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Supplementary Material

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

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S1. Materials and methods

Chromatin stability was assessed by SCSA (Sperm Chromatin Structure Assay), which has been thoroughly described in many articles (e.g., Evenson and Jost, 2000). The technique is based in the denaturalization of broken DNA and in the properties of acridine orange (AO), whose fluorescence shifts from green (dsDNA) to red (ssDNA) depending on the degree of DNA denaturation. Green and red fluorescence are recorded, and each spermatozoon receive two scores. The first score is related to the DNA fragmentation and is termed DFI (DNA Fragmentation Index), calculated as the red / total fluorescence ratio (×100). The second score is termed HDS (High DNA Stainability), and it is simply the intensity of the green fluorescence of the acridine orange, inversely related to the amount of chromatin compaction, and therefore with increase histone retention or reduced sperm nuclear maturity. Therefore, a sperm sample can be defined according to several variables obtained from the DFI values, and another variable from the HDS. We obtained the standard deviation of DFI values (a measurement of DNA fragmentation heterogeneity), termed SD-DFI. The percentage of spermatozoa with increased DFI (threshold typically at 25, as used in this study) is termed %DFI. %HDS is the percentage of spermatozoa with HDS above a certain threshold (750 green fluorescence in this study).

For the protocol, we followed strictly the guidelines from reference articles (Evenson and Jost, 2000). Samples were diluted in TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl and 1 mM disodium EDTA, pH 7.4) at 2×10 mL, and stored at -80C. For analysis, the samples were thawed on crushed ice. A volume of 200 µL was pipetted in a flow cytometry tube, and immediately mixed with 0.4 mL of the acid-detergent solution (0.08 M HCl, 0.15 M NaCl, and 0.1% Tritón X-100, pH 1.2). After 30 s, 1.2 mL of the staining orange solution (6 µg/mL 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 acridine orange was chromatographically purified, as described in the reference protocols (Polysciences Inc., Warrington, PA, USA; cat. no. 04539).

The tube was kept on ice for 3 min before flow cytometry analysis. We used a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA) and the acquisition software CellQuest v. 3. We used the Ar-ion 488 nm laser (15 mW) for exciting the acridine orange. Fluorescence was detected in the FL1 and FL3 photomultiplier tubes (PMT). According to the FASCcalibur Instrument Guide, scattered light first passes through a mirror (560 SP), which passes green and yellow-green fluorescence and reflects longer wavelengths. The passed light goes to the FL1 (green/yellow-green) PMT with a 10% fraction split off to provide the side scatter signal to the next PMT (SSC). The reflected light goes back to a second mirror (640 LP) that passes long wavelength red light to the FL3 PMT. FL1 is behind a 530/30 filter (green fluorescence of dsDNA-bound AO), and FL3 is behind a 650 long-pass filter (red fluorescence of ssDNA-bound AO).

The acquisition template consisted in a forward vs. scatter (FSC-H vs. SSC-H) dot plot (logarithmic scales), a red vs. green fluorescence (FL3 vs. FL1) dot plot (linear scale) with a region enclosing all the area from channel 2 to channel 1023 (linear range 1–1024), except for the lower left angle (excluding all below from [2, 350] to [300, 2], approximately). No compensation is required for this analysis.

Gating was carried out as indicated by (Evenson and Jost, 2000). The FSC/SSC plot (Fig. S1a) allowed checking for the spermatozoa population according to forward and side scatter and the amount of debris (but no gating was necessary at this stage). Voltage values for FSC and SSC (logarithmic scale) were set at standard values for spermatozoa, with the FSC at E00 (cytometer predefined value, for the FSC photodiode) and the SSC at 414. FSC was selected as the trigger parameter, setting the threshold at channel 280.

Fluorescence events were plotted in a FL3-H vs. FL1-H scattergram (linear scales, Fig. S1b). A gate was defined based in the region described above, and gated events were plotted in another FL3-H vs. FL1-H scattergram (Fig. S1c). Previously to each analysis session and each 12 experimental tubes, FL1 and FL3 voltages (linear scales, gain set to 1) were adjusted according to a standard sample. This sample was obtained from the same ejaculate and prepared in the same manner han the experimental samples. Around 100 tubes

containing each 200 μL of sample were prepared and stored at -80C, therefore the same standard sample was used throughout the experiment. Mean±SD of FL1-H and FL3-H for all the experiment were 587±10 and 749±31, respectively.

At least 5 000 spermatozoa were analyzed per sample. Data was saved in flow cytometry standard (FCS) v. 2 files, which were processed using the open source R statistical environment (R Core Team, 2016) with the flowCore package (Bioconductor repository, Ellis et al., 2016). Data were processed for obtaining SCSA variables as indicated in the bibliography (Evenson and Jost, 2000; Evenson et al., 2002), producing SD-DFI, %DFI and %HDS as indicated by the beginning of this section. These variables were used for further analysis in this study.

S2. Results

This supplementary material shows profile plots for each male (figures S2–S4, regarding CASA and subpopulation analyses. The plots allow to visualize the variability between males.

References

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

Evenson, D. P., Larson, K. L., and Jost, L. K. (2002). Sperm chromatin structure assay: its clinical use for detecting sperm dna fragmentation in male infertility and comparisons with other techniques. *J. Androl.* **23**, 25–43.

R Core Team (2016). 'R: A Language and Environment for Statistical Computing.' (R Foundation for Statistical Computing: Vienna, Austria)

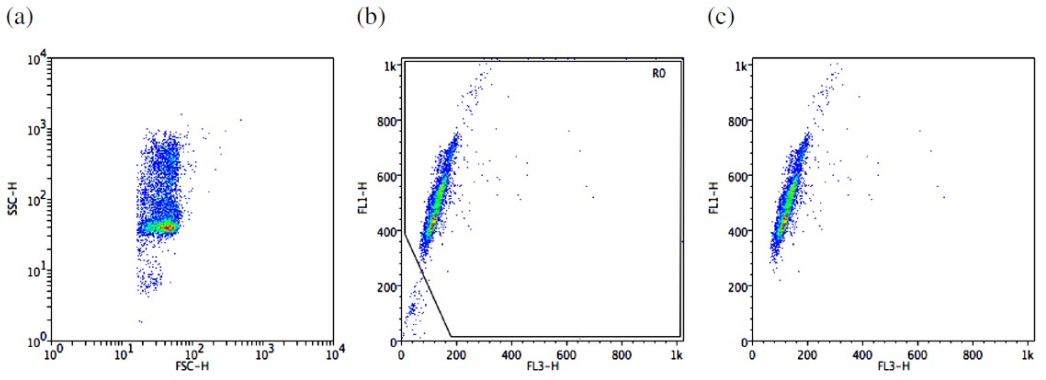
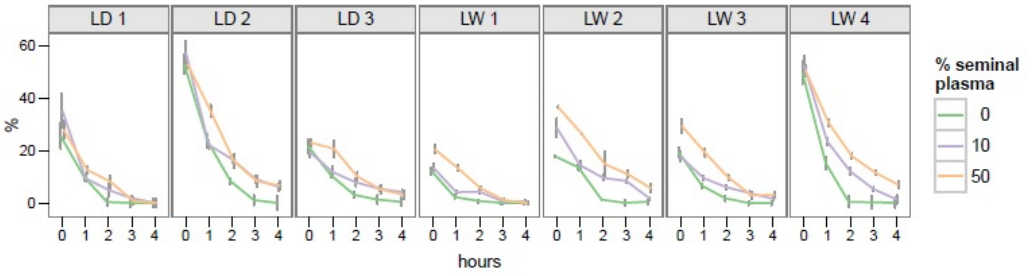
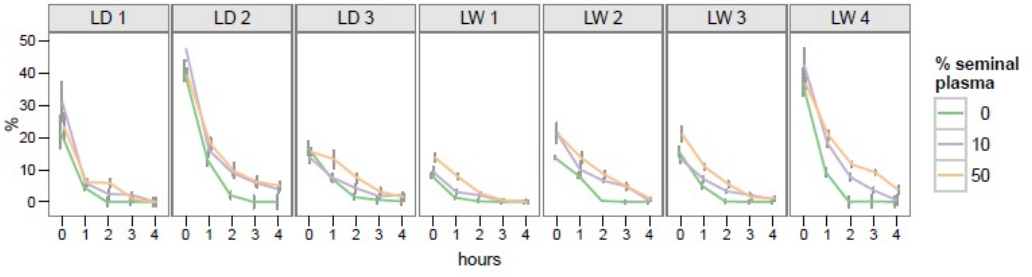


Fig. S1. Cytograms and gating strategy for the SCSA technique. Cytogram (a) shows the events according to their FSC-H vs. SSC-H signals. This cytogram was used for thresholding and confirming the presence of the sperm population. Cytogram (b) shows the events according to the FL3-H vs. FL1-H (red vs green fluorescence) signal intensity, defining a diagonal cloud of events corresponding to the sperm population (moderate to high green fluorescence, and mainly low red fluorescence). The events with both very low green and red fluorescence correspond to debris. A region (R0) was drawn in order to define a gate for excluding events close to the axes (debris and cell clumps) and the lower left corner grouping most of the debris. Cytogram (c) shows the gated FL3-H vs. FL1-H plot. Cytogram (c) was used for flow cytometry adquisition and adjustment with the standard sample. Ungated data was similarly processed in the R statistical environment (R Core Team, 2016), obtaining the SCSA variables from the gated FL1-H and FL3-H intensities.

(a) Total motility



(b) Progressive motility



(c) VCL (curvilinear velocity)

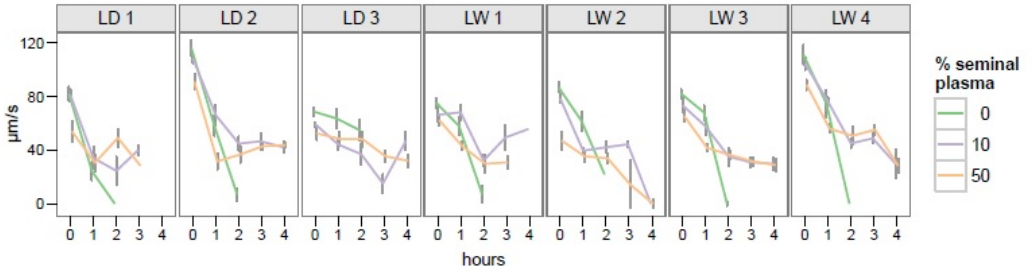
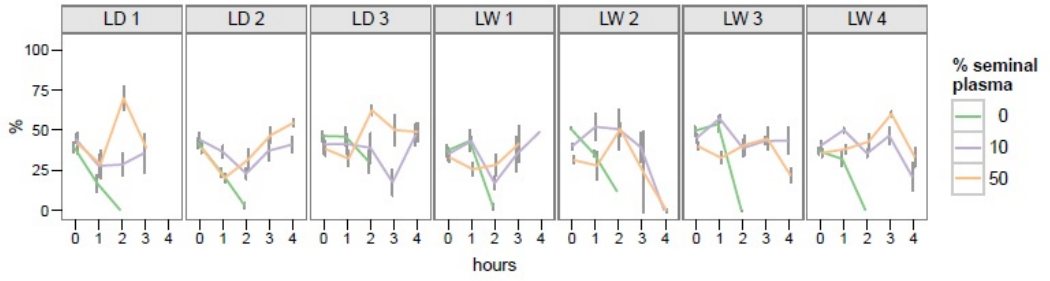
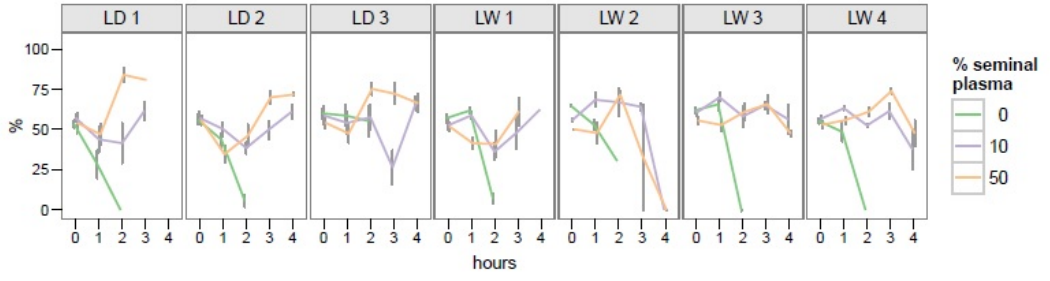


Fig. S2. Motility results showing the high variability among males (total and progressive motility, and VCL), despite of a similar effect of incubation time and seminal plasma, showed in Figure 1 (continued in Figure S3). Each male is identified by the breed (Landrace —LD— and Large White —LW—) and a correlative number. We could not detect a significant effect of breed, although it might be masked by the high between-male variability.

(a) LIN (linearity)



(b) WOB (wobble)



(c) ALH (lateral head displacement)

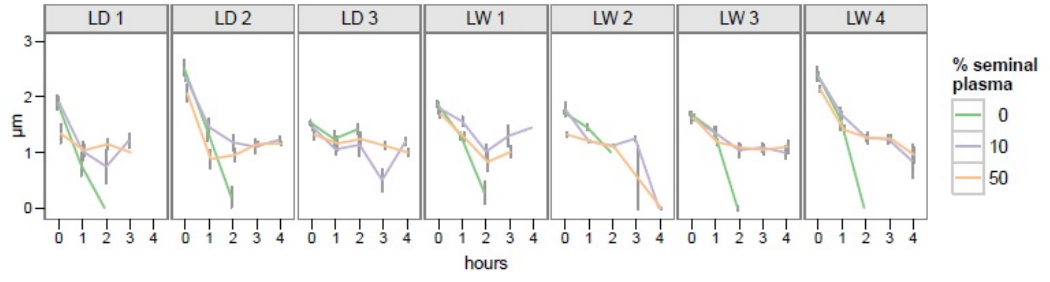
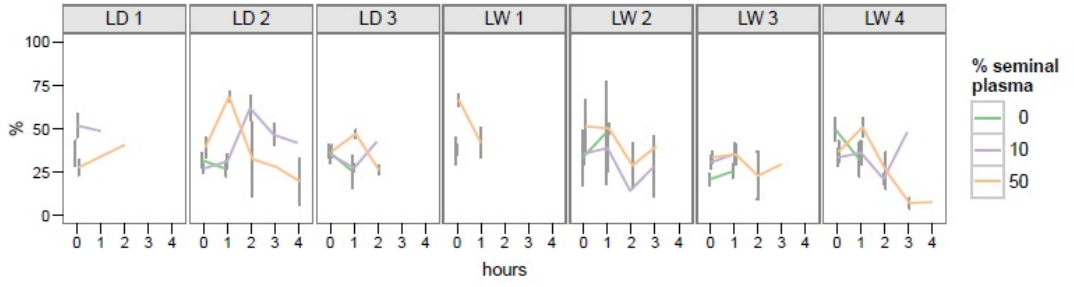
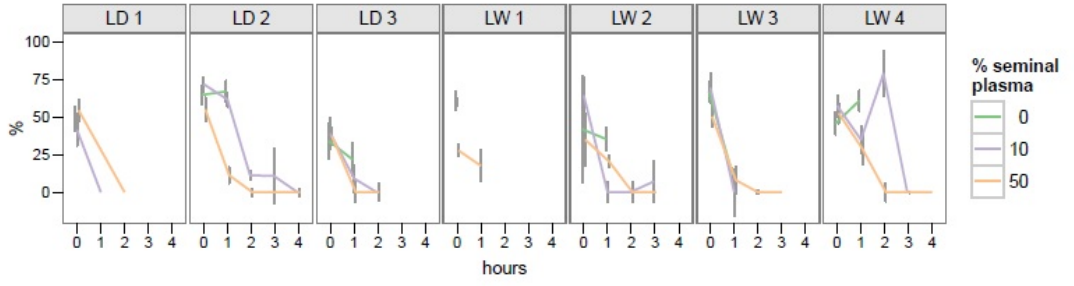


Fig. S3. Motility results per male (LIN, WOB and ALH), continued from Fig. S2. Samples show very variable profiles, depending on the male, but they followed the trend as showed in the main paper.

(a) Subpopulation 1



(b) Subpopulation 2



(c) Subpopulation 3

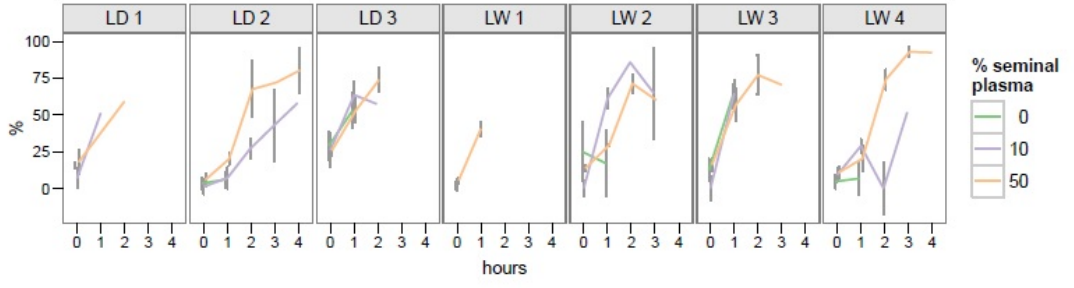


Fig. S4. Subpopulation changes within each male (see Fig. S2 caption for label descriptions). The quick drop of motility with time, especially in samples with 0% seminal plasma, hindered the analysis of between-male variability. The effect of the male on the subpopulation pattern was significant in the case of subpopulation 1. Interestingly, despite of between-male differences, the general trend in subpopulations 2 and 3 was very similar for most males.