Biological Rhythm Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713734219

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To cite this Article Singh, Shiv S. andHaldar, Chandana(2007) 'Biological significance of daily variation in immunity of *Perdicula asiatica*: role of melatonin and testosterone', Biological Rhythm Research, 38: 2, 95 — 106
To link to this Article: DOI: 10.1080/09291010600902652
URL: http://dx.doi.org/10.1080/09291010600902652

Biological Rhythm Research
April 2007; 38(2): 95 – 106

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Biological significance of daily variation in immunity of *Perdicula asiatica*: role of melatonin and testosterone

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Abstract
Daily variation of plasma melatonin affects daily activity pattern of many birds, but daily immunoprotective activity of melatonin in any seasonally breeding avian species is lacking. We report the influence of endogenous melatonin and testosterone on the daily variation in immunity of the Indian tropical bird *Perdicula asiatica* during reproductively active (RAP) and inactive (RIP) periods when the level of melatonin was high and in the former case. Daily variation in levels of melatonin, testosterone and immune parameters was noted during RAP and RIP. Maximum immune activity was noted at 2:00 hrs during RAP and at 14:00 hrs during RIP. During RAP, high testosterone in the circulation suppressed melatonin levels and immune parameters. A high basal level of melatonin during RIP was responsible for the suppression of testosterone resulting in high immune activity. Therefore, along with testosterone, melatonin acts like a major temporal synchronizer to maintain not only the reproductive rhythm but also daily immune adaptability of this avian species.

Keywords: *Melatonin, testosterone, total leukocyte count, daily variation, blastogenic response, immune status*

Introduction

The pineal gland is responsible for the transformation of external signals, mainly photoperiodic information, into a hormonal output, melatonin in a rhythmical fashion. The circadian correlation of pineal gland and melatonin level has been noted with that of the daily activity pattern in birds (Gaston 1971; Menaker et al. 1997). In these species the cyclicity in pineal melatonin content reveals a consistent phase relationship with cyclical changes in several environmental factors.

Neuroendocrine and lymphoid cells share a number of neurotransmitters, neuromodulatory substances, hormones and their receptors in common, supporting the existence of bi-directional regulation between the neuroendocrine and immune system (Fabris 1994; Skwarlo-Sonta 2002; Skwarlo-Sonta et al. 2003). On the other hand, products of the immune system like lymphokines and monokines modulate the neuroendocrine function, while various endocrine signals also affect the rhythmic immune functions. The daily/circadian correlation

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ISSN 0929-1016 print/ISSN 1744-4179 online © 2007 Taylor & Francis
DOI: 10.1080/09291010600902652

between melatonin and immune activity is less established in birds. Our recent study on the seasonal cycle of Indian jungle bush quail *Perdicula asiatica* suggest that plasma melatonin, gonadal activity (Haldar & Rai 1997; Sudhakumari et al. 2001) and immune status present an annual rhythm where melatonin and immune status is inversely related to gonadal steroids (Haldar & Singh 2001). However, the literature presents a lacuna regarding the studies on daily variations in plasma melatonin of tropical and subtropical avian species in relation to circadian variations in immune parameters.

In the present study, our aim was to note the inter-relationship between daily variation in pineal gland activity (plasma melatonin level), gonadal steroid (testosterone) with immune status (spleen weight, total leukocyte count (TLC), lymphocyte count (LC) and % stimulation ratio (%SR)) of male tropical Indian jungle bush quail *Perdicula asiatica*, during two important reproductive phases i.e. reproductively active (RAP) and inactive phase (RIP) when this tropical bird faces drastic environmental changes (photoperiod, temperature and humidity) and environmental threats (seasonal diseases, lack of food and shelter) to their life.

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.

The experiments were conducted with adult male birds (body weight 35 – 40 g) during reproductively active (June: photoperiod approx. 14L:10D; maximum and minimum temp. $37 \pm 5^{\circ}$C and $26 \pm 5^{\circ}$C) and inactive phases (January: photoperiod approx. 11L:13D; maximum and minimum temperature $15 \pm 5^{\circ}$C and $6 \pm 3^{\circ}$C). The birds were collected from the vicinity of the Varanasi (Lat. 25°, 18′ N, Long. 83°, 01′ E) and acclimatized for two weeks in an open-air fenced aviary exposed to normal environmental conditions. They were fed with millet seeds (*Pennisetum typhoid*) and other seasonal grain with water *ad libitum*.

Sample collection

To study the circadian correlation of melatonin and immune status, male birds were selected randomly ($n = 5$), and sacrificed by decapitation at four-hour intervals starting from 06.00 hrs; 10.00 hrs, 14.00 hrs, 18.00 hrs, 22.00 hrs, 02.00 hrs and 06.00 hrs of a 24 hr circadian timescale. The birds were sacrificed by decapitation and blood was taken in a heparinized tube. The spleen was dissected out on ice and weighed on a Sartorius balance then processed for culture to observe the blastogenic response.

Hormonal analysis

Collected blood was centrifuged at 1000 g for 15 minutes to collect the plasma. Plasma was stored at -20°C for hormonal assay of melatonin and testosterone. Melatonin RIA was performed following the method of Rollag and Niswender (1976) using Guildhey anti-melatonin antibody (Guildhey, Surrey, UK). Plasma testosterone was measured following the modified radioimmunoassay technique of Kime and Manning (1982). The recovery, accuracy and sensitivity for the melatonin RIA were 92%, 0.987, and 10 pg/ml respectively, while for testosterone, it was 95%, 0.997 and 6 pg/ml respectively. Intra- and inter-assay variation were between 9.0% and 15.0% and 4.2% and 5.1% for melatonin and testosterone, respectively.

Hematological parameters

Blood film was prepared for lymphocyte count. Total leukocyte count was done in a Neubauer hemocytometer. Lymphocyte count was conducted following Leishman's staining method.

Reagent and culture medium for blastogenic response

Tissue culture medium RPMI-1640 and all other chemicals 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 processed for preparation of single cell suspensions. The number of cells was adjusted to 1×10^6 cells/ml in complete medium. Two millilitres of cell suspension was placed in duplicate culture tubes and kept at 37°C in a 5% CO_2 incubator for 72 h. Blastogenic response was measured in terms of [^{3}H] thymidine (specific activity 8.9 Ci/mM) uptake against stimulation by Con A of the splenocytes (Pauly & Sokel 1972).

Statistical analysis

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

Results

Daily variation in plasma melatonin

Plasma melatonin showed significant daily changes during both reproductively inactive and active phases. During the reproductively inactive phase, maximum plasma levels of melatonin were noted at 22:00 hrs in the night and the minimum plasma level of melatonin was noted at 06:00 hrs (Figure 1a). During the reproductively active phase, the maximum plasma level of melatonin was noted at 02:00 hrs at night and a minimum plasma level of melatonin was noted at 06:00 hrs (Figure 1a).

Daily variation in plasma testosterone

Plasma testosterone showed significant daily changes during both reproductively inactive and reproductively active phases. During reproductively inactive and active phases, the maximum plasma level of testosterone was noted at 10:00 hrs with a low basal level during the reproductively inactive phase and a high basal level during the reproductively active phase. Minimun plasma testosterone was noted at 0:02 hrs during both reproductively phases (Figure 1b).

Daily variation in spleen weight

Significant daily variation was noted during both reproductively inactive and reproductively active phases. During reproductively inactive and active phases maximum spleen weight was noted at 14:00 hrs and minimum spleen weight was noted at 10:00 hrs (Figure 2).

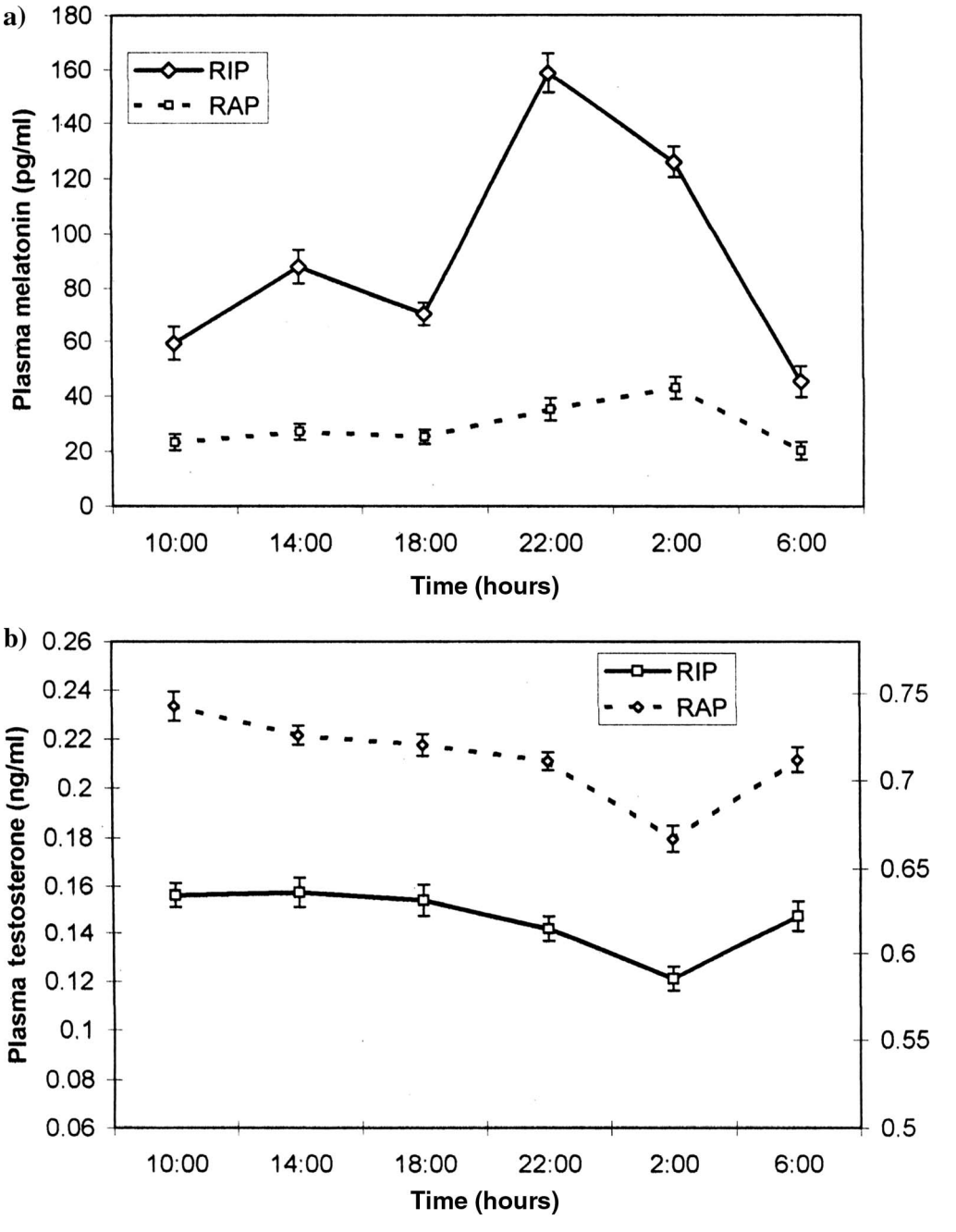

Figure 1. (a) Daily variation of plasma melatonin level of Indian Jungle bush quail, *Perdicula asiatica* during reproductively inactive and reproductively active phases (mean ± SEM). (b) Daily variation of plasma testosterone level of Indian Jungle bush quail *Perdicula asiatica* during reproductively inactive and reproductively active phases (mean ± SEM).

Daily variation in total leukocyte count (TLC)

Significant daily variation was noted in total leukocyte count during the reproductively inactive phase, being maximum at 14:00 hrs and a minimum total leukocyte count was noted

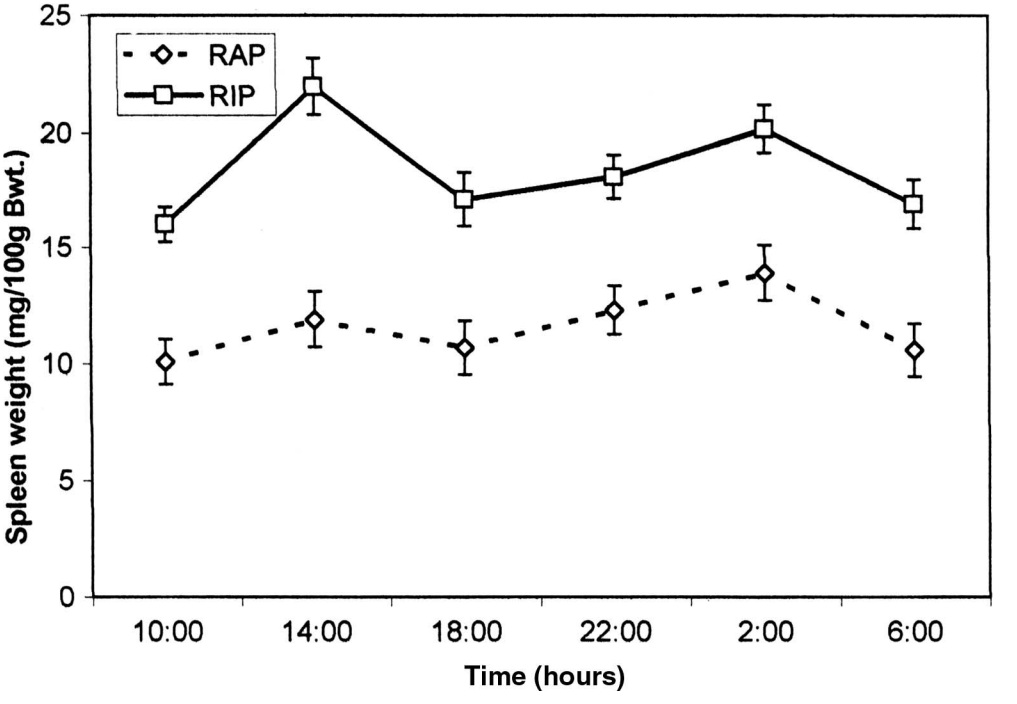

Figure 2. Daily variation of spleen weight of Indian Jungle bush quail, *Perdicula asiatica*, during reproductively inactive and reproductively active phases (mean ± SEM).

at 06:00 hrs (Figure 3a). During the reproductively active phase total leukocyte count presented no significant rhythm (Figure 3a).

Daily variation in lymphocyte count (LC)

Significant daily variation was noted in lymphocyte count during reproductively inactive and active phases. During the reproductively inactive phase, the maximum lymphocyte count was noted at 14:00 hrs and minimum lymphocyte count was noted at 10:00 hrs (Figure 3b). During the reproductively active phase, the maximum lymphocyte count was noted at 02:00 hrs during night and the minimum lymphocyte count was noted at 10:00 hrs (Figure 3b).

Daily variation in blastogenic response of splenocyte

Blastogenic response was observed in the terms of basal as well as T-cell mitogen concanavalin A (Con A) induced splenocyte proliferation in culture. Significant daily changes were observed in the blastogenic response of splenocyte during both reproductively inactive and active phases. During the reproductively inactive phase, maximum splenocyte blastogenesis (both basal and mitogen stimulated) was observed at 14:00 hrs and minimum was observed at 06:00 hrs (Figure 4a). During the reproductively active phase, maximum splenocyte blastogenesis (both basal and mitogen stimulated) was observed at 02:00 hrs at night and a minimum was observed at 06:00 hrs (Figure 5a).

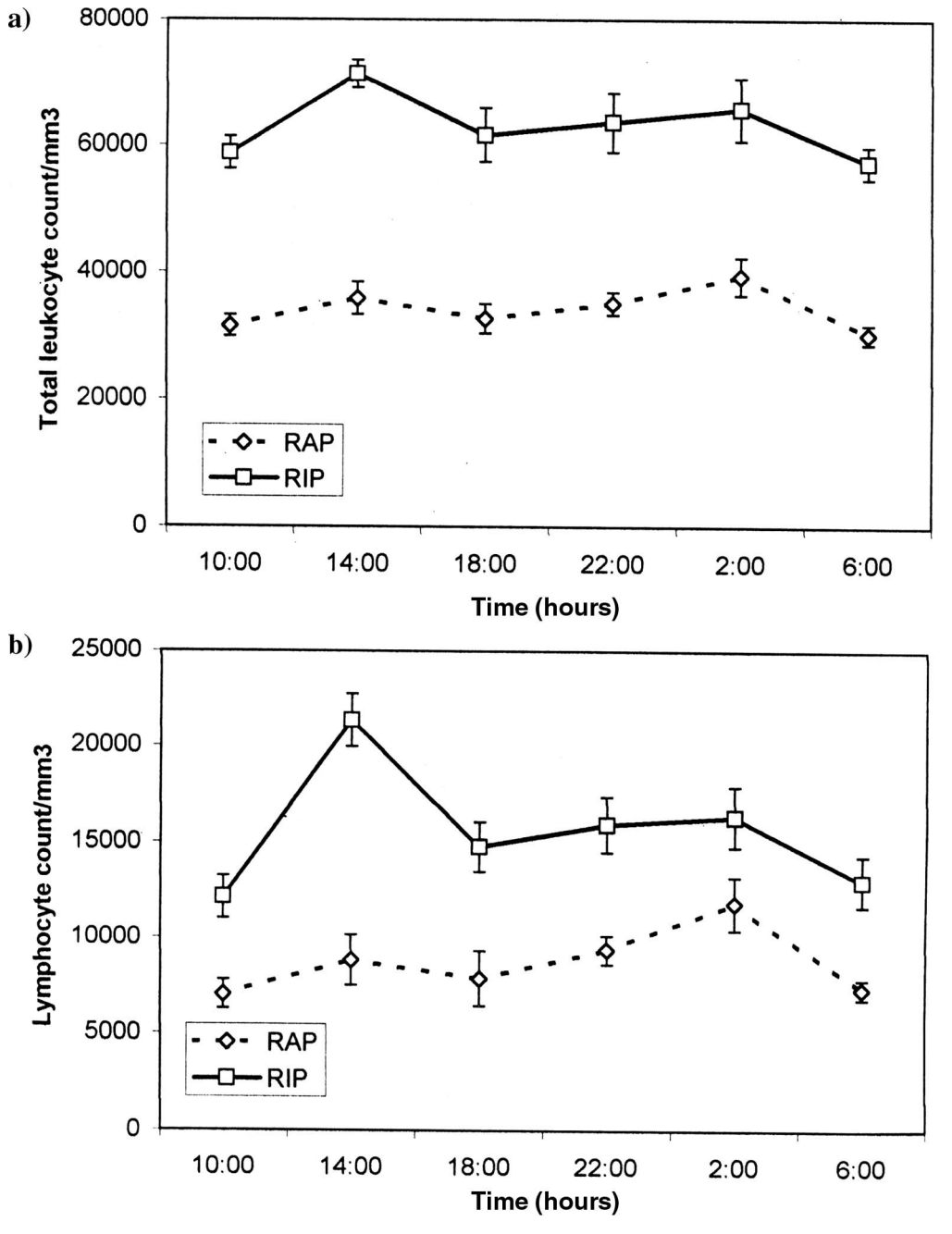

Figure 3. (a) Daily variation of total leukocyte count (TLC) of Indian Jungle bush quail, *Perdicula asiatica*, during reproductively inactive and reproductively active phases (mean ± SEM). (b) Daily variation of lymphocyte count (LC) of Indian Jungle bush quail *Perdicula asiatica*, during reproductively inactive and reproductively active phases (mean ± SEM).

Daily variation in percentage stimulation ratio (%SR)

Significant daily changes were noted in percentage stimulation ratio during both reproductively inactive and active phases. During the reproductively inactive phase, the

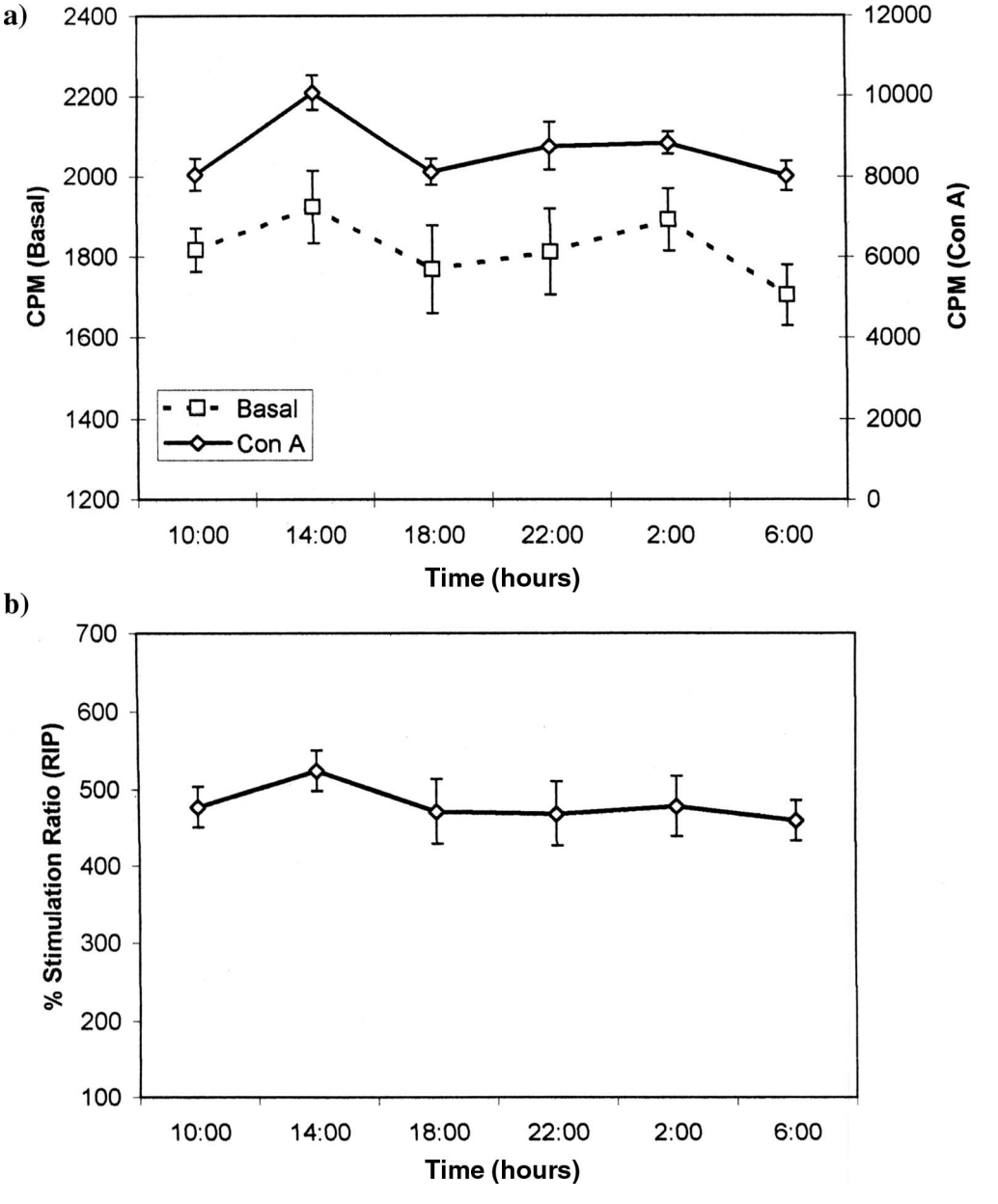

Figure 4. (a) Daily variation of basal and mitogen (Con A) induced blastogenic response of splenocytes of Indian Jungle bush quail, *Perdicula asiatica* during reproductively inactive phase (mean ± SEM). (b) Daily variation of percent stimulation ratio (%SR) of splenocytes of Indian Jungle bush quail, *Perdicula asiatica* during reproductively inactive phase (mean ± SEM).

maximum percent stimulation ratio was noted at 14:00 hrs and minimum percent stimulation ratio was noted at 06:00 hrs (Figure 4b). During the reproductively active phase, maximum percent stimulation ratio was noted at 02:00 hrs at night and minimum percent stimulation ratio was noted at 06:00 hrs (Figure 5b).

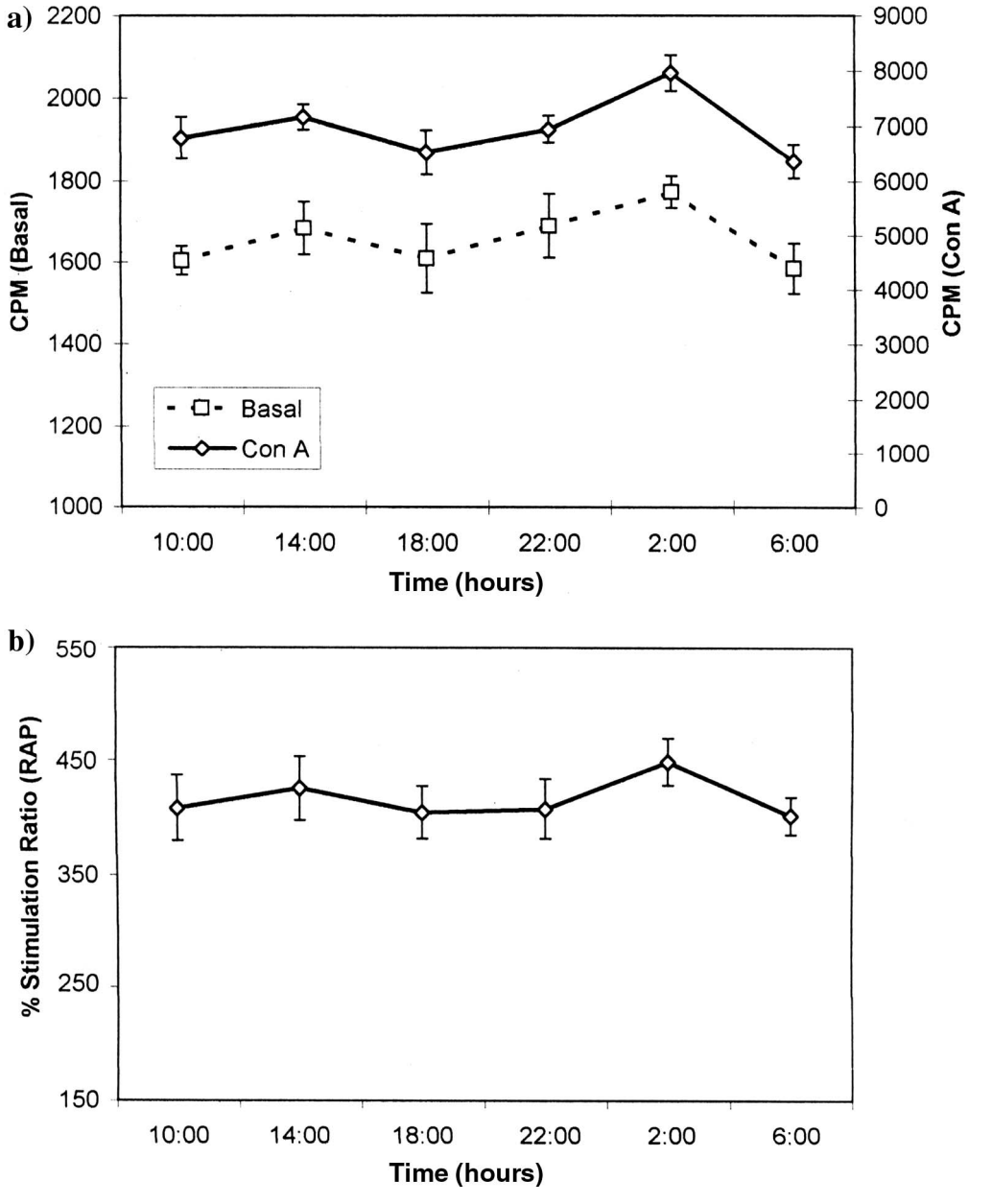

Figure 5. (a) Daily variation of basal and mitogen (Con A) induced blastogenic response of splenocytes of Indian Jungle bush quail, *Perdicula asiatica* during reproductively active phase (mean ± SEM). (b) Daily variation of percent stimulation ratio (%SR) of splenocytes of Indian Jungle bush quail *Perdicula asiatica* during reproductively active phase (mean ± SEM).

Discussion

The role of pineal in controlling behavioural and activity rhythms has already been established (Gaston 1971). Furthermore, the rhythmic synthesis and secretion of the pineal hormone,

melatonin, is suggested to be the mechanism by which pineal controls circadian oscillator(s) elsewhere for various other functional rhythm(s) in birds (Takahashi et al. 1989).

Demas and Nelson (1996) suggested that in contrast to melatonin, which peaks during the night both in diurnal and nocturnal species, the cyclicity of several immune parameters becomes correlated with the pattern of the animals' locomotor activity. The immune parameters that peak at one time of the day for a diurnal species peak about 12 hours later for a nocturnal species (Hayashi & Kikuchi 1985). Moreover, various immune parameters peak at various times points, anticipating an encounter with a pathogen during the period of activity while undergoing energetically expensive resolution of the immune response during the resting period (Nelson & Demas 2004). Further, daily and seasonal cyclicity of the immune functions are temporally integrated with other physiological and behavioural processes and all of them are regulated and coordinated with daily and seasonal changes of an external environment by the neuroendocrine homeostatic system (Zapata et al. 1992; Nelson & Demas 1997), proving once more the biological significance of the rhythms in immunity for survival.

This report, therefore, brings for the first time a daily variation in melatonin secretion and gonadal hormone along with daily variation in immune status (TLC, LC and %SR) of a seasonally breeding tropical avian species *Perdicula asiatica*. The experiment on daily variation was performed during two reproductive phases, i.e. active and inactive phases when not only the gonadal status but also the environmental factors such as photoperiod and temperature present drastic changes which, in turn, influence the neuroendocrine axis including pineal activity. During these two reproductive phases, the natural photoperiod and temperature conditions were completely different and hence the melatonin peak was at least 4 hours advanced (phase advanced) during the reproductively inactive phase than during the active phase. This shift in peak phase of melatonin level could be due to the decreased length of daytime (i.e. photoperiod in the month of January vs. June, Haldar et al. 2006).

Reports suggest that the pattern of nocturnal pineal production of melatonin varies with species (Haldar 1996), i.e. the peak may occur late in the dark period, near the middle of the dark period or throughout the dark period etc. (Binkley 1981). In the present avian species, we have noted peak plasma melatonin during the middle of the dark period as independent of the season and reproductive phase. The melatonin rhythm of this bird showed a typical tropical pattern (Haldar & Ghosh 1990; Haldar 1996). Slightly high melatonin levels were noted at 14:00 hours due to high tropical afternoon temperatures leading to afternoon siestas and energy balance (Bubenik 2002). This high melatonin level decreases again at 18.00 hours during winter and reaches a peak at midnight. No such variation was noted in summer, as the basal level of melatonin was already low (Figure 1a). Recent studies also provide evidence that the gastrointestinal tract (GIT) acts as a reservoir of melatonin and activates the digestive processes during the afternoon (Bubenik et al. 1999) GIT is also known as a significant contributor of melatonin to peripheral circulating levels particularly during midday (DeBoer 1988; Huether 1994; Bubenik et al. 1996). A sudden elevation in melatonin concentrations in blood observed by us may account for a hypothermic condition (Dollins et al. 1994) leading to sleepiness after meals and siesta (Bubenik et al. 2000). Further, Bubenik (2002) has mentioned that due to the hypothermic effect siesta is most popular in the hot, periequatorial reason of the earth as in the cases of tropical animals.

Our study reveals that during the reproductively active phase, the immune parameters (total leukocyte count, lymphocyte count and % stimulation ratio) showed parallel rhythmicity as noted for the plasma melatonin. During this phase the plasma level of testosterone was very high, hence, a steroid induced suppression of the immune parameters was noted during daytime while at night-time a small elevation in the immune parameters occurred due to the increase of high night-time melatonin levels in plasma along with low testosterone level.

However, favourable environmental conditions of summer (food availability, shelter, low frequency in diseases) and high testosterone level in plasma protected the birds from seasonal infections and reduced the rate of mortality for which we have proposed recently the ''Trade off'' hypothesis (Rai et al. 2005).

We propose that the immune variation noted in this bird is essential for adaptation (Haldar & Singh 2001) but a circadian relationship among immune function, melatonin and gonadal steroids during different reproductive phases appears equally important and biologically significant for daily adaptation. This is because the wild birds of the tropical zones face a maximum challenge from nature in winter in terms of hypothermia, lack of food, shelter and with several seasonal diseases, such as dermal and eye infections, leading to high rates of mortality. During the reproductively inactive phase, the basal level of immune parameters was higher than during the reproductively active phase. Low plasma steroids and slightly high daytime basal levels of plasma melatonin in the circulation favoured the peak value of immune parameters in the daytime. However, the peak value of plasma levels of melatonin in the night-time favoured the increase in the immune parameters during the reproductively inactive phase for stronger winter stress adaptation of this bird as suggested by Kliger et al. (2000) in broiler chicken. It indicates that immune parameters are enhanced in short days to counteract stress-mediated immune suppression occurring during the winter (Demas & Nelson 1996).

In this bird, a short photoperiod caused gonadal inactivity and enhanced melatonin levels in circulation (Haldar & Rai 1997), which might have enhanced immune parameters (leukocyte count, lymphocyte count and percent stimulation ratio) as noted by us. The high melatonin along with high status of immune activity protects the bird from adverse conditions of winter and diseases.

The direct effect of the reproductive state on immune function was also suggested in mammals (Bentley et al. 1998). In this bird, reproductive activity and immune status appear to have co-evolved as the mechanism for control of reproductive function by steroid hormone that is responsible not only for the high metabolic cost of birds but is also involved in supporting the reduced immune status. Furthermore, melatonin receptors are present on lymphoid organs as well as on lymphocytes, suggesting a direct effect of melatonin on the immune system and a key role in immunomodulation (Singh 2003).

Our results suggest that the biological significance of the daily rhythm of this bird can be correlated with the rhythm of immune parameters, but can also be directly correlated with circulating melatonin levels, which act like a major temporal synchronizer to maintain the hormonal and immune adaptability of this avian species.

Acknowledgements

The authors are thankful to the Council of Scientific and Industrial Research and University Grant Commission for financial support. Equipment gift from the Alexander von Humboldt Foundation, Germany is gratefully acknowledged.

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