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Original article

Morphological defects of epididymal spermatozoa in male roe deer (*Capreolus capreolus*) during the reproductive season

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Abstract

The aim of this study was to analyse the morphology of the epididymal spermatozoa of male roe deer obtained postmortem at the beginning (May), peak (July/August) and the end (September) of the reproductive season. Spermatozoal abnormalities were divided into major (associated with impaired fertility) and minor (not associated with impaired fertility) defects. The highest percentage of abnormal spermatozoa was observed in May (17.78 ± 1.88%), with a much higher proportion of major (12.35 ± 1.11%) than minor defects (5.43 ± 1.59%) being observed. The percentage of abnormal spermatozoa was lowest during the peak of the reproductive season (4.97 ± 1.13%), with the proportion of major (2.68 ± 0.78%) and minor defects (2.28 ± 0.45%) being comparable during this period. The percentage of abnormal spermatozoa increased again in September (11.05 ± 1.60%), with the major defects (6.15 ± 1.04%) slightly surpassing the minor defects (4.90 ± 0.77%); however, total abnormalities still remained lower than those found in May. These differences were statistically significant, with the exception of the difference in minor defects between the pre-rut and post-rut periods. These results indicate that the best period to collect epididymal spermatozoa from roe deer postmortem is the peak of the reproductive season (July/August); however, they can also be recovered at the end of the reproductive season (September), as the percentage of major defects is relatively low at this time. This study provides the basis for further research to determine optimal methods for the storage and cryopreservation of epididymal spermatozoa in this species.

Key words: epididymal spermatozoa, sperm defects, male roe deer, reproduction season

Introduction

During the winter months spermatogenesis in male roe deer is arrested and only the spermatogonia and Sertoli cells are present within the seminiferous tubules at this time. In February the spermatogonia start to proliferate, and spermatogenesis begins. The highest prevalence of tetraploid (4C) cells, indicating high mitotic activity in the testes, occurs in April. In May, the spermatogenic cells representing all stages of spermatogenesis are detectable and the first spermatozoa can be found in the lumens of the seminifer-

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ous tubules. The highest percentage of haploid (1C) cells (spermatids and spermatozoa) appears during the rut in July and August (Blottner et al. 1996, Roelants et al. 2002, Schön et al. 2004). Roe deer semen collected by electroejaculation throughout the year has shown that the highest number of sperm, of the best vitality and mobility, as well as the lowest percentage of defective spermatozoa occurs during the rut period (July/August) (Goeritz et al. 2003).

The recovery of semen from wild animals using electroejaculation or artificial vaginas or collecting semen immediately postmortem presents considerable difficulties. A viable alternative to these methods is the postmortem recovery of spermatozoa during the hunting season. It has been proven that semen from wild ruminants, including roe deer, that is kept at low temperatures (5°C) in the epididymis postmortem and is subsequently obtained from the cauda epididymis, maintains good quality for a long time after the death of the animal (Martinez-Pastor et al. 2005a, Martinez-Pastor et al. 2005b, Soler et al. 2005). The effect of season on epididymal spermatozoa recovered postmortem has also been investigated. The sperm concentration, progressive movement, viability, acrosomal status and HOS test reactivity showed the highest values during the rut period in roe deer and other cervids (Willard and Randel 2002, Martinez-Pastor et al. 2005a, Martinez-Pastor et al. 2005c).

An important parameter influencing the normal process of fertilization of oocytes is sperm morphology. Spermatozoa without defects have a greater chance of fertilizing an oocyte during natural fertilization or assisted reproductive methods (Malo et al. 2005, Al-Makhzoomi et al. 2008). The presence of sperm head abnormalities is especially important as they adversely affect the binding of spermatozoa to the oocyte and their passage through the zona pellucida (Parkinson 2004, Al-Makhzoomi et al. 2008). Morphological abnormalities in spermatozoa can be classified into major and minor defects according to their effect on fertility (Blom 1973). Major sperm defects include most abnormalities of the head and midpiece and proximal cytoplasmic droplets, which are associated with impaired fertility. Minor sperm defects include mainly looped tails, detached sperm heads and distal cytoplasmic droplets, which are not associated with impaired fertility. This study was undertaken because of the current lack of detailed analysis of the morphology of the epididymal spermatozoa of roe deer. By analysing the morphology of roe deer epididymal spermatozoa collected postmortem at the beginning (May), peak (July/August) and end (September) of the reproductive season, it can be determined whether the frequency of the major and minor defects changes over this time.

Materials and Methods

Spermatozoa for this study were collected from 36 mature male roe deer (bucks) 5 to 8 years old (based on dentition) during hunts occurring from 2007 to 2010 in the Podkarpacie region (50°1' N; 22°0' E) of Poland in Central Europe. The deer were collected during three periods: (1) from 10 to 31 May (pre-rut period); (2) from 15 July to 15 August (rut period, oestrus period in females according to Sempere et al. (1998)) and (3) from 10 to 31 September (post-rut period, when females are no longer in oestrus), with 12 deer being harvested during each experimental period. Only wild deer were used.

Morphological examination of epididymal spermatozoa

The testes and epididymides were cut out immediately postmortem, placed in PBS (phosphate-buffered saline, pH 7.4) and transported to the laboratory at 5°C within 2 hours of removal from the animal. The spermatozoa were subsequently collected by making several incisions in the cauda of the epididymis with a surgical blade according to a method described by Martinez-Pastor et al. (2006). The extracted sample was diluted 1:1 with PBS (phosphate-buffered saline, pH 7.4) and epididymal spermatozoal smears were prepared, fixed in 4% buffered formaldehyde and stained according to the Watson's method (Watson 1975). Five hundred spermatozoa were counted from each animal using a light microscope at a 1000 times magnification and the abnormalities were classified according to a system developed by Blom (1973). Distal cytoplasmic droplets were not considered a defect because they are a normal occurrence in the epididymal spermatozoa of ruminants (Cooper 2005). The number of spermatozoa showing each class of abnormality was expressed as a percentage of the total counted spermatozoa. All sperm defects were imaged using a light microscope (Olympus CX41) equipped with digital camera (ARTCAM-500MI).

Statistical analysis

Spermatozoal defects were expressed as a percentage of the total sperm count (mean ± SD). The means were compared using a one-way analysis of variance (ANOVA) followed by Tukey's test. A significance level of $p < 0.01$ was assumed for statistical analysis. If statistically significant differences were detected, the groups were identified using different letters (a, b, c). The calculations were performed with

Table 1. The percentage (mean ± SD%) of spermatozoa with major and minor defects obtained postmortem from the cauda epididymis of roe deer during the pre-rut (n = 12), rut (n = 12) and post-rut (n = 12) periods.

	Sperm defects (%)	Pre-rut period (May)	Rut period (July/August)	Post-rut period (September)
Major defects	underdeveloped	0.92 ± 0.08^a	0.17 ± 0.05^b	0.25 ± 0.04^b
	decapitate	4.37 ± 0.39^a	0.57 ± 0.16^b	1.23 ± 0.21^c
	diadem defect	0.25 ± 0.02^a	0.05 ± 0.01^b	0.15 ± 0.03^a
	pyriform (pear-shaped) head	2.37 ± 0.21^a	0.42 ± 0.12^b	0.93 ± 0.16^c
	narrow at base	0.32 ± 0.02^a	0.02 ± 0.01^b	0.09 ± 0.01^b
	head with abnormal contour	0.25 ± 0.02^a	0.03 ± 0.08^b	0.18 ± 0.03^a
	small and abnormal head	0.33 ± 0.03^a	0.07 ± 0.02^b	0.15 ± 0.03^c
	loose and abnormal head	0.23 ± 0.02^a	0.07 ± 0.02^b	0.22 ± 0.04^a
	corkscrew midpiece	0.50 ± 0.05^a	0.23 ± 0.07^b	0.82 ± 0.14^c
	other midpiece defect	0.24 ± 0.02	0.20 ± 0.06	0.30 ± 0.05
	proximal cytoplasmic droplet	1.68 ± 0.15^a	0.50 ± 0.15^b	1.18 ± 0.20^c
	pseudodroplet	0.47 ± 0.04^a	0.23 ± 0.07^b	0.48 ± 0.08^a
	„Dag" defect (strongly coiled or folded tail)	0.37 ± 0.07^a	0.12 ± 0.03^b	0.17 ± 0.03^b
Minor defects	narrow head	0.17 ± 0.05^a	0.15 ± 0.03^a	0.22 ± 0.03^b
	small head but normal	0.12 ± 0.03^a	0.18 ± 0.04^b	0.26 ± 0.04^b
	wide and giant head	0.25 ± 0.07^a	0.13 ± 0.03^b	0.32 ± 0.05^a
	loose and normal head	2.45 ± 0.72^a	0.39 ± 0.08^b	1.53 ± 0.24^c
	abaxial tail position	1.48 ± 0.44^a	0.65 ± 0.13^b	1.22 ± 0.19^c
	single bent tail	0.63 ± 0.18^a	0.38 ± 0.07^b	1.13 ± 0.18^c
	terminally coiled tail	0.30 ± 0.08^a	0.40 ± 0.08^b	0.22 ± 0.03^a

[a,b,c] Means in the same row with different superscript letters differ significantly from one another ($p < 0.01$).

Excel and Statistica 7.1 for Windows (StatSoft, Poland) computer software.

Results

The morphological analysis of abnormalities in spermatozoa from male roe deer during the pre-rut (May), rut (July/August) and post-rut periods (September) showed that spermatozoa with both major and minor defects were present in all three periods (Table 1). Among the major defects observed were underdevelopment (Fig. 1b), decapitation (Fig. 1c), diadem defects (Fig. 1d), heads with abnormal contours (Fig. 1d), pyriform (pear-shaped) heads (Fig. 1e), narrow at base (Fig. 1f), small and abnormal heads (Fig. 1g), loose and abnormal heads (Fig. 1h), corkscrew-shaped midpieces (Fig. 1i) and other midpiece defects (Fig. 1j), proximal cytoplasmic droplets (Fig. 1j), pseudodroplets (Fig. 1k) and „Dag" defect (strongly coiled or folded tails) (Fig. 1l). Some of the minor defects observed were narrow heads (Fig. 2a), small but normal heads (Fig. 2b), wide and giant heads (Fig. 2c), loose and normal heads (Fig. 2d), abaxial tail position (Fig. 2e), single bent tails (Fig. 2f) and terminally coiled tails (Fig. 2f). There were statistically significant differences in the total number of abnormal spermatozoa (the sum of major and minor defects) between all three periods examined (Fig. 3). The highest percentage of defective spermatozoa was observed in the pre-rut period (17.78 ± 1.88%) and the lowest in the rut period (4.97 ± 1.13%), with the post-rut period being intermediate (11.05 ± 1.60%). The highest percentage of major (12.35 ± 1.11%) and minor (5.43 ± 1.59%) defects was present in the pre-rut period. The lowest percentage of major (2.68 ± 0.78%) and minor (2.28 ± 0.45%) defects was noted during the rut period. In the post-rut period the percentage of major defects was 6.15 ± 1.04%, whereas 4.90 ± 0.77% of the observed spermatozoa had minor defects during this period. Apart from the difference in the percentage of minor defects between the pre- and post-rut periods all other differences were statistically significant (Fig. 3).

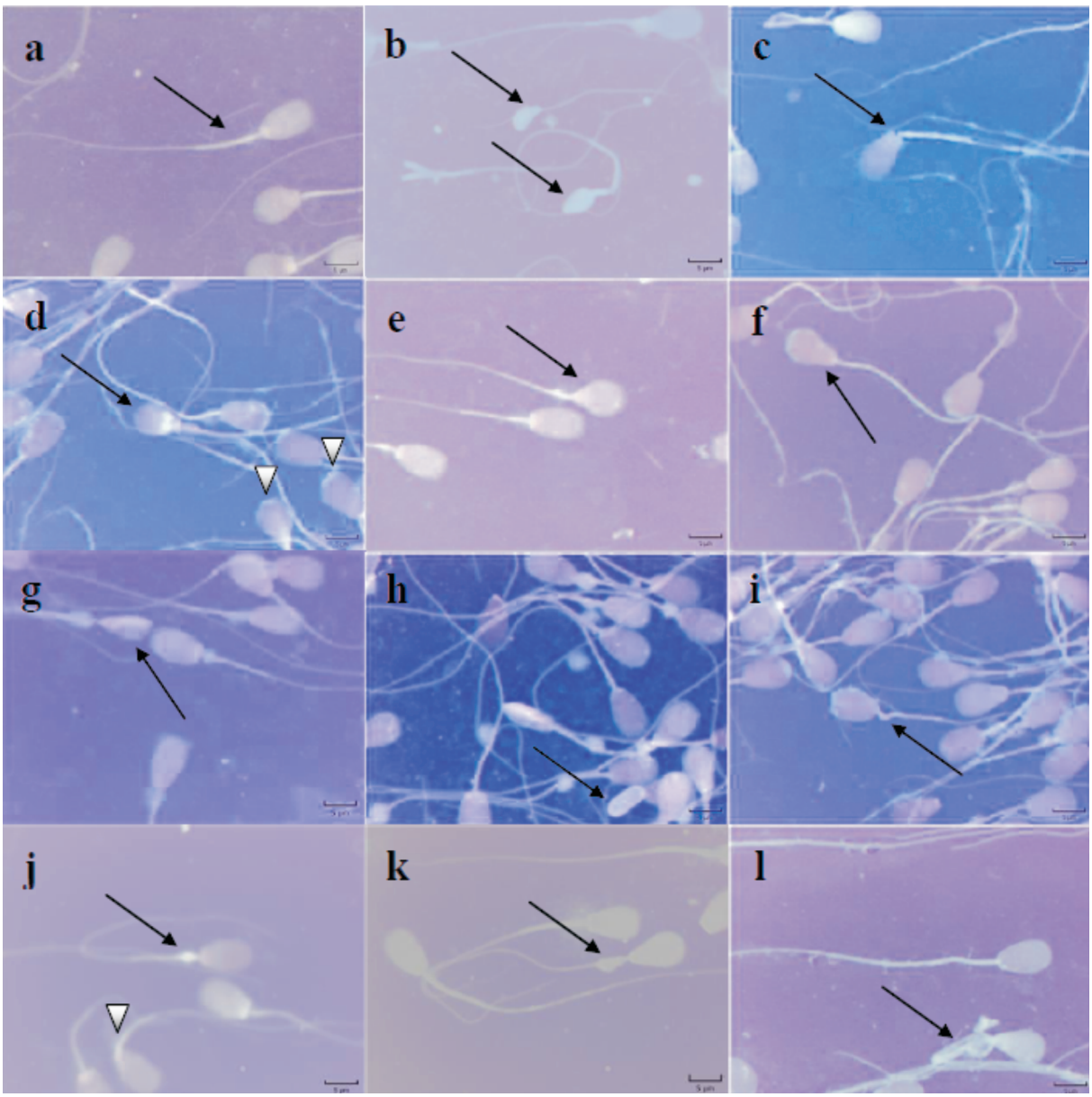

Fig. 1. Major defects in spermatozoa collected postmortem from the cauda epididymis of male roe deer during the reproductive season: (a) normal sperm (↑); (b) underdeveloped (↑); (c) decapitated (↑); (d) diadem defect (↑), head with abnormal contour (Δ); (e) pyriform (pear-shaped) head (↑); (f) narrow at base (↑); (g) small and abnormal head (↑); (h) loose and abnormal head; (i) corkscrew midpiece (↑); (j) proximal cytoplasmic droplet (↑), other midpiece defect (Δ); (k) pseudodroplet (↑); (l) „Dag" defect (strongly coiled or folded tail) (↑). Watson stained smears. Scale bars = 5 μm.

Discussion

Seasonal variation in the testicular activity of cervids contributes to fluctuations in both the quantity and quality of the spermatozoa produced (Goeritz et al. 2003, Giżejewski 2004, Martinez-Pastor et al. 2005c). The semen collected during the period of highest spermatogenesis activity and testosterone production, which coincides with the period of rut in females, showed the best quality. It contained the highest concentration of sperm and sperm with the highest vitality and motility, and the smallest percentage of abnormal sperm. Such semen provides high fertility to males and is ideal for storage and cryopreservation (Umapathy et al. 2007, Gomendio and Roldan 2008, Garcia-Alvarez et al. 2009).

An important part of semen analysis is examining sperm morphology. The percentage of morphologically normal spermatozoa is positively related to fertility. Spermatozoa without morphological deviations show better efficacy in fertilizing an oocyte both *in vivo* and *in vitro* (Al-Makhzoomi et al. 2008, Malo

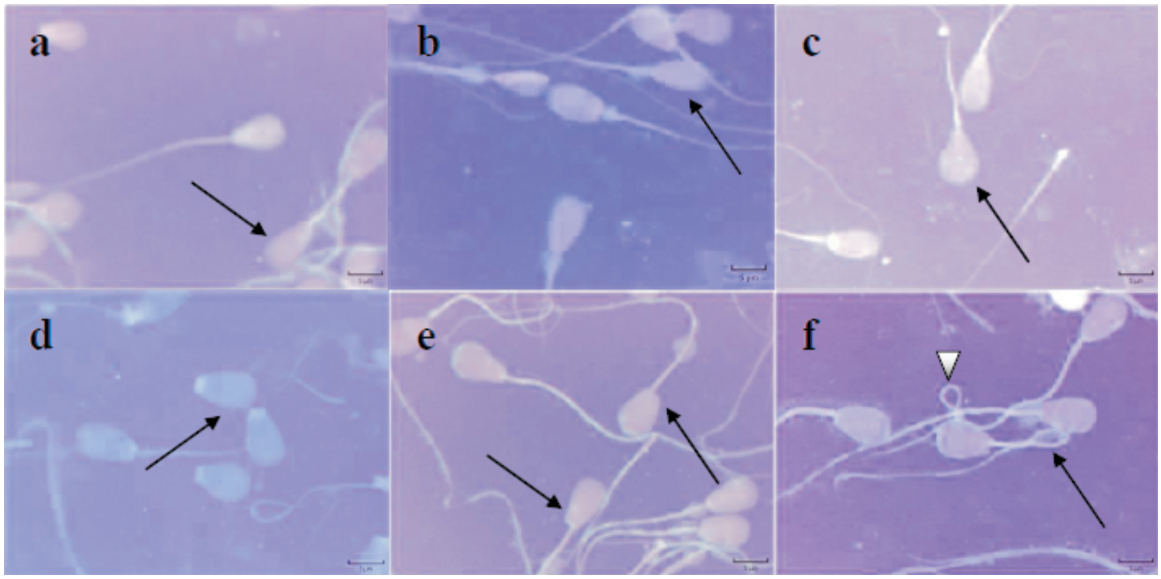

Fig. 2. Minor defects in spermatozoa collected postmortem from the cauda epididymis of male roe deer during the reproductive season: (a) narrow head (↑); (b) small but normal head (↑); (c) wide and giant head (↑); (d) loose and normal head (↑); (e) abaxial tail position (↑); (f) single bent tail (↑), terminally coiled tail (Δ).Watson stained smears. Scale bars = 5 μm.

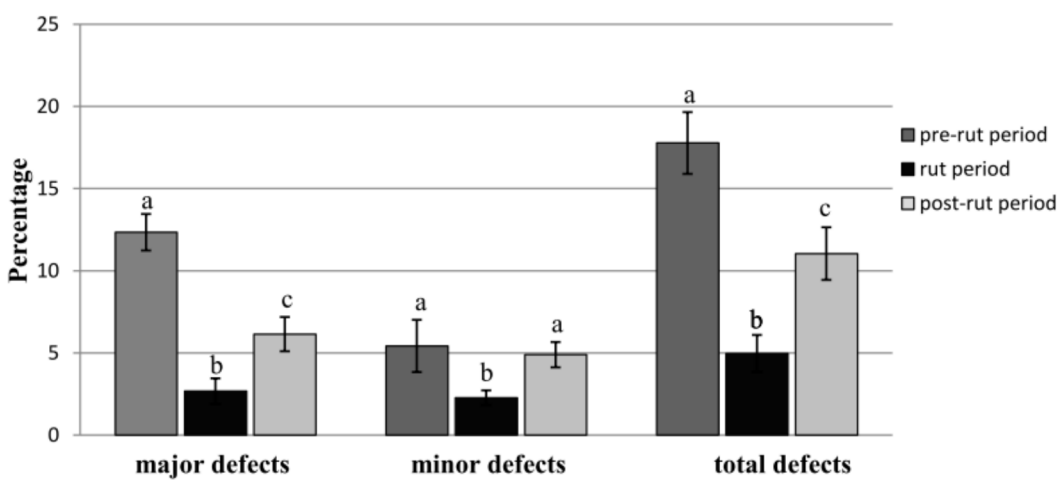

Fig. 3. The percentage (mean ± SD) of major, minor and total defective spermatozoa obtained postmortem from the cauda epididymis of roe deer during the pre-rut (n = 12), rut (n = 12) and post-rut (n = 12) periods (Different letters indicate statistically significant differences ($p < 0.01$)).

et al. 2005). According to Blom (1983), the presence of spermatozoa with major defects in excess of 15% is correlated with decreased fertility and may indicate disorders in the spermatogenesis and/or the epididymal maturation processes. Such disturbances may occur during the pre-rut period (May), judging by the high percentage of spermatozoa with major defects observed during this time. Previous studies have also shown that the steroidogenesis process was less active and the tissues of the testes and epididymides were less sensitive to androgens during this period than when the animals were in rut (Kozioł and Koziorowski 2013). Furthermore, the highest levels of steroid hormones as well as the highest expression of androgen receptors in testicular and epididymal cells were observed during the rut period (July/August), which coincides with the lowest percentage of major defects observed during the same period in this study. Elevated concentrations of steroid hormones and androgen receptors are therefore very important for normal spermatogenesis and epididymal maturation, and thus the morphology of the epididymal spermatozoa in male roe deer. In the post-rut period (September), there was a decrease in the steroidogenesis activity,

and a slight decrease in the amount of androgen receptors in the testes and epididymides. This may have contributed to the slight increase in the major defects of the epididymal spermatozoa at this time. The number of androgen receptors in the cells of the gonads and epididymides was statistically significantly lowest in the pre-rut (May) period. There were no statistically significant differences between the rut and post-rut periods (Kozioł and Koziorowski 2013), which may explain the good morphological quality of the spermatozoa observed in September relative to that collected in May. Besides, numerous studies show that oestrogens play an important role in the male reproductive system and that their presence is required for proper fertility (Robertson et al. 2001, Carreau and Hess 2010), including roe deer (Schön and Blottner 2008). Also studies revealed the presence of androgen and oestrogen receptors in mature sperm in many species (Solakidi et al. 2005, Rago et al. 2007, Kotula-Balak et al. 2012). The expression of steroid hormone receptors in the spermatozoa clearly indicate that steroid hormones can directly regulate sperm functional and morphological properties (Baldi et al. 2009).

Goeritz et al. (2003) analysed the morphology of spermatozoa from male roe deer during different seasonal periods. The smallest percentage of abnormal spermatozoa was observed in the rut period. However, the percentage of defective spermatozoa reached higher values in the rut (30%), pre-rut (80%) and post-rut periods (60%) than was found in this study. This discrepancy may be a result of differences in the method of sperm collection. In this study, spermatozoa were obtained postmortem from the cauda epididymis, whereas Goeritz et al. (2003) used electroejaculation under general anaesthesia. Martinez et al. (2008) compared the characteristics of electroejaculated Iberian red deer (*Cervus elaphus hispanicus*) spermatozoa to epididymal samples. The percentage of abnormal forms was slightly higher for epididymal spermatozoa (29%) compared with electroejaculated spermatozoa (18.8%). In contrast, spermatozoa from sika deer (*Cervus nippon*) that were collected from the epididymides postmortem and stored at 4ºC for 1 to 7 days were characterized by low percentages of morphological abnormalities. The percentage of abnormal spermatozoa increased from 2.1% when initially collected to 2.9% after 1 day of storage, subsequently increasing to 5.2% and 8.6% after 4 and 7 days of storage, respectively (Hishinuma et al. 2003).

Giżejewski (2004) examined the effect of season on the morphology of spermatozoa in red deer (*Cervus elaphus*), collecting semen using a modified artificial vagina and classifying spermatozoal abnormalities according to the methodology developed by Blom (1973). In red deer the lowest percentage of major defects (< 5%) was recorded during the rut period (September/October), whereas a higher percentage (> 10%) was recorded during the pre-rut (August) and post-rut (December to April) periods. The number of minor sperm defects was the lowest in the period of maximum libido (September/October), and ranged from 6% to 12% (Giżejewski 2004). These results are comparable to the results obtained in this study for roe deer. Increases in the number of morphologically abnormal spermatozoa during the pre-rut and post-rut periods have also been observed in other cervids, such as fallow deer (*Dama dama*) (Gosch and Fischer 1989), wapiti (*Cervus elaphus*) (Haigh et al. 1984), Eld's deer (*Cervus eldi thamin*) (Monfort et al. 1993) and spotted deer (*Axis axis*) (Umapathy et al. 2007).

Recent studies have reported that semen stored in the cauda epididymis maintains good quality for a long time postmortem in roe deer (Matinez-Pastor et al. 2005b) and other cervids (Hishinuma et al. 2003, Matinez-Pastor et al. 2005b, Soler et al. 2005). This is in agreement with the results of this study, as the percentage of abnormal spermatozoa found was relatively low. This would facilitate the creation of germplasm banks of not only endangered species (Ptak et al. 2002, Kozdrowski et al. 2011) but also those that could be at risk in the future (Holt and Pickard 1999). In addition, epididymal spermatozoa collected postmortem could be used for artificial insemination (Garde et al. 2006, Morrow et al. 2009), *in vitro* fertilization (Garcia-Alvarez et al. 2009) and *in vitro* embryo production (Comizzoli et al. 2001, Martins et al. 2007).

Conclusion

The epididymal spermatozoa of male roe deer collected postmortem at the beginning of the reproductive season (May) were characterized by poor quality sperm morphology. During this period the percentage of spermatozoa with major defects, which indicates disturbances in the process of spermatogenesis and/or epididymal maturation, was the highest. The best time to obtain epididymal spermatozoa is during the peak of the reproductive season (July/August), as the lowest percentage of spermatozoa with major defects was found during this time. At the end of the reproductive season (September) the number of spermatozoa with major defects increased slightly; however this is also a good period for the postmortem recovery of spermatozoa from the cauda epididymis in this species.

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