DE GRUYTER OPEN

Bull Vet Inst Pulawy 59, 279-286, 2015
DOI: 10.1515/bvip-2015-0041

Boar sperm quality in relation to presence of sp32-like protein in spermatozoa - preliminary studies

Aleksandra Orzołek, Paweł Wysocki, Jerzy Strzeżek, Magdalena Koziorowska-Gilun, Anna Dziekońska, Władysław Kordan

Department of Animal Biochemistry and Biotechnology
University of Warmia and Mazury in Olsztyn, 10-719 Olsztyn, Poland
aleksandra.deszczka@uwm.edu.pl

Received: December 19, 2014 Accepted: June 3, 2015

Abstract

The aim of the study was to analyse sperm proteomes of ejaculates from Polish Large White (PLW) and Polish Landrace (PL) boars and to identify differences which putatively influence semen quality. Spermatozoa protein profiles were analysed by electrophoretic methods followed by selected techniques to evaluate semen quality on the following factors: sperm motility, lipid peroxidation levels (MDA production), ATP content, activities of superoxide dismutase (SOD) and catalase (CAT), total antioxidant status (TAS), and total oxidant status (TOS) of seminal plasma. A protein with an estimated molecular weight of 30 kDa was found in spermatozoa of selected ejaculates. Mass spectrometry demonstrated that this polypeptide is most similar to proacrosin binding protein (sp32). The presence of the protein was more frequently observed in sperm extracts obtained in spring-summer period. Ejaculates containing sp32-like protein demonstrated significantly higher spermatozoa motility, lower inhibition of MDA production by seminal plasma, and higher SOD activity in seminal plasma. Boar semen which included sp32-like protein also demonstrated lower ATP levels in spermatozoa as well as higher TAS and lower TOS of seminal plasma, though the differences were not statistically significant. Ejaculates from PLW boars, with sp32-like protein present in sperm, were characterised by significantly higher sperm motility, lower ATP content in spermatozoa, and higher TAS of seminal plasma. The diminished parameters of semen quality were observed in ejaculates from PL boars that also contained the discussed protein, but the differences were not statistically significant. These findings suggest that the presence of sp32-like protein in boar spermatozoa could influence semen quality.

Keywords: boar, spermatozoa, proacrosin binding protein, semen quality.

Introduction

Spermatozoa derived from different boars may vary in viability, motility, plasma membrane integrity, and fertilising ability (22). A complete analysis of an ejaculate should include a standard spermocytogram (percentage of viable and motile spermatozoa) as well as a biochemical evaluation of seminal plasma composition (27). Standard methods of assessing semen quality are no longer sufficient (13). Boar ejaculates may contain vast percentages of spermatozoa with morphological and structural changes that could form specific subpopulations of dysfunctional cells. The size of dysfunctional cell populations varies between individuals as well as between ejaculates from the same animal. A high percentage of abnormal gametes is associated with low fertilising capability, lower embryo quality, and lower farrowing rate (19). Ejaculate quality can be improved with the involvement of a two-pronged approach by eliminating abnormal/non-functional spermatozoa or diagnosing factors that contribute to low semen quality (6). The aim of contemporary andrology is to search for individual differences in ejaculates (8). Some evidence suggests that differences in semen quality are determined by the protein and lipid content of the sperm plasma membrane, the content of seminal plasma, and the function of accessory sex glands (28). The transcriptomes and proteomes of boar testes and epididymides have not yet been fully studied, and the existing studies focus on the identification and characteristics of spermatozoa proteins (12). The sperm

© 2015 A. Orzołek et al. This is an open access article distributed under the Creative Commons Attribution-NonCommercial-NoDerivs license (http://creativecommons.org/licenses/by-nc-nd/3.0/)

proteome contains everything a spermatozoon needs to survive and function correctly (23). Previous studies demonstrated numerous relations between the presence of selected proteins and sperm maturity (6)**,** and between protein phosphorylation and the capacitation state of boar spermatozoa (11, 16, 20). Recent research indicates that proteins involved in capacitation (*e.g*. phospholipid hydroperoxide glutathione peroxidase - PHGPx, mitochondrial pyruvate dehydrogenase E1 component subunit β – PDHB, or acrosin binding protein - ACRBP) can be used in the process of designing biomarkers for predicting the quality of boar sperm. Such studies are essential for the identification and development of valid biomarkers of male fertility and, consequently, semen quality (16).

To date, several sperm-specific proteins have been identified with the involvement of proteomic approaches, such as two-dimensional polyacrylamide gel electrophoresis, mass spectrometry, and differential in-gel electrophoresis (23). For this reason, the main aim of this study was to analyse sperm proteomes derived from Polish Large White (PLW) and Polish Landrace (PL) boars, and to identify differences which putatively influence semen quality.

Material and Methods

Animals and semen collection. The experimental animals were sexually mature PLW (5 individuals) and PL (5 individuals) boars. The animals were fed a standard diet and were kept under standard housing conditions with exposure to natural daylight. All ejaculates were collected by the gloved-hand technique. Semen samples were obtained in 2 collection periods: spring-summer (April to September) and autumn-winter (October to March). During the experiment, 50 sperm-rich fractions (with 5 ejaculates from each individual) were collected and evaluated in both periods. Sperm-rich fractions had an estimated volume of 120 mL each. This approach supported the acquisition of samples with higher sperm concentrations and an almost complete spectrum of seminal plasma proteins.

Sperm extracts. The collected semen was filtered through sterile gauze to remove the gel fraction. Two millilitres of fresh sperm-rich fractions were centrifuged at 3000 × g at room temperature for 5 min to remove seminal plasma, which was then stored at –20°C for further analyses. Sperm pellets were twice rinsed with 2 mL of 0.85% NaCl and centrifuged at 3000 × g, followed by 10000 × g. Supernatants were removed. Sperm proteins were extracted with 2 mL of buffer containing 10 mM Tris-HCl, 1 mM sodium orthovanade, and 1% Triton X-100 (pH 7.4). Such a methodical approach led to obtaining a sizeable group of sperm proteins in their intact and native state. The prepared sperm extracts were frozen at –20°C prior further analyses.

Total protein content. Total protein content was determined according to the method proposed by Lowry *et al.* (18) with bovine serum albumin (BSA, Serum and Vaccine Production, Cracow, Poland) as the standard.

SDS-PAGE electrophoresis. Sperm proteins were separated in 12% SDS-PAGE gels in 50 mM Tris, 250 mM glycine, and 0.5% SDS (pH 8.3) buffer in the Mini Protean II Cell apparatus (Bio-Rad, USA) according to Laemmli (17). Concentrated lysis buffer (1 M Tris-HCl, 20% SDS, 20% glycerol, 2% β-mercaptoethanol, 2% bromophenol blue, pH 6.8) was added to samples containing 1.25 mg of protein/1 mL. The probes were heated at 95ºC for 5 min. Protein separation was conducted at constant voltage of 150 V. Precision Plus Protein Standards (Bio-Rad, USA) served as molecular mass standards. Protein bands obtained in SDS-PAGE electrophoresis were stained with silver (13).

Isoelectric focusing (IEF). IEF was conducted in the Mini-Protean II Cell (Bio-Rad, USA) apparatus. After 30 min of polymerisation, IEF gels (38% urea, 10% glycerol, 2% Triton X-100, 5.5% acrylamides, 6% ampholytes 3/10, 0.1% TEMED, 0.375% ammonium persulfate) were subjected to pre-electrophoresis at 300 V for 15 min and at 600 V for 30 min. The samples were prepared to obtain protein concentration of 1.25 mg/mL and were diluted 1:1 with buffer containing 24% urea, 25% glycerol, 2.5% DTT, 10% ampholytes 3/10, and 3% Triton X-100. After 30 min of incubation, probes were loaded onto gels. The anode buffer consisted of 20 mM CH_3COOH and 20 mM NaOH served as the cathode buffer. Isoelectric focusing was performed at 800 V for 3.5 h.

The gels were stained with colloidal Coomassie Blue solution (0.1% Coomassie Brilliant Blue G-250, 3% orthophosphoric acid, 20% ethanol, 10% ammonium sulphate) for 12 h. They were transferred to destaining buffer (10% ethanol, 2.5% ortophosphoric acid) and washed for several times in deionised H_2O.

Mass spectrometry. Gel sections containing selected polypeptides were cut out with a scalpel. Proteins were identified by trypsin degradation and liquid chromatography (LC). The results were compared against the protein sequence database on the Mascot server (Matrix Science Inc., USA).

Assessment of sperm motility. Sperm motility was evaluated under a light microscope (200×) (Nikon OPTIPHOT-2, Japan) equipped with a heated stage (37°C) (Makler Chamber, Sefi Medical Instruments, Israel). Sperm motility was assessed with ±5% accuracy. Fresh semen with minimum 70% motility, measured after caffeine treatment, was used in further analyses.

Determination of lipid peroxidation levels. Lipid peroxidation levels were determined according to

the method described by Tappel and Zalkin (25) with some modifications (24). The evaluated parameter was measured spectrophotometrically by the production of malondialdehyde (expressed as the amount of nM MDA produced by 1×10^8 spermatozoa after 1 h incubation at 37°C). The capacity of the seminal plasma to inhibit MDA production (% inhibition) was also determined.

Assessment of sperm ATP content. The ATP content of sperm was measured in accordance with the instructions given in the Bioluminescence Assay Kit CLSH (Roche Molecular Biochemical). ATP was extracted from spermatozoa by adding 900 µL of buffer (100 mM Tris, 4 mM EDTA; pH 7.75) to 100 µL of diluted semen. The prepared samples were heated to 100°C for 10 min, then cooled to room temperature and stored for further analyses at -20ºC. Bioluminescence was measured with the Junior Bioluminometer (Berthold Technologies, Germany). ATP content was determined with the use of an ATP standard curve and expressed in nmolATP/10^8 spermatozoa.

Assessment of superoxide dismutase (SOD) activity. SOD activity was measured in seminal plasma. The enzyme activity was estimated with the use of the RANSOD reagent kit (RANDOX Laboratories Ltd., CrumLin, UK) according to the manufacturer's instructions and expressed in U/mL.

Assessment of catalase (CAT) activity. CAT activity was measured in seminal plasma. The measurement was conducted with the use of a commercial reagent kit (Sigma-Aldrich Inc., USA) according to the manufacturer's instructions and expressed in µM/mL/min.

TAS and TOS measurements. The total antioxidant status (TAS) of seminal plasma was measured with the use of the TAS reagent kit (RANDOX Laboratories, UK) according to the manufacturer's instructions. The total oxidant status (TOS) of seminal plasma was assessed according to the method described by Erel (10). Absorbance was measured at 600 nm with the Beckman DU 62 spectrophotometer (Beckman Coulter, USA). The result was determined from a standard curve and expressed in terms of hydrogen peroxide micromoles in 1 L (µmol H_2O_2 Equiv/L).

Statistical analysis. Semen quality parameters associated with the presence of sp32-like protein were evaluated by Student's *t*-test. The data were averaged over 5 samples within both periods for each individual. The results were presented as means and standard errors of the means (SEM). The data were processed in the Statistica programme version 12.0 (StatSoft, Poland). Statistical differences were estimated for $P \leq 0.05$.

Results

SDS-PAGE electropherograms of sperm extracts revealed approximately 15 protein bands of molecular weights ranging from 10 to more than 200 kDa. SDS electrophoresis demonstrated the presence of protein bands with an estimated molecular weight of 30 kDa in selected sperm extracts (Fig. 1). IEF revealed that the presence of the analysed polypeptide was correlated with the season. The protein was more frequently observed in sperm extracts collected in spring-summer period (Fig. 2). The presence of the 30 kDa protein fraction was not confirmed in the majority of autumn-winter period sperm extracts (Fig. 2). The discussed protein appeared more frequently in ejaculates from PLW boars (Fig. 1 and 2).

The protein with an estimated molecular weight of 30 kDa was subjected to mass spectrometry, which revealed that the analysed polypeptide was most similar to proacrosin binding protein (sp32) and acrosin binding protein (ACRBP) (Table 1). In the literature, proacrosin binding protein is often used interchangeably with acrosin binding protein, although the two differ in their mode of action.

Table 1. Similarity of the examined polypeptide to selected proteins in boar (*Sus scrofa*) semen identified by mass spectrometry

Protein	MW (kDa)	pI	Score	NCBInr
Proacrosin-binding protein (sp32)	61641	4.82	1240	Q29016
Acrosin-binding protein	61929	4.85	1240	XP003126 581
Fibronectin isoform 2	275640	5.41	378	XP003133690
AWN-1 spermadhesin	14995	9.33	251	AAB21990
Sperm surface protein (sp47)	46722	6.15	220	P79385
Leucine-rich repeat-containing protein (37A2-like)	106049	7.08	143	XP003131631
Inactive serine protease 54-like	43324	5.95	102	XP003355 844
Chain B of PSPI/PSPII spermadhesins	12866	8.72	100	1SPPB
AQN-3 spermadhesin	13104	8.91	76	P24020
PSP-I spermadhesin	12203	7.82	68	S23942

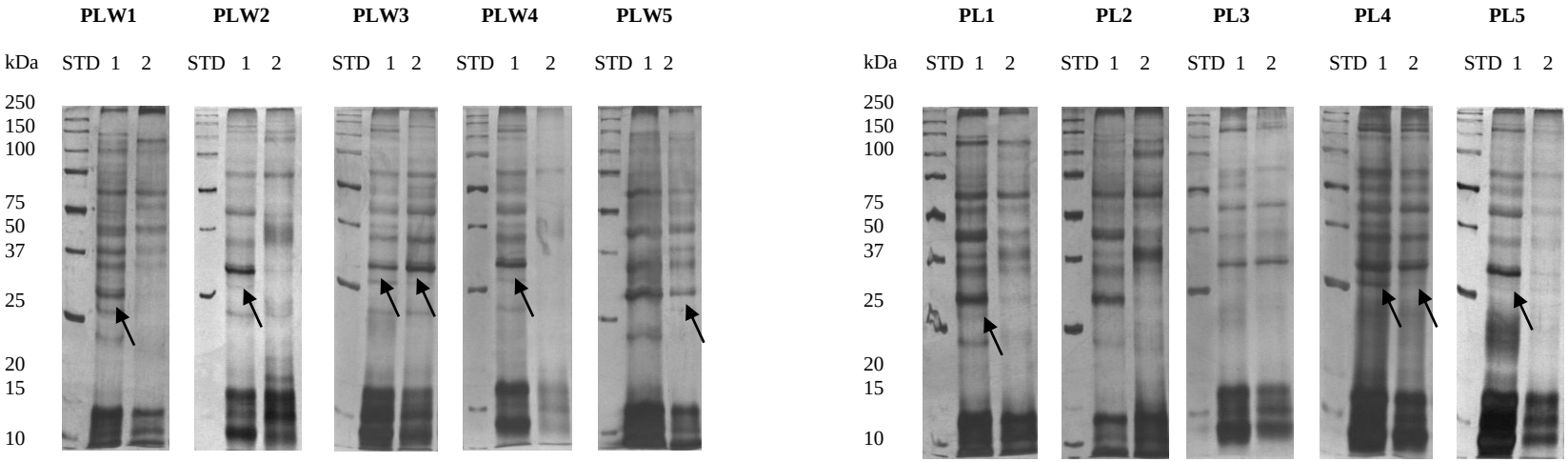


Fig. 1. Selected electropherograms of sperm extracts of fresh ejaculates from PLW and PL boars; 1 – spring-summer period; 2 – autumn-winter period; STD – Precision Plus Protein Standards; 30 kDa protein fractions are indicated by arrows

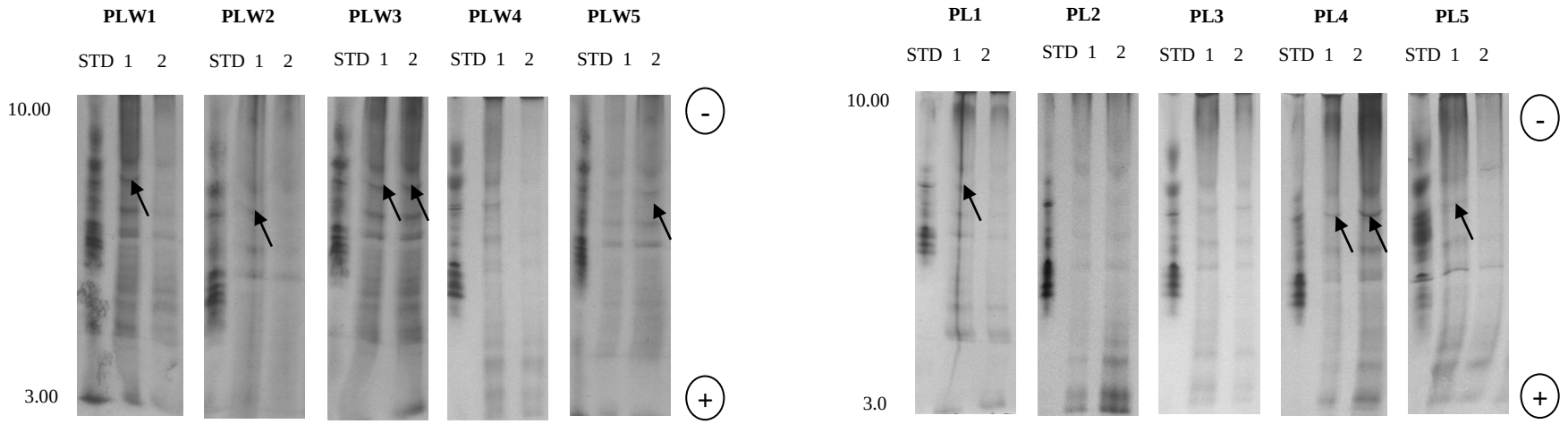


Fig. 2. Selected isoelectrofocusing profiles of sperm extract proteins from ejaculates of PLW and PL boars; 1 – spring-summer period; 2 – autumn-winter period; STD – Haemoglobin; 30 kDa protein fractions are indicated by arrows

The presence of sp32-like protein in sperm extracts was associated with significantly higher sperm motility, higher level of superoxide dismutase activity in seminal plasma, and lower capacity of seminal plasma to inhibit MDA production. The presence of the discussed polypeptide was also associated with higher MDA production by spermatozoa, higher level of catalase activity in seminal plasma, lower ATP content in spermatozoa, higher total antioxidant status, and lower total oxidant status of fresh semen (Table 2). It should also be noted that semen quality parameters associated with the presence/absence of sp32-like protein did not differ in boars of the 2 evaluated breeds. Ejaculates from PLW boars containing sp32-like protein were characterised by significantly higher sperm motility, lower ATP content, and higher TAS value of seminal plasma. Among PLW ejaculates there were also ascertained higher MDA production and lower capacity of seminal plasma to inhibit MDA production, higher SOD and CAT activities levels in seminal plasma, and lower TOS value of seminal plasma, though the differences were not statistically significant (Table 3). The diminished parameters of semen quality were observed in ejaculates from PL boars, which also contained mentioned protein (Table 3). In case of the ejaculates obtained from PL boars, statistical differences were not revealed.

Table 2. Relations between the presence of sp32-like protein and selected semen parameters

Parameter	Presence of sp32-like protein	Absence of sp32-like protein
Sperm motility (- caffeine)	71.8 ± 1.2^{a}	69.1 ± 1.2^{b}
Sperm motility (+ caffeine)	82.0 ± 1.2^{a}	79.7 ± 1.2^{b}
MDA production (nM/10^8 sperm)	22.0 ± 1.1	20.3 ± 1.0
Seminal plasma's capacity to inhibit MDA production (%)	38.6 ± 3.3^{a}	50.1 ± 3.7^{b}
ATP content (nM/10^8 sperm)	6.7 ± 0.5	8.0 ± 0.5
SOD activity (U/mL)	47.2 ± 6.8^{a}	25.1 ± 6.4^{b}
CAT activity (μM/mL/min)	2.5 ± 0.2	2.1 ± 0.2
TAS (mM)	0.83 ± 0.08	0.65 ± 0.06
TOS (μM H_2O_2)	5.2 ± 0.6	5.8 ± 0.5

Values are expressed as means ±SEM
Values with different superscripts (a, b) differ significantly at $P \leq 0.05$ within columns

Table 3. Relations between the presence of sp32-like protein, boar breed, and selected semen parameters

Parameter	Polish White Large (PLW)		Polish Landrace (PL)	
	Presence of sp32-like protein	Absence of sp32-like protein	Presence of sp32-like protein	Absence of sp32-like protein
Sperm motility (- caffeine)	74.2 ± 1.4^{a}	67.3 ± 1.5^{b}	69.3 ± 1.9	69.0 ± 1.0
Sperm motility (+ caffeine)	83.8 ± 1.2	80.4 ± 1.8	80.9 ± 2.0	78.9 ± 1.2
MDA production (nM/10^8 sperm)	23.1 ± 1.5	20.9 ± 1.8	20.9 ± 1.8	21.8 ± 1.4
Seminal plasma's capacity to inhibit MDA production (%)	34.6 ± 4.3	48.1 ± 4.8	43.1 ± 4.7	51.4 ± 4.9
ATP (nM/10^8 sperm)	7.0 ± 0.5^{a}	8.98 ± 0.7^{b}	6.5 ± 0.8	7.1 ± 0.5
SOD activity (U/mL)	51.6 ± 10.8	27.7 ± 12.0	42.4 ± 9.8	22.9 ± 4.2
CAT activity (μM/mL/min)	2.6 ± 0.2	2.3 ± 0.2	2.5 ± 0.3	2.0 ± 0.2
TAS (mM)	1.0 ± 0.1^{a}	0.6 ± 0.1^{b}	0.64 ± 0.1	0.62 ± 0.1
TOS (μM H_2O_2)	5.3 ± 0.6	5.8 ± 0.6	5.2 ± 0.7	5.8 ± 0.5

Values are expressed as means ±SEM
Values with different superscripts (a, b) differ significantly at $P \leq 0.05$ within columns for PLW boars
Values with different superscripts (c, d) differ significantly at $P \leq 0.05$ within columns for PL boars

Discussion

Recent research has focused on the development of reliable fertility markers at genome and proteome level. Seminal plasma proteins contribute to fertilisation success, and they are good candidates for male fertility markers. Sperm plasma membrane proteomes also play an important role by transporting spermatozoa through the female genital tract and binding spermatozoa to oolemma (21). It is generally believed that the identification of sperm proteins can expand our knowledge of regulatory mechanisms responsible for male fertility and infertility (23). Current research indicates that sperm proteomes differ between individuals and their composition is putatively associated with semen quality.

Acrosin (EC 3.4.21.10) is a serine protease which is found in acrosomes in the form of an inactive proenzyme – proacrosin. Acrosin is responsible for the proteolysis of zona pellucida glycoproteins, whereas proacrosin binds with oolemma polysaccharides. The above indicates that proacrosin plays an important role by recognising gametes and their interactions in the first stage of fertilisation. Purified acrosin from boar spermatozoa is a mixture of proteins with a molecular weight of 55 and 53 kDa. The 55 kDa enzyme is auto-activated to a 53 kDa enzyme, whereas the latter is auto-activated to 3 active forms: 49, 35, and 25 kDa, referred to as α, β, and γ acrosin respectively. Acrosin β is the most stable form of the enzyme (3). In boars, sperm acrosomal enzymes also include proacrosin-binding proteins. The presence of 32 kDa protein was observed in extracts of ejaculated spermatozoa. The above protein is capable of binding proacrosin and acrosin with a molecular weight of 55, 53 and 49 kDa. It accelerates proacrosin auto-activation in basic pH and affects subsequent stages of activation. The biological role of proacrosin-binding protein in acrosome functioning, spermatozoa maturation, and fertilisation has not been fully explained. Its role is limited to male reproductive system because its gene is only expressed in the testes (4). Bailey *et al.* (5) examined the phosphorylation status of capacitated boar spermatozoa and demonstrated that 32 kDa protein is released from sperm cells after incubation with Triton X-100 detergent. This outcome suggests that unlike other polypeptides subjected to intensified phosphorylation, the discussed polypeptide does not bind to the cell cytoskeleton. The analysed protein was reported in the cytosolic fraction of sperm extracts with Triton X-100. In the present study, sp32-like protein was also easily extracted from sperm extracts with Triton X-100.

The amount of sp32 can differ between individuals. In the study involving 2-D gel electrophoresis and liquid chromatography tandem mass spectrometry (LC MS/MS), Thepparat *et al.* (26) observed that sp32 can be expressed in 2 different ways. In 6 out of 9 bulls, the sp32 protein appeared in the form of a big spot identified as Y1 (32 kDa, pI 5.3), and in the remaining 3 individuals – in the form of 2 little spots: Y1 and Y2 (32 kDa, pI 5.3 and 31.5 kDa, pI 5.5 respectively). Both polypeptides were identified as acrosomal proacrosin-binding proteins (sp32). The cited authors hypothesised that an additional spot (sp32 at spot Y2; 31.5 kDa, pI 5.5) which was observed in their study could be a tyrosine phosphorylated form of sp32 (p32) resulting from spontaneous capacitation. In our study, the amount of sp32-like protein in boar ejaculate differed between individuals and was probably associated with the season. The seasonal presence of sp32-like protein in semen could be related to acrosin activity in boar spermatozoa. Ciereszko *et al.* (8) demonstrated that acrosin activity levels were stable between March and July. They increased significantly in August and slowly declined between September and February. A similar trend was observed in the presence of sp32-like protein in spermatozoa. The band with a molecular weight of 30 kDa was most visible in electrophoretic protein profiles in the summer months (especially between June and August). Because sp32 is involved in acrosin system stabilisation, it could be assumed that acrosin and sp32-like protein secretions are mutually coupled. The amount of sp32-like protein in ejaculate could also be associated with boar race. The discussed polypeptide was more frequently noted in ejaculates from PLW boars, although differences in acrosin activity had not been previously observed between breeds (8). Further work is needed to confirm the validity of the above observations.

In this study, sperm motility was significantly higher when the 30 kDa protein was present in the sperm. Sperm motility is an important parameter of ejaculate quality. Numerous authors have demonstrated the presence of a correlation between the motility and fertilising ability of the sperm. In comparison with 60%-70% sperm motility, 80% sperm motility is associated with lower farrowing rate, but a higher number of live born piglets (7). Despite the above, the presence of sp32-like protein was negatively related to the amount of ATP in fresh spermatozoa. High amounts of ATP are required for the maintenance of a healthy cell structure, internal ion levels, and, in particular, sperm motility (14). Our findings validate the above thesis only partially because ejaculates characterised by higher motility had lower ATP content. In the study by Dziekońska *et al.* (9), ATP levels in boar spermatozoa differed between spring-summer and autumn-winter periods. Statistically higher ATP levels were observed in spermatozoa collected in autumn-winter. Our results indicate that ATP levels are lower in spring-summer months when sp32-like protein is present in spermatozoa. It should be noted that ATP concentration in sperm is inhibited by hydrogen peroxide (product of superoxide dismutase and substrate for catalase reaction), which lowers energy metabolism of spermatozoa (2). Our research demonstrated that ejaculates containing proacrosin

binding-like protein were characterised by higher superoxide dismutase (SOD) and catalase (CAT) activities in seminal plasma. Higher activity of SOD in seminal plasma could be related to intracellular enzyme leakage caused by destabilisation of sperm membranes. During storage, SOD activity probably decreases in spermatozoa but increases in seminal plasma (29). Therefore, the catalytic pathway of CAT, where H_2O_2 is the only substrate, is the predominant pathway at higher concentrations of H_2O_2, which can be observed in oxidative burst or over-expression of SOD (15). The absence of sp32-like protein in spermatozoa was probably related to greater seminal plasma capacity to inhibit lipid peroxidation. The above indicates that seminal plasma has higher antioxidant capacity. Am-In *et al.* (1) demonstrated that seminal plasma from ejaculates with high sperm motility was characterised by higher total antioxidant capacity than seminal plasma from ejaculates with low sperm motility (TAS levels were also positively correlated with viability, morphology and plasma membrane status of spermatozoa). In our study, this hypothesis was confirmed only partially in PLW boars.

The results of our study indicate that the presence of sp32-like protein in boar spermatozoa could influence semen quality. However, the mechanism responsible for the above remains unknown. The relations between sp32-like protein and semen quality parameters did not differ relevantly between the 2 examined breeds, however, the mentioned polypeptide was more frequently demonstrated in ejaculates gained from PLW boars. Further studies involving different analytical methods are required to provide more conclusive data on the correlations between sp32-like protein and semen quality.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: The research and the article were funded from University funds (no. 11.610.003-110) and the NCBiR research grant (N R12 0014 04).

Animal Rights Statement: The experiments on animals were conducted in accordance with local Ethical Committee laws and regulations as regards care and use of laboratory animals.

References

1. Am–In N., Kirkwood R.N., Techakumphu M., Tantasuparuk W.: Lipid profiles of sperm and seminal plasma from boars having normal or low sperm motility. Theriogenology 2011, 75, 897–903.
2. Armstrong J.S., Rajasekaran M., Chamulitrat W., Gatti P., Hellstrom W.J., Sikka S.C.: Characterization of reactive oxygen species induced effects on human spermatozoa movement and energy metabolism. Free Radic Biol Med 1999, 26, 869–880.
3. Baba T., Kashiwabara S., Watanabe K., Itoh H., Michikawa Y., Kimura K., Takada M., Fukamizu A., Arai Y.: Activation and maturation mechanisms of boar acrosin zymogen based on the deduced primary structure. J Biol Chem 1989, 264, 11920–11927.
4. Baba T., Niida Y., Michikawa Y., Kashiwabara S., Kodaira K., Takenaka M., Kohno N., Gerton G.L., Arai Y.: An acrosomal protein, sp32, in mammalian sperm is a binding protein specific for two proacrosins and an acrosin intermediate. J Biol Chem 1994, 269, 10133–10140.
5. Bailey J.L., Tardif S., Dube Ch., Beaulieu M., Reyes-Moreno C., Lefievre L., Leclerc P.: Use of phosphoproteomics to study tyrosine kinase activity in capacitating boar sperm. Kinase activity and capacitation. Theriogenology 2005, 63, 599–614.
6. Bonet S., Briz M.D., Yeste M.: A proper assessment of boar sperm function may not only require conventional analyses but also others focused on molecular markers of epididymal maturation. Reprod Dom Anim 2012, 47, 52–64.
7. Broekhuijse M.L.W.J., Sostarić E., Feitsma H., Gadella B.M.: The value of microscopic semen motility assessment at collection for a commercial artificial insemination center, a retrospective study on factors explaining variation in pig fertility. Theriogenology 2012, 77, 1466–1479.
8. Ciereszko A., Ottobre J.S., Glogowski J.: Effects of season and breed on sperm acrosin activity and semen quality of boars. Anim Reprod Sci 2000, 64, 89–96.
9. Dziekońska A., Fraser L., Koziorowska-Gilun M., Strzezek J., Koziorowski M., Kordan W.: Seasonal-dependent variations in metabolic status of spermatozoa and antioxidant enzyme activity in the reproductive tract fluids of wild boar/domestic pig hybrids. Pol J Vet Sci 2014, 17, 307–313.
10. Erel O.: A new automated colorimetric method for measuring total oxidant status. Clin Biochem 2005, 38, 1103–1111.
11. Flesch F.M., Colenbrander B., Van Golde L.M.G., Gadella B.M.: Capacitation induces tyrosine phosphorylation of proteins in the boar sperm plasma membrane. *Biochem Biophys Res Commun* 1999, 262, 787–792.
12. Gatti J.L., Castella S., Dacheux F., Ecroyd H., Metayer S., Thimon V., Dacheux J.L.: Post-testicular sperm environment and fertility. Anim Reprod Sci 2004, 82–83: 321–339.
13. Heukeshoven J., Dernick R.: Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. Electrophoresis 1985, 6, 103–112.
14. Kamp G., Busselmann G., Jones N., Wiesner B., Lauterwein J.: Energy metabolism and intracellular pH in boar spermatozoa. Reproduction 2003, 126, 517–525.
15. Koziorowska-Gilun M., Koziorowski M., Fraser L., Strzeżek J.: Antioxidant defence system of boar cauda epididymidal spermatozoa and reproductive tract fluids. Reprod Dom Anim 2011, 46, 527–533.
16. Kwon W.S., Rahman M.S., Lee J.S., Kim J., Yoon S.J., Park Y.J., You Y.A., Hwang S., Pang M.G.: A comprehensive proteomic approach to identifying capacitation related proteins in boar spermatozoa. BMC Genomics 2014, doi:10.1186/1471–2164–15–897.
17. Laemmli U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970, 227, 680–685.
18. Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J.: Protein measurement with the Folin phenol reagent. J Biol Chem 1951, 193, 265–275.
19. Martinez-Alborcia M.J., Valverde A., Parilla I., Vázquez J.M., Martinez E.A., Roca J.: Detrimental effects of non-functional spermatozoa on the freezability of functional spermatozoa from boar ejaculate. PLoS ONE, http://www.plosone.org 2012, 7, e36550.
20. Naz R.K., Rajesh P.B.: Role of tyrosine phosphorylation in sperm capacitation/acrosome reaction. Reprod Biol Endocrinol 2004, 2: 75.
21. Novak S., Smith T.A., Paradis F., Burwash L., Dyck M.K., Foxcroft G.R., Dixon W.T.: Biomarkers of *in vivo* fertility in

sperm and seminal plasma of fertile stallions. Theriogenology 2010, 74, 956–967.
22. Okazaki T., Shimada M.: New strategies of boar sperm cryopreservation: development of novel freezing and thawing methods with a focus on the roles of seminal plasma. Anim Sci J 2012, 83, 623–629.
23. Rahman M.S., Lee J.S., Kwon W.S., Pang M.G.: Sperm proteomics: road to male fertility and contraception. Inter J Endo 2013, doi:10.1155/2013/360986.
24. Strzeżek J., Fraser L., Demianowicz W., Kordan W., Wysocki P., Hołody D.: Effect of depletion tests (DT) on the composition of boar semen. Theriogenology 2000, 54, 949–963.
25. Tappel A.L., Zalkin H.: Lipid peroxidation in isolated mitochondria. Arch Biochem Biophys 1959, 80, 326–332.
26. Thepparat T., Katawatin S., Vongpralub T., Duangjinda M., Thammasirirak S., Utha A.: Markedly different expression in mature bovine proacrosin binding protein (sp32). Thai J Vet Med 2012, 42, 463–469.
27. Wetterauer U.: Recommended biochemical parameters for routine semen analysis. Urol Res 1986, 14, 241–246.
28. Yeste M., Estrada E., Casas I., Bonet S., Rodriguez-Gil J. E.: Good and bad freezability boar ejaculates differ in the integrity of nucleoprotein structure after freeze-thawing but not in ROS levels. Theriogenology 2013, 79, 929–939.
29. Zakošek Pipan M., Mrkun J., Kosec M., Nemec Svete A., Zrimšek P.: Superoxide dismutase: a predicting factor for boar semen characteristics for short-term preservation. BioMed Res Inter 2014, doi:10.1155/2014/105280.