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Immune responses of Indian Jungle Bush Quail, *P. asiatica*, to different photoperiodic regimens during the reproductively inactive phase

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Effects of different photoperiod regimens: Natural Day Length (NDL), Constant Light (LL), Constant Dark (DD), 16 h Light:8 h Dark (16L:8D), 14 h Light:10 h Dark (14L:10D), 12 h Light:12 h Dark (12L:12D) on immunity was noted during the reproductively inactive phase of a tropical bird, *Perdicula asiatica*. Extreme exposure of photoperiod i.e. LL and DD has a drastic effect on the immune status of the birds while two long photoperiods 16L:8D and 14L:10D have a significant effect when compared with the immune status of NDL birds. The immune status and melatonin level had a direct relationship but shared an inverse relationship with that of the gonadal activity of the birds. Thus, even in the tropical zone (where the photoperiod is having slight difference) the immunity relies on photoperiodic time measurement mechanisms that include the pineal gland and melatonin to relay photoperiodic changes in physiological and behavioural components of immune function.

Keywords: photoperiod; melatonin; steroid; immune status

Introduction

Birds were the first vertebrates in which photoperiodic reactions were described (Rowan 1925) and there is now an ever-increasing literature on photoperiodism in this vertebrate group. Photoperiodic effect was found in more than 50 species of birds (Farner 1975; Murton and Westwood 1977), while the functions measured to indicate photoperiodic effects is usually reproduction, molt, fat deposition and migratory restlessness. Melatonin appears to mediate seasonal adjustments in immune function and is hypothesized to have been co-opted throughout evolution in seasonally breeding animals to enhance immune function at a time when energetic constraints pose a danger for survival (Nelson and Demas 1996; Haldar and Singh 2001; Singh and Haldar 2007).

Manipulation in photoperiod is noninvasive and may influence the immune status of several organisms. In male ring doves (*Streptopelia risoria*), pinealectomy suppresses both the number and function of white blood cells (Rodriguez and Lea 1994). In addition, melatonin prevents the suppression of immune function by photostimulation in European starlings (*Sturnus vulgaris*) (Bentley et al. 1998). It is known that periods of constant darkness and constant light cause stimulation and inhibition of melatonin secretion from the pineal gland and hence can influence immune responses. Several studies demonstrate a

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relation of immune status to the manipulation of photoperiod in various tropical and temperate zone mammals (Brainard et al. 1985; Blom et al. 1994; Demas and Nelson 1996), but to date no information is available regarding the immune status of the tropical birds with respect to the photoperiodic manipulations.

In birds immunity is affected by melatonin administration (Haldar and Singh 2001; Singh and Haldar 2005, 2007; Singh et al. 2006), however, the conventional way of increasing and decreasing the melatonin level is possible via photoperiods and can thus be used to promote and inhibit immunity, which can be experimentally accessed. We therefore have noted the effects of different photoperiod regimens on the immune response of male and female *Perdicula asiatica*, Indian jungle bush quail. We have included both the sexes in order to note the sex dependent variation if any in photoperiodic responses of immunity.

Materials and methods

Animal care and its maintenance

Male and female Indian jungle bush quails *P. asiatica* were used in this study. The birds are abundant in the vicinity of Varanasi (Lat 25° 18′ N; Long 83° 01′ E) and were collected during its reproductively inactive phase (winter, November; 10L: 14D). They were maintained in an aviary exposed to ambient conditions and acclimatized in this condition for two weeks. The birds were fed with millet seeds (*Pennisetum typhoides)* and water *ad libitum*. After two weeks both male and female birds were divided into six groups and exposed to different photoperiodic regimens for the study (Table 1).

At the end of experiment prior to the day of sacrificing, each bird was bled through the pectoral vein during night time (10:00–11:30 pm) under dim red light. Blood was collected and centrifuged; plasma was collected and stored at –20°C to perform radioimmunoassay (RIA) of melatonin, estradiol and testosterone. On the following day the birds were sacrificed by decapitation. The spleen was dissected out on ice and weighed on a Sartorius balance and was processed for splenocyte culture to assess the cell mediated immune response by measuring splenocyte proliferation in response to the T-cell mitogen Concanavalin A (Con A) separately for male and female birds.

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

Radioimmunoassay

RIA of estradiol and testosterone were performed with the help of a commercial kit purchased from Leuco Diagnostic Inc., MI, USA. Melatonin RIA was performed

Table 1. Experimental design.

	Photoperiod Regimens	Total no. of birds	Time period of the exposure
I	Control; NDL (Natural day length)	14 (7 males & 7 females)	8 weeks
II	LL (Constant Light)	14 (7 males & 7 females)	8 weeks
III	DD (Constant Dark)	14 (7 males & 7 females)	8 weeks
IV	16L:8D (16 h Light:8 h Dark)	14 (7 males & 7 females)	8 weeks
V	14L:10D (14 h Light:10 h Dark)	14 (7 males & 7 females)	8 weeks
VI	12L:12D (12 h Light:12 h Dark)	14 (7 males & 7 females)	8 weeks

according to Rollag and Niswender (1976), using Guildhey anti-melatonin antibody (Guildhey, Surrey, UK).

Hematological parameters

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 Gention violet) and the white blood cells counted (no./mm^3) in Neubauer's counting chamber (Spencer, USA) under the microscope. A thin film of blood was prepared and stained with Leishman's stain and differential lymphocytes were counted under an oil immersion lens of a Leitz MPV3 microscope. Lymphocyte counts (no./mm^3) were determined from the total and differential lymphocyte count using the following formula:

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

Splenocyte culture

Tissue culture medium RPMI-1640 and all other chemicals were purchased from Sigma-Aldrich Chemicals, USA. The culture medium was supplemented with 100 μg/ml Streptomycin, 100 U/ml Penicillin and 10% fetal calf serum. Spleen was dissected out and processed for the preparation of single cell suspensions. The number of cells was adjusted to 1×10^6 cells/ml in culture medium. Two millilitres of spleen cell suspension were cultured on the culture plates for 72 h in a fully humified 5% CO_2 atmosphere, in 41°C Heraeus, Hera cell CO_2 incubator. Prior to harvesting 10 μCi of ^{3}H-thymidine was added to each plate. Blastogenic responses of splenocytes were measured in terms of [^{3}H] thymidine (BARC, India; specific activity 8.9 Ci/mM) uptake against stimulation by Concanavalin A (Con A; T cell mitogen; SIGMA, USA) of the splenocytes (Pauly and Sokal 1972).

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

Statistical analysis

Organ masses were presented as relative values. All values were presented as mean $\pm$ SEM. Data was analysed by one way ANOVA followed by Student Newman-Keul's test. Differences of means were considered significant when $P < 0.05$ and highly significant when $P < 0.01$.

Results

Body weight

The body weight of the birds of all the experimental five groups was not affected by any photoperiodic regimen when compared with control Group I birds kept under Natural Day Length (NDL) of November. However, females were heavier than males (Figure 1a).

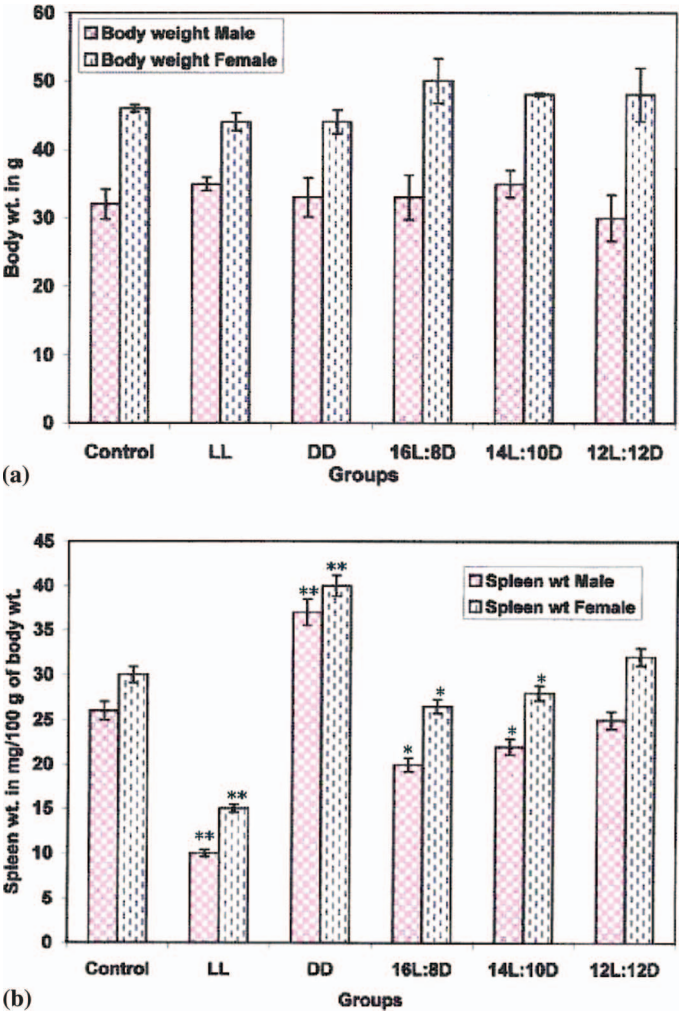


Figure 1. Effect of different photoperiod regimens on (a) body weight; (b) spleen weight of male and female *P. asiatica* during reproductively inactive phase (Nov–Jan). Histograms represent mean ± SEM, $n = 7$ for each group within this experiment. Con = Control, DD = Constant dark, LL = Constant light, 16L:8D = 16 h light:8 h dark, 14L:10D = 14 h light:10 h dark, 12L:12D = 12 h Light:12 h dark. *$P < 0.05$, **$P < 0.01$ Con vs. DD, Con vs. LL, Con vs. 16L:8D, Con vs. 14L:10D, Con vs. 12L:12D.

Spleen weight

A significantly ($P<0.01$) high spleen weight was noted in Group III birds experiencing constant dark (DD) when compared with control Group I birds experiencing natural day length (NDL; 10L:14D). Group II birds experiencing constant light (LL), showed significantly ($P<0.01$) lowest spleen weight, Group IV (16L:8D) and Group V (14L:10D) birds also showed significantly ($P<0.05$) less spleen weight when compared with control Group I birds (NDL; 10L:14D), whereas no significant difference was noted in the spleen weight of Group VI birds experiencing (12L:12D) when compared with control Group I birds experiencing natural day length (NDL; 10L:14D) (Figure 1b).

Total leukocyte count and lymphocyte count (TLC and LC)

Significantly ($P<0.01$) high TLC and LC were noted in Group III birds experiencing constant dark (DD) when compared with control Group I birds experiencing natural day length (NDL; 10L:14D). Group II birds experiencing constant light (LL) showed the lowest TLC and LC ($P<0.01$) number, while Group IV (16L:8D) and Group V (14L:10D) birds also showed significantly ($P<0.05$) less TLC and LC when compared with control Group I birds (NDL; 10L:14D). No significant difference was noted in the TLC and LC of Group VI birds experiencing (12L:12D) when compared with control Group I birds experiencing natural day length (NDL; 10L:14D) (Figure 2a and 2b).

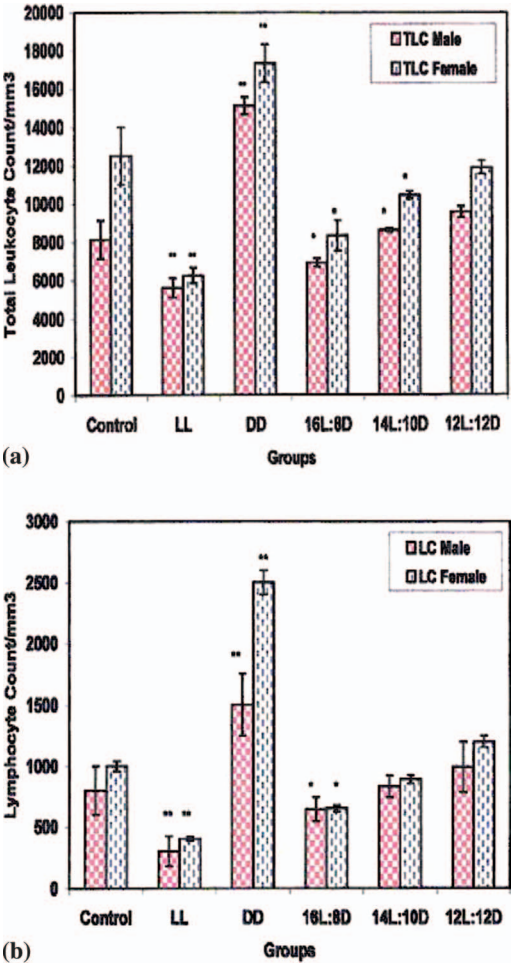


Figure 2. Effect of different photoperiod regimens on (a) total leukocyte count; (b) lymphocyte count of male and female *P. asiatica* during the reproductively inactive phase (Nov–Jan). Histograms represent mean ± SEM, $n = 7$ for each group within this experiment. Con = Control, DD = Constant dark, LL = Constant light, 16L:8D = 16 h light:8 h dark, 14L:10D = 14 h light:10 h dark, 12L:12D = 12 h Light:12 h dark. $^{*}P < 0.05$, $^{**}P < 0.01$ Con vs. DD, Con vs. LL, Con vs. 16L:8D, Con vs. 14L:10D, Con vs. 12L:12D.

Blastogenic response of splenocytes and percent stimulation ratio (% SR)

Significantly high basal (male and female: $P<0.05$) and mitogen Con A induced (male and female: $P<0.01$) blastogenic response was noted in Group III birds experiencing constant dark (DD) when compared with control Group I birds experiencing natural day length (NDL; 10L:14D). Group II birds experiencing constant light (LL) showed significantly less basal (male and female: $P<0.005$) and mitogen Con A induced (male and female: $P<0.01$) blastogenic response when compared with control Group I birds experiencing natural day length (NDL; 10L:14D). In Group IV (16L:8D) and Group V (14L:10D) birds of both sexes the difference in the basal count was not significant but the difference in mitogen Con A induced blastogenic response in both the groups was significant (male and female: $P<0.05$) when compared with control Group I birds (NDL; 10L:14D). No significant difference was noted in both the basal as well as mitogen Con A induced blastogenic response in Group VI birds of both sexes experiencing (12L:12D) when compared with control Group I birds experiencing natural day length (NDL; 10L:14D). The percentage stimulation ratio (% SR) of splenocytes was significantly high (male and female: $P<0.05$) in Group III birds experiencing constant dark (DD) and significantly low (male and female: $P<0.05$) in Group II birds experiencing constant light (LL) when compared with control Group I birds experiencing natural day length (NDL; 10L:14D). The difference in the % SR of splenocytes in Group IV, V and VI birds of both sexes experiencing (16L:8D) and (14L:10D) and 12L:12D) respectively was not significant when compared with control Group I birds experiencing natural day length (NDL; 10L:14D) (Figure 3a–c).

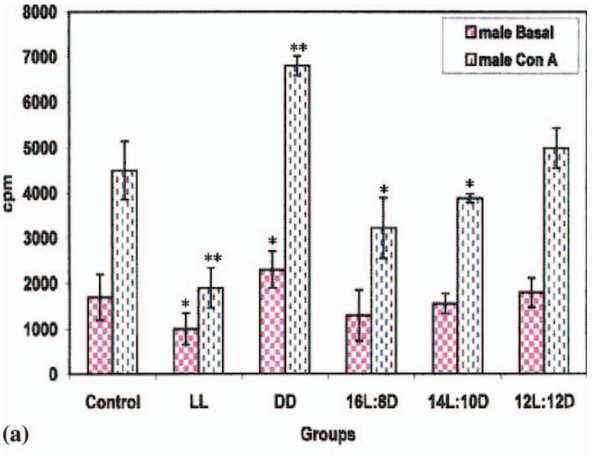
Circulating level of melatonin and gonadal steroids

A significantly high circulating level of plasma melatonin was noted in Group III birds of both sexes experiencing constant light (DD), whereas the lowest level of circulating plasma melatonin was noted in the Group II birds of both sexes experiencing constant light (LL) when compared with control Group I birds experiencing natural day length (NDL; 10L:14D). Group IV (16L:8D) and Group V (14L:10D) birds of both sexes showed a decrease in the circulating level of melatonin when compared with control Group I birds (NDL; 10L:14D). The circulating level of plasma melatonin in Group VI birds of both sexes was almost similar to that of circulating plasma melatonin in control Group I birds (NDL; 10L:14D). The opposite was noted for the circulating levels of testosterone and estradiol in male and female bird of all the experimental groups respectively (Figure 4).

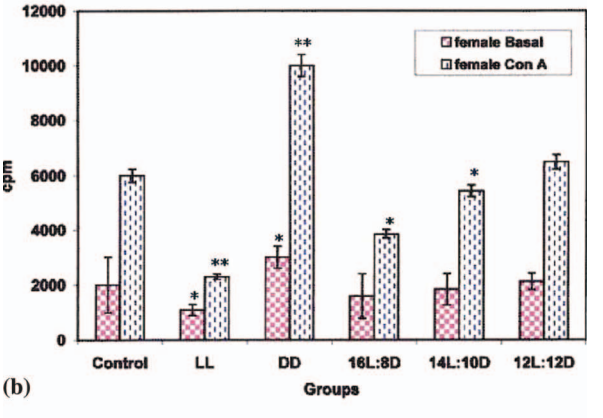
Figure 3. Effect of different photoperiod regimens on basal and mitogen Con A induced blastogenic response of (a) male and (b) female *P. asiatica* during the reproductively inactive phase (Nov–Jan). Histograms represent mean $\pm$ SEM, $n = 7$ for each group within this experiment. Con = Control, DD = Constant dark, LL = Constant light, 16L:8D = 16 h light:8 h dark, 14L:10D = 14 h light:10 h dark, 12L:12D = 12 h Light:12 h dark. $^{*}P < 0.05$, $^{**}P < 0.01$ Con vs. DD, Con vs. LL, Con vs. 16L:8D, Con vs. 14L:10D, Con vs. 12L:12D. (c) Effect of different photoperiod regimens on percentage stimulation ratio of splenocytes of male and female *P. asiatica* during the reproductively inactive phase (Nov–Jan). Histograms represent mean $\pm$ SEM, $n = 7$ for each group within this experiment. Con = Control, DD = Constant dark, LL = Constant light, 16L:8D = 16 h light:8 h dark, 14L:10D = 14 h light:10 h dark, 12L:12D = 12 h Light:12 h dark. $^{*}P < 0.05$, $^{**}P < 0.01$ Con vs. DD, Con vs. LL, Con vs. 16L:8D, Con vs. 14L:10D, Con vs. 12L:12D.

Discussion

The present study proposes that peripheral melatonin level is regulated by photoperiod, which in turn controls the immune status of the birds. We observed that the body weight of the birds of all the experimental groups was not affected under different photoperiodic



(a)



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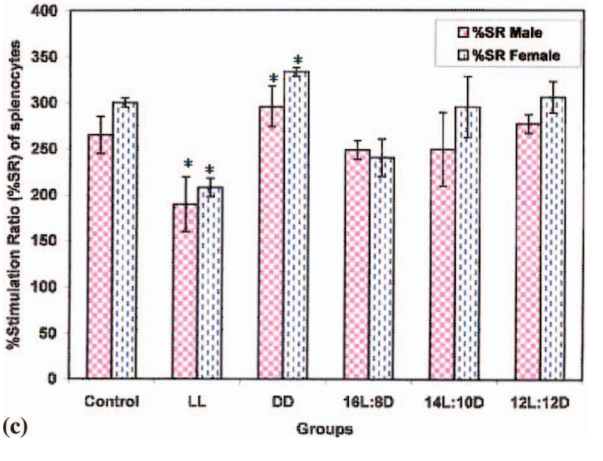

(c)

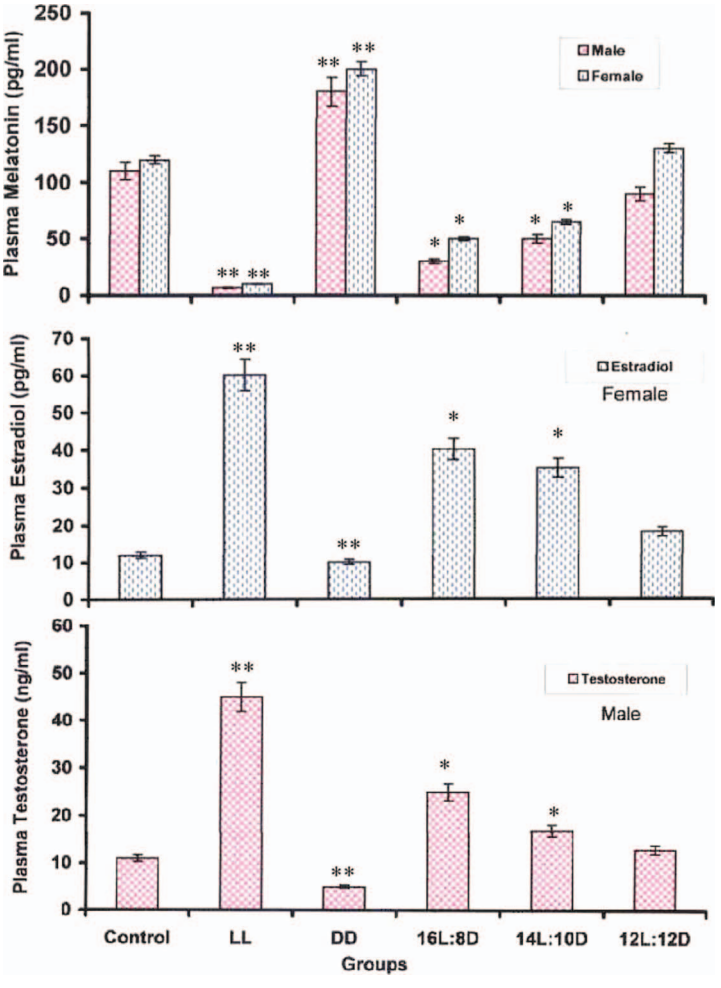

Figure 4. Effect of different photoperiod regimens on circulating plasma levels of melatonin, estradiol and testosterone of male and female *P. asiatica* during the reproductively inactive phase (Nov–Jan). Histograms represent mean ± SEM, $n = 7$ for each group within this experiment. Con = Control, DD = Constant dark, LL = Constant light, 16L:8D = 16 h light:8 h dark, 14L:10D = 14 h light:10 h dark, 12L:12D = 12 h Light:12 h dark. $^{*}P < 0.05$, $^{**}P < 0.01$. Con vs. DD, Con vs. LL, Con vs. 16L:8D, Con vs. 14L:10D, Con vs. 12L:12D.

regimens when compared with the body weight of the birds exposed to natural day length of the November group experiencing the photoperiod of ~10L:14D which were treated as control Group I. Stability in the body weight of both male and female birds exposed to different photoperiodic regimens indicates that the birds were healthy and were not under stress. The spleen is one of the most important immune organs in adult birds, hence, spleen weight indicates the immune status of the bird.

In *Perdicula asiatica* the variations in the different immune parameters showed a particular pattern in different photoperiodic regimens. The variation in the immune responses of the birds of the control group experiencing natural day length of November, photoperiod of 10L:14D, were almost similar to those of experimental Group VI birds experiencing 12L:12D. Similarities in the immune responses were observed in Group VI

birds experiencing 16L:8D and Group V experiencing 14L:10D. From the results it implies that photoperiods 10L:14D and 12L:12D were acting as short photoperiods (<12.5 h) for this bird, whereas 16L:8D and 14L:10D photoperiods were acting as long photoperiods (>12.5 h) for this bird. The 2 h variation in short and long photoperiod does not have any significant effect on the immune status (under observed parameters) of this bird.

The purpose of this experiment was to test the hypothesis that photoperiodic changes in enumerative measures of immune function are dependent on pineal melatonin secretion i.e. in turn duration of daylength. Total leukocyte counts served as an omnibus indicator of whether photoperiod was registered by the immune system following the experimental manipulations (day length, pinealectomy). High spleen weight, TLC, LC, basal as well as mitogen Con A induced blastogenic response was noted in the birds exposed to constant darkness, whereas minima were noted in the birds exposed to constant light. Similar variation in the circulating plasma melatonin level implies that constant dark induces the secretion of pineal melatonin and thereby increases the immunity of the birds by increasing the spleen weight, TLC, LC, basal as well as blastogenic response of the splenocytes of the birds. Constant light caused a decrease in the circulating plasma melatonin level which thereby decreases the immune functions of the bird. Lower circulating level of gonadal steroids and high melatonin were recorded in the birds experiencing constant dark. It means that gonadal steroids are acting as inhibitors for the immune function of the bird and melatonin is immunostimulator, hence enhancing the immune status of this bird. Hence, our results supported the earlier findings that melatonin and gonadal steroids share an inverse relationship and melatonin acts as an immunostimulator (Mase and Oishi 1991; Wichman et al. 1996; Nelson and Drazen 1999; Moore and Siopes 2000, 2005; Haldar and Singh 2001; Singh and Haldar 2005). Melatonin treatment mediates adaptive physiologic responses to photoperiod in Siberian hamsters (Yellon 2007).

In virtually all studies reported to date, melatonin has been shown to enhance immune function (Caroleo et al. 1992; Guerrero and Reiter 1992; Giordano 1993; Giordano et al. 1993; Pioli et al. 1993; Poon et al. 1994; Nelson et al. 1995; Singh and Haldar 2005) and it is also supported from our observation of DD and LL results. It is therefore possible that photoperiod may influence immune function via influencing the peripheral level of melatonin. Although it is not clear how melatonin exerts its effect on the immune system, melatonin receptors have been identified on circulating lymphocytes and splenocytes of mammals and birds (Lopez-Gonzales et al. 1993; Calvo et al. 1995; Rafii-El-Idrissi et al. 1995; Markowska et al. 2002; Skwarlo-Sonta et al. 2003).

The results of the present study are comparable to several other laboratory studies which suggest that seasonal changes in immune function of an organism appear to be mediated by photoperiod for example, short days enhance immune function in several species of rodents including deer mice (*Peromyscus maniculatus*), Syrian hamsters (*Mesocrietus auratus*), and voles (*Microtus pennsylvanicus*; *Microtus ochrogaster*) (Vriend and Lauber 1973; Brainard et al. 1985; Blom et al. 1994; Nelson et al. 1995; Nelson and Nelson 1996). In Siberian hamsters and most other photoperiodic mammals, it is well established that the secretion of pineal melatonin is necessary for the majority of seasonal responses to changes in day length. Very little is known about the extent to which photoperiodic changes in immune function rely on the endogenous production of pineal melatonin signals. Pineal dependence of innate immunity has been inferred from a study which maintained hamsters in constant light, suppressing endogenous melatonin secretion (Yellon et al. 2005).

In Siberian hamsters exposure to short days also attenuated both the magnitude and the duration of two major consequences of bacterial infection, replicating earlier work in this species (Bilbo et al. 2002; Prendergast et al. 2004a) which demonstrated that exposure to SD enhances the number of circulating leukocytes (and thus capacity for immunosurveillance) and mitigates symptoms of infection. In Siberian hamsters (*Phodopus sungorus*) surgical pinealectomy abolished the effects of short photoperiods on circulating leukocyte counts and on the behavioural and somatic sickness responses to LPS (Wen et al. 2007).

Thus the immune system relies on photoperiodic time measurement mechanisms that include the pineal gland to engage photoperiodic changes in physiological and behavioural components of immune function. Circulating leukocytes were enumerated by staining. Photoperiodic conditions have activational effects on the immune system (Prendergast et al. 2004b). There is a growing literature supporting the immunoenhancing effects of melatonin (Nelson and Drazen 2000). Comparing the results of this experiment with above-mentioned studies we can say that short photoperiods 12L:12D, 10L:14D (Control) enhance immune function, whereas long photoperiods 16L:8D, 14L:10D inhibit the immune response in this photoperiodic tropical bird *Perdicula asiatica*. In birds exposed to constant light (LL), there is a decrease in circulating melatonin level and also an increase in gonadal steroid levels, hence there is a decrease in immune response and vice versa was noted in the case of birds exposed to constant dark (DD).

Disturbed rhythms in melatonin production may miscue physiological responses and deter appropriate adaptations to seasons. As a result, this could lead to increased rates of disease and mortality. These findings raise the possibility that the pineal gland may play a critical role in adjusting immune response capabilities to anticipate seasonal diseases and infection in the human and other species. Exposure to short days, typical of winter day lengths, enhances spleen mass, thymic mass, leukocyte counts, and wound healing rates (Blom et al. 1994; Mahmoud et al. 1994; Nelson and Blom 1994). Thus, photoperiod modulation of immune cell activity is highly selective and may not be predictive of the response capabilities by other immune cells (Yellon et al. 1999).

The components of immune function, i.e. cell mediated immunity among Collared lemmings (*Dicrostonyx groenlandicus*) are responsive to changes in day length i.e. they respond both physiologically and behaviourally to changes in day length (Weil et al. 2006, 2007). Different photoperiodic regimens and *in vitro* treatment of melatonin enhances various immune parameters in male Broiler mhickens (Klieger et al. 2000).

The present study showed that non-conventional increases or decreases in melatonin by the different photoperiodic regimes play an important role in down- and upregulation of the immune responses of the bird by the photoperiod and hence could be of high adaptive importance. We performed our experiments in winter months when photoperiod in nature was short i.e. 10.45 h and hence their peripheral level of melatonin is also high with high immunity, but after experiencing long photoperiod regimens birds presented decreased immunity. Constant dark conditions induced the basal melatonin level and hence, induced the immune status of *P. asiatica* as judged by the increased TLC, spleen weight and blastogenic response of the splenocyte in response to T cell mitogen Con A, while constant light caused physiological pinealectomy conditions in birds where the basal level of melatonin reduced and hence suppressed the immune status. Further, we found that the immune response of *P. asiatica* is independent of sex.

Understanding the *in vitro* mechanism of action of melatonin will help us to understand the role of melatonin *in vivo* on neuro-immunomodulation. Further, we can say that photoperiod plays an important role for the survival of the birds whether it is in term of foraging period, reproduction and growth of young ones. From the results of the present study it can be proposed that the immunity responds to photoperiod and this effect could be due to an increase/decrease in melatonin levels as in nature melatonin is peripherally high in winter when days are short and this high melatonin helps the birds to overcome the winter-bound stress and diseases. The immune response of female birds was high compared to the male birds but the difference was not significant. Our study, by exposing the birds to a summer time long photoperiod during winter time, suggests that the immune system is sensitive to all the long photoperiodic regimes and decreased the immunity as if the birds have entered into summertime. Therefore, melatonin acts as not only a clock and calendar for the reproductive function of birds, but also on the immune status.

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