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Lack of Evidence for a Trade-off Between Immune and Reproductive Systems in a Seasonally-Breeding Desert Rodent

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Abstract: Immune and reproductive activities require significant energy. Therefore, many previous studies suggest the existing of trade-offs between reproduction and immune function, with immunity being compromised during the breeding seasons. This concept motivated the present study which was designed to address the trade-off between reproductive activity and immune function in a seasonally breeding desert rodent, the lesser gerbil (*Gerbillus gerbillus*). Various immune parameters were assessed in wild-caught male and female gerbils during the early spring when animals having maximum reproductive activity and in autumn when they have minimum reproductive activity. Also the effect of seasonal reproductive status on the Bcl-2 apoptotic pathway was evaluated in spleen tissues by immunohistochemical staining. All measured immune parameters were not suppressed by increased seasonal reproductive activity in gerbils. Specifically, spleen mass, differential leukocyte count, serum concentrations of cytokines (IFNγ, TNFα, or IL-6) and serum concentrations of IgE were not affected by seasonal reproductive status. Unexpectedly, serum concentrations of IgG were higher in reproductively active gerbils than in reproductively inactive gerbils. In addition, seasonal reproductive status had no remarkable effects on the histological architecture of spleen or the percentages of lymphocytes in splenic white pulp as measured by image analysis technique. Reproductively active gerbils had higher apoptotic rates, as judged by suppressed Bcl-2 levels, compared to reproductively inactive gerbils. These findings are in contrast to expected results, but they offer preliminary evidence that reproductive status in *Gerbillus gerbillus* is not predictive of seasonal variation in immune function, and probably seasonal changes in energy availability may accurately predicts the direction of alteration. According to available knowledge, this is the first report of seasonal immune function in *Gerbillus gebrillus* and the first attempt to determine the trade-offs between reproductive and immune systems in this species.

Key words: seasonal · Immune · Reproduction · Cytokines · Immunoglobulins · Bcl-2 · *Gerbillus gebrillus*

INTRODUCTION

The immune system activity is one of the physiological capabilities most responsible for the survival of an individual and its subsequent fitness [1]. However, mounting an immune response and maintaining maximal immune function are energetically costly processes and necessitate resources that could otherwise allocated to other physiological processes such as reproduction, thermogenesis, or growth [2]. Therefore seasonal changes in immune function are common among vertebrate species, perhaps to facilitate survival in terms of annual cycles in energy availability and pathogen prevalence [3]. Other factors that may account for seasonal variation in immune function include sex differences in investment in immune responses and differences in seasonal environmental factors such as day length, ambient temperature, humidity and rainfall [4]. The importance of such factors for alterations in the immune function and subsequent survival has recently increased the interest in eco-immunology research. Besides, eco-immunologists in recent times are mainly paying a great deal of attention to the energy balance between the immune system and other biological processes, especially reproduction.

Small mammals occupying non-tropical zones often breed seasonally as they are faced with marked seasonal fluctuations in energy availability and requirements. This seasonality is to ensure that offspring are born and raised in the most favourable conditions for their survival [5].

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Generally, at the temperate regions, spring and summer represent favourable conditions in which individual of most mammalian species can flourish. Alternatively, winter is energetically challenging because of low temperature and limited food availability, so animals may stop breeding to conserve energy [4]. Beyond this generality, there are documented instances of reproduction being suppressed during the summer in temperate zone mammals [5].

Virtually all preceding studies that investigated immune parameters across seasons have demonstrated remarkable seasonal changes in lymphoid tissue size and immune function [6]. Many species show higher immune response in winter (i.e. non-breeding season) than in summer [7]. That is, on the contrary to the winter-decrease in reproductive activity, immune function tends to be increased at this time of year, possibly to promote the survival of individuals [8]. Since reproductive and immune activities require significant energy, a competition for energy may exist during winter between these two physiological processes [4]. For this reason, it has been suggested that seasonal changes in immune functions are mediated, in part, by the physiological trade-offs between the reproductive and immune system. The trade-offs are defined as direct and indirect opposing interactions between two physiological processes, which are most responsible for the survival and fitness of individuals in fluctuating environmental conditions [3].

The concept that the immune function is compromised during the breeding seasons motivated the present study which was designed to address the seasonal trade-off between reproductive activity and immune function in a desert rodent, the lesser gerbil (*Gerbillus gerbillus*). Although some information has already been accumulated on seasonal variation in reproductive activity of desert rodents, almost no attention has been given to the details of the immune response in these animals and to the best of available knowledge, no data are available on the physiological trade-offs between the reproductive and immune system in these species. Desert environments are harsh and require adaptations to extreme temperature and precipitation; therefore the existence of trade-offs between the reproductive and immune system is highly predictable in order to face the harmful environmental conditions.

The Egyptian lesser gerbil (*Gerbillus gerbillus*) is a desert rodent inhabits the Eastern and Western deserts of Egypt (~30 No latitude). It exhibits seasonal reproductive cycles [9] in which the onset of reproductive endocrine activity is concurrent with the lowest temperature and the shortest photoperiod in winter (December-January). This activity increases with increasing day lengths and temperatures until it reaches highest value in spring (April). The reproductive system of lesser gerbils is regressed in summer and minimum reproductive endocrine activity is detected in late summer and autumn [10].

Therefore the present study assessed various immune parameters in wild-caught male and female *G. gerbillus* during the early spring when animals having maximum reproductive activity and in autumn when they have minimum reproductive activity. These immune measures included spleen mass, differential white blood cell count, serum concentrations of various cytokines (IFNγ, TNFα, IL-6), serum levels of immunoglobulins (IgG and IgE), assessment of splenic architecture and quantification of splenic lymphocyte population using image analysis technique. Additionally, because apoptosis is involved in homoeostasis of organs and tissues, the effect of seasonal reproductive status on the Bcl-2 apoptotic pathway was evaluated in spleen tissues by immunohistochemical staining.

MATERIALS AND METHODS

Animals: Adult male and female lesser gerbils, *Gerbillus gerbillus*, were trapped from Western desert, Egypt (~30N° latitude), in early spring (breeding season; n=14) and autumn (non-breeding season n=16) and brought to the department of Zoology, Faculty of Science, Minia University, Egypt, for study. In all animals, the reproductive functions were judged by using the criteria of Osborn and Helmy [9] and Khammar and Brudieux [10] for *Gerbillus gerbillus*. The animals were housed for one week under normal laboratory conditions to omit the stress of transportation and as a quarantine period. All procedures were conducted in accordance with institutional guidelines and follow the Guide for Care and Use of Laboratory Animals.

Blood and Tissue Sampling: The animals were weighed and killed by decapitation under deep ether anesthesia. Blood samples were collected individually from the jugular vein of each gerbil in heparinized and non-heparinized tubes for measuring various immunological parameters. Non-heparinized blood samples were allowed to clot at room temperature. After that, they were centrifuged for 30 min at 4000 rpm. Serum aliquots were extracted and stored in micro-centrifuge tubes at -08 °C until assayed. After blood sampling, spleens were immediately harvested from each animal, weighed (mg) and fixed in neutral formalin solution (10%).

Differential Leukocyte Counts: The effector cells of innate and adaptive immune responses include granulocytes (neutrophils, eosinophils and basophils), monocytes and lymphocytes; therefore, differential leukocyte count was determined by the method of Dacie and Lewis [11] to assess the percentages of these cells in the peripheral blood of gerbils. Briefly, thin blood films were prepared, allowed to dry rapidly in air, fixed in methanol for 10 min and then stained with freshly prepared Giemsa's stain for about 30 min. The stain was then drained off in a stream of distilled water and the slides were allowed to air dry. Stained slides were examined microscopically and the percentage of each type of leucocytes in relation to the total number of leucocytes was calculated.

Measurement of Cytokines: Serum IFNγ, TNFα, IL-6 and were measured using enzyme-linked immunosorbent assay (ELISA) kits (ALPCO Diagnostics, Salem, New Hampshire, USA; Catalog # 61-IFGRT-E01, 75-TNFRT-E01 and 61-IL6RT-E01, respectively) according to the manufacturer's instructions. The results were expressed as picograms per milliliter.

Measurement of IgG and IgE: Humoral immune response was determined by measuring serum IgG and IgE concentrations using two-site enzyme-linked immunosorbent assay (ELISA) kits (ALPCO Diagnostics, Salem, NH, USA; Catalog # 41-IGGRT-E01, 41-IGERT-E01, respectively) following the manufacturer's instructions. The results are expressed as nanograms per milliliter.

Histological Study and Image Analysis: Formalin-fixed spleen tissues were dehydrated, embedded in Paraplast (Sigma, m.p. 56-58), cut at 5 µm and stained with hematoxylin and eosin using standard procedures. Stained sections were investigated using Leica DM 750 microscope equipped with computerized image analysis system (Leica Application Suit; LAS version 3.8 Buil: 878). Images were captured at a magnification of x 400 and were transferred by means of the RGB (red, green, blue) system. The regions of the tissue sections that have debris or poor histological quality were interactively excluded from analysis to increase the accuracy. Image analysis was applied to the white pulp because it has B and T lymphocytes and any change in these cell populations can be reflected in this area. Therefore, images were automatically segmented to select the white pulp; within the white pulp images were automatically segmented to select the lymphocytes. In order to count equal areas in every tissue section, fixed frames comprising exclusively the white pulp histological compartments of interest were applied, and lymphocyte population was counted and expressed as a percentage of the total count. Percentages reported were average measurements of different compartments of the white pulp. The image analysis was performed by experienced technician naïve to the treatment regimen.

Immunohistochemical Study: Immunohistochemical staining of spleen sections (5 µm) was performed using the avidin-biotin-peroxidase technique. The sections were deparaffinized in xylene, passed through graded alcohols and rehydrated with water. They were.incubated with 3% hydrogen peroxidase for 10 min to quench endogenous peroxidase activity, blocked with goat serum in PBS for 20 min and incubated overnight at 4°C with the primary antibody, BCl-2 (diluted 1: 100; Santa Cruz Biotechnology, Inc. USA). Sections were then incubated for 1h at room temperature with biotinylated goat anti-rabbit IgG (Sigma-Aldrich Co. Germany) followed by avidin-biotinylated-peroxidase complex (ABC; Vector Laboratories Ltd, UK) for 1h at room temperature. Finally, sections were incubated in a solution of 0.02% 3,3'diaminobenzidine (DAB) and 0.03% hydrogen peroxide until the brown color developed. The sections were then counterstained with hematoxylin, dehydrated, cover-slipped and were investigated using Olympus CX21 microscope equipped with HDCE 50B digital camera.

Bcl-2 immunopositivity was assessed semiquantitatively according to the method of Resendes *et al*. [12] with some modifications. In this method morphometrical analysis was performed by taking the histological compartments of spleen (white and red pulp) into account, Briefly, cells expressing Bcl-2 were counted in 5 random fields (x 400 magnification) of each compartment of spleen tissue from each animal. Then morphometric results from each compartments were grouped into three categories, according to the mean number of cells expressing Bcl-2 per field: low (= 5 cells), moderate (> 5 =10 cells) and high (> 10 cells).

Statistical Analysis: Data were presented as mean ± SEM (standard error of the mean). All data were analyzed using independent Student's *t*-tests (SPSS software, version 13.0; SPSS Inc., Chicago, IL, USA. Differences between group means were considered statistically significant if $P \leq 0.05$.

RESULTS

No statistical differences were found between males and females in any of the parameters measured ($P > 0.05$), therefore data from males and females were pooled

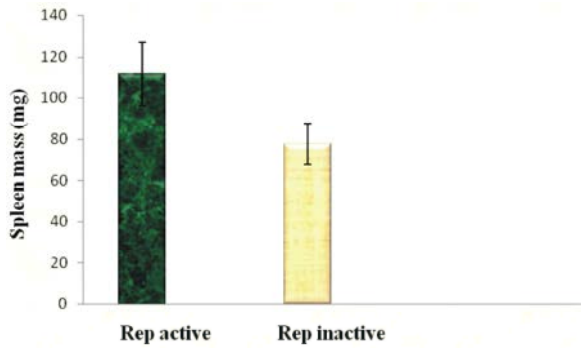

Fig. 1: Effect of seasonal reproductive status on spleen mass of wild-cought gerbils. Values are means ± SEM. Rep active = reproductively active gerbils trapped during spring; Rep inactive = reproductively inactive gerbils trapped in fall.

Table 1: Effect of seasonal reproductive status on differential leukocyte counts (mean percentage ± SEM) of wild-caught gerbils

Parameter	Rep. active	Rep. inactive
Lymphocytes	56.25 ± 2.17	55 ± 2.42
Neutrophils	39.25 ± 1.70	40.75 ± 2.46
Monocytes	2.75 ± 0.48	2.25 ± 0.25
Eosinophils	1.75 ± 0.25	2 ± 0.41
Basophils	0 ± 0	0 ± 0

Rep. active = reproductively active gerbils trapped during spring; Rep inactive = reproductively inactive gerbils trapped in fall.

together for statistical analysis. Also, no significant seasonal alterations were seen in body weight of gerbils (data not shown).

Spleen Mass: As shown in Fig. (1), there was no evidence that seasonal reproductive status exert any effect on spleen mass; specifically no significant differences ($P > 0.05$) in spleen mass were noticed between gerbils trapped in spring (*i.e.*, during the period of maximum reproductive activity) and gerbils trapped in fall (*i.e.*, during the period of minimum reproductive activity). However, gerbils trapped in spring had higher values of spleen mass than gerbils trapped in fall, but this increase was not statistically significant.

Differential Leukocyte Counts: Seasonal reproductive status had no significant effect on the percentages of different leucocytes from peripheral blood. As a result, the percentages of lymphocytes, neutrophils, monocytes, eosinophils and basophils did not vary significantly ($P > 0.05$) in both groups of gerbils (Table 1).

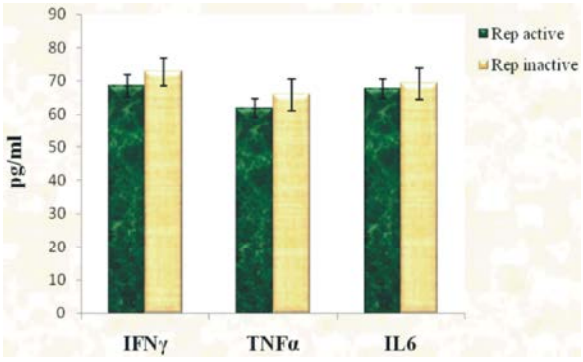

Fig. 2: Mean ± SEM. serum cytokine concentrations (pg/ml) in wild-caught gerbils trapped during spring in which reproductive activity show maximum values (Rep active) or during fall in which reproductive activity show minimum values (Rep inactive). IFNγ = interferon γ; TNFα = tumor necrosis factor α; IL6 = interleukin 6.

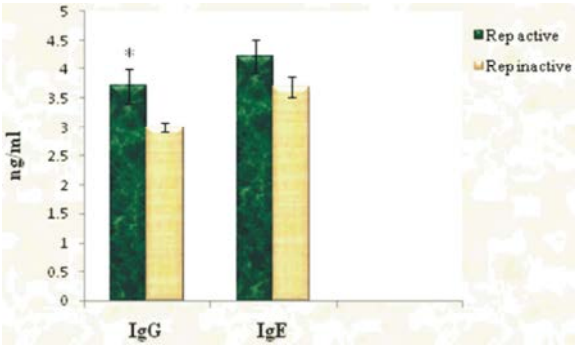

Fig. 3: Effect of seasonal reproductive status on seum immunoglobulin G (IgG) or immunoglobulin E (IgE) concentrations (ng/ml) of wild-cought gerbils. Data represent means ± SEM. Rep active = reproductively active gerbils trapped during spring; Rep inactive = reproductively inactive gerbils trapped in fall. * = $P < 0.05$ *vs.* reproductively inactive gerbils.

Serum Levels of Cytokines: There was no significant effect of seasonal reproductive status on serum concentrations of cytokine internferon γ (IFNγ), Tumor necrosis factor α (TNFα) or interleukin 6 (IL-6). That is, spring-caught reproductively active gerbils showed no significant difference in serum levels of cytokines ($P > 0.05$) compared with reproductively inactive gerbils caught during fall (Fig. 2).

Serum Concentrations of Immunoglobulins: There is a statistically significant effect of seasonal breeding status on serum immunoglobulin G (IgG) concentrations (Fig. 3);

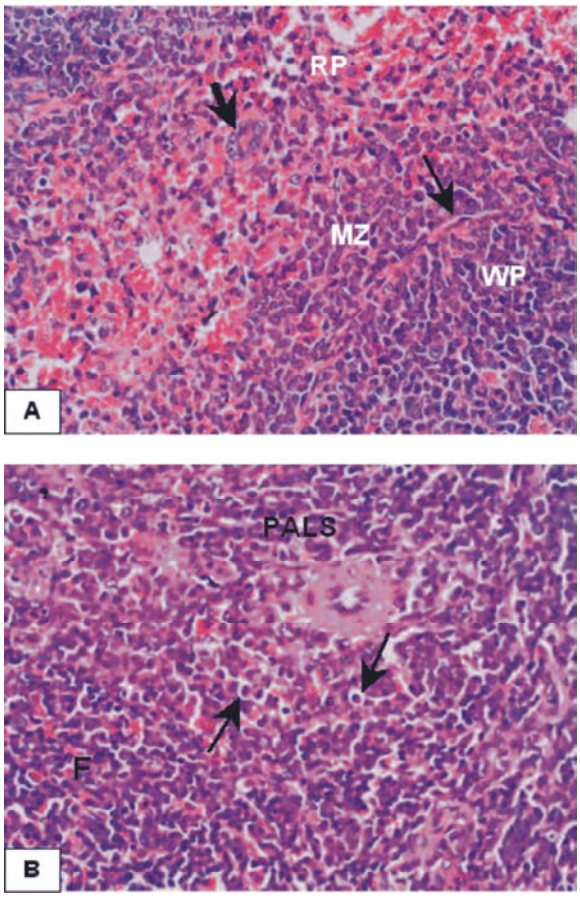

Fig. 4: Spleen sections from spring-caught reproductively active gerbils. (A) Showing clear distinction between the red pulp (RP) and the white pulp (WP); mature erythrocytes and hematopoietic cells (thick arrow) are evident in the red pulp; the marginal zone (MZ) as well as the marginal sinus and its sinus lining cells (thin arrow) are obvious. (B) Showing white pulp area with periarteriolar lymphoid sheath (PALS) and lymphoid follicles (F) densely populated with lymphocytes; apoptotic cells and tangible body macrophages (arrows) are occasionally seen within this area.(H & E. x 400).

IgG levels were significantly higher ($P < 0.05$) in serum from gerbils captured in spring (i.e. during the period of maximum reproductive activity) compared with gerbils captured in fall (i. e. during the period of minimum reproductive activity). On the other hand, serum levels of immunoglobulin E (IgE) were not season dependent, in that there were no statistically significant differences ($P > 0.05$) in serum concentrations of IgE between gerbils from breeding (spring) or non breading (fall) seasons (Fig.3).

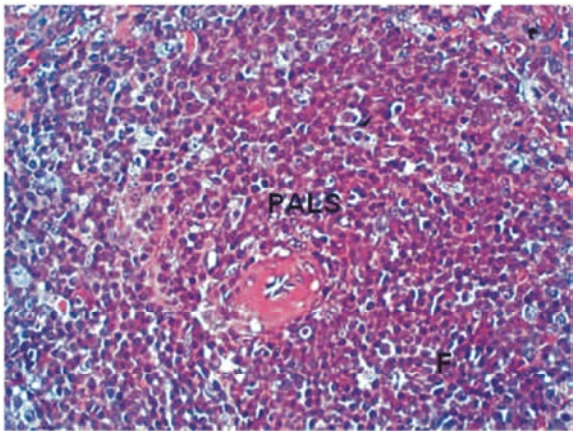

Fig. 5: Spleen section from a fall-caught reproductively inactive gerbil revealing white pulp with periarteriolar lymphoid sheath (PALS) and a part of lymphoid follicle (F). Note the slight decrease in the follicular lymphocyte density and the increase in macrophages. (H & E. x 400).

Histological Studies and Image Analysis: The spleen of gerbils captured during spring showed fairly normal histological architecture, composed of two distinct compartments, the red pulp and the white pulp (Fig. 4A & B). The red pulp consisted of splenic cords and venous sinuses. It contained many sells including macrophages, erythrocytes, granulocytes, monocytes lymphocytes, plasma cells and hematopoietic cells (Fig. 4A).

The white pulp was composed of periarteriolar lymphoid sheath (PALS), adjacent lymphoid follicles and prominent marginal zone located between the white and red pulp (Fig. 4A & B). The follicles were inconspicuously demarcated from the PALS (Fig. 4B). The White pulp was populated by small and medium lymphocytes, interdigitating dendritic cells, macrophages and plasma cells (Fig. 4B). Occasionally some apoptotic cells and tangible body macrophages engulfing apoptotic bodies were detected in the white pulp. (Fig.4 B). Pigment-laden macrophages were sometimes seen in the red pulp and white pulp (Fig. 4A & B). Conspicuous sinusoids were observed as well in the white pulp.

The spleen of reproductively inactive gerbils, trapped in fall during did not show remarkable structural changes in histological architecture compared to those of reproductively active animals. There was a slight decrease in the density of the follicular lymphocyte population associated with increase in macrophages throughout the white pulp (Fig 5). No other obvious changes were noticed in the PALS, lymphoid follicles, marginal zone or red pulp.

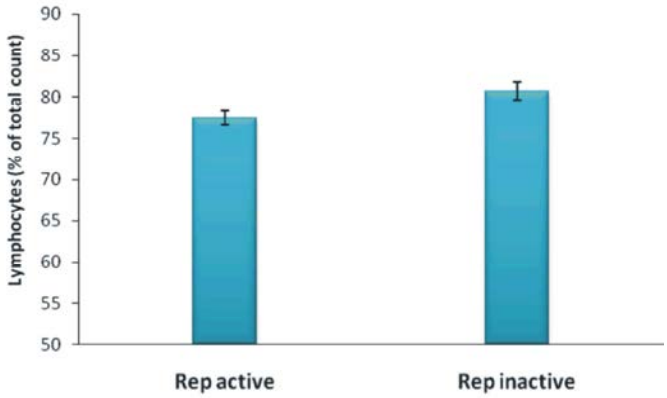

Fig. 6: Quantitative image analysis of hematoxylin and eosin-stained sections of spleen from spring-caught reproductively active gerbil (Rep active) and fall-caught reproductively inactive gerbil (rep inactive), with count of lymphocytes expressed as a percentage of the total count of white pulp cells. Bars represent Mean (± SEM).

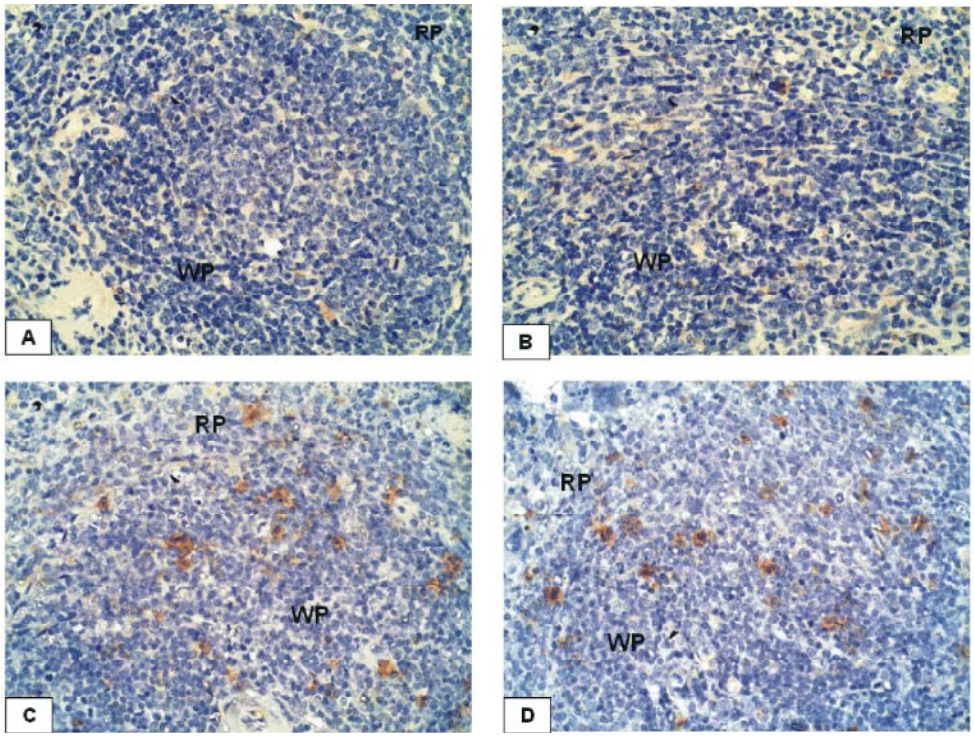

Fig. 7: (A, B) immunohistochemistry with anti-Bcl-2 antibodies revealing Bcl-2 immunoreactivity in spleen sections from spring-caught reproductively active gerbils. Note that Bcl-2 immunolabeling is scarcely detected in both white pulp (WB) and red pulp (RB) areas. (C, D) immunohistochemical staining of Bcl-2 in spleen sections from fall-caught reproductively inactive gerbils. Note the presence of extensively stained positive Bcl-2 cells in the white pulp (WP) and red pulp (RP).

Immunohistochemical Findings: Semiquantitative evaluation of the immunoreactivity of Bcl-2 protein in splenic tissue of spring-caught reproductively active gerbils revealed the presence of low rates of scattered faintly labeled Bcl-2 positive cells in both white and red pulp areas (Fig. 7A and B). On the other hand, spleen of fall-caught reproductively inactive gerbils showed high rates of extensively labeled Bcl-2 positive cells in the white pulp area; red pulp area contained moderate rates of such cells (Fig. 7C and D).

DISCUSSION

The present data demonstrate that all measured immune parameters were not suppressed by increased seasonal reproductive activity in desert lesser gerbil (*Gerbillus gerbillus*). Specifically, spleen mass, differential leukocyte count and serum concentrations of cytokines (IFNγ, TNFα, or IL-6) were not affected by seasonal reproductive status. Serum concentrations of IgG were higher in reproductively active gerbils trapped in spring than in reproductively inactive gerbils trapped in fall, whereas serum concentrations of IgE were not affected by differences in seasonal reproductive status. In addition, seasonal reproductive status had no effect on the percentages of lymphocytes in the splenic white pulp. The histological architecture of spleen showed slight changes in reproductively inactive gerbils manifested by slight decrease in the density of the follicular lymphocyte population associated with an increase in the macrophages throughout the white pulp. These findings are surprising and are in contrast to expected results. Based on the notion of energetic trade-offs between the reproductive and immune system, it was predicted that the immune function would be diminished or completely inhibited in gerbils having high reproductive activity and vice versa. According to the best of available knowledge, this is the first report of seasonal immune function in *Gerbillus gebrillus* and the first attempt to determine the trade-offs between reproductive and immune systems in this species.

In all mammalian species, the immune system consists of two main components, innate and adaptive immune responses. Innate immune response is non-specific and does not require pre-exposure to antigens to be elicited; therefore, it is the first line of defence against invading pathogens [13]. The effector cells of the innate immune response are natural killer (NK) cells, granulocytes (neutrophils, eosinophils and basophils), macrophages, monocytes and dendritic cells. They exert their effects by production of cytokines (e.g., IFNγ {NK cells}, TNFα, IL-β and IL-6 {macrophages and monocytes}), or by phagocytosis of antigens (macrophages, neutrophils and monocytes), or by lysis of infected cells (NK cells) [14]. On the other hand, adaptive immune response is more specific and needs pre-exposure to antigens to produce a full response; it involves cell-mediated and humoral immune responses. Cell-mediated immune responses entail T lymphocytes, whereas humoral immune responses entail the antibody production by B lymphocytes [15]. Within the T lymphocyte population, two subsets of cells are recognized, helper T lymphocytes (Th or CD4+ cells) and Cytotoxic/suppressor T lymphocytes (Tc/Ts or CD8+ cells). CD4+ cells are the key elements in the regulation and coordination of non-specific and specific responses through the production of a great variety of cytokines (e.g., IFNγ, IL-2, IL-4, IL-10). CD8+ cells directly destroy foreign or infected cells and secret cytokines as well [14]. It is well established that, various cytokines have pleiotropic actions as they act on numerous target cells. For example, IFN γ play an important role in specific and innate immune responses; TNFα is an essential mediator for pro-inflammatory responses, but it also has a role in activating T-cells; IL-6 stimulates the differentiation of T and B-lymphocytes, activates macrophages and NK cells and has anti-inflammatory properties [16].

It is noticed in the present experiments that different immune parameters varied independently in different seasons. That is, the only significant effect of seasonal reproductive status per se was a non-expected elevation in humoral immune responses in spring-caught reproductively active gerbils, as indicated by increased IgG concentrations. Other immune measures, including another arm of humoral immunity (Ig E), did not show any significant seasonal changes. Independent variation in immune parameters on seasonal basis has been well-documented in a number of mammalian species, without taking the reproductive status into consideration. For example, cotton rats (*Sigmodon hispidus*) exhibited a decreased number of peripheral white blood cells, but an augmented number of plaque forming cells (PFCs) in response to Sheep red blood cells in winter, compared with summer values [17]. Moreover, Female C57/B6C3F1 mice showed increased levels of PFCs in summer, but heaviest spleen and thymus masses in spring [18]. In rats, rabbits and dogs, significant seasonal variations were noticed in specific immune responses such as IgM antibodies, with elevation detected during autumn and winter, compared with spring and summer; non-remarkable changes were found in Ig E values or E-rosette-forming cell numbers [19]. The underlying mechanism for this independent variation in immune function across seasons is not exactly known, but it has been suggested that, at least in cotton rats, the seasonal changes in humoral and cell-mediated immunity are driven by changes in specific splenocyte subpopulation [20].

Despite the fact that all animals would benefit from strong immune defences against invading pathogens at all times of their lives, seasonal variation in immune responses is common [4]. It has been hypothesized that

immune functions vary seasonally because it is too expensive to use when other physiological activities are operating at maximum [21]. Additionally, it is well established that reproduction is energetically expensive [22]. Hence, many previous studies in both ectotherms and endotherms suggest the existence of trade-offs between reproduction and immune function, with immunity being compromised during energetically demanding reproductive periods [23, 24, 3, 25]. This hypothesis is not supported by the present study in which immune parameters were not suppressed in spring-caught reproductively active gerbils, but some measures as IgG levels were increased. The detection of high concentrations of IgG in serum of gerbils trapped during the period of maximum reproductive activity is intriguing and further investigation may uncover the mechanisms for such increase in humoral immunity during this period of high energy demands. Increased circulating IgG levels may reflect either elevated infection (i. e., a state of immunological activation) or elevated antibody secretion in preparation for coping with antigens (*i.e*., immunosurveillance, a state of immunological readiness) [26]. No signs of infections were observed in these gerbils, therefore, augmented IgG concentrations may interpreted as an increased immunosurveillance.

The present data, however, are in line with the observations of Xu *et al*. [27] that total IgG and anti-keyhole limpet hemocyanin (KLH) IgG have been significantly increased by increased reproductive investment (i.e., during lactation) in voles; other immune responses have not been suppressed in lactating voles as well. These findings support the lack of both physiological and fitness costs of immune activity, previously reported in different species [28, 29] and indicate that immune function may not always energetically expensive. It is difficult to explain these contradictory results, but such discrepancies may reflect the complexity of the vertebrate immune system. The development, maintenance and use of the immune system impart distinct costs. For example, the development of adaptive immune system is potentially the greatest immunological investment in vertebrate's life, but the cost of its use is modest [30]. In this context, it has been found that generating antibodies in domestic chickens (*G. gallus*) requires a small amount of the amino acid lysine compared with other physiological processes [30]. This may explain the present observation of utilization of humoral immunity function in gerbils during the most energetically demanding periods of their lives.

In contrast to earlier studies of seasonal changes in spleen mass in wild animals [4], no major differences were found in the splenic mass between reproductively active gerbils and reproductively inactive gerbils wild-trapped during the course of this study. Although there was a trend toward increasing splenic mass in reproductively active gerbils, this was not statistically significant. Similarly, no obvious seasonal changes were found in the splenic histological architecture or the lymphocyte number of splenic white pulp in these two groups of gerbils. Spleen is the largest second lymphoid organ; it is a critical immune organ that provides disease resistance through rapid production of antibodies, filtration of pathogens, lymphocyte recirculation and phagocytosis [31]. Therefore, It was expected to see patterns of seasonal changes in splenic mass and splenic architecture that were related to trends in immune function alterations during the periods of maximum and minimum reproductive activities. As noted earlier, spring-caught and fall-caught gerbils did not show any remarkable differences in these parameters.

Apoptosis is a form of cell death that is genetically regulated (programmed cell death). It is characterized by typical morphological signs including cellular shrinkage, membrane blebbing, nuclear condensation and fragmentation. It permits elimination of excess, damaged and aged cells that have accomplished their intended biological function [32]. It is well known that mitochondria play an essential role in apoptotic process. Death signals increase the permeability of the outer mitochondrial membrane, which sequentially causes apoptosis [33]. The permeability of the outer mitochondrial membrane is regulated by various proteins, the most important of which are the proteins of the Bcl-2 family. In this family, some proteins are pro-apoptotic and others are anti-apoptotic. A well-studied pro-apoptotic member of the Bcl-2 family is Bax. It causes the release of cytochrome c from the mitochondrial membrane into the cytoplasm. Cytochrome c in turn activates caspase and initiates the apoptotic process. On the other hand, Bcl-2 is an anti-apoptotic protein. It prevents Bax insertion into the mitochondrial membrane, thus inhibiting cytochrome c release [33]. It is well documented that the process of apoptosis is activated by suppressing Bcl-2 [34, 35]. In the present study, the expression of Bcl-2 in the spleen of spring-caught reproductively active gerbils and fall-caught reproductively inactive gerbils was shown by immunohistochemical staining. Reproductively active gerbils had higher apoptotic rates, as judged by suppressed Bcl-2 levels, compared to reproductively

inactive gerbils. One possible explanation for these results is that such suppression of Bcl-2 could reflect immune dysfunction linked to some factors such as increased oxidative stress and associated with mitochondrial dysfunction. No evidence from measured immune parameters in this study, including differential leukocyte counts, splenic mass, cytokine production, antibody secretion, spleen histological architecture and splenic white pulp lymphocyte percentages, support this hypothesis. Actually, as recorded earlier, the immune function was not suppressed in the reproductively active gerbils, but some immune parameters (IgG level) were increased. It is known that in normal lymphoid tissues, apoptosis and proliferation are related phenomena by which the total lymphocyte population is kept at a relatively constant level [36, 37]. Therefore, indirectly apoptosis can be interpreted as an indicator of proliferative activity [12]. The current results somehow strengthen this hypothesis and lymphocyte proliferation could be responsible for the increased levels of IgG. Unfortunately, no immunohistochemical studies concerning apoptotic changes that may develop in lymphoid tissues of *Gerbillus gerbillus* on seasonal basis are available to add more support to this suggestion.

Probably the most important conclusion that can be drawn from this study is simply that seasonal reproductive activity and immune function are not linked in *Gerbillus gerbillus*. Also seasonal changes in many immune parameters were not observed in this species. However, it is noteworthy to mention that, seasonal changes in immunity were not examined in the present study during the two extreme seasons (winter and summer), because it was out of the scope of this study. It is possible that *Gerbillus gerbiluus* experience seasonal fluctuations in immune parameters during summer and winter months regardless of reproductive status. These possibilities need to be further examined in future studies.

There is compelling evidence that variation in energy resource availability may drive the link between the reproductive and immune system, with functional trade-offs between these two systems occurring only when resources are limited; hence, animals can maintain both reproductive activity and immune function when given abundant resources [24]. The Egyptian Lesser Gerbils (*Gerbillus gerbillus*) breed during the winter and spring months in Eastern and Western deserts of Egypt [9]; their breeding period, from January to May coincides with the availability of rain, green food, reduced ambient temperature and short day lengths. These environmental conditions ensure peak energy availability. Taking this information into account, the present results that the reproductive status was not crucial for seasonal alterations in immune function, suggest the importance of resource availability in the occurrence of trade-offs between reproduction and immune systems in gerbils and support the suggestion of French *et al*. [24] that, trade-offs between reproductive and immune systems are not mandatory responses to physiological alterations during reproduction, but are facultative responses to resource availability; variance in species resource requirements, environmental resource availability and individual energy can influence the trade-offs and yield different results, depending on the experimental conditions

Taken together, the results presented here offer preliminary evidence that reproductive status in *Gerbillus gerbillus* is not predictive of seasonal variation in immune function and probably seasonal changes in energy availability may accurately predicts the direction of alteration. These results also provide evidence that *Gerbillus gerbillus* promise to be a useful animal model to further explore the hypothesis that link the importance of resource availability to both reproductive and immune function.

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