DAILY AND SEASONAL REGULATION OF STEROIDOGENESIS IN TREE SPARROW (*PASSER MONTANUS*)

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DAILY AND SEASONAL REGULATION OF STEROIDOGENESIS IN TREE SPARROW (*PASSER MONTANUS*)

BY

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DEPARTMENT OF ZOOLOGY

Under the Supervision of

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Submitted

In partial fulfillment of the requirement of the Degree of Doctor of Philosophy in Zoology of Mizoram University, Aizawl

CERTIFICATE

I certify that the thesis entitled "**Daily and Seasonal Regulation of Steroidogenesis in Tree Sparrow (*Passer Montanus*)**." submitted to the Mizoram University for the award of the degree of Doctor of Philosophy in Zoology by **Subu Yatung** is a record of research work carried out during the period of 2020-2025 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 **Subu Yatung**, hereby declare that the subject matter of this thesis is the record of the work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or 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/Institute.

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GENERAL INTRODUCTION

Animal behavior is profoundly impacted by environmental variations and the impending change that they convey. The internal biological clock regulates the cyclical variations that occur in an animal's physiological and biological processes. These biological clocks enable species to synchronize with the cyclical events in nature, such as daytime and nighttime, and then the seasons. Photoperiod is the proximate environmental time cue capable of entraining biological clocks. It allows the phase of cyclic events in the environment to be set with the phase of endogenous rhythms (Dunlap et al., 2004). Hence, this environmental time cue, or zeitgeber, produces a series of signals allowing endogenous timers to orchestrate with the biological cycles, generating a persistent phase relationship between the zeitgebers and rhythm (Cowan et al., 2017). Consequently, most birds display seasonal variability in their behavioral, physiological, and reproductive activities, such as a change in body weight, molt in feathers, bill color, song production, hormonal changes, gonadal status, and migration. Some species are continuous breeders, while others are seasonal breeders whose reproductive cycles are hormonally controlled. Seasonal birds are excellent models for studying seasonal reproduction due to their rapid and dramatic response to changes in photoperiod. The key role of photoperiod (day length) in seasonal time measurement was conclusively demonstrated in various organisms in the 1920s, including plants (Garner & Allard, 1920), insects (Marcovitch, 1923), and birds (Rowan, 1925).

Besides, light properties such as intensity and duration, different wavelengths of the spectrum can also influence numerous avian life history functions, such as behavior, development, reproduction, as well as metabolism etc. (Dakan, 1934; Kumar & Rani, 1996; Kumar et al., 2000; Malik et al., 2002; Malik et al., 2004; Rani et al., 2002). Numerous birds have four single cone types for color perception and are UV-sensitive (Bennett & Cuthill, 1994; Osorio & Vorobyev, 2008). Additionally, their pineal gland and brain include photoreceptors that allow them to perceive light outside of their eyes (Cassone, 2014). According to several studies, penetration of the skull is more easily obtained by a longer wavelength than by shorter ones. They also have a greater ability to trigger seasonal photoperiodic responses (Hartwig & van Veen, 1979), promote gonad development, and affect body fattening (Malik et al., 2002).

Seasonally, long photoperiods stimulate opsin-containing deep-brain photoreceptors and initiate transcription of *Dio2 (type 2 deiodinase)* via the *TSH (Thyroid-stimulating hormone)* receptor signaling pathway and monitor gonadal activities that take place downstream (Yoshimura et al., 2003; Nakao et al., 2008; Perfito et al., 2012; Pérez et al., 2019; Pérez et al., 2023). The hypothalamic pituitary-gonad axis (HPGa), which is essential for processes like sex differentiation, gonad growth, and spawning, governs the timing of reproduction and the biosynthesis of sex steroid hormones (Gothilf et al., 1997; Falcón et al., 2007). Nonetheless, it is well known that sex steroids are critical for testicular function, controlling the reproductive processes by promoting either spermatogenesis or spermiogenesis. (e.g., acrosome biogenesis) or the development/differentiation in addition to the maintenance of secondary sexual characteristics in all vertebrates, particularly those with seasonal reproductive cycle (Di Fiore et al., 2005; Carreau et al., 2011).

The biosynthesis of all the steroid hormones is controlled by the two key functional classes of steroidogenic enzymes: cytochrome P450 and hydrosteroid dehydrogenase (Schiffer et al., 2019). The steroidogenic pathway begins in mitochondria, where Scp2*,* a sterol carrier protein and an activator of mitochondrial steroidogenesis, stimulates the transfer of cholesterol as well as that of phospholipids between membranes in vitro (Poorthuis et al., 1981; Scallen et al., 1985), and might be involved in the intracellular transport or metabolism of phospholipids and cholesterol (Teerlink et al., 1984; Chanderbhan et al., 1982; Seltman et al., 1985). A mitochondrial protein, StAR (steroidogenic acute regulatory protein), helps transport cholesterol to mitochondria (Soccio & Breslow, 2003). A nuclear receptor (NUR77/Nr4a1) is present in steroidogenic cells, and its expression is induced by hormones known to activate StAR expression (Martin et al., 2008). StAR acts on the mitochondrial outer membrane (OMM) to enable the conversion of cholesterol into pregnenolone by facilitating its movement from the OMM to the inner mitochondrial membrane. Further, StAR interacts with voltage-dependent anion channel 1 (Vdac1) on the OMM, facilitating processing of the 37-kDa phospho-*StAR* to the 32-kDa intermediate. StAR then interacts with cholesterol and, by the enzyme Cyp11a1 (cytochrome P450 side-chain cleavage enzyme), is converted to pregnenolone. In

smooth endoplasmic reticulum, pregnenolone is then transformed into dehydroepiandrosterone and is modified into androstenedione by 3β-hsd (3β-hydroxysteroid dehydrogenase). Later, 17β-hsd (17β-hydroxysteroid dehydrogenase) catalyzes the conversion of androstenedione to testosterone (T). Testosterone can be converted both to 17β-estradiol (E2) by cytochrome P450 aromatase (P450 arom) or to 5 alpha-dihydrotestosterone (DHT) by 5 alpha-reductase (Moeller & Adamski, 2009; Carreau et al., 2011; Papadopoulos & Miller, 2012). Later, 17α-hydroxy pregnenolone is converted to 17β-oh-progesterone by Cytochrome P450, family 21, subfamily A, polypeptide 2 (Cyp21) and is further converted to Cortisol by Cytochrome P450 family 11 subfamily B (Cyp11b) and to Cortisone by Hydroxysteroid 11-beta Dehydrogenase 2 (Hsd11b2). Sex hormones are necessary for an individual's survival and level of fitness. Therefore, biological processes that determine an individual's fitness and survival, such as steroidogenesis and reproduction, operate in parallel.

Present thesis

The present thesis studied the tree sparrow (*Passer montanus*) population that inhabits the area between 23°N and 92°E on the campus of Mizoram University. The study examines the daily and seasonal variations of the selected steroidogenic gene markers, Photoperiodic regulation of genes involved in steroidogenesis and gonadal function in both adult male and female tree sparrows, linking these findings to their annual reproductive cycles. Also, the effect of dLAN and corticosterone treatment on steroidogenesis. The detailed objectives of the thesis are discussed below.

OBJECTIVE 1: ***Daily expression of key genes involved in steroidogenesis and gonadal function***

This section includes the study of the daily rhythmicity of several steroidogenic gene markers and gonadal function in male and female adult tree sparrows at six time points in a 24-hour cycle and the comparison between daily expressions of mRNA transcripts involved in steroidogenesis in the hypothalamus and gonads of male and female tree sparrows.

OBJECTIVE 2: ***Photoperiodic regulations of genes involved in steroidogenesis and gonadal function.***

The section contains results from the following different experiments executed to examine the responses under different conditions of the light properties.

Experiment 1: Effect of day length on steroidogenesis.

This experiment examines the effect of different day-length conditions on the key genes involved in steroidogenesis and gonadal function of male and female tree sparrows.

Experiment 2: Effect of light intensity on steroidogenesis.

This experiment investigates the effects of differential light intensity on steroidogenesis and gonadal function in male and female tree sparrows.

Experiment 3: Effect of light spectra on steroidogenesis.

The experiment covers the differential effects of various compositions of light spectra on key genes involved in steroidogenesis and gonadal function in male and female tree sparrows.

OBJECTIVE 3: ***Annual life history state-dependent expression of steroidogenic genes.***

The section involves the seasonal and monthly variations of several steroidogenic enzyme expressions and their function in the gonads.

OBJECTIVE 4: ***Effect of short-term exposure to dim light at night on steroidogenesis.***

The study examines the effect of dim light as low as 10 lux at night on the steroidogenic gene expression and several metabolic gene expressions.

OBJECTIVE 5: ***Effect of temperature on steroidogenesis.***

This section investigates whether the effect of high and cold temperatures during the photorefractory stage influences photoperiodic responses after subsequent exposure to the photo-stimulatory phase in male tree sparrows

OBJECTIVE 6: ***Effect of corticosterone treatment on steroidogenesis and reproductive-linked processes.***

This study examines whether the corticosterone treatment affects the physiology and mRNA transcripts of steroidogenic genes and reproductive genes in male tree sparrows.

GENERAL MATERIALS AND METHODS

Tree sparrow (*Passer montanus*)

To execute the study, both genders of adult Eurasian tree sparrows, otherwise known as tree sparrows, which belong to the Passeriformes and are found throughout the Indian subcontinent, from Bangladesh to Baluchistan, Eastern Ghats, and Northeast India (Erard, 1999). This species does not have sexual dimorphism. Besides, an adult bird has a dull chestnut crown and nape, a whitish cheek, a black ear patch, a throat with a small black patch not extending onto the breast,

Animal acquisition and maintenance

The Birds were procured in and around the Mizoram University campus, brought to the laboratory, and kept in a wooden cage (L x W x H = 3 × 2.5 × 3 fits) in the room. Food (paddy, *Oryza sativa*; mealworm, *Tenebrio molitor*; and boiled egg) was provided. Mealworm was cultured in laboratory conditions. After procurement, the birds were acclimatized under natural daylight conditions (NDL). Food and water were changed twice daily in the morning and evening, and the cages were cleaned every day. The birds were housed in wooden chambers under laboratory conditions, and the 9-watt CFL, Phillips fluorescent light bulbs were used at the appropriate intensity and wavelength as stated in the respective experiments. During the nocturnal hours, no light was available. Besides, an automatic time switch (Frontier digital timer) was used to control the periods of light and dark transition. In a cage, five sparrows were kept for the experiments. The ventilation was maintained through air circulators.

Study design

The sections have specific experimental protocols, and the detailed protocols are specified in each respective experiments in section. Several physiological

parameters were measured before and after the end of the experiments, and the detailed explanation of the measurements is specified in the respective experiments.

Body mass

The body mass of an individual bird was recorded using a top pan balance with an accuracy of 0.1g. Initially, a very thin cotton bag was weighed and tared, and was then weighed by keeping a bird inside the bag.

Gonad size

Gonad size was recorded using a procedure called unilateral laparotomy reported by (Kumar et al., 2001), where birds were initially anesthetized (Ketamine and xylazine), and a very minor incision was made in between the last two ribs on the left flank and the testis/ovary was tracked by using a spatula within the abdominal cavity, length, and width of the left testis and diameter of the largest ovarian follicles was measured and recorded. Further, the testicular volume was calculated using the formula $4/3\pi ab2$, where a and b represent half of the long (length) and short (width) axes, respectively.

Bill color

Bill color was recorded according to Trivedi *et al*. (2006). Bill's color was evaluated in a score index from 0-5, where 0 represents a stubble bill color, score 1 indicates a bill that is stubble in color with a little hint of blackness, score 2 specifies a slightly blackish bill, whereas score 3 reflects a bill having a comparable percentage of stubble and black, score 4 suggests a bill black with very less stubble patch while score 5 is given to a completely black bill.

Molt

Primaries feathers were scored in between 0–5: where, 0 – indicates a damaged or old feather, 1 – is given to a lost feather that is just released, 2 – is given to a new feather papilla developing up to the accomplishment of one-third of the growth, 3 – to a new feather that has reached two-third of the growth, 4 – to a new partial grown feather, 5 – to a new fully grown feather. For the body molt, the whole body was divided into 11 different regions: 1 – head, 2 – neck, 3 –shoulder, 4 – posterior, 5 – pelvic, 6 – throat, 7 – trunk, 8 – abdomen, 9 – flank, 10 – shank, and 11 – sub-caudal.

Any of the regions could have a score from 0 (old feathers) or 1 (newly emerged feathers), and hence the total body molt score could be in between 0–11 (Trivedi et al., 2006).

RNA isolation and cDNA synthesis

Total RNA was extracted from tissue samples using TRIzol Reagent (Invitrogen by Thermo Fisher Scientific). The purity and concentration of total RNA were determined using (Nanodrop Spectrophotometer Thermo Fisher Scientific, Wilmington, DE). 2 µg RNA was used to process cDNA synthesis. RQ1 DNase (Promega M6101; Madison, WI, USA) kit was used to eliminate any probable genomic DNA contamination. And for cDNA synthesis, the iScript™ cDNA Synthesis Kit (BIO-RAD, USA, 1708891) was used.

Table 1a. Reproductive gene primers

Genes	Forward primer	Reverse primer	Publication
18S	5'GACGCGTGCATTTATCAGAC 3'	3'GCACAGACAGTACCATCGAAAG5'	Yatung & Trivedi 2024
Tshβ	5'ACGTACAAGGAGATGCTGTACC 3'	3'TGTTGCACTTGCCACACTT 5'	Yatung & Trivedi 2024
Dio2	5'AAAGATGTGCAGCTGCTCAC 3'	3'TTGTTGTCCATGCAGTCAGC 5'	Yatung & Trivedi 2024
Dio3	5'AGACCCCTCATCCTCAACTTC 3'	3'ACGGGTGTGCTTCTTCAATG 5'	Yatung & Trivedi 2024
GnRH	5'AATTAGAGGAGGTGCAGCAGAG 3'	3'TCTCCATGGCTTCCTTCAGATC 5'	Yatung & Trivedi 2024
GnIH	5'AAGGTATCACACAGGCTTGGG 3'	3'ATGTCTCGAGGCAGATTGACAG5'	Yatung & Trivedi 2024
Eya 3	5'ACCCAGACCTACGGACTACC3'	3'CAGGTCCCAGAGAAACACCC 5'	Yatung & Trivedi 2024

Table 1b. Steroidogenic gene primers

Genes	Forward primer	Reverse primer	Publication
StAR	5'ACAAAGTGCTGAGCAGGTG 3'	3'TTCTTTGACGTTGGGGTTCC 5'	Yatung & Trivedi 2024

Scp2	5’TGCAAGTGCTGGCAAAGAAC 3’	3’ACGGGTTGTTGGTTGAATGG 5’	Yatung & Trivedi 2024
Cyp11a1	5’GACTTCCGCAACAAACCCTA C 3’	3’TCATTGTCCTCATCTCCATG GC5’	Yatung & Trivedi 2024
Nr4a1	5’GCAAGGGCTTCTTCAAGGTA AG 3’	3’CAAAGCGCTGTGGGAACAT C 5’	Yatung & Trivedi 2024
Cyp11b	5’AGGCCGAGATGATGCTGTTC 3’	3’CATGAGGATGAAGCGGAAC AC5’	Yatung & Trivedi 2024
Cyp17a1	5’TGACCCTGCTGAAGAAATGC 3’	3’AGGCTTCCTTGTGTTCAGTG 5’	Yatung & Trivedi 2024
Srd5a1	5’TGAGTATGTCAGTGGAGCCA AC3’	3’AGCTTTCAATGGTGCAGCAG 5’	Yatung & Trivedi 2024
Hsd11b2	5’TCCTGCCTGGCTACTACAAA AC 3’	3’TGAACTGCTGGTTGATCTCC TC5’	Yatung & Trivedi 2024
Er	5’GAGGGCATGGTGGAAATCTT TG3’	3’TGTGATGCCGTGTTGTTGTG 5’	Yatung & Trivedi 2024
Vdac1	5’AGTTTGGCGGCTCCATTTAC 3’	3’TTGGCTGCTATTCCAAAGCG 5’	Yatung & Trivedi 2024
Foxl2	5’ACAAGAAAGGCTGGCAGAA C 3’	3’TTGCCCTTCTCGAACATGTC 5’	Yatung & Trivedi 2024
Cyp19a1	5’TCGGCCCTTTTTCACCAAAG 3’	3’TTGAGCACGTTGATGTTGCC 5’	Yatung & Trivedi 2024

Table 1c. Metabolic gene primers

Genes	Forward Primer	Reverse primer	Accession no.
Acaca	5’TGGTTTGCACCTTGTGAAG C3’	3’GCTGCAATGCCATTGTTTG C5’	XM_064394767.1
Fasn	5’ATGCAGCCCATTTGTGAAC C3’	3’TTTCAGTGCCAACCAGTGT G5’	XM_039715859.1

Hmgcs 2	5’TCTGTCCTCTGAGTACCCG G3’	3’CGTAGGAGAAGGCACCGA TC5’	XM_039699 320.1
Sirt1	5’TCCTTTGCAACAGCTTCCT G3’	3’ACACAATGTCCGGCTTCAT G5’	XM_064429 683.1
Hmcg	5’GCAGATGGGATGACTCGA GG3’	3’ATGTTTGCTGCATGTGCGT T5’	XM_039729 717.1
Cs	5’TGACCATGCTGGACAACTT C3’	3’AACTTGCTCTCGCTGTTGA G5’	XM_039716 566.1
Aco1	5’TGCTGCAGTGGACCAAAA AG3’	3’TGTCGGTCTGGTGCAATTT G5’	XM_064403 680.1
Idh2	5’TCATCAAGTTTGCGCAGAC G3’	3’TCAGCTTCACATTGGCAAG G5’	XM_039722 937.1
Suclg2	5’AAAGGATCATCAGGCGTTG C3’	3’ATTTGATCTGCTGCCTGCT G5’	XM_039734 373.1
Sdhaf4	5’TCATCACTGCTTTGCCACT C3’	3’ATCAAACCTCCCCACTGGT AAC5’	XM_039730 784.1
Sdhaf2	5’AAAACTGCATCCTGCTCAG C3’	3’AAATGTCCCAGTCGTTGCT G5’	XM_039703 387.1
Sdhc	5’TGTGTCCGTGTTCTCTCTG G3’	3’TTCCAGGTGTGGTAGCACA AG5’	XM_039730 380.1
Fh	5’AACACAAGGGTTGGCTTT GC3’	3’TTGGAGCAGTGACAAAAG GC5’	XM_039710 976.1
Mdh	5’AATCCTGGTTGGTTCCATG C3’	3’TTGCTGGATTCCCAACAAC C5’	XM_039718 827.1
Pygl	5’CATCATCCGCCGCTTCAAA G3’	3’GGTGATCCCGTTGGTCTTG T5’	XM_039698 082.1
Hmcgl	5’GAGTGAAGGTGGTGGAGG TG3’	3’TCGTAGCACCCCATGGAGT A5’	XM_039733 805.1
Foxo1	5’AGCCACCCCACAACAAAA TG3’	3’ACGTGTGTGACATGGTGTT C5’	XM_039716 687.1
Vip	5’CACGCAGACGGGCTCTTC3 ’	3’TCTGTATCTTCCTTCATGTC TGCA5’	XM_039706 557.1

Quantitative (real-time) RT-PCR (qPCR)

Gene-specific primers were designed from the sequences using Primer 3 plus (online primer design program, Boston, MA, USA). qPCR was performed on Quant-Studio 5 (Applied Biosystem by Thermo Fisher Scientific, Foster City, CA, USA). Briefly, PCR reaction of the volume of 7 µl for each gene comprised 1 µl each of cDNA, gene-specific forward and reverse primers, 3 µl Power UpTM SYBR Green Master Mix (Applied Biosystem by Thermo Fisher Scientific, Foster City, CA, USA, A25742), and 1µl of nuclease-free water (Ambion, AM9938). qPCR cycle conditions included 1 cycle at 95°C (20 s), 35 cycles at 95°C (01 s), 60°C (20 s) and 95°C (01 s), additional melt curve plot step included 1 cycle of 60°C (20 s) and 1 cycle of 95°C (01 s). The ΔΔCt method (Livak & Schmittgen, 2001) was applied for the analysis of gene expression. All the samples were run in duplicate.

Hormonal and biochemical assay

Each hormonal and biochemical assays were measured using the protocol provided, and the detailed explanations are specified in the respective experiments.

Statistical analysis

The student's t-test was used to compare values between the two time points. One-way ANOVA (One-way analysis of variance), followed by Tukey's multiple comparison test, was used as a post hoc test for multiple comparisons of the effect of various treatments over a period of time. Likewise, two-way ANOVA was also used to determine the effects when two factors were taken into account. Significant difference was always taken at $P < 0.05$. All statistical analyses were made using GraphPad Prism version 8.

SECTION 1: DAILY EXPRESSION OF KEY GENES INVOLVED IN STEROIDOGENESIS AND GONADAL FUNCTION

1. ABSTRACT

Population responds to environmental variability, largely determined by the dynamic interactions between fitness components within- and among-individual variation in the expression of the environmentally sensitive phenotype. The tree sparrow is one example of a seasonal breeder that has adapted to the shifting environment. The present study was conducted on daily changes in the transcript levels of steroidogenic markers in both adult male and female tree sparrows. The birds were procured (n=5/time points/sex) using a mist net during the breeding season, i.e, in April. Afterwards, the birds were sampled at 6 time points, i.e., ZT1, ZT5, ZT9, ZT13, ZT17, and ZT21 (ZT 0 = sun rise time; 5:14 am) during a 24-hour cycle. Immediately, brain and gonads were excised and stored at -80ºC for gene expression analysis. The study reveals higher expressions were observed in the light phase when the birds are more active, and gradually lower in the light switch-off period in the testis and ovary tissue. However, higher expressions of most of these genes in the hypothalamic tissue were observed in the dark phase in comparison to the light phase, exhibiting tissue-specific 24-hour rhythmicity in the steroidogenic activity. These results show that mRNA levels of steroidogenic genes exhibit daily rhythmicity both in the hypothalamus and gonads of male and female tree sparrows.

2. INTRODUCTION

Changes in the environment and the resulting variance greatly impact animal behavior. Their physiological and behavioral processes exhibit cyclical changes, which can be attributed to their internal biological clock. Animals with biological clocks can synchronize with natural cyclical occurrences, such as day and night and seasons. The primary time cues that might entrain biological clocks are changes in the photoperiod or the 24-hour light-dark cycle. It makes it possible to match the phase of cyclic events in the environment with endogenous rhythms (Dunlap et al., 2004). This environmental time cue, or zeitgeber, produces a series of signals that, once acquired, allow endogenous timers to coordinate with environmental cycles, exhibiting a constant phase relation between the endogenous rhythm and the zeitgeber (Cowan et

al., 2017). Anatomical structures known as oscillators or pacemakers are responsible for the formation and/or regulation of the biological rhythms. According to Welsh et al. (2010), the hypothalamic suprachiasmatic nucleus (SCN) serves as the main pacemaker in mammals. Self-sustained molecular clocks control circadian rhythms, and the so-called clock genes form the core of the molecular clock. Transcription factors *CLOCK* and *BMAL*, which heterodimerize and trigger the transcription of other genes, constitute the positive loop. The negative loop is formed by genes from the *Per* and *Cry* families that are triggered by CLOCK (*BMAL*), and the negative feedback loop is closed when *PER* and *CRY* go to the nucleus and suppress the expression of *Clock* and *Bmal* (Reppert & Weaver 2002; Vatine et al. 2011). According to Panda & Sassone-Corsi (2002), the circadian system has three parts: the oscillator, input, and output pathways. The pacemaker receives input signals from an external or internal synchronizer, which regulates the pacemaker's rhythm by entraining its phase. In addition, the circadian system is influenced by several factors, including temperature, food, and tides, but light is the primary external environmental cue (Rensing & Ruoff 2002; Mistlberger 2009; Tessmar-Raible et al. 2011). These clock genes control the regular expression of several genes, including those related to steroidogenesis. According to research, for example, *Cyp11a1* and *StAR* in rodents' adrenal glands have diurnal expression patterns that peak during the animal's active phase of the circadian cycle (Son et al., 2008). Likewise, *Cyp17a1* expression in fish gonadal tissues has been connected to circadian rhythms, which affect when fish mate (Zhang et al., 2016). In the hypothalamic–pituitary cascade, some hypothalamic neuroendocrine cells regulate the adenohypophysis's hormone production and release (Löhr & Hammerschmidt, 2011). By producing and secreting their hormones, the adenohypophyseal cells in turn can affect target organs or peripheral glands, including the pituitary and hypothalamus, through negative feedback loops (Ooi et al., 2004; Levavi-Sivan et al., 2010). Many vital processes in vertebrates, such as growth, metabolism, reproduction, energy homeostasis, stress reactions, and osmoregulation, are controlled by the hypothalamic-pituitary axis (Machluf et al., 2011). This axis is usually broken down further into multiple axes based on the primary physiological effects and the target organs or endocrine glands (Ooi et al., 2004; Löhr & Hammerschmidt, 2011). In addition, steroidogenic enzymes play a dynamic role in the functioning of sex steroids. The

genetic machinery of reproductive hormones and related processes primarily controls the follicular growth in avian species, and the steroid hormone synthesis and secretion are key factors leading to reproductive activity (Ren et al., 2019). It controls spermatogenesis and spermiogenesis, or the preservation of secondary sexual traits in vertebrates with seasonal reproduction, and is essential for the testis in males (Rosati et al., 2017). The essential steroid hormones that control follicle development are progesterone (P4), testosterone (T), and estradiol (E2) (Paitz & Cagney, 2019). Additionally, seasonal animals' breeding habits can be correlated with the levels of steroid hormones produced. In particular, these animals exhibit large gonadal development, high levels of sex steroids, and reproductive behaviors during the breeding season; in contrast, they show no reproductive activity, low levels of sex steroid hormones, and regressed gonadal development during the non-breeding season (Voigt & Leitner, 2008; Ansel et al., 2011; Forlano et al., 2015). Steroidogenesis consists of cholesterol mobilization from lipid droplets and the plasma membrane, transport into mitochondria, and the formation of pregnenolone into final steroids (Hu et al., 2010). The biosynthesis of all the steroid hormones is controlled by the two key functional classes of steroidogenic enzymes (cytochrome P450 and hydrosteroid dehydrogenase) (Schiffer et al., 2019). A mitochondrial protein, StAR (steroidogenic acute regulatory protein), helps transport cholesterol to mitochondria, further leading to subsequent enzymatic processes for steroid hormone production (Soccio & Breslow, 2003).

As a result of adaptations to various ecological niches and reproductive tactics, many species exhibit variable levels of daily expression of steroidogenic genes. When the active phase begins in mammals like mice and humans, the adrenal glands' production of glucocorticoids peaks in the early morning (for diurnal species) or early evening (for nocturnal species). The coordinated expression of *StAR*, *Cyp11a1*, and *Cyp21a2* is what drives this rhythm (Lightman et al., 2008). *Cyp17a1* and *Hsd3b*, on the other hand, exhibit peak expression during the light phase, supporting egg-laying cycles, and steroidogenic gene expression in the gonads is controlled by photoperiod in avian species, including chickens (Nakao et al., 2007). Also, daily variations in steroidogenic enzymes were reported in *Gallus gallus domesticus L*. (Chustecka et al., 2021). Daily rhythms in HPG gene expression and sex steroids in the plasma of tilapia

(*Oreochromis niloticus*) have been studied (de Alba et al., 2019). In the amphibian (*Pelophylax esculentus*), upregulation of the steroidogenic enzyme gene expression controls cyclic endocrine activity in the testes (Santillo et al., 2017). In adult zebra finches (*Taeniopygia guttata*), expression of the steroidogenic enzymes *Cyp11a1*, *3β-hsd*, *Cyp17a1*, and *Cyp19* has been reported in adrenals and gonads (Freking et al., 2000). Furthermore, steroidogenic genes' daily expression in the brain, especially in areas like the hippocampus and hypothalamus, points to a function in neurosteroidogenesis, which regulates behavior and neural plasticity. For example, circadian shifts in anxiety-like behaviors have been connected to daily variations in *Cyp11a1* expression in the rat brain (Higo et al., 2011).

Though the characterization, localization, and expression levels of the key genes involved in steroidogenesis have been extensively studied in many vertebrates, their daily rhythms have been poorly examined. The present study advocates that no prior research has been conducted on this species concerning daily steroidogenic enzyme gene expression. The study's prime objective is to inspect if the steroidogenic enzyme gene expression exhibits daily rhythmicity in adult male and female Tree sparrows. For this preliminary study, we hypothesized that gene expression varies daily and seasonally. Therefore, we examined the daily and seasonal variations in steroidogenic enzyme gene expression and correlated them with yearly reproductive cycles in male and female tree sparrows.

3. MATERIALS AND METHODS

3.1. Animals

The study was conducted using both male and female adult tree sparrows procured in and around the Mizoram University campus (Tanhril, Aizawl, Mizoram, India, 23° 44'10'' N to 92° 39'51'' E) using a mist net in April 2022, during the breeding season. After procurement, the birds were immediately transferred to the laboratory in an animal house, Department of Zoology, Mizoram University, and kept in an indoor aviary under natural day-length conditions (NDL) in captivity. Food, along with mealworms and water, was provided *ad libitum*. Since this species has no sexual dimorphism, Laparotomy was performed according to the report (Kumar et al., 2001). The next day, sampling was done (n=5/sex/timepoints) at every 4-hour intervals during

a 24-hour cycle at the following 6 time points: ZT1, ZT5, ZT9, ZT13, ZT17, and ZT21 (ZT 0=sunrise time, 5:14 am). Birds were sampled by decapitation, and the brains and gonads were excised and stored at -80°C for gene expression analysis. The hypothalamus and pituitary were later removed from the individual brain by cutting it with a surgical blade. The thick coronal section was cut laterally from the optic chiasma to the infundibular region and dorsally, beginning about TrSm. The protocols were approved by the institutional animal ethics committee of Mizoram University (No: MZU/IAEC/2021-22/12).

3.2. Gene transcription

The mRNA transcripts coding for steroidogenic genes (Table 1b) *StAR, Nr4a1, Er, Scp2, Cyp17a1, Cyp11a1, Hsd11b2, Cyp11b, Srd5a1,* and *Vdac1* were measured in male hypothalamus and testis whereas*, StAR, Nr4a1, Er, Scp2, Cyp17a1, Cyp11a1, Hsd11b2, Cyp11b, Vdac1, Foxl2,* and *Cyp19a1* were measured in the female hypothalamus and ovary tissues for daily transcript expression profile. *18S* as a reference gene was used to determine the relative expression of the transcripts (Yatung & Trivedi, 2024; 2025).

3.3. RNA isolation and cDNA synthesis

Total RNA extraction was done from the desired tissue samples using TRIzol Reagent (Invitrogen by Thermo Fisher Scientific). The purity and concentration of total RNA were determined using (Nanodrop Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). 2 µg RNA was used to process cDNA synthesis. RQ1 DNase (Promega M6101; Madison, WI, USA) kit was used to eliminate any probable genomic DNA contamination. For cDNA synthesis, the iScript™ cDNA Synthesis Kit (BIO-RAD, USA, 1708891) was used.

3.4. Quantitative (real-time) RT-PCR (qPCR)

Gene-specific primers were designed from the sequences using Primer 3 plus (online primer design program, Boston, MA, USA). qPCR was performed on Quant-Studio 5 (Applied Biosystem by Thermo Fisher Scientific, Foster City, CA, USA) as described in our previous publications (Renthlei & Trivedi, 2019; Borah et al., 2020;

Renthlei et al., 2021; Borah et al., 2022; Lalpekhlui et al., 2022; Yatung & Trivedi, 2024). Briefly, PCR reaction of the volume of 7 µl for each gene comprised 1 µl each of cDNA, 1 µl each of gene-specific forward and reverse primers, 3 µl Power UpTM SYBR Green Master Mix (Applied Biosystem by Thermo Fisher Scientific, Foster City, CA, USA, A25742), and 1µl of nuclease-free water (Ambion, AM9938). qPCR cycle conditions included 1 cycle at 95°C (20 s), 35 cycles at 95°C (01 s), 60°C (20 s) and 95°C (01 s), additional melt curve plot step included 1 cycle of 60°C (20 s) and 1 cycle of 95°C (01 s). The ΔΔCt method (Livak & Schmittgen, 2001) was used for gene expression analysis. Each sample was run in duplicate.

3.5. Statistical analysis

One-way ANOVA (one-way analysis of variance) was used to determine the significant variation in the daily profile, and Tukey's multiple comparison tests were applied if ANOVA displayed a significant difference, and $P < 0.05$ was considered a significant difference. Based on report by (Cuesta et al., 2009), values of daily variation of steroidogenic genes were subjected to cosinor analyses based on unimodal cosinor regression $y=A+(B.\cos(2\pi(x-C)/24))]$, where A, B, and C denote the mean level (mesor), amplitude, and acrophase of the rhythm, respectively. The significance of regression analysis was calculated using the number of samples, R2 values, and numbers of predictors (mesor, amplitude, and acrophase; Soper, 2013; http://www.danielsoper.- com/statcalc3/calc.aspx? id 1⁄4 15). In addition, correlation was calculated by Pearson's correlation coefficient (r) to display the correlation between the reproductive gene expression and steroidogenic markers. All the analysis was done using GraphPad Prism version 8.

4. RESULTS

24-hour changes in the expression levels of steroidogenic transcripts in the hypothalamus and gonads of male and female tree sparrows were observed. In males, mRNA transcript of *StAR* gene exhibited daily changes in the hypothalamus ($P=0.0023$; One-Way ANOVA; Table 2a; Fig. 1a) and in the testis ($P<0.0001$; One-Way ANOVA; Table 2a; Fig. 2a). *StAR* mRNA levels displayed variable expression, showed highest expression at ZT5 in the hypothalamus and at ZT13 in the testis. *Scp2*

mRNA transcript (P=0.0001; One-Way ANOVA; Table 2a; Fig. 1b) showed a significant variance in the hypothalamus and peaked at ZT21, whereas no daily variation was observed in the testis (P=0.1749; One-Way ANOVA; Table 2a; Fig. 2b), *Cyp11a1* exhibited daily changes in the hypothalamus (P = 0.0014; One-Way ANOVA; Table 2a; Fig. 1c) as well as in the testis (P<0.0001; One-Way ANOVA; Table 2a; Fig. 2c). Peak expression was observed at ZT21 in the hypothalamus and at ZT5 and ZT13 in the testis. *Nr4a1* presented a significant difference in the hypothalamus (P=0.0001; One-Way ANOVA; Table 2a; Fig. 1d) as well as in the testis (P<0.0001; One-Way ANOVA; Table 2a; Fig. 2d). Peak expression time for *Nr4a1* was at ZT5 and ZT21 in the hypothalamus, whereas, ZT13 in the testis.

Table 2a. Values of One-way ANOVA of daily expression of steroidogenic gene transcription in male tree sparrows

Transcript	Hypothalamus	Testis
StaR	$F_{5,42}$= 4.491, *P*=0.0023	$F_{5,42}$= 10.68, *P*<0.0001
Scp2	$F_{5,42}$= 6.674, *P*=0.0001	$F_{5,42}$= 1.623, *P*=0.1749
Cyp11a1	$F_{5,42}$= 4.848, *P*=0.0014	$F_{5,42}$= 6.988, *P*<0.0001
Nr4a1	$F_{5,42}$= 6.604, *P*=0.0001	$F_{5,40}$= 19.99, *P*<0.0001
Cyp11b	$F_{5,40}$= 4.037, *P*=0.0046	$F_{5,40}$= 2.071, *P*=0.0893
Cyp17a1	$F_{5,42}$= 5.258, *P*=0.0008	$F_{5,42}$= 8.485, *P*<0.0001
Srd5a1	$F_{5,40}$= 6.219, *P*=0.0002	$F_{5,40}$= 3.412, *P*=0.0116
Hsd11b2	$F_{5,38}$= 7.632, *P*<0.0001	$F_{5,42}$= 13.03, *P*<0.0001
Er	$F_{5,42}$= 6.565, *P*=0.0001	$F_{5,42}$= 2.257, *P*=0.0662
Vdac1	$F_{5,42}$= 4.141, *P*=0.0038	$F_{5,40}$= 4.164, *P*=0.0039

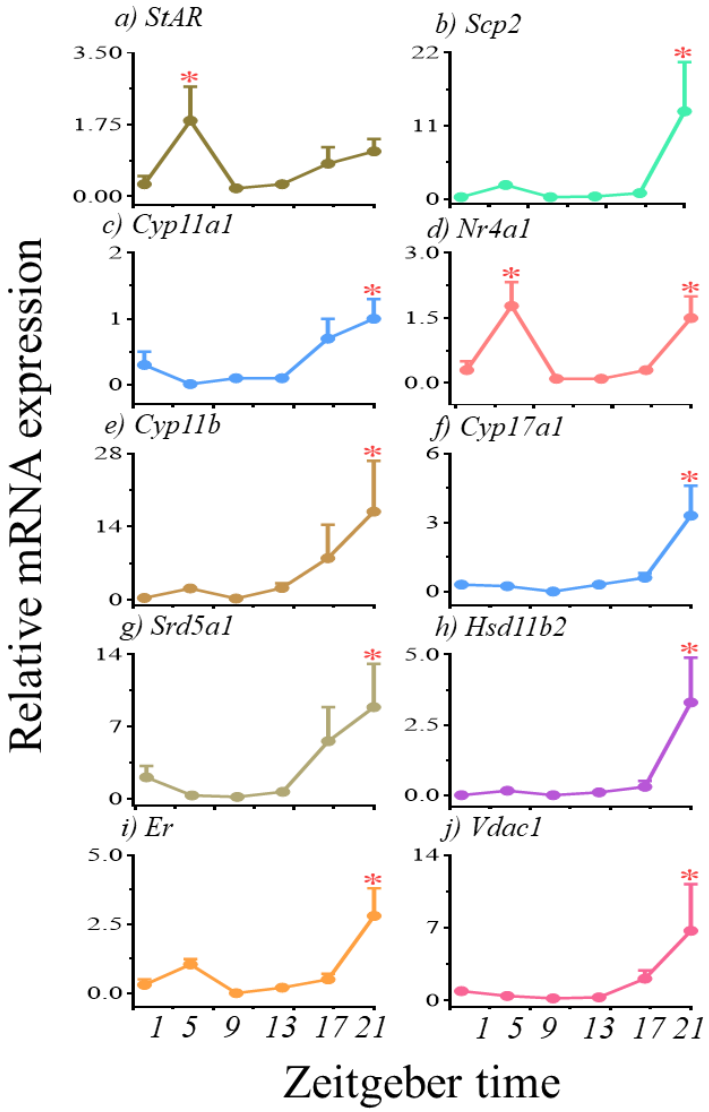

Fig. 1. Data are presented as a mean (± SE, n = 5/month). The mRNA levels of steroidogenic transcripts in the hypothalamus of an adult male tree sparrow collected in April. The X-axis shows the zeitgeber time (ZT 0 represents sunrise). Significant changes across time points were determined using one-way ANOVA and Tukey's multiple comparison test (*, $P < 0.05$).

Cyp11b transcript presented a significant difference in the hypothalamus (P=0.0046; One-Way ANOVA; Table 2a; Fig. 1e) showing the highest expression at ZT21, while no differences were observed in testis (P=0.0893; One-Way ANOVA; Table 2a; Fig. 2e). *Cyp17a1* showed a significant difference in the hypothalamus (P=0.0008; One-Way ANOVA; Table 2a; Fig. 1f) and testis (P<0.0001; One-Way

ANOVA; Table 2a; Fig. 2f). The expression peaked in the hypothalamus was observed at ZT21 whereas, at ZT5 in the testis. *Srd5a1* transcript showed a significant difference in testis (P=0.0116; One-Way ANOVA; Table 2a; Fig. 2g) with peaked expression at ZT5 whereas, peak expression was observed at ZT21 in the hypothalamus (P=0.0002; One-Way ANOVA; Table 2a; Fig. 1g). *Hsd11b2* transcript showed a significant difference in the testis (P<0.0001; One-Way ANOVA; Table 2a; Fig. 2h) and peak expression was observed at ZT5. A significant difference in the hypothalamus (P<0.0001; One-Way ANOVA; Table 2a; Fig. 1h) was observed and peaked at ZT21. *Er* displayed a significant difference in the hypothalamus (P=0.0001; One-Way ANOVA; Table 2a; Fig. 1i) and the expression peaked at ZT21, whereas, no differences in testis were observed (P=0.0662; One-Way ANOVA; Table 2a; Fig. 2i). Transcript expression of *Vdac1* showed a significant difference in the testis (P=0.0039; One-Way ANOVA; Table 2a; Fig. 2j) as well as in the hypothalamus (P=0.0223; One-Way ANOVA; Table 2a; Fig. 1j) and the highest expression of the transcript was observed at ZT13 in the testis and at ZT21 in the hypothalamus.

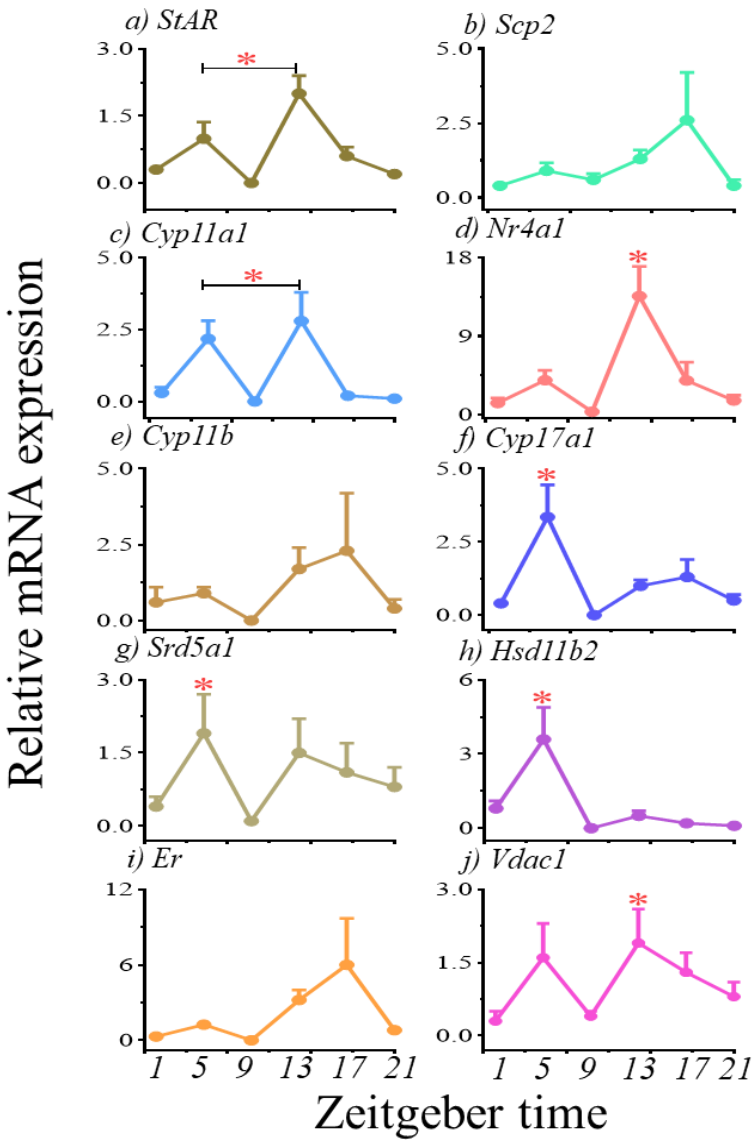

Fig. 2. Data are presented as a mean (± SE, n = 5/month). The mRNA levels of steroidogenic transcripts in the testis of an adult male tree sparrow collected in April. The X-axis shows the zeitgeber time (ZT 0 represents sunrise). Significant changes across time points were determined using one-way ANOVA and Tukey's multiple comparison test (*, $P < 0.05$).

Similarly, daily variations of steroidogenic transcripts in the hypothalamic and ovarian tissues were also measured. The mRNA transcript of *StAR* showed daily variation in the hypothalamic tissue (P= 0.0062; One-Way ANOVA; Table 2b; Fig. 3a) and in the ovary (P= 0.0142; One-Way ANOVA; Table 2b; Fig. 4a) of females. *StAR*

mRNA levels displayed the highest expression at ZT9 both in the hypothalamus and ovary ($P<0.05$; Tukey's multiple comparison tests; Fig. 3a, 4a), while, *Scp2* mRNA levels ($P=0.0158$; One-Way ANOVA; Table 2b Fig. 3c) showed a significant variance and peaked at ZT21 in the hypothalamus, whereas, in the ovary ($P=0.0026$; One-Way ANOVA; Table 2b; Fig. 4c) peaked at ZT5 ($P<0.05$; Tukey's multiple comparison test; Fig. 4c). *Cyp11a1* exhibited daily variations in the hypothalamus ($P=0.0002$; One-Way ANOVA; Table 2b; Fig. 3e) and in the ovary ($P=0.0001$; One-Way ANOVA; Table 2b; Fig. 4e). Peak expression was observed in the hypothalamus at ZT21 and in the ovary at ZT1 and ZT5 ($P<0.05$; Tukey's multiple comparison tests; Fig. 3e, 4e). *Nr4a1* mRNA showed significant variances in the hypothalamus ($P<0.0001$; One-Way ANOVA; Table 2b; Fig. 3b) and ovary ($P=0.0062$; One-Way ANOVA; Table 2b; Fig. 4b).

Table 2b. Values of One-way ANOVA of daily expression of steroidogenic gene transcription in female tree sparrows

Transcripts	Hypothalamus	Ovary
StaR	$F_{5,40}= 3.835$, $P= 0.0062$	$F_{5,18}= 3.911$, $P=0.0142$
Scp2	$F_{5,40}= 3.209$, $P=0.0158$	$F_{5,40}= 4.440$, $P=0.0026$
Cyp11a1	$F_{5,42}= 6.284$, $P=0.0002$	$F_{5,42}= 6.761$, $P=0.0001$
Nr4a1	$F_{5,38}= 7.331$, $P<0.0001$	$F_{5,18}= 4.724$, $P=0.0062$
Cyp11b	$F_{5,40}= 6.653$, $P=0.0001$	$F_{5,42}= 6.865$, $P<0.0001$
Cyp17a1	$F_{5,42}= 7.857$, $P<0.0001$	$F_{5,42}= 3.082$, $P=0.0185$
Foxl2	$F_{5,42}= 6.709$, $P=0.0001$	$F_{5,42}= 3.778$, $P=0.0065$
Cyp19a1	$F_{5,42}= 3.389$, $P=0.0116$	$F_{5,42}= 2.628$, $P=0.0372$
Hsd11b2	$F_{5,42}= 1.810$, $P=0.1317$	$F_{5,40}= 2.189$, $P=0.0745$
Er	$F_{5,36}= 5.026$, $P=0.0014$	$F_{5,42}= 4.418$, $P=0.0025$
Vdac1	$F_{5,42}= 4.599$, $P=0.0020$	$F_{5,41}= 7.776$, $P<0.0001$

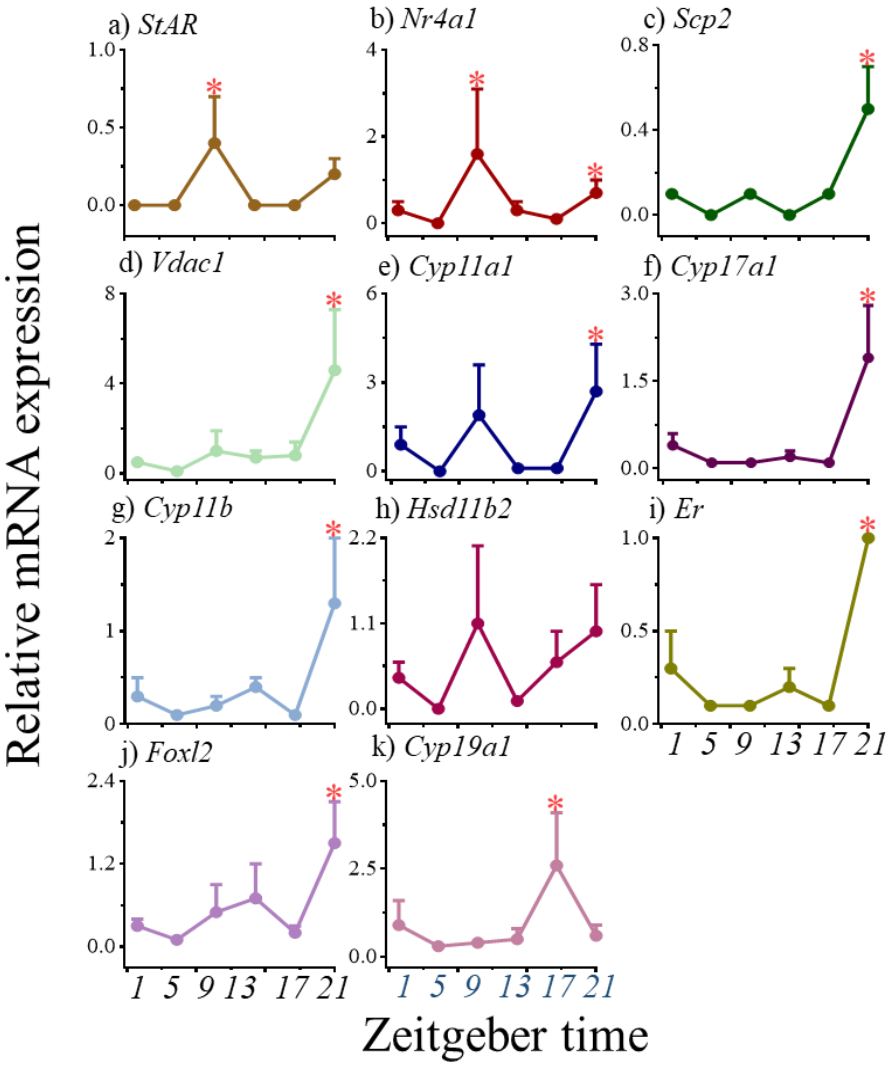

Fig. 3. Data are presented as a mean (± SE, n = 5/month). The mRNA levels of steroidogenic transcripts in the hypothalamus of an adult female tree sparrow collected in April. The X-axis shows the zeitgeber time (ZT 0 represents sunrise). Significant changes across time points were determined using one-way ANOVA and Tukey's multiple comparison test (*, $P < 0.05$).

Highest expression timing for *Nr4a1* was at ZT9 and ZT21 both in the hypothalamus ($P<0.05$; Tukey's multiple comparison tests; Fig. 3b) and in the ovary at ZT1 ($P<0.05$; Tukey's multiple comparison tests; Fig. 4b). *Cyp11b* mRNA exhibited a significant difference in the hypothalamus ($P=0.0001$; One-Way ANOVA; Table 2b; Fig. 3g) displaying the highest expression at ZT21 ($P<0.05$; Tukey's multiple

comparison tests; Fig. 3g) whereas, in ovary at ZT5 ($P<0.0001$; One-Way ANOVA; Table 2b; Fig. 4g). Further, *Cyp17a1* appeared to have a significant difference in the hypothalamus ($P<0.0001$; One-Way ANOVA; Table 2b; Fig. 3f) as well as in the ovary ($P=0.0185$; One-Way ANOVA; Table 2b; Fig. 4f). In the hypothalamic tissue, the peaked expression was observed at ZT21 ($P<0.05$; Tukey's multiple comparison tests; Fig. 3f). However, *Hsd11b2* transcript did not show significant difference in the ovary ($P=0.0745$; One-Way ANOVA; Table 2b; Fig. 4h) as well as in the hypothalamus ($P=0.1317$; One-Way ANOVA; Table 2b; Fig. 3h). A significant difference in the *Er* transcript was observed in the hypothalamus ($P=0.0014$; One-Way ANOVA; Table 2b; Fig. 3i) and peaked at ZT21 ($P<0.05$; Tukey's multiple comparison tests; Fig. 3i) and in the ovary ($P=0.0025$; One-Way ANOVA; Table 2b; Fig. 4i) at ZT1 and ZT9 ($P<0.05$; Tukey's multiple comparison tests; Fig. 4i).

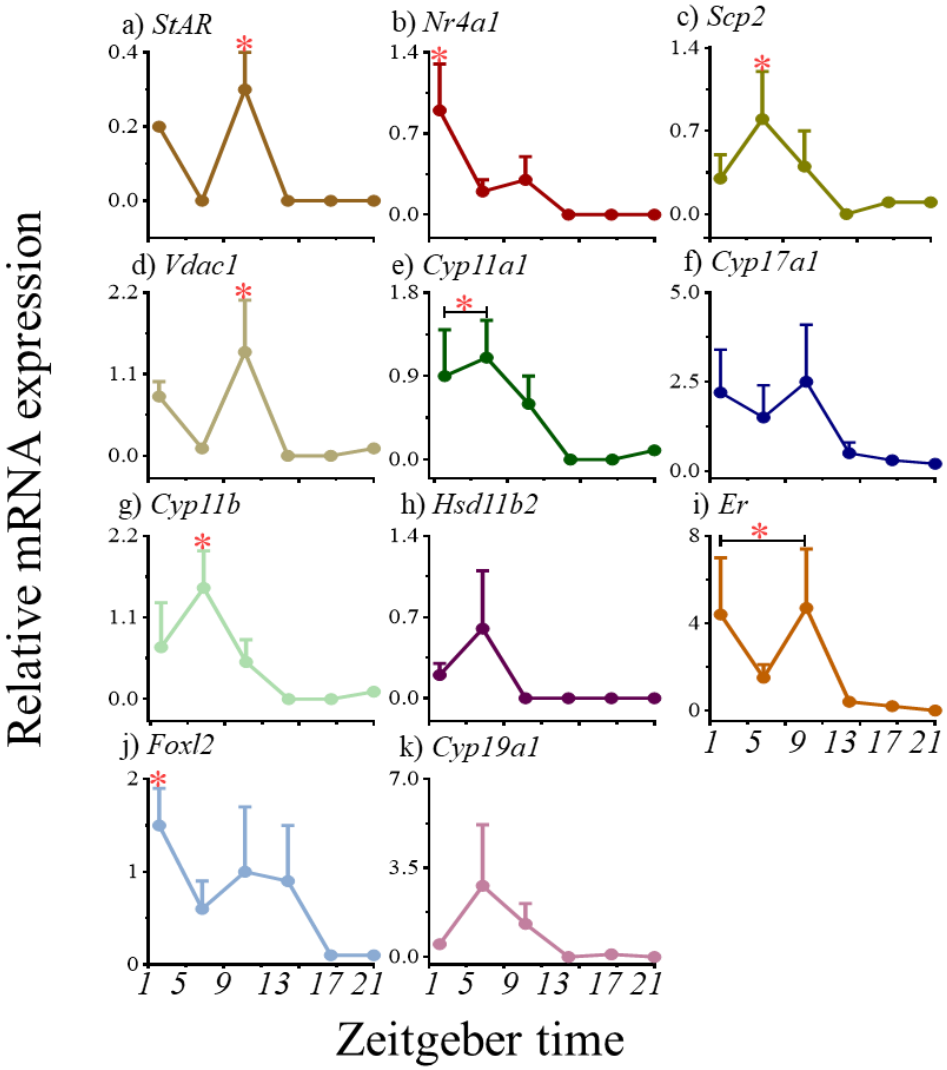

Fig. 4. Data are presented as a mean (± SE, n = 5/month). The mRNA levels of steroidogenic transcripts in the ovary of an adult female tree sparrow collected in April. The X-axis shows the zeitgeber time (ZT 0 represents sunrise). Significant changes across time points were determined using one-way ANOVA and Tukey's multiple comparison test (*, $P < 0.05$).

Transcript expression of *Vdac1* displayed a significant variance in the ovarian tissue (P<0.0001; One-Way ANOVA; Table 2b; Fig. 4d) and in the hypothalamic tissue (P=0.0020; One-Way ANOVA; Table 2b; Fig. 3d); the highest expression of the *Vdac1* was observed at ZT9 in the ovary (P<0.05) and at ZT21 in the hypothalamus (P<0.05). *Foxl2* mRNA transcript exhibited significant difference in the hypothalamus (P=0.0001; One-Way ANOVA; Table 2b; Fig. 3j) showing peaked expression at ZT21(P<0.05) while, at ZT1 in ovary (P=0.0065; One-Way ANOVA; Table 2b; Fig. 4j). Further, transcript expression of *Cyp19a1* was significant in the hypothalamus (P=0.0116; One-Way ANOVA; Table 2b; Fig. 3k) showing highest expression at ZT17(P<0.05) whereas, no significant differences were observed in ovary (P=0.0372; One-Way ANOVA; Table 2b; Fig. 4k).

5. DISCUSSION

Seasonal breeders produce more sex steroid hormones throughout reproductive seasons, causing major physiological and anatomical changes. Tree sparrows are seasonal breeders that adapt to shifting environments. Studies on daily variation in the steroidogenic gene expressions are very limited. The study investigated transcript expressions of the several genes such as *StAR, Nr4a1, Er, Scp2, Cyp17a1, Cyp11a1, Hsd11b2, Cyp11b, Srd5a1, Vdac1, Foxl2, and Cyp19a1* which are known to be involved in steroidogenic pathways were measured both in the hypothalamic tissue and gonads of the adult male and female tree sparrows, a long-day strong seasonal breeder. Renthlei et al. (2021) also covered the significance of temperature and photoperiod in controlling seasonal reproduction. The diurnal expression of several genes involved in steroidogenesis has been the subject of very limited investigations in avian and other species (Di Rosa et al., 2016; de Alba et al., 2019; Chustecka et al., 2021). *StAR*, a component of Leydig cells in males; granulosa and theca cells in the ovary, which aids in the movement of cholesterol across the mitochondria. *StAR* exhibited a significant diurnal rhythm with their acrophases showing expression at ZT5 and ZT13 in the testis and at ZT5 in the hypothalamus during the light phase, suggesting the secretion of the enzyme being maximum in the daytime, which is consistent with findings in nile tilapia (de Alba et al., 2019), where enzyme release is highest during the light phase. Consistent with the previous study (Di Rosa et al.,

2016), no daily variation was observed in the expression level of the *Cyp11b* gene in the testis. Nonetheless, *Cyp11b* transcript expression throughout the current investigation represents daily fluctuations in the hypothalamus; besides *StAR*, *Cyp11a1*, *Nr4a1*, *Cyp17a1*, *Hsd11b2*, and *Srd5a1* enzyme mRNA expression were shown to be higher during the light phase, peaked at ZT5, and then gradually decreased during the night phase following sunset in the present study (Yatung & Trivedi, 2024).

Similar responses of daily variation of steroidogenic gene markers were observed in the hypothalamus and ovary of female tree sparrows. The female ovary's Granulosa cells include the gene *StAR*, which helps move cholesterol through the mitochondria. where *StAR* plays a pivotal role in 24-h rhythmicity with their acrophases revealing peaked expression at ZT9, during the light switched-on period, indicating the secretion of the maximum enzyme during the light phase, which is also evident in male tree sparrows in a study reported by (Yatung & Trivedi, 2024). Similarly, *Cyp17a1*, *Cyp19a1*, and, *Foxl2* also presented significant daily rhythms with their acrophases located in the first half of the light phase (ZT1, 5, and 9, respectively) which is consistent with study findings conducted in Nile Tilapia (de Alba et al., 2019; Di Rosa et al., 2016). In the current investigation, nearly all the steroidogenic genes such as (*StAR, Nr4a1, Scp2, Vdac1, Cyp11a1, Cyp17a1, Cyp11b, Hsd11b2, Er, Cyp19a1,* and *Foxl2*) showed light-dependent expression in the ovary during the light phase and at the dark phase in the hypothalamic tissue and are consistent with the study (Di Rosa et al., 2016; Yatung &Trivedi, 2024). A study on Yangzhou goose (*Anser cygnoides*) suggests that both circadian clock genes and genes involved in progesterone synthesis exhibit rhythmic expression patterns in ovarian preovulatory granulosa cells, and *Bmal1* regulates the transcription of the *StAR* gene (Chen et al., 2023). In addition, the expression of steroidogenic genes exhibits diurnal changes during a 24-h cycle in the gonads and brain tissues of zebra fishes and rhythmic expression of aromatase (*cyp19a1a*), antimüllerian hormone (amh), *foxl2*, and *dmrt1* was observed in gonads, while *cyp19a1*b and *cyp11b* exhibited daily differences in the brain (Di Rosa et al., 2016).

Steroidogenesis, the biological process of synthesizing steroid hormones, is crucial for reproductive and physiological functions in bird species. While ovarian and testicular steroidogenesis share central similarities, they differ in their regulation, hormonal production, and roles in avian reproduction. In females, the primary organ for steroidogenesis is the theca cells (which produce androgens (e.g., testosterone, androstenedione) and granulosa cells (which produce progesterone, especially in preovulatory follicles, but also contribute to estrogen synthesis in smaller follicles, while in males in the Leydig cells (which is the primary site of androgen production e.g., testosterone, androstenedione) and in Sertoli cells (which support spermatogenesis but are less directly involved in steroidogenesis) (Wingfield, 1993). The steroidogenesis in both organs is closely regulated by the hypothalamic-pituitary-ovarian/testicular axis (HPG).

The daily expression of steroidogenic genes was observed in the hypothalamus of male and female tree sparrows (Yatung & Trivedi, 2024, 25). *StAR*, which plays a crucial role in shuttling cholesterol into mitochondria, leading to the beginning of the steroidogenic pathway, showed its peak expression at ZT 5 (Fig. 1a) in the male hypothalamus, whereas in females, the peak expression was observed at ZT 9 (Fig. 3a) and similar response were observed in *Nr4a1* expression in hypothalamus and gonad of both sexes (Fig. 1d and Fig. 3d) *Scp2*, a sterol protein which helps in the movement of cholesterol, *Cyp11a1* (helps in the formation of pregnenolone), *Cyp11b* (converts to 17β-oh-progesterone into cortisol), *Cyp17a1*(essential for producing glucocorticoids and androgens), *Hsd11b2* (primarily plays a role in regulating cortisol and corticosterone bioavailability), *Er* (regulates the production of estrogen), and *Vdac1* (helps in the translocation of StAR) were expressed highest at ZT21 in both male and female hypothalamus. However, *Srd5a1,* which is crucial for the metabolic conversion of testosterone into dihydrotestosterone (DHT), and this Conversion is essential for proper male sexual development in birds, peaked at ZT21. Also, Foxl2 (associated with the transcriptional regulation of CYP19A1 in female gonads) and *Cyp19a1* (aromatize testosterone into estrogen) showed peaks at ZT1 and ZT5 in female hypothalamus.

In the testicular tissue, expression of the *StAR* peaks at ZT13 (Fig. 2a), whereas it was expressed in advanced at ZT9 (Fig. 4a) in the ovarian tissue. No differences

were noticed in testicular *Scp2* transcripts (Fig. 3b). However, a peak expression was noted at ZT5 (Fig. 4c) in the ovary. Several peaks were observed in *Cyp11a1* at ZT5 and ZT13 (Fig. 2c) in the testis and the ovary at ZT1 and ZT5 (Fig. 4e). *Nr4a1* peaked at ZT13 in the testis, while advanced expression was observed at ZT1 in the ovary. There was no significant difference in *Cyp11b* and *Er* (Fig. 2e, i) in the testis, however, in the ovary *Cyp11b* peaked at ZT5 (Fig. 4g) and *Er* at ZT1, ZT9 (Fig. 4i). *Cyp17a1* displayed maximum expression at ZT5 (Fig. 2f) in the testis, however no variance was observed in the ovary. In the testicular tissue, *Hsd11b2* transcript levels were maximum at ZT5 (Fig. 2h), however, no variance was observed in the ovary. *Vdac1* showed several peaks, showing the highest expression at ZT13 (Fig. 2j), and in the ovary, maximum expression was observed at ZT9 (Fig. 4d).

6. CONCLUSIONS

The study may conclude that the results demonstrate key genes known to be involved in steroidogenesis for the production of sex hormones that are expressed in the hypothalamic tissues and the gonads of the tree sparrows and reveal diurnal rhythmicity, showing maximum expression in the light phase. The results of the experiments indicate that food intake and cholesterol biosynthesis may be indicators of diurnal fluctuations in the expression of steroidogenic genes. Besides, differential expression of these transcripts at different times suggests tissue-specific and time-dependent regulation of steroidogenesis in both male and female tree sparrows.

SECTION II: PHOTOPERIODIC REGULATION OF GENES INVOLVED IN STEROIDOGENESIS AND GONADAL FUNCTION

1. ABSTRACT

Avian species are greatly impacted by the light, and the light source is the most prominent environmental cue to which these species rely to time their life history stages, such as reproduction, behaviour, and endocrine processes. Light comprises three fundamental characteristics: duration, intensity, and spectrum. Almost all bird species are diurnal, and they experience variations in light intensity that correspond to daily, lunar, or seasonal cycles. These physiological changes are governed by the interaction between the endogenous circadian rhythm and the ecological variables such as temperature, resource accessibility, and photoperiod (light source). Besides, Photoperiod is one of the most critical environmental cues regulating seasonal variations in reproductive physiology and behavior, and long day (LD) photoperiods in spring stimulate gonadal recrudescence and a resulting increase in plasma concentrations of gonadal steroids. An effective season-related neuroendocrine mechanism appears to be mediated by appropriate reproduction timing and HPG-axis activity linked with reinitiation, maintenance, and regression of spermatogenesis and steroidogenesis. In the present study, we examined the outcome of differential effects of photoperiodic regimes, intensity, and spectra on the steroidogenic gene markers expression in the hypothalamus tissue and gonads of male and female tree sparrows and linked with the seasonal reproduction. The study was carried out in three experiments. Photosensitive adult male and female birds were used and procured using mist nets. Birds, n=5/sex/group, were used in each experiment, respectively. In experiment one, birds were divided into three groups. Group 1 was treated with short photoperiod 8L:16D, Group 2 was treated with equinox photoperiod 12L:12D, and Group 3 was treated with long photoperiod 16L:8D. For female birds, a similar experimental design was used. The experiment was run for 30 days. Body weight, bill's color, and gonad size were recorded at the beginning and end of the experiment. The birds were sampled in the middle of their light phase. Hypothalamus and gonads were excised and kept in -80ºC for further analysis. In experiment two, both male and female birds were divided into 3 groups each. Group 1 was exposed to 10 lux. Group

2 was exposed to 50 lux, and Group 3 was exposed to 100 lux intensity of light. All the groups were kept under photoperiodic chambers of 16L:8D, and the experiment was run for 30 days. Body mass, gonad size, and bill color were recorded before and after the experiment. Later hypothalamus and gonad tissue were harvested for gene expression analysis. In experiment three birds (n=5 birds/group/sex) were used and divided into four groups, each sex. Group one was exposed to red spectrum (650 nm), Group two was exposed to blue spectrum (450 nm), Group 3 was exposed to green spectrum (520 nm), and Group four was exposed to white light, and served as a control group. The intensity used was 0.78 W/m^2. All the groups were exposed to a long photoperiod of 16L:8D, and the experiment was run for 30 days. Body mass, color of the bill, and gonadal size were recorded before and at the end of the experiment. Hypothalamus and gonads were harvested and immediately kept at -80ºC for gene expression analysis. All the birds were sampled at the mid-point of their light phase. The results of experiment one reveal the differential effect of photoperiod on reproductive-linked steroidogenesis. No significant difference was observed in body mass; however slight increase in bill color was observed in 12L:12D and 16L:8D. In addition, higher expressions of reproductive stimulatory genes such as *Tshβ*, *Dio2* and *GnRH* were observed in 16L:8D followed by 12L:12D in the hypothalamus, however, higher expressions of reproductive inhibitory genes such as *Dio3* and *GnIH* were observed in 8L:16D. Besides, highest expressions of steroidogenic gene markers (*StAR, Nr4a1, Er, Scp2, Cyp17a1, Cyp11a1, Hsd11b2, Cyp11b, Vdac1, Srd5a1, Foxl2,* and *Cyp19a1*) were observed in the birds exposed to 16L:8D, which mimics the long day experienced during breeding season, followed by 12L:12D, an equinox photoperiod, suggesting 12 hours of light reflects the optimal day length to stimulate reproductive activity. However, the inhibitory effects of reproductive activity were observed in 8L:16D, which reflects a short photoperiod. Experiment two showed no significant effects of differential intensity on body mass and bill color in females; however, bill color was slightly darker in the 50 and 100 lux groups. Gonadal growth was highest in the 100 lux group compared to the 10 lux and 50 lux groups in both sexes. In both the male and female hypothalamus, higher expressions of reproductive genes were observed in the 100 lux of light intensity group, followed by 50 lux, and lowest in the 10 lux intensity group. Similarly, the maximum expression of

steroidogenic genes was observed in the 100-lux intensity group, then 50 lux, compared to 10 lux, both in the hypothalamus and testicular tissue of both sexes. Experiment four reveals the differential outcome of light spectra treatment on tree sparrows. In contrast to white light exposure, gonadal growth was more advanced in the red-light group. We did not observe any gonadal growth in the blue light group. Besides, the light spectra do not affect body mass and bill color in both sexes. Whereas we observed higher expressions of reproductive genes in the red-light group, in contrast to green and blue light. Also, maximum expressions of several steroidogenic transcripts were witnessed in the red-light group and were lower in the blue light group. Overall, the present study reveals that light intensity stimulates the gonadal growth in both male and female tree sparrows. Highest reproductive and steroidogenic transcript levels were observed at 100 lux intensity, indicating the positive influence on reproductive-linked steroidogenic processes. In addition, the study suggests that a long photoperiod modulates steroidogenic responses and a short photoperiod inhibits the steroidogenic functions in this species. Moreover, longer-wavelength (red light) has a stimulatory effect, while short-wavelength (blue light) has an inhibitory effect on photoperiodic responses in tree sparrows

2. INTRODUCTION

Annual changes in season are surrounded by different ecological factors such as photoperiod, temperature, and food resources, which may contribute to an animal's seasonal changes in behavior, physiology, and survival. The seasonal change in day length (Photoperiod) is the predominant extrapolative factor that avian species use to time seasonal rhythms (Dawson, 2001; Tolla & Stevenson, 2020). Photoperiod has a similar effect on reproduction and steroidogenesis in non-avian species like mammals; however, the processes differ. For example, in seasonal breeders such as sheep and hamsters, photoperiodic melatonin secretion patterns modulate *GnRH* expression, regulating gonadal steroid synthesis (Chemineau et al., 2007). In fish and amphibians, photoperiod influences spawning and steroid hormone levels, often in concert with temperature cues (Pankhurst & Porter, 2003). A circadian rhythm of photoperiodic photosensitivity, or CRPP, is involved in the photoperiodic modulation of reproductive processes in birds (Rani & Kumar, 1999; Dawson et al., 2001). Hence, seasonal

responses are the outcome of seasonal variations in the phase relationship between CRPP and environmental factors. When the photoperiod occurs within the photoinducible phase of an endogenous circadian rhythm, the photoperiodic feedback takes place. Additionally, circannual rhythm output is an endogenous rhythm that lasts for around a year and controls various behavioral and physiological systems associated with the yearly cycles of birds. Based on photoperiodic responses, the photosensitive species are categorized as; Long day breeders: Such birds, prolonged daylight (e.g., 16L:8D) increases gonadal development reproductive activity, whereas short day lengths (such as 8 hours of light a day) are inhibitory example, Indian weaver bird (*Ploceus philippinus*), redheaded bunting (*Emberiza bruniceps*), black-headed bunting (*Emberiza melanocephala*), common Indian rosefinch (*Carpodacus erythrinus*), crested bunting (*Melophus lathami*), yellow-throated sparrow (*Petronia xanthocollis*), house sparrow (*Passer domesticus*), brahminy myna (*Sturnia pagodarum*) ((Thapliyal & Saxena, 1964b; Thapliyal & Tewary, 1964; Singh & Chandola, 1981; Chakravorty & Chandola, 1985; Tewary & Tripathi, 1983; Rani, 1999; Tewary & Kumar, 1982; Misra et al., 2004; Kumar & Tewary, 1983b; Tewary et al., 1983; Tewary & Kumar, 1983a; Tewary & Tripathi, 1985; Tewary & Dixit, 1986; Trivedi et al., 2006; Kumar & Kumar, 1991; 1993). Both long and short-day breeders: Species under this group of exhibit gonadal recurrence during both long and short photoperiods (e.g., 16L:8D and 8L:16D), such as Blackheaded munia (*Lonchura malacca*) (Thapliyal & Saxena, 1964a; Pandha & Thapliyal, 1969). Short day breeders: In this group, short photoperiod (8L:16D) can cause gonadal growth. However, long photoperiod (16L:8D) increases pituitary activity, as evidenced by the emergence of LH-dependent plumage. Such as Lal munia, *Estrilda amandava* (Thapliyal & Tewary, 1963; Tewary & Thapliyal, 1965), and lastly, very short-day breeders: Species in this group, a very short photoperiod (such as 3L:21D) can stimulate gonadal growth. Such as Spotted munia, *Lonchura punctulata* (Chandola et al., 1975; Thapliyal et al., 1975). These variations highlight the evolutionary conservation of photoperiodic regulation, adapted to species-specific ecological niches.

Light is a key environmental factor that can affect physiological and behavioral processes and welfare in birds (Manser, 1996; Deep et al., 2010). Physiological roles

and effects of light include facilitating sight, regulating reproductive hormone release, and affecting social behavior. Most bird species are diurnal, meaning they experience variation in light intensity that corresponds to daily, lunar, or seasonal cycles. Unsurprisingly, light has a significant impact on many areas of avian physiology, biology, and ecology. Organs (eyes, pineal organ, and hypothalamus) govern the photoperiodism and circadian rhythms in avian species (Oishi et al., 2001; Underwood et al., 2001). Photoperiodic induction is influenced by both light wavelength and intensity (Rani & Kumar, 2000; Malik et al., 2002; Misra et al., 2004). The wavelength and intensity (irradiance) of daily light influence the endogenous time-keeping system such that the circadian output is matched with the ideal time of day and/or annually (Malik et al., 2004).

Apart from the photoperiod, the intensity refers to the number of photons available in the light source, and color is the sensation experienced due to the activation of certain classes of photoreceptors by selected wavelengths of the visible light spectrum. Light quality is defined and manifested by photoperiod, intensity/brightness, and spectrum, and plays a pivotal role as an environmental factor activating reproductive functions. Besides, the influence of light on bird reproduction has been studied for many decades to accelerate productivity. In addition, the terms light intensity and light brightness are mistakenly mixed due to a misinterpretation of light perception in avian species. The intensity of any electromagnetic radiation (visual light is a subset of electromagnetic radiation) is measured in watts/m2, whereas brightness (a unit that reflects the effect of light on retinal photoreceptors) is measured in lux (lx), foot candle, and lumen. The third component of light quality is the light source's spectrum output, which is measured in nanometers. In most bird species, light perception occurs at two prime sites: the eye through the retina (retinal photoreceptors) and various locations in the brain via extra-retinal photoreceptors (ERPRs). Several investigations identified deep brain photoreceptors in sites other than the retina, including the pineal gland, olfactory bulb, and hypothalamus (Scanes & Dridi, 2021). However, in mammalian species, retinal photoreceptors are the only ones that regulate the circadian rhythm (Tosini et al., 2016). Light is a radiant energy that excites the retina and produces a visual sensation. In mammals, eyes are the only photoreceptive

structure but in non-mammalian vertebrates, besides the eyes (retinal photoreceptors), the extra-retinal photoreceptors (e.g. lateral eyes, pineal and parapineal organs, and deep brain photoreceptors) also serve as the sites of encephalic photoreception These multiple photoreceptive structures interact and aids to these animals' diurnal or seasonal timing (Foster & Soni 1998; Kumar et al. 2004). Moreover, Light interacts with objects and is reflected in different directions and intensities at various wavelengths, depending on the structures and material compositions of different objects. Correspondingly, the eyes of all birds have the same basic structure, but there is variation that presumably reflects their ecological needs (Martin & Osorio, 2008). Visual systems are generally used for detecting intensity and spectral differences in light reflected from objects in space and deriving a perception of contrasts between them. Contrasts derived purely by a difference in intensity hold no color information and are called achromatic contrasts. The "visible light" corresponds to a wavelength range of 380–760 nm and is represented as VIBGYOR (violet, indigo, blue, green, yellow, orange, and red) (Kumar et al., 2017). The wavelengths of ultraviolet and infrared radiation are shorter than those of visible violet and red light, respectively. A blend of visible spectrum colors makes up white light, whereas complete darkness is represented by black light. This indicates that red light has a lower frequency than blue light due to its longer wavelength (~622–780 nm) than blue light (~455–492 nm). This variation in wavelength causes them to penetrate animal tissues at varying strengths, which results in distinct biological consequences (Kumar et al., 2000). Light quality is defined and represented by photoperiod, intensity/brightness, and spectrum, and it plays a crucial role in triggering reproduction.

Birds' color vision is mediated by four types of cone photoreceptors (Olsson, 2016), meaning that birds have an extra dimension of color information. The light source is detected through the retinal and extra-retinal photoreceptors in the avian species. (Siopes & Wilson, 1980; Sharp, 1993; Dawson et al., 2001). The retina allows birds to see and further synchronize to their environment and exhibits the effect of light on their behavior and development (Wilson & Lindstrom, 2011). The retina contains 2 types of photoreceptors, cones and rods. Lewis et al. (2007) reported that cones have the highest sensitivity to blue (450 nm), green (550 nm), red (700 nm), or

violet (415 nm) wavelengths. Extra-retinal photoreceptors are situated in the pineal gland and the hypothalamus (Siopes & Wilson, 1980; Dawson et al., 2001) and are responsible for controlling birds' circadian rhythm via the melatonin secretion (Pelham et al., 1972; Pang et al., 1974; Nir et al., 1987; Kumar & Rani, 1999). The hypothalamus directly controls or is involved in the control of most homeostatic and physiological processes, including reproduction, and is located within the brain tissue. Hence, in avian species, light is a very crucial environmental factor that not only aids vision but also influences key physiological responses.

In photoperiodic species, long photoperiods stimulate opsin-containing deep-brain photoreceptors. Consistently, VA opsin also plays a pivotal role along with OPN5 and OPN4, which stimulate the thyroid signaling in the hypothalamus (Yoshimura et al., 2003; Nakao et al., 2008; Nakane et al., 2010; Perfito et al., 2012; Pérez et al., 2019; Pérez et al., 2023). Further, *TSH* within the MBH (Medial Basal Hypothalamus) acts on the ECs (Ependymal cells), induces transcription of *Dio2* via the TSH receptor pathway, and an increase in the *Dio2* transcription further leads to downstream progressions at the gonadal levels (Nakao et al. 2008). Studies on black-headed bunting suggest that photoperiodic initiation is influenced by both light wavelength and intensity (Kumar & Rani 1996; Rani & Kumar 2000; Malik et al. 2002). Malik et al. (2004) also displayed that longer wavelengths are more effective than short wavelengths. Buntings display comparable patterns of photoperiod response, particularly in their reproductive phenotypes. According to Stevenson et al. (2022), Japanese quail exhibit exceptionally strong alterations in their neuroendocrine response and reproductive physiology in response to an extended photoperiod. A study by Sharp (1993) concluded that the photoperiod is the most significant cue that affects the concentrations of *FSH (follicle-stimulating hormone)* and *LH (luteinizing hormone)* (Dittami et al., 1985; Dawson et al., 2001). The secretion of FSH and LH regulates the production of Estradiol (E_2), which is essential for the development of the ovary (Bacon et al., 1980). Similarly, the physiology and reproductive behavior of vertebrates are primarily regulated by sex steroid hormones (Nelson et al., 1990). The genetic system of reproductive hormones and associated functions primarily controls avian follicular growth, and steroid hormone synthesis and secretion (Ren et al., 2019).

Studies have reported that the reproductive performance of domestic birds is highly dependent on proper light manipulation. This involves both the quantity (duration and intensity) and light's quality (color or wavelength). According to Rostati et al. (2019), vital steroid hormones such as progesterone (P4), testosterone (T), and Estradiol (E2) regulates follicular growth in females and; in males, it controls the spermatogenesis and spermiogenesis in the testis, or the maintenance of secondary sexual characteristics in vertebrates with seasonal reproduction (Rosati et al., 2019). Thus, the sex-steroid hormones have a significant role as regulators of vertebrates' reproductive physiology and behavior. The expression of key enzymes ultimately determines the different sex- and season-specific patterns, and since these hormones are essential for reproduction in a wide range of vertebrates, their removal prevents reproduction (Meitzen & Thompson, 2008). Besides, Steroidogenesis, the biosynthesis of steroid hormones, is tightly regulated by photoperiod-induced changes in enzyme activity (e.g., aromatase and 5α-reductase) and gene expression within gonadal and adrenal tissues (Ubuka et al., 2013). Disruptions in photoperiod, such as those caused by artificial lighting, can impair reproductive success and steroid hormone balance, with implications for conservation and aquaculture (Pankhurst & Porter, 2003).

A seasonal alteration in light intensity levels may influence the timing of the annual testicular and molt cycles in equatorial stonechats, *Saxicola torquata* (Gwinner & Scheuerlein, 1998). In humans, light intensity controls circadian rhythms through the stimulation of photoreceptors in the retina, influencing melatonin production and sleep-wake cycles (Czeisler et al., 1995). In aquatic ecosystems, the vertical distribution of phytoplankton is determined by the light intensity gradients, which form the base of the marine food web (Kirk, 2011). Similarly, terrestrial animals, such as insects and birds, exhibit behavioral responses to light intensity, including phototaxis and foraging patterns (Warrant & Nilsson, 2006). These examples underline the universal importance of light intensity in determining biological systems across taxonomic groups. It has been demonstrated that wavelength and intensity affect diverse functions of birds such as behavior, growth pattern, immune system, and stress status (Scott & Siopes 1994; Senaratna et al. 2008). It affects energy expenditure, in terms of oxygen consumption and carbon dioxide production, and different behaviors,

viz., activity, rest, and feeding in chickens (Kim et al. 2014). Also, the magnetic compass orientation in Australian silvereyes (*Zosterops lateralis*), European robins (*Erithacus rubecula*), carrier pigeons (*Columba livia f. domestica*), garden warblers (*Sylvia borin*), and domestic chickens (*Gallus gallus*) is influenced by light wavelength (Wiltschko et al., 1993, 2007). In addition, low light intensity significantly decreases locomotor activity and increases time sitting or lying on the litter. Ultimately, dim light can negatively affect leg health, produce hock and breast blisters, and patchy areas of poor-quality litter (Bessei, 2006). Since the broiler chickens show prominent daily rhythmic behavior and appear to display more content behaviors under brighter light, hence, bright light has been suggested to improve the welfare of birds (Alvino et al., 2009). Besides, when reared with higher intensity, such as (180-200 lux), birds become more active compared to lower intensity of (5-6 lux) of light (Newberry et al., 1988; Blatchford et al., 2009). Furthermore, it is suggested that prolonged light exposure may negatively affect the melatonin release by the pineal gland (Martínez-Soriano et al., 1999). Its secretion rises during the dark hours, which impairs animal reproductive efficiency by inhibiting *GnRH* secretion and upregulating *GnIH* secretion (Mousa-Balabel & Mohamed, 2011; Palacin et al., 2011; Celi et al., 2013). *GnRH* is recognized to exert a positive influence on reproductive function, as well as the production and secretion of gonadotropins from the pituitary (Sun et al., 2017). Therefore, an adequate light intensity is required for establishing a clear distinction between day and night periods, and this light intensity serves as a signal to commence or cease the biosynthesis of melatonin hormone, which is a biological stimulus in animals that signals changes in day and night cycle that contribute in the regulation of reproductive processes. Besides, other than avian species, a study in rabbits observed that 80-90 lux of light intensity could improve the pregnancy rate of rabbit does compared with the use of 60 lux (Ren et al., 2014). Also, a study on Pekin ducks (*Anas platyrhynchos domesticus*) showed, increased light intensity improved reproductive performance. Besides, under a 12L:12D photoperiod, the 900lux group of Shan-partridge ducks (*Anas platyrhynchos*) laid more than the 600lux group (Porter et al., 2018; Liu-Fu et al., 2024). These studies reveal that light intensity has an effect on poultry laying performance. However, the mechanism by which light intensity regulates ovarian development in avian species is still unknown.

In addition to light intensity and duration, the spectrum structure of light can also influence many physiological processes, including growth, metabolism, reproduction, and behavior (Dakan, 1934; Scott & Payne, 1937; Ringoen, 1942; Benoit & Otto, 1944; Ishibashi & Kato, 1951; Benoit & Assenmacher, 1966; Morris, 1967; Woodard et al., 1968; Harrison et al., 1970; Hollwich, 1979; Kumar & Rani, 1996; Kumar et al., 2000; Malik et al., 2002, 2004; Malik et al., 2014; Borah et al., 2018). Photoperiodic control of daily and seasonal responses has been exhibited in the bunting (Malik et al., 2014), as well as circadian behavior such as the Indian Weaver bird (Pandey & Bhardwaj, 2011). A study on Japanese quail revealed that red light and green light enhance the gonadal development, whereas under blue light, a continuous regression of gonads has been observed (Yadav & Chaturvedi, 2015). In the context of wavelength, it was determined that red light stimulated gonadal growth and development in the European starling, *Sturnus vulgaris* (Bissonnette et al., 1932), English sparrow (Ringoen, 1942), and fowl (Ishibashi & Kato, 1951), but blue or green light had an inhibitory effect. Also, gonadal development in the duck responded primarily to the red wavelengths (Benoit & Ott, 1944). Also, a study reported on Turkey (*Meleagris*) suggests, white light or the long wavelengths of red induced sexual maturity months in advance of the breeding season (Scott & Payne,1937). Mobarkey et al. (2013) demonstrated that, in contrast to the use of red light, selective photo stimulation under green light led to a notable delay in the beginning of egg production in hens. Apart from the avian species, the reproductive physiology, energy metabolism, and growth and development have been observed in tropical damselfish, *Chrysiptera cyanea* (Bapary et al., 2011). In juvenile barfin flounder, *Verasper moseri*, the somatic growth is stimulated by green spectral light (Yamanome et al., 2009), while rainbow trout is negatively impacted by blue spectral light, and juvenile gilthead seabream (*Sparus aurata*), inhibited by red spectral light (Karakatsouli et al., 2007). However, in the subtropical sapphire devil (*Chrysiptera cyanea*), ovarian growth is stimulated by red light, which has a greater gonadosomatic index (GSI) than white light (Bapary et al., 2011).

Most of the studies have evidenced the effect of different photoperiods and intensities on the reproductive activity, behavioral, and hormonal changes in avian

species. However, less is known regarding the effect of exposure to differential photoperiod regimes and how the variation in light intensity affects steroidogenesis in a seasonal breeder such as tree sparrows. Moreover, less is known about the effect of different spectra on the several steroidogenic gene marker expressions in this species. Therefore, the present study examines the effect of steroidogenic transcript expression levels on being exposed to variable photoperiodic regimes, differential light intensity, and light spectra on the reproductive-linked steroidogenic regulations in the brain and at the gonadal level in tree sparrows.

3. MATERIALS AND METHODS

3.1. Animal procurement and maintenance

The investigation was carried out on both male and female adult photosensitive tree sparrows procured in late November using mist nets. All the procedures were approved by the institutional animal ethics committee of Mizoram University (No. MZU/IAEC/2021-22/12). The study was categorized into three experiments.

3.1.1. Experiment 1: Effect of different photoperiod regimes on steroidogenesis

In experiment one, birds were divided into three groups (n=5/sex/group) and transferred into a wooden cage (46 × 46 × 46 cm). The cage was placed inside a photoperiodic chamber (L x W x H = 3 × 2.5 × 3 fits) exposed to different photoperiod regimes. Group 1 was exposed to a short photoperiod of 8L:16D (8 hours of light and 16 hours of darkness), Group 2 was exposed to an equal photoperiod of 12L:12D (12 hours of light and 12 hours of darkness), and Group 3 was exposed to a stimulatory photoperiod of 16L:8D (16 hours of light and 8 hours of darkness). The experiment was run for 30 days. Body mass, Bill colour, and gonad size were recorded before and at the end of the experiment. And after 30 days, all the birds were sampled at the middle of their respective light phase. Hypothalamus and gonad tissue were collected for analysis of gene expression.

3.1.2. Experiment 2: Effect of light intensity on steroidogenesis

In experiment two, birds (n=5/sex/group) were categorized into 3 groups and transferred into a wooden cage. The cages were kept under a photoperiodic chamber exposed to a long photoperiod (16L: 8D; 16h light and 8h dark) but with varying light

intensity. Group 1 was treated with 10 lux; Group 2 was treated with 50 lux, and Group 3 was treated with 100 lux intensity. The experiment was run for 30 days. At the end of the study, all the birds were sacrificed under the dim light in the middle of their light phase. Body mass, color of the bill, and gonad size were measured before and after the end of the experiment. Hypothalamus and gonads were collected and stored at -80ºC for gene expression analysis.

3.1.3. Experiment 3. Effect of light spectra on steroidogenesis

In this experiment, birds were categorized into four groups (n=5/sex/group) and kept in a cage consisting of 5 birds each group. The cages were kept inside a photoperiodic chamber exposed to a long stimulatory photoperiod (16L: 8D; 16h light and 8h darkness) with the same light intensity but with different light quality (spectra). Intensity was maintained at 0.78/W/m^2 measured at the perch level. Group 1 was subjected to red light (650 nm), Group 2 was subjected to blue light (450 nm), Group 3 was subjected to green light (520 nm), and Group four was subjected to white light, which also served as a control group. All the groups were exposed to the same photoperiod of 16L:8D, and the experiment was run for 30 days. The physiological parameters, such as body weight, color of bill, and gonadal growth, were recorded before and at the end of the experiment. After the termination of the study, all the birds were sacrificed under dim light in the middle of their light phase. The brains and gonads were harvested and used for a gene expression study.

Later, the hypothalamus, along with the pituitary, was harvested from the individual brain by cutting the brain with a surgical blade having a thick coronal section trimmed dorsally (starting approximately at TrSm) and laterally extending from the optic chiasma to the infundibular area.

3.2. Gene transcription

In the hypothalamus of male and female tree sparrows, the mRNA transcription levels of reproductive genes encoding for *Tshβ*, *Dio2*, *Dio3*, *GnRH*, and *GnIH* were assessed. In addition, for the effect of different photoperiodic regimes, intensity and spectra of the mRNA transcription levels of steroidogenic genes encoding for *StAR*, *Nr4a1*, *Er*, *Scp2*, *Cyp17a1*, *Cyp11a1*, *Hsd11b2*, *Cyp11b*, *Srd5a1*, and *Vdac1* were

measured in the male hypothalamus and testis, and the female tree sparrow's hypothalamus and ovary corresponding to *StAR*, *Nr4a1*, *Er*, *Scp2*, *Cyp17a1*, *Cyp11a1*, *Hsd11b2*, *Cyp11b*, *Vdac1*, *Foxl2*, and *Cyp19a1*. The relative expression of the transcripts was determined using *18S* as a reference gene.

3.3. RNA isolation and cDNA synthesis

From the hypothalamus and gonad tissue, total RNA was extracted using TRIzol Reagent (Invitrogen by Thermo Fisher Scientific). The purity and concentration of the RNA were determined using (Nanodrop Spectrophotometer Thermo Fisher Scientific, Wilmington, DE). And for cDNA synthesis, a starting RNA of 2 µg was used to process. RQ1 DNase (Promega M6101; Madison, WI, USA) kit was used to eliminate any probable genomic DNA contamination. For cDNA synthesis, the iScript™ cDNA Synthesis Kit (BIO-RAD, USA, 1708891) was used.

3.4. Quantitative (real-time) RT-PCR (qPCR)

The Primer 3 plus (an online primer designing program, Boston, MA, USA was used to design gene-specific primers from the sequences. Real-time PCR (qPCR) was performed on Quant-Studio 5 (Applied Biosystem by Thermo Fisher Scientific, Foster City, CA, USA). Briefly, PCR reaction of the volume of 7 µl for each gene comprised 1 µl each of cDNA, 1 µl each of gene-specific forward and reverse primers, 3 µl Power UpTM SYBR Green Master Mix (Applied Biosystem by Thermo Fisher Scientific, Foster City, CA, USA, A25742), and 1µl of nuclease-free water (Ambion, AM9938). qPCR cycle conditions included 1 cycle at 95°C (20 s), 35 cycles at 95°C (01 s), 60°C (20 s) and 95°C (01 s), additional melt curve plot step included 1 cycle of 60°C (20 s) and 1 cycle of 95°C (01 s) as reported in the previous publications (Renthlei & Trivedi, 2019; Borah et al., 2020; Renthlei et al., 2021; Borah et al., 2022; Lalpekhlui et al., 2022; Yatung & Trivedi, 2004, 25). The ΔΔCt method was used for gene expression analysis reported by (Livak & Schmittgen, 2001). Each sample was run in duplicate.

3.5. Statistical analysis

All statistics were performed by using GraphPad Prism version 8.0, San Diego, CA, USA. One-way ANOVA was used to determine the significance of differences between groups in terms of transcript expression, and two-way ANOVA was used to

determine the physiological parameter when two time points were taken into consideration. Followed by Tukey's multiple comparison test, if ANOVA showed a significant difference. Significant differences were always taken at $P<0.05$.

4. RESULTS

4.1. Experiment 1: Effect of day length on steroidogenesis

In male tree sparrows (Table 3a), 30 days of treatment with short or long photoperiod does not alter the body mass (Photoperiod: P=0.0918; Time: P=0.5145; Interaction: P= 0.0003; Two-way ANOVA; Fig. 5a). However, bill color showed a significant difference (Photoperiod: P<0.0001; Time: P=0.0064; Interaction: P=0.0001; Two-way ANOVA; Fig. 5b). The color of the bill was observed to be darker in the 12L:12D and 16L:8D compared to 8L:16D. A significant difference was observed in testicular volume (Photoperiod: P<0.0001; Time: P<0.0001; Interaction: P<0.0001; Two-way ANOVA; Fig. 5c). Increased testicular volume was observed in 16L:8D and 12L:12D ($P<0.05$) in comparison to 8L:16D.

Table 3a. Values of the Two-way ANOVA of physiological parameters in male tree sparrows (photoperiod)

	Time	Treatment	Interaction
Body mass	$F_{(1,24)}$=0.4378, P=0.5145	$F_{(2,24)}$ =2.642, P=0.0918	$F_{(2,24)}$ =11.69, P=0.0003
Bill color	$F_{(1,24)}$ = 8.909, P= 0.0064	$F_{(2,24)}$ =69.27, P<0.0001	$F_{(2,24)}$ =13.27, P= 0.0001
Testicular volume	$F_{(1,24)}$ =26.72, P<0.0001	$F_{(2,24)}$ =16.80, P<0.0001	$F_{(2,24)}$ =16.47, P<0.0001

Table 3b. Values of the Two-way ANOVA of physiological parameters of female tree sparrows (photoperiod)

	Time	Treatment	Interaction
Body mass	$F_{(1,22)}$ =0.01011, P=0.9208	$F_{(2,22)}$ =2.104, P=0.1459	$F_{(2,22)}$ = 0.2259, P=0.7997
Bill color	$F_{(1,22)}$ =4.006, P=0.0578	$F_{(2,22)}$ =10.62, P=0.0006	$F_{(2,22)}$ =1.057, P=0.3646
Testicular volume	$F_{(1,22)}$ = 15.47, P= 0.0007	$F_{(2,22)}$ =6.463, P=0.0062	$F_{(2,22)}$ =8.384, P=0.0020

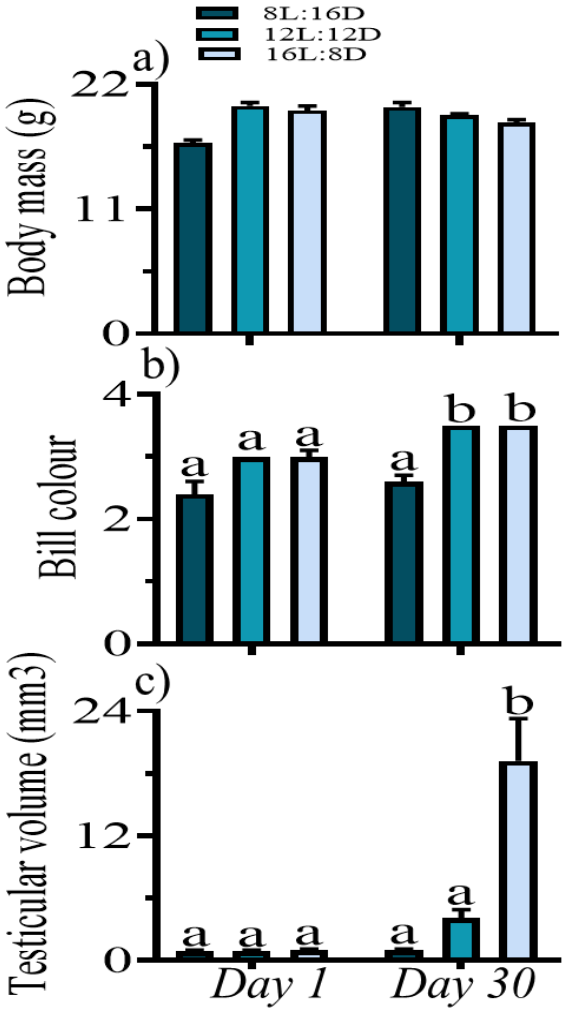

Fig. 5. Data are represented as mean (± SE, n=5/group). Body mass (a), bill color (b), and testicular volume (c) of photosensitive male tree sparrows when exposed to 8L:16D, 12L:12D, and 16L:8D for 30 days. Two-way ANOVA followed by Tukey's multiple comparison test was applied to assess whether there is a significant difference between the groups. Lowercase letters represent the significant difference ($P < 0.05$).

Also, in females (Table 3b), 30 days of treatment of short or long photoperiod does not alter the body mass (Photoperiod: P=0.1459; Time: P=0.9208; Interaction: P=0.7997; Two-way ANOVA; Fig. 6a). However, a significant difference was observed between birds exposed to 8L and 16L of light regimes on bill color (Photoperiod: P=0.0006; Time: P=0.0578; Interaction: P=0.3646; Two-way ANOVA,

Fig. 6b). Darker bill color was observed in the birds exposed to long day i.e., 16L:8D photoperiod ($P < 0.05$). Similarly, time and treatment affected follicular growth. After 30 days of photoperiod exposure, a significant increase in the follicular diameter was observed (Photoperiod: P=0.0062; Time: P=0.0007; Interaction: P=0.0020; Two-way ANOVA; Fig. 6c) of birds exposed to lengthy photoperiods, 12L: 12D and 16L: 8D ($P < 0.05$) in comparison to 8L: 16D ($P > 0.05$).

Table 3c. Values of One-way ANOVA of the reproductive gene transcripts in male tree sparrows (photoperiod)

Transcripts	Hypothalamus
Tshβ	$F_{(2, 11)} = 1.037$, P=0.3869
Dio2	$F_{(2, 12)} = 11.20$, P=0.0018
Dio3	$F_{(2, 12)} = 23.85$, P<0.0001
GnRH	$F_{(2, 12)} = 14.01$, P=0.0007
GnIH	$F_{(2, 12)} = 1.899$, P=0.1920

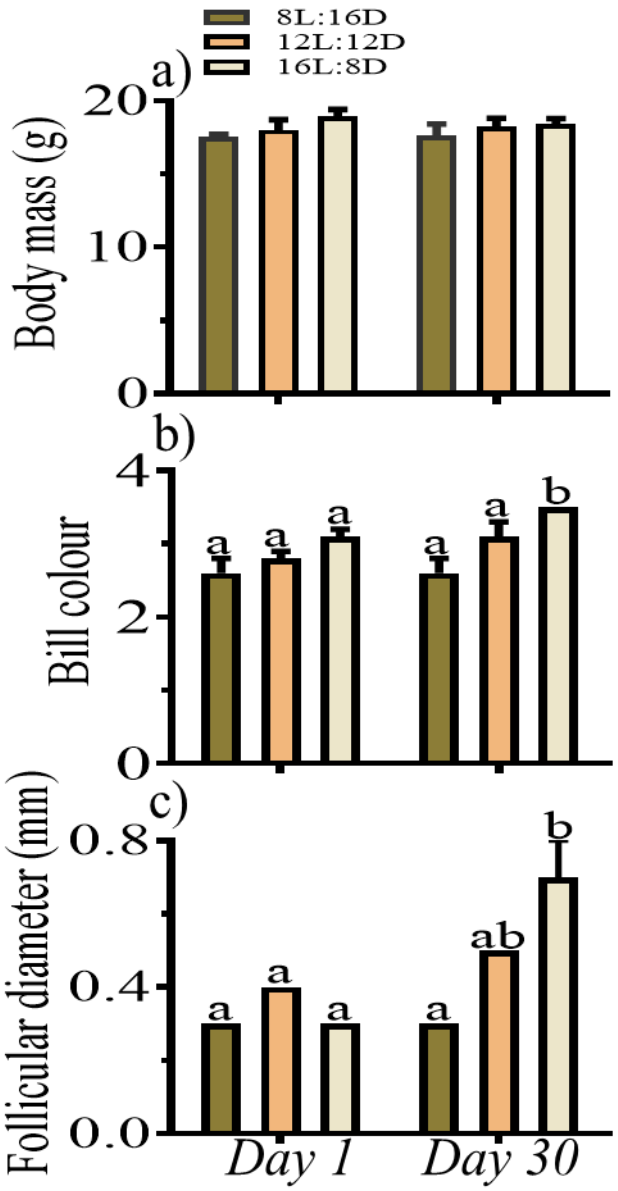

Fig. 6. Data are represented as mean (± SE, n=5/group). Body mass (a), bill color (b), and follicular diameter (c) of photosensitive male tree sparrows when exposed to 8L:16D, 12L:12D, and 16L:8D for 30 days. Two-way ANOVA followed by Tukey's multiple comparison test was applied to assess whether there is a significant difference between the groups. Lowercase letters represent the significant difference ($P < 0.05$).

Similarly, 30 days of treatment of short and long photoperiod resulted in differential expression of the steroidogenic transcripts in the hypothalamus of male

tree sparrow (Fig. 7). Long day exposed birds (12L:12D and 16L:8D) resulted in higher expression of *Dio2* (P= 0.0018; One-way ANOVA; Table 3c; Fig. 7b), and *GnRH* (P= 0.0007; One-way ANOVA; Table 3c; Fig.7d), While higher expression of *Dio3* (P <0.0001; One-way ANOVA; Table 3c; Fig.7c) was observed in short photoperiod (8L:16D) exposed birds compared to 12L:12D and 16L:8D group. However, no significant differences were observed in *Tshβ* (P = 0.3869; One-way ANOVA; Table 3c; Fig. 7a) and *GnIH* (P = 0.1920; One-way ANOVA; Table 3c; Fig. 7e) across all photoperiod-exposed groups.

Table 3d. Values of One-way ANOVA of the reproductive gene transcripts in female tree sparrows (photoperiod)

Transcripts	Hypothalamus
Tshβ	$F_{(2, 12)} = 9.331$, P=0.0036
Dio2	$F_{(2, 12)} = 1.493$, P=0.2637
Dio3	$F_{(2, 10)} = 3.512$, P=0.0699
GnRH	$F_{(2, 11)} = 7.886$, P=0.0075
GnIH	$F_{(2, 12)} = 0.3132$, P=0.7369

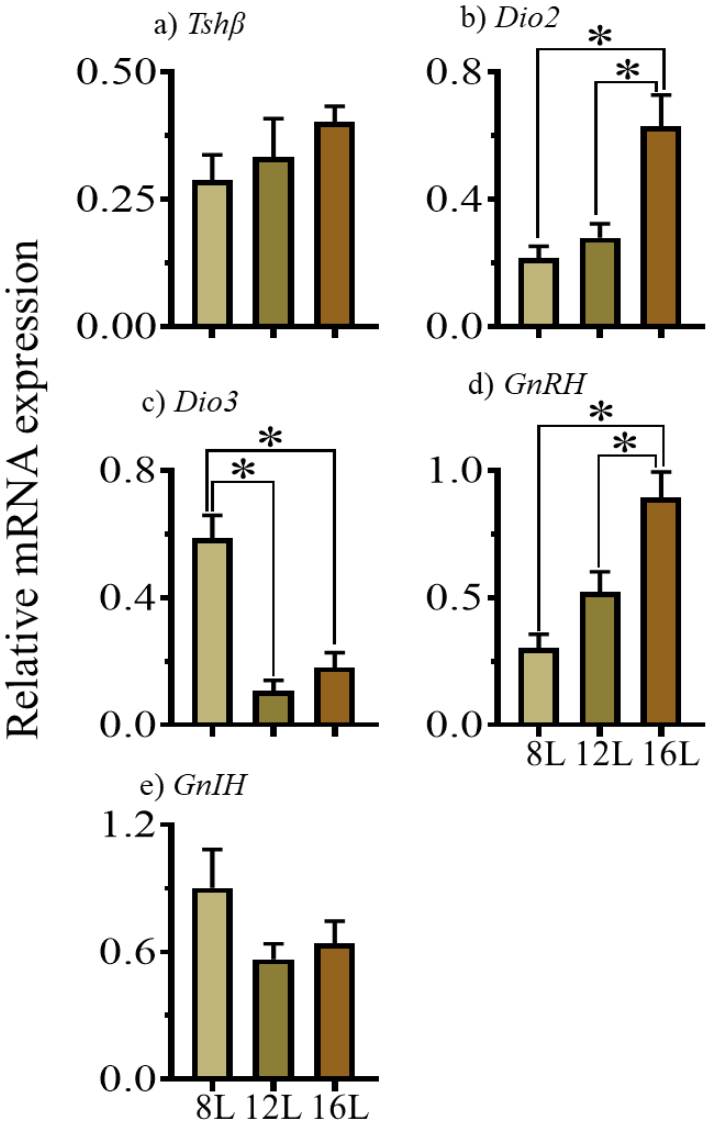

Fig. 7. Data are represented as mean (± SE, n =5/group). Relative mRNA expression of hypothalamic reproductive genes in adult male tree sparrows exposed to different photoperiods. One-way ANOVA followed by Tukey's multiple comparison test to determine significant differences. * represents a significant difference ($P < 0.05$).

Similarly, the transcripts known to be involved in seasonal reproduction were also assessed in the hypothalamic tissue of adult female tree sparrows, where a significant difference was observed in *Tshβ* (P= 0.0036; One-way ANOVA; Table 3d; Fig.8a). Higher expression of *Tshβ* was found in birds exposed to 16L:8D and 12L:12D groups compared to 8L:16D group. While, higher expression of *GnRH* (P= 0.0075;

One way ANOVA; Table 3d; Fig.8d) was found in birds exposed to 16L:8D when compared to 8L:16D and 12L:12D. However, there was no significant differences in the transcript's expression of *Dio2* (P= 0.2637; One-way ANOVA; Table 3d; Fig.8b), *Dio3* (P= 0.0699; One-way ANOVA; Table 3d; Fig.8c) and *GnIH* (P= 0.7369; One-way ANOVA; Table 3d; Fig.8e).

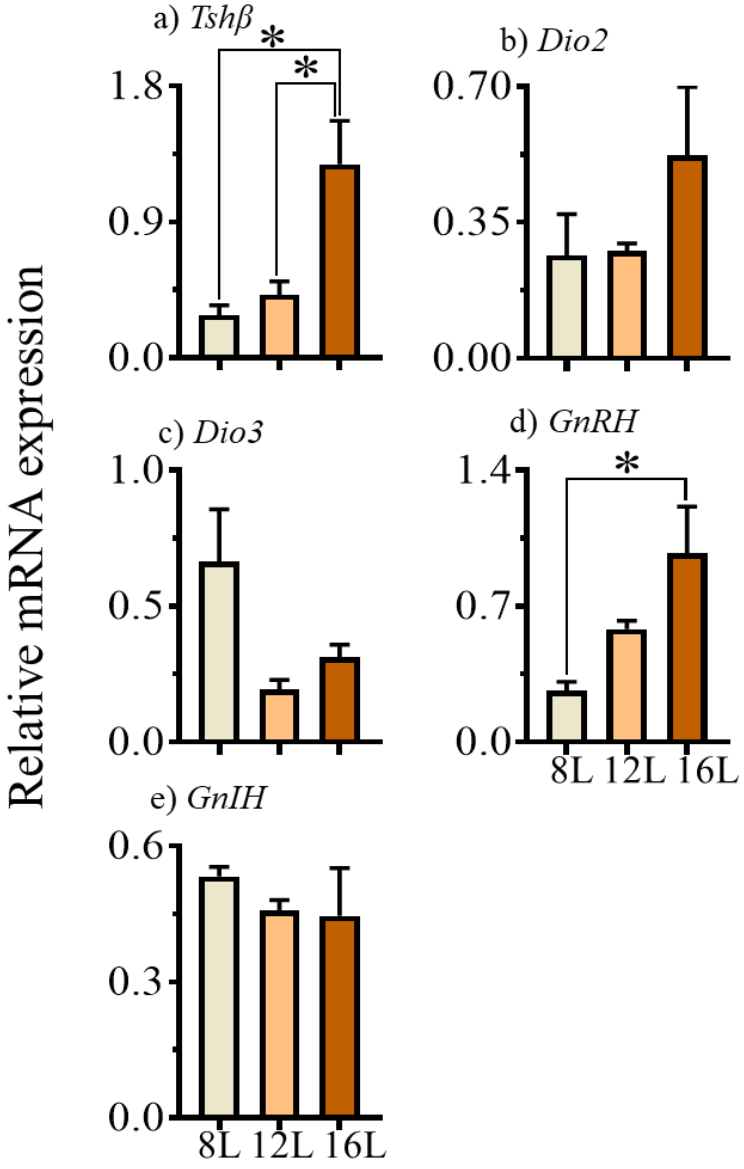

Fig. 8. Data are represented as mean (± SE, n =5/group). Relative mRNA expression of hypothalamic reproductive genes in adult female tree sparrows exposed to different photoperiods. One-way ANOVA followed by Tukey's multiple comparison test to determine significant differences. * represents a significant difference ($P < 0.05$).

Several transcripts involved in steroidogenesis were also observed in the hypothalamus and testis tissue of adult male tree sparrows. In the hypothalamus, higher expression of mRNA transcripts of *Er* (P= 0.0026; One-way ANOVA; Table 3e;

Fig.9c), *Scp2* (P<0.0001; One-way ANOVA; Table 3e; Fig.9d), *Hsd11b2* (P= 0.0107; One-way ANOVA; Table 3e; Fig.9h), *Vdac1* (P<0.0001; One-way ANOVA; Table 3e; Fig.9i) and *Srd5a1*(P<0.0001; One-way ANOVA; Table 3e; Fig.9j) were observed in 12L:12D and highest in 16L:8D exposure groups in comparison to 8L:16D group. However, no differences were found in mRNA transcripts of *StAR* (P= 0.3833; One-way ANOVA; Table 3e; Fig.9a), *Nr4a1* (P= 0.1453; One-way ANOVA; Table 3e; Fig.9b), *Cyp11a1* (P= 0.2525; One-way ANOVA; Table 3e; Fig.9e), *Cyp17a1* (P= 0.0930; One-way ANOVA; Table 3e; Fig.9f), *Cyp11b* (P= 0.4682; One-way ANOVA; Table 3e; Fig.9g) in all the groups.

Table 3e. Values of One-way ANOVA of the steroidogenic gene transcripts in male tree sparrows (photoperiod)

Transcripts	Hypothalamus	Testis
StaR	$F_{(2, 21)} = 1.004$, P=0.3833	$F_{(2, 23)} = 5.655$, P=0.0101
Scp2	$F_{(2, 21)} = 16.13$, P<0.0001	$F_{(2, 21)} = 1.371$, P=0.2758
Cyp11a1	$F_{(2, 21)} = 1.471$, P=0.2525	$F_{(2, 21)} = 5.161$, P=0.0150
Nr4a1	$F_{(2, 21)} = 2.117$, P=0.1453	$F_{(2, 21)} = 7.783$. P=0.0030
Cyp11b	$F_{(2, 21)} = 0.7869$, P=0.4682	$F_{(2, 21)} = 10.25$, P=0.0008
Cyp17a1	$F_{(2, 21)} = 2.665$, P=0.0930	$F_{(2, 21)} = 1.193$, P=0.3232
Srd5a1	$F_{(2, 21)} = 18.24$, P<0.0001	$F_{(2, 21)} = 9.590$, P=0.0011
Hsd11b2	$F_{(2, 21)} = 5.672$, P=0.0107	$F_{(2, 21)} = 9.664$, P=0.0011
Er	$F_{(2, 21)} = 7.982$, P=0.0026	$F_{(2, 21)} = 22.51$, P<0.0001
Vdac1	$F_{(2, 21)} = 18.70$, P<0.0001	$F_{(2, 21)} = 14.68$, P=0.0001

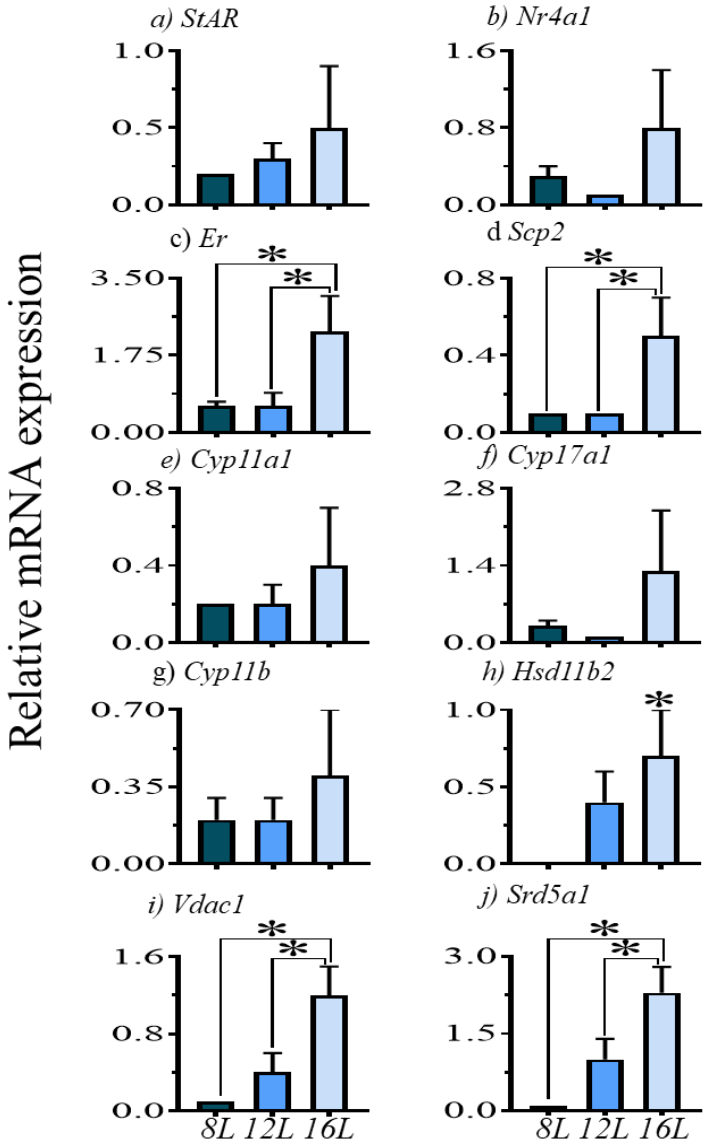

Fig. 9. Data are represented as mean (± SE, n=5/group). Relative mRNA expression of steroidogenic genes in the hypothalamus of adult male tree sparrows exposed to different photoperiods for 30 days. One-way ANOVA was applied, followed by Tukey's multiple comparison test to determine the significant differences between the groups. * represents a significant difference ($P < 0.05$).

Besides, in the testis tissue, *StAR* (P= 0.0101; One-way ANOVA; Table 3e; Fig.10a), *Nr4a1* (P= 0.0030; One-way ANOVA; Table 3e; Fig.10b), *Cyp11b* (P= 0.0008; One-way ANOVA; Table 3e; Fig.10d), *Er* (P<0.0001; One-way ANOVA; Table 3e; Fig.10e), *Cyp11a1*(P= 0.0150; One-way ANOVA; Table 3e; Fig.10g),

Hsd11b2 (P= 0.0011; One-way ANOVA; Table 3e; Fig.10h), *Vdac1* (P= 0.0001; One-way ANOVA; Table 3e; Fig.10i) and *Srd5a1*(P= 0.0011; One-way ANOVA; Table 3e; Fig.10j) displayed a significant difference and higher expression of these genes were observed in 16L: 8D exposed group followed by 12L:12D in comparison to 8L:16D. However, the expression does not differ in *Cyp17a1* (P= 0.3232; One-way ANOVA; Table 3e; Fig.10c) and *Scp2* (P= 0.2758; One-way ANOVA; Table 3e; Fig.10f) transcripts in all the photoperiod exposed groups.

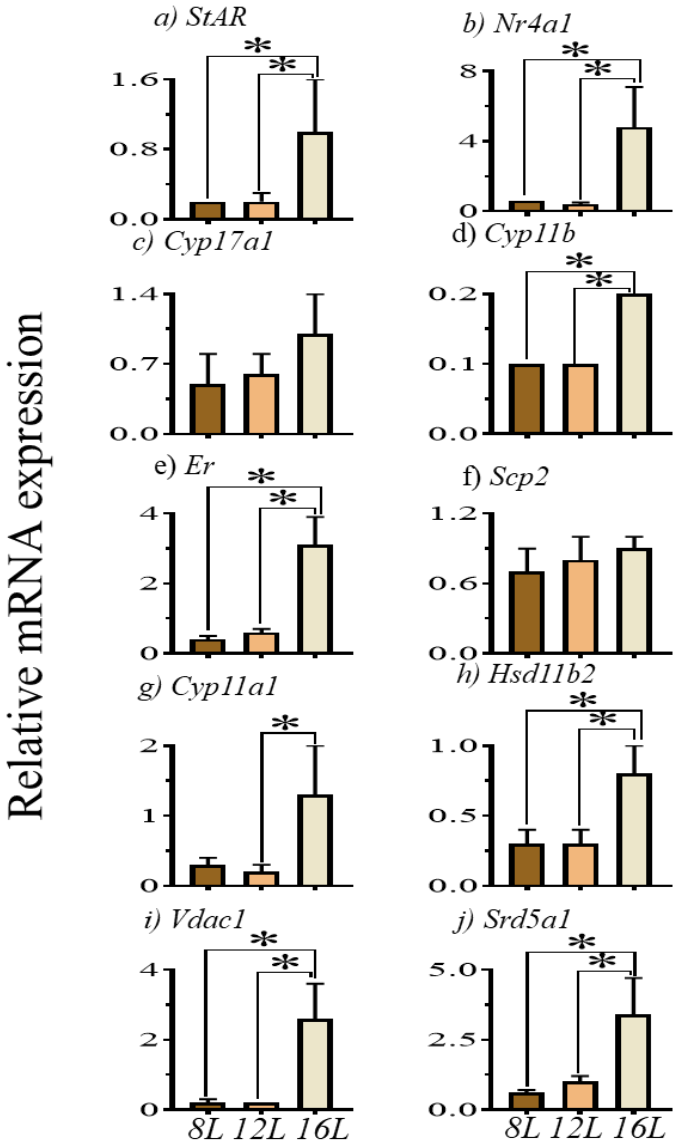

Fig. 10. Data are represented as mean (± SE, n=5/group). Relative mRNA expression of steroidogenic genes in the testis of adult male tree sparrows exposed to different photoperiods for 30 days. One-way ANOVA was applied, followed by Tukey's multiple comparison test to determine the significant differences between the groups. * represents a significant difference ($P < 0.05$).

Similarly, in the female hypothalamus mRNA transcripts of *StAR* (P= 0.0434; One-way ANOVA; Table 3f; Fig.11a), *Nr4a1*(P= 0.0285; One-way ANOVA; Table 3f; Fig.11b), *Scp2* (P<0.0001; One-way ANOVA; Table 3f; Fig.11c), *Cyp11a1* (P<0.0001; One-way ANOVA; Table 3f; Fig.11e), *Cyp17a1* (P<0.0001; One-way ANOVA; Table

3f; Fig.11f), *Cyp11b* (P= 0.0056; One-way ANOVA; Table 3f; Fig.11g), *Hsd11b2* (P<0.0001; One-way ANOVA; Table 3f; Fig.11h), *Er* (P<0.0001; One-way ANOVA; Table 3f; Fig.11i), and *Foxl2* (P<0.0001; One-way ANOVA; Table 3f; Fig.11j) showed a significant difference, and expression was higher in 16L:8D followed by 12L:12L exposed groups in comparison to 8L:16D. However, transcript expression does not differ in *Vdac1* (P= 0.0649; One-way ANOVA; Table 3f; Fig.11d) and *Cyp19a1* (P= 0.6367; One-way ANOVA; Table 3f; Fig.11k).

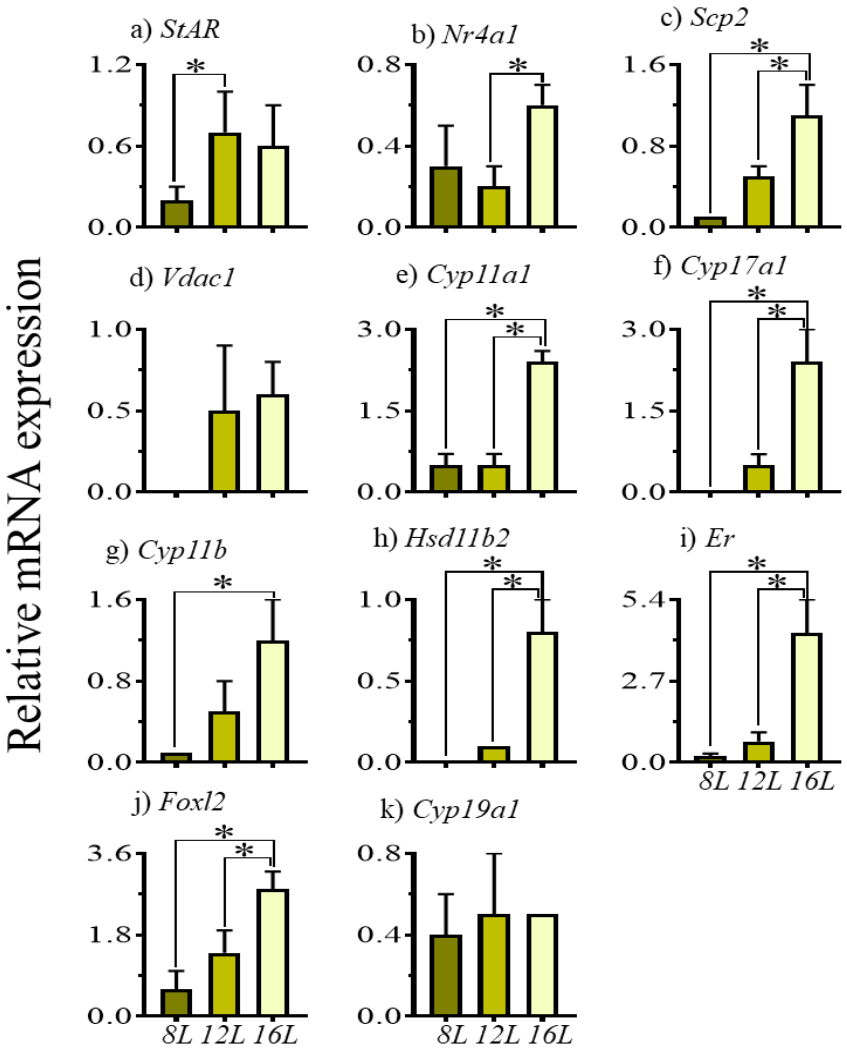

Fig. 11. Data are represented as mean (± SE, n=5/group). Relative mRNA expression of steroidogenic genes in the hypothalamus of adult female tree sparrows exposed to different photoperiods for 30 days. One-way ANOVA was applied, followed by Tukey's multiple comparison test to determine the significant differences between the groups. * represents a significant difference ($P < 0.05$).

Similarly, in the ovarian tissue, higher expressions of transcript expression of *StAR* (P<0.0001; One-way ANOVA; Table 3f; Fig.12a), *Scp2* (P=0.0002; One-way ANOVA; Table 3f; Fig.12c), *Vdac1* (P=0.0006; One-way ANOVA; Table 3f; Fig.12d), *Cyp11a1* (P=0.0196; One-way ANOVA; Table 3f; Fig.12e), *Cyp17a1* (P<0.0001; One-way ANOVA; Table 3f; Fig.12f), *Cyp11b* (P= 0.0003; One-way ANOVA; Table 3f;

Fig.12g), *Hsd11b2* (P=0.0092; One-way ANOVA; Table 3f; Fig.12h), and *Cyp19a1* (P= 0.0060; One-way ANOVA; Table 3f; Fig.12k) were observed in 16L:8D exposed group followed by 12L:12D and lower expressions in 8L:16D exposed group. However, transcript expressions do not differ in the case of *Nr4a1*(P= 0.0743; One-way ANOVA; Table 3f; Fig. 12b), *Er* (P= 0.0405; One-way ANOVA; Table 3f; Fig.12i), and *Foxl2* (P= 0.1053; One-way ANOVA; Table 3f; Fig.12j)

Table 3f. Values of One-way ANOVA of the steroidogenic gene transcripts in female tree sparrows (Photoperiod)

Transcripts	Hypothalamus	Ovary
StaR	$F_{(2, 21)} = 3.657$, P=0.0434	$F_{(2, 21)} = 14.97$, P<0.0001
Scp2	$F_{(2, 21)} = 15.65$, P<0.0001	$F_{(2, 21)} = 12.65$, P=0.0002
Cyp11a1	$F_{(2, 21)} = 84.31$, P<0.0001	$F_{(2, 21)} = 4.772$, P=0.0196
Nr4a1	$F_{(2, 21)} = 4.235$, P=0.0285	$F_{(2, 21)} = 2.950$, P=0.0743
Cyp11b	$F_{(2, 21)} = 6.714$, P=0.0056	$F_{(2, 21)} = 12.50$, P=0.0003
Cyp17a1	$F_{(2, 21)} = 29.16$, P<0.0001	$F_{(2, 21)} = 18.75$, P<0.0001
Hsd11b2	$F_{(2, 21)} = 25.65$, P<0.0001	$F_{(2, 21)} = 5.913$, P=0.0092
Er	$F_{(2, 21)} = 27.10$, P<0.0001	$F_{(2, 21)} = 3.749$, P=0.0405
Vdac1	$F_{(2, 21)} = 3.125$, P=0.0649	$F_{(2, 21)} = 10.87$, P=0.0006
Cyp19a1	$F_{(2, 21)} = 0.4613$, P=0.6367	$F_{(2, 21)} = 2.511$, P=0.1053
Foxl2	$F_{(2, 21)} = 18.57$, P<0.0001	$F_{(2, 21)} = 6.580$, P=0.0060

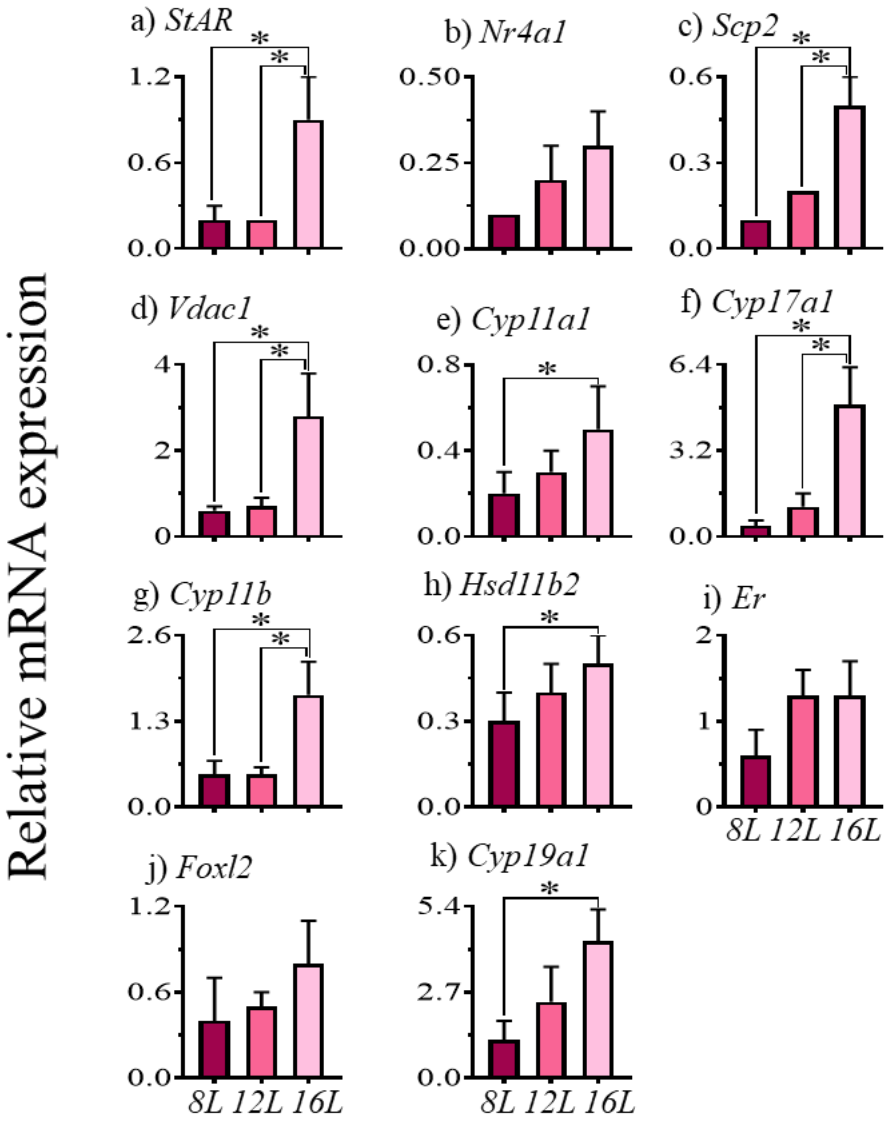

Fig. 12. Data are represented as mean (± SE, n=5/group). Relative mRNA expression of steroidogenic genes in the ovary of an adult female tree sparrows exposed to different photoperiods for 30 days. One-way ANOVA was applied, followed by Tukey's multiple comparison test to determine the significant differences between the groups. * represents a significant difference ($P < 0.05$).

4.2. Experiment 2: Effect of light intensity on steroidogenesis

In an adult male tree sparrow, body mass did not show any significant difference at the beginning and on day 30 at the termination of the experiment (Table 4a), (Intensity: P=0.0561; Time: P=0.0505; Interaction: P=0.4472; Two-way ANOVA Fig. 13a). However, a slight increase in the bill color was observed in the 100 lux group compared to 10 and 50 lux intensity (Intensity: P=0.0005: Time: P=0.8514; Interaction: P=0.0911; Two-way ANOVA Fig. 13b) and a significant increase in the testicular volume was observed in 100 lux intensity group than the 10 lux and 50 lux intensity and a significant difference in the time and interaction (Intensity: P<0.0001; Time:P<0.0001; Interaction: P=0.0001; Two-way ANOVA Fig. 13c).

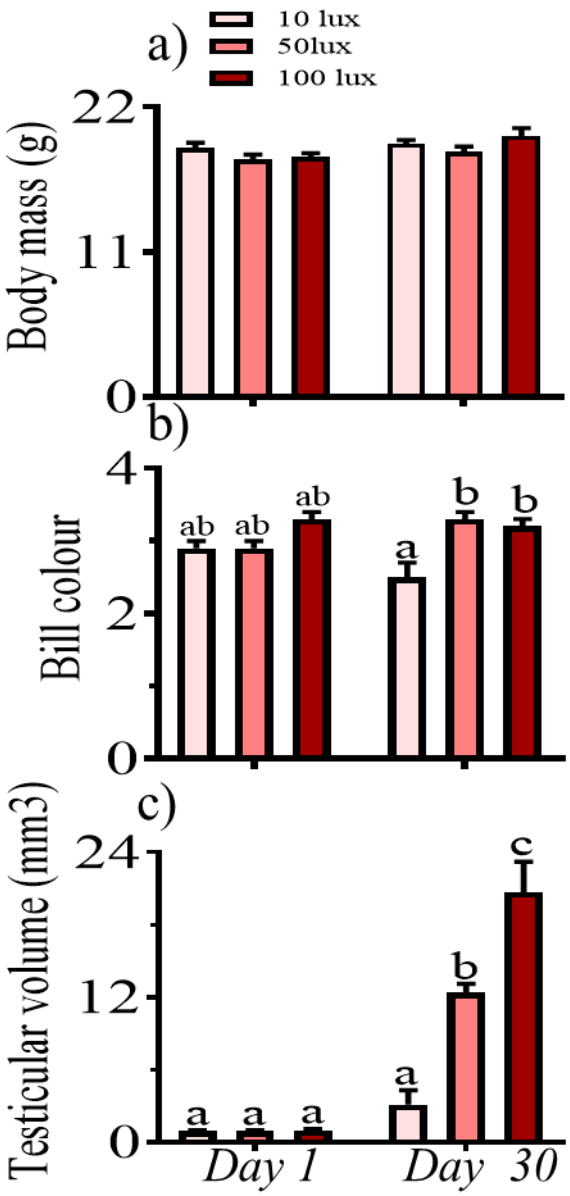

Figure 13. Data are represented as mean (± SE, n =5/group) body mass (a), bill color (b), and testicular volume (c) of photosensitive male tree sparrows exposed to different light intensities for 30 days. Two-way ANOVA followed by Tukey's multiple comparison test to determine significant differences between the groups. Alphabet indicates significant difference ($P < 0.05$).

Similar responses were observed in female tree sparrow (Table 4b), where, there is no significant difference in body mass (Intensity: P=0.6946; Time: P=0.7792; Interaction: P=0.4149; Two-way ANOVA Fig. 14a) and bill color (Intensity: P=0.8820; Time: P=0.0584; Interaction: P=0.3058; Two-way ANOVA Fig. 14b). However, a significant difference was observed in follicular diameter between day1 and day 30 and highest growth was observed in 100 lux intensity group in comparison to 10 and 50 lux. Also, there a significant difference in time and interaction between the three groups (Intensity: P=0.0036; Time: P=0.0368; Interaction: P=0.0043; Two-way ANOVA Fig. 14c).

Table 4a. Values of Two-way ANOVA of physiological parameters in male tree sparrows (Intensity)

	Time	Treatment	Interaction
Body mass	$F_{(1,12)}$ =5.271, P=0.0405	$F_{(2,12)}$ =3.697, P=0.0561	$F_{(2,12)}$ =0.8611, P=0.4472
Bill color	$F_{(1,11)}$= 0.03679, P=0.8514	$F_{(2,11)}$ =16.38, P=0.0005	$F_{(2,11)}$ =3.002, P=0.0911
Testicular volume	$F_{(1,11)}$ =120.9, P<0.0001	$F_{(2,11)}$ =24.60, P<0.0001	$F_{(2,11)}$=23.82, P=0.0001

Table 4b. Values of Two-way ANOVA of physiological parameters in female tree sparrows (Intensity)

	Time	Treatment	Interaction
Body mass	$F_{(1,11)}$=0.08254,P=0.7792	$F_{(2,11)}$ =0.3768, P=0.6946	$F_{(2,11)}$ =0.9541, P=0.4149
Bill color	$F_{(1,11)}$=10.26, P=0.0084	$F_{(2,11)}$ =0.1271, P=0.8820	$F_{(2,11)}$ =1.322, P=0.3058
Follicular diameter	$F_{(1,11)}$ =5.641, P=0.0368	$F_{(2,11)}$ =9.828,P=0.0036	$F_{(2,11)}$=9.330, P=0.0043

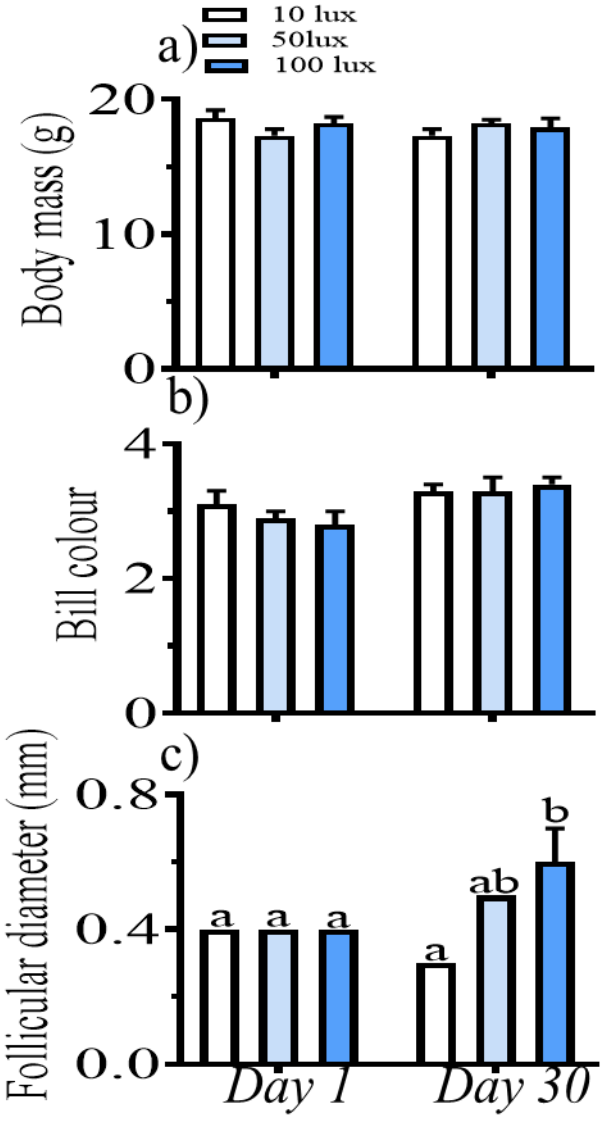

Fig. 14. Data are represented as mean (± SE, n=5/group) body mass (a), bill color (b), and follicular diameter (c) of photosensitive female tree sparrows exposed to different light intensities for 30 days. Two-way ANOVA followed by Tukey's multiple comparison test was applied to determine any significant differences between the groups. Alphabet indicates significant difference ($P < 0.05$).

Several mRNA transcripts involved in the seasonal reproduction were also measured in the hypothalamic tissue of male and female tree sparrows. In males, a significant difference was found in *Tshβ* (P= 0.0002; One-way ANOVA; Table 4c; Fig. 15a) and *Dio2* (P= 0.0462; One-way ANOVA; Table 4c; Fig. 15b). Both *Tshβ* and

Dio2 were upregulated in the 100 lux intensity group than in 10 lux and 50 lux groups. However, transcript expression of *GnIH* (P= 0.0311; One-way ANOVA; Table 4c; Fig. 15e) was downregulated in the 100 lux and 50 lux intensity groups and upregulated in the 10 lux intensity group. Whereas, we did not observe a significant difference in the transcript expressions of *Dio3* (P= 0.0833; One-way ANOVA; Table 4c; Fig. 15c) and *GnRH* (P= 0.2762; One-way ANOVA; Table 4c; Fig. 15d).

Table 4c. Values of One-way ANOVA of the reproductive gene transcripts in male tree sparrows (Intensity)

Transcripts	Hypothalamus
Tshβ	$F_{(2, 12)} = 18.85$, P=0.0002
Dio2	$F_{(2, 12)} = 4.015$, P=0.0462
Dio3	$F_{(2, 12)} = 3.079$, P=0.0833
GnRH	$F_{(2, 10)} = 1.467$, P=0.2762
GnIH	$F_{(2, 12)} = 4.699$, P=0.0311

Table 4d. Values of One-way ANOVA of the reproductive gene transcripts in female tree sparrows (Intensity)

Transcripts	Hypothalamus
Tshβ	$F_{(2, 11)} = 78.99$, P<0.0001
Dio2	$F_{(2, 11)} = 7.446$, P=0.0090
Dio3	$F_{(2, 12)} = 2.190$, P=0.1546
GnRH	$F_{(2, 11)} = 8.394$, P=0.0061
GnIH	$F_{(2, 11)} = 11.41$, P=0.0021

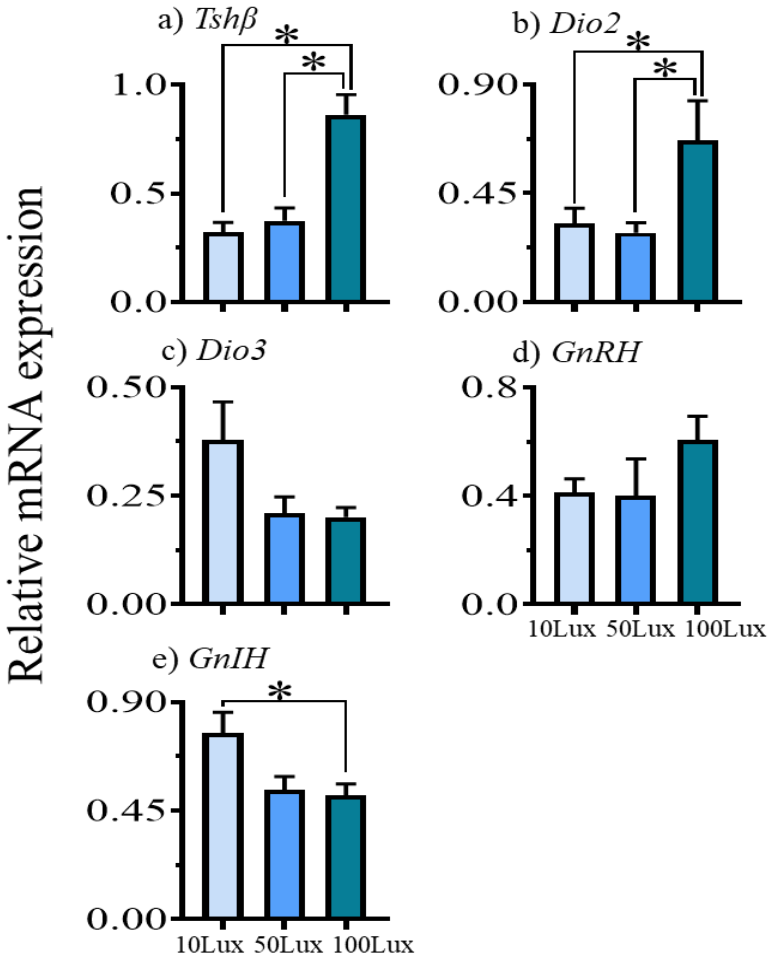

Fig. 15. Data are represented as mean (± SE, n =5/group). Relative mRNA expression of reproductive genes in the hypothalamic tissue of adult male tree sparrows exposed to different intensities of light. One-way ANOVA followed by Tukey's multiple comparison test to determine significant differences between the groups. * represents significant differences ($P < 0.05$).

Similarly, in female hypothalamus a significant difference was observed in *Tshβ* ($P<0.0001$; One-way ANOVA; Table 4d; Fig. 16a), *Dio2* ($P= 0.0090$; One-way ANOVA; Table 4d; Fig. 16b) and *GnRH* ($P= 0.0061$; One-way ANOVA; Table 4d; Fig. 16d). These stimulatory reproductive were upregulated in 100 lux intensity group compared to 10 lux and 50 lux intensity group. Whereas, *GnIH* ($P= 0.0021$; One-way ANOVA; Table 4d; Fig. 16e) was significantly upregulated in birds exposed to 10 lux

intensity group and downregulated in 50 lux and 100 lux intensity groups, and no difference was observed in *Dio3* (P= 0.1546; One-way ANOVA; Table 4d; Fig. 16c).

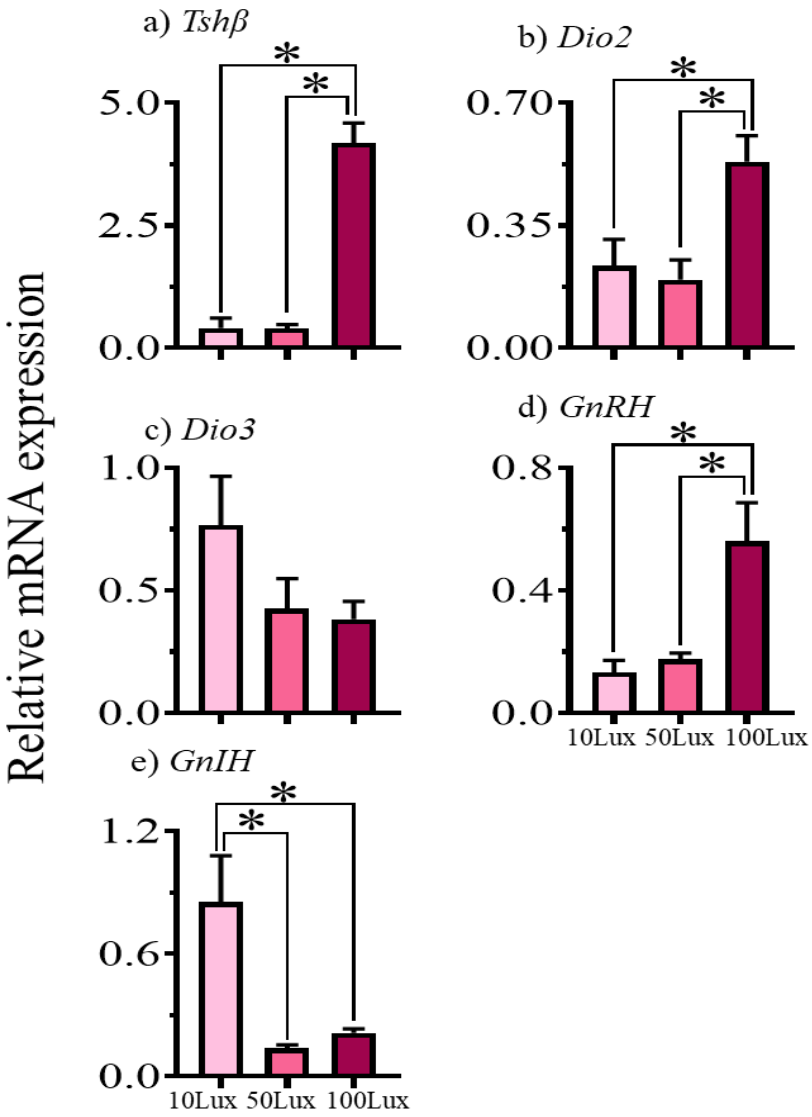

Fig. 16. Data are represented as mean (± SE, n=5 birds/group). Relative mRNA expression of reproductive genes in the hypothalamic tissue of adult female tree sparrows exposed to different intensities of light for 30 days. One-way ANOVA followed by Tukey's multiple comparison test to determine significant differences between the groups. * represents a significant difference ($P < 0.05$).

Also, several genes known to be involved in steroidogenesis were measured in the hypothalamus and gonad tissue of both sexes. In male hypothalamus, a significant difference was observed in *StAR* (P= 0.0059; One-way ANOVA; Table 4e; Fig. 17a), *Scp2* (P= 0.0200; One-way ANOVA; Table 4e; Fig. 17b), *Cyp11a1* (P= 0.0004; One-way ANOVA; Table 4e; Fig. 17c), *Nr4a1* (P<0.0001; One-way ANOVA; Table 4e; Fig. 17d), *Cyp11b* (P= 0.0092; One-way ANOVA; Table 4e; Fig. 17e), *Cyp17a1* (P<0.0001; One-way ANOVA; Table 4e; Fig. 17f), *Srd5a1* (P= 0.0022; One-way ANOVA; Table 4e; Fig. 17g), *Hsd11b2* (P<0.0001; One-way ANOVA; Table 4e; Fig. 17h), and *Vdac1*(P= 0.0275; One-way ANOVA; Table 4e; Fig. 17j). These steroidogenic transcripts were upregulated in 100 lux treated intensity group followed by 50 lux group in comparison to 10 lux intensity group. However, no significant variance was found in transcript expression of *Er* (P= 0.0943; One-way ANOVA; Table 4e; Fig. 17h).

Table 4e. Values of One-way ANOVA of the steroidogenic gene transcripts in male tree sparrows (Intensity)

Transcripts	Hypothalamus	Testis
StaR	$F_{(2, 21)} = 6.629$, P=0.0059	$F_{(2, 33)} = 4.262$, P=0.0226
Scp2	$F_{(2, 21)} = 4.738$, P=0.0200	$F_{(2, 21)} = 2.588$, P=0.0989
Cyp11a1	$F_{(2, 21)} = 11.90$, P=0.0004	$F_{(2, 21)} = 1.182$, P=0.3262
Nr4a1	$F_{(2, 21)} = 17.01$, P<0.0001	$F_{(2, 27)} = 6.946$, P=0.0037
Cyp11b	$F_{(2, 21)} = 5.916$, P=0.0092	$F_{(2, 27)} = 7.746$, P=0.0022
Cyp17a1	$F_{(2, 21)} = 16.42$, P<0.0001	$F_{(2, 27)} = 7.003$, P=0.0035
Hsd11b2	$F_{(2, 30)} = 26.62$, P<0.0001	$F_{(2, 21)} = 6.881$, P=0.0050
Er	$F_{(2, 21)} = 2.648$, P=0.0943	$F_{(2, 27)} = 7.647$, P=0.0023
Vdac1	$F_{(2, 21)} = 4.286$, P=0.0275	$F_{(2, 25)} = 4.076$, P=0.0294
Srd5a1	$F_{(2, 21)} = 8.313$, P=0.0022	$F_{(2, 21)} = 4.508$, P=0.0235

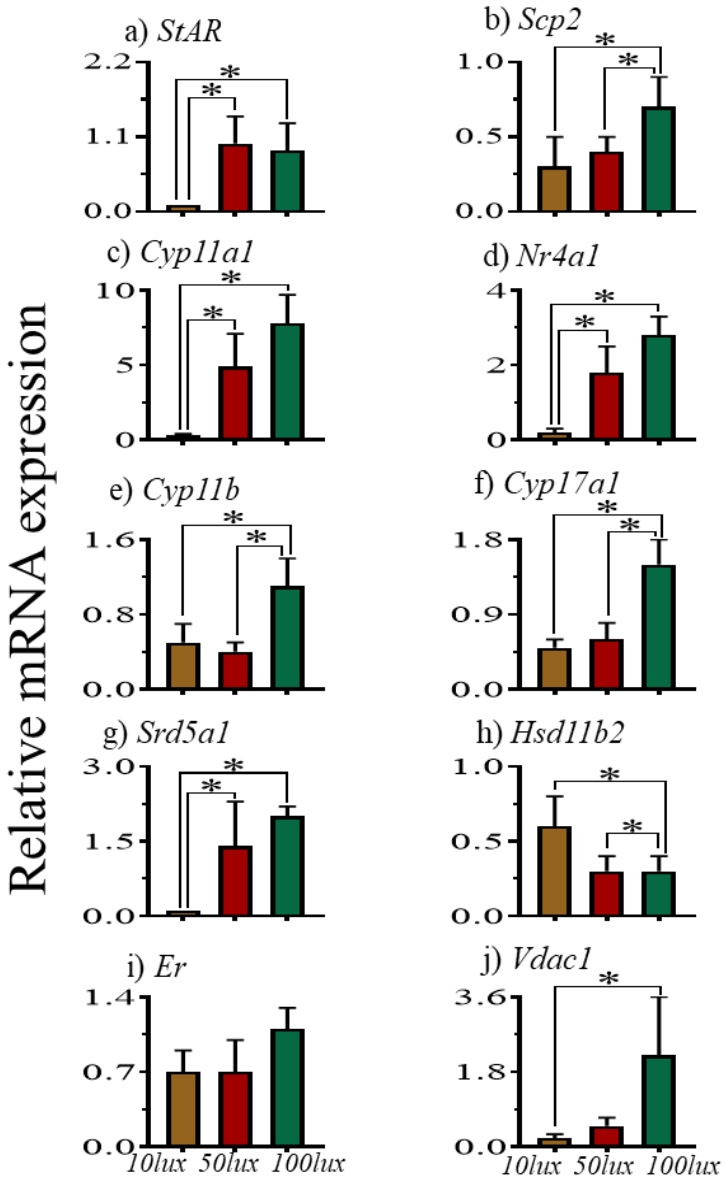

Fig. 17. Data are represented as mean (± SE, n=5 birds/group). Relative mRNA expression of steroidogenic genes in the hypothalamus of male tree sparrows treated with different intensities of light for 30 days. One-way ANOVA was followed by Tukey's multiple comparison test to determine significant differences between the groups. * represents a significant difference ($P < 0.05$).

In the testis tissue, higher expression of transcripts *StAR* (P= 0.0226; One-way ANOVA; Table 4e; Fig. 18a), *Nr4a1* (P= 0.0037; One-way ANOVA; Table 4e; Fig.

18d), *Cyp11b* (P= 0.0022; One-way ANOVA; Table 4e; Fig. 18e), *Cyp17a1* (P= 0.0035; One-way ANOVA; Table 4e; Fig. 18f), *Srd5a1* (P= 0.0235; One-way ANOVA; Table 4e; Fig. 18g), *Hsd11b2* (P= 0.0050; One-way ANOVA; Table 4e; Fig. 18h), *Er* (P= 0.0023; One-way ANOVA; Table 4e; Fig. 18i) and *Vdac1* (P= 0.0294; One-way ANOVA; Table 4e; Fig. 18j) were observed in 100 lux intensity treated group followed by 50 lux compared to 10 lux intensity group. However, *Scp2* (P= 0.0989; One-way ANOVA; Table 4e; Fig. 18b) and *Cyp11a1* (P= 0.0989; One-way ANOVA; Table 4e; Fig. 18c) did not exhibit any significant difference among the three groups.

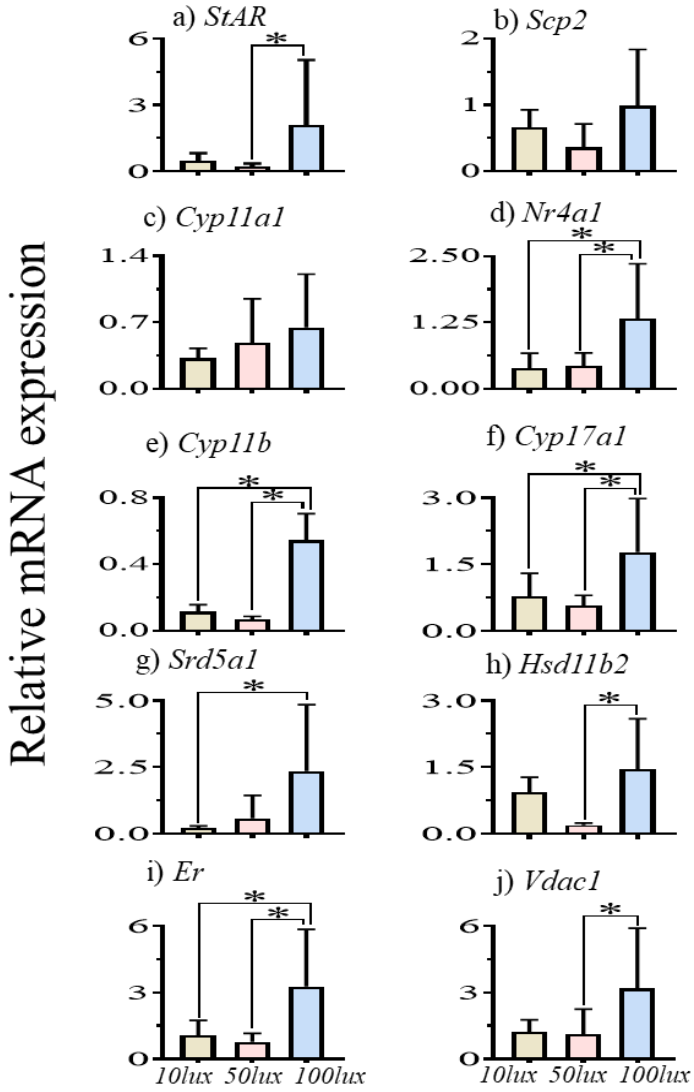

Fig. 18. Data are represented as (mean±SE, n=5 birds/group). Relative mRNA expression of steroidogenic genes in the testis tissue of male tree sparrows treated with different intensities of light for 30 days. One-way ANOVA was applied, followed by Tukey's multiple comparison test to determine significant differences between the groups. * represents a significant difference ($P < 0.05$).

Similar responses were observed in steroidogenic mRNA transcripts in the female hypothalamus and ovarian tissue. Higher expression of transcripts *StAR* (P= 0.0183; One-way ANOVA; Table 4f; Fig. 19a), *Nr4a1* (P= 0.0106; One-way ANOVA; Table 4f; Fig. 19b), *Vdac1* (P= 0.0101; One-way ANOVA; Table 4f; Fig. 19d),

Cyp11a1 (P= 0.0060; One-way ANOVA; Table 4f; Fig. 19e), Cyp17a1 (P<0.0001; One-way ANOVA; Table 4f; Fig. 19f), *Cyp11b* (P= 0.0151; One-way ANOVA; Table 4f; Fig. 19g), Er (P= 0.0046; One-way ANOVA; Table 4f; Fig. 19i), *Foxl2* (P= 0.0366; One-way ANOVA; Table 4f; Fig. 19j), and *Cyp19a1* (P= 0.0096; One-way ANOVA; Table 4f; Fig. 19k) were observed in 100 lux intensity group in comparison to 50 lux and 10 lux intensity group. However, transcript expression of *Scp2* (P= 0.1915; One-way ANOVA; Table 4f; Fig. 19c) and *Hsd11b2* (P= 0.0837; One-way ANOVA; Table 4f; Fig. 19h) showed no significant difference in all three groups.

Table 4f. Values of One-way ANOVA of the steroidogenic gene transcripts in female tree sparrows (Intensity)

Transcripts	Hypothalamus	Ovary
StaR	$F_{(2, 21)} = 4.868$, P=0.0183	$F_{(2, 21)} = 2.145$, P=0.1420
Scp2	$F_{(2, 21)} = 1.790$, P=0.1915	$F_{(2, 21)} = 2.337$, P=0.1212
Cyp11a1	$F_{(2, 21)} = 6.583$, P=0.0060	$F_{(2, 21)} = 1.683$, P=0.2100
Nr4a1	$F_{(2, 21)} = 5.686$, P=0.0106	$F_{(2,21)} = 0.7585$, P=0.4808
Cyp11b	$F_{(2, 21)} = 5.151$, P=0.0151	$F_{(2, 21)} = 1.320$, P=0.2884
Cyp17a1	$F_{(2, 19)} = 58.57$, P<0.0001	$F_{(2, 21)} = 1.544$, P=0.2368
Hsd11b2	$F_{(2, 21)} = 2.798$, P=0.0837	$F_{(2, 18)} = 1.545$, P=0.2403
Er	$F_{(2, 21)} = 7.018$, P=0.0046	$F_{(2, 21)} = 5.907$, P=0.0092
Vdac1	$F_{(2, 21)} = 5.764$, P=0.0101	$F_{(2,21)} = 0.03303$, P=0.9676
Foxl2	$F_{(2, 21)} = 3.887$, P=0.0366	$F_{(2,21)} = 0.6895$, P=0.5128
Cyp19a1	$F_{(2, 21)} = 5.843$, P=0.0096	$F_{(2, 25)} = 4.333$, P=0.0242

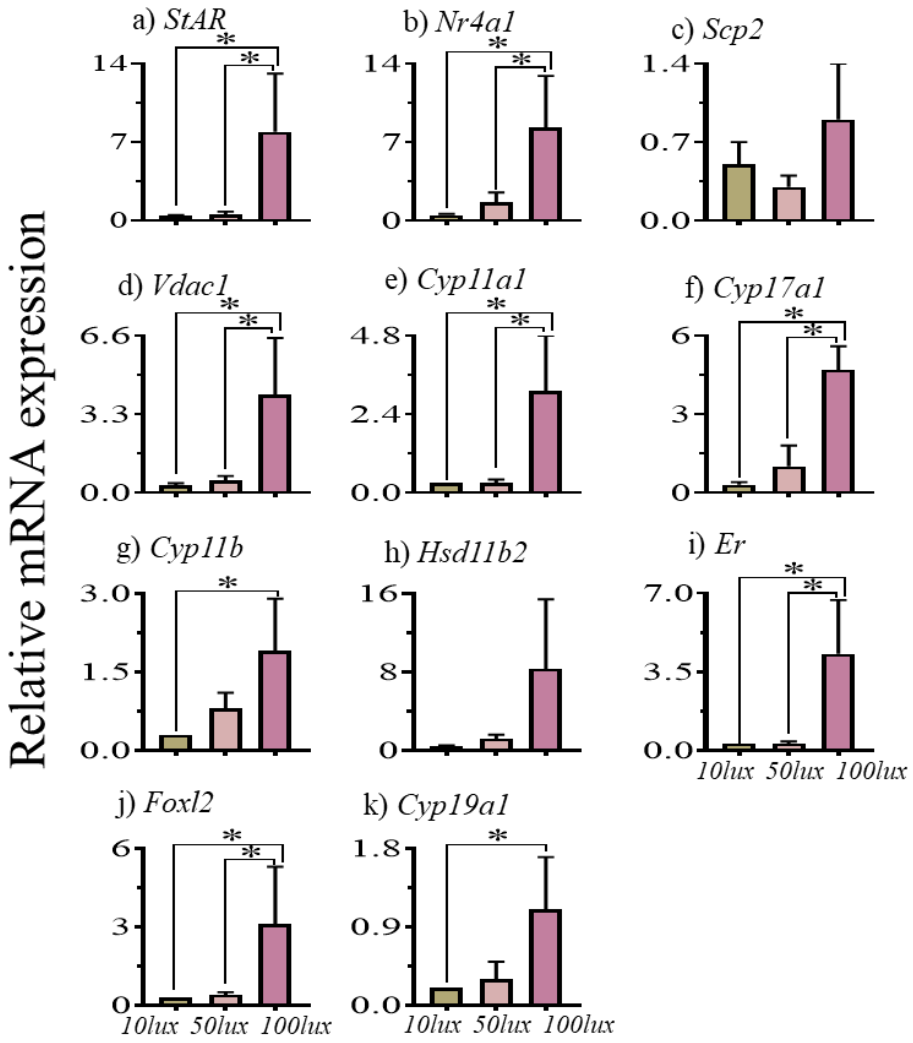

Fig. 19. Data are represented as (mean ± SE, n=5 birds/group). Relative mRNA expression of steroidogenic genes in the hypothalamus of female tree sparrows treated with different intensities of light for 30 days. One-way ANOVA was followed by Tukey's multiple comparison test to determine significant differences between the groups. * represents a significant difference ($P < 0.05$).

In ovarian tissue, higher expression of mRNA transcript was observed in *Er* (P= 0.0092; One-way ANOVA; Table 4f; Fig. 20i) in 100 lux intensity group followed by 50 lux in comparison to 10 lux intensity group and *Cyp19a1* expression (P= 0.0242; One-way ANOVA; Table 4f; Fig. 20k) in 100 lux intensity compared to 50 lux and 10

lux intensity group. Whereas, no difference was observed in all the other steroidogenic transcripts in ovary i.e., *StAR* (P= 0.1420; One-way ANOVA; Table 4f; Fig. 20a), *Nr4a1* (P= 0.4808; One-way ANOVA; Table 4f; Fig. 20b), *Scp2* (P= 0.1212; One-way ANOVA; Table 4f; Fig. 20c), *Vdac1* (P= 0.9676; One-way ANOVA; Table 4f; Fig. 20d), *Cyp11a1* (P= 0.2100; One-way ANOVA; Table 4f; Fig. 20e), *Cyp17a1* (P= 0.2368; One-way ANOVA; Table 4f; Fig. 20f), *Cyp11b* (P= 0.2884; One-way ANOVA; Table 4f; Fig. 20g), *Hsd11b2* (P= 0.2403; One-way ANOVA; Table 4f; Fig. 20h) and *Foxl2* (P= 0.5128; One-way ANOVA; Table 4f; Fig. 20i).

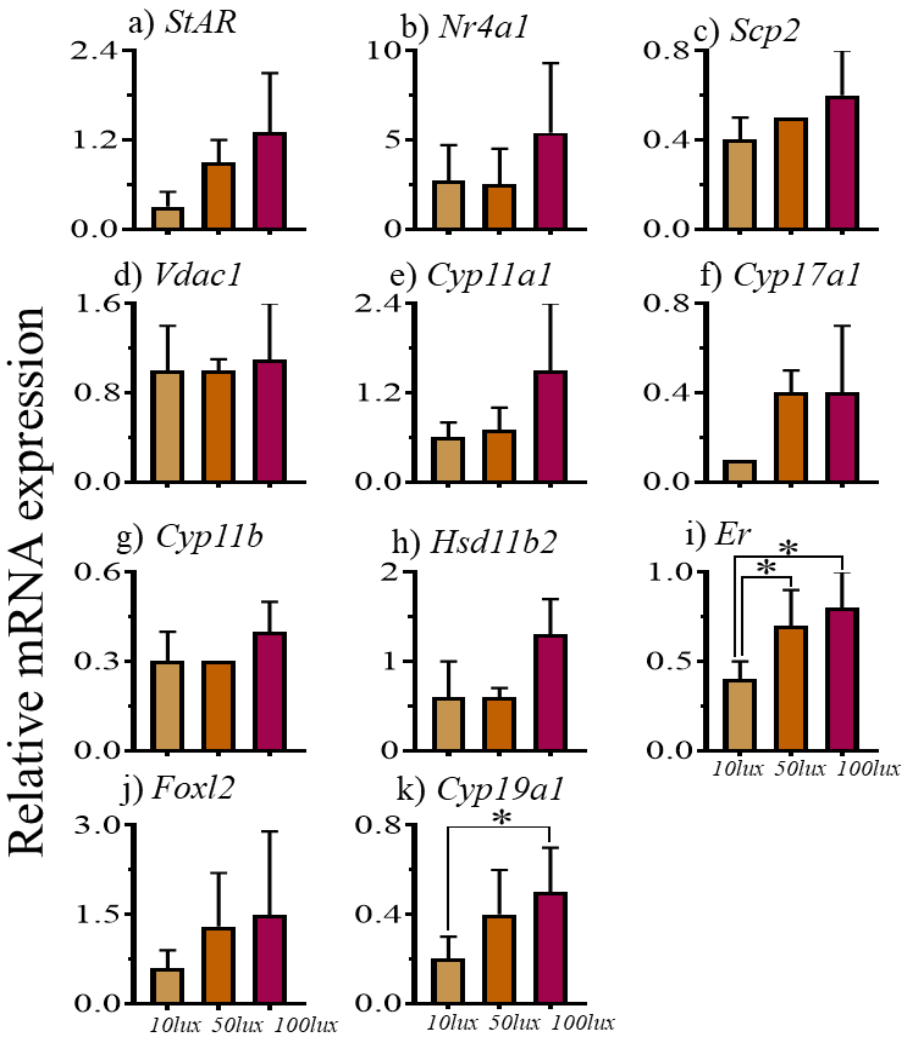

Fig. 20. Data are represented as (mean ± SE, n=5 birds/group). Relative mRNA expression of steroidogenic genes in the ovary of female tree sparrows exposed to different intensities of light for 30 days. One-way ANOVA was applied, followed by Tukey's multiple comparison test to determine significant differences between the groups. * represents a significant difference ($P < 0.05$).

4.3. Experiment 3. Effect of light spectra on steroidogenesis

The effect of different light spectra was measured in both male and female tree sparrows. In males, no significant difference was observed in body mass in all the spectrum-treated groups at the beginning of the experiment and the end (Table 5a), (Spectra: P= 0.8671; Time: P=0.0569; Interaction: P=0.8897; Fig. 21a). In bill color significant difference was observed in time between day 1 and day 30 (Spectra: P= 0.3286; Time: P= 0.0015; Interaction: P= 0.0563; Fig. 21b). However, there was significant increase in testicular volume (Spectra: P=0.0080; Time: P=0.0001; Interaction: P=0.0111; Fig. 21c). Highest testicular growth was observed in red light group followed by white light compare to green and blue light.

Table 5a. Values of Two-way ANOVA of the physiological parameters in male tree sparrows (Spectra)

	Time	Treatment	Interaction
Body mass	$F_{(1,32)}$ =5.38, P=0.0269	$F_{(3,32)}$ =0.2409, P=0.8671	$F_{(3,32)}$ =0.2086, P=0.8897
Bill color	$F_{(1,16)}$ =14.70, P=0.0015	$F_{(3,16)}$ =1.238, P=0.3286	$F_{(3,16)}$ =3.101, P=0.0563
Testicular volume	$F_{(1,16)}$ =25.92, P=0.0001	$F_{(3,16)}$ =5.614, P=0.0080	$F_{(3,16)}$ =5.141, P=0.0111

Table 5b. Values of Two-way ANOVA of the physiological parameters in female tree sparrows (Spectra)

	Time	Treatment	Interaction
Body mass	$F_{(1,20)}$ =0.00250, P=0.9606	$F_{(3,20)}$ =2.039, P=0.1408	$F_{(3,20)}$ =1.045, P=0.3942
Bill color	$F_{(1,21)}$ =2.398, P=0.1364	$F_{(3,21)}$ =1.553, P=0.2304	$F_{(3,21)}$=7.224, P=0.0016
Testicular volume	$F_{(1,21)}$ =29.48, P<0.0001	$F_{(3,21)}$ =11.03, P=0.0001	$F_{(3,21)}$ =11.03, P=0.0001

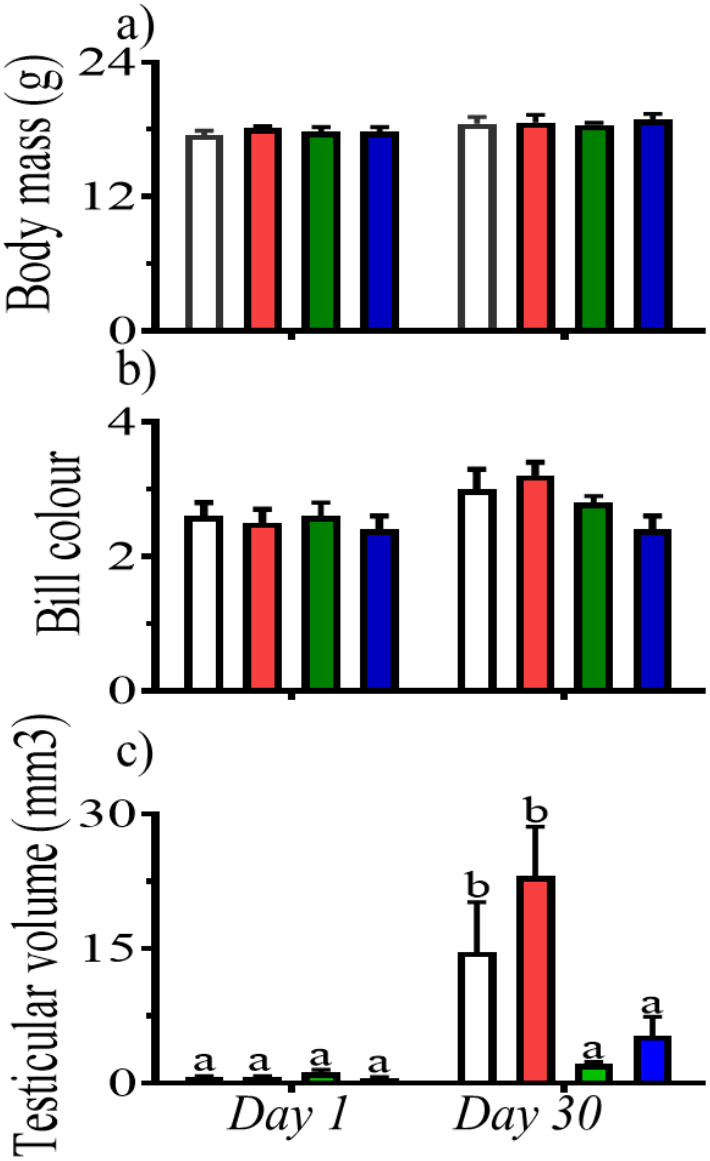

Fig. 21. Data are represented as (mean ± SE, n =5 birds/group). Body mass (a), bill color (b), and testicular volume (c) of photosensitive adult male tree sparrows treated with different spectra for 30 days. Two-way ANOVA followed by Tukey's multiple comparison test was applied to determine significant differences between the groups. Alphabet represents a significant difference ($P < 0.05$).

In the female tree sparrow, a similar response was observed (Table 5b), where body mass did not show any significant variance (Spectra: P= 0.1408; Time: P= 0.9606; Interaction: P=0.3942; Fig. 22a) and bill color (Spectra: P=0.1140; Time: P=0.2292; Interaction: P=0.4292; Fig. 22b). However, a significant difference in the follicular growth was observed (Spectra: P= 0.0001; Time: P<0.0001; Interaction: P= 0.0001; Fig. 22c).

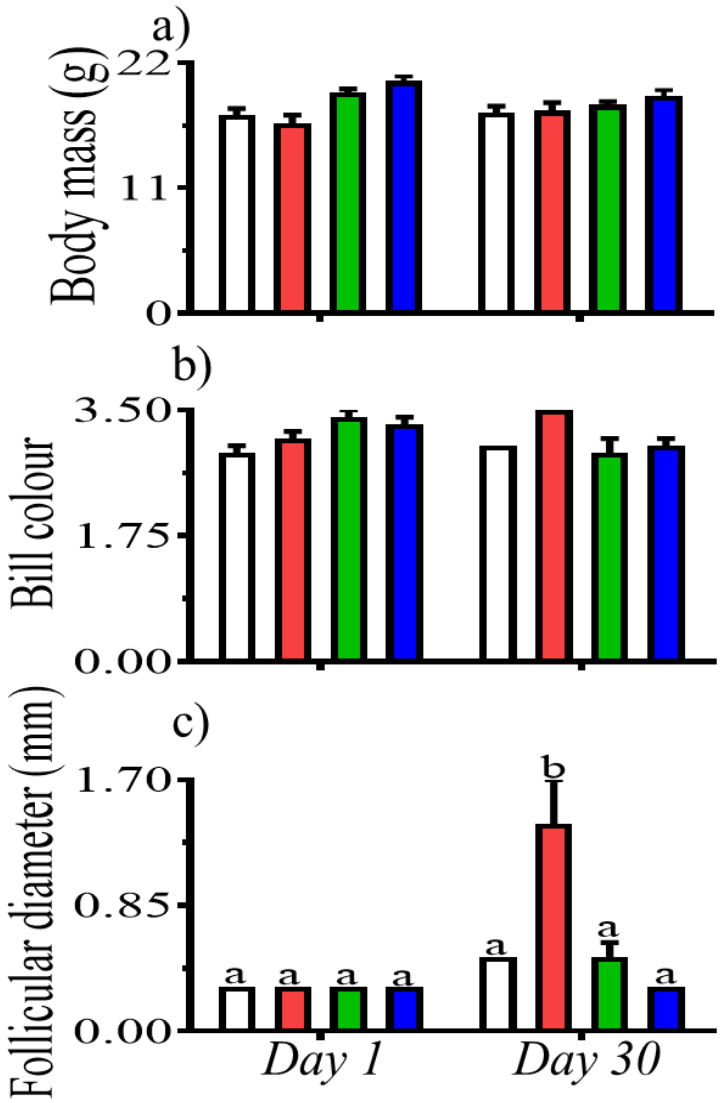

Fig. 22. Data are represented as (mean ± SE, n=5/group). Body mass (a), bill color (b), and follicular diameter (c) of photosensitive female tree sparrows exposed to White, Red, Green, and Blue wavelengths for 30 days. A Two-way ANOVA followed by Tukey's multiple comparison test was used to determine significant differences between the groups. Lower case indicates significant difference ($P < 0.05$).

The reproductive genes believed to be involved in seasonal reproduction were observed in the hypothalamus. In males, significant upregulation of *Tshβ* ($P<0.0001$; One-way ANOVA; Table 5c; Fig. 23a), *Dio2* ($P= 0.0013$; One-way ANOVA; Table 5c; Fig. 23b), and *GnRH* ($P<0.0001$; One-way ANOVA; Table 5c; Fig. 23d) were

observed in red light group followed by white light compared to green and blue light group. However, reproductive inhibitory transcripts *Dio3* (P= 0.0122; One-way ANOVA; Table 5c; Fig. 23c) and *GnIH* (P<0.0001; One-way ANOVA; Table 5c; Fig. 23e).

Table 5c. Values of One-way ANOVA of the reproductive gene transcripts in male tree sparrows (Spectra)

Transcripts	Hypothalamus
Tshβ	$F_{(3, 16)} = 14.89$, P<0.0001
Dio2	$F_{(3, 16)} = 8.482$, P=0.0013
Dio3	$F_{(3, 14)} = 5.257$, P=0.0122
GnRH	$F_{(3, 14)} = 23.82$, P<0.0001
GnIH	$F_{(3, 16)} = 16.56$, P<0.0001

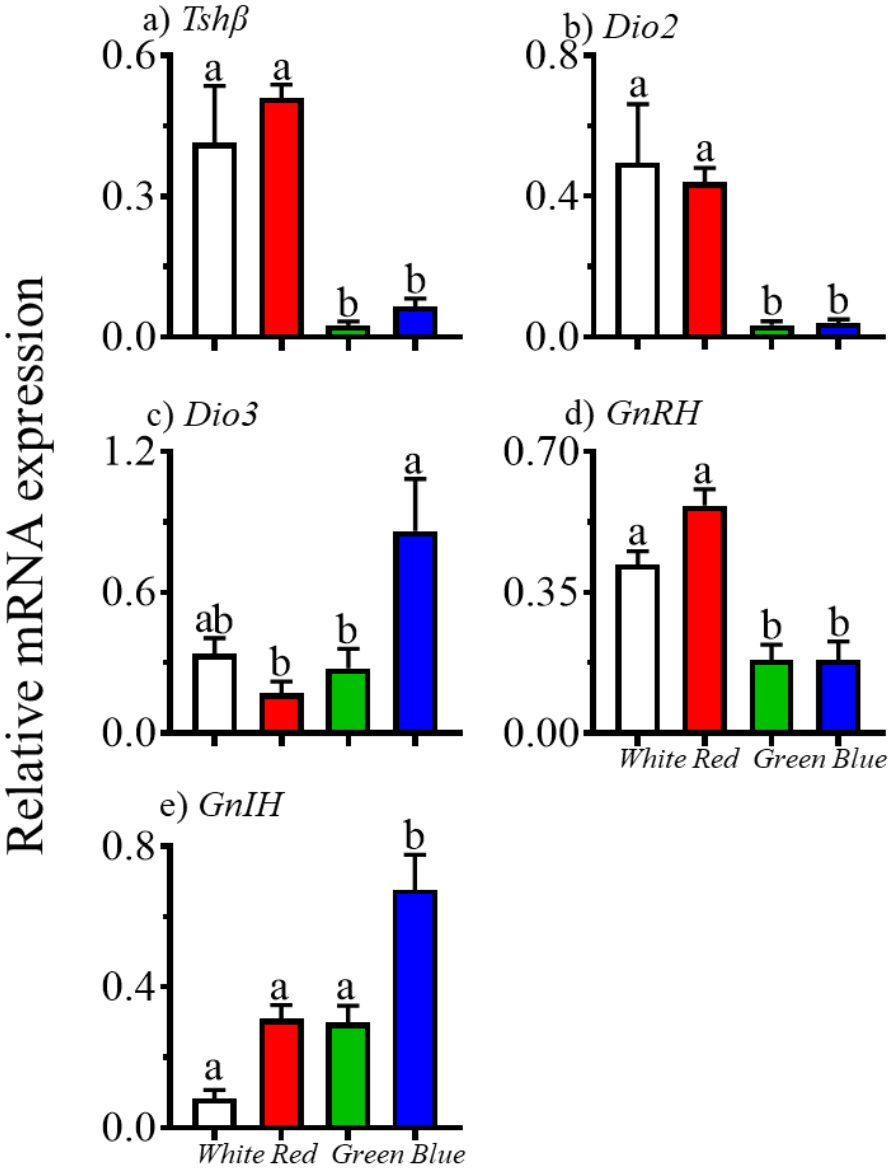

Fig. 23. Data are represented as (mean ± SE, n=5 birds/group). Relative mRNA expression of reproductive genes in the hypothalamic tissue of adult male tree sparrows treated with different light spectra. One-way ANOVA followed by Tukey's multiple comparison test to determine significant differences between the groups. Alphabet represents significant differences ($P < 0.05$).

Similarly, the female hypothalamus showed a significant upregulation of reproductive transcripts *Tshβ* (P= 0.0120; One-way ANOVA; Table 5d; Fig. 24a), *Dio2* (P<0.0001; One-way ANOVA; Table 5d; Fig. 24b), *GnRH* (P= 0.0037; One-way

ANOVA; Table 5d; Fig. 24d) in red light group compared green and blue light. However, *GnIH* (P= 0.0316; One-way ANOVA; Table 5d; Fig. 24e) expression was maximum in the blue light-treated group. Whereas, no significant variance was observed in the transcript expression of *Dio3* (P= 0.1111; One-way ANOVA; Table 5d; Fig. 24c).

Table 5d. Values of One-way ANOVA of the reproductive gene transcripts in female tree sparrows (Spectra)

Transcripts	Hypothalamus
Tshβ	$F_{(3, 15)} = 5.150$, P=0.0120
Dio2	$F_{(3, 15)} = 15.18$, P<0.0001
Dio3	$F_{(3, 15)} = 2.374$, P=0.1111
GnRH	$F_{(3, 16)} = 6.760$, P=0.0037
GnIH	$F_{(3, 15)} = 3.852$, P=0.0316

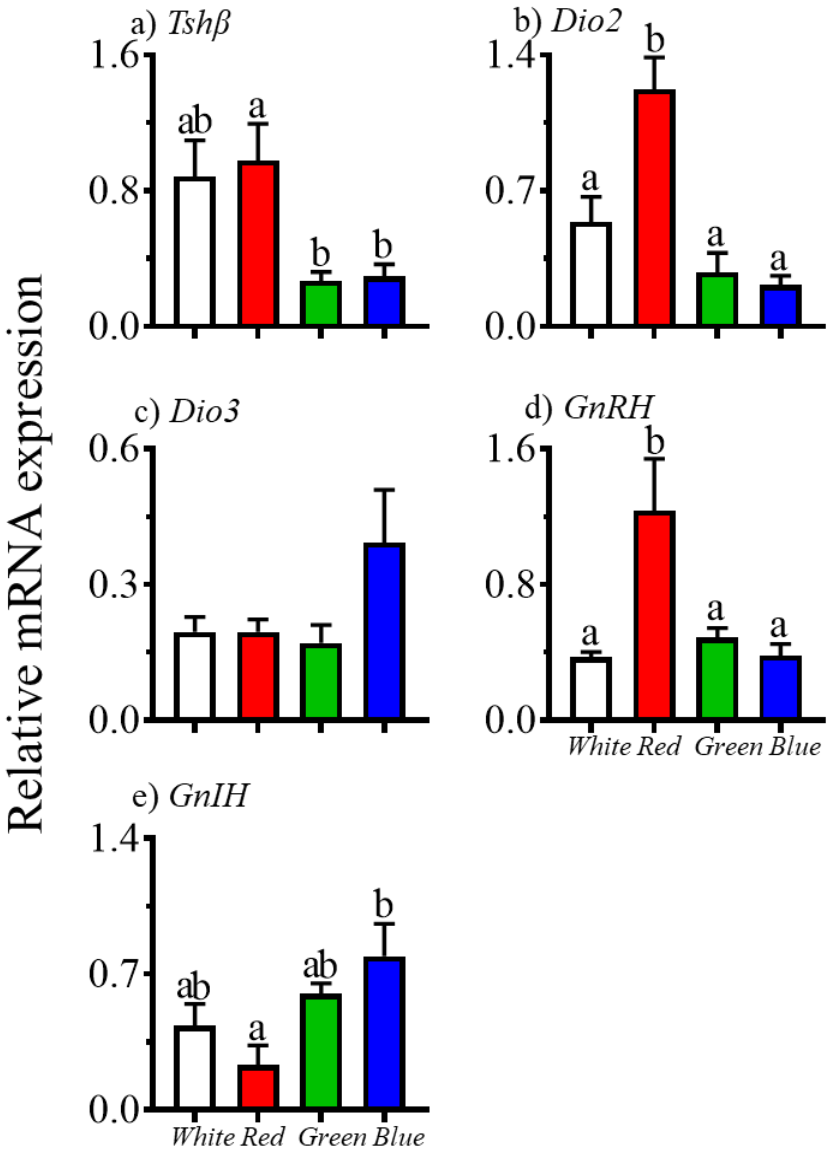

Fig. 24. Data are represented as (mean ± SE, n=5 birds/group). Relative mRNA expression of reproductive genes in the hypothalamic tissue of adult female tree sparrows exposed to different light spectra for 30 days. One-way ANOVA followed by Tukey's multiple comparison test was applied to determine significant differences between the groups. Alphabet represents a significant difference ($P < 0.05$).

Also, the transcript expression of steroidogenic genes was measured in the hypothalamus and gonads of both male and female tree sparrows. In male hypothalamus, the transcripts *StAR* (P= 0.0103; One-way ANOVA; Table 5e; Fig.

25a), *Nr4a1* (P= 0.0035; One-way ANOVA; Table 5e; Fig. 25d), and *Srd5a1* (P= 0.0018; One-way ANOVA; Table 5e; Fig. 25g) displayed a significant difference and upregulated in the red-light group compared to the white, green, and blue light groups. Whereas, *Cyp11b* (P= 0.0020; One-way ANOVA; Table 5e; Fig. 25e) was upregulated in the white light group compared to all the other spectrum groups. However, transcripts *Scp2* (P= 0.0690; One-way ANOVA; Table 5e; Fig. 25b), *Cyp17a1* (P= 0.6270; One-way ANOVA; Table 5e; Fig. 25f), *Hsd11b2* (P= 0.5384; One-way ANOVA; Table 5e; Fig. 25h), *Er* (P= 0.0373; One-way ANOVA; Table 5e; Fig. 25i), and *Vdac1* (P= 0.0382; One-way ANOVA; Table 5e; Fig. 25j) did not show significant difference in all the spectrum groups.

Table 5e. Values of One-way ANOVA of the steroidogenic gene transcripts in male tree sparrows (Spectra)

Transcripts	Hypothalamus	Testis
StaR	$F_{(3, 32)} = 4.427$, P=0.0103	$F_{(3, 32)} = 5.610$, P=0.0033
Scp2	$F_{(3, 17)} = 2.837$, P=0.0690	$F_{(3, 32)} = 3.665$, P=0.0224
Cyp11a1	$F_{(3, 28)} = 2.991$, P=0.0478	$F_{(3, 32)} = 3.536$, P=0.0256
Nr4a1	$F_{(3, 32)} = 5.538$, P=0.0035	$F_{(3, 32)} = 6.369$, P=0.0016
Cyp11b	$F_{(3, 32)} = 6.135$, P=0.0020	$F_{(3, 28)} = 4.912$, P=0.0072
Cyp17a1	$F_{(3, 32)} = 0.5885$, P=0.6270	$F_{(3, 32)} = 6.214$, P=0.0019
Hsd11b2	$F_{(3, 32)} = 0.7359$, P=0.5384	$F_{(3, 32)} = 2.563$, P=0.0720
Er	$F_{(3, 18)} = 3.490$, P=0.0373	$F_{(3, 32)} = 3.233$, P=0.0351
Vdac1	$F_{(3, 32)} = 3.154$, P=0.0382	$F_{(3, 32)} = 3.088$, P=0.0410
Srd5a1	$F_{(3, 18)} = 7.536$, P=0.0018	$F_{(3, 32)} = 2.722$, P=0.0606

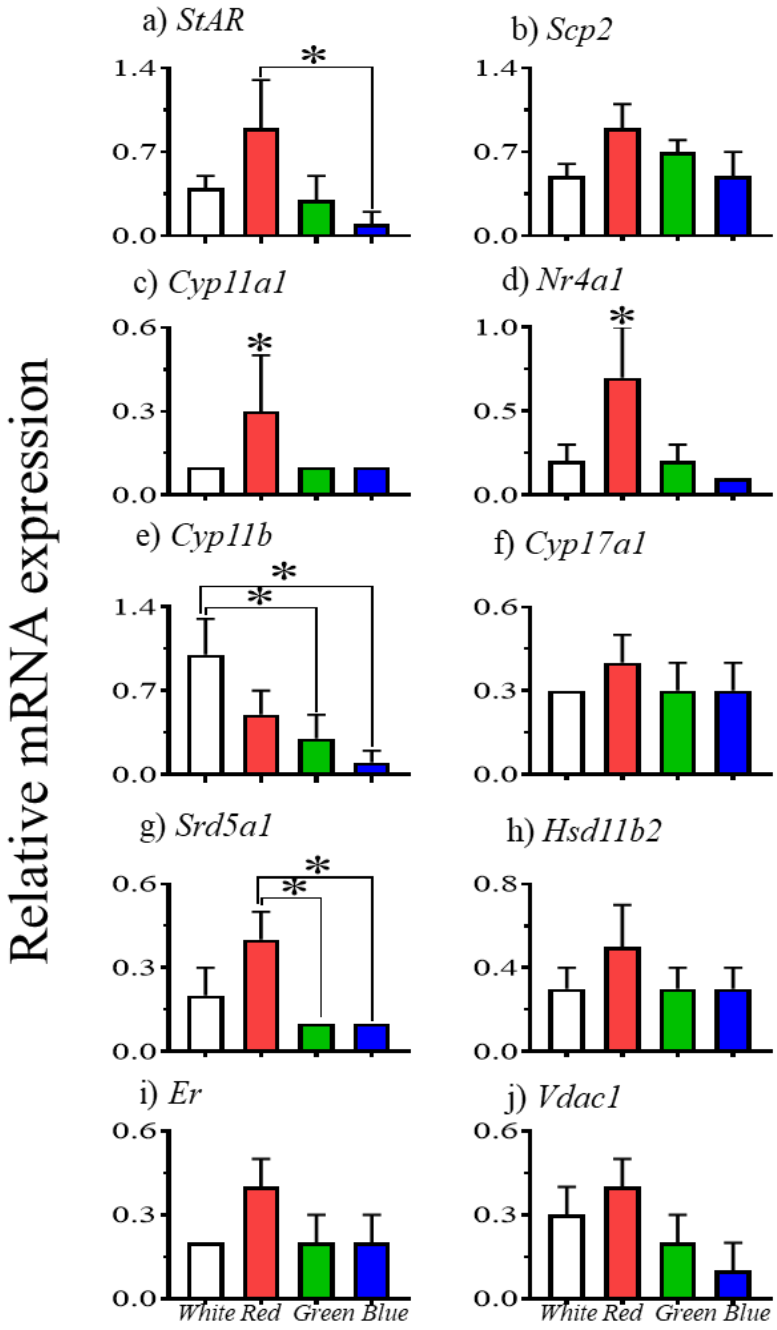

Fig. 25. Data are represented as (mean ± SE, n=5 birds/group). Relative mRNA expression levels of steroidogenic genes in the hypothalamus tissue of male tree sparrows exposed to distinct light spectra for 30 days. One-way ANOVA followed by Tukey's multiple comparison test to determine significant differences between the groups. * represents a significant difference ($P < 0.05$).

In the testicular tissue, a significant difference was observed in *StAR* (P= 0.0033; One-way ANOVA; Table 5e; Fig. 26a), *Cyp11a1* (P= 0.0256; One-way ANOVA; Table 5e; Fig. 26c), *Nr4a1* (P= 0.0016; One-way ANOVA; Table 5e; Fig. 26d), *Cyp11b* (P= 0.0072; One-way ANOVA; Table 5e; Fig. 26e), *Cyp17a1* (P= 0.0019; One-way ANOVA; Table 5e; Fig. 26f), *Srd5a1* (P= 0.0606; One-way ANOVA; Table 5e; Fig. 26g), *Er* (P= 0.0351; One-way ANOVA; Table 5e; Fig. 26i), and *Vdac1* (P= 0.0410; One-way ANOVA; Table 5e; Fig. 26j) in red light group followed by white light in comparison to green and blue light group. However, no significant variance was found in *Scp2* (P= 0.0624; One-way ANOVA; Table 5e; Fig. 26b) and *Hsd11b2* (P= 0.0720; One-way ANOVA; Table 5e; Fig. 26h) transcripts.

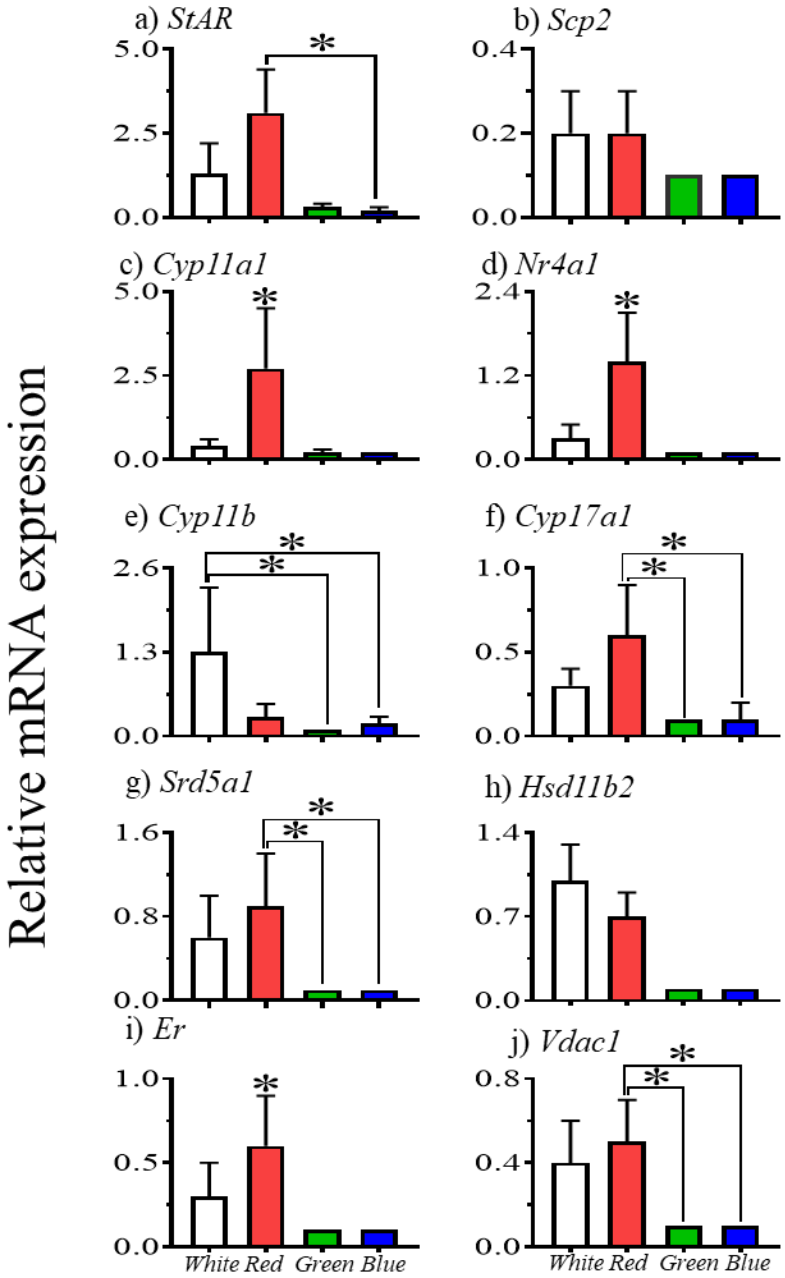

Fig. 26. Data are represented as mean (± SE, n =5/group). Relative mRNA levels of steroidogenic genes in the testis of male tree sparrows exposed to different light spectra for 30 days. One-way ANOVA was followed by Tukey's multiple comparison test to determine significant differences between the groups. * indicates a significant difference ($P<0.05$).

Similarly, in the female hypothalamus, higher expressions of mRNA transcripts *StAR* (P= 0.0118; One-way ANOVA; Table 5f; Fig. 27a), *Cyp11a1* (P= 0.0246; One-way ANOVA; Table 5f; Fig. 27c), *Nr4a1* (P= 0.0259; One-way ANOVA; Table 5f; Fig. 27d), *Cyp17a1* (P<0.0001; One-way ANOVA; Table 5f; Fig. 27f), *Hsd11b2* (P= 0.0063; One-way ANOVA; Table 5f; Fig. 27g), *Er* (P<0.0001; One-way ANOVA; Table 5f; Fig. 27h), *Foxl2* (P= 0.0016; One-way ANOVA; Table 5f; Fig. 27j), and *Cyp19a1* (P= 0.0111; One-way ANOVA; Table 5f; Fig. 27k) were observed to be upregulated in red light group followed by white light then green and blue light group, whereas, *Vdac1* (P= 0.0013; One-way ANOVA; Table 5f; Fig. 27i) was upregulated in red light followed by green light compared to white and red light. However, there was no difference in the transcript expression of *Scp2* (P= 0.0923; One-way ANOVA; Table 5f; Fig. 27c) and *Cyp11b* (P= 0.0704; One-way ANOVA; Table 5f; Fig. 27e).

In the ovarian tissue, higher expressions of transcripts *StAR* (P= 0.0001; One-way ANOVA; Table 5f; Fig. 28a), *Cyp17a1* (P= 0.0028; One-way ANOVA; Table 5f; Fig. 28f), *Vdac1* (P= 0.0123; One-way ANOVA; Table 5f; Fig. 28i) and *Cyp19a1* (P= <0.0001; One-way ANOVA; Table 5f; Fig. 28k) was observed and was upregulated in red light compared to other spectrum groups. Whereas, *Scp2* (P<0.0001; One-way ANOVA; Table 5f; Fig. 28c), and *Er* (P<0.0001; One-way ANOVA; Table 5f; Fig. 28h) were upregulated in white light than in the other groups. However, no significant variance was found in transcript expressions of *Cyp11a1* (P= 0.0058; One-way ANOVA; Table 5f; Fig. 28c), *Nr4a1* (P= 0.0007; One-way ANOVA; Table 5f; Fig. 28d), *Cyp11b* (P= 0.0056; One-way ANOVA; Table 5f; Fig. 28e), *Hsd11b2* (P= 0.0004; One-way ANOVA; Table 5f; Fig. 28g) and *Foxl2* (P= 0.0001; One-way ANOVA; Table 5f; Fig. 28j).

Table 5f. Values of the One-way ANOVA of the steroidogenic gene transcripts in female tree sparrows (Spectra)

Transcripts	Hypothalamus	Ovary
StaR	$F_{(3, 14)} = 5.313$, P=0.0118	$F_{(3, 28)} = 10.24$, P=0.0001

Scp2	$F_{(3, 28)} = 2.366$, P=0.0923	$F_{(3, 28)} = 16.07$, P<0.0001
Cyp11a1	$F_{(3, 28)} = 3.641$, P=0.0246	$F_{(3, 28)} = 5.155$, P=0.0058
Nr4a1	$F_{(3, 28)} = 3.591$, P=0.0259	$F_{(3, 28)} = 7.680$, P=0.0007
Cyp11b	$F_{(3, 28)} = 2.621$, P=0.0704	$F_{(3, 28)} = 5.190$, P=0.0056
Cyp17a1	$F_{(3, 28)} = 15.25$, P<0.0001	$F_{(3, 28)} = 5.956$, P=0.0028
Hsd11b2	$F_{(3, 28)} = 5.070$, P=0.0063	$F_{(3, 28)} = 8.451$, P=0.0004
Er	$F_{(3, 28)} = 12.50$, P<0.0001	$F_{(3, 28)} = 14.78$, P<0.0001
Vdac1	$F_{(3, 28)} = 6.855$, P=0.0013	$F_{(3, 28)} = 4.351$, P=0.0123
Foxl2	$F_{(3, 28)} = 6.661$, P=0.0016	$F_{(3, 28)} = 9.740$, P=0.0001
Cyp19a1	$F_{(3, 16)} = 5.147$, P=0.0111	$F_{(3, 28)} = 11.93$, P<0.0001

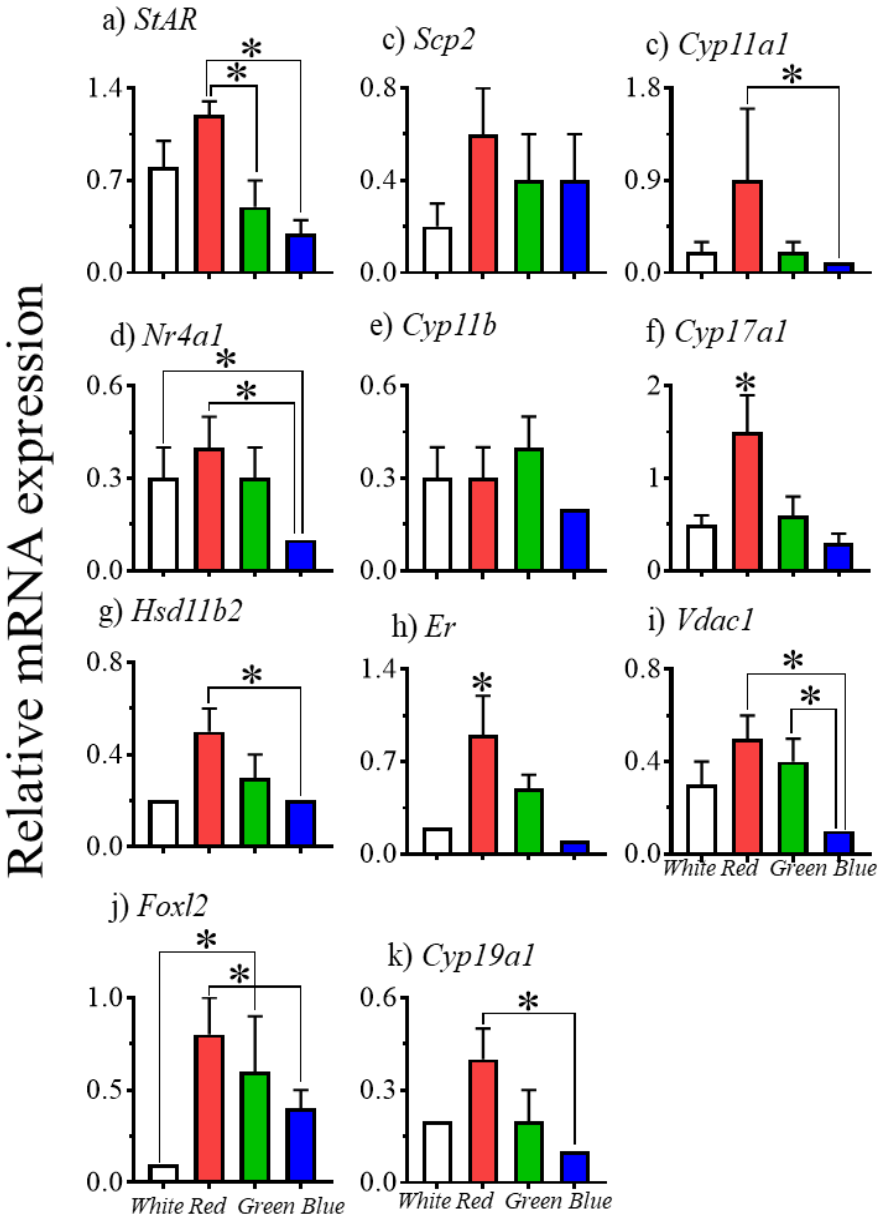

Fig. 27. Data are represented as (mean ± SE, n=5 birds/group). Relative mRNA expression of steroidogenic genes in the hypothalamic tissue of female tree sparrows when subjected to different light spectra for 30 days. One-way ANOVA was followed by Tukey's multiple comparison test to evaluate significant differences between the groups. * represents a significant difference ($P < 0.05$).

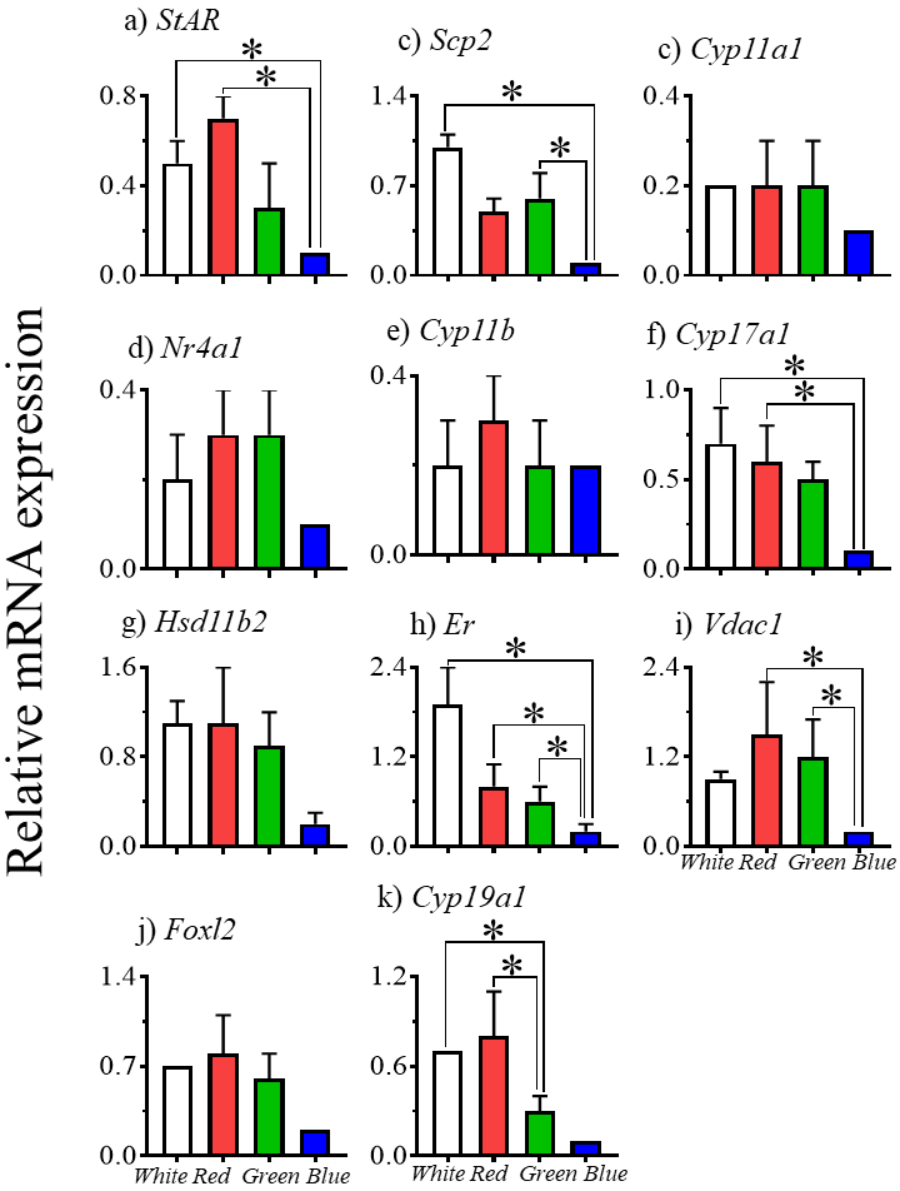

Fig. 28. Data are represented as mean (± SE, n=5 birds/group). Relative mRNA expression of steroidogenic genes in the ovary of female tree sparrows subjected to different light spectra for 30 days. One-way ANOVA was followed by Tukey's multiple comparison test to determine significant differences between the groups. * represents significant difference ($P < 0.05$).

5. DISCUSSION

Change in the photoperiod (day length) in nature drastically influences the positive and negative impact concerning to an individual's physiology, behavior, and reproduction as evidenced by various studies in the past few decades. The present experiment reveals the photoperiodic control responses in tree sparrows. In male and female tree sparrows, photoperiod does not have a significant effect across all the photoperiod regimes (8L:16D, 12L:12D, and 16L:8D), suggesting, in the experimental conditions, body mass is not directly influenced by the change in day length in this species which is consistent to the studies on other passerines where body mass is closely linked to the energy balance and food availability than photoperiod alone (Hau, 2001). However, bill color showed a significant difference, and darker bill color was observed in the long photoperiod group of 12L:12D and 16L:8D (Fig. 5b, 6b), possibly reflecting the increase in LH and testosterone level (Renthlei et al., 2021). A darker bill color is influenced by the hormonal changes that reflect the increased melanin deposition, which is a testosterone-dependent trait in various avian species (Dawson et al., 2001; Bókony et al., 2008). Also, photoperiod influences the color variation of the bills of zebra finches, where male zebra finches under long photoperiods of (14L:10D) developed darker, redder bills according to Burley et al. (1982). This suggests that long photoperiods, most likely via testosterone-mediated carotenoid production, improve sexual dimorphism in bill coloration. Besides, females also exhibit photoperiod-dependent reproductive responses where darker bill color under long photoperiod stimulates estrogen production, which is consistent with research on other bird species (Silverin et al., 1997). Similarly, there was an increase in the testicular volume and follicular diameter in birds exposed to equinox and long photoperiods 12L:12D and 16L:8D, which can stimulate the HPG axis. However, birds under SD (8L:16D) photoperiod did not respond to the photoperiodic induction, signifying that 8L:16D is beneath the threshold day length that can stimulate the system. The stimulation of the testicular and follicular growth in long photoperiod (16L:8D) reflects an increase in LH and FSH, leading to higher gonadal activity, and increased spermatogenesis and folliculogenesis (Dawson et al., 2001; Renthlei et al., 2021; Yatung & Trivedi, 2024,25). Also, Lewis et al. (2007) reported that transferring broilers from 11-h to 16-h photoperiods could upregulate plasma LH concentration.

Also, higher expressions in 12L:12D suggest, 12 hours of daylight is the optimal time of the day to stimulate the gonadal development in this species which is consistent with the study reported in domestic chicken where, the shortest saturation day length to stimulate LH release in egg-laying birds is between 12.75 h and 15.25 h (Dunn & Sharp, 1990).

Photoperiod is the proximate time cue that regulates physiological processes (Dixit & Singh, 2011). And this regulation is mediated through the HPG axis, which organizes the production of steroid hormones vital for reproductive processes such as gonadal growth, secondary sexual characteristics, and gametogenesis (Dawson et al., 2001). Higher expressions of *Dio2* and *GnRH* in male tree sparrows exposed to equinox and long photoperiods of 12L:12D and 16L:8D reflect the stimulation of the HPG axis, and this shift enhanced thyroid hormone signaling, upregulating *GnRH-I* and *Fshβ* transcripts, which drove testicular growth and spermatogenesis (Renthlei et al., 2021), whereas higher expression of *Dio3* and *GnIH* under short photoperiod (8L:16D) suggests an increase in *Dio3* inactivates T3, suppressing reproduction in short photoperiods. In the female tree sparrows, the increased mRNA transcripts of *Tshβ* and *GnRH* in the 12L and 16L groups indicate that the TSH signaling plays a more prominent role in female tree sparrows compared to males, where *Tshβ* expression was not significantly altered (Fig. 7a). In the pituitary TSH which expresses in pars tuberalis, a key site for photoperiodic signal transduction in avian species as described in the studies on quail and sparrows (Nakao et al., 2008). However, no significant changes in the *Dio2* (Fig. 8b) transcript in females indicate potential sex-specific variances in thyroid hormone regulation.

We have also observed differential expression of steroidogenic genes in the hypothalamic tissue of adult male and female tree sparrows. In male tree sparrows, higher transcript expression of *Er*, *Scp2*, *Hsd11b2*, *Vdac1,* and *Srd5a1* (Fig. 9) was observed in birds exposed to equinox and long photoperiods of 12L:12D and 16L:8D, indicating reproductive activity paralleled with enhanced steroid signaling and metabolism. Upregulation of *Er* transcript suggests that *Er* mediates estrogen response in the hypothalamus to regulate *GnRH* expression, which is consistent with the study report on this species and red-headed buntings (Renthlei et al., 2021; Mishra et al.,

2023; Yatung & Trivedi, 2024; Yatung & Trivedi, 2025), *Er* mRNA was significantly upregulated in the hypothalamus under long photoperiods (12L:12D and 16L:8D) suggesting enhanced *GnRH* secretion in response to estrogens, indirectly stimulating testicular steroidogenesis by increasing LH and FSH secretion and also, ER-mediated regulation of aromatase activity. *Scp2* is a sterol carrier protein, and *Vdac1* is a voltage-dependent anion-selective channel 1 that helps transport *StAR* for steroidogenesis. Higher expression of *Scp2* was observed in birds exposed to 12L:12D and 16L:8D, suggesting the beginning of the higher steroidogenic activity. Similarly, *Hsd11b2* and *Srd5a1* (Fig. 9h,j) were upregulated in the equinox and long photoperiod groups of 12L:12D and 16L:8D, reflecting their positive role in steroidogenesis. *Hsd11b2* regulates the inactivation of glucocorticoid, possibly reducing stress-related suppression of reproduction, whereas *Srd5a1* converts testosterone to dihydrotestosterone, amplifying androgenic effects (Tokarz et al., 2013). However, transcript expression did not show any differences in *StAR*, *Nr4a1*, *Cyp11a1*, *Cyp17a1*, and *Cyp11b* in all the photoperiodic regimes. This may be because of the delay in their peak expression time due to the short duration of exposure or indicates that these transcripts may be less responsive to photoperiod in this tissue or regulated post-transcriptionally (Stevenson et al., 2012). In the testis tissue, higher expression of transcripts *StAR*, *Nr4a1*, *Cyp11b*, *Er*, *Cyp11a1*, *Hsd11b2*, *Vdac1*, and *Srd5a1* was found in the stimulatory photoperiod exposed group 16L:8D, followed by 12L:12D, when compared to a short day treated group of 8L:16D. Given that *StAR* and *Cyp11a1* start steroidogenesis by translocating cholesterol and cleaving it to pregnenolone, respectively, this is consistent with testicular recrudescence under extended photoperiods (Mishra et al., 2023; Yatung & Trivedi, 2024). While upregulation of transcript *Cyp11b* aids corticosterone synthesis, potentially modulating stress responses, and higher expression of *Nr4a1* in the study reflects regulation of steroidogenic gene transcription (Bassett et al., 2004).

In the female hypothalamus, higher expression of mRNA transcripts (Fig. 11) was observed under the long photoperiod of 16L:8D and 12L:12D, indicating a robust activation of reproductive-linked steroidogenic pathways, and this response is similar to the male tree sparrows, suggesting a conserved mechanism in both sexes. *StAR* and

Scp2 transcript upregulation in long-day conditions (16L:8D) suggests enhanced steroid hormone production in response to increased reproductive activity that is triggered by longer photoperiods than the critical day length. This aligns with the study reported in seasonal breeders, where long-day photoperiods stimulate HPG axis activity, increasing gonadotropin release and steroidogenesis (Dawson et al., 2001; Yatung & Trivedi, 2025). Also, significant upregulation of transcripts *Cyp11a1*, *Cyp17a1*, *Cyp11b*, and *Hsd11b2* in 16L:8D suggests that the hypothalamus may contribute to local steroid production or metabolism, potentially modulating reproductive and stress responses. This is consistent with findings in this species and other species, such as rodents, where photoperiod influences hypothalamic expression of steroidogenic enzymes, correlating with seasonal reproductive cycles (Taves et al., 2011; Yatung & Trivedi, 2025). Besides, higher expressions of regulatory genes such as *Nr4a1* under longer photoperiod (16L:8D) indicate a role in coordinating the photoperiodic response of the HPG axis, potentially by enhancing *GnRH* expression, while upregulation *Er* in 16L:8D photoperiod suggests sensitivity to estrogen, which may contribute to hypothalamic responsiveness to circulating sex hormones and facilitate reproductive activities and *Foxl2* a transcription factor critical for ovarian development and function is typically associated with gonadal tissue, whereas its upregulation in the hypothalamus suggests a novel role in neuroendocrine regulation, possibly by influencing *GnRH* neuron activity and is supported by recent study (Ellsworth et al., 2006). In addition, upregulation of ovarian *StAR*, *Scp2*, *Vdac1*, *Cyp11a1*, *Cyp17a1*, *Cyp11b*, *Hsd11b2*, and *Cyp19a1* in long-day conditions suggests enhanced ovarian steroidogenesis (Fig. 12), likely leading to increased production of progesterone, androgens, and estrogens. This is consistent with the role of long photoperiods in stimulating ovarian reproductive activity in the tree sparrow (Yatung & Trivedi, 2024, 25), where the expression of steroidogenic genes was upregulated during the breeding season when the birds experience longer day length. Whereas, higher expression of *Cyp19a1* in the ovary is distinct from its lack of change in the hypothalamus, indicating tissue-specific regulation. In the ovary, *Cyp19a1* expression is critical for estrogen synthesis, and its upregulation in 16L:8D aligns with increased follicular activity and estrogen production, as reported in (Wang et al., 2023; Yatung & Trivedi, 2025).

Furthermore, the present work reveals the differential effects of light intensities on the tree sparrow. No significant variations were observed in the body mass in both male and female tree sparrows in all three groups in the 30-day experiment. The tree sparrow does not show significant variations in body mass since it is a resident bird and does not retain a lot of fat, which is consistent with the other studies (Renthlei et al., 2021; Yatung & Trivedi, 2024, 25). However, the bill color was slightly darker in the 100 lux group in comparison to the 10 lux and 50 lux groups, suggesting 100 lux is adequate to stimulate the secondary sexual characteristics. Whereas, a significant increase in the testicular (Fig. 13c) and follicular growth (Fig. 14c) was observed in the 100 lux intensity group, followed by 50 lux in comparison to the 10 lux group, indicating higher intensity is required to stimulate gonadal development, and suggests higher intensity of 100 lux amplifies the duration of daylength i.e 16L:8D exposure. This result aligns with the well-established study by Dawson et al. (2008), where higher photoperiod is required to initiate reproductive activity.

In the hypothalamic tissue of male and female tree sparrows, higher expressions of *Tshβ* and *Dio2* at 100 lux intensity indicate the upregulation of thyroid hormone signaling in response to photoperiodic induction of reproduction. The TSH begins to initiate *Dio2* through the TSH receptor signalling pathway and leading to downstream signaling processes at the gonadal levels (Yoshimura et al., 2003; Nakao et al., 2008). No significant variation in the expression of *Dio3* and *GnRH* (Fig. 15c,d) indicates a delay in the stimulation of these genes' upregulation. Also, higher expression of the *GnRH* (Fig. 16c) in females under 100 lux intensity, unlike males, indicates sex specific regulation of the HPG axis. Higher expression of *GnIH* in the 10-lux intensity group and lower in the 100-lux group shows the inhibitory nature of *GnIH*, which downregulates the *GnRH* expression, suggesting that lower intensities may suppress the reproductive activity (Tsutsui et al., 2000).

We have also observed the effect of differential light intensities on steroidogenesis in both male and female tree sparrows (Fig. 17-20). In the hypothalamus, upregulation of steroidogenic genes (*StAR*, *Cyp11a1*, Cyp17a1, *Nr4a1*, *Cyp11b*, *Srd5a1*, *Hsd11b2*, *Vdac1*) under 100 lux of intensity indicates the increased steroid hormone synthesis, such as testosterone and estrogen production, which then

stimulates the gonadal development and reproductive behaviour. *StAR* aids in the transportation of cholesterol across the mitochondria, commencing the steroidogenesis (Freking et al., 2000; Ings et al., 2006; Rosati et al., 2019; Wang et al., 2021; Wang et al., 2023; Yatung & Trivedi, 2024, 25). Higher expression of *StAR* in the hypothalamic tissue may reflect the initiation of reproductive activity in the brain leading to enhanced local synthesis of neurosteroids in the brain tissue. *Cyp11a1* converts the cholesterol into pregnenolone, the very first step in the steroid synthesis pathway, and higher expression of this transcript under 100 lux reflects the start of the steroid signalling pathway and is comparable with the study reported by Santillo et al. (2017), where the steroidogenic enzyme expression in brain regions showed to be higher in the breeding season when the photoperiod intensity is longer.

Similarly, higher expression of *Cyp17a1*, *Cyp11b*, *Srd5a1*, and *Hsd11b2* in the 100 lux group compared to 10 or 50 lux group reflects that the 100 lux intensity is adequate to stimulate the synthesis of steroidogenic genes with enhance sex hormone production and higher reproductive activity and is consistent with the study conducted on a japanese quail where steroidogenic genes such as *StAR*, *3β-hsd, 17β-hsd, p450, ar*, and *5α-Red* were constantly present in the somatic (Leydig and Sertoli cells) and, germ cells (spermatogonia, spermatocytes I and II, spermatids, and spermatozoa) and the maximum expressions were noted during the reproductive phase (Rosati et al., 2019). Similarly, neurosteroid levels in the brain of edible frogs fluctuate during their reproductive stages. In addition, seasonal variations in gene expression levels of *hsd3b1*, *hsd17b1*, *srd5a1*, and *cyp19a1* were recognized in the dienencephalon-mesencephalon area of both sexes, and these transcript expression levels were increased during the reproductive phase compared to the post-reproductive period. Besides, *Vdac1*, the voltage-dependent anion channel, facilitates cholesterol to the inner mitochondrial membrane (Prasad et al., 2015), and higher *Vdac1* expression reflects more cholesterol transportation for steroidogenesis to begin. *Nr4a1* expression is controlled by many agents, including Ca2+, growth factors (Li et al., 2006), and *Nr4a1* modulates the expression levels of several genes involved in steroidogenesis, such as *StAR, Cyp17a1, Hsd3b2*, and *Cyp11b2* (Zhang & Mellon, 1997; Bassett et al., 2004; Martin et al., 2008). Additionally, it is well recognized that sex steroids have a

major impact on the testis, regulating the reproductive processes by either promoting spermatogenesis and spermiogenesis or by supporting seasonal breeders to produce, differentiate, and preserve secondary sex traits (Angelini & Botte, 1992; Di Fiore et al., 2005; Carreau et al., 2011) and are consistent with the results of the present study, where higher expressions of transcript *StAR, Cyp11a1, Nr4a1, Cyp11b, Cyp17a1, Srd5a1, Hsd11b2, Er, and Srd5a1* in 100 lux intensity group in the gonads suggests the stimulation of enhanced steroid hormone production, particularly testosterone and estrogen, which are critical for gonadal development and reproductive behavior.

Similar responses were observed in the female hypothalamus and ovary, where upregulation of steroidogenic genes (e.g., *StAR, Cyp11a1, Cyp17a1, Nr4a1, Cyp11b, Srd5a1, Hsd11b2, Vdac1*) under 100 lux intensity reflects enhanced steroid hormone production. A rate-limiting stage in steroidogenesis, the transport of cholesterol into mitochondria is facilitated by the *StAR* gene (Stocco, 2001). Likewise, *Nr4a1* controls the expression of steroidogenic genes, while *Cyp11a1* and *Cyp17a1* encode enzymes essential for the conversion of cholesterol to sex steroids (Zhang & Mellon, 1997; Bassett et al., 2004; Martin et al., 2008). *Scp2* and *Cyp11a1* in male testes and several ovarian steroidogenic genes do not significantly alter, indicating tissue-specific regulation that may be brought about by variations in local enzymatic activity or feedback mechanisms. Increased estrogen synthesis is indicated by the overexpression of *Cyp19a1* (aromatase) in the female hypothalamus and ovary under 100 lux, which is in line with improved follicular development (Freking et al., 2000).

There is considerable literature and studies conducted on the effect of light wavelengths on various physiological and developmental processes. Whereas, the present study results also indicate that light's spectral component (wavelength) plays a crucial role in regulating physiological responses during photoperiods. We have observed a differential effect of light spectra on physiological parameters such as bill color. Bill color is associated with secondary sexual characteristics and reproductive readiness (Yatung & Trivedi, 2024, 2025). A darker bill color was noted in the red-light group and white light in comparison to the green and blue light groups (Fig. 21), suggesting reproductive readiness in this species. In addition, a faster and significant increase in the testicular volume (Fig. 21c) and follicular diameter (Fig. 22c) was

observed under red light, followed by white light, and lower under green and blue light; however, no gonadal development was witnessed in blue light, irrespective of the long stimulatory photoperiod of 16L:8D exposure to all the birds, suggesting red light with the longest wavelength has a stimulatory effect on the gonadal growth. Besides, the responses were slower in white light in comparison to red light which is observed to be more stimulatory, (Fig. 21c, 22c), indicating a light's wavelength-dependent inductive effect in tree sparrows, and is consistent with other bird species (Kumar & Rani, 1996; Rani & Kumar, 2000; Malik et al., 2004). However, body mass was not affected by any of the spectrum treatments, which is in contrast with the study by Renthlei (2020), where they observed a slight increase in body mass when the spectral treatment was run for 180 days; however, the present study was conducted under a short-term experiment which may be the reason for no change in the body mass. Also, since tree sparrows are resident birds that store very small amounts of fat, and do not show significant variations in their body weight, unlike migratory species (Trivedi et al., 2006; Yatung & Trivedi, 2024, 25).

The longer wavelengths of light (i.e., red light) have more inductive effects in numerous bird species (Oishi & Lauber, 1973; Glass & Lauber, 1981; Benoit, 1964; Foster & Follett, 1985; Siopes & Wilson, 1980). The photoperiodic gonadal response of red light could be because of the number of photons in longer wavelengths able to penetrate the skull and reach the brain tissue obtained by the deep brain photoreceptors situated deep in the brain (Vriend & Lauber, 1973). In birds, the photoperiodic signal transduction system involves these deep-brain photoreceptors correspondingly stimulating TSH signalling and triggering subsequent gonadal processes (Yamamura et al., 2006; Nakao et al., 2008). The red light is observed to stimulate the gonadal growth, potentially enhancing testosterone and progesterone synthesis. To confirm this, we have also observed expression of transcripts governing seasonal reproduction in avian species in the hypothalamus of both male and female tree sparrows. Highest expressions of *Tshβ*, *Dio2* and *GnRH* were observed in the red-light group, followed by white light in both male and female, suggesting initiation of TSH signaling in the hypothalamus leading to transcription of *Dio2* and further increased gonadotropin release, which is also similar with the study (Yatung & Trivedi, 2024, 2025), where

higher expressions of these genes were observed in the hypothalamic tissue when the birds experienced long stimulatory photoperiod during the breeding season. However, the upregulation of *Dio3* and *GnIH* (Fig. 23, 24) in response to blue light suggests an inhibition of reproductive activity. The upregulation of stimulatory reproductive genes in red light and downregulation in the green and blue light in the initiation/inhibition of reproductive system has been extensively studied in many avian species (the European starling, Bissonnette, 1932; English sparrow, Ringoen, 1942; duck, Benoit & Otto, 1944; fowl, Ishibashi & Kato, 1951; Japanese quail, Woodard et al., 1969, Yadav & Chaturvedi, 2015; red-headed bunting, Rani & Kumar, 2000; black-headed bunting, Malik et al., 2004; geese, Chang et al., 2015).

The available literature on the effect of light spectra on steroidogenic gene expression is very limited, and broader effects of light wavelength on physiological processes, including circadian rhythms and hormone production, with specific studies on steroidogenic gene expression are very scarce. Therefore, we have further measured the effect of different light spectra on several steroidogenic markers in the hypothalamic tissue and gonadal tissue (testis and ovary) in the adult tree sparrows. It is known that reproductive activity is connected with enhanced steroidogenesis (Freking et al., 2000; Rosati et al., 2019; Wang et al., 2021; Yatung & Trivedi, 2024, 2025). In the hypothalamus of male tree sparrows, we have observed higher expressions of *StAR*, *Cyp11a1*, *Nr4a1*, and *Srd5a1* in the red light-treated group in comparison to white, green, and blue reconfirms the role of red light as being more inductive modulator of the reproductive activity. Whereas, *Cyp11b* was upregulated in the white light compared to the other groups. However, no changes were observed in the transcript analysis of *Scp2*, *Cyp17a1, Hsd11b2, Er,* and *Vdac1* (Fig. 25).

Also, similar responses were observed in testis tissue, where almost all of these genes were upregulated in red light in comparison to white, green, and blue light (Fig. 26), as red light wavelength is photo-inducible (Yadav & Chaturvedi, 2015), reflecting enhanced steroidogenesis and metabolism in this species. Furthermore, the steroidogenic process in gonads displays distinct sex and season-specific patterns as well as crucial enzyme expression, and sex steroids have a significant impact on

vertebrates' reproductive behavior. Other species have also been shown to exhibit higher levels of sex steroids during the long stimulatory photoperiod, which occurs before or during breeding (Yatung & Trivedi, 2024). Similarly, in the female hypothalamus, upregulation of transcripts *StAR*, Cyp11a1, *Nr4a1*, *Cyp17a1*, *Hsd11b2*, and *Cyp19a1* was observed to be highest in the red-light group, suggesting upregulation of neurosteroidogenesis in the brain areas, which is also relevant to the study by (Yatung & Trivedi 2024, 2025), where higher expressions of several steroidogenic genes were observed in the hypothalamus during the breeding phase when birds experienced stimulatory long photoperiods. However, no differences were observed in transcript expression of *Scp2* and *Cyp11b*, which might be due to a delay in their expression time under a short-term experiment. In addition, transcript expression of *Vdac1* and *Foxl2* was observed to be higher in the red light and green light groups in comparison to white and blue light. This indicates that the red light is the most stimulatory, and apart from red light, green light also has a stimulatory effect on hypothalamic steroidogenesis, since the deep brain photoreceptors are most sensitive to short-wavelength (around 500 nm, green light; Foster & Follett, 1985) and are comparable with the study (Rozenboim et al., 2004), where green light stimulated growth of birds at an early age. Also, Green light has been shown to stimulate muscle growth (Halevy et al., 1998), indicating a potential mechanism for growth acceleration by light stimulation. Further in the ovarian tissue, higher expressions of transcripts *StAR*, *Scp2*, *Cyp17a1*, *Er*, *Vdac1*, and *Cyp19a1* were observed, leading to higher steroidogenic activity, suggesting increased folliculogenesis (Krasnow, 1991; Yatung & Trivedi, 2025). However, no differences were observed in the expression levels of *Cyp11a1*, *Nr4a1*, *Cyp11b*, *Hsd11b2*, and *Foxl2* (Fig. 28) in the ovary and *Scp2*, *Cyp17a1*, *Hsd11b2*, *Er*, and *Vdac1* (Fig. 25) in the hypothalamus of male reflecting tissue-specific, sex dependant, and time-dependent expression.

6. CONCLUSIONS

In conclusion, the findings reveal that photoperiod has a considerable impact on the expression of steroidogenic and its regulatory genes in both hypothalamus and gonads tissue of male and female tree sparrows, with long-day conditions (16L:8D) promoting higher expression of most of the genes, followed by equinox (12L:12D)

and inhibitory effects in short-day (8L:16D) conditions, These findings align with the known effects of photoperiod on reproductive physiology in seasonal breeders and highlight tissue-specific differences in gene regulation. Comparisons with previous studies confirm the function of long-day photoperiods in influencing HPG axis activity, with possible species- and tissue-specific changes. Also, differential expression of reproductive and steroidogenic transcripts was observed when treated with different intensities of light. 100 lux of light intensity is adequate to stimulate the HPG axis, leading to higher reproductive activity and sex steroid production. Moreover, similar responses in most of the gene expression were observed in both sexes, indicating a conserved mechanism in this species. Moreover, the lack of expression of some genes in both the hypothalamus and the gonads reflects tissue-specific and sex-specific regulation of reproduction. And the effect of light spectra on gene expression, specifically concerning steroidogenic activity in avian species, is very limited. In conclusion, the study reveals differential effects of light wavelength on reproductive activity and linked steroidogenic gene expression, where red light has the most stimulatory effect, enhancing reproductive-linked steroidogenesis, followed by white light and green light, whereas blue light has an inhibitory effect, hence downregulates the steroidogenesis in both male and female tree sparrow.

SECTION III: ANNUAL LIFE HISTORY STATE-DEPENDENT EXPRESSION OF STEROIDOGENIC GENES

1. ABSTRACT

The population responds to environmental variability, largely determined by the dynamic interactions between fitness components within- and among-individual variation in the expression of the environmentally sensitive phenotype. Seasonal breeders display elevated sex steroid hormone production during reproductive seasons, resulting in significant physiological and structural alterations. One such seasonal breeder adapted to the changing environment is the Tree sparrow. The study aims to investigate annual variations in the expression of reproductive activity and linked steroidogenic gene markers of adult male and female tree sparrows and corresponding seasonal changes in the physiological characters in both sexes. In the experiment, birds (n = 5/month/sex) were sampled every month at midday for a year. Body mass, bill color, testis size, and molt in feathers were recorded. The hypothalamus and gonad tissues were harvested and kept at -80ºC and later used for gene expression studies. Blood plasma cholesterol, testosterone levels, and progesterone levels were measured. Higher testicular volumes were recorded from March to May, whereas maximum molt was observed during the post-breeding phase. Plasma cholesterol levels were highest before the breeding phase. Higher testosterone levels corresponded with the breeding phase. Higher expressions of *Tshβ*, *Dio2*, and *GnRH* during the breeding phase and higher expression of *Dio3* and *GnIH* were observed during the non-breeding phase. Similarly, in females, larger follicular size occurs during May and June. Whereas, maximum molt was observed during the post-reproductive phase. Cholesterol levels were highest before the breeding phase, and higher progesterone levels paralleled larger follicular size. While higher levels of *GnIH* and *Dio3* were observed during the non-breeding phase, elevated expression of *Tshβ*, *Dio2,* and *GnRH* was noted during the reproductive period. The steroidogenic transcripts showed seasonal changes in their expression in the hypothalamic and gonadal tissue and were upregulated either during the pre-breeding or breeding phase. The study reveals that mRNA levels of steroidogenic transcripts (*StAR, Nr4a1, Er, Scp2, Cyp17a1, Foxl2, Cyp11a1, Hsd11b2,*

Cyp11b, Cyp19a1, and *Vdac1*) show seasonal variations that correspond to the annual reproductive cycle of both male and female tree sparrows.

2. INTRODUCTION

Changing environment greatly affects the physiological and biological attributes of vertebrates. Predicting how individuals and populations will react to environmental variation and change, as well as figuring out the processes sustaining or limiting the important phenotypic variation, requires quantifying impacts (Chevin et al., 2015; Arnold et al., 2019; Fox et al., 2019). Subsequently, the degree to which an individual's phenotype and the fitness implications of phenotype vary within and between seasons, whereas, years determine the population's outcome and are indicative of labile plasticity and temporal fluctuation in selection (Siepielski et al., 2009; Chevin et al., 2010; Nettle & Bateson, 2015). The HPG axis, which is crucial to several processes like sex differentiation, gonad growth, and spawning, regulates the timing of reproduction and the generation of sex steroids (Gothilf et al., 1997; Falcón et al., 2007). Environmental factors, such as photoperiod, temperature, and resource availability, are responsible for avian species' physiological and behavioral variations. Photoperiod is one of the most vital environmental cues to which avian species correspond to time their life history stages: Reproduction, molting, and migration. The prime role of photoperiod in seasonal time measurement was conclusively demonstrated in numerous organisms (Garner & Allard, 1920; Marcovitch, 1923; Rowan, 1925). The internal biological clock is responsible for the cyclical variations in an animal's physiological and behavioral processes. Biological clocks allow animals to correspond with cyclic events in nature, such as day and night/season. In birds, independent clocks are present at a minimum of three levels: the retina of the eyes, the pineal gland, and the hypothalamus (Yoshimura et al., 2000; Steele et al., 2003; Yasuo et al., 2003; Kumar et al., 2004; Karaganis et al., 2008).

Long photoperiods have been shown to activate deep-brain photoreceptors in photoperiodic species that include opsin (Yoshimura et al., 2003; Nakao et al., 2008; Perfito et al., 2012; Pérez et al., 2019; Pérez et al., 2023). According to Pérez et al. (2023), a new study on Japanese quail indicates that VA opsin, in addition to OPN5

and OPN4, is critical in stimulating thyroid signaling in the hypothalamus. Further, *TSH* acts on the ECs within the MBH, and initiates transcription *Dio2* via the *TSH* receptor signalling pathway, and leads to downstream developments at gonadal levels (Nakao et al., 2008). A decrease in transcription of the gene coding for *GnIH*, inhibits *GnRH* release (Tsutsui et al., 2000; Chowdhury et al., 2010). These molecules have also been shown to be up- or down-regulated depending on the photoperiod and physiological state of the avian species such as Red-headed bunting, Black-headed bunting, and Tree sparrow (Majumdar et al., 2015; Mishra et al., 2017; Dixit & Byrast, 2018; Zhang et al., 2019; Trivedi et al., 2019; Renthlei & Trivedi, 2019; Renthlei et al., 2021).

Steroidogenic enzymes play a vital role in the functioning of sex steroids. As avian follicle growth is primarily regulated by the genetic machinery of reproductive hormones and associated functions, steroid hormone synthesis and secretion play a dominant role (Ren et al., 2019). Besides, in males, it plays a crucial role in the testis and regulates spermatogenesis and spermiogenesis, or the maintenance of secondary sexual characteristics in vertebrates having seasonal reproductive cycles (Rosati et al., 2017). Progesterone, testosterone, and estradiol are important steroid hormones regulating follicle development (Kawashima et al., 1994; Paitz & Cagney, 2019). Sex steroid hormones are influential regulators of reproductive behavior and physiology in vertebrates, and steroidogenesis has discrete sex- and season-specific patterns ultimately dictated by the expression of key enzymes. (Lincoln et al., 2023) and are necessary for reproduction in a wide variety of vertebrates, and reproduction does not occur if these hormones are removed (Arnold & Breedlove, 1985; Balthazart & Foidart, 1993; Meitzen & Thompson, 2008; Antonio-Cabrera & Paredes, 2014). Seasonally breeding animals have natural changes to steroid hormone levels and breeding behavior. In particular, these animals have large gonadal growth, produce high levels of sex steroids, and show reproductive behaviors throughout the breeding season, with regressed gonadal growth, low sex steroid hormone levels, and no reproductive activity during the non-breeding season (e.g., Voigt & Leitner, 2008; Ansel et al., 2011; Forlano et al., 2014). Hence, steroidogenesis is likely regulated in seasonally breeding animals to produce seasonal changes in circulating steroid

hormones. Gonadal steroidogenesis is under the control of the HPG axis system, such that circulating LH triggers the gonadal production of steroidogenic acute regulatory protein (*StAR*) (Clark et al., 1994).

The steroidogenic pathway consists of cholesterol mobilization from lipid droplets and the plasma membrane, transport into mitochondria, and the formation of pregnenolone into final steroids (Hu et al., 2010). The biosynthesis of all the steroid hormones is controlled by the two key functional classes of steroidogenic enzymes (cytochrome P450 and hydrosteroid dehydrogenase) (Schiffer et al., 2019). In the mitochondria, *Scp2*, a sterol carrier protein and an activator of mitochondrial steroidogenesis, stimulates the transfer of cholesterol as well as that of phospholipids between membranes in vitro (Bloj & Zilversmit, 1977; Poorthuis et al., 1981; Scallen et al., 1985), and it may play a role in the intracellular transport or metabolism of phospholipids and cholesterol (Chanderbhan et al., 1982; Teerlink et al., 1984; Seltman et al., 1985). A mitochondrial protein, *StAR,* helps transport cholesterol to mitochondria (Soccio & Breslow, 2003). A nuclear receptor (Nur77/Nr4a1) is present in steroidogenic cells, and its expression is induced by hormones known to activate StAR expression (Martin et al., 2008). *StAR* acts on the outer mitochondrial membrane (OMM) to facilitate the movement of cholesterol from the OMM to the inner mitochondrial membrane to be converted to pregnenolone. Further, *StAR* interacts with *Vdac1* on the OMM, which then facilitates processing of the 37-kDa phospho-*StAR* to the 32-kDa intermediate. *StAR* then interacts with cholesterol and is converted to pregnenolone by the *Cyp11a1*. In smooth endoplasmic reticulum, pregnenolone is then transformed into dehydroepiandrosterone and is modified into androstenedione by 3β-hsd. Later, 17β-hsd catalyzes the conversion of androstenedione to testosterone (T). Testosterone can be converted both in 17β-estradiol by cytochrome P450 aromatase (P450 arom) or to 5 alpha-dihydrotestosterone (DHT) by 5 alpha-reductase (Moeller & Adamski, 2009; Carreau et al., 2011; Papadopoulos & Miller, 2012). Later, 17α-hydroxy pregnenolone is converted to 17β-oh-progesterone by *Cyp21* and is further converted to Cortisol by *Cyp11b* and to Cortisone by *Hsd11b2*. In males, testosterone is produced by Leydig cells, and testosterone directly regulates both spermatogenesis and secondary sexual characteristics (Zhou et al., 2019). Typically, the ovary expresses *Cyp11a1*, *3β-hsd*, *Cyp17*, and *Cyp19,* so the primary terminal secretion is estrogen.

Foxl2 is responsible for transcriptional regulation of p450arom in female gonads because *Foxl2* and p450arom mRNA are co-expressed in the medullary region of the female gonad during sex differentiation (13-15 days incubation) in chicken (Govoroun et al., 2004). The ultimate steroidal signal secreted by the tissue depends upon which steroidogenic enzymes are expressed.

The present study advocates that no prior research has been conducted on this species concerning seasonal steroidogenic enzyme gene expression. The study's primary objective is to examine whether the steroidogenic enzyme gene expression varies annually in an adult male and female Tree Sparrow. For this preliminary study, we hypothesized that gene expression does vary seasonally. Therefore, we examined the seasonal variations in steroidogenic enzyme gene expression and correlated them with annual reproductive cycles

3. MATERIALS AND METHODS

3.1. Animal procurement and maintenance

The study was conducted on both male and female adult Tree sparrows procured locally using mist nets. Adult male and female birds (N=5/month) were collected monthly throughout the year (January to December 2022). Immediately after procurement, birds were transferred to the laboratory and kept in NDL (natural day-length conditions in captivity). Food, along with the mealworms and water, was provided ad libitum. Body weight was recorded using a top pan balance to the accuracy of 0.1 g. For individual birds, the molt in body and primary flight feathers, and the bill color. Bill's color was assessed based on a subjective criterion as reported earlier (Trivedi et al., 2006). Briefly, bill color was assessed in an index of 0–5 where 0 represents a straw color bill (S), score 1 indicates a bill that is straw in color with a little tinge of blackness (ratio- SSS: B), score 2 indicates a slightly blackish bill (ratio- SS: B), score 3 reflects a bill having similar percentage of straw and black (ratio- S: B), score 4 suggests a bill is black with very little straw patch (ratio- S: BB) while score 5 is given when a bill is completely black (B). In primary flight (wing primaries) and body feathers, a molt was recorded (Trivedi et al., 2006). Primaries feathers were scored 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. So, each primary could have a maximum score of 5. The maximum score for one wing can be up to 45 (9 × 5 = 45); for each bird, the feather score could total up to 90 (2 × 45 = 90). While the minimum score could be as low as 0. For body molt, the whole bird's body was divided into 11 different regions: 1 – head, 2 – neck, 3 – shoulder, 4 – back, 5 – pelvic, 6 – throat, 7 – chest, 8 – abdomen, 9 – flank, 10 – shank, and 11 – sub-caudal. Any region could have a score of either 0 (old feathers) or 1 (new feathers emerged), and hence the total body molt score could be in the range of 0–11, depending on the number of regions that had scores of 0 or 1. Since tree sparrow does not have sexual dimorphism, a unilateral laparotomy was performed (Kumar et al., 2001) The gonadal size was recorded by performing a laparotomy, where a small incision was made between the last two ribs on the left flank and the left testis was located within the abdominal cavity with the help of a spatula. The dimensions of the left testis were recorded, and testicular volume (TV) was calculated using the formula 4/3πab2, where a and b denote half of the long (length) and short (width) axes, respectively. Birds were sampled during the middle of the day. Blood (200–300 μL) from the trunk of each bird was collected in heparinized tubes, and plasma was harvested and stored at -20 °C for biochemical and hormonal assays. Immediately after sampling, the brain and gonads were excised and stored at -80°C for gene expression analysis. Later, the hypothalamus, along with the pituitary, was harvested from the individual brain by slicing the brain with a surgical blade having a thick coronal section trimmed dorsally (starting approximately at TrSm) and laterally extending from the optic chiasma to the infundibular area. The protocols were approved by the institutional animal ethics committee of Mizoram University (No MZU/IAEC/2021-22/12).

3.2.Cholesterol assay

Using the manufacturer's protocol, male and female plasma cholesterol levels were measured using the CHOLESTEROL KIT (Coral Clinical Systems, India). Briefly, clean test tubes were labelled as blank, standard, and test. After that, 1 ml of working reagent was pipetted into blank, standard, and test sample tubes. 10 μL of distilled water was pipetted into a blank test tube, followed by 10 μL of cholesterol

standard into a standard test tube and 10 µL of plasma sample into a sample test tube. After adding all the required reagents to the test tubes, it was mixed well and incubated at room temperature (25°C) for 15 minutes. The absorbance of standard and test samples was measured against the blank within fifteen minutes at 505 nm wavelength using the SpectraMax MINI system (Multimode Microplate Reader, AFL System Molecular Devices, USA). Each sample was run in duplicate.

3.3. Testosterone assay

Testosterone levels were measured using enzyme immunoassay kits (Testosterone, Diametra, DKO002) as per the manufacturer's protocol. This assay has been validated and used for the measurement of testosterone in this and other species (Biswas et al., 2010; Pathak & Lal, 2010; Dixit & Singh, 2014). Briefly, before use, all the reagents were kept at room temperature for 30 minutes. Plasma samples were allowed to thaw on ice. The wash solution was diluted as per the protocol provided. All calibrators, controls, test samples, and blanks were run in duplicate. 25 µL of sample or control and 25 µL of calibrator were pipetted into each respective well, and 100 µL of conjugate was added to each calibrator and sample well. After adding the reagents, the plate was incubated at 37 °C for 1 hour. After one hour, the content was removed from each well and washed three times with 300 µL of diluted wash solution every 5-minute intervals. The residual buffer was blotted by tapping the plate upside down on absorbent paper. Next, a TMB substrate of 100 µL was added to each well and incubated at room temperature (25 °C) for 15 minutes in the dark, followed by adding a stop solution of 100 µL, mixed gently. Absorbance was taken using a Multimode ELISA reader, SpectraMax MINI system at 450 nm against the Blank within 5 minutes. Each sample was run in duplicate.

3.4. Progesterone assay

Progesterone levels were measured using enzyme immunoassay kits (Progesterone, Diametra, DKO006) as per the manufacturer's protocol. Briefly, before use, all the reagents were kept at room temperature for 30 minutes. Plasma samples were allowed to thaw on ice. The wash solution was diluted as per the protocol provided. All calibrators, controls, test samples, and blank were run in duplicate. 50

μL of sample or control and 50 μL of calibrator were pipetted into each respective well, and 100 μL of conjugate was added to each calibrator and sample well. After adding the reagents, the plate was incubated at 37 °C for 1 hour. After one hour, the content was removed from each well and washed three times with 300 μL of diluted wash solution every 5-minute interval. The residual buffer was blotted by tapping the plate upside down on absorbent paper. Next, a TMB substrate of 100 μL was added to each well and incubated at room temperature for 15 minutes in the dark, followed by adding a stop solution of 100 μL, mixed gently. Absorbance was taken using a Multimode ELISA reader, SpectraMax MINI system at 450 nm against the Blank within 5 minutes.

3.5. Gene transcription

The mRNA transcription levels of reproductive genes coding for *Tshβ, Dio2, Dio3, GnRH,* and *GnIH* were measured in the hypothalamus, and mRNA transcription of steroidogenic genes coding for *StAR, Nr4a1, Er, Scp2, Cyp17a1, Cyp11a1, Hsd11b2, Cyp11b, Srd5a1, Vdac1* were measured both in hypothalamus and testis and steroidogenic genes coding for *StAR, Nr4a1, Er, Scp2, Cyp17a1, Cyp11a1, Hsd11b2, Cyp11b, Foxl2, Vdac1 and Cyp19a1* for daily and seasonal studies. *18S* was used as a reference gene to determine the relative expression of transcripts.

3.6. RNA isolation and cDNA synthesis

Total RNA was extracted from tissue samples using TRIzol Reagent (Invitrogen by Thermo Fisher Scientific). The concentration and purity of total RNA were determined using (Nanodrop Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). 2 μg RNA was used to process cDNA synthesis. RQ1 DNase (Promega M6101; Madison, WI, USA) kit was used to remove any possible genomic DNA contamination. For cDNA synthesis, the iScript™ cDNA Synthesis Kit (BIO-RAD, USA, 1708891) was used.

3.7. Quantitative (real-time) RT-PCR (qPCR)

Gene-specific primers were designed from the sequences using Primer 3 plus (online primer design program, Boston, MA, USA; Supplementary Table 1). qPCR was performed on Quant-Studio 5 (Applied Biosystem by Thermo Fisher Scientific,

Foster City, CA, USA) as described in our previous publications (Renthlei & Trivedi, 2019; Borah et al., 2020; Renthlei et al., 2020; 2021; Borah et al., 2022; Lalpekhlui et al., 2022). Briefly, PCR reaction of the volume of 7 µl for each gene comprised 1 µl each of cDNA, gene-specific forward and reverse primers, 3 µl Power UpTM SYBR Green Master Mix (Applied Biosystem by Thermo Fisher Scientific, Foster City, CA, USA, A25742), and 1µl of nuclease-free water (Ambion, AM9938). qPCR cycle conditions included 1 cycle at 95°C (20 s), 35 cycles at 95°C (01 s), 60°C (20 s) and 95°C (01 s), additional melt curve plot step included 1 cycle of 60°C (20 s) and 1 cycle of 95°C (01 s). The ΔΔCt method (Livak & Schmittgen, 2001) was used for gene expression analysis. Each sample was run in duplicate.

3.8. Statistics

To determine the significant difference in the monthly profile, one-way ANOVA (one-way analysis of variance) was used, followed by Bonferroni's and Tukey's multiple comparison tests were applied if ANOVA showed a significant difference. Significant difference was always taken at $P<0.05$. Analysis was done using GraphPad Prism version 8.

4. RESULTS

In the males, no significant change in body mass was observed across the year ($P=0.1863$; One Way ANOVA; Table 6a; Fig. 29a). Annual variations in bill color ($P<0.0001$; One Way ANOVA; Table 6a; Fig. 29b), molt in body feathers ($P<0.0001$; One Way ANOVA; Table 6a; Fig. 29d), primary flight feathers ($P<0.0001$; One Way ANOVA; Table 6a; Fig. 29f), and testicular volume ($P<0.0001$; One Way ANOVA; Table 6a; Fig. 29c) were also observed. Higher bill color score was observed from April till July ($P<0.05$; Bonferroni's multiple comparison tests; Fig. 29b), having the highest score during June ($P<0.05$; Bonferroni's multiple comparison tests; Fig. 29b). Molt in body feathers and primary flight feathers begin in July and completed in October ($P<0.05$; Bonferroni's multiple comparison tests; Fig. 29d, f) with maximum molt during September (Fig. 29d, f). Significantly higher gonadal sizes were observed from March till May, began to regress in June, and were fully regressed from July onwards (Fig. 29c). Plasma cholesterol levels also showed seasonal fluctuation ($P<0.0001$; One Way ANOVA; Table 6a; Fig. 29g). Elevated plasma cholesterol levels

were observed in January, were significantly higher in February ($P<0.05$; Bonferroni's multiple comparison tests; Fig. 29g), and started to decline from March onwards (Fig. 29g). Minimum cholesterol levels were observed during July and August ($P<0.05$; Bonferroni's multiple comparison tests; Fig. 29g). Annual changes in plasma testosterone levels were also observed ($P<0.0001$; One Way ANOVA; Table 6a; Fig.29e). Plasma testosterone levels started to elevate in March, maximum levels were observed during April ($P<0.05$; Bonferroni's multiple comparison tests; Fig. 29e), and began to decline from May onwards (Fig. 29e).

Table 6a. Values of One-way ANOVA of the monthly physiological parameters in male tree sparrows

Body mass	$F_{(11, 46)} = 1.444$, $P=0.1863$
Bill color	$F_{(11, 46)} = 9.555$, $P<0.0001$
Testicular volume	$F_{(11, 46)} = 12.31$, $P<0.0001$
Plasma testosterone level	$F_{(11, 48)} = 12.16$, $P<0.0001$
Plasma cholesterol level	$F_{(11, 43)} = 24.05$, $P<0.0001$
Bod ymolt	$F_{(11, 46)} = 7.298$, $P<0.0001$
Primary feather molt	$F_{(11, 46)} = 14.24$, $P<0.0001$

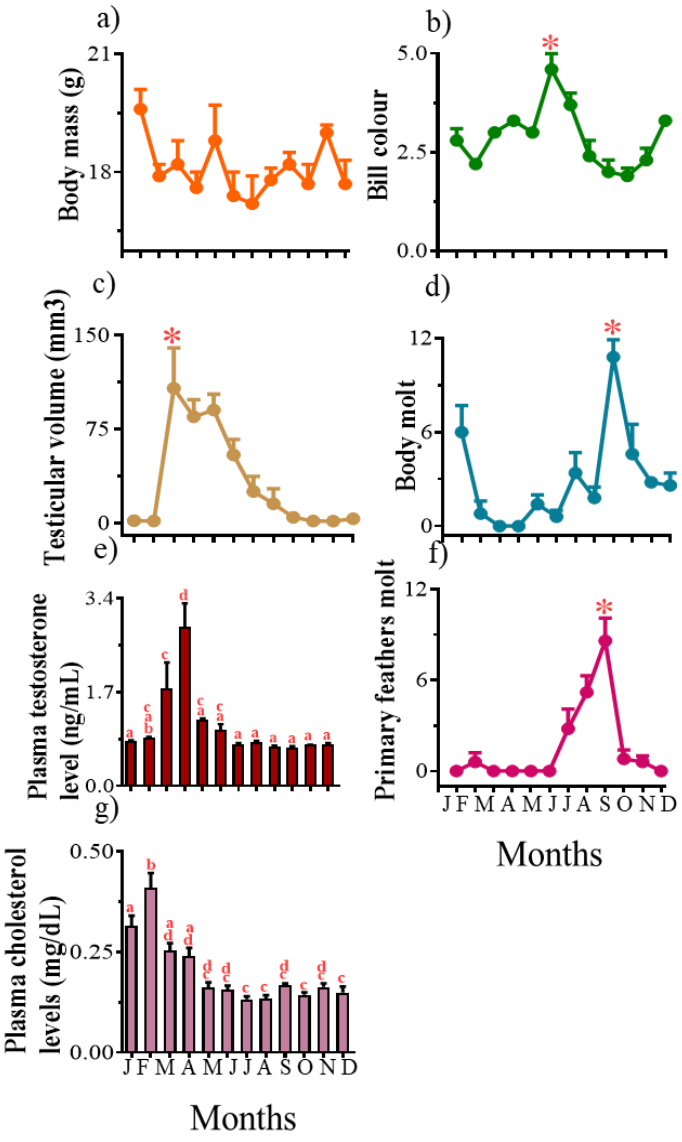

Fig. 29. Data is represented as mean (± SE, n = 5/month). Parameters (a) body mass (b) bill color, (c) testicular volume, (d) Body molt, (e) Plasma testosterone levels (f) molt in primary flight feathers, and (g) plasma cholesterol levels from adult male tree sparrows collected every month for 12 months. * and lower case alphabets indicate significant differences ($P < 0.05$) among the months.

Transcripts known to be involved in seasonal reproduction were measured in the hypothalamus of male adult tree sparrows. All the transcripts studied had annual variation in the hypothalamic tissue; *Tshβ* ($P<0.0001$; One Way ANOVA; Table 6b; Fig. 30a), *Dio2* ($P<0.0001$; One Way ANOVA; Table 6b; Fig. 30d), *GnRH* ($P<0.0001$; One Way ANOVA; Table 6b; Fig. 30e), *Dio3* ($P=0.0011$; One Way ANOVA; Table 6b; Fig. 30b), and *GnIH* ($P<0.0001$; One Way ANOVA; Table 6b; Fig. 30c). Maximum *Tshβ* expression was observed during March ($P<0.05$; Bonferroni's multiple comparison test; Fig. 30a), while the levels were minimum from May onwards ($P<0.05$; Bonferroni's multiple comparison test; Fig. 30a). *Dio2* levels started elevated by March with peak expression during April and May ($P<0.05$; Bonferroni's multiple comparison test; Fig. 30d). Levels were minimum during June and these low levels were maintained rest of the year ($P<0.05$; Bonferroni's multiple comparison test; Fig. 30d). *GnRH* expression levels corresponded with *Dio2*, and elevated mRNA levels were observed in March with peak expression during April, subsequent fall in transcription levels during June and low levels were observed rest of the year ($P<0.05$; Bonferroni's multiple comparison test; Fig. 30e). *Dio3* and *GnIH* levels were in antiphase of *Tshβ*, *Dio2*, and *GnRH* expression (Fig. 30). Higher levels of *Dio3* were observed in July till January, with maximum expression during October till January ($P<0.05$; Bonferroni's multiple comparison test; Fig. 30b). Similarly, higher *GnIH* levels were observed during September till January ($P<0.05$; Bonferroni's multiple comparison test; Fig. 30c).

Table 6b. Values of One-way ANOVA of the monthly expressions of steroidogenic genes in male tree sparrows

Transcripts	Hypothalamus
Tshβ	$F_{(11, 36)} = 15.65$, $P<0.0001$
Dio2	$F_{(11, 36)} = 12.22$, $P<0.0001$
Dio3	$F_{(11, 27)} = 4.233$, $P= 0.0011$
GnRH	$F_{(11, 35)} = 7.208$, $P<0.0001$
GnIH	$F_{(11, 36)} = 9.413$, $P<0.0001$

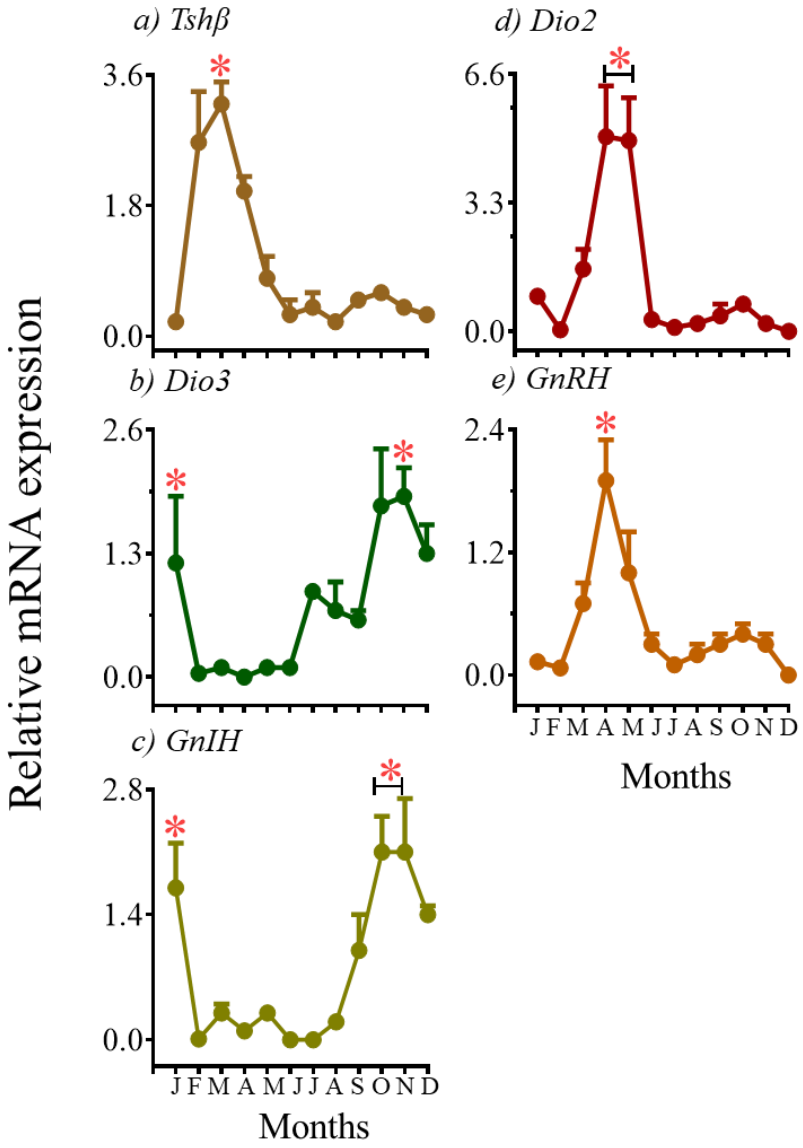

Fig. 30. Data are represented as mean (± SE, n = 5/month). Relative mRNA levels of reproductive genes in the hypothalamic tissues of adult male tree sparrows were collected every month for 12 months. One-way ANOVA was followed by Tukey's multiple comparison test to determine significant differences. * indicates significant differences ($P < 0.05$) among the months.

Hypothalamic expression of steroidogenic markers had seasonal changes (Fig. 31). qPCR analysis of these transcripts in hypothalamus, revealed seasonal fluctuations in the expression levels of *StAR* (P=0.0036; One Way ANOVA; Table 6c; Fig. 31a), *Scp2* (P<0.0001; One Way ANOVA; Table 6c; Fig. 31b), *Cyp11a1* (P=0.0020; One Way ANOVA; Table 6c; Fig. 31c), *Nr4a1* (P<0.0001; One Way ANOVA; Table 6c; Fig. 31d), *Cyp11b* (P<0.0001; One Way ANOVA; Table 6c; Fig. 31e), *Cyp17a1* (P=0.0003; One Way ANOVA; Table 6c; Fig. 31f), *Srd5a1*(P=0.0022; One Way ANOVA; Table 6c; Fig. 31g), *Hsd11b2* (P=0.0087; One Way ANOVA; Table 6c; Fig. 31h), *Er* (P<0.0001; One Way ANOVA; Table 6c; Fig. 31i), and *Vdac1* (P=0.0045; One Way ANOVA; Table 6c; Fig. 31j). Maximum expression levels of *StAR* and *Scp2* transcripts were observed in the month of April (P<0.05; Bonferroni's multiple comparison test; Fig. 31) while levels were low at other months of the year (P<0.05; Bonferroni's multiple comparison test; Fig. 31). Higher expression of *Cyp11a1* was observed prior to breeding phase i.e., January till March while levels were low at other times of the year (P<0.05; Bonferroni's multiple comparison test; Fig. 31c). Expression levels of *Nr4a1*, *Cyp11b*, and *Vdac1* peaked during March while levels were low during the other times of the year (P<0.05; Bonferroni's multiple comparison test; Fig. 31). In the hypothalamus, peak expression of *Cyp17a1* was observed in the month of January (P<0.05; Bonferroni's multiple comparison test; Fig. 31f). Multiple peaks in expression levels of *Srd5a1* were observed throughout the year (Fig. 31g). However, amplitude of peaks was lower during post breeding phase (P<0.05; Bonferroni's multiple comparison test; Fig. 31g). *Hsd11b2* expression was maximum in the month of March while levels were low at other times of the year (P<0.05; Bonferroni's multiple comparison test; Fig. 31h). Higher levels of *Er* were observed from January till April, with peaked expression during January and February in the hypothalamus (P<0.05; Bonferroni's multiple comparison test; Fig. 31i).

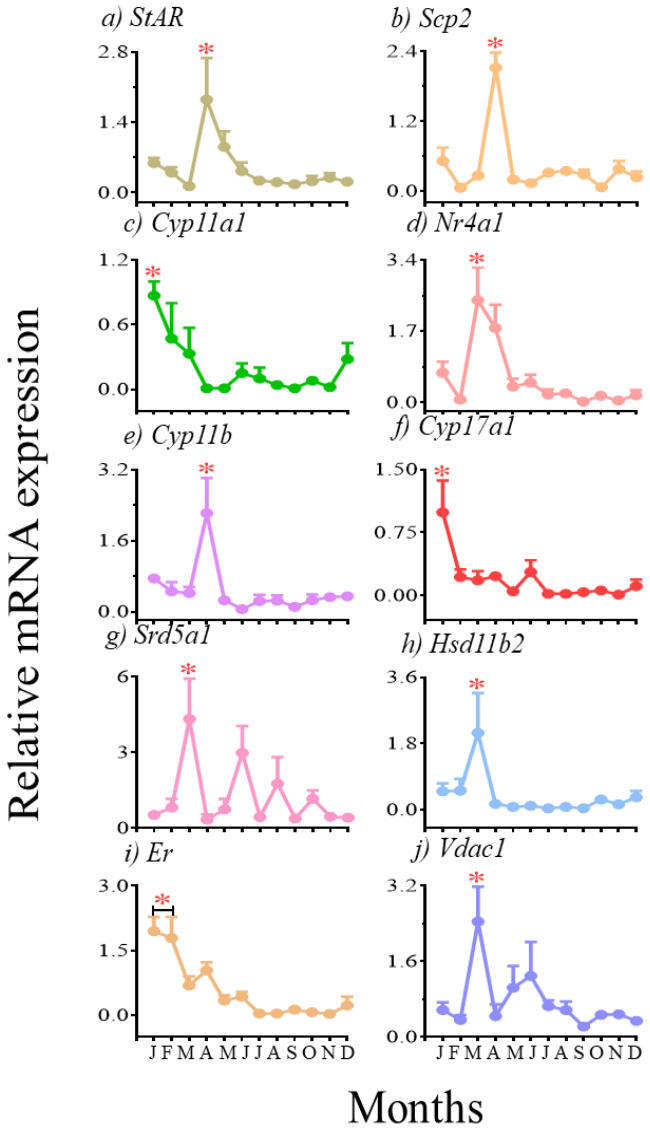

Fig. 31. Data is represented as mean (± SE, n = 5/month). Relative mRNA levels of steroidogenic transcripts in the hypothalamus of adult male tree sparrows were collected every month for 12 months. One-way ANOVA was followed by Tukey's multiple comparison test to determine significant differences. * indicates significant differences ($P < 0.05$) among the months.

Seasonal variation in the expression levels of *StAR* (P<0.0001; One Way ANOVA; Table 6c; Fig. 32a), *Scp2* (P<0.0001; One Way ANOVA; Table 6c; Fig. 32b), *Cyp11a1* (P<0.0001; One Way ANOVA; Table 6c; Fig. 32c), *Nr4a1* (P<0.0001; One Way ANOVA; Table 6c; Fig. 32d), *Cyp11b* (P=0.0007; One Way ANOVA; Table 6c; Fig. 32e), *Cyp17a1* (P<0.0001; One Way ANOVA; Table 6c; Fig. 32f), *Srd5a1*(P=0.0018; One Way ANOVA; Table 6c; Fig. 32g), *Hsd11b2* (P< 0.0001; One Way ANOVA; Table 6c; Fig. 32h), *Er* (P=0.0026; One Way ANOVA; Table 6c; Fig. 32i), and *Vdac1* (P<0.0001; One Way ANOVA; Table 6c; Fig. 32j) was also reflected in testes of tree sparrows (Fig. 32). In testis, *StAR*, *Scp2*, *Nr4a1*, and *Cyp17a1* peaked in May (P<0.05; Bonferroni's multiple comparison test; Fig. 32). Both *Hsd11b2* and *Er* peaked during April (Fig 32). However, we also observed a second peak in *Er* transcripts during post-breeding in September (Fig. 32i). Expression levels of *Cyp11a1* began to rise from February, and peak expression was observed during June (P<0.05; Bonferroni's multiple comparison tests; Fig. 32c). *Srd5a1* and *Vdac1* peaked during February (P<0.05; Bonferroni's multiple comparison tests; Fig. 32g, j), while *Cyp11b* peaked during post-breeding during October (P<0.05; Bonferroni's multiple comparison test; Fig. 32e).

Table 6c. Values of One-way ANOVA of the monthly expressions of steroidogenic genes in male tree sparrows

Transcripts	Hypothalamus	Testis
StaR	$F_{11,36}$= 3.254, P=0.0036	$F_{11,43}$= 8.100, P<0.0001
Scp2	$F_{11,36}$= 21.37, P<0.0001	$F_{11,43}$= 5.546, P<0.0001
Cyp11a1	$F_{11,36}$= 3.539, P=0.0020	$F_{11,43}$= 5.038, P<0.0001
Nr4a1	$F_{11,31}$= 7.037, P<0.0001	$F_{11,43}$= 24.10, P<0.0001
Cyp11b	$F_{11,35}$= 5.474, P<0.0001	$F_{11,35}$= 4.047, P=0.0007
Cyp17a1	$F_{11,36}$= 4.425, P=0.0003	$F_{11,43}$= 5.524, P<0.0001
Srd5a1	$F_{11,35}$= 3.513, P=0.0022	$F_{11,35}$= 3.604, P=0.0018
Hsd11b2	$F_{11,36}$= 2.850, P=0.0087	$F_{11,43}$= 8.060, P<0.0001
Er	$F_{11,36}$= 10.93, P<0.0001	$F_{11,41}$= 3.303, P=0.0026
Vdac1	$F_{11,36}$= 3.153, P=0.0045	$F_{11,35}$= 5.462, P<0.0001

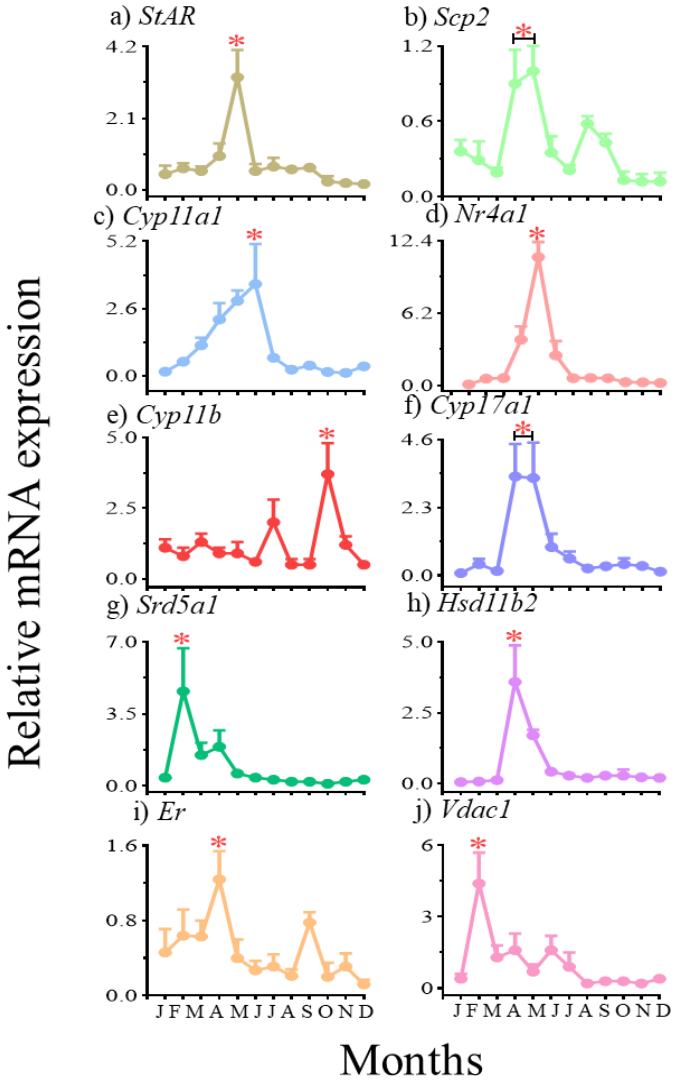

Fig. 32. Data are represented as mean (± SE, n = 5/month). Relative mRNA levels of steroidogenic in the testis of adult male tree sparrows were collected every month for 12 months. One-way ANOVA followed by Tukey's multiple comparison test if ANOVA showed significant differences. * indicates significant differences ($P < 0.05$) among the months.

A correlation of reproductive gene transcript with steroidogenic markers was observed in male tree sparrows. A positive correlation was observed between hypothalamic *Tshβ* with *Nr4a1*(r = 0.6832, p = 0.0146; Fig. 33b) and *Hsd11b2* (r= 0.7251, p= 0.0076; Fig. 33h), where the mRNA expression of *Tshβ* is directly proportional to the mRNA expression level of *Nr4a1* and *Hsd11b2*. While a negative correlation was observed between *Tshβ* and *Cyp17a1* expression (r= -0.02581, p=

0.9365; Fig. 33f). However, no correlation was observed between *Tshβ* with *StaR* (r= 0.2200, p= 0.4921; Fig. 33a), *Scp2* (r= 0.2194, p= 0.4933; Fig. 33c), *Vdac1* (r= 0.4934, p= 0.1030; Fig. 33d), *Cyp11a1* (r= 0.1562, p= 0.6278; Fig. 33e), *Cyp11b* (r= 0.3728, p= 0.2326; Fig. 33g), *Er* (r= 0.4503, p= 0.1419; Fig. 33i), and *Srd5a1* (r= 0.4280, p= 0.1652; Fig. 33j).

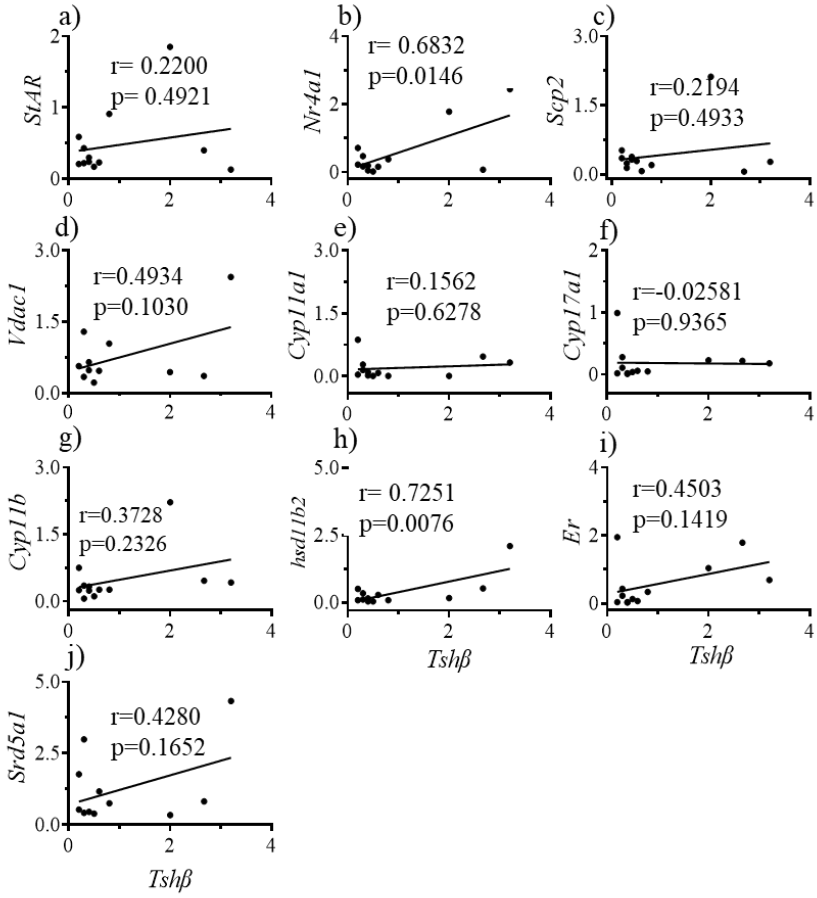

Fig. 33. Correlation of hypothalamic *Tshβ* expression with hypothalamic expression of *StAR, Scp2, Cyp11a1, Nr4a1, Cyp11b, Cyp17a1, Hsd11b2, Er,* and *Vdac1*. Significant differences were taken at $p<0.05$. For correlation, r=1 is a positive correlation, r>1 is a negative correlation.

In the testicular tissue, a positive correlation was observed between the hypothalamic *Tshβ* with *Vdac1* (r= 0.6819, p= 0.0146; Fig. 34d) and *Srd5a1* (r=

0.7869, p= 0.0024; Fig. 34j). While, a negative correlation was observed in *Cyp11b* (r= -0.02702, p= 0.9336; Fig. 34g). However, no correlation was observed in transcript expression of *Tshβ* with *StAR* (r= 0.06390, p= 0.8436; Fig. 34a), *Nr4a1* (r= 0.03838, p= 0.9057; Fig. 34b), *Scp2* (r=0.06560, p=0.8395; Fig. 34c), *Cyp11a1* (r= 0.1285, p= 0.6907; Fig. 34e), *Cyp17a1* (r= 0.1589, p= 0.02525; Fig. 34f), *Hsd11b2* (r= 0.2277, p= 0.4766; Fig. 34h), and *Er* (r= 0.5938, p= 0.0418; Fig. 34i).

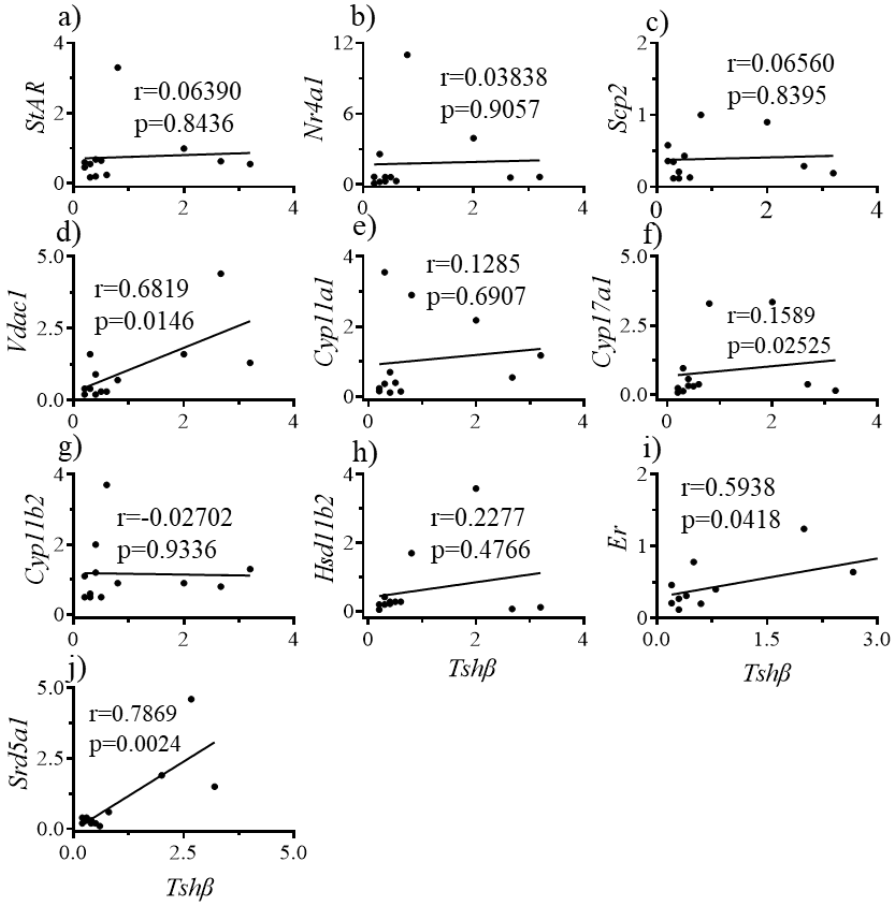

Fig. 34. Correlation of hypothalamic *Tshβ* expression with testicular expression of *StAR, Scp2, Cyp11a1, Nr4a1, Cyp11b, Cyp17a1, Hsd11b2, Er,* and *Vdac1*. Significant differences were taken at $p<0.05$. For correlation, r=1 is a positive correlation, r>1 is a negative correlation.

A significant positive correlation in the hypothalamus was observed for reproductive gene *Dio2* with *StAR* (r= 0.8436, p= 0.0006; Fig. 35a), *Scp2* (r= 0.6297, p= 0.0282; Fig. 35c) and *Cyp11b* (r=0.6349, p=0.0265; Fig. 35g). Further, negative

correlation was observed for *Dio2* with *Cyp11a1* (r= -0.2473, p= 0.4383; Fig. 35e) and *Srd5a1* (r= -0.08245, p= 0.7989; Fig. 35j). Whereas, no correlation was observed in expression level of *Dio2* with *Nr4a1* (r= 0.5040, p= 0.0948; Fig. 35b), *Vdac1* (r= 0.1803, p= 0.5750; Fig. 35d), *Cyp17a1* (r= 0.01703, p= 0.9581; Fig. 35f), *Hsd11b2* (r= 0.004924, p= 0.9871; Fig. 35h) and *Er* (r= 0.1430, p= 0.6574; Fig. 35i).

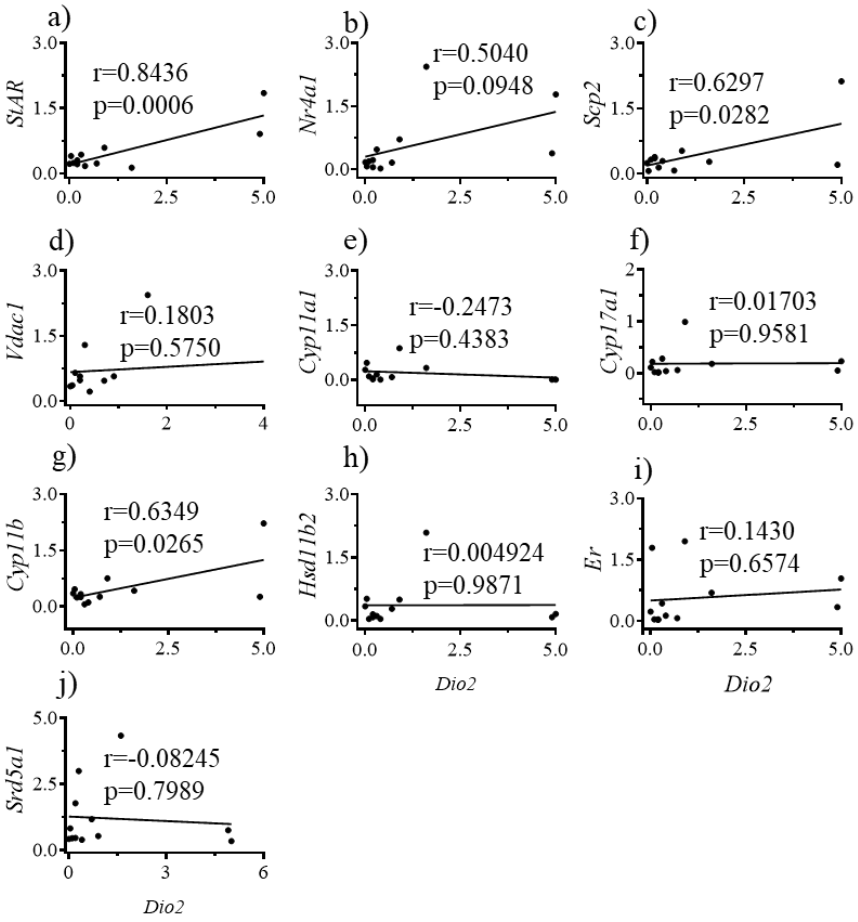

Fig. 35. Correlation of hypothalamic *Dio2* expression with hypothalamic expression of *StAR, Scp2, Cyp11a1, Nr4a1, Cyp11b, Cyp17a1, Hsd11b2, Er,* and *Vdac1*. Significant differences were taken at p<0.05. For correlation, r=1 is a positive correlation, r>1 is a negative correlation.

Further, a positive correlation was observed between the hypothalamic expression of *Dio2* with testicular expression of *StAR* (r=0.7501, p=0.0050; Fig. 36a), *Nr4a1* (r= 0.8130, p= 0.0013; Fig. 36b), *Scp2* (r= 0.8420, p= 0.0006; Fig. 36c), *Cyp11a1* (r= 0.5822, p= 0.0470; Fig. 36e), *Cyp17a1* (r= 0.9330, P< 0.0001; Fig. 36f)

and *Hsd11b2* (r= 0.8840, p= 0.0001; Fig. 36h). Whereas, a negative correlation was observed between *Dio2* and *Cyp11b* (r= -0.06679, p= 0.8366; Fig. 36g), however, no correlation was observed between *Dio2* with *Er* (r= 0.5740, p= 0.0510; Fig. 36i) and *Srd5a1* (r= 0.1154, p= 0.7211; Fig. 36j).

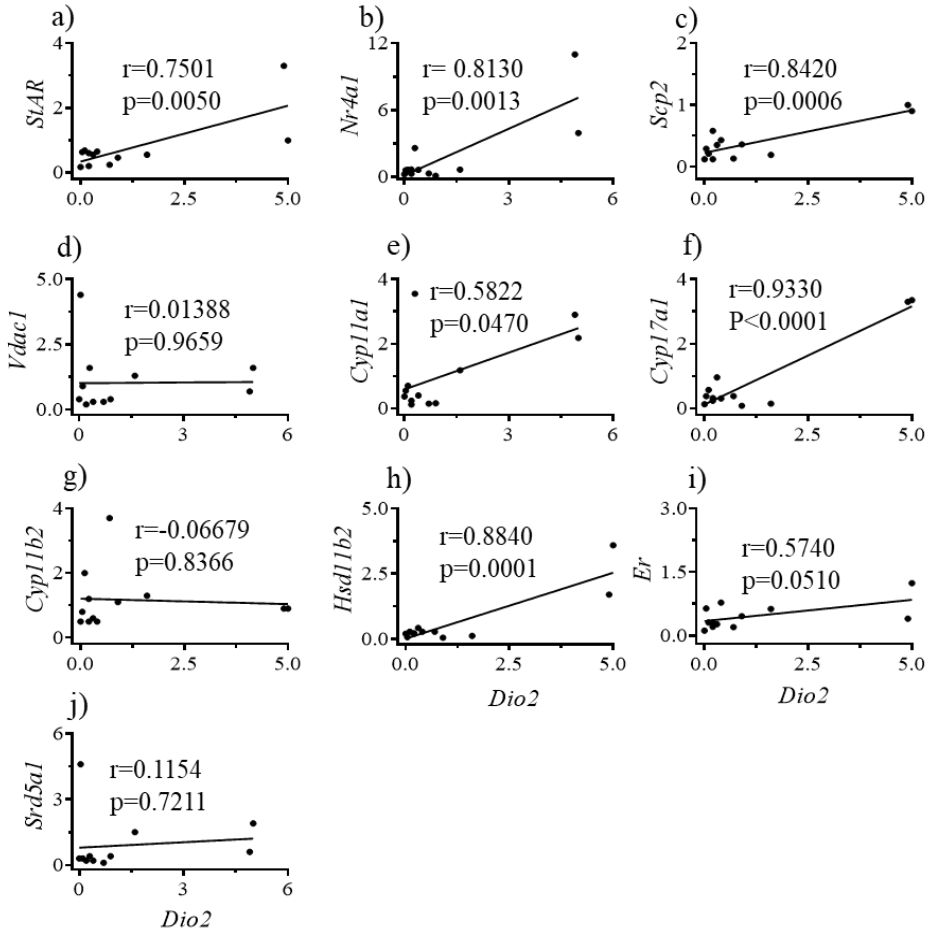

Fig. 36. Correlation of hypothalamic *Dio2* expression with testicular expression of *StAR, Scp2, Cyp11a1, Nr4a1, Cyp11b, Cyp17a1, Hsd11b2, Er,* and *Vdac1*. Significant differences were taken at p<0.05. For correlation, r=1 is a positive correlation, r>1 is a negative correlation.

Also, we have observed a significant negative correlation of the inhibitory reproductive gene *Dio3* with hypothalamic expression of *StAR* (r= -0.4127, p= 0.1825; Fig. 37a), *Nr4a1* (r= -0.4663, p= 0.1265; Fig. 37b), *Scp2* (r= -0.2504, p= 0.4326; Fig. 37c), *Vdac1* (r= -0.4194, p= 0.1747; Fig. 37d), *Cyp17a1* (r= -0.01732, p= 0.9574; Fig.

37f), *Cyp11b* (r= -0.2629, p=0.4090; Fig. 37g), *Hsd11b2* (r= -0.2307, p= 0.4707; Fig. 37h), *Er* (r=-0.3372, p=0.2837; Fig. 37i) and *Srd5a1* (r=-0.3788, p=0.2246; Fig. 37j).

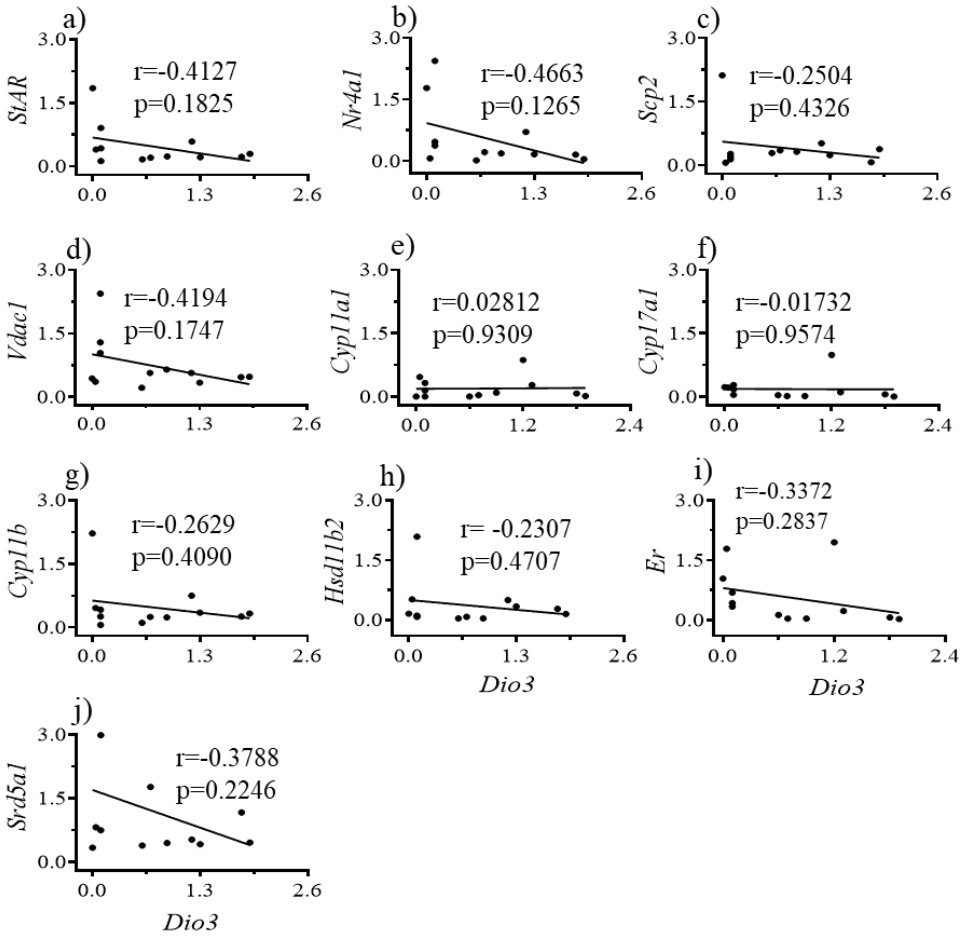

Fig. 37. Correlation of hypothalamic *Dio3* expression with hypothalamic expression of *StAR, Scp2, Cyp11a1, Nr4a1, Cyp11b, Cyp17a1, Hsd11b2, Er,* and *Vdac1*. Significant differences were taken at $p<0.05$. For correlation, r=1 is a positive correlation, r>1 is a negative correlation.

Similarly, a significant negative correlation was observed between hypothalamic expression of *Dio3* with testicular expression of *StAR* (r= -0.4854, p= 0.1097; Fig. 38a), *Nr4a1* (r= -0.4790, p= 0.1152; Fig. 38b), *Scp2* (r= -0.5912, p= 0.0429; Fig. 38c), *Vdac1* (r= -0.5935, p= 0.0419; Fig. 38d), *Cyp11a1* (r= -0.6771, p= 0.0156; Fig. 38e), *Cyp17a1* (r= -0.5019, p= 0.0964; Fig. 38f), *Hsd11b2* (r=-0.4263, p=0.1670; Fig. 38h), *Er* (r= -0.5583, p= 0.0592; Fig. 38i) and *Srd5a1* (r= -0.5454, p=

0.2975; Fig. 38j). However, no correlation was observed for Cyp11b (r= 0.5011, p= 0.0970; Fig. 38g).

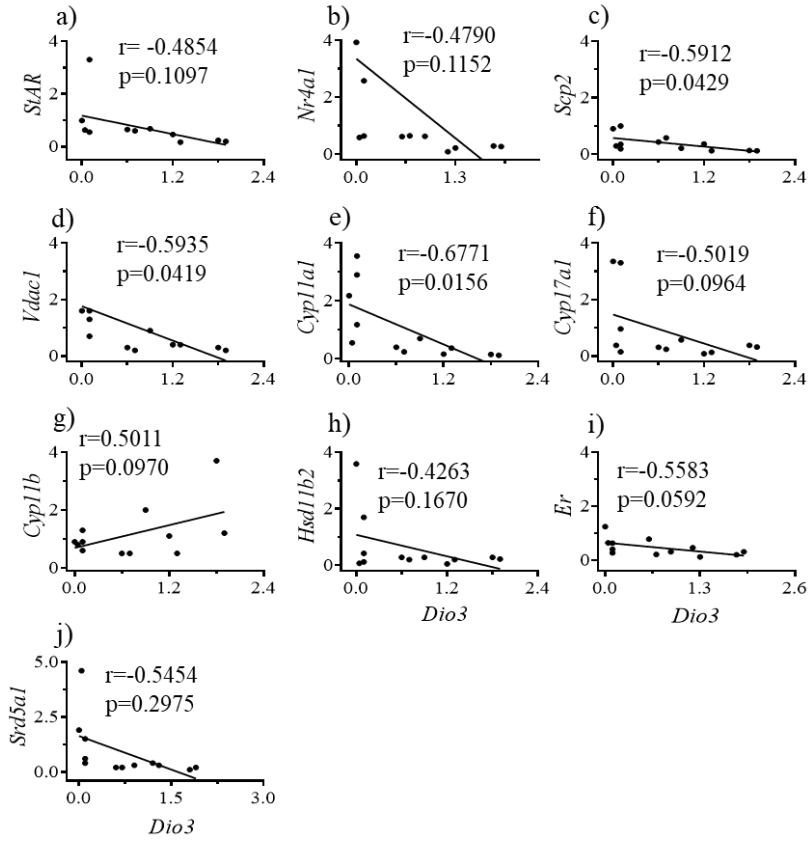

Fig. 38. Correlation of hypothalamic *Dio3* expression with testicular expression of *StAR, Scp2, Cyp11a1, Nr4a1, Cyp11b, Cyp17a1, Hsd11b2, Er,* and *Vdac1*. Significant differences were taken at $p<0.05$. For correlation, r=1 is a positive correlation, r>1 is a negative correlation.

Further, a significant positive correlation was observed between hypothalamic expression of *GnRH* with hypothalamic expression of *StAR* (r= 0.8690, p= 0.0002; Fig. 39a), *Nr4a1* (r= 0.6325, p= 0.0273; Fig. 39b), *Scp2* (r= 0.8048, p= 0.0006; Fig. 39c) and *Cyp11b* (r= 0.6325, p= 0.0273; Fig. 39g). Whereas, a negative correlation was observed in hypothalamic *GnRH* with *Cyp11a1* (r=-0.3637, p=0.2452; Fig. 39e), and *Cyp17a1* (r= -0.06780, p= 0.8342; Fig. 39f). However, no correlation was observed for *Vdac1* (r=0.1807, p=0.5742; Fig. 39d), *Hsd11b2* (r= 0.05806, p= 8578;

Fig. 39h), *Er* (r= 0.08537, p= 0.7919; Fig. 39i) and *Srd5a1* (r= 0.01127, p= 0.9723; Fig. 39j).

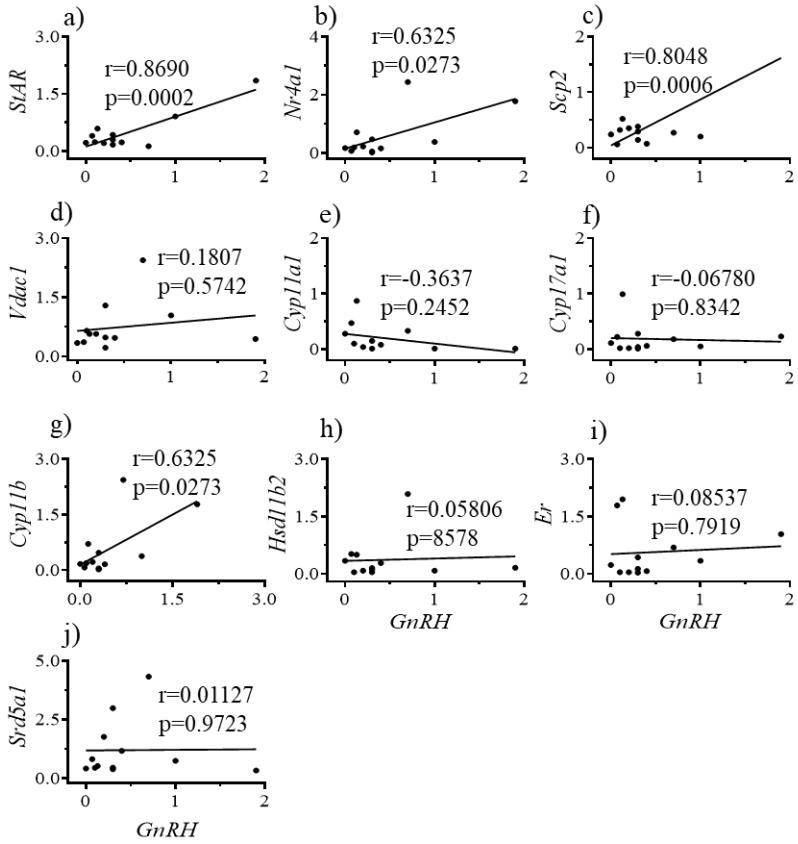

Fig. 39. Correlation of hypothalamic *GnRH* expression with hypothalamic expression of *StAR, Scp2, Cyp11a1, Nr4a1, Cyp11b, Cyp17a1, Hsd11b2, Er,* and *Vdac1*. Significant differences were taken at p<0.05. For correlation, r=1 is a positive correlation, r>1 is a negative correlation.

Similarly, a significant positive correlation was observed between hypothalamic expression of *GnRH* with testicular expression of *Nr4a1* (r= 0.5782, p= 0.0489; Fig. 40b), *Scp2* (r= 0.7208, p= 0.0082; Fig. 40c), *Cyp17a1* (r= 0.8553, p= 0.0004; Fig. 40f), *Hsd11b2* (r= 0.9393, P< 0.0001; Fig. 40h), *Er* (r= 0.7253, p= 0.0076; Fig. 40i) and *Srd5a1* (r= 0.1527, p= 0.6356; Fig. 40j). Whereas, a negative correlation was observed with *Cyp11b* (r= -0.02070, p= 0.9491; Fig. 40g) and no correlation with *StAR* (r= 0.4692, p= 0.1239; Fig. 40a), *Vdac1* (r= 0.05072, p= 0.8756; Fig. 40d), and *Cyp11a1* (r= 0.5305, p= 0.0760; Fig. 40e).

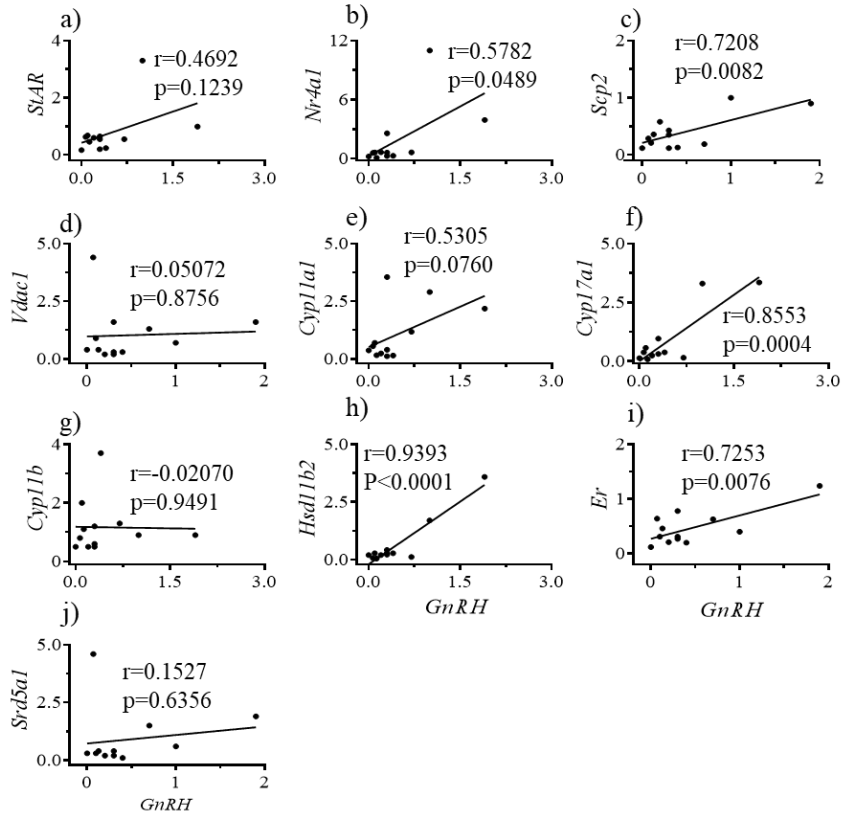

Fig. 40. Correlation of hypothalamic *GnRH* expression with testicular expression of *StAR, Scp2, Cyp11a1, Nr4a1, Cyp11b, Cyp17a1, Hsd11b2, Er,* and *Vdac1*. Significant differences were taken at $p<0.05$. For correlation, r=1 is a positive correlation, r>1 is a negative correlation.

Further, we have also measured the correlation of the reproductive gene *GnIH* with hypothalamic steroidogenic gene expressions. A significant negative correlation was observed between *GnIH* with *StAR* (r= -0.2712, p= 0.3938; Fig. 41a), *Nr4a1* (r= -0.3008, p= 0.3420; Fig. 41b), *Scp2* (r= -0.1808, p= 0.5740; Fig. 41c), *Vdac1* (r= -0.3405, p= 0.2788; Fig. 41d), *Cyp11b* (r= -0.1471, p= 0.6483; Fig. 41g), *Hsd11b2* (r= -0.07813, p= 0.8093; Fig. 41h), *Er* (r= -0.1245, p= 0.6999; Fig. 41i), and *Srd5a1* (r= -0.3310, p= 0.2934; Fig. 41j). However, no correlation was observed in *Cyp11a1* (r= 0.1613, p= 0.6166; Fig. 41e) and Cyp17a1 (r= 0.1696, p= 0.5983; Fig. 41f).

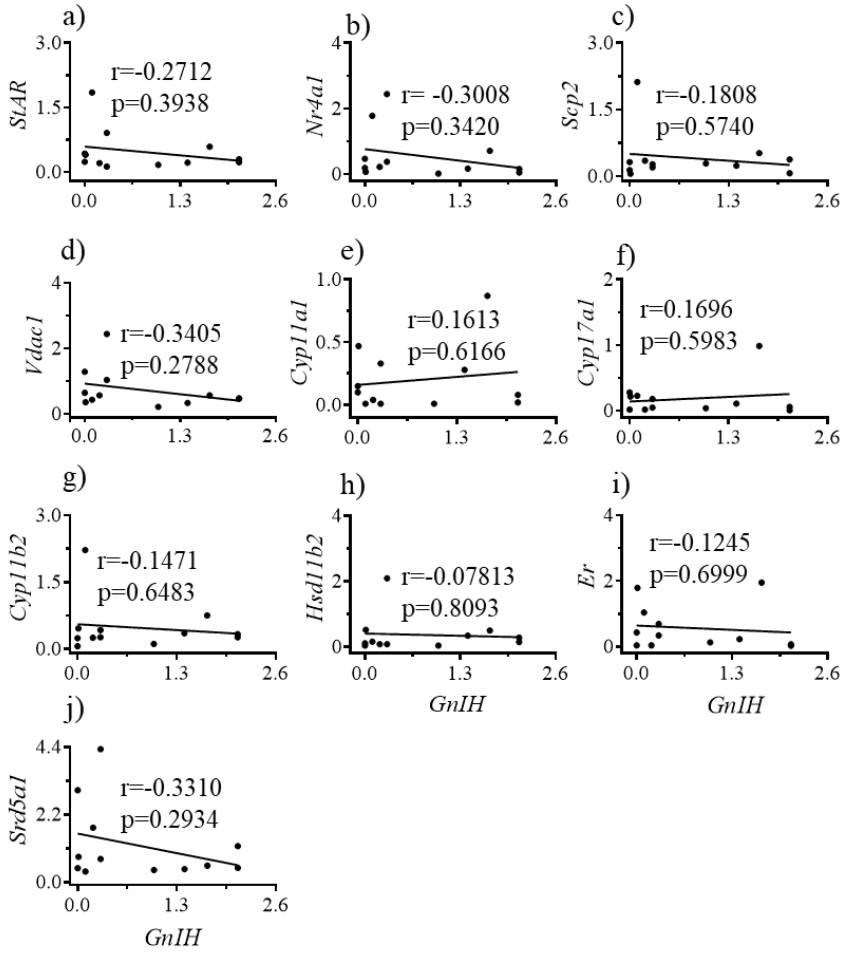

Fig. 41. Correlation of hypothalamic *GnIH* expression with hypothalamic expression of *StAR, Scp2, Cyp11a1, Nr4a1, Cyp11b, Cyp17a1, Hsd11b2, Er,* and *Vdac1*. Significant differences were taken at $p < 0.05$. For correlation, r= 1 indicates a positive correlation, while r > 1 indicates a negative correlation.

Similarly, a significant negative correlation was observed between hypothalamic expression of *GnIH* with testicular expression of *StAR* (r= -0.3803, p= 0.2226; Fig. 42a), *Nr4a1* (r= -0.3539, p= 0.2591; Fig. 42b), *Scp2* (r= -0.4688, p= 0.1242; Fig. 42c), *Vdac1* (r= -0.5462, p= 0.0662; Fig. 42d), *Cyp11a1* (r= -0.5784, p= 0.0488; Fig. 42e), *Cyp17a1* (r= -0.3911, p= 0.2087; Fig. 42f), *Hsd11b2* (r= -0.3174, p= 0.3148; Fig. 42h), *Er* (r= -0.3378, p= 0.2828; Fig. 42i) and *Srd5a1* (r= -0.4470, p= 0.1451; Fig. 42j). However, no correlation was observed between *Dio2* and *Cyp11b* (r= 0.4122, p= 0.1831; Fig. 42g).

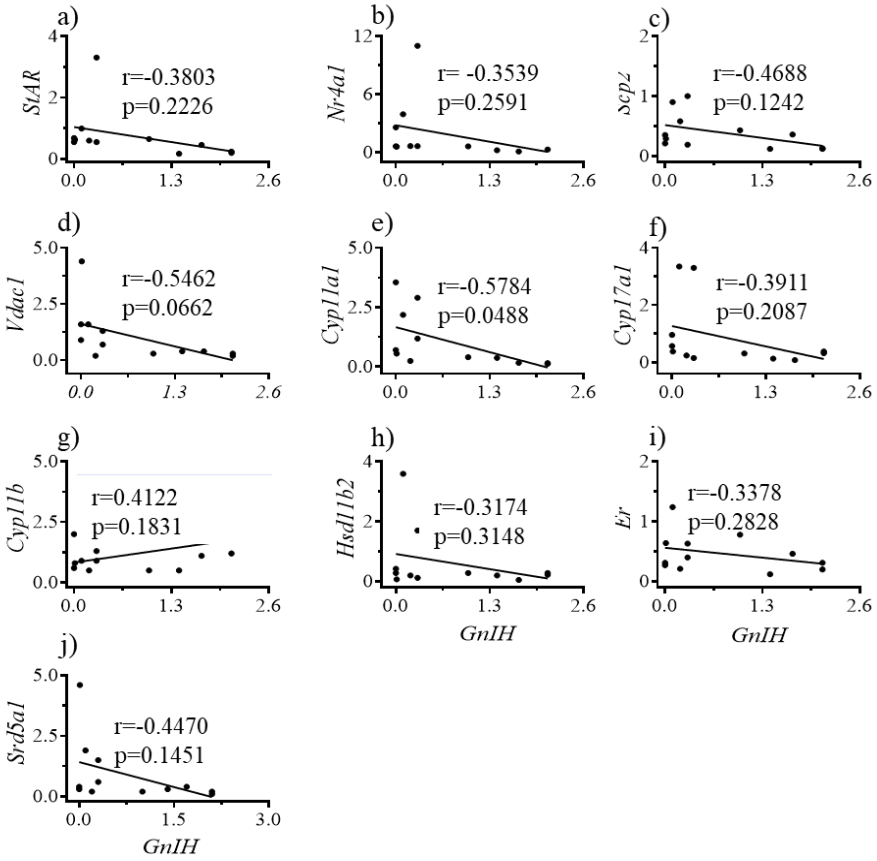

Fig. 42. Correlation of hypothalamic *GnIH* expression with testicular expression of *StAR, Scp2, Cyp11a1, Nr4a1, Cyp11b, Cyp17a1, Hsd11b2, Er,* and *Vdac1*. Significant differences were taken at $p < 0.05$. For correlation, r=1 is a positive correlation, r>1 is a negative correlation.

The study also examined the physiological changes and seasonal variations in the steroidogenic genes in the adult female tree sparrows. In females, no significant change in the body mass was observed throughout the year (P=0.1507; One Way ANOVA; Table 6d; Fig. 43a). Annual variations in bill color (P<0.0001; One Way ANOVA; Table 6d; Fig. 43b), molt in body feathers (P<0.0001; One Way ANOVA; Table 6d; Fig. 43f), primary flight feathers (P=0.0051; One Way ANOVA; Table 6d; Fig. 43g), and follicular diameter (P<0.0001; One Way ANOVA; Table 6d; Fig. 43e) were also observed. Higher bill color score was observed from April till June (P<0.0001; Tukey's multiple comparison tests; Table 6d; Fig. 43b), having the highest score during June (P<0.05; Tukey's multiple comparison tests; Fig. 43b). Molt in body feathers begin in July and completed in January (P<0.0001; One Way ANOVA; Table 6d; Fig. 43f) and primary flight feathers start at July and completed in October (P=0.0051; Tukey's multiple comparison tests; Table 6d; Fig. 43g) with both having maximum molt during August and September (P<0.05; Tukey's multiple comparison tests; Table 6d; Fig. 43f, g). Significantly higher follicular sizes were observed from April till June, began to regress in July, and fully regressed from August onwards (P<0.0001; One Way ANOVA; Table 6d; Fig. 43e). Plasma Cholesterol levels also showed seasonal fluctuation (P=0.0005; One Way ANOVA; Table 6d; Fig. 43c). Elevated plasma cholesterol levels were observed in February and April, were significantly higher in February (P<0.05; Tukey's multiple comparison tests; Table 6d; Fig. 43c), and started to decline from May onwards (Fig. 43c). Minimum cholesterol levels were observed during June till September (P<0.05; Tukey's multiple comparison tests; Table 6d; Fig. 43c). Annual changes in plasma progesterone levels were also observed (P<0.0001; One Way ANOVA; Table 6d; Fig. 43e). Plasma progesterone levels started to elevate in March, maximum levels were observed during June (P<0.05; Tukey's multiple comparison tests; Table 6d; Fig. 43d), and began to decline from July onwards (Fig. 43d).

Table 6d. Values of One-way ANOVA of the monthly physiological parameters in female tree sparrows

Body mass	$F_{(11, 45)} = 1.541$, P=0.1507
Bill color	$F_{(11, 45)} = 16.33$, P<0.0001

Follicular diameter	$F_{(11, 45)} = 14.57$, $P<0.0001$
Plasma progesterone level	$F_{(11, 48)} = 4.267$, $P=0.0002$
Plasma cholesterol level	$F_{(11, 100)} = 3.376$, $P=0.0005$
Bod ymolt	$F_{(11, 46)} = 6.801$, $P<0.0001$
Primary feather molt	$F_{(11, 45)} = 2.947$, $P=0.0051$

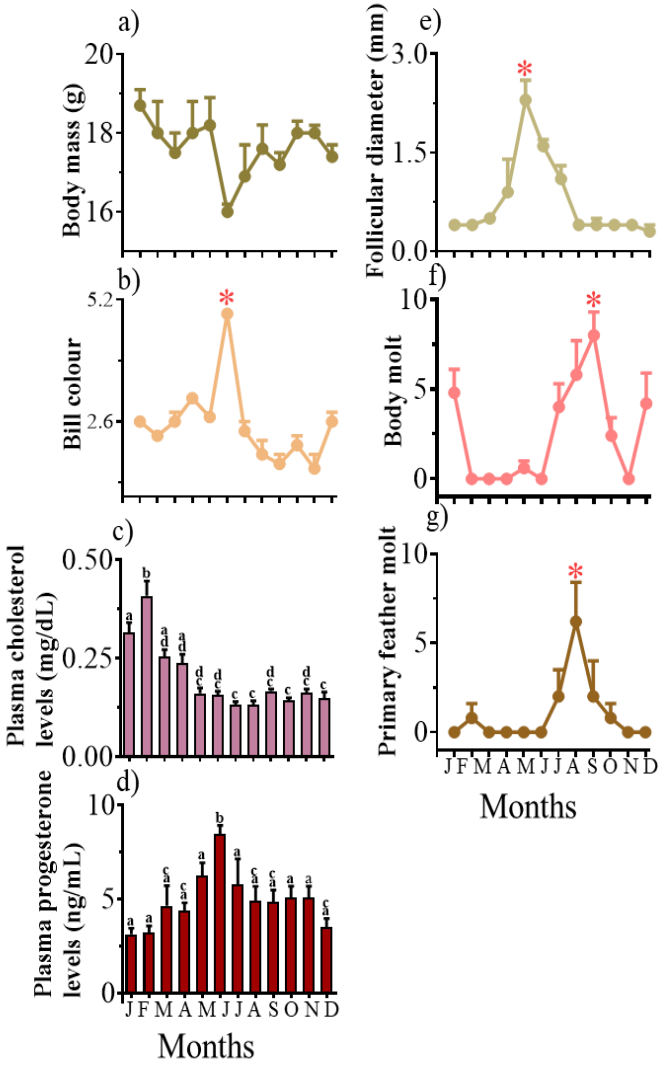

Fig. 43. Data is represented as mean (± SE, n = 5/month). Parameters (a) body mass (b) bill color, (c) plasma cholesterol levels, (d) Plasma progesterone, (e) Follicular diameter (f) Body molt, and (g) molt in primary flight feathers from adult female tress sparrows collected every month for 12 months. * and lower case alphabets indicate significant differences ($P < 0.05$) among the months.

Transcripts known to be involved in seasonal reproduction were measured in the hypothalamus of male adult tree sparrows. All the transcripts studied had annual variation in the hypothalamic tissue; *Tshβ* ($P<0.0001$; One Way ANOVA; Table 6e; Fig. 44a), *Dio2* ($P<0.0001$; One Way ANOVA; Table 6e; Fig. 44e), *GnRH* ($P<0.0001$; One Way ANOVA; Table 6e; Fig. 44d), *Dio3* ($P=0.0210$; One Way ANOVA; Table 6e;

Fig. 44b), and *GnIH* (P=0.0008; One Way ANOVA; Table 6e; Fig. 44c). Maximum *Tshβ* expression was observed during May (P<0.05; Tukey's multiple comparison test; Table 6e; Fig. 44a), while the levels were minimum from June onwards (P<0.05; Tukey's multiple comparison test; Fig. 44a). *Dio2* levels started elevated by February with peak expression during April and May (P<0.05; Tukey's multiple comparison test; Table 6e; Fig. 44e). Levels were minimum during June and these low levels were maintained rest of the year (P<0.05; Tukey's multiple comparison test; Fig. 44e). *GnRH* expression levels corresponded *Dio2*, and elevated mRNA levels were observed in March with peak expression during April, subsequent fall in transcription levels during May and low levels were observed rest of the year (P<0.05; Tukey's multiple comparison test; Table 6e; Fig. 44d). *Dio3* and *GnIH* levels were in antiphase of *Tshβ*, *Dio2*, and *GnRH* expression (Fig. 44). Higher levels of *Dio3* were observed in October till January, with maximum expression during November till January (P<0.05; Tukey's multiple comparison test; Table 6e; Fig. 44b). Similarly, higher *GnIH* levels were observed in January during pre-breeding phase (P<0.05; Tukey's multiple comparison test; Table 6e; Fig. 44c).

Table 6e. Values of One-way ANOVA of the monthly expressions of reproductive genes in female tree sparrows

Transcripts	Hypothalamus
Tshβ	$F_{11,36} = 5.627$, P<0.0001
Dio2	$F_{11,36} = 26.95$, P<0.0001
Dio3	$F_{11,36} = 2.452$, P<0.0001
GnRH	$F_{11,36} = 10.62$, P<0.0001
GnIH	$F_{11,84} = 3.332$, P=0.0008

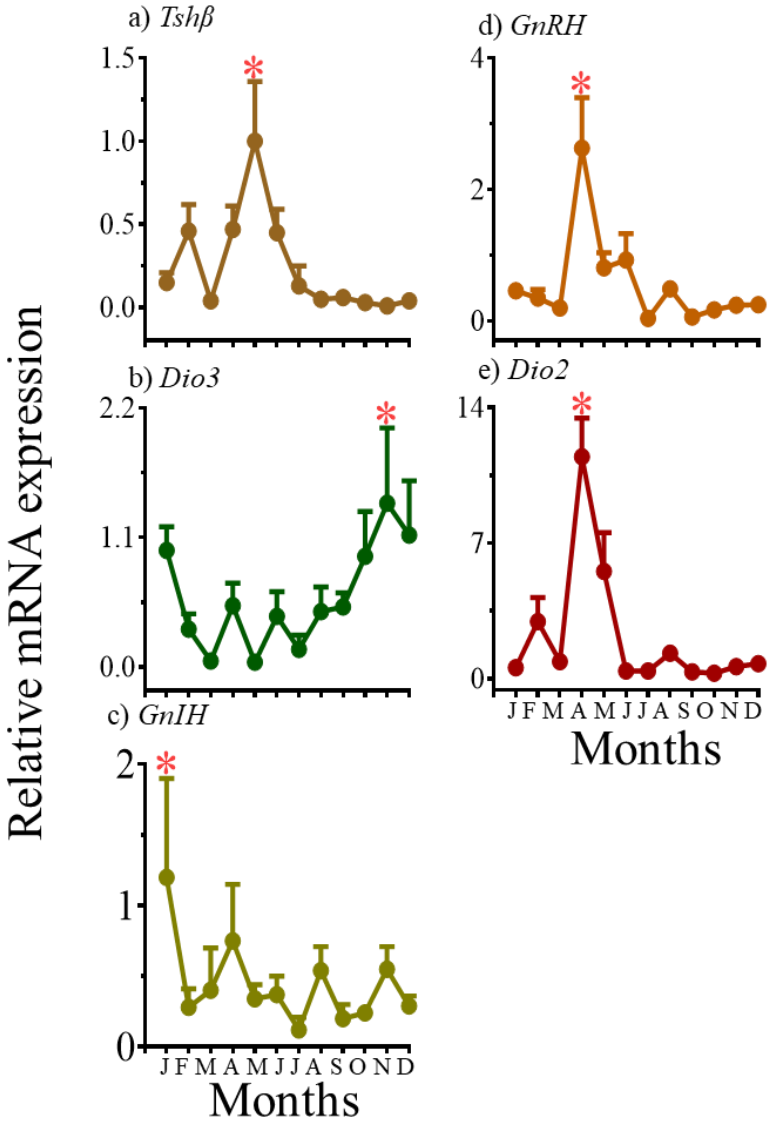

Fig. 44. Data is represented as mean (± SE, n = 5/month). Relative mRNA levels of reproductive genes in the hypothalamic tissues of adult female tree sparrows were collected every month for 12 months. One-way ANOVA followed by Tukey's multiple comparison test if ANOVA showed significant differences. * indicates significant differences ($P < 0.05$) among the months.

Hypothalamic expression of steroidogenic markers revealed seasonal changes (Fig. 45). qPCR analysis of these transcripts in hypothalamus, revealed seasonal variations in the expression levels of *StAR* ($P<0.0001$; One Way ANOVA; Table 6f; Fig. 45a), *Scp2* ($P<0.0001$; One Way ANOVA; Table 6f; Fig. 45c), *Cyp11a1*

(P=0.0160; One Way ANOVA; Table 6f; Fig. 45e), *Nr4a1*(P=0.0008; One Way ANOVA; Table 6f; Fig. 45b), *Cyp11b* (P<0.0001; One Way ANOVA; Table 6f; Fig. 45g), *Cyp17a1* (P=0.0037; One Way ANOVA; Table 6f; Fig. 45f), *Hsd11b2* (P<0.0001; One Way ANOVA; Table 6f; Fig. 45h), *Er* (P=0.0003; One Way ANOVA; Table 6f; Fig. 45i), and *Vdac1* (P<0.0001; One Way ANOVA; Table 6f; Fig. 45d). *Cyp19a1* (P<0.0001; One Way ANOVA; Table 6f; Fig. 45k), *Foxl2* (P<0.0001; One Way ANOVA; Table 6f; Fig. 45j).

Table 6f. Values of One-way ANOVA of the monthly expressions of steroidogenic genes in female tree sparrows

Transcripts	Hypothalamus	Ovary
StAR	$F_{11,36}$= 12.66, P<0.0001	$F_{11,36}$= 5.100, P<0.0001
Scp2	$F_{11,36}$= 10.09, P<0.0001	$F_{11,84}$= 5.042, P<0.0001
Cyp11a1	$F_{11,36}$= 2.574, P=0.0160	$F_{11,80}$= 4.415, P<0.0001
Nr4a1	$F_{11,36}$= 3.998, P=0.0008	$F_{11,84}$= 3.184, P=0.0012
Cyp11b	$F_{11,36}$= 57.62, P<0.0001	$F_{11,82}$= 4.801, P<0.0001
Cyp17a1	$F_{11,36}$= 3.238, P=0.0037	$F_{11,80}$= 3.239, P=0.0011
Foxl2	$F_{11,36}$= 7.205, P<0.0001	$F_{11,36}$= 5.334, P<0.0001
Cyp19a1	$F_{11,84}$= 4.104, P<0.0001	$F_{11,35}$= 6.088, P<0.0001
Hsd11b2	$F_{11,36}$= 7.512, P<0.0001	$F_{11,82}$= 5.758, P<0.0001
Er	$F_{11,84}$= 3.604, P=0.0003	$F_{11,36}$= 6.341, P<0.0001

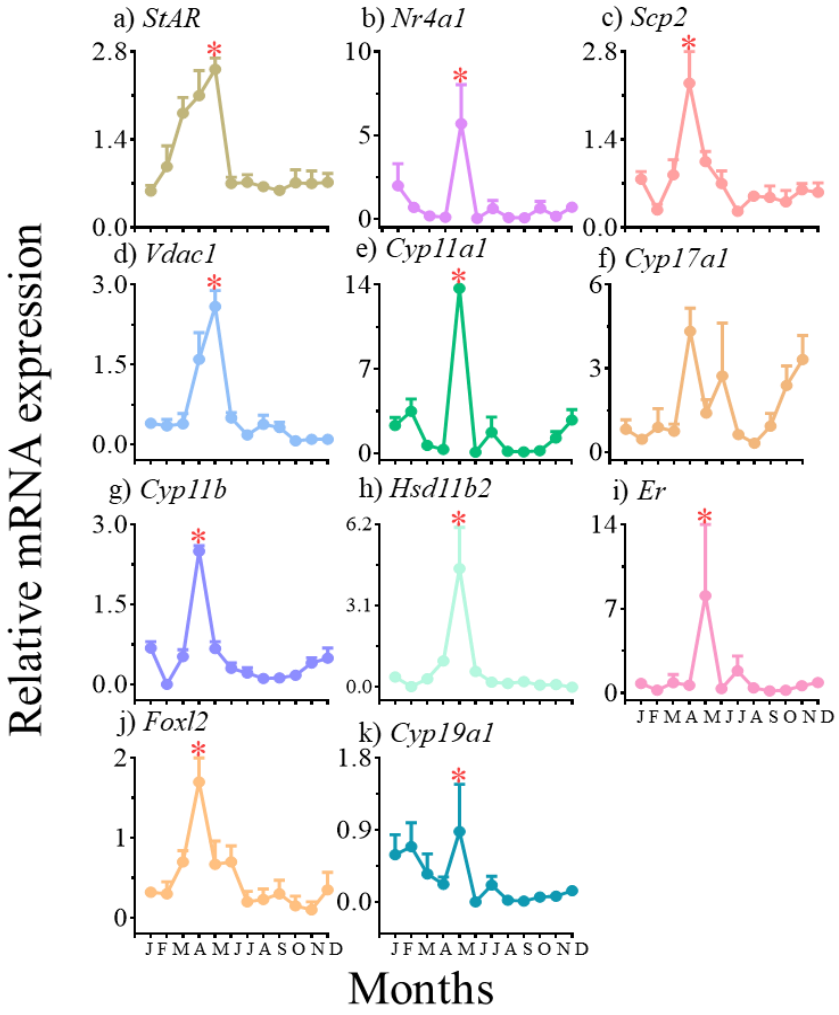

Fig. 45. Data is represented as mean (± SE, n = 5/month). Relative mRNA levels of steroidogenic transcripts in the hypothalamus of adult female tree sparrows were collected every month for a year. One-way ANOVA followed by Tukey's multiple comparison test if ANOVA showed significant differences. * indicates significant differences ($P < 0.05$) among the months.

Maximum expression levels of *StAR* were observed in May, and *Scp2* transcripts were observed in April ($P<0.05$; Tukey's multiple comparison test; Fig. 45a, c) while levels were low at other months of the year ($P>0.05$; Tukey's multiple comparison test; Fig. 45a, c). Higher expression of *Cyp11a1* was observed during the breeding phase i.e., in May while levels were low at other times of the year ($P<0.05$; Tukey's multiple comparison tests; Fig. 45e). Expression levels of *Cyp11b*, and *Foxl2*

peaked during April while levels were low during the other times of the year (P<0.05; Tukey's multiple comparison tests; Fig. 45g, j). In the hypothalamus, peak expression of *Nr4a1, Hsd11b2, Vdac1,* and *Er* was observed in May (P<0.05; Tukey's multiple comparison test; Fig. 45b, h,d,i). Multiple peaks in expression levels of *Cyp17a1* were observed in May, July, November, and December with the highest expression level in May (Fig. 45f). However, the amplitude of peaks was lower during the pre-breeding phase (P>0.05; Tukey's multiple comparison tests; Fig. 45f). Transcript levels of *Cyp19a1* were observed during pre-breeding phase in January and February, showing peaked expression in May in the breeding phase and lower levels in the rest of the year. (P<0.05; Tukey's multiple comparison test; Fig. 45k).

Seasonal variation in the ovarian transcript expression levels of *StAR* (P<0.0001; One Way ANOVA; Table 6c; Fig. 46a), *Scp2* (P<0.0001; One Way ANOVA; Table 6c; Fig. 46c), *Cyp11a1* (P<0.0001; One Way ANOVA; Table 6c; Fig. 46e), *Nr4a1* (P=0.0012; One Way ANOVA; Table 6c; Fig. 46b), *Cyp11B* (P<0.0001; One Way ANOVA; Table 6c; Fig. 46g), *Cyp17a1* (P=0.0011; One Way ANOVA; Table 6c; Fig. 46f), *Hsd11b2* (P<0.0001; One Way ANOVA; Table 6c; Fig. 46h), *Cyp19a1* (P<0.0001; One Way ANOVA; Table 6c; Fig. 46k), *Foxl2* (P<0.0001; One Way ANOVA; Table 6c; Fig. 46j), *Er* (P<0.0001; One Way ANOVA; Table 6c; Fig. 46i), and *Vdac1* (P<0.0001; One Way ANOVA; Table 6c; Fig. 46d) were also observed. In ovary, *StAR* transcript levels peaked in March (P<0.05; Tukey's multiple comparison test; Fig. 46a). *Nr4a1* levels were highest in May during breeding phase (P<0.05; Tukey's multiple comparison test; Fig. 46b). *Er* peaked during April and subsequent fall from June onwards (Fig. 46i). *Hsd11b2* levels peaked in March (pre-breeding season) and September during post-breeding (Fig. 46i). Expression levels of *Cyp11a1* peak during January and February during the pre-breeding phase, and lower in the rest of the year (P<0.05; Tukey's multiple comparison tests; Fig. 46e). *Cyp19a1* and *Foxl2* peaked during February (P<0.05; Tukey's multiple comparison tests; Fig. 46j, k), while *Cyp17a1 and Scp2* show multiple peaks during breeding phase during January, March, April and May (P<0.05; Tukey's multiple comparison tests; Fig. 46c, f). *Cyp11b* transcript shows peaked expression in the pre- and post-breeding season, i.e., January and September (P<0.05; Tukey's multiple comparison tests; Fig. 46c, f). However, the

Vdac1 expression level was high in the post-breeding phase in August (P<0.05; Tukey's multiple comparison tests; Fig. 46d).

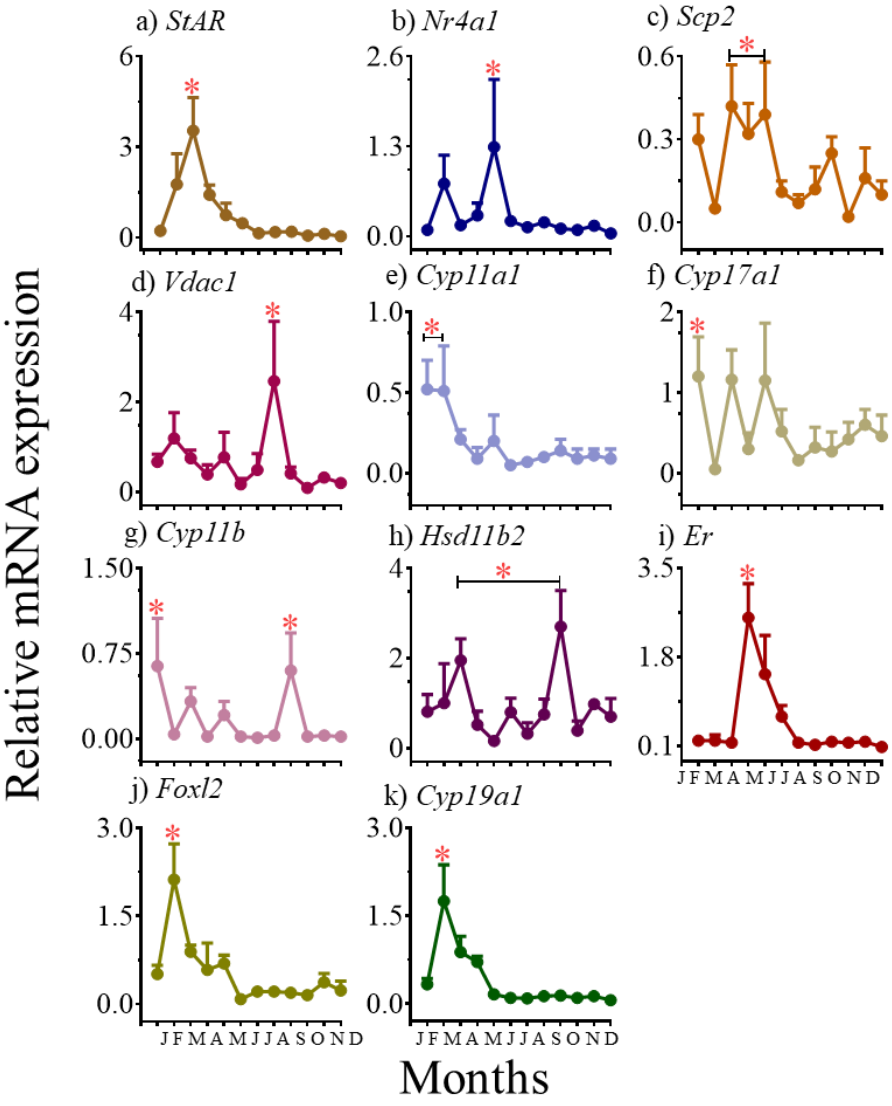

Fig. 46. Data is represented as mean (± SE, n = 5/month). Relative mRNA levels of steroidogenic in the ovary of adult female tree sparrows were collected every month for a year. One-way ANOVA followed by Tukey's multiple comparison test if ANOVA showed significant differences. * indicates significant differences (P < 0.05) among the months.

A correlation was observed between reproductive gene transcripts and steroidogenic markers. Significant positive correlation of *Tshβ* and *StAR* transcripts in the hypothalamus was observed (r = 0.7034, P = 0.0107; Fig. 47a), whereas no significant differences were observed in mRNA expression levels in the ovary (r = 0.1477, P = 0.6468; Fig. 48a). A positive correlation of *Tshβ* and *Nr4a1* in the hypothalamus (r = 0.7343, P = 0.0065; Fig. 47b) and ovary (r = 0.9008, P < 0.0001; Fig. 48b), *Tshβ* and *Er* in the hypothalamus (r = 0.7577, P = 0.0043; Fig. 47i) and ovary (r = 0.6724, P = 0.0166; Fig. 48i) was observed. A significant correlation of *Tshβ* with *Hsd11b2* (r = 0.8599, P = 0.0003; Fig. 47h), *Vdac1*(r = 0.8853, P = 0.0001; Fig. 47d), *Cyp11a1*(r = 0.7822, P = 0.0062; Fig. 47e), and *Cyp19a1*(r = 0.6749, P = 0.0161; Fig. 47k) in the hypothalamus was observed, whereas no significant correlations were observed for *Tshβ* with *Scp2* (r = 0.4144, P = 0.1805; Fig. 47c), *Foxl2*(r = 0.4800, P = 0.1142; Fig. 47j), *Cyp17a1* (r = 0.3984, P = 0.1996; Fig. 47f), *Cyp11a1*(r = 0.7822, P= 0.0062; Fig. 47e), and *Cyp19a1*(r = 0.6749, P = 0.0161; Fig. 47k) in the hypothalamus.

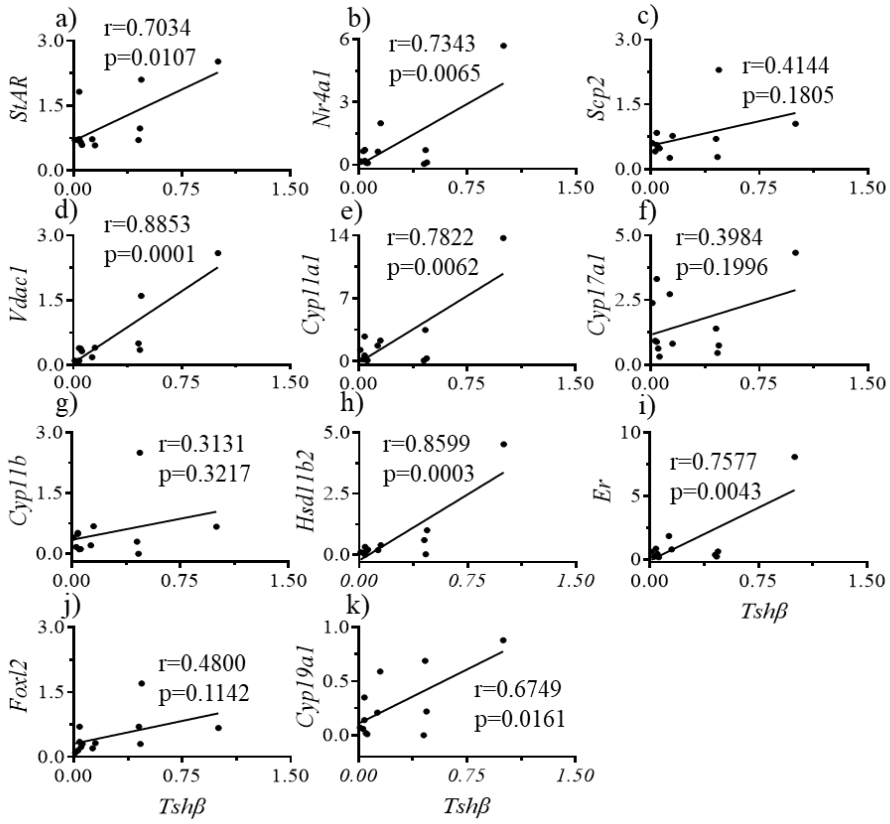

Fig. 47. Correlation of hypothalamic *Tshβ* expression with hypothalamic expression of *StAR, Scp2, Cyp11a1, Nr4a1, Cyp11b, Cyp17a1, Hsd11b2, Er, Vdac1, Foxl2,* and *Cyp19a1*. Significant differences were taken at $p < 0.05$. For correlation, $r = 1$ indicates a positive correlation, while $r > 1$ indicates a negative correlation.

Also, *Tshβ* with *Scp2*($r = 0.3406$, $P = 0.2787$; Fig. 48c), *Vdac1*($r = 0.01309$, $P = 0.9678$; Fig. 48d), *Foxl2*($r = 0.3522$, $P = 0.2616$; Fig. 48j), *Cyp17a1*($r = 0.2055$, $P = 0.5218$; Fig. 48f), *Cyp11a1*($r = 0.1688$, $P = 0.6000$; Fig. 48e), and *Cyp19a1*($r = 0.2151$, $P = 0.5020$; Fig. 48k) in the ovary were observed. Further, in the ovary, there were significant negative correlations of *Tshβ* with *Hsd11b2* ($r = -0.4011$, $P = 0.1963$; Fig. 48h) and *Cyp11b* ($r = -0.1008$, $P = 0.7552$; Fig. 48g).

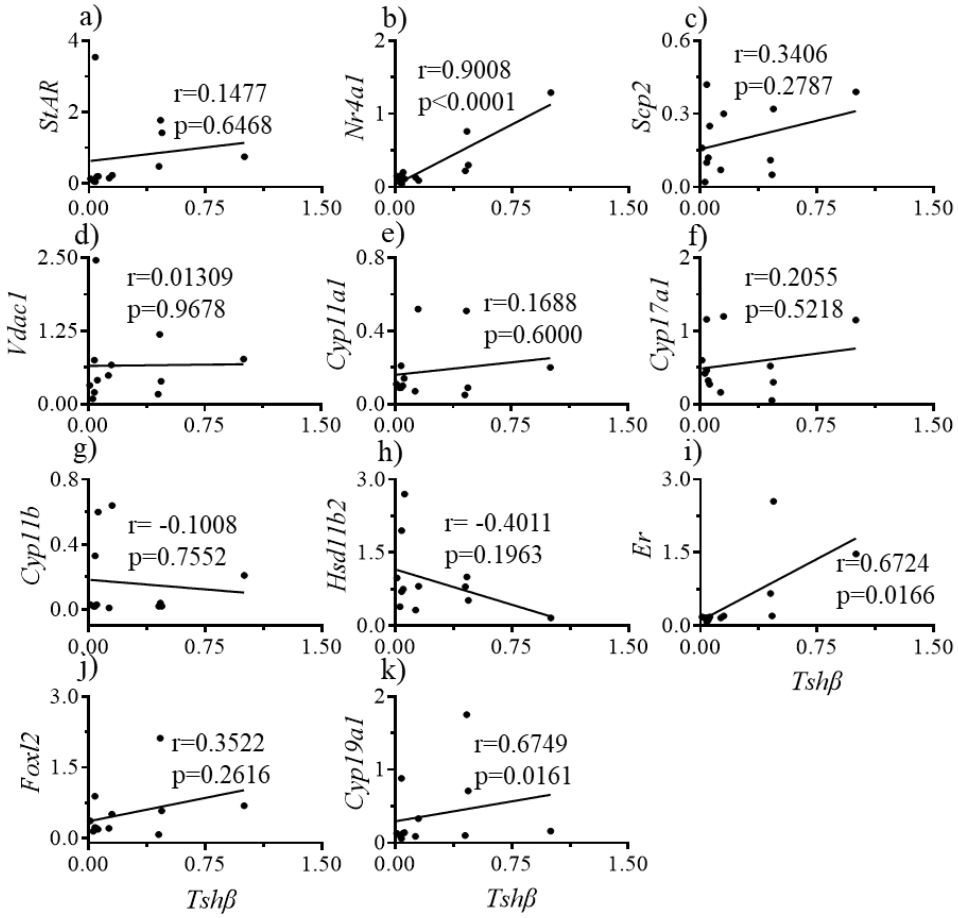

Fig. 48. Correlation of hypothalamic *Tshβ* expression with ovarian expression of *StAR, Scp2, Cyp11a1, Nr4a1, Cyp11b, Cyp17a1, Hsd11b2, Er, Vdac1, Foxl2,* and *Cyp19a1*. Significant differences were taken at $p < 0.05$. For correlation, r=1 is a positive correlation, r>1 is a negative correlation.

A significant positive correlation in the hypothalamus was observed for reproductive gene *Dio2* with *StAR* ($r = 0.7487$, $P = 0.0051$; Fig. 49a), *Scp2* ($r = 0.8906$, $P = 0.0001$; Fig. 49c), *Foxl2* ($r = 0.8650$, $P = 0.0003$; Fig. 49j), *Vdac1* ($r = 0.7488$, $P = 0.0051$; Fig. 49d), and *Cyp11b* ($r = 0.8739$, $P = 0.0002$; Fig. 49g), whereas no significant correlation was observed between *Dio2* with *Hsd11b2* ($r = 0.4719$, $P = 0.1214$; Fig. 49h), *Nr4a1* ($r = 0.2388$, $P = 0.4549$; Fig. 49b), *Er* ($r = 0.3052$, $P = 0.3347$; Fig. 49i), *Cyp17a1* ($r = 0.03657$, $P = 0.9102$; Fig. 49f), *Cyp11a1* (r = 0.2798, $P = 0.3784$; Fig. 49e), and *Cyp19a1* ($r = 0.3201$, $P = 0.3104$; Fig. 49k).

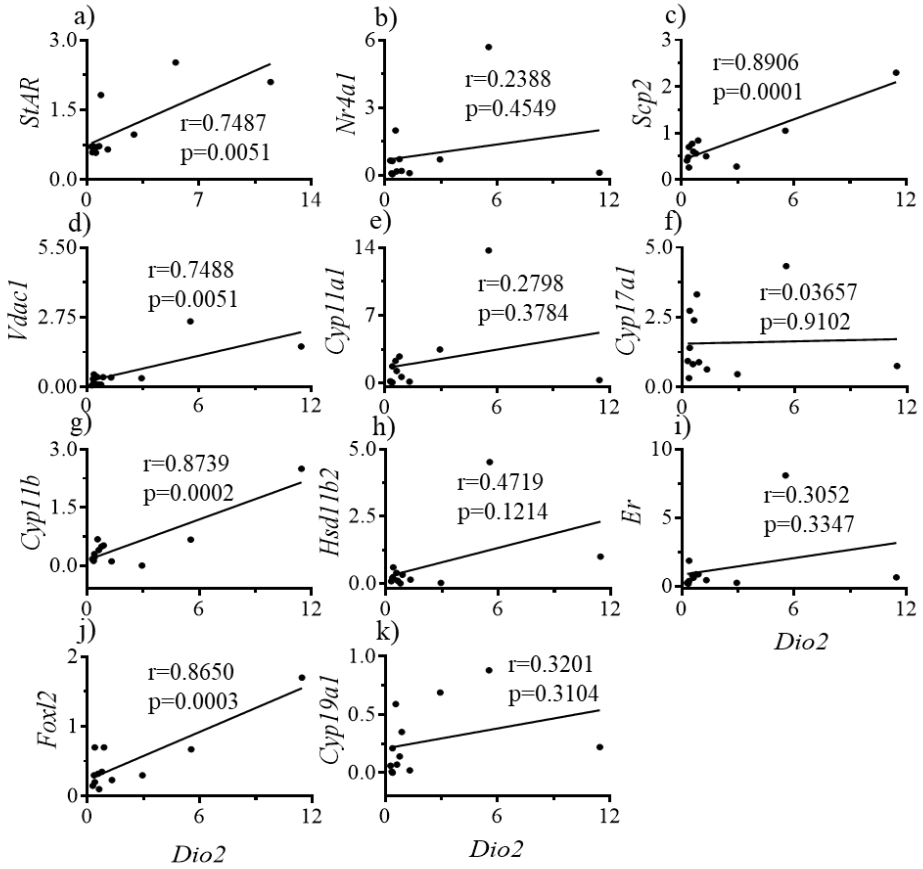

Fig. 49. Correlation of hypothalamic *Dio2* expression with hypothalamic expression of *StAR, Scp2, Cyp11a1, Nr4a1, Cyp11b, Cyp17a1, Hsd11b2, Er, Vdac1, Foxl2* and *Cyp19a1*. Significant differences were taken at $p < 0.05$. For correlation, $r = 1$ indicates a positive correlation, while $r > 1$ indicates a negative correlation.

Besides, a significant negative correlation between *Dio2* gene expression with steroidogenic markers *Hsd11b2* ($r = -0.3028$, $P = 0.3388$; Fig. 50h), *Cyp11b* ($r = -0.2019$, $P = 0.5292$; Fig. 50g), *Cyp17a1* ($r = -0.05247$, $P = 0.8713$; Fig. 50f), and *Cyp11a1* ($r = -0.02128$, $P = 0.9477$; Fig. 50e) was observed in the ovary. However, no significant correlation was observed for *StAR* ($r = 0.2767$, $P = 0.3840$; Fig. 50a), *Scp2* ($r = 0.4188$, $P = 0.1755$; Fig. 50c), *Foxl2* ($r = 0.2658$, $P = 0.4037$; Fig. 50j), *Vdac1* ($r = 0.01986$, $P = 0.9512$; Fig. 50d), *Nr4a1* ($r = 0.4559$, $P = 0.1364$; Fig. 50b), and *Cyp19a1* ($r = 0.3172$, $P = 0.3151$; Fig. 50k).

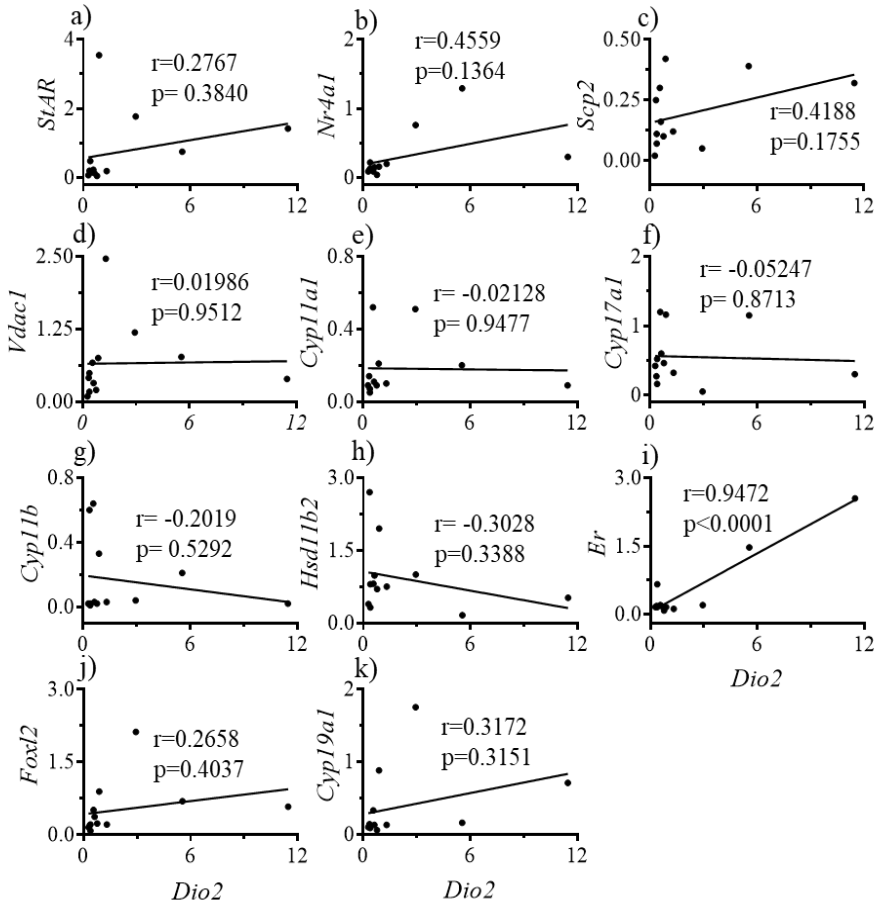

Fig. 50. Correlation of hypothalamic *Dio2* expression with ovarian expression of *StAR, Scp2, Cyp11a1, Nr4a1, Cyp11b, Cyp17a1, Hsd11b2, Er, Vdac1, Foxl2* and *Cyp19a1*. Significant differences were taken at $p < 0.05$. For correlation, r = 1 indicates a positive correlation, while r>1 indicates a negative correlation.

A significant negative correlation was observed in the hypothalamus between *Dio3* and *StAR* ($r = -0.5366$, $P = 0.0720$; Fig. 51a), *Scp2* ($r = -0.09695$, $P = 0.7644$; Fig. 51c), *Foxl2* ($r = -0.3219$, $P = 0.3085$; Fig. 51j), *Hsd11b2* ($r = -0.4271$, $P = 0.1662$; Fig. 51h), *Vdac1* ($r = -0.4607$, $P = 0.1317$; Fig. 51d), *Nr4a1* ($r = -0.2671$, $P = 0.4012$; Fig. 51b), *Er* ($r = -0.4177$, $P = 0.1767$; Fig. 51i), *Cyp17a1* (r = -0.00047, $P = 0.9988$; Fig. 51f), *Cyp11a1* ($r = -0.3201$, $P = 0.3104$; Fig. 51e), and *Cyp19a1* ($r = -0.3795$, $P = 0.2237$; Fig. 51k).

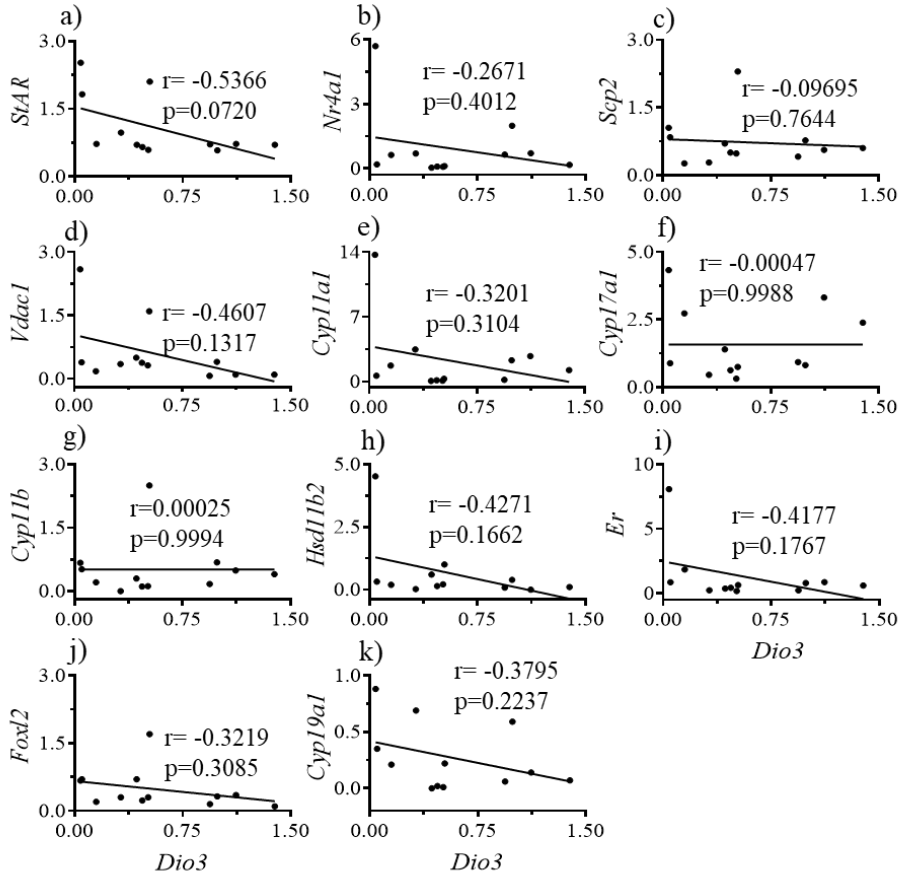

Fig. 51. Correlation of hypothalamic *Dio3* expression with hypothalamic expression of *StAR, Scp2, Cyp11a1, Nr4a1, Cyp11b, Cyp17a1, Hsd11b2, Er, Vdac1, Foxl2* and *Cyp19a1*. Significant differences were taken at $p < 0.05$. For correlation, r = 1 indicates a positive correlation, while r > 1 indicates a negative correlation.

Similarly, in the ovary, *Dio3* shows a significant negative correlation with all the steroidogenic markers *StAR* ($r = -0.5425$, $P = 0.0684$; Fig. 52a), *Scp2* ($r = -0.3360$, $P = 0.2856$; Fig. 52c), *Foxl2* (r = −0.3200, P = 0.3105; Fig. 52j), *Hsd11b2* ($r = -0.07713$, $P = 0.8117$; Fig. 52h), *Vdac1* ($r = -0.3168$, $P = 0.3157$; Fig. 52d), *Cyp11b* (r = -0.03794, $P = 0.9068$; Fig. 52g), *Nr4a1* (r = -0.5073, $P = 0.0923$; Fig. 52b), *Er* ($r = -0.2574$, $P = 0.4193$; Fig. 52i), *Cyp17a1* (r = -0.03795, $P = 0.9068$; Fig. 52f), *Cyp11a1* ($r = -0.03431$, $P = 0.09157$; Fig. 52e) and *Cyp19a1* ($r = -0.3313$, $P = 0.2928$; Fig. 52k).

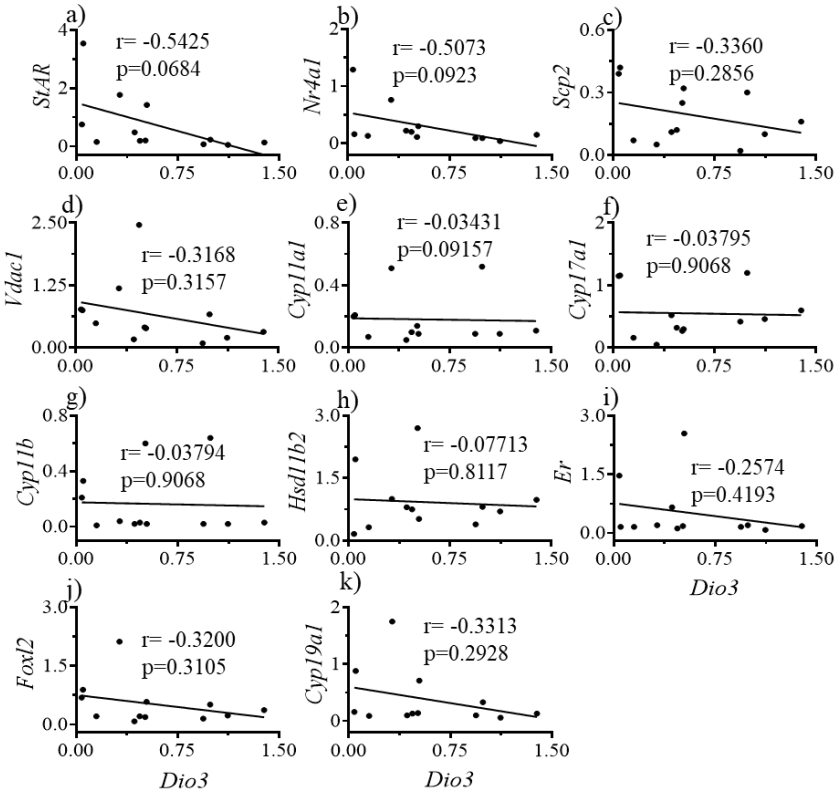

Fig. 52. Correlation of hypothalamic *Dio3* expression with ovarian expression of *StAR, Scp2, Cyp11a1, Nr4a1, Cyp11b, Cyp17a1, Hsd11b2, Er, Vdac1, Foxl2* and *Cyp19a1*. Significant differences were taken at $p < 0.05$. For correlation, r=1 is a positive correlation, r>1 is a negative correlation.

However, no significant correlation in the hypothalamus was observed for *Cyp11b* (r = 0.00025, P = 0.9994; Fig. 52g). In the hypothalamus, a significant correlation was observed between *GnRH* steroidogenic markers *Scp2* (r = 0.9336, P < 0.0001; Fig. 53c), *Foxl2* (r = 0.9248, P < 0.0001; Fig. 53j), *Vdac1* (r = 0.6095, P = 0.0354; Fig. 53d), *Cyp11b* (r = 0.9107, P < 0.0001; Fig. 53g), and a negative correlation was observed with *Cyp17a1* (r = −0.09715, P = 0.7639; Fig. 53f). Whereas, no significant correlation was observed of *GnRH* with *Hsd11b2* (r = 0.3100, P = 0.3267; Fig. 53h), *Nr4a1* (r = 0.03475, P = 0.9146; Fig. 53b), *Er* (r = 0.08865, P = 0.7841; Fig. 53i), *Cyp11a1* (r = 0.02749, P = 0.9324; Fig. 53e), and *Cyp19a1* (r = 0.07125, P = 0.8258; Fig. 53k).

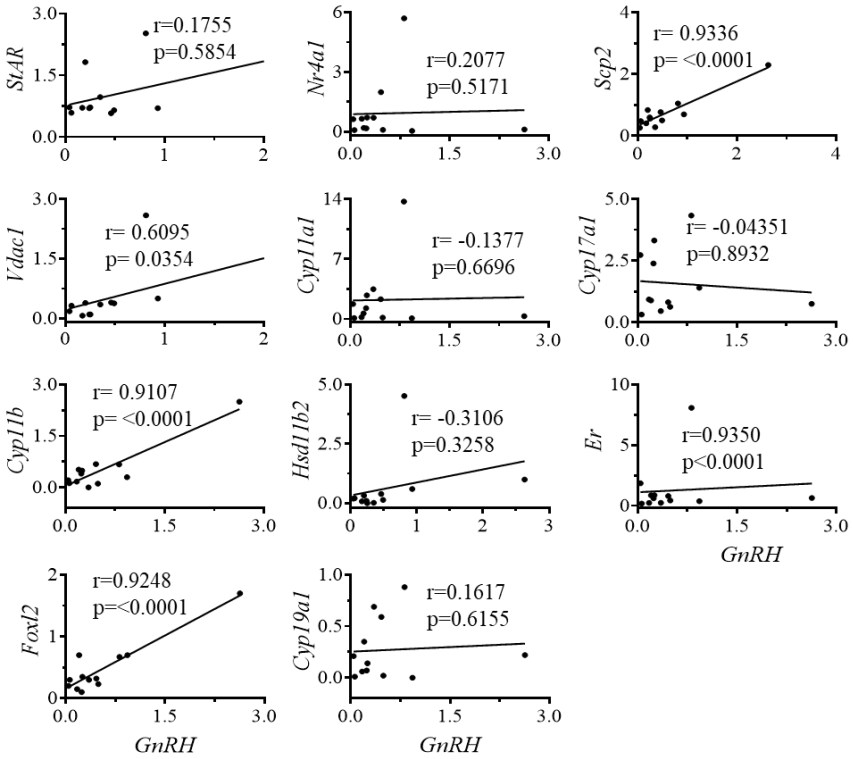

Fig. 53. Correlation of hypothalamic *GnRH* expression with hypothalamic expression of *StAR, Scp2, Cyp11a1, Nr4a1, Cyp11b, Cyp17a1, Hsd11b2, Er, Vdac1, Foxl2,* and *Cyp19a1*. Significant differences were taken at p< 0.05. For correlation, r= 1 indicates a positive correlation, while r > 1 indicates a negative correlation.

In the ovary, a negative correlation was observed with *Hsd11b2* ($r = -0.3106$, $P = 0.3258$; Fig. 54h), *Vdac1* ($r = -0.05871$, $P = 0.8562$; Fig. 54d), *Cyp11b* (r = -0.2218, $P = 0.4883$; Fig. 54g), *Cyp17a1* (r = -0.04351, $P = 0.8932$; Fig. 54f), *Cyp11a1* ($r = -0.1377$, $P = 0.6696$; Fig. 54e), and a positive correlation with *Er* (r = 0.9350, $P < 0.0001$; Fig. 54i). However, no correlation was observed with *StAR* (r = 0.1755, $P = 0.5854$; Fig. 54a), *Scp2* (r = 0.3407, $P = 0.2784$; Fig. 54c), *Foxl2* (r = 0.03780, $P = 0.9072$; Fig. 54j), *Nr4a1* ($r = 0.2077$, $P = 0.5171$; Fig. 54b), and *Cyp19a1* ($r = 0.1617$, $P = 0.6155$; Fig. 54k) in the ovary.

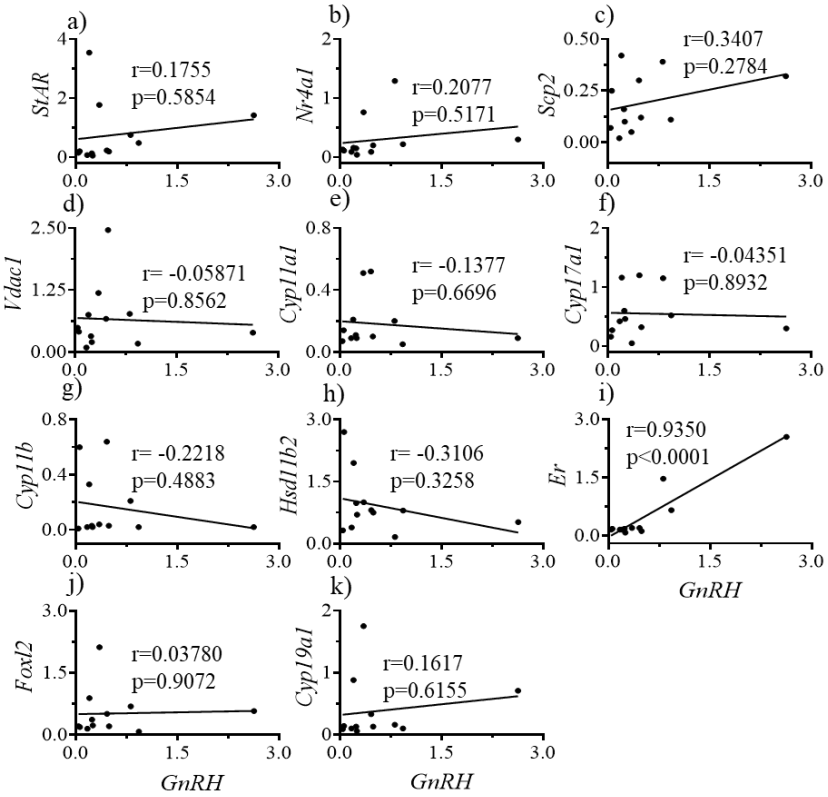

Fig. 54. Correlation of hypothalamic *GnRH* expression with ovarian expression of *StAR, Scp2, Cyp11a1, Nr4a1, Cyp11b, Cyp17a1, Hsd11b2, Er, Vdac1, Foxl2,* and *Cyp19a1*. Significant differences were taken at p< 0.05. For correlation, r=1 is a positive correlation, r>1 is a negative correlation.

A negative correlation was observed in the hypothalamus between *GnIH* with steroidogenic markers *Hsd11b2* ($r = -0.001419$, $P = 0.9965$; Fig. 55h), *Er* ($r = -0.1125$, $P = 0.7279$; Fig. 55i), *Cyp17a1* ($r = -0.2592$, $P = 0.4159$; Fig. 55f), *Cyp11a1* ($r = -0.08023$, $P = 0.8042$; Fig. 55e), and in the ovary with *StAR* ($r = -0.00076$, $P = 0.9981$; Fig. 16a), *Hsd11b2* ($r = -0.1211$, $P = 0.7078$; Fig. 56h), and *Nr4a1* ($r = -0.1401$, $P = 0.6641$; Fig. 56b). However, no correlation was observed with *StAR* ($r = 0.03767$, $P = 0.9075$; Fig. 55a), *Scp2* ($r = 0.4613$, $P = 0.1311$; Fig. 55c), *Foxl2* ($r = 0.2757$, $P = 0.3857$; Fig. 55j), *Vdac1* ($r = 0.1312$, $P = 0.6845$; Fig. 55d), *Cyp11b* ($r = 0.4794$, $P =$

0.1148; Fig. 55g), *Nr4a1* (*r* = 0.08162, *P* = 0.8009; Fig. 55b), *Cyp19a1* (r = 0.2192, *P* = 0.4936; Fig. 55k) in the hypothalamus.

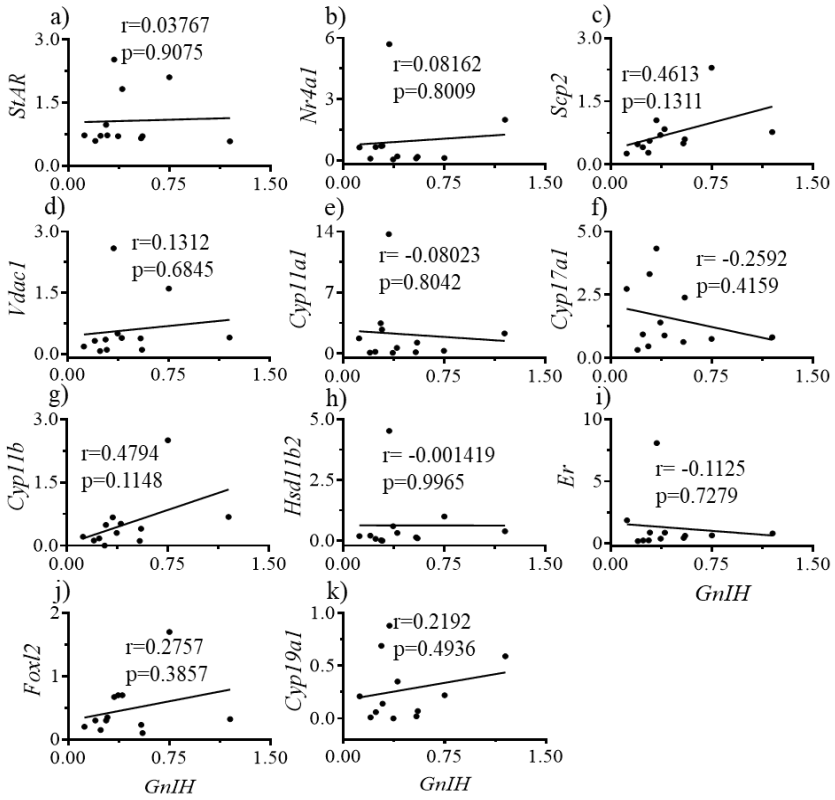

Fig. 55. Correlation of hypothalamic *GnIH* expression with hypothalamic expression of *StAR, Scp2, Cyp11a1, Nr4a1, Cyp11b, Cyp17a1, Hsd11b2, Er, Vdac1, Foxl2,* and *Cyp19a1*. Significant differences were taken at $p < 0.05$. For correlation, r = 1 indicates a positive correlation, while r > 1 indicates a negative correlation.

And *Scp2* (*r* = 0.4108, *P* = 0.1846; Fig. 56c), *Foxl2* (*r* = 0.001315, *P* = 0.9968; Fig. 56j), *Vdac1* (*r* = 0.1422, *P* = 0.6593; Fig. 56d), *Cyp11b* (*r* = 0.3973, *P* = 0.2009;

Fig. 56g), *Er* (*r* = 0.2472, *P* = 0.4385; Fig. 56i), *Cyp17a1* (*r* = 0.4806, *P* = 0.1137; Fig. 56f) and *Cyp19a1* (r = 0.04174, *P* = 0.8975; Fig. 56k) in the ovary.

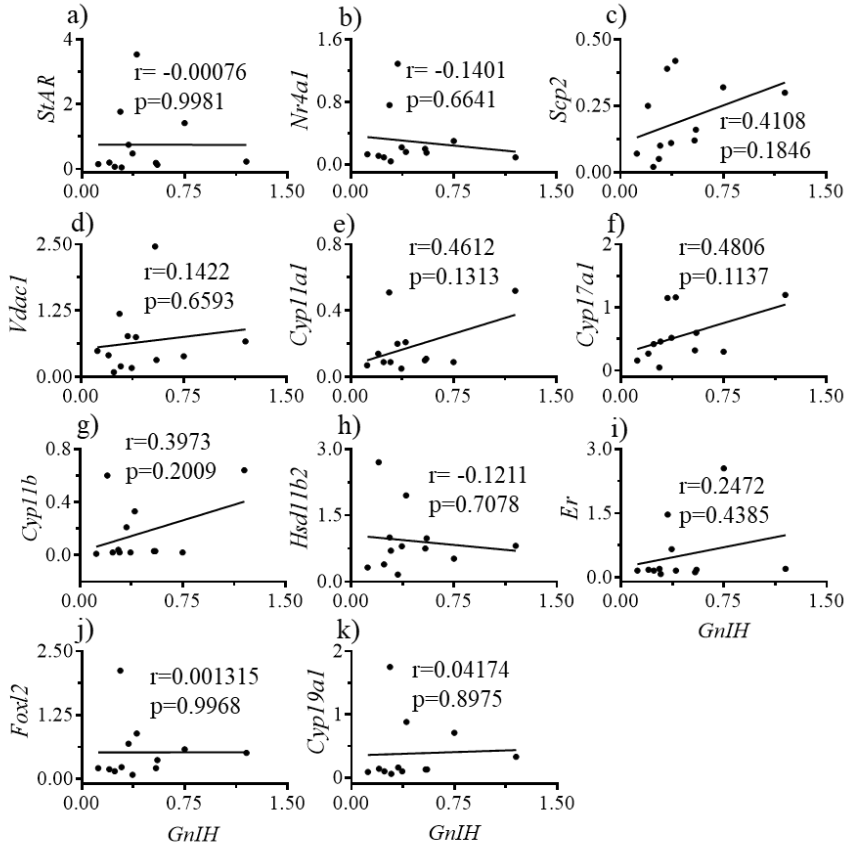

Fig. 56. Correlation of hypothalamic *GnIH* expression with ovarian expression of *StAR, Scp2, Cyp11a1, Nr4a1, Cyp11b, Cyp17a1, Hsd11b2, Er, Vdac1, Foxl2,* and *Cyp19a1*. Significant differences were taken at p < 0.05. For correlation, r=1 is a positive correlation, r>1 is a negative correlation.

5. DISCUSSION

Sex steroid hormones are primarily synthesized within reproductive organs, and once secreted, they modulate different physiological events in target tissues; besides, seasonal fluctuations in environmental conditions directly influence physiology, leading to changes in seasonal breeders' reproductive functions. Small fluctuations in annual changes in body mass were observed in tree sparrows (Fig. 29a). This is consistent with our previous observations (Renthlei et al., 2019). Resident birds store very small amounts of fat, reflecting limited annual variations in body mass (Renthlei et al., 2019; Trivedi et al., 2006). Seasonal birds exhibit annual changes in primary sexual characteristics, such as gonadal growth, and secondary sexual characteristics, such as bill color and molt in feathers (Renthlei et al., 2019). Our study shows reproductive phase-dependent changes in bill color. A darker bill color was observed during the breeding phase (Fig. 29b). Bill coloring is associated with plasma testosterone levels. During the breeding season, when the bill color is at its darkest, there is also a significant increase in testosterone levels. Bill coloration is associated with testosterone levels, as evidenced by the low levels of plasma testosterone and gonad size observed throughout the post-breeding season (Laucht et al., 2010; Green et al., 2022). Gonadal size-dependent change in bill color has been reported for seasonally breeding avian species (Trivedi et al., 2006). Seasonally breeding birds do not retain fully grown testes during the non-breeding period. Our results are consistent with these findings (Fig. 29c). We recorded several fold changes in testicular size from March to May (Fig. 29c), which is the breeding time for this species at this latitude (Renthlei et al., 2019; 2021). The highest plasma testosterone level was observed during April, which corresponds to the bigger testes and higher reproductive activities and is consistent with the previous reports (Dixit et al., 2016). Seasonally breeding birds exhibit a dramatic change in the size of gonads during the breeding season than the non-breeding season because of the higher gonadal activity during the breeding period (Renthlei et al., 2019; 2021; Trivedi et al., 2006). Besides, higher gonadal activity also reflects higher steroidogenesis-linked reproductive activity in tree sparrows. Our study shows the highest plasma cholesterol level (February) just before the higher testicular activity (March onward), validating the role of cholesterol in the steroidogenic enzymes' biosynthesis. Cholesterol helps maintain the integrity and

fluidity of cell membranes and serves as a precursor for synthesizing substances vital for the organism, including steroid hormones, bile acids, and vitamin D (Zampelas & Magriplis, 2019). Energy obtained from food resources forms the fundamental basis of biological activity; thus, energy intake is essential in evaluating an animal's nutritional state (Robbins, 2012). In our study, maximum molt in body and primary flight feathers was observed during the post-breeding phase, i.e., during September, and is consistent with reports on this and other seasonally breeding avian species (Renthlei et al., 2019; Trivedi et al., 2006). Higher expression of *Tshβ*, *Dio2*, and *GnRH* in the hypothalamus of tree sparrows during the reproductively active phase is consistent with the previous reports for this species (Renthlei et al., 2021) and other seasonally breeding avian species (Trivedi et al., 2019). These findings reconfirm the critical role played by these transcripts in regulating seasonal reproduction in seasonally breeding species (Yoshimura et al., 2003; Nakao et al., 2008; Trivedi et al., 2019). Further, a decrease in the transcription levels of *Dio3* and *GnIH* during the reproductive phase and an increase in these transcripts during the post-reproductive phase (September to December) reconfirms the involvement of the product of these transcripts in inhibiting reproduction (Fig. 30). These results are consistent with the previous reports on the inhibitory nature of these transcripts on gonadal activity (Tsutsui et al., 1999; Majumdar et al., 2015; Dixit & Byrsat, 2018; Zhang et al., 2019; Trivedi et al., 2019). However, an alternate paradigm is suggested by a recent study on Japanese quail. A transcriptome-based analysis suggests that the photoperiod-independent regulation of follicle-stimulating hormone-β (*fshβ*) expression and supplementary environmental cues, such as temperature, can regulate the timing of seasonal reproduction. The study suggests that photoinduced increase in *fshβ* expression precedes increases in prolactin, thyrotropin-stimulating hormone-β, and testicular growth during the simulated spring equinox (Majumdar et al., 2023). Further, our findings are consistent with those of Japanese quail. The study shows that short photoperiods (less than 12 h light per day) lead to gonadal involution, and long-term exposure to short photoperiods results in significant upregulation of dio3 expression and vimentin immunoreactivity in the median eminence (Majumdar et al., 2023). Our findings are consistent with it and we observed higher mRNA expression of *Dio3* during the breeding phase from July till January (Fig. 30b). The study also suggests

that stimulatory daylengths longer than 12 h induce thyrotropes to increase *Tshβ* leading to a cascade of molecular events in the MBH that permit *GnRH* to stimulate gonadotropes to release *fshβ* and initiate gonadal development. We did not study the expression of *fshβ*; however, higher expression of *Tshβ* was observed during the breeding phase (Fig. 30a).

To assess the steroidogenic pathways that govern the seasonal reproductive cycle, we have investigated the expression of transcripts of a gene (StAR) along with a series of steroidogenic enzymes (*Er, Cyp17a1, Nr4a1, Scp2, Cyp11a1, Hsd11b2, Vdac1, Cyp11b, and Srd5a1*) in the hypothalamus, as well as circulating testosterone titers throughout the year (Fig. 31,32). Neurons and glial cells of the brain can locally synthesize biologically active steroids de novo (Baulieu, 1998; Compagnone & Mellon, 2000; Do Rego et al., 2009). This study shows expression of these transcripts in the hypothalamic tissues with annual variations (Fig. 31). Expression of these steroidogenic transcripts is directly influenced by the annual reproductive stage of tree sparrows. In the hypothalamus, higher expression of *Cyp11a1, Nr4a1, Cyp11b, Cyp17a1, Hsd11b2, Er,* and *Vdac1* was observed during the pre-breeding phase (Fig. 31), while higher expression of *StAR* in the hypothalamus corresponds with growing testes (Fig. 29 and 31). Multiple peaks were observed for *Srd5a1* in the hypothalamus (Fig. 31). A study conducted on Japanese quail shows similarity with the current study where *StAR, 3β-hsd, 17β-hsd, p450, ar*, and 5α-Red were always present in the somatic (Leydig and Sertoli cells) and germ cells (spermatogonia, spermatocytes I and II, spermatids, and spermatozoa) and highest expressions were observed during the reproductive phase (Rosati et al., 2019). Such evidence from other organisms suggests there are physiology-dependent changes in these transcripts' expression. In edible frogs, brain levels of neurosteroids oscillate simultaneously with the reproductive cycle. Seasonal fluctuations in gene expression levels of *hsd3b1, hsd17b1, srd5a1*, and *cyp19a1* were observed in the dienchephalon-mesencephalon region of both sexes of the edible frog, and higher transcript levels were observed in the reproductive period than in post-reproductive period (Santillo et al., 2017). Our results show that the steroidogenic process could be quite relevant during spermiogenesis and is consistent with findings of other long-day seasonal breeders, i.e., quail (Rosati et al., 2019). In seasonal breeders, the differential expression of steroidogenic enzymes during the

reproductive and non-reproductive cycles could be the local key in the control of spermatogenesis. In tree sparrows, expression of these steroidogenic transcripts showed reproductive stage-linked expression in the testes (Fig. 32). These transcripts were upregulated either before or during the reproductive stage (Fig. 29 and 32). These sex steroids are strong regulators of reproductive behavior in vertebrates. The steroidogenesis in gonads shows distinct sex- and season-specific patterns and expression of key enzymes. Higher expression of sex steroids during the pre-breeding or breeding phase has been shown in other species. In seasonally breeding vertebrate species mating behavior, elevated gametogenesis, and peak circulating sex steroid concentrations coincide temporally (Crews, 1984). In the toad (*Bufo japonicus*), circulatory sex steroid levels fluctuate seasonally and coincide with major reproductive events in species with distinct breeding seasons (Itoh & Ishii, 1990). We observed seasonal fluctuations in the *StAR* transcripts in the testes having peak expression during the breeding phase (Fig. 32). Maximum expression of *StAR* was also observed in the testes of garter snakes (*Thamnophis sirtalis parietalis*) during the spring mating season (Lincoln et al., 2023). The StAR protein is involved in shuttling cholesterol from the outer to the inner mitochondrial membrane, an essential step for initiating steroidogenesis. Higher expression of *StAR* mRNA in testes before/during the reproductive phase corresponds to higher steroidogenic activity and is consistent with our findings. StAR proteins also interact with voltage-dependent anion channel (Vdac1) at the mitochondria-associated endoplasmic reticulum membrane to facilitate cholesterol to the inner mitochondrial membrane (Prasad et al., 2015). We also observed peak expression of *Nr4a1* mRNA in testes during May, corresponding with higher testicular activities in tree sparrows. Expression of *Nr4a1* is modulated by several agents, including Ca2+, and growth factors (Li et al., 2006). In gonads, *Nr4a1* regulates the expression of several genes involved in steroidogenesis, including *StAR* (Martin et al., 2007), *Cyp17a1* (Zhang et al., 1997), *Hsd3b2* (Bassett et al., 2004a), and *Cyp11b2* (Bassett et al., 2004a). We observed higher expression of *Scp2* mRNA before/during the peak testicular activity (Fig. 32b). The *Scp2* gene, having two distinct transcription initiation sites, encodes a 15 kDa pro-Scp2 protein and the 58 kDa SCPx, which have identical C-termini (Ohba et al., 1995). Scp2 and SCPx proteins are highly expressed in all tissues with high cholesterol metabolism rates

(VAHOUNY et al., 1987). These proteins transport and metabolize cholesterol and other lipids (Li et al., 2016). We observed higher expression of *Cyp11a1* during the breeding phase (Fig. 32c). The enzymes Cyp11a1 and Cyp11b reside in the mitochondria, and their deficiency leads to impaired steroidogenesis (Parajes et al., 2013). *Srd5a1* expression is widely distributed in the body in nervous and non-nervous tissues (Rył et al., 2017). *Srd5a1* and *Hsd3b1* catalyze the conversion of testosterone into androgen DHT and DHT metabolism (Hernández et al., 2015). The enzymes are present in both microsomal and nuclear fractions. Higher expression of *Srd5a1* transcripts during the breeding season may directly indicate higher testosterone catabolism. We observed higher expression of *Er* transcripts before the breeding phase (Fig. 32i). Estrogen differentially modulates reproductive and non-reproductive responses via their receptors (Kuiper et al., 1998). ERs are required for the normal development of reproductive organs and for the expression of both male and female sexual behavior (Rissman et al., 1999). Peak expression of *Cyp17a1* transcripts was observed during April and May, which correspond to the peak testicular size in this species (Fig. 29). Highest expression of *Cyp17a1* has been shown in breeding testis in garter snakes (Lincoln et al., 2023). These observations reveal active involvement of steroidogenesis when mating behavior is maximal in male tree sparrows. Sex steroids are known to play a significant role in the testis, controlling the reproductive processes by either promoting spermatogenesis and spermiogenesis or developing, differentiating, and maintaining secondary sex characteristics in seasonal breeders (Carreau et al., 2011; Angelini & Botte, 1992; Di Fiore et al., 2005). Seasonal changes in spermatogenesis and testicular steroidogenesis have also been reported in wild male raccoon dogs (*Nyctereutes procynoides*) and steroidogenic markers P450sec and P450c17 peaked during mating season. Evidence has been shown in other species such as Japanese quail, where Western blot analysis of steroidogenic genes of 17βhsd, p450 aromatase, and 5α-Red showed the highest mRNA expression levels during the breeding season when the testis is reproductively active. The study supports that the steroidogenic process in quail testis directly corresponds to the seasonal variation stimulating androgenic and estrogenic pathways in the reproductive period and their synergic mechanism in the regulation of spermatogenesis (Rosati et al., 2019). Other than bird species, in sea cucumber, real-time qPCR analysis of steroidogenic genes

(*StAR, Cyp10, 3β-Hsd, 17β-Hsd*) shows increasing levels of mRNA transcripts during gonadal maturation (Thongbuakaew et al., 2021).

Similarly, we have also examined the daily and seasonal variations of several genes involved in reproduction and steroidogenesis in adult female tree sparrows. The tree sparrow is a resident bird and does not store much fat, unlike migratory birds, which use the fat as stored food and fuel for migration and, hence, have restricted annual variations in body mass, which is also consistent in male tree sparrows (Yatung & Trivedi, 2024). In the present study, slight variations in the body mass of female tree sparrows were observed annually (Fig. 43a) and are consistent with previous findings (Trivedi et al., 2006; Yatung & Trivedi, 2024). Tree sparrows exhibited seasonal variations in secondary sexual traits, including bill color and molting, as well as primary sexual traits like gonadal growth (Renthlei & Trivedi, 2019). This study shows reproductive phase-dependent variations in the bill color; a darker bill color was observed during the breeding phase (Fig. 43b). A recent study on male tree sparrows (Yatung & Trivedi, 2024) also found that the gonadal size is correlated with bill coloring. Low plasma progesterone levels and small follicular sizes are recorded throughout the post-breeding season and vice-versa; and when the bill color is at its darkest, the follicular size also significantly increases during the breeding season (Wang et al., 2023). A gonadal size-dependent change in bill color in seasonally breeding avian species house sparrow has also been shown (Trivedi et al., 2006; Yatung & Trivedi, 2024). Seasonally breeding birds do not retain fully grown gonadal growth during the non-breeding period. Meanwhile, our results are consistent with these findings (Fig. 43e). Several-fold changes in follicular size from April to July were recorded (Fig. 43e), which is the breeding time for this species at this latitude (Renthlei et al., 2021; Yatung & Trivedi, 2024). Seasonally breeding birds show a marked shift in gonadal size from the non-breeding phase to the breeding phase, correlated with increased gonadal activity. Furthermore, increased gonadal activity in tree sparrows corresponds to increased steroidogenesis-linked reproductive activity observed in male tree sparrows and the study shows the highest plasma cholesterol level (February) before the higher gonadal activity (April onwards) which is also parallel with the plasma cholesterol level in male tree sparrows, thus validating the role of cholesterol in the steroidogenic enzymes' biosynthesis (Yatung & Trivedi,

2024). In females, during the process of ovulation, progesterone plays a vital role. Therefore, elevated progesterone in pre-breeding and breeding seasons may help the coming nesting and egg-laying behaviors (Licht et al., 1982). The highest plasma progesterone level was observed during June (reproductive phase), which corresponds with the larger follicular size and higher reproductive activities and is consistent with reports in other species such as olive ridley sea turtle (*Lepidochelys olivacea*), Jinding duck (*Anas platyrhynchos*) (Licht et al., 1982; Cui et al., 2021). In this study, maximum molt in body and primary flight feathers was observed during the post-breeding phase, i.e., August and September, and is consistent with reports on this and other seasonally breeding avian species (Trivedi eta al., 2006; Yatung & Trivedi, 2024). Higher mRNA expression levels of *Tshβ*, *Dio2*, and *GnRH* in the hypothalamus during the active reproductive phase are consistent with the existing reports of tree sparrows and other seasonally breeding avian species (Trivedi et al., 2019; Renthlei et al., 2021; Yatung & Trivedi, 2024). These results reaffirm these transcripts' critical function in controlling seasonal reproduction in animals that breed seasonally (Yoshimura et al., 2003; Nakao et al., 2008; Trivedi et al., 2019). Moreover, a decrease in the transcription levels of *Dio3* during the reproductive phase and an increase in these transcripts during the post reproductive phase (October to December) reconfirm the involvement of the product of these transcripts in inhibiting reproduction (Fig. 44). However, *GnIH* transcript showed multiple fluctuations in expression and the highest peak was observed during post-breeding phase. (Fig. 44). These results are consistent with previous reports on the repressive nature of these transcripts on gonadal activity (Tsutsui et al., 2000; Yoshimura et al., 2003; Nakao et al., 2008; Ubuka et al., 2008; Majumdar et al., 2015; Dixit & Byrsat, 2018; Trivedi et al., 2019; Zhang et al., 2019). Further, a study on Japanese quail suggests a different pattern in the timing of seasonal reproduction, which can be controlled by other environmental factors such as temperature and photoperiod-independent control of expression of *Fshβ*. The findings indicate that during the artificial spring equinox, surges in prolactin and thyrotropin-stimulating hormone-β, in addition, testicular development, occur before photoinduced increases in *Fshβ* expression. Furthermore, our findings are consistent with those of another long-day breeding species Japanese quail. The study demonstrates that

gonadal development is caused by short photoperiods (less than 12 h of light per day) and that vimentin immunoreactivity and *Dio3* expression are significantly upregulated in the median eminence after prolonged exposure to short photoperiods (Majumdar et al., 2023). Our results support it, and we observed increased *Dio3* mRNA expression from July through January, during the breeding season (Fig. 44b). The existing literature suggests that day lengths that exceed 12 h increase the thyrotrope production and start a series of biochemical reactions in the MBH that enable *GnRH* to trigger gonadotropes to release *Fshβ*, to start the process of gonadal development. Nonetheless, our investigation identified a higher level of *Tshβ* expression throughout the breeding period.

To evaluate the steroidogenic pathways that govern the seasonal reproductive cycle, we have investigated the expression of transcripts of enzymes (*StAR, Er, Cyp17a1, Nr4a1, Scp2, Cyp11a1, Hsd11b2, Vdac1, Cyp11b, Cyp19a1,* and *Foxl2*) as well as circulating progesterone titers throughout the year (Figs. 45‑46). Neurons and glial cells of the brain can locally synthesize biologically active steroids de novo (Baulieu, 1998; Compagnone & Mellon, 2000; Do Rego et al., 2009). This study shows the expression of these transcripts in the hypothalamic tissues with annual variations (Fig. 45). The yearly reproductive stage of tree sparrows directly influences the expression of these steroidogenic transcripts. Higher level expression of the *StAR, Cyp11a1, Nr4a1, Cyp11b, Hsd11b2, Foxl2, Cyp19a1, Er*, and *Vdac1* was observed during the breeding phase in the hypothalamus, and numerous peaks in the hypothalamus were perceived for *Cyp17a1* (Fig. 45). Moreover, evidence from the recent publication shows a similar pattern in the expression of these steroidogenic enzymes peaked during the breeding phase (Yatung & Trivedi, 2024). Also, other organisms suggest physiology-dependent changes in these transcripts' expression, such as in edible frogs, brain levels of neurosteroids oscillate simultaneously with the reproductive cycle. Seasonal fluctuations in gene expression levels of *Hsd3b1, Hsd17b1, Srd5a1,* and *Cyp19a1* were observed in the Diencephalon‑Mesencephalon region of both sexes of the edible frog, and higher transcript levels were observed in the reproductive period than in post post-reproductive period (Santillo et al., 2017). The study depicts that the steroidogenic process and seasonal variation in these

steroidogenic enzymes are potentially relevant during folliculogenesis, as reported by Krasnow (1991). In seasonal breeders, the differential expression of steroidogenic enzymes during the reproductive and nonreproductive cycles could be the local key to controlling follicular development. In adult female tree sparrows, expression of these steroidogenic transcripts showed reproductive stage-linked expression in the ovary (Fig. 46). These transcripts were upregulated before or throughout the reproductive stage (Figs. 43,46). These sex steroids exert a strong influence on the reproductive behavior of vertebrates. The steroidogenic process in gonads displays discrete sex and season-specific patterns and key enzyme expression. Higher expression of sex steroids during the pre-breeding or breeding phase has also been demonstrated in other species. In seasonally breeding vertebrate species, mating behavior, elevated gametogenesis, and peak circulating sex steroid concentrations coincide temporally (Crews, 1984). In the toad, circulatory sex steroid levels fluctuate seasonally and coincide with major reproductive events in species with distinct breeding seasons (Itoh & Ishii, 1990). In the ovary, peak expression of *StAR* transcript was observed during the pre-breeding phase (Fig. 46a). Maximum expression of *StAR* was also observed in the ovary of green anole lizards (*Anolis carolinensis*) during the spring mating season (Peek & Cohen, 2018). The gene *StAR* is a vital stimulant for steroidogenesis and plays a critical role in the process of transporting cholesterol from the outer to the inner membrane of the mitochondria. In line with our findings, higher ovarian *StAR* mRNA expression before or during the reproductive phase correlates with higher steroidogenic activity. This is also visible in the testis tissues of male tree sparrows (Yatung & Trivedi, 2024). *StAR* proteins are also associated with a voltage-dependent anion channel (*Vdac2*) at the mitochondria-associated endoplasmic reticulum membrane to enable the transport of cholesterol to the inner mitochondrial membrane (Maurya & Mahalakshmi, 2016). We also observed peak expression of *Nr4a1* mRNA in the ovary during May, corresponding with higher gonadal activities in tree sparrows. Expression of *Nr4a1* is modulated by several agents, including Ca2+ and growth factors (Li et al., 2010). In gonads, *Nr4a1* controls the expression of several genes involved in steroidogenesis, including *StAR* (Martin et al., 2008), *Cyp17a1* (Zhang & Mellon, 1997), *Hsd3b2* (Bassett et al., 2004a), and *Cyp11b2* (Bassett et al., 2004b). Higher expression of *Scp2* mRNA before/during the peak follicular growth and activity was observed (Fig. 46c).

The *Scp2* gene, having two distinct transcription initiation sites, encodes a 15 kDa pro-Scp2 protein and the 58 kDa SCPx, which have identical C-termini (Ohba et al., 1995). *Scp2* and *SCPx* proteins are highly expressed in all tissues with increased cholesterol metabolism rates (Vahouny et al., 1987). The pre-breeding phase showed higher expression of *Cyp11a1* and *Cyp19a1* than the post-breeding phase (Fig. 46e), which aligns with reports (Wang et al., 2021). Impaired steroidogenesis results from a lack of the mitochondrial enzymes *Cyp11a1* and *Cyp11b* (Parajes et al., 2013). We observed higher expression of *Er* transcripts during the breeding phase (Fig. 46j). Estrogen differentially controls reproductive and non-reproductive responses through its receptors (Kuiper et al., 1998). We observed that *Cyp19a1* transcript levels were higher during the pre-breeding phase. *Cyp19a1* is a key enzyme that converts testosterone to androgen, a steroid hormone necessary to induce feminization (Andrews et al., 1997; Akazome et al., 2002). *Foxl2* mRNA levels also showed peak expression in February during the pre-breeding phase. More recently, it has been claimed that *Foxl2* is responsible for the transcriptional regulation of *Cyp19a1* in female gonads because *Foxl2* and *Cyp19a1* mRNA are co-expressed in the medullary region of female gonads during sex differentiation in the chicken (Govoroun et al., 2004). Several-fold expression of *Cyp17a1* transcripts was observed during January, March, and May during pre-/post-breeding season (Fig. 46f). *Cyp17a1* is the enzyme involved in the production of some androgens by converting 17α-hydroxy pregnenolone and 17α-hydroxyprogesterone and eventually metabolized to testosterone, a substrate for p450arom. Therefore, higher expression of this enzyme in females may indicate a contribution to estrogen production. This is consistent with previous findings (Nettle & Bateson, 2015). These observations demonstrate that when male tree sparrows are mating at their peak, steroidogenesis is actively involved. Other than bird species, in sea cucumber, real-time qPCR analysis of steroidogenic genes (*StAR, cyp10, 3βhsd, 17βhsd*) shows increasing levels of mRNA transcripts during gonadal maturation (Thongbuakaew et al., 2021).

6. CONCLUSIONS

The study may conclude that the key enzymes involved in steroidogenesis for sex hormone production are expressed in the hypothalamus and gonads (ovary in

females and testis in males) tree sparrows. Seasonal variations in these genes' regulation in the brain and gonads imply the possibility that seasonal distinctions in gene expression control tissue-specific steroidogenic capacity. Furthermore, a recent study on adult male tree sparrows found that the steroidogenic transcript expression patterns in adult female tree sparrows connected with the annual reproductive cycle are comparable (Yatung & Trivedi, 2024). Moreover, the present study advocates for male and female tree sparrows with the conserved mechanism. In addition, the tree sparrow's conjunctive mechanism for the growth and regulation of the gonadal system is shown by the seasonal variations in the stimulation of both androgenic and estrogenic pathways during the reproductive phase in the steroidogenic process. Furthermore, presenting research opportunities on the molecular mechanism of steroidogenesis regulation and reproductive capacity is crucial. This includes understanding the factors that influence the unique expression of these steroidogenic genes and what triggers their tissue-specific and time-dependent expression.

SECTION IV: EFFECT OF SHORT-TERM EXPOSURE TO DIM LIGHT AT NIGHT ON STEROIDOGENESIS

1. ABSTRACT

Anthropogenic factors, particularly urbanization, have immense implications on the natural light-dark transition cycle of the species. Additionally, since the majority of birds breed seasonally and rely on environmental cues to synchronize their seasonal activities, any disruption to this link has a detrimental impact on the birds' physiological processes. The present study examines the effects of dim light during nighttime hours on the transcriptional expression of reproductive, steroidogenic, and metabolic gene markers in adult male tree sparrows. Adult male tree sparrows were procured using a mist net, and after procurement immediately brought into the laboratory. Later, birds were categorized into two groups (n=6 birds/group). Group 1 was exposed to 12 hours of light and 12 hours of darkness, and also served as a control. Group 2 was also exposed to 12 hours of light and 12 hours of darkness, but with a constant dim light (10 lux) during the dark hours. The experiment was run for 2 weeks. At the end of the experiment, birds were sampled, and the hypothalamus, liver, and testis tissue were harvested and used for gene expression analysis. Blood plasma was used for hormonal and biochemical assays. The findings suggest that 2 weeks of exposure did not significantly change the body mass, plasma cholesterol, plasma glucose, and plasma testosterone levels. However, an increase in testicular volume was observed in dLAN-treated birds. Furthermore, elevation in hypothalamic transcripts (*Tshβ, Dio2, GnRH, and Eya3*) involved in seasonal reproduction, along with an increase in steroidogenic genes (*StAR, Scp2, Srd5a1, Hsd11b2*, and *Er*) in the testis, was observed; besides, liver metabolic transcript levels (*Acaca, Fasn, Hmcg, Idh2, Sdhaf4, Sdhaf2, Sdhc, Fh, Mdh, Foxo1, and Vip*) were also elevated in the dLAN-treated group compared to the control group. The study reveals that even a short-term exposure of 2 weeks duration to the lower intensity of dim light at night of 2 weeks can stimulate the hypothalamic gonadal axis with enhanced steroidogenic activity and liver metabolism in tree sparrows. These findings may help us comprehend how nighttime light affects the physiology of diurnal species.

2. INTRODUCTION

Urbanization is undoubtedly having a significant impact on the animal kingdom in a world where it is growing rapidly. One of the key effects of urbanization is anthropogenic light pollution (Shochat et al., 2006), which seems to be an imminent hazard to urban wildlife (Longcore & Rich, 2004). Most of the birds are seasonal breeders and use environmental cues to time their seasonal processes (Renthlei et al., 2022). Many of these birds make an exceptional study model to examine the effects of ALAN (Artificial light at night) and are diurnal species that depend on circadian light cycles as a trigger for essential biological rhythms (Gaston et al., 2014; Trivedi et al., 2014). The circadian system regulates homeostatic functions in many organisms, and Circadian rhythms are naturally occurring 24-hour rhythms in physiology and behavior that are most strongly coordinated by light information. It is increasingly becoming clear that disrupting the circadian clock with exposure to light at night has substantial physiological consequences. Variation in this light and dark cycle controls the initiation of reproduction through the HPG axis, and a rise in day length leads to elevated secretion of GnRH-I from the median eminence of the hypothalamus. This triggers the synthesis and release of LH and FSH. These hormones, in turn, are secreted into the bloodstream and induce gonadal maturation, and matured gonads secrete Testosterone and Estradiol , which are critical for both gamete maturation and the initiation and maintenance of reproductive behaviors (Yamamura et al., 2006; Dawson, 2008).

Over the last decades, the dramatic surge in ALAN has altered the chronological partitioning of day-night cycles. Consequently, the circadian clock-regulated biological processes, including hormone secretions, metabolism, physiology, cognition, and daily cycles in activity-rest and feeding patterns, are affected (Fonken et al., 2010; Fonken et al., 2013; Fonken et al., 2014; Gaston et al., 2015; Dominoni et al., 2018; Ouyang et al., 2018; Taufique et al., 2018; Renthlei & Trivedi, 2019; Alaasam et al., 2021; Sangma & Trivedi, 2023; Sangma et al., 2024). A study of captive European blackbirds (*Turdus merula*) demonstrated that exposure to even extremely low light intensities at night can affect the timing of reproductive functioning (Dominoni et al., 2013).

The maintenance of secondary sexual traits in vertebrates' seasonal reproductive cycles, known as spermatogenesis and spermiogenesis, is regulated by sex steroids, which are essential for the testis function. The biosynthesis of the steroid hormones initiates with the transportation of cholesterol into the mitochondria via the *StAR* gene, leading to sequential enzymatic reactions producing sex hormones (Yatung & Trivedi, 2024). Besides, the association between sex hormones such as estrogen, testosterone, progesterone, and metabolic genes is a complex and bidirectional relationship that influences the species' reproduction, metabolism, and overall physiology.

Besides, the fatty acid synthesis initiates through carboxylation of acetyl-CoA to malonyl-CoA, an irreversible step catalyzed by the enzyme Acetyl-CoA carboxylase (ACC). Subsequently, fatty acid synthase (FASN) generates palmitate. Similarly, within the mitochondria, the enzyme HMGCS2 catalyzes the formation of ketone bodies by combining acetyl-CoA and acetoacetyl-CoA to produce HMG-CoA (HMGC), which has a crucial role in lipid metabolism and cholesterol production, a rate-limiting enzyme in ketogenesis, where the body produces ketone bodies for energy when there is a lack of carbohydrates (Asif et al., 2022). Besides, the molecule acetyl-CoA, a key intermediate in lipid and cholesterol pathways, fatty acids from lipid breakdown are converted into acetyl-CoA via beta-oxidation, then enter the TCA cycle to be entirely oxidized for energy production.

DLAN, even at low intensities (e.g., 10 lux), can alter these pathways by suppressing melatonin and disrupting circadian rhythms, potentially upregulating steroidogenic and metabolic gene expression (Cho et al., 2016). A metabolic pathway, such as in the TCA cycle, CS (citrate synthase) initiates the very first step: the acetyl-CoA condenses with oxaloacetate to form citrate. Citrate is then isomerized to isocitrate by enzyme ACO1 (Aconitase 1), transforming one molecule into a structurally different molecule that proceeds on to the next step in the cycle. IDH2 (isocitrate dehydrogenase) catalyzes the conversion of isocitrate to alpha-ketoglutarate (α-KG), a vital phase in the production of cellular energy; basically, IDH2 enables the oxidative decarboxylation of isocitrate, which helps the mitochondria produce NADPH. Further, the SUCLG2 (succinate-CoA ligase GDP-forming subunit beta)

enzyme converts succinyl-CoA to succinate while simultaneously generating GTP through substrate-level phosphorylation. Besides, SDHAF4 (succinate dehydrogenase complex assembly factor 4) and SDHAF2 (succinate dehydrogenase complex assembly factor 2) facilitate the assembly of succinate dehydrogenase (SDHC), a key enzyme within the cycle that activates the oxidation of succinate to fumarate, thus enabling the cycle to proceed and contribute to energy production by linking it to the electron transport chain. Later, FH (Fumarate Hydratase) catalyzes the conversion of fumarate to malate, and MDH1(Malate Dehydrogenase 1) catalyzes the reversible conversion of malate to oxaloacetate, facilitating the transfer of reducing equivalents within the cycle and contributing to the malate-aspartate transport that moves metabolites across the mitochondrial membrane. PYGL, a liver glycogen phosphorylase, plays a vital part in carbohydrate metabolism by breaking down glycogen (stored glucose) into glucose-1-phosphate, contributing to the readily available glucose supply, particularly during fasting states; basically, it is responsible for mobilizing liver glycogen stores to keep blood sugar levels stable. The HMGCL gene provides instructions for making an enzyme called 3-hydroxymethyl-3-methylglutaryl-CoA lyase (HMG-CoA lyase). HMG-CoA lyase plays a critical role in breaking down proteins and fats from the diet (Veerappa & McClure, 2020; Martínez-Reyes & Chandel, 2020).

A study by Batra et al. (2019) validates a dLAN induced adverse effect on zebra finch metabolism in the liver, which is the primary center of metabolic homeostasis. In particular, elevated *Egr1* and *StAR* expressions indicated improved cholesterol production and lipid metabolism, and elevated *G6pc* and *Foxo1* mRNA transcripts indicated enhanced gluconeogenesis. Similarly, increased gluconeogenesis and cholesterol production under dLAN are indicators of protection against metabolic harm, as indicated by overexpressed Sirtuin1 (Sirt1). *Sirt1* communicates with transcription factors, including *Foxo1*, *Srebp*, and *Pgc-α*, to regulate the inclusive cellular lipid as well as glucose metabolism (Ye et al., 2016).

Likewise, exposure to dLAN reduces total neuronal densities in selected brain areas (Moaraf et al., 2020) and alters the gene expression associated with glucose and lipid metabolism in avian species (Zhu et al., 2023; Kumar et al., 2024). According to

some research, the onset of LH secretion in urban tree sparrow populations is accelerated by artificial anthropogenic light sources. Additionally, dLAN (dim light at night) has intensity-dependent effects on the activation/suppression of the HPG axis (Zhang et al. 2014; Zhang et al. 2019). Previously, we have shown the effect of urban environment on daily pineal machinery and clock genes expression (Renthlei & Trivedi, 2019), season-dependent effects of urban environment on the circadian clock of tree sparrow (Renthlei et al., 2020), and the effect of urban environment on the timing of progression of testicular recrudescence in tree sparrow (Renthlei et al., 2021). In all the above studies, birds were directly obtained from the urban and rural habitats, and hence various environmental factors may be involved in the contribution to the alterations of the physiology. However, Aizawl (23.73°N) has a smaller photoperiod variation (~11–13 hours), and tree sparrows potentially rely on ALAN as a dominant cue. To better understand the consequences of anthropogenic light at night on the physiology of diurnal birds, more controlled laboratory experiments are required. Therefore, the present study intended to understand the short-term exposure of dimly illuminated light at night (dLAN of 10 lux) on reproduction and metabolism-linked phenomena of tree sparrows under laboratory conditions. Moreover, several studies report on the effect of dim light at night, such as (Dominoni et al., 2013a,b; Fudickar et al., 2016; Cho et al., 2016; Fonken, L.K., et al., 2013) have reported 0.3-10 lux artificial light illuminance in the night time environment but with the exposure of long/short photoperiod for longer duration, i.e., 4 to 8 weeks, and observed a drastic effect of dim light at night on the physiological and behavioral processes.

As it is evident from the studies, long photoperiod can stimulate the HPG axis in long-day seasonal breeders. However, the present study hypothesizes that at the equinox photoperiod of 12L:12D, coupled with 10 lux of intensity for a shorter duration, can stimulate the reproductive-linked processes in this species. Therefore, the study examines the effect of 2-week exposure, which is shorter than most avian dLAN studies (e.g., 4–8 weeks in Dominoni et al., 2013a, b; long-term in Dominoni et al., 2013c) to dLAN of 10 lux on reproduction and metabolic-linked processes in this species.

3. MATERIALS AND METHODS

3.1. Animal Maintenance and Experiment

The study was approved by the Institutional Animal Ethics Committee (IAEC) of Mizoram University (No. MZU/IAEC/2023–24/05). Male adult tree sparrows were used in this study. Photosensitive adult birds (n=12) were collected locally using mist nets during late November, and transferred immediately to the animal house facility, and held in cages (28.5 x 22.1 x 14 cm) for acclimatization under NDL (Natural daylength conditions). Food (paddy), along with mealworms, was fed, and water was provided *ad libitum*. After a week of acclimatization, body mass was recorded using a top pan balance with an accuracy of 0.1 g. Bill's color and gonad size were also recorded (Yatung & Trivedi, 2024). Following the week of acclimatization, the birds were categorized into two groups (n=6/group), i.e, experimental and control groups, and birds of both groups were exposed to a daily light and dark cycle of 12L:12D, of which an experimental group being dimly illuminated with dLAN (Dim light at night) of 0.049 W/m^2. The experiment was run for 2 weeks, and after that, birds were sampled during the middle of the light phase (6 hr after lights on). Brain, liver, and gonads were harvested and stored at -80ºC for gene expression analysis. Blood (200–300 µL) from the trunk of each bird was collected in heparinized tubes, and plasma was harvested and stored at -20ºC for biochemical and hormonal assays.

3.2. Cholesterol assay

Using CHOLESTEROL KIT (Coral Clinical Systems, India), plasma cholesterol levels were measured as detailed previously (Yatung & Trivedi, 2024). In brief, cleaned test tubes were labeled as blank, standard, and test. Afterward, 1 mL volume of working reagent was pipetted out into blank, standard, and test sample tubes. 10 µL of water (distilled) was pipetted into a blank test tube, 10 µL of cholesterol standard into a standard test tube, followed by 10 µL of plasma sample into a sample test tube. After adding all the essential reagents to the test tubes, it was properly mixed and incubated at room temperature (25ºC) for 15 minutes. The absorbance value of the standard and test samples was measured against the blank within fifteen minutes at 505 nm wavelength using the SpectraMax MINI system (Multimode Microplate Reader, AFL System Molecular Devices, USA). All the sample was run in duplicate.

3.3. Glucose assay

Plasma glucose levels were determined using GLUCOSE KIT (Coral Clinical Systems, India) as per the protocol provided. Each sample was run twice. Cleaned test tubes were marked as blank, standard, and test. Then, 1 mL of glucose working reagent was pipetted into the blank, standard, and test sample tubes. Then, 10 µL of distilled water was added to a bank test tube, 10 µL of glucose standard into the standard test tube, and 10 µL of plasma into the sample test tubes. It was mixed properly and was incubated for 30 minutes at room temperature (25ºC). Later, within 60 minutes, absorbance was measured against the blank at 505 nm wavelength using a Multimode ELISA reader, SpectraMax MINI system.

3.4. Testosterone assay

As per manufacturer protocol, plasma testosterone levels were measured using enzyme immunoassay kits (Testosterone, Diametra, DKO002; Yatung & Trivedi, 2024). To quantify testosterone in this and other species, this assay has been validated (Pathak & Lal, 2010; Dixit & Singh, 2014). All the reagents were left at room temperature for 30 minutes before use. Plasma samples were kept on ice to thaw. The wash solution was diluted following the given protocol. All calibrators, controls, test samples, and blanks were run in duplicate. 25 µL of sample or control and 25 µL of calibrator were pipetted into each well, and 100 µL of conjugate was added to each calibrator and sample well. After adding the reagents, the plate was incubated for 1 hour at 37 °C temperature. After one hour, all the content was removed from each well by washing three times with 300 µL of diluted wash solution at every 5-minute intervals. The residual buffer was completely removed by tapping the plate upside down on an absorbent paper. After adding 100 µL of TMB substrate to each well and incubating at room temperature (25 °C) for 15 minutes in the dark, 100 µL of stop solution was added and gently mixed. Absorbance was taken using a Multimode ELISA reader, SpectraMax MINI system at 450 nm against the Blank within 5 minutes. Each sample was run in duplicate.

3.5. Gene transcription

The mRNA transcript expression of reproductive genes encoding for *Tshβ*, *Dio2*, *Dio3*, *GnRH*, and *GnIH* were measured in the hypothalamus tissue, and mRNA transcripts of steroidogenic genes encoding for *StAR*, *Nr4a1*, *Er*, *Scp2*, *Cyp17a1*, *Cyp11a1*, *Hsd11b2*, *Srd5a1* was measured in the testis tissue, also metabolic mRNA transcript expressions of (*Hmcg*, *Pygl*, *Sirt1*, *Acaca*, *Suclg2*, *Foxo1*, *Cs*, *Mdh*, *Aco1*, *Fasn*, *Vip*, *Sdhaf2*, *Idh2*, *Sdhc*, *Hmgcs2*, *Hmcgl*, *Sdhaf2*) were measured in the liver tissue. *18S* as a reference gene was used to determine the relative expression of transcripts (Yatung & Tivedi, 2024).

3.6. RNA isolation, cDNA synthesis, and qPCR analysis

Total RNA was pulled from tissue samples using TRIzol Reagent (Invitrogen by Thermo Fisher Scientific). The purity and concentration of total RNA were measured using (Nanodrop Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). And 2 µg RNA was used to process cDNA synthesis. RQ1 DNase (Promega M6101; Madison, WI, USA) kit was used to eradicate any probable genomic DNA contamination. For cDNA synthesis, the iScript™ cDNA Synthesis Kit (BIO-RAD, USA, 1708891) was used.

qPCR analysis was performed on Quant-Studio 5 (Applied Biosystem by Thermo Fisher Scientific, Foster City, CA, USA) as described in our former publications (Renthlei & Trivedi, 2019; Renthlei et al., 2019, 2021, Borah et al., 2019, 2020, 2022; Lalpekhlui et al., 2022; Sangma et al., 2024; Yatung & Trivedi, 2024; Yatung & Trivedi, 2025). Briefly, PCR reaction of the volume of 7 µL for each gene comprised 1 µL each of cDNA, gene-specific forward and reverse primers, 3 µL Power UpTM SYBR Green Master Mix (Applied Biosystem by Thermo Fisher Scientific, Foster City, CA, USA, A25742), and 1 µL of nuclease-free water (Ambion, AM9938). qPCR cycle conditions included 1 cycle at 95ºC (20 s), 35 cycles at 95ºC (01 s), 60ºC (20 s), and 95ºC (01 s), additional melt curve plot step included 1 cycle of 60ºC (20 s), and 1 cycle of 95ºC (01 s). The ΔΔCt method was used for gene expression analysis (Schmittgen, 2001). Each sample was run in duplicate.

3.7. Data Analysis

To determine the significant difference between the control and dLAN-treated group, an unpaired Student's t-test was used. $P<0.05$ was considered a significant difference. Analysis was done using GraphPad Prism version 8.

4. RESULTS

No difference was noted in the body mass ($p = 0.8066$; Student t-test; Fig. 57a), plasma cholesterol levels (p 0.5928; Student t-test; Fig. 57c), plasma glucose levels (p 0.4155; Student t-test; Fig. 57e), and plasma testosterone levels ($p = 0.7088$; Student t-test; Fig. 57d) before and at the end of the study. However, a significant difference was shown in the testicular volume between the control and dLAN group ($p=0.0160$; Student t-test; Fig. 57b). Higher testicular volume was observed in the dLAN group as compared to the control group ($p<0.05$).

All the reproductive transcripts measured showed significant differences in the hypothalamic tissue. Higher expression of reproductive stimulatory transcripts *Tshβ* ($p=0.0059$; Student t-test; Fig. 58a), *Dio2* ($p = 0.0348$; Student t-test; Fig. 58b), *GnRH* ($p = 0.0348$; Student t-test; Fig. 58c), and *Eya3* ($p = 0.0159$; Student t-test; Fig. 58f) was observed in the dLAN group than in the control group. However, lower expressions of inhibitory transcripts *Dio3* ($p = 0.0143$; Student t-test; Fig. 58d) and *GnIH* ($p = 0.0035$; Student t-test; Fig. 58e) were observed in the dLAN group in comparison to the control group.

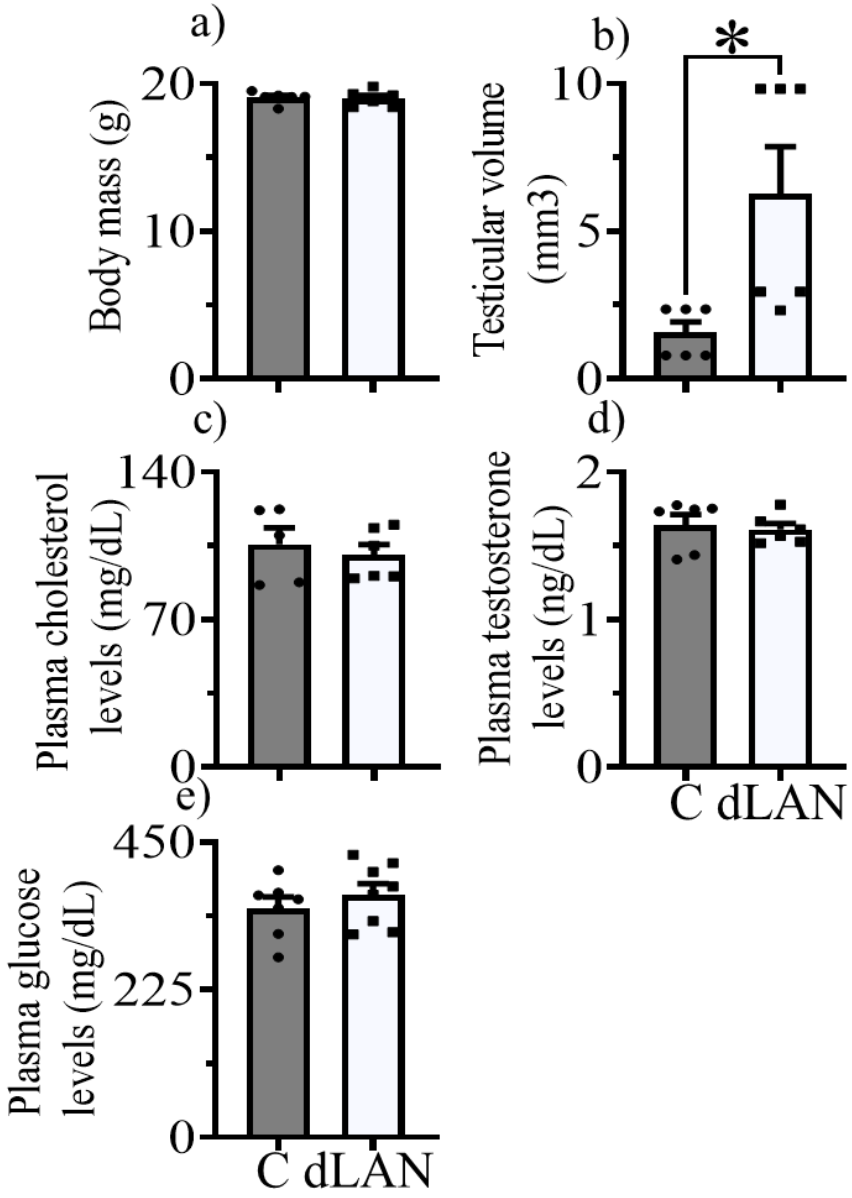

Fig. 57. Change in body mass (a), testicular volume (b), plasma cholesterol levels (c), plasma testosterone levels (d), and plasma glucose levels (e) in adult male tree sparrow after two weeks of exposure to equinox photoperiod (12L:12D) without night light (gray bar) or with dim light of 0.049 W/m^2 at night (white bar). An unpaired Student's t-test was used to determine significant differences between the groups. * represents a significant difference between the two groups.

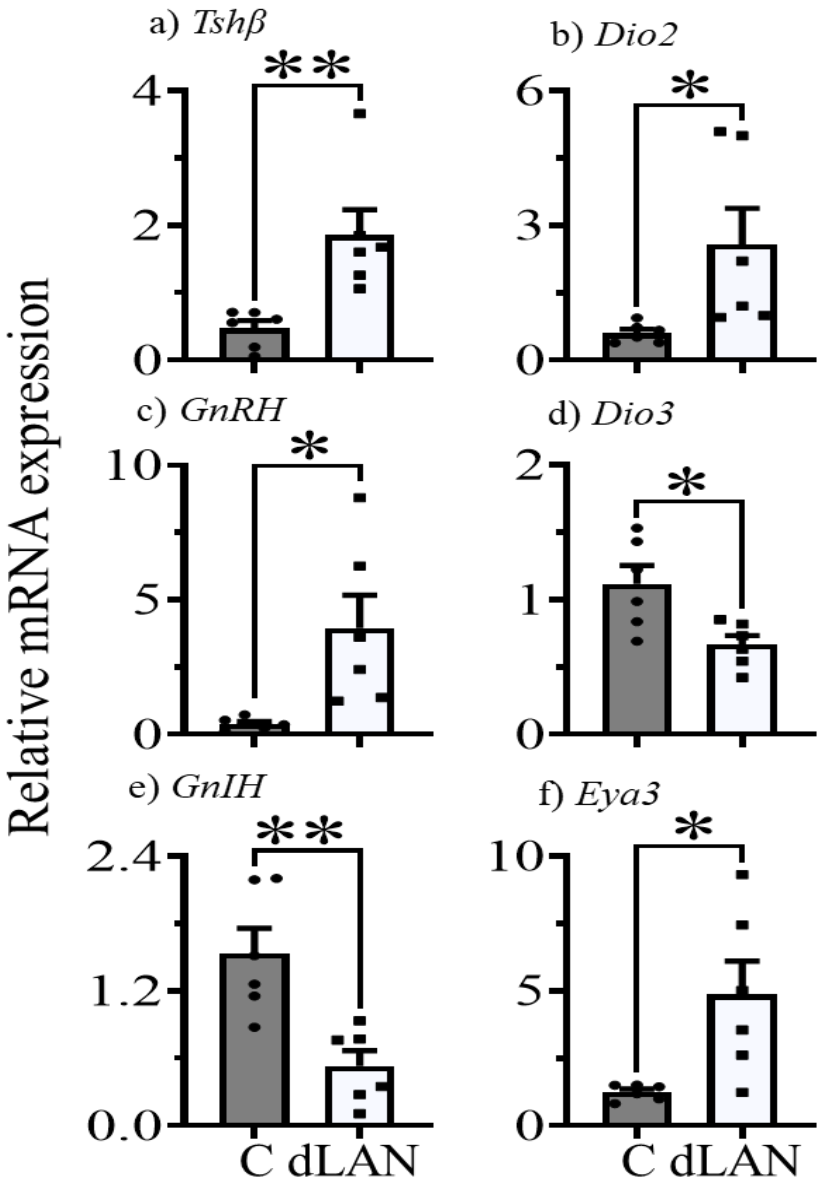

Fig. 58. Change in relative mRNA levels of reproductive genes in the hypothalamic tissues of adult male tree sparrow exposed to equinox photoperiod (12L:12D) without night light (gray bar) or with dim light of 0.049 W/m^2 at night (white bar). An unpaired t-test was used to determine significant differences between the groups. * represents a significant difference between the two groups.

Also, several steroidogenic markers were measured in the testis in the dLAN and control groups. In comparison to the LD controls, dLAN exposure groups showed significant increase in mRNA expression of enzyme *StAR* ($p = 0.0148$; Student t-test; Fig. 59a), *Scp2* ($p = 0.0151$; Student t-test; Fig. 59b), *Srd5a1* ($p = 0.0003$; Student t-test; Fig. 59f), *Hsd11b2* ($p = 0.0411$; Student t-test; Fig. 59g), and *Er* ($p = 0.0255$; Student t-test; Fig. 59h). However, no changes were observed between LD controls and dLAN exposed groups in mRNA expression of *Cyp11a1* ($p = 0.1067$; Student t-test; Fig. 59c), *Nr4a1* ($p = 0.3986$; Student t-test; Fig. 59d), and *Cyp17a1* ($p = 0.8259$; Student t-test; Fig. 59e).

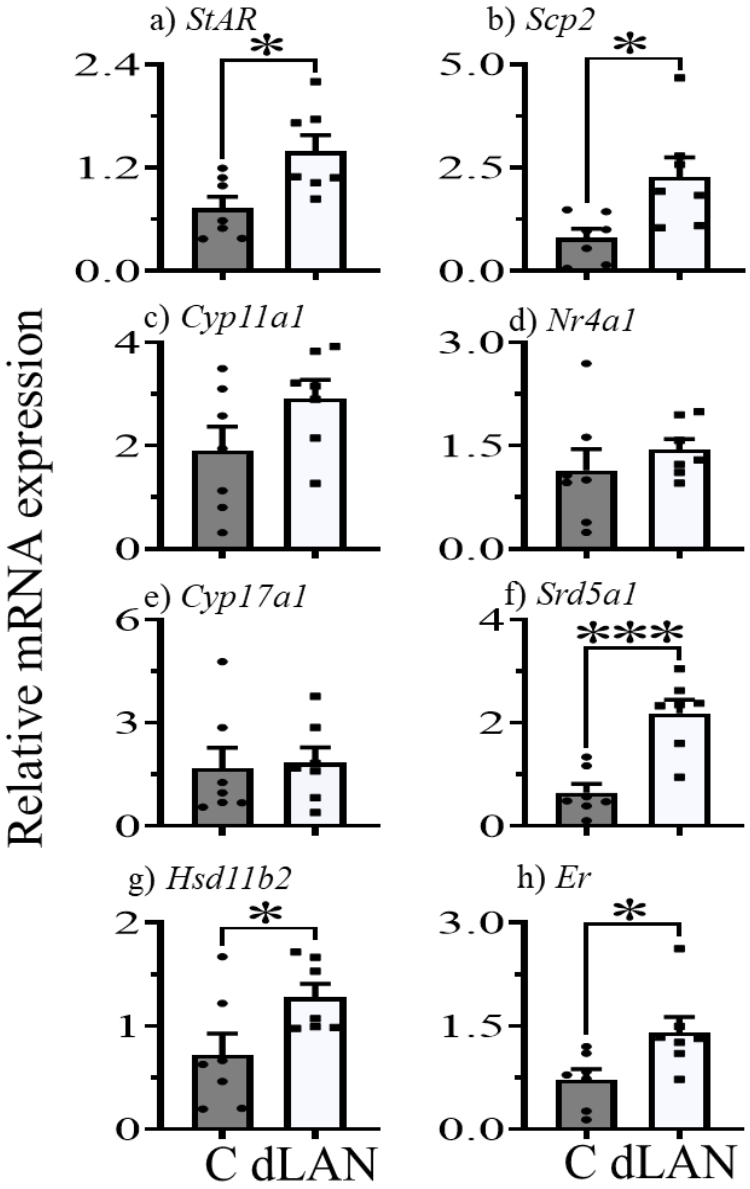

Fig. 59. Change in relative mRNA levels of steroidogenic genes in the testis tissues of adult male tree sparrows exposed to equinox photoperiod (12L:12D) without night light (gray bar) or with dim light of 0.049 W/m^2 at night (white bar). An unpaired t-test was used to determine significant differences between the groups. * represents a significant difference between the two groups.

Several gene markers known to be involved in lipid metabolism were also measured in the liver tissue of both the LD and dLAN-treated groups. A significant difference was observed in several genes studied in the liver tissue. Increased mRNA levels were observed in the enzymes *Acaca* ($p = 0.0153$; Student t-test; Fig. 60a), *Fasn* ($p = 0.0350$; Student t-test; Fig. 60b), and *Hmcg* ($p = 0.0039$; Student t-test; Fig. 60e). However, no significant change was observed in mRNA levels of enzymes *Sirt1* ($p = 0.8407$; Student t-test; Fig. 60d) and *Hmgcs2* ($p = 0.1107$; Student t-test; Fig. 60c). Similarly, mRNA levels of metabolic genes involved in carbohydrate, protein, TCA cycle, and gastrointestinal metabolism were also measured in the liver tissue. Higher mRNA levels were observed in the treated group for enzymes *Idh2* ($p = 0.0207$; Student t-test; Fig. 61c), *Sdhaf4* ($p = 0.0077$; Student t-test; Fig. 61e), *Sdhaf2* ($p = 0.0085$; Student t-test; Fig. 61f), *Sdhc* ($p = 0.0489$; Student t-test; Fig. 61g), *Fh* ($p = 0.0278$; Student t-test; Fig. 61h), *Mdh* ($p = 0.0265$; Student t-test; Fig. 61i), *foxo1* ($p = 0.0368$; Student t-test; Fig. 61l) and, *Vip* ($p = 0.0180$; Student t-test; Fig. 61m). Whereas, no difference was observed between the LD and dLAN group for the genes *Pygl* ($p = 0.5503$; Student t-test; Fig. 61j), *Cs* ($p = 0.1215$; Student t-test; Fig. 61a), *Hmcgl* ($p = 0.6057$; Student t-test; Fig. 61k), *Suclg2* ($p = 0.1591$; Student t-test; Fig. 61d) and *Aco1* ($p = 0.1636$; Student t-test; Fig. 61b).

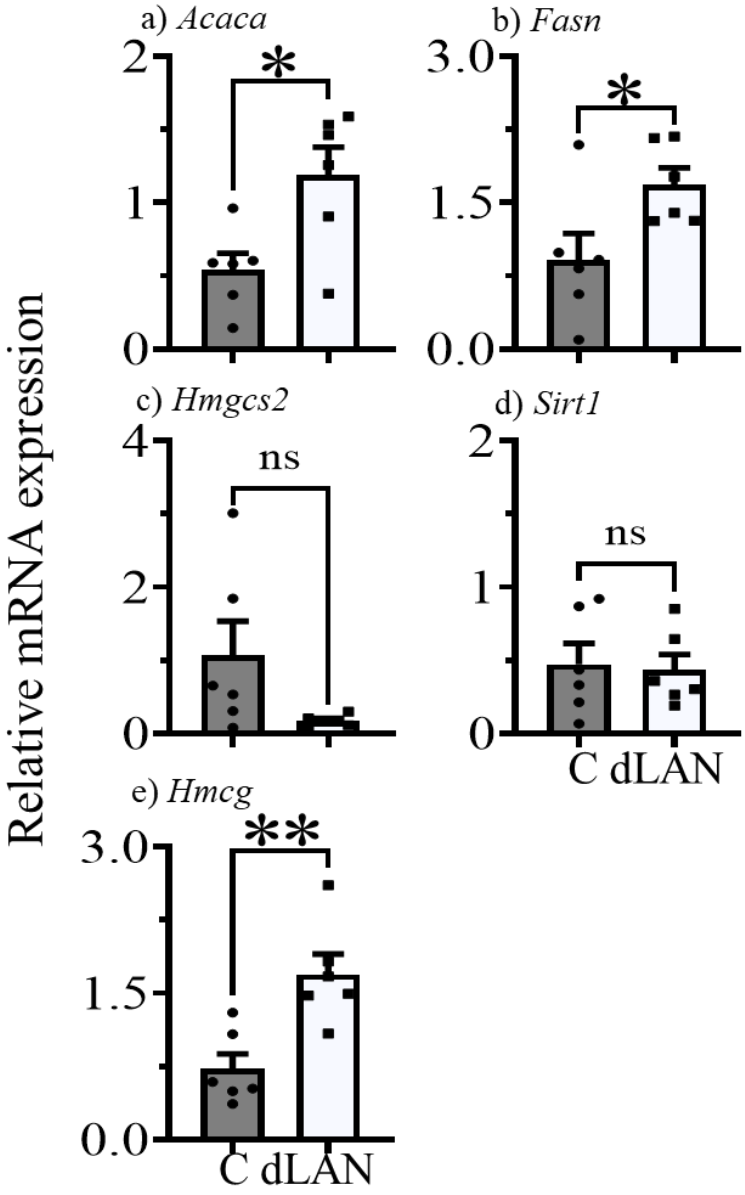

Fig. 60. Change in relative mRNA expression of hepatic transcripts involved in metabolism in the liver tissues of adult male tree sparrows exposed to equinox photoperiod (12L:12D) without night light (gray bar) or with dim light of 0.049 W/m^2 at night (white bar). An unpaired t-test was used to determine significant differences between the groups. * represents a significant difference between the two groups.

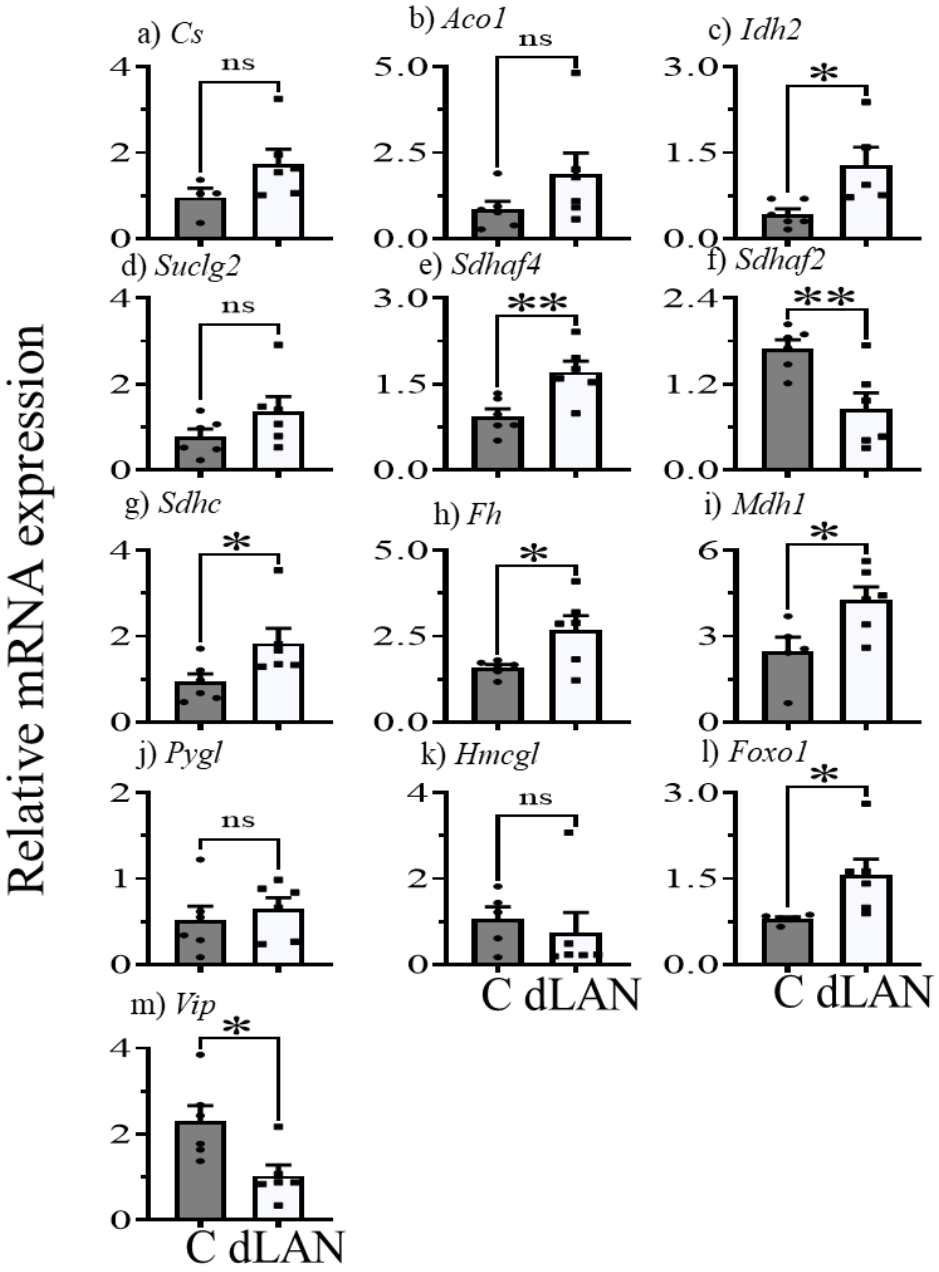

Fig. 61. Changes in relative mRNA expression of hepatic transcripts involved in metabolism in the liver tissues of adult male tree sparrows exposed to an equinox photoperiod (12L:12D) with or without night light (gray bar) or with dim light of 0.049 W/m² at night (white bar). An unpaired t-test was used to determine significant differences between the groups. * represents a significant difference.

5. DISCUSSION

The light/dark cycle is vital for an organism's behavioral and physiological processes. Besides, it is well known that artificial lights raise nighttime luminance, generating the most evident effect of light pollution. Exposure to dim light, dLAN of 10 lux at night for two weeks, significantly altered the reproductive and metabolic processes in tree sparrows, advancing gonadal development and shifting metabolic gene expression. These results suggest that dLAN acts as a potent environmental disruptor, representing the effects of extended photoperiods and overriding the natural light-dark cycles, which are crucial for synchronizing physiological rhythms. Studies have reported that dim light at night (dLAN) as low as 0.3 lux intensity can advance the reproductive physiology in European blackbirds, developing functional testes earlier (Dominoni et al., 2013). This observation is consistent with the present study, where higher testicular volume was observed when the birds were exposed to dLAN (10 lux) in comparison to the LD control birds. However, the study reveals no significant changes in body mass, plasma cholesterol, plasma glucose, and plasma testosterone levels (Fig. 57a, c, d,e), which could be the result of short-term exposure to dLAN (2 weeks) and higher intensity (10 lux), as testosterone elevation may require longer exposure or lower intensities to avoid feedback inhibition (Navara & Nelson, 2007). Whereas, the observed increase in testicular volume and upregulation of the hypothalamic reproductive mRNA transcripts (*Tshβ*, *Dio2*, *GnRH*, *Eya3*) with simultaneous downregulation of inhibitory hormones (*Dio3*, *GnIH*) point to a disruption of melatonin signaling as a central mechanism. Greives et al. (2012), reported continuous administration of melatonin can delay the lay time of wild great tits (*Parus major*). Melatonin, suppressed by even low-intensity light (Dominoni et al., 2013). In addition, McGuire et al. (2011) discussed that melatonin is known to induce the *GnIH*, whose effect, in turn, is to inhibit the development and maintenance of the gonads. Therefore, in dLAN-treated birds, reduced melatonin likely disinhibited *GnRH*, accelerating testicular growth (Fig. 57c). This is supported by elevated expression of steroidogenic genes (*StAR*, *Scp2*, *Srd5a1*, *Hsd11b2*, *Er*) in the testes, which enhance cholesterol transport and steroid hormone synthesis, facilitating gonadal maturation. This is also consistent with this species, where birds from urban habitats had a delay in the onset of melatonin release with attenuated amplitude

(Renthlei & Trivedi, 2019). Further, the urban environment also alters phase, amplitude, or both in the expression of transcripts for *Mel1a*, *Mel1 b,* and *Aanat* in tree sparrows (Renthlei & Trivedi, 2019). Correspondingly, downregulating the transcript levels of reproductive inhibitory hormones such as *Dio3* (Fig. 57d) and *GnIH* (Fig. 57e) is also evident in the study. The absence of significant changes in plasma testosterone, cholesterol, and glucose levels may reflect the short-term (2-week) exposure, as longer durations or lower intensities might be required to overcome feedback inhibition (Navara & Nelson, 2007).

Evaluation of the seasonal reproductive cycle is regulated by steroidogenic pathways. The study also examined the transcript expressions of genes (*StAR*, *Nr4a1*, *Er*, *Scp2*, *Cyp17a1*, *Cyp11a1*, *Hsd11b2*, *Srd5a1*) in the testis of dLAN and LD control. Expression of these steroidogenic transcripts is directly influenced by the annual reproductive stage of tree sparrows (Yatung & Trivedi, 2024). During the study, higher expression of *StAR* (Fig. 59a) was observed in the dLAN group than in the LD control. *StAR* is known to be involved in the transportation of cholesterol into mitochondria for steroidogenesis to commence. The higher expression of *StAR* corresponds with the larger testicular volume and increased steroidogenic activity. Moreover, increased mRNA transcripts of *Scp2* (Fig. 59b) were observed in the dLAN group. *Scp2* is a sterol protein that associates with *StAR* to enhance the movement of cholesterol between vesicles and mitochondria, hence, stimulating mitochondrial pregnenolone synthesis. *Scp2* transcripts are more consistent in the potential steroidogenic compartments of the ovary and tropic hormones that stimulate steroidogenesis and increase *Scp2* gene expression (Pfeifer et al., 1993), which is consistent with our findings. Higher expression of *Srd5a1*, *Hsd11b2*, and *Er* was found in the dLAN group as compared to the LD control. *Srd5a1* (Fig. 59f) plays a fundamental role in the catabolism of testosterone and DHT (dihydrotestosterone) (Liu et al., 2020), whereas estrogen modulates reproductive and non-reproductive responses through its receptors differentially (Kuiper et al., 1998). Also, it is vital for the normal development of reproductive organs and the expression of both male and female reproductive behavior (Rissman et al., 1999). These increased expressions of reproductive-linked steroidogenic markers suggest that exposure to light at night advances gonadal

function and increases steroidogenic activity. However, no significant variation was found in the transcript expressions of *Cyp11a1*, *Nr4a1,* and *Cyp17a1* (Fig. 59c-e) between dLAN and LD control.

Several genes involved in metabolism were also evaluated. The study examined the gene markers that are known to be involved in lipid, carbohydrate, protein, TCA cycle, and gastrointestinal metabolism in the liver tissue. Upregulation of the transcript expression of metabolic genes involved in fat synthesis and TCA cycle was observed in DLAN-treated birds, such as *Acaca*, *Fasn, Hmgc Fh*, *Mdh1*, *Sdhaf4*, *Idh2*, *Sdhc,* and *Sdhaf2,* suggesting the breakdown of lipids (through beta-oxidation) to produce acetyl-CoA, which is the shared intermediate between fat synthesis and TCA cycle initiation. Metabolically, dLAN upregulated genes involved in lipid synthesis (*Acaca, Fasn, Hmgc*) and the TCA cycle (*Fh*, *Mdh1*, *Sdhaf4*, *Idh2*, *Sdhc*, *Sdhaf2*) indicate enhanced fatty acid and cholesterol production. These changes likely support the increased steroidogenic demands of advanced gonadal function, as cholesterol is a precursor for steroid hormones. The elevated *Foxo1* expression suggests enhanced gluconeogenesis, potentially reflecting a metabolic shift to meet the energy demands under disrupted circadian conditions. The lack of changes in other metabolic genes (*Aco1, Sirt1, Hmgcs2, Cs, Suclg2*) may indicate a delayed or context-specific response, warranting further investigation into exposure duration and intensity. Hence, the activity of lipid metabolism directly influences the function of the TCA cycle. Thus, the expression of genes encoding for the enzymes involved in the TCA cycle can be regulated based on the availability of lipids and the need for energy production, therefore suggesting genes expression involved in lipid synthesis is directly parallel to TCA cycle (Satapati et al., 2012; Bender et al., 2015; Yoon et al., 2021). Whereas, no significant changes in the transcript expression of *Cs*, *Suclg2* were observed under dLAN. Higher transcript expression of *Foxo1* (Fig. 61b) suggested enhanced gluconeogenesis under dLAN-treated birds as compared to LD control and is consistent with the report (Fonken et al., 2010; Batra et al., 2019).

6. CONCLUSIONS

In conclusion, the present work reveals the effect of DLAN on multiple tissues (hypothalamus, liver, testes, and blood). Brief exposure to a dimly illuminated night light alters the light/dark cycle-induced reproduction and metabolism-linked phenomenon and upregulates the steroidogenic processes at the gonadal level and metabolic processes in the liver. This upregulation could be a mismatch with the interval milieu among various physiological functions and could be potentially harmful for wild populations.

SECTION V: EFFECT OF TEMPERATURE ON STEROIDOGENESIS

1. ABSTRACT

Day length is one of the most consistent environmental factors on which animal depends for breeding and survival. Apart from the photoperiod (day length), other factors such as food availability and temperature can also affect their physiology and behaviour. The present study hypothesized that exposure to high and low temperatures at the photorefractory stage affects photoperiodic responses during the photo-stimulatory phase. For this study, adult male photorefractory birds were captured using a mist net. Birds (n=10/group) were divided into two groups. Group one was exposed to short photoperiod (8L:16D) but either high (30 ± 2ºC) or low temperature (20 ± 2ºC) for five or seven weeks and then later transferred to a long photoperiod of (16L:8D) for another 30 days. Group two was also exposed to high and low temperatures as group one, but the duration of short photoperiod was maintained for 7 weeks, followed by a long photoperiod of (16L:8D) for another 30 days. At the end of the experiment, all birds were sampled in the middle of the light phase. Brain and testis tissue were harvested for gene expression analysis. Body mass and testicular volume were recorded before and after the end of the experiment. The result reveals no significant changes in the body mass of both the 5-week and 7-week groups. However, a significant increase in the testicular volume was observed in the low temperature-treated group under the 5-week SD exposure. Also, mRNA levels of *Tshβ*, *Dio2*, *Dio3*, *GnRH*, *GnIH*, and *Eya3* were measured in the hypothalamus, and expression levels of *StAR*, *Er*, *Cyp17a1*, *Cyp11b*, *Foxl2*, and *Nr4a1* were measured in the testes. Exposure to 5 weeks of high temperature coupled with short photoperiod suppresses expression of *Tshβ*, *Dio2*, and *GnRH* in the hypothalamus and *StAR*, *Er*, *Cyp17a1*, and *Cyp11b* in the testes under long days. No such effects were observed in birds exposed to high temperatures along with SD for seven weeks. The study findings suggest that exposure to low temperature during the photorefractory stage regulates photoperiodic responses during the photo-stimulatory stage in time time-dependent manner.

2. INTRODUCTION

Seasonality is the physiological processes' regular start, stop, and restart (Renthlei et al., 2022). The survival of many species is dependent on seasonal responses. The light-dark cycle predominantly controls these seasonal processes (Liddle et al., 2022). Non-photic cues, on the other hand, may also be important modulators of the physiological responses. This annual seasonal transition determines the reproductive success of many species. In addition, to ensure breeding when offspring survival is at its highest, seasonal breeders only produce during specific months. Besides, photosensitivity and photorefractoriness are the two physiological states of reproduction that many bird species experience annually. Photosensitive long-day species undergo gonadal growth regression cycles on exposure to long days, pass from photosensitive to photostimulatory, and finally become photorefractory (Dawson et al., 2001). If a species experiences photorefractoriness, gonads will not grow further until exposed to a short photoperiod for a certain duration (Renthlei et al., 2022).

According to the proposed molecular mechanism of avian seasonal reproduction, the pars tuberalis (PT) of the anterior pituitary gland and the ependymal cells (ECs) on the ventrolateral walls of the third ventricle in the MBH regulate reproduction (Yoshimura et al., 2003; Nakao et al., 2008). *TSH*, thyrotropin, secreted by PT under long stimulatory photoperiod, acts on the ECs in the MBH via the *TSH* receptor signaling pathway to initiate transcription *Dio2*. *Dio2* is involved in encoding the enzyme that activates the thyroid hormone, whereas *Dio3* is involved in encoding the enzyme that deactivates thyroid hormone. As a result, *GnRH* nerve terminals and the glial endfeet in the median eminence (Yoshimura et al., 2003) undergo structural alterations. It controls downstream processes at the gonadal level and regulates *GnRH* secretion from the hypothalamus to the portal capillary system (MacDougall et al., 2009). *GnRH* activity is inhibited by transcription of another gene that encodes *GnIH* (Tsutsui et al., 2000). Although the current model of seasonal reproduction is only shown in the domestic Japanese quail, a relative photorefractory species, these molecules have also been shown to be up- or down-regulated depending on photoperiod and physiological state in absolute photorefractory avian species (Dixit & Byrsat, 2018; Zhang et al., 2019; Trivedi et al., 2019; Renthlei et al., 2021).

However, a very recent study on Japanese quail suggests an alternative model (Majumdar et al., 2023). A transcriptome analysis revealed that the expression of *FSHβ* is regulated independently of photoperiod, and that additional environmental cues, such as temperature, may modulate when seasonal reproduction happens. According to Majumdar et al. (2023), the study shows that during the supplementary spring equinox, increases in prolactin, thyrotropin-stimulating hormone-β, and testicular development occur after photoinduced increases in *FSHβ* expression. In vertebrates, sperm development and maturation are directly regulated by gonadal steroid hormone production. Changes in *StAR* and *Cyp17* likely influence overall steroidogenic production during the reproductive cycle in male rainbow trout (Kusakabe et al., 2006). There is growing evidence that suggests *Foxl2* plays a role in the transcriptional regulation of *Cyp19a1* and *CYPp17* (Wang et al., 2007) and is sensitive to thermal exposure (Yamaguchi et al., 2007).

Additionally, environmental factors like temperature, photoperiod, and food availability alter gene expression and cause physiological changes. Also, the environment is changing, as well as urbanization and city growth, both drastically increasing. Moreover, the changing environment over the past few decades has altered the phenological swings in occurrences across trophic levels. (Parmesan et al., 2003; Thackeray et al., 2010). The shift of phenology, or the timing of seasonal occurrences, is the most apparent consequence of urbanization (Parmesan et al., 2003). An important environmental change that has altered biological systems is a temperature rise (Walther et al., 2002). Temperature variation in the spring might shift many tropical birds' breeding time (Both & Visser, 2001). Past ambient temperature also helps decide the timing of birds' egg-laying dates (Marcel et al., 2003; Salvante & Williams, 2003). The direct or indirect effects of temperature on photoperiodically induced gonadal development remain unclear. Under normal environmental circumstances, the cycles of temperature and duration are inseparable. Moreover, day length alone may not be enough to control seasonal behaviors; temperature may also play a role alongside day length. In the earlier study, we showed that high temperatures reduce the growth of testis in photosensitive tree sparrows, and that high temperatures during the photosensitive stage can change testicular responses during the photostimulatory phase (Renthlei et al., 2021). Here, we have investigated the effects

of exposing photorefractory tree sparrows to a short photoperiod and either high or low temperatures for variable lengths of time after subsequent exposure to long days of photostimulatory responses.

3. MATERIALS AND METHODS

The study was approved by the Institutional Animal Ethics Committee (IAEC) of Mizoram University (No. MZU/IAEC/2023–24/05). Adult tree sparrows were procured locally using a mist-net in September when they were in the photorefractory stage (Renthlei, 2021). The study was conducted on adult male birds. Birds were randomly divided into two groups (n= 10/group). All the birds were exposed to a daily photoperiod of 8 h light and 16 h dark (8 L:16D; SD) but either to high temperature (30±2ºC; HT) or low temperature (20±2ºC; LT). After five weeks of respective treatment (SD with high or low-temperature conditions), half of the birds (n=5/group) from each group were transferred to long photoperiod (16 L:8D; LD) for 30 days. The remaining birds (n=5/group) of both groups were transferred to long photoperiod after seven weeks of respective treatment (SD with high or low-temperature conditions). Body mass and testicular volume of individual birds were assessed at the beginning, after 5 or 7 weeks of SD exposure, and after 30 days of LD exposure. At the end of the study, all birds were sampled during the middle of the light phase. The hypothalamus and testes were excised from individual birds and stored at -80ºC till samples were processed for gene expression studies.

3.1. Gene transcription

The mRNA transcripts of genes coding for *Tshβ*, *Dio2*, *Dio3*, *GnRH*, and *GnIH* were measured in the hypothalamus, while mRNA transcripts of genes coding for steroidogenic genes (*StAR*, *Er*, *Cyp17a1*, *Cyp11b*, *Foxl2*, and *Nr4a1*) were measured in the testis. *18S* was used as a reference gene.

3.2. RNA isolation, cDNA synthesis, and RT-PCR (qPCR)

Tri reagent solution (Invitrogen; USA) was used to homogenize the tissues, and the phenol-chloroform method was followed to extract the total RNA (Renthlei et al., 2019). Extracted RNA was quantified (ND-ONE NanoDrop one spectrophotometer; ThermoFisher Scientific, USA), and 1-µg RNA was used to

prepare cDNA. To remove any potential genomic DNA contamination, RQ1 DNase (Promega M6101; Wisconsin, USA) kit was applied. The iScript™ cDNA synthesis kit (Bio-Rad) was used to synthesize cDNA. qPCR amplification was done using Quant-Studio 5 (Applied Biosystem by Thermo Fisher Scientific; USA) machine as detailed in a previous publication from our lab (Renthlei & Trivedi, 2019; Renthlei et al., 2020; Borah et al., 2020; 2022; Lalpekhlui et al., 2022; Trivedi et al., 2022; Yatung & Trivedi, 2024; 25). *18S* was used as a control gene (Renthlei & Trivedi, 2019; Yatung & Trivedi, 2024; 25). The relative transcription of genes was determined using the ΔΔCt method (Livak & Schmittgen, 2001).

3.3. Statistical analysis

Data are presented as mean (mean±SE). GraphPad Prism version 8.0 was used for preparing graphs and statistics. The student's t-test was used to compare two-point values. Two-way analysis of variance (Two-way ANOVA) followed by a post-test Bonferroni test was used to determine the significant differences between the groups comparing two factors together (high vs. low temperature and time).

4. RESULTS

Five weeks of temperature treatment did not affect the body mass as no significant differences in body mass in both high and low temperature treated groups were observed at the beginning and the end of the study ($F_{5,29} = 2.611$, $P = 0.421$; two Way ANOVA; Fig. 62a). However, exposure of 5 weeks of short photoperiod (8L:16D) coupled with high and low temperature had a differential effect on the testicular growth after the exposure to a long photoperiod of (16L:8D) for 30 days ($F_{5,29} = 15.742$, $P = 0.0042$; Two-way ANOVA; Fig. 62b). After 30 days of a long day exposure testicular growth was significantly higher in the low-temperature treated group than high-temperature treated group ($P<0.05$; Bonferroni's multiple comparison test; Fig. 62b). Also, the effect of temperature treatment was further reflected at the transcription levels. A significantly higher expression of *Tshβ* ($P < 0.05$; Student t-test, Fig. 63a), *Dio2* ($P < 0.05$; Student t-test, Fig. 63b), and *GnRH* ($P < 0.05$; Student t-test, Fig. 63c) in the hypothalamus of the low-temperature treated group was observed; whereas, no differences in the expression levels of *Dio3* ($P > 0.05$; Student t-test, Fig.

63d), *GnIH* ($P > 0.05$; Student t-test, Fig. 63e), and *Eya3* ($P > 0.05$; Student t-test, Fig. 63f) was observed. Further, the effect of temperature treatment was also reflected at the expression levels of steroidogenic gene markers in the testis tissue (Fig. 64). A significantly higher expression of transcripts *StAR* ($P < 0.05$; Student t-test, Fig. 64a), *Er* ($P < 0.05$; Student t-test, Fig. 64b), *Cyp17a1* ($P < 0.05$; Student t-test, Fig. 64d), and *Cyp11b* ($P < 0.05$; Student t-test, Fig. 64f) were observed in birds treated with low temperature compared to high temperature group. However, no effect of prior temperature treatment on expression levels of *Foxl2* ($P > 0.05$; Student t-test, Fig. 64c) and *Nr4a1* ($P > 0.05$; Student t-test, Fig. 64e) was observed.

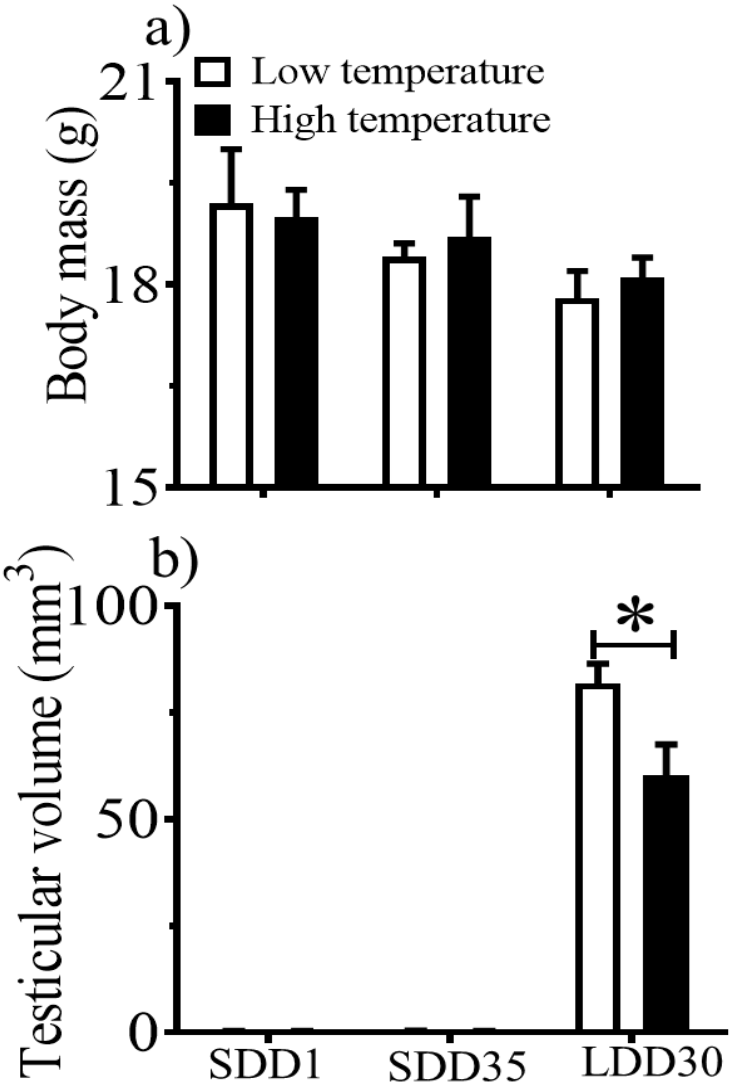

Fig. 62. Effect of five weeks of high (20±2ºC) or high (30±2ºC) temperature treatment in SD followed by 30 days LD exposure on body mass and testis volume of adult male tree sparrow, where the closed bar represents low temperature and the open bar represents high temperature treatment. Data (n=5 birds) is described as mean (±SE). * indicates a significant difference ($P < 0.05$).

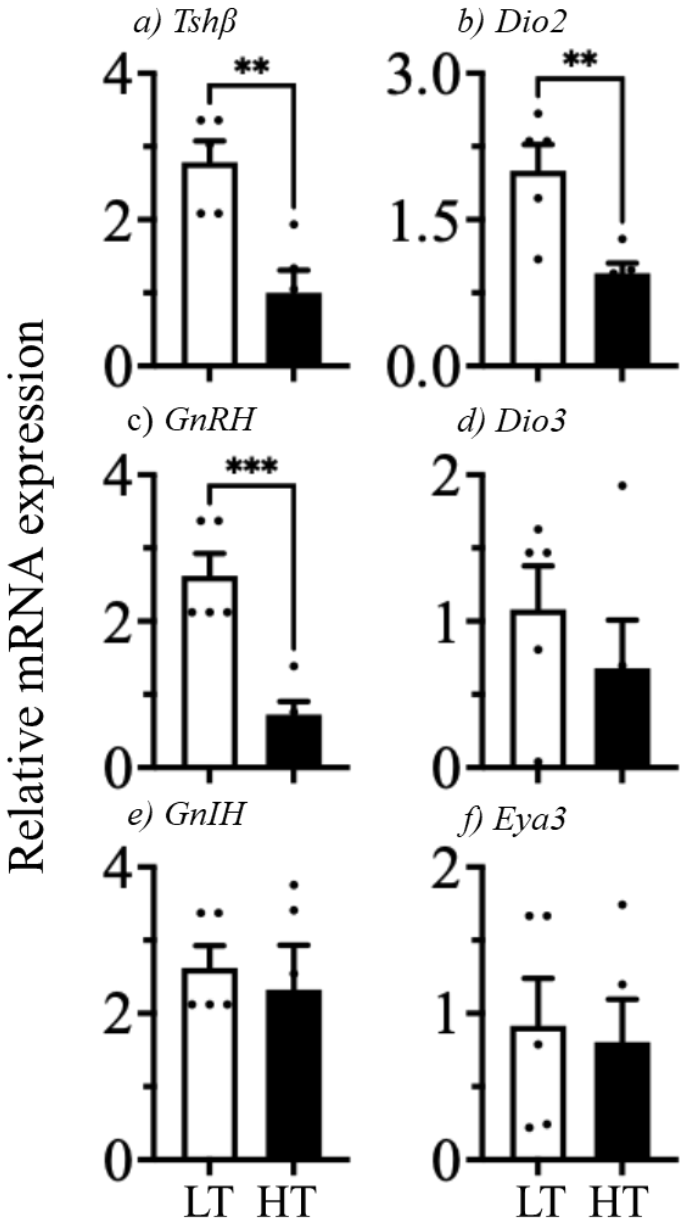

Fig. 63. Effect of five weeks of high (30±2ºC) or low (20±2ºC) temperature treatment in SD followed by 30 days LD exposure on relative mRNA levels of *Tshβ*, *Dio2*, *Dio3*, *GnRH*, *GnIH,* and *Eya3* in the hypothalamus of birds on day 30 of long day exposure (16 L:8D). * indicates a significant difference ($P < 0.05$) in the response between the two groups.

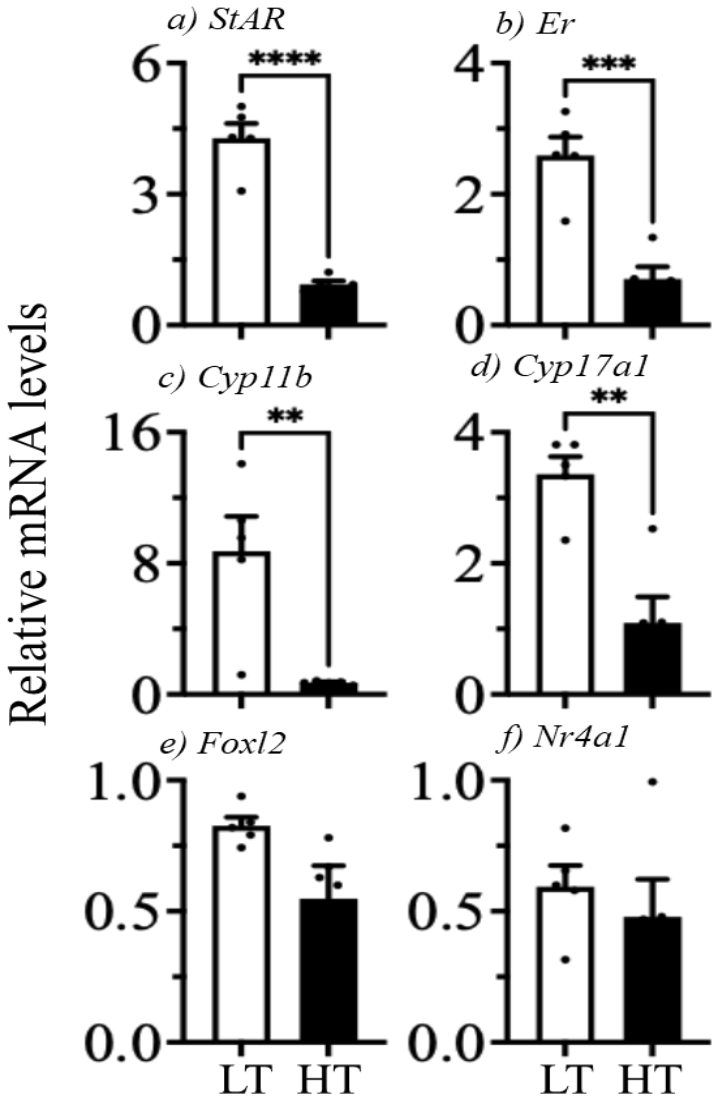

Fig. 64. Effect of five weeks of high (30±2ºC) or low (20±2ºC) temperature treatment in SD followed by 30 days of LD exposure on relative mRNA levels of *StAR*, *Er*, *Foxl2*, *Cyp17a1*, *Nr4a1*, and *Cyp11b* in testis of birds on day 30 of long day exposure (16 L:8D). Asterisks show significant difference ($P < 0.05$) in the response between the two groups.

In the 7 weeks of the treatment group, birds exposed to short photoperiod (8 L;16D) coupled with high (30±2ºC) or low (20±2ºC) temperature for 7 weeks, did not show any differential effect of temperature treatment on the body mass ($F_{5,29} = 3.11$, $P = 0.221$; two Way ANOVA; Fig. 65a) and testicular volume ($F_{5,29} = 2.9124$, $P =$

0.3121; two Way ANOVA; Fig. 65b). Also, no significant differences in the expression levels of transcripts in the hypothalamus of both high and low temperature treatment groups; *Tshβ* ($P > 0.05$; Student t-test, Fig. 66a), *Dio2* ($P > 0.05$; Student t-test, Fig. 66b), *GnRH* ($P > 0.05$; Student t-test, Fig. 66c), *Dio3* ($P > 0.05$; Student t-test, Fig. 66d), *GnIH* ($P > 0.05$; Student t-test, Fig. 66e), and *Eya3* ($P > 0.05$; Student t-test, Fig. 66f) were observed. Similarly, steroidogenic gene markers did not differ in expression levels in the testes of both high and low temperature treated groups; *StAR* ($P > 0.05$; Student t-test, Fig. 67a), *ER* ($P > 0.05$; Student t-test, Fig. 67b), *Foxl2* ($P > 0.05$; Student t-test, Fig. 67c), *Cyp17a1* ($P > 0.05$; Student t-test, Fig. 67d), *Nr4a1* ($P > 0.05$; Student t-test, Fig. 67e), and *Cyp11b* ($P > 0.05$; Student t-test, Fig. 67f).

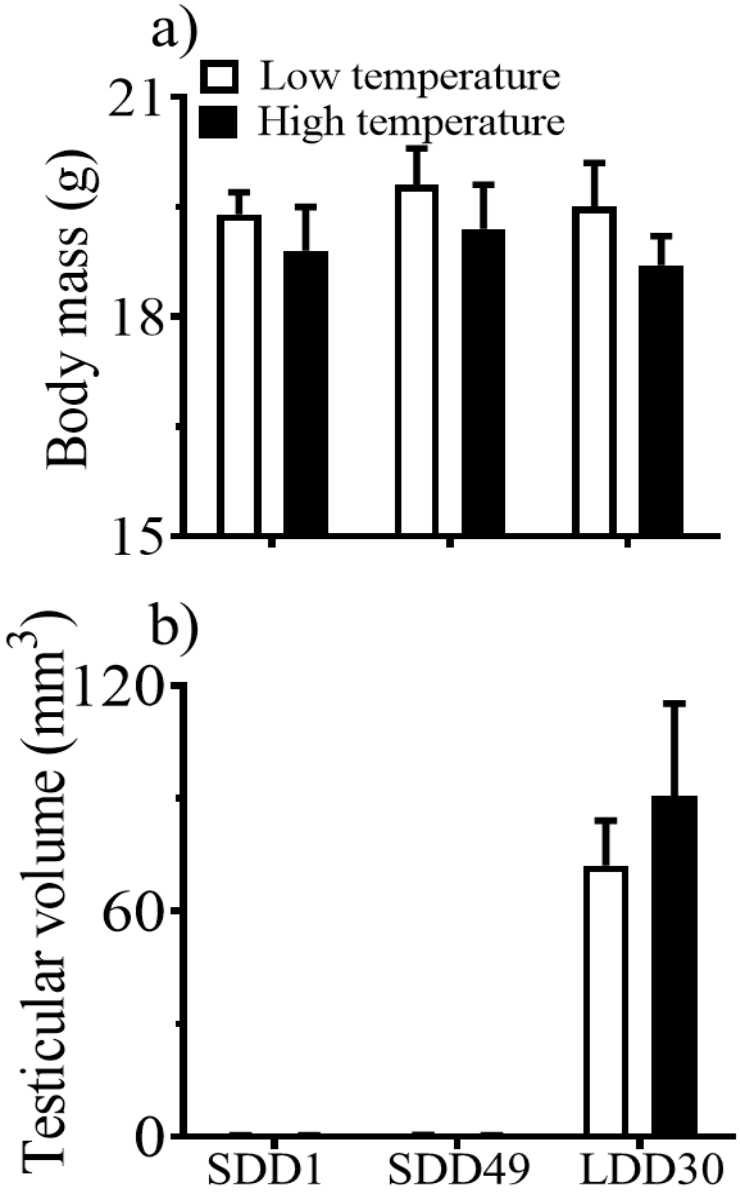

Fig. 65. Effect of seven weeks of high (20±2ºC) or high (30±2ºC) temperature and short photoperiod (SD) of 7 weeks followed by 30 days LD exposure on body mass and testis volume of adult male tree sparrow, where the closed bar represents low temperature and the open bar represents high temperature treatment. Data (n=5 birds) is represented as mean (±SE).

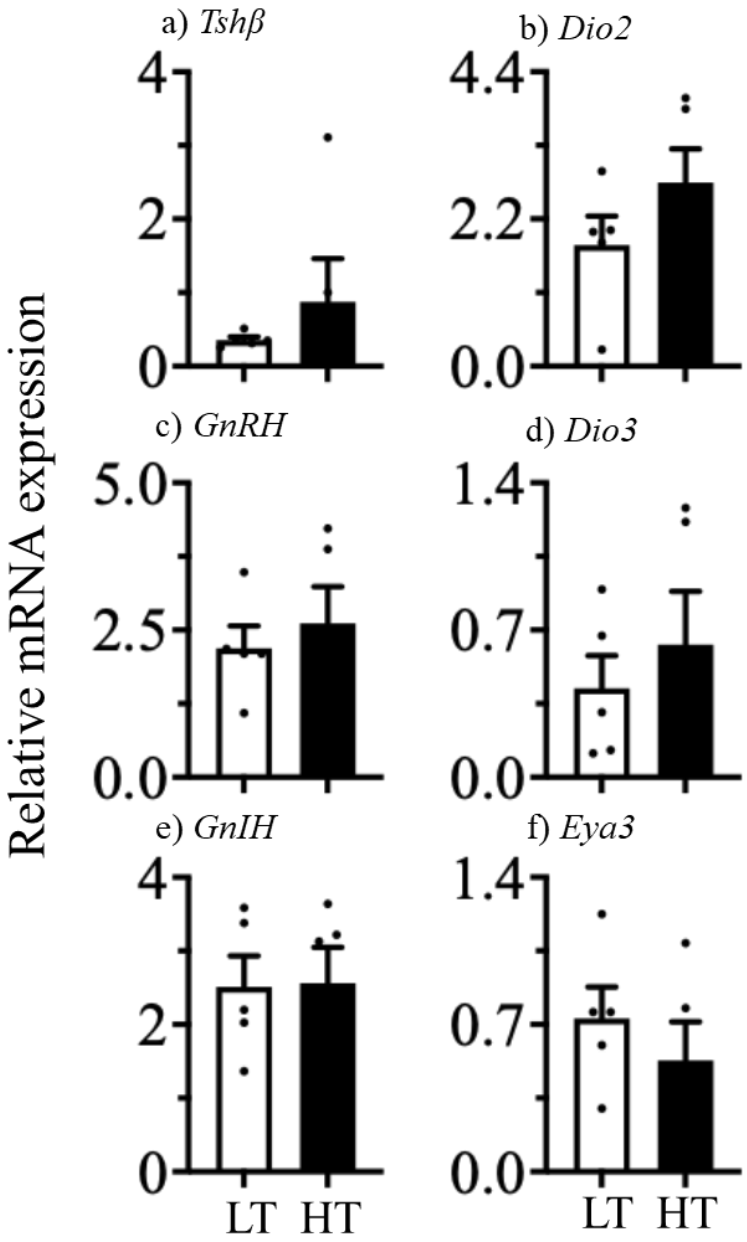

Fig. 66. Effect of seven weeks of high (30±2ºC) or low (20±2ºC) temperature treatment in SD followed by 30 days of LD exposure on mRNA levels of *Tshβ*, *Dio2*, *Dio3*, *GnRH*, *GnIH* and *Eya3* in the hypothalamus of birds on day 30 of long day exposure (16 L:8D). Asterisks show significant difference ($P < 0.05$) in the response between the two groups.

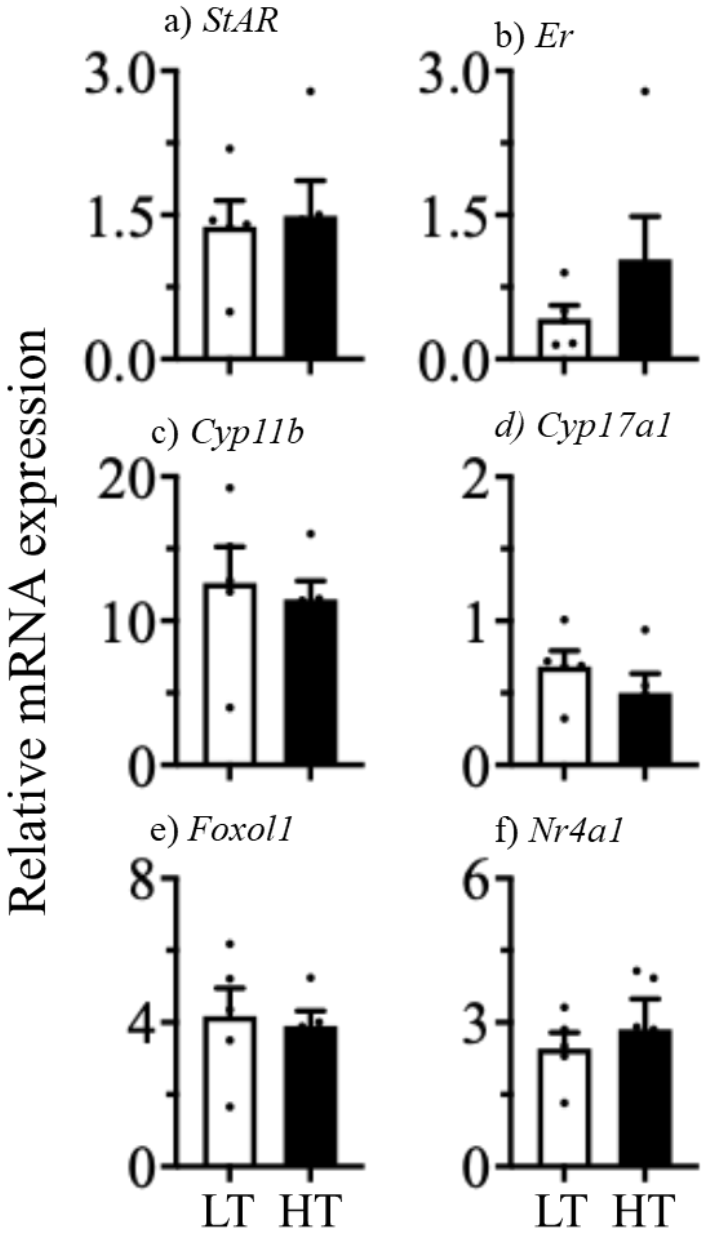

Fig. 67. Effect of seven weeks of high (30±2ºC) or low (20±2ºC) temperature treatment in SD followed by 30 days of LD exposure on mRNA levels of *StAR*, *Er*, *Foxl2*, *Cyp17a1*, *Nr4a1*, and *Cyp11b* in testis of birds on day 30 of long day exposure (16 L:8D). Asterisks show significant difference ($P < 0.05$) in the response between the two groups.

5. DISCUSSION

Rise in temperature is one of the major causes that is negatively affecting the environment and hence animals' physiology and behavior. Several studies of environmental change affecting the phenology of animals have reported (Parmesan & Yohe, 2003; Root et al., 2003; Thackeray et al., 2010; Hällfors et al., 2020). The present study suggests that exposure to different temperatures and short photoperiods during the photorefractory stage affects subsequent photoperiodic responses during the photostimulatory stages. However, this effect depends on the duration of exposure to the respective temperature treatment. Exposure to high temperature (30±2°C) for five weeks during the photorefractory stage attenuates testicular growth in comparison to birds exposed to low temperature (20±2°C) on subsequent exposure to a long stimulatory photoperiod (16 L:8D; Fig. 62). We speculate that differential response of high and low-temperature treatment coupled with short photoperiod could be because of the differential termination of photorefractoriness due to different temperature treatment. At the beginning of the experiment, these birds were photorefractory. Therefore, exposure to a short day was essential to terminate the photorefractoriness. For this species, exposure of 5–7 weeks of short photoperiod can lead to the termination of photorefractoriness and the regaining of photosensitivity (Renthlei, 2021). It seems that exposure to high temperature could not completely terminate the birds' photorefractoriness; hence, subsequent exposure to long photoperiod had delayed/attenuated the photoperiodic response. Exposure to high temperatures during the termination of photorefractoriness in nature (September to November) could be a conflicting environmental situation where birds expect to have low temperatures but experience high temperatures, and hence could be a potentially stressful condition. Although we have not measured serum corticosterone levels in this study, a previous study has shown that exposure to high temperature during the photostimulatory stage results in considerably greater corticosterone levels (Renthlei et al., 2021). It has been proposed that the birds' hypothalamic-pituitary-thyroid (HPT) axis plays a role in both development and reproduction (McNabb et al., 2007). Previously, we have also demonstrated the impact of temperature differences on this species' testicular development regression cycle and transcription levels of related genes (Renthlei et al.,

2021). In photosensitive birds under long photoperiod, we do not see a temperature-dependent effect on body mass (Renthlei et al., 2021).

Unlike migratory species, resident birds do not significantly increase the amount of fat in their adipose tissues (Trivedi et al., 2006). However, migratory species gather a sizable amount of reserve food in their bodies before beginning the real migration, which serves as fuel during their spring migration (Trivedi et al., 2014). For resident species, there is no such necessity; hence, the body mass cycle is less accurate. The effect of time-dependent differential temperature treatment is also reflected in the hypothalamic transcripts involved in reproduction, i.e., *Tshβ*, *Dio2*, and *GnRH* (Figs. 63) and testis steroidogenesis markers; *StAR*, *Cyp17a1*, and *Cyp11b* (Figs. 64). However, such an effect is not revealed after seven weeks of treatment of high and low temperature coupled with short photoperiod either at the gonadal level or at the transcription level (Figs. 66,67). Temperature-dependent photoperiodic effects on *Tshβ*, *Dio2*, and *GnRH* mRNA expressions support the modulatory role of temperature in reproductive behaviors in seasonal breeders (Nakane & Yoshimura, 2014; Majumdar et al., 2015). These data further suggest that hypothalamic deiodinase system expression is not limited to photoperiodic control of gonadal maturation in seasonally breeding animals but also depends on the environmental temperature and temperature appears to have modulatory effects on transcripts involved in seasonal reproduction both during photosensitive (Trivedi et al., 2019; Renthlei et al., 2021) and photosensitivity recovery stage. Higher expression levels of steroidogenic markers in the testes of the low temperature-treated group could indicate ongoing steroidogenic activities in the testes.

6. CONCLUSIONS

In conclusion, the findings firmly support the fact that temperature cues considerably impact the photoperiodic regulation of seasonal reproduction. High temperatures (30 ± 2ºC) coupled with short photoperiod can directly affect the time and amount of gonadal growth and development in tree sparrows during the upcoming photostimulatory stage. The molecular data on the transcript levels of *Tshβ*, *Dio2*, *GnRH,* and steroidogenic markers in the testes further confirm our findings that the effects of temperature are substantial enough to affect the activity of the reproductive

axis under long days. However, how temperature cues reach the ependymal layer, or the pars tuberalis, is unknown. The study has its limitations. The study includes single-point sampling, and hence, we do not know if the time of sampling impacts the mRNA expression profile observed. Further, exposure to 30 days of photostimulation alters the rate of the photoperiodic response, but we do not know if it accelerates responses under low temperatures or delays the response under high temperatures. These findings are limited to male tree sparrows, and how female birds respond needs to be further examined.

SECTION VI: EFFECT OF CORTICOSTERONE TREATMENT ON STEROIDOGENESIS AND REPRODUCTIVE-LINKED PROCESSES

1. ABSTRACT

In all the avian species documented to date, there is immediate activation of adrenocortical tissue in response to a range of stressor stimuli (e.g., malnutrition, handling, restraint, severe temperature, etc.) that leads to a rise in circulating corticosterone. Corticosterone is the primary glucocorticoid in avian species, which is secreted by the adrenal cortex of the adrenal gland and, in response to environmental stressors, regulates the hypothalamic-pituitary-adrenal (HPA) axis, leading to physiological, reproductive, and behavioural changes. Melatonin is a neurohormone, predominantly secreted by the pineal gland in the brain, which is a key modulator of circadian rhythms, seasonal reproduction, responses to environmental cues such as photoperiod, and has antioxidant properties (an antagonist to corticosterone). Here in the present study, we procured photorefractory adult male tree sparrow in the month of September. Physiological parameters such as body mass, bill color, and testicular volume were recorded before and after the end of the experiment. Later, the birds (n=7/group) were divided into three groups. Group one was treated with normal ethanolic saline, which served as a control. Group two was treated with corticosterone (2 µg/100 µl), and Group three was treated with corticosterone (2 µg/100 µl) coupled with melatonin (10 µg/100 µl). All the groups were exposed to 8L:16D (8 hours of light and 16 hours of darkness), and the experiment was run for 4 weeks. Here, melatonin treatment was given half an hour before the light switch-off time. After 4 weeks of the respective treatment, all the groups were again exposed to 16L:8D for another 30 days. At the end of the experiment, all the birds were sampled in the middle of their light phase, and brain and testis tissue were harvested and kept at -80°C for gene expression analysis. The study reveals no variation in the body mass and bill color among all the groups; however, testicular volume was significantly increased after a long day exposure of 30 days, irrespective of the treatment and control, compared to day 1. Besides, downregulation of the reproductive genes was observed in the corticosterone-treated group, whereas upregulation of these genes was observed in the corticosterone coupled with melatonin treatment group and the control group.

Also, several steroidogenic genes were also observed to be downregulated in the corticosterone-treated group compared to the corticosterone coupled with melatonin and control group. These results reveal a negative effect of corticosterone on reproductive activity and steroidogenic activity in this species. However, melatonin is an antagonist of corticosterone and retains the negative effect of corticosterone, hence improving the reproductive-linked steroidogenesis in adult male tree sparrows.

2. INTRODUCTION

A glucocorticoid, i.e., corticosterone, is produced by the adrenal cortex in the adrenal gland in an avian species. It is regulated by the hypothalamic-pituitary-adrenal (HPA) axis in response to environmental stressors, affecting physiological, reproductive, and behavioural changes. The glucocorticoid synthesis pathways of birds are quite similar to those in mammals. Besides, the stored cholesterol is necessary for adrenal steroid synthesis. Avian adrenocortical cells contain cholesterol- and ester-rich lipid droplets initially derived from plasma lipoproteins. Transport proteins within the adrenocortical cells bind to specific steroid intermediates present within the lipid droplets and shuttle them across the mitochondrial and smooth endoplasmic reticulum membranes (Hess, 2002). Studies of avian adrenocortical cells have shown that adrenocortical steroidogenic activity differs with avian species and age. In response to stress, Corticotropin-releasing factor (CRF) from the hypothalamus excites the secretion of ACTH from the pituitary gland. ACTH, which is the predominant stimulus for avian corticosteroid secretion is and causes a rapid, dose-dependent, receptor-mediated release of corticosteroids from the avian adrenal glands. Circulating T3 concentrations and corticosterone production have an inverse correlation, with T3 production in the majority of bird species modulating adrenocortical activity. In reaction to metabolic, psychological, or environmental stressors that disrupt homeostasis, the hypothalamus's corticotropin-releasing factor (CRF) triggers the pituitary gland to release ACTH (Hess, 2002). Besides, avian species exhibit a repeatable daily pattern in their plasma corticosteroid concentrations, and concentrations are found to be highest at the transition between dark and light phases of the day or during the light-dark phase. In addition, birds' regular daily variation in corticosterone concentration reflects changes in the secretion of CRF, ACTH, and

hypothalamic neurons. Seasonal changes, not only in light cycle but also food availability, may help regulate diurnal changes in corticosterone, since fasting eliminates the normal daily variation in corticosterone concentration. Seasonally driven changes in circulating plasma corticosterone concentration help birds adapt to physiologic changes (such as altered reproductive status and molting) and to environmental changes (such as limited food availability and territoriality).

One of the processes by which organisms regulate body homeostasis is the release of glucocorticoid steroid hormones such as corticosterone in birds. The hypothalamic-pituitary-adrenal (HPA) axis's reaction to stressors results in these outcomes (Romero, 2004; Charmandari et al., 2005). The effects of corticosterone on behaviour and physiology have been extensively studied in free-living and domesticated species. Glucocorticoids can have diverse effects, including mobilization of glucose through gluconeogenesis, suppression of the immune system, and alteration of behavioral patterns (Holmes & Phillips, 1976; Axelrod & Reisine, 1984; Munck et al., 1984; Wingfield, 1994). Higher glucocorticoid levels signal the presence of stressors that might disrupt homeostasis and lead to physiological impairment, such as chronic stress impairs the cognitive and learning abilities in vertebrates (Sapolsky et al. 2000), decreases body mass (Malheiros et al., 2003), increases lipid peroxidation levels and decreases antioxidant capacity in different body tissues (Behl et al. 1997; McIntosh et al. 1998a; Orzechowski et al. 2002). Also, corticosterone can decline aggression, parental behavior, and reproductive behavior in tetrapods (Silverin, 1986), increase foraging (Wingfield *et al.,* 1990), and increase or decrease feeding rate and locomotor activity, depending on prior food availability (Astheimer *et al.,* 1992). Busch et al. (2008) reported that Plasma corticosterone can be amplified for minutes to hours by applying corticosterone to the skin in birds, injecting corticosterone into mealworms before ingestion (Breuner et al., 1998; Lohmus et al., 2006), or by single injections of corticosterone (Lin et al., 2004b). A study reported by Costantini et al. (2008) showed that corticosterone treatment in birds had impaired antioxidant capacity, leading to increased oxidative stress.

In contrast, Melatonin is a broad-spectrum antioxidant, a neurohormone, predominantly produced by the pineal gland in the brain, which is a key modulator of

circadian rhythms, seasonal reproduction, and responses to environmental cues such as photoperiod. Its level fluctuates, being highest in the dark and lower during the day, having a rhythmic pattern in the LD cycle (Ball & Balthazart, 2009; Yoshimura, 2013). Melatonin's antioxidant qualities have been extensively established in the past recent years. (Chang et al., 2008). Melatonin is synthesized in the dark hours of a light/dark transition, is thought to play a vital role in providing defense against the free radicals' toxic effects (Reiter et al., 2001). A study by Yadav & Haldar (2014) on *P. asiatica* reveals that melatonin improves stress-induced oxidative damage and the suppressed immune status via expression of its membrane receptors (Mel1a and Mel1 b). Similarly, a study on the ring dove (*Streptopelia risoria*) suggests that melatonin, coupled with corticosterone, where melatonin release during stress may counteract glucocorticoid-induced adverse effects, perhaps due to its antioxidant properties (Barriga et al., 2002). Other than avian species, melatonin may modulate metabolic performance and reproductive behavior under corticosterone-mediated stress conditions in red-sided garter snakes (*Thamnophis sirtalis parietalis*) and cane toad (*Rhinella marinus*) (Lutterschmidt et al., 2004; Jessop et al., 2014).

Corticosterone impacts have been extensively studied in several species involved in behavioural, reproductive, and metabolic processes. However, limited studies have been conducted on the effect of corticosterone treatment and counter-regulatory effects of melatonin hormone on reproductive-linked steroidogenic gene expression in the tree sparrow. Therefore, the study investigates the effect of corticosterone-induced stress response in steroidogenesis and the counter-effect of the melatonin treatment in the adult male tree sparrows and examined the effect of corticosterone on several reproductive and steroidogenic gene expressions in this species.

3. MATERIALS AND METHODS

This work was approved by the Institutional Animal Ethics Committee (IAEC) of Mizoram University (No. MZU/IAEC/2023–24/05). For this study, adult male tree sparrows were used. The male adult birds were obtained locally in September when the birds were experiencing photorefractoriness in nature. To identify the male birds, laparotomy was performed (Kumar et al., 2001). Later, after a week of acclimatization,

birds were divided into three groups (n= 7/group). The body weight, color of the bill, and testicular size were recorded before and at the end of the experiment. All the birds were exposed to a daily short photoperiod of 8 h light and 16 h dark (8L:16D) divided into three groups with different treatments. The body mass, bill color, and testicular volume were recorded at the start of the treatment and the end of the experiment. Group one was treated with ethanolic saline 100 µl (which served as a control). Group two was treated with Corticosterone (2µg/100 µl), and Group three was treated with Corticosterone (2 µg/100µl) coupled with Melatonin (10µg/100µl) half an hour before the lights went off. Briefly, Corticosterone was dissolved in 30% ethanolic saline so that 0.1 ml of solution contained the desired steroid concentration. Control birds were injected with 30% ethanolic saline, and the dose was selected according to the previous study (Chaturvedi & Suresh, 1990) and a known amount of melatonin was dissolved in 100% ethanol and diluted in saline (0.9 % NaCl) such that each injection in a 100 µl volume was 0.1 % ethanolic saline containing 10 µg of hormone. Melatonin injections were prepared as reported by Trivedi et al. (2004). The treatment was given through subcutaneous injection, and all the injections were given in 0.1 ml of solution for over 4 weeks. After the respective treatments for 4 weeks, all the groups were exposed to a stimulatory long photoperiod of 16L: 8D for another 30 days. Later, after 30 days of long-day exposure, all birds were sampled during the middle of the light phase. The hypothalamus and testes were excised from the individual birds and stored at -80ºC till samples were processed for gene expression studies.

3.1. Gene transcription

The transcripts mRNA of genes coding for *Tshβ*, *Dio2*, *Dio3*, *GnRH*, and *GnIH* were measured in the hypothalamic tissue, in addition, mRNA transcripts of genes ecoding for steroidogenesis (*StAR*, *Nr4A1, Scp2, Vdac1, Cyp11a1*, *Cyp17a1*, *Cyp11b*, *Hsd11b2*, *Er* and *Srd5a1*) were measured in the testis tissue. *18s* was used as a reference gene (Yatung & Trivedi, 2024, 2025).

3.2. RNA isolation and cDNA synthesis

From the hypothalamus and gonad tissue, total RNA was pulled out using TRIzol Reagent (Invitrogen by Thermo Fisher Scientific). The purity and concentration of the RNA were determined using (Nanodrop Spectrophotometer

Thermo Fisher Scientific, Wilmington, DE). For cDNA synthesis, a starting RNA of 2 µg was used to process. RQ1 DNase (Promega M6101; Madison, WI, USA) kit was used to eliminate any probable genomic DNA contamination. For cDNA synthesis, the iScript™ cDNA Synthesis Kit (BIO-RAD, USA, 1708891) was used.

3.3. Quantitative (real-time) RT-PCR (qPCR)

The Primer 3 plus (an online primer designing program, Boston, MA, USA; Supplementary Tables 1-2) was used to design gene-specific primers from the sequences. Real-time PCR (qPCR) was performed on Quant-Studio 5 (Applied Biosystem by Thermo Fisher Scientific, Foster City, CA, USA). Briefly, PCR reaction of the volume of 7 µl for each gene comprised 1 µl each of cDNA, 1 µl each of gene-specific forward and reverse primers, 3 µl Power UpTM SYBR Green Master Mix (Applied Biosystem by Thermo Fisher Scientific, Foster City, CA, USA, A25742), and 1µl of nuclease-free water (Ambion, AM9938). qPCR cycle conditions included 1 cycle at 95°C (20 s), 35 cycles at 95°C (01 s), 60°C (20 s) and 95°C (01 s), additional melt curve plot step included 1 cycle of 60°C (20 s) and 1 cycle of 95°C (01 s) as reported in the previous publications (Renthlei & Trivedi, 2019; Borah et al., 2020; Renthlei et al., 2020; 2021; Borah et al., 2022; Lalpekhlui et al., 2022; Yatung & Trivedi, 2024; Yatung & Trivedi, 2025). The ΔΔCt method was used for gene expression analysis reported by (Livak & Schmittgen, 2001). Each sample was run in duplicate.

3.4. Statistical analysis

Data are presented as mean (mean ± SE). GraphPad Prism version 8.0 was used for preparing graphs and statistics. Two-way analysis of variance (Two-way ANOVA) followed by Tukey's multiple comparison test was used to determine the significance of the difference between the groups comparing two factors together (photoperiod vs time). A one-way ANOVA was used to determine whether there was a significant difference among the groups. Tukey's multiple comparison tests were applied if ANOVA showed a significant difference, and if one-way ANOVA revealed a significant difference, $p < 0.05$ was considered a significant difference.

4. RESULTS

In male tree sparrows, 4 weeks of treatment coupled with 30 days of long day does not affect the body mass (Treatment: P=0.5069; Time: P=0.1685; Interaction: P=0.5585; Two-way ANOVA; Table 7a; Fig. 68a). However, in bill color significant difference was observed in time and interaction but does not have effect of the treatment on bill color (Treatment: P=0.5707; Time: P=0.0038; Interaction: P=0.0082; Two-way ANOVA; Table 7a; Fig. 68b). A significant difference was observed in the testicular volume over time but no difference in the treatment and interaction (Treatment: P=0.9385; Time: P<0.0001; Interaction: P=0.9385; Two-way ANOVA; Table 7a; Fig. 68c).

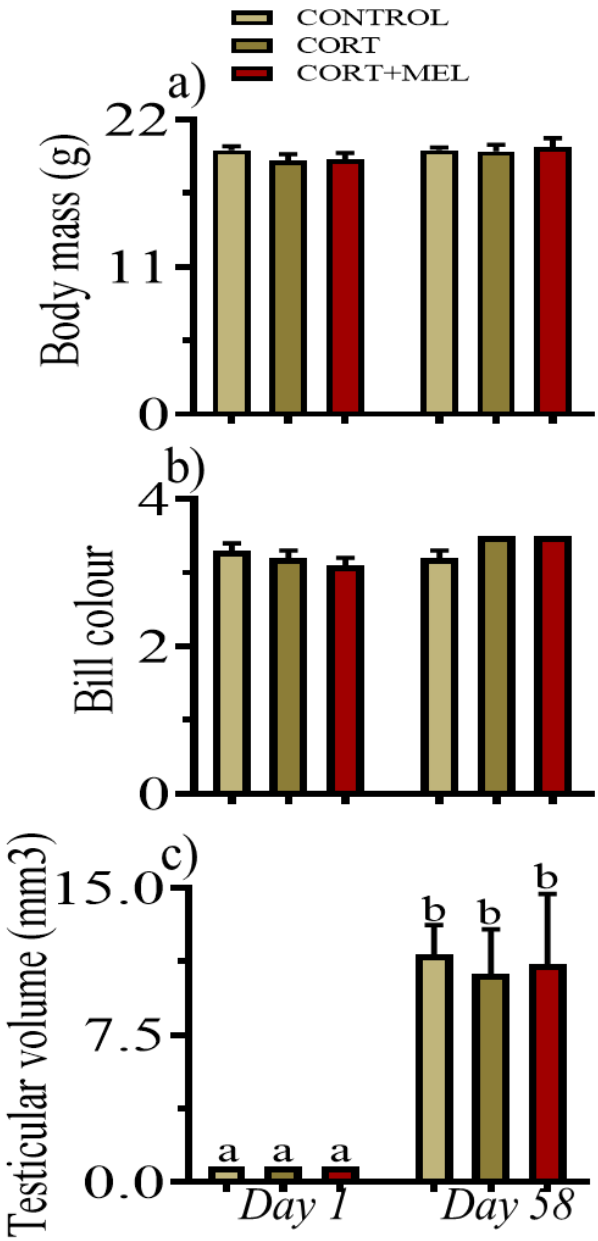

Fig. 68. Data are represented as (mean ±SE, n= 7/group). Parameters (a) body mass, (b) Bill color, and (c) Testicular volume of adult male tree sparrows. Two-way ANOVA followed by Tukey's multiple comparison test to determine significant differences. The alphabet represents significant differences ($P < 0.05$) among the groups.

Transcripts involved in avian reproduction were also measured in the hypothalamus of the tree sparrow. A significant difference was observed in mRNA transcript expressions of *Tshβ* (P= 0.0082; Table 7b; One-way ANOVA; Fig. 69a), *Dio2* (P< 0.0001; Table 7b; One-way ANOVA; Fig. 69b), *GnRH* (P= 0.0039; Table 7b; One-way ANOVA; Fig. 69d) and *Eya3* (P= 0.0097; Table 7b; One-way ANOVA; Fig. 69f). The transcript expressions of these genes were significantly downregulated in the birds treated with corticosterone, however significantly upregulated in the control group and corticosterone coupled with melatonin treated group. Whereas transcript expression of *GnIH* (P= 0.0030; Table 7b; One-way ANOVA; Fig. 69e) was upregulated in the corticosterone-treated group in comparison to the control and corticosterone coupled with melatonin group. However, no differences were observed in the transcript expression of *Dio3* (P= 0.6921; Table 7b; One-way ANOVA; Fig. 69c) in all the treatment groups.

Table 7a. Values of the Two-way ANOVA of physiological parameters

	Time	Treatment	Interaction
Body mass	$F_{(1,13)}$=2.127, P=0.1685	$F_{(2,13)}$= 0.7161, P=0.5069	$F_{(2,13)}$ =0.6094, P=0.5585
Bill color	$F_{(1,13)}$=12.32, P= 0.0038	$F_{(2,13)}$ = 0.5858, P=0.5707	$F_{(2,13)}$=7.121, P=0.0082
Testicular volume	$F_{(1,13)}$=49.24, P<0.0001	$F_{(2,13)}$=0.06380, P=0.9385	$F_{(2,13)}$=0.06380, P=0.9385

Table 7b. Values of One-way ANOVA of the reproductive gene transcripts in the hypothalamus

Transcripts	Hypothalamus
Tshβ	$F_{(2, 15)}$ = 6.736, P=0.0082
Dio2	$F_{(2, 15)}$ = 20.66, P<0.0001
Dio3	$F_{(2, 15)}$ = 0.3772, P=0.6921
GnRH	$F_{(2, 14)}$ = 8.465, P=0.0039
GnIH	$F_{(2, 15)}$ = 8.784, P=0.0030
Eya3	$F_{(2, 15)}$ = 6.408, P=0.0097

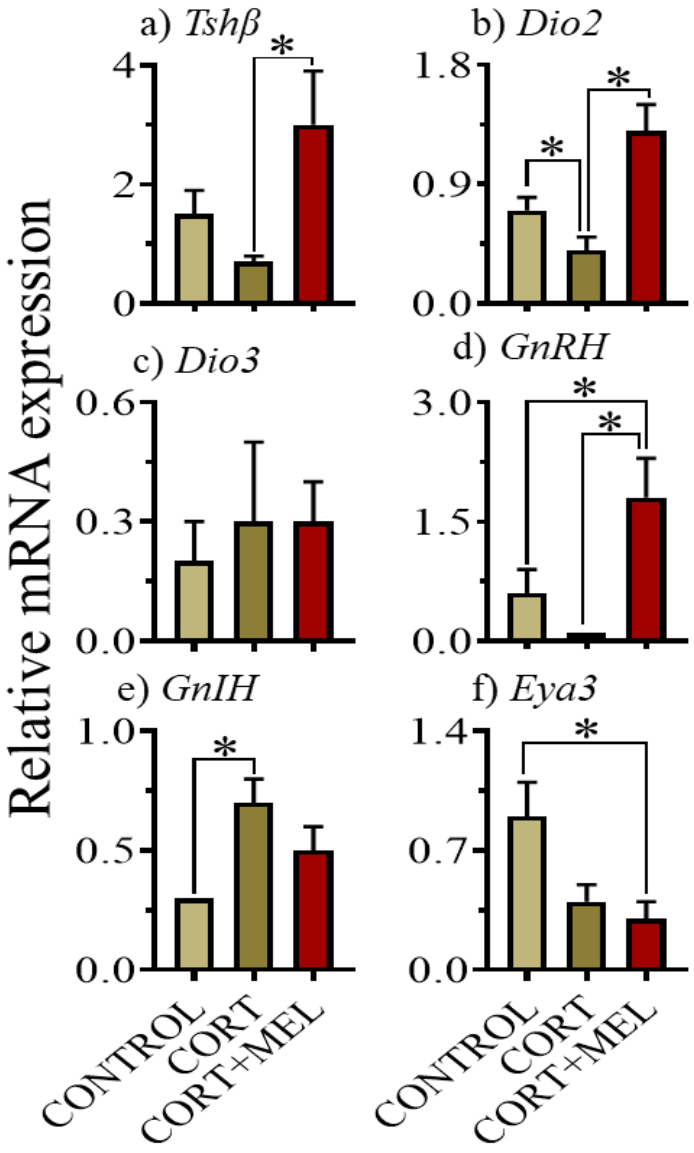

Fig. 69. Data are represented as the (mean ± SE n=7/group). Relative mRNA expression of reproductive genes in the hypothalamic tissues of adult male tree sparrows. One-way ANOVA followed by Tukey's multiple comparison test to determine significant differences among the groups. * represents significant differences ($P < 0.05$).

Similarly, we have also examined the mRNA expressions of several steroidogenic genes in the testicular tissue. A significant variance was found in the

mRNA transcripts of *StAR* (P= 0.0311; Table 7c; One-way ANOVA; Fig. 70a), *Vdac1*(P= 0.0016; Table 7c; One-way ANOVA; Fig. 70d), *Cyp11a1* (P= 0.0387; Table 7c; One-way ANOVA; Fig. 70e), *Cyp17a1* (P= 0.0156; Table 7c; One-way ANOVA; Fig. 70f), *Cyp11b* (P= 0.0011; Table 7c; One-way ANOVA; Fig. 70g), *Hsd11b2* (P= 0.0263; Table 7c; One-way ANOVA; Fig. 70h) and *Srd5a1* (P= 0.0085; Table 7c; One-way ANOVA; Fig. 70j). Higher expressions of these genes were found in the control group and corticosterone coupled with melatonin group and downregulated in the corticosterone group. However, no variance was observed in transcript expression of *Nr4a1* (P= 0.3242; Table 7c; One-way ANOVA; Fig. 70b), *Scp2* (P= 0.0559; Table 7c; One-way ANOVA; Fig. 70c), and *Er* (P= 0.3859; Table 7c; One-way ANOVA; Fig. 70i) in all the treatment groups.

Table 7c. Values of One-way ANOVA of the steroidogenic gene expressions in the testis

Transcripts	Testis
StAR	$F_{(2, 16)} = 4.345$, P=0.0311
Nr4a1	$F_{(2, 14)} = 1.222$, P=0.3242
Scp2	$F_{(2, 16)} = 3.473$, P=0.0559
Vdac1	$F_{(2, 16)} = 9.851$, P=0.0016
Cyp11a1	$F_{(2, 15)} = 4.072$, P=0.0387
Cyp17a1	$F_{(2, 15)} = 5.566$, P=0.0156
Cyp11b	$F_{(2, 33)} = 8.485$, P=0.0011
Hsd11b2	$F_{(2, 15)} = 4.682$, P=0.0263
Er	$F_{(2, 16)} = 1.011$, P=0.3859
Srd5a1	$F_{(2, 15)} = 6.666$, P=0.0085

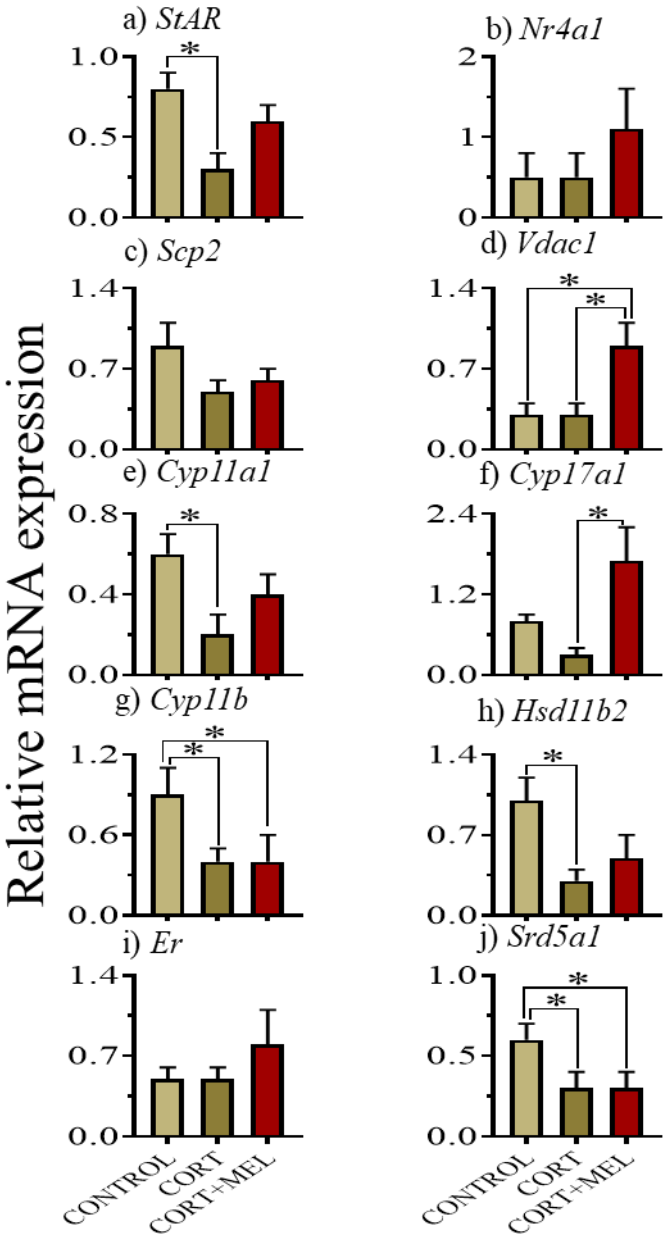

Fig. 70. Data are represented as mean (± SE, n=7/group). Relative mRNA expression of steroidogenic genes in the testicular tissue of adult male tree sparrows. One-way ANOVA followed by Tukey's multiple comparison test was applied to determine significant differences between the groups. * represents significant differences ($P < 0.05$) among the groups.

5. DISCUSSION

The corticosterone in birds (glucocorticoid) is the end-product of the HPA axis produced in response to variable environmental stressors, and higher levels of corticosterone disrupt the homeostasis and affect the balance between pro-oxidants and antioxidants evident in the studies reported by (Sies, 1991; Finkel & Holbrook, 2000; Halliwell & Gutteridge, 2007). Similarly, the present study reveals the effect of corticosterone treatment on several reproductive and steroidogenic mRNA transcript expressions in the hypothalamus and testis tissue. Corticosterone, a stress hormone produced in response to various environmental stressors, can damage biomolecules by increasing free radicals in the body (Yadav & Haldar, 2014). After 4 weeks of corticosterone treatment, body mass does not alter in control, corticosterone, and corticosterone coupled with melatonin groups (Fig. 68a). This could be because of the short-term exposure of 4 weeks treatment, which is consistent with the study reported on (Astheimer et al., 2000), where corticosterone implants in white-crowned sparrows did not constantly affect body mass under controlled conditions, signifying that short-term glucocorticoid exposure may not alter the energy homeostasis in some birds. The bill color does not show any significance in all the treatment groups, but shows distinction throughout long-day exposure (Fig. 68b). Since in the avian species, bill color is a secondary sexual character, a trait linked to reproductive readiness and testosterone levels. Hence, the significant time effect suggests enhanced bill coloration, under long-day exposure, which mimics the breeding season, may be via increased testosterone or carotenoid mobilization (McGraw et al., 2006; Yatung & Trivedi, 2024, 2025). The significant difference in the effect of interaction indicates the relationship between the corticosterone and melatonin effects over time, potentially by regulating the testosterone production and carotenoid metabolism. Since corticosterone is known to suppress testosterone production (Deviche et al., 2014), it shows a significant interaction with melatonin, which counteracts the suppressive effect of corticosterone. This is consistent with melatonin's role in modulating reproductive physiology (Sainz et al., 1999; Trivedi et al., 2004). No significant difference was observed in testicular volume at the beginning and after 4 weeks treatment; however, 4 weeks of respective treatment coupled with long-day exposure, showed a significant increase in testicular growth over time, which suggests exposure

to long photoperiods initiates the HPG axis, enhancing reproductive activity and is consistent with the study on this species (Dawson et al., 2001; Dixit & Singh, 2011; Yatung & Trivedi, 2024). The lack of treatment effect suggests that corticosterone, or corticosterone with melatonin, does not significantly alter the photoperiod-driven process in tree sparrows.

Also, the study examined mRNA transcripts of genes involved in seasonal reproduction in the hypothalamic tissue of adult male tree sparrows. The genes *Tshβ*, *Dio2*, and *GnRH* showed a notable difference and were downregulated in the corticosterone-treated group in comparison to the control and corticosterone with melatonin group, where it is upregulated. These genes are stimulatory and are critical for photoperiodic regulation of reproduction. *Tshβ* and *Dio2* are involved in the TSH signaling pathway that facilitates reproductive activation under long-day conditions in birds (Nakao et al., 2008), and *GnRH* stimulates gonadotropin secretion, enhancing testicular growth, whereas *Eya3* is a transcriptional co-activator linked to photoperiodic responses (Dardente et al., 2010). Downregulation of these transcripts in the corticosterone-treated group suggests a stress-induced suppression of reproductive signaling (Fig. 69f), which is consistent with its role in inhibiting the HPG axis (Sapolsky et al., 2000). However, these genes' upregulation in the corticosterone coupled with melatonin treatment group suggests an antagonist effect of melatonin in altering the stress-induced suppression and further retaining the reproductive capability in this species (Fig. 69a,b,d), and is consistent with the report (Yadav & Haldar, 2014). The mechanisms of the antagonistic effects of melatonin and glucocorticoids are still unclear, but seemingly, melatonin inhibits the mRNA expression of the glucocorticoid receptor (Sainz et al., 1999). However, a significant upregulation of *GnIH* indicates the melatonin function in regulating stress-mediated inhibition of reproductive activity, also reported in other species (Calisi et al., 2008; Chowdhury et al., 2010). Melatonin's ability to lessen this effect shows that it may inhibit corticosterone's stress response, presumably by regulating *GnIH* receptor signaling.

The study also examined the effect of corticosterone treatment on several steroidogenic markers (*StAR*, *Nr4a1, Scp2, Vdac1, Cyp11a1*, *Cyp17a1*, *Cyp11b*, *Hsd11b2*, *Er,* and *Srd5a1*) in the testicular tissue, revealing significant effects of corticosterone and melatonin. *StAR* helps the transportation of cholesterol across mitochondria, the rate-limiting step in the steroidogenic process (Stocco, 2001), and *Nr4a1* is a nuclear receptor that regulates *StAR* transcription. Downregulation of these genes in the corticosterone-treated group suggests, corticosterone inhibits *NR4a1*-dependent transactivation of the *StAR* promoter in Leydig cells by reducing *Nr4a1*, and inhibition of the steroidogenic activity in the testis through suppression of glucocorticoid receptor-mediated transcription. This is also consistent with the study reported on rodent Leydig cells (Badrinarayanan et al., 2006; Martin & Tremblay, 2008). Similarly, downregulation of transcripts *Scp2, Vdac1, Cyp11a1*, *Cyp17a1*, *Cyp11b*, *Hsd11b2*, *Er,* and *Srd5a1* in the corticosterone group indicates glucocorticoid receptor-mediated transcription suppression leading to inhibition of testosterone production and are similar with the studies where, corticosterone also leads to oxidative stress generates free radicals, it can oxidize critical cellular components (e.g., mitochondrial membranes, enzymes), directly damaging steroidogenic pathways (Hales et al., 2000; Orzechowski et al., 2002; Costantini et al., 2008; Hu et al., 2009). Whereas, upregulation of these transcripts in the corticosterone combined with melatonin group suggests that melatonin aids in suppression of corticosterone's negative effect with its antioxidant properties (Chang et al., 2008) and may have retained the steroidogenic activity in this species. Melatonin's restoration of expression may reflect its antioxidant and anti-glucocorticoid properties, protecting steroidogenic pathways (Reiter et al., 2016). The downregulation in transcript expression of *Cyp11b, Hsd11b2, Srd5a1,* in corticosterone-treated groups suggests complex regulatory feedback where corticosterone may suppress local glucocorticoid metabolism or androgen conversion. The lack of melatonin's protective effect here could indicate specificity in its action. Since corticosterone primarily affects androgen-related pathways, the lack of a substantial change in *Er* transcript expression indicates that it has no impact on estrogen signaling in testicular tissue.

6. CONCLUSIONS

In summary, the present study reveals the differential regulation of steroidogenic genes on melatonin-corticosterone interactions in the tree sparrow, while the existing pieces of literature on steroidogenesis focus on mammals or fish, very limited research has been done on avian species. This present study elucidates the counter-regulatory effects of melatonin hormone on corticosterone-induced alterations in male tree sparrows, and melatonin co-treatment effectively reverses corticosterone effects for most genes involved in reproduction and steroidogenesis, restoring expression levels, thus highlighting its protective role against stress-induced reproductive and steroidogenic suppression. These findings emphasize melatonin's capacity to lessen stress-induced suppression of reproduction in birds and serve as a foundation for further research into the molecular mechanisms and ecological consequences of seasonal breeding.

REFERENCES

Akazome, Y., Abe, T., & Mori, T. (2002). Differentiation of chicken gonad as an endocrine organ: expression of LH receptor, FSH receptor, cytochrome P450c17 and aromatase genes. *REPRODUCTION-CAMBRIDGE-*, *123*(5), 721-728.

Alaasam, V. J., Liu, X., Niu, Y., Habibian, J. S., Pieraut, S., Ferguson, B. S., ... & Ouyang, J. Q. (2021). Effects of dim artificial light at night on locomotor activity, cardiovascular physiology, and circadian clock genes in a diurnal songbird. *Environmental Pollution*, *282*, 117036.

Alvino, G. M., Blatchford, R. A., Archer, G. S., & Mench, J. A. (2009). Light intensity during rearing affects the behavioural synchrony and resting patterns of broiler chickens. *British Poultry Science*, *50*(3), 275-283.

Andrews, J. E., Smith, C. A., & Sinclair, A. H. (1997). Sites ofEstrogen ReceptorandAromataseExpression in the Chicken Embryo. *General and comparative endocrinology*, *108*(2), 182-190. https://doi.org/10.1006/gcen.1997.6978.

Angelini, F., & Botte, V. (1992). Spermatogenesis in reptiles, dynamic and regulatory aspect. *Sex origin and evolution*, *6*, 211-230.

Ansel, L., Bentsen, A. H., Ancel, C., Bolborea, M., Klosen, P., Mikkelsen, J. D., & Simonneaux, V. (2011). Peripheral kisspeptin reverses short photoperiod-induced gonadal regression in Syrian hamsters by promoting GNRH release. *Reproduction*, *142*(3), 417. https://doi.org/10.1530/rep-10-0313.

Antonio-Cabrera, E., & Paredes, R. G. (2014). Testosterone or oestradiol implants in the medial preoptic area induce mating in noncopulating male rats. *Journal of Neuroendocrinology*, *26*(7), 448-458. https://doi.org/10.1111/jne.12164.

Arnold, P.A., Nicotra, A.B. and Kruuk, L.E., 2019. Sparse evidence for selection on phenotypic plasticity in response to temperature. *Philosophical Transactions*

of the Royal Society B, *374*(1768), p.20180185. https://doi.org/10.1098/rstb.2018.0185.

Asif, S., Kim, R. Y., Fatica, T., Sim, J., Zhao, X., Oh, Y., ... & Kim, K. H. (2022). Hmgcs2-mediated ketogenesis modulates high-fat diet-induced hepatosteatosis. *Molecular metabolism*, *61*, 101494. https://doi.org/10.1016/j.molmet.2022.101494.

Astheimer, L. B., Buttemer, W. A., & Wingfield, J. C. (1992). Interactions of corticosterone with feeding, activity and metabolism in passerine birds. *Ornis Scandinavica*, 355-365.

Astheimer, L. B., Buttemer, W. A., & Wingfield, J. C. (2000). Corticosterone treatment has no effect on reproductive hormones or aggressive behavior in free-living male tree sparrows, Spizella arborea. *Hormones and Behavior*, *37*(1), 31-39.

Axelrod, J., & Reisine, T. D. (1984). Stress hormones: their interaction and regulation. *Science*, *224*(4648), 452-459.

Badrinarayanan, R., Rengarajan, S., Nithya, P., & Balasubramanian, K. (2006). Corticosterone impairs the mRNA expression and activity of 3 β-and 17 β-hydroxysteroid dehydrogenases in adult rat Leydig cells. *Biochemistry and cell biology*, *84*(5), 745-754.

Ball, G. F., & Balthazart, J. (2009). Neuroendocrine regulation of reproductive behavior in birds.

Bapary, M. A. J., Amin, M. N., Takeuchi, Y., & Takemura, A. (2011). The stimulatory effects of long wavelengths of light on the ovarian development in the tropical damselfish, Chrysiptera cyanea. *Aquaculture*, *314*(1-4), 188-192.

Barriga, C., Marchena, J. M., Lea, R. W., Harvey, S., & Rodríuez, A. B. (2002). Effect of stress and dexamethasone treatment on circadian rhythms of melatonin and corticosterone in ring dove (Streptopelia risoria). *Molecular and Cellular Biochemistry*, *232*, 27-31.

Bassett, M. H., Suzuki, T., Sasano, H., De Vries, C. J., Jimenez, P. T., Carr, B. R., & Rainey, W. E. (2004a). The orphan nuclear receptor NGFIB regulates transcription of 3β-hydroxysteroid dehydrogenase: implications for the control of adrenal functional zonation. *Journal of Biological Chemistry*, *279*(36), 37622-37630. 10.1074/jbc.M405431200.

Bassett, M. H., Suzuki, T., Sasano, H., White, P. C., & Rainey, W. E. (2004b). The orphan nuclear receptors NURR1 and NGFIB regulate adrenal aldosterone production. *Molecular Endocrinology*, *18*(2), 279-290. https://doi.org/10.1210/me.2003-0005.

Batra, T., Malik, I., & Kumar, V. (2019). Illuminated night alters behaviour and negatively affects physiology and metabolism in diurnal zebra finches. *Environmental Pollution*, *254*, 112916. https://doi.org/10.1016/j.envpol.2019.07.084.

Baulieu, E. E. (1998). Neurosteroids: a novel function of the brain. *Psychoneuroendocrinology*, *23*(8), 963-987. https://doi.org/10.1016/s0306-4530(98)00071-7.

Behl, C., Lezoualc'h, F., Trapp, T., Widmann, M., Skutella, T., & Holsboer, F. (1997). Glucocorticoids enhance oxidative stress-induced cell death in hippocampal neurons in vitro. *Endocrinology*, *138*(1), 101-106. https://doi.org/10.1210/endo.138.1.4835.

Bender, D. A., & Mayes, P. A. (2015). The citric acid cycle: the central pathway of carbohydrate, lipid & amino acid metabolism. *Harper's Illustrated Biochemistry. McGraw-Hill Education*, 161-167.

Bennett, A. T., & Cuthill, I. C. (1994). Ultraviolet vision in birds: what is its function?. *Vision research*, *34*(11), 1471-1478.

Benoit J, Otto L. (1994). External and internal factors in sexual activity. Effect of irradiation with different wavelengths on the mechanisms of photostimulation

of the hypophysis and on testicular growth in the immature duck. *Yale J Biol Med*. 17: 22 – 46.

Bessei, W. (2006). Welfare of broilers: a review. *World's Poultry Science Journal*, *62*(3), 455-466.

Bissonnette, T. H. (1932). Studies on the sexual cycle in birds. VI. Effects of white, green, and red lights of equal luminous intensity on the testis activity of the European starling (Sturnus vulgaris). *Physiological Zoology*, *5*(1), 92-123.

Biswas, A., Mohan, J. and Sastry, K.V.H., 2010. Age-dependent variation in hormonal concentration and biochemical constituents in blood plasma of Indian native fowl. *Veterinary Medicine International*, *2010*. https://doi.org/10.4061/2010/737292.

Blatchford, R. A., Klasing, K. C., Shivaprasad, H. L., Wakenell, P. S., Archer, G. S., & Mench, J. A. (2009). The effect of light intensity on the behavior, eye and leg health, and immune function of broiler chickens. *Poultry science*, *88*(1), 20-28.

Bloj, B. and Zilversmit, D.B., 1977. Rat liver proteins capable of transferring phosphatidylethanolamine. Purification and transfer activity for other phospholipids and cholesterol. *Journal of Biological Chemistry*, *252*(5), pp.1613-1619. https://doi.org/10.1016/S0021-9258(17)40593-X.

Borah, B. K., Renthlei, Z., & Trivedi, A. K. (2019). Seasonality in terai tree frog (Polypedates teraiensis): Role of light and temperature in regulation of seasonal breeding. *Journal of Photochemistry and Photobiology B: Biology*, *191*, 44-51. https://doi.org/10.1016/j.jphotobiol.2018.12.005.

Borah, B. K., Renthlei, Z., & Trivedi, A. K. (2020). Hypothalamus but not liver retains daily expression of clock genes during hibernation in terai tree frog (Polypedates teraiensis). *Chronobiology International*, *37*(4), 485-492. https://doi.org/10.1080/07420528.2020.1726373.

Borah, B. K., Renthlei, Z., Tripathi, A., & Trivedi, A. K. (2022). Molecular and epigenetic regulation of seasonal reproduction in Terai tree frog (Polypedates teraiensis). *Photochemical & Photobiological Sciences*, *21*(6), 1067-1076. https://doi.org/10.1007/s43630-022-00195-2.

Both, C., & Visser, M. E. (2001). Adjustment to climate change is constrained by arrival date in a long-distance migrant bird. *Nature*, *411*(6835), 296-298.

Breuner, C. W., Greenberg, A. L., & Wingfield, J. C. (1998). Noninvasive corticosterone treatment rapidly increases activity in Gambel's white-crowned sparrows (Zonotrichia leucophrys gambelii). *General and comparative endocrinology*, *111*(3), 386-394.

Burrone, L., Santillo, A., Pinelli, C., Baccari, G. C., & Di Fiore, M. M. (2012). Induced synthesis of P450 aromatase and 17β-estradiol by d-aspartate in frog brain. *Journal of Experimental Biology*, *215*(20), 3559-3565. https://doi.org/10.1242/jeb.073296.

Busch, D. S., Sperry, T. S., Wingfield, J. C., & Boyd, E. H. (2008). Effects of repeated, short-term, corticosterone administration on the hypothalamo–pituitary–adrenal axis of the white-crowned sparrow (Zonotrichia leucophrys gambelii). *General and Comparative Endocrinology*, *158*(3), 211-223.

Calisi, R. M., Rizzo, N. O., & Bentley, G. E. (2008). Seasonal differences in hypothalamic EGR-1 and GnIH expression following capture-handling stress in house sparrows (Passer domesticus). *General and comparative endocrinology*, *157*(3), 283-287.

Carreau, S., Bouraima-Lelong, H. and Delalande, C., 2011. Estrogens–new players in spermatogenesis. *Reproductive biology*, *11*(3), pp.174-193.https://doi.org/10.1016/s1642-431x(12)60065-5.

Cassone, V. M. (2014). Avian circadian organization: a chorus of clocks. *Frontiers in neuroendocrinology*, *35*(1), 76-88.

Celi, I., Gatica, M. C., Guzmán, J. L., Gallego-Calvo, L., & Zarazaga, L. A. (2013). Influence of the male effect on the reproductive performance of female Payoya goats implanted with melatonin at the winter solstice. *Animal Reproduction Science*, *137*(3-4), 183-188.

Chakravorty K, Chandola-Saklani A. (1985). Termination of seasonal breeding in weaver finch, *(Ploceus philippinus)*: Role of photoperiod. J Exp Zool. 235: 381 – 386.

Chanderbhan, R., Noland, B.J., Scallen, T.J. and Vahouny, G.V., 1982. Sterol carrier protein2. Delivery of cholesterol from adrenal lipid droplets to mitochondria for pregnenolone synthesis. *Journal of Biological Chemistry*, *257*(15), pp.8928-8934. https://doi.org/10.1016/S0021-9258(18)34222-4.

Chandola A, Pavnaskar J, Thapliyal JP. (1975). Scoto/Photoperiodic responses of a subtropical finch (spotted munia) in relation to seasonal breeding cycle. *J Interdiscipl Cycle Res*. 6: 189 – 202.

Chang, H. M., Huang, Y. L., Lan, C. T., Wu, U. I., Hu, M. E., & Youn, S. C. (2008). Melatonin preserves superoxide dismutase activity in hypoglossal motoneurons of adult rats following peripheral nerve injury. *Journal of pineal research*, *44*(2), 172-180.

Chang, S. C., Lin, M. J., Zhuang, Z. X., Huang, S. Y., Lin, T. Y., Jea, Y. S., ... & Lee, T. T. (2015). Effect of monochromic light-emitting diode light with different color on the growth and reproductive performances of breeder geese. *Asian-Australasian journal of animal sciences*, *29*(6), 830.

Charmandari, E., Tsigos, C., & Chrousos, G. (2005). Endocrinology of the stress response. *Annu. Rev. Physiol.*, *67*, 259-284. https://doi.org/10.1146/annurev.physiol.67.040403.120816.

Chaturvedi, C. M., & Suresh, P. K. (1990). Effects of corticosterone, metapyrone, and ACTH on testicular function at different stages of the breeding cycle in

migratory redheaded bunting, Emberiza bruniceps. *General and comparative endocrinology*, *78*(1), 1-11.

Chemineau, P., Malpaux, B., Brillard, J. P., & Fostier, A. (2007). Seasonality of reproduction and production in farm fishes, birds, and mammals. *Animal*, *1*(3), 419-432.

Chen, R., Qin, Y., Du, J., Liu, J., Dai, S., Lei, M., & Zhu, H. (2023). Circadian clock gene BMAL1 regulates STAR expression in goose ovarian preovulatory granulosa cells. *Poultry Science*, *102*(12), 103159. https://doi.org/10.1016/j.psj.2023.103159.

Chevin, L. M., Lande, R., & Mace, G. M. (2010). Adaptation, plasticity, and extinction in a changing environment: towards a predictive theory. *PLoS biology*, *8*(4), e1000357. https://doi.org/10.1371/journal.pbio.1000357.

Chevin, L. M., Visser, M. E., & Tufto, J. (2015). Estimating the variation, autocorrelation, and environmental sensitivity of phenotypic selection. *Evolution*, *69*(9), 2319-2332. https://doi.org/10.1111/evo.12741.

Cho, C. H., Lee, H. J., Yoon, H. K., Kang, S. G., Bok, K. N., Jung, K. Y., ... & Lee, E. I. (2016). Exposure to dim artificial light at night increases REM sleep and awakenings in humans. *Chronobiology international*, *33*(1), 117-123.

Chowdhury, V. S., Yamamoto, K., Ubuka, T., Bentley, G. E., Hattori, A., & Tsutsui, K. (2010). Melatonin stimulates the release of gonadotropin-inhibitory hormone by the avian hypothalamus. *Endocrinology*, *151*(1), 271-280. https://doi.org/10.1210/en.2009-0908.

Chustecka, M., Blügental, N., Majewski, P. M., & Adamska, I. (2021). 24 hour patterning in gene expression of pineal neurosteroid biosynthesis in young chickens (Gallus gallus domesticus L.). *Chronobiology International*, *38*(1), 46-60. https://doi.org/10.1080/07420528.2020.1823404.

Clark, B. J., Wells, J., King, S. R., & Stocco, D. M. (1994). The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR). *Journal of biological chemistry*, *269*(45), 28314-28322. https://doi.org/10.1016/S0021-9258(18)46930-X.

Compagnone, N.A. and Mellon, S.H., (2000). Neurosteroids: biosynthesis and function of these novel neuromodulators. *Frontiers in neuroendocrinology*, *21*(1), pp.1-56. https://doi.org/10.1006/frne.1999.0188.

Costantini, D., Fanfani, A., & Dell'Omo, G. (2008). Effects of corticosteroids on oxidative damage and circulating carotenoids in captive adult kestrels (Falco tinnunculus). *Journal of Comparative Physiology B*, *178*, 829-835. https://doi.org/10.1007/s00360-008-0270-z.

Cowan, M., Azpeleta, C., & López-Olmeda, J. F. (2017). Rhythms in the endocrine system of fish: a review. *Journal of Comparative Physiology B*, *187*, 1057-1089. https://doi.org/10.1007/s00360-017-1094-5.

Crews, D. (1984). Gamete production, sex hormone secretion, and mating behavior uncoupled. *Hormones and behavior*, *18*(1), 22-28. https://doi.org/10.1016/0018-506X(84)90047-3.

Cuesta, M., Clesse, D., Pévet, P., & Challet, E. (2009). From daily behavior to hormonal and neurotransmitters rhythms: comparison between diurnal and nocturnal rat species. *Hormones and behavior*, *55*(2), 338-347. https://doi.org/10.1016/j.yhbeh.2008.10.015.

Cui, Y. M., Wang, J., Zhang, H. J., Qi, G. H., & Wu, S. G. (2021). Effects of photoperiod on performance, ovarian morphology, reproductive hormone level, and hormone receptor mRNA expression in laying ducks. *Poultry Science*, *100*(4), 100979. https://doi.org/10.1016/j.psj.2021.01.002.

Czeisler, C. A., Shanahan, T. L., Klerman, E. B., Martens, H., Brotman, D. J., Emens, J. S., ... & Rizzo, J. F. (1995). Suppression of melatonin secretion in some blind

patients by exposure to bright light. *New England Journal of Medicine*, *332*(1), 6-11.

Dakan, E. L. (1934). Light absorption, feed consumption, and the urge to lay.

Dardente, H., Wyse, C. A., Birnie, M. J., Dupré, S. M., Loudon, A. S., Lincoln, G. A., & Hazlerigg, D. G. (2010). A molecular switch for photoperiod responsiveness in mammals. *Current Biology*, *20*(24), 2193-2198.

Dawson, A. (2008). Control of the annual cycle in birds: endocrine constraints and plasticity in response to ecological variability. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *363*(1497), 1621-1633. https://doi.org/10.1098/rstb.2007.0004.

Dawson, A., King, V. M., Bentley, G. E., & Ball, G. F. (2001). Photoperiodic control of seasonality in birds. *Journal of biological rhythms*, *16*(4), 365-380.

de Alba, G., Mourad, N. M. N., Paredes, J. F., Sánchez-Vázquez, F. J., & López-Olmeda, J. F. (2019). Daily rhythms in the reproductive axis of Nile tilapia (Oreochromis niloticus): Plasma steroids and gene expression in brain, pituitary, gonad and egg. *Aquaculture*, *507*, 313-321. https://ui.adsabs.harvard.edu/link_gateway/2019Aquac.507..313D/doi:10.1016/j.aquaculture.2019.04.047.

Deep, A., Schwean-Lardner, K., Crowe, T. G., Fancher, B. I., & Classen, H. L. (2010). Effect of light intensity on broiler production, processing characteristics, and welfare. *Poultry science*, *89*(11), 2326-2333.

Deviche, P., Beouche-Helias, B., Davies, S., Gao, S., Lane, S., & Valle, S. (2014). Regulation of plasma testosterone, corticosterone, and metabolites in response to stress, reproductive stage, and social challenges in a desert male songbird. *General and Comparative Endocrinology*, *203*, 120-131.

Di Fiore, M.M., Chieffi Baccari, G., Rastogi, R.K., DI MEGLIO, M., Pinelli, C. and Iela, L., 2005. Hormonal regulation on secondary sexual characters. In *Amphibian biology* (pp. 2228-2249). Surrey Beatty & Sons.

Di Rosa, V., López-Olmeda, J. F., Burguillo, A., Frigato, E., Bertolucci, C., Piferrer, F., & Sánchez-Vázquez, F. J. (2016). Daily rhythms of the expression of key genes involved in steroidogenesis and gonadal function in zebrafish. *PloS one*, *11*(6), e0157716. https://doi.org/10.1371%2Fjournal.pone.0157716.

Dixit, A. S., & Byrsat, S. (2018). Photoperiodic control of GnRH-I expression in seasonal reproduction of the Eurasian tree sparrow. *Photochemical & Photobiological Sciences*, *17*, 934-945. https://doi.org/10.1039/c8pp00153g.

Dixit, A. S., & Singh, N. S. (2014). Photoperiodic control of testicular growth, histomorphology and serum testosterone levels in the male Eurasian tree sparrow: Involvement of circadian rhythm. *General and Comparative Endocrinology*, *208*, 5-11. https://doi.org/10.1016/j.ygcen.2014.09.003.

Dixit, A. S., & Singh, N. S. (2016). Seasonality in circadian locomotor activity and serum testosterone level in the subtropical tree sparrow (Passer montanus). *Journal of Photochemistry and Photobiology B: Biology*, *158*, 61-68. https://doi.org/10.1016/j.jphotobiol.2016.02.014.

Do Rego, J. L., Seong, J. Y., Burel, D., Leprince, J., Luu-The, V., Tsutsui, K., ... & Vaudry, H. (2009). Neurosteroid biosynthesis: enzymatic pathways and neuroendocrine regulation by neurotransmitters and neuropeptides. *Frontiers in neuroendocrinology*, *30*(3), 259-301. https://doi.org/10.1016/j.yfrne.2009.05.006.

Dominoni, D. M., & Nelson, R. J. (2018). Artificial light at night as an environmental pollutant: An integrative approach across taxa, biological functions, and scientific disciplines. *Journal of experimental zoology. Part A, Ecological and integrative physiology*, *329*(8-9), 387.

Dominoni, D., Quetting, M., & Partecke, J. (2013). Artificial light at night advances avian reproductive physiology. *Proceedings of the Royal Society B: Biological Sciences*, *280*(1756), 20123017. https://doi.org/10.1098/rspb.2012.3017.

Dunlap, J. C., Loros, J. J., & DeCoursey, P. J. (2004). *Chronobiology: biological timekeeping*. Sinauer Associates.

Dunn, I. C., & Sharp, P. J. (1990). Photoperiodic requirements for LH release in juvenile broiler and egg-laying strains of domestic chickens fed ad libitum or restricted diets. *Reproduction*, *90*(1), 329-335.

Ellsworth, B. S., Egashira, N., Haller, J. L., Butts, D. L., Cocquet, J., Clay, C. M., ... & Camper, S. A. (2006). FOXL2 in the pituitary: molecular, genetic, and developmental analysis. *Molecular Endocrinology*, *20*(11), 2796-2805.

Erard, C. (1999). Grimmett, R, Inskipp, C. & Inskipp, T.—Birds of the Indian subcontinent. Helm Identification Guides. Christopher Helm, A. & C. Black, London. 1998. *Revue d'Écologie (La Terre et La Vie)*, *54*(4), 394-395.

Falcón, J., Besseau, L., Sauzet, S. and Boeuf, G., 2007. Melatonin effects on the hypothalamo–pituitary axis in fish. *Trends in Endocrinology & Metabolism*, *18*(2), pp.81-88. https://doi.org/10.1016/j.tem.2007.01.002.

Finkel, T., & Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *nature*, *408*(6809), 239-247. https://doi.org/10.1038/35041687.

Fonken, L. K., & Nelson, R. J. (2014). The effects of light at night on circadian clocks and metabolism. *Endocrine reviews*, *35*(4), 648-670.

Fonken, L. K., Aubrecht, T. G., Meléndez-Fernández, O. H., Weil, Z. M., & Nelson, R. J. (2013). Dim light at night disrupts molecular circadian rhythms and increases body weight. *Journal of biological rhythms*, *28*(4), 262-271. https://doi.org/10.1177/0748730413493862.

Fonken, L. K., Workman, J. L., Walton, J. C., Weil, Z. M., Morris, J. S., Haim, A., & Nelson, R. J. (2010). Light at night increases body mass by shifting the time of food intake. *Proceedings of the National Academy of Sciences*, *107*(43), 18664-18669. https://doi.org/10.1073/pnas.1008734107.

Forlano, P. M., Sisneros, J. A., Rohmann, K. N., & Bass, A. H. (2015). Neuroendocrine control of seasonal plasticity in the auditory and vocal systems of fish. *Frontiers in neuroendocrinology*, *37*, 129-145. https://doi.org/10.1016%2Fj.yfrne.2014.08.002.

Fox, R.J., Donelson, J.M., Schunter, C., Ravasi, T. and Gaitán-Espitia, J.D., 2019. Beyond buying time: the role of plasticity in phenotypic adaptation to rapid environmental change. *Philosophical transactions of the Royal Society B*, *374*(1768), p.20180174. https://doi.org/10.1098/rstb.2018.0174.

Freking, F., Nazairians, T. and Schlinger, B.A., 2000. The expression of the sex steroid-synthesizing enzymes CYP11A1, 3β-HSD, CYP17, and CYP19 in gonads and adrenals of adult and developing zebra finches. *General and comparative endocrinology*, *119*(2), pp.140-151. https://doi.org/10.1006/gcen.2000.7503.

G. Majumdar, A.K. Trivedi, N.J. Gupta, V. Kumar, Circadian synchronization determines critical day length for seasonal responses, Physiol. Behav. 147 (2015) 282–290.

Garner, W. W., & Allard, H. A. (1920). Effect of the relative length of day and night and other factors of the environment on growth and reproduction in plants. *Monthly Weather Review*, *48*(7), 415-415.

Gaston, K. J., Duffy, J. P., Gaston, S., Bennie, J., & Davies, T. W. (2014). Human alteration of natural light cycles: causes and ecological consequences. *Oecologia*, *176*, 917-931. https://doi.org/10.1007/s00442-014-3088-2.

Gaston, K. J., Visser, M. E., & Hölker, F. (2015). The biological impacts of artificial light at night: the research challenge. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *370*(1667), 20140133. https://doi.org/10.1098/rstb.2014.0133.

Gothilf, Y., Meiri, I., Elizur, A. and Zohar, Y., 1997. Preovulatory changes in the levels of three gonadotropin-releasing hormone-encoding messenger ribonucleic acids (mRNAs), gonadotropin I-subunit mRNAs, plasma gonadotropin, and steroids in the female gilthead seabream, Sparus aurata. *Biology of Reproduction*, *57*(5), pp.1145-1154. https://doi.org/10.1095/biolreprod57.5.1145.

Govoroun, M. S., Pannetier, M., Pailhoux, E., Cocquet, J., Brillard, J. P., Couty, I., ... & Cotinot, C. (2004). Isolation of chicken homolog of the FOXL2 gene and comparison of its expression patterns with those of aromatase during ovarian development. *Developmental dynamics: an official publication of the American Association of Anatomists*, *231*(4), 859-870. https://doi.org/10.1002/dvdy.20189.

Green, P. A., George, E. M., Rosvall, K. A., Johnsen, S., & Nowicki, S. (2022). Testosterone, signal coloration, and signal color perception in male zebra finch contests. Ethology, 128(2), 131-142. https://doi.org/10.1111%2Feth.13247.

Greives, T. J., Kingma, S. A., Beltrami, G., & Hau, M. (2012). Melatonin delays clutch initiation in a wild songbird. *Biology Letters*, *8*(3), 330-332. https://doi.org/10.1098/rsbl.2011.1100.

Gwinner, E., & Scheuerlein, A. (1998). Seasonal changes in day-light intensity as a potential zeitgeber of circannual rhythms in equatorial Stonechats. *Journal of Ornithology*, *139*(4), 407-412.

Hällfors, M. H., Antão, L. H., Itter, M., Lehikoinen, A., Lindholm, T., Roslin, T., & Saastamoinen, M. (2020). Shifts in timing and duration of breeding for 73

boreal bird species over four decades. *Proceedings of the National Academy of Sciences*, *117*(31), 18557-18565.

Halliwell, B., & Gutteridge, J. M. (2015). *Free radicals in biology and medicine*. Oxford university press.

Hartwig, H. G., & Van Veen, T. (1979). Spectral characteristics of visible radiation penetrating into the brain and stimulating extraretinal photoreceptors: Transmission recordings in vertebrates. *Journal of comparative physiology*, *130*(3), 277-282.

Hau, M. (2001). Timing of breeding in variable environments: tropical birds as model systems. *Hormones and behavior*, *40*(2), 281-290.

Henríquez-Hernández, L.A., Valenciano, A., Foro-Arnalot, P., Álvarez-Cubero, M.J., Cozar, J.M., Suárez-Novo, J.F., Castells-Esteve, M., Fernández-Gonzalo, P., De-Paula-Carranza, B., Ferrer, M. and Guedea, F., 2015, July. Genetic variations in genes involved in testosterone metabolism are associated with prostate cancer progression: A Spanish multicenter study. In *Urologic Oncology: Seminars and Original Investigations* (Vol. 33, No. 7, pp. 331-e1). Elsevier. https://doi.org/10.1016/j.urolonc.2015.04.003.

Hess, L. (2002, April). Corticosteroid synthesis and metabolism in birds. In *Seminars in Avian and Exotic Pet Medicine* (Vol. 11, No. 2, pp. 65-70). WB Saunders.

Higo, S., Hojo, Y., Ishii, H., Komatsuzaki, Y., Ooishi, Y., Murakami, G., ... & Kawato, S. (2011). Endogenous synthesis of corticosteroids in the hippocampus. *PLoS One*, *6*(7), e21631. https://doi.org/10.1371/journal.pone.0021631.

Holmes, W. N. (1976). The adrenal cortex of birds. *General and comparative endocrinology of the adrenal cortex*, 293-420.

Hu, J., Zhang, Z., Shen, W.J. and Azhar, S., 2010. Cellular cholesterol delivery, intracellular processing and utilization for biosynthesis of steroid

hormones. *Nutrition & metabolism*, *7*, pp.1-25. https://doi.org/10.1186/1743-7075-7-47.

Ings, J. S., & Van Der Kraak, G. J. (2006). Characterization of the mRNA expression of StAR and steroidogenic enzymes in zebrafish ovarian follicles. *Molecular Reproduction and Development: Incorporating Gamete Research*, *73*(8), 943-954. https://doi.org/10.1002/mrd.20490.

Ishibashi, T., & Kato, Y. (1951). Effects of irradiation with different wave-lengths on the development of the gonad in the fowl.

Itoh, M., & Ishii, S. (1990). Changes in plasma levels of gonadotropins and sex steroids in the toad, Bufo japonicus, in association with behavior during the breeding season. *General and comparative endocrinology*, *80*(3), 451-464. https://doi.org/10.1016/0016-6480(90)90194-Q.

Jalabert, C., Ma, C. and Soma, K.K., 2021. Profiling of systemic and brain steroids in male songbirds: Seasonal changes in neurosteroids. *Journal of neuroendocrinology*, *33*(1), p.e12922. https://doi.org/10.1111/jne.12922.

Jessop, T. S., Dempster, T., Letnic, M., & Webb, J. K. (2014). Interplay among nocturnal activity, melatonin, corticosterone and performance in the invasive cane toad (Rhinella marinus). *General and Comparative Endocrinology*, *206*, 43-50.

Karaganis, S. P., Kumar, V., Beremand, P. D., Bailey, M. J., Thomas, T. L., & Cassone, V. M. (2008). Circadian genomics of the chick pineal gland in vitro. *BMC genomics*, *9*, 1-17.

Karakatsouli, N., Papoutsoglou, S. E., Pizzonia, G., Tsatsos, G., Tsopelakos, A., Chadio, S., ... & Papadopoulou-Daifoti, Z. (2007). Effects of light spectrum on growth and physiological status of gilthead seabream Sparus aurata and rainbow trout Oncorhynchus mykiss reared under recirculating system conditions. *Aquacultural Engineering*, *36*(3), 302-309.

Kotula-Balak, M., Slomczynska, M., Fraczek, B., Bourguiba, S., Tabarowski, Z., Carreau, S. and Bilinska, B., 2003. Complementary approaches demonstrate that cellular aromatization in the bank vole testis is related to photoperiod. *European Journal of Histochemistry*, *47*(1), pp.55-62. https://doi.org/10.4081/807.

Krasnow, J. (1991, November). Advances in understanding ovarian physiology: regulation of steroidogenic enzymes in ovarian follicular differentiation. In *Seminars in reproductive endocrinology* (Vol. 9, No. 04, pp. 283-302). Copyright© 1991 by Thieme Medical Publishers, Inc. https://doi.org/10.1055/s-2007-1019421.

Kuiper, G. G., Lemmen, J. G., Carlsson, B. O., Corton, J. C., Safe, S. H., Van Der Saag, P. T., ... & Gustafsson, J. A. (1998). Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. *Endocrinology*, *139*(10), 4252-4263. https://doi.org/10.1210/endo.139.10.6216.

Kumar BS, Kumar V. (1993). Photoperiodic control of annual reproductive cycle in subtropical brahminy myna *(Sturnus pagodarum)*. *Gen Comp Endocrinol*. 89: 149 –160.

Kumar V, Kumar BS. (1991). The development of photorefractoriness in termination of the breeding season in the tropical brahminy myna: Role of photoperiod. *Reprod Nutr Dev*. 31: 27 – 36.

Kumar, M., Kumar, A., Tripathi, V., Prabhat, A., & Bhardwaj, S. K. (2024). Dimly illuminated nights alter behavior and negatively affect fat metabolism in adult male zebra finches. *Photochemical & Photobiological Sciences*, *23*(12), 2201-2210. https://doi.org/10.1007/s43630-024-00659-7.

Kumar, V., & Follett, B. K. (1993). The circadian nature of melatonin secretion in Japanese quail (Coturnix coturnix japonica). *Journal of pineal research*, *14*(4), 192-200. https://doi.org/10.1111/j.1600-079x.1993.tb00502.x.

Kumar, V., & Rani, S. (1996). Effects of wavelength and intensity of light in initiation of body fattening and gonadal growth in a migratory bunting under complete and skeleton photoperiods. *Physiology & behavior*, *60*(2), 625-631.

Kumar, V., & Rani, S. (1999). Light sensitivity of the photoperiodic response system in higher vertebrates: wavelength and intensity effects.

Kumar, V., Rani, S., & Malik, S. (2000). Wavelength of light mimics the effects of the duration and intensity of a long photoperiod in stimulation of gonadal responses in the male blackheaded bunting (Emberiza melanocephala). *Current Science*, 508-510.

Kumar, V., Rani, S., Malik, S., Trivedi, A. K., Schwabl, I., Helm, B., & Gwinner, E. (2007). Daytime light intensity affects seasonal timing via changes in the nocturnal melatonin levels. *Naturwissenschaften*, *94*, 693-696. 10.1007/s00114-007-0243-1.

Kumar, V., Singh, B. P., & Rani, S. (2004). The bird clock: a complex, multi-oscillatory and highly diversified system. *Biological Rhythm Research*, *35*(1-2), 121-144.

Kumar, V., Singh, S., Misra, M., & Malik, S. (2001). Effects of duration and time of food availability on photoperiodic responses in the migratory male blackheaded bunting (Emberiza melanocephala). *Journal of experimental Biology*, *204*(16), 2843-2848. https://doi.org/10.1242/jeb.204.16.2843.

Kumar, V., Wingfield, J. C., Dawson, A., Ramenofsky, M., Rani, S., & Bartell, P. (2010). Biological clocks and regulation of seasonal reproduction and migration in birds. *Physiological and Biochemical Zoology*, *83*(5), 827-835. https://doi.org/10.1086/652243.

Kusakabe, M., Nakamura, I., Evans, J., Swanson, P., & Young, G. (2006). Changes in mRNAs encoding steroidogenic acute regulatory protein, steroidogenic enzymes and receptors for gonadotropins during spermatogenesis in rainbow trout testes. *Journal of Endocrinology*, *189*(3), 541-554.

Lalpekhlui, R., Renthlei, Z., & Trivedi, A. K. (2022). Molecular expression of clock genes in central and peripheral tissues of white-rumped munia (Lonchura striata). *Chronobiology International*, *39*(8), 1058-1067. https://doi.org/10.1080/07420528.2022.2062374.

Laucht, S., Kempenaers, B., & Dale, J. (2010). Bill color, not badge size, indicates testosterone-related information in house sparrows. Behavioral ecology and sociobiology, 64, 1461-1471. https://doi.org/10.1007%2Fs00265-010-0961-9.

Levavi-Sivan, B., Bogerd, J., Mañanós, E. L., Gómez, A., & Lareyre, J. J. (2010). Perspectives on fish gonadotropins and their receptors. *General and comparative endocrinology*, *165*(3), 412-437. https://doi.org/10.1016/j.ygcen.2009.07.019.

Lewis, P. D., & Morris, T. R. (2006). *Poultry lighting: the theory and practice*. Northcot.

Lewis, P. D., Gous, R. M., Ghebremariam, W. K., & Sharp, P. J. (2007). Broiler breeders do not respond positively to photoperiodic increments given during the laying period. *British Poultry Science*, *48*(3), 245-252.

Li, M., Xue, K., Ling, J., Diao, F. Y., Cui, Y. G., & Liu, J. Y. (2010). The orphan nuclear receptor NR4A1 regulates transcription of key steroidogenic enzymes in ovarian theca cells. *Molecular and Cellular Endocrinology*, *319*(1-2), 39-46. https://doi.org/10.1016/j.mce.2010.01.014.

Li, N. C., Fan, J., & Papadopoulos, V. (2016). Sterol carrier protein-2, a nonspecific lipid-transfer protein, in intracellular cholesterol trafficking in testicular Leydig cells. *PLoS One*, *11*(2), e0149728. https://doi.org/10.1371/journal.pone.0149728.

Li, Q. X., Ke, N., Sundaram, R., & Wong-Staal, F. (2006). NR4A1, 2, 3 an orphan nuclear hormone receptor family involved in cell apoptosis and carcinogenesis. *Histology and histopathology*. https://doi.org/10.14670/hh-21.533.

Licht, P., Owens, D. W., Cliffton, K., & Penaflores, C. (1982). Changes in LH and progesterone associated with the nesting cycle and ovulation in the olive ridley sea turtle, Lepidochelys olivacea. *General and Comparative Endocrinology*, *48*(2), 247-253. https://doi.org/10.1016/0016-6480(82)90023-5.

Liddle, T. A., Stevenson, T. J., & Majumdar, G. (2022). Photoperiodic regulation of avian physiology: From external coincidence to seasonal reproduction. *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology*, *337*(9-10), 890-901.

LIN, H., DECUYPERE, E. & BUYSE, J. (2004b) Oxidative stress induced by corticosterone administration in broiler chickens (Gallus gallus domesticus) 2. Short-term effect. C

Lincoln, J. M., Barlowe, M. L., Rucker, H. R., & Parker, M. R. (2023). Reconsidering reproductive patterns in a model dissociated species, the red-sided garter snake: Sex-specific and seasonal changes in gonadal steroidogenic gene expression. *Frontiers in Endocrinology*, *14*, 1135535. https://doi.org/10.3389/fendo.2023.1135535.

Liu, X., Wei, D., Jiang, J., Liu, X., Tu, R., Luo, Z., ... & Mao, Z. (2020). Associations of SRD5A1 gene variants and testosterone with dysglycemia: Henan Rural Cohort study. *Nutrition, Metabolism and Cardiovascular Diseases*, *30*(4), 599-607. https://doi.org/10.1016/j.numecd.2019.11.011

Liu-Fu, S., Pan, J. Q., Sun, J. F., Shen, X., Jiang, D. L., Ouyang, H. J., ... & Huang, Y. M. (2024). Effect of immunization against OPN5 on the reproductive performance in Shan Partridge ducks under different photoperiods. *Poultry Science*, *103*(4), 103413.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *methods*, *25*(4), 402-408.. https://doi.org/10.1006/meth.2001.1262.

Lohmus, M., Sundström, L. F., & Moore, F. R. (2006). Non-invasive corticosterone treatment changes foraging intensity in red-eyed vireos Vireo olivaceus. *Journal of Avian Biology*, *37*(5), 523-526.

Löhr, H., & Hammerschmidt, M. (2011). Zebrafish in endocrine systems: recent advances and implications for human disease. *Annual review of physiology*, *73*(1), 183-211. https://doi.org/10.1146/annurev-physiol-012110-142320.

Longcore, T., & Rich, C. (2004). Ecological light pollution. *Frontiers in Ecology and the Environment*, *2*(4), 191-198. http://dx.doi.org/10.1890/1540-9295(2004)002[0191:ELP]2.0.CO;2.

Lutterschmidt, D. I., LeMaster, M. P., & Mason, R. T. (2004). Effects of melatonin on the behavioral and hormonal responses of red-sided garter snakes (Thamnophis sirtalis parietalis) to exogenous corticosterone. *Hormones and Behavior*, *46*(5), 692-702.

MacDougall-Shackleton, S. A., Stevenson, T. J., Watts, H. E., Pereyra, M. E., & Hahn, T. P. (2009). The evolution of photoperiod response systems and seasonal GnRH plasticity in birds. *Integrative and comparative biology*, *49*(5), 580-589.

Machluf, Y., Gutnick, A., & Levkowitz, G. (2011). Development of the zebrafish hypothalamus. *Annals of the New York Academy of Sciences*, *1220*(1), 93-105. https://doi.org/10.1111/j.1749-6632.2010.05945.x.

Majumdar, G., Liddle, T. A., Stewart, C., Marshall, C. J., Bain, M., & Stevenson, T. (2023). FSHβ links photoperiodic signaling to seasonal reproduction in Japanese quail. *Elife*, *12*, RP87751. https://doi.org/10.7554/eLife.87751.3.

Majumdar, G., Trivedi, A. K., Gupta, N. J., & Kumar, V. (2015). Circadian synchronization determines critical day length for seasonal responses. *Physiology & Behavior*, *147*, 282-290. https://doi.org/10.1016/j.physbeh.2015.05.005.

Malheiros, R. D., Moraes, V. M., Collin, A., Decuypere, E., & Buyse, J. (2003). Free diet selection by broilers as influenced by dietary macronutrient ratio and corticosterone supplementation. 1. Diet selection, organ weights, and plasma metabolites. *Poultry science*, *82*(1), 123-131. https://doi.org/10.1093/ps/82.1.123.

Malik, S., Rani, S., & Kumar, V. (2004). Wavelength dependency of light-induced effects on photoperiodic clock in the migratory blackheaded bunting (Emberiza melanocephala). *Chronobiology International*, *21*(3), 367-384.

Malik, S., Rani, S., Kumar, V. (2002). The influence of light wavelength on phasedependent responsiveness of the photoperiodic clock in migratory blackheaded bunting. Biol. Rhythm Res. 33:65–73.

Malik, S., Yadav, G., Rani, S., & Kumar, V. (2014). Light wavelength dependent circadian and seasonal responses in blackheaded bunting.

Malpaux, B., Migaud, M., Tricoire, H., & Chemineau, P. (2001). Biology of mammalian photoperiodism and the critical role of the pineal gland and melatonin. *Journal of biological rhythms*, *16*(4), 336-347.

Manser, C. E. (1996). Effects of lighting on the welfare of domestic poultry: a review. *Animal Welfare*, *5*(4), 341-360.

Marcel, E. V., Adriaensen, F., Van Balen, J. H., Blondel, J., Dhondt, A. A., Van Dongen, S., ... & Thomson, D. L. (2003). Variable responses to large-scale climate change in European Parus populations. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *270*(1513), 367-372.

Marcovitch, S. (1923). Plant lice and light exposure. *Science*, *58*(1513), 537-538.

Martin, L. J., Boucher, N., Brousseau, C., & Tremblay, J. J. (2008). The orphan nuclear receptor NUR77 regulates hormone-induced StAR transcription in Leydig cells through cooperation with Ca2+/calmodulin-dependent protein kinase

I. *Molecular endocrinology*, *22*(9), 2021-2037. https://doi.org/10.1210/me.2007-0370.

Martínez-Reyes, I., & Chandel, N. S. (2020). Mitochondrial TCA cycle metabolites control physiology and disease. *Nature communications*, *11*(1), 102.

Martínez-Soriano, F., Hernández-Gil de Tejada, T., López Bigorra, M., Ballester Carmona, S., & Vollrath, L. (1999). Circadian and seasonal changes of synaptic bodies in different parts of the rabbit pineal gland. *Histology and histopathology*, *14*(4), 1079-1092.

Maurya, S. R., & Mahalakshmi, R. (2016). VDAC-2: Mitochondrial outer membrane regulator masquerading as a channel?. *The FEBS journal*, *283*(10), 1831-1836. https://doi.org/10.1111/febs.13637.

McGraw, K. J., Correa, S. M., & Adkins-Regan, E. (2006). Testosterone upregulates lipoprotein status to control sexual attractiveness in a colorful songbird. *Behavioral Ecology and Sociobiology*, *60*, 117-122.

McGuire, N. L., Kangas, K., & Bentley, G. E. (2011). Effects of melatonin on peripheral reproductive function: regulation of testicular GnIH and testosterone. *Endocrinology*, *152*(9), 3461-3470. https://doi.org/10.1210/en.2011-1053.

McIntosh, L. J., Cortopassi, K. M., & Sapolsky, R. M. (1998). Glucocorticoids may alter antioxidant enzyme capacity in the brain: kainic acid studies. *Brain research*, *791*(1-2), 215-222. https://doi.org/10.1016/s0006-8993(98)00115-2.

McNabb, F. A. (2007). The hypothalamic-pituitary-thyroid (HPT) axis in birds and its role in bird development and reproduction. *Critical reviews in toxicology*, *37*(1-2), 163-193.

Meitzen, J., & Thompson, C. K. (2008). Seasonal-like growth and regression of the avian song control system: neural and behavioral plasticity in adult male

Gambel's white-crowned sparrows. *General and comparative endocrinology*, *157*(3), 259-265. https://doi.org/10.1016/j.ygcen.2008.03.014.

Menaker, M., & Underwood, H. (1976). Extraretinal photoreception in birds. *Extraretinal Photoreception*, 299-306.

Mishra, I., Agarwal, N., Rani, S., & Kumar, V. (2023). Photoperiodic regulation of seasonal reproduction in migratory birds: Molecular insights from hypothalamic and gonadal gene expression. Molecular Reproduction and Development, 90(7), 548–561. https://doi.org/10.1002/mrd.23607

Mishra, I., Singh, D., & Kumar, V. (2017). Seasonal alterations in the daily rhythms in hypothalamic expression of genes involved in the photoperiodic transduction and neurosteroid-dependent processes in migratory blackheaded buntings. *Journal of Neuroendocrinology*, *29*(5). https://doi.org/10.1111/jne.12469.

Misra, M., Rani, S., Singh, S., Kumar, V. (2004). Regulation of seasonality in the migratory male blackheaded bunting (Emberiza melanocephala). Reprod. Nutr. Dev. submitted.

Mistlberger, R. E. (2009). Food-anticipatory circadian rhythms: concepts and methods. *European Journal of Neuroscience*, *30*(9), 1718-1729. https://doi.org/10.1111/j.1460-9568.2009.06965.x.

Moaraf, S., Vistoropsky, Y., Pozner, T., Heiblum, R., Okuliarová, M., Zeman, M., & Barnea, A. (2020). Artificial light at night affects brain plasticity and melatonin in birds. *Neuroscience Letters*, *716*, 134639. https://doi.org/10.1016/j.neulet.2019.134639.

Moeller, G., & Adamski, J. (2009). Integrated view on 17beta-hydroxysteroid dehydrogenases. Molecular and cellular endocrinology, 301(1-2), 7-19. https://doi.org/10.1016/j.mce.2008.10.040.

Mousa-Balabel, T. M., & Mohamed, R. A. (2011). Effect of different photoperiods and melatonin treatment on rabbit reproductive performance. *Veterinary Quarterly*, *31*(4), 165-171.

Munck, A., Guyre, P. M., & Holbrook, N. J. (1984). Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. *Endocrine reviews*, *5*(1), 25-44.

Nakane, Y., & Yoshimura, T. (2014). Universality and diversity in the signal transduction pathway that regulates seasonal reproduction in vertebrates. *Frontiers in neuroscience*, *8*, 115.

Nakao, N., Ono, H., Yamamura, T., Anraku, T., Takagi, T., Higashi, K., ... & Yoshimura, T. (2008). Thyrotrophin in the pars tuberalis triggers photoperiodic response. *Nature*, *452*(7185), 317-322. https://doi.org/10.1530/REP-08-0041.

Nakao, N., Yasuo, S., Nishimura, A., Yamamura, T., Watanabe, T., Anraku, T., ... & Yoshimura, T. (2007). Circadian clock gene regulation of steroidogenic acute regulatory protein gene expression in preovulatory ovarian follicles. *Endocrinology*, *148*(7), 3031-3038. https://doi.org/10.1210/en.2007-0044.

Naomi, K., Yonju, H., Akira, T., Noboru, S., & Kiyoshi, S. (2008). Profiles of mRNA expression of FOXL2, P450arom, DMRT1, AMH, P450c17, SF1, ERα and AR, in relation to gonadal sex differentiation in duck embryo. *日本家禽学会誌*, *45*(2), 132-138. https://doi.org/10.2141/jpsa.45.132.

Navara, K. J., & Nelson, R. J. (2007). The dark side of light at night: physiological, epidemiological, and ecological consequences. *Journal of pineal research*, *43*(3), 215-224.

nee Pathak, N. D., & Lal, B. (2010). Seasonality in expression and distribution of nitric oxide synthase isoforms in the testis of the catfish, Clarias batrachus: role of nitric oxide in testosterone production. *Comparative Biochemistry and*

Physiology Part C: Toxicology & Pharmacology, *151*(3), 286-293. https://doi.org/10.1016/j.cbpc.2009.11.008.

Nelson, R. J., Badura, L. L., & Goldman, B. D. (1990). Mechanisms of seasonal cycles of behavior. *Annual review of psychology*, *41*(1), 81-108. https://doi.org/10.1146/annurev.ps.41.020190.000501.

Nettle, D., & Bateson, M. (2015). Adaptive developmental plasticity: what is it, how can we recognize it and when can it evolve?. *Proceedings of the Royal Society B: Biological Sciences*, *282*(1812), 20151005. https://doi.org/10.1098/rspb.2015.1005.

Newberry, R. C., Hunt, J. R., & Gardiner, E. E. (1988). Influence of light intensity on behavior and performance of broiler chickens. *Poultry Science*, *67*(7), 1020-1025.

Nir, I., Allen, A. E., & Pang, S. F. (1987). Pineal, Retinal, Serum Melatonin, and N-Acetylserotonin Rhythms in Chicks on Reversing Light Regimen Eye Covering. *Journal of pineal research*, *4*(3), 305-314.

Ohba, T., Holt, J. A., Billheimer, J. T., & Strauss III, J. F. (1995). Human sterol carrier protein x/sterol carrier protein 2 gene has two promoters. *Biochemistry*, *34*(33), 10660-10668. https://doi.org/10.1021/bi00033a042.

Oishi, T., Yamao, M., Kondo, C., Haida, Y., Masuda, A., & Tamotsu, S. (2001). Multiphotoreceptor and multioscillator system in avian circadian organization. *Microscopy research and technique*, *53*(1), 43-47.

Okuliarova, M., Rumanova, V. S., Stebelova, K., & Zeman, M. (2020). Dim light at night disturbs molecular pathways of lipid metabolism. *International journal of molecular sciences*, *21*(18), 6919. https://doi.org/10.3390/ijms21186919.

Olsson, P. E. T. E. R. (2016). *Colour Vision in Birds*.

Ooi, G. T., Tawadros, N., & Escalona, R. M. (2004). Pituitary cell lines and their endocrine applications. *Molecular and cellular endocrinology*, *228*(1-2), 1-21. https://doi.org/10.1016/j.mce.2004.07.018.

Orzechowski, A., Ostaszewski, P., Wilczak, J., Jank, M., Bałasińska, B., Waręski, P., & Fuller Jr, J. (2002). Rats with a glucocorticoid-induced catabolic state show symptoms of oxidative stress and spleen atrophy: the effects of age and recovery. *Journal of Veterinary Medicine Series A*, *49*(5), 256-263. https://doi.org/10.1046/j.1439-0442.2002.00443.x.

Osorio, D., & Vorobyev, M. (2008). A review of the evolution of animal colour vision and visual communication signals. *Vision research*, *48*(20), 2042-2051.

Ouyang, J. Q., Davies, S., & Dominoni, D. (2018). Hormonally mediated effects of artificial light at night on behavior and fitness: linking endocrine mechanisms with function. *Journal of Experimental Biology*, *221*(6), jeb156893.

Paitz, R. T., & Cagney, E. (2019). In ovo metabolism of progesterone to 5β-pregnanedione in chicken eggs: Implications for how yolk progesterone influences embryonic development. *General and Comparative Endocrinology*, *282*, 113221. https://doi.org/10.1016/j.ygcen.2019.113221.

Palacín, I., Forcada, F., & Abecia, J. A. (2011). Meta-analysis of the efficacy of melatonin implants for improving reproductive performance in sheep. *Spanish Journal of Agricultural Research*, *9*(3), 730-743.

Pandha SK, Thapliyal JP. (1969). Light and the gonadal cycle of the blackheaded munia, *Munia malacca malacca*. *Endocrinol Japonica*. 16: 157 – 161.

Pankhurst, N. W., & Porter, M. J. R. (2003). Cold and warm water fish: Photoperiod and reproduction. *Aquaculture, 224*(1-4), 1-16. https://doi.org/10.1016/S0044-8486(03)00205-9

Papadopoulos, V., & Miller, W. L. (2012). Role of mitochondria in steroidogenesis. *Best practice & research Clinical endocrinology & metabolism*, *26*(6), 771-790. https://doi.org/10.1016/j.beem.2012.05.002.

Parajes, S., Griffin, A., Taylor, A. E., Rose, I. T., Miguel-Escalada, I., Hadzhiev, Y., ... & Krone, N. (2013). Redefining the initiation and maintenance of zebrafish interrenal steroidogenesis by characterizing the key enzyme cyp11a2. *Endocrinology*, *154*(8), 2702-2711. https://doi.org/10.1210/en.2013-1145.

Parmesan, C., & Yohe, G. (2003). A globally coherent fingerprint of climate change impacts across natural systems. *nature*, *421*(6918), 37-42.

Peek, C. E., & Cohen, R. E. (2018). Seasonal regulation of steroidogenic enzyme expression within the green anole lizard (Anolis carolinensis) brain and gonad. *General and Comparative Endocrinology*, *268*, 88-95. https://doi.org/10.1016/j.ygcen.2018.08.005.

Pelham, R. W., Ralph, C. L., & Campbell, I. M. (1972). Mass spectral identification of melatonin in blood. *Biochemical and Biophysical Research Communications*, *46*(3), 1236-1241.

Pérez, J. H., Tolla, E., Bishop, V. R., Foster, R. G., Peirson, S. N., Dunn, I. C., ... & Stevenson, T. J. (2023). Functional inhibition of deep brain non-visual opsins facilitates acute long day induction of reproductive recrudescence in male Japanese quail. *Hormones and Behavior*, *148*, 105298. https://doi.org/10.1016/j.yhbeh.2022.105298.

Pérez, J. H., Tolla, E., Dunn, I. C., Meddle, S. L., & Stevenson, T. J. (2019). A comparative perspective on extra-retinal photoreception. *Trends in Endocrinology & Metabolism*, *30*(1), 39-53. https://doi.org/10.1016/j.tem.2018.10.005.

Perfito, N., Jeong, S.Y., Silverin, B., Calisi, R.M., Bentley, G.E. and Hau, M., 2012. Anticipating spring: wild populations of great tits (Parus major) differ in

expression of key genes for photoperiodic time measurement. *PloS one*, *7*(4), p.e34997. https://doi.org/10.1371/journal.pone.0034997.

Pfeifer, S. M., Furth, E. E., Ohba, T., Chang, Y. J., Rennert, H., Sakuragi, N., ... & Strauss III, J. F. (1993). Sterol carrier protein 2: a role in steroid hormone synthesis?. *The Journal of Steroid Biochemistry and Molecular Biology*, *47*(1-6), 167-172. https://doi.org/10.1016/0960-0760(93)90071-4.

Poorthuis, B.J., Glatz, J.F., Akeroyd, R. and Wirtz, K.W., 1981. A new high-yield procedure for the purification of the non-specific phospholipid transfer protein from rat liver. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, *665*(2), pp.256-261. https://doi.org/10.1016/0005-2760(81)90010-2.

Porter, L., Porter, A., Potter, H., Alenciks, E., Fraley, S. M., & Fraley, G. S. (2018). Low light intensity in Pekin duck breeder barns has a greater impact on the fertility of drakes than hens. *Poultry Science*, *97*(12), 4262-4271.

Prasad, M., Kaur, J., Pawlak, K.J., Bose, M., Whittal, R.M. and Bose, H.S., 2015. Mitochondria-associated endoplasmic reticulum membrane (MAM) regulates steroidogenic activity via steroidogenic acute regulatory protein (StAR)-voltage-dependent anion channel 2 (VDAC2) interaction. *Journal of Biological Chemistry*, *290*(5), pp.2604-2616. https://doi.org/10.1074%2Fjbc.M114.605808.

Qiang, W., Murase, T. and Tsubota, T., 2003. Seasonal changes in spermatogenesis and testicular steroidogenesis in wild male raccoon dogs (Nyctereutes procynoides). *Journal of Veterinary Medical Science*, *65*(10), pp.1087-1092. https://doi.org/10.1292/jvms.65.1087.

Rani S, Kumar V. (1999). Time course of sensitivity of the photoinducible phase to light in the redheaded bunting. *Biol Rhythm Res*. 30: 555 – 562.

Rani, S., & Kumar, V. (2000). Phasic response of the photoperiodic clock to wavelength and intensity of light in the redheaded bunting, Emberiza bruniceps. Physiology & behavior, 69(3), 277-283.

Rani, S., Singh, S., & Kumar, V. (2002). Light sensitivity of the biological clock. In *Biological rhythms* (pp. 232-243). Berlin, Heidelberg: Springer Berlin Heidelberg.

Reiter, R. J., Mayo, J. C., Tan, D. X., Sainz, R. M., Alatorre-Jimenez, M., & Qin, L. (2016). Melatonin as an antioxidant: under promises but over delivers. *Journal of pineal research*, *61*(3), 253-278.

Reiter, R. J., Tan, D. X., Manchester, L. C., & Qi, W. (2001). Biochemical reactivity of melatonin with reactive oxygen and nitrogen species: a review of the evidence. *Cell biochemistry and biophysics*, *34*, 237-256.

Ren, J., Sun, C., Chen, L., Hu, J., Huang, X., Liu, X., & Lu, L. (2019). Exploring differentially expressed key genes related to development of follicle by RNA-seq in Peking ducks (Anas Platyrhynchos). *PLoS One*, *14*(6), e0209061. https://doi.org/10.1371/journal.pone.0209061.

Rensing, L., & Ruoff, P. (2002). Temperature effect on entrainment, phase shifting, and amplitude of circadian clocks and its molecular bases. *Chronobiology international*, *19*(5), 807-864. https://doi.org/10.1081/cbi-120014569.

Renthlei, Z., & Trivedi, A. K. (2019). Effect of urban environment on pineal machinery and clock genes expression of tree sparrow (Passer montanus). *Environmental Pollution*, *255*, 113278. https://doi.org/10.1016/j.envpol.2019.113278.

Renthlei, Z., Borah, B. K., & Trivedi, A. K. (2021). Urban environment alter the timing of progression of testicular recrudescence in tree sparrow (Passer montanus). *Environmental Science and Pollution Research*, *28*, 31097-31107. https://doi.org/10.1007/s11356-021-12918-6.

Renthlei, Z., Borah, B.K., Gurumayum, T. and Trivedi, A.K. (2020). Season dependent effects of urban environment on circadian clock of tree sparrow (Passer montanus). *Photochemical & Photobiological Sciences*, *19*, pp.1741-1749. https://doi.org/10.1039/d0pp00257g.

Renthlei, Z., Gurumayum, T., Borah, B. K., & Trivedi, A. K. (2019). Daily expression of clock genes in central and peripheral tissues of tree sparrow (Passer montanus). *Chronobiology International*, *36*(1), 110-121. https://doi.org/10.1080/07420528.2018.1523185.

Renthlei, Z., Yatung, S., Lalpekhlui, R., & Trivedi, A.K. (2022). Seasonality in tropical birds. *Journal of Experimental Zoology- Part A Ecological and Integrative Physiology*. 337(9-10): 952-966. https://doi.org/10.1002/jez.2649.

Reppert, S. M., & Weaver, D. R. (2002). Coordination of circadian timing in mammals. *Nature*, *418*(6901), 935-941. https://doi.org/10.1038/nature00965.

Richardson, J. T. (2011). Eta squared and partial eta squared as measures of effect size in educational research. *Educational research review*, *6*(2), 135-147.

Ringoen, A. R. (1942). Effects of continuous green and red light illumination on gonadal response in the English sparrow, Passer domesticus (Linnaeus).

Rissman, E. F., Wersinger, S. R., Fugger, H. N., & Foster, T. C. (1999). Sex with knockout models: behavioral studies of estrogen receptor α. *Brain research*, *835*(1), 80-90. https://doi.org/10.1016/s0006-8993(99)01452-3.

Robbins, C. (2012). *Wildlife feeding and nutrition*. Elsevier.

Romero, L. M. (2004). Physiological stress in ecology: lessons from biomedical research. *Trends in ecology & evolution*, *19*(5), 249-255. https://doi.org/10.1016/j.tree.2004.03.008.

Root, T. L., Price, J. T., Hall, K. R., Schneider, S. H., Rosenzweig, C., & Pounds, J. A. (2003). Fingerprints of global warming on wild animals and plants. *Nature*, *421*(6918), 57-60.

Rosati, L., Agnese, M., Di Fiore, M. M., Andreuccetti, P., & Prisco, M. (2016). P450 aromatase: a key enzyme in the spermatogenesis of the Italian wall lizard, Podarcis sicula. *Journal of Experimental Biology*, *219*(15), 2402-2408. https://doi.org/10.1242/jeb.135996.

Rosati, L., Di Fiore, M. M., Prisco, M., Di Giacomo Russo, F., Venditti, M., Andreuccetti, P., ... & Santillo, A. (2019). Seasonal expression and cellular distribution of star and steroidogenic enzymes in quail testis. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, *332*(6), 198-209. https://doi.org/10.1002/jez.b.22896.

Rosati, L., Santillo, A., Di Fiore, M. M., Andreuccetti, P., & Prisco, M. (2017). Testicular steroidogenic enzymes in the lizard Podarcis sicula during the spermatogenic cycle. *Comptes Rendus Biologies*, *340*(11-12), 492-498. https://doi.org/10.1016/j.crvi.2017.10.001.

Rosvall, K. A., Bergeon Burns, C. M., Jayaratna, S. P., Dossey, E. K., & Ketterson, E. D. (2016). Gonads and the evolution of hormonal phenotypes. *Integrative and comparative biology*, *56*(2), 225-234. https://doi.org/10.1093/icb/icw050.

Rowan, W. (1925). Relation of light to bird migration and developmental changes. *Nature*, *115*(2892), 494-495.

Rozenboim, I., Biran, I., Chaiseha, Y., Yahav, S., Rosenstrauch, A., Sklan, D., & Halevy, O. (2004). The effect of a green and blue monochromatic light combination on broiler growth and development. *Poultry science*, *83*(5), 842-845.

Rył, A., Rotter, I., Grzywacz, A., Małecka, I., Skonieczna-Żydecka, K., Grzesiak, K., ... & Laszczyńska, M. (2017). Molecular analysis of the SRD5A1 and SRD5A2 genes in patients with benign prostatic hyperplasia with regard to metabolic

parameters and selected hormone levels. *International journal of environmental research and public health*, *14*(11), 1318.p.1318. https://doi.org/10.3390%2Fijerph14111318.

Sainz, R. M., Mayo, J. C., Reiter, R. J., Antolín, I., Esteban, M. M., & Rodríguez, C. (1999). Melatonin regulates glucocorticoid receptor: an answer to its antiapoptotic action in thymus. *The FASEB journal*, *13*(12), 1547-1556.

Salvante, K. G., & Williams, T. D. (2003). Effects of corticosterone on the proportion of breeding females, reproductive output and yolk precursor levels. *General and comparative endocrinology*, *130*(3), 205-214.

Sangma, J. T., & Trivedi, A. K. (2023). Light at night: effect on the daily clock, learning, memory, cognition, and expression of transcripts in different brain regions of rat. *Photochemical & Photobiological Sciences*, *22*(10), 2297-2314. https://doi.org/10.1007/s43630-023-00451-z.

Sangma, J. T., Renthlei, Z., & Trivedi, A. K. (2024). Bright daylight produces negative effects on affective and cognitive outcomes in nocturnal rats. *Journal of Photochemistry and Photobiology B: Biology*, *253*, 112885. https://doi.org/10.1016/j.jphotobiol.2024.112885.

Santillo, A., Falvo, S., Chieffi, G., Di Giacomo Russo, F., Rosati, L., & Di Fiore, M. M. (2018). Testis and brain steroidogenesis during the reproductive cycle of Pelophylax esculentus. In *Amphibians: Biology, ecology and conservation* (pp. 39-54). Nova Science Publishers, Inc..

Santillo, A., Falvo, S., Di Fiore, M. M., & Baccari, G. C. (2017). Seasonal changes and sexual dimorphism in gene expression of StAR protein, steroidogenic enzymes and sex hormone receptors in the frog brain. *General and Comparative Endocrinology*, *246*, 226-232. http://dx.doi.org/10.1111/azo.12169.

Sapolsky, R. M., Romero, L. M., & Munck, A. U. (2000). How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory,

and preparative actions. *Endocrine reviews*, *21*(1), 55-89. https://doi.org/10.1210/edrv.21.1.0389.

Satapati, S., Sunny, N. E., Kucejova, B., Fu, X., He, T. T., Méndez-Lucas, A., ... & Burgess, S. C. (2012). Elevated TCA cycle function in the pathology of diet-induced hepatic insulin resistance and fatty liver. *Journal of lipid research*, *53*(6), 1080-1092. https://doi.org/10.1194/jlr.M023382.

Scallen, T. J., Noland, B. J., Gavey, K. L., Bass, N. M., Ockner, R. K., Chanderbhan, R., & Vahouny, G. V. (1985). Sterol carrier protein 2 and fatty acid-binding protein. Separate and distinct physiological functions. *Journal of Biological Chemistry*, *260*(8), 4733-4739.

Scanes, C. G., and Dridi, S. (Editors) (2021). Sturkie's avian physiology (Massachusetts, United States: Academic Press).

Schiffer, L., Barnard, L., Baranowski, E. S., Gilligan, L. C., Taylor, A. E., Arlt, W., ... & Storbeck, K. H. (2019). Human steroid biosynthesis, metabolism and excretion are differentially reflected by serum and urine steroid metabolomes: A comprehensive review. *The Journal of steroid biochemistry and molecular biology*, *194*, 105439. https://doi.org/10.1016/j.jsbmb.2019.105439.

Seltman, H., Diven, W., Rizk, M., Noland, B. J., Chanderbhan, R., Scallen, T. J., ... & Sanghvi, A. (1985). Regulation of bile-acid synthesis. Role of sterol carrier protein2 in the biosynthesis of 7α-hydroxycholesterol. *Biochemical Journal*, *230*(1), 19-24.https://doi.org/10.1042/bj2300019.

Sharp, P. M., Averof, M., Lloyd, A. T., Matassi, G., & Peden, J. F. (1995). DNA sequence evolution: the sounds of silence. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, *349*(1329), 241-247.

Shochat, E., Warren, P. S., Faeth, S. H., McIntyre, N. E., & Hope, D. (2006). From patterns to emerging processes in mechanistic urban ecology. *Trends in ecology & evolution*, *21*(4), 186-191. https://doi.org/10.1016/j.tree.2005.11.019.

Siepielski, A. M., DiBattista, J. D., & Carlson, S. M. (2009). It's about time: the temporal dynamics of phenotypic selection in the wild. *Ecology letters*, *12*(11), 1261-1276. https://doi.org/10.1111/j.1461-0248.2009.01381.x.

Sies, H. (1991). Oxidative stress: from basic research to clinical application. *The American journal of medicine*, *91*(3), S31-S38. https://doi.org/10.1016/0002-9343(91)90281-2.

Silverin, B. (1986). Corticosterone-binding proteins and behavioral effects of high plasma levels of corticosterone during the breeding period in the pied flycatcher. *General and Comparative Endocrinology*, *64*(1), 67-74.

Silverin, B., Kikuchi, M., & Ishii, S. (1997). Seasonal changes in follicle-stimulating hormone in free-living great tits. *General and comparative endocrinology*, *108*(3), 366-373.

Singh S, Chandola A. (1981). Photoperiodic control of seasonal reproduction in tropical weaver bird. *J Exp Zool*. 216: 293 – 298.

Singh, J., Rani, S., & Kumar, V. (2012). Functional similarity in relation to the external environment between circadian behavioral and melatonin rhythms in the subtropical Indian weaver bird. *Hormones and behavior*, *61*(4), 527-534. https://doi.org/10.1016/j.yhbeh.2012.01.015.

Siopes, T. D., & Wilson, W. O. (1980). Participation of the eyes in the photostimulation of chickens. *Poultry Science*, *59*(5), 1122-1125.

Soccio, R.E. and Breslow, J.L., 2003. StAR-related lipid transfer (START) proteins: mediators of intracellular lipid metabolism. *Journal of Biological Chemistry*, *278*(25), pp.22183-22186. https://doi.org/10.1074/jbc.r300003200.

Son, G. H., Chung, S., Choe, H. K., Kim, H. D., Baik, S. M., Lee, H., ... & Kim, K. (2008). Adrenal peripheral clock controls the autonomous circadian rhythm of glucocorticoid by causing rhythmic steroid production. *Proceedings of the*

National Academy of Sciences, *105*(52), 20970-20975. https://doi.org/10.1073/pnas.0806962106.

Soper, D. S. (2013). F-value and p-value calculator for multiple regression. *See http://www. danielsoper. com/statcalc*.

Steele, C. T., Zivkovic, B. D., Siopes, T., & Underwood, H. (2003). Ocular clocks are tightly coupled and act as pacemakers in the circadian system of Japanese quail. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *284*(1), R208-R218.

Stevenson, T. J., Bernard, D. J., McCarthy, M. M., & Ball, G. F. (2012). Photoperiodic regulation of seasonal reproduction in birds: Integrating environmental and endocrine mechanisms. *Hormones and Behavior*, 62(3), 229–239. https://doi.org/10.1016/j.yhbeh.2012.03.004

Stocco, D. M. (2001). StAR protein and the regulation of steroid hormone biosynthesis. *Annual review of physiology*, *63*(1), 193-213.

Sun, L., Wu, Z., Li, F., Liu, L., Li, J., Zhang, D., & Sun, C. (2017). Effect of light intensity on ovarian gene expression, reproductive performance and body weight of rabbit does. *Animal reproduction science*, *183*, 118-125.

Taufique, S. T., Prabhat, A., & Kumar, V. (2018). Illuminated night alters hippocampal gene expressions and induces depressive-like responses in diurnal corvids. *European Journal of Neuroscience*, *48*(9), 3005-3018. https://doi.org/10.1111/ejn.14157.

Taves, M. D., Ma, C., Heimovics, S. A., Saldanha, C. J., & Soma, K. K. (2011). Measurement of steroid concentrations in brain tissue: methodological considerations. *Frontiers in Endocrinology*, *2*, 39.

Teerlink, T., Van Der Krift, T.P., Van Heusden, G.P.H. and Wirtz, K.W., 1984. Determination of nonspecific lipid transfer protein in rat tissues and Morris hepatomas by enzyme immunoassay. *Biochimica et Biophysica Acta (BBA)-*

Lipids and Lipid Metabolism, *793*(2), pp.251-259. https://doi.org/10.1016/0005-2760(84)90327-8.

Tessmar-Raible, K., Raible, F., & Arboleda, E. (2011). Another place, another timer: marine species and the rhythms of life. *Bioessays*, *33*(3), 165-172. https://doi.org/10.1002/bies.201000096.

Tewary PD, Dixit AS. (1986). Photoperiodic regulation of the reproduction in subtropical yellow-throated sparrows (Gymnorhis xanthocollis). Condor. 88: 70 – 73.

Tewary PD, Kumar V, Prasad BN. (1983). Influence of photoperiod in subtropical migratory finch, the common Indian rosefinch, *Carpodacus erythrinus*. *Ibis*. 125: 115-120.

Tewary PD, Kumar V. (1982). Photoperiodic responses of a subtropical migratory finch, the blackheaded bunting *(Emberiza melanocephala)*. *Condor*. 84: 168–171.

Tewary PD, Kumar V. (1983a). Effect of light on the gonad and body weight in the crested bunting (*Melophus lathami*). *Environ Control Biol*. 21(1): 7 – 10.

Tewary PD, Thapliyal JP. (1965). Effect of long days on the hypophyseal and gonadal cycles of lal munia (*Estrilda amandava*). *Am Zool*. 5: 657 – 658.

Tewary PD, Tripathi BK. (1983). Photoperiodic control of reproduction in female redheaded bunting. *J Exp Zool*. 226: 269 – 272.

Tewary PD, Tripathi BK. (1985). Photoperiodic induction of testicular growth in the subtropical yellow-throated sparrows (*Gymnorchis xanthocollis*). *Arch Biol*. 96: 425 – 439.

Tewary PD. (1967). Sexual cycle and secondary sex character of some Indian birds. Ph.D. Thesis. Banaras Hindu University, Varanasi, India.

Thackeray, S.J., Sparks, T.H., Frederiksen, M., Burthe, S., Bacon, P.J., Bell, J.R., et al., 2010. Trophic level asynchrony in rates of phenological change for marine, freshwater and terrestrial environments. Glob. Change Biol. 16, 3304–3313.

Thapliyal JP, Chandola A, Pavnaskar J. (1975). Effects of continuous light and very short photoperiods of the gonadal function of a subtropical finch, spottedmunia, *Lonchura punctulata*. *J Endocrinol*. 65: 141 – 142.

Thapliyal JP, Saxena RN. (1964a). Gonadal development of the male blackheaded munia under constant nine-hour (short) days. *J Exp Zool*. 156: 153 –156.

Thapliyal JP, Saxena RN. (1964b). Absence of refractory period in the common weaver bird. *Condor*. 66: 199 – 208.

Thapliyal JP, Tewary PD. (1963). Effect of nine hour short days on the sexual cycle of lal munia, *Estrilda amandava*. Proc XVI Int Congr Zool. Washington D.C. 1:40.

Thapliyal JP, Tewary PD. (1964). Effect of light on the pituitary, gonad and plumage pigmentation in the avadavat, *Estrilda amandava*, and Baya Weaver, *Ploceus philippinus*. *Proc Zool Soc London*. 142: 67 – 71.

Thongbuakaew, T., Suwansa-Ard, S., Chaiyamoon, A., Cummins, S.F. and Sobhon, P., 2021. Sex steroids and steroidogenesis-related genes in the sea cucumber, Holothuria scabra and their potential role in gonad maturation. *Scientific reports*, *11*(1), p.2194. https://doi.org/10.1038/s41598-021-81917-x.

Tokarz, J., Mindnich, R., Norton, W., Möller, G., De Angelis, M. H., & Adamski, J. (2012). Discovery of a novel enzyme mediating glucocorticoid catabolism in fish: 20beta-hydroxysteroid dehydrogenase type 2. *Molecular and Cellular Endocrinology*, *349*(2), 202-213.

Tomy, S., Wu, G. C., Huang, H. R., Dufour, S., & Chang, C. F. (2007). Developmental expression of key steroidogenic enzymes in the brain of protandrous black

porgy fish, Acanthopagrus schlegeli. *Journal of neuroendocrinology*, *19*(8), 643-656. https://doi.org/10.1111/j.1365-2826.2007.01572.x.

Tosini, G., Ferguson, I., & Tsubota, K. (2016). Effects of blue light on the circadian system and eye physiology. *Molecular vision*, *22*, 61.

Trivedi, A. K., Kumar, J., Rani, S., & Kumar, V. (2014). Annual life history–dependent gene expression in the hypothalamus and liver of a migratory songbird: insights into the molecular regulation of seasonal metabolism. *Journal of biological rhythms*, *29*(5), 332-345.

Trivedi, A. K., Rani, S., & Kumar, V. (2004). Melatonin blocks inhibitory effects of prolactin on photoperiodic induction of gain in body mass, testicular growth and feather regeneration in the migratory male redheaded bunting (Emberiza bruniceps). *Reproductive Biology and Endocrinology*, *2*, 1-8.

Trivedi, A. K., Rani, S., & Kumar, V. (2006). Control of annual reproductive cycle in the subtropical house sparrow (*Passer domesticus*): evidence for conservation of photoperiodic control mechanisms in birds. *Frontiers in Zoology*, *3*, 1-12. http://dx.doi.org/10.1186/1742-9994-3-12.

Trivedi, A. K., Vanlalmalsawmi, E., Renthlei, Z., & Jongte, L. (2022). Food, not the photoperiod, entrains the circadian rhythms in the liver of Winstar albino rats. Journal of Applied and Natural Science, 14(2), 559.

Trivedi, A.K., Rani, S., & Kumar, V. (2014). Circadian adaptation to seasons: Effects on activity *behavior in subtropical House Sparrow, Passer domesticus. Biological* Rhythm Research. 45(3); 465-475. https://doi.org/10.1080/09291016.2013.850888.

Trivedi, A.K., Sur, S., Sharma, A., Taufique, S.T., Gupta, N.J. and Kumar, V. (2019). Temperature alters the hypothalamic transcription of photoperiod responsive genes in induction of seasonal response in migratory redheaded buntings. *Molecular and cellular endocrinology*, *493*, p.110454. https://doi.org/10.1016/j.mce.2019.110454.

Tsubota, T., Howell-Skalla, L., Nitta, H., Osawa, Y., Mason, J.I., Meiers, P.G., Nelson, R.A. and Bahr, J.M., 1997. Seasonal changes in spermatogenesis and testicular steroidogenesis in the male black bear Ursus americanus. *Reproduction*, *109*(1), pp.21-27. https://doi.org/10.1530/jrf.0.1090021.

Tsutsui, K., Saigoh, E., Ukena, K., Teranishi, H., Fujisawa, Y., Kikuchi, M., Ishii, S. and Sharp, P.J. (2000). A novel avian hypothalamic peptide inhibiting gonadotropin release. *Biochemical and biophysical research communications*, *275*(2), pp.661-667. https://doi.org/10.1006/bbrc.2000.3350.

Tsutsui, K., Ukena, K., Takase, M., Kohchi, C. and Lea, R.W. (1999). Neurosteroid biosynthesis in vertebrate brains. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, *124*(2), pp.121-129. https://doi.org/10.1016/s07428413(99)00065-1.

Ubuka, T., Kim, S., Huang, Y.C., Reid, J., Jiang, J., Osugi, T., Chowdhury, V.S., Tsutsui, K. and Bentley, G.E. (2008). Gonadotropin-inhibitory hormone neurons interact directly with gonadotropin-releasing hormone-I and-II neurons in European starling brain. *Endocrinology*, *149*(1), pp.268-278. https://doi.org/10.1210/en.2007-0983.

Underwood, H., Steele, C. T., & Zivkovic, B. (2001). Circadian organization and the role of the pineal in birds. *Microscopy research and technique*, *53*(1), 48-62.

Vahouny, G. V., Chanderbhan, R., Kharroubi, A., Noland, B. J., Pastuszyn, A., & Scallen, T. J. (1987). Sterol carrier and lipid transfer proteins. *Advances in lipid research*, *22*, 83-113. https://doi.org/10.1016/B978-0-12-024922-0.50007-2.

Vatine, G., Vallone, D., Gothilf, Y., & Foulkes, N. S. (2011). It's time to swim! Zebrafish and the circadian clock. *FEBS letters*, *585*(10), 1485-1494. https://doi.org/10.1016/j.febslet.2011.04.007.

Veerappa, S., & McClure, J. (2020). Intermediary metabolism. *Anaesthesia & Intensive Care Medicine*, *21*(3), 162-167.

Voigt, C., & Leitner, S. (2008). Seasonality in song behaviour revisited: seasonal and annual variants and invariants in the song of the domesticated canary (Serinus canaria). *Hormones and behavior*, *54*(3), 373-378. https://doi.org/10.1016/j.yhbeh.2008.05.001.

Vriend, J., & Lauber, J. K. (1973). Effects of light intensity, wavelength and quanta on gonads and spleen of the deer mouse. *Nature*, *244*(5410), 37-38.

Walther, G. R., Post, E., Convey, P., Menzel, A., Parmesan, C., Beebee, T. J., ... & Bairlein, F. (2002). Ecological responses to recent climate change. *Nature*, *416*(6879), 389-395.

Wang, D. S., Kobayashi, T., Zhou, L. Y., Paul-Prasanth, B., Ijiri, S., Sakai, F., ... & Nagahama, Y. (2007). Foxl2 up-regulates aromatase gene transcription in a female-specific manner by binding to the promoter as well as interacting with ad4 binding protein/steroidogenic factor 1. *Molecular Endocrinology*, *21*(3), 712-725.

Wang, Y., Guo, Z. Y., Zhang, C., Miao, D. Z., Mao, X. Y., Lu, S. M., ... & Wang, Z. Y. (2023). Characterization of ovarian follicles, serum steroid hormone concentration, and steroidogenic gene expression profiles in the developing ovarian follicles in White King pigeons. *Poultry Science*, *102*(7), 102673. https://doi.org/10.1016/j.psj.2023.102673.

Wang, Y., Liu, X., Li, W., Zhao, J., Liu, H., Yu, L., & Zhu, X. (2021). Reproductive performance is associated with seasonal plasma reproductive hormone levels, steroidogenic enzymes and sex hormone receptor expression levels in cultured Asian yellow pond turtles (Mauremys mutica). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *254*, 110566. https://doi.org/10.1016/j.cbpb.2021.110566.

Welsh, D. K., Takahashi, J. S., & Kay, S. A. (2010). Suprachiasmatic nucleus: cell autonomy and network properties. *Annual review of physiology*, *72*(1), 551-577. https://doi.org/10.1146/annurev-physiol-021909-135919.

Wilson, M., & Lindstrom, S. H. (2011). What the bird's brain tells the bird's eye: the function of descending input to the avian retina. *Visual Neuroscience*, *28*(4), 337-350.

Wingfield, J. C. (1993). Endocrinology of reproduction in wild species. *Avian biology*, 163-327.

Wingfield, J. C., Schwabl, H., & Mattocks Jr, P. W. (1990). Endocrine mechanisms of migration. In *Bird migration: physiology and ecophysiology* (pp. 232-256). Berlin, Heidelberg: Springer Berlin Heidelberg.

Wingfileld, J. (1994). Modulation of the adrenocortical response to stress in birds. *Perspectives in comparative endocrinology*, 520-528.

Woodard, A. E., Moore, J. A., & Wilson, W. O. (1969). Effect of wave length of light on growth and reproduction in Japanese quail (Coturnix coturnix japonica). *Poultry Science*, *48*(1), 118-123.

Yadav, S. K., & Haldar, C. (2014). Experimentally induced stress, oxidative load and changes in immunity in a tropical wild bird, Perdicula asiatica: involvement of melatonin and glucocorticoid receptors. *Zoology*, *117*(4), 261-268.

Yadav, S., & Chaturvedi, C. M. (2015). Light colour and intensity alters reproductive/seasonal responses in Japanese quail. *Physiology & behavior*, *147*, 163-168.

Yamaguchi, T., Yamaguchi, S., Hirai, T., & Kitano, T. (2007). Follicle-stimulating hormone signaling and Foxl2 are involved in transcriptional regulation of aromatase gene during gonadal sex differentiation in Japanese flounder, Paralichthys olivaceus. *Biochemical and Biophysical Research Communications*, *359*(4), 935-940.

Yamamura, T., Yasuo, S., Hirunagi, K., Ebihara, S., & Yoshimura, T. (2006). T3 implantation mimics photoperiodically reduced encasement of nerve terminals

by glial processes in the median eminence of Japanese quail. *Cell and tissue research*, *324*(1), 175-179. https://doi.org/10.1007/s00441-005-0126-8.

Yamanome, T., Mizusawa, K., Hasegawa, E. I., & Takahashi, A. (2009). Green light stimulates somatic growth in the barfin flounder Verasper moseri. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, *311*(2), 73-79.

Yasuo, S., Watanabe, M., Okabayashi, N., Ebihara, S., & Yoshimura, T. (2003). Circadian clock genes and photoperiodism: comprehensive analysis of clock gene expression in the mediobasal hypothalamus, the suprachiasmatic nucleus, and the pineal gland of Japanese quail under various light schedules. *Endocrinology*, *144*(9), 3742-3748.

Yatung, S & Trivedi, A.K. (2024). Daily and seasonal changes in steroidogenic markers in the hypothalamus and testes of tree sparrow (*Passer montanus*). *Journal of Neuroendocrinology*. https://doi.org/10.1111/jne.13478.

Yatung, S., & Trivedi, A. K. (2025). Time- and season-dependent changes in the steroidogenic markers in female tree sparrow (Passer montanus). *Photochemical & Photobiological Sciences*, 1-22.

Ye, X., Li, M., Hou, T., Gao, T., Zhu, W. G., & Yang, Y. (2016). Sirtuins in glucose and lipid metabolism. *Oncotarget*, *8*(1), 1845. https://doi.org/10.18632/oncotarget.12157.

Yoon, H., Shaw, J. L., Haigis, M. C., & Greka, A. (2021). Lipid metabolism in sickness and in health: Emerging regulators of lipotoxicity. *Molecular cell*, *81*(18), 3708-3730. https://doi.org/10.1016/j.molcel.2021.08.027.

Yoshimura, T., Suzuki, Y., Makino, E., Suzuki, T., Kuroiwa, A., Matsuda, Y., ... & Ebihara, S. (2000). Molecular analysis of avian circadian clock genes. *Molecular brain research*, *78*(1-2), 207-215.

Yoshimura, T., Yasuo, S., Watanabe, M., Iigo, M., Yamamura, T., Hirunagi, K. and Ebihara, S. (2003). Light-induced hormone conversion of T4 to T3 regulates photoperiodic response of gonads in birds. *Nature*, *426*(6963), pp.178-181. https://doi.org/10.1038/nature02117.

Zampelas, A. and Magriplis, E., 2019. New insights into cholesterol functions: a friend or an enemy? *Nutrients*, *11*(7), p.1645. https://doi.org/10.3390%2Fnu11071645.

Zhang, P., & Mellon, S. H. (1997). Multiple orphan nuclear receptors converge to regulate rat P450c17 gene transcription: novel mechanisms for orphan nuclear receptor action. *Molecular Endocrinology*, *11*(7), 891-904. https://doi.org/10.1210/mend.11.7.9940.

Zhang, S., Chen, X., Zhang, J., & Li, H. (2014). Differences in the reproductive hormone rhythm of tree sparrows (Passer montanus) from urban and rural sites in Beijing: The effect of anthropogenic light sources. *General and Comparative Endocrinology*, *206*, 24-29. https://doi.org/10.1016/j.ygcen.2014.05.020.

Zhang, X., Yang, W., Liang, W., Wang, Y., & Zhang, S. (2019). Intensity dependent disruptive effects of light at night on activation of the HPG axis of tree sparrows (Passer montanus). *Environmental Pollution*, *249*, 904-909. https://doi.org/10.1016/j.envpol.2019.03.008.

Zhou, R., Wu, J., Liu, B., Jiang, Y., Chen, W., Li, J., ... & He, Z. (2019). The roles and mechanisms of Leydig cells and myoid cells in regulating spermatogenesis. Cellular and molecular life sciences, 76, 2681-2695. https://doi.org/10.1007/s00018-019-03101-9.

Zhou, X., Xu, Y., Fang, C., Ye, C., Liang, W., Fan, Z., ... & Luo, Q. (2024). Integrated Transcriptomic–Metabolomic Analysis Reveals the Effect of Different Light Intensities on Ovarian Development in Chickens. *International Journal of Molecular Sciences*, *25*(16), 8704.

Zhu, N., Shang, J., & Zhang, S. (2023). Short-term night lighting disrupts lipid and glucose metabolism in Zebra Finches: Implication for urban stopover birds. *Avian Research*, *14*, 100138. https://doi.org/10.1016/j.avrs.2023.100138.

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Sl. No	Title with page nos.	Name of the Journal/Book	ISSN/ISBN No.	Year of Publication	Remark
1.	Seasonality In Tropical Birds	Journal of Experimental Zoology Part A: Ecological and Integrative Physiology	2471-5646	2022	Scopus Indexed
2.	Daily And Seasonal Changes in Steroidogenic Markers in The Hypothalamus and Testes of the Tree Sparrow (*Passer montanus*).	Journal of Neuroendocrinology	1365282 6/ 0953819 4	2024	Scopus Indexed
3.	High Temperature During the Photorefractory Stage Attenuates Photoperiodic Responses During Photostimulator y Stage in Male Tree Sparrows (*Passer montanus*)	Theriogenology Wild	2773-093X	2024	Scopus Indexed
4.	Time And Season-Dependent Changes in the Steroidogenic Markers in Female Tree Sparrow (*Passer montanus*)	Photochemical & Photobiological Sciences	1474-9092	2025	Scopus Indexed
5.	Short-term exposure to dim light at night affects reproduction and	Comparative Biochemistry and Physiology, Part A	1531-4332	2025	Scopus Indexed

	metabolism-linked processes in adult male tree sparrows (Passer montanus)				
6.	Melatonin modulates corticosterone-induced effects on steroidogenesis and reproductive-linked processes in male tree sparrow (passer montanus)	General and Comparative Endocrinology	1095-6840	2025	Scopus Indexed

Conferences/workshops participated:

Sl. No	Title of Conference/Seminar	Organised by	Whether International/ National/ State/Regional/ college
1.	National Symposium on Avian Biology	CUSB, Gaya, Bihar (2022)	National
2.	National symposium on Chronobiology: Biological Timing and Health Challenges in Conjunction with the Biennial meeting of the Indian Society for Chronobiology	Department of Zoology, Mizoram University, Aizawl, Mizoram (2023)	National
3.	InSc Workshop on Mechanisms Underlying Daily and Seasonal Biological Processes	Mizoram University, Aizawl (2024)	National
4.	13th International Symposium on Avian Endocrinology	Department of Zoology, Chaudhary Charan Singh University, Meerut (2024)	International

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Daily and seasonal changes in steroidogenic markers in the hypothalamus and testes of tree sparrow (*Passer montanus*)

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Abstract

The population responds to environmental variability largely determined by the dynamic interactions between fitness components within- and among-individual variation in the expression of the environmentally sensitive phenotype. The study was conducted on daily and seasonal changes in the expression of steroidogenic gene markers and corresponding seasonal changes in the physiological characters in adult male tree sparrows. Two experiments were performed. In experiment one, birds ($n = 5$/time points) were sampled during the breeding season at 6-time points, i.e., ZT1, ZT5, ZT9, ZT13, ZT17, and ZT21 [Zeitgeber time (ZT) 0 = sun rise time at the respective time of the year], and daily variation in expression of steroidogenic markers was observed in hypothalamus and testes tissues. In experiment two, birds ($n = 5$/month) were sampled every month at mid-day for a year. Body mass, bill color, testes size, and molt in feathers were recorded. The hypothalamus and testes tissues were used for gene expression studies. Blood plasma cholesterol and testosterone levels were measured. Higher testicular volumes were recorded from March to May, whereas maximum molt was observed during the post-breeding phase. Plasma cholesterol levels were highest before the breeding phase. Higher testosterone levels corresponded with the breeding phase. Higher expressions of *thyroid-stimulating hormone subunit beta* (*tshβ*), *type 2 deiodinase* (*dio2*), and *gonadotropin-releasing hormone* (*gnrh*) during the breeding phase and higher expression of *type 3 deiodinase* (*dio3*) and *gonadotropin-inhibitory hormone* (*gnih*) were observed during the non-breeding phase. The steroidogenic transcripts showed seasonal changes in their expression in the hypothalamic and testicular tissue and were upregulated either during the pre-breeding or breeding phase. The study reveals that mRNA levels of steroidogenic enzymes exhibit daily rhythmicity both in the hypothalamus and testis tissues. Further, steroidogenic transcripts show seasonal variations that correspond to the annual reproductive cycle of the tree sparrow (*Passer montanus*).

KEYWORDS
gonadotropins, seasonality, star, steroidogenesis, testosterone

1 | INTRODUCTION

Changing environment greatly affects the physiological and biological attributes of vertebrates. Predicting how individuals and populations will react to environmental variation and change and figuring out the processes sustaining or limiting the important phenotypic variation requires quantifying impacts.[1–3] Subsequently, the degree to which an individual's phenotype and the fitness implications of phenotype vary

within and between seasons, whereas years determine the population's outcome and are indicative of labile plasticity and temporal fluctuation in selection.[4–6]

Animal behavior is greatly influenced by environmental variation and the forthcoming change it brings. The internal biological clock is responsible for the cyclical variations in their physiological and behavioral processes. Biological clocks allow the animals to correspond with the cyclic events such as day and night/season in nature. Photoperiod (24-h LD cycle) is the most dominant environmental cue that entrains the biological clocks. It allows the phase of endogenous rhythms to be set with the phase of cyclic events in the environment.[7] This environmental time cue, or zeitgebers, produces a series of signals that, once acquired, allow endogenous clocks to synchronize with environmental cycles, exhibiting a constant phase relation between the rhythm and the zeitgeber.[8] Accordingly, most birds exhibit seasonality in their behavioral, physiological, and reproductive functions, such as a change in body weight, bill color, molt in feathers, song production, hormonal changes, and gonadal status.[9]

The timing of reproduction and production of sex steroids is controlled by the hypothalamic–pituitary–gonad axis (HPGa), which is necessary for several processes like sex differentiation, gonad growth, and spawning.[10,11] In photoperiodic species, long photoperiods stimulate opsin-containing deep-brain photoreceptors.[12–16] Correspondingly, a recent study on Japanese quail (*Coturnix japonica*) suggests that *vertebrate ancient* (VA) opsin also plays a pivotal role along with neuropsin (OPN5) and melanopsin (OPN4), which stimulates the thyroid signaling in the hypothalamus.[16] Further, thyroid-stimulating hormone (TSH) acts on the ependymal cells (ECs) within the mediobasal hypothalamus (MBH) initiates transcription of type 2 deiodinase (*dio2*) via the TSH receptor signalling pathway and leads downstream processes at gonadal levels.[13] A decrease in transcription of the gene coding for *gonadotropin-inhibitory hormone* (*gnih*) inhibits *gnrh* release.[17,18] These molecules have also been shown to be up or downregulated depending on the photoperiod and physiological state of the avian species such as red-headed bunting (*Emberiza bruniceps*), black-headed bunting (*Emberiza melanocephala*), and tree sparrow (*Passer montanus*).[19–25]

Steroidogenic enzymes play a vital role in the functioning of sex steroids. In males, it plays a crucial role in the testis and regulates spermatogenesis and spermiogenesis or the maintenance of secondary sexual characters in vertebrates having seasonal reproductive cycles.[26] It consists of cholesterol mobilization from lipid droplets and plasma membrane, transport into mitochondria, and the formation of pregnenolone into final steroids.[27] The biosynthesis of all the steroid hormones is controlled by the two key functional classes of steroidogenic enzymes (cytochrome p450 and hydrosteroid dehydrogenase).[28] In the mitochondria, where Scp2 a sterol carrier protein and an activator of mitochondrial steroidogenesis stimulates the transfer of cholesterol as well as that of phospholipids between membranes in vitro,[29–31] and it may play a role in the intracellular transport or metabolism of phospholipids and cholesterol.[32–34] A mitochondrial protein StAR (steroidogenic acute regulatory protein) helps transport cholesterol to mitochondria.[35] A nuclear receptor (Nur77/Nr4a1) is present in steroidogenic cells, and its expression is induced by hormones known to activate StAR expression.[36] StAR acts on the outer mitochondrial membrane (OMM) to facilitate the movement of cholesterol from the OMM to the inner mitochondrial membrane to be converted to pregnenolone. Further StAR interacts with voltage-dependent anion channel 1 (Vdac1) on the OMM, which then facilitates processing of the 37-kDa phospho-StAR to the 32-kDa intermediate. StAR then interacts with cholesterol and is converted to pregnenolone by the cytochrome P450 side-chain cleavage enzyme (Cyp11a1). In smooth endoplasmic reticulum, pregnenolone is then transformed into dehydroepiandrosterone and is modified into androstenedione by 3β-hydroxysteroid dehydrogenase (3β-HSD). Later 17β-hydroxysteroid dehydrogenase (17β-HSD) catalyzes the conversion of androstenedione to testosterone (T). Testosterone can be converted both in 17β-estradiol (E2) by cytochrome P450 aromatase (P450 arom) or in 5 alpha-dihydrotestosterone (DHT) by 5 alpha-reductase.[37–39] Later, 17α hydroxy pregnenolone is converted to 17β OH-progesterone by Cyp21 and is further converted to cortisol by Cyp11b and to cortisone by Hsd11b2. In males, testosterone is produced by Leydig cells, and testosterone directly regulates both spermatogenesis and secondary sexual characteristics.[40]

Daily variations in steroidogenic enzymes were reported in chicken (*Gallus gallus domesticus L*).[41] Daily rhythms in HPG gene expression and plasma sex steroids in tilapia (*Oreochromis niloticus*) have also been studied.[42] Further, in the amphibian green frog (*Pelophylax esculentus*), upregulation of the steroidogenic enzyme gene expression controls cyclic endocrine activity in the testes.[43] In adult zebra finches (*Taeniopygia guttata*), expression of the sex steroid-synthesizing enzymes *cyp11a1*, *3β-hsd*, *cyp17a1*, and *cyp19* have been studied in gonads and adrenals.[44] Song sparrows (*Melospiza melodia*) express high rates of steroid synthesis in specific brain regions such as neurocutaneous melanocytosis (NCM), ventral tegmental area (VTA), hypothalamic preoptic area (POA), anterior hypothalamic area (AH), lateral septum (LS), and taeniae of the amygdala (TnA).[45] In Japanese quail, the steroidogenic process shows seasonal variation with the development of both androgenic and estrogenic pathways in the reproductive phase, suggesting their synergic mechanism in regulating spermatogenesis.[46] Seasonal changes of steroidogenic enzymes that influence steroidogenesis and spermatogenesis have been reported in some wild species, the green frog (*P. esculentus*),[47,48] Italian wall lizard (*Podarcis sicula*),[49] dark-eyed Junco (*Junco hyemalis*),[50] raccoon dogs (*Nyctereutes procynoides*),[51] American black bears (*Ursus americanus*),[52] and Bank vole (*Clethrionomys glareolus*).[53]

Though the characterization, localization, and expression of the key genes involved in steroidogenesis have been extensively studied in many vertebrates, their daily rhythms have been poorly examined. The present study advocates that no prior research has been conducted on this species, concerning daily and seasonal steroidogenic enzyme gene expression. The study's primary objective is to examine if the steroidogenic enzyme gene expression varies daily and annually in a male tree sparrow. For this preliminary study, we hypothesized that gene expression does vary daily and seasonally. Therefore, we examined the daily and seasonal variations in steroidogenic enzyme

gene expression and correlated it with annual reproductive cycles in male tree sparrows.

2 | MATERIALS AND METHODS

2.1 | Animals

The study was conducted on adult male tree sparrows (*Passer montanus*) procured locally (Tanhril, Aizawl, Mizoram, India, 23° 44′10″ N to 92° 39′51″ E) using mist nets. Two experiments were performed:

2.1.1 | Experiment 1: Daily changes in steroidogenic markers in the hypothalamus and testis

Birds ($n = 30$) procured in April 2022 were used in this study. Immediately after procurement, birds were transferred to the laboratory and kept in NDL (natural day-length conditions in captivity) in cages ($3 \times 2.5 \times 3$ fit, five birds per cage). Food along with mealworms and water were provided ad libitum. The next day, sampling was done ($n = 5$/timepoints) at every 4 h of intervals during a 24-h cycle at the following time points: ZT1, ZT5, ZT9, ZT13, ZT17, ZT21 [Zeitgeber time (ZT) 0 = sunrise time at the respective time of the year i.e. 05:11 a.m.]. The brain and testes tissues were excised and immediately stored at −80°C for gene expression analysis.

2.1.2 | Experiment 2: Monthly changes in steroidogenic markers in the hypothalamus and testis

Birds ($n = 5$/month) were collected monthly throughout the year (January to December 2022). Immediately after procurement, birds were transferred to the laboratory and kept in NDL in cages ($3 \times 2.5 \times 3$ fit, five birds per cage). Food along with the mealworms and water were provided ad libitum. Body weight was recorded using a top pan balance to the accuracy of 0.1 g. For individual birds, molt in body and primary flight feathers, and bill color was recorded. Bill's color was assessed based on a subjective criterion as reported earlier.[54] Briefly, bill color was assessed in an index of 0–5, where 0 represents a straw color bill (S), score 1 indicates a bill that is straw in color with a little tinge of blackness (ratio: SSS: B), score 2 indicates a slightly blackish bill (ratio: SS: B), score 3 reflects a bill having similar percentage of straw and black (ratio: S: B), score 4 suggests a bill is black with very little straw patch (ratio: S: BB), while score 5 is given when a bill is completely black (B). In primary flight (wing primaries) and body feathers, a molt was recorded.[54] Primaries feathers were scored 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, and 5—new feather fully grown. So, each primary could have a maximum score of 5. The maximum score for one wing can be up to 45 ($9 \times 5 = 45$); for each bird, the feather score could total up to 90 ($2 \times 45 = 90$). While the minimum score could be as low as 0. For body molt, the whole bird's body was divided into 11 different regions: 1—head, 2—neck, 3—shoulder, 4—back, 5—pelvic, 6—throat, 7—chest, 8—abdomen, 9—flank, 10—shank, and 11—sub-caudal. Any region could have a score of either 0 (old feathers) or 1 (new feathers emerged), and hence the total body molt score could be in the range of 0–11, depending on the number of regions that had scores of 0 or 1. The gonadal size was recorded by performing unilateral laparotomy,[55] where a small incision was made between the last two ribs on the left flank and the left testis was located within the abdominal cavity with the help of a spatula. The dimensions of the left testis were recorded, and testicular volume (TV) was calculated using the formula 4/3πab2, where a and b denote half of the long (length) and short (width) axes, respectively. Birds were sampled during the middle of the day. Blood (200–300 μL) from the trunk of each bird was collected in heparinized tubes, and plasma was harvested and stored at −20°C for biochemical and hormonal assay. Immediately after sampling, the brain and testes were excised and stored at −80°C for gene expression analysis. Later, the hypothalamus, along with the pituitary, was harvested from the individual brain by slicing the brain with a surgical blade having a thick coronal section trimmed dorsally (starting approximately at TrSm) and laterally extending from the optic chiasma to the infundibular area. The protocols were approved by the institutional animal ethics committee of Mizoram University (No. MZU/IAEC/2021-22/12).

2.2 | Cholesterol assay

Using manufacturer protocol, plasma cholesterol levels were measured using CHOLESTEROL KIT (Coral Clinical Systems, India). Briefly, clean test tubes were labeled as blank, standard, and test. After that, 1 mL of working reagent was pipetted into blank, standard, and test sample test tubes. 10 μL of distilled water was pipetted into a blank test tube, followed by 10 μL of cholesterol standard into a standard test tube and 10 μL of plasma sample into a sample test tube. After adding all the required reagents in the test tubes, it was mixed well and incubated at room temperature (25°C) for 15 min. The absorbance of standard and test samples was measured against the blank within fifteen minutes at 505 nm wavelength using the SpectraMax MINI system (Multimode Microplate Reader, AFL System Molecular Devices, USA). Each sample was run in duplicate.

2.3 | Testosterone assay

Testosterone levels were measured using enzyme immunoassay kits (Testosterone, Diametra, DKO002) as per manufacturer protocol. This assay has been validated and used for the measurement of testosterone in this and other species.[56–58] Briefly, before use, all the reagents were kept at room temperature for 30 min. Plasma samples were allowed to thaw on ice. The wash solution was diluted as per the

protocol provided. All calibrators, control, test samples, and blank were run in duplicate. 25 µL of sample or control and 25 µL of calibrator were pipetted into each respective well, and 100 µL of conjugate was added to each calibrator and sample well. After adding the reagents, the plate was incubated at 37°C for 1 h. After one hour, the content was removed from each well and washed three times with 300 µL of diluted wash solution every 5-min intervals. The residual buffer was blotted by tapping the plate upside down on absorbent paper. Next, a TMB substrate of 100 µL was added to each well and incubated at room temperature (25°C) for 15 min in the dark, followed by adding a stop solution of 100 µL, mixed gently. Absorbance was taken using a Multimode ELISA reader SpectraMax MINI system at 450 nm against the Blank within 5 min. Each sample was run in duplicate.

2.4 | Gene transcription

The mRNA transcription levels of reproductive genes coding for *tshβ*, *dio2*, *dio3*, *gnrh*, and *gnih* were measured in the hypothalamus, and mRNA transcription of steroidogenic genes coding for *star*, *nr4a1*, *er*, *scp2*, *cyp17a1*, *cyp11a1*, *hsd11b2*, *cyp11b*, *srd5a1*, *vdac1* was measured both in hypothalamus and testis for daily and seasonal studies. *18 s* was used as a reference gene to determine the relative expression of transcripts.

2.5 | RNA isolation and cDNA synthesis

Total RNA was extracted from tissue samples using TRIzol Reagent (Invitrogen by Thermo Fisher Scientific). The concentration and purity of total RNA were determined using (Nanodrop Spectrophotometer, Thermo Fisher Scientific, Wilmington, DE). 2 µg RNA was used to process cDNA synthesis. RQ1 DNase (Promega M6101; Madison, WI, USA) kit was used to remove any possible genomic DNA contamination. For cDNA synthesis, iScript™ cDNA Synthesis Kit (BIO-RAD, USA, 1708891) was used.

2.6 | Quantitative (real-time) RT-PCR (qPCR)

Gene-specific primers were designed from the sequences using Primer 3 plus (online primer design program, Boston, MA, USA; Table S1). qPCR was performed on Quant-Studio 5 (Applied Biosystem by Thermo Fisher Scientific, Foster City, CA, USA) as described in our previous publications[24,25,59–62] Briefly, PCR reaction of the volume of 7 µL for each gene comprised 1 µL each of cDNA, gene-specific forward and reverse primers, 3 µL Power UpTM SYBR Green Master Mix (Applied Biosystem by Thermo Fisher Scientific, Foster City, CA, USA, A25742), and 1 µL of nuclease-free water (Ambion, AM9938). qPCR cycle conditions included 1 cycle at 95°C (20 s), 35 cycles at 95°C (01 s), 60°C (20 s), and 95°C (01 s), additional melt curve plot step included 1 cycle of 60°C (20 s), and 1 cycle of 95°C (01 s). ΔΔCt method[63] was used for gene expression analysis. Each sample was run in duplicate.

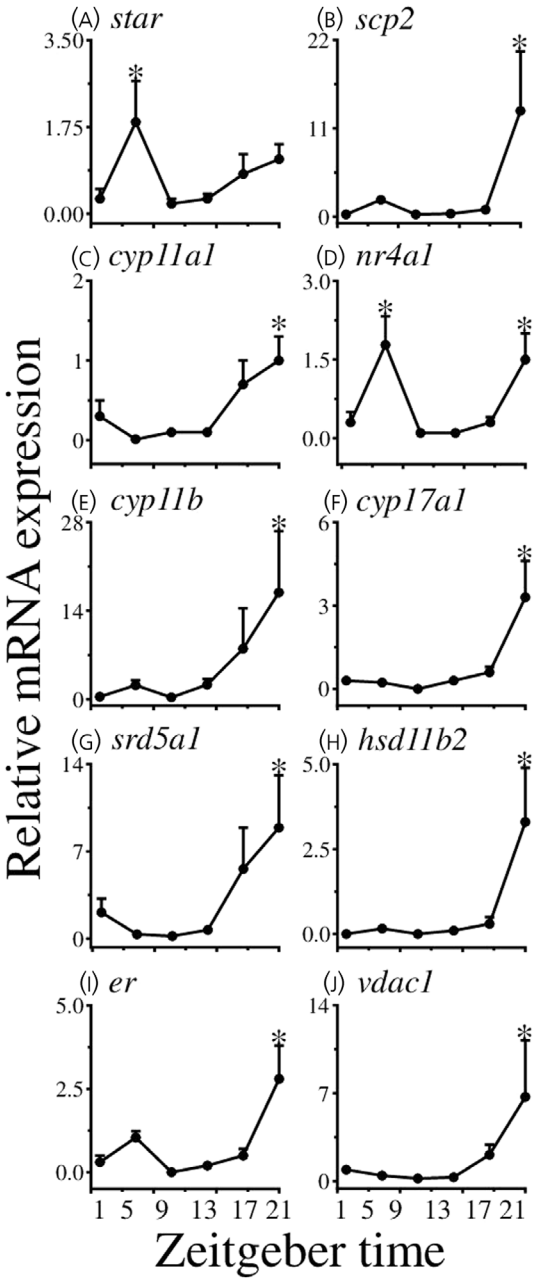

FIGURE 1 The data shows relative mRNA levels of steroidogenic transcripts (*star*, *scp2*, *cyp11a1*, *nr4a1*, *cyp11b*, *cyp17a1*, *srd5a1*, *hsd11b2*, *er*, and *vdac1*) in the hypothalamus of adult male tree sparrow (*Passer montanus*) collected in April (A–J). The *X*-axis indicates the zeitgeber time (ZT 0 is sunrise time) *Indicates significant differences among the time points.

3 | DATA ANALYSIS

To determine the significant difference in monthly profile, one-way ANOVA (one-way analysis of variance) was used. Bonferroni's

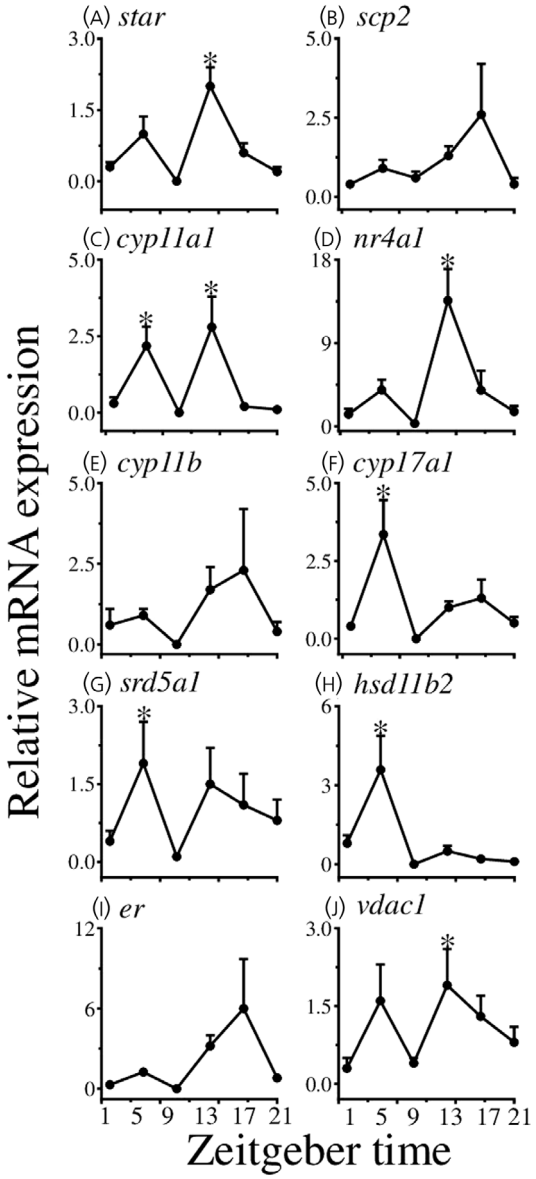

FIGURE 2 The figure depicts relative mRNA levels of steroidogenic transcripts (*star*, *scp2*, *cyp11a1*, *nr4a1*, *cyp11b*, *cyp17a1*, *srd5a1*, *hsd11b2*, *er*, and *vdac1*) in the testis of adult male tree sparrow (*Passer montanus*) collected in April (A–J). The *X*-axis indicates the zeitgeber time (ZT 0 is sunrise time) *Indicates significant differences among the time points.

multiple comparison tests were applied if ANOVA showed a significant difference. Analysis was done using graph pad Prism version 9. Values of daily steroidogenic genes were subjected to cosinor analyses based on unimodal cosinor regression $y = A+(B.\cos(2\pi(x-C)/24))$, where A, B, and C denote the mean level (mesor), amplitude, and acrophase of the rhythm, respectively.[64] The significance of regression analysis was calculated using the number of samples, R^2 values, and numbers of predictors (mesor, amplitude, and acrophase; http://www.danielsoper.-com/statcalc3/calc.aspx?id1/415).[65]

4 | RESULTS

4.1 | Experiment 1: Daily changes in steroidogenic markers in the hypothalamus and testis

Daily variations were observed in the expression levels of steroidogenic transcripts in the hypothalamus and testis of tree sparrows. *star* mRNA transcript showed daily variation in the hypothalamus ($p = .0023$; one-way ANOVA; Table S2; Figure 1A) and in the testis ($p < .0001$; one-way ANOVA; Table S2; Figure 2A). *star* mRNA levels showed variable expression having the highest expression at ZT5 in the hypothalamus and at ZT13 in the testis. *scp2* mRNA levels in the hypothalamus ($p = .0001$; one-way ANOVA; Table S2; Figure 1B) showed a significant difference and peaked at ZT21, whereas in testis ($p = .1749$; one-way ANOVA; Table S2; Figure 2B), no daily variation was observed. *cyp11a1* exhibited daily changes in the hypothalamus ($p = .0014$; one-way ANOVA; Table S2; Figure 1C) and in the testis ($p < .0001$; one-way ANOVA; Table S2; Figure 2C). Peak expression was observed at ZT21 in the hypothalamus and at ZT5 and ZT13 in the testis. *nr4a1* mRNA showed significant differences in the hypothalamus ($p = .0001$; one-way ANOVA; Table S2; Figure 1D) and testis ($p < .0001$; one-way ANOVA; Table S2; Figure 2D). Peak expression timing for *nr4a1* was highest at ZT5 and ZT21 in the hypothalamus, whereas ZT13 in the testis. *cyp11b* transcript showed a significant difference in the hypothalamus ($p = .0046$; one-way ANOVA; Table S2; Figure 1E) showing the highest expression at ZT21 but no significant differences were observed in testis ($p = .0893$; one-way ANOVA; Table S2; Figure 2E). *cyp17a1* showed a significant difference in the hypothalamus ($p = .0008$; one-way ANOVA; Table S2; Figure 1F) and testis ($p < .0001$; one-way ANOVA; Table S2; Figure 2F). In the hypothalamus, the expression peaked at ZT21 while in the testis at ZT5. *srd5a1* transcript showed a significant difference in testis ($p = .0116$; one-way ANOVA; Table S2; Figure 2G) with peaked expression at ZT5, whereas peak expression was observed at ZT21 in the hypothalamus ($p = .0002$; one-way ANOVA; Table S2; Figure 1G). *hsd11b2* transcript showed a significant difference in the testis ($p < .0001$; one-way ANOVA; Table S2; Figure 2H) and peak expression was observed at ZT5, whereas a significant difference in the hypothalamus ($p < .0001$; one-way ANOVA; Table S2; Figure 1H) was observed and expression peaked at ZT21. *er* transcript showed a significant difference in the hypothalamus ($p = .0001$; one-way ANOVA; Table S2; Figure 1I), and peaked expression was observed at ZT21, whereas no significant differences were observed in testis ($p = .0662$; one-way ANOVA; Table S2; Figure 2I). *vdac1* transcript showed a significant difference in the testis ($p = .0039$; one-way ANOVA; Table S2; Figure 2J) and hypothalamus ($p = .0038$; one-way ANOVA; Table S2; Figure 1J) and the highest expression of the transcript was observed at ZT13 in the testis and at ZT21 in the hypothalamus. Cosinor analyses confirmed all the transcripts studied expressed rhythmically in the hypothalamus and testis tissues.

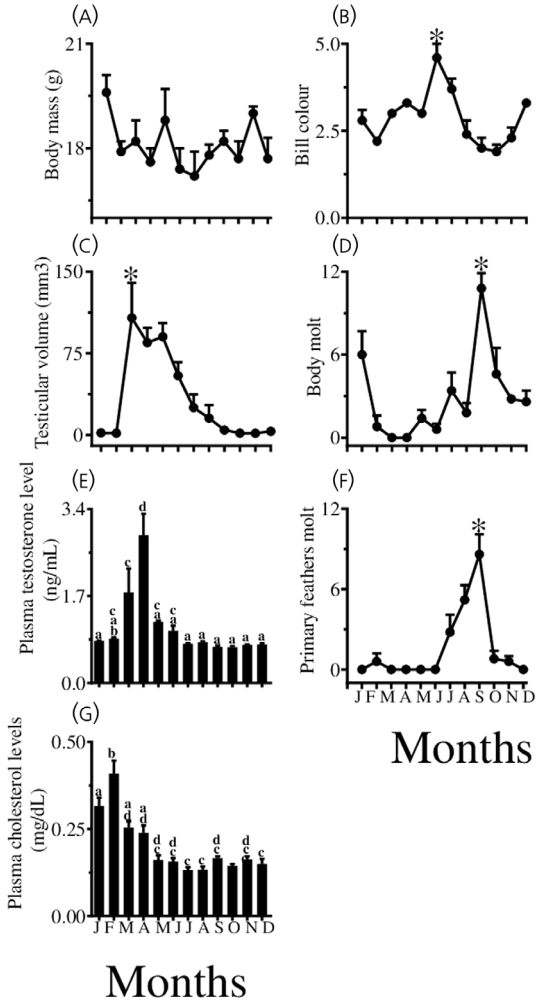

FIGURE 3 The above figure displays parameters (A) body mass (B) bill color, (C) testicular volume, (D) Body molt, (E) Plasma testosterone levels (F) molt in primary flight feathers, and (G) plasma cholesterol levels from adult male tress sparrows (*Passer montanus*) collected every month for 12 months. *Indicates significant differences among the months.

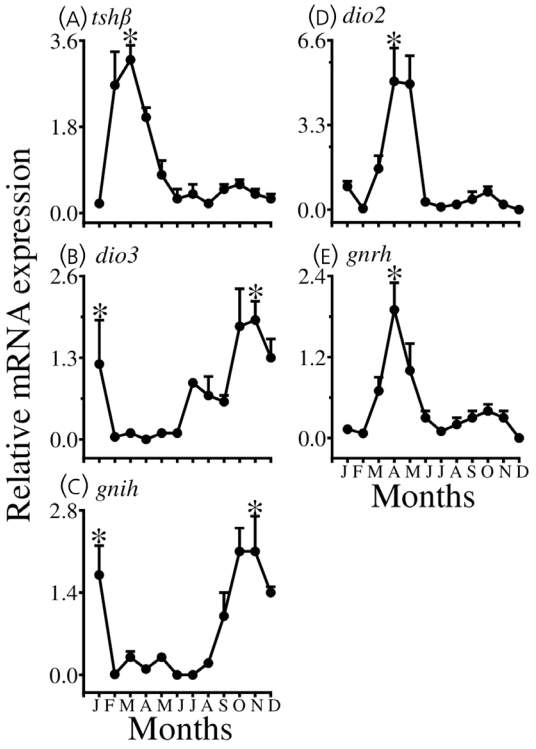

FIGURE 4 The figure shows relative mRNA levels of reproductive genes (*tshβ*, *dio2*, *dio3*, *gnrh*, and *gnih*) in the hypothalamic tissues of adult male tree sparrow (*Passer montanus*) collected every month for 12 months (A–E). * Indicates significant differences among the months.

4.2 | Experiment 2: Monthly changes in steroidogenic markers in the hypothalamus and testis

No significant change in body mass was observed across the year ($p = .1863$; one-way ANOVA; Table S3a; Figure 3A). Annual variations in bill color ($p < .0001$; one-way ANOVA; Table S3a; Figure 3B), molt in body feathers ($p < .0001$; one-way ANOVA; Table S3a; Figure 3D), primary flight feathers ($p < .0001$; one-way ANOVA; Table S3a; Figure 3F), and testicular volume ($p < .0001$; one-way ANOVA; Table S3a; Figure 3C) were also observed. Higher bill color score was observed from April till July ($p < .05$; Bonferroni's multiple comparison tests; Figure 3B), having the highest score during June ($p < .05$; Bonferroni's multiple comparison tests; Figure 3B). Molt in body feathers and primary flight feathers begin in July and completed in October ($p < .05$; Bonferroni's multiple comparison tests; Figure 3D,F) with maximum molt during September (Figure 3D,F). Significantly higher gonadal sizes were observed from March till May, began to regress in June, and were fully regressed from July onward (Figure 3C). Plasma cholesterol levels also showed seasonal fluctuation ($p < .0001$; one-way ANOVA; Table S3a; Figure 3G). Elevated plasma cholesterol levels were observed in January, were significantly higher in February ($p < .05$; Bonferroni's multiple comparison tests; Figure 3G), and started to decline from March onward (Figure 3G). Minimum cholesterol levels were observed during July and August ($p < .05$; Bonferroni's multiple comparison tests; Figure 3G). Annual changes in plasma testosterone levels were also observed ($p < .0001$; one-way ANOVA; Table S3a; Figure 3E). Plasma testosterone levels started to elevate in March, maximum levels were observed during April ($p < .05$; Bonferroni's multiple comparison tests; Figure 3E), and began to decline from May onward (Figure 3E).

Transcripts known to be involved in seasonal reproduction were measured in the hypothalamus of male adult tree sparrows. All the transcripts studied had annual variation in the hypothalamic tissue; *tshβ* ($p < .0001$; one-way ANOVA; Table S3b; Figure 4A), *dio2* ($p < .0001$; one-way ANOVA; Table S3b; Figure 4D), *gnrh* ($p < .0001$; one-way ANOVA; Table S3b; Figure 4E), *dio3* ($p = .0011$; one-way ANOVA; Table S3b; Figure 4B), and *gnih* ($p < .0001$; one-way ANOVA; Table S3b; Figure 4C). Maximum *tshβ* expression was observed during March ($p < .05$; Bonferroni's multiple comparison test; Figure 4A), while the levels were minimum from May onward ($p < .05$;

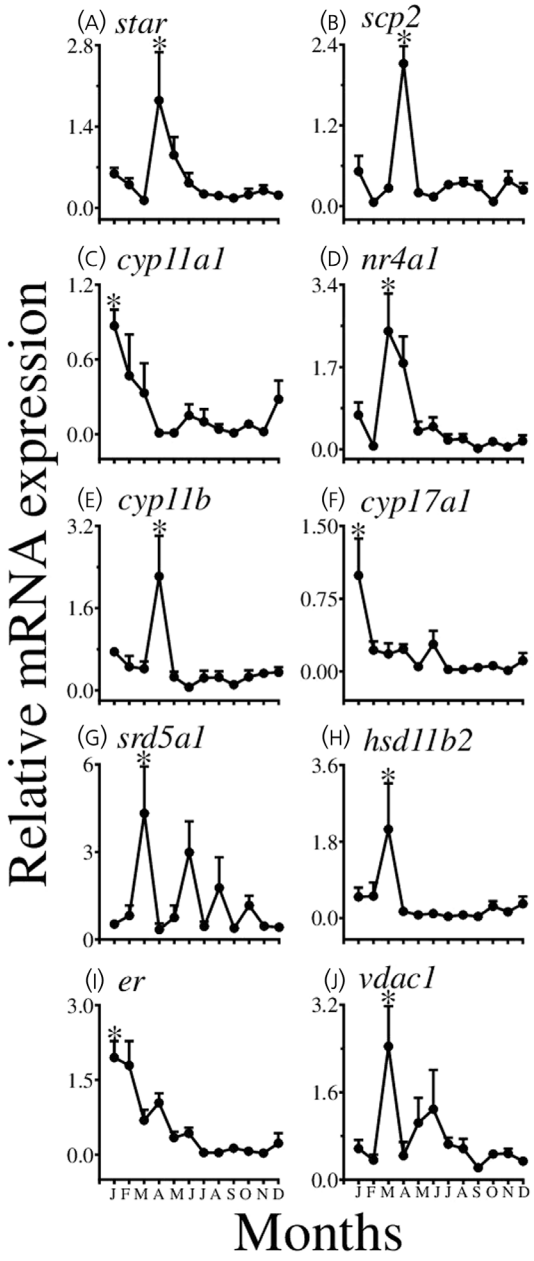

FIGURE 5 The figure depicts relative mRNA levels of steroidogenic transcripts (*star*, *scp2*, *cyp11a1*, *nr4a1*, *cyp11b*, *cyp17a1*, *srd5a1*, *hsd11b2*, *er*, and *vdac1*) in the hypothalamus of adult male tree sparrow (*Passer montanus*) collected every month for 12 months (A–J). *Indicates significant differences among the months.

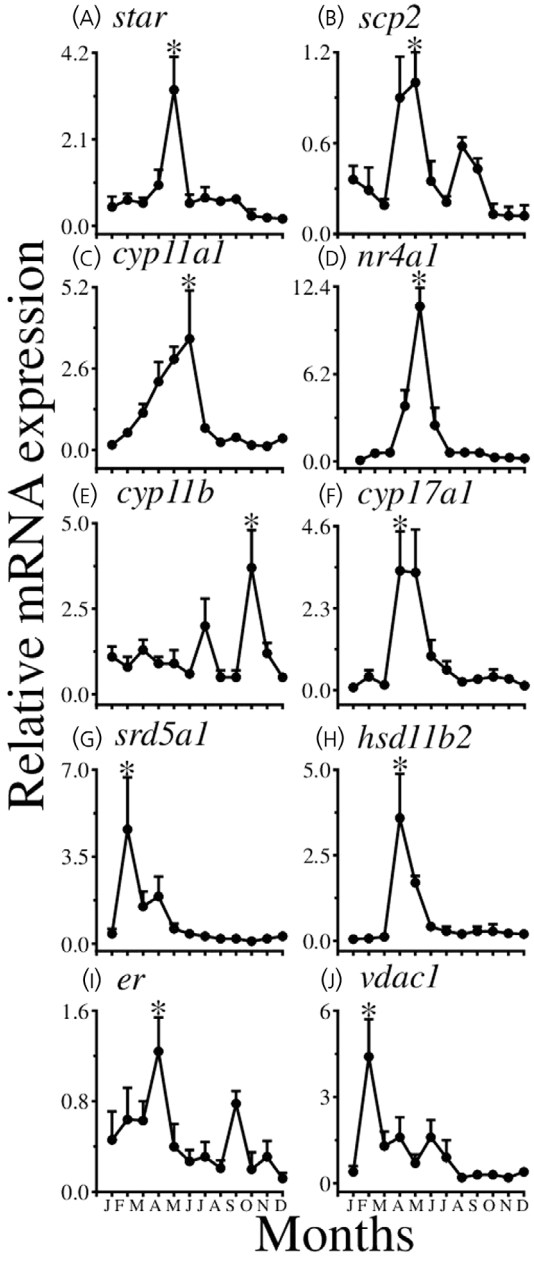

FIGURE 6 The above figure shows relative mRNA levels of steroidogenic transcripts (*star*, *scp2*, *cyp11a1*, *nr4a1*, *cyp11b*, *cyp17a1*, *srd5a1*, *hsd11b2*, *er*, and *vdac1*) in the testes of adult male tree sparrow (*Passer montanus*) collected every month for 12 months (A–J). *Indicates significant differences among the months.

Bonferroni's multiple comparison test; Figure 4A). *dio2* levels started elevated by March with peak expression during April and May ($p < .05$; Bonferroni's multiple comparison test; Figure 4D). Levels were minimum during June, and these low levels were maintained rest of the year ($p < .05$; Bonferroni's multiple comparison test; Figure 4D). *gnrh* expression levels corresponded with *dio2*, and elevated mRNA levels were observed in March with peak expression during April, subsequent fall in transcription levels during June and low levels were observed rest of the year ($p < .05$; Bonferroni's multiple comparison test; Figure 4E). *dio3* and *gnih* levels were in antiphase of *tshβ*, *dio2*, and *gnrh* expression (Figure 4). Higher levels of *dio3* were observed in July till January, with maximum expression during October till January ($p < .05$; Bonferroni's multiple comparison test; Figure 4B). Similarly, higher *gnih* levels were observed during September till January ($p < .05$; Bonferroni's multiple comparison test; Figure 4C).

Hypothalamic expression of steroidogenic markers had seasonal changes (Figure 5). qPCR analysis of these transcripts in hypothalamus revealed seasonal fluctuations in the expression levels of *star* ($p = .0036$; one-way ANOVA; Table S4; Figure 5A), *scp2* ($p < .0001$; one-way ANOVA; Table S4; Figure 5B), *cyp11a1* ($p = .0020$; one-way ANOVA; Table S4; Figure 5C), *nr4a1* ($p < .0001$; one-way ANOVA;

Table S4; Figure 5D), *cyp11b* ($p < .0001$; one-way ANOVA; Table S4; Figure 5E), *cyp17a1* ($p = .0003$; one-way ANOVA; Table S4; Figure 5F), *srd5a1*($p = .0022$; one-way ANOVA; Table S4; Figure 5G), *hsd11b2* ($p = .0087$; one-way ANOVA; Table S4; Figure 5H), *er* ($p < .0001$; one-way ANOVA; Table S4; Figure 5I), and *vdac1* ($p = .0045$; one-way ANOVA; Table S4; Figure 5J). Maximum expression levels of *star* and *scp2* transcripts were observed in the month of April ($p < .05$; Bonferroni's multiple comparison test; Figure 5), while levels were low at other months of the year ($p < .05$; Bonferroni's multiple comparison test; Figure 5). Higher expression of *cyp11a1* was observed prior to breeding phase, that is, January till March, while levels were low at other times of the year ($p < .05$; Bonferroni's multiple comparison test; Figure 5C). Expression levels of *nr4a1*, *cyp11b*, and *vdac1* peaked during March while levels were low during the other times of the year ($p < .05$; Bonferroni's multiple comparison test; Figure 5). In the hypothalamus, peak expression of *cyp17a1* was observed in the month of January ($p < .05$; Bonferroni's multiple comparison test; Figure 5F). Multiple peaks in expression levels of *srd5a1* were observed throughout the year (Figure 5G). However, amplitude of peaks was lower during post breeding phase ($p < .05$; Bonferroni's multiple comparison test; Figure 5G). *hsd11b2* expression was maximum in the month of March while levels were low at other times of the year ($p < .05$; Bonferroni's multiple comparison test; Figure 5H). Higher levels of *er* were observed from January till April, with peaked expression during January and February in the hypothalamus ($p < .05$; Bonferroni's multiple comparison test; Figure 5I).

Seasonal variation in the expression levels of *star* ($p < .0001$; One Way ANOVA; Table S4; Figure 6A), *scp2* ($p < .0001$; One Way ANOVA; Table S4; Figure 6B), *cyp11a1* ($p < .0001$; One Way ANOVA; Table S4; Figure 6C), *nr4a1* ($p < .0001$; One Way ANOVA; Table S4; Figure 6D), *cyp11b* ($p = .0007$; One Way ANOVA; Table S4; Figure 6E), *cyp17a1* ($p < .0001$; One Way ANOVA; Table S4; Figure 6F), *srd5a1*($p = .0018$; One Way ANOVA; Table S4; Figure 6G), *hsd11b2* ($p < .0001$; One Way ANOVA; Table S4; Figure 6H), *er* ($p = .0026$; One Way ANOVA; Table S4; Figure 6I), and *vdac1* ($p < .0001$; One Way ANOVA; Table S4; Figure 6J) was also reflected in testes of tree sparrows (Figure 6). In testis, *star*, *scp2*, *nr4a1*, and *cyp17a1* peaked in May ($p < .05$; Bonferroni's multiple comparison test; Figure 6). Both *hsd11b2* and *er* peaked during April (Figure 6). However, we also observed a second peak in *er* transcripts during post-breeding in September (Figure 6I). Expression levels of *cyp11a1* began to rise from February, and peak expression was observed during June ($p < .05$; Bonferroni's multiple comparison tests; Figure 6C). *srd5a1* and *vdac1* peaked during February ($p < .05$; Bonferroni's multiple comparison tests; Figure 6G,J), while *cyp11b* peaked during post-breeding during October ($p < .05$; Bonferroni's multiple comparison test; Figure 6E).

5 | DISCUSSION

Experiment one investigated transcript expression of the *star* gene and several enzymes (*nr4a1*, *er*, *scp2*, *cyp17a1*, *cyp11a1*, *hsd11b2*, *cyp11b*, *srd5a1*, *vdac1*) of steroidogenic pathway both in the hypothalamus and in the testes of the tree sparrows, a long-day seasonal breeder. Limited studies examined the daily expression of selected steroidogenic enzymes.[41,42,66] Enzyme *star* is present in the Leydig cells, which aids in transferring cholesterol across the mitochondria. *star* displayed significant daily rhythms with their acrophases showing expression at ZT5 and ZT13 during the light phase, suggesting the secretion of the enzyme being maximum in the daytime, which is consistent with findings in nile tilapia (*Oreochromis niloticus*).[42] Consistent with the previous study,[66] no daily variation was observed in the expression of *cyp11b* enzyme in the testis. However, during the present study, the transcript expression of *cyp11b* reflects daily variation in the hypothalamus. In our study, higher expression of *star*, *nr4a1*, *cyp11a1*, *scp2*, *cyp17a1*, *hsd11b2*, *srd5a1* mRNA were observed higher during the light phase, peaked at ZT5, and gradually lowered at night phase after sunset in the testis. These higher expressions of transcripts during the middle of the day could also result from the availability of post-feeding cholesterol substrate. A study on Yangzhou goose (*Anser cygnoides*) suggests that both circadian clock genes and genes involved in progesterone synthesis exhibit rhythmic expression patterns in ovarian preovulatory granulosa cells and Bmal1 regulates the transcription of *star* gene.[67] The expression of steroidogenic genes exhibits daily changes during a 24-h cycle in the gonads and brain tissues of zebra fishes.[66] Rhythmic expression of aromatase (*cyp19a1a*), antimüllerian hormone (amh), *foxl2*, and *dmrt1* was observed in gonads, while *cyp19a1b* and *cyp11b* exhibited daily differences in the brain.[66]

Seasonal fluctuations in environmental conditions directly influence physiology, leading to changes in seasonal breeders' reproductive functions. Small fluctuations in annual changes in body mass were observed in tree sparrows (Figure 3A). This is consistent with our previous observations.[24] Resident birds store very small amounts of fat, reflecting limited annual variations in body mass.[24,54] Seasonal birds exhibit annual changes in primary sexual characteristics such as gonadal growth and secondary sexual characteristics such as bill color and molt in feathers.[24] Our study shows reproductive phase-dependent changes in bill color. Darker bill color was observed during the breeding phase (Figure 3B). Bill coloring is associated with plasma testosterone levels. During the breeding season, when the bill color is at its darkest, there is also a significant increase in testosterone levels. Bill coloration is associated with testosterone levels, as evidenced by the low levels of plasma testosterone and gonad size observed throughout the post-breeding season.[68,69] Gonadal size-dependent change in bill color has been reported for seasonally breeding avian species.[54] Seasonally breeding birds do not retain fully grown testes during the non-breeding period. Our results are consistent with these findings (Figure 3C). We recorded several fold changes in testicular size from March to May (Figure 3C), which is the breeding time for this species at this latitude.[24,25] The highest plasma testosterone level was observed during April, which corresponds to the bigger testes and higher reproductive activities and is consistent with the previous reports.[70] Seasonally breeding birds exhibit a dramatic change in the size of gonads during the breeding season than the non-breeding season because of the higher gonadal activity during the breeding

period.[24,25,54] Besides, higher gonadal activity also reflects higher steroidogenesis-linked reproductive activity in tree sparrows. Our study shows the highest plasma cholesterol level (February) just before the higher testicular activity (March onward), validating the role of cholesterols in the steroidogenic enzymes' biosynthesis. Cholesterol helps maintain the integrity and fluidity of cell membranes and serves as a precursor for synthesizing substances vital for the organism, including steroid hormones, bile acids, and vitamin D.[71] Energy obtained from food resources forms the fundamental basis of biological activity; thus, energy intake is essential in evaluating an animal's nutritional state.[72] In our study, maximum molt in body and primary flight feathers was observed during the post-breeding phase, i.e., during September, and is consistent with reports on this and other seasonally breeding avian species.[24,54]

Higher expression of *tshβ*, *dio2*, and *gnrh* in the hypothalamus of tree sparrows during the reproductively active phase is consistent with the previous reports for this species,[25] and other seasonally breeding avian species.[23] These findings reconfirm the critical role played by these transcripts in regulating seasonal reproduction in seasonally breeding species.[12,13,23] Further, a decrease in the transcription levels of *dio3* and *gnih* during the reproductive phase and an increase in these transcripts during the post-reproductive phase (September to December) reconfirms the involvement of the product of these transcripts in inhibiting reproduction (Figure 4). These results are consistent with the previous reports on inhibitory nature of these transcripts on gonadal activity.[19,21–23,73] However, an alternate paradigm is suggested by a recent study on Japanese quail.[74] A transcriptome-based analysis suggests that the photoperiod-independent regulation of follicle-stimulating hormone-β (*fshβ*) expression and supplementary environmental cues, such as temperature, can regulate the timing of seasonal reproduction. The study suggests that photoinduced increase in *fshβ* expression precedes increases in prolactin, thyrotropin-stimulating hormone-β, and testicular growth during the simulated spring equinox.[74] Further, our findings are consistent with those of Japanese quail.[74] The study shows that short photoperiods (less than 12 h light per day) lead to gonadal involution and long-term exposure to short photoperiods results in significant upregulation of *dio3* expression and vimentin immunoreactivity in the median eminence.[74] Our findings are consistent with it and we observed higher mRNA expression of *dio3* during the breeding phase from July till January (Figure 4B). The study also suggests that stimulatory daylengths longer than 12 h induce thyrotropes to increase *tshβ* leading to a cascade of molecular events in the MBH that permit *gnrh* to stimulate gonadotropes to release *fshβ* and initiate gonadal development. We did not study the expression of *fshβ*; however, higher expression of *tshβ* was observed during the breeding phase (Figure 4A).

To assess the steroidogenic pathways that govern the seasonal reproductive cycle, we have investigated the expression of transcripts of a gene (*star*) along with a series of steroidogenic enzymes (*er*, *cyp17a1*, *nr4a1*, *scp2*, *cyp11a1*, *hsd11b2*, *vdac1*, *cyp11b*, and *srd5a1*), as well as circulating testosterone titers throughout the year (Figures 3–6). Neurons and glial cells of the brain can locally synthesize biologically active steroids de novo.[75–77] This study shows expression of these transcripts in the hypothalamic tissues with annual variations (Figure 5). Expression of these steroidogenic transcripts is directly influenced by the annual reproductive stage of tree sparrows. In the hypothalamus, higher expression of *cyp11a1*, *nr4a1*, *cyp11b*, *cyp17a1*, *hsd11b2*, *er*, and *vdac1* was observed during the pre-breeding phase (Figure 5), while higher expression of *star* in the hypothalamus corresponds with growing testes (Figures 3 and 5). multiple peaks were observed for *srd5a1* in the hypothalamus (Figure 5). A study conducted on Japanese quail shows similarity with the current study where *star*, *3β-hsd*, *17β-hsd*, *p450*, *ar*, and *5α-Red* were always present in the somatic (Leydig and Sertoli cells) and germ cells (spermatogonia, spermatocytes I and II, spermatids, and spermatozoa) and highest expressions were observed during the reproductive phase.[46] Such evidence from other organisms suggests there are physiology-dependent changes in these transcripts' expression. In edible frogs (*Pelophylas esculentus*), brain levels of neurosteroids oscillate simultaneously with the reproductive cycle. Seasonal fluctuations in gene expression levels of *hsd3b1*, *hsd17b1*, *srd5a1*, and *cyp19a1* were observed in the dienchephalon-mesencephalon region of both sexes of the edible frog, and higher transcript levels were observed in the reproductive period than in post-reproductive period.[43]

Our results show that the steroidogenic process could be quite relevant during spermiogenesis and is consistent with findings of other long-day seasonal breeders, i.e., quail.[46] In seasonal breeders, the differential expression of steroidogenic enzymes during the reproductive and non-reproductive cycles could be the local key in the control of spermatogenesis. In tree sparrows, expression of these steroidogenic transcripts showed reproductive stage-linked expression in the testes (Figure 6). These transcripts were upregulated either before or during the reproductive stage (Figures 3 and 6). These sex steroids are strong regulators of reproductive behavior in vertebrates. The steroidogenesis in gonads shows distinct sex- and season-specific patterns and expression of key enzymes. Higher expression of sex steroids during the pre-breeding or breeding phase has been shown in other species. In seasonally breeding vertebrate species mating behavior, elevated gametogenesis, and peak circulating sex steroid concentrations coincide temporally.[78] In the toad (*Bufo japonicus*), circulatory sex steroid levels fluctuate seasonally and coincide with major reproductive events in species with distinct breeding seasons.[79]

We observed seasonal fluctuations in the *star* transcripts in the testes having peak expression during the breeding phase (Figure 6A). Maximum expression of *star* was also observed in the testes of garter snakes (*Thamnophis sirtalis parietalis*) during the spring mating season.[80] The StAR protein is involved in shuttling cholesterol from the outer to the inner mitochondrial membrane, an essential step for initiating steroidogenesis. Higher expression of *star* mRNA in testes before/during the reproductive phase corresponds to higher steroidogenic activity and is consistent with our findings. StAR proteins also interact with voltage-dependent anion channel (Vdac1) at the mitochondria-associated endoplasmic reticulum membrane to facilitate cholesterol to the inner mitochondrial membrane.[81] We also observed peak expression of *nr4a1* mRNA in testes during May,

corresponding with higher testicular activities in tree sparrows. Expression of Nr4a1 is modulated by several agents, including Ca^{2+}, and growth factors.[82] In gonads, Nr4a1 regulates the expression of several genes involved in steroidogenesis, including *star*,[36] *cyp17a1*,[83] *hsd3b2*,[84] and *cyp11b2*.[85]

We observed higher expression of *scp2* mRNA before/during the peak testicular activity (Figure 6B). The *scp2* gene, having two distinct transcription initiation sites, encodes a 15 kDa pro-*Scp2* protein and the 58 kDa SCPx, which have identical C-termini.[86] Scp2 and SCPx proteins are highly expressed in all tissues with high cholesterol metabolism rates.[87] These proteins transport and metabolize cholesterol and other lipids.[88] We observed higher expression of *cyp11a1* during the breeding phase (Figure 6C). The enzymes Cyp11a1 and Cyp11b reside in the mitochondria, and their deficiency leads to impaired steroidogenesis.[89] *srd5a1* expression is widely distributed in the body in nervous and non-nervous tissues.[90] Srd5a1 and Hsd3b1 catalyze the conversion of testosterone into androgen DHT and DHT metabolism.[91] The enzymes are present in both microsomal and nuclear fractions. Higher expression of *srd5a1* transcripts during the breeding season may directly indicate higher testosterone catabolism. We observed higher expression of *er* transcripts before the breeding phase (Figure 6I). Estrogen differentially modulates reproductive and non-reproductive responses via their receptors.[92] ERs are required for the normal development of reproductive organs and for the expression of both male and female sexual behavior.[93]

Peak expression of *cyp17a1* transcripts was observed during April and May, which correspond to the peak testicular size in this species (Figure 3). Highest expression of *cyp17a1* has been shown in breeding testis in garter snakes.[80] These observations reveal active involvement of steroidogenesis when mating behavior is maximal in male tree sparrows. Sex steroids are known to play a significant role in the testis, controlling the reproductive processes by either promoting spermatogenesis and spermiogenesis or developing, differentiating, and maintaining secondary sex characteristics in seasonal breeders.[38,94,95] Seasonal changes in spermatogenesis and testicular steroidogenesis have also been reported in wild male raccoon dogs (*Nyctereutes procyonoides*) and steroidogenic markers P450sec and P450c17 peaked during mating season. Evidence has been shown in other species such as Japanese quail, where Western blot analysis of steroidogenic genes of 17βhsd, p450 aromatase, and 5α-Red showed the highest mRNA expression levels during breeding season when the testis is reproductively active. The study supports that the steroidogenic process in quail testis directly corresponds to the seasonal variation stimulating androgenic and estrogenic pathways in the reproductive period and their synergic mechanism in the regulation of spermatogenesis.[46] Other than bird species, in sea cucumber (*Holothuria scraba*), real-time qPCR analysis of steroidogenic gene (*star*, *cyp10*, *3β-hsd*, *17β-hsd*) shows increasing levels of mRNA transcripts during gonadal maturation.[96]

The results of the experiments indicate that dietary intake and cholesterol synthesis may be indicators of daily fluctuations in the expression of steroidogenic enzymes. Further, seasonal fluctuations in the patterns of steroidogenic gene expression in the hypothalamus and testis related to the reproductive cycle. The study shows key enzymes involved in steroidogenesis for sex hormone production, are expressed in the hypothalamus and the testis of the tree sparrows. Moreover, the study revealed that the steroidogenic process both in the hypothalamus and in the testis of tree sparrow exhibits seasonal variations and stimulates both androgenic and estrogenic pathways in the reproductive period, suggesting their synergic mechanism in the spermatogenesis regulation in this species. Besides, the study prospects the seasonal reproductive linked mechanism of steroidogenesis; how steroidogenic genes are influenced, and factors that affect the genes to express at distinct periods of the year, may be further examined. Our study has limitations. The present study exclusively involved male birds. Is the mechanism conserved in the female birds need to be explored in future studies.

AUTHOR CONTRIBUTIONS

Subu Yatung: Investigation; writing – original draft; formal analysis; data curation. **Amit Kumar Trivedi:** Conceptualization; funding acquisition; writing – review and editing; supervision.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interest.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

REFERENCES

1. Chevin LM, Visser ME, Tufto J. Estimating the variation, autocorrelation, and environmental sensitivity of phenotypic selection. *Evolution*. 2015;69(9):2319-2332. doi:10.1111/evo.12741
2. Arnold PA, Nicotra AB, Kruuk LE. Sparse evidence for selection on phenotypic plasticity in response to temperature. *Philos Trans R Soc B*. 2019;374(1768):20180185. doi:10.1098/rstb.2018.0185
3. Fox RJ, Donelson JM, Schunter C, Ravasi T, Gaitán-Espitia JD. Beyond buying time: the role of plasticity in phenotypic adaptation to rapid environmental change. *Philos Trans R Soc B*. 2019;374(1768):20180174. doi:10.1098/rstb.2018.0174
4. Siepielski AM, DiBattista JD, Carlson SM. It's about time: the temporal dynamics of phenotypic selection in the wild. *Ecol Lett*. 2009;12(11):1261-1276. doi:10.1111/j.1461-0248.2009.01381.x

5. Chevin LM, Lande R, Mace GM. Adaptation, plasticity, and extinction in a changing environment: towards a predictive theory. *PLoS Biol*. 2010;8(4):e1000357. doi:10.1371/journal.pbio.1000357
6. Nettle D, Bateson M. Adaptive developmental plasticity: what is it, how can we recognize it and when can it evolve? *Proc R Soc B-Biol Sci*. 2015;282(1812):20151005. doi:10.1098/rspb.2015.1005
7. Dunlap JC, Loros JJ, DeCoursey PJ. *Chronobiology: Biological Timekeeping*. Sinauer Associates; 2004.
8. Cowan M, Azpeleta C, López-Olmeda JF. Rhythms in the endocrine system of fish: a review. *J Comp Physiol B*. 2017;187:1057-1089. doi:10.1007/s00360-017-1094-5
9. Renthlei Z, Yatung S, Lalpekhlui R, Trivedi AK. Seasonality in tropical birds. *J Exp Zool A Ecol Integr Physiol*. 2022;337(9–10):952-966. doi:10.1002/jez.2649
10. Gothilf Y, Meiri I, Elizur A, Zohar Y. Preovulatory changes in the levels of three gonadotropin-releasing hormone-encoding messenger ribonucleic acids (mRNAs), gonadotropin I-subunit mRNAs, plasma gonadotropin, and steroids in the female gilthead seabream, Sparus aurata. *Biol Reprod*. 1997;57(5):1145-1154. doi:10.1095/biolreprod57.5.1145
11. Falcón J, Besseau L, Sauzet S, Boeuf G. Melatonin effects on the hypothalamo–pituitary axis in fish. *Trends Endocrinol Metab*. 2007;18(2):81-88. doi:10.1016/j.tem.2007.01.002
12. Yoshimura T, Yasuo S, Watanabe M, et al. Light-induced hormone conversion of T4 to T3 regulates photoperiodic response of gonads in birds. *Nature*. 2003;426(6963):178-181. doi:10.1038/nature02117
13. Nakao N, Ono H, Yoshimura T. Thyroid hormones and seasonal reproductive neuroendocrine interactions. *Reproduction*. 2008;136(1):1-8. doi:10.1530/REP-08-0041
14. Perfito N, Jeong SY, Silverin B, Calisi RM, Bentley GE, Hau M. Anticipating spring: wild populations of great tits (Parus major) differ in expression of key genes for photoperiodic time measurement. *PLoS One*. 2012;7(4):e34997. doi:10.1371/journal.pone.0034997
15. Pérez JH, Tolla E, Dunn IC, Meddle SL, Stevenson TJ. A comparative perspective on extra-retinal photoreception. *Trends Endocrinol Metab*. 2019;30(1):39-53. doi:10.1016/j.tem.2018.10.005
16. Pérez JH, Tolla E, Bishop VR, et al. Functional inhibition of deep brain non-visual opsins facilitates acute long day induction of reproductive recrudescence in male Japanese quail. *Horm Behav*. 2023;148:105298. doi:10.1016/j.yhbeh.2022.105298
17. Tsutsui K, Saigoh E, Ukena K, et al. A novel avian hypothalamic peptide inhibiting gonadotropin release. *Biochem Biophys Res Commun*. 2000;275(2):661-667. doi:10.1006/bbrc.2000.3350
18. Chowdhury VS, Yamamoto K, Ubuka T, Bentley GE, Hattori A, Tsutsui K. Melatonin stimulates the release of gonadotropin-inhibitory hormone by the avian hypothalamus. *Endocrinology*. 2010;151(1):271-280. doi:10.1210/en.2009-0908
19. Majumdar G, Trivedi AK, Gupta NJ, Kumar V. Circadian synchronization determines critical day length for seasonal responses. *Physiol Behav*. 2015;147:282-290. doi:10.1016/j.physbeh.2015.05.005
20. Mishra I, Singh D, Kumar V. Seasonal alterations in the daily rhythms in hypothalamic expression of genes involved in the photoperiodic transduction and neurosteroid-dependent processes in migratory blackheaded buntings. *J Neuroendocrinol*. 2017;29(5). doi:10.1111/jne.12469
21. Dixit AS, Byrsat S. Photoperiodic control of GnRH-I expression in seasonal reproduction of the Eurasian tree sparrow. *Photochem Photobiol Sci*. 2018;17:934-945. doi:10.1039/c8pp00153g
22. Zhang X, Yang W, Liang W, Wang Y, Zhang S. Intensity dependent disruptive effects of light at night on activation of the HPG axis of tree sparrows (*Passer montanus*). *Environ Pollut*. 2019;249:904-909. doi:10.1016/j.envpol.2019.03.008
23. Trivedi AK, Sur S, Sharma A, Taufique ST, Gupta NJ, Kumar V. Temperature alters the hypothalamic transcription of photoperiod responsive genes in induction of seasonal response in migratory redheaded buntings. *Mol Cell Endocrinol*. 2019;493:110454. doi:10.1016/j.mce.2019.110454
24. Renthlei Z, Trivedi AK. Effect of urban environment on pineal machinery and clock genes expression of tree sparrow (*Passer montanus*). *Environ Pollut*. 2019;255:113278. doi:10.1016/j.envpol.2019.113278
25. Renthlei Z, Borah BK, Trivedi AK. Urban environment alter the timing of progression of testicular recrudescence in tree sparrow (*Passer montanus*). *Environ Sci Pollut Res*. 2021;28:31097-31107. doi:10.1007/s11356-021-12918-6
26. Rosati L, Santillo A, Di Fiore MM, Andreuccetti P, Prisco M. Testicular steroidogenic enzymes in the lizard Podarcis sicula during the spermatogenic cycle. *C R Biol*. 2017;340(11–12):492-498. doi:10.1016/j.crvi.2017.10.001
27. Hu J, Zhang Z, Shen WJ, Azhar S. Cellular cholesterol delivery, intracellular processing and utilization for biosynthesis of steroid hormones. *Nutr Metab*. 2010;7:1-25. doi:10.1186/1743-7075-7-47
28. Schiffer L, Barnard L, Baranowski ES, et al. Human steroid biosynthesis, metabolism and excretion are differentially reflected by serum and urine steroid metabolomes: a comprehensive review. *J Steroid Biochem Mol Biol*. 2019;194:105439. doi:10.1016/j.jsbmb.2019.105439
29. Bloj B, Zilversmit DB. Rat liver proteins capable of transferring phosphatidylethanolamine. Purification and transfer activity for other phospholipids and cholesterol. *J Biol Chem*. 1977;252(5):1613-1619. doi:10.1016/S0021-9258(17)40593-X
30. Poorthuis BJ, Glatz JF, Akeroyd R, Wirtz KW. A new high-yield procedure for the purification of the non-specific phospholipid transfer protein from rat liver. *Biochim Biophys Acta, Lipids Lipid Metab*. 1981;665(2):256-261. doi:10.1016/0005-2760(81)90010-2
31. Scallen TJ, Noland BJ, Gavey KL, et al. Sterol carrier protein 2 and fatty acid-binding protein. Separate and distinct physiological functions. *J Biol Chem*. 1985;260(8):4733-4739.
32. Chanderbhan R, Noland BJ, Scallen TJ, Vahouny GV. Sterol carrier protein2. Delivery of cholesterol from adrenal lipid droplets to mitochondria for pregnenolone synthesis. *J Biol Chem*. 1982;257(15):8928-8934. doi:10.1016/S0021-9258(18)34222-4
33. Teerlink T, Van Der Krift TP, Van Heusden GPH, Wirtz KW. Determination of nonspecific lipid transfer protein in rat tissues and Morris hepatomas by enzyme immunoassay. *Biochim Biophys Acta, Lipids Lipid Metab*. 1984;793(2):251-259. doi:10.1016/0005-2760(84)90327-8
34. Seltman H, Diven W, Rizk M, et al. Regulation of bile-acid synthesis. Role of sterol carrier protein2 in the biosynthesis of 7α-hydroxycholesterol. *Biochem J*. 1985;230(1):19-24. doi:10.1042/bj2300019
35. Soccio RE, Breslow JL. StAR-related lipid transfer (START) proteins: mediators of intracellular lipid metabolism. *J Biol Chem*. 2003;278(25):22183-22186. doi:10.1074/jbc.r300003200
36. Martin LJ, Boucher N, Brousseau C, Tremblay JJ. The orphan nuclear receptor NUR77 regulates hormone-induced StAR transcription in Leydig cells through cooperation with Ca^{2+}/calmodulin-dependent protein kinase I. *Mol Endocrinol*. 2008;22(9):2021-2037. doi:10.1210/me.2007-0370
37. Moeller G, Adamski J. Integrated view on 17beta-hydroxysteroid dehydrogenases. *Mol Cell Endocrinol*. 2009;301(1–2):7-19. doi:10.1016/j.mce.2008.10.040
38. Carreau S, Bouraima-Lelong H, Delalande C. Estrogens–new players in spermatogenesis. *Reprod Biol*. 2011;11(3):174-193. doi:10.1016/s1642-431x(12)60065-5
39. Papadopoulos V, Miller WL. Role of mitochondria in steroidogenesis. *Best Pract Res Clin Endocrinol Metab*. 2012;26(6):771-790. doi:10.1016/j.beem.2012.05.002
40. Zhou R, Wu J, Liu B, et al. The roles and mechanisms of Leydig cells and myoid cells in regulating spermatogenesis. *Cell Mol Life Sci*. 2019;76:2681-2695. doi:10.1007/s00018-019-03101-9

41. Chustecka M, Blügental N, Majewski PM, Adamska I. 24 hour patterning in gene expression of pineal neurosteroid biosynthesis in young chickens (Gallus gallus domesticus L.). *Chronobiol Int*. 2021; 38(1):46-60. doi:10.1080/07420528.2020.1823404
42. de Alba G, Mourad NMN, Paredes JF, Sánchez-Vázquez FJ, López-Olmeda JF. Daily rhythms in the reproductive axis of Nile tilapia (Oreochromis niloticus): plasma steroids and gene expression in brain, pituitary, gonad and egg. *Aquaculture*. 2019;507:313-321. https://ui.adsabs.harvard.edu/link_gateway/2019Aquac.507.313D. doi:10.1016/j.aquaculture.2019.04.047
43. Santillo A, Falvo S, Chieffi Baccari G, Di Fiore MM. Seasonal changes in gene expression of steroidogenic enzymes, androgen and estrogen receptors in frog testis. *Acta Zoologica*. 2017;98(2):221-227. doi:10.1111/azo.12169
44. Freking F, Nazairians T, Schlinger BA. The expression of the sex steroid-synthesizing enzymes CYP11A1, 3β-HSD, CYP17, and CYP19 in gonads and adrenals of adult and developing zebra finches. *Gen Comp Endocrinol*. 2000;119(2):140-151. doi:10.1006/gcen.2000.7503
45. Jalabert C, Ma C, Soma KK. Profiling of systemic and brain steroids in male songbirds: seasonal changes in neurosteroids. *J Neuroendocrinol*. 2021;33(1):e12922. doi:10.1111/jne.12922
46. Rosati L, Di Fiore MM, Prisco M, et al. Seasonal expression and cellular distribution of star and steroidogenic enzymes in quail testis. *J Exp Zool B Mol Dev Evol*. 2019;332(6):198-209. doi:10.1002/jez.b.22896
47. Santillo A, Falvo S, Chieffi G, Di Giacomo Russo F, Rosati L, Di Fiore MM. Testis and brain steroidogenesis during the reproductive cycle of Pelophylax esculentus. *Amphibians: Biology, Ecology and Conservation*. Nova Science Publishers, Inc; 2018:39-54.
48. Burrone L, Santillo A, Pinelli C, Baccari GC, Di Fiore MM. Induced synthesis of P450 aromatase and 17β-estradiol by d-aspartate in frog brain. *J Exp Biol*. 2012;215(20):3559-3565. doi:10.1242/jeb.073296
49. Rosati L, Agnese M, Di Fiore MM, Andreuccetti P, Prisco M. P450 aromatase: a key enzyme in the spermatogenesis of the Italian wall lizard, Podarcis sicula. *J Exp Biol*. 2016;219(15):2402-2408. doi:10.1242/jeb.135996
50. Rosvall KA, Bergeon Burns CM, Jayaratna SP, Dossey EK, Ketterson ED. Gonads and the evolution of hormonal phenotypes. *Integr Comp Biol*. 2016;56(2):225-234. doi:10.1093/icb/icw050
51. Qiang W, Murase T, Tsubota T. Seasonal changes in spermatogenesis and testicular steroidogenesis in wild male raccoon dogs (Nyctereutes procynoides). *J Vet Med Sci*. 2003;65(10):1087-1092. doi:10.1292/jvms.65.1087
52. Tsubota T, Howell-Skalla L, Nitta H, et al. Seasonal changes in spermatogenesis and testicular steroidogenesis in the male black bear Ursus americanus. *Reproduction*. 1997;109(1):21-27. doi:10.1530/jrf.0.1090021
53. Kotula-Balak M, Slomczynska M, Fraczek B, et al. Complementary approaches demonstrate that cellular aromatization in the bank vole testis is related to photoperiod. *Eur J Histochem*. 2003;47(1):55-62. doi:10.4081/807
54. Trivedi AK, Rani S, Kumar V. Control of annual reproductive cycle in the subtropical house sparrow (Passer domesticus): evidence for conservation of photoperiodic control mechanisms in birds. *Front Zool*. 2006;3:1-12. doi:10.1186/1742-9994-3-12
55. Kumar V, Singh S, Misra M, Malik S. Effects of duration and time of food availability on photoperiodic responses in the migratory male blackheaded bunting (Emberiza melanocephala). *J Exp Biol*. 2001; 204(16):2843-2848. doi:10.1242/jeb.204.16.2843
56. Biswas A, Mohan J, Sastry KVH. Age-dependent variation in hormonal concentration and biochemical constituents in blood plasma of Indian native fowl. *Vet Med Int*. 2010;2010:1-5. doi:10.4061/2010/737292
57. nee Pathak ND, Lal B. Seasonality in expression and distribution of nitric oxide synthase isoforms in the testis of the catfish, Clarias batrachus: role of nitric oxide in testosterone production. *Comp Biochem Physiol C Toxicol Pharmacol*. 2010;151(3):286-293. doi:10.1016/j.cbpc.2009.11.008
58. Dixit AS, Singh NS. Photoperiodic control of testicular growth, histomorphology and serum testosterone levels in the male Eurasian tree sparrow: involvement of circadian rhythm. *Gen Comp Endocrinol*. 2014;208:5-11. doi:10.1016/j.ygcen.2014.09.003
59. Borah BK, Renthlei Z, Trivedi AK. Hypothalamus but not liver retains daily expression of clock genes during hibernation in terai tree frog (Polypedates teraiensis). *Chronobiol Int*. 2020;37(4):485-492. doi:10.1080/07420528.2020.1726373
60. Renthlei Z, Borah BK, Gurumayum T, Trivedi AK. Season-dependent effects of urban environment on circadian clock of tree sparrow (*Passer montanus*). *Photochem Photobiol Sci*. 2020;19:1741-1749. doi:10.1039/d0pp00257g
61. Borah BK, Renthlei Z, Tripathi A, Trivedi AK. Molecular and epigenetic regulation of seasonal reproduction in Terai tree frog (Polypedates teraiensis). *Photochem Photobiol Sci*. 2022;21(6):1067-1076. doi:10.1007/s43630-022-00195-2
62. Lalpekhlui R, Renthlei Z, Trivedi AK. Molecular expression of clock genes in central and peripheral tissues of white-rumped munia (Lonchura striata). *Chronobiol Int*. 2022;39(8):1058-1067. doi:10.1080/07420528.2022.2062374
63. Schmittgen TD. Real-time quantitative PCR. 2001. doi:10.1006/meth.2001.1262
64. Cuesta M, Clesse D, Pévet P, Challet E. From daily behavior to hormonal and neurotransmitters rhythms: comparison between diurnal and nocturnal rat species. *Horm Behav*. 2009;55(2):338-347. doi:10.1016/j.yhbeh.2008.10.015
65. Soper DS. F-value and p-value calculator for multiple regression. 2013 See http://www.danielsoper.com/statcalc
66. Di Rosa V, López-Olmeda JF, Burguillo A, et al. Daily rhythms of the expression of key genes involved in steroidogenesis and gonadal function in zebrafish. *PLoS One*. 2016;11(6):e0157716. doi:10.1371/journal.pone.0157716
67. Chen R, Qin F, Du J, et al. Circadian clock gene BMAL1 regulates STAR expression in goose ovarian preovulatory granulosa cells. *Poult Sci*. 2023;102(12):103159. doi:10.1016/j.psj.2023.103159
68. Laucht S, Kempenaers B, Dale J. Bill color, not badge size, indicates testosterone-related information in house sparrows. *Behav Ecol Sociobiol*. 2010;64:1461-1471. doi:10.1007/s00265-010-0961-9
69. Green PA, George EM, Rosvall KA, Johnsen S, Nowicki S. Testosterone, signal coloration, and signal color perception in male zebra finch contests. *Ethology*. 2022;128(2):131-142. doi:10.1111/eth.13247
70. Dixit AS, Singh NS. Seasonality in circadian locomotor activity and serum testosterone level in the subtropical tree sparrow (*Passer montanus*). *J Photochem Photobiol B Biol*. 2016;158:61-68. doi:10.1016/j.jphotobiol.2016.02.014
71. Zampelas A, Magriplis E. New insights into cholesterol functions: a friend or an enemy? *Nutrients*. 2019;11(7):1645 10.3390%2Fnu11071645
72. Robbins C. *Wildlife Feeding and Nutrition*. Elsevier; 2012.
73. Tsutsui K, Ukena K, Takase M, Kohchi C, Lea RW. Neurosteroid biosynthesis in vertebrate brains. *Comp Biochem Physiol C Toxicol Pharmacol Endocrinol*. 1999;124(2):121-129. doi:10.1016/s07428413(99)00065-1
74. Majumdar G, Liddle TA, Stewart C, Marshall CJ, Bain M, Stevenson T. FSHβ links photoperiodic signaling to seasonal reproduction in Japanese quail. *Elife*. 2023;12:RP87751. doi:10.7554/eLife.87751.3
75. Baulieu EE. Neurosteroids: a novel function of the brain. *Psychoneuroendocrinology*. 1998;23(8):963-987. doi:10.1016/s0306-4530(98)00071-7
76. Compagnone NA, Mellon SH. Neurosteroids: biosynthesis and function of these novel neuromodulators. *Front Neuroendocrinol*. 2000; 21(1):1-56. doi:10.1006/frne.1999.0188

77. Do Rego JL, Seong JY, Burel D, et al. Neurosteroid biosynthesis: enzymatic pathways and neuroendocrine regulation by neurotransmitters and neuropeptides. *Front Neuroendocrinol*. 2009;30(3):259-301. doi:10.1016/j.yfrne.2009.05.006
78. Crews D. Gamete production, sex hormone secretion, and mating behavior uncoupled. *Horm Behav*. 1984;18(1):22-28. doi:10.1016/0018-506X(84)90047-3
79. Itoh M, Ishii S. Changes in plasma levels of gonadotropins and sex steroids in the toad, Bufo japonicus, in association with behavior during the breeding season. *Gen Comp Endocrinol*. 1990;80(3):451-464. doi:10.1016/0016-6480(90)90194-Q
80. Lincoln JM, Rucker HR, Parker MR. Reconsidering reproductive patterns in a model dissociated species, the red-sided garter snake: sex-specific and seasonal changes in gonadal steroidogenic gene expression. *Front Endocrinol*. 2023;14:1135535. doi:10.3389/fendo.2023.1135535
81. Prasad M, Kaur J, Pawlak KJ, Bose M, Whittal RM, Bose HS. Mitochondria-associated endoplasmic reticulum membrane (MAM) regulates steroidogenic activity via steroidogenic acute regulatory protein (StAR)-voltage-dependent anion channel 2 (VDAC2) interaction. *J Biol Chem*. 2015;290(5):2604-2616. doi:10.1074/jbc.M114.605808
82. Li QX, Ke N, Sundaram R, Wong-Staal F. NR4A1, 2, 3 an orphan nuclear hormone receptor family involved in cell apoptosis and carcinogenesis. *Histol Histopathol*. 2006;21(5):533-540. doi:10.14670/hh-21.533
83. Zhang P, Mellon SH. Multiple orphan nuclear receptors converge to regulate rat P450c17 gene transcription: novel mechanisms for orphan nuclear receptor action. *Mol Endocrinol*. 1997;11(7):891-904. doi:10.1210/mend.11.7.9940
84. Bassett MH, Suzuki T, Sasano H, et al. The orphan nuclear receptor NGFIB regulates transcription of 3β-hydroxysteroid dehydrogenase: implications for the control of adrenal functional zonation. *J Biol Chem*. 2004a;279(36):37622-37630. doi:10.1074/jbc.m405431200
85. Bassett MH, Suzuki T, Sasano H, White PC, Rainey WE. The orphan nuclear receptors NURR1 and NGFIB regulate adrenal aldosterone production. *Mol Endocrinol*. 2004b;18(2):279-290. doi:10.1210/me.2003-0005
86. Ohba T, Holt JA, Billheimer JT, Strauss JF III. Human sterol carrier protein x/sterol carrier protein 2 gene has two promoters. *Biochemistry*. 1995;34(33):10660-10668. doi:10.1021/bi00033a042
87. Vahouny GV, Chanderbhan R, Kharroubi A, Noland BJ, Pastuszyn A, Scallen TJ. Sterol carrier and lipid transfer proteins. *Adv Lipid Res*. 1987;22:83-113. doi:10.1016/b978-0-12-024922-0.50007-2
88. Li NC, Fan J, Papadopoulos V. Sterol carrier protein-2, a nonspecific lipid-transfer protein, in intracellular cholesterol trafficking in testicular Leydig cells. *PLoS One*. 2016;11(2):e0149728. doi:10.1371/journal.pone.0149728
89. Parajes S, Griffin A, Taylor AE, et al. Redefining the initiation and maintenance of zebrafish interrenal steroidogenesis by characterizing the key enzyme cyp11a2. *Endocrinology*. 2013;154(8):2702-2711. doi:10.1210/en.2013-1145
90. Rył A, Rotter I, Grzywacz A, et al. Molecular analysis of the SRD5A1 and SRD5A2 genes in patients with benign prostatic hyperplasia with regard to metabolic parameters and selected hormone levels. *Int J Environ Res Public Health*. 2017;14(11):1318 10.3390%2Fijerph14111318
91. Henríquez-Hernández LA, Valenciano A, Foro-Arnalot P, et al. Genetic variations in genes involved in testosterone metabolism are associated with prostate cancer progression: a Spanish multicenter study. *Urologic Oncology: Seminars and Original Investigations*. Vol 33, No. 7. Elsevier; 2015, July:331-e1. doi:10.1016/j.urolonc.2015.04.003
92. Kuiper GG, Shughrue PJ, Merchenthaler I, Gustafsson JÅ. The estrogen receptor β subtype: a novel mediator of estrogen action in neuroendocrine systems. *Front Neuroendocrinol*. 1998;19(4):253-286. doi:10.1006/frne.1998.0170
93. Rissman EF, Wersinger SR, Fugger HN, Foster TC. Sex with knockout models: behavioral studies of estrogen receptor α. *Brain Res*. 1999;835(1):80-90. doi:10.1016/s0006-8993(99)01452-3
94. Angelini F, Botte V. Spermatogenesis in reptiles, dynamic and regulatory aspect. *Sex Origin Evolution*. 1992;6:211-230.
95. Di Fiore MM, Chieffi Baccari G, Rastogi RK, Di Meglio M, Pinelli C, Iela L. Hormonal regulation on secondary sexual characters. *Amphibian Biology*. Surrey Beatty & Sons; 2005:2228-2249.
96. Thongbuakaew T, Suwansa-Ard S, Chaiyamoon A, Cummins SF, Sobhon P. Sex steroids and steroidogenesis-related genes in the sea cucumber, Holothuria scabra and their potential role in gonad maturation. *Sci rep*. 2021;11(1):2194. doi:10.1038/s41598-021-81917-x

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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ORIGINAL PAPERS

Time- and season-dependent changes in the steroidogenic markers in female tree sparrow (*Passer montanus*)

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Abstract

Seasonal breeders display elevated sex steroid hormone production during reproductive seasons, resulting in significant physiological and structural alterations. One such seasonal breeder adapted to the changing environment is a Tree sparrow (*Passer montanus*). The study aims to investigate 24-h rhythmicity and annual variations in the expression of steroidogenic gene markers of adult female tree sparrows. Two experiments were conducted; in experiment one, birds ($n = 5$ birds/time points) were sampled at six time points, i.e., ZT1, ZT5, ZT9, ZT13, ZT17, and ZT21 (ZT = Zeitgeber time, ZT0 = sunrise time) during the reproductive stage; subsequently, hypothalamus and ovary were harvested for gene expression analysis. In experiment two, birds ($n = 5$/month) were sampled at mid-day every month for a year. Feather molt, follicular diameter, body mass, and bill coloration were recorded. The hypothalamus and ovary were harvested for gene expression studies. Blood plasma cholesterol and progesterone were also measured. The study indicates a larger follicular size during May and June. Whereas, maximum molt was observed during the post-reproductive phase. Cholesterol levels were highest prior breeding phase and higher progesterone levels paralleled larger follicular size. While higher levels of *GnIh* (*gonadotropin-inhibitory hormone*) and *Dio3* (*type 3 deiodinase*) were observed during the non-breeding phase, elevated expression of *Tshβ* (*thyroid stimulating hormone subunit beta*), *Dio2* (*type 2 deiodinase*), and *GnRh* (*gonadotropin-releasing hormone*) was noted during the reproductive period. The study also reveals 24-h rhythmicity in selected steroidogenic markers (*StAR, Nr4a1, Er, Scp2, Cyp17a1, Foxl2, Cyp11a1, Hsd11b2, Cyp11b, Cyp19a1,* and *Vdac1*) and seasonal variations directly influence steroidogenesis, which connects with the annual reproductive cycle.

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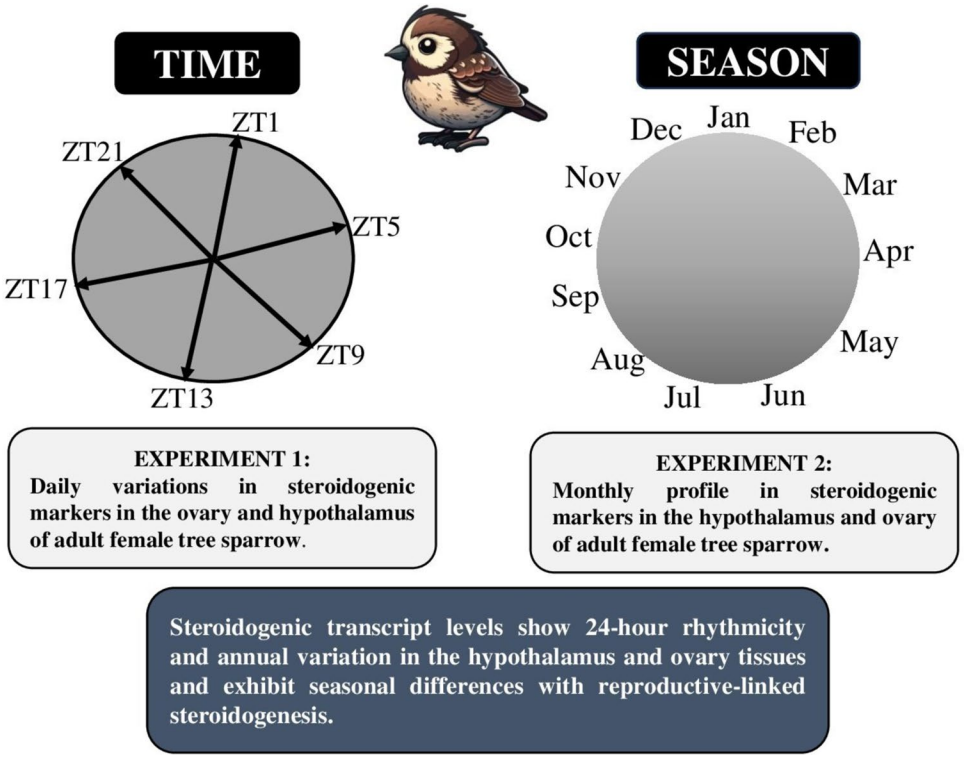
Graphical abstract



Keywords Tree sparrow · Seasonality · Steroidogenesis · StAR · Gonadotropins · Progesterone

1 Introduction

Environmental factors, including temperature, day length, and food accessibility, have a reflective influence on the physiology and behavior of avian species, especially in terms of seasonal events (reproduction, migration, and molt) and daily cycles (activity, metabolism, etc.) and exhibit physiological and behavioral rhythmicity alterations driven by the internal biological timekeeping system. These clocks permit the animals to correspond with the cyclic events such as day and night/season in nature. They use changes in day length as the clock and calendar to interpret the time of day and year, respectively [1–3]. Thus, day length (photoperiod) is a main component in providing individual periodic information about the environment [4]. Environmental time cues (zeitgebers) initiate a sequence of signals that enable biological clocks to synchronize with ecological cycles, ensuring a consistent phase relationship between the internal rhythm and external cues. Rhythms in neuroendocrine factors exist at all levels of the HPG (hypothalamus–pituitary–gonadal) axis to ensure the synchronization of reproduction rhythms and the environment so that it occurs at the most convenient time for offspring success [5]. Similarly, most avian species exhibit seasonality in their behavioral, physiological, and reproductive functions [6].

Similarly, sex steroid hormones predominantly control vertebrates' reproductive behavior and physiology [7]. Avian follicle growth is mainly regulated by the genetic machinery of reproductive hormones and associated functions, steroid hormone synthesis and secretion play a dominant role [8]. In males, it plays a crucial role in the testis and regulates spermatogenesis and spermiogenesis, or the maintenance of secondary sexual characters in vertebrates having seasonal reproductive cycles [9]. Progesterone (P4), testosterone (T), and estradiol (E2) are vital steroid hormones regulating follicle development [10]. Thus, sex steroid hormones are influential regulators of reproductive behavior and physiology in vertebrates, and have distinct sex- and season-specific patterns ultimately dictated by the expression of key enzymes [11] and are critical for reproduction in a wide-ranging variety of vertebrates, reproduction does not occur if these hormones are removed [12, 13]. Also, the breeding behavior of seasonal animals corresponds to levels of steroid hormones. Specifically, during the

breeding season, these animals exhibit large gonadal development, high levels of sex steroids, and reproductive behaviors; in contrast, during the non-breeding season, they exhibit regressed gonadal development, low levels of sex steroid hormones, and no reproductive activity [14–16]. Therefore, in animals that reproduce periodically, steroidogenesis is possibly controlled to result in seasonal variations in the levels of circulating steroid hormones. The hypothalamic–pituitary–gonadal (HPG) axis system regulates gonadal steroidogenesis, whereby circulating luteinizing hormone (LH) stimulates the gonadal production of steroidogenic acute regulatory protein (StAR) [17].

The initial step in steroidogenesis involves the conversion of cholesterol into pregnenolone by the cytochrome P450 enzyme CYP11A1. Pregnenolone can then be metabolized along two primary pathways. The first pathway leads to the synthesis of androgens, beginning with the conversion of pregnenolone to dehydroepiandrosterone (DHEA) by CYP17. DHEA is subsequently converted to androstenedione (AE) by 3β-HSD. The second pathway leads to the synthesis of progestins, starting with the conversion of pregnenolone to progesterone by 3β-HSD. Progesterone can then be converted to AE by CYP17. Androstenedione can be further metabolized into the more potent androgen testosterone (T) by 17β-HSD. Finally, T can be aromatized into the estrogen, estradiol (E2) by CYP19. The expression of these steroidogenic enzymes is regulated by the specific steroid hormone produced by the tissue, ensuring the appropriate synthesis of hormones to meet the physiological needs of an organism. FOXL2 is responsible for transcriptional regulation of CYP19A1 in female gonads because FOXL2 and CYP19A1 mRNA are co-expressed in the medullary region of the female gonad during sex differentiation (13–15 days incubation) in chicken (*Gallus gallus domesticus L*) [18].

A study on steroidogenic enzyme's daily rhythmicity in the brain and gonads has been detailed in the limited existing literature on chicken (*Gallus gallus domesticus L*), Nile tilapia (*Oreochromis niloticus*), and male tree sparrow (*Passer montanus*) [19–21]. Many studies also have shown that steroidogenic enzyme plays a significant role in gonadal development and reproductive activity across seasonally breeding animals' zebrafish (*Danio rerio*), cherry valley duck (*Anas platyrhynchos domesticus*), quail (*Coturnix coturnix*), Italian wall lizard (*Podarcis sicula*) [9, 22–24].

A tree sparrow is a long-day seasonal breeder whose biological activities are highly associated with the natural photoperiod. In a recent work reported by [21], the primary study was dedicated to principal genes involved in steroidogenesis, and their daily rhythm, annual variation in adult male tree sparrows, and the relationship between steroidogenic enzyme expression levels and reproductive capability in male tree sparrows are known. Henceforth, the primary aim of this study is to examine if the selected steroidogenic markers also exhibit 24-h rhythmicity and yearly variations in the expression of selected transcript levels in adult female tree sparrows. Moreover, the study investigated the 24-h rhythmicity and seasonal variations in the gene's expression encoding steroidogenic enzymes related to the female tree sparrows' reproductive cycles, and a correlation was carried out of the female with the results of male tree sparrows.

2 Experiments

2.1 Experiment one: daily rhythmicity in steroidogenic markers in the hypothalamus and ovary

The study was conducted on adult female tree sparrows (*Passer montanus*) procured locally (Tanhril, Aizawl, Mizoram, India, 23 44′10'' N to 92 39′51'' E) using mist nets. Adult female birds ($n = 30$) were acquired during the breeding period in April. Upon procuring, the birds were transferred to the laboratory and caged ($3 \times 2.5 \times 3$ fits), five birds in each cage, exposed to natural daylight environments (NDL) in captivity, with access to paddy (*Oryza Sativa)*, mealworms (*Tenebrio molitor*), and water ad libitum. Birds ($n = 5$/timepoints) were sacrificed the next day (by decapitation) at six time points: ZT1, ZT5, ZT9, ZT13, ZT17, and ZT21 (ZT0 = 05:11 a.m, sunrise time at the respective time of the year) during a 24-h cycle. Brain and ovary tissues were removed and promptly stored at −80 °C for subsequent gene expression analysis.

2.2 Experiment two: annual variation in steroidogenic markers in the hypothalamus and ovary

Adult female tree sparrows (n = 5/month) procured locally (Tanhril, Aizawl, Mizoram, India, 23 44′10'' N to 92 39′51'' E) using mist nets monthly for a year. The birds were transferred to the laboratory instantly upon acquisition and housed in an indoor aviary in natural length conditions (NDL) with water ad *libitum* and access to paddy and mealworms. Body weight was recorded using a top pan balance with an accuracy of 0.1 g. Body molt and molt in primary flight feathers were recorded for individual birds, and bill color was evaluated according to a previously stated criterion [25]. In brief, the bill color was measured using a guide ranging from 0 to 5, where 0 denotes a straw-colored bill (S), 1 specifies a straw bill color with a minor black color (SSS: B), 2 defines a somewhat blackish bill (SS: B), 3 specifies a bill with a similar proportion of black and straw

(ratio—S: B), 4 defines a black bill with a small straw cover (ratio—S: BB), and 5 specifies a full black colored bill (B). Molt was observed in the body and primary flight (wing primaries). The primaries' feathers were scored between 0 and 5: 0—damaged or old feather, 1—lost feather (just released), 2—from the emergence of a new feather papilla till one-third development is attained, 3—new feather attained two-thirds growth, 4—new feather grownup, but is not completed, 5—fully fledged new feather. For the molt in body, the bird's whole body was divided into 11 regions: 1 = head, 2 = neck, 3 = shoulder, 4 = back, 5 = pelvic, 6 = throat, 7 = chest, 8 = abdomen, 9 = flank, 10 = shank, and 11 = sub-caudal. A score of 0 (old feathers) or 1 (newly emerged feathers) could be given to any region, and hence the total body molt score could be in the range of 0–11, depending on the number of regions that had scores of 0 or 1. The follicular diameter was recorded by performing unilateral laparotomy [26], where a small incision was made between the last two ribs on the left flank, and the ovary was located within the abdominal cavity with the help of a spatula. Birds were sampled during the middle of the day. Blood samples (200–300 µL) were collected from each bird into heparinized tubes. The blood was centrifuged to separate the plasma, which was then stored at -20 °C for subsequent biochemical and hormonal analysis. The brain and ovary tissues were excised from each bird and immediately stored at -80 °C for gene expression analysis. The protocols were approved by the institutional animal ethics committee of Mizoram University (No. MZU/IAEC/2021–22/12).

2.3 Cholesterol level assessment

Plasma cholesterol levels were measured using CHOLESTEROL KIT (Coral Clinical Systems, India) as per the manufacturer's protocol. The test tubes were cleansed and labeled as blank, standard, and test. After that, 1000 µL of the working reagent was then pipetted into test tubes for the test sample, standard, and blank. Distilled water of 10 µL was pipetted into a blank test tube, followed by 10 µL of cholesterol standard into a standard test tube and 10 µL of plasma sample into a sample test tube. After the required reagents were added to the test tubes, it was properly mixed and kept incubated at room temperature (25 °C) for 15 min. Within 15 min, the absorbance of the standard and test samples at a wavelength of 505 nm was measured against the blank using the SpectraMax MINI system (Multimode Microplate Reader, AFL System Molecular Devices, USA). Each sample was run in duplicate.

2.4 Progesterone level assessment

Plasma progesterone levels were measured using enzyme immunoassay kits (Progesterone, Diametra, DKO006) as per manufacturer protocol. Briefly, before use, all the reagents were kept at room temperature for 30 min. The blood plasma samples were placed on ice to thaw. The wash solution was diluted as per the protocol provided. All calibrators, control, test samples, and blank were run in duplicate. 50 µL of sample or control and 50 µL of calibrator were pipetted into each respective well, and for each calibrator and sample well, 100 µL of conjugate was added. Following the addition of all the reagents, the plate was incubated at 37 °C for 1 h. After 1 h, the content was removed from each well and washed three times with 300 µL of diluted wash solution every 5-min interval. The remaining buffer was removed by tapping the plate on absorbent paper upside down. After adding 100 µL of TMB substrate to each well and letting it sit at room temperature for 15 min in the dark, 100 µL of a stop solution was added and gently mixed. Then absorbance was taken using a Multimode ELISA reader SpectraMax MINI system at 450 nm against the Blank within 5 min.

2.5 Gene transcription

The relative mRNA transcript levels of reproductive genes coding for *Tshβ, Dio2, Dio3, GnRh,* and *GnIh* were measured in the hypothalamus, and mRNA transcript levels of the steroidogenic gene coding for *StAR* and *Nr4a1, Er, Scp2, Cyp17a1, Foxl2, Cyp11a1, Hsd11b2, Cyp11b, Cyp19a1,* and *Vdac1* was measured both in hypothalamus and ovary for daily and seasonal studies. 18 s was selected as the control gene to determine the relative expression of transcripts [21].

2.6 RNA isolation and cDNA synthesis

Total RNA was extracted from tissue samples using TRIzol Reagent (Invitrogen by Thermo Fisher Scientific). The concentration and purity of total RNA were determined (Nanodrop Spectrophotometer, Thermo Fisher Scientific, Wilmington, DE). The cDNA synthesis was processed using 2 µg of RNA. RQ1 DNase (Promega M6101; Madison, WI, USA) kit was used to remove any possible genomic DNA contamination. For cDNA synthesis, iScript™ cDNA Synthesis Kit (BIO-RAD, USA, 1,708,891) was used.

2.7 Quantitative (real-time) RT-PCR (qPCR)

Gene-specific primers were designed from the sequences using Primer 3 plus (online primer design program, Boston, MA, USA; Supplementary Table 1). qPCR was performed on Quant-Studio 5 (Applied Biosystem by Thermo Fisher Scientific, Foster City, CA, USA) as described in our previous publications [21, 27–31]. Briefly, PCR reaction of 7 µl total volume for each gene comprised 1 µl each of cDNA, 1 µl gene-specific forward and reverse primers each, 3 µl Power UpTM SYBR Green Master Mix (Applied

Biosystem by Thermo Fisher Scientific, Foster City, CA, USA, A25742) and 1 µl of nuclease-free water (Ambion, AM9938). qPCR cycle conditions included 1 cycle at 95 °C (20 s), 35 cycles at 95 °C (01 s), 60 °C (20 s) and 95 °C (01 s), additional melt curve plot step included 1 cycle of 60 °C (20 s) and 1 cycle of 95 °C (01 s). ΔΔCt method [32], was used for gene expression analysis. Every sample was run in duplicate.

2.8 Statistical analysis

To determine the significant difference in 24-h and monthly profiles of the selected steroidogenic markers, one-way ANOVA (one-way analysis of variance) was used. Analysis was done using graph pad Prism version 9. Values of daily steroidogenic genes were subjected to 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 [33]. Based on Ref. [34], the significance of regression analysis was calculated using the number of samples, R2 values, and numbers of predictors (mesor, amplitude, and acrophase; http://www.danielsoper.- com/statcalc3/calc.aspx? id 1⁄4 15). The effect size was calculated in eta square (η^2), where η^2 = treatment sum of squares/total sum of squares [35]. Tukey's multiple comparison tests were applied if ANOVA showed a significant difference, and if one-way ANOVA revealed a significant difference. $P < 0.05$ was considered a significant difference. Correlation was calculated by Pearson's correlation coefficient (r) to show relationships between the reproductive gene expression and steroidogenic markers.

3 Results

3.1 Experiment one: daily rhythmicity in steroidogenic markers in the hypothalamus and ovary

Daily variations were observed in the expression of steroidogenic mRNA transcript levels in the hypothalamus and ovary of tree sparrows. *StAR* mRNA transcript showed daily variation in the hypothalamus ($P = 0.0062$; one-way ANOVA; $\eta^2 = 0.32$; Supplementary Table 2; Fig. 1a) and in the ovary ($P = 0.0142$; one-way ANOVA; $\eta^2 = 0.52$; Supplementary Table 2; Fig. 2a). *StAR* mRNA levels showed highest expression at ZT9 both in the hypothalamus and ovary ($P < 0.05$; Tukey's multiple comparison tests; Fig. 1a, 2a). *Scp2* mRNA levels in the hypothalamus ($P = 0.0158$; one-way ANOVA; $\eta^2 = 0.28$; Supplementary Table 2; Fig. 1c) showed a significant difference and peaked at ZT21, whereas in the ovary ($P = 0.0026$; one-way ANOVA; $\eta^2 = 0.35$; Supplementary Table 2; Fig. 2c) peaked at ZT5 ($P < 0.05$; Tukey's multiple comparison tests; Fig. 2c). *Cyp11a1* exhibited daily changes in the hypothalamus ($P = 0.0002$; one-way ANOVA; $\eta^2 = 0.42$; Supplementary Table 2; Fig. 1e) and in the ovary ($P = 0.0001$; one-way ANOVA; $\eta^2 = 0.44$; Supplementary Table 2; Fig. 2e). Peak expression was observed at ZT21 in the hypothalamus and at ZT5 in the ovary ($P < 0.05$; Tukey's multiple comparison tests; Fig. 1e, 2e). *Nr4a1* mRNA showed significant differences in the hypothalamus ($P < 0.0001$; one-way ANOVA; $\eta^2 = 0.49$; Supplementary Table 2; Fig. 1b) and ovary ($P = 0.0062$; one-way ANOVA; $\eta^2 = 0.56$; Supplementary Table 2; Fig. 2b). Peak expression timing for *Nr4a1* was highest at ZT9 in the hypothalamus ($P < 0.05$; Tukey's multiple comparison tests; Fig. 1b) and ZT1 in the ovary ($P < 0.05$; Tukey's multiple comparison tests; Fig. 2b). *Cyp11b* transcript showed a significant difference in the hypothalamus ($P = 0.0001$; one-way ANOVA; $\eta^2 = 0.45$; Supplementary Table 2; Fig. 1g) showing the highest expression at ZT21 ($P < 0.05$; Tukey's multiple comparison tests; Fig. 1g), whereas significant differences were observed

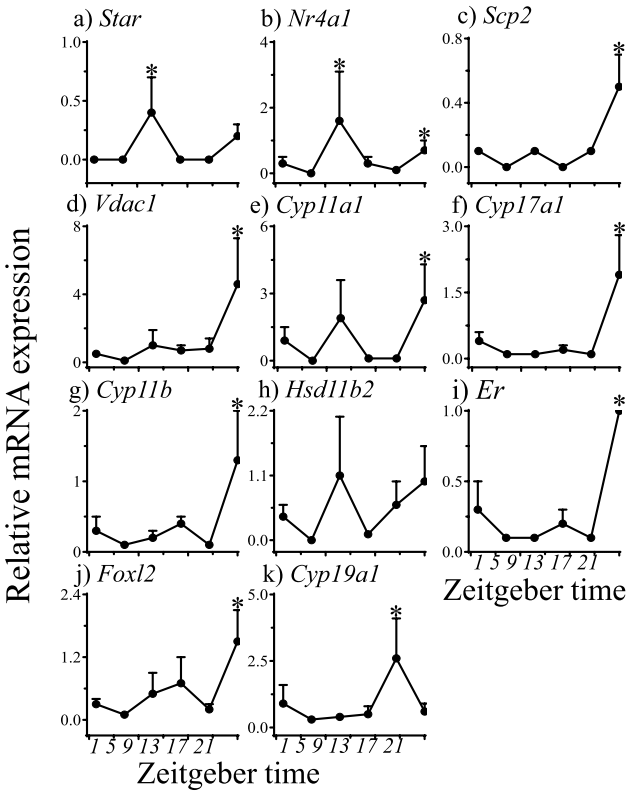


Fig. 1 The data display relative mRNA levels of steroidogenic transcripts (*StAR*, *Scp2*, *Cyp11a1*, *Nr4a1*, *Cyp11b*, *Cyp17a1*, *Foxl2*, *Cyp19a1*, *Hsd11b2*, *Er*, and *Vdac1*) in the hypothalamus of adult female tree sparrow (*Passer montanus*) collected in April (Fig. 1a-k). The x-axis indicates the zeitgeber time (ZT 0 is sunrise time) * Indicates significant differences among the time points

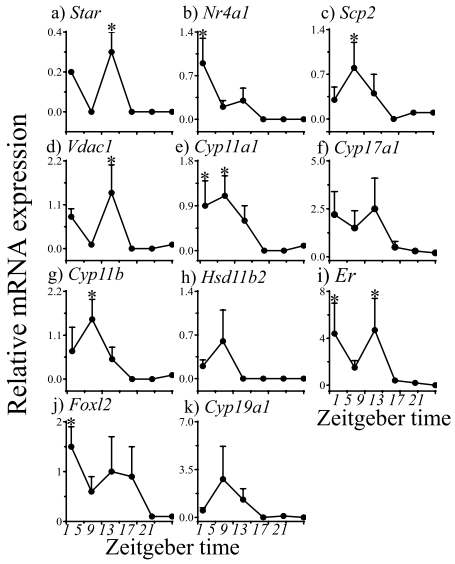


Fig. 2 Relative mRNA levels of steroidogenic transcripts (*StAR*, *Scp2*, *Cyp11a1*, *Nr4a1*, *Cyp11b*, *Cyp17a1*, *Foxl2*, *Cyp19a1*, *Hsd11b2*, *Er*, and *Vdac1*) in the ovary of adult female tree sparrow (*Passer montanus*) collected in April (Fig. 2a-k). The x-axis indicates the zeitgeber time (ZT 0 is sunrise time) * Indicates significant differences among the time points

in ovary at ZT5 ($P < 0.0001$; one-way ANOVA; $\eta^2 = 0.44$; Supplementary Table 2; Fig. 2g). *Cyp17a1* showed a significant difference in the hypothalamus ($P < 0.0001$; one-way ANOVA; $\eta^2 = 0.48$; Supplementary Table 2; Fig. 1f) and expression peaked at ZT21 ($P < 0.05$; Tukey's multiple comparison tests; Fig. 1f). Whereas no significant difference was observed in the ovary ($P = 0.0185$; one-way ANOVA; $\eta^2 = 0.26$; Supplementary Table 2; Fig. 2f). In the hypothalamus and ovary, the *Hsd11b2* transcript showed no significant difference ($P = 0.1317$; one-way ANOVA; $\eta^2 = 0.17$; Supplementary Table 2; Fig. 1h) and ($P = 0.0745$; one-way ANOVA; $\eta^2 = 0.21$; Supplementary Table 2; Fig. 2h). *Er* transcript showed a significant difference in the hypothalamus ($P = 0.0014$; one-way ANOVA; $\eta^2 = 0.41$; Supplementary Table 2; Fig. 1i) and peaked expression was observed at ZT21 ($P < 0.05$; Tukey's multiple comparison tests; Fig. 1i) and in the ovary ($P = 0.0025$; one-way ANOVA; $\eta^2 = 0.34$; Supplementary Table 2; Fig. 2i), it peaked at ZT1 and ZT9 ($P < 0.05$; Tukey's multiple comparison tests; Fig. 2i). *Vdac1* transcript showed a significant difference in the ovary ($P < 0.0001$; one-way ANOVA; $\eta^2 = 0.48$; Supplementary Table 2; Fig. 2d) and hypothalamus ($P = 0.0020$; one-way ANOVA; $\eta^2 = 0.35$; Supplementary Table 2; Fig. 1d), and the highest expression of the transcript was observed at ZT9 in the ovary ($P < 0.05$; Tukey's multiple comparison tests; Fig. 2d) and ZT21 in the hypothalamus ($P < 0.05$; Tukey's multiple comparison tests; Fig. 1d). *Foxl2* transcript showed significant difference in the hypothalamus ($P = 0.0001$; one-way ANOVA; $\eta^2 = 0.44$; Supplementary Table 2; Fig. 1j) showing highest expression at ZT21 ($P < 0.05$; Tukey's multiple comparison tests; Fig. 1j), whereas at ZT1 in ovary ($P = 0.0065$; one-way ANOVA; $\eta^2 = 0.31$; Supplementary Table 2; Fig. 2j). *Cyp19a1* transcript showed significant difference in the hypothalamus ($P = 0.0116$; one-way ANOVA; $\eta^2 = 0.28$; Supplementary Table 2; Fig. 1k) showing highest expression at ZT17 ($P < 0.05$; Tukey's multiple comparison tests; Fig. 1k), whereas in ovary, no significant differences were observed ($P = 0.0372$; one-way ANOVA; $\eta^2 = 0.23$; Supplementary Table 2; Fig. 2k).

3.2 Experiment two: annual variations in steroidogenic markers in the hypothalamus and ovary

No significant variation in the body mass was observed over the year ($P = 0.1507$; one-way ANOVA; $\eta^2 = 0.27$; Supplementary Table 3a; Fig. 3a). Annual variations in bill color ($P < 0.0001$; one-way ANOVA; $\eta^2 = 0.79$; Supplementary Table 3a; Fig. 3b), molt in body feathers ($P < 0.0001$; one-way ANOVA; $\eta^2 = 0.61$; Supplementary Table 3a; Fig. 3f), primary flight feathers ($P = 0.0051$; one-way ANOVA; $\eta^2 = 0.41$; Supplementary Table 3a; Fig. 3g), and follicular diameter ($P < 0.0001$; one-way ANOVA; $\eta^2 = 0.78$; Supplementary Table 3a; Fig. 3e) were also observed. Higher bill color score was observed from April till June ($P < 0.0001$; Tukey's multiple comparison tests; Fig. 3b), having the highest score during June ($P < 0.05$; Tukey's multiple comparison tests; Fig. 3b). Molt in body feathers began in July and completed in December (Supplementary Table 3a; Fig. 3f) and primary flight feathers started at July and completed in October with both having maximum molt during August and September ($P < 0.05$; Tukey's multiple comparison tests; Fig. 3f,g). Significantly higher follicular sizes were observed from April till June, began to regress in July, and fully regressed from August onwards. Plasma cholesterol levels also showed seasonal fluctuation ($P = 0.0005$; one-way ANOVA; $\eta^2 = 0.27$; Supplementary Table 3a; Fig. 3c). Elevated plasma cholesterol levels were observed in February and April, were significantly higher in February ($P < 0.05$; Tukey's multiple comparison tests; Fig. 3c), and started to decline from May onwards (Fig. 3c). Minimum cholesterol levels were observed during June till September ($P < 0.05$; Tukey's multiple comparison tests; Fig. 3c). Annual changes in plasma progesterone levels were also observed ($P < 0.0001$; one-way ANOVA; $\eta^2 = 0.49$; Supplementary Table 3a; Fig. 3e). Plasma progesterone levels started to elevate in March, maximum levels were observed during June ($P < 0.05$; Tukey's multiple comparison tests; Fig. 3d), and began to decline from July onwards (Fig. 3d).

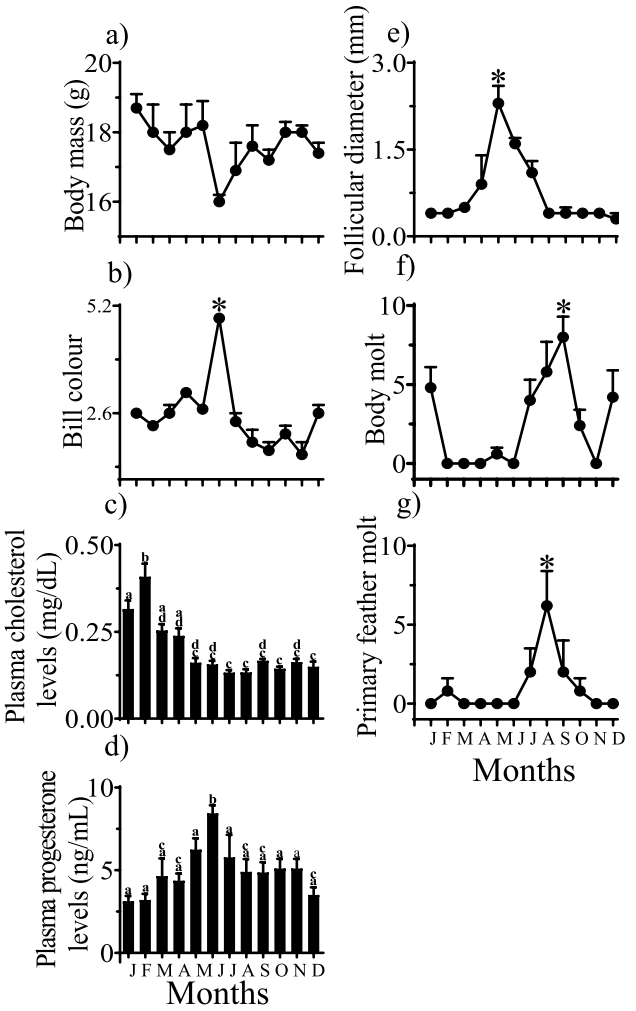


Fig. 3 Parameters: **a** body mass, **b** bill color, **c** plasma cholesterol levels, **d** plasma progesterone levels, **e** follicular diameter, **f** body molt, and **g** molt in primary flight feathers, from adult male tress sparrows (*Passer montanus*) collected every month for 12 months. * and the lowercase alphabet indicate significant differences among the months

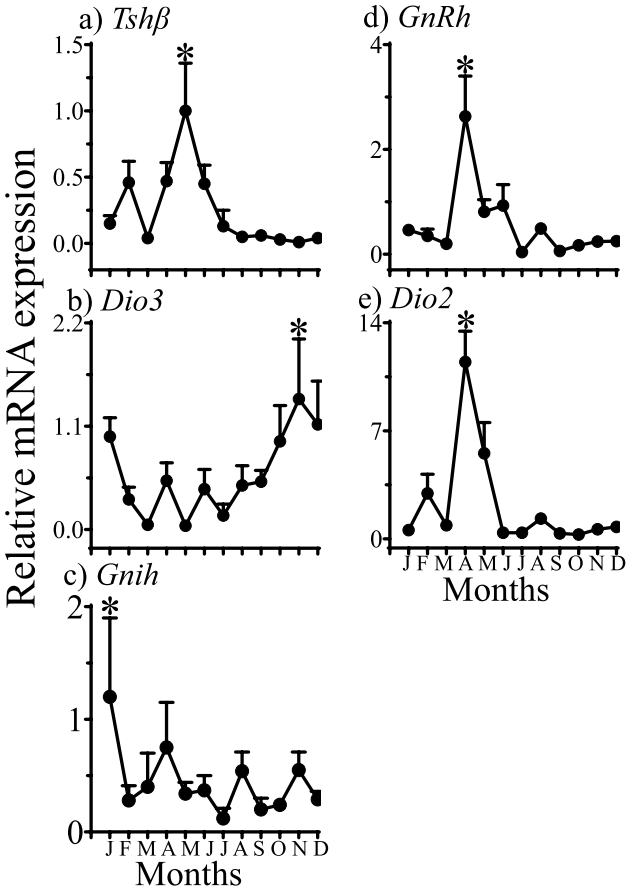


Fig. 4 Relative mRNA levels of reproductive genes (*Tshβ, Dio3, GnIh, Dio2,* and *GnRh*) in the hypothalamic tissues of adult female tree sparrow (*Passer montanus*) collected every month for a year (Fig. 4a-e). * Indicates significant differences among the months

Transcripts associated with seasonal reproduction were measured in the hypothalamus of adult female tree sparrows. All the transcripts studied had annual variation in the hypothalamic tissue; *Tshβ* ($P < 0.0001$; one-way ANOVA; $\eta^2 = 0.63$; Supplementary Table 3b; Fig. 4a), *Dio2* ($P < 0.0001$; one-way ANOVA; $\eta^2 = 0.89$; Supplementary Table 3b; Fig. 4e), *GnRh* ($P < 0.0001$; one-way ANOVA; $\eta^2 = 0.76$; Supplementary Table 3b; Fig. 4d), *Dio3* ($P = 0.0210$; one-way ANOVA; $\eta^2 = 0.42$; Supplementary Table 3b; Fig. 4b), and *GnIh* ($P = 0.0008$; one-way ANOVA; $\eta^2 = 0.30$; Supplementary Table 3b; Fig. 4c). Maximum *Tshβ* expression was observed during May ($P < 0.05$; Tukey's multiple comparison test; Fig. 4a), while the levels were minimum from June onwards ($P < 0.05$; Tukey's multiple comparison test; Fig. 4a). *Dio2* levels peaked during April and May ($P < 0.05$; Tukey's multiple comparison test; Fig. 4e). Levels were minimum during June and these low levels were maintained rest of the year ($P < 0.05$; Tukey's multiple comparison test; Fig. 4e). *GnRh* expression levels corresponded *Dio2*, and elevated mRNA levels were observed during April, subsequent fall in transcription levels during May and low levels were observed rest of the year ($P < 0.05$; Tukey's multiple comparison test; Fig. 4d). *Dio3* and *GnIh* levels were in antiphase of *Tshβ*, *Dio2*, and *GnRh* expression (Fig. 4). Higher levels of *Dio3* were observed in October till January, with maximum expression during November till January ($P < 0.05$; Tukey's multiple comparison test; Fig. 4b). Similarly, higher *GnIh* levels were observed in January during pre-breeding phase ($P < 0.05$; Tukey's multiple comparison test; Fig. 4c).

Hypothalamic expression of steroidogenic markers revealed seasonal changes (Fig. 5). qPCR analysis of these transcripts in hypothalamus revealed seasonal variations in the expression levels of *StAR* ($P < 0.0001$; one-way ANOVA; $\eta^2 = 0.79$; Supplementary Table 4;

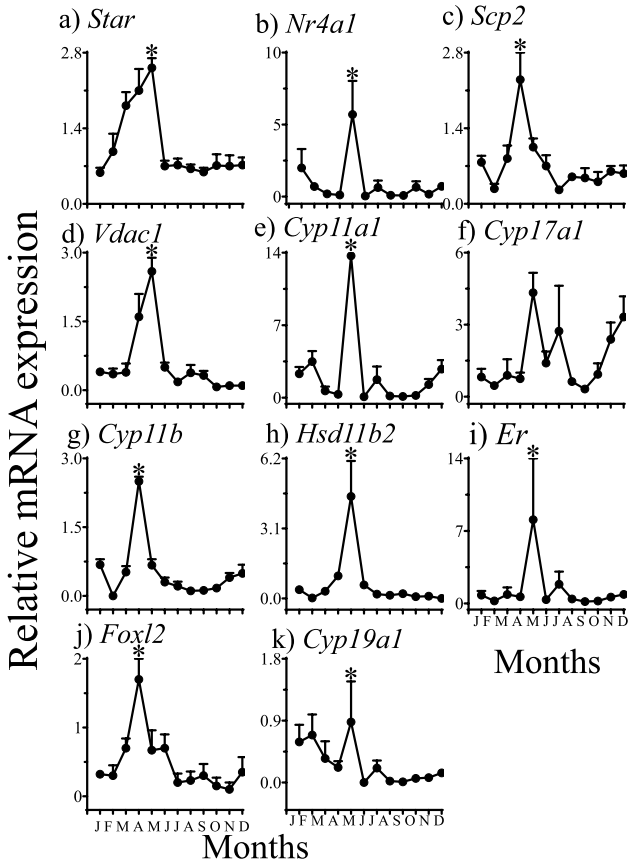


Fig. 5 Relative mRNA levels of steroidogenic transcripts (*StAR*, *Scp2*, *Cyp11a1*, *Nr4a1*, *Cyp11b*, *Cyp17a1*, *Foxl2*, *Cyp19a1*, *Hsd11b2*, *Er*, and *Vdac1*) in the hypothalamus of adult female tree sparrow (*Passer montanus*) collected every month for 12 months (Fig. 5a-k). * Indicates significant differences among the months

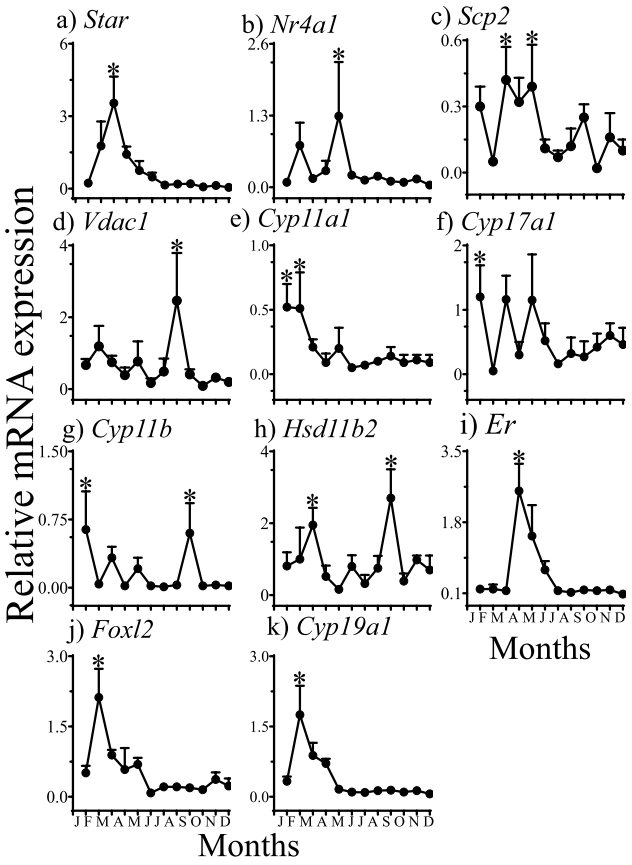


Fig. 6 Relative mRNA levels of steroidogenic transcripts (*StAR*, *Scp2*, *Cyp11a1*, *Nr4a1*, *Cyp11b*, *Cyp17a1*, *Foxl2*, *Cyp19a1*, *Hsd11b2*, *Er*, and *Vdac1*) in the ovary of adult female tree sparrow (*Passer montanus*) collected every month for 12 months (Fig. 6a-k). * Indicates significant differences among the months

Fig. 5a), *Scp2* ($P < 0.0001$; one-way ANOVA; $\eta^2 = 0.75$; Supplementary Table 4; Fig. 5c), *Cyp11a1* ($P = 0.0160$; one-way ANOVA; $\eta^2 = 0.44$; Supplementary Table 4; Fig. 5e), *Nr4a1*($P = 0.0008$; one-way ANOVA; $\eta^2 = 0.54$; Supplementary Table 4; Fig. 5b), *Cyp11b* ($P < 0.0001$; one-way ANOVA; $\eta^2 = 0.94$; Supplementary Table 4; Fig. 5g), *Cyp17a1* ($P = 0.0037$; one-way ANOVA; $\eta^2 = 0.49$; Supplementary Table 4; Fig. 5f), *Hsd11b2* ($P < 0.0001$; one-way ANOVA; $\eta^2 = 0.69$; Supplementary Table 4; Fig. 5h), *Er* ($P = 0.0003$; one-way ANOVA; $\eta^2 = 0.32$; Supplementary Table 4; Fig. 5i), *Vdac1* ($P < 0.0001$; one-way ANOVA; $\eta^2 = 0.79$; Supplementary Table 4; Fig. 5d), *Cyp19a1* ($P < 0.0001$; one-way ANOVA; $\eta^2 = 0.34$; Supplementary Table 4; Fig. 5k), and *Foxl2* ($P < 0.0001$; one-way ANOVA; $\eta^2 = 0.68$; Supplementary Table 4; Fig. 5j). Maximum expression levels of *StAR* were observed in the month of May and *Scp2* transcripts were observed in the month of April ($P < 0.05$; Tukey's multiple comparison test; Fig. 5a, c), while levels were low at other months of the year ($P > 0.05$; Tukey's multiple comparison test; Fig. 5a, c). Higher expression of *Cyp11a1* was observed during the breeding phase, i.e., in May while levels were low at other times of the year ($P < 0.05$; Tukey's multiple comparison tests; Fig. 5e). Expression levels of *Cyp11b* and *Foxl2* peaked during April, while levels were low during the other times of the year ($P < 0.05$; Tukey's multiple comparison tests; Fig. 5g, j). In the hypothalamus, peak expression of *Nr4a1, Hsd11b2, Vdac1,* and *er* was observed in May ($P < 0.05$; Tukey's multiple comparison test; Fig. 5b, h,d,i). Several peaks in expression levels of *Cyp17a1* were detected in May, July, November, and December with the highest expression level in May (Fig. 5f). However, the amplitude of peaks was lower during the pre-breeding phase ($P > 0.05$; Tukey's multiple comparison tests; Fig. 5f). Transcript levels of *Cyp19a1* were observed during the pre-breeding phase in January and February, showing peaked expression in May in the breeding phase and lower levels in the rest of the year. ($P < 0.05$; Tukey's multiple comparison test; Fig. 5k).

Seasonal variation in the ovarian transcript expression levels of *StAR* ($P < 0.0001$; one-way ANOVA; $\eta^2 = 0.60$;

Supplementary Table 4; Fig. 6a), *Scp2* ($P < 0.0001$; one-way ANOVA; $\eta^2 = 0.39$; Supplementary Table 4; Fig. 6c), *Cyp11a1* ($P < 0.0001$; one-way ANOVA; $\eta^2 = 0.37$; Supplementary Table 4; Fig. 6e), *Nr4a1* ($P = 0.0012$; one-way ANOVA; $\eta^2 =$ Supplementary Table 4; Fig. 6b), *Cyp11b* ($P < 0.0001$; one-way ANOVA; $\eta^2 =$ Supplementary Table 4; Fig. 6g), *Cyp17a1* ($P = 0.0011$; one-way ANOVA; $\eta^2 =$ Supplementary Table 4; Fig. 6f), *Hsd11b2* ($P < 0.0001$; one-way ANOVA; $\eta^2 =$ Supplementary Table 4; Fig. 6h), *Cyp19a1* ($P < 0.0001$; one-way ANOVA; $\eta^2 =$ Supplementary Table 4; Fig. 6k), *Foxl2* ($P < 0.0001$; one-way ANOVA; $\eta^2 =$ Supplementary Table 4; Fig. 6j), *Er* ($P < 0.0001$; one-way ANOVA; $\eta^2 =$ Supplementary Table 4; Fig. 6i), and *Vdac1* ($P < 0.0001$; one-way ANOVA; $\eta^2 =$ Supplementary Table 4; Fig. 6d) was observed. In ovary, *StAR* transcript levels peaked in March ($P < 0.05$; Tukey's multiple comparison test; Fig. 6a). *Nr4a1* levels were highest in May during breeding phase ($P < 0.05$; Tukey's multiple comparison test; Fig. 6b). *Er* peaked during April and subsequent fall from June onwards (Fig. 6i). *Hsd11b2* levels peaked in March (pre-breeding season) and September during post-breeding (Fig. 6i). Expression levels of *Cyp11a1* peak during January and February during the pre-breeding phase, and lower in the rest of the year ($P < 0.05$; Tukey's multiple comparison tests; Fig. 6e). *Cyp19a1* and *Foxl2* peaked during February ($P < 0.05$; Tukey's multiple comparison tests; Fig. 6j, k), while *Cyp17a1* and *Scp2* show multiple peaks during breeding phase during January, March, April, and May ($P < 0.05$; Tukey's multiple comparison tests; Fig. 6c, f). *Cyp11b* transcript shows peaked expression in the pre- and post-breeding season, i.e., January and September ($P < 0.05$; Tukey's multiple comparison tests; Fig. 6c, f). However, the *Vdac1* expression level was high in the post-breeding phase in August ($P < 0.05$; Tukey's multiple comparison tests; Fig. 6d).

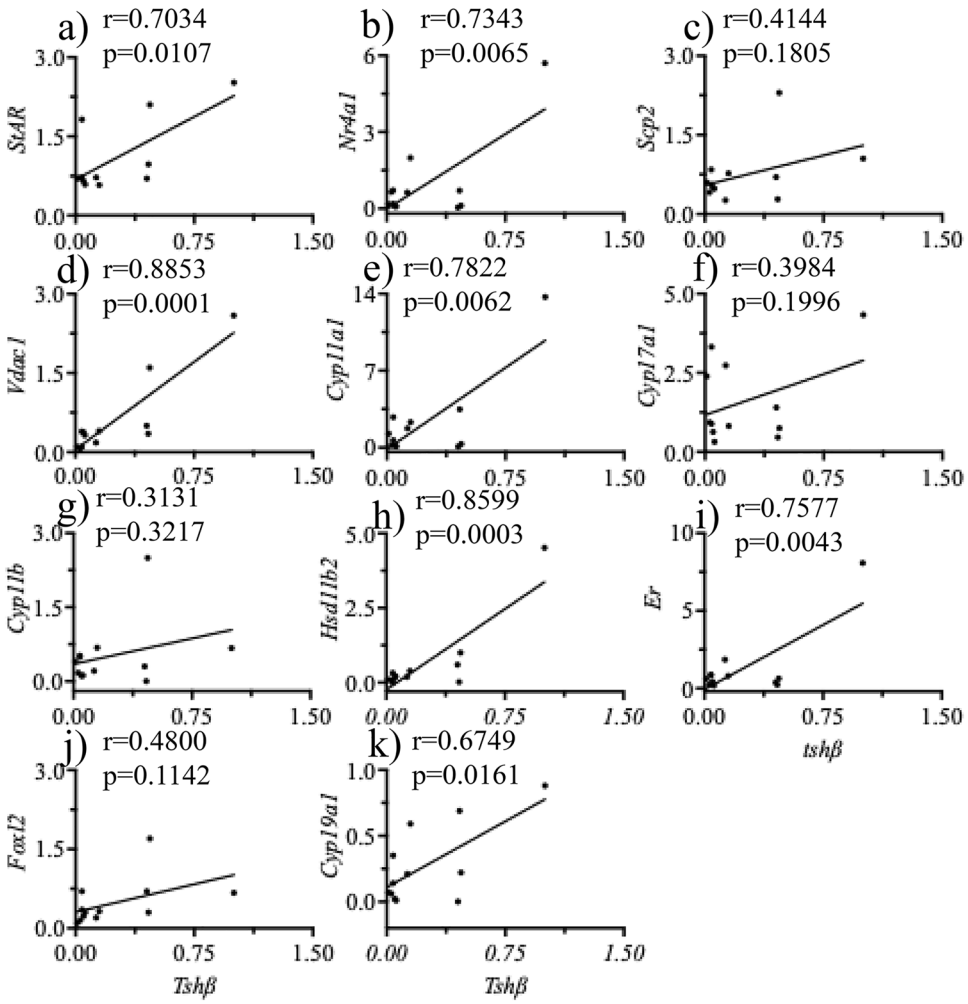


Fig. 7 Correlation of hypothalamic *Tshβ* expression with hypothalamic expression of *StAR*, *Scp2*, *Cyp11a1*, *Nr4a1*, *Cyp11b*, *Cyp17a1*, *Foxl2*, *Cyp19a1*, *Hsd11b2*, *Er*, and *Vdac1*

3.2.1 Annual reproductive transcript level correlation with steroidogenic markers in the hypothalamus and ovary

A correlation of reproductive gene transcript with steroidogenic markers was observed. Significant positive correlation of *Tshβ* and *StAR* transcripts in the hypothalamus was observed ($r = 0.7034$, $P = 0.0107$; Fig. 7a), whereas no significant differences were observed in mRNA expression levels in the ovary (r = 0.1477, $P = 0.6468$; Fig. 8a). A positive correlation of *Tshβ* and *Nr4a1* in the hypothalamus (r = 0.7343, $P = 0.0065$; Fig. 7b) and ovary (r = 0.9008, $P < 0.0001$; Fig. 8b), *Tshβ* and *Er* in the hypothalamus ($r = 0.7577$, $P = 0.0043$; Fig. 7i) and ovary ($r = 0.6724$, $P = 0.0166$; Fig. 8i) was observed. A significant correlation of *Tshβ* with *Hsd11b2* (r = 0.8599, $P = 0.0003$; Fig. 7h), *Vdac1*($r = 0.8853$, $P = 0.0001$; Fig. 7d), *Cyp11a1*($r = 0.7822$, $P = 0.0062$; Fig. 7e), and *Cyp19a1*($r = 0.6749$, $P = 0.0161$; Fig. 7k) in the hypothalamus was observed, whereas no significant correlations were observed for *Tshβ* with *Scp2* (r = 0.4144, $P = 0.1805$; Fig. 7c), *Foxl2*(r = 0.4800, $P = 0.1142$; Fig. 7j), *Cyp17a1* ($r = 0.3984$, $P = 0.1996$; Fig. 7f), *Cyp11a1*($r = 0.7822$, $P = 0.0062$; Fig. 7e), and *Cyp19a1*($r = 0.6749$, $P = 0.0161$; Fig. 7k) in the hypothalamus and *Tshβ* with *Scp2*($r = 0.3406$, $P = 0.2787$; Fig. 8c), *Vdac1*($r = 0.01309$, $P = 0.9678$; Fig. 8d), *Foxl2*($r = 0.3522$, $P = 0.2616$; Fig. 8j), *Cyp17a1*($r = 0.2055$, $P = 0.5218$; Fig. 8f), *Cyp11a1*(r = 0.1688, $P = 0.6000$; Fig. 8e), and *Cyp19a1*($r = 0.2151$, $P = 0.5020$; Fig. 8k). Further, there were significant negative correlations of *Tshβ* with *Hsd11b2* ($r = -0.4011$, $P = 0.1963$; Fig. 8h) and *Cyp11b* ($r = -0.1008$, $P = 0.7552$; Fig. 8g).

A significant positive correlation in the hypothalamus was observed for reproductive gene *Dio2* with *StAR* ($r = 0.7487$, $P = 0.0051$; Fig. 9a), *Scp2* ($r = 0.8906$, $P = 0.0001$; Fig. 9c), *Foxl2* ($r = 0.8650$, $P = 0.0003$; Fig. 9j), *Vdac1* ($r = 0.7488$, $P = 0.0051$; Fig. 9d), and *Cyp11b* ($r = 0.8739$, $P = 0.0002$; Fig. 9g), whereas no significant correlation was observed between *Dio2* with *Hsd11b2* ($r = 0.4719$, $P = 0.1214$; Fig. 9h), *Nr4a1* ($r = 0.2388$, $P = 0.4549$; Fig. 9b), *Er* ($r = 0.3052$, $P = 0.3347$; Fig. 9i), *Cyp17a1* ($r = 0.03657$, $P = 0.9102$; Fig. 9f), *Cyp11a1* (r = 0.2798, $P = 0.3784$;

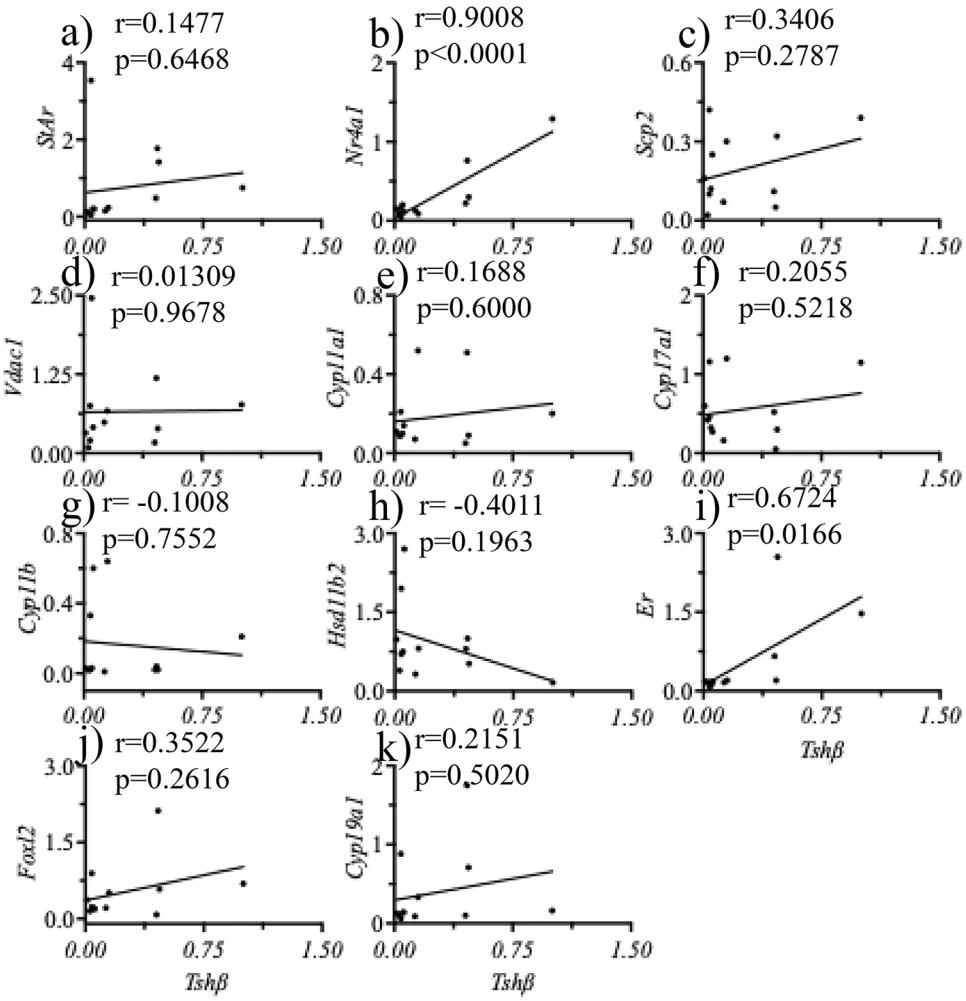


Fig. 8 Correlation of hypothalamic *Tshβ* expression with ovarian expression of *StAR*, *Scp2*, *Cyp11a1*, *Nr4a1*, *Cyp11b*, *Cyp17a1*, *Foxl2*, *Cyp19a1*, *Hsd11b2*, *Er*, and *Vdac1*

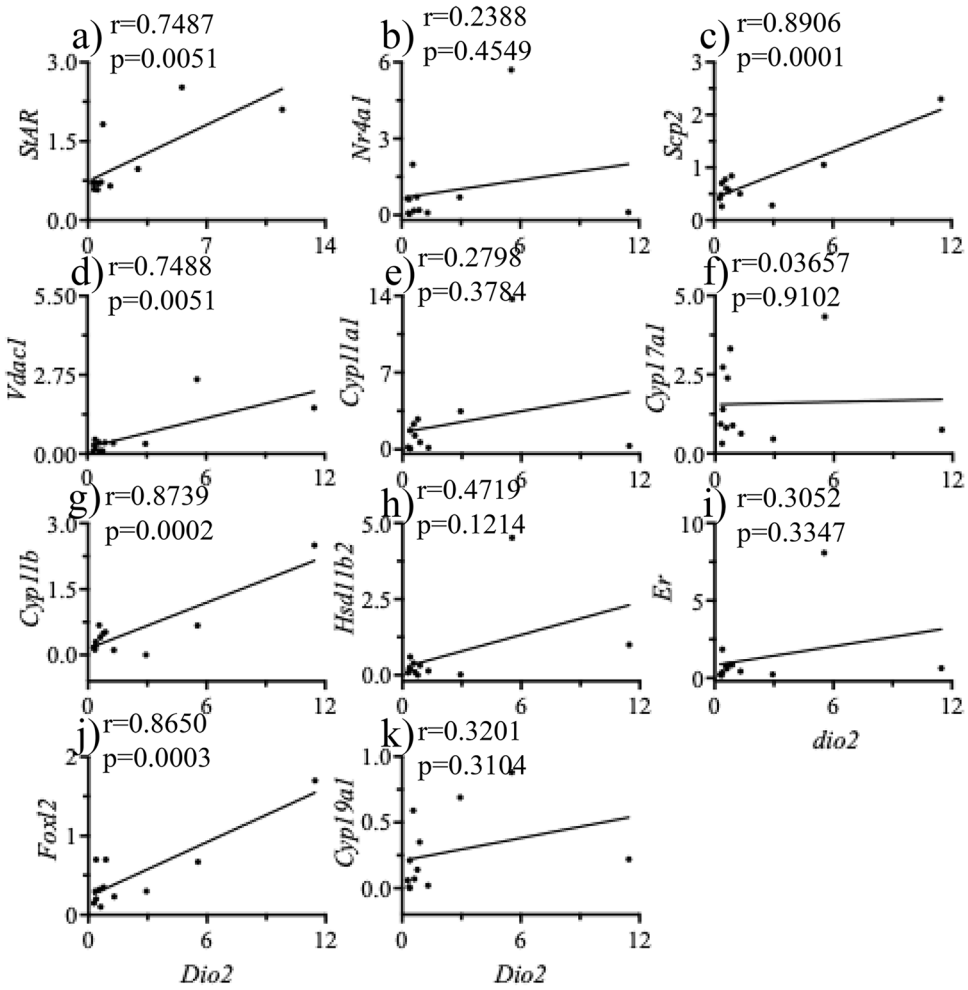


Fig. 9 Correlation of hypothalamic *Dio2* expression with hypothalamic expression of *StAR*, *Scp2*, *Cyp11a1*, *Nr4a1*, *Cyp11b*, *Cyp17a1*, *Foxl2*, *Cyp19a1*, *Hsd11b2*, *Er*, and *Vdac1*

Fig. 9e), and *Cyp19a1* ($r=0.3201$, $P=0.3104$; Fig. 9k). Besides, a significant negative correlation between *Dio2* gene expression with steroidogenic markers *Hsd11b2* ($r=-0.3028$, $P=0.3388$; Fig. 10h), *Cyp11b* ($r=-0.2019$, $P=0.5292$; Fig. 10g), *Cyp17a1* ($r=-0.05247$, $P=0.8713$; Fig. 10f), and *Cyp11a1* ($r=-0.02128$, $P=0.9477$; Fig. 10e) was observed in the ovary. However, no significant correlation was observed for *StAR* ($r=0.2767$, $P=0.3840$; Fig. 10a), *Scp2*($r=0.4188$, $P=0.1755$; Fig. 10c), *Foxl2* ($r=0.2658$, $P=0.4037$; Fig. 10j), *Vdac1* ($r=0.01986$, $P=0.9512$; Fig. 10d), *Nr4a1* ($r=0.4559$, $P=0.1364$; Fig. 10b), and *Cyp19a1* ($r=0.3172$, $P=0.3151$; Fig. 10k).

A significant negative correlation was observed in the hypothalamus between *Dio3* and *StAR* ($r=-0.5366$, $P=0.0720$; Fig. 11a), *Scp2* ($r=-0.09695$, $P=0.7644$; Fig. 11c), *Foxl2* ($r=-0.3219$, $P=0.3085$; Fig. 11j), *Hsd11b2* ($r=-0.4271$, $P=0.1662$; Fig. 11h), *Vdac1* ($r=-0.4607$, $P=0.1317$; Fig. 11d), *Nr4a1* ($r=-0.2671$, $P=0.4012$; Fig. 11b), *Er* ($r=-0.4177$, $P=0.1767$; Fig. 11i), *Cyp17a1* (r=-0.00047, $P=0.9988$; Fig. 11f), *Cyp11a1* ($r=-0.3201$, $P=0.3104$; Fig. 11e), and *Cyp19a1* ($r=-0.3795$, $P=0.2237$; Fig. 11k). Similarly, in the ovary, *Dio3* shows a significant negative correlation with all the steroidogenic markers *StAR* ($r=-0.5425$, $P=0.0684$; Fig. 12a), *Scp2* ($r=-0.3360$, $P=0.2856$; Fig. 12c), *Foxl2* ($r=-0.3200$, $P=0.3105$; Fig. 12j), *Hsd11b2* ($r=-0.07713$, $P=0.8117$; Fig. 12h), *Vdac1* ($r=-0.3168$, $P=0.3157$; Fig. 12d), *Cyp11b* (r=-0.03794, $P=0.9068$; Fig. 12g), *Nr4a1* (r=-0.5073, $P=0.0923$; Fig. 12b), *Er* ($r=-0.2574$, $P=0.4193$; Fig. 12i), *Cyp17a1* (r=-0.03795, $P=0.9068$; Fig. 12f), *Cyp11a1* ($r=-0.03431$, $P=0.09157$; Fig. 12e) and *Cyp19a1* ($r=-0.3313$, $P=0.2928$; Fig. 12k). However, no significant correlation in the hypothalamus was observed for *Cyp11b* ($r=0.00025$, $P=0.9994$; Fig. 12g).

In the hypothalamus, a significant correlation was observed between *GnRh* steroidogenic markers *Scp2* ($r=0.9336$, $P<0.0001$; Fig. 13c), *Foxl2* ($r=0.9248$, $P<0.0001$; Fig. 13j), *Vdac1* ($r=0.6095$, $P=0.0354$; Fig. 13d), *Cyp11b* ($r=0.9107$, $P<0.0001$; Fig. 13g), and a negative correlation was observed with *Cyp17a1* ($r=-0.09715$, $P=0.7639$; Fig. 13f). Whereas, no significant correlation was observed of *GnRh* with *Hsd11b2*

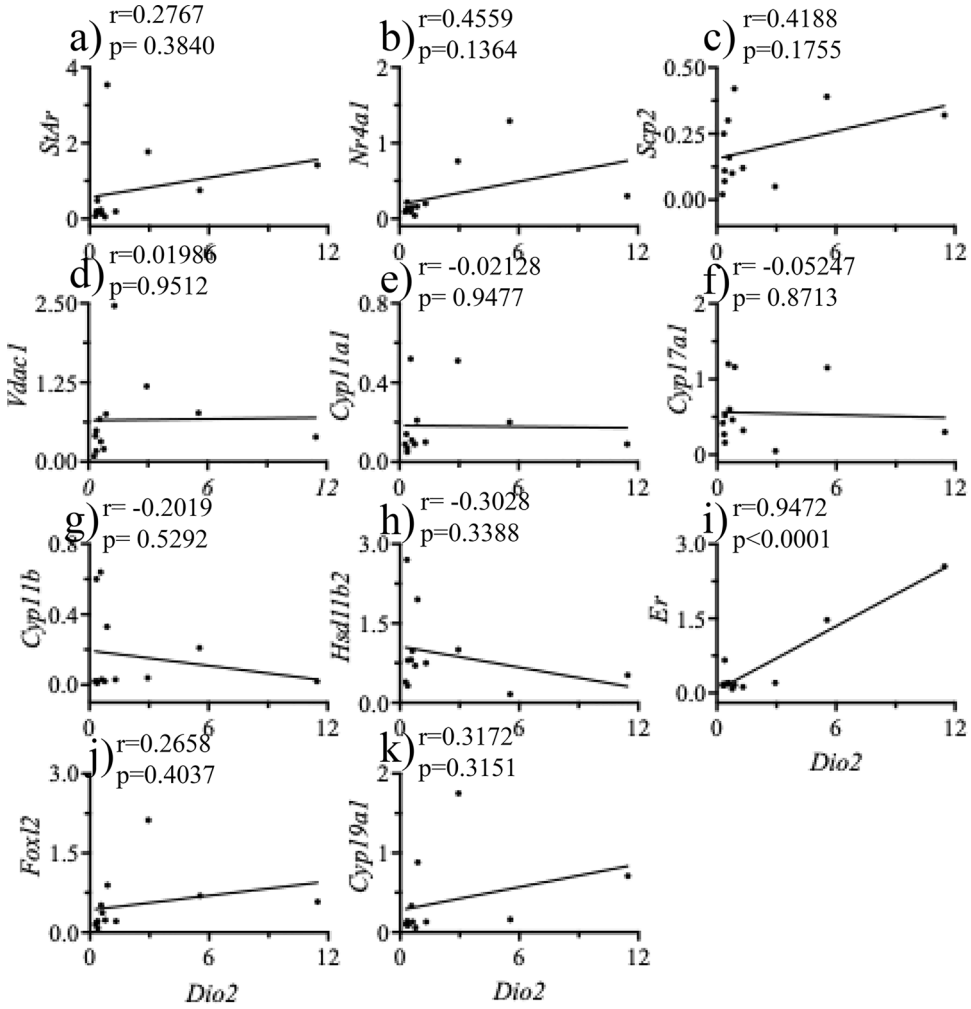


Fig. 10 Correlation of hypothalamic *Dio2* expression with ovarian expression of *StAR*, *Scp2*, *Cyp11a1*, *Nr4a1*, *Cyp11b*, *Cyp17a1*, *Foxl2*, *Cyp19a1*, *Hsd11b2*, *Er*, and *Vdac1*

($r = 0.3100$, $P = 0.3267$; Fig. 13h), *Nr4a1* ($r = 0.03475$, $P = 0.9146$; Fig. 13b), *Er* ($r = 0.08865$, $P = 0.7841$; Fig. 13i), *Cyp11a1* ($r = 0.02749$, $P = 0.9324$; Fig. 13e), and *Cyp19a1* ($r = 0.07125$, $P = 0.8258$; Fig. 13k). In the ovary, a negative correlation was observed with *Hsd11b2* ($r = -0.3106$, $P = 0.3258$; Fig. 14h), *Vdac1* ($r = -0.05871$, $P = 0.8562$; Fig. 14d), *Cyp11b* (r = -0.2218, $P = 0.4883$; Fig. 14g), *Cyp17a1* (r = -0.04351, $P = 0.8932$; Fig. 14f), *Cyp11a1* ($r = -0.1377$, $P = 0.6696$; Fig. 14e), and a positive correlation with *Er* (r = 0.9350, $P < 0.0001$; Fig. 14i). However, no correlation was observed with *StAR* (r = 0.1755, $P = 0.5854$; Fig. 14a), *Scp2* (r = 0.3407, $P = 0.2784$; Fig. 14c), *Foxl2* (r = 0.03780, $P = 0.9072$; Fig. 14j), *Nr4a1* ($r = 0.2077$, $P = 0.5171$; Fig. 14b), and *Cyp19a1* ($r = 0.1617$, $P = 0.6155$; Fig. 14k) in the ovary.

A negative correlation was observed in the hypothalamus between *GnIh* with steroidogenic markers *Hsd11b2* ($r = -0.001419$, $P = 0.9965$; Fig. 15h), *Er* ($r = -0.1125$, $P = 0.7279$; Fig. 15i), *Cyp17a1* ($r = -0.2592$, $P = 0.4159$; Fig. 15f), *Cyp11a1* ($r = -0.08023$, $P = 0.8042$; Fig. 15e), and in the ovary with *StAR* ($r = -0.00076$, $P = 0.9981$; Fig. 16a), *Hsd11b2* ($r = -0.1211$, $P = 0.7078$; Fig. 16h), and *Nr4a1* ($r = -0.1401$, $P = 0.6641$; Fig. 16b). However, no correlation was observed with *StAR* ($r = 0.03767$, $P = 0.9075$; Fig. 15a), *Scp2* ($r = 0.4613$, $P = 0.1311$; Fig. 15c), *Foxl2* ($r = 0.2757$, $P = 0.3857$; Fig. 15j), *Vdac1* ($r = 0.1312$, $P = 0.6845$; Fig. 15d), *Cyp11b* ($r = 0.4794$, $P = 0.1148$; Fig. 15g), *Nr4a1* ($r = 0.08162$, $P = 0.8009$; Fig. 15b), *Cyp19a1* (r = 0.2192, $P = 0.4936$; Fig. 15k) in the hypothalamus and *Scp2* ($r = 0.4108$, $P = 0.1846$; Fig. 16c), *Foxl2* ($r = 0.001315$, $P = 0.9968$; Fig. 16j), *Vdac1* ($r = 0.1422$, $P = 0.6593$; Fig. 16d), *Cyp11b* ($r = 0.3973$, $P = 0.2009$; Fig. 16g), *Er* ($r = 0.2472$, $P = 0.4385$; Fig. 16i), *Cyp17a1* ($r = 0.4806$, $P = 0.1137$; Fig. 16f) and *Cyp19a1* (r = 0.04174, $P = 0.8975$; Fig. 16k) in the ovary.

4 Discussion

The study reveals 24-h and seasonal profiling of the steroidogenic genes coding for *StAR, Nr4a1, er, Scp2, Cyp17a1, Cyp11a1, Hsd11b2, Cyp11b, Foxl2, Cyp19a1*,

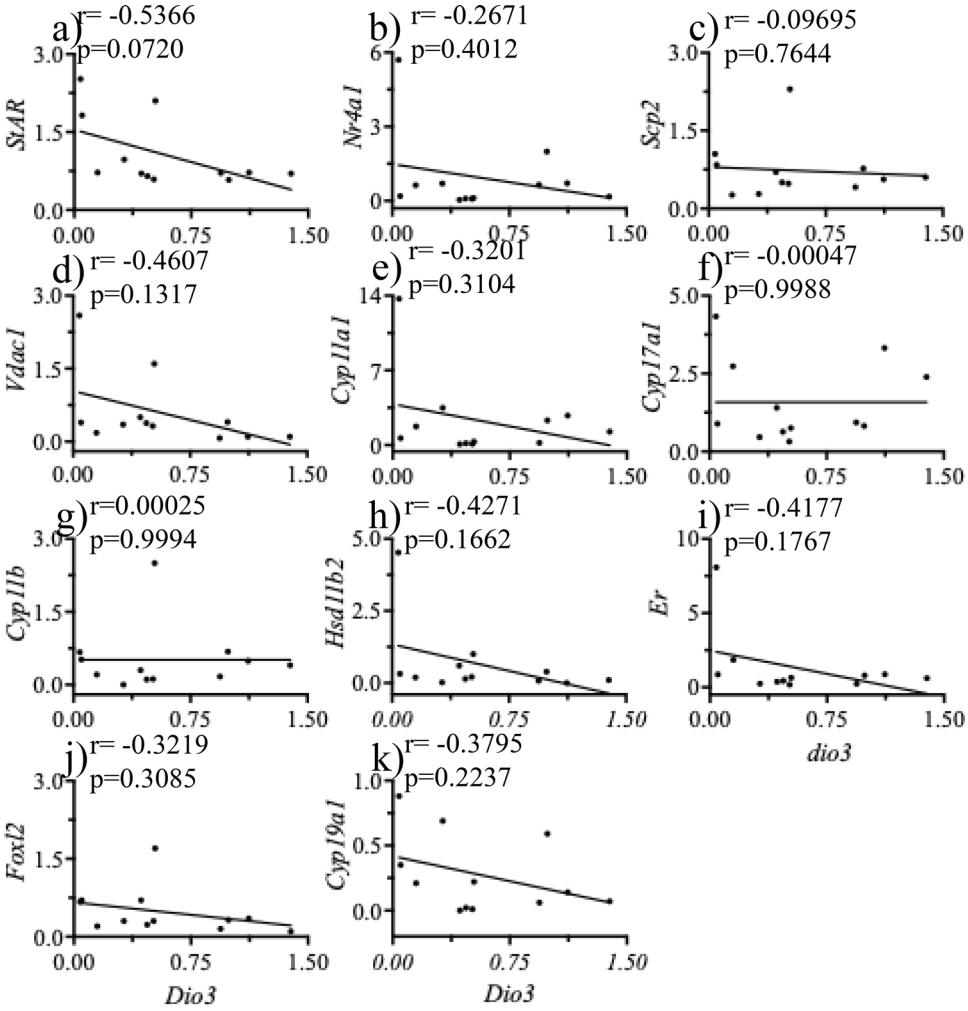


Fig. 11 Correlation of hypothalamic *Dio3* expression with hypothalamic expression of *StAR*, *Scp2*, *Cyp11a1*, *Nr4a1*, *Cyp11b*, *Cyp17a1*, *Foxl2*, *Cyp19a1*, *Hsd11b2*, *Er*, and *Vdac1*

and *Vdac1* enzymes in the hypothalamus and ovary of an adult female tree sparrows. Experiment one examines the transcript levels of some enzymes involved in the steroidogenesis pathway in both the hypothalamus and the ovary of the adult tree sparrows. Limited studies have revealed the daily expression of steroidogenic enzymes in birds and other species [19–21, 36], StAR enzymes play a pivotal role in 24-h rhythmicity with their acrophases revealing peaked expression at ZT9, during the light switched-on period, indicating the secretion of the maximum enzyme during the light phase which is also evident in male tree sparrows in a study reported by [21]. Likewise, these steroidogenic markers presented significant 24-h rhythms with their peak activity occurring during the early part of the daylight period (ZT1, ZT5, ZT9, and ZT13 h, respectively) which is also constant with Nile tilapia (*Oreochromis niloticus*) [19], Zebrafish (*Danio rerio*) [36] and Tree sparrow (*Passer montanus*) [21]. In the current investigation, nearly all the steroidogenic genes (*StAR, Nr4a1, Scp2, Vdac1, Cyp11a1, Cyp17a1, Cyp11b, Hsd11b2, Er, Cyp19a1,* and *Foxl2*) showed light-dependent expression. They peaked at the first half of the light phase which is associated with the recent study conducted on male Tree sparrows (*Passer montanus*), and also in other species like Zebrafish (*Danio rerio*) [21, 36].

In the brain, *StAR* and *Nr4a1* expression showed statistically significant differences and was expressed during the initial part of the light phase. Although, expression levels *of Scp2, Vdac1, Cyp11a1, Cyp17a1, Cyp11b, Hsd11b2, Er, Cyp19a1*, and *Foxl2* were high after the sunset, i.e., dark phase which is similar to that of male tree sparrow [21]. Cyp19a1 which helps in the biosynthesis of estrogens and is a vital factor in sexual development and Foxl2 and Steroidogenic Factor-1 (SF-1) interact in granulosa cells to regulate the transcription of *Cyp17* and *Cyp19* genes are present in ovaries. Their expression adheres to a rhythmic pattern; hence, the peak expression level of gonadal aromatase occurs during the light phase. However, in this study, expression of *Cyp19a1* was observed highest during the dark phase in the hypothalamus. While the role of aromatase in the gonads has been extensively studied, many questions

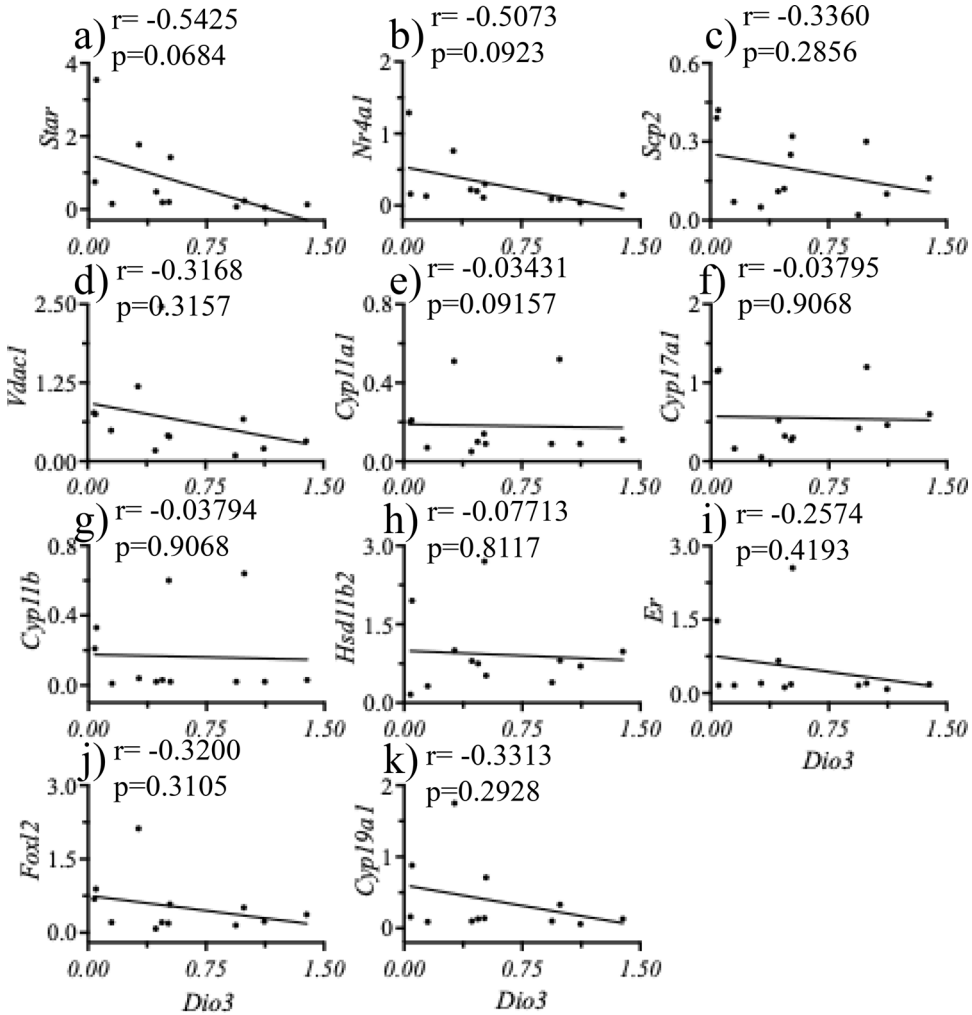


Fig. 12 Correlation of hypothalamic *Dio3* expression with ovarian expression of *StAR*, *Scp2*, *Cyp11a1*, *Nr4a1*, *Cyp11b*, *Cyp17a1*, *Foxl2*, *Cyp19a1*, *Hsd11b2*, *Er*, and *Vdac1*

regarding its function in the brain remain unanswered. There is a piece of evidence that aromatase plays a role in the control of reproduction by the brain [37] and shows different activities depending on the sex and life stage. Further, hormonal rhythmicity is connected directly to rhythms of biological and behavioral processes, such as reproduction and spawning, which usually accord with the phase of the LD cycle that shows the greatest locomotor activity [36]. Also, these higher expressions of transcripts during the middle of the day could result from the availability of a post-feeding cholesterol substrate. In Yangzhou Goose (*Anser cygnoides*), the ovarian preovulatory granulosa cells have rhythmic expression patterns for both progesterone synthesis and circadian clock genes, and *bmal1* controls the transcription of the *StAR* [38]. Similarly, gonads and brain tissues of zebrafish (*Danio rerio*) show daily fluctuations in the expression of steroidogenic genes throughout a 24-h cycle [36]. Daily changes were also reported in the brain tissue for *Cyp19a1b* and *Cyp11b*, while the gonads exhibited rhythmic expression of aromatase (Cyp19a1a), anti-Müllerian hormone (amh), *Foxl2*, and *Dmrt1* [39].

The tree sparrow is a resident bird and does not store much fat, unlike migratory birds which use the fat as stored food and fuel for migration and, hence, have restricted annual variations in body mass. In the present study, slight variations in the body mass of female tree sparrows were observed annually (Fig. 3a) and are consistent with previous findings [21, 25, 27]. Tree sparrows exhibited seasonal variations in secondary sexual traits including bill color and molting, as well as primary sexual traits like gonadal growth [27]. This study shows reproductive phase-dependent variations in the bill color; a darker bill color was observed during the breeding phase (Fig. 3b). A recent study on male tree sparrows [21] also found that the gonadal size is correlated with bill coloring. Low plasma progesterone levels and small follicular sizes are recorded throughout the post-breeding season and vice-versa; and when the bill color is at its darkest, the follicular size also significantly increases during the breeding season [40]. A gonadal size-dependent

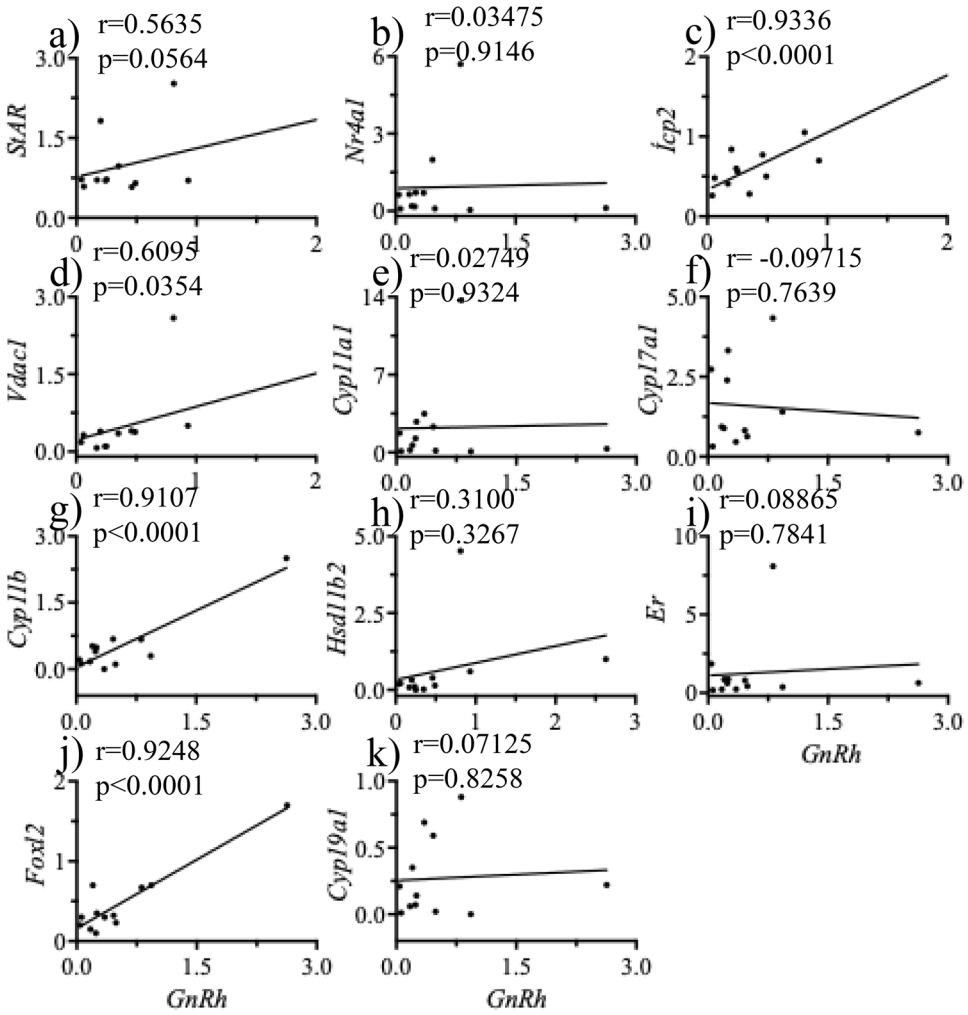


Fig. 13 Correlation of hypothalamic *GnRh* expression with hypothalamic expression of *StAR*, *Scp2*, *Cyp11a1*, *Nr4a1*, *Cyp11b*, *Cyp17a1*, *Foxl2*, *Cyp19a1*, *Hsd11b2*, *Er*, and *Vdac1*

change in bill color in seasonally breeding avian species house sparrow (*Passer domesticus*) has also been shown [25]. Seasonally breeding birds do not retain fully grown gonadal growth during the non-breeding period. Meanwhile, our results are consistent with these findings (Fig. 3e). Several-fold changes in follicular size from April to July were recorded (Fig. 3e), which is the breeding time for this species at this latitude [30]. Seasonally breeding birds show a marked shift in gonadal size from the non-breeding phase to the breeding phase, correlated with increased gonadal activity. Furthermore, increased gonadal activity in tree sparrows corresponds to increased steroidogenesis-linked reproductive activity observed in male tree sparrows and the study shows the highest plasma cholesterol level (February) before the higher gonadal activity (April onwards) which is also parallel with the plasma cholesterol level in male tree sparrows, thus validating the role of cholesterol in the steroidogenic enzymes' biosynthesis [21]. In females, during the process of ovulation, progesterone plays a vital role. Therefore, elevated progesterone in pre-breeding and breeding seasons may help the coming nesting and egg-laying behaviors [41]. The highest plasma progesterone level was observed during June (reproductive phase), which corresponds with the larger follicular size and higher reproductive activities and is consistent with reports in other species such as olive ridley sea turtle (*Lepidochelys olivacea*), Jinding duck (*Anas platyrhynchos*) [41, 42]. In this study, maximum molt in body and primary flight feathers was observed during the post-breeding phase, i.e., August and September, and is consistent with reports on this and other seasonally breeding avian species [21, 25].

Higher mRNA expression levels of *Tshβ*, *Dio2*, and *GnRh* in the hypothalamus during the active reproductive phase are consistent with the existing reports of tree sparrows and other seasonally breeding avian species [21, 30, 43]. These results reaffirm these transcripts' critical function in controlling seasonal reproduction in animals that breed seasonally [43–45]. Moreover, a decrease in the transcription levels of *Dio3* during the reproductive phase and an increase in these transcripts during the post-reproductive phase (October to December) reconfirm the involvement of the product of these transcripts in inhibiting

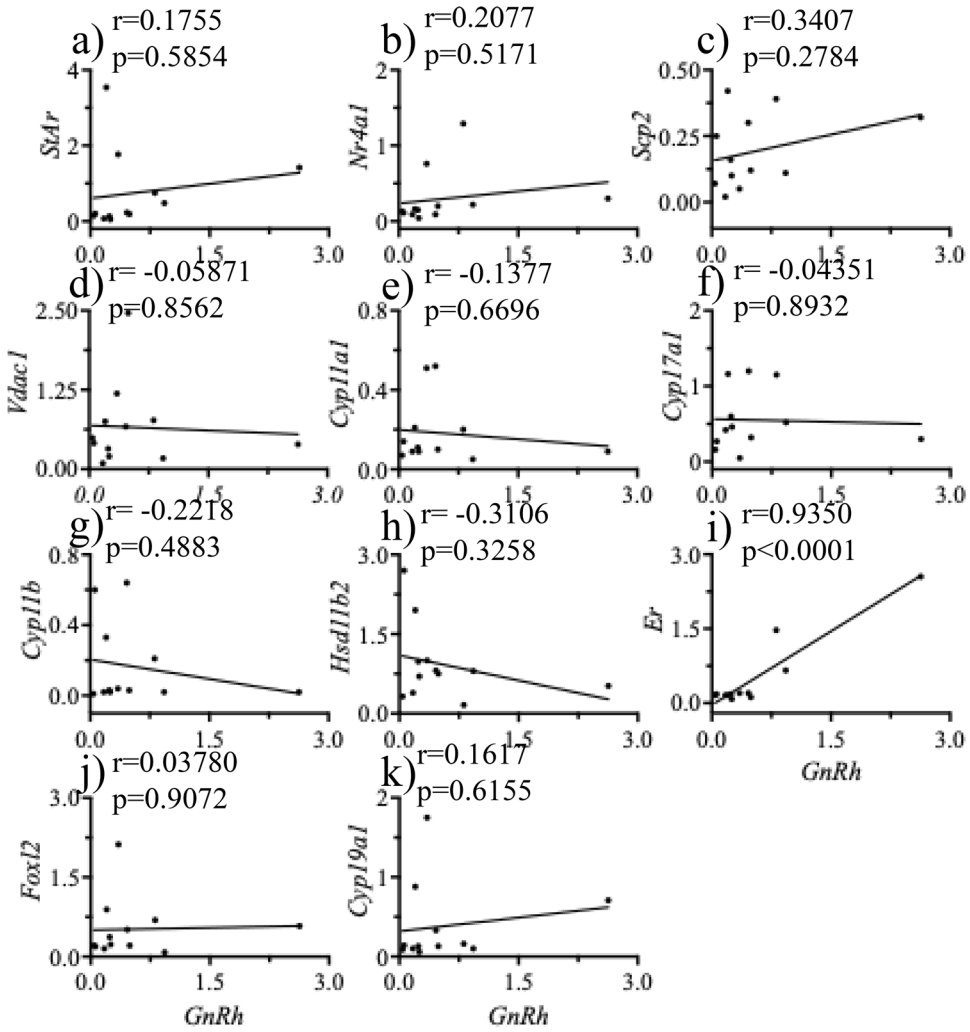


Fig. 14 Correlation of hypothalamic *GnRh* expression with ovarian expression of *StAR*, *Scp2*, *Cyp11a1*, *Nr4a1*, *Cyp11b*, *Cyp17a1*, *Foxl2*, *Cyp19a1*, *Hsd11b2*, *Er*, and *Vdac1*

reproduction (Fig. 4). However, *GnIh* transcript showed multiple fluctuations in expression and the highest peak was observed during post-breeding phase. (Fig. 4). These results are consistent with previous reports on the repressive nature of these transcripts on gonadal activity [43–50].

A recent study on Japanese quail (*Coturnix japonica*) suggests a different pattern in the timing of seasonal reproduction which can be controlled by other environmental such as temperature and photoperiod-independent control of expression follicle-stimulating hormone-β (*Fshβ*). The findings indicate that during the artificial spring equinox, surges in prolactin, and thyrotropin-stimulating hormone-β, in addition, testicular development occurs before photoinduced increases in *Fshβ* expression. Furthermore, our findings are consistent with those of another long-day breeding species Japanese quail. The study demonstrates that gonadal development is caused by short photoperiods (less than 12 h of light per day) and that vimentin immunoreactivity and *Dio3* expression are significantly upregulated in the median eminence after prolonged exposure to short photoperiods [51]. Our results support it, and we observed increased *Dio3* mRNA expression from July through January, during the breeding season (Fig. 4b). The existing literature suggests that day lengths that exceed 12 h increase the thyrotrope production and start a series of biochemical reactions in the MBH that enable *GnRh* to trigger gonadotropes to release *Fshβ*, to start the process of gonadal development. Nonetheless, our investigation identified a higher level of *Tshβ* expression throughout the breeding period.

To evaluate the steroidogenic pathways that govern the seasonal reproductive cycle, we have investigated the expression of transcripts of enzymes (*StAR, Er, Cyp17a1, Nr4a1, Scp2, Cyp11a1, Hsd11b2, Vdac1, Cyp11b, Cyp19a1,* and *Foxl2*) as well as circulating progesterone titers throughout the year (Figs. 3–6). Neurons and glial cells of the brain can locally synthesize biologically active steroids de novo [52–54]. This study shows the expression of these transcripts in the hypothalamic tissues with annual variations (Fig. 5). The yearly reproductive stage

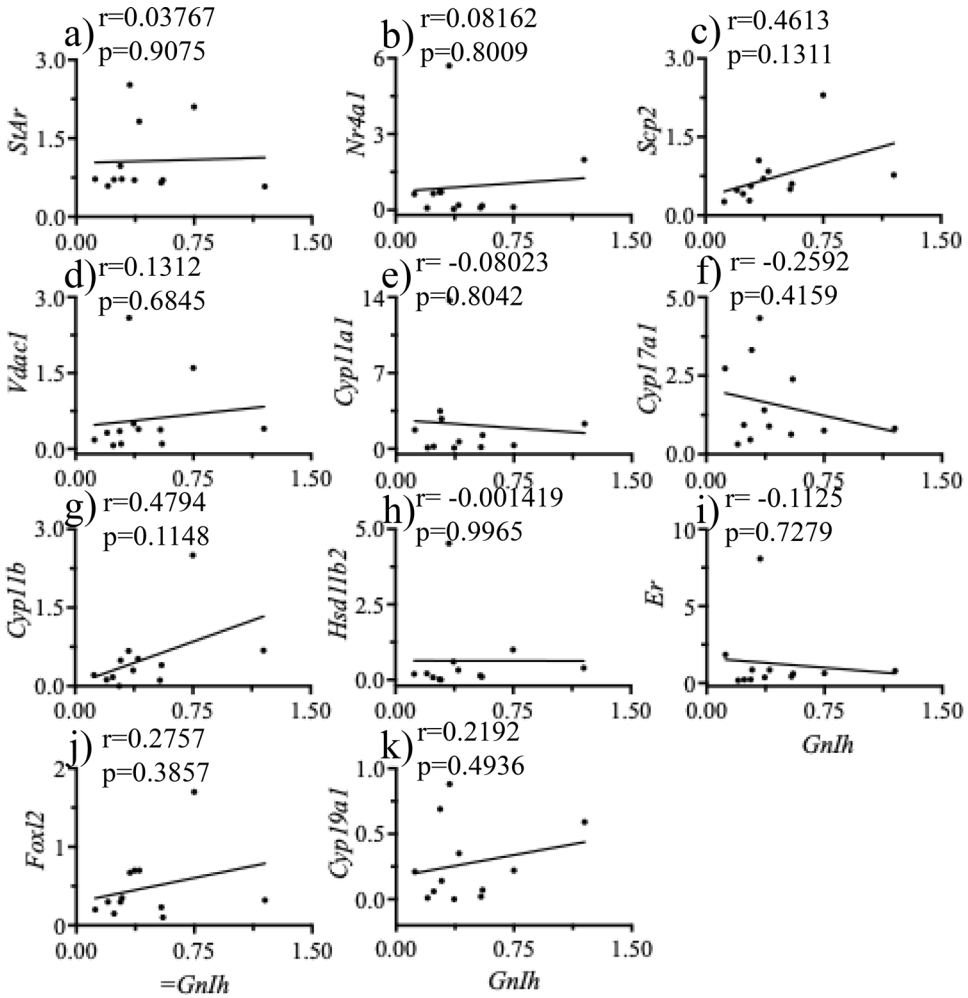


Fig. 15 Correlation of hypothalamic *GnIh* expression with hypothalamic expression of *StAR*, *Scp2*, *Cyp11a1*, *Nr4a1*, *Cyp11b*, *Cyp17a1*, *Foxl2*, *Cyp19a1*, *Hsd11b2*, *Er*, and *Vdac1*

of tree sparrows directly influences the expression of these steroidogenic transcripts. Higher level expression of the *StAR, Cyp11a1, Nr4a1, Cyp11b, Hsd11b2, Foxl2, Cyp19a1, Er*, and *Vdac1* was observed during the breeding phase in the hypothalamus, and numerous peaks in the hypothalamus were perceived for *Cyp17a1* (Fig. 5). Moreover, evidence from the recent publication shows a similar pattern in the expression of these steroidogenic enzymes peaked during the breeding phase [21]. Also, other organisms suggest physiology-dependent changes in these transcripts' expression such as in edible frogs (*Pelophylax esculentus*), brain levels of neurosteroids oscillate simultaneously with the reproductive cycle. Seasonal fluctuations in gene expression levels of *hsd3b1, hsd17b1, srd5a1,* and *cyp19a1* were observed in the Diencephalon–Mesencephalon region of both sexes of the edible frog, and higher transcript levels were observed in the reproductive period than in post-reproductive period [55].

The study depicts that the steroidogenic process and seasonal variation in these steroidogenic enzymes are potentially relevant during folliculogenesis as reported by [56]. In seasonal breeders, the differential expression of steroidogenic enzymes during the reproductive and non-reproductive cycles could be the local key to controlling follicular development. In adult female tree sparrows, expression of these steroidogenic transcripts showed reproductive stage-linked expression in the ovary (Fig. 6). These transcripts were upregulated before or throughout the reproductive stage (Figs. 3,6). These sex steroids exert a strong influence on the reproductive behavior of vertebrates. The steroidogenic process in gonads displays discrete sex- and season-specific patterns and key enzyme expression. Higher expression of sex steroids during the pre-breeding or breeding phase has also been demonstrated in other species. In seasonally breeding vertebrate species, mating behavior, elevated gametogenesis, and peak circulating sex steroid concentrations coincide temporally [57]. In the toad (*Bufo japonicus*), circulatory sex steroid levels fluctuate seasonally and coincide with major reproductive events in species with distinct breeding seasons [58].

In the ovary, peak expression of *StAR* transcript was observed during the pre-breeding phase (Fig. 6a).

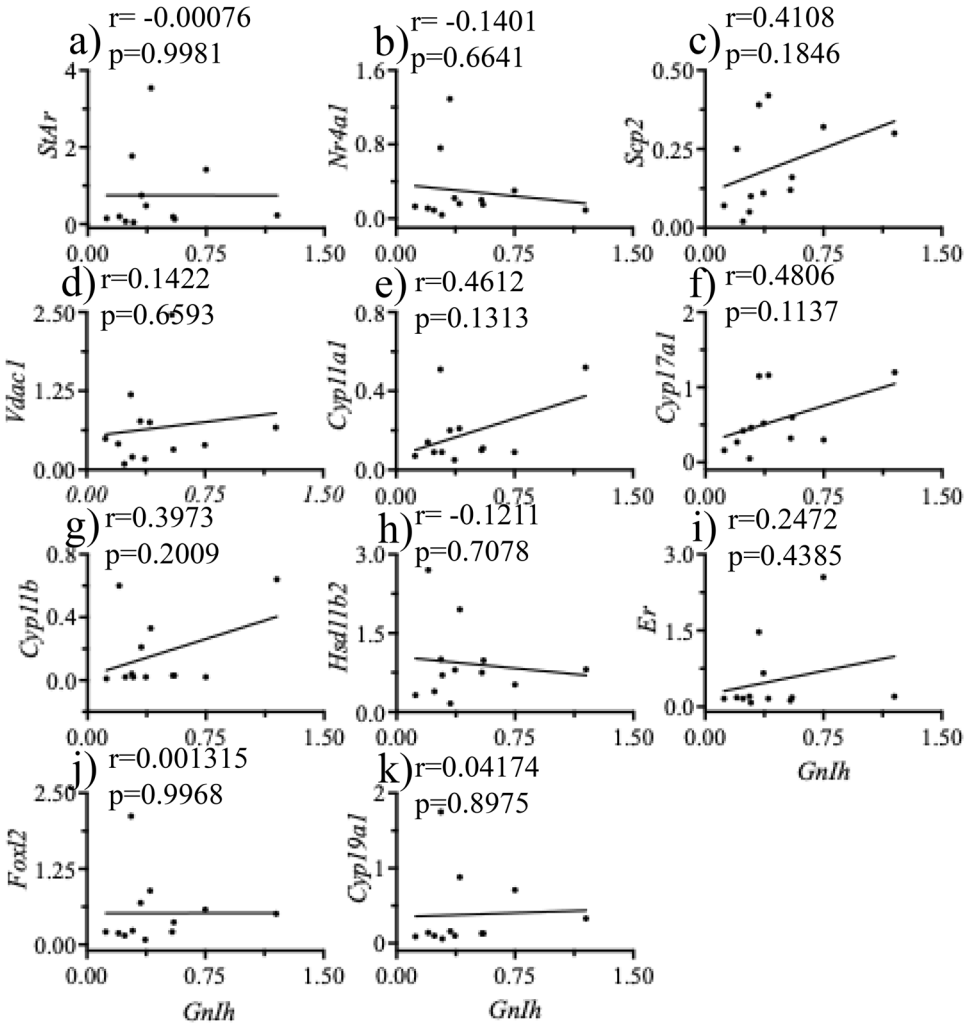


Fig. 16 Correlation of hypothalamic *GnIh* expression with ovarian expression of *StAR*, *Scp2*, *Cyp11a1*, *Nr4a1*, *Cyp11b*, *Cyp17a1*, *Foxl2*, *Cyp19a1*, *Hsd11b2*, *Er*, and *Vdac1*

Maximum expression of the *StAR* was also observed in the ovary of green anole lizards (*Anolis carolinensis*) during the spring mating season [59]. The gene *StAR* is a vital stimulant for steroidogenesis and plays a critical function in the process of transporting cholesterol from the outer to the inner membrane of the mitochondria. In line with our findings, higher ovarian *StAR* mRNA expression before or during the reproductive phase correlates with higher steroidogenic activity. This is also visible in the testis tissues of male tree sparrows [21]. *StAR* proteins are also associated with a voltage-dependent anion channel (*vdac2*) at the mitochondria-associated endoplasmic reticulum membrane to enable the transport of cholesterol to the inner mitochondrial membrane [60]. We also observed peak expression of *Nr4a1* mRNA in the ovary during May, corresponding with higher gonadal activities in tree sparrows. Expression of *Nr4a1* is modulated by several agents, including Ca2 + , and growth factors [61]. In gonads, *Nr4a1* controls the expression of several genes involved in steroidogenesis, including *StAR* [62], *Cyp17a1* [63], *hsd3b2* [64], and *Cyp11b2* [65].

Higher expression of *Scp2* mRNA before/during the peak follicular growth and activity were observed (Fig. 6c). The *Scp2* gene, having two distinct transcription initiation sites, encodes a 15 kDa pro-Scp2 protein and the 58 kDa SCPx, which have identical C-termini [66]. *Scp2* and *SCPx* proteins are highly expressed in all tissues with increased cholesterol metabolism rates [67]. The pre-breeding phase showed higher expression of *Cyp11a1* and *Cyp19a1* than the post-breeding phase (Fig. 6e), which aligns with reports [68]. Impaired steroidogenesis results from a lack of the mitochondrial enzymes *Cyp11a1* and *Cyp11b* [69]. We observed higher expression of Er transcripts during the breeding phase (Fig. 6j). Estrogen differentially controls reproductive and non-reproductive responses through their receptors [70].

We observed that *Cyp19a1* transcript levels were higher during the pre-breeding phase. *Cyp19a1* is a key enzyme

that converts testosterone to androgen, a steroid hormone necessary to induce feminization [71, 72]. *Foxl2* mRNA levels also showed peak expression in February during the pre-breeding phase. More recently, it has been claimed that Foxl2 is responsible for the transcriptional regulation of *Cyp19a1* in female gonads because *Foxl2* and *Cyp19a1* mRNA are co-expressed in the medullary region of female gonads during sex differentiation in the chicken [18]. Several-fold expression of *Cyp17a1* transcripts was observed during January, March, and May during pre/post-breeding season (Fig. 6f). *Cyp17a1* is the enzyme involved in the production of some androgens by converting 17α-hydroxy pregnenolone and 17α-hydroxyprogesterone and eventually metabolized to testosterone, a substrate for p450arom. Therefore, higher expression of this enzyme in females may indicate a contribution to estrogen production. This is consistent with previous findings [39]. These observations demonstrate when male tree sparrows are mating at their peak, steroidogenesis is actively involved. Other than bird species, in sea cucumber *Holothuria scabra*, real-time qPCR analysis of steroidogenic gene (*StAR, cyp10, 3βhsd, 17βhsd*) shows increasing levels of mRNA transcripts during gonadal maturation [73].

5 Conclusion

The study's results may conclude that the key enzymes involved in steroidogenesis for sex hormone production are expressed in the hypothalamus and the ovary of tree sparrows. Seasonal variations in these genes' regulation in the brain and gonads imply the possibility that seasonal distinctions in gene expression control tissue-specific steroidogenic capacity. Furthermore, a recent study on adult male tree sparrows found that the steroidogenic transcript expression patterns in adult female tree sparrows connected with the annual reproductive cycle are comparable [21]. The principal findings of the study advocate that the steroidogenic enzyme exhibits 24-h patterning and seasonal variation in steroidogenic gene expression patterns linked with annual reproduction in adult female tree sparrows. Moreover, the present study advocates male and female tree sparrows with the conserved mechanism. In addition, the tree sparrow's conjunctive mechanism for the growth and regulation of the gonadal system is shown by the seasonal variations in the stimulation of both androgenic and estrogenic pathways during the reproductive phase in the steroidogenic process. Furthermore, presenting research opportunities on the molecular mechanism of steroidogenesis regulation and reproductive capacity is crucial. This includes understanding the factors that influence the unique expression of these steroidogenic genes and what triggers their tissue-specific and time-dependent expression.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s43630-025-00711-0.

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Authors' contributions AKT conceived the ideas and designed the methodology; SY collected and analyzed the data; SY executed the writing of the manuscript. AKT revised and finalized the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Data availability Data are available and may be provided if required. *Nucleotide sequence data* These above gene sequences have been submitted to the GenBank (http://www.ncbi.nlm.nih.gov) databases under accession numbers SUB14302313, SUB14300140, SUB14300315, SUB14300332, SUB14300339, SUB13960522, SUB13960499, SUB13960560, SUB13960832, SUB13960845, SUB14300328, SUB14474972.

Declarations

Conflict of interest The authors declare that they have no conflict of interest with the publication of this article.

References

1. Kumar, V., & Follett, B. K. (1993). The circadian nature of melatonin secretion in Japanese quail (*Coturnix coturnix japonica*). *Journal of Pineal Research, 14*(4), 192–200. https://doi.org/10.1111/j.1600-079x.1993.tb00502.x
2. Kumar, V., Rani, S., Malik, S., Trivedi, A. K., Schwabl, I., Helm, B., & Gwinner, E. (2007). Daytime light intensity affects seasonal timing via changes in the nocturnal melatonin levels. *Naturwissenschaften, 94*, 693–696. https://doi.org/10.1007/s00114-007-0243-1
3. Singh, J., Rani, S., & Kumar, V. (2012). Functional similarity in relation to the external environment between circadian behavioral and melatonin rhythms in the subtropical Indian weaver bird. *Hormones and Behavior, 61*(4), 527–534. https://doi.org/10.1016/j.yhbeh.2012.01.015
4. Kumar, V., Wingfield, J. C., Dawson, A., Ramenofsky, M., Rani, S., & Bartell, P. (2010). Biological clocks and regulation of seasonal reproduction and migration in birds. *Physiological and Biochemical Zoology, 83*(5), 827–835. https://doi.org/10.1086/652243
5. Cowan, M., Azpeleta, C., & López-Olmeda, J. F. (2017). Rhythms in the endocrine system of fish: A review. *Journal of Comparative Physiology B, 187*, 1057–1089. https://doi.org/10.1007/s00360-017-1094-5
6. Renthlei, Z., Yatung, S., Lalpekhlui, R., & Trivedi, A. K. (2022). Seasonality in tropical birds. *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology, 337*(9–10), 952–966. https://doi.org/10.1002/jez.2649
7. Nelson, R. J., Badura, L. L., & Goldman, B. D. (1990). Mechanisms of seasonal cycles of behavior. *Annual Review of*

Psychology, 41(1), 81–108. https://doi.org/10.1146/annurev.ps.41.020190.000501
8. Ren, J., Sun, C., Chen, L., Hu, J., Huang, X., Liu, X., & Lu, L. (2019). Exploring differentially expressed key genes related to development of follicle by RNA-seq in Peking ducks (Anas Platyrhynchos). *PLoS ONE, 14*(6), e0209061. https://doi.org/10.1371/journal.pone.0209061
9. Rosati, L., Santillo, A., Di Fiore, M. M., Andreuccetti, P., & Prisco, M. (2017). Testicular steroidogenic enzymes in the lizard Podarcis sicula during the spermatogenic cycle. *Comptes Rendus Biologies, 340*(11–12), 492–498. https://doi.org/10.1016/j.crvi.2017.10.001
10. Paitz, R. T., & Cagney, E. (2019). In ovo metabolism of progesterone to 5β-pregnanedione in chicken eggs: Implications for how yolk progesterone influences embryonic development. *General and Comparative Endocrinology, 282*, 113221. https://doi.org/10.1016/j.ygcen.2019.113221
11. Lincoln, J. M., Barlowe, M. L., Rucker, H. R., & Parker, M. R. (2023). Reconsidering reproductive patterns in a model dissociated species, the red-sided garter snake: Sex-specific and seasonal changes in gonadal steroidogenic gene expression. *Frontiers in Endocrinology, 14*, 1135535. https://doi.org/10.3389/fendo.2023.1135535
12. Meitzen, J., & Thompson, C. K. (2008). Seasonal-like growth and regression of the avian song control system: Neural and behavioral plasticity in adult male Gambel's white-crowned sparrows. *General and Comparative Endocrinology, 157*(3), 259–265. https://doi.org/10.1016/j.ygcen.2008.03.014
13. Antonio-Cabrera, E., & Paredes, R. G. (2014). Testosterone or oestradiol implants in the medial preoptic area induce mating in noncopulating male rats. *Journal of Neuroendocrinology, 26*(7), 448–458. https://doi.org/10.1111/jne.12164
14. Voigt, C., & Leitner, S. (2008). Seasonality in song behaviour revisited: Seasonal and annual variants and invariants in the song of the domesticated canary (*Serinus canaria*). *Hormones and Behavior, 54*(3), 373–378. https://doi.org/10.1016/j.yhbeh.2008.05.001
15. Ansel, L., Bentsen, A. H., Ancel, C., Bolborea, M., Klosen, P., Mikkelsen, J. D., & Simonneaux, V. (2011). Peripheral kisspeptin reverses short photoperiod-induced gonadal regression in Syrian hamsters by promoting *GNRH* release. *Reproduction, 142*(3), 417. https://doi.org/10.1530/rep-10-0313
16. Forlano, P. M., Sisneros, J. A., Rohmann, K. N., & Bass, A. H. (2015). Neuroendocrine control of seasonal plasticity in the auditory and vocal systems of fish. *Frontiers in Neuroendocrinology, 37*, 129–145.
17. Clark, B. J., Wells, J., King, S. R., & Stocco, D. M. (1994). The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR). *Journal of Biological Chemistry, 269*(45), 28314–28322.
18. Govoroun, M. S., Pannetier, M., Pailhoux, E., Cocquet, J., Brillard, J. P., Couty, I., & Cotinot, C. (2004). Isolation of chicken homolog of the FOXL2 gene and comparison of its expression patterns with those of aromatase during ovarian development. *Developmental Dynamics: An Official Publication of the American Association of Anatomists, 231*(4), 859–870.
19. de Alba, G., Mourad, N. M. N., Paredes, J. F., Sánchez-Vázquez, F. J., & López-Olmeda, J. F. (2019). Daily rhythms in the reproductive axis of Nile tilapia (*Oreochromis niloticus*): Plasma steroids and gene expression in brain, pituitary, gonad and egg. *Aquaculture, 507*, 313–321.
20. Chustecka, M., Blügental, N., Majewski, P. M., & Adamska, I. (2021). 24-hour patterning in gene expression of pineal neurosteroid biosynthesis in young chickens (*Gallus gallus domesticus* L.). *Chronobiology International, 38*(1), 46–60.
21. Yatung, S., & Trivedi, A. K. (2024). Daily and seasonal changes in steroidogenic markers in the hypothalamus and testes of tree sparrow (*Passer montanus*). *Journal of Neuroendocrinology*. https://doi.org/10.1111/jne.13478
22. Ings, J. S., & Van Der Kraak, G. J. (2006). Characterization of the mRNA expression of StAR and steroidogenic enzymes in zebrafish ovarian follicles. *Molecular Reproduction and Development: Incorporating Gamete Research, 73*(8), 943–954. https://doi.org/10.1002/mrd.20490
23. Naomi, K., Yonju, H., Akira, T., Noboru, S., & Kiyoshi, S. (2008). Profiles of mRNA expression of FOXL2, P450arom, DMRT1, AMH, P450c17, SF1, ERα and AR, in relation to gonadal sex differentiation in duck embryo. *The Journal of Poultry Science, 45*(2), 132–138. https://doi.org/10.2141/jpsa.45.132
24. Rosati, L., Di Fiore, M. M., Prisco, M., Di Giacomo Russo, F., Venditti, M., Andreuccetti, P., & Santillo, A. (2019). Seasonal expression and cellular distribution of StAR and steroidogenic enzymes in quail testis. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution, 332*(6), 198–209.
25. Trivedi, A. K., Rani, S., & Kumar, V. (2006). Control of annual reproductive cycle in the subtropical house sparrow (*Passer domesticus*): Evidence for conservation of photoperiodic control mechanisms in birds. *Frontiers in Zoology, 3*, 1–12. https://doi.org/10.1186/1742-9994-3-12
26. Kumar, V., Singh, S., Misra, M., & Malik, s. (2001). Effects of duration and time of food availability on photoperiodic responses in the migratory male blackheaded bunting (*Emberiza melanocephala*). *Journal of Experimental Biology, 204*(16), 2843–2848. https://doi.org/10.1242/jeb.204.16.2843
27. Renthlei, Z., & Trivedi, A. K. (2019). Effect of urban environment on pineal machinery and clock genes expression of tree sparrow (*Passer montanus*). *Environmental Pollution, 255*, 113278.
28. Borah, B. K., Renthlei, Z., & Trivedi, A. K. (2020). Hypothalamus but not liver retains daily expression of clock genes during hibernation in terai tree frog (*Polypedates teraiensis*). *Chronobiology International, 37*(4), 485–492. https://doi.org/10.1080/07420528.2020.1726373
29. Renthlei, Z., Borah, B. K., Gurumayum, T., & Trivedi, A. K. (2020). Season dependent effects of urban environment on circadian clock of tree sparrow (*Passer montanus*). *Photochemical & Photobiological Sciences, 19*, 1741–1749. https://doi.org/10.1039/d0pp00257g
30. Renthlei, Z., Borah, B. K., & Trivedi, A. K. (2021). Urban environment alter the timing of progression of testicular recrudescence in tree sparrow (*Passer montanus*). *Environmental Science and Pollution Research, 28*, 31097–31107. https://doi.org/10.1007/s11356-021-12918-6
31. Lalpekhlui, R., Renthlei, Z., & Trivedi, A. K. (2022). Molecular expression of clock genes in central and peripheral tissues of white-rumped munia (*Lonchura striata*). *Chronobiology International, 39*(8), 1058–1067.
32. Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *Methods, 25*(4), 402–408.
33. Cuesta, M., Clesse, D., Pévet, P., & Challet, E. (2009). From daily behavior to hormonal and neurotransmitters rhythms: Comparison between diurnal and nocturnal rat species. *Hormones and behavior, 55*(2), 338–347. https://doi.org/10.1016/j.yhbeh.2008.10.015
34. Soper, D. S. (2013). F-value and p-value calculator for multiple regression. See. http://www. danielsoper. com/statcalc
35. Richardson, J. T. (2011). Eta squared and partial eta squared as measures of effect size in educational research. *Educational Research Review, 6*(2), 135–147.

36. Di Rosa, V., López-Olmeda, J. F., Burguillo, A., Frigato, E., Bertolucci, C., Piferrer, F., & Sánchez-Vázquez, F. J. (2016). Daily rhythms of the expression of key genes involved in steroidogenesis and gonadal function in zebrafish. *PLoS ONE, 11*(6), e0157716.
37. Tomy, S., Wu, G. C., Huang, H. R., Dufour, S., & Chang, C. F. (2007). Developmental expression of key steroidogenic enzymes in the brain of protandrous black porgy fish, Acanthopagrus schlegeli. *Journal of Neuroendocrinology, 19*(8), 643–656. https://doi.org/10.1111/j.1365-2826.2007.01572.x
38. Chen, R., Qin, Y., Du, J., Liu, J., Dai, S., Lei, M., & Zhu, H. (2023). Circadian clock gene BMAL1 regulates STAR expression in goose ovarian preovulatory granulosa cells. *Poultry Science, 102*(12), 103159. https://doi.org/10.1016/j.psj.2023.103159
39. Nettle, D., & Bateson, M. (2015). Adaptive developmental plasticity: What is it, how can we recognize it and when can it evolve? *Proceedings of the Royal Society B: Biological Sciences, 282*(1812), 20151005. https://doi.org/10.1098/rspb.2015.1005
40. Wang, Y., Guo, Z. Y., Zhang, C., Miao, D. Z., Mao, X. Y., Lu, S. M., & Wang, Z. Y. (2023). Characterization of ovarian follicles, serum steroid hormone concentration, and steroidogenic gene expression profiles in the developing ovarian follicles in White King pigeons. *Poultry Science, 102*(7), 102673.
41. Licht, P., Owens, D. W., Cliffton, K., & Penaflores, C. (1982). Changes in LH and progesterone associated with the nesting cycle and ovulation in the olive ridley sea turtle. *Lepidochelys olivacea. General and Comparative Endocrinology, 48*(2), 247–253. https://doi.org/10.1016/0016-6480(82)90023-5
42. Cui, Y. M., Wang, J., Zhang, H. J., Qi, G. H., & Wu, S. G. (2021). Effects of photoperiod on performance, ovarian morphology, reproductive hormone level, and hormone receptor mRNA expression in laying ducks. *Poultry Science, 100*(4), 100979. https://doi.org/10.1016/j.psj.2021.01.002
43. Trivedi, A. K., Sur, S., Sharma, A., Taufique, S. T., Gupta, N. J., & Kumar, V. (2019). Temperature alters the hypothalamic transcription of photoperiod responsive genes in induction of seasonal response in migratory redheaded buntings. *Molecular and Cellular Endocrinology, 493*, 110454. https://doi.org/10.1016/j.mce.2019.110454
44. Yoshimura, T., Yasuo, S., Watanabe, M., Iigo, M., Yamamura, T., Hirunagi, K., & Ebihara, S. (2003). Light-induced hormone conversion of T4 to T3 regulates photoperiodic response of gonads in birds. *Nature, 426*(6963), 178–181. https://doi.org/10.1038/nature02117
45. Nakao, N., Ono, H., & Yoshimura, T. (2008). Thyroid hormones and seasonal reproductive neuroendocrine interactions. *Reproduction, 136*(1), 1. https://doi.org/10.1530/REP-08-0041
46. Tsutsui, K., Saigoh, E., Ukena, K., Teranishi, H., Fujisawa, Y., Kikuchi, M., Ishii, S., & Sharp, P. J. (2000). A novel avian hypothalamic peptide inhibiting gonadotropin release. *Biochemical and biophysical research communications, 275*(2), 661–667. https://doi.org/10.1006/bbrc.2000.3350
47. Ubuka, T., Kim, S., Huang, Y. C., Reid, J., Jiang, J., Osugi, T., Chowdhury, V. S., Tsutsui, K., & Bentley, G. E. (2008). Gonadotropin-inhibitory hormone neurons interact directly with gonadotropin-releasing hormone-I and-II neurons in European starling brain. *Endocrinology, 149*(1), 268–278. https://doi.org/10.1210/en.2007-0983
48. Majumdar, G., Trivedi, A. K., Gupta, N. J., & Kumar, V. (2015). Circadian synchronization determines critical day length for seasonal responses. *Physiology & Behavior, 147*, 282–290. https://doi.org/10.1016/j.physbeh.2015.05.005
49. Dixit, A. S., & Byrsat, S. (2018). Photoperiodic control of *GnRH*-I expression in seasonal reproduction of the Eurasian tree sparrow. *Photochemical & Photobiological Sciences, 17*, 934–945. https://doi.org/10.1039/c8pp00153g
50. Zhang, X., Yang, W., Liang, W., Wang, Y., & Zhang, S. (2019). Intensity dependent disruptive effects of light at night on activation of the HPG axis of tree sparrows (*Passer montanus*). *Environmental pollution, 249*, 904–909. https://doi.org/10.1016/j.envpol.2019.03.008
51. Majumdar, G., Liddle, T. A., Stewart, C., Marshall, C. J., Bain, M., & Stevenson, T. (2023). FSHβ links photoperiodic signaling to seasonal reproduction in Japanese quail. *eLife, 12*, 87751.
52. Baulieu, E. E. (1998). Neurosteroids: A novel function of the brain. *Psychoneuroendocrinology, 23*(8), 963–987. https://doi.org/10.1016/s0306-4530(98)00071-7
53. Compagnone, N. A., & Mellon, S. H. (2000). Neurosteroids: Biosynthesis and function of these novel neuromodulators. *Frontiers in Neuroendocrinology, 21*(1), 1–56. https://doi.org/10.1006/frne.1999.0188
54. Do Rego, J. L., Seong, J. Y., Burel, D., Leprince, J., Luu-The, V., Tsutsui, K., & Vaudry, H. (2009). Neurosteroid biosynthesis: enzymatic pathways and neuroendocrine regulation by neurotransmitters and neuropeptides. *Frontiers in Neuroendocrinology, 30*(3), 259–301.
55. Santillo, A., Falvo, S., Chieffi Baccari, G., & Di Fiore, M. M. (2017). Seasonal changes in gene expression of steroidogenic enzymes, androgen and estrogen receptors in frog testis. *Acta Zoologica, 98*(2), 221–227.
56. Krasnow, J. (1991). Advances in understanding ovarian physiology: regulation of steroidogenic enzymes in ovarian follicular differentiation. *Seminars in Reproductive Medicine, 9*(4), 283–302.
57. Crews, D. (1984). Gamete production, sex hormone secretion, and mating behavior uncoupled. *Hormones and Behavior, 18*(1), 22–28. https://doi.org/10.1016/0018-506X(84)90047-3
58. Itoh, M., & Ishii, S. (1990). Changes in plasma levels of gonadotropins and sex steroids in the toad, Bufo japonicus, in association with behavior during the breeding season. *General and comparative endocrinology, 80*(3), 451–464. https://doi.org/10.1016/0016-6480(90)90194-Q
59. Peek, C. E., & Cohen, R. E. (2018). Seasonal regulation of steroidogenic enzyme expression within the green anole lizard (Anolis carolinensis) brain and gonad. *General and Comparative Endocrinology, 268*, 88–95. https://doi.org/10.1016/j.ygcen.2018.08.005
60. Maurya, S. R., & Mahalakshmi, R. (2016). VDAC-2: Mitochondrial outer membrane regulator masquerading as a channel? *The FEBS Journal, 283*(10), 1831–1836. https://doi.org/10.1111/febs.13637
61. Li, M., Xue, K., Ling, J., Diao, F. Y., Cui, Y. G., & Liu, J. Y. (2010). The orphan nuclear receptor NR4A1 regulates transcription of key steroidogenic enzymes in ovarian theca cells. *Molecular and Cellular Endocrinology, 319*(1–2), 39–46. https://doi.org/10.1016/j.mce.2010.01.014
62. Martin, L. J., Boucher, N., Brousseau, C., & Tremblay, J. J. (2008). The orphan nuclear receptor NUR77 regulates hormone-induced StAR transcription in Leydig cells through cooperation with Ca2+/calmodulin-dependent protein kinase I. *Molecular Endocrinology, 22*(9), 2021–2037. https://doi.org/10.1210/me.2007-0370
63. Zhang, P., & Mellon, S. H. (1997). Multiple orphan nuclear receptors converge to regulate rat P450c17 gene transcription: Novel mechanisms for orphan nuclear receptor action. *Molecular Endocrinology, 11*(7), 891–904. https://doi.org/10.1210/mend.11.7.9940
64. Bassett, M. H., Suzuki, T., Sasano, H., De Vries, C. J., Jimenez, P. T., Carr, B. R., & Rainey, W. E. (2004). The orphan nuclear receptor NGFIB regulates transcription of 3β-hydroxysteroid dehydrogenase: Implications for the control of adrenal functional

zonation. *Journal of Biological Chemistry, 279*(36), 37622–37630. https://doi.org/10.1074/jbc.M405431200
65. Bassett, M. H., Suzuki, T., Sasano, H., White, P. C., & Rainey, W. E. (2004). The orphan nuclear receptors NURR1 and NGFIB regulate adrenal aldosterone production. *Molecular Endocrinology, 18*(2), 279–290. https://doi.org/10.1210/me.2003-0005
66. Ohba, T., Holt, J. A., Billheimer, J. T., & Strauss, J. F., III. (1995). Human sterol carrier protein x/sterol carrier protein 2 gene has two promoters. *Biochemistry, 34*(33), 10660–10668. https://doi.org/10.1021/bi00033a042
67. Vahouny, G. V., Chanderbhan, R., Kharroubi, A., Noland, B. J., Pastuszyn, A., & Scallen, T. J. (1987). Sterol carrier and lipid transfer proteins. *Advances in Lipid Research, 22*, 83–113. https://doi.org/10.1016/B978-0-12-024922-0.50007-2
68. Wang, Y., Liu, X., Li, W., Zhao, J., Liu, H., Yu, L., & Zhu, X. (2021). Reproductive performance is associated with seasonal plasma reproductive hormone levels, steroidogenic enzymes and sex hormone receptor expression levels in cultured Asian yellow pond turtles (*Mauremys mutica*). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 254*, 110566. https://doi.org/10.1016/j.cbpb.2021.110566
69. Parajes, S., Griffin, A., Taylor, A. E., Rose, I. T., Miguel-Escalada, I., Hadzhiev, Y., & Krone, N. (2013). Redefining the initiation and maintenance of zebrafish interrenal steroidogenesis by characterizing the key enzyme cyp11a2. *Endocrinology, 154*(8), 2702–2711.
70. Kuiper, G. G., Lemmen, J. G., Carlsson, B. O., Corton, J. C., Safe, S. H., Van Der Saag, P. T., & Gustafsson, J. A. (1998). Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. *Endocrinology, 139*(10), 4252–4263.
71. Andrews, J. E., Smith, C. A., & Sinclair, A. H. (1997). Sites of estrogen receptor and aromatase expression in the chicken embryo. *General and Comparative Endocrinology, 108*(2), 182–190. https://doi.org/10.1006/gcen.1997.6978
72. Akazome, Y., Abe, T., & Mori, T. (2002). Differentiation of chicken gonad as an endocrine organ: Expression of LH receptor, FSH receptor, cytochrome P450c17 and aromatase genes. *REPRODUCTION-CAMBRIDGE, 123*(5), 721–728.
73. Thongbuakaew, T., Suwansa-Ard, S., Chaiyamoon, A., Cummins, S. F., & Sobhon, P. (2021). Sex steroids and steroidogenesis-related genes in the sea cucumber, Holothuria scabra and their potential role in gonad maturation. *Scientific Reports, 11*(1), 2194. https://doi.org/10.1038/s41598-021-81917-x

ISAE 2024

13th International Symposium on Avian Endocrinology

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Mizoram University, India

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Particulars of the candidate

NAME OF THE CANDIDATE : **SUBU YATUNG**

DEGREE : Doctor of Philosophy (Ph.D.)

DEPARTMENT : Zoology, School of Life Sciences

TITLE OF THE THESIS : Daily and Seasonal Regulation of Steroidogenesis in Tree Sparrow (*Passer montanus*)

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ABSTRACT

DAILY AND SEASONAL REGULATION OF STEROIDOGENESIS IN TREE SPARROW (*PASSER MONTANUS*)

AN ABSTRACT SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

SUBU YATUNG
MZU REGISTRATION NO.: 1800149
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DEPARTMENT OF ZOOLOGY
SCHOOL OF LIFE SCIENCES
JUNE, 2025

DAILY AND SEASONAL REGULATION OF STEROIDOGENESIS IN TREE SPARROW (*PASSER MONTANUS*)

BY

SUBU YATUNG

Department of Zoology

Supervisor

Dr. Amit Kumar Trivedi

Submitted

In partial fulfillment of the requirement of the Degree of Doctor of Philosophy in Zoology of Mizoram University, Aizawl

Environmental factors, including temperature, day length, and food accessibility, have a reflective influence on the physiology and behavior of avian species, especially in terms of seasonal events (reproduction, migration, and molt) and daily cycles (activity, metabolism, etc.) and exhibit physiological and behavioral rhythmicity alterations driven by the internal biological timekeeping system. The internal biological clock regulates the cyclical variations that occur in an animal's physiological and biological processes. These biological clocks enable species to synchronize with the cyclical events in nature, such as day and night, and the seasons. Photoperiod is the proximate environmental time cue capable of entraining biological clocks. It allows the phase of cyclic events in the environment to be set with the phase of endogenous rhythms, thereby producing a series of signals that enable endogenous timers to synchronize with biological cycles, establishing a persistent phase relationship between the zeitgebers and the rhythm. Consequently, most birds display seasonal variability in their behavioral, physiological, and reproductive activities, such as a change in body weight, molt in feathers, bill color, song production, hormonal changes, gonadal status, and migration.

Besides, light properties such as intensity and duration, different wavelengths of the spectrum can also influence numerous avian life history functions, such as behavior, development, reproduction, as well as metabolism, etc. In addition, the hypothalamic pituitary-gonad axis (HPGa), which is essential for processes like sex differentiation, gonad growth, etc., governs the timing of reproduction and the biosynthesis of sex steroid hormones. Nonetheless, it is well known that sex steroids are critical for testicular function, controlling the reproductive processes by promoting either spermatogenesis or spermiogenesis or the development/differentiation, in addition to the maintenance of secondary sexual characteristics in all vertebrates, particularly those with a seasonal reproductive cycle. The biosynthesis of all the steroid hormones is controlled by the two key functional classes of steroidogenic enzymes: cytochrome P450 and hydrosteroid dehydrogenase. Besides, Sex hormones are necessary for an individual's survival and level of fitness. Therefore, biological processes that determine an individual's fitness and survival, such as steroidogenesis and reproduction, operate in parallel.

Present thesis

The present thesis studied the tree sparrow (*Passer montanus*) population that inhabits the area between 23°N and 92°E on the campus of Mizoram University. The study includes daily and seasonal variations of the selected steroidogenic gene markers and Photoperiodic regulation of genes known to be involved in the biosynthesis of steroidogenesis and gonadal function in both adult male and female tree sparrows, and links it with their annual reproductive cycles. Also, the effect of dLAN and corticosterone treatment on steroidogenesis. Several studies conducted are summarised in the following sections.

SECTION 1: DAILY EXPRESSION OF KEY GENES INVOLVED IN STEROIDOGENESIS AND GONADAL FUNCTION

Population responds to environmental variability, largely determined by the dynamic interactions between fitness components within- and among-individual variation in the expression of the environmentally sensitive phenotype. Their physiological and behavioral processes exhibit cyclical changes, which can be attributed to their internal biological clock. These biological clocks can synchronize with natural cyclical occurrences, such as day and night and seasons. Besides, the primary time cues that might entrain biological clocks are changes in the photoperiod or the 24-hour light-dark cycle. It makes it possible to match the phase of cyclic events in the environment with endogenous rhythms. The tree sparrow is one example of a seasonal breeder that has adapted to the shifting environment. The present study was conducted on daily changes in the transcript levels of steroidogenic markers in both adult male and female tree sparrows. The birds were procured locally (n=5/time points/sex) using a mist net during the breeding season, i.e, in April. Afterwards, the birds were sampled at 6 time points, i.e., ZT1, ZT5, ZT9, ZT13, ZT17, and ZT21 (ZT 0 = sun rise time; 5:14 am) during a 24-hour cycle. Immediately, brain and gonads were excised and stored at -80ºC for gene expression analysis. The study reveals higher expressions were observed in the light phase when the birds are more active, and gradually lower in the light switch-off period in the testis and ovary tissue. However, higher expressions of most of these genes in the hypothalamic tissue were observed in the dark phase in comparison to the light

phase, exhibiting tissue-specific 24-hour rhythmicity in the steroidogenic activity. These results demonstrate key genes known to be involved in steroidogenesis for the production of sex hormones that are expressed in the hypothalamic tissues and the gonads of the tree sparrows and reveal diurnal rhythmicity, showing maximum expression in the light phase. Besides, differential expression of these transcripts at different times suggests tissue-specific and time-dependent regulation of steroidogenesis in both male and female tree sparrows.

SECTION II: PHOTOPERIODIC REGULATION OF GENES INVOLVED IN STEROIDOGENESIS AND GONADAL FUNCTION

Avian species are greatly impacted by the light, and the light source is the most prominent environmental cue to which these species rely to time their life history stages, such as reproduction, behaviour, and endocrine processes. Light comprises three fundamental characteristics: duration, intensity, and spectrum. Almost all bird species are diurnal, and they experience variations in light intensity that correspond to daily, lunar, or seasonal cycles. These physiological changes are governed by the interaction between the endogenous circadian rhythm and the ecological variables such as temperature, resource accessibility, and photoperiod (light source). Besides, Photoperiod is one of the most critical environmental cues regulating seasonal variations in reproductive physiology and behavior, and long day (LD) photoperiods in spring stimulate gonadal recrudescence and a resulting increase in plasma concentrations of gonadal steroids. An effective season-related neuroendocrine mechanism appears to be mediated by appropriate reproduction timing and hypothalamic-pituitary-gonadal axis (HPG-a) activity linked with reinitiation, maintenance, and regression of spermatogenesis and steroidogenesis. In the present study, we examined the outcome of differential effects of photoperiodic regimes, intensity, and spectra on the steroidogenic gene markers expression in the hypothalamus tissue and gonads of male and female tree sparrows and linked with the seasonal reproduction. The study was carried out in three experiments. Photosensitive adult male and female birds were used and procured using mist nets. Birds, n=5/sex/group, were used in each experiment, respectively. In experiment one, birds were divided into three groups. Group 1 was treated with short photoperiod

8L:16D, Group 2 was treated with equinox photoperiod 12L:12D, and Group 3 was treated with long photoperiod 16L:8D. For female birds, a similar experimental design was used. The experiment was run for 30 days. Body weight, bill's color, and gonad size were recorded at the beginning and end of the experiment. Hypothalamus and gonads were excised and kept in -80ºC for further analysis. In experiment two, both male and female birds were divided into 3 groups each. Group 1 was exposed to 10 lux. Group 2 was exposed to 50 lux, and Group 3 was exposed to 100 lux intensity of light. All the groups were kept under photoperiodic chambers of 16L:8D, and the experiment was run for 30 days. Body mass, gonad size, and bill color were recorded before and after the experiment. Later hypothalamus and gonad tissue were harvested for gene expression analysis. In experiment three birds (n=5 birds/group/sex) were used and divided into four groups, each sex. Group one was exposed to red spectrum (650 nm), Group two was exposed to blue spectrum (450 nm), Group 3 was exposed to green spectrum (520 nm), and Group four was exposed to white light, and served as a control group. The intensity used was 0.78 W/m^2. All the groups were exposed to a long photoperiod of 16L:8D, and the experiment was run for 30 days. Body mass, color of the bill, and gonadal size were recorded before and at the end of the experiment. Hypothalamus and gonads were harvested and immediately kept at -80ºC for gene expression analysis. All the birds were sampled at the mid-point of their respective light phase. The results of experiment one reveal the differential effect of photoperiod on reproductive-linked steroidogenesis. No significant difference was observed in body mass; however slight increase in bill color was observed in 12L:12D and 16L:8D. In addition, higher expressions of reproductive stimulatory genes such as *Tshβ*, *Dio2* and *GnRH* were observed in 16L:8D followed by 12L:12D in the hypothalamus, however, higher expressions of reproductive inhibitory genes such as *Dio3* and *GnIH* were observed in 8L:16D. Besides, highest expressions of steroidogenic gene markers (*StAR*, *Nr4a1*, *Er*, *Scp2*, *Cyp17a1*, *Cyp11a1*, *Hsd11b2*, *Cyp11b*, *Vdac1*, *Srd5a1*, *Foxl2*, and *Cyp19a1*) were observed in the birds exposed to 16L:8D, which mimics the long day experienced during breeding season, followed by 12L:12D, an equinox photoperiod, suggesting 12 hours of light reflects the optimal day length to stimulate reproductive activity. However, the inhibitory effects of reproductive activity were observed in 8L:16D,

which reflects a short photoperiod. Experiment two showed no significant effects of differential intensity on body mass and bill color in females; however, bill color was slightly darker in the 50 and 100 lux groups. Gonadal growth was highest in the 100 lux group compared to the 10 lux and 50 lux groups in both sexes. In both the male and female hypothalamus, higher expressions of reproductive genes were observed in the 100 lux of light intensity group, followed by 50 lux, and lowest in the 10 lux intensity group. Similarly, the maximum expression of steroidogenic genes was observed in the 100-lux intensity group, then 50 lux, compared to 10 lux, both in the hypothalamus and testicular tissue of both sexes. Experiment four reveals the differential outcome of light spectra treatment on tree sparrows. In contrast to white light exposure, gonadal growth was more advanced in the red-light group. We did not observe any gonadal growth in the blue light group. Besides, the light spectra do not affect body mass and bill color in both sexes. Whereas we observed higher expressions of reproductive genes in the red-light group, in contrast to green and blue light. Also, maximum expressions of several steroidogenic transcripts were witnessed in the red-light group and were lower in the blue light group. Overall, the present study reveals that light intensity stimulates the gonadal growth in both male and female tree sparrows. Highest reproductive and steroidogenic transcript levels were observed at 100 lux intensity, indicating the positive influence on reproductive-linked steroidogenic processes. In addition, the study suggests that a long photoperiod modulates steroidogenic responses and a short photoperiod inhibits the steroidogenic functions in this species. Moreover, longer-wavelength (red light) has a stimulatory effect, while short-wavelength (blue light) has an inhibitory effect on photoperiodic responses in tree sparrows.

SECTION III: ANNUAL LIFE HISTORY STATE-DEPENDENT EXPRESSION OF STEROIDOGENIC GENES

The population responds to environmental variability, largely determined by the dynamic interactions between fitness components within- and among-individual variation in the expression of the environmentally sensitive phenotype. Seasonal breeders display elevated sex steroid hormone production during reproductive

seasons, resulting in significant physiological and structural alterations. The HPG axis, which is crucial to several processes like sex differentiation, gonad growth, and spawning, regulates the timing of reproduction and the production of sex steroids and steroidogenic enzymes play a vital role in the functioning of sex steroids. As avian follicle growth is primarily regulated by the genetic machinery of reproductive hormones and associated functions, steroid hormone synthesis and secretion play a dominant role. Besides, in males, it plays a crucial role in the testis and regulates spermatogenesis and spermiogenesis, or the maintenance of secondary sexual characteristics in vertebrates having seasonal reproductive cycles, moreover, the ultimate steroidal signal secreted by the tissue depends upon which steroidogenic enzymes are expressed. One such seasonal breeder adapted to the changing environment is the Tree sparrow. The present study advocates that no prior research has been conducted on this species concerning seasonal steroidogenic enzyme gene expression. The study's primary objective is to examine whether the steroidogenic enzyme gene expression varies annually in an adult male and female Tree Sparrow. Therefore, present the study aims to investigate annual variations in the expression of reproductive activity and linked steroidogenic gene markers of adult male and female tree sparrows and corresponding seasonal changes in the physiological characters in both sexes. In the experiment, birds (n = 5/month/sex) were sampled every month at midday for a year. Body mass, bill color, testis size, and molt in feathers were recorded. The hypothalamus and gonad tissues were harvested and kept at -80ºC and later used for gene expression studies. Blood plasma cholesterol, testosterone levels, and progesterone levels were measured. Higher testicular volumes were recorded from March to May, whereas maximum molt was observed during the post-breeding phase. Plasma cholesterol levels were highest before the breeding phase. Higher testosterone levels corresponded with the breeding phase. Higher expressions of *Tshβ*, *Dio2*, and *GnRH* during the breeding phase and higher expression of *Dio3* and *GnIH* were observed during the non-breeding phase. Similarly, in females, larger follicular size occurs during May and June. Whereas, maximum molt was observed during the post-reproductive phase. Cholesterol levels were highest before the breeding phase, and higher progesterone levels paralleled larger follicular size. While higher levels of *GnIH* and *Dio3* were observed during the non-breeding phase,

elevated expression of *Tshβ*, *Dio2,* and *GnRH* was noted during the reproductive period. The steroidogenic transcripts showed seasonal changes in their expression in the hypothalamic and gonadal tissue and were upregulated either during the pre-breeding or breeding phase. The study reveals that mRNA levels of steroidogenic transcripts (*StAR, Nr4a1, Er, Scp2, Cyp17a1, Foxl2, Cyp11a1, Hsd11b2, Cyp11b, Cyp19a1,* and *Vdac1*) show seasonal variations that correspond to the annual reproductive cycle of both male and female tree sparrows. Seasonal variations in these genes' regulation in the brain and gonads imply the possibility that seasonal distinctions in gene expression control tissue-specific steroidogenic capacity. Moreover, the present study advocates for male and female tree sparrows with the conserved mechanism. In addition, the tree sparrow's conjunctive mechanism for the growth and regulation of the gonadal system is shown by the seasonal variations in the stimulation of both androgenic and estrogenic pathways during the reproductive phase in the steroidogenic process.

SECTION IV: EFFECT OF SHORT-TERM EXPOSURE TO DIM LIGHT AT NIGHT ON STEROIDOGENESIS

Anthropogenic factors, particularly urbanization, have immense implications on the natural light-dark transition cycle of the species. In addition, since the majority of birds breed seasonally and rely on environmental cues to time their seasonal activities, any disruption to this link has a detrimental impact on the birds' physiological processes. The circadian system regulates homeostatic functions in many organisms, and Circadian rhythms are naturally occurring 24-hour rhythms in physiology and behavior that are most strongly coordinated by light information. It is increasingly becoming clear that disrupting the circadian clock with exposure to light at night has substantial physiological consequences. The present study deliberates on the effects of dim light during the night hours on the transcript expression of reproductive, steroidogenic, and metabolic gene markers in adult male tree sparrows. Adult male tree sparrows were procured using a mist net, and after procurement immediately brought into the laboratory. Later, birds were categorized into two groups (n=6 birds/group). Group 1 was exposed to 12 hours of light and 12 hours of darkness, and also served as a control. Group 2 was also exposed to 12 hours of light

and 12 hours of darkness, but with a constant dim light (10 lux) during the dark hours. The experiment was run for 2 weeks. At the end of the experiment, birds were sampled, and the hypothalamus, liver, and testis tissue were harvested and used for gene expression analysis. Blood plasma was used for hormonal and biochemical assays. The findings suggest that 2 weeks of exposure did not significantly change the body mass, plasma cholesterol, plasma glucose, and plasma testosterone levels. However, an increase in testicular volume was observed in dLAN-treated birds. Furthermore, elevation in hypothalamic transcripts (*Tshβ, Dio2, GnRH, and Eya3*) involved in seasonal reproduction, along with an increase in steroidogenic genes (*StAR, Scp2, Srd5a1, Hsd11b2*, and *Er*) in the testis, was observed; besides, liver metabolic transcript levels (*Acaca, Fasn, Hmcg, Idh2, Sdhaf4, Sdhaf2, Sdhc, Fh, Mdh, Foxo1, and Vip*) were also elevated in the dLAN-treated group compared to the control group. The study reveals that even a short-term exposure of 2 weeks duration to the lower intensity of dim light at night of 2 weeks can stimulate the hypothalamic gonadal axis with enhanced steroidogenic activity and liver metabolism in tree sparrows. These findings may help us comprehend how nighttime light affects the physiology of diurnal species.

SECTION V: EFFECT OF TEMPERATURE ON STEROIDOGENESIS

Day length is one of the most consistent environmental factors on which animal depends for breeding and survival. Apart from the photoperiod (day length), other factors such as food availability and temperature can also affect their physiology and behaviour. Also, the environment is changing, as well as urbanization and city growth, both drastically increasing. Moreover, the changing environment over the past few decades has altered the phenological swings in occurrences across trophic levels. Also, an important environmental change that has altered biological systems is a temperature rise. Temperature variation in the spring might shift many tropical birds' breeding time. The direct or indirect effects of temperature on photoperiodically induced gonadal development remain unclear. Under normal environmental circumstances, the cycles of temperature and duration are inseparable. Moreover, day length alone may not be enough to control seasonal behaviors; temperature may also play a role alongside day length. Therefore, present

study hypothesized that exposure to high and low temperatures at the photorefractory stage affects photoperiodic responses during the photo-stimulatory phase. For this study, adult male photorefractory birds were captured using a mist net. Birds (n=10/group) were divided into two groups. Group one was exposed to short photoperiod (8L:16D) but either high (30 ± 2ºC) or low temperature (20 ± 2ºC) for five or seven weeks and then later transferred to a long photoperiod of (16L:8D) for another 30 days. Group two was also exposed to high and low temperatures as group one, but the duration of short photoperiod was maintained for 7 weeks, followed by a long photoperiod of (16L:8D) for another 30 days. At the end of the experiment, all birds were sampled in the middle of the light phase. Brain and testis tissue were harvested for gene expression analysis. Body mass and testicular volume were recorded before and after the end of the experiment. The result reveals no significant changes in the body mass of both the 5-week and 7-week groups. However, a significant increase in the testicular volume was observed in the low temperature-treated group under the 5-week SD exposure. Also, mRNA levels of *Tshβ*, *Dio2*, *Dio3*, *GnRH*, *GnIH*, and *Eya3* were measured in the hypothalamus, and expression levels of *StAR*, *Er*, *Cyp17a1*, *Cyp11b*, *Foxl2*, and *Nr4a1* were measured in the testes. Exposure to 5 weeks of high temperature coupled with short photoperiod suppresses expression of *Tshβ*, *Dio2*, and *GnRH* in the hypothalamus and *StAR*, *Er*, *Cyp17a1*, and *Cyp11b* in the testes under long days. No such effects were observed in birds exposed to high temperatures along with SD for seven weeks. The study findings suggest that exposure to low temperature during the photorefractory stage regulates photoperiodic responses during the photo-stimulatory stage in time time-dependent manner.

SECTION VI: EFFECT OF CORTICOSTERONE TREATMENT ON STEROIDOGENESIS AND REPRODUCTIVE-LINKED PROCESSES

In all the avian species documented to date, there is immediate activation of adrenocortical tissue in response to a range of stressor stimuli (e.g., malnutrition, handling, restraint, severe temperature, etc.) that leads to a rise in circulating corticosterone. Corticosterone is the primary glucocorticoid in avian species which is

secreted by the adrenal cortex of the adrenal gland and in the in response to environmental stressors regulates the hypothalamic-pituitary-adrenal (HPA) axis, leading to physiological, reproductive, and behavioural changes. Studies of avian adrenocortical cells have shown that adrenocortical steroidogenic activity differs with avian species and age. In response to stress, Corticotropin-releasing factor (CRF) from the hypothalamus excites the secretion of ACTH from the pituitary gland. ACTH, which is the predominant stimulus for avian corticosteroid secretion is and causes a rapid, dose-dependent, receptor-mediated release of corticosteroids from the avian adrenal glands. In contrast, Melatonin is a broad-spectrum antioxidant, a neurohormone, predominantly produced by the pineal gland in the brain, which is a key modulator of circadian rhythms, seasonal reproduction, and responses to environmental cues such as photoperiod. Its level fluctuates, being highest in the dark and lower during the day, having a rhythmic pattern in the LD. Melatonin's antioxidant qualities have been extensively established in the past recent years and it is synthesized in the dark hours of a light/dark transition, is thought to play a vital role in providing defense against the free radicals' toxic effects. Corticosterone and melatonin impacts have been extensively studied in several species involved in behavioural, reproductive, and metabolic processes. However, limited studies have been conducted on the effect of corticosterone treatment and counter-regulatory effects of melatonin hormone on reproductive-linked steroidogenic gene expression in the tree sparrows. Therefore, the study investigates the effect of corticosterone-induced stress response in steroidogenesis and the counter-effect of the melatonin treatment in the adult male tree sparrows and examined the effect of corticosterone on several reproductive and steroidogenic gene expressions in this species. For this study, we procured photorefractory adult male tree sparrow in the month of September. Physiological parameters such as body mass, bill color, and testicular volume were recorded before and after the end of the experiment. Later, the birds (n=7/group) were divided into three groups. Group one was treated with normal ethanolic saline, which served as a control. Group two was treated with corticosterone (2 µg/100 µl), and Group three was treated with corticosterone (2 µg/100 µl) coupled with melatonin (10 µg/100 µl). All the groups were exposed to 8L:16D (8 hours of light and 16 hours of darkness), and the

experiment was run for 4 weeks. Here, melatonin treatment was given half an hour before the light switch-off time. After 4 weeks of the respective treatment, all the groups were again exposed to 16L:8D for another 30 days. At the end of the experiment, all the birds were sampled in the middle of their light phase, and brain and testis tissue were harvested and kept at -80°C for gene expression analysis. The study reveals no variation in the body mass and bill color; however, testicular volume was significantly increased at the end of the experiment. Besides, downregulation of the reproductive genes (*Tshβ, Dio2, GnRH,* and *Eya3*) was observed in the corticosterone-treated group, whereas upregulation of these genes was observed in the corticosterone coupled with melatonin treatment group and the control group. Also, several steroidogenic genes (*StAR*, *Nr4A1, Scp2, Vdac1, Cyp11a1*, *Cyp17a1*, *Cyp11b*, *Hsd11b2*, *Er* and *Srd5a1*) were also observed to be downregulated in the corticosterone-treated group compared to the corticosterone coupled with melatonin and control group. These results reveal a negative effect of corticosterone on reproductive activity and steroidogenic activity in this species. However, elucidates the counter-regulatory effects of melatonin hormone on corticosterone-induced alterations in male tree sparrows, and melatonin co-treatment effectively reverses corticosterone effects for most genes involved in reproduction and steroidogenesis, restoring expression levels, thus highlighting its protective role against stress-induced reproductive and steroidogenic suppression.