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# OXYGRAPHIC EVALUATION OF ACTIVITY OF RESPIRATORY ENZYMES IN BOAR'S SEMEN

# OKSYGRAFICZNA OCENA AKTYWNOŚCI ENZYMÓW ODDECHOWYCH PLEMNIKÓW W NASIENIU KNURA

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**Streszczenie.** W pracy opisano zalety zastosowania metody oksygraficznej do analizy metabolicznej mitochondrialnych enzymów oddechowych plemników knurów. Badania wykonano na nasieniu pobranym od 36 knurów 4 ras, w wieku od 8 do 40 miesięcy. Wszystkie próby nasienia charakteryzowały się przynajmniej 80-procentową zawartością plemników o ruchu postępowym. Zużycie tlenu mierzono za pomocą sondy tlenowej typu Clarka, sprzężonej z rejestratorem analogowo-cyfrowym. Pomiary wykonano w temperaturze 37°C, a szybkość zużycia tlenu przez plemniki (v) wyrażano w nmol $O_2 \cdot min^{-1}$ na $10^8$ plemników. Podstawę analizy stanowiły wartości szybkości oddychania po podaniu bursztynianu ($v_S$) i PMS ($v_P$). Uzyskane wyniki wskazują, że opisana w pracy metoda oksygraficznej oceny aparatu ruchu plemnika umożliwia precyzyjne badanie stanu układu oddechowego plemników i tym samym pozwala na selekcję osobników o najlepszych parametrach nasienia, z uwzględnieniem ich cech indywidualnych, rasy i wieku; powinna stanowić uzupełnienie badań nasienia podczas selekcji knurów do stacji unasienniania, po ich chorobie, w badaniach okresowych nasienia, a także przy podejmowaniu decyzji o eliminacji knura.

**Key words:** boar, insemination, spermatozoa, mitochondria, respiratory chain.
**Słowa kluczowe:** knur, inseminacja, plemniki, mitochondria, łańcuch oddechowy.

## INTRODUCTION

Boars sperm undergo extensive testing, and semen parameters such volume, concentration, consistency and color of ejaculate, pH, motility and morphology are routinely determined. However, each of these studies has certain limitations and therefore can't be regarded as an unambiguous measure of assessing the ability of sperm to fertilize. With the development of analytical techniques there is the possibility of using tests that were not previously possible. Such tests should be oxygraphic measuring the activity of enzymes of the respiratory chain in mitochondria of spermatozoa.

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Each cell, in particular gametes, needs a considerable amount of chemical energy to perform its biological program. In the case of spermatozoa, practically all pool of ATP synthesized in a cell (70–80%) is consumed in the processes associated with the movement (Bielański 1979; Strzeżek 1996). This means that sperm motility test should be given priority. This is confirmed by research of Tardiff et al. (1999), who used semen doses with the sperm concentration lower than the standard recommended, showed a positive correlation between the number of motile sperm and fertility of boars.

The main place "manufacturing" of this energy are mitochondria, where oxidative processes occur and the associated synthesis of ATP. The substrates are cytoplasmic products of fructolysis and molecular oxygen. The energy transformation of substrates in ATP energy takes place in the respiratory chain, positioned in the inner membrane of the mitochondria. The mitochondrial respiratory chain is composed of four distinct multi-subunit complexes (complex I, II, III and IV) and two electron shuttle molecules (ubiquinone and cytochrome c) – Lenaz and Genova (2010). The first and the largest component of the mitochondrial respiratory chain is complex I – oxidoreductase NADH: Q (EC 1.6.5.3) – Koopman et al. (2010). Succinate represents the substrate of succinate dehydrogenase or complex II (dehydrogenase succinate; EC 1.3.5.1) – Rutter et al. (2010). Complex III (CoQHa-cytochrome *c* reductase; EC 1.10.2.2) or cytochrome bc1 complex operates as a homodimer in the inner mitochondrial membrane (Zara et al. 2009). The last component of the mitochondrial respiratory chain is complex IV, or cytochrome c oxidase (EC 1.9.3.1) – Fontanesi et al. (2008). Sometimes ATP synthase (EC 3.6.1.34) is described as complex V of the electron transport chain, responsible for the synthesis of ATP (Boyer 1997).

Due to a very high demand on ATP spermatozoa formed in the course of evolution a efficient apparatus of oxygen assimilation and oxygen consumption. The performance of this apparatus is differentiated species specific. For example, the boars spermatozoa are capable of absorbing the oxygen with about 30% greater speed than the bull sperm under comparable conditions (Kordan and Strzezek 1997; Piasecka et al. 2004).

Assuming that the knowledge of the metabolic efficiency of the energy system of sperm for artificial insemination facilitates, the decision meeting of the breeder in the rational use of the breeding materials, we made a study whose purpose was to demonstrate the usefulness of the oxygraphic method to measure the activity of enzymes of the mitochondrial respiratory system of boar spermatozoa.

## MATERIAL AND METHODS

All reagents used in the work were of analytical grade – potassium chloride (0205/6, Chempur, Poland), magnesium chloride (779118-6, Standard, Poland), potassium phosphate (742020112, 742100117, POCH, Poland), EDTA (POCH, Poland ), Tris (Chempur, Poland), BSA (A7906, Sigma, USA), sodium hydrosulfite (POCH, Poland), ascorbic acid (A5960, Sigma, USA), TMPDA- N, N, N ', N'-tetramethyl-p-phenylenediamine (T3134, Sigma, USA), rotenone (R8875, Sigma , USA), antimycin A (A8674, Sigma, USA), KCN (Analchem, Poland), succinic acid (Roanal, Hungary), PMS- phenazine methosulphate (P9625, Sigma, USA).

The study was performed on semen collected from 36 boars, 4 breeds: Polish Landrace – 17 ejaculates, Polish Large White – 9 ejaculates, Duroc x Pietrain – 8 ejaculates and Per-An--Lan – 2 ejaculates, aged 8 to 40 months, coming from the Breeding and Insemination Station. All attempts sperm were characterized by at least 80% content of motile spermatozoa. Fresh semen was transported in a thermo-container at 17 ± 2°C about 2 hours after the collection to the biochemical laboratory of the Department of Physiological Chemistry of Westpomeranian University of Technology in Szczecin, where they were prepared for oxygraphic analysis. The analytical procedure was initiated not later than 3 hours from time of collection of the semen.

It included five successive steps: 1) separation of sperm from seminal fluid, 2) the removal of the cytoplasmic membrane from the middle piece and the unveiling of mitochondria, 3) rinsing the remains of the cytoplasmic membrane and enzymes as well as endogenous respiratory substrates, 4) spermatozoa counting and dilution, 5) evaluation of mitochondrial membrane integrity.

In the first step semen was centrifuged for 10 minutes at 800 rpm, spermatozoa were collected, and then suspended in isotonic phosphate buffer pH 7.4 (12.5 mmol · $dm^{-3}$ , $K_2HPO_4$, 2.5 mmol · $dm^{-3}$ $K_2HPO_4$, 113.0 mmol · $dm^{-3}$ KC1, 3.0 mmol · $dm^{-3}$ $MgCl_2$, 20.0 mmol · $dm^{-3}$ Tris, 0.4 mmol · $dm^{-3}$ EDTA) and centrifuged. In order to bind free fatty acids that are present in seminal plasma, bovine serum albumin (BSA, 0.2% w/w) was added to the buffer. The process of rinsing and centrifugation was repeated three times. In the second step, the sperm were resuspended in hypotonic (150 mOsm) phosphate buffer pH 7.4 (1.134 g $K_2HPO_4$ + 0.290 g $K_2HPO_4$) and kept on ice for 90 minutes (Piasecka et al. 2001). The incubation in hypotonic buffer allows the cytoplasmic membrane of the sperm to tear and expose the underlying mitochondria, which are associated with middle piece, without destruction of the integrity of mitochndrial membranes. The separation of the cytoplasmic membrane makes it easier to contact exogenous substrates and reagents with the mitochondrial enzymes of the respiratory chain (Baccetti 1984; Hrudka 1987, Willoughby et al. 1996; Jones 1997; Vazquez et al. 1997). In the third step, spermatozoa lacking the cytoplasmic membrane were washed three times in isotonic phosphate buffer pH 7.4, EDTA and BSA (Piasecka et al. 2001).

In the fourth step the concentration of spermatozoa in the resulting formulations was determined in Burker's chamber and diluted with isotonic phosphate buffer at pH 7.4 (without EDTA, and BSA) in such a manner that the final concentration of sperm in each sample was $10^8$ spermatozoa · $cm^{-3}$ *(*Keyhani and Storey 1973; Strzeżek 1996*)* The resultant preparations – spermatozoa suspended in isotonic phosphate buffer pH 7.4 (without EDTA and BSA) – were subjected to two tests designed to confirm: 1) the removal of the cytoplasmic membrane, 2) maintain the integrity of mitochondrial membranes. The first test is to observe the color of the sperm suspension in the presence of ascorbic acid and TMPDA. Lipophilic TMPDA, unlike the ascorbate ion, easily passes across the cytoplasmic membrane and tries to mitochondria where it is oxidized by cytochrome oxidase, and becomes blue. The lack of the cytoplasmic membrane allows for immediate reduction of the oxidized form of TMPD by the ascorbate and elimination of color (Kimelberg and Nichols 1969). Missing staining of the sample examined demonstrates successful removal of the cytoplasmic membrane.

The second test is designed to exclude damage to mitochondrial membranes during sample preparation. The test consists of measuring the rate of oxygen consumption by the mitochondria

incubated in media containing ascorbate (200 μmol · $dm^{-3}$), and TMPDA (40 μmol · $dm^{-3}$). The catalyst for this reaction is cytochrome oxidase interacts with cytochrome c. In the space between the membranes of mitochondria is located the pool of endogenous cytochrome c; in consequence of the disruption of the mitochondrial outer membrane, he would reach the environment, thus preventing the reaction progress. In this case, the administration of exogenous cytochrome c should increase the rate of reaction. In contrast, no acceleration of reaction after the administration of exogenous cytochrome c shows the maintaining of integrity of mitochondrial membranes during the preparation (Piasecka et al. 2001).

The rate of oxygen consumption was measured using a Clark-type oxygen probe (YSI 5331, USA), coupled to the analog-to-digital recorder. The measurements were performed at 37°C. The electrode was calibrated against water saturated with air at a temperature of 37°C to obtain a complete aerobic conditions and in the presence of sodium hydrosulfite ($Na_2S_2O_4$) added to obtain anaerobic conditions. It was assumed that oxygen concentration in the pure water at temp. 37°C is 215.01 μmol · $cm^{-3}$ (Carpenter 1966; Djakiew and Cardullo 1986). The measuring system consisted of a thermostated glass cells of a volume of 1.5 $cm^3$, a magnetic stirrer, oxygen probe connected with an abundance corks and a place to insert the tip of the capillary pipette, analogue-digital transformation unit with a sensitivity 10–15 $O_2$ · $dm^{-3}$ and computer with corresponding software (Medson, Poland), allowed the recording and digital processing of the signal (Fig. 1). Measurements were made in the sperm suspension in an isotonic buffer, pH 7.4 (without EDTA, and BSA) in 37°C. The substrates and/or inhibitors were introduced with a capillary tip, which allowed to provide solutions in a volume of 10 μl.

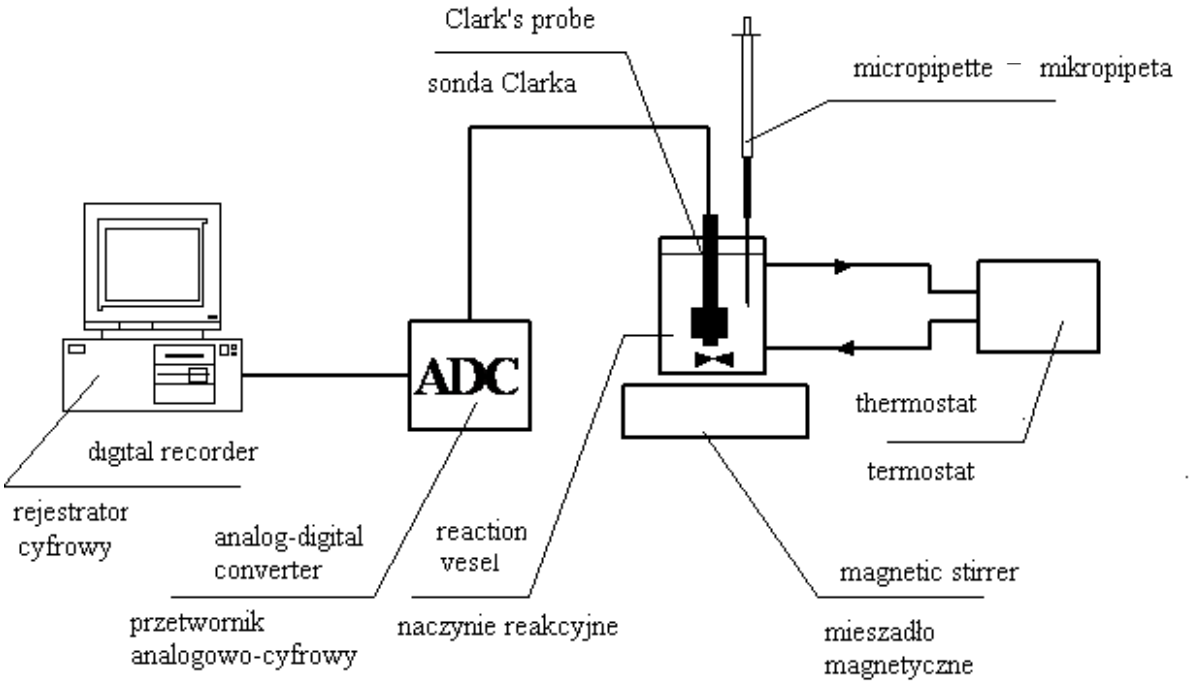


Fig. 1. Diagram of the measuring system to be tested mitochondrial respiratory activity
Ryc. 1. Schemat układu pomiarowego przeznaczonego do badania aktywności oddechowej mitochondriów

The rate of oxygen consumption by the sperm (v) are expressed in nmol $O_2 \cdot min^{-1} \cdot 10^{-8}$ sperm cells. In order to verify the effectiveness of the substrates, inhibitors or artificial (non--physiological) electron acceptors in each series of measurements, control measurements were performed to confirm the specificity of each enzyme reaction and the entrance of substrates to the respiratory chain.

In the first phase of the measurement, during the pre-incubation of sperm, to the reaction mixture was added rotenone (6 μmol · $dm^{-3}$) – an inhibitor of NADH oxidoreductase Q (complex I of the respiratory chain). In the actual examination, after stabilization of the system (2–4 min), to the reaction mixture was added succinate (4 mmol · $dm^{-3}$) – specific substrate of the complex II of respiratory chain. Then the activation of complexes II, III, IV takes place. After the registration of the oxygen consumption by the presence of succinate the process was inhibited with either antimycin A (10 μmol · $dm^{-3}$) – inhibitor of the complex III or with potassium cyanide (1 mmol · $dm^{-3}$) – inhibitor of the complex IV of the respiratory chain. The reaction stopped because of Inhibition of the oxidoreductase $QH_2$: ferrocytochrome c or cytochrome oxidase. Then, after equilibration, to the reaction mixture was added PMS (500 μmol · $dm^{-3}$) – artificial electron acceptor, "collecting" electrons from the inner mitochondrial membrane and transferring them to oxygen. This procedure allowed the inhibition of complex I, III, IV, and to examine only the oxidative activity of the complex II. An example result of an experiment are presented in Fig. 2.

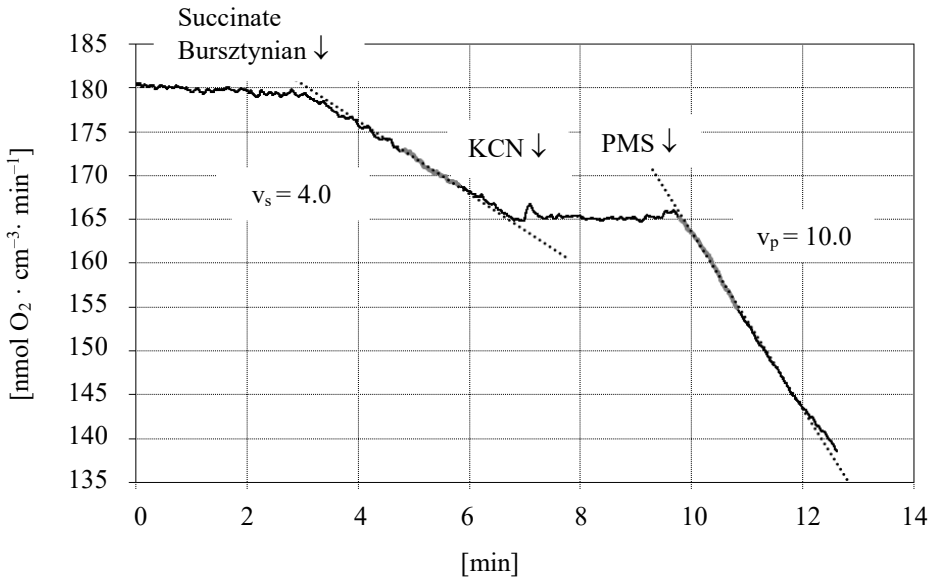


Fig. 2. The rate of oxygen consumption by mitochondria of boar's spermatozoa ($10^8$ spermatozoa per 1 $cm^3$). The horizontal line in the first phase of the graph means no endogenous substrates in the measurement system; following administration of succinate reaction occurs involving oxidoreductase succinate $O_2$; administration of KCN results in the inhibition of cytochrome oxidase and stop the reaction; PMS application restores oxygen consumption involving oxidoreductase succinate : PMS $O_2$. The measure of the reaction rate is the slope of the oxygen consumption after administration of the substrate and the electron acceptor $v_S$ – succinate $v_P$ – PMS

Ryc. 2. Szybkość zużycia tlenu przez mitochondria plemników knura ($10^8$ plemników w 1 $cm^3$). Pozioma linia w pierwszej części wykresu oznacza brak substratów endogennych w układzie pomiarowym; po podaniu bursztynianu następuje reakcja z udziałem oksydoreduktazy bursztynian : $O_2$; podanie KCN prowadzi do inhibicji oksydazy cytochromowej i zatrzymania reakcji; podanie PMS przywraca zużycie tlenu z udziałem oksydoreduktazy bursztynian: PMS → $O_2$. Miarą szybkości reakcji jest nachylenie linii zużycia tlenu po podaniu substratu i akceptora elektronów: $v_S$ – bursztynianu, $v_P$ – PMS

The obtained data were statistically processed using the software MedCalc v4.15a. The structure of the tested population is represented by the average value (Mean) and standard deviation (SD). For comparison of means the t-test was used. It was assumed that two unrelated average variable significantly different when the calculated probability (Pu-unpaired) is less than the value of 0.05.

## RESULTS AND DISCUSSION

This paper presents the measurement results of two mitochondrial respiratory activities: oxidoreductase succinate: O2 oxidoreductase and succinate: PMS —> O2 (Fig. 3).

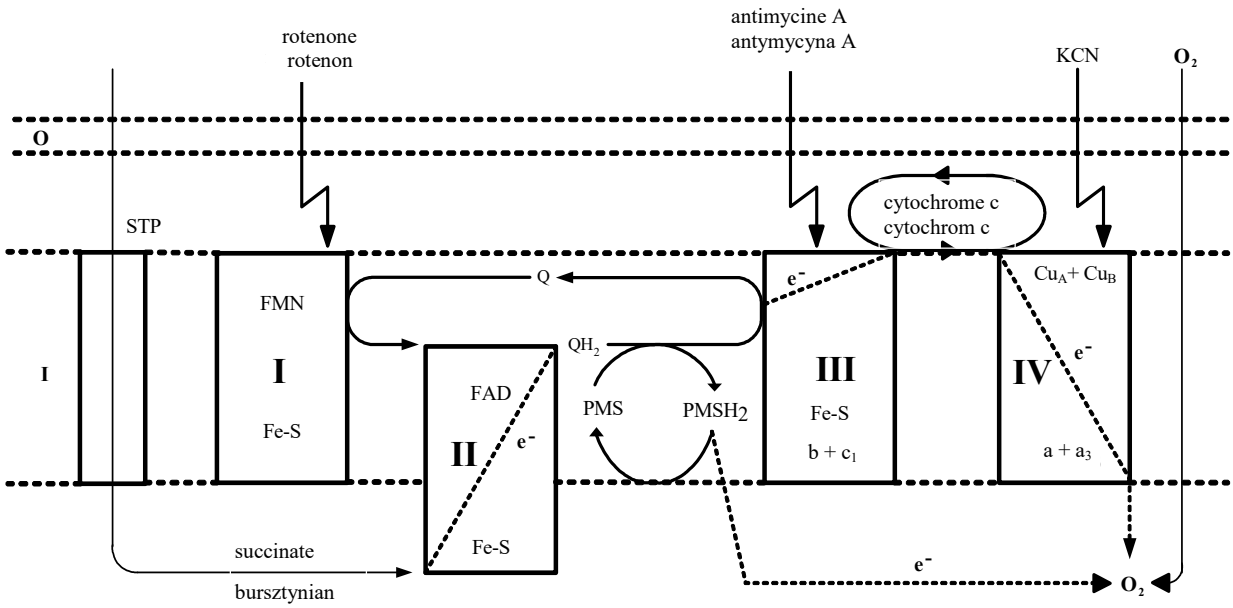


Fig. 3. Diagram of measurement of mitochondrial respiratory activity: oxidoreductase succinate : $O_2$ oxidoreductase and succinate : PMS $\rightarrow$ $O_2$. Z – the outer mitochondrial membrane; W – inner mitochondrial membrane; STP – transport protein succinate; $e^-$ – electrons; $H^+$ – translocated protons; Q $\rightarrow$ $QH_2$ – ubiquinone (CoQ); FMN – flavin mononucleotide; FAD – flavin-adenin dinucleotide; Fe-S – group of iron-sulfur; b + $c_1$, c, a + $a_3$ – cytochromes; $Cu_A$+ $Cu_B$ – prosthetic atoms; PMS – artificial oxygen scavenger; succinate – substrate succinate dehydrogenase (II)
Ryc. 3. Schemat pomiaru mitochondrialnych aktywności oddechowych: oksydoreduktazy bursztynian : : $O_2$ i oksydoreduktazy bursztynian : PMS $\rightarrow$ $O_2$. Z – zewnętrzna błona mitochondrialna; W – wewnętrzna błona mitochondrialna; STP – białko transportujące bursztynian; $e^-$ – elektrony; $H^+$ – translokowane protony; Q $\rightarrow$ $QH_2$ – ubichinon (CoQ); FMN – mononukleotyd flawinowy, FAD – dinukleotyd flawinowo-adeninowy; Fe-S – grupy żelazowo-siarkowe; b + $c_1$, c, a + $a_3$ – cytochromy; $Cu_A$ + $Cu_B$ – atomy prostetyczne; PMS – sztuczny akceptor tlenu; succinate – substrat dehydrogenazy bursztynianowej (II)

Both measurements were performed sequentially in the same reaction system. The efficiency of the energy system of boar spermatozoa were determined by the rate of mitochondrial respiration after adding succinate ($V_S$) and PMS ($v_p$). The values are summarized in Table 1.

Table 1. Activity of oxidoreductase succinate : $O_2$ ($v_S$) and oxidoreductase succinate : PMS → $O_2$ ($v_P$) in boar's spermatozoa depending on the breed and age
Tabela 1. Badanie aktywności oksydoreduktazy bursztynian : $O_2$ ($v_S$) i oksydoreduktazy bursztynian : : PMS → $O_2$ ($v_P$) w plemnikach knurów z podziałem na rasy i grupy wiekowe

| Breed – Rasa | Age – Wiek [months – – miesiące] | n | Spermatozoa [nmol $O_2 \cdot min^{-1}$ per $10^8$] [nmol $O_2 \cdot min^{-1}$ na $10^8$ plemników] | | | | $v_S/v_P$ | |
|---|---|---|---|---|---|---|---|---|
| | | | $v_S$ | | $v_P$ | | | |
| | | | mean średnia | SD | mean średnia | SD | mean średnia | SD |
| Polish Landrace Polska Biała Zwisłoucha | 8–12 | 2 | 6.0 | 0.99 | 7.6 | 1.99 | 0.79 | 0.70 |
| | 13–30 | 9 | 2.8 | 0.74 | 5.5 | 2.42 | 0.50 | 0.17 |
| | 31–40 | 6 | 2.0 | 0.76 | 6.1 | 2.82 | 0.35 | 0.15 |
| Polish Large White Wielka Biała Polska | 8–12 | 5 | 4.6 | 0.94 | 6.9 | 2.43 | 0.68 | 0.19 |
| | 13–30 | 4 | 3.5 | 0.22 | 5.7 | 2.57 | 0.45 | 0.04 |
| Duroc × Pietrain | 8–12 | 8 | 2.7 | 1.21 | 5.8 | 3.19 | 0.58 | 0.11 |
| Pen-Ar-Lan | 13–30 | 2 | 3.6 | 0.60 | 3.9 | 0.59 | 0.94 | 0.29 |

The analysis of numerical ratio $V_S/V_P$ presented in Table I indicating a dependency of the race and does not preclude its correlation with age of boars. In all cases studied, the $V_S/V_P$ values characterized by a negative trend in relation to age of the boar. Because of the small number of measurements, this relationship was not statistically secured. However, an analysis of probability of the null hypothesis – Pu obtained for samples with the highest numbers (Polish Landrace, Pu = 0.1592; Polish Large White, Pu = 0.2065) suggests that the $V_S/V_P$ could prove differences in respective age groups to be significant at a larger number of animals. More clearly seems a relationship between $V_S/V_P$ and breed of boars. For example, in the age group 13–30 months for the breeds Pen Ar Lan and Polish Landrace.

The $v_s$ and $V_P$ values shown are connected to each other by the activity of succinate dehydrogenase. Standard tests of enzyme activity (substrate conversion rate in products) is recommended to measure with an excess of substrate. The values obtained then correspond to the full activity of the examined enzyme (maximum catalytic rate) and are – in compliance with the stable external measurement conditions – proportional to the amount of enzyme to be measured. Such assumptions meet the $V_P$ value measurement. Therefore, this value can be taken as a measure of the activity of succinate dehydrogenase (associated with the inner mitochondrial membrane) in the test system.

Oxygraphic method allows independent investigation of several kinetic processes of oxygen consumption by intact mitochondria. A prerequisite for this is to modify the catalytic' machinery of the mitochondrial respiratory chain appropriate so that a part of enzymes and / or electron-transporting proteins is "excluded" (inhibited) and respiratory substrates (physiological or non-physiological) are added. Selected measurement strategies allow the assessment of the activity of both individual enzymes as well as of all catalytic routes. The collected empirical data can be used as a basis for the conclusion both catalytic performance of single enzymes as well as about the condition of the mitochondrial apparatus of the energy transformation. The results of investigation of Ferramosca et al. (2007) in human showed a proper functionality of the various mitochondrial respiratory chain complexes and a tight coupling between respiration and phosphorylation. They found significant decrease in the mitochondrial respiratory efficiency in asthenozoospermic subjects.

The analysis of the experimental data from the Table 1 leads to the conclusion that the machinery of energy transformation in boar spermatozoa is characterized by considerable variability. Although the results indicate a certain dependence on the breed and age of boars, but these relations were not confirmed statistically due to the small size of compared groups. Other factors, e.g. season (Koprianiuk et al. 2014), and individual properties can't be excluded. Also the oxidative stress may play a role, because Ferramosca et al. (2013) found its negative effect on human sperm mitochondrial respiration The sperm from boars of various breeds are characterized with different number of mitochondria (different values of $V_P$). In young animals, both the activity of the respiratory enzymes and the $V_S/V_P$ ratio were high. With age decreases the number of active mitochondria and – considerably clearer – the activity of cytochrome-t oxidase, an enzyme which is responsible for the oxygen utilization in the cell. Since the loss of $v_s$ speed is greater than $v_p$, it can be concluded that the respiratory enzymes lose activity unevenly, and that the cytochrome c oxidase (complex IV of the respiratory chain) is the enzyme, its activity is more dependent on the age. Less efficient oxygen use is directly linked with the efficiency reduction of ATP production, which result in a reduction of the ability of spermatozoa to exercise longer work. Since in the measuring system is an equal number of spermatozoa, and the carrier of the enzyme which are situated in the mitochondria, the value of $V_P$ measures is the amount of metabolically active mitochondria, present in a single cell. Complemented by the study is to measure the value of $v_s$, which brings information on the efficiency of the electron transport system in the provides respiratory chain of mitochondria. In fact, the $v_s$ -speed is the speed of the slowest stage of the system transferred the electrons from succinate transferred to molecular oxygen. We believe that the limiting factor is the activity of cytochrome oxidase. Such a conclusion is justified by the ratio $v_S/v_P \rightarrow 1$. If this ratio is determined in the same study, it may allow relative assessment of the activity of at least two enzymes and represents a good measure of catalytic efficiency of different semen samples. In our studies the value of this ratio was always less than 1. This means that the limiting factor for the speed of the oxidation succinate can't be the activity of succinate dehydrogenase.

## CONCLUSIONS

Investigation of the mitochondrial bioenergetics of sperm will provide more information on the role of sperm mitochondria in motility and on the overall quality of the gametes. The introduction of the of the above presented method in the examination of boar sperm, due to its sensitivity and independence from the subjective assessment of the laboratory, will help to improve the performance of insemination and selecting boars with superior sperm. It can produce clear and repeatable test results, and allow better assess individual ejaculates than the commonly used tests based on the microscopically observation of motility. It ensures a precise examination of the respiratory system condition of the sperm and thus allows the selection of the boars with the best sperm characteristics. This method can be recommended as a supplementary sperm tests in boars after diseases, in periodic sperm tests and in deciding on the eliminate of the boar from insemination.

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**Abstract.** In the article advantages of the use of oxygraphic method for metabolic examination of mitochondrial respiratory enzymes in boar spermatozoa were described. Investigations were performed on semen samples taken from 36 boars of 4 breeds, at the age from 8 to 40 months. All samples of semen characterized at least 80% content of motile spermatozoa. The rate of oxygen uptake was measured with the use of Clark type electrode connected with analog-digital recorder. Measurements were executed in temperature 37°C, and the rate of oxygen uptake (v) was expressed in nmol $O_2 \cdot min^{-1}$ per $10^8$ spermatozoa. The values of oxygen consumption rate after application of succinate ($v_s$) and PMS ($v_p$) made up the basis of analysis. The results show that the method of oxygraphic evaluation of spermatozoa movement system described in the paper makes possible the precise investigation of the spermatozoa respiratory chain status and enables selection of boars with the best parameters of semen. This method can be taken in consideration at semen examination in the course of boar's selection for the AI stations, after boar's reproductive disease, in periodical semen tests and also when taking decision about the boar's elimination.