CSIRO PUBLISHING

Reproduction, Fertility and Development
http://dx.doi.org/10.1071/RD15530

Thawing boar semen in the presence of seminal plasma improves motility, modifies subpopulation patterns and reduces chromatin alterations

AQ1

Rocío Fernández-Gago[A], *Manuel Álvarez-Rodríguez*[B], *Marta E. Alonso*[C], *J. Ramiro González*[A], *Beatriz Alegre*[A], *Juan Carlos Domínguez*[A,D] *and Felipe Martínez-Pastor*[D,E,F]

[A]Department of Medicine, Surgery and Veterinary Anatomy, University of León, 24071 León, Spain.
[B]IKE (Department of Clinical and Experimental Medicine), Linköping University, SE-581 83 Linköping, Sweden.
[C]Department of Animal Production, University of León, 24071 León, Spain.
[D]INDEGSAL (Institute for Animal Health and Cattle Development), University of León, 24071 León, Spain.
[E]Molecular Biology (Cell Biology), University of León, 24071 León, Spain.
[F]Corresponding author. Email: felipe.martinez@unileon.es

Abstract. Seminal plasma could have positive effects on boar semen after thawing. In the present study we investigated changes in the motility and chromatin structure in spermatozoa over 4 h incubation (37°C) of boar semen thawed in the presence of 0%, 10% or 50% seminal plasma from good-fertility boars. Cryopreserved doses were used from seven males, three of which were identified as susceptible to post-thawing chromatin alterations. Motility was analysed by computer-aided sperm analysis every hour, and data were used in a two-step clustering, yielding three subpopulations of spermatozoa (slow non-linear, fast non-linear, fast linear). Chromatin structure was analysed using a sperm chromatin structure assay and flow cytometry to determine the DNA fragmentation index (%DFI) as a percentage, the standard deviation of the DFI (SD-DFI) and the percentage of high DNA stainability (%HDS), indicating chromatin compaction. Thawing without seminal plasma resulted in a rapid loss of motility, whereas seminal plasma helped maintain motility throughout the incubation period and preserved the subpopulation comprising fast and linear spermatozoa. The incidence of chromatin alterations was very low in samples from non-susceptible males, whereas samples from males susceptible to post-thawing chromatin alterations exhibited marked alterations in %DFI and %HDS. Seminal plasma partly prevented these alterations in samples from susceptible males. Overall, 50% seminal plasma was the most efficient concentration to protect motility and chromatin. Some changes were concomitant with physiological events reported previously (e.g., semen thawed with 50% seminal plasma increased the production of reactive oxygen species and yielded higher fertility after AI). Thawing in the presence of seminal plasma could be particularly useful in the case of samples susceptible to post-thawing chromatin damage.

Additional keywords: cryopreservation, flow cytometry, sperm physiology.

Received 14 December 2015, accepted 18 July 2016, published online xx xxxxx xxxx

Introduction

Seminal plasma plays an important role in maintaining sperm viability and modulating both sperm physiology and the response of the female genital tract to the presence of spermatozoa (Maxwell *et al.* 2007; Kaczmarek *et al.* 2013). Although these secretions are critical for the natural fertilisation process, they are often detrimental for the purposes of artificial reproductive techniques (ART). Thus, seminal plasma is removed and/or samples are extended with solutions that neutralise or protect spermatozoa against these detrimental components (Manjunath *et al.* 2007; Rodríguez-Martínez *et al.* 2008; Okazaki *et al.* 2009). Nevertheless, a whole field of research into the identification, isolation and production of seminal plasma components with beneficial properties for reproductive technologies has flourished (Caballero *et al.* 2008; Serrano *et al.* 2013).

Okazaki *et al.* (2009) found that the removal of seminal plasma was associated with improved post-thawing quality

of boar semen, especially in samples with poor freezability. Moreover, when semen was thawed in the presence of seminal plasma from high-fertility boars, both quality (with 5–20% seminal plasma) and fertility after AI (with 10% seminal plasma) improved notably. Previously, we found that the presence of 50% seminal plasma in the thawing medium prevented the decreases in pregnancy rate and total litter size observed with cryopreserved semen (Garcia *et al.* 2010). In contrast with the findings reported by Okazaki *et al.* (2009), the viability and motility of thawed semen were not higher for samples thawed in the presence of 10% seminal plasma compared with samples thawed without seminal plasma (Garcia *et al.* 2010). In an effort to understand how seminal plasma affects the physiology of boar spermatozoa, we undertook a flow cytometry study (Fernández-Gago *et al.* 2013). The most pronounced change during 4 h of incubation at 37°C occurred when we used 50% seminal plasma, with a rapid increase in membrane fluidity and a delayed increase in intracellular reactive oxygen species (ROS). Other changes were less evident (acrosomal status, extracellular free thiols etc.), and samples thawed in the presence of 10% seminal plasma only underwent subtle changes. Indeed, few studies have focused on these physiological variables. For example, Vadnais *et al.* (2005) reported that capacitation could be reverted when using 10% or 20% seminal plasma. Another study using 10% seminal plasma did not find any differences regarding membrane fluidity, but tyrosine phosphorylation and annexin V externalisation were prevented by the addition of seminal plasma to the thawed semen incubated under capacitating conditions (Vadnais and Althouse 2011).

The aim of the present study was to enhance our understanding of the processes occurring in thawed boar spermatozoa exposed to seminal plasma in order to better explain the increased fertility of these samples (Okazaki *et al.* 2009; Garcia *et al.* 2010). Because motility and chromatin status are critical for sperm fertilising ability, we focused on these parameters in the present study. Thus, we assessed thawed samples using computer-aided sperm analysis (CASA) and subpopulation analysis (Martínez-Pastor *et al.* 2011), as well as a sperm chromatin structure assay (SCSA; Hernández *et al.* 2006). There is evidence that seminal plasma can affect sperm motility (Saravia *et al.* 2007; Rodríguez-Martínez *et al.* 2008), so we expected to detect changes in the motility pattern of thawed spermatozoa exposed to 10% or 50% seminal plasma.

Considering sperm chromatin, part of the fertility results could be explained by this uncompensable trait (Evenson 1999). Indeed, it has been found that samples from 'bad freezer' boars tend to have lower chromatin quality (Hernández *et al.* 2006). Previous studies have highlighted that samples from 'bad freezers' could be especially vulnerable to chromatin damage (Hernández *et al.* 2006; Yeste *et al.* 2013) and that seminal plasma could be particularly beneficial for these types of samples (Okazaki *et al.* 2009). Thus, we used semen samples from two groups of males, all of them producing high-quality semen but differing in chromatin status after cryopreservation.

We tested two hypotheses with regard to thawing boar semen in the presence of 10% or 50% seminal plasma: (1) supplementation with seminal plasma can preserve the motility pattern (both the proportion of motile spermatozoa and the swimming trajectories and velocities, including subpopulation structure; Martínez-Pastor *et al.* 2011); and (2) changes in sperm chromatin occurring after thawing could be prevented by seminal plasma. Moreover, we propose that this protection could be more important in samples from 'bad freezers', defined as those samples with more damaged sperm chromatin after thawing. To perform this experiment, we surveyed our cryobank, identifying males with low levels of chromatin changes after thawing (low susceptibility) and those with high levels of chromatin changes (high susceptibility).

Materials and methods

Experimental design

The cryopreserved doses used in the present study were obtained from seven boars (three different freezing sessions for each boar). The males were selected based on previous analyses, with samples from four males having a low incidence of chromatin alterations after thawing and those from three males being highly susceptible to chromatin changes after thawing. Low-susceptibility males were defined as those with samples that exhibited low levels of chromatin alterations just after thawing, as determined by SCSA, with values for the DNA fragmentation index (%DFI) $<6\%$ or high DNA stainability (%HDS) $<15\%$, whereas high-susceptibility males were defined as those with samples exhibiting high levels of chromatin alterations after thawing (%DFI $\geq 6\%$ or %HDS $\geq 15\%$). These cut-off values for the SCSA variables were chosen based on previous work on boars (Boe-Hansen *et al.* 2008; Didion *et al.* 2009). In each experimental session, three straws were thawed per boar and freezing session, which were then pooled. Semen was then diluted to a concentration of 25×10^6 spermatozoa mL^{-1} with MR-A extender (Kubus) supplemented with heterologous seminal plasma at concentrations of 0%, 10% or 50%. The samples were kept at 37°C and assessed for motility and chromatin status 0, 1, 2, 3 and 4 h after supplementation. Each experimental session for each boar × freezing combination was repeated three times.

Reagents and media

All chemicals were at least of reagent grade and acquired from Sigma. Acridine orange (AO) was obtained from Polysciences (chromatographically purified). Flow cytometry equipment, software and consumables were purchased from Becton Dickinson.

Semen collection and preservation

Semen was collected by the gloved-hand method from three Landrace and four Large White mature boars, housed at the Technological Centre of Artificial Insemination (Campo de Villavidel, León, Spain). The males were part of the routine semen production system of the centre and were between 12 and 24 months of age. Males selected for the experiment (both for semen and seminal plasma) had good fertility (average prolificacy >12 piglets per litter). The sperm quality of the fresh ejaculate was assessed visually, and only ejaculates containing

>85% motile spermatozoa and <25% abnormal spermatozoa were processed.

Semen was cryopreserved as described previously (Fernández-Gago *et al.* 2013). Briefly, sperm concentration was assessed spectrophotometrically, 60×10^6 spermatozoa were diluted in MR-A at 32.5°C, left at room temperature (20–22°C) for 1 h and then at 15°C for 3 h. The supernatant was removed after centrifugation (800*g*, 10 min, 4°C) and the pellet was resuspended in lactose–egg yolk extender (80% v/v of an 11.0% w/v lactose monohydrate solution and 20.0% v/v hen's egg yolk) to a concentration of 1.5×10^9 spermatozoa mL^{-1}. After 2 h at 5°C, the semen was slowly mixed with freezing extender (89.55% v/v lactose–egg yolk extender, 8.95% v/v glycerol and 1.5% v/v Equex STM; Minitüb) at two parts semen to one part extender (final concentrations 3% glycerol and 1.0×10^9 spermatozoa mL^{-1}). The semen was packaged in 0.25-mL straws and frozen in a programmable freezer (Ice Cube 1810; Sy-Laboratory) at a rate of -3°C min^{-1} from +5 to −6°C, held for 1 min and then cooled at a rate of -20°C min^{-1} from −6°C to −100°C. Samples were then plunged into liquid nitrogen for storage. The straws were thawed in water at 50°C for 12 s.

Seminal plasma

Seminal plasma was obtained after double centrifugation (800*g*, 10 min, 25°C) of a semen pool derived from 11 boars (Duroc, Large White and Landrace). These boars were between 12 and 24 months of age and had good semen quality and prolificacy, as in the case of the boars from which semen was obtained for cryopreservation. Semen samples were checked for quality as described above for semen collection. Seminal plasma aliquots were stored at −20°C until needed. The seminal plasma was thawed at 37°C in a water bath until all the ice had melted.

CASA analysis

Spermatozoa were diluted to a concentration of $10–20 \times 10^6$ spermatozoa mL^{-1} with phosphate-buffered saline (PBS) and loaded into a Makler counting chamber (10 μm depth; Sefi Medical Instruments, Haifa, Israel) at 37°C. The CASA system consisted of a triocular optical phase contrast microscope (Nikon Eclipse 80i; Nikon), equipped with a warming stage at 37°C and a Basler A302fs digital camera (Basler Vision Technologies). The camera was connected to a computer by an IEEE 1394 interface. Images were captured and analysed using ISAS software (Proiser). Sampling was performed using a ×10 negative phase contrast objective (no intermediate magnification). Image sequences (at least three fields with more than 200 spermatozoa analysed) were saved and analysed afterwards. Software settings were adjusted to boar spermatozoa. The standard parameter settings were as follows: 53 frames s^{-1}; 20–90 μm for head area; and curvilinear velocity (VCL) >10 μm s^{-1} to classify a spermatozoon as motile. For each spermatozoon, the software rendered the percentage of motile spermatozoa, three velocity parameters (VCL, straight line velocity (VSL) and average path velocity (VAP)), three track linearity parameters (linearity (LIN), straightness (STR) and wobble (WOB)), the amplitude of lateral head displacement (ALH) and beat cross frequency (BCF).

Sperm chromatin structure assay

Chromatin stability was assessed by SCSA (Evenson and Jost 2000). Full descriptions of the technique, cytometer configuration and gating strategy are provided in the supplementary material (section S1 and Fig. S1). Briefly, the technique is based on the denaturalisation of broken DNA and on the properties of AO, whose fluorescence shifts from green (double-stranded (ds) DNA) to red (single-stranded (ss) DNA) depending on the degree of DNA denaturation. Samples were diluted in TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl and 1 mM disodium EDTA, pH 7.4) to a final concentration of 2×10^6 spermatozoa mL^{-1} and stored at −80°C. For analysis, samples were thawed on crushed ice. A 200-μL aliquot of the sample was pipetted into a flow cytometry tube and immediately mixed with 0.4 mL acid-detergent solution (0.08 M HCl, 0.15 M NaCl and 0.1% Triton X-100, pH 1.2). After 30 s, 1.2 mL staining orange solution (6 μg mL^{-1} AO in 0.1 M citric acid, 0.2 M Na_2HPO_4, 1 mM disodium EDTA and 0.15 M NaCl, pH 6.0) was added to the tube. The tube was kept on ice for 3 min before flow cytometry analysis. A FACScalibur flow cytometer (Becton Dickinson) was used for analysis with acquisition software CellQuest version 3 (Becton Dickinson). In all, 5000 spermatozoa were analysed per sample, exciting the AO with an Ar ion laser at 488 nm and using a 530/30 filter for the green fluorescence of dsDNA-bound AO and a 650 long-pass filter for the red fluorescence of ssDNA-bound AO. Data were saved in flow cytometry standard (FCS) version 2 files, which were processed using the R statistical environment (R Development Core Team 2016). The DFI was calculated for each spermatozoon as the ratio of red fluorescence to total (red + green) fluorescence. From the %DFI, the standard deviation of DFI (SD-DFI) and the percentage of spermatozoa with a high fragmentation index (DFI >25%; %hDFI) were determined. In addition, the percentage of HDS, defined as those events with green fluorescence above Channel 750, was also determined.

Statistical analysis

Statistical analyses were performed in the R statistical environment (R Development Core Team 2016). Data were analysed using linear mixed-effects models. Incubation time and seminal plasma concentration were included as fixed factors, with male and ejaculate used to group random effects in the models. To study the effect of male, it was as a fixed factor, leaving ejaculate as the random effect in the model. Results are presented as mean ± s.e.m.

In addition, clustering analysis was performed on motility data in order to classify spermatozoa in groups characterised by distinctive motility patterns. A two-step procedure was performed, as described previously (Martínez-Pastor *et al.* 2011; Gallego *et al.* 2015). Briefly, two successive hierarchical clustering steps were performed on the data, the first for each individual analysis and the second using the median values of the first set of clusters. We ended up with a reduced set of clusters, using the proportion of spermatozoa in each cluster for subsequent analyses.

Table 1. Significance table for the tested models (main effects and their interactions) when incubation time, seminal plasma (SP) treatment and male were included as fixed effects in the statistical models

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. MOT, total motility; PROG, progressive motility; VCL, curvilinear velocity; LIN, linearity; WOB, wobble; ALH, amplitude of lateral head displacement; SP1, SP2, SP3, sperm subpopulations 1, 2 and 3 respectively (see Table 2); SD-DFI, standard deviation of the DNA Fragmentation index (DFI); %DFI, proportion of spermatozoa with DFI >25; %HDS, proportion of spermatozoa with high DNA stainability (green fluorescence above Channel 750)

Variable	Time	SP	Male	Time × SP	TIME × male	SP × male	time × SP × male
MOT	***	***	*	n.s.	***	n.s.	n.s.
PROG	***	***	*	n.s.	***	n.s.	n.s.
VCL	***	***	*	***	***	**	n.s.
LIN	n.s.	**	n.s.	***	**	***	n.s.
WOB	n.s.	***	n.s.	***	***	***	n.s.
ALH	***	*	*	***	***	*	n.s.
SP1	n.s.	n.s.	n.s.	**	n.s.	*	n.s.
SP2	***	***	n.s.	n.s.	n.s.	n.s.	n.s.
SP3	***	**	n.s.	n.s.	n.s.	n.s.	n.s.
s.d.-DFI	n.s.	**	n.s.	n.s.	*	***	n.s.
%DFI	n.s.	***	n.s.	n.s.	***	***	n.s.
%HDS	n.s.	***	n.s.	***	***	***	***

Results

Variability of motion parameters

All motility parameters were clearly affected by incubation time, with a noticeable reduction in the proportion of motile spermatozoa and the quality of movement (Table 1; Fig. 1). However, this effect was highly modulated by the presence of seminal plasma. Samples from different males differed in motility characteristics, although the use of seminal plasma had a positive effect overall (see Figs S2 and S3, available as Supplementary Material to this paper).

Total motility (Fig. 1*a*) and progressive motility (Fig. 1*b*) decreased with incubation time (the total motility of most samples fell below 10% by 3 h), with 50% seminal plasma slowing this decline ($P < 0.001$ for time and seminal plasma as main effects, no interaction). Total and progressive motility differed across the three treatment groups, with 0% seminal plasma resulting in the lowest mean values and 50% seminal plasma resulting in the highest ($P < 0.001$). Kinematic parameters were significantly affected by interactions between time and treatment. These interactions were due primarily to the early loss of motility in the 0% seminal plasma treatment group, resulting in no kinematic data available after 2 h. The trends in VCL (Fig. 1*c*) and ALH (Fig. 1*f*) resembled those of total motility, decreasing with time in the three treatment groups ($P < 0.001$). However, the presence of seminal plasma abolished the decrease in VCL and ALH after 1 h incubation. Different profiles were observed for LIN (Fig. 1*d*) and WOB (Fig. 1*e*), with significant but less marked effects of incubation time. LIN and WOB in samples incubated with 0% seminal plasma decreased, on average, to <20% at 2 h (no motile spermatozoa remaining afterwards), whereas they tended to remain the same when seminal plasma was present. However, LIN and WOB were significantly higher in samples incubated in the presence of 10% seminal plasma than 50% seminal plasma at 0 and 1 h ($P < 0.05$), a situation that was reversed after 1 h.

The loss of motility at advanced incubation times (after 1 h) limited the analysis of sperm subpopulations because little information could be extracted beyond 1 h, especially for the 0% seminal plasma treatment (<10% motility at 2 h; Fig. 1*a*). We were able to obtain three subpopulations with very different kinematics (Table 2). Subpopulation (SP) 1 was defined by relatively low values of the kinematic parameters and comprised slow spermatozoa, with low linearity and wobbling trajectories (low WOB). SP2 was characterised by fast, low-linear wobbling trajectories, with a wide 'yaw' (ALH). Finally, SP3 showed intermediate velocity, but high linearity and low wobbling (high WOB) and yaw (low ALH).

Incubation with seminal plasma affected the subpopulation pattern. SP1 (Fig. 2*a*) was initially predominant in samples incubated in the presence of 50% seminal plasma, decreasing subsequently (time effect $P = 0.014$), whereas the proportion of SP1 tended to remain the same at all sampling times in the 10% seminal plasma treatment group. In contrast, SP2 and SP3 (Fig. 2*b*, *c*) were strongly affected by incubation time in all treatments, following opposite trends: the proportion of SP2 decreased, whereas that of SP3 increased. In general, samples treated with 10% seminal plasma had the highest proportion of SP2, whereas samples treated with 50% seminal plasma had the highest proportion of SP3, at least by the middle of the incubation period (2 h).

In addition, very high variability was detected among males. The profile plots of individual males (Figs S2 and S3) showed different responses to incubation time and seminal plasma. We confirmed a significant male effect for most variables after including 'male' as a fixed effect in the models, either as a main effect or as an interacting term with time or seminal plasma (Table 1). The factor 'male' exhibited significant interaction with time for total and progressive motility (Fig. S2*a*, *b*), indicating that it affected the rate of motility loss. This interaction was affected by initial motility, which was much higher for

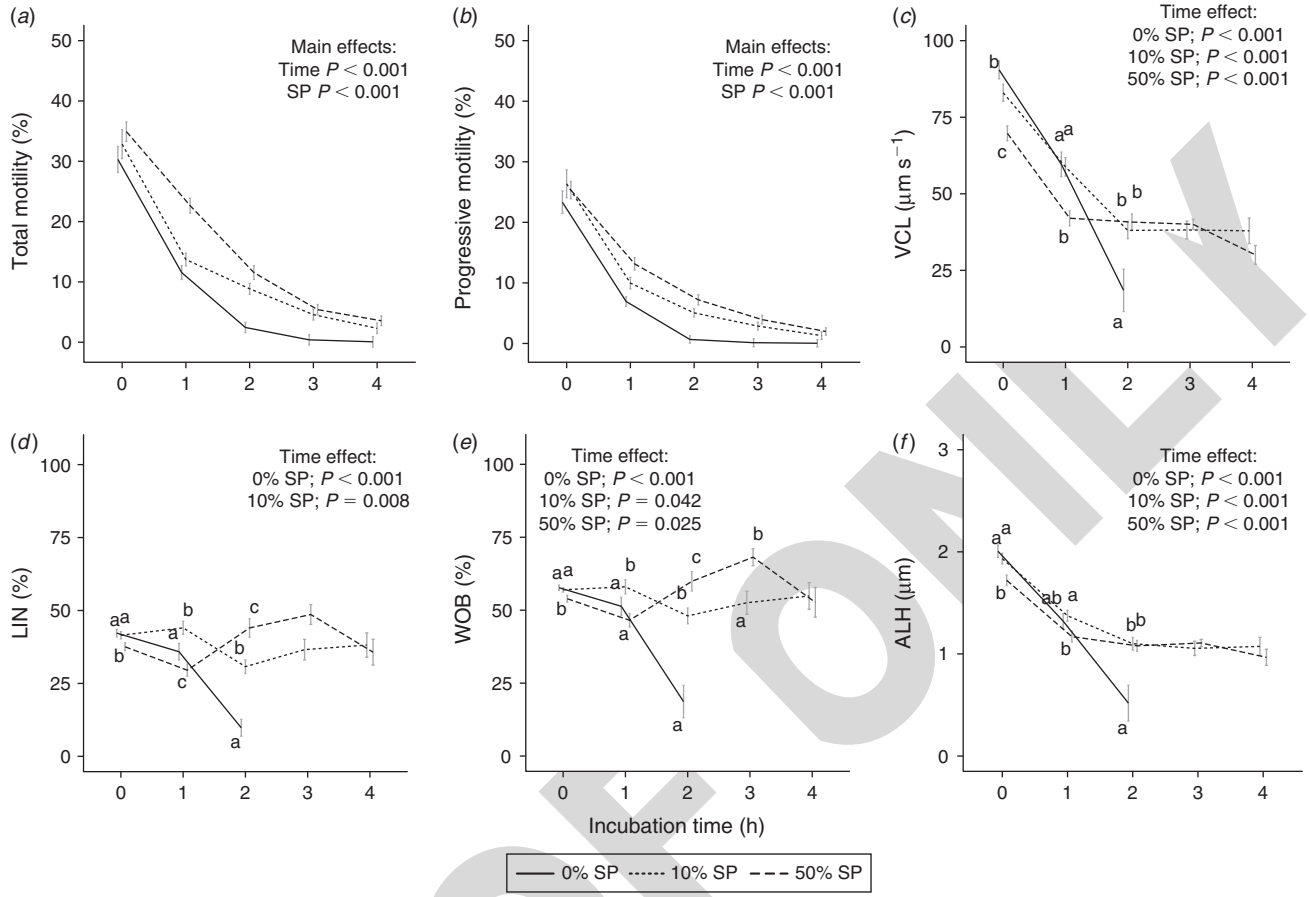

Fig. 1. Computer-aided sperm analysis (CASA) revealed a decrease in (*a*) total and (*b*) progressive motility, as well as (*c*) curvilinear velocity (VCL) with incubation time. The addition of seminal plasma (SP), especially at a concentration of 50%, improved sperm motility. Less marked changes were observed in the linearity variables of (*d*) linearity (LIN) and (*e*) wobble (WOB), whereas (*f*) amplitude of lateral head displacement (ALH) decreased in a similar manner to VCL. The time × SP interaction was significant for motility variables, except for total and progressive motility. When the interaction was significant, *P*-values are given for the time effect within each SP treatment, if $P < 0.05$. Data are the mean ± s.e.m. Different letters on the graphs indicate significant differences between SP treatments at each time point.

Table 2. Sperm subpopulations obtained from the computer-aided sperm analysis dataset

Mean ± s.d. values for kinetic parameters are given. In all, 17 125 motile spermatozoa from 246 samples were used in the clustering analysis. VCL, curvilinear velocity; LIN, linearity; WOB, wobble; ALH, amplitude of lateral head displacement; SP1, SP2, SP3, sperm subpopulations 1, 2 and 3 respectively

Subpopulation	VCL (μm s^{-1})	LIN (%)	WOB (%)	ALH (μm)
SP1	56.0 ± 31.4	27.7 ± 15.7	50.4 ± 10.8	1.6 ± 0.6
SP2	113.1 ± 38.8	49.1 ± 13.3	59.7 ± 10.2	2.4 ± 0.9
SP3	67.3 ± 33.2	67.8 ± 14.2	79.8 ± 11.9	1.4 ± 0.3

LD2 (Landrace) and LW4 (Large White) males. For the rest of the kinematic parameters, the factor 'male' interacted with both time and seminal plasma treatment. Indeed, samples from LD1, LD2, LW1, LW3 and LW4 males tended to maintain values of the kinematic variables throughout the incubation period incubated in the presence of seminal plasma. There was a significant interaction between male and seminal plasma when analysing the proportion of SP1 (Fig. S4*a*). In the case of SP2 and SP3, the overall effects of time and seminal plasma were by far more important than any effect due to between-male variability (Fig. S4*b*, *c*).

Variability of chromatin structure

Seminal plasma protected sperm chromatin against post-thawing alterations, but only in samples from susceptible males (LD3, LW1 and LW3; Fig. 3). Nevertheless, this susceptibility was not associated with motility parameters. SD-DFI (Fig. 3*a*) and %DFI (Fig. 3*b*) were little affected by incubation time, and the estimated change was small, even when the effect was significant. Adding seminal plasma to samples decreased SD-DFI only for LD3, the male with the highest values of SD-DFI in samples incubated without the addition of seminal plasma (0%; Fig. 3*a*). Differences were more marked for %DFI (Fig. 3*b*), with samples from all susceptible males showing significantly lower values

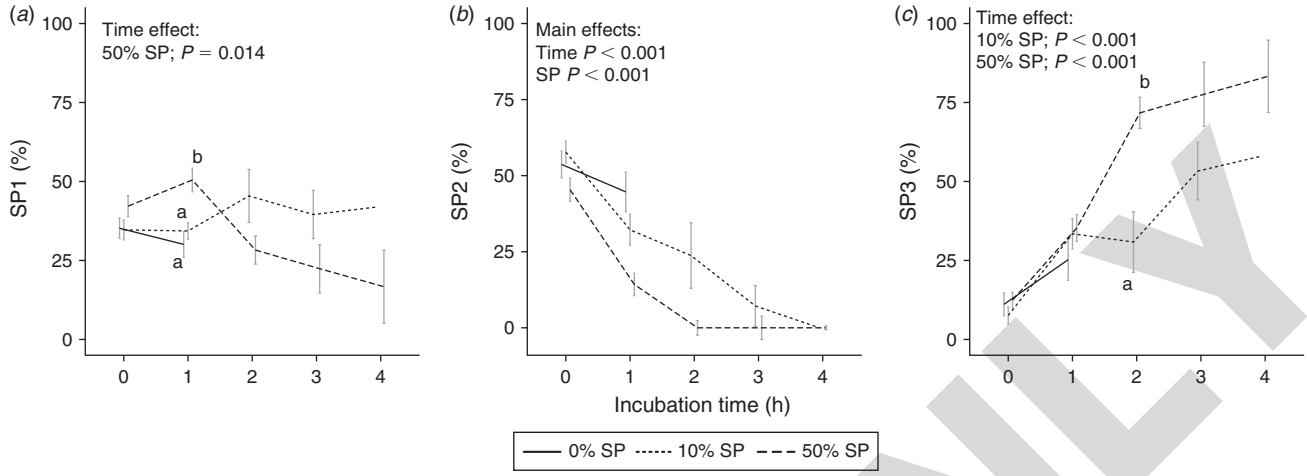

Fig. 2. Different trends in the proportion of the three sperm subpopulations (SP1, SP2 and SP3) obtained from computer-aided sperm analysis (CASA) data clustering with incubation time. There was a decrease in the proportion of SP2 (*b*), but an increase in SP3 (*c*). There was little change in the proportion of SP1 (*a*), except for a sustained decrease in samples incubated with 50% seminal plasma (SP). Samples treated with 50% SP showed an overall higher proportion of SP3 and a lower proportion of SP2 than samples treated with 10% SP. In all cases, the time × SP interaction was significant. For each interaction, *P*-values are given for the time effect within each SP treatment, if $P < 0.05$. Data are the mean ± s.e.m. Different letters on the graphs indicate significant differences between SP treatments at each time point.

after incubation in the presence of 10% and 50% seminal plasma. Overall, for these males, incubation in the presence of 10% and 50% seminal plasma reduced %DFI by $-4.64 \pm 0.72\%$ and $-6.47 \pm 0.71\%$ respectively ($P < 0.001$ vs 0% seminal plasma; $P = 0.026$ between 10% and 50% seminal plasma). However, the effects of seminal plasma and incubation time differed between males, with %DFI increasing over time for samples from LW3 incubated with 10% seminal plasma. For non-susceptible males, even though some significant effects of seminal plasma or time were detected, the biological effects were not significant, with %DFI being very low ($1.54 \pm 0.09\%$ overall).

The effects of seminal plasma were also evident for HDS (Fig. 3*c*). Non-susceptible males had very low mean %HDS ($1.91 \pm 0.09\%$), and although time and seminal plasma showed significant effects, these were very small. Samples from susceptible LW1 and LW3 males exhibited slightly increased %HDS in 0% seminal plasma ($2.87 \pm 0.37\%$), which was <1% in samples incubated with either 10% or 50% seminal plasma. Results for samples from LD3 stood out because of a significant interaction between time and treatment. In this case, not only was the initial %HDS in 0% seminal plasma higher than in any other treatments at that time ($6.44 \pm 2.49\%$ vs $1.91 \pm 0.09\%$ overall), but it also increased considerably during incubation, reaching >15% by 3 h. Incubation of samples from this male in the presence of seminal plasma decreased %HDS to values similar to those in non-susceptible males and prevented the increase in %HDS with incubation time.

Discussion

Seminal plasma has considerable effects on boar spermatozoa after thawing (Okazaki *et al.* 2009; Garcia *et al.* 2010; Vadnais and Althouse 2011; Fernández-Gago *et al.* 2013). In the present study, we confirmed that adding whole seminal plasma from good-fertility boars not only delayed the loss of motility during incubation, especially of linear-swimming spermatozoa, but also prevented changes in the sperm chromatin of samples from susceptible boars. Others have highlighted the benefits of thawing boar spermatozoa in the presence of seminal plasma, although they favoured using the first fraction of the ejaculate, which has a different protein profile (Saravia *et al.* 2009). This may explain why some studies have reported good results using only 20% seminal plasma (Vadnais *et al.* 2005; Okazaki *et al.* 2009; Vadnais and Althouse 2011), compared with the 50% seminal plasma used in the present study. Using all the ejaculate may require a higher proportion of seminal plasma to obtain similar benefits (i.e. to contribute an equivalent amount of protective components). Nevertheless, this must be confirmed by directly comparing different proportions of the first fraction and whole seminal plasma.

A previous study (Saravia *et al.* 2009) has reported negative effects of using seminal plasma from the second fraction of the ejaculate, whereas others (Fraser *et al.* 2007) have suggested that low molecular weight components could be detrimental for freezing boar semen, recommending dialysing the seminal plasma. Nevertheless, we observed no negative effects when using whole seminal plasma (first and second fractions, not dialysed), and seminal plasma prepared using the same protocol was reported to increase field fertility (Garcia *et al.* 2010). A possible explanation for this apparent discrepancy is that the negative effects observed by Saravia *et al.* (2009) and Fraser *et al.* (2007) were due to the omission of seminal plasma from the first fraction, rather than including the second fraction, and it may be that the dialysable fraction of seminal plasma has a negligible effect when spermatozoa are exposed to it after thawing. Of course, we cannot discount the possibility that both omitting the second fraction of the ejaculate and dialysing the seminal plasma could further improve our results, and future studies could be aimed at testing these hypotheses.

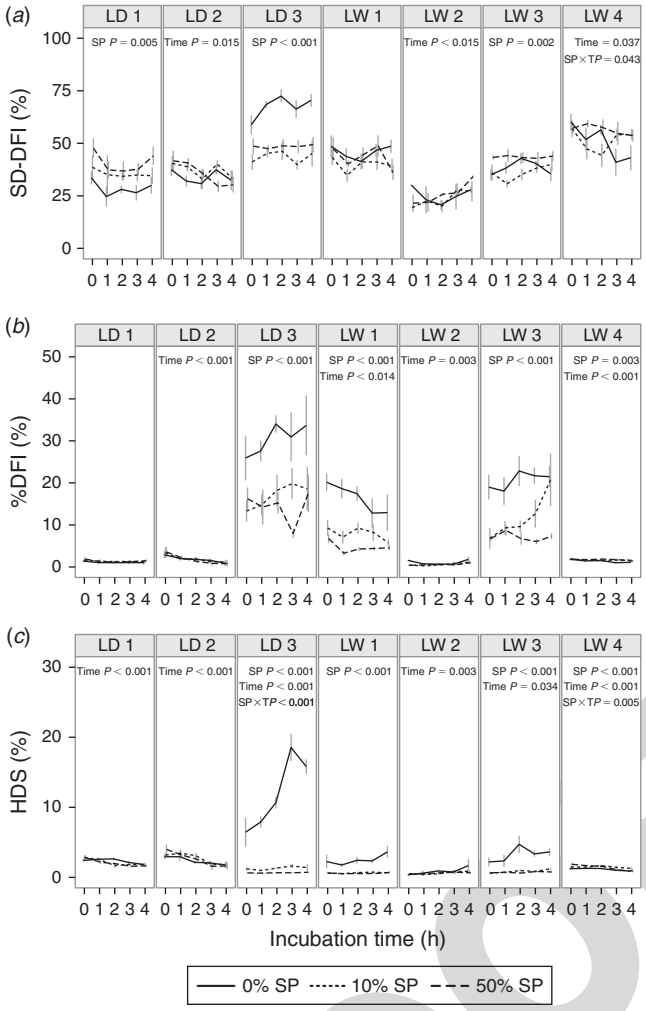

Fig. 3. The sperm chromatin structure assay (SCSA) demonstrated a clear effect of seminal plasma (SP) treatment on chromatin, with an important effect of between-male differences (LD1, LD2, LW2 and LW4, boars determined to be non-susceptible to chromatin changes after thawing; LD3, LW1 and LW3, boars susceptible to chromatin changes after thawing). (*a*) The pattern of the standard deviation in the DNA fragmentation index (SD-DFI) was highly variable between males. (*b*) In the presence of 50% SP, the lowest results for the percentage of spermatozoa with a high fragmentation index (DFI >25%; %DFI) were observed in samples from susceptible males, which nevertheless had the highest values in all cases. (*c*) High DNA stainability (HDS) was especially high for the LD3 boar, and increased with incubation time. However, incubation of samples from LD3 in both 10% and 50% SP reduced these elevated values to levels similar or even lower than in other males. *P*-values are shown for the effects of incubation time, seminal plasma or their interaction when significant (exact values are shown). It should be noted that even if these factors were significant, the magnitude of the effect sizes (amount of change due to time or SP) was almost negligible in many cases, especially for samples from non-susceptible males.

It is interesting to consider our results together with our previous report on the effect of seminal plasma on physiological variables, assessed by flow cytometry (Fernández-Gago *et al.* 2013). In that study, we considered membrane permeability and fluidity, mitochondrial and acrosomal status, intracellular $[Ca^{2+}]$ and intracellular ROS concentrations ($[ROS]_i$), as well as presence of extracellular free thiols as a proxy for oxidative stress. We noted that 10% seminal plasma only caused small modifications in these variables, if any. Moreover, after multiparametric analysis, samples thawed with 10% seminal plasma tended to cluster with samples thawed without seminal plasma. However, when 50% seminal plasma was used, a clear increase in membrane fluidity was observed, followed by an increase in $[ROS]_i$ and free thiols. In the present study, we noted that 10% seminal plasma had a protective effect on both motility and chromatin, even if the effect sizes were not as large as those produced by 50% seminal plasma. Thus, given the protective effects on motility and chromatin status observed in the present study, it is possible that 10% seminal plasma indeed modifies sperm molecular pathways. Components of the seminal plasma protect spermatozoa and modulate intracellular signalling pathways by interacting with the plasma membrane and possibly at other levels (Muiño-Blanco *et al.* 2008; Mendoza *et al.* 2013). Moreover, many seminal plasma proteins are involved in the stabilisation of the acrosome and in the interaction of spermatozoa with the oviduct and zona pellucida (Caballero *et al.* 2008; Töpfer-Petersen *et al.* 2008; Luna *et al.* 2015*a*). Because cryopreservation could induce the shedding of seminal plasma proteins from the plasma membrane, post-thawing supplementation could improve the fertilising ability of the spermatozoon by renewing this coating. Other seminal plasma proteins, specifically the spermadhesins PSP-I and PSP-II (porcine seminal plasma proteins) could have an immunosuppressive role, preparing the uterine environment for embryos (Töpfer-Petersen *et al.* 2008), although the amount added during AI would be negligible compared with physiological ejaculation.

Taking into consideration the findings of the present study, the higher $[ROS]_i$ detected in samples thawed in the presence of 50% seminal plasma (Fernández-Gago *et al.* 2013) could be associated with the motility changes reported herein. Linearity-related kinematic parameters and the proportion of SP3 increased in samples incubated with 50% seminal plasma by 2 h, coinciding with the time when an increase in $[ROS]_i$ was observed. Therefore, we can support our preliminary hypothesis that 50% seminal plasma not only protects thawed boar spermatozoa, but may also activate different pathways leading to an increase in sperm survival. The increase in $[ROS]_i$ could be the consequence of modulation of intracellular pathways (de Lamirande and O'Flaherty 2008) and not related to oxidative stress. This is also supported by our previous findings (Fernández-Gago *et al.* 2013), which show that the increase in $[ROS]_i$ was simultaneous with an increase in free thiols, when the opposite would be expected in a scenario of oxidative stress.

It is likely that the protection of sperm motility could have played a role in increasing the fertility of samples thawed in 50% seminal plasma (Garcia *et al.* 2010). However, the subpopulation pattern found in samples thawed in the presence of 50% seminal plasma seemed paradoxically less favourable at 0 and 1 h (predominance of SP1) than that observed for samples incubated with 0% or 10% seminal plasma. One possible explanation is that total sperm motility was better preserved at all times in the presence of 50% seminal plasma, and it is possible that the lowest-quality spermatozoa (SP1) would have

a better chance of survival under those treatment conditions. In contrast, spermatozoa in SP1 would have died more rapidly in the samples in the 0% and 10% seminal plasma treatment groups, yielding a subpopulation pattern that apparently indicates better quality (lower SP1). We have observed this effect in ruminants, where some treatments cause an effect in contrast to with the expected effects on the subpopulation pattern because of a better overall protection of low-quality spermatozoa (Martínez-Pastor *et al.* 2005, 2008; Domínguez-Rebolledo *et al.* 2009). The positive effect of 50% seminal plasma on the subpopulation pattern became evident by 2 h, when these samples showed a rapid surge of SP3 (linearly swimming spermatozoa), coinciding with the increase in $[ROS]_i$ (Fernández-Gago *et al.* 2013). This kind of moderately fast and linearly swimming spermatozoa has been related to higher overall quality, and even fertility, in previous studies (Martinez-Pastor *et al.* 2005; Ferraz *et al.* 2014; Yániz *et al.* 2015) and it may be related to the higher fertility of boar spermatozoa thawed with 50% seminal plasma (Garcia *et al.* 2010). In fact, a recent study in sheep found that the addition of seminal plasma causes changes in the subpopulation pattern (Luna *et al.* 2015*b*).

The use of seminal plasma could be critical for samples from some males because it might remediate, at least in part, the effect of or susceptibility to chromatin changes. The present study supports previous observations indicating that DNA fragmentation seems to be low in boar semen, even after thawing, and that it does not seem to increase even after several hours of incubation (Gosálvez *et al.* 2011). In fact, in a study of 692 semen samples (only liquid stored) from 79 Piétrain boars in an AI centre, the authors only found six samples with increased DFI, with only five of these above a 5% threshold (Waberski *et al.* 2011). This could be the reason for the apparent lack of biologically significant effects of seminal plasma on samples from males with initially good chromatin conditions. Indeed, this prompted us to select males from our cryobank that had shown elevated DFI after thawing, despite showing a good performance after AI with liquid-stored samples. Although we still know little about the effects of chromatin alterations on the fertility of thawed samples from production boars, Didion *et al.* (2009) suggested that a DFI of 6% could be used as a threshold for increased risk of fertility loss (suggesting that our susceptible samples would have a very high risk of failure if used for AI after thawing). Others have highlighted the importance of the overall chromatin structure in this species, beyond DNA fragmentation (Yeste *et al.* 2013; Estrada *et al.* 2014). The protamine organisation (e.g. the integrity of disulfide bonds) could have a major role in chromatin status in this species, and may be related to HDS. Indeed, susceptible males in the present study showed a distinctive HDS pattern, especially LD3.

Although the protective mechanism of seminal plasma in sperm chromatin remains unknown, it is clear whole seminal plasma from good-fertility boars partly prevented DNA fragmentation and chromatin alterations in thawed spermatozoa from susceptible males. The interactions detected in our models between male and seminal plasma or incubation time (looking at both CASA and SCSA data) are particularly interesting, because they support the notion that some treatments could favour individual samples. This highlights the need to focus on groups of males, and even on individuals, with specific problems regarding sperm conservation and application, as pointed out by many authors (Yeste *et al.* 2013, 2015; Rickard *et al.* 2014).

In conclusion, thawing boar spermatozoa in the presence of seminal plasma from good-fertility boars had very positive effects on sperm motility, including the preservation of a potentially important sperm subpopulation. Seminal plasma also prevented, at least in part, chromatin alterations in samples from susceptible males. Overall, adding 50% seminal plasma had positive effects, in agreement with previous studies.

Acknowledgements

This work was supported by Project DPI2009-08424 (Diputación de León, Spain). Rocío Fernández-Gago was supported by a Ph.D. grant (Junta de Castilla y León, Spain) and F. Martínez-Pastor was supported by the Ramón y Cajal program (RYC-2008-02560; Ministry of Science and Innovation, Spain). The authors thank CENTROTEC (León, Spain) for housing the animals and providing the semen samples, and Alejandra Besa, Diana Sánchez-Paniagua and Haritz Martín for their help with the analyses.

References

Boe-Hansen, G. B., Christensen, P., Vibjerg, D., Nielsen, M. B. F., and Hedeboe, A. M. (2008). Sperm chromatin structure integrity in liquid stored boar semen and its relationships with field fertility. *Theriogenology* **69**, 728–736. doi:10.1016/J.THERIOGENOLOGY.2007.12.004

Caballero, I., Vazquez, J. M., García, E. M., Parrilla, I., Roca, J., Calvete, J. J., Sanz, L., and Martínez, E. A. (2008). Major proteins of boar seminal plasma as a tool for biotechnological preservation of spermatozoa. *Theriogenology* **70**, 1352–1355. doi:10.1016/J.THERIOGENOLOGY.2008.07.013

de Lamirande, E., and O'Flaherty, C. (2008). Sperm activation: role of reactive oxygen species and kinases. *Biochim. Biophys. Acta* **1784**, 106–115. doi:10.1016/J.BBAPAP.2007.08.024

Didion, B. A., Kasperson, K. M., Wixon, R. L., and Evenson, D. P. (2009). Boar fertility and sperm chromatin structure status: a retrospective report. *J. Androl.* **30**, 655–660. doi:10.2164/JANDROL.108.006254

Domínguez-Rebolledo, A. E., Fernández-Santos, M. R., García-Alvarez, O., Maroto-Morales, A., Garde, J. J., and Martínez-Pastor, F. (2009). Washing increases the susceptibility to exogenous oxidative stress in red deer spermatozoa. *Theriogenology* **72**, 1073–1084. doi:10.1016/J.THERIOGENOLOGY.2009.06.027

Estrada, E., Rodríguez-Gil, J. E., Rocha, L. G., Balasch, S., Bonet, S., and Yeste, M. (2014). Supplementing cryopreservation media with reduced glutathione increases fertility and prolificacy of sows inseminated with frozen–thawed boar semen. *Andrology* **2**, 88–99. doi:10.1111/J.2047-2927.2013.00144.X

Evenson, D. P. (1999). Loss of livestock breeding efficiency due to uncompensable sperm nuclear defects. *Reprod. Fertil. Dev.* **11**, 1–15. doi:10.1071/RD98023

Evenson, D., and Jost, L. (2000). Sperm chromatin structure assay is useful for fertility assessment. *Methods Cell Sci.* **22**, 169–189. doi:10.1023/A:1009844109023

Fernández-Gago, R., Domínguez, J. C., and Martínez-Pastor, F. (2013). Seminal plasma applied post-thawing affects boar sperm physiology: a flow cytometry study. *Theriogenology* **80**, 400–410. doi:10.1016/J.THERIOGENOLOGY.2013.05.003

Ferraz, M. A. M. M., Morató, R., Yeste, M., Arcarons, N., Pena, A. I., Tamargo, C., Hidalgo, C. O., Muiño, R., and Mogas, T. (2014). Evaluation of sperm subpopulation structure in relation to *in vitro* sperm–oocyte interaction of frozen–thawed semen from Holstein bulls. *Theriogenology* **81**, 1067–1072. doi:10.1016/J.THERIOGENOLOGY.2014.01.033

Fraser, L., Dziekońska, A., Strzezek, R., and Strzezek, J. (2007). Dialysis of boar semen prior to freezing–thawing: its effects on post-thaw sperm characteristics. *Theriogenology* **67**, 994–1003. doi:10.1016/J.THERIOGENOLOGY.2006.12.002

Gallego, V., Vílchez, M. C., Peñaranda, D. S., Pérez, L., Herráez, M. P., Asturiano, J. F., and Martínez-Pastor, F. (2015). Subpopulation pattern of eel spermatozoa is affected by post-activation time, hormonal treatment and the thermal regimen. *Reprod. Fertil. Dev.* **27**, 529–543. doi:10.1071/RD13198

Garcia, J. C., Dominguez, J. C., Pena, F. J., Alegre, B., Gonzalez, R., Castro, M. J., Habing, G. G., and Kirkwood, R. N. (2010). Thawing boar semen in the presence of seminal plasma: effects on sperm quality and fertility. *Anim. Reprod. Sci.* **119**, 160–165. doi:10.1016/J.ANIREPROSCI.2009.11.001

Gosálvez, J., López-Fernández, C., Fernández, J. L., Gouraud, A., and Holt, W. V. (2011). Relationships between the dynamics of iatrogenic DNA damage and genomic design in mammalian spermatozoa from eleven species. *Mol. Reprod. Dev.* **78**, 951–961. doi:10.1002/MRD.21394

Hernández, M., Roca, J., Ballester, J., Vázquez, J. M., Martínez, E. A., Johannisson, A., Saravia, F., and Rodríguez-Martínez, H. (2006). Differences in SCSA outcome among boars with different sperm freezability. *Int. J. Androl.* **29**, 583–591. doi:10.1111/J.1365-2605.2006.00699.X

Kaczmarek, M. M., Krawczynski, K., and Filant, J. (2013). Seminal plasma affects prostaglandin synthesis and angiogenesis in the porcine uterus. *Biol. Reprod.* **88**, 72. doi:10.1095/BIOLREPROD.112.103564

Luna, C., Colás, C., Casao, A., Serrano, E., Domingo, J., Pérez-Pé, R., Cebrián-Pérez, J. A., and Muiño-Blanco, T. (2015*a*). Ram seminal plasma proteins contribute to sperm capacitation and modulate sperm-zona pellucida interaction. *Theriogenology* **83**, 670–678. doi:10.1016/J.THERIOGENOLOGY.2014.10.030

Luna, C., Yeste, M., Rivera Del Alamo, M. M., Domingo, J., Casao, A., Rodriguez-Gil, J. E., Pérez-Pé, R., Cebrián-Pérez, J. A., and Muiño-Blanco, T. (2015*b*). Effect of seminal plasma proteins on the motile sperm subpopulations in ram ejaculates. *Reprod. Fertil. Dev.* doi:10.1071/RD15231

Manjunath, P., Bergeron, A., Lefebvre, J., and Fan, J. (2007). Seminal plasma proteins: functions and interaction with protective agents during semen preservation. *Soc. Reprod. Fertil. Suppl.* **65**, 217–228.

Martinez-Pastor, F., Diaz-Corujo, A. R., Anel, E., Herraez, P., Anel, L., and de Paz, P. (2005). Post mortem time and season alter subpopulation characteristics of Iberian red deer epididymal sperm. *Theriogenology* **64**, 958–974. doi:10.1016/J.THERIOGENOLOGY.2005.01.003

Martínez-Pastor, F., Cabrita, E., Soares, F., Anel, L., and Dinis, M. T. (2008). Multivariate cluster analysis to study motility activation of *Solea senegalensis* spermatozoa: a model for marine teleosts. *Reproduction* **135**, 449–459. doi:10.1530/REP-07-0376

Martínez-Pastor, F., Tizado, E. J., Garde, J. J., Anel, L., and de Paz, P. (2011). Statistical series: opportunities and challenges of sperm motility subpopulation analysis. *Theriogenology* **75**, 783–795. doi:10.1016/J.THERIOGENOLOGY.2010.11.034

Maxwell, W. M. C., de Graaf, S. P., Ghaoui, R. E. -H., and Evans, G. (2007). Seminal plasma effects on sperm handling and female fertility. *Soc. Reprod. Fertil. Suppl.* **64**, 13–38.

Mendoza, N., Casao, A., Pérez-Pé, R., Cebrián-Pérez, J. A., and Muiño-Blanco, T. (2013). New insights into the mechanisms of ram sperm protection by seminal plasma proteins. *Biol. Reprod.* **88**, 149. doi:10.1095/BIOLREPROD.112.105650

Muiño-Blanco, T., Pérez-Pé, R., and Cebrián-Pérez, J. A. (2008). Seminal plasma proteins and sperm resistance to stress. *Reprod. Domest. Anim.* **43**, 18–31. doi:10.1111/J.1439-0531.2008.01228.X

Okazaki, T., Abe, S., Yoshida, S., and Shimada, M. (2009). Seminal plasma damages sperm during cryopreservation, but its presence during thawing improves semen quality and conception rates in boars with poor post-thaw semen quality. *Theriogenology* **71**, 491–498. doi:10.1016/J.THERIOGENOLOGY.2008.08.014

R Development Core Team (2016). 'R: A Language and Environment for Statistical Computing.' (R Foundation for Statistical Computing: Vienna.) Available at http://www.R-project.org/doc/manuals/fullrefman.pdf [Verified 26 July 2016].

Rickard, J. P., Schmidt, R. E., Maddison, J. W., Bathgate, R., Lynch, G. W., Druart, X., and de Graaf, S. P. (2014). Variation in seminal plasma alters the ability of ram spermatozoa to survive cryopreservation. *Reproduction* **148**, 469–478. doi:10.1530/REP-14-0285

Rodríguez-Martínez, H., Saravia, F., Wallgren, M., Roca, J., and Peña, F. J. (2008). Influence of seminal plasma on the kinematics of boar spermatozoa during freezing. *Theriogenology* **70**, 1242–1250. doi:10.1016/J.THERIOGENOLOGY.2008.06.007

Saravia, F., Hernández, M., Wallgren, M., Johannisson, A., and Rodríguez-Martínez, H. (2007). Controlled cooling during semen cryopreservation does not induce capacitation of spermatozoa from two portions of the boar ejaculate. *Int. J. Androl.* **30**, 485–499. doi:10.1111/J.1365-2605.2006.00741.X

Saravia, F., Wallgren, M., Johannisson, A., Calvete, J. J., Sanz, L., Peña, F. J., Roca, J., and Rodríguez-Martínez, H. (2009). Exposure to the seminal plasma of different portions of the boar ejaculate modulates the survival of spermatozoa cryopreserved in MiniFlatPacks. *Theriogenology* **71**, 662–675. doi:10.1016/J.THERIOGENOLOGY.2008.09.037

Serrano, E., Pérez-Pé, R., Calleja, L., Guillén, N., Casao, A., Hurtado-Guerrero, R., Muiño-Blanco, T., and Cebrián-Pérez, J. A. (2013). Characterization of the cDNA and *in vitro* expression of the ram seminal plasma protein RSVP14. *Gene* **519**, 271–278. doi:10.1016/J.GENE.2013.02.016

Töpfer-Petersen, E., Ekhlasi-Hundrieser, M., and Tsolova, M. (2008). Glycobiology of fertilization in the pig. *Int. J. Dev. Biol.* **52**, 717–736. doi:10.1387/IJDB.072536ET

Vadnais, M. L., and Althouse, G. C. (2011). Characterization of capacitation, cryoinjury, and the role of seminal plasma in porcine sperm. *Theriogenology* **76**, 1508–1516. doi:10.1016/J.THERIOGENOLOGY.2011.06.021

Vadnais, M. L., Kirkwood, R. N., Specher, D. J., and Chou, K. (2005). Effects of extender, incubation temperature, and added seminal plasma on capacitation of cryopreserved, thawed boar sperm as determined by chlortetracycline staining. *Anim. Reprod. Sci.* **90**, 347–354. doi:10.1016/J.ANIREPROSCI.2005.02.007

Waberski, D., Schapmann, E., Henning, H., Riesenbeck, A., and Brandt, H. (2011). Sperm chromatin structural integrity in normospermic boars is not related to semen storage and fertility after routine AI. *Theriogenology* **75**, 337–345. doi:10.1016/J.THERIOGENOLOGY.2010.09.004

Yániz, J. L., Palacín, I., Vicente-Fiel, S., Sánchez-Nadal, J. A., and Santolaria, P. (2015). Sperm population structure in high and low field fertility rams. *Anim. Reprod. Sci.* **156**, 128–134. doi:10.1016/J.ANIREPROSCI.2015.03.012

Yeste, M., Estrada, E., Casas, I., Bonet, S., and Rodríguez-Gil, J. E. (2013). Good and bad freezability boar ejaculates differ in the integrity of nucleoprotein structure after freeze–thawing but not in ROS levels. *Theriogenology* **79**, 929–939. doi:10.1016/J.THERIOGENOLOGY.2013.01.008

Yeste, M., Estrada, E., Rocha, L. G., Marín, H., Rodríguez-Gil, J. E., and Miró, J. (2015). Cryotolerance of stallion spermatozoa is related to ROS production and mitochondrial membrane potential rather than to the integrity of sperm nucleus. *Andrology* **3**, 395–407. doi:10.1111/ANDR.291

AUTHOR QUERIES

AQ1: *Is Carlos a middle name or part of the surname?*

AQ2: Section S1 – this was not included in the supplementary material. Please supply it.