogenous rhythm with a period of approximately one year regulates various physiological and behavioral functions associated with the annual cycles of birds. It is in question whether, in birds, the photoperiodic regulation of seasonality involves an endogenous circadian or circannual rhythm exclusively, or they might interact closely, albeit as per the adaptive needs of the species. It is also argued that circannual events are manifestations of circadian rhythms, as is evident from the circadian rhythm involvement during initiation and termination of gonadal growth and photoperiodism and circannual rhythm generation are evolved as separate mechanisms. The Indian species (both resident and migratory), which have been investigated, are photosensitive, although they may exhibit differential responses to photoperiods. Based on photoperiodic responses, they can be categorized as follows:

***Long day breeders*:** In these birds, long day lengths (e.g., 15L:9D) induce gonadal growth and development, while short day lengths (photoperiods less than 9 h per day) are inhibitory. Examples: Indian weaver bird (Thapliyal and Saxena, 1964b; Thapliyal and Tewary, 1964; Singh and Chandola, 1981; Chakravorty and Chandola, 1985), redheaded bunting (Tewary and Tripathi, 1983; Rani, 1999), black-headed bunting (Tewary and Kumar, 1982; Misra *et al*., 2004), common Indian rosefinch (Kumar and Tewary, 1982b; Tewary *et al.*, 1983), crested bunting (Tewary and Kumar, 1983a), yellow-throated sparrow (Tewary and Tripathi, 1985; Tewary and Dixit, 1986), house sparrow (Trivedi *et al*., 2006) and brahminy myna (Kumar and Kumar, 1991, 1993).

Both long and short day breeders: Birds of this category show gonadal recrudescence under both long and short day lengths (e.g., 15L:9D and 9L:15D). Example: Blackheaded munia, *Munia malacca malacca* (Thapliyal and Saxena, 1964a; Pandha and Thapliyal, 1969).

Short day breeders: Here, short day lengths (e.g., 9L:15D) can induce gonadal development, but long day lengths (e.g., 15L:9D) activates pituitary function as indicated by the growth of LH-dependent plumage. Example: Lal munia, *Estrilda amandava* (Thapliyal and Tewary, 1963; Tewary and Thapliyal, 1965; Tewary, 1967).

Very short day breeders: Very short photoperiods (e.g., 3L:21D) can induce gonadal development. Example: Spotted munia, *Lonchura punctulata* (Chandola *et al*., 1975; Thapliyal *et al*., 1975).

Continuous exposure of inductive long photoperiods induces the development of a period of insensitivity to day lengths that were initially stimulatory. This reproductively insensitive stage is referred to as photorefractoriness, characterized by spontaneous regression of gonads under continued exposure to stimulatory photoperiods (Nicholls *et al*., 1988). A similar situation can occur in other photoperiod-induced seasonal processes, such as fat deposition-depletion (Farner *et al*., 1983; Nicholls *et al*., 1988; Kumar, 1997). Photorefractoriness can be terminated naturally by winter day lengths in the wild and by exposure to short day lengths (9:15D) in the laboratory condition (Farner *et al*., 1983; Nicholls *et al*., 1988; Kumar, 1997). Photorefractoriness is an adaptive phase in the annual cycle of a vertebrate. It limits the reproduction of a species to the best suited time of the year ensuring maximal reproductive success, permits sufficient time for replenishment of energy stores for post-breeding maintenance activities, such as the postnuptial molt and preparation for migration (Farner, 1964; Farner and Follett, 1966, 1979), and avoids wastage of the reproductive potential (Farner and Lewis, 1971).

Resource availability is an essential determinant of the demography and distribution of many species. The availability of food resources is critical for the optimization of breeding schedules and the reproductive performance of birds (Kumar *et al*., 2001: Mishra and Kumar, 2019). The limitations of food in nature can vary in time and across species, breeding stages, and environments. Food limitation can be defined as when a shortage of food or any critical nutrients results in lowering the reproductive and/or reduced survival (Martin, 1987; Sinclair, 1989;

Newton, 1964) that ultimately leads to a decline in the growth rate of the population. Food limitation is less critical for organisms where predation or critical resources other than food keeps populations below their carrying capacity (Osenberg and Mittelbach, 1996). However, food availability can modulate the timing of the onset of the breeding, mating, and egg-laying in a large group of seasonally reproducing animals, including birds. Availability of food resources can have short (e.g., reproductive effort) and long-term (e.g., adult survival and future reproduction) consequences. Therefore, variation in food availability in time and space can be an important cue for organisms to adapt their reproductive decisions; it can provide information on where and when to breed (Martin, 1987; Asdell, 1964; Lack, 1966). Restricted food availability delays and/or cause sub-optimal gonadal development in European starlings (*Sturnus vulgaris*; Meijer, 1991) and Abert's towhees (*Melozone aberti*; Davies *et al*., 2015). Time-restricted food availability affected the gonadal growth regression cycle of testes in buntings (Kumar *et al*., 2001). Birds with a supplemented diet advanced in egg-laying dates and enhanced breeding success, as reflected by egg-laying frequency, clutch size, hatching success, brood size and the number of fledglings (Reynolds *et al*., 2003; Rutstein *et al*., 2004; Robb *et al*., 2008; Kaiser *et al*., 2014).

On the other hand, food restriction at one annual life history state can modulate the photoperiodic responses at other states in redheaded buntings (Budki *et al*., 2008). Further, temporal food restriction can also have differential effects of reproductive performances as food availability for 5 h in the evening, but not in the morning, coupled with long stimulatory photoperiod of 16 h light per day depressed by almost half the photostimulated testicular recrudescence in both migratory black-headed buntings (Kumar *et al*., 2001), and resident house sparrows, *Passer domesticus* (Bhardwaj and Anushi, 2004). Similarly, layers fed twice a day laid more eggs than those who were fed once a day (Spradley *et al*., 2008).

Here, we investigated the effect of a long and short photoperiod on gonadal recrudescence on tree sparrows at two different times of the year, i.e., during March

and September when in nature they experience equinox photoperiod. Further, we also investigated the effect of food restriction on the growth of ovarian follicles.

3. MATERIAL AND METHODS

Two experiments were performed:

3.1 Experiment 1: Role of day length in the regulation of the seasonal gonadal cycle

We examined the role of artificial photoperiod on testicular growth. Adult male birds were used for this study. Birds (n= 20) were procured locally during two times of the year, i.e., during March and September 2018 when they were experiencing equinox photoperiod (12L:12D). Each time, birds were divided into two groups and exposed to either long photoperiod (16L:10D) or short photoperiod (8L:16D). Changes in body mass, bill color, and testes size were recorded.

3.2 Experiment 2: Role of food in the regulation of seasonal reproduction

The study evaluated the role of food in the regulation of reproductive cycles. Adult female photosensitive birds (n = 8 each) were used for this study. Birds were divided into two groups. Both the groups were exposed to stimulatory photoperiod of 14L:10D (14 h light: 10h dark). However, group 1 was exposed to restricted food availability (the first 7 hr) of the light phase, while group 2 had food ad libitum. Changes in body mass, bill color, and follicle diameter were recorded.

3.3 Statistical analysis

Data are presented as mean ± SE. They were analyzed using a 1-way analysis of variance (1-way ANOVA) followed by Newman-Keuls post hoc tests if ANOVA indicated a significance of the difference. 2-way analysis of variance was employed to analyze the effects when two factors (e.g., treatment and time) were taken into consideration, and the Bonferroni test was used as a post hoc test for group comparisons. Significance was always taken at $P < 0.05$.

4. RESULTS

4.1 Experiment 1: Role of day length in the regulation of seasonal gonadal cycle

Exposure to long or short photoperiod did not affect the body weight in March birds (photoperiod: : $F_{2,24}$ = 3.796, P = 0.0632; time: $F_{2,24}$ = 1.836, P = 0.1811 and interaction: $F_{2,24}$ = 0.06002, P = 0.9419; 2-way ANOVA; Fig. 8a). There was only effect of time on bill colour but not of photoperiod (photoperiod: : $F_{2,24}$ = 0.2361, P = 0.6315; time: $F_{2,24}$ = 15.82, P < 0.0001 and interaction: $F_{2,24}$ = 1.121, P = 0.3423; 2-way ANOVA; Fig. 8b). Effect of photoperiod was observed on testicular volume (photoperiod: : $F_{2,24}$ = 3.109, P = 0.0321; time: $F_{2,24}$ = 6.246, P = 0.0066 and interaction: $F_{2,24}$ = 5.769, P = 0.0090; 2-way ANOVA; Fig. 8c). Birds under LD had signficantly higher ($P < 0.05$; Bonferroni's multiple comnparison test; Fig. 8c) testicular volume on day 30 of experiment. On day 60 of experiment, birds had fully regressed testes (Fig. 8c). Birds under SD did not show any significant change in testicular volume (Fig. 8c).

Birds exposed to LD or SD during September did not show any affect of photoperiod on body mass (photoperiod: $F_{2,24}$ = 2.349 P = 0.9962; time: $F_{2,24}$ = 3.325, P = 0.0531 and interaction: $F_{2,24}$ = 0.0596, P = 0.9422; 2-way ANOVA; Fig. 8d), bill colour (photoperiod: : $F_{2,24}$ = 0.0098, P = 0.9219; time: $F_{2,24}$ = 2.492, P = 0.1039 and interaction: $F_{2,24}$ =0.1323, P = 0.8767; 2-way ANOVA; Fig. 8e) and testicular volume (photoperiod: $F_{2,24}$ = 2.497, P = 0.1272; time: $F_{2,24}$ = 1.227, P = 0.3110 and interaction: $F_{2,24}$ = 2.190, P = 0.1338; 2-way ANOVA; Fig. 8f). Birds exposed to LD had slight gain in testicular volume on day 60 (Fig. 8f).

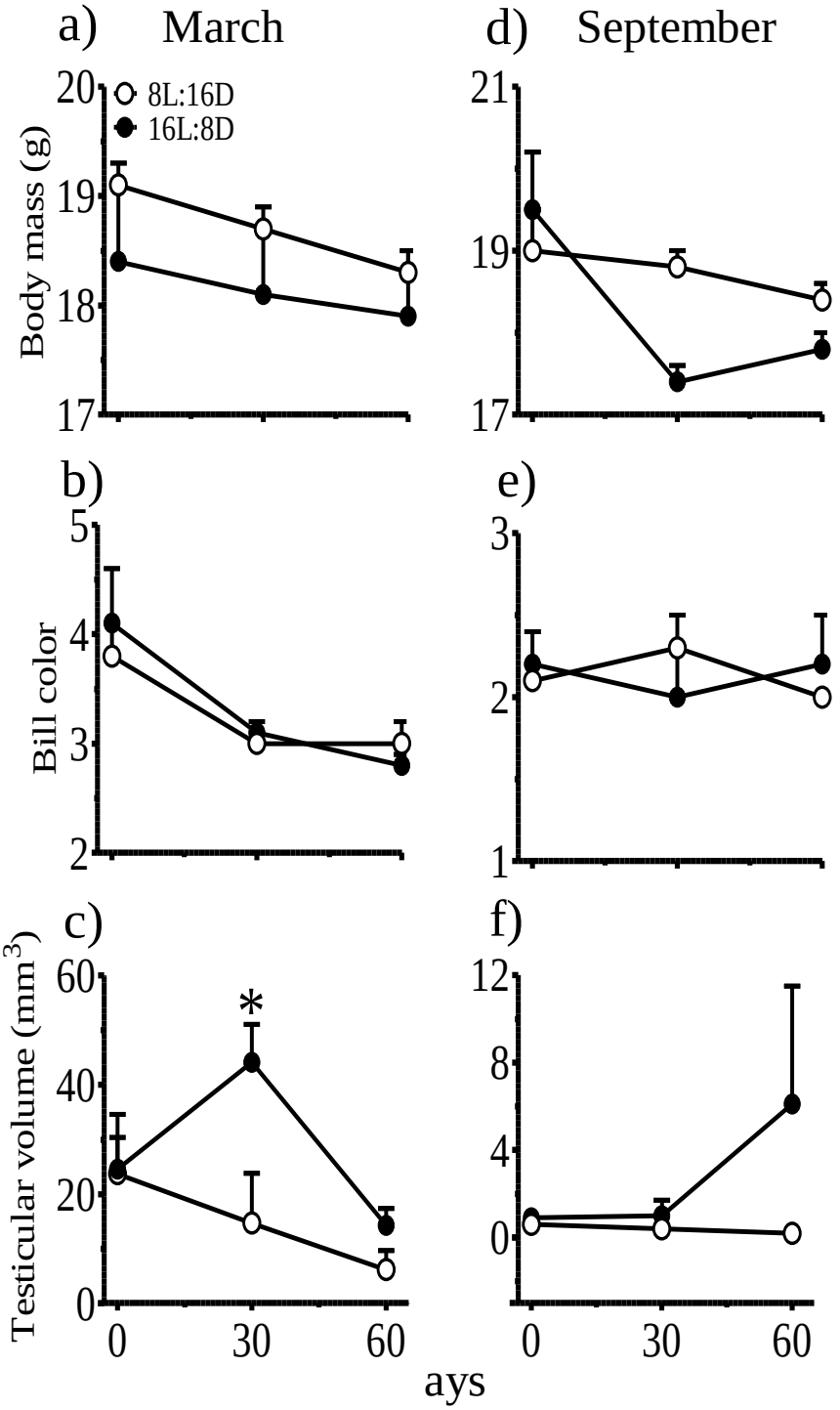

Figure 8: Mean (±SE) body mass (a,d), bill color (b,e) and testicular volume of male tree sparrows at two time i.e., during March and September. Asterisks show significant difference ($P < 0.05$) between two groups at that point of time.

4.2 Experiment 2: Role of food in the regulation of seasonal reproduction

Effect of food restriction was observed on body mass (food restriction: $F_{1,16}$ = 3491, P < 0.0001; time: $F_{1,16}$ = 5.274, P = 0.0170 and interaction: $F_{2,24}$ = 5.691, P = 2.4616; 2-way ANOVA; Fig. 9a). There was a significant loss in body mass in food restricted group on day 30 of experiment (P < 0.05; Bonferroni's multiple comnparison test; Fig. 9a). We did not see any effect of food restriction on bill colour (food restriction: $F_{1,16}$ =1.213, P = 0.9900; time: $F_{1,16}$ = 1.163, P = 0.1635 and interaction: $F_{2,24}$ = 0.201,5 P = 0.50465; 2-way ANOVA; Fig. 9b). However, effect of restricted food availability was observed on FD (food restriction: $F_{1,16}$ = 8.533, P = 0.0100; time: $F_{1,16}$ = 3.133, P = 0.01635 and interaction: $F_{2,24}$ = 19.20, P = 0.0005; 2-way ANOVA; Fig. 9c). Follicular diameter was significantly higher (P < 0.05; Bonferroni's multiple comnparison test; Fig. 9c) in food ad libitum group in comparison to food restricted group on day 30.

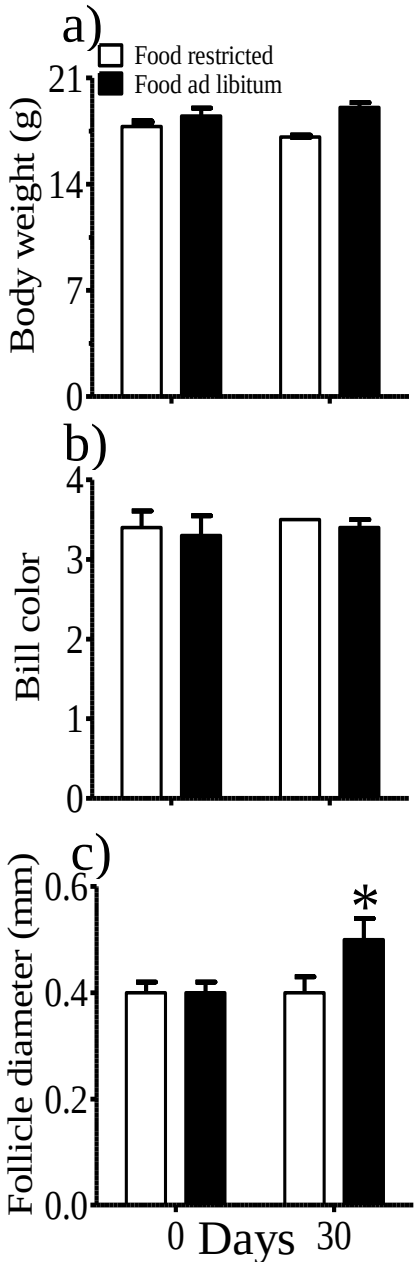

Figure 9: Mean (±SE) body mass (a), bill colour (b), and follicle diameter (c) of female tree sparrows exposed to either food restriction or food ad libitum conditions. Asterisks show significant difference ($P < 0.05$) in the response between the two conditions.

5. DISCUSSION

Our results show the annual life history state-dependent effect of photoperiod on the testicular growth cycle. When birds were exposed to LD and SD during March, birds exposed to LD exhibited testicular recrudescence within 30 days of exposure, and on day 60, fully regressed testes were observed (Fig. 8c). On the other hand, birds under SD did not show any significant change in testicular volume until the end of the study (Fig. 8c). However, when the birds procured during September were exposed to LD and SD, no testicular growth was observed on day 30, while a minimal change in testicular volume was observed on day 60 but only in the birds exposed to LD (Fig. 8f). Our results are in agreement of being tree sparrow as a long day breeder (Dixit and Singh, 2011, 2012). Birds procured from wild during March are photosensitive birds, and therefore exposure to long days led to testicular growth and regression (Fig. 8c). However, birds exposed to SD (8L:16D) photoperiod did not show any photoperiodic induction suggesting that this photoperiod is below critical day length. On the other hand, birds procured during September were physiologically refractory for the reproductive processes. Hence, even exposure to long photoperiod was not able to induce the gonadal growth regression cycle (Fig. 8f).

Responses of photosensitive tree sparrows to stimulatory long photoperiod is comparable to that reported in the house sparrows (Trivedi *et al.*, 2006), Indian weaver bird (Singh and Chandola, 1981), the brahminy myna (Kumar and Kumar, 1991) and redheaded buntings (Rani and Kumar, 2000). Exposure to LD during September could not induce testicular recrudescence indicates an actual loss of sensitivity in tree sparrows to long day photostimulation. These observations suggest that the termination of the breeding season in the tree sparrow is due to the development of photorefractoriness, and the photorefractoriness is followed by a recovery period, at the end of which birds regain their responsiveness to stimulatory day lengths (for review see Murton and Westwood, 1977; Farner *et al.*, 1983; Nicholls *et al.*, 1988). In nature, till September, birds were not able to regain their photosensitivity, suggesting that more prolonged exposure to short day length is required to break photorefractoriness in this species.

The evidence that artificial long day length (14L:10D) induces a photoperiodic response that is usually seen under increasing NDL (as evident from section 1), suggesting that the photoperiod regulates gonadal cycle and associated events in the tree sparrow. This suggests that under long day lengths induction of a photoperiodic response occurs in this species as it has been observed in other species (Follett *et al.*, 1973; Lewis, 1975; Kumar and Tewary, 1982b, 1983; Tewary and Kumar, 1982; Tewary and Tripathi, 1983; Tewary *et al.*, 1983; Tewary and Dixit, 1986; Kumar and Kumar, 1991; Trivedi *et al.*, 2006). However, a typical short day (8L:16D) was not inductive to tree sparrows. A similar photoperiodic response has been reported for other species as in house sparrows (Trivedi *et al.*, 2006) and the Indian weaver bird, *Ploceus philippinus* (Pandey and Bhardwaj, 2011). However, in house sparrows (Trivedi *et al.*, 2006) and Indian weaver bird exposed to a long photoperiod (15L:9D), beginning from September showed partially regressed testes. The pituitary was activated as indicated by changes in the LH-dependent plumage pigmentation, androgen-dependent darkening of the bill, and gonadal volume and histology (Thapliyal and Tewary, 1964; Chandola *et al.*, 1974; Singh and Chandola, 1981). Thus, the tree sparrow's photoperiodic response system is selectively responsive to long day lengths. After fully gonadal recrudescence, they undergo a photorefractory period indicating the end of the breeding season. Photoperiod-dependent regulation of seasonality has been reported in many species (Murton and Westwood, 1977; Farner and Follett, 1979; Wingfield and Farner, 1980; Kumar and Tewary, 1982b, 1983; Tewary and Kumar, 1982; Tewary and Tripathi, 1983; Tewary *et al.*, 1983; Follett, 1984; Tewary and Tripathi, 1985; Gwinner, 1987; Lal and Pathak, 1987; Gwinner *et al.*, 1988, Chaturvedi and Thapliyal, 1979; Maitra, 1987; Kumar and Kumar 1991; Trivedi *et al.*, 2006; Dixit *et al.*, 2011; 2012).

Our results from timed restricted food availability suggest that there is an effect of restricted feeding on body mass and follicle diameter of the female tree sparrows (Fig. 9c). Therefore, restricted food availability suggests the role of food in the photoperiodic induction of physiological processes in tree sparrow. Although these birds were exposed to a highly stimulatory photoperiod (14L:10D), they still lost the body mass. Further, attenuated follicle size was also observed (Fig. 9c). Our

results are consistent with the findings on black-headed bunting. In buntings, the effects of the duration and time-restricted food availability was examined in responses to gain in body fattening and body mass, growth, and development of testes under long stimulatory days. The results suggest that the duration of food supply and/or the time of day at which food is available to affect photoperiodic responses. The loss in body mass and attenuated follicular growth suggest that the time-restricted feeding was unable to meet out the costs of physiological events like lipogenesis and/ or gametogenesis induced by long day lengths. Reduction in the availability of food reduces the storage of extra calories in adipose tissues as fat and subsequently causes a decrease in body mass in migratory species (e.g., spotted flycatcher *Muscicapa striata*; Biebach, 1985; garden warbler Sylvia borin, Gwinner *et al*., 1985; bunting, Kumar *et al*., 2001).

The mechanism involved in the time-restricted food availability influence reproduction is mostly unknown. The involvement of food availability in the synchronization of circadian functions is known (Hau and Gwinner, 1996), which are photo periodically regulated. There may be a close association of light and food cues in the timing of a physiological event, such as the growth and development of gonads. It has been shown in the regulation of the annual testicular cycle of an opportunistic breeder, the red crossbill *Loxia curvirostra* (Hahn, 1995). Along with food availability, at what time of day food is available can also modulate photoperiodic responses. In buntings, when food was available for 8 h, coinciding with the end of the lights-on period had differential effects on different physiology. There was an intermediate effect on fattening and gain in body mass, but the growth and development of the testes were unaffected. However, food-availability restricted to 4 h period at the same phase affected the Food restrictions of 4h effects reduces sex steroid and mesotocin levels, but not general metabolism, as indicated by the absence of a difference in thyroxin and triiodothyronine levels in zebra finches (*Taeniopygia guttata*; Mishra *et al*., 2019a). Further, GnRH-II and TH levels were significantly lower in restricted feeding compared to the group having food ad libitum. Further, GnRH-II and TH- levels were negatively and positively correlated

with egg-laying latency and reproductive success (offspring/brood/pair), of zebra finches (Mishra *et al*., 2019b).

In conclusion, the present findings support the idea that photoperiodism in a seasonally breeding species is affected by light and food availability. Long photoperiods can induce gonadal responses in photosensitive birds but not in photorefractory birds. Food can have direct effects, and restricted food availability can result in loss of body mass and attenuated reproductive responses.

SECTION III: ROLE OF LIGHT SPECTRUM ON REPRODUCTION

1. ABSTRACT

Light is an essential component of the environment and regulates many physiological processes. Three fundamental characteristics of light include duration, intensity, and spectrum. The majority of studies focused on the effects of daylength and light intensity on seasonal regulation of physiological processes. However, the light spectrum could also be critical in the involvement of avian physiology. Light can be classified into ultraviolet light, visible light, and infrared light according to spectral components. The visible range of light spectrum composed of seven colors of the rainbow (VIBGYOR) ranging from violet to red, having a shorter wavelength of 380 nm (violet), and a longer wavelength of 750 nm (red). Differential effects of these spectrum lights have been reported on vertebrates and invertebrate systems. Here we investigated the effects of exposure of photosensitive tree sparrows to long stimulatory days coupled with a long and short wavelength while white light served as control. Change in body mass, bill color, and testicular volume was assessed at a regular interval. We observed the effect of long-wavelength (red light) on body mass, bill color, and testicular growth regression cycle.

In comparison to white light, testicular growth was faster in red light. We did not see any testicular growth in blue light. Our results suggest that long-wavelength (red light) is stimulatory while short wavelength (blue light) in inhibitory for photoperiodic responses in tree sparrows.

2. INTRODUCTION

Light is an essential part of the geophysical environment and controls many physiological processes (Deep *et al*., 2012; Olanrewaju *et al*., 2006). Three components of light include duration (photoperiod), intensity, and color (Kim *et al*., 2013). A number of studies have emphasized the effects of photoperiod and light intensity on the behavior of animals, hormone concentrations, and reproductive performances in birds (Ahmad *et al*., 2011; Busso *et al*., 2013; Molino *et al*., 2015). Light can be classified into ultraviolet light, visible light, and infrared light according to spectral components. The visible range of light spectrum composed of seven colors of the rainbow (VIBGYOR) ranging from violet to red, having the shorter wavelength (380 nm; violet), and the longer wavelength (750 nm; red).

Along with light intensity and duration spectral composition of light can also affect various physiological processes, viz. growth, metabolism, reproduction, and behavior (Dakan, 1934; Scott and Payne, 1937; Ringoen, 1942; Benoit and Otto, 1944; Ishibashi and Kato, 1951; Benoit and Assenmacher, 1966; Morris, 1967; Woodard *et al*., 1968; Harrison *et al*., 1970; Hollwich, 1979; Kumar and Rani, 1996; Kumar *et al*., 2000; Rani *et al*., 2002; Malik *et al*., 2002, 2004; Malik et al., 2014; Renthlei *et al*., 2016; Borah *et al*., 2018). Previous studies have shown that different spectrum of light can influence the behavior (Parvin *et al*., 2014), development (Rozenboim *et al*., 2004; Renthlei *et al*., 2016; Borah *et al*., 2018), age of sexual organs, puberty and sexual maturity (Hassan *et al*., 2013; Baxter *et al*., 2014). The study from black-headed bunting (*Emberiza melanocephala*) suggests that the photoperiodic response system can discriminate between different wavelengths of light and requires a minimum light intensity threshold for photo-stimulation (Malik *et al*., 2014). Wavelength and intensity-dependent photoperiodic regulations of daily and seasonal responses have been documented in the bunting (Malik *et al*., 2014) and circadian behavior in the Indian Weaver bird, *Ploceus philippinus* (Pandey and Bhardwaj, 2011).

Differential effects of these spectral lights have been observed. Red light is found to be stimulatory whereas blue and green light is inhibitory for gonadal growth in the European starling (*Sturnus vulgaris*; Bissonnette, 1932), English

sparrow (*Passer domesticus*; Rington, 1942), duck (Benoit and Otto, 1944), fowl (Ishibashi and Kato, 1951), quail (Oishi and Lauber, 1973), migratory bunting (Kumar and Rani, 1996; 1999), black-headed bunting (*E. melanocephala;* (Malik *et al*., 2004; Kumar *et al*., 2000; Rani *et al*., 2001) and red-headed bunting (*Emberiza bruniceps*; Rani and Kumar, 2000). Response to the LD cycle may vary with the wavelength of the light, and the circadian system appears to be differentially sensitive to light at different times of the day. However, wavelength alone does not determine the photoperiodic responses of gonadal growth and development, as blue light at high irradiance was also photo-inductive, although at lower irradiance, the longer wavelength was inductive and the shorter one (blue) was not inductive (Ishibashi and Kato, 1951). Further, green and blue lights stimulate growth in broilers (Rozenboim *et al*., 2004). Green to blue or green to green-blue mixed lighting at the time of rearing in female broilers improve body and muscle growth and meat quality (Karakaya *et al*., 2009). During embryogenesis, green light enhances the weight gain after hatching of male broilers, increased breast muscle growth, and improves the feed conversion ratio, but do not cause a change in the chemical composition of breast muscle or meat quality (Zhang *et al*., 2012). This knowledge is directly applicable to improve growth and productive performance in broilers (Cao *et al*., 2012). Similarly, a combination of red to green light in comparison to monochromatic red light enhances egg production in laying hens (Hassan *et al*., 2013).

Light spectra can influence growth and development, energy metabolism, and reproductive physiology of tropical damselfish, *Chrysiptera cyanea* (Bapary *et al*., 2011). The green spectral light stimulates the somatic growth of juvenile barfin flounder, *Verasper moseri* (Yamanome *et al*., 2009) and the blue spectral light can have a negative effect on the growth performance of rainbow trout (*Oncorhynchus mykiss*), while the red spectral light can reduce the growth of juvenile gilthead seabream, *Sparus aurata* (Karakatsouli *et al*., 2007). The gonadosomatic index (GSI) is higher in the red light in comparison to white light, and ovarian development is induced in a subtropical sapphire devil, *Chrysiptera cyanea* (Bapary *et al*., 2011) while the maturation of oocyte in grass puffer (*Takifugu niphobles*) can

be accelerated by exposure to the spectrum of green (Choi *et al*., 2018). However, the growth rate of rockfish (*Sebastes inermis*) in the juvenile stage is higher in the green light spectra than red light or the natural light (Shin *et al*., 2015).

The effect of the different spectral light composition is not limited to only growth and development but may influence other behavioral/physiological processes. Removing wavelengths shorter than ~500 nm from broad-spectrum white light suppresses melatonin during nocturnal light exposure (Kayumov *et al*., 2005; Souman *et al*., 2018), prevents alterations in central and peripheral clock gene expression (Rahman *et al*., 2008, 2011) and, results in circadian phase resetting in an irradiance dependent manner (Gladanac *et al*., 2019).

Tree sparrows breed under long stimulatory photoperiod in nature. Here we tested the effects of short (blue light) and long-wavelength (red light) on the testicular growth regression cycle of male tree sparrows.

3. MATERIAL AND METHODS

The study was started in February 2017. Adult male photosensitive tree sparrows were used for this study. Birds (n=21) were divided into three groups and were kept in cages (28.5 x 22.1 x 14 cm). All three groups were subjected to artificial light-dark cycles of the same photoperiod (14L:10D; light on 06:00; light off 20:00) but of different wavelengths of light. Group one was exposed to red light (wavelength; 640 nm), group two was exposed to blue light (wavelength; 450 nm), and group three was exposed to white light and served as control. Light intensity was maintained at 0.7W/m^2 at the perch level. Food (seeds of kakuni, *Setaria italica*, and paddy, *Oryza sativa*), mealworm; *Tenebrio molitor*, cultured in the laboratory), and water was available ad libitum. The light was provided by 14W fluorescent CFL at the top of the cage. An automatic timer regulated the light cycle. The temperature was maintained at 24 ± 2 ^{0}C. Observations on change in body mass, bill color, and testicular size were made on an interval of 30 days.

3.1 Statistical analysis

The data is represented as mean ± SE. One-way analysis of variance (1-way ANOVA), followed by Newman-Keuls post hoc tests if ANOVA indicated the significance of difference, was employed to examine the effect of various treatment over a period of time. Similarly, two-way ANOVA was also applied to analyze effects when two factors (e.g., light quality and duration of the treatment) were taken into consideration, and the Bonferroni test was used as a post hoc test for group comparisons. Significance was always taken at $P < 0.05$.

4. RESULTS

Figure 10 shows change in body weight, bill colour and testicular volume over a period of time under different spectral conditions. There was a significant change in body weight under all spectral conditions (red: $F_{(6,34)}= 2.819$, $P = 0.0246$; blue:

$F_{(6,37)}$= 9.778, P< 0.0010 and white: $F_{(3,35)}$= 3.029, P=0.0142; 1-way ANOVA; Fig.10a). In whilte light maximum body weight was observed at the end of the experiment i.e., on day 180 (Fig. 10a). Birds under blue light initially did not show any change but started losing body weight after day 90 of experiment (Fig. 10a). In red light group higher body mass was recorded on day 120 in comparison to day 0 (Fig. 10a). Two Way ANOVA revealed that there is an effect of spectral treatment, time and interaction of spectral treatment x time on body weight (treatment: $F_{(2,63)}$= 76.63, P< 0.0001; time: $F_{(2,63)}$= 38.53, P< 0.0001; interaction of treatment x time: $F_{(2,63)}$= 47.19, P< 0.0001; Two Way ANOVA; Fig. 10a). There was no difference between body weight of red and whie light group (P > 0.05; Bonferroni's multiple comnparison test; Fig.xx) but in comparison to red and white light there was a significantly reduced (P < 0.05; Bonferroni's multiple comnparison test; Fig. 10a) body weight in blue light on day 120 onwards. As time progressed bill colour became darker in all three groups (red: $F_{(6,34)}$= 9.948, P< 0.0001; blue: $F_{(6,37)}$= 5.935, P= 0.0004 and white: $F_{(3,35)}$= 11.31, P< 0.0001; 1-way ANOVA; Fig. 10a).

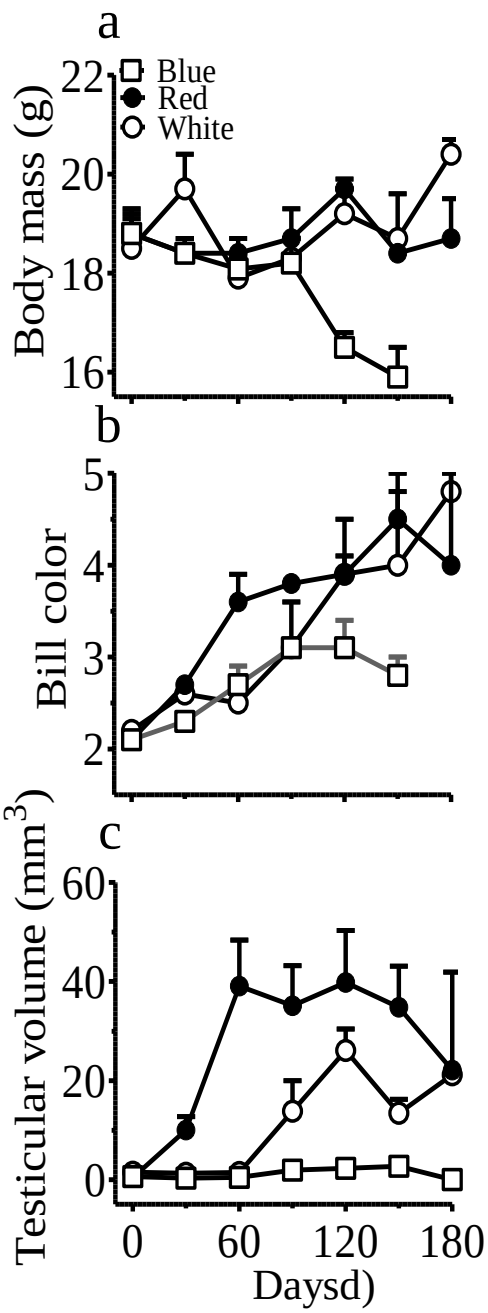

Figure 10: Change in body mass (a), bill color (b) and testicular volume of adult photosensitive male tree sparrows exposed to red, blue and white light conditions for 180 days. Asterisks show significant difference ($P < 0.05$) among the groups at that point of time.

As time progressed there was gain in bill colour in all the groups (red: $F_{(6,34)}$= 9.948, P < 0.0001; blue: $F_{(6,37)}$= 5.395, P = 0.0004 and white: $F_{(3,35)}$= 11.31, P < 0.0001; 1-way ANOVA; Fig. 10b). Two Way ANOVA revealed that there is an effect of spectral treatment, time and interaction of spectral treatment x time on bill color (treatment: $F_{(2,63)}$= 119.8, P< 0.0001; time: $F_{(2,63)}$= 52.63, P< 0.0001; interaction of treatment x time: $F_{(2,63)}$= 79.22, P< 0.0001; Two Way ANOVA; Fig. 10b). Day 60 onwards, the bill color was significantly higher in the red group (P < 0.05; Bonferroni's multiple comparison tests; Fig. 10b) in comparison to blue and white light. On day 120 onwards, there was no difference in bill color of the red and white group, but both of these groups had higher bill scores than the blue group on day 150 onwards till the end of the experiment (Fig. 10b). There was change in testicular volume in all three groups as time progressed (red: $F_{(6,34)}$= 5.077, P= 0.0008; blue: $F_{(6,37)}$= 7,781, P< 0.0001 and white: $F_{(3,35)}$= 16.53, P< 0.0001; 1-way ANOVA; Fig. 10c). Two Way ANOVA revealed that there is an effect of spectral treatment, time and interaction of spectral treatment x time on body weight (treatment: $F_{(2,63)}$= 30.23, P< 0.0001; time: $F_{(2,63)}$= 5.048, P= 0.0003; interaction of treatment x time: $F_{(2,63)}$= 2.654, P= 0.0061; Two Way ANOVA; Fig. 10c). A light spectrum dependent effect was observed on testicular growth (Fig. 10c). Fastest testicular growth was observed in red light, and on day 60, birds attained a peak in testicular volume (Fig. 10c) while in white light, testicular growth was slower. Peak testes volume was achieved by day 120 (Fig. 10c) while there was a slight gain in testicular volume by day 120, but it was not fully grown till the end of the experiment (Fig. 10c).

5. DISCUSSION

Our results suggest that the spectral component of light (wavelength) could be the essential characteristic of light in photoperiodic regulation of physiological responses. The effects of the spectral composition of light on body mass, bill color, and testicular growth regression cycle of male tree sparrows were observed (Fig. 10). Under red and white light, there was a slight gain in body mass, but on the other hand, under blue light, there was no gain but a loss in body mass as time progressed (Fig. 10a). These results suggest that blue light was not strong enough to induce

photoperiodic response for the induction of gain in body mass. Similar results have been observed in migratory black-headed buntings (*Emberiza melanocephala*). When buntings were exposed to skeleton photoperiod, it was noninductive if both of its light periods were of short wavelengths (blue light) while photoperiodic induction took place if they were exposed to both light periods of an SKP was of long wavelengths (red light; Malik *et al*., 2004). Our result from bill color and testicular growth regression cycle also suggests that red light in more inductive in comparison to white and blue light for photoperiodic responses. Maximum and faster testicular growth was observed under red light while no testicular growth regression cycle was observed in blue light, irrespective of that all birds were exposed to long stimulatory photoperiod of 14L:10D. In comparison to red light, responses were slower in white light (Fig. 10c). There is a wavelength-dependent inductive effect of light on the CRPP in the tree sparrows and is consistent with other avian species (Malik *et al*., 2004; Rani and Kumar, 2000; Kumar and Rani, 1996).

The long wavelengths of light (e.g., red light) are photo periodically inductive in many birds (Oishi and Lauber, 1973; Glass and Lauber, 1981; Benoit, 1964; Foster and Follett, 1985; Siopes and Wilson, 1980) although, the deep brain photoreceptors are most sensitive to short-wavelength (around 500, green light; Foster and Follett, 1985). The potential reason could be that the photoperiodic gonadal response is faster in long-wavelength (red light) than in short wavelengths (blue light) of light because of the difference in the number of photons able to cross through the skull and received by the photoreceptors lying deep in the brain (Vriend and Lauber, 1973). As at equal energy levels, the number of photons is larger in long than in short wavelengths and, also, the penetration to brain tissues, thereby access to the photoreceptors of the long-wavelength is far faster and deeper than of the short wavelength. Alternately differential response of different spectral light could be because of the presence of different types of deep brain photoreceptor molecules as reported for various avian species.

Among all the different DBPs identified, Opn5 expression is localized in CSF-contacting neurons while VA-opsin expression is in the anterior and medial

hypothalamus (Halford *et al*., 2009; Nakane *et al*., 2010). In birds, these two opsins are involved in the photoperiodic signal transduction pathway from the beginning with the activation of DBPs to end with the neuroendocrine outputs underlying seasonal reproduction in birds. Information about the photoperiodic environment is detected by the Opn5 neurons in the PVO region and translated via G protein-coupled receptor into a physiological action into the external zone of the ME, juxtaposed to pars tuberalis, PT, site of the thyroid-stimulating hormone-beta subunit (Nakane *et al*., 2010). Tshβ released from PT thyrotrophs binds to Tsh receptors in the ependymal cell lining the ventrolateral walls of the third ventricle (Yoshimura *et al*., 2003). It acts on the ECs within the MBH initiates transcription of type 2 deiodinase (*Dio2*) and suppresses type 3 deiodinase (*Dio3*) via the TSH receptor signaling pathway (Nakao *et al*., 2008). *Dio2* encodes thyroid hormone (TH) activating enzyme, while *Dio3* is responsible for encoding the TH inactivating enzyme. This TH induces structural changes between the GnRH nerve terminals and the glial endfeet in the median eminence (ME) (Yamamura *et al*., 2006) and modulates seasonal gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus to the portal capillary system which leads downstream processes at gonadal levels (Yoshimura *et al*., 2003; Yamamura *et al*., 2006). The simultaneous decrease in the transcription of a gene coding for gonadotropin-inhibitory hormone (GnIH) suggests its involvement in seasonal reproduction via the inhibition of GnRH activity (Tsutsui *et al*., 2000). Therefore, it's a high probability that under different spectral composition, these deep brain photoreceptors get activated, and as a response, differential growth in gonads takes place. Whether similar types of deep brain photoreceptor molecules are present in tree sparrows needs to be investigated.

In conclusion, our results confirm that a long wavelength of light (red light) is highly stimulatory. In contrast, the short-wavelength (blue light) is inhibitory for the induction of the photoperiodic responses in tree sparrows.

SECTION IV: EFFECT OF TEMPERATURE ON REPRODUCTION

1. ABSTRACT

The majority of birds use environmental cues to time their reproduction. Photoperiod is the most dominant cue, but other environmental factors may play a critical role in successful reproduction. Here we tested if the temperature can modulate the reproductive responses of tree sparrows. Two experiments were performed on adult male birds. In experiment 1, birds (n=5/group) were exposed to either high (30 ± 2 ^{0}C) or low temperature (20 ± 2 ^{0}C). Change in body mass, bill color, and testes volume was recorded every 30 days for 120 days. In experiment 2, a similar temperature protocol was followed, but birds were sacrificed after 30 days. In experiment 2 immediately, after sacrificing birds, blood samples were collected, serum was used for hormone assay, of serum CORT, T_3, and T_4 levels. mRNA levels of thyroid-stimulating hormone-β (*Tshβ*), type 2 deiodinase (*Dio2*), type 3 deiodinase (*Dio3*), gonadotropin-releasing hormone (*GnRH*), and gonadotropin inhibitory hormone (*GnIH*) were measured. Results from experiment 1 show that high temperature attenuates the testicular responses and accelerates the timing of regression. Experiment 2 shows that on day 30, there is no difference in body weight, bill color, and testicular volume of the birds of two groups, but serum CORT and T_4 levels were significantly higher in the high-temperature group while T_3 levels were higher in the low-temperature group. Together, these findings suggest that high temperature modulates reproductive responses of tree sparrow.

2. INTRODUCTION

Along with the expansions of cities, urbanization is increasing drastically and leading to a change in the environment. One of the significant changes in the environment is an increase in temperature (global warming) and has affected the biological systems (Walther *et al*., 2002). During the past few decades, environmental change has led to change in phenological shifts in events across trophic levels (Parmesan and Yohe, 2003; Root *et al*., 2003; Thackeray *et al*., 2010).

The most evident effect of urbanization is in the alterations of phenology or timing of seasonal events (Parmesan and Yohe, 2003; Brown *et al*., 1999; Crick *et al*., 1997). Birds are one of the best-known environmental indicators. The mechanism of seasonal avian reproduction was deciphered for the first time in Japanese quail (*Coturnix coturnix japonica*). The ependymal cells (ECs) of the ventrolateral walls of the third ventricle within the mediobasal hypothalamus (MBH) and pars tuberalis (PT) of the anterior pituitary gland are neuronal centers to regulate reproduction (Yoshimura *et al*., 2003; Nakao *et al*., 2008). Under long stimulatory photoperiod, PT secretes thyroid-stimulating hormone (TSH, thyrotropin), and it acts on the ECs within the MBH initiating transcription of type 2 deiodinase (*Dio2*) and suppressing type 3 deiodinase (*Dio3*) via the TSH receptor signaling pathway (Nakao *et al*., 2008). *Dio2* involves encoding thyroid hormone (TH) activating enzyme while *Dio3* is involved in encoding the TH inactivating enzyme. This TH results in structural changes between the GnRH nerve terminals and the glial endfeet in the median eminence (ME) (Yamamura *et al*., 2006). It modulates gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus to the portal capillary system (Yoshimura *et al*., 2003; Nakao *et al*., 2008) and leads downstream processes at gonadal levels. On the other hand, there is a decrease in the transcription of a gene coding for gonadotropin-inhibitory hormone (GnIH) and inhibits GnRH activity (Tsutsui *et al*., 2000; Chowdhury *et al*., 2010; Surbhi *et al*., 2015; Ubuka *et al*., 2008). These molecules have also been shown to be up or down-regulated depending photoperiod and physiological state of the avian species (Mishra *et al*., 2017; Dixit and Byrsat, 2018; Zhang *et al*., 2019; Trivedi *et al*., 2019; Majumdar *et al*., 2015).

Most of the avian species breed seasonally (Kumar, 1997; Renthlei and Trivedi, 2017) and use environmental cues to time their seasonal reproduction (Dawson *et al*., 2001). This actual timing of breeding is crucial for birds as hatching must be synchronized with a narrow window of maximal food abundance for the best chances of survival of offspring. Daylength is the proximate factor used to time reproduction linked phenomenon (Kumar, 1997), but there can be considerable inter-annual variability in the timing of breeding behavior of a given population

within or between the habitats (Brown *et al.*, 1999; Both *et al*., 2007; Sanz *et al*., 2003; Visser *et al*., 2006; Thomas *et al*., 2010). As photoperiod is always fixed at a given latitude/altitude, this annual variation could be due to change in other factors (ultimate factors). These secondary factors provide supplementary but critical information to allow birds to more closely synchronize the timing of breeding (Visser *et al*., 1998; Both *et al*., 2006; Wilson *et al*., 2004; Budki *et al*., 2008; Perfito *et al*., 2008: Caro *et al*., 2013). The effect of daylength in the regulation of seasonal reproduction of tree sparrows is known. Tree sparrows (*Passer montanus*) breed under long stimulatory photoperiod (Dixit and Singh, 2011; 2012; Zhang *et al*., 2019). What could be the effects of altered environmental temperature on the mechanism of the reproduction of tree sparrow is mostly unknown. Previously, at the physiological level, it has been shown that temperature, coupled with long photoperiod, delays the gonadal regression at a lower temperature (17 ^{0}C; Dixit *et al*., 2018). But the mechanism, involved in the processes is not known. Further, is it the species-specific phenomena or local environmental conditions can modulate the effects of temperature is in question. To address these questions, we studied the reproduction and linked phenomena and the candidate genes known to be involved in the seasonal reproduction in the tree sparrow under two different temperature conditions.

3. MATERIALS AND METHODS

3.1 Animals and Tissue Collection

Birds were procured locally using mist-net during late January when these birds are in photosensitive stage. These birds were maintained in indoor aviary under non-stimulatory photoperiod (8L:16D; 8h light 16hdark/24h). Laparotomy (Kumar *et al*., 2001) was done for selecting the male birds before the experiment. Food (kakuni, *Setaria italica*, and Asian rice, *Oryza sativa*) and water were available ad libitum during experiments. Two experiments were performed. In experiment one, birds (n = 5 per group) were divided into two groups and exposed to long photoperiod 14L:10D (14h light and 10h dark) but two different temperature conditions, i.e., high temperature (30 ± 2 ^{0}C) and low temperature (20 ± 2 ^{0}C). Change in body mass, bill color, and testis size was recorded at the interval of 30 days until the end of the study. In experiment two, birds received similar treatment as of experiment 1; but were sacrificed during the middle of the day after 30 days of long photoperiod and high (30 ± 2 ^{0}C) or low (20 ± 2 ^{0}C) temperature treatment. Blood was collected; serum was harvested and stored at -20 ^{0}C until assayed for serum corticosterone, thyroxine, and tri-iodothyronine. Hypothalamus was collected from freshly dissected-out brains in RNA later solution (Thermo Fisher Scientific, USA, AM 7020) and first kept at 4 ^{0}C overnight and then stored at -80 ^{0}C.

3.2 Hormone assay

Serum corticosterone (CS), thyroxine (T4) and tri-iodothyronine (T3) levels were measured using chicken specific immune assay enzyme kits (KINESISDx cat No K16-0020, 0021 and 0016, respectively) following manufacturer's protocol. First, serum samples were allowed to thaw on ice. Standards were diluted using provided standard concentration and standard diluents as per protocol. 50 µL of standards and 40 µL samples were transferred into respective wells of 96 well plates provided, followed by 10 µL of Biotin conjugate into each sample well. 50 µL of HRP conjugate was added into each sample and standards well and incubated for 1hr at 37 °C. Then, washed four times with 1X Wash Buffer and the residual buffer were blotted by firmly tapping the plate upside down on paper towels. Substrate A 50 µL,

and Substrate B 50 µL were added to each well, including blank. The plate was covered with aluminum foil and incubated for 10 min at 37 °C in the dark. After that, 50 µL of Stop Solution was added, and the absorbance was taken at 450 nm with a SpectraMax M2e (Molecular devices, USA) within 15 minutes after adding the Stop Solution.

3.3 Gene transcription:

mRNA transcription of genes coding for *Tshβ*, *Dio2*, *Dio3*, *GnRH*, and *GnIH* were measured in the hypothalamus of each individual as described previously (Renthlei and Trivedi, 2019; Renthlei *et al*., 2019). Primer sequences used are mentioned in Table 1 and were based on previously published studies.

3.4 RNA Isolation, cDNA Synthesis and quantitative (Real-time) RT-PCR (qPCR)

Tri reagent solution (Ambion AM9738; USA) was used to extract RNA. 1-µg RNA (quantified on ND-ONE NanoDrop one spectrophotometer; ThermoFisher Scientific, USA) was used to prepare cDNA. RQ1 DNase (Promega M6101; Wisconsin, USA) kit was used to remove genomic DNA contamination. cDNA was synthesized using the first-strand cDNA synthesis kit (Thermo Scientific, K1622; Lithuania, Europe). A Quant-Studio 5 (Applied Biosystem by Thermo Fisher Scientific; USA) machine was used for qPCR amplification as detailed in general material and methods. 18S was used as a reference gene. The ΔΔCt method (Livak and Schmittgen, 2001) was followed to determine the relative transcription of genes.

3.5 Statistical analysis

All statistics and graphs were prepared using Graph pad prism 6.0. Data are presented as mean (mean and SE). The student t-test was used to compare two point values. The significance of difference within the groups was tested using a one-way analysis of variance (1-way ANOVA) followed by post hoc test (Newman-Keuls). To determine the significance of the difference between the groups comparing two factors together (high vs. low temperature and time), Two-way analysis of variance (2-way ANOVA) followed by post-test (Bonferroni) was applied. Significance was always taken at $P < 0.05$.

Gene	*F primer*	*R Primer*	*Accession No.*
Tshβ	F: 5-CCA CCA TCT GCG CTG GAT-3	R: 5-GCC CGG AAT CAG TGC TGT T-3	NM205063
Dio2	**5'–CCT ACA AGC AGG TCA AAC TGG –3'**	**5'–ACT TGC CAC CAA CGT TCT TC–3'**	KX951490.1
Dio3	**5'–CCT ACA ACA TCC CCA AGC AC–3'**	**5'–AAG CAT TGT CCA TGG TGT CC–3'**	KX951491.1
GnRh	F: 5-ATC GCA AAC GAA ATG GAA AG-3	R: 5-CTG GCT TCT CCT TCG ATC AG-3	NM204321
GnIh	**5'–CCA GGA GGT GCA AAT GA–3'**	**5'–CTT GTT CTT CCA TCA GCC T–3'**	KT351598.1
18S	5' –GAC GCG TGCATT TATCAG**3'**	**5'** GTT GAT AGG GCA GAC ATT **3'**	D38344.1

Table1: Sequences of gene specific primers used for qPCR analysis.

4. RESULTS

Experiment 1: There was only an effect of time ($F_{4,49}$ = 13.58, P < 0.0001; 2-way ANOVA; Fig 11a) and interaction of time and temperature ($F_{4,49}$ = 6.450, P = 0.0003; 2-way ANOVA; Fig. 11a) but not of temperature alone (temperature: $F_{1,49}$ = 1.666, P = 0.2028; 2-way ANOVA; Fig. 11a) on change in body mass. Minimum body mass was recorded on day 60 of experiment (Fig. 11a). An effect of time, treatment of temperature and interaction of two factors was observed on bill color (time: $F_{4,49}$ = 19.85, P < 0.0001, temperature: $F_{1,49}$ = 5.975, P = 0.0183; interaction of habitat and time: $F_{4,49}$ = 5.097, P = 0.0017; 2-way ANOVA; Fig. 11b). Bill color score was low during the beginning of study and maximum score (darkest bill color) was observed on day 90 of experiment (Fig. 11b). However, drop in bill color was slower in low temperature treatment birds and bill color score of low temperature treated birds had higher score than high temperature treated birds on day 120 (Fig. 11b). Temperature treatment affected the testicular growth regression cycle (Fig. 10c). High temperature treated birds showed attenuated testicular growth (temperature: $F_{1,49}$ = 6.101, P = 0.0178; time: $F_{4,49}$ = 8.5583, P = 0.0031, interaction of habitat and time: $F_{4,49}$ = 0.4124, P = 0.0162; 2-way ANOVA; Fig. 10c). Testicular growth in high temperature group was significantly less on day 60 and 90 of experiment (Fig. 11c); P < 0.05; Bonferroni test). Further, testicular regression was also faster in high temperature treated birds and on day 120 high temperature birds had fully regressed testes (2.2 ± 1.1 mm^3) while in low temperature treated birds were not fully regressed (17.3 ± 0.8 mm^3).

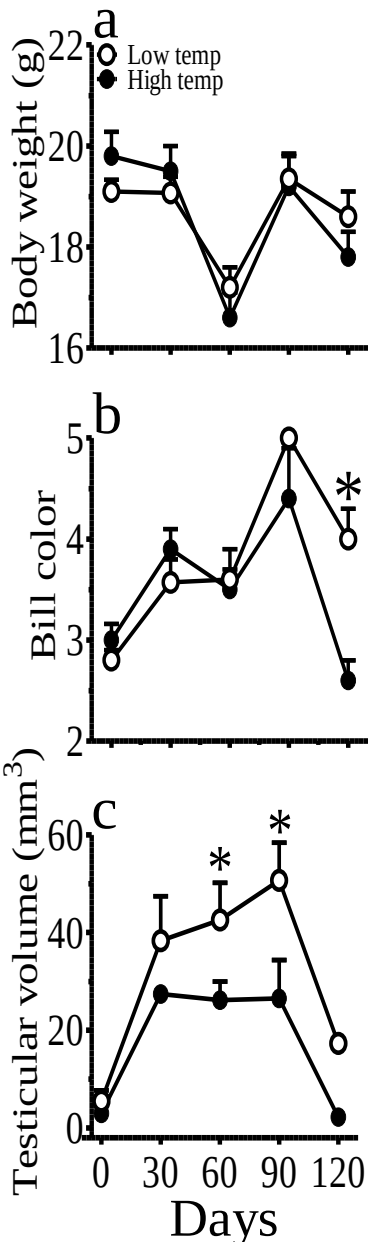

Figure 11: Change in body mass (a), bill color (b) and testicular volume of adult photosensitive male tree sparrows exposed to high (30 ±2 0C) and low temperature (30 ±2 0C). Asterisks show significant difference ($P < 0.05$) between the groups at that point of time.

In experiment 2, there was no change in body weight (temperature: $F_{1,19}$ = .4974, P = 0.4892; time: $F_{1,19}$ = 0.0021, P = 0.9636, interaction of habitat and time: $F_{1,19}$ = 3.844, P = 0.0648; 2-way ANOVA; Fig. 12a) and bill color (temperature: $F_{1,19}$ = 4.054, P = 0.0584; time: $F_{1,19}$ = 0.0631, P = 0.9373, interaction of habitat and time: $F_{1,19}$ = 0.0039, P = 0.9843; 2-way ANOVA; Fig. 12b) on day 30 of experiment. However, in testicular volume there was an effect of time alone (temperature: $F_{1,19}$ = 1.303, P = 0.2694; time: $F_{1,19}$ = 17.49, P = 0.0006, interaction of habitat and time: $F_{1,19}$ = 0.3230, P = 0.5772; 2-way ANOVA; Fig. 12c) and high testicular volume was observed in both groups on day 30. On day 30 CS, T_3, and T_4 levels differed between high and low-temperature groups ((Fig. 12d-f). CS levels were significantly higher (P = 0.0008; Student t-test; Fig. 11d) in high temperature group. Serum T_3 levels were significantly higher (P = 0.0009; Student t-test; Fig. 12e) while T_4 levels were significantly lower (P = 0.0151; Student t-test; Fig. 12f) in low temperature group. Treatment dependent effects on candidate genes were observed. After 30 days of respective treatment, mRNA levels of *Tshβ* (P = 0.0005; Unpaired T-test; Fig. 13a), *Dio2* (P = 0.0077; Unpaired T-test; Fig. 13b) and *GnRH* (P = 0.0113; Unpaired T-test; Fig. 13d) were significantly higher in low temperature treated groups while mRNA levels of *Dio3* (P = 0.0375; Unpaired T-test; Fig. 13c) and *GnIH* (P = 0.0427; Unpaired T-test; Fig. 13e) were significantly lower in low temperature treated groups.

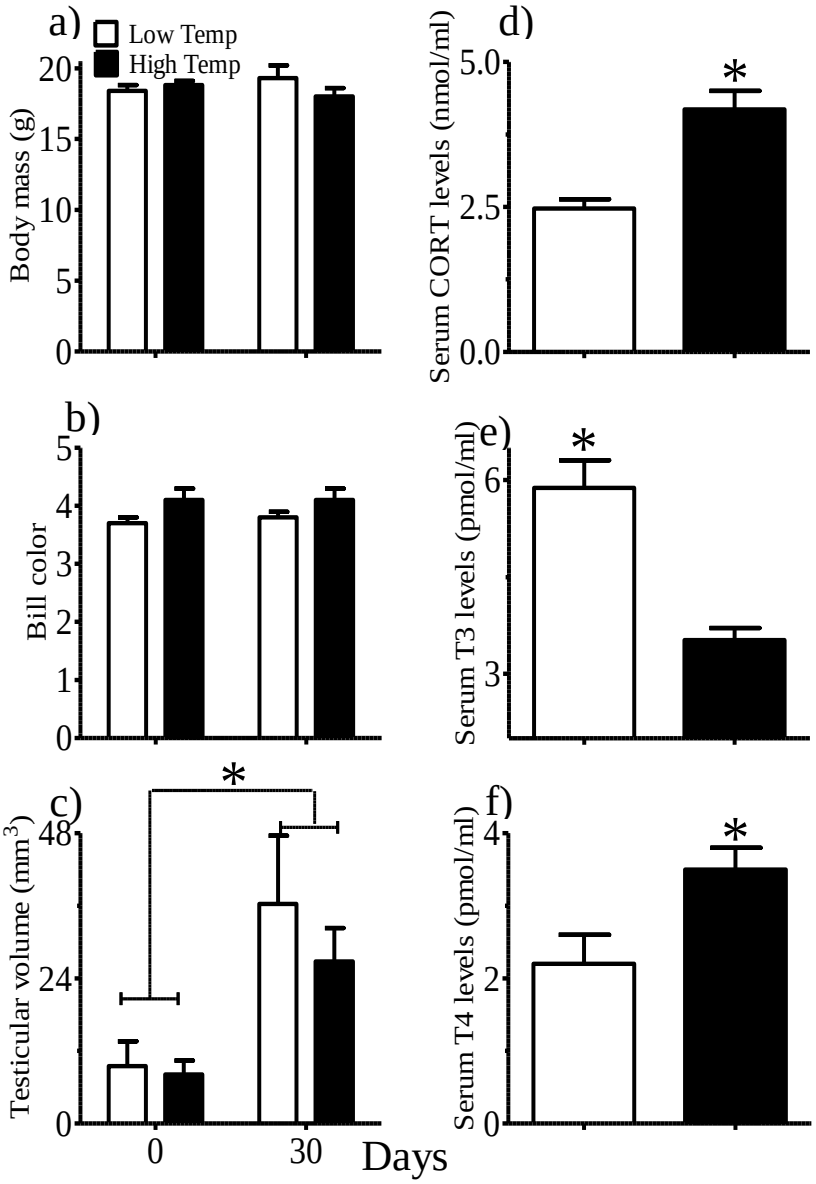

Figure 12: Mean (±SE) body mass (a), bill colour (b), and testis volume (c) of tree sparrows under low temperature (hallow circle) and high temperature (solid circle) after 30 days of treatment. No change in body mass (a) and bill colour (b) was observed after 30 days. High testicular volume (c) was observed in both the groups on day 30. Change in serum CORT (d), tri-iodothyronine (T3) and thyroxine (T4) levels on day 30. High CORT and T4 level and low T3 levels were observed in high temperature group. Asterisks show significant difference ($P < 0.05$) in the response between the two conditions (d, c and e) while only effect of time in testes.

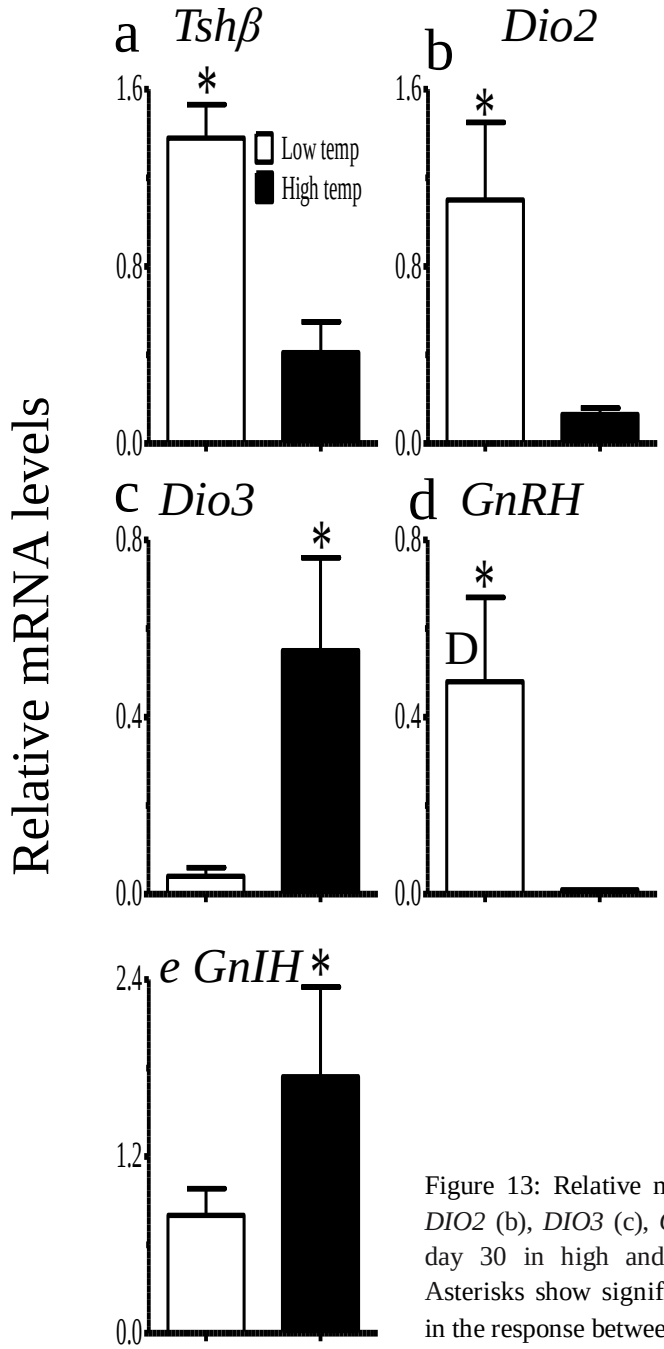

Figure 13: Relative mRNA levels of *TSH*β (a), *DIO2* (b), *DIO3* (c), *GnRH* (d) and *GnIH* (e) on day 30 in high and low temperature groups. Asterisks show significant difference ($P < 0.05$) in the response between the two conditions.

5. DISCUSSION

Our study shows the effect of temperature on testicular growth regression cycle and transcription levels of associated reproductive genes. Response specific effects of temperature were observed in this study. Under long stimulatory photoperiod of 14L:10 D, we do not see any temperature-dependent effect on body mass as no difference in body mass was observed between two groups (low temperature vs. high temperature). On the other hand, high temperature (30 °C) affected the testicular growth regression cycle (Fig. 11). Attenuated testicular growth and faster testicular regression were observed in the high-temperature group (30 °C) than 22 °C (Fig. 11). Experiment 2 was conducted to see early effects (when a response is not reflected at gonadal levels) of temperature treatment on candidate molecules involved in seasonal reproduction. After 30 days of respective temperature treatment we observed change in CS, T_3 and T_4 levels and transcript levels of *Tsh*β, *Dio2*, *Dio3*, *GnRH* and *GnIH* in both the groups where higher levels of serum T_3 and transcript levels of *Tsh*β, *Dio2* and *GnRH* were upregulated while T_4 and transcripts of *Dio3* and *GnIH* were downregulated in a low-temperature group. Further, corticosterone levels were significantly higher in the high-temperature group. Our results are consistent with the role of these transcripts in seasonal breeders (Yoshimura *et al*., 2003; Nakao *et al*., 2008; Yamamura *et al*., 2006 Tsutsui *et al*., 2000; Chowdhury *et al*., 2010; Surbhi *et al*., 2015; Ubuka *et al*., 2008; Mishra *et al*., 2017; Dixit and Byrsat, 2018; Zhang *et al*., 2019; Trivedi *et al*., 2019; Majumdar *et al*., 2015). Similar response specific effects of temperature have been observed in black-headed buntings (Singh *et al*., 2011). In black-headed bunting, the temperature had a differential impact on the fat deposition and testicular recrudescence where under high-temperature buntings did not achieve full fat deposition but attained full testicular maturation. Still, there was a shortened reproductive active phase, delayed maturation, and advanced regression of the testes (Singh *et al*., 2011). Our observations on the tree sparrow contrast with previous reports on the effects of temperature on the gonadal cycle of this species (Dixit *et al*., 2018). In the previous report under three different temperature conditions (17 ^{0}C, 25 ^{0}C, and 30 ^{0}C) under stimulatory photoperiod, gonadal growth regression cycles were observed at all the

temperatures, but the birds had greater growth rate at higher temperatures (25 and 30 °C) compared to the lower temperature (17 °C) (Dixit *et al*., 2018). The difference between the two studies could be due to differences in the temperature used. In our study, we used two temperature conditions; a low temperature of 20 ± 2 ^{0}C and a high temperature of 30 ± 2 ^{0}C while Dixit et al. used a lower temperature of 17 ^{0}C and a high temperature of 30 ^{0}C. So, in our study, the lower temperature used (20 ± 2 ^{0}C) was not as low as of other studies. Further, in their study, they also observed that in comparison to 30 ^{0}C, birds exposed to 25 ^{0}C achieved the gonadal peak earlier, suggesting that a high temperature of 30 ^{0}C is delaying the timing of gonadal progression (in comparison to 25 ^{0}C). Some other studies also suggest that temperature modulates timing of gonadal growth regression cycle as reported for the white-crowned sparrows and European starlings where the accelerated rate of photoperiodic induction at higher temperatures was observed (Wingfield *et al*., 2003; Dawson, 2005).

We do know the exact mechanism involved in the temperature-dependent seasonal reproduction (gonadal growth regulation cycle) but will discuss the possibilities of the mechanism of action of temperature. One of the possibilities is that continuous exposure to high temperature acts as a stressor to gonadal induction as reflected by higher CS levels in a high-temperature group (experiment 2; Fig. 12d). Corticosteroids suppress the activity of the hypothalamic–pituitary–gonadal axis. Chronic administration of corticosteroids leads to the inhibition of the pulsatile release of GnRH from the hypothalamus (Oakley *et al*., 2009). Corticosteroids reduce the responsiveness of pituitary cells to GnRH (Li and Wagner, 1983), which in turn, result in a reduction of the amplitude of the luteinizing hormone (LH) response. Corticosteroids itself can act directly on the gonads to suppress reproduction by reducing androgen synthesis by the testes (Welsh *et al*., 1982).

Furthermore, corticosteroid administration can inhibit breeding in animals (Rivier and Rivest, 1991; Salvante and Williams, 2003; Wingfield and Sapolsky, 2003; Spée *et al*., 2011). Another possibility is that temperature response for this species is habitat-specific, and therefore we see the difference in the gonadal response of tree sparrows from the previous study (Dixit *et al*., 2018). Habitat

dependent effects of temperature have been shown by song sparrow (*Melospiza melodia morphna*), where the mountain population shows a relatively stronger effect of temperature on the reproductive phenology than in the coastal population (Perfito *et al*., 2005). Altogether our findings suggest that in comparison to 20 ± 2 ^{0}C, 30 ± 2 ^{0}C suppresses the gonadal growth and reproduction linked molecules in tree sparrows.

In conclusion, our results strongly support the idea that the temperature cues exhibit a significant effect on photoperiodic regulation of seasonal reproduction. In tree sparrows, high temperature (30 ± 2 ^{0}C) can exert direct effects on the timing and amount of gonadal growth and development. Our results also indicate that the after-effects of temperature are strong enough to influence the activity of the reproductive axis under long days, which is further supported by the molecular data of transcript levels of *Tsh*β, *Dio2*, *Dio3*, *GnRH* and *GnIH*. To sum up, the current findings suggest that along with the photoperiod temperature is critical for seasonally breeding trees sparrows, and high temperature might potentially affect reproduction.

SECTION V: STUDY OF DAILY RHYTHMS IN CLOCK GENES

1. ABSTRACT

Almost all organisms live in a fluctuating environment. To achieve synchrony with the fluctuating environment, organisms have evolved with time tracking mechanism commonly known as biological clocks. This circadian clock machinery has been identified in almost all cells of vertebrates and categorized as central and peripheral clocks. In birds, three independent circadian clocks reside within the nervous tissues in the hypothalamus, pineal, and retina, which interact with each other and produce circadian time at a functional level. There is limited knowledge available of the molecular clockwork, and integration between central and peripheral clocks in birds. Here we studied the daily expression of canonical clock genes (*Bmal1*, *Clock*, *Per2*, *Per3*, *Cry1*, and *Cry2*) and clock-controlled gene (*Npas2*) in all three central tissues (hypothalamus, pineal, and retina) and peripheral tissues (liver, intestine, and muscle). Wild-caught adult male tree sparrows were exposed to equinox photoperiod (12L:12D) for two weeks, and after that, birds were sacrificed (N=5 per time point) at six-time point (ZT1, ZT5, ZT9, ZT13, ZT17, and ZT21; ZT0 is lights on). Daily expression of clock genes was studied using qPCR. *Bmal1*, *Clock*, *Per2*, *Per3*, *Cry1*, *Cry2*, and *Npas2* showed daily oscillation in all tissues except *Cry2* in the hypothalamus, pineal, and intestine. We observed tissue-specific expression patterns for all clock and clock-controlled genes. *Bmal1* transcripts expressed during the early phase of the night. *Clock* acrophase was observed during the middle or late day time in the central clock while during the early to the middle phase of the night in peripheral tissues. *Npas2* expression pattern was similar to *Bmal1*. *Per* genes peaked either late at night or early during day time. However, *Cry* genes were peaked either at late day time (*Cry1*in retina, liver, and intestine; *Cry2* in the liver and intestine) or early night phase (*Cry1* in hypothalamus, pineal, and muscle; *Cry2* in hypothalamus, pineal, retina, and muscle). Our results are consistent with the autoregulatory circadian feedback loop and suggest a conserved tissue-level circadian time generation in tree sparrows. Change in peak expression timing of these genes in different tissues implicates tissue-specific contribution of individual clock genes in the circadian time generation.

2. INTRODUCTION

Almost all organisms live in an environment, which is fluctuating daily as well as seasonally. To better adapt to a fluctuating environment, organisms need to keep track of time so that they perform daily and seasonal processes at the best time as a mismatch may have fitness consequences. To govern daily and seasonal cycles in their physiology and behavior, most vertebrates exhibit circadian (*circa* = about, *dian* = day) and circannual (*circa* = about, *annum* = year) clocks (Kumar *et al*., 2010), respectively. In mammals, the suprachiasmatic nucleus (SCN) of anterior hypothalamus functions as a circadian pacemaker and regulates many behavioral and physiological functions (Rusak and Zucker, 1979; Welsh *et al*., 1995). However, in non-mammalian vertebrates, circadian pacemakers are not confined at one place but are distributed at more than one place within nervous tissues. In birds, at least three separate circadian oscillators have been attributed, which reside within the hypothalamus, pineal, and retina of the nervous tissue. These circadian pacemakers are photoreceptive**,** interact with each other and with the external environment to produce timing at the functional level (Gwinner and Brandstatter, 2001; Kumar *et al*., 2004). However, the degree of self-sustainment and contribution to circadian time generation for these three circadian oscillators can vary among avian species (Cassone and Menaker, 1984; Gwinner and Brandstatter, 2001; Kumar *et al*., 2004).

A vertebrate circadian clock exhibits a transcriptional-translational feedback loop (TTFL). The genes designated as clock genes are arranged in an interacting positive (circadian locomotor output cycles kaput, *Clock*/ neuronal PAS domain protein 2, *Npas2* and Brain and muscle arnt like protein 1, *Bmal1*) and negative (period, *Per* and cryptochrome, *Cry*) limbs (Reppert and Weaver, 2002). Several other genes are also involved in stabilizing TTFL (Yin *et al*., 2010). These circadian genes are present in almost all body cells and exhibit differences in phase and amplitude with the central tissues suggesting tissue-level timekeeping in vertebrates (Yamazaki *et al*., 2000; Abraham *et al*., 2002; Yasuo *et al*., 2002; Karaganis *et al*., 2008; Singh *et al*., 2013, 2015; Trivedi *et al*., 2016).

Most of the knowledge of circadian clock genes comes either from mammalian (rat and mice) or from insect (*Drosophil*a) models. However, in the past

one and half decades, the avian orthologs of canonical and clock-controlled circadian genes have also been sequenced in few species. Still, most of the knowledge of avian clock genes have been limited to certain species; particularly chicken (*Gallus domesticus*; Bailey *et al*., 2003, 2004; Doi *et al*., 2001; Karaganis *et al*., 2008, 2009; Jiang *et al*., 2017), zebra finches (*Taenopygia guttata*; Pinzon-Rodriguez *et al*., 2018), Japanese quail (*Coturnix Coturnix. japonica*; Yasuo *et al*., 2003; Yoshimura *et al*., 2000), house sparrow (*Passer domesticus*; Abraham *et al*., 2002, 2003; Brandstatter *et al*., 2001; Helfer *et al*., 2006) and buntings (*Emberiza species*; Singh *et al*., 2013; 2015; Trivedi *et al*., 2016). Further, most of these studies have been focused on the expression of the clock genes within the nervous tissues. For example, *Per2* mRNA expression is determined in the eye, pineal, and hypothalamus of quail (Yasuo *et al*., 2003; Yoshimura *et al*., 2000) in pineal and retina of chicken (Bailey *et al*., 2003, 2004; Doi *et al*., 2001; Karaganis *et al*., 2008) and in the hypothalamus of house sparrow (Abraham *et al*., 2002; Helfer *et al*., 2006). On the other hand, *Bmal1*, *Clock* and cryptochromes (*Cry1* and *Cry2*) oscillations have been determined in the hypothalamus of quail and sparrow, in the retina of chicken and quail and in the pineal gland of chicken and quail (Bailey *et al*., 2003, 2004; Doi *et al*., 2001; Haque *et al*., 2002; Karaganis *et al*., 2008; Yasuo *et al*., 2003; Yoshimura *et al*., 2000). In some studies, *Cry1* and *Cry2* oscillations have also been reported in liver, skin, muscle, heart, testes, and lung of Japanese quail and chicken (Fu *et al*., 2002; Karaganis *et al*., 2009). In migratory species (*Emberiza bruniceps* and *Emberiza melanocephala*), the oscillation of clock and clock-controlled genes have been shown both in central and peripheral tissues (Singh *et al*., 2013, 2015; Trivedi *et al*., 2016).

Comparing the diversity of Class Aves still there is a scarcity of knowledge of molecular clockwork and its relationship within and among the tissues. In the present study, we aimed to investigate oscillation of the canonical clock genes (*Bmal1*, *Clock*, *Per2*, *Per3*, *Cry1*, and *Cry2*) and clock-controlled gene (*Npas2*) in the central (hypothalamus, pineal, and retina) and peripheral (liver, intestine, and muscle) tissues of songbird, tree sparrow (*Passer montanus*) exposed to equinox photoperiod (12L:12D) under laboratory conditions. We studied the 24-h mRNA

levels of circadian clock genes. Further, we determined the phase relationship in the expression of clock genes within the tissue and among the tissues.

3. MATERIALS AND METHODS

3.1 Animals and Tissue Collection

The study was performed on adult male tree sparrows (*Passer montanus*). Birds were procured locally (Aizawl, Mizoram, 23^0N, and 92^0E) using mist nets during the first week of September 2017. Initially, birds were kept in cages (3 x 2.5 x 3 fit, four birds per cage) and acclimatized under NDL (natural day length conditions) conditions for one week before the beginning of the experiment. The experiment started during the third week of September 2017. After acclimatization, birds were brought indoor photoperiodic chambers (2 x 2 x 2 fit, 3 birds per cage) and exposed to equinox photoperiod 12L:12D (12h light:12h darkness) for two weeks. Food and water were provided ad libitum, and the temperature was maintained at 22 ^{0}C. On the day15th, birds (N=5 per time point) were sacrificed by decapitation at six times: ZT1, ZT5, ZT9, ZT13, ZT17, and ZT21 (lights on = ZT0, zeitgeber time 0). The brain, retina, liver, gut, and muscle tissues were immediately removed and collected in RNA later solution (Thermo Fisher Scientific, USA, AM 7020) and kept at 4 ^{0}C overnight and then stored at -80 ^{0}C till RNA was extracted. Later, the pineal was harvested by removing meninges from the brain and hypothalamus was excised out from respective brains by slicing the brain using a surgical blade in such a way that a thick coronal section trimmed dorsally (starting approximately at TrSm) and laterally extends from the optic chiasma to infundibular area.

3.2 RNA Isolation, cDNA Synthesis

Tri reagent (Ambion AM9738; USA) was used to extract total RNA. Extracted RNA was quantified using a spectrophotometer (Eppendorf Biophotometer Plus), and 1-µg RNA were used to make cDNA. To remove the genomic contamination RQ1 DNase (Promega M6101; Wisconsin, USA) kit was used. cDNA was synthesized by the first-strand cDNA synthesis kit (Thermo Scientific, K1622; Lithuania, Europe).

3.3 Quantitative (Real-time) RT-PCR (qPCR)

Gene-specific primers (Table 2) were designed from the sequences using Primer 3 plus online primer design program. qPCR was performed on Quant-Studio 5

(Applied Biosystem by Thermo Fisher Scientific; USA). PCR reaction volume of 7 µL for each gene comprised 1 µL each of cDNA, gene-specific forward and reverse primers, 3 µL Power Up^{TM} SYBR Green Master Mix (Applied Biosystem by Thermo Fisher Scientific; US, A25742) and 1µL of nuclease-free water (Ambion, AM9938). qPCR cycle conditions included 1 cycle at 95 ^{0}C (20 sec), 35 cycles at 95 ^{0}C (01 s), 60 ^{0}C (20 s), 95 ^{0}C (01 s), additional melt curve plot step included 1 cycle of 60 ^{0}C (20 s) and one cycle of 95 ^{0}C (01 s). We used the 18S gene as a reference gene for determining the relative expression levels of specific target genes. We ran each sample in duplicate along with non-template and negative RT controls. The relative expression of genes was determined by the $\Delta\Delta C_t$ method (Livak & Schmittgen, 2001).

3.4 Statistical analysis

One-way analysis of variance (one-way ANOVA) followed by Newman-Keuls post hoc test was used to determine the significance of difference among the six-time points of the daily profile. To test the daily variation, we performed cosinor analyses based on unimodal cosinor regression [y=A+(B.cos(2π(x-C)/24))], where A, B, and C denote the mean level (mesor), amplitude, and acrophase of the rhythm, respectively (Cuesta *et al*., 2009). Statistical analysis was done using graph pad prism version 5. Further, the significance of regression analysis was calculated using the number of samples, R^2 values, and numbers of predictors (mesor, amplitude, and acrophase; Soper, 2013; http://www.danielsoper.- com/statcalc3/calc.aspx?id 1⁄4 15).

Gene	F Primer	R Primer	GenBank submission/accession ID
Bmal1	**5' –TCT GCA GGA TGA AGT GCA AC –3'**	**5' -GCG GTC TGC TTT CTT CTT TG –3'**	**MH715485.1**
Clock	**5' –TAG CAT GTG GAG CGG TAA TG – 3'**	**5' -GCA GCA AAA GTG GGA TAA GC – 3'**	**MH715483.1**
Per2	**5' –GGG GAT CTG GTA AGT CAT CATC – 3'**	**5' – ATG TGC TTC AGG ATC CCA TC –3'**	**MH715481.1**
Per3	**5' –TAT GCG TCC AGA CAG CAA AG – 3'**	**5' –TTT CCA GGC TCT GAA TGA CC – 3'**	**MH715482.1**
Cry1	**5' –ACA GCC AGC AGA TGT TTT CC – 3'**	**5' –ATCCGAACA ATG ACC TCC AC – 3'**	**MH715487.1**
Cry2	**5' –GCA ATC ATG ACC CAA CTG AG – 3'**	**5' –CCA GCA ACA GCTCAT CAA AC- 3'**	**MH715484.1**
Npas2	**5' –TCG GTT TCT GAC CAA AGG AC – 3'**	**5' –CAC AAT GAA CTC TGG CTT GG – 3'**	**MH715486.1**
18S	5' –GAC GCG TGCATT TATCAG**3'**	**5' –**GTT GAT AGG GCA GAC ATT **–3'**	**D38344.1**

Table 2: Sequence of primers used for qPCR analysis

4. RESULTS

4.1 Daily expression of clock genes in central and peripheral tissues

Figure 14 shows daily variations in the level of transcripts expression in the central (hypothalamus, pineal and retina) and peripheral tissues (liver, intestine, and muscle) of tree sparrow at six times of the day under 12L:12D light dark conditions. *Bmal1* mRNA levels showed daily variations in all the tissues studied (hypothalamus: $F_{5,24} = 5.696$, $P = 0.0013$; pineal: $F_{5,24} = 3.924$, $P = 0.0097$; retina: $F_{5,24} = 16.49$, $P < 0.0001$; liver: $F_{5,24} = 9.161$, $P < 0.0001$; intestine: $F_{5,24} = 8.456$, $P = 0.0001$ and muscle: $F_{5,24} = 13.49$, $P < 0.000$; one-way ANOVA; Fig. 14, 15). In central tissues (hypothalamus, pineal and retina), *Bmal1* peaked slightly after lights off (Fig. 14). In liver tissue we observed peak levels of *Bmal1* transcripts just before lights off while in intestine and muscle after lights off (Fig. 15, 16). Peak expression timing (cosinor analyses) of *Bmal1* mRNA levels varied and showed tissue specific expression timing as ZT13.6 (hypothalamus; Fig. 16A; Table 4), ZT12.6 (pineal; Fig. 16A; Table 3), ZT14.1 (retina; Fig. 16A; Table 3), ZT9.2 (liver; Fig. 16A; Table 3), ZT10.6 (intestine; Fig. 16A; Table 3) and ZT15.6 (muscle; Fig. 16A; Table 3).

Clock transcripts also expressed in all the tissues significantly and showed daily variations (hypothalamus: $P = 0.0010$; pineal: $P < 0.0001$; retina: $P = 0.0002$; liver: $P = 0.0009$; intestine: $P = 0.0013$ and muscle: $P < 0.000$; one-way ANOVA; Fig.14, 15). *Clock* transcripts showed variable peak expression times among tissues (Table 3). Acrophase of *Clock* transcript was recorded during middle of day in pineal (ZT6.9; Fig. 16B; Table 3), retina (ZT6.3; Fig. 16B; Table 3) and liver (ZT6.4; Fig. 16B; Table 3), late in the day in hypothalamus (ZT10; Fig. 16B; Table 3) and in intestine (ZT9.8; Fig. 16B; Table 3) and during early night hours (ZT14.2; Fig. 16B; Table 3) in muscle. Period genes (*Per2* and *Per3*) also underwent significant daily variations in all the tissues (hypothalamus: *Per2*: $P < 0.0010$; *Per3*: $P = 0.0010$; pineal: *Per2*: $P = 0.0001$; *Per3*: $P < 0.0001$; retina: *Per2*: $P = 0.0008$; *Per3*: $P < 0.0001$; liver: *Per2*: $P < 0.0001$; *Per3*: $P = 0.0001$; intestine: *Per2*: $P =$

0.0005; *Per3*: P < 0.0001 and muscle: *Per2*: P = 0.0387; *Per3*: P = 0.0010; one-way ANOVA; Fig. 14, 15). Whereas *Per2* expression peaked during late in the night (between ZT18 and ZT0; Table 3) in hypothalamus (ZT18.6; Fig. 16D; Table 3), retina (ZT23.8; Fig. 16D; Table 3) and in muscle (ZT19.5; Fig. 16D; Table 3), in rest tissues it peaked during early day time; pineal (ZT3.2; Fig. 16D; Table 3), liver (ZT3.2; Fig. 16D; Table 3) and intestine (ZT0.5; Fig. 16D; Table 3). However, *Per3* acrophase was observed during the late night between ZT18 and ZT0 as follows: hypothalamus (ZT21.95; Fig. 16E; Table 3), retina (ZT21.7; Fig. 16E; Table 3), intestine (ZT23.9; Fig. 16E; Table 3) and muscle (ZT18.2; Fig. 16E; Table 3) except in pineal and liver where acrophase was recorded during the early day time (pineal: ZT0.3; liver 1ZT2.6; Fig. 16E; Table 3).

Like period genes cryptochrome genes also showed daily variation in the expression of their transcripts in all tissues (hypothalamus: *Cry1*: P = 0.0069; *Cry2*: P = 0.0414; pineal: *Cry1*: P = 0.0001; *Cry2*: P = 0.0155; retina: *Cry1*: P < 0.0010; *Cry2*: P = 0.0005; liver: *Cry1*: P = 0.0111; *Cry2*: P = 0.0003; intestine: *Cry1*: P = 0.0016; and muscle: *Cry1*: P < 0.0010; *Cry2*: P = 0.0001; one-way ANOVA; Fig. 15, 16) except *Cry2* in intestine (P = 0.1158; one-way ANOVA; Fig. 15b.6). Peak expression timing of *Cry1* was different in different tissues. It peaked during early night phase in hypothalamus (ZT12.4; Fig. 16F; Table 3), pineal (ZT13.9; 16F; Table 3) and muscle (ZT14.9; 16F; Table 3) while during late daytime in retina (ZT10.3; 16F; Table 3), liver (ZT7.0; 16F; Table 3) and intestine (ZT8.8; 16F; Table3). *Npas2* transcripts had daily variations in all tissues (hypothalamus: P = 0.0346; pineal: P < 0.0001; retina: P = 0.0005; liver: P < 0.0001; intestine: P = 0.0012 and muscle: P = 0.0146; one-way ANOVA; Fig. 14, 15). In general, *Npas2* peaked during dark hours between ZT12 to ZT19 [hypothalamus (ZT13.3; Fig. 16C; Table 3), pineal (ZT12.7; Fig. 16C; Table 3), retina (ZT14.7; Fig. 16C; Table3), intestine (ZT13.8; Fig. 16C; Table 3and muscle (ZT 8.9; Fig. 16C; Table3)] except liver where acrophase was recorded during middle of the day (ZT4.8; Fig. 16C; Table 3). Although *Cry2* showed daily variations in mRNA in all tissues except intestine; cosinor analysis revealed that it did not oscillate in hypothalamus, pineal and intestine (Fig 14, 15).

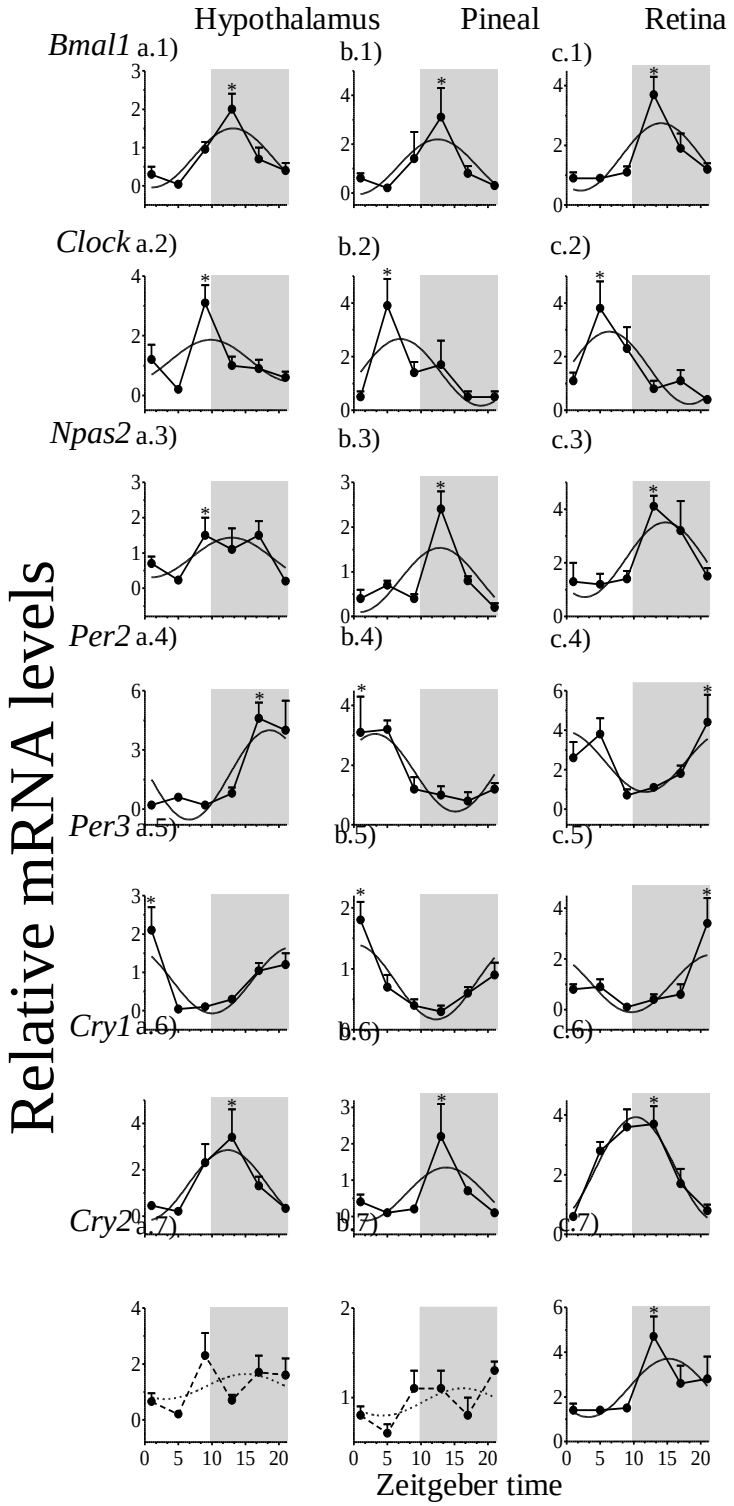

Figure 14: Figure 1 shows daily expression profiles of canonical clock genes (*Bmal 1, Clock, Per2, Per3, Cry1* and *Cry2*) and clock controlled gene (*Npas2*) in the hypothalamus (a1–a7), pineal (b1–b7) and retina (c1–c7) of the tree sparrow (*Passer montanus*). Daily oscillation at the interval of 4 h were measured in tissues collected at six times (ZT1, ZT5, ZT9, ZT13, ZT17, and ZT21; ZT0 is lights on) in sparrows exposed to 12 h:12 h light-dark (LD) cycle (grey part in each graph night time). One-way ANOVA determined the significance of difference among the six time points of the daily profile. The asterisk shows maximal expression of a gene in a particular tissue. A cosinor regression curve through time points was drawn to validate daily rhythm. Dotted line indicates there is no daily oscillation. Each data point represents mean and the vertical line on it indicates the standard error. Significance level: $p<0.05$, post hoc Newman-Keuls test.

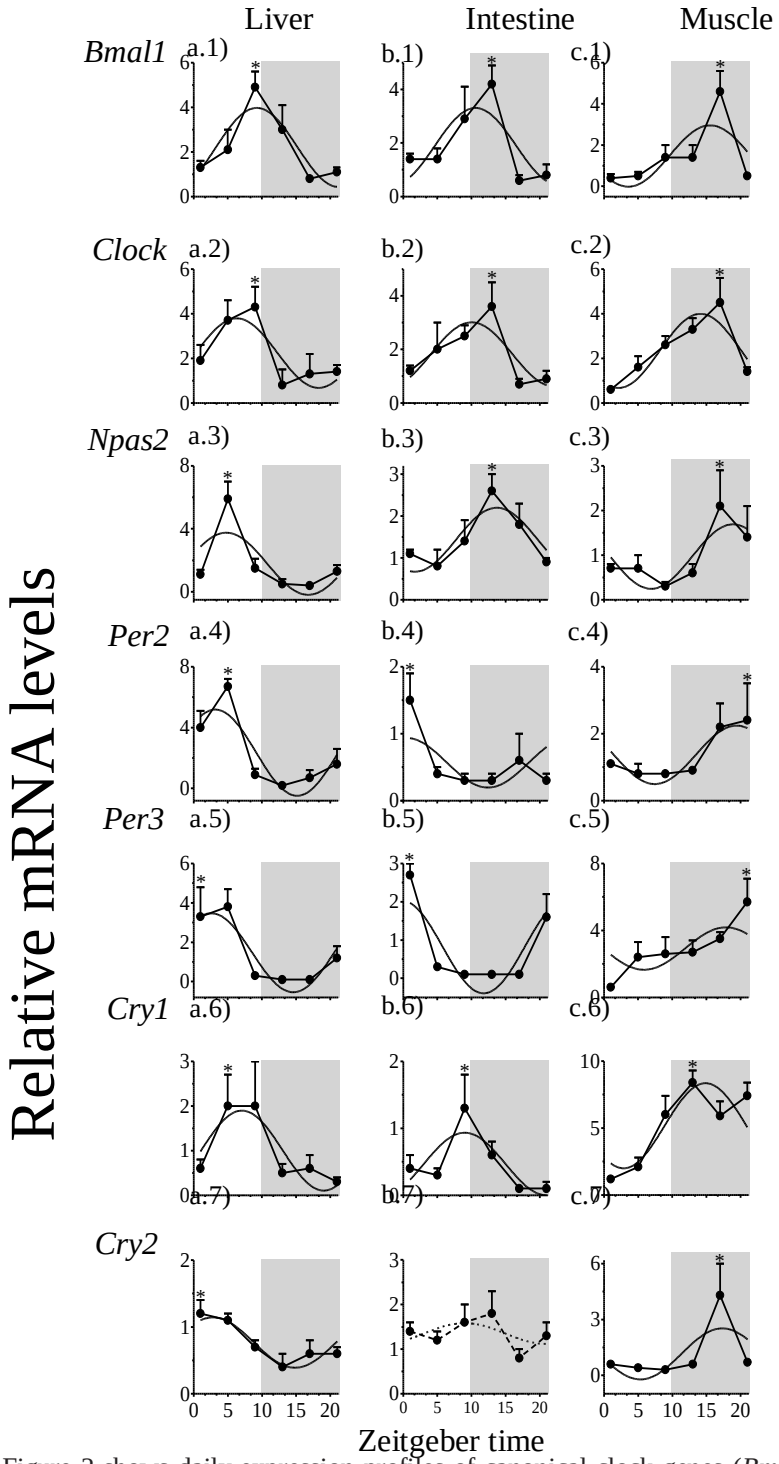

Figure 15: Figure 2 shows daily expression profiles of canonical clock genes (*Bmal1*, *Clock*, *Per2*, *Per3*, *Cry1* and *Cry2*) and clock controlled gene (*Npas2*) in the liver (a1–a7), intestine (b1–b7) and muscle (c1–c7) of the tree sparrow (*Passer montanus*). Daily oscillation at the interval of 4 h were measured in tissues collected at six times (ZT1, ZT5, ZT9, ZT13, ZT17, and ZT21; ZT0 is lights on) in sparrows exposed to 12 h:12 h light-dark (LD) cycle (grey part in each graph night time). One-way ANOVA determined the significance of difference among the six time points of the daily profile. The asterisk shows maximal expression of a gene in a particular tissue. A cosinor regression curve through time points was drawn to validate daily rhythm. Dotted line indicates there is no daily oscillation. Each data point represents mean and the vertical line on it indicates the standard error. Significance level: $p<0.05$, post hoc Newman-Keuls test.

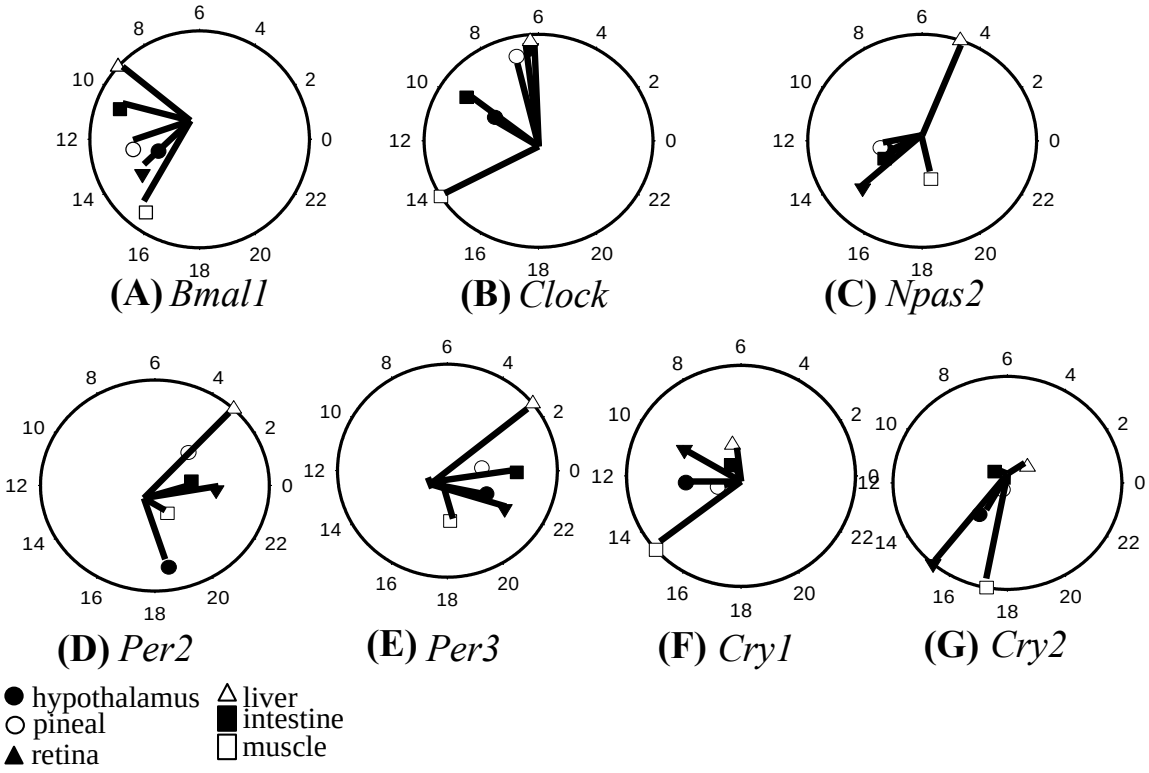

Figure 16. Relative presentation of acrophase and amplitude of clock genes [*Bmal1* (A), *Clock* (B), *Npas2* (C), P*er2* (D), *Per3* (E), *Cry1* (F) and *Cry2* (G)] and oscillations in different tissues. Central clock (hypothalamus, pineal, retina and peripheral (liver, intestine and muscle) tissues under 12l:12D light dark conditions in tree sparrows (n = 5) as determined by Cosinor analysis. Longer line indicates higher amplitude of mRNA expression.

4.2 Tissue-specific phase relationships between circadian genes

We determined the relationship of acrophase among genes within the tissue in relation to *the Per2* gene. In the hypothalamus, in comparison to the *Per2* gene, *Bmal1* and *Clock* transcripts peaked later, while *Per3*, *Cry1*, *Cry2*, and *Npas2* peaked earlier (Fig. 14; Table 4a). Acrophase was delayed in *Bmal1* (5.5 h) and *Clock* (8.6 h) while it was advanced in *Per3* (3.3 h), *Cry1* (6.2 h), *Cry2* (3.2h), and *Npas2* (5.3 h) (Fig. 14; Table 4a). In pineal except *for Per3* (2.9 h advance), acrophase of all genes was delayed (*Bmal1* 9.4 h, *Clock* 3.7h, *Cry1* 10.7 h, *Cry2* 12.7 h and *Npas2* 10.1 h; Fig. 14; Table 4a). Similar responses were observed in the retina where except *Per3* (2.1h advance) acrophase of all genes were also delayed (*Bmal1* 14.3 h, *Clock* 6.5 h, *Cry1* 10.5 h, *Cry2* 15.4 h and *Npas2* 14.9 h). In liver acrophase of *Per3* and *Cry2* was slightly advanced, and it was 0.6 h and 0.4 h, respectively (Fig. 15; Table 4a). For rest genes, acrophase was delayed. For *Bmal1*, it was delayed by 6 h, *Clock* by 3.2 h, *Cry1* by 3.8 h, and *Npas2* by 1.6 h (Fig. 15; Table 4a). However, in intestine acrophase of all genes were delayed (*Bmal1* 10.1 h, *Clock* 9.4 h, *Cry1* 8.3 h, *Cry2* 8.7 h and *Npas2* 13.3 h) except *Per3* which was advanced by 0.6 h (Fig. 15; Table 4a). In muscle, all genes peaked before *Per2* and were advanced 3.9 h for *Bmal1*, 5.2 h for *Clock*, 1.3 h for *Per3*, 4.7 h for *Cry1*, 2.1 h for *Cry2*, and 0.6 h for *Npas2* (Fig. 15; Table 4a).

Gene	Tissue	Mesor (A)	Amplitude (B)	Acrophase (C)
pPer2	Hypothalamus	1.7	2.3	18.6
	Pineal	1.8	1.3	3.2
	Retina	2.4	1.5	23.8
	Liver	2.4	2.8	3.2
	Intestine	0.6	0.4	0.5
	Muscle	1.4	0.9	19.5
pPer3	Hypothalamus	0.8	0.8	21.9
	Pineal	0.8	0.6	0.3
	Retina	1.0	1.2	21.7
	Liver	1.5	2.0	2.6
	Intestine	0.8	1.2	23.9
	Muscle	2.8	1.0	18.2
pBmal1	Hypothalamus	0.8	0.7	13.1
	Pineal	1.1	1.1	12.6
	Retina	1.6	1.2	14.1
	Liver	2.2	1.8	9.2
	Intestine	1.9	1.4	10.6
	Muscle	1.5	1.5	15.6
pClock	Hypothalamus	1.2	0.7	10.0
	Pineal	1.4	1.2	6.9
	Retina	1.6	1.3	6.3
	Liver	2.2	1.6	6.4
	Intestine	1.9	1.2	9.9
	Muscle	2.3	1.6	14.2
pCry1	Hypothalamus	1.4	1.5	12.4
	Pineal	0.6	0.8	13.9
	Retina	2.2	1.9	10.3
	Liver	1.0	0.9	7.0
	Intestine	0.5	0.5	8.8
	Muscle	5.2	3.2	14.8
pCry2	Hypothalamus	1.3	0.5	15.4
	Pineal	1.0	0.2	15.9
	Retina	2.4	1.3	15.2

	Liver	0.8	0.4	2.8
	Intestine	1.3	0.2	9.2
	Muscle	1.2	1.4	17.4
Npas2	Hypothalamus	0.9	0.6	13.3
	Pineal	0.8	0.7	12.7
	Retina	2.1	1.4	14.7
	Liver	1.8	2.0	4.8
	Intestine	1.4	0.8	13.8
	Muscle	1.0	0.7	18.9

Table 3: Rhythm parameters of all six genes in different tissues of tree sparrow, as determined by cosinor analyses

Tissue	*Bmal1*	*Clock*	*Per3*	*Cry1*	*Cry2*	*Npas2*
Hypothalamus	-5.5	-8.6	3.3	6.2	3.2	5.3
Pineal	-9.4	-3.7	2.9	-10.7	-12.7	-10.1
Retina	-14.3	-6.5	2.1	-10.5	-15.4	-14.9
Liver	-6	-3.2	0.6	-3.8	0.4	-1.6
Intestine	-10.1	-9.4	0.6	-8.3	-8.7	-13.3
Muscle	3.9	5.2	1.3	4.7	2.1	0.6

Table 4a: Table demonstrates relationship in time of peak expression (acrophase) within the tissue in reference of *Per2* acrophase. Values are represented in hour. Negative (-) sign represents delay while no sign represents advance in acrophase with reference of *Per2* acrophase.

Tissue	*Bmal1*	*Clock*	*Per2*	*Per3*	*Cry1*	*Cry2*	*Npas2*
Pineal	0.4	3	-8.6	-2.4	-1.5	-0.5	0.6
Retina	-1	3.7	-5.2	0.2	2.1	0.2	-1.4
Liver	3.4	3.6	-8.6	-4.7	5.4	12.6	8.5
Intestine	2	1	-5.9	-2	3.6	6.2	-0.5
Muscle	-3	-4.2	-0.9	3.7	-2.5	-2	-5.6

Table 4b: Relationship in time of peak expression (acrophase) between tissues in reference of acrophase of hypothalamus. Values are represented in hour. Negative (-) sign represents delay while no sign represents advance in acrophase with reference of acrophase in hypothalamus.

4.3 Phase relationships of circadian genes between different tissues

We used expression timing in the hypothalamus as a reference to determine the relationship in peak expression timing of genes in different tissues considering the hypothalamus is the central clock of avian complex clock system. When we compared with reference to peak expression time in the hypothalamus, *the Bmal1* peak was slightly advanced in the pineal by 0.4 h, in the liver by 3.4 h and intestine by 2 h. However, it was delayed in retina and muscle by 1 h and 3 h, respectively (Fig. 14, 15; Table 4b). *Clock* peak was advanced in all the tissues, and it was ranged between 0.1 to 3.7 h. in pineal it was advanced by 3 h, in the retina by 3.7 h, in the liver by 3.6 h and in the intestine by 0.1 h, while it was delayed in muscle by 4.2 h (Fig. 14, 15; Table 4b). *Per2* peak was delayed in all other tissues in comparison to the hypothalamus, and it ranged between 0.9 h to 8.6 h. In pineal and liver, it was delayed by 8.6 h, in the retina by 5.2 h, in the intestine by 5.9 h and in muscle by 0.9 h (Fig. 14, 15; Table 4b). Unlike *Per2, Per3* peak was more varied and advanced in retina and muscle by 0.2 h and 3.7 h, respectively. In rest tissues, *the Per3* peak was delayed, in pineal by 2.4 h, in the liver by 4.7 h and in the intestine by 2 h (Fig. 14, 15; Table 4b). *Cry1* peak was delayed in pineal and muscle by 1.5 h and 2.5 h, respectively. In rest tissues, it advanced; in the retina by 2.1 h, in the liver by 5.4 h and in the intestine by 3.6 h (Fig. 14, 15; Table 4b). Like *Cry1, Cry2* peak was also delayed in pineal and muscle by 0.5 h and 2 h, respectively. In rest tissues, it advanced; in the retina by 0.2 h, in the liver by 12.6 h and in the intestine by 6.2 h (Fig. 14, 15; Table 4b). *Npas2* peak was delayed by 1.4 h in the retina, 0.5 h in the intestine, and 5.6 h in muscles and advanced by 0.6 h in the pineal and 8.5 h in the liver (Fig. 14, 15; Table 4b).

We have also calculated the mesor and amplitude of all the genes in all the tissues (Table 3). In general, we observed higher amplitude and mesor for all genes in liver tissue except *Cry* genes (Fig. 16; Table 3). A significant variation in the rhythmic expression of genes was analyzed by cosinor analysis, and all the genes in all the tissues except *Cry2* in the hypothalamus, pineal, and intestine, showed daily rhythm in the expression of their respective transcripts.

5. DISCUSSION

The present study demonstrates the molecular clockwork of circadian clock genes and their phase relationships within and between tissues in the central and peripheral tissues in a resident songbird tree sparrow (*Passer montanus*) inhabiting high altitudes. Expression profiles of mRNA transcripts of canonical clock genes and clock-controlled gene showed daily variations, and it is in correspond with those reported in other avian species such as house sparrow (Abraham *et al*., 2002, 2003; Helfer *et al*., 2006); Japanese quail (Yasuo *et al*., 2003; Yoshimura *et al*., 2001), chicken (Doi *et al*., 2001; Karagensi *et al*., 2008) and buntings (Singh *et al*., 2013; 2015, Trivedi *et al*., 2016).

Bmal1, the component of the positive limb of the transcriptional-translational feedback loop (TTFL), shows daily oscillations in both central and peripheral tissues. In our study under the equinox photoperiod, *Bmal1* transcripts peaked just after light off in both central (hypothalamus, pineal, and retina) and peripheral tissue (intestine and muscle) except in muscle where it peaked just before lights off (Fig. 14, 15). A similar oscillation pattern of *the Bmal1* transcript has been reported in night migratory buntings (*Emberiza bruniceps* and *Emberiza melanocephala*). In red-headed bunting, *rbBmal1* transcripts were high during late day time and peaked around the light-dark transition time. In different tissues, *rbBmal1* acrophased between ZT9 and ZT17 (Singh *et al*., 2013). Similar results have been reported in black-headed bunting (*Emberiza melanocephala*; Singh *et al*., 2015). Unlike *Bmal1*, *Clock* the other component of a positive limb of the transcriptional- translational feedback loop had acrophase either during the middle of the day (pineal, retina, and liver) or during the late day (hypothalamus and intestine) except in muscle where it peaked during early night phase (Fig. 14, 15, Table 3). Some avian studies report temporal regulation of *Clock* mRNA. In the hypothalamus of a sparrow, *Clock* acrophase has been reported at ZT9.5 (Helfer *et al*., 2006). However, it did not oscillate in the quail brain (Yoshimura et al., 2000). Variable peak expression times of *Clock* transcripts were reported among tissues of red-headed bunting (Singh *et al*., 2013). Similar to our findings in red-headed bunting, the daily *rbClock* expression was around mid-day in the retina (ZT5.1) and

late in the day in the hypothalamus (ZT11.8). More variable acrophase was observed in other peripheral tissues (Singh *et al*., 2013). In *Drosophila* Clock transcripts oscillate with two peaks, one during the middle of a day at ZT5 and another during a late night at ZT23 (Darlington *et al.*, 1998). In many mammalian tissues, *Clock* transcripts did not show daily oscillations. However, it has been reported in the rat SCN (Abe *et al*., 1999).

Like the component of positive limb (*Bmal1* and *Clock*) of TTFL; period genes (*Per2* and *Per3*) also showed daily oscillation in all central and peripheral tissues studied (Fig. 14, 15) with their peak either late at night or during the early day time in a tissue-specific manner (Fig. 16). Similar responses of *Per2* transcripts had been reported in red-headed bunting (Singh *et al*., 2013; Trivedi *et al*., 2016), black-headed bunting (Singh *et al*., 2015), Japanese quail (Yoshimura *et al*., 2000), chicken (Bailey *et al*., 2004; Okano *et al*., 2001) and in mice (Albrecht *et al*., 1997). It has been suggested that PER abundance is critical for sustaining circadian oscillations (Chen *et al*., 2009; Yamamoto *et al*., 2005). *Cry* genes (*Cry1* and *Cry2*), another partner of negative-limb genes also had daily rhythms in their oscillation (Fig. 14). We observed the tissues specific expression pattern of *the Cry1* gene, which peaked between ZT 7 and ZT 15 (Fig. 14, 15). Similar observations have been noted in the red-headed bunting where *Cry1* peaked during late days between ZT8 and ZT10 (Singh *et al*., 2013), in the retina of chicken during ZT8 (Haque *et al*., 2002), in sparrow hypothalamus at ZT6.5 (Helfer *et al*., 2006) and in quail tissues (Fu *et al*., 2002; Yasuo *et al*., 2003). We did not find oscillation of *Cry2* in the hypothalamus, pineal, and intestine (Fig. 14, 15). Our findings are in consistent with the reports in quail where *Cry2* did not oscillate in pineal, eye, muscle, liver, heart, and skin tissues (Fu *et al*., 2002). The lack of oscillation of *Cry2* in various central and peripheral tissues implicated relatively less involvement as a negative element (Oster *et al*., 2002) of the feedback loop underlying circadian time generation, or it could be due to a tissue-specific contribution of *Cry2* to the circadian clock mechanism.

In general, we observed a phase relationship between *Bmal1* and *Per* (*Per2* and *Per3*) genes and their oscillations were antiphase in central (hypothalamus,

pineal, and retina) and peripheral (liver, intestine, and muscle) tissues (Fig. 14, 15), as reported in buntings (Singh *et al*., 2013; 2015), chicken pineal (Okano and Fukada, 2001), quail SCN, MBH, and pineal (Yasuo *et al*., 2003) and in rat SCN and peripheral tissues (Oishi et al., 1998). Similarly, *Clock* mRNA showed a phase relationship with *Per* genes in both central and peripheral tissues (Fig. 14). In comparison to *Per2*, *Bmal1* and *Clock* transcripts were always delayed in their acrophase except in muscle tissue (Fig. 14, 15, and Table 3). Further, *Cry1* and *Npas2* also delayed in peak expression in comparison to *Per2* except in the hypothalamus and muscle (Fig. 14, 15 and Table 3). More variability was observed in the case of *Cry2*, which was peaked earlier in the hypothalamus, liver, and muscle while delayed in the pineal, retina, and intestine (Fig. 14, 15 and Table 3).

Npas2 had a significant rhythm in both central (hypothalamus, pineal, and retina) and peripheral tissues (Fig. 14, 15). Previously we have shown a similar expression pattern in red-headed buntings where both in the hypothalamus and retina *Npas2* transcripts peaked after lights off (Trivedi *et al*., 2016). Thus, *Npas2* and *Per* expression peaks were phased (Fig. 14, 15; Table 3).

Using cosinor analysis, we calculated the mesor and amplitude of all the genes in all the tissues. Messor and amplitude of different transcripts were higher in the liver tissue in general except for *Cry* genes (Fig. 16, Table 4). Higher values of mesor and amplitude in the liver suggests its higher expression in this tissue. The liver being a metabolic center, is an important organ. Several studies demonstrated the significance of peripheral tissue clocks in different physiological processes such as glucose homeostasis, energy regulation, renal plasma flow, urine production, blood pressure and heart rate (Rudic *et al*., 2004; Oishi *et al*., 2006; Le Martelot *et al*., 2009). The maintaining of circadian rhythmicity in the peripheral tissues is probably to optimize the conservation of resources or to serve to optimize cellular physiology (Schibler *et al*., 2003). The canonical clock genes *Cry1* and *Cry2* drives glucose metabolism and control gluconeogenesis in the liver (Zhang *et al*., 2010). *Clock* drives glycogen synthesis in the liver through triggering *Gys2* transcription (Doi *et al*., 2010), *KLF10*, a transcription factor encoded by a clock-controlled gene, hinders hepatic glucose production decreasing *Pepck* expression (Guillaumond *et al*., 2010).

The daily oscillation of *Cyp7a1* expression is regulated by *Reverbα, Dbp/E4Bp4*, and *Dec2* (Lavery and Schibler, 1993). RORa is involved in binding cholesterol and its metabolites, with transcriptional activity modulation (Wang *et al*., 2009), and triggers the expression of low-density lipoprotein component (apo C-III) (Raspe *et al*., 2001), whose transcription is regulated by *Reverbα* (Raspe, 2002). Recent studies in mammals demonstrated that peripheral tissues maintain highly different rhythmic characteristics with respect to the phase of circadian expressed genes (Harbour *et al*., 2014). These results implicate a tissue-specific circadian regulation that probably involves in local tissue-specific functions.

Together, our findings support the existence of the autoregulatory transcription-translation feedback loop mechanism, both in the central and peripheral tissues of tree sparrow. These results implicate that tissue-level circadian time generation as acrophase of different genes is different in different tissues. We speculate a tissue-specific contribution of individual genes in circadian time generation in tree sparrow, as suggested in other avian species due to differential phase relationships in genes of positive and negative limbs of the TTFL. Whether this tissue-specific circadian time relationship varies during a different time of year needs to be further investigated.

SECTION VI: EFFECT OF URBAN ENVIRONMENT ON CIRCADIAN CLOCK

1. ABSTRACT

Increasing urbanisation is altering the physiology of wild animals and the mechanisms involved are largely unknown. We hypothesised that altering the physiology of urban organisms is due to the effect of extra light at night on the circadian clock by modulating the expression of pineal machinery and clock genes. Two experiments were performed. In Experiment 1, immediately after being procured from their respective sites (urban and rural sites), birds were released individually in LL_{dim} light conditions. Circadian rhythm period, activity duration, and total activity count were calculated and did not differ between urban and rural birds. In Experiment 2, birds (from urban and rural habitats) were sampled at six time points at regular four-hour intervals, beginning one hour after sunrise. We measured daily variations in plasma melatonin levels. We also analysed the expression levels of *Aanat*, *Mel1A* and *Mel1B* as an indicator of melatonin biosynthesis and action machinery. Clock and clock-controlled genes (*Bmal1*, *Clock*, *Per2*, *Per3*, *Cry1* and *Npas2*) were studied in the hypothalamus, the pineal gland, and retina to investigate the effects of urban habitats on the circadian clock. Our results show that there is a lower expression of *Aanat* in the pineal gland and relatively low plasma melatonin levels in urban birds. Further, clock genes are also differentially expressed in all three central tissues of urban birds. We propose that alterations in the melatonin biosynthesis machinery and the expression of clock genes could result in miscalculations in the internal timing of the organism, with environmental timings leading to altered physiology in urban wild animals.

2. INTRODUCTION

Organisms predominantly live in an environment fluctuating daily/seasonally and use their endogenous clock (circadian and/or circannual) to track the time of day and year to perform daily and seasonal events at the most appropriate time (Kumar *et al*., 2010). Mismatches between the endogenous clock and exogenous environment may have severe fitness consequences. In birds, circadian pacemakers are distributed at more than one place and have been identified as residing within the hypothalamus, pineal, and retina of the nervous tissue (Gwinner and Brandstatter, 2001).

Artificial light at night is a rapidly increasing phenomenon and has been associated with alterations in temporal activity patterns and physiology in vertebrates (Kempenaers *et al*., 2010; Dominoni *et al*., 2013a). During the last decade, the consequences of night lights on ecological aspects, particularly in the context of effects on wildlife balance, have received great interest (Rich and Longcore, 2006). The effect of city lights at night has been illustrated using compositional differences on the foraging behaviour of shorebirds (Santos *et al*., 2010), survival rates and stress responses in juvenile Pacific bluefin tuna (*Thunnus orientalis*; Honryo et al., 2012) and the commuting strategies of bats (Stone et al., 2009). Ecological research has emphasised the impact of urbanisation on animal populations and community dynamics (McKinney, 2006). New interest has emerged in understanding the mechanisms of individual responses to urbanisation (Shochat *et al*., 2010) and scattered information is available on the different aspects of animal physiology e.g. stress and reproductive physiology (Partecke *et al*., 2004, 2006; Dominoni *et al*., 2013a; Zhang *et al*., 2019), temporal and spatial activity patterns (Riley *et al*., 2013; Spoelstra *et al*., 2018), metabolism (Liker *et al*., 2008), behavior (Rees *et al*., 2009), social cues (Dominoni *et al*., 2014), sleep (Raapa *et al*., 2017) and on endocrine system (Dominoni *et al*., 2013b; Zhang *et al*., 2014).

It has been hypothesised that night lights may alter the detection of day-length (Dominoni *et al*., 2013b; Titulaer *et al*., 2012; Longcore and Rich, 2004), but at present we do not have an understanding of the physiological mechanisms involved in the process. As urban environments lead to extra light after sunset and clock genes are sensitive to the availability of light, we hypothesised that changes in

the physiology of urban birds is due to alterations in the expression of clock and clock-controlled genes. To address this, we studied the effects of urban night light habitats on the daily clock of tree sparrows living in an urban environment and compared it with birds living in nearby rural habitats in Aizawl, Mizoram, India.

3. MATERIALS AND METHODS

3.1 Animals and Tissue Collection

Study was conducted in accordance with guidelines of the Institutional Animal Ethics Committee (IAEC) of Mizoram University. Adult male tree sparrows (*Passer montanus*) were procured during third week of March 2018 at two locations, urban and rural sites. Urban site was selected in the core area of city (Aizawl, Mizoram, India; 23.7^0 N 92.7^0 E) having maximum human activities (population density: 737 individuals/km^2) while rural site was selected within Mizoram University campus (23.7^0 N 92.6^0 E), with limited human activities (population density: 375 individuals/ km^2). Mizoram University campus is approximately 15 km away from city of Aizawl and is situated at the west side of city. University campus is spread in 395.86 ha with lush greenery and hills. The University campus contains regenerating tropical wet evergreen and semi-evergreen forests, including a protected forested water catchment reserve in the north. A number of streams flow through the campus. Four sites were randomly selected within 100 range of urban and rural site of bird procurement and night light intensity was measured.. Average night light intensity recorded was 13.3 lux and 8.6 lux at urban and rural site respectively. Birds were captured using mist nets. Within two hours of procurement birds were transported back to outdoor aviaries. Immediately after procurement, birds were transported to the lab and kept in room of NDL conditions (L × W × H = 427 × 579 × 304 cm) having two windows (L x W x H = 2 x 5 x 5 feet) facing northeast direction. Birds (N=4) were kept in the cages (L x W x H = 2 x 2 x 2 feet) in the room. Two experiments were performed:

3.2 Experiment 1: Effect of habitats on circadian rhythm characteristics

Here we investigated the effects of habitat on circadian rhythms in activity behaviour. Within few hours after procurement (N=6 each from urban and rural site), birds were moved indoors to activity recording facility under LL_{dim} light condition (light intensity was maintained at 0.8±0.1 lux at perch level of cage). General activity of each bird was monitored as previously described in Trivedi *et al.*,

2014a. The circadian period (tau, τ), duration and distribution of activity over 10 circadian days were determined with reference to activity onsets after 7 days of start of experiment using Chronobiology Kit software program of Stanford Software Systems (Stanford, USA).

3.3 Experiment 2: Effect of habitats on daily clock of tree sparrow

For this experiment immediately after procurement; birds were transported to the lab and were kept in the NDL (natural day length conditions) in outdoor aviary and were sacrificed same day of the procurement. Birds (N=30 each site) were sacrificed by decapitation (N=5 per time point per site) at six times: ZT1, ZT5, ZT9, ZT13, ZT17, and ZT21 (ZT0 = sunrise time); dark hour samplings were done under dim red light. Blood was collected and serum was harvested and stored at –20 ^{0}C till hormone assay for melatonin was performed. The pineal, hypothalamus and retina tissues were immediately excised and stored in RNA later solution (Thermo Fisher Scientific, USA, AM 7020) and kept at 4 ^{0}C overnight and then stored at -80 ^{0}C till RNA was extracted from individual tissue.

3.4 Melatonin assay

Assay was performed as per the manufacturers' protocol using an immunoassay kit from IBL international (Hamburg, Germany; cat. no. RE54021). Briefly, 100 µl of each extracted standard, control and samples were pipetted into respective wells in a 96 well plate. Then, subsequently 100 µl of melatonin biotin and 100µl of melatonin antiserum were added in the well. Plate was incubated overnight at 4 °C. Next day, each well was rinsed three times using 250 µl of wash buffer (1X), then 150µl of enzyme conjugate was added to respective well. Plate was incubated at 500 rpm for 2 h at 25 °C. Further, following three washes with wash buffer, 200 µl of PNPP (p-nitrophenyl phosphate) substrate solution was added. After 40 min of incubation at 500 rpm and 25 °C, 50µl of PNPP stop solution was and optical density was measured at 405 nm by BioSpectrometer microplate reader (Eppendorf, Germany).

3.5 Gene expression

mRNA expression of genes coding for *Aanat*, two melatonin receptor subtypes (*Mel1A* and *Mel1B*), and clock genes (*Bmal1, Clock, Npas2, Per2, Per3* and *Cry1*) were measured in the retina, pineal and hypothalamus of each individual procured from urban and rural sites at 4 h intervals, beginning from 1 h after sun rise (ZT 1, 5, 9, 13, 17 and 21), as described previously for gene expression studies.

3.6 RNA Isolation, cDNA Synthesis

RNA was extracted using Tri reagent solution (Ambion AM9738; USA) as per the manufacturers protocol. Quantification was done using spectrophotometer (Biophotometer Plus, Eppendorf, Germany) and 1-µg RNA was used to prepare cDNA. RQ1 DNase (Promega M6101; Wisconsin, USA) kit was used to remove genomic DNA contamination if any. First strand cDNA synthesis kit (Thermo scientific, K1622; Lithuania, Europe) was used to synthesize cDNA.

3.7 Quantitative (Real-time) RT-PCR (qPCR)

Gene-specific primers for melatonin receptors (*Mel1a* and *Mel1b*) and gene encoding enzyme *Aanat* (Table 5) were designed from the sequences using Primer 3 plus online primer design tool. Primers for clock and clock-controlled genes (*Bmal1, Clock, Per2, Per3, Cry1* and *Npas2*) were used as mentioned in Table 5. Quant-Studio 5 (Applied Biosystem by Thermo Fisher Scientific; USA) was used to perform qPCR amplifications as described in Renthlei et al., 2019. $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001) using 18S as a reference gene (Renthlei *et al*., 2019) was used to determine the relative expression of genes.

3.8 Statistical Analysis

Student t- test was used to compare characteristics of activity rest behavior. One-way analysis of variance (1-way ANOVA) followed by Newman-Keuls post hoc test was used to determine the significance of difference among the six time points of daily profile. Cosinor analyses based on unimodal cosinor regression [$y=A+(B.\cos(2\pi(x-C)/24)$], where A, B, and C denote mean level (mesor), amplitude, and acrophase of the rhythm, respectively (Cuesta *et al.*, 2009) was used to test the daily variation. Two-way analysis of variance (2-way ANOVA) followed

by Bonferroni post hoc test was applied to determine the significance of difference when two factors (factor one: habitat and factor two: time) were considered together. Statistical analysis was done using graph pad prism version 6. Significance of regression analysis was calculated using number of samples, R^2 values, and numbers of predictors (mesor, amplitude, and acrophase; Soper, 2013; http://www.danielsoper.- com/statcalc3/calc.aspx?id 1⁄4 15).

Gene	***F primer***	***R Primer***	***Accession No.***
Bmal1	5’ TCT GCA GGA TGA AGT GCA AC 3’	5’ GCG GTC TGC TTT CTT CTT TG 3’	MH715485.1
Clock	5’ TAG CAT GTG GAG CGG TAA TG –3’	5’ GCA GCA AAA GTG GGA TAA GC 3’	MH715483.1
Per2	5’ GGG GAT CTG GTA AGT CAT CATC 3’	5’ ATG TGC TTC AGG ATC CCA TC 3’	MH715481.1
Per3	5’ TAT GCG TCC AGA CAG CAA AG 3’	5’ TTT CCA GGC TCT GAA TGA CC 3’	MH715482.1
Cry1	5’ ACA GCC AGC AGA TGT TTT CC 3’	5’ ATCCGAACA ATG ACC TCC AC 3’	MH715487.1
Npas2	5’ TCG GTT TCT GAC CAA AGG AC 3’	5’ CAC AAT GAA CTC TGG CTT GG 3’	MH715486.1
Aanat	5’ GAGGGAGCCAATGATGAAAG 3’	5’ ATATCGGTTTCTGGGGACTG 3’	MK900680.1
Mel1A	5’ ACATTTGCCCAGTCTGTGAG 3’	5’ CGAGGATCCATATTCGCAAG 3’	MK900681.1
Mel1B	5’ TGTTTCACCCAGTGTCCATC 3’	5’ CAGGTAACGCATTTGTGGTG 3’	MK900682.1
18S	5’ –GAC GCG TGCATT TATCAG3’	5’ GTT GAT AGG GCA GAC ATT 3’	D38344.1

Table 5: Gene specific primers used for qPCR analysis

4. RESULTS

4.1 Experiment 1: Effect of habitats on circadian rhythm characteristics

After releasing in aperiodic environment (LL_{dim} light condition), all individuals expressed the circadian clock with their own endogenous period. Irrespective of their habitat, all individual free ran with period less than 24h (Fig. 17a and b). There was no effect ($P > 0.05$, Student t- test) of habitat on total activity count (Fig. 17c), activity duration (Fig. 1d) and period (Fig. 17e).

4.2 Experiment 2: Effect of habitats on daily clock of tree sparrow

4.2.1 Effect of habitats on daily plasma melatonin levels

There was a significant difference between day and night time plasma melatonin levels in the birds (rural: $F_{5,29} = 97.47$, $P < 0.0001$; urban: $F_{5,29} = 68.42$, $P < 0.0001$; 1-way ANOVA; Fig. 18). Cosinor analyses confirmed daily oscillation in plasma melatonin levels. We found effects of habitat, time of day and interaction (habitat: $F_{1,48} = 51.35$, $P < 0.0001$, time: $F_{5,48} = 159.8$, $P < 0.0001$, interaction of habitat x time: $F_{5,48} = 23.38$, $P < 0.0001$, 2-way ANOVA; Fig. 18) on plasma melatonin level. In comparison to rural bird's night time melatonin levels were significantly reduced ($P < 0.05$; Bonferroni's multiple comparison test; Fig. 18) in urban birds.

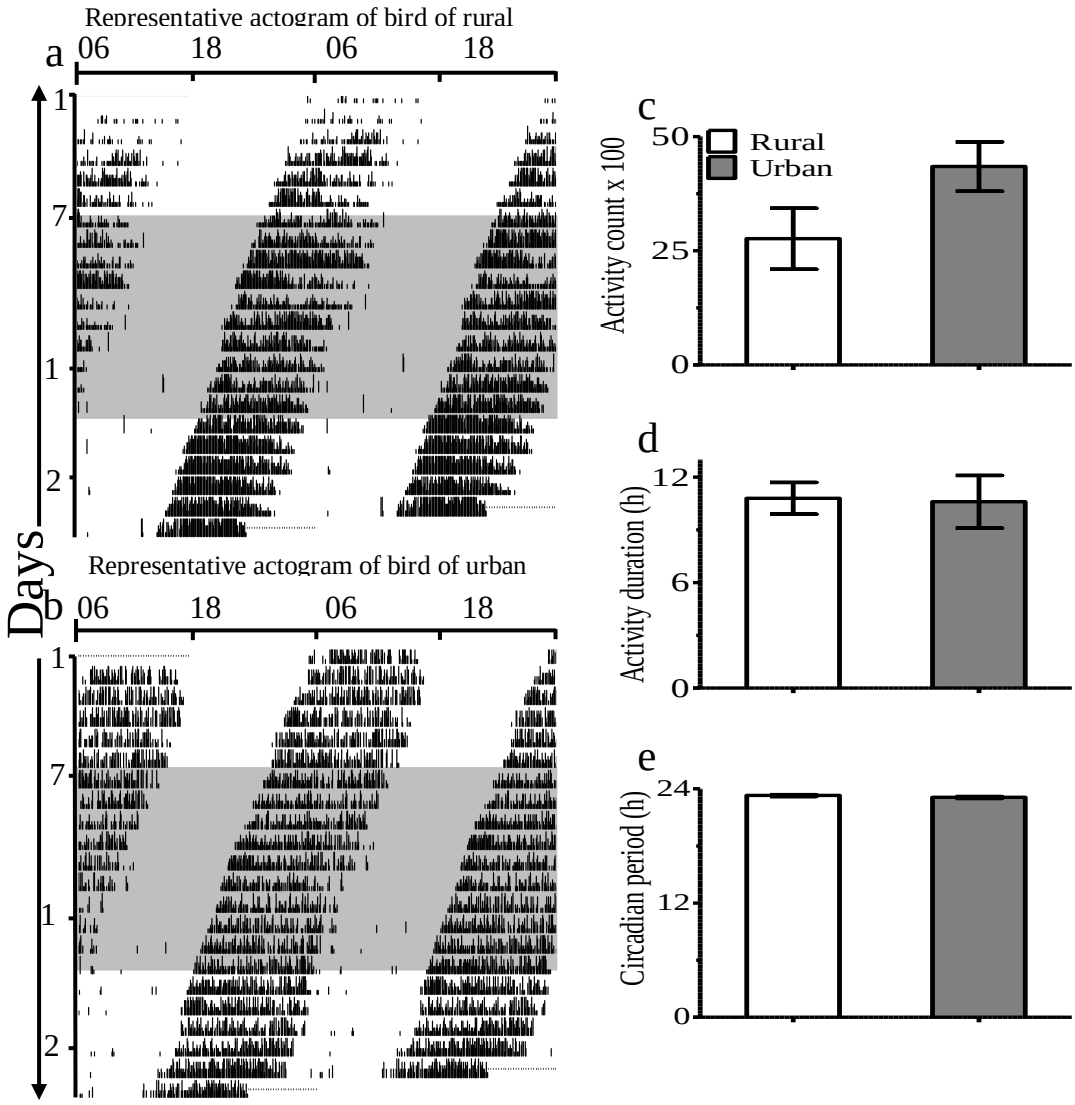

Figure 17. Representative double-plotted activity record (actograms) of free running condition of bird procured from rural (a) and urban (b) habitat. Data are represented as Mean (± SE, N = 6/ time point. Total activity count (c), activity duration (h) and period (e) during 10 days' time beginning after 7 days of start of experiment. Gray boxed portion of

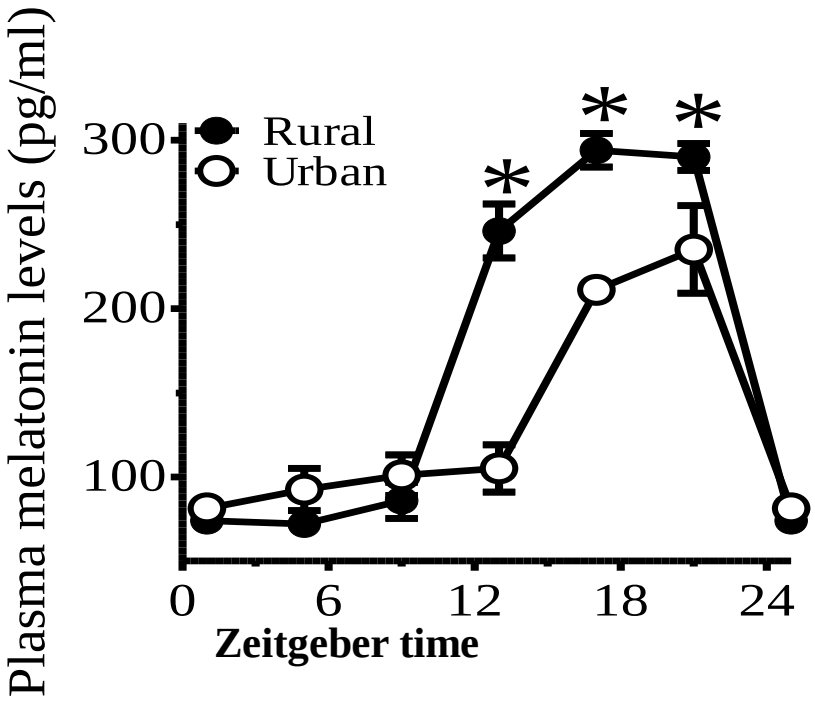

Figure 18. Daily variations in plasma melatonin levels in the birds procured from rural and urban habitat. Mean (± SE, N = 5/ time point) plasma melatonin levels measured at 4 h intervals beginning from one hour after sunrise over 24 h in tree sparrow (*Passer montanus*). Solid circle represents rural birds while hallow circle represents urban birds. A cosinor waveform represents a significant daily rhythm in plasma melatonin levels. Asterisk (*) represents significant difference between the two habitats as determined by Bonferroni posttest, following two-way ANOVA. For statistical significance, the alpha was set at 0.05.

4.2.2 Effect of habitats on daily expression of melatonin receptors and *Aanat* mRNA

4.2.2.1 Retina

Habitat-dependent daily variations in mRNA expression of *Mel1A, Mel1B* and *Aanat* in retina of sparrows (Fig. 19a) were observed from both rural (*Mel1A*: $F_{5,29}$= 5.646, P = 0.0014; *Mel1B*: $F_{5,29}$= 6.605, P = 0.0005 and *Aanat*: $F_{5,29}$= 14.31, P< 0.0001; 1way ANOVA; Fig. 19a) and urban birds (*Mel1A*: $F_{5,29}$= 15.68, P< 0.0001; *Mel1B*: $F_{5,29}$= 2.895, P = 0.0349 and *Aanat*: $F_{5,29}$= 8.521, P< 0.0001; 1-way ANOVA; Fig. 19a). The cosinor analysis also revealed daily oscillations in the *Mel1A, Mel1B* and *Aanat* transcripts from either habitat. We found effects of habitat, time and interaction of time and habitat for *Mel1B* (habitat: $F_{1,48}$= 90.65, P< 0.0001; time: $F_{5,48}$= 5.191, P = 0.0149 and interaction of time x habitat: $F_{5,48}$= 7.484, P< 0.0001; 2-way ANOVA; Fig. 19a) but only of time and interaction for *Mel1A* (habitat: $F_{1,48}$= 3.194, P= 0.0802; time: $F_{5,48}$= 3.636, P = 0.0072 and interaction of time x habitat: $F_{5,48}$= 13.54, P< 0.0001; 2-way ANOVA) and *Aanat* (habitat: $F_{1,48}$= 2.239, P= 0.1411; time: $F_{5,48}$= 9.909, P< 0.0001 and interaction of time x treatment: $F_{5,48}$= 14.33, P< 0.0001; 2-way ANOVA). There were differences in the parameters of rhythm waveform, the acrophase, mesor and amplitude of daily oscillations (Table 6; Fig. 19a). The acrophase of *Mel1A, Mel1B* and *Aanat* oscillation was at night in rural habitat (hour 15.4, hour 14 and hour 13.5 respectively) but during middle to late day time in urban population (*Mel1A* hour 8.3, *Mel1B* hour 6.6 and *Aanat* hour 8.8).

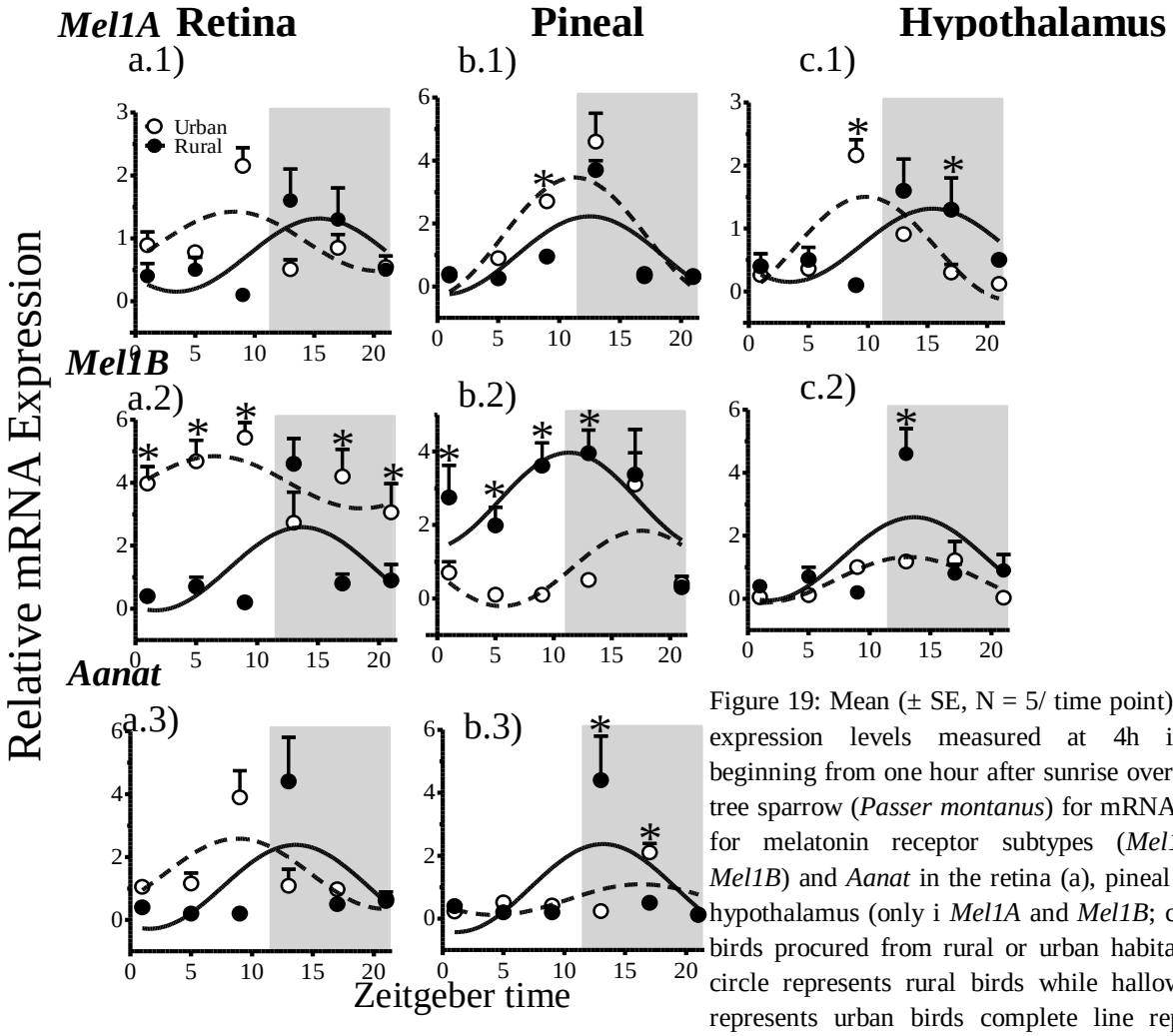

Figure 19: Mean (± SE, N = 5/ time point) mRNA expression levels measured at 4h intervals beginning from one hour after sunrise over 24 h in tree sparrow (*Passer montanus*) for mRNA coding for melatonin receptor subtypes (*Mel1A* and *Mel1B*) and *Aanat* in the retina (a), pineal (b) and hypothalamus (only i *Mel1A* and *Mel1B*; c) of the birds procured from rural or urban habitat. Solid circle represents rural birds while hallow circle represents urban birds complete line represents best fitted curve for rural birds while dotted line represents best fitted curve for urban birds. Asterisk (*) represents significant difference between the two habitats as determined by Bonferroni posttest, following two-way ANOVA. For statistical significance, the alpha was set at 0.05.

4.2.2.2 Pineal gland

All three mRNA showed a significant 24 h variation in birds of both habitats, rural (*Mel1A*: $F_{5,29}$= 22.46, P< 0.0001; *Mel1B*: $F_{5,29}$= 3.761, P = 0.0118 and *Aanat*: $F_{5,29}$= 6.240, P= 0.0008; 1-way ANOVA; Fig. 19b) and urban (*Mel1A*: $F_{5,29}$= 74.01, P< 0.0001; *Mel1B*: *P* < 0.0001 and *Aanat*: $F_{5,29}$= 8.271, P= 0.0001; 1-way ANOVA; Fig. 19b). Habitat effect was also observed on expression of all three genes, *Mel1A* (habitat: $F_{1,48}$= 9.293, P= 0.0037; time: $F_{5,48}$= 19.79, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 30.90, P< 0.0001; 2-way ANOVA; Fig. 19b), *Mel1B* (habitat: $F_{1,48}$= 8.095P= 0.0149; time: $F_{5,48}$= 5.716, P= 0.0003 and interaction of time x habitat: $F_{5,48}$= 4.714, P= 0.0149; 2-way ANOVA; Fig. 19b) and *Aanat* (habitat: $F_{1,48}$= 5.617,P= 0.0209; time: $F_{5,48}$= 5.380, P=0.0156 and interaction of time x habitat: $F_{5,48}$= 4.436, P= 0.0011; 2-way ANOVA; Fig. 19b). There was a difference in the acrophase, mesor and amplitude of daily oscillations (Table 6; Fig. 19b). The peak acrophase time was different in the two populations; *Mel1A* (rural: hour 17.5, urban hour 9.9), *Mel1B* (rural: hour 17.2, urban hour 13.5) and *Aanat* (rural: hour 16.9, urban hour 8.8).

4.2.2.3 Hypothalamus

The two receptor subtypes *Mel1A* and *Mel1B* had daily variations in the hypothalamus of birds of either habitat, rural (*Mel1A*: $F_{5,29}$ = 59.18, *P* < 0.0001 and *Mel1B*: $F_{5,29}$ = 11.20, *P* = 0.0007; 1-way ANOVA; Fig. 19c) an urban (*Mel1A*: $F_{5,29}$ = 33.12, *P* < 0.0001 and *Mel1B*: $F_{5,29}$ = 5.628, *P* = 0.0014; 1-way ANOVA). The cosinor analysis revealed daily oscillation of mRNA of two receptor subtypes and *Aanat*. There was an effect of habitat, time and interaction of habitat x time on both the receptors, *Mel1A* (habitat: $F_{1,48}$= 16.53, P= 0.0002; time: $F_{5,48}$= 75.53, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 3.697, P= 0.0065; 2-way ANOVA; Fig. 19c) and *Mel1B* (habitat: $F_{1,48}$= 38.56, P< 0.0001; time: $F_{5,48}$= 7.595, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 4.443, P= 0.0021; 2-way ANOVA; Fig. 19c). In urban birds *Mel1A* peaked 0.8h advance while *Mel1B* peaked 4h delay in comparison to rural birds.

4.3.3 Effect of habitat on daily expression of clock and clock-controlled genes

4.3.3.1 Retina*:* Clock genes had daily variations and rhythmic oscillation in retina of birds from both rural (*Bmal1*: $F_{5,29}$= 10.03, P< 0.0001; *Clock*: $F_{5,29}$= 9.904, P< 0.0001, *Npas2*: $F_{5,29}$= 13.54, P< 0.0001; *Per2*: $F_{5,29}$= 4.401, P= .0055; *Per3*: $F_{5,29}$= 36.60, P< 0.0001, *Cry1*: $F_{5,29}$= 4.862, P= 0.0033; 1-way ANOVA; Fig. 20a) and urban habitat (*Bmal1*: $F_{5,29}$= 8.120, P= 0.0001; *Clock*: $F_{5,29}$= 3.342, P= 0.0197, *Npas2*: $F_{5,29}$= 3.493, P= 0.0163; *Per2*: $F_{5,29}$= 3.461, P= .0170; *Per3*: $F_{5,29}$= 2.909, P= 0.0343, *Cry1*: $F_{5,29}$= 6.632, P= 0.0005; 1-way ANOVA; Fig. 20a). Overall, we found significant effects of habitat, time of day and their interaction on clock genes *Bmal1* (habitat: $F_{1,48}$= 5.763, P = .0203; time: $F_{5,48}$= 10.53, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 5.810, P = 0.0003; 2-way ANOVA; Fig. 20a), *Clock* (habitat: $F_{1,48}$= 7.224, P = 0.0099; time: $F_{5,48}$= 7.009, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 8.173, P< 0.0001; 2-way ANOVA; Fig. 20a), *Npas2* (habitat: $F_{1,48}$= 27.13, P< 0.0001; time: $F_{5,48}$= 6.227, P = 0.0002 and interaction of time x habitat: $F_{5,48}$= 3.769, P = 0.0059; 2-way ANOVA; Fig. 20a), *Per2* (habitat: $F_{1,48}$= 14.46, P = 0.0004; time: $F_{5,48}$= 4.065, P = 0.0032 and interaction of time x habitat: $F_{5,48}$= 3.571, P = 0.0080; 2-way ANOVA; Fig. 20), *Per3* (habitat: $F_{1,48}$= 42.87, P< 0.0001; time: $F_{5,48}$= 33.24, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 32.07, P< 0.0001; 2-way ANOVA; Fig. 20a) and *Cry1* (habitat: $F_{1,48}$= 6.965, P = 0.0112; time: $F_{5,48}$= 7.649, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 5.337, P = 0.0006; 2-way ANOVA). The parameters of rhythm waveform showed a difference in the acrophase, mesor and amplitude of daily oscillations (Table 6; Fig. 20a). The peak acrophase time was different in the birds of two habitat *Clock* (rural: hour 4.3, urban hour 11.8), *Npas2* (rural: hour 12.2, urban hour 7.9), *Per2* (rural: hour 22.9, urban hour 2.4), *Per3* (rural: hour 21.8, urban hour 3.2), *Cry1* (rural: hour 9.6, urban hour 7.0) except *Bmal1* (rural: hour 7.4, urban hour 7.5).

4.3.3.2 Pineal gland

Clock genes also had daily variations and oscillation in the pineal gland of birds of both habitat, rural (*Bmal1*: $F_{5,29}$= 10.03, P< 0.0001; *Clock*: $F_{5,29}$= 6.775, P= 0.0005, *Npas2*: $F_{5,29}$= 6.221, P= 0.0008; *Per2*: $F_{5,29}$= 6.328, P= .0007; *Per3*: $F_{5,29}$= 19.55, P< 0.0001, *Cry1*: $F_{5,29}$= 19.28, P< 0.0001; 1-way ANOVA; Fig. 20b) and urban habitat

(*Bmal1*: $F_{5,29}$= 32.07, P< 0.0001; *Clock*: $F_{5,29}$= 72.36, P< 0.0001, *Npas2*: $F_{5,29}$= 17.07, P< 0.0001; *Per2*: $F_{5,29}$= 5.867, P= 0.0011; *Per3*: $F_{5,29}$= 30.82, P< 0.0001, *Cry1*: $F_{5,29}$= 7.768, P= 0.0002; 1-way ANOVA; Fig. 20b). Cosinor analysis revealed daily oscillations in clock genes in pineal glands of birds of both habitats. 2-way ANOVA revealed the effect of habitat on expression of *Per3* (habitat: $F_{1,48}$= 14.34, P= 0.0004; time: $F_{5,48}$= 30.49, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 10.86, P< 0.0001; 2-way ANOVA; Fig. 20b) and *Cry1* (habitat: $F_{1,48}$= 14.37, P= 0.0004; time: $F_{5,48}$= 12.30, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 18.90, P< 0.0001; 2-way ANOVA; Fig. 20b). Other clock genes showed only effect of time and interaction of time and habitat but not of habitat alone, *Bmal1* (habitat: $F_{1,48}$= .0098, P= 0.9216; time: $F_{5,48}$= 50.53, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 13.51, P< 0.0001; Fig. 20b), *Clock* (habitat: $F_{1,48}$= 1.551, P= 0.2190; time: $F_{5,48}$= 29.12, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 5.288, P= 0.0006; 2-way ANOVA; Fig. 20b), *Per2* (habitat: $F_{1,48}$= 0.4332, P= 0.5136; time: = $F_{5,48}$= 7.813, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 4.480, P= 0.0020; 2-way ANOVA; Fig. 20b) and *Npas2* (habitat: $F_{1,48}$= 1.292, P= 0.2613; time: $F_{5,48}$= 4.442, P= 0.0021 and interaction of time x habitat: $F_{5,48}$= 15.09, P< 0.0001; 2-way ANOVA; two-way ANOVA; Fig. 20b). In general, mesor values of clock genes were similar in both the population (Table 6). There were differences in acrophase of clock genes from the two habitats; *Bmal1* (rural: hour 7.0, urban hour 5.5), *Clock* (rural: hour 7.7, urban hour 9.4), *Npas2* (rural: hour 17.5, urban hour 9.6), *Per2* (rural: hour 1.1, urban hour 23.4), *Per3* (rural: hour 1.0, urban hour 23.5) and *Cry1* (rural: hour 12.8, urban hour 7.9).

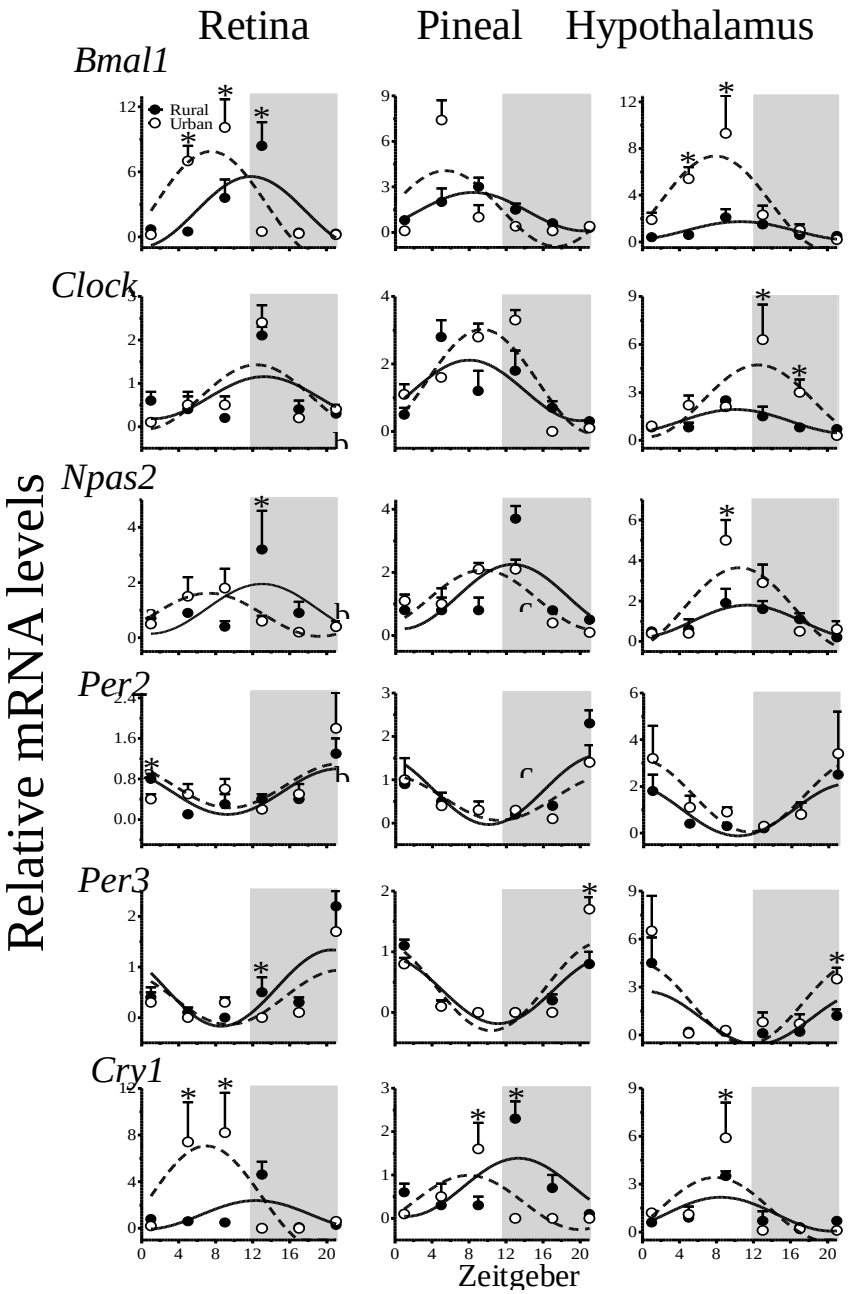

Figure20: Mean (± SE, N = 5/ time point) mRNA expression levels measured at 4h intervals beginning from one hour after sunrise over 24 h in tree sparrow (*Passer montanus*) for mRNA coding for clock and clock-controlled transcripts (*Bmal1, Clock, Npas2, Per2, Per3 and Cry1*) in the retina (a), pineal (b) and hypothalamus of the birds procured from rural or urban habitat. Solid circle represents rural birds while hallow circle represents urban birds complete line represents best fitted curve for rural birds while dotted line represents best fitted curve for urban birds. Asterisk (*) represents significant difference between the two habitats as determined by Bonferroni posttest, following two-way ANOVA. For statistical significance, the alpha was set at 0.05.

4.3.3.3 Hypothalamus

There was a daily variation and rhythmic oscillation in the clock genes in hypothalamic tissue of birds of both the habitats, rural (*Bmal1*: $F_{5,29}$= 14.63, P< 0.0001; *Clock*: $F_{5,29}$= 17.49, P< 0.0001, *Npas2*: $F_{5,29}$= 3.797, P= 0.0113; *Per2*: $F_{5,29}$= 2.869, P= 0.0361; *Per3*: $F_{5,29}$= 13.69, P< 0.0001, *Cry1*: $F_{5,29}$= 5.609, P= 0.0015; 1-way ANOVA; Fig. 20c) and urban habitat (*Bmal1*: $F_{5,29}$= 9.198, P< 0.0001; *Clock*: $F_{5,29}$= 7.238, P= 0.0003, *Npas2*: $F_{5,29}$= 18.45, P< 0.0001; *Per2*: $F_{5,29}$= 3.225, P = 0.0229; *Per3*: $F_{5,29}$= 11.30, P< 0.0001, *Cry1*: $F_{5,29}$= 10.08, P< 0.0001; 1-way ANOVA; fig. 20c). Except *Per2* mRNA (habitat: $F_{1,48}$= 0.4994, P= 0.4832; time: $F_{5,48}$= 4.939, P= 0.0010 and interaction of time x habitat: $F_{5,48}$= 1.040, P= 0.4051; 2-way ANOVA), all other transcripts had an effect of habitat on their expression, *Bmal1* (habitat: $F_{1,48}$= 23.68, P< 0.0001; time: $F_{5,48}$= 14.79, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 4.501, P= 0.0019; 2-way ANOVA; Fig. 20c), *Clock* (habitat: $F_{1,48}$= 8.906P= 0.0045; time: $F_{5,48}$= 5.886, P= 0.0003 and interaction of time x habitat: $F_{5,48}$= 2.940, P= 0.0215; 2-way ANOVA; Fig. 20c), *Npas2* (habitat: $F_{1,48}$= 5.998, P= 0.0182; time: $F_{5,48}$= 16.30, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 8.063, P< 0.0001; 2-way ANOVA; Fig. 20c), *Per3* (habitat: $F_{1,48}$= 10.69, P= 0.0020; time: $F_{5,48}$= 15.39, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 6.169, P= 0.0002; 2-way ANOVA; Fig. 20c) and *Cry1* (habitat: $F_{1,48}$= 5.434, P= 0.0240; time: $F_{5,48}$= 41.37, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 2.546, P= 0.0402; 2-way ANOVA; Fig. 20c). There was a phasing in the peak timing of hypothalamic clock genes expression between rural and urban birds. Except *Clock* (1.2h advance) and *Cry1* (0.8h advance) all other clock genes showed phase delay in urban birds in peak expression timing (*Bmal1*: 2h, *Npas*2: 5h; *Per2*: 2.5h and *Per3*: 1.7h). 24 h rhythm also showed habitat-dependent differences in the rhythm waveform parameters (Table 6; fig. 20c). Acrophase time was different in birds of two habitat, *Bmal1* (rural: hour 7.0, urban hour 5.5), *Clock* (rural: hour 11.3, urban hour 12.5), *Npas2* (rural: hour 15.3, urban hour 10.5), *Per2* (rural: hour 21, urban hour 23.5), *Per3* (rural: hour 21.7, urban hour 23.4) and *Cry1* (rural: hour 8.7, urban hour 7.9).

Tissue	Gene	Environment	Mesor	Amplitude	Acrophase
Retina	*Mel1A*	Rural	0.8	0.6	15.4
		Urban	1.0	0.5	8.3
	Mel1B	Rural	1.1	0.9	14
		Urban	4.0	0.8	6.6
	Aanat	Rural	1.1	1.3	13.5
		Urban	1.4	1.0	8.8
	Bmal1	Rural	1.5	1.0	7.4
		Urban	3.1	4.8	7.5
	Clock	Rural	1.0	1.0	4.3
		Urban	0.5	0.4	11.8
	Npas2	Rural	1.9	0.9	12.2
		Urban	1.0	0.6	7.9
	Per2	Rural	0.9	0.4	22.9
		Urban	0.5	0.1	2.4
	Per3	Rural	0.8	1.3	21.8
		Urban	0.2	0.1	3.2
	Cry1	Rural	1.1	0.6	9.6
		Urban	2.7	4.3	7.0
Pineal	*Mel1A*	Rural	1.2	1.6	17.5
		Urban	0.7	0.8	9.9
	Mel1B	Rural	1.1	0.7	17.2
		Urban	0.6	0.7	13.5
	Aanat	Rural	0.7	0.6	16.9
		Urban	0.5	0.5	8.8
	Bmal1	Rural	1.6	1.1	7.0
		Urban	1.6	2.5	5.5
	Clock	Rural	1.3	1.1	7.7
		Urban	1.5	1.6	9.4
	Npas2	Rural	1.3	.6	17.5
		Urban	1.1	.9	9.6
	Per2	Rural	.7	.5	1.1
		Urban	.6	.5	23.4
	Per3	Rural	.4	.5	1.0
		Urban	.2	.3	23.5
	Cry1	Rural	1.0	1.0	12.8
		Urban	.4	.6	7.9
	Mel1A	Rural	.9	1.2	12.5
		Urban	1.5	1.9	11.3

Hypothalamus	*Mel1B*	Rural	2.6	1.3	11.3
		Urban	0.8	1.0	17.5
	Bmal1	Rural	1.0	1.1	9.9
		Urban	3.3	4.0	7.9
	Clock	Rural	1.1	9.4	11.3
		Urban	2.5	2.2	12.5
	Npas2	Rural	1.0	.6	15.3
		Urban	1.6	2.0	10.5
	Per2	Rural	2.0	2.1	21.0
		Urban	1.6	1.6	23.5
	Per3	Rural	0.8	1.0	21.7
		Urban	1.9	2.5	23.4
	Cry1	Rural	1.1	1.5	8.7
		Urban	1.1	2.0	7.9

Table 6: Rhythm parameters of all nine genes in different tissues under different habitat of tree sparrow, as determined by cosinor analyses.

5. DISCUSSION

This is the first comprehensive study conducted to decipher the effects of urban environments (with light at night) on the circadian clock at the level of behaviour (locomotor activity rest behaviour), biochemical (melatonin hormone), and molecules (melatonin machinery; *Aanat*, *Mel1A* and *Mel1B* and clock genes; *Bmal1*, *Clock*, *Npas2*, *Per2*, *Per3* and *Cry1*). We did not find any effect of the urban environment on locomotor activity-rest pattern of these birds as reflected by period, activity duration, and total activity count. Similar results, albeit under laboratory conditions, were obtained from a study conducted on great tits (*Parus major*) where the timing of the onset of activity in DD was independent of whether birds were kept at "LD (light dark cycle) or LDim (dim light at night) before being exposed to DD". Advanced activity in night light environments in great tits was a direct effect of light rather than a phase shift of the internal clock (Spoelstra *et al*., 2018).

Irrespective of their habitat, clocks were expressed with daily variations in birds on both sites (Figs. 18-20). In both rural and urban birds, plasma melatonin levels were low during the daytime and started to rise as the dark started. Peak levels of melatonin were observed during later phases of the night and are consistent with other songbirds (Kumar and Follett, 1993; Brandstätter *et al*., 2000, 2001; Malik *et al*., 2015). Elevations in the melatonin levels were found during the light-to-dark transition and similar reductions during the dark-to-light transition are consistent with the idea that the endogenous circadian clock controls melatonin secretion, as reported in other vertebrates (see Trivedi and Kumar, 2014; Trivedi *et al*., 2017). Further, we observed a reduction in the amplitude levels of plasma melatonin in sparrows from urban habitats, suggesting that urban environments are affecting the expression/secretion levels of melatonin biosynthesis machinery. Reductions in melatonin levels in urban-like night illuminations have also been demonstrated in European blackbirds (*Turdus merula*) (Dominoni *et al*., 2013b). (8.8h; Suppl. Table S4), but during the middle of the night in rural birds (pineal 16.9 h, retina 13.5h; Suppl. Table S4).

mRNA of *Mel1A* and *Mel1B* receptors were rhythmically expressed both in the pineal and retina in urban and rural birds (Fig. 3); this is consistent with previous

finding (Jones *et al*., 2012). However, in comparison to the rural birds, urban birds showed a 4-7 hour advance in the acrophase of mRNA of *Mel1A* and *Mel1B* receptors (Fig. 19). Mismatches between the timing of peak melatonin secretion and peak melatonin receptor activity could influence physiological responses and may lead to fitness consequences in the organisms in urban habitats. We also observed the altered expression pattern in melatonin receptor transcripts in birds in urban habitats. The effect of light duration on melatonin receptors is documented. *Mel1A* melatonin receptor expression is higher during long days in comparison to short days (Dardente et al., 2003). Similarly, constant light exposure during subjective nights elevates expression patterns in *Mel1A* melatonin receptors (Wagner et al., 2007). Further, melatonin receptor expression was regulated by SD-dLAN (short day-dim light at night) in Siberian hamsters, suggesting that dLAN not only suppresses melatonin secretion, but also alters the melatonin processing pathway (Ikeno *et al*., 2014). Vertebrates use their melatonin secretion profile to decode night length (Brandstätter *et al*., 2000, 2001) and hence daytime. Decoding daylength is essential for seasonal animals as their physiology is photoperiod dependent (Kumar, 1997). Subsequently, extra night light in urban environments could mislead animals' photoperiodic processes and cause altered physiological responses at inappropriate times of day and year, as reported in certain avian species (Partecke *et al*., 2005; Schoech *et al*., 2003; Chamberlain *et al*., 2009; Deviche and Davies, 2014; de Jong *et al*., 2017).

Clock and clock-controlled genes (*Bmal1*, *Clock*, *Per2*, *Per3*, *Cry1* and *Npas2*) oscillated in all three neural tissues (hypothalamus, pineal and retina) of the tree sparrows procured from either urban or rural habitats and is consistent with our previous findings (Renthlei *et al*., 2019). However, there were alterations in the expression pattern of clock genes in the hypothalamus of birds in rural and urban habitats. *Bmal1*, *Clock*, *Per2* and *Per3* transcripts peaked earlier (1 to 5 h), while *Npas2* and *Cry1* genes were delayed (1 to 5 h) in their peak expression timing in urban birds compared to rural birds (Suppl. Table S4). Laboratory experiments suggest that dim lights at night can influence the circadian system. In Siberian hamsters, light exposure changes the expression pattern of *Per1* in SCN (Grone *et*

al., 2011). Similarly, when exposed to dim lights at night, higher expression levels in the mRNA of *Per1* and *Mel1A* are present (Ikeno *et al*., 2014). Chronic exposure to dim lights (5 lux) at night alters circadian clock genes in both the SCN and peripheral tissues in mice and attenuates the rhythm in *Per* genes and protein expression in the SCN around the light/dark transition (Shuboni and Yan, 2010; Fonken and Aubrecht, 2017). Exposure to extra light at night can result in metabolic disorders (van Amelsvoort *et al*., 1999; Parkes, 2002; Obayashi *et al*., 2013) and hence the fitness of the organisms. Like house sparrows, tree sparrows also prefer to live in close proximity of human habitat and hence in cities these birds are more prone to expose to night light. However, even living in urban sites, sparrows could also have a dark roosting site. Thus avoiding such lighting sites through behavioral regulation/adaptation is also possible. We don't know exactly at individual levels what light intensity these birds were exposed at night as we did not record their individual night light exposure using light sensors as performed by Dominoni *et al*., 2013a.

In conclusion, we provide an extensive characterisation of the circadian clock and the expression of clock-controlled genes in the central circadian oscillators in the nervous tissue of tree sparrows from rural and urban habitats. These findings are significant as they provide evidence to support the effect of urban environment on circadian functions. Our results from tree sparrows demonstrate that urban environments alter melatonin biosynthesis machinery; the expression of clock genes could thus result in misalignments in the internal timing of the organism with the outside environment. Correct time measurements are critical for organisms and facilitate their seasonal adaptations; therefore, our study suggests that light pollution could be one of the reasons behind the recent reports of altered reproductive behaviours in urban birds.

SECTION VII: SEASON DEPENDENT EFFECTS OF URBAN ENVIRONMENT ON CIRCADIAN CLOCK

1. ABSTRACT

In the recent past, much emphasis has been given to understanding the effect(s) of urban environments on the circadian and seasonal physiology of wild animals, and the mechanisms involved are largely unknown. Most of the laboratory studies and a few studies on wild animals suggest alterations in physiological functions of organisms in urban habitats. Here, we addressed the effects of the interaction of seasons and urban environments on the expression of clock genes in three central tissues of tree sparrows (*Passer montanus*). Tree sparrows (N=30/site/time of the year) were procured from rural and urban habitats at three times corresponding to their three physiological states, i.e., June (longest photoperiod; reproductive phase), September (equinox photoperiod; refractory phase) and December (shortest photoperiod; sensitive phase). Birds (N=5/time/site/month) were sampled at six time points: ZT1, ZT5, ZT9, ZT13, ZT17, and ZT21 (ZT0 = sunrise time) and expression of clock genes (*Bmal1*, *Clock*, *Npas2*, *Per2* and *Cry1*) in the hypothalamus, pineal gland and retina were studied. Our results show that there is persistence of the circadian clock in both rural and urban birds throughout the year. However, there are season and tissue-specific effects on the expression of clock genes. In comparison to rural birds, in urban birds, *Bmal1*, *Npas2*, *Per2* and *Cry1* acrophases were advanced by 0.1 to 7 h while the *Clock* acrophase was delayed by 0.7 h to 4.8 h, depending on the tissue and time of the year. This difference could be because of changes in the availability, duration, and intensity of sunlight during different times of the year and/or due to differential sensitivities of the photoreceptors/differential physiological states or a combination of all these factors. These results are important as they reveal, for the first time, season-dependent effects of an urban environment on the molecular machinery of the circadian clock in any species.

2. INTRODUCTION

Life on Earth evolved under the sustained influence of regular cycles of light and darkness, with a daily periodicity of approximately 24 h. The majority of organisms use their endogenous mechanism (circadian and/or circannual clocks) to time daily and seasonal processes at the most appropriate time of the day/year (Kumar *et al*., 2010). The circadian clocks are adoptive in nature. The anticipation of changes by circadian clock(s) associated with day and night seemed to benefit the survival of most of the organisms because these circadian clocks are equipped to measure circadian periods and regulate the temporal coordination of all physiological processes across the molecular and biochemical levels of the body's organization (Chaix *et al*., 2016). In mammals, the circadian system is hierarchical, with master circadian pacemakers residing in the suprachiasmatic nucleus (SCN) of the hypothalamus (Takahashi, 2016). However, in birds, it comprises complex circuitry in which at least three neuronal tissues – the SCN, pineal gland and retinae – contribute to the constitution of a complex clock (Gwinner and Brandstatter, 2001; Kumar *et al*., 2004). These pacemakers generate self-sustained rhythms in gene expression and electrical activity that oscillate with a circadian period of approximately 24 h and produce the humoral or electrical timing signals that are distributed throughout the body (Dibner *et al*., 2010). The clockwork mechanism underlying this endogenous periodicity is based on the transcriptional/translational negative and positive feedback loops of clock and clock-controlled genes, such as *Bmal1, Clock, Period, Cryptochromes, Rev-erbα, Rorα and Npas2* (Takahashi, 2016). Although these pacemakers are capable of generating circadian rhythms even in complete darkness, to be synchronized with the environment, daily exposure to the solar light/dark cycle is essential. The daily exposure to light allows them to be synchronized with the latitude and season.

Light at night is an ever-growing phenomenon that has been associated with the alteration of various physiological processes of organisms (Kempenaers *et al*., 2010; Rotics *et al*., 2011; Dominoni *et al*., 2013a; Hatori and Panda, 2015; McKinney, 2006; Perry *et al*., 2008; Rich and Longcore, 2006; Sanders and Gaston, 2018; Davies *et al*,. 2012; Santos *et al*., 2010; Dominoni *et al*., 2013b) .It has been

hypothesised that night lights may alter the detection of day length, hence physiology (Dominoni *et al*., 2013b; Titulaer *et al*., 2012; Longcore and Rich, 2004) . Therefore, more and more studies need to be conducted to address how organisms are responding/coping with this global challenge. In previous study, we found that circadian timing in the central tissues (pineal, retina and hypothalamus) varies according to physiological conditions/time of the year (Singh *et al*., 2015). Further, we showed the effects of urban environments on the circadian clock of the tree sparrow, demonstrating that urban birds exhibit lower expressions of *Aanat* mRNA in the pineal gland and relatively low plasma melatonin levels (Renthlei and Trivedi, 2019). Also, clock genes are differentially expressed in the pineal gland, retina and hypothalamus of urban birds (Renthlei and Trivedi, 2019).

The rotation of the Earth around its own axis leads to daily changes in light intensity while the tilt of the Earth's rotational axis as it orbits the Sun leads to seasonal variations. A change in the zeitgeber signal is critical for the entrainment of the circadian clock. The range of entrainment is correlated with zeitgeber strength and photoperiod. It has been shown that the phase of entrainment varies with season and latitude (Schmal *et al*., 2020). Since urban environments lead to extra light after sunset, clock genes are sensitive to the availability of light, and the expression pattern of clock genes is variable throughout the year (Singh *et al*., 2015), we asked whether there is an interactive effect of season and urban environment on the circadian timing of the birds living in urban habitats.

3. MATERIALS AND METHODS

3.1 Animals and Tissue Collection

The guidelines of the Institutional Animal Ethics Committee (IAEC) of Mizoram University were followed to conduct the study. Mist nets were used to procure adult male tree sparrows (*Passer montanus*) during the third week of June, September and December 2018 at two locations – urban and rural sites. These three times of the year were selected on the basis of the differences in their daylength at this latitude (23.7^0 N 92.7^0 E) as June has the longest daylength, September has an equinox photoperiod while December corresponds to the shortest daylength. Further, these three times of the year also correspond to different physiological conditions of the tree sparrow: June is their breeding phase, September corresponds to the refractory phase, while December is the time of the year when these birds regain their sensitivity for reproduction (Dixit and Singh 2011). An urban site was selected in the core area of a city (Aizawl, Mizoram, India; 23.7^0 N 92.7^0 E) with maximum human activities (population density: 737 individuals/km^2) and a rural site was selected within the Mizoram University campus (23.7^0 N 92.6^0 E), where there are limited human activities (population density: 375 individuals/ km^2), as previously described in genear materian and method section. Immediately after procurement, birds were transported to the lab and kept in a room with natural day length (NDL) conditions (L × W × H = 427 × 579 × 304 cm) and were sampled on the day of procurement. Birds (N=30 each site) were sacrificed (N=5/time/per site/month) at six times: ZT1, ZT5, ZT9, ZT13, ZT17, and ZT21 (ZT0 = sunrise time at the respective time of the year). The hypothalamus, pineal and retina tissues were immediately collected and stored in RNAlater solutions (Thermo Fisher Scientific, USA, AM 7020) first at 4^0C overnight and then at -80^0C.

3.2 Gene expression

mRNA expression of genes coding for clock genes (*Bmal1*, *Clock*, *Npas2*, *Per2* and *Cry1*) were measured in the hypothalamus, pineal and retina tissue of each bird, as described previously.

3.3 RNA Isolation, cDNA Synthesis

Total RNA was extracted using Tri Reagent solution (Ambion AM9738; USA) Quantification of RNA was performed using NanoDrop One spectrophotometer (Thermo Electron Scientific Instruments, LLC; USA), and 1-µg RNA was used to prepare cDNA. RQ1 DNase (Promega M6101; Wisconsin, USA) kit was used to remove genomic DNA contamination. A cDNA synthesis kit (Thermo Scientific, K1622; Lithuania, Europe) was applied to synthesize cDNA.

3.4 Quantitative (Real-time) RT-PCR (qPCR)

Primers for clock and clock-controlled genes (*Bmal1*, *Clock*, *Npas2*, *Per2* and *Cry1*) were used as mentioned in Table 5. The QuantStudio 5 (Applied Biosystem by Thermo Fisher Scientific; USA) system was used to conduct qPCR amplifications, as described in our previous publications (Renthlei and Trivedi, 2019; Renthlei *et al*., 2019; Borah *et al*., 2020). The $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001) using 18S as a control gene was used to determine the relative expression of genes (Renthlei and Trivedi, 2019; Renthlei *et al*., 2019).

3.5 Statistical analysis

Cosinor analyses based on unimodal cosinor regression [$y=A+(B.\cos(2\pi(x-C)/24))$], where A, B, and C denote mean level (mesor), amplitude, and acrophase of the rhythm, respectively was used to assess daily variation (Cuesta *et al*., 2009). Two-way analysis of variance (2-way ANOVA) was used to determine the significance of difference when two factors (factor one: habitat and factor two: time) were considered together. Further, Bonferroni post hoc test was applied if 2-way ANOVA showed differences. Statistical analysis was done using graph pad prism version 6.

4. RESULTS

4.1 Effect of habitat on daily and seasonal expression of clock and clock-controlled genes

4.1.1 Hypothalamus

Clock genes had daily variation and rhythmic oscillation in hypothalamus both in rural and urban birds (Fig. 21; Cosinor analyses). When we compared the gene expression among three times of the year (within the habitat), significant effects of time of the year, time of day and their interaction was observed on rural; *Bmal1* (months: $F_{2,72}$= 13.85, P< 0.0001; time: $F_{5,72}$= 22.48, P< 0.0001 and interaction of time of day x months: $F_{10,72}$= 11.19, P< 0.0001; 2-way ANOVA; Fig. 21a.1), *Clock* (months: $F_{2,72}$= 38.96, P< 0.0001; time: $F_{5,72}$= 4.920, P = 0.0006 and interaction of time of day x months: $F_{10,72}$= 6.873, P< 0.0001; 2-way ANOVA; Fig. 21a.2), *Npas2* (months: $F_{2,72}$= 49.42, P< 0.0001; time: $F_{5,72}$= 20.48, P< 0.0001 and interaction of time of day x months: $F_{10,72}$= 10.91, P< 0.0001; 2-way ANOVA; Fig. 21a.3), *Per2* (months: $F_{2,72}$= 6.290, P= 0.0030; time: $F_{5,72}$= 18.00, P< 0.0001 and interaction of time of day x months: $F_{10,72}$= 2.277, P= 0.0222; 2-way ANOVA; Fig. 11a.4) and *Cry1* (months: $F_{2,72}$= 76.63, P< 0.0001; time: $F_{5,72}$= 68.79, P< 0.0001 and interaction of time of day x months: $F_{10,72}$= 25.70, P< 0.0001; 2-way ANOVA; Fig. 21a.5) and urban birds; *Bmal1* (months: $F_{2,72}$= 20.94, P< 0.0001; time: $F_{5,72}$= 21.74, P< 0.0001 and interaction of time of day x months: $F_{10,72}$= 14.16, P< 0.0001; 2-way ANOVA; Fig. 21b.1), *Clock* (months: $F_{2,72}$= 7.787, P= 0.0009; time: $F_{5,72}$= 33.51, P< 0.0001 and interaction of time of day x months: $F_{10,72}$= 22.55, P< 0.0001; 2-way ANOVA; Fig. 21b.2), *Npas2* (months: $F_{2,72}$= 26.00, P< 0.0001; time: $F_{5,72}$= 16.88, P< 0.0001 and interaction of time of day x months: $F_{10,72}$= 11.15, P< 0.0001; 2-way ANOVA; Fig. 21b.3) and *Cry1* (months: $F_{2,72}$= 17.70, P< 0.0001; time: $F_{5,72}$= 10.29, P< 0.0001 and interaction of time of day x months: $F_{10,72}$= 7.989, P< 0.0001; 2-way ANOVA; Fig. 21b.5) except *Per2* where we did not see any effect of time of the year (months: $F_{2,72}$= 5.229, P= 0.0581; time: $F_{5,72}$= 16.79, P< 0.0001 and interaction of time of day x months: $F_{10,72}$= 4.174, P= 0.0001; 2-way ANOVA; 21b.4).

The parameters of rhythm waveform showed differences in the acrophase, mesor and amplitude of daily oscillations (Table 7; Fig. 21). In birds procured from rural habitat, *Bmal1* peak expression timing (cosinor analyses) varied from mid-day to late day time having time of the year specific expression, June (ZT 6.8 Fig. 21a.1), September (ZT 10.4 Fig. 21a.1) and December (ZT 8.1; Fig. 21a.1) while in urban birds it peaked earlier than rural birds, June (ZT 6.8 Fig. 21b.1), September (ZT 7.3 Fig. 21b.1) and December (ZT 5.9; Fig. 21b1). *Clock* genes peaked during middle to late day time in rural birds, June (ZT 11.3 Fig. 21a.2), September (ZT 7.5 Fig. 21a.2) and December (ZT 10.7; Fig. 21a2) while it was delayed in urban birds with correspond to their rural conspecifics at respective months, June (ZT 12.9 Fig. 21b.2), September (ZT 9.2; Fig. 21b.2) and December (ZT 14.5; Fig. 21b.2). In rural birds, *Npas2* peak timing was time of the year dependent, June (ZT 14.4 Fig. 21a.3), September (ZT 5.0 Fig. 21a.3) and December (ZT 12.5; Fig. 21a.3) and in urban birds it was relatively advanced, June (ZT 9.2 Fig.1b.3), September (ZT 5.7 Fig. 21b.3) and December (ZT 10.0; Fig. 21b.3). Peak expression timing of *Per2* was observed either late night or early

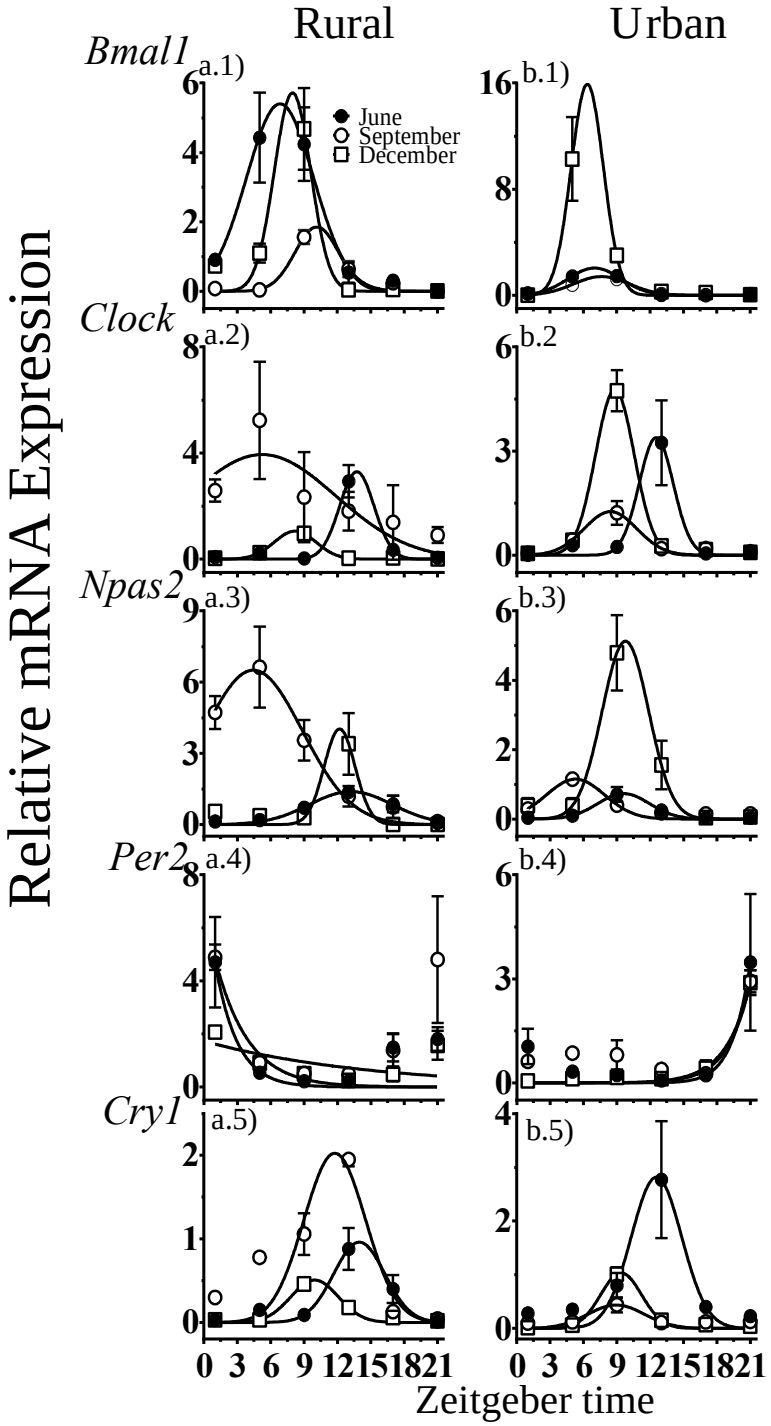

Figure 21: Mean (± SE, N = 5/ time point/site/month) mRNA transcript levels measured at 4h intervals beginning from one hour after sunrise (respective month of the year) over 24 h in tree sparrow (*Passer montanus*) for mRNA coding for clock and clock-controlled transcripts (*Bmal1, Clock, Npas2, Per2, and Cry1*) in the hypothalamus of the birds procured from rural or urban (a.1–a.5) habitat during June (solid circle), September (hallow circle) and December (rectangle). Lines represent best fitted curve.

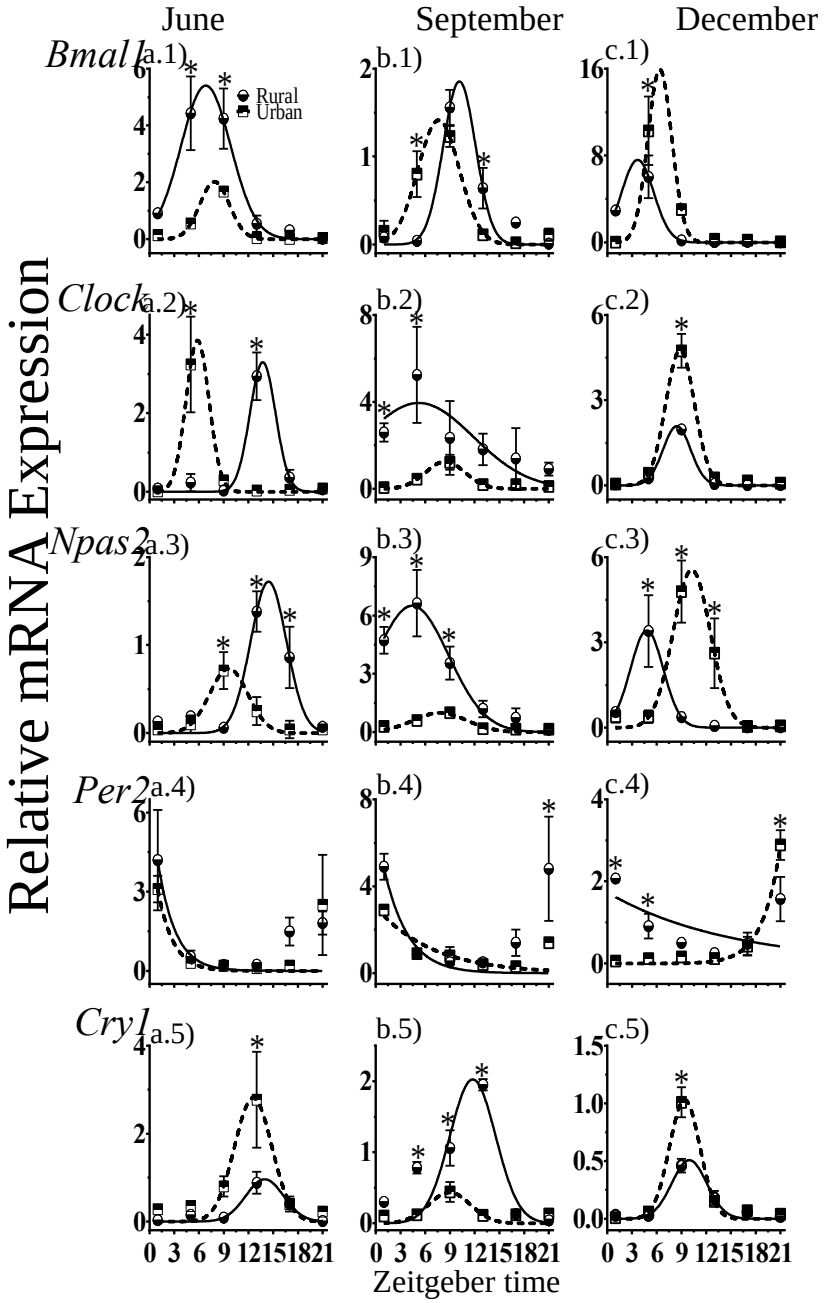

Figure 22: Mean (± SE, N = 5/ time point/site/month) mRNA transcript levels measured at 4h intervals beginning from one hour after sunrise (respective month of the year) over 24 h in tree sparrow (*Passer montanus*) for mRNA coding for clock and clock-controlled transcripts (*Bmal1, Clock, Npas2, Per2, and Cry1*) in the hypothalamus of the birds procured from rural (circle) and urban (rectangle) habitat during June (a.1–a.5), September (b.1–b.5) and December (c.1–c.5). Solid line represents best fitted curve for rural birds while dotted line represents best fitted curve for urban birds. Asterisk (*) represents significant difference between the two habitats at that point determined by Bonferroni posttest, following two-way ANOVA. For statistical significance, the alpha was set at 0.05.

daytime in rural birds, June (ZT 23.1 Fig. 21a.4), September (ZT 0.5 Fig.1a.4) and December (ZT 0.1; Fig. 21a.4) while in urban birds it was slight advanced, June (ZT 22.0; Fig. 21b.4), September (ZT 23.5; Fig. 21b.4) and December (ZT 20.6; Fig. 21b.4). *Cry1* peaked during mid-day to late day time in rural birds, June (ZT 13.6; Fig. 21a.5), September (ZT 10.7; Fig. 21a.5) and December (ZT 10.2; Fig. 21a.5) while urban birds were slightly advanced, June (ZT 12.4; Fig. 21b.5), September (ZT 10.0; Fig. 21b.5) and December (ZT 9.5; Fig. 21b.5; Table 7).

There was an effects of habitat, time of day and their interaction on clock genes, June: *Bmal1* (habitat: $F_{1,48}$= 36.47, P< 0.0001; time: $F_{5,48}$= 22.73, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 8.000, P< 0.0001; 2-way ANOVA; Fig. 22a.1), *Clock* (habitat: $F_{1,48}$= 04035, P= 0.0185; time: $F_{5,48}$= 11.61, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 19.83, P< 0.0001; 2-way ANOVA; Fig. 22a.2), *Npas2* (habitat: $F_{1,48}$= 12.43, P< 0.0001; time: $F_{5,48}$= 12.04, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 15.05, P< 0.0001; 2-way ANOVA; Fig. 22a.3) and *Cry1* (habitat: $F_{1,48}$= 12.95, P< 0.0001; time: $F_{5,48}$= 12.30, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 3.666, P= 0.0069; 2-way ANOVA; Fig. 22a.5), September: *Bmal1* (habitat: $F_{1,48}$= 4.088, P= 0.0176; time: $F_{5,48}$= 23.11, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 5.530, P= 0.0004; 2-way ANOVA; Fig. 22b.1), *Clock* (habitat: $F_{1,48}$= 41.47, P< 0.0001; time: $F_{5,48}$= 4.858, P= 0.0011 and interaction of time x habitat: $F_{5,48}$= 3.786, P= 0.0057; 2-way ANOVA; Fig. 22b.2), *Npas2* (habitat: $F_{1,48}$= 87.45, P< 0.0001; time: $F_{5,48}$= 17.02, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 13.17, P< 0.0001; 2-way ANOVA; Fig. 22b.3), *Per2* (habitat: $F_{1,48}$= 9.864, P= 0.0029; time: $F_{5,48}$= 12.83, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 2.981, P=0.0201; 2-way ANOVA; Fig. 22b.4) and *Cry1* (habitat: $F_{1,48}$= 154.1, P< 0.0001; time: $F_{5,48}$= 66.53, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 53.19, P< 0.0001; 2-way ANOVA; Fig. 22b.5) and December: *Bmal1* (habitat: $F_{1,48}$= 5.487, P=0.0121; time: $F_{5,48}$= 27.87, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 4.441, P=0.0021; 2-way ANOVA), *Clock* (habitat: $F_{1,48}$= 78.76, P< 0.0001; time: $F_{5,48}$= 121.6, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 53.90, P< 0.0001; 2-way ANOVA; Fig. 22c.2), *Npas2* (habitat:

$F_{1,48}$= 5.696, P=0.0210; time: $F_{5,48}$= 10.07, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 14.85, P< 0.0001; 2-way ANOVA; Fig. 22c.3), *Per2* (habitat: $F_{1,48}$= 9.229, P= 0.0038; time: $F_{5,48}$= 33.04, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 16.15, P< 0.0001; 2-way ANOVA; Fig. 22c.4) and *Cry1* (habitat: $F_{1,48}$= 19.09, P< 0.0001; time: $F_{5,48}$= 113.0, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 18.12, P< 0.0001; 2-way ANOVA; Fig. 22c.5) except *Per2* (habitat: $F_{1,48}$= 2.860, P= 0.0973; time: $F_{5,48}$= 8.207, P< 0.0001, and interaction of time x habitat: $F_{5,48}$= 4.110, P= 0.0035; 2-way ANOVA; Fig. 22a.4) during June.

The parameters of rhythm waveform had difference in the acrophase, mesor and amplitude of daily oscillations (Table 7). Peak acrophase time differed between rural and urban birds. In urban birds it was advanced in *Bmal1* (June: 0 h, September: 3.1 h and December: 2.2 h; Suppl. Table 4c), *Npas2* (June: 5.2 h, September: -0.7 h and December: +2.5 h), *Per2* (June: 1.1 h, September: 1.0 h and December: 3.5 h) and *Cry1* (June: 1.2 h, September: 0.7 h and December: 0.7 h) while delay in peak expression timing of *Clock* (June: -2.4 h, September: -3.3 h and December: -0.7 h; Table 8c).

4.1.2 Pineal gland

Clock genes had daily variations and rhythmic oscillation in pineal in birds of rural and urban habitat (Fig. 23; Cosinor analyses). Gene expression during three time of the year (within the habitat) had significant effects of time of the year, time of day and their interaction on rural birds; *Bmal1* (months: $F_{2,72}$= 5.294, P= 0.0280; time: $F_{5,72}$= 27.48, P< 0.0001 and interaction of time of day x months: $F_{10,72}$= 8.169, P< 0.0001; 2-way ANOVA; Fig. 23a.1), *Clock* (months: $F_{2,72}$= 16.37, P< 0.0001; time: $F_{5,72}$= 6.789, P< 0.0001 and interaction of time of day x months: $F_{10,72}$= 6.046, P< 0.0001; 2-way ANOVA; Fig. 23a.2), *Npas2* (months: $F_{2,72}$= 6.214, P= 0.0130; time: $F_{5,72}$= 7.217, P< 0.0001 and interaction of time of day x months: $F_{10,72}$= 13.00, P< 0.0001; 2-way ANOVA; Fig. 23a.3), *Per2* (months: $F_{2,72}$= 30.54, P< 0.0001; time: $F_{5,72}$= 22.09, P< 0.0001 and interaction of time of day x months: $F_{10,72}$= 26.74, P< 0.0001; 2-way ANOVA; Fig. 23a.4) and *Cry1* (months: $F_{2,72}$= 3.255, P=- 0.0443; time: $F_{5,72}$= 9.370, P< 0.0001 and interaction of time of day x months: $F_{10,72}$= 10.56,

P< 0.0001; 2-way ANOVA; Fig. 23a.5). Birds from urban habitat exhibited effect of time of the year, time of day and their interaction on clock genes; *Bmal1* (months: $F_{2,72}$= 6.1594, P=0.0285; time: $F_{5,72}$= 32.65, P< 0.0001 and interaction of time of day x months: $F_{10,72}$= 26.31, P< 0.0001; 2-way ANOVA; Fig. 23b.1), *Clock* (months: $F_{2,72}$= 7.554, P= 0.0011; time: $F_{5,72}$= 17.79, P< 0.0001 and interaction of time of day x months: $F_{10,72}$= 7.903, P< 0.0001; 2-way ANOVA; Fig. 23b.2), *Npas2* (months: $F_{2,72}$= 6.7318, P=0.0261; time: $F_{5,72}$= 26.68, P< 0.0001 and interaction of time of day x months: $F_{10,72}$= 10.34, P< 0.0001; 2-way ANOVA; Fig. 23b.3), *Per2* (months: $F_{2,72}$= 19.72, P< 0.0001; time: $F_{5,72}$= 22.32, P< 0.0001 and interaction of time of day x months: $F_{10,72}$= 19.89, P< 0.0001; 2-way ANOVA; Fig. 23b4) and *Cry1* (months: $F_{2,72}$= 28.72, P< 0.0001; time: $F_{5,72}$= 82.68, P< 0.0001 and interaction of time of day x months: $F_{10,72}$= 14.22, P< 0.0001; 2-way ANOVA; Fig. 23b.5).

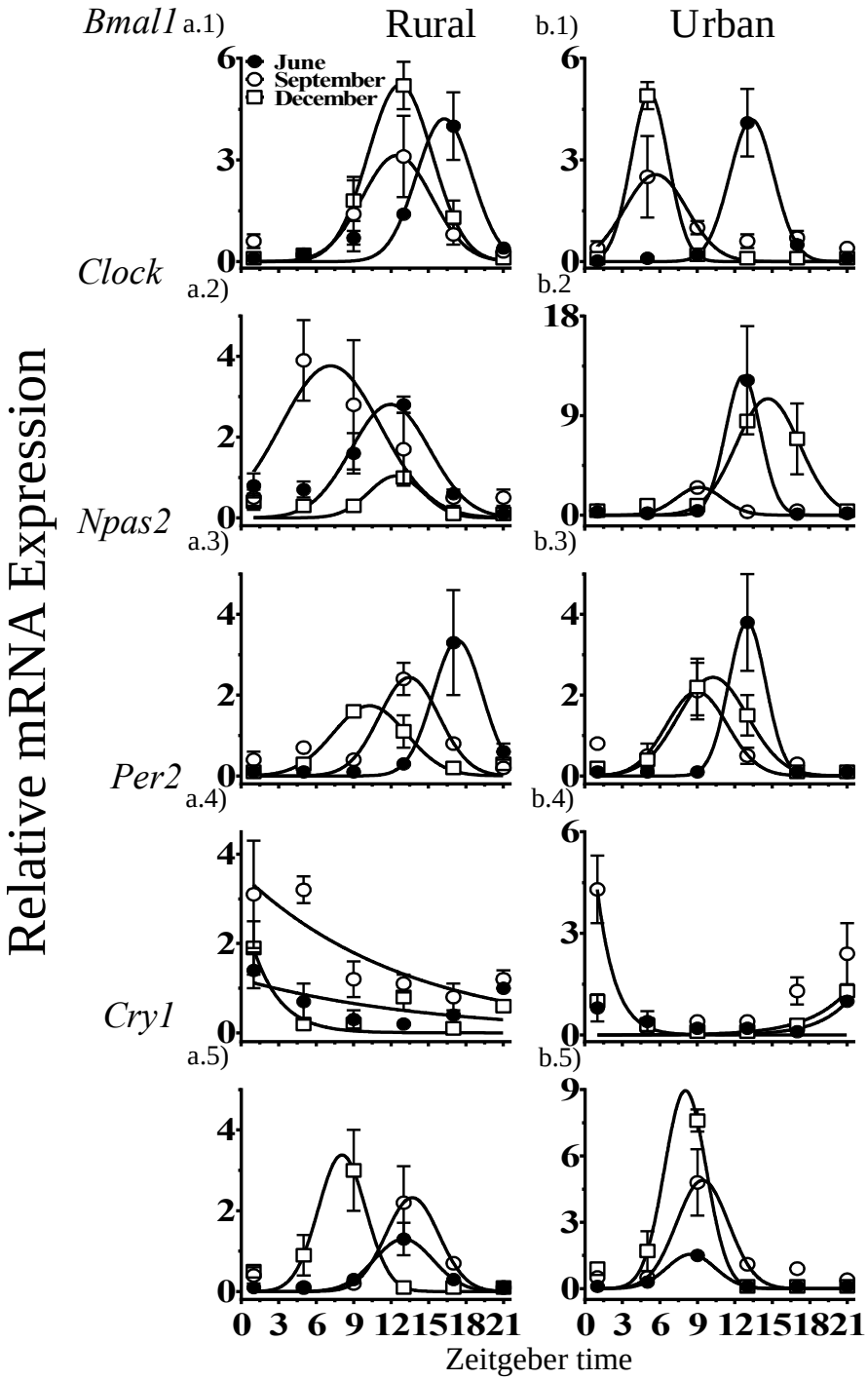

Figure 23: Mean (± SE, N = 5/ time point/site/month) mRNA transcript levels measured at 4h intervals beginning from one hour after sunrise (respective month of the year) over 24 h in tree sparrow (*Passer montanus*) for mRNA coding for clock and clock-controlled transcripts (*Bmal1, Clock, Npas2, Per2, and Cry1*) in the pineal of the birds procured from rural or urban (a.1–a.5) habitat during June (solid circle), September (hallow circle) and December (rectangle). Lines represent best fitted s curve.

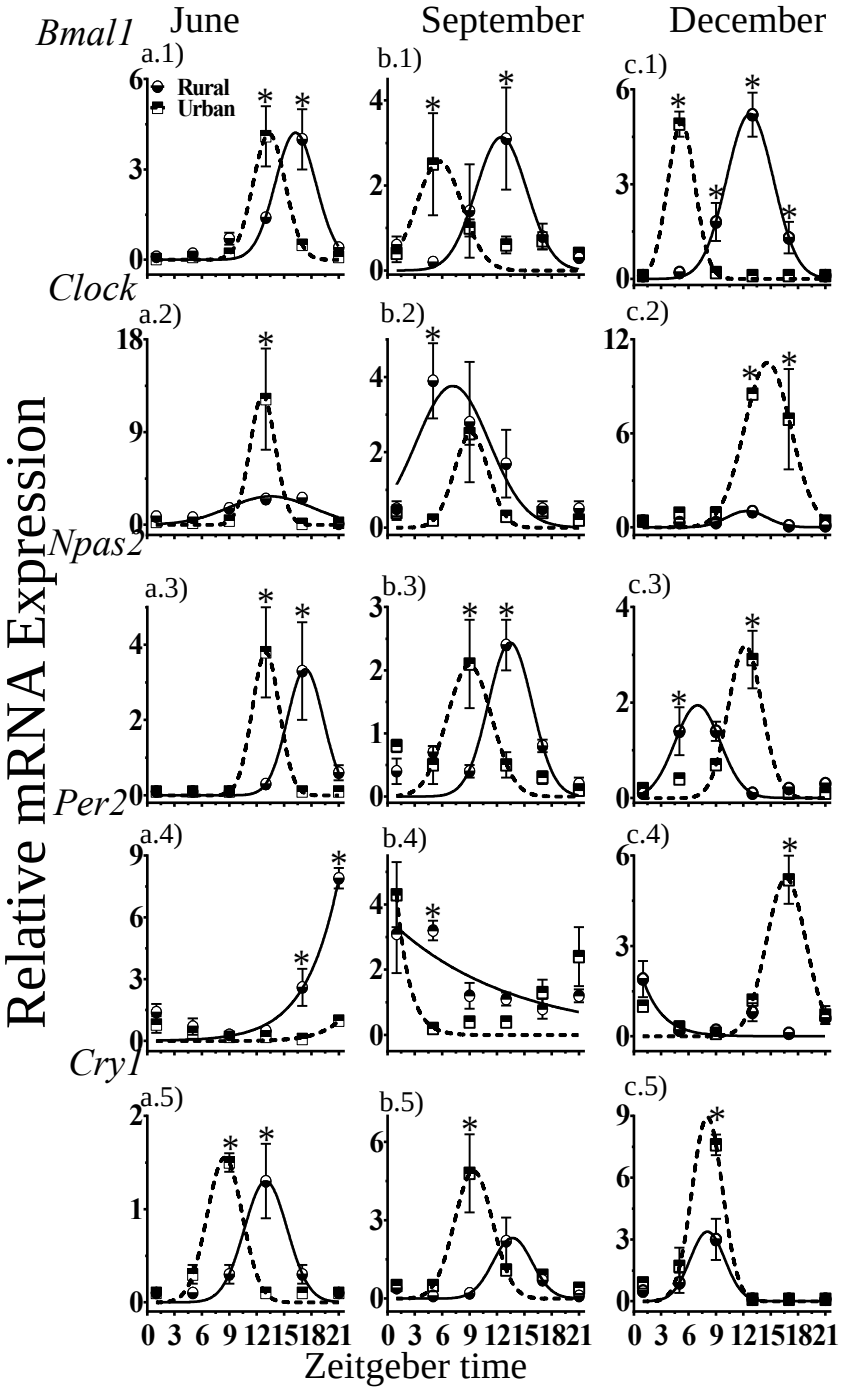

Figure 24: Mean (± SE, N = 5/ time point/site/month) mRNA transcript levels measured at 4h intervals beginning from one hour after sunrise (respective month of the year) over 24 h in tree sparrow (*Passer montanus*) for mRNA coding for clock and clock-controlled transcripts (*Bmal1, Clock, Npas2, Per2, and Cry1*) in the pineal of the birds procured from rural (circle) and urban (rectangle) habitat during June (a.1–a.5), September (b.1–b.5) and December (c.1–c.5). Solid line represents best fitted curve for rural birds while dotted line represents best fitted curve for urban birds. Asterisk (*) represents significant difference between the two habitats at that point determined by Bonferroni posttest, following two-way ANOVA. For statistical significance, the alpha was set at 0.05.

We observed time of the year dependent clock genes expression (Fig. 23) in pineal tissue. In birds procured from rural habitat, *Bmal1* peak expression timing (cosinor analyses) varied from mid-day to late day hours, June (ZT 15.8; Fig. 23a.1), September (ZT 12.6 Fig; Fig. 23a.1) and December (ZT 12.7; Fig. 23a.1) while in urban birds it peaked earlier than rural birds, June (ZT 13.2; Fig. 23b.1), September (ZT 5.7 Fig; Fig. 3b.1) and December (ZT 5.1; Fig. 23b.1). *Clock* gene peaked during middle of the day time in rural birds, June (ZT 11.3; Fig. 23a.2), September (ZT 7.5; Fig. 23a.2) and December (ZT 10.7; Fig. 23a.2) while it was delayed in peak timing in urban birds in correspond to their rural conspecifics in respective months, June (ZT 12.9; Fig. 23b.2), September (ZT 9.2; Fig. 23b.2) and December (ZT 14.5; Fig. 3b.2). *Npas2* peak timing was time of the year dependent, in rural birds it peaked in June (ZT 17.4; Fig. 23a.3), September (ZT 12.7; Fig. 23a.3) and December (ZT 10.5; Fig. 23a.3) while in urban birds it was advanced, June (ZT 17; Fig. 23a.3), September (ZT 8.1; Fig. 23a.3) and December (ZT 10.2; Fig. 23a.3). *Per2* peaked during early daytime throughout the year in rural birds, June (ZT 0.2; Fig. 23a.4), September (ZT 3.2; Fig. 23a.4) and December (ZT 0.2; Fig. 23a.4) while urban birds showed slight advance in peak timing, June (ZT 23.9; Fig. 23b.4), September (ZT 22.9; Fig. 3b.4) and December (ZT 22.6; Fig. 23b.4). *Cry1* peaked during late day time in rural birds, June (ZT 12.8; Fig. 23a.5), September (ZT 13.9; Fig. 23a.5) and December (ZT 7.6; Fig. 23a.5) while urban birds were slightly advanced in peak timing of *Cry1* expression, June (ZT 8.4; Fig. 23b.5), September (ZT 9.7; Fig. 23b.5) and December (ZT 8.0; Fig. 23b.5; Table 7).

2-way ANOVA revealed the effects of habitat, time of day and their interaction on clock genes, June: *Bmal1* (habitat: $F_{1,48}$= 3.901, P= 0.0251; time: $F_{5,48}$= 31.18, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 21.53, P< 0.0001; 2-way ANOVA; Fig. 24a.1; Suppl. Table 2), *Clock* (habitat: $F_{1,48}$= 1.716, P= 0.0196; time: $F_{5,48}$= 12.42, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 8.078, P< 0.0001; 2-way ANOVA; Fig. 24a.2), *Npas2* (habitat: $F_{1,48}$= 0.0492, P=0.8253; time: $F_{5,48}$= 2.716, P=0.0306 and interaction of time x habitat: $F_{5,48}$= 5.323, P=0.0006; 2-way ANOVA; Fig. 24a.3), *Per2* (habitat: $F_{1,48}$= 109.7, P< 0.0001; time: $F_{5,48}$= 56.61, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 40.71, P< 0.0001; 2-way

ANOVA; Fig. 24a.4) and *Cry1* (habitat: $F_{1,48}$= 4.2792, P=0.0159; time: $F_{5,48}$= 33.62, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 37.60, P< 0.0001; 2-way ANOVA; Fig. 24a.5), September: *Bmal1* (habitat: $F_{1,48}$= 5.2599, P=0.0012; time: $F_{5,48}$= 2.716, P=0.0306 and interaction of time x habitat: $F_{5,48}$= 5.323, P=0.0006; 2-way ANOVA; Fig. 24b.1), *Clock* (habitat: $F_{1,48}$= 13.28, P=0.0007; time: $F_{5,48}$= 8.315, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 4.388, P=0.0023; 2-way ANOVA; Fig. 24b.2), *Npas2* (habitat: $F_{1,48}$= 3.9267, P=0.0340; time: $F_{5,48}$= 11.37, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 6.507, P= 0.0001; 2-way ANOVA; Fig. 24b.3), *Per2* (habitat: $F_{1,48}$= 3.181, P=0.0282; time: $F_{5,48}$= 12.53, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 53.69, P< 0.0001; 2-way ANOVA; Fig. 24b.4) and *Cry1* (habitat: $F_{1,48}$= 11.68, P= 0.0013; time: $F_{5,48}$= 10.69, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 12.62, P< 0.0001; 2-way ANOVA; Fig. 24b.5) and December: *Bmal1* (habitat: $F_{1,48}$= 15.19, P= 0.0003; time: $F_{5,48}$= 46.70, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 87.86, P< 0.0001; 2-way ANOVA; Fig. 24c.1), *Clock* (habitat: $F_{1,48}$= 39.83, P< 0.0001; time: $F_{5,48}$= 14.32, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 11.94, P< 0.0001; 2-way ANOVA; Fig. 24c.2), *Npas2* (habitat: $F_{1,48}$= 4.337, P=0.0132; time: $F_{5,48}$= 26.01, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 34.20, P< 0.0001; 2-way ANOVA; Fig. 24c.3), *Per2* (habitat: $F_{1,48}$= 25.14, P< 0.0001; time: $F_{5,48}$= 86.95, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 53.69, P< 0.0001; 2-way ANOVA; Fig. 24c.4) and *Cry1* (habitat: $F_{1,48}$= 25.56, P< 0.0001; time: $F_{5,48}$= 76.84, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 15.43, P< 0.0001; 2-way ANOVA; Fig. 24c.5).

The parameters of rhythm waveform showed a difference in the acrophase, mesor and amplitude of daily oscillations (Table 7). The peak acrophase time was different in urban birds in compare to rural birds. Like hypothalamus there was advance in peak timing in *Bmal1*(June: 2.6 h, September: 6.9 h and December: 7.6 h), *Npas2* (June: 0.4 h, September: 4.6 h and December: 0.3 h), *Per2* (June: 0.3 h, September: 4.3 h and December: 1.6 h) and *Cry1* (June: 4.4 h, September: 4.2 h and December: - 0.4 h) while delay in *Clock* acrophase (June: -1.6 h, September: -1.7 h and December: -4.8 h; Table 7).

4.1.3 Retina

Clock genes had daily variations and rhythmic oscillation in retina of birds throughout the year both in rural and urban habitat (Fig. 25; Cosinor analyses). When we compared the gene expression during three time of the year (within the habitat), significant effects of time of the year, time of day and their interaction was observed in clock gene expression of rural birds; *Bmal1* (months: $F_{2,72}$= 49.75, P< 0.0001; time: $F_{5,72}$= 46.52, P< 0.0001 and interaction of time of day x months: $F_{10,72}$= 38.22, P< 0.0001; 2-way ANOVA; Fig. 25a.1), *Clock* (months: $F_{2,72}$= 17.54, P< 0.0001; time: $F_{5,72}$= 18.80, P< 0.0001 and interaction of time of day x months: $F_{10,72}$= 13.12, P< 0.0001; 2-way ANOVA; Fig. 25a.2), *Npas2* (months: $F_{2,72}$= 3.567, P= 0.0333; time: $F_{5,72}$= 24.39, P< 0.0001 and interaction of time of day x months: $F_{10,72}$= 5.580, P< 0.0001; 2-way ANOVA; Fig. 25a.3), *Per2* (months: $F_{2,72}$= 5.854, P= 0.0164; time: $F_{5,72}$= 6.103, P< 0.0001 and interaction of time of day x months: $F_{10,72}$= 11.77, P< 0.0001; 2-way ANOVA; Fig. 25a.4) and *Cry1* (months: $F_{2,72}$= 59.79, P< 0.0001; time: $F_{5,72}$= 151.9, P< 0.0001 and interaction of time of day x months: $F_{10,72}$= 25.40, P< 0.0001; 2-way ANOVA; Fig. 25a.5). Birds from urban habitat also had significant effects of time of the year, time of day and their interaction on clock genes *Bmal1* (months: $F_{2,72}$= 12.14, P< 0.0001; time: $F_{5,72}$= 33.90, P< 0.0001 and interaction of time of day x months: $F_{10,72}$= 17.90, P< 0.0001; 2-way ANOVA; Fig. 25b.1), *Clock* (months: $F_{2,72}$= 11.08, P< 0.0001; time: $F_{5,72}$= 14.78, P< 0.0001 and interaction of time of day x months: $F_{10,72}$= 11.75, P< 0.0001; 2-way ANOVA; Fig. 25b.2), *Npas2* (months: $F_{2,72}$= 19.27, P< 0.0001; time: $F_{5,72}$= 11.00, P< 0.0001 and interaction of time of day x months: $F_{10,72}$= 5.263, P< 0.0001; 2-way ANOVA; Fig. 25b.3), *Per2* (months: $F_{2,72}$= 7.525, P= 0.0011; time: $F_{5,72}$= 10.07, P< 0.0001 and interaction of time of day x months: $F_{10,72}$= 4.922, P< 0.0001; 2-way ANOVA; Fig. 25b.4) and *Cry1* (months: $F_{2,72}$= 103.5, P< 0.0001; time: $F_{5,72}$= 85.71, P< 0.0001 and interaction of time of day x months: $F_{10,72}$= 78.28, P< 0.0001; 2-way ANOVA; Fig. 25b.5).

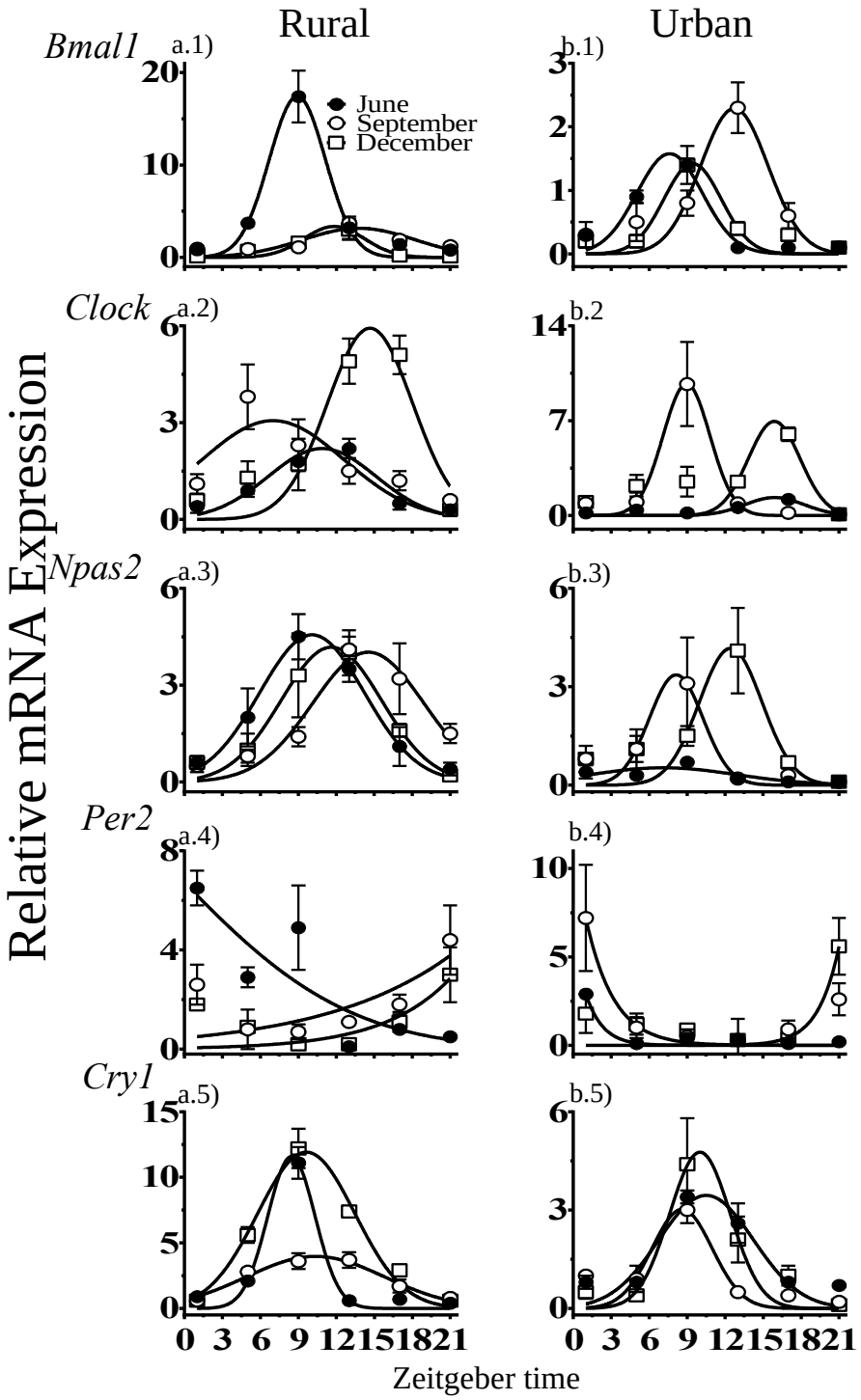

Figure 25: Mean (± SE, N = 5/ time point/site/month) mRNA transcript levels measured at 4h intervals beginning from one hour after sunrise (respective month of the year) over 24 h in tree sparrow (*Passer montanus*) for mRNA coding for clock and clock-controlled transcripts (*Bmal1, Clock, Npas2, Per2, and Cry1*) in the retina of the birds procured from rural or urban (a.1–a.5) habitat during June (solid circle), September (hallow circle) and December (rectangle). Lines represents best fitted s curve.

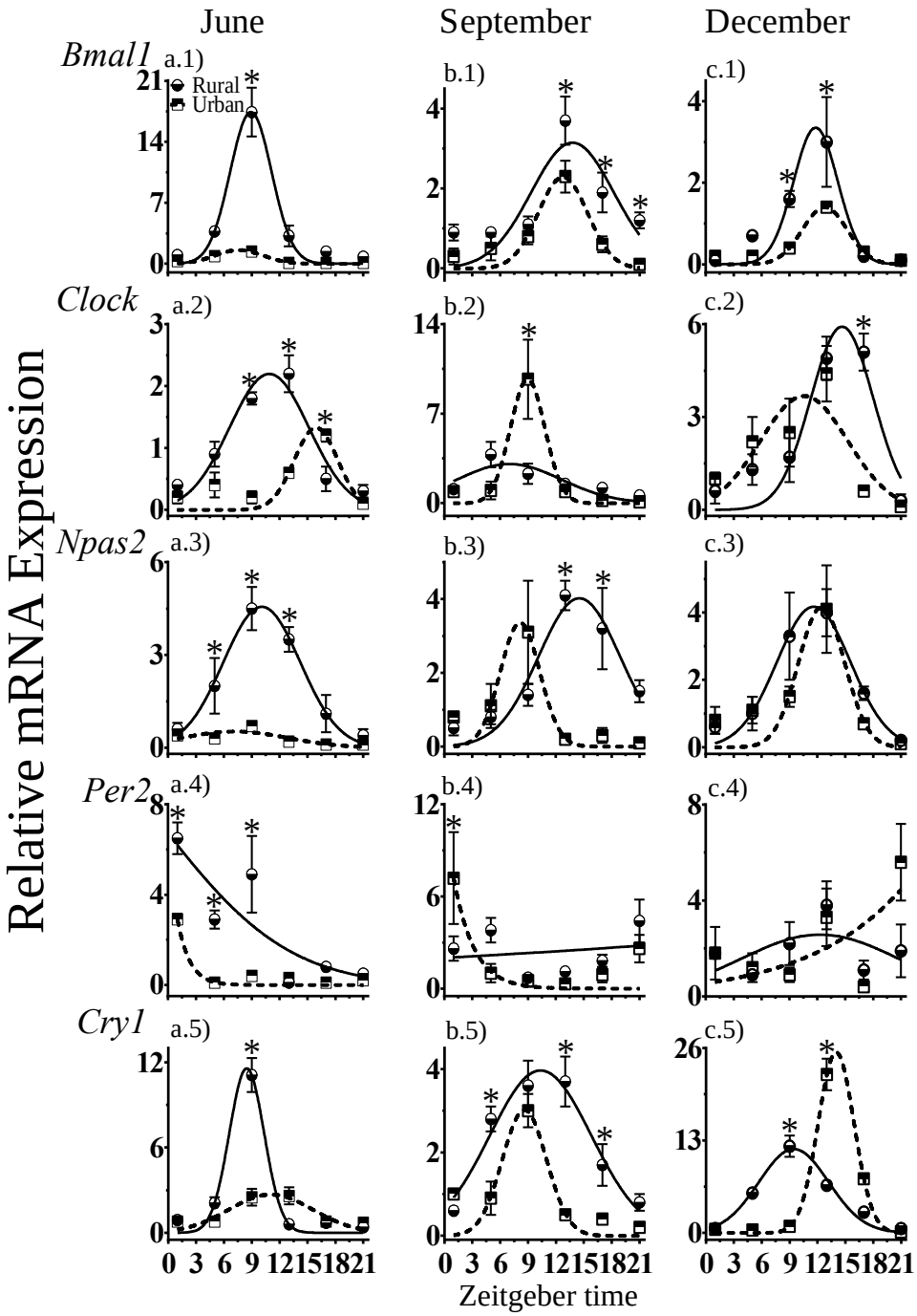

Figure 26: Mean (± SE, N = 5/ time point/site/month) mRNA transcript levels measured at 4h intervals beginning from one hour after sunrise (respective month of the year) over 24 h in tree sparrow (*Passer montanus*) for mRNA coding for clock and clock-controlled transcripts (*Bmal1, Clock, Npas2, Per2, and Cry1*) in the retina of the birds procured from rural (circle) and urban (rectangle) habitat during June (a.1–a.5), September (b.1–b.5) and December (c.1–c.5). Solid line represents best fitted curve for rural birds while dotted line represents best fitted curve for urban birds. Asterisk (*) represents significant difference between the two habitats at that point determined by Bonferroni posttest, following two-way ANOVA. For statistical significance, the alpha was set at 0.05.

We observed time of the year dependent clock genes expression (Fig. 25). In birds procured from rural habitat, *Bmal1* peak expression timing (cosinor analyses) varied from mid-day to late day hours and showed time of the year specific expression, June (ZT 9.0; Fig. 25a.1; Suppl. Table 3), September (ZT14.1; Fig. 25a.1) and December (ZT 11.2; Fig. 5a.1;). *Clock* peaked during mid-day to late day hours during June (ZT 10.6; Fig. 25a.2), September (ZT 7.0; Fig. 5a.2) and December (ZT 9.8; Fig. 25a.2). *Npas2* peak expression timing was observed during late day hours, June (ZT 10.1; Fig. 25a.3), September (ZT 14.6; Fig. 25a.3) and December (ZT 11.5; Fig. 25a.3). *Per2* expression peaked during late night hours, September (ZT 23.8; Fig. 25a.4) and December (ZT 22.7; Fig. 25a.4) except June where it peaked during early day time (ZT 4.2; Fig. 25a.4). *Cry1* peaked was observed during late day time throughout the year, June (ZT 8.5; Fig. 25a.5), September (ZT 10.3; Fig. 25a.5) and December (ZT 9.8; Fig. 25a.5). There were differences in peak expression timing of clock genes in urban birds in comparison to the birds from rural habitat. *Bmla1* expression was around 1-2 h advanced during June (ZT 6.8; Fig. 25b.1), September (ZT 12.1; Fig. 25b.1) and December (ZT9.6; Fig. 25b.1). Peak *Clock* timing was delayed in urban birds throughout the year, June (ZT 15.1; Fig. 25b.2), September (ZT 8.7; Fig. 5b.2) and December (ZT 10.2; Fig. 25b.2). *Npas2* peak expression was advanced in urban birds in comparison to rural birds, June (ZT 6.6; Fig. 25b.3), September (ZT 7.6; Fig. 5b.3) except December (ZT 11.4; Fig. 25b.3). *Per2* expression timing was slightly advanced during June (ZT 1.3; Fig. 25b.4), September (ZT 22.4; Fig. 25b.4) and December (ZT 20.3; Fig. 25b.4). However, Cry1 was peaked slightly earlier in urban birds during September (ZT 7.8; Fig. 25b.5) but no effect was observed during June (ZT 8.9; Fig. 25b.5) and December (ZT 10.4; Fig. 25b.5; Table 7).

Overall, we found significant effects of habitat, time of day and their interaction on clock genes, June: *Bmal1* (habitat: $F_{1,48}$= 107.0, P< 0.0001; time: $F_{5,48}$= 49.72, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 36.97, P< 0.0001; 2-way ANOVA; Fig. 26a.1), *Clock* (habitat: $F_{1,48}$= 70.00, P< 0.0001; time: $F_{5,48}$= 30.29, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 28.76, P< 0.0001; 2-way ANOVA; Fig. 26a.2), *Npas2* (habitat: $F_{1,48}$= 87.14, P< 0.0001; time: $F_{5,48}$= 16.11,

P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 11.44, P< 0.0001; 2-way ANOVA; Fig. 26a.3), *Per2* (habitat: $F_{1,48}$= 65.66, P< 0.0001; time: $F_{5,48}$= 36.24, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 11.08, P< 0.0001; 2-way ANOVA; Fig. 26a.4) and *Cry1* (habitat: $F_{1,48}$= 34.98, P< 0.0001; time: $F_{5,48}$= 86.95, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 53.69, P< 0.0001; 2-way ANOVA; Fig. 26a.5), September: *Bmal1* (habitat: $F_{1,48}$= 38.81, P< 0.0001; time: $F_{5,48}$= 29.98, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 2.279, P= 0.0614; 2-way ANOVA; Fig. 26b.1), *Clock* (habitat: $F_{1,48}$= 9.722, P=0.0139; time: $F_{5,48}$= 14.50, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 10.44, P< 0.0001; 2-way ANOVA; Fig. 26b.2), *Npas2* (habitat: $F_{1,48}$= 13.91, P< 0.0001; time: $F_{5,48}$= 4.934, P= 0.0010 and interaction of time x habitat: $F_{5,48}$= 10.95, P< 0.0001; 2-way ANOVA; Fig. 26b.3), *Per2* (habitat: $F_{1,48}$= 3.398, P=0.0351; time: $F_{5,48}$= 7.473, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 4.273, P= 0.0027; 2-way ANOVA; Fig. 26b.4) and *Cry1* (habitat: $F_{1,48}$= 21.58, P< 0.0001; time: $F_{5,48}$= 10.78, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 3.841, P< 0.0001; 2-way ANOVA; Fig. 26b.4) and December: *Bmal1* (habitat: $F_{1,48}$= 11.43, P= 0.0014; time: $F_{5,48}$= 18.43, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 3.497, P= 0.0089; 2-way ANOVA; Fig. 26c.1), *Clock* (habitat: $F_{1,48}$= 4.239, P= 0.0182; time: $F_{5,48}$= 20.75, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 8.341, P< 0.0001; 2-way ANOVA; Fig. 26c.2), *Npas2* (habitat: $F_{1,48}$= 1.900, P=0.1745; time: $F_{5,48}$= 17.34, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 1.299, P=0.2801; 2-way ANOVA; Fig. 26c.3), *Per2* (habitat: $F_{1,48}$= 3.420, P=0.0319; time: $F_{5,48}$= 7.014, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 3.334, P= 0.0115; 2-way ANOVA; Fig. 26c.4) and *Cry1* (habitat: $F_{1,48}$= 6.369, P=0.0247; time: $F_{5,48}$= 94.44, P< 0.0001 and interaction of time x habitat: $F_{5,48}$= 71.53, P< 0.0001; 2-way ANOVA; Fig. 26c.4).

The parameters of rhythm waveform showed a difference in the acrophase, mesor and amplitude of daily oscillations (Table 7). The peak acrophase time was different in urban birds in compare to rural birds. In urban birds it was advanced for *Bmal1*(June: 2.2 h, September: 2.0 h and December: 1.6 h), *Npas2* (June: 3.5 h, September: 7.0 h except December: 0.1 h), *Per2* (June: 2.8 h, September: 1.4 h and December: 2.4 h) and *Cry1* (June: -0.4; September: 2.5 h and December: - 0.6 h)

except *Clock* (June: -4.5 h, September: -1.7 h and December: -0.8 h; Table 7) where it was delayed in urban birds.

4.2 Tissue-specific phase relationships between circadian genes

We determined the relationship of acrophase among genes within the tissue in relation to *Per2* gene. We observed effect of time of the year. In hypothalamus, in comparison of *Per2* gene, *Bmal1* transcripts peaked later (Fig. 21; Table 8a). Acrophase was delayed in *Bmal1* during June (7.7 h) September (9.9 h) and December (8.0 h). *Clock* transcripts were advanced in June (10.3 h) but delayed during September (4.9 h) and December (8.2 h). *Npas2* peaked advance in June (8.7 h) and December (11.6 h) but delayed during September (10.1 h) while *Cry1* was delayed during September (10.2 h) and December (10.1 h) but advanced (9.5 h) during June. Like hypothalamus, in pineal, in comparison of *Per2* gene, *Bmal1* transcripts were delayed during June (8.4 h) and September (9.4 h) but not in December (11.5 h). *Clock* transcripts were delayed all the three time (June: 11.1 h June, September: 4.3 h and December: 10.5 h). Both *Npas2*, and *Cry1* peaked earlier during June (*Npas2*: 6.8 h; *Cry1*: 11.4 h) but delayed during September (*Npas2*: 9.5 h; *Cry1*: 10.1 h and December (*Npas2*: 10.1 h; *Cry1*: 7.4 h). In retina, *Bmal1* peak was delayed during June (8.9 h; Suppl. Table 5a) but phase advanced during September (9.7 h) and December (11.5 h). Both Clock and *Cry1* were delayed all three times; June (*Clock*: 6.5 h; *Cry1*: 4.4 h September (*Clock*: 7.2 h h; *Cry1*: 10.5 h) and December (*Clock*: 10.5 h; *Cry1*: 11.1 h) while *Npas2* was delayed during June (6.0 h) but phase advanced during September (9.2 h) and December (11.2 h; Table 8a).

4.3 Phase relationships of circadian genes between different tissues

When we compared with reference of peak expression time in the hypothalamus, *Bmal1* peak was delayed both in the pineal (June: 9.0 h, September: 2.2 h and December: 4.6 h; Table 8b) and retina (June: 2.2 h, September: 3.7 h and December: 3.1 h) during all three time of the year. *Clock* peak was advanced in pineal and retina during June (pineal: 1.5 h, retina 2.2 h) but delayed in both tissues at the other two time of the year i.e., September (pineal: 2.1 h, retina 1.6 h) and December (pineal: 2.4 h, retina 1.5 h). *Npas2* peak was delayed in June in pineal (3.0 h) and in both

pineal (7.7 h) and retina (9.6 h) in September but it was advanced in June (4.3 h) in retina and in December both in pineal (2.0 h) and retina (1.0 h). *Per2* peak was delayed in pineal during all three times (June: 1.1 h, September: 2.7 h and December: 0.1 h) but only during June (5.0 h) in retina. In retina *Per2* peak was advance during September (0.7h) and December (1.4 h). *Cry1* peak was advanced in pineal (June: 0.8 h, December: 2.6 h) and retina (June: 5.1 h, September: 0.4 h and December: 0.4 h) throughout the year except in pineal during September (3.2 h; Table 8b).

4.4 Season dependent relationships among circadian genes

We used September as a reference point to compare acrophase among different circadian transcripts. In both hypothalamus and retina *Bmal1* acrophase was advance during June (hypothalamus: 3.6 h, retina: 5.1 h; Table 8d) and December (hypothalamus: 2.3 h, retina: 2.9 h) while it was delayed pineal (June: -3.2 h and December: -0.1 h). Acrophase in *Clock* was always delayed both during June (hypothalamus: -7.4 h, pineal: -3.7 h, retina: -3.6 h) and December (hypothalamus: -2.3 h, pineal: -3.2 h, retina: -2.8 h). *Npas2* acrophase was delayed during June in hypothalamus (-9.7h; Suppl. Table 4d) and pineal (-4.7 h) but only during December in hypothalamus (-7.5 h) while it was advanced during December (2.2 h) in pineal and both during June (4.5h) and December (3.1 h) in retina. *Per2* acrophase was delayed in hypothalamus (June: -1.4 h, December: -0.4 h) only during June (-4.3 h) in retina but was advanced during December (1.1 h) in retina and both June (3.0 h) and December (3.0 h) in pineal. Except June (-2.9 h) hypothalamus *Cry1* acrophase was always advanced, December (Hypothalamus: 0.5 h), June (pineal: 1.1 h, retina: 1.8h; Suppl. Table 4d) and December (pineal: 6.3 h, retina: 0.5 h; Table 8d).

Tissue	Gene	Site	June			September			December		
			Mesor	Amplitude	Acrophase	Mesor	Amplitude	Acrophase	Mesor	Amplitude	Acrophase
Hypothalamus	*Bmal1*	Rural	1.7	2.4	6.8	0.4	0.6	10.4	1.3	2.1	8.1
		Urban	0.4	0.7	6.8	0.4	0.6	7.3	2.3	3.9	5.9
	Clock	Rural	0.6	1.1	12.8	2.4	1.7	5.4	0.2	0.4	8.3
		Urban	0.6	1.0	5.2	0.4	0.4	8.7	1.0	1.6	9.0
	Npas2	Rural	0.5	0.6	14.4	2.8	3.1	5.0	0.7	1.3	12.5
		Urban	0.2	0.3	9.2	0.3	0.2	5.7	1.4	2.1	10.0
	Per2	Rural	1.4	1.6	23.1	2.6	2.5	0.5	0.8	0.5	0.1
		Urban	0.9	1.3	22.0	0.9	0.4	23.5	0.6	1.0	20.6
	Cry1	Rural	0.3	0.4	13.6	0.7	.0.9	10.7	0.1	0.2	10.2
		Urban	0.8	1.0	12.4	0.2	0.1	10.0	0.2	0.4	9.5
Pineal	*Bmal1*	Rural	1.1	1.6	15.8	1.1	1.1	12.6	1.4	2.2	12.7
		Urban	0.8	1.4	13.2	0.9	0.8	5.7	0.9	1.6	5.1
	Clock	Rural	1.1	0.9	11.3	1.7	1.7	7.5	0.4	0.2	10.7
		Urban	2.2	4.0	12.9	.6	0.7	9.2	3.0	4.1	14.5
	Npas2	Rural	0.7	1.2	17.4	0.8	0.7	12.7	0.6	0.7	10.5
		Urban	1.9	2.5	17	0.7	0.7	8.1	0.7	1.0	10.2
	Per2	Rural	2.2	3.0	0.2	1.7	1.3	3.2	0.6	0.5	0.2
		Urban	0.6	0.4	23.9	1.5	1.7	22.9	0.5	0.6	22.6
	Cry1	Rural	0.4	0.5	12.8	0.6	0.8	13.9	0.7	1.1	7.6

		Urban	0.3	0.5	8.4	1.4	1.5	9.7	1.7	2.8	8.0
Retina	*Bmal1*	Rural	4.5	6.3	9.0	1.6	1.2	14.1	1.0	1.3	11.2
		Urban	0.4	0.7	6.8	0.8	0.8	12.1	0.4	.0.5	9.6
	Clock	Rural	1.0	1.0	10.6	1.8	1.3	7.0	2.4	1.7	9.8
		Urban	0.4	0.3	15.1	2.1	3.3	8.7	1.8	1.8	10.2
	Npas2	Rural	2.0	2.1	10.1	1.9	1.7	14.6	1.8	1.9	11.5
		Urban	0.3	0.3	6.6	0.9	1.1	7.6	1.4	1.4	11.4
	Per2	Rural	2.6	2.6	4.1	2.4	1.5	23.8	1.1	1.0	22.7
		Urban	0.7	0.9	1.3	1.1	0.9	22.4	2.2	1.2	20.3
	Cry1	Rural	2.6	4.0	8.5	2.2	1.8	10.3	4.8	5.4	9.8
		Urban	1.0	0.7	8.9	1.0	.97	7.8	1.3	1.7	10.4

Table 7: Rhythm parameters of all nine genes in different tissues under different habitat of tree sparrow, as determined by cosinor analyses.

Gene	Hypo			Pineal			Retina		
	June	Sep	Dec	June	Sep	Dec	June	Sep	Dec
Bma1	-7.7	-9.9	-8.0	-8.4	-9.4	11.5	-8.9	9.7	11.5
Clock	10.3	-4.9	-8.2	-11.1	-4.3	-10.5	-6.5	-7.2	-11.1
Npas2	8.7	-4.5	11.6	6.8	-9.5	-10.3	-6.0	9.2	11.2
Cry1	9.5	-10.2	-10.1	11.4	-10.1	-7.4	-4.4	-10.5	-11.1

Table 8a: Relationship in time of peak expression of clock gens within the tissue in reference to *Per2* acrophase

Gene	Pineal			Retina		
	June	Sep	Dec	June	Sep	Dec
Bma1	-9	-2.2	-4.6	-2.2	-3.7	-3.1
Clock	1.5	-2.1	-2.4	2.2	-1.6	-1.5
Npas2	-3	-7.7	2	4.3	-9.6	1.0
Per2	-1.1	-2.7	-0.1	-5.0	0.7	1.4
Cry1	0.8	-3.2	2.6	5.1	0.4	0.4

Table 8b: Relationship in time of peak expression of clock gens between tissues in reference to hypothalamus

Gene	Hypo			Pineal			Retina		
	June	Sep	Dec	June	Sep	Dec	June	Sep	Dec
Bma1	0	3.1	2.2	2.6	4.6	0.3	2.2	2.0	1.6
Clock	-2.4	-3.3	-0.7	-1.6	-1.7	-4.8	-4.5	-1.7	-0.8
Npas2	5.2	-0.7	2.5	0.4	4.6	0.3	3.5	7	0.1
Per2	1.1	1.0	3.5	0.3	4.3	1.6	2.8	1.4	2.4
Cry1	1.2	0.7	0.7	4.4	4.2	-0.4	-0.4	2.5	-0.6

Table 8c: Acrophase of clock genes in urban birds in three central tissues at three time of the year with reference to rural birds

Gene	Hypo			Pineal			Retina		
	June	Dec		June	Dec		June	Sep	Dec
Bmal1	3.6	2.3		-3.2	-0.1		5.1		2.9
Clock	-7.4	-2.3		-3.7	-3.2		-3.6		-2.8
Npas2	-9.4	-7.5		-4.7	2.2		4.5		3.1
Per2	-1.4	-0.4		3	3		-4.3		1.1
Cry1	-2.9	0.5		1.1	6.3		1.8		0.5

Table 8d: Acrophase of clock genes during June and December in rural birds with reference to September month

5. DISCUSSION

Seasonal mRNA expression patterns were investigated for clock genes in three central tissues (hypothalamus, pineal and retina) of wild-caught *P. montanus* at six daily time-points during three different times of the year, i.e., June, September and December. Our results show the persistence of circadian clock (transcriptional level) oscillations in three central tissues (hypothalamus, pineal and retina) at three different times (corresponding to three different physiological stages) of the year (Fig. 1-6). Our results indicate that the expression patterns of clock genes are tissue-specific and are differently affected by changes in the photoperiodic environment (time of the year). In rural birds, in comparison to September (equinox time when day and night lengths are approximately the same), both June and December birds had phase advances in peak expression timing (acrophase) of *Bmal1* in the hypothalamus and retina, but it was delayed in the pineal gland (Suppl. Table 4d). Acrophases of *Clock* genes were delayed in all three tissues in June and December in comparison to September (Table 8d). In the hypothalamus, *Npas2* peak expression timing was delayed in June and December; in the pineal gland, it was delayed during June but advanced during December; and in the retina, it was advanced both in June and December. In reference to September, *Per2* peak expression timing was delayed during June and December in the hypothalamus, during June in the retina, but advanced during June and December in the pineal gland and during December in retina. *Cry1* peak expression timing was advanced both in June and December in all three tissues except for June in the hypothalamus. The association of photoperiod and/or season with clock gene transcription have been observed in few previous studies on mammals (Tournier *et al*., 2003), birds (Singh *et al*., 2015), fish (Davie *et al*., 2009; Herrero and Lepesant, 2014) and insects (Goto and Denlinger, 2002). In natural conditions, the lengthening and shortening of photoperiods affect the phases of both dark (D)/light (L) and L/D transitions, and corresponding changes in the peak timing of the clock genes were observed in our study. We used expression timing in the hypothalamus as a reference for determining the relationship to peak expression timing of genes in pineal and retina tissues. We observed phasing in peak timing between the

hypothalamus and the other two neural tissues, and this is consistent with our previous report for this species (Renthlei *et al*., 2019) and other species (Singh *et al*., 2013; Agarwal *et al*., 2018).

The main objective of this study was to see if there were season-dependent effects of urban environments on clock gene expression. We clearly observed the effects of season/time of the year on clock gene expression. *Bmal1* mRNA always peaked in advance in urban birds (Fig. 2,4,6; Table 7). The maximum change in peak timing of *Bmal1* was observed during September in the hypothalamus (3.1 h) and the pineal gland (4.6 h) but in June in the retina (2.2 h), while the minimum change in peak was observed during December (hypothalamus: 2.2 h, pineal: 0.3 h and retina: 1.6 h; Table 8). There was no difference in peak timing in the hypothalamus during June. Similarly, *Per2* and *Npas2* peaks were always advanced in urban birds except for *Npas2* during September in the hypothalamus (Table 7). Our results show that the peaks of *Clock* transcripts were always delayed in urban birds while mixed responses were observed for *Cry1* where it was advanced in all tissues most of the time, except in June in the retina and both in the pineal gland and retina during December.

Differences in peak timing of clock genes between urban and rural birds were documented in our previous study which compared the clock gene expression in the hypothalamus, pineal and retina tissues of urban and rural tree sparrows[21] during the month of March. In our previous study, when we compared the expression of clock genes at only one time of the year, we observed that *Bmal1*, *Clock* and *Per2* transcripts peaked earlier (1 to 5 h), while *Npas2* and *Cry1* genes were delayed (1 to 5 h) in their peak expression timing in urban birds. In this study, *Bmal1, Npas2, Per2* and *Cry1* acrophases were advanced 0.1 to 7 h, while the *Clock* acrophase was delayed by 0.7 h to 4.8 h, depending on the tissue and time of the year (Supplementary Table 3), suggesting that season/time of the year modulates peak timing of the clock gene. Here, two factors are critical: (1) availability, duration and intensity of sunlight during different times of the year; and (2) urban environments differ from rural environments because of the availability of extra light at night in urban environments. These two factors could be the potential causes

of differing expression patterns in clock genes of the birds in the two habitats. Previous laboratory studies support the hypothesis that dim lights at night can affect the circadian system. In Siberian hamsters, acute light exposure changes the expression pattern of *Per1* in SCN (Grone *et al*., 2011), while exposure to dim lights at night leads to higher expression levels in the mRNA of *Per1* and *Mel1A* (Ikeno *et al*., 2014). Similarly, chronic exposure to dim lights at night affects circadian clock genes in both the SCN and peripheral tissues in mice and attenuates the rhythm in *Per* genes and protein expression in the SCN during the light/dark transition (Shuboni and Yan, 2010). Some limited evidences also suggest the adverse effects of exposure to extra light at night can lead to metabolic disorders (van Amelsvoort *et al*., 1999; Parkes, 2002; Obayashi *et al*., 2013) and hence decrease the fitness of the organisms. Another reason for the differential effects of urban environments on clock gene expression could be due to differential sensitivity of the photoreceptors during the different times of the year (Majumdar *et al*., 2015) or differential physiological states (Singh *et al*., 2015) or a combination of both as tree sparrows live in close proximity to human habitats, and hence, the chances of exposure to cities' night lights are high for urban birds.

In conclusion, we have provided an extensive characterisation of the clock and clock-controlled genes in the central circadian oscillators (hypothalamus, pineal gland and retina) of tree sparrows from rural and urban habitats during three different times (corresponding to different physiological conditions), i.e., June (long days), September (equinox photoperiod) and December (short days). These findings are significant as they provide the first evidence in support of the effect of season-dependent influences of the urban environment on circadian functions.

SECTION VIII: SEASON DEPENDENT EFFECTS OF URBAN ENVIRONMENT ON SEASONAL CLOCK

1. ABSTRACT

Urbanization is a rapidly growing phenomenon that affects wildlife. Laboratory studies show the effects of night light on the physiology of the organisms. Limited studies have been conducted on birds in their natural habitat. Here we studied the effects of the urban environment on reproduction linked phenomenon and molecules involved in the regulation of seasonal breeding. Birds (N=5/time/site) were procured from urban and rural sites at specific times, i.e., in March (stimulatory phase), June (reproductive phase), September (refractory phase), and December (sensitive phase) of 2018. Immediately after procurement, birds were brought to the laboratory. Bodyweight, bill color, molt in body feathers, and testes size were recorded. The next day all the birds were sacrificed in the middle of the day. Blood was collected and serum was used for ELISA of corticosterone, triiodothyronine (T3) and thyroxine (T4). mRNA levels of thyroid-stimulating hormone-β (*Tshβ*), type 2 deiodinase (*Dio2*), type 3 deiodinase (*Dio3*), gonadotropin-releasing hormone (*GnRh*) and gonadotropin inhibitory hormone (*GnIh*) were measured in hypothalamic tissue. Urban birds showed higher levels of corticosterone during the stimulatory phase. There was a delay in the initiation of testicular growth in urban birds and it was supported by reduced levels of T3 in blood plasma and relatively lower transcription of *Dio2* and *GnRH mRNA* in urban birds. Our findings suggest that the urban environment delays the timing of reproduction in birds and could be the consequence of local environmental conditions.

2. INTRODUCTION

Cities are better at providing amenities for human beings but pose a greater threat to biodiversity by altering the population and composition of flora and fauna of the ecosystem (McKinney, 2002, 2006; McDonnell and Hah, 2008; Shochat *et al*., 2010; Aronson *et al*., 2014). Anthropogenic light pollution is a major consequence of urbanization (Shochat *et al*., 2006) and is a significant threat to the urban wildlife population (Longcore and Rich, 2004). The effects of the urban/artificial night light environment have been studied on various physiological parameters; foraging behavior (Santos *et al*., 2010; Fuirst *et al*., 2018), survival rate and stress responses (Honryo *et al*., 2012), community dynamics (McKinney, 2006), reproductive physiology (Partecke *et al*., 2005; 2006; Dominoni *et al*., 2013a; Dominoni *et al*., 2014; Zhang *et al*., 2014), activity patterns (Riley *et al*., 2003; Spoelstra *et al*., 2018), metabolism (Liker *et al*., 2008; Batra *et al*., 2019), behavior (Rees *et al*., 2009; Dominoni *et al*., 2014), sleep (Raap *et al*., 2017) and endocrine system (Dominoni *et al*., 2013b). Recently we have also shown the effects of urban night light environment on the circadian clock of tree sparrow (Renthlei and Trivedi, 2019).

Birds are predominantly seasonal breeders. In the last two decades, the mechanism involved in the regulation of seasonal reproduction has been revealed. In Japanese quail (*Coturnix coturnix japonica*), it has been demonstrated that the ependymal cells (ECs) lining the ventrolateral walls of the third ventricle within the mediobasal hypothalamus (MBH) and pars tuberalis (PT) of the anterior pituitary gland are upstream key centers for seasonal reproduction (Yoshimura *et al*., 2003; Nakao *et al*., 2008). Under stimulatory photoperiod, PT secretes thyroid-stimulating hormone (TSH, thyrotropin). It acts on the ECs within the MBH initiates transcription of type 2 deiodinase (*Dio2*) and suppresses type 3 deiodinase (*Dio3*) via the TSH receptor signalling pathway (Nakao *et al*., 2008). *Dio2* encodes thyroid hormone (TH) activating enzyme while *Dio3* is responsible for encoding the TH inactivating enzyme. This TH induces structural changes between the GnRH nerve terminals and the glial endfeet in the median eminence (ME) (Yamamura *et al*., 2006) and modulates seasonal gonadotropin-releasing hormone (GnRH) secretion

from the hypothalamus to the portal capillary system which leads downstream processes at gonadal levels (Yoshimura *et al*., 2003; Yamamura *et al*., 2006). The simultaneous decrease in the transcription of a gene coding for gonadotropin-inhibitory hormone (GnIH) advocates its involvement in seasonal reproduction via the inhibition of GnRH activity (Tsutsui *et al*., 2000). A similar mechanism has been suggested for other avian species, as these molecules are regulated by photoperiod and their expression is physiology dependent (Dixit and Byrsat, 2018; Zhang *et al*., 2019; Trivedi *et al*., 2019).

Tree sparrows (*Passer montanus*) are long-day breeders (Dixit and Singh, 2011, 2012) and the involvement of these candidate genes in the regulation of seasonal reproduction has been shown earlier (Dixit and Byrsat, 2018; Zhang *et al*., 2019; Dixit *et al*., 2017). Artificial anthropogenic light sources advance the onset of luteinizing hormone (LH) secretion in urban tree sparrow populations (Zhang *et al*., 2014) and the intensity-dependent effects of dLAN (dim light at night) on the activation/suppression of the hypothalamic-pituitary-gonadal axis (HPG axis) (Zhang *et al*., 2019) has been reported. These previous studies addressed the effects of dLAN on the HPG axis under laboratory conditions by exposing the animals to artificial light-dark conditions (Dominoni and Nelson, 2018; Dominoni *et al*., 2020; Helm *et al*., 2013; Salmón *et al*., 2018; Salmón *et al*., 2017). How the urban environment is affecting the reproduction in the wild is largely unknown. Seasonal breeders are highly sensitive to change in photoperiodic conditions. As an urban environment provides extra light at night, therefore, we predicted that it may alter the timing of reproduction. To address this, we selected tree sparrows as an experimental model because of two reasons (i) tree sparrows prefer to live in close proximity with human habitat and (ii) this species is widely available in the core city areas of Aizawl (study site). Here we studied physiological (body mass, bill color, body molt, and testicular volume), biochemical (corticosteroid, triiodothyronine, and thyroxine) and molecular parameters (*Tshβ, Dio2, Dio3, GnRH,* and *GnIH*) related with the seasonal reproductive physiology of the birds living in an urban environment and compared it with birds living in the nearby rural habitat of Aizawl, Mizoram, India.

3. MATERIAL AND METHODS

3.1 Animals and Tissue Collection

Using mist nets, tree sparrows (*Passer montanus*) were captured in the middle of the month at four times of the year (March, June September and December 2018) at two locations, urban and rural sites as described previously (Renthlei and Trivedi , 2019). The urban site was situated in the city of Aizawl (Mizoram, India; 23.7° N 92.7° E; 1130 m above sea level; mean temperature recorded at the urban site was 27.2 °C [March], 32.5 °C [June], 30.5 °C [September] and 27.6 °C [December]) and the rural site was selected as Mizoram University campus (23.7° N 92.6° E; 800 m above sea level; mean temperature recorded at the rural site was 29.7 °C [March], 34.4 °C [June], 32.5 °C [September] and 27.5 °C [December]). The population density of humans was approximately 737 individuals/km^2 in urban habitat while 375 individuals/ km^2 in rural habitat. Mizoram University campus is approximately 15 km from the west side of the city of Aizawl. The university campus is spread over an area of approximately 395 hectares, in an environment of regenerating tropical wet evergreen and semi-evergreen forests. The average night light intensity recorded was 13.3 lux and 8.6 lux at the urban and the rural sites respectively (Renthlei and Trivedi, 2019). The four times of the year selected in the study correspond to the birds' annual reproductive cycle, i.e., March corresponds to the stimulatory phase (growing gonads), June corresponds to the reproductive phase (gonads are at their maximum size), September corresponds to the refractory phase (exposure to stimulatory photoperiod will not lead gonads to grow or maintain their size) while December corresponds to the sensitive phase (stimulatory photoperiod induces gonadal growth (Dixit and Singh, 2011; Macdougall-Shackleton *et al*., 2009). Immediately after collection, the birds were transported to the lab and kept in NDL (natural daylength conditions but in captivity). Bodyweight was recorded using a top pan balance to the precision of 0.1 g. Body molt was recorded as per Trivedi *et al*., 2006. The color of the bill was assessed using a subjective criterion as reported earlier (Trivedi, 2005). As tree sparrows are sexually monomorphic, laparotomy was performed to identify the gender of the birds. The size of the left testis was recorded by unilateral laparotomy as described by Kumar *et al*., 2001.

Laparotomy is a minor surgical process during which a small incision was made between the last two ribs on the left flank. The left testis was located within the abdominal cavity with the help of a spatula. We measured the length and width of the left testis and testis volume (TV) was calculated using the formula 4/3πab2, where a and b denote half of the long (length) and short (width) axes, respectively. The next day male birds (N=5/time/site) were killed by decapitation in the middle of the day. Blood was collected; serum was harvested and stored at -20 °C. Hypothalamus was harvested, stored in RNA later solution (Thermo Fisher Scientific, USA, AM 7020) first overnight at 4 °C later on at –80 °C.

3.2 Hormone assay

Serum corticosterone (CS), thyroxine (T4) and tri-iodothyronine (T3) levels were measured using chicken specific immune assay enzyme kits (KINESISDx cat No K16-0020, 0021 and 0016, respectively) with an automated Biospectrometer microplate reader (Eppendorf, Germany) following manufacturer's instructions. Before starting the assay, plasma samples were allowed to thaw on ice. Standards were diluted using provided standard concentration and standard diluents as per protocol provided. Thereafter, 50 µL of standards (ran in triplicate) and 40 µL samples (ran in duplicate) were transferred into respective wells of 96 well plate followed by 10 µL of Biotin conjugate into each sample well. 50 µL of HRP conjugate was pipetted into each sample and standards well and incubated for 1hr at 37 °C. Then, washed four times with 1X Wash Buffer and the residual buffer was blotted by firmly tapping the plate upside down on absorbent paper. Substrate A 50 µL and Substrate B 50 µL were added to each well including blank and incubated for 10 min at 37 °C in dark. 50 µL of Stop Solution was added and wells turned from blue to yellow in color. The absorbance was taken at 450 nm within 15 minutes after adding the Stop Solution. Intra assay variability was 5.2%, 6.1%, and 5.7% for CS, T4, and T3 levels respectively.

3.3 Gene transcription:

mRNA transcription of genes coding for *Tshβ, Dio2, Dio3, GnRh*, and *GnIh* were measured in the hypothalamus of each individual procured from urban and rural sites at the four times of the year, as described previously for gene transcription studies (Trivedi *et al*., 2014; Renthlei *et al*., 2019). Primer sequences used for *Tshβ* and *GnRh* (Zhang *et al*., 2019) and *18S* (Renthlei and Trivedi, 2019; Renthlei *et al*., 2019) were selected from previously published studies. Primer sequences of *Dio2*, *Dio3*, and *GnIh* are mentioned in Table 9.

3.4 RNA Isolation, cDNA Synthesis

Tri reagent solution (Ambion AM9738; USA) was used to extract RNA following the manufacturer's protocol. We used 1-µg RNA (quantified on Nanodrop; Thermo Scientific, USA) to prepare cDNA. RQ1 DNase (Promega M6101; Wisconsin, USA) kit was used to remove any genomic DNA contamination present. cDNA was synthesized using the first-strand cDNA synthesis kit (Thermo Scientific, K1622; Lithuania, Europe).

3.5 Quantitative (Real-time) RT-PCR (qPCR)

A Quant-Studio 5 (Applied Biosystem by Thermo Fisher Scientific; USA) machine was used for qPCR amplification as detailed in the previous publication from our lab (Renthlei and Trivedi , 2019; Renthlei *et al*., 2019; Borah *et al*., 2020). 18S was used as a reference gene (Renthlei and Trivedi , 2019; Renthlei *et al*., 2019). We followed the $\Delta\Delta C_t$ method (Livak and. Schmittgen, 2001) to determine the relative transcription of genes. qPCR protocol included enzyme activation at 95 ^{0}C 3 min (1 cycle), denaturation at 95 ^{0}C 15 sec, annealing at 60 ^{0}C for 1 min, extension at 95 ^{0}C for 15 sec (denaturation to extension was done for 40 cycles).

3.6 Statistical analysis

All statistics and graphs were prepared using Graph pad prism 6.0. Data are presented as mean (mean and SE). The student t-test was used to compare two point

values. The significance of difference within the groups was tested using a one-way analysis of variance (1-way ANOVA) followed by post hoc test (Newman-Keuls). To determine the significance of the difference between the groups comparing two factors together (urban vs. rural site and time of year), Two-way analysis of variance (2-way ANOVA) followed by post-test (Bonferroni) was applied.

Gene	*F primer*	*R Primer*	*Accession No.*
Dio2	5'–CCT ACA AGC AGG TCA AAC TGG –3'	5'–ACT TGC CAC CAA CGT TCT TC–3'	KX951490.1
Dio3	5'–CCT ACA ACA TCC CCA AGC AC–3'	5'–AAG CAT TGT CCA TGG TGT CC–3'	KX951491.1
GnRh	F: 5-ATC GCA AAC GAA ATG GAA AG-3	R: 5-CTG GCT TCT CCT TCG ATC AG-3	NM204321
GnIh	5'–CCA GGA GGT GCA AAT GA–3'	5'–CTT GTT CTT CCA TCA GCC T–3'	KT351598.1
Tshβ	F: 5-CCA CCA TCT GCG CTG GAT-3	R: 5-GCC CGG AAT CAG TGC TGT T-3	NM205063
18S	5' –GAC GCG TGCATT TATCAG3'	5' GTT GAT AGG GCA GAC ATT 3'	D38344.

Table 9: Gene specific primers used for qPCR analysis

3. RESULTS

3.1 Annual Reproductive cycle and affiliated behavior

There was no effect of habitat, time and interaction of two factors on change in the body mass (habitat: $F_{1,72}$ = 1.323, P = 0.2537; time: $F_{1,72}$ = 0.1112, P = 9533 and Interaction $F_{1,72}$ = 0.6341, P = 5954; 2-way ANOVA; Fig 27a;). We observed an effect of habitat, time and interaction of habitat and time on change in bill color (habitat: $F_{1, 72}$ = 36.14, P < 0.0001; time: $F_{3,72}$ = 49.05, P < 0.0001 and Interaction: $F_{3,72}$ = 5.788, P = 0.0013; 2-way ANOVA; Fig. 27b). Bill color showed reproduction dependent changes and dark-colored bills were observed during the breading phase (March and June) while straw-colored bills were observed during the post-breeding phase (September and December; Fig. 27b). Further, in December bill score was significantly higher in rural birds then urban birds (P < 0.01, Bonferroni test). The annual testicular growth and regression cycle was also observed in birds of both sites (Fig. 27c). There was an effect of habitat, time and interaction of habitat and time on testicular growth regression cycle (habitat: $F_{1,72}$ = 4.215, P = 0.01386; time: $F_{1,72}$= 80.55, P < 0.0001 and Interaction $F_{1,72}$ = 9.023, P < 0.0001; 2-way ANOVA; Fig. 27c). The higher testicular activity was observed during March and June in birds from both sites (Fig. 27c). Interestingly, testicular growth was slightly delayed in urban birds and during March testicular volume was significantly low in urban birds (P = 0.002; Bonferroni multiple comparison test). Rest time of the year (June, September, and December) we did not see the effect of habitat, and testicular volume was similar in birds of both habitat (Fig. 1c). The interaction results suggest that the testicular responses of the urban birds depend on the habitat and time of the year (Fig. 28). Molt in body feathers reflected seasonal variations, both in rural and urban birds, and followed the annual reproductive cycle. There was an effect of time and interaction of habitat x time but not of habitat alone on body moult (habitat: $F_{1,72}$ = 3.246, P = 0.0758; time: $F_{3,72}$ = 26.61, P < 0.0001 and Interaction: $F_{3,72}$ = 3.474, P = 0.0204; 2-way ANOVA; Fig. 27d). No molt in body feathers was observed during the breeding phase (March and June) and high molt in body feathers was recorded during the post-breeding phase (September), by December molting was completed (Fig. 27d). Although, we do not see the main effect on

feather molt but the interaction result suggest that the molt in birds depends on the habitat and time of the year (Fig. 27).

3.2 Hormone Assay

Corticosterone levels varied significantly between rural and urban birds. There was an effect of habitat, time and Interaction of habitat and time on corticosterone levels (habitat: $F_{1,32}$ = 7.455, P = 0.0102; time: $F_{3,32}$ = 6.963, P = 0.0010 and Interaction of habitat and time $F_{3,32}$ = 3.780, P = 0.0369; 2-way ANOVA; Fig. 28a;). In March corticosterone levels were significantly higher (P < 0.001; Bonferroni post-test) in urban birds than rural birds. Both rural and urban birds showed seasonal variations in corticosterone levels and minimum corticosterone levels were observed during September (Fig. 28a). Irrespective of habitat, birds showed seasonal changes in plasma T3 and T4 levels. Effect of time, habitat and their interaction was observed on plasma T3 levels (habitat: $F_{1,32}$ = 10.32, P = 0.0028; time: $F_{3,32}$ = 22.08, P < 0.0001 and Interaction of habitat and time: $F_{3,32}$ = 3.222, P = 0.0355; 2-way ANOVA; Fig 28b) but only of time on T4 levels (habitat:, $F_{1,32}$ = 0.2550, P = 0.6170; time: $F_{3,32}$ = 10.05, P < 0.0001 and Interaction of habitat and time: $F_{3,32}$ = 0.6171, P = 0.6090; 2-way ANOVA; Fig. 28c). Levels of plasma T3 were significantly higher (P < 0.001; Bonferroni post-test) in rural birds in March then urban birds.

3.3 Gene transcription

All reproductive genes studied showed seasonal changes in transcription from either habitat. There was an effect of habitat on transcription of *Dio2* (habitat: $F_{1,32}$ = 7.750, P = 0.0089; time: $F_{3,32}$ = 14.32, P < 0.0001, Interaction of habitat and time: $F_{3,32}$ = 6.251, P = 0.0018; 2-way ANOVA; Fig. 29) and *GnRh* (habitat: $F_{1,32}$ = 6.096, P = 0.0302; time: $F_{3,32}$ = 26.45, P < 0.0001, Interaction of habitat and time: $F_{3,32}$ = 3.222, P = 0.0355; 2-way ANOVA; Fig.29) but not on transcription of *Dio3* (habitat: $F_{1,32}$ = 0.3487, P = 0.5590; time: $F_{3,32}$ = 9.896, P < 0.0001, Interaction of habitat and time: $F_{3,32}$ = 0.3167, P = 0.8132; 2-way ANOVA; Fig. 29), *GnIh* (habitat: $F_{1,32}$ = 1.026, P = 0.3186; time: $F_{3,32}$ = 7.353, P = 0.0007, Interaction of habitat and time: $F_{3,32}$ = 13.18, P < 0.0001; 2-way ANOVA; Fig. 29) and *Tshβ* (habitat: $F_{1,32}$ = 1.966, P = 0.1705; time: $F_{3,32}$ = 12.25, P < 0.0001, Interaction of

habitat and time: $F_{3,32} = 0.6467$, $P = 0.5906$; 2-way ANOVA; Fig. 29). mRNA levels of *Dio2* and *GnRh* were significantly higher in rural birds in comparison to urban birds during March (Fig. 29). Transcript levels of *Dio2*, *Tshβ* and *GnRh* were significantly higher in March, while *Dio3* levels were higher during December (Fig. 29). *GnIh* levels showed maximum transcription during September in both rural and urban birds (Fig. 29d).

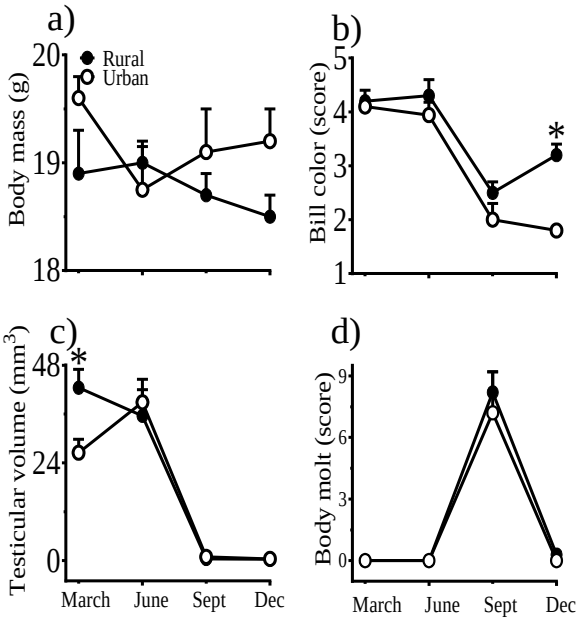

Figure 27: Data is represented as mean (mean ± SE). Change in body mass (a), bill colour (b), testicular volume (c) and molt in body feathers (d) at four time (March, June, September and December) of the year. * indicates difference between the group at that point (2- way ANOVA).

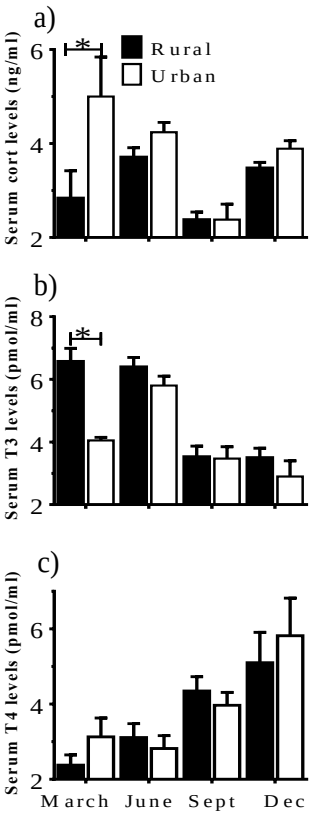

Figure 28: Data is represented as mean (mean ± SE). Change in serum corticosterone levels during March (a), T3 levels (b) and T4 levels (c) in rural and urban birds at four time (March, June, September and December) of the year. * indicates difference between groups at that time point (2- way ANOVA).

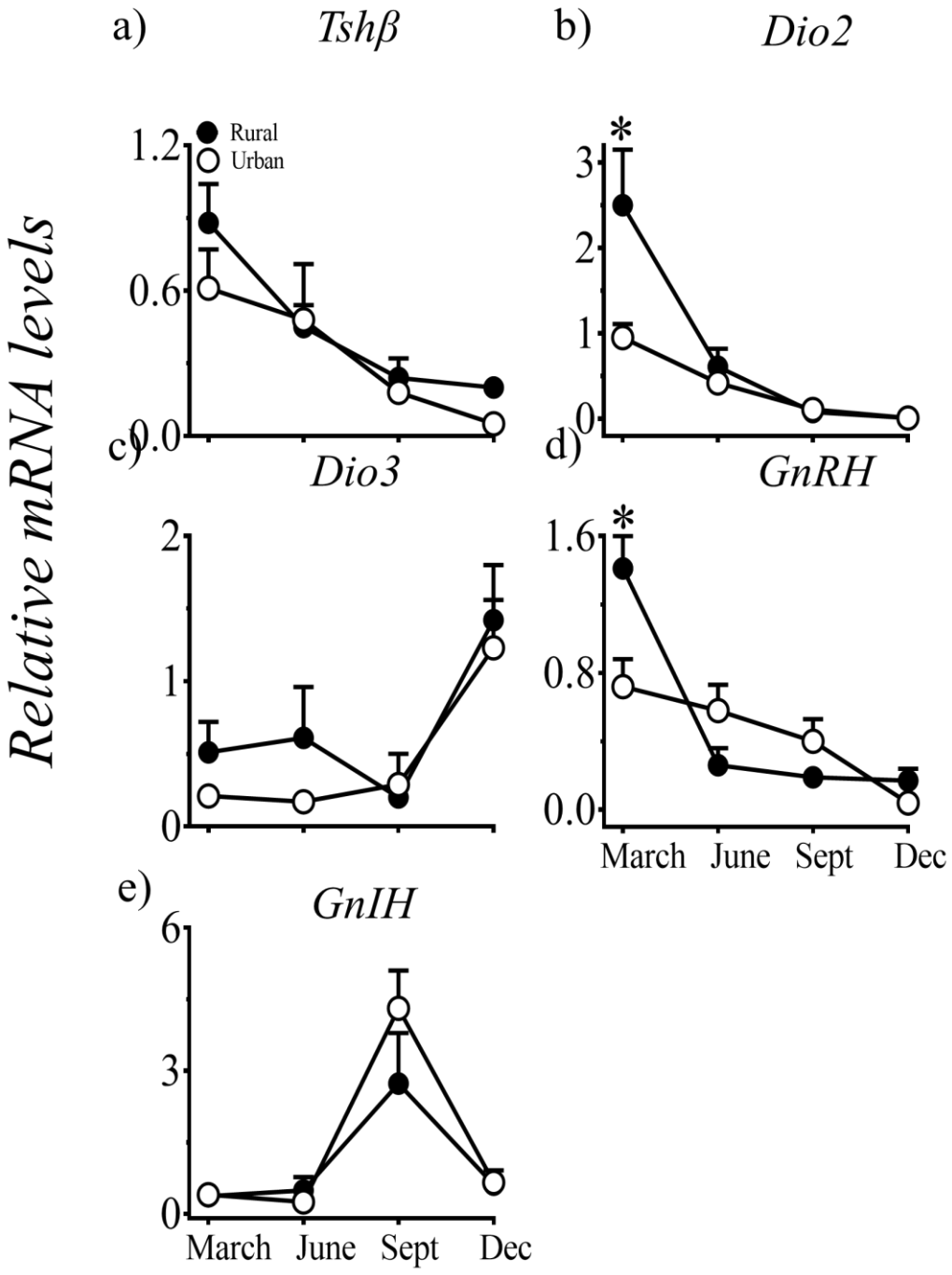

Figure 29: : Data is represented as mean (mean SE). Change in transcript levels of Tsh (a) Dio2 (b), Dio3 (c) GnRH (d) and GnIH (e) in rural and urban birds at four time (March, June, September and December) of the year. * indicates difference between groups at that time point (2- way ANOVA)

4. DISCUSSION

Our results confirm that tree sparrows are seasonal breeders (Fig. 27) and are consistent with previous findings (Dixit and Singh, 2011, 2012). There was no change in body mass as time progressed and it is consistent with many resident avian species which either do not go annual change in body mass or show a slight difference in their body mass (Trivedi *et al*., 2006; Bhardwaj and Anushi, 2006). Testes size and bill color changed in line with the season with darker bill color and greater testes size corresponding to the breeding phase in nature (March and June), while straw bill color and smaller testes size were found during the non-breeding phase (September and December; Fig. 27). These findings are in accordance with previous reports on the tree sparrow (Dixit and Singh, 2011). Post-breeding molt in body feathers is also consistent for this species (Dixit and Singh, 2011) and with the majority of other avian species (Boswell, 1991; Trivedi *et al*., 2006). We observed differences in bill color between rural and urban birds only during the time of sensitive phase (December), where rural birds had a higher score (darker bill color) than urban birds suggesting that rural birds start preparing for next year's reproduction in advance then urban birds.

Here we studied two aspects of the corticosteroids, one seasonal variation in the CORT levels and other is differences in the CORT level between birds of rural and urban habitats. Corticosterone levels varied seasonally in birds of both rural and urban habitat (Fig. 28a) and are consistent with other avian species (Wingfield, 1994). Tree sparrows showed maximum corticosterone levels during June and minimum corticosterone levels during September which corresponds to their refractory stage when the birds had maximum molt in body feathers (Fig. 28a). We also observed higher levels of corticosterone in urban birds then rural birds (Fig. 28a) during the stimulatory/pre-reading phase. In a variety of arctic passerines, elevated corticosterone levels are reported during breeding (Romero *et al*., 1998), with the lowest corticosterone levels being recorded during molt (Dawson and Howe, 1983). Corticosteroids are not limited to help animals in regulating energy balance but also in a variety of other functions like immune responses, behaviour, and reproduction (Sapolsky *et al*., 2000). Urban environment exhibit unnatural

conditions where extra light is available after Sunset. This unfavorable environmental conditions could cause increased corticosteroid release directly (de Bruijn and Romero, 2013) or indirectly (Voigt *et al.*, 2011), which increases corticosteroid levels (McEwen and Wingfield, 2003). Corticosteroids can act at multiple levels to suppress the activity of the hypothalamic–pituitary–gonadal axis. Chronic administration of corticosteroids results in the inhibition in the pulsatile release of GnRH from the hypothalamus (Oakley *et al*., 2009), and reduce the responsiveness of pituitary cells to GnRH (Li and Wagner, 1983), which reduces the amplitude of the luteinizing hormone (LH) response. Further, corticosteroids can act directly on the gonads to suppress reproduction by reducing androgen synthesis by the testes (Welsh *et al*., 1982). Furthermore, corticosteroid administration can inhibit breeding in animals (Rivier and Rivest, 1991; Salvante and Williams, 2013; Wingfield and Sapolsky, 2013; Silverin, 1986; Spée *et al*., 2011). Now we have a conundrum, at one end higher levels of corticosteroid are observed during breeding than then post-breeding phase (Dawson and Howe, 1983; Sapolsky *et al*., 2000) but at the same time elevated levels of CORT can compromise reproduction (Rivier and Rivest, 1991; Salvante and Williams, 2013; Wingfield and Sapolsky, 2013; Silverin, 1986; Spée *et al*., 2011). This can be explained on the basis of the "CORT flexibility hypothesis" (Lattin *et al*., 2016). This hypothesis proposes that “pre-breeding” is a special period of annual life history state which is characterized by the priming of corticosteroid function, which allows animals to respond flexibly to non-optimal environments. During the pre-breeding animal has enhanced sensitivity in its corticosteroid system, so that if it encounters a negative supplemental factor (night light environment could potentially act as a negative factor) it is primed to respond strongly in order to pause reproductive behavior and physiology and it could be a potential cause of slight delay in the gonadal maturation in the urban birds in our study. Studies on glucocorticoid levels in the context of urbanization are contradictory. Some previous studies report higher levels of glucocorticoids in urban birds (Oakley *et al*., 2009; Li and Wagner, 1983; Welsh *et al*., 1982; Rivier and Rivest, 1991; Salvante and Williams, 2013; Wingfield and Sapolsky, 2013; Silverin, 1986; Spée *et al*., 2011; Lattin *et al*., 2016; Bonier *et al*., 2007; Fokidis *et al*., 2009;

Zhang *et al*., 2011) while others suggest lower stress-induced glucocorticoid levels (Partecke *et al*., 2006; Fokidis *et al*., 2009; Atwell, 2012) in urban populations, but not for all species (Fokidis *et al*., 2009; Atwell, 2012). This higher glucocorticoid level in the urban population probably promotes survival by mobilizing and redirecting energy away from long-term investments toward more immediately essential processes (Wingfield and Kitaysky, 2002). Therefore, the glucocorticoid stress response may play a critical role in individuals' responses to coping with urban challenges (Wingfield and Kitaysky, 2002). We propose that differences in corticosterone levels between urban and rural birds are physiologically dependent where corticosterone levels differ during the stimulatory phase but not during other reproductive phases (Fig. 28a).

We observed differential levels of serum thyroid hormones (T3 and T4) at a different time of year, corresponding to the reproductive states involved (Fig. 28). We observed higher levels of T3 in March and June (Fig. 28b,c), which is the breeding phase for this species (Dixit and Singh, 2011), and higher levels of T4 during September and December (Fig. 28c), which corresponds to the post-reproductive phase for tree sparrows (Dixit and Singh, 2011). We also observed differences in serum T3 levels between rural and urban birds during March (Fig 28b). Higher levels of T3 in rural birds suggest earlier initiation of gonadal growth in these birds. Thyroid hormones are an essential component of the reproductive system (Pèczely, 1985) and THs are required for the onset of the seasonal reproductive cycle as well as for the development of photorefractoriness in birds (Bentley, 1997; Follett and Nicholls, 1988; Yoshimura, 2010). Differences in the reproductive hormones in rural and urban tree sparrows have been reported previously (Zhang *et al*., 2014). In urban tree sparrows, LH secretion began earlier but no difference was observed in testosterone (T) and estradiol (E2) secretion. On the other hand, T and E2 levels were lower at urban sites (Zhang *et al*., 2014). We observed different patterns between body weight and testes volume in urban habitat; suggests that there may be differential sensitivity of different physiological functions to local human population density/urban environment. In comparison to a metabolic system (as reflected by the change in body mass), reproductive physiology (change

in testes volume) appears to be more sensitive for urban gradients. Up to what extent it is correct needs further investigation.

Our study shows differences in the initiation of testes recrudescence in birds from both habitats. Interestingly, there is a slight advance in the initiation of testes growth in rural birds in comparison to urban birds (Fig. 27c). This change in initiation in testicular growth is also reflected at the molecular level (Fig. 29). Higher levels of *Dio2* and *GnRH* were observed in rural birds in comparison to urban birds in March (Fig. 29a,b). However, there was no difference in the transcription of *Tshβ, Dio3* and *GnIH* levels between rural and urban birds (Fig. 29). Elevated levels of *Dio2* and *GnRH* and lower levels of *Dio3* and *GnIH* correspond to their breeding and non-breeding phases respectively, as has been suggested in a number of avian species (Yoshimura *et al*., 2003; Yamamura *et al*., 2006; Dixit and Byrsat, 2018; Trivedi *et al*., 2019). Our results on the early initiation of testicular growth in rural birds are not in accordance with previous studies. Previous studies on European blackbirds (*Turdus merula*) and on scrub-jays (*Aphelocoma coerulescens*) report that urban populations of these bird species exhibit an earlier onset of breeding compared with their rural counterparts (Partecke *et al*., 2005; Schoech and Bowman, 2003). Differences in the outcome in these studies could also be because of local environmental conditions. Studies on birds suggest that, along with photoperiod, the temperature is a critical environmental cue playing role in the regulation of seasonal reproduction (Trivedi *et al*., 2019; Dixit and Singh, 2012). It has been shown that in comparison to low temperature (17 °C), high temperature (25 °C) slightly advances the initiation of gonadal recrudescence in tree sparrows (Dixit *et al*., 2018), white-crowned sparrows (Wingfield *et al*., 2003) and European starlings (Dawson, 2005) which exhibit an accelerated rate of photoperiodic induction at higher temperatures. The two sites selected in this study differ slightly in their altitudes. The slightly higher altitude of urban sites (1130 m above sea level) has 2 – 4 °C lower temperature in comparison to rural habitat (800 m above sea level). This difference in temperature could be a potential cause of a slight delay in the initiation of the gonadal recrudescence in urban birds. In our study we do not have behavioral data on the mating system of rural and urban birds, therefore, we do

not know whether male used in the present study were successful breeders or not but we do not see differences in the testicular volume during June only differences we observed during the March suggesting initiation timing is different in the birds of two habitats.

In conclusion, our findings are significant as they show differences from previous studies on birds due to delay in the initiation of gonadal recrudescence in urban birds. Further, it provides evidence to support the effect of the urban environment on reproductive functions. Our results from tree sparrows demonstrate that urban environments lead to a slight delay in the initiation of the reproductive cycle and hence probably put extra pressure on birds to complete reproduction in time as offspring need to grow enough before they encounter adverse conditions of winter.

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- National Symposium on Avian Biology in conjunction with annual meeting of Association Of Avian Biologists in India (AABI), Department of Zoology, Mizoram University, Aizawl, October 22-24,2018.

Publications:

1. **Renthlei Z**, Hmar L, Kumar Trivedi A (2020). High temperature attenuates testicular responses in tree sparrow (*Passer montanus*). ***Gen Comp Endocrinol***. **(IF- 2.426)**

2. **Renthlei Z**, Borah BK, Gurumayum T, Trivedi AK (2020). Season dependent effects of urban environment on circadian clock of tree sparrow (*Passer montanus*). ***Photochem Photobiol Sci***. **(IF- 2.831)**
3. BK Borah, **Z Renthlei** and AK Trivedi (2020). Hypothalamus but not liver retains daily expression of clock genes during hibernation in terai tree frog *(Polypedates teraiensis*) ***Chronobiology International***. **(IF- 2.486)**
4. **Z Renthlei** and AK Trivedi (2019). Effect of urban environment on pineal machinery and clock genes expression of tree sparrow (*Passer montanus***).** ***Environmental Pollution***. **(IF -6.792)**
5. S Sanjeev, M K Murthy, M S Devi, M Khushboo, **Z Renthlei**, K S Ibrahim, N S Kumar, VK Roy and G Gurusubramanian (2019). Isolation, characterization, and therapeutic activity of bergenin from malberry (*Ardisia colorata* Roxb.) leaf on diabetic testicular complications in Wistar albino rats. ***Environmental Science and Pollution Research***. **(IF- 3.056)**
6. BK Borah, **Z Renthlei**, and AK Trivedi (2018). Seasonality in terai tree frog (*Polypedates teraiensis*): Role of light and temperature in regulation of seasonal breeding. ***Journal of Photochemistry & Photobiology, B: Biology***. **(IF- 4.383)**
7. BK Borah, L Hauzel, **Z Renthlei**, and AK Trivedi (2018). Photic and non photic regulation of growth and development of *Rhacophorus maximus* tadpole. ***Biological Rhythm Research***. **(IF- 0.826)**

8. **Z Renthlei**, T Gurumayum, BK Borah and AK Trivedi (2018). Daily Expression of Clock Genes in Central and Peripheral Tissues of tree sparrow (*Passer montanus*). ***Chronobiology International***. **(IF- 2.486)**
9. **Z Renthlei**, BK Borah and AK Trivedi (2017). Photoperiod induce developmental effects on silkmoth, *Bombyx mori*. ***Biological Rhythm Research***. 48(1):121-128. **(IF- 0.826)**
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Z Renthlei and AK Trivedi: Regulation of seasonal reproduction in higher vertebrates. (C. Haldar, S Gupta, S Goswami., eds.). Publishing Cell Banaras Hindu University, Varanasi, India.

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DEPARTMENT : ZOOLOGY

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Seasonality represents the initiation-termination-reinitiation of any physiological processe. It is an adaptation for survival in most of the species in the fluctuating environment. Most of the birds fall under category of seasonal breeders and exhibit seasonality in several other physiological functions, for example, change in body weight, deposition in body fat, season dependent parasitic load, hormone levels, change in the bill and plumage color, molt in feathers, immune system and migration. Seasonality in reproduction and affiliated behaviors is species-specific. Reproduction is timed in such a way that when actual breeding happens, plenty of food resources should be available for both parents and offspring so that chances of survival are maximum. Light at night (LAN) is an increasing phenomenon and has been associated with alterations in temporal activity patterns and physiology in vertebrates. The effects of LAN on ecological aspects are receiving great interest. The effect of urban lights at night has been illustrated using compositional differences on the foraging behavior of shorebirds, survival rates and stress responses in juvenile Pacific bluefin tuna and the commuting strategies of bats. Ecological research has revealed the impact of urbanization on animal populations and community dynamics. New interest has emerged in understanding the mechanisms of individual responses to urbanization and scattered information is available on the different aspects of animal physiology e.g., stress and reproductive physiology, temporal and spatial activity patterns, metabolism, behavior, social cues, sleep and on the endocrine system.

Present thesis includes investigations on the population of tree sparrow (*Passer montanus*) that lives in and around Mizoram University campus (23.7ºN and 92.7º E). In particular, the emphasis is placed on studying seasonal responses of tree sparrows in the natural environment, the effect of light quality and temperature on gonadal recrudescence, and the regression cycle under laboratory conditions. We also studied the molecular clockwork in central and peripheral tissues of tree sparrows and compared the daily and seasonal responses of tree sparrows from rural and urban habitat. We also compared the various physiological responses of these birds with the birds living in the urban environment. Various studies conducted are summarized in the following sections.

SECTION 1: SEASONAL PATTERNS IN BREEDING AND ASSOCIATED BEHAVIOR

Class Aves represents approximately 10000 living species world wide and approximately 1346 species have been reported from India. Northeast part of India is a hotspot for biodiversity, and around 900 species have been reported from this region. However, the breeding biology of most of the species is not known. Most of the avian species that have been studied thus far exhibit seasonality in many physiological functions, including body mass, food intake, change in plumage color, variations in bill color
, gonadal growth regression cycle, photorefractoriness, molt in feathers, various hormonal levels, production of the song, immune functions, migration, etc. In general, the annual change in body mass is exhibited by both migratory and resident bird species. However, in comparison to resident species, migratory birds show many dramatic changes in the body mass as these species deposit heavy fat deposition, which serves as "fuel" for migration. Further, nonmigratory birds reflect some changes in body mass during the sexual phase than in post-reproductive phase of the annual cycle and during the sexual period, males weigh higher than females. Reproduction is a fundamental characteristic of living organisms. An essential attribute of any surviving species or population is the ability to reproduce successfully and produce offspring. Although a large number of avian species are known, seasonality in reproduction and linked phenomenon has been studied in only a limited number of species. The specific aim of this study was to describe changes in body mass, bill colors, gonadal growth and development, and molt in tree sparrows collected every month over a period of one year from the populations living in and around Mizoram University campus (rural habitat; 23.7^0N, 92.6^0E) and compared these responses with observations recorded at similar intervals on birds from the core area of the city of Aizawl (urban habitat; 23.7^0N, 92.7^0E) to see the effects of the urban environment on the annual reproductive cycle and affiliated responses.

The study included adult birds of both sexes procured locally during the middle of each month, beginning from January 2017 till December 2017. The study includes two components. In the first component, we studied the annual changes in various

morphological and physiological characters in birds procured in and around the core area of the city of Aizawl (urban site; 23.7^0N, 92.7 ^{0}E). In the second part, we compared all these characteristics with the conspecific procured at the same time but from rural habitat (23.7^0N, 92.6 ^{0}E). We measured change in body mass and bill colo in both male and female birds (n = 10). Corresponding changes in day-length, temperature, and humidity were also recorded during each month. In order to know the seasonal variations in gonadal status, the size of the testis and ovarian follicles were measured. Sperm count was done (only urban birds), as mentioned in the general materials and method section. Data was analyzed using a 1-way analysis of variance (1-way ANOVA) followed by Newman-Keuls posthoc tests if ANOVA indicated a significance of the difference. 2-way analysis of variance was employed to analyze the effects when two factors (e.g., habitat and time) were taken into consideration, and the Bonferroni test was used as a post hoc test for group comparisons. Significance was taken at $P < 0.05$. Our results demonstrate that both male and female tree sparrows essentially have a similar pattern in annual cycles of body mass, bill color, gonads, and molt. Birds had significantly larger gonads between March and July from either population but in comparison to rural birds gonadal growth regression cycle was slightly delayed in urban birds. Further, molt followed reproductive cycle and similarly delayed in the molt cycle was observed in urban birds.

SECTION 2: SEASONAL CYCLES UNDER LABORATORY CONDITIONS

Like other organisms birds are equipped with endogenous time-keeping mechanism (circadian/circannual clock) which they utilize to track daily changes in the timing of sunrise and sunset. Seasonal cycles of various physiological and behavioral functions in birds seem to be regulated by two mechanisms viz. photoperiodism and circannual rhythm generation. In seasonal breeders, the photoperiod regulates seasonal rhythms by stimulating and terminating the physiological processes like gonadal growth and development, feathers molt, and change in bill color and migration, in most photoperiodic birds, cycle between periods of photosensitivity and photorefractoriness. In long-day breeders, initiation of gonadal growth happens during long photoperiod, and then birds

became photorefractory under this long stimulatory photoperiod. This photorefractoriness gets terminated, and birds subsequently recovered their photosensitivity under short days. Both long and short days are critical for seasonal reproductive physiology in these birds, although they are used to control different physiologies. Therefore, these species must have some mechanism for the measurement of day length in order to have a successful interaction with day length at appropriate phases of the endogenous clocks. The photoperiodic control of reproductive functions in birds involves a circadian rhythm of photoperiodic photosensitivity (CRPP). The phase relationships between CRPP and environmental factors change with the season, leading to seasonal responses. The photoperiodic response occurs when the photoperiod coincides with the photoinducible phase of an entrained endogenous circadian rhythm. In circannual rhythm generation, a self-sustained endogenous rhythm with a period of approximately one year regulates various physiological and behavioral functions associated with the annual cycles of birds. It is in question whether, in birds, the photoperiodic regulation of seasonality involves an endogenous circadian or circannual rhythm exclusively, or they might interact closely, albeit as per the adaptive needs of the species. It is also argued that circannual events are manifestations of circadian rhythms, as is evident from the circadian rhythm involvement during initiation and termination of gonadal growth and photoperiodism and circannual rhythm generation are evolved as separate mechanisms. Here, we investigated the effect of a long and short photoperiod on gonadal recrudescence on tree sparrows at two different times of the year, i.e., during March and September when in nature they experience equinox photoperiod. Further, we also investigated the effect of food restriction on the growth of ovarian follicles.

Two experiments were performed. In experiment 1, we examined the role of artificial photoperiod on testicular growth. For this birds were procured locally during two times of the year, i.e., during March and September when they were experiencing equinox photoperiod (12L:12D) and were divided into two groups and exposed to either long photoperiod (16L:10D) or short photoperiod (8L:16D). Changes in body mass, bill color, and testes size were recorded. Our results show that birds procured during March

showed long photoperiod induced gonadal growth regression cycle but not in September. Short photoperiod was not inductive at either time of the year. In experiment 2, female birds were exposed to long stimulatory photoperiod but either food ad libitum condition or food limited to the first 7 h of light phase. Our result suggests that food restriction compromise the gonadal growth as reflected by reduced follicle diameter in size in food restriction group.

SECTION 3: ROLE OF LIGHT SPECTRUM ON REPRODUCTION

Light is an essential component of the geophysical environment and controls many physiological processes. Three components of light include duration (photoperiod), intensity, and color. A number of studies have emphasized the effects of photoperiod and light intensity on the behavior of animals, hormone concentrations, and reproductive performances in birds. Light can be classified into ultraviolet light, visible light, and infrared light according to spectral components. The visible range of light spectrum composed of seven colors of the rainbow (VIBGYOR) ranging from violet to red, having the shorter wavelength (380 nm; violet), and the longer wavelength (750 nm; red). Along with light intensity and duration spectral composition of light can also affect various physiological processes, viz. growth, metabolism, reproduction, and behavior. Previous studies have shown that different spectrum of light can influence the behavior, age of sexual organs, puberty and sexual maturity. The study from black-headed bunting (*Emberiza melanocephala*) suggests that the photoperiodic response system can discriminate between different wavelengths of light and requires a minimum light intensity threshold for photo-stimulation. Wavelength and intensity-dependent photoperiodic regulations of daily and seasonal responses have been documented in the bunting and circadian behavior in the Indian Weaver bird, *Ploceus philippinus*. Tree sparrows breed under long stimulatory photoperiod in nature. Here we tested the effects of short (blue light) and long-wavelength (red light) on the testicular growth regression cycle of male tree sparrows. Adult male photosensitive tree sparrows were used for this study. Birds were divided into three groups. All three groups were subjected to artificial

light-dark cycles of the same photoperiod (14L:10D; light on 06:00; light off 20:00) but of different wavelengths of light. Group one was exposed to red light (wavelength; 640 nm), group two was exposed to blue light (wavelength; 450 nm), and group three wasexposed to white light and served as control. Light intensity was maintained at $0.7W/m^2$ at the perch level. Observations on change in body mass, bill color, and testicular size were made on an interval of 30 days. Our results suggest that the spectral component of light (wavelength) could be the essential characteristic of light in photoperiodic regulation of physiological responses. The effects of the spectral composition of light on body mass, bill color, and testicular growth regression cycle of male tree sparrows were observed. Under red and white light, there was a slight gain in body mass, but on the other hand, under blue light, there was no gain but a loss in body mass as time progressed. These results suggest that blue light was not strong enough to induce photoperiodic response for the induction of gain in body mass.

SECTION 4: EFFECT OF TEMPERATURE ON REPRODUCTION

As the urbanization is increasing drastically it's leading to a change in the environment. One of the significant changes in the environment is an increase in temperature (global warming) and has affected the biological systems. During the past few decades, environmental change has led to change in phenological shifts in events across trophic levels. Most of the avian species breed seasonally and use environmental cues to time their seasonal reproduction. This actual timing of breeding is crucial for birds as hatching must be synchronized with a narrow window of maximal food abundance for the best chances of survival of offspring. Daylength is the proximate factor used to time reproduction linked phenomenon, but there can be considerable inter-annual variability in the timing of breeding behavior of a given population within or between the habitats. As photoperiod is always fixed at a given latitude/altitude, this annual variation could be due to change in other factors (ultimate factors). These secondary factors provide supplementary but critical information to allow birds to more closely synchronize the timing of breeding. The effect of daylength in the regulation of seasonal reproduction of

tree sparrows is known. Tree sparrows (*Passer montanus*) breed under long stimulatory photoperiod. What could be the effects of altered environmental temperature on the mechanism of the reproduction of tree sparrow is mostly unknown. Previously, at the physiological level, it has been shown that temperature, coupled with long photoperiod, delays the gonadal regression at a lower temperature (17 ^{0}C). But the mechanism, involved in the processes is not known. Further, is it the species-specific phenomena or local environmental conditions can modulate the effects of temperature is in question. To address these questions, we studied the reproduction and linked phenomena and the candidate genes known to be involved in the seasonal reproduction in the tree sparrow under two different temperature conditions.

Two experiments were performed. In experiment one, birds were divided into two groups and exposed to long photoperiod 14L: 10D (14h light and 10h dark) but two different temperature conditions, i.e., high temperature (30 ± 2 ^{0}C) and low temperature (20 ± 2 ^{0}C). Change in body mass, bill color, and testis size was recorded at the interval of 30 days until the end of the study. In experiment two, birds received similar treatment as of experiment 1; but were sacrificed during the middle of the day after 30 days of long photoperiod and high (30 ± 2 ^{0}C) or low (20 ± 2 ^{0}C) temperature treatment. Blood was collected; serum was harvested and stored at -20 ^{0}C until assayed for serum corticosterone, thyroxine, and tri-iodothyronine. Hypothalamus was collected from freshly dissected-out brains in RNA later solution (Thermo Fisher Scientific, USA, AM 7020) and first kept at 4 ^{0}C overnight and then stored at -80 ^{0}C. Our study shows the effect of temperature on testicular growth regression cycle and transcription levels of associated reproductive genes. Response specific effects of temperature were observed in this study. Under long stimulatory photoperiod of 14L:10 D, we do not see any temperature-dependent effect on body mass as no difference in body mass was observed between two groups (low temperature vs. high temperature). On the other hand, high temperature (30 °C) affected the testicular growth regression cycle. Attenuated testicular growth and faster testicular regression were observed in the high-temperature group (30 °C) than 22 °C. Experiment 2 was conducted to see early effects (when a response is not

reflected at gonadal levels) of temperature treatment on candidate molecules involved in seasonal reproduction. After 30 days of respective temperature treatment we observed change in CS, T_3 and T_4 levels and transcript levels of *Tsh*β, *Dio2*, *Dio3*, *GnRH* and *GnIH* in both the groups where higher levels of serum T_3 and transcript levels of *Tsh*β, *Dio2* and *GnRH* were upregulated while T_4 and transcripts of *Dio3* and *GnIH* were downregulated in a low-temperature group. Further, corticosterone levels were significantly higher in the high-temperature group.

SECTION 5: STUDY OF DAILY RHYTHMS IN CLOCK GENES

Organisms live in a fluctuating environment. To better adapt to a fluctuating environment, organisms need to keep track of time so that they perform daily and seasonal processes at the best time as a mismatch may have fitness consequences. To govern daily and seasonal cycles in their physiology and behavior, most vertebrates exhibit circadian (*circa* = about, *dian* = day) and circannual (*circa* = about, *annum* = year) clocks, respectively. In birds, at least three separate circadian oscillators have been attributed, which reside within the hypothalamus, pineal, and retina of the nervous tissue. These circadian pacemakers are photoreceptive**;** interact with each other and with the external environment to produce timing at the functional level. However, the degree of self-sustainment and contribution to circadian time generation for these three circadian oscillators can vary among avian species. Most of the knowledge of circadian clock genes comes either from mammalian (rat and mice) or from insect (*Drosophil*a) models. However, in the past one and half decades, the avian orthologs of canonical and clock-controlled circadian genes have also been sequenced in few species. Still, most of the knowledge of avian clock genes has been limited to certain species. Comparing the diversity of Class Aves still there is a scarcity of knowledge of molecular clockwork and its relationship within and among the tissues. In the present study, we aimed to investigate oscillation of the canonical clock genes (*Bmal1*, *Clock*, *Per2*, *Per3*, *Cry1*, and *Cry2*) and clock-controlled gene (*Npas2*) in the central (hypothalamus, pineal, and retina) and peripheral (liver, intestine, and muscle) tissues of songbird, tree sparrow (*Passer*

montanus) exposed to equinox photoperiod (12L:12D) under laboratory conditions. We studied the 24-h mRNA levels of circadian clock genes. Further, we determined the phase relationship in the expression of clock genes within the tissue and among the tissues. The study was performed on adult male tree sparrows (*Passer montanus*). Birds were exposed to equinox photoperiod 12L:12D (12h light:12h darkness) for two weeks. Food and water were provided ad libitum, and the temperature was maintained at 22 ^{0}C. On the day15th, birds (N=5 per time point) were sacrificed by decapitation at six times: ZT1, ZT5, ZT9, ZT13, ZT17, and ZT21 (lights on = ZT0, zeitgeber time 0). The brain, retina, liver, gut, and muscle tissues were immediately removed and collected in RNA later solution (Thermo Fisher Scientific, USA, AM 7020) and kept at 4 ^{0}C overnight and then stored at -80 ^{0}C till RNA was extracted. Our findings support the existence of the autoregulatory transcription-translation feedback loop mechanism, both in the central and peripheral tissues of tree sparrow. These results implicate that tissue-level circadian time generation as acrophase of different genes is different in different tissues. We speculate a tissue-specific contribution of individual genes in circadian time generation in tree sparrow, as suggested in other avian species due to differential phase relationships in genes of positive and negative limbs of the TTFL. Whether this tissue-specific circadian time relationship varies during a different time of year needs to be further investigated.

SECTION 6: EFFECT OF URBAN ENVIRONMENT ON CIRCADIAN CLOCK

Artificial light at night is a rapidly increasing phenomenon and has been associated with alterations in temporal activity patterns and physiology in vertebrates. During the last decade, the consequences of night lights on ecological aspects, particularly in the context of effects on wildlife balance, have received great interest. The effect of city lights at night has been illustrated using compositional differences on the foraging behaviour of shorebirds, survival rates and stress responses in juvenile Pacific bluefin tuna and the commuting strategies of bats. Ecological research has emphasised the impact of urbanisation on animal populations and community dynamics. It has been hypothesised that night lights may alter the detection of day-length, but at present we do not have an

understanding of the physiological mechanisms involved in the process. As urban environments lead to extra light after sunset and clock genes are sensitive to the availability of light, we hypothesised that changes in the physiology of urban birds is due to alterations in the expression of clock and clock-controlled genes. To address this, we studied the effects of urban night light habitats on the daily clock of tree sparrows living in an urban environment and compared it with birds living in nearby rural habitats in Aizawl. Adult male tree sparrows (*Passer montanus*) were procured during third week of March at two locations, urban and rural sites. Urban site was selected in the core area of city (Aizawl, Mizoram, India; 23.7^0 N 92.7^0 E) having maximum human activities (population density: 737 individuals/km^2) while rural site was selected within Mizoram University campus (23.7^0 N 92.6^0 E), with limited human activities (population density: 375 individuals/ km^2).

We did not find any effect of the urban environment on locomotor activity-rest pattern of these birds as reflected by period, activity duration, and total activity count. mRNA of *Mel1A* and *Mel1B* receptors were rhythmically expressed both in the pineal and retina in urban and rural birds. However, in comparison to the rural birds, urban birds showed a 4 – 7 hour advance in the acrophase of mRNA of *Mel1A* and *Mel1B* receptors. Delay in onset of release of melatonin and attenuated peak was observed in urban birds. We also observed the altered expression pattern in melatonin receptor transcripts in birds in urban habitats. Clock and clock-controlled genes oscillated in all three neural tissues (hypothalamus, pineal and retina) of the tree sparrows procured from either urban or rural habitats. However, there were alterations in the expression pattern of clock genes in the hypothalamus of birds in rural and urban habitats.

SECTION 7: SEASON DEPENDENT EFFECTS OF URBAN ENVIRONMENT ON CIRCADIAN CLOCK

The rotation of the Earth around its own axis leads to daily changes in light intensity while the tilt of the Earth's rotational axis as it orbits the Sun leads to seasonal variations. A change in the zeitgeber signal is critical for entrainment of the circadian clock. The

range of entrainment is correlated with zeitgeber strength and photoperiod. It has been shown that the phase of entrainment varies with season and latitude. Since urban environments lead to extra light after sunset, clock genes are sensitive to the availability of light, and the expression pattern of clock genes is variable throughout the year, we asked whether there is an interactive effect of season and urban environment on the circadian timing of the birds living in urban habitats. Birds were procured from two locations – urban and rural sites at three time of the year. These three times of the year were selected on the basis of the differences in their daylength at this latitude (23.7^0 N 92.7^0 E) as June has the longest daylength; September has an equinox photoperiod while December corresponds to the shortest daylength. Birds were sampled on the day of procurement. Birds (N=30 each site) were sacrificed (N=5/time/per site/month) at six times: ZT1, ZT5, ZT9, ZT13, ZT17, and ZT21 (ZT0 = sunrise time at the respective time of the year). The hypothalamus, pineal and retina tissues were immediately collected and stored in RNAlater solutions (Thermo Fisher Scientific, USA, AM 7020) first at 4^0C overnight and then at -80^0C. Our results provide an extensive characterisation of the clock and clock-controlled genes in the central circadian oscillators (hypothalamus, pineal gland and retina) of tree sparrows from rural and urban habitats during three different times (corresponding to different physiological conditions), i.e., June (long days), September (equinox photoperiod) and December (short days). We observed tissue and season dependent expression of clock genes in rural and urban birds. These findings are significant as they provide the first evidence in support of the effect of season-dependent influences of the urban environment on circadian functions.

SECTION 8: SEASON DEPENDENT EFFECTS OF URBAN ENVIRONMENT ON SEASONAL CLOCK

Cities are better at providing amenities for human beings but pose a greater threat to biodiversity by altering the population and composition of flora and fauna of the ecosystem. Anthropogenic light pollution is a major consequence of urbanization and is a significant threat to the urban wildlife population. The effects of the urban/artificial

night light environment have been studied on various physiological parameters; foraging behavior, survival rate and stress responses, community dynamics, reproductive physiology, activity patterns, metabolism and reproduction. Birds are predominantly seasonal breeders. As an urban environment provides extra light at night, therefore, we predicted that it may alter the timing of reproduction. Here we studied physiological (body mass, bill color, body molt, and testicular volume), endocrine (corticosteroid, triiodothyronine, and thyroxine) and molecular parameters (*Tshβ*, *Dio2*, *Dio3*, *GnRH*, and *GnIH*) related with the seasonal reproductive physiology of the birds living in an urban environment and compared it with birds living in the nearby rural habitat of Aizawl.

Birds were captured in the middle of the month at four times of the year (March, June September and December 2018) at two locations, urban and rural sites as described previously. Immediately after collection, the birds were transported to the lab and kept in NDL (natural daylength conditions but in captivity). Bodyweight was recorded using a top pan balance to the precision of 0.1 g. Body molt was recorded. The color of the bill was assessed using a subjective criterion as reported earlier. As tree sparrows are sexually monomorphic, laparotomy was performed to identify the gender of the birds. The size of the left testis was recorded by unilateral laparotomy. The next day male birds (N=5/time/site) were killed by decapitation in the middle of the day. Blood was collected; serum was harvested and stored at -20 °C. Hypothalamus was harvested, stored in RNA later solution (Thermo Fisher Scientific, USA, AM 7020) first overnight at 4 °C later on at –80 °C.

Our study shows differences in the initiation of testes recrudescence in birds from both habitats. Interestingly, there is a slight advance in the initiation of testes growth in rural birds in comparison to urban birds. This change in initiation in testicular growth is also reflected at the molecular level. Higher levels of *Dio2* and *GnRH* were observed in rural birds in comparison to urban birds in March. However, there was no difference in the transcription of *Tshβ, Dio3* and *GnIH* levels between rural and urban birds. Elevated levels of *Dio2* and *GnRH* and lower levels of *Dio3* and *GnIH* correspond to their

breeding and non-breeding phases respectively, as it has been suggested in a number of avian species.