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Peripheral melatonin modulates seasonal immunity and reproduction of Indian tropical male bird *Perdicula asiatica*

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Abstract

Seasonal changes in pineal function are well coordinated with seasonal reproductive activity of tropical birds. Further, immunomodulatory property of melatonin is well documented in seasonally breeding animals. Present study elucidates the interaction of peripheral melatonin with seasonal pattern of immunity and reproduction in Indian tropical male bird *Perdicula asiatica*. Significant seasonal changes were noted in pineal, testicular and immune function(s) of this avian species. Maximum pineal activity along with high immune status was noted during winter month while maximum testicular activity with low immune status was noted in summer. During summer month's long photoperiod suppressed pineal activity and high circulating testosterone suppressed immune parameters, while in winter short photoperiod elevated pineal activity and high circulating melatonin maintained high immune status and suppressed gonadal activity. Therefore, seasonal levels of melatonin act like a major temporal synchronizer to maintain not only the seasonal reproduction but also immune adaptability of this avian species.

Keywords: Seasonal changes; Pineal gland; Melatonin; Testosterone; Immune function

1. Introduction

It has been suggested that maintenance of positive energy balance is critical for survival and reproductive success (Bronson and Heidman, 1994; Nelson and Demas, 1996; Nelson, 2004). Several stressful environmental conditions such as reduced food availability, low ambient temperature, over-crowding, lack of shelter or increased number of predator can lead to seasonal fluctuation in immune function among individuals and seasonal changes in population-wide-diseases and death rates of vertebrates (Nelson, 2004).

Species that modify their physiological state on annual/seasonal basis are believed to use the pineal gland and its hormone, melatonin to prepare and respond to upcoming seasons (Haldar and Saxena, 1988). One of the systems that is receiving increasing support is the seasonal and photoperiodic regulation of the immune system. The pineal gland being a photoneuroendocrine organ is implicated as a major participant in the photo-immunomodulation (Giordano et al., 1993; Pioli et al., 1993; Maestroni, 1993; Poon et al., 1994; Guerrero and Reiter, 2002; Skwarlo-Sonta et al., 2003; Carrillo-Vico et al., 2005; Wen et al., 2006).

The Indian jungle bush quail, *Perdicula asiatica* is a seasonal breeder. Its breeding season extend from late March to July. Substantial work has been performed regarding the reproductive aspect of this species (Haldar and Ghosh, 1990; Sudhakumari et al., 2001), where as no work has been done regarding inter-action between seasonal variation in pineal, gonadal and immune status of any tropical bird. Therefore, present investigation has been performed to access the peripheral level of melatonin in regulation of seasonal immune function (lymphoid organ weight, total leukocyte count, lymphocyte count and % stimulation ratio) along with testicular function (testis weight and testosterone level) of male *P. asiatica* throughout the year.

2. Materials and methods

All experiments were conducted in accordance with institutional practice and within the framework of revised Animals (Scientific Procedures) Act of 2002 of Govt. of India on Animal Welfare.

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The experiments were conducted with adult male birds (*P. asiatica* Galliformes: Phasianidae) (body mass 35–40 g) for two consecutive years and mean of the data is presented. The birds were collected from the vicinity of the Varanasi (Lat. 25°, 18′ N, Long. 83°, 01′ E) in the first week of the each month of the year and acclimatized for two weeks in an open-air fenced aviary exposed to normal environmental conditions. They were fed with millet seeds (*Pennisetum typhoideum*) and other seasonal grain and water *ad libitum*.

2.1. Sample collection

The male birds were selected randomly ($n=7$), and sacrificed by decapitation in red light in the last week of the each month. One night (at 22.00 h) prior to sacrifice, birds were bled through pectoral vein for radioimmunoassay of melatonin and then sacrificed on the following day (at 11.00 h). The daytime collected blood was processed for radioimmunoassay of testosterone. The blood was taken in heparinized tube and plasma was separated out and stored at −20 °C for radioimmunoassay of melatonin and testosterone. Spleen was dissected out on ice and weighed on Sartorius balance and processed for splenocyte culture to observe the blastogenic response. Pineal gland and testis was dissected out on ice and weighed.

2.2. Hormonal analysis

The plasma contents of melatonin and testosterone in respective animals groups were estimated using the modified radioimmunoassay method of Rollag and Niswender (1976) for melatonin and RIA kit from Immunochemical Corporation, Carson, USA for testosterone. The validation of radioimmunoassay was performed as described earlier (Haldar and Rai, 1997; Sudhakumari et al., 2001). The intra and inter assay variation for melatonin was 9 and 15% and for testosterone 4.5 and 5.6% respectively. The sensitivity for melatonin RIA was 18–20 pg/mL and for testosterone RIA was 6 pg/mL. The recovery of melatonin and testosterone RIA was 92% and 95% respectively.

2.3. Hematological parameters

Following sacrifice blood was taken in a WBC pipette and diluted 20 times in Turk's fluid (2.0 mL Glacial acetic acid, 0.1 g mercuric chloride, one drop aniline, and 0.2 g gentian violet). The number of white blood cells was counted (no./mm^3) in Neubauer's counting chamber (Spencer, USA) under the Nikon microscope. Thin film of blood was prepared and stained with Leishman's stain and differential leukocyte (lymphocyte) number was counted under oil immersion lens of Leitz MPV3 microscope. Lymphocyte count (no./mm^3) was determined from the total and differential leukocyte count by using the following formula:

$$\text{Lymphocyte Count} = \frac{\text{TLC} \times \text{Lymphocyte percentage}}{100}$$

2.4. Reagent and culture medium for blastogenic response

Tissue culture medium RPMI-1640 and all other chemical were purchased from Sigma Chemicals, USA. The culture medium was supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal calf serum. Spleen was dissected out and processed for preparation of single cell suspensions. The number of cells was adjusted to 1×10^6 cells/mL in culture medium. Two milliliters of cell suspension was placed in duplicate culture tubes and kept at 37 °C in a 5% CO_2 incubator for 72 h. The control culture tubes were incubated without mitogen whereas test culture tubes were incubated with mitogen concanavalin A (Con A; T cell mitogen; Sigma-Aldrich, USA; 10 μg/mL). Eighteen hours before harvesting, 1 μCi of tritiated thymidine (^{3}H) (BARC, India; specific activity 8.9 Ci/mM) was added to each culture tube. Culture tubes were harvested after 72 h of incubation following the method of Pauly and Sokal (1972). Blastogenic response was measured in terms of [^{3}H] thymidine uptake against stimulation by Con A of the splenocytes.

$$\%\text{SR} = \frac{\text{CPM with Con } A}{\text{CPM without Con } A} \times 100$$

2.5. Statistical analysis

Statistical analysis of the data was performed with one-way ANOVA followed by Newman Keuls' multiple range test. The differences were considered significant when $P<0.05$.

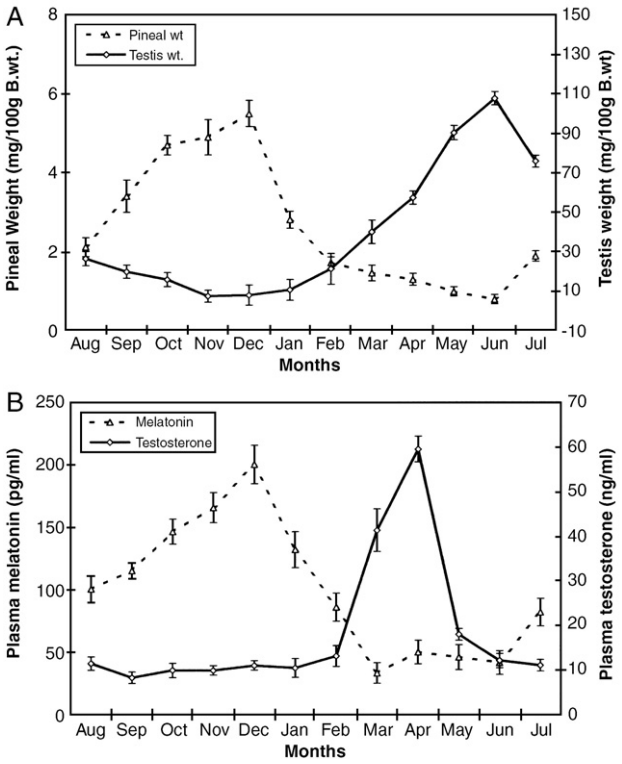

Fig. 1. A. Seasonal variation of pineal weight and testis weight of Indian Jungle bush quail, *Perdicula asiatica*. (Mean±SEM). B. Seasonal variation of plasma melatonin and plasma testosterone level of Indian Jungle bush quail, *Perdicula asiatica*. (Mean±SEM).

3. Result

3.1. Seasonal variation in pineal gland weight and plasma melatonin

Variations in pineal gland weight and plasma melatonin level were noted through out the year. Maximum pineal gland weight and peripheral melatonin level was noted in the month of December (5.5 mg/100 g b.wt. and 200 pg/mL, respectively). Minimum pineal gland weight was noted in June (0.79 mg/100 g b.wt.) and maximum peripheral melatonin level was noted in the March (33.6 pg/mL) with low basal level of plasma melatonin from May to July (Fig. 1A,B).

3.2. Seasonal variation in testis weight and plasma testosterone

Testis weight and circulating testosterone showed variations throughout the year. Maximum testis weight was noted in the month of June (107.33 mg/100 g b.wt.) and minimum was noted in the month of November (7.44 mg/100 g b.wt.). Maximum testosterone in circulation was noted in month of April (59.63 ng/mL) and a low basal level of plasma testosterone was noted rest of the year (Fig. 1A,B).

3.3. Seasonal variation in spleen weight

Variation in spleen weight was noted throughout the year. Maximum spleen mass was noted in the month of January

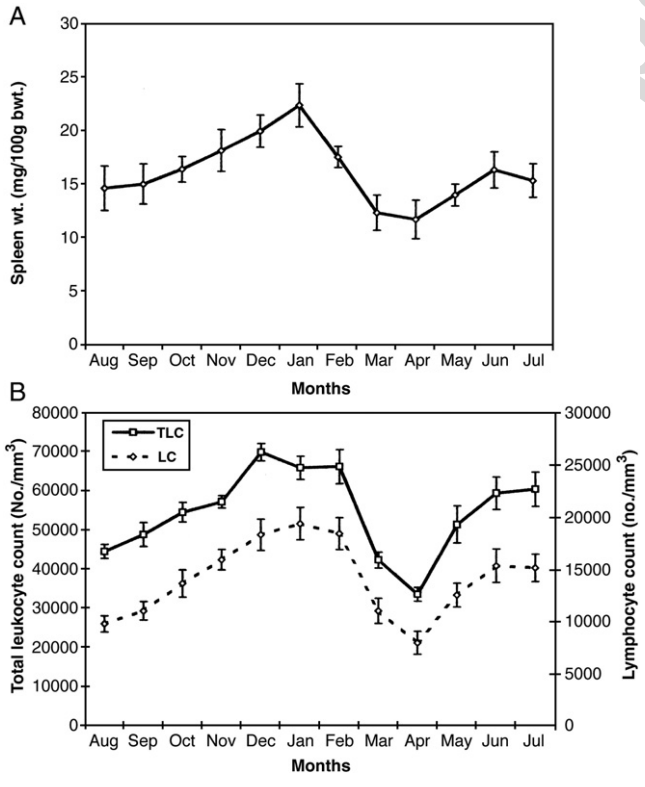

Fig. 2. A. Seasonal variation of spleen weight of Indian Jungle bush quail, *Perdicula asiatica,* (Mean±SEM). B. Seasonal variation of total leukocyte count (TLC) and lymphocyte count (LC) of Indian Jungle bush quail, *Perdicula asiatica*, (Mean±SEM).

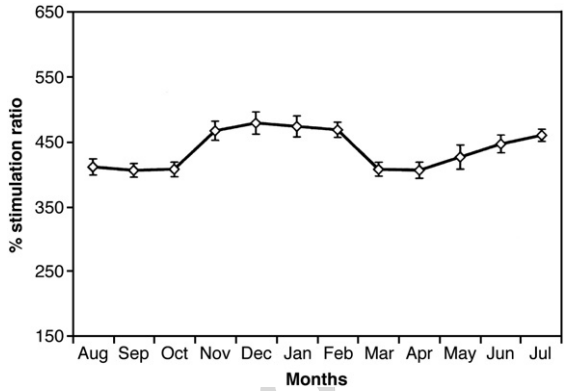

Fig. 3. Seasonal variation of percent stimulation ratio (%SR) of splenocytes of Indian Jungle bush quail, *Perdicula asiatica* (Mean±SEM).

(22.4 mg/100 g b.wt). A sharp decline was observed in the month of March being minimum in the month of April (11.72 mg/100 g b.wt) further, it increased from May to June, and a small decline was noted in the month of July (Fig. 2A).

3.4. Seasonal variation in total leukocyte count (TLC) and lymphocyte count (LC)

Variations in the TLC and LC were noted throughout the year being maximum total leukocyte count in the month of December (69900/mm^3) and lymphocyte count in the month of January (19394/mm^3). Minimum total leukocyte (33600/mm^3) and lymphocyte count (7944.8/mm^3) was noted in the month of April (Fig. 2B).

3.5. Seasonal variation in percent stimulation ratio (% SR)

Variation in blastogenic response of splenocyte was noted throughout the year. From November to February an increased percent stimulation ratio was noted with maximum in the month of December (478.8%). Declined in percent stimulation ratio was observed from March being minimum in the month of April (406%) (Fig. 3).

4. Discussion

Tropical changes in the environmental factors are significantly different than temperate zones. Indian tropical bird, *P. asiatica* is a summer breeder, whereas pineal gland activity showed opposite relationship with gonadal activity (Haldar and Ghosh, 1990). The maximum reproductive activity in this bird was reported in April to July when environmental conditions were favourable for mating and brooding of young once. Along with long photoperiod, during this phase harvesting is in full swing thus providing excess of food grains in the field justifying the status of summer breeder for this granivourous species. They store energy and grow their young one for forthcoming winter. Further, pineal gland weight and plasma melatonin showed a parallel seasonal cyclicity. Maximum pineal gland weight and highest plasma melatonin concentration was reported in December when reproductively inactive state of

this bird was noted (Sudhakumari et al., 2001). From the month of February to August, decreased pineal gland weight and comparatively low plasma melatonin concentration was noted proving inverse relationship between pineal gland and gonadal function.

The seasonal cyclicity in the development, regression and regeneration has been reported in thymus and spleen of various mammals and bursa of Fabricius of birds (Zapata et al., 1992; Nelson and Demas, 1996; Haldar and Singh, 2001). The annual changes in spleen weight presented an apparent variation, being inversely related with gonadal function and directly related with peripheral melatonin level.

Circulating total leukocyte and lymphocyte count, which accounts for immune status showed changes parallel to each other and directly proportional to blastogenesis in lymphoid organs i.e. spleen. When we looked upon the environmental factors i.e. photoperiod, temperature, relative humidity, through out the year, we found that environmental factors also have an impact on the various immune indices. Photoperiod and temperature probably play a role in the regulation of the lymphoid organs such as thymus, spleen and other parameters of immune system i.e. total leukocyte count, lymphocyte count and blastogenic response of the splenocytes in mammals (Champney and Mc Murray, 1991; Bilbo et al., 2003; Demas et al., 2003).

Moreover, photoperiod alone play an important immuno-regulatory role was evidenced in mammals. Mahmoud and his co-workers (1994) reported that the rats maintained at complete darkness for four weeks showed increased lymphatic mass when compared with rats maintained at normal day length (LD condition). Further, studies suggested that short days treatment increases splenic mass when compared with long day treated Syrian hamsters and deer mice (*Peromyscus maniculatus*) (Demas and Nelson, 1996; Drazen et al., 2002). Kirby and Froman (1991) suggested that cockerels grown under constant lighting had a lower anti-SRBC titer than those grown under 12L: 12D condition.

However, photoperiod does not affect all aspects of immune function i.e. humoral immunity, as assessed by serum antibody concentration and innate immunity (as assessed by blood neutrophil counts; Blom et al., 1994). Our data on spleen weight, total leukocyte count and splenocytes proliferative response to the mitogen Con A showed clearly a short photoperiodic dependent increase from October to January (~10L: 14D) onwards favouring the earlier reports. This suggests that slight changes in photoperiod of tropical zone as noted during dawn and dusk can affect immune status of this bird (Singh and Haldar, in press).

Our study of splenocytes proliferative response to the mitogen Con A in the different month throughout the year showed lowest splenocytes proliferation as well as % stimulation ratio in the month of April when days were long (~13L:11D). However, maximum splenocytes proliferation was noted in the month of December when days were short, supporting once more the effect of photoperiod on immune status. Kliger and his co-workers (2000) reported that in male broiler chickens the manipulation in photoperiod i.e. intermittent lighting had higher splenocytes proliferative activity than the chickens exposed to constant light condition.

Demas and Nelson (1996) suggested that winter associated stressors (restricted food, low ambient temperature) appeared to counteract short day enhancement of immune function by melatonin. In our avian species short photoperiod of winter enhanced melatonin level and this high melatonin level coincided with high immune status as depicted by high spleen weight, total leukocyte number, lymphocyte number and splenocytes proliferation to the Con A. In other words increasing trend of melatonin concentration in serum during the winter suppressed the strong effect of winter stressors, enhanced the immune parameters and helped the bird to remain healthy and fit in order to combat with winter born diseases (sudden death syndrome, chick flue, conjunctivitis etc.).

Seasonal changes in photoperiod and temperature influences the interior milieu i.e. hormonal concentration, which required for the various seasonal adjustments of metabolic activities. This alteration could be due to internal level of melatonin and gonadal/adrenal steroids. During summer days i.e. long photoperiod, higher gonadal steroids in circulation is responsible for reproductive activity in this birds and thereby decreases the immune status as steroid suppresses immunity in general (Schuurs and Verheul, 1990; Singh and Haldar, 2005; Weil et al., 2006).

From the above observation we may suggest that variation in peripheral melatonin by natural light conditions acts as a bolster to the immune function on one hand and suppressed the gonadal activity in winter on the other to help the individuals to fight with seasonal stressors (food scarcity and low ambient temperature). Hence, fluctuating immune function noted throughout the year might be responsible for adaptations that have evolved to decrease the odds of surviving possibilities and hormonal availability for reproduction.

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