

## Journal of the Hellenic Veterinary Medical Society

Vol 70, No 4 (2019)



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doi: [10.12681/jhvms.22238](https://doi.org/10.12681/jhvms.22238)

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### To cite this article:

MICHOS, I., TSAKMAKIDIS, I., TSANTARLIOTOU, M., TSOUSIS, G., HADWEH, P., & BOSCOS, C. (2020). Effect of seasonal infertility period on boar sperm proteins and quality characteristics. *Journal of the Hellenic Veterinary Medical Society*, 70(4), 1875–1882. <https://doi.org/10.12681/jhvms.22238>

## Effect of seasonal infertility period on boar sperm proteins and quality characteristics

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**ABSTRACT.** Swine seasonal infertility reduces the productivity and profitability of a pig farm. The main causes of this condition are elevated environmental temperatures and long photoperiod during the summer season. The aim of this study was to investigate which sperm proteins and parameters are affected during the period of seasonal infertility. Depending on the environmental temperatures, the period from October to June was considered as cold and the period from July to September as warm season. A total of 65 ejaculates from 18 boars were collected over a year. Each semen sample was evaluated for kinetics (Computer Assisted Semen Analyzer), morphology (Sperm Blue stain), viability (Propidium Iodide - Calcein AM stain), mitochondrial membrane potential (Rhodamine 123 – Propidium Iodide stain), membrane integrity and functionality (Hypo-osmotic swelling test) and sperm DNA integrity (Acridine Orange Test). Moreover, selected proteins (HSP90, GPX5, OPN) were detected and quantified. The kinetic parameters VSL, LIN and the midpiece abnormalities were significantly higher in the warm compared to the cold season ( $p < 0.05$ ), while a strong tendency towards higher values for HSP90 and GPX5 was observed in warm compared to cold season ( $p = 0.07$  and  $p = 0.06$ , respectively). In conclusion, among the boar sperm characteristics tested in our study, seasonal infertility period negatively affected VSL and LIN kinetics, while GPX5 seminal plasma enzyme and HSP90 sperm surface protein increased their sperm protective effects.

**Keywords:** boar semen, semen analysis, HSP90, GPX5, seasonal infertility

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*Date of initial submission: 23-05-2019*  
*Date of revised submission: 03-06-2019*  
*Date of acceptance: 05-06-2019*

## INTRODUCTION

Seasonal differences in pig productivity have been repeatedly reported, particularly in geographical areas with tropical and hot climate (Peña et al., 2016). The summer is usually characterized as the season of infertility in swine industry. It is accompanied by anestrus or low expression and abnormal duration of estrus, prolonged or abnormal weaning to estrus intervals, high return to estrus rates, low pregnancy and farrowing rates, smaller litter sizes and lower boar fertilizing ability (Peltoniemi et al., 1999; De Rensis and Kirkwood, 2016).

Seasonal infertility has been attributed to heat stress, photoperiod, humidity, genetic background and management systems (De Rensis et al., 2017). Although some researchers suggest that heat stress is the main cause of this condition (Prunier et al., 1994), others indicate the photoperiod as the most important factor. Flowers (1997, 2015) found significant semen degradation when boars were kept at 34° C for 8-16 hours daily for 11 weeks or at 26-29° C for 10-14 weeks. However, Peltoniemi et al. (1999) reported seasonal infertility effects in Finland at ambient temperature that did not exceed 25° C, thus implying the photoperiod as a more important cause.

Regarding the effects of heat stress on boar sperm, most studies investigated basic semen parameters and reported lower volume and concentration, lower sperm motility and higher percentage of morphological abnormalities during warm periods (Egbunike and Dede 1980; Barranco et al., 2013). Peña et al. (2019) found that tropical summer induces boar sperm DNA damage; however, no further studies have investigated effects of seasonality on specific, functional semen parameters.

Seminal plasma proteins are involved in spermatozoa's motion, capacitation and stress protection, thus influencing their function (Gonzalez-Gadavid et al., 2014). Killian et al. (1993) found osteopontin (OPN), a seminal plasma protein (55 kDA), to be associated with high fertility in bulls. In swine IVF the use of the OPN improved embryo development (Hao et al., 2008) and reduced polyspermy rates (Hao et al., 2006). Moreover, some membrane proteins play a crucial role to sperm regulation, communication and protection from oxidative stress (Strzeczek, 2005). Among them, Heat Shock Protein (HSP) group helps the cells withstand extreme temperature variations. HSP90 is the most noted sperm surface protein. Its low concentration has been correlated to low quality boar sperm during warm periods (Huang et al., 2000;

Valencia et al., 2017). Furthermore, it is well known that boar spermatozoa are susceptible to oxidative damage due to their relative high content of unsaturated fatty acids. Glutathione peroxidase-5 (GPX5) is an H<sub>2</sub>O<sub>2</sub>-scavenging enzyme identified in boar seminal plasma. GPX5 prevents premature capacitation. It is anchored on sperm plasma membrane or free into the epididymal fluid (Drevet, 2006). Vilagran et al. (2016) found that GPX5 of seminal plasma is positive related to sperm quality and Barranco et al. (2016) found a positive correlation between sperm quality and fertilization outcome after artificial insemination with liquid-stored boar semen.

Thus, the aim of the present study was to investigate which of the boar sperm parameters and proteins are affected by seasonality. Throughout a whole year, under commercial pig farming conditions, we evaluated the changes of semen analysis parameters and sperm proteins in order to detect changes between the seasonal infertility (warm) and normal productivity (cold) periods.

## MATERIALS AND METHODS

All reagents and chemicals used in this study were purchased from Sigma-Aldrich, (St Louis, MO, USA), unless otherwise specified.

### Animals, management and data recording

The study was approved by the Ethics Committee on Animal Use of the School of Veterinary Medicine, Aristotle University of Thessaloniki, Greece and all operations were carried out according to the University's Guidelines for Animal Research.

As cold season was considered the period of semen collections between October and June (mean temperature 12.5° C), while as warm season was considered the period between July and September (mean temperature 23.9° C).

A healthy population of 18 crossbred sexually matured boars (2-3 years old) was used as sperm donors. All boars were used for artificial insemination (AI) in routine basis twice a week with a 3-day interval between collections. A total of 65 ejaculates (3 ejaculates per boar at average) were collected and tested in this study, over a 12-month period. The animals were properly housed in a commercial pig farm (Imathia region, Greece, 40°36'52.9"N, 22°22'08.1"E). Water was provided *ad libitum*, and animals were fed according to the standard nutrition protocols for adult boars.

### Semen collection, separation of seminal plasma and spermatozoa

Sperm-rich ejaculate fractions were collected once per week using the gloved-hand technique. The sperm rich fraction was filtered through gauze and divided into aliquots. The first aliquot was extended with a commercial extender (M III<sup>®</sup>, Minitube, Germany) to a final concentration of  $3 \times 10^7$  spermatozoa/ml, divided to doses ready for insemination and stored at 17° C. Later a dose was transported to the laboratory (within an hour), inside an air-conditioned isothermal box (Minitube, Germany) adjusted at 17° C for further analysis, while the remaining doses were used for AI.

A second aliquot of the collected semen was used for the assessment of sperm and seminal plasma proteins. Semen was immediately mixed with a protease inhibitor cocktail [4-(2-aminoethyl) benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, aprotinin] as proposed by González-Cadavid et al. (2014). Then, it was centrifuged at 640 x g for 15 minutes at 17° C to separate sperm and seminal plasma. Seminal plasma was centrifuged once more at 10,000 x g for 15 min at 17° C and supernatant was finally stored at -80° C until further examination.

Sperm pellet was washed with Phosphate Buffer Solution (PBS) and diluted at  $1.5 \times 10^9$  spermatozoa per ml (determined photometrically). According to Vilagran et al. (2013), sperm was once again pelleted at 640 x g for 3 minutes at 17° C, washed with 10mL PBS and re-centrifuged at 800 x g for 5 minutes at 17° C. Pellets were resuspended with HAM F-10 1X (ThermoFisher<sup>®</sup> Scientific, USA) and samples were once again centrifuged and supernatant was discarded. Spermatozoa were solubilized in NP-40 lysis buffer [50 mM Tris-HCL pH 7.4, 250 mM NaCl, 5 mM EDTA, 1% Glycerol, 0.5% NP-40, 1 mM DTT, 1 mM PMSF 100 mM, 1× protease inhibitor cocktail (Roche)] and the sample was stored at -80° C until further analysis.

### Semen evaluation

Upon the arrival at the laboratory, each sperm sample was warmed at 37° C and a Computer Assisted Sperm Analyzer (CASA) was used (Sperm Class Analyzer<sup>®</sup>, Microptic S.L., Barcelona, Spain) to assess motility parameters such as sperm total motility (TM, %), progressive motility (PM, %), straight line velocity (VSL,  $\mu\text{m/s}$ ), average path velocity (VAP,  $\mu\text{m/s}$ ), curvilinear velocity (VCL,  $\mu\text{m/s}$ ), beat cross frequency (BCF, Hz), amplitude of lateral head dis-

placement (ALH,  $\mu\text{m}$ ), straightness (STR, VSL/VAP, %), linearity (LIN, VSL/VCL, %), wobble (WOB, VAP/VCL, %), rapid, medium and slow moving spermatozoa ( $10 < \text{slow} < 25 < \text{medium} < 45 < \text{rapid} \mu\text{m/sec}$ ), and hyperactivation (sperm subpopulation of increased VCL  $> 97 \mu\text{m/sec}$ , ALH  $> 3.5 \mu\text{m}$  and LIN  $< 0.32\%$ ). CASA configuration was set at 25 frames/sec, region of particle control 10-18 microns, depth of field 10 microns, progressive movement of  $> 45\%$  of the indicator STR, circumferential movement  $< 50\%$  LIN (Karageorgiou et al., 2016). An aliquot of sperm sample (10 $\mu\text{l}$ ) was placed on a pre-warmed Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel) and the evaluation was performed according to manufacturer's instructions. Pictures of at least 5 random fields were taken (x10), with a minimum of 1000 analyzed spermatozoa. Sperm evaluation was performed by a phase-contrast microscope with a thermal plate attached (Zeiss, Axio, Scope A1. Germany) and consecutive images were obtained by digital camera (Olympus BX 41, Japan), digitized and analyzed with Sperm Class Analyzer<sup>®</sup> software.

Morphology was estimated by Spermblye<sup>®</sup> staining method (Microptic S.L., Barcelona, Spain) according to manufacturer's instructions. Magnification x400 was used and 200 spermatozoa per slide were estimated and counted while results were expressed in % ratio. Spermatozoa were classified as normal and abnormal. As abnormal spermatozoa were considered those with morphological abnormalities such as abnormal heads, midpieces, tails and proximal or distal cytoplasmic droplets. Spermatozoa with abnormal acrosomes or detached heads were also classified as abnormal.

The viability was assessed by Propidium Iodide (PI; 0.75 mmol/L) - Calcein AM (1 mmol/L) double staining method (Basioura et al., 2018). A total of 200 spermatozoa were scored and results were expressed in % ratio.

According to Najafi (2013), Rhodamine 123 (Rh123) and PI dual fluorescent staining is used to provide critical information about mitochondrial membrane potential. Digital photos were instantly taken under fluorescent microscope and a total of 200 spermatozoa were scored. The percentage of sperm with functional mitochondria was identified by R123 high fluorescence and no PI fluorescence and results were expressed in % ratio.

Hypo-osmotic swelling test (HOST) was performed to evaluate sperm membranes' biochemical

activity and functionality. Aliquots of each semen sample (0.1 ml) were added to 0.9 ml of the hypotonic solution (150 mOsm/L) and incubated at 37° C for 60 min. A wet smear was made and left to air dry. Later the sample was examined under a phase contrast microscope at x400 magnification. A total of 200 spermatozoa was examined and categorized as positive or negative and results were expressed in % ratio. As positive spermatozoon with intact membranes was considered that with a swelling at the flagellum or was curled at any point. As negative were considered spermatozoa without no morphological alterations after the subjection of hypo-osmotic swelling test. Results were expressed in % ratio and a total of 200 spermatozoa were scored.

Acridine orange test (AOT) was used to estimate sperm DNA integrity as described by Tejada et al. (1984). Spermatozoa with normal double-stranded DNA displayed green fluorescence, whereas denatured single stranded DNA displayed as yellow-orange to red fluorescence. A total of 200 spermatozoa were examined under fluorescent microscope and the results were expressed in % ratio.

### **Seminal plasma and sperm protein extraction and quantification by Western Blot (WB)**

In order to assess spermatozoa and seminal plasma proteins, a frozen aliquot of each sample was thawed and the Bradford method (Bradford, 1976) was used to determine the total protein concentration using a spectrophotometer (Quick Start™ Bradford Protein Assay; Bio-rad), and bovine albumin was used for the construction of the standard curve.

The sperm pellet was washed three times with PBS and centrifuged at 800 x g for 1 minute. Pellets were lysed in 5xSDS loading buffer (Tris 250mM, SDS 10%, Glycerol 50%, β-Mercaptoethanol 15%), boiled for 5 minutes and equal amount of protein lysates were loaded onto 12% slab gels for electrophoresis (SDS-PAGE). Electrophoresis was performed using a Mini Protean 3 Cell apparatus (Bio-Rad, Berkley, CA) with 50 mA/gel constant current for 1 h.

The proteins were then transferred to nitrocellulose membranes (GE Healthcare) under 50mA/300V for 1h (PowerPac 1000, Bio-Rad). Following this procedure, membrane was washed three times with TBST (20 mM Tris, 500 mM NaCl, 0.05% Tween; pH 7.5) and incubated with blocking solution (5% skimmed milk powder in PBST) for 1 h. Afterwards,

the membrane was washed for 5 minutes in TBST and then incubated for 2 h with the following primary antibodies: rabbit polyclonal anti-HSP 90 (AP-22747PU-N; Acris; diluted 1:1000 with TTBS), rabbit polyclonal anti-GPX5 (18731-1-AP; Proteintech Europe; diluted 1:500) and rabbit polyclonal anti- beta actin (ab8227, Abcam). Membranes were washed 3 times and subsequently incubated with a horseradish peroxidase-conjugated polyclonal goat anti-rabbit immunoglobulin (SC-2004; Santa Cruz; 1:2000). Pierce™ ECL Plus Western Blotting Substrate (Thermoscientific, IL, USA) was used for development and membranes were scanned with Typhoon FLA 7000 (GE Healthcare). Protein levels were expressed as “band volume”. That is the total signal intensity measured inside the boundary of a band in pixel intensity units. Protein bands were quantified using ImageJ (v.1.8.0), with actin serving as a normalizing factor. Their ratio was given as the level of proteins, according to Vilagran et al. (2013). The mean of three different measurements for each sample was taken into account.

According to previously published data (Valencia et al., 2017), SDS-PAGE, Western Blot and quantification of seminal plasma proteins were likewise carried out. Anti-OPN (GTX 37582; GeneTextech; diluted 1:200) was used for the detection of osteopontin while a non-specific band from Ponceau S staining was used as a normalizing factor. Both bands were quantified with ImageJ (v.1.8.0) and their ratio was used for graphical presentation. The mean of three different measurements for each seminal plasma sample was taken into account.

### **Statistical analysis**

Statistical analysis was conducted using the Statistical Analysis System V9.3 (SAS Institute Inc., Cary, NC, USA). The Shapiro–Wilk test was performed in all outcome variables to test for the underlying distribution of the data. The distribution of total motility, VCL, ALH, hyperactivation, head, midpiece and tail abnormality cytoplasmatic droplet, HSP90, GPX5, OPN70 and OPN12 was different from normal distribution; thus, a Wilcoxon’s two sample non-parametric tests was applied. All other parameters showed a normal distribution. For these variables a two-sample t-test for independent observations was used. Data are presented as mean ± standard deviation for data with normal distribution and as median ± median absolute deviation (MAD) for data not normally distributed. All analyses were considered to be statistically significant at  $P < 0.05$  and tended to differ if  $0.10 > P \geq 0.05$ .



**RESULTS**

The results of motility and kinetics in the two seasons are presented in table 1. Significant lower values of VSL and LIN were noticed in warm compared to

cold season ( $p=0.04$  and  $p=0.03$ , respectively), while the remaining parameters were not significantly different ( $p>0.05$ ).

**Table 1.** Boar sperm motility and kinetics after CASA analysis during warm and cold season (mean or median  $\pm$  standard deviation or MAD)

Variable	Warm season (n=11)	Cold season (n=54)	p-value
Total motility (%)	88.60 $\pm$ 3.29	90.79 $\pm$ 5.73	0.23
Non progressive motility (%)	39.26 $\pm$ 9.01	40.06 $\pm$ 10.68	0.82
Progressive motility (%)	47.58 $\pm$ 13.15	48.88 $\pm$ 15.12	0.79
Rapid moving spermatozoa (%)	48.19 $\pm$ 17.59	51.78 $\pm$ 22.58	0.62
Medium moving spermatozoa (%)	21.05 $\pm$ 6.56	19.45 $\pm$ 9.39	0.59
Slow moving spermatozoa (%)	17.61 $\pm$ 7.85	17.70 $\pm$ 10.42	0.98
VCL	56.34 $\pm$ 12.90	55.02 $\pm$ 12.26	0.83
VSL	19.26 $\pm$ 6.20	23.91 $\pm$ 7.54	0.04
VAP	33.91 $\pm$ 9.11	41.09 $\pm$ 12.53	0.08
LIN	35.47 $\pm$ 4.39	40.45 $\pm$ 12.81	0.03
STR	56.56 $\pm$ 7.79	58.59 $\pm$ 9.99	0.53
WOB	63.22 $\pm$ 7.04	68.36 $\pm$ 14.36	0.09
ALH	2.02 $\pm$ 0.13	2.11 $\pm$ 0.28	0.19
BCF	9.68 $\pm$ 2.91	9.37 $\pm$ 4.23	0.82
Hyperactivated spermatozoa (%)	0.97 $\pm$ 0.24	1.59 $\pm$ 0.15	0.19

Abbreviations: VCL: curvilinear velocity ( $\mu\text{m}/\text{sec}$ ); VSL: straight line velocity ( $\mu\text{m}/\text{sec}$ ); VAP: average path velocity ( $\mu\text{m}/\text{sec}$ ); LIN: linearity (VSL/VCL); STR: straightness (VSL/VAP); WOB: wobble (VAP/VCL); ALH: amplitude of lateral head displacement ( $\mu\text{m}$ ); BCF: beat cross frequency (Hz).

No significant differences were observed in boar sperm morphological and functional characteristics between seasons ( $p>0.05$ ), except for midpiece ab-

normalities which were higher in the warm period ( $p=0.01$ , table 2).

**Table 2.** Boar sperm morphological and functional characteristics during warm and cold season (mean or median  $\pm$  standard deviation or MAD)

Variable	Warm season (n=11)	Cold season (n=54)	p-value
Normal morphology (%)	75.59 $\pm$ 12.84	76.06 $\pm$ 9.92	0.89
Head abnormalities (%)	9.5 $\pm$ 4.5	6.5 $\pm$ 3	0.68
Midpiece abnormalities (%)	2.0 $\pm$ 1.0	1.0 $\pm$ 0.5	0.01
Tail abnormalities (%)	9.0 $\pm$ 6.0	12.5 $\pm$ 6.0	0.33
Cytoplasmic droplets (%)	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.92
Viability (%)	81.86 $\pm$ 5.84	83.01 $\pm$ 8.08	0.66
HOST+ spermatozoa (%)	40.14 $\pm$ 13.59	38.61 $\pm$ 12.37	0.72
Activated Mitochondria (%)	86.50 $\pm$ 3.97	84.49 $\pm$ 7.90	0.52
Sperm DNA damaged (%)	1.9 $\pm$ 0.34	1.6 $\pm$ 0.33	0.79

No significant differences were found in sperm protein quantities ( $p>0.05$ , table 3). However, a strong tendency towards higher values of HSP90 and GPX5

in warm compared to cold period was found ( $p=0.07$  and  $p=0.06$ , respectively).

**Table 3.** Boar sperm and seminal plasma proteins during warm and cold season (median  $\pm$  MAD)

Variable	Warm season (n=11)	Cold season (n=54)	p-value
HSP90	1.58 $\pm$ 1.27	1.06 $\pm$ 0.80	0.07
GPX5	3.59 $\pm$ 1.38	1.98 $\pm$ 1.39	0.06
OPN70	0.60 $\pm$ 0.59	1.95 $\pm$ 1.1	0.19
OPN12	2.38 $\pm$ 2.37	2.01 $\pm$ 1.97	0.81

## DISCUSSION

Seasonal infertility in swine reduces significantly the productivity of a pig farm. This is mainly the result of the decrease in reproductive parameters, i.e. lower farrowing rates, lower number of live born piglets and decreased litter sizes (Love, 1978; Peltoniemi et al. 1999). Females and males are both affected by seasonal infertility. Concerning the boar, previous studies reported that heat stress results in reduced libido and decreased semen volume and concentration (Cameron and Blackshaw, 1980; Flowers, 1997).

The present study found seasonal differences of semen parameters which can be contributed to the outcome of AI. Among kinetics, VSL and LIN were lower in warm compared to cold period. It is known that good sperm kinetics can positively affect fertility. These results can influence the seasonal reproductive differences in a farm, since VSL is correlated with the total number of live born piglets (Broekhuijse et al., 2012). Lee et al. (2014) proposed that VSL should be considered to select semen for AI. Although LIN is not a sensitive indicator of sperm motion, Hirai et al. (2001) found a higher non-return to estrus rate in boars with significantly higher LIN. Additionally, Casas et al. (2009), reported that a combination of LIN and STR can be advantageous for the freezability of boar sperm. Moreover, it is well known that sperm motility is one of the most important indicators of field fertilizing ability. It correlates with litter size in pigs, even though an AI with a low number of spermatozoa per dose takes place (Tardif et al., 1999). In the present study no significant differences between warm and cold season were found regarding total and progressive motility. This finding agrees with the study of Popwell and Flowers (2004), who monitored three boars' semen quality with significantly different *in vitro* and *in vivo* fertility for 40 weeks, but they did not reveal total motility differences. Although most of the published studies agree that motility and kinetics play a crucial role in the fertilization process, there are reports that show different results. Reproductive efficiency can be attributed to other important factors, such as boar genetics, farm management, enriched nutrition and animals' care. Moreover, the results of the kinetics CASA measurements can be affected by many factors, i.e. the sample preincubation time, the different sperm counting chamber, the software settings and the user training (Yeste et al., 2018). Therefore, only the results which emerged following similar methodology can be compared.

In our study no effect of season on sperm quality and functional characteristics was noticed, with the excep-

tion of the percentage of midpiece abnormalities, indicating that motility parameters are more susceptible. Midpiece abnormalities have been related to Reactive Oxygen Species (ROS) generation, lipid peroxidation and increase in creatine kinase (CK) activity (Huszar and Vigue, 1994). CK, which is a marker of sperm maturity, has been related to low sperm fertilizing capacity (Hallak et al., 2001), while ROS hyperproduction and oxidative damage impair the normal function of spermatozoa (Radomil et al., 2011). Heat stress has also been associated with testicular dysfunction and oxidative stress (Hamilton et al., 2016). The determination of oxidative parameters was not within the scopes of the present study. However, GPX5 and HSP90 were assessed, demonstrating as a tendency higher values in warm compared to cold season. Hydrogen peroxide ( $H_2O_2$ ) is considered as the major damaging ROS for boar sperm, while Glutathione Peroxidases (GPXs) are the responsible enzymes that neutralize  $H_2O_2$  (Awda et al., 2009). Moreover, GPX5 protects boar spermatozoa during their route into the female genital tract, decreasing the harmful effects of ROS generated by uterine tissues. According to Barranco et al. (2016), GPX5 affects fertility after AI with liquid-stored boar semen. Based on the findings of our study, it is probable that boar spermatozoa exhibit higher GPX5 to overcome the extended ROS production during the periods of seasonal infertility. Additionally, HSP90 is the most noted sperm surface protein (Valencia et al., 2017) and it is reduced when sperm quality is low (Huang et al., 2000). In accordance with GPX5 changes, HSP90 was also increased in the warm period of our study to support a defensive mechanism of boar spermatozoa against heat stress. Many genomic regions responsible for heat tolerance have been identified in pigs (Riquet et al., 2017) reflecting the boar genetic improvement that has been succeeded up to today.

## CONCLUSION

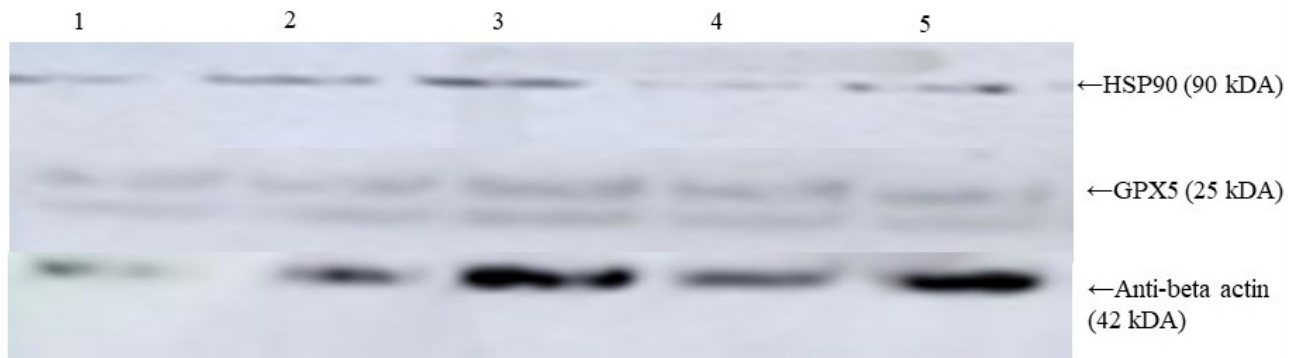
In conclusion, among the boar sperm characteristics tested in our study, seasonal infertility period negatively affected VSL and LIN kinetics, while GPX5 seminal plasma enzyme and HSP90 sperm surface protein increased their sperm protective effects.

## ACKNOWLEDGEMENTS

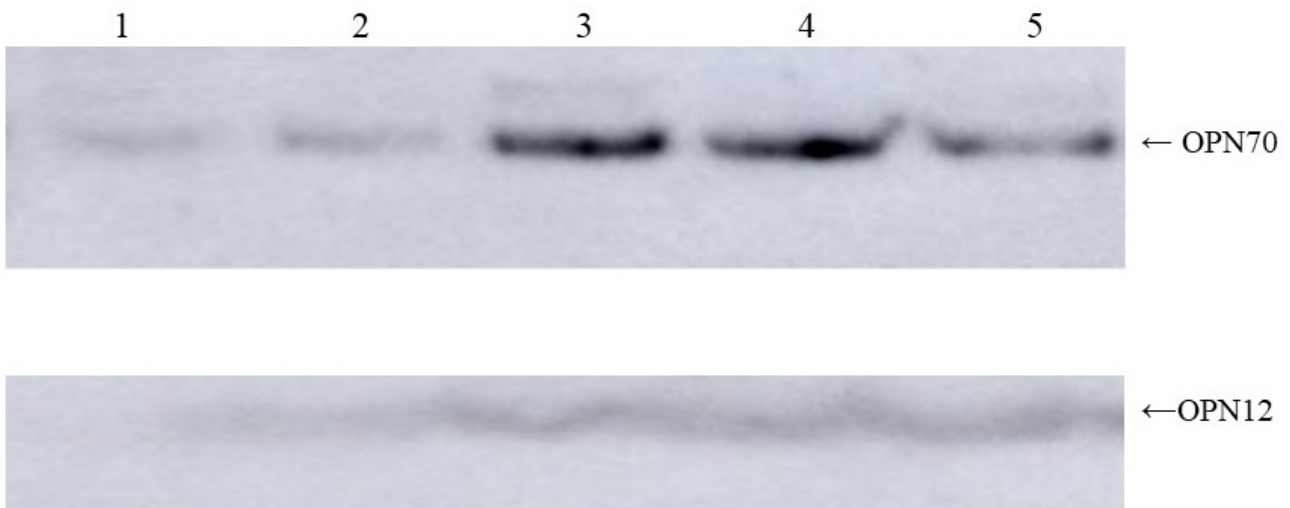
The authors would like to thank the staff of the pig farm Karanikas LTD for their valuable help to the completion of this study.

## CONFLICT OF INTEREST

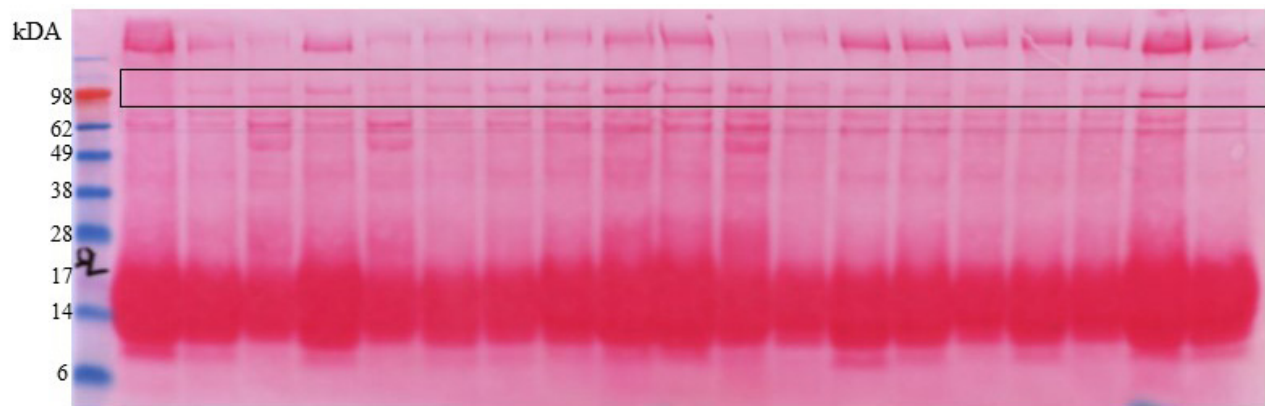
The authors declare no conflict of interest.



**Fig 1.** Representative band patterns from WB analysis of HSP90 and GPX5 from the solubilized membranous fraction of boar spermatozoa



**Fig 2.** Representative band patterns from WB analysis of OPN from the boar seminal plasma



**Fig 3.** Nitrocellulose blot stained with Ponceau S. The protein band used as OPN control is highlighted



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