Supplementary Information

Comprehensive functional analysis reveals that acrosome integrity and viability are key variables distinguishing AI bulls of varying fertility

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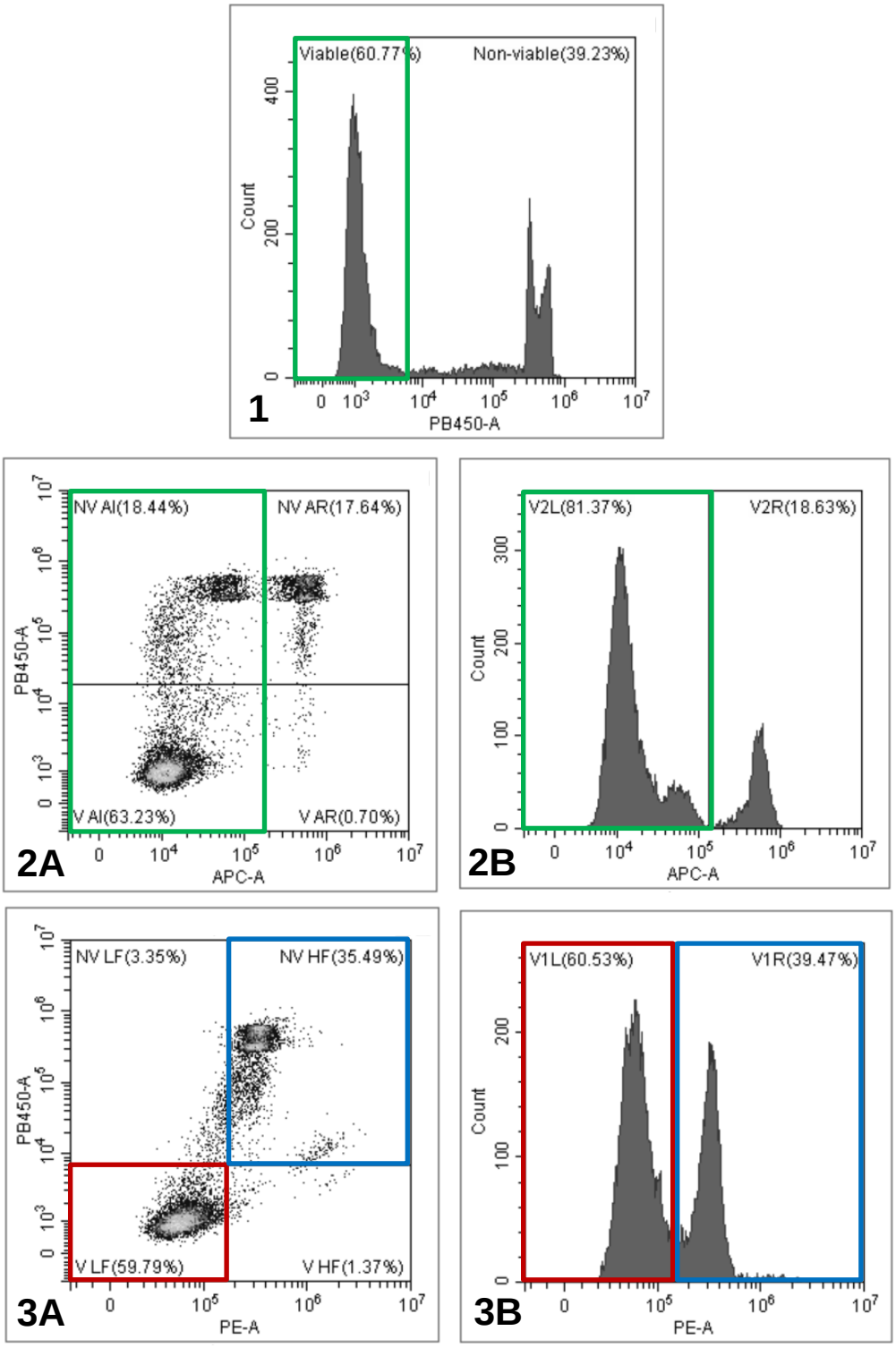
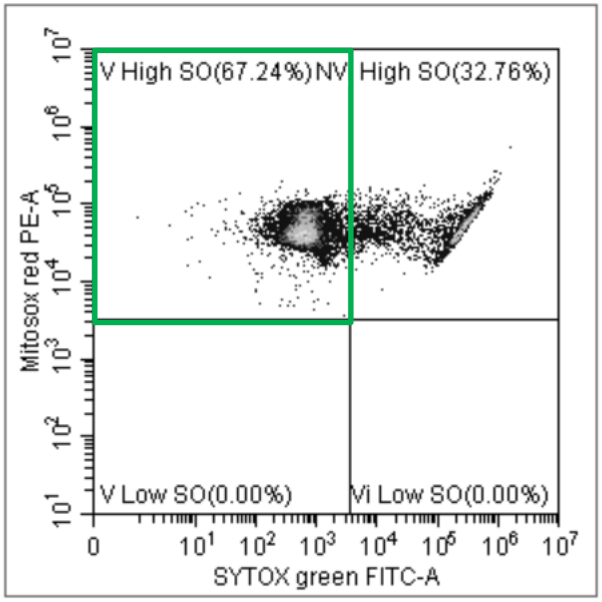
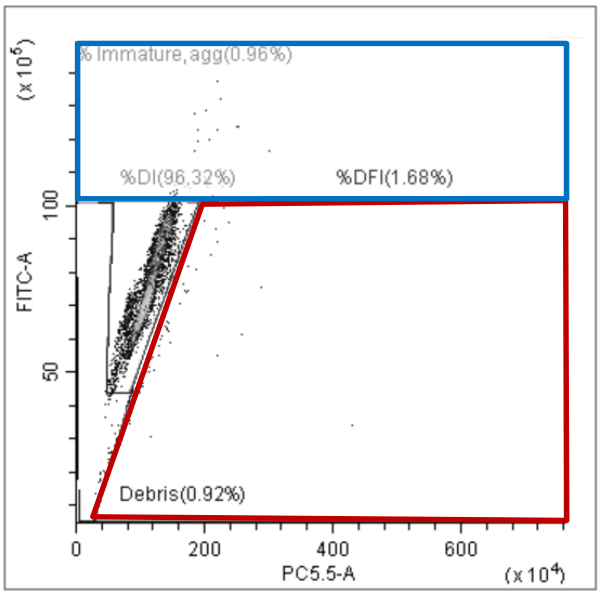


Supplementary Figure 1. Representative histograms and scatter plots obtained following the simultaneous assessment of viability (1), acrosome integrity (2A and B) and membrane fluidity (3A and B) using DAPI, AF647 and M540, respectively. The areas of the histograms or plots highlighted by coloured boxes identify the populations analysed in this study. For acrosome integrity, the green coloured boxes highlight all acrosome intact spermatozoa, as observed by the scatter plot and histogram (2A and B). For membrane fluidity, the red coloured box highlights viable spermatozoa with low membrane fluidity (3A), whereas the blue box indicates non-viable spermatozoa with high membrane fluidity (3B). The median fluorescence of M540 was also assessed in the population of viable spermatozoa with low membrane fluidity.



Supplementary Figure 2. Representative scatter plot obtained following the simultaneous assessment of viability and superoxide production using Sytox Green and MitoSOX Red, respectively. In this plot, the highlighted population indicated by the green coloured box identifies viable spermatozoa with high superoxide production. The median fluorescence of MitoSOX Red was also assessed in this sperm population.



Supplementary Figure 3. Representative scatter plot obtained following the assessment of DNA integrity using Acridine Orange. In this plot, the highlighted population indicated by the red coloured box identifies spermatozoa with DNA fragmentation, whereas the blue box indicates spermatozoa with high DNA staining.

Supplementary Table 1. Range of post-thaw sperm functional, morphological and intracellular variables assessed in Holstein Friesian bulls of high and low fertility (n = 10 bulls per phenotype; in Experiment 1a) at 0, 3 and 6 h incubation (with the exception of morphology and DNA integrity).

	High Fertility[1]			Low Fertility[1]		
	0 h	3 h	6 h	0 h	3 h	6 h
Parameter						
CASA						
Total motility (%)	50.0±6.0^{a} (*20.4-82.4*)	45.9±5.7^{ab} (*15.9-76.3*)	41.8±4.2^{b} (*15.7-66.5*)	43.6±6.0^{a} (*9.7-82.5*)	40.5±4.4^{ab} (*10.8-69.8*)	37.4±3.9^{b} (*9.0-63.3*)
Progressive motility (%)	34.6±5.2^{a} (*10.1-64.0*)	32.7±5.1^{ab} (*8.5-61.4*)	28.5±3.6^{b} (*10.6-52.6*)	33.5±4.7^{a} (*7.7-61.8*)	30.0±3.4^{ab} (*8.3-45.9*)	26.4±3.3^{b} (*4.1-46.9*)
Curvilinear velocity (VCL; µm/s)	66.6±4.8^{a} (*36.3-93.2*)	65.9±5.4^{a} (*33.5-100.1*)	61.7±4.8^{a} (*35.2-102.0*)	75.9±5.1^{a} (*44.9-101.3*)	70.1±4.8^{b} (*40.4-101.9*)	65.2±5.2^{b} (*37.1-92.5*)
Straight-line velocity (VSL; µm/s)	40.8±4.9^{a} (*13.8-75.3*)	37.7±5.5^{a} (*14.3-74.9*)	32.3±4.6^{b} (*12.1-74.7*)	49.7±4.8^{a} (*20.8-78.3*)	44.0±4.7^{a} (*14.1-73.3*)	35.7±3.9^{b} (*17.3-60.2*)
Average path velocity (VAP; µm/s)	45.7±4.6^{a} (*22.3-80.0*)	42.0±5.0^{a} (*19.0-79.8*)	37.3±3.9^{b} (*17.9-79.5*)	55.2±4.7^{a} (*32.2-82.4*)	48.3±4.3^{b} (*23.3-78.4*)	40.4±3.8^{c} (*22.4-66.2*)
Linearity (LIN; %)	51.5±3.6^{a} (*29.5-73.7*)	49.7±3.9^{ab} (*29.7-75.9*)	46.0±3.0^{b} (*32.2-69.1*)	58.8±3.1^{a} (*36.0-76.7*)	56.4±3.9^{a} (*29.0-76.8*)	49.5±2.5^{b} (*32.6-61.9*)
Straightness (STR; %)	71.6±3.3^{a} (*48.1-86.5*)	73.0±3.4^{a} (*49.2-92.8*)	71.8±2.8^{a} (*52.9-88.7*)	78.1±2.6^{a} (*57.2-91.7*)	77.8±3.3^{a} (*50.2-91.1*)	75.2±2.4^{a} (*56.7-87.4*)
Amplitude of lateral head movement (ALH; µm)	2.5±0.1^{a} (*1.9-3.3*)	2.6±0.1^{ab} (*1.8-3.6*)	2.7±0.1^{b} (*1.8-3.6*)	2.6±0.1^{ab} (*1.8-3.1*)	2.6±0.1^{a} (*1.7-3.3*)	2.8±0.2^{b} (*1.7-3.5*)
Beat cross frequency (BCF; Hz)	7.7±0.6^{a} (*3.7-11.1*)	8.3±0.7^{a} (*4.4-13.5*)	7.9±0.6^{a} (*4.8-13.2*)	8.8±0.5^{ab} (*4.9-11.8*)	9.2±0.7^{a} (*3.7-12.8*)	8.3±0.5^{b} (*5.2-11.3*)
Wobble (WOB; %)	67.0±2.1^{a} (*56.7-81.5*)	63.9±2.5^{b} (*51.7-80.9*)	60.7±1.9^{c} (*50.8-75.8*)	71.3±1.9^{a} (*58.6-83.6*)	68.2±2.5^{b} (*54.1-83.1*)	62.3±1.6^{c} (*53.8-70.7*)
Morphology (0 h only)						
Normal (%)	68.4±2.3 (*57.5-79.2*)			63.7±3.1 (*51.5-74.8)*		
Head abnormalities (%)	5.7±0.7 (*3.3-9.7)*			8.2±1.5 (*3.0-19.2)*		
Acrosome abnormalities (%)	18.7±2.1 (*11.3-28.3*)			19.2±2.3 (*9.5-33.5*)		
Mid-piece abnormalities (%)	3.3±0.8 (*1.2-8.7*)			3.2±0.9 (*0.8-13.2*)		
Tail abnormalities (%)	2.5±0.6 (*1.2-7.2*)			3.3±0.7 (*1.3-6.8*)		

Cytoplasmic droplets (Proximal and Distal; %)	0.3±0.1 (*0.0-0.7*)			0.7±0.3 (*0.0-3.0*)		
Flow cytometry						
Viable (%)	51.1±3.1[a] (*20.1-71.1*)	47.1±2.8[ab] (*22.3-68.0*)	44.5±2.7[b] (*24.9-65.9*)	36.9±5.4[a] (*7.5-68.9*)	33.1±6.1[ab] (*1.1-66.0*)	32.3±6.0[b] (*0.2-61.6*)
Acrosome intact (%)	73.6±2.1[a] (*59.4-86.0*)	76.1±1.9[b] (*22.3-92.7*)	76.7±2.0[b] (*24.9-92.8*)	65.8±3.8[a] (*37.3-83.6*)	66.5±4.4[a] (*32.1-84.2*)	66.8±4.6[a] (*32.5-84.6*)
Viable, low membrane fluidity (%)	43.7±3.1[a] (*18.6-62.7*)	39.3±2.8 [b] (*18.5-52.8*)	36.7±2.7[b] (*22.2-52.4*)	33.3±5.0[a] (*6.9-65.7*)	28.6±5.6[b] (*0.7-60.0*)	28.2±5.5[b] (*0.1-55.2*)
Viable, high membrane fluidity (%)	3.7±1.5[a] (*0.2-36.0*)	5.2±1.4[a] (*0.9-30.9*)	5.3±1.3[a] (*1.2-28.7*)	2.4±0.7[a] (*0.4-8.5*)	2.8±1.0[a] (*0.3-10.5*)	2.3±1.0[a] (*0.1-10.2*)
M540 fluorescence intensity in viable cells (MFU, 000s)	66.2±13.9[a] (*10.7-234.6*)	66.4±11.0[a] (*14.2-197.9*)	61.3±10.2[a] (*13.4-183.8*)	56.0±10.6[a] (*11.1-132.4*)	59.8±10.2[a] (*17.1-134.3*)	56.8±8.6[a] (*14.4-125.1*)
MitoSOX fluorescence intensity in viable cells (MFU, 000s)	51.5±4.3[a] (*37.6-92.4*)	47.8±2.3[a] (*32.5-59.2*)	46.4±2.2[a] (*28.1-58.4*)	54.9±4.6[a] (*40.6-99.5*)	50.6±2.7[a] (*33.2-63.1*)	49.0±2.8[a] (*31.7-66.4*)
DNA fragmentation (%)	1.7±0.4[a] (*0.6-9.6*)		1.8±0.4[a] (*0.6-7.8*)	2.7±0.5[a] (*1.2-6.2*)		2.4±0.3[a] (*1.1-4.5*)
High DNA staining (%)	0.5±0.2[a] (*0.1-5.1*)		0.6±0.2[a] (*0.1-5.8*)	0.6±0.2[a] (*0.2-4.5*)		0.7±0.2[a] (*0.1-3.5*)

[1]Results are presented as the mean ± s.e.m. and the range of each measured variable is given in parentheses. If the results were log transformed, the geometric mean ± back-transformed s.e.m. is provided. Superscripts denote differences across the incubation period within a fertility phenotype (P<0.05).

Supplementary Table 2. *In vitro* fertilisation variables assessed in Experiment 1b across a subpopulation of Holstein Friesian bulls of high (n = 6) and low fertility (n = 4).

Parameter[1]	High fertility[2]	Low fertility[2]	Difference in fertility (P value)[3]
48 hpi (%)	77.1±4.0 (*50.0-96.4*)	49.2±18.8 (*15.6-93.1*)	NS
Day 6 (%)	9.4±2.6 (*4.0-19.5*)	4.1±5.4 (*0.0-15.4*)	NS
Day 7 (%)	18.8±2.6 (*7.0-33.3*)	12.7±6.8 (*0.0-28.0*)	NS
Day 8 (%)	19.8±3.5 (*9.3-40.6*)	11.9±6.6 (*0.0-33.3*)	NS

[1]Presumptive zygotes were assessed 48 hours post-insemination (48 hpi) and embryo development assessed on Day 6, 7 and 8 post-insemination.
[2]Results are presented as the mean ± s.e.m and the range of each measured variable is given in parentheses.
[3]NS = non-significant.