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Seasonal variation of testicular activity in European brown hare *Lepus europaeus*

Steffen BLOTTNER, Detlef FABER and Hannelore ROELANTS

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Testicular activity in brown hare *Lepus europaeus* Pallas, 1778 was studied during the annual cycle. Testicular mass, spermatozoa and testosterone were estimated. Percentages of haploid, diploid and tetraploid cells were monitored using DNA flow cytometry and the proportions of somatic and spermatogenetic cells were determined after selective labelling of somatic cells. Testis mass was high from January to July and declined thereafter to the nadir in September. Testis growth was reactivated significantly in December. Changes in testis mass corresponded with the spermatogenic efficacy (spermatozoa/g testis). High spermatogenic activity was characterized by intensive meiotic transformation of spermatocytes to spermatids, high percentages of haploid cells and low proportions of cells in the G2/M phase of mitosis. Proportions of haploid cells declined rapidly during the testis involution. Spermatogenesis was newly activated from November. The proportions as well as the ratios of spermatogenic and somatic cells differed significantly between the periods of testis involution and recrudescence. Testosterone level showed a pronounced increase in autumn preceding the intensification of spermatogenesis; the lowest concentration was found during prominent testis involution in August. The results suggest that the regulation of seasonal testicular activity is characterized by co-ordinated shifts in the relationships between mitosis, meiosis and testosterone production.

Institute for Zoo Biology and Wildlife Research, PF 601103, 10252 Berlin, Germany, e-mail: blottner@izw-berlin.de (SB, HR); Institute for Animal Breeding and Farm Animal Genetic, Justus-Liebig University Gießen, 35392 Gießen, Germany (DF)

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Introduction

Seasonal variation in reproduction is common in mammals as an adaptation to annual changes in the environment. The role of environmental signals in synchronization has been described in many studies, especially the circannual regulation of pituitary and gonadal function by photoperiod (for reviews see Bronson 1988, 1989, Lincoln 1989, Bartke *et al.* 1991). The final purpose of the complex interacting control mechanisms in males is the optimal timing for generation of functionally competent spermatozoa. Seasonal changes of cellular composition and processes in testes have been studied in several mammalian species such as hamsters (Bergmann *et al.* 1990, Lerchl *et al.* 1993, Van Haaster *et al.* 1993), bank vole (Tähkä *et al.* 1983, 1993, 1997), roe deer (Hartung and Schoppmeyer 1986, Marchlewska-Koj and

Kruczek 1988, Gentile *et al.* 1989, Blottner *et al.* 1996), Eld's deer (Monfort *et al.* 1993) and horse (Johnson 1991). Cycles of complete testicular regression and recrudescence in seasonally breeding species result from the fine-tuning of alternating inhibition and activation of controlling factors. Therefore, such species are valuable models to study basic mechanisms for the regulation of spermatogenesis.

The European brown hare *Lepus europaeus* Pallas, 1778 is characterized by a protracted breeding season extending from January to August or September and a non-breeding period between September and December. Consequently, mature males show cyclic transitions between highly activated and totally arrested spermatogenesis. Several studies in brown hare have focused on this annual cycle of involution and recrudescence of testes in connection with plasma hormone levels (Lincoln 1974, 1976, Broekhuizen and Maaskamp 1981, Caillol *et al.* 1989, Ciberej *et al.* 1991). However, a quantitative characterization of changing cellular composition and the timing of mitotic and meiotic activities connected with hormone production in testes of brown hare is, to our knowledge, not available. Therefore, the aim of this study was to measure seasonal changes of different cell types and to determine the mitotic and meiotic activity as well as the variance in testosterone concentration in testes of the European brown hare.

Material and methods

Animals

A total of 58 adult brown hares was examined from November 1995 until April 1998. The testes were collected immediately after hunting, cooled to 4–6°C for transport and prepared in the laboratory within 24 h after removal from the animals. For estimation of testosterone additional samples were available from earlier studies (Blottner *et al.* 1995), and data of testis weight and spermatozoa/g testis include results of earlier spermatological investigations (Hingst *et al.* 1995). The variable sample sizes for the measurement of different parameters are indicated in the results.

Preparation of testes

The testes and epididymides were separated and the testes were weighed. In order to count the number of testicular spermatozoa, 0.2 g of decapsulated testis were carefully pressed through a 28-μm nylon mesh and suspended in 4-ml medium M199 supplemented with 0.4% (w/v) BSA (Sigma, Deisenhofen). The germ cell suspension was diluted in the same volume of water and counted with a haemocytometer. Results are expressed in spermatozoa per gram testis.

One-parameter flow cytometric DNA-analysis

Testicular cells were prepared according to the procedure described earlier (Blottner *et al.* 1996) The cells were dispersed by finely mincing 0.1 g of decapsulated testicular tissue in 1 ml 100 mM citric acid and 0.5% (v/v) Tween 20. The released nuclei were dispersed by gentle agitation for 20 minutes and stained with 5 ml of 0.175% 4',6-diamidino-2-phenylindol (DAPI; Sigma, Deisenhofen) in 400 mM Na_2HPO_4. The flow cytometric analysis was performed in a PAS III flow cytometer (Partec GmbH, Münster) with a mercury lamp using a wavelength of 360 nm for excitation and of 420 nm for emission. Approximately 5–10 $\times$ 10^4 cells were measured in each sample. The DNA histograms obtained were analysed by DPAC computer software to determine the proportions of cells in each peak. The contents of haploid (spermatids and spermatozoa), diploid (spermatogonia, secondary spermatocytes

and somatic cells) and tetraploid cells (all cells in the G2/M phase of cell cycle, mainly primary spermatocytes) were expressed as 1C, 2C and 4C percentages.

Dual-parameter flow cytometry

The indirect immunofluorescence method was used according to Roelants (1997, Roelants and Blottner 1997). Briefly, suspended cells were fixed by adding of –20°C cold ethanol (96%) and stored for ≥ 24 hours before further preparation. The samples were washed twice with PBS and resuspended in PBS. The sample was incubated with monoclonal anti-vimentin antibody (clone V9; Sigma, Deisenhofen) at a dilution of 1:40 for 60 min. Subsequently the cells were washed twice with PBS (plus 2% BSA) and resuspended in PBS. They were incubated with fluorescein isothiocyanate (FITC)-conjugated anti--mouse antibody (final dilution 1:50) for 30 min in darkness. After two wash steps with PBS/2% BSA, the pellet was resuspended in a solution of 1 mg/ml ribonuclease (Sigma) and 50 μg propidium iodide (Sigma) and incubated at 37°C for 30 min in the dark. The samples were filtered through a 28-μm nylon mesh before analysis with the flow cytometer.

Fluorescence distributions were analysed using the PAS III illuminated with an argon laser at 488 nm wavelength. A negative control omitting the primary antibody was included for each sample. The calculation of vimentin-positive cells was corrected for the low unspecific fluorescence. Vimentin--positive and vimentin-negative cells represent the somatic and spermatogenic types, respectively. They were calculated by peak analysis using the PAS III computer software.

Low numbers of animals obtainable in some months and improper storage (cooling conditions) of testes from some males limited the tissue samples available for analyses using the vimentin-antibody. The cell cycle analysis by measurement of DNA is very robust, but the vimentin antigen-antibody reaction is very sensitive. Therefore, an accurate analysis was not possible for all seasons, but it included males from periods at the beginning (November, n = 10 and December, n = 10), the highly active (April, n = 5) and the end of spermatogenesis (July, n = 9).

Estimation of testosterone by EIA

Testicular testosterone was measured by enzyme-immunoassay with a double antibody technique as described earlier (Meyer and Hoffmann 1987). Briefly, triplicates of 200 μg tissue were extracted with 1 ml ethanol/water 70/30 (v/v). The extract was diluted with assay buffer as required; then 2 × 20 μl were analysed. The assay used a polyclonal antibody raised in rabbits against testosterone--11-hemisuccinate-BSA, and the label was testosterone-3-carboxymethyl-oxime-horse radish peroxidase. The testosterone standard curve ranged from 0.4 pg/20 μl to 50 pg/20 μl and the crossreactivity with testosterone was 100%, with 5α-dihydrotestosterone 10%, with androstenedione 2%, with estradiol < 0.1% and with progesterone < 0.1%. The results were given in ng testosterone per gram testis (ng/g testis).

Statistical analysis

The measured parameters were assessed over the course of the annual cycle. Therefore, results of animals obtained within 5 to 8 days were combined (means of 3–14 animals per date). Selected parameters were subjected to the non-parametric U-test to examine the significance level of differences between seasonal periods.

Results

Testis weight remained above 10 g from February until July. Subsequently it declined approximately six-fold to the nadir in September (mean ± SEM = 2.06 ± 0.30 g) and increased again in November and December. Gonadal size corresponded to numbers of testicular spermatozoa/g testis indicating changes in both mass and efficacy of seminiferous epithelium (Fig. 1).

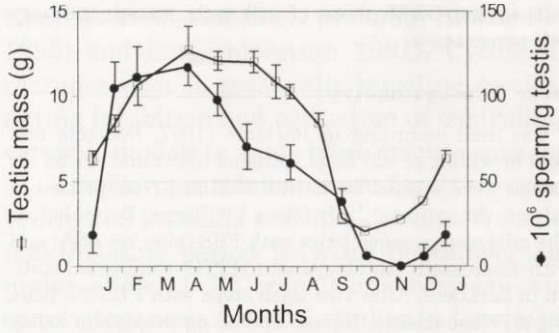

Fig. 1. Testis mass and testicular spermatozoa concentration in brown hare during the annual cycle. The results are expressed as means ± SEM. Where no error bar is shown it is within the symbol. Total number of animals: $n = 102$; number per date varies between $n = 3$ (January, February) and $n = 14$ (November, December).

The proportions of haploid (1C), diploid (2C) and tetraploid cells (4C) in the testis parenchyma also changed over time with high percentages of 1C cells in the breeding period (Fig. 2). This period of high spermatogenic activity (January–July) is characterized by low proportions of cells in the G2/M phase of mitosis (4C cells). During the involution of testes proportions of haploid cells declined rapidly.

The ratio of 1C:4C cells showed the maximum in February (11.94 ± 2.35, $n = 3$) and a rapid drop during the testis involution in late summer to the lowest value in September (0.59 ± 0.30, $n = 3$). This ratio is indicative of the meiotic transformation of primary spermatocytes to spermatids. However, it includes the spermatogenic as well as somatic cells (mainly Sertoli cells, Leydig and other interstitial cells) in the 4C cell percentages. Therefore, the correct meiotic transformation and the total germ cell transformation (ratio of 1C:2C cells) were estimated after differentiation of spermatogenic 4C and 2C cells, respectively. The results showed higher values of germ cell transformations at the end of the breeding season (July) in comparison to the starting (November and December) or highly activated (April) spermatogenesis (Fig. 3). The differences of 1C:2C ratios between April and July were not significant, all other differences were above the significance level ($p < 0.001$). The situation for the 1C:4C ratios were the same with the exception of the difference between April and December ($p < 0.05$).

The total proportions as well as the ratios of somatic and spermatogenic cells within diploid and tetraploid peaks of flow cytometric histograms demonstrate

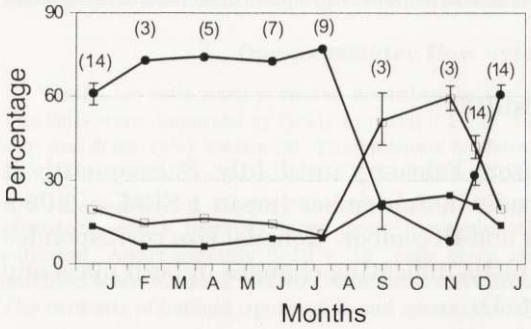

Fig. 2. Seasonally changing proportions of testicular haploid (●), diploid (□) and tetraploid (■) cells in brown hare analysed by flow cytometry (means ± SEM). Error bars that are not shown are contained within the data point (number in parenthesis = number of animals).

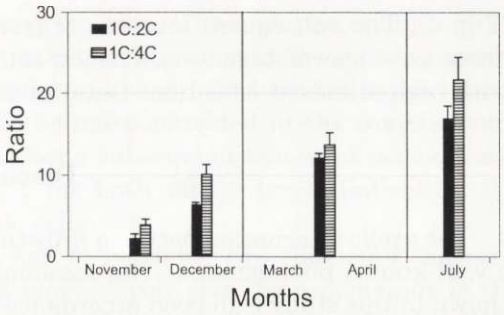

Fig. 3. Total germ cell transformation (ratio of haploid : spermatogenic diploid cell percentages) and meiotic transformation (ratios of haploid : spermatogenic tetraploid cell percentages) in testes of brown hare from seasonal periods at the start, the maximum and the end of spermatogenesis (means ± SEM). The sample sizes are the same as in Table 1.

significant differences between the periods at the start and at the end of active spermatogenesis (Table 1). In early April the ratio of spermatogenic to somatic cells in the G2/M phase was the highest. This ratio decreased in July. In late November both cell types occur in enhanced percentages. Within a short period the 2C and 4C percentages are diminished and the ratios of spermatogenic to somatic cells increase for both ploidy levels in December.

Testosterone level showed a peak during the spermatogenic active period in spring and the lowest concentration during pronounced testis involution in August

Table 1. Percentages (means ± SEM) and ratios of germinative and somatic cells within the diploid and tetraploid peaks of flow cytometric histograms in testes of brown hares from periods at the active (April), the ending (July) and newly starting (November, December) spermatogenesis. Percentages of germinative cells in the same column with different superscripts are significantly different ($p < 0.01$).

Period	n	Diploid cells			Tetraploid cells		
		Germinative	Somatic	Ratio	Germinative	Somatic	Ratio
April 3	5	$6.15^{a} \pm 0.23$	6.06 ± 0.56	1.02	$5.73^{a} \pm 0.62$	3.45 ± 0.61	1.66
July 15	9	$4.95^{a} \pm 0.54$	5.26 ± 0.50	0.94	$4.16^{a} \pm 0.62$	5.31 ± 0.52	0.78
November 30	10	$18.93^{b} \pm 2.38$	23.93 ± 2.65	0.79	$9.0^{b} \pm 0.56$	12.53 ± 0.60	0.72
December 15	10	$10.23^{c} \pm 0.54$	7.82 ± 1.05	1.31	$7.06^{b} \pm 0.58$	5.97 ± 0.82	1.18

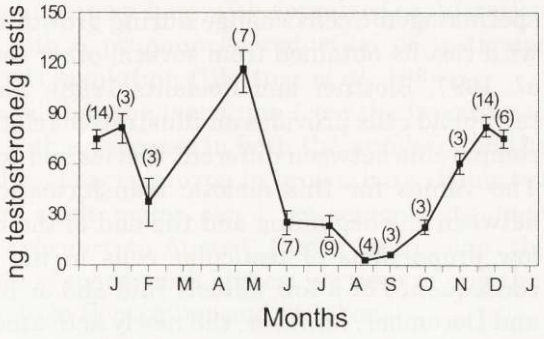

Fig. 4. Seasonal changes of testicular testosterone concentration in brown hare (means ± SEM). Error bars that are not shown are contained within the data point (number in parenthesis = number of animals).

(Fig. 4). The subsequent increase of testosterone in autumn preceded the recrudescence of spermatogenesis. The low concentration in February was a single point with high standard deviation. Data for March and April were not available.

Discussion

The cyclic alternation between growth and involution of testes in brown hare is a well-known phenomenon. The seasonality of testis mass and sperm production shown in this study is in good accordance with several other reports (Lincoln 1974, 1976, Möller 1980, Broekhuizen and Maaskamp 1981, Zörner 1981, Ciberej *et al.* 1991). Changes observed in the male gonads of brown hare were characteristic of a period of sexual activity between December and July and a subsequent period of depressed spermatogenesis between August and November. In some years however, deviations from the normal annual reproduction pattern can occur and some individual males can also deviate from the mean of their population (Möller 1980).

Our results represent new data about changes of intratesticular cells and demonstrate the interacting alterations of tissue growth, spermatogenesis and hormone production during the annual cycle. The flow cytometric analysis of quantitative changes in cellular composition provides an exact estimation of mitotic and meiotic steps in the testicular tissue. DNA flow cytometry has been established as a sensitive and precise method to determine parameters of spermatogenesis (Hacker-Klom *et al.* 1986, Hellstrom *et al.* 1990, Suresh *et al.* 1992, Hirsch *et al.* 1993, Spano and Evenson 1993). The discrimination between somatic and spermatogenic cells by dual staining of testicular cells with propidium iodide and FITC-labelled anti-vimentin antibody provides additional information about the proportions of germinative and somatic cells (Roelants and Blottner 1997, Roelants *et al.* 1997, Blottner and Roelants 1998).

The percentages of haploid, diploid and tetraploid cells showed a marked annual fluctuation, with the highest value of haploid cells in the breeding period, whereas diploid cells reached their peak in the non-breeding period. Most mitotic active cells (G2/M phase) were found during the period preceding the breeding season.

The presented data of dual-parameter flow cytometric analysis demonstrate that the total percentages as well as the relative proportions of somatic and spermatogenic cells change during growth and involution of testes. These data fit with results obtained from several other seasonal breeding mammals (Roelants *et al.* 1997, Blottner and Roelants 1998). The ratio of haploid and spermatogenic tetraploid cells provides an illustrative relative parameter of meiotic activity and is comparable between different species and conditions (Blottner and Roelants 1998). The values for this meiotic transformation show quite clearly the differences between the beginning and the end of the breeding period. They suggest that the low proportions of testicular cells in the G2/M phase (4C) in July could be a consequence of a low mitosis rate and/or high transformation rate. In November and December, however, the newly activated testis contains high proportions of 4C

cells, though with only a low rate of transformation. The high percentages of 2C and 4C cells are indicative of strongly activated proliferation in the growing testes. The ratios of spermatogenic to somatic cells are below 1 for both cell types late in November. So, the proliferation seems to be more activated in the somatic compartment during this phase. But already after a subsequent two-week period these ratios increase to values greater than 1 for both ploidy levels indicating the stimulated proliferation of spermatogenic cells.

The interpretation of these results has to consider two important aspects. Firstly, the values of cells with different ploidy levels give the proportions of the different cell types and not the absolute numbers of cells with different DNA content. Secondly, there are fundamental differences between a steady state spermatogenesis and the continuously changing situation in a seasonal breeder. The circannual changes of testicular activity include situations with high relative amounts of spermatocytes and lacking (not yet developed) spermatids as well as low proportions of spermatocytes (ceased proliferation) and high numbers of spermatids. At the end of reproductive season the ratio of 1C:4C cell ratio is especially high, because the proliferation of spermatogonia/spermatocytes is already diminished, but the differentiation of developed spermatids seems to continue.

The high numbers of spermatids and spermatozoa produced during breeding season could be a result of both stimulated proliferation and reduced apoptotic cell death of premeiotic cells. Apoptosis is a basic mechanism for signal-induced, selective cell elimination. This process is considered a normal, hormonally controlled phenomenon in the mammalian testis (Kerr 1992, Dym 1994, Dunkel *et al.* 1997). The output of spermatozoa is determined largely by the shifts in degeneration vs survival of dividing and developing germ cells (Bartke 1995). The survival of spermatogenic cells is dependent on gonadotropins as well as on intratesticular androgens induced by LH (Tapanainen *et al.* 1993, Troiano *et al.* 1994, Billig *et al.* 1995, Marathe *et al.* 1995). Testosterone plays an essential role in preventing apoptotic cell death in androgen-dependent tissues (reviewed in Tenniswood *et al.* 1992, Thompson 1994). This assumption is supported by reports of enhanced testicular apoptosis during photoperiodically induced testis regression in Djungarian hamsters (Furuta *et al.* 1994) and of the inverse relationship between apoptosis and proliferation within the annual cycle of roe deer (Blottner *et al.* 1996). In previous studies, similar situations were observed in brown hare with depressed proliferation and intensified apoptosis coinciding with a pronounced reduction of testicular testosterone concentrations during testis involution (Blottner *et al.* 1995).

Furthermore, earlier reports revealed that the transition from the breeding to the non-breeding season is associated with a decrease in both the number and the functional competence of cauda epididymal spermatozoa in brown hare (Hingst *et al.* 1995). It has been described that adult males can have semen with high quantities of vital spermatozoa from February to August. Due to regression, the epididymis contained the lowest number of sperm with highest portions of morphologically and functionally abnormal cells in September and October.

The time-curve of testosterone level showed a shift in comparison with the spermatogenic activity and the growth of testes. In autumn the prominent increase of testosterone concentration precedes these processes. The testosterone level reaches its highest value during the breeding period. Subsequently it decreases to its lowest value during pronounced testis involution in August, even before testis mass and testicular spermatozoa are at their minimum. The transient decrease of the testosterone concentration in February is not significant. In addition to the high error of this single point estimations for March/April are lacking. Consequently, more data could result in a continuous curve during the spring. Nevertheless, the results of this study suggest that the increase of testosterone concentration is not simply paralleled to the testicular growth.

In conclusion, the production of spermatozoa in brown hare is intensified by enlargement of gonads as well as by enhanced efficacy of spermatogenesis during the breeding season. The significant involution of testes after this period is associated with a reduction of proliferation resulting in pronounced changes in cellular composition of testicular tissue preceded by a decline in testosterone concentration. The results suggest that regulation of seasonal testicular activity is characterized by co-ordinated shifts in the dynamic equilibrium between mitosis, meiosis and testosterone production.

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