Identification of optimal assisted aspiration conditions of oocytes for use in porcine in vitro maturation: a re-evaluation of the relationship between the cumulus oocyte complex and oocyte quality

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Identification of optimal assisted aspiration conditions of oocytes for use in porcine *in vitro* maturation: a re-evaluation of the relationship between the cumulus oocyte complex and oocyte quality

Short running title: Pig oocyte collection and selection for *in vitro* maturation

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Abstract

The quality of porcine oocytes for use in IVF is commonly graded according to the number of layers of cumulus cells (CCs) surrounding the oocyte; together these form the cumulus oocyte complex (COC). At least three compact layers of CCs is regarded as important for efficient IVP. To test this, oocytes were scored according to cumulus investment, with grade A representing COCs with three or more cumulus layers including granulosa cell-cumulus oocyte complexes, grade B those with an intact corona radiata surrounded by another layer of cumulus cells and grades C and D representing COCs with lower CC investment. These oocytes were then monitored for *in vitro* maturation (IVM), as assessed by tubulin immunostaining for meiotic progression, the development of a cortical granule ring, and by glutathione levels. Results indicate that grading correlates closely with nuclear maturation and cytoplasmic maturation, suggesting that grading oocytes by cumulus investment is a reliable method to predict IVM success. Importantly, Grade A and B oocytes showed no significant differences in any measure and hence using a cut-off of two or more CC layers may be optimal. We also determined the effect of assisted aspiration for oocyte retrieval, comparing the effect of needle size and applied pressure on the retrieval rate. These data indicated that both variables affected oocyte recovery rates and the quality of recovered oocytes. In combination, these experiments indicate that grade A and B oocytes have a similar developmental potential and that the recovery of oocytes of these grades is maximised by use of an 18-gauge needle and 50mmHg aspiration pressure.

Keywords: Oocyte; Pig; *In vitro* production; Morphology

Introduction

Pig embryo *in vitro* production (IVP) often involves oocyte collection from the ovaries of abattoir animals. Usually, oocyte donors will not have undergone pharmacological treatments to regulate or induce the production of mature oocytes. As such, their gametes will be immature and require laboratory culture, a process known as *in vitro* maturation (IVM). Following slaughter, oocyte retrieval can be achieved using either follicular aspiration or ovary slicing. Aspiration methods can be either manual, using a syringe barrel and needle, or assisted, using a negative pressure aspiration pump and attached needle. Automated aspiration procedures generally allow for a more consistent collection environment when compared to manual aspiration, which is prone to inter-operator variability (Marques et al., 2015). The oocytes retrieved for IVM are found in combination with cumulus cells (CCs), forming the cumulus oocyte complex (COC). CCs and oocytes share a complex network of interactions (Gilchrist, Ritter, & Armstrong, 2004) and there is a strong correlation between the number of CCs and the ability of an oocyte to complete both nuclear and cytoplasmic maturation (Dang-Nguyen et al., 2011; Lin, Oqani, Lee, Shin, & Jin, 2016; Nagai, Ding, & Moor, 1993).

The oocyte is maintained in meiotic arrest by the CCs, which supply it with stable levels of the meiotic progress inhibitor cyclic adenosine monophosphate (Anderson & Albertini, 1976; Racowsky, 1985) and of the phosphodiesterase inhibitor, cyclic guanosine monophosphate (Norris et al., 2009). Interestingly, the CCs also control meiosis resumption in response to high luteinising hormone levels (Mattioli & Barboni, 2000; Norris et al., 2008). Further to this, CCs

promote the migration of cortical granules (CGs) towards the periphery of the oocyte (Galeati, Modina, Lauria, & Mattioli, 1991), a key element of cytoplasmic maturation. Cytoplasmic maturation has also been shown to be enhanced by high levels of glutathione (GSH) which CCs actively synthesise and transport to the oocyte (Maedomari et al., 2007; You, Kim, Lim, & Lee, 2010), where it acts as a scavenger of reactive oxygen species (ROS) (Tatemoto, Sakurai, & Muto, 2000) and increases amino acid transport and protein synthesis (Lafleur, Hoorweg, Joenje, Westmijze, & Retèl, 1994). Furthermore, high levels of GSH seem important for correct male pronuclear formation upon fertilisation (Niwa, 1993; Yoshida, Ishigaki, Nagai, Chikyu, & Pursel, 1993). Due to the many functional roles of CCs, the morphology of the COC is commonly used to determine candidates for IVM in pigs and in other farm animals (Alvarez, Dalvit, Achi, Miguez, & Cetica, 2009; Nagano, Katagiri, & Takahashi, 2006). Laboratory based retrieval methods and aspiration pressures used in ovum pickup from live animals have shown that aspiration pressure also has an impact on the morphology of the COC (Brüssow, Torner, Ratky, Hunter, & Nürnberg, 1997; Marques et al., 2015).

In pigs, full oocyte meiotic competence is achieved in follicles with a diameter of more than three mm and a positive correlation has been observed between follicle size and oocyte *in vitro* developmental competence (Marchal, Vigneron, Perreau, Bali-Papp, & Mermillod, 2002). Interestingly, oocytes from gilts display a reduced developmental potential when compared to oocytes from sows (Lechniak et al., 2007); this may be because the average follicle size is smaller in gilts (Bagg, Nottle, Armstrong, & Grupen, 2007). Given these results, primary oocytes for pig IVP are usually recovered from follicles ranging between three and eight mm (Bagg et al., 2007);

it is however difficult for operators to judge follicle size accurately (Lin et al., 2016), and as such appropriate criteria for post retrieval oocyte selection are essential. Even though it is often recommended that only COCs formed of multiple compact layers of CCs should be selected for IVM (Bagg et al., 2007; Esaki et al., 2004; Fowler, Mandawala, Griffin, Walling, & Harvey, 2018; Lee, Hyun, & Lee, 2012; Lin, Lee, Shin, Oqani, & Jin, 2015; Long, Dobrinsky, & Johnson, 1999; Rath, Niemann, & Tao, 1995; Sherrer, Rathbun, & Davis, 2004), this practice results in wastage as COCs formed of three of more layers form only a proportion of the total yield (Lin et al., 2016). With this in mind, here we investigate how maturation, as assessed by meiotic progression, the development of a CG ring, and by GSH levels, varies in COCs with different numbers and morphologies of their CC layers. We have also investigated how specific combinations of aspiration pressure and needle gauge affect COC recovery and morphology. These analyses indicate that oocytes with three or more cumulus layers including granulosa cell-cumulus oocyte complexes (Grade A) and those with intact corona radiata surrounded by another layer of CCs (Grade B) have similar developmental potentials and that the recovery of oocytes of these grades is maximised by use of an 18-gauge needle and 50mmHg aspiration pressure.

Materials and Methods

Ovaries were collected from unsynchronised animals on an abattoir line by trained staff from JSR Genetics Ltd. and were stored and transported to the laboratory in phosphate buffered saline (PBS) at 38.5°C within six hours of collection. PBS, and all other chemicals, were acquired from Sigma-Aldrich (Gillingham, UK) except when stated otherwise. Upon arrival, ovaries were decanted into an autoclaved 500ml beaker, and maintained at 38.5°C until use. For all

experiments, follicles ranging between three and eight mm in diameter were aspirated (see below for details) to recover oocytes. The number of ovaries received in each batch varied depending on availability (between 30 and 50 in total).

Following aspiration, the collected follicular fluid was transferred into a pre-warmed (38.5˚C) petri dish and all COCs were recovered using a stereomicroscope and an EZ-grip pipette (Origio) equipped with a 290µm EZ-tip (Origio). Collected COCs were washed twice in tyrode lactate buffered 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (TL-HEPES) polyvinyl-alcohol (PVA) medium and categorized as A, B, C or D (Figure 1) according to the number and morphology of their CC layers and ooplasm quality (granular patterns, colour and density): Grade A, three or more cumulus layers including granulosa cell-cumulus oocyte complexes, even cytoplasm; Grade B, intact corona radiata surrounded by another layer of CCs, even cytoplasm; Grade C, incomplete corona radiata or partially denuded oocyte, uneven cytoplasm; Grade D: denuded oocyte, uneven cytoplasm. COC grading was replicated by two independent operators.

Oocyte maturation

To assess how maturation varies in COCs that that differ in the number and morphology of their CC layers, oocytes were manually aspirated using a 5ml syringe and a 19-gauge needle with the aid of a small volume of TL-HEPES-PVA medium. Comparisons of nuclear maturation potential were repeated using oocytes obtained from five separate deliveries, with assays of cytoplasmic maturation potential repeated on oocytes from six separate deliveries.

IVM of graded oocytes was undertaken in North Carolina State University (NCSU)-23 media (Petters & Wells, 1993) supplemented with 10% porcine follicular fluid (pFF) that had been collected from a separate batch of abattoir-derived ovaries. pFF was prepared by centrifugation for 30 minutes at 3,000rpm, and subsequent filter sterilisation of the supernatant using a Minisart single use filter (0.2mm). pFF was stored at -20°C until use. Graded oocytes were matured separately in groups of 50 in 500µl media under 6% CO_2 at 37°C. For the first 22 hours, the culture medium was supplemented with hormones (1:100 PG600, Intervet, Milton Keys, UK), 0.8mM L-cysteine, 10ng/ml EGF, 1mM db-cAMP and 50µM β-mercaptoethanol. Oocytes were then transferred to fresh medium for a further 22 h with the hormones and db-cAMP excluded. The IVM oocytes produced were then tested as described below.

To assess the nuclear maturation potential of oocytes of different grades, the meiotic spindles of IVM oocytes were immunostained with anti α-tubulin. IVM oocytes were denuded of CCs by incubation for 10 minutes in 0.2%w/v Hyaluronidase Type VIII from bovine testis and passing them several times through a 125-µm tip. These oocytes were then fixed overnight at 4°C in 4% PFA in PHEM buffer (60mM PIPES, 25mM HEPES, 10mM EGTA, 4mM $MgSO_4$, pH 7.0). Fixed oocytes were then rinsed three times in PBS for five minutes and permeabilised for ten minutes in 1% Triton X-100 in PHEM buffer. After rinsing in PBS, the oocytes were blocked in 20% fetal bovine serum (FBS) PHEM buffer for one hour at room temperature. Oocytes were then stained for 30 minutes in 1:200 anti-α tubulin-Alexa 488 (ab195887, Abcam, UK), 5% FBS in PHEM buffer. After rinsing in PBS, oocytes were counterstained in 1 µg/ml Hoechst 33342 in PBS, mounted on slides with an antifade agent (Fluoroshield). Meiotic stage was classified as previously published

153 (Ma, Hou, Sun, Sun, & Wang, 2003), with oocytes that had reached at least anaphase I considered
154 to have achieved full nuclear maturation.
155
156 Two approaches were taken to assess the cytoplasmic maturation potential of oocytes of
157 different grades. Firstly, CGs in post IVM oocytes (N = 107, 64, 93 and 68 for Grades A, B, C and
158 D, respectively) were stained with peanut agglutinin (PNA) lectin-Alexa 488. Secondly, the levels
159 of intrinsic GSH were measured in four replicates of 120 oocytes for each oocyte grade to
160 investigate the capability of the oocytes to cope with oxidative stress.
161
162 PNA staining, using a modification of the method of Zhang and colleagues (Zhang et al., 2010)
163 was undertaken on IVM oocytes that had been denuded and fixed as described above. Oocytes
164 were rinsed three times in 0.3% bovine serum albumin (BSA) in PBS for five minutes, followed by
165 permeabilization for five minutes in 0.1% Triton X-100 in PBS. Permeabilized oocytes were rinsed
166 twice in PBS and stained in 100µg/mL PNA lectin-Alexa 488 (L21409, Life Technologies, Paisley,
167 UK) in PBS. After three washes of five minutes in 0.3% BSA, 0.01% Triton X-100 in PBS, oocytes
168 were counterstained with 1µg/ml Hoechst 33342, mounted with Fluoroshield and observed.
169 Oocytes were defined as cytoplasmically mature if they showed a clear, continuous ring of CGs
170 close to their membrane rather than a homogeneously dispersed pattern throughout the
171 cytoplasm.
172
173 GSH content measurement was undertaken on IVM oocytes that had been denuded as described
174 above, then washed three times in PBS to eliminate any possible thiol carryover from the culture

media (mainly from β-mercaptoethanol and L-Cysteine). Measurements were completed as described previously (Funahashi, Cantley, Stumpf, Terlouw, & Day, 1994) in a final volume of 1ml and using 0.25U GSH reductase from baker's yeast (G3664). A Biomate 3S spectrophotometer (ThermoScientific, Waltham, MA) was set for continuous reading at 412nm and measurements were taken every 20 seconds for two minutes. The oocyte GSH content was then estimated from a linear calibration curve employing 1, 0.1, and 0.01nmol reduced GSH per reaction.

All fluorescence observations were completed at x200 total magnification using an Olympus BX60 fluorescence microscope equipped with standard DAPI and Fluorescein isothiocyanate (FITC) bandpass filters. Images were captured using a Hamamatsu ORCA-03G camera and processed through the software SmartCapture (version 3; Digital Scientific, Cambridge, UK), but CG stained oocyte images are not shown as the static images obtained were not as clear as observations seen by eye.

Rates of nuclear maturation and of cytoplasmic maturation as assessed by PNA staining were analysed by chi-squared test, with differences between treatments determined by false discovery rate (FDR) corrected pairwise tests (Benjamini & Hochberg, 1995) . The GSH content of matured oocytes of different grades were compared by Kruskal-Wallis, with pairwise Mann-Whitney U tests used for *post hoc* comparisons. Data analysis was performed in R version 3.3.4 (R Core Team, 2018).

Oocyte recovery

For analysis of how specific combinations of aspiration pressure and needle gauge affect COC recovery and morphology, oocytes were collected using an aspirator pump (Labotect Aspirator 3). The rubber tubing attaching the needle to the aspirant collection tube was primed with TL-HEPES-PVA (Funahashi, Cantley, & Day, 1997). A needle of appropriate diameter (18, 19, 20, 21 and 23-gauge) (BD Microlance 3) was attached to the aspirator pump, set to an appropriate pressure setting (25, 50, 75, 100, 125 or 150mmHg), connected to a 50ml aspirant collection tube sealed with a rubber bung. TL-HEPES-PVA media was flushed through the rubber tube prior to oocyte collection and subsequently periodically to ensure all COCs were recovered into the aspirant collection tube. All 25 combinations of needle and pressure could not be tested on each batch of ovaries as this would either limit sample size per treatment or make the collection period too long. Therefore, for each round of oocyte collection, between three and six different combinations of needle and pressure were tested, with follicles harvested from ten random ovaries from the shipment for each combination. The effects of needle gauge and aspiration pressure on the recovery of 1) oocytes, 2) grade A COCs, and 3) grade A and B COCs, from the follicles aspirated was assessed by using a chi-squared test using Bonferroni corrected *p* values for different aspiration pressures within each needle gauge. For significant tests, differences between aspiration pressures were then determined by false discovery rate (FDR) corrected pairwise tests (Benjamini & Hochberg, 1995). Differences between the standardised Pearson residuals were used to determine pressures associated with increased recovery rates. In total, 38,595 follicles were aspirated using a range of pressures and needle gauges, yielding 26,370 oocytes.

Ethical approval process

Oocytes were obtained from abattoir derived ovaries. No specific ethical approval was required.

Results

Oocyte maturation

To assess how maturation varies in COCs that that differ in the number and morphology of their cumulus cell layers, quality and both nuclear and cytoplasmic maturation were assessed in COCs recovered by manual aspiration. These COCs were categorised as grade A, B, C or D according to the number and morphology of their CC layers: Grade A, three or more cumulus layers including granulosa cell-cumulus oocyte complexes; Grade B, intact corona radiata surrounded by another layer of CCs; Grade C, incomplete corona radiata or partially denuded oocyte; Grade D: denuded oocyte (see Figure 1 for examples of these categories).

Nuclear maturation in IVM oocytes was evaluated by visualising the metaphasic spindles with α-tubulin immunostaining (Figure 2). This showed that the standard diagnostic features of meiosis could be identified and that the oocytes were maturing. Analysis of these data indicates that IVM oocytes of different grades differ in their rates of nuclear maturation ($\chi^2 = 10.05$, df = 3, $p = 0.018$, and $\chi^2 = 35.19$, df = 3, $p < 0.0001$ for the percentage assessed as metaphase I and metaphase II, respectively), with higher grade oocytes displaying higher levels of nuclear maturation (Figure 3i and B). These analyses indicate that the maturation potential of Grade D oocytes is very limited, with only a small percentage (mean of 2.9%) reaching metaphase I (Figure 3i) and no oocytes of this grade assessed as having achieved full nuclear maturation (Figure 3ii). These analyses also

241 indicate that, in terms of nuclear maturation, there is no significant difference between the
242 development of Grade A and B oocytes (Figure 3i and 3ii).
243
244 Cytoplasmic maturation of IVM oocytes as assessed by PNA lectin-Alexa 488 staining of CGs
245 indicated that maturation rates differed between oocyte grades ($\chi^2 = 26.64$, df = 3, $p < 0.0001$)
246 (Figure 4i). Here, oocytes in complex with two or more complete layers of CCs (Grades A and B)
247 displayed a continuous peripheral ring of CGs more often than partially or fully denuded oocytes
248 (Grades C and D) (Figure 4i). Levels of GSH also differed between oocytes classes (Kruskal-Wallis H
249 = 11.89, df = 3, $p = 0.01$), with higher levels of GSH seen in Grade A and Grade B oocytes (6.3 ±
250 0.5 pmol/oocyte and 5.8 ± 1.9 pmol/oocyte, respectively) than in Grade C and Grade D oocytes
251 (2.2 ± 0.3 pmol/oocyte and 0.9 ± 0.3 pmol/oocyte, respectively) (Figure 4ii).
252
253 *Oocyte recovery*
254 The aspiration of 38,595 follicles, using a range of pressures and needle gauges yielded a total of
255 26,370 oocytes, with the recovered oocytes unequally distributed across the quality grades
256 (Grade A: 8.1%; Grade B: 24.1%; Grade C: 35.7%; Grade D: 32.1%) (Figure 5). These data were
257 analysed to investigate three questions (Table 1). Specifically, which combinations of aspiration
258 pressure and needle gauge recovered the maximum percentage of 1) oocytes, 2) grade A and B
259 COCs, and 3) grade A COCs, from the follicles aspirated (Figure 5, Table 1). These analyses
260 indicated that, for nearly all needle gauges, the aspiration pressure affects recovery and quality
261 of oocyte (Table 1). The exception to this is for the 23-gauge needle, where no difference
262 between aspiration pressures in the percentages of grade A COCs recovered was identified (Table

1). However, oocyte recovery is maximised at higher aspiration pressures (Table 1) and the worst recovery is seen when using 23-gauge needles (Table 1, Figure 5). We also observed that the recovery of high-quality oocytes (either grade A only, or grades A and B combined) is the highest when low aspiration pressures are used (Table 1). For example, if grade A and B COCs are required, these data suggest that recovery is maximised using an 18-gauge needle and an aspiration pressure of 50mmHg.

Discussion

The global population is predicted to rise to 9.8 billion by the year 2050 (Bruinsma, 2002). This growth, in combination with changes in dietary preferences, is increasing the global demand for animal protein, with the livestock sector challenged to find new ways of accommodating this need. The global shipment of genetically advanced breeding stock to upgrade or replace local genetic lines involve high production, environmental and logistical costs. In some circumstances movement of live animals is not permitted because of the perceived risk of the introduction of disease into the territory, for example, there are regulations around boar movement and pig trade because African swine fever virus infection risk (Taylor et al., 2020). Taken together, these factors have resulted in the *in vitro* production (IVP) of pig embryos being of increased interest to producers, given that great financial and environmental benefits could be made. Pig IVP has the potential to become a viable alternative to artificial insemination for agricultural and biomedical purposes, however for this to be achieved oocyte quality is paramount, and systematic comparisons between oocytes matured *in vitro* and *in vivo* are still needed. The acquisition of adequate numbers of competent oocytes is the first, critical step to successful

subsequent *in vitro* embryo production; it is imperative that the maximum number of the highest quality oocytes can be recovered. Here we have investigated the both the number and quality of oocytes that can retrieved from abattoir derived ovaries, and have determined that CC investment can act as a predictor of oocyte developmental competence. Further to this, we have also shown that both needle size and applied pressure alters the retrieval rate of developmentally competent COCs.

Our results indicate that nuclear maturation and cytoplasmic maturation is variable across the four grades of oocytes (Figure 1). Grade A and B oocytes were observed to mature at high rates whilst fewer Grade C and D oocytes were assessed as having completed maturation (Figure 4). Interestingly, fully denuded oocytes (Grade D), which were never observed to complete nuclear maturation, were occasionally observed to have progressed to full cytoplasmic maturation, therefore indicating that these two processes are not necessarily linked. Alternatively, as the developmental capabilities of denuded oocytes can be rescued by co-culture with COCs (Luciano et al., 2005), the limited development seen here may have been the result of the presence of a limited number of CCs in the wells in which Grade D oocytes were cultured.

The IVM system in this study included the use of both cysteine and β-mercaptoethanol. It has been reported that the mean GSH content in pig oocytes increased from 7.9 ± 0.6 pmol/oocyte to 10.4 ± 2.8 pmol/oocyte (means ± standard errors) in the presence of 50 μM β-mercaptoethanol (Abeydeera, Wang, Cantley, Prather, & Day, 1998), whilst another found that supplementing the maturation medium with 0.57 mM cysteine increased oocyte GSH from 4.0 ±

0.8 pmol/oocyte to 15 ± 0.3 pmol/oocyte (means ± standard errors) (Yoshida et al., 1993). Our findings indicated that Grade A and B oocytes have levels of GSH comparable to these levels, while Grade C and D oocytes appear largely depleted of GSH (Figure 4). This shows the presence of cysteine and β-mercaptoethanol alone during IVM is not sufficient to increase intracellular GSH levels in pig oocytes as CCs are required to complete the process (Tatemoto et al., 2000). Moreover, the absence of enough GSH in Grade C and D oocytes could well explain their reduced cytoplasmic and nuclear maturation potentials.

In our investigation of the recovery rates of oocytes, we found that A and B oocytes formed only a minority of the total yield (8.1% and 24.1% of A and B, respectively, Figure 5), which is in line with previous findings (Lin et al., 2016). Our comparisons of oocytes recovered using different aspiration pressures and needle gauges (Figure 5, Table 1) indicate that both factors affect oocyte recovery; this supports previous work that identified a negative correlation between the size of the COCs retrieved and the aspiration pressure, with higher pressures increasing the incidence of denuded oocytes (Brüssow et al., 1997; Marques et al., 2015). Further, these data suggest the existence of a compromise between recovery rates and oocyte quality, with quality maximised by lower pressures. Given that CCs support both nuclear and cytoplasmic oocyte maturation (Tanghe, Van Soom, Nauwynck, Coryn, & de Kruif, 2002), it is desirable to minimise disruption or damage to the COC when retrieving oocytes from follicles.

Conclusions

Taken together, our previous observations on the maturational competence of oocytes concur with the well-established link between COC investment and oocyte developmental competence (Alvarez et al., 2009; Bagg et al., 2007; Kim et al., 2010; Lin et al., 2016; Marchal et al., 2002; Nagano et al., 2006), and suggest that previous studies may have been too stringent in COC selection for subsequent IVP. Here, optimum results were achieved by using an 18-gauge needle and 50mmHg aspiration pressure (Figure 5). Given that Grade A and B COCs are equivalent in their developmental competence, it is possible to maximise both the number, and the quality of oocytes retrieved with this combination. If however other considerations in future investigations indicate that only Grade A oocytes are required for IVP, then there are a broader range of needle and pressure combinations that can be considered (Figure 5). Going forward, it would be interesting to determine whether the developmental potential of Grades C and D pig oocytes could be rescued using meiotic inhibition and an extended IVM protocol, in concordance with other work in both pigs and cattle (Li et al., 2016; Park et al., 2016; Sugimura et al., 2018). This would, in principle, afford these oocytes more time to grow and develop fully. It is important that future work aims to support these findings with subsequent *in vitro* embryo development data.

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Conflicts of interest

The authors declare no competing interests.

Authors' contributions

Author contributions: Conceptualization: D.K.G., K.E.H., P.J.I.E., G.A.W.; Methodology: D.K.G., K.E.H., P.J.I.E.; Formal analysis: S.C.H.; Investigation: G.S., C.C.R., R.L.G., K.E.H.; Writing – Original draft: G.S., C.C.R., S.C.H., K.E.H., Writing – Review & Editing: G.S., C.C.R., S.C.H., R.L.G., G.A.W., K.E.H., P.J.I.E., D.K.G., Funding acquisition: D.K.G., Resources: G.A.W.; Supervision: D.K.G., and K.E.H.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Table 1: The effect of needle gauge and aspiration pressure on the recovery rates and quality of recovered oocytes. Shown for comparisons of 1) oocyte recovery, 2) recovery of A and B grade oocytes, and 3) recovery of A grade oocytes only, are the χ^2 values and Bonferroni corrected *p* values for tests of different aspiration pressures within each needle gauge, and results of *post hoc* comparisons, where aspiration pressures that differ significantly do not share a number. The aspiration pressures associated with recovery of increased numbers of oocytes, or of higher proportions of high-quality oocytes (grades A and B in 2 and grade A only in 1), are also listed (as determined by inspection of the standardised Pearson residuals). Where multiple pressures are listed, the order indicates the strength of the association with the first pressure listed having the greatest effect.

Comparison	Needle Gauge	χ^2 (*p value)*	Aspiration pressure						Pressure(s) with highest recovery
			25	50	75	100	125	150	
1) Oocytes	18	603.5 (*p*<0.001)	1	2	3	2	4	5	125, 150
	19	472.13 (*p*<0.001)	1	1	1	1	2	3	125, 150, 50, 100
	20	323.1 (*p*<0.001)	1	2	3	3	4	5	150, 100, 75
	21	177.3 (*p*<0.001)	1	2	3	2	4	1	100, 50, 75
	23	282.3 (*p*<0.001)	1	2	2	2,4	3	4	150, 50, 100, 75
2) A and B grade	18	351.3 (*p*<0.001)	1	2	3	1	4	1	50, 75
	19	141.3 (*p*<0.001)	1	1	2	3	3	2	50, 25
	20	351.3 (*p*<0.001)	1	2	3	1	4	1	50, 75
	21	98.4 (*p*<0.001)	1	1,3	2	2,3	2	4	25, 50, 100
	23	100.0 (*p*<0.001)	1	2	2	2	3	1	75, 50, 100

3) A grade	18	195.1 (p<0.001)	1	2	2	1	2	1	50, 75
	19	127.9 (p<0.001)	1	2	2	3	3	2	25, 50
	20	53.0 (p<0.001)	1,2	2	1,3	3,4	4	4	50, 25, 75
	21	97.4 (p<0.001)	1	2	2	2	3	3	25, 50, 75
	23	25.5 (p=0.03)	-	-	-	-	-	-	-

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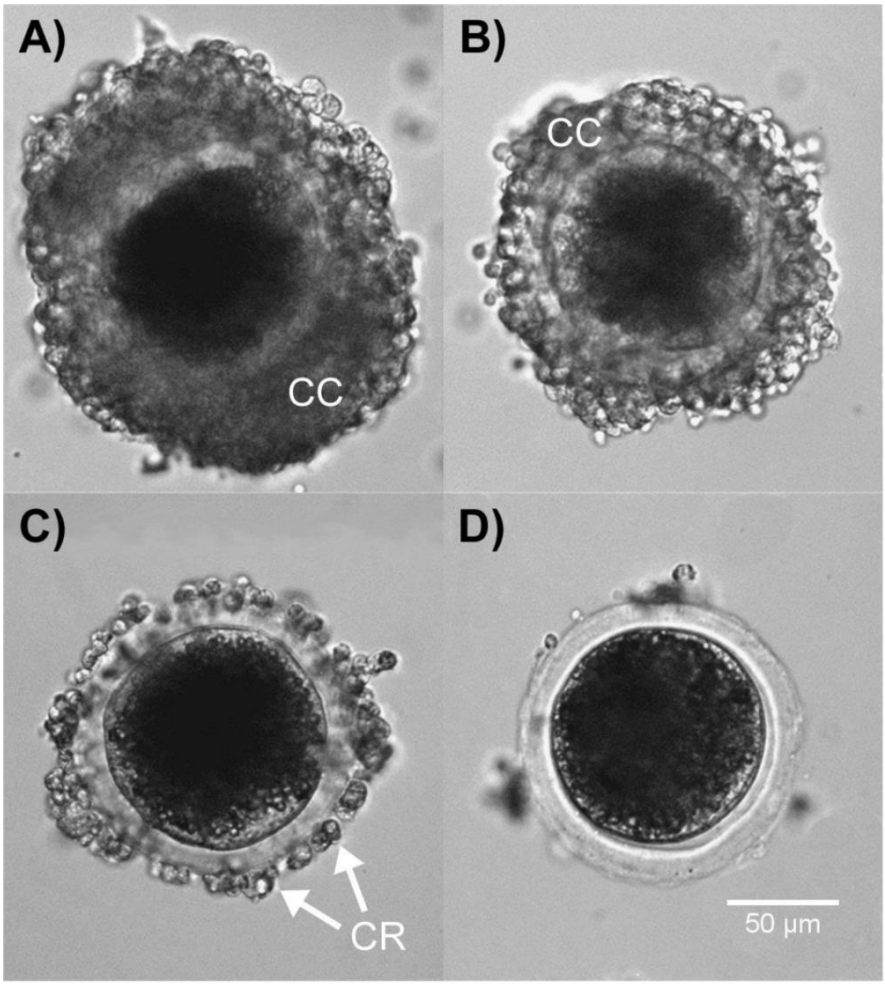

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535 **Figure 1: Representative examples of oocyte morphological categorisation.** A: grade A cumulus
536 oocyte complex; several layers of CCs and even cytoplasm, B: grade B cumulus oocyte complex;
537 intact corona radiata surrounded by another layer of CCs and even cytoplasm, C: grade C cumulus
538 oocyte complex; incomplete corona radiata or partially denuded oocyte and uneven cytoplasm,
539 and D: grade D, denuded oocyte with an uneven cytoplasm. CC = cumulus cells; CR = corona
540 radiata.

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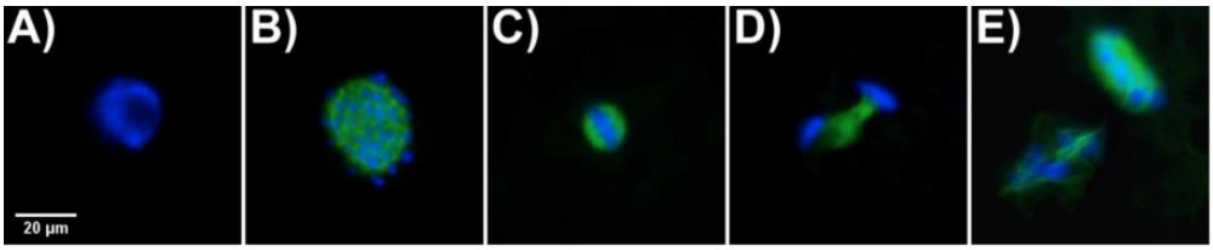
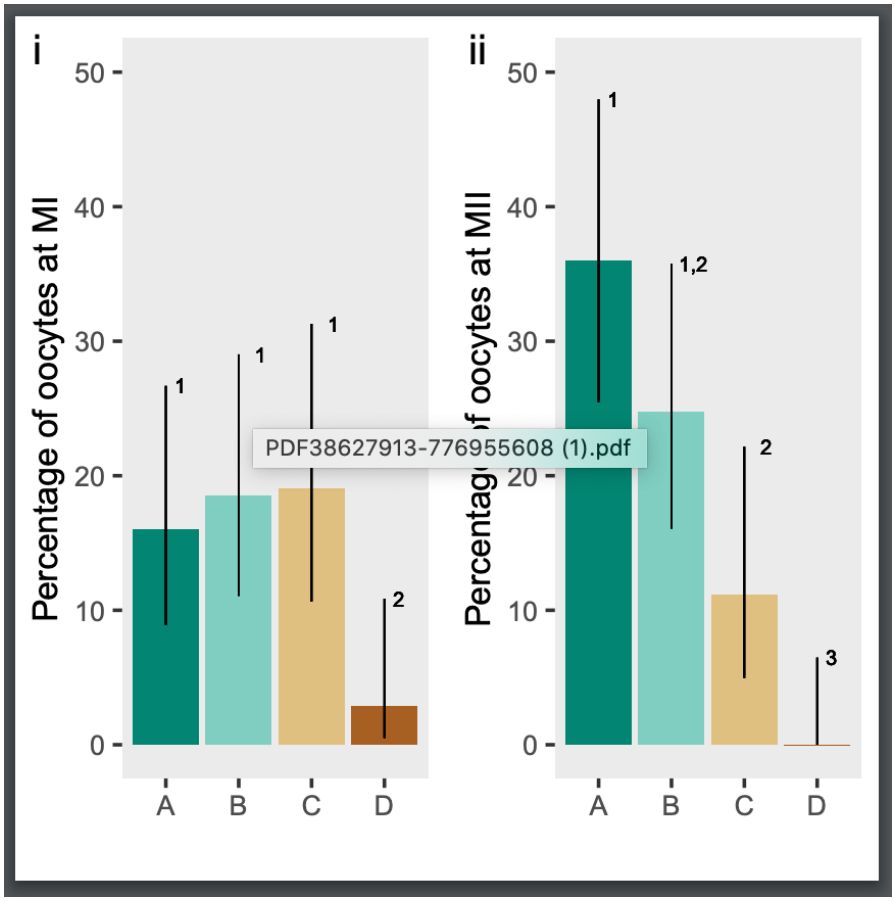


Figure 2: Meiotic stage of pig oocytes visualised by α-tubulin immunostaining**.** Shown are examples of immunostained oocytes, with tubulin shown in green (Alexa488) and DNA shown in blue (Hoechst 33342). A) Immature oocyte at the germinal vesicle stage, no spindle is detectable. B) Prometaphase I, a spindle is forming. C) Metaphase I, a clear tubulin spindle has assembled around the chromosomal compartment. D) Anaphase I, two sets of chromosomes are separated by a tubulin bridge. E) Metaphase II, two independent spindles can be detected belonging to either the oocyte or its polar body. Images captured by fluorescence microscopy at x200 total magnification.



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553 **Figure 3: Oocyte grade affects potential for nuclear maturation.** Comparison of maturation rates
554 in IVM oocytes of different grades. Shown are the percentage of oocytes achieving a particular
555 stage, with the error bars showing the 95% confidence intervals. This indicates that the
556 percentage of oocytes developing to both i) metaphase I and to ii) metaphase II differ. Numbers
557 associated with each oocyte grade relate to *post hoc* tests, with oocyte grades that do not share
558 numbers being significantly different (FDR corrected pairwise tests, $p < 0.05$).

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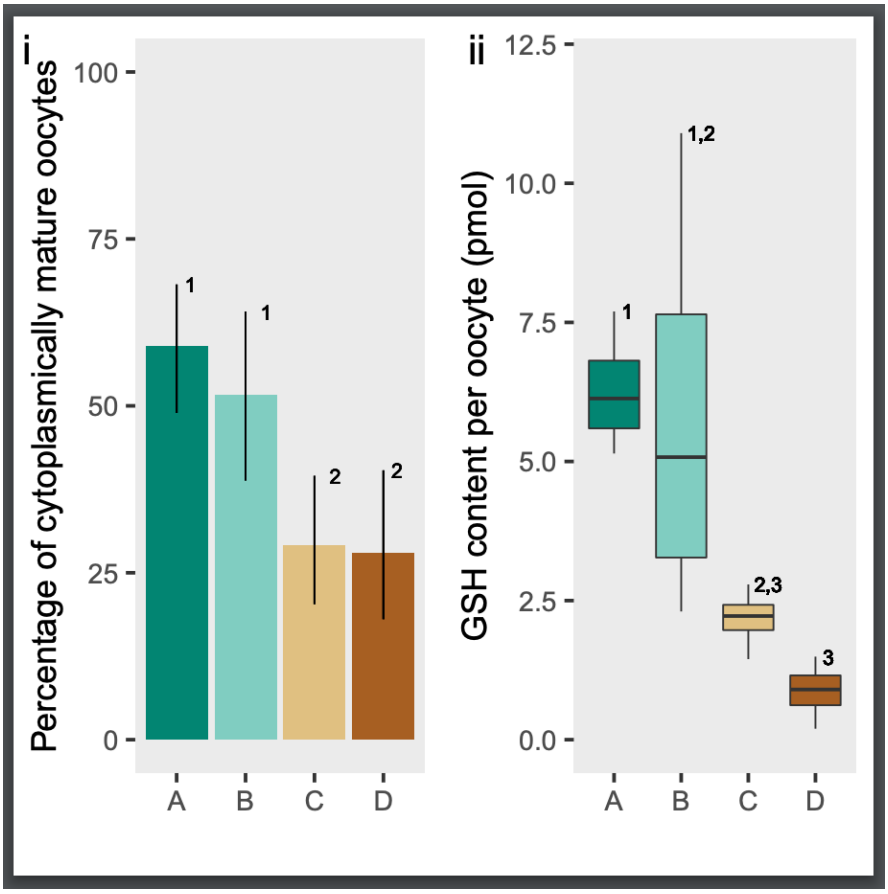

Figure 4: Oocyte grade affects potential for cytoplasmic maturation. i) The percentage, with the error bars showing the 95% confidence intervals, of oocytes assessed as mature by CG staining (N = 107, 64, 93 and 68 for Grades A, B, C and D, respectively). Numbers associated with each oocyte grade relate to *post hoc* tests, with oocyte grades that do not share numbers being significantly different (FDR corrected pairwise tests, $p < 0.05$. ii) Box plots showing the glutathione content per oocyte, with the central bars representing the median value, the lower and upper hinges corresponding to the first and third quartiles and the whiskers extending from the hinge to the largest value no further than 1.5 of the interquartile range from the hinge, numbers associated with each oocyte grade relate to *post hoc* tests, with oocyte grades that do not share numbers being significantly different (FDR corrected pairwise tests, $p < 0.05$).

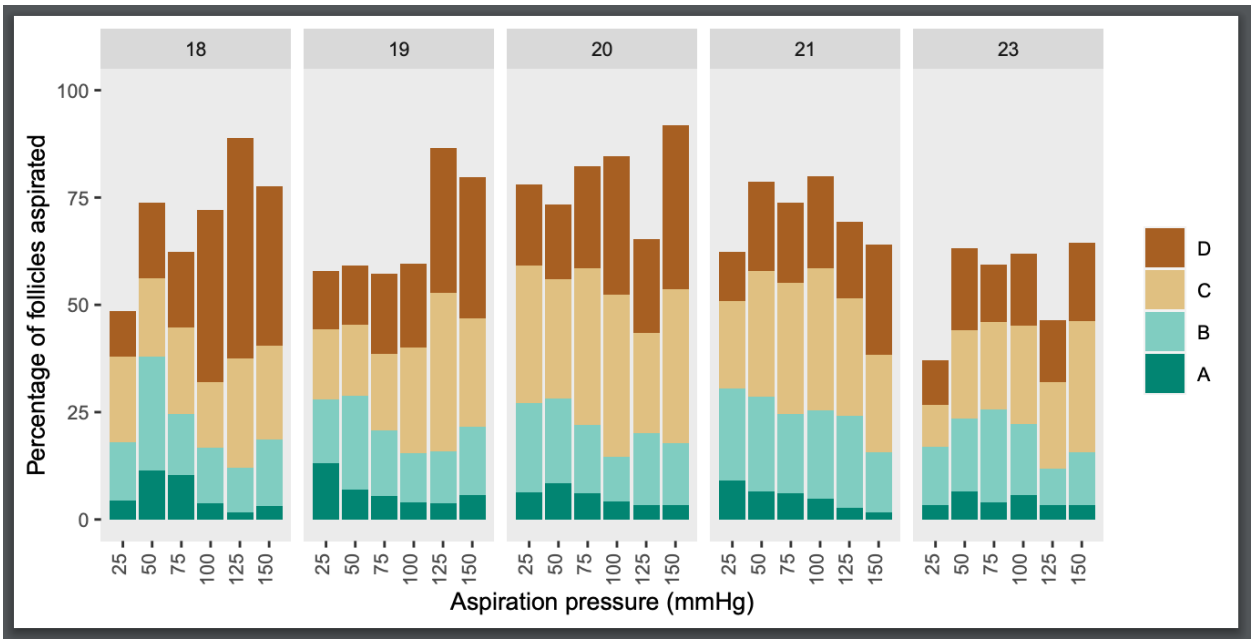

Figure 5: The effect of needle gauge and aspiration pressure on the quality of recovered oocytes. Shown is the percentage of aspirated follicles that yielded oocytes of different grades for the various combinations of needle gauges and aspiration pressures. In each column, the top of the D grade oocyte bar presents the total percentage of aspirated follicles from which an oocyte was recovered.