General materials and methods

Model species

The present work was carried out on both the sexes of tree sparrow, *Passer montanus*. This bird has been chosen because it is mainly granivorous, easy to maintain and survives well under laboratory conditions. Most of all, it showed clear photoperiodic responses. Besides, previous studies from our laboratory on this species helped us in designing newer experiments. Tree sparrows are present in temperate as well as tropical/subtropical regions and are, therefore, a widely distributed avian species (Wong, 1983). Its native range expands throughout Central and Southern Europe, Central Asia and parts of South-East Asia (Sibley and Monroe, 1990). It is a small bird of about 12.5-14 cm in length and about 24 g in weight (Linnaeus, 1758) and is roughly 10% smaller than the house sparrow (Snow and Perrins, 1998). It can adapt to a wide variety of habitat types (Field et al., 2008). In India, tree sparrow is a resident bird distributed abundantly in the hilly regions of the North-Eastern part of the country and confined mostly along with the human habitats. It is a passerine bird belonging to the family Passeridae. It has distinctive characters of rich chestnut crown and nape, and a kidney-shaped black patch near ear covert on each pure white cheeks. The area between the bill and throat are black. The wings are brown in colour with two distinct narrow white bars. Sexual dimorphism is not distinct; however, the young birds can be easily differentiated from adults as they are dull in colouration (Mullarney et al., 1999). They usually remain in flocks and are noisy, except during breeding season when they keep in pairs. Main food includes grass, weed-seeds and cereal grains. Food also comprises of fruit and flower buds, tender shoots, kitchen scraps and insects. Nestlings are exclusively fed on proteinaceous foods, including invertebrates, especially insects, woodlice, millipedes, caterpillars, spiders, harvestmen etc. (Field and Anderson, 2004). The untidy nest is usually made of straw,
fibres, grass, wool, cotton threads and lined with feathers (Garcia-Navas et al., 2008) to improve the thermal insulation (Pinowski et al., 2006) placed in the ceilings of abandoned houses or verandah, electric boxes, holes and cracks of houses or the crown of a palm tree. A typical clutch is usually of 5 to 6 eggs. Nest building is performed by both sexes, but the female does most of the incubation and both parents are involved in parental care (Singh, 2012).

**Birds capture, maintenance and care**

Tree sparrows were caught from their wild habitat in and around the hills of Shillong (Latitude 25°34’N, Longitude 91°53’E), Meghalaya using mist net. They were kept in an outdoor aviary (size = 300 x 250 x 250 cm) for acclimatization to captive conditions. The aviary, housing birds, is located in the vicinity of our department in an open area surrounded by natural vegetation and receiving natural light and temperature conditions. Green plants, grasses, sands, nest boxes and wooden perches were provided inside the aviary to create a natural environment as far as possible. The greens were regularly replaced so that the aviary environment looked always fresh. Here, birds received natural day light, temperature and humidity close to that they receive in nature. The birds from this stock were used for different experiments. These birds were first transferred and acclimatised to the laboratory conditions for a fortnight before they were subjected to experimental conditions. There, they were exposed to natural variations of photoperiod, temperature and humidity. They were then transferred to short day length of 9L/15D for eight weeks to eliminate photorefractoriness, if they had any in nature, and to ensure their photosensitivity at the time of commencement of various experiments. Laparotomy (surgical opening of abdominal wall between the last two ribs) at four weeks intervals during the pretreatment period confirmed that they had regressed gonads and normal body weight. These photosensitive birds were used for different photoperiodic
investigations. Male and female birds were separated using laparotomy. Only adult birds were used in the present study.

**Experimental conditions**

Birds, in photoperiodic experiments, were kept in the light proof wooden chambers (210 x 120 x 135 cm) illuminated by artificial light available from compact fluorescent tubes (CFL, Phillips) at an intensity of ~ 400 lux at the perch level. This light, like the natural light, was the broad-spectrum radiation and referred to loosely as ‘white’. The dark periods or nights were either completely dark or had very dim light illumination depending upon the experimental paradigm indicated in respective experiments. Lights on and off were controlled by automatic digital time switches (Crono Digital Time Switches, Larsen and Toubro LTD., India). The photoperiodic chambers were well aerated through inlets and outlets connected to air circulators. The temperature and humidity of experimental chambers, as recorded by HOBO data logger, varied in the ranges of about 17°C (December) - 24°C (June) and 55-75%, respectively in a year. The temperature of photoperiodic chambers did not vary more that 2°C from the temperature of the bird room. The first experimental photophase in all experiments was in phase with the pretreatment schedule and commenced at 6:00 h. Food (kakuni, *Setaria italica* and paddy, *Oriza sativa*) and water were provided ad libitum and were replenished once daily during the light period. Supplementary food (prepared by mixing bread crumbs, boiled eggs and cheese) was also given to the birds twice a week. They were also provided glucose (Glucon-D, Heinz India Pvt. Ltd.), vitamins (Vimeral, Virbac Animal Health India Pvt. Ltd, Mumbai) and antibiotics (Tetracycline hydrochloride, Intervet India Pvt. Ltd) mixed in their drinking water once every month for five consecutive days. Birds enjoyed similar husbandry conditions and maintained good health under captive conditions throughout the course of various experiments.
Experimental design and data collection

Each section of the thesis consists of some experiments and each experiment is having a specific experimental design which is detailed in respective experiments. Data from different experiments were collected at the beginning and at the end and at appropriate intervals during the course of the experiment. The effects of treatments were determined using the measurements which are categorized as follows:

Morphological

The following measurements were taken:

(i) Gonadal size

Measurement of the gonadal size was considered as an index of gonadal growth and regression (Trivedi, 2005). For example, changes in dimensions of the left testis in male and largest ovarian follicle in female birds were considered accounting for sum-effects of the photoperiodic treatment over a period of time on gonadotropin secretion (Lofts, 1975). The gonadal size was recorded by performing unilateral laparotomy under local anaesthesia using a subcutaneous injection of 2% xylocaine (Astra-IDL Ltd. Bangalore, India) as per the procedure described in Kumar et al. (2001). Briefly, laparotomy was performed by the surgical opening of the abdominal wall between the last two ribs on the left side. In the male, testis was located within the abdominal cavity with the help of a spatula and the length and width of the left testis were measured using a calliper with reference to markings on a graph sheet (1 cm x 1 cm with 100 squares; each square is 0.01 mm$^2$). Testis volume was calculated using the formula $\frac{4}{3}\pi ab^2$, where $a$ and $b$ denote half of the long (length) and short (width) axes, respectively. In the female, the diameter of the largest follicle was measured (Kumar et al., 2001). The regressed ovary with indistinct follicles was assigned a follicular diameter (FD) of 0.3 mm to make the data statistically comparable with the stimulated follicles. This
procedure takes few minutes, and the incision was stitched by a surgical thread. An antibacterial skin ointment (Soframycin skin cream, Aventis Pharma Ltd.) was applied on the wound. Wound healing was very rapid as there was no sign of the post-operative infections. A subjective grading of the testis size was also done to explain the response:

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TV = 0.33 \text{ to } < 2.35 \text{ mm}^3 \text{- no response; 2.35 to } < 9.82 \text{ mm}^3 \text{- initiation of response; 9.82 to } < 18.86 \text{ mm}^3 \text{- small response; 18.86 to } < 41.9 \text{ mm}^3 \text{- moderate response; 41.9 mm}^3 \text{ and above- full response (Singh et al., 2002).}
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**(ii) Bill colour**

The bill colour was assessed using subjective criteria and was scored in an index of 0-5 as described in Rani et al. (2007). Briefly, the score pattern was as follows:

0- Bill straw in colour (S)

1- Bill straw in colour but with a little tinge of blackness (ratio-SSS: B)

2- Bill slightly blackish in colour (ratio-SS: B)

3- Bill straw and black in approximately 50:50 patches (ratio-S: B)

4- Bill black with very little straw patch left (ratio-S: BB)

5- Bill completely black (B)

**(iii) Moult**

The moult pattern was recorded by observations on body feathers and primary wing feathers (called primaries).

**(a) Body feather moult**

Body feather moult was recorded as outlined by Budki et al. (2008). Whole body of the bird was divided into 12 different regions: 1= head, 2= neck, 3= shoulder, 4= back, 5= pelvic, 6= caudal, 7= throat, 8= chest, 9= abdomen, 10= flank, 11= shank and 12=...
sub-caudal. If these regions had old feathers, the score was 0, but when new feathers emerged they were scored as 1. Each region could have a score of either 0 (no moult, fully grown or old feather) or 1 (moult: no feather or new feathers emerging) and hence, the total body moult score could be in the range of 0-12.

(b) Primary feather moult

For primaries, we followed the scoring pattern as outlined by Boswell (1991) in a scale of 0-5 as per the following:

0- worn or old feather
1- missing feather (just dropped)
2- from a new feather papilla emerging up to one-third growth
3- new feather attaining two-third growth
4- new feather has grown, but still, the growth is not fully complete
5- fully grown new feather

Thus, each primary feather can possess a minimum score of 0 and maximum of 5. As there are nine primaries on each wing, the maximum score per wing could be up to 45 (9x5=45), and for each bird, a maximum score of 90 (2x45) could be expected. The minimum score could be as low as 0.

(iv) Body weight

Birds were individually weighed on a top pan digital balance to the nearest accuracy of 0.1 g. For this, the bird was placed in a cotton bag, the weight of which was tared to zero. Then the bird with the cotton bag was weighed. Record of body weight at regular intervals enabled us to describe its changes under various experimental conditions.
**Behavioural**

*(i) Locomotor activity recording*

Activity movements of birds were measured by locomotor activity recording. Each bird was caged in a specially designed activity cage (60 x 45 x 35 cm) that was furnished with two perches and mounted with a Napoleon miniature passive infrared detector (Maximum electronic Ltd., Israel) with a range of 16 m and wide angle (100°) field of view. Each sensor was connected to a separate computer channel of a window XP-compatible computer and the computerized recording of the activity and collection and analysis of activity data were performed using the software program of the Chronobiology Kit (Release Version 1c, © 1998-2004) of the Stanford Software Systems, Stanford, CA, USA. The record of general activity by an individual within the cage was considered reflecting the response of its circadian system (Malik et al., 2004). Activity records (actogram) are shown as double plots in figures. Each day is duplicated along the horizontal axis, whereas subsequent days are shown underneath in the increasing order. Activity records (actograms) over the experimental period were generated, and from them, the activity profile was calculated and plotted to show a better illustration of the effects. The significance of periodicity was determined using chi-square periodogram analysis (Sokolove and Bushell, 1978). The onset and end of activity during the daytime was calculated with reference to the times of sunrise and sunset, respectively. The phase relationships between activity and the LD cycle were described as the phase angle difference (ψ). The number of movements of each individual was counted and compared.
Endocrine

(i) Hormonal Assay

Serum levels of testosterone and estradiol-17β were measured using ELISA in blood samples collected by puncturing the wing vein in a small volume of about 100-150 µl using a 26 gauge needle. This type of blood sampling is almost non-invasive, has no risk to bird health and bird survives till the end of the experiments. Blood samples were kept at room temperature for 1.5 h and then centrifuged at 1500 rpm for 15 minutes in a refrigerated microcentrifuge (Eppendorf, 5430 R) at 4°C. The serum was collected and frozen at -20°C until used for the hormone assay. The serum was used in the quantitative determination of testosterone and estradiol-17β concentration by the competitive immunoenzymatic colorimetric method using ELISA kit procured from DiaMetra, Italy. The ELISA plate included in the kit comprised of 96 wells flat bottomed polystyrene microplate (coated microplate; coated with anti-testosterone IgG). A standard curve was constructed by using 5 standard solutions, viz. the concentrations of 0, 0.2, 1.0, 4.0 and 16.0 ng/ml for testosterone. Standards and samples were analyzed in duplicate. Briefly, one blank and 25 µl of standards and samples solution were taken in separate wells. Thereafter, 100 µl of the diluted conjugate (Testosterone-HRP conjugate) was added into each well leaving the blank. The contents in each well were properly mixed by shaking the plate slowly between two fingers and incubated at 37°C for 1 h in an oven. Then, non-bound antibodies were removed by emptying the plate and washing it repeatedly with 300 µl distilled water. In order to measure the amount of labelled steroid bound to the antibody, 100 µl of TMB-Substrate (H₂O₂-TMB 0.26 g/L) were added to each well, and then incubated for an additional 15 mins at room temperature (22-28°C) in the dark until the reaction was stopped by the addition of 100 µl of stop solution (H₂SO₄ 0.15 mol/L). The absorbance was recorded at 450 nm with an automatic microplate reader.
(BioRad iMark™ Microplate reader) for each well containing standard or sample solution. A similar procedure was adopted for the estimation of estradiol-17β taking a standard sample of 0, 20, 120, 300, 600 and 2000 pg/ml. Serum testosterone and estradiol-17β concentrations were expressed as ng/ml and pg/ml, respectively. Serial dilution of blood serum samples from both male and female sparrows showed parallelism with their respective standard concentrations. This assay has been validated, standardized and used for the measurement of testosterone and estradiol-17β in other species, including tree sparrows (Biswas et al., 2010; Dixit and Singh, 2013; Dixit et al., 2017).

**Statistical analyses**

The data from different experiments are presented as mean ± S.E.M. They were analyzed using One-way analysis of variance with repeated measures (One-way RM ANOVA), as required, followed by Newman-Keul’s Multiple range ‘t’ test if ANOVA indicated a significance of difference. Two-way ANOVA was also used to compare when two factors (e.g. photoperiod and duration of treatment) were involved followed by post hoc Bonferroni test for group comparisons. Multiple-way ANOVA was also used when more than two factors were involved (for e.g. photoperiod, wavelength or intensity and duration of treatment). Significance was taken at 95% confidence limit. The data from the birds that died during the experiments were not included in the statistical analysis. The statistical analysis for one- and two- way ANOVA was done using GraphPad Prism 5.01 software and SPSS 20 was used for multiple-way ANOVA.

The specific materials and methods as applied to a particular experiment are mentioned in the respective experiments.