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Seasonal variations in the morphometric analysis of the testis, testosterone production, and occurrence of pathological spermatozoa in the brown hare (*Lepus europaeus*)

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ABSTRACT

Seasonal variations in the morphometric analysis of the testis, variations in testosterone production, and occurrence of pathological spermatozoa in the brown hare (*Lepus europaeus*) were studied.

In evaluating the relative volume of germinal epithelium, interstitium and lumen in hare testis we found the highest relative volume of germinal epithelium in the winter (84.14%). The lowest level was found in the summer, but the highest relative volumes of interstitium (12.47%) and lumen (18.11%) were in the summer. The germinal epithelium achieved a maximum height in the winter (35.47 μm), when the diameter of the seminiferous tubules was also at a maximum (320.75 μm). These data are supported by analysis of testosterone production, which shows that the highest concentration of testosterone in blood was found in the winter (5.72 ng/ml), the lowest in the summer (0.87 ng/ml). This difference was significant ($P<0.01$).

Analysis of pathological spermatozoa showed that their occurrence ranged from 11.50 to 24.17%, with the highest value in the spring. As the spermatozoa were collected from the epididymis, the most frequently found pathological form was spermatozoa with retention of the cytoplasmic drop.

KEY WORDS: hare (*Lepus europaeus*), testis, spermatozoa, testosterone, season

INTRODUCTION

The European or Brown hare (*Lepus europaeus* Pallas 1778) is a large animal, averaging 3.8 kg (3-5 kg) in weight, of relatively uniform appearance. The fur is long and curled on the back, with a tawny or rusty colour over the chest and sides, darker above, white below. The tail is large and conspicuous, black on top and white underneath. The tips of the ears have a large triangular black patch on the back contrasting with the paler grey of the rest of the ear. In winter there is some white on the sides of the head and base of the ears, and grey on the haunches (Chapman and Flux, 1990).

Great attention is being paid to applied research on the brown hare, which was once the most plentiful game in Slovakia (Hell and Slamečka, 1999), in connection with the critical decline of the population during the last ten years. However, attention should be paid to fundamental research as well, because it can help solve this problem in practical terms.

The literature contains very little data about the morphometry, structure and secretory activity of sexual organs in the hare. The uterine enlargement (Calliol et al., 1989a), increased progesterone (Calliol and Martinet, 1976; Calliol et al., 1991) and estradiol (Caillol et al., 1989a, 1991; Semizorová et al., 1990) levels in the blood of females have been reported. In males, growth of testicles and increased blood testosterone levels (Calliol et al., 1989b) during the reproductive cycle have been described.

The purpose of this study was to analyse the variations in the microscopic structure of the testis, occurrence of pathological spermatozoa and to determine testosterone production in the brown hare (*Lepus europaeus*) in relation to the season.

MATERIAL AND METHODS

The samples were collected as described by Massányi et al. (2000). In a one-year period, 37 males were analysed. In the spring we collected samples in March, April and May (9 animals), in the summer in June, July and August (9 animals), in autumn in September, October and November (9 animals), and in winter in December, January and February (10 animals).

The procedure of preparing testis samples was described in a previous paper (Massányi et al., 2000). From microphotographs (Docuval, Carl Zeiss Jena) based on micromorphological criteria (Weibel et al., 1966; Vrzgulová et al., 1979; Toman and Massányi, 1997) the quantitative values of testicular structure were evaluated in each sample.

In testis we analysed the qualitative microscopic structure and the relative volume (%) of the germinal epithelium, interstitium and lumen. Further, we analysed the height of the germinal epithelium and the diameter of seminiferous tubules; both are expressed in µm.

For documentation of ultrastructure by scanning electron microscopy, the testes samples were washed in PBS and fixed in 1% glutaraldehyde in 0.125 mol cocodylate buffer (pH 7.3) at an ambient temperature for 1 h. Next, the samples fixed for 12 h at 4°C and postfixed in 1% OsO_4 for 1 h in cocodylate buffer (pH 7.3). After dehydratation with ethanol the samples were dried using a critical-point drying technique in Freon 13 and were strewed by an aurum layer (Fléchon et al., 1986). The samples were examined using a JEM 100 CX-II JOEL electron microscope at 80 kV and photographed.

The level of sexual hormones in blood of hares was assessed by RIA (radioimmunoassay) using kits (the Institute for Radioecology and Use of Nuclear Technique, Košice, Slovak Republic) according to the manufacturer's instructions. The sensitivity of RIA to testosterone was 50 pg/ml, the cross-reaction of the used antiserum with dihydrotestosterone was 7.2% and with progesterone, estradiol and cortisol lower than 0.004 %.

Samples obtained from the head region of the epididymis (the *caput epididymis*) were used to assess the occurrence of pathological spermatozoa. A drop of the epididymidal content was diluted with a drop of physiological saline and then placed on a slide tilted at a 45° angle. The samples were dried at 38°C, fixed in Hancock's solution and rinsed in distilled water. The samples were stained as described by Hancock (1957) and viewed under a light microscope at a magnification of 500x. In each season 4 000 spermatozoa were evaluated (Gamčík et al., 1992; Massányi et al., 1996). We found the following pathological changes in spermatozoa: separated tail (ST), knob twisted tail (KT), retention of cytoplasmic drop (RD), broken tail (BT), other forms of pathological spermatozoa (tail ball, tail torso, club bag tumour, small or large head, teratogenic changes).

To compare these treatment means the analysis of variance as well as Student's t-test and Scheffe's test were applied (SAS, 1989) and EXCEL (1998).

RESULTS

Testes

The testis of the brown hare (*Lepus europaeus*) is completely enclosed by the *tunica albuginea*, which is thickened posteriorly to form the mediastinum of the testis, projecting some way into the body of the testis. Blood and lymphatic vessels, and the channels carrying spermatozoa pass through this area. Fibrous septa form the mediastinum divide the body of the testis into lobules, each lobule contains seminiferous tubules (Figure 1).

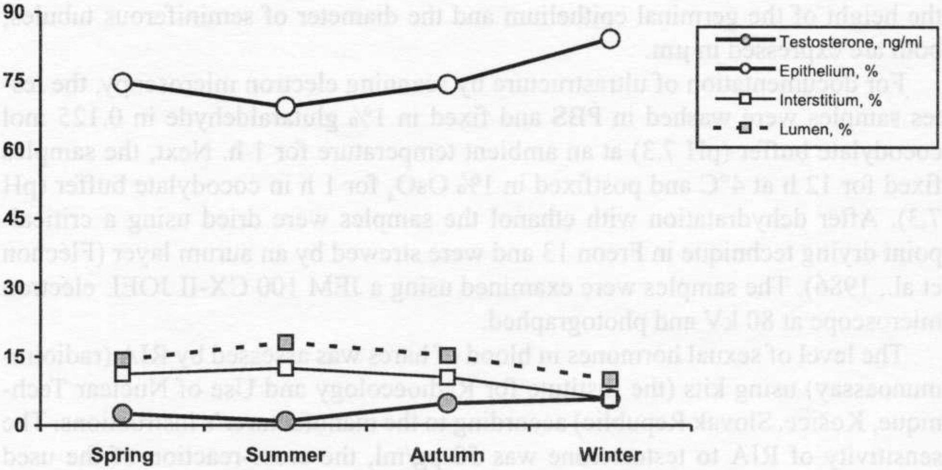

Figure 1. Testicular structure in brown hare in winter period. Seminiferous tubules (T) are filled with germinal epithelium (G) and in centre is lumen (L). Between the tubules interstitium (S) is present (H-E, 200x)

A seminiferous tubule is a coiled, unbranching closed loop, both ends of which open into a system of channels (*rete testis*) at the posterior hilum of the testis, close to the mediastinum. Each seminiferous tubule has a central lumen lined by an actively replicating epithelium - the germinal or seminiferous epithelium, mixed with a population of supporting cells - the Sertoli cells.

Morphometric analysis of testis in relation to season showed that the highest relative volume of germinal epithelium is in the winter (84.14%), the lowest in the summer (Table 1, Figures 1 and 2). On the other hand, the lowest relative volume of interstitium was found in the winter (5.81%) and the highest in the summer (12.47%). The relative volume of lumen ranged from 10.06 to 18.11%

TABLE 1

The structure of hare testis in relation to season′

Season	Relative volume					
	germinal epithelium		interstitium		lumen	
	x	s	x	s	x	s
Spring	74.53	7.53	11.19	2.52	14.28	5.60
Summer	69.31	12.24	12.47	5.69	18.11	9.03
Autumn	74.21	3.80	10.54	2.96	15.25	3.15
Winter	84.14	4.02	5.81	1.53	10.06	3.50

during the year. Seasonal differences were not significant. Also the height of the germinal epithelium reached the highest value in the winter, as did the diameter of the seminiferous tubules (320.75 μm). There were no significant differences between the studied seasons , but all morphometric data suggest that the highest activity of the testis (spermatogenesis) was in the winter and the lowest in the summer (Tables 1 and 2).

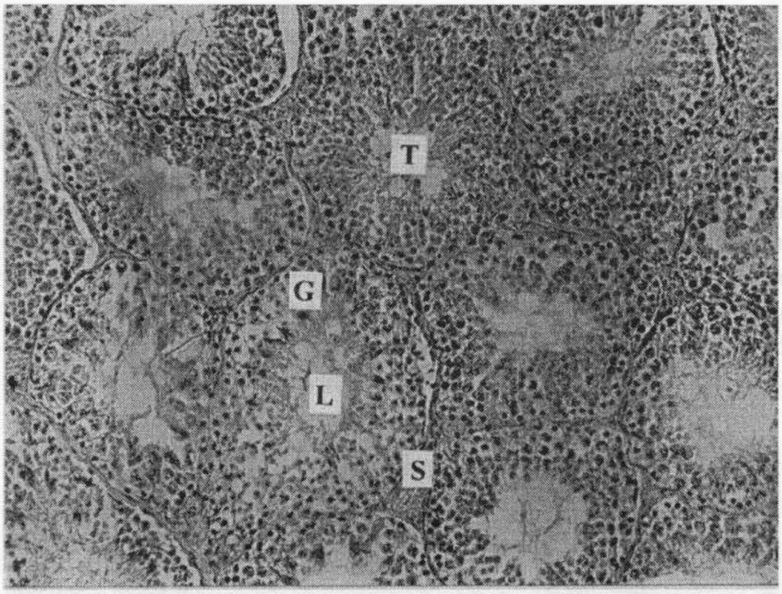

Figure 2. In summer period the activity of testis is decreased. Relative volume of germinal epithelium (G) is decreased. Higher stages of spermatogenesis are missing. Higher relative volume of lumen (L) is present (H-E, 200x)

TABLE 2

Analysis of the height of germinal epithelium and diameter of seminiferous tubules in hare in relation to season

Season	Height of germinal epithelium μm		Diameter of seminiferous tubules μm	
	x	s	x	s
Spring	33.72	5.87	260.82	125.90
Summer	32.20	5.77	247.89	101.67
Autumn	25.20	4.41	157.77	46.99
Winter	35.47	6.58	320.75	141.04

Testosterone

The morphometric data is very well supported by results of blood testosterone assays (Figure 4). The highest concentration of testosterone was found in the winter (5.72±1.22 ng/ml), lower values were found in the autumn (4.83±1.02 ng/ml) and spring (2.52±0.54 ng/ml). The lowest concentration of testosterone was in the summer (0.87 ± 0.12 ng/ml) and this value was significantly lower in comparison with the remaining seasons ($P<0.01$).

Spermatozoa

In the analysis of seasonal occurrence of pathological spermatozoa we analysed the spermatozoa obtained from the tail of the epididymis. Over the year we found 11.50-24.17% pathological spermatozoa. The highest percentage was in the spring. This analysis showed that the most frequent abnormality was retention of a cytoplasmic drop (8.25-14.50%), which is a typical sign of developing spermatozoa (Figure 3). Spermatozoa with separated tail accounted for 1.30-5.80%, spermatozoa with knob twisted tail, for 1.00-1.90%, spermatozoa with broken tail, 0.90-1.35%; other forms of pathological spermatozoa reached 1.00-1.60% (Table 3). No significant differences were found among the seasons.

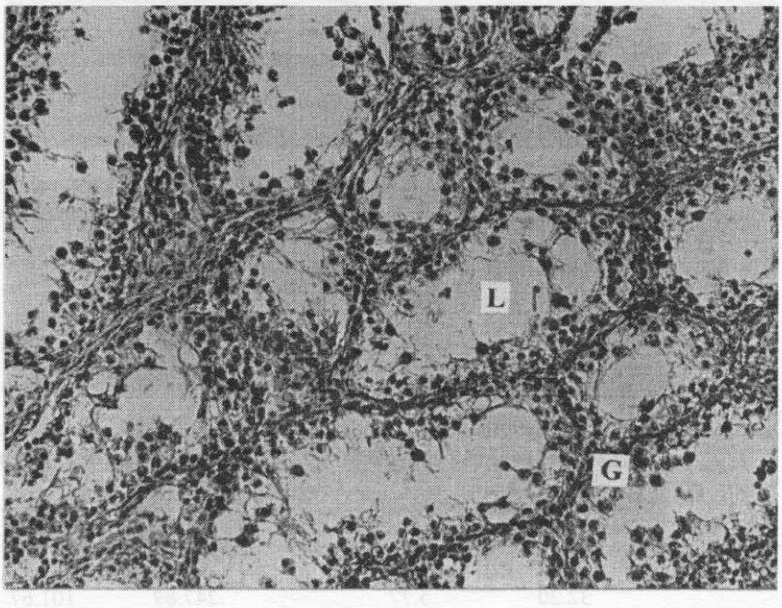

Figure 3. In testis as well as epididymis spermatozoa with cytoplasmatic drop (D) were often found (SEM, 5900x)

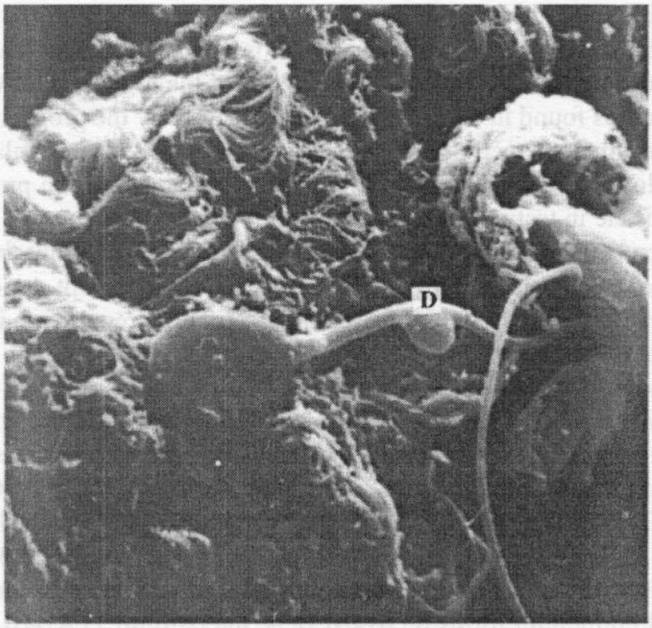

Figure 4. Relation of the level of testosterone to the structure of testis. Values of testosterone ng/ml, the relative volume of germinal epithelium, interstitium and lumen, %

TABLE 3

Seasonal occurrence of pathological spermatozoa in brown hare, %

Season	The form of pathological spermatozoa					Total
	ST	KT	RD	BT	OF	
Spring	5.80	1.57	14.50	1.20	1.10	24.17
Summer	1.60	1.00	8.25	0.70	1.00	12.55
Autumn	1.30	1.90	6.30	0.90	1.10	11.50
Winter	4.50	1.65	10.20	1.35	1.60	19.30

ST – separated tail, KT – knob twisted tail, RD – retention of cytoplasmatic drop, BT – broken tail, OF – other forms of pathological spermatozoa

DISCUSSION

Generally there is very little data describing reproduction in the brown hare and data on microscopic structure is particularly scarce.

In this study, seasonal variations in the morphometric analysis of the testis are reported. We found the highest relative volume of germinal epithelium, height of germinal epithelium, diameter of seminiferous tubules as well as the highest concentration of testosterone in the winter, which is a clear sign of high activity of the brown hare during this season. We also report that the highest relative volume of the interstitium is found in the summer, caused mainly by the proliferation of connective tissue since the activity of endocrine Leydig cells is not enhanced. Also, the smaller lower diameter of seminiferous tubules supports the proliferation of connective tissue in testicular interstitium.

It has been reported that European hares start breeding at about the winter solstice in all nine countries where they have been studied except Argentina (Flux, 1965; Amaya et al., 1979; Slamečka et al., 1997). These reports are in correspondence with our results.

Many studies describe seasonal variations in the blood parameters of the brown hare (Črep and Švický, 1993; Slamečka et al., 1997) but morphology is not well studied. In contrast, the morphological structure of reproductive organs in farm animals under various conditions is well studied (Vrzgulová et al., 1979, 1980; Kolodzieyski et al., 1991; Kolodzieyski and Danko, 1995; Jantošovičová et al., 1996).

Analysis of the testes showed that germinal epithelium forms 69.31-84.14% of this gland, interstitium 5.81-12.47% and lumen 10.06-18.11%. In the fallow deer the germinal epithelium forms 63.61-76.18%, interstitium 11.47-20.05% and lumen 12.35-16.34%, so it can be seen that the fallow deer has a higher amount of interstitium, but the relative volume of germinal epithelium is in the same range (Massányi et al., 1999). Also in *Apodemus sylvaticus* and *Apodemus flavicollis*, members of wild *Muridae*, similar data were reported (Jančová et al., 1999). In *A. sylvaticus* the germinal epithelium forms 67.55 and in *A. flavicollis*, 76.90%. In rabbits the germinal epithelium forms 77.75%, interstitium 12.27% and lumen 9.99% (Toman and Massányi, 1997). In rats, seminiferous epithelium makes up 82.43% and in rams, 70.5% (Mori and Christensen, 1980; Cigánková et al., 1996, 1998). In ICR mice germinal epithelium forms 89.13±4.24% and interstitium 10.87±4.24% (Massányi et al., 2000). These data show that the differences among these species are not significant.

The diameter of seminiferous tubules in the brown hare is in the range of 157.77-320.75 μm. These results are similar in comparison with fallow deer (143.08-228.91 μm), *Apodemus sylvaticus* and *Apodemus flavicollis* (140.20 and 142.50 μm), rabbits (118.69 μm) and other species (Toman and Massányi, 1997; Jančová et al., 1999; Massányi et al., 1999). Finally, we can conclude that, on the one hand, there are some insignificant differences in the morphometric analysis of testis among various animal species, but on the other hand testicular structure is very sensitive to various conditions such as the season, as reported in this study, which affects it significantly.

A relatively high percentage of pathological spermatozoa occurring seasonally was found (11.50-24.17%). The high percentage can be attributed to the fact that the spermatozoa were collected from the epididymis, where maturation of spermatozoa occurs. The most frequent registered pathological form of spermatozoa was spermatozoa with cytoplasmic drop, a typical form found in the epidydimis (Massányi, 1992). If we do not take this form into consideration, the value of pathological spermatozoa will be 4.3-9.67%. These figures are good in comparison with rabbits, where the minimum activity after freezing the ejaculate has to be 30% (Gamčík et al., 1992).

Generally, it is well known that climate and time of year are the main factors of sexual activity in wild animals, and these factors control the development of testes and their functions. This study confirms evident seasonal variation in the male reproductive function evaluated by morphometric analysis in the hare, a wild animal species.

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STRESZCZENIE

Sezonowe zmiany w morfometrycznej ocenie jąder, produkcji testosteronu oraz występowania patologicznych (schorzałych) plemników u zająca (*Lepus europaeus*)

Badaniami objęto 37 samców, po 9 wiosną (marzec do maja), latem (czerwiec do sierpnia) i jesienią (wrzesień do listopada) oraz 10 w okresie zimowym (grudzień do lutego).

Względna objętość nabłonka kanalików nasiennych jąder była największa zimą (84,14%), namniejsza latem, podczas gdy w miesiącach letnich największa była względna objetość tkanki śródmiąższowej (12,47%) oraz światło kanalików (18,11%). Wysokość nabłonka nasieniotwórczego (35,47 μm) oraz średnica kanalików nasiennych (320,75 μm) były największe zimą. Dane te znajdują potwierdzenie w produkcji testosteronu, którego stężenie w krwi było największe także zimą (5,72 ng/ml), a najmniejsze w miesiącach letnich (0,87 ng/ml); różnice te były statystycznie istotne ($P<0,01$).

Przypadki wystepowania zmienionych patologicznie plemników wahały się od 11,50 do 24,17%, przy czym najczęściej w okresie wiosennym. Przy pobieraniu plemników z najądrza częściej spotykano formy patologiczne, w których najczęściej obserwowano plemniki z kroplą cytostatyczną.