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RESEARCH ARTICLE

Role of GnIH in photoperiodic regulation of seasonal reproduction in the Eurasian tree sparrow

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ABSTRACT

Seasonal reproductive cycles of most birds are regulated by photoperiod via neuroendocrine control. Gonadotropin-inhibitory hormone (GnIH) in the hypothalamus has been reported to act as neuroendocrine integrator of photoperiodic cues. In this study, both captive and field investigations were carried out to understand the effects of photoperiod and seasonality on GnIH expression in subtropical tree sparrows. Monthly observations of GnIH mRNA and peptide expression in wild birds over a year revealed a significant increase in GnIH mRNA level and number of GnIH-ir neurons during the non-breeding season when compared with their expression in the breeding season. GnIH-ir neurons were found primarily in the paraventricular nucleus (PVN) with their fibers projecting into the median eminence and some other areas of the brain. In an 8 month-long experiment, birds exposed to short days had higher GnIH expression compared with birds exposed to long days regardless of sampling month. Long-day birds with regressed testes had similar GnIH levels to short-day birds. Though the number of GnIH peptide-expressing neurons ran almost parallel to the levels of GnIH mRNA, they were inversely related to gonadal size in both sexes under natural and artificial photoperiodic conditions. These results clearly indicate an inhibitory role of GnIH in photoperiodic regulation of seasonal reproduction in the tree sparrow.

KEY WORDS: Gonadotropin-inhibitory hormone (GnIH), Paraventricular nucleus (PVN), Neuroendocrine circuitry, Photoperiod, Testicular cycle

INTRODUCTION

Most birds confine their reproduction to the time of year when environmental conditions are most favorable for successful breeding (Dixit and Singh, 2011). Regression of the gonads during the non-breeding season reduces energetic demands associated with maintenance of reproductive tissues, which helps individuals survive during the harshest time of the year (Demas, 2004). Seasonally reproducing birds undergo various life-history stages (Dixit et al., 2014). These stages are closely coupled and are temporally spaced in order to avoid physiological conflict (Kumar et al., 2006; Dixit and Singh, 2013). As photoperiod, among different environmental factors, is the most reliable predictor of season, it is used by the majority of birds to forecast local conditions and initiate physiological preparations for reproduction well in advance of the optimum environmental conditions (Dawson et al., 2001; Hau et al., 2004; Bradshaw and Holzapfel, 2007; Dixit and Singh, 2012). The role of photoperiod in the regulation of seasonal reproduction in birds has been well established (Dawson, 2007).

Recent efforts have focused on uncovering neuroendocrine mechanisms by which a photoperiodic signal drives reproductive changes (Ikegami and Yoshimura, 2013; Wood and Loudon, 2014). The available evidence suggests that the hypothalamic dodecapeptide gonadotropin-inhibitory hormone (GnIH) is an important component of the neuroendocrine circuitry regulating seasonal reproductive cycles in birds (Tsutsui et al., 2013; Ubuka et al., 2013). GnIH was identified in the Japanese quail (*Coturnix japonica*) by Tsutsui et al. (2000) as a regulatory molecule that plays an inhibitory role in the release of pituitary gonadotropins in a dose-dependent manner, giving a new perspective on the neuroendocrine control of avian reproduction. This study was later extended and confirmed in a few other song birds including Gambel's white-crowned sparrow (*Zonotrichia leucophrys gambelii*; Osugi et al., 2004) and rufous-winged sparrow (*Aimophila carpalis*; Small et al., 2008), where GnIH has been reported to play an important role in regulating seasonal reproduction by down-regulating the release of pituitary gonadotropins via action on the hypothalamic-pituitary–gonadal (HPG) axis (Osugi et al., 2004; Bentley et al., 2006a; Ubuka et al., 2006; Johnson et al., 2007). GnIH cell bodies are primarily localized to the paraventricular nucleus (PVN) area of the avian brain (Tsutsui et al., 2000; Osugi et al., 2004). GnIH fibers, presumably from the cell bodies localized to the PVN, extend to the median eminence and hypothalamus, and caudally through the brain to at least the brain stem (Bentley et al., 2006b). GnIH fibers have also been localized in close proximity to gonadotropin-releasing hormone (GnRH) neurons in the preoptic area of the brain of birds (Bentley et al., 2003). Further, the GnIH receptor is a G-protein-coupled receptor (Yin et al., 2005) and mRNA for this peptide has been found localized to the hypothalamus (both GnRH-I and -II neurons), pituitary, testes and epididymis in quail, and to the testes, ovaries and oviduct in European starlings (*Sturnus vulgaris*; Yin et al., 2005; Bentley et al., 2008) as well as gonadotropes in the quail pituitary (Chowdhury et al., 2010). These data, combined with functional studies, indicate that GnIH is able to alter the activity of the HPG axis via direct action on GnRH neurons (Smith and Clarke, 2007; Bentley et al., 2008) as well. It is well established that GnRH upregulates the secretion of the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Sharp et al., 1990; Greives et al., 2008). These gonadotropins induce gonadal growth and development and stimulate the production of sex steroids, which, in turn, act on the brain to induce appropriate sexual behavior (Greives et al., 2008) in the regulation of seasonal reproduction. GnIH neuronal fibers not only terminate in the median eminence but also remain distributed in multiple brain areas, suggesting their

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Received 13 June 2017; Accepted 10 August 2017

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Journal of Experimental Biology (2017) 220, 3742-3750 doi:10.1242/jeb.164541

multiple roles within the CNS. The close proximity of GnIH-immunoreactive (GnIH-ir) fibers to c-GnRH-II neurons in the CNS suggests that GnIH or its related peptides might have direct effects on reproductive behavior (Bentley et al., 2006a; Ubuka et al., 2012a). GnIH has been found to inhibit socio-sexual behaviors of male quail by activating aromatase and increasing neuroestrogen synthesis (Ubuka et al., 2014). Important transition points during the parental care stage have been found to be associated with significant changes in the abundance of GnIH cells in the hypothalamus in the European starling (Calisi et al., 2016). GnIH may serve as a modulator of reproductive behaviors in response to social environment in starlings (Calisi et al., 2011). Further, changes in abundance of GnIH cells throughout the breeding season (Calisi et al., 2011) mirror changes in neuronal soma size and estimated peptide concentration in this species (Amorin and Calisi, 2015). GnIH and its receptor have also been reported in interstitial cells and secondary spermatocytes of house sparrow testes (McGuire and Bentley, 2010) and in the theca and granulosa layers in starling ovary (Bentley et al., 2008). However, the role of GnIH in the testes is not well understood (Bentley et al., 2008).

In photoperiodic songbirds, including American tree sparrows (*Spizella arborea*) and Eurasian tree sparrows (*Passer montanus*), long days cause gonadal growth and maturation as well as regression to the quiescent stage (Dawson et al., 2001; Dixit and Singh, 2011, 2012). Annual reproductive cycles and photoperiodic responses of some low-latitude avian species often resemble the seasonal breeding strategies of temperate birds (Wikelski et al., 2003). However, it still remains unsolved to what extent the mechanisms regulating seasonal reproduction in temperate and tropical birds are similar to or different from each other (Hau, 2001). Despite decades of research on photoperiodic regulation of avian reproduction, the physiological mechanisms by which the reproductive system is activated and inactivated in the breeding and non-breeding season, respectively, are not completely understood and need further investigation. Specifically, the neuroendocrine signals relaying photoperiodic information that regulate seasonal reproductive cycles are unclear. The role of GnIH in down-regulating the release of gonadotropins along with accompanying changes following photoperiodic manipulations suggest that this peptide may be responsible for key modulatory input required for photoperiodic control of seasonal reproduction (Wingfield, 2008). While considerable progress has been made in elucidating the role of GnIH in the regulation of reproduction, especially in mammals, our understanding of its role in the control of seasonal reproduction in birds is in its infancy. Moreover, previous studies on GnIH in a couple of temperate species and semi-domestic Japanese quail were only short term and did not address the different stages of the reproductive cycle. A longer term study involving different phases of the reproductive cycle and different photoperiodic conditions may provide more pertinent conclusions on the neuroendocrine regulation of seasonal reproduction and the role of GnIH. Therefore, we studied the role of GnIH in the photoperiodic regulation of seasonal reproduction in the Eurasian tree sparrow, *Passer montanus* (Linnaeus 1758), a resident bird found abundantly distributed in the hilly regions of North-East India.

MATERIALS AND METHODS

The following experiments were performed using both sexes of tree sparrow, captured in and around the hills of Shillong, Meghalaya, India (latitude 25°34′N, longitude 91°53′E) using mist nets.

Experiment 1 – expression of GnIH mRNA and peptide under natural photoperiod

This experiment was done to investigate seasonal expression of the GnIH gene in relation to annual variations in day length and gonadal size. Adult birds were captured from their wild habitat in the middle of every month of the year and used in the present study. mRNA expression was recorded in the brain tissue of both sexes (n=4 each per month) while the number of GnIH-ir neurons was recorded only in male birds (n=4 per month).

Experiment 2 – expression of GnIH mRNA and peptide under artificial photoperiod

This experiment was performed to find out whether GnIH mRNA and peptide expression is photoperiodically regulated and also to investigate the relationship among photoperiod, GnIH expression levels and gonadal size. Wild birds, captured in November 2013, were kept in an outdoor open aviary with unrestricted access to natural light, temperature and humidity. These birds were then acclimatized to laboratory conditions for a fortnight. There, they were subjected to natural variations of photoperiod, temperature and humidity. They were then transferred to short day length (9 h light/15 h dark, 9L/15D) for 8 weeks to eliminate any naturally occurring photorefractoriness, and to ensure their photosensitivity at the time of commencement of the experiment. Laparotomy (surgical opening of the abdominal wall between the last two ribs) at 4 week intervals during this pretreatment period revealed that they had regressed gonads. These photosensitive birds were used for investigation under different artificial photoperiods. Male and female birds were separated and each sex was divided into two groups (n=80 each). For each sex, one group was transferred to short day length (9L/15D: corresponding to the shortest day length of Shillong) and the other group to long day length (14 h light/10 h dark, 14L/10D: corresponding to the longest day length of Shillong). Birds were kept in lightproof wooden chambers (2.10 m×1.20 m×1.35 m) illuminated by CFL bulbs (Philips Electronics India Ltd, Kolkata, India) providing light of an intensity of ∼400 lx at the perch level with automated control of light on and off. These photoperiodic chambers were well aerated through inlets and outlets connected to air circulators. Food and water were available *ad libitum* and were replenished only during the light phase of the cycle. Periodic observations of gonadal size, GnIH mRNA level and number of GnIH-ir neurons (only in males) were recorded at an interval of 30 days for 8 months.

Measurements

GnIH mRNA expression

Birds were decapitated and their skulls were removed to expose the brain. The PVN was dissected out, cut into pieces, placed in TRI Reagent (Ambion Inc., Austin, TX, USA, cat no.74123) and frozen at −80°C. The frozen tissue was thawed and homogenized in TRI Reagent. Total RNA was then extracted following the manufacturer's protocol and purity was assessed on a Nanodrop. A 1 µg sample of total RNA was reverse transcribed to cDNA using the Verso cDNA synthesis kit (ThermoFisher Scientific, Waltham, MA, USA, cat. no. AB1453A). The degenerate primers (forward: 5′-TCCAGCTGSARAGCAGAG-3′ and reverse: 5′-GACTTCCC-RAATCTCTGTG-3′) used for amplification of GnIH in the present study were taken from the study by Majumdar et al. (2015) on redheaded bunting, *Emberiza bruniceps*. Briefly, the PCR amplification cycle included initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 55.6°C for 1 min, extension at 72°C for 1 min and final

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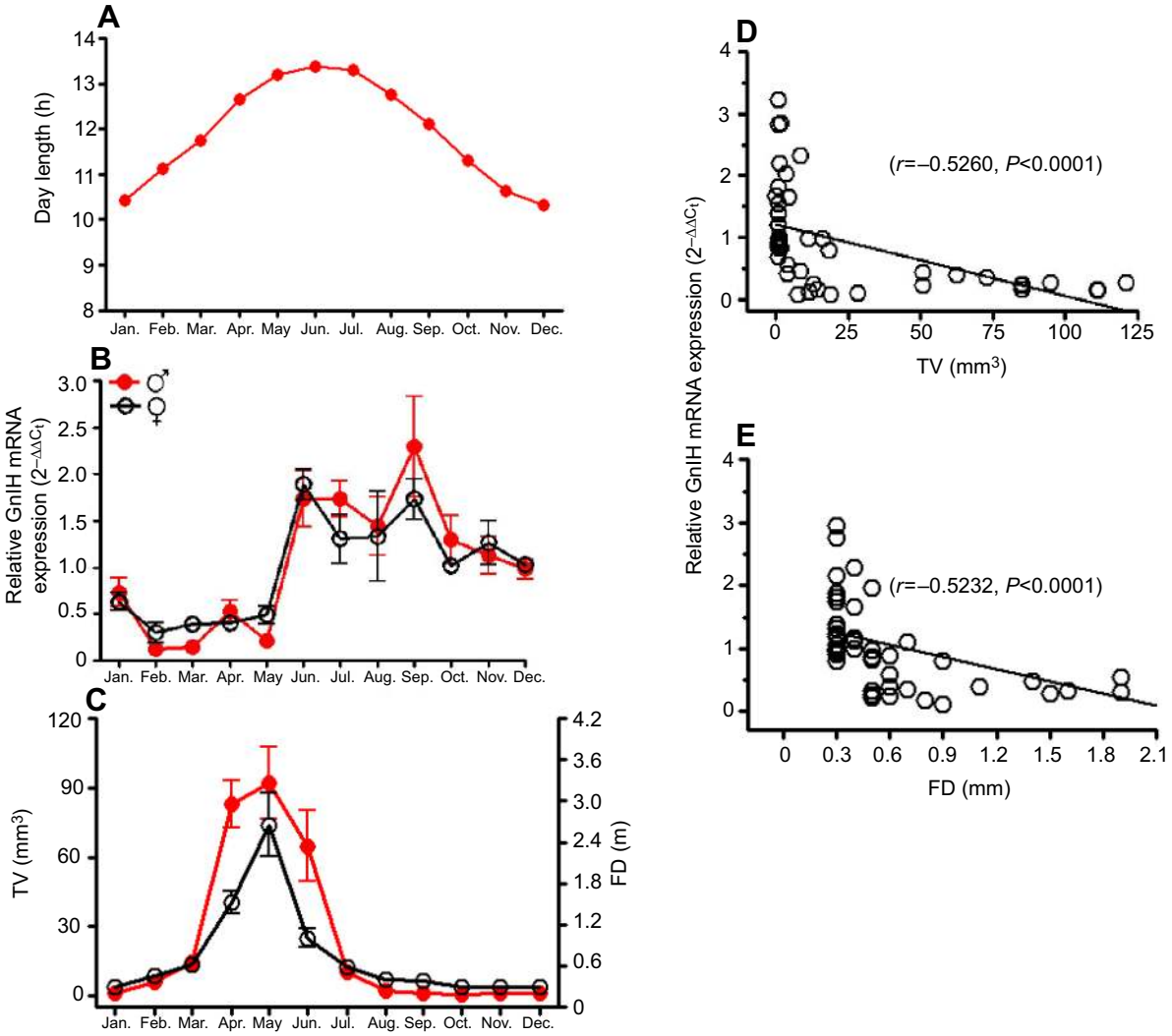

Fig. 1. Seasonal gonadotropin-inhibitory hormone (GnIH) expression in the Eurasian tree sparrow, *Passer montanus*. (A) Annual changes in day length at Shillong, India. (B,C) Relative GnIH mRNA expression (B) and annual gonadal cycles (C) in relation to day length in the sparrow. TV, testicular volume; FD, follicular diameter. (D,E) Negative correlation of relative GnIH mRNA expression with TV (D) and FD (E).

extension at 72°C for 7 min. Amplification of the PCR product was confirmed by 1% agarose gel electrophoresis and visualization by gel documentation. The band of the correct size was cut out of the gel and the PCR product eluted with Qiagen Gel Extraction Kit (Hilden, Germany, cat. no. 28704). The PCR product was then subcloned into the pGEM-T cloning vector (pGEM-T Easy Vector System, Promega, Madison, WI, USA). Following transformation, the plasmids were confirmed by digestion and then commercially sequenced. The nBLAST program (NCBI) was used to analyze and compare the predicted amino acid sequence with available amino acid sequences in GenBank for sequence homology, revealing similarity with *GnIH* of *Passer domesticus* (98%), *Sturnus vulgaris* (96%), *Zonotrichia leucophrys gambelli* (95%), *Ploceus phillippinus* (94%) and *Emberiza bruniceps* (94%), among others. The partial cDNA sequence of *P. montanus GnIH* was then submitted to GenBank (accession no. KT351598.1). Similarly, the partial sequence of the *β-actin* gene of *P. montanus* (accession no. KT351599) was obtained using degenerate primers (forward: 5′-CCCTGAAGTACCCCATTGAA-3′ and reverse: 5′-GCTGTGA-TCTCCTTCTGCATC-3′) (Singh et al., 2013; Srivastava, 2015). For real-time PCR (qPCR), gene-specific primers for *GnIH* (forward: 5′-TGGAGAGCAGAGAAGACAATGATG-3′ and reverse: 5′-TGTCTTTTGTTCCCCAGTCTTCCA-3′) and *β-actin* (forward: 5′-GGATTTCGAGCAGGAGATGG -3′ and reverse: 5′-GGGCACCTGAACCTCTCATT-3′) were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/). Possible primer efficiency, d-imers and hairpins were checked with Oligo Analyzer 3.1. A 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) was used to perform quantitative *GnIH* gene expression analysis with *β-actin* as the reference gene. Amplification of the *GnIH* and *β-actin* genes in the PCR reaction was carried out using Power SYBER® Green (Applied Biosystems, cat. no.1301388). The standardized primer concentration in the total reaction volume attained a slope of −3.3 to −3.4, showing a good melt curve and efficiency of the primer. The threshold cycle (C_t) values were then normalized against C_t values for *β-actin*. $\Delta\Delta C_t$ values were plotted as the negative power to 2 ($2^{-\Delta\Delta C_t}$) and were calculated as per Majumdar et al. (2015).

Immunocytochemical analysis of GnIH peptide

Immunocytochemistry was used to localize and measure GnIH peptide in terms of the number of neurons showing expression.

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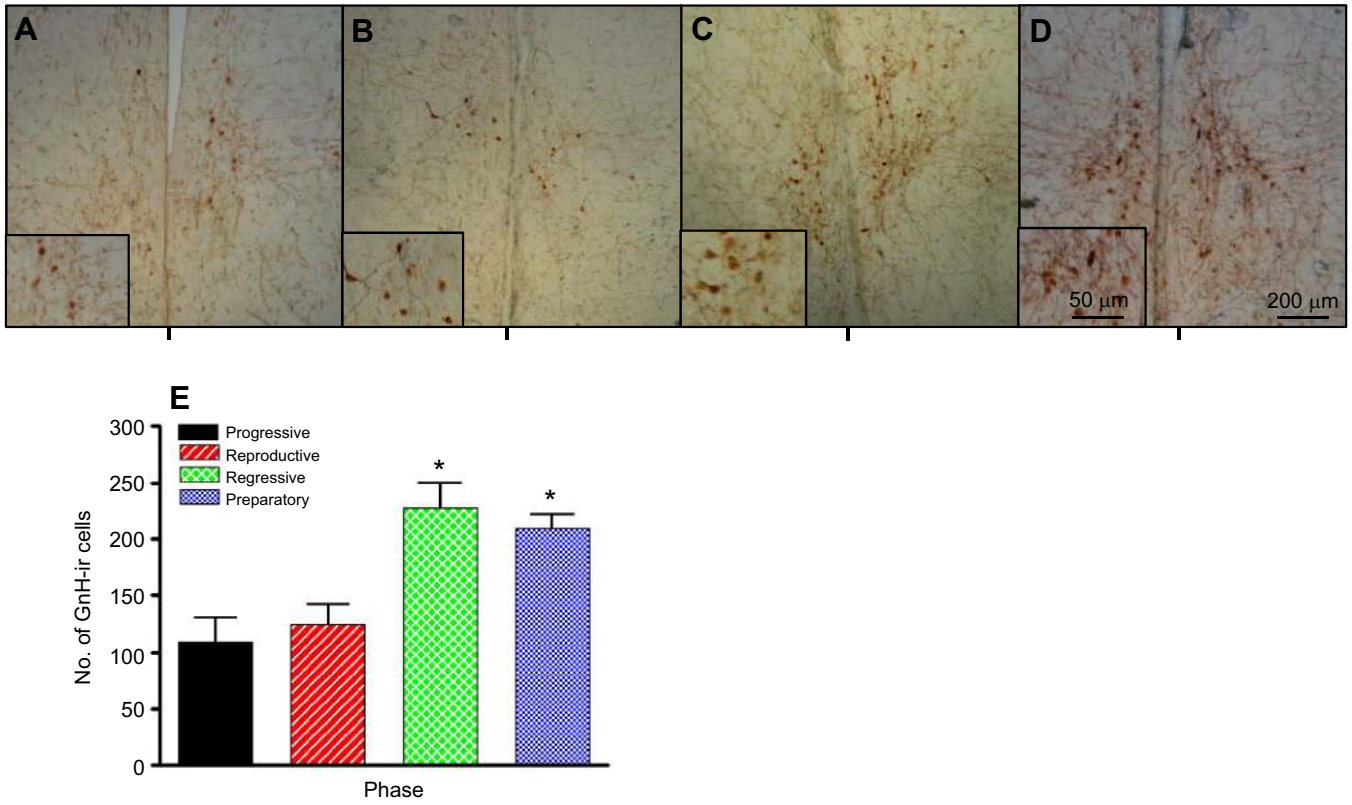

Fig. 2. GnIH immunoreactivity in the paraventricular nucleus (PVN) during the annual testicular cycle in *P. montanus*. (A–D) GnIH expression in the progressive (A), reproductive (B), regressive (C) and preparatory (D) phases of the testicular cycle. (E) Number of GnIH-ir cells in the PVN according to phase. *P<0.05.

Birds were terminally anesthetized (subcutaneous injection of ketamine–xylazine solution at 0.003 ml g^{-1} body mass) and perfused transcardially with ice-cold saline (pH 7.4) followed by 4% paraformaldehyde solution (0.1 mol l^{-1} phosphate buffer at pH 7.4). Brains were dissected out and stored in paraformaldehyde solution overnight at 4°C. They were then post-fixed by transfer to serial grades (10%, 20% and 30%) of cryoprotectant sucrose solution until the tissues sank to the bottom of the 50 ml Falcon tube at 4°C. Subsequently, brains were transferred to 15% polyvinylpyrrolidone solution (PVP, HiMedia, Mumbai, India) in a 50 ml Falcon tube and stored at −80°C until further processing. All samples were collected between 09:00 h and 10:00 h. Brain tissue was thawed on ice before use, then mounted in the cryostat tissue holder with the help of 15% PVP inside the cryostat chamber, which was maintained at −18 to −20°C. The whole brain was sectioned serially in the coronal plane at 30 µm thickness using the cryostat (Leica CM 1850, Wetzlar, Germany). These sections were then processed for the GnIH immunocytochemical study as described in Rastogi et al. (2011). Anti-quail serum (dilution 1:20,000; generously gifted by Dr K. Tsutsui, Waseda University, Japan) was used in the present study. This anti-serum cross-reacts with song sparrow (*Melospiza melodia*) and house sparrow (*Passer domesticus*) GnIH (Bentley et al., 2003). The specificity of the antibody has also been demonstrated in previous studies (Tsutsui et al., 2000; Ubuka et al., 2008; Rastogi et al., 2013; Surbhi et al., 2015). However, in order to confirm the specificity of this GnIH antibody in our birds, a control was also run to check for non-specific immunoreactivity. The absence of primary antibody in the reaction resulted in the total loss of immunoreactivity, while the presence of it resulted in strong immunoreactivity at up to 1:20,000 µl dilution. Slides were examined in a trinocular bright-field microscope (Motic, Hong Kong, China) and digital images of immunoreactive cells were captured using a high megapixel camera (Motic cam) at 10× and 40× magnification. Photography was done using standard illumination. The images were adjusted for size, contrast and brightness as required using Motic image version 2 analyzer software. Both strongly (bright) and weakly (faint) stained GnIH-ir cells were counted in all sections of the PVN region to avoid any staining-intensity bias (Rastogi et al., 2013). Thus, the number of GnIH-ir cells obtained from all brains was averaged and their mean (±s.e.m.) was calculated.

Gonadal size

Gonadal development was measured in terms of changes in testicular volume and follicle diameter. The length and width of the left testis was measured with respect to divisions on the graph paper using a caliper. Testicular volume (TV) was calculated using the formula $4/3\pi ab^2$, where a and b denote half the long (length) and short (width) axes, respectively. Ovarian growth was measured in terms of the diameter of the largest follicle. A regressed ovary with an indistinct follicle was assigned a follicular diameter (FD) of 0.3 mm to make the data statistically comparable with the stimulated follicles. Subsequently, the changes in TV and FD were converted into growth rate k (day^{-1}) ($\ln b - \ln a/t$, where a and b denote initial and final gonadal size, respectively, and t is time) to compare the data between male and female birds.

The procedures used in these studies were approved by the Institutional Animal Ethics Committee, North-Eastern Hill University, Shillong, India.

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Statistical analyses

Data are presented as means±s.e.m. They were analyzed using one-way, two-way and multiple-way ANOVA as required, followed by Bonferroni *post hoc* mean comparison test, if ANOVA indicated a significant difference. Regression analysis was also done to see the relationship between gonadal size and GnIH expression. Significance was taken at the 95% confidence level.

RESULTS

Experiment 1 – expression of GnIH mRNA and peptide under natural photoperiod

The annual changes in day length at Shillong range from 10.29 h in December to 13.44 h in June (Fig. 1A). A 317 base pair *GnIH* cDNA was identified and its peptide was found to be primarily localized in the neuronal cell bodies restricted to the hypothalamic PVN and in fibers projecting to different regions of the brain of tree sparrow. Our data indicated temporal regulation of GnIH mRNA. Two-way ANOVA showed significant annual variation in the levels of GnIH mRNA in both sexes under natural day length (month: $F_{11,60}$=17.18, P<0.001; Fig. 1B). GnIH mRNA expression was lowest from February to May during the reproductive phase of the gonadal cycle in both sexes, when the gonads were increasing gradually in size, reaching a peak in May, with increasing natural day length. A sharp and significant (P<0.001) increase in mRNA expression was noted in June, when the gonads started regressing in both sexes. The higher levels of GnIH mRNA were fairly constant thereafter until January, when the gonads entered their quiescent stage. Maximum mRNA level was reached in September. No significant difference (sex: $F_{1,60}$=0.3157, P=0.5763) in the expression of GnIH mRNA was observed between the sexes. The interaction between month and sex ($F_{11,60}$=0.8082, P=0.6316: Fig. 1B) was also not significant. Further, both sexes exhibited significant variation in gonadal size throughout the year (Fig. 1C; TV: $F_{11,29}$=8.735, P<0.0001; FD: $F_{11,31}$=8.893, P<0.0001; one-way ANOVA). TV and FD increased gradually from February onwards, reaching a peak in May. Gonadal regression was observed in June, which proceeded gradually through July, reaching quiescent conditions in August, which were maintained till January (Fig. 1C). Further, no significant difference was observed in gonadal growth rate between male and female birds ($F_{1,60}$=2.319, P=0.1322). However, there was a significant difference among months ($F_{11,60}$=27.57, P<0.0001) and a significant interaction between sex and month ($F_{11,60}$=5.503, P<0.0001). Thus, the annual reproductive cycle of tree sparrows can be divided into progressive (February–March), reproductive (April–May), regressive (June–August) and preparatory (September–January) stages. A comparison between gonadal size and mRNA levels revealed a negative correlation (TV and mRNA expression: r=−0.5260, P<0.0001; FD and mRNA expression: r=−0.5232, P<0.0001) in both sexes, indicating that the increase in gonadal size is inversely related to mRNA expression levels in both sexes of tree sparrow (Fig. 1D,E). Densely immunoreactive GnIH cell bodies were observed only in the PVN area of the tree sparrow brain (Figs 2 and 4). One-way ANOVA indicated a significant difference ($F_{3,11}$=10.19, P<0.0001) in the number of GnIH-ir neurons during different phases of the reproductive cycle in the tree sparrow (Fig. 2). The number of GnIH-ir neurons was significantly (P<0.001) higher during the regressive and preparatory phases compared than during the progressive and reproductive phases of the testicular cycle (Fig. 2E). Further, the maximum number of GnIH-ir neurons was observed during the regressive phase, while the minimum number occurred in the progressive phase. There was an inverse relationship between gonadal size and the number of GnIH-ir neurons in the PVN during different phases of the reproductive cycle of tree sparrows.

Experiment 2 – expression of GnIH mRNA and peptide under artificial photoperiod

There were significant variations in the levels of GnIH mRNA (month: $F_{8,108}$=2.703, P=0.012; photoperiod: $F_{1,107}$=73.383, P<0.0001; interaction sex×photoperiod: $F_{1,107}$=4.350, P=0.041, photoperiod×month: $F_{8,107}$=8.739, P<0.0001; multiple-way ANOVA; Fig. 3A) and number of GnIH-ir neurons (month: $F_{4,20}$=4.373, P=0.0106; photoperiod: $F_{1,20}$=10.93, P=0.0035; interaction photoperiod×month: $F_{4,20}$=3.696, P=0.0207; two-way ANOVA; Fig. 4) in the PVN region of the brain. Tree sparrows exhibited significant changes in both GnIH mRNA and peptide expression only under long day length. GnIH mRNA expression dropped sharply and reached a minimal level with increases in gonadal size after 1 month of exposure to long day length in both sexes. The minimal level of GnIH mRNA was maintained till the end of the third and fourth months in female and male birds, respectively. GnIH mRNA expression level increased gradually thereafter with gonadal regression till the end of the experiment (eighth month) in both sexes (male: $F_{8,35}$=29.05, P<0.0001 and female: $F_{8,35}$=27.09, P<0.0001; one-way ANOVA; Fig. 3). A similar response was observed at the protein level in the sparrow hypothalamus. Birds maintained under long day length showed a significant (P<0.001) decrease in the number of GnIH-ir neurons at the end of the second and fourth month, followed by an increase after 6 months of exposure, which remained fairly constant until the end of the experiment (Fig. 4A–E,K). Thus, the decrease in GnIH gene expression was coincident with the increase in gonadal size during the reproductive phase of the gonadal cycle and vice versa, indicating an inhibitory role of GnIH in seasonal reproduction of the

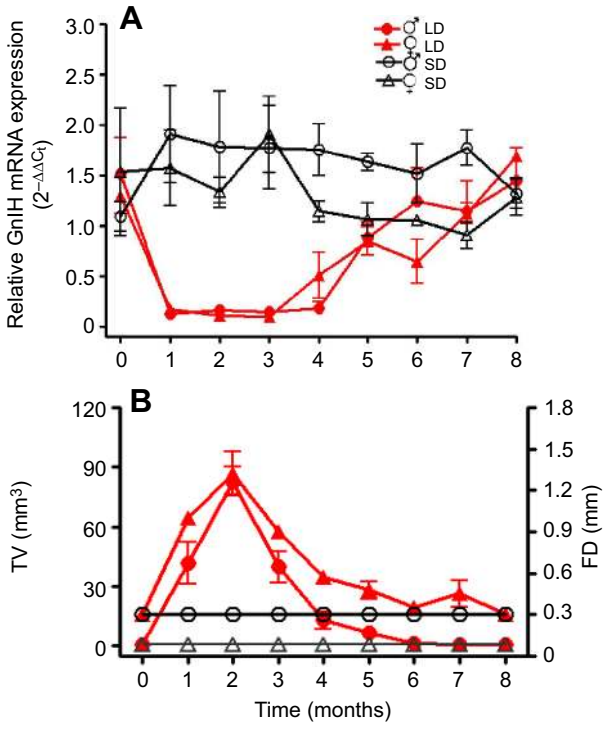

Fig. 3. Expression of GnIH under artificial photoperiod in *P. montanus*. Tree sparrows were maintained under artificial long (LD) and short day lengths (SD) for 8 months. (A) Relative GnIH mRNA expression. (B) Gonadal size.

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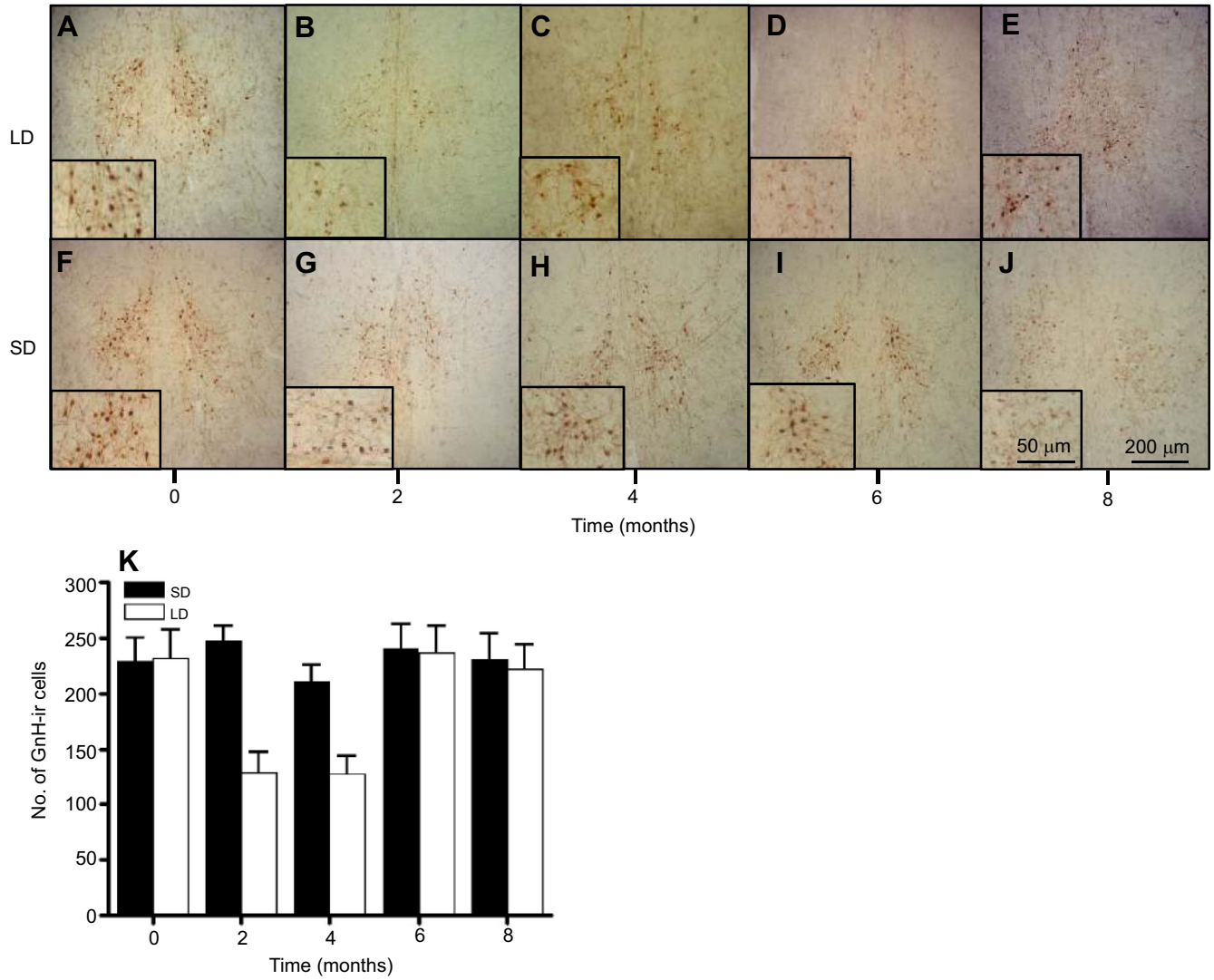

Fig. 4. GnIH immunoreactivity in the PVN under artificial photoperiod in *P. montanus*. GnIH expression is shown for birds maintained for 8 months under LD (A–E) or SD (F–J) conditions. (K) Number of GnIH-ir cells in the PVN under LD and SD.

tree sparrow. In contrast, no significant change in either GnIH mRNA or peptide expression was observed under short day length and the birds maintained significantly higher GnIH expression and minimal gonadal size (male: $F_{8,35}$=0.8500, P=0.5685 and female: $F_{8,35}$=0.7500, P<0.6480; one-way ANOVA; Figs 3 and 4F–K) throughout their exposure (8 months).

A comparison between gonadal size and GnIH mRNA expression level revealed a significant negative correlation in birds of both sexes (male: r=−0.6432, P=0.0003; female: r=−0.7294, P<0.0001; Fig. 5A,C) exposed to long day length, while there was no correlation under short day length (male: r=0.1765, P=0.3764; female: r=0.0761, P=0.7095; Fig. 5B,D), confirming that gonadal size and GnIH mRNA expression levels are inversely related in the tree sparrow. Gonadal growth rate showed significant variation (month: $F_{8,143}$=26.78, P<0.0001; sex: $F_{1,143}$=8.223, P=0.005; photoperiod: $F_{1,143}$=15.906, P<0.0001; and interactions sex×photoperiod: $F_{1,143}$=9.227, P<0.0001; sex×month: $F_{8,143}$=16.589, P<0.0001; photoperiod×month: $F_{8,143}$=28.538, P<0.0001; and sex×photoperiod×month: $F_{8,143}$=18.210, P<0.0001).

DISCUSSION

The isolated partial *GnIH* gene sequence of the tree sparrow showed a close similarity with the *GnIH* gene sequence of *P. domesticus*, *S. vulgaris*, *Z. l. gambelii*, *P. phillippinus* and *E. bruniceps*. GnIH peptide was primarily localized in the PVN region of the hypothalamus, and exhibited seasonal variation in its expression. Increasing day length in spring (February) triggered gonadal growth in both the sexes that proceeded gradually, reaching to peak in May during the progressive and reproductive phases of the annual gonadal cycle. Birds showed low but relatively constant levels of GnIH mRNA and peptide during this period of gonadal growth and development. However, a sharp and significant increase in the levels of GnIH mRNA and peptide was noted with the onset of gonadal regression in June and the high levels remained fairly constant throughout the regressive and preparatory phases when the gonads were regressing and/or attained quiescent stage. These results clearly indicate an inverse relationship between GnIH mRNA and peptide levels and the growth and development of gonads in both sexes of tree sparrow. A comparison of annual changes in gonadal size and *GnIH* gene

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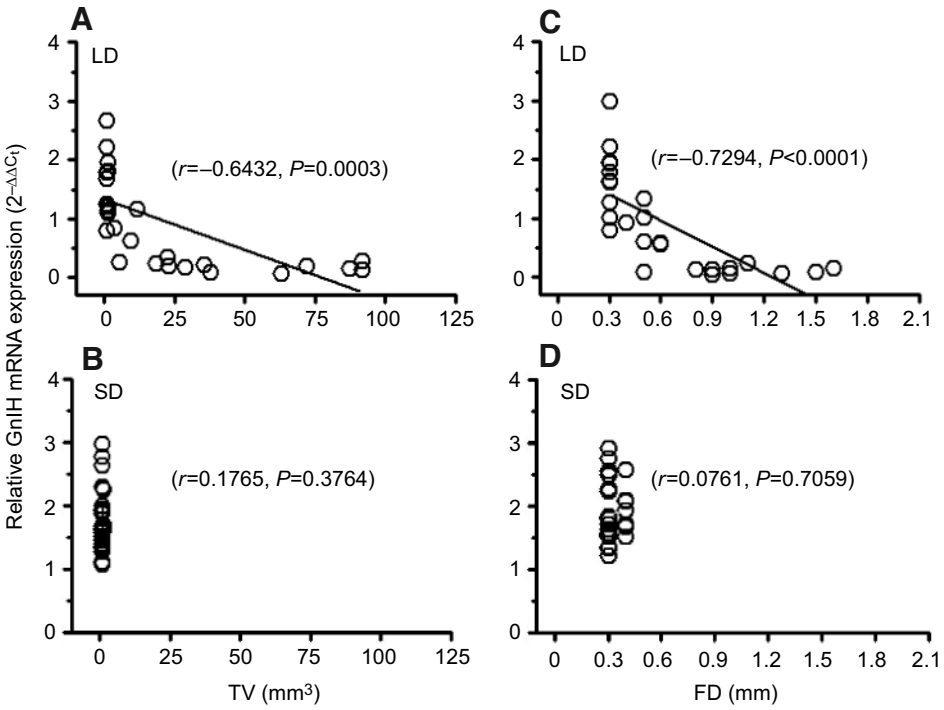

Fig. 5. Relative GnIH mRNA expression and gonadal size under artificial photoperiod in *P. montanus*. (A,B) TV and (C,D) FD in sparrows maintained under artificial long (LD; A,C) and short (SD; B,D) day lengths.

expression with the annual variations in day length at Shillong revealed the possibility of their photoperiodic regulation in the tree sparrow (Figs 1 and 2). Tree sparrows exposed to artificial long day length (14L/10D) underwent a significant gonadal growth–regression cycle and exhibited *GnIH* gene expression that was inversely related to gonadal size. In contrast, no significant change in either GnIH mRNA and protein levels or gonadal size was noted in birds maintained under short day length (9L/15D). Thus, the photoperiod-induced changes in GnIH expression were evident and measurable at both the transcription and translation levels in our study bird. These changes may play an important role in the processing of photoperiodic information in the brain and in the regulation of the HPG axis controlling seasonal reproduction in the tree sparrow.

The number of GnIH-ir neurons in the PVN area decreased during the initiation and progression of reproductive activities and increased during the termination of breeding in tree sparrows exposed to both natural and long day length, suggesting temporal photoperiodic regulation of this neuropeptide. GnIH has also been reported to display changes in the pattern of expression of mRNA and peptide in relation to reproductive stages and changing photoperiod in some seasonally breeding birds. Our findings on GnIH mRNA expression in the tree sparrow are consistent with those observed in the subtropical spotted munia (*Lonchura punctulata*), a circannual species (Budki et al., 2012) that exhibits high levels of GnIH mRNA during the quiescent phase and lower levels during the reproductive phase under natural day length and continuous light (Srivatava, 2015). A similar pattern has also been observed in house sparrows (Calisi et al., 2008). Although testis volume changed significantly under both short and long day length, GnIH mRNA expression varied significantly only in the long day condition in munia. In contrast to tree sparrows, munia exhibit a time-dependent response instead of photoperiod-dependent effects on testes growth and GnIH mRNA expression. This reveals how a circannual and a photoperiodic species differ in the way they respond to changing day length to adjust their reproductive physiology (Srivatava, 2015). However, blackheaded buntings (*Emberiza melanocephla*) show inconsistencies in relation to gonadal growth and GnIH mRNA expression, as gene expression levels were low in the non-stimulatory groups exposed to T-photocycles of $T24_{11L}$ (6L/4D/1L/13D) and $T26_{12L}$ (6L/5D/1L/14D) (Majumdar et al., 2015). Song sparrows display increased GnIH immunoreactivity when becoming photorefractory to long days (Bentley et al., 2003). Photoperiodic studies in Japanese quail also confirmed the regulatory influence of GnIH: short days correspond to high levels of expression and long days to low levels of expression (Ubuka et al., 2006). In contrast to our observations on tree sparrows, no significant differences were observed in either GnIH-ir cell number and size or expression of GnIH mRNA between breeding and non-breeding periods in zebra finches (Perfito et al., 2011). The disparity between the responses of tree sparrows and zebra finches might reflect differences in their reproductive strategy, with the former having distinct breeding seasons and the latter adopting a more opportunistic breeding strategy. Further, no significant difference in the levels or pattern of GnIH mRNA expression was noted between male and female tree sparrows under natural or artificial photoperiods. However, in a different study, Ubuka et al. (2013) showed that the GnIH mRNA expression and sexual activities of male and female song birds are negatively correlated. It was also reported that GnIH-R mRNA expression was significantly higher in the pituitary of sexually immature chickens relative to sexually mature chickens (Maddineni et al., 2008).

Clusters of GnIH-ir neurons were observed primarily in the PVN region of the hypothalamus of tree sparrows, with bundles of fibers projecting into the median eminence and other regions of the brain. The presence of GnIH-ir neurons in the external median eminence indicates the potential for this neuropeptide to influence the synthesis and/or release of hormones from the anterior pituitary. Further, GnIH-ir neuronal fibers were found to be widely distributed in the diencephalic and mesencephalic regions in the tree sparrow. Similar observations have also been reported in some other avian

species, e.g. Japanese quail (*C. japonica*; Tsutsui et al., 2000), white-crowned sparrow (*Z. l. gambelii*; Osugi et al., 2004), song sparrow (*M. melodia*) and house sparrow (*P. domesticus*) (Bentley et al., 2003). As in the tree sparrow, GnIH-ir neurons have been found abundantly located in the PVN region of the hypothalamus in all these birds, regardless of sex (Ubuka et al., 2003; Bentley et al., 2006b). GnIH-ir neuronal fibers have been reported in the diencephalic and mesencephalic regions of the brain in Japanese quail (Ukena et al., 2003), European starlings (Ubuka et al., 2008) and white-crowned sparrows (Ubuka et al., 2012a). They were also observed in the ventral paleostriatum, septal area, preoptic area, optic tectum and the dorsal motor nucleus of the vagus in some birds (Ubuka et al., 2012b). The distribution of GnIH fibers in different regions of the avian brain clearly suggests that GnIH not only regulates pituitary function but also plays significant roles in behavioral and autonomic mechanisms in birds (Ubuka et al., 2012a).

GnIH may act only at the level of the anterior pituitary gland (Tsutsui et al., 2000) or it could inhibit GnRH release by acting on GnRH fiber terminals in the median eminence (Bentley et al., 2006a). Interestingly, the number of GnIH neuronal cells was higher during the non-breeding season than during the breeding season in our study birds. Similarly, Bentley et al. (2003) working on house and song sparrows reported that GnIH-containing neurons were more numerous in birds during the end of the reproductive season. GnIH-ir neurons were found in significantly greater numbers at the termination of breeding under both natural and artificial long day length in the tree sparrow. The increase in the number of GnIH-ir neurons and the level of GnIH mRNA in the non-breeding phase and their decline in the breeding phase in tree sparrows indicate the inhibitory role of GnIH in the regulation of reproduction. Also, GnIH may play a significant role in the termination of breeding in some songbirds, including tree sparrows. Thus, GnIH seems to be an important part of the photoperiodic machinery regulating seasonal reproduction in these birds.

The identified *GnIH* cDNA sequence and the localization of its transcript primarily to the PVN region of the brain reveal a high degree of homology between tree sparrows and other galliform and passerine birds. The decrease in the number of GnIH neurons in the PVN during initiation and progression of reproductive activities and their increase during the termination of breeding suggest temporal regulation of this neuropeptide in the tree sparrow. Long photoperiods, either natural or artificial, stimulate gonadal growth and development in the tree sparrow by downregulating *GnIH* gene expression at both the transcription and translation level in the neurons residing in the PVN area 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 hyper-regulation of *GnIH* gene transcription and translation in these neurons. Thus, GnIH seems to be an important part of a mechanism by which photoperiod regulates seasonal reproduction in the tree sparrow.

Acknowledgements
We are grateful to our collaborator Professor Kazuyoshi Tsutsui (Laboratory of Integrative Brain Sciences, Department of Biology, Waseda University, Tokyo, Japan) for kindly providing the anti-quail GnIH serum and guidance.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.S.D.; Methodology: N.S.S.; Formal analysis: N.S.S., S.B.; Investigation: N.S.S.; Resources: N.S.S., S.B.; Writing - original draft: A.S.D.; Writing - review & editing: N.S.S., S.B.

Funding
Financial support from the Departments of Science and Technology and Department of Biotechnology, Government of India, New Delhi are gratefully acknowledged.

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