J Endocrinol Reprod 18 (2014) 2 : 87-94 ***Original Research***

Photic stress modulates cholesterol content and oxidative load of steroidogenic organs (adrenal, ovary and testis) of Indian spotted finch *Lonchura punctulata*

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Summary

Steroidogenic organs such as adrenal gland and gonad are of vital importance in birds, as the adrenal gland regulates stress by modulating the levels of corticosterone, while gonads synthesize gonadal steroids that regulate breeding cycle. Cholesterol content in both adrenal and gonad reflect the physiological status of these organs for stress management and reproduction, respectively. Birds, being photosensitive, experience phototoxicity in the nature due to the rapid urbanization. If photic stress induced by constant dark (DD) and constant light (LL) influences steroidogenesis in terms of cholesterol content and total antioxidant status (TAS%) as a marker of stress was experimentally assessed in Indian spotted finch *Lonchura punctulata* along with measurement of peripheral corticosterone levels. DD was stressful and, hence, caused decrease of ovary / testis weight and increase of adrenal gland weight, while cholesterol content decreased significantly due to inhibition of steroidogenesis. LL stimulated gonadal function but caused decrease of cholesterol content suggesting active steroidogenesis. TAS% increased under DD and decreased under LL. The phototoxicity, whether in term of DD or LL, was stressful to Indian spotted finch as evident from increased adrenal gland weight and decreased cholesterol content that can be correlated with increased synthesis of corticosterone. It may be concluded that cholesterol content is an important marker of streroidogenic status, and extremes of photoperiod influence cholesterol content, corticosterone level and TAS%.

Key words: Cholesterol, Corticosterone, Photic-stress, Spotted-finch, Steroidogenic-organs, TAS%.

Introduction

Cholesterol is an integral component of every cell and accounts for the integrity of cellular membrane as well as for metabolic activities of cell such as in facilitating cell signalling (Norris, 2013). Further, cholesterol synthesis and content are tissue-specific. Cholesterol is an important aspect of the reproductive system, not only for the structure and functioning of the cell membrane but also for steroidogenesis (Heard et al., 1956). Adrenal gland contains considerable amount of cholesterol, which is needed for glucocorticoid secretion and stress management; hence, cholesterol is of vital importance for survival of living beings (Sharaw et al., 1979). Light pollution is known to affect important biological functions, including daily and annual cycles, reproductive status and stress regulation of wild animals (Dominoni et al., 2013). Being photosensitive, birds are highly susceptible to different kinds of environmental stresses, the most important one being photoperiod (Niekamp et al., 2007) or phototoxicity caused due to urban development which has brought the need for artificial lighting of roadways, shopping centres, stadiums and homes. Photic disturbances that alter the natural light cycle may produce elevated physiological and behavioral effects in birds (Navara & Nelson, 2007). Further, measurement of cholesterol content and level of oxidative stress appear to be important tools for deep understanding of the functional profile of adrenal gland and gonad since cholesterol content would account for corticosterone level which in turn would lead to increased oxidative load (Pravosudov et al., 2002). Cholesterol content in ovary and testis signify different functional profiles on account of their physiological importance in modulation of reproduction (Singh & Singh, 1979) in relation to seasonal photoperiodic changes at least in birds. Differential response of gonad and adrenal gland in terms of cholesterol content and total antioxidant status (TAS%) to photic stress induced by constant dark (DD)/ constant light conditions (LL), in birds is not yet clearly known. Therefore, we estimated cholesterol content of

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adrenal gland and gonads, the total antioxidant status (TAS%) and the circulatory level of corticosterone as indirect markers of oxidative stress, in the common finch *Lonchura punctulata* under conditions of constant dark (DD) / constant light (LL).

Materials and methods

Maintenance of animals

All the experiments were performed in accordance with institutional animal ethics practices and within the framework of revised Animal Protection Amendment Act of 2007 of Government of India. Indian spotted finch *Lonchura punctulata* are abundant on cultivated fields in the vicinity of Varanasi (Lat. 25°18'N; Long. 83°01'E). They were collected during first week of every month and acclimated to laboratory conditions for 14 days in an open-air aviary. The birds were fed with millet seeds (*Pennisetum typhoides*) along with seasonally available grains and water *ad-libitum*. Laparotomy was performed for idntifying males and females as *L. punctulata* does not exhibit sexual dimorphism.

Experimental groups and sample collection

Five adult birds of each sex weighing 13-14 g were kept in wire-net cages in photo-chambers fitted with digital automatic incandescent light-regulating unit in which the intensity of light was the same as environmental light (app. 300 Lux) and served as control. A similar number of birds of both sexes were kept in LL (constant light with incandescent bulb) and DD (constant dark with dim red light to help them in feeding) conditions in respective photo-chambers. After a fortnight of photoperiodic treatment their body weight was recorded, the birds were sacrificed under deep anaesthesia and thoracic blood was collected in heparinized tubes. The adrenal, ovary and testis were dissected out, wiped on filter paper, weighed in an electrical balance (Sartorius) and then processed for histological observation. Plasma was separated out by centrifugation of blood at 3000 rpm and stored at -20°C for assay of corticosterone.

To study the annual variation in adrenal and gonadal activities, five birds of each sex were sacrificed by decapitation between 10.00 h and 11.00 h in the second week of every month starting from August of the first year to July of the subsequent year. Meteorological data, such as the day length, ambient temperature and relative humidity, were obtained from the Department of Geophysics, Banaras Hindu University, Varanasi.

Histology

The adrenal, testis and ovary were fixed in Bouin's fluid and processed for dehydration followed by paraffin infiltration. Sections of 5 μm thickness obtained in a microtome (Leica Microsystem Inc., USA) were spread on glass slides and stained with hematoxylin and eosin, observed in a microscope (Leica MPV-3, Germany) and appropriate fields were photographed.

Cholesterol content

The cholesterol content of the adrenal gland was determined adopting the method of Sackett (1925) which is based on Liebermann-Burchard reaction. Cholesterol reacts with acetic anhydride-H_2SO_4 mixture (20:1) and produces a green-colored complex. This color was read colorimetrically at 680 nm. Standard cholesterol (stock solution-0.01 mg/ml) was prepared in chloroform. Chloroform was used as the blank. Adrenal, ovary and testis were homogenized in ethanol-ether mixture (3:1), and centrifuged. The supernatant was evaporated to dryness, reconstituted in 5 ml of chloroform and processed according to the standard protocol. The standard curve of cholesterol was prepared by plotting absorbance against concentration at 680 nm.

Determination of total antioxidant status (TAS %)

The total antioxidant statuses of adrenal, testis and ovary were evaluated by ABTS assay following the method of Rice-Evans et al. (1994) with slight modification. The free radical scavenging activity of the antioxidant for ABTS radical cation was measured according to the method of Re et al. (1999). A stock solution of ABTS radical cation was prepared by mixing 5 ml of 7 nM potassium persulfate, and kept in dark at room temperature for 16 h. The stock solution of ABTS radical cations was then diluted with distilled water till its absorbance reached 0.7 at 734nm. ABTS radical cation was generated by oxidation of ABTS with potassium persulfate when 2.95 ml of ABTS radical cation solution was mixed with 50 µl of 10% tissue homogenate and the decrease in absorbance was monitored at 734 nm for 60 min at 6 min intervals. The activity was compared with the ABTS radical cation solution using ascorbic acid as the standard. Appropriate solvent blanks were run with each assay. Total antioxidant capacity (TAS%) was expressed as percent inhibition of bleaching in relation to aqueous control following the equation: Inhibition of A734 (%) = (1-Af/Ao) × 100 (where, Ao = absorbance of uninhibited radical cation and Af = absorbance measured 6 minutes after addition of the test sample).

Enzyme-linked immunosorbent assay (ELISA) of corticosterone

Corticosterone was measured using an ELISA kit (Immuno-Technology & Steroid Laboratory, NIHFW, New Delhi, India) according to the manufacturer's instructions. Plasma samples were thawed and applied to microplate. Following incubation, the plates were read in the microplate reader at 405 nm and the values were arrived at as described elsewhere (Yadav and Haldar, 2014).

Statistical analysis

Statistical analysis of the data was performed adopting one-way ANOVA followed by Student Newman-Keul's multiple range tests. The results are expressed as mean ± SEM. The differences were considered significant when pd"0.05 and highly significant when p<0.01.

Results

Annual changes in meteriological parameters

The Indian subcontinent lies in the tropical zone where annual variation of day length shows difference with respect to long days of summer (April-June) and short days of winter (November-January). A markedly high difference in humidity (relative humidity) was recorded during the monsoon seasons (July-September) followed by heavy rainfall. Significantly high temperature was recorded during the summer months (April-June) compared to winter months (December-January) (Fig. 1).

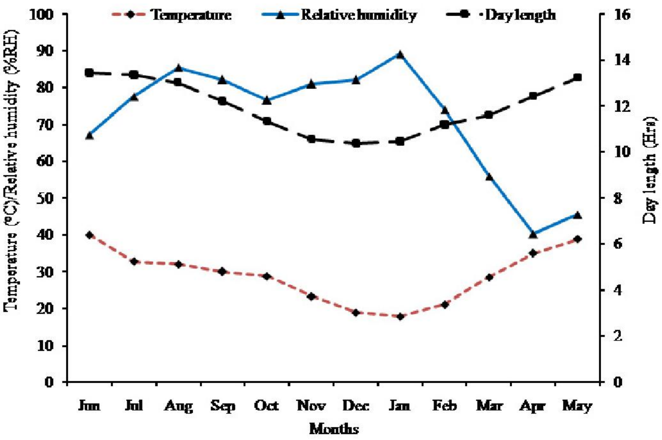

Fig.1: Annual variation in biometeriological parameters viz., temp (°C), relative humidity (%), and day length (hr). Vertical bar on each histogram represents standard error of mean.

Annual variation in body-, adrenal-, testis- and ovary weight

The body weight of *L. punctulata* did not vary much through the year except for a slight increase during August–September, which period coincided with the reproductively active phase of these birds. Adrenal gland weight showed fluctuation throughout year which can be correlated with the environmental stress and reproductive activity. Adrenal gland weighed the highest during December-January, which might be due to the winter stress to the birds and another peak of adrenal weight was recorded during the onset of reproductive activity (June) and offset of reproductive activity before winter (October, Fig. 2). The testis weight and testis volume as well as ovary weight showed only one peak. Testis and ovary weights increased from June onwards and the maximum weight was recorded during September. The testis volume matched the trend in testis weight. Decrease in testis weight, testis volume and ovary weight began in November and continued till May (Fig. 3).

Histological observation of adrenal, testis and ovary under constant dark (DD) / light (LL) conditions.

The control birds showed the normal dispersed adrenal cortex and medulla tissue while under DD and LL condition the adrenal cortical tissue regressed when

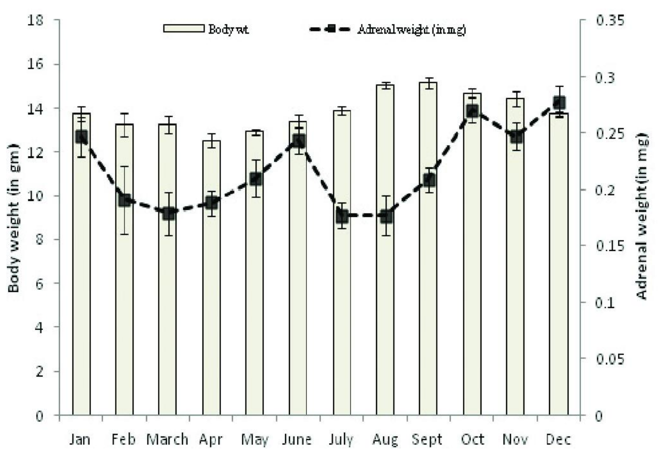

Fig.2: Annual variation in body weight (g) and adrenal weight (mg) of *L. punctulata*. Histogram represents Mean ± SEM (N= 5). Vertical bar on each histogram represents standard error of mean.

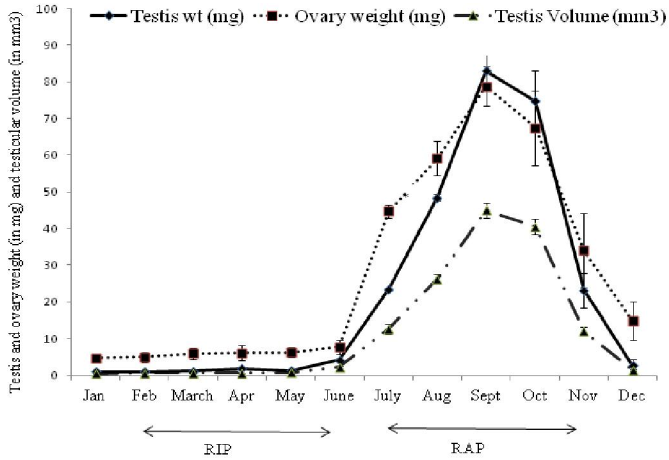

Fig. 3: Annual variation in weight of testis and ovary (mg) and testicular volume (mm^3) of *L. punctulata*. Histogram represents Mean ± SEM (N= 5). RIP stands for reproductively inactive phase and RAP for reproductively active phase. Vertical bar on each histogram represents standard error of mean.

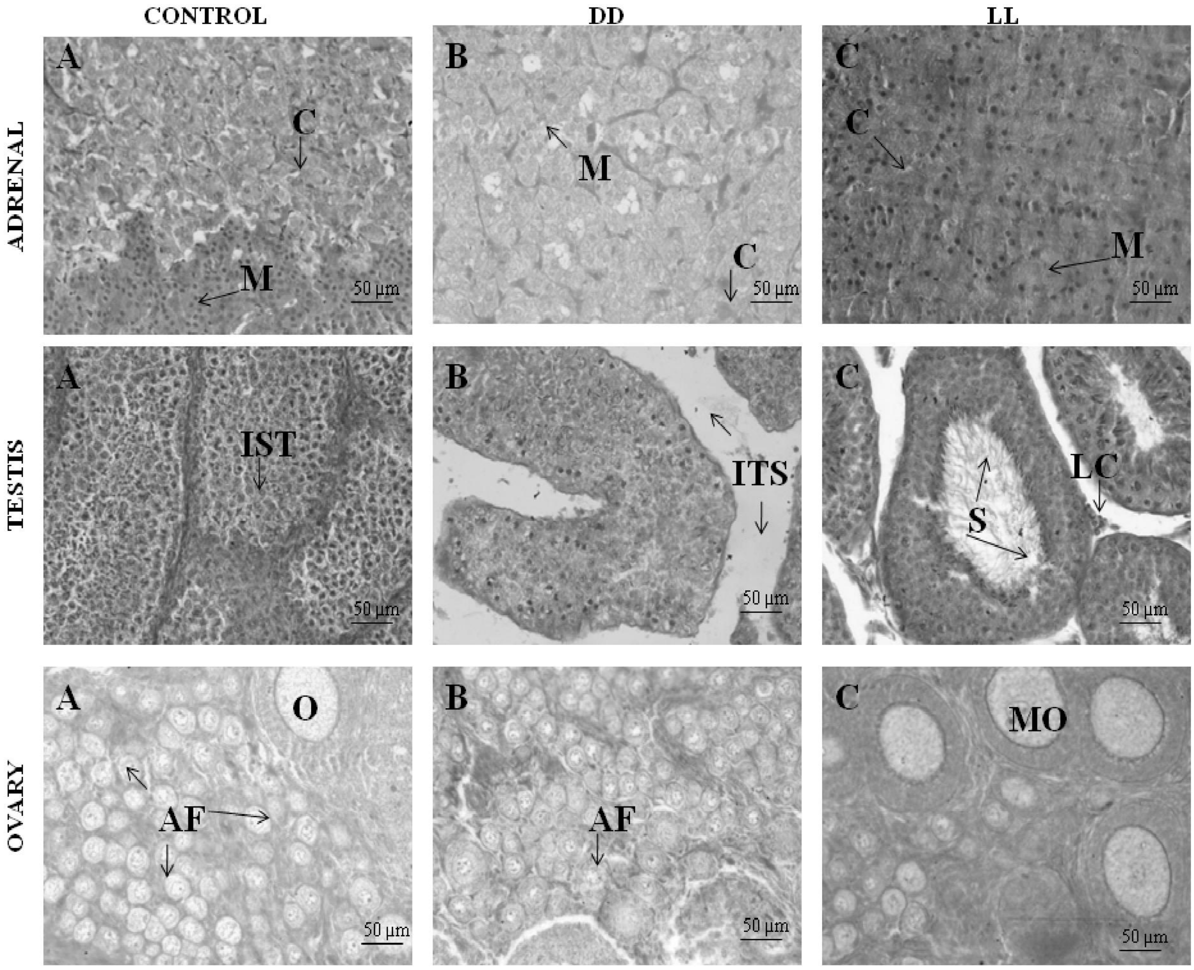

Fig.4: Photomicrograph showing transverse sections of adrenal, testis and ovary under control, DD and LL conditions. (A) Histoarchitecture under control condition. (B) Histoarchitecture under DD condition. (C) Histoarchitecture under LL condition. (M, medulla; C, cortex; IST, inactive seminiferous tubule; ITS, intertubular space; S, spermatozoa; LC, Leydig cell; O, oocyte; AF, atretic follicle; MO, mature follicle). Scale Bar = 50 µm

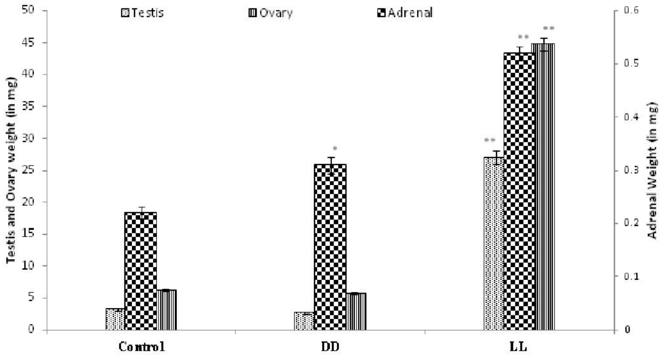

Fig. 5: Variation in weight of adrenal gland, testis and ovary (in mg) of birds *L. punctulata* exposed to constant dark (DD) / light (LL) conditions. Histogram represents Mean ± SEM (N= 5). Vertical bar on each histogram represents standard error of mean. Significance of difference *p<0.05 and **p<0.01 control vs. DD and LL.

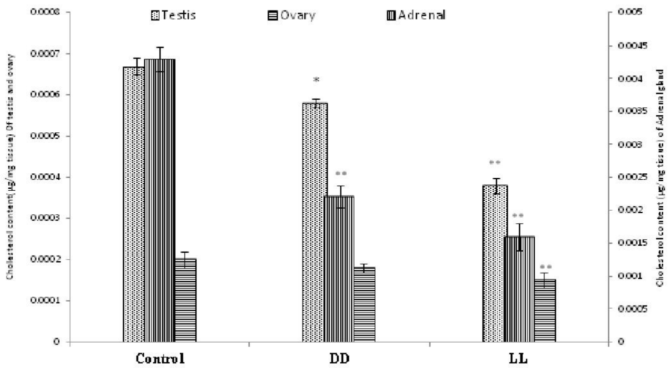

Fig. 6: Cholesterol content in adrenal gland, testis and ovary of *L. punctulata* exposed to constant dark (DD) / light (LL) conditions. Histogram represents Mean ± SEM, (N= 5). Vertical bar on each histogram represents standard error of mean. Significance of difference *p<0.05 and **p<0.01 control vs. DD and LL.

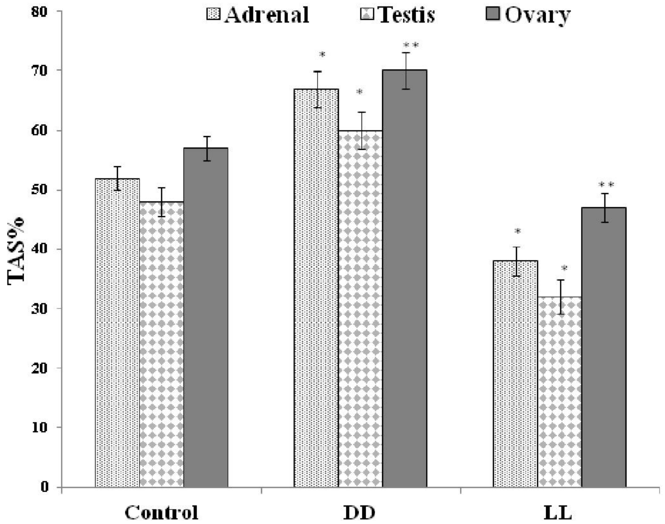

Fig. 7: Total antioxidant status (TAS %) of adrenal gland, testis and ovary of birds exposed to constant dark (DD) / light (LL) conditions. Histogram represents Mean ± SEM (N=5). Vertical bar on each histogram represents standard error of mean. Significance of difference *p<0.05 and **p<0.01 control vs. DD and LL.

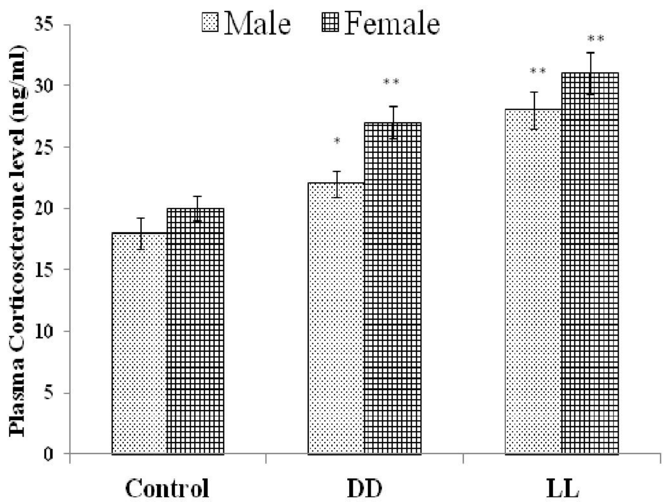

Fig. 8: Plasma corticosterone level of birds exposed to constant dark (DD) / light (LL) conditions. Histogram represent Mean ± SEM (N=5). Vertical bar on each histogram represents standard error of mean. Significance of difference *p<0.05 and **p<0.01 control vs. DD and LL.

the change in the medulla was very prominent. The testis of control birds did not show spermatogenic activity beyond round cells. Exposure to DD caused decrease of diameter of seminiferous tubules and the intertubular spaces were increased. LL condition caused stimulation of spermatogenesis resulting in large number of spermatozoa and active Leydig cells. The ovary of control birds showed total inactivity. Under DD there were a large number of atretic follicles. LL condition induced folliculogenesis in ovary, and there were many mature oocytes (Fig. 4).

Weight and cholesterol content of adrenal gland, testis and ovary under constant dark (DD) / light (LL) conditions

The weight of adrenal gland, testis and ovary showed moderately higher values under various experimental conditions as compared to control (Fig. 5). Cholesterol content (cc) of control adrenal gland was moderately high as compared to cc of adrenal gland of birds kept in conditions of constant light (LL) which in turn was remarkably high as compared to that under constant dark (DD). Cc of ovary was lowest in LL than DD and control groups. But the ratio of this fluctuation was not parallel to the cc as was observed in adrenal gland (Fig. 6). In testis, the cholesterol content (cc) in control group was significantly higher as compared to that of DD groups but was lowest in LL group birds (Fig. 6).

Total antioxidant status (TAS %) of adrenal gland, testis and ovary under constant dark (DD)/ light (LL) conditions

The total antioxidant status (TAS%) of adrenal gland, testis and ovary were high in birds kept under constant dark condition (DD) as compared to control. On the other hand the TAS% values were significantly low in case of LL groups as compared to control birds (Fig.7).

Corticosterone level under constant dark (DD) / light (LL) conditions

Eposure to DD/LL conditions produced significant increase in the coticosterone level of the birds. Here, LL condition caused much higher level of corticosterone than in DD-exposed birds (Fig. 8).

Discussion

Changes in the tropical environmental factors could be significantly different, i.e., drastic changes in humidity due to monsoon rainfall and high temperature in summer rather than day length of summer and winter as observed in the temperate zone. For these diurnal birds, the major food sources are grains and, hence, they visit wheat or paddy fields. Thus, their foraging reduces both shelter and food resources and, hence, *L. punctulata* face stress in nature. Consequently adrenal gland weight increases significantly during those days and in months of winter (short days with winter stress).

Steroid hormones are crucial for the proper reproduction and general physiology wherein the hormones mediate a wide variety of vital functions. Steroids are synthesized and secreted by endocrine glands such as the adrenal cortex and gonads (ovary and testis) for which cholesterol acts as the precursor, and its content in the gland is therefore an indicator of reproductive status. The environmental conditions during December to June caused reduction in gonadal activity of *L. punctulata* but adrenal activity was increased. Sudden decline in adrenal activity in July coincided with peak gonadal activity in summer months (July-October), suggesting a probable involvement of environmental factors, long photoperiod and high temperature, as stimulators of gonadal function. During the breeding phase of birds (July-October) adrenal activity was low, and gonad reflected sensitivity to long days and high temperature suggesting that spotted finch are long day breeders.

The abundant literature suggest photoperiodic influence on gonadal function of birds, but the role of photic stimuli in modulation of adrenal activity has received little attention (Sudhakumari, 1995; Sudhakumari and Haldar, 2001, Yadav and Haldar, 2014). Our observation suggests that this finch is certainly photoperiod-dependent, and along with gonadal function the adrenal gland is also influenced by photoperiod which would cause environmental stress and affect gonadal steroids. Light exposure can also produce adverse eûects indirectly through promotion of oxidative stress. To establish this, we conducted experiment by exposing *L. punctulata* to extreme photoperiodic conditions such as constant light (LL) and constant dark (DD) that acts as photic-stress. The experiment was performed during reproductively inactive phase when gonads were in inactive phase and adrenal was in active condition.

Annual body weight cycle of *L. punctulata* showed increase from August-October due to the availability of ample of food grains and insect larvae till the early monsoon. In the present study we proposed to correlate the gonadal and adrenal gland function as both are steroidogenic in nature. We, therefore, measured cholesterol content as a marker of steroidogenic function. We observed that during moderately short photoperiod (October-January) gonadal function decreased from recrudescent to completely inactive. From February to June, when day length increased wherein it was longest in June, the adrenal activity significantly decreased with increased gonadal activity. As soon as monsoon commenced (July/August) adrenal function decreased and gonadal function increased (September-October). But at the end of this phase (September onwards) adrenal activity started to increase and reached the peak and, hence, regression of reproduction. Thus, our results show that the adrenal has a direct relationship with testicular and ovarian functions during certain period of the year as noted in other birds (Sudhakumari and Haldar, 2001). This clearly establishes an inverse functional relationship between gonad and adrenal gland of *L. punctulata*.

We found that the gonadal activity decreased following exposure to DD while LL increased testicular and ovarian functions significantly. Interestingly under LL conditions adrenal weight and corticosterone level were also high which might be due to the photic stress. Long day (LL) exposure increased gonadal function which was evident from the cholesterol content as marker of steroidogenic activity.

Glucocorticoids are involved in numerous physiological processes such as survival under stress conditions, cellular proliferation, differentiation and apoptosis (Franchimont, 2004). Further, it has been reported that corticosterone level under stress enhances short-term survival of birds (Wingfield et al., 1998) and also accelerates the generation of free radicals (Stier et al., 2009). Our study also showed that experimental LL and DD increased circulatory level of corticosterone and also oxidative load in tissues due to enhanced free radical generations.

Oxidative stress is combated through numerous physiological mechanisms responsible for maintaining an oxidant-antioxidant balance within the body. Under normal conditions, the generation of excessive free radicals were related with damages at cellular levels being controlled by cellular antioxidant defence systems. Melatonin is a well-known antioxidant, playing an important role in antioxidant defence and regulating antioxidant enzyme activity and their production. Therefore, total antioxidant status (TAS %) of organs is related to metabolic activity and melatonin level (Reiter, 2001, 2003). Thus, the constant dark (DD) condition that increased melatonin level could be the factor responsible for enhanced TAS%. On the other hand, inhibition of melatonin synthesis by LL (physiological pinealectomy) might have resulted in decreased level of TAS% in adrenal, testis and ovary. Our present study reveals for the first time a direct adrenal-gonad relationship in spotted finch *L. punctulata*. The phototoxicity induced by constant LL/DD conditions induces stress and modulates cholesterol content in adrenal and gonads along with circulatory corticosterone level. Furthermore, experimental or natural photoperiod modulates adrenal and gonadal functions through pineal gland hormone melatonin, thus facilitating the survival of bird under changing seasonal conditions.

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