Study 1A: Food intake effects on circadian activity rhythm

In this study we determined the effect of food restriction on locomotor activity pattern and gonadal growth on black-headed munia (*Lonchura malacca malacca*) exposed to12L: 12D. Two groups of male and female munia (*n* = 5 pairs each) were exposed to 12L: 12D (During day time when light was on, light intensity L = 265 lux; and in Dark when light was off, the photo intensity was very slow D = 0.3 lux) for day 1–7. On day 8, group one received 4 h food (ZT 0–4; ZT 0 = Zeitgeber time, light onset) and group two received food *ad libitum* (ZT 0–24 h). Locomotor activity pattern and gonadal growth were recorded over the experiment period of one year. Birds were highly active in group one, with a high daily and total activity. However, activity behaviour was fully synchronized with light onset in the group two, when food was available *ad libitum*. Gonadal maturation was initiated in both the groups but the maturation of testis and ovarian follicle was noticed only in group two (food *ad libitum*).

Introduction

In natural environment day length, food availability and other environmental factor regulate the circadian and seasonal processes in several avian species. Among all behavioral and seasonal processes, the pattern of feeding and circadian behavior is mostly affected by day-to-day variation in light–dark cycle (Newton, 1998; Trnka and Prokop, 2006). However, the birth of young is coincides with maximum food availability. So, food as an ultimate cue plays a significant effect on photoperiodic regulation of seasonal reproduction. In rats (Bolles and Duncan, 1969; Krieger, 1974) and mice, limited food availability entrained the core temperature (T_c) rhythm in the

absence of light, when food presented at predictable time (Nelson *et al.*, 1975). In some mammals (rats) displayed ultradian feeding rhythm with a 4-h inter-meal interval (Richter, 1927) but during food deprivation, their frequency of feeding and the size of meals are increased (Le Magnen and Tallon, 1968; Levitsky, 1970).

Mostly, birds are diurnal animals and they are highly active during morning and evening time. They show the bimodal activity pattern in daily feeding and locomotor activity pattern (Daan and Aschoff, 1975; McNamara *et al.*, 1994; Pravosudov and Grubb, 1997; Polo and Bautista, 2006). Their locomotor activity coincides with food availability under artificial condition. Previous finding of Budki *et al.* (2009) suggests that food deprivation affects the timing of gonadal growth and regression during non-breeding phase of redheaded bunting (*Emberiza bruniceps*). They reported that the food restriction during photosensitive phase attenuated the gonadal growth under long photoperiod. Some other findings are based on different experimental regimes on food manipulation; such as food timing and food duration (Kumar *et al.*, 2001), food quantity (Munn *et al.*, 2010) and the interruptions to foraging (Dall and Witter, 1998) have demonstrated its effect on daily and seasonal responses. Also, the nutritional differences during the early developmental phases affect the physiological responses in adult zebra finch (Criscuolo *et al.*, 2008; Prior *et al.*, 2013).

Some findings have shown the effect of food and light on daily locomotor activity pattern on temperate and tropical birds (Hau and Gwinner, 1997; Low *et al.*, 2008; Schoech and Hahn, 2008; Rani *et al.*, 2009; Yom-Tov, 2011; Singh *et al.*, 2012). Our previous finding on subtropical a species demonstrates that light intensity stimulates the rate of circadian and seasonal responses but does not alter the

critical day length for photoperiodic responses in weaver birds (Pandey and Bhardwaj, 2011; Bhardwaj and Pandey, 2015). Recent finding of Srivastava *et al.* (2015) suggests that the 2-h food deprivation under different zeitgeber timing (ZT) schedule under 12L:12D on two interrelated behaviors, the feeding (food intake) and locomotion in subspecies of munia (Spotted Munia; *Lonchura punctulata*), the locomotor activity of birds increased during food deprivation and it reduced when food available at *ad libitum*. However, less number of finding have reported, how different behaviors (feeding and daily locomotor activity) may interact with different phases of food deprivation. Here, we aimed to investigate whether different timing of food availability affects the circadian (locomotor activity) and seasonal responses (gonadal development) in blackheaded munia. In these consequences, the present study aimed to find out the effect of food deprivation during early light onset on daily activity pattern and photoperiodic induction in black-headed munia (*Lonchura malacca malacca*).

Methods

Two groups of birds (n = 5 pair/group) were subjected to 12L:12D (12 h light: 12 h dark; L = 265 lux; D = 0.3 lux) and constant temperature conditions (24 ± 2°C) for 70 days. Both group of birds received similar experimental regime with food *ad libitum* condition at day 1–7. On day 8, birds of group-1received food *ad libitum* for the entire duration of experiment (ZT 0–24; zeitgeber time, ZT 0; time of light onset), but group two birds were given restricted feeding schedule, food present only 4 h of the day (ZT 0–4). These feeding schedules continued until end of experiment (day 70). Birds were housed in pair (male and female) in activity cages (size = 61 × 31 × 40 cm) kept inside the photoperiodic boxes (size =74×56×72 cm).

Each activity cage had two perches and mounted with an infrared motion detector (Napoleon miniature passive IR Detectors; Maximum Comp. Ltd. Israel) (figure 1).

The results of activity are shown on selected days on actogram and activity count. The observation of testes and ovarian growth were measured by laparotomy (Kumar *et al.*, 2001). The daily and total counts in a 24 h day were plotted as mean ± SE for each group. The difference in the testicular and ovarian growth was analyzed by the one-way analysis of variance with repeated measures (one-way RM ANOVA). The difference in day and night activity counts of food *ad libitum* and restriction was analyzed by the unpaired student's *t*-test. Significance was taken at $P < 0.05$. All the statistical analyses were done using GraphPad Prism software ver. 3.0 (GraphPad Software, San Diego, CA).

Results

Results are shown in figures 2-5. Figure 2 and 3 (i) and (ii) shows representative actograms (pair; male-female) for selected days (day 1–70) of birds under two food conditions food ad libitum (ZT: 0–24) and food restricted (ZT: 0–4). Birds in both conditions showed high activity during the day (ZT: 0–12) [figure 1 (i) & (ii)]. In initial days (days 1–7), both group of birds showed similar locomotor activity and activity counts of daily profile under 12L:12D photoperiods. The daily locomotor activity showed significantly high activity on light phase and completely diminished activity during dark phase so, the black-headed munia showed complete diurnal rhythm. During food restriction (ZT: 0–4) phase at day 8, activity of these birds declined gradually from dawn to dusk and statistically significant difference occurred on food *ad libitum* group ($P= 0.0231$; unpaired student's t-test) [figure 2 and 3(a) and (b)].

The total counts of daytime activity were decreased per day of group two (food restricted) when food was available on 4 h till light onset and activity of these birds were restricted during the food available period (figure 5a). However, the night time activity total counts were similar in both groups (figure 5a). In the food intake, *ad libitum* and restricted group showed no significant difference (P= 0.0898; unpaired student's *t*-test) but the food *ad libitum* group (food intake: 4.74 ± 0.32) take more food compared with food restricted group (food intake: 3.87 ± 0.22) during the course of entire period of experiment (figure 5b).

Testes failed to under growth-regression cycle in the birds held under food restricted but a slight induction in testicular growth was observed between days 120–180. Under similar photoperiod, testes were significant larger on the birds of food *ad libitum* group than food restricted at day 90–180 (P< 0.05; Bonferroni *post test*) (figure 4c). There was an effect of the food, duration of exposure and interaction between them on testicular response at food *ad libitum* group (food: $F_{1,21}$ = 16.42, P= 0.0006; duration of exposure: $F_{6,21}$ = 12.63, P < 0.0001; food x duration of exposure: $F_{6,21}$ = 1.470, P= 0.2364; two-way ANOVA) (figure 4c). There was no significant maturation on ovarian follicles were noticed the food restricted group during entire period of experiment, but significant follicular growth was observed on the birds of food *ad libitum* group on day 150 ($F_{6,24}$ = 25.98, P< 0.0001; one-way RM ANOVA) (figure 4d). There was an effect of the food, duration of exposure and interaction between them on ovarian follicles maturation on food *ad libitum* group (food: $F_{1,21}$ = 8.409,P= 0.0086; duration of exposure: $F_{6,21}$ = 2.948, P= 0.0301; food x duration of exposure: $F_{6,21}$ = 1.613, P= 0.1930; two-way ANOVA) (figure 4d).

Discussion

This study examines both the indirect and direct effects of food *ad libitum* and restriction condition on groups of black-headed munia housed in a pair (male/female). Since the black-headed munia feeds during the daytime, food availability was changed from an *ad libitum* (ZT 0**–**24) to restriction phase (ZT 0**–**4) only during the daytime (L = 12 h). Whereas the daily locomotor activity (circadian behavior) was considered to show indirect effects of the food restriction regimes, changes in gonadal growth (testes/ovary) to show their direct effects. Our results demonstrated that the food restriction affected allocation of locomotor activity and feeding behavior of black-headed munia. During food *ad libitum* condition, the activity levels increased and completely distributed entire period of light phase but in opposite, it was mostly restricted only few hours when food available at a particular time [figure 2 and 3 (i) & (ii)]. So, the food presence or absence modulates the locomotor activity of black-headed munia. But in quail, food restriction increases the locomotor activity than control groups (Boon *et al.*, 1999). Our results showed that total food intake was depending upon the food availability. There was only marginal difference in total food intake in both control (food available for 24 h) and treatment (food available for 4 h) group (figure 5 b).

The present finding suggests that food availability pursued by food deprivation induced higher intake. So, the food intake in several birds, in response to intermittent feeding schedules, depends on their daily energy requirements as determined by the length of fasting prior to feeding (Boon *et al.,* 1999). Our results also support the earlier finding on other bird species shown that the physiological and behavioral correlation under unpredictable and variable food conditions

(Hurly, 1992). Food restriction or starvation has been reduced the energy expenditure in several birds (MacLeod *et al.*, 1993) and mammals (Hervey and Tobin, 1982). Food restriction also stimulates the daily locomotor activity pattern (Singh *et al.*, 2012) as well as plasma metabolites (Fokidis *et al.*, 2011) and cognitive functions (Shettleworth, 2003). Finding of Piccione *et al.* (2002) demonstrated that 4-day food deprivation caused a significant fall in the body temperature of ruminant mammals (goats and sheep).

The results in figure 2c and d conclude the food availability accelerate the gonadal maturation. There was no significant change in testes and ovarian follicular growth when food was available only 4 h during the daytime (ZT 0–4; ZT 0 = onset of light). However, significant change in gonadal growth (testis/ovary follicle diameter) was noticed when food available at *ad libitum* during the entire period of day. Similar finding on zebra finch was reported by Prior *et al.*, (2013) during water restriction. Their finding shows that the water restriction affects the physiology, endocrinology (hormonal secretion) and social behaviour in both male and female zebra finches during the non-breeding phase (Prior *et al.*, 2013). The recent finding by Srivastava *et al.* (2015) on spotted munia showed that the two behaviors (feeding and locomotion) of spotted munia are re-allocated temporally by food deprivation.

Study 1B: To analyse seasonal rhythmicity in gonadal cycle

To investigate the seasonality in photoperiodic response, experiment was performed on adult male blackheaded munia. Birds were captured by mist nets and kept in out-door aviary from September 2013 to June 2014. On each season of the year (both equinoxes and solstice), three groups of birds (n=6 each) were exposed in short day length (SD), long day length (LD) and natural day length (NDL). Daytime light intensity was 460 lux and night time received dim light illuminations (~0.3 lux) in light regimes (SD and LD condition). A group was maintained under natural day lengths (NDL) served as control. Observations on body molt, body mass and testis size were taken from beginning to the end at appropriate intervals during the experiments.

Introduction

Most organisms are facing rhythmic changes in an environment called the physical periodicities or seasons, almost all organisms, which have been investigated, are light sensitive and in many organisms, especially those living away from the equator, the annual solar cycle has been found influencing various seasonal functions (Murton and Westwood, 1977; Thapliyal, 1981; Hoffman, 1981). At the equator where changes in daily light are small, the changes in daytime light intensity across seasons can influence seasonal responses (Gwinner and Scheuerlein, 1998).

According to Jain and Kumar (1995) and Deviche and Small (2001) most of the birds exhibit well-defined seasonality in their various physiological and behavioral functions including gonadal growth and development, molt, body mass, bill coloration. The behavioural and physiological functions centre on reproduction

that occurs at the most appropriate time of the year when the food availability and survival of the young are at a maximum level (Dawson, 2007). So, the timing of actual reproduction is critical for the species. Since change in photoperiod is entirely predictable at given latitude, both within and between years, it is used as a most reliable cue to time the physiological preparations for three major life-history stages: reproductive cycle, molt and migration (Goldman, 2001; Gwinner, 2003). Thus, reproductive cycles of most avian species are controlled by the annual change in photoperiod with the birds cycling between physiological states of photosensitivity, photostimulation and photorefractoriness. It is considered as a powerful 'initial predictive cue' that initiates or terminates reproduction in many temperate, subtropical and tropical species (Dawson *et al.,* 2001; Rani *et al.,* 2002; Kumar *et al.,* 2004). Other external zeitgeber cues such as temperature, rainfall, food abundance, and social stimuli may also affect the timing of life cycle stages, but provide only short term predictive information. Such evidence is not suggested to all species of birds, which the time of egg-laying varies with spring temperature. It is less clear whether this is a consequence of a direct effect of temperature on photoperiodically induced gonadal maturation.

Although attempts were made to assess the role of temperature on photoperiodic induction of gonadal growth, the results, showing positive or negative effects, were inconclusive (Wingfield *et al.,* 2003). Therefore, it is logical to perform more experiments with different species at various latitudes to ascertain the role of temperature on photoperiodic regulation of reproduction and associated events in birds. Reproduction and plumage molt are high energy demanding processes in the life cycle of birds (Klaassen, 1995). Feather loss may impair flight performance

making molt and reproduction incompatible (Langston and Rohwer, 1996). Accordingly, both the events are so timed that they do not overlap as a molt breeding overlap is inherently costly (Hemborg *et al.*, 2001). Further, birds show a diversity of breeding schedules that can be correlated with the diversity of photo-response systems. Interspecific variation in the parameters of photoperiod response systems could give rise to different breeding schedules. Some of these parameters include: (a) the photoperiod required for photostimulation in spring, (b) the time of establishment of photorefractoriness, (c) if photorefractoriness can be terminated by short day treatment, and (d) whether the photorefractoriness is absolute, relative, or absent (Hahn and MacDougall-Shackleton, 2007). Variation in these parameters could provide birds with (i) highly seasonal breeding schedule with termination of reproductive functions prior to summer solstice, (ii) long, more flexible breeding schedule, or (iii) opportunistic breeding with little influence of photoperiod.

The testes are very small and usually avascular structure. It's shape, oblong or cylindrical, smooth on the surface and creamy-white in color, although they may be partially or totally pigmented. In a mature bird, the testes can vary in size and greatly enlarge during the breeding season. Basically testes perform two important functions, first to produce sperm and the secondary secrete the male hormone, testosterone which is responsible for various secondary sexual characteristics such as male sexual behavior (including song), color and feather formation (if different from the female) and also the development of a comb and wattles in some species. Testosterone is produced by cells, known as interstitial cells of leydig. These cells are located in the spaces between seminiferous tubules.

Microscopically, the testis consists almost entirely of tubular structures known as seminiferous tubules. These tubular structures contain two types of cell line, spermatogonia cells and sertoli cells. The spermatogonia cells proliferate and differentiate through definite stages of development to form sperm. Spermatogonia initially multiply and grow to form considerably enlarged cells called primary spermatocytes. Primary spermatocytes cells then enter a period of maturation in which the first maturation division forms secondary spermatocytes and the second maturation division forms the spermatids. Each spermatid develops into a spermatozoan. Spermatids are produced by meiotic division, that is, without replication of chromosomes, merely a division of those already present. Therefore, each spermatid has half of the normal complement of chromosomes, none of them paired. Sertoli cells are large cells interspaced between spermatogonia which extend from the base of the seminiferous epithelium to the interior of the tubules. Spermatids attach themselves to the sertoli cells and some specific relationship seems to exist between the two cell types which cause the spermatids to change into active sperm. Seminiferous tubules of immature males are small and lined by a single layer of cells. The mature testis has large irregular-shaped tubules with a multi-layered germinal epithelium consisting of cells representing all stages of spermatogenesis.

The annual reproductive cycle of Indian birds can be divided into distinct phases i.e., (i) Preparatory or regenerative phase, (ii) progressive phase, (iii) breeding phase, and (iv) regressive phase. The duration of each phase varies according to species to species (Saxena, 1964; Pandha, 1966; Tewary, 1967; Garg, 1968; Chaturvedi, 1976; Kumar, 1981; Lal, 1982; Singh, 1982; Dixit, 1987; Tripathi, 1987). After regression phase, the regenerative (Marshall, 1961) or preparatory (Wolfson,

1959 a, b) phase is started immediately. During the preparatory phase, size and weight of testis are minimum also the seminiferous tubules are of minimum diameter and contain 1-3 layers of spermatogonia only. Interstitial cells are present in groups in the inter-tubular spaces, and they do not secrete any secretory substances. The preparatory phase is further converted on the progressive phase, during this phase the testicular volume and weight increases gradually. The seminiferous tubules widen in diameter, and spermatogonial cells became dividing in mitotically. In the late progressive phase, the formation of spermatocytes and spermatids is performed by the successive division of the spermatogonia. The initiation of spermiogenesis marks the end of the progressive phase and initation of breeding phase. Seminiferous tubules are highly stretched due to increase in population of dividing germinal cells during the breeding phase, and contain bunches of spermatozoa in the lumen. The maximum testicular volume characterizes the peak of breeding phase. The testis attains the minimum weight and volume at the end of the regressive phase and enters into the preparatory phase.

Few histological studies have been done on avian species to show the effect of photoperiodic factors, various drugs and chemicals on gonad of birds (Bhavna and Geeta, 2010; Singh and Dixit, 2012; Lan *et al.*, 2011). Studies also demonstrate the characteristics of annual gonadal cycle and gametogenesis on carps (Hassanin *et al.*, 2002; Carballo *et al.*, 2005). Here, we carried the histological studies on testis of blackheaded munia bird, under this study 1B, the details of which have been given elsewhere to understand the testicular activity at histological level.

Many empirical studies have shown the role of day length in the control of avian seasonality (Dawson *et al.*, 2001; Dawson, 2002). Most studies have been

confined to the resident or short distance migratory species of the temperate zone where the time during the year is immediately apparent from regular sinusoidal change in day length and the species experiencing these conditions appear to be photoperiodic. In contrast, birds living in tropical/subtropical regions experience relatively shorter change in photoperiod and have been considered to be unable to use day length as a cue for controlling seasonality (Dittami and Gwinner, 1985). However, evidences suggest that even tropical species have evolved the ability to respond to photoperiodic change. The fact that both high and low latitude species are capable of fine discrimination of even small photoperiodic changes reveals that they represent an adaptation in inhabiting different photoperiodic environments. Thus, it is more interesting to study photoperiodic adaptations in the bird species having wide distribution covering various latitudes.

Therefore, it is proposed to study the detailed pattern of seasonality, including the effect of photoperiod and whether annual variation of day length at Meerut, India, play an important role in the timing of seasonal breeding and associated events in the blackheaded munia. In this experiment, we have described annual seasonal cycles in the captive birds under natural conditions and investigated seasonal cycles under programmed photoperiodic schedules (9L:15D and 15L:9D) during different seasons of the year.

Methods

Adult male birds were captured from wintering and kept in an outdoor aviary (3.6 m × 3.6 m × 2.5 m) for 2 weeks, aviary receiving natural light-dark cycle. Study of annual seasonal cycles was conducted on these birds. Two groups (n = 7-8) of birds were exposed under short day length (9L:15D) and long day length (15L:9D)

for 360 days on different time of the year; 22 September 2013, 21 December 2013, 20 March 2014 and 21 June 2014. All groups were received 460 lux light intensity during day time and dim light illumination during night time (0.3 lux). The observations on body mass, molt body, molt primaries and gonadal size were recorded at weekly and monthly intervals (figure 6).

Results

Results from this experiment show whether photoperiodic changes affect the physiological and photoperiodic inductions of seasonal responses in the blackheaded munia during different seasons of life history stages. Changes in body mass, gonadal growth/development, and molt in body and primary wing feathers of blackheaded munia during the period of pre-breeding phase [winter solstices (December), or vernal equinox (March), or summer solstices (June) breeding phase and in post–breeding phase September (Autumnal equinoxes)] and of the year, compared with these observations on birds, held under natural day length (NDL). Day length increases after its minimum to maximum induction in December until summer solstice and the longest day length of 13.44 h is attained in June. It starts decreasing thereafter to reaches the minimum (10.29 h) by winter solstice (December). Thus, the day length varies over the range of 3.15 h annually.

Results demonstrate that mean body mass of male blackheaded munia gradually increased and decreased throughout the experiment. There was no significant change in the body mass in June, September, December and March groups [September (SD group) $F_{24,96} = 4.676$, $P < 0.0001$ (LD group) $F_{24,96} = 1.321$, $P = 0.1718$; December (SD group) $F_{24,96} = 0.6141$, $P = 0.9138$ (LD group) $F_{24,96} = 75.73$, $P = 0.7789$; March (SD group) $F_{24,96} = 7.779$, $P < 0.0001$(LD group) $F_{24,96} =$

4.680, P < 0.0001; June (SD group) $F_{24,96}$ = 2.737, P < 0.0003 (LD group) $F_{24,96}$ = 1.429, P = 0.1144; 1-way RM ANOVA)(figure 7 a, c, e and g]. The mean testicular volume in each group attained a peak value and then gradually decreased. There was significant change in the testis volume in June, September, December and March groups [September (SD group) $F_{24, 48}$=10.49, P<0.0001; (LD group) $F_{24,48}$=13.49, P<0.0001; December (SD group) $F_{24,48}$=0.4710, P=0.9186 (LD group) $F_{24,48}$=27.86, P < 0.0001; March (SD group) $F_{24,48}$=1.239, P=0.2857; (LD group) $F_{24,48}$ = 3.947, P<0.0003; June (SD group) $F_{24,48}$=22.76, P < 0.0001; (LD group) $F_{24,48}$=4.083, P<0.0002; 1-way RM ANOVA) (figure 7 b, d, f & h)].

The mean body molt and molt primaries increased throughout the experiments in each groups (figure 13). There was significant change in the body molt in September, December, March and June groups [September (SD group) $F_{6,12}$ =1.982, P =0.1476 (LD group) $F_{6,12}$ =23.81, P<0.0001; December (SD group) $F_{6,12}$ =1.121, P=0.4062; (LD group) $F_{6,12}$ =1.625, P=0.2232; March (SD group) $F_{6,12}$=1.055, P=0.4390; (LD group) $F_{6,12}$=2.245, P=0.1099; June (SD group) $F_{6,12}$ =3.861, P<0.0222 (LD group) $F_{6,12}$ =19.64, P<0.0001; 1-way RM ANOVA) (figure a, c, e and g). And molt primary in September, December, March and June groups [September (SD group) $F_{3,6}$ =1.671, P=0.2711 (LD group) $F_{3,6}$=2.399, P = 0.1665; December (SD group) $F_{3,6}$ =6.512, P<0.0257 (LD group) $F_{3,6}$= 6.615, P< 0.0249; March (SD group) $F_{3,6}$=8.614, P<0.0136 (LD group) $F_{3,6}$ =3.520, P= 0.0887; June (SD group) $F_{3,6}$ =24.58, P<0.0009; (LD group) $F_{3,6}$ =24.58, P<0.0009; 1-way RM ANOVA) (figure 8b, d, f and h).

The photo micrographic details of season (September, December March and June) and NDL groups are given in Plate I. In NDL group of September and

December month, testis was reduced, but in March birds during pre-breeding phase testis show the preparatory phase plate I b and c. Seminiferous tubules were narrow and lined by a single or double-layered spermatogonial cells. Tunica albuginea was fibrous and thick. The intertubular spaces are wide containing interstitial cells. In June month bird was in breeding phase, the seminiferous tubules are highly stretched due to maximum with increase in population of dividing germinal cells, the inter tubular space reduced and seminiferous tubule gradually increased and spermatocytes, spermatogonial cells are present, spermatids and sperm maturation become started. Spermatogenesis is absent in September month and testis regression was started.

In LDL groups of September month, testis remains in reduced form due to their photorefractory stage. Seminiferous tubule becomes enlarge and intertubular space reduced in LDL group of December, March and June months. In December group of LDL, bunches of spermatozoa are attached to cells of sertoli in seminiferous tubules. Spermatogenetic activity seems to be maximum in wide lumen. The interstitial tissue between adjacent tubules has become greatly compressed. In June month LDL group, reproductive activity was in peak. Bunches of spermatozoa are present in wide lumen. Intertubular spaces are much reduced. In September, showed the end of reproductive activity regressive change becomes much distinct. Tubules are narrow and tunica propria is indistinct. But the end of experiment, SDL groups of September, December, March and June groups, testis was observed in reduced form (Plate I).

Discussion

Photoperiodism is the mechanism appears to be involved in regulation of seasonality and in the circannual rhythm generation which a self-sustained endogenous rhythmicity of approximately 1-year times. These mechanisms may not be mutually exclusive and, in fact, might interact closely, albeit as per adaptive needs of the species. However, many would argue an opposite proposition; Photoperiodism and circannual rhythm generation are evolved as separate mechanisms. A commonly held view is that a photoperiodic species lacks a strong circannual component, and a circannual species lacks a strong photoperiodic component. Part of this widely held assumption stems from studies that (i) show maintenance of the post-reproductive photorefractoriness until a long day photoperiodic bird species is kept under stimulatory long day lengths (Sansum and King, 1976) and (ii) shows circannual rhythm generation in low-latitude and equatorial species that are not considered typically photoperiodic species (Gwinner, 1986). This suggests the possibility that photoperiodism and circannual rhythm generation mechanisms coexist in the same photoperiodic species.

Experiments on this species suggest that the blackheaded munia is a photosensitive species. In natural condition, the annual cycles of body mass and testes correspond to annual variations in day length, similar to a number of temperate and tropical/subtropical species, reported by several investigator (Tewary, 1967; Murton and Westwood, 1977; Kumar and Tewary, 1983; Chandola *et al.*, 1983; Dittami and Gwinner, 1985; Dittami and Knauer, 1986; Kumar and Kumar, 1991, 1993; Deviche and Small, 2001). Photoperiods of 15h light per day trigger initiation of fattening and gonadal growth in the spring, but the fat depletes, body mass declines and mature

gonads fully regress by August, when day lengths are still longer than those initiated response in spring, suggesting the onset of refractoriness to stimulatory effects of long day length. This is a general phenomenon in avian photoperiodism for the termination of seasonality in photoperiodic species (Murton and Westwood, 1977; Farner *et al.,* 1983; Nicholls *et al.,* 1988). The annual cycles in body mass and testis size was studied on different species of birds earlier also by Kumar and Tewary (1982b) and Jain and Kumar (1995) in black headed buntings, but these were done either by exposing a group (n=4) of birds every month from a batch of birds maintained in small cages (Kumar and Tewary, 1982a) or making observations for 8 months (January to September) in a group of birds kept in small cages (Jain and Kumar, 1995).

Role of photoperiodic and seasonal rhythmic induction in gonadal cycle show that the response under natural and long photoperiods of the blackheaded munia can easily and reliably be mimicked under artificial day length. These observations clearly mean that blackheaded munia uses the length of daily photoperiod as source of temporal information for regulating their seasonal cycles. The exposure of birds to non-stimulatory photoperiods in which birds will never undergo gonadal development would also ensure that they never become photorefractory. If there was one, the response of birds to long day length should vary depending on the time of the year, irrespective of the fact that birds are continuously maintained on non-stimulatory short day lengths. This study provided the evidence that artificial long day lengths (15L: 9D) reproduce a photoperiodic response that is normally seen under increasing natural day length, introducing that the day length regulates gonadal cycle and associated events in the blackheaded munia. This suggests that under long day lengths

induction of a photoperiodic response was faster. As is true of several other species (Lewis, 1975; Kumar and Tewary, 1982b, 1983; Tewary and Kumar, 1982; Tewary and Tripathi, 1983; Tewary *et al.,* 1983; Tewary and Dixit, 1986; Kumar and Kumar, 1991), a typical short day (8L:16D) was not inductive to blackheaded munia. A similar photoperiodic response to such photoperiods (8L and 16L) is reported in another species, the Indian weaver bird at 25^0N, 83^0E that often shares habitat with the house sparrow. When exposed to a long photoperiod (16L:8D), beginning from September, partially regressed testes recrudesced and the pituitary was activated as indicated by changes in the LH-dependent plumage pigmentation, androgen-dependent darkening of the bill, and gonadal volume and histology (Chandola *et al.*, 1974; Singh and Chandola, 1981).

In some species, long photoperiods (16 h or longer light per day) can keep gonads active for at least a year. Examples are: rufous collared sparrow, *Zonotrichia capensis* (Lewis *et al.*, 1974), *Zonotrichia capensis costaricensis* (Epple *et al.*, 1972) and Indian weaver bird, *Ploceus philippinus* (Thapliyal and Saxena, 1964b). Equatorial redbilled quelea (*Quelea quelea*) is also photoperiodic and responds to photostimulation with a brief refractory period dissipated spontaneously regardless of the exogenous photoperiods (Lofts, 1962). A similar report exists for the equatorial fiscal shrikes, *Lanius collaris* (Dittami and Knauer, 1986). Tropical stonechats (*Saxicola torquata axillaris*) also react to photoperiods (Gwinner and Dittami, 1984; Dittami and Gwinner, 1985) and use light intensity as *zeitgeber* in regulation of the annual cycles in testes and molt (Gwinner and Scheuerlein, 1998). Interesting responses are seen in the blackheaded munia, which shows testicular development both under short and long photoperiods (Thapliyal and Saxena, 1964a; Pandha and

Thapliyal, 1969), and spotted munia, which responds to only unnaturally short photoperiods (<6h) (Chandola *et al.*, 1975). Bhatt *et al.* (1986) suggested that photoperiod acts only as synchronizer of the endogenous circannual rhythm of breeding cycle of the spotted munia.

Study 2: To investigate responsiveness to changing photoperiods

A series of experiments were performed on the blackheaded munia during June, September, December and March month. A groups of photosensitive birds (n = 6-8 per group) was exposed to long day length (16L: 8D). After 4 weeks, this group transferred to decreasing photoperiod sequentially; 15L: 9D, 14L: 10D, 13L: 11D, 12L: 12D, 11L: 13D, 10L: 14D, 9L: 15D and 8L: 16D. Another group of photosensitive birds (n = 6-8) was exposed to short day length(8L: 16D). After 4 weeks this groups was transferred to increasing photoperiod sequentially; 9L:15D, 10L:14D, 11L:13D, 12L:12D, 13L:11D, 14L:10D, 15L:9D and 16L:8D.

Introduction

Day length regulates the seasonal responses in many vertebrates, including bird species. The seasonal change in day length provides the most reliable source of temporal information about the environment and has been adopted by birds in the course of evolution as the main proximate environmental cues for synchronizing reproduction, moult and migration with favourable environmental conditions. Some other ultimate factors serve as supplementary cues for fine-tuning the rate of gonadal growth and the timing of breeding with local phonological conditions (Wingfield *et al.*, 1992, 1993; Hahn *et al.*, 1997; Budki *et al.*, 2012). In birds, the change in day length is the most important environmental cue used for synchronizing breeding, moult and migration with recurrent seasonal fluctuation in environmental conditions (Coppack and Pulido, 2004).

According to Rowan studies (1925) on slate-colored Juncos (*Junco hyemalis*), the role of day length (or photoperiod) as a major source of temporal information

regulating seasonal responses has been demonstrated in many bird species (Murton and Westwood, 1977; Follett, 1984; Wingfield and Farner, 1993; Kumar, 1997; Gwinner and Hau, 2000; Dawson *et al.*, 2001). Relatively less is known of photoperiodism in tropical birds (Thapliyal and Gupta, 1989; Hau, 2001). Light is a major source of temporal information for circadian and seasonal responses in many species of birds. The regulation of annual periodicity in physiological and behavioral processes in wild birds depends upon the appropriate relationship with phase, among several endogenous components, including secretion of several hormones. Many, if not all, endogenous components need entrainment with the environmental variable(s), which is (are) precise in occurrence, to keep them in proper pace with seasons of the year.

Earlier findings suggest that the role of long day length in stimulation of fat deposition and/or gonadal growth in several species of temperate (Rowan, 1928; Wolfson, 1940; Murton and Westwood, 1977; Farner and Follett, 1979; Follett, 1984; Dittami and Gwinner, 1985; Nicholls *et al.*, 1988; Gwinner and Hau, 2000) and tropical bird species (Thapliyal, 1981; Tewary *et al.*, 1982; Tewary and Kumar, 1982; Kumar and Tewary, 1983; Tewary and Tripathi, 1983; Tewary and Dixit, 1986; Kumar and Kumar, 1991, 1993; Kumar *et al.*, 1993; Kumar, 1997; Budki *et al.*, 2009; Dixit and Singh, 2011; Singh *et al.*, 2012; Bhardwaj *et al*, 2011, 2012; Kumar *et al.*, 2012; Singh and Dixit, 2012; Sharma and Bhardwaj, 2012). Relatively less attention is paid on photoperiod-induced effects on fat deposition (Tewary and Kumar, 1982; Kumar and Tewary, 1983; Kumar, 1986, 1988, 1997).

In the photoperiodic literature, it appears that there is a threshold photoperiod for the gonadal response; testicular growth, within a range, occurs at a rate

proportional to day length (Farner and Wilson, 1957; Farner *et al.*, 1966). Fat deposition preceding breeding period (called vernal fattening), in contrast, appears to be example of an 'on-off' process, and hence the rate of photoinduced fattening is independent of day length (King and Farner, 1965). Also, several studies have suggested that the photoperiodic response system has a minimum light intensity threshold (Rani *et al.*, 2002). In the earlier photoperiodic literature, different types of LD cycle paradigm (light and dark periods in different combinations in 24 h and non 24 h periods) have been used frequently to investigate how light information is used by the organisms in regulating their seasonal responses.

The testes are very small and usually avascular structure. It's shape, oblong or cylindrical, smooth on the surface and creamy-white in color, although they may be partially or totally pigmented. In a mature bird, the testes can vary in size and greatly enlarge during the breeding season. Basically testes perform two important functions, first to produce sperm and the secondary secrete the male hormone, testosterone which is responsible for various secondary sexual characteristics such as male sexual behavior (including song), color and feather formation (if different from the female) and also the development of a comb and wattles in some species. Testosterone is produced by cells, known as interstitial cells of Leydig. These cells are located in the spaces between seminiferous tubules.

The electrophoresis patterns of avian serum proteins

More than 50 years, it has been known that particular proteins characterize every species of plants and animals and their phylogenetic relationships are reflected in protein structure. The first application of this fact to taxonomic studies was by Nuttall (1904) who used the precipitin reaction of immune sera to test degrees of

relationship in over 500 species of animals. With refinements in technique have come many more serological studies and the results have justified the statement by Landsteiner (1945) that 'chemical differences parallel the variation in structure' and hence are useful in classification.

The development of other methods for protein characterization has suggested that these too might be applied for systematics. Soon after Tiselius (1937) described his apparatus for the electrophoretic separation of colloidal mixtures Landsteiner *et al.* (1938) used it to compare the egg albumins and hemoglobins of five species of birds. Within the next few years there followed the studies by Moore (1945) Deutsch and Goodloe (1945) and Deutsch and McShan (1949). These authors investigated the plasma proteins of several species of reptiles, amphibians, fish, birds, mammals, and some invertebrates. They showed that electrophoresis could detect the species specific qualities of proteins and that similarity in proteins paralleled evolutionary relationships. With the development of filter paper electrophoresis the procedure has been simplified and the study of Dessauer and Fox (1956) on the plasma proteins of more than 100 species and subspecies of reptiles and amphibians has been the most extensive to date. Others who have used paper electrophoresis include Zweig and Crenshaw (1957) who found specific characters in the serum proteins of turtles of the genus Pseudemys, Starr and Fosberg (1958) who published the serum protein patterns of several species of sharks. Woods *et al.* (1958) used starch gel electrophoresis in a study of the sera of 19 species of invertebrates. The egg white proteins of birds have also been shown to be species specific and to produce excellent electrophoretic profiles. The papers by Bain and Deutsch (1947) and McCabe and Deutsch (1952) are the principal ones to date. The latter reported on 37 species of birds and concluded

that the method was applicable to taxonomic problems. Sibley (1960) has used paper electrophoresis in a study of the albumin proteins of egg of more than 300 species and has found the conclusions of McCabe and Deutsch (1952) fully justified.

Methods

Adult male and female munia were captured from wintering and kept in an outdoor aviary (3.6 m × 3.6 m × 2.5 m) for 2 weeks, aviary receiving natural light-dark cycle. Study of annual seasonal cycles was conducted on these birds. A series of experiments were performed on the blackheaded munia (both male and female) during 20 June, 22 September, 23 December 2014 and 23 March 2015. Groups of photosensitive birds (n = 6-8 per group) were exposed to long day lengths (16L: 8D). After 4 weeks, this group was transferred to decreasing photoperiod sequentially to 15L: 9D, 14L: 10D, 13L: 11D, 12L: 12D, 11L: 13D, 10L: 14D, 9L: 15D and 8L: 16D for next consecutive 4 weeks. Another group of photosensitive birds (n = 6-8) were exposed to short day length 8L: 16D and then transferred to increasing photoperiod sequentially for each month to 9L: 15D, 10L: 14D, 11L: 13D, 12L: 12D, 13L: 11D, 14L: 10D, 15L: 9D and 16L: 8D photoperiod. Both groups received 460 lux light intensity during the daytime at perch level. Observations on body mass, moults and gonadal size (testis and follicular size) was taken at the beginning and at appropriate intervals during the experiment. The blood sampling was performed in three times during the experimental period; in beginning, mid and at the end of experiment in both groups (figure 9).

Results

Results are shown in figure 10. There was significant change observed in body mass of male blackheaded munia during March ($F_{18,72}$=6.174, P<0.0001), and

June ($F_{18,72}$=3.805, P<0.0001; 1-way RM ANOVA) month. But there was no significant response occurred in September ($F_{18,72}$=08280, P=0.6622) and December ($F_{18,72}$=0.8268, P=0.6636; 1-way RM ANOVA) groups under LD (15L: 9D) to SD (9L:15D) transferred group (figure 10a, c, e, g). There was no significant change in body mass was noticed in SD (9L: 15D) to LD (15L: 9D) transferred group (March: $F_{18,72}$=3.577, P<0.0001, June: $F_{18,72}$=2.733, P<0.0001, September: $F_{18,72}$=2.970, P=<0.0001 and December: $F_{18,72}$=4.507, P<0.0001; 1-way RM ANOVA) (figure 10a, c, e, g).

Changes in testis volume are presented in the figure 10 (b, d, f and h). There was a significant induction in testis size was noticed in both groups subjected to sequentially transferred under long day length to short day and short day to long day length (March: $F_{9,36}$=19.88, P<0.0001, June: $F_{9,36}$=34.31, P<0.0001 and December: $F_{9,36}$=34.31, P<0.0001; 1-way RM ANOVA). And under short day length to long day length transferred group (March: $F_{9,36}$=51.49, P<0.0001, June $F_{9,36}$=18.91, P<0.0001, September $F_{9,36}$=18.91, P<0.0001 and December: $F_{9,36}$=103.3, P<0.0001; 1-way RM ANOVA). But in September month, LD to SD transferred group showed no significant change ($F_{9,36}$=1.566, P=0.0001; 1-way RM ANOVA). Testes were fully enlarged in the September and December groups during the experiment period (figure 10b, d, f and h).

The molt cycle of both (body feathers and wing primaries) in male blackheaded munia followed testicular cycle. Complete molt cycles of the feathers occurred in both LD to SD transferred group (March: body molt, $F_{40,160}$=1.541, P<0.0323; wing primaries, $F_{40,160}$=27.99, P<0.0001, June: body feathers, $F_{40,160}$=27.21, P<0.0001; wing primaries, $F_{40,160}$=32.76, P<0.0001: September: body

feathers, $F_{40,160}$=22.13, P<0.0001; wing primaries, $F_{40,160}$=27.98, P<0.0001 and December: body feathers, $F_{40,160}$=6.707, P<0.0001; wing primaries, $F_{40,160}$=27.61, P<0.0001; 1-way RM ANOVA) (figure 11a, c, e and g). In SD to LD transferred groups (March: body feathers, $F_{40,160}$=41.30, P<0.0001; wing primaries, $F_{40,160}$=21.76, P<0.0001, June: body feathers, $F_{40,160}$=35.70, P<0.0001; wing primaries, $F_{40,160}$=69.88, P<0.0001, September: body feathers, $F_{40,160}$=36.72, P<0.0001; wing primaries, $F_{40,160}$=45.19, P<0.0001 and December: body feathers, $F_{40,160}$=3.881, P<0.0001; wing primaries, $F_{40,160}$=59.86, P<0.0001; 1-way RM ANOVA) (figure 11b, d, f and h).

Figure 12 (a, c, e and g) shows the results of body mass in female munia. Except March and December group (March: $F_{18,36}$=1.745, P<0.0763 and December: $F_{18,36}$=1.745, P=<0.0001; 1-way RM ANOVA), no significant change was observed in body mass of LD (15L: 9D) to SD (9L:15D) transferred female birds in June ($F_{18,36}$=0.2722, P=0.9977) and September groups ($F_{18,36}$=5.609, P=0.6665). But in SD (9L: 15D) to LD (15L: 9D) transferred group result showed no significant change in March ($F_{18,36}$=0.6783, P=0.8089) and June groups ($F_{18,36}$=2.091 P=0.0294). But the significant change occurred in September ($F_{18,36}$=3.602, P<0.0005) and December groups ($F_{18,36}$=4.507, P<0.0001; 1-way RM ANOVA) (figure 12a, c, e and g).

Changes in follicular size are presented in figure 12 (b, d, f and h). There was a significant induction occurred in follicular size in blackheaded munia subjected to both groups long day to short day length and short day to long day length sequentially transferred photoperiodic groups [LD to SD (March) $F_{9,36}$=2.651, P<0..974 (June) $F_{9,18}$=3.796, P<0.0077 (December) $F_{9,18}$=4.038, P<0.0057; 1-way RM ANOVA) except September month group ($F_{9,18}$=0.5567, P=0.8143)]. In short day to long day

length transferred group no significant change noticed during experiment period (March: $F_{9,18}$=2.012, P=0.0988, September: $F_{9,18}$=1.522, P=0.2140 and December: $F_{9,18}$=1.156, P<0.3774). But significant change observed in June month group ($F_{9,18}$=4.333, P<0.0040; 1-way RM ANOVA).

The molt cycle of both (body feathers and wing primaries) in female blackheaded munia followed gonadal growth-regression cycle. Complete molt cycles on the body and wing feathers occurred in both LD to SD and SD to LD transferred groups [LD to SD (March: body feathers, $F_{40,80}$=11.96, P<0.0001; wing primaries, $F_{40,80}$=50.07, P<0.0001, June: body feathers, $F_{40,80}$=1.146, P=0.2977; wing primaries, $F_{40,80}$=13.16, P<0.0001, September: body feathers, $F_{40,80}$=14.79, P<0.0001; wing primaries, $F_{40,80}$=19.10, P<0.0001 and December: body feathers, $F_{40,80}$=2.523, P<0.0002; wing primaries, $F_{40,80}$=8.389, P<0.0001) SD to LD (March: body feathers, $F_{40,80}$=13.26, P<0.0001; wing primaries, $F_{40,80}$=6.001, P<0.0001, June: body feathers, $F_{40,80}$=4.885, P<0.0001; wing primaries, $F_{40,80}$=7.053, P<0.0001, September: body feathers, $F_{40,80}$=15.94, P<0.0001; wing primaries, $F_{40,80}$=8.389, P<0.0001, and December: body feathers, $F_{40,80}$=9.508, P<0.0001; wing primaries, $F_{40,80}$=90.23, P<0.0001 1-way RM ANOVA)] (figure 13a- h).

Histological data of plate II revealed the data of testis volume. Photomicrograph A-D shows transverse sections of SD to LD December transfer group of blackheaded munia. Photomicrograph A showed the initial size of testis. Seminiferous tubules were narrow and lined by a single or double-layered spermatogonial cells. Tunica propria was thin and distinct. Tunica albuginea was fibrous and thick. The intertubular spaces were wide containing interstitial cells. Photomicrograph B shows to pre-breeding phase and size of testes just started to

increase. In photomicrograph C shows the breeding phase, seminiferous tubules are highly stretched due to maximum width, increase in population of dividing germinal cells during the breeding phase. Spermatogonial cells are present but sperm formation was unidentified. Photomicrograph D shows the narrow tubules, tunica propria is indistinct the bunches of spermatozoa are attached to cells of sertoli in seminiferous tubules. Spermatogenetic activity seems to be maximum with wide lumen. The interstitial tissue between adjacent tubules has become greatly compressed. Intertubular spaces are triangular in shape. In the photomicrograph of E-H shows testis histology of LD to SD December transfer groups starting of experiment, birds were in pre breeding phase. Their testes were in reduced form. In photomicrograph E, shows the highly reduced seminiferous tubules with complete regression of testis. Intertubular spaces were wider due to the complete reduction of seminiferous tubules. Tunica albuginea was thick and appeared clearly, In photomicrograph F, the gonadal size gradually increased and went to pre-breeding phase, photomicrograph G, testis are in breeding phase, Photomicrograph H testis are in post breeding phase seminiferous tubules become reduced, and inter tubular space increase (Plate II).

Besides gonadal response, body molt and molt primaries, the blood plasma proteins are an obvious choice for investigation of different photoperiodic response because it was easy to collect and because a great deal is known about their properties and functions. Plasma is the fluid portion of blood in which the blood cells are suspended. It is a complex mixture of proteins, carbohydrates, lipids, steroids, and free ions whose composition varies with sex, age, starvation and seasons, etc. (Moore, 1948; Clegg *et al.*, 1951; Vanstone *et al.*, 1955; Dessauer and Fox, 1956; Saito, 1957a), to test different photoperiodic stress factor, blood was

collected of March group in both (LD to SD and SD to LD transfer group) and a September (LD-SD transfer) group. Birds of March group showed pre-breeding phase. No any stress protein was observed, in LD-SD transfer group. The stress factors (130_{kDa} protein bands) were observed in male and female munia by LD-SD photoperiodic induction. While in month of september (post-breeding phase) 112-130_{kda} stress proteins bands were observed in changing photoperiods. This stress factor effects the whole body but eating disorders, skin and feather problems are a few of the outward symptoms of stress. When photoperiod was sequentially increased one hour every 30 day from short day to long day there was no stress proteins found (figure 14 and 15).

Discussion

The role of light as a major source of information is fully recognized in several long-term physiological processes in birds and other vertebrates. Seasonal reproduction in many birds is regulated by the annual cycle in day length (Murton and Westwood, 1977; Follett, 1984; Kumar, 1997). In long day species, long photoperiods of spring cause increased gonadotropin secretion and gonadal growth. This is followed by a period of refractoriness, and thereafter intervening periods of short day lengths restore the photosensitivity. Day length is used in temporal control of the annual reproductive cycle in many temperate birds (Farner and Follett, 1979; Follett, 1984; Nicholls *et al.*, 1988). In the tropics, many species which breed during spring and summer react to photoperiod in a similar fashion to temperate species (Thapliyal, 1981; Dittami and Gwinner, 1985; Tewary and Dixit, 1986). In many species, the breeding season ends with the development of a state of refractoriness which results in spontaneous gonadal collapse and loss of response to the stimulatory

effects of long day lengths (Farner *et al.*, 1983; Nicholls *et al.*, 1988). Once the birds are refractory to long day photostimulation, exposure for a time to short day lengths is required to render them photosensitive again (Burger, 1947; Hamner, 1964, 1968; Farner *et al.*, 1983).

In our study long day length to short day length transferred birds body mass was increased on day initial to 90 days. In this period testicular growth was initiated. But during maturation period body mass was decreased or maintained during the pre-breeding and breeding phase. So the results suggest that the body fattening is physiological associated events. In September month, the testicular growth and maturation process was initiated only short day length to long day length transferred group. But the long day length to short day length transferred group was unstimulated during the experimental period. These birds showed testicular recrudescence entire the period of experiment.

A long day response is believed to result from the interaction of long light pulse simultaneously with two different phases of the circadian rhythm of photoperiodic photosensitivity. It is assumed that the beginning of the day a long light pulse entrains CRPP, and later in the day when extends into the photosensitive of CRPP it stimulates the neuroendocrine circuitry resulting in the photoperiod-induced physiological responses (Follett, 1984; Kumar and Follett, 1993). So some pulses introduced at during initiation of the day and offset of light, respond as the long day.

The present data (figure 10 and 12) support that the March and September group of LD to SD and SD to LD transferred group, 16L:8D and 8L:16D evoke a long day response (gonadal growth, development and termination of testis). In June group birds were refractory; they showed the termination of breeding cycle. In

September group, after March month, slight gonadal growth started after 120 days (figure 10f). In December month group transferred under SD to LD condition, testis remain small and unstimulated during 180 days under short photoperiod (8L:16D). These results suggest that the rate of recovery of the responsivity to short day length to long day length photostimulation, in some case, is related to shortness of short days as well as the period of treatment with short days (Burger, 1947; Hamner, 1968; Turek, 1975; Nicholls and Storey, 1977; Farner *et al.*, 1983). Finding of Bhardwaj and Pandey (2015) in baya weaver birds reported that in post-breeding phase (August) under stimulatory photoperiodic condition (13L: 11D), testicular growth was noticed after 90 days. So, this clearly indicates that the birds were non-sensitive under stimulatory effects of these photoperiods and perceiving them as short days.

There are a few studies in which the duration- and/or intensity-dependent inductive effect of light have been investigated. In common Indian rose finches, testis growth under 3L: 3D was not different from that under 4L: 4D or 6L: 6D (Kumar *et al.*, 1983) which negates a relationship between the course of illumination and the degree of photostimulation. However, the absence of differential photostimulation in rose finches could be due to the fact that a 3h illumination at high intensity used in that study was well above the limit for duration of illumination of and intensity of light. Besides this, different light pulses of light duration/cycle in Japanese quail induced the photo-stimulation (rise in luteinizing hormone, LH, and/or testis growth) (Wilson and Siopes, 1976; Siopes and Wilson, 1980; Follett and Milette, 1982). These photoperiodic responses is regulated by the circadian system (Kumar and Follett, 1993), and that there can be a time course for magnitude of the response to light in components of the circadian timing system (suprachiamatic nuclei, SCN,

intereniculate leaflet, IGL, retina etc.; Rusak *et al.*, 1990; Rea, 1992; Teclemariam-Mesbah *et al.*, 1995). However the claim of time-dependent photoinductiveness needs to be further confirmed using rapid markers of photoperiodic responses (eg., rise in LH following single day photostimulation) since the testis growth response is the summation of the inductive effects of light related responses over a number of days (Lofts, 1975).

It appears that the subtropical blackheaded munia use photoperiodic strategy in regulation of its seasonal testicular responses similar to that is reported by some earlier finding on house sparrow (Trivedi, 2004; Anushi and Bhardwaj, 2006). Light sensitivity of the circadian activity rhythm faithfully predicts the photosensitive phase of the CRPP in photoperiodic species like house finch (Hamner and Enright, 1967), golden hamster (Elliott *et al.*, 1972) and black headed bunting (Kumar and Rani, 1999). In summary, photoperiodic transfer of short to long day length and long to short day length regulated by photoperiodic machinery of blackheaded munia birds.

Study 3: To determine the critical day length

We investigated the effect of different combination of photoperiods, during pre-breeding phase of testis growth and regression, along with other associated physiological changes reflected in body mass, molt of primary wing and body feathers in blackheaded munia. Birds (n = 70) were subjected to short day length (8L: 16D) in November. Experiment started in February 2016 during the pre-breeding phase for blackheaded munia. Birds were divided into seven groups (n = 6-8) were exposed to different photoperiods, group 1 to 9 exposed under increasing photoperiods (group 1- 9L:15D, group 2- 10L:14D, group 3- 11L:13D, group 4- 12L:12D, group 5- 13L:11D, group 6- 14L:10D, group 7- 15L: 9D and group 8- 16L:8D). During the light phase, all birds received 460 lux at perch level. Observations on body mass, moulting and gonadal size were taken at the beginning, weekly, and monthly intervals. The blood sample was collected during the beginning and end of experiment. The glucose, cholesterol and protein were estimated, groups 1 to group 4 serum of blood was pooled due to no response during the experiment and group 5 to group 9 proteins which administered photoperiodic response were individually analysed.

Introduction

In seasonally breeding animals, the breeding season is restricted to the optimum period for raising young, which varies widely to suit the ecological needs of each species. A critical day length (or threshold photoperiod) is referred to as the 'minimum' day light period that will induce a photoperiodic response to half maximum. In nature, the daily light period changes across seasons and so this study

will let us understand if this species has a photoperiodic switch to distinguish between short and long photoperiods. This study was done in an experiment, which explains role of photoperiod in the termination of breeding season. In most species of birds from temperate latitudes, in which reproduction usually beings sometime during spring, the breeding season ends by the development of a state of photorefractoriness which results in spontaneous gonadal collapse and loss of response to stimulatory day lengths; once the birds become photorefractory exposure for a time to short day lengths is necessary to render them photosensitive again (Murton and Westwood, 1977; Farner *et al.*, 1983; Follett, 1984; Stokkan and Sharp, 1984; Nicholls *et al.*, 1988). Similar results have been found in some spring/summer breeders that reproduce at high latitudes but overwinter in the tropics (Tewary and Kumar, 1982; Kumar and Tewary, 1983; Tewary and Tripathi, 1983).

The role of photoperiod and photorefractoriness is fully investigated in some birds. Examples are: blackheaded bunting, *Emberiza melanocephala* (Kumar and Tewary, 1984); brahminy myna, *Sturnus pagodarum* (Kumar and Kumar, 1991); canary, *Serinus canarius* (Storey *et al.*, 1980; Hurley *et al.*, 2008); castrated willow ptarmigan, *Lagopus lagopus lagopus* (Stokkan and Sharp, 1984); european starling, *Sturnus vulgaris* (Wieselthier and Tienhoven, 1970; Dawson and Goldsmith, 1983; Goldsmith and Nicholls, 1984; Nicholls *et al.*, 1984, 1988; Dawson, 1987, 1989, 1991, 2001; Dawson *et al.*, 1985, 1986; Falk and Gwinner 1988; Bentley *et al.*, 1997; Dawson and Sharp, 1998); equatorial bird, *Quelea quelea* (Lofts, 1962); house sparrow, *Passer domesticus* (Murton *et al.*, 1970; Hahn and Ball, 1995); house finch, *Carpodacus mexicanus* (Hamner, 1968); japanese quail, *Coturnix cotrunix japonica* (Robinson and Follett, 1982; Follett and Nicholls, 1984); redheaded bunting,

Emberiza bruniceps (Prasad and Tewary, 1982); siberian hamster, *Phodopus sungorus* (Michael and Zucker, 1995); spotted ant birds, *Hylophylax naevioides* (Beebe *et al.*, 2005); turkey hen, *Meleagril gallopavo* (Proudman and Siopes, 2002, 2004), white crowned sparrow, *Zonotrichia leucophrys gambelii* (Hahn *et al.*, 2008); white-throated sparrow, *Zonotrichia albicollis* (Turek *et al.*, 1980) Indian weaver bird (Chandola-Saklani *et al.*, 1983; Bhardwaj and Pandey, 2015; Pandey and Bhardwaj, 2015).

In the tropics, breeding seasons among birds are spread out over the entire year and correspond to existent favourable conditions, but breeding cycle of some species reported seasonally (Chandola-Saklani *et al.*, 1983). A number of species breed during spring and summer, and some of them react to photoperiods similar to those of temperate species (Epple *et al.*, 1972; Lewis *et al.*, 1974; Thapliyal, 1981; Gwinner and Dittami, 1984; Dittami and Gwinner, 1985; Tewary and Dixit, 1986). However, the mechanisms controlling the termination of breeding season in these photosensitive species are divergent and remain controversial. While photorefractoriness is lacking in some long day breeders (Thapliyal and Saxena, 1964a; Miller, 1965; Epple *et al.*, 1972; Lewis *et al.*, 1974), but in some birds spontaneous gonadal regression are reported under continuous long day lengths (Tewary and Dixit, 1986).

Photorefractoriness is characterized by the spontaneous gonadal collapse and loss of response to the long day lengths. At this time, there is complete cessation of steroidogenesis, as evident by the presence of degenerated leydig cells (Lam and Farner, 1976; Kumar, 1981; Rohss and Silverin, 1983) and absolutely minimal (almost non-existent) circulating levels of gonadal steroid (Temple, 1974; Lincoln *et*

al., 1980; Dawson, 1983). In order to regain responsivity, the refractory birds should be subjected for a time to short day lengths prior to long day photostimulation (Lofts and Murton, 1968; Murton and Westwood, 1977; Farner and Follett, 1979; Follett, 1984). As a consequence of short day exposure, potentially functional leydig cells reappear (Storey, 1979) and begin producing a very small quantity of gonadal steroids which apparently suppress the secretion of gonadotropins and the subsequent gonadal growth under short day lengths.

Thus photo periodically controlled reproductive cycles in seasonally breeding species can be classified into three main categories. In the first category are the birds like wood pigeon (*Columba palumbus*) lacking refractoriness in which reproduction is symmetrical with respect to the photoperiodic changes : gonads begin to develop in May (day length = 12-13hrs) and remain active until October when critical day length is passed again (Murton and Westwood, 1977). In the second category are species having short breeding season in which gonadal regression begins after summer solstice while day lengths are still longer than the inductive photoperiods-these species are called as absolutely photorefractory (Wilson and Follett, 1974; Storey, 1979; Nicholls *et al.,* 1988). Third category includes species which exhibit only a relative form of photorefractoriness in which gonads of sexually mature birds under long photoperiods regress on transfer to a relatively shorter photoperiod (18L:6D to 13L:11D) although latter is itself photostimulatory.

Methods

Birds were subjected to short day length (8L: 16D) in the last week of November 2015. Experiment started in first week of February 2016 during the pre-breeding phase of blackheaded munia. Photosensitive birds (n = 70) were divided into

seven groups (n = 6-8 each/group) and exposed to different photoperiods (group 1- 9L:15D, group 2- 10L:14D, group 3- 11L:13D, group 4- 12L:12D, group 5- 13L:11D, group 6- 14L:10D, group 7- 15L: 9D and group 8- 16L:8D) (figure 16). During the light phase, all birds received 460 lux at perch level. Observations on body mass, moulting and gonadal size were taken at the beginning, weekly, monthly and also at appropriate intervals. The blood samples were collected during the beginning and the end of experiment in all groups. The glucose, cholesterol and protein were estimated, groups 1 to 4 serums of blood during the experiment and group 5 to 8 proteins which administered photoperiodic response were individually analysed. The SDS-PAGE was performed initially and in responded groups.

Results

Results from this experiment have shown the mean body mass gradually increased and decreased throughout the experiment (figure 17a, c). There was significant change in the male body mass among all the groups (1- way RM ANOVA: group 1, $F_{6,18}$=0.4887, P=0.8087; group 2, $F_{6,18=}$1.689, P=0.1811; group 3, $F_{6,18}$=2.901, P=0.0370; group 4, $F_{6,18}$=2.445, P=0.0661; group 5, $F_{6,18}$=1.241, P=0.3322; group 6, $F_{6,18}$=2.405, P=0.0696; group 7, $F_{6,18}$=6.952, P<0.0006 and group 8, $F_{6,18}$=6.952, P <0.0006). The testis underwent growth and regressed throughout the experiment. There was a significant change in the testis volume of all the groups (1-way RM ANOVA: group 1, $F_{3,12}$=1.005, P<0.4241; group 2, $F_{3,12}$=0.1047, P=0.9557; group 3, $F_{3,12}$=4.8121, P<0.0200; group 4, $F_{3,12}$=0.007599, P<0.9990; group 5, $F_{3,12}$=2.610, P=0.0996 group 6, $F_{3,12}$=31.79, P <0.0001), group 7, $F_{3,12}$=26.90, P <0.0001), and group 8, $F_{3,12}$=26.90, P <0.0001) (figure 17b and d).

The molt cycle of body feathers and wing primaries in male blackheaded munia followed testicular cycle. Complete molt cycles of the feathers occurred in 16L, 15L, 14L, and 13L group (16L group: body feathers, $F_{16,112}$=53.37, P<0.0001; wing primaries, $F_{16,112}$=35.79, P<0.0001, 15L group: body feathers, $F_{16,112}$=18.43, P<0.0001; wing primaries, $F_{16,112}$=52.19, P<0.0001, 14L group: body feathers, $F_{16,112}$=5.973, P<0.0001; wing primaries, $F_{16,112}$=27.13, P<0.0001, 13L group body feathers, $F_{16,112}$=8.245, P<0.0001; wing primaries, $F_{16,112}$=49.63, P<0.0001; 1-way RM ANOVA) (figure 18a and c). Molting pattern of body feathers also showed the similar pattern in 12L, 11L, 10L, and 9L group (12L group: body feathers, $F_{16,112}$=4.267, P<0.0001; wing primaries, $F_{16,112}$=15.40, P<0.0001, 11L group: body feathers, $F_{16,112}$=3.621, P<0.0001; wing primaries, $F_{16,112}$=13.12, P<0.0001, 10L group: body feathers, $F_{16,112}$=4.244, P<0.0001; wing primaries, $F_{16,112}$=26.62, P<0.0001, 9L group: body feathers, $F_{16,112}$=0.7783, P=0.7068; wing primaries, $F_{16,112}$=8.994, P<0.0001; 1-way RM ANOVA) (figure 18b and d).

Body mass in female birds of all the groups was not significantly increased during the experiment (group 1: $F_{6,12}$=1.982, P =0.1476; group 2: $F_{6,12=}$1.810, P=1.675; group 3: $F_{6,12}$=1.121, P=0.4062; group 4: $F_{6,12}$=1.625, P=0.2232; group 5: $F_{6,12}$=1.055, P=0.4390; group 6: $F_{6,12}$=2.245, P=0.1099; group 7: $F_{6,12}$=3.861, P-=0.222 and group 8: $F_{6,12}$=5.749, P <0.0050; 1- way RM ANOVA) (figure 19a and c). Changes in follicular diameter are shown in figure (19b and d). The follicles underwent growth and regressed throughout the experiment. There was a significant change in the follicular diameter of all the groups (group 1: $F_{3,6}$=1.671, P =0.2711; group 2: $F_{3,6}$=2.399, P=0.1665; group 3: $F_{3,6}$=6.512, P<0.0257; group 4: $F_{3,6}$=6.615, P=0.0249; group 5: $F_{3,6}$=8.614, P<0.0136; group 6: $F_{3,6}$=3.520, P=0.0887; group 7:

$F_{3,6}$=24.58, P<0.0009 and group 8: $F_{3,6}$=3.797, P <0.0773; 1-way RM ANOVA) (figure 19b and d).

The molt cycle of body feathers and wing primaries in female blackheaded munia did not follow the cycle of follicular growth. The significant changes in molt cycles of the feathers occurred only under 16L and 10L groups ($F_{16,48}$=20.95, P<0.0001; $F_{16,48}$=4.929, P<0.0001; 1-way RM ANOVA). But the feather regeneration was not significant in 15L, 14L, 13L, 12L, 11L, and 9L group (15L: $F_{16,48}$=0.9648, P=0.5133, 14L: $F_{16,48}$=0.3900, P=0.9755, 13L: $F_{16,48}$=2.529, P=0.0121, 12L: $F_{16,48}$=0.8975, P=0.5781, 11L: $F_{16,48}$=0.6205, P=0.8514; and 9L: $F_{16,48}$=0.7607, P=0.7144; 1-way RM ANOVA) (figure 20a and c). But the wing primaries regeneration cycle change significantly in all groups 16L,15L, 14L, 13L, 12L, 11L, and 10L groups (16L: $F_{16,48}$=63.47, P<0.0001, 15L: $F_{16,48}$=17.14, P<0.0001, 14L: $F_{16,48}$=5.285, P<0.0001, 13L: $F_{16,48}$=13.18, P<0.0001, 12L: $F_{16,48}$=26.37, P<0.0001; 11L: $F_{16,48}$=4.246, P<0.0001 and 10L: F16,48=7.716, P<0.0001; 1-way RM ANOVA) except 9L group ($F_{16,48}$=1.773, P=0.082; 1-way RM ANOVA) (figure 20b and d).

Initially, in each group, gonads of all birds were reduced. Seminiferous tubules were narrow and lined by a single or double-layered spermatogonial cells. Tunica propria was thin and distinct. Tunica albuginea was fibrous and thick. The Intertubular spaces were wide containing interstitial cells but after 90 days, at the end of experiment result observed that were histological data of the critical day length in plate III and IV. In the photomicrograph of plate III has given the detail of testis. The photomicrograph of group 9L, 10L, 11L, and 12L did not show the mature seminiferous tubules and intertubular spaces also showed the enough spaces. But in the groups 13L, 14L, 15L and 16L testis size showed breeding phase, seminiferous

tubules were highly stretched due to maximum width, increase in population of dividing germinal cells during the breeding phase. Spermatogonial cells were present; 13L photomicrograph shows testicular size changed, become much distinct. Tubules narrow and tunica propria were indistinct, bunches of spermatozoa attached to cells of sertoli in seminiferous tubules but sperms were not much active; In 14L, and 15L group photomicrograph showed the maximum spermatogenetic activity in wide lumen. The interstitial tissues between adjacent tubules become greatly compressed. Intertubular spaces were in triangular shape. In 16L group Photomicrograph attain the peak of reproductive activity. Bunches of spermatozoa were present in wide lumen. Intertubular space much reduced, and was confined to only triangular areas (Plate III).

In plate IV given the detail of female gonads (ovaries). Photomicrograph of group 9L, 10L, 11L, and 12L did not show the any mature secondary follicles, in 12L group developing oocytes was present, two or more follicles were in same size and the germinal epithelium layer was very thin, but 13L, 14L, 15L and 16L groups showed breeding phase. In 13L photoperiodic group showed changed follicular size, become much distinct graffian follicle, the germinal epithelium become thick; In 14L, and 15L groups Photomicrograph showed the maximum follicular size, theca interna, theca externa and antrum of graffian follicle were clearly visible.

In month of February all eight groups of birds were exposed different photoperiods. The pooled plasma of birds (male and female) of 9L and 10L photoperiodic group was poured in P1 column of gel. While in column P2 plasma of 11L and12L photoperiodic birds; in column P13, P14, P15 and P16 result of group 13L, 14L, 15L and 16L photoperiodic group has given respectively. By the SDS-PAGE result was concluded, that in column P1 and P2 no any stress proteins were

found because of their short photoperiod. In P13, P14, P15 and P16 column (130kDa protein band) were found in male and female blackheaded munia (figure 21).

Discussion

The present results support the previous findings on photoperiodic control of annual reproductive cycle (Thapliyal, 1981; Thapliyal and Gupta, 1989; Kumar, 1997) by showing the modulating effects of light intensity on photoperiod- induced testicular cycle in blackheaded munia. The brighter the light, the faster is the testicular recrudescence and the earlier the regression under a stimulatory long photoperiod (Kumar, 1997; Nicholls *et al.,* 1988; Bhardwaj and Pandey, 2015). However, light intensity could not modulate the duration of the photoperiods. This is evidenced by the comparison of the response under blackheaded munia exposed to different stimulatory photoperiods. Whereas birds exposed to 13L, 14L and 15L photoperiod exhibited comparable responses at 460 lux light intensity, they did not respond to the lower photoperiod when exposed to 11L and 12L photoperiods (figure 17 and 19b and d). Also, testicular response to 14L and 15L was half-maximal at similar light intensity (figure 17 and 19b and d), and 2/5 birds did show initiation of testicular recrudescence under 14L photoperiod 460 lux light intensity. These results are similar to those reported in the Japanese quail (*Coturnix c. japonica*; Follett and Maung, 1978), white-crowned sparrow (*Zonotrichia leucophrys gambelii*; Morton *et al.,* 1985), European starling (Bentley *et al.,* 1998), migratory blackheaded bunting (Misra *et al.,* 2004) and redheaded bunting (Rani *et al.,* 2005). In all these species, there was a photoperiod-dependent, intensity dependent in the blackheaded and redheaded buntings in photoperiod induction of the seasonal responses. In European starlings, the testicular recrudescence–regression under long days was found to be

clearly dependent on light intensities (3, 13, 45 and 108 lux) and photoperiod (11 L, 13 L, 16 L and 18 L) (Bentley *et al.*, 1998).

Consistent with testis response, the molt pattern also exhibited photoperiodic dependent, but with a greater inter-individual difference between longer photoperiods (figures 18 & 20). This was not surprising since, compared to gonadal growth–involution cycle, the periodicity in molt is variable and more scattered (hence less synchronized), especially in the laboratory environment of light–dark cycles, as reported in tropical stonechats (*Saxicola torquata axillaris*; Gwinner and Scheuerlein 1998) and subtropical spotted munia (*Lonchura punctulata*; Budki *et al.*, 2012, 2014). It may be noted that, between stonechats and spotted munia, the former exhibits a much tighter circannual molt cycle than the latter (Budki *et al.*, 2012). There could also be species-specific differences in the exhibition of annual cycles and its synchronization to the environmental photoperiod. For example, white-crowned sparrows (*Zonotrichia leucophrys*) show a circannual testicular cycle, but not with a corresponding post-nuptial wing primary molt under 12L: 12D (Farner *et al.*, 1983). In general, photoperiodic drive refers to the degree of switching-on of reproductive function (egg production) by light, whereas photorefractoriness refers to the degree of switching-off of reproductive responses to light. These have been addressed for the domestic hen (Sharp, 1993) and other avian species (Sharp, 1996). Photorefractoriness is associated with gonadal regression and subsequent molt and remains a major mechanism limiting the continuous production of semen and eggs in birds. Both photoperiodic drive and photorefractoriness are activated at the start of exposure of photosensitive birds to photoperiods long enough to induce gonadal

development (Nicholls *et al.*, 1988; Wilson and Reinert, 1993, 1996; Reinert and Wilson, 1996).

An important observation of this experiment is the difference in response between body mass and gonadal growth of male and female blackheaded munia, as has been argued earlier in other species (Kumar, 1997, 1988). Body mass of all groups have similar response but the induced testis recrudescence was noticed in only 13L: 11D, 14L: 10D, 15L: 9D and 16L: 8D response (figure 17 and 19b and d). Also, the increase in testis size was relatively similar in 14L: 10D and 15L: 9D but relatively small under 16L:8D. An increase in body mass under different photoperiod, which were otherwise non-inductive or weakly inductive in terms of gonadal growth response, was consistent with the idea that birds gain in body mass prior to gonadal growth in order to support the activities associated with reproduction. But in some migratory species, the blackheaded bunting (*Emberiza melanocephala*), the increase in body mass precedes testicular recrudescence. In the wild, blackheaded munia begins to recrudescence their testes in March/April when day length is ~ 12.5 h, and show full gonadal development in June/ July when day length is ~ 14 h. induction in body mass could be independent of the reproductive response to a photoperiod in a non-migratory species. In summary, longer photoperiod influences the photoperiod induced response cycle but does not alter the critical photoperiod for induction in the subtropical blackheaded munia.