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ORIGINAL ARTICLE

Seasonal variation and innate immune responses of spleen in fresh-water snake, *Natrix piscator*

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Innate immunity provides first line defense in all animals against pathogens and parasites. There is seasonal variation in pathogen prevalence and disease because of the seasonal lifecycle of the parasite and due to annual variation in the infectivity of pathogens. Organisms face seasonal stress by regulating their internal physiology, i.e. by secreting hormones. Melatonin and sex steroids contribute to the seasonal redistribution of immunological activity including winter-time up-regulation of some immune responses, and reproduction-related immunosuppression. Present study aims to understand seasonal variation in splenocyte innate immune response in the fresh-water snake, *Natrix piscator*. Reptiles represent the pivotal phylogenic group as they were the ancestor of both birds and mammals and they are the only ectothermic amniotes providing the key link between ectothermic anamniotic fishes and amphibians, and endothermic amniotic birds and mammals; a greater study of reptilian innate immune response will provide important insights into the evolutionary history of vertebrate immunity. Animals were mildly anaesthetized and the spleen was isolated aseptically. Spleen was used for calculating splenosomatic index, cellularity and macrophage phagocytosis. Spleen size has a trend to be high in autumn and winter months and low in spring and summer, though data were not significant. 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. It is concluded that seasonal variation in splenocyte immune response provides a mechanism that suites best to the organism and which might coincide with the pathogen prevalence. Seasonal cycle of immune response is helpful in understanding the disease processes in animals and the direct implication of this study could be utilized for the endangered species living in captivity.

Key words: Seasonal variation, spleen, immune response, phagocytosis, snake

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Changes in environmental factors throughout the year determine season of the year. The photoperiod is primary cue 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).

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 non tropical 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 stresses. Many animals, however, die indirectly from opportunistic diseases that seem to overwhelm immunological defense, presumably at times when these defenses are low (Nelson and Demas, 1996; Lochmiller and Deerenberg, 2000).

To increase survival, one strategy is to enhance immune function prior to the onset of the poor conditions that may compromise immune function. Maintaining maximal immune function is energetically expensive; the cascade of dividing immune cells, the onset and maintenance of inflammation and fever, and the production of humoral immune factors, all require significant energy (Demas *et al*., 1997). Mounting an immune response requires resources that could otherwise be allocated to other biological functions (Sheldon and Verhulst, 1996). Thus, maintaining a positive energy balance is required for survival and reproductive success. Individuals may partition resources among the immune system and other biological processes, such as reproduction, growth, or thermogenesis. Consequently animals may attain the highest level of immune function that is energetically possible given the constraints of processes essential for survival, growth, reproduction, thermogenesis, foraging and other activities (Deerenberg *et al*., 1997).

Recent evidences suggest that immune function varies substantially on a seasonal basis (Lochmiller *et al*., 1994; Nelson and Demas, 1996), and a direct link between seasonal fluctuation in the environment and specific immunological changes has been established in several studies that have provided evidence for reduced immune function and increased death rates from infectious diseases during the winter (Nelson *et al*., 1995; Nelson and Demas, 1996; Sinclair and Lochmiller, 2000).

Seasonal cycles in the development, regression, and regeneration of the thymus, spleen, and bursa of Fabricius (in birds) have been described in many vertebrate species (Nelson *et al*., 2002); relatively less is known for non-mammalian vertebrates. In reptiles, earlier studies have demonstrated that these are also endowed with an advanced type of immune system: bone marrow, thymus, spleen and gut-associated lymphoid tissues are well developed (Hussein *et al*., 1979; Mansour *et al*., 1980). The functional relevance of the reptilian spleen has been demonstrated, as splenectomy in lizards causes an acute inhibition of several immune responses (Jayaraman and Muthukkaruppan, 1977), and spleen is the major site of immune responses to blood-borne antigens and is also a site of hematopoiesis (Batista and Harwood, 2009).

Reptiles have an extended terrestrial lifestyle and they show direct development without metamorphosis. Reptiles are generally long-lived, with an extended period of growth and maturation early in life. However, reptiles are unable to

internally regulate their body temperature, and undergo strong seasonal change in behavior associated with environmental temperatures. Collectively, these characteristics may have profound effects on how reptiles partition energy resources to self-maintenance activities. A greater knowledge of reptilian immunity is required to understand the evolutionary history of vertebrate immunity (Zimmerman *et al*., 2010). This warrants more and more studies in reptiles.

MATERIALS AND METHODS

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 housed in vivarium (wood and wire net cages; size 50x30x30 cm). 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 guideline 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.

Chemicals

Culture medium (RPMI-1640), 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 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.

Experiment

To study seasonal variation in immune functions, the requisite number of animals were sacrificed under mild anesthesia during mid of each month. Spleen was excised aseptically, weighed immediately, and Spleno-somatic index (SSI: spleen weight per 100 g body weight) was calculated. Excised spleens were kept in cool (4°C) culture medium and soon after utilized to study cellularity and macrophage phagocytosis. In all study, sample size was 5 to 6.

Preparation of spleen single cell suspension

Under aseptic conditions, excised spleen was immediately macerated through a nylon strainer of pore size <100 µm into complete culture medium (2 ml/spleen) to get single cell suspension under a sterile laminar flow hood. Cell viability was checked light microscopically through trypan blue exclusion test, which exceeded 95%. Spleen cellularity (number of splenocytes mg^{-1} tissue) was determined with help of hemocytometer and light microscope.

Preparation of macrophage monolayer

Splenic macrophage monolayer was prepared, and phagocytic assay was performed following the method of Mondal and Rai (1999a, 1999b, 2001). Briefly, splenic cell suspension (200 µl) was flooded onto individual pre-washed and sterilized glass slides. Phagocytic macrophages were allowed to adhere by incubating the slides at 25°C in humidified CO_2 atmosphere for 90 min. Non

adherent cells were washed off with 0.2 M phosphate buffer saline (PBS; pH 7.2). The splenic macrophage monolayer was prepared in duplicate from each spleen. In the adherent cell population, more than 90% of the cells were macrophages as judged by their morphology.

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 three times in PBS and finally suspended in the complete culture medium to get a concentration of $1x10^8$ cells ml^{-1}.

The prepared macrophage monolayer, as above, was flooded with yeast cell suspension, and phagocytosis was allowed to proceed. After 90 min incubation at 25°C in humidified CO_2 atmosphere, the slides were rinsed three times in PBS, fixed in methanol, stained with Giemsa, and examined under oil immersion. For each slide, a total of 100 macrophages were examined randomly without any predetermined sequence. The phagocytic index was determined by calculating the average number of yeast cells engulfed by single macrophage. The percent phagocytosis was calculated by dividing the number of macrophages showing phagocytosis by 100.

Statistical analysis

Data are presented as Mean ± SEM. Means were compared by ANOVA.

RESULTS AND DISCUSSION

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 (Fig. 1). Spleen cellularity was recorded high in winter months and again in September; while it remained low during rest of the year (Fig. 2). No definite pattern was observed in phagocytosis by splenic macrophages. The percent phagocytosis varied between 42 to 60 %, being highest in month of February (Fig. 3).

Lymphatic organs like spleen, thymus and bursa of Fabricius (in birds) are important immune organs. Seasonal cycles of lymphatic organ mass have been recognized well before the immunological functions of these organs identified. In various studies, early as well as recent, the immune status of the animal has also been assessed indirectly through changes in gross lymphatic organ mass that presumably reflect immunologic activity of the tissue. It is logical also to assume that lymphatic organ mass positively correlates with organ function; this assumption is common with that associated with the seasonal changes in reproductive organ mass. A relatively large number of studies have reported seasonal changes in lymphoid tissue, with a trend toward regression of lymphoid tissue during the mating period and winter, compared with other times of the year.

In the present study, spleen size and spleen cellularity were found to have a trend to be high in autumn and winter months and low in spring and summer. El Ridi *et al*. (1981) have reported seasonal variation in the spleen of snake, *Psammophis schokari*. Red pulp is well developed; while the white pulp had only a few scattered aggregates during winter. However, during summer, the red pulp was indistinct with lymphatic tissue regressing in size. In avian species, relative splenic size undergoes a reduction at the beginning of breeding

season in both migratory and non migratory birds; regeneration of spleen occurs during subsequent incubation and feeding of the hatchlings (Oakson, 1956; Fange and Silverin, 1985). Reduction in splenic mass during winter compared to summer and spring is recorded in short-tailed voles, *Microtus agrestis* (Newson, 1962). On the other hand, increase in splenic mass during winter is reported in deer mice (Demas *et al*., 1997). Elevated splenic mass has been reported during September and October in red-backed mice, *Clethrionomus rutilus* (Sealander and Bickerstaff, 1967). Splenic and thymic sizes have been reported to be minimal in several avian species when the gonads begin the process of vernal recrudescence (Oakeson, 1956; Fange and Silverin, 1985). In avian species also, thymus has been reported to be minimal when the gonads were undergoing vernal recrudescence (Nelson *et al*., 2002).

Phagocytes are the most important components of the non-specific cell-mediated immune system (Neumann *et al*., 2001), as they play a major role in clearing foreign particles in tissues. We found higher percentage phagocytosis during spring and summer; while phagocytic index was higher in spring 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 at temperature, 12 ^{0}C than at higher temperatures, 20 or 28 ^{0}C. A similar pattern was observed 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).

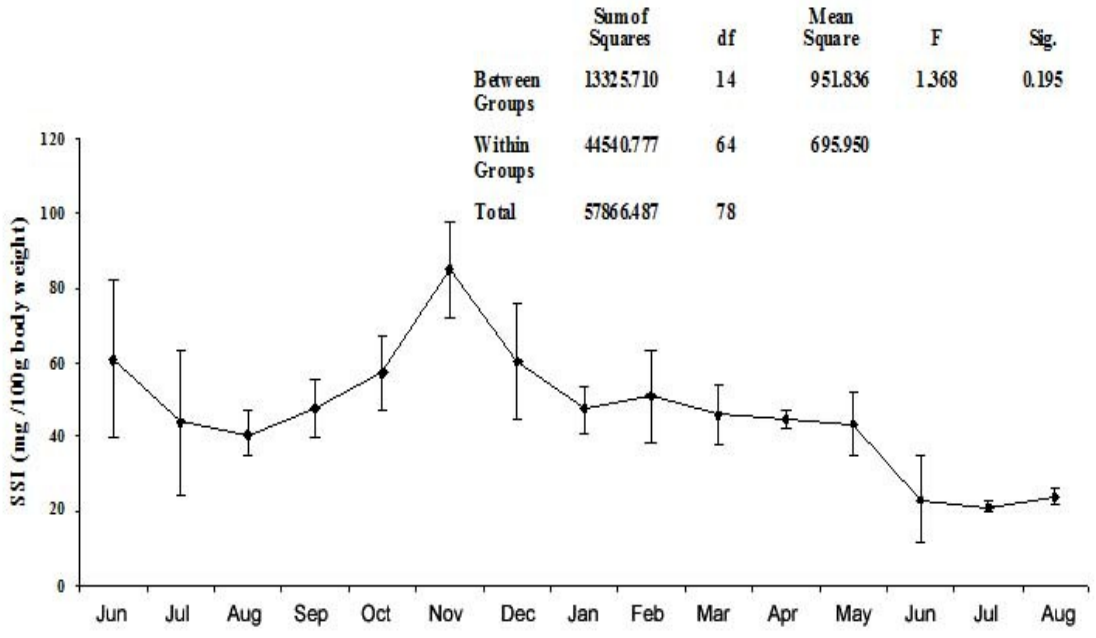

Figure 1 : Seasonal variation in spleen mass (Mean ± SEM) in the fresh-water snake, *Natrix piscator*. Data were analyzed by ANOVA.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	13.061	14	0.933	3.079	0.001
Within Groups	16.969	56	0.303		
Total	30.029	70			

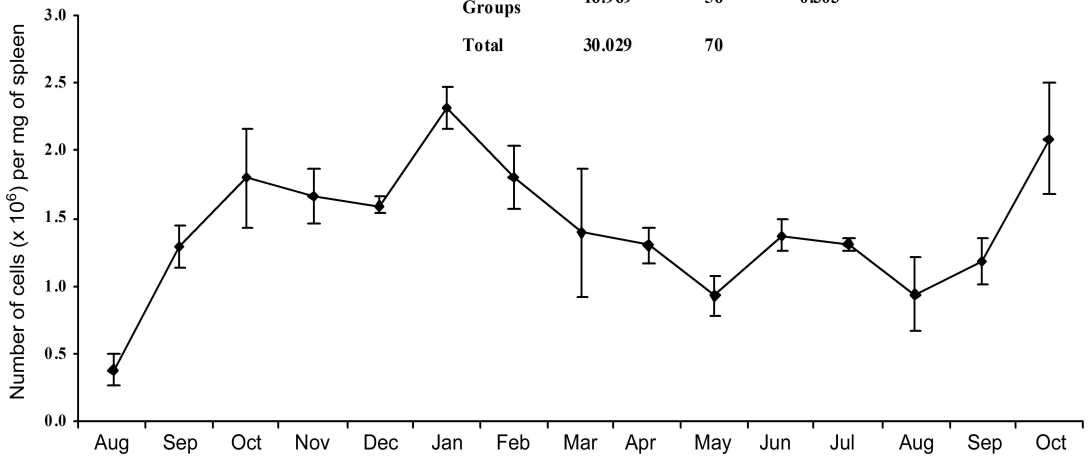

Figure 2. Seasonal variation in spleen cellularity (Mean ± SEM) in the fresh-water snake, *Natrix piscator*. Data were analyzed by ANOVA.

Percentage phagocytosis						Phagocytic index					
	Sum of Squares	df	Mean Squar	F	Sig.		Sum of Squares	df	Mean Squar	F	Sig.
Between	1901.6	14	135.83	3.1922	0.002	Between	1.5011	14	0.1072	1.4425	0.183
Within	1872.2	44	42.551			Within	2.7502	37	0.0743		
Total	3773.9	58				Total	4.2514	51			

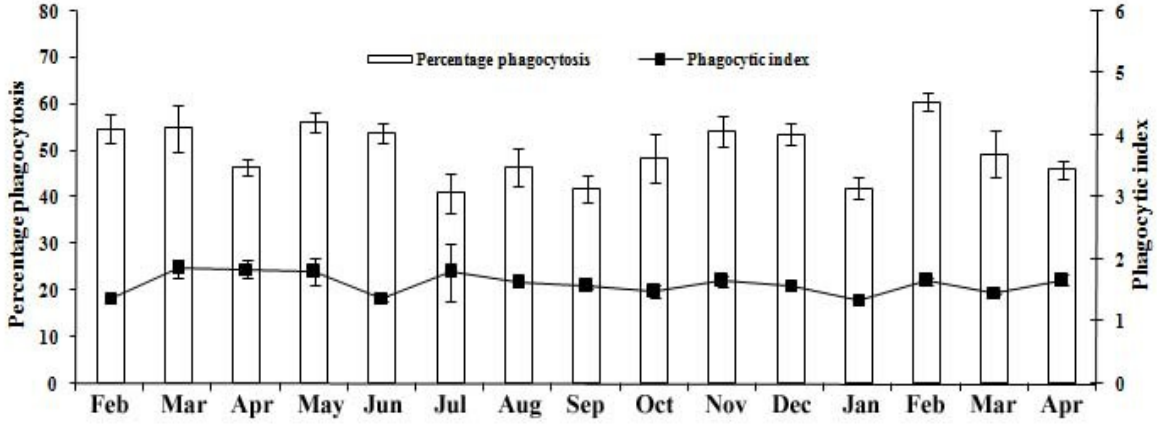

Figure 3. Seasonal variation in splenic macrophage phagocytosis in the fresh-water snake, *Natrix piscator.* Data were analyzed by ANOVA.

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*., 2002). The current consensus is that reproduction-associated alterations couples with the adaptation to winter conditions are the driving factors for seasonal changes in immune defenses (Martin *et al*., 2007a). Furthermore, 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 28° 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 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.

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