

## Supplementary information

### **Sperm function in vitro and fertility after antibiotic-free, hypothermic storage of liquid preserved boar semen**

Dagmar Waberski<sup>1</sup>, Anne-Marie Luther<sup>1</sup>, Benita Grünther<sup>1</sup>, Helen Jäkel<sup>1</sup>, Heiko Henning<sup>1,2</sup>, Charlotte Vogel<sup>3</sup>, Wolfgang Peralta<sup>4</sup> & Karl Fritz Weitze<sup>1</sup>

1 Unit for Reproductive Medicine of Clinics/Clinic for Pigs and Small Ruminants, University of Veterinary Medicine Hannover, Bünteweg 15, D-30559 Hannover, Germany

2 Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 112, 3584 CM Utrecht, The Netherlands

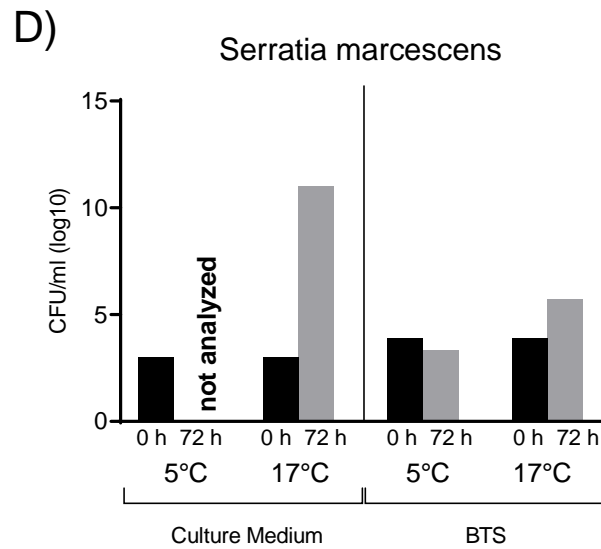
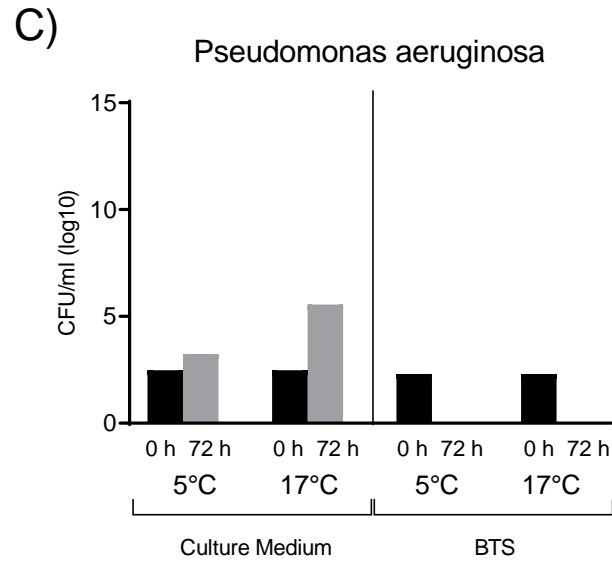
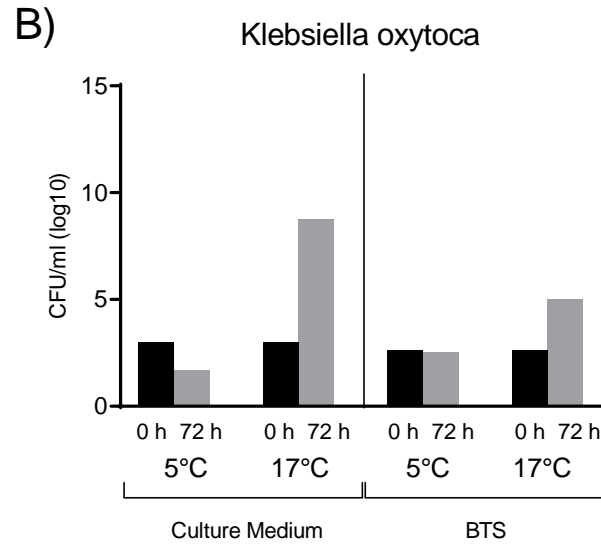
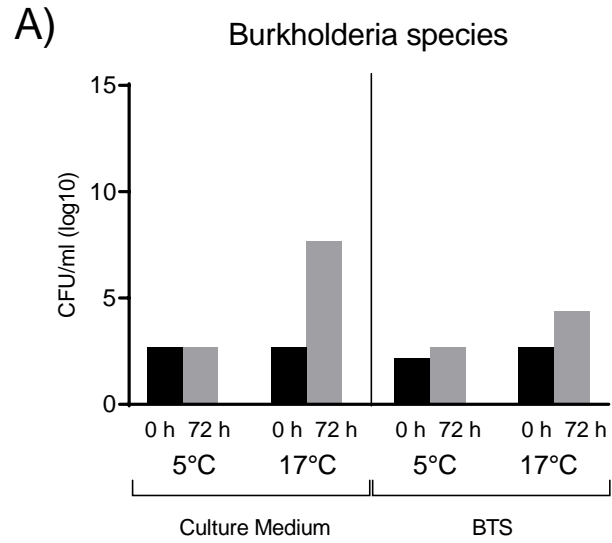
3 Institute for Biometry, Epidemiology and Information Processing (IBEI), University of Veterinary Medicine Hannover, Bünteweg 2, D-30559 Hannover, Germany

4 Agricola Super Ltda., Rancagua, Chile

\* Corresponding author: [dagmar.waberski@tiho-hannover.de](mailto:dagmar.waberski@tiho-hannover.de)

### Supplemental Figure S1

Bacteria growth in culture media and antibiotic-free BTS semen extender after storage for 72 h at 5°C and 17°C. Sterile media were inoculated with  $10^3$  CFU (colony forming units)/ml with four typical mesophilic gram-negative bacteria strains isolated from boar semen.



Supplemental Table S1:

Results of the two-way repeated measures ANOVA and selected pairwise comparisons within temperature/extender treatment (A) and storage time point (B). Global p values of the two-way repeated measures ANOVA are reported in brackets (trt = temperature-extender treatment, time = storage time, trt x time = temperature-extender x time interaction). Bonferroni post-hoc tests were carried out within each level of temperature-extender treatment and each level of storage time. Estimated differences are reported with 95% confidence intervals. MI = Membrane integrity (propidium iodide and FITC-PNA negative sperm), DFI= DNA fragmentation index, \*P ≤ 0.05, \*\*P ≤ 0.01, \*\*\*P ≤ 0.001.

A)

Comparison	24 h	72 h	144 h
<b>Motility %</b> (trt: p<0.001, time: p<0.001, trt x time: p=0.033)			
17/APrem - 17/BTS	5.3 [ -1.2, 11.8]	3.3 [ -3.2, 9.8]	6.4 [ -0.1, 12.9]
17/APrem - 5/APrem	7.9 [ 1.4, 14.3]**	4.3 [ -2.2, 10.8]	6.2 [ -0.3, 12.7]
17/APrem - 5/BTS	17.3 [ 10.8, 23.8]***	18.4 [ 11.9, 24.9]***	22.1 [ 15.6, 28.6]***
17/BTS - 5/APrem	2.5 [ -4.0, 9.0]	1.0 [ -5.5, 7.5]	-0.2 [ -6.7, 6.3]
17/BTS - 5/BTS	12.0 [ 5.5, 18.5]***	15.1 [ 8.6, 21.6]***	15.7 [ 9.2, 22.2]***
5/APrem - 5/BTS	9.4 [ 2.9, 15.9]***	14.1 [ 7.6, 20.6]***	15.9 [ 9.4, 22.4]***
<b>MI %</b> (trt: p<0.001, time: p=0.238, trt x time: p=0.0272)			
17/APrem - 17/BTS	0.7 [ -3.8, 5.2]	1.8 [ -2.7, 6.2]	2.1 [ -2.4, 6.6]
17/APrem - 5/APrem	1.0 [ -3.5, 5.5]	2.9 [ -1.5, 7.4]	1.9 [ -2.6, 6.4]
17/APrem - 5/BTS	4.9 [ 0.4, 9.4]*	9.3 [ 4.8, 13.8]***	5.7 [ 1.2, 10.2]**
17/BTS - 5/APrem	0.3 [ -4.2, 4.8]	1.2 [ -3.3, 5.7]	-0.2 [ -4.6, 4.3]
17/BTS - 5/BTS	4.1 [ -0.3, 8.6]	7.6 [ 3.1, 12.1]***	3.6 [ -0.9, 8.1]
5/APrem - 5/BTS	3.9 [ -0.6, 8.3]	6.4 [ 1.9, 10.9]***	3.8 [ -0.7, 8.3]
<b>DFI</b> (trt: p=0.048)			
17/APrem - 17/BTS	.	0.1 [ -0.1, 0.3]	.
17/APrem - 5/APrem	.	-0.0 [ -0.2, 0.2]	.
17/APrem - 5/BTS	.	0.2 [ -0.0, 0.4]	.
17/BTS - 5/APrem	.	-0.1 [ -0.3, 0.1]	.
17/BTS - 5/BTS	.	0.1 [ -0.1, 0.3]	.
5/APrem - 5/BTS	.	0.2 [ -0.0, 0.4]	.

B)

Comparison	17/APrem	17/BTS	5/APrem	5/BTS
<b>Motility %</b>				
24 h – 72 h	2.7 [ -1.9, 7.4]	0.7 [ -3.9, 5.3]	-0.9 [ -5.5, 3.8]	3.8 [ -0.8, 8.4]
24 h – 144 h	3.8 [ -0.9, 8.4]	4.8 [ 0.2, 9.5]*	2.1 [ -2.5, 6.8]	8.6 [ 4.0, 13.2]***
72 h – 144 h	1.0 [ -3.6, 5.6]	4.1 [ -0.5, 8.8]	3.0 [ -1.6, 7.6]	4.8 [ 0.2, 9.4]*
<b>MI %</b>				
24 h – 72 h	-1.3 [ -5.7, 3.2]	-0.2 [ -4.7, 4.2]	0.7 [ -3.8, 5.1]	3.2 [ -1.3, 7.7]
24 h – 144 h	-1.4 [ -5.8, 3.1]	-0.0 [ -4.5, 4.4]	-0.5 [ -4.9, 4.0]	-0.5 [ -5.0, 3.9]
72 h – 144 h	-0.1 [ -4.6, 4.4]	0.2 [ -4.2, 4.7]	-1.1 [ -5.6, 3.3]	-3.7 [ -8.2, 0.7]

17/APrem: Semen stored at 17°C in AndroStar Premium extender with antibiotic.

17/BTS: Semen stored at 17°C in BTS extender with antibiotic.

5/APrem: Semen stored at 5°C in AndroStar Premium extender without antibiotic.

5/BTS: Semen stored at 5°C in BTS extender with antibiotic.

Supplemental Table S2:

Sperm quality by temperature/extender treatment and storage time point (24 h, 72 h, 144 h). All results (n=12) are mean  $\pm$  SEM. DFI was measured at 72 h only. MI = Membrane integrity (propidium iodide and FITC-PNA negative sperm), DFI= DNA fragmentation index.

	24 h	72 h	144 h
<b>Motility %</b>			
17/APrem	92.6 $\pm$ 0.9	89.8 $\pm$ 0.7	88.8 $\pm$ 0.9
17/BTS	87.2 $\pm$ 1.29	86.5 $\pm$ 1.39	82.4 $\pm$ 2.29
5/APrem	84.7 $\pm$ 1.69	85.6 $\pm$ 2.39	82.6 $\pm$ 2.39
5/BTS	75.3 $\pm$ 2.5	71.5 $\pm$ 2.1	66.7 $\pm$ 2.79
<b>MI %</b>			
17/APrem	90.8 $\pm$ 1.2	92.1 $\pm$ 0.5	92.2 $\pm$ 0.5
17/BTS	90.1 $\pm$ 1.0	90.3 $\pm$ 0.5	90.1 $\pm$ 0.7
5/APrem	89.8 $\pm$ 1.2	89.2 $\pm$ 1.2	90.3 $\pm$ 0.9
5/BTS	86.0 $\pm$ 1.5	82.8 $\pm$ 1.9	86.5 $\pm$ 1.1
<b>DFI %</b>			
17/APrem	.	1.7 $\pm$ 0.2	.
17/BTS	.	1.6 $\pm$ 0.2	.
5/APrem	.	1.7 $\pm$ 0.2	.
5/BTS	.	1.5 $\pm$ 0.2	.

17/APrem: Semen stored at 17°C in AndroStar Premium extender with antibiotic.

17/BTS: Semen stored at 17°C in BTS extender with antibiotic.

5/APrem: Semen stored at 5°C in AndroStar Premium extender without antibiotic.

5/BTS: Semen stored at 5°C in BTS extender with antibiotic.

Supplemental Table S3:

Results of the two-way repeated measures ANOVA and selected pairwise comparisons of live, low calcium (propidium iodide and Fluo-3-AM negative) spermatozoa (%) within temperature/extender treatment (A) and incubation time (B). Global p values of the two-way repeated measures ANOVA are reported in brackets (trt = temperature-extender treatment, time = storage time, trt x time = temperature-extender x time interaction). Bonferroni post-hoc tests were carried out within each level of temperature-extender treatment and each level of storage time. Estimated differences are reported with 95% confidence intervals. \*P ≤ 0.05, \*\*P ≤ 0.01, \*\*\*P ≤ 0.001.

A)

Comparison	3 min	60 min	120 min	180 min
<b>Tyrode C</b> (trt: p<0.001, time: p<0.001, trt x time: p=0.081)				
17/APrem - 17/BTS	3.2 [ -4.0, 10.4]	1.4 [ -6.6, 9.3]	1.8 [ -7.8, 11.4]	2.8 [ -8.4, 14.0]
17/APrem - 5/APrem	4.0 [ -3.2, 11.2]	3.6 [ -4.4, 11.5]	5.2 [ -4.4, 14.8]	5.8 [ -5.4, 17.0]
17/APrem - 5/BTS	20.5 [ 13.3, 27.7]***	20.1 [ 12.1, 28.0]***	23.8 [ 14.2, 33.4]***	24.9 [ 13.7, 36.1]***
17/BTS - 5/APrem	0.8 [ -6.4, 8.0]	2.2 [ -5.8, 10.1]	3.4 [ -6.2, 13.0]	3.0 [ -8.2, 14.2]
17/BTS - 5/BTS	17.3 [ 10.1, 24.5]***	18.7 [ 10.8, 26.7]***	22.0 [ 12.4, 31.6]***	22.1 [ 10.9, 33.3]***
5/APrem - 5/BTS	16.5 [ 9.3, 23.7]***	16.5 [ 8.6, 24.5]***	18.7 [ 9.1, 28.3]***	19.1 [ 7.9, 30.3]***
<b>Tyrode A</b> (trt: p<0.001, time: p<0.001, trt x time: p=0.118)				
17/APrem - 17/BTS	3.8 [ -3.1, 10.8]	8.9 [ -5.4, 23.2]	13.7 [ -8.2, 35.5]	15.3 [ -11.3, 41.9]
17/APrem - 5/APrem	4.1 [ -2.9, 11.0]	7.4 [ -6.9, 21.7]	5.1 [ -16.8, 26.9]	1.9 [ -24.8, 28.5]
17/APrem - 5/BTS	21.2 [ 14.2, 28.2]***	26.9 [ 12.6, 41.1]***	27.1 [ 5.2, 48.9]**	24.6 [ -2.1, 51.2]
17/BTS - 5/APrem	0.2 [ -6.8, 7.2]	-1.5 [ -15.8, 12.8]	-8.6 [ -30.4, 13.2]	-13.4 [ -40.1, 13.2]
17/BTS - 5/BTS	17.4 [ 10.4, 24.3]***	18.0 [ 3.7, 32.2]**	13.4 [ -8.4, 35.2]	9.3 [ -17.4, 35.9]
5/APrem - 5/BTS	17.1 [ 10.2, 24.1]***	19.5 [ 5.2, 33.7]***	22.0 [ 0.2, 43.8]*	22.7 [ -3.9, 49.3]

B)

Comparison	17/APrem	17/BTS	5/APrem	5/BTS
<b>Tyrode C</b>				
3 min – 60 min	0.9 [ -1.2, 2.9]	-1.0 [ -3.0, 1.1]	0.4 [ -1.6, 2.4]	0.4 [ -1.6, 2.5]
3 min – 120 min	1.5 [ -2.6, 5.6]	0.1 [ -4.1, 4.2]	2.6 [ -1.5, 6.8]	4.8 [ 0.7, 9.0]**
3 min – 180 min	2.8 [ -2.1, 7.6]	2.4 [ -2.5, 7.3]	4.6 [ -0.3, 9.4]	7.2 [ 2.3, 12.0]***
60 min – 120 min	0.6 [ -2.7, 4.0]	1.1 [ -2.3, 4.4]	2.2 [ -1.1, 5.6]	4.4 [ 1.1, 7.7]**
60 min – 180 min	1.9 [ -2.2, 6.0]	3.4 [ -0.7, 7.5]	4.2 [ 0.1, 8.3]*	6.7 [ 2.6, 10.8]***
120 min – 180 min	1.3 [ -1.0, 3.6]	2.3 [ 0.0, 4.6]*	1.9 [ -0.3, 4.2]	2.3 [ 0.1, 4.6]*
<b>Tyrode A</b>				
3 min – 60 min	11.3 [ 2.7, 19.9]**	16.4 [ 7.8, 25.0]***	14.7 [ 6.1, 23.3]***	17.0 [ 8.4, 25.6]***
3 min – 120 min	20.2 [ 6.4, 34.1]***	30.1 [ 16.2, 43.9]***	21.3 [ 7.4, 35.1]***	26.1 [ 12.3, 39.9]***
3 min – 180 min	26.7 [ 9.3, 44.0]***	38.1 [ 20.8, 55.5]***	24.4 [ 7.1, 41.8]***	30.0 [ 12.7, 47.4]***
60 min – 120 min	8.9 [ 1.5, 16.3]**	13.7 [ 6.3, 21.1]***	6.6 [ -0.8, 14.0]	9.1 [ 1.7, 16.5]**
60 min – 180 min	15.3 [ 4.2, 26.5]***	21.7 [ 10.6, 32.8]***	9.8 [ -1.4, 20.9]	13.0 [ 1.9, 24.1]**
120 min – 180 min	6.4 [ 0.7, 12.1]*	8.0 [ 2.4, 13.7]***	3.2 [ -2.5, 8.8]	3.9 [ -1.8, 9.6]

17/APrem: Semen stored at 17°C in AndroStar Premium extender with antibiotic.

17/BTS: Semen stored at 17°C in BTS extender with antibiotic.

5/APrem: Semen stored at 5°C in AndroStar Premium extender without antibiotic.

5/BTS: Semen stored at 5°C in BTS extender with antibiotic.

Supplemental Table S4:

Changes in live, low calcium (propidium iodide and Fluo-3-AM negative) spermatozoa (%) by temperature/extender treatment and storage time point. All results (n=6) are mean  $\pm$  SEM.

	3 min	60 min	120 min	180 min
<b>Tyrode C</b>				
17/APrem	86.1 $\pm$ 0.6	85.3 $\pm$ 0.7	84.6 $\pm$ 0.6	83.3 $\pm$ 0.9
17/BTS	82.9 $\pm$ 0.7	83.9 $\pm$ 0.5	82.8 $\pm$ 0.6	80.5 $\pm$ 0.7
5/APrem	82.1 $\pm$ 1.9	81.7 $\pm$ 2.3	79.4 $\pm$ 2.0	77.5 $\pm$ 2.5
5/BTS	65.6 $\pm$ 2.0	65.2 $\pm$ 2.1	60.8 $\pm$ 3.2	58.4 $\pm$ 3.6
<b>Tyrode A</b>				
17/APrem	85.8 $\pm$ 0.7	74.5 $\pm$ 1.1	65.6 $\pm$ 2.3	59.2 $\pm$ 3.5
17/BTS	82.0 $\pm$ 0.7	65.6 $\pm$ 3.2	51.9 $\pm$ 6.0	43.9 $\pm$ 7.4
5/APrem	81.8 $\pm$ 1.6	67.1 $\pm$ 2.1	60.5 $\pm$ 3.1	57.3 $\pm$ 4.1
5/BTS	64.6 $\pm$ 2.1	47.6 $\pm$ 4.2	38.5 $\pm$ 5.2	34.6 $\pm$ 5.7

17/APrem: Semen stored at 17°C in AndroStar Premium extender with antibiotic.

17/BTS: Semen stored at 17°C in BTS extender with antibiotic.

5/APrem: Semen stored at 5°C in AndroStar Premium extender without antibiotic.

5/BTS: Semen stored at 5°C in BTS extender with antibiotic.



Supplemental Table S5:

Distribution of parities of female pigs in the two insemination groups

Insemination group	Parity at the time of insemination							Total	
	0 (gilts)	1	2	3	4	5	6		
<b>17°C/with AB</b>									
n	71	63	80	70	46	42	33	405	
%	17.5	15.6	19.8	17.3	11.4	10.4	8.2		
<b>5°C/without AB</b>									
n	87	78	73	68	41	45	18	410	
%	21.2	19.0	17.8	16.6	10.0	11.0	4.4		
<b>Total</b>	<b>n</b>	158	141	153	138	87	87	51	815*

\*Parities of two sows, one in each insemination group, were not recorded and therefore were not considered in the table.

AB: =Antibiotic in semen extender