Annexure I

Abstract/Synopsis

Reptiles are ectothermic amniotes, providing the key link between anamniotic ectotherms, fishes and amphibians, and amniotic endotherms, birds and mammals. A greater understanding of reptilian immunity will provide important insights into the evolutionary history of vertebrate immunity. Hence, this study was undertaken to examine the effect of melatonin and testosterone hormones and photoperiodic modulation on immune responses of splenic macrophages. Also, seasonal as well as circadian variation in immune responses of splenic macrophages in the fresh-water snake was studied. Following immune parameters were taken into consideration:

- Spleen mass and cellularity
- Splenic macrophage Phagocytosis
- Quantitative NBT reduction
- Nitrite release
- Splenic lymphocyte proliferation

To study the effect of melatonin hormone, melatonin injections in saline vehicle (dose: 5 µg /g body weight and 10 µg /g body weight) were given during evening hours, and alteration in lymphoid organ mass, splenic macrophage phagocytosis, Nitric oxide (NO) production (Nitrite assay), superoxide production (NBT reduction assay) and lymphoproliferation (MTT assay) were studied on the day following 10^{th} and 20^{th} injection. No consistent and significant change was observed in phagocytic response of splenic macrophages harvested from snakes treated with either dose of melatonin for different duration. Nitrite release and superoxide production by splenic macrophages were significantly ($p<0.05$) higher in snakes receiving melatonin injections. Exogenous melatonin enhanced the mitogen-induced splenic lymphocyte proliferation only at the dose 10 µg/ g body weight, but not at that of 5 µg one. *In vitro* melatonin also invariably has enhanced the mitogen-induced splenic lymphocyte proliferation, and vigorous response has been observed in splenocytes harvested from snakes receiving 10 µg /g body weight melatonin injection for 20 days, when splenocytes were induced by mitogens: 10 µg mL^{-1} of Con A and PHA and by 20 µg mL^{-1} of LPS. Taken together, the

results of the present study demonstrate that *in vivo* melatonin treatment enhances innate immunity, in general except phagocytosis, in the fresh-water snake, *N. piscator*.

To study seasonal variation in immune functions, the requisite number of animals were sacrificed under mild anaesthesia during mid of each month. In all assay, sample size was 5 to 6. Analysis of variance reveals that there was no significant variation in splenic mass of the fresh-water snake during different months of study period, though spleen size has a trend to be high in autumn and winter months and low in spring and summer. Spleen cellularity was recorded high in winter months and again in September; while it remained low during rest of the year. No definite pattern was observed in phagocytosis by splenic macrophages. The percent phagocytosis varied between 42 to 60 %, being highest in month of February. Analysis of variance revealed that free radical production and lymphoproliferation varied significantly during different months. Super oxide production, as judged by NBT reduction, was found to be high during spring season. Nitrite release by splenic macrophages was high in months of January, May and September - October. The splenic lymphocyte proliferation, both basal as well as Con A induced, was highest in month of May, followed by a little low in January and October: it remained low during rest of the year.

In the study of circadian variation, Cosinor analysis of the data revealed that no significant circadian rhythm was validated in fluctuation of splenosomatic index and spleen cellularity in all the three seasons. The percentage phagocytosis had significant rhythm of 24 h in summer and spring seasons, but not in winter; and no significant circadian rhythm was validated in phagocytic index. NBT reduction and nitrite release by splenic macrophages of fresh-water snake also varied significantly in circadian manner during winter season. But, significant circadian rhythm was absent in NBT reduction and nitrite release during summer season. A significant circadian rhythm was observed in splenocyte proliferation (Basal) and splenocyte proliferation (Con A10 stimulated) in all three seasons: summer, winter and spring. A significant phase shift in splenocyte proliferation (Con A10 stimulated) was obtained in all three seasons with a trend of delayed phase shift from winter to spring and spring to summer.

Most formal studies on seasonality have focused on day lengths i.e., photoperiod, as the environmental cue used by animals to coordinate intrinsic rhythms with extrinsic seasonal environmental changes. To study the effect of photoperiodic manipulation on immune parameters, snakes were maintained in 24h dark (DD), 24h light (LL) and at normal day length (10L:14D). Spleen mass was increased, but insignificantly, in the animals subjected to DD. No effect was observed in the animals kept under continuous light, i.e. LL, when compared with control ones. On other hand, spleen cellularity was also increased, but insignificantly, in the animals of both groups kept in DD as well as LL. No significant ($p<0.05$) change was observed in percentage phagocytosis and phagocytic index in animals kept under DD or LL, as compared with animals kept in 10L: 14D. Super oxide production, as measured by NBT reduction assay, was reduced significantly ($p<0.05$) in the animals kept either in 24D or 24L. Production of nitric oxide by snakes' splenic macrophages, as measured by nitrite assay, was significantly ($p<0.05$) decreased in animals kept under LL; while no change in nitrite release was observed in animals kept in DD, when compared with animals kept in 10L:4D. There was significant ($p<0.05$) enhancement of mitogen induced splenocytes proliferation in cultures obtained from animals kept under DD. To study whether melatonin could induce the splenocytes proliferation, splenocytes cultures obtained from all group animals were treated with i*n vitro* melatonin (500 pg ml^{-1}, final concentration). *In vitro* melatonin significantly ($p<0.05$) enhanced the splenocyte proliferation of the cultures obtained from animals kept in LL, as compared with cultures from 10L:14D animals, but not in those of DD animals.

To study the effect of *in vitro* testosterone, splenic macrophages monolayer prepared on slides was exposed to different concentrations male sex hormone, (0.1, 1, 10, 50 and 100 ng ml^{-1}). After 4 hour of incubation, the monolayers were washed three times to remove testosterone, and phagocytosis was allowed to proceed. Effect of different concentrations of *in vitro* testosterone was also studied on NBT reduction, nitrite release and splenic lymphocyte proliferation (basal as well as mitogen induced). It was observed that percentage phagocytosis decreased significantly in correlation with the *in vitro* testosterone concentration. Maximum reduction of percentage phagocytosis was obtained

at 100 ng ml^{-1} concentration of testosterone. Effect of *in vitro* testosterone on NBT reduction was found to be differential: as NBT reduction was decreased at 10 ng ml^{-1} concentrations, but no decrease at 100ng ml^{-1}, and again decreased at 1000 ng ml^{-1}. The decrease was maximum at 1000 ng ml^{-1} testosterone concentration. Nitrite release was also significantly ($p<0.05$) reduced by *in vitro* testosterone treatment in a concentration dependent manner. When the macrophages were pre-incubated with testosterone receptor antagonist, Cyproterone Acetate (CPA), the decrease in Nitrite release was alleviated, as Nitrite release was comparable to that of control splenocytes incubated in medium alone. There was no change in basal proliferation of splenic lymphocytes in relation to *in vitro* testosterone treatment; while mitogen induced proliferative response reduced significantly ($p<0.05$) by *in vitro* testosterone. Present finding reveals testosterone as inhibitory to splenocytes immune responses in the fresh-water snake, *Natrix piscator*.

Based on the results in the present work, it may be suggested that immune function in reptiles varies in different seasons. Pineal gland, by diurnal rhythm of synthesis and release of its principal hormone, melatonin, transmits signals to the immune system, and thus, regulates the immune responses. Melatonin injection enhanced the innate immune functions, and thus may be considered as an immunoregulatory factor in the development and maturation of immune system and in the progression of the immune response in our animal model, *Natrix piscator*. Our study also suggests there is circadian variation in innate immune parameters, some of which also show a clear phase shift according to the season. Further, *in vitro* testosterone reduces immune response in a manner dependent to testosterone concentration used. Differential effect of photoperiodic regimens, in immune parameters studied, is also established in our study. It is concluded that relationship exists between external environmental factors, melatonin, sex steroids and immune system which might have led to the adaptations to best fit the animal in the challenging conditions present.