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Maturation conditions and boar affect timing of cortical reaction in porcine oocytes

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Abstract

The cortical reaction induces changes at the egg's Zona pellucida (ZP), perivitelline space and/or oolemma level, blocking polyspermic fertilization. We studied the timing of sperm penetration and cortical reaction in pig oocytes matured under different conditions and inseminated with different boars. Immature (germinal vesicle stage) and *in vitro* matured (IVM) (metaphase II stage) oocytes were inseminated and results assessed at different hours post insemination. Penetrability and polyspermy rates increased with gamete coincubation time and were higher in IVM oocytes. A strong boar effect was observed in IVF results. Cortical reaction (assessed as area occupied by cortical granules) and galactose-β(1-3)-Nacetylgalactosamine residues on ZP (area labeled by peanut agglutinin lectin, PNA) were assessed in IVM and *in vivo* matured (IVV) oocytes at different hours post insemination. After maturation, IVM and IVV oocytes displayed similar area occupied by cortical granules and it decreased in fertilized oocytes compared to unfertilized ones. Cortical reaction was influenced by boar and was faster in polyspermic than in monospermic oocytes, and in IVM than in IVV oocytes. The outer ZP of inseminated oocytes appeared stained by PNA and the labeled area increased along with gamete coculture time. This labeling was also observed after insemination of isolated ZP, indicating that this modification in ZP carbohydrates is not induced by cortical reaction. The steady and maintained cortical reaction observed at 4 to 5 h post insemination in IVV monospermic oocytes might reflect the physiological time course of this important event in pigs. Both maturation conditions and boar affect cortical granules release.

Keywords: Pig oocytes; Cortical reaction; Epididymal sperm; Monospermy

1. Introduction

The release of cortical granule (CG) contents into the perivitelline space, the so-called "cortical reaction" prevents polyspermy by inducing changes at the zona pellucida (zona reaction), perivitelline space and/or oolemma level [1]. The CG are unique oocyte organelles. They migrate to the peripheral oolemma during maturation and once they are released by appropriate stimulus their content is not synthesized again [1,2]. Thus, it could be assumed that an efficient block against polyspermic penetration implies that once the spermatozoon sperm fuses with the oolemma, the oocyte displays a temporary adequate cortical reaction (CR) with a complete release of the CG contents followed by a homogeneous distribution into the perivitelline space.

In pigs, the high incidence of polyspermy is still an unresolved problem (reviewed by [3]), which has been associated with "failures" in the CR, such as i) delayed

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and incomplete CR [4], ii) lack of distribution of released CG contents into the perivitelline space [5], iii) delayed zona reaction [6], iv) undefined differences between *in vitro* (IVM) and *in vivo* (IVV) matured oocytes [5] such as incomplete zona pellucida maturation in the former ones.

In rodents CR has been described as a fast phenomenon; after a spermatozoon fuses with the oolemma it takes 9 min for completion in hamster [7] or 10 to 60 min in mouse [8]. In pigs there is still a lack of data about the physiological basis and timing of the CR. Most studies are conducted by inducing the CR with chemicals [6,9] or assessing the CR only at one or two specific time points after insemination [9–11] rather than in a wide time frame. *In vitro*, an important decrease in CG density is observed about 3 h post insemination (hpi) [11], parallel to sperm penetration [12]. It has been hypothesized that CR in pigs could not be as fast as in other mammals since the majority of *in vitro* matured (IVM) oocytes showed no CR up to 6 hpi [4]. Comparison between the different reports on porcine CR timing is difficult because both IVF medium [10] and IVF conditions [13] affect CG density. The information about CR timing of IVM and *in vivo* (IVV) matured oocytes is controversial. Studies from the same group report that IVM and ovulated oocytes display similar CR pattern [12] but that IVM oocytes have a delayed CR that causes polyspermy [14]. Later studies from the same group and others [5,6] reported that high incidence of polyspermy was not due to a delayed CR but differences in CG contents distribution in the perivitelline space between IVM and IVV oocytes [5,6].

The temporal dependence between sperm penetration and establishment of the zona-mediated block to polyspermy might vary significantly. The specific modifications of the zona pellucida (ZP) induced by CR have not been fully described so far, but it is known that released CG-enzymes induce changes to the ZP carbohydrate, inducing enzymatic removal of glycan ligands [15]. Under physiological conditions the fusion of the penetrating spermatozoon triggers CG release and, despite the boar effect on IVF results being well documented [16,17], its direct role on the CR has, to our knowledge, never been investigated thoroughly. It is also unknown whether sperm cells from different boars would trigger CR timing and patterns differently. Epididymal spermatozoa have some advantages over ejaculated ones as they have never been in contact with seminal plasma and its decapacitation molecules, thus resulting in consistent IVF rates with lower variability [11,18]. Therefore, a study of the time frame of sperm-induced CR in differently matured oocytes after insemination with spermatozoa from different boars would broaden the knowledge about the role of CR in fertilization.

The objective of this work was to study the effect of maturation conditions and boar on oocyte penetrability and CR after insemination with epididymal spermatozoa. Modifications at galactose-β(1-3)-Nacetylgalactosamine residues on ZP were measured by the degree of peanut agglutinin (PNA) binding.

2. Materials and methods

2.1. Experimental design

Two experiments were performed to study the effects of maturation conditions and boar on penetrability and the cortical reaction. In Experiment 1, penetrability data were obtained by doing homologous *in vitro* penetration assays both with immature (germinal vesicle stage) and *in vitro* matured (metaphase II) oocytes. Both kinds of oocytes were inseminated *in vitro* with epididymal spermatozoa from three boars. At 2, 3, 4, 5, 6, 7 and 18 h post insemination (hpi), samples were fixed (15 oocytes per group) and penetrability results assessed. Four and five replicates were done with immature and *in vitro* matured oocytes, respectively. In Experiment 2, *in vitro* and *in vivo* matured oocytes; and zona pellucidae collected from *in vitro* matured oocytes were inseminated *in vitro* with the same boars and IVF conditions as in Experiment 1. After adding spermatozoa, samples (4–6 oocytes per group) were processed at 0.5, 1, 2, 4, 5, 6, 7 and 18 hpi to assess cortical granule density and zona pellucida labeling with fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA). Seven and five replicates were carried out with *in vitro* (IVM) and *in vivo* (IVV) matured oocytes, respectively.

2.2. Culture media

Unless otherwise indicated, all the chemicals used in this study were purchased from Sigma-Aldrich (Munich, Germany).

Oocyte maturation medium was NCSU-37 supplemented with 0.57 mM cysteine, 1 mM dibutyryl cAMP, 5 μg/mL insulin, 50 μM β-mercaptoethanol, 1 mM glutamine, 10 IU/mL eCG (Folligon, Intervet International B.V., Boxmeer, Holland), 10 IU/mL hCG (Chorulon, Intervet International B.V., Boxmeer, Holland), 10 ng/mL EGF and 10% (v/v) porcine follicular fluid. The fertilization medium was modified TALP [19] with

2 mM caffeine and supplemented with 3 mg/mL fatty acid-free BSA and 1.10 mM sodium pyruvate.

2.3. Oocyte collection and in vitro maturation (IVM)

Ovaries from Landrace × Large White crossbred gilts were collected at the slaughterhouse and transported to the laboratory in saline at 38.5 °C. Methods for cumulus-oocyte complexes (COCs) collection and *in vitro* maturation have been described previously [11]. Briefly groups of 50 COCs were cultured in 500 μL of NCSU-37 medium for 22 h at 38.5 °C under 5% CO_2 in air. After culture, oocytes were washed twice with fresh maturation medium without dibutyryl cAMP, eCG, and hCG and cultured for an additional 20 to 22 h.

2.4. Collection of in vivo matured oocytes

Landrace X Large White crossbred prepubertal gilts 5 to 6 mo of age and weighing approximately 90 kg were superovulated with 2000 IU eCG (Intervet, Germany) followed by 500 IU hCG (Intervet, Germany) 72 h later. With this superovulation protocol, gilts are expected to ovulate 40 to 42 h after hCG administration. Thirty-eight h later gilts were killed and genital tracts immediately transported (≤10 min) in a thermos flask with saline at 38.5 °C to the nearby laboratory. Follicles close to ovulation (≥11-mm diameter) were aspirated and *in vivo* matured oocytes collected in warm PBS under a stereomicroscope. Only COCs showing expanded cumulus cells were used. All animal procedures were approved by the Federal Research Institute for Animal Health (FLI).

2.5. Sperm preparation and in vitro fertilization (IVF)

Methods for *in vitro* fertilization were those described previously [18]. COCs were completely denuded by gently pippetting in IVF medium before insemination. Frozen epididymal spermatozoa from three boars (Large White X Landrace) were used in all the experiments. Briefly, on the day of IVF, three 0.25-mL straws were thawed (20 s at 38 °C), diluted in 10 mL Androhep (Minitüb, Tiefenbach, Germany) and centrifuged at 800 g for 3 min. The supernatant was discharged and the sperm pellet re-suspended in modified TALP medium equilibrated overnight in an incubator at 38.5 °C under 5% CO_2. Final sperm concentration was adjusted to 2000 spermatozoa in 10 μL and were added to 40-μL droplets containing 5 denuded oocytes (ratio 400 spermatozoa per oocyte). After sperm preparation, motility was estimated under a phase-contrast microscope (X 200, Nikon, Tokyo, Japan) by placing two sample aliquots from each boar on warm glass slides (38.5 °C). The percentage of motile sperm was estimated to the nearest 5% and the forward progressive motility (FPM) using an arbitrary scale from 0 to 5. Only samples with ≥70% motility and ≥4 were used for insemination.

Putative zygotes were fixed and stained (1% wt/v orcein) at 2, 3, 4, 5, 6, 7 and 18 h post insemination (hpi). Samples were examined under a phase-contrast microscope (X 400, Nikon Diaphot 300, Tokyo, Japan) for sperm penetration and pronucleus formation.

2.6. Assessment of CG density and zona pellucida labeling

In vitro and *in vivo* matured oocytes were inseminated with spermatozoa from three different boars under the same conditions and sperm concentration as mentioned before (5 oocytes per droplet and 400 spermatozoa per oocyte). After adding sperm, oocytes were fixed at 0.5, 1, 2, 4, 5, 6, 7 and 18 hpi and processed for confocal microscopy analysis. Control oocytes (*in vitro* and *in vivo* matured) were kept in IVF medium without sperm for 2, 3, 4, 5, 6, 7 and 18 h. This experiment was replicated 7 times for IVM and 5 times for IVV oocytes.

A modified method of CG visualization was based on that previously described [11,20]. Briefly, oocytes and putative zygotes were washed several times in PBS without calcium chloride (PBS-w/o) to eliminate remaining cumulus cells and sperm bound to the ZP. Thereafter, samples were fixed with freshly prepared 3.7% paraformaldehyde in PBS-w/o (30 min, room temperature) and washed in PBS-w/o. This was followed by treatment with freshly prepared 1% Triton X-100 in PBS-w/o for 5 min and three washes in PBS-w/o. Samples were then incubated in the dark with 10 μg/mL fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) in PBS-w/o for 30 min and 100 μg/mL propidium iodide (PI) for 5 min to stain the DNA. After staining, oocytes were washed in PBS-w/o and equilibration buffer medium (Slow fade, Antifade kit, Molecular Probes). An adhesive ring was attached to a clean slide, 5 to 7 oocytes transferred inside the ring with 4 μL antifade mounting medium and finally protected with a cover slide sealed with nail polish. Samples were kept in the dark at 4 °C until confocal assessment within 48 h.

Oocytes were examined from the outer ZP in one oocyte's pole to the opposite pole under an X 63 magnification oil objective of a confocal laser-scanning microscope equipped with argon and helium–neon lasers (Carl Zeiss, Germany) using the following set up parameters: 1-mW laser power (FITC was excited at

480 nm and emission collected at 530 nm; PI was excited at 514 nm and emission collected at 603 nm), 1-μs pixel dwell, 1 Airy unit pinhole aperture and 1-μm thickness section. Nuclear stage, sperm penetration and number of sperm cells inside ooplasm were recorded by means of PI fluorescence.

The fluorescent emissions from the oocytes were recorded and saved as TIFF files using the software attached to the microscope. The largest diameter section (equator of the oocyte) was used for further image analysis with MIP-4.5 Microm Image Processing software (Consulting de Imagen Digital S.L., Microm, Barcelona, Spain). CG distribution (FITC fluorescence) was studied by selecting the FITC-PNA stained portion (area occupied by CG) in the area directly below the oocyte plasma membrane as previously described [10,11]. Briefly, on each image the oocyte's cortical area was selected for image analysis by drawing two concentric circles with the program-drawing tool. The area occupied by CG as a percentage of the total cortical area was calculated as the FITC-PNA labeled area divided by the entire cortical selected area × 100. The largest diameter section (equator of the oocyte) was also used for the ZP analysis. To calculate the portion of the outer ZP stained by FITC-PNA, the external third of its area was selected by two concentric circles and the portion of the fluorescence area was calculated as the FITC-PNA labeled area divided by the entire ZP area × 100. From each oocyte, data of cortical area occupied by CG (%) and the outer ZP area (%) were calculated and saved for further analysis.

2.7. Isolation of the of the zona Pellucidae (ZPs)

In order to elucidate whether the observed PNA labeling on the outer ZP was derived from CG contents or acrosomal shrouds, ZPs from IVM oocytes were isolated and incubated with sperm under the same conditions as described above (5 ZPs per droplet and 400 spermatozoa per ZP). After IVM period, oocytes were denuded by pipetting and washed in PBS. Oocytes were transferred individually to PBS microdroplets and investigated at X 200 magnification under an inverted microscope (Nikon Diaphot 300, Tokyo, Japan) with attached micromanipulators (TransferMan NK, Eppendorf, Hamburg, Germany). A holding pipette fixed each oocyte and the ZP and oolemma were penetrated with an injection pipette. After aspiration of the whole cytoplasmic content the injection pipette was removed from the oocyte. Isolated ZPs were then gently washed

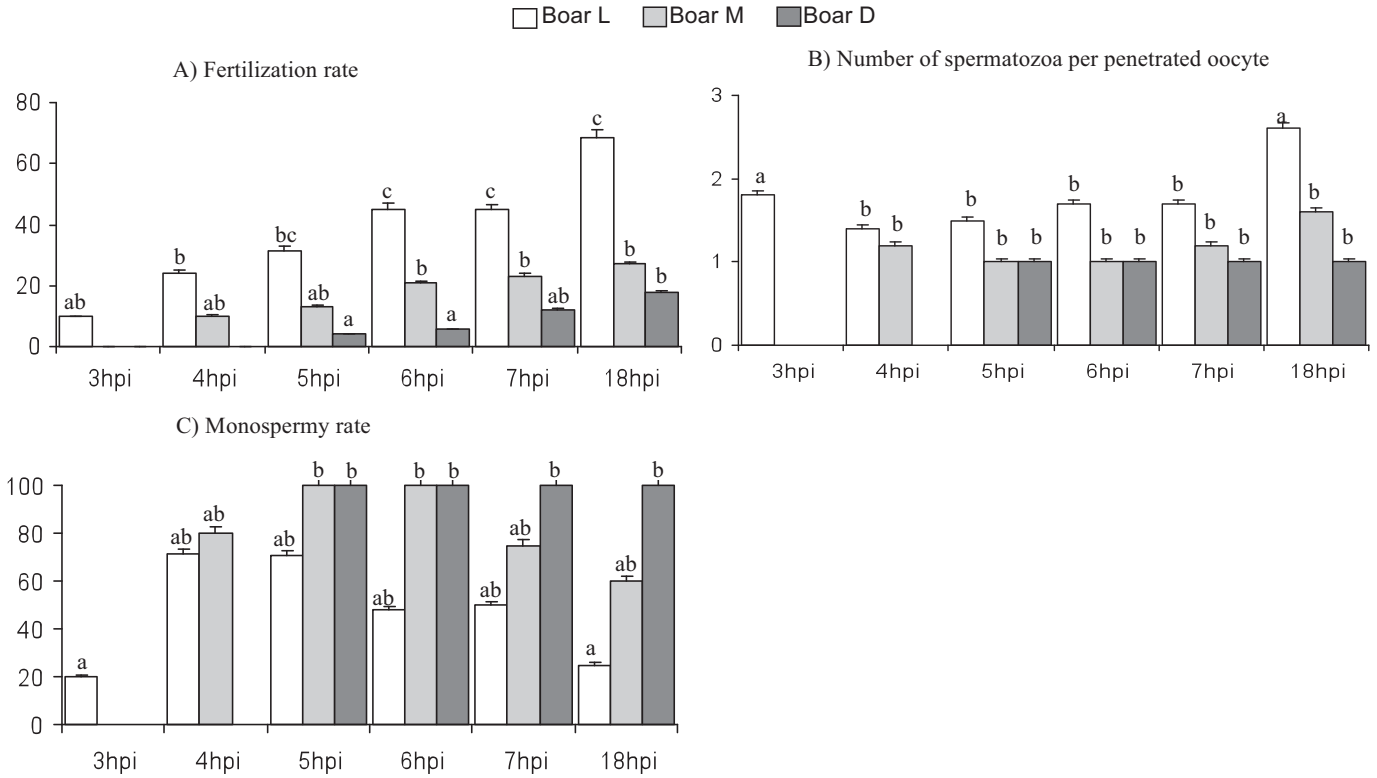

Fig. 1. *In vitro* fertilization results for immature pig oocytes inseminated with epididymal spermatozoa from three different boars and assessed at different hours post insemination (hpi). Results are represented as mean ± SEM. Data with different letters differ significantly ($P < 0.05$).

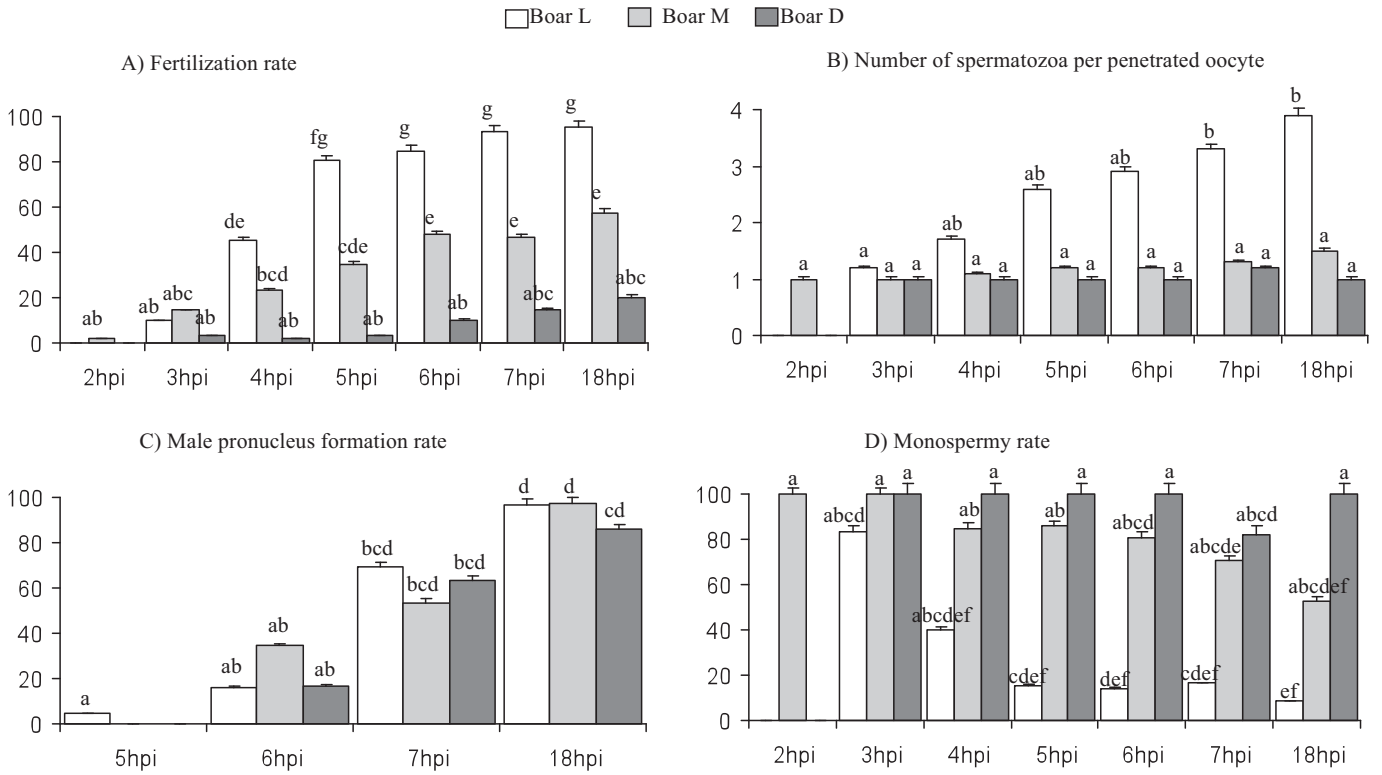

Fig. 2. *In vitro* fertilization results for *in vitro* matured pig oocytes inseminated with epididymal spermatozoa from three different boars and assessed at different hpi. Results are represented as mean ± SEM. Data with different letters differ significantly ($P < 0.05$).

in TALP medium and immediately cocultured with spermatozoa. ZPs were fixed and processed for confocal microscopy analysis after 0.5, 1, 2, 4, 5, 6, 7 and 18 hpi of adding spermatozoa. Five ZPs were examined at each time point.

2.8. Statistical analysis

Data are presented as mean ± SEM, and all rates were analyzed with a binomial model of parameters. Data were analyzed by ANOVA and, when a significant effect was revealed, values were compared using the Tukey test. A P value <0.05 was taken to denote statistical significance.

3. Results

3.1. Fertilization rate, timing of sperm penetration, and monospermy depend on boar and oocyte source

Penetration results for immature and IVM oocytes indicated a variable time course of penetration among the tested boars. Thus, fertilization rate and timing of

Table 1
Area occupied by CG and ZP area labeled by FITC-PNA in *in vitro* (IVM) and *in vivo* (IVV) matured porcine oocytes kept in IVF medium without sperm for different periods of time.

	IVM OOCYTES		IVV OOCYTES	
Hours	% CG area (N)	% stained ZP area (N)	% CG area (N)	% stained ZP area (N)
0	20.1 ± 0.5 (34)[a]	0.2 ± 0.2 (34)	20.4 ± 2.8 (27)	0.4 ± 0.2 (27)
0.5	20.1 ± 0.8 (30)[a]	0.3 ± 0.1 (30)	19.1 ± 3.6 (15)	0.4 ± 0.2 (15)
1	20.4 ± 0.4 (39)[a]	0.5 ± 0.1 (39)	20.5 ± 1.7 (17)	0.3 ± 0.2 (17)
2	19.1 ± 0.5 (38)[a]	0.3 ± 0.1 (38)	19.2 ± 2.4 (14)	0.1 ± 0.1 (14)
4	18.2 ± 0.7 (42)[a]	0 (42)	18.9 ± 1.6 (16)	0.3 ± 0.2 (16)
5	15.3 ± 0.4 (39)[b]	0.4 ± 0.1 (39)	16.7 ± 1.2 (19)	0.4 ± 0.2 (19)
18	12.7 ± 0.5 (29)[b]	0.2 ± 0.1 (29)	13.2 ± 1.9 (17)	0.1 ± 0.1 (17)

N (number of evaluated oocytes). Results are presented as mean ± SEM. Data with different letters differ significantly ($P < 0.001$).

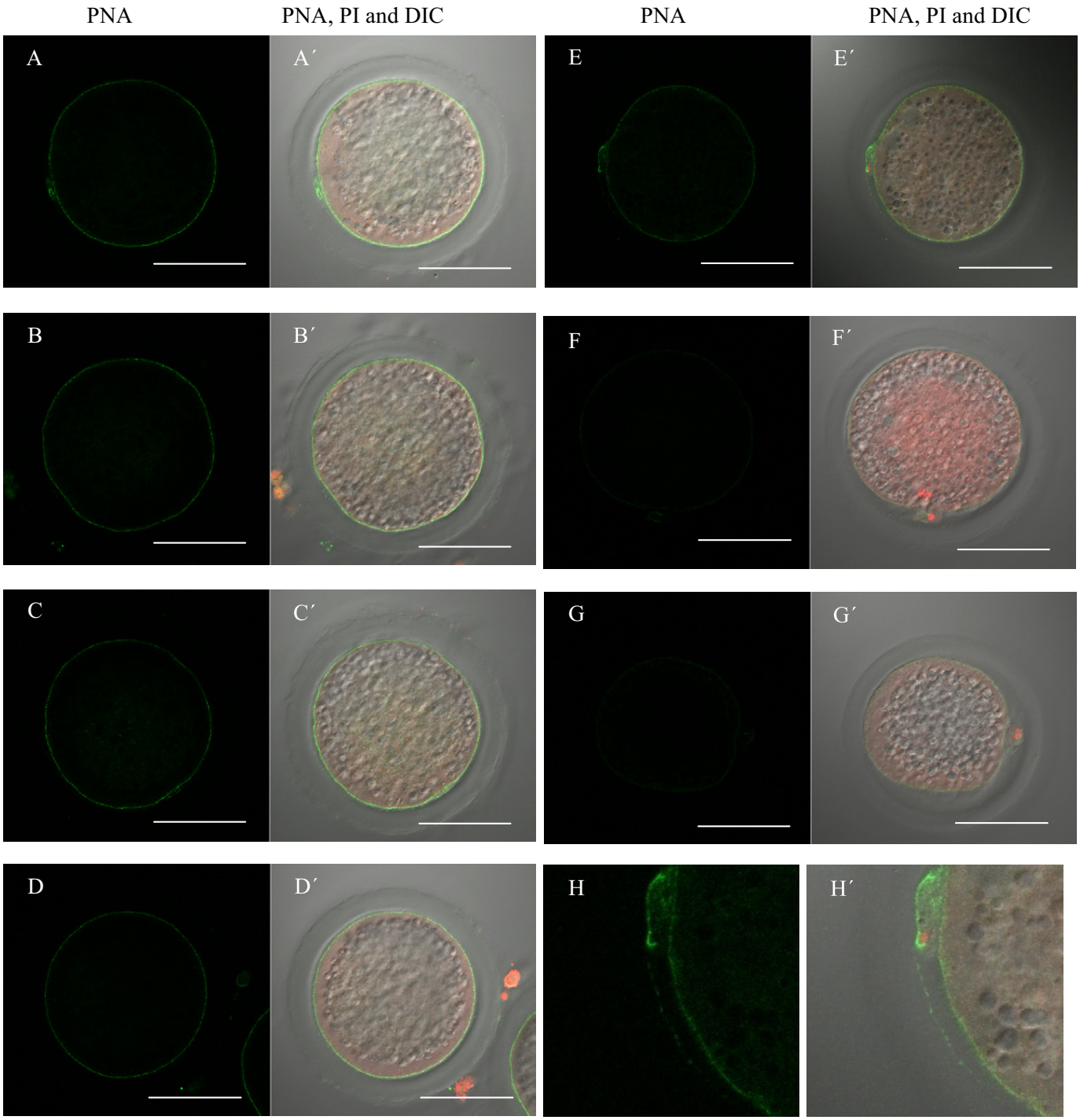

Fig. 3. Multilaser fluorescence and differential interference contrast (DIC) confocal images of *in vitro* matured (IVM) oocytes cultured in IVF medium for different intervals in the absence of spermatozoa. Labeling by FITC-PNA lectin and propidium iodide (PI). cortical granule (CG) monolayer beneath the oolemma and absence of stained material on the outer Zona pellucida (ZP) is observed. A–A', Oocyte just after IVM. Oocytes after different intervals in IVF medium: B–B′, 0.5 h, C–C′, 1 h, D–D′, 2 h, E–E′, 4 h, F–F′, 5 h and G–G′, 18 h. H–H′, Magnification of oocyte after 5 h in culture showing labeled material deposited in the perivitelline space. Scale bar indicates 50 μm.

sperm penetration in immature oocytes was significantly different among boars ($P < 0.001$, Fig. 1A, 1B). First sperm penetration was observed at 3 (boar L), 4 (boar M) or 5 hpi (boar D). Time of gamete incubation also affected the penetration rate increasing with incubation time ($P < 0.001$), whereas monospermy rate decreased with incubation time ($P < 0.001$, Fig. 1C).

When IVM oocytes were used, boars behaved equal keeping their fertilization speed and monospermic/polyspermic profiles ($P < 0.001$, Fig. 2). As was

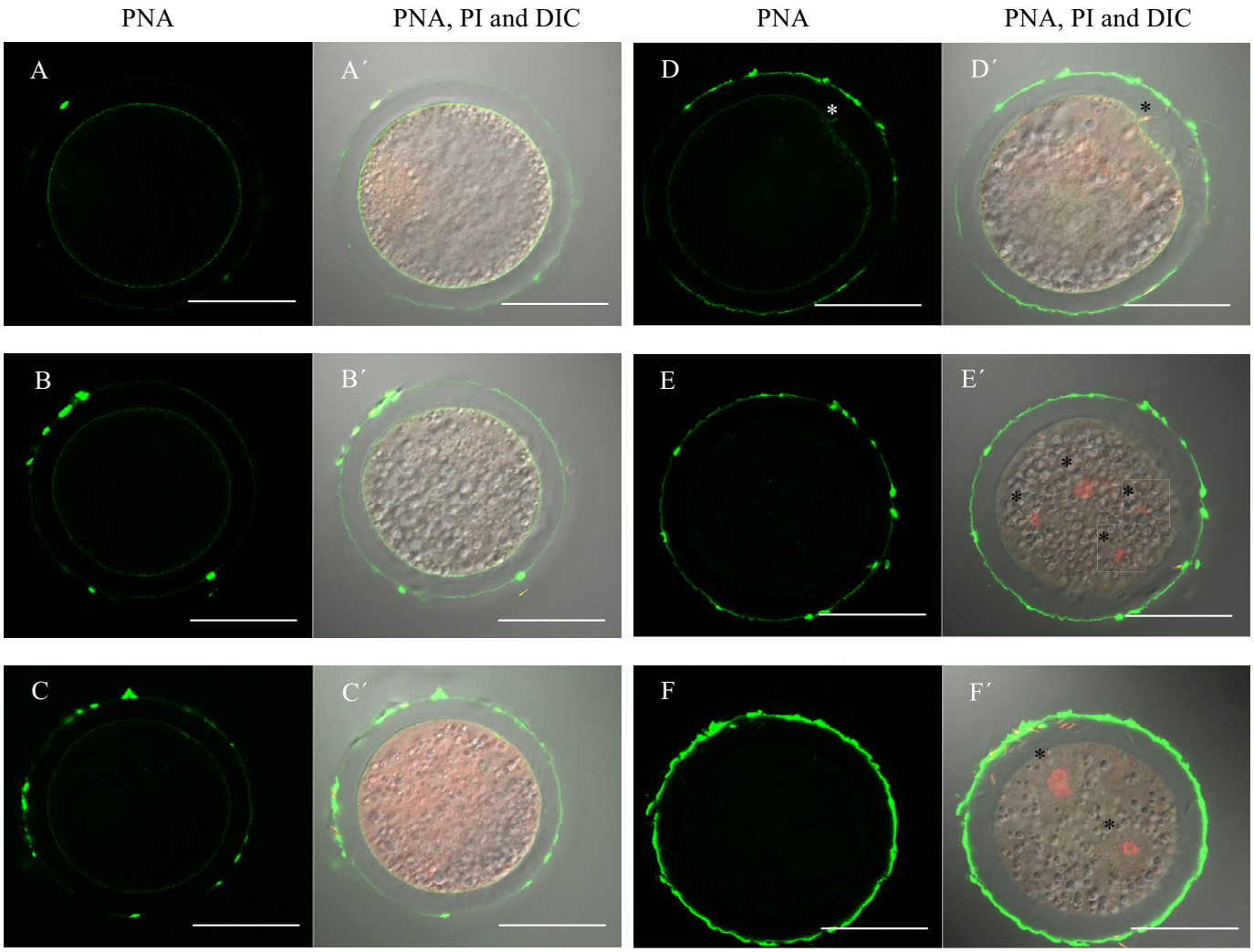

Fig. 4. Multilaser fluorescence and DIC confocal images of oocytes inseminated with epididymal spermatozoa assessed at different hpi after staining with FITC-PNA lectin and PI. A–A′, Unfertilized oocyte after 0.5 hpi with a clear CG monolayer beneath oolema and sperm bound to ZP. B–B′, Unfertilized oocyte after 1 hpi with a CG monolayer and several sperm bound to ZP. C–C′, Unfertilized oocyte after 2 hpi with an evident CG monolayer and numerous sperm bound to ZP. D–D′, Oocyte after 4 hpi with a spermatozoon (asterisk) contacting the oolema and disappearing CG monolayer. E–E′, Polyspermic oocyte with several sperm heads (asterisk) after 5 hpi with a less distinctive CG monolayer and intensively stained outer ZP. F–F′, Polyspermic oocyte after 18 hpi lacking CG, with a very intensively stained outer ZP and many sperm trapped in the thickness of ZP. Scale bar indicates 50 μm.

observed in immature oocytes, boar L showed the faster (2 hpi) and highest penetration rate (95.1 ± 2.8) with the lowest monospermy rate (8.6 ± 3.7). However, boar M reached 52.9 ± 8.7 monospermy rate at 18 hpi. IVM oocytes inseminated with D boar were always monospermic. Monospermy rate was affected by boar and coincubation time ($P < 0.05$). First male pronuclear (MPN) formation was observed at 5 hpi and over 85% of oocytes had formed an MPN at 18 hpi. No boar effect was observed for this parameter.

Overall IVM oocytes were penetrated faster and to a higher degree than immature oocytes.

3.2. Multiple sperm penetration accelerates the release of cortical granules

After the maturation period, both IVM and IVV control oocytes showed a similar cortical area occupied by CG (20.1 ± 0.5 and 20.4 ± 2.8%, respectively, Table 1). After being kept for 5 h in TALP medium, IVM oocytes showed a CG decrease (15.3 ± 0.4, $P < 0.001$, Table 1, Fig. 3) that was not significant in IVV oocytes (16.7 ± 1.2, $P > 0.05$, Table 1). This decrease of CG area observed in non inseminated (control) oocytes might be a consequence of a partial CG release since after 5 h in TALP medium some oocytes from both had FITC-PNA fluorescent material deposited in the perivitelline space (Fig. 3E).

Table 2
Area occupied by CG in unfertilized and fertilized *in vitro* (IVM) and *in vivo* (IVV) matured porcine oocytes inseminated with different boars and assessed at different hours post insemination (hpi).

Boar	hpi	IVM OOCYTES		IVV OOCYTES	
		Unfertilized (N)	Fertilized (N)	Unfertilized (N)	Fertilized (N)
L	2	19.67 ± 0.43 (28)		20.64 ± 2.28 (17)[a]	7.98 ± 0.56 (2)[b]
	4	19.64 ± 0.68 (11)[a]	6.66 ± 0.65 (17)[b]	20.86 ± 2.13 (9)[a]	2.20 ± 0.53 (18)[b]
	5	16.14 ± 1.94 (4)[a]	2.17 ± 0.66 (23)[b]	16.49 ± 0.71 (2)[a]	1.68 ± 0.26 (24)[b]
	18		1.87 ± 0.43 (25)	12.55 (1)[a]	1.73 ± 0.55 (32)[b]
M	4	18.76 ± 0.42 (20)[a]	8.12 ± 0.59 (4)[b]	19.95 ± 1.76 (12)[a]	9.65 ± 2.36 (13)[b]
	5	16.77 ± 0.35 (20)[a]	4.91 ± 0.67 (7)[b]	20.84 ± 2.53 (22)[a]	8.03 ± 2.87 (9)[b]
	18	12.81 ± 1.32 (6)[a]	2.05 ± 0.44 (21)[b]	10.56 ± 1.18 (12)[a]	0.89 ± 0.26 (16)[b]
D	4	18.64 ± 0.32 (22)[a]	2.46 ± 0.69 (5)[b]	18.47 ± 4.65 (10)[a]	6.64 ± 0.80 (4)[b]
	5	16.26 ± 0.64 (31)[a]	1.01 ± 0.48 (6)[b]	17.20 ± 2.22 (14)[a]	5.26 ± 0.94 (5)[b]
	18	12.98 ± 0.55 (24)[a]	1.14 ± 0.36 (11)[b]	11.94 ± 0.47 (10)[a]	0.79 ± 0.45 (8)[b]

N (number of oocytes evaluated). Results are represented as mean ± SEM. Data from the same source of oocytes with different letters between fertilized/unfertilized and for the same boar differ significantly ($P < 0.05$).

Regarding the CR in the fertilized oocytes, the CG band started to disappear as sperm contacted or fused with the oolemma (Fig. 4) and by 4 hpi, the CG disappearance was around 60% independently of the oocyte source (IVM or IVV) and the boar (Table 2). The direct comparison between IVM and IVV oocytes showed that at 5 hpi IVV oocytes had a significantly higher CG area than IVM and at 18 hpi their exocytosis of CG was almost full with <1% of CG area remaining ($P < 0.05$). Among monospermic and polyspermic penetrated oocytes at the different times (4, 5 and 18 hpi), the monospermic displayed a slower disappearance of the CG area than polyspermic ones (Fig. 5A). Finally, the individual boar significantly affected the CR pattern ($P < 0.05$, Fig. 6) and two patterns were observed: whereas IVV oocytes inseminated with L boar ("polyspermic" boar) showed a fast decrease of CG area at 4 to 5 hpi, M and D boars ("monospermic" boars) provoked a steady and continuous release of CG over time.

Regarding the presence of ZP-galactose-β(1-3)-Nacetylgalactosamine moieties, no labeling of the outer or inner ZP by FITC-PNA was observed in noninseminated (control) oocytes (Table 1) in spite of some oocytes partially releasing their CG contents to the perivitelline space after 5 h in culture medium (Fig. 3F). However, when oocytes were inseminated, the rate of stained ZP significantly increased along with culture time both in unfertilized and fertilized oocytes (see Suppl. Table 1). This was coincident with a longer time of gamete coincubation and thus a higher number of spermatozoa bound to ZP (Fig. 7). At each specifically studied time point, the FITC-PNA labeled ZP area was the same between IVM and IVV oocytes; and between monospermic and polyspermic oocytes (Fig. 5B). A boar effect was not observed on labeled ZP areas at these time points. The area occupied by CG was not correlated to the percentage of stained ZP ($P > 0.05$).

To confirm that the positive labeling of ZP with PNA was due to acrosomal content deposited during gamete coculture and not due to ZP reaction because of CG contents, isolated *in vitro* matured ZPs lacking of ooplasm content inside were inseminated. Results showed positive PNA labeling on the outer ZP surface (Fig. 8). Labeling increased with gamete coculture time and was concomitant with an increasing number of bound spermatozoa on the ZP surface. This can be clearly observed when ZPs are assessed at 0.5 hpi (Fig. 8B) and 18 hpi (Fig. 8F). As ooplasm had been completely removed, contribution of CG contents to ZP labeling by PNA was not possible.

4. Discussion

Since the discovery of CG in mammalian eggs, much work has been carried out on their role in fertilization. Prevention of polyspermy, zona reaction, and changes of the cell surface have been assigned to CG and their contents (reviewed by [1]).

The effect of boar and incubation time has been widely studied in porcine *in vitro* fertilization (IVF) systems, both with immature and *in vitro* matured (IVM) oocytes. Frozen-thawed epididymal spermatozoa have shown consistent IVF rates with minimal variability compared to ejaculate ones [11,18] and were therefore employed in this study. A marked effect of boar and incubation time on *in vitro* penetrability was observed. This is in concordance with other studies employing ejaculated spermatozoa, where IVF results were also strongly influenced by the boar [16,21,22]. In

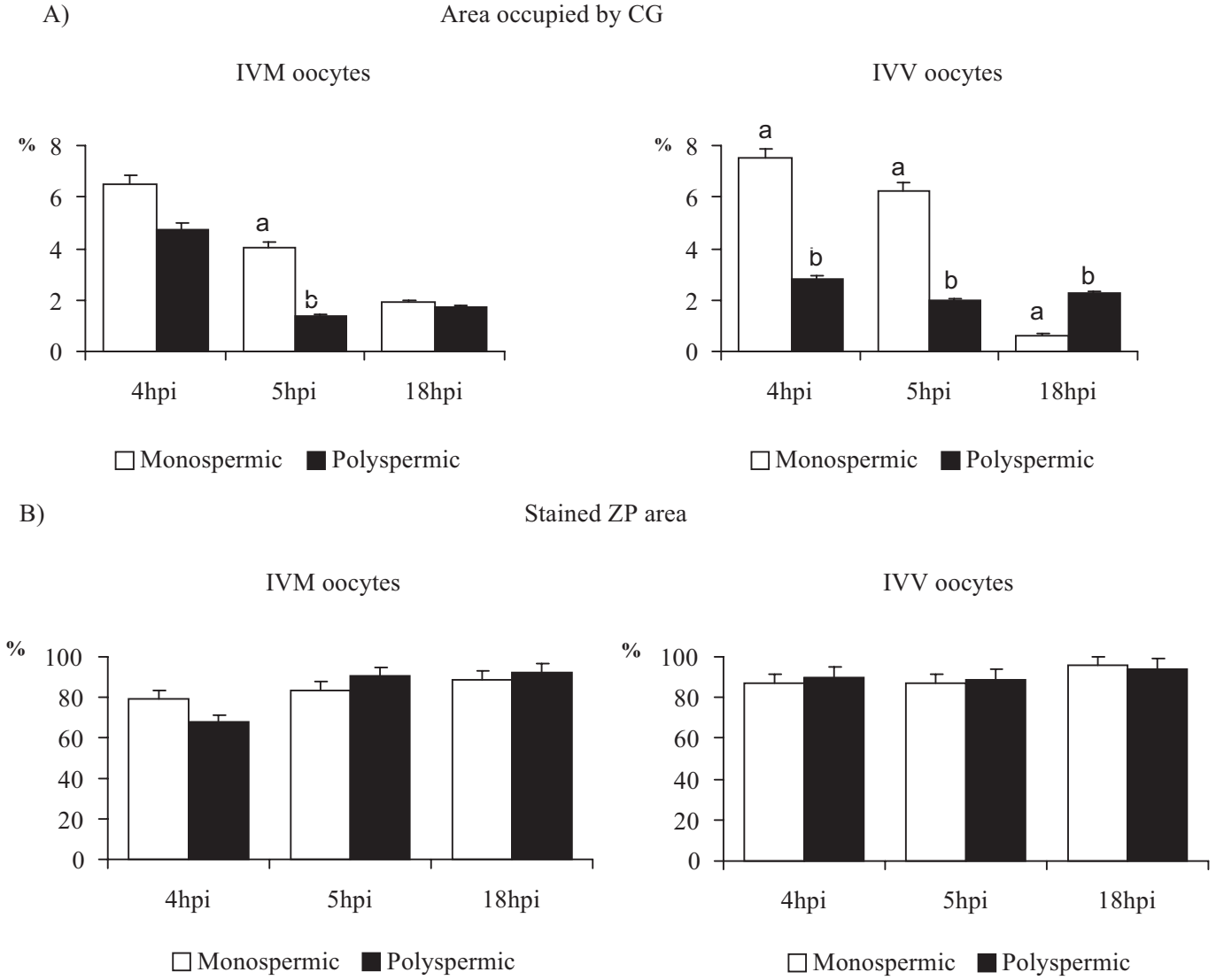

Fig. 5. Area occupied by CG (A), and ZP area labeled by FITC-PNA (B), in monospermic and polyspermic oocytes after being *in vitro* (IVM) or *in vivo* (IVV) matured. Results are represented as mean ± SEM. Data with different letters at the same hpi differ significantly ($P < 0.05$).

this study, the first fertilized oocytes were observed at 2 hpi (IVM oocytes) and 3 hpi (immature oocytes), while frozen-thawed ejaculated boar spermatozoa started to penetrate IVM oocytes at 3 hpi [23]. When ejaculated sperm are used, the boar is known to influence male pronucleus formation [16]. We did not observe such an effect with epididymal spermatozoa, which could be explained by the improved cytoplasmic maturation of oocytes achieved in the current IVM systems [24]. Individual sperm samples with faster sperm penetration also reached the highest polyspermy rate and one boar reached 100% monospermy independently of coculture time. Likely reasons for the boar effect are explained later.

In the present study, IVM and *in vivo* matured (IVV) oocytes showed similar cortical areas covered by CG (around 20%) coinciding with our previous studies in IVM oocytes [10,11] and with others describing that, once the maturation period is complete, IVM and IVV oocytes do not differ in the number of CG [12]. However, we observed that IVM oocytes have a decrease of their CG area over time when they are kept in TALP medium in the absence of spermatozoa and also in those inseminated but not fertilized. Several reasons could explain this decrease in lectin binding, such as i) changes in the content and/or type of CG, ii) oocyte overmaturation and aging, iii) CG internalization, and iv) effect of IVF medium compounds. Cran and Cheng [25] described that at 42 h of maturation the majority of CG were light-type and at 50 h dark-type CG were more abundant. They explained their transmission electron microscopy studies as a degeneration and overma-

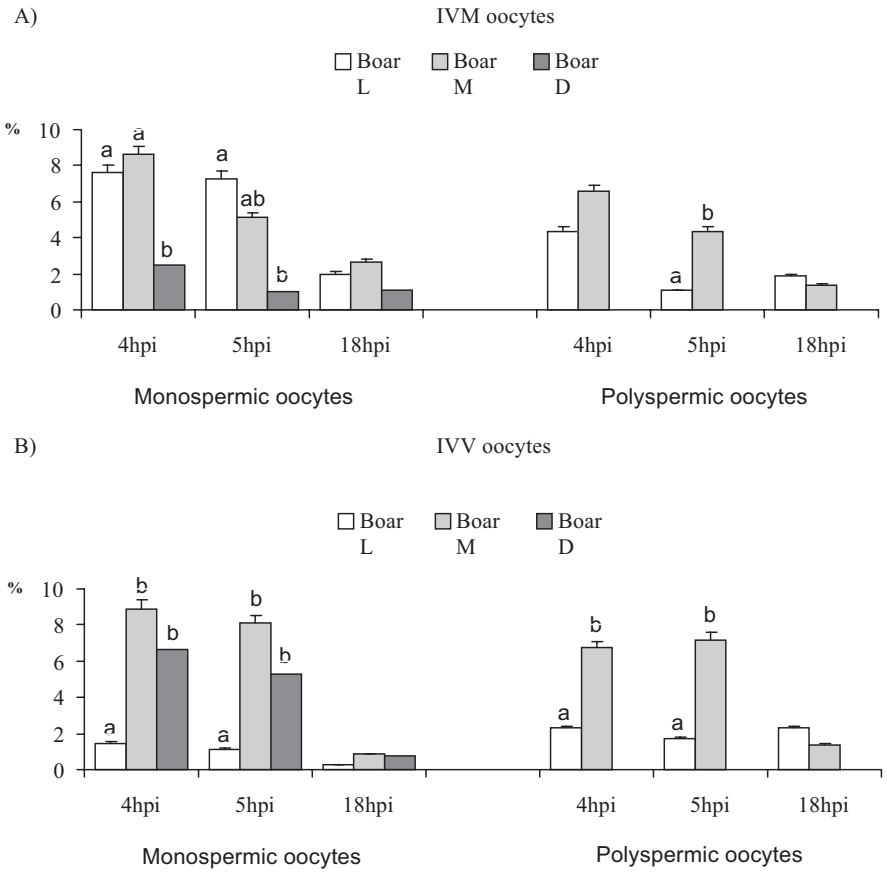

Fig. 6. Area occupied by CG in *in vitro* (IVM; A) and *in vivo* (IVV; B) matured oocytes inseminated with three different boars. Results are represented as mean ± SEM. Data with different letters at the same hpi differ significantly ($P < 0.05$).

turation of CG contents. Thus, in our study, PNA labeling of oocytes kept in culture for 5 and 18 h (corresponding to 49 and 62 h of maturation, respectively) was different from those oocytes freshly assessed after 44 h of maturation. Moreover, aged oocytes showed a decreased number of CG [26] and an internalization of these organelles into ooplasm [27]. Because we only selected the cortical area for image analysis, the likely internalized CG would not be taken into account for analysis.

Regarding the CR, it has been reported for pig oocytes that the triggering of a "fast" CR is necessary to block polyspermy effectively and that a "delayed" CR could account for higher polyspermy rates [4]. However, for other authors a delayed CG exocytosis is unlikely to be a major factor for an insufficient zona block [5]. Concerning the oocyte's maturation (*in vivo* vs. *in vitro*), some studies have observed a different dispersal of CG between IVM and IVV oocytes [5], whereas others showed a similar CR pattern between both kind of oocytes at 6 hpi [12] and 18 hpi [14]. The conflicting information about CR in pigs might be explained by the low number of oocytes used in the studies, the assessment of CR at a specific time point instead of a complete time window and the use of oocytes from gilts and sows [12]. In our study all oocytes were collected from prepubertal animals and our results are in agreement with the data from Cran and Cheng [5] who suggested differences between CR in IVM and IVV oocytes. Under monospermic penetration we observed that at 4 hpi both type of oocytes had similar CG areas (around 6–8%), whereas at 5 hpi the CG decrease was higher in IVM oocytes. In addition, at 18 hpi the CG release from IVV oocytes was complete (CG area 0.64%), whereas IVM oocytes still had CG in 2% of the cortical area. The complete release of GC and full CR observed in IVV oocytes is of great importance, since it has been described that CG-derived proteins play not only a role in the zona block but also on further preimplantation embryo development [28]. We also observed that CR is slower in monospermic than polyspermic oocytes (Fig. 5A), and this steady CR is evident in IVV oocytes. At all time points studied, the polyspermic oocytes showed a lower CG area than monospermic ones.

Our results showed a boar effect both on IVF parameters and CR. After fertilization, a soluble activating factor (phospholipase C-zeta) in the spermatozoon

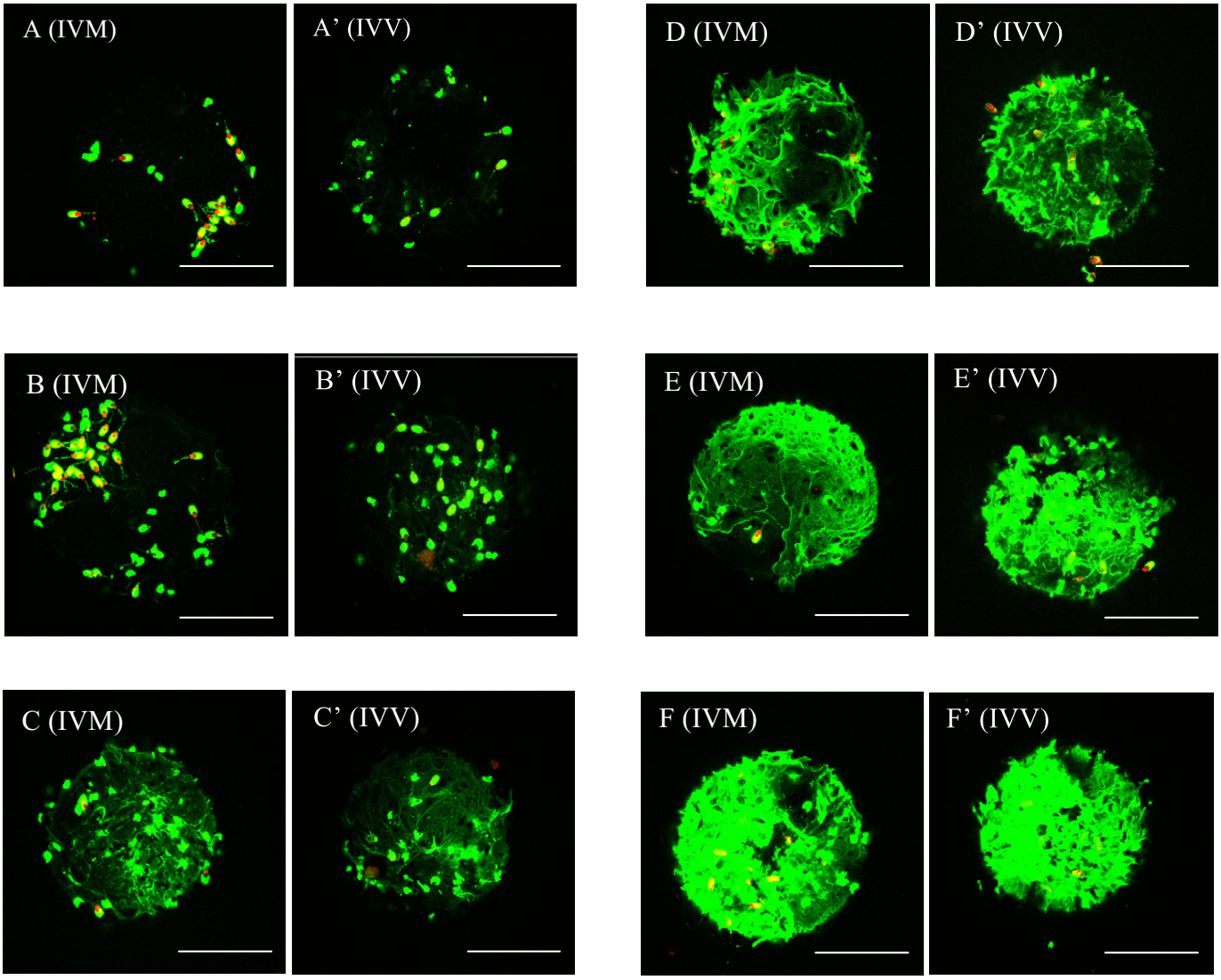

Fig. 7. Projection of acquired confocal images of *in vivo* (IVV) and *in vitro* matured (IVM) oocytes inseminated with epididymal spermatozoa and assessed at different hpi. Staining by FITC-PNA and PI. Sperm bound to ZP and labeling of ZP surface by PNA is observed. A, A′, Oocytes after 0.5 hpi, B, B′, 1 hpi, C, C′, 2 hpi, D, D′, 4 hpi, E, E′, 5 hpi and F, F′, 18 hpi. Scale bar indicates 50 μm.

is delivered to the cytosol inducing oocyte activation, a highly complex process that involves different players (reviewed by [29]). Penetrability has been attributed to the boar breed [30] as well as post thawing sperm quality (reviewed by [31]). All boars in the present study belonged to the same breed and post-thawing motility was similar (around 70%, data not shown). However, differences in the time course of sperm penetration and CR were still present. The explanation might involve differences between sperm protein profiles in the individual boars and different induction of Ca^{2+} oscillations [32,33]. This hypothesis should be further investigated.

From our study, and others mentioned above, it could be concluded that pig CR starts at 3 to 4 hpi with a first exocytosis of about 60% of the CG. It continues for at least 1 h more, and by 18 hpi the CG occupy only 1 to 2% of cortical area. If we combine the more physiological conditions from our study (IVV oocytes and monospermic penetration) it is clear that these oocytes display a steady and time-released CR. We hypothesized that progressively released CR could contribute more efficiently to the block of polyspermy than an "immediate, fast and massive release" of CG content permitting a better dispersion of CG contents and also a longer interaction with oolemma and/or inner surface of ZP. In hamster it has been demonstrated that the dispersal of the CG contents is slow and it does not occur immediately after exocytosis [34] and the "massive" release of CG content could account for the difficulties of dispersal in the perivitelline space that have been reported in IVM pig oocytes [5]. After CG release,

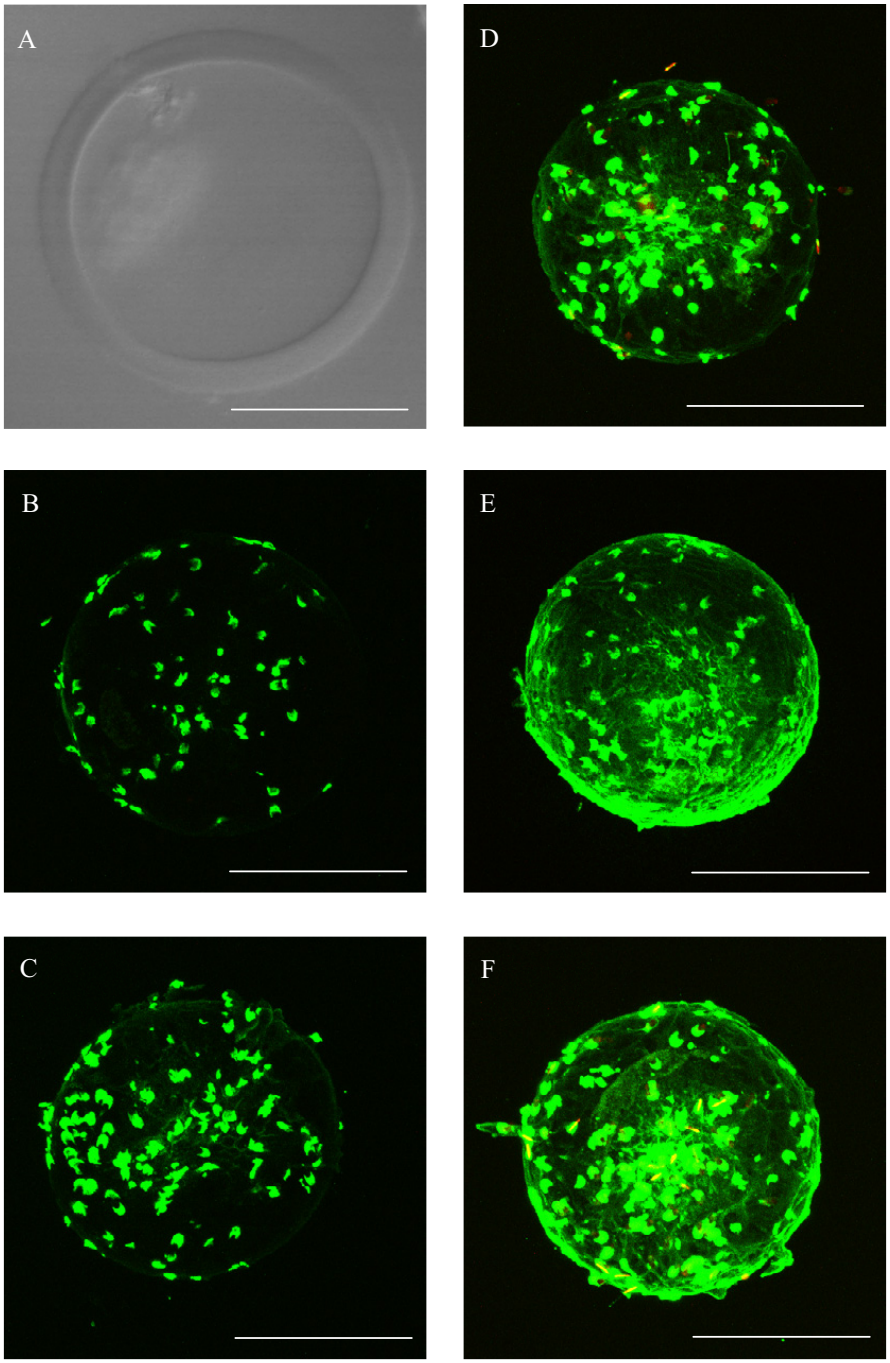

Fig. 8. Projection of acquired confocal images of *in vitro* matured ZPs inseminated with epididymal spermatozoa and assessed at different hpi. FITC-PNA labeling. A, Isolated ZP after aspiration of ooplasm. B, ZP after 1 hpi, (C) 2 hpi, (D) 4 hpi, (E) 5 hpi and (F) 18 hpi. Scale bar indicates 50 μm.

porcine ZP requires a prolonged time in order to complete ZP modifications [35] confirming the beneficial effects of a steady CR to complete the ZP reaction and block polyspermy. Moreover, a steady CR would prolong the time for the endocytotic process that has been described to take place after CR and has great physiological importance [34,36]. In our opinion it is not excessive to suggest a slowed down CR in pigs since we demonstrate in recent studies that the final block to polyspermy in this species also relies on other mechanisms, such as gamete contact with oviductal secretion [37,38].

We observed an increase in PNA binding sites after insemination resulting in strong ZP labeling by FITC-PNA lectin. This interesting finding was also observed by Li, et al. [13]. Since porcine CG, but not ZP, are

specifically stained with PNA [20] this observation could have two possibilities: i) CG contents released from oocytes may diffuse into the ZP; thus, the ZP would also be stained by FITC-PNA; ii) ZP may be modified by the enzymes released from CG; thus, the modified ZP would be labeled by FITC-PNA. The first possibility is unlikely since the staining pattern was observed in inseminated ZPs (without ooplasm) and inseminated, but not fertilized, oocytes. Although the second possibility cannot be completely ruled out, we attribute this ZP surface labeling to the presence of acrosomal shrouds as other authors have proposed [39], since the boar acrosome is labeled by PNA [40,41]. We observed that ZP reactivity increased along with gamete coculture time when a high number of sperm have contacted the ZP and reacted.

In conclusion, our study shows that after IVF with epididymal spermatozoa penetrability is higher in IVM than in immature oocytes and there is a strong boar effect on different IVF parameters, where the faster boar sperm penetrating an oocyte is related to the highest polyspermy rate and faster male pronucleus formation. The CR pattern is affected by boar, oocyte maturation (IVV vs. IVM) and degree of sperm penetration (monospermic vs. polyspermic). After insemination, the outer ZP strongly increases its affinity to PNA lectin along with gamete coincubation time. This carbohydrate residue modification is not due to CG contents but to the presence of acrosomal shrouds. The set of our observations shows that a maintained and steady release of CG after 4 to 5 h post-insemination would be necessary for a further effective block of polyspermy and boar selection should be taken into consideration to achieve an appropriate CR.

Appendix A Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.theriogenology.2012.05.009.

Acknowledgments

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Suppl. Table 1
Rate of ZP area labeled by FITC-PNA in *in vitro* (IVM) and *in vivo* (IVV) matured porcine oocytes after coincubation for different hours with epididymal spermatozoa from different boars (L, M, D).

Boar	Hours	IVM OOCYTES		IVV OOCYTES	
		Fertilized (N)	Unfertilized (N)	Fertilized (N)	Unfertilized (N)
L	0.5		25.2 ± 5.6 (28)[a]		13.4 ± 3.4 (24)[a]
	1		40.9 ± 6.3(26)[ab]		27.5 ± 4.3 (22)[a]
	2		57.2 ± 5.4 (28)[b]	95.92 ± 3.98 (2)	70.3 ± 4.5 (17)[b]
	4	75.0 ± 4.8 (17)[a]	80.9 ± 4.5 (11)[c]	91.79 ± 3.51 (18)	73.2 ± 7.8 (9)[b]
	5	89.5 ± 2.6 (23)[b]	84.8 ± 1.6 (4)[c]	90.03 ± 1.36 (4)	87.9 ± 8.4 (2)[b]
	18	92.9 ± 1.3 (25)[b]		95.90 ± 0.77 (32)	87.0 (1)[b]
M	0.5		13.2 ± 4.0 (26)[a]		13.1 ± 3.1 (18)[a]
	1		38.1 ± 5.8 (24)[b]		21.0 ± 5.1 (22)[a]
	2		49.3 ± 4.3 (26)[b]		43.1 ± 5.2 (22)[b]
	4	76.5 ± 1.8 (4)[a]	76.1 ± 3.6 (20)[c]	83.32 ± 3.65 (13)	63.9 ± 6.8 (12)[b]
	5	83.3 ± 1.7 (7)[b]	82.8 ± 2.0 (20)[c]	83.61 ± 5.13 (9)	62.5 ± 5.4 (22)[b]
	18	87.9 ± 1.8 (21)[b]	85.7 ± 3.1 (6)[c]	92.00 ± 1.65 (16)	65.8 ± 7.1 (12)[b]
D	0.5		47.42 ± 4.66 (28)[a]		39.4 ± 7.2 (10)[a]
	1		58.77 ± 3.71 (29)[a]		23.7 ± 6.1 (10)[a]
	2		76.68 ± 2.88 (29)[b]		73.2 ± 8.2 (10)[b]
	4	82.6 ± 3.8 (5)	74.90 ± 4.23 (22)[b]	92.94 ± 4.18 (4)	88.9 ± 3.6 (10)[b]
	5	86.2 ± 4.4 (6)	82.27 ± 2.81 (31)[b]	88.98 ± 2.74 (5)	85.5 ± 4.9 (14)[b]
	18	90.4 ± 2.3 (11)	88.97 ± 3.13 (24)[b]	95.93 ± 1.65 (8)	94.3 ± 1.9 (10)[b]

N (number of oocytes evaluated). Results are presented as mean ± SEM. Fertilized and unfertilized oocytes were analyzed separately. A two-way ANOVA was performed considering boar and hpi as main effects. When a significant effect was revealed, values were compared using the Tukey test. Different letters in the same column and boar differ significantly ($P < 0.02$).