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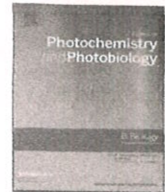

Circadian rhythm in photoperiodic expressions of GnRH-I and GnIH regulating seasonal reproduction in the Eurasian tree sparrow, *Passer montanus*

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ABSTRACT

The present study investigates the involvement of circadian rhythm in photoperiodic expressions of *GnRH-I* and *GnIH* in the hypothalamus controlling seasonal reproduction in the Eurasian tree sparrow (*Passer montanus*). Groups of photosensitive birds were exposed for four weeks to resonance light dark cycles comprising of a light phase of 6 h (L) combined with dark phase of different durations (D) such that the period of LD cycles varied by 12 h increments viz. 12- (6 L/6D), 24- (6 L/18D), 36- (6 L/30D), 48- (6 L/42D), 60- (6 L/54D) and 72- (6 L/66D) h. In addition, a control group (C) was maintained under long day length (14 L/10D). Observations, recorded at the beginning and end of experiment, revealed significant testicular growth with corresponding increase in the hypothalamic expression of GnRH-I peptide but low levels of *GnIH* mRNA and peptide in the birds exposed to resonance cycles of 12, 36 and 60 h which were read as long days. On the other hand, birds experiencing resonance cycles of 24, 48 and 72 h read them as short days wherein they maintained their quiescent gonads and low levels of GnRH-I peptide but exhibited significant increase in *GnIH* mRNA and peptide expressions. Thus, sparrows responded to resonance light dark cycles differently despite the fact that each of them contained only 6 h of light. These findings suggest that an endogenous circadian rhythm is involved in photoperiodic expressions of above molecules and indicate a shift in their expressions depending upon whether the light falls in the photoinducible or non photoinducible phase of an endogenous circadian rhythm.

1. Introduction

The measurement of day length is critical in a photoperiodic species for exact timing of physiological transitions between life history stages [1–3]. Birds have developed precise time-keeping mechanism to design transition through their yearly life cycle which helps them to exploit favorable conditions and survive during the harshest time of the year. The photoperiodic time measurement allows them to anticipate and prepare for the favorable season in advance of its arrival. This has great relevance for maintaining their fitness in the seasonal environments [4]. Since the pioneer discovery of Rowan [5], revealing the importance of day length in control of seasonal reproductive cycles in birds, various attempts have been made to understand the mechanism by which they measure day length to time the physiological preparations for successful reproduction and related seasonal events. Some birds adapt to daily light dark cycle by using their endogenous "clock" to exactly time their physiological and behavioral functions. The interaction of day length with the above clock induces a seasonal response [6–8]. The above endogenous program enables birds in timing switch on (photoinduction) and switch off (photorefractoriness) of their physiological mechanisms. This ensures the occurrence of seasonal events at the most appropriate time of the year when resources in the wild are optimally present and the chances of survival of offspring are maximum. Several studies have shown the participation of a circadian rhythm of photoperiodic photosensitivity (CRPP) in timing initiation and termination of gonadal responses during photoperiodic control of reproductive cycles in some birds [2,8–11]. Bunning [12] formulated that the response of CRPP to light is phase dependent. Further, it is explainable on the basis of an external coincidence model [13] which predicts that a photoperiodic response results due to the coincidence of light with the photosensitive phase or more precisely photoinducible phase of an entrained endogenous circadian rhythm occurring early in subjective night. Light plays dual role in the above model i.e., entrainer as well as inducer [14]. Thus, a photoperiodic response in a long day breeder

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results when light coincides with the phase of maximum inducibility of the endogenous clock that occurs about 12 h after the sunrise. This is evident in spring and summer months when the photoperiod is read as "long days". The above coincidence fails to occur during winter months when the light period remains shorter than 12 h and is read as "short day" [15]. However, the short days terminate photorefractoriness by inducing the recovery of the photosensitivity in refractory birds [16].

Recent studies have revealed the mediobasal hypothalamus (MBH) as the site of photoperiodic induction in birds [17–19]. Long photoperiod stimulates synthesis of thyrotropin-stimulating hormone beta (TSH-β) in the pars tuberalis [18] that leads to increase in expression of gene encoding type 2 iodothyronine deiodinase (*DIO2*). DIO2 is a thyroid hormone activating enzyme that converts T_4 to T_3 causing an increase in local production of triiodothyronine (T_3) [17,20]. T_3 further alters the structural arrangement of the gonadotropin releasing hormone-I (GnRH-I) nerve terminals at the median eminence, where the glial end feet that ensheath the terminals retract allowing increase in secretion of GnRH-I [21,22]. GnRH-I further acts on the anterior pituitary to stimulate gonadotropins (luteinizing hormone, LH; follicle-stimulating hormone, FSH) synthesis and release that lead to seasonal changes in reproductive physiology, gonadal growth and functions and behavior in birds. The above physiological cascade is triggered when light falls in a specific time window or photoinducible phase of an endogenous circadian rhythm. In majority of avian species, the annual changes in photoperiod cause marked changes in the GnRH-I secretion [6,23–26]. The increase in day length during spring and summer months progressively engages the photoinducible phase and induces a photoperiodic response. However, under short photoperiod, increased synthesis of type 3 iodothyronine deiodinase (DIO3), a thyroid hormone inactivating enzyme that converts T_4 and T_3 to inactive metabolites rT_3 and T_2, respectively [13,18] inhibits GnRH-I synthesis [27] and a photoperiodic response fails to occur. In majority of avian species, the annual changes in photoperiod cause marked changes in the GnRH-I secretion [23,26]. However, recently the invention of another hypothalamic neuropeptide called gonadotropin-inhibitory hormone (GnIH) has challenged the prime role of GnRH-I in control of reproduction [28]. GnIH inhibits synthesis and release of gonadotropins by its direct action on the pituitary gland and also indirectly by decreasing GnRH-I neuronal activity. It has been reported that GnIH acts as neuroendocrine integrator of photoperiodic cue where it integrates external and internal environmental information and regulates gonadotropin secretion to time seasonal reproduction in birds [29–33]. Both GnRH-I and GnIH respond to a variety of environmental signals and act as significant components of the neuronal circuitry in the brain controlling seasonal reproductive responses across avian species.

Despite reasonable research on molecular basis of photoperiodic control of reproduction via HPG axis, the mechanisms by which the expressions of the regulatory molecules are controlled with particular emphasis on the possible role of an endogenous circadian rhythm is not clearly understood and needs further investigations. The cloning of homologues of mammalian circadian clock genes in birds has provided a better way to examine the molecular link between the circadian clock and photoperiodism in birds [34,35]. However, the mechanism of seasonal time measurement still remains unclear, and questions regarding the mechanism by which the circadian clock determines the photoinducible and non-photoinducible phases remain unanswered. Further, due to limited investigations at the mechanistic level, our understanding for the circadian control of molecular mechanisms underlying photoperiodic regulation of reproduction in birds is still in its infancy. Therefore, it is proposed to study the involvement of an endogenous circadian rhythm in photoperiodic expressions of GnRH-I and GnIH regulating seasonal reproduction in a photoperiodic species, the tree sparrow. In our earlier investigations on this species, we have reported that the tree sparrows possess a definite annual reproductive cycle. Gonadal growth in them is triggered by increasing day lengths of spring in March reaching to peak in May. The gonads regress in summer month (June) when the day lengths are still longer than the spring months indicating the onset of photorefractoriness. They are photosensitive and use day length in regulation of their seasonal reproduction [2,8,36]. The initiation of gonadal growth in this species is a long day phenomenon, while the termination of photorefractoriness and recovery of photosensitivity is a short day phenomenon [2,36]. Further, an endogenous circadian rhythm is involved in induction of gonadal growth and consequent increase in plasma levels of gonadal steroids [8,37]. The present study is a step forward to investigate the circadian control of molecular mechanism at the hypothalamic level underlying photoperiodic control of seasonal gonadal cycle in the Eurasian tree sparrow.

2. Materials and Methods

2.1. *Animal Model and Experiment*

The Eurasian tree sparrow is a widely distributed avian species occupying different latitudes, including temperate as well as tropical and sub- tropical regions [38]. Its native range expands throughout central and southern Europe, central Asia, and parts of south–east Asia [39]. In India, tree sparrows are plentifully spread in the hilly regions of the North-East India, including Shillong, Meghalaya (Latitude 25°34′N, Longitude 91°53′E) having a maximum height of 1966 feet MSL with predominant subtropical moist pine forests and an average temperature ranging from 7 °C (winter) to 25 °C (summer) and highest rainfall in the month of July. The day length varies in the range of 3 h 15 m annually in Shillong with minimum of 10 h 29 m in December and maximum of 13 h 44 m in June. Tree sparrows are passerine birds of non-migratory nature and mostly found in the residential areas [40] making their nest in the roof cavities in houses, ceiling of verandas, cavity in tree, pole, fence post etc. They feed on seeds, grains and insects.

Adult male tree sparrows were captured from their wild habitat in and around Shillong by using mist net. They were acclimatized to laboratory conditions for a fortnight by exposing them to natural variations of photoperiod, temperature and humidity. These birds were then subjected to short photoperiod (9 L/15D) for 2 months in order to eliminate photorefractoriness, if they had any in nature and also to make the birds photosensitive before the beginning of experiments. Observations on testicular size and body weight at four-weeks intervals during the above treatment revealed maintenance of quiescent gonads and normal body weights. The above photosensitive birds were divided into seven groups ($n = 12$ each) comprising one control and six experimental. The control group (C) was subjected to long day length (14 L/10D) while the experimental groups were exposed to resonance light dark cycles for four weeks. Briefly, in resonance light dark cycles, birds were exposed to a short fixed photophase of 6 h in combination with dark phases of varying durations so that the periods of light–dark cycle lengthened systematically by 12 h increments like 12-(6 L/6D), 24-(6 L/18D), 36-(6 L/30D), 48-(6 L/42D), 60- (6 L/54D) and 72-(6 L/66D) h. Observations at the beginning and end of the experiment were made on the levels of expressions of GnRH-I peptide and *GnIH* mRNA and peptide using immunohistochemistry and realtime-PCR (qPCR). In addition, measurement of testicular volume was also done at each observation to note gonadal development. Birds, under different photoperiodic treatments, were kept in lightproof wooden chambers (2.10 m × 1.20 m × 1.35 m) which were illuminated by light of an intensity of ~400 lx at the perch level provided from CFL bulbs (Philips Electronics India Limited, Kolkata, India) with automated control. The first light ON was in phase with the pre-treatment schedule and commenced at 06:00 h in all photoperiodic regimes. The photoperiodic chambers were well aerated by air circulator. Food (kakuni, *Setaria italica* and Asian rice, *Oryza sativa*) and water were available ad libitum and were replenished only during the light phase of the cycle.

2.2. Measurements

2.2.1. Testicular Size

The testicular development was measured in terms of changes in its volume. Briefly, the testicular volume was recorded in situ by opening abdominal wall between the last two ribs to locate left testes after the brain was taken out from the birds following anesthesia and perfusion as described below. Then length and width of the testes were measured with respect to divisions on the graph paper using a calliper. The calculation of testicular volume (TV) was done using the formula $4/3\pi ab^2$, where a and b denote half of the long (length) and short (width) axes, respectively.

2.2.2. Gene Expression

The birds ($n = 4$/group) were decapitated and their brains were exposed by removing skulls for the study of GnIH mRNA expression. The hypothalamus was dissected out, cut into pieces and kept in Trireagent (Ambion Inc., Cat No.74123) at −80 °C. The freezed tissue was thawed and homogenized to extract total RNA following the TRIreagent manufacturer's protocol. The total RNA isolated was suspended on DEPC treated water and its purity was assessed on a nanodrop. 1 µg of total RNA was reverse transcribed to cDNA using cDNA synthesis kit (Thermo scientific, Verso, Cat. No. AB1453A).

For qPCR, gene specific primers of GnIH (forward: 5′-TGGAGAGC AGAGAAGACAATGATG-3′ and reverse: 5′- TGTCTTTTGTTCCCCAGTC TTCCA-3′) and *β-Actin* (forward: 5′-GGATTTCGAGCAGGAGATGG -3′ and reverse: 5′- GGGCACCTGAACCTCTCATT-3′) were designed from partial sequences available on GenBank (Accession number: GnIH-KT351598 and β-Actin-KT351599) by using Primer3 (freely available software online). The possible primer efficiency, its dimer and hairpin were checked with the help of OligoAnalyzer 3.1. A 7500 real-time PCR system (Applied Biosystems) was used to perform the quantitative expression of *GnIH* gene. In brief, each PCR tube had a total reaction mixture of 10 µl comprising of 1 µl of cDNA, 0.3 µl each of forward and reverse primers, 5 µl of Power SYBR Green Master mixture (Applied Biosystems, Cat. No.1301388) and 3.4 µl of nucleases-free water. The standardized primer concentration along with a total reaction volume attained a slope of −3.3 to −3.4 showing a good melt curve and efficiency of the primer. Relative expression level of the target gene was determined by taking *β-Actin* as a reference gene. The Ct value of *GnIH* gene was normalized against the Ct value of the *β-Actin*. ΔΔCt values were calculated as per Majumdar et al. [41] and plotted as negative power to 2.

2.2.3. Immunohistochemistry of GnRH-I and GnIH Peptides

This was done to measure the expression levels of GnRH-I and GnIH peptides in terms of number, area and density of neurons expressing them. For this study, birds ($n = 4$ each peptides/group) were first given subcutaneous injection of general anesthesia (ketamine-xylazine solution of 0.003 ml/g body weight) and then perfused transcardially with 50 ml ice-cold saline (pH 7.4) followed by 50 ml of 4% paraformaldehyde solution (0.1 M phosphate buffer at pH 7.4). Their brains were dissected out and stored for overnight in paraformaldehyde solution at 4 °C and then post-fixed by transferring to serial grades of cryoprotectant sucrose solutions of 10%, 20% and 30% under 4 °C. Finally, brains were transferred to 15% polyvinylpyrrolidone solution (PVP, Himedia) and stored at −80 °C until further processing. The brain tissue was taken out from deep freezer and thawed by keeping on ice and then mounted on the cryostat tissue holder with the help of 15% PVP inside the cryostat chamber. The sectioning of the brain was done serially in the coronal plane at 30 µm thicknesses using cryostat (Leica CM 1850). The above brain sections were then processed for immunohistochemical study as per the protocol described in Rastogi et al. [42]. At the end, tissue sections were visualize by adding two different staining agent i.e. DAB and nickel (DAB 4100, Vector labs.). DAB was used for staining GnRH-I (brownish-red) while nickel was used for GnIH (dark-blue) peptides. The anti-quail serum, gifted by Dr. K. Tsutsui, Waseda University, Japan, was used at 1: 20,000 dilution for the detection of GnIH peptide. This anti-serum has been found to cross-react with GnIH of song sparrow (*Melospiza melodia*) and house sparrow (*Passer domesticus*) [29]. The specificity of this antibody has already been demonstrated in some previous studies [19,28,33,43,44]. HU60 bleed (dilution 1:18000) was used as primary antibody for GnRH and was gifted by Dr. Henryk F. Urbanski, Oregon Health and Sciences University, Portland, Oregon, USA. This antibody was generated in rabbits against mammalian GnRH and shows high specificity for GnRH [44,45]. The details about characteristics of this antibody have been mentioned in Urbanski et al. [46] and Urbanski [47]. However, controls with tissues labelled with these two antibodies were also run to test non-specific immunoreactivity. The presence of primary antibody in the reaction resulted in strong immunoreactivity while its absence caused total loss of immunoreactivity. The control protocol of exclusion of the primary antisera from the reaction, as well as replacing the antisera with buffer or BSA have been performed to verify the specificity of the immunoreaction. Both these procedures resulted in the total loss of immunoreactivity [33].

The examination of desired brain sections were done using a trinocular bright-field microscope (Motic) and the digital images of immunoreactive cells were captured by a high megapixel camera (Motic cam). The images of the specified region were captured at 10× and 40× magnifications. Photography was done using standard illumination. As per requirement, the images were adjusted for size, contrast and brightness using the Motic image version 2 analyzer software [33]. GnRH-I and GnIH immunoreactive cells were counted in the entire preoptic area (POA) and paraventricular nucleus (PVN) regions, respectively taking into consideration strongly (bright) as well as weakly (faint) stained cells to avoid any bias for staining-intensity [19] The numbers of -ir cells in the POA and PVN regions of all brains were summed up separately and their means (± SE) were calculated. Additional measures of immunoreactions i.e. % cell area, cell area and cell OD were also quantified in the above brain regions as per Rastogi et al., [19]. The number of cells and % cell area indicate density whereas cell area and cell OD are measures of peptide content of a specific neuronal population detected by the primary antibody [19,23,48]. The % cell area, cell area and cell OD were measured by capturing images at 40× magnification using ImageJ (NIH) software. In brief, a well-defined frame of 200×150 µm^2 was chosen to measure above three parameters [49]. Calculations were done by making outlines of all individual cells covered in a frame in every fourth section. Cell body areas were summed up and averaged for right and left halves of the brain. The sum total (total cell area) and average (mean cell area) were taken as one reading for an individual brain within a group. However, % cell area was calculated using equation: total cell area/frame area × 100. The average of the percentage cell areas for images from all the sections gave a single value for each bird. Relative cell OD (intensity above background) was obtained by subtracting background intensity (average staining intensity of five regions lacking cell bodies and fibres) from the average OD. Finally, the mean ± SE for the group was calculated.

The procedures used in this study were approved by the Institutional Animal Ethics Committee of the North-Eastern Hill University, Shillong.

2.3. Statistical Analyses

The data, presented as mean ± S.E.M, were analysed using one-way ANOVA followed by Newman-Keuls multiple comparison post-hoc test in cases where ANOVA indicated a significance of difference. Correlation analyses were also performed to investigate the relationship between number of GnRH-I and GnIH-ir cells and also between testicular size and the relative *GnIH* mRNA expression. Significance of difference was taken at $P < 0.05$. Graph Pad Prism software (version 6.0, Sandiego, CA, USA) was used for statistical analyses.

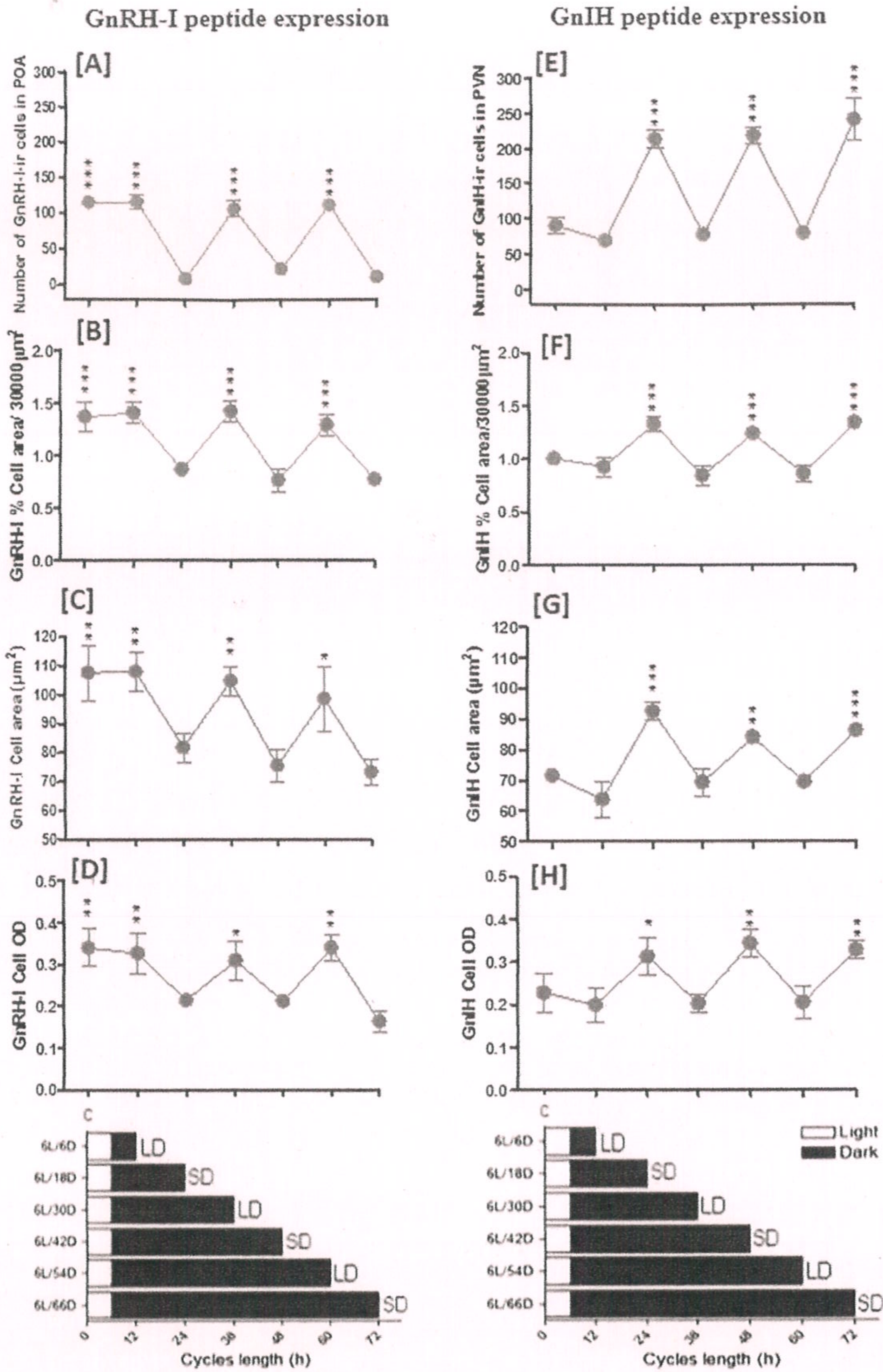

Fig. 1. Expression of GnRH-I (A-D) and GnIH (E-H) peptides under different light dark cycles. Light-dark cycles are represented by bars. Closed bars represent different durations of dark phase preceded by 6 h of light phase (open bars). C represents a control group while LD and SD indicate long day and short day, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

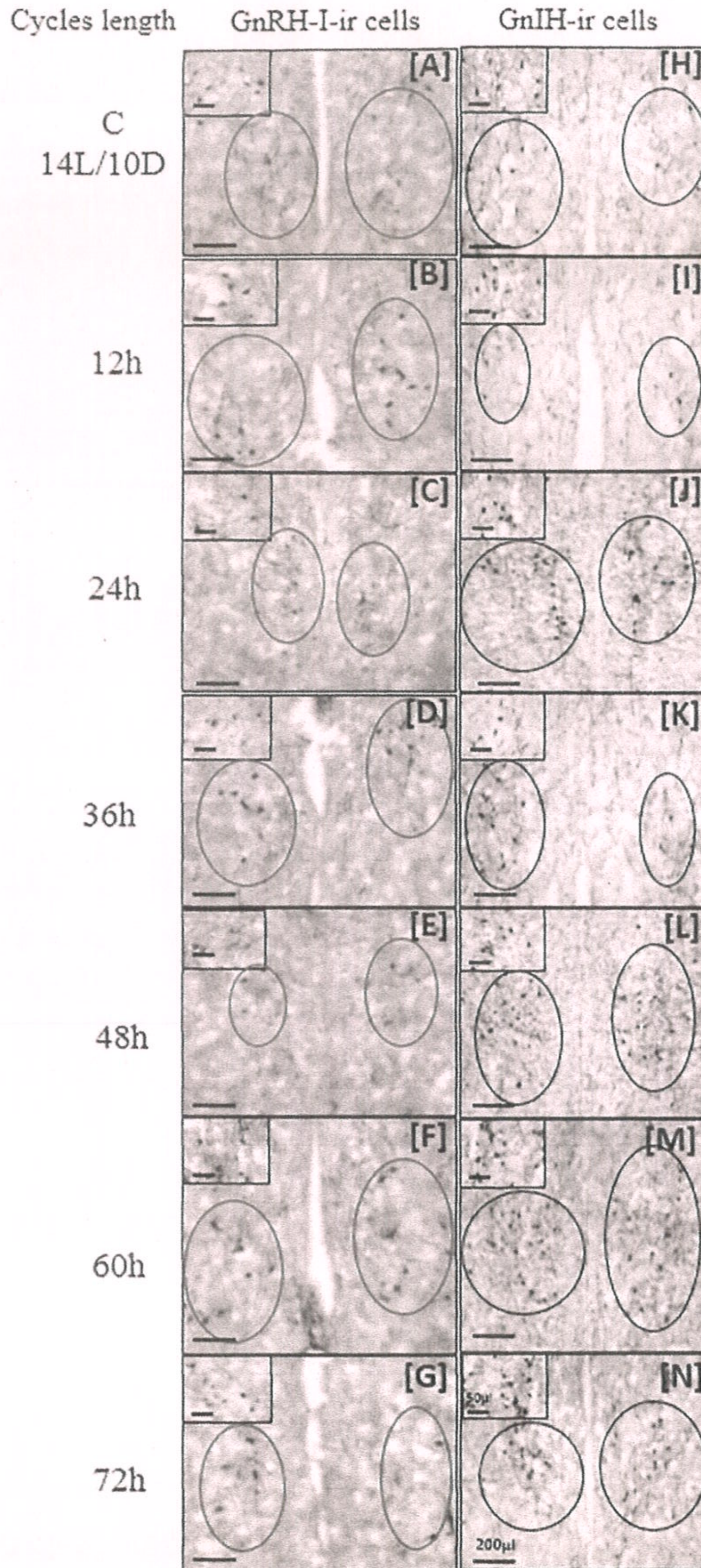

Fig. 2. GnRH-I (A-G) and GnIH (H-N) immunoreactivity in the preoptic area (POA) and paraventricular nucleus (PVN), respectively, in the brain of the tree sparrow under different resonance light dark cycles and control (C). Scale bar: general view-200 μm, magnified view-50 μm.

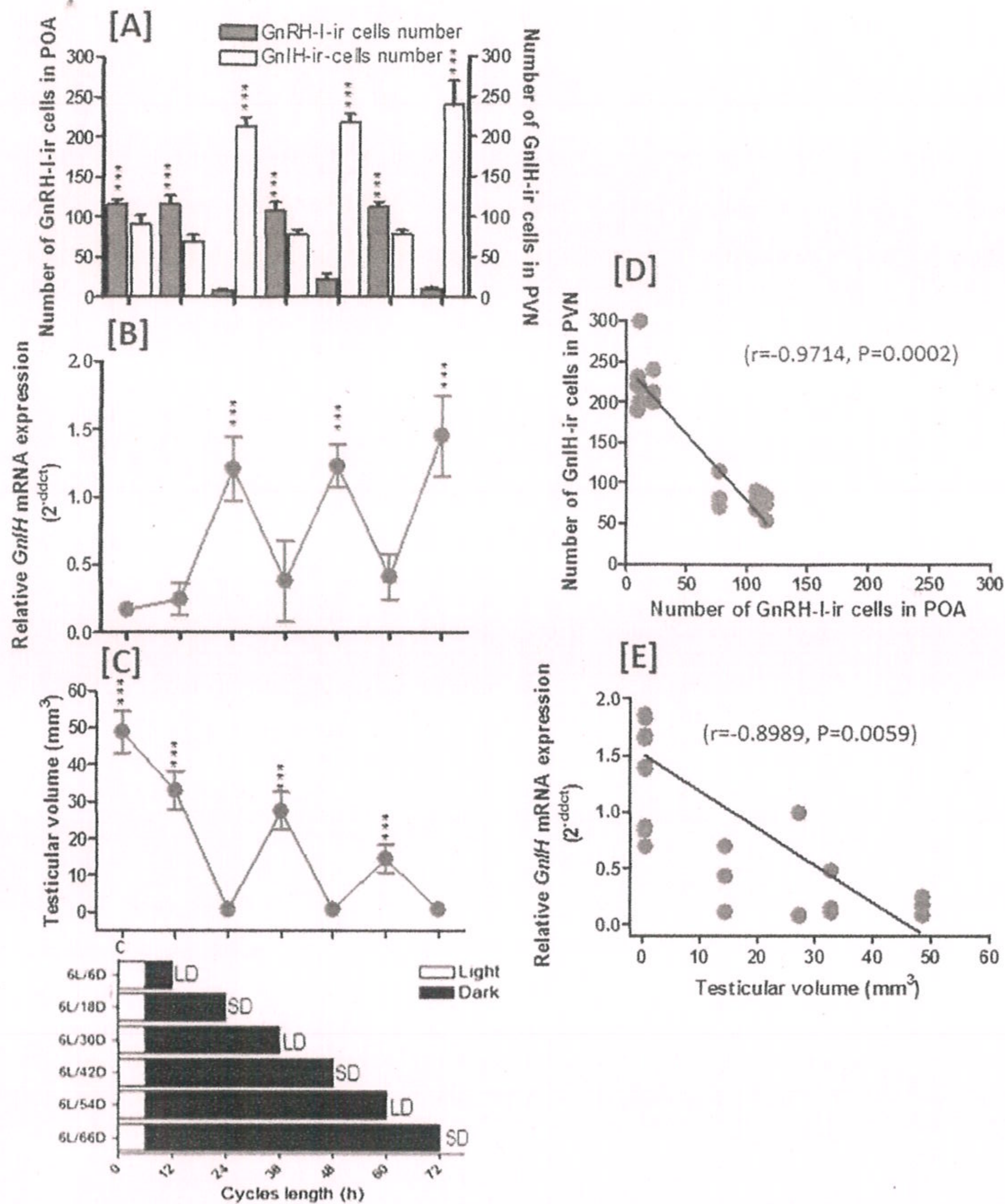

Fig. 3. Number of GnRH-I-Ir and GnIH-Ir cells (A); Relative GnIH mRNA expression (B); testicular volume (C); Correlation between GnIH-Ir cells in the paraventricular nucleus (PVN) and GnRH-I-Ir cells in the preoptic area (POA) of the hypothalamus (D); and Correlation between relative GnIH mRNA expression and testicular volume (E) in the tree sparrow under various resonance light dark cycles. ***P $<$ 0.001.

3. Results

The results are presented in Figs. 1–4. The birds of control group exposed to long day length (14 L/10D) exhibited significant testicular growth ($P < 0.001$) confirming their photosensitivity at the time of their exposure to experimental light dark cycles (Fig. 3C). Significant GnRH-I peptide expression (GnRH-I-ir cells number; % cell area; cell area: $P < 0.0001$ and cell OD: $P = 0.0002$) in the hypothalamic preoptic area (POA) and the consequent increase in testicular volume were observed in the birds exposed to resonance cycles of 12, 36 and 60 h while those experiencing 24, 48 and 72 h cycles failed ($P > 0.05$) to show any response (Figs. 1A–D and 2A–G). Conversely, significant expressions of both *GnIH* mRNA (P $<$ 0.0001) and peptide (GnIH-ir cells number; % cell area; cell area: P $<$ 0.0001 and cell OD: $P = 0.0009$) in the paraventricullar nucleus (PVN) of hypothalamus were noticed only in the resonance cycles of 24, 48 and 72 h ($P < 0.05$) but not in the cycles of 12, 36 and 60 h and in control group (Figs. 1E–H, 2H–N and 3A–B). The numbers of GnRH-I and GnIH immunoreactive (ir) cells in the POA and PVN of brain, respectively were found negatively correlated ($r = -0.9714$, $P = 0.0002$; Fig. 3D). Similarly, a negative correlation ($r = -0.8989$, $P = 0.0059$) also existed between *GnIH* mRNA expression and testicular size of the birds under experimentation (Fig. 3E). The expression of GnRH-I was found running parallel to gonadal size while that of *GnIH* mRNA and peptide ran antiparallel to both GnRH-I expression and gonadal size in various light dark cycles. Thus, resonance cycles of 12, 36 and 60 h acted as long days showing significant increase in GnRH-I expression and gonadal size while those of 24, 48 and 72 h acted as short days exhibiting significantly higher GnIH expression and quiescent gonads though each of them had only 6 h light period per cycle.

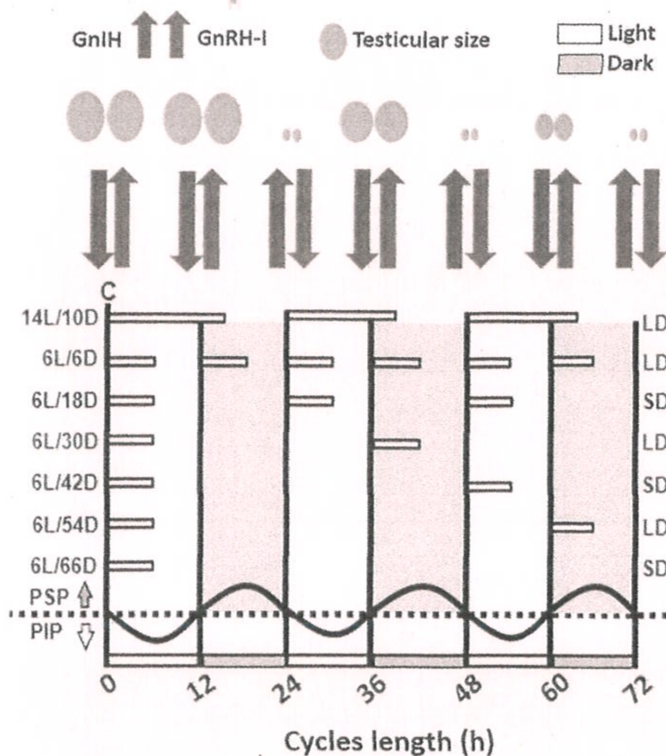

Fig. 4. Schematic representation of photoperiodic response under various resonance light dark cycles showing position of the light relative to photosensitive and photoinsensitive phases of the circadian rhythm. Photosensitive phase (PSP); Photoinsensitive phase (PIP); Long day (LD); Short Day (SD).The white bar represents the duration and the position of the light period falling in the PSP/ PIP.

Immunohistochemical examination revealed significant ($F_{6, 27} = 71.06$: $P < 0.0001$; Figs. 1A and 2A–G) variation in the number of GnRH-I immunoreactive cells (GnRH-I ir cells) in the POA area of the hypothalamus of the birds under experimentation. Further, the birds under resonance cycles of 12, 36, 60 h and control group (14l: 10D) showed significant increase ($P < 0.001$) in number of GnRH-I-ir cells suggesting photostimulatory nature of these cycles which acted as long days and induced gonadal growth while there was no significant difference in GnRH-I-ir cells in the cycles of 24, 48 and 72 h indicating that these cycles acted as short days and failed to induce photoperiodic response. The numbers of GnRH-I-ir cells in the birds under resonance cycles of 12, 36 and 60 h and control (14 L/ 10D) were significantly ($P < 0.001$) higher than those of the birds under 24, 48 and 72 h cycles. Further, no significant difference was observed in number of GnRH-I-ir cells among the birds of 12, 36 and 60 h cycles and control group ($P > 0.05$). Further, the changes in the % cell area ($F_{6, 27} = 17.78$: $P < 0.0001$; Fig. 1B), cell area ($F_{6, 27} = 8.679$: $P < 0.0001$; Fig. 1C) and cell OD ($F_{6, 27} = 7.654$: $P = 0.0002$; Fig. 1D) of GnRH-I-ir cells in the POA of the hypothalamus followed the trend as observed in GnRH-I-ir cell number. There was significant difference in the number of GnIH-ir cells in the PVN area of the brain of the birds under various light dark cycles ($F_{6, 27} = 46.48$: $P < 0.0001$; Fig. 1E). The birds exhibited significantly ($P < 0.001$) higher number of GnIH-ir cells in PVN and quiescent testes under 24, 48 and 72 h cycles when compared to their number (Figs. 1E and 3C) in the birds under 12, 36 and 60 h cycles confirming that these cycles acted as short days and failed to induce gonadal growth. Further, no difference in the number of GnIH-ir cells ($P > 0.05$) was noticed either among the birds of 12 h, 36 h and 60 h cycles or 24 h, 48 h and 72 h cycles. The changes in the % cell area ($F_{6, 27} = 19.59$: $P < 0.0001$; Fig. 1F), cell area ($F_{6, 27} = 17.50$: $P < 0.0001$; Fig. 1G) and cell OD ($F_{6, 27} = 6.041$: $P = 0.0009$; Fig. 1H) of GnIH-ir cells in the PVN of the hypothalamus followed the trend as observed in GnIH-ir cell number. A compression on the number of immunoreactive cells of GnRH-I in the POA and GnIH in PVN under various light dark cycles following correlation analysis ($r = -0.9714$, $P = 0.0002$) indicated that the number of GnIH-I-ir cells increase with the decrease in the number of GnRH-ir cells and vice versa, thus, both run antiparallel to each other (Fig. 3 A and D). The *GnIH* mRNA expression ran almost parallel to that of the GnIH-ir peptide expression (Fig. 3A and B) in the birds under various resonance cycles. Tree sparrows exposed to different light dark cycles exhibited significant difference in their testicular size ($F_{6, 27} = 47.38$: $P < 0.0001$; Fig. 3C) showing significant increase in gonadal size under 12, 36 and 60 h cycles and quiescent gonads in 24, 48 and 72 h cycles, thus, following the trend observed for GnRH-I peptide expression (Fig. 3A) but opposite to that of *GnIH* mRNA and peptide expressions (Fig. 3A–C). Further, a correlation analysis ($r = -0.8989$, $P = 0.0059$) indicated a significant decrease in testicular volume with the increase in the expression of the *GnIH* mRNA and vice versa in the tree sparrow (Fig. 3E).

4. Discussion

The results obtained from the present experiment clearly suggest that the tree sparrows utilize a photosensitive rhythm with a period of about 24 h to regulate photoperiodic expressions of *GnRH-I* and *GnIH* genes in control of their reproductive responses (Figs. 1–4). The resonance cycles of 12, 36 and 60 h acted as long days by inducing significant testicular growth as a consequence of increased hypothalamic expression of GnRH-I and maintenance of decreased levels of *GnIH* transcripts. On the other hand, resonance cycles of 24, 48 and 72 h acted as short days and maintained quiescent gonads by stimulating hypothalamic expressions of *GnIH* transcripts and inhibiting GnRH-I. The tree sparrows respond to resonance light dark cycles differently despite the fact that each of them contained only 6 h of light. Further, the above genes express in an anti-phasic manner in photoperiodic regulation of reproductive responses in the tree sparrow. The expressions of GnRH-I was found running parallel to gonadal size while the expression of *GnIH* mRNA and peptide ran antiparallel to both GnRH-I expression and gonadal size. These observations clearly suggest circadian control of hypothalamic expressions of *GnRH-I* and *GnIH* in photoperiodic regulation of gonadal responses involving Hypothalamus-Pituitary- Gonadal (HPG) axis in the tree sparrow. They seem to utilize their endogenous circadian rhythm during photoperiodic time measurement to differentiate between long and short photoperiods in control of hypothalamic expressions of GnRH-I and GnIH.

Further, the above observations are consistent with the Bunning hypothesis and are interpretable on the basis of an external coincidence model of photoperiodic time measurement [13,50,51]. According to this model, the circadian rhythm believed to be involved in photoperiodic time measurement consists of two different phases. The former phase is photoinsensitive or non-photoinducible phase (subjective day) and the latter is photosensitive or photoinducible phase (subjective night). The birds are insensitive and sensitive to their photoperiodic responses in the above two phases, respectively. A photoperiodic response depends upon weather the light coincides or fails to coincide with the photoinducible phase of an entrained endogenous circadian rhythm. Thus, long day responses occur as a result of extension of light into the photoinducible phase of an entrained circadian rhythm. On the other hand, light remains restricted to non-photoinducible (photoinsensitive) phase of the rhythm under short days and photoinduction fails to occur [52,53]. Our results are indicative of the involvement of circadian rhythm in photoperiodic expressions of *GnRH-I* and *GnIH* genes at the hypothalamic level in the tree sparrow. They further indicate a shift in the expression of above genes depending upon whether the light falls in the photoinducible or non-photoinducible phase of an endogenous circadian rhythm. The light falling in the "photoinducible"

phase (as in cycles of 12, 36 and 60 h) triggers the expression of GnRH-I and inhibits that of GnIH leading to activation of HPG axis and consequent testicular growth. On the other hand, restriction of light only in the "non-photoinducible" phase (as in cycles of 24, 48 and 72 h) activates GnIH expression but inhibits GnRH-I expression leading to down regulation of HPG axis and maintenance of quiescent testes in the tree sparrows. Thus, the photoperiodic expressions of *GnRH-I* and *GnIH* genes in the hypothalamus of tree sparrow depend upon the coincidence of light with two different phases of the circadian rhythm i.e., photoinducible and non-photoinducible, respectively.

A careful examination of our data with respect to the above framework reveals that there is daily coincidence of light with the photoinducible phase of circadian rhythm in the resonance cycle of 12 h (6 L: 6D) and in long day control (14 L/ 10D) while it occurs at an alternate cycle or after every two days in the resonance cycles of 36 (6 L/ 30D) and 60 h (6 L/ 54D), respectively, resulting in an increased expression of GnRH-I in POA and decreased expression of GnIH in PVN areas of the hypothalamus leading to testicular growth. On the other hand, light remained confined to non-photoinducible phase in the resonance cycles of 24, 48 and 72 h resulting in the inhibition of GnRH-I expression but activationof GnIH expression leading to maintenance of quiescent gonads (Fig. 4). The variations in the hypothalamic expressions of above peptides and consequent variations in the testicular growth in the birds under gonadostimulatory resonance cycles (i.e., 12, 36 and 60 h) might be due to the fact that none of the above cycles provided a customary long day treatment like control group (14 L: 10D). The birds exposed to cycles of 12 and 36 h responded well by showing higher testicular growth when compared to gonadal response under 60 h cycle. This might be due to the fact that extent of gonadal growth and the number of intervening short days in a resonance light dark cycle are inversely related [54]. However, no significant difference was noticed either in GnRH-I expression among the gonadostimulatory cycles or in GnIH expression among the non-gonadostimulatory cycles indicating lack of differentiation at the gene expression levels. Further, increased GnRH-I expression and gonadal stimulation were evident only in the resonance cycles of 12, 36 and 60 h despite the fact that each of them comprised same duration of light i.e. 6 h that was near half of the critical photoperiod (11 h/day) for testicular stimulation in tree sparrow [2]. Thus, our data suggest that it is not the absolute durations of light and dark or the ratio of light to dark in the resonance cycle, which is responsible for a positive response in the tree sparrow rather it is the position of light with respect to phases of the circadian rhythm which is important. The *GnRH-I* and *GnIH* genes, in the tree sparrow, express in an anti-phasic manner and show a shift in their expression depending upon whether the light falls in the photoinducible or non-photoinducible phase of an endogenous circadian rhythm.

Our earlier investigations on the tree sparrow revealed that the photoperiodic control mechanism regulating seasonal reproduction in this photoperiodic species depends on the changes in hypothalamic expressions of *GnRH-I* and *GnIH* transcripts in the POA and PVN areas of their brain, respectively. The results further suggest the neuronal link between photoperiod and the GnRH-I and GnIH system in the processing of photoperiodic information in the brain of the tree sparrow, which regulates the HPG axis and ultimately controls seasonal reproduction. In different experiments on photoperiod-induced changes in GnRH-I and GnIH expressing neurons in the POA and PVN, respectively together with changes in testicular size under both natural and artificial photoperiodic conditions, we found that the sparrows possess definite seasonal cycles of expressions of GnRH-I and GnIH peptides that follow an antiphasic pattern. Long photoperiods, either natural or artificial, stimulate gonadal growth and development in the tree sparrow by upregulating *GnRH-I* and downregulating *GnIH* genes expressions at both the transcription and translation levels in the neurons residing in the POA and PVN areas of the hypothalamus. In contrast, gonadal regression and development of photorefractoriness following continued exposure to long day lengths or maintenance of quiescent gonads under short day lengths is characterized by hyporegulation of *GnRH-I* and hyper-regulation of *GnIH* genes. Further, tree sparrows possess a critical photoperiod and show significant increase in expression of GnRH-I and decline in GnIH with gonadal growth only when the daily photoperiod is 11 h or more. These results clearly indicate that tree sparrows are capable of fine discrimination of photoperiodic information and use day length for *GnRH-I* and *GnIH* expressions to control their seasonal reproduction [33,55]. Sparrows respond to some photoperiods (those above 11 h) and not to all further their photoperiodic responses change with the change in photoperiods suggesting that they can discriminate photoperiodic information in terms of expressions of GnRH-I and GnIH expression with considerable degree of accuracy. By employing resonance protocols, we have revealed that the tree sparrows measure photoperiodic time using their endogenous circadian rhythm in regulation of reproductive responses including gonadal growth, histomorphology and serum levels of gonadal steroids [8,37,56]. Moving a step forward, the present finding clearly suggests the importance of position of light with respect to photoinducible and non-photoinducible phases of the circadian rhythm in regulation of molecular circuitry involving GnRH-I and GnIH expressions at the hypothalamic level leading to photoperiodic stimulation / inhibition of HPG axis and gonadal response accordingly. Our results are consistent with those reported in Japanese quail that reveal an increase in the expression of TSH-β in the pars tuberalis (PT) upon coincidence of light in the photoinducible phase which is followed by a sustained reciprocal switching of two thyroid enzymes viz. DIO2 and DIO3 that finally leads to activation of reproductive axis [57]. The Japanese quail and black-headed bunting show induction of gonadotropin secretion, if light phase coincides with the photoinducible phase [58,59]. Thus, coincidence of light with the photoinducible phase triggers molecular cascade in the hypothalamus leading to stimulation of GnRH-I synthesis and release and inhibition of GnIH. It has been proven in both mammals and birds that the core oscillator of the circadian clock genes viz. Period (Per), Cryptochrome (Cry), Bmal1 and Clock express rhythmically not only in the suprachiasmatic nucleus (SCN) but also in brain regions such as the MBH, pituitary gland and pineal gland. In Japanese quail, the localization of circadian clock has been demonstrated in the MBH that house the centre regulating photoperiodic time measurement [60].

5. Conclusions

The "resonance" model has been previously documented in some avian species. The present study is a step forward in understanding the mechanistic details of the regulation of important components of neuroendocrine circuitry regulating photoperiodic responses in the trees sparrow. It provides further an unequivocal evidence for an external-coincidence circadian mechanism in controlling seasonal gonadal cycle mediated by altered expression of opposing stimulatory/inhibitory hypothalamic neuropeptides namely, the GnRH-I and GnIH in the tree sparrows. However, the mechanism by which the circadian clock determines the photoinducible and non-photoinducible phases remains unanswered and needs further investigation. In addition to anticipating environmental change through transduction of photoperiodic information and modifying reproductive state accordingly, circadian expressions of GnRH-I and GnIH are also positioned to regulate acute changes in reproductive status should unpredictable conditions manifest throughout the year.

Many organisms use their circadian system for measuring photoperiod to regulate annual events including seasonal reproduction. The circadian clock helps the organism to anticipate daily environmental changes and prepare accordingly as an adaptation for a rotating world. Whether circadian rhythms have an adaptive significance is an intriguing question. The circadian systems in the organisms that match the periodic environment are adaptive. They are the result of a response to selection forces and therefore have a significant adaptive value. Some seasonal species, in their natural environment, exhibit adaptations in

their circadian systems that correlate with living at different latitudes. In addition, some show plasticity in their circadian systems to match their physical and social environment. Thus, there is an adaptive advantage to have circadian activity if daily conditions are fluctuating. However, in constant environmental conditions, there may be no advantage to restricting activity to certain times of day.

Declaration of Competing Interest

The authors declares that there is no conflict of interest.

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