Boar spermatozoa proteomic profile varies in sperm collected during the summer and winter

David Martín-Hidalgo[a,b,#], Beatriz Macías-García[a,d,#], Luis Jesús García-Marín[a,e], María Julia Bragado[a,c], Lauro González-Fernández[a,c*]

[a]*Research Group of Intracellular Signalling and Technology of Reproduction (Research Institute INBIO G+C), University of Extremadura, Cáceres, Spain*

[b]*Unit for Multidisciplinary Research in Biomedicine (UMIB), Laboratory of Cell Biology, Department of Microscopy, Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto, Porto, Portugal*

[c]*Department of Biochemistry and Molecular Biology and Genetics, Faculty of Veterinary Sciences, University of Extremadura, Cáceres, Spain*

[d]*Department of Animal Medicine, Faculty of Veterinary Sciences, University of Extremadura, Cáceres, Spain*

[e]*Department of Physiology, Faculty of Veterinary Sciences, University of Extremadura, Cáceres, Spain*

*Corresponding Author: Lauro González-Fernández, PhD. E-mail: lgonfer@unex.es

Research group of Intracellular Signalling and Technology of Reproduction (Research Institute INBIO G+C). Avda. de la Universidad s/n, University of Extremadura, 10003 Cáceres, Spain

[#]These authors contributed equally to the present work and should be regarded as co-first authors

ABSTRACT

Boar sperm quality is less during the summer as a result of the different photoperiod or ambient temperatures as compared with the winter. The present study was conducted to elucidate possible variations in proteomic profiles of boar spermatozoa collected during the summer and winter. Effects of season on sperm viability, total motility, progressive motility, acrosome status, mitochondrial membrane potential and plasma membrane lipid organization were also analyzed. Only sperm viability and mitochondrial membrane potential were less during the summer ($P < 0.05$). Spermatozoa were processed and evaluated using the nano LC–MS/MS QTof procedures. A total of 1,028 proteins were identified in sperm collected during both seasons of the year (False Discovery Rate < 0.01) and, among the total, 85 proteins differed in sperm collected in the winter and summer, with there being a lesser abundance of these proteins when there were ejaculate collections during the summer (q-value ≤ 0.05). The results from enrichment assessments for these protein networks utilizing UniProtKB procedures for determining reproductive processes indicates there were 23 proteins that were less abundant in the summer than winter. These proteins have essential functions in spermatogenesis, sperm motility, acrosome reaction and fertilization. These results are the first where there was ascertaining of proteomic differences in boar spermatozoa collected in the summer and winter. These results might help to

explain the decreased sperm quality and prolificity when semen of boars is used for artificial insemination that is collected during the season of the year when ambient temperatures are relatively greater.

Keywords: Spermatozoa; Heat stress; Pig; Sperm proteome

1. Introduction

Swine are an important food producing animals in most countries (McGlone, 2013). There, therefore, is a great effort to improve assisted reproductive technologies (ARTs) in pigs with artificial insemination (AI) being the most common ART used. There are greater than 90% of all gilts and sows in commercial pig meat production enterprises inseminated using this technique (Knox, 2016). Different factors can impair the quality of ejaculates, and thus the quality of the seminal doses produced with one of these factors being the season of the year in which ejaculates are collected (Cameron and Blackshaw, 1980; Trudeau and Sanford, 1986; Ciereszko et al., 2000; Murase et al., 2007). Specifically, during the summer, there is a lesser sperm quality in ejaculates and a resulting lesser than optimal fertility when seminal doses are used for AI, which leads to a negative economic impact.

This decrease in the quality of sperm in ejaculates has been associated with the effect of the increased photoperiod during the summer months (Knecht et al., 2013) and the thermal stress that occurs during the seasons of the year when ambient temperatures are greater (Cameron and Blackshaw, 1980; Trudeau and Sanford, 1986; Murase et al., 2007). In European countries such as Spain, where

ambient temperatures during the summer can be as great as 40 °C, there is a marked decrease in the quality of sperm in seminal doses collected in AI centers (Pinart et al., 2013; Martinez Pastor et al., 2019), which leads to a reduction in litter sizes and number of piglets born alive (Pinart et al., 2013).

Proteomic analysis has been recognized to be a very useful technique for investigation of sperm quality in all species. There has been detection of divergent proteomic profiles in 1) fresh and cryopreserved human spermatozoa (Wang et al., 2014); 2) relatively lesser and greater motile human sperm subpopulations (Martin-Hidalgo et al., 2020); 3) the main fractions of the pig ejaculate (Perez-Patino et al., 2016); and 4) in ram spermatozoa with different capacitation statuses (Peris-Frau et al., 2019). To the best of our knowledge, there have been no previous studies aimed at identifying differences in the boar sperm proteome during the seasons of the year when ambient temperatures are greatest and least. In the present study, therefore, there was the aim of describing and comparing the proteomic profile and sperm quality of pig spermatozoa when ambient temperatures were greatest and least (i.e., winter and summer).

2. Material and methods

2.1. Chemical products and sources

The sperm density gradient was prepared using PureSperm® 100 and PureSperm® Buffer from Nidacon International AB (Mölndal, Sweden). Propidium iodide (PI), SYBR-14, Merocyanine-540 (M540), Yo-Pro-1 and Mitotracker® orange probes were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The other reagents were purchased from Sigma-Aldrich® (St Louis, MI, USA).

2.2. Boar semen collection

Ejaculates were collected from Duroc boars (2-4 years of age) and were subsequently diluted in Vitasem extender (Magapor S.L., Zaragoza, Spain) at 30 × 10^6 spermatozoa/mL. This semen was from a commercial boar stud (Tecnogenext S.L., Mérida, Spain). All boars were housed in conditions that are consistent with the regional and European regulations in individual pens in an environmentally controlled building (15-25 °C) with natural lighting and received the same diet. Semen collected during the winter season was purchased in February and March, whereas the semen processed during the summer was purchased in June and July.

2.3. Evaluation of sperm motility

Sperm motility was evaluated using a CASA system (ISAS®, Proiser R+D, Paterna, Valencia, Spain). Aliquots of 500 µL of semen were incubated for 15 min at 38.5 °C, and 2 µL of the sample were placed in a pre-warmed counting chamber at 38.5 °C (Leja Products B.V., Nieuw-Vennep, The Netherlands). Sperm motility was analyzed using a microscope (Eclipse 50i; Nikon, Tokyo, Japan) equipped with a 10 x negative-phase contrast objective and a heated stage at 38.5 °C. Analysis was based on the examination of 25 consecutive digitalized images and at least 300 spermatozoa per sample from four representative fields in a random distribution were analyzed. Spermatozoa with an average path velocity (VAP) > 15 µm/s were considered motile (TM) and spermatozoa with a straightness (STR) greater than 80% were considered as progressively motile (PM).

2.4. Flow cytometry

Flow cytometry analyses were performed using an ACEA NovoCyte® flow cytometer (ACEA Biosciences, Inc., San Diego, CA, USA) equipped with a blue/red laser (488/640 nm) and three detection channels: BL-1 channel (530 ± 30 nm band pass filter); BL-2 channel (572 ± 28 nm band pass filter) and BL-4 channel (675 ± 30 nm band pass filter). Flow cytometer performance was ensured using fluorescent validation particles (NovoCyte™ Quality Control (QC) Particles; ACEA Biosciences, Inc., San Diego, CA, USA) to assess the mean fluorescence intensity (MFI) and coefficient of variance (CV) of FSC, BL-1 channel, BL-2 channel and BL-4 channel. Forward scatter (FSC) and side scatter (SSC) were used to gate the sperm population and to exclude debris. Samples were analyzed at 400 to 800 cells/s, and data were collected for 10,000 cells in each sample. The data were acquired in a logarithmic scale. Flow cytometry experiments and data analyses were performed using ACEA Novo Express® software (ACEA Biosciences, Inc., San Diego, CA, USA).

2.4.1. Analysis of sperm viability

Sperm viability was performed as described previously (Gallardo-Soler et al., 2019). Semen samples were diluted at 3×10^6 spermatozoa/ml (500 µL final volume) in phosphate buffer saline (PBS) and were incubated with 20 nM of SYBR-14 and 5 µM of PI at room temperature (RT; 20 - 25 °C) with placement in the darkened area for 15 min. After excitation at 488 nm, fluorescence was detected using a 530 ± 30 nm band pass filter for SYBR-14 and 675 ± 30 nm band pass

filter for PI. Viable spermatozoa were considered to be the average of the percentage of SYBR14-positive and PI-negative spermatozoa (LIVE).

2.4.2. Analysis of mitochondrial membrane potential

The mitochondrial membrane potential was evaluated using the specific probe Mitotracker® orange as described previously (Gallardo-Soler et al., 2019). Samples were incubated with 50 nM of Mitotracker® orange at 38.5 °C after placement in a darkened area for 10 min. After excitation at 488 nm, fluorescence was determined using a 572 ± 28 nm band pass filter and the percentage of orange-stained cells in the entire population was detected. Results are expressed as the average of the percentage of spermatozoa having a relatively greater mitochondrial membrane potential (hMMP).

2.4.3. Evaluation of the integrity of the acrosome membrane

The acrosomal status of spermatozoa was assessed using PNA-FITC and PI probes as described previously (Calle-Guisado et al., 2017). There were 100 µL of the sample incubated with PNA-FITC at 2 µg/mL and 5 µM of PI at RT after placement in a darkened area for 5 min and 400 µL of PBS were added before flow cytometry analysis. The fluorescence of PNA-FITC was detected using a 530 ± 30 nm band pass filter and 675 ± 30 nm band pass filter for PI. Viable spermatozoa with a reacted or damaged acrosome are expressed as the average of the percentage of PNA-positive and PI-negative spermatozoa (LAR).

2.4.4. Evaluation of the plasma membrane lipid organization

Sperm plasma membrane lipid organization was assessed using M540 and Yo-Pro-1 probes as described previously (González-Fernández et al., 2018). Samples were incubated with 75 nM of Yo-Pro-1 and 6 µM of M540 at 38.5 °C in a darkened area for 15 min. After excitation at 488 nm, fluorescence was detected using a 572 ± 28 nm band pass filter for M540 and 530 ± 30 nm band pass filter for Yo-Pro-1. Results are expressed as the average of the percentage of live spermatozoa with plasma membrane lipid disorganization: M540-positive and Yo-Pro-1-negative (PMLD).

2.5. Protein extraction for proteomic analysis

To minimize individual boar variations, ejaculates from three animals were pooled using semen from nine boars in different combinations (three pools for winter and three pools for summer). Four mL of the pooled semen from the ejaculates were layered onto 2 mL of 40% PureSperm® and centrifuged at 600 *g* for 10 min at RT to obtain a purified sperm population. The colloid was subsequently removed, and spermatozoa were washed with 5 mL of phosphate buffered saline (PBS) by centrifugation at 300 *g* for 5 min at RT. Sperm samples were subsequently washed a second time for 3 min with centrifugation at 10,000 *g* and re-suspended in lysis buffer [1% (w/v) C7BzO (3-(4-heptyl)phenyl-(3-hydroxypropyl) dimethylammoniopropanesulfonate), 7 M urea, 2 M thiourea, and 40 mM Tris (pH 10.4)] as described previously (Netherton et al., 2018). After 1 h at 4 °C in constant shaking, samples were centrifuged at 18,000 *g* and 4 °C for 15 min. The resulting supernatants were subsequently lyophilized and frozen at -80 °C

189 until analysis. A total of six samples (three for winter and three for summer) were
190 analyzed using a nano LC–MS/MS QTof (Triple Tof).
191
192 *2.6. Sample preparation for LC-MS/MS analysis*
193 A label-free quantitative proteomic analysis was conducted following a
194 bottom-up strategy, with three biological replicates for each season of the year
195 (summer or winter). Samples were subjected to methanol-chloroform precipitation
196 to isolate proteins, remove interfering substances and completely dried. Protein
197 extracts were dissolved in a solution containing 7 M urea, 2 M thiourea and 100
198 mM triethylammonium bicarbonate, subsequently reduced with 50 mM tris (2-
199 carboxyethyl) phosphine (TCEP), and alkylated by addition of cysteine-blocking
200 reagent (200 mM methyl methanethiosulfonate). Samples were further diluted and
201 digested with trypsin at an enzyme to protein ratio of 1:20, at 37 °C overnight.
202
203 *2.7. Protein identification using nano LC–MS/MS QTof (Triple Tof) analysis*
204 Peptide samples were analyzed using a nano liquid chromatography system
205 (Eksigent Technologies nanoLC Ultra 1D plus; AB SCIEX, Foster City, CA, USA)
206 coupled to a 5600 Triple TOF mass spectrometer (ABSCIEX, Foster City, CA,
207 USA) with a nanoelectrospray ion source. Samples were injected in a C18 PepMap
208 trap column (5 µm, 100 µm I.D. x 2 cm; Thermo Scientific, Waltham, MA, USA) at 2
209 µL/min, in 0.1% formic acid in water, and the trap column was switched on-line to a
210 C18 nanoACQUITY BEH analytical column (1.7 µm, 100 Å, 75 µm I.D. x15 cm,
211 Waters). Liquid chromatography was performed using as mobile phases A and B,
212 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. Peptide

elution was accomplished by using a 200 min gradient from 5% to 60% B, at 250 nL/min. The mass spectrometer was operated in data-dependent acquisition mode. For TOF scans, the accumulation time was set to 250 ms (MS1), and as many as 10 precursor ions were acquired per cycle (100 ms for each MS2).

2.8. Proteomic data analysis

The MS/MS spectra were exported to mgf format using Peak View v1.2.0.3 and searched using Mascot Server 2.6.1, OMSSA 2.1.9, X!TANDEM Alanine 2017.2.1.4 and Myrimatch 2.2.140 against a composite target/decoy database developed from sequences in the *Sus scrofa* reference proteome at Uniprot Knowledgebase (UP000008227, Last modified: 20 September 2018), together with commonly occurring contaminants. Search engines were configured to match potential peptide candidates with a mass error tolerance of 25 ppm and fragment ion tolerance of 0.02 Da, allowing for as many as two missed tryptic cleavage sites and a maximum isotope error (13C) of 1, considering fixed MMTS modification of cysteine and the following variable modifications: oxidation of methionine, possible pyroglutamic acid from glutamine or glutamic acid at the peptide N-terminus, and acetylation of the protein N-terminus. Score distribution models were used to compute peptide-spectrum match *P*-values (Ramos-Fernandez et al., 2008) and spectra recovered by a False Discovery Rate (FDR) < 0.01 (peptide-level) filter were selected for quantitative analysis. The least quality signals were removed prior to further analysis.

2.9. Statistical analysis

Sperm quality variables were assessed for normality using a Shapiro-Wilk test and analyzed for equal variances using a Levene´s test. A t-test was used to compare normal data and, if the data did not follow a gaussian distribution, a Mann-Whitney U test was used. Analyses were performed using SigmaPlot software (ver. 12.0) for windows (Systat Software, Chicago, IL, USA).

Peptide ion signals were paired for different processing times to generate intensity ratios, which were then subjected to log2 transformation and normalization. Linear mixed effects models with intensity-based weights and residual variance stratification were used to estimate protein-treatment interaction effects. *P*-values were computed using parametric bootstrap, and differences in mean values were assessed using false discovery rate (FDR) procedures similar to those previously described (Storey and Tibshirani, 2003). A q-value ≤ 0.05 threshold was used for determination of mean differences. Proteomic analyses were conducted using software from Proteobotics (Madrid, Spain).

2.10. Bioinformatic analysis

Protein functional annotation analyses were performed using free available bioinformatics annotation procedures and databases such as the Protein Analysis Through Evolutionary Relationships database (PANTHER) (http://www.pantherdb.org/) and the UniProt Knowledgebase (UniProtKB) (https://www.uniprot.org/).

3. Results

3.1. Effect of season on sperm quality variables

Results from analysis of sperm quality variables indicated there was a lesser percentage of live spermatozoa and those having a relatively greater mitochondrial membrane potential in the summer compared with winter season (Fig. 1, $P < 0.05$). Total and progressive sperm motility, percentage of live spermatozoa with reacted or damaged acrosomes and percentage of live spermatozoa with plasma membrane lipid disorganization did not vary between seasons when semen was collected (Fig. 1).

3.2. Effect of season on boar sperm protein profile

Results from proteomic analyses allowed for the identification of a total of 1,028 characterized proteins that were present when semen was collected in both seasons of the year. A list of boar sperm proteins that were identified in semen samples collected during both seasons of the year and distribution of molecular functions based on the classification using PANTHER (v14.1) are presented in Supplemental Table 1 and are depicted in Supplemental Figure 1. Results when there was a comparative analysis, indicated there were 85 proteins that were different in abundance as a result of season of the year when semen was collected (q-value ≤ 0.05; Table 1). Interestingly, all these proteins were less abundant when semen was collected in the summer compared with the winter. The results from the analysis of these proteins using PANTHER indicated these were related to sperm binding, catalytic activity, regulation of molecular function, structural molecule activity and transporter activity (Fig. 2). When there was use of UniProtKB procedures for enrichment of the biological processes into categories that focused

on reproductive processes, there were 23 proteins that were less abundant when semen was collected in the summer as compared with the winter (Table 2).

Using UniProtKB analyses procedures, there were six proteins identified using the software for “reviewed: records with information extracted from literature and computational data" [(namely outer dense fiber protein 1 (ODF1), phospholipid hydroperoxide glutathione peroxidase (PHGPx), seminal plasma protein pB1 (pAIF-1), major seminal plasma glycoprotein (PSP-I), acrosin and 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase zeta-1 (PLCZ1)] and 17 as “unreviewed: computationally analyzed but awaiting for full manual annotation”. Regarding the first six proteins identified, the reproduction-related main functions were spermatogenesis, single fertilization, acrosome reaction, capacitation and egg activation (Table 2).

4. Discussion

The results of the present study indicate that there are different proteomic profiles of boar spermatozoa collected during the winter and summer and that minor associations of these proteins with overall semen quality exist. Although there were differences in the proteomic profile of spermatozoa between the semen collected during the different seasons of the year, there were not any marked differences in values for sperm quality variables between semen samples collected during these two seasons. There was a lesser sperm viability and mitochondrial membrane potential in semen samples collected during the summer compared with the winter while there were no differences in sperm motility (Fig. 1). Recently, Peña et al. (2019) reported that there was a greater DNA fragmentation in boar

spermatozoa collected during tropical summer while values for motility variables were not different, indicating that, when conducting sperm motility assessments, there may not be detection of spermatozoa with compromised fertilization capacity. For this reason, other approaches and/or variables should be analyzed to ascertain the quality/fertilizing capacity of boar spermatozoa. In the present study, the value of proteomic analyses techniques for identification of new biomarkers was determined.

One of the proteins that is in lesser abundance when semen is collected during the summer compared with the winter is PLCZ1. This protein has important functions in inducing egg activation after the sperm has penetrated the zona pellucida of the oocyte during the fertilization process (Yelumalai et al., 2015; Nomikos et al., 2017). Furthermore, in the conditions imposed in the present study, there was a lesser abundance of acrosin when the semen was collected during the summer. This protein is involved in the acrosome reaction and in the disassociation of cumulus cells from the zona pellucida prior to sperm-oocyte interaction (Isotani et al., 2017). It is noteworthy that acrosin activity in semen samples stored at 17 °C has been reported to be less when the semen is collected during the summer, and that this lesser abundance of acrosin is associated with a reduction in farrowing rates and litter sizes (Pinart et al., 2013). These previous results are consistent with findings in the present study because there was a lesser abundance of acrosin in semen samples collected during the summer, which could explain why there was lesser acrosin activity in sperm from these samples (Pinart et al., 2013). Hence, the lesser abundance of PLCZ1 and acrosin could contribute to the reduced boar sperm fertilizing capacity in semen samples collected during the summer.

Oxidative stress is one of the greatest insults that negatively affect mammalian sperm quality and is inversely associated with sperm fertility (reviewed in Aitken and Drevet, 2020). To counteract this insult, spermatozoa possess antioxidant enzymes such as superoxide dismutase (SOD), phospholipid hydroperoxide glutathione peroxidase (PHGPx), peroxiredoxin (PRDX), thioredoxin (TRX) or thioredoxin reductase (TRD) (reviewed in Martin-Hidalgo et al., 2019). Results from the present study indicate there is a lesser abundance of PHGPx in boar spermatozoa collected during the summer months, possibly resulting in boar sperm not having the capacity to efficiently adapt to conditions when there is oxidative stress during the summer as compared with the winter. This lesser PHGPx abundance could also be associated with a reduction in boar sperm quality and fertility, because the feeding of diets supplemented with antioxidants when there are tropical summer conditions mitigates the negative effect on DNA integrity in boar spermatozoa (Peña et al., 2019). In mature sperm, PHGPx functions as a structural protein maintaining the morphology of these cells (Ursini et al.,1999). In this regard supplementation of boar diets with organic selenium leads to increases in the abundance of PHGPx and there are subsequent decreases in the percentage of head and midpiece abnormalities along with proximal droplets in boar spermatozoa (Martins et al., 2014; Martins et al., 2015).

The proteomics results from the present study indicate that the abundance of PSP-I is lesser in semen samples of boars collected during the periods of the year when ambient temperatures and photoperiod are greatest. The abundance of this protein is associated with the prevalence of monospermic fertilization (Table 2). The relative abundance of PSP-I mRNA transcripts in a previous study was

356 negatively correlated with boar fertility and litter size (Kang et al., 2019). The
357 results from this previous study were inconsistent with those from the present
358 study; however, in this previous study boar semen samples were selected based
359 on previously collected data for litter size and in the present study pooled semen
360 was used for the proteomic evaluations. Another important difference is that in the
361 present study there was analysis of the abundance of PSP-I and not the
362 abundance of mRNA transcript for the gene encoding for the protein. Another
363 protein that was in lesser abundance when semen was collected when ambient
364 temperatures were greater was ODF-1, which is implicated in the axoneme
365 stability, spermatogenesis and in the maintenance of sperm motility (Lehti and
366 Sironen, 2017; Zhao et al., 2018). This protein has been associated with sperm
367 fertility because there is a lesser abundance of ODF-1 in asthenozoospermic men
368 (Chen et al., 2009; Zhao et al., 2018). There was also a lesser abundance of
369 seminal plasma protein pB1 (pAIF-1) in semen samples collected during the
370 summer in the present study. This protein has important functions in sperm
371 capacitation inducing sperm cholesterol efflux in cattle (Therien et al., 1998).
372 Interestingly, there is an association between larger abundances of BSP1 in bull
373 spermatozoa and enhanced embryo cleavage and development (Rodriguez-
374 Villamil et al., 2016). The lesser abundance of PSP-I, ODF-1 and pAIF-1 in the
375 present study when semen was collected during the summer, as compared with
376 the winter, may also be associated with the lesser fertility occurring when there is
377 AI using boar spermatozoa collected when ambient temperatures are greater.
378 In addition, there were the 17 other proteins identified using UniProtKB
379 procedures. Interestingly, these proteins are related to spermatid development,

spermatogenesis, meiosis I, cell motility, single fertilization, sperm capacitation and binding of sperm to zona pellucida and were in lesser abundance in semen samples collected during the summer than winter (Table 2). The involvement of these proteins in spermatogenesis and in fertilization-related processes could be associated with the reduced total sperm count (Huang et al., 2000) and fertilizing capacity of boar sperm from ejaculates collected when ambient temperatures are greater.

Although, the semen evaluated in the present study was collected in a commercial boar stud equipped with ventilation and cooling systems to maintain a constant temperature, when ambient temperatures were greater during the summer months, fluctuations in the barn temperature occurred. When the ambient temperature was greater, there was a greater temperature in the barn where the boars were housed during the summer in the present study (ranging from 21 to 27 °C), even when boar studs were equipped with a cool-cell system (Lugar et al., 2019). These temperatures have been classically considered to be in the thermoneutral zone for pigs (Stone, 1982), however, results from some studies indicate temperatures of greater than 23 °C are beyond those of the thermoneutral zone for pigs (Brown-Brandl et al., 2013). It, therefore, is likely that the temperature variations during the summer and/or the changing photoperiod could be responsible for the changes in values for semen variables as a result of season of year in the present study.

5. Conclusions

403 The present study is the first in which results indicate that boar sperm
404 proteome differs in semen collected during different seasons of the year: summer
405 and winter. The divergent protein abundance described in the present study could
406 be highly relevant in explaining the lesser fertility during the summer in pigs.
407 Further research is required to study the functions of the sperm proteins that differ
408 among seasons; this will help to establish which proteins are contributing to the
409 reduction of boar sperm fertility during the summer and if there can be use of these
410 proteins as biomarkers to accurately predict boar fertility.
411

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Declaration of interest

Authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Author contribution statement

D. Martín-Hidalgo: Data curation, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review and editing; B. Macías-García: Data curation, Writing - original draft, Writing - review and editing. L.J. García-Marín: Review and editing, Funding acquisition; M.J. Bragado: Review and editing, Funding acquisition. L. González-Fernández: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing - original draft, Writing - review and editing.

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Table 1

List of boar sperm proteins that were identified to be in lesser abundance in the summer than winter (q-value ≤ 0.05); Fold change (FC) is expressed as log2 ratio of protein abundance in the summer compared with the winter

Protein ID	Protein Name	Gene name	FC
A0A286ZWH7	A-kinase anchoring protein 4	AKAP4	-1.532
A0A287AFU3	Dynein axonemal heavy chain 17	DNAH17	-0.62
F1RR82	Outer dense fiber of sperm tails 2	ODF2	-0.648
A0A287AZU8	Mannosidase alpha class 2C member 1	MAN2C1	-0.673
F1STE2	Testis specific 10	TSGA10	-2.783
D5K8A2	Mitochondria-eating protein	SPATA18	-1.333
Q2I373	Fascin	FSCN3	-1.74
F1S605	Glutathione S-transferase	GSTM3	-0.566
P36968	Phospholipid hydroperoxide glutathione peroxidase	GPX4	-0.993
K7GLT8	ATP synthase subunit beta	ATP5F1B	-0.559
A0A287BCL9	Glutamine rich 2	QRICH2	-1.082
F1SA46	Actin like 9	ACTL9	-0.569
D5K8A4	Spermatogenesis associated 3	SPATA3	-2.831
P08001	Acrosin	ACR	-0.467
F1RH80	Hormone-sensitive lipase	LIPE	-0.746
Q29077	Outer dense fiber protein 1	ODF1	-1.727
I3LT05	Ropporin-1B isoform X1	ROPN1	-0.672
F1RJB0	Actin related protein T2	ACTRT2	-0.512
F1RPA7	Glycoprotein 2	GP2	-1.498
A0A287AHL5	Dynein axonemal heavy chain 7	DNAH7	-0.417
F1RQD7	Family with sequence similarity 71 member B	FAM71B	-0.563
F1SLZ5	Coenzyme Q10A	COQ10A	-3.747
F1S5N4	Glutathione S-transferase omega 2	GSTO2	-1.548
A0A286ZTA6	Sulfatase domain-containing protein	ARSA	-0.698
K7GSU4	EF-hand domain containing 2	EFHC2	-0.446
I3LAZ9	Maestro heat-like repeat-containing protein family member 1 isoform 1	N/A	-0.944
F1RYX9	Armadillo repeat containing 12	ARMC12	-0.791
F1SR82	Galactosidase beta 1 like	GLB1L	-0.593
F1RL08	Tektin 5	TEKT5	-0.304
F1SE27	Voltage-dependent anion-selective channel protein 3	VDAC3	-0.542
I3LUR5	Acyl-CoA dehydrogenase family member 9	ACAD9	-0.567
K7GPW3	MICOS complex subunit	APOO	-1.423
Q9MZ15	Voltage-dependent anion-selective channel protein 2	VDAC2	-0.727
F1SDE8	Tektin 3	TEKT3	-0.348
F1RSL2	Spermatogenesis and centriole associated 1	SPATC1	-0.745
F1SEB3	Chromosome 9 open reading frame 24	C9orf24	-0.409
I3LC15	Angiotensin-converting enzyme	LOC100515049	-0.368

F1SMS2	Carboxypeptidase A1	CPA1	-0.804
F1SP29	Actin like 7A	ACTL7A	-0.357
F1RRW5	Angiotensin-converting enzyme	ACE	-0.381
F1RFM8	Dynein axonemal heavy chain 10	DNAH10	-0.65
A0A287AND7	Cytochrome c oxidase subunit	COX6B2	-0.623
I3LNF2	Dynein axonemal heavy chain 1	DNAH1	-0.519
P80964	Seminal plasma protein pB1	N/A	-0.891
F1SIK7	Dpy-19 like 2	DPY19L2	-0.803
I3LMV8	ATP binding cassette subfamily B member 8	ABCB8	-1.697
F1SP93	V-type proton ATPase catalytic subunit A	ATP6V1A	-0.532
D5K8A1	Spermatogenesis associated 6	SPATA6	-1.165
A0A287AY47	RAN guanine nucleotide release factor	RANGRF	-1.311
A0A287B8Z2	Fructose-bisphosphate aldolase	ALDOC	-2.666
D3K5J6	Acrosomal vesicle protein 1	ACRV1	-1.987
O79875	NADH-ubiquinone oxidoreductase chain 2	MT-ND2	-1.497
F1S814	Phosphoglucomutase 1	PGM1	-0.652
Q5S233	Mitochondrial associated cysteine-rich protein	SMCP	-1.617
F1RPB7	Developmentally-regulated GTP-binding protein 1	DRG1	-2.428
I3LDJ2	Spermatogenesis-associated protein 19	SPATA19	-1.121
A0A287A3S5	Tektin-4 isoform 1	LOC110259951	-0.31
P00889	Citrate synthase, mitochondrial	CS	-0.417
F1RH17	Family with sequence similarity 71 member E1	FAM71E1	-1.084
A0A287AZF9	Cytochrome c1	CYC1	-0.63
F1SQ16	Golgin B1	GOLGB1	-2.357
F1SCH1	NADH dehydrogenase ubiquinone 1 beta subcomplex subunit 7	NDUFB7	-0.771
F1S8P1	Sacchrp_dh_NADP domain-containing protein	SCCPDH	-0.354
F1RZ12	T-complex 11	TCP11	-1.459
F1SN46	Chromosome 15 open reading frame 48	C15orf48	-0.556
F1S3C7	AP-1 complex subunit gamma	AP1G1	-2.341
F1RMB9	Nudix hydrolase 18	NUDT18	-1.101
A0A287BLC7	Long-chain-fatty-acid--CoA ligase 6 isoform X1	ACSL6	-0.386
F1S5Q9	Theg spermatid protein	THEG	-0.543
K9IVI1	2-oxoglutarate dehydrogenase	OGDH	-0.359
F1SDN3	Dynein regulatory complex subunit 1	DRC1	-1.144
F1SI60	Zinc finger and SCAN domain containing 29	ZSCAN29	-2.152
D3K5M3	A-kinase (PRKA) anchor protein 3	AKAP3	-1.455
A0A287AN02	26S proteasome non-ATPase regulatory subunit 2	ECE2	-0.617
F1S110	Solute carrier family 9 member B1	SLC9B1	-1.616
F1RVX7	Family with sequence similarity 166 member A	FAM166A	-0.356
C8C4M8	Zona pellucida binding protein 2	ZPBP2	-0.329
I3LNB4	Multifunctional fusion protein	ALDH4A1	-0.474
F1SDH8	Phospholipid-transporting ATPase	ATP8B3	-0.658
A0A287ARZ5	Pyridoxal dependent decarboxylase domain containing 1	PDXDC1	-2.043
P35495	Major seminal plasma glycoprotein PSP-I	N/A	-0.805
F1ST73	Glyoxylate and hydroxypyruvate reductase	GRHPR	-2.008

F1RGC9	Proteasome 26S subunit, non-ATPase 13	PSMD13	-1.057
Q866A8	Fertilin beta	FTNB	-0.817
Q7YRU3	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase zeta-1	PLCZ	-0.364

643 **Table 2**

644 List of proteins that regulate reproductive processes and distribution in reproductive-

645 related functions groups based on the UniProtKB database (www.uniprot.org)

646 assessments; Proteins marked with asterisk (*) are classified in UniProtKB, while the other

647 proteins (without asterisk) are classified as unreviewed

648

Protein ID	Protein name	Reproduction-related function	FC
Q29077*	Outer dense fiber protein 1	Spermatogenesis	-1.727
P36968*	Phospholipid hydroperoxide glutathione peroxidase	Spermatogenesis	-0.993
P80964*	Seminal plasma protein pB1	Single fertilization; sperm capacitation	-0.891
P35495*	Major seminal plasma glycoprotein PSP-I	Single fertilization	-0.805
P08001*	Acrosin	Single fertilization; acrosome reaction	-0.467
Q7YRU3*	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase zeta-1	Egg activation	-0.364
F1STE2	Testis specific 10	Spermatogenesis	-2.783
Q2I373	Fascin	Spermatid development; spermatogenesis	-1.74
Q5S233	Mitochondrial associated cysteine-rich protein	Flagellated sperm motility	-1.617
A0A286ZWH7	A-kinase anchoring protein 4	Flagellated sperm motility	-1.532
D5K8A1	Spermatogenesis associated 6	Spermatogenesis	-1.165
A0A287BCL9	Glutamine rich 2	Flagellated sperm motility	-1.082
F1RGC9	Proteasome 26S subunit, non-ATPase 13	Meiosis I	-1.057
Q866A8	Fertilin beta	Single fertilization	-0.817
F1SIK7	Dpy-19 like 2	Spermatid development	-0.803
I3LT05	Ropporin-1B isoform X1	Sperm capacitation	-0.672
F1SDH8	Phospholipid-transporting ATPase	Binding of sperm to zona pellucida	-0.658
F1S5Q9	Theg spermatid protein	Spermatogenesis	-0.543
I3LNF2	Dynein axonemal heavy chain 1	Flagellated sperm motility	-0.519
F1RRW5	Angiotensin-converting enzyme	Spermatogenesis	-0.381
F1SDE8	Tektin 3	Flagellated sperm motility	-0.348
C8C4M8	Zona pellucida binding protein 2	Binding of sperm to zona pellucida	-0.329
A0A287A3S5	Tektin-4 isoform 1	Cilium movement involved in cell motility	-0.31

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650

Fig.1. Effect of season on sperm quality variables; hMMP: high mitochondrial membrane potential; TM: Total motility; PM: Progressive motility; LAR: Live spermatozoa with acrosome reacted or damaged; PMLD: Plasma membrane lipid disorganization; Values are expressed as mean ± standard error of the mean, SEM; *$P < 0.05$. ($n = 6$, six seminal doses from six different boars)

Fig. 2. Pie charts representing the distribution of molecular functions of proteins in lesser abundance in boar spermatozoa collected during the summer compared with winter based on the classifications utilizing PANTHER (v14.1)

Supplemental Table 1

List of boar sperm proteins identified in both seasons

Supplemental Fig. 1

Pie charts representing the distribution of molecular functions of boar sperm proteins detected in semen samples collected during both seasons of the year based on the classification using PANTHER (v14.1)

Figure 1

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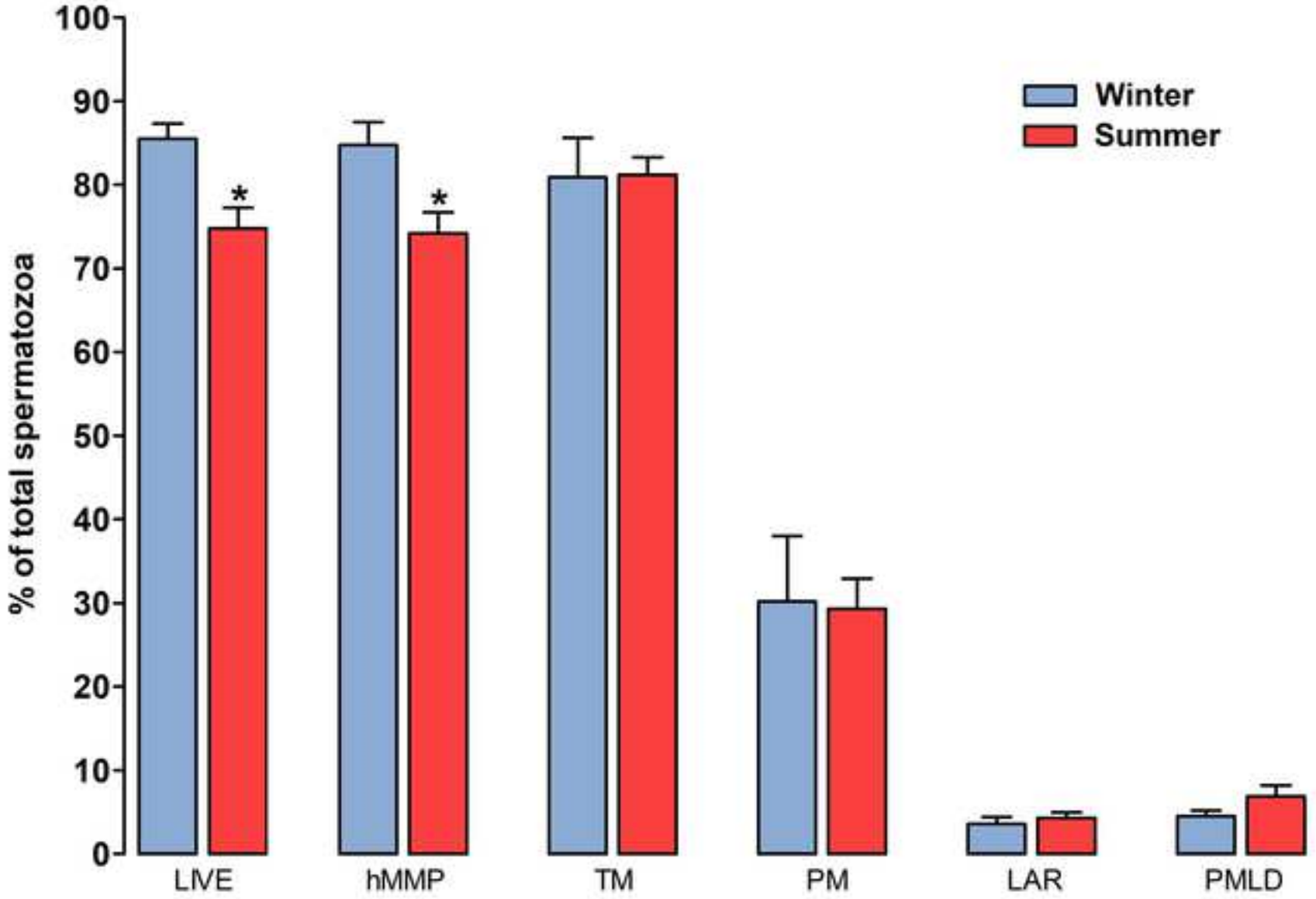
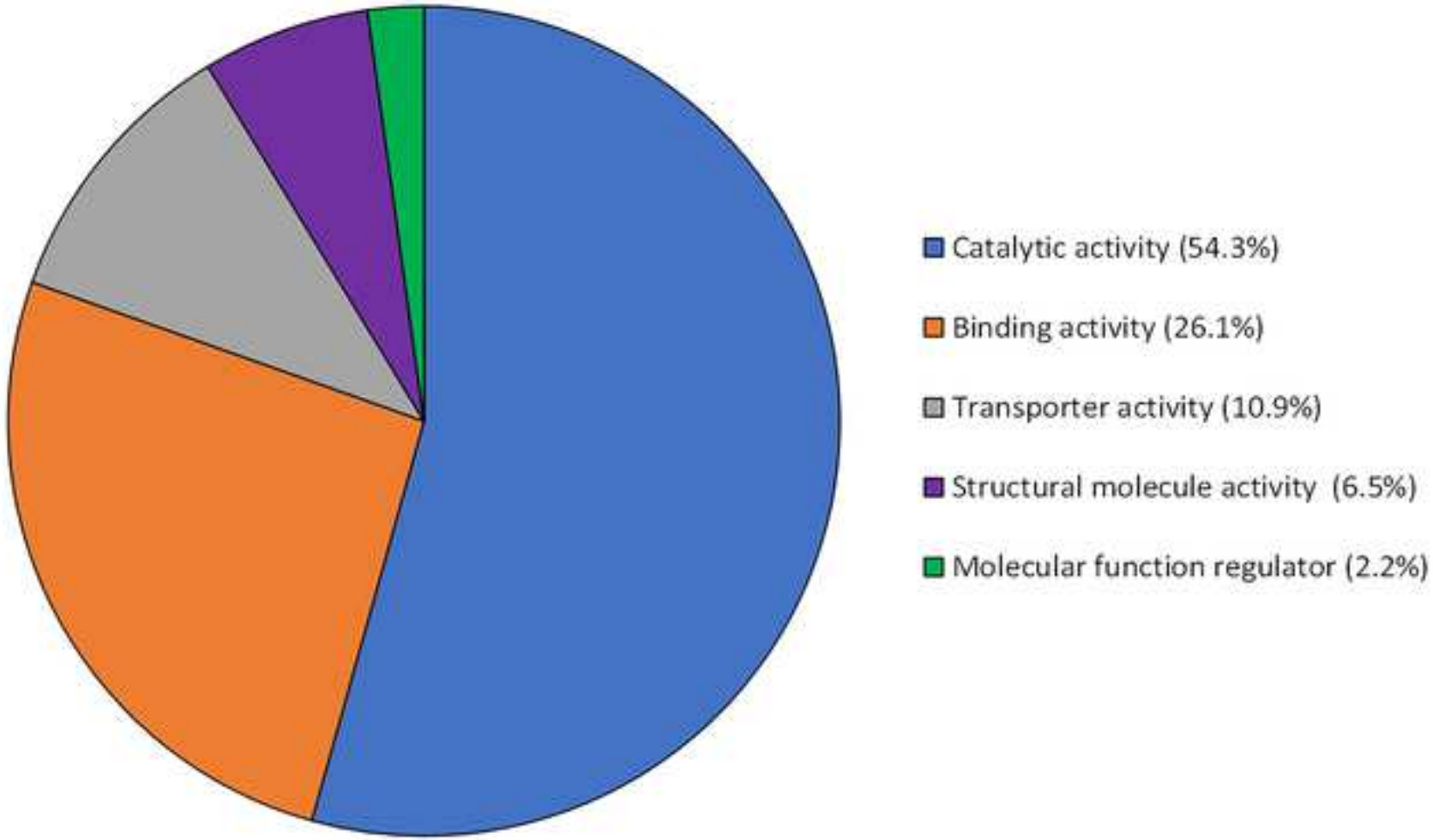


Figure 2



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