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Original article

Storage-dependent changes in sperm motility, membrane integrity and morphology following preservation of Duroc boar semen

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Abstract

The aim of this study was to evaluate the qualitative parameters of sperm during storage of the semen of Duroc boars. The ejaculates were diluted, and insemination doses were then prepared and stored at 17°C. Analyses were performed four times: after collection, after one day of storage, after four and after eight days. Parameters of sperm motility, morphology and morphometry, cell membrane integrity, mitochondrial membrane potential and DNA integrity were evaluated. Analysis using the CASA system revealed a decrease in the percentage of motile sperm and sperm showing progressive motility during liquid storage of boar semen samples. The greatest differences, which were statistically significant, were shown between the results on the day of ejaculate collection and after eight days of semen storage. In addition, the percentage of sperm with head and tail defects increased, and differences were noted in the morphometric dimensions of the sperm during preservation of the semen. The length, width and area of the sperm heads increased over storage time. Preservation of Duroc boar semen at 17°C for eight days resulted in a decrease in the percentage of sperm with an integral cell membrane, sperm with high mitochondrial membrane potential, and DNA integrity. The breakthrough moment was the fourth day of sperm conservation. After then, the semen quality of Duroc boars deteriorated significantly. This study is particularly important since it provides a more complete analysis of the qualitative traits of the sperm of Duroc boars during semen preservation. However, there is a need for further study to determine the relationship between the qualitative parameters of the semen of Duroc boars evaluated during liquid storage of semen samples and fertility data.

Keywords: boar, motility, morphology, semen quality, semen storage time

Introduction

Reproductive performance in pigs is the result of multiple factors, including environmental factors, breed characteristics and individual traits. Artificial insemination in pigs usually involves the use of semen collected directly from the boar or after a few days of storage at 15-17°C (Johnson et al. 2000, Knox 2016, Yeste 2017, Wiebke et al. 2022). Semen stored in liquid form is widely used for artificial insemination in this species. This method of semen preservation allows for a wider insemination reach in pigs. Sperm metabolism decreases at 15-17°C, which is a necessary condition for extending storage time (Althouse et al. 1998, Lopez Rodriguez et al. 2017). It has been observed that changes in the permeability of the sperm cell membrane occur during storage of liquid boar semen and initiate capacitation (Petrunkina et al. 2005). Therefore the assessment of the integrity of the cell membrane (Yeste et al. 2010) as well as the mitochondrial membrane potential (Wysokińska 2020) is particulary important. Because of its structure, the cell membrane of boar sperm is more susceptible to damage than the cell membrane of the sperm of other animal species. The ability of sperm to move through the female reproductive tract is fundamental to fertility, since only fully functional sperm are able to reach the oocyte and fertilize it (Muvhali et al. 2022). Total sperm motility in the ejaculate is the most commonly used parameter for assessing male fertility (Popwell and Flowers 2004). The traditional method of evaluating sperm motility is subjective, based on the use of a light microscope with phase contrast and a heated stage. Assessment of sperm motility using the CASA (Computer Assisted Sperm Analysis) system is gaining wider adoption. This system was developed in the 1980s to enable objective measurements of sperm motility (Aitken et al. 1985). The CASA system provides a more standardized and objective measurement of sperm motility; it is also faster and simpler to use (Maside et al. 2023). Furthermore, CASA can be used to determine all parameters defining the motility of each sperm cell individually or the average measurements for each ejaculate (van der Horst et al. 2018, Luther et al. 2020). However, due to species differences in sperm characteristics, the CASA system requires species-specific calibration of measurements, combined with careful sample preparation before analysis and some expertise of the operator (Amann and Waberski 2014).

Another important aspect of determination of semen quality is assessment of the morphological structure of sperm cells, which may be influenced by numerous factors and processes taking place in the animal's body (Wysokińska et al. 2023). The most significant abnormalities and anomalies in sperm structures occur in the testes during spermatogenesis and in the epididymis during maturation. They may also arise as a consequence of an unfavorable environment for sperm in the ejaculate, including temperature, osmotic pressure, pH, and staining time (Dziekońska et al. 2013, Gacem et al. 2021). During laboratory handling of the ejaculate, changes may be generated in the sperm cell structures, which can affect their ability to fertilize the oocyte (Schulze et al. 2015, Schulze et al. 2017). The occurrence of sperm with abnormal morphological structure and varied size in semen may be a predisposing factor for the reduced effectiveness of fertilization, as some studies have shown a link between sperm dimensions and male fertility (Yániz et al. 2015, Garcia-Vazquez et al. 2016).

However, research on morphology is most often conducted on freshly collected ejaculates. Since boar semen used for artificial insemination is stored for a certain time in liquid form at 17°C, its quality may change during storage. There is also the occurrence of individual and inter-breed variability in semen quality characteristics, which affects the need to undertake research in relation to a specific breed of sire. There are no comprehensive studies characterizing the semen quality of Duroc boars. Therefore, the aim of our study was to evaluate the qualitative parameters of sperm, i.e. their motility, cell membrane integrity, mitochondrial membrane potential, morphology and morphometry, and DNA integrity during storage of the semen of Duroc boars, as well as the correlations between these parameters.

Material and Methods

This study comprises accepted methods and standard operation procedures for boar semen processing. The study was carried out in compliance with Directive 63/2010/EU and the Journal of Laws of the Republic of Poland of 2015 on the protection of animals used for scientific or educational purposes. Approval for the research was obtained from the Polish Society for Laboratory Animal Sciences (No. 3401/2015).

The material for the study was ejaculates collected from ten healthy Duroc boars used for artificial insemination, at the age of 24-30 months. Ejaculates from these boars were collected at regular intervals of 4-5 days. Boars were kept individually in pens with an area of 6 m^2. They were fed a complete feed intended for this group of animals in the amount of 3 kg of food per day per animal. Ejaculates were collected manually in the morning (about 6.00-7.00 a.m.) by a single individual. The study included two ejaculates collected from each

boar at one-week intervals in January-February (total of 20 ejaculates). Each ejaculate was evaluated for volume, sperm concentration, sperm motility, and the number of sperm in the ejaculate, after which the degree of dilution was established and the ejaculates were diluted using standard Biosolwens Plus extender (Biochefa, Sosnowiec, Poland) (Trzcińska and Bryła 2022). The ejaculates were than placed in 90 ml plastic blisters, with 2.8 x 109 sperm in each. Insemination doses prepared in this manner were stored at 17°C. Analyses were carried out after collection (0 days), after one day of storage, after four days, and after eight days. A different insemination dose was opened each time to avoid microbial contamination. A total of 80 insemination doses were analysed.

Sperm motility

Sperm motility was evaluated using the CASA system (SCA system, Microptic S.L., Barcelona, Spain) and a Nikon Eclipse E200 microscope (Japan) with a heated stage and a Basler acA1300-200uc camera (Germany). The microscope used was equipped with a 10x negative-phase contrast objective (AN 0.25). From each insemination dose, 5 µl of semen was collected and placed on a GoldCyto slide with a chamber depth of 20 µm, heated to 37°C. The analysis was carried out using at least five fields of view, and at least 500 cells per sample were analysed. The sperm motility parameters measured were total motility (%), progressive motility (%), non-motile sperm (%), fast motility (%), medium motility (%), slow motility (%), straight-line velocity (VSL, µm/s), curve-linear velocity (VCL, µm/s), average-path velocity (VAP, µm/s), straightness (STR: VSL/VAP x 100, %), linearity (LIN: VSL/VCL x 100, %), wobble (WOB: (VAP/VCL x 100, %), amplitude of lateral head displacement (ALH, µm), and beat-cross frequency (BCF, Hz). The software settings for boar sperm motility analysis were as follows: number of frames: 50; frames per sec: 60 Hz; minimum contrast: 50; minimum cell size: 6 pixels; contrast with static cells: 30; straightness: 80%.

Sperm cell membrane integrity

Sperm cell membrane integrity assessment was performed using SYBR-14 and propidium iodide (PI) fluorochromes using the Live/Dead Sperm Viability Kit (Molecular Probes Inc., Leiden, The Netherlands). Test preparations were made in accordance with the methodology given in Wysokińska and Szablicka (2021). In each preparation 200 sperm were analyzed. The assessment identified three groups of sperm: sperm stained green, identified as live cells (with an integral cell membrane, stained with SYBR-14), sperm stained red, identified as dead (with a damaged cell membrane, stained with PI) and sperm stained green and red, identified as dying sperm.

Mitochondrial membrane potential (MMP)

Fluorochrome JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolocarbocyanine iodide) (Sigma Aldrich, USA) was used to analyze the mitochondrial membrane potential (MMP) of sperm as described by Wysokińska (2020) with modifications. In each slide 200 sperm cells were assessed, distinguishing those with active mitochondria with high membrane potential (with JC-1 aggregates with high ΔΨm; sperm emitting orange fluorescence from the midpiece), sperm with active mitochondria with moderate membrane potential (with JC-1 aggregates with moderate ΔΨm; sperm emitting orange and green fluorescence from the midpiece), and sperm with active mitochondria with low membrane potential (with JC-1 aggregates with low ΔΨm; sperm emitting green fluorescence from the midpiece). A Nikon Eclipse E200 fluorescence microscope (Tokyo, Japan) was used to make the assessment.

Sperm DNA integrity

Sperm DNA integrity was assessed using an Acridine Orange (Sigma-Aldrich, St. Louis, MO, USA) according to the methodology described in the works of Katiyar et al. (2022, 2024). In each slide at least 200 sperm cells were assessed, distinguishing sperm with intact DNA (sperm heads emitting green fluorescence) and sperm with demaged DNA (sperm heads emitting orange to red fluorescence). A Nikon Eclipse E200 fluorescence microscope (Tokyo, Japan) was used to make the assessment (exicitation 465-495 and 540 nm).

Sperm morphology

Sperm morphology was evaluated on slides stained using the SpermBlue method (van der Horst and Maree (2009). Semen in the amount of 5 µl was applied to a microscope slide, and a smear was prepared. After the slide had dried, it was immersed in SpermBlue stain (Microptic S.L., Barcelona, Spain) for 15 min and then washed in distilled water and left to dry at room temperature. On each slide 500 sperm were evaluated, distinguishing sperm with normal structure and sperm with morphological anomalies, including major and minor defects (Blom, 1981). Sperm morphology was assessed by a single individual to avoid the influence of the individual on the result. In addition, sperm with morphological defects were divided into three subgroups: sperm with head defects, sperm with tail defects, and sperm with a cytoplasmic droplet. The eva-

luation was carried out using a Nicon Eclipse E200 microscope (Tokyo, Japan) at 1000× magnification.

Sperm morphometry

Morphometric measurements of sperm were made on the microscope slides prepared for the evaluation of sperm morphology stained with SpermBlue. Sperm morphometry was analysed using the Sperm Class Analyzer system (Microptic S.L., Barcelona, Spain) and a Nikon Eclipse E200 microscope (Tokyo, Japan) with a Basler acA1300-200uc camera (Germany) configured to work with the Sperm Class Analyzer system, version 6.5.091. The microscope was equipped with a 60× bright field lens. For better accuracy, individual parts of the sperm cell were measured by manual selection. Due to the detailed nature of the measurements and the long analysis time, 30 sperm were measured in one sample (total of 2,400 sperm). All measurements were performed by one person. The following measurements were made in each spermatozoon: the length, width, area, and perimeter of the head; tail length; and total sperm length. Based on the results of the morphometric measurements, shape indices were calculated for the sperm heads: ellipticity (length/width), elongation (length-width)/(length+width), roughness (4π*surface area/perimeter$^{2)}$ and regularity (π*length*width/4*surface area).

Statistical analysis

The results are presented as mean and standard error of the mean (SEM). Normal distribution of the data was confirmed using the Shapiro-Wilk test at $p>0.05$. Calculations were made using STATISTICA v. 3.21 software (StatSoft, Tulsa, USA). The influence of different storage times on sperm motility parameters, on the frequency of morphological defects, morphometric characteristics, integrity of the cell membrane, potential of the mitochondrial membrane and DNA integrity was determined using analysis of variance (ANOVA). The significance of differences between means was determined using Tukey's test at $p\leq0.05$. Pearson correlation coefficients were determined between sperm motility and sperm morphology and morphometry on each day of semen storage. The significance of the correlation coefficients was determined for $p<0.05$.

Results

Table 1 presents the results of evaluation of sperm motility parameters in semen stored at 17°C. The data indicate that sperm motility parameters in the semen of Duroc boars decrease over storage time. The greatest differences, which were statistically significant, were observed between the results on the day of ejaculate collection and after eight days of semen storage. These differences were confirmed for total motility, progressive motility, sperm with fast motility, and non-motile sperm. The lowest percentages of motile sperm (71.72%) and sperm with progressive motility (28.72%) were shown after eight days of preservation of Duroc boar semen, with a marked increase in the number of sperm showing no motility compared to the day the ejaculate was collected ($p\leq0.05$). The VSL, VCL, VAP, STR, LIN and BCF values decreased slightly during storage, but the differences were not confirmed statistically.

Data characterizing the integrity of sperm cell membranes, the mitochondrial membrane potential and the DNA integrity are presented in Table 2. It was found that the integrity of sperm cell membranes changes with the sperm storage time. On the eighth day of semen storage, there were over 18% fewer sperm with an integral cell membrane than on day 0 ($p\leq0.05$). An increase in the percentage of sperm with damaged cell membrane during semen preservation was observed. On the fourth day of semen storage, there were over 19% of such sperm, and on the eighth day of storage, over 23%. The percentage of dying sperm remained at a similar level on individual days of semen storage. The share of sperm with high mitochondrial membrane potential on the eighth day of liquid semen storage decreased by more than 10% compared to the day of collection (day 0). On the eighth day of semen storage, significantly more sperm with medium and low mitochondrial membrane potential were detected compared to the other periods examined.

Table 3 presents the percentages of sperm with normal structure and sperm with morphological defects in the semen of Duroc boars. The frequency of sperm with morphological defects was shown to change over semen storage time. The percentage of sperm with normal morphology was significantly lower on the eighth day of semen storage ($p\leq0.05$). The percentages of sperm with major and minor defects increased with semen storage time (from 1.40% on the day of ejaculate collection to 5.70% on the eighth day of storage and from 2.37% to 12.65%, respectively). In the first 24 hours, the frequency of sperm with these defects remained similar, after which it began to increase to 5.70% (major defects at 8 days of preservation) and 12.65% (minor defects at 8 days) ($p\leq0.05$). The percentage of sperm with head and tail defects increased over semen storage time. The greatest differences were noted between one and eight days of preservation ($p\leq0.05$). No changes were noted in the percentage of sperm with a cytoplasmic droplet during storage of semen of Duroc boars.

Table 1. Sperm motility depending on storage time of semen of Duroc boars (mean±SEM).

Item	Semen storage time (days)			
	0	1	4	8
Total motility (%)	91.78±0.58^{a}	81.90±2.77^{ab}	76.75±5.80^{b}	71.72±7.09^{b}
Progressive motility (%)	53.00±3.95^{a}	42.27±6.45^{ab}	31.92±3.80^{b}	28.72±4.26^{b}
Non-motile sperm (%)	8.21±0.58^{a}	18.09±2.77^{ab}	23.25±5.80^{ab}	28.30±7.09^{b}
Fast motility (%)	61.09±2.55^{a}	48.99±6.83^{ab}	37.33±9.74^{b}	32.40±7.67^{b}
Medium motility (%)	14.49±1.85^{a}	13.00±1.46^{a}	12.60±1.20^{a}	11.52±2.00^{a}
Slow motility (%)	16.19±1.85^{a}	19.92±3.28^{a}	26.81±5.63^{a}	27.78±3.00^{a}
VSL (µm/s)	43.88±1.51^{a}	38.35±2.37^{a}	37.33±2.48^{a}	39.95±2.75^{a}
VCL (µm/s)	125.87±8.61^{a}	115.45±3.24^{a}	112.08±5.85^{a}	118.27±6.79^{a}
VAP (µm/s)	64.23±3.44^{a}	57.42±2.65^{a}	56.43±4.27^{a}	61.20±4.00^{a}
STR (%)	68.73±1.70^{a}	66.29±1.11^{a}	66.20±0.89^{a}	64.55±1.10^{a}
LIN (%)	36.80±1.81^{a}	34.08±1.68^{a}	34.09±1.39^{a}	34.65±1.21^{a}
WOB (%)	52.39±1.09^{a}	50.31±1.85^{a}	50.64±2.24^{a}	52.51±1.60^{a}
ALH (µm)	3.28±0.24^{a}	3.02±11.00^{a}	3.05±0.13^{a}	3.12±0.16^{a}
BCF (Hz)	11.91±0.78^{a}	11.80±1.82^{a}	9.41±0.62^{a}	9.41±0.70^{a}

a,b – Values in rows with different letters are significantly different at p≤0.05.

Table 2. Sperm cell membrane integrity, mitochondrial membrane potential (MMP) and DNA integrity depending on storage time of semen of Duroc boars (mean±SEM).

Item	Semen storage time (days)			
	0	1	4	8
Sperm cell membrane integrity (%):				
Sperm with intact cell membrane	88.42±1.38^{a}	86.25±2.41^{a}	74.92±5.18^{b}	70.00±5.16^{b}
Sperm with damaged cell membrane	5.83±1.03^{a}	6.00±1.13^{a}	19.17±5.54^{b}	23.33±5.40^{b}
Moribund spermatozoa	5.75±1.84^{a}	7.75±2.83^{a}	5.91±1.87^{a}	6.67±1.45^{a}
Mitochondrial membrane potential (%)				
High	86.00±2.07^{a}	80.58±2.21^{ab}	84.92±2.25^{a}	75.42±2.18^{b}
Medium	5.67±1.07^{a}	7.08±0.58^{a}	8.25±2.45^{a}	14.75±1.19^{b}
Low	8.33±2.17^{a}	12.33±2.48^{b}	6.83±0.47^{a}	9.83±1.46^{c}
DNA integrity (%)	96.75±0.76^{a}	96.00±0.89^{ab}	95.25±0.81^{ab}	93.42±0.92^{b}

a,b,cValues in rows with different letters are significantly different at P≤0.05.

Table 3. Percentage of sperm with normal structure and with morphological defects depending on storage time of semen of Duroc boars (mean±SEM).

Item	Semen storage time (days)			
	0	1	4	8
Sperm with normal structure (%)	96.23±0.56^{a}	94.58±0.96^{a}	90.38±1.14^{a}	82.25±3.28^{b}
Sperm of morphologically abnormal (%):				
Major abnormalities	1.40±0.34^{a}	1.78±0.56^{a}	3.48±0.91^{ab}	5.70±1.03^{b}
Minor abnormalities	2.37±0.45^{a}	3.65±1.03^{a}	6.48±1.22^{ab}	12.65±3.46^{b}
Sperm with:				
Head defects (%)	1.15±0.32^{a}	2.00±0.36^{ab}	4.63±0.91^{b}	7.90±1.54^{c}
Tail defects (%)	1.87±0.31^{a}	2.15±0.34^{a}	3.58±0.43^{a}	8.17±1.98^{b}
Cytoplasmic droplet (%)	0.77±0.16^{a}	1.25±0.56^{a}	1.78±0.51^{a}	2.53±0.98^{a}

a,b,cValues in rows with different letters are significantly different at p≤0.05.

Table 4. Morphometric dimensions of sperm depending on storage time of semen of Duroc boars (mean±SEM).

Item	Semen storage time (days)			
	0	1	4	8
Number of analyzed cells	600	600	600	600
Head				
Length (µm)	9.10±0.06^{a}	9.26±0.06^{ab}	9.37±0.07^{bc}	9.45±0.07^{c}
Width *(µm)*	4.85±0.04^{a}	4.93±0.04^{ab}	4.94±0.05^{ab}	4.97±0.05^{b}
Area *(µm²)*	37.92±0.35^{a}	39.11±0.27^{b}	39.50±0.30^{b}	40.67±0.33^{c}
Perimeter *(µm)*	23.95±0.11^{a}	24.29±0.12^{ac}	24.70±0.13^{b}	24.60±0.14^{bc}
Tail				
Length *(µm)*	46.22±0.25^{a}	46.90±0.20^{a}	46.68±0.28^{a}	46.67±0.20^{a}
Sperm total length *(µm)*	55.33±0.27^{a}	56.16±0.22^{b}	56.05±0.30^{ab}	56.12±0.19^{b}
Shape indices				
Ellipticity	1.88±0.02^{a}	1.89±0.02^{a}	1.90±0.02^{a}	1.91±0.02^{a}
Elongation	0.30±0.00^{a}	0.31±0.00^{a}	0.31±0.00^{a}	0.31±0.00^{a}
Rugosity	0.83±0.01^{a}	0.83±0.01^{a}	0.82±0.01^{a}	0.84±0.01^{a}
Regularity	0.92±0.01^{a}	0.92±0.01^{a}	0.92±0.01^{a}	0.91±0.01^{a}

a,b,cValues in rows with different letters are significantly different at $p \leq 0.05$.

Table 4 presents the results for the morphometric dimensions of sperm depending on the storage time of liquid semen of Duroc boars. The data indicate differences in sperm dimensions during preservation of semen. These differences were most evident between the day the ejaculate was collected (0 days) and after eight days of storage. These differences mainly concerned the sperm head dimensions. The length, width and area of the sperm heads increased with semen storage time. After eight days of storage, the sperm head dimensions were significantly larger than after collection (0 days). The dimensions of the sperm heads on the eighth day of semen storage were: head length 9.45 µm, width 4.97 µm, area 40.67 µm², whereas on the day of ejaculate collection they were 9.10 µm, 4.85 µm and 37.92 µm2, respectively.

The correlation coefficients between the total motility and the morphological structure of sperm depending on semen storage time are presented in Table 5. Positive, significant correlations were noted between total and progressive motility: r=0.76 after one day of storage, r=0.85 after four days, and r=0.94 after eight days. Positive correlations were noted between sperm head dimensions and total motility. Significant relationships were shown between total motility and width of sperm heads after one day of storage of liquid semen, between total motility and sperm with intact cell membrane, head length after four days, and between total motility and the surface area, perimeter and length of sperm heads at eight days. Interestingly, at four and eight days of preservation of liquid semen, negative correlations were shown between sperm motility and tail length (r=-0.83 and r=-0.94).

Discussion

This study is particularly important for assessing the quality of the semen of Duroc boars. The results of the study indicate that evaluation of the qualitative parameters of the spermatozoa of Duroc boars during storage of liquid semen is important and necessary for correct diagnostics of ejaculates used for artificial insemination. The analysis carried out using the CASA system revealed that the percentage of motile sperm and sperm with progressive motility decreased during storage of liquid boar semen. Motility is one of the main characteristics associated with the fertilization capacity of sperm. Decreased motility of sperm in the semen has been shown to be correlated with lower fertilization rates (Yániz et al. 2018). According to Keller and Kerns (2023), conventional analysis of sperm motility and morphology is not sufficient to predict male fertility. According to their research, combining the analysis of sperm capacitation with motility and morphology can increase the effectiveness of determining an ejaculate fertility potential. In our study, we did not analyze the process of sperm capacitation, but based on the decreasing percentage of sperm with normal morphology and progressive motility during semen storage, it can be assumed that such sperm will have a lower ability for capacitation. Sperm motility (total and progressive) and concentration are the main ejaculate parameters evaluated to determine the number of insemination doses per ejaculate (Caldeira et al. 2019). Adenosine triphosphate (ATP) concentration has been shown to be linked to sperm motility. A decrease in the percentage of motile sperm in semen may be due to potentially reduced ATP

Table 5. Pearson correlation coefficients between total motility and sperm morphology and morphometry, cell membrane integrity, mitochondrial membrane potential of sperm and DNA integrity at different times of storage of boar semen (*p<0.05).

Item	Semen storage time (days)			
	0	1	4	8
Progressive motility	0.63	0.76	0.85*	0.94*
Normal morphology	0.10	0.88*	-0.70	-0.31
Major abnormalities	0.07	-0.04	-0.06	-0.42
Minor abnormalities	-0.17	-0.73	0.55	0.35
Head defects	-0.16	-0.13	0.61	0.31
Tail defects	-0.08	-0.90*	0.07	0.22
Cytoplasmic droplet	0.22	-0.86*	-0.09	0.42
Head area	0.01	0.56	0.27	0.82*
Head perimeter	-0.44	0.78	0.59	0.87*
Head length	0.67	0.66	0.78	0.90*
Head width	-0.56	0.90*	0.62	0.64
Tail length	0.83*	0.43	-0.83*	-0.94*
Sperm total length	0.91*	0.64	-0.77	-0.79
Sperm with intact cell membrane	-0.05	-0.36	0.84*	0.60
Sperm with demaged cell membrane	-0.60	-0.23	-0.75	-0.43
Moribund spermatozoa	0.38	0.40	-0.09	-0.55
High MMP	-0.54	0.09	-0.06	-0.08
Medium MMP	0.59	-0.07	0.09	-0.08
Low MMP	0.23	-0.06	-0.21	0.19
DNA integrity	-0.08	-0.36	-0.24	-0.11

production (Li et al. 2016). Adenosine triphosphate (ATP) in semen may decrease as a result of storage of semen in various extenders (Fraser et al. 2001, Gogol et al. 2009). The observed decrease in the percentage of motile sperm during storage of liquid boar semen could be indicative of a decrease in ATP in the sperm midpiece. However, there are studies in which, despite the decrease in sperm motility, no decrease in ATP content was demonstrated in semen stored in a liquid state (Dziekońska et al. 2009). In the study by Tremoen et al. (2018), clear differences in sperm motility and ATP concentration between day 0 and day 4 of semen storage were indicated. Therefore, it is important to analyze semen stored for several days, because often diluted boar semen is often transported long distances. Maintaining the correct mitochondrial membrane potential guarantees adequate ATP production by mitochondria (Luo et al. 2013). In our previous studies, we indicated that the percentage of sperm with a high mitochondrial membrane potential remains similar (not statistically significantly different) until the fourth day of sperm storage, after which it decreases significantly. It can therefore be assumed that after the fourth day of sperm preservation, the mitochondrial membrane potential weakens. Mitochondria have been indicated to be the main determinants of cell life or death. Induction of apoptosis causes the opening of mitochondrial pores, which results in a decrease in the mitochondrial membrane potential (Trzcińska et al. 2011).

The literature contains few studies evaluating parameters of boar semen during preservation. Our previous research on the semen of Landrace boars showed that the percentage of sperm with morphological defects and the percentage of sperm with a damaged cell membrane increased during semen storage at 17°C (Wysokińska et al. 2023). In other studies, it was indicated that in the semen of Duroc boars, during the preservation of liquid semen, a decrease in the number of sperm with the correct structure of the sperm cell membrane was observed with a greater intensity than in crossbred boars and Pietrain boars (Wysokińska and Szablicka 2021). According to Prieto-Martínez et al. (2014) sperm cell membrane integrity is affected by a high level of bacterial contamination. A high storage temperature for boar semen is conducive to more rapid microbial growth (Morell 2016). Therefore, analysis of semen quality should take into account a variety of factors which may be important in semen diagnostics. Semen characteristics show high variation resulting from genetic factors, environmental factors, and individual traits (Dziekońska and Strzeżek 2011, Schulze et al. 2018, Hafemeister et al. 2022). In the present study,

we analysed semen of Duroc boars, which some studies have shown to be characterized by smaller ejaculate volume but a higher sperm concentration than the semen of boars of other breeds (Smital et al. 2004). For this reason, the ratio between seminal plasma and spermatozoa in the semen of Duroc boars is smaller than in the semen of boars of other breeds (Wysokińska and Szablicka 2021). Seminal plasma contains various proteins whose concentrations in the semen influence fertility (Flowers et al. 2016). Dilution of boar semen is believed to decrease the content of proteins and natural antioxidants, as well as other components of the seminal plasma essential to the normal functioning and integrity of the sperm cell membrane (Maxwell and Johnson 1999). Thus the changes in the quality of Duroc boar semen observed in the present study may be due to natural processes taking place during storage of liquid semen. Nevertheless, our data show that the qualitative characteristics of sperm change during semen preservation, which may reduce the effectiveness of fertilization. Our study showed that the percentage of sperm with head and tail defects increases during storage of liquid semen of Duroc boars, and differences in the morphometric dimensions of sperm are observed. Perhaps the tendency of decreased sperm motility during semen storage observed in our studies is caused by morphological changes occurring in the sperm tail, especially marked on the eighth day of preservation.

Some studies have found that sperm head dimensions may be associated with male fertility (Saravia et al. 2007, Hidalgo et al. 2008, Barquero et al. 2021). Males producing sperm with smaller heads have been shown to be more fertile (Hirai et al. 2001). The size and shape of the sperm head depends on the size of the cell nucleus, its shape, and the shape of the acrosome. These traits may be determined genetically (Thurston et al. 2001), and thus may depend on the breed of boar (Kondracki et al. 2012, Wysokińska and Kondracki 2019). Saravia et al. (2007) demonstrated that sperm head dimensions are greater in Duroc boars than in boars of other breeds. Only the semen of Duroc boars was analysed in the present study. The current findings indicate a need to investigate how storage factors interact with breed-specific characteristics. According to some researchers, changes in the shape of the sperm head may arise as a result of chromatin condensation disorders and DNA fragmentation (Ostermeier et al. 2001). Morphometric characteristics play an important role in the functioning of individual sperm structures (Maroto-Morales et al. 2016). Some authors indicate that the morphometric dimensions of sperm depend on the frequency of sperm with morphological defects in the head (Casey et al. 1997) and on sperm motility (Malo et al. 2006). Investigating specific mechanisms responsible for the correlation between sperm motility and sperm morphometry (mainly the length of the sperm tail) may be the basis for future research. Some studies have indicated that sperm with longer tails are more active in terms of motility, but their activity is shorter than that of sperm with shorter tails (Gomendio and Roldan 1991). Our study showed no effect of the duration of semen storage on the length of the sperm tail, but it showed significant negative correlations between sperm motility (total motility) and the length of the tail and of the entire sperm after four and eight days of semen preservation. Thus it can be expected that during storage of liquid Duroc boar semen, the motility of sperm and their energy state may be reduced. Sperm with a longer mitochondrial sheath (midpiece) of the sperm tail may be more competitive than sperm with a shorter mitochondrial sheath and can reach the egg cell faster. Such sperm can produce more energy necessary for sperm movement. However, as shown in the research of Kerns et al. (2020), there is a negative correlation between the length of the mitochondrial sheath of the sperm tail and the number of piglets born in the litter. The authors of these studies conclude that this relationship may be related to the process taking place immediately after fertilization, since the oocyte may have difficulty eliminating paternal mitochondria without proceeding to embryogenesis. Hence there is a need to monitor and analyse the qualitative parameters of boar sperm during semen storage, which may also be an indicator of their potential to reach the oocyte.

To sum up, preservation of the semen of Duroc boars at 17°C for eight days reduced the qualitative parameters of the sperm: total motility, progressive motility, percentage of sperm with fast motility, the percentage of sperm with normal morphology, the share of sperm with an integral cell membrane and sperm with a high potential mitochondrial membrane. The breakthrough moment is the fourth day of sperm conservation. After the fourth day, the semen quality of Duroc boars deteriorates significantly. Changes in sperm dimensions are observed during storage of liquid semen, especially in the heads. The length, width and surface area of the sperm heads increase with preservation time. The proportion of sperm with morphological defects, i.e. changes in the head and tail, increases as well. These parameters were evaluated using the CASA system, which enabled an objective analysis of the qualitative characteristics of the sperm of Duroc boars during the preservation of liquid semen. The analysis of many structural parameters of sperm gives more information on the changes occurring during storage of liquid boar semen and provides valuable information about the fertility potential of the sire. However, to fully

assess the impact of storage on fertilization potential, further research is needed to investigate the relationship between these qualitative parameters and fertility data in Duroc boars.

References

Aitken RJ, Sutton M, Warner P, Richarson DW **(1985)** Relationship between the movement characteristics of human spermatozoa and their ability to penetrate cervical mucus and zona-free hamster oocytes. J Reprod Fertil 73: 441-449.

Althouse GC, Wilson ME, Kuster C, Parsley M **(1998)** Characterization of lower temperature storage limitations of fresh-extended porcine semen. Theriogenology 50: 535-543.

Amann RP, Waberski D **(2014)** Computer-assisted sperm analysis (CASA): capabilities and potential developments. Theriogenology 81: 5-17.

Barquero V, Roldan ER, Soler C, Yániz JI, Camacho M, Valverde A **(2021)** Predictive capacity of boar sperm morphometry and morphometric subpopulations on reproductive success after artificial insemination. Animals (Basel) 11: 920.

Blom E **(1981)** The morphological estimation of the spermatozoa defects of bull II. The proposal of new classification of spermatozoa defects. Med Weter 37: 239-242.

Caldeira C, Hernández-Ibánez S, Valverde A, Martin P, Herranz-Jusdado JG, Gallego V, Asturiano JF, Dzyuba B, Pšenička M, Soler C **(2019)** Standardization of sperm motility analysis by using CASA-Mot for Atlantic salmon (Salmo salar), European eel (Anguilla anguilla) and Siberian sturgeon (Acipenser baerii). Aquaculture 502: 223-231.

Casey PJ, Gravance CG, Davis RO, Chabot DD, Liu IK **(1997)** Morphometric differences in sperm head dimensions of fertile and subfertile stallions. Theriogenology 47: 575-582.

Dziekońska A, Fraser L, Strzeżek J **(2009)** Effect of different storage temperatures on the metabolic activity of spermatozoa following liquid storage of boar semen. J Anim Feed Sci 18: 638-649.

Dziekońska A, Fraser L, Majewska A, Lecewicz M, Zasiadczyk Ł, Kordan W **(2013)** Effect of commercial long-term extenders on metabolic activity and membrane integrity of boar spermatozoa stored at 17 degrees C. Pol J Vet Sci 16: 517-525.

Dziekońska A, Strzeżek J **(2011)** Boar variability affects sperm metabolism activity in liquid stored semen at 5 degrees C. Pol J Vet Sci 14: 21-27.

Flowers WL, Deller F, Stewart KR **(2016)** Use of heterospermic inseminations and paternity testing to evaluate the relative contributions of common sperm traits and seminal plasma proteins in boar fertility. Anim Reprod Sci 174: 123-131.

Fraser L, Gorszczaruk K, Strzeżek J **(2001)** Relationship between motility and membrane integrity of boar spermatozoa in media varying in osmolality. Reprod Domest Anim 36: 325-329.

Gacem S, Catalán J, Yánez-Ortiz I, Soler C, Miró J **(2021)** New Sperm Morphology Analysis in Equids: Trumorph® Vs Eosin-Nigrosin Stain. Vet Sci 8: 79.

Garcia-Vazquez FA, Gadea J, Matas C, Holt WV **(2016)** Importance of sperm morphology during sperm transport and fertilization in mammals. Asian J Androl 18: 844-850.

Gogol P, Szczęśniak-Fabiańczyk B, Wierzchoś-Hilczer A **(2009)** The photon emission, ATP level and motility of boar spermatozoa during liquid storage. Reprod Biol 9: 39-49.

Gomendio M, Roldan ER **(1991)** Sperm competition influences sperm size in mammals. Proc Biol Sci 243: 181-185.

Hafemeister T, Schulze P, Bortfeldt R, Simmet C, Jung M, Fuchs-Kittowski F, Schulze M **(2022)** Boar Semen Shipping for Artificial Insemination: Current Status and Analysis of Transport Conditions with a Major Focus on Vibration Emissions. Animals (Basel) 12: 1331.

Hidalgo M, Rodríguez I, Dorado J, Soler C **(2008)** Morphometric classification of Spanish thoroughbred stallion sperm heads. Anim Reprod Sci 103: 374-378.

Hirai M, Boersma A, Hoeflich A, Wolf E, Foll J, Aumüller TR, Braun J **(2001)** Objectively measured sperm motility and sperm head morphometry in boars (Sus scrofa): relation to fertility and seminal plasma growth factors. J Androl 22: 104-110.

Johnson LA, Weitze KF, Fiser P, Maxwell WM **(2000)** Storage of boar semen. Anim Reprod Sci 62: 143-172.

Katiyar R, Chakravarty H, Deori S, Singh M, Abedin S, Rautela R, Khargharia G, Mishra VK **(2024)** Oxyrase-Mediated Improvement in the Quality and Fertility of Crossbred Boar Spermatozoa During Liquid Storage. Reprod Domest Anim 59: e14735.

Katiyar R, Ghosh SK, Karikalan M, Kumar A, Pande M, Gemeda AI, Rautela R, Dhara SK, Bhure SK, Srivastava N, Patra MK, Chandra V, Devi HL, Singh M. **(2022)** An Evidence of Humanin-Like Peptide and Humanin Mediated Cryosurvival of Spermatozoa in Buffalo Bulls. Theriogenology 194: 13-26.

Keller A, Kerns K **(2023)** Sperm capacitation as a predictor of boar fertility. Mol Reprod Dev 90: 594-600.

Kerns K, Jankovitz J, Robinson J, Minton A, Kuster C, Sutovsky P **(2020)** Relationship between the length of sperm tail mitochondrial sheath and fertility traits in boars used for artificial insemination. Antioxidants (Basel) 9: 1033.

Knox RV **(2016)** Artificial insemination in pigs today. Theriogenology 85: 83-93.

Kondracki S, Iwanina M, Wysokińska A, Huszno M **(2012)** Comparative analysis of Duroc and Pietrain boar sperm morphology. Acta Vet Brno 81: 195-199.

Li X, Wang L, Li Y, Zhao N, Zhen L, Fu J, Yang Q **(2016)** Calcium regulates motility and protein phosphorylation by changing cAMP and ATP concentrations in boar sperm in vitro. Anim Reprod Sci 172: 39-51.

Lopez Rodriguez A, Van Soom A, Arsenakis I, Maes D **(2017)** Boar management and semen handling factors affect the quality of boar extended semen. Porcine Health Manag 3: 15.

Luo SM, Schatten H, Sun QY **(2013)** Sperm mitochondria in reproduction: good or bad and where do they go? J Genet Genomics 40: 549-556.

Luther I, Maree L, Kotze A, Hildebrandt T, Göritz F, Hermes R, Van der Horst G **(2020)** Sperm motility, kinematics, morphometry and morphology over two seasons in free-ranging African elephants (Loxodonta africana). Reprod Fertil Dev 32: 425-438.

Malo AF, Gomendio M, Garde J, Lang-Lenton B, Soler AJ, Roldan ERS **(2006)** Sperm design and sperm function. Biol Lett 2: 246-249.

Maroto-Morales A, Garcia-Alvarez O, Ramon M, Martinez-Pastor F, Fernandez-Santos MR, Soler AJ, Garde JJ **(2016)** Current status and potential of morphometric sperm analysis. Asian J Androl 18: 863-870.

Maside C, Recuero S, Salas-Huetos A, Ribas-Maynou J, Yeste M **(2023)** Animal board invited review: an update on the methods for semen quality evaluation in swine – from farm to the lab. Animal 17: 100720.

Maxwell WM, Johnson LA **(1999)** Physiology of spermatozoa at high dilution rates: the influence of seminal plasma. Theriogenology 52: 1353-1362.

Morrell JM **(2016)** Antimicrobials in boar semen extenders – a risk/benefit analysis. J Antimicro 2: 107.

Muvhali PT, Bonato M, Malecki IA, Cloete SW **(2022)** Mass Sperm Motility Is Correlated to Sperm Motility as Measured by Computer-Aided Sperm Analysis (CASA) Technology in Farmed Ostriches. Animals (Basel) 12: 1104.

Ostermeier GC, Sargeant GA, Yandell BS, Evenson DP, Parrish JJ **(2001)** Relationship of bull fertility to sperm nuclear shape. J Androl 22: 595-603.

Petrunkina AM, Volker G, Weitze KF, Beyerbach M, Topfer-Petersen E, Waberski D **(2005)** Detection of cooling-induced membrane changes in the response of boar sperm to capacitating conditions. Theriogenology 63: 2278-2299.

Popwell JM, Flowers WL **(2004)** Variability in relationships between semen quality and estimates of in vivo and in vitro fertility in boars. Anim Reprod Sci 81: 97-113.

Prieto-Martinez N, Bussalleu E, Garcia-Bonavila E, Bonet S, Yeste M **(2014)** Effects of Enterobacter cloacae on boar sperm quality during liquid storage at 17°C. Anim Reprod Sci 148: 72-82.

Quintero-Moreno A, Rigau T, Rodriguez-Gil JE **(2004)** Regression analyses and motile sperm subpopulation structure study as improving tools in boar semen quality analysis. Theriogenology 61: 673-690.

Saravia F, Núnez-Martínez I, Morán JM, Soler C, Muriel A, Rodríguez-Martínez H, Pena FJ **(2007)** Differences in boar sperm head shape and dimensions recorded by computer-assisted sperm morphometry are not related to chromatin integrity. Theriogenology 68: 196-203.

Schulze M, Ammon C, Schaefer J, Luther AM, Jung M, Waberski D **(2017)** Impact of different dilution techniques on boar sperm quality and sperm distribution of the extended ejaculate. Anim Reprod Sci 182: 138-145.

Schulze M, Kuster C, Schäfer J, Jung M, Grossfeld R **(2018)** Effect of production management on semen quality during longterm storage in different European boar studs. Anim Reprod Sci 190: 94-101.

Schulze M, Rüdiger K, Waberski D **(2015)** Rotation of boar semen doses during storage affects sperm quality. Reprod Domest Anim 50: 684-687.

Smital J, De Sousa LL, Mohsen A **(2004)** Differences among breeds and manifestation of heterosis in AI boar sperm output. Anim Reprod Sci 80: 121-130.

Thurston LM, Watson PE, Mileham AJ, Holt WV **(2001)** Morphologically distinct sperm subpopulations defined by Fourier shape descriptors in fresh ejaculates correlate with variation in boar semen quality following cryopreservation. J Androl 22: 382-394.

Tremoen NH, Gaustad AH, Andersen-Ranberg I, van Son M, Zeremichael TT, Frydenlund K, Grindflek E, Vage DI, Myromslien FD **(2018)** Relationship between sperm motility characteristics and ATP concentrations, and association with fertility in two different pig breeds. Anim Reprod Sci 193: 226-234.

Trzcińska M, Bryła M **(2022)** Assessment of high hydrostatic pressure for the cryopreservation procedure of boar semen with low initial sperm motility. Anim Sci Pap Rep 40(2): 159-170.

Trzcińska M, Bryła M, Smorąg Z **(2011)** Apoptotic-like changes in the spermatozoa of fresh and stored boar semen and the quality of embryos produced in vivo. Anim Reprod Sci 124: 90-97.

van der Horst G, Maree L **(2009)** SpermBlue: a new universal stain for human and animal sperm which is also amenable to automated sperm morphology analysis. Biotech Histochem 84: 299-308.

van der Horst G, Maree L, du Plessis SS **(2018)** Current perspectives of CASA applications in diverse mammalian spermatozoa. Reprod Fertil Dev 30: 875-888.

Wiebke M, Hensel B, Nitsche-Melkus E, Jung M, Schulze M **(2022)** Cooled storage of semen from livestock animals (part I): boar, bull, and stallion. Anim Reprod Sci 246: 106822.

Wysokińska A **(2020)** Effect of sperm concentration on boar spermatozoa mitochondrial membrane potential and motility in semen stored at 17 °C. Acta Vet Brno 89: 333-340.

Wysokińska A, Kondracki S **(2019)** Heterosis for morphometric characteristics of sperm cells from Duroc x Pietrain crossbred boars. Anim Reprod Sci 211: 106217.

Wysokińska A, Szablicka D **(2021)** Integrity of Sperm Cell Membrane in the Semen of Crossbred and Purebred Boars during Storage at 17 °C: Heterosis Effects. Animals (Basel) 11: 3373.

Wysokińska A, Szablicka D, Dziekońska A, Wójcik E **(2023)** Analysis of changes in the morphological structures of sperm during preservation of liquid boar semen in two different seasons of the year. Anim Reprod Sci 256: 107297.

Yániz JL, Silvestre MA, Santolaria P, Soler C **(2018)** CASA-Mot in mammals: an update. Reprod Fertil Dev 30: 799-809.

Yániz JL, Soler C, Santolaria P **(2015)** Computer assisted sperm morphometry in mammals: a review. Anim Reprod Sci 156: 1-12.

Yeste M **(2017)** State-of-the-art of boar sperm preservation in liquid and frozen state. Anim Reprod 14: 69-81.

Yeste M, Briz M, Pinart E, Sancho S, Bussalleu E, Bonet S **(2010)** The osmotic tolerance of boar spermatozoa and its usefulness as sperm quality parameter. Anim Reprod Sci 119: 265-274.