



## RELATIONSHIP OF ARCTIC FOX (*ALOPEX LAGOPUS* L.) SPERM MORPHOLOGY WITH AGE OF MALES, SPERM CONCENTRATION, EJACULATE VOLUME AND ACROSIN ACTIVITY\*

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### Abstract

The aim of the study was to determine the relationship of sperm morphology with age of males, ejaculate concentration and volume, as well as with acrosin activity determined in sperm acrosome extracts. The study used manually collected ejaculates from 9 male arctic foxes, including 6 young males aged one year and 3 older males (between 3 and 5 years of age). All of the 39 ejaculates used in the study were classified as normal based on motility exceeding 70%. The ejaculates collected from the foxes were evaluated for volume, sperm concentration and frequency of morphological changes including primary and secondary defects. The spermiograms of the male arctic foxes were classified according to a six-grade subjective scale. In addition, acrosin activity was determined in the sperm acrosome extracts. The data were analysed using the criteria of male age, sperm concentration, ejaculate volume, and acrosin activity. The morphology of arctic fox spermatozoa was dependent on the age of the male. A greater number of morphologically altered spermatozoa tended to occur in the ejaculates of young foxes, which were in their first breeding season. In addition, statistical analysis revealed positive relationships between the frequency of morphological changes in sperm and their ejaculate concentration. In contrast, there were no significant correlations between the percentages of morphologically changed spermatozoa and the ejaculate volume and the content of acrosin, which is an indicator of acrosomal integrity. Semen quality is dependent on the number of sperm in ejaculate with morphological defects which prevent oocyte fertilization. Therefore, morphological assessment of semen, which covers both the number and type of morphological changes, is highly useful when selecting appropriate males for reproduction.

**Key words:** arctic fox, semen, morphology, spermatozoa

Reproductive efficiency of male mammals is determined by the quality of their semen. Semen quality is most often evaluated by determining the physical properties of ejaculate, namely ejaculate volume, sperm concentration in the ejaculate, and sperm motility. Analysis of sperm morphology is central to the assessment (Oet-

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tlé, 1993). Sperm morphology test shows the percentage of spermatozoa with morphological abnormalities, and indirectly enables inferences to be made on testicular function and the normal course of spermatogenesis and spermiogenesis (Brodzki et al., 2015). Only morphologically normal spermatozoa are able to bind to the zona pellucida, to trigger the acrosomal reaction, and to adhere to the oolemma (Marnet et al., 2000). The proportion and type of sperm morphological abnormalities reflect the extent of abnormalities during the course of spermatogenesis (Waberski et al., 2006; Wolf, 2009).

A high proportion of morphologically altered spermatozoa may lead to a temporary or permanent deterioration of the biological function of semen and its fertilizing capacity. It is therefore important to minimize the proportion of morphologically altered spermatozoa in the ejaculates accepted for insemination. According to the classification of Blom (1981), any sperm structural abnormalities may be recognized as main (primary) or minor (secondary) defects. Abnormal forms of spermatozoa may develop during both spermatogenesis and epididymal maturation, and may have a genetic background or be influenced by environmental factors (Brodzki et al., 2015). The frequency of sperm morphological changes may be due to the impact of seasonal factors (Purwantara et al., 2010), genetic factors (Chenoweth, 2005; Wysokińska and Kondracki, 2013) and individual predispositions (Boersma et al., 1999). The incidence of morphologically altered spermatozoa depends also on nutritional factors (Bonet, 1990) and housing conditions (Kunavongkit et al., 2005; Kowalewski et al., 2016). Large differences were also revealed in the frequency of sperm morphological changes depending on the age of sire (Jankeviciute and Zilinskas, 2002).

The sperm head (which contains the sperm's genetic material) and the adjacent acrosome have been ascribed the principal role in the fertilizing process. Even the slightest damage to cytoplasmic membrane covering the sperm head contributes to the appearance of different enzymatic proteins, located in the sperm acrosome, in the seminal plasma (Bronicka and Dembiński, 1999). One of these enzymes is acrosin, which is involved in fertilization by catalyzing the reaction of hydrolytic breakdown of the zona pellucida of the oocyte. This leakage of enzymes leads to changes in chemical composition and in metabolic activity of spermatozoa (Marí et al., 2003).

In order to fully exploit the reproductive potential of males, insemination is increasingly used because foxes are monoestrous animals (they only have one breeding season), and poor quality semen may exclude females from breeding in a given year. Hence the need to study the factors determining the quality of their semen.

The aim of the study was to determine the relationship of arctic fox (*Alopex lagopus* L.) sperm morphology with age of males, ejaculate concentration and volume, as well as with acrosin activity determined in sperm acrosome extracts.

## Material and methods

Semen from 9 male arctic foxes (*Alopex lagopus* L.) was investigated. The experimental foxes were raised in a fur farm where artificial insemination of foxes is

practised. The semen was obtained from 6 males aged one year (young males) and 3 males aged 3 to 5 years (older males). The samples were manually collected from the arctic foxes during the period of increased sexual activity (from mid-February to mid-April). Semen was collected five times at regular intervals (every 2 weeks). All of the ejaculates ( $n=39$ ) used in the study were classified as normal based on motility exceeding 70%. The experiment was conducted with the approval of the Local Ethics Committee in Bydgoszcz.

The ejaculates collected from the foxes were evaluated for volume (measured using a calibrated tube) and sperm concentration (measured in a Bürker chamber). Acrosin activity was measured using a clinical assay developed for human spermatozoa (Kennedy et al., 1989) after modification for fox spermatozoa by Stasiak et al. (2012). Microscopic preparations made from ejaculate samples were stained using eosin and gentian violet (Kondracki et al., 2006). A thin semen smear was made on defatted glass slide warmed to around 36°C. After drying, the smear was fixed in 96% ethanol solution for around 5 minutes. The fixed smear was washed in distilled water and then stained with 10% aqueous bluish eosin solution over 20–60 seconds. The stained preparations were again washed in distilled water and stained for 3 to 5 minutes in gentian violet consisting of 2 g methyl blue, 0.75 g gentian violet, 5 ml glycerol, and 100 ml distilled water. Next, the preparations were rinsed in distilled water and dried in open air. The morphology of 500 spermatozoa was evaluated in each preparation, specifying morphologically normal spermatozoa and sperm with primary and secondary changes according to Blom's classification (1981). The spermograms of the male arctic foxes were classified according to a six-grade and subjective scale developed by Kondracki et al. (2011 a).

The collected material was subgrouped according to four criteria: age of males, sperm concentration, ejaculate volume, and acrosin activity. Based on the criterion of sperm concentration, the ejaculates were divided into five groups: group I – ejaculates with <100 million sperm per  $\text{cm}^3$ ; group II – ejaculates with 101 to 150 million sperm per  $\text{cm}^3$ ; group III – ejaculates with 151 to 200 million sperm per  $\text{cm}^3$ ; group IV – ejaculates with 201 to 300 million sperm per  $\text{cm}^3$ ; and group V – ejaculates with >300 million sperm per  $\text{cm}^3$ . Based on the criterion of ejaculate volume, the following four groups were identified: group I – ejaculates with a volume of <0.2  $\text{cm}^3$ ; group II – ejaculates with a volume of 0.2 to 0.4  $\text{cm}^3$ ; group III – ejaculates with a volume of 0.4 to 0.6  $\text{cm}^3$ ; and group IV – ejaculates with a volume of >0.6  $\text{cm}^3$ . Four groups were identified using the criterion of acrosin activity: group I – ejaculates with acrosin activity <1.5 mU/million sperm; group II – ejaculates with acrosin activity of 1.5 to 2.5 mU/million sperm; group III – ejaculates with acrosin activity of 2.5 to 3.5 mU/million sperm; and group IV – ejaculates with acrosin activity >3.5 mU/million sperm.

The results were collated and statistically analysed using STATISTICA 13.1 software (StatSoft, USA). The data did not meet the assumptions of normality and homogeneity of variance required for parametric statistics. Shapiro-Wilk test and analysis of normal probability graphs showed a non-normal distribution for most variables. The results for percentage of morphologically normal spermatozoa, and percentage of spermatozoa with primary and secondary defects are presented as me-

dian  $\pm$  quartiles values. All results are shown in graphs. Mann-Whitney U test was used to determine significant differences between the two groups (young and older males) and Kruskal-Wallis test was used for more than two groups. The interrelationships between the analysed parameters were evaluated based on Spearman's rank correlation coefficients with Bonferroni correction. To perform a Bonferroni correction, the critical P value ( $P=0.05$ ) was divided by number of comparisons being made (12). Differences between the groups were considered significant when  $P<0.004$ .

## Results

Table 1 presents the results of spermograms classified according to a six-grade scale developed by Kondracki et al. (2011 a). The data show that around 79% of male arctic fox spermograms were classified into the group of ejaculates highly suitable for insemination, of which 23 ejaculates (59%) were grade 5 (very good) and 8 ejaculates (20%) were grade 4 (no objections). The other ejaculates were classified as grade 3 (medium quality) – 5 ejaculates (12.8%), grade 2 (dubious quality) – 1 ejaculate (2.6%), and grade 1 (low quality) – 2 ejaculates (5.6%). No spermograms were graded as 0 (unacceptable). Therefore, data in Table 1 suggest that the majority of experimental male arctic foxes produced semen of very good or good quality, and only 3 out 39 classified ejaculates were considered unsuitable for insemination (spermogram grades 1 and 2).

Table 1. Classification of male arctic fox spermograms based on a six-grade scale (Kondracki et al., 2011 a)

Semen grade	Percentage of normal spermatozoa	Percentage of spermatozoa with primary changes	No. of accepted ejaculates
0 Unacceptable	$\leq 65$	$20 >$	0
1 Low quality	65–73	15–20	2
2 Doubtful quality	73–85	10–15	1
3 Medium quality	73–85	$< 10$	5
4 No objections	$85 \geq$	3–5	8
5 Very good	$90 \geq$	$\leq 3$	23

Figure 1 shows the frequency of sperm morphological changes in ejaculates of young and older male arctic foxes. No significant differences were observed in the frequency of spermatozoa with primary and secondary morphological changes in the semen of young and older male arctic foxes. It is notable, however, that the total frequency of morphological changes (primary changes + secondary changes) was considerably higher in young males (7.9%) than in older males (5.8%).

Figure 2 presents the frequency of primary and secondary sperm changes depending on the concentration of spermatozoa in fox ejaculates. These data support the conclusions derived from Table 2 that the increase in sperm concentration in the ejaculate was paralleled by an increasing percentage of morphologically altered

spermatozoa. Most sperm morphological changes occurred in the group of ejaculates with highest sperm concentration (more than 200 million/cm<sup>3</sup>) and this concerned both primary and secondary morphological changes.

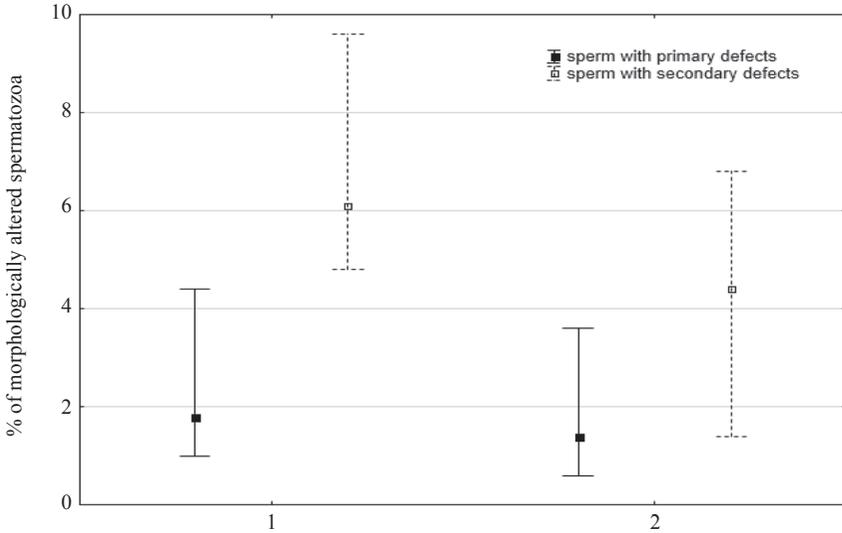


Figure 1. Frequency (median ± quartiles) of morphologically altered spermatozoa in the ejaculates of male arctic foxes in relation to the age of the male; 1 – ejaculates of young males (n=28), 2 – ejaculates of older males (n=11)

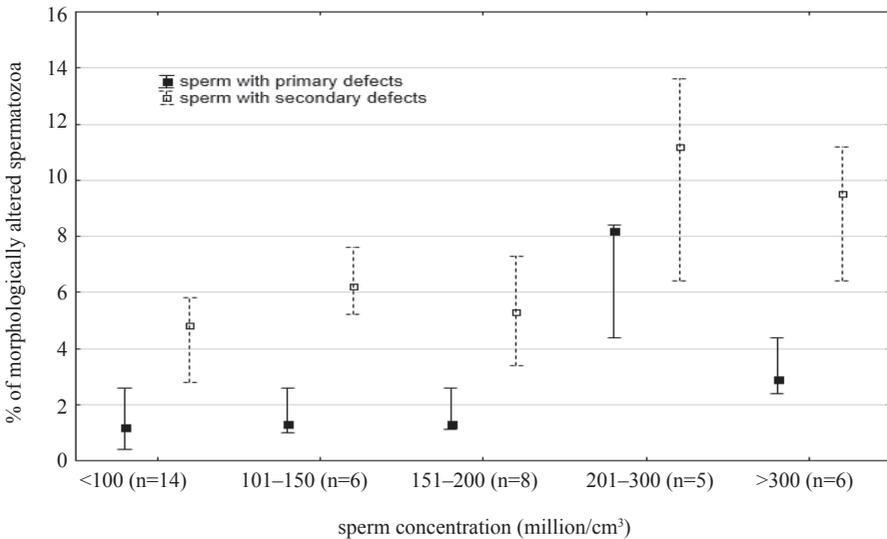


Figure 2. Frequency (median ± quartiles) of morphologically altered spermatozoa in the ejaculates of male arctic foxes in relation to sperm concentration in the ejaculate

Table 2. Frequency of sperm morphological changes as related to acrosin activity and physical properties of the ejaculate of male arctic foxes (Spearman's coefficients of correlation)

	Percentage of normal spermatozoa	Percentage of spermatozoa with primary changes	Percentage of spermatozoa with secondary changes	Ejaculate volume	Sperm concentration	Acrosin activity
Ejaculate volume	0.35	-0.16	-0.35	-	-0.01	-0.01
Sperm concentration	-0.46*	0.42	0.44	-0.01	-	-0.74*
Acrosin activity	0.42	-0.30	-0.44	-0.01	-0.74*	-

\*Significant at  $P < 0.004$ .

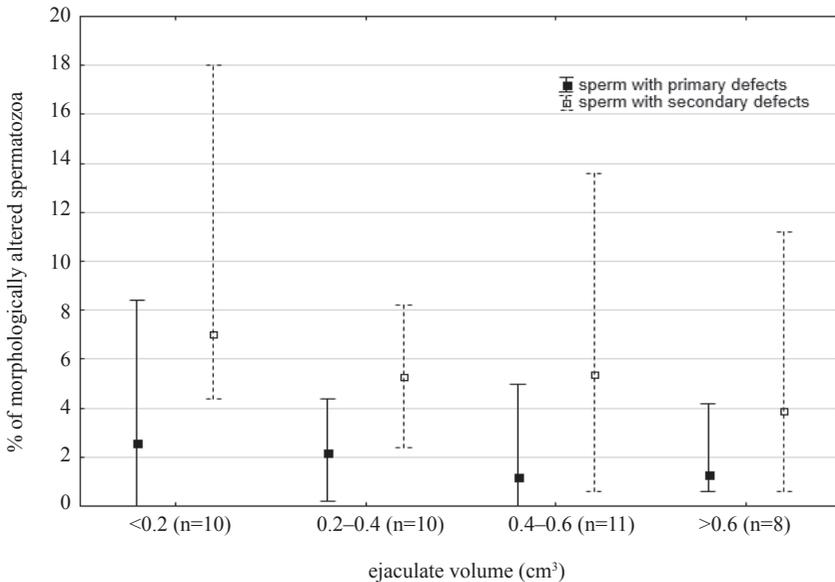


Figure 3. Frequency (median  $\pm$  quartiles) of morphologically altered spermatozoa in the ejaculates of male arctic foxes in relation to ejaculate volume

The effect of ejaculate volume on the frequency of sperm morphological changes is presented in Figure 3. The data suggest that the frequency of sperm morphological changes (secondary changes in particular) declines as ejaculate volume increases. However, the statistical analysis carried out did not confirm this impact.

One of the major enzymes involved in the fertilization process is acrosin. The effect of acrosin activity on the frequency of sperm morphological changes is presented in Figure 4. The ejaculates with the lowest acrosin activity were characterized by the highest frequency of sperm morphological changes. As acrosin activity increased, the frequency of morphological changes in sperm decreased. However, these differences were not significant.

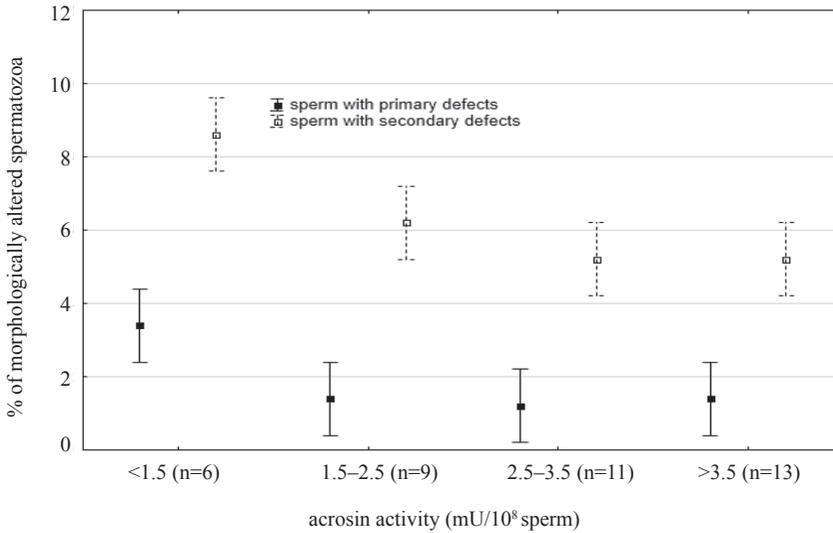


Figure 4. Frequency (median  $\pm$  quartiles) of morphologically altered spermatozoa in the ejaculates of male arctic foxes in relation to acrosin activity

## Discussion

As is apparent from data in Figure 1, the percentage of morphologically altered spermatozoa did not exceed 7.9%, and in the group of older males (aged 3 to 5 years) it was even lower (5.8%). In both age groups, the average percentage of morphologically normal spermatozoa exceeded 90%. Thus, semen quality of the experimental male arctic foxes is considered very high, much above the quality requirements for canine semen. Jalkanen (1993) showed in male silver foxes (*Vulpes vulpes*) accepted for insemination that the percentage of morphologically normal spermatozoa in their ejaculates was 87.55%. Dog ejaculates eligible for insemination should contain at least 80% of structurally sound sperm (Martínez, 2004). According to Oettlé (1993), the lowest percentage of morphologically normal spermatozoa (<60%) adversely affects sire fertility. In our study, the percentage of morphologically abnormal spermatozoa in the ejaculates of male arctic foxes was low and never exceeded 35%, which made these ejaculates acceptable for insemination. Neither did the proportion of morphologically altered spermatozoa in the arctic fox ejaculates exceed the values reported for the ejaculates of other male canids: red wolf (*Canis rufus*) – 35%, silver fox (*Vulpes vulpes*) – 12.5%, and the domestic dog (*Canis lupus familiaris*) – 11.6–18.3% (Koehler et al., 1998; Silva et al., 2003). Morphological diversity of spermatozoa in the ejaculates obtained from animals of one family, may be indicative of their species specificity. In addition to genetically based causes, sperm morphology in male mammals is influenced by a number of environmental factors. The most common include ambient temperature and photoperiodism (Wysokińska et al.,

2009; Knecht et al., 2014; Kowalewski et al., 2016). Excessive temperature lowers thermoregulatory capacity of the testes, which leads to mitotic disturbances and causes the atrophy of primary spermatocytes (Bierła and Giżejowski, 2007). Disturbances in spermatogenesis and spermiogenesis may also occur as a result of lower concentrations of the androgens testosterone and androsterone (Brodzki et al., 2015).

The males of most animal species utilized by humans are used from a very young age, soon after reaching sexual maturity. Ejaculatory efficiency of young sires is generally lower than that of adult males which completed their sexual development (Banaszewska and Kondracki, 2012). Spermatogenesis persists throughout the male's life, from attainment of sexual maturity to loss of fertility due to ageing. During this period, gonadal function changes as the male becomes older (Kidd et al., 2001; Kühnert and Nieschlag, 2004). The data from our study show that the ejaculates of adult males contain fewer morphologically altered spermatozoa compared to the ejaculates of younger (one-year-old) male arctic foxes. These data, although the differences are not significant, agree with an earlier study which showed that age has an effect on the quality of male fox semen (Stasiak and Janicki, 2014). Compared to younger animals which are in their first season, older animals produce ejaculates with higher volume but lower sperm concentration. This results from the fact that sexual development in male mammals does not end at sexual maturity or with the beginning of reproduction but continues, and they reach maximum ejaculatory efficiency at an age immediately preceding the attainment of somatic maturity (Smital, 2009; Banaszewska and Kondracki, 2012). In our study, no significant differences were established despite the noticeable difference in the evaluation of ejaculates from both age groups. This is not conclusively supported in the literature concerning members of the family *Canidae*. According to Oetllé (1993), the age of male dogs does not adversely affect their fertility. Only a proper proportion of normal spermatozoa (more than 60%) ensures successful insemination. In turn, Rijsselaere et al. (2007) reported that the percentage of morphologically normal spermatozoa decreases with the age of male dogs.

Our study demonstrated that only sperm concentration contributes significantly to the incidence of morphologically altered spermatozoa. In the ejaculates with higher sperm concentration, more spermatozoa exhibited morphological changes. According to Rijsselaere et al. (2007), this is caused by the epididymides, in which spermatozoa become mature and acquire fertilizing capacity. When sperm concentration is too high, their preliminary selection in the epididymides is limited (misshapen spermatozoa are phagocytosed by macrophages). The relationship between sperm morphological traits and sperm concentration in the ejaculate has been reported for dogs (Rijsselaere et al., 2004), bulls (Kondracki et al., 2012), stallions (Davis et al., 1993) and boars (Banaszewska et al., 2009; Kondracki et al., 2011 b).

As the data in Figure 2 indicate, the frequency of sperm morphological changes increases with increasing sperm concentration in the ejaculate. Most morphologically altered spermatozoa were observed in group IV, where sperm concentration in the ejaculate was 201–300 million/cm<sup>3</sup>. These ejaculates showed the greatest percentage of spermatozoa with primary abnormalities (8.2%), while in the other groups this figure did not exceed 1.3%, and the proportion of spermatozoa with secondary changes in this group of ejaculates was 11.2%.

A higher frequency of sperm morphological changes in canine ejaculates with a high sperm concentration was reported by Rijsselaere et al. (2004). They showed that ejaculates of male domestic dogs with lower sperm concentration contain fewer spermatozoa with morphological changes, but the proportion of morphological abnormalities increases with increasing sperm concentration. Our data also show that the frequency of sperm morphological changes increases with increasing sperm concentration in the ejaculate, which is broadly in line with the observations of Rijsselaere et al. (2004). Higher sperm concentration in the ejaculate normally also indicates their higher concentration in the excurrent ducts, where they are stored and undergo changes that determine their fertilizing capacity. The high density of spermatozoa in the excurrent ducts may degrade sperm development conditions and increase the number of spermatozoa with morphological defects. Our results and those of other authors (Rijsselaere et al., 2004; Kondracki et al., 2013) appear to support this thesis.

Not without significance is also the type of sperm morphology changes found in male semen. In our study, most spermatozoa with secondary changes (spermatozoa with distal protoplasmic droplets, spermatozoa with a single loop of the tail, spermatozoa with damaged acrosome) were observed in the ejaculates with the highest sperm concentration (over 200 million/cm<sup>3</sup>). Jalkanen (1993) demonstrated that tail defects account for more than half of all sperm morphological changes in the ejaculates of silver foxes. According to Kondracki et al. (2011 b), sperm morphological traits depend on sperm count in the ejaculate, which has an effect on the size and shape of sperm head. In the ejaculates with lower sperm concentration, sperm heads are larger, longer and wider.

According to Kondracki et al. (2006) and Smital et al. (2009), the increasing ejaculate volume of male mammals is accompanied by the decreasing sperm concentration in the ejaculate. Recognizing this fact, it could not be expected that ejaculate volume and sperm concentration in the ejaculate will be equally relevant for the frequency of sperm morphological changes. Indeed, the data presented in Figure 3 show that the frequency of sperm morphological changes decreased with increasing ejaculate volume, whereas the increasing sperm concentration in the ejaculate has the opposite effect (Figure 2). Our data suggest that the higher the ejaculate volume, the fewer the morphologically altered spermatozoa, and although the differences between the groups were not significant, this trend is clear. The fact that sperm morphological changes are associated with ejaculate volume was also reported for other animal species (Górski et al., 2016).

Figure 4 shows that the frequency of sperm morphological changes decreases as the activity acrosin increases. The total percentage of sperm morphological changes (primary and secondary), which in the group of ejaculates with low acrosin activity (below 1.5 mU/million spermatozoa) was 12.0%, decreased almost two-fold in the group of ejaculates with the highest acrosin activity (above 3.5 mU/million spermatozoa) to reach only 6.6%. This may indicate, on the one hand, that the type of defect has changed, and, on the other, that the acrosome structural damage is greater. According to Niżański (2004), who investigated dog semen, the more the spermatozoa with normal acrosome, the fewer the spermatozoa with primary ( $r = -0.58$ ,  $P \leq 0.001$ ) and secondary defects ( $r = -0.30$ ,  $P \leq 0.05$ ).

In summary it is concluded that sperm morphology in male arctic foxes is only related to their concentration in the ejaculate. This is reflected in the coefficients of correlation ( $P < 0.004$ ). The frequency of sperm morphological changes in the ejaculate is directly proportional to sperm concentration in the ejaculate. Sperm morphology in male arctic foxes depends also on the age of the males. A greater number of morphologically altered spermatozoa tended to occur in the ejaculates of young foxes, which were in their first breeding season. A complete morphological assessment of sperm, including both the number and nature of the morphological changes in spermatozoa, may be useful for assessing breeding soundness in male arctic foxes.

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