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**THE EFFECT OF AGE ON THE MORPHOMETRIC SPERM TRAITS
OF DOMESTIC PIGS (*Sus scrofa domestica*)**

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Abstract: The experiment was conducted using 20 male domestic pigs, which were in 2 (equal-sized) age groups: under 14 months old and over 18 months old. At least 5 ejaculates from each male were taken, and in each ejaculate, morphometric measurements of 50 spermatozoa were made. The measured parameters were head area, head length and width, and flagellum length. For each ejaculate, the basic physical traits and the frequency of occurrence of developmental anomalies of the spermatozoa were examined. It was found that sperm dimensions tended to increase along with the boars' age. Considerable and statistical differences in head area and flagellum length were proved. Spermatozoa collected from older boars (above 18 months of age) had a head area larger by $0.49 \mu\text{m}^2$ ($P \leq 0.01$) and a flagellum longer by $0.67 \mu\text{m}$ ($P \leq 0.01$) than spermatozoa collected from younger boars (under 14 months of age). The differences in head length and width between the spermatozoa of the tested boars were considerably smaller and were not statistically provable. Significant correlations between the head area of spermatozoa and the head length ($r = 0.56$, $P \leq 0.05$) and head width ($r = 0.36$, $P \leq 0.05$) were found. However, the head length was not significantly directly correlated with its width ($r = 0.18$, $P \leq 0.05$), and flagellum length was negatively correlated with spermatozoan head width ($r = -0.71$, $P \leq 0.05$). Slight correlations between the morphometric traits of the sperm and the physical traits of the ejaculates ($r = -0.11$ to 0.16) were found, although in most cases, the correlations were not statistically provable.

Key Words: Spermatozoa, Boar, Morphometry, Age, Sperm Dimensions

INTRODUCTION

The study of semen intended for insemination was more often than not limited to the examination of a few basic physical traits, including the volume of ejaculate,

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the sperm concentration and the percentage of sperm motility. In experiments carried out on human spermatozoa, the effect of sperm morphology on fertility was shown [1-3].

The sperm morphology of boar spermatozoa has been a subject of study considerably less frequently. The improvement of boar semen traits usually goes together with the animals' sexual development [4, 5]. This is associated with the progressive development of the inner structure of the testicle, which determines the number of spermatozoa, as well as with the development of accessory sex glands, which define ejaculative volume. A dynamic increase in the efficiency of the seminiferous epithelium of the seminiferous tubules of the testicles [6] and the improvement of ejaculative traits [5, 7] in the first months of performance were proved. The levels of sexual hormones, including testosterone [8], which play a substantial role in spermatogenesis, also change. Many scientific studies showed that each individual differed in the frequency of occurrence of spermatozoa with morphological changes [9, 10]. It was also found that physical traits had a considerable effect on the semen's fertilizing capacity and preservation ability [11, 12]. In addition, ejaculates which contained spermatozoa characterized by a lower coefficient of oocyte penetration *in vitro* contained far more morphologically changed spermatozoa, especially with cytoplasmic droplets [13].

Sperm dimensions and shapes could affect semen ability. Sperm dimensions are characterized by large variability, not only in males of different species [14, 15], but also in males of the same species [16, 17]. Buendia *et al.* [18] distinguished 3 categories of head size in the semen of alpaca (normal: 50%, small: 26% and large: 24%) and 5 categories in relation to the shape of spermatozoa (normal: 47%, long: 29%, short: 20%, pear-shaped: 3% and round: 1%). Thurston *et al.* [19] separated 3 subpopulations of boar spermatozoa differing in head shape; they were defined as rectangular-shaped sperm heads, sharply tapering sperm heads and slightly tapering sperm heads. The authors also noticed considerable differences in the intensity and forms of spermatozoa motility in relation to head shape. Moreover, the authors showed an effect of the trait on the semen preservation ability. A correlation between sperm dimensions and male fertility in some animal species was also found [20-23]. Genetic differences, mostly dependent on sire breed, and the age of the boar are among the most important factors determining the variation in boar insemination ability. Boar breeds may not only differ in ejaculate volume but also produce ejaculates with different sperm concentrations and spermatozoan activity, and different fertilizing capacity [24-26]. Apart from species, breed and individual variability, there are many factors that could affect the morphometric traits of spermatozoa [27-30]. The significance and effect of such factors have not yet been thoroughly elaborated on. The aim of this study was to determine the effect of the age of domestic male pigs on the morphometric traits of spermatozoa, the correlation of those traits with the physical traits of ejaculates, and the frequency of occurrence of spermatozoa with morphological changes.

MATERIALS AND METHODS

Biological material

The experiment was conducted using 20 domestic male pigs, which were in 2 (equal-sized) age groups: under 14 months old (I group) and over 18 months old (II group). The boars were chosen based on an analysis of origin in order to eliminate the effect of paternity. At least 5 ejaculates from each boar were collected and assessed. The ejaculates were taken by means of the gloved-hand technique [31]. Immediately after collection, the semen was filtered through 4 layers of sterile gauze into a prewarmed beaker to remove gel particles. The filtered semen was maintained at room temperature until needed for slide preparation for morphology and morphometry. Slides were prepared within 15 minutes of collection. In each ejaculate, morphometric measurements of 50 randomly selected spermatozoa were made.

Ejaculate assessment

Each ejaculate was subjected to standard macro- and microscopic assessment, covering the basic physical traits, including the volume of ejaculate, spermatozoa concentration, percentage of spermatozoa with proper motility, and total number of spermatozoa in one ejaculate. The number of insemination doses that could be prepared from each ejaculate was also calculated. The ejaculate volume (without the gelatinous fraction) was measured and related to the ejaculate weight. This measurement was carried out using an electronic balance. Spermatozoa concentration was assessed using the colorimetric method. The method was based on the measurement of light intensity let through the suspended spermatozoa in an isotonic solution of chlorine or sodium citrate. The percentage of spermatozoa with proper motility was determined at a magnification of x 200 under a microscope with a warmed stage (37°C). The total number of spermatozoa per ejaculate was determined optically (within a single field) under a microscope at a magnification of x 200.

Morphological assessment of spermatozoa and their morphometric measurements

Morphological assessment of spermatozoa. Semen slides were prepared within 15 minutes of collection. Smears were prepared by carefully dragging a drop of the fresh sperm across a degreased microscopic slide heated to 37°C. The slides were allowed to air dry for a minimum of 2 h, and were then prepared and preserved in a 96 % ethanol solution during a 5-minute exposure. After 30 min., the preserved slides were washed in distilled water, and then coloured with a 10 % aqueous solution of eosin during a 20- to 60-second exposure. The coloured slides were washed in distilled water and coloured with gentian pigment during a 3- to 5-minute exposure. After colouring, the slides were washed and dried. The slides were gently rinsed with distilled water for 2 min. to remove debris. This procedure led to a clean background, and thus a good contrast against the stained

spermatozoa. All the reagents used were purchased from Sigma Chemical (Germany). The slides were prepared and assessed at the same time, and by the same person using a microscope. In each slide, the morphology of 500 spermatozoa, including spermatozoa with proper proportions and showing major and minor abnormalities, was assessed.

Spermatozoon morphometric measurements were conducted on the same slides as those for the estimation of sperm morphology. Briefly, the morphometry system consisted of a microscope, a video camera and a computer. A Nikon Eclipse E-400 microscope (Japan) was equipped with a x 100 bright-field oil-immersion objective and a green filter, which was placed below the stage condenser to enhance the contrast of the acquired bright-field. In the field of the microscope, spermatozoa were chosen with the proper morphological proportions, then measurements of spermatozoa were done by hand. A Panasonic Industrial colour CCD camera model GP-KR-222 (Japan) video camera was attached to the microscope and linked up to the computer. The sperm image analysis system was calibrated against a stage micrometer, and the final image magnification on the computer monitor was x 4761. Morphometric tests were carried out employing SCREEN MEASUREMENT v. 4.1 for computer analysis of a picture. The following sperm dimensions were determined:

- the area of the spermatozoon head, established by measuring the area of the figure confined by a curve drawn on the edge of the microscopic image of the spermatozoon head (Fig. 1A);
- the length of the spermatozoon head, established as the length of an a-b line where a is the point where the head and the connecting piece (the flagellum neck) adjoin, and b is the farthest point from a at the tip of the head (Fig. 1B);

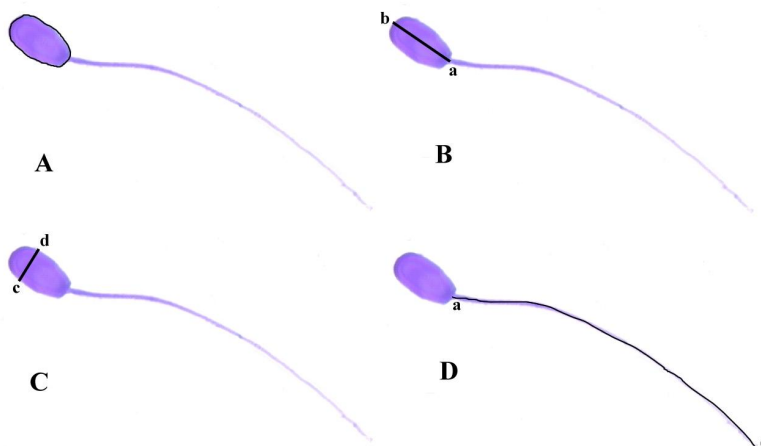


Fig. 1. The way of measuring morphometric sperm dimensions. A – head area, B – head length, C – head width, D – flagellum length.

- the width of the spermatozoon head, set as the length of a c-d line where c and d are the farthest sticking points from each other on the edge of spermatozoa head, and the line was drawn perpendicular to the long axis of the spermatozoon head at the height of $\frac{1}{2}$ an acrosome (Fig. 1C);

- the length of the spermatozoon flagellum, measured as the length of an a-e curve drawn along the long axis of the flagellum, where a is defined as above, and e is the flagellum tip (Fig. 1D).

The data for the morphometric measurements of the spermatozoa were stored in a database and exported for further statistical analysis.

Statistical analysis

The analysis of variability of spermatozoa morphometric traits was carried out according to the following mathematical model:

$$y_{ijk} = \mu + a_i + b_j + ab_{ij} + e_{ijk}$$

where: y_{ijk} – tested trait value; μ – population average; a_i – effect of boar's age; b_j – effect of the individual; ab_{ij} – effect of cooperation of controlled factors; e_{ijk} – error.

The significance of the differences between groups was concluded on the grounds of Tukey's test. Phenotypic correlation coefficients between each of the morphometric traits of the spermatozoa, and correlations between the morphometric traits of spermatozoa and the physical traits of the ejaculates and between the morphometric traits and the frequency of occurrence of developmental abnormalities of the spermatozoa were analysed.

RESULTS AND DISCUSSION

The traits of ejaculates and the tested morphometric traits of the spermatozoa in relation to the age of the boars are shown in Tab. 1.

The ejaculates of younger boars were smaller in volume and contained fewer sperm, but had larger concentrations than the ejaculates of older boars. We did not find differences in sperm motility and in the percentage of sperm abnormalities with regard to the age of the boars. Older boars (II group) had spermatozoa with larger head areas (on average by $0.49 \mu\text{m}^2$, $P \leq 0.01$) and with longer flagella (on average by $0.67 \mu\text{m}$, $P \leq 0.01$) than the younger boars. We did not find differences in the length and width of the spermatozoon head between the boars of different ages; however, the visual dimensions seemed to be larger in the case of the older boars.

The phenotypic correlation coefficients between the morphometric traits of spermatozoa are presented in Tab. 2. Significant correlations between the area of the spermatozoon head and its length ($P \leq 0.05$) and its width ($P \leq 0.05$), and also between the width of the spermatozoon head and its length ($P \leq 0.05$) were proved. A negative correlation between the head width and flagellum length ($P \leq 0.05$) was found.

Tab. 1. Traits of ejaculates and morphometric traits of the spermatozoa in relation to the age of the boars^a.

Specification	I group (under 14 months of age)	II group (over 18 months of age)
Number of boars	10	10
Number of ejaculates	55	56
Ejaculate volume (ml)	151.81 ± 48.3a	290.86 ± 68.8b
Sperm concentration (x 10 ⁶ spermatozoa)	500.91 ± 84.00a	452.81 ± 62.2b
Number of sperm in ejaculate (x 10 ⁹ spermatozoa)	66.60 ± 20.95a	108.02 ± 26.33b
Motility (%)	79.81 ± 1.35a	79.42 ± 3.67a
Sperm with major abnormalities (%)	2.28 ± 1.15a	1.91 ± 0.64a
Sperm with minor abnormalities (%)	7.92 ± 2.75a	9.03 ± 3.21a
Head area (µm ²)	40.68 ± 2.58a	41.17 ± 2.68b
Head length (µm)	9.35 ± 0.35	9.37 ± 0.44
Head width (µm)	4.85 ± 0.33	4.88 ± 0.28
Flagellum length (µm)	45.59 ± 3.14a	46.26 ± 1.75b

^aData are expressed as means ± S.D. Means followed by different letters within the rows are significantly different (a,b – P≤0.01).

Tab. 2. Phenotypic correlation coefficients between the morphometric traits of spermatozoa.

	Head area	Head length	Head width	Flagellum length
Head area	1.0	0.56*	0.36*	-0.02
Head length	0.56*	1.00	0.18*	0.07
Head width	0.36*	0.18*	1.00	-0.71*
Flagellum length	-0.02	0.07	-0.71*	1.00

* P≤0.05

Tab. 3. Phenotypic correlation coefficients between the morphometric traits and frequency of occurrence of developmental anomalies of spermatozoa and the physical traits of ejaculates.

	Head area	Head length	Head width	Flagellum length
Percentage of normal spermatozoa	0.09	-0.06	0.08	-0.07
Percentage of spermatozoa with major abnormalities	-0.11	-0.05	-0.09	-0.05
Percentage of spermatozoa with minor abnormalities	-0.6	0.08	-0.06	0.09

The correlations between the morphometric traits and the frequency of occurrence of morphological abnormalities of the spermatozoa were not stated

(Tab. 3). The correlations were low ($r \leq 0.11$) and they were not statistically proved.

Comparatively low correlation coefficients concerning the relationship between the sperm dimensions and the physical traits of ejaculates were observed (Tab. 4). We only found correlations between the ejaculate volume and the head length of spermatozoa ($r=0.16$, $P \leq 0.05$).

Tab. 4. Phenotypic correlation coefficients between the physical traits of the ejaculate and sperm dimensions.

	Head area	Head length	Head width	Flagellum length
Ejaculate volume	0.11	0.16*	-0.09	-0.02
Concentration of spermatozoa	0.06	-0.11	-0.02	0.04
Percentage of spermatozoa with progressive motility	-0.06	-0.06	0.04	0.01

* $P \leq 0.05$

The presented data showed that even after reaching breeding maturity, the young boars were still sexually developing. This was indicated by the increasing sperm dimensions, which in males of over 18 months old were larger than those of under 14 months old. The differences in relation to the head area and the flagellum length of spermatozoa were statistically proved. The head dimensions of morphologically normal boar spermatozoa are qualified as 8 μm long and 5 μm wide [32]. The obtained results for the width were similar to the norm; however, in the experiment, considerably larger head lengths of spermatozoa were found.

Compared to Hirai *et al.* [33], the spermatozoa of the tested boars had a head area larger by over 5 μm^2 , and the head length and width were larger by approximately 0.2 μm . The head length (larger by over 1 μm) and the total spermatozoon length (larger by approximately 2 μm) were also proved in relation to the results presented by Morrow and Gage [34]. Comparative studies showed that the competitive level of spermatozoa within female reproductive organs was relevant to their dimensions [34-36]. Spermatozoa with a longer connecting piece and flagellum were shown to have more powerful flagella [37]. Some researchers explained that the differences in head dimensions were caused by the variability of chromatin structure. The chromatin density of cat spermatozoa was significantly larger in case of cells with abnormally changed heads [38]. It was also found that the variability of head dimensions in bulls depended on abnormal chromatin structure [39]. Moreover, the changes in chromatin structure of spermatozoa isolated from the bull scrotum were connected with a growth in the content of morphologically abnormal spermatozoa [40]. The variability of the morphological forms of spermatozoa was found to be related to genetic factors. The increase in the content of spermatozoa with morphological abnormalities was often the result of the increase in the coefficient of inbreeding [41].

Comparatively high correlations between sperm dimensions showed the possibility of constructing indices of spermatozoa structure based on the interactions between the head length, head width, head area and flagellum length of spermatozoa. Thurston *et al.* [19] showed that spermatozoa could differ considerably in head shape, and that this was related to the important traits of semen. The results obtained in this study also indicate considerable differences in the morphological structure of boar spermatozoa. This was evidenced by the significant and high correlation between the flagellum length and head width of spermatozoa. The negative correlation ($r=-0.71$) suggested that in the tested population, a large number of spermatozoa with different structures, i.e. relatively short and with wider heads, could be found.

It seemed that the morphometric traits of the spermatozoa of domestic male pigs were not directly related to the frequency of occurrence of morphological abnormalities, as the correlation between sperm dimensions and the frequency of major and minor abnormalities of spermatozoa was not proved. The obtained results did not permit the definition of the direct relationship between the physical parameters of the ejaculates and the morphometric traits of the spermatozoa in the ejaculates.

To sum up, the age of sexually mature domestic male pigs had a significant effect on the morphometric traits of their spermatozoa – older boars had spermatozoa with longer heads and longer flagella. The interactions between particular sperm dimensions indicated the occurrence of gametes differing in shape. However, a direct relationship between the morphometric structure and the frequency of occurrence of morphological abnormalities of spermatozoa was not found.

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