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Zona pellucida birefringence correlates with developmental capacity of bovine oocytes classified by maturational environment, COC morphology and G6PDH activity

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Abstract. In the present study we aimed to analyse structural changes during *in vitro* maturation of the bovine zona pellucida (ZP) by scanning electron microscopy (SEM) ands zona pellucida birefringence (ZPB). Here we show that alterations during *in vitro* maturation invasively analysed by SEM are reflected in ZPB. *In vivo*-matured oocytes displayed significantly lower birefringence parameters and significantly higher blastocyst rates compared with *in vitro*-derived oocytes (39.1% vs 21.6%). The same was observed for *in vitro*-matured oocytes with cumulus–oocyte complex (COC) Quality 1 (Q1) compared with Q3-COCs with respect to zona birefringence and developmental capacity. Immature oocytes with Q1-COCs displayed higher ZPB values and a higher developmental capacity to the blastocyst stage (27.7% vs 16.9%) compared with immature Q3-COCs. Considering *in vitro*-matured oocytes, only those with Q1-COC showed a trend for ZPB similar to *in vivo*-matured oocytes. Therefore, a decreasing trend for ZPB during *in vitro* maturation seems to be typical for high-quality oocytes and successful cytoplasmic maturation. In accordance, fully-grown immature oocytes reached significantly higher blastocyst rates (32.0% vs 11.5%) and lower ZPB values compared with still-growing ones. In conclusion, we successfully evaluated the applicability of zona imaging to bovine oocytes: alterations during *in vitro* maturation invasively analysed by scanning electron microscopy were reflected in the birefringence of the zona pellucida of bovine oocytes affecting developmental capacity at the same value. Therefore ZPB measurement by live zona imaging has potential to become a new tool to assess correctness of *in vitro* maturation and to predict developmental competence.

Additional keywords: development, *in vitro* maturation, IVP, scanning electron microsopy, viability.

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Introduction

Developmental competence of bovine *in vitro*-produced embryos remains significantly lower compared with their *in vivo*-derived counterparts. Despite intensive research a large proportion of bovine oocytes fail to develop to the blastocyst stage following maturation, fertilisation and *in vitro* culture (Farin and Farin 1995). It is generally accepted that oocyte quality is a key factor for optimising the efficiency of reproductive techniques in farm animals as well as for human assisted reproductive technologies (Coticchio *et al.* 2004; Sirard *et al.* 2006; Telfer and McLaughlin 2007; Van Soom *et al.* 2007; Wang and Sun 2007). Therefore, identification of credible predictors for developmental competence of bovine oocytes for *in vitro* production is indispensible.

A variety of cellular and subcellular parameters have been investigated to determine whether they are related to developmental competence, such as gene expression pattern (Wrenzycki *et al.* 2007), mitochondrial status (Stojkovic *et al.* 2001), calcium stores and calcium current activity (Boni *et al.* 2002), apoptotic index (Yuan *et al.* 2005), gene expression profiles in cumulus cells (Assidi *et al.* 2008; Tesfaye *et al.* 2009) and factors present in the follicular fluid (Nicholas *et al.*

2004; Van Soom *et al.* 2007; Sinclair *et al.* 2008). Unfortunately, these techniques are often complex, time-consuming and, most importantly, invasive, which excludes further development of the oocyte. Therefore, non-invasive criteria to evaluate oocytes potency based on morphology were investigated during the last decade, including homogeneity of ooplasm, thickness and diameter of oocytes as well as compactness of surrounding cumulus layers (Blondin and Sirard 1995; Nagano *et al.* 2006; Santos *et al.* 2008). However, the predictive value of these morphological characteristics as evaluated by light microscopy is controversial, due to subjectivity and inaccuracy (Lonergan 2007; Wang and Sun 2007; Nagy 2008).

The intrinsic quality of an oocyte, which is acquired during folliculogenesis, relying on vascularisation, oxygen content and cumulus cell characteristics, is a great factor affecting subsequent development of an embryo (Van Blerkom *et al.* 1997; Corn *et al.* 2005). Thereby, any negative effect during folliculogenesis will harm the oocyte, resulting in substantial morphological alterations like discoloration (Esfandiari *et al.* 2006), shape anomaly (Ebner *et al.* 2008), zona splitting (Shen *et al.* 2008) or changes to its three-dimensional structure.

It has been reported that the mean difference in thickness between zona pellucida (ZP) from human conception cycles and failed ones was ~1 μm (Shen *et al.* 2005); a value that is, however, beyond the limit of verifiability of most systems designed for measuring cells. In contrast to light microscopic analysis of bovine oocytes, scanning electron microscopic (SEM) analysis of bovine oocytes has already identified clear relationships between oocyte morphology and developmental competence: Using SEM, the structure of the ZP in human oocytes matured *in vitro* is seen as a large multilayered network resembling a sponge, whereas in immature and atretic oocytes the ZP has a compact and smooth surface (Familiari *et al.* 2006). Santos *et al.* (2008) reported that the number of pores on the ZP surface varies with the quality of the oocyte. Furthermore, significant differences were ascertained in the thickness of the ZP between species, varying from 5 μm in the mouse to 27 μm in the cow (Dunbar *et al.* 1994), as well as between *in vitro* and *in vivo* embryogenesis (Michelmann *et al.* 2007). Recent investigations revealed that matured bovine oocytes with high-quality cumulus–oocyte complex (COC), classified by stereomicroscope, had significantly smaller zona pore diameters compared with those of low-quality oocytes (Santos *et al.* 2008). However, application of SEM does not allow further development of the analysed oocytes because of its invasive nature.

In contrast, the recent introduction of polarisation light microscopy opens a new window for non-invasive assessment of morphological zona pellucida properties. Zona pellucida imaging at the metaphase-II stage of oocyte development was established successfully as a predictive marker for human oocyte quality in several studies (Rama Raju *et al.* 2007; Montag *et al.* 2008; Madaschi *et al.* 2009; Ebner *et al.* 2010).

Although oocyte maturation remains a poorly understood process, the follicle can be considered as the reproductive unit of the ovary. Initiation of germinal vesicle breakdown and completion of nuclear changes lead to the extrusion of the first polar body and arrangement of the second metaphase plate (Lin and Hwang 2006). The formation of metaphase-II stage oocytes is closely linked to completion of nuclear changes, but do not correlate with full developmental competence based on molecular and structural maturity in all oocytes (Trounson *et al.* 2001). Any factor affecting follicular recruitment and growth may influence the secretion of cumulus cells and oocytes (Qi *et al.* 2002). Hence, the physiology of folliculogenesis (Pelletier *et al.* 2004) and the quality of *in vitro* culture might affect the texture of the zona pellucida. Coincidentally, characteristics and appearance of the ZP may reflect the history of folliculogenesis (Qi *et al.* 2002) and be useful as markers of correct folliculogenesis and normal oocyte maturation and predictors for subsequent developmental competence.

Previous studies speculated that properties of the zona layers might reflect the history of human oocyte cytoplasmic maturation (Liu *et al.* 2003) whereupon, the zona pellucida birefringence (ZPB) from germinal vesicle (GV)-stage oocytes showed a significantly decreasing trend to MII-stage oocytes (Cheng *et al.* 2010). In accordance, a higher percentage of high-birefringence oocytes was observed in human oocytes being in prophase I compared with metaphase I stage. Interestingly, the percentages of high-birefringence oocytes did not change when comparing oocytes before and after *in vitro* maturation for both prophase I and metaphase I oocytes (de Almeida Ferreira Braga *et al.* 2010). If ZPB indeed correlates with developmental competence that would imply that developmental competence is already fixed before *in vitro* maturation. That, in turn, would suggest that a better selection of oocytes rather than improving *in vitro* maturation conditions is necessary to improve overall *in vitro* developmental rates. However, all studies published so far focussed on human assisted reproductive technologies (ART) and it remains an open question if these results could be transferred to the cow.

Taking into account that oocytes are usually of unknown origin with inhomogeneous developmental competence in bovine *in vitro* production (IVP), any correlation between zona pellucida characteristics with age of donor, follicular origin, maturational stage as well as maturation environment could be fruitful to select more homogenous groups of oocytes bearing higher developmental competence. However, the relationship between oocyte cytoplasmic maturation and ZPB in the cow is still inexplicit.

The aim of the present study was therefore to evaluate the applicability of zona imaging to bovine oocytes. Therefore we analysed subpopulations of bovine oocytes bearing variable prospective developmental competence, classified according to their environment of maturation (*in vivo* vs *in vitro*), their cumulus cell investment and their glucose-6-phosphate dehydrogenase (G6PDH) activity with respect to zona pellucida properties and their developmental competence, simultaneously. As an invasive technique, we analysed the zona pellucida of *in vivo*- and *in vitro*-derived oocytes by scanning electron microscopy to visualise alterations of the morphological structure before and after maturation related to developmental competence. Using polarisation light microscopy as a non-invasive technique, we consequently aimed to find out whether these structural changes are also reflected in the zona birefringence.

Materials and methods

Oocyte collection

Bovine ovaries were obtained from a local slaughterhouse and transported in warm (30–35°C) physiological saline solution within 1–3 h. Antral follicles (2 to 8 mm in diameter) were aspirated using an 18-gauge needle attached to a 10-mL syringe and collected into a 50-mL conical tube. COCs with evenly granulated oocyte cytoplasm surrounded by more than three compact layers of cumulus cells were selected and transferred to modified tissue culture medium (TCM199; Sigma, Taufkirchen, Germany) supplemented with 4.4 mM Hepes, 33.9 mM $NaCHO_3$, 2 mM pyruvate, 2.9 mM calcium lactate, 55 μg mL^{-1} gentamycin and 12% heat-inactivated oestrous cow serum (OCS).

Assessment of cumulus–oocyte complex (COC) quality

The assessment of COC morphology was performed as follows. Briefly, oocytes with presence of clear and compact cumulus cells with more than three layers were allocated to Quality 1 (Q1), oocytes with a compacted cumulus with two or three layers were classified as Quality 2 (Q2) and oocytes with one or fewer layers of cumulus cells were judged as Quality 3 (Q3).

Assessment of glucose-6-phosphate dehydrogenase (G6PDH) activity

We performed the brilliant cresyl blue (BCB) stain to separate oocytes undergoing growth and those that have completed their growth phase as described in our previous study (Ghanem *et al.* 2007). Briefly, immature oocytes with high-quality COCs (Q1) were subjected to 26 μM BCB (Sigma-Aldrich, Taufkirchen, Germany) diluted in mDPBS for 90 min at 38.5°C in humidified air atmosphere. After washing, stained COCs were examined under a stereomicroscope and categorised into two groups according to their cytoplasmic staining: oocytes with any degree of blue colouration in the cytoplasm (BCB+) were classified into the low G6PDH activity group, representing fully-grown immature oocytes, and oocytes without visual blue colouration (BCB–) were classified into the high G6PDH activity group, representing still-growing immature oocytes.

In vitro *maturation*

COCs were washed and incubated (in groups of 50) in 400 mL of maturation medium that consisted of TCM-199 (Sigma) with Earle salts buffered with 4.43 mM HEPES (Sigma) and 33.9 mM sodium bicarbonate (Sigma) supplemented with 12% OCS, 0.5 mM L-glutamine, 0.2 mM pyruvate, 50 mg mL^{-1} gentamycin sulfate and 10 μL mL^{-1} FSH (Folltropin; Vetrepharm, London, ON, Canada) in four-well dishes (Nunc, Roskilde, Denmark). The maturation medium was covered with mineral oil (Sigma-Aldrich) and was pre-incubated under the maturation conditions for a minimum of 1 h (38.7°C, 5% CO_2 in air with maximum humidity) and then incubated for 22 h after oocytes were added.

In vitro *fertilisation and* in vitro *culture*

After maturation COCs were co-incubated with spermatozoa (2×10^6 spermatozoa mL^{-1}) in a fertilisation medium consisting of Fert-TALP medium (Parrish *et al.* 1988) supplemented with 10 mM sodium lactate, 1 mM sodium pyruvate, 6 mg mL^{-1} BSA, 1 μg mL^{-1} heparin, 10 μM hypotaurine, 20 μM penicillamine and 2 μM epinephrine at 38.7°C in 5% CO_2 in air.

Eighteen hours after insemination the presumed zygotes were denuded from cumulus cells. Nearly fifty cumulus-free presumptive zygotes were washed three times in CR1 aa (Rosenkrans *et al.* 1993) supplemented with 12% heat inactivated oestrous cow serum (OCS) and then cultured in 400 μL of the same medium in four-well dishes (Nunc) under mineral oil at 38.7°C in 5% CO_2 in humidified air.

Collection of in vivo*-matured oocytes*

Pre-synchronisation was performed by intra-muscular administration of 500 μg cloprostenol (PGF2α, Estrumate; Essex Tierarznei, Munich, Germany) twice within 11 days. Two days after each of the PGF2α treatments animals received 10 μg gonadotrophin-releasing hormone (GnRH, Receptal; Intervet, Boxmeer, Netherlands). Twelve days after the last GnRH injection heifers received the first of eight consecutive FSH-injections over 4 days in decreasing doses (in total 300–400 mg FSH equivalent according to bodyweight; Stimufol, University of Liege, Liege, Belgium). Two PGF2α treatments were performed 58 and 72 h after the initial FSH. Finally, 40 h after the first PGF2α application, 10 μg GnRH was given to each animal. Endoscopic guided follicle aspiration to collect *in vivo*-matured MII-oocytes was performed 16–20 h after GnRH administration.

After restraining the recipients, administering 5 mL of a 2% lidocaine solution (Xylanest Purum1; Richter Pharma,Wels, Austria) for epidural anaesthesia and disinfecting the vulva (Octenisept1; Schülke/Mayer, Vienna, Austria), a trocar set consisting of an universal metal tube (12.5 mm × 52 cm; Storz, Vienna, Austria) and an atraumatic mandrin was placed caudo-dorsal of the fornix vaginae. The mandrin was replaced by a sharp trocar and the trocar set was inserted through the vaginal wall into the peritoneal cavity. The trocar was replaced by a shaft bearing the endoscope (5.5 mm 08 forward Hopkins endoscope; Storz) and the punctation line in the dorsal cavity of the vagina. The site was illuminated by a fibreoptic cold light (250 W; Storz) and visualised with a camera (Telecam PAL-Endovision; Storz) connected to a monitor. The aspiration line consisted of a single lumen needle (diameter 17 G, length 70 cm; William Cook Europe GmbH, Mönchengladbach, Germany) connected to a 50-mL Falcon tube by 100 cm of Teflon tubing. Vacuum pressure was provided by a regulated vacuum pump (V-MAR-5000; William Cook) and adjusted to create a flow rate of 16–20 mL min^{-1}. The collection medium consisted of Hepes-buffered TCM supplemented with 50 mg L^{-1} gentamicin (Sigma, Taufkirchen, Germany), 60 mg L^{-1} heparin (Sigma) and 1% fetal calf serum (FCS; Biochrom, Berlin, Germany).

After bringing the ovary into the right position by transrectal manipulation, the aspiration line was advanced to puncture the ovarian follicle. The follicular contents of all follicles of each heifer were aspirated individually and kept at 39°C in thermos. Finally, the follicular fluid contents were poured

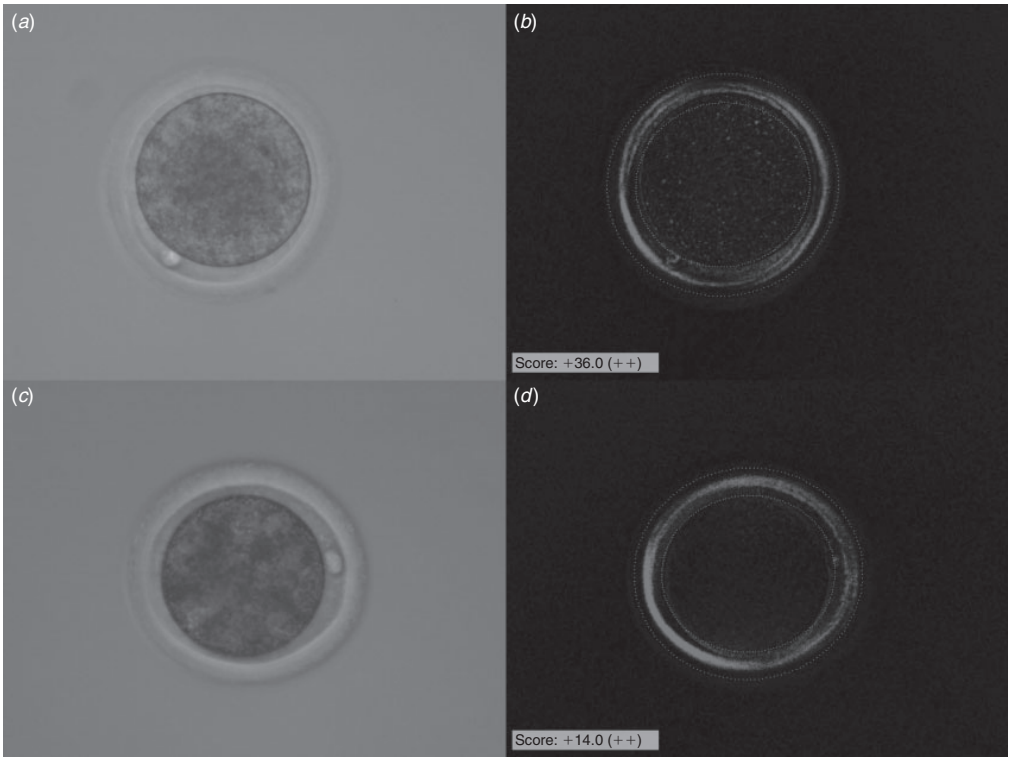

Fig. 1. Imaging of matured *in vivo*-derived metaphase II stage oocyte by (*a*) conventional light microscopy and (*b*) polarised light microscopy. Similarly, images (*c*) and (*d*) show *in vitro*-derived metaphase II oocytes.

into a square grid dish to facilitate finding of oocytes under a stereomicroscope.

Preparation of oocytes for scanning electron microscopy (SEM)

For SEM analysis oocytes were placed in fixation medium composed of 2.5% glutaraldehyde for one hour and washed afterwards in 0.1 M sodium cacodylate for 10 min three times, before two hours' fixation in 2% osmiumtetroxide solution. After repeating the washing step, the samples were dehydrated by plunging them into ethyl alcohol at different concentrations (50–100%) and acetone. Following dehydration the samples were dried in a critical-point dryer (Polaron, Watford, UK). After drying they were coated with 30-nm gold by a Balzers sputtering device (Balzers Union, Balzers, Liechtenstein). SEM observations were conducted with ESEM XL 30 FEG, FEI (Philips, Eindhoven, Netherlands) in the institute for pathology RWTH Aachen.

Live zona imaging

Live zona imaging of individual oocytes was performed non-invasively on a Leica DM IRB inverted microscope equipped with 10×, 20× and 40× Hoffmann interference optics, 20× and 40× stain-free objectives, a circular polarisation filter and liquid-crystal analyser optics. The birefringence analysis including autocalibration was fully controlled by a polarisation imaging software module (OCTAX ICSI Guard; OCTAX Microscience GmbH, Altdorf, Germany) implemented in an imaging software system (OCTAX Eyeware).

In detail, the image processing on the birefringence image (Fig. 1*b*, *d*) extracted several birefringence intensity profiles ($n > 20$) across the inner zona layer. For each of the profiles, i.e. along the entire zona layer, a cumulated birefringence value (CV-Mean) and the average thickness of the inner zona layer (WT-Mean) were calculated. These values (which were computed for each intensity profile, i.e. over the entire cell's circumference) were averaged and resulted in the values CV-Mean and WT-Mean. As plastic dishes interfere with polarised light, glass-bottom dishes (WillCo, Amsterdam, Netherlands) were used for examination. Denuded oocytes were separated in 4-μL drops of HEPES-modified tissue culture medium and imaged at 200× magnification. Each drop was covered with mineral oil; screening in groups of 10 did not last longer than two minutes.

Statistical analysis

Allocation of oocytes into different morphological groups according to their zona characteristics analysed by scanning electron microscopy and developmental rates of embryos generated by *in vitro* fertilisation were analysed by χ^2-test. Analysis

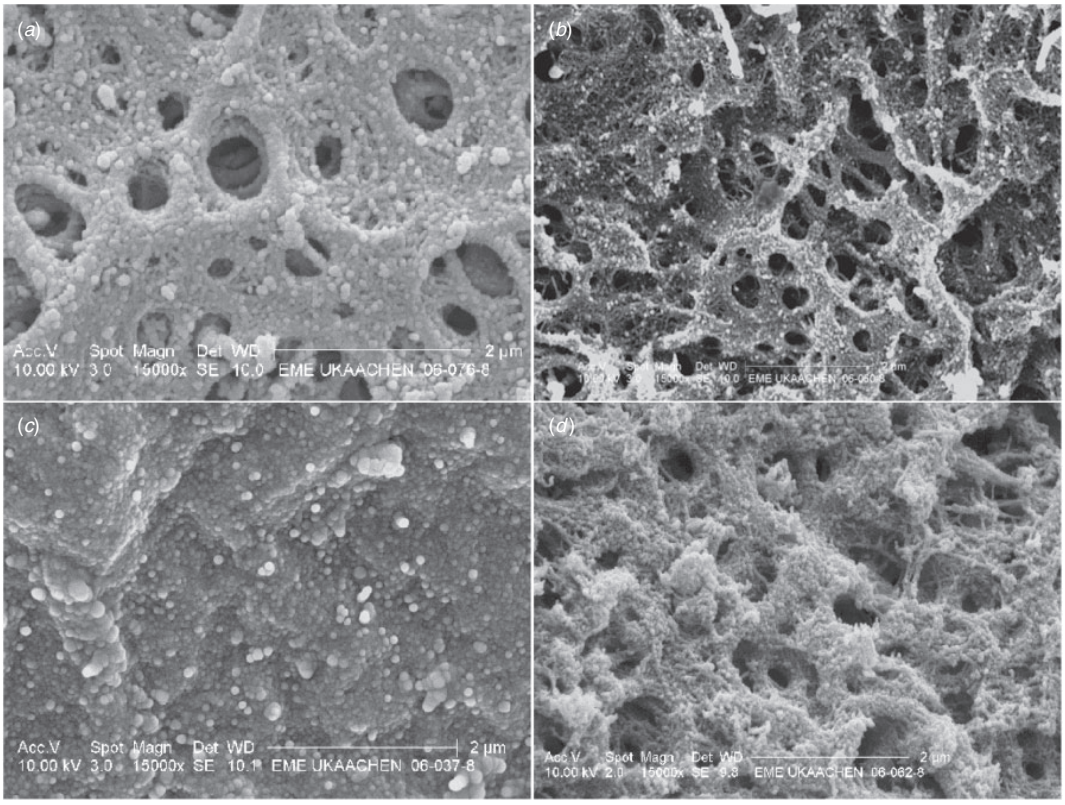

Fig. 2. Representative zona pellucida scanning electron microphotographs of oocytes at different stages of maturity: (*a*) porous surface with a typical pore structure of an immature GV-stage oocyte, (*b*) porous surface with an irregular degraded emerging pore structure of an immature GV-stage oocyte, (*c*) poreless surface of an *in vitro*-matured metaphase II oocyte and (*d*) porous surface with a reticular fine-meshed pore structure of an *in vivo*-matured metaphase II oocyte. All images are 15 000× magnification.

of variance (ANOVA, two tailed *t*-test) was performed for comparison of mean values of zona pellucida evaluation. Differences of $P < 0.05$ were considered to be statistically significant.

Experimental design

In the present work we performed four subsequent experiments. In the first experiment we recapitulated the correlation of the maturational environment (*in vivo* vs *in vitro*) and the COC quality (Quality 1–3) on the bovine zona pellucida structure measured by scanning electron microscopy (SEM). In the second experiment we analysed the effect of the maturational environment and the COC quality on the subsequent *in vitro* developmental competence after *in vitro* fertilisation. In Experiment 3 we aimed to check whether maturational environment and COC quality are reflected in the bovine zona pellucida structure of GV- and metaphase II-stage oocytes analysed by zona pellucida birefringence (ZPB). Finally, in the fourth experiment we compared zona pellucida characteristics measured by ZPB and subsequent developmental competence of immature bovine oocytes classified according to their G6PDH activity as a proven indicator for subsequent developmental competence.

Results

Experiment 1: effect of maturational environment and COC quality on bovine zona pellucida structure measured by SEM

A total of 21 bovine GV-stage oocytes were analysed by SEM. All GV-stage oocytes from Q1+Q2 ($n = 15$) and Q3 ($n = 6$) COCs showed a porous zona pellucida. Considering oocytes of COC Qualities 1+2, a total of 10 (66.6%) displayed a typical fine-meshed reticular pore structure (Fig. 2*a*), whereas the zona of five oocytes (33.3%) showed a pore structure of irregular appearance (Fig. 2*b*). Oocytes of COC Quality 3 showed a tendency for a higher proportion of pore structures of irregular appearance (66.7%) as shown in Table 1. A significantly ($P < 0.05$) lower proportion of metaphase-II oocytes showed a porous zona structure compared with GV-stage oocytes (31.3% vs 100%) irrespective of COC quality, with 100% showing typical fine-meshed reticular pores. A significantly higher proportion of *in vivo*-matured oocytes displayed a porous zona

Table 1. Effect of maturational environment and COC quality on bovine zona pellucida structure measured by scanning electron microscopy
Values with different superscripts within columns differ significantly ($P < 0.05$)

Oocyte group	Quality	*n*	Aporous		Porous		Structure of pores			
							Typical		Irregular	
			n	%	*n*	%	*n*	%	*n*	%
GV	Q1+Q2	15	0	0.0	15	100.0	10	66.7	5	33.3
GV	Q3	6	0	0.0	6	100.0	2	33.3	4	66.7
	Total	21	0	0.0[a]	21	100.0[a]	12	57.1	9	42.9
In vitro MII	Q1+Q2	8	5	62.5	3	37.5	3	100.0	0	0.0
In vitro MII	Q3	8	6	75.0	2	25.0	2	100.0	0	0.0
	Total	16	11	68.8[b]	5	31.3[b]	5	100.0	0	0.0
In vivo MII	Total	6	0	0.0[a]	6	100.0[a]	6	100.0	0	0.0

Table 2. Effect of maturational environment and COC quality on *in vitro* developmental competence of bovine oocytes
Values with different superscripts within columns differ significantly ($P < 0.05$)

Oocyte group	Quality	*n*	Cleavage rate		Blastocyst rate	
			n	%	*n*	%
In vitro MII	Q1	126	104	82.5[a]	35	27.7[b]
In vitro MII	Q2	120	86	71.6[b]	24	20.0[a]
In vitro MII	Q3	124	85	68.5[b]	21	16.9[a]
In vitro MII	Total	370	275	74.3[ab]	80	21.6[a]
In vivo MII	Total	68	52	76.5[ab]	27	39.1[c]

Table 3. Effect of maturational environment and COC quality on zona pellucida birefringence of bovine MII stage oocytes measured by polarised light microscopy
Values with different superscripts within columns differ significantly (a, b; A, B, C; $P < 0.05$)

Oocyte group	Quality	*n*	CV-Mean Mean ± s.e.m.	WT-Mean Mean ± s.e.m.
In vitro MII	Q1	79	20.23 ± 2.69[A]	12.03 ± 1.50[A]
In vitro MII	Q2	91	20.39 ± 3.54[AB]	12.22 ± 1.98[A]
In vitro MII	Q3	77	21.75 ± 3.56[B]	12.95 ± 2.19[B]
In vitro MII	Total	247	20.76 ± 3.02[a]	12.39 ± 1.91[a]
In vivo MII	Total	21	16.54 ± 2.41[b]	10.72 ± 1.48[b]

structure with typical fine-meshed reticular pores (Fig. 2*d*) compared with *in vitro*-matured oocytes (31.3% vs 100%) as presented in Table 1. Likewise, significantly more *in vitro*-derived MII oocytes showed an aporous structure (68.8% vs 0%; Fig. 2*c*). Pores with irregular structure were only observed in immature oocytes.

Experiment 2: effect of maturational environment and COC quality on in vitro *developmental competence of bovine oocytes*

When 438 oocytes were fertilised *in vitro*, *in vivo*-matured bovine oocytes ($n = 68$) reached a similar cleavage rate compared with *in vitro*-matured oocytes ($n = 370$, 76.5% vs 74.3%). Significantly, more *in vivo*-matured oocytes reached the blastocyst stage compared with *in vitro*-matured oocytes (39.1% vs 21.6%) as shown in Table 2. Considering *in vitro*-matured oocytes of different COC qualities, Quality 1 COCs reached a significantly higher cleavage rate compared with Quality 2 and 3 COCs (82.5%, 71.6% and 68.5%, respectively) as well as higher subsequent blastocyst rates (27.7%, 20.0% and 16.9%, respectively; Table 2).

Experiment 3: effect of maturational environment and COC quality on bovine zona pellucida structure of immature and matured oocytes measured by ZPB

The mean birefringence (CV-Mean) of *in vivo*-matured oocytes ($n = 21$) was significantly lower ($P < 0.05$) compared with their *in vitro*-matured counterparts ($n = 247$; 16.54 ± 2.41 vs 20.76 ± 2.69). Within different COC qualities of *in vitro*-matured oocytes a significantly lower birefringence was observed for COC Quality 1 ($n = 79$) compared with Quality 3 ($n = 77$; 20.23 ± 2.69 vs 21.75 ± 3.56) as presented in Table 3. Accordingly, the thickness of the inner layer (WT-Mean) of *in vivo*-matured oocytes was significantly thinner compared with *in vitro*-derived oocytes (10.72 ± 1.48 vs 12.39 ± 2.19). With respect to COC quality, oocytes from Quality 1 COCs displayed a significantly thinner inner zona layer compared with oocytes of Quality 3 COCs (12.3 ± 1.50 vs 12.95 ± 2.19) as shown in Table 3.

When we analysed the zona birefringence and thickness of the inner zona layer of immature oocytes, significant differences for both parameters were observed between oocytes of different COC qualities (Q1–Q3). The mean birefringence (CV-Mean) significantly decreased from COC Quality 1 to COC Quality 3 (20.95 ± 3.79, 19.44 ± 2.91 and 18.03 ± 2.85, respectively; Table 4). The thickness of the inner zona layer (WT-Mean) showed the same trend considering COC Quality 1 to COC Quality 3 (11.44 ± 1.92, 10.79 ± 1.54 and 10.55 ± 1.73, respectively; $P < 0.01$, Table 4).

Putting the results of the immature (Table 4) and the matured oocytes (Table 3) into relation, the trend from GV- to MII-stage in terms of zona birefringence (CV-Mean) and thickness of the inner zona layer (WT-Mean) were significantly different ($P < 0.05$) between different maturational environments and

Table 4. Effect of COC quality of bovine GV-stage oocytes on zona pellucida birefringence measured with polarised light microscopy
Values with different superscripts within columns differ significantly ($P < 0.05$)

Oocyte group	Quality	*n*	CV-Mean Mean ± s.e.m.	WT-Mean Mean ± s.e.m.
GV	Q1	79	20.95 ± 3.79^{a}	11.44 ± 1.92^{a}
GV	Q2	85	19.44 ± 2.91^{b}	10.97 ± 1.54^{ab}
GV	Q3	77	18.03 ± 2.85^{c}	10.55 ± 1.73^{b}

individual COC qualities as presented in Fig. 3. *In vivo* maturation leads to a decrease in ZPB whereas *in vitro* maturation overall increases birefringence (Fig. 3*a*). With respect to COC quality, only oocytes of high-quality COCs (Q1) follow the trend of *in vivo*-matured oocytes whereas oocytes of Quality 2 and 3 COCs increased in birefringence (CV-Mean, Fig. 3*b*). *In vivo* maturation also led to a decrease in the thickness of the inner zona layer in contrast to the average of *in vitro*-matured oocytes (Fig. 3*c*). Oocytes of all individual COC qualities increased in thickness of the inner zona layer; however, the increase in Quality 3 COCs was the strongest and in Quality 1 COCs the most moderate (Fig. 3*d*).

Experiment 4: effect of G6PDH activity of immature bovine oocytes on in vitro *developmental competence and zona pellucida structure measured by ZPB*

Immature oocytes with low G6PDH activity (BCB+) reached a significantly higher cleavage rate compared with oocytes with low G6PDH activity (BCB–; 75.5% vs 65.1%) as well as a significantly higher blastocyst rate (32.0% vs 11.5%) as presented in Table 5. Concurrently, the mean birefringence of oocytes with high G6PDH activity and the thickness of the inner layer were significantly lower ($P < 0.05$) compared with oocytes with low G6PDH activity (18.83 ± 3.50 vs 20.37 ± 4.49 and 10.84 ± 1.95 vs 11.77 ± 2.35, respectively; Table 6).

Discussion

The characteristics of the ZP have been proposed to reflect the developmental competence of follicles and oocytes in human (Qi *et al.* 2002). Moreover, it is known that during follicular development in secondary follicles, the developing oocyte and follicular cells secrete the zona pellucida surrounding the plasma membrane of mammalian eggs (Dunbar *et al.* 1994).

This implies that any harm to the oocyte or to the surrounding cumulus cells caused by suboptimal conditions within the follicle at time of growth or maturation could alter the secretion and patterning of the extracellular coat (Shen *et al.* 2005). In other words, ZP properties could function as a marker of correct folliculogenesis and oocyte maturation. Studies performing scanning electron microscopy (SEM) reported that the number of ZP pores on the surface varies with oocyte quality (Santos *et al.* 2008), with high-quality oocytes having a greater number of pores than low-quality oocytes in the cow. With the aim to recapitulate the results of Santos *et al.* (2008) and to analyse further whether zona characteristics are affected by maturational environment we compared the zona characteristics of *in vivo*- and *in vitro*-derived MII-stage oocytes.

In the present study we observed differences in zona characteristics analysed by SEM between immature oocytes with cumulus cell investments of different qualities, oocytes of different maturational stages and MII-stage oocytes matured either *in vivo* or *in vitro*. The COC quality of immature oocytes correlates with morphological structures of the zona pellucida. A higher proportion of good-quality COCs (Q1–2) had a typical fine-meshed pore structure whereas COCs of lower quality (Q3) were found to exhibit an irregular degraded emerging structure at higher proportions. That is in line with recent findings showing that COC quality affects zona characteristics (Santos *et al.* 2008). That study reported a strong and negative correlation between COC quality and pore diameter, including a high number of pores with a significantly smaller size for Quality 1 oocytes compared with those of low quality (Santos *et al.* 2008). Additionally, that study showed that the number of pores and the meshed structure of the outer surface alters drastically during *in vitro* maturation. Moreover we observed alterations in the surface structure as a result of *in vitro* maturation to such an extent that the zona of all immature oocytes was found to be porous whereas only 31% of *in vitro*-matured oocytes were porous. However, all *in vitro*-matured porous oocytes exhibited a typical wide meshed, fibrous network with deep pores whereas 43% of all immature porous oocytes showed an irregular pore structure. In contrast, all *in vivo*-matured oocytes in the present study showed a homogenous structure with a typical reticular, fine-meshed surface with a very high number of small pores. This is in line with the observations of Santos *et al.* (2008) who reported that *in vitro* maturation affects zona characteristics. Moreover, strong differences between the morphology of *in vivo*- and *in vitro*-matured oocytes were observed recently in porcine oocytes; *in vivo*-matured porcine oocytes showed a fine-meshed and rough surface with a high number of pores, whereas *in vitro*-matured oocytes had a smooth and tight surface (Funahashi *et al.* 2000).

Taken together, all immature oocytes were found to be porous with only one-third of them showing a regular pore structure. After *in vitro* maturation, the zona of only one-third of all oocytes were found to be porous; however, all of them were of typical structure. Thus, we suggest that irregular pores of immature oocytes disappear during maturation. Macchiarelli *et al.* (1992) suspected that the pores are generated through penetration of cytoplasm appendages of corona radiata cells, which are in contact with the plasma membrane. Hence, explanations for the alterations during *in vitro* maturation could be due to inadequate contact with corona radiata cells, less distinct penetration or an early retraction of cytoplasm appendages in a proportion of oocytes, which may lead to a nearly poreless surface. Only one-third of all oocytes entering maturation resulted in matured oocytes with regular pores. Interestingly, this is very similar to the rate of development to the blastocyst stage usually obtained after *in vitro* fertilisation (Rizos *et al.* 2002). Having that in mind, we speculate that a certain proportion of oocytes entering our *in vitro* maturation system are not suitable for maturation because of insufficient contact with surrounding cumulus cells.

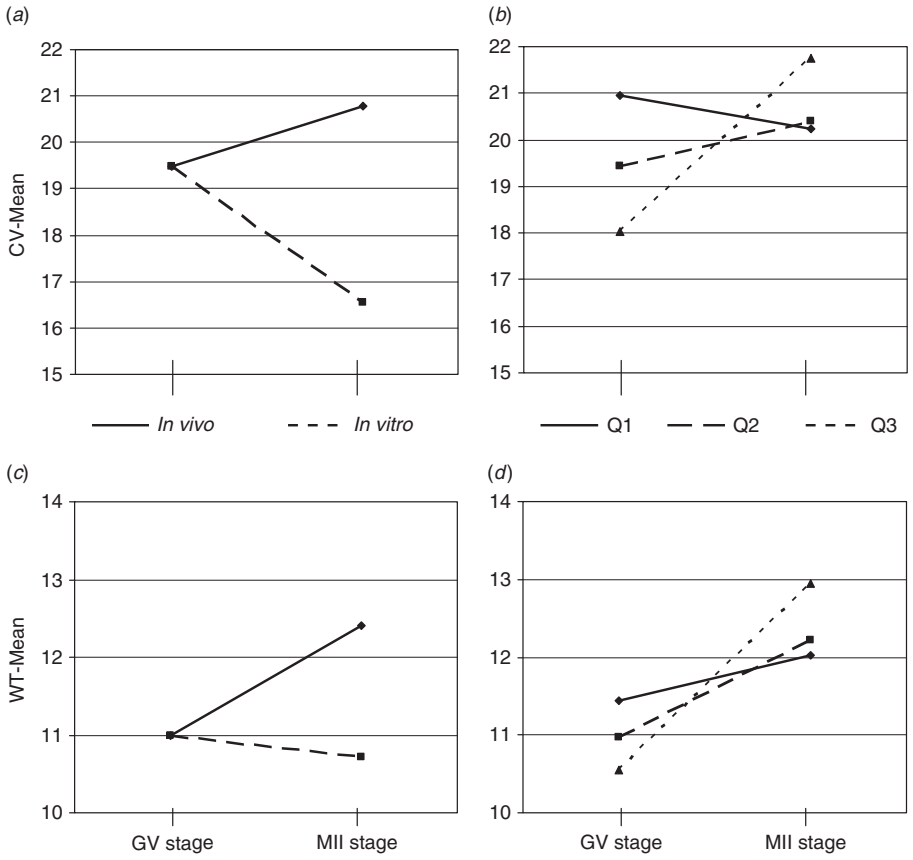

Fig. 3. Dynamics from GV stage to MII in terms of zona birefringence (CV-Mean) and thickness of the inner zona layer (WT-Mean) with respect to maturational environment and COC quality. (*a*) *In vivo* maturation leads to a decrease in ZPB whereas *in vitro* maturation overall increases birefringence. (*b*) With respect to COC quality, only oocytes of high-quality COCs (Q1) follow the trend of *in vivo*-matured oocytes, whereas oocytes of Q1 and Q2 COCs increase in birefringence. (*c*) *In vivo* maturation also leads to a decrease in the thickness of the inner zona layer in contrast to the average of *in vitro*-matured oocytes. (*d*) Oocytes of all individual COC qualities increased in thickness of the inner zona layer; however, Quality 3 COCs increased the most and Quality 1 COCs the least.

Table 5. Effect of G6PDH activity of immature bovine oocytes on subsequent *in vitro* developmental competence

Values with different superscripts within columns differ significantly ($P < 0.05$)

G6PDH activity	Growth status	*n*	Cleavage rate		Blastocyst rate	
			n	%	*n*	%
Low	Fully grown	416	314	75.5[a]	133	32.0[a]
High	Still growing	358	233	65.1[b]	41	11.5[c]
Average	Ordinary	197	145	73.6[a]	47	23.9[b]

Table 6. Effect of G6PDH activity of immature bovine oocytes on zona pellucida birefringence measured with polarised light microscopy

Values with different superscripts within columns differ significantly ($P < 0.05$)

G6PDH activity	Growth status	*n*	CV-Mean Mean ± s.e.m.	WT-Mean Mean ± s.e.m.
Low	Fully grown	105	18.83 ± 3.50[a]	10.84 ± 1.95[a]
High	Still growing	98	20.37 ± 4.49[b]	11.77 ± 2.35[b]

Our results also confirm that the maturational environment as well as morphological features of the COCs are useful as predictors for subsequent developmental competence. *In vivo*-matured oocytes reached significant higher blastocyst rates compared with *in vitro*-matured oocytes and high-quality COCs reached higher rates compared with lower quality COCs. Whereas high-quality COCs reached significantly higher cleavage rates compared with low-quality COCs, no differences in terms of cleavage rate were obtained between *in vivo*- and *in vitro*-matured oocytes.

These outcomes are completely comparable to those of another study, in which no difference in cleavage rate between

in vivo- and *in vitro*-matured groups but significant differences in the blastocyst yield were reported (Rizos *et al.* 2002). However, although zona characteristics correlate with developmental competence, it has not been possible to introduce this invasive technique to identify oocytes of superior developmental competence into laboratory routine because it completely damages the oocytes.

To circumvent this problem we therefore aimed to test whether zona properties predictive for developmental competence could be evaluated through zona pellucida birefringence (ZPB) measurement, a new technique that has recently been introduced into human ARTs (Shen *et al.* 2005; Rama Raju *et al.* 2007; Montag *et al.* 2008). Likewise, the present study shows that during maturation the structural changes visualised by SEM are reflected in the ZPB. To our knowledge, the present study is the first to analyse the environmental influence on zona properties of bovine oocytes by polarised light microscopy. In addition, a direct comparison between SEM and ZPB has not been reported so far.

When comparing the ZPB of immature oocytes of different COC quality, COCs of high quality reached significantly higher values compared with oocytes from COCs of lower quality. The higher birefringence is correlated with the thicker internal layer of the zona pellucida (Kilani *et al.* 2006). Therefore, immature oocytes from high-quality COCs have higher birefringence values and higher developmental competence at the same value. These results are in line with recent studies performed in human reproduction in which a positive correlation between thicker inner layers and high birefringence scores on the one side and higher developmental potential and a higher development to term on the other side were reported (Rama Raju *et al.* 2007; Montag *et al.* 2008; Madaschi *et al.* 2009; Ebner *et al.* 2010). In contrast, we observed a lower birefringence for *in vivo*-matured versus *in vitro*-matured oocytes and matured oocytes of high-quality COCs had significantly lower values compared with their lower-quality counterparts. Due to the higher developmental competence of *in vivo*-matured oocytes, a thinner inner layer could be interpreted as being predictive for better developmental potential. This is in accordance with a recent study that reported that low mean values for zona birefringence parameters were related to superior zygote quality and subsequently led to better preimplantation development following artificial activation or IVF (Koester *et al.* 2011). However, these results are not in agreement with a recent study performed in humans in which a positive correlation between a thicker inner layer and better developmental potential in human oocytes was reported (Rama Raju *et al.* 2007). Analysing the ZPB measurements of immature oocytes compared with matured ones, we found a strong decreasing trend from GV stage oocytes to *in vivo*-matured oocytes. Within the groups of all *in vitro*-matured oocytes only oocytes with high-quality COCs (Q1) showed a comparable trend for birefringence whereas oocytes from COCs of lower quality as well as *in vitro*-matured oocytes showed an increasing trend. An explanation considering both observations could be that high ZPB values in immature oocytes are an indicator for high quality of immature oocytes and a decrease in ZPB during the process of maturation could be an indicator for successful maturation. Likewise a recent study in human reproductive cycles showed that MI oocytes yielded a higher percentage of high-birefringence oocytes compared with MII stage oocytes, indicating that zona birefringence decreases as oocyte nuclear maturation takes place (de Almeida Ferreira Braga *et al.* 2010). These outcomes are comparable with those of Cheng *et al.* (2010) who analysed the birefringence of human oocytes during IVF cycles. Similarly to our results, a decreasing trend regarding zona parameters from GV stage to MII was reported (Bhojwani *et al.* 2007). The positive correlation between decreasing zona thickness and mean birefringence demonstrated for the *in vivo*-matured oocytes corresponds with results reported previously (Shen *et al.* 2005).

Thus, our results clearly show that maturational environment as well as quality of the cumulus cell investment of immature oocytes affect developmental competence as well as zona properties at the same value. However, although classification of oocyte quality by morphological characteristics could provide valuable information for the preselection of oocytes with higher developmental competence, this kind of method is not very precise. Therefore, we aimed to investigate whether ZPB parameters as an indicator for subsequent developmental competence of immature oocytes could be related to glucose-6-phosphate-dehydrogenase (G6PDH) activity, representing a molecular and subcellular predictor for oocyte quality of proven value (Pujol *et al.* 2004; Alm *et al.* 2005; Bhojwani *et al.* 2007). In the present study, we observed significant differences between immature oocytes with contrasting G6PDH activities. Our experiments confirmed a higher developmental competence of oocytes with low G6PDH activity compared with oocytes with high activity in terms of cleavage rate and blastocyst rate. This is in line with observations in various species reporting different molecular and subcellular characteristics of oocytes due to contrasting G6PDH activities (Ghanem *et al.* 2007; Torner *et al.* 2008) as well as different developmental capacities including pig (Roca *et al.* 1998; El Shourbagy *et al.* 2006; Wongsrikeao *et al.* 2006), goat (Rodríguez-González *et al.* 2003; Urdaneta *et al.* 2003), mouse (Mangia and Epstein 1975; Wu *et al.* 2007), buffalo (Manjunatha *et al.* 2007) and cow (Pujol *et al.* 2004; Alm *et al.* 2005; Bhojwani *et al.* 2007).

At the same value, immature oocytes with low G6PDH activity exhibited a lower birefringence and a thinner inner layer compared with their counterparts with high G6PDH activity. This result seems to conflict with the results of Experiments 1–3, which brought us to the suggestion that higher ZPB values for immature oocytes are predictive for high developmental capacity. Indeed, immature oocytes showing low G6PDH activity are correlated with higher developmental competence and lower ZPB values compared with immature oocytes with high G6PDH activity. However, we have to keep in mind that immature oocytes with low G6PDH activity are presumed to have completed their growth phase whereas oocytes with high activity are presumed to be still undergoing growth (Cheng *et al.* 2010). Torner *et al.* (2008) reported a higher proportion of oocytes with low G6PDH activity being in progressed diakinesis stage, whereas immature oocytes with high G6PDH activity were with higher probability retarded in diplotene stage. Thus, on a hypothetic time line, growing oocytes (high G6PDH activity) are developmentally retarded

compared with ordinary immature oocytes and thereby might be less suitable for *in vitro* maturation as analysed in Experiment 3. Lower development capacity and higher ZPB scores approved the trend for ZPB during maturation by time, as observed in Experiments 1–3. Concurrently, oocytes having completed their growth phase (lower G6PDH activity) are developmentally progressed compared with ordinary immature oocytes. Therefore, the results of Experiment 4 fit well rather than being conflictive. Collectively, classification of immature oocytes by COC morphology and G6PDH activity are not comparable since oocytes are at different growth stages.

Taken together, *in vivo*-matured M-II oocytes reached higher subsequent developmental competence and lower ZPB values compared with *in vitro*-derived M-II oocytes. Considering *in vitro*-matured oocytes, MII oocytes derived from high-quality COCs classified according to cumulus investment, reached a higher developmental competence as well as lower ZPB values compared with those of lower quality COCs. Likewise, immature oocytes derived from high-quality COCs reached higher developmental competence as well as higher ZPB values compared with immature oocytes of lower quality COCs. In accordance, fully-grown immature oocytes reached higher developmental competence and lower ZPB scores compared with growing oocytes. In summary, we suggest a decreasing trend for ZPB during *in vitro* maturation to be typical for high-quality oocytes. To our knowledge, this is the first work correlating zona pellucida birefringence of bovine oocytes with different environmental backgrounds as well as developmental competence. Moreover, by correlating ZPB with G6PDH activity, representing a molecular predictor of oocyte quality, we have overcome a limitation of similar studies into ZPB in human oocytes and provided a link between molecular characteristics, zona pellucida properties and developmental capacity.

In conclusion, we successfully evaluated the applicability of zona imaging to bovine oocytes: alterations during *in vitro* maturation invasively analysed by scanning electron microscopy were reflected in the birefringence of the zona pellucida. Our results show that maturational environment and quality of immature oocytes classified by COC investment and G6PDH activity correlate with developmental competence and ZPB at the same value. Therefore, polarised light microscopy is a useful tool offering some opportunities to improve selection of competent oocytes in assisted reproduction. However, further studies are necessary to improve the power of this promising new technique.

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