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ORIGINAL PAPERS

Hypothalamic expression of *GnRH-I* and *GnIH* in the Eurasian tree sparrow over a single long day

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Abstract

Seasonal reproductive cycles of most birds are regulated by photoperiod via neuroendocrine control. The present study aims to investigate the role of a single long day in triggering hypothalamic expressions of *GnRH-I* and *GnIH* in the Eurasian tree sparrow (*Passer montanus*). Sparrows were divided into two groups ($n = 24$ each) and pre-treated under short days (9L: 15D) for 4 days. On the fifth day, one group was exposed to long day (14L: 10D), while other was continued under short day for another 1 day. Birds of both the groups were sacrificed and perfused on fifth day at different time points, i.e., ZT 14, ZT 16 and ZT 18 and the expressions of *GnRH-I* and *GnIH* mRNAs and peptides were studied using real-time PCR and immunohistochemistry, respectively. In addition, testicular size was measured to know testicular development. Observations revealed that birds exposed to a single long day (14L: 10D) showed an increase in hypothalamic expressions of *GnRH-I* mRNA and peptide and decrease in levels of *GnIH* mRNA only at ZT 16 and ZT 18 with no significant change in *GnIH* peptide. However, no significant change in *GnRH-I* or *GnIH* expression was observed at any time point under short day and birds maintained high and low expression levels of *GnIH* and *GnRH-I*, respectively. Our results clearly indicate that the photoperiodic response system of sparrow is highly sensitive to light and responds even to single long day. Furthermore, they suggest that the *GnRH-I* and *GnIH* are expressed in the hypothalamus of tree sparrow in an anti-phasic manner and switching over of their expression occurs at late hours of exposure of birds to single long day.

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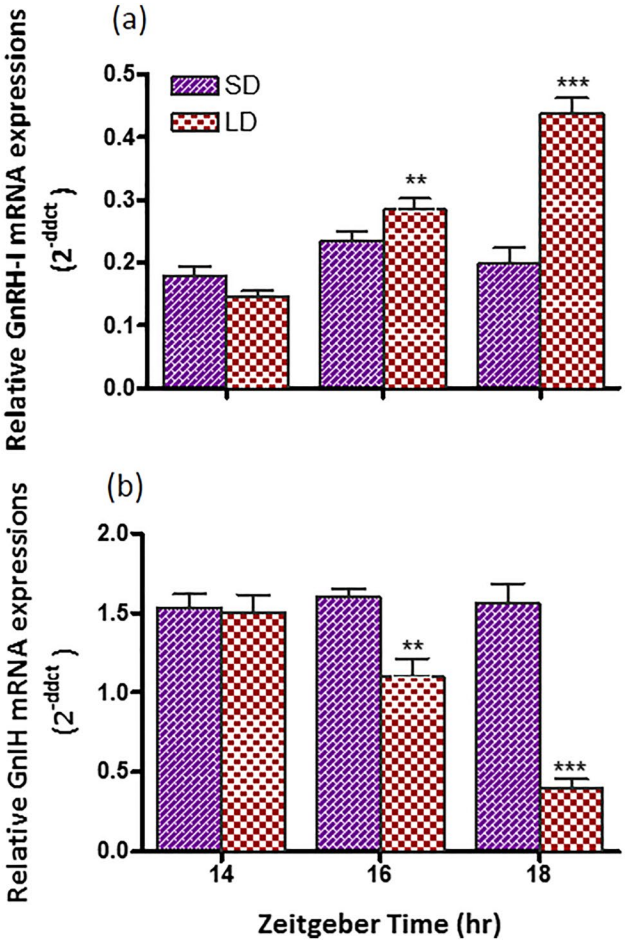
Graphical abstract



Keywords GnRH-I · GnIH · Tree sparrow · Day length · Expressions

1 Introduction

The day length measurement is extremely crucial for photoperiodic birds for timing of life history stages such as reproduction, molt and migration [1–3]. To avoid physiological conflict, the above stages are temporally spaced and closely coupled [4, 5]. Majority of birds use day length as the most reliable environmental information to predict favourable season and to begin physiological preparations in advance of the conducive conditions in the environment for successful reproduction [6–9]. The role of day length as a primary environmental factor regulating seasonal reproduction is well established in a variety of avian species belonging to mid as well as high latitudes [2, 10]. The day length triggers neuroendocrine system of birds and initiates reproduction by subsequently activating the hypothalamus–pituitary–gonad (HPG) axis.

Some investigations directed to uncover the neuroendocrine mechanisms involved in photoperiodic regulation of reproductive responses have yielded significant results [11, 12]. The mediobasal hypothalamus (MBH) has been suggested as the site of photoperiodic induction in birds [13–15]. Long photoperiod stimulates synthesis of thyroid stimulating hormone beta (TSH-β) in the pars tuberalis leading to increase in production of type-2 iodothyronine deiodinase (DIO2) that converts tetraiodothyronine (T_4) to triiodothyronine (T_3). T_3 changes the structural arrangement in the nerve terminals of gonadotropin-releasing hormone-I (GnRH-I) expressing neurons at the median eminence causing retraction of glial end feet encasement around GnRH nerve terminals, thus, allowing access of nerve terminals to the basal lamina as well as portal blood supply to the pituitary. GnRH-I further stimulates gonadotropins (luteinizing hormone, LH and follicle-stimulating hormone, FSH) synthesis and release from the anterior pituitary causing

seasonal variation in reproductive physiology, gonadal development and behaviour in birds. Another neurohormone, known as gonadotropin-inhibitory hormone (GnIH), has been reported to exert gonado-inhibitory effects [16]. It acts as a neuroendocrine integrator of photoperiodic cue and regulates gonadotropins secretion to time avian seasonal reproduction [15, 17–19]. GnIH acting on the anterior pituitary and GnRH-I neurons inhibits the synthesis and release of gonadotropins in a direct and indirect manner, respectively, leading to gonadal regression and inhibition of reproductive behaviours. Thus, GnRH-I and GnIH are two significant components of the neuronal circuitry in the avian brain that play important role in the regulation of their reproductive responses.

The "Single long-day induction paradigm" is quite useful in uncoding the photoperiodic molecular circuitry at the level of brain in birds. A recent study, using this paradigm on white-throated sparrows, *Zonotrichia albicollis*, revealed an activation of GnRH-I neurons after exposure to just one long day [20] thereby indicating the rapidness of photoperiodic induction. Another study reported a significant rise in plasma LH in Japanese quail, *Coturnix coturnix japonica* exposed to single long day at 18 or 20 h after lights-on [21, 22]. Also, significant differences between Swedish and German great tit populations have been reported in the expression of key genes involved in measurement of day length (*PER2, CRY1*) and photoperiodic response (*DIO2, DIO3, GnRH* and *FSH-b*) on exposure to a single long day [3]. Furthermore, *EYA3* and *TSH-β* were induced at 14 h as the first-wave genes in the pars tuberalis (PT) followed by induction of the second-wave genes including *DIO2* at 18 h in ependymal cells (ECs) lining the third ventricle on exposure of Japanese quail to the first long day [14]. In spite of these studies, it still remains unclear whether a single long day can trigger hypothalamic expressions of above genes sufficient enough to activate and/or inhibit the downstream events via pituitary in control of seasonal reproduction across avian species.

Our understanding of the molecular mechanism involved in photoperiodic control of seasonal reproduction in birds is still in its infancy due to limited investigation at the mechanistic level [23, 24]. Although reports are available on photoperiodic induction of expression of few molecules under long day, only little is known about the immediate effect of single long day in triggering the expressions of two very important hypothalamic neuropeptides, the GnRH-I and GnIH in birds [3, 14, 25]. Furthermore, the earlier studies were mainly confined to birds of temperate regions and were conducted especially on migratory species. The investigations on birds inhabiting tropical or sub-tropical regions, particularly involving local resident species, are fewer in view of large number of birds inhabiting these latitudes. In the light of the above, it would be interesting to carry out experiments on widely distributed and resident avian species that occupies temperate as well as tropical or sub-tropical regions [26]. Therefore, the present study was carried out on the Eurasian tree sparrow which is a typical multiple-brooded species and native to the Eurasian continent [27, 28]. It is an intensively studied resident species having broad distribution covering different latitudes and exposed to various environments of the Eurasian continent [29].

Therefore, this study aims to investigate the expressions of two important hypothalamic neuropeptides, i.e., GnRH-I and GnIH during exposure to single long day in the sub-tropical population of the Eurasian tree sparrow (*Passer montanus*) at Shillong. In our previous investigations, we have reported that the tree sparrows exhibit a seasonal reproductive cycle. The gonadal growth, in sparrows, is triggered by the increasing day lengths of spring (March) and the gonadal regression sets in during summer months (June) after gonads attain peak growth in May indicating the onset of photorefractoriness. The tree sparrows are photoperiodic and use long day length (11 h/day or more) for initiation of gonadal growth. A minimum of 6-week exposure of sparrows to short day lengths terminates photorefractoriness [2, 9, 30]. Recent studies in our laboratory have revealed photoperiodic expression of hypothalamic neuropeptides, i.e., GnRH-I and GnIH in control of reproduction in the tree sparrows [15, 19, 31].

2 Experimental methods

2.1 Animal model

The Eurasian tree sparrow is a small, passerine bird of non-migratory nature which is abundant in the hilly regions of North-East India, including Shillong (Latitude 25°34′N, Longitude 91°53′E), Meghalaya, India. It has wide distribution covering different latitudes which include temperate and tropical/sub-tropical regions [32]. They are mostly present in the residential areas [33] and make nest in the roof cavities in houses, ceilings of verandas, cavities in trees, poles, fence posts, etc. Their food includes seeds, grains and insects.

2.2 Experiment

Adult male birds were captured from their wild habitat during the month of December when they remain in photosensitive stage of their annual reproductive cycle with quiescent gonads [9]. They were kept in an open outdoor aviary with an unrestricted availability of natural light, humidity and temperature. The birds were then brought indoors for acclimatization to laboratory conditions by exposing them to natural variations of temperature, photoperiod and humidity for a fortnight. The acclimatized birds were given a

pre-treatment of short day length (9L/15D) for 2 months to dissipate photorefractoriness, if the birds had any in nature and also to ensure their photosensitivity at the beginning of the experiment. The testicular size, recorded at 4-week intervals during the pre-treatment, revealed that the birds had quiescent testes throughout the period of pre-treatment. Experiments were performed using these photosensitive birds. The photosensitive birds ($n = 48$) were divided into two groups ($n = 24$ each) and both the groups were exposed for 4 consecutive days to short day length (9L/15D). One group was transferred to long day length (14L/10D), while the other group was continued under the short day length (9L/15D) for one complete day. The birds ($n = 8$ per observation each group) from both the groups were sacrificed and perfused in the dark phase at three different time points viz. ZT14, ZT16 and ZT18 (ZT0 = light on at 6 AM) on the same day for the measurement of mRNAs and peptides expressions of *GnRH-I and GnIH* using real-time PCR (qPCR) and immunohistochemistry, respectively. In addition, testicular volume was measured to record testicular development. In the photoperiodic experiments, birds were kept in wooden chambers (2.10 m × 1.20 m × 1.35 m) which were lightproof, well aerated by air circulators and illuminated by CFL bulbs with automated control of light on and off. Birds were provided food and water ad libitum which were replenished daily in the light phase.

2.3 Measurements

2.3.1 mRNA expression

For the study of *GnRH-I* and *GnIH* mRNA expressions, skulls of birds were removed after decapitation to expose their brains. The hypothalamus was taken out and stored at − 80 °C in TRIreagent (Ambion Inc., Cat No.74123) after cutting into pieces. The hypothalamic tissue was homogenized after thawing and the total RNA was extracted as per the TRIreagent manufacturer's protocol. The isolated total RNA was dissolved and suspended on DEPC-treated water and Nanodrop was used to assess the purity. The cDNA synthesis kit (Thermo scientific, Verso, Cat. No. AB1453A) was used to reverse transcribe 1 µg of total RNA to cDNA.

Gene-specific primers of *GnRH-I* (forward: 5′- TGGAGA AATTAGAGGAGGAGCA-3′) and (reverse: 5′-CATGGC TTCCTTCAGAGCC-3′), *GnIH* (forward: 5′-TGGAGA GCAGAGAAGACAATGATG-3′) and (reverse: 5′- TGT CTTTTGTTCCCCAGTCTTCCA-3′) and *β-Actin* (forward: 5′-GGATTTCGAGCAGGAGATGG -3′) and (reverse: 5′- GGGCACCTGAACCTCTCATT-3′) were designed from partial sequences available on GenBank (Accession Number: GnRH-I -MH427011, GnIH-KT351598 and β-Actin-KT351599) using Primer3 (freely available online software) for qPCR. The Oligo Analyzer 3.1 was used to check the primer efficiency, its dimer and hairpin. A 7500 real-time PCR system (Applied Biosystems) was used for performing quantitative expression of *GnRH-I* and *GnIH* mRNA. Power SYBR® Green (Applied Biosystems, Cat. No.1301388) was used for carrying out amplification of the *GnRH-I* and *GnIH* genes in the PCR reaction. For the above, the detailed procedure as described in Majumdar et al. [34] were followed.

2.3.2 GnRH-I and GnIH immunohistochemistry

GnRH-I and GnIH peptides expression levels were measured in terms of the number, area and density of neurons expressing them using immunohistochemistry. Birds were deeply anaesthetized using ketamine-xylazine solution (0.003 ml/g body weight). The transcardial perfusion of birds was done using ice-cold saline (pH 7.4) water which was followed by 4% paraformaldehyde solution (0.1 M phosphate buffer at pH 7.4). The whole procedure was performed under the dark phase of the light–dark cycle. Then the birds' brains were quickly removed and kept in paraformaldehyde solution at 4 °C for overnight. Thereafter, the brains were cryoprotected by transferring them to various grades of sucrose solutions viz. 10%, 20% and 30% at 4 °C. Then the brain tissue was stored in 15% polyvinylpyrrolidone solution (PVP, Himedia) at − 80 °C for future use. These tissues were thawed and then mounted using 15% PVP on the cryostat tissue holder. The brain sectioning was done in the coronal plane at 30 µm thicknesses using cryostat (Leica CM 1850). The processing of brain sections for immunohistochemistry of GnRH-I and GnIH was done using the protocol described in Rastogi et al., [35] with some modification. The diaminobenzidine (DAB) and nickel (DAB 4100, Vector labs.) were used for staining GnRH-I (brownish-red) and GnIH (dark-blue) peptides, respectively, in the tissue sections. The primary antibodies for GnRH (HU60 bleed) and GnIH (anti-quail serum) used in this study were kindly gifted by Dr. Henryk F. Urbanski, Oregon Health and Sciences University, USA and Dr. K. Tsutsui, Waseda University, Japan, respectively. The GnIH anti-quail serum at 1: 20,000 dilutions was used for the detection of GnIH peptide. This serum cross-reacts with GnIH of house sparrow (*Passer domesticus*) and song sparrow (*Melospiza melodia*) [36]. Moreover, some previous studies have already demonstrated the specificity of this antibody [15, 16, 19, 37–39]. The primary antibody for GnRH, HU60 bleeds, was used at 1: 18,000 dilution. This antibody has high specificity for GnRH and was generated against mammalian GnRH in rabbit [39, 40]. The detailed nature of above antibody is described in Urbanski et al. [41] and Urbanski [42]. The specificity of both the above antibodies has already been tested in our earlier studies on tree sparrow [15, 19, 31]. However, the specificity of the immunoreactions was tested through control procedures that involved the

removal of the primary antisera from the reaction and also its replacement by buffer or BSA. There was total loss of immunoreactivity in both the above procedures [15, 19, 31].

The desired brain sections were examined using a trinocular bright-field microscope (Motic) and the digital images of immunoreactive (-ir) cells were captured by a high megapixel camera (Motic cam) at 10 × and 40 × magnifications using standard illumination. Motic image version 2 analyzer software was employed to adjust size, contrast and brightness of the captured image as per requirement [19]. The counting of GnRH-I-ir and GnIH-ir cells were done in the entire preoptic area (POA) and paraventricular nucleus (PVN) regions, respectively. As an additional measures for immunoreactions, the recording of % cell area, cell area and cell optical density (OD) were also done [38]. The cell number and % cell area signify density, while cell area and cell OD denote peptide content of particular neuronal population identified by antibody [43]. The counting of cell number and measurement of % cell area, cell area and cell OD were done using ImageJ (NIH) software following the procedures described in Surbhi et al. [44].

2.3.3 Testicular volume

The left testes was located with the help of a spatula by opening abdominal wall and the testicular volume (TV) was recorded in situ after removal of brain from the birds for immunohistochemical studies. The testicular length and width were measured using a calliper and the calculation of testicular volume was done by the formula $4/3ab^2$, where a and b indicate half of the length and width, respectively.

All protocols employed in this study were approved by the Institutional Animal Ethics Committee, North-Eastern Hill University, Shillong (1886 of 04. 12. 2014).

2.3.4 Statistical analyses

The data, presented as mean ± S.E.M, were analysed employing two-way ANOVA followed by Newman-Keul's multiple comparison post hoc test in cases where ANOVA indicated a significance of difference. Significant difference was taken at 95% confidence level. For all statistical analyses, a Graph Pad Prism software (version 6.0, Sandiego, CA, USA) was used.

3 Results

Significant variation in the hypothalamic expression of GnRH-I mRNA was noticed in the tree sparrow exposed to short day (SD) and long day (LD) lengths for a single day (Table 1, Fig. 1a). A significant and gradual increase in GnRH-I mRNA expression was noticed at ZT 16 ($P < 0.01$) and ZT 18 ($P < 0.001$) when compared to the expression at ZT 14 under LD. The expression at ZT18 was significantly higher ($P < 0.001$) than that of ZT16. However, no significant change in the expression of GnRH-I mRNA was observed and the birds maintained low levels of expression throughout their exposure to SD (Fig. 1a). A significant variation was also observed in the expression of GnRH-I peptide in the POA of tree sparrows exposed to single SD and LD in cell number, cell area, % cell area and relative cell OD (Table 1, Figs. 2a–d and 3a–f). Although the sparrows maintained under single LD showed a remarkable increase in GnRH-I peptide expression under ZT 16 and ZT 18, a significant increase was noticed only at ZT18. The number of GnRH-I cells increased significantly ($P < 0.01$) in the birds at ZT 18 under LD. Furthermore, the changes in % cell area ($P < 0.01$), cell area ($P < 0.05$), and relative cell OD ($P < 0.01$) of GnRH-I-ir cells were found similar to the trend noticed in GnRH-I-ir cells number in the POA of the hypothalamus (Figs. 2a–d and 3d–f). The above observations reveal that the exposure of sparrows to even a single long day (LD) is sufficient to trigger GnRH-I mRNA and peptides expressions at the later hour i.e. ZT 16 and ZT 18. On the other hand, birds receiving continued exposure to short day length (9L/15D) for an additional day (control group) failed to exhibit significant change in all the above parameters of GnRH-I expression at all the three observed time points viz. ZT 14, ZT 16 and ZT 18 ($P > 0.05$) and they maintained almost constant low level of GnRH-I (Figs. 2a–d and 3a–c).

There was a significant variation in GnIH mRNA expression in the tree sparrows exposed to single LD and SD (Table 1). A compression of *GnIH* mRNA expression under SD and LD at various time points of observation revealed a significant decline in the expression level only at ZT16 ($P < 0.01$) and ZT18 ($P < 0.001$) while no significant change was noticed at ZT 14. Furthermore, there was a significant decrease in the *GnIH* mRNA expression at ZT16 ($P < 0.01$) and ZT18 ($P < 0.001$) when compared to the expression at ZT14 in the birds exposed to single LD. Besides, the expression level at ZT18 was significantly lower ($P < 0.01$) when compared to the expression at ZT16 under single LD. On the contrary, a significantly high level of *GnIH* mRNA expression was maintained at all observations under single SD with the birds showing no significant difference in *GnIH* expression levels at various observations (Fig. 1b). Furthermore, no significant difference in the GnIH peptide expression was observed in the birds exposed to single LD and SD at any time point of observation and in any parameter including cell number, cell area, % cell area and relative cell OD (Table 1. 3 g-l). Unlike GnRH-I peptide, the expression of GnIH peptide under single LD did not show any significant differences ($P > 0.05$) when compared to the expressions under single SD at all the three time points of observation (i.e. ZT 14, 16 and 18) suggesting that the GnIH peptide expression might take longer

Table 1 Summary of two-way AVOVA for the hypothalamic expression of GnRH-I and GnIH under single long day

Parameters	Source of variations	Degree of freedom	*F* value	*P* value
Hypothalamic expression of *GnRH-I mRNA*	Photoperiod	1	31.64	0.001
	ZT	2	35.41	0.001
	Photoperiod * ZT	2	28.45	0.001
Expression of GnRH-I peptide in the POA (Cell No.)	Photoperiod	1	7.408	0.018
	ZT	2	0.987	0.040
	Photoperiod * ZT	2	1.283	0.031
Expression of GnRH-I peptide in the POA (Cell Area)	Photoperiod	1	3.803	0.074
	ZT	2	7.403	0.049
	Photoperiod * ZT	2	3.806	0.052
Expression of GnRH-I peptide in the POA (% Cell Area)	Photoperiod	1	14.74	0.002
	ZT	2	1.558	0.250
	Photoperiod * ZT	2	4.926	0.027
Expression of GnRH-I peptide in the POA (OD)	Photoperiod	1	4.838	0.048
	ZT	2	2.216	0.151
	Photoperiod * ZT	2	4.615	0.032
Hypothalamic expression of *GnIH mRNA*	Photoperiod	1	52.02	0.001
	ZT	2	16.08	0.001
	Photoperiod * ZT	2	17.52	0.001
Expression of GnIH peptide in the PVN (Cell No.)	Photoperiod	1	0.010	0.366
	ZT	2	0.814	0.600
	Photoperiod * ZT	2	0.504	0.297
Expression of GnIH peptide in the PVN (Cell Area)	Photoperiod	1	0.094	0.764
	ZT	2	0.195	0.825
	Photoperiod * ZT	2	0.316	0.734
Expression of GnIH peptide in the PVN (% Cell Area)	Photoperiod	1	0.172	0.685
	ZT	2	0.223	0.803
	Photoperiod * ZT	2	1.201	0.334
Expression of GnIH peptide in the PVN (OD)	Photoperiod	1	0.017	0.896
	ZT	2	0.265	0.771
	Photoperiod * ZT	2	0.174	0.842
Testicular Volume	Photoperiod	1	0.164	0.299
	ZT	2	0.616	0.910
	Photoperiod * ZT	2	3.979	0.404

time and may occur at the later hours of exposure to single long day. There was no significant change in the testicular size of tree sparrows under single LD and SD at all observed time points and the testes remained quiescent throughout the experiment (Table 1). Furthermore, a negative correlation was found between relative mRNA expressions of GnRH-I and GnIH in the hypothalamus of the tree sparrows exposed to single SD and LD (Fig. 4).

4 Discussion

Our results clearly suggest that a single long day (LD) is sufficient to trigger the expressions of GnRH-I and GnIH genes through involvement of an endogenous circadian rhythm in the tree sparrow. Earlier studies on photoperiod-induced GnRH release in birds provide evidence about the

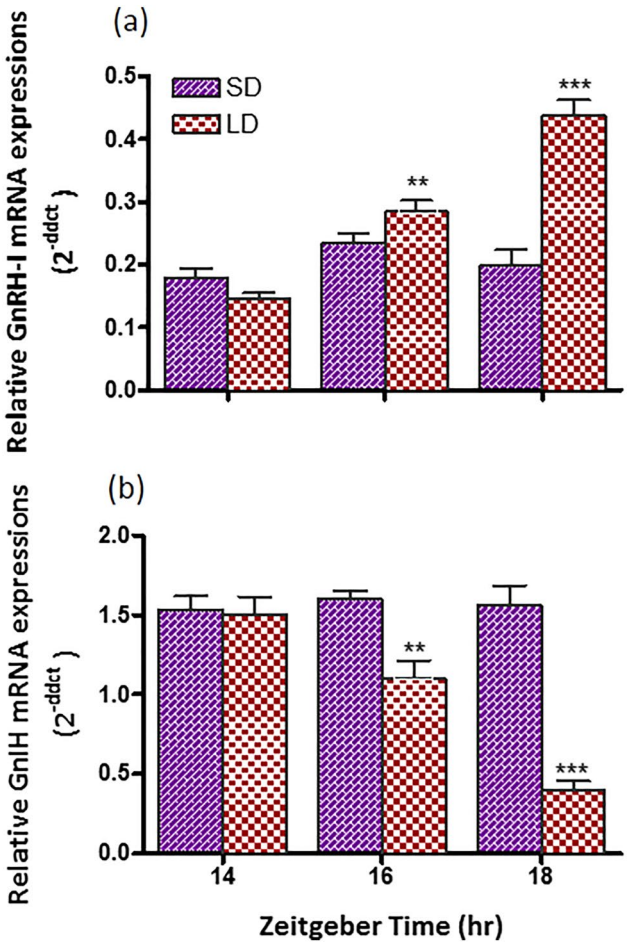


Fig. 1 Relative expressions of *GnRH-I* (**a**) and *GnIH* (**b**) mRNAs at various time points of observation in the tree sparrows exposed to single long day (LD) and short day (SD). Zeitgeber time (ZT0 = 6 AM) $^{**}P < 0.01$, $^{***}P < 0.001$

active role of GnRH axon terminals and less involvement of somata of the medial septo-preoptic area in response to photic cues [23, 45, 46]. However, recent studies suggest an active role of GnRH cell bodies in response to photostimulation [20, 47]. Our observations on the GnRH-I expression in the tree sparrow are consistent with the findings reported in the white-throated sparrows (*Zonotrichia albicolis*), in showing activation of GnRH neurons when subjected to single long day. Similar results were also obtained in chicken (*Gallus gallus*) and turkey (*Maleagris gallapavo*) where GnRH transcription and synthesis increased after exposure to a single long day [48–50]. Interestingly, studies suggest that single long day does not directly activate GnRH neurons instead it first activates the immediate early genes (IEG) that lead to GnRH activation. In Japanese quail (*Coturnix coturnix japonica*) and white-throated sparrows, single long day can cause upregulation of the protein products of IEGs in the MBH adjacent to the GnRH axons terminals. After exposure to light for a single long day, immunoreactivity of two IEG (Fos and Egr-1) proteins increased in GnRH neurons in the septo-preoptic area [20, 51]. But, the separate distribution of c-Fos and GnRH neurons in the POA of migratory redheaded bunting (*Emberiza bruniceps*) suggests that GnRH neurons are not directly photoresponsive in this migratory species [52]. Our observations in the present study exhibited an increase in the expression *GnRH-I* mRNA as well as in all the measures of GnRH-I immunoreactivity including cell number, % cell area, cell area and cell OD in the POA at ZT 16 and ZT18 under single long day when compared to single short day in the tree sparrow. Similar observations were also recorded in Indian weaver bird (*Ploceus philippinus*), that exhibits *GnRH-I* mRNA expression at ZT 20 on exposure to single long day [53]. The increase in GnRH expression upon exposure to single long day in typical photoperiodic birds including the tree sparrow could be explained in two possible ways. First, the direct integration of photic cues with the GnRH neurons could lead to the photoperiodic induction. Second, there could be an involvement of machinery (EGR-1) required to replenish the GnRH release during early response. The tree sparrow exhibit an enhanced expression of GnRH-I at late hours i.e., ZT 16 and ZT 18 on exposure to single long day but not in a single short day. This reveals the robustness of photoperiod in inducing molecular expressions involved in regulation of seasonal reproduction in the tree sparrow.

The tree sparrows showed a significant decrease in the expression of GnIH mRNA in the PVN area of hypothalamus only at ZT16 and ZT18, while the expression of GnIH peptide remained unaffected. Srivastava [54] reported that the spotted munia (*Lonchura punctulata*; a circannual species) do not show any difference in *GnIH* mRNA expression at ZT 4 and ZT 20 when exposed to single long day. However, Indian weaver birds (a relative photorefractory bird) exhibit higher *GnIH* mRNA level at ZT 4 as compared to ZT 20 under first long day. These findings together with our results on the tree sparrow, an absolute photorefractory species, that shows higher GnIH mRNA expression only during late hours of the day i.e., ZT 16 and ZT 18 suggest that the photoperiodic responses to changes in day length differs among circannual, relative photorefractory and absolute photorefractory birds. However, their responses seem to depend on both the transcriptional and translational levels of the first long day. The single long day failed to induce any significant change in the expression of GnIH peptide in the tree sparrows. The possible explanation for this can be given in two ways. First, transcription and translation are not coupled in eukaryotes and translation begins after a lag period once the transcription is over. Therefore, significant variation in GnIH-immunoreactivity might be observed in later hours. Unfortunately, in the present study, the GnIH

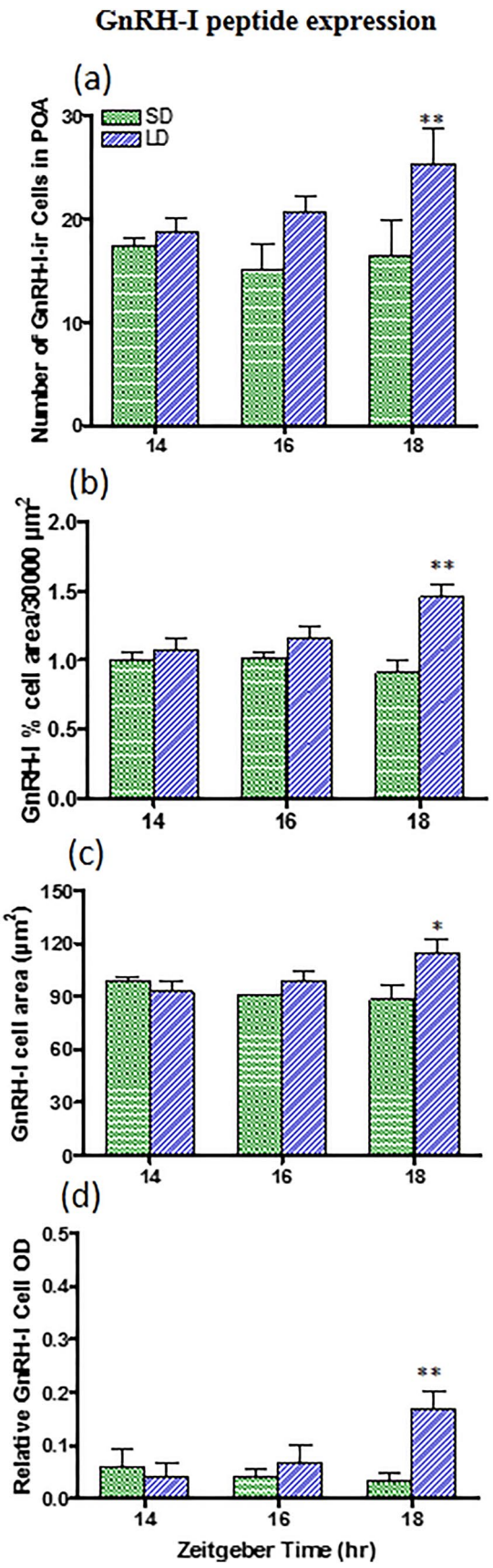


Fig. 2 Expression of GnRH-I peptide in terms of cell number (**a**), % cell area (**b**), cell area (**c**) and relative cell OD (**d**) in the preoptic area (POA) of hypothalamus of the tree sparrows exposed to single long day (LD) and short day (SD). *ZT* Zeitgeber time (ZT0 = 6 AM). $^{*}P < 0.05$, $^{**}P < 0.01$

peptide expression was not recorded beyond ZT 18. Second, it could be that a single long day is sufficient in triggering the GnIH expression only at the transcriptional but not at translational level in the tree sparrow. Furthermore, the decrease in *GnIH* mRNA level in tree sparrow coincided with an increase in *GnRH-I* mRNA and peptide at ZT 16 and ZT 18 (Fig. 1a, b). The negative correlation between *GnRH-I* and *GnIH* mRNA expressions under a single long day suggests their anti-phasic expression in the hypothalamus under both SD as well as LD (Fig. 4). The above observations support our earlier findings wherein the tree sparrows exposed to long (14L: 10 D) and short (9L: 15D) day lengths for 8 months and to the resonance light dark cycles exhibited an anti-phasic pattern of expression of *GnRH-I* and *GnIH* genes [19, 31]. Similar observations were also reported in the Japanese quail that shows opposite pattern for hypothalamic expression of *GnRH-I* and *GnIH*. Furthermore, the sexually regressed quails showed decreased hypothalamic *GnRH-I* and increased *GnIH* expressions [55]. Our observations on the tree sparrow suggest that there might be some initial changes at the upstream level in the hypothalamus upon exposure of birds to single long day that finally regulate *GnRH-I* and *GnIH* expressions in the POA and PVN, respectively. The "first day release" model has proven quite useful for better understanding of the photoperiodic molecular reactions taking place in the brain of few avian species including Japanese quail [14, 46], blackheaded bunting [38] and redheaded bunting [52].

In photoperiodic birds, the photoperiodic regulation of reproductive responses are mediated by a circadian rhythm of photoperiodic photosensitivity (CRPP) [6] that responds to light in a phase-dependent manner. Such a concept was originally formulated by Bunning [56] and involves the operation of an external coincidence model [57] which predicts that photoperiodic induction occurs when the light coincides with the photosensitive phase or more precisely photoinducible phase of an entrained endogenous circadian rhythm. In the present study, significant changes in the expression of both *GnRH-I* and *GnIH* were observed in the late hours of single long day i.e., ZT 16 and ZT 18 when the light coincided with the photoinducible phase of an entrained endogenous circadian rhythm. However, the failure of response at ZT 14 in the tree sparrow suggests that the response of above two genes might occur at the later hours of photoinducible phase upon exposure of birds to a single long day. The above results are consistent with our earlier finding

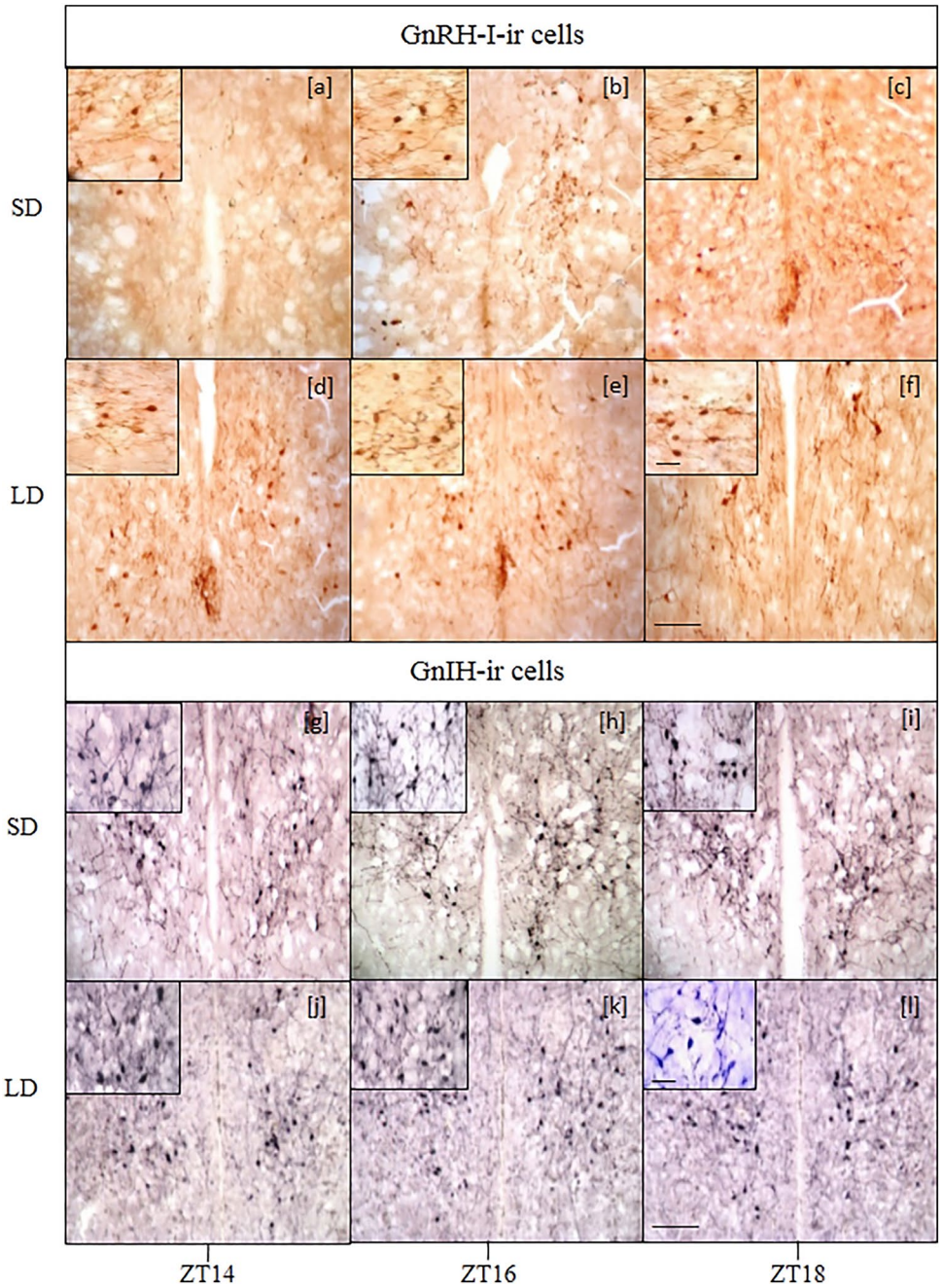


Fig. 3 GnRH-I (**a**–**f**) and GnIH (**g**–**l**) immunoreactivity in the preoptic area (POA) and paraventricular nucleus (PVN), respectively, in the hypothalamus of the tree sparrow exposed to single long day (LD) and short day (SD). *ZT* Zeitgeber time. Scale bar: general view = 200 µm, magnified view = 50 µm

on the tree sparrow using resonance protocol that suggested that an endogenous circadian rhythm is involved in photoperiodic expressions of *GnRH-I* and *GnIH* with a shift in their expressions depending upon whether the light falls in the photoinducible or non-photoinducible phase of an endogenous circadian rhythm [15]. Our findings on tree sparrow are consistent with the observation reported in female turkey (*Maleagris gallapavo*) in which GnRH neurons are sensitive to light stimulation only during the photoinducible phase [49].

5 Conclusions and perspectives

In conclusion, our findings suggest that the neuroendocrine machinery involving *GnRH-I and GnIH* expressions in the tree sparrow gets activated upon exposure of birds even to single long day. A significant increase and decrease in the expressions of *GnRH-I* and *GnIH*, respectively, at ZT16 and ZT18 in the hypothalamus of tree sparrow revealed that the initial response of these two genes might occur

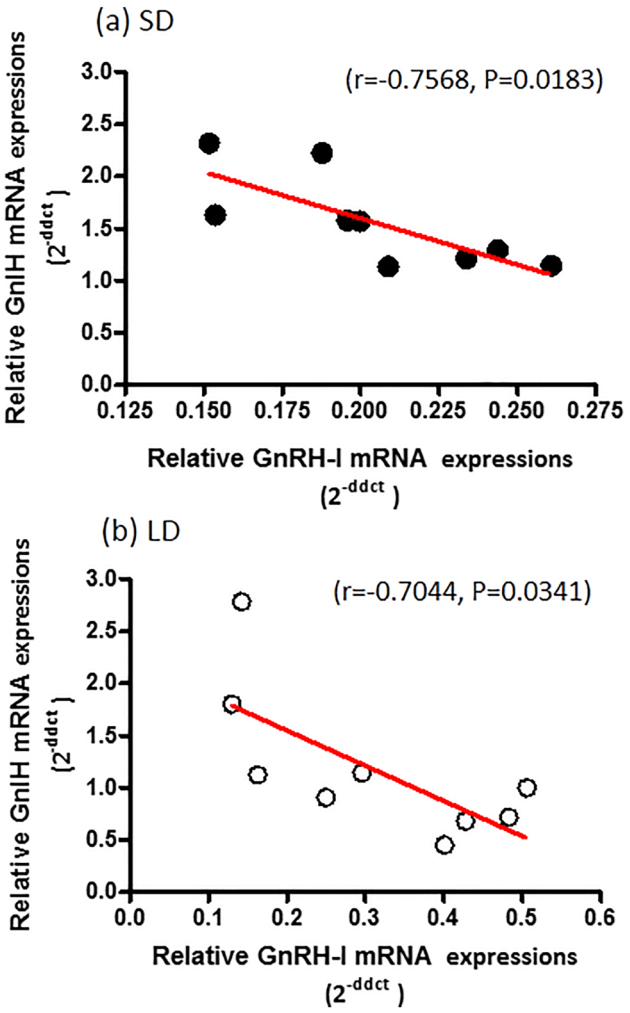


Fig. 4 Graph showing correlation between *GnRH-I* and *GnIH* mRNAs expression in the tree sparrows exposed to single short day: SD (**a**) and single long day: LD (**b**)

at the late hours of exposure to a single long day. Furthermore, the *GnRH-I* and *GnIH* express in an anti-phasic manner in the POA and PVN of the hypothalamus of tree sparrow, respectively, in control of the downstream events regulating seasonal reproduction. Thus, the tree sparrows exhibit photoperiod-induced changes in *GnRH-I* and *GnIH* expressions which are evident and measurable at transcriptional and/or translational levels during late hours upon their exposure to first long day. Furthermore, though a single long day is able to trigger the expressions of *GnRH-I* and *GnIH* genes, it is not sufficient to induce testicular responses in the tree sparrow. The present study, thus, contributes significantly to understand the mechanistic details of the photoperiodic regulation of the expression of two important components of nueroendocrine circuitry (i.e., GnRH-I and GnIH) in the brain controlling seasonal reproduction in the tree sparrow. However, further investigations aimed to define the photoinducible phase and interrelationships of the expressions of *GnRH-I*, *GnIH* and related genes involved in the molecular circuitry in the brain of tree sparrow would be interesting to completely reveal the mechanism of photoperiodic responses.

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Author contributions ASD conceived the idea, directed the research and written the manuscript. SB and BK performed the experiment and analysed the data.

Declarations

Conflict of interest The authors declare that there is no conflict of interest.

References

1. Paul, M., Zucker, I., & Schwartz, W. J. (2008). Tracking the seasons: The internal calendars of vertebrates. *Philosophical Transactions of Royal society B: Biological Sciences, 363*, 341–361.
2. Dixit, A. S., & Singh, N. S. (2011). Photoperiod as a proximate factor in control of seasonality in the subtropical male Tree Sparrow, *Passer montanus*. *Frontiers in Zoology, 8*, 1–12.
3. Perfito, N., Jeong, S. Y., Silverin, B., Calisi, R. M., Bentley, G. E., & 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*, 3499.
4. Kumar, V., Rani, S., & Singh, B. P. (2006). Biological clocks help reduce the physiological conflicts in avian migrants. *Journal of Ornithology, 147*, 281–286.
5. Dixit, A. S., & Singh, N. S. (2013). Participation of endogenous circadian rhythm in photoperiodic time measurement during ovarian responses of the subtropical tree sparrow, *Passer montanus*. *Indian Journal of Experimental Biology, 52*, 496–503.
6. Dawson, A., King, V. M., Bentley, G. E., & Ball, G. F. (2001). Photoperiodic control of seasonality in birds. *Journal of Biological Rhythms, 16*, 365–380.
7. Hau, M., Wikelski, M., Gwinner, H., & Gwinner, E. (2004). Timing of reproduction in a Darwin's finch temporal opportunism under spatial constraints. *Oikos, 106*, 489–500.
8. Bradshaw, W. E., & Holzapfel, C. M. (2007). Genetic response to rapid climate change: It's seasonal timing that matter. *Molecular Ecology, 17*, 157–166.
9. Dixit, A. S., & Singh, N. S. (2012). Seasonal variation in sensitivity of the photoperiodic response system in the subtropical tree sparrow (*Passer montanus*). *Journal of Experimental Zoology, 317*, 488–498.
10. Dawson, A. (2007). Seasonality in a temperate zone bird can be entrained by near equatorial photoperiods. *Proceedings of the Royal Society B: Biological Sciences, 274*, 721–725.
11. Ikegami, K., & Yoshimura, T. (2013). Seasonal time measurement during reproduction. *Journal of Reproduction and Development, 59*, 327–333.
12. Wood, S., & Loudon, A. (2014). Clocks for all seasons: Unwinding the roles and mechanisms of circadian and interval timers in

the hypothalamus and pituitary. *Journal of Endocrinology, 222*, 39–59.
13. Yoshimura, T., Yasuo, S., Watanabe, M., Iigo, M., Yamamura, T., & Hirunagi, K. (2003). Light-induced hormone conversion of T_4 to T_3 regulates photoperiodic response of gonads in birds. *Nature, 426*, 178–181.
14. Nakao, N., Ono, H., Yamamura, T., Anraku, T., Takagi, T., & Higashi, K. (2008). Thyrotrophin in the pars tuberalis triggers photoperiodic response. *Nature, 452*, 317–322.
15. Dixit, A. S., Byrsat, S., & Singh, N. S. (2020). Circadian rhythm in photoperiodic expressions of GnRH-I and GnIH regulating seasonal reproduction in the Eurasian tree sparrow, *Passer montanus*. *Journal of Photochemistry and Photobiology B: Biology, 211*, 111993.
16. 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*, 661–667.
17. Johnson, M. A., Tsutsui, K., & Fraley, G. S. (2007). Rat RFamide-related peptide-3 stimulates GH secretion, inhibits LH secretion, and has variable effects on sex behaviour in the adult male rat. *Hormones and Behavior, 51*, 171–180.
18. Small, T. W., Sharp, P. J., Bentley, G. E., Millar, R. P., Tsutsui, K., & Mura, E. (2008). Photoperiod-independent hypothalamic regulation of luteinizing hormone secretion in a free-living Sonoran desert bird, the Rufous-winged Sparrow (*Aimophila carpalis*). *Brain, Behavior and Evolution, 71*, 127–142.
19. Dixit, A. S., Singh, N. S., & Byrsat, S. (2017). Role of GnIH in photoperiodic regulation of seasonal reproduction in the Eurasian tree sparrow. *Journal of Experimental Biology, 220*, 3742–3750.
20. Saab, S. S., Lange, H. S., & Maney, D. L. (2010). Gonadotrophin-releasing hormone neurones in a photoperiodic songbird express fos and Egr-1 protein after a single long day. *Journal of Neuroendocrinology, 22*, 196–207.
21. Nicholls, T. J., Follett, B. K., & Robinson, J. E. (1983). A photoperiodic response in gonadectomized Japanese quail exposed to a single long day. *Journal of Endocrinology, 97*, 121–126.
22. Kumar, V., Jain, N., & Follett, B. K. (1996). The photoperiodic clock in blackheaded buntings (*Emberiza melanocephala*) is mediated by a self-sustaining circadian system. *Journal of Comparative Physiology A, 179*, 59–64.
23. Yoshimura, T. (2006). Molecular mechanism of the photoperiodic response of gonads in birds and mammals. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology, 144*, 345–350.
24. Nishiwaki-Ohkawa, T., & Yoshimura, T. (2016). Molecular basis for regulating seasonal reproduction in vertebrates. *Journal of Endocrinology, 229*(3), R117–R127.
25. Follett, B. K., Davies, D. T., & Gledhill, B. (1977). Photoperiodic control of reproduction in Japanese quail: Changes in gonadotrophin secretion on the first day of induction and their pharmacological blockade. *Journal of Endocrinology, 74*(3), 449–460.
26. Wong, M. (1983). Effect of unlimited food availability on the breeding biology of wild Eurasian tree sparrow in West Malaysia. *The Wilson Bulletin, 95*, 287–294.
27. Sibley, C. G., & Monroe, B. L., Jr. (1990). *Distribution and taxonomy of birds of the world*. Yale University Press.
28. Nabi, G., Hao, Y., Liu, X., Sun, Y., Wang, Y., Jiang, C., & Li, D. (2020). Hypothalamic–pituitary–thyroid axis crosstalk with the hypothalamic–pituitary–gonadal axis and metabolic regulation in the Eurasian tree sparrow during mating and non-mating periods. *Frontiers in Endocrinology*. https://doi.org/10.3389/fendo.2020.00303
29. Li, D., Zhang, X., Li, Y., Hao, C., Zhang, J., & Wu, Y. (2012). Stress responses of testosterone and corticosterone-binding globulin in a multi-brooded species, Eurasian Tree Sparrows *(Passer montanus*): Does CBG function as a mediator? *Hormones and Behavior, 61*(4), 582–589.
30. 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.
31. Dixit, A. S., & Byrsat, S. (2018). Photoperiodic control of GnRH-I expression in seasonal reproduction of the Eurasian tree sparrow. *Photochemical and Photobiological Sciences, 17*, 934.
32. Summers-Smith, D. (2009). Eurasian tree sparrow (*Passer montanus*). In J. del Hoyo, A. Elliott, & D. A. Christie (Eds.), *Handbook of the birds of the world.* (Vol. 14). Barcelona: Lynx Edicions.
33. Dixit, A. S., & Singh, N. S. (2016). Seasonality in circadian locomotor activity and serum testosterone level in subtropical tree sparrow (*Passer montanus*). *Journal of Photochemistry and Photobiology B, 158*, 61–68.
34. Majumdar, G., Trivedi, A. K., Gupta, N. J., & Kumar, V. (2015). Circadian synchronization determines critical day length for seasonal responses. *Physiology and Behavior, 147*, 282–290.
35. Rastogi, A., Kumari, Y., Rani, S., & Kumar, V. (2011). Phase inversion of neural activity in the olfactory and visual systems of a night-migratory bird during migration. *European Journal of Neuroscience, 34*, 99–109.
36. Bentley, G. E., Perfito, N., Ukena, K., Tsutsui, K., & Wingfield, J. C. (2003). Gonadotropin-inhibitory peptide in song sparrows (*Melospiza melodia*) in different reproductive conditions, and in house sparrows (*Passer domesticus*) relative to chicken-gonadotropin-releasing hormone. *Journal of Neuroendocrinology, 15*, 794–802.
37. 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*, 268–278.
38. Rastogi, A., Kumari, Y., Rani, S., & Kumar, V. (2013). Neural correlates of migration: Activation of hypothalamic clock(s) in and out of migratory state in the blackheaded bunting (*Emberiza melanocephala*). *PLoS ONE, 8*, 10.
39. Rastogi, A., Rani, S., & Kumar, V. (2015). Seasonal plasticity in the peptide neuronal systems: potential roles of gonadotrophin-releasing hormone, gonadotrophin-inhibiting hormone, neuropeptide y and vasoactive intestinal peptide in the regulation of the reproductive axis in subtropical Indian weaver birds. *Journal of Neuroendocrinology, 27*, 357–369.
40. Ubuka, T., & Bentley, G. E. (2009). Identification, localization, and regulation of passerine GnRH-I messenger RNA. *Journal of Endocrinology., 201*, 81–87.
41. Urbanski, H. F., Kim, S. O., & Connolly, M. L. (1990). Influence of photoperiod and 6-methoxy benzoxazolinone on the reproductive axis of inbred LSH/Ss Lak male hamsters. *Reproduction, Fertility and Development, 26*, 437–444.
42. Urbanski, H. F. (1992). Photoperiod and modulation of luteinizing hormone secretion in orchidectomized Syrian hamsters and the influence of excitatory amino acids. *Endocrinology, 131*, 1665–1669.
43. Perfito, N., Zann, R., Ubuka, T., Bentley, G. E., & Hau, M. (2011). Potential roles for GnIH and GnRH-II in reproductive axis regulation of an opportunistically breeding songbird. *General and Comparative Endocrinology, 173*, 20–26.
44. Rastogi, A., Malik, S., Rani, S., & Kumar, V. (2016). Changes in brain peptides associated with reproduction and energy homeostasis in photosensitive and photorefractory migratory redheaded buntings. *General and Comparative Endocrinology, 230–231*, 67–75.
45. Dawson, A., Follett, B. K., Goldsmith, A. R., & Nicholls, T. J. (1985). Hypothalamic gonadotropin-releasing hormone and

pituitary and plasma FSH and prolactin during photostimulation and photorefractoriness in intact and thyroidectomized starlings (*Sturnus vulgaris*). *Journal of Endocrinology, 105*, 71–77.
46. Meddle, S. L., & Follett, B. K. (1997). Photoperiodically driven changes in Fos expression within the basal tuberal hypothalamus and median eminence of Japanese quail. *The Journal of Neuroscience, 17*, 8909–8918.
47. Saldanha, C. J., Silverman, A. J., & Silver, R. (2001). Direct innervation of GnRH neurons by encephalic photoreceptors in birds. *Journal of Biological Rhythms, 16*, 39–49.
48. Kang, S. W., Thayananuphat, A., Rozenboim, I., Millam, J. R., Proudman, J. A., & El Halawani, M. E. (2006). Expression of hypothalamic GnRH-I mRNA in the female turkey at different reproductive states and following photostimulation. *General and Comparative Endocrinology, 146*, 91–99.
49. Thayananuphat, A., Kang, S. W., Bakken, T., Millam, J. R., & El Halawani, M. E. (2007). Rhythm-dependent light induction of the c-fos gene in the turkey hypothalamus. *Journal of Neuroendocrinology, 19*, 407–417.
50. Li, H., & Keunzel, W. J. (2008). A possible neural cascade involving the photoneuroendocrine system (PNES) responsible for regulating gonadal development in an avian species, *Gallus gallus*. *Brain Research Bulletin, 76*, 586–596.
51. Blahser, S., Oksche, A., & Farner, D. S. (1986). Projection of fibres immunoreactive to an antiserum against gonadoliberin (LHRH) into the pineal stalk of the white-crowned sparrow, *Zonotrichia leucophrys gambelii*. *Cell and Tissue Research, 244*, 193–196.
52. Majumdar, G., Yadav, G., Rani, S., & Kumar, V. (2014). A photoperiodic molecular response in migratory redheaded bunting exposed to a single long day. *General and Comparative Endocrinology, 204*, 104–113.
53. Trivedi, N. (2015). Role of GnIH in photoperiodic regulation of seasonal breeding in a passerine finch. PhD thesis, Department of Zoology, University of Lucknow, UP, India.
54. Srivastava, A. (2015). Role of GnRH and GnIH in regulation of seasonal breeding of spotted munia (*Lonchura punctulata*). PhD thesis, Department of Zoology, University of Lucknow, UP, India.
55. Banerjee, S. (2016). Neuroendocrine mechanism of reproduction and energy regulation in Japanese quail, *Coturnix coturnix japonica*. Ph. D. thesis, Banaras Hindu University, Varanasi, India.
56. Bunning, E. (1936). Die endogene Tagesrhythmik als Grundlage der photoperiodische Reaktion. *Berichte der Deutschen Botanischen Gesellschaft, 54*, 590–607.
57. Pittendrigh, C. S., & Minis, D. H. (1964). The entrainment of circadian oscillations by light and their role as photoperiodic clocks. *The American Naturalist, 98*(902), 261–294.