

1 ***In vitro* assessment of sperm from bulls of high and low field fertility**

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16

17 **Abstract**

18

19 The aim of this study was to investigate the reasons for differences in field fertility of
20 bulls following insemination with frozen-thawed semen. The study was carried out in
21 two separate parts over two years and comparisons were made between 5 high and 4
22 low fertility Holstein Friesian bulls as determined by their either 90 day non-return
23 rate (Year 1) or calving rate (Year 2). Two high fertility Limousin bulls were included
24 in Year 1 for comparative purposes. The ability of sperm from each bull to penetrate
25 artificial mucus was assessed (Year 1 = 7 replicates; Year 2 = 5 replicates). Glass
26 capillary tubes (2 per bull per replicate) were filled with artificial mucus and
27 incubated with sperm stained in 1% Hoechst 33342 for 30 min at 37 °C. The number
28 of sperm were subsequently counted at 10 mm intervals along the tube between 40
29 and 80 mm markers. Sperm mitochondrial activity of each bull was assessed by the
30 MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay (4
31 replicates in each year). Sperm were incubated with MTT for 1 h at 37°C following
32 which the absorbance of formazan was read using a spectrophotometer. Sperm
33 viability after thawing was assessed for each bull using a live/dead sperm viability kit
34 (Year 1 = 3 replicates; Year 2 = 4 replicates). A minimum of 250 cells were assessed
35 per bull in each replicate and classified as either live or dead. Finally, the ability of
36 sperm to fertilise oocytes *in vitro* and their ability to develop to blastocyst stage
37 embryos were assessed (5 replicates in each year involving 220 to 306 oocytes per
38 bull). Data transformation to normalise residuals was required for mucus sperm
39 penetration (square root) and IVF (cleavage and blastocyst rate) results (arcsin). The
40 mean number of sperm counted at each 10 mm mark between 40 and 80 mm was
41 higher in the high fertility (56.0; 95%CI 39.5 to 75.3) compared to the low fertility

42 (42.9; 95% CI 29.3 to 59.1) Holstein Friesian bulls but the difference did not reach
43 formal significance ($P = 0.09$). Fertility status had no effect on the ability of sperm to
44 reduce MTT to formazan (mean absorbance 0.34 ± 0.051 and 0.30 ± 0.044) or on the
45 percentage of live sperm per straw (mean 47.3 ± 5.47 and 32.4 ± 4.66) for high and
46 low fertility Holstein Friesian bulls respectively. Oocyte cleavage rate following
47 insemination with sperm from high fertility Holstein Friesian bulls was significantly
48 higher than with sperm from low fertility Holstein Friesian bulls [76.7% (95%CI 60.9
49 to 89.4) and 55.3 (95%CI 40.4 to 69.7) respectively, $P = 0.04$]. There was no
50 significant effect of bull fertility on blastocyst rate [34.7% (95%CI 21.1 to 49.6) and
51 24.2 % (95%CI 14.1 to 36.0) for the high and low fertility Holstein Friesian bulls,
52 respectively; $P = 0.2$]. In conclusion, sperm from high fertility bulls tended to be more
53 effective in penetrating artificial mucus and to have an increased ability to fertilise
54 oocytes *in vitro*; however, once fertilisation occurred subsequent embryo
55 development was not significantly affected by fertility status.

56

57 *Keywords:* Bull semen, field fertility, artificial mucus, IVF, MTT

58

59 **1. Introduction**

60

61 The introduction of artificial insemination (AI) to the dairy industry in the 1950s
62 revolutionised cattle breeding and has displaced natural service as the preferred
63 method of breeding in most developed countries. More recent developments in
64 molecular biology have enabled the genomic selection of young elite bulls for
65 inclusion in AI programmes [1]. This technology is now commercially available and
66 coupled with AI allows for faster genetic progress [2]. However, one of the problems

67 of using young bulls in artificial insemination programmes is that, by definition, their
68 field fertility has not been proven. The fertility of a bull has traditionally been
69 evaluated by test inseminations in the field and while this method is considered
70 reliable, it is expensive and time-consuming [3]. Consequently, it would be of benefit
71 to the cattle breeding industry to have an accurate, simple and efficient *in vitro* method
72 of predicting the potential fertility of semen, where aspects such as time, cost and
73 practicability are considered. In order to develop such a test it is first necessary to
74 understand why frozen-thawed semen from some bulls results in a higher pregnancy
75 rate than that from other bulls. Several hypotheses exist as to possible reasons for such
76 differences. Frozen-thawed sperm from low fertility bulls may exhibit an abnormal
77 change in morphology or metabolic activity, be unable to transverse the female
78 reproductive tract to the site of fertilisation in sufficient numbers, or their ability to
79 fertilise the oocyte or yield a developmentally competent embryo may be impaired.

80

81 Conventional *in vitro* evaluation of semen quality following the freeze thaw process,
82 such as the assessment of concentration, motility and morphology are of limited value
83 in assessing field fertility [4]. Various fluorescent staining techniques have also been
84 used to evaluate sperm viability [5,6,7], capacitation status [8,9], membrane integrity
85 [10], chromatin integrity [11], acrosome status [5,12] and mitochondrial activity
86 [5,13], and while these measurements are useful for *in vitro* assessment of sperm they
87 have limited ability to predict field fertility. The rate at which specific stains can be
88 reduced by the mitochondria of sperm has also been used as an assessment of
89 metabolic status [14]. It has been suggested that MTT (3- (4,5-Dimethylthiazol-2-yl) -
90 2,5- diphenyl- tetrazolium bromide) may be used as a reliable indicator for bovine [14]
91 and equine [15] sperm fertility. MTT is a yellow, water soluble tetrazolium salt which

92 is reduced to water-insoluble purple formazan crystals in the mitochondria of living
93 cells. The amount of formazan can be measured spectrophotometrically and therefore
94 gives an estimate of the number of living cells in a sample. This method was first
95 reported by Mosmann [16], who considered it to be a simple and inexpensive method
96 to assess viability, and has been used widely on many cells types [14,17,18,19].

97

98 It has been reported that *in vivo* barriers to sperm transport can be mimicked *in vitro*
99 using sperm migration tests as a tool to examine sperm quality [20,21]. These tests
100 have been used for determination of sperm function in humans [22], goats [23] and
101 bulls [24,25]. Recently, artificial mucus composed of hyaluronic acid [26] or
102 polyacrylamide gel [25] has been used as a suitable and more consistent alternative to
103 natural mucus. On the other hand other authors have suggested that there is no
104 relationship between mucus penetration and field fertility [21,27,28].

105

106 Some authors have reported that *in vitro* fertilisation (IVF) can be used as a tool to
107 predict the field fertility of a bull or to discriminate among bulls of different field
108 fertility in terms of both cleavage and blastocyst formation rates [29,30,31,32]. Others
109 have reported a correlation between field fertility and cleavage rate alone [33,34] or
110 blastocyst formation rate alone [35,36], while some authors have reported that IVF is
111 not a useful predictor of field fertility [37,38,39].

112

113 The aim of the present study was to investigate whether differences in field fertility of
114 bulls is reflected in differences (i) in the metabolic activity of sperm, in terms of their
115 ability to penetrate mucus, their mitochondrial activity as well as their ability to

116 survive the freeze-thaw process or (ii) in the ability to fertilise oocytes and produce
117 viable embryos.

118

119 **2. Materials and Methods**

120 *2.1 Experimental Design*

121 All bulls used in this study were located in the National Cattle Breeding Centre
122 (NCBC), Enfield, Co Meath, Ireland and were used for commercial AI in Ireland.
123 Semen was diluted and then frozen in 0.25 mL straws, each containing approximately
124 80×10^6 spermatozoa per 1 mL. The study was carried out over two years and
125 involved a separate sample of bulls each year. In Year 1, semen from six bulls was
126 used of which three bulls (1 Holstein Friesian and 2 Limousin) were classified as
127 having ‘high fertility’ and three Holstein Friesian bulls were classified as having ‘low
128 fertility’. Given the different breeds in the high fertility group, the experiment was
129 repeated in Year 2 using semen from five Holstein Friesian bulls of which three were
130 classified as having ‘high fertility’ and two were classified as having ‘low fertility’. In
131 Year 1 the field fertility of the 6 bulls was calculated as 90-day non-return rate (NRR;
132 Table 1). Due to an updated recording system used by the NCBC NRR was not
133 available for the bulls in Year 2. Instead the field fertility was available as pregnancy
134 rate adjusted for various factors, including semen type (frozen, fresh), parity of cow,
135 month of service, day of the week when serviced, individual cow effects and AI
136 technician effect, and weighted for number of service records [44].

137

138 *2.2. Assessment of sperm motility and concentration*

139 Straws were thawed at 37 °C for 30 s in a water bath and semen was held in a 15 mL
140 polypropylene conical tube during processing. As a quality control check a 5 µL

141 sample was placed on a pre-warmed slide and motility was subjectively assessed on a
142 scale of 0 to 5 (0 = no motility, 5 = progressively motile) for each bull prior to each
143 replicate in each experiment. Sperm concentration was assessed for each bull using a
144 haemocytometer and dilutions were performed using Tyrode's albumin lactate
145 pyruvate (TALP) media as appropriate [40].

146

147 *2.3. Mucus penetration test*

148 The ability of sperm to penetrate artificial mucus was evaluated using the method
149 described by O'Hara et al. [41]. Artificial mucus was prepared by diluting a solution
150 containing sodium hyaluronate (MAP-5, Labstock MicroServices, Ireland) with
151 phosphate-buffered saline to give a final concentration of 6 mg sodium hyaluronate
152 per 1 mL.

153

154 The penetration test was replicated (7 times in Year 1 and 5 times in Year 2). For each
155 replicate one straw per bull was thawed and held in a water bath at 37 °C for
156 processing. Each bull used in a given year was represented in each replicate in that
157 year and was kept separate throughout. Semen was diluted to a final sperm
158 concentration of 10×10^6 per mL in TALP medium containing Hoechst 33342
159 fluorescent stain (10 mg/mL in 2.3% sodium citrate) and incubated at 37 °C for 5 min
160 to ensure uptake of the stain.

161

162 Flattened capillary tubes (0.3 mm x 3.0 mm x 100 mm; Composite Metal Services
163 Ltd, UK) were marked at 10 mm intervals between 10 and 80 mm. These were then
164 filled with artificial mucus using an adapted 5 mL syringe. Two capillary tubes were
165 placed vertically in a 1.5 mL eppendorf containing a 100 μ L aliquot of the stained

166 sperm from one bull. Therefore, in each replicate each of the bulls were represented
167 by two capillary tubes. The tubes were incubated in a dry oven for 30 min at 37 °C.
168 After incubation the tubes were placed on a hotplate at 45 °C for 1 min to immobilize
169 the sperm. Sperm were counted from wall to wall across the tube, within the width of
170 a single field of view, at each 10 mm interval using a fluorescent microscope (40x;
171 Olympus BX 60). Because the test yielded an excessive number of sperm at 10, 20
172 and 30 mm positions, which made an accurate count impossible, these data points
173 were excluded from the analysis.

174

175 *2.4. Assessment of sperm cell viability*

176 Sperm viability status of each bull was assessed using a live/dead sperm viability kit
177 (SYBR 14/ Propidium Iodide (PI); Molecular Probes L-7011). One straw was
178 analysed per bull in each of three replicates in Year 1 and in each of four replicates in
179 Year 2. Semen was diluted 1:30 with Synthetic Oviduct Fluid (SOF) medium and 5
180 µL of SYBR-14 stain was added to 995 µL of the diluted sample in an eppendorf to
181 give a final SYBR-14 concentration of 100 nM. The stained sample was vortexed and
182 incubated for 10 min at 37 °C in 95% O₂ and 5% CO₂. Following this, 5 µL of PI was
183 added to give a final concentration of 12 µM PI and the sample was then incubated for
184 an additional 5 min at 37 °C as above. Two aliquots, each of 4 µL were then placed on
185 a pre-warmed slide, covered with a cover slip and viewed using a fluorescent
186 microscope (40x; Olympus BX 60). A minimum of 250 cells were assessed per
187 droplet and classified as either live (stained green with SYBR 14) or dead (stained red
188 with propidium iodide).

189

190 *2.5. MTT Assay*

191 The MTT assay was performed according to the method of Aziz [14]. Four replicates
192 were performed in each of Years 1 and 2. One straw per bull was thawed, motility
193 was assessed and sperm were diluted to 20×10^6 per mL in PBS + 3% bovine serum
194 albumin (BSA) and 100 μ L of this suspension was aliquoted into a well of a 96-well
195 plate (2 to 3 wells per bull per replicate). MTT (10 μ L of a 5 mg/mL solution) was
196 added to each well and the plate was incubated at 37 °C for 1 h in a dry oven.
197 Following the incubation dimethyl sulfoxide (80 μ L per well) was added; the plate
198 was covered in tinfoil and agitated for 1 min to dissolve any formazan crystals. The
199 optical density of the samples was then read using a spectrophotometer at both 550
200 (maximum absorbance of formazan) and 690 nm (to account for background
201 absorbance) and the results subtracted from each other.

202

203 *2.6. In vitro fertilisation*

204 *2.6.1. Oocyte collection and in vitro maturation (IVM)*

205 Cumulus–oocyte complexes (COCs) were obtained by aspirating follicles from the
206 ovaries of slaughtered heifers. A total of 5 batches of COCs were used each year and
207 all bulls within a year were evaluated in each batch (replicate) for that year. After four
208 washes in PBS supplemented with pyruvate (36 μ g/mL), gentamycin (50 μ g/mL) and
209 BSA (0.5 mg/mL; Sigma, St Louis, MO), groups of up to 50 COCs were placed in 500
210 μ L maturation medium in four-well dishes (Nunc, Roskilde) and cultured for 24 h at
211 39 °C under an atmosphere of 5% CO₂ in air with maximum humidity. The maturation
212 medium was TCM-199 supplemented with 10% fetal calf serum (FCS) and 10 ng/mL
213 epidermal growth factor (EGF) [42].

214

215 *2.6.2. Sperm preparation and in vitro fertilisation*

216 For IVF, COCs were washed four times in fertilisation medium before being randomly
217 assigned to treatment and transferred, in groups of up to 50, into four-well dishes
218 containing 250 μ L fertilisation medium (Tyrode's medium with 25 mmol bicarbonate,
219 22 mmol sodium lactate, 1 mmol sodium pyruvate and 6 mg/mL fatty acid-free BSA).
220 In addition, heparin–sodium salt (10 μ g/mL; Calbiochem, San Diego, CA) was added.
221 Motile spermatozoa were obtained by centrifugation of frozen–thawed semen. One
222 straw per bull in each replicate was centrifuged on a discontinuous Percoll (Pharmacia,
223 Uppsala) density gradient (2.5 mL 45% (v/v) Percoll over 2.5 mL 90% (v/v) Percoll)
224 at 2000 g for 9 min at room temperature. Viable spermatozoa collected at the bottom
225 of the 90% fraction were washed in HEPES-buffered Tyrode's medium and pelleted by
226 centrifugation at 1000 g for 5 min. The spermatozoa were counted in a
227 haemocytometer and diluted in the appropriate volume of fertilisation medium to give
228 a concentration of 2×10^6 spermatozoa per 1 mL. A 250 μ L aliquot of this suspension
229 was added to each fertilisation well to obtain a final concentration of 1×10^6
230 spermatozoa per 1 mL. The plates were incubated for 20 h at 39 °C under an
231 atmosphere of 5% CO₂ in air with maximum humidity.

232

233 *2.6.3. Embryo culture after fertilisation*

234 At approximately 20 h after insemination, presumptive zygotes were denuded by
235 gentle vortexing and washed four times in PBS before they were transferred to 25 μ L
236 culture droplets of SOF (25 embryos per droplet) [43] under mineral oil. FCS (10%)
237 was added 24 h after placement in culture. The dishes were incubated in an
238 atmosphere of 5% CO₂, 5% O₂ and 90% N₂ at 39 °C. Cleavage rates were recorded at
239 48 h post insemination and the proportion of embryos developing to the blastocyst
240 stage was recorded from Day 6 to 8.

241

242 *2.7. Statistical analysis*

243 Data from Years 1 and 2 were pooled for statistical analysis using mixed model
244 procedures [45]. While data for the two Limousin bulls evaluated in Year 1 were
245 retained in the analyses, the effect of fertility status was evaluated from the contrast
246 between ‘high’ and ‘low’ status Holstein Friesian bulls.

247

248 The number of sperm counted per capillary tube at each 10 mm point between 40 and
249 80 mm, inclusive, was averaged and this value, after square root transformation to
250 normalise the residuals, was analysed using a model with year, and breed by fertility
251 class as fixed effects and bull and year x replicate as random terms. Thus, the error
252 term for evaluating the difference between fertility classes had 7 degrees of freedom.

253 Data on the percentage of live sperm and MTT were analysed using the same model
254 but without any transformation. The data from IVF [cleavage rate and blastocyst rate
255 (number of blastocysts relative to the number of cleaved oocytes)] were subjected to
256 arcsin transformation prior to analysis using the same model as for the other variables.

257 Where transformations were involved the means are presented after back-
258 transformation along with associated 95% confidence intervals (95%CI). In the other
259 cases least squares means are presented with associated standard errors.

260

261 **3. Results**

262

263 *3.1. Mucus penetration test*

264 Sperm from high fertility Holstein Friesian bulls penetrated artificial mucus in greater
265 numbers than sperm from low fertility Holstein Friesian bulls, however, this

266 difference did not reach significance ($P = 0.09$). The mean number of sperm at each
267 10 mm point was 56.0 (95%CI 39.5 to 75.3) and 42.9 (95%CI 29.3 to 59.1) for high
268 and low fertility Holstein Friesian bulls, respectively (Fig. 1). The corresponding
269 value for the high fertility Limousin bulls was 90.8 (95%CI 52.8 to 111.5).

270

271 *3.2. Assessment of sperm cell viability and mitochondrial metabolic activity*

272 The percentage of live sperm for the high and low fertility Holstein Friesian bulls was
273 47.3 ± 5.47 and 32.4 ± 4.66 respectively; the difference did not reach statistical
274 significance ($P = 0.08$). The percentage of live sperm for the high fertility Limousin
275 bulls was 29.1 ± 8.22 . In addition, there was no difference in the ability of sperm from
276 high and low fertility Holstein Friesian bulls to reduce MTT to formazan (mean
277 absorbance 0.34 ± 0.051 and 0.30 ± 0.044 for the high and low fertility Holstein
278 Friesian bulls respectively; $P = 0.6$). The mean absorbance for the high fertility
279 Limousin bulls was 0.30 ± 0.072 .

280

281 *3.3. In vitro fertilisation*

282 A higher percentage of oocytes were cleaved following insemination with sperm from
283 high fertility compared with sperm from low fertility Holstein Friesian bulls: 76.7
284 (95%CI 60.9 to 89.4) and 55.3 (95%CI 40.4 to 69.7) respectively, ($P = 0.04$; Fig. 2).
285 There was no evidence for an effect of fertility on the percentage of cleaved oocytes
286 that developed to the blastocyst stage: 34.7 (95%CI 21.1 to 49.6) and 24.2 (95%CI
287 14.1 to 36.0) for the high and low fertility Holstein Friesian bulls, respectively ($P =$
288 0.2 ; Fig. 2). The cleavage and blastocyst rates for the high fertility Limousin bulls
289 were 63.7 (95%CI 39.1 to 46.4) and 25.1 (95%CI 8.8 to 46.4), respectively.

290

291 **4. Discussion**

292

293 Understanding the basis for differences in field fertility between individual bulls is an
294 important objective towards developing a predictive *in vitro* test of male field fertility.

295 In the current study, spermatozoa from bulls with superior field fertility displayed an
296 increased ability to fertilise oocytes *in vitro* as well as an increased ability to penetrate
297 artificial mucus. However, these differences were not associated with differences in
298 sperm mitochondrial metabolic activity, as determined by the MTT assay.

299

300 Sperm, *in vivo*, must penetrate viscous mucus secreted by the mucosal epithelial layer
301 as they navigate towards the site of fertilisation in the ampulla of the oviducts. This
302 represents a major barrier and can be mimicked by *in vitro* migration tests to evaluate
303 sperm quality [20,21]. In the current study, sperm from high fertility bulls tended to
304 be more capable of penetrating artificial mucus than those from low fertility bulls,
305 however, this did not reach formal significance. The reason why sperm from high
306 fertility bulls penetrated artificial mucus in greater numbers was not due to the
307 increased mitochondrial metabolic activity, as determined by the MTT assay. In
308 addition, it was not due to a greater number of live sperm in the incubated sample as
309 was shown by the percentage live following staining. The number of live sperm in all
310 samples, from both high and low fertility bulls, was low reflecting the relatively poor
311 survivability of bull sperm following the freeze-thaw process.

312

313 The ability of sperm to reach the site of fertilisation represents only part of their
314 challenge on the journey to establishing a viable pregnancy. They must then penetrate
315 and fertilise the oocyte and produce a viable embryo. The outcome of IVF has

316 previously been positively correlated with field fertility in cattle [29,30,32,46-48] and
317 sheep [49]; however, others found no such relationship [37-39]. In the current study,
318 the significant differences in cleavage rate but not blastocyst rate, following selection
319 of motile sperm, indicates that the freeze-thaw process inherently reduces the
320 fertilising ability of motile sperm of low fertility bulls to a greater degree but once
321 fertilisation has been achieved there is no evident difference between bulls in the
322 ability of their sperm to yield developmentally competent embryos.

323

324 In conclusion, sperm from low fertility bulls exhibited a reduced ability to penetrate
325 mucus as well as to fertilise oocytes *in vitro*. However, once fertilisation occurred
326 subsequent embryo development to the blastocyst stage proceeded normally
327 irrespective of bull fertility level. These differences were not due to any difference in
328 the mitochondrial metabolic activity of the sperm, as determined by the MTT assay,
329 but may be due to other sub-cellular damage caused by the freeze thaw process.

330

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332

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337

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339

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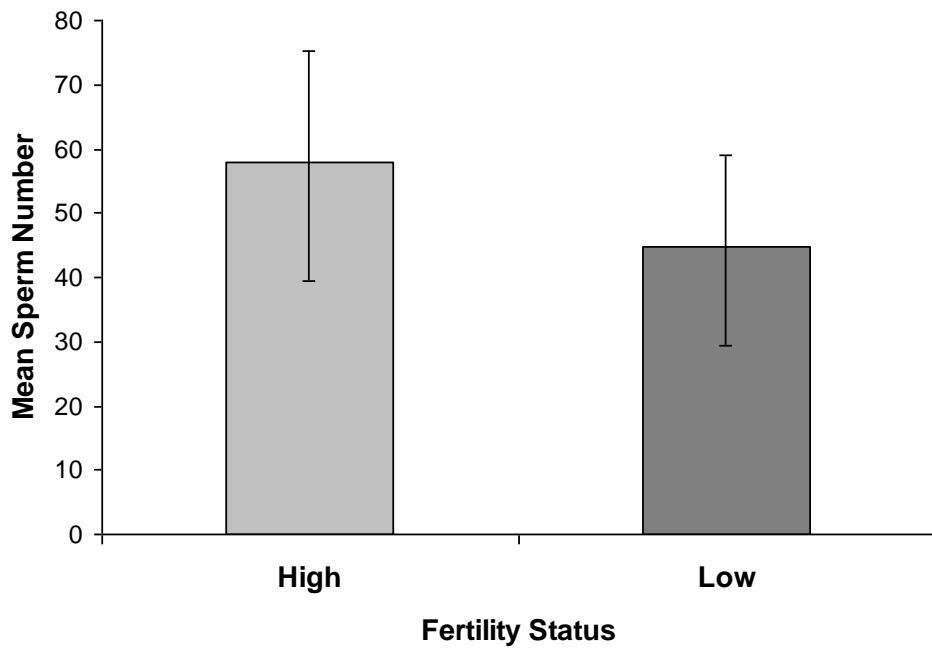
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484 Table 1: *In vivo* fertility data of bulls of high and low fertility as determined by non
 485 return rate (NRR; Year 1) and pregnancy rate (Year 2).

Year	Bull number	Breed of bull	Number of inseminations	90 day NRR (%)	Pregnancy rate (%)	Fertility status
1	2	Holstein	688	75.3		High
1	5	Holstein	1490	65.4		Low
1	6	Holstein	1308	65.1		Low
1	4	Holstein	3594	66.2		Low
1	1	Limousin	483	75.8		High
1	3	Limousin	720	75.6		High
2	7	Holstein	637		51.8	High
2	8	Holstein	3055		54.7	High
2	9	Holstein	430		53.3	High
2	10	Holstein	364		26.6	Low
2	11	Holstein	601		23.1	Low

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489 Fig.1. The mean (back transformed) number of sperm in artificial mucus (counted per

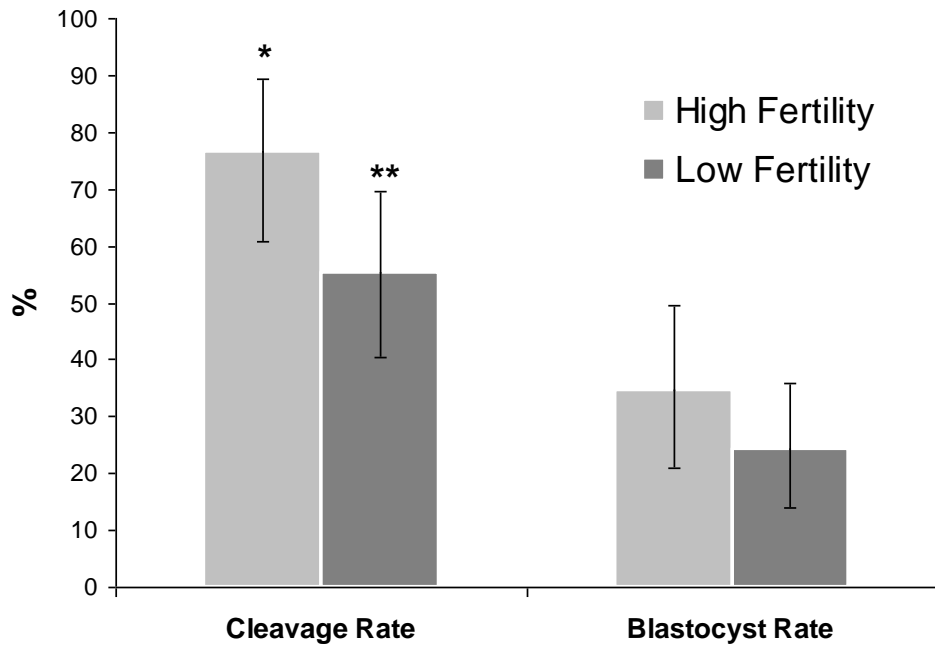
490 10 mm mark between 40 and 80 mm) for high and low fertility Holstein Friesian

491 bulls. Vertical bars represent the 95% confidence interval.

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495 Fig.2. Cleavage and blastocyst rates following *in vitro* fertilisation of oocytes with
 496 sperm from high and low fertility Holstein Friesian bulls. Blastocyst rate was assessed
 497 on Day 8 and is expressed as a % of cleaved oocytes. Vertical bars represent the 95%
 498 confidence intervals. * Bars with different superscripts differ significantly.

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