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PRELIMINARY RESEARCH ON EVALUATION OF SPERM MORPHOMETRY AND CHROMATIN STRUCTURE IN THE SEMEN OF SILVER FOX (*VULPES VULPES*)

WSTĘPNE BADANIA MORFOMETRII I STRUKTURY CHROMATYNY PLEMNIKÓW LISA SREBRZYSTEGO (*VULPES VULPES*)

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Streszczenie. Ocena nasienia jest niezbędna w przewidywaniu płodności samców. Dokładność oceny morfologii plemników zależy od staranności przygotowania preparatów, utrwalania i barwienia plemników, ponieważ ma to wpływ na morfometrię główki i całego plemnika. Celem badań była szczegółowa analiza morfometryczna oraz ocena struktury chromatyny plemników lisa srebrzystego (*Vulpes vulpes*). Przedmiotem badań były plemniki jednorocznych hodowlanych lisów srebrzystych. Preparaty z ejakulatów poddano trzem technikom barwienia: aniline blue (AB), chromomycin (CMA3) i acridine orange (AO). Zastosowane techniki umożliwiły identyfikację plemników z nieprawidłową retencją histonów. W wyniku pomiarów wyznaczono średnie wartości parametrów morfometrycznych plemników lisa: długość i szerokość główki wynosiły odpowiednio 6,59 µm i 4,39 µm, obwód główki – 17,98 µm, pole główki – 21,69 µm^2, pole akrosomu – 11,2 µm^2, zasięg akrosomu – 51,69%, długość wstawki – 12,84 µm, zasięg wstawki – 19,72%, długość witki – 65,11 µm, długość plemnika – 71,70 µm, indeks eliptyczności główki – 1,51, wydłużenie główki – 0,2, pofałdowanie główki – 0,84, regularność główki – 1,05. Brak ustalonych standardów użycia różnych technik barwienia jest stale aktualnym tematem, w odniesieniu do literatury dotyczącej różnicowego barwienia plemników na potrzeby oceny morfologicznej.

Key words: fox, sperm, protamination, morphometry, Tygerberg criteria.
Słowa kluczowe: lis, plemnik, protaminacja, morfometria, kryterium Tygerberg.

INTRODUCTION

Semen analysis is a way to predict male fertility, being of great importance in terms of maximization of male reproductive performance, both in natural and assisted reproduction. The most important parameters in semen evaluation include sperm concentration, motility,

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and morphology. Sperm morphology is regarded as the most reliable image of male fertility, especially if accompanied by a detailed morphometric analysis (Maree et al. 2010, Lasiene et al. 2013). Many authors bind the concept of sperm morphology almost inextricably with sperm dimensions, which vary within the standard range for a given species, and seek correlations between sperm morphometry and fertility (Esteso et al. 2006; Núñez-Martínez et al. 2007; Banaszewska et al. 2015b, c). Traditionally, sperm morphology analysis is the first step in the evaluation of male fertility. Unfortunately, some abnormalities remain undetected at the level of morphological and morphometric analysis. These include pathological changes in the chromatin structure, which most often result from a malfunctioning replacement of histones by protamines (Bianchi et al. 1996; Iranpour et al. 2000). The spermatozoa afflicted by such defects may have normal morphology still being dysfunctional in terms of genetic material quality (Nagvenkar et al. 2005; Kazerooni et al. 2009; Banaszewska et al. 2015a).

In the laboratory practice, various staining techniques are applied to help in the analysis of morphology and morphometry of the sperm. The recommended method depends on the species. SpermBlue® (Van der Horst and Maree 2009) is used in both human and animal semen analysis, although the Papanicolaou method is recommended for human sperm (WHO 2010). According to the Society for Theriogenology (SFT) guidelines, stallion sperm morphology analysis should be performed on fresh, unstained smears using a microscope equipped with phase contrast (Kenney et al. 1983). Most andrology or veterinary labs, however, lack such equipment and stain equine semen using an array of techniques, often those recommended for other species. The eosin-nigrosin stain is recommended by SFT for bovine semen analysis (Chenoweth et al. 1992). Other authors, on the other hand, propose eosin-gentian violet staining (Blom 1981; Kondracki et al. 2012).

The quality of sperm morphology evaluation depends on the diligence put in the preparation of smears, their fixation and cell staining, since these may affect the morphometry of the head and the entire sperm alike (Menkveld 2007; Łukaszewicz et al. 2008). Here is where an important question arises: which staining technique should be chosen for the particular sample; namely, the impact on the cells being stained should be minimized (Maree et al. 2010) without sacrificing the visibility of any important details in the sperm structure. Sperm morphology analysis is a subjective, difficult to standardize process, which is due to the fact that each technique affects the stained cells differently, since different reagents are used.

As subjects of genetics research, farm foxes appear quite sporadically in the literature. The reports involve studies in cytogenetics, molecular genetics, or genomics (Szczerbal et al. 2003, 2006; Jakubczak et al. 2009, 2011; Jeżewska-Witkowska et al. 2012). Canine spermatozoa – and the fox sperm in particular – are not very often described in articles dealing with reproduction. Increasingly more common artificial insemination of animals prompts the necessity to improve the accuracy of sperm morphology and morphometry analysis and to determine dyeing techniques that would be most appropriate for canine semen.

The lack of clear recommendations in relation to sperm staining, as well as the lack of morphometric reference values for canine sperm, inspired us to undertake this study, which was aimed at a detailed morphometric analysis and chromatin structure evaluation in the sperm cells of the silver fox (*Vulpes vulpes*).

MATERIAL AND METHODS

The material involved the sperm of year-old captive silver foxes. The semen was collected manually during the on-farm artificial insemination procedures. Immediately after the collection, semen was subjected to a macroscopic and microscopic assessment. Samples containing at least 75% of progressively motile sperm were diluted with M III extender to a concentration of $15 \cdot 10^6$ sperm cells / 1 ml of semen. For our analyses, the semen collected from 4 males was additionally diluted to final concentration of $20 \cdot 10^3$ sperm using the same M III extender. Smears were made on glass slides and left to dry up at room temperature. The slides were stained using one of the following dyes: aniline blue (AB), chromomycin (CMA3), and acridine orange (AO). The slides were viewed under the fluorescent microscope Olympus BX50 with the lens UPlanApo 100x/1.35/Oil Iris/∞/0.17. A total of 500 sperm cells stained with each method were evaluated from each male.

AB staining was done according to Franken et al. (1999). Smears were pre-incubated in 3% buffered glutaraldehyde at room temperature and then stained with 5% AB solution in 4% acetic acid (pH 3.5) for 5 minutes. We focused on the presence of spermatozoa with normal histone content (bright blue color) and an excessive content of histones (intense blue). CMA3 staining was performed according to the procedure described by Lolis et al. (1996). The slides were pre-incubated in Carnoy's fixative at 40°C for 5 minutes, and then immersed in 100 µl of CMA3 solution (0.25 mg/ml in pH 7.0 McIlvaine buffer, containing 10 mM $MgCl_2$) for 20 minutes. The slides were washed in the buffer and left in buffered glycerol. The analysis covered the percentage of sperm with normal (dull green fluorescence) and abnormal chromatin packaging (bright green fluorescence). AO staining was carried out following the procedure described by Tejda et al. (1984). The staining solution was prepared by mixing 10 ml of stock solution (1 mg AO in 1000 ml of distilled water) with 40 ml of 0.1 M citric acid and 2.5 ml of 0.3 M $Na_2 HPO_4$. The slides were stained with 2–3 ml of the solution for 10 minutes and washed in distilled water. Wet smears were cover-slipped and sealed with rubber cement. Microscopic analysis of the slides was focused on identification of sperm with the normal DNA structure (green fluorescence) and defective, single-stranded DNA (orange fluorescence).

AB-stained sperm cells were subjected to detailed measurements. For each of the males, 30 sperm with normal morphology were evaluated. Using the MultiScan measuring package (Computer Scanning Systems), the following measurements were taken: head length, width and perimeter, acrosome area and coverage, mid-piece length and coverage, tail length, tail end-piece length, and sperm length. Applying the Tygerberg strict criteria (Maree et al. 2010), we determined the parameters that most accurately describe the head of the sperm (Table 1).

Table 1. Formulas used in the calculation of the sperm head morphometry measurements
Tabela 1. Formuły użyte do obliczenia pomiarów morfometrycznych główki plemnika

Variable – Parametr	Formula – Formuła
Head length – Długość główki [µm]	L
Head width – Szerokość główki [µm]	W
Head perimeter – Obwód główki [µm]	P
Head area – Pole główki [μm^2]	A
Ellipticity – Eliptyczność	L/W
Elongation – Wydłużenie	(L – W) / (L + W)
Roughness – Pofałdowanie	$4\pi(A / P^2)$
Regularity – Regularność	$\pi(L \cdot W / 4 \cdot A)$

The parameters we obtained were next characterized using descriptive statistics: mean, standard deviation, and coefficient of variability. We used the statistics package STATISTICA version 10.0 PL (StatSoft Inc.) for processing the data.

RESULTS

Acridine orange allows distinguishing between the sperm with the normal, double-stranded DNA structure and those with a damaged, single-stranded DNA. The sperm viewed on all the analyzed slides demonstrated green fluorescence of the head, which implies that no sperm with a damaged genetic material structure were present in the studied specimens. Aniline blue enables identification of sperm with normal and abnormal histone persistence. The staining revealed prevailing sperm with normal histone retention; the mean content of sperm with abnormal, elevated histone concentration was found at a level of 2%. Chromomycin A3 staining was aimed to test the regularity of the protamination process. The percentage of sperm showing improper protamination in our specimens was very low, 0.3%. Table 2 presents the detailed results of sperm staining with acridine orange, aniline blue and chromomycin.

Table 2. Analysis of smears stained with acridine orange, aniline blue and chromomycin A3
Tabela 2. Analiza preparatów barwionych oranżem akrydyny, aniliną blue i chromomycyną A3

Fox Lis	AB		CMA3		AO	
	normal spermatozoa plemniki prawidłowe	sperm with abnormalities plemniki nieprawidłowe	normal spermatozoa plemniki prawidłowe	sperm with abnormalities plemniki nieprawidłowe	normal spermatozoa plemniki prawidłowe	sperm with abnormalities plemniki nieprawidłowe
1	487	13	497	3	500	0
2	492	8	499	1	500	0
3	485	15	498	2	500	0
4	496	4	500	0	500	0
Total Łącznie	1960	40	1994	6	2000	0

In the case of fox sperm staining, aniline blue reveals each element of the sperm structure very clearly (Fig. 1).

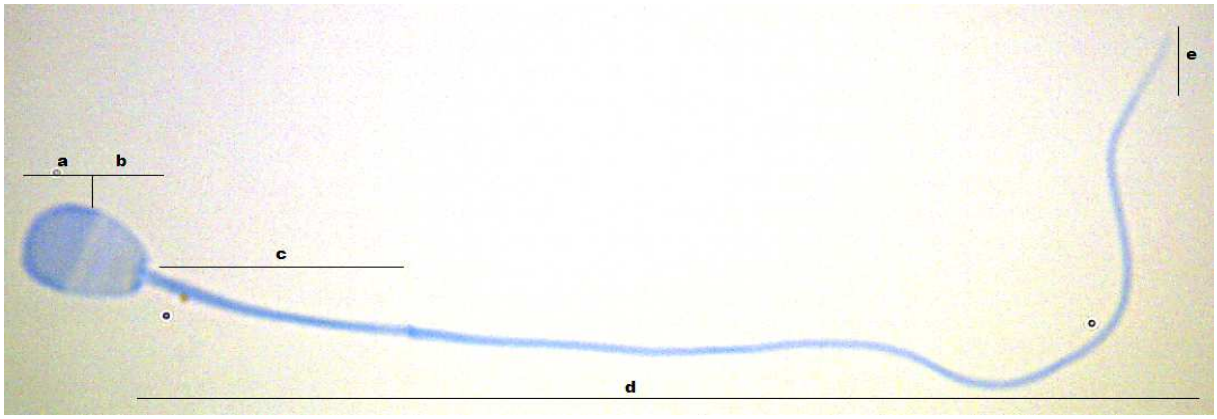

Fig. 1. Fox sperm – aniline blue staining (a – acrosome, b – distal postacrosomal region, c – mid-piece, d – tail, e – end of tail)
Ryc. 1. Plemnik lisa – barwienie aniliną blue (a – akrosom, b – region zaakrosomowy, c – wstawka, d – witka, e – końcówka witki)

The measurements resulted in the following mean values of the fox sperm morphometric parameters: head length and width: 6.59 µm and 4.39 µm, respectively, head perimeter: 17.98 µm, head area: 21.69 µm^2, acrosome area: 11.2 µm^2, acrosome coverage: 51.69%, mid-piece length: 12.84 µm, mid-piece coverage: 19.72%, tail length: 65.11 µm, sperm length: 71.70 µm, head ellipticity index: 1.51, head elongation: 0.2, head roughness: 0.84, and head regularity: 1.05. The detailed overview of the measurements for each male fox are presented in Table 3.

Table 3. Sperm morphometric traits in the studied male foxes
Tabela 3. Cechy morfometryczne plemników badanych lisów

Parameter Parametr		Fox – Lis 1	 2	 3	 4	Total Łącznie
Head length	$\bar{x}$ ± SD	6.61 ± 0.26	6.56 ± 0.21	6.61 ± 0.21	6.58 ± 0.26	6.59 ± 0.23
Długość główki [µm]	V%	3.98	3.21	3.11	3.98	3.57
Head width	$\bar{x}$ ± SD	4.35 ± 0.27	4.38 ± 0.20	4.53 ± 0.29	4.31 ± 0.22	4.39 ± 0.26
Szerokość główki [µm]	V%	6.30	4.59	6.34	5.00	5.88
Head perimeter	$\bar{x}$ ± SD	17.88 ± 0.68	17.98 ± 0.60	18.22 ± 0.74	17.84 ± 0.70	17.98 ± 0.69
Obwód główki [µm]	V%	3.78	3.35	4.05	3.93	3.83
Head area	$\bar{x}$ ± SD	21.23 ± 1.17	21.75 ± 1.12	22.23 ± 1.03	21.54 ± 1.49	21.69 ± 1.25
Pole główki [µm^2]	V%	5.51	5.17	4.61	6.91	5.78
Acrosome area	$\bar{x}$ ± SD	11.17 ± 0.72	10.94 ± 0.61	11.25 ± 0.77	11.45 ± 1.06	11.20 ± 0.82
Pole akrosomu [µm^2]	V%	6.40	5.61	6.88	9.24	7.30
Acrosome coverage	$\bar{x}$ ± SD	52.67 ± 2.69	50.34 ± 2.34	50.60 ± 2.77	53.16 ± 3.31	51.69 ± 3.03
Zasięg akrosomu [%]	V%	5.10	4.66	5.47	6.22	5.86
Ellipticity	$\bar{x}$ ± SD	1.53 ± 0.02	1.50 ± 0.10	1.46 ± 1.10	1.53 ± 0.09	1.51 ± 0.11
Eliptyczność	V%	7.66	6.39	7.12	6.14	6.98
Elongation	$\bar{x}$ ± SD	0.21 ± 0.04	0.20 ± 0.03	0.19 ± 0.04	0.21 ± 0.03	0.20 ± 0.03
Wydłużenie	V%	17.79	15.02	18.75	13.84	16.71
Roughness	$\bar{x}$ ± SD	0.84 ± 0.04	0.85 ± 0.05	0.84 ± 0.05	0.85 ± 0.03	0.84 ± 0.04
Pofałdowanie	V%	5.31	6.23	5.53	3.57	5.22
Regularity	$\bar{x}$ ± SD	1.06 ± 0.05	1.04 ± 0.04	1.06 ± 0.05	1.03 ± 0.04	1.05 ± 0.04
Regularność	V%	4.34	3.96	4.50	3.60	4.25
Mid-piece length	$\bar{x}$ ±SD	12.65 ± 0.35	13.03 ± 0.31	13.11 ± 0.27	12.56 ± 0.25	12.84 ± 0.38
Długość wstawki [µm]	V%	2.78	2.36	2.08	2.00	2.95
Mid-piece coverage	$\bar{x}$ ± SD	19.65 ± 0.58	19.77 ± 0.48	19.96 ± 0.53	19.50 ± 0.43	19.72 ± 0.53
Zasięg wstawki [%]	V%	2.94	2.45	2.64	2.21	2.68
Tail length	$\bar{x}$ ± SD	64.42 ± 0.94	65.88 ± 0.86	65.72 ± 1.01	64.40 ± 1.02	65.11 ± 1.18
Długość witki [µm]	V%	1.46	1.31	1.53	1.59	1.81
Tail end length	$\bar{x}$ ± SD	3.20 ± 0.29	3.16 ± 0.26	3.35 ± 0.25	3.56 ± 0.32	3.32 ± 0.32
Długość końcówki witki [µm]	V%	8.75	8.16	7.57	9.03	9.64
Sperm length	$\bar{x}$ ± SD	71.03 ± 0.87	72.44 ± 0.84	72.33 ± 1.11	70.98 ± 1.00	71.70 ± 1.18
Długość plemnika [µm]	V%	1.23	1.06	1.53	1.41	1.64

DISCUSSION

The importance of basic proteins that stabilize the structure of sperm DNA is increasingly more often emphasized in the reports from studies on human and animal reproduction. Some infertility cases are directly caused by irregularities in spermiogenesis related to histone replacement. Evaluation of the chromatin arrangement in the sperm is an indication of normality of both the spermatogenesis and the paternal genome and epigenome (Martianov et al. 2005; Enciso et al. 2011).

The histone-protamine replacement is a critical moment in the life of the reproductive cell. Nucleosomes in the sperm are more tightly packaged than in somatic cells. Protamins protect the sperm DNA against damage by nucleases and polymerases. Due to the tight

packaging of chromatin by protamins, any changes to protamins, or lack thereof, lead to aberrations in the sperm nucleus, which affects the morphological quality of semen and its fertilizing potential. Low quality semen often reveals sperm with too loosely packaged chromatin or damaged DNA (Saxena et al. 2008; Banaszewska et al. 2015a).

In the early round spermatids, the number of histones replaced by their transition variants exceeds 50%. Still, the classical nucleosome structure is preserved and transcriptional activity is underway. It stops only after dissociation of the H1t histone and most histones, as well as with the emergence of transition proteins, TP (Hammoud et al. 2009). After this chromatin rearrangement, only about 10–15% of the histones remain in the mature sperm (Kimmins and Saccone-Corsi 2005). The presence of histones means that the structure of the nucleosome will be maintained. More recent studies by Hammoud et al. (2009) revealed that this pertains to 4% of the haploid genome (Hammoud et al. 2009).

It has been demonstrated that early miscarriages and embryonic mortality are correlated with an increased level of histones in the sperm (Kazerooni et al. 2009). An elevated level of histones was found in infertile individuals or those suffering from oligospermia, teratozoospermia, or asthenozoospermia (Zhang et al. 2006; Zini et al. 2008).

Staining with acridine orange allows simple and quick testing sperm for DNA defects. Bound with a double-stranded DNA molecule, the dye gives green fluorescence, whereas with RNA or a single strand of DNA, the fluorescence is red. Evenson et al. (1980) observed that the presence of sperm with denatured, single-stranded DNA reduced fertilizing potential and the quality of *in-vitro* created embryos. Despite the fact that orange has been applied in sperm analysis of various species, the level of pathological DNA fragmentation was determined only in human semen. The rate of defective DNA sperm up to 15% is considered normal, 15–25% means reduced fertility, whereas above 25% is considered as posing a high risk of infertility (Evenson et al. 1999, 2000). It has been observed in bulls that fertility dropped when the percentage of defective sperm did not exceed 10% (Bochenek et al. 2001). No reference values have been determined for other species (Banaszewska et al. 2015a).

Abnormal chromatin condensation (Boitrelle et al. 2011; Franco et al. 2012) or DNA fragmentation (Oliviera et al. 2010; de Almeida Ferreira Braga et al. 2011) lead to emergence of nuclear vacuoles, which – if present in the sperm head – affect fertility and, in consequence, deteriorate the quality of embryos (Leandri et al. 2013; Banaszewska et al. 2015b).

The relation between sperm morphometry and fertility is the subject of research primarily related to human fertility. Scarce reports deal on this topic in horses (Hidalgo et al. 2008, Phetudomsinsuk et al. 2008), whose fertility disorders are positively correlated with enlarged sperm heads (Gravance et al. 1996). The problem was also addressed in relation to male fertility in swine (Peña et al. 2005; Saravia et al. 2007; Banaszewska et al. 2011), cattle (Boersma et al. 1999; Kondracki et al. 2012), or dogs (Núñez-Martínez et al. 2007).

Sperm morphometry is an important indicator of male reproduction performance. According to Katz et al. (1986), the sperm of infertile men were larger, as measured along the long and the short axis of the head; also the ratio of head length to head width in infertile men was higher. The results of the experiments on human sperm correspond to the data obtained in veterinary medicine (Klimowicz et al. 2005; Niżański and Klimowicz 2005). Ample differences in the sizes of sperm heads between fertile and infertile males were found in stallions (Gravance et al. 1996), boars (Banaszewska et al. 2011) and dogs (Núñez-Martínez

et al. 2007). The animals that produced sperm with smaller heads were characterized by higher fertility. The studies revealed also that it is not only the head size that is associated with fertility; the dimensions of the tail and the mid-piece are also important. The spermatozoa with a longer tail have a better chance to fertilize due to increased motor abilities (Banaszewska et al. 2015b). A problem in comparisons is the lack of reference material. The morphometric standard for the human sperm has been described (WHO 2010). In relation to sperm of livestock animals, there are numerous reports that can be treated as a comparative material. Unfortunately, when it comes to foxes, there are no reference values and the literature does not provide much information.

Functionally, the sperm consists of three regions: the head, the mid-piece, and the tail. Precise morphological and morphometric analysis of sperm enables development of the normal sperm standard for the semen of a given species. During spermiogenesis, due to the loss of most typical organelles and cytoplasm, the volume of the cell decreases and, in consequence, the sperm's aerodynamic properties increase, which potentially facilitates fertilization (Ramalho-Santos et al. 2007). Gage (1998) explains that the shape of the head is an important factor of its hydrodynamic properties and presumes that the sperm with more slender and oval heads are characterized by a higher motor efficiency. One may therefore seek the relationship between the shape of the head and sperm motility observing, whether the sperm with more oval heads have also longer mid-pieces, whose organelles undoubtedly affect the motility (Banaszewska et al. 2015b, c).

Tygerberg strict criteria specify four additional indexes characterizing the shape of the sperm head: ellipticity, elongation, roughness, and regularity. The ellipticity index differentiates the heads into thin and tapering ones. Its value equal 1 is characteristic for the circle; hence, the higher ellipticity index, the thinner the head of the sperm. Elongation determines the degree of the head's roundness. For the elongation index equal zero, the head would be round. The roughness specifies heads whose cellular membrane is uneven, which are rough or amorphous. The heads with a lower value of this index are more shapeless. Regularity, on the other hand, describes the normality of the shape and identifies the 'pear-shaped' heads (Maree et al. 2010).

Besides the precise identification of sperm's head and tail, optimization of histological staining techniques more often emphasizes the evaluation and identification of the acrosome and the mid-piece. There is also a trend to include these to structures to the standard semen diagnostics (Henkel et al. 2008; Maree et al. 2010). It is known that the functional capability of sperm depends on the proper functioning of the acrosome (Nikolettos et al. 1999). The acrosome coverage of the sperm head is a valuable parameter in fertility prediction (Menkveld et al. 2011). It allows determining the ability of the sperm to properly undergo the acrosome reaction and, in consequence, to penetrate through the zona pellucida during fertilization. In order to precisely measure the acrosome coverage, a proper staining technique must be applied that will clearly visualize the head (Maree et al. 2010). Another structure that is not particularly paid attention to in routine semen analysis is the mid-piece. The mid-piece contains the mitochondrial spiral (which represents up to 80% of the mid-piece) to supply the sperm with energy it needs to move (Sutovsky and Manandhar 2006). If mitochondrial defects in the semen are relatively common, its fertility may be reduced or even

total sterility may occur (Piasecka 2004a, b). Accordingly, besides acrosomal evaluation, detailed morphological analysis of the mid-piece in conjunction with a functional diagnosis of the mitochondria should constitute another extension of the routine semen diagnostics.

CONCLUSIONS

According to the literature on differential sperm staining for morphological evaluation purposes, the lack of established standards and the use of a variety of dyeing techniques appear as a current issue. The authors often point out that there is a need to develop and establish the staining methodology that would enable an accurate and precise analysis of animal sperm morphology and morphometry. Moreover, a standard smear preparation procedure should be developed for sperm morphology analysis. This would allow comparisons of result data between laboratories, which would again improve the value of sperm analysis in terms of fertility prediction and assessment. Tygerberg strict criteria may be used as a biomarker of the sperm function and be part in developing a uniform morphometric standard of silver fox sperm. Although the chromatin structure of the sperm is crucial for fertilization and embryo development, a standard semen evaluation is still limited solely to detection of morphological abnormalities in the sperm structure.

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Abstract. Semen analysis is a way to predict male fertility. The quality of sperm morphology evaluation depends on the diligence put in the preparation of smears, their fixation and cell staining, since these may affect the morphometry of the head and the entire sperm alike. The aim of the study was a detailed morphometric analysis and chromatin structure evaluation in the sperm cells of the silver fox (*Vulpes vulpes*). The material involved the sperm of year-old captive silver foxes. The slides were stained using one of the following dyes: aniline blue (AB), chromomycin (CMA3), and acridine orange (AO). The staining revealed prevailing sperm with normal histone retention. The measurements resulted in the following mean values of the fox sperm morphometric parameters: head length and width: 6.59 μm and 4.39 μm, respectively, head perimeter: 17.98 μm, head area: 21.69 μm^2, acrosome area: 11.2 μm^2, acrosome coverage: 51.69%, mid-piece length: 12.84 μm, mid-piece coverage: 19.72%, tail length: 65.11 μm, sperm

length: 71.70 μm, head ellipticity index: 1.51, head elongation: 0.2, head roughness: 0.84, and head regularity: 1.05. According to the literature on differential sperm staining for morphological evaluation purposes, the lack of established standards and the use of a variety of dyeing techniques appear as a current issue.