Chapter-2

SEASONAL VARIATION IN IMMUNE RESPONSES OF BLOOD LEUCOCYTES IN THE FRESH-WATER SNAKE, *NATRIX PISCATOR*

2.1. Introduction

Changes in environmental factors throughout the year determine season of the year. The photoperiod length is used by most of the organisms as a seasonal marker, because it is the most reproducible and predictable sign of the changing season (Goldman, 2001; Prendergast *et al.*, 2002). Nevertheless, the changes of ambient temperature, humidity or food access may also be used as seasonal cues. The environmental changes drive the organism's seasonal rhythms in some species; whereas in others, these merely synchronize the endogenous circannual rhythms with the season of the year (Hoffman and Reiter, 1965).

Varying environmental factors in relation to season may impose fluctuation in non-specific immune function, besides other physiological functions. Organisms face seasonal challenges by regulating their internal physiology and by behavioral adaptations. Physiologically, to adapt to the annual changes, the organisms display seasonal rhythms. Seasonal adaptation involves the changes in reproductive status, feeding behavior, fur colour and quality, and thermoregulation. These changes increase the viability of organisms during the unfavourable seasons of the year. Breeding, molting, migration, territorial defense and other energetically expensive activities are synchronized to coincide with the availability of food or favorable local conditions; these typically occur during long days (Bronson, 1989; Wingfield and Kenagy, 1991; Wingfield and Farner, 1993).

Further, several physiological processes, including growth, cellular maintenance, immune function, thermogenesis, and reproductive processes are energy demanding.(Merino *et al.*, 2000; Sinclair and Lochmiller, 2000). Most species, not buffered from potentially dramatic seasonal changes in their environment, may presumably become sick and die from a direct failure to balance their energetic demands due to exposure to extreme seasonal conditions.

Maintaining optimal immune function related to survival is energetically expensive; the cascade of mitotic activities among immune cells, the onset and

maintenance of inflammation and fever, and the production of humoral immune factors, all require significant energy (Kelley, 1985). Mounting an immune response requires resources that could otherwise be allocated to other biological functions (Sheldon and Verhulst, 1996). Thus, activation of immune function is costly (Nelson *et al.*, 2002; Demas *et al.*, 2003a). Consequently, animals may maintain the highest level of immune function that is energetically possible, given the constrains of processes essential for survival, growth, reproduction, thermogenesis and other activities (Festa-Bianchet, 1989; Richner *et al.*, 1995; Deerenberg *et al.*, 1997). It is observed that immune function fluctuates seasonally and is compromised during times of breeding, migration and molt (Zuk, 1990; John, 1994; Zuk and Johnsen, 1998). The trade off between investing in reproduction and survival has been documented in mammalian species (Sheldon and Verhulst, 1996; Lochmiller and Deerenberg, 2000; Norris and Evans, 2000).

Numerous field studies have reported seasonal alterations in immune function in a variety of species; whereas laboratory studies have focused on the role of photoperiod in modulating immune function (Nelson and Demas, 1996; Hotchkiss and Nelson, 2002). Typically immune function is enhanced prior to the onset of demanding winter conditions (Nelson *et al.*, 1995; Nelson and Demas, 1996; Demas and Nelson, 1998b; Hotchkiss and Nelson, 2002). Reduction in cell mediated and humoral immune functions as well as decrease in mass of lymphoid organs during winter months have been reported in birds, mammals and some reptiles (Nelson *et al.*, 1994, 2002).

Reptiles are ectothermic amniotes, providing the key link between ectothermic anamniotic fishes and amphibians, and endothermic amniotic birds and mammals. A greater understanding of reptilian immunity will provide important insights into the evolutionary history of vertebrate immunity (Zimmerman *et al.*, 2010).

Seasonal variation in lymphoid organ mass has been reported in reptiles: *Clemnys leprosa, Spalerosophis diadema, Testudo mauritonica, Scincus scincus, Psammophis schokari and Mauremys capsica* (Aime, 1912; Hussein *et*

al., 1979a, b; El Ridi *et al.*, 1981; Leceta and Zapata, 1985; Zapata, 1996). Effect of seasonal changes on the structure of lymphoid organs, cell viability, proportion of T and B cells, antibody titers, responses to mitogens and mixed leucocyte reactions have been reported in Egyptian Lizards (El Ridi *et al.*, 1981). Seasonal variations in Cell-Mediated and Humoral Immunity have been reported in the snake, *Psammophis schokari* (El Ridi *et al.*, 1981); the, lizard, *Scincus scincus* (Hussein *et al.*, 1979b); the turtle, *Mauremys capsica* (Leceta and Zapata, 1986). Peripheral blood leucocyte function, mitogen induced peripheral blood lymphoproliferation, is reported to vary seasonally in the turtle, *Mauremys capsica* (Munoz *et al.*, 2000; Munoz and Fuente, 2001, 2003).

Reptilian immunity is complex but overall, there is considerably less known about immune function in reptiles. The aim of the present study was to understand seasonal changes in blood leucocytes innate immune responses in fresh water snake *Natrix piscator*. In the present study, profile of leucocytes, phagocytosis, superoxide anion production, NO production and lymphocyte proliferation were taken into account as the parameter of nonspecific immune response for study.

2.2. Materials and Methods

2.2.1. Animals

Sexual dimorphism is reported in innate immune responses of lizard (Mondal and Rai, 1999a, b); therefore, only male individuals were used in this study throughout. Fresh-water snakes, weighing 80-120 g, were obtained in beginning of each month from a local supplier who collected these animals in the suburbs of Varanasi (28^0 18' N; 83^0 1' E). Animals were brought to the unconditioned laboratory. Animals were housed in vivarium (wood and wire net cages; size 50x30x30 cm). Each cage had wooden floor and frame with wire net sides, one side being window. Each cage had an earthen bowl (4 L capacity) filled with water to accommodate 4-5 snakes. Snakes were fed on small fishes once a week. Cages were cleaned, and bowl water was changed next day following feeding. Animals were acclimated to the laboratory

conditions for two weeks, and experiments were performed. The guidelines of the committee for the purpose of control and supervision of experiment on animals (CPCSEA), Ministry of Statistics & Programme Implementation, Government of India, were followed in maintenance and sacrifice of animals.

2.2.2. Chemicals

MTT [3-(4, 5-dimethylthiozol-2-yl)-2, 5 diphenyl tetrazolium bromide], NBT (Nitroblue Tetrazolium salt), Melatonin, and mitogens (Con A, Concanavalin A and LPS, Liposaccharide) were purchased from Sigma Chemicals. Culture medium (RPMI-1640), lymphocyte separation medium (HiSep), L-glutamine, Gentamycin, fetal bovine serum (FBS), dimethyl sulfoxide (DMSO), and other chemicals were purchased from Himedia Laboratories Pvt. Ltd. (India).

The culture medium was supplemented with 1.0 µl ml^{-1} Gentamycin, 10 µl ml^{-1} of 200 mM L-glutamine, 10 µl ml^{-1} anti-anti (Gibco) and 5% FBS and referred to as complete culture medium.

2.2.3. Experiments

To study seasonal variation in immune functions, the requisite number of animals was sacrificed under mild anaesthesia during mid of each month. Blood was isolated through cardiac puncture. Blood was kept at 4°C and soon after it was utilized to study the following immune parameters:

- TLC
- DLC
- Blood Phagocytosis
- NBT slide assay
- Quantitative NBT reduction assay
- Nitrite assay
- Splenic lymphocyte proliferation

In all assay, sample size was 5 to 6.

2.2.4. Total leucocyte count (TLC)

For TLC, 20 µl of blood was diluted twenty times with Turk's fluid (0.2% gentian violet solution in 3% acetic acid). The diluted blood was applied on Haemocytometer Neubauer counting chamber (ROHEM, India), and cells from the four chambers of a square millimeter were counted under the microscope, and number of leucocytes/mm^3 was determined.

2.2.5. Differential leucocyte count (DLC)

For DLC, a uniform blood film was smeared on clean glass slide. The blood smear was air dried and stained in a mixture of Giemsa and Leishman stain. After washing under tap water, the slides were dried, dehydrated, cleared in xylene and mounted in DPX. The purpose of a differential leucocyte count is to establish the relative percentage frequency of each cell type. Stained slide was observed under oil immersion objective from the upper edge of the smear to the extreme lower edge. Hundred leucocytes were identified and counted. Once the relative percentage frequency of each type of cells was obtained, their number is calculated in mm^3 blood from total leucocyte count.

2.2.6. Phagocytic assay

For phagocytic assay, the yeast cells were used as target cell. The yeast cell suspension was prepared by mixing 20 mg of commercial baker's yeast (*Saccharomyces cerevisae*) in 10 ml of 0.2 M PBS. The suspension was kept at 80°C for 15 min. The cells were washed thrice in PBS and finally suspended in complete culture medium to get a concentration of $1x10^8$ cells ml^{-1}.

Equal amount of blood and yeast cells were mixed and incubated for 30 min and 60 min. After incubation, a thin smear was made on a clean glass slide, air dried, fixed in methanol, stained with Giemsa, and examined under oil immersion. For each slide, a total of 100 phagocytes were examined randomly without any predetermined sequence. The phagocytic index was determined by calculating the average number of yeast cells engulfed by single phagocyte. The percent phagocytosis was calculated by dividing the number of phagocytes showing phagocytosis by 100.

2.2.7. Isolation of leucocytes

Peripheral Blood Leucocytes (PBL) were collected from the buffy coat (the layer of PBLs between the plasma and RBCs) using a slow spin technique as described by Keller *et al.* (2005). The tubes were centrifuged at 42xg for 25 min at 8°C. The PBLs were collected by gently swirling the buffy coat into the plasma and transferring the cells into a new tube. Following centrifugation at 200xg for 10 min, the plasma was removed and the cell pellet was gently resuspended in 1 ml of complete culture medium.

2.2.8. Quantitative NBT reduction assay

Superoxide anion production by phagocyte was determined as the reduction of NBT. NBT is a water soluble, yellow colored and membrane permeable dye. It is reduced into NBT-diformazan (Purple color) by superoxide. NBT assay was performed following the methods of Berger and Slapnickova (2003).

Leucocytes were counted and adjusted to $2x10^6$ cells ml^{-1} in complete RPMI. Cell viability was checked through trypan blue exclusion test, which exceeded 95%. 50 µl of leucocyte cell suspension ($1x10^5$ cells) was mixed with 50 µl of RPMI containing NBT (1 mg ml^{-1}) in culture plate (96 well) in triplicates from each animal. Well with culture medium (100 ml) without cells in triplicates served as blank. Plate was then incubated in CO_2 atmosphere at 25°C for 2 hr, centrifuged at 700xg, washed with PBS and fixed in 70% methanol. 20 µl of 0.1% triton X-100 was mixed in each well. The formazan crystals, present inside the cells, were dissolved by mixing 120 µl KOH (2 M) and 140 µl DMSO in each well. Optical density was measured at 620 nm with the help of ELISA plate reader (Thermo Multiscan). Following blank subtraction, triplicates were averaged.

2.2.9. Nitrite assay

Nitric oxide (NO) is a major effector molecule of cellular cytotoxicity. It is a highly unstable compound produced from L-arginine by enzyme Nitric oxide synthase (NOS). Soon after production, NO decomposes to other nitrogen oxides such as nitrate (NO_3^-) and nitrite (NO_2^-) popularly known as

RNI (Jorens *et al.*, 1995). So, nitrite was assayed as a marker of cytotoxicity. Nitrite content was measured by the method of Ding *et al.* (1988).

Briefly, 100 µl of leucocytes ($1x10^6$ cells ml^{-1}) was added in each well of 96 well culture plate. Plates were incubated in CO_2 atmosphere at 25°C for 24 hours, then centrifuged at 200xg and supernatant was collected. Equal volume of supernatant and Griess reagent (1% sulfanilamide in 3N HCl and 0.1% naphthylenediamine dihydrochloride in distilled water) are mixed and optical density of the solution was measured at 540 nm with the help of ELISA plate reader (Thermo Multiscan). Culture medium alone without any cell served as blank. All the samples were taken in triplicate. Following blank subtraction, triplicates were averaged.

2.2.10. Lymphocyte proliferation assay

The lymphocyte proliferation was assessed using colorimetric assay based on membrane permeable dye (tetrazolium salt, MTT), following the methods of Berridge *et al.* (2005). The colorimetric method, utilizing tetrazolium salts, has been an advantageous alternative method measuring lymphoproliferation (Mosmann, 1983). In metabolically active cells, tetrazolium salts are incorporated into active mitochondria, the tetrazolium rings of MTT is cleaved by mitochondrial dehydrogenase enzyme and bioreduced into dark blue formazan crystals which are impermeable to the cell membrane. Solubilisation of cells by the addition of a detergent results in liberation of the crystals. The quantity of formazan product as measured by amount of absorbance 570 nm is directly proportional to the number of living cells in culture (Cory *et al.*, 1991). Thus, quantifying the conversion of salts by mitochondrial dehydrogenases in blue coloured formazan product provides a measure cell number (not mitoses *per se*) during last hours of *in vitro* culture. The accumulation of coloured formazan products is positively correlated with incorporation of ^{3}H-thymidine into cellular DNA in the S-Phase of cell division during last hours of *in vitro* culture, which is a direct measure of blastogenesis under the conditions of mitogenic stimulation (Gieni *et al.*, 1995).

Blood lymphocytes were isolated by density gradient centrifugation using HiSep (Density 1.077 g ml^{-1}). Whole blood (1ml) was overlaid on 1ml HiSep in RIA vial and centrifuged at 400xg for 30 minutes with brakes off at 8°C. Using a clean glass Pasteur pipette, the lymphocytes layer, at the interface between medium and HiSep, was carefully aspirated, washed three times with PBS, counted and assessed for viability on a haemocytometer by trypan blue exclusion. Viable cells (> 95%) were adjusted to $2x10^6$ cells ml^{-1} with culture medium.

Basal as well as mitogen induced *in vitro* lymphocyte proliferation was assessed in the presence of T cell mitogen [concanavalin A (Con A)] and B cell mitogen [lipopolysaccharide (LPS)]. Stock solution of mitogens was made in 0.2 M PBS (pH 7.2) at a concentration of 1 mg ml^{-1}. Further dilution was made in culture medium: 10 μg ml^{-1}, final concentration for ConA and 20 μg ml^{-1}, final concentration for LPS (these respective concentration of mitogen were found sufficient to induce proliferation, **see Chapter 1**). Flat bottom 96 well culture plates were used. 50 μl of mitogen and 50 μl of cell suspension ($2x10^6$ cells ml^{-1}) was seeded into well of culture plate. To study spontaneous or basal proliferation, 50 μl cell suspension was seeded into well of culture plate along with 50 μl of mitogen-free culture medium. Additional well containing only 100 μl of culture medium served as blank. All assays were made in triplicates form each experimental animal.

Plates were incubated in humidified CO_2 atmosphere at 25°C for 48 hr, after which 10 μl of MTT reagent (5 mg ml^{-1}) was added to each well, and plates were again incubated overnight in humidified CO_2 atmosphere at 25°C. Following incubation, the supernatant was aspirated, and 100 μl of DMSO was added to each well to solubilize the formazan crystals. Absorbance was measured at 570 nm with the help of ELISA plate reader (Thermo Multiscan). Following blank subtraction, triplicates were averaged.

2.2.11. Statistical analysis

Data are presented as mean ± SEM. Means were compared by Analysis of Variance (ANOVA).

2.3. Result

When the variation in total leucocyte count was analyzed with ANOVA, it was found to be significant. Total leucocytes count was found to be in the range of 3400 per mm^3 to 7333 per mm^3 in the snake during the whole period of study (Fig. 2.1). It started increasing from February and remained more or less in higher side up to month of August, decreased abruptly to the lowest in September. Almost similar trend was observed in number of lymphocytes (Fig. 2.2), immunologically important white blood cell type, and its proportion was also found to be highest among leucocyte in blood of snake.

Data analysis (ANOVA) revealed a significant variation in monocyte count in different months. Monocyte was found to be lowest in January. Higher count of it was recorded from February to August. It decreased abruptly to the lowest in September and remained lower during October to December (Fig. 2.3).

The proportion of important granulocyte, heterophil, varied in number throughout the annum. Highest count was recorded in spring months and lowest in September (Fig.2.4). Eosinophil count in snake blood varied greatly between 249/mm^3 to 13240/mm^3; and significantly up and down were noted during whole period of study. As shown in Fig. 2.5, eosinophil number was found to remain in similar range from the month of January to June, then to increase in July and August, again decreasing in September to October and abruptly increased during November.

Data analysis (ANOVA) revealed a significant variation in basophil count in different months. Basophilic granulocyte proportion is generally lowest among the peripheral blood leucocytes. In snake, Basophil number varied from 17 – 228 per mm^3 during different months of study; however, it was not recorded at all in the blood smear in the month, July (Fig. 2.6).

TLC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	39.194	14	2.799	5.327	0.001
Within Groups	15.765	30	0.525		
Total	54.959	44			

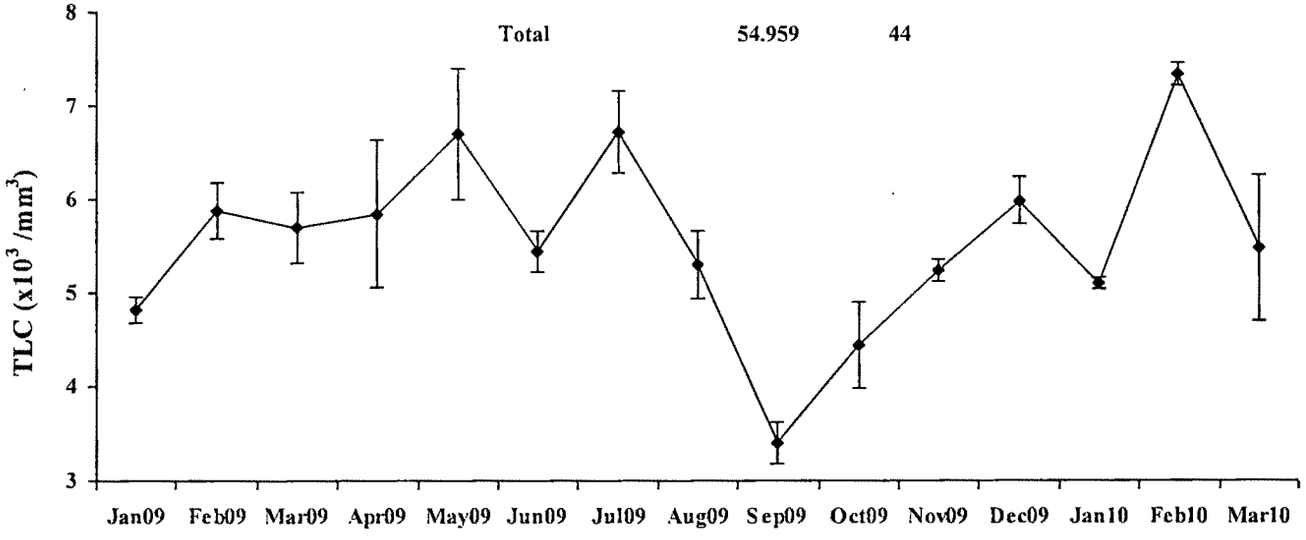

Fig. 2.1 Seasonal variation in total leucocyte count (TLC) in the fresh-water snake, *Natrix piscator*. Data were analyzed by ANOVA.

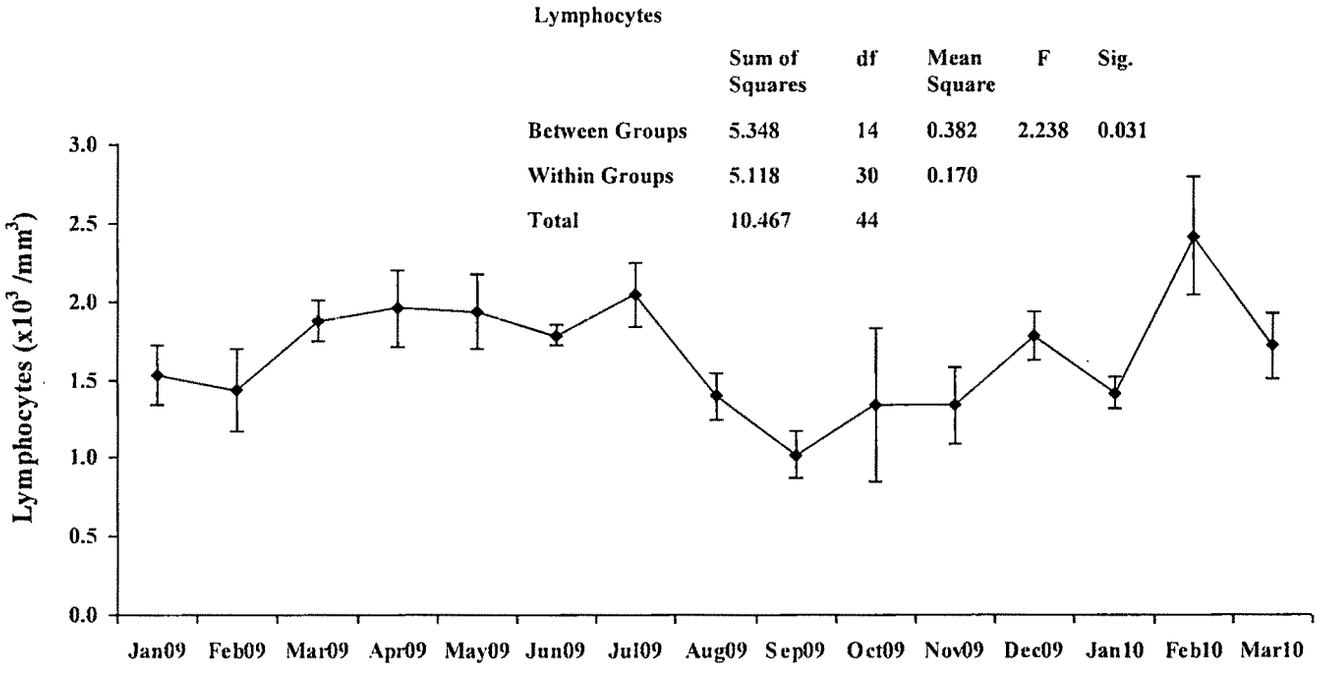

Fig. 2.2 Seasonal variation in lymphocyte count in the fresh-water snake, *Natrix piscator*. Data were analyzed by ANOVA.

Monocytes

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8.355	14	0.596	5.865	0.001
Within Groups	3.052	30	0.101		
Total	11.407	44			

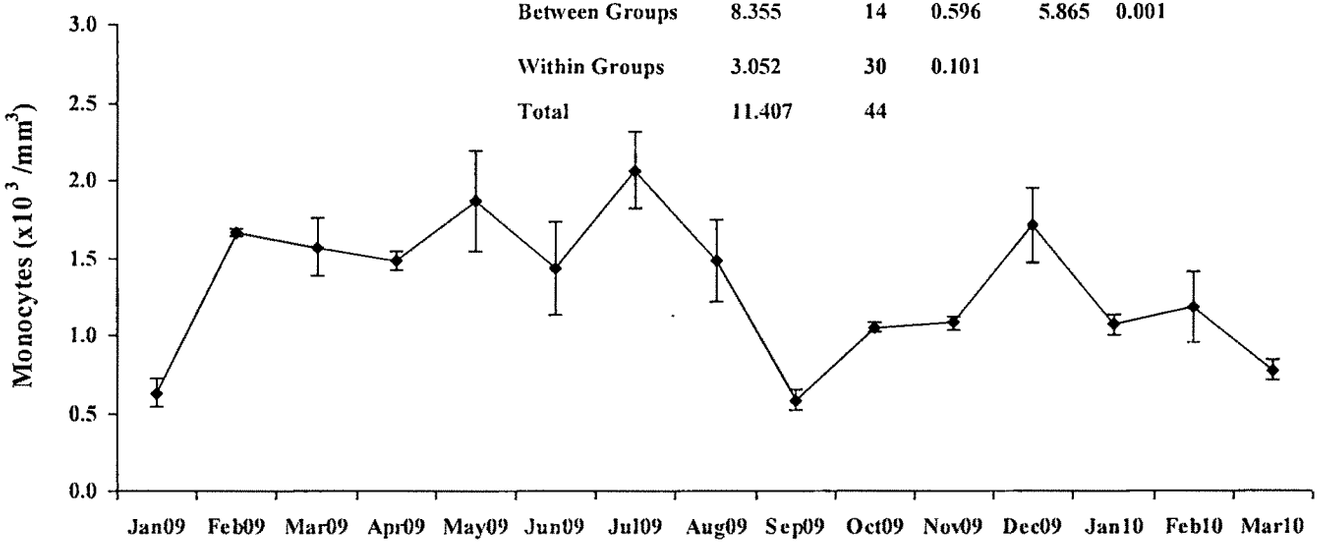

Fig. 2.3 Seasonal variation in monocyte count in the fresh-water snake, *Natrix piscator*. Data were analyzed by ANOVA.

Heterophil

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11.291	14	0.806	2.836	0.008
Within Groups	8.530	30	0.284		
Total	19.822	44			

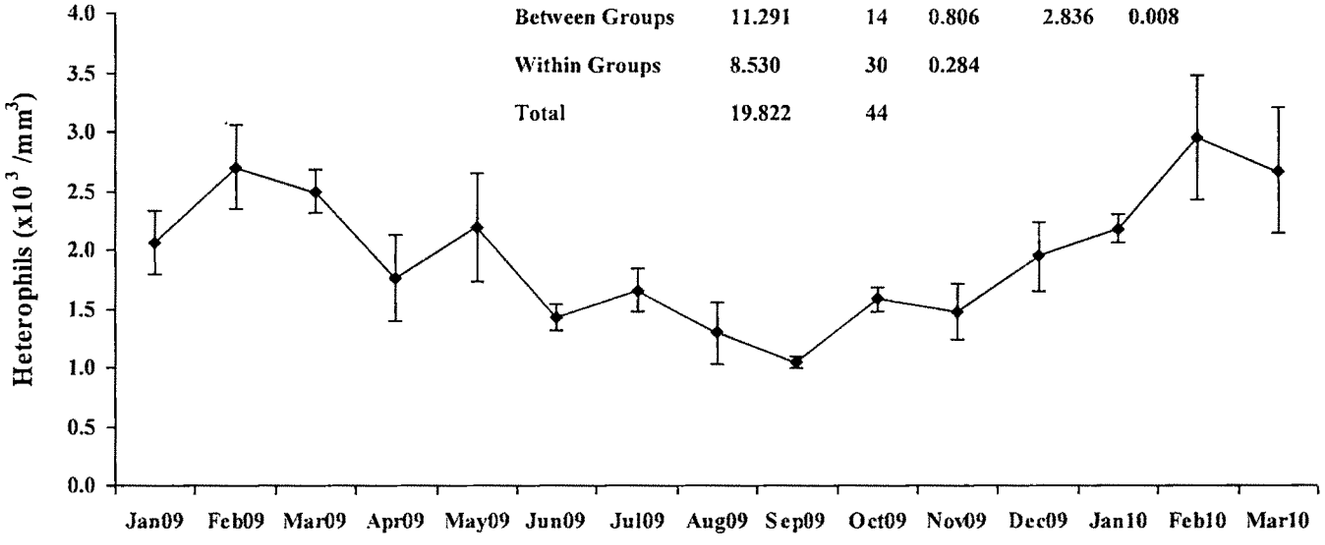

Fig. 2.4 Seasonal variation in neutrophil count in the fresh-water snake, *Natrix piscator*. Data were analyzed by ANOVA.

Eosinophil

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.199	14	0.228	2.343	0.025
Within Groups	2.925	30	0.097		
Total	6.125	44			

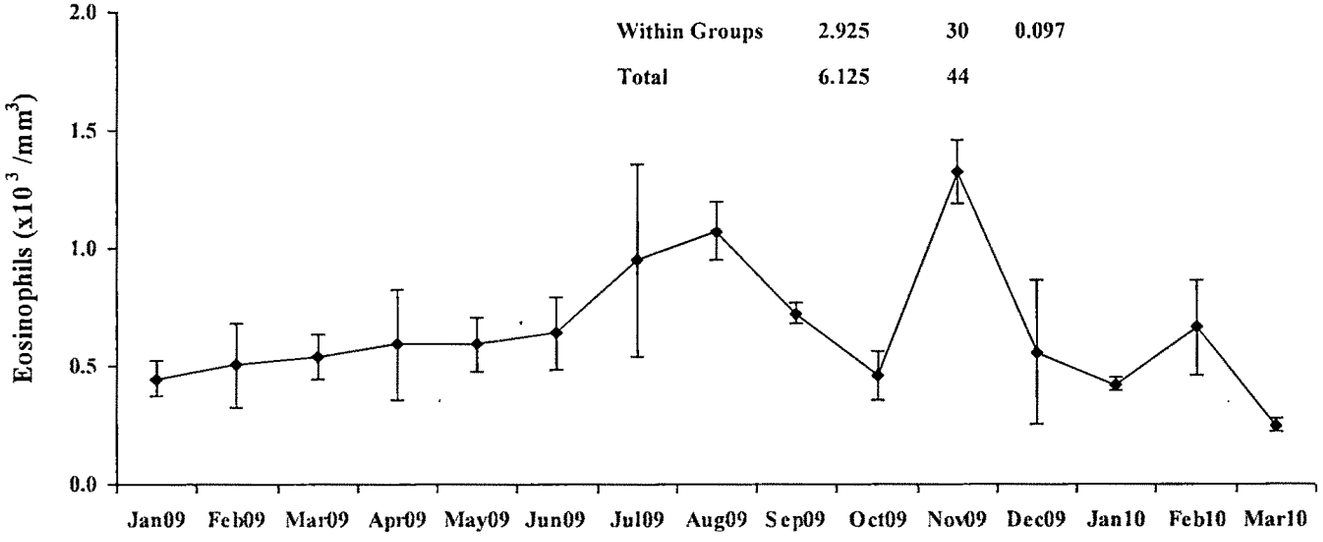

Fig. 2.5 Seasonal variation in eosinophil count in the fresh-water snake, *Natrix piscator*. Data were analyzed by ANOVA.

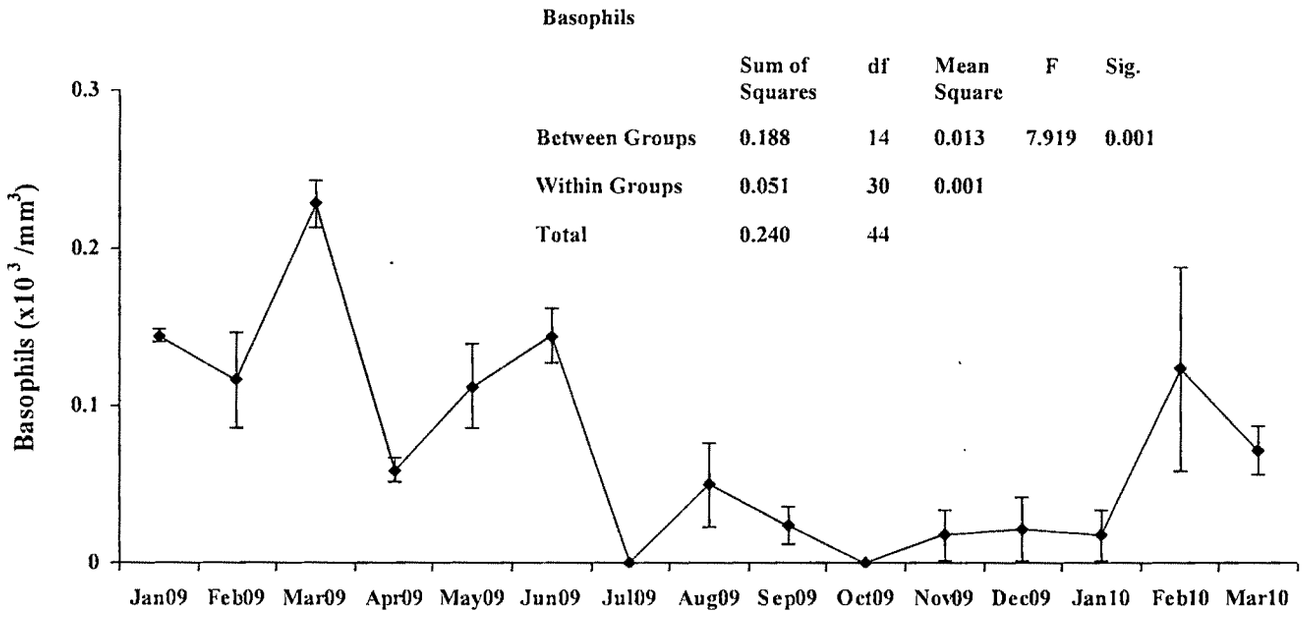

Fig. 2.6 Seasonal variation in basophil count in the fresh-water snake, *Natrix piscator*. Data were analyzed by ANOVA.

Percentage phagocytosis

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5012	14	358	8.76	0.001
Within Groups	1226	30	40.866		
Total	6238	44			

Phagocytic index

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11.249	14	0.803	5.868	0.001
Within Groups	4.107	30	0.136		
Total	15.356	44			

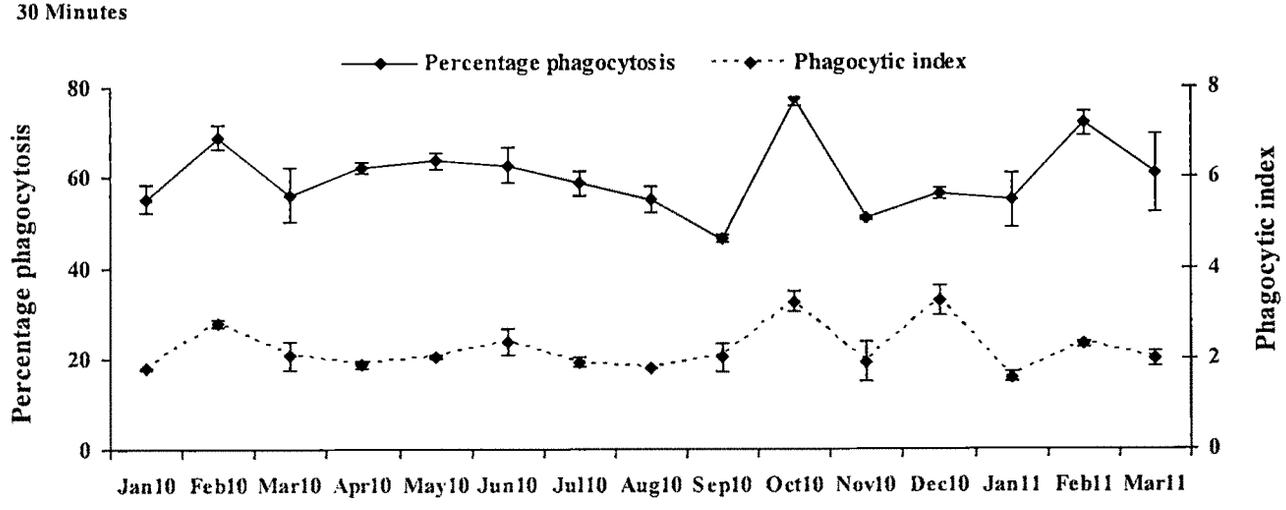

Fig. 2.7 Seasonal variation in leucocyte phagocytosis in the fresh-water, *Natrix piscator*. Data were analyzed by ANOVA.

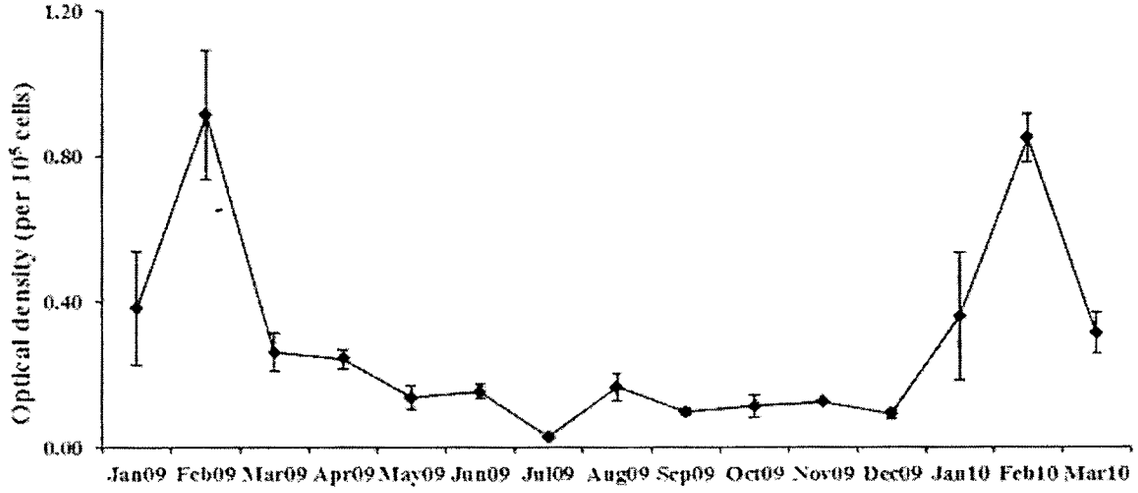

Fig.2.8 Seasonal variation in NBT reduction by leucocytes in the fresh-water snake, *Natrix piscator*. Data were analyzed by ANOVA.

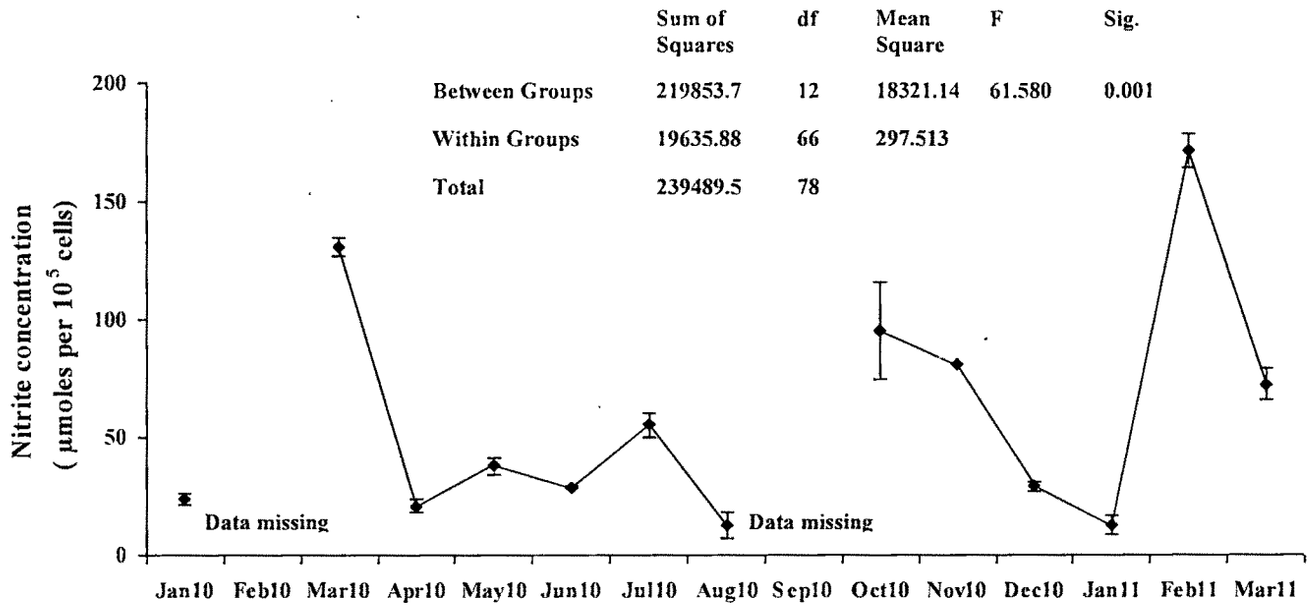

Fig.2.9 Seasonal variation in nitrite release by leucocytes in the fresh-water snake, *Natrix piscator*. Data were analyzed by ANOVA.

ConA	Sum of	df	Mean	F	Sig.
Between	16.11	14	1.151	2.719	0.011
Within Groups	12.698	30	0.423		
Total	28.809	44			

LPS	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	26.562	14	1.897	2.916	0.007
Within	19.517	30	0.651		
Total	46.08	44			

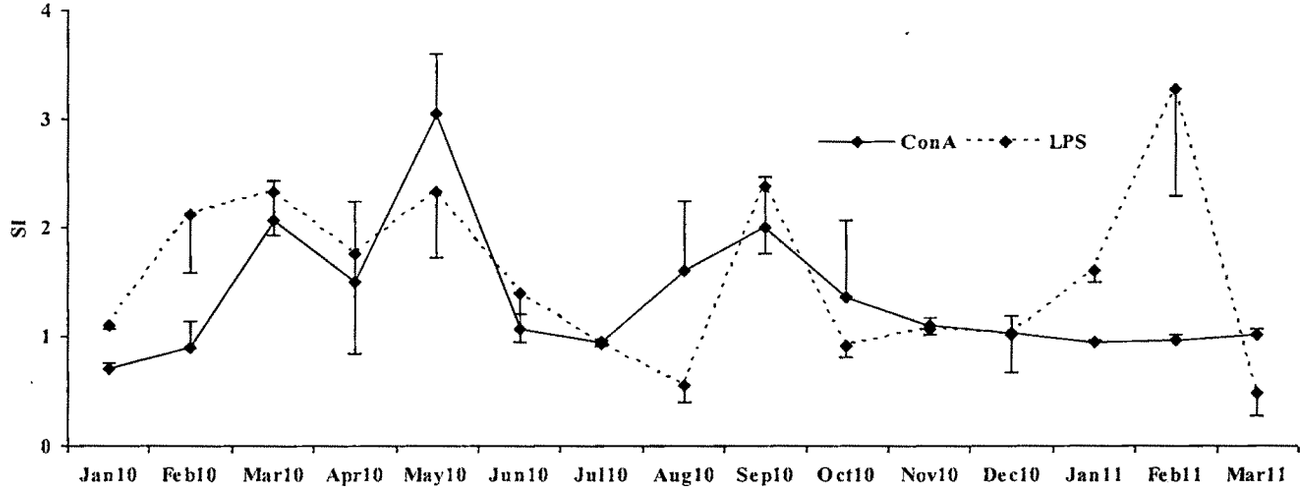

Fig. 2.10 Seasonal variation in mitogens (Con A – Concanavalin A and LPS- Lipopolysaccharide) induced lymphocyte proliferation in *Natrix piscator*. Data were analyzed by ANOVA.

To study the phagocytosis, leucocytes were incubated with target cells for 30 and 60 min. No significant difference was found in phagocytosis between two incubation periods. Hence, phagocytosis at 30 min incubation is presented. Percentage phagocytosis was high in the month of February. It decreased in March, remained low up to September and reached to the highest in October. Phagocytic index was highest in month of October and December, as compared to other months. During rest of the year, it remained more or less in the same range (Fig. 2.7). Super oxide production, as judged by NBT assay, and nitrite release by leucocytes was found to high in spring month and remained low during rest of the year (Figs. 2.8 & 2.9).

Proliferatitive response of blood lymphocyte to the mitogen, Con A, was low during October to February. It was recorded increasing in March, reached to the greatest in May, again decreased in June – July and then increased in August – September. On the other hand, LPS – induced lymphocyte proliferation was recorded greater during February-May, then decreased during June –August. The response was greatest in September and decreased to the low during October-January (Fig. 2.10).

2.4. DISCUSSION

In this investigation, so far the peripheral blood leukocyte is concerned, each type of leucocyte showed a characteristic annual variation. Total leucocyte count was found low in spring and summer months and high during rest of the year. The relatively uncommon basophilic granulocyte, lymphocyte and monocyte showed no definite pattern of annual variation. Neutrophil count was found to increase from autumn month onwards and remained high in winter and spring; while the eosinophil count showed up and down throughout the year.

There are very few reports on seasonal fluctuations or annual variation of leucocytes, the primary immune cells. In reptilian species, as reviewed by Duguy (1970), the basophil, lymphocyte and neutrophils are found to be minimum during month of hibernation and high during summer months,

whereas eosinophils showed reverse pattern, being maximum during hibernation and minimum during summer. In recent observation on the living fossil, tuatara, *Sphenodon*, Burnham *et al.* (2006), have reported total leucocyte count as well as lymphocyte and heterophil to be higher in winter and spring month.

In one of the first study of seasonal variation in immunity among healthy individuals, circulatory lymphocyte numbers were counted and were reportedly highest in the winter month among healthy subjects in France (Reinberg, 1977; Maes *et al.*, 1994). Subsequent studies, characterizing changes in the subsets of lymphocytes have supported the initial finding that the number of immune cells in circulation changes seasonally, and the seasonal rhythm of circulating lymphocyte varies depending on the subsets examined: the proportion of viable B-cells significantly elevated; while that of T-cells depleted in winter, as compared to summer. (MacMurray *et al.*, 1983; Van Rood *et al.*, 1991).

So far non human mammals is concerned, significant fluctuation in WBC count has also been reported, but fluctuations are independent of specific seasons in cotton rats, *Sigmodon hispidus* (Lochmiller *et al.*, 1994). In the same species, reduction in peripheral white blood cell count in response to sheep blood cells injection has been reported in winter, as compared with that in summer. Mann *et al.* (2000), have reported that circulating number of WBC and neutrophil are higher in winter than summer. Periodic changes in the number of peripheral white blood cells and its type might results due to several factors like distribution of circulatory and marginal cells, components of tissues, influx from storage site, cell proliferation and release of de novo cells into circulation as well as cell distribution and removal.

In common with the seasonal changes in lymphatic organ mass among birds, mammals, and reptiles, there also exist seasonal changes in immune functions (reviewed in Nelson and Demas, 1996). Seasonal changes in immune function appear common among reptiles (reviewed in Zapata *et al.*, 1992). Phagocytes are the most important components of the non-specific cell-

mediated immune system (Finco-Kent and Thune, 1987; Secombes and Fletcher, 1992; Neumann *et al.*, 2001), as they play a major role in clearing foreign particles in tissues. We found higher percentage phagocytosis spring and summer; while phagocytic index was higher in winter in *N. piscator*. *In vivo* experiments by Le Morvan *et al.* (1997) showed that head kidney macrophages of carp (*Cyprinus carpio*) display better phagocytic capacity in normal temperature (12 ^{0}C) than in higher temperatures (20 or 28 ^{0}C). A similar pattern was observed *in vitro* in *T. tincta*, where the blood granulocytes showed higher capacity to phagocytose latex beads and to produce oxidative anions in 12 ^{0}C than in 22 ^{0}C (Collazos *et al.* 1994b). In *Clarias batrachus*, super oxide production was highest at a lower temperature of 19 ^{0}C during winter than at higher temperature of 28-31 ^{0}C during rainy and autumn seasons (Kumari *et al.*, 2006). Kortet *et al.* (2003a) found that seasonal patterns of certain immune parameters including white cell counts, chemotaxis, and respiratory burst of head kidney phagocytes and spleen size differed between population and sex in roach *R. rutilus*. In general, results of various studies support the idea of winter-time up-regulation of cellular immune defenses in fish.

In the present study, super oxide production and nitrite release by leucocytes was found to high in spring month and remained low during rest of the year. ConA induced lymphocyte proliferation was low during autumn and winter. It was recorded increasing in March, reached to the greatest in May, again decreased in June – July and then increased in August – September. LPS – induced lymphocyte proliferation was recorded greater during February-May, then decreased during June –August. The response was greatest in September and decreased to the low during October-January. Farag and El Ridi (1985) has reported that snakes tested in summer display a stronger lymphoproliferative response to mixed leucocyte cultures, compared with winter caught animals. In turtle, *M. caspica*, chemotaxis and proliferation of lymphocytes, and the activity of natural killer (NK) cells showed high winter-time levels (Munoz *et al.*, 2000). However, various functions of splenocytes in *M. caspica* showed different patterns during the seasonal cycle. Proliferation of

lymphocytes from the Caspian pond turtle (*Mauremys caspica*) in response to ConA was strongest in spring, but was significantly diminished in summer, autumn and winter (Munoz and Fuentz, 2001). Lochmiller *et al.* (1994) have shown that lymphocyte proliferation to T-cell mitogens Con A and *Phytolacca americana*, an extract of pokeweed mitogen (PKW), are elevated in February relative to other months of the year. Mann *et al.* (2000) have reported that lymphocyte proliferation in response to mitogens is higher in winter than summer.

Taken together, the present study shows improved cell-mediated innate immune functions in winter-time in fresh-water snake *N. piscator*. That the reproduction-associated alterations coupled with the adaptation to winter conditions are the driving factors for seasonal changes in immune defenses has been suggested by Martin *et al.* (2007a). Most of the studies have correlated the variation in blood parameters with fluctuations in environmental factors like temperature, food, thyroid and gonadal activity. Environmental factors play an important role in body physiology, oxidation, and intermediary metabolism and gonadal activity of all the vertebrates, including reptiles. Temperature at Varanasi (L 25° 18'N and L 83° 1' E) during the year of study varied significantly. In addition, some of the other elements of climate, viz., rainfall and relative humidity also showed marked annual variations. The minimum temperature was recorded down up to 10° C during December to mid January and maximum was up to 45° C during May-June. It is obvious that animals living at Varanasi have to adapt to the above variations in environmental conditions during a year. The variation in ecofactors, of course, are not of the same magnitude as are in temperate countries, but are probably enough to influence significantly the physiology of the animals and cause variations in the immune parameters. More comparative studies between animals that differ in their life-history strategies will throw more light on this subject.

****