

## Effect of Heat Stress on the Antioxidant Activity of Boar Ejaculate Revealed by Spectroscopic and Electrochemical Methods

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An effect of heat stress on antioxidant activity and overall quality of boar ejaculates was monitored. The samples of boar ejaculates were divided into two groups according to ejaculate quality. In the group A, regarding antioxidant activity a decreased value of *N,N*-dimethyl -1,4-diaminobenzene method by 71 %, an increase in 2,2-diphenyl-1-picrylhydrazyl method by 104 %, and decrease in superoxide dismutase activity by 39 % was determined. Results of the metal content were as follows decrease in levels of copper by 60 % and zinc by 52 %. Also decreases in metallothionein content by 64 %, and in pH by 11 % were recorded. In the group B, in antioxidant activity again a decreased value of DMPD by 68 %, and an increase in DPPH by 207 % was recorded. In addition reduced levels of copper by 78 %, zinc by 30 %, metallothionein by 64 %, and value of pH by 11 % were determined. The results show that heat stress induces an increase in protection against oxidative stress; in addition, levels of metallothionein, zinc and copper in the boar ejaculates in both groups are decreased when compared to day 0.

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**Keywords:** Boar ejaculates; Heat stress; Antioxidant activity; Quality of ejaculates

## 1. INTRODUCTION

The quality of boar ejaculates varies significantly during the season. The highest fluctuations are observed in all species of male livestock in the summer months at higher temperatures [1]. Also stress itself may influence the body temperature [2,3]. Quality of boar ejaculates decreases at increased heat load. A reduction of motility and concentration of sperms as well as an increase of percentage of abnormal sperm have been recorded [4]. Reduced sperm motility is caused by a lower level of ATP [5-7]. In the boars, the quality of ejaculate is largely influenced by season. The best quality of ejaculate is observed in spring and autumn. Conversely, in the summer, when the animals are exposed to high temperatures, significantly worse qualitative and quantitative indicators of the ejaculate are recorded. An influence of breed has also been recorded [8,9]. High temperature may also cause an excessive loading the organism with oxygen radicals and decrease in activity of antioxidant enzymes [10]. In some cases, overproduction of free radicals causes a damage of biomolecules and cancer [11] [12]. Reactive oxygen species [13] negatively affect the quality of sperms. Negative impact of these reactive oxygen species causes peroxidation of lipids that create the outer shell of sperms [14]. Reactive oxygen species may affect the metabolism of proteins in the ejaculate [15-17]. High temperature also affects the reproductive performance of breeding pigs. Selenium is part of the sperm and affects its mobility. Selenium deficiency reduces sperm motility. Zinc and copper are essential metals, which affect the concentration and motility of sperms. [18,19].

The aim of this experiment was to evaluate the effect of heat stress on biochemical parameters of ejaculate of breeding boars focusing on changes in antioxidant status (at boars with good or poor quality of ejaculates). The aim of the experiment was also to evaluate the effect of heat stress on ejaculate quality of boars.

## 2. EXPERIMENTAL PART

### 2.1. Animals

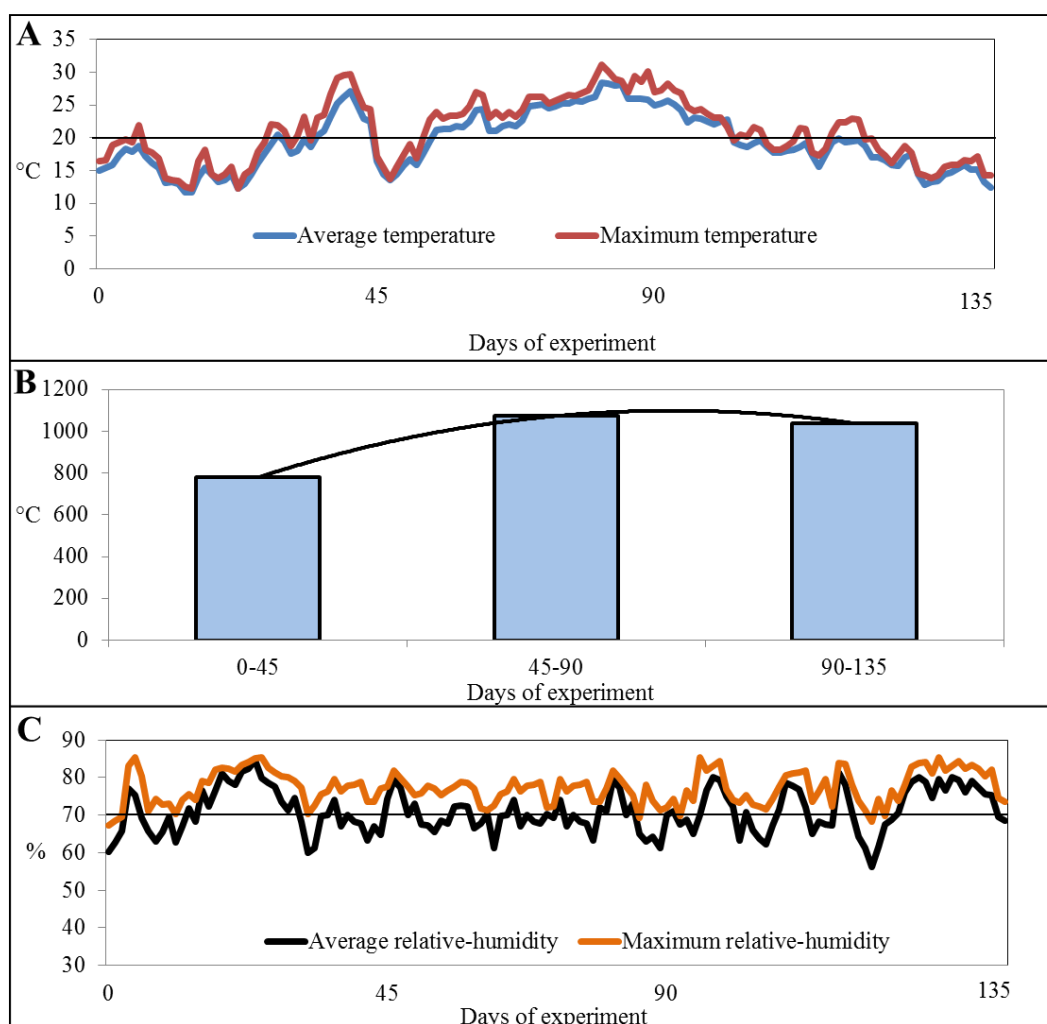
The experiment was performed in the sperm collection centre of boars in Velke Mezirici (Czech Republic). Twenty Duroc boars breeding stocks were included in the experiment (*Sus scrofa domestica*). The average age was  $2 \pm 0.3$  years and the average weight of boars was  $250 \pm 20$  kg. The length of the experiment was set at 135 days (May to September). Experimental animals were stalled individually (2.5 x 2.5 m) and were supplemented with water *ad libitum*. All animals were fed with 3.3 kg per day of basic feed mixture (Tab. 1).

**Table 1.** The composition of the feed mixture for boars

Component	% in feed mixture
Barley grain	36.00
Wheat grain	20.36

Oat grain	20.00
SBM (soybean meal)	14.50
EKPO T	3.00
BergaFat	2.10
Calcium carbonate	1.50
Monocalciumphosphate	1.20
Mineral vitamin premix for boars 0.5%	0.50
Sodium chloride	0.40
Magnesium oxide	0.15
L-Lysine HCl	0.14
L- Threonine	0.09
Methionine DL	0.06

Bergafat (Berg + Schmidt, Germany) – palm oil; EKPO T (Delika – Pet, Czech Republic) – biscuit meal



**Figure 1.** Development of temperature, which acts on both experimental groups of boars during the experimental observation (A); the sum of the temperatures during individual parts of the experiment (B), the development of the relative humidity (C)

Content of MEp (metabolizable energy of pigs) was  $12.6 \text{ MJ.kg}^{-1}$  of diet. Boars were divided into two groups. The first group (n = 10) of boars (group A) had values of ejaculate without pathological changes (motility of sperm cells 72 %, concentration of sperm cells  $499\,000.\text{mm}^{-3}$ ; ejaculate volume 203 ml; percentage of pathological sperm cells below 6 %). The second group (group B) contained boars (n = 10), which had problems with the quality of ejaculate produced (low sperm motility 67 %, and a low concentration of sperm cells  $430\,000.\text{mm}^{-3}$ ). During the whole experiment, the temperature was registered by monitoring the environment in which the boars were located. The temperature was monitored with a data logger (Voltcraft DL-121TH, Germany), which was placed at the level of the animals (1 m above the ground). The data logger recorded the actual temperature in hourly intervals; the average temperature for each day was calculated from these values. Figures 1A and 1B show average temperature and the sum of temperature values during the experiment. Figure 1C shows average and maximal relative humidity of the environment in which the experiment was carried out. Ejaculates were collected weekly, after collection they were frozen to  $-80\text{ }^{\circ}\text{C}$  and they were thawed immediately before analyses. The ejaculates, which were taken at the beginning of the experiment (day 0 -control samples) and at 45<sup>th</sup>, 90<sup>th</sup> and 135<sup>th</sup> day of the experiment, were used for analyses. The ejaculates were obtained from boars using a “jump mare” [20]

Determination of the ejaculate volume, sperm concentration, its motility, and percentage of abnormal sperms were carried out according to Lovercamp et al. [20]. Ejaculate volume was determined using a measuring cylinder. Determination of sperm motility was performed within 15 minutes after collection from microscopically-mixed semen. Sperm concentration was determined photometrically. The percentage of pathological sperms was determined microscopically and they were collected prior to the start of the experiment; Photographs of sperms were taken using an inverted Olympus microscope IX 71 S8F-3 (Tokyo, Japan). Records were taken using a camera Olympus DP73 and processed with Stream Basic 1.7 software. Magnification: 600x, Resolution:  $1600 \times 1200$  pixels.

## 2.2. Preparation of samples for biochemical analyses

Firstly, 0.5 ml volume of native thawed ejaculate was pipetted with subsequent addition of 2 ml of liquid nitrogen and 0.5 ml of phosphate buffer. Subsequently, the sample was homogenized in an ULTRA-TURRAX T8 homogenizer (IKA, Königswinter, Germany) at 3000 rpm for 2 minutes. After homogenization, 1 ml of phosphate buffer was added. Sample modified like this was homogenized in a vortex (Vortex-2 Genie Scientific Industries, New York, NY, USA) at 2000 rpm for 15 minutes. Subsequently, the sample was centrifuged in a Universal 32 R centrifuge (Hettich-Zentrifugen GmbH, Tuttlingen, Germany) at 16000 rpm at  $4\text{ }^{\circ}\text{C}$  for 20 minutes. Finally, supernatant was removed and used for analyses (1.5 ml).

## 2.3. Determination of glutathione peroxidase (GPx)

A Glutathione Peroxidase Cellular Activity Assay Kit (CGP1, Sigma Aldrich, USA) was used for the GPx assay, determination was done in native ejaculate. For the determination of GPx activity, a

BS 400 automated spectrophotometer (Mindray, China) was used. The experimental protocol was as it follows. A 260  $\mu\text{l}$  volume of reagent R1 (0.3 mM NADPH in GPx buffer) was pipetted into a plastic cuvette with subsequent addition of 10  $\mu\text{l}$  of sample and after mixing, a 30  $\mu\text{l}$  volume of reagent R2 (3 mM tert-butyl hydroperoxide) was added to the cuvette which starts the reaction. The decrease in absorbance was measured at 340 nm using kinetic program for 126 seconds.

#### 2.4. Determination of SOD

Kit 19160 SOD (Sigma Aldrich, USA) was used for assay of superoxide dismutase (SOD, EC 1.15.1.1.). A 200  $\mu\text{L}$  volume of reagent R1 (WTS solution diluted 20 times with buffer) was pipetted into a plastic cuvette and agent was incubated at 37 °C for 108 s. Afterwards, a 20  $\mu\text{L}$  volume of sample was pipetted and in 378 s, the reaction was started by adding a 20  $\mu\text{L}$  volume of reagent R2 (enzyme solution 167 times diluted with buffer). It was incubated for 72 s and then absorbance was measured at  $\lambda = 450$  nm. Kinetic reaction was measured for 180 s and absorbance was read every 9 s.

#### 2.5. Determination of antioxidant activity

Solutions of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and free radical (FR) assays were prepared according to Sochor et al. [21]. Brief description of the assays is below.

#### 2.6. Determination of antioxidant activity using the ABTS test

The ABTS radical method is one of the most used assays for the determination of the concentration of free radicals. It is based on the neutralization of a radical-cation arising from the one-electron oxidation of the synthetic chromophore 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS):  $\text{ABTS}^{\bullet} - e^- \rightarrow \text{ABTS}^{++}$ . This reaction is monitored spectrophotometrically by the change of the absorption value.

A 150  $\mu\text{l}$  volume of reagent was mixed with 3  $\mu\text{l}$  of sample. Absorbance was measured at 660 nm. For calculating of the antioxidant activity, difference between absorbance at the last (12<sup>th</sup>) minute and at the second minute of the assay procedure was used.

#### 2.7. Determination of antioxidant activity by Ferric Reducing Antioxidant Power (FRAP) method

The FRAP method is based on the reduction of complexes of 2, 4, 6-tripyridyl-*s*-triazine (TPTZ) with ferric chloride hexahydrate ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ), which are almost colourless, and eventually slightly brownish. This chemical forms blue ferrous complexes after its reduction. Reagent preparation: Solution 1: 10  $\text{mmol} \cdot \text{L}^{-1}$  solution of TPTZ in 40  $\text{mmol} \cdot \text{L}^{-1}$  of hydrochloric acid. Solution 2: 20  $\text{mmol} \cdot \text{L}^{-1}$  solution of ferric chloride hexahydrate in ACS water. Solution 3: 20  $\text{mmol} \cdot \text{L}^{-1}$  acetate buffer, pH 3.6. These three solutions (TPTZ,  $\text{FeCl}_3$ , acetate buffer) are mixed in a 1:1:10 ratio. A 150

$\mu\text{L}$  volume of reagent was injected into a plastic cuvette with subsequent addition of a 3  $\mu\text{L}$  sample. Absorbance was measured at 605 nm for 12 minutes. Difference between absorbance at the last (12<sup>th</sup>) minute and second minute of the assay procedure was used for calculating of the antioxidant activity.

#### 2.8. Determination of antioxidant activity by the *N,N*-dimethyl -1,4-diaminobenzene (DMPD) method

The compound DMPD is converted in solution to a relatively stable and coloured radical form (DMPD<sup>•+</sup>) by the action with ferric salt. After addition of a sample containing antioxidants, DMPD<sup>•+</sup> radicals are scavenged and as a result of this scavenging, the coloured solution is decolourized [22]. Procedure for the determination was taken from Sochor et al. [23,24]. A 160  $\mu\text{l}$  volume of reagent (200 mM DMPD, 0.05 M FeCl<sub>3</sub>, 0.1 M acetate buffer pH 5.25) was injected into a plastic cuvette with subsequent addition of 4  $\mu\text{l}$  sample. Absorbance was measured at 505 nm. Difference between absorbance at the 10<sup>th</sup> minute and second minute of the assay procedure was used for calculating of the antioxidant activity.

#### 2.9. Determination of antioxidant activity using the DPPH<sup>•</sup> test

The DPPH test is based on the ability of the stable 2,2-diphenyl-1-picrylhydrazyl free radical to react with hydrogen donors. The DPPH<sup>•</sup> radical displays an intense UV-VIS absorption spectrum. In this test, a solution of radical is decolourized after reduction with antioxidant (AH) or a radical (R<sup>•</sup>) in accordance with the following scheme: DPPH<sup>•</sup> + AH  $\rightarrow$  DPPH-H + A<sup>•</sup>, DPPH<sup>•</sup> + R<sup>•</sup>  $\rightarrow$  DPPH-R.

A 150  $\mu\text{l}$  volume of reagent was incubated with 15  $\mu\text{l}$  of sample. Absorbance was measured at 505 nm for 12 minutes. Output ratio was achieved by the calculation of difference of absorbance at the last (12<sup>th</sup>) minute and at the second minute of the assay procedure.

#### 2.10. Determination of antioxidant activity by the FR method

This method is based on ability of chlorophyllin (the sodium-copper salt of chlorophyll) to accept and donate electrons with a stable with a stable change of maximum absorption. This effect is conditioned by an alkaline environment and the addition of catalyst.

A 150  $\mu\text{l}$  volume of reagent was injected into a plastic cuvette with subsequent addition of a 6  $\mu\text{l}$  sample. Absorbance was measured at 450 nm at the second minute of assay and the last (12<sup>th</sup>) minute. Difference between these two absorbancies was considered as an outputting value and served for the calculation of antioxidant activity.

#### 2.11. Determination of zinc

Determination of zinc by differential pulse voltammetry was performed using a 797 VA Computrace instrument connected to 813 Compact Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes. A hanging mercury drop electrode (HMDE) with a drop area of 0.4

mm<sup>2</sup> was the working electrode. An Ag/AgCl/3M KCl electrode was the reference and platinum electrode was the auxiliary. For data processing, 797 VA Computrace software by Metrohm CH was employed. The analysed samples were deoxygenated prior to measurements by purging with argon (99.9%). Acetate buffer (0.2M CH<sub>3</sub>COONa + CH<sub>3</sub>COOH, pH 5) as a supporting electrolyte was used. The supporting electrolyte was exchanged after each analysis. The parameters of the measurement were as follows: initial potential of -1.2 V, end potential -0.7 V, deoxygenating with argon 90 s, deposition 420 s, time interval 0.04 s, step potential 5 mV, modulation amplitude 25 mV, adsorption potential -1.2 V, volume of injected sample: 10 µl, volume of measurement cell 2 ml (10 µl of sample, 1990 µl of acetate buffer) [25,26].

### 2.12. Preparation of samples for electrochemical determination of copper – microwave digestion

To 10 µl ejaculate 500 µl of digestion mixture (350 µl HNO<sub>3</sub> + 150 µl H<sub>2</sub>O<sub>2</sub>) was added. Samples were digested in MW Anton Paar, rotor MG-65. The program (SUP 6) begins and ends with the same ten-minute-long-step, beginning with the power of 50 W and ending with the power of 0 W. Microwave power was 100 W in the main part of the program (30 min.).

### 2.13. Determination of metallothionein

Differential pulse voltammetric measurements were performed with a 747 VA Stand instrument connected to 693 VA Processor and an 695 Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes and cooled sample holder and measurement cell to 4 °C (Julabo F25, JulaboDE). A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm<sup>2</sup> was the working electrode. An Ag/AgCl/3M KCl electrode was the reference and glassy carbon electrode was the auxiliary. For data processing VA Database 2.2 by Metrohm CH was employed. The analysed samples were deoxygenated prior to measurements by purging with argon (99.999 %) saturated with water for 120 s. Brdicka supporting electrolyte containing 1 mM Co(NH<sub>3</sub>)<sub>6</sub>Cl<sub>3</sub> and 1 M ammonia buffer (NH<sub>3</sub>(aq) + NH<sub>4</sub>Cl, pH = 9.6) was used. The supporting electrolyte was exchanged after each analysis. The parameters of the measurement were as follows: initial potential of -0.7 V, end potential of -1.75 V, modulation time 0.057 s, time interval 0.2 s, step potential 2 mV, modulation amplitude -250 mV, Eads = 0 V, volume of injected sample: 10 µl, volume of measurement cell 2 ml (10 µl of sample, 1990 µl of Brdicka solution) [27-31].

### 2.14. Determination of concentration of selenium in ejaculate

The concentration of selenium was determined using differential pulse voltammetry by a 797 VA Computrace and an 889 IC Sample Center (Metrohm, Switzerland) using a standard cell with three electrodes. A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm<sup>2</sup> was the working electrode. An Ag/AgCl/3M KCl electrode was the reference and glassy carbon electrode was the auxiliary. The analysed samples were deoxygenated prior to measurements by purging with argon

(99.999 %) saturated with water for 120 s. The parameters of the measurement were as follows: deposition potential -0.6 V, accumulation time 200 s, pulse amplitude 0.03 V, pulse time 0.05 s, voltage step 0.006 V, voltage step time 0.1 s, sweep rate 0.06 V/s, equilibration time 30 s. Total volume of the measuring vessel was 2 ml (1980  $\mu\text{l}$  of electrolyte and 20  $\mu\text{l}$  of sample). The electrolyte for selenium was prepared by using 0.015 mM ammonium sulphate with addition of copper sulphate; the final concentration of  $\text{CuSO}_4$  in solution was 0.05 mM. pH of this electrolyte was adjusted to 2.2 using sulphuric acid. The scan was in the range of potentials -0.4 V to -0.9 V and the characteristic peak of selenium was recorded at potential of -0.7 V.

### 2.15. Photometric determination of malondialdehyde

The principle of malondialdehyde determination is reaction between malondialdehyde (MDA) with thiobarbituric acid (TBA) under formation of TBA-MDA-TBA adduct that absorbs strongly at 535 nm. Trichloroacetic acid (TCA) is added to the sample because of its ability to precipitate proteins, bilirubin, unsaturated fatty acids and lipoproteins. A 300  $\mu\text{L}$  sample of blood plasma was mixed with 10  $\mu\text{L}$  0.5 M solution of butylated hydroxytoluene (BHT) in 96 % ethanol (*v/v*) and 310  $\mu\text{L}$  20 % TCA (*v/v*) prepared in 0.6 M HCl. After 20 min incubation on ice mixture was centrifuged at 11,000 rpm for 15 min. Subsequently, 400  $\mu\text{L}$  of supernatant was mixed with 800  $\mu\text{L}$  of 30 mM TBA and mixture was incubated in a thermomixer Comfort (Eppendorf, Germany) at 90 °C for 30 min. After cooling in ice MDA absorbance was measured using a spectrophotometer at 535 nm and the concentration was subtracted from the calibration curve [32].

### 2.16. Determination of glucose

Glucose is oxidized by glucose oxidase to gluconolactone in the presence of oxygen. The resulting hydrogen peroxide is oxidized by 4-aminoantipyrine and phenol to quinone red in the presence of peroxidase (POD). The intensity of generated red colour is directly proportional to the concentration of glucose and can be determined photometrically. Into plastic cuvettes, 200  $\mu\text{l}$  reagent (Greiner, Germany, 0.1 M phosphate buffer pH 7.5, 0.75 mM phenol, 0.25 mM 4-aminoantipyrin, glucose oxidase  $\geq 15\text{kU} / \text{l}$  peroxidase  $\geq 1.5 \text{ U} / \text{l}$ ) was added followed by 20  $\mu\text{l}$  of the sample. The absorbance was measured at  $\lambda = 505$  after an incubation lasting 10 minutes. The absorbance values of reagents and absorbance values after 10 minutes of incubation with the sample were used to calculate glucose content in the sample.

### 2.17. Determination of pH

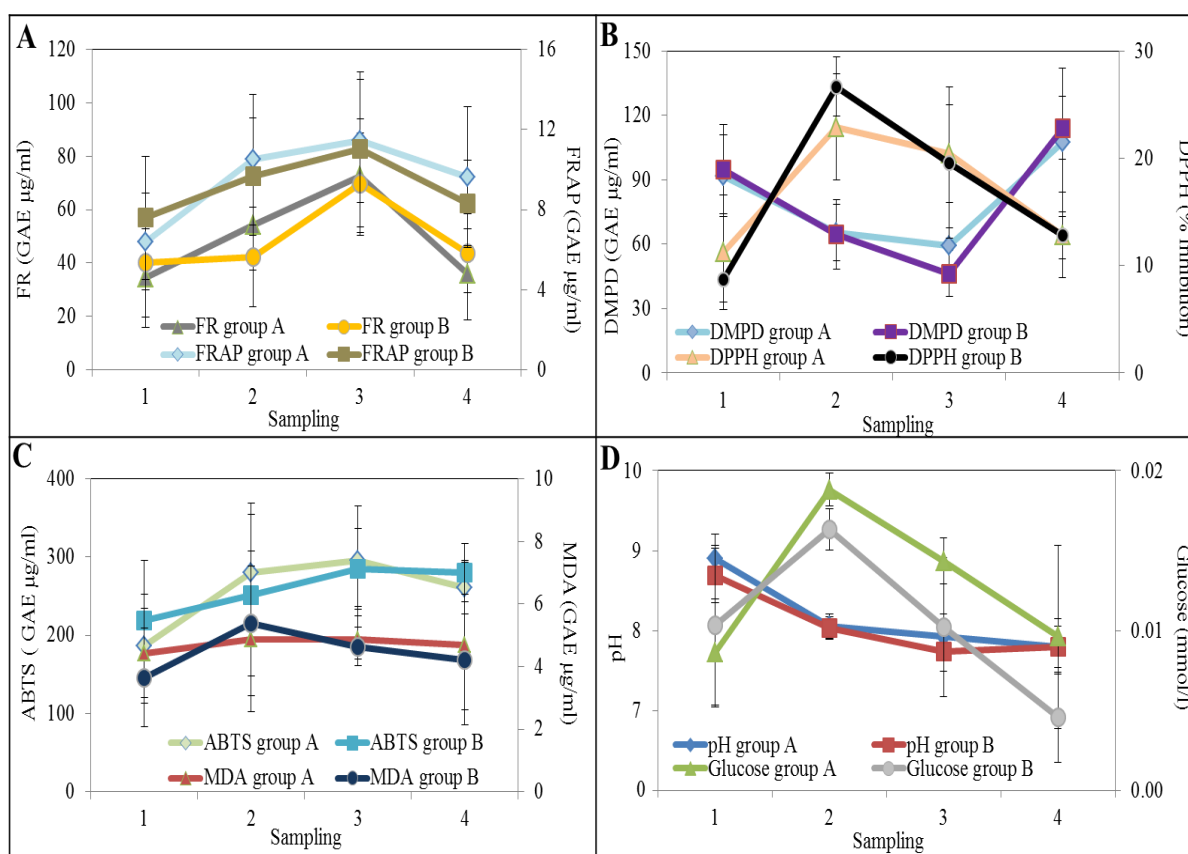
pH in the ejaculate was determined using a pH meter inoLab level 3 (WTW GmbH, Weilheim, Germany).



2.18. Statistic

The data were processed statistically using STATISTICA.CZ, version 10.0 (Czech Republic). Results are expressed as mean from 3 measurements  $\pm$  standard deviation. Statistical significance was determined by examining the basic differences among individual samples using ANOVA and Scheffé's method (two-factor analysis) for the parameters GPx, SOD; DMPD; DPPH; FRAP; ABTS; FR; MDA; glucose; pH; metallothionein; levels selenium; zinc and copper; quality of ejaculate volume of ejaculate; motility of sperms; concentration of sperms and abnormal sperms. Differences with  $P < 0.05$  were considered significant.

3. RESULTS

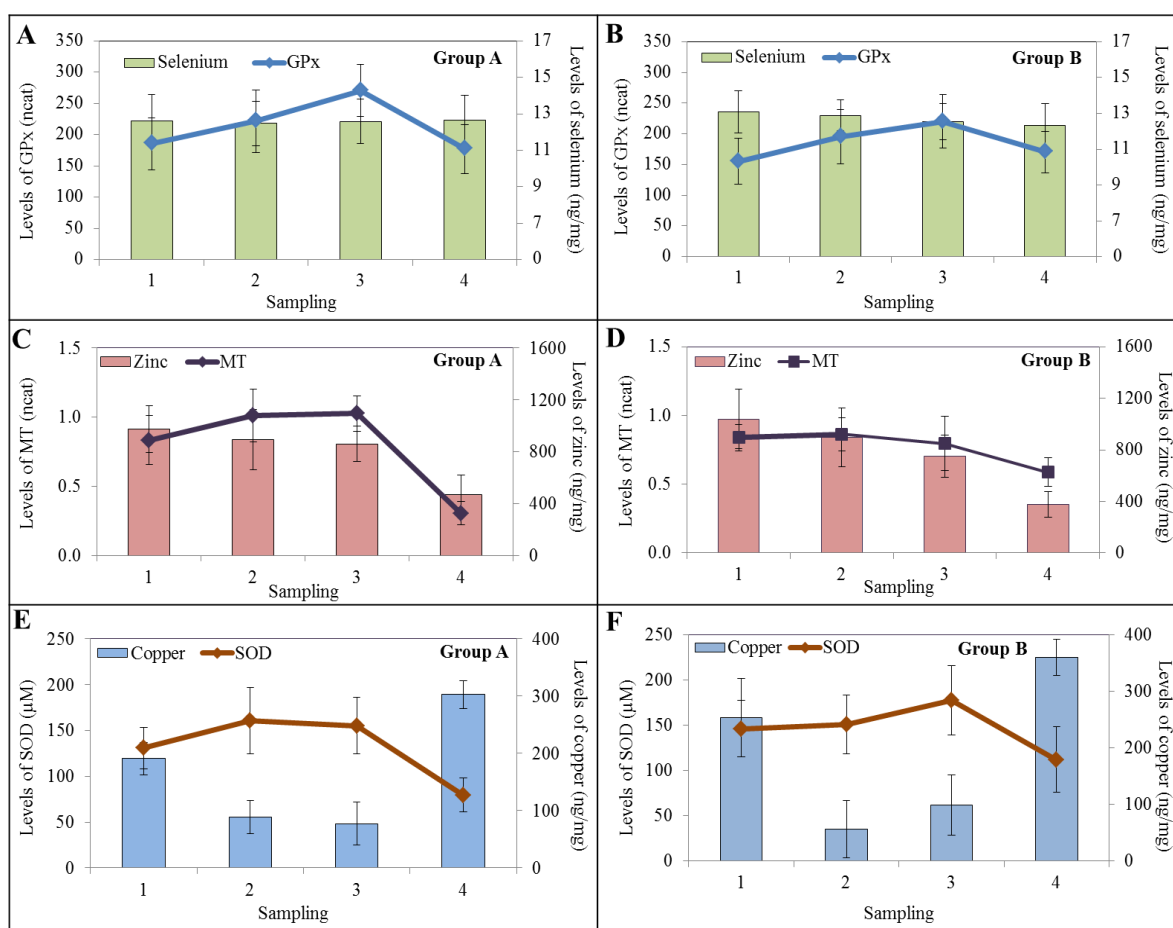


**Figure 2.** Antioxidant activity measured by methods FRAP - Ferric Reducing Antioxidant Power and FR - Free Radicals (A); DMPD - N-dimethyl -1,4-diaminobenzene and DPPH-2,2-diphenyl-1-picrylhydrazyl (B); ABTS-2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid and lipid peroxidation measures as a content of MDA - malondialdehyde (C); pH and glucose (D). Results are expressed as mean from 3 measurements  $\pm$  standard deviation;  $P < 0.05$ .

Experiment lasted 135 days and no health problems were observed in experimental animals. During this period, the average daily temperature of air varied from 12.3 to 29.2 °C. Samples of boar ejaculates at the beginning of the experiment were divided into two groups according to ejaculate

quality. The group A with a good quality of ejaculate (number of animals = 10; motility of sperm cells 72 %, and their concentration  $499\ 000\ \text{mm}^{-3}$ ; percentage of pathological sperm cells 6 %). On the other hand the group B produced ejaculate of bad quality with low sperm motility and concentration (number of animals = 10; motility of sperm cells 67 %, and their concentration was below  $430\ 000\ \text{mm}^{-3}$ ; percentage of pathological sperm cells 6 %).

No statistical significant changes in the volume of the ejaculate in both experimental groups of boars were observed (Fig. 2A). Motility of sperms (Fig. 2B) differed only minimally in the group A and remained at the same level during the whole experiment. On the other hand, significant reduction of motility of sperms was observed in the group B in the 90<sup>th</sup> and 135<sup>th</sup> day by 7 % and 8 %, respectively. Concentration of sperms (Fig. 2C) had a non-significant downward trend in the group A. The group B of boars showed also no changes in the concentration of sperms during the experiment.



**Figure 3.** Activities of antioxidant enzymes and concentration of metals in ejaculate of boars in both experimental groups A and B; glutathion peroxidase - GPx (A); selenium (B), superoxid dimutase - SOD (C); copper (D); metalothionein (E); zinc (F); pH (G); glucose (H). Results are expressed as mean from 3 measurements  $\pm$  standard deviation;  $P < 0.05$ .

In the evaluation of abnormal sperms (Fig. 2D), non-significant increase in the 135<sup>th</sup> day compared to the day 0 was observed in the group B. In the group A of boars, gradual increase in the

percentage of abnormal sperms was recorded - by 16 % in the 45<sup>th</sup> day, by 48 % in the 90<sup>th</sup> day and by 81 % in the 135<sup>th</sup> day when compared to the day 0.

During the experiment, the abnormalities in sperm cells under the action of heat stress were observed. Most often damage of acrosome and sperm integrity was observed. Sperm thus loses its fertilizing ability. Pathological changes that occur most numerous in boars during heat stress are shown in Fig. 3. These are mainly damage to the acrosome and flagellum.

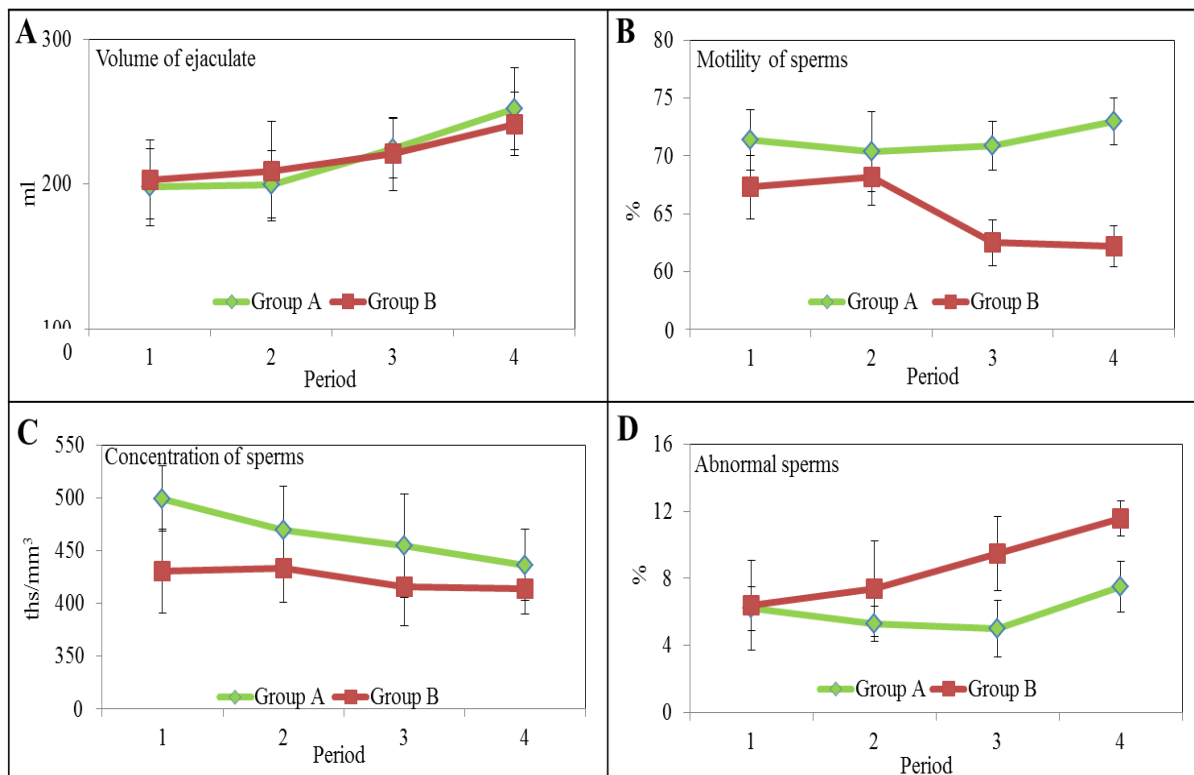
When measuring antioxidant activity using the FRAP method, no significant differences in both experimental groups were observed (Fig. 4A). The highest values were in both groups of boars (A and B) after 90 days of heat stress, 11.0 and 11.4 GAE (gallic acid equivalents)  $\mu\text{g/ml}$  which was increased by 64 % or 78 %, respectively. Values of FR had similar trend as FRAP values and reached maximal values also in the 90<sup>th</sup> day of heat stress (Fig. 4A) and were in the group A increased by 111 % and in the group B by 174 % in comparison with the day 0. On the other hand DMPD values (Fig. 4B) were significantly decreased in both groups (A and B) by 29 % and 32 %, respectively in the 45<sup>th</sup> day. In the 90<sup>th</sup> day, a decrease in the group A and B by 36% and 51%, respectively was observed when compared to the control. When measuring the antioxidant activity using DPPH method, a significant increase in the 45<sup>th</sup> day by 204 % in the group A and by 307 % in the group B compared to the control was observed. In the 90<sup>th</sup> day, DPPH values were in the group A significantly increased by 181 % and in the group B by 225% in comparison with control (Fig. 2B).

Antioxidant status determined by the ABTS method (Fig. 4C) was in both experimental groups of boars without statistical significance. Level of lipid peroxidation measured using the MDA method (Fig. 4C) was highest in the group B of experimental animals in the 45<sup>th</sup> day (by 47%). No significant changes in MDA values were determined in the experimental group A of boars during the whole experiment (Fig. 4C). Value of pH in ejaculate (Fig. 4D) decreased significantly in the group A in the 45<sup>th</sup>, 90<sup>th</sup> and 135<sup>th</sup> day by 10 %, 11 % and by 12 % respectively, compared to the control sampling. Similar trend was well evident in the group B, when a decrease in pH value was by 8 % in the 45<sup>th</sup> day, by 11 % in the 90<sup>th</sup> day, and by 10 % in the 135<sup>th</sup> day. Levels of glucose in ejaculate (Fig. 4D) showed significant differences during the experiment. The highest concentrations of glucose in both experimental groups A and B were determined in the 45<sup>th</sup> day, when an increase in their levels was by 119 and 158 %, respectively. At the end of the experiment (135<sup>th</sup> day), the group A showed an increase in the concentration of glucose only by 12 %, on the other hand, a decrease in the group B by 56 % in comparison with the control was well evident.

Next, values of antioxidant enzymes, metallothionein and metals were investigated. GPx is a selenoprotein [33] so results of GPx activity and selenium content of the boar ejaculates are shown together in figure 5A, B. Activity of GPx (Fig. 5A) increased from the beginning of the experiment in the group A of boars by 20 % in the 45<sup>th</sup> day and by 46 % in the 90<sup>th</sup> day without statistical significance. In the 135<sup>th</sup> day, activity of GPx was at the same level when compared with the control sampling (day 0). In the group B (Fig. 5B), the increase in GPx activity by 26 % in the 45<sup>th</sup> day and by 42 % in the 90<sup>th</sup> day was observed. Level of selenium (Fig. 5A, B) in the ejaculate was without significant difference in both experimental groups and remained at the same level.

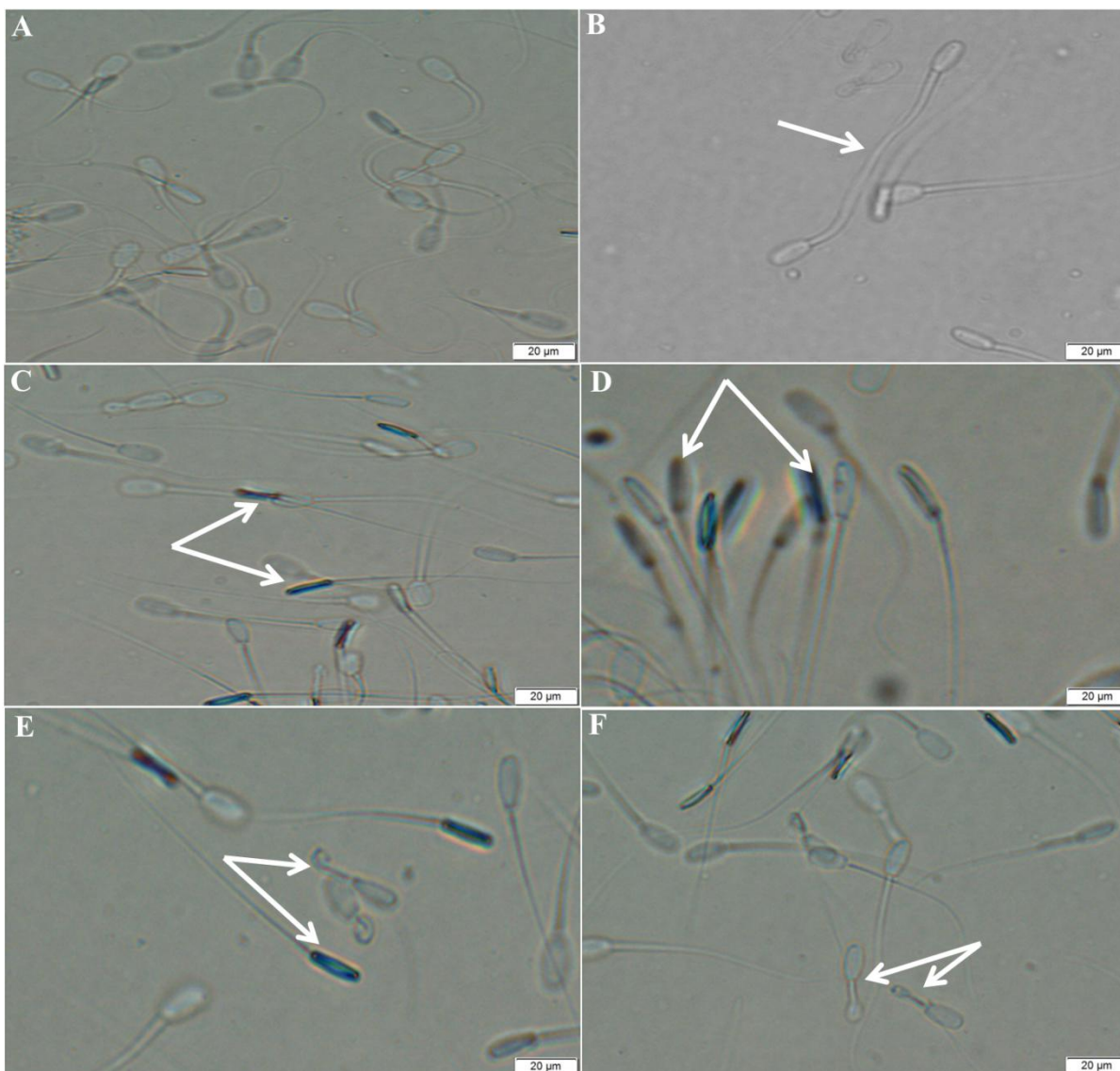
Metallothionein plays a key role in the homeostasis of heavy metals (especially zinc) and protection of cells from oxidative stress [34]. Concentration of metallothionein (Fig. 5C) increased in

the group A in the 45<sup>th</sup> and 90<sup>th</sup> day by 21 and by 23 %, respectively while a decrease in the 135<sup>th</sup> day (at the end of the experiment) by 64 % when compared with the day 0 was observed. On the other hand in the group B (Fig. 5D), a gradual decrease in metallothionein content was observed and in the 135<sup>th</sup> day reached 64 %. Amount of zinc in the ejaculate (Fig. 5C) gradually decreased and in the 135<sup>th</sup> day was by 52 % when compared to the control in the group A. Level of zinc in boar ejaculates of the group B (Fig. 5D) was stable in the 45<sup>th</sup> and 90<sup>th</sup> day. In the 135<sup>th</sup> day, a decrease by 30 % was well evident.



**Figure 4.** Indicators of quality of ejaculate of experimental boars of both groups A and B; volume of ejaculate (A); motility of sperms (B); concentration of sperms (C); abnormal sperms (D). Results are expressed as mean from 3 measurements ± standard deviation; P<0.05.

Copper is bound to the active site of SOD [33], so results of copper content and SOD activity are again shown together. Activity of SOD (Fig. 5E, F) was in the 90<sup>th</sup> day increased in both experimental groups A and B by 19 and 21 %, respectively in comparison with the control group. At the end of the experiment, activity of SOD was reduced by 39 % in the group A. On the other hand activity of SOD in the group B was reduced only by 23 %. Concentration of copper in the ejaculate was also significantly reduced in the 45<sup>th</sup> and 90<sup>th</sup> day (Fig. 5E, F). In the 45<sup>th</sup> day, a decrease in both groups A and B by 53 % and 77 % respectively was observed. In the 90<sup>th</sup> day, concentration of copper was reduced by 60 % in the group A. Similar trend was observed in the group B, too; a decrease by 61 % in comparison with the day 0.



**Figure 5.** Representation of normal (A) and abnormal sperms (B) double sperm (C, D, E) header damage of sperm (F) damage to the sperm flagellum that occur at heat stress.

#### 4. DISCUSSION

If the boar is exposed to stress conditions, whether it is physical stress or high temperatures, it produces a high amount of oxygen radicals [35]. This fact has been confirmed indirectly by our experiment and experimental data due to the fact that the boars were exposed to temperatures that are considered to be stressful according to Orzolek et al. [36]; the authors consider the temperature of 20 °C to be a threshold. At this temperature activity of SOD in ejaculate is reduced; this fact has not been confirmed in our experiment. Incubation of sperms of rams at 37 °C led to a decrease in the sperm motility ( $P < 0.05$ ) and a reduction of sperm viability. Authors observed also increased lipid peroxidation evaluated as MDA [37]. Incubation of ejaculate at 37 °C caused increased damage in DNA of sperms [38]. In the study, which examined the effect of the summer season on reproductive

performance of boars, decrease in sperms motility ( $P < 0.05$ ) and sperm concentration was detected similarly as in our study [39].

In an experiment, in which the temperature was monitored throughout the year, it has been found that the lowest quality of boar ejaculate occurs from August to September, when sperm motility, sperm concentration, and the percentage of abnormal sperm decreases significantly ( $P < 0.05$ ) [40]. Similarly, in our experiment we observed significant decrease in sperm motility and an increase in the rate of abnormal sperms ( $P < 0.05$ ) in these months. When examined mice that were exposed for 21 days to temperature of 35 °C, significantly reduced insemination index in females that were fertilized by this ejaculate has been shown [41]. According to some authors, feed intake at high temperatures is responsible for the decline in ejaculate quality [42].

In another experimental monitoring, in which the effects of season on reproductive performance of boar ejaculates was compared, it has been found that when the temperature rises above 25 °C, volume of the ejaculate decreases; however, we observed the opposite trend, the volume of ejaculate increased in both experimental groups of boars. High temperature in the summer months also reduces feed intake in pigs [43]. Furthermore, a decline in sperm motility ( $P < 0.05$ ) and an increase in the percentage of abnormal sperms ( $P < 0.05$ ) [44] was observed similarly as in our experiment (especially in the group B of boars), similar conclusions have been shown by Egbunike et al. [45].

In an experiment in which the antioxidant activity of ejaculate of breeding bulls was evaluated, it has been found that increased production of free radicals decreases sperm motility significantly [46]. In our experiment, we observed increased antioxidant activity determined by FRAP, FR, DPPH, and ABTS and also increased activities of GPx and SOD in both experimental groups (A and B) at higher temperature; this fact could indicate that organisms activate their protective antioxidant mechanisms against free radicals. Significant reduction of sperm motility was well evident in the group B of boars that had also the lowest level of antioxidant activity measured by the methods FR, ABTS, and decreased activity of GPx and reduced level of metallothionein in comparison with the group A.

According to Maya-Soriano et al. [47] and Meyers [48], free radicals affect especially mitochondria that serve as an energetic source for sperm and enable its movement. Damage of mitochondria leads to the decreased sperm motility. Increased production of SOD shown as an increase in SOD activity is able to increase the motility and viability of sperms [49]. In our experiment, the activity of SOD was increased only slightly at the second and third samplings; after it, it decreased in the boars of both groups at the end of the experiment. However, we did not record an increase in the motility and concentration of sperms. Nevertheless, it is possible that significant decrease in level of copper and zinc ( $P < 0.05$ ) is caused also by relatively lower activity of SOD. Increased production of free radicals may be caused also by lower temperatures [50]. A range of temperature between 10 – 17 °C is optional in terms of low production of free radicals and keeping the quality of ejaculate [51].

One of the basic principles is the good management [52]. At higher temperatures (above 27 °C), animals are restless and the overall performance of the organism can decrease [53]. Antioxidant activity of ejaculate may be increased by supplementation of animals with selenium. Activity of GPx and antioxidant activity measured by ABTS and FR methods can be effectively increased in this way [54]. Similar results were observed also in sows after birth, when the supplementation with selenium in organic form significantly increased antioxidant status measured using FRAP, FR, ABTS, and GPx

activity during this period of heat stress [55]. Antioxidant status of animals can be improved also by other dietary supplements, such as zinc and copper [56], vitamin E [57], or taurine [58].

## 5. CONCLUSIONS

During this period, the average daily temperature was monitored; average temperature varied from 12.3 to 29.2 °C during the experiment. The quality of ejaculate in the first group A (n=10) was good. The second group B (n=10) produced ejaculate with low motility and low sperm concentration. In the group A, a decreased value of DMPD by 71.4 % (P <0.05), an increase in DPPH for 104 % (P<0.05), a decrease in SOD activity by 39.2 % (P<0.05), decrease in levels of copper by 59.5 % (P<0.05), zinc by 51.9 % (P<0.05), metallothionein by 63.9 % (P<0.05), and pH by about 11.0 % (P<0.05) were recorded. In the group B, a decreased value of DMPD by 68.0 % (P<0.05), an increase in DPPH by 207 % (P<0.05), reduced levels of copper by 77.6 % (P<0.05), metallothionein by 63.9 % (P<0.05), zinc by 30.1 % (P<0.05), and value of pH by 11.0 % (P<0.05) were determined. In the evaluation of quality of ejaculate, a decrease of sperm motility by 7.6 % (P<0.05) and an increase of number of abnormal sperms by 81.3 % (P<0.05) were observed in the group B.

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## Conflict of interest

The authors have declared no conflict of interest

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