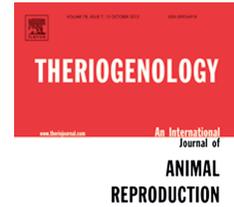


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Oviduct Binding Ability of Porcine Spermatozoa Develops in the Epididymis and can be Advanced by Incubation with Caudal Fluid

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1 **Oviduct Binding Ability of Porcine Spermatozoa Develops in the Epididymis and**
2 **can be Advanced by Incubation with Caudal Fluid**
3

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16

17 **Abstract.** The sperm reservoir is formed when spermatozoa bind to the
18 epithelium of the utero-tubal junction and caudal isthmus of the oviduct. It is an
19 important mechanism that helps synchronize the meeting of gametes by regulating
20 untimely capacitation and polyspermic fertilisation. This study investigated the
21 influence of epididymal maturation and caudal fluid on the ability of spermatozoa to
22 bind to oviduct epithelium using a model porcine oviduct explant assay. Spermatozoa
23 from the rete testis, middle caput (E2-E3), middle corpus (E6) and cauda (E8) of
24 Large White or Large White x Landrace boars at 10-14 months of age were diluted in
25 modified Androhep solution and incubated with porcine oviduct explants. Results
26 reported in this study support our hypothesis that testicular spermatozoa need to pass
27 through the regions of the epididymis in order to acquire the ability to bind to the
28 oviduct. There was a sequential increase in the number of spermatozoa that bound to
29 oviduct explants from the rete testis to caudal epididymis. Binding of caudal
30 spermatozoa to isthmus explants was the highest (15.0 ± 1.2 spermatozoa per 1.25
31 mm^2 ; mean \pm standard error of the mean; $P \leq 0.05$) and lowest by spermatozoa from
32 the rete testis (2.0 ± 0.3 per 1.25 mm^2), and higher to isthmus from sows compared to
33 gilts (35.8 ± 6.7 per 1.25 mm^2 vs. 14.8 ± 3.0 per 1.25 mm^2 ; $P \leq 0.05$). Binding of
34 ejaculated spermatozoa to porcine isthmus was higher than for caudal spermatozoa
35 (26.3 ± 1.4 per 1.25 mm^2 vs. 15.0 ± 0.8 per 1.25 mm^2 ; $P \leq 0.05$), and higher to
36 porcine than to bovine isthmus (26.3 ± 2.3 per 1.25 mm^2 vs. 18.8 ± 1.9 per 1.25 mm^2 ;

37 $P \leq 0.05$). Incubation of spermatozoa from the caput and corpus in caudal fluid
38 increased the ability of spermatozoa to bind to oviduct epithelium ($P \leq 0.05$). In
39 conclusion, the capacity of testicular spermatozoa to bind to oviduct epithelium
40 increases during their maturation in the epididymis, and can be advanced by
41 components of the caudal fluid.

42

43 *Extra keywords:* boar, epididymis, sperm-oviduct binding, sperm reservoir, caudal
44 fluid

45 Abridged title: Epididymal maturation and caudal fluid increase porcine sperm-
46 oviduct binding

47

48 **Introduction**

49 Maturation of spermatozoa in the epididymis is just as important in
50 fertilisation as production in the testis. Characteristics essential for fertilisation in the
51 female reproductive tract, such as motility and the ability to penetrate the oocyte,
52 cannot be acquired by testicular spermatozoa without undergoing significant
53 maturation within the epididymis [1, 2].

54 While millions of spermatozoa are deposited into the female reproductive tract
55 during coitus or after artificial insemination, only a few thousand pass through the
56 utero-tubal junction and reach the caudal isthmus [3]. Those spermatozoa which are
57 morphologically abnormal are phagocytized before they gain access to the oviducts
58 [4]. Some spermatozoa reach the ampulla within minutes after insemination, but do
59 not necessarily participate in fertilising oocytes [5-7]. Instead, a second population
60 reaches the oviduct several hours after insemination and most are trapped in the
61 isthmus and are held until ovulation is eminent. During this time, spermatozoa bind to
62 ciliated epithelial cells of the isthmus forming what is called the sperm reservoir or
63 oviductal reservoir [8, 9]. In the pig, at least 4,000 - 5,000 spermatozoa are present in
64 the isthmus before ovulation occurs [10]. The sperm reservoir regulates the release of
65 appropriate numbers of capacitated spermatozoa at the proper physiological time to
66 ensure successful monospermic fertilisation.

67 Compared to ejaculated spermatozoa, epididymal spermatozoa from the boar
68 show a reduced ability to bind to oviduct epithelium *in vitro* [11]. However, it is still

69 largely unknown where and when spermatozoa develop this ability to bind to
70 oviductal epithelium and hence form the sperm reservoir. It is not clear whether this
71 capacity begins to develop in the testis or during sperm maturation in different regions
72 of the epididymis. It is known that the maturation processes that occur to spermatozoa
73 during their passage in the epididymal tract contribute to the biochemical changes to
74 their plasma membrane [12]. It is possible that the changes could include the
75 formation of molecules responsible for binding of spermatozoa to epithelia of the
76 isthmus. While a complex array of proteins and secretory products in the epididymis
77 have been identified [13], a detailed understanding of how they influence the cellular
78 changes that occur to spermatozoa at different sites of the epididymis is still largely
79 unknown.

80 The caudal epididymis and caudal fluid in particular, provide an important
81 environment that supports sperm survival during storage and the acquisition of
82 fertilising capacity [14, 15]. Numerous studies have shown the unique composition of
83 caudal fluid when compared to secretions in proximal segments of the epididymis.
84 These include different secretory proteins either native to caudal fluid or transported
85 from proximal regions and accumulated in this fluid [12, 16-18], enzymes [19], Ca^{2+}
86 concentrations and signalling mechanisms [20], sperm association or formation [21],
87 and chemical characteristics of the fluid itself [22]. Given the above factors and the
88 amount of time that spermatozoa spend in the cauda prior to ejaculation, it is logical
89 to assume that the cauda and caudal fluid may play a significant role in developing the
90 ability of spermatozoa to bind to oviduct epithelium.

91 We hypothesize that testicular spermatozoa must pass through the different
92 regions of the epididymis in order to gain the ability to bind to oviduct epithelium.
93 Moreover, we speculate that this ability predominantly develops in the cauda
94 mediated by components unique to caudal fluid. Thus, the aim of this study was to
95 compare the binding potential of boar spermatozoa from the rete testis and different
96 regions of the epididymis to the isthmus and ampulla of porcine oviducts using an
97 oviduct explant assay. Moreover, the effect of caudal fluid on oviduct binding in
98 immature spermatozoa was also investigated.

99

100 **Materials and Methods**

101 *Boars*

102 Large White or Large White x Landrace boars either purchased from a
103 commercial piggery at 16 weeks of age or born at the College of Public Health,
104 Medical & Vet Sciences, James Cook University, Townsville, were reared until 10-14
105 months of age in the animal facilities of the College. Approval to conduct experiments
106 was provided by the James Cook University Animal Ethics Committee (Approval
107 number A1007).

108

109 *Preparation of spermatozoa*

110 Fresh chilled ejaculated boar semen was used in a preliminary experiment to
111 compare the binding capacity of boar spermatozoa to bovine versus porcine oviducts.
112 The semen was obtained from the same boar (Large White PPG 114), supplied by a
113 commercial breeder (Premier Pig Genetics, Wacol, Australia). The semen was
114 shipped in a polystyrene esky with an ice pack and usually arrived at the laboratory
115 the day before an experiment was undertaken. Before use, the semen was examined
116 for motility and concentration using a computer-aided semen analyser (CASA)
117 (Hamilton Thorne Research, Beverly, MA, USA) and was directly diluted to 5×10^6
118 spermatozoa per ml with modified Androhep solution (pH 7.4 and 290 mOsm/kg)
119 containing 144.0 mM glucose, 27.2 mM tri-sodium citrate-2-hydrate, 14.3 mM
120 sodium bicarbonate and 37.0 mM HEPES in Nano-Pure deionised water [11].

121 For remaining experiments, sperm samples were prepared from the testes and
122 epididymides of seven boars. The left testis and epididymis was obtained by unilateral
123 castration and the right when the boar was slaughtered four to five weeks later.

124 Castrations were performed to coincide with the delivery of oviducts to the
125 laboratory. Boars were pre-medicated based on estimated body weight with 5 mg/kg
126 atropine sulphate (atropine 0.6 mg/ml; Apex Laboratories Pty Ltd, Somersby, NSW,
127 Australia) followed 5 min later by 6 mg/kg ketamine hydrochloride i.m. (Ketamine
128 100 mg/ml; Parnell Laboratories Pty Ltd, Alexandria, NSW, Australia) and 1 mg/kg
129 xylazine hydrochloride (Ilium Xylazet 100 mg/ml; Troy Laboratories Pty Ltd,
130 Smithfield, NSW, Australia). Once anaesthetised, the scrotum was aseptically
131 prepared and the left testicle extruded via a single incision. Large haemostats were
132 used while three sutures (Ethicon 3, 5 metric chromic catgut; Johnson & Johnson

133 Medical Pty. Ltd., North Ryde, NSW, Australia) where applied to the spermatic cord
134 before removal of the testicle. The parietal vaginal tunic was closed with interrupted
135 sutures (Ethicon 3, 5 metric chromic catgut) and the scrotal skin was closed with
136 mattress sutures (Ethicon 3.0 metric Vicryl). Boars were given 1200 mg
137 oxytetracycline i.m. (Engemycin; Intervet Australia Pty Ltd, Bendigo, VIC,
138 Australia). The castrated testis was transported to the laboratory in a polystyrene esky
139 containing an ice pack.

140 After oviductal explants had been prepared, the testis with attached epididymis
141 was dissected from the tunica vaginalis. Sperm samples were collected from the rete
142 testis by longitudinally cutting the testicle to expose the mediastinum. Epididymal
143 spermatozoa were collected by making a small incision in the middle caput (E2 to
144 E3), middle corpus (E6) and cauda (E8) [23]. Spermatozoa from the rete testis and
145 cauda were aspirated using a 1 ml sterile tuberculin syringe, while spermatozoa from
146 the middle caput and middle corpus were collected from the incision by gentle
147 scraping using the blunt end of a scalpel blade. Collection of spermatozoa from the
148 rete testis and epididymis took about 15 minutes. Within a minute of collection, each
149 sample was diluted in 1 ml modified Androhep solution, analysed for sperm
150 concentration and motility characteristics, and then adjusted to 5×10^6 spermatozoa
151 per ml as described for ejaculated spermatozoa.

152

153 *Determination of motility characteristics by Computer Assisted Sperm Analysis*

154 Concentration and motility characteristics of epididymal spermatozoa were
155 analysed using a computer-aided semen analyser (CASA) (IVOS version 10,
156 Hamilton Thorne Research. Beverly, MA, USA). The CASA software was calibrated
157 to the following settings: analysis set-up #7: BOAR; frames acquired, 40/sec; frame
158 rate, 50 Hz; minimum contrast, 60%; minimum cell size, 2 pixels; minimum static
159 contrast, 30%; straightness threshold, 71.4%; low VAP cut-off, 5.0 $\mu\text{m}/\text{sec}$; medium
160 VAP cut-off, 22.0 $\mu\text{m}/\text{sec}$; low VSL cut-off, 11.0 $\mu\text{m}/\text{sec}$; head size (non-motile), 2
161 pixels; head intensity (non-motile), 70 pixels; static head size, 0.10 to 10.0 pixels;
162 static head intensity, 0.10 to 0.95 pixels; static elongation, 0 to 60; count slow cells as
163 motile, YES; magnification, 3.20; video source, camera; video frequency, 50; bright
164 field, NO; and illumination intensity, 2381. The temperature of the slide chamber was

165 set to 39⁰ C. Definitions used for the various motility parameters were based on those
166 described previously [24].

167

168 *Preparation of oviductal explants for the binding assay*

169 The procedures were modified from Petrunkina *et al.* [11] and Wagner *et al.*
170 [25]. Oviducts were obtained from gilts slaughtered at an abattoir in Charters Towers,
171 about 130 km from James Cook University, Townsville. Gilts were approximately 20
172 weeks old and non-cycling as determined by the absence of corpora lutea. Both
173 oviducts were removed from each gilt and placed in a 30 ml container filled with
174 phosphate buffered saline solution (PBS; pH 7.4 and 280 mOsm/kg) containing 150
175 mM NaCl, 11.7 mM NaH₂PO₄ and 2.5 mM KH₂PO₄. Samples were transported to the
176 laboratory by air-conditioned car in a polystyrene esky containing an ice pack.

177 In the laboratory, the mesentery of the oviduct was removed to straighten the
178 oviducts and to help distinguish the isthmus and ampulla. One end of the oviduct was
179 pinned with a 19G needle to a sterile platform while the other end was held with fine
180 forceps and opened longitudinally through the length of the oviduct with small fine
181 scissors. Small pieces (2-3 mm²) of oviductal mucosa including the underlying stroma
182 were cut from the isthmus and the ampulla with a scalpel blade and placed
183 individually in 96-well flat bottom culture dishes (NUNCLON, Thermo Scientific,
184 Scoresby, VIC, Australia). Explants were incubated in modified Tyrode's solution
185 (TALP; pH 7.4 and 300 mOsm/kg) consisting of 96.0 mM NaCl, 3.1 mM KCl, 0.4
186 mM magnesium sulphate, 2.0 mM CaCl₂, 5.0 mM glucose, 0.3 mM sodium
187 dihydrophosphate, 15.0 mM sodium bicarbonate, 21.6 mM sodium lactate, 2.2 mg/ml
188 sodium pyruvate, 20.0 mM HEPES and 6.0 mg/ml BSA (A4378; Sigma, Sydney,
189 NSW, Australia) in Nano-Pure deionised water. Oviducts that were not used
190 immediately were stored for up to two hours at 4⁰ C until use.

191

192 *Co-incubation of spermatozoa and explants*

193 Each explant from the isthmus and ampulla was pre-equilibrated for 20 min in
194 a 60 µl droplet of TALP at 39⁰ C in a humidified atmosphere containing 5% CO₂ in
195 air before adding spermatozoa. Viability of explants was examined before use by
196 assessing movement in the cilia of the epithelium. Sperm suspended in modified

197 Androhep solution was also pre-equilibrated for at least 5 min under the same
198 conditions, then 20 μl of the sperm suspension (1×10^5 spermatozoa) was added to
199 each explant and incubated for 15 min at 39°C in a humidified atmosphere containing
200 5% CO_2 in air. The average time interval between the collection of oviducts and
201 addition of spermatozoa to explants was about six hours. After incubation, explants
202 were immediately washed twice with TALP using a fine strainer in a small dish to
203 free loosely attached spermatozoa.

204

205 *Fixation and counting of bound spermatozoa*

206 The explants were fixed overnight at 4°C in 2% formaldehyde in 0.1 M
207 sodium phosphate buffer plus 0.01% CaCl_2 at pH 7.3. The next day, explants were
208 rinsed in three changes of 10 mM phosphate-buffered saline solution (pH 7.3) and
209 stained with Gill's Haematoxylin for fifteen seconds, followed by rinsing a further
210 five times. Gill's Haematoxylin consisted of 25% ethylene glycol, 0.2%
211 Haematoxylin (Cl 75290; Sigma), 0.02% sodium iodate, 1.76% aluminium sulphate
212 and 2% glacial acetic acid in distilled water. Explants were then mounted on glass
213 slides flooded with sufficient glycerol to prevent drying of tissues during examination
214 under the microscope. Slides were covered with coverslips immobilized by petroleum
215 jelly (Vaseline) as a support, and examined for bound spermatozoa with a light
216 microscope at 400X magnification. A graticule was used to aid the counting of
217 spermatozoa. Bound spermatozoa were counted in 20 fields at 0.0625 mm^2 per field,
218 giving an area of 1.25 mm^2 per explant.

219

220 *Comparison of the binding capacity of ejaculated boar spermatozoa to bovine and* 221 *porcine oviducts*

222 In this preliminary experiment, 36 oviducts were collected from 18 non-
223 pregnant cows slaughtered at the Australian Meat Holdings Abattoir, Townsville.
224 Cows were in the mid-luteal phase of the oestrous cycle, as determined by the
225 presence of a mature corpus luteum on the ovaries, to ensure a relative comparison to
226 a similar number of oviducts collected from 18 non-cycling pre-pubertal gilts. The
227 procedures used to prepare oviductal explants for cows and pigs were the same and
228 are described earlier. The experimental design consisted of four to six oviducts from

229 two to three animals each week. Three explants were taken from both the isthmus and
230 the ampulla of each oviduct, giving a total of 108 oviductal explants from each region
231 of the oviduct per species. In addition, 36 tracheal explants each were prepared as
232 controls from six cows and six gilts using the same procedure except that only the
233 mucosa was used. Spermatozoa from eight ejaculates of the same commercial boar
234 (PPG114) were used in this experiment as described earlier.

235

236 *The binding of boar epididymal spermatozoa to porcine oviducts*

237 A total of 112 oviducts were collected from 56 non-cycling gilts. Each
238 experimental setup consisted of epididymal spermatozoa from one boar (n=7 boars
239 total) and four oviducts from two gilts. Explants were sampled from the oviduct as
240 described above, and yielded 84 explants from the isthmus or ampulla for incubation
241 with each sperm sample (i.e. from the rete testis, caput, corpus and cauda). Moreover,
242 each sperm sample was also incubated with 21 tracheal explants as control.

243

244 *Comparison of the binding capacity of epididymal boar spermatozoa to the oviducts*
245 *of sows and gilts*

246 In addition to the binding of epididymal boar spermatozoa to oviducts from
247 readily obtainable gilts as described previously, a separate experiment was conducted
248 to examine the binding capacity of epididymal boar spermatozoa to the oviducts of
249 sows (which were more difficult to obtain). Four oviducts were obtained and explants
250 pooled from each of two sows and two gilts that were slaughtered at the same time.
251 One sow was raised at the College of Public Health, Medical & Vet Sciences and the
252 other was obtained from a commercial piggery. Upon examination of their ovaries,
253 the sows were found to be in the follicular phase, but their oviducts were used since
254 luteal phase sow oviducts were not available at the time of study. The gilts were non-
255 cycling as described previously. In this experiment, 10 to 12 explants from both the
256 isthmus and ampulla of gilts were incubated with spermatozoa from each region of
257 the epididymis (i.e. caput, corpus and cauda), and compared to that from sows.

258

259 *The binding of epididymal spermatozoa to gilt oviducts after incubation in caudal*
260 *fluid*

261 Six caput and seven corpus epididymides were used in this experiment. Boars
262 were unilaterally castrated at the College of Public Health, Medical & Vet Sciences,
263 and oviductal explants were prepared as described previously. In this experimental
264 setup, epididymal spermatozoa were exposed to different pre-treatments then each
265 was incubated with a total of 36 oviductal explants from both the isthmus and
266 ampulla. Pre-treatments included: (i) caput spermatozoa in modified Androhep; (ii)
267 caput spermatozoa in caudal fluid; (iii) corpus spermatozoa in modified Androhep;
268 (iv) corpus spermatozoa in caudal fluid; and (v) caudal spermatozoa in modified
269 Androhep. The contents of the caudal epididymis was first collected into small vials
270 and centrifuged for 30 min at 1200 g, then centrifuged for a further 30 min to fully
271 extract the caudal fluid. In the interim, oviductal explants were prepared as described
272 earlier and pre-equilibrated in TALP for at least 15 min at 39⁰ C in a humidified
273 atmosphere containing 5% CO₂ in air. The caudal fluid supernatant was collected into
274 Eppendorf tubes and divided between the specific caput and corpus treatment groups
275 outlined above. Caudal spermatozoa were only diluted with modified Androhep.
276 Sperm samples were incubated for 30 min at 39⁰ C in a humidified atmosphere
277 containing 5% CO₂ in air before being analysed for sperm concentration and motility
278 characteristics by CASA. Thereafter, sperm samples were centrifuged for 10 min at
279 600 g and the supernatant replaced with the modified Androhep solution to yield a
280 final concentration of 5 x 10⁶ sperm/ml. Sperm samples were then pre-equilibrated for
281 at least 5 min at 39⁰ C in a humidified atmosphere containing 5% CO₂ in air, before
282 20 µl (1 x 10⁵ spermatozoa) was added to each oviductal explant and further
283 incubated for 15 min under the same conditions. Thereafter, explants were fixed,
284 mounted on slides and examined as described previously.

285

286 *Data analyses and presentation*

287 Data were analysed using the Statistical Package for Social Sciences (SPSS)
288 software version 11. Graphs were plotted using Microsoft Excel 2003. Statistical
289 comparisons between two variables (i.e. isthmus vs. ampulla, porcine vs. bovine
290 oviducts, ejaculated vs. epididymal spermatozoa, and sow vs. gilt oviducts) were

291 calculated using the Student's T-test. Analysis of variance (ANOVA) was used to
292 compare binding capacity of spermatozoa across the four regions of the
293 testis/epididymis, sperm binding capacity across different boars, and the motility
294 characteristics generated by CASA. A post-hoc Tukey test for multiple comparisons
295 of means was used to determine homogeneous subsets in variables tested by ANOVA.
296 \log_{10} transformation were performed for motility data by CASA, binding results
297 between porcine vs. bovine isthmus, ejaculated vs. epididymal spermatozoa as well as
298 between boars in order to normalise distribution of data prior to analyses. Normality
299 was not achieved after the \log_{10} transformation for binding between porcine and
300 bovine ampulla, thus a non-parametric test (Mann-Witney U test) was used. The level
301 of significant difference was set at $P \leq 0.05$.

302

303 **Results**

304 In preliminary studies, a comparison was made of the binding of ejaculated boar
305 spermatozoa to oviductal epithelium from cows and gilts to determine if explants of
306 isthmus and ampulla from cows could be used in place of those from gilts. More
307 ejaculated boar spermatozoa attached to the isthmus than ampulla of porcine but not
308 bovine explants ($P \leq 0.05$; Fig. 1), while fewer ($P \leq 0.05$) spermatozoa were bound to
309 tracheal control explants of both species. Moreover, more ($P \leq 0.05$) boar
310 spermatozoa bound to the porcine isthmus than to the bovine isthmus. The mean
311 number of spermatozoa bound to the other explant types did not differ between
312 species.

313 The mean percentage of motile spermatozoa from the rete testis and the three
314 regions of the epididymis was determined immediately after collection (Fig. 2). The
315 percentage of motile spermatozoa was greater in samples from the cauda and corpus
316 and lowest ($P \leq 0.05$) in samples from the caput and rete testis. Mean values for other
317 sperm motility characteristics did not differ across all regions of the epididymis but
318 were different ($P \leq 0.05$) from the rete testis (Table 1).

319 More ($P \leq 0.05$) spermatozoa from the cauda bound to the isthmus explants
320 from sows, while more spermatozoa from the corpus bound to the ampullary explants
321 from gilts ($P \leq 0.05$; Fig. 3a and b). The number of sperm bound to oviductal explants
322 did not differ between sows and gilts for spermatozoa from any other epididymal

323 region. Moreover, in both sows and gilts, more ($P \leq 0.05$) caudal spermatozoa bound
324 to explants than caput spermatozoa irrespective of explant.

325 The number of spermatozoa that bound to the oviductal epithelium increased
326 progressively ($P \leq 0.05$) from the rete testis to the cauda (Fig. 4). With the exception
327 of corpus spermatozoa, more spermatozoa were bound to isthmic than ampullary
328 explants. The same was true for ampullary explants except that the number of bound
329 spermatozoa from the cauda did not differ to those from the corpus. With the
330 exception of spermatozoa from the rete testis that bound to the ampulla, the mean
331 number of spermatozoa bound to tracheal controls was less ($P \leq 0.05$) than other
332 explants for all sperm samples.

333 In order to determine if seminal fluid influenced binding capacity of mature
334 spermatozoa as has been observed in cattle [26, 27], caudal and ejaculated
335 spermatozoa were also compared (Fig. 5). More ($P \leq 0.05$) ejaculated spermatozoa
336 attached to both the isthmus and ampulla than caudal spermatozoa.

337 No difference was observed in the mean number of spermatozoa from the rete
338 testis that bound to the isthmus between the seven boars (Fig. 6a). However,
339 epididymal spermatozoa from boar S4, S5 and S9 appeared to have lower binding
340 capacity relative to other boars while caudal spermatozoa from boar S3 had a
341 particularly higher binding capacity to the isthmus than spermatozoa from boar S4, S5
342 and S9 ($P \leq 0.05$).

343 The mean number of spermatozoa from the rete testis and caput that bound to
344 the ampulla was similar between boars, while marked differences were found in
345 corpus and caudal spermatozoa (Fig. 6b). Specifically, spermatozoa from the corpus
346 and cauda of boar S5 and caudal spermatozoa from boar S9 appeared to have
347 particularly lower binding to ampullary explants than for other boars. Of all boars, the
348 epididymal spermatozoa from boar S5 had the lowest binding capacity to the ampulla
349 ($P \leq 0.05$). Moreover, the number of spermatozoa that bound to isthmic and
350 ampullary explants generally did not differ between the left and right testicle of each
351 boar (data not shown).

352 The percentage of motile epididymal spermatozoa as well as their motility
353 characteristics were assessed immediately after incubation with either modified
354 Androhep or caudal fluid (Fig. 7 and Table 2). Caudal spermatozoa had the highest

355 mean percentage of motile spermatozoa in Androhep (higher than caput; $P \leq 0.05$)
356 followed by spermatozoa from the corpus, then caput. However, when spermatozoa
357 from the caput and the corpus were incubated with caudal fluid, there was a reduction
358 in their motility (significant for corpus; $P \leq 0.05$) when compared to spermatozoa
359 incubated with modified Androhep medium (Fig. 7). The average path velocity,
360 straight-line velocity and curvilinear velocity of spermatozoa from the caput were
361 higher ($P \leq 0.05$) in modified Androhep medium than in caudal fluid. In the corpus,
362 only the curvilinear velocity and the beat cross frequency were higher ($P \leq 0.05$) in
363 modified Androhep medium than in caudal fluid (Table 2).

364 As previously described, the binding of caudal spermatozoa pre-incubated in
365 Androhep to either the isthmus or the ampulla prepared from gilt oviducts was greater
366 ($P \leq 0.05$) than spermatozoa from other regions of the epididymis exposed to the
367 same treatment (Fig. 8a and b). However, the binding capacity of spermatozoa from
368 either the caput or corpus to both isthmic and ampullary explants increased ($P \leq 0.05$)
369 when pre-incubated with caudal fluid. Surprisingly, oviduct binding of corpus
370 spermatozoa increased to levels equivalent to that observed for caudal spermatozoa.
371 In all cases, the binding of epididymal spermatozoa to explants from the isthmus was
372 higher ($P \leq 0.05$) than to explants from the ampulla.

373

374 **Discussion**

375 There are limited reports in the literature on the binding of epididymal
376 spermatozoa to oviductal epithelium and, to our knowledge, this is the first study to
377 compare oviduct binding of spermatozoa from different regions of the epididymis. We
378 report here that the capacity of testicular spermatozoa to bind to the oviduct and form
379 the sperm reservoir appears to develop progressively during maturation in the
380 epididymis. In addition, we demonstrate for the first time that caudal fluid can
381 enhance oviduct binding of immature epididymal spermatozoa to levels equivalent to
382 that of mature caudal spermatozoa. The isthmus of the oviduct was found to bind
383 spermatozoa most effectively and this appears to be most prominent during the
384 follicular phase in sexually mature animals. Moreover, binding to the isthmus occurs
385 preferentially in a species-specific manner. Lastly, considerable variability among
386 males exists in the oviduct binding capacity of their spermatozoa.

387 There was a sequential increase in the number of spermatozoa from the rete
388 testis to the caudal epididymis that bound to the epithelium of the isthmus and
389 ampulla; with the highest binding found with caudal spermatozoa to the isthmus.
390 These results imply that spermatozoa undergo developmental changes as they pass
391 through the epididymis which appear to increase their capacity to bind to oviductal
392 epithelium. Given that evidence in the literature indicates carbohydrate-recognition
393 mechanisms are involved in sperm-oviduct binding [28, 29], it is likely that
394 carbohydrate-binding molecules are involved such as spermadhesin AWN secreted by
395 the rete testis [30], that appears to accumulate on spermatozoa as they travel along the
396 epididymis [31]. Alternatively, secretions of the epididymal epithelium may
397 structurally modify pre-existing molecules on the apical region of the sperm plasma
398 membrane such that they acquire the ability to bind to carbohydrates on the surface of
399 oviductal epithelium [12].

400 There are many products in caudal fluid, some of which are secreted under the
401 influence of androgens [32]. Among the important secretory products found to be
402 highly expressed in the cauda, is the cysteine-rich secretory protein (CRISP) family of
403 proteins that are involved in spermiogenesis, capacitation and binding of the
404 spermatozoon to the oocyte [33]. Factors have been also identified in caudal fluid that
405 are associated with fertility in dairy bulls [34], and appear to sustain motility of
406 bovine spermatozoa *in vitro* [35]. In our study, 30 minutes pre-incubation of caput
407 and corpus spermatozoa with caudal fluid significantly increased the binding of
408 spermatozoa both to the isthmus and ampulla when compared to caput and corpus
409 spermatozoa maintained in modified Androhep. This potentially indicates that caudal
410 fluid contains distinct factors that could directly or indirectly enhance the interaction
411 between spermatozoa and oviduct epithelium. Importantly, it demonstrates how
412 caudal fluid can accelerate binding capacity (and potential fertility) of immature
413 sperm, which could have important implications in animal production systems. It
414 would be interesting to determine the optimum duration required for immature
415 spermatozoa to be exposed to caudal fluid in order to acquire maximum binding
416 capacity. Caudal sperm in modified Androhep served as positive control and no
417 preparations were made where caudal sperm was pre-incubated with caudal fluid.
418 This is because the spermatozoa was extracted from the cauda where essentially it had

419 already been in extensive contact with the caudal constituents. Interestingly,
420 comparable rates of binding can be observed between corpus spermatozoa incubated
421 in caudal fluid and caudal spermatozoa (Fig. 8). While this comparison is not ideal, it
422 demonstrates that even 30 min pre-incubation in caudal fluid is sufficient to confer
423 oviductal binding capacity equivalent to that of mature spermatozoa. Whether longer
424 pre-incubation will further improve binding capacity in caput spermatozoa remains to
425 be determined. .

426 Glycoprotein binding receptors are present on the sperm head in order to bind
427 with carbohydrate ligands on the oviductal epithelium [25]. Thus, it is likely that the
428 presence of these binding sites on spermatozoa differs between regions of the
429 epididymis; as shown by the differences in their ability to bind to oviduct epithelium.
430 Caudal fluid contains a number of glycoconjugates that can be detected in lectin-
431 binding studies [36] and it is likely that caput and corpus spermatozoa acquired these
432 binding molecules in the present study during incubation with caudal fluid. While not
433 all glycoconjugates are directly produced in the cauda, some from the proximal
434 epididymis may be transported in epididymal plasma to the cauda and made available
435 to spermatozoa during storage. Incubation of caput and corpus spermatozoa in caudal
436 fluid has been shown to facilitate the acquisition of fertility-related glycoproteins [37].
437 Considerable reorganization of sperm plasma membrane glycoproteins does occur
438 during maturation in the epididymis, which can be mediated by a direct interaction
439 with epididymal proteins [38, 39]. Moreover, incubation of bovine spermatozoa in
440 caudal fluid facilitates acquisition of a low molecular weight protein capable of
441 stimulating calcium uptake, particularly with caput spermatozoa [40]. Interestingly in
442 cows, the binding of spermatozoa to Lewis-a trissacharide on the oviduct epithelium
443 is mediated by Ca^{2+} [41]. While not yet investigated in the pig, this may be one
444 putative explanation for the increased binding of immature boar spermatozoa after
445 incubation in caudal fluid.

446 In addition, specific proteins rich in sphingomyelin (and with a high
447 cholesterol/phospholipid ratio) are known to be secreted by the epididymal
448 epithelium, and are capable of regulating both sperm motility and fertilising ability
449 [42]. These are associated with epididymosomes. Examples include enzymes involved
450 in the polyol pathway and a cytokine (MIF; macrophage migration inhibitory factor)

451 believed to be selectively transferred to spermatozoa during epididymal transit.
452 Similar to epididymosomes are prostasomes (prostate-derived small membrane
453 vesicles) that are found along the male reproductive tract and particularly in
454 ejaculated semen [43]. Epididymosomes and prostasomes can greatly influence the
455 environment through which spermatozoa pass by allowing the transfer of new
456 biologically active proteins, as well as contributing to lipid and cholesterol content.
457 These in turn allow spermatozoa to gain new adhesion molecules that could facilitate
458 inter-cellular communication between the sperm surface and the oviduct epithelium
459 and in return promote binding [44]. Specifically, prostasomes secreted in a timely
460 manner under hormonal control are believed to be involved with post-testicular sperm
461 maturation due to their immunosuppressive activity, improvement in sperm motility,
462 and their modulation of capacitation [reviewed in 43, 45].

463 The number of caudal spermatozoa however, that bind to oviductal explants in
464 the pig is about half that of ejaculated spermatozoa [11], which is consistent with
465 results obtained in this current study. This suggests that seminal plasma must contain
466 factors, including prostasomes that further enhance oviduct binding in these otherwise
467 structurally mature spermatozoa. Using indirect immunofluorescence, Manásková *et*
468 *al.* [46] demonstrated that boar seminal plasma protein, DQH, binds to the oviducts.
469 Specific proteins known to promote oviduct binding and subsequent formation of the
470 sperm reservoir, have also been identified in the seminal plasma of the bull [26, 27].

471 The use of a heterologous system for studying sperm binding to oviductal
472 epithelium has been examined in several species including the binding of human
473 spermatozoa to the oviducts of cows and macaques [47], canine spermatozoa to
474 porcine oviducts [48] and stallion spermatozoa to bovine oviductal cells [49].
475 Heterologous systems are mainly used for logistical reasons, particularly in humans
476 where an adequate supply of disease-free oviduct tissues is not always available [47].
477 When variation between species is minimal; much of the effort, time and cost of the
478 study can be reduced. This study is the first to examine the ability of porcine
479 spermatozoa to bind to the oviductal epithelium of cows. Bovine oviducts were
480 considered for use in this study because they were readily availability from a large
481 cattle abattoir in close proximity to the laboratory. By contrast, the nearest source of
482 porcine oviducts was from an abattoir 130 km away. While the number of canine

483 spermatozoa that bound to canine and porcine oviducts was similar [48], the number
484 of ejaculated boar spermatozoa in our study that bound to the isthmus (but not
485 ampulla) was significantly less in cows than gilts. This result suggests that binding is
486 preferentially species-specific because carbohydrate-binding lectins and
487 glycoconjugates present on the plasma membrane of the sperm head and surface of
488 oviductal epithelium may vary considerably among species [29, 50]. Thus we
489 concluded that it was necessary to use porcine oviducts for remaining experiments
490 despite the increased cost and logistical difficulties associated with such an
491 experimental set-up.

492 However, differences in oviduct receptivity caused by the reproductive cycle
493 (luteal phase cows vs. non-cycling gilts) cannot be excluded. Different results have
494 been reported on the effect of (i) the region of the oviduct, (ii) steroid hormones, and
495 (iii) the reproductive status of the animal on the capacity of spermatozoa to bind to the
496 oviductal epithelium. While no significant difference in the binding of spermatozoa to
497 oviductal explants from either follicular or luteal phase pig oviducts has been
498 observed, the addition of exogenous oestradiol was found to enhance sperm binding
499 to both the isthmus and ampulla [51]. In our study, it was necessary to use oviducts
500 from pre-pubertal gilts because a consistent supply of sow oviducts could not be
501 assured from the abattoir, which primarily slaughtered pigs up to about 20 weeks of
502 age. Moreover, previous literature reported no difference in the number of ejaculated
503 boar spermatozoa that bound to the oviducts of gilts compared to cycling sows [11],
504 although the authors didn't specify the age nor pre/post-pubertal status of gilts used.
505 Nevertheless, there was an opportunity to compare the binding capacity of
506 spermatozoa to the oviducts from two sows of known history with that of gilts. In
507 contrast to previous results in pigs [11] and cows [52], we found preferential binding
508 of caudal spermatozoa to the isthmus but not ampulla of follicular-phase sows
509 compared to non-cycling gilts. This is consistent with studies in the horse [53] in
510 which the presence of oestrus (but not diestrus) concentrations of steroids in the
511 medium increased the percentage of spermatozoa attaching to both the isthmus and
512 ampulla of the oviduct. These results imply the significant involvement of increased
513 levels of oestrogen in the binding of spermatozoa to oviducts of sexually mature sows

514 compared to pre-pubertal gilts. However due to the small samples size, oviducts from
515 more sows need to be examined to confirm this result.

516 Different strategies have been employed to conduct binding assays using
517 oviductal epithelium. The use of hormone-supplemented oviductal epithelial
518 monolayers cultured *in vitro* have been successfully demonstrated in various animals
519 [28, 54]. *In vitro* culture of oviduct epithelium has the advantage of a ready supply of
520 epithelial cells that saves time in the conduct of research work, but may differ
521 considerably to the oviduct *in vivo*. Epithelial cultures also suffer from overgrowth by
522 non-epithelial cells [55], as well as reduced binding capacity upon repeated culture
523 [28]. For these reasons and to mimic the *in vivo* conditions as closely as possible, we
524 used an explant method to preserve the integrity of the oviduct mucosa.

525 More ejaculated or epididymal spermatozoa bound to the isthmus than the
526 ampulla. The reason for this may be attributed to differences in the epithelial
527 structure, regional secretions and biochemical features that exist between the isthmus
528 and ampulla. Studies report no differences in the binding capacity of spermatozoa to
529 the isthmus and ampulla of pigs [11, 51] or cattle [52], although Raychoudhury and
530 Suarez [55] found more porcine spermatozoa bound to the isthmus (10.8 ± 0.4
531 spermatozoa per 0.3 mm^2) than to the ampulla (5.6 ± 0.4 spermatozoa per 0.3 mm^2).
532 They suggested that the presence of a high concentration of oestrogen during oestrus
533 favoured the binding of spermatozoa to isthmus explants. Moreover, spermatozoa
534 from the horse and human have also been reported to bind in greater numbers to the
535 isthmus than to the ampulla [56, 57]. It is important to realise that regional differences
536 in the expression of glycoconjugates are apparent between segments of the porcine
537 oviduct, and across the different stages of the oestrous cycle, thereby affecting the
538 available binding sites [58]. It seems logical that the difference observed with binding
539 is consistent with the normal physiological functions of the oviduct in these two
540 regions: i.e. sperm storage and reservoir formation in the utero-tubal junction and
541 isthmus, *versus* sperm-oocyte binding, acrosome reaction and fertilization in the
542 ampulla. Thus one would expect binding sites to be reduced in the ampulla because
543 this is where sperm must locate and fertilise oocytes without binding to false targets
544 such as the epithelium.

545 The current study found significant differences between boars in the capacity
546 of spermatozoa to bind to oviductal epithelium. A similar observation has been made
547 by other workers in pigs [11] and in the horse [56]. These differences imply that
548 individual variation in the level of fertility between boars could be attributed to the
549 number of spermatozoa that form the sperm reservoir. Interestingly, Waberski *et al.*
550 [59] demonstrated differences among boars in binding capacity of spermatozoa to
551 oviduct epithelium after 72 h storage *in vitro*. Known sub-fertile boars and those with
552 a higher proportion of morphologically abnormal spermatozoa showed lower binding
553 index potential, suggesting that sperm-oviduct binding assays could be used as a
554 potential tool in assessing male fertility.

555 The acquisition of motility by spermatozoa during their maturation in the
556 epididymis is well established [4]. The current study found a significant increase in
557 the motility of spermatozoa from the corpus and caudal epididymis when compared to
558 spermatozoa from the rete testis and caput. This indicates that motility of boar
559 spermatozoa predominantly develops from the corpus onwards. This is consistent
560 with several other maturational changes that occur during epididymal transit that
561 facilitate sperm motility [15]. These include changes in cAMP concentrations
562 between epididymal regions [60]; decrease in intracellular pH [61]; decrease in free
563 calcium ion concentration and glucose transport into spermatozoa [62]; and a decrease
564 in the exchange of calcium ions into mitochondria [63]. Acott and Hoskins [64]
565 demonstrated that when cAMP was added to immature bovine spermatozoa from the
566 caput, sperm motility increased and was further enhanced by the addition of forward
567 motility protein. Moreover, forward motility protein binds to spermatozoa in the caput
568 and becomes concentrated on spermatozoa in the caudal epididymis. Thus, in addition
569 to the capacity for binding to the oviductal epithelium, the acquisition of sperm
570 motility during epididymal maturation is critical to successfully establish the
571 functional sperm reservoir prior to fertilization.

572 Spermatozoa from the caudal epididymis need to be stored in an immotile
573 state to avoid exhaustion of energy reserves. It is not yet fully understood how this is
574 mediated by caudal fluid [14], however pH and bicarbonate concentration are known
575 to play a role [65]. In the boar, the pH of the caudal fluid (pH 6.5) is lower than in
576 more proximal regions (pH 7.2), while the concentration of bicarbonate (3-4 mM) is

577 considerably less than in rete testis fluid (30 mM) [65]. It is therefore not surprising in
578 the present study that the proportion of motile spermatozoa and their motility
579 characteristics after incubation in caudal fluid were significantly less than those
580 incubated in modified Androhep medium. It is important to note that in our study
581 motility parameters predominantly associated with velocity (i.e. VAP, VSL and VCL;
582 see Table 2) were affected. This is consistent with a reduction in progressive
583 motility/sperm metabolic rate possibly by factors in caudal fluid that may act to
584 prolong sperm storage in the cauda, rather than as a consequence of sperm death *per*
585 *se*.

586 In conclusion, this study has demonstrated the importance of the epididymis
587 and factors in the caudal fluid for the capacity of immature spermatozoa to bind to the
588 oviductal epithelium and form the sperm reservoir. Whether this is due to structural
589 modification of the glycocalyx or addition of glycoproteins or oligosaccharides to the
590 plasma membrane of spermatozoa requires further investigation.

591

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Table 1. Motility characteristics of epididymal spermatozoa immediately after collection

Data are presented as mean percentages (\pm SEM). VAP, average path velocity; VSL, straight-line velocity; VCL, curvilinear velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; STR, straightness; LIN, linearity. Different letters indicate a significant difference between testicular regions ($P \leq 0.05$); $n = 7$ testicles.

Motility Parameter	Rete testis	Caput	Corpus	Cauda
VAP	9.85 \pm 6.28 ^a	30.89 \pm 8.18 ^b	35.66 \pm 10.84 ^b	32.51 \pm 5.28 ^b
VSL	8.20 \pm 5.34 ^a	24.18 \pm 6.90 ^b	25.33 \pm 8.51 ^b	23.85 \pm 4.46 ^b
VCL	17.88 \pm 11.96 ^a	52.86 \pm 13.39 ^b	59.04 \pm 15.66 ^b	56.38 \pm 8.44 ^b
ALH	1.22 \pm 0.79 ^a	2.72 \pm 0.74 ^b	2.95 \pm 0.74 ^b	2.30 \pm 0.57 ^b
BCF	5.90 \pm 3.87 ^a	9.11 \pm 2.82 ^b	14.81 \pm 4.92 ^b	16.00 \pm 3.07 ^b
STR	28.33 \pm 18.01 ^a	53.90 \pm 11.95 ^b	48.42 \pm 10.95 ^b	66.80 \pm 7.89 ^b
LIN	88.00 \pm 11.57 ^a	35.80 \pm 8.00 ^b	29.67 \pm 7.04 ^b	40.70 \pm 5.53 ^b

Table 2. Motility characteristics of epididymal spermatozoa after 30 min incubation in either modified Androhep medium or caudal fluid

Data are presented as mean percentages (\pm SEM). Different letters indicate a significant difference between modified Androhep medium (mAndro) and caudal fluid (CF) within an epididymal region ($P \leq 0.05$); $n = 7$ epididymides.

Motility Parameter	Caput		Corpus		Cauda
	mAndro	CF	mAndro	CF	mAndro
VAP	62.14 \pm 10.51 ^a	24.06 \pm 6.40 ^b	46.25 \pm 3.38	31.63 \pm 6.73	50.92 \pm 3.81
VSL	45.88 \pm 8.87 ^a	19.56 \pm 5.08 ^b	32.95 \pm 2.54	24.32 \pm 5.36	35.63 \pm 2.14
VCL	101.98 \pm 12.81 ^a	36.66 \pm 9.79 ^b	85.45 \pm 3.71 ^a	49.92 \pm 10.45 ^b	95.32 \pm 7.16
ALH	4.42 \pm 0.37	2.64 \pm 0.93	4.93 \pm 0.18	3.27 \pm 0.71	5.02 \pm 0.05
BCF	15.60 \pm 4.25	21.48 \pm 8.05	19.35 \pm 1.64 ^a	9.95 \pm 2.34 ^b	20.50 \pm 0.89
STR	72.80 \pm 2.84	65.20 \pm 16.18	70.50 \pm 1.65	62.50 \pm 12.57	69.67 \pm 1.12
LIN	46.20 \pm 3.76	45.60 \pm 11.52	41.17 \pm 2.75	41.17 \pm 8.57	39.33 \pm 1.05

Figure captions

Figure 1. The mean (+ SEM) binding of ejaculated boar spermatozoa to porcine and bovine oviductal and tracheal explants. Different letters indicate a significant difference between explant types. Different numbers indicate a significant difference between species ($P \leq 0.05$). $n = 108$ explants for each region of the oviduct from 18 gilts and 18 cows; 36 tracheal explants from each species; 8 ejaculates from a Large White boar (PPG 114).

Figure 2. The mean (+ SEM) percentage of motile spermatozoa from the rete testis and different regions of the epididymis. Different letters indicate a significant difference between testicular regions ($P \leq 0.05$). $n = 7$ boars

Figure 3. The mean (+ SEM) binding of epididymal spermatozoa to a) isthmic explants and b) ampullary explants of sows and gilts. Different letters indicate a significant difference between epididymal regions within each animal type (i.e. sow or gilt), while different numbers indicate a significant difference between sows and gilts within each epididymal region. $n = 10$ -12 explants each from 2 sows and 2 gilts for spermatozoa from each region of the epididymis.

Figure 4. The mean (+ SEM) binding of boar spermatozoa from the rete testis and different regions of the epididymis to isthmic and ampullary explants, and tracheal controls. Different letters indicate a significant difference between different testicular regions within an explant type, while different numbers indicate a significant difference between explant types within a testicular region ($P \leq 0.05$). $n = 84$ isthmic or ampullary explants and 21 tracheal explants for each sperm sample; 7 testicles.

Figure 5. Comparison of ejaculated and caudal spermatozoa binding to isthmic and ampullary explants (mean + SEM). Different numbers indicate a significant difference between sperm samples within an explant type ($P \leq 0.05$). $n = 121$ and 84 isthmic or ampullary explants for ejaculated and caudal spermatozoa respectively; 8 ejaculates used from a Large White boar (PPG 114) and 7 testicles used for caudal spermatozoa.

Figure 6. Comparison between individual boars (S1-S9) in the binding of spermatozoa from the rete testis and different regions of the epididymis to a) isthmic explants and b) ampullary explants (mean + SEM). Different letters indicate a significant difference between boars within a testicular region ($P \leq 0.05$). $n = 12$ explants for each sperm sample per boar; 7 boars.

Figure 7. The mean (+ SEM) percentage of motile spermatozoa from different regions of the epididymis after incubation in modified Androhep medium *versus* caudal fluid. Different letters indicate a significant difference between epididymal regions per treatment, while different numbers indicate a significant difference between caudal fluid and modified Androhep medium within an epididymal region ($P \leq 0.05$). $n = 7$ epididymides.

Figure 8. The influence of pre-incubation in modified Androhep medium *versus* caudal fluid on binding of epididymal spermatozoa to a) isthmic explants and b) ampullary explants (mean + SEM). Different letters indicate a significant difference between epididymal regions per treatment, while different numbers indicate a significant difference between modified Androhep medium and caudal fluid within an epididymal region ($P \leq 0.05$). n = 36 explants for each pre-incubation treatment per epididymal region; 6 caput and 7 corpus epididymides respectively.

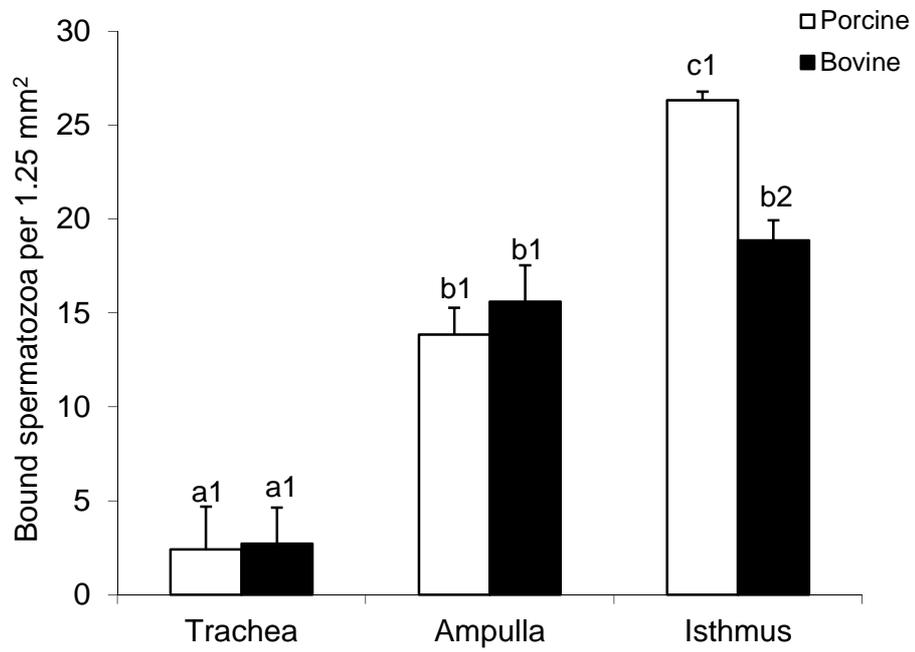
Figure 1

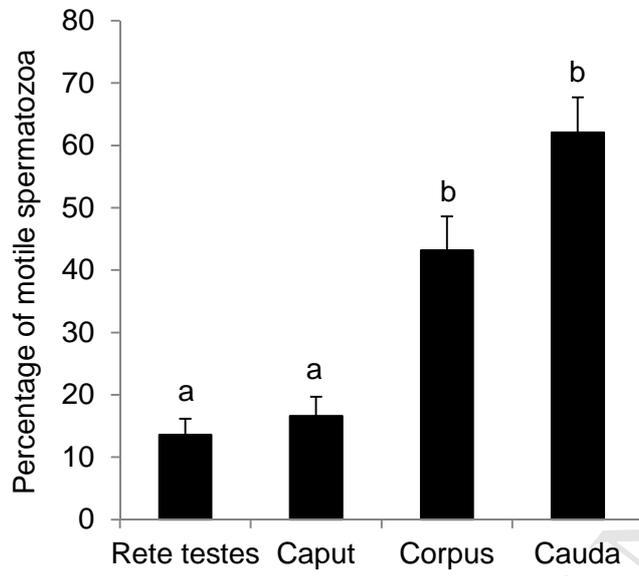
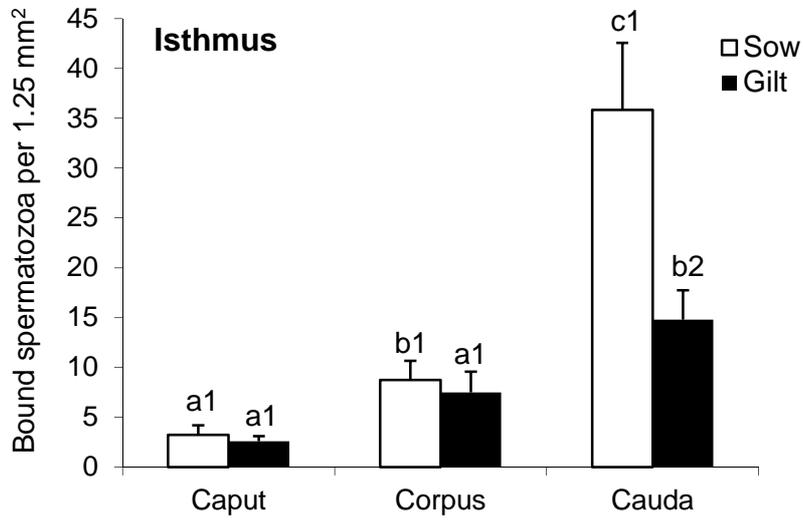
Figure 2

Figure 3

a)



b)

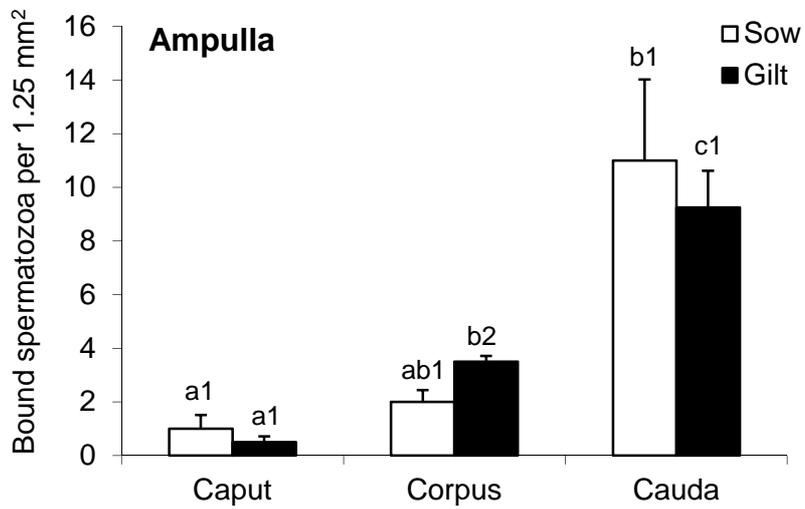


Figure 4

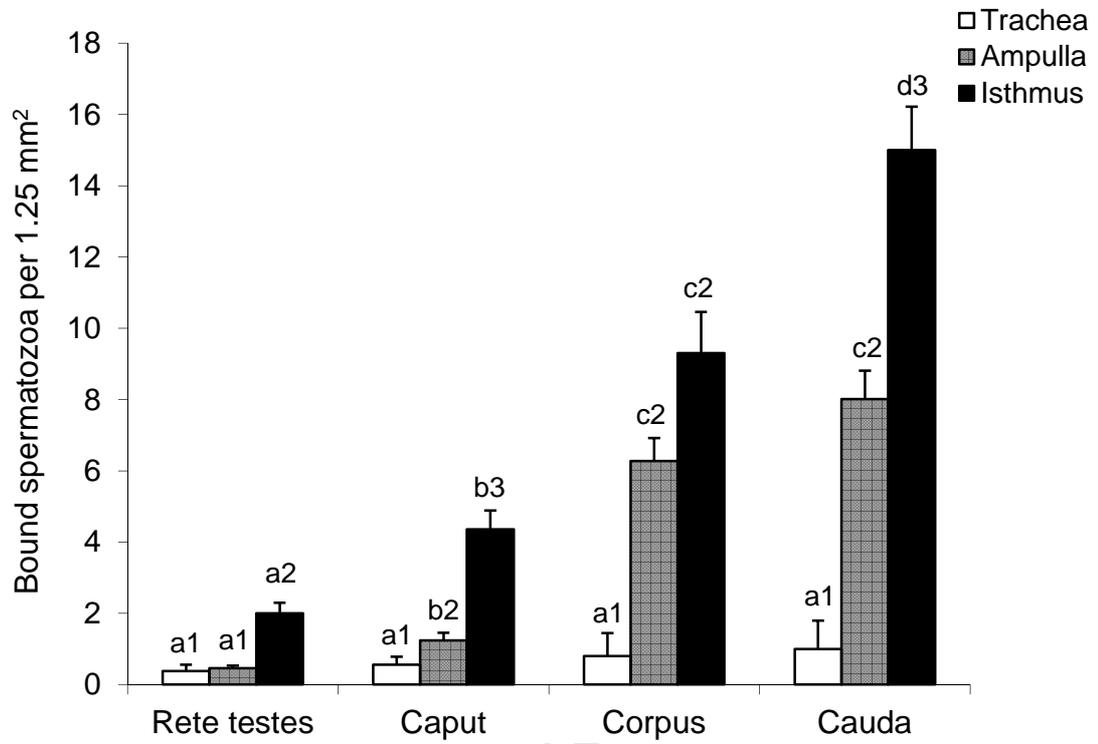


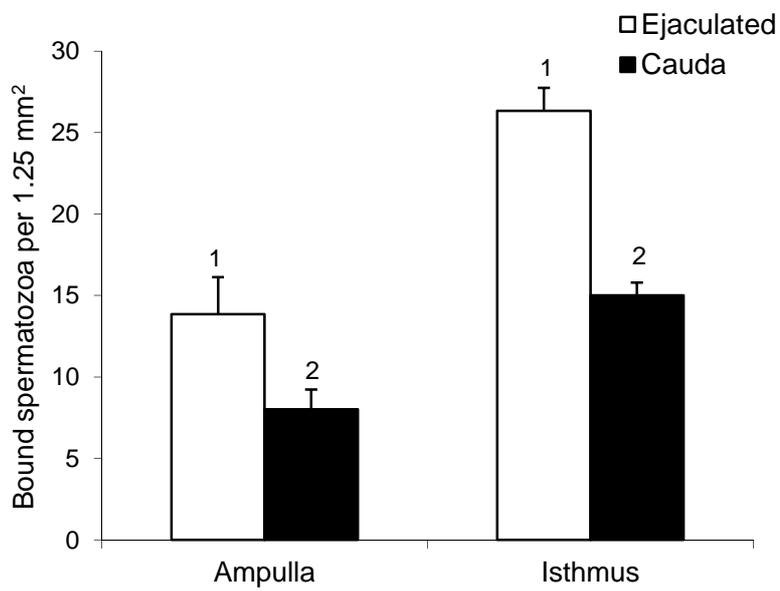
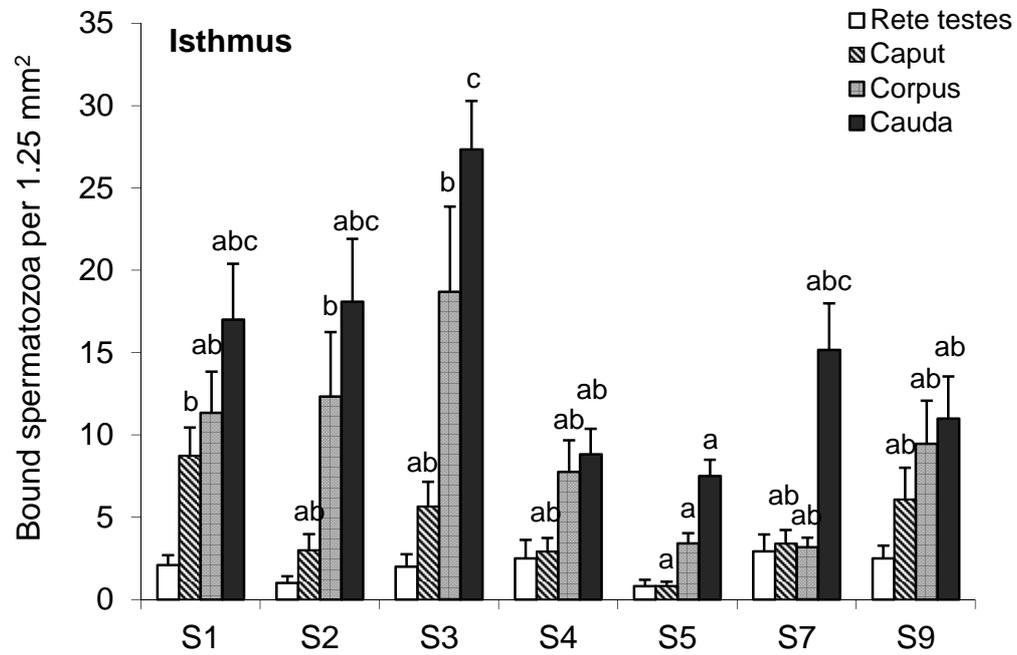
Figure 5

Figure 6

a)



b)

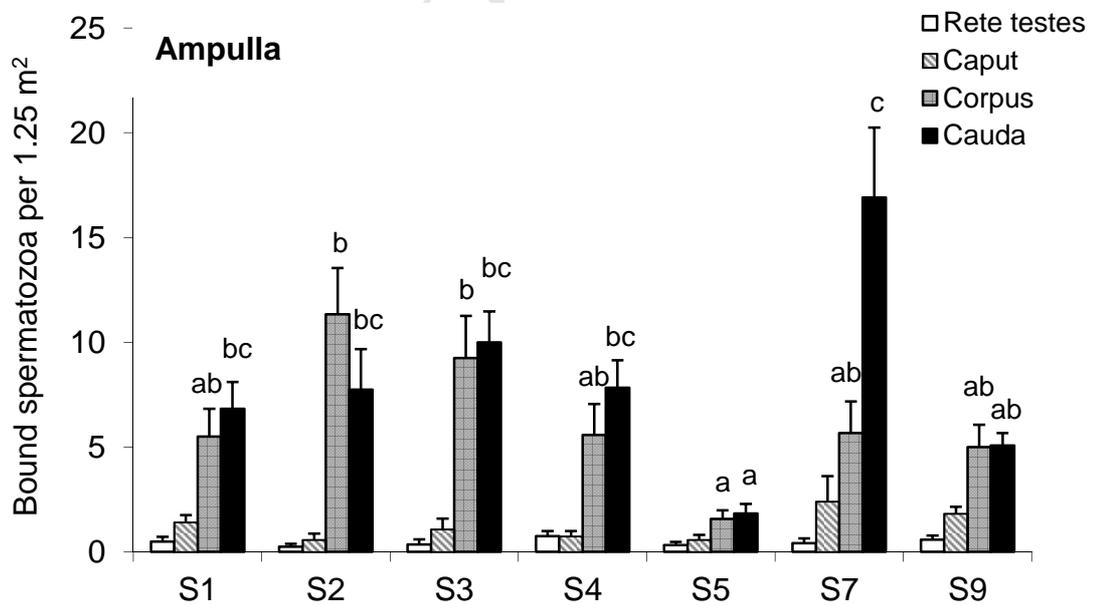


Figure 7

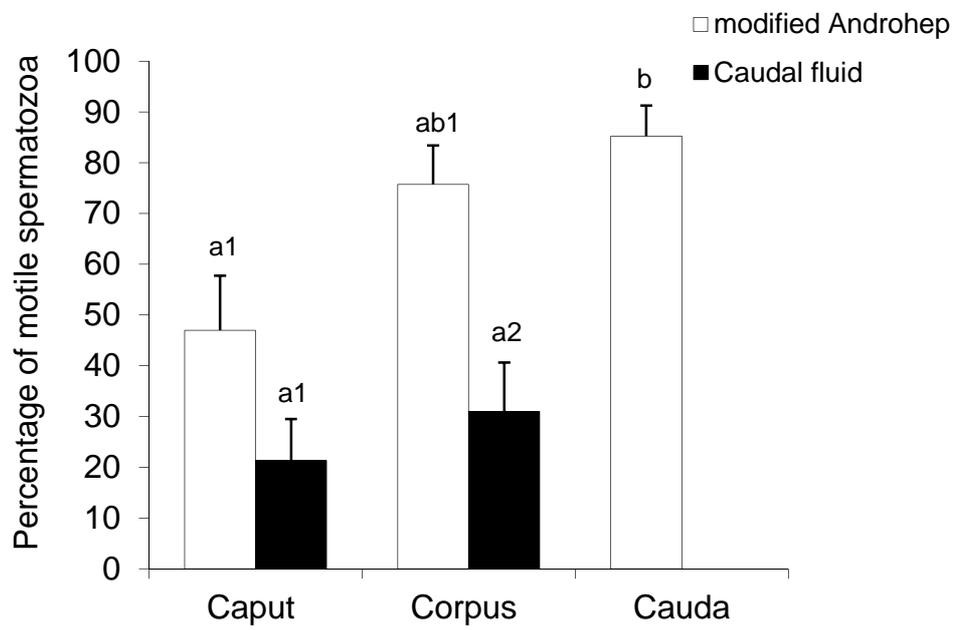
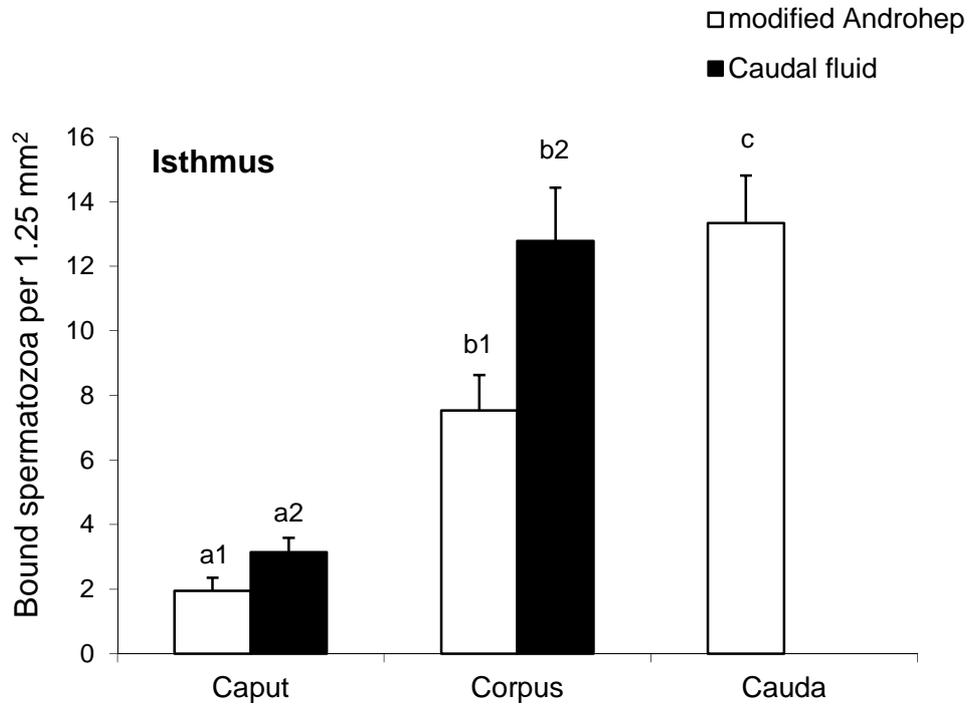
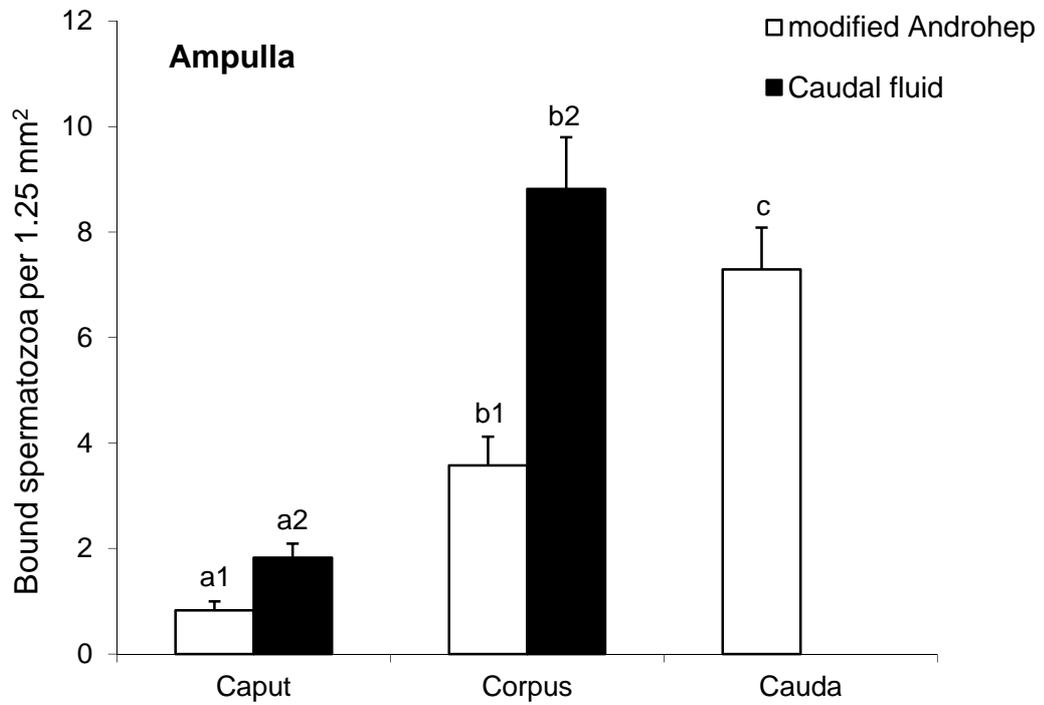


Figure 8

a)



b)



Oviduct Binding Ability of Porcine Spermatozoa Develops in the Epididymis and can be Advanced by Incubation with Caudal Fluid

-Highlights-

- Testicular spermatozoa must pass through the different regions of the epididymis in order to gain the ability to bind to oviduct epithelium.
- The ability to bind to oviduct epithelium predominantly develops in the cauda mediated by components unique to caudal fluid.
- There was significant sequential increase in the number of spermatozoa that bound to oviduct explants from the rete testis to caudal epididymis.
- Binding of epididymal spermatozoa was significantly higher to porcine oviducts than to bovine oviducts, to oviducts from sows than to oviducts of gilts, and to the isthmus than to ampulla.