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# Photoperiodic control of GnRH-I expression in seasonal reproduction of the Eurasian tree sparrow

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Day length has been shown to be a major source of temporal information regulating seasonal reproduction in the Eurasian tree sparrow (*Passer montanus*). The present study aims to investigate the photoperiodic expression of gonadotropin-releasing hormone-I (GnRH-I), and how it mediates seasonal reproduction in male tree sparrows. In different experiments, we studied photoperiod-induced changes in GnRH-I expressing neurons in the hypothalamic preoptic area (POA), together with changes in testicular size under both natural and artificial photoperiodic conditions. Experiment 1, which involved studying changes in GnRH-I expression and testicular volume during different phases of the annual reproductive cycle under natural day length (NDL), revealed that sparrows possess a definite seasonal cycle of GnRH-I peptide expression that runs parallel to testicular size. Birds showed significantly higher levels of GnRH-I expression in the breeding phase when compared to the non-breeding phase. In experiment 2, photosensitive birds were exposed to artificial short (SD: 9L/15D) and long (LD: 14L/10D) day lengths for 240 days to investigate the photoperiodic regulation of GnRH-I expression. They exhibited a significant increase followed by a decrease in GnRH-I expression and testicular size under LD but not SD, suggesting photoperiodic regulation. In experiment 3, when photosensitive birds were exposed to increasing photoperiods (9L/15D, 10L/14D, 10.5L/13.5D, 11L/13D, 12L/12D) for 30 days to find out the critical photoperiod for GnRH-I expression, they responded only when the daily photoperiod was 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 expression to control their seasonal reproduction.

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## Introduction

Many birds time their reproduction when the environmental conditions are most favourable for successful breeding.[1] Among different environmental factors, the photoperiod can be viewed as an initial cue that drives changes in the neuroendocrine system controlling reproduction in birds.[2–5] The photoperiod acts as the most reliable predictor of the season in the majority of bird species to forecast local conditions and initiate physiological preparations for reproduction well in advance of the optimum environmental conditions.[3,6–8] Supplementary cues, such as rainfall, temperature and food availability, integrate to fine-tune the timing of breeding to match variation in the local environment.[5,9]

It is generally accepted that the photoperiod initiates the reproductive process by activating the hypothalamo-pituitary-gonadal (HPG) axis. It exerts neuroendocrine control of the annual reproductive cycle by promoting changes in the secretion of GnRH. Based on its location in the brain, GnRH is recognised in two functionally different isoforms: GnRH-I in the preoptic area (POA) and GnRH-II in the midbrain.[10] It is well established that GnRH-I primarily controls reproductive physiology in birds by stimulating gonadotropin synthesis and release from the anterior pituitary, whereas GnRH-II is possibly involved in controlling reproductive behaviours.[11–13] In birds, the main populations of GnRH-I-ir cell bodies are bilaterally clustered in the POA, which extends from the anterior commissure to the supracommissural division of the organum vasculosum of the lamina lateralis.[14,15] GnRH-I immunoreactive fibres project into the median eminence, allowing for the regulation of pituitary gonadotropin release. Thus, the GnRH-I neuronal system provides the key link between the brain and the reproductive endocrine system.[16] In the majority of avian species, the annual changes in photoperiod cause marked changes in GnRH-I secretion. Greater numbers of immunoreactive GnRH-I neurons have been detected in the brain during the breeding season and a dramatic decrease has

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been observed in non-breeding seasons.[3,17,18] In seasonal species, variation in photoperiod is associated with marked changes in brain GnRH-I and testicular size.[19] In photoperiodic birds, long days stimulate gonadal maturation, whereas short days fail to do so.[5] This is due to the induced and inhibited secretion of GnRH-I under long and short day conditions, respectively.[20] However, the molecular mechanism regulating seasonal GnRH-I secretion is not completely understood in songbirds.

In order to effect a change in reproductive physiology and behaviour, day length needs to be perceived, assessed, transduced, and finally translated in a neuroendocrine response in birds.[21] The primary sensory system that detects photoperiodic cues in birds is thought to be deep brain photoreceptors (DBPs).[21] DBPs have been proposed to localise at four different sites in the lateral septal region of avian brains, including the lateral septal organ (LSO), the paraventricular nucleus (PVN), premammillary nucleus (PMN), and the paraventricular organ (PVO).[22–24] It is possible that all of the above brain areas possess DBPs and coordinate in the detection of photoperiodic information. The available evidence suggests that the most plausible site associated with the photoperiodic response is the mediobasal hypothalamus (MBH), operating *via* the direct innervation of GnRH neurons by encephalic photoreceptors,[25] or *via* the pars tuberalis (PT) of the adenohypophysis.[26] The cross-talk within the DBP-containing neurons and GnRH-I neurons involves the major manner in which GnRH-I neurons are regulated.[27] Until recently, some DBPs including rhodopsin, melanopsin, neuropsin and vertebrate ancient (VA) opsin have been identified in various photoperiodic birds. Neuropsin (Opn5), which is expressed in the anterior pituitary and medial hypothalamus, has been linked to the receptor-mediated neuroendocrine signalling that regulates the seasonal breeding in birds.[28]

There is evidence suggesting that long photoperiods stimulate the synthesis of thyrotropin-stimulating hormone beta (TSH-β) in the pars tuberalis,[29,30] which leads to an increase in the expression of the gene encoding type 2 iodothyronine deiodinase (DiO2). DiO2 is a thyroid hormone activating enzyme that converts thyroxine ($T_4$) into triiodothyronine ($T_3$), which leads to an increase in the local production of $T_3$.[31–33] $T_3$ further alters the structural arrangement of the GnRH-I nerve terminals at the median eminence, where the glial end feet that ensheath the terminals retract, promoting an increase in the secretion of GnRH-I.[34,35] However, in short photoperiods, the 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,[29,31] causes the inhibition of GnRH-I synthesis.[36] A long day length has two separate effects on GnRH-I neurones.[20] The first is a photoperiod based effect on GnRH-I release in the median eminence, where longer the day length greater is the release rate of GnRH-I causing increasing secretion of pituitary gonadotropins leading to gonadal growth and development. Secondly, a long day length has an effect on GnRH-I synthesis, where chronic exposure to long days downregulates GnRH-I synthesis, thereby inducing photorefractoriness. Conversely, GnRH-I levels increase as photorefractoriness is terminated and photosensitivity is resumed during short photoperiods.[16] Thus, long days have gonado-stimulatory as well as gonado-inhibitory effects. The gonado-inhibitory effects are due to the direct action of a neurohormone known as gonadotropin-inhibitory hormone (GnIH)[37] on GnRH-I activity by acting *via* its specific receptor (GPR147) located on GnRH-I neurons.[38] Thus, GnIH has the potential to influence the HPG axis activity through its action on GnRH-I neurons, ultimately leading to the regulation of seasonal reproduction in birds.

In our earlier investigations on Eurasian tree sparrows, we revealed that they are photosensitive and use day length to control their seasonal reproduction. The initiation of gonadal growth in this species is a long day phenomenon, while the termination of photorefractoriness and consequent recovery of photosensitivity is a short day phenomenon. Furthermore, their annual reproductive cycle can be divided into four distinct phases, *i.e.*, preparatory (December–January), progressive (February–March), reproductive (April–May) and regressive (June–November), with a single annual reproductive peak in May.[1,39,40]

Despite decades of research into photoperiodic regulation of avian reproduction, the physiological mechanisms by which the reproductive system is activated and deactivated in breeding and non-breeding seasons, respectively, are not completely understood and need further investigation. Moreover, various studies on GnRH-I peptide expression in different avian species have mostly been short-term and fragmented. Investigations into the neuroendocrine regulation of seasonal reproduction and the role of GnRH-I using experiments performed during different phases of the reproductive cycle, as well as under different artificial photoperiodic conditions for a longer duration, may give a better understanding of the photoperiodic control of avian reproduction *via* the GnRH-I system.

## Materials and methods

### Animal model

Adult male tree sparrows, a non-migratory resident species inhabiting the north-eastern part of India, were captured in and around the hills of Shillong using a mist net. Three experiments were performed using these birds, which are described below.

### Experiments

**Experiment I: Expression of GnRH-I peptide under natural day length (NDL).** This experiment was performed from January to December, 2015, to study the seasonal expression of GnRH-I peptide in relation to annual variations in testicular size in tree sparrows and day length at Shillong. Birds ($n = 4$) captured from their wild habitat in the middle of the months during four different phases of their annual reproductive cycle, *i.e.*, preparatory (December–January), progressive (February–

March), reproductive (April–May) and regressive (June–November), were used in this study. At each observation, they were first anaesthetised and then perfused to remove the brain for further processing for immunohistochemical studies. GnRH-I peptide expression was measured in terms of cell number, cell area, % cell area and cell optical density (OD) of GnRH-I expressing neurones in the hypothalamus. In addition, the testes of birds were located in the body cavity and their sizes were measured to determine their reproductive status. Data for annual variations in day length were obtained from the meteorological department at Shillong.

**Experiment II: Expression of GnRH-I peptide under artificial day length.** This experiment was performed to investigate whether the expression of GnRH-I peptide in the tree sparrow is photoperiodically regulated. In this experiment, which began in January 2016, photosensitive birds ($n$ = 70) were exposed to two different artificial photoperiods, *i.e.*, 9L/15D ($n$ = 35) and 14L/10D ($n$ = 35), which were close to the shortest and longest day lengths that birds experience in Shillong during the year, respectively. Data on the expression of GnRH-I-ir cells (cell number, cell area, % cell area and cell OD) in the POA and testicular size were recorded at an interval of 30 days during the course of the experiment, which continued for 240 days. At each observation, four birds were randomly selected from each light regime to record the above data.

**Experiment III: Critical day length (CDL) for GnRH-I expression.** This experiment was carried out to find out the minimum photoperiod required to induce GnRH-I peptide expression in the tree sparrow. In this experiment, groups of photosensitive birds ($n$ = 4 per group) were exposed to various combinations of light and dark over a 24 h cycle with an increasing proportion of light periods, *i.e.*, 9L/15D, 10L/14D, 10.5L/13.5D, 11L/13D and 12L/12D, for 30 days. GnRH-I peptide expression and testicular size were recorded at the beginning and end of the experiment. A group of four birds was sacrificed on day 0 to record initial data, while all of the birds were sacrificed at the end of the experiment for immunohistochemical studies and measurement of testicular size.

### Experimental conditions

The birds subjected to artificial photoperiods were captured from their wild habitat in November 2015 and were kept in an outdoor open aviary with unrestricted access to natural light, temperature and humidity for three days. These birds were then brought indoors and acclimatised to the laboratory conditions for a fortnight. There, they were subjected to natural variations of photoperiod, temperature and humidity. These birds were then transferred to the short day length (9L/15D) condition for eight weeks to eliminate photorefractoriness, if they had any in nature, and to ensure their photosensitivity at the time of commencement of the various experiments. Laparotomy (surgical opening of abdominal wall between the last two ribs) at four-week intervals during this pretreatment period revealed that they had regressed and sexually immature testes. These photosensitive birds were used for investigations under different artificial photoperiods. They were housed in light proof wooden chambers (2.10 m × 1.20 m × 1.35 m) illuminated by CFL bulbs (Philips Electronics India Limited, Kolkata, India) providing light with an intensity of ~400 lux at the perch level with automated control of light on and off. Our photoperiodic chambers were well aerated through inlets and outlets connected to air circulators. The temperature and humidity of the experimental chambers, as recorded by a HOBO data logger, varied in the ranges of about 17 °C (December) to 24 °C (June) and 55–75%, respectively, in a year. Food and water were available *ad libitum* and were replenished only during the light phase of the cycle. Periodic observations were recorded on cell number, cell area, % cell area and cell OD of GnRH-I peptide expressing neurones and testicular size.

### Measurement of testicular size

Testicular development was measured in terms of changes in volume. Briefly, testicular size was recorded *in situ* by opening the abdominal wall, locating the left testis with the help of a spatula and measuring its length and width with respect to divisions on graph paper using a calliper after the brain was taken out following anaesthesia and perfusion as described below. Testicular volume (TV) was calculated using the formula $4/3\pi ab^2$, where $a$ and $b$ denote half of the long (length) and short (width) axes, respectively.

### Measurement of GnRH-I peptide expression

**Tissue preparation.** For tissue preparation, we used the protocol described by Rastogi *et al.* (2011).[41] Briefly, birds deeply anesthetised with ketamine-xylazine solution (0.003 ml per g body weight) were transcardially perfused successively with 25 ml chilled saline water and 25 ml fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) under the light phase of the light–dark cycle. Quickly dissected brains were then post-fixed overnight using same fixative, and thereafter cryoprotected in a gradient of sucrose (Merck) solutions (10%, 20% and 30%) at 4 °C. Finally, the brains were embedded in 15% polyvinylpyrrolidone (PVP; PVP40 T, Sigma) and stored at −80 °C until further processing.

**Immunohistochemistry of GnRH-I peptide.** All of the brain tissues were processed together for immunohistochemistry studies of GnRH-I peptide to minimize differences in the staining intensity using a standard avidin–biotin protocol with slight modifications to the method described by Rastogi *et al.* (2011).[41] In brief, the whole brain was sectioned in the coronal plane at 30 μm thickness on a cryostat (Leica CM 1850). These sections were collected separately in a 24-well tissue culture plate containing phosphate-buffered saline (PBS, pH 7.4), with a total of five sections per well. These sections were then given three 10 min rinses with PBS. They were then successively treated for 30 min with 0.3% hydrogen peroxide ($H_2O_2$) dissolved in methanol to block any endogenous peroxidase activity. After washing the sections in PBS three times, they were incubated for 30 min in 1% normal bovine serum

albumin (BSA) dissolved in PBS containing 0.3% Triton-X100 (PBSBT) to block any unspecific binding. Thereafter, sections were incubated with GnRH primary antibody (rabbit anti-GnRH; dilution 1 : 18 000; HU60H) overnight at 4 °C. The next day, three subsequent washes in PBS were followed with incubation for 2 h in biotinylated goat anti-rabbit IgG secondary antibody (1 : 200; B2770; Invitrogen, Eugene, USA). After washing, they were then incubated for another 2 h in avidin–biotin complex (1 : 110; Elite ABC Kit; Vector Laboratories, Burlingame, CA). Finally, all sections were washed with PBS and visualized for the antigen–antibody reaction after treatment with diaminobenzidine solution (DAB; D4293, Sigma) for 3–5 min. The colour reaction was stopped after the appearance of a minimal background reaction by adding more PBS. The sections were washed in distilled water, ordered and mounted onto glass slides coated with poly-L-lysine. They were then dehydrated in the ascending grades of alcohol, cleared in xylene and cover-slipped in DPX.

The primary antibody used for this study, HU60 bleed, was kindly gifted by Dr Henryk F. Urbanski, Oregon Health and Sciences University, Portland, Oregon, USA. The characteristics of this antibody have been described in Urbanski *et al.* (1990) and Urbanski (1992).[42,43] This antibody is generated in rabbits against mammalian GnRH. It is highly specific for GnRH, as reported in various studies.[10,44] Although the antibody does not distinguish between GnRH-I and GnRH-II neurons, they can be identified on the basis of their separate locations and from their distinctive appearance. GnRH-I neurons are located in the preoptic area, while GnRH-II neurons are localised in the midbrain. Also, GnRH-II neurons are smaller in size and stubby with thinner neuritis.[45] To confirm the specificity of the GnRH antibody in our birds, a control was also run along with the GnRH antibody-labelled tissue to investigate the possibility of non-specific immunoreactivity. The absence of a primary antibody in the reaction resulted in a total loss of immunoreactivity, while the presence of it showed a strong immunoreactivity with an efficiency up to 1 : 18 000 µl dilutions. Control procedures were performed to verify the specificity of the immunoreaction. This included the omission of the primary antisera from the reaction, as well as the replacement of the antisera with buffer or BSA. Both of these procedures resulted in a total loss of immunoreactivity.

**Quantification of GnRH-I peptide.** The desired brain sections were examined in a trinocular bright-field microscope (Motic) and digital images of immunoreactive cells were captured using a high megapixel camera (Motic cam). Images of the specified region were captured at 10× and 40× magnifications. Photography was done using standard illumination. The images were adjusted for size, contrast and brightness as per requirement using the Motic image version 2 analyser software.[5] GnRH-I immunoreactive cells were counted in the entire POA, starting from the division of tractus septomesencephalicus (TSM) to the caudal extent of the anterior commissure, until no GnRH-I cell bodies were detected. We counted both strongly (bright) and weakly (faint) stained GnRH-I-ir cells to avoid any staining-intensity bias.[46] Thus, the numbers of GnRH-I-ir cells in the POA obtained from all brains were summed and their mean (±SE) was calculated. In the same area, additional measures of immunoreactions, cell area, % cell area and cell OD were also quantified.[46] The number of cells and % cell area reflect the density of a specific neuronal population, whereas cell area and cell OD serve as a measure of peptide content as detected by the primary antibody in a cell.[19,46,47] We measured cell area, % cell area and cell OD by capturing images at 40× magnification using ImageJ (NIH) software. A defined frame of 200 × 150 $\mu m^2$ was chosen to measure all of these three parameters.[48] Calculations were performed by making outlines of all individual cells covered in a frame in every fourth section, while cell body areas were summed up and averaged for the 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 from the total cell area/frame area multiplied by 100, and an average value of the % cell area for images from all of the sections gave a single value for each bird. Relative cell OD (intensity above background) was obtained by substracting the 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 study complied with all institutional and national guidelines, as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) under the Prevention of Cruelty to Animals Act, 1960, of the Government of India. The protocol was approved by the Institutional Animal Ethics Committee of the North-Eastern Hill University, Shillong (1886 of 04. 12. 2014) and all participants provided written informed consent.

### Statistical analyses

All data are presented as mean ± S.E.M. They were analysed using one- or two-way ANOVA as per the experimental design, followed by a Bonferroni *post-hoc* mean comparison test, if ANOVA indicated a significant difference. Regression and correlation analyses were also performed to investigate the relationship between testicular size and the number of GnRH-I-ir cells. All statistical analyses were performed using GraphPad Prism software (version 6.0, Sandiego, CA, USA). Significance was taken at the 95% confidence level.

## Results

The data are presented in Fig. 1–5.

### Experiment I: Expression of GnRH-I peptide under natural day length (NDL)

Day length is recorded at a minimum (10.29 h) during the winter solstice in December. It starts increasing thereafter until the summer solstice and the longest day length of

13.44 h is attained in June. This is followed by a decrease in day length until its minimum in December. Thus, the annual variation in day length at Shillong is 3 h 15 m (Fig. 1a).

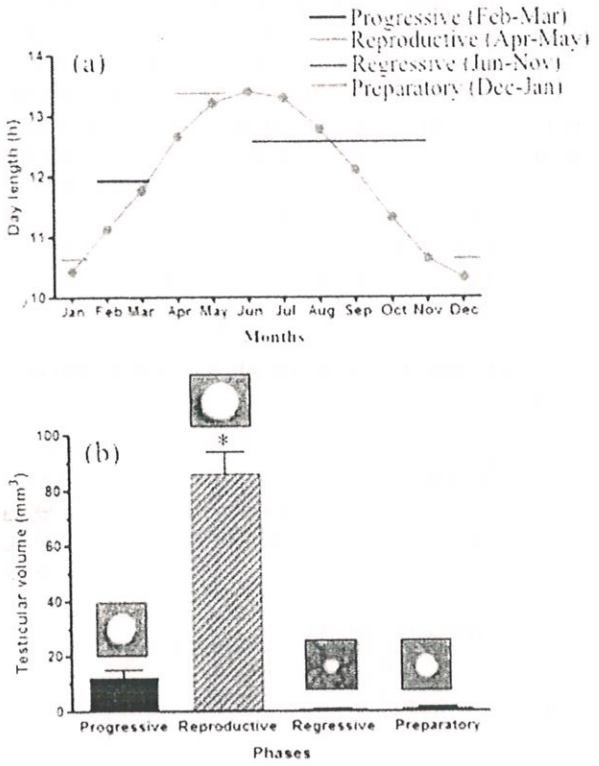


Fig. 1 Annual changes in day length at Shillong (a) and testicular volume (b) during different phases of the annual gonadal cycle of the tree sparrow. *$P < 0.05$.

Observations of GnRH-I immunoreactive cell bodies in different areas of the brain revealed that they are located primarily in the hypothalamic POA of the brain in tree sparrows (Fig. 2A–D). The annual variations in GnRH-I expression (cell number: $F_{3,15} = 209.1$, $P < 0.0001$; % cell area: $F_{3,15} = 4.964$, $P = 0.0182$; cell area: $F_{3,15} = 9.091$, $P = 0.0021$; cell OD: $F_{3,15} = 43.47$, $P < 0.0001$; One-way ANOVA) during different phases of the annual reproductive cycle under natural day length were found to run parallel to annual changes in testicular size ($F_{3,9} = 97.80$, $P < 0.0001$; One-way ANOVA) and day length at Shillong (Fig. 1b and 2). Furthermore, all four parameters for the measurement of GnRH-I expression (*i.e.*, cell number, % cell area, cell area and cell OD) varied more or less in the same fashion during different phases of the annual reproductive cycle of the tree sparrow. A significant and gradual increase in GnRH-I expression was noticed in the preparatory (December–January; cell number: $P < 0.001$, % cell area: $P < 0.05$, cell area: $P < 0.01$ and cell OD: $P < 0.01$) and progressive (February–March; cell number: $P < 0.001$, % cell area: $P < 0.01$, cell area: $P < 0.05$ and cell OD: $P < 0.001$; One-way ANOVA) phases with the increase in testicular size and day length at Shillong. Peptide expression reached its peak in the reproductive phase (April–May: cell number: $P < 0.001$, % cell area: $P < 0.05$, cell area: $P < 0.05$ and cell OD: $P < 0.01$) when the testes attained their maximum size. This was followed by a decline in GnRH-I expression (cell number: $P < 0.001$, % cell area: $P > 0.05$, cell area: $P < 0.05$ and cell OD: $P < 0.05$) with testicular regression ($P < 0.01$) reaching a minimum in the regressive phase (June–November), indicating the onset and persistence of photorefractoriness (Fig. 1b and 2a–d). Observations on GnRH-I expression (cell number, % cell area, cell area and cell OD) in the POA were found to be significantly higher in the reproductive phase when compared to the preparatory, progressive and regressive phases (Fig. 2). Furthermore, a comparison of the

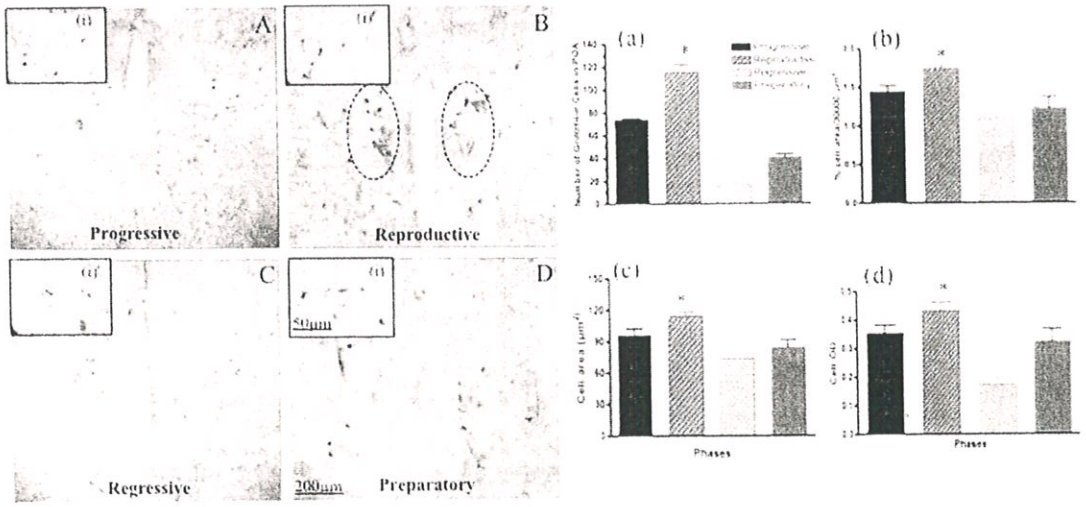


Fig. 2 GnRH-I immunoreactivity in the POA of the brains of tree sparrows during different phases of the annual gonadal cycle (A–D). Changes in cell number (a), % cell area (b), cell area (c) and cell OD (d) of GnRH-I-ir cells during different phases of the annual testicular cycle of tree sparrows. *$P < 0.05$.

numbers of GnRH-I-ir neurons in the POA with testicular volume during different phases of the reproductive cycle of the tree sparrow revealed a positive correlation (Fig. 5a).

### Experiment II: Expression of GnRH-I peptide under artificial day length

Two-way ANOVA revealed significant variations in GnRH-I expression in the POA of tree sparrows exposed to artificial short and long day lengths (cell number: photoperiod-$F_{1,36}$ = 190.2, $P < 0.0001$, days-$F_{8,36}$ = 79.77, $P < 0.0001$, interaction-photoperiod*days-$F_{8,36}$ = 71.76; % cell area: photoperiod-$F_{1,54}$ = 0.01322, $P = 0.0089$, days-$F_{8,54}$ = 2.504, $P = 0.0216$, interaction-photoperiod*days-$F_{8,54}$ = 3.238, $P = 0.0044$; cell area: photoperiod-$F_{1,54}$ = 2.722, $P = 0.0048$, days-$F_{8,54}$ = 15.05, $P < 0.0001$, interaction-photoperiod*days-$F_{8,54}$ = 3.238, $P = 0.0044$ and cell OD: photoperiod-$F_{1,54}$ = 4.032, $P = 0.0497$, days-$F_{8,54}$ = 1.776, $P = 0.0022$, interaction-photoperiod*days-$F_{8,54}$ = 0.7304, $P = 0.6641$, Fig. 3a, b and d–g) and testicular volume (photoperiod: $F_{1,54}$ = 131.5, $P < 0.0001$; days: $F_{8,54}$ = 59.71, $P < 0.0001$; interaction-photoperiod*days: $F_{8,54}$ = 59.82, $P < 0.0001$, Fig. 3c). Sparrows, under long day length, showed a significant increase ($P < 0.001$) in GnRH-I expression (cell number, % cell area, cell area and OD) with testicular growth, followed by a decrease in GnRH-I expression with testicular regression leading to their minimal values (Fig. 3a and d–g). On the other hand, birds exposed to a short day length failed to show the above responses over a total duration of 240 days (Fig. 3b and d–g). A significant increase in GnRH-I expression was observed under long day length on day 30 (cell number: $P < 0.001$; % cell area: $P < 0.05$; cell area: $P < 0.05$ and cell OD: $P < 0.05$) leading to maximum expression on day 60 (cell number: $P < 0.001$; % cell area: $P < 0.001$; cell area: $P < 0.001$ and cell OD: $P < 0.001$). A significant decline in peptide expression to a minimum value was noticed on day 90, which was maintained until the end of the experiment on day 240 (cell number: $P > 0.05$; % cell area: $P > 0.05$; cell area: $P > 0.05$; cell OD: $P > 0.05$, Fig. 3a and d–g). Sparrows exhibited a positive correlation between GnRH-I cell number and testicular volume both under LD and SD (Fig. 5b and c).

### Experiment III: Critical day length (CDL) for GnRH-I peptide expression

One-way ANOVA exhibited significant variations in GnRH-I expression (cell number: $F_{4,14}$ = 21.57, $P < 0.0001$; % cell area: $F_{4,9}$ = 2.032, $P = 0.0081$; cell area: $F_{4,9}$ = 2.151, $P = 0.0114$ and cell OD: $F_{4,9}$ = 1.447, $P = 0.0024$) and testicular volume ($F_{4,14}$ =

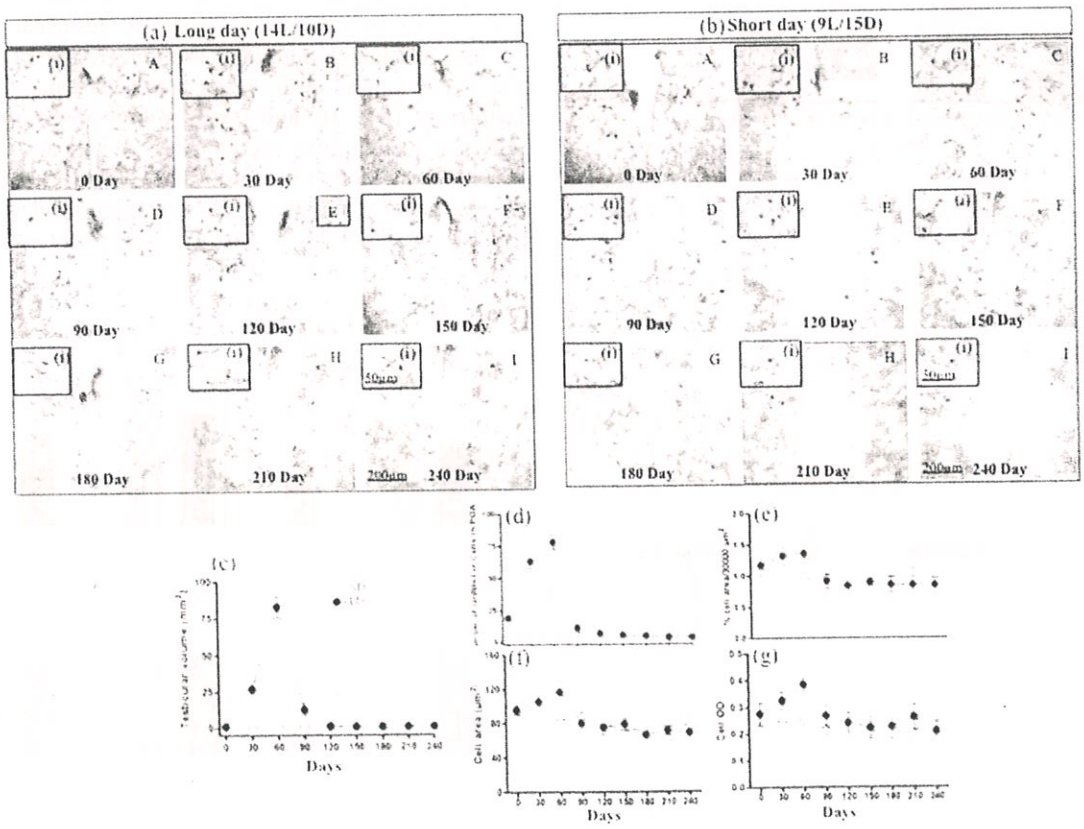


Fig. 3 GnRH-I immunoreactivity in the POA of the brains of tree sparrows under long (a: A–I) and short (b: A–I) day lengths. Testicular response under LD and SD (c). Changes in cell number (d), % cell area (e), cell area (f) and cell OD (g) of GnRH-I-ir cells in the POA of tree sparrows under LD and SD.

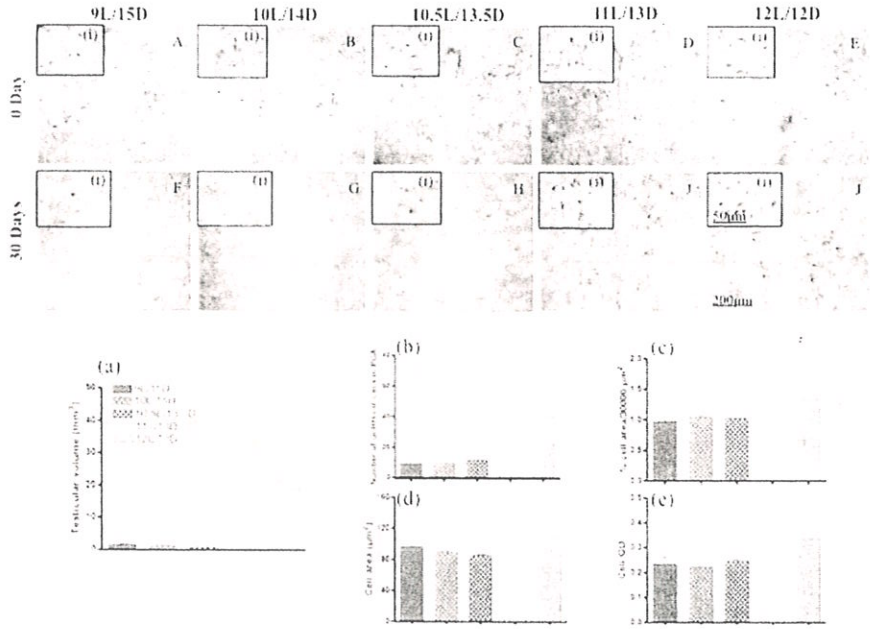


Fig. 4 GnRH-I immunoreactivity in the POA of the brains of tree sparrows under increasing daily photoperiods (A–J). Changes in testicular volume (a), cell number (b), % cell area (c), cell area (d) and cell OD (e) under different photoperiods.

30.15, $P < 0.0001$) in the sparrows exposed to various light dark cycles. Birds did not respond to daily photoperiods of 9, 10 and 10.5 h, but significant peptide expression ($P < 0.001$) and testicular growth were observed in the birds exposed to 11L/13D, 12L/12D. Furthermore, the rates of peptide expression and testicular growth were found to be greater under longer photoperiod. The mean peptide expression (cell number: $P < 0.001$; % cell area: $P < 0.01$; cell area: $P < 0.01$ and cell OD: $P < 0.01$) and testicular volume ($P < 0.001$) in the birds under 11L/13D and 12L/12D were significantly higher than those in the birds under 9L/15D, 10L/14D and 10.5L/13.5D. However, no significant difference was observed in either peptide expression or testicular volume between the birds under 11/13D and 12L/12D ($P > 0.05$, Fig. 4). Furthermore, the birds exhibited testicular development only under the photoperiods that induced peptide expression (GnRH-I-ir cell number), indicating a positive correlation between the two (Fig. 5d). These results clearly suggest that light exposure for 11 h per day or more is important in inducing GnRH-I expression and testicular growth in the tree sparrow.

## Discussion

The GnRH-I-ir neurons, which are found primarily in the hypothalamic POA area of the brain, exhibit seasonal variations in their expression of GnRH-I peptide. A positive correlation was found between the expression of GnRH-I peptide and testicular size in the tree sparrow. Increasing day length in spring was observed to trigger testicular growth, alongside an increase in the neuronal expression of GnRH-I during the progressive phase (February–March), which continued to increase further and peaked in the reproductive phase (April–May). However, a significant drop in the expression of GnRH-I peptide was noticed with the onset of testicular regression (June) when the day length was still longer than that in the spring months that triggered gonadal development and an increase in peptide expression, indicating the onset of photorefractoriness. Thus, the onset of photorefractoriness in the tree sparrow is at the hypothalamic level due to decreased expression of GnRH-I, leading to a minimum level, which is ultimately translated into testicular regression. The minimal level of GnRH-I and quiescent testes were almost maintained until the end of the regressive phase (November). This was followed by the preparatory phase (December–January) when an increase in GnRH-I expression was noticed but the birds did not show testicular growth as they were still experiencing short day length in nature. A comparison of testicular size and levels of expression of GnRH-I peptide during different phases of the annual reproductive cycle with annual variations in day length at Shillong reveals the possibility of their photoperiodic regulation in the tree sparrow (Fig. 1 and 2). Furthermore, sparrows, when exposed to artificial long (LD:14L/10D) day length, exhibited an increase followed by a decrease in GnRH-I peptide expression with testicular growth and regression, respectively. On the other hand, no significant change in either GnRH-I peptide or testicular size was noticed in the birds maintained under short day length (SD:9L/15D; Fig. 3). Thus, an increase in GnRH-I peptide expression was evident and was found to run parallel to an increase in testicular size during the spring and summer

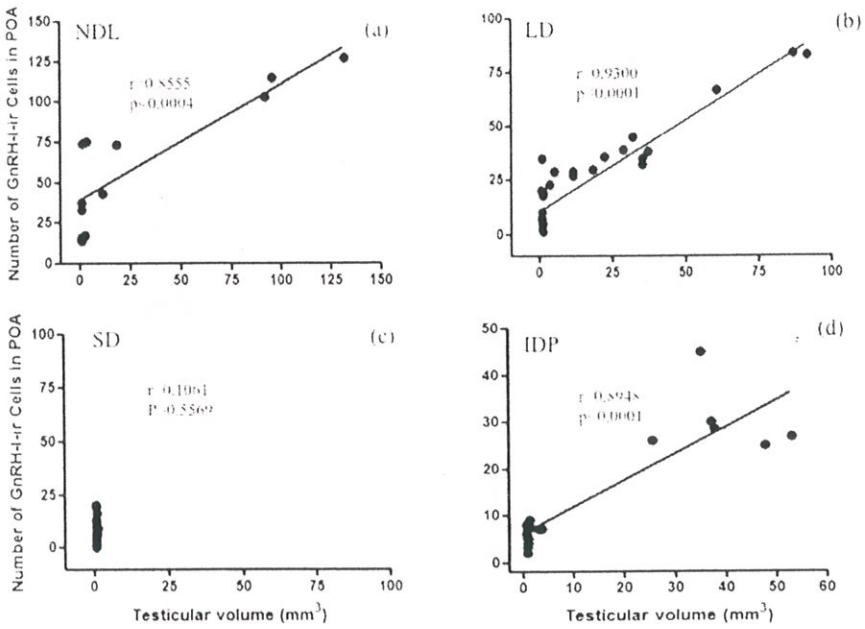


Fig. 5 Correlation of testicular volume with the number of GnRH-I expressing cells in the POA of tree sparrows under (a) natural day length (NDL), (b) long day length (LD), (c) short day length (SD) and (d) increasing daily photoperiod (IDP).

months, or under artificial LD (before photorefractoriness) when the tree sparrows were experiencing long day lengths. Meanwhile, the birds experiencing short day lengths either in the laboratory or in nature had regressed gonads and minimum GnRH-I expression. These results clearly suggest that photoperiodic changes in GnRH-I expression in the hypothalamic GnRH-I-ir neurons of the POA play an important role in processing photoperiodic information in the brains of tree sparrows, leading to regulation of the HPG axis and ultimately, the control of seasonal reproduction.

Tree sparrows exhibited a significant increase in all measures of GnRH-I immunoreactivity (*i.e.* cell number, % cell area, cell area and cell OD) in the pre-breeding and breeding phases, followed by a decrease in the refractory and non-breeding states under both NDL and LD (Fig. 2 and 3a, d–g). GnRH-I has also been reported to display a similar expression pattern with respect to reproductive stages and changing photoperiod in some other seasonally breeding birds, such as the European starling (*Sturnus vulgaris*), Gambel's white-crowned sparrow (*Zonotrichia leucophrys gambelli*), American tree sparrow (*Spizella arborea*), house finch, (*Carpodacus mexicanus*) and dark eyed junco (*Junco hyemalis*).[49–53] There are reports on significant changes in GnRH-I synthesis and content[54] in the POA as a function of the reproductive condition. In general, birds that are undergoing gonadal regression or are photorefractory have less hypothalamic GnRH-I compared to photosensitive ones.[49,52] The present study supports and extends the above findings. As observed in the tree sparrow, low GnRH-I levels are associated with the onset of absolute photorefractoriness (post-breeding) in several birds,[55] including the European starling,[49] Gambel's white-crowned sparrow,[50] house sparrow (*Passer domesticus*),[47] American tree sparrow,[51] house finch,[52] dark eyed junco[53] and redheaded bunting (*Emberiza bruniceps*),[48] in which the decline in hypothalamic GnRH-I levels run parallel to post-breeding testicular regression.[20,45] Interestingly, there are differences in seasonal changes in GnRH-I expression between absolute and relative refractory species.[56] Absolute refractory birds, *i.e.*, the tree sparrow, white-crowned sparrow,[57] house sparrow,[47] American tree sparrow,[51] house finch,[52] dark-eyed junco[53] and rufous-winged sparrow (*Aimophila carpalis*),[58] show significant variations in brain GnRH-I expression levels between the photosensitive, photostimulated and early photorefractory states. However, in relative photorefractory birds, such as the domestic chicken (*Gallus domesticus*), Japanese quail (*Coturnix coturnix japonica*) and Indian weaver bird (*Ploceus philippinus*), there is a lack of seasonal plasticity in GnRH-I peptide expression.[10] In these species, the development of photorefractoriness is not associated with a decrease in GnRH-I levels.[55] The difference in hypothalamic GnRH-I expression between absolute and relative refractoriness suggests that the neuronal systems in birds may function in different ways causing diversity in photoperiodic reproductive responses. However, our birds exhibited an increase in GnRH-I expression at the end of photorefractoriness, which has also been observed in some other songbirds.[30,59,60] The above-mentioned variations in the immunoreactivity of GnRH-I-ir cells with seasonal reproductive states in the tree sparrow thus suggest the involvement of GnRH-I in the regulation of the annual testicular cycle.

Sparrows, when exposed to long day length, show a significant increase followed by a decrease in both GnRH-I peptide and testicular size, suggesting dual role of long day length in inducing photostimulation and photorefractoriness. Photosensitive birds exhibit a modest number of GnRH-I-ir cells; however, exposure to long day length enhances this number, leading to increased peptide expression, which is followed by a marked decrease in expression to almost non-detectable levels upon prolonged exposure (Fig. 3a and d–g). This is pivotal in establishing that the decline in GnRH-I levels may be a critical step required for the development of photorefractoriness in this bird species. Together, the findings provide a framework in which long days not only stimulate GnRH-I expression, but also initiate an inhibitory process leading to photorefractoriness. A similar phenomenon may occur upon exposure to short days, in which the initial exposure to short days initiates GnRH-I re-expression, but also initiates another series of events necessary for the subsequent receptivity to stimulatory long days. The exact signal produced during the exposure to short days that gives rise to the resumption of GnRH-I peptide expression is currently unknown, but may include the involvement of either an endogenous timing system or the removal of direct light stimulation during long days. However, there have been studies performed on certain birds that suggest an active participation of extraretinal photoreceptors (DBPs) in controlling the reproductive responses to seasonal changes in day length.[61,62] In the Japanese quail, significant expression of DBPs (Opn5 and VA opsin) in the POA was observed in photosensitive birds, while there was moderate expression of these photopigments in scotorefractory (refractory to the inhibitory effects of short days) birds. The expression of these photopigments was found to be significantly reduced in the hypothalamus of both photorefractory and scotosensitive (sensitive to inhibitory effects of short days) quails.[63] Thus, it seems that these specialised avian DBP neurons that detect photoperiodic information in quails could also be part of a key neural circuit with connectivity to GnRH-I neurons in other birds including the tree sparrow.

Our results further suggest seasonality in the responsivity of the endogenous system to annual changes in natural as well as artificial day lengths. They also confirm the seasonal expression of GnRH-I peptide and testicular development in the tree sparrow cycle between periods of photosensitivity and photorefractoriness. The initiation of GnRH-I expression, consequent gonadal growth and the onset of photorefractoriness characterised by a decline in peptide expression and testicular size after attaining their maxima are long day phenomena in the tree sparrow. Meanwhile, the termination of photorefractoriness and consequent recovery of photosensitivity expressed as reinitiation of GnRH-I expression and testicular growth are short day phenomena in this species. Thus, both long and short days are important for photoperiodic regulation of seasonal responses, including GnRH-I expression and testicular development in the tree sparrow, although the bird uses them for different purposes. The decline in GnRH-I expression under the short day condition and prolonged exposure to the long day condition may be due to the inhibitory effect of GnIH. Immunohistochemical studies in the brain of the house sparrow and European starling showed that GnRH-I neurons are in contact with GnIH axons, suggesting the direct role of GnIH in controlling the activity of GnRH-I neurons.[45,64] This is supported by the finding in the Japanese quail, where the photorefractory and scotosensitive birds showed the opposite expression pattern for GnRH-I and GnIH in the hypothalamus. In this species, the sexually regressed birds exhibited decreased hypothalamic GnRH-I and increased GnIH.[63] This may also be the case in the tree sparrow, in which a high level of GnIH[5] expression and low level of GnRH-I (present study) expression in the hypothalamus have been observed during the regressive and photorefractory phases, while the opposite was found in the case of photosensitive and photostimulated conditions.

The data presented in Fig. 4 indicate that light exposure for 11 h is important in inducing GnRH-I peptide expression and testicular growth in the tree sparrow. Daily photoperiods of 9 h, 10 h and even 10.5 h failed to induce GnRH-I expression and consequent gonadal growth in sparrows, while the birds experiencing 11 h and 12 h of light per day responded significantly. Further, the rates of these responses were found to be greater under the longer photoperiod (12 h). Thus, the tree sparrow, in the present study, may have a photoperiodic threshold equal to or even bit less than 11 h for the activation of neuroendocrine machinery, leading to GnRH-I expression and consequent gonadal growth and function. Tree sparrows in the wild show initiation of GnRH-I expression and gonadal growth during the progressive phase in February. The day length in this month at Shillong is recorded to be about 11 h per day. Thus, the natural time for GnRH-I expression and testicular growth in sparrows is almost similar to the photoperiodic threshold (11 h) for these activities observed under laboratory conditions. Further increases in day length in the subsequent months from March (progressive phase; day length: 12.01 h) to May (reproductive phase; day length: 13.34 h) accelerate the above responses to their maxima. These observations clearly support our findings in which the birds exhibited greater responses under the longer photoperiod of 12 h per day when compared to the shorter day length of 11 h per day (Fig. 4). Thus, despite the small annual variation in day length in the tropics and subtropics (3 h and 15 min at Shillong, India; 25°34′N, 91°53′E), increasing day length in spring stimulates the synthesis and release of GnRH-I from the hypothalamus, regulating reproductive seasonality in the tree sparrow.

## Conclusions

In conclusion, our study suggests that the photoperiodic control mechanism regulating seasonal reproduction in the tree sparrow depends on changes in hypothalamic GnRH-I expression in the POA of their brain. The results further confirm the neuronal link between photoperiod and the GnRH-I system in the processing of photoperiodic information

 

in the brain of the tree sparrow, which regulates the HPG axis and ultimately controls seasonal reproduction. Furthermore, this study provides a better understanding of the seasonal plasticity of GnRH-I in absolute photorefractory birds.

## Conflicts of interest

There are no conflicts of interest to declare.

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