Season- and gender-dependent changes in the immune function of Siberian hamsters (*Phodopus sungorus*)

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Abstract

OBJECTIVES: Siberian hamsters are photoperiodic animals exhibiting seasonality of reproduction and other physiological functions. Thus, the influence of photoperiod on the *in vitro* activity of selected immune cells from male and female hamsters challenged with peritoneal inflammation was examined.

METHODS: Animals were housed for 8–10 weeks in LD (L:D=18:6) or SD (L:D=6:18). Peritoneal leukocytes (PTLs) and splenocytes were isolated form male and female and testicular macrophages (TMs) from male hamsters, intact or challenged with zymosan-induced peritonitis. PTL and TM activity was assessed by the level of reactive oxygen species (ROS) measured with the use of flow cytometry and splenocyte activity – by the spontaneous and mitogen-stimulated proliferation measured with the use of ^{3}H-tymidine incorporation test.

RESULTS: Results obtained indicate that the immune system of Siberian hamsters is highly sexually dimorphic. Experimentally evoked peritonitis developed differently in males and females: only in LD male hamsters an increase in PTL activity was observed after zymosan treatment. Also, in LD males, PTL activity was higher in LD than in SD. Developing peritonitis exerted in these animals a stimulatory effect on splenocyte proliferation but had no influence on cells residing in the immune privileged testes. Splenocyte proliferation, both spontaneous and PHA-stimulated, depended on the photoperiod studied: in LD it was significantly higher than in SD in animals of both sexes.

CONCLUSIONS: Innate immunity of Siberian hamsters studied on the peritoneal inflammation model, seems to be gender- and photoperiod dependent. Moreover, local inflammation may affect other lymphoid organs but does not influence immune-privileged sites.

Abbreviations & units
2-ME – 2-mercaptoethanol
AA – antibiotic antimycotic
cpm – counts per minute
FCS – foetal calf serum
GnRH – gonadotropin releasing hormone
LD – long day photoperiod (L:D=16:8)
MEM – minimal Eagle medium
NK – natural killer
PBS – phosphate buffered saline
PHA – phytohemagglutinin
PTL – peritoneal leukocytes
SD – short day photoperiod (L:D=8:16)
LH – luteotropic hormone
MEL – melatonin
TM – testicular macrophages

ORIGINAL ARTICLE

Introduction

Animals of temperate climates exhibit a wide range of adaptations to survive unfavorable winter conditions. Of many environmental indicators, photoperiod seems to be the most important in the regulation of animals' seasonality, the breading activity in particular. Siberian hamsters (*Phodopus sungorus*) are long-day breeders [6]. Processes of photoinduction and photorefraction in this species depend mainly on the light history of the animal and the direction of the change – whether the day-length increases or decreases [15]. Short day evokes gradual regression of reproductive organs in both males and females of this species [6, 12] followed by body-weight reduction and the change of fur color. Finally, after 10–12 weeks, episodes of torpor may be observed [10].

Also in the immune system of Siberian hamsters, seasonal changes are observed. Briefly, male Siberian hamsters housed for several weeks in SD exhibit enhanced T-lymphocytes and NK cells activity and lowered B-lymphocyte and non antigen specific (macrophages and neutrophils) responses (for review see: [19]). Little research has been conducted on female hamster immune system, in which seasonal changes seem to be far less evident than in males [2, 8]. At present, sexual dimorphism of the immune system, resulting mainly from the immunomodulatory properties of sex steroid hormones cannot be questioned [7].

The aim of this study was to examine the effect of photoperiod (LD vs. SD) on the immune system of male and female Siberian hamsters, control or challenged with zymosan-induced peritonitis.

Material and Methods

Animals. Experiments were conducted on adult (14–16 week old) Siberian hamsters (*Phodopus sungorus*), which have attained maturity in LD (L:D=16:8). At age of 6 weeks, animals were either put in SD (L:D=8:16) or left in LD, in constant temperature (22°C), with free access to food and water. Hamsters were submitted to treatment according to the Polish regulations concerning experiments on animals.

Reagents. Zymosan, PHA, 2-ME, MEL, glucose, and EDTA were purchased from Sigma. AA, HEPES buffer and sodium pyruvate were obtained form Gibco BRL. FCS was obtained from Alab (Warsaw, Poland). CM-H_2DCFDA was purchased from Merck. MEM and PBS were obtained from Polfa (Lublin, Poland) and ^{3}H-thymidine from UVVVR (Prague, Czech Republic).

Induction of Peritonitis. After 8–10 weeks of the adaptation to selected photoperiod, in some animals (ZYM) an inflammatory reaction was evoked by a single intraperitoneal (ip) injection of zymosan solution in PBS (2 mg/kg body weight; 0.5 ml per hamster, at 9.00 AM – 4th hour of light in LD or 1st hour of light in SD) according to the protocol established for the experiments on mice [11]. Control hamsters were left intact (INT). Animals were killed 24 hours later by the overdose of pentobarbital injected i.p., peritoneal leukocytes (PTL), testicular macrophages (TM) and splenocytes were collected for further *in vitro* assays.

Cell preparation. Peritoneal cavity was flushed with PBS (10 ml per hamster) and PTLs were collected and counted. Both testicles from male hamsters were removed, weighted and TM were washed out with 5 ml PBS per testicle, pooled within groups and counted. PTLs and TMs were centrifuged (8 min, 500 g) and re-suspended in PBS supplemented with glucose (90 mg/100 ml PBS), EDTA (20 mg/100 ml PBS) and AA (1 ml/100 ml PBS) – modified PBS – to obtain final suspensions of 2*10^6 cells/ml. Spleens were removed aseptically, pooled within groups, and homogenised in MEM. Erythrocyte lysis was conducted twice with the use of 0.17 M Tris/0.16 M NH_4Cl buffer, pH=7.2 (15 min in 4°C). Thereafter, cells were washed twice with MEM, counted and re-suspended in MEM supplemented with heat-inactivated FCS (10 ml/100 ml MEM), HEPES buffer (2 ml/100 ml MEM), AA (1 ml/100 ml MEM), sodium pyruvate (1 ml/100 ml MEM) and 2-ME (2*10^{-5} M) to obtain the final cell suspension of 2*10^6 cells/ml.

Reactive oxygen species (ros) level measurement. The method is based on the fluorimetric measurement of the concentration of dichlorofluorescein, the product of the reduction of dichloro-dihydro-fluorescein diacetate (CM-H_2DCFDA) by ROS synthesised in the cells examined. For each essay, 1*10^6 cells (PTLs and TMs)

Table I : Table I. The influence of photoperiod and zymosan-induced peritonitis on selected parameters of Siberian hamsters. *p<0.05, ** p<0.01 LD vs. SD, n=6-8.

	Male				Female			
Selected parameters	LD		SD		LD		SD	
Body weight (g)	31.81±0.92		30.55±1.1		26.09±0.01		25.1±1.1	
	INT	ZYM	INT	ZYM	INT	ZYM	INT	ZYM
Paired testicle weight (g)	0.47±0.02	0.42±0.01	0.045±0.004**	0.05±0.01**	–	–	–	–
PTL number (*10^6) 24h postinjection	4.09±1.5	1.75±0.6	5.59±2.8	3.92±1.1	2.22±0.1	5.57±3.4	7.7±3.3	3.58±1.4
TM number (*10^6) per paired testicles	0.52	0.31	2.78	3.15	–	–	–	–
Relative TM number (*10^6/g tissue)	1.25	0.77	76.42	63.32	–	–	–	–
splenocyte number (*10^6) per spleen	10.88	18.63	35.16	77.25	26.88	49.88	46.5	27.8

were used. Cells were incubated with CM-H_2DCFDA (0,5 μg per $1*10^6$ cells) for 45 min in 37°C, centrifuged, re-suspended in modified PBS and analyzed in a cytofluorometer [18 with modification].

Cell culture. Splenocyte cultures were conducted according to the routine established by Champney *et al* [3] with modifications. Cells ($2*10^5$ cells/well) were cultured in 96-well microtiter plates (Falcon) in the supplemented MEM medium alone (spontaneous proliferation) or in presence of PHA (3–12μg/ml) for 72 h in fully humidified 5% CO_2 atmosphere, in 37°C. Prior to harvesting with semiautomatic cell harvester (Scatron Instruments, Lier, Norway) onto the glass-fiber filters, 1 μCi of ^{3}H-thymidine was added to each well for the last 18 h [13]. ^{3}H-thymidine incorporation was measured by liquid scintillation spectrometry (Beckman) and expressed as counts per minute (cpm).

Phagocytic activity measurement. The activity of TMs isolated from LD male hamsters was measured using latex beads incorporation test established by Rodriguez *et al*. [16]. Phagocytic index (PI) was calculated as the number of latex beads ingested by 100 macrophages

Statistical analysis. A Student's non-paired *t*-test was used for statistical analysis and results were expressed as mean ± S.E. A value of $p<0.05$ was taken as significant.

Results

After 8–10 weeks of housing in SD conditions, an apparent change in the pelage color of hamsters of both sexes was observed. Although there was no reduction in body weight, in males a significant decrease of testicle weight was noted (table I). No differences in the number of residential PTLs (INT animals) was observed between either the two sexes or photoperiods. Contrarily, in both photoperiods the number of splenocytes isolated from females was higher than from males. SD conditions increased the number of splenic leukocytes in both sexes as well as the number of TMs in males. In both sexes, the induction of peritonitis did not influence the number of PTLs 24 h postinjection, but it decreased TM number in LD males and increased the number of splenocytes in males housed in both photoperiods.

In intact animals, a gender-dependent difference between PTLs was observed: in males it was a homogenous population, contrarily to females, where a distinct subpopulation described as "macrophages" was seen (Figure 1 A and C, only exemplary results for LD animals are given). This phenomenon was similar in both photoperiods. Zymosan treatment did not influence PTL subpopulations in females Figure 2 C and D), while in males two distinct groups of cells described as "granulocytes" and "macrophages" appeared 24 hours postinjection (Figure 1 A and B).

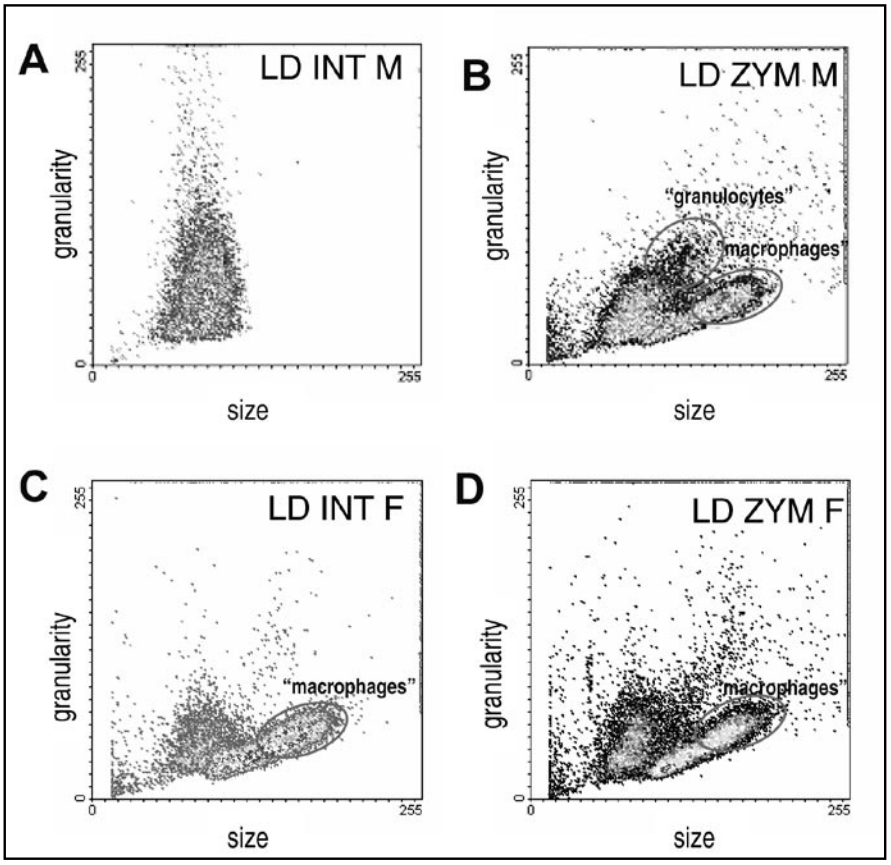

Figure 1. Exemplary dot-plots showing differences in the subpopulations of PTLs retrieved from intact LD male (A) and female (C) hamsters and in their response to zymosan treatment (B and D) 24 h postinjection.

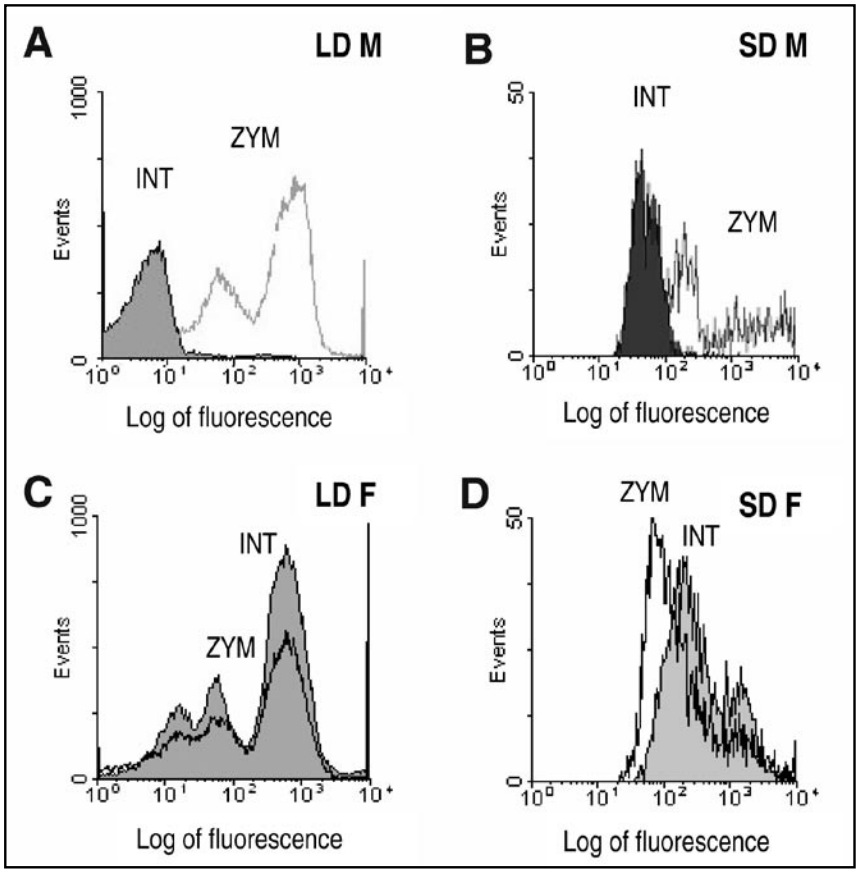

Figure 2. Exemplary histograms showing gender-dependent (A vs. C, B vs. D) and seasonal (A vs. B, C vs. D) differences in the effect of zymosan injection in PTLs after 24 h. The shift to the right along the vertical axis illustrates the increase in ROS level.

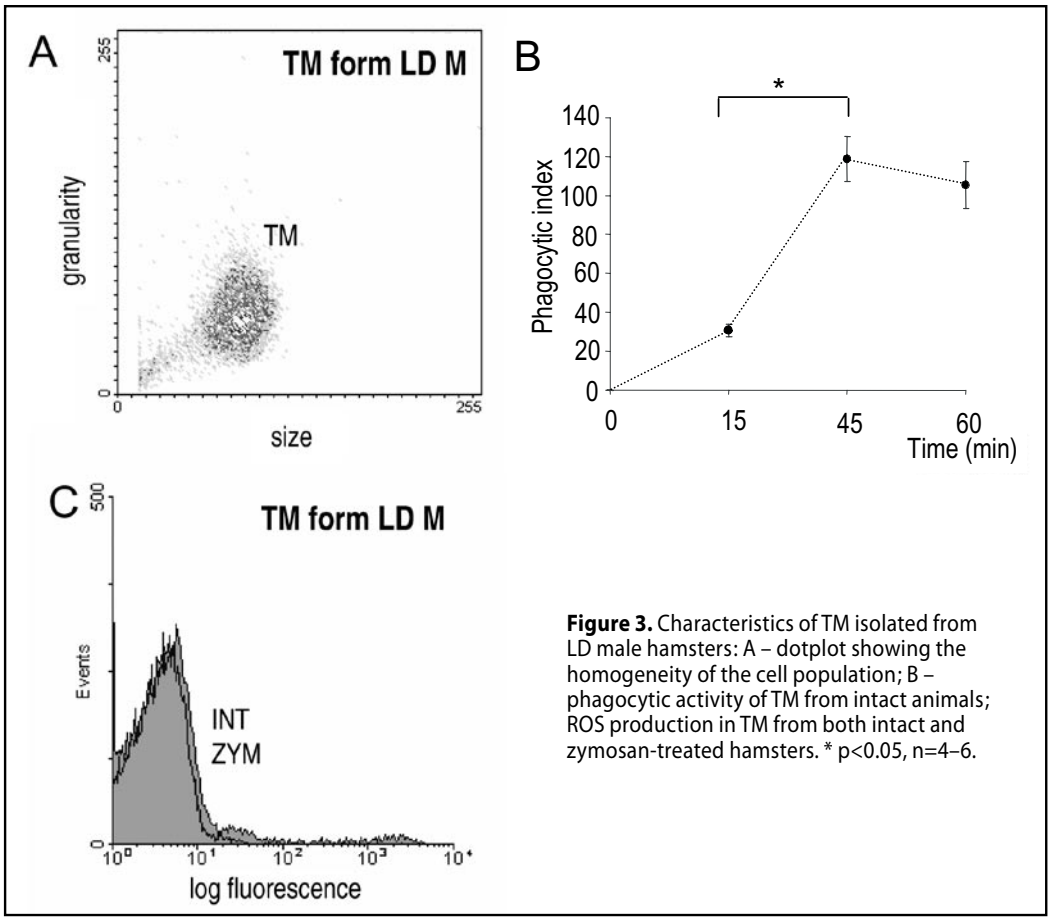

Figure 3. Characteristics of TM isolated from LD male hamsters: A – dotplot showing the homogeneity of the cell population; B – phagocytic activity of TM from intact animals; ROS production in TM from both intact and zymosan-treated hamsters. * $p<0.05$, $n=4–6$.

Induction of peritonitis increased the activity of PTLs isolated from male hamsters 24 hours postinjection (Figure 2 A), while zymosan-treatment had no influence on ROS production in PTLs retrieved from females (Figure 2 B). This effect was similar in both photoperiods studied, however in males it was much more pronounced in LD compared to SD (Figure 2 A and B).

A homogenous population of TM was isolated from LD male hamsters (Figure 3 A). TM exhibited latex-bead stimulated phagocytic activity (Figure 3 B) and spontaneous ROS synthesis, which, contrarily to PTL activity, was not affected by the zymosan injection (Figure 3 C).

Both spontaneous and mitogen-stimulated splenocyte proliferation was higher in LD compared to SD in both sexes (Figure 4 A vs. B and 4 C vs. D) and only in LD male hamsters it was significantly increased by zymosan injection (Figure 4 A vs. B, C and D).

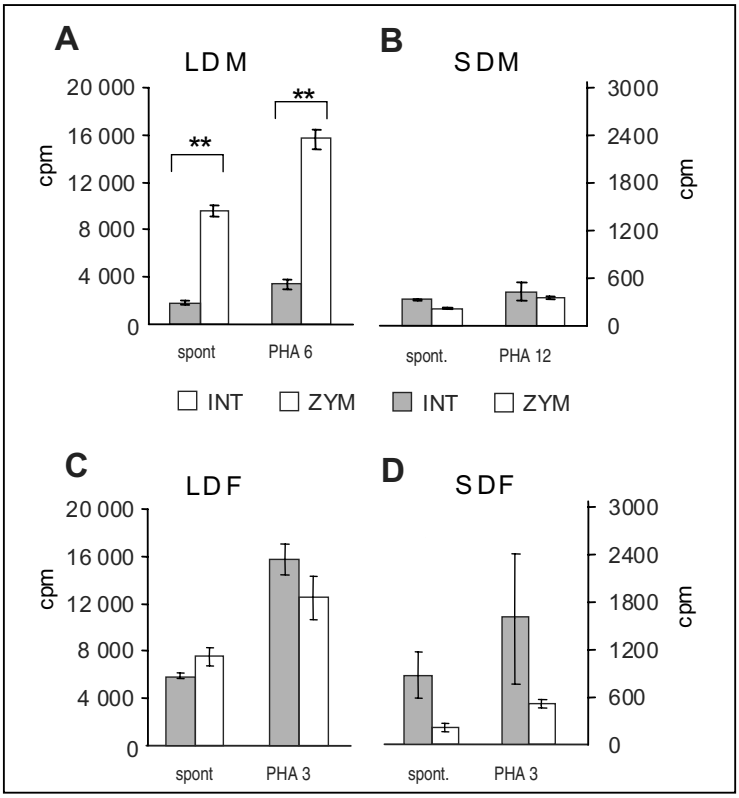

Figure 4. The effect of zymosan treatment on the spontaneous and PHA-stimulated splenocyte proliferation in male (A and B) and female (C and D) hamsters housed in LD or SD conditions. ** p<0.01, n=4–6.

Discussion

A number of studies indicate the existence of photoperiodic changes in the immune system of Siberian hamsters, however to our knowledge, there are no papers concerning experimental peritonitis in this species. In our experiments the activity of selected immune cells isolated from intact animals or challenged with zymosan-induced peritonitis were investigated in relation to sex and season.

In the experimental model applied, hamsters housed in SD for 8–10 weeks underwent physiological adaptations typical for "winter" conditions such as the apparent change in pelage color and the testicle regression in males. However, no difference in body weight between LD and SD animals of both sexes was noted, which is probably due to the same standard diet for laboratory rodents used in both lighting conditions.

Some studies indicate that in Siberian hamsters the innate immunity (assessed by phagocytosis and oxidative burst measurements and pro-inflammatory cytokines expression) is diminished in SD [19, 1]. On the other hand, the effect of photoperiod on leukocyte proliferation has been contradictory. In male Siberian hamsters, blood leukocyte proliferation [19] and the activity of naive (as opposed antigen-challenged) lymphocytes from auricular nodes [20] were decreased in LD compared to SD, whereas concanavalin A-stimulated splenocyte proliferation was significantly higher in LD than SD [4]. Our data indicate that both innate (peritoneal inflammation) and acquired (splenocyte proliferation) immunity are enhanced in LD compared to SD. In hamsters of both sexes spontaneous and PHA-stimulated splenocyte proliferation, was significantly higher in LD (Figure 3). Moreover, splenocytes isolated from SD animals were less sensitive to PHA (data not shown), which is why higher doses of mitogen in the experiments on SD male hamsters were needed for obtaining the stimulation of proliferation (Figure 4 A and B). Interestingly, in intact hamsters of both sexes, the number of splenocytes per spleen was higher in SD than in LD (table I). We hypothesize that at the end of the summer season an amount of lymphocytes is produced and stored in the lymphatic organs to facilitate and quicken the immune response in winter.

Peritoneal inflammation is a convenient model, used also in our laboratory to investigate changes in the innate immunity of birds [13]. Surprisingly, in male hamsters, the total number of PTLs did not increase 24 h after zymosan injection (table I) in either of the photoperiods studied, however we observed the increase in ROS synthesis by PTLs (compared to intact animals), which was important in LD and much less evident in SD (Figure 2 A and B). Not only did the zymosan-treatment influence the activity of PTLs, but also it changed PTL subpopulation subsets: new subpopulations named "granulocytes" and "macrophages" basing on their size and granularity, appeared (Figure 1 A and B). Moreover, splenocyte activity, both spontaneous and PHA-stimulated, was significantly increased in animals with the inflammatory reaction compared to intact hamsters (Figure 4 A) but this was observed only in LD. This suggests that in males, in the given experimental conditions, peritoneal inflammation is not a local reaction but may affect other lymphoid tissues, excluding however immune privileged testes. We have found that although TMs isolated form LD animals are functional phagocytes (Figure 3 A) and have a very low spontaneous ROS synthesis, their activ-

ity remains unaffected (contrarily to PTLs activity) by zymosan injection (Figure 3 C).

We planned to perform experiments on TMs isolated from both LD and SD male hamsters in relation to the dramatic seasonal changes observed in the reproductive system of these animals. Unfortunately, even though the number of TMs retrieved form SD hamsters was higher than that of LD animals (table I), we were unable to conduct *in vitro* assays on these cells. For the reasons yet unknown, the efficacy of the purification procedure of SD TMs was dramatically lower than that of LD TMs. Further investigation is necessary, but so far we hypothesize that their adherence may be lowered in SD.

In female hamsters, interestingly, no effect of zymosan injection on either the PTL number (table I) or ROS synthesis (Figure 2 C and D) was observed. Moreover, PTL subpopulations retrieved form both intact and zymosan-treated females (LD and SD) were similar (Figure 1 C and D). These results indicate that zymosan injection cannot induce peritoneal inflammation in females, or that it develops differently and different time-points should be chosen for *in vitro* experiments. This sexual dimorphism of the development of experimentally-induced peritonitis has already been reported in birds [18] and similar gender-related differences are found in human blood leukocytes activity and explained by the immunoenhancing properties of estrogens [5].

To conclude, in this study we demonstrated that the immune system of Siberian hamsters is sexually dimorphic, especially when the peritoneal inflammation is concerned. Further investigation with the use of specific antibodies is necessary, but the results presented herein indicate, that the peritoneal inflammation in male Siberian hamsters may not consist in the high influx of immune cells but rather in the stimulation of the activity of the cells already residing in the peritoneum. In male hamsters, locally evoked peritoneal inflammation stimulated splenocyte proliferation having no influence on cells residing in the immune privileged testes. Photoperiod affected both the intensity of peritoneal inflammation in males as well as splenocyte proliferation in animals of both sexes.

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