

Original Paper

**ADAPTIVE SIGNIFICANCE OF ANNUAL VARIATION IN IMMUNE PARAMETERS AND ENDOGENOUS HORMONES (MELATONIN AND THYROXINE) OF A TROPICAL RODENT *FUNAMBULUS PENNANTI***

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**ABSTRACT**

The significance of annual variation in metabolic (thyroxine) and chronobiotic (melatonin) hormones in relation to immune adaptation of the Indian palm squirrel was investigated. The squirrel is summer breeder and, hence, remains healthy during reproductively active phase (Feb – Aug) when melatonin level is low and thyroxine level is high. Our data show that the circulating levels of melatonin (MEL) and thyroxine ( $T_4$ ) have their respective annual rhythms, which are inversely related. Thyroxine level is low during the winter months (Sep-Jan) when metabolic activity of this rodent is also low. During this period melatonin level is high because of the short photoperiod and the squirrels indicate poor reproductive activity, with low gonadal steroids, which could be due to high circulating melatonin. The immune parameters also present an annual rhythmicity, which is parallel with circulating melatonin level, but inversely related to thyroxine level. This suggests that the metabolic hormone, thyroxine, and the chronobiotic hormone, melatonin, might be responsible for maintenance of the immune system to adapt the rodent for the rigors of the seasonal environmental changes.

**Key words:** Annual variation, immune parameters, melatonin, squirrel, thyroxine.

**INTRODUCTION**

Most of the animals experience a rhythmic change in the important environment-dependent variables. Seasonally breeding animals use photoperiodic cues to initiate or terminate specific seasonal activities in order to maintain a positive balance (1) and, ultimately, enhance survival and presumably increase fitness (2). It has been suggested that pineal is involved in the regulation of various circadian rhythms (3). The thyroid gland and its hormone thyroxine have been reported to influence metabolism and reproduction (4) and pineal and its hormone melatonin to influence thyroid function (5) and lymphatic tissue sizes (6). The pineal responds to changes in the photoperiod with alteration in synthesis of melatonin and has been proposed to be a major role player in immune-modulation (7). Reports are available in this regard with respect to the laboratory animals such as rat, mouse and hamster (8). However, the relationship of bone marrow, which is an immune marker for the bone marrow macrophages, with melatonin has received little attention (9). Bone marrow is an extra-pineal source of melatonin (10).

*Funambulus pennanti* is a seasonal breeder and its breeding season extends from Feb to Aug (long photoperiod and high ambient temperature) (11). The gonads regress during late Sep to Nov (short photoperiod and low ambient temperature), followed by a short phase of reproductive quiescence in Nov-Dec (11). In order to

find the relationship between annual rhythmicity of melatonin and thyroxine, on the one hand, and the rhythmicity of immune cells of thymus, spleen and bone marrow, on the other, of this tropical seasonal breeder this investigation was taken up. Monthly data (first week, every month) on immune parameters such as bone marrow and peripheral blood lymphocytes, total leukocyte count and blastogenic response as % stimulation ratio of thymocytes and splenocytes were taken into consideration to record the immune status, and correlated with the circulating melatonin and thyroxine levels for two consecutive years.

**MATERIALS AND METHODS**

All the experiments were conducted in accordance with the Institutional Practices and within the framework of revised Animals (Specific Procedure) Act of 2002 of Government of India on animal welfare. Young adult male (body wt.  $120.00 \pm 5.00$ g) squirrels were collected from the vicinity of Varanasi (Latitude  $25^\circ, 18' N$ , Longitude  $83^\circ 01' E$ ) at the beginning (first week) of each month for two consecutive years. The squirrels were acclimated for 10 –15 days to the laboratory conditions of light, temperature and humidity.

**Sample collection**

To study the annual variation of melatonin, thyroxine and immune status, male squirrels were selected at random ( $n=5$ ) at the end of each month throughout the

year. Five male squirrels were sacrificed by decapitation, under mild pentathol anesthesia, in the first week of every month. Total leukocyte (TLC) and lymphocyte count (LC) of peripheral blood and percent lymphocyte count and macrophages of bone marrow cells were noted. Blood was collected and centrifuged at 3000 rpm for 20 min and plasma was separated for determination of melatonin and thyroxine. Spleen and thymus were dissected and weighed in a digital balance and processed for culture to observe the blastogenic response in terms of percent stimulation ratio (%SR) (12).

### Hormone analysis

The plasma melatonin was determined adopting radio-immunoassay (RIA) according to Rollag and Niswender (13). RIA of thyroxine was conducted using the commercial kit (TT4 RIA; Immunotech, Czech Republic). RIAs for melatonin and thyroxine were validated with respect to specificity, precision (intra- and inter-assay variations) and accuracy. Validation of melatonin RIA in the squirrel has already been reported (14, 15). In the present experiment the lowest sensitivity of plasma melatonin was found to be 0.02ng/ml. The intra- and inter-assay co-efficient of variation were recorded to be 9% and 15 %, respectively. The lowest sensitivity of plasma thyroxine was 15ng/ml. The intra- and inter assay co-efficient of variation were recorded as 10% and 15 %, respectively.

### Percent lymphocyte count (% LC) of peripheral blood and bone marrow

Lymphocytes were counted following the procedure published elsewhere (16).

### Separation of lymphocytes from thymocytes and splenocytes

Thymus and spleen were dissected out in chilled phosphate-buffered saline (PBS). The cell viability was checked adopting trypan blue exclusion method.

### Blastogenic response of the thymocytes and splenocytes

For the study of blastogenic response, the cell suspension was divided into aliquots of 2ml each ( $10^6$  cells/ml) and the control tubes were cultured in the absence of the mitogen whereas the test cultures were stimulated with mitogen Con A at the concentration (5mg/ml) following the method of Pauley and Sokal (11). The lymphoid cell proliferation was assayed by pulse labeling with tritiated thymidine ( $^3\text{H}$ -TdR; specific activity 8.9ci mM; BARC Mumbai, India), 18 hr before the end of the incubation period. A 0.1ml aliquot was counted using a liquid scintillation counter (Packard, USA). Results are expressed

as  $^3\text{H}$ -TdR incorporation counts in splenocytes per min. The percent stimulation was calculated from the ratio of uptake of  $^3\text{H}$ -TdR against stimulation by Con A of splenocytes and thymocytes (16).

### Statistical analysis

Statistical analysis of the data was performed adopting one and two-way ANOVA followed by a student Newman Keul's test. The differences were considered significant when  $P < 0.05$ .

## RESULTS

### Annual variation in the weight of lymphoid organs

Spleen and thymus weights increased from Sept onwards and reached the maximum in Nov. There was a sharp decrease in these lymphoid organs weights in Dec, reaching the lowest in Jan. This was followed by an increasing trend in weight of spleen and thymus from Feb to Mar, followed by a slight decline in thymus weight in Apr. The weight of spleen continued to increase till Jun and it decreased again in Jul (Fig. 1a)

### Annual variation in plasma melatonin and thyroxine

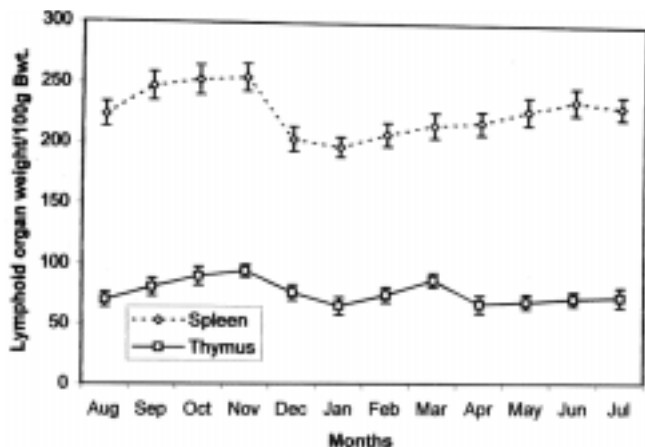
Plasma melatonin recorded the highest in Dec. There was a sharp decrease in plasma melatonin in Feb and the level was maintained until May. It increased in Jun, decreased during Jul-Aug and then increased to reach the peak in Dec (Fig. 1b). An increase was noted in plasma thyroxine from Jun to reach the highest in Aug. It was followed by a decline to reach the lowest in Nov and then increased to reach a second peak in Apr, decreased till Jun and then increased to reach the highest peak in Aug (Fig. 1b).

### Annual variation in total leukocyte (TLC) and lymphocyte counts (LC)

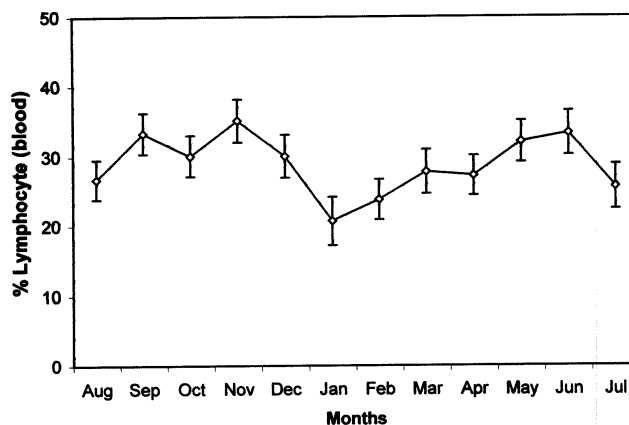
The lowest counts of total leukocytes and lymphocytes were recorded in Jan. However, an increasing trend in LC was found from Feb to Jun and then a sharp decline was observed. An increase in TLC was recorded from Feb to Apr and a slight decrease was found in May, which was followed by increase in Jun but again decreased to the minimum in Jul. Higher the values of TLC and LC were found in Sep, reaching the maximum in Nov. Again a sharp decrease was noticed from Dec onwards (Fig. 2a).

### Annual variation in percent (%) lymphocyte of blood and bone marrow

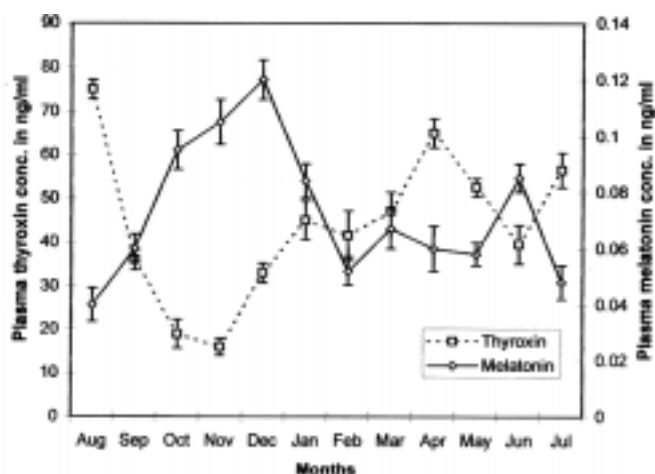
The highest values of % lymphocytes of peripheral blood and bone marrow were found in Nov, followed by a decreasing trend from Dec through Jan. It was followed



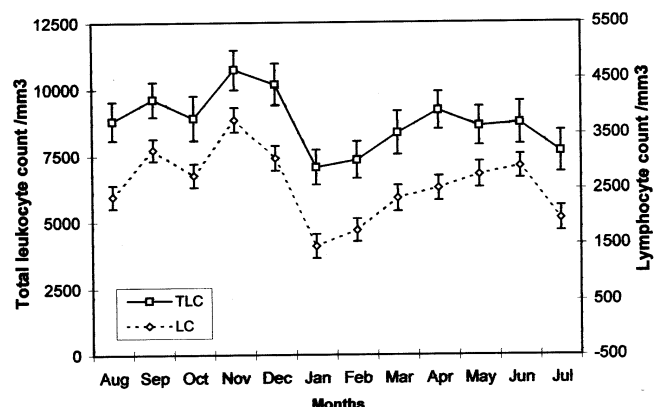
**Fig.1a.** Annual variation in spleen and thymus weight (relative; mg/100g body weight) of Indian palm squirrel, *Funambulus pennanti*. Data represent Mean  $\pm$  SEM.



**Fig.2b.** Annual variation in percent lymphocyte count (% LC) of Indian palm squirrel, *Funambulus pennanti*. Data represent Mean  $\pm$  SEM.



**Fig.1b.** Annual variation in plasma melatonin and thyroxin levels (ng/ml) of Indian palm squirrel, *Funambulus pennanti*. Data represent Mean  $\pm$  SEM.



**Fig.2a.** Annual variation in total leukocyte count (TLC/mm<sup>3</sup>) and lymphocyte count (LC/mm<sup>3</sup>) of Indian palm squirrel, *Funambulus pennanti*. Data represent Mean  $\pm$  SEM.

by increase in Feb and Mar. Again an increase in % lymphocyte of blood was found in Jun. Almost a similar pattern was observed in % lymphocyte count of bone marrow except that from Dec to May there was little change and, suddenly, a slight decrease in Jun followed by an increase in Jul was observed (Fig. 2b, 3a).

**Annual variation in percent (%) macrophage of bone marrow**

There was gradual increase in the % bone marrow macrophages from Aug onwards, reaching the maximum in Nov. A fluctuating trend of decrease and increase was noted from Dec onwards and the lowest % macrophages was found in Jul (Fig. 3a).

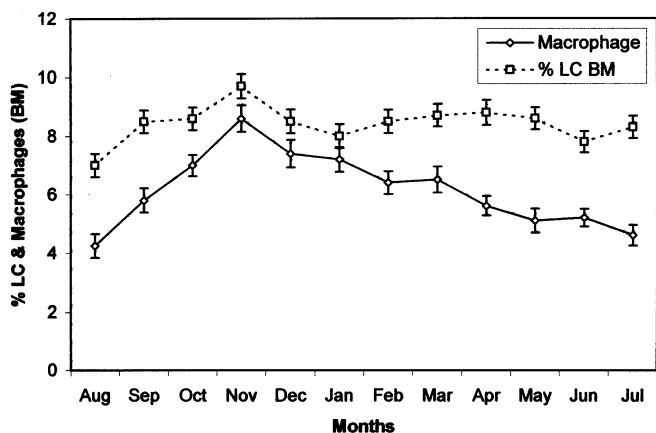
**Annual variation of blastogenic response in terms of percent stimulation ratio (% SR) of thymocytes and splenocytes**

The blastogenic responses of thymocytes and splenocytes were observed in terms of basal as well as T-cell mitogen concanavalin A (Con A)-induced blastogenesis in thymocytes and splenocytes in culture. Stimulation ratio (%SR) of thymocytes and splenocytes showed an increasing trend from Aug to Nov and again from Feb to Mar with a clear decrease in Dec to January. Again, from Apr onwards a slight increase in % SR was noted (Fig. 3b).

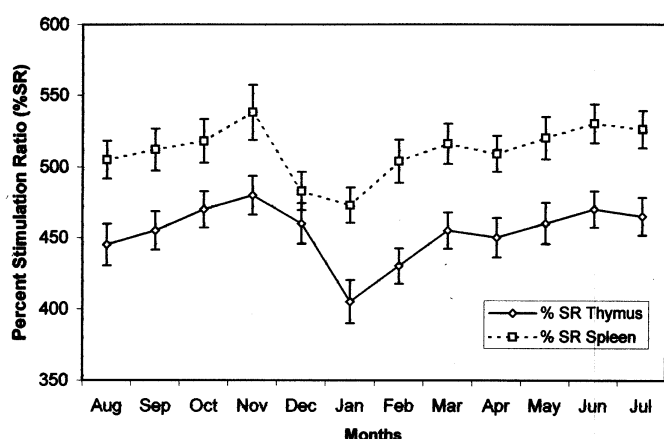
**DISCUSSION**

The ambient environmental factors, such as humidity, rainfall and temperature, in the tropical countries vary significantly during the year. In the present study the relationship between the pineal gland and immune parameters, including hematological parameters of blood and bone marrow, macrophages of bone marrow, lymphoid

organ weight analysis and blastogenic response of thymocytes and splenocytes were studied, through two consecutive years but the data pertaining to only one year is presented since there was no significant difference between the years.. The study was conducted on the squirrels experiencing natural environmental conditions and, hence, the impact of the climatic factors on pineal and immune status was also correlated. The increase of humidity in Jul to Oct, due to the monsoon, would limit the availability of food in nature and the consequent restricted activity of the squirrels would decrease the energy status, which would provide for infections and diseases. During this period the pineal gland becomes active by secreting melatonin, an immuno-modulatory hormone, which would enhance the immune status of the squirrel.



**Fig.3a.** Annual variation in percent lymphocyte and bone marrow macrophages count (% LC & % Macrophages) of Indian palm squirrel, *Funambulus pennanti*. Data represent Mean  $\pm$  SEM.



**Fig.3b.** Annual variation in percent stimulation ratio (% SR) of thymocytes and splenocytes of Indian palm squirrel, *Funambulus pennanti*. Data represent Mean  $\pm$  SEM.

A large-sized spleen during the reproductively inactive phase (Nov – Dec) of this squirrel may, therefore, be interpreted as an enhanced capacity to respond effectively to infection or increased immunological activity from already established infections in contrast to the breeding-related involution of the spleen in the reproductively active phase (Mar – Jul). It is pertinent to point out in this connection that, in addition to immune function, the spleen is known to act as an erythrocyte reservoir (17).

Plasma thyroxine was found to be very low in Nov. However, the peak activity of thyroid, as found in Aug, occurred during the peak of the breeding phase. The period of low plasma thyroxine, Nov - Dec, coincided with the high melatonin. In other words, an inverse relationship between melatonin and thyroxine is maintained throughout the year. We assessed the seasonal rhythmicity in the immune status and found that TLC, LC and % lymphocytes were the highest between Sep and Nov and lowest between Dec and Mar. In addition to assessing the annual variation of cellular immunity, we measured the blastogenic response of thymus and spleen to mitogen Con A. The lowest blastogenic response of thymocytes and splenocytes was found in Apr, whereas % stimulation ratio for both these cells was found in Jan. Other than this, a free running pattern was observed in the blastogenic response and % stimulation ratio. The blastogenic response and % stimulation ratio of thymus and spleen to mitogen Con A exhibited an annual variation as TLC, LC and % lymphocyte of bone marrow and peripheral blood. The bone marrow macrophages were the highest in Nov and there-after, a free running pattern was observed.

The plasma melatonin level was the highest in Dec and lowest in Feb and Jul. While assessing the environmental impact on melatonin rhythm we found that the comparatively short day length, along with decreased temperature, humidity and rainfall, from Oct to Dec, contributes to the peak level of melatonin. On the other hand, longer day length, along with high temperature from Feb to May would have brought about decreased melatonin level to the lowest. The inhibitory effect of sex steroids during the reproductively active phase on pineal/melatonin in this squirrel would regulate the annual melatonin rhythm, which is inversely phased with the gonadal hormones (5, 15). According to the immunocompetence handicap hypothesis the secretion of testosterone during the breeding phase may bring about a decrease in immune function (19). Buchanan (20) suggested that breeding itself might contribute to a physiological stress, which can affect immune function.

The impact of physical factors of the environment since Apr resulted in an increase in all the immune parameters. It could be due to the immune system of this rodent responding positively to the environmental stresses so as to maintain a high level of resistance needed for the conditions prevailing during Oct to Nov when the day length is short and the temperature is low (21, 22). Further, the sudden decrease in all the immune parameters in Dec to Jan could be due to additional stress exerted by the shortened day length, low temperature and increased rainfall during the winter season, resulting in stress and increasing the basal level of melatonin until the end of the winter (Mar). In addition to the impact of environmental factors, the inhibitory effect of sex steroids on the immune system could be another reason for the basal level of immunity during the reproductively active phase, since it has been noted for other mammals that testosterone (23) and estradiol (24) significantly inhibit immunity. Thus, the seasonal variation in immune function is the result of the endogenous annual rhythms of testosterone, corticosterone and thyroxine that are synchronized by melatonin, which also may control the seasonal breeding activity pattern of this squirrel. Seasonal changes in the immune system have been found in a wide range of animal species as reviewed by Zapata and Cooper (25) and Nelson and Demas (26). The view that temporal change in immune-competence is correlated with a change in breeding activity is widely accepted, though it has been poorly studied in wild populations.

When we looked at the seasonal variation in the bone marrow lymphocytes and macrophages we found that high plasma level of melatonin has a strong impact on immune parameters since the latter exhibited a parallel relationship with melatonin. Thus, the high level of melatonin and the maximum value of % lymphocytes and macrophages of bone marrow may be related to the protective mechanism of this rodent against environmental insults. In addition, it is known that melatonin has immunoenhancing property and counteracts immune-depression (if raised by internal level of testosterone and corticosterone) that follows acute stress (27).

Our study reveals parallel/direct relationship between melatonin and immune status of this tropical rodent. It appears that the response to the environment by the pineal gland plays a positive role in maintenance of a seasonal pattern in immunity of the squirrel by an annual rhythmic secretion of melatonin as reported earlier for the other mammals (28). In view of the need of resistance against environmental stress, melatonin would help

restoration of immunity during winter when the squirrel is reproductively quiescent (Sep to Nov). On the other hand, it could be that during late winter (the early reproductively active phase, Jan-Feb) the reduced level of endogenous melatonin is unable to exert any impact on the immune system of the squirrel but the health is maintained by the high gonadal steroid, *i.e.*, testosterone.

The above data of melatonin variation throughout the year clearly shows that short photoperiod cues increased secretion of melatonin, which coincides with enhanced immune parameters such as percent lymphocytes of bone marrow as well as peripheral blood, total leukocyte count, lymphocyte count and splenocyte and thymocyte proliferation in response to mitogen Con A. Our data get strengthened from the earlier reports of short photoperiodic modulation of immune function in this rodent (16).

On the basis of these findings, we conclude that secretion of melatonin, which is induced by short photoperiod, acts as a blaster to the immune function in winter to help the individuals to cope up with seasonal stresses (low ambient temperature) that would otherwise compromise immune function to critical levels. Fluctuating immune function found throughout the year may represent an adaptation that has evolved to increase the possibilities of survival, with energy and hormonal availability. Further, species-specific studies are needed to investigate the natural patterns of immune function and to test if alterations in these rhythms are caused by breeding or differences in the challenges to the immune system at certain time of the year.

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