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ORIGINAL ARTICLE

On the winter enhancement of adaptive humoral immunity: hypothesis testing in desert hamsters (*Phodopus roborovskii*: Cricetidae, Rodentia) kept under long-day and short-day photoperiod

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Abstract

We tested the winter immunity enhancement hypothesis (WIEH) on male desert hamsters (*Phodopus roborovskii*) kept under long-day (LD) and short-day (SD) photoperiods. We assumed that under SD in a laboratory, the adaptive humoral immune responsiveness to the antigenic challenge would be enhanced due to the lack of winter physical stressors and food shortages and/or because of the action of an endogenous winter bolstering mechanism, while under LD the immune responsiveness would be suppressed by the activity of the reproductive system. The results support the WIEH in part. We did not find a difference in antibody production in response to sheep erythrocytes between SD and LD hamsters, but SD males had the lower number of granulocytes and the higher number of lymphocytes in white blood cell counts. Reproductive activity was lower in SD males. These males demonstrated an increase in their mass-specific resting metabolic rate, their mass-specific maximal metabolic rate and their level of cortisol. The result of a generalized linear model analysis indicates the negative effect on secondary immunoresponsiveness to sheep erythrocytes of mid-ventral gland size, the organ characterizing individual reproductive quality, and designates a tradeoff between antibody production and reproductive effort. The mass-independent maximal metabolic rate also negatively affected antibody production, indicating a tradeoff between maximal aerobic performance and the adaptive immune function. The higher stress in SD males seems to be the most likely reason for the lack of the effect of daylight duration on antibody production.

Key words: adaptive humoral immunity, desert hamster, photoperiodicity, winter immunoenhancement

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INTRODUCTION

It is well known that in seasonally breeding vertebrates, the day length triggers and regulates reproduction (Elliott & Goldman 1981; Bronson 1988; Dawson

et al. 2001; Goldman 2001; Hazlerigg 2012). With regard to seasonal changes in immunity, the effect of day length is not so uniform. The results of studies are ambiguous and vary depending on which branch of immunity (innate, adaptive cellular or humoral) is examined (Martin *et al*. 2008; Zysling *et al*. 2009; Adelman *et al*. 2013; Stevenson & Prendergast 2015; Schults *et al*. 2017).

At the same time, in accordance with the winter immunoenhancement hypothesis (Sinclair & Lochmiller 2000), immunocompetence should increase in winter due to the action of an endogenous bolstering mechanism (Nelson & Demas 1996; Sinclair & Lochmiller 2000; Nelson 2004) or to the tradeoff between reproduction and energetically costly immunity functions (Martin *et al*. 2004, 2006a; Greenman *et al*. 2005). The first explanation is based on the assumption that winter stressors activate higher production of glucocorticoid hormones by the adrenal cortex, which suppress specific aspects of immunity (e.g. McEwen *et al*. 1997; Demas & Nelson 1998; Dhabhar 2000; Padgett & Glaser 2003). The endogenous bolstering suggests the existence of an endogenous buffer mechanism that enhances immune function in winter when the influence of physical stressors and nutritional deficiencies increases. According to the second explanation, the actual level of immune activity is the result of a tradeoff between costly functions competing for common and limited resources. Primarily, it is a tradeoff between immunity and reproduction. Although both mechanisms may act simultaneously, which makes them difficult to verify (Martin *et al*. 2008), in its general terms, the winter immunoenhancement hypothesis can be tested under standardized conditions of a laboratory experiment.

By providing contrasting photoperiods and other standardized conditions, we can expect immune function to be higher in short daylight (SD), because in a laboratory we have winter physical stressors and nutritional deficiencies removed, whereas in long daylight (LD) the mechanisms of immune defense will be suppressed by the activity of the reproductive system.

We tested this assumption in an experiment on same-aged, mature, virgin desert hamster males [*Phodopus roborovskii* (Satunin, 1903)] from a laboratory-bred population. We expected that with SD, reproductive functions would be suppressed, and the adaptive immune response to the antigenic load would be stronger. At the same time, the background level of stress would be higher with LD because the action of external stressors not related to reproduction is excluded. We also assumed that the cost of activation of adaptive humoral immunity would be high, because it is associated with the proliferation of antibody-producing cells and the production of antibodies in response to infections (Demas *et al*. 1997; Svensson *et al*. 1998; Ilmonen *et al*. 2000; Ots *et al*. 2001). That is why we studied the effect of the duration of daylight on the responsiveness of adaptive (acquired) humoral immunity.

We also analyzed blood cell counts on smears of peripheral blood. Changes in leukocyte counts (WBC) depending on SD and LD photoperiods can characterize the response both of innate and adaptive immunity (Prendergast *et al*. 2003). The higher number of lymphocytes in baseline WBC in SD photoperiod may testify to the enhancement of adaptive immunity (Blom *et al*. 1994; Yellon *et al*. 1999; Bilbo *et al*. 2002, 2003). Acute stressors induce trafficking of lymphocytes and monocytes out of the blood, and the process goes faster in SD than in LD (Bilbo *et al*. 2002). Accordingly, the neutrophils (heterophils in birds)-to-lymphocytes ratio in peripheral blood smears was repeatedly used as an index characterizing the stress level in free-range animals (Davis *et al*. 2008).

We set up our experiment on males because of the repeatedly discussed negative relationship between androgens and the functional activity of the adaptive immunity system, including the possibility of exerting a direct inhibitory effect of testosterone (e.g. Grossman 1985; Schuurs & Verheul 1990; Klein 2000; Roberts *et al*. 2004; Mills *et al*. 2009; Giefing-Kröll *et al*. 2015).

MATERIALS AND METHODS

Animals

The desert hamster is a small, dusk-active and night-active, non-hibernating, polyestrous, sexually dimorphic rodent that feeds on seeds and insects. It inhabits deserts and semi-deserts of Central Asia, while being common to South Tuva, Mongolia and China (Flint & Golovkin 1961; Sokolov & Orlov 1980; Ma *et al*. 1987; Ross 1994). At maturity, female weight ranged from 18–22 g and male weight ranged from 20–28 g. Males have a large specific mid-ventral gland, the testosterone-dependent organ (Mitchell 1965; Sokolov & Chernova 2001), which is seasonally variable in size and activity depending on participation of the male in reproduction (Zhang *et al*. 2015). Hamsters use a secretion of the gland to mark territory and to communicate with each other.

There is no published information on reproduction, mating system and territorial relations in nature. According to our observations in Trans-Altai Gobi Mongolia, reproduction is limited to the warm period of the year. Seasonally restricted reproduction is confirmed by the dynamics of sex hormone concentrations in hamsters kept outdoors year-round (Feoktistova & Meschersky 2005; Feoktistova *et al*. 2010), as well as by the observation of seasonal variation in sex hormonal response to conspecific odors (Feoktistova & Naidenko 2006).

The experimental hamsters originated from a laboratory population at the A.N. Severtsov Institute of Ecology and Evolution RAS, descended from the founders, caught in the Mongolian Trans-Altai Gobi Desert in the mid-1980s. Although the seasonality of breeding in a vivarium under standard conditions with a photoperiod of 14 h light : 10 h dark and a temperature of 23 ± 2 °C was lost over the years, once the animals were relocated outdoors, they soon demonstrated seasonal patterns of breeding (Feoktistova & Meschersky 2005; Feoktistova *et al*. 2010; Vasilieva, unpubl. data).

The animals were born and grew up in a vivarium under a fixed photoperiod of 14 h light, which provided normal female cycles and breeding all year-round. Food (mixed fodder for rats, mice and hamsters: oats, rye bread, beets, cabbage, with the addition of sunflower seeds and low-fat cottage cheese once a week) and water *ad libitum*. Wood chips were used as litter. The litter was replaced once every 10 days but not less than a week before any manipulation with the animals. Each cage was equipped with a shelter and a running wheel.

Design of the experiment

The experimental males lived with their parents and siblings up to the age of 1.5 months (Fig. 1). We kept males in sexually uniform groups of 10 individuals each in cages (70 × 40 × 40 cm^3). At the age of 3.5–4.5 months we placed the males into separate plastic cages (34 × 26 × 22 cm^3). A month later, all males were immunized with sheep erythrocytes (SRBC; see below), and the primary humoral immune responses to SRBC were estimated (see below). We took blood samples from the animals' sublingual vein (Graievskaya *et al*. 1986) on the 7th day after immunization, when the response to SRBC was already high. According to the preliminary testing, there was no difference in immune response between 6 and 11 days after immunization (Mann–Whitney *U*-test: $Z = 1.47$, $P = 0.14$, $n_1 = 34$, $n_2 = 10$). This

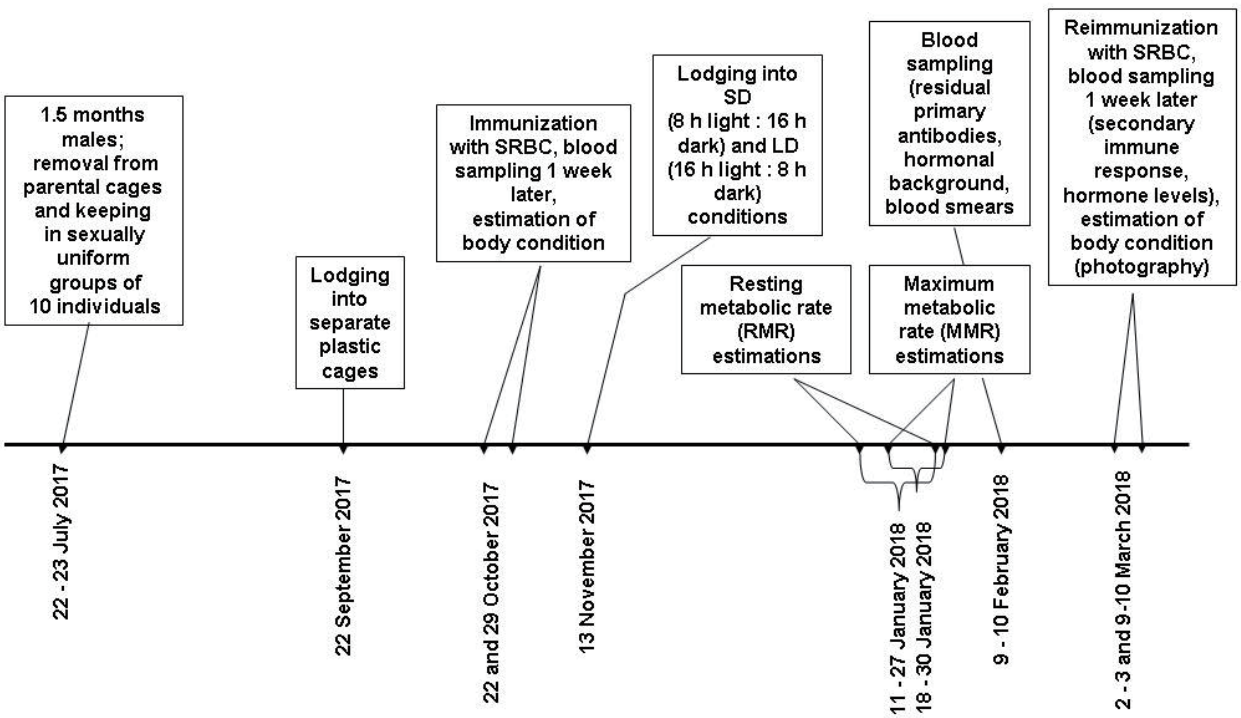

Figure 1 Experiment schedule.

pattern in desert hamsters is similar to what we found in *Phodopus campbelli* (Rogovin *et al*. 2014).

Two weeks later, and after estimation of primary immune responses, we divided the pool of males into 2 identical groups with equally represented immune responses and body masses. Subsequently, 35 males were kept in individual cages in a room with a photoperiod of 8 h light : 16 h dark (SD mode), and 34 males in a room with a photoperiod of 16 h light : 8 h dark (LD mode). In both rooms, the air temperature was maintained at 23 ± 2 °C and fluctuated synchronously.

At the end of a 2-month period of adaptation to the light regime, we estimated the resting metabolic rate (RMR) in animals of both groups. SD and LD individuals were used in equal proportion for measurements on different days with conduction tests in the daytime, when the animals' activity was minimal. The maximum metabolic rate (MMR) in the acute cooling test was estimated using a similar scheme with intervals after individual RMR measurements from 4 to 7 days. Each hamster was tested only once in each test to avoid adaptation.

We took blood samples again from the animals' sublingual veins 9–10 days after completing the metabolism measurements to assess the retained primary immune response and background concentrations of cortisol and testosterone. Within the first 5 s after puncturing the venous sinus, we made 2 blood slides. Slides were air dried for the subsequent staining (see below).

One month after completing the maximum-metabolism measurements and 3 weeks after taking blood to estimate background hormone levels, we reimmunized the males with SRBC. On the 7th day after reimmunization, blood samples were taken to evaluate the antibody titer, as well as serum cortisol and testosterone concentrations at the peak of the immune response.

In total, 3 blood samples of 300–350 μL were taken from each hamster. Blood was always taken at the beginning of the light phase. The procedure continued no longer than 1.5 min, which is half the time of the glucocorticoid signal in the blood in response to a restrained stressor. The serum was separated by centrifuging the blood for 15 min at 4136 g, frozen and stored at −19 °C until analysis.

After the last blood sampling, the males were photographed with a Nikon D7000 SLR camera against a ruler scale in the fixed standard position, belly up. The head–body length (from the tip of the nose to the anus, mm), the size of the mid-ventral gland (length multiplied by width, mm^2), and the ano-genital distance (mm) were then measured on a computer screen from digital images (Shekarova *et al*. 2011). We estimated body mass with an accuracy of 0.1 g at the beginning and at the end of the experiment, and during the metabolism estimations. The mid-ventral gland and ano-genital distance are secondary sexual traits of male dwarf hamsters (Vasilieva *et al*. 2015). We used the gland size and the distance between the base of the penis and the anterior margin of the anus as the most definite indicators of the reproductive status of the male. Recovery of reproductive activity in photoperiodic rodents is thought to begin after 20 weeks on SD (Reiter 1972; Prendergast & Nelson 2001; Prendergast *et al*. 2002). This is 3 weeks longer than the duration of our experiment.

Resting metabolic rate

We measured RMR using the indirect calorimetry method in an open-circuit system from 1000 to 1600 hours. The animals were placed in 1.3-L cylindrical polypropylene chambers, which were placed in a thermostat with a set temperature within the thermoneutral zone of the species (T = 30 ± 0.5 °C) (Zhan & Wang 2004). A little bedding from the individual nest was put into each chamber.

Five independent membrane pumps pushed outside air at a speed of approximately 300 mL/min through chambers with KSMG color-indicating silica gel to dry the air from water vapor and then through the chambers with hamsters. To evaluate the gas exchange in several animals within 1 session, an airflow changeover system was used, which alternately sent air from the chambers with hamsters and from the empty chamber to the gas analyzers in an automatic mode (for more details, see Rogovin *et al*. 2014). The gas exchange measurement system was assembled on the basis of 2 integrated CO_2 and O_2 gas analyzers (Sable Systems, USA, FoxBox-C), each of which contained a variable pump and mass flow meter. The air coming out of the animal chambers first passed through the chamber with a Drierite desiccant (V = 50 mL) and then through the flow meter. After that, the airflow was divided, and a part of it at a rate of 100 mL/min was directed to O_2 and CO_2 gas analyzers of both FoxBox respirometers. The registration of the relative concentrations of oxygen and carbon dioxide as well as the speed of the airflow occurred every 3 s on both respirometers.

Four hamsters were used in each measurement session; the gas exchange of each hamster then was measured twice for 35 min. We always discarded the first 5

min of measurements, as the chamber still contained air from the previous hamster, and it took time for the gas concentrations to reach a new stable value. The volume of oxygen consumed was calculated from the relative concentrations of O_2 and CO_2 in accordance with the Haldane transformation principle (Luft *et al*. 1973; Wagner 1973). As an indicator of RMR (mL/min), an 8-min portion of the gas-exchange curve was used, which was found using the minimum moving average method. After completion of the experiment, we removed the hamsters from their chambers and weighed them with an accuracy of 0.1 g. Individual body masses were later used to calculate mass-independent RMR (residuals from the regression of RMR on body mass) and mass-specific RMR, which was estimated as oxygen consumption (mL) per unit mass (g) per hour.

Maximum metabolic rate

The MMR was assessed in a test of short-term cooling, where exposure to cold in a helium–oxygen atmosphere was used as a stressful stimulus (Rosenmann & Morrison 1974; Wang 1980). The measurements were carried out in a portable close-circuit respirometer designed and made by D. V. Petrovski (Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences). The hamsters were placed in a helium–oxygen (80%:20%) atmosphere for 18 min at an ambient temperature of 7 °C. In similar experiments with desert hamsters, no correlation was found between oxygen consumption and ambient temperature within 6–10 °C (Moshkin *et al*. 2002). Consequently, the oxygen consumption values obtained at 7 °C can be considered as an indicator of the maximum aerobic performance. After placing the animal in a chamber of 1.2-L volume, a calibrated helium–oxygen mixture from the gas tank was pumped through it for 3 min. The total volume of this mixture was at least 3.5 times greater than the working volume of the system. After aeration, its supply with the gas mixture was terminated, and the gas mixture remaining in the system circulated through the hermetically sealed circuit consisting of the pump, the chamber with the animal, and the container with alkali (KOH). Every minute the pressure in the system was brought to atmospheric pressure, and the working mixture was enriched with pure oxygen. Oxygen consumption readings were automatically recorded every minute in accordance with standard conditions (STPD). As a measure of MMR (mL/min), a 5-min interval of the oxygen consumption curve was used. It was found using the maximum moving average method.

After completion of the experiment, the hamsters were weighed with an accuracy of 0.1 g. Individual body masses were used to calculate a mass independent MMR (residuals from the regression of MMR on body mass) and mass specific MMR (mL O_2/g × h).

Humoral adaptive immunoresponsiveness

To determine the immune status, the animals were intraperitoneally injected with a 2% suspension of SRBC in isotonic saline solution at the rate of 2 μL/g of body mass and a blood sample was taken on the 7th day after immunization. Immediately before the injection, SRBC were centrifuged at 4136 g for 10 min and rinsed from Alsever's solution 3 times (whole fresh ram's blood preserved in Alsever's solution certified by the K.I. Skryabin Moscow State Academy of Veterinary Medicine and Biotechnology was used). The level of antibodies in the serum of immunized hamsters was determined by hemagglutination reaction (Wegmann & Smithies 1966) in the wells of a 96-well immunological plate by titrating serum samples in the wells and by adding to serially diluted samples 0.5% suspension of SRBC in saline. The antibody titer (ABT) in the serum was assessed visually by the number of the last well of the plate, in which, at successive multiple dilutions, the amount of antibodies was still sufficient for hemagglutination. The number of the last well was used as an indicator of the intensity of the immune response. In the case of a value intermediate between 2 adjacent wells, 0.5 was added to the number of the previous well.

Blood smears and counts of blood cells

Air-dried blood smears were stained with May–Grünwald and subsequently with azure-eosin for further estimation of blood cell counts (Nikitin 1949). We rejected methanol fixation before staining and used simply air-dried smears. Robertson and Maxwell (1990) showed that methanol fixation could decrease the stainability of basophile granules, resulting in a risk of miscounting basophils as lymphocytes.

Microscopy of imbued smears was done under 10 × 100 magnifications with oil immersion. We estimated the numbers of neutrophils, eosinophils, basophils, monocytes and lymphocytes and the neutrophil-to-lymphocyte ratio counting different cell types in a sample of 100 leukocytes. Heterophil-to-lymphocyte or neutrophil-to-lymphocyte (H : L or N : L) ratios were used extensively to characterize physiological stress levels in birds and mammals in the past, and were rehabilitated as a reliable assessments of stress in all vertebrate taxa

after critical analysis of the published literature (Davis *et al*. 2008). The white blood cell count (WBC) was estimated by counting the number of leukocytes per 10 000 erythrocytes. The number of erythrocytes and the number of leukocytes were counted using a mechanical cell counter. As soon as the number of red blood cells reached 10 000 within sectorized non-overlapping fields of vision, we stopped counting leukocytes and recorded the result. We did not make a precise hemocytometric calculation of the numbers of red and white blood cells in relation to blood-volume unit.

Hormones

Serum testosterone and cortisol concentrations were assessed by means of an enzyme-linked immunosorbent assay (ELISA). We used ready-made test systems, "IEA-TS (testosterone)" and "IEA-cortisol" ("ZAO Immunotekh," Moscow, Russia). The cross-reaction of cortisol with testosterone for these kits was 0.08%. Optical density was measured with a plate spectrophotometer "iMark" at a wavelength of 450 nm. If the testosterone concentration was higher than the sensitivity limit of the test system proposed by the manufacturer, we diluted the serum with a buffer solution for dilution of blood serum from the same manufacturer.

Data samples

Of the 69 males in the experiment, we lost 3 males: 1 male escaped, 1 died in the SD group and 1 died in the LD group. In a few cases we could not estimate metabolic rate, immunity response or concentration of hormones. The sample sizes included in the analysis are given in Table. 1. Analysis of the effects of predictor variables on the immunoresponsiveness of males was carried out using the most complete dataset, which included 60 individuals (29 SD and 31 LD).

Variables for analysis and statistical methods

To compare 2 independent male groups kept in the SD and LD photoperiods, we used the following list of variables (Table 1) : (i) the intensity of the immune response to SRBC after reimmunization (ABT 2, c.u.); (ii) the difference between the magnitude of the response after reimmunization and the values of the retained primary response, measured before reimmunization (ΔABT_{1-2}, c.u.); (iii) the background level of cortisol (Cort 1, nm/L); (iv) the background level of testosterone (Testr 1, nm/L); (v) cortisol level after reimmunization with SRBC (Cort 2, nm/L); (vi) testosterone level after reimmunization with SRBC (Testr 2, nm/L); (vii) resting metabolic rate, by the volume of oxygen consumed per minute (RMR, mL O_2/min); (viii) mass-specific resting metabolic rate (SRMR, mL O_2/g × h); (ix) maximum metabolic rate (MMR, mL O_2/min); (x) mass-specific maximum metabolic rate (SMMR, mL O_2/g × h); (xi) body mass when measuring RMR (BMAS RMR, g); (xii) body mass when measuring MMR (BMAS MMR, g); (xiii) head-and-body length (L, mm); (xiv) mid-ventral gland size (MVG, product of length by width in mm^2); and (xv) ano-genital distance (AnGen, mm).

The mid-ventral gland and ano-genital distance represented secondary sexual traits. Body mass directly characterized fatness. We found it to be the best indicator of fatness as compared with commonly used body condition indexes (Peig & Green 2010). White fat deposits estimated in 15 SD and 16 LD males euthanized with CO_2 in June 2018 after the end of the experiment were highly correlated with body mass. Pearson's r between log-transformed data was for SD $r = 0.95$, $P < 0.001$ $y = 1.21 + 0.37x$ and for LD $r = 0.88$, $P < 0.001$, $y = 1.27 + 0.3x$. We used the difference between the antibody titer after repeated immunization with SRBC and the remaining titer of the first immunization to characterize the direct production of antibodies after reimmunization. The ΔABT_{1-2} was inversely related to the retained primary immune response (Fig. 2).

The analysis was carried out based on the STATISTICA 7.0 software package (StatSoft, USA). To estimate the significance of the deviation from the normal distribution, we applied the Shapiro–Wilk test as the main test and the Kolmogorov–Smirnov test with the Lilliefors correction (Lilliefors 1967) as an additional test in parallel with a visual assessment of histograms. To assess the homogeneity of the distributions, we used Levene's test for homogeneity of variances. We applied a log-transformation of values of hormone concentrations to normalize distributions. To compare the normally distributed characteristics of SD and LD males, we used mean values with the standard errors and the Student's t-test for independent samples. In cases of significant deviations from normality, the Mann–Whitney U-test was used. For the variables whose distributions met the Kolmogorov–Smirnov criterion with the Lilliefors amendment, but did not pass the Shapiro–Wilk criterion, both parametric and non-parametric statistics are given (Table 1). Means with standard errors and medians with limits of variation are given for all cases.

To assess the effect of a set of independent variables (potential predictors) on the immunoresponsive-

Table 1 Morpho-physiological and physiological characteristics of desert hamster males on SD and LD

Characteristics	SD		:	LD			Statistics			
	mean ± SE †	median (min–max) ‡	*N*	mean ± SE	median (min–max)	*N*	t_{St}	*p*	Z_{M-W}	*p*
Testr 1, nm/L	0.58 ± 0.14§	2 (0–130)	32	1.06 ± 0.14§	15 (0–163)	33	–2.22	0.03	–2.15	0.03
Testr 2, nm/L	0.81 ± 0.12§	7 (1–125)	32	1.18 ± 0.09§	15 (1–135)	34	–2.43	0.018		
MVG, mm^2	97.8 ± 7.3	97.8 (32–182)	32	132.6 ± 7.3	138 (30–208)	34	–3.38	0.001		
AnGen, mm	10.4 ± 0.3	10.3 (6.0–14.5)	32	12.1 ± 0.3	12.0 (8.5–14.0)	33	–4.37	<0.001	–4.00	<0.001
L, mm	84.7 ± 0.5	85 (78–94)	32	87.9 ± 0.6	88 (80–95)	34	–4.07	<0.001		
BMAS RMR, g	21.5 ± 0.8	20.4 (16.7–45.0)	34	26.6 ± 0.9	25.8 (19.0–38.0)	33	–4.29	<0.001	–5.04	<0.001
BMAS MMR, g	22.2 ± 0.8	21.3 (17.0–46.1)	34	26.3 ± 0.8	24.8 (19.4–36.8)	33	–3.50	<0.001	–4.31	<0.001
RMR, mL O_2/min	0.52 ± 0.01	0.51 (0.35–0.84)	34	0.58 ± 0.02	0.58 (0.39–0.80)	33	–2.91	0.005	–3.07	0.002
SRMR, mL O_2/g × h	1.45 ± 0.03	1.45 (1.11–1.71)	34	1.31 ± 0.02	1.31 (1.14–1.53)	33	4.57	<0.001		
MMR, mL O_2/min	5.41 ± 0.21	5.39 (2.43–7.94)	34	5.69 ± 0.18	5.80 (3.5–7.54)	33	–0.93	0.356		
SMMR, mL O_2/g × h	14.78 ± 0.49	15.46 (7.24–19.38)	34	13.18 ± 0.43	13.17 (8.34–17.14)	33	2.47	0.016	2.58	0.01
Cort 1, nm/L	2.06 ± 0.05§	126 (25–439)	32	1.67 ± 0.05§	40 (20–323)	33	5.73	<0.001		
Cort 2, nm/L	2.04 ± 0.06§	113 (36–498)	32	1.82 ± 0.04§	63 (16–218)	34	3.07	0.003		
ABT 2, c.u.	10.1 ± 0.2	10 (7–12)	32	9.9 ± 0.3	10 (2–13)	34	0.43	0.67	0.17	0.87
ΔABT_{1-2}, c.u.	6.2 ± 0.4	6.5 (2.5–11.5)	29	6.1 ± 0.3	6 (1–10)	33	0.28	0.78		

† Mean value and standard error. ‡ Median and limits (in parentheses). § Decimal logarithm of hormone concentration. Abbreviations: Testr 1 – the background level of testosterone, Testr 2 – testosterone level after reimmunization with SRBC, MVG – mid-ventral gland size, AnGen - ano-genital distance, L – head-and-body length, BMAS RMR – body mass when measuring RMR, BMAS MMR – body mass when measuring MMR, RMR – resting metabolic rate, by the volume of oxygen consumed per minute, SRMR – mass-specific resting metabolic rate, MMR – maximum metabolic rate, SMMR – mass-specific maximum metabolic rate, Cort 1 – the background level of cortisol, Cort 2 – cortisol level after reimmunization with SRBC, ABT 2 – the intensity of the immune response to SRBC after reimmunization, ΔABT_{1-2} – the difference between the magnitude of the response after reimmunization and the values of the retained primary response, measured before reimmunization. *N*, sample size; *P*, significance level; t_{St}, Student's *t*-values for independent samples, Z_{M-W}, *Z*-statistic in Mann–Whitney *U*-test.

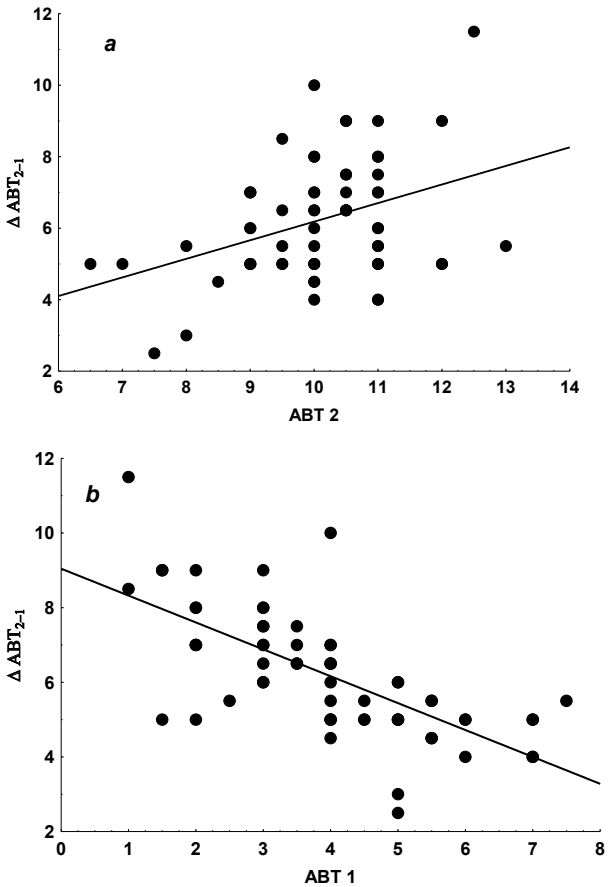

Figure 2 Dependence of the intensity (a) of a pure secondary immune response (ΔABT_{2-1}) on the intensity of a total immune response (ABT 2) after repeated immunization of desert hamster males with SRBC ($y = 0.9833 + 0.52x$; $r = 0.3746$; $P < 0.003$) and (b) of a pure secondary immune response on the residual intensity of a primary immune response, before reimmunization with SRBC ($y = 9.04 - 0.72x$; $r = -0.68$; $P < 0.001$).

ness of male hamsters, a module of Generalized Linear and Nonlinear Models (GLZ) in Statistica 7.0 was used with the condition for "normal distribution with identity link function." The intensity of the secondary humoral immune response of reimmunized hamsters and the difference between the secondary and retained primary responses were used as dependent variables. The set of potential predictors included the lighting duration (a categorical variable represented by 2 states: SD and LD) and a number of continuous variables, given above. Because there was no difference between RMR body mass and MMR body mass ($P > 0.05$) in GLZ, we included mean values between 2 body masses. The mass-specific values of metabolic rates and the regression residuals of RMR and MMR values on the corresponding body mass values were used in a separate model. Two-way interactions were also considered. We used decimal logarithms of hormone concentrations, body mass and metabolism values. Values of all variables were standardized. The significance of predictors was assessed by Wald statistic. To select the best model among the candidate models, the method of stepwise elimination of variables (Backward stepwise elimination) was used.

Ethical principles of working with animals

In our study, we adhered to the recommendations given in "Guidelines for the treatment of animals in behavioral research and teaching" (Buchanan *et al*. 2012) and "Principles of Laboratory Animal Care" (1996), and in legislation of the Russian Federation. This research project was approved by the Commission on Bioethics under the A. N. Severtsov Institute of Ecology and Evolution RAS, Protocol No. 23, dated 31 January 2018.

RESULTS

Reproductive characteristics and metabolic rates

Long-day males seemed to be reproductively active. These males had a basal testosterone level in blood serum 30% higher and a postimmunization testosterone level 19% higher. They had 8% larger ano-genital distance, a 15% larger mid-ventral gland (at a qualitative level of assessment there was more secretion produced), a greater head-and-body length, and greater body mass (Table 1). They had a more intense RMR, estimated by the amount of oxygen consumed per minute. However, the mass-specific RMR and the mass-specific MMR were 5% and 6% higher in SD males correspondingly and the serum cortisol concentrations were higher in SD males as well (10% for basal level and 6% for postimmunization level; Table 1).

Humoral immune responsiveness

We did not detect significant differences between SD and LD males in terms of the intensity of the humoral immune response to SRBC after the second immunization, both when using the cumulative values of immune response and when using the residual values after elimination of the remains of the primary immune response.

The GLZ analysis of the effect on the cumulative

secondary immune response (ABT 2) of a set of potential predictors (characteristics of males kept under SD and LD photoperiods and the day length mode [categorical variable]) did not reveal significant effects of any of the variables in the model. When we used the difference between the cumulative secondary immune response and the retained primary response as a dependent variable, the size of the mid-ventral gland and the maximum metabolism had statistical support (Table 2). At the same time, there was no statistically significant effect on body mass (Table 2). When the residuals of regression of RMR and MMR on the corresponding body mass were inserted into the model, the result was similar (MMR residual: $b = -0.26 \pm 0.13$, Wald statistic = 4.1, $P = 0.04$; MVG: $b = -0.48 \pm 0.18$, Wald statistic = 6.46, $P = 0.01$). The result was similar when the mass-specific MMR was used as an independent variable (SMMR: $b = -0.83 \pm 0.34$, Wald statistic = 5.93, $P = 0.01$; MVG: $b = -0.64 \pm 0.28$, Wald statistic = 5.22, $P = 0.02$). The procedure of stepwise elimination of variables left only these 2 variables in the final model with negative coefficients, signifying a negative relationship between the intensity of the secondary immune response and the predictors identified. The day length, used as a categorical variable, did not affect the intensity of the secondary immune response in any of the options for including variables in the model (either by itself or in relation to its possible interaction with another variable).

Table 2 The result of GLZ analysis of the effect on the pure secondary humoral immunoresponsiveness of a set of morpho-physiological and physiological characteristics in desert hamster males on long-day (LD) and short-day (SD) photoperiods

Variables	Parameter	*SE*	Wald statistic	*P*
Intersept	4.52	4.64	0.95	0.33
SD and LD mode	0.04	0.16	0.07	0.79
MVG	–0.50	0.19	7.30	0.01
AnGen	0.35	0.21	2.69	0.10
Cort 1	0.02	0.15	0.02	0.88
Testr 1	0.19	0.12	2.39	0.12
Cort 2	0.06	0.14	0.18	0.67
Testr 2	0.08	0.12	0.39	0.53
BMAS[†]	0.34	0.23	2.15	0.14
RMR	–3.32	3.38	0.96	0.33
MMR	–0.32	0.15	4.69	0.03

[†] BMAS is the mean value between BMAS RMR and BMAS MMR. For other abbreviations see Table 1

Counts of blood cells

We found a higher mean number of lymphocytes in white blood cell counts in SD males. The mean number of granulocytes in WBC counts was higher in LD males, mainly due to the higher mean number of eosinophils. There were no significant differences with respect to other blood cell types, in the N : L ratio and in the number of WBC per 10^4 RBC (Table 3).

Table 3 Counts of blood cells in desert hamster males on long-day (LD) and short-day (SD) photoperiods

Blood cell type[§]	SD		:	LD			Statistics			
	Mean ± SE[†]	Median (min–max)[‡]	*N*	Mean ± SE	Median (min–max)	*N*	t_{St}	*p*	Z_{M-W}	*P*
Neutrophils	31.3 ± 1.5	32 (11–48)	33	33.2 ± 1.7	33 (14–59)	33	−0.84	0.403		
Eosinophils	10.2 ± 0.9	10 (2-23)	33	13.7 ± 0.8	14 (4–22)	33	−2.98	0.004		
Basophils	0.4 ± 0.1	0 (0–2)	33	0.4 ± 0.1	0 (0–3)	33			0.19	0.845
Granulocytes (sum)	41.8 ± 2.0	44 (19–66)	33	47.3 ± 1.9	47 (28–68)	33	−2.04	0.045		
Monocytes	6.7 ± 0.6	7 (1–14)	33	7.7 ± 0.8	6 (2–19)	33			−0.58	0.562
Lymphocytes	51.5 ± 2.0	52 (29–76)	33	45.0 ± 1.7	45 (28–67)	33	2.43	0.018		
N : L	0.68 ± 0.06	0.63 (0.17–1.53)	33	0.82 ± 0.07	0.67 (0.22–2.11)	33			−1.28	0.199
WBC per 10^4 RBC	26.0 ± 1.1	27 (11–39)	33	23.8 ± 1.4	26 (10–38)	33	1.25	0.217		

[§]The numbers of different cell types in a sample of 100 leukocytes. [†]Mean value and standard error. [‡]Median and limits (in parentheses). *N*, sample size; N : L, neutrophils to lymphocytes ratio; *P*, significance level; t_{St}, Student's *t*-values for independent samples; WBC per 10^4 RBC, number of leukocytes per 10^4 erythrocytes; Z_{M-W}, *Z*-statistic in Mann–Whitney *U*-test.

DISCUSSION

We expected that with an SD photoperiod, the adaptive humoral immunity would look enhanced, because under laboratory conditions there are no physical stressors or food shortages, while with a long daylight period immune responsiveness to the antigenic challenge would be suppressed by the activity of the reproductive system. In a recent study of the effect of photoperiod on energy consumption, thermogenesis and reproduction in the desert hamster, it was shown that under SD conditions, animals reduced their body mass; their reproductive function was inhibited, and adaptive non-shivering thermogenesis was intensified (Zhang *et al*. 2015). These results are consistent with the results we obtained for desert hamster males kept under SD and LD photoperiods. In our study, the SRMR in LD males corresponds to estimations obtained earlier for hamsters of the same species kept permanently in LD conditions. The SRMR in SD males in our study was much lower than in cold acclimated hamsters (Chi & Wang 2011)

Contrary to our expectations, we did not observe differences in the humoral adaptive immunoresponsiveness. There were no differences in responsiveness to SRBC after repeated immunization between SD and LD males, despite the activation of reproductive function in LD males. The latter is confirmed by an increased level of testosterone, a greater expression of secondary sexual characteristics (mid-ventral gland and ano-genital distance), a greater body mass and greater head-and-body length.

Nevertheless, we found clear evidence of a conflict between reproduction and antibody production. We found a statistically significant tendency towards a negative relationship between the size of the testosterone-dependent mid-ventral gland, reflecting the activity of the reproductive system, and the pure secondary immune response (ΔABT_{1-2}) to the SRBC antigen.

It is more difficult to interpret the negative effect of a maximum metabolic rate. Indicators of metabolism and adaptive immunity could be interrelated because both systems use the same signaling molecules, such as glucocorticoids (Zera & Harshman 2001; Martin *et al*. 2008; Robinson *et al*. 2010) and can compete for resources (i.e. energy and substrates; Sibly & Calow 1986; Stearns 1989).

Metabolic rate and antibody production

The association of basal metabolic rate (BMR) or related resting metabolic rate with immune functions has been noted in several papers (Raberg *et al*. 2002; Książek *et al*. 2003; Tieleman *et al*. 2005; Robinson *et al*. 2010; Versteegh *et al*. 2012). However, unlike the maximum level of aerobic performance (MMR), in our study we did not find a correlation between RMR and the intensity of adaptive humoral immune response. In this respect, our result coincides with the results of research on the wild red voles (*Myodes rutilus*), in which it was shown that immunization with SRBC, or infestation with a taiga tick (*Ixodes persulcatus*), or infection with nematode (*Heligmosomum mixtum*) did not affect RMR (Novikov *et al*. 2015). The relationship between BMR and adaptive (acquired) or non-specific (innate) immunity was also not detected in experiments with laboratory mice selected for high and low mass-independent MMR over several generations (Downs *et al*. 2013). Selection for high mass-independent MMR (maximal locomotory load) suppressed innate but not adaptive immunity. However, results of analysis at the individual level appear to support our results indicating a negative correlation between MMR and adaptive immune function. An evolutionary enhancement of aerobic performance increases individual endurance but can lead to a weakening of costly immune functions. High MMR can be associated with low immunoresponsiveness if there is competition for limited energy resources. It is also possible that in our case the effect of MMR may indirectly indicate a conflict between the specific humoral immunity system and the male's reproductive quality.

Photoperiod, humoral adaptive immunity and stress

Why did the duration of daylight in our experiment not affect the hamsters' adaptive humoral immunoresponsiveness? There may be several reasons for this, but one seems to be the most likely: the increased level of stress in SD hamsters. Physiological stress provides mobilization and relocation of vital resources of an organism for survival (Wingfield *et al*. 1998) and provides rearrangement of physiology in accordance with changes in the environment to maintain homeostasis (McEwen & Wingfield 2003). Acute stress over short intervals can, in some cases, enhance immune functions (Dhabhar 2009). However, over long intervals, chronic stress has a negative effect on both reproduction and immunity (Wingfield & Romero 2001; Romero 2002; Nelson *et al*. 2002; Padgett & Glaser 2003).

We expected that in the SD mode in the absence of winter stressors, glucocorticoid levels would be low-

er compared to LD. However, in the experiment, we observed an increased level of cortisol in SD male hamsters. An increased level of stress manifested itself against the background of a higher mass-specific RMR and mass-specific MMR in SD males and, possibly, reflected the functioning of an organism according to the winter scenario. It is likely that the higher background stress in SD males leveled the effect of short day on the adaptive humoral immunity system of male hamsters. In our case, the absence of differences in immunoresponsiveness between SD and LD males could be explained by a suppression of the immune function by the activity of the reproductive system in LD males, and by stress due to the functioning of the metabolic system according to the winter scenario in SD males.

Higher GC are known to accompany an increase in fat deposits (Epel *et al*. 2000; Adam & Epel 2007; Kuo *et al*. 2007; Mujica-Parodi *et al*. 2008), and it could be supposed that the higher cortisol level in SD males might be due to putting on fat stores. This was not the case in our study because body mass, being highly correlated with the reserves of white fat, was found to be lower on a short day. Thus, it appears that the higher stress in SD was associated with energetic performance of winter type. A decrease in fat deposits and body mass in SD is common for photoperiodic Siberian dwarf hamsters (Zysling *et al*. 2009).

Effect on white blood cell counts

We found a higher mean number of lymphocytes and lower mean number of eosinophils in white blood cell counts in association with higher hormonal stress in SD males. The mean number of all granulocytes in WBC counts was higher in LD males.

A decrease in the number of circulating eosinophils had been proposed by Selye (1950) as a constant sign of the "alarm response," the first stage of stress syndrome. Now it is well established that corticosteroids can reduce eosinophils. Although in many studies eosinopenia has been described as a relatively short-term effect of acute stressors (Karaconstantis *et al*. 2018), it is possible that the chronic stress may cause eosinopenia as well (Altman *et al*. 1981). An increased absolute number of circulating blood leukocytes and lymphocytes was reported for SD deer mice (*Peromyscus maniculatus*; Blom *et al*. 1994) and for SD Siberian hamsters (*Phodopus sungorus*; Bilbo *et al*. 2002). In the latter species, this was associated with increased glucocorticoid concentrations, confirming our finding on higher stress in SD males. It was also shown for Siberian hamsters that enhanced basal lymphocyte proliferation was common for an SD photoperiod, whereas phagocytosis and oxidative burst activity by both granulocytes and monocytes were enhanced in hamsters exposed to an LD photoperiod (Yellon *et al*. 1999). Publications supporting the winter adaptive immunoenhancement in mammals and birds usually report results of testing the cutaneous inflammatory responses to antigenic challenges (e.g. Demas & Nelson 1998; Sinclair & Lochmiller 2000; Bilbo *et al*. 2002; Martin *et al*. 2004, 2006a,b; Prendergast *et al*. 2005), which is more associated with T-cells attraction and proliferation. For B-cell (humoral) immunity in rodents the SD photoperiod caused both an increase (Saino *et al*. 2000) and a decrease (Sidky *et al*. 1972) in antibody production. Thus, the results we obtained do not contradict the winter immunity enhancement hypothesis. The enhancement could be provided mostly by T-lymphocytes but not by antibody-producing B-lymphocytes.

In our study, the higher neutrophil-to-lymphocyte ratio did not relate to the higher cortisol level in SD males. Although this ratio was recommended as a reliable assessment of stress in mammals (or heterophil-to-lymphocyte ratios in other vertebrate taxa) by Davis *et al*. (2008), we did not find a difference in this index between SD and LD hamsters. Moreover, as an index of stress, the granulocyte-to-lymphocyte ratio signals the opposite relation to the level of cortisol. This might be due to the fact that the N : L ratio as a stress index reflects the response to acute short-term stressors; that is, more rapid trafficking of lymphocytes out of the blood (Bilbo *et al*. 2002), rather than the effect of long-term chronic stressors in our experiment (Dhabhar & McEwen 1999; Dhabhar *et al*. 2012; Dhabhar 2013).

Other possible explanations

We also cannot ignore other possible explanations for the lack of the effect of day length on the adaptive humoral immunoresponsiveness in desert hamsters in our experiment.

1. It is possible that the day length was not a direct signaling factor for the system of adaptive humoral immunity. The day length effect on the immune system is species-specific and can be considerably mediated by the activity of the reproductive system (Martin *et al*. 2008; Stevenson & Prendergast 2015).

2. Even if testosterone is capable of exerting a direct inhibitory effect on adaptive humoral immunity (Schuurs &Verheul 1990; Folstad & Karter 1992; Klein 2000; Muehlenbein & Bribiescas 2005), the remaining

question is at what threshold level of the hormone its effect on immunity becomes visible (Mills *et al*. 2009). Above the background of activated reproductive function during the long-day photoperiod in our experiment, the threshold concentration of testosterone for the immune system might not be reached.

3. In our study, we proceeded from the assumption that humoral adaptive immunity is a costly branch of immunity in terms of the energy and substrates intake (Demas *et al*. 1997; Svensson *et al*. 1998; Ilmonen *et al*. 2000; Ots *et al*. 2001; Hanssen *et al*. 2004; Muehlenbein *et al*. 2010; see criticism from Ksiażek *et al*. 2003; McKean & Lazzaro 2011). If, in reality, the cost of production of antibodies after repeated immunization with SRBC is relatively low, then we have no serious reason to expect a tradeoff between adaptive humoral immunity and reproduction. It is also necessary to add that a decline in metabolic rate in some cases may be part of an individual defense strategy (Klasing 2004).

4. Our hamsters were bred in captivity under fixed photoperiods for many years before this experiment, and under standardized LD photoperiods all year-round. Although they demonstrated pronounced photoperiodic dependence when introduced to LD and SD environments, we cannot fully discount the possible effect on our results of long-term breeding in the standardized conditions of a vivarium. Yet the data on laboratory breeding of reproductively non-photoperiodic inbred strains of mice and rats suggest that the immune system continues to be sensitive to photoperiod modulation (Yellon & Tran 2002; Prendergast *et al*. 2007).

Summarizing the above, it seems to us that a more promising approach to analyzing the effect of photoperiod duration on the immunity of desert hamsters, as well as of other photoperiodic rodents, would be to study the changes in immune activity in a situation when this activity is modulated additionally: for example, by castration of males with subsequent therapy using androgens. In this case, it would be possible to eliminate the direct effect of testosterone and activated reproduction. One might also consider the responses in other branches of the overall immune system.

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