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CIRCADIAN VARIATION IN PERIPHERAL BLOOD LEUKOCYTES, THE PRIMARY IMMUNE CELLS, IN THE GARDEN LIZARD, *CALOTES VERSICOLOR* (DAUDIN)

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ABSTRACT
The present study was undertaken to explore the circadian rhythmicity in leucocytes in *Calotes versicolor* (Daudin). Adult male lizards were acclimatized to the unconditioned laboratory for a week, lizards (N = 5) were weighed, anaesthetized mildly and sacrificed at each of the following time point: 8 a.m., 12 noon, 04 p.m., 8 p.m., 12 midnight, 4 a.m. and 8 a.m. Blood was collected for determining Total Leukocyte Count (TLC) and Differential Leukocyte Count (DLC).During 24h, TLC ranged between 7.27 ± 1.32 to 12.05 ± 2.69; Monocyte (M) count between 2.22 ± 0.67 to 0.83 ± 0.11; Lymphocyte (L) count between 4.21 ± 1.03 to 2.15 ± 0.25. Amongst the granulocytes, basophil (B) count ranged between 1.83 ± 0.54 to 0.34 ± 0.10; eosinophil (E) count between 5.45 ± 1.36 to 0.40 ± 0.13; neutrophil (N) count between 2.48 ± 0.46 to 0.69 ± 0.28. However, cosinor analysis revealed that only one variable, eosinophil, exhibited statistically validated circadian rhythm; (plotted against 95% confidence limit), Acrophase being at 2040h.

INTRODUCTION

Behavioral and physiological functions, including immune function, are expressed rhythmically across day and night. Most of these rhythms appear to be endogenous, but are generally synchronized with light: dark cycle. These daily rhythms provide a temporal frame necessary for adequate homeostasis and are referred to as circadian rhythm. Circadian rhythm enables organisms to adapt to the daily environmental changes such as, light, temperature etc., and serve to synchronize multiple molecular and biochemical processes with each other.

Peripheral blood leucocytes are primary immune cells and important elements of non specific immunity. Variation in these is widely taken into consideration as functional assessment of immune status of the animals. The circulating formed elements in the peripheral blood show highly reproducible circadian rhythms. Not only the number but also the reactivity of peripheral blood leucocytes varies predictably as a function of time, as shown for circadian rhythm in proliferative response of lymphocyte *in vitro* to mitogens like phytohemagglutinin (PHA) and pokeweed mitogen (PWM) reviewed by Haus *et al.* (1983), in natural killer cell activity (Arjona and Sarkar, 2006, 2008) and in relative numbers of circulating white blood cells and their subsets (Kawate *et al*., 1981; Dimitrov *et al*., 2009). Circadian variation in leukocyte and some lymphocyte subsets plays a part in the development and regulation of immune response as reviewed by Berger (2004). It is also possible that local clocks in immune cells directly control cellular immune functions (Keller *et al*., 2009).

Reptiles occupy a crucial berth in the phylogeny of vertebrate evolution however; chronobiological studies on peripheral blood leucocytes and its reactivity are meager in reptiles (Pati *et al*., 1987). Barring few papers, diurnal and seasonal variations reported so far in reptiles are of questionable validity, because of classical and macroscopic methods of data handling (Vivien-Roels *et al*., 1979). Circadian rhythm in eosinophil in snake has been reported (Pati and Thapliyal, 1984; Pati, 1989). More studies are required to throw light on reptilian leucocytes and its reactivity. Hence, this study was undertaken to explore the circadian rhythmicity in leucocytes count in the garden lizard, *Calotes versicolor*.

MATERIALS AND METHODS

Animals and experiment

Adult male garden lizards, *Calotes versicolor* (Daudin), were caught locally in suburbs of Varanasi (25°18′NL and 83°1′EL) during the month of June. They measured snout - vent length 10 ± 2cm and average body weight 30 ± 2g. They were immediately brought to laboratory, housed in vivarium (wire net cages of size 18 x12x 10″). They were provided with food (crickets, maggots, flies) and water *ad libitum*. All cages were kept in a well ventilated room experiencing natural day length and temperature. They were acclimatized to the above conditions for a week before sampling. Sampling was done during the mid of June. A group of five lizards were weighed and anaesthetized under mild anesthesia at each of the following time point; 8a.m., 12noon, 04p.m., 8p.m., 12

midnight, 4a.m. and 8a.m. Blood was collected in heparinized syringe with a hypodermic 20 gauze needle through cardiac puncture and used for estimation of total leukocyte count (TLC) and differential leukocyte counts (DLC). Data were presented as mean ± SEM and expressed as number of cells x 10^3 / mm^3.

Statistical analysis

The circadian rhythms were characterized by the following parameters, obtained by Cosinor analysis (Bingham *et al.,* 1982; Cornelissen and Halberg, 1994): mesor (rhythm adjusted mean; when the interval of time between data sampling is constant, it equals the arithmetic mean), amplitude (A, semi difference between the highest and the lowest value), acrophase (ϕ, timing of overall high value), and p, indicating the significance of the oscillation.

RESULTS AND DISCUSSION

In the present study on a lacertilian species, circadian variation in peripheral blood leukocyte counts is demonstrated. Observations on different peripheral blood leucocytes at various time – point during 24h are presented in chronogram (Figs. 1, 2). As shown in chronograms, TLC ranged between 7.27 ± 1.32 to 12.05 ± 2.69, during 24h, being the lowest at 4.00 hours and the highest at 16.00h. TLC was, however, lower in morning hours. Monocyte (M) count was highest (2.22 ± 0.67) at 16.00 h and lowest (0.83 ± 0.11) at 24.00 h. The number of Lymphocyte (L) was found to be highest (4.21 ± 1.03) at 8.00 h and lowest (2.15 ± 0.25) at 20.00 h. Amongst the granulocytes: basophil (B) count was highest (1.83 ± 0.54) at 20.00 h and lowest (0.34 ± 0.10) at 8.00 h; eosinophil (E) count was highest (5.45 ± 1.36) at 24.00 h and lowest (0.40 ± 0.13) at 8.00 h; neutrophil (N) count was highest (2.48 ± 0.46) at 8.00 h and lowest (0.69±0.28) at 4.00 h. However, when data was analyzed with the help of cosinor analysis to characterize the circadian rhythm, it was found that only one variable, eosinophil, exhibited statistically validated circadian rhythm (plotted against 95% confidence limit), Acrophase being at 2040 h and Amplitude was 1498.09. Peaks of TLC and B occurred in evening hour; that of N and M, in afternoon hours; while peak of L occurred in forenoon hours (Fig. 3). Cosinor Rhythmometry of circadian variation in different leucocytes is presented in Table 1.

The present observation corroborates with that of Pati and Thapliyal (1984) in an ophidian species. The circannual variation in the timing of eosinophil circadian rhythm in snake is also reported under natural condition as well under constant condition of light and temperature (Pati and Thapliyal, 1984;

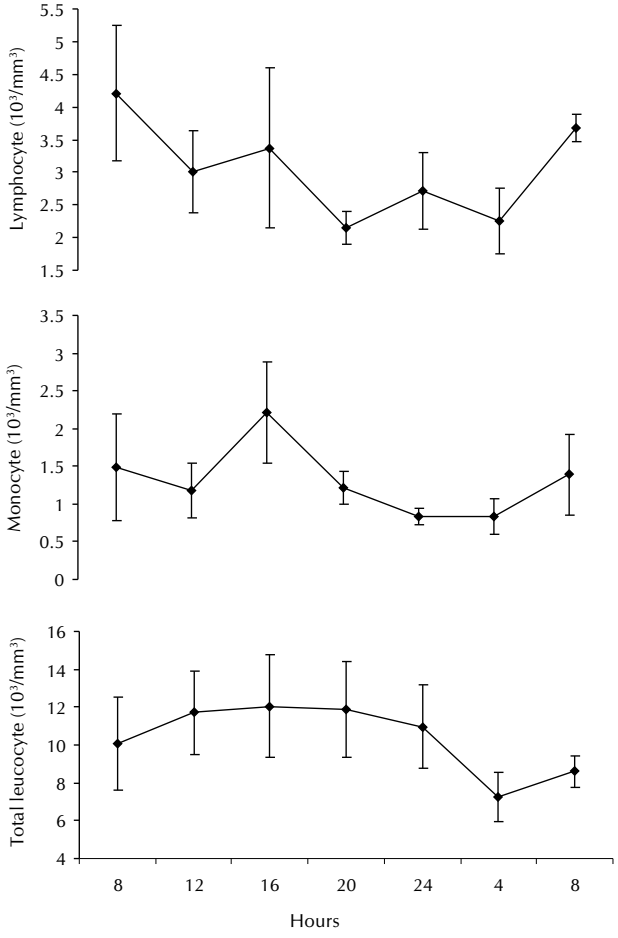

Figure 1: Chronomap of lymphocyte, monocyte and total leucocytes in the garden lizard, *Calotes versicolor*

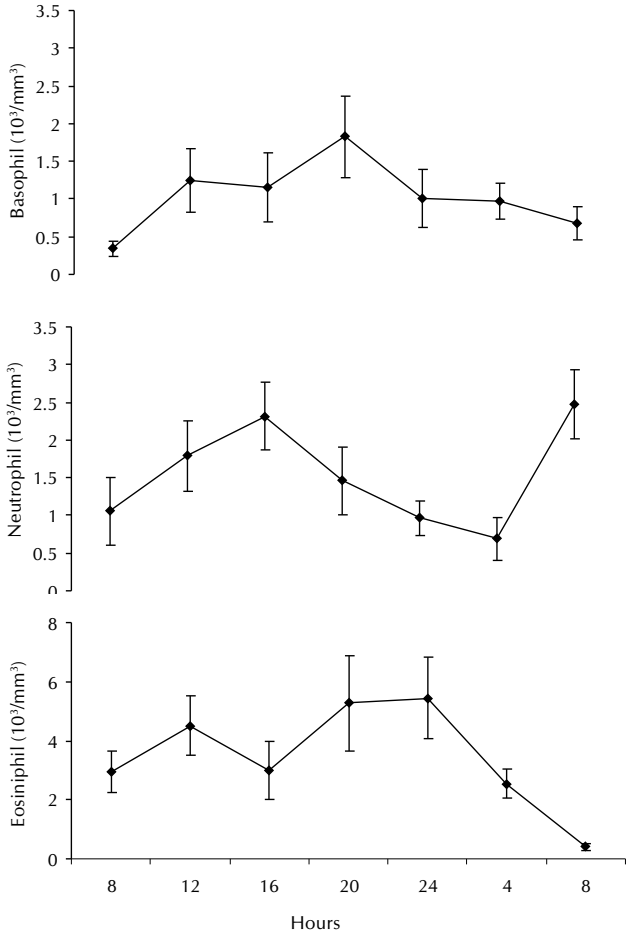

Figure 2: Chronomap of basophil, neutrophil and eosinophil in the garden lizard, *Calotes versicolor*

Table 1: Parameters of Cosinor Rhythmometry of circadian variation in leucocytes count

Variable	Data	Rhythm	Mean, M ± SE	Amplitude, A	Acrophase ϕ in h
	Point	detection		(95% CL)	(95% CL)
Total Leucocyte count	34	0.32	10586.83 ± 985.31	2144.27	16.81
Eosinophil	34	0.04	3705.12 ± 428.36	1498.09 (2815.03,2973.25)	20.4 (14.9, 25.7)
Neutrophil	34	0.08	1508.69 ± 203.25	705.46	14.0
Basophil	34	0.17	1099.15 ± 189.42	492.18	19.0
Lymphocyte	34	0.21	2966.06 ± 282.57	710.37	10.3
Monocyte	34	0.36	1308.38 ± 231.22	501.83	14.3

Pati, 1989): the acrophase of eosinophil rhythm usually occurred between 1400 and 1700 h.

Periodic changes in the number of leukocytes circulating in the peripheral blood might result from several factors. These include the distribution of circulating and marginal cell components of tissues and organs, influx from storage sites, cell proliferation, and release of *de novo* cells into the circulation, and cell destruction and removal (Haus and Smolansky, 1999). Circadian variation in the number of leukocytes circulating in the peripheral blood might also result due to some endogenous and exogenous factors. The endogenous factor like endocrine hormone, that itself show circadian rhythmicity, may be responsible for variation in leucocyte count. In cold blooded animals, like fishes, adrenocorticoids decrease the number of lymphocytes (Robertson *et al.*, 1963; McLeay, 1973). The number of total leucocytes of vertebrates in the circulating blood is reported to be generally related with adrenal cortical and medullary hormones. Cortisol and epinephrine is reported to control circadian rhythm in T cell subsets (Dimitrov *et al.*, 2009) Thus, adrenal cortical and medullary hormones may be responsible for circadian variation in the number of leukocytes circulating in the peripheral blood of the lizard, *Calotes*.

Further, since melatonin is an internal synchronizer of circadian oscillation (Siopes and Underwood, 2008), one can look for a melatonin influence on blood and haemopoetic cells to explain the rhythm in these cells. Immunomodulatory role of melatonin has been well documented (Maestroni, 1999; Reiter *et al.*, 2000; Skwarlo-Sonta, 2002). Melatonin administered *in vivo* increases the leucocyte counts (Karimungi and Joshi, 1996). Rogers *et al*. (1997) have reported that melatonin not only affect lymphocyte proliferation, but also potentiate corticosteroidal inhibition of lymphocyte proliferation. Thus, it may be suggested that in the present lacertilian model *Calotes* the neuroendocrine and immunological interactions can be a component of the endogenous regulation of circadian rhythm in blood system.

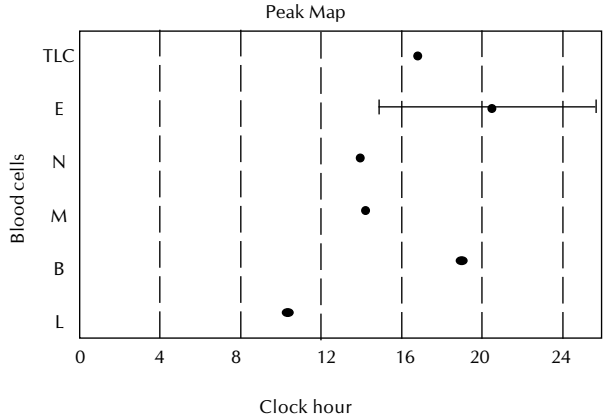

Figure 3: Peak map showing average peak occurrence of rhythms in total leucocyte count (TLC), eosinophil (E), neutrophil (N), monocyte (M), basophil (B) and lymphocyte (L). One variable (E) exhibited statistically validated circadian rhythm (Plotted with 95% confidence limits). The peaks of TLC, N, M and B occurred in afternoon hours, while that of the E and lymphocyte occurred in the evening and morning hours, respectively

So far exogenous factor is concerned, recently Berger (2004) has suggested, "Visible light (400-700 nm) can penetrate epidermal and dermal layers of the skin and may directly interact with circulating lymphocytes to modulate the immune function". In contrast to visible light, *in vivo* exposure to UVB (280-320 nm) and UVA (320-400 nm) radiation can alter the normal human immune function only by a skin mediated response (Roberts, 2000). Thus, visible light, UVA and UVB may influence the circulating lymphocyte number in the lacertilian model *Calotes*.

In the present study it was also found that when eosinophils were minimal, the lymphocytes were maximal and vice versa. This may reflect a difference in their origin. Eosinophils in mammals and other vertebrates are supposed to be derived from bone-marrow only and lymphocytes from lymphoid tissues as well as bone marrow.

Whether the circadian rhythmicity in leucocyte counts in this lacertilian species is the reflection of the rhythmicity of melatonin together with endogenous hormones or the influence of exogenous factor, light, needs further exhaustive investigation.

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