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Journal article

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

4

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

6

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21

22 **Abstract**

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

41

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

43 **Introduction**

44

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

59

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

65 promote the migration of cortical granules (CGs) towards the periphery of the oocyte (Galeati,
66 Modina, Lauria, & Mattioli, 1991), a key element of cytoplasmic maturation. Cytoplasmic
67 maturation has also been shown to be enhanced by high levels of glutathione (GSH) which CCs
68 actively synthesise and transport to the oocyte (Maedomari et al., 2007; You, Kim, Lim, & Lee,
69 2010), where it acts as a scavenger of reactive oxygen species (ROS) (Tatemoto, Sakurai, & Muto,
70 2000) and increases amino acid transport and protein synthesis (Lafleur, Hoorweg, Joenje,
71 Westmijze, & Retèl, 1994). Furthermore, high levels of GSH seem important for correct male
72 pronuclear formation upon fertilisation (Niwa, 1993; Yoshida, Ishigaki, Nagai, Chikyu, & Pursel,
73 1993). Due to the many functional roles of CCs, the morphology of the COC is commonly used to
74 determine candidates for IVM in pigs and in other farm animals (Alvarez, Dalvit, Achi, Miguez, &
75 Cetica, 2009; Nagano, Katagiri, & Takahashi, 2006). Laboratory based retrieval methods and
76 aspiration pressures used in ovum pickup from live animals have shown that aspiration pressure
77 also has an impact on the morphology of the COC (Brüssow, Torner, Ratky, Hunter, & Nürnberg,
78 1997; Marques et al., 2015).

79

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

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

102

103 **Materials and Methods**

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

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

112

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

123

124 *Oocyte maturation*

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

130

131 IVM of graded oocytes was undertaken in North Carolina State University (NCSU)-23 media
132 (Petters & Wells, 1993) supplemented with 10% porcine follicular fluid (pFF) that had been
133 collected from a separate batch of abattoir-derived ovaries. pFF was prepared by centrifugation
134 for 30 minutes at 3,000rpm, and subsequent filter sterilisation of the supernatant using a
135 Minisart single use filter (0.2mm). pFF was stored at -20°C until use. Graded oocytes were
136 matured separately in groups of 50 in 500µl media under 6% CO₂ at 37°C. For the first 22 hours,
137 the culture medium was supplemented with hormones (1:100 PG600, Intervet, Milton Keys, UK),
138 0.8mM L-cysteine, 10ng/ml EGF, 1mM db-cAMP and 50µM β-mercaptoethanol. Oocytes were
139 then transferred to fresh medium for a further 22 h with the hormones and db-cAMP excluded.
140 The IVM oocytes produced were then tested as described below.

141
142 To assess the nuclear maturation potential of oocytes of different grades, the meiotic spindles of
143 IVM oocytes were immunostained with anti α-tubulin. IVM oocytes were denuded of CCs by
144 incubation for 10 minutes in 0.2%w/v Hyaluronidase Type VIII from bovine testis and passing
145 them several times through a 125-µm tip. These oocytes were then fixed overnight at 4°C in 4%
146 PFA in PHEM buffer (60mM PIPES, 25mM HEPES, 10mM EGTA, 4mM MgSO₄, pH 7.0). Fixed
147 oocytes were then rinsed three times in PBS for five minutes and permeabilised for ten minutes
148 in 1% Triton X-100 in PHEM buffer. After rinsing in PBS, the oocytes were blocked in 20% fetal
149 bovine serum (FBS) PHEM buffer for one hour at room temperature. Oocytes were then stained
150 for 30 minutes in 1:200 anti-α tubulin-Alexa 488 (ab195887, Abcam, UK), 5% FBS in PHEM buffer.
151 After rinsing in PBS, oocytes were counterstained in 1 µg/ml Hoechst 33342 in PBS, mounted on
152 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

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

181

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

188

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

195

196 *Oocyte recovery*

197 For analysis of how specific combinations of aspiration pressure and needle gauge affect COC
198 recovery and morphology, oocytes were collected using an aspirator pump (Labotect Aspirator
199 3). The rubber tubing attaching the needle to the aspirant collection tube was primed with TL-
200 HEPES-PVA (Funahashi, Cantley, & Day, 1997). A needle of appropriate diameter (18, 19, 20, 21
201 and 23-gauge) (BD Microlance 3) was attached to the aspirator pump, set to an appropriate
202 pressure setting (25, 50, 75, 100, 125 or 150mmHg), connected to a 50ml aspirant collection tube
203 sealed with a rubber bung. TL-HEPES-PVA media was flushed through the rubber tube prior to
204 oocyte collection and subsequently periodically to ensure all COCs were recovered into the
205 aspirant collection tube. All 25 combinations of needle and pressure could not be tested on each
206 batch of ovaries as this would either limit sample size per treatment or make the collection period
207 too long. Therefore, for each round of oocyte collection, between three and six different
208 combinations of needle and pressure were tested, with follicles harvested from ten random
209 ovaries from the shipment for each combination. The effects of needle gauge and aspiration
210 pressure on the recovery of 1) oocytes, 2) grade A COCs, and 3) grade A and B COCs, from the
211 follicles aspirated was assessed by using a chi-squared test using Bonferroni corrected p values
212 for different aspiration pressures within each needle gauge. For significant tests, differences
213 between aspiration pressures were then determined by false discovery rate (FDR) corrected
214 pairwise tests (Benjamini & Hochberg, 1995). Differences between the standardised Pearson
215 residuals were used to determine pressures associated with increased recovery rates. In total,
216 38,595 follicles were aspirated using a range of pressures and needle gauges, yielding 26,370
217 oocytes.
218

219 *Ethical approval process*

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

221

222 **Results**

223 *Oocyte maturation*

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

231

232 Nuclear maturation in IVM oocytes was evaluated by visualising the metaphasic spindles with α -
233 tubulin immunostaining (Figure 2). This showed that the standard diagnostic features of meiosis
234 could be identified and that the oocytes were maturing. Analysis of these data indicates that IVM
235 oocytes of different grades differ in their rates of nuclear maturation ($\chi^2 = 10.05$, $df = 3$, $p = 0.018$,
236 and $\chi^2 = 35.19$, $df = 3$, $p < 0.0001$ for the percentage assessed as metaphase I and metaphase II,
237 respectively), with higher grade oocytes displaying higher levels of nuclear maturation (Figure 3i
238 and B). These analyses indicate that the maturation potential of Grade D oocytes is very limited,
239 with only a small percentage (mean of 2.9%) reaching metaphase I (Figure 3i) and no oocytes of
240 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 ($6.3 \pm$
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

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

269

270 **Discussion**

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

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

291
292 Our results indicate that nuclear maturation and cytoplasmic maturation is variable across the
293 four grades of oocytes (Figure 1). Grade A and B oocytes were observed to mature at high rates
294 whilst fewer Grade C and D oocytes were assessed as having completed maturation (Figure 4).
295 Interestingly, fully denuded oocytes (Grade D), which were never observed to complete nuclear
296 maturation, were occasionally observed to have progressed to full cytoplasmic maturation,
297 therefore indicating that these two processes are not necessarily linked. Alternatively, as the
298 developmental capabilities of denuded oocytes can be rescued by co-culture with COCs (Luciano
299 et al., 2005), the limited development seen here may have been the result of the presence of a
300 limited number of CCs in the wells in which Grade D oocytes were cultured.

301
302 The IVM system in this study included the use of both cysteine and β -mercaptoethanol. It has
303 been reported that the mean GSH content in pig oocytes increased from 7.9 ± 0.6 pmol/oocyte
304 to 10.4 ± 2.8 pmol/oocyte (means \pm standard errors) in the presence of $50 \mu\text{M}$ β -
305 mercaptoethanol (Abeydeera, Wang, Cantley, Prather, & Day, 1998), whilst another found that
306 supplementing the maturation medium with 0.57 mM cysteine increased oocyte GSH from $4.0 \pm$

307 0.8 pmol/oocyte to 15 ± 0.3 pmol/oocyte (means \pm standard errors) (Yoshida et al., 1993). Our
308 findings indicated that Grade A and B oocytes have levels of GSH comparable to these levels,
309 while Grade C and D oocytes appear largely depleted of GSH (Figure 4). This shows the presence
310 of cysteine and β -mercaptoethanol alone during IVM is not sufficient to increase intracellular
311 GSH levels in pig oocytes as CCs are required to complete the process (Tatemoto et al., 2000).
312 Moreover, the absence of enough GSH in Grade C and D oocytes could well explain their reduced
313 cytoplasmic and nuclear maturation potentials.

314

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

326

327 **Conclusions**

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

343

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347

348 **Conflicts of interest**

349 The authors declare no competing interests.

350

351 **Authors' contributions**

352 Author contributions: Conceptualization: D.K.G., K.E.H., P.J.I.E., G.A.W.; Methodology: D.K.G.,
353 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
354 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.,
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356 K.E.H.

357

358 **Data availability statement**

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

361

362 **References**

- 363 Abeydeera, L. R., Wang, W. H., Cantley, T. C., Prather, R. S., & Day, B. N. (1998). Presence of β -
364 mercaptoethanol can increase the glutathione content of pig oocytes matured in vitro
365 and the rate of blastocyst development after in vitro fertilization. *Theriogenology*, *50*(5),
366 747–756. [https://doi.org/https://doi.org/10.1016/S0093-691X\(98\)00180-0](https://doi.org/https://doi.org/10.1016/S0093-691X(98)00180-0)
- 367 Alvarez, G. M., Dalvit, G. C., Achi, M. V., Miguez, M. S., & Cetica, P. D. (2009). Immature oocyte
368 quality and maturational competence of porcine cumulus-oocyte complexes
369 subpopulations. *Biocell*, *33*(3), 167–177.
- 370 Anderson, E., & Albertini, D. F. (1976). Gap junctions between the oocyte and companion
371 follicle cells in the mammalian ovary. *The Journal of Cell Biology*, *71*(2), 680–686.

372 <https://doi.org/https://doi.org/10.1083/jcb.71.2.680>

373 Bagg, M. A., Nottle, M. B., Armstrong, D. T., & Grupen, C. G. (2007). Relationship between
374 follicle size and oocyte developmental competence in prepubertal and adult pigs.
375 *Reproduction, Fertility and Development*, 19(7), 797–803.
376 <https://doi.org/https://doi.org/10.1071/RD07018>

377 Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: a practical and
378 powerful approach to multiple testing. *Journal of the Royal Statistical Society: Series B*
379 *(Methodological)*, 57(1), 289–300. [https://doi.org/https://doi.org/10.1111/j.2517-](https://doi.org/https://doi.org/10.1111/j.2517-6161.1995.tb02031.x)
380 [6161.1995.tb02031.x](https://doi.org/https://doi.org/10.1111/j.2517-6161.1995.tb02031.x)

381 Bruinsma, J. (2002). *World Agriculture: towards 2015/2030: summary report*. Food and
382 Agriculture Organization of the United Nations (FAO).

383 Brüssow, K. P., Torner, H., Ratky, J., Hunter, M. G., & Nürnberg, G. (1997). Ovum pick up in
384 swine: the influence of aspiration vacuum pressure on oocyte recovery from preovulatory
385 follicles. *Acta Veterinaria Hungarica*, 45, 189–196.

386 Dang-Nguyen, T. Q., Somfai, T., Haraguchi, S., Kikuchi, K., Tajima, A., Kanai, Y., & Nagai, T.
387 (2011). In vitro production of porcine embryos: current status, future perspectives and
388 alternative applications. *Animal Science Journal*, 82(3), 374–382.
389 <https://doi.org/https://doi.org/10.1111/j.1740-0929.2011.00883.x>

390 Esaki, R., Ueda, H., Kurome, M., Hirakawa, K., Tomii, R., Yoshioka, H., ... Nagashima, H. (2004).
391 Cryopreservation of porcine embryos derived from in vitro-matured oocytes. *Biology of*
392 *Reproduction*, 71(2), 432–437.
393 <https://doi.org/https://doi.org/10.1095/biolreprod.103.026542>

394 Fowler, K. E., Mandawala, A. A., Griffin, D. K., Walling, G. A., & Harvey, S. C. (2018). The
395 production of pig preimplantation embryos in vitro: Current progress and future
396 prospects. *Reproductive Biology*. <https://doi.org/10.1016/j.repbio.2018.07.001>

397 Funahashi, H., Cantley, T. C., & Day, B. N. (1997). Synchronization of meiosis in porcine oocytes
398 by exposure to dibutyl cyclic adenosine monophosphate improves developmental
399 competence following in vitro fertilization. *Biology of Reproduction*, *57*(1), 49–53.
400 <https://doi.org/https://doi.org/10.1095/biolreprod57.1.49>

401 Funahashi, H., Cantley, T. C., Stumpf, T. T., Terlouw, S. L., & Day, B. N. (1994). In vitro
402 development of in vitro-matured porcine oocytes following chemical activation or in vitro
403 fertilization. *Biology of Reproduction*, *50*(5), 1072–1077.
404 <https://doi.org/https://doi.org/10.1095/biolreprod50.5.1072>

405 Galeati, G., Modina, S., Lauria, A., & Mattioli, M. (1991). Follicle somatic cells influence pig
406 oocyte penetrability and cortical granule distribution. *Molecular Reproduction and
407 Development*, *29*(1), 40–46. <https://doi.org/https://doi.org/10.1002/mrd.1080290107>

408 Gilchrist, R. B., Ritter, L. J., & Armstrong, D. T. (2004). Oocyte–somatic cell interactions during
409 follicle development in mammals. *Animal Reproduction Science*, *82*, 431–446.

410 Kim, J., You, J., Hyun, S., Lee, G., Lim, J., & Lee, E. (2010). Developmental competence of
411 morphologically poor oocytes in relation to follicular size and oocyte diameter in the pig.
412 *Molecular Reproduction and Development: Incorporating Gamete Research*, *77*(4), 330–
413 339. <https://doi.org/https://doi.org/10.1002/mrd.21148>

414 Lafleur, M. V. M., Hoorweg, J. J., Joenje, H., Westmijze, E. J., & Retèl, J. (1994). The ambivalent
415 role of glutathione in the protection of DNA against singlet oxygen. *Free Radical Research*,

416 21(1), 9–17. <https://doi.org/https://doi.org/10.3109/10715769409056550>

417 Lechniak, D., Warzych, E., Pers-Kamczyc, E., Sosnowski, J., Antosik, P., & Rubes, J. (2007). Gilts
418 and sows produce similar rate of diploid oocytes in vitro whereas the incidence of
419 aneuploidy differs significantly. *Theriogenology*, 68(5), 755–762.
420 <https://doi.org/https://doi.org/10.1016/j.theriogenology.2007.06.012>

421 Lee, J., Hyun, S.-H., & Lee, E. (2012). A comparative study on the parthenogenetic development
422 of pig oocytes cultured in north carolina state university-23 and porcine zygote medium-
423 3. *J. Emb. Trans*, 27, 121–126.

424 Li, H. J., Sutton-McDowall, M. L., Wang, X., Sugimura, S., Thompson, J. G., & Gilchrist, R. B.
425 (2016). Extending prematuration with cAMP modulators enhances the cumulus
426 contribution to oocyte antioxidant defence and oocyte quality via gap junctions. *Human*
427 *Reproduction*, 31(4), 810–821. <https://doi.org/https://doi.org/10.1093/humrep/dew020>

428 Lin, T., Oqani, R. K., Lee, J. E., Shin, H. Y., & Jin, D. Il. (2016). Coculture with good-quality COCs
429 enhances the maturation and development rates of poor-quality COCs. *Theriogenology*,
430 85(3), 396–407. <https://doi.org/https://doi.org/10.1016/j.theriogenology.2015.09.001>

431 Long, C. R., Dobrinsky, J. R., & Johnson, L. A. (1999). In vitro production of pig embryos:
432 comparisons of culture media and boars. *Theriogenology*, 51(7), 1375–1390.
433 [https://doi.org/https://doi.org/10.1016/S0093-691X\(99\)00081-3](https://doi.org/https://doi.org/10.1016/S0093-691X(99)00081-3)

434 Luciano, A. M., Lodde, V., Beretta, M. S., Colleoni, S., Lauria, A., & Modina, S. (2005).
435 Developmental capability of denuded bovine oocyte in a Co-culture system with intact
436 cumulus-oocyte complexes: Role of cumulus cells, cyclic adenosine 3', 5'-monophosphate,
437 and glutathione. *Molecular Reproduction and Development: Incorporating Gamete*

438 *Research*, 71(3), 389–397. <https://doi.org/https://doi.org/10.1002/mrd.20304>

439 Ma, W., Hou, Y., Sun, Q. Y., Sun, X. F., & Wang, W. H. (2003). Localization of centromere
440 proteins and their association with chromosomes and microtubules during meiotic
441 maturation in pig oocytes. *Reproduction*, 126(6), 731–738.

442 Maedomari, N., Kikuchi, K., Ozawa, M., Noguchi, J., Kaneko, H., Ohnuma, K., ... Kashiwazaki, N.
443 (2007). Cytoplasmic glutathione regulated by cumulus cells during porcine oocyte
444 maturation affects fertilization and embryonic development in vitro. *Theriogenology*,
445 67(5), 983–993. <https://doi.org/https://doi.org/10.1016/j.theriogenology.2006.11.012>

446 Marchal, R., Vigneron, C., Perreau, C., Bali-Papp, A., & Mermillod, P. (2002). Effect of follicular
447 size on meiotic and developmental competence of porcine oocytes. *Theriogenology*,
448 57(5), 1523–1532. [https://doi.org/https://doi.org/10.1016/S0093-691X\(02\)00655-6](https://doi.org/https://doi.org/10.1016/S0093-691X(02)00655-6)

449 Marques, M. G., de Barros, F. R. O., Goissis, M. D., Giassetti, M. I., Assumpção, M. E. O. D., &
450 Visintin, J. A. (2015). Effect of oocyte recovery techniques on in vitro production of swine
451 embryos. *Open Journal of Animal Sciences*, 5(04), 467.
452 <https://doi.org/10.4236/ojas.2015.54048>

453 Mattioli, M., & Barboni, B. (2000). Signal transduction mechanism for LH in the cumulus–oocyte
454 complex. *Molecular and Cellular Endocrinology*, 161(1–2), 19–23.
455 [https://doi.org/https://doi.org/10.1016/S0303-7207\(99\)00218-X](https://doi.org/https://doi.org/10.1016/S0303-7207(99)00218-X)

456 Nagai, T., Ding, J., & Moor, R. M. (1993). Effect of follicle cells and steroidogenesis on
457 maturation and fertilization in vitro of pig oocytes. *Journal of Experimental Zoology*,
458 266(2), 146–151. <https://doi.org/https://doi.org/10.1002/jez.1402660208>

459 Nagano, M., Katagiri, S., & Takahashi, Y. (2006). Relationship between bovine oocyte

460 morphology and in vitro developmental potential. *Zygote*, 14(1), 53–61.

461 <https://doi.org/https://doi.org/10.1017/S0967199406003510>

462 Niwa, K. (1993). Effectiveness of in vitro maturation and in vitro fertilization techniques in pigs.

463 *Journal of Reproduction and Fertility. Supplement*, 48, 49–59.

464 Norris, R. P., Freudzon, M., Mehlmann, L. M., Cowan, A. E., Simon, A. M., Paul, D. L., ... Jaffe, L.

465 A. (2008). Luteinizing hormone causes MAP kinase-dependent phosphorylation and

466 closure of connexin 43 gap junctions in mouse ovarian follicles: one of two paths to

467 meiotic resumption. *Development*, 135(19), 3229–3238.

468 <https://doi.org/10.1242/dev.025494>

469 Norris, R. P., Ratzan, W. J., Freudzon, M., Mehlmann, L. M., Krall, J., Movsesian, M. A., ... Jaffe, L.

470 A. (2009). Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and

471 meiosis in the mouse oocyte. *Development*, 136(11), 1869–1878.

472 <https://doi.org/10.1242/dev.035238>

473 Park, B., Lee, H., Lee, Y., Elahi, F., Lee, J., Lee, S. T., ... Lee, E. (2016). Cilostamide and forskolin

474 treatment during pre-IVM improves preimplantation development of cloned embryos by

475 influencing meiotic progression and gap junction communication in pigs. *Theriogenology*,

476 86(3), 757–765. <https://doi.org/https://doi.org/10.1016/j.theriogenology.2016.02.029>

477 Petters, R. M., & Wells, K. D. (1993). Culture of pig embryos. *Journal of Reproduction and*

478 *Fertility. Supplement*, 48, 61–73.

479 Racowsky, C. (1985). Effect of forskolin on maintenance of meiotic arrest and stimulation of

480 cumulus expansion, progesterone and cyclic AMP production by pig oocyte—cumulus

481 complexes. *Journal of Reproduction and Fertility*, 74(1), 9–21.

482 <https://doi.org/https://doi.org/10.1530/jrf.0.0740009>

483 Rath, D., Niemann, H., & Tao, T. (1995). In vitro maturation of porcine oocytes in follicular fluid
484 with subsequent effects on fertilization and embryo yield in vitro. *Theriogenology*, *44*(4),
485 529–538. [https://doi.org/https://doi.org/10.1016/0093-691X\(95\)00224-V](https://doi.org/https://doi.org/10.1016/0093-691X(95)00224-V)

486 Sherrer, E. S., Rathbun, T. J., & Davis, D. L. (2004). Fertilization and blastocyst development in
487 oocytes obtained from prepubertal and adult pigs. *Journal of Animal Science*, *82*(1), 102–
488 108. <https://doi.org/https://doi.org/10.2527/2004.821102x>

489 Sugimura, S., Yamanouchi, T., Palmerini, M. G., Hashiyada, Y., Imai, K., & Gilchrist, R. B. (2018).
490 Effect of pre-in vitro maturation with cAMP modulators on the acquisition of oocyte
491 developmental competence in cattle. *Journal of Reproduction and Development*.
492 <https://doi.org/https://doi.org/10.1262/jrd.2018-009>

493 Sun, Q.-Y., & Nagai, T. (2003). Molecular mechanisms underlying pig oocyte maturation and
494 fertilization. *Journal of Reproduction and Development*, *49*(5), 347–359.
495 <https://doi.org/https://doi.org/10.1262/jrd.49.347>

496 Tanghe, S., Van Soom, A., Nauwynck, H., Coryn, M., & de Kruif, A. (2002). Minireview: functions
497 of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. *Molecular*
498 *Reproduction and Development*, *61*(3), 414–424.
499 <https://doi.org/https://doi.org/10.1002/mrd.10102>

500 Tatemoto, H., Sakurai, N., & Muto, N. (2000). Protection of porcine oocytes against apoptotic
501 cell death caused by oxidative stress during in vitro maturation: role of cumulus cells.
502 *Biology of Reproduction*, *63*(3), 805–810.
503 <https://doi.org/https://doi.org/10.1095/biolreprod63.3.805>

504 Taylor, R., Condoleo, R., Simons, R., Gale, P., Kelly, L., & Snary, E., (2020). The risk of infection by
505 African swine fever virus in European swine through boar movement and legal trade of
506 pigs and pig meat. *Frontiers in Veterinary Science*, 6, 486.
507 <https://doi.org/10.3389/fvets.2019.00486>

508 Yoshida, M., Ishigaki, K., Nagai, T., Chikyu, M., & Pursel, V. G. (1993). Glutathione concentration
509 during maturation and after fertilization in pig oocytes: relevance to the ability of oocytes
510 to form male pronucleus. *Biology of Reproduction*, 49(1), 89–94.
511 <https://doi.org/https://doi.org/10.1095/biolreprod49.1.89>

512 You, J., Kim, J., Lim, J., & Lee, E. (2010). Anthocyanin stimulates in vitro development of cloned
513 pig embryos by increasing the intracellular glutathione level and inhibiting reactive
514 oxygen species. *Theriogenology*, 74(5), 777–785.
515 <https://doi.org/https://doi.org/10.1016/j.theriogenology.2010.04.002>

516 Zhang, X., Miao, Y., Zhao, J.-G., Spate, L., Bennett, M. W., Murphy, C. N., ... Prather, R. S. (2010).
517 Porcine oocytes denuded before maturation can develop to the blastocyst stage if
518 provided a cumulus cell-derived coculture system. *Journal of Animal Science*, 88(8),
519 2604–2610. <https://doi.org/doi.org/10.2527/jas.2009-2714>

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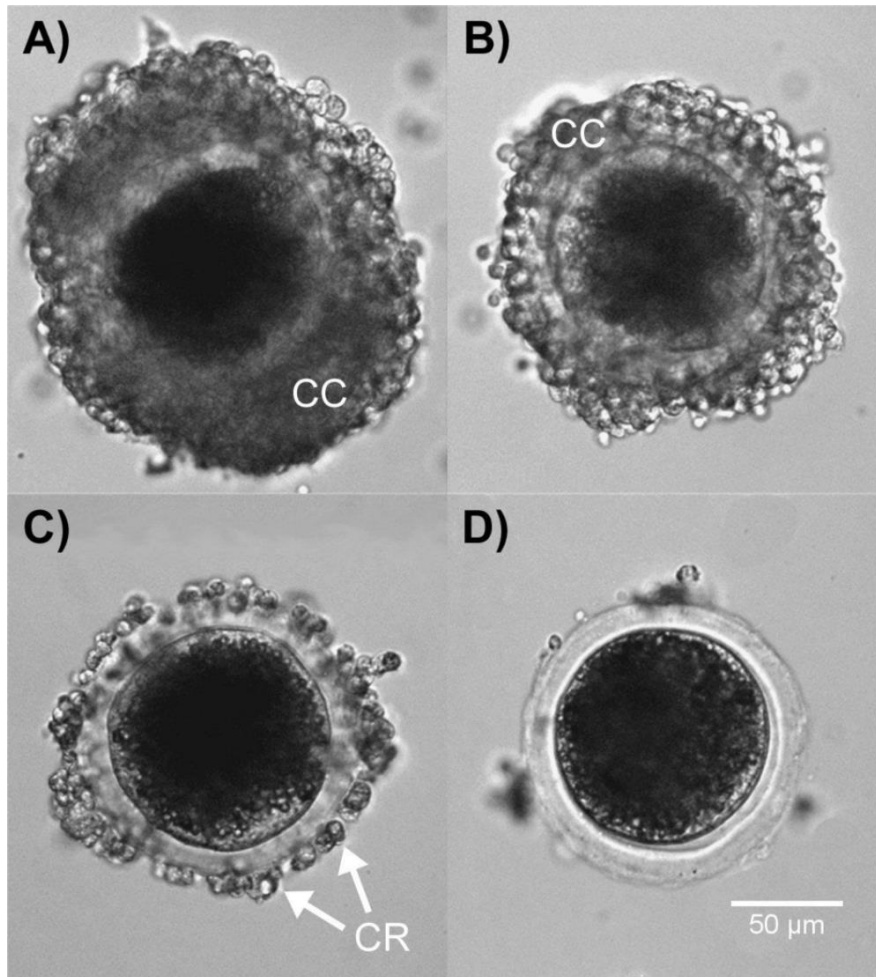
522 **Table 1: The effect of needle gauge and aspiration pressure on the recovery rates and quality**
523 **of recovered oocytes.** Shown for comparisons of 1) oocyte recovery, 2) recovery of A and B grade
524 oocytes, and 3) recovery of A grade oocytes only, are the χ^2 values and Bonferroni corrected p
525 values for tests of different aspiration pressures within each needle gauge, and results of *post*
526 *hoc* comparisons, where aspiration pressures that differ significantly do not share a number. The
527 aspiration pressures associated with recovery of increased numbers of oocytes, or of higher
528 proportions of high-quality oocytes (grades A and B in 2 and grade A only in 1), are also listed (as
529 determined by inspection of the standardised Pearson residuals). Where multiple pressures are
530 listed, the order indicates the strength of the association with the first pressure listed having the
531 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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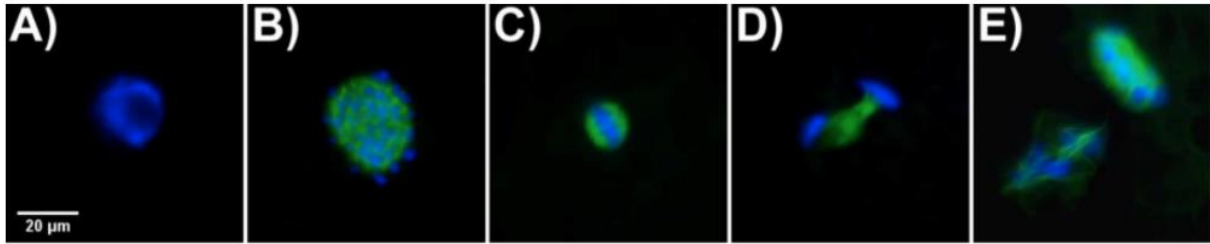
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534

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.

541



542

543 **Figure 2:** Meiotic stage of pig oocytes visualised by α -tubulin immunostaining. Shown are

544 examples of immunostained oocytes, with tubulin shown in green (Alexa488) and DNA shown in

545 blue (Hoechst 33342). A) Immature oocyte at the germinal vesicle stage, no spindle is detectable.

546 B) Prometaphase I, a spindle is forming. C) Metaphase I, a clear tubulin spindle has assembled

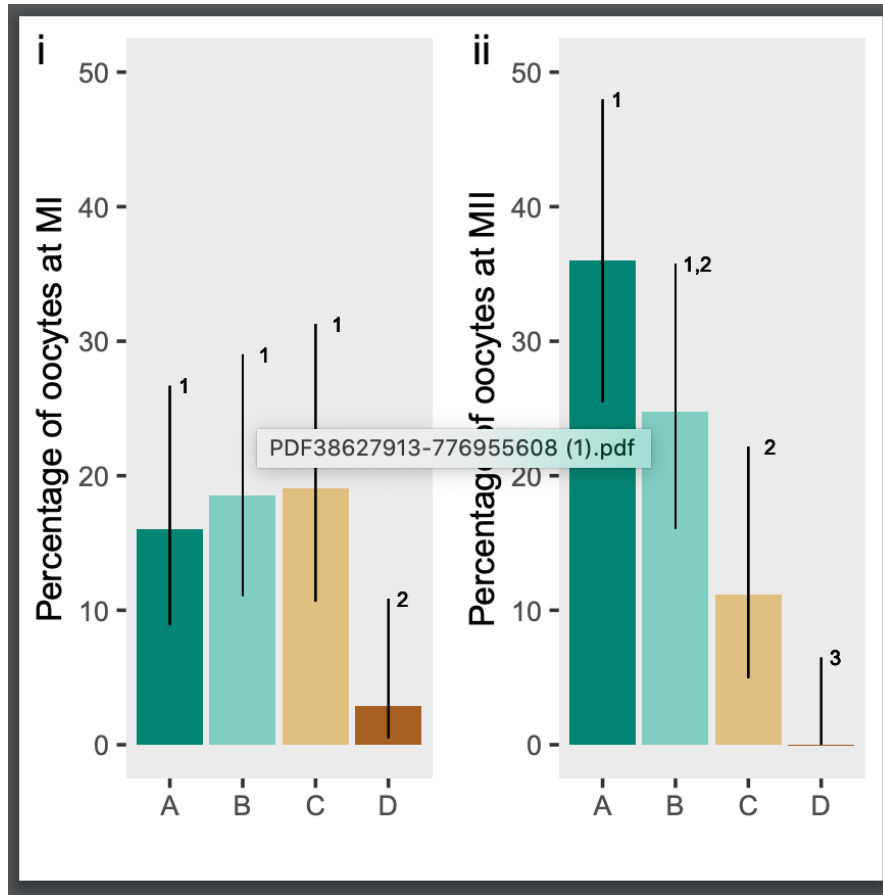
547 around the chromosomal compartment. D) Anaphase I, two sets of chromosomes are separated

548 by a tubulin bridge. E) Metaphase II, two independent spindles can be detected belonging to

549 either the oocyte or its polar body. Images captured by fluorescence microscopy at x200 total

550 magnification.

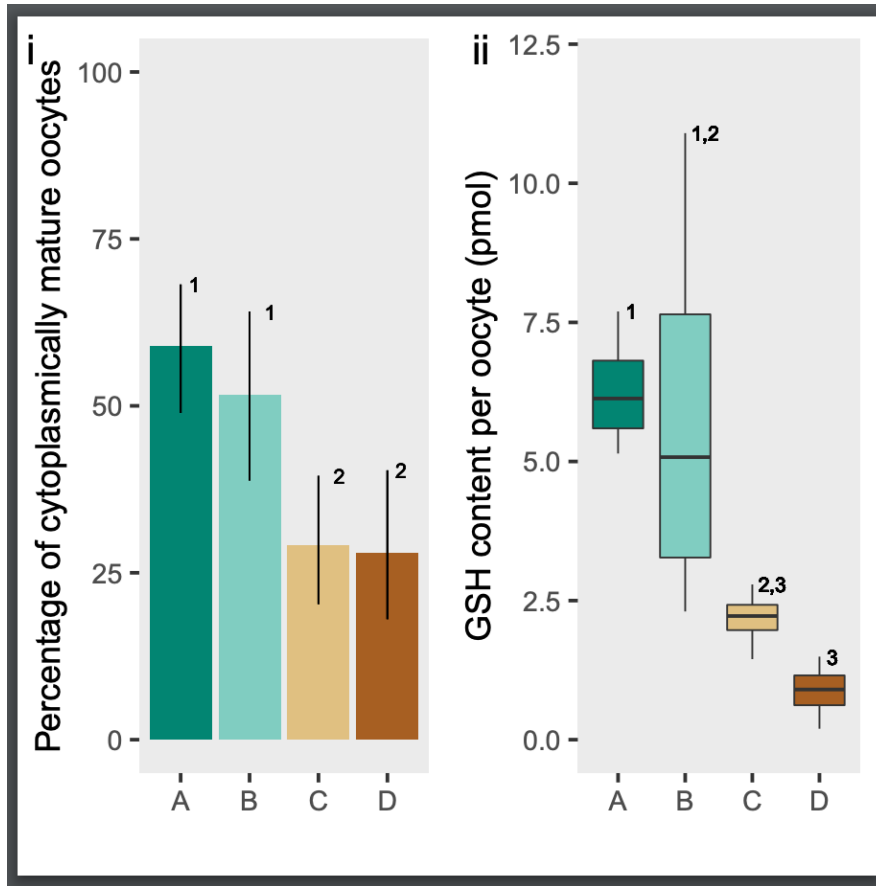
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552

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, $p < 0.05$).

559



560

561 **Figure 4: Oocyte grade affects potential for cytoplasmic maturation.** i) The percentage, with the

562 error bars showing the 95% confidence intervals, of oocytes assessed as mature by CG staining

563 (N = 107, 64, 93 and 68 for Grades A, B, C and D, respectively). Numbers associated with each

564 oocyte grade relate to *post hoc* tests, with oocyte grades that do not share numbers being

565 significantly different (FDR corrected pairwise tests, $p < 0.05$. ii) Box plots showing the glutathione

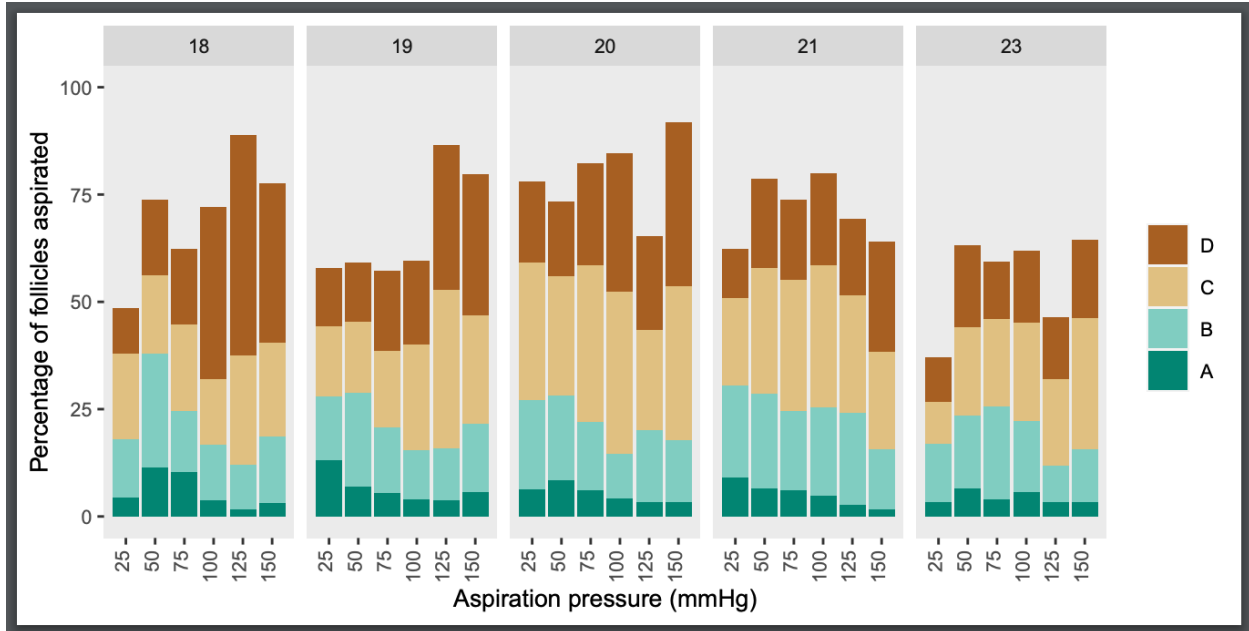
566 content per oocyte, with the central bars representing the median value, the lower and upper

567 hinges corresponding to the first and third quartiles and the whiskers extending from the hinge

568 to the largest value no further than 1.5 of the interquartile range from the hinge, numbers

569 associated with each oocyte grade relate to *post hoc* tests, with oocyte grades that do not share

570 numbers being significantly different (FDR corrected pairwise tests, $p < 0.05$).



571

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