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*Original article*

# Seasonal-dependent variations in metabolic status of spermatozoa and antioxidant enzyme activity in the reproductive tract fluids of wild boar/domestic pig hybrids

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## Abstract

This study investigated seasonal changes in the metabolic performance of spermatozoa and activity of the antioxidant enzymes in the seminal plasma of three wild boar/domestic pigs (aged 1.5 to 2.5 years) and the activity of the antioxidant enzymes in fluids of the cauda epididymidis and vesicular glands from 16 wild boar/domestic pig hybrids (aged 1 to 3 years). Parameters of the sperm metabolic activity, such as total motility, mitochondrial functions, and measurements of oxygen uptake, ATP content and L-lactate production, were analyzed during the spring-summer and autumn-winter periods. Besides these sperm metabolic parameters, the sperm membrane integrity was also assessed. Total protein content and activity of the antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), were measured in the reproductive tract fluids. There were no marked significant differences ( $P > 0.05$ ) between the seasonal periods in terms of sperm motility, mitochondrial function and oxygen uptake; however, spermatozoa collected during the autumn-winter period exhibited higher ( $P < 0.05$ ) ATP content and L-lactate production than those harvested during the spring-summer period. It was found that the vesicular gland fluid exhibited a higher level of SOD activity during the spring-summer period compared with the autumn-winter period. Furthermore, CAT activity in the seminal plasma and vesicular gland fluid was greater during the autumn-winter. Total protein content was significantly higher in the vesicular gland fluid, whereas the cauda epididymidal fluid exhibited greater SOD and GPx activities, irrespective of the seasonal period. The findings of this study confirmed seasonal-related differences in the metabolic performance of spermatozoa and activity of antioxidant enzymes of the reproductive tract of the boar/domestic pig hybrids.

**Key words:** spermatozoa, wild boar/domestic pig hybrids, metabolic activity, antioxidant enzymes

## Introduction

Seasonal effects in both the male and female pigs have been documented in numerous studies (Claus and Weiler 1985, Claus et al. 1985, Mauget and Boissin 1987, Strzezek et al. 2000, Sancho et al. 2004). Photoperiod fluctuations have been considered to be one of main environmental factors that is responsible for reproductive seasonality in the pig (Claus and Weiler 1985, Peltoniemi et al. 2000). Accumulating evidence has been shown that seasonal changes have a marked effect on the quality of semen of the European wild boar (Kozdrowski and Dubiel 2004a, Klimas et al. 2012) and domestic boar (Kondracki et al. 1997, Ciereszko et al. 2000, Strzezek et al. 2000, Sancho et al. 2004).

In the European wild boar seasonal changes in the reproductive cycle were associated with fluctuations in hormone levels, as evident in high testosterone concentrations during the autumn-winter period (Mauget and Boissin 1987). According to Kozdrowski and Dubiel (2004a), ejaculates collected from the European wild boar in the late autumn exhibited better semen quality, in terms of ejaculate volume, sperm concentration and activity of seminal plasma enzymes. Moreover, in the wild boar/domestic pig hybrid semen collected during the autumn-winter period was characterized by higher total number of spermatozoa in the ejaculate, sperm motility and morphology (Kozdrowski and Dubiel 2004b).

The antioxidant defense system of the male reproductive tract comprises mostly three enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), which provide protection to cellular structures against oxidative damage (Zini and Schlegel 1996, Peeker et al. 1997, Aitken and Vernet 1998, Strzezek 2002, Kozirowska-Gilun et al. 2011a,b). Studies in our laboratory have shown that seasonal changes have a marked effect on the antioxidant defense system of the boar reproductive tract (Kowalowka et al. 2008, Kozirowska-Gilun et al. 2011b). This study reported on the effect of seasonal variations on the metabolic performance of spermatozoa and activity of the antioxidant enzymes of the seminal plasma, and fluids of the cauda epididymidis and vesicular glands of the wild boar/domestic pig hybrids.

## Materials and Methods

### Animals and semen collections

This study was divided into two experiments: (i) Experiment 1 (Exp. 1) was based on the analysis of

the sperm metabolic activity and (ii) Experiment 2 (Exp. 2) comprised analysis of the antioxidant enzyme activity in the seminal plasma and fluids of the cauda epididymidis and vesicular glands of the wild boar/domestic pig hybrids. Also, seminal plasma, obtained from the 3 pig hybrids in Exp. 1, was used for the analysis of the antioxidant enzyme activity (in Exp. 2).

In Exp. 1 ejaculates were collected manually from three wild boar/domestic pigs (aged 1.5 to 2.5 years), housed at the laboratory research facility of the Department of Animal Biochemistry and Biotechnology, Olsztyn (Poland), for a period of 12 months. The ejaculates were allocated in two seasonal periods: spring-summer (April through September) and autumn-winter (October through March). A total of 15 ejaculates were collected from the 3 pig hybrids in each seasonal period. Following collections, the semen samples were subjected to macro- and microscopical analyses.

In Exp. 2 the fluids of the cauda epididymis and vesicular glands were collected from 16 wild boar/domestic pig hybrids (aged 12-36 months), stationed at the Experimental Farm Branch Campus of Faculty of Biotechnology, University of Rzeszów, Werynia near Kolbuszowa (Poland). The fluids of the cauda epididymidis and vesicular glands were collected from 8 pig hybrids during the spring-summer and autumn-winter periods, respectively, according to a previously described method (Kozirowska-Gilun et al. 2011a). Permission to conduct these studies was granted by the Local Ethics Committee.

### Assessment of the sperm metabolic status

#### Motility evaluations

Aliquots of semen samples were placed on pre-warmed slides, covered with a glass cover slide and examined under a light microscope (Olympus Bx 40, Tokyo, Japan). Total sperm motility was evaluated at 200 × magnification under a light microscope, equipped with a heated stage (37°C).

#### Mitochondrial function

The percentage of spermatozoa with functional mitochondria was assessed in semen samples ( $30 \times 10^6$  spermatozoa/ml), using dual fluorescent staining with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (Molecular Probes, Eugene, USA) with propidium iodide (PI), according to a previously described method (Thomas et al. 1998),

with some modifications (Dziekońska et al. 2009). Aliquots (10  $\mu$ l) of stained sperm cells were examined under a fluorescence microscope (Olympus CH 30 RF-200, Tokyo, Japan). Sperm cells displaying only orange-red fluorescence in the mid-piece region were classified as viable spermatozoa with functional mitochondria. Two slides were assessed per sample, and approximately 100 spermatozoa were evaluated per slide.

### Oxygen uptake

Oxygen uptake was measured polarographically using a YSI Model 5300 Biological Oxygen Monitor (Yellow Springs Instrument, Co., Inc., Yellow Springs, Ohio, USA), with the application of a Clarke's electrode, according to the manufacturer's protocol. Oxygen measurements were expressed as  $\mu$ L O<sub>2</sub>/10<sup>8</sup> spermatozoa/1h/37°C.

### ATP content

The ATP content in spermatozoa was determined according to a protocol provided by the Bioluminescence Assay Kit (Roche Molecular Biochemical Company), using a Junior bioluminometer (Berthold Technologies, GmbH & Co. KG, Germany). The ATP content in spermatozoa was calculated from a standard ATP curve and expressed as nmol/10<sup>8</sup> spermatozoa.

### L-lactate production

The rate of L-lactate production in spermatozoa was determined according to a previously described method (Rodriguez-Gil and Rigau 1995), with some modifications (Dziekońska et al. 2009). Sperm pellets (100  $\times$  10<sup>6</sup> spermatozoa/ml) were re-suspended in a buffer containing 2.94% (w/v) sodium citrate and 5.4% (w/v) fructose (pH 7.4), and incubated for 1h at 37°C. Following incubation, the mixture was centrifuged (600  $\times$  g, 5 min at room temperature), and L-lactate production was measured in the supernatant using a reagent kit (Pointe Scientific Inc., Canton, Michigan, USA). Measurements were performed using a spectrophotometer (Beckman DU<sup>®</sup> - 62) at a wavelength of 550 nm and L-lactate production was expressed as nmol/10<sup>8</sup>spermatozoa/1h/37°C.

### Assessment of plasma membrane integrity

The fluorochrome PI (Sigma, St. Louis, MO, USA) was used to assess sperm plasma membrane

integrity, according to a previously described method (Fraser et al. 2002). Membrane-intact spermatozoa did not emit any fluorescence, whereas membrane-damaged spermatozoa displayed red fluorescence. Plasma membrane integrity was defined as the percentage of spermatozoa with intact membrane. Aliquots (10  $\mu$ l) of stained sperm cells were examined under a fluorescence microscope (Olympus CH 30 RF-200, Tokyo, Japan). Two slides were assessed per sample, and approximately 100 spermatozoa were evaluated per slide.

### Biochemical analysis of the reproductive tract fluids

Measurements of total protein content and activity of the antioxidant enzymes were performed in the seminal plasma obtained from the 3 pig hybrids in Exp. 1, and fluids of the cauda epididymidis and vesicular glands (Exp. 2).

The seminal plasma was separated from the ejaculates of the pig hybrids by centrifugation at 1000  $\times$  g for 15 min at room temperature. The recovered seminal plasma was further centrifuged at 10 000  $\times$  g for 10 min at room temperature and stored at -80°C, until required for further analysis.

### Protein content

The total protein content was determined by the biuret protein assay (Weischelbaum, 1946), using serum bovine albumin (BSA, Serum and Vaccine Production, Cracow, Poland), as a standard.

### Activity of antioxidant enzymes

The activities of SOD, CAT and GPx were measured using commercial kits, according to the manufacturers' instructions. All assays were performed in duplicates.

### SOD assay

The activity of SOD was measured using Randox RANSOD assay (Randox Laboratories, Crumlin, Great Britain). This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. One unit (U) of SOD activity was defined as the amount of the enzyme that caused 50%

Table 1. Seasonal effects on metabolic activity and plasma membrane integrity of spermatozoa of the wild boar/domestic pig hybrids.

Sperm parameters	Seasonal periods	
	spring-summer (n=15)	autumn-winter (n=15)
Total motility	72.55 ± 0.59	74.27 ± 0.68
Mitochondrial function (%)	95.22 ± 0.27	95.3 ± 0.77
Oxygen uptake (µl O <sub>2</sub> /10 <sup>8</sup> spermatozoa/1h /37°C)	23.59 ± 1.45	25.66 ± 5.56
ATP content (nmol /10 <sup>8</sup> spermatozoa)	13.89 ± 0.48 <sup>x</sup>	16.89 ± 0.66 <sup>y</sup>
L-lactate (nmol /10 <sup>8</sup> spermatozoa/1h /37°C)	749.23 ± 38.78 <sup>x</sup>	1017.58 ± 99.89 <sup>y</sup>
Plasma membrane integrity (%)	92.56 ± 1.22	94.02 ± 0.86

Values represent the mean (± SEM) of 15 ejaculates from 3 wild boar/domestic pigs. Spring-summer vs Autumn—winter values with different letters (x, y) are significant at P < 0.05.

Table 2. Seasonal effects on total protein content and activity of antioxidant enzymes of the reproductive fluids of the wild boar/domestic pig hybrids.

Parameters	Seminal plasma		Cauda epididymidal fluid		Vesicular gland fluid	
	Spring-summer (n=15)	Autumn-winter (n=15)	Spring-summer (n=8)	Autumn-winter (n=8)	Spring-summer (n=8)	Autumn-winter (n=8)
Total protein content (mg/ml)	52.41 ± 8.51 <sup>a</sup>	46.83 ± 5.8 <sup>a</sup>	19.50 ± 0.28 <sup>b</sup>	22.14 ± 2.00 <sup>b</sup>	114.33 ± 21.00 <sup>c</sup>	143.00 ± 5.74 <sup>c</sup>
SOD (U/ml)	1.47 ± 0.03 <sup>a</sup>	1.40 ± 0.03 <sup>a</sup>	8.08 ± 0.06 <sup>b</sup>	7.66 ± 0.19 <sup>b</sup>	1.61 ± 0.27 <sup>x,a</sup>	0.95 ± 0.16 <sup>y,a</sup>
CAT (U/ml)	0.66 ± 0.01 <sup>x,a</sup>	1.24 ± 0.20 <sup>y,a</sup>	1.44 ± 0.20 <sup>b</sup>	1.26 ± 0.20 <sup>a</sup>	0.83 ± 0.10 <sup>x,ab</sup>	5.83 ± 1.00 <sup>y,b</sup>
GPx (U/ml)	0.26 ± 0.05 <sup>a</sup>	0.23 ± 0.05 <sup>a</sup>	0.59 ± 0.05 <sup>b</sup>	0.54 ± 0.05 <sup>b</sup>	0.30 ± 0.09 <sup>a</sup>	0.20 ± 0.07 <sup>a</sup>

Seminal plasma analysis represents the mean (± SEM) of 15 ejaculates from 3 wild boar/domestic pigs. Analysis of the fluids of the cauda epididymidis and vesicular gland represents the mean (± SEM) 8 wild boar/domestic pigs. Spring-summer vs Autumn-winter values with different letters (x, y) are significant at P < 0.05. Within seasonal period, values with different letters (a, b, c) are significant at P < 0.05. SOD: superoxidase dismutase; CAT: catalase; GPx: glutathione peroxidase.

inhibition in the rate of reduction of INT at 37°C (pH 7.0). The activity of SOD was measured spectrophotometrically at 505 nm and expressed as U/ml.

### CAT assay

A commercial kit (Sigma Aldrich Corp., St. Louis, MO, USA) was used to measure CAT activity by monitoring the decrease in hydrogen peroxide. One unit (U) of catalase decomposed 1.0 micromole of hydrogen peroxide to oxygen and water per minute at 25°C (pH 7.0). The activity of CAT was measured at 520 nm and expressed as U/ml.

### GPx assay

The activity of GPx was determined, using the Ransel Glutathione Peroxidase kit (Randox Labora-

tories, UK). In this assay, GPx catalyzes the oxidation of glutathione (GSH) with cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH, oxidized glutathione (GSSG) is converted to GSH with a concomitant oxidation of NADPH to NADP<sup>+</sup>. The decrease in absorbance was measured at 340 nm at 37°C (pH 7.2), and the GPx activity was expressed as U/ml.

### Statistical analysis

The results are expressed as the mean ± standard error of the mean (SEM), and were analyzed by the analysis of variance (ANOVA) followed by Duncan's multiple range test (P < 0.05). All data were analyzed with the Statistica software package (StatSoft Incorporation, Tulsa, OK, USA). A significant difference was denoted at P < 0.05.

## Results

### Experiment 1 (Exp. 1)

There were no significant seasonal variations ( $P > 0.05$ ) in sperm total motility, mitochondrial function, rate of oxygen uptake and plasma membrane integrity (Table 1). Spermatozoa sampled in the autumn-winter period were characterized by a significantly higher ( $P < 0.05$ ) ATP content and L-lactate production than those collected during the spring-summer period (Table 1).

### Experiment 2 (Exp. 2)

Table 2 shows the effect of seasonal variations on total protein content and activity of the antioxidant enzymes of the reproductive tract fluids of the wild boar/domestic pig hybrids. No marked seasonal differences in total protein content, SOD and GPx activities in the seminal plasma were observed. However, CAT activity was significantly higher ( $P < 0.05$ ) in the seminal plasma during the autumn-winter period compared with the spring-summer period. There were marked seasonal differences in SOD and CAT activities in the vesicular gland fluid. It was found that SOD activity in the vesicular gland fluid was approximately 1.7-fold higher during the spring-summer period compared with the autumn-winter period. In contrast, the vesicular gland exhibited about 7-fold higher level of CAT activity during the autumn-winter period. The cauda epididymidal fluid did not exhibit any marked seasonal differences ( $P > 0.05$ ) neither in the total protein content nor in the analyzed activities of the antioxidant enzymes. Total protein content was significantly higher ( $P < 0.05$ ) in the vesicular gland fluid, whereas the cauda epididymidal fluid exhibited greater ( $P < 0.05$ ) SOD and GPx activities, irrespective of the seasonal period. Also, CAT activity was highest ( $P < 0.05$ ) in the vesicular gland during the autumn-winter period compared with the seminal plasma and cauda epididymidal fluid.

## Discussion

This study investigated, for the first time, seasonal variations in some selected markers of the metabolic activity of spermatozoa of the wild boar/domestic pig hybrids. Even though there were not any marked seasonal changes in sperm motility, mitochondrial function and the rate of oxygen uptake, it was observed that spermatozoa collected during the autumn-winter period exhibited higher ATP content and L-lactate

production than those obtained during the spring-summer period. According to Halang et al. (1985), approximately 80% of the energy generated in the form of ATP by mitochondrial oxidative phosphorylation and glycolysis is used to maintain the functionality of the sperm motility apparatus. It should be noted that assessments of the sperm mitochondrial function and measurements of the rate of oxygen uptake have been used to evaluate the efficiency of oxidative phosphorylation (Dziekońska et al. 2009), whereas the sperm glycolytic activity has been monitored by the measurements of the rate of L-lactate production in spermatozoa, using fructose as the exogenous substrate (Rigau et al. 1996). Previous studies showed that boar spermatozoa could produce L-lactate during glycolysis under aerobic conditions (Jones 1997, Dziekońska et al. 2009). In the current study, it is therefore possible that glycolysis (fructolysis) was the predominant metabolic pathway for spermatozoa of the wild boar/domestic pig hybrids during the autumn-winter period, as indicated by the significantly higher L-lactate production and ATP content. In the boar it was reported that increased sperm metabolic performance during the autumn-winter period had a beneficial effect on the quality of liquid-stored semen (Fraser et al. 2003). However, it should be emphasized that high ATP content in boar spermatozoa does not always coincide with enhanced sperm motility (Dziekońska et al. 2009), as observed in the findings of this study.

The findings of this study showed that there were seasonal changes in the antioxidant defense system of wild boar/domestic pig hybrids. In the boar, the bulk of the antioxidant protection in the different reproductive tract fluids is provided by superoxide dismutases (Kowalowka et al. 2008, Kozirowska-Gilun et al. 2011a,b). The superoxide dismutases, a family of metalloenzymes which scavenge superoxide radicals, consist of three isoforms: cytosolic (Cu/Zn SOD, SOD1), mitochondrial (Mn-SOD, SOD2) and extracellular SOD (Ec-SOD, SOD3) (Pecker et al. 1997, Aitken and Vernet 1998, Mruk et al. 2002, Strzezek 2002, Kowalowka et al. 2008). The Randox RANSOD assay used in this study showed that seasonal changes did not have any significant effect on SOD activity in the seminal plasma and cauda epididymidal fluid. However, the finding of this study and those of another study (Kozirowska-Gilun et al. 2011b) showed that there was a large variability in SOD activity between the different reproductive tract fluids of the boar and wild boar/domestic pig hybrids, regardless of the seasonal period. It is possible that such variation in SOD activity might be due to differences in the assay procedure of the enzyme extraction. The Randox RANSOD assay is specific for SOD1 (Skrzycki et

al. 2009), whereas the methods of Beauchamp and Fridovich (1971) and Kowalowka et al. (2008) measure primarily the activity of SOD3, which is abundant in large amounts in the boar reproductive tract. Moreover, higher SOD activity observed in the cauda epididymidal fluid compared with the vesicular gland fluid might be in response to the production of high level of reactive oxygen species (ROS) in the cauda epididymidis of the wild boar/domestic pig hybrids. Similar assertions have been suggested for boar cauda epididymidis, which contributes larger amounts of SOD to the seminal plasma compared with the vesicular glands (Koziorowska-Gilun et al. 2011b). These findings reaffirm the role of SOD as a major antioxidant scavenging enzyme of the reproductive tract of the pig hybrids.

The antioxidant enzymes, CAT and GPx, are involved in the conversion of hydrogen peroxide to water and are implicated in providing protection against ROS-mediated attack (Zini and Schlegel 1996, Aitken and Vernet 1998). The results of this study have confirmed that the vesicular gland fluid contributes large amounts of CAT to the seminal plasma during the autumn-winter period, whereas GPx activity was stable in the reproductive tract fluids, regardless of the seasonal period. It is noteworthy that, similarly to SOD activity, greater GPx activity in the cauda epididymidal fluid suggests the important role of the antioxidant enzyme in protecting cellular tissues and spermatozoa of the cauda epididymidis of the wild boar/domestic pig hybrids.

The secretory activity of the reproductive organs of the wild boar is seasonal and marked effect, in terms of better semen quality, was observed in the late autumn (Kozdrowski and Dubiel 2004a). It has been reported that the semen quality of the wild boar or domestic boar does not differ from that of the wild boar/domestic pig hybrids in the autumn and winter (Kozdrowski and Dubiel 2004a,b). In our study seasonal-related differences in the sperm metabolic performance, characterized by L-lactate production and ATP content, were more marked during the autumn-winter period in the wild boar/domestic pig hybrids. As regard the antioxidant enzymes, seasonal changes were more evident in the vesicular gland fluid for SOD and CAT activity during the spring-summer and the autumn-winter periods, respectively. However, further research studies are needed on a large number of animals to fully explain the effect of seasonality on the characteristics of semen and antioxidant defense system of the reproductive organs of the wild boar/domestic pig hybrids.

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