ANDROLOGY

Intracellular calcium movements of boar sperm during 'in vitro' capacitation and subsequent acrosome exocytosis follow a multiple-storage place, extracellular calcium-dependent model

Journal:	*Andrology*
Manuscript ID:	ANDR-2015-0042.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Yeste, Marc; University of Oxford, Department of Obstetrics & Gynaecology Fernández-Novell, Josep; University of Barcelona, Department of Biochemistry Ramió-Lluch, Laura; Autonomous University of Barcelona, Department of Animal Medicine and Surgery Estrada, Efrén; Autonomous University of Barcelona, Animal Medicine and Surgery Rocha, Luiz; Autonomous University of Barcelona, Animal Medicine and Surgery Cebrian-Perez, Jose; Universidad de Zaragoza Mui&#65533;o-Blanco, Teresa; University of Zaragoza, Biochemistry and Molecular and Cellular Biology; University of Zaragoza, Departde Bioquimica y Biologia Molec Concha, Ilona; Universidad Austral de Chile, Bioquímica y Microbiología Ramírez-Reveco, Alfredo; Universidad Austral de Chile, Instituto de Ciencia Animal, Facultad de Ciencias Veterinarias Rodríguez-Gil, Joan E.; Autonomous University of Barcelona, Animal Medicine and Surgery
Key Words:	other < animal models, sperm function, CASA (Computer-Assisted Semen Analysis), acrosome, sperm capacitation < sperm quality parameters

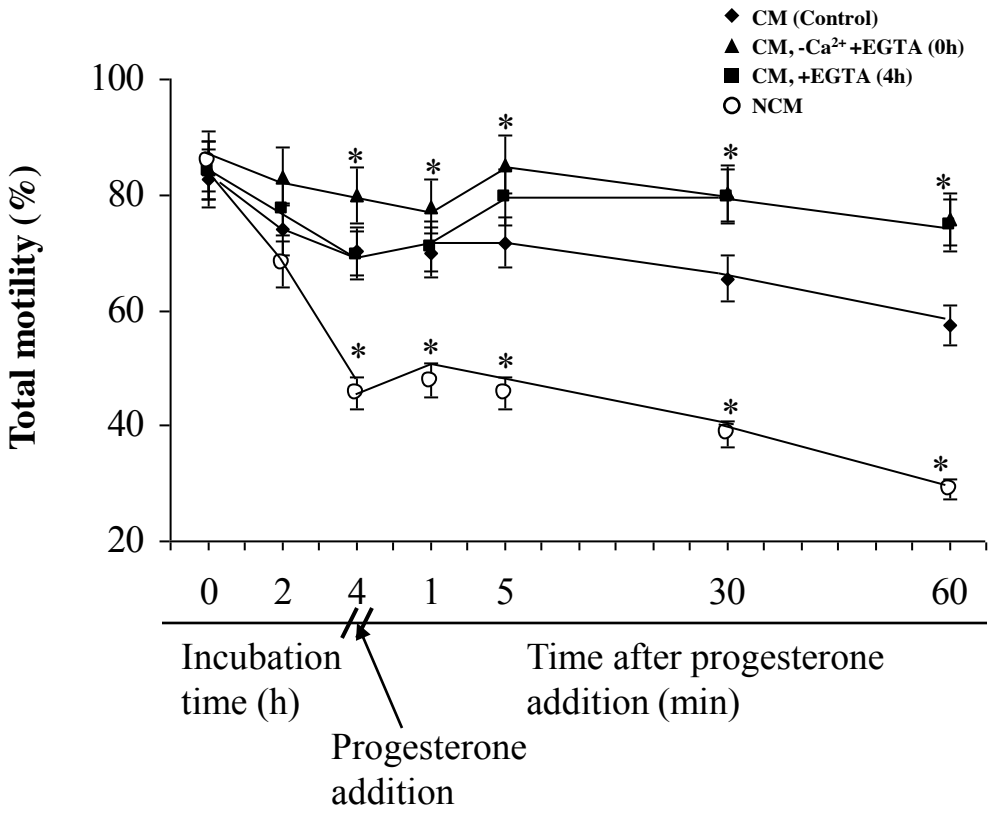

CM (Control)
CM, -Ca2+ +EGTA (0h)
CM, +EGTA (4h)
NCM
Total motility (%)
100
80
60
40
20
0
2
4
1
5
30
60
Incubation time (h)
Time after progesterone addition (min)
Progesterone addition

Figure 1

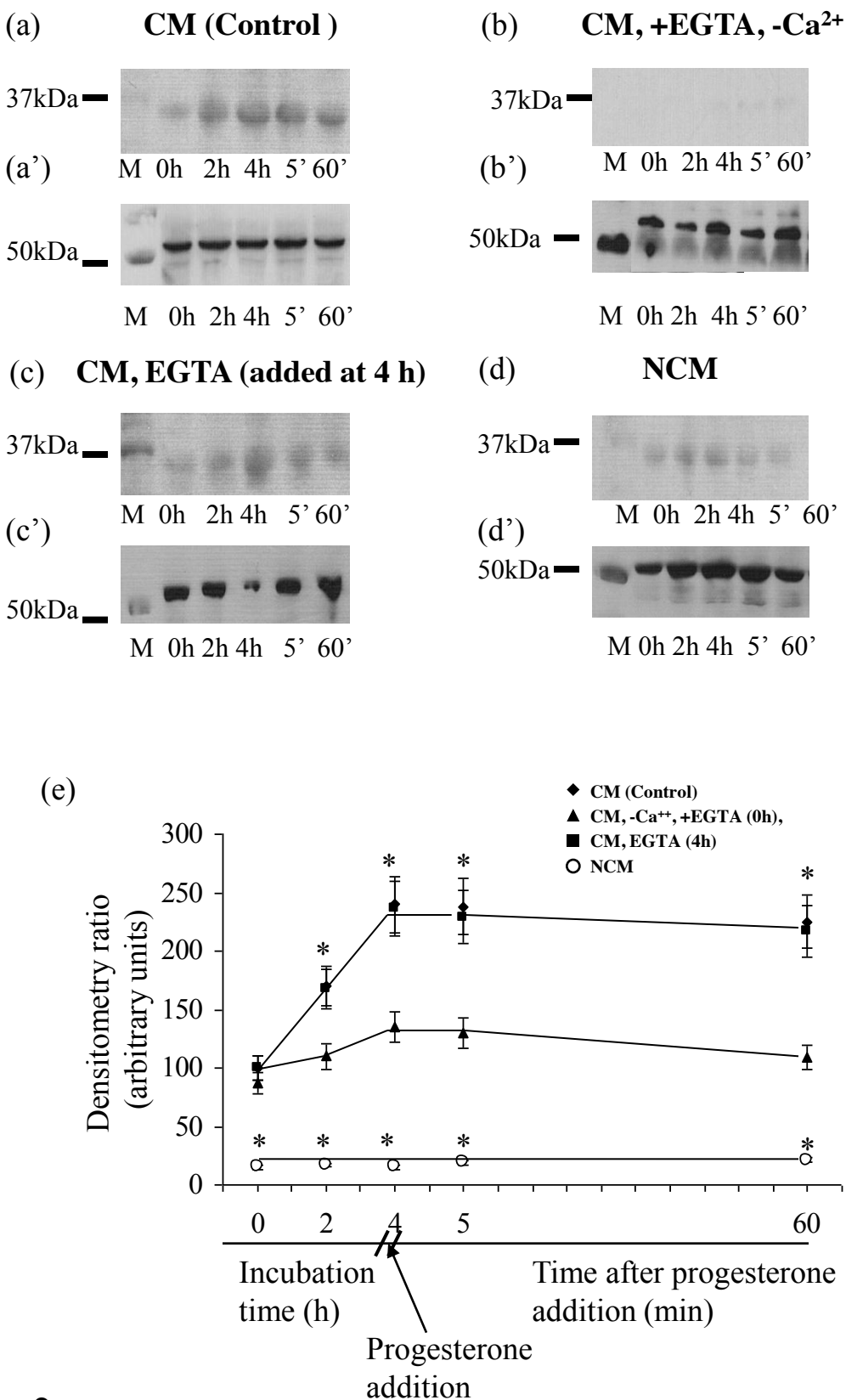

(a) CM (Control)
37kDa
M 0h 2h 4h 5' 60'
(a')
50kDa
M 0h 2h 4h 5' 60'
(b) CM, +EGTA, -Ca2+
37kDa
M 0h 2h 4h 5' 60'
(b')
50kDa
M 0h 2h 4h 5' 60'
(c) CM, EGTA (added at 4 h)
37kDa
M 0h 2h 4h 5' 60'
(c')
50kDa
M 0h 2h 4h 5' 60'
(d) NCM
37kDa
M 0h 2h 4h 5' 60'
(d')
50kDa
M 0h 2h 4h 5' 60'
(e)
CM (Control)
CM, -Ca++, +EGTA (0h),
CM, EGTA (4h)
NCM
Densitometry ratio (arbitrary units)
300
250
200
150
100
50
0
0 2 4 5 60
Incubation time (h)
Time after progesterone addition (min)
Progesterone addition

Figure 2

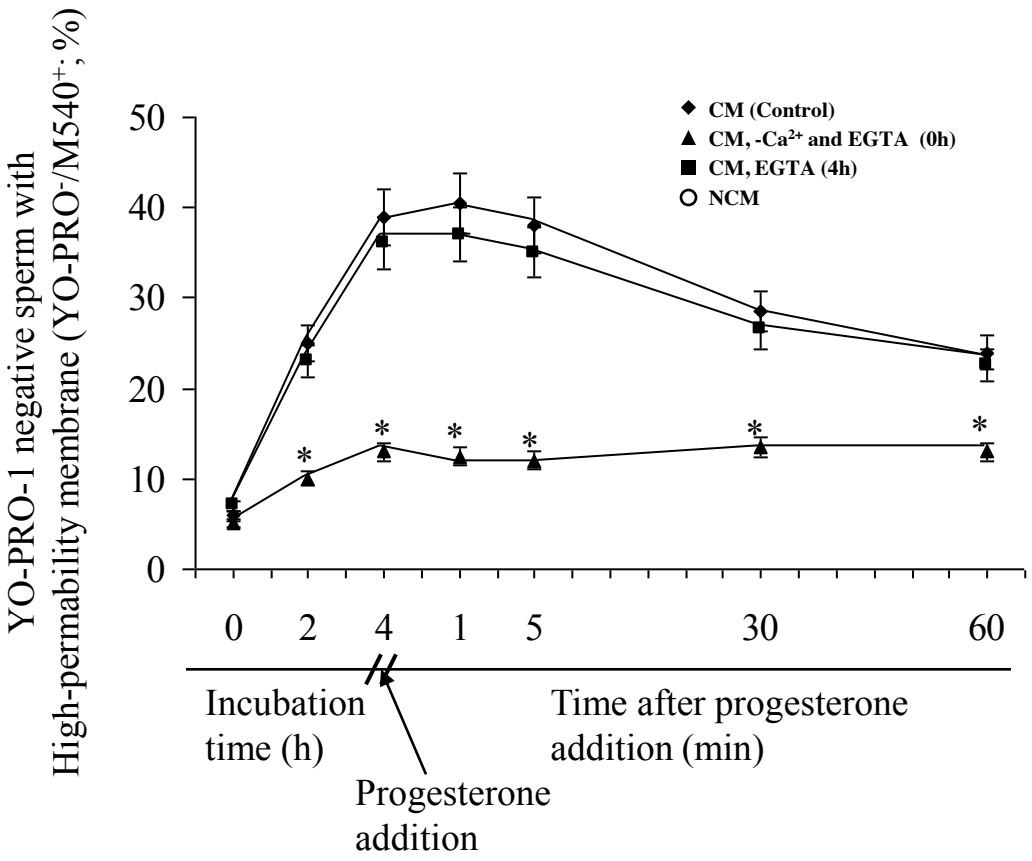
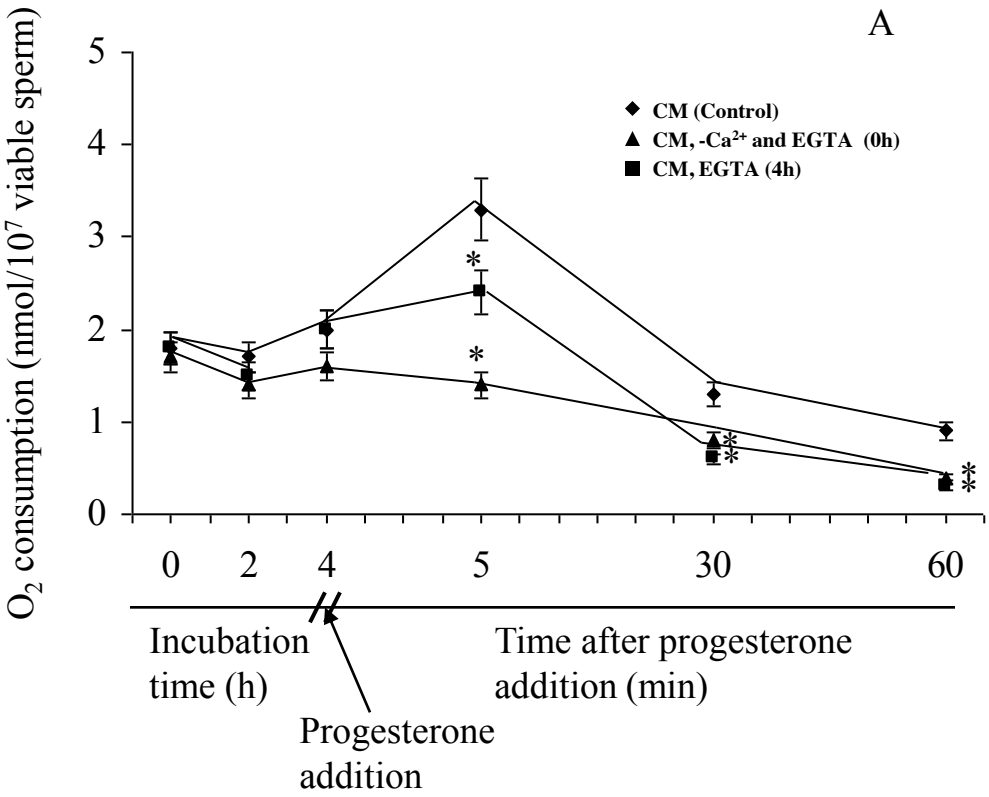

YO-PRO-1 negative sperm with
High-permability membrane (YO-PRO-/M540+; %)
50
40
30
20
10
0
CM (Control)
CM, -Ca2+ and EGTA (0h)
CM, EGTA (4h)
NCM
*
0
2
4
1
5
30
60
Incubation
time (h)
Time after progesterone
addition (min)
Progesterone
addition

Figure 3


A
CM (Control)
CM, -Ca2+ and EGTA (0h)
CM, EGTA (4h)
O2 consumption (nmol/10^7 viable sperm)
0
1
2
3
4
5
0
2
4
5
30
60
Incubation time (h)
Time after progesterone addition (min)
Progesterone addition

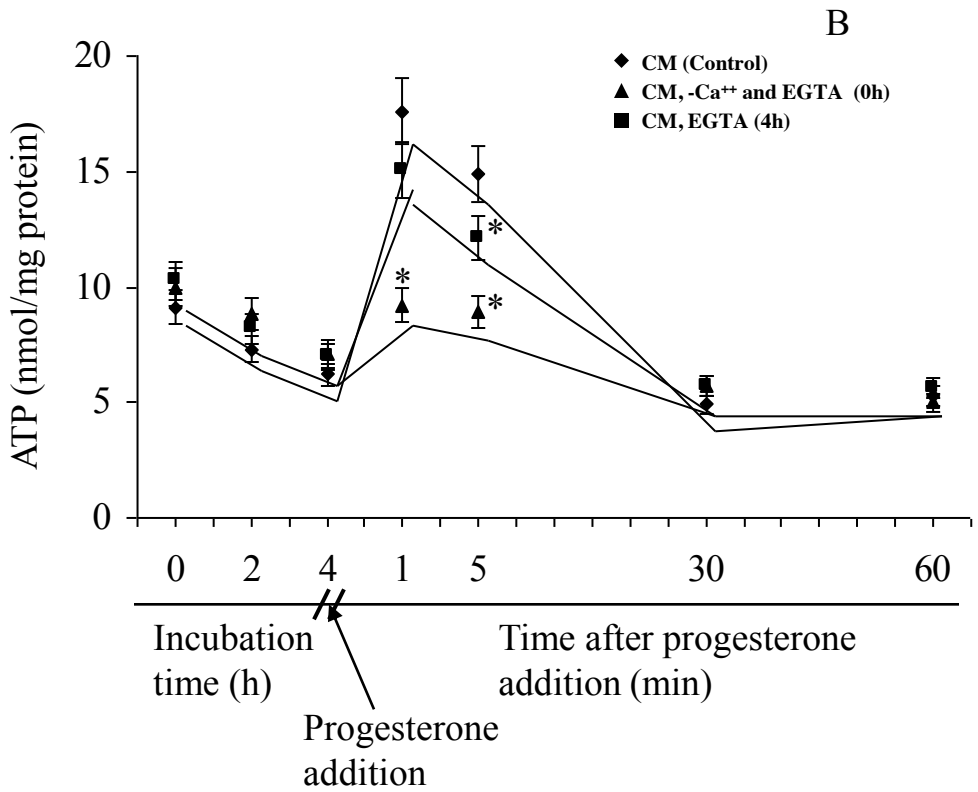

B
CM (Control)
CM, -Ca++ and EGTA (0h)
CM, EGTA (4h)
ATP (nmol/mg protein)
0
5
10
15
20
0
2
4
1
5
30
60
Incubation time (h)
Time after progesterone addition (min)
Progesterone addition

Figure 4

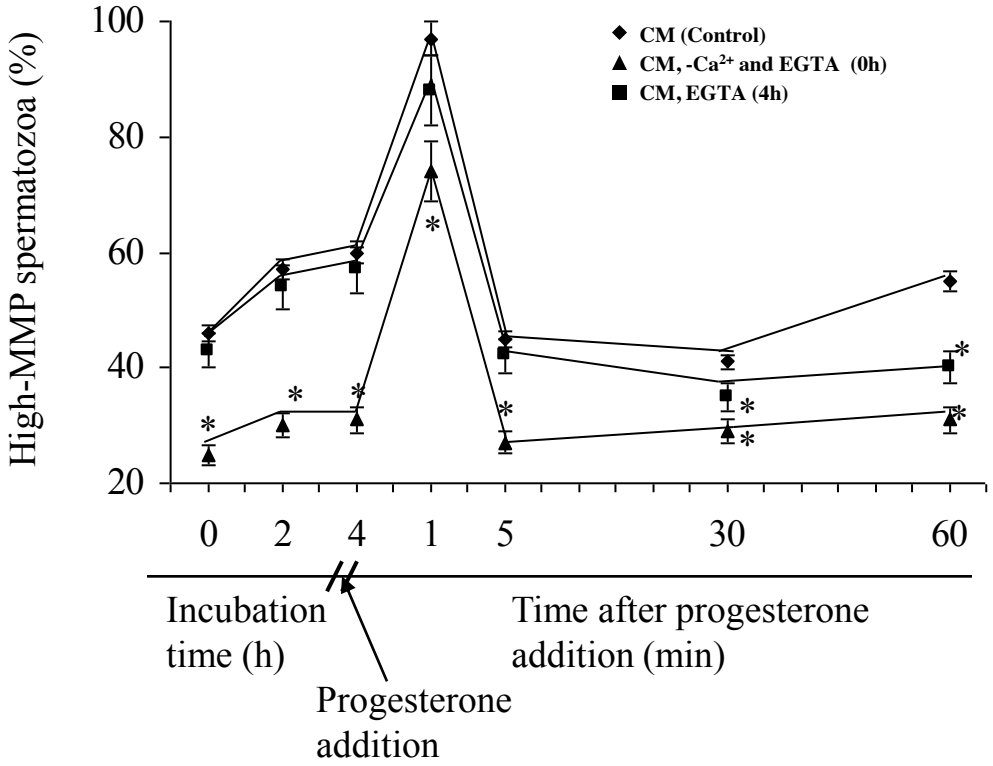

High-MMP spermatozoa (%)
100
80
60
40
20
CM (Control)
CM, -Ca2+ and EGTA (0h)
CM, EGTA (4h)
0
2
4
1
5
30
60
Incubation time (h)
Time after progesterone addition (min)
Progesterone addition

Figure 5

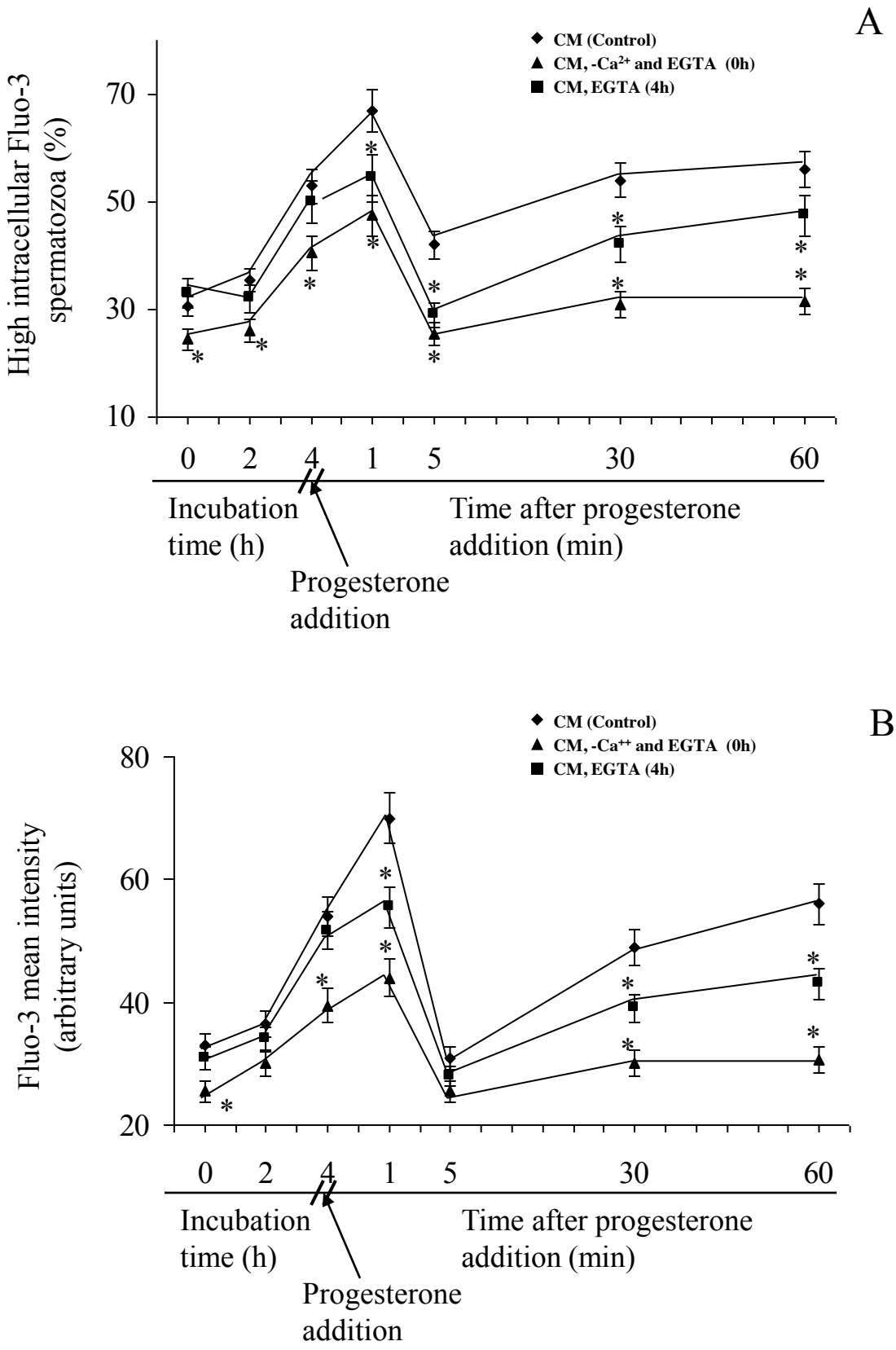

A
CM (Control)
CM, -Ca2+ and EGTA (0h)
CM, EGTA (4h)
High intracellular Fluo-3 spermatozoa (%)
70
50
30
10
0
2
4
1
5
30
60
Incubation time (h)
Time after progesterone addition (min)
Progesterone addition
B
CM (Control)
CM, -Ca++ and EGTA (0h)
CM, EGTA (4h)
Fluo-3 mean intensity (arbitrary units)
80
60
40
20
0
2
4
1
5
30
60
Incubation time (h)
Time after progesterone addition (min)
Progesterone addition

Figure 6

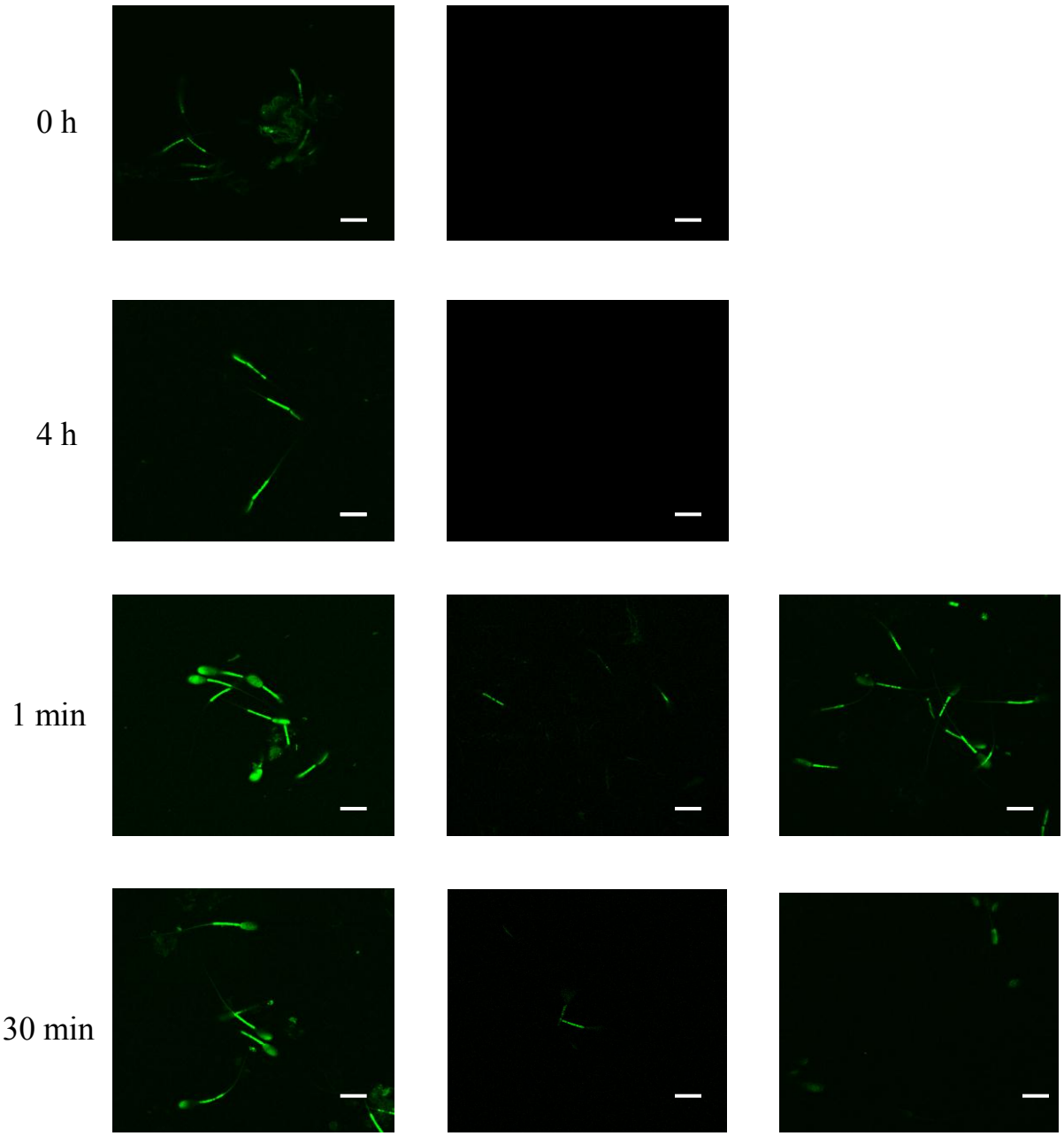
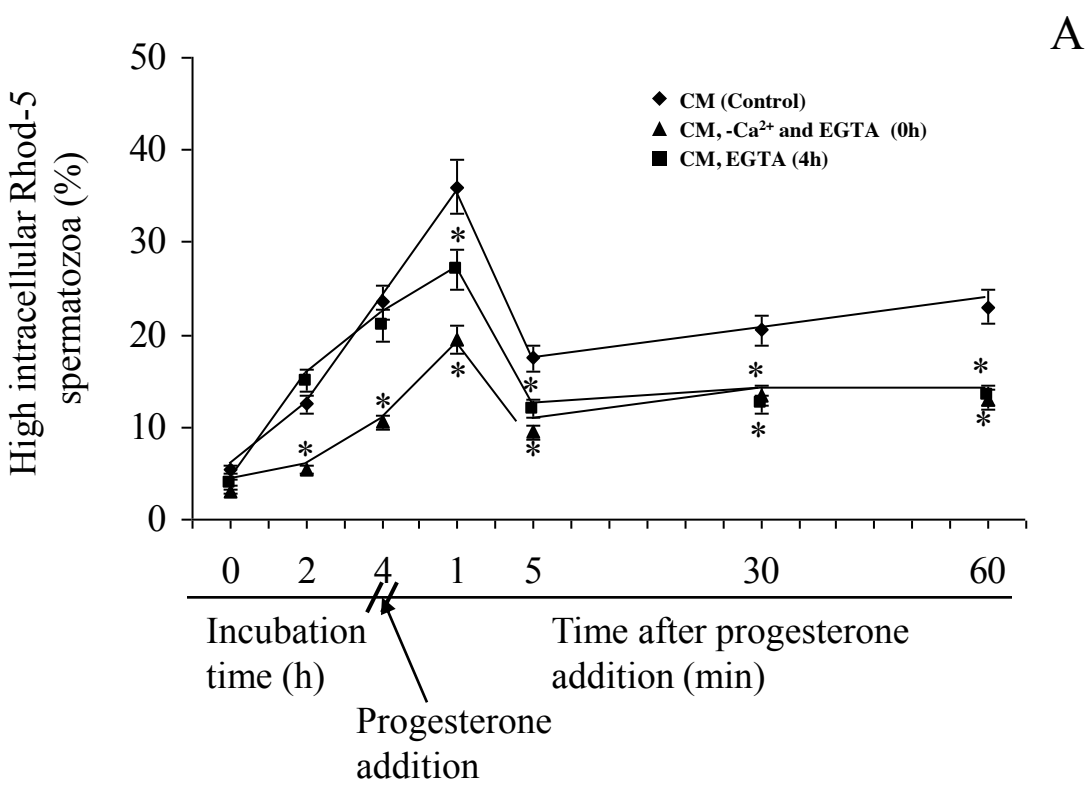


Figure 7


A
High intracellular Rhod-5 spermatozoa (%)
50
40
30
20
10
0
CM (Control)
CM, -Ca2+ and EGTA (0h)
CM, EGTA (4h)
0
2
4
1
5
30
60
Incubation time (h)
Time after progesterone addition (min)
Progesterone addition

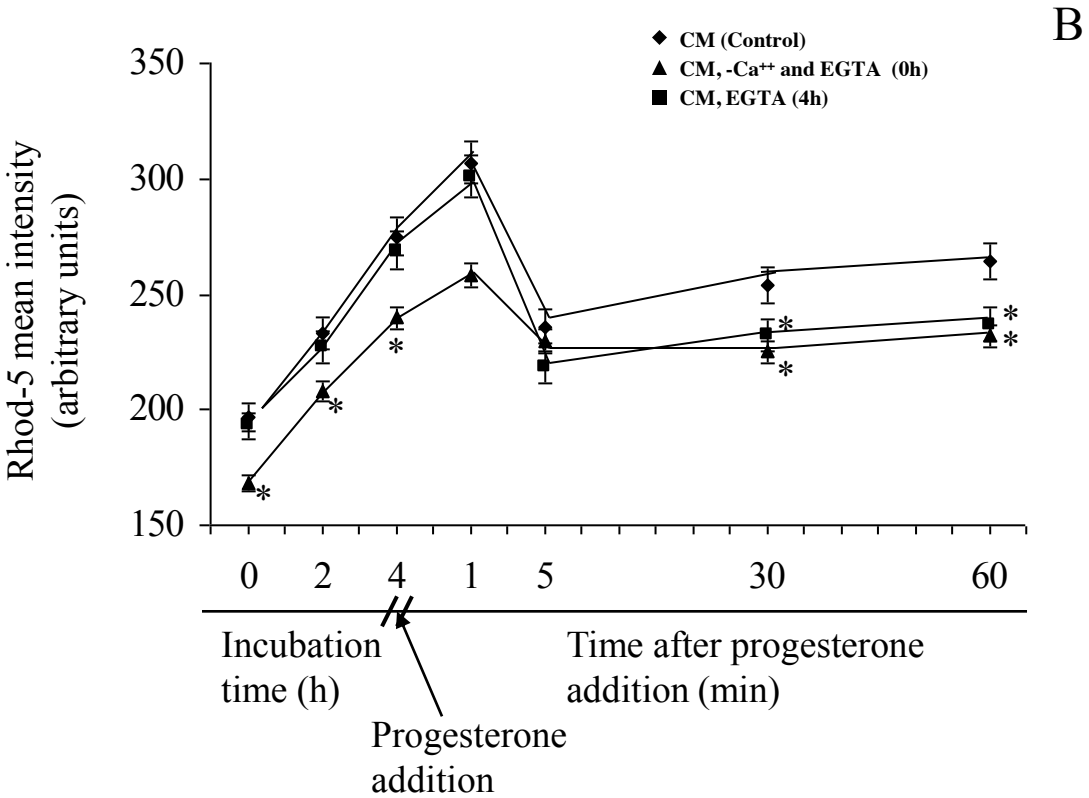

B
Rhod-5 mean intensity (arbitrary units)
350
300
250
200
150
CM (Control)
CM, -Ca++ and EGTA (0h)
CM, EGTA (4h)
0
2
4
1
5
30
60
Incubation time (h)
Time after progesterone addition (min)
Progesterone addition

Figure 8

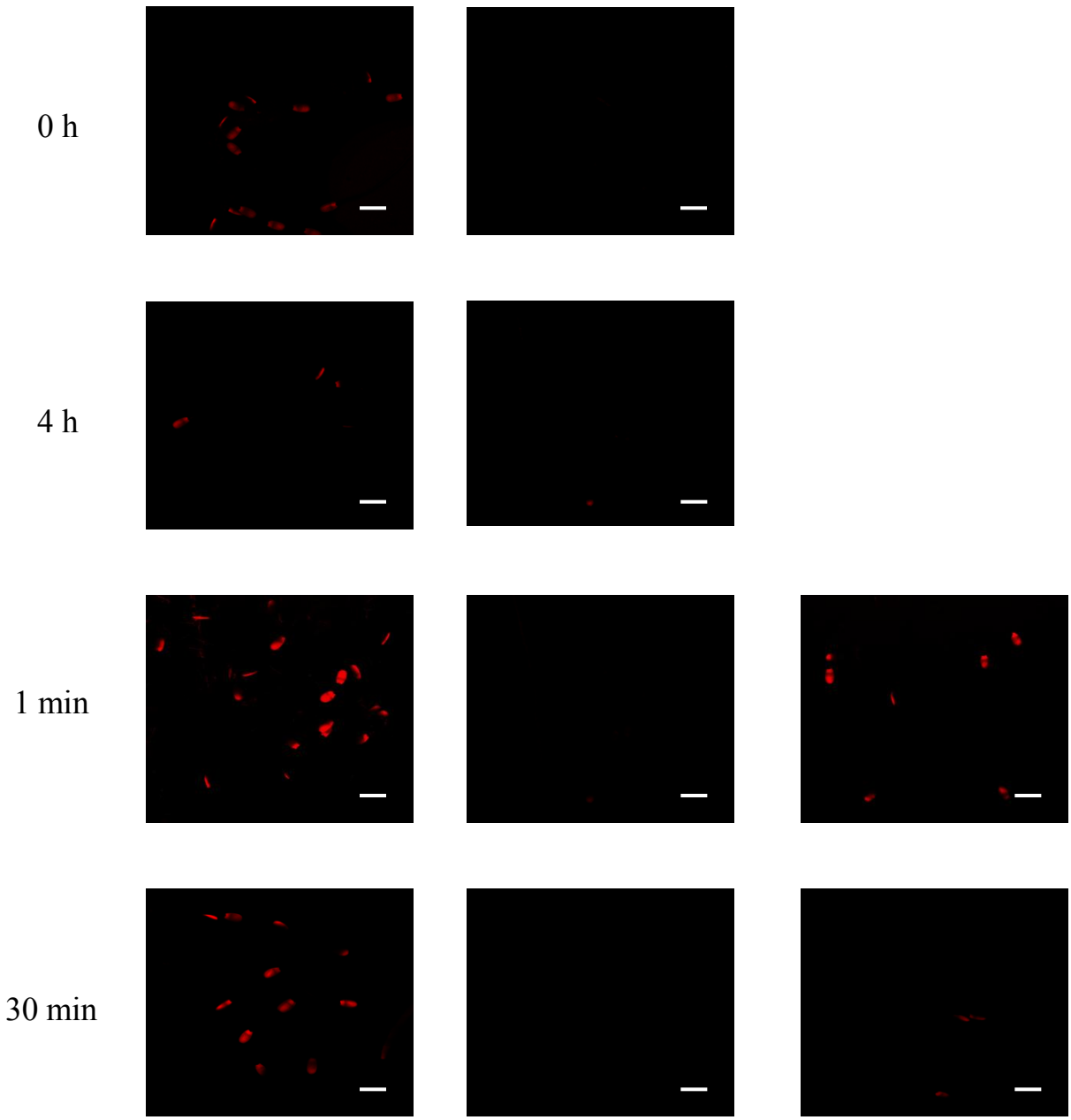


Figure 9

Table 1 Extracellular calcium levels of boar spermatozoa subjected to *in vitro* capacitation (IVC) and further progesterone-induced *in vitro* acrosome exocytosis (IVAE) in the presence or absence of EGTA. NCM: spermatozoa incubated in NCM medium. CM: spermatozoa incubated in standard CM medium. –Ca CM: spermatozoa incubated in a CM medium without Ca^{2+} but with EGTA. CM-EGTA: spermatozoa incubated in standard CM medium (with Ca^{2+}) that EGTA and progesterone are added together at 4h. N.D.: Non-detectable levels of free Ca^{2+} ions (these values were considered as 0 in the statistical analyses). Results are expressed as means±S.E.M. for 11 separate experiments. Different superscripts (*a, b, c*) indicate significant ($P<0.05$) differences between columns (time points) within the same row. Asterisks (*) indicate significant ($P<0.05$) differences between rows (treatments) when compared to their respective CM value within a given column (time point).

	IVC		IVAE		
Incubation time	0h	4h	1 min	5 min	60 min
CM	2.55 ± 0.22^{a}	1.39 ± 0.09^{b}	1.25 ± 0.10^{b}	1.34 ± 0.14^{b}	1.32 ± 0.13^{b}
-Ca CM	$N.D.^{a*}$	$N.D.^{a*}$	$N.D.^{a*}$	$N.D.^{a*}$	$N.D.^{a*}$
CM-EGTA	2.53 ± 0.20^{a}	1.49 ± 0.10^{b}	$0.32\pm0.10^{c*}$	$N.D.^{d*}$	$N.D.^{d*}$
NCM	2.57 ± 0.21^{a}	$2.55\pm0.19^{a*}$	$2.49\pm0.17^{a*}$	$2.53\pm0.21^{a*}$	$2.51\pm0.22^{a*}$

Table 2 Mean values of viability and true acrosome exocytosis of boar spermatozoa subjected to 'in vitro' capacitation and further progesterone-induced 'in vitro' acrosome exocytosis in the presence or absence of EGTA. Results of true acrosome exocytosis are shown as the percentage of cells with true acrosome exocytosis, with respect to the total number of viable cells, and not as a percentage, with respect to the total, viable and non-viable, analysed cells. Groups labelled with 'IVC' indicate that cells were incubated in a medium without calcium and with 2 mM EGTA added from the start of the experiment. Groups labelled with 'IVAE' indicate that cells were incubated with 2 mM EGTA added together with 10 $\mu g \cdot mL^{-1}$ progesterone after 4h incubation in the standard capacitation medium. Groups labelled with 'Negative Control' indicate spermatozoa incubated in a medium similar to the capacitation medium but without BSA. These cells were utilised as negative controls of capacitation. Results are expressed as means±S.E.M. for 11 separate experiments. Different superscripts in a row indicate significant ($P<0.05$) differences among groups. Asterisks indicate significant ($P<0.05$) differences, when compared with the respective Control values.

	IVC			IVAE			
Incubation Time	0h	2h	4h	1 min	5 min	30 min	60 min
Viability Negative Control (%)	85.7 ± 2.7^{a}	59.4 ± 3.6^{b}	49.7 ± 1.9^{c}	48.1 ± 2.9^{c}	48.4 ± 2.1^{d}	40.3 ± 2.8^{d}	$31.6\pm2.1^{e*}$
Viability Control (%)	86.0 ± 2.2^{a}	64.7 ± 4.2^{b}	62.9 ± 4.5^{b}	62.5 ± 4.0^{b}	61.0 ± 2.0^{b}	55.3 ± 4.7^{b}	54.6 ± 4.6^{b}
Viability EGTA IVC (%)	87.1 ± 1.5^{a}	64.4 ± 5.0^{b}	62.9 ± 4.2^{c}	58.3 ± 4.9^{cd}	54.2 ± 5.3^{d}	57.7 ± 4.9^{d}	50.2 ± 5.6^{d}
Viability EGTA IVAE (%)	85.4 ± 1.4^{a}	62.0 ± 5.8^{b}	63.9 ± 5.6^{b}	63.8 ± 5.9^{b}	64.3 ± 5.7^{b}	45.0 ± 6.2^{c}	49.2 ± 6.2^{d}
True acrosome exocytosis Negative Control (%)	4.9 ± 2.4^{a}	4.4 ± 2.8^{a}	3.8 ± 1.3^{a}	3.2 ± 1.9^{a}	$3.0\pm2.3^{a*}$	$3.4\pm2.2^{a*}$	$3.0\pm2.9^{a*}$
True acrosome exocytosis Control (%)	5.1 ± 3.3^{a}	4.6 ± 3.5^{a}	4.6 ± 3.9^{a}	8.7 ± 4.1^{a}	20.7 ± 4.2^{b}	68.4 ± 6.2^{c}	72.3 ± 6.2^{c}
True acrosome exocytosis EGTA IVC (%)	4.6 ± 2.0^{a}	3.2 ± 0.6^{a}	4.8 ± 1.2^{a}	9.3 ± 1.9^{b}	14.0 ± 1.5^{c}	$15.0\pm0.9^{c*}$	$15.4\pm3.5^{c*}$

True acrosome exocytosis EGTA IVAE (%)	3.8±0.8[a]	4.1±0.6[a]	3.9±0.9[a]	8.5±1.6[b]	21.0±2.1[c]	25.9±2.0[c*]	17.9±3.8[d*]

Table 3 Mean values of velocity parameters and ALH of boar spermatozoa subjected to 'in vitro' capacitation and further progesterone-induced 'in vitro' acrosome exocytosis in the presence or absence of EGTA. Groups labelled with 'IVC' indicate that cells were incubated in a medium without calcium and with 2 mM EGTA added from the start of the experiment. Groups labelled with 'IVAE' indicate that cells were incubated with 2 mM EGTA added together with 10 $\mu g \cdot mL^{-1}$ progesterone after 4h incubation in the standard capacitation medium. Groups labelled with 'Negative Control' indicate spermatozoa incubated in a medium similar to the capacitation medium but without BSA. These cells were utilised as negative controls of capacitation. Results are expressed as means±S.E.M. for 11 separate experiments. Different superscripts in a row indicate significant ($P<0.05$) differences among groups. Asterisks indicate significant ($P<0.05$) differences, when compared with the respective Control values.

	IVC			IVAE			
Incubation Time	0h	2h	4h	1 min	5 min	30 min	60 min
VCL Negative Control ($\mu m \cdot s^{-1}$)	71.6 ± 2.0^{a}	48.3 ± 4.2^{b}	45.9 ± 3.9^{b}	45.2 ± 3.8^{b}	45.3 ± 3.3^{b}	41.8 ± 2.9^{c}	39.7 ± 3.3^{c}
VCL Control ($\mu m \cdot s^{-1}$)	74.7 ± 1.3^{a}	80.5 ± 1.6^{b}	88.9 ± 1.0^{c}	91.2 ± 2.1^{c}	86.2 ± 1.8^{c}	88.8 ± 2.8^{c}	83.6 ± 2.5^{bc}
VCL EGTA IVC ($\mu m \cdot s^{-1}$)	$87.6\pm2.1^{a*}$	$88.6\pm2.7^{a*}$	$102.5\pm3.0^{c*}$	100.6 ± 2.8^{c}	$96.2\pm2.3^{c*}$	$109.6\pm3.7^{d*}$	$92.2\pm3.3^{e*}$
VCL EGTA IVAE ($\mu m \cdot s^{-1}$)	74.3 ± 1.3^{a}	82.5 ± 1.9^{b}	93.2 ± 3.6^{c}	91.5 ± 3.8^{bc}	85.6 ± 3.1^{b}	94.3 ± 3.4^{c}	84.0 ± 3.2^{b}
VSL Negative Control ($\mu m \cdot s^{-1}$)	29.8 ± 1.1^{a}	25.5 ± 2.3^{ab}	24.9 ± 1.9^{b}	25.6 ± 2.0^{ab}	25.5 ± 2.5^{ab}	22.7 ± 1.8^{b}	19.9 ± 1.1^{b}
VSL Control ($\mu m \cdot s^{-1}$)	30.9 ± 1.0^{a}	37.7 ± 2.3^{b}	42.7 ± 2.6^{c}	58.1 ± 2.3^{d}	44.3 ± 1.5^{c}	44.6 ± 1.7^{c}	41.9 ± 2.4^{c}
VSL EGTA IVC ($\mu m \cdot s^{-1}$)	30.6 ± 0.9^{a}	$47.2\pm2.4^{b*}$	$49.6\pm2.4^{b*}$	$51.8\pm2.7^{b*}$	$55.8\pm2.5^{b*}$	$62.4\pm3.0^{c*}$	$56.0\pm2.7^{b*}$
VSL EGTA IVAE ($\mu m \cdot s^{-1}$)	30.7 ± 0.8^{a}	39.6 ± 1.8^{b}	$44.6\pm1.9.^{c}$	$42.9\pm2.0^{c*}$	$39.1\pm1.1^{c*}$	$36.2\pm1.5^{c*}$	36.0 ± 1.9^{c}
VAP Negative Control ($\mu m \cdot s^{-1}$)	46.7 ± 1.2^{a}	45.6 ± 1.3^{a}	43.0 ± 2.3^{a}	43.7 ± 2.8^{a}	43.8 ± 2.9^{a}	39.1 ± 2.5^{a}	37.2 ± 3.0^{a}

VAP Control (μm·s^{-1})	48.0±0.9^{a}	57.1±0.8^{b}	65.3±1.4^{c}	59.8±1.4^{d}	63.2±1.7^{c}	63.8±1.3^{c}	59.6±1.1^{bc}
VAP EGTA IVC (μm·s^{-1})	49.6±1.1^{a}	61.3±2.8^{b}	75.7±2.3^{c*}	72.9±2.6^{c*}	73.1±1.8^{c*}	74.8±2.0^{c*}	70.7±1.3^{c*}
VAP EGTA IVAE (μm·s^{-1})	47.6±1.6^{a}	57.7±1.1^{b}	64.6±1.6^{c}	61.6±2.3^{cd}	58.2±2.2^{d}	58.1±2.5^{d}	54.5±2.5^{d*}
LIN Negative Control (%)	33.9±1.5^{a}	43.1±2.2^{b}	45.3±2.4^{b}	47.6±2.1^{b}	45.9±1.9^{b}	44.2±2.3^{b}	40.5±1.7^{b}
LIN Control (%)	33.6±1.7^{a}	45.4±1.4^{b}	49.3±1.3^{c}	45.7±1.8^{b}	48.8±1.8^{c}	50.1±1.0^{c}	47.6±1.3^{bc}
LIN EGTA IVC (%)	33.5±1.4^{a}	43.9±2.0^{b}	56.1±0.8^{c*}	57.9±1.1^{c*}	54.7±1.6^{c*}	56.6±0.6^{c*}	55.2±1.2^{c*}
LIN EGTA IVAE (%)	32.8±1.1^{a}	44.9±1.5^{b}	51.1±1.7^{c}	49.3±2.0^{bc}	46.7±2.5^{b}	47.7±2.3^{b}	48.3±2.8^{b}
STR Negative Control (%)	64.8±1.1^{a}	56.2±1.0^{b}	56.9±1.1^{b}	55.9±1.3^{b}	57.2±2.1^{b}	54.3±1.0^{b}	48.5±1.0^{c}
STR Control (%)	63.9±1.0^{a}	75.6±1.3^{b}	75.9±1.4^{b}	71.7±1.9^{c}	74.5±1.7^{b}	78.6±1.1^{bd}	79.8±1.3^{d}
STR EGTA IVC (%)	66.5±0.7^{a*}	77.0±2.4^{b}	85.5±2.1^{c*}	85.7±1.9^{c*}	85.6±1.5^{c*}	87.1±1.7^{c*}	86.4±1.9^{b*}
STR EGTA IVAE (%)	63.2±1.0^{a}	73.6±1.1^{b}	77.2±0.8^{c}	71.7±1.8^{b}	69.3±1.7^{b*}	72.8±1.0^{b*}	73.0±1.0^{b*}

Supplementary Table 1. Mean values of complementary motility parameters of boar spermatozoa subjected to *in vitro* capacitation and further progesterone-induced *in vitro* acrosome exocytosis in the presence or absence of EGTA. Motility parameters have been defined in the Material and Methods section. Groups labeled with 'IVC' indicate that cells were incubated in a medium without calcium and with 2 mM EGTA added from the start of the experiment. Groups labeled with 'IVAE' indicate that cells were incubated or with 2 mM EGTA added together with 10 µg/mL progesterone after 4 h incubation in the standard capacitation medium. Groups labelled with "Negative Control" indicate spermatozoa incubated in a medium similar to the capacitation medium but without BSA. These cells were utilised as negative controls of capacitation. Results are expressed as means±S.E.M. for 11 separate experiments. Different superscripts in a row indicate significant (P<0.05) differences among groups. Asterisks indicate significant (P<0.05) differences, when compared with the respective Control values. Statistical analyses were performed as described in the Material and Methods section.

	IVC			IVAE			
Incubation time	0 h	2 h	4 h	1 min	5 min	30 min	60 min
ALH Negative Control (µm)	3.6 ± 0.1^{a}	2.4 ± 0.1^{b}	2.1 ± 0.2^{b}	2.2 ± 0.1^{b}	2.2 ± 0.2^{b}	2.0 ± 0.1^{c}	2.0 ± 0.1^{c}
ALH Control (µm)	4.1 ± 0.2^{a}	3.8 ± 0.3^{a}	4.1 ± 0.3^{a}	4.8 ± 0.3^{b}	4.5 ± 0.2^{b}	4.4 ± 0.3^{ab}	4.3 ± 0.2^{ab}
ALH EGTA IVC (µm)	4.0 ± 0.1^{a}	3.7 ± 0.2^{a}	4.4 ± 0.1^{b}	4.8 ± 0.2^{c}	$4.9\pm0.1^{c*}$	$5.0\pm0.1^{d*}$	4.7 ± 0.1^{b}
ALH EGTA IVAE (µm)	4.2 ± 0.2^{a}	3.8 ± 0.1^{a}	4.2 ± 0.1^{a}	$4.3\pm0.2^{a*}$	4.3 ± 0.1^{ab}	4.5 ± 0.2^{b}	4.5 ± 0.1^{b}
WOB Negative Control (%)	53.9 ± 1.1^{a}	77.9 ± 2.8^{b}	77.3 ± 3.0^{b}	78.6 ± 2.6^{b}	80.2 ± 3.0^{b}	76.5 ± 3.1^{b}	76.3 ± 3.2^{b}
WOB Control (%)	52.9 ± 0.7^{a}	60.6 ± 0.6^{b}	66.3 ± 1.2^{c}	69.6 ± 1.9^{c}	63.5 ± 0.8^{bc}	65.2 ± 0.7^{c}	63.1 ± 0.9^{bc}
WOB EGTA IVC (%)	53.5 ± 1.7^{a}	61.1 ± 1.5^{b}	67.3 ± 1.4^{c}	67.4 ± 1.9^{c}	$67.3\pm1.0^{c*}$	$68.1\pm0.8^{c*}$	$67.8\pm1.0^{c*}$

WOB EGTA IVAE (%)	53.2±1.0[a]	59.1±1.3[b]	66.9±1.5[c]	64.3±1.1[bc*]	62.8±0.9[b]	63.8±1.2[bc]	61.9±0.7[b]
BCF Negative Control (Hz)	6.8±0.2[a]	6.2±0.1[b]	6.1±0.1[b]	6.2±0.3[b]	6.3±0.3[b]	6.7±0.3[a]	7.2±0.3[a]
BCF Control (Hz)	6.3±0.2[a]	6.2±0.1[a]	6.3±0.1[a]	6.9±0.1[b]	6.4±0.1[c]	6.4±0.2[a]	6.3±0.2[a]
BCF EGTA IVC (Hz)	6.5±0.2[a]	6.3±0.3[a]	7.1±0.2[b*]	7.3±0.2[b]	7.2±0.2[b*]	7.5±0.2[b*]	6.8±0.3[b]
BCF EGTA IVAE (Hz)	6.4±0.2[a]	6.3±0.2[a]	6.3±0.2[a]	6.8±0.2[ab]	6.7±0.2[ab]	7.1±0.1[b*]	6.1±0.1[a]

Title

Intracellular calcium movements of boar sperm during 'in vitro' capacitation and subsequent acrosome exocytosis follow a multiple-storage place, extracellular calcium-dependent model

Running header (Short title)

Calcium storage during boar sperm capacitation

Authors and affiliations

Marc Yeste[1, 2, *], Josep M. Fernández-Novell[3], Laura Ramió-Lluch[1], Efrén Estrada[1], Luiz G. Rocha[1], José A. Cebrián-Pérez[4], Teresa Muiño-Blanco[4], Ilona I. Concha[5], Alfredo Ramírez[5], Joan E. Rodríguez-Gil[1, *]

[1]*Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, Autonomous University of Barcelona, Bellaterra (Cerdanyola del Vallès), Spain.*

[2]*Nuffield Department of Obstetrics and Gynaecology, University of Oxford, Level 3, Women's Centre, John Radcliffe Hospital, Headington, Oxford, United Kingdom.*

[3]*Department of Biochemistry and Molecular Biology, University of Barcelona, Barcelona, Spain.*

[4]*Department of Biochemistry and Molecular and Cell Biology, IUCA, School of Veterinary Medicine, University of Zaragoza-IUCA, Zaragoza, Spain.*

[5]*Institute of Biochemistry and Microbiology and Institute of Animal Science, Universidad Austral de Chile, Valdivia, Chile.*

***Correspondence should be addressed to:**

Joan E. Rodríguez-Gil, Department of Animal Medicine and Surgery, Autonomous University of Barcelona, E-08193 Bellaterra (Cerdanyola del Vallès), Spain. Phone: +34 935 811 045. Fax: +34 935 812 006. Electronic address: juanenrique.rodriguez@uab.cat

Marc Yeste, Nuffield Department of Obstetrics and Gynaecology, University of Oxford, Level 3, Women's Centre, John Radcliffe Hospital, Headington, Oxford, OX3 9DU, United Kingdom. Tel: +44 (0)1865 782829; Fax: +44 (0)1865 769141. Electronic address: marc.yeste@obs-gyn.ox.ac.uk

Abstract

This work analysed intracellular calcium stores of boar sperm subjected to 'in vitro' capacitation (IVC) and subsequent progesterone-induced acrosome exocytosis (IVAE). Intracellular calcium was analysed through two calcium markers with different physico-chemical properties, Fluo-3 and Rhod-5N. Indicative parameters of IVC and IVAE were also evaluated. Fluo-3 was located at both the midpiece and the whole head. Rhod-5N was present at the sperm head. This distribution did not change in any of the assayed conditions. Induction of IVC was concomitant with an increase in both head and midpiece Ca^{2+} signals. Additionally, while IVC induction was concurrent with a significant (P<0.05) increase in sperm membrane permeability, no significant changes were observed in O_2 consumption and ATP levels. Incubation of boar sperm in the absence of calcium showed a loss of both Ca^{2+} labellings concomitantly with the sperm's inability to achieve IVC. The absence of extracellular calcium also induced a severe decrease in the percentage of sperm exhibiting high mitochondrial membrane potential (hMMP). The IVAE was accompanied by a fast increase in both Ca^{2+} signalling in control spermatozoa. These peaks were either not detected or much lessened in the absence of calcium. Remarkably, Fluo-3 marking at the midpiece increased after progesterone addition to sperm cells incubated in a medium without Ca^{2+}. The simultaneous addition of progesterone with the calcium chelant EGTA inhibited IVAE, and this was accompanied by a significant (P<0.05) decrease in the intensity of progesterone Ca^{2+}-induced peak, O_2 consumption and ATP levels. Our results suggest that boar spermatozoa present different calcium deposits with a dynamic equilibrium among them and with the extracellular environment. Additionally, the modulation role of the intracellular calcium in sperm function seems to rely on its precise localisation in boar sperm.

Keywords: boar sperm, calcium, motility, capacitation, acrosome exocytosis.

Introduction

Signalling ways relying on calcium metabolism are of paramount importance in mammalian spermatozoa, since this second messenger regulates a myriad of crucial processes, including sperm capacitation, hyperactivated motility, chemotaxis and acrosome reaction (Publicover et al., 2007; Costello et al., 2009; Aitken & Nixon, 2013). Pathways by which calcium exerts these important regulatory and modulatory effects vary, since the Ca^{2+} ion can exert its effects through a wide array of separate transductional signalling pathways. In fact, mammalian sperm present a wide array of voltage-gated and non-voltage-gated Ca^{2+} channels, such as CatSper and VOCCs, which are known to be involved in the regulation of sperm motility, capacitation and the achievement of the progesterone-induced acrosome exocytosis (Blackmore et al., 1990; García & Meizel, 1999; Breitbart, 2002; Jagannathan et al., 2002; Teves et al., 2006; Lishk et al., 2011). This wide variety of Ca^{2+}-linked regulatory pathways implies that the comprehension of mammalian sperm calcium metabolism, as a whole, is very difficult, with further studies devoted to this point being required.

Intracellular calcium in eukaryotic cells is distributed through separate stores that are in a dynamic equilibrium amongst each other and with the extracellular environment (Costello, et al., 2009). In somatic cells, some of these intracellular calcium deposits, such as the endoplasmic reticulum or mitochondria, are well-known (See Kroemer (1999) for a review). In the case of mammalian spermatozoa, intracellular calcium has been reported to be mainly stored at either the sperm head or connecting/midpiece (Costello, et al., 2009). These separate calcium stores suggest that the role of head and midpiece, mitochondria-related calcium stores may be different. In this manner, while head calcium could be involved in the modulation of progesterone-induced acrosome exocytosis, midpiece calcium could be more closely related to mitochondria-modulated processes, such as sperm motility, mitochondria-based energy production and early capacitation steps (Costello et al., 2009; Gunter et al., 2004). However, this hypothesis needs further confirmation.

Both ‘in vitro’ capacitation (IVC) and ‘in vitro’ acrosome exocytosis (IVAE) in boar sperm are accompanied by Ca^{2+} fluxes similar to those described in other mammalian species (See Töpfer-Petersen et al., 1985 and Dubé et al., 2003 as examples). Indeed, incubation of boar sperm with the Ca^{2+} chelant EGTA (ethylene glycol-bis(2-aminoethylether)-N,N,N’,N’,-tetraacetic acid), is known to inhibit the specific tyrosine-phosphorylation of P32 protein, a hallmark of IVC achievement (Dubé et al., 2003). Previous studies support the idea that a

relationship between roles and deposits of intracellular calcium exists. For example, non-mitochondrial Ca^{2+} is involved in head-to-head agglutination in boar sperm (Harayama et al., 2003), thereby indicating that the head-accumulated calcium can play other roles than those linked to the launching of IVAE.

Against this background, the present study seeks to determine: a) the dynamics of separate calcium deposits of boar sperm subjected to IVC and subsequent progesterone-induced IVAE, and b) the role that extracellular calcium may play in the development of that dynamics. With this purpose, the presence of separate calcium deposits in boar sperm was analysed through incubation with a capacitation medium in the presence or absence of extracellular calcium. These separate calcium deposits were determined through the utilisation of two separate calcium markers, Fluo-3 and Rhod-5N, which stain calcium deposits with different physico-chemical characteristics. The Fluo-3 stain has a very high affinity for calcium, although it is unable to cross polarised cell membranes (Takahashi et al., 1999). On the contrary, Rhod-5N specifically labels calcium stored in deposits surrounded by polarised membranes, and its calcium-affinity is lower than that of Fluo-3 (Takahashi et al., 1999). We also analysed different IVC and IVAE markers, and mitochondria-related energy management was evaluated through the rate of O_2 consumption, mitochondrial membrane potential (MMP), and intracellular ATP concentration (Ramió-Lluch et al., 2014).

Materials and Methods

Suppliers

All reagents were of analytical grade and came from Sigma, Boehringer-Mannheim (Mannheim, Germany) and Merck (Darmstadt, Germany). In the case of fluorochromes, and unless otherwise stated, all were purchased from Molecular Probes® (Invitrogen; Eugene, Oregon, USA) and were diluted with dimethyl sulfoxide (DMSO; Sigma).

Semen samples

In this study, boars were not handled by us; the semen was obtained from a local farm (Servicios Genéticos Porcinos, S.L.; Roda de Ter, Barcelona, Spain). Thus, these ejaculates were initially dedicated for artificial insemination purposes, and we just bought them for our experimental purposes. Despite the aforementioned, and even though it was not required as the authors did not manipulate any boar, the experimental protocol was specifically approved by the Ethics Committee of our institution. This ethics committee is known as the 'Bioethics Commission of the Autonomous University of Barcelona' (Bellaterra, Cerdanyola del Vallès, Spain). Furthermore, handling of boars by the local farm were performed in accordance with the EU Directive 2010/63/EU for animal experiments and the Animal Welfare Law issued by the Regional Government of Catalonia (Generalitat de Catalunya, Spain).

A total of ten different ejaculates coming from ten different boars of proven fertility were obtained from a commercial farm (Servicios Genéticos Porcinos, S.L.; Roda de Ter, Spain). Boar semen was manually collected by the gloved-hand method. The sperm-rich fraction was diluted to 2×10^7 sperm·mL^{-1} with a commercial extender for refrigerated semen (MR-A Extender; Kubus, S.A.; Majadahonda, Spain), divided into seminal doses of 50mL, and cooled down to 17ºC. Three seminal doses per ejaculate were randomly chosen and immediately transported in a portable refrigerator at 17ºC for approximately 45 minutes, which was the time required to arrive at the laboratory. All experiments described below were thus repeated ten times, using a total of 30 seminal doses coming from ten boar ejaculates.

'In vitro' Capacitation and Acrosome Exocytosis Procedures

For each replicate, the three AI seminal doses coming from the same ejaculate were pooled upon arrival and washed thrice through centrifugation (600×*g* at 16ºC for 5 min) and resupension with phosphate buffered saline (PBS). This series of washing steps allowed for

eliminating any traces of seminal plasma and commercial extender. After washing, samples were re-diluted in a medium which consisted of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer (pH=7.4), containing 112 mM NaCl, 3.1 mM KCl, 5 mM glucose, 21.7 mM L-lactate, 1 mM sodium pyruvate, 0.3 mM Na_2HPO_4, 0.4 mM $MgSO_4$ and 4.5 mM $CaCl_2$ (NCM). The osmolarity was 304±5 $mOsm \cdot Kg^{-1}$, and pH was adjusted to 7.4. After the last wash, the sperm was resuspended in a capacitating medium (CM), which consisted of NCM supplemented with 5 $mg \cdot mL^{-1}$ of bovine serum albumin (BSA), to a final concentration of 20×10^6 spermatozoa$\cdot mL^{-1}$. Incubation in CM was maintained in a Heracell® 150 incubator (Heraeus Instruments GmbH, Osterode, Germany) at 38.5 ºC and 5% CO_2 for 4h, as described by Ramió-Lluch et al. (2011). Simultaneously, a semen aliquot from the same sample was also subjected to the same washing and centrifugation process. After the final wash, notwithstanding, this aliquot was again resuspended in NCM and was subsequently subjected to the same incubation process at 38.5ºC for 4h in a 5% CO_2 atmosphere. This aliquot was utilised as a negative control for the achievement of IVC and subsequent IVAE.

The induction of IVAE was carried out through incubation with progesterone, as described before (Jiménez et al., 2003; Ramió et al., 2008). With this purpose, progesterone was added to a final concentration of 10 $\mu g \cdot mL^{-1}$ to boar sperm previously incubated in either CM or NCM for 4 h at 38.5 ºC in a 5% CO_2 atmosphere. After a thorough mixing, spermatozoa were further incubated for an additional hour in the same conditions (i.e. 38.5ºC, 5% CO_2 atmosphere). Sperm aliquots of 1.5 mL each were taken at 0h, 2h and 4h of capacitation, and after 1 min, 5 min, 15 min, 30 min and 60 min of the induction of IVAE. In the determination of O_2 consumption rates, the 1-min point after IVAE induction was not available due to insurmountable technical restrictions intrinsic to the evaluation technique.

To test the effects of the lack of extracellular calcium, spermatozoa were incubated following two separate experimental designs. The first one was based up on the incubation of spermatozoa in a standard CM without $CaCl_2$ and with the chelating agent EGTA at a final concentration of 2 mM. The second design was based upon the addition of 2 mM EGTA together with that of progesterone (to a final concentration of 10 $\mu g \cdot mL^{-1}$) after 4h of incubation in the standard CM, and allowed for the evaluation of the effect of Ca^{2+} chelation on capacitated sperm subjected to IVAE.

Evaluation of the Achievement of Capacitation and Progesterone-Induced Acrosome Exocytosis Status

The evaluation of both IVC and IVAE was performed through the analysis of previously described IVC- and IVAE-linked parameters (Ramió et al., 2008; Ramió-Lluch et al., 2011). These parameters were the percentage of viable spermatozoa subjected to progesterone-induced acrosome exocytosis (true acrosome exocytosis), the mean values of motility parameters after evaluation using a computer-assisted sperm-analysis system (CASA), the determination of tyrosine phosphorylation of the p32 protein through the Western blot technique and the analysis of changes in cell-membrane lipid disorder and intracellular calcium levels, both through Fluo-3 and Rhod-5N staining. These latter analyses were performed through flow cytometry as detailed in a subsection below. Cytometry analyses were not applied in samples incubated in NCM.

The percentage of true acrosome exocytosis was analysed through the simultaneous evaluation of sperm viability and acrosome integrity using three different fluorochromes: Hoescht 33258, propidium iodide and Alexa Fluor® 488-conjugated lectin trypsin-inhibitor from soybean (SBTI) staining, as described Yeste et al. (2008a). Briefly, an aliquot of sperm suspension was incubated with a solution of 15mM Hoescht 33258 (proportion 1:1000, v/v) for 10 min at 37ºC. Afterwards, spermatozoa were incubated with propidium iodide (final concentration: 12µM) at 37ºC for 5 min. Spermatozoa were subsequently centrifuged at 600×*g* for 10 min and the supernatant was discarded. The obtained sperm pellet was resuspended in 1 mL of IVC medium without BSA, containing Alexa Fluor® 488-conjugated SBTI (final concentration: 15µM). Samples were incubated at 37ºC for 20 min and then centrifuged at 600×*g* for 12 min. The resultant supernatant was discarded, and the sperm pellet was resuspended in 1 mL of IVC medium without BSA at 37ºC. Finally, the sperm suspension was spread onto slides and fluorescence was immediately determined under a Zeiss Axioskop-40 fluorescence microscope (Karl Zeiss GmbH; Jena, Germany) with the appropriate filters. Viability and altered acrosome percentages were determined after counting three replicates (slides) of 100 spermatozoa each at 400× magnification. The corresponding means ± standard error of the mean (SEM) were calculated per sample and time-point. Unaltered acrosomes were considered to be those that showed a faint-to-moderate and uniform STBI lectin stain. Altered acrosomes showed a very faint and non-uniform stain. Sperm subjected to a true acrosome reaction were considered to be those which showed, after

the stimulation of IVAE, positive viability (blue stain of the sperm head, and non-PI labelling) and an intense and non-uniform SBTI lectin stain. Finally, non-viable spermatozoa showed an intense red stain of the head.

CASA analysis was performed by using a commercial system (Integrated Sperm Analysis System V1.0; Proiser; Valencia, Spain). Briefly, samples were previously warmed at 37ºC for 5 min in a water bath, and 15-µL aliquots of samples were then placed onto a warmed (37ºC) Neubauer chamber (Paul Marienfeld GmbH & Co. KG; Lauda-Konigshofen, Germany). Three replicates per sample and time point were evaluated, prior to calculating the corresponding mean ± SEM. Our CASA system was based upon the analysis of 25 consecutive, digitalised photographic images obtained from a single field at a magnification of 100× in a negative phase-contrast field. These 25 consecutive photographs were taken at a velocity of image-capturing of one photograph every 40 msec. Three separate fields were taken for each sample. Sperm motility descriptors obtained were those described in Yeste et al. (2008b). The analysed parameter ranges were: curvilinear velocity (VCL), the mean path velocity of the sperm head along its actual trajectory ($\mu m \cdot s^{-1}$); linear velocity (VSL), the mean path velocity of the sperm head along a straight line from its first to its last position ($\mu m \cdot s^{-1}$); mean velocity (VAP), the mean velocity of the sperm head along its average trajectory ($\mu m \cdot s^{-1}$); linearity coefficient (LIN), (VSL/VCL) × 100 (%); straightness coefficient (STR): (VSL/VAP) × 100 (%); wobble coefficient (WOB), (VAP/VCL) × 100 (%); mean amplitude of lateral head displacement (ALH), the mean value of the extreme side-to-side movement of the sperm head in each beat cycle (µm); and frequency of head displacement (BCF), the frequency with which the actual sperm trajectory crosses the average path trajectory (Hz). Total motility was defined as the percentage of spermatozoa showing a VAP above 10 $\mu m \cdot s^{-1}$. Settings applied for the CASA analysis were the following: range of area particles: 10 μm^2–80 μm^2; connectivity: a minimum of 11 images for all parameters, but a minimum of 10 images for the mean amplitude of lateral head displacement (ALH).

The analysis of tyrosine phosphorylation levels of the p32 protein (Tyr-Phos P32) was performed with 1.5-mL aliquots of boar sperm suspensions. Cells were first centrifuged at 10,000×*g* for 30 sec at 15ºC and the resultant pellet was immediately frozen at -196ºC in liquid N_2 and stored at -80ºC before use. When stated, sperm pellets were homogenised by sonication in 200 mL of ice-cold 50mM Tris-HC buffer (pH 7.4) containing 1mM EDTA, 10mM EGTA, 25mM DTT, 1.5% (w/v) Triton X-100, 1mM phenylmethyl sulfonyl fluoride

(PMSF), 1mM benzamidin,10 μg·mL^{-1} leupeptin and 1mM Na_2VO_4, this latter added to avoid changes in the overall phosphorylation of the homogenates. Samples were then centrifuged at 13,000×*g* for 15 min at 4ºC. Supernatants were recovered and total protein content of the samples was determined through the Bradford method (Bradford, 1976) by using a commercial kit (Bio-Rad Laboratories). Supernatants were immediately stored at -80ºC until use. The sperm protein samples were boiled for 1 min before being transferred to the sodium dodecyl sulfate (SDS) gel, and polyacrylamide gel electrophoresis in the presence of SDS (SDS–PAGE) was carried out following the standard protocol established by Laemmli (1970). The total amount of protein loaded in each lane was 15 μg.

Western blot analysis was carried out following the standard protocol of transferring the SDS–PAGE to nitrocellulose membranes (Burnette, 1981). Transference was tested through the staining of membranes with red Poinceau stain (Bannur et al., 1999), which also allowed for the determination that the presence of BSA in the medium did not interfere with the position of p32 (data not shown). Transferred samples were tested by applying an anti-phospho-Tyr antibody (PY-20; Chemicon International; Temecula, CA, USA) as in Medrano et al. (2006). The final dilution of the primary antibody was of 1:1000 (v/v). Immunoreactive proteins were detected by using peroxidase-conjugated anti-mouse secondary antibody (Amersham; Buckinghamshire, UK). The reaction was developed with an ECL-Plus detection system (Amersham). Previous works showing the specificity of the anti-pTyr antibody used have already been published (Ramió-Lluch et al., 2011; 2012), indicating the suitability of this specific antibody. The membranes were subsequently stripped off and subjected to a further Western blot analysis against β-tubulin. With this purpose, membranes were incubated with an anti β-3-tubulin antibody (Life Technologies; Madrid, Spain) at a final dilution of 1:1000 (v/v). Again, immunoreactive proteins were detected through a peroxidase conjugated anti-mouse secondary antibody (Amersham) and the reaction was developed with an ECL-Plus detection system (Amersham). The intensities of Tyr-Phos P32 and β-tubulin bands were quantified using specific software for image analysis of blots and arrays (Multi Gauge® v3.0; Fujifilm Europe; Düsseldorf, Germany). In all cases, the background, defined as the surrounding area of the band (width=1 mm), was utilised to adjust the intensity value of each band. Ratios between the intensity values of Tyr-Phos P32 and their corresponding β-tubulin bands were calculated using the following formula: $\frac{P-BP}{T-BT}$ where *P* was the intensity value

(in arbitrary units) obtained from the Tyr-Phos P32 band, *BP* was the intensity of the corresponding background for Tyr-Phos P32, *T* was the intensity value obtained from the β-tubulin band, and *BT* was the intensity of the corresponding background for β-tubulin. Then, values were corrected to obtain a basal value of 100 arbitrary units for the control point, which corresponded to the samples incubated in standard CM at the beginning of the experiment (0h of incubation). All the other ratio values were subsequently adjusted against this control, basal point of 100 arbitrary units.

Extracellular Calcium Levels Analysis

The extracellular calcium levels of all media were analyzed spectrophotometrically by using the Arsenazo III technique that specifically evaluates free calcium ions (Bauer, 1981; commercial kit: Calcium Arsenazo III Kit; Ref. OSR61117; Olympus, Sabadell, Spain). This detection system allowed us to differentiate the free Ca^{2+} ions from those previously chelated in the presence of EGTA or even trapped by substances such as BSA. In this form, this technique is useful to determine the chelation dynamics of calcium when EGTA is added to the medium. For this purpose, at the appropriate incubation times, 500 µL-aliquots of each analyzed point were centrifuged at 10.000×*g* and15ºC for 30 sec. Afterwards, supernatants were taken and calcium levels were immediately determined in these supernatants. To determine the trapping rhythm of EGTA when this substance is added together with progesterone to the standard CM after 4h of incubation, calcium measurements were performed after 1 min of the addition of both progesterone and EGTA following the procedure described above to a 5 mL-aliquot of CM. Other measurements were performed after 5 min and 60 min of the addition of both progesterone and EGTA, also following the procedure described above to a 5 mL-aliquot of CM. Measurements were repeated in 11 separate replicates and at each experimental point.

Oxygen Consumption Measurement

Sperm oxygen consumption was measured with a Clark oxygen electrode (Oxytherm Hansatech Instruments Ltd.; Norfolk, UK) linked to a recorder system programme (Oxygraph, Hansatech Instruments) as in Ramió-Lluch et al. (2011). Circulating water at 38.5ºC passed throughout the DW1 oxygen electrode chamber under constant stirring to ensure a homogeneous O_2 distribution. The zero point was set by adding a few grains of $Na_2S_2O_4$ to

the chamber, which contained 700 µL of distilled water. Measurements were made by mixing 100 µL of the sperm solution and 900 µL of CM (with or without calcium and EGTA, depending on the analysed point) previously warmed at 38.5ºC. The O_2 consumption was monitored for approximately 3 min. The mean sperm concentration in the DW1 chamber was 8×10^6 sperm·mL^{-1}. Data are presented as nmol O_2 consumed/10^7 viable spermatozoa. A total of three replicates per sample and time-point were evaluated, and then means ± SEM were calculated.

Analysis of Intracellular ATP Levels

To determine the intracellular content of ATP, 250-µL aliquots of the sperm suspensions were taken and immediately centrifuged at 1,000×*g* for 1 min. Supernatants were discarded and the resultant cellular pellets were immediately frozen in liquid N_2. Samples were stored at -80ºC until analysis. In all cases, a separate 10-µL aliquot was also collected for analysis of total protein content. To determine ATP concentrations, the resultant frozen pellets were homogenised by sonication in 200 µL of ice-cold 10% (v:v) $HClO_4$, carefully avoiding an accidental thawing of pellets before the addition of $HClO_4$. Resultant homogenates were then centrifuged at 10,000×*g* for 15 min at 4ºC, and the resulting supernatants were neutralised with 5M K_2CO_3 before analysis. The ATP content of all samples was determined immediately after neutralisation through the enzymatic technique described by Lambrecht & Transtschold (1998). Total protein content of the corresponding aliquots samples was determined by the Bradford method (Bradford, 1976) using a commercial kit (Bio-Rad Laboratories; Hercules, CA).

Flow Cytometry Analyses

Information concerning flow cytometry analyses is given according to the recommendations of the International Society for Advancement of Cytometry (ISAC; see Lee et al., 2008). Flow cytometry was used to evaluate sperm-membrane lipid disorder, potential of mitochondrial membrane and intracellular calcium levels in all samples and time-points. At each relevant time point, the sperm concentration in each treatment was adjusted to 1×10^6 spermatozoa·mL^{-1} in a final volume of 0.5 mL as in Yeste et al. (2013). Spermatozoa were then stained with the appropriate combinations of fluorochromes, following the protocols described below.

Samples were evaluated through a Cell Laboratory QuantaSC™ cytometer (Beckman Coulter; Fullerton, California, USA), after excitation through an argon ion laser (488 nm) set at a power of 22 mW. Cell diameter/volume was directly measured with a Cell Lab Quanta™ SC cytometer employing the Coulter principle for volume assessment. This system has forward scatter (FS) replaced by electronic volume (EV). The EV channel was periodically calibrated using 10-µm Flow-Check fluorospheres (Beckman Coulter) by positioning this size of the bead at channel 200 on the volume scale. A total of three different optical filters were used with the following characteristics: FL1 (green fluorescence): Dichroic/Splitter, DRLP: 550 nm, BP filter: 525 nm, detection width 505-545 nm; FL2 (orange fluorescence): DRLP: 600 nm, BP filter: 575 nm, detection width: 560-590 nm); FL3 (red fluorescence) LP filter: 670 nm, detection width: 655-685 nm. Signals were logarithmically amplified and photomultiplier settings were adjusted to particular staining methods. FL-1 was used to detect green fluorescence (YO-PRO-1, Fluo-3, JC-1 monomers), FL2 was utilised to detect JC-1 aggregates and FL3 was used to detect red fluorescence (Merocyanine-540, Rhod-5N).

Sheath flow-rate was set at 4.17 $\mu l \cdot min^{-1}$ in all analyses, and EV and side-scatter (SS) were recorded in a linear mode (in EE *vs.* SS dot plots) for a minimum of 10,000 events per replicate. The analyser threshold was adjusted in the EV channel to exclude subcellular debris (particle diameter<7µm) and cell aggregates (particle diameter>2µm). Therefore, the sperm-specific events were positively gated on the basis of EV and SS distributions, while the others were gated out. In some protocols, compensation was used to minimise spill-over of green fluorescence into the red channel, as described below. Dot-plots (FL1 *vs.* FL3; FL2 *vs.* FL3) were analysed through Cell Lab Quanta®SC MPL Analysis Software (version 1.0; Beckman Coulter). In addition, data obtained from flow-cytometry experiments were corrected according to the procedure described by Petrunkina et al. (2010), as stated at the end of this section. Each assessment per sample and parameter was repeated three times in independent tubes, prior to calculating the corresponding mean ± SEM.

Analysis of membrane lipid changes (M-540/YO-PRO-1)

Membrane lipid changes were assessed using the co-staining protocol for Merocyanine-540 (M-540) and YO-PRO-1 described in Harrison et al. (1996). Spermatozoa stained with M-540 ($M\text{-}540^{+}$) were those that presented an increased membrane lipid disorder, and that is why these cells are described here as having a high membrane lipid disorder. Concomitantly, the

YO-PRO-1 stain indicated early changes in sperm membrane permeability. Those cells that were only positive for the YO-PRO-1 stain (YO-PRO-1^{+}/M540^{-}) were those that showed degenerative changes not directly associated with IVC achievement. For this reason, we only show percentages of sperm cells which are positive for the M-540 stain and negative for the YO-PRO-1 stain (M-540^{+}/YO-PRO-1^{-}), as these are the cells subjected to membrane changes compatible with the achievement of a proper IVC. Sperm samples were incubated at 38ºC in the dark for 10 min with M-540 and YO-PRO-1 at final concentrations of 2.6µM and 25nM, respectively. The fluorescence of M-540 was detected through FL-3, while that of YO-PRO-1 was detected through FL-1. Data were not compensated.

Analysis of Mitochondrial Membrane Potential

The potential of mitochondrial membrane (MMP) was determined following the protocol described by Garner & Johnson (1995). Briefly, samples were incubated with JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'tetraethylbenzimidazolylcarbocyanine iodide (final concentration: 0.3µM) at 38ºC for 30 min in the dark. Two different emission filters (FL-1 and FL-2) were used to differentiate two sperm populations: a) spermatozoa with high MMP (JC-1 aggregates), and b) spermatozoa with low MMP (JC-1 monomers; Gillan et al., 2005). The percentage of spermatozoa with high MMP corresponded to the orange-stained spermatozoa, which appeared in the upper half of the diagram in FL1 *vs*. FL2 dot-plots. Data were not compensated.

Analysis of Intracellular Calcium

As described above, intracellular calcium of spermatozoa was determined by using two separate, specific stains, Fluo-3 and Rhod-5N. While Fluo-3 staining was performed following the protocol described by Harrison et al. (1993) and modified by Kardivel et al. (2009), Rhod-5N labelling was performed for the first time in mammalian spermatozoa and was thus required to be adapted from somatic cells (Hayato et al., 2011; De la Fuente et al., 2012)

A total of three different analyses were performed. In the first case, intracellular calcium levels in boar sperm were stained with Fluo-3, and sperm membrane integrity was evaluated with PI. Sperm samples were incubated for 10 min at 38ºC with Fluo-3-AM (final concentration: 1µM) and PI (final concentration: 12µM). The FL-1 detector was used for

collecting the fluorescence of Fluo-3 and the FL-3 detector was utilised to determine the PI-fluorescence. A total of four sperm populations were identified: i. viable spermatozoa with low levels of intracellular calcium (Fluo-3^-/PI^-); ii. viable spermatozoa with high levels of intracellular calcium (Fluo-3^+/PI^-); iii. non-viable spermatozoa with low levels of intracellular calcium (Fluo-3^-/PI^+), and iv. non-viable spermatozoa with high levels of intracellular calcium (Fluo-3^+/PI^+). Unstained and single-stained samples were used for setting the EV-gain, FL-1 and FL-3 PMT voltages and for compensating Fluo-3 spill over into the FL3-channel (2.45%) and PI spill-over into the FL1-channel (28.72%).

In the second case, samples were stained with Rhod-5N for evaluating intracellular calcium levels, and YO-PRO-1 was used to evaluate sperm membrane integrity. With this purpose, boar spermatozoa were stained with Rhod-5N-AM (final concentration: 5µM) and YO-PRO-1 (final concentration: 25nM) and incubated at 38ºC for 10 min. The FL-3 detector was used for Rhod-5N fluorescence, whereas that of YO-PRO-1 was collected through the FL-1 detector. Again, a total of four sperm populations were identified: i. viable spermatozoa with low levels of intracellular calcium (Rhod-$5N^-$/YO-$PRO\text{-}1^-$); ii. viable spermatozoa with high levels of intracellular calcium (Rhod-$5N^+$/YO-$PRO\text{-}1^-$); iii. non-viable spermatozoa with low levels of intracellular calcium (Rhod-$5N^-$/YO-$PRO\text{-}1^+$), and iv. non-viable spermatozoa with high levels of intracellular calcium (Rhod-$5N^+$/YO-$PRO\text{-}1^+$). Fluorescence from Rhod-5N was compensated for through the FL1-channel (3.16%).

Finally, intracellular calcium levels in boar spermatozoa were evaluated simultaneously with Fluo-3-AM and Rhod-5N-AM. Final concentrations were 1µM and 5µM, respectively, and staining was performed at 38ºC for 10 min. Whereas the FL-1 detector collected the Fluo-3-fluorescence, FL-3 collected that of Rhod-5N. Four sperm populations were identified as follows: i. Fluo-3^-/Rhod-$5N^-$; ii. Fluo-3^+/Rhod-$5N^-$; iii. Fluo-3^-/Rhod-$5N^+$, and iv. Fluo-3^+/Rhod-$5N^+$. Geometric means of Fluo-3 and Rhod-5N were also recorded. Compensation consisted of Fluo-3 spill-over into the FL3-channel (2.45%) and Rhod-5N spill-over into the FL1-channel (3.16%).

Correction of Cytometric Data: Identification of non-DNA-Containing Particles

Data from all cytometric assessments were corrected following the protocol described by Petrunkina et al. (2010). This procedure determines the percentage of non-DNA-containing particles (alien particles) and avoids an overestimation of sperm particles. Briefly, 5 µL of

each analysed sperm aliquot were diluted with 895 µL of milliQ®-distilled water. Samples were then stained with PI to a final concentration of 12µM and incubated at 38ºC for 3 min. Percentages of alien particles (f) were used to correct the percentages of non-stained spermatozoa (q_1) in each sample in a dual-staining analysis according to the following formula: $q_1' = \frac{q_1 - f}{100 - f} \times 100$, where q_1' is the percentage of non-stained spermatozoa after correction.

Detection of both Fluo-3 and Rhod-5N stains through confocal microscope analysis

Spermatozoa stained with Fluo-3 and Rhod-5N was also evaluated by laser confocal microscopy. The evaluation was performed at the same time-points as for flow cytometry assessments and sperm were stained following the same conditions (final concentrations, Fluo-3-AM: 1µM, Rhod-5N-AM: 5µM; incubation conditions: 38ºC for 10 min). Samples were visualised under a Leica TCS 4D confocal scanning microscope (Leica Lasertechnik; Vertrieb, Germany) adapted to an inverted Leitz DMIRBE microscope and a 63× (NA=1.4 oil) Leitz Plan-Apo lens (Leitz; Stuttgart, Germany). The light source was an argon/krypton laser (74 mW), and fluorescence detection was performed using excitation wavelengths of 506 nm (Fluo-3) and 554 nm (Rhod-5N). A series of confocal slices of images (image thickness of 0.5µm) was integrated to get three-dimensional images that were recorded in TIFF-format. Since confocal images were only intended for the determination of signal location in sperm, they were not subjected to any intensity-analysis processing. Thus, quantification of the calcium intensity signal was only determined through cytometry.

Statistical analyses

Statistical analyses were performed using a statistical package (IBM SPSS for Windows version 21.0; SPSS Inc.; Chicago, Illinois, USA), and data from all assessments are presented as the mean ± standard error of the mean (SEM). Data were first tested for normality and variance homogeneity through Shapiro-Wilk and Levene tests, respectively. When required, data (x) were transformed using the arcsine square root (arcsin √x) before a general mixed model (i.e. with repeated measures) was run. In this model, the intersubject factor was the treatment (i.e. composition of capacitation media) and the intrasubject factor was the incubation time (i.e. 0h, 2h, 4h, 4h 1 min, 4h 5 min, 4h 15 min, 4h 30 min and 4h 60 min). In

all cases, each sperm functional parameter was the dependent variable, and multiple post-hoc comparisons were calculated using Sidak's test.

When no transformation remedied the normality, non-parametric procedures were used with raw data. Friedman's test and the Wilcoxon matched-pairs test were performed as non-parametric alternatives to repeated measures ANOVA. In all statistical analyses, the minimal level of significance was set at $P<0.05$.

Results

Evaluation of extracellular calcium levels in CM and NCM

The extracellular Ca^{2+} levels of boar sperm incubated in both standard CM and NCM after 0h of incubation were 2.55 mM±0.22 mM and 2.57 mM±0.21 mM, respectively (mean ± SEM for 11 separate experiments). There were no significant differences in this value in boar sperm incubated in NCM in any of the tested points, both before and after the addition of progesterone (Table 1). Notwithstanding, there was a significant ($P<0.05$) decrease in free extracellular calcium ions in cells incubated in CM, these extracellular Ca^{2+} levels dropping to 1.39 mM±0.09 mM after 4h of incubation (Table 1). This decrease was maintained after the addition of progesterone, showing values of 1.32 mM±0.13 mM after 1h of the hormone addition (Table 1). Extracellular Ca^{2+} was not detectable in the samples incubated in medium without calcium and added with EGTA at the start of the incubation period (Table 1). This lack of detection was observed during all of the tested incubation period, both before and after the addition of progesterone. Finally, the addition of both progesterone and Ca^{2+} to sperm incubated in CM for 4h induced a fast and very intense decrease in extracellular Ca^{2+} levels, which reached values of 0.32 mM±0.1 mM after 1 min. Five min after the addition of progesterone and EGTA, and up to the end of experimental period, extracellular calcium levels were undetectable (Table 1).

Effects of EGTA on Sperm Viability and True Acrosome Exocytosis

Incubation of boar spermatozoa in standard CM induced a progressive decrease in the percentage of viable spermatozoa, which dropped from 86.0%±2.2% at 0h to 62.9%±4.5% after 4h of incubation (means±SEM; Table 2). The subsequent addition of progesterone was followed by a further decrease in viability, which reached values of 54.6%±4.5% at the end of the experiment (i.e. 60 min after progesterone addition, Table 2). A more intense drop in viability was observed in cells incubated in NCM throughout all of the determined incubation time (Table 2). The incubation of sperm cells in a modified CM that did not contain calcium but did contain EGTA, or in standard CM in which EGTA was added together with progesterone at 4h, did not significantly modify sperm viability in any of the tested points when compared with cells incubated in standard CM (Table 2).

As expected, the addition of progesterone to cells incubated for 4 h in standard CM induced a progressive increase in the percentage of acrosome exocytosis, which reached maximal

values after 60 min of progesterone addition (Table 2). This increase was not observed in cells incubated in NCM (Table 2). Remarkably, the incubation in a calcium-depleted medium prevented this increase (Table 2). A similar lack of acrosome exocytosis stimulation was observed when EGTA was added together with progesterone after 4h of incubation in standard CM (Table 2).

Effects of EGTA on Sperm Motility

A progressive decrease in total sperm motility was observed when boar spermatozoa were incubated in standard CM for 4h. Indeed, this parameter went from about 85% at the beginning of the experiment to about 70% after 4h of incubation. Subsequent progesterone addition was related to a more intense decrease of this parameter, which reached values of about 55% after 60 min of progesterone addition (Fig. 1). As expected, the time-dependent drop of motility was more intense in cells incubated in NCM during the entire experiment (Fig. 1).

In contrast, incubation of spermatozoa in a modified CM medium that did not contain calcium, but rather EGTA, had no effect on total sperm motility after 4h of incubation. Additionally, the aforementioned decrease in total sperm motility after progesterone addition was significantly less intense ($P<0.05$) when spermatozoa were incubated with a calcium-depleted medium (modified CM medium) and when EGTA was added together with progesterone to standard CM medium at 4h than in control treatment (Fig. 1).

Again, as expected, the incubation in standard CM medium for 4 h induced a progressive increase in most of the sperm kinetic parameters: VCL, VSL, VAP, LIN, STR and WOB (Table 3). These data indicated that, as previously published, under our experimental conditions boar spermatozoa presented a typical motility pattern of capacitated boar spermatozoa (See García-Herreros et al. (2000)). Subsequent progesterone addition had little effect on these kinetic parameters, although an overall, slight decrease was observed after 60 min of incubation (Table 3 and Supplementary Table 1). In contrast, incubation in NCM induced a constant and intense drop of VCL that was accompanied by more gradual, and less intense, time-linked parallel decreases of VSL, VAP, STR and ALH (Table 3 and Supplementary Table 1). These decreases were accompanied, in cells incubated in NCM, by a progressive, time-linked increase in both LIN and WOB (Table 3).

Surprisingly, the incubation of sperm cells in the modified CM that did not contain calcium, but rather had EGTA, induced a significant (P<0.05) increase in almost all of the analysed motion parameters. This increase differed at different incubation times and depended on each sperm parameter. Indeed, while a lack of calcium induced an immediate increase in VCL and STR, VSL was only significantly (P<0.05) increased after 2h of incubation, and VAP, LIN and BCF were significantly (P<0.05) augmented after 4h of incubation (Table 3).

The addition of EGTA together with that of progesterone after incubating sperm with standard CM for 4h had a different result. In this case, the addition of progesterone and EGTA at that time-point induced an immediate, rapid decrease in VSL, STR and WOB, whereas a significant (P<0.05) decrease in VAP was seen after 60 min of incubation, and a transient increase of BCF was observed after 30 min of progesterone addition (Table 3). Furthermore, the observed decrease in WOB was only transient, and it disappeared 5 min after the addition of progesterone and EGTA (Table 3).

Effects of EGTA on Tyrosine Phosphorylation levels of the p32 Protein

As expected, incubation in CM induced a time-dependent, progressive increase in tyrosine phosphorylation levels of the p32 protein, reaching maximal values after 4h of incubation (Fig. 2). Subsequent progesterone addition did not seem to significantly modify this intensity. On the contrary, samples subjected to incubation in NCM did not show any significant increase in p32 tyrosine phosphorylation after 4h of incubation. Meanwhile, sperm incubated in the absence of extracellular calcium showed a very faint, or even total, absence of p32 tyrosine phosphorylation during all of the tested incubation times (Fig. 2). Finally, the addition of both progesterone and EGTA together to sperm incubated during 4h in standard CM did not significantly affect p32 phospho-tyrosine levels when compared with cells in which only progesterone was added (Fig. 2).

Effects of EGTA on Sperm Membrane Fluidity

Sperm cells incubated in standard CM showed a steady increase in membrane fluidity, as the YO-PRO-1/M-540 test indicated. Indeed, the percentage of viable spermatozoa with high membrane fluidity (YO-PRO-1$^-$/M-540$^+$) increased from about 6% at 0h to about 38% after 4h of incubation (Fig. 3). The subsequent progesterone addition did not immediately modify this tendency, although there was a progressive decrease in the percentage of YO-PRO-1$^-$/M-

540^+ cells, reaching values of about 25% after 1h of the progesterone addition (Fig. 3). Incubation of spermatozoa in the modified CM (i.e. without calcium but with EGTA) had a marked effect in the percentage of YO-PRO-1^-/M-540^+ sperm. As shown in Fig. 3, the absence of Ca^{2+} significantly lowered (P<0.05) this percentage, reaching values of about only 13% after 4h of incubation. This effect was maintained after the addition of progesterone (Fig. 3). In contrast, the simultaneous addition of EGTA and progesterone to sperm cells incubated for 4h in standard CM did not have any significant effect on M-540-determined membrane fluidity when compared with control cells (Fig. 3).

Effects of EGTA on O_2 Consumption and Intracellular ATP Levels

At the beginning of the experiment, boar spermatozoa showed a low O_2 consumption when incubated in standard CM (1.8±0.4 nmol/10^7 viable cells; see Fig. 4A). This value did not significantly change over the first 4h of incubation. The addition of progesterone to standard CM induced a rapid, intense, and transient peak in O_2 consumption, which reached values of 3.3±0.7 nmol/10^7 viable cells after 5 min of hormone addition (Fig. 4A). Afterwards, O_2 consumption decreased, reaching minimal values 60 min after the progesterone addition (Fig. 4A).

Incubation of spermatozoa in a Ca^{2+}-depleted medium did not induce any significant change in the rhythm of O_2 consumption after 4h of incubation (Fig. 4A). However, when progesterone was added, no peak in O_2 consumption was observed (Fig. 4A). Similarly, the induced O_2 consumption peak in cells incubated in standard CM for 4h, in which EGTA together with progesterone were added, was significantly (P<0.05) lower than that observed in control cells (2.4±0.5 nmol/10^7 viable cells after 5 min of progesterone+EGTA addition *vs.* 3.3±0.7 nmol/10^7 viable cells in control samples; see Fig. 4A).

The dynamics of ATP intracellular levels followed a similar pattern to that observed in the rhythm of O_2 consumption. Thus, as shown in Fig. 4B, boar spermatozoa did not significantly modify ATP levels after 4h of incubation in standard CM, which went from 9.1±1.8 nmol/mg protein at 0 h to 6.5±1.3 nmol/mg protein at 4h. The addition of progesterone after 4h of incubation in standard CM induced a rapid, significant (P<0.05) and intense increase in ATP levels, which reached values of 17.6±2.9 nmol/mg protein after 1 min of progesterone addition (Fig. 4B). Subsequently, ATP levels were progressively decreased, and values of 5.3±0.4 nmol/mg protein were reached after 60 min of progesterone addition (Fig. 4B). The

incubation in modified CM without Ca^{2+}, but with EGTA (Ca^{2+}-depleted medium), did not modify intracellular ATP levels over the first 4h. However, the progesterone-induced ATP increase was not detected in those spermatozoa incubated in this Ca^{2+}-depleted medium (Fig. 4B). Furthermore, while the addition of progesterone together with EGTA after 4h of incubation in CM did not significantly ($P>0.05$) decrease the progesterone-induced ATP increase after 1 min of the addition of the effector, ATP values after 5 min of progesterone+EGTA addition were significantly lower ($P<0.05$) than those observed in control cells (Fig. 4B).

Effects of EGTA on mitochondrial membrane potential

Incubation of boar sperm with standard CM significantly ($P<0.05$) increased the percentage of spermatozoa with high mitochondrial membrane potential (MMP) following the JC-1 staining method (from 46.1%±3.2% at 0h to 60.9%±3.7% at 4h of incubation, see Fig. 5). The addition of progesterone to standard CM after 4h of incubation induced a very rapid increase in this percentage, which reached values of 97.3%±5.7% after 1 min of progesterone addition (Fig. 5). Subsequently, there was a rapid decrease in the percentage of sperm with high MMP that was maintained until the end of the experimental period.

Sperm incubation in modified CM that did not contain calcium, but had EGTA, prevented the aforementioned increase in the percentage of spermatozoa with high MMP observed in standard CM (Fig. 5). Notwithstanding, the addition of progesterone to a calcium-depleted medium after 4h of incubation also augmented the percentage of spermatozoa with high MMP. However, the extent of that increase was significantly ($P<0.05$) lower than that observed in the control medium (74.5%±6.1% in calcium-depleted medium *vs.* 97.3%±5.7% in standard CM; see Fig. 5). Afterwards, the percentage of spermatozoa with high MMP rapidly decreased, reaching similar values to those observed before the progesterone addition.

When progesterone was added together with EGTA to standard CM after 4h, a significant ($P<0.05$) increase in the percentage of spermatozoa with high MMP was found but, again, the extent of this increase was significantly ($P<0.05$) lower than that of control cells after 30 min and 60 min of progesterone addition (Fig. 5).

Effects of EGTA on Fluo-3-marked intracellular Ca^{2+}

The percentage of viable spermatozoa with a positive Fluo-3 (Fluo-3^+) signal increased progressively when spermatozoa were incubated in standard CM. Indeed, as shown in Fig. 6A, this percentage went from 30.5%±2.0% at 0h to 53.7%±2.9% after 4h of incubation. The addition of progesterone induced a rapid, significant increase in this percentage, reaching maximal values (67.2%±3.7%) after 1 min of progesterone addition. This peak was followed by a quick drop in the percentage of Fluo-3^+-spermatozoa (42.5%±3.7% after 5 min of progesterone addition). This percentage was subsequently progressively increased up to 56.4%±3.9% after 60 min of progesterone addition (Fig. 6A).

Spermatozoa incubated in a calcium-depleted medium (i.e. without Ca^{2+} and with EGTA) showed significantly ($P<0.05$) lower percentages of viable spermatozoa with a positive Fluo-3 signal. This percentage also increased progressively during a 4h-incubation, but not to the same extent as with control cells (40.6%±3.1% at 4h). It is also worth noting that no peak in Fluo-3^+-spermatozoa was observed after 1 min of progesterone addition when cells were incubated in a calcium-depleted medium (Fig. 6A). In spite of this, the percentage of viable spermatozoa with a positive Fluo-3 signal decreased, reaching values of 25.6%±2.4% and 31.7%±2.3% after 5 min and 60 min of progesterone addition, respectively (Fig. 6A). The addition of EGTA together with that of progesterone after 4h of incubation in standard CM also prevented the significant increase in the percentage of Fluo-3^+-sperm, a similar dynamics to that observed in a calcium-depleted medium after 5 min of progesterone addition (Fig. 6A).

With regard to the geometric mean of Fluo-3^+ intensity, the pattern was similar to that described for percentages of Fluo-3^+-spermatozoa. Thus, sperm cells incubated in standard CM showed a time-dependent increase of this parameter, reaching values of 54.8 ±5.3 arbitrary units after 4h of incubation (Fig. 6B). Again, progesterone induced a rapid peak, reaching maximal values after 1 min that were followed by a quick decrease of Fluo-3^+ fluorescence intensity, which was minimal (31.6±5.3 arbitrary units) after 5 min of progesterone addition. After this time-point, these values partially recovered, reaching levels of 56.8±5.7 arbitrary units after 60 min of progesterone addition (Fig. 6B).

Sperm incubation both in a modified CM (with EGTA and without calcium) and in a standard CM with added progesterone and EGTA prevented the progesterone-induced peak in Fluo-3^+ fluorescence intensity after 1 min of incubation. The subsequent depletion-recovering sequence was similar to that observed for control cells, but the values of mean fluorescence intensity were significantly ($P<0.05$) lower (Fig. 6B).

As far as the confocal microscope analysis is concerned, Fluo-3^+-staining was mainly found at the midpiece. In addition, a less intense marking was also found in the sperm head at the start of incubation with standard CM (Fig. 7). No changes in Fluo-3^+ localisation were observed. Although the confocal signal was not intended for quantification, it is worth mentioning that immediately after progesterone addition (4h 1 min), there was a noticeable increase in the intensity of Fluo-3 marking at the sperm head (Fig. 7). The Fluo-3 signal of sperm incubated in a modified medium without Ca^{2+} but containing EGTA was very low or even practically absent in most of the sperm throughout all of the incubation period. Strikingly, the addition of progesterone to sperm incubated for 4h in modified CM without Ca^{2+} and with added EGTA was concomitant with the appearance of a slight but evident Fluo-3 signal at the midpiece in a great percentage of sperm. This signal was maintained until the end of the incubation time (Fig. 7 and data not shown). Finally, the addition of progesterone together with EGTA to standard CM did not induce the increase in the head signal detected in sperm incubated in standard CM (Fig. 7).

Effects of EGTA on Rhod-5N-marked intracellular Ca^{2+}

In a similar manner to that observed for the Fluo-3 signal, the percentage of viable cells with a positive Rhod-5N signal progressively increased when sperm was incubated in standard CM. Indeed, as shown in Fig. 8A, this percentage increased from 5.7%±2.5% at 0h to 23.4%±3.1% after 4h of incubation. The addition of progesterone induced a rapid increase of this percentage, reaching values of 36.9%±3.8% after 1 min of progesterone addition. However, this peak was followed by a fast drop in the percentage of Rhod-$5N^+$ cells, which was 17.4%±3.1% after 5 min of progesterone addition. Subsequently, this proportion progressively increased, reaching final values of 23.5%±2.7% 60 min after of progesterone addition (Fig. 8A).

In the case of calcium-depleted medium (i.e. without Ca^{2+} but with EGTA), there was a similar percentage of Rhod-$5N^+$-spermatozoa to that observed in standard CM at 0h of incubation. However, the lack of extracellular Ca^{2+} partially prevented the increase in the percentage of Rhod-$5N^+$-spermatozoa observed in standard CM (Fig. 8A), and only 10.4%±2.9% of spermatozoa presented a positive Rhod-5N signal after 4h of incubation (Fig. 7A). Again, and similarly to that observed for Fluo-3 staining, no significant increase in the percentage of Rhod-$5N^+$-spermatozoa was observed after progesterone addition in this calcium-depleted medium (Fig. 8A). This percentage did not change significantly until the

end of the incubation period, reaching values of 13.8±3.3% 60 min after of progesterone addition (Fig. 8A).

When EGTA was added together with progesterone to standard CM, the expected progesterone-induced increase in the percentage of Rhod-5N$^+$-spermatozoa was partially inhibited although it followed a similar dynamics to that observed in calcium-depleted cells (Fig. 8A).

With regard to Rhod-5N$^+$-fluorescence intensity (geometric mean, arbitrary units), the pattern was similar to that observed for Fluo-3 marking. Thus, cells incubated in a standard CM showed a time-dependent increase in this parameter, reaching values of 275.7±9.2 arbitrary units after 4h of incubation (Fig. 8B). Again, progesterone addition induced a rapid peak of Rhod-5N$^+$ fluorescence intensity, its geometric mean reaching maximal values after 1 min. This was followed by a rapid decrease, and minimal values of 236.7±9.3 arbitrary units were observed after 5 min of progesterone addition. These values partially recovered thereafter, reaching final levels of 264.5±9.1 arbitrary units after 60 min of progesterone addition (Fig. 8B).

Sperm incubation in a modified medium that did not contain Ca^{2+} but had EGTA prevented the appearance of a significant peak in Rhod-5N$^+$ after progesterone addition, and no variations were seen throughout the incubation period (Fig. 8B). On the contrary, the addition of EGTA together with that of progesterone to standard CM did not prevent the progesterone-induced peak observed in control cells (Fig. 8B). However, the subsequent recovering dynamics in the mean intensity values of Rhod-5N$^+$ was significantly (P<0.05) lower than that observed in control cells, reaching values of 237.3±8.9 arbitrary units after 60 min of progesterone+EGTA addition (Fig. 8B).

When evaluated through confocal a microscope, the Rhod-5N$^+$ signal was mainly detected in both acrosomal and post-acrosomal regions of boar sperm at the start of the incubation in CM (Fig. 9). This localisation remained unchanged throughout all of the incubation time in CM (Fig. 9). Progesterone addition to standard CM did not modify the Rhod-5N$^+$ signal location (Fig. 9). The Rhod-5N$^+$ signal in sperm incubated in a modified CM without Ca^{2+} and with EGTA was practically absent in most spermatozoa during all of the experimental period (Fig. 9). The addition of EGTA together with that of progesterone after 4h of incubation in standard CM seemed to be accompanied by a slight decrease in Rhod-5N$^+$-intensity, without modifying the signal location (Fig. 9).

Discussion

Our results indicate that intracellular calcium in boar spermatozoa both before and after the achievement of feasible IVC and subsequent, progesterone-induced IVAE is distributed in separate deposits with specific physico-chemical characteristics. Apart from agreeing with Costello et al. (2009), our data suggest, for the first time, that these separate calcium deposits are in a dynamic equilibrium amongst each other. In addition, this dynamic equilibrium seems to be greatly dependent upon extracellular calcium levels. Regarding IVC, it must be stressed that in our experimental conditions capacitation status was achieved in a medium without bicarbonate. This is important since bicarbonate is a well-known and potent capacitation inductor in practically all mammalian species, including boar (Boatman & Robins, 1991). However, previous work from our laboratory has demonstrated that IVC can also be achieved in boar sperm in a medium without bicarbonate, including only BSA as a capacitating factor (Ramió et al., 2008; Ramió-Lluch et al., 2011; 2014]. These results have also been proved in this study, in which the attainment of a feasible IVC in CM without bicarbonate is demonstrated by the determination of viability, motility, p32 tyrosine phosphorylation, cytometry-analysed parameters such as membrane changes and, above all, the positive attainment of IVAE after progesterone induction. In fact, the feasible induction of IVAE by progesterone is the most positive proof indicating the attainment of previous IVC. This is especially evident when comparing the obtained results with those acquired in cells incubated in NCM, in which IVAE was not attained after progesterone induction, as indicated by the p32 tyrosine phosphorylation and the values of true acrosome exocytosis. Thus, our results indicate that although bicarbonate is a potent capacitating factor of boar spermatozoa, it is not an absolute requirement to attain IVC in this species, in which BSA can act as the sole capacitating factor in our conditions. This is different to that observed in the majority of the studied species, such as bovine, mice and human, in which bicarbonate is absolutely needed to attain IVC (Visconti et al., 1995; Smaili and Russell, 1999; Battisone et al., 2013). In fact, this is not the only difference in the attainment of IVC among species. In this way, mice sperm shows a much more rapid instauration of the progesterone-induced IVAE than that observed in boar cells, both in the presence and the absence of bicarbonate (Boatman & Robins, 1991; Ramió et al., 2008; Ramió-Lluch et al., 2011; 2014). Additionally, there are other species such as horse in which, although bicarbonate has been considered as a vital factor to achieve IVC (Rathi et al., 2001), the fact is that bicarbonate alone is not enough by itself to induce the capacitated status, since the presence of other

substrates such as procaine are required to achieve this point (McPartlin et al., 2009). All of these data clearly indicate that despite the fact that the general molecular mechanisms underlying the attainment of IVC would be common among species, the specific importance of these mechanisms would change depending on the species. In this manner, our results suggest that, in boar sperm, BSA would be enough to initiate the whole succession of events that ultimately render a feasible IVC and the bicarbonate-launched events can be mimicked through other ways in this species. Finally, as a corollary, the observed effects of the lack of extracellular calcium in the attainment of IVC and subsequent IVAE would be caused by affecting this BSA-started cascade of events.

In centring on calcium, the results of this study have revealed several calcium deposits, and they allow us to address some issues. First of all, the progesterone-induced rapid calcium peak observed after the induction of IVAE implies the accumulation of calcium from the extracellular environment in both Fluo-3- and the Rhod-5N-marked Ca^{2+} deposits. Indeed, the Ca^{2+} influx observed immediately after IVAE induction is present in both the sperm head and midpiece, as staining of Fluo-3 and Rhod-5N deposits shows. Related to this, calcium influx to the sperm head is known to play a crucial role while triggering the acrosome exocytosis (See Breitbart (2002) for a review). However, no previous study showed the IVAE-linked influx of extracellular Ca^{2+} into the boar-sperm midpiece. Although the exact role of this calcium influx to the midpiece is yet to be reported, we can suggest two hypotheses. The first one is that this Ca^{2+} entry into the midpiece may be involved in the mitochondrial metabolism change that occurs during IVAE launching. Our results showed that the progesterone induction of IVAE resulted in a higher proportion of spermatozoa with high mitochondrial membrane potential (MMP), concomitant with a sudden increase in both the rhythm of O_2 consumption and the intracellular ATP levels, which is consistent with previous reports (Ramió-Lluch et al., 2011; 2014). Moreover, the observed failure in the progesterone-induction of IVAE in the medium without Ca^{2+} was concomitant with a total inhibition in the IVAE-linked increase of O_2 consumption and ATP levels. These results would agree with those of De Marchi et al. (2014), who have determined a strong regulatory action of extracellular calcium on oxidative metabolism and ATP synthase-dependent respiration in mitochondria from pancreatic β-cells. In contrast, the decrease in the percentage of high-MMP cells was less apparent. This contradiction between O_2 consumption rhythm, ATP levels and MMP could be explained from the complex regulation of MMP, which results from a myriad of processes that are not exclusively related to Krebs cycle

and electronic chain-chemiosmosis. Indeed, MMP can also be modulated by other factors, such as changes in the rhythm of ion exchanges and antiporters across mitochondrial membranes, modification of intra- and extra-mitochondrial pH, or variations in the permeability of mitochondrial transition pores (Baysal et al., 1991; Smaili & Russell, 1999; Vander Heiden et al., 2000; Santo-Domingo & Demaurex, 2012). Additionally, the majority, if not all, of these MMP-modulatory factors can also be modified by changes in intramitochondrial Ca^{2+} levels (Smaili & Russell, 1999; Odagiri et al., 2009). This indicates that while modifications in the activity of oxidative phosphorylation induced by changes in the concentration of modulators, such as Ca^{2+} and bicarbonate, ultimately affect the MMP, this MMP could be affected, in our conditions, by factors other than these modulators. This would occur regardless of the importance of bicarbonate, which would act through direct modulation of the electronic chain activity (Acín-Pérez et al., 2009; Mizrahi & Breitbart, 2014). The close relationship between mitochondrial calcium and the control of mitochondrial energy production is a well-known phenomenon. In eukaryotic cells, intra-mitochondrial calcium plays a prominent activating role in the regulation of ATP production through a modulating oxidative phosphorylation rhythm (Gunter et al, 2011). In this way, the observed calcium peak in the sperm midpiece, revealed by the Fluo-3^{+}-signal immediately after IVAE, would induce a parallel augmentation in the rhythm of oxidative phosphorylation, which would, in turn, be reflected by a direct increase in O_2 consumption, ATP levels and percentage of high-MMP sperm.

The second hypothesis, and in a similar way to that described for the relationship between the midpiece calcium deposit and mitochondrial activity, changes in the midpiece calcium content could also be related to the observed modification in motility patterns. A relationship between the electronic chain activity, the corresponding mitochondrial calcium content and sperm motility has already been reported in human, dog, mouse and bull spermatozoa (Krzyzosiak et al., 1999; Mukai et al., 2004; Nascimento et al., 2008; Mizrahi & Breitbart, 2014). However, the relationship between the midpiece calcium content and sperm motility seems to not be solely based on the modulatory calcium role on mitochondrial ATP production. Indeed, we expected that incubating the spermatozoa with a modified CM without calcium would have led to a drop of sperm motility, or even to complete sperm immobilisation, since Ca^{2+} ions are known to be crucial in the maintenance of the proper flagellum contractibility (Rathi et al., 2001; Lesich et al., 2012). In contrast, we observed that not only did the lack of extracellular calcium not reduce the percentage of motile spermatozoa, but rather some motion parameters, such as VCL and linearity

and straightness increased, thereby indicating that sperm was more rapid and with a more linear trajectory. These results were accompanied by non-significant changes in the percentages of viability and altered acrosomes, thus indicating that these motility changes were not associated with other sperm alterations. These results were more evident over the first 4h of incubation. During this time, both the intracellular calcium content in the midpiece and the percentage of spermatozoa with high MMP were lower when incubation was performed in a medium without calcium, despite both the rhythm of O_2 consumption and the intracellular ATP levels remaining unaffected. Thus, while the increase in both linearity and straightness appeared together with a decrease in the electronic chain activity and the midpiece calcium content, it seemed to not be associated with modifications in the rhythm of the Krebs cycle and overall ATP synthesis. The effects of lack of extracellular calcium on sperm motility could be explained by its role in the activity of calcium-dependent kinases such as protein kinase C, which is a well-known modulator of sperm motility (Naor & Breitbart, 2004). In fact, our flow cytometry data suggest that the lack of extracellular calcium does not induce a complete depletion of midpiece Ca^{2+}, since levels of Fluo-3-marked calcium were measurable. In addition, confocal results on Fluo-3-staining after IVAE induction indicated a signal of Fluo-3-marked calcium only after the addition of progesterone in a medium without calcium. This post-IVAE induction marking might be due to a re-distribution of intracellular calcium from deposits that were not detectable by confocal microscopy in our conditions before the addition of progesterone. This result could indicate that during IVAE, boar spermatozoa try to maintain adequate and minimal calcium intracellular levels at the midpiece in a Fluo-3 detected location. This minimal amount of midpiece calcium could be required to modulate progesterone-induced changes in functions such as motility. This would be logical, taking into account that sperm motility depends on calcium-modulated structures and mechanisms linked to the flagellum (Patel-King et al., 2004). Additionally, the exact sperm-motion pattern could depend on several factors such as the Ca^{2+}-linked activity status of protein kinases, e.g. PKC (Naor & Breitbart, 2004), and/or the activity of mitochondrial function proteins such as the olygomicin-sensitive mitochondrial ATP-synthase (Ramió-Lluch et al., 2014). All of these data support the hypothesis of the necessity to maintain a midpiece intracellular calcium deposit, especially in situation in which sudden changes in motion parameters are required, such as after progesterone-induced IVAE.

From the results obtained when progesterone and EGTA were added together to sperm incubated for 4h in an standard CM medium, it appears that this experimental design induces an

intermediate effect between the results obtained in CM and in CM without calcium and added with EGTA. This interpretation can be explained by the fact that the simultaneous addition of EGTA with progesterone was not able to induce an immediate and complete abolishment of extracellular calcium, although there was a significant decrease of this parameter at incubation times as short as 1 min after EGTA addition. We are not completely sure about the mechanism by which EGTA fails to induce a complete removal of free extracellular Ca^{2+} ions. However, our results suggest that an important percentage of Ca^{2+} of CM medium is trapped by the BSA included in this medium, since free Ca^{2+} ions in CM after 4h of incubation are lower than those found at the beginning of the experiment. In this context, we can assume that the addition of EGTA would immediately chelate all free Ca^{2+} ions present in the extracellular environment. At that moment, BSA would release the trapped-Ca^{2+} in an attempt to buffer the elimination of extracellular calcium. Therefore, this BSA-buffering effect would be brief, since the chelating effect of EGTA would be much fast and intense than the buffering ability of BSA to trap Ca^{2+} ions. In any case, after progesterone addition, sperm incubated in these conditions have to take extracellular Ca^{2+} ions from a medium that rapidly decreases its extracellular free Ca^{2+} ions. Therefore, since sperm are in a medium with suboptimal extracellular calcium conditions at the time of progesterone-induced IVAE, the effects are less apparent than those observed in the standard CM.

At first glance, the comparison of results obtained through flow cytometry and confocal microscopy could not be in total agreement. This is especially evident when comparing the results obtained in a calcium-depleted medium, in which flow cytometry detected measurable calcium levels after utilising both Fluo-3 and Rhod-5N, but confocal microscopy did not detect any calcium signal or only a very faint one in the majority of the analysed points. This apparent discrepancy has a methodological explanation. Flow cytometry is more sensitive than is confocal microscopy in the detection of low levels of fluorochrome-marked substrates. This greater sensitivity is even accentuated because the technician operating confocal microscopy has to set the baseline limits against a background that is often much more intense than that detected in a cytometric analysis. Taking this into account, the interpretation of results in flow cytometry and confocal analysis may differ. Flow cytometry indicates quantitative changes in intracellular calcium levels as a whole. Meanwhile, confocal microscopy was intended to establish the exact location of the specifically marked calcium in the sperm cell. In this regard, it is worth noting that the combined utilisation of both Fluo-3 and Rhod-5N markings yields a more integrative

overview of calcium dynamics during boar sperm IVC and IVAE. We must remember that Fluo-3 has very high sensitivity to calcium, as it is able to detect low amounts of intracellular Ca^{2+} (Takahashi et al., 1999). This high sensitivity implies that Fluo-3 is not especially useful to detect small changes in calcium content. As a consequence, the changes detected by cytometry can be interpreted as very intense variations in Fluo-3-marked calcium levels. Moreover, Fluo-3 exhibits fairly poor penetration ability through highly polarised membranes, thus being relatively unable to detect calcium included in this type of structures (Takahashi et al., 1999). This property seems to be contradictory with the presence of a clear Fluo-3 signal at the midpiece, since mitochondrial membranes, especially the inner one, are usually highly polarised (Saraste, 1999). A possible explanation for this might be that boar-sperm mitochondrial membranes are less polarised than are mitochondria from other cell types, thereby facilitating a better penetration and marking of Fluo-3 in mitochondria. This possibility is reinforced from the results obtained with Rhod-5N. The Rhod-5N fluorochrome has the ability to cross highly polarised membranes, and it is accumulated in the inner membrane of highly polarised cell organelles (Takahashi et al., 1999). Rhod markers have been used as effective markers for mitochondrial calcium (See Simpson & Russell (1996) and Hoth et al. (1997) as examples), although only in restrictive experimental conditions are the Rhod family markers completely specific for mitochondria (Takahashi et al., 1999). In any case, our results indicate that boar-sperm mitochondria seem to not present a highly polarised membrane able to easily trap Rhod-5N in their inner side. On the contrary, the boar sperm head shows the presence of an area surrounded by a highly polarised membrane that is able to maintain Rhod-5N-marked calcium inside it. Thus, the fact that boar sperm mitochondria did not show any Rhod-5N marking strongly suggests that their specific characteristics of membrane polarisation differ from mitochondria in other cellular types, including spermatozoa from other mammalian species.

Regarding the effect of calcium in the induction of IVC, it is worth noting that the lack of extracellular calcium in the CM was concomitant with the inability of boar sperm to modify its cell-membrane fluidity and, finally, to achieve a proper progesterone-induced IVAE. The effect of the lack of calcium on membrane fluidity is important, since changes in this fluidity mainly caused by an efflux of membrane cholesterol are one of the most important characteristics for the attainment of boar sperm IVC (Cross, 1998). One hypothesis that could explain this effect would be that the lack of extracellular calcium could block the capacitation-linked activation of sperm phospholipases, such as calcium-dependent phospholipase A (PLA) and several isotypes of

phospholipase C (PLC). This would be significant since PLA and PLC are key enzymes for cholesterol efflux and the start of acrosome exocytosis (Bennet et al., 1987; Breibart & Naor, 1999; Kurokawa et al., 2007). The calcium-induced blocking of sperm phospholipases could be performed either by a direct way, such as protein kinase C, or indirectly through altering ion-membrane channels, such as CatSper (Ren & Xia, 2010) and protein kinase A (Ickowiz et al., 2012). The inhibition of these protein kinases would thus prevent the achievement of the capacitation status not only by blocking the capacitation-linked membrane changes, but also by preventing changes in the phosphorylation status of key proteins in sperm capacitation, such as p32, which is phosphorylated through a calcium-dependent mechanism (Dubé et al., 2003). Summarising, the alteration of the synchrony in the activation of the separate pathways involving protein phospho-dephosphorylation could ultimately inhibit the achievement of a feasible capacitation status.

Regarding IVAE, our results not only are in accordance with those previously published elsewhere (See Publicover et al. (2007), Costello et al. (2009) and Aitken & Nixon (2013) as reviews), but they also provide new important information. In the present work, the presence of very low or non-detectable extracellular Ca^{2+} ions after the simultaneous addition of progesterone and EGTA to CM also prevented the achievement of progesterone-induced IVAE. This is an expected result, since calcium mobilisation is instrumental in the achievement of the process (Talbot et al., 1976). The simultaneous comparison of cytometry results obtained with Fluo-3 and Rhod-5N staining showed a parallel dynamics in both calcium populations, indicating that the calcium located at both Fluo-3- and Rhod-5N-marked points follows a similar dependence on extracellular calcium. Furthermore, the presence of a simultaneous marking of both Fluo-3 and Rhod-5N at the sperm head after the progesterone addition is worth mentioning. As indicated, the dynamics of both markings is parallel, suggesting that both Fluo-3 and Rhod-5N would mark calcium from the same calcium store. The simultaneous marking for the same location with both probes indicates that while sperm-head membranes were polarised enough to retain the Rhod-5N stain, they also presented a low enough polarity to allow Fluo-3 to enter. Microscope images also show that the sperm-head marking for both fluorochromes is not limited to the acrosomal area, but is also found in the post-acrosomal zone. This suggests that the acrosome is not the only head compartment where calcium can be found. In addition, since Rhod-5N exclusively marks sperm-head calcium and Fluo-3 marks both head and midpiece deposits, we suggest that those

investigations focusing on calcium in the sperm head preferentially use Rhod markers rather than Fluo-family ones.

In conclusion, our results indicate that boar sperm presents different calcium deposits with specific physical-chemical properties. These deposits are placed at the whole head and at the midpiece. These separate calcium deposits seem to have a dynamic equilibrium between them and between the extracellular calcium. Finally, the exact role of intracellular calcium in the modulation of different processes seems to be linked to its precise localisation, namely the head or the midpiece.

Declaration of interest

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

Acknowledgments

We wish to thank Mr. Chuck Simmons for his accurate revision of the English grammar of this manuscript. Marc Yeste was funded by Ministry of Economy and Competitiveness, Spain (Grant: JCI-2010-08620) and FP7-People Programme, Marie Curie-IEF, European Commission (Grant: 626061).

Authors' Contribution

MY, JMFN, LRL, EE, LGR, JACP, TMB, IIC, AR and JERG participated in the research design and the acquisition, analysis and interpretation of the data. MY and JERG wrote the manuscript. All authors revised critically and approved the final version of the paper.

References

Acín-Pérez R, Salazar E, Kamenetsky M, Buck J, Levin LR & Manfredi G. (2009) Cyclic AMP produced inside mitochondria regulates oxidative phosphorylation. *Cell Metab* 9, 265-276.

Aitken RJ & Nixon B. (2013) Sperm capacitation: a distant landscape glimpsed but unexplored. *Mol Hum Reprod* 19, 785-793.

Bannur SV, Kulgod SV, Metkar SS, Mahajan SK & Sainis JK. (1999) Protein determination by Ponceau S using digital colour image analysis of protein spots on nitrocellulose membranes. *Anal Biochem* 267, 382-389.

Battistone MA, Da Ros VG, Salicioni AM, Navarrete FA, Krapf D, Visconti PE & Cuasnicú PS. (2013) Functional human sperm capacitation requires both bicarbonate-dependent PKA activation and down-regulation of Ser/Thr phosphatases by Src family kinases. *Mol Hum Reprod* 19, 570-580.

Bauer PJ. (1981) Affinity and stoichiometry of calcium binding by arsenazo III. *Anal Biochem* 110, 61-72.

Baysal K, Brierley GP, Novgorodov S & Jung DW. (1991) Regulation of the mitochondrial Na^+/Ca^{2+} antiport by matrix pH. *Arch Biochem Biophys* 291, 383-389.

Bennet PJ, Moatti JP, Mansat A, Ribbes H, Cayrac JC, Pontonnier F, Chap H & Douste-Blazy L. (1987) Evidence for the activation of phospholipases during acrosome reaction of human sperm elicited by calcium ionophore A23187. *Biochim Biophys Acta* 919, 255-265.

Blackmore PF, Beebe SJ, Danforth DR & Alexander N. (1990) Progesterone and 17 +alpha-hydroxyprogesterone. Novel stimulators of calcium influx in human sperm. *J Biol Chem* 265, 1376–1380.

Boatman DE & Robbins RS. (1991) Bicarbonate: carbon dioxide regulation of sperm capacitation, hyperactivated motility, and acrosome reactions. *Biol Reprod* 44, 806-813.

Bradford MM. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* 72, 248–254.

Breitbart H & Naor Z. (1999) Protein kinases in mammalian sperm capacitation and the acrosome reaction. *Rev Reprod* 4, 151-159.

Breitbart H. (2002) Intracellular calcium regulation in sperm capacitation and acrosome reaction. *Mol Cell Endocrinol* 187, 139-144.

Burnette WN. (1981) "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulphate-plyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein. *Anal Biochem* 112, 195-203.

Costello S, Michelangeli F, Nash K, Lefievre L, Morris J, Machado-Oliveira G, Barratt C, Kirkman-Brown J & Publicover S. (2009) Ca^{2+}-stores in sperm: their identities and functions. *Reproduction* 138, 425-437.

Cross NL. (1998) Role of cholesterol in sperm capacitation. *Biol Reprod* 59, 7-11.

De la Fuente S, Fonteriz RI, Montero M & Alvarez J. (2012) Dynamics of mitochondrial [$Ca^{(2+)}$] measured with the low-$Ca^{(2+)}$-affinity dye Rhod-5N. *Cell Calcium* 51, 65-71.

De Marchi U, Thevenet J, Hermant A, Dioum E, Wiederehr A. (2014) Calcium co-regulates oxidative metabolism and ATP synthase-dependent respiration in pancreatic beta cells. J Biol Chem 289, 9182-9184.

Dubé C, Tardif S, Leclerc P & Bailey JL. (2003) The importance of calcium in the appearance of p32, a boar sperm tyrosine phosphoprotein, during in vitro capacitation. *J Androl* 24, 727-733.

García MA & Meizel S. (1999) Progesterone-mediated calcium influx and acrosome reaction of human spermatozoa: pharmacological investigation of T-type calcium channels. *Biol Reprod* 60, 102-109.

García-Herreros M, Aparicio IM, Núñez I, García-Marín LJ, Gil MC & Peña Vega FJ. (2005) Boar sperm velocity and motility patterns under capacitating and non-capacitating incubation conditions. *Theriogenology* 63, 795-805.

Garner DL & Johnson LA. (1995) Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. *Biol Reprod* 53, 276-284.

Gillan L, Evans G & Maxwell WM. (2005) Flow cytometric evaluation of sperm parameters in relation to fertility potential. *Theriogenology* 63, 445-57

Gunter TE, Yule DI, Gunter KK, Eliseev RA & Salter JD. (2004) Calcium and mitochondria. *FEBS Lett* 567, 96-102.

Harayama H, Okada K & Miyake M. (2003) Involvement of cytoplasmic free calcium in boar sperm: head-to-head agglutination induced by a cell-permeable cyclic adenosine monophosphate analog. *J Androl* 24, 91-99.

Harrison RAP, Ashworth PJ & Miller NGA. (1996) Bicarbonate/CO_2, an effector of capacitation, induces a rapid and reversible change in the lipid architecture of boar sperm plasma membranes. *Mol Reprod Dev* 45, 378–391.

Harrison RAP, Mairet B & Miller NGA. (1993) Flow cytometric studies of bicarbonate-mediated Ca^{2+} influx in boar sperm populations. *Mol Reprod Dev* 35, 197–208.

Hayato R, Higure Y, Kuba M. Nagai H, Yamashita H & Kuba K. (2011) β_3-adrenergic activation of sequential $Ca^{(2+)}$ release from mitochondria and the endoplasmic reticulum and the subsequent $Ca^{(2+)}$ entry in rodent brown adipocytes. *Cell Calcium* 49, 400-414.

Hoth M, Fanger CN & Lewis RS. (1997) Mitochondrial regulation of store-operated calcium signaling in T lymphocytes. *J Cell Biol* 137, 633–648.

Ickowiz D, Finkelstein M & Breitbart H. (2012) Mechanism of sperm caapcitation and the acrosome reaction: role of protein kinases. *Asian J Androl* 14, 816-821.

Jagannathan S, Publicover SJ & Barrat CLR. (2002) Voltage-operated calcium channels in male germ cells. Reproduction 123, 203-215.

Jiménez I, González-Márquez H, Ortiz R, Herrera JA, García A, Betancourt M & Fierro R. (2003) Changes in the distribution of lectin receptors during capacitation and acrosome reaction in boar spermatozoa. *Theriogenology* 59, 1171–1180.

Kadirvel G, Kumar S, Kumaresan A & Kathiravan P. (2009) Capacitation status of fresh and frozen-thawed buffalo spermatozoa in relation to cholesterol level, membrane fluidity and intracellular calcium. *Anim Reprod Sci* 116, 244–253.

Kroemer G. (1999) Mitochondrial control of apoptosis: an overview. *Biochem Soc Symp* 66, 1-15.

Krzyzosiak J, Molan P & Vishwanath R. (1999) Measurements of bovine sperm velocities under true anaerobic and aerobic conditions. *Anim Reprod Sci* 55, 163–173.

Kurokawa M, Yoon SY, Alfandari D, Fukami K, Sato K & Fissore RA. (2007) Proteolytic processing of phospholipase Czeta and [Ca^{2+}]i oscillations during mammalian fertilization. *Dev Biol* 312, 407-418.

Laemmli UK. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

Lambrecht M & Transtschold D. (1998) ATP determination with hexokinase and glucose 6-phosphate dehydrogenase. In: Methods of enzymatic analysis, edited by Bergmeyer HU: Weinheim: Verlag Chemie: pp 543–551.

Lee JA, Spidlen J, Boyce K, Cai J, Crosbie N, Dalphin M, Furlong J, Gasparetto M, Goldberg M, Goralczyk EM, Hyun B, Jansen K, Kollmann T, Kong M, Leif R, McWeeney S, Moloshok TD, Moore W, Nolan G, Nolan J, Nikolich-Zugich J, Parrish D, Purcell B, Qian Y, Selvaraj B, Smith C, Tchuvatkina O, Wertheimer A, Wilkinson P, Wilson C, Wood J, Zigon R; International Society for Advancement of Cytometry Data Standards Task Force, Scheuermann RH & Brinkman RR. (2008) The minimum information about a flow cytometry experiment. *Cytometry A* 73, 926–930.

Lesich KA, Kelsch CB, Ponichter KL, Dionne BJ, Dang L, Lindemann CB. (2012) The calcium response of mouse sperm flagella: role of calcium ions in the regulation of dynein activity. *Biol Reprod* 86, 105.

Lishk PV, Botchkina IL & Kirichok Y. (2011) Progesterone activates the principal Ca^{2+} channel of human sperm. *Nature* 471, 387–391.

McPartlin LA, Suárez SS, Czaya CA, Hinricks K & Bedford-Guaus, SJ. (2009) Hyperactivation of stallion sperm is required for successful in vitro fertilization of equine oocytes. *Biol Reprod* 81, 199-206.

Medrano A, Fernández-Novell JM, Ramió L, Álvarez J, Goldberg E, Rivera MM, Guinovart JJ, Rigau T & Rodríguez-Gil JE. (2006) Utilisation of citrate and lactate through a lactate dehydrogenase and ATP-regulated pathway in boar spermatozoa. *Mol Reprod Develop* 73, 369-378.

Mizrahi R & Breitbart H. (2014) Mitochondrial PKA mediates sperm motility. *Biochem Biophys*

Acta 1840, 3404-3412

Mukai C & Okuno M. (2004) Glycolysis plays a major role for adenosine triphosphate supplementation in mouse sperm flagellar movement. *Biol Reprod* 71, 540-547.

Naor Z & Breitbart H. (2004) Protein kinase C and mammalian spermatozoa acrosome reaction. *Trends Endocrinol Metab* 8, 337-342.

Nascimento JM, Shi LZ, Tam J, Chandsawangbhuwana C, Durrant B, Botvinick EL & Berns MW. (2008) Comparison of glycolysis and oxidative phosphorylation as energy sources for mammalian sperm motility, using the combination of fluorescence imaging, laser tweezers, and real-time automated tracking and trapping. *J Cell Physiol* 217, 745–751.

Odagiri K, Katoh H, Kawashima H, Tanaka T, Ohtani H, Saotome M, Urushida T, Satoh H & Hayashi H. (2009) Local control of mitochondrial membrane potential, permeability transition pore and reactive oxygen species by calcium and calmodulin in rat ventricular myocytes. *J Mol Cell Cardiol* 46, 989-997.

Patel-King RS, Gorbatyuk O, Takebe S & King SM. (2004) Flagellar radial spokes contain a Ca^{2+}-stimulated nucleoside diphosphate kinase. *Mol Biol Cell* 15, 3891-3902.

Petrunkina AM, Waberski D, Bollwein H & Sieme H. (2010) Identifying non-sperm particles during flow cytometric physiological assessment: a simple approach. *Theriogenology* 73, 995-1000.

Publicover S, Harper CV & Barratt C. (2007) [Ca^{2+}]-signalling in sperm: making the most of what you've got. *Nature Cell Biol* 9, 235-242.

Ramió L, Rivera MM, Ramírez A, Concha II, Peña A, Rigau T & Rodríguez-Gil JE. (2008) Dynamics of motile-sperm subpopulation structure in boar ejaculates subjected to "in vitro" capacitation and further "in vitro" acrosome reaction. *Theriogenology* 69, 501-512.

Ramió-Lluch L, Fernández-Novell JM, Peña A, Colás C, Cebrián-Pérez JA, Muiño-Blanco T, Ramírez A, Concha II, Rigau T & Rodríguez-Gil JE. (2011) 'In vitro' capacitation and acrosome reaction are concomitant with specific changes in mitochondrial activity in boar sperm: evidence for a nucleated mitochondrial activation and for the existence of a capacitation-sensitive subpopulational structure. *Reprod Domest Anim* 46, 664–673.

Ramió-Lluch L, Fernández-Novell JM, Peña A, Ramírez A, Concha II & Rodríguez-Gil JE. (2012) 'In vitro' capacitation and further 'in vitro' progesterone-induced acrosome exocytosis are linked to specific changes in the expression and acrosome location of protein phosphorylation in serine residues of boar spermatozoa. *Reprod Domest Anim* 47, 766-776.

Ramió-Lluch L, Yeste M, Fernández-Novell JM, Estrada E, Rocha L, Cebrián-Pérez JA, Muiño-Blanco T, Concha II, Ramírez A & Rodríguez-Gil JE. (2014) Oligomycin A-induced inhibition of mitochondrial ATP-synthase activity suppresses boar sperm motility and in vitro capacitation achievement without modifying overall sperm energy levels. *Reprod Fertil Dev* 26, 883-897.

Rathi R, Colenbrander B, Bevers MM & Gadella BM. (2001). Evaluation of in vitro capacitation of stallion spermatozoa. *Biol Reprod* 65, 462-470.

Ren D & Xia J. (2010) Calcium signalling through CatSper channels in mammalian fertilization. *Physiology* 25, 165-175.

Santo-Domingo J & Demaurex N. (2012) The renaissance of mitochondrial pH. *J Gen Physiol* 139: 415-423.

Saraste M. (1999) Oxidative phosphorylation at the “fin de siècle”. *Science* 283, 1488–1493.

Simpson PB & Russell JT. (1996) Mitochondrial support inositol 1,4,5-triphosphate-mediated Ca21 waves in cultured oligodendrocytes. *J Biol Chem* 271, 33493–33501.

Smaili SS & Russell JT. (1999) Permeability transition pore regulates both mitochondrial membrane potential and agonist-evoked Ca^{2+} signals in oligodendrocyte progenitors. *Cell Calcium* 26, 121-130.

Takahashi A, Camacho P, Lechleiter JD & Herman B. (1999) Measurement of intracellular calcium. *Physiol Rev* 79, 1089-1125.

Talbot P, Summers RG, Hylander BL, Keough EM & Franklin LE. (1976) The role of calcium in the acrosome reaction: an analysis using ionophore A23187. *J Exp Zool* 198, 383-392.

Teves ME, Barbano F, Guidobaldi HA, Sanchez R, Miska W & Giojalas LC. (2006) Progesterone at the picomolar range is a chemoattractant for mammalian spermatozoa. *Fertil Steril* 86, 745-749.

Töpfer-Petersen E, Friess AE, Sinowatz F, Biltz S & Schill WB. (1985) Immunocytological characterization of the outer acrosomal membrane (OAM) during acrosome reaction in boar. *Histochemistry* 82, 113-120.

Vander Heiden MG, Chandel NS, Li XX, Schumacker PT, Colombini M & Thompson CB. (2000) Outer mitochondrial membrane permeability can regulate coupled respiration and cell survival. *Proc Nat Acad Sci USA* 97, 4666-4671.

Visconti PE, Bailey JL, Moore GD, Pan D, Olds-Clarke P & Kopf GS. (1995) Capacitation of mouse spermatozoa I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development* 121, 1129-1137.

Yeste M, Briz M, Pinart E, Sancho S, Garcia-Gil N, Badia E, Bassols J, Pruneda A, Bussalleu E, Casas I & Bonet S. (2008a) Hyaluronic acid delays boar sperm capacitation after 3 days of storage at 15 degrees C. *Anim Reprod Sci* 109, 236-250.

Yeste M, Briz M, Pinart E, Sancho S, Garcia-Gil N, Badia E, Bassols J, Pruneda A, Bussalleu E, Casas I & Bonet S. (2008b) Boar spermatozoa and prostaglandin F2alpha. Quality of boar sperm after the addition of prostaglandin F2alpha to the short-term extender over cooling time. *Anim Reprod Sci* 108, 180-195.

Yeste M, Estrada E, Casas I, Bonet S & Rodríguez-Gil JE. (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.

Figure legends

Figure 1. Percentages of total motility of boar sperm subjected to 'in vitro' capacitation and subsequent 'in vitro' acrosome exocytosis in either a standard capacitation medium or a Ca^{2+}-depleted capacitation medium. Boar sperm were incubated for 4h and then 10 $\mu g \cdot mL^{-1}$ progesterone were added and subjected to further incubation for 60 min, as described in the Material and Methods section. ◆: Control cells. ▲: Spermatozoa incubated in capacitation medium without $CaCl_2$, and with 2 mM EGTA added from the beginning of the experiments. ■: Spermatozoa incubated in a standard capacitation medium for 4h and subsequently with 10 $\mu g \cdot mL^{-1}$ progesterone and 2 mM EGTA added together. ○: Spermatozoa incubated in a medium similar to the capacitation medium but without BSA. These cells were utilised as negative controls of capacitation, as indicated in the results shown here (constant and intense drop in total motility when compared with spermatozoa incubated in standard capacitation medium). Results are expressed as means±S.E.M. for 11 separate experiments. Asterisks indicate significant (P<0.05) differences, when compared with the respective Control values. The scale of the X axis is different between results before and after the addition of progesterone in order to clearly show the early events appearing after IVAE induction.

Figure 2. Tyrosine phosphorylation of boar-sperm P32 protein of cells subjected to 'in vitro' capacitation and subsequent 'in vitro' acrosome exocytosis in either a standard capacitation medium or a Ca^{2+}-depleted capacitation medium. Boar sperm were incubated for 4h and then 10 $\mu g \cdot mL^{-1}$ progesterone were added and subjected to further incubation for 60 min, as described in the Material and Methods section. Spermatozoa incubated in NCM. These were utilised as a negative control of the achievement of capacitation. (a, b, c, d) Western blots against tyrosine phosphorylation of the P32 boar sperm protein. (a', b', c', d') Western blots against β-tubulin. These are shown as samples of the utilisation of β-tubulin as an internal control of the total amount of protein loaded in each lane. M: Molecular weight markers lane. The molecular weight of the marker showed in the Figure is of 37 kDa for phosphorylated P32 and of 50 kDa for β-tubulin. 0h, 2h, 4h: incubation time of sperm in the corresponding media. 5 min, 60 min: time after the addition of either 10 μg/mL progesterone or of 10 μg/mL progesterone and 2 mM EGTA together. Subsequently to 4h of incubation in the corresponding media. (e) Results of densitometry ratios for the coefficient P32 Tyrosine phosphorylation/β-tubulin, taking the ratio of

incubation in standard capacitation medium at 0h of incubation as a basal, arbitrary value of 100. ◆: Control cells. ▲: Spermatozoa incubated in capacitation medium without $CaCl_2$, and with 2 mM EGTA added from the beginning of the experiments. ■: Spermatozoa incubated in a standard capacitation medium for 4 h and subsequently with 10 $\mu g \cdot mL^{-1}$ progesterone and 2 mM EGTA added together. ○: Spermatozoa incubated in a medium similar to the capacitation medium but without BSA. These cells were utilised as negative controls of capacitation, as indicated in the results shown here (lack of phosphorylation of the boar sperm P32 protein when compared with spermatozoa incubated in the standard capacitation medium). Data shown are representative images for eleven separate experiments.

Figure 3. Percentages of viable boar sperm with high-permeability cell membranes (merocyanine-540-positive cells) concomitantly to the negative YO-PRO 1 stain (YO-PRO-1^-/M-540^+ cells) after the induction of 'in vitro' capacitation and the subsequent 'in vitro' acrosome reaction in either a standard capacitation medium or a Ca^{2+}-depleted capacitation medium. Boar sperm were incubated for 4h, and then 10 $\mu g \cdot mL^{-1}$ progesterone were added and subjected to further incubation for 60 min, as described in the Material and Methods section. ◆: Control cells. ▲: Spermatozoa incubated in capacitation medium without $CaCl_2$, and with 2 mM EGTA added from the beginning of the experiments. ■: Spermatozoa incubated in a standard capacitation medium for 4h, and subsequently with 10 $\mu g \cdot mL^{-1}$ progesterone and 2 mM EGTA added together. Results are expressed as means±S.E.M. for 11 separate experiments. Asterisks indicate significant (P<0.05) differences, when compared with the respective Control values. The scale of the X axis is different between results before and after the addition of progesterone in order to clearly show the early events appearing after IVAE induction.

Figure 4. Rhythm of O_2 consumption and intracellular ATP levels of boar sperm subjected to 'in vitro' capacitation and the subsequent 'in vitro' acrosome reaction in either a standard capacitation medium or a Ca^{2+}-depleted capacitation medium. Boar sperm were incubated for 4h, and then 10 $\mu g \cdot mL^{-1}$ progesterone were added and subjected to further incubation for 60 min, as described in the Material and Methods section. A): Rhythm of O_2 consumption. ◆: Control cells. ▲: Spermatozoa incubated in capacitation medium without $CaCl_2$, and with 2 mM EGTA added from the beginning of the experiments. ■: Spermatozoa incubated in a standard capacitation medium for 4h, and subsequently with

10 μg·mL^{-1} progesterone and 2 mM EGTA added together. B): Intracellular ATP levels. ◆: Control cells. ▲: Spermatozoa incubated in capacitation medium without $CaCl_2$, and with 2 mM EGTA added from the beginning of the experiments. ■: Spermatozoa incubated in a standard capacitation medium for 4h, and subsequently with 10 μg·mL^{-1} progesterone and 2 mM EGTA added together. Results are expressed as means±S.E.M. for 11 separate experiments. Asterisks indicate significant (P<0.05) differences, when compared with the respective Control values. The scale of the X axis is different between results before and after the addition of progesterone in order to clearly show the early events appearing after IVAE induction.

Figure 5. Percentages of viable boar sperm with high mitochondrial membrane potential following the JC-1 stain and mean intensity levels of the JC-1 stain of boar sperm with high mitochondrial membrane potential after the induction of 'in vitro' capacitation and subsequent 'in vitro' acrosome reaction in either a standard capacitation medium or a Ca^{2+}-depleted capacitation medium. Boar sperm were incubated for 4h, and then 10 μg·mL^{-1} progesterone were added and subjected to further incubation for 60 min, as described in the Material and Methods section. ◆: Control cells. ▲: Spermatozoa incubated in capacitation medium without $CaCl_2$, and with 2 mM EGTA added from the beginning of the experiments. ■: Spermatozoa incubated in a standard capacitation medium for 4h, and subsequently with 10 μg·mL^{-1} progesterone and 2 mM EGTA added together. Results are expressed as means±S.E.M. for 11 separate experiments. Asterisks indicate significant (P<0.05) differences, when compared with the respective Control values. The scale of the X axis is different between results before and after the addition of progesterone in order to clearly show the early events appearing after IVAE induction.

Figure 6. Percentages of viable boar sperm with high intracellular Ca^{2+} levels and mean intensity levels of the Fluo-3 stain of boar sperm with high intracellular Ca^{2+} levels following the Fluo-3 stain after the induction of 'in vitro' capacitation and the subsequent 'in vitro' acrosome reaction in either a standard capacitation medium or a Ca^{2+}-depleted capacitation medium. Boar sperm were incubated for 4h, and then 10 μg·mL^{-1} progesterone were added and subjected to further incubation for 60 min, as described in the Material and Methods section. A): Percentages of boar sperm with high intracellular Ca^{2+} levels. ◆: Control cells. ▲: Spermatozoa incubated in capacitation medium without $CaCl_2$, and with 2 mM EGTA added from the beginning of the experiments. ■:

Spermatozoa incubated in a standard capacitation medium for 4h, and subsequently with 10 $\mu g \cdot mL^{-1}$ progesterone and 2 mM EGTA added together. B): Mean intensity levels of the Fluo-3 stain of boar sperm with high intracellular Ca^{2+} levels. ◆: Control cells. ▲: Spermatozoa incubated in capacitation medium without $CaCl_2$, and with 2 mM EGTA added from the beginning of the experiments. ■: Spermatozoa incubated in a standard capacitation medium for 4h, and subsequently with 10 $\mu g \cdot mL^{-1}$ progesterone and 2 mM EGTA added together. Results are expressed as means±S.E.M. for 11 separate experiments. Asterisks indicate significant ($P<0.05$) differences, when compared with the respective Control values. The scale of the X axis is different between results before and after the addition of progesterone in order to clearly show the early events appearing after IVAE induction.

Figure 7. Confocal microscope images of Fluo-3-marked intracellular calcium of boar sperm subjected to 'in vitro' capacitation and the subsequent 'in vitro' acrosome reaction in either a standard capacitation medium or a Ca^{2+}-depleted capacitation medium. Boar sperm were incubated for 4h, and then 10 $\mu g \cdot mL^{-1}$ progesterone were added and subjected to further incubation for 60 min, as described in the Material and Methods section. Control: Control cells. EGTA IVC: Spermatozoa incubated in capacitation medium without $CaCl_2$, and with 2 mM EGTA added from the beginning of the experiments. EGTA IVAE: Spermatozoa incubated in a standard capacitation medium for 4h, and subsequently with 10 $\mu g \cdot mL^{-1}$ progesterone and 2 mM EGTA added together. 0h; 4h: time of incubation in the CM medium with or without Ca^{2+}. 1 min; 30 min: time after the addition of progesterone either with or without EGTA. Images are representative for 8 separate experiments. Bars indicate a size of 15 µm.

Figure 8. Percentages of viable boar sperm with high intracellular Ca^{2+} levels and mean intensity levels of the Rhod-5N stain of boar sperm with high intracellular Ca^{2+} levels following the Rhod-5N stain after the induction of 'in vitro' capacitation and subsequent 'in vitro' acrosome reaction in either a standard capacitation medium or a Ca^{2+}-depleted capacitation medium. Boar sperm were incubated for 4h, and then 10 $\mu g \cdot mL^{-1}$ progesterone were added and subjected to further incubation for 60 min, as described in the Material and Methods section. A): Percentages of boar sperm with high intracellular Ca^{2+} levels. ◆: Control cells. ▲: Spermatozoa incubated in capacitation medium without $CaCl_2$, and with 2 mM EGTA added from the beginning of the experiments. ■:

Spermatozoa incubated in a standard capacitation medium for 4,h and subsequently with 10 $\mu g \cdot mL^{-1}$ progesterone and 2 mM EGTA added together. B): Mean intensity levels of the Rhod-5N stain of boar sperm with high intracellular Ca^{2+} levels. ◆: Control cells. ▲: Spermatozoa incubated in capacitation medium without $CaCl_2$, and with 2 mM EGTA added from the beginning of the experiments. ■: Spermatozoa incubated in a standard capacitation medium for 4h, and subsequently with 10 $\mu g \cdot mL^{-1}$ progesterone and 2 mM EGTA added together. Results are expressed as means±S.E.M. for 11 separate experiments. Asterisks indicate significant ($P<0.05$) differences, when compared with the respective Control values. The scale of the X axis is different between results before and after the addition of progesterone in order to clearly show the early events appearing after IVAE induction.

Figure 9. Confocal microscope images of Rhod-5N-marked intracellular calcium of boar sperm subjected to 'in vitro' capacitation and subsequent 'in vitro' acrosome reaction in either a standard capacitation medium or a Ca^{2+}-depleted capacitation medium. Boar sperm were incubated for 4h, and then 10 $\mu g \cdot mL^{-1}$ progesterone were added and subjected to further incubation for 60 min, as described in the Material and Methods section. Control: Control cells. EGTA IVC: Spermatozoa incubated in capacitation medium without $CaCl_2$, and with 2 mM EGTA added from the beginning of the experiments. EGTA IVAE: Spermatozoa incubated in a standard capacitation medium for 4h, and subsequently with 10 $\mu g \cdot mL^{-1}$ progesterone and 2 mM EGTA added together. 0h; 4h: time of incubation in CM with or without Ca^{2+}. 1 min; 30 min: time after the addition of progesterone either with or without EGTA. Images are representative for 8 separate experiments. Bars indicate a size of 15 µm.