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

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

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

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

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

Introduction

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

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

Compared to ejaculated spermatozoa, epididymal spermatozoa from the boar show a reduced ability to bind to oviduct epithelium *in vitro* [11]. However, it is still largely unknown where and when spermatozoa develop this ability to bind to oviductal epithelium and hence form the sperm reservoir. It is not clear whether this capacity begins to develop in the testis or during sperm maturation in different regions of the epididymis. It is known that the maturation processes that occur to spermatozoa during their passage in the epididymal tract contribute to the biochemical changes to their plasma membrane [12]. It is possible that the changes could include the formation of molecules responsible for binding of spermatozoa to epithelia of the isthmus. While a complex array of proteins and secretory products in the epididymis have been identified [13], a detailed understanding of how they influence the cellular changes that occur to spermatozoa at different sites of the epididymis is still largely unknown.

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

We hypothesize that testicular spermatozoa must pass through the different regions of the epididymis in order to gain the ability to bind to oviduct epithelium. Moreover, we speculate that this ability predominantly develops in the cauda mediated by components unique to caudal fluid. Thus, the aim of this study was to compare the binding potential of boar spermatozoa from the rete testis and different regions of the epididymis to the isthmus and ampulla of porcine oviducts using an

oviduct explant assay. Moreover, the effect of caudal fluid on oviduct binding in immature spermatozoa was also investigated.

Materials and Methods

Boars

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

Preparation of spermatozoa

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

For remaining experiments, sperm samples were prepared from the testes and epididymides of seven boars. The left testis and epididymis was obtained by unilateral castration and the right when the boar was slaughtered four to five weeks later. Castrations were performed to coincide with the delivery of oviducts to the laboratory. Boars were pre-medicated based on estimated body weight with 5 mg/kg atropine sulphate (atropine 0.6 mg/ml; Apex Laboratories Pty Ltd, Somersby, NSW,

Australia) followed 5 min later by 6 mg/kg ketamine hydrochloride i.m. (Ketamine 100 mg/ml; Parnell Laboratories Pty Ltd, Alexandria, NSW, Australia) and 1 mg/kg xylazine hydrochloride (Ilium Xylazet 100 mg/ml; Troy Laboratories Pty Ltd, Smithfield, NSW, Australia). Once anaesthetised, the scrotum was aseptically prepared and the left testicle extruded via a single incision. Large haemostats were used while three sutures (Ethicon 3, 5 metric chromic catgut; Johnson & Johnson Medical Pty. Ltd., North Ryde, NSW, Australia) where applied to the spermatic cord before removal of the testicle. The parietal vaginal tunic was closed with interrupted sutures (Ethicon 3, 5 metric chromic catgut) and the scrotal skin was closed with mattress sutures (Ethicon 3.0 metric Vicryl). Boars were given 1200 mg oxytetracycline i.m. (Engemycin; Intervet Australia Pty Ltd, Bendigo, VIC, Australia). The castrated testis was transported to the laboratory in a polystyrene esky containing an ice pack.

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

Determination of motility characteristics by Computer Assisted Sperm Analysis

Concentration and motility characteristics of epididymal spermatozoa were analysed using a computer-aided semen analyser (CASA) (IVOS version 10, Hamilton Thorne Research. Beverly, MA, USA). The CASA software was calibrated to the following settings: analysis set-up #7: BOAR; frames acquired, 40/sec; frame

rate, 50 Hz; minimum contrast, 60%; minimum cell size, 2 pixels; minimum static contrast, 30%; straightness threshold, 71.4%; low VAP cut-off, 5.0 µm/sec; medium VAP cut-off, 22.0 µm/sec; low VSL cut-off, 11.0 µm/sec; head size (non-motile), 2 pixels; head intensity (non-motile), 70 pixels; static head size, 0.10 to 10.0 pixels; static head intensity, 0.10 to 0.95 pixels; static elongation, 0 to 60; count slow cells as motile, YES; magnification, 3.20; video source, camera; video frequency, 50; bright field, NO; and illumination intensity, 2381. The temperature of the slide chamber was set to 39^0 C. Definitions used for the various motility parameters were based on those described previously [24].

Preparation of oviductal explants for the binding assay

The procedures were modified from Petrunkina *et al*. [11] and Wagner *et al*. [25]. Oviducts were obtained from gilts slaughtered at an abattoir in Charters Towers, about 130 km from James Cook University, Townsville. Gilts were approximately 20 weeks old and non-cycling as determined by the absence of corpora lutea. Both oviducts were removed from each gilt and placed in a 30 ml container filled with phosphate buffered saline solution (PBS; pH 7.4 and 280 mOsm/kg) containing 150 mM NaCl, 11.7 mM NaH_2PO_4 and 2.5 mM KH_2PO_4. Samples were transported to the laboratory by air-conditioned car in a polystyrene esky containing an ice pack.

In the laboratory, the mesentery of the oviduct was removed to straighten the oviducts and to help distinguish the isthmus and ampulla. One end of the oviduct was pinned with a 19G needle to a sterile platform while the other end was held with fine forceps and opened longitudinally through the length of the oviduct with small fine scissors. Small pieces (2-3 mm^2) of oviductal mucosa including the underlying stroma were cut from the isthmus and the ampulla with a scalpel blade and placed individually in 96-well flat bottom culture dishes (NUNCLON, Thermo Scientific, Scoresby, VIC, Australia). Explants were incubated in modified Tyrode's solution (TALP; pH 7.4 and 300 mOsm/kg) consisting of 96.0 mM NaCl, 3.1 mM KCl, 0.4 mM magnesium sulphate, 2.0 mM $CaCl_2$, 5.0 mM glucose, 0.3 mM sodium dihydrophosphate, 15.0 mM sodium bicarbonate, 21.6 mM sodium lactate, 2.2 mg/ml sodium pyruvate, 20.0 mM HEPES and 6.0 mg/ml BSA (A4378; Sigma, Sydney,

NSW, Australia) in Nano-Pure deionised water. Oviducts that were not used immediately were stored for up to two hours at 4^0 C until use.

Co-incubation of spermatozoa and explants

Each explant from the isthmus and ampulla was pre-equilibrated for 20 min in a 60 µl droplet of TALP at 39^0 C in a humidified atmosphere containing 5% CO_2 in air before adding spermatozoa. Viability of explants was examined before use by assessing movement in the cilia of the epithelium. Sperm suspended in modified Androhep solution was also pre-equilibrated for at least 5 min under the same conditions, then 20 µl of the sperm suspension (1×10^5 spermatozoa) was added to each explant and incubated for 15 min at 39^0 C in a humidified atmosphere containing 5% CO_2 in air. The average time interval between the collection of oviducts and addition of spermatozoa to explants was about six hours. After incubation, explants were immediately washed twice with TALP using a fine strainer in a small dish to free loosely attached spermatozoa.

Fixation and counting of bound spermatozoa

The explants were fixed overnight at 4^0 C in 2% formaldehyde in 0.1 M sodium phosphate buffer plus 0.01% $CaCl_2$ at pH 7.3. The next day, explants were rinsed in three changes of 10 mM phosphate-buffered saline solution (pH 7.3) and stained with Gill's Haematoxylin for fifteen seconds, followed by rinsing a further five times. Gill's Haematoxylin consisted of 25% ethylene glycol, 0.2% Haematoxylin (Cl 75290; Sigma), 0.02% sodium iodate, 1.76% aluminium sulphate and 2% glacial acetic acid in distilled water. Explants were then mounted on glass slides flooded with sufficient glycerol to prevent drying of tissues during examination under the microscope. Slides were covered with coverslips immobilized by petroleum jelly (Vaseline) as a support, and examined for bound spermatozoa with a light microscope at 400X magnification. A graticule was used to aid the counting of spermatozoa. Bound spermatozoa were counted in 20 fields at 0.0625 mm^2 per field, giving an area of 1.25 mm^2 per explant.

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

In this preliminary experiment, 36 oviducts were collected from 18 non-pregnant cows slaughtered at the Australian Meat Holdings Abattoir, Townsville. Cows were in the mid-luteal phase of the oestrous cycle, as determined by the presence of a mature corpus luteum on the ovaries, to ensure a relative comparison to a similar number of oviducts collected from 18 non-cycling pre-pubertal gilts. The procedures used to prepare oviductal explants for cows and pigs were the same and are described earlier. The experimental design consisted of four to six oviducts from two to three animals each week. Three explants were taken from both the isthmus and the ampulla of each oviduct, giving a total of 108 oviductal explants from each region of the oviduct per species. In addition, 36 tracheal explants each were prepared as controls from six cows and six gilts using the same procedure except that only the mucosa was used. Spermatozoa from eight ejaculates of the same commercial boar (PPG114) were used in this experiment as described earlier.

The binding of boar epididymal spermatozoa to porcine oviducts

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

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

In addition to the binding of epididymal boar spermatozoa to oviducts from readily obtainable gilts as described previously, a separate experiment was conducted to examine the binding capacity of epididymal boar spermatozoa to the oviducts of sows (which were more difficult to obtain). Four oviducts were obtained and explants pooled from each of two sows and two gilts that were slaughtered at the same time.

One sow was raised at the College of Public Health, Medical & Vet Sciences and the other was obtained from a commercial piggery. Upon examination of their ovaries, the sows were found to be in the follicular phase, but their oviducts were used since luteal phase sow oviducts were not available at the time of study. The gilts were non-cycling as described previously. In this experiment, 10 to 12 explants from both the isthmus and ampulla of gilts were incubated with spermatozoa from each region of the epididymis (i.e. caput, corpus and cauda), and compared to that from sows.

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

Six caput and seven corpus epididymides were used in this experiment. Boars were unilaterally castrated at the College of Public Health, Medical & Vet Sciences, and oviductal explants were prepared as described previously. In this experimental setup, epididymal spermatozoa were exposed to different pre-treatments then each was incubated with a total of 36 oviductal explants from both the isthmus and ampulla. Pre-treatments included: (i) caput spermatozoa in modified Androhep; (ii) caput spermatozoa in caudal fluid; (iii) corpus spermatozoa in modified Androhep; (iv) corpus spermatozoa in caudal fluid; and (v) caudal spermatozoa in modified Androhep. The contents of the caudal epididymis was first collected into small vials and centrifuged for 30 min at 1200 g, then centrifuged for a further 30 min to fully extract the caudal fluid. In the interim, oviductal explants were prepared as described earlier and pre-equilibrated in TALP for at least 15 min at 39^0 C in a humidified atmosphere containing 5% CO_2 in air. The caudal fluid supernatant was collected into Eppendorf tubes and divided between the specific caput and corpus treatment groups outlined above. Caudal spermatozoa were only diluted with modified Androhep. Sperm samples were incubated for 30 min at 39^0 C in a humidified atmosphere containing 5% CO_2 in air before being analysed for sperm concentration and motility characteristics by CASA. Thereafter, sperm samples were centrifuged for 10 min at 600 g and the supernatant replaced with the modified Androhep solution to yield a final concentration of 5×10^6 sperm/ml. Sperm samples were then pre-equilibrated for at least 5 min at 39^0 C in a humidified atmosphere containing 5% CO_2 in air, before

20 µl (1 x 10^5 spermatozoa) was added to each oviductal explant and further incubated for 15 min under the same conditions. Thereafter, explants were fixed, mounted on slides and examined as described previously.

Data analyses and presentation

Data were analysed using the Statistical Package for Social Sciences (SPSS) software version 11. Graphs were plotted using Microsoft Excel 2003. Statistical comparisons between two variables (i.e. isthmus *vs.* ampulla, porcine *vs.* bovine oviducts, ejaculated *vs.* epididymal spermatozoa, and sow *vs.* gilt oviducts) were calculated using the Student's T-test. Analysis of variance (ANOVA) was used to compare binding capacity of spermatozoa across the four regions of the testis/epididymis, sperm binding capacity across different boars, and the motility characteristics generated by CASA. A post-hoc Tukey test for multiple comparisons of means was used to determine homogeneous subsets in variables tested by ANOVA. Log_{10} transformation were performed for motility data by CASA, binding results between porcine *vs.* bovine isthmus, ejaculated *vs.* epididymal spermatozoa as well as between boars in order to normalise distribution of data prior to analyses. Normality was not achieved after the log_{10} transformation for binding between porcine and bovine ampulla, thus a non-parametric test (Mann-Witney U test) was used. The level of significant difference was set at $P \leq 0.05$.

Results

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

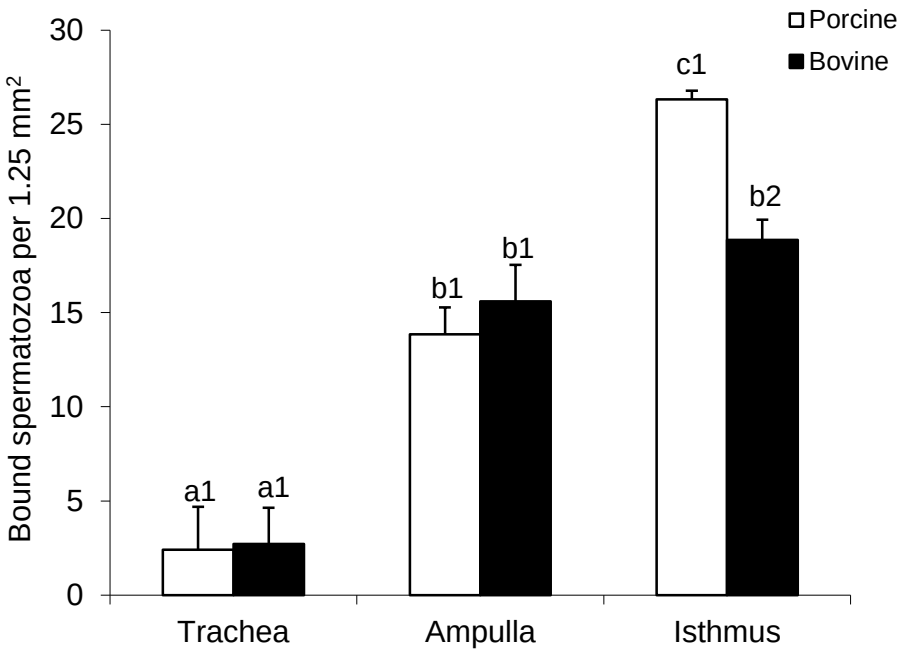


Figure 1. The mean (+ SEM) binding of ejaculated boar spermatozoa to porcine and bovine oviductal and tracheal explants. Different letters indicate a significant difference between explant types. Different numbers indicate a significant difference between species ($P \leq 0.05$). n = 108 explants for 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).

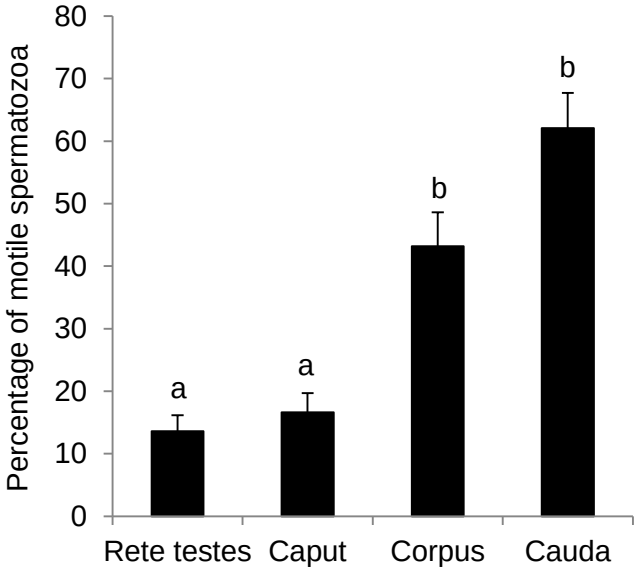


Figure 2. The mean (+ SEM) percentage of motile spermatozoa from the rete testis and different regions of the epididymis. Different letters indicate a significant difference between testicular regions ($P \leq 0.05$). n = 7 boars

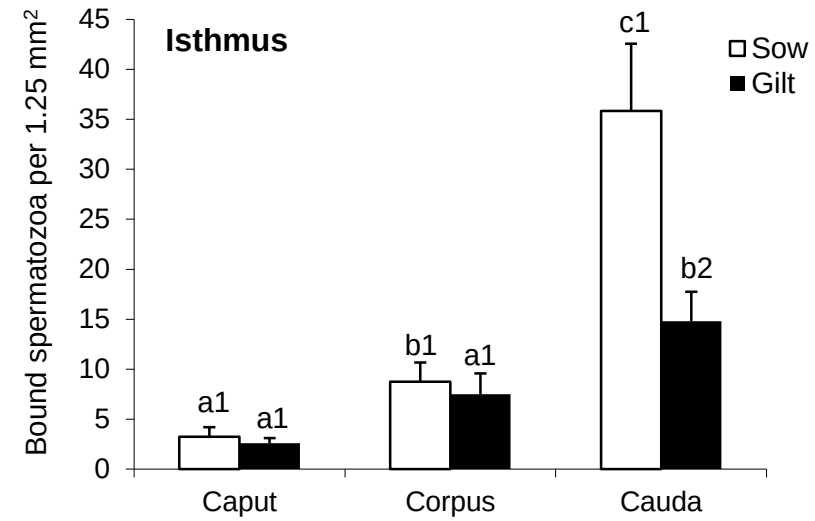
The mean percentage of motile spermatozoa from the rete testis and the three regions of the epididymis was determined immediately after collection (Fig. 2). The percentage of motile spermatozoa was greater in samples from the cauda and corpus and lowest ($P \leq 0.05$) in samples from the caput and rete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

Table 1. Motility characteristics of epididymal spermatozoa immediately after collection
Data are presented as mean percentages (± 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±6.28[a]	30.89±8.18[b]	35.66±10.84[b]	32.51±5.28[b]
VSL	8.20±5.34[a]	24.18±6.90[b]	25.33±8.51[b]	23.85±4.46[b]
VCL	17.88±11.96[a]	52.86±13.39[b]	59.04±15.66[b]	56.38±8.44[b]
ALH	1.22±0.79[a]	2.72±0.74[b]	2.95±0.74[b]	2.30±0.57[b]
BCF	5.90±3.87[a]	9.11±2.82[b]	14.81±4.92[b]	16.00±3.07[b]
STR	28.33±18.01[a]	53.90±11.95[b]	48.42±10.95[b]	66.80±7.89[b]
LIN	88.00±11.57[a]	35.80±8.00[b]	29.67±7.04[b]	40.70±5.53[b]

More ($P \leq 0.05$) spermatozoa from the cauda bound to the isthmic explants from sows, while more spermatozoa from the corpus bound to the ampullary explants from gilts ($P \leq 0.05$; Fig. 3a and b). The number of sperm bound to oviductal explants did not differ between sows and gilts for spermatozoa from any other epididymal region. Moreover, in both sows and gilts, more ($P \leq 0.05$) caudal spermatozoa bound to explants than caput spermatozoa irrespective of explant.

a)



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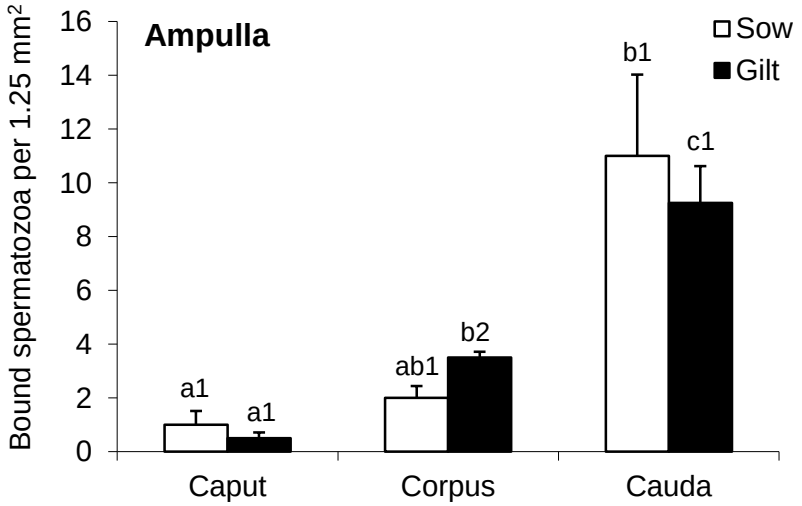


Figure 3. The mean (+ SEM) binding of epididymal spermatozoa to a) isthmic explants and b) ampullary explants of sows and gilts. Different letters indicate a significant difference between epididymal regions within each animal type (i.e. sow or gilt), while different numbers indicate a significant difference 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.

The number of spermatozoa that bound to the oviductal epithelium increased progressively ($P \leq 0.05$) from the rete testis to the cauda (Fig. 4). With the exception of corpus spermatozoa, more spermatozoa were bound to isthmic than ampullary

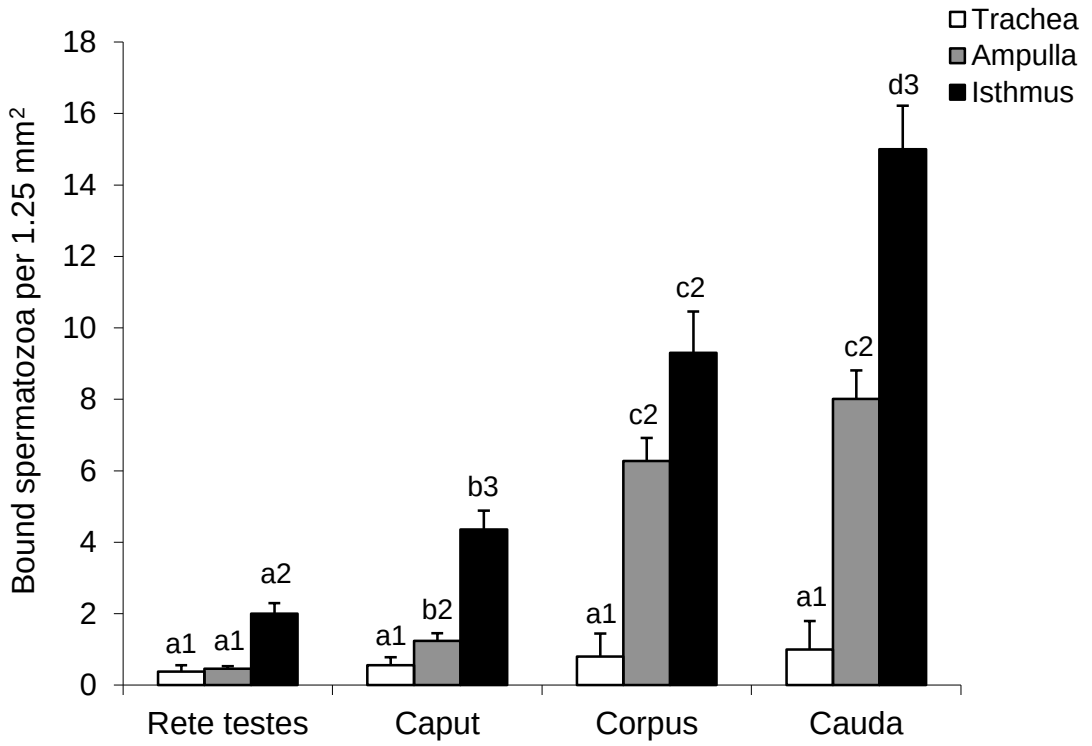


Figure 4. The mean (+ SEM) binding of boar spermatozoa from the rete testis and different regions of the epididymis to isthmic and ampullary explants, and tracheal controls. Different letters indicate a significant difference between different testicular regions within an explant type, while different 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.

explants. The same was true for ampullary explants except that the number of bound spermatozoa from the cauda did not differ to those from the corpus. With the exception of spermatozoa from the rete testis that bound to the ampulla, the mean number of spermatozoa bound to tracheal controls was less ($P \leq 0.05$) than other explants for all sperm samples.

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

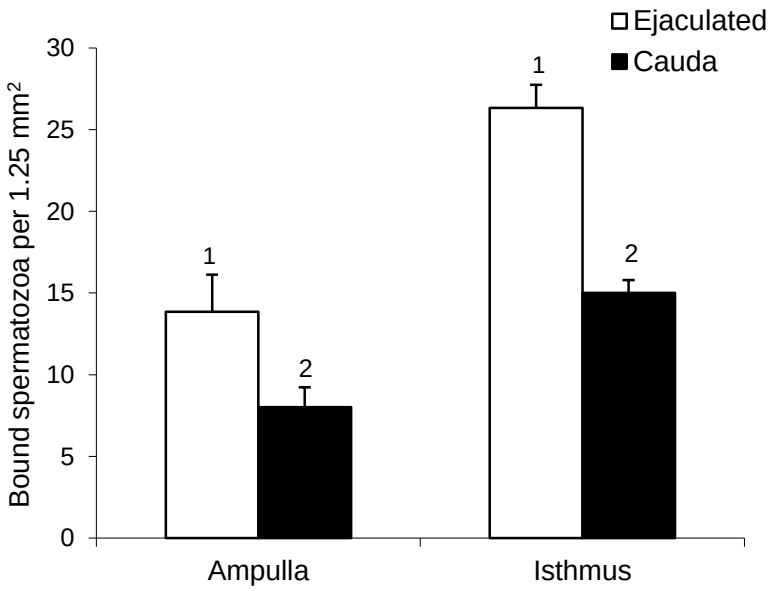


Figure 5. Comparison of ejaculated and caudal spermatozoa binding to isthmic and ampullary explants (mean + SEM). Different numbers indicate a significant difference between sperm samples within an explant type ($P \leq 0.05$). n = 121 and 84 isthmic or ampullary explants for ejaculated and caudal spermatozoa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

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

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

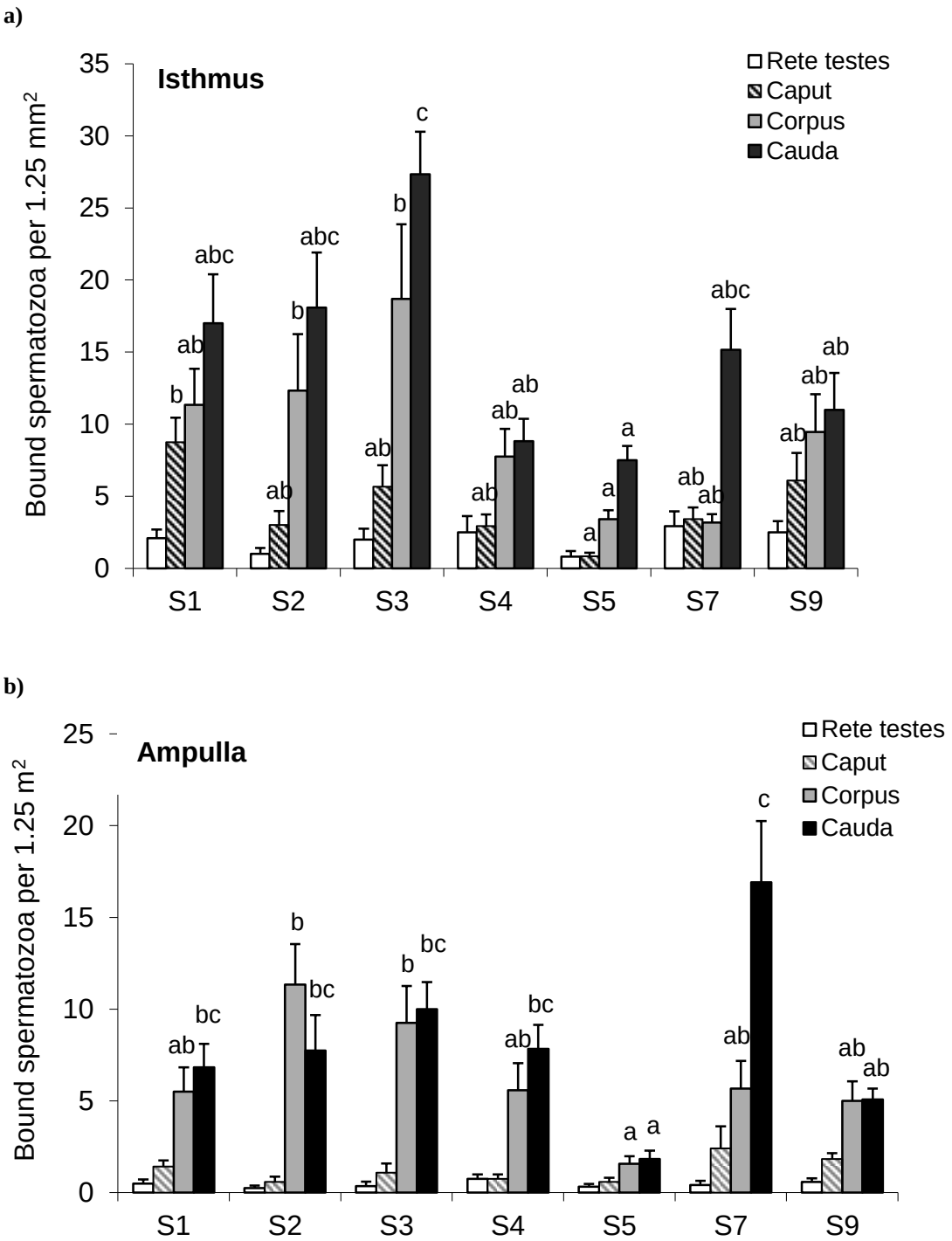


Figure 6. Comparison between individual boars (S1-S9) in the binding of spermatozoa from the rete testis and different regions of the epididymis to a) isthmic explants and b) ampullary explants (mean + SEM). Different letters indicate a significant difference between boars within a testicular region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

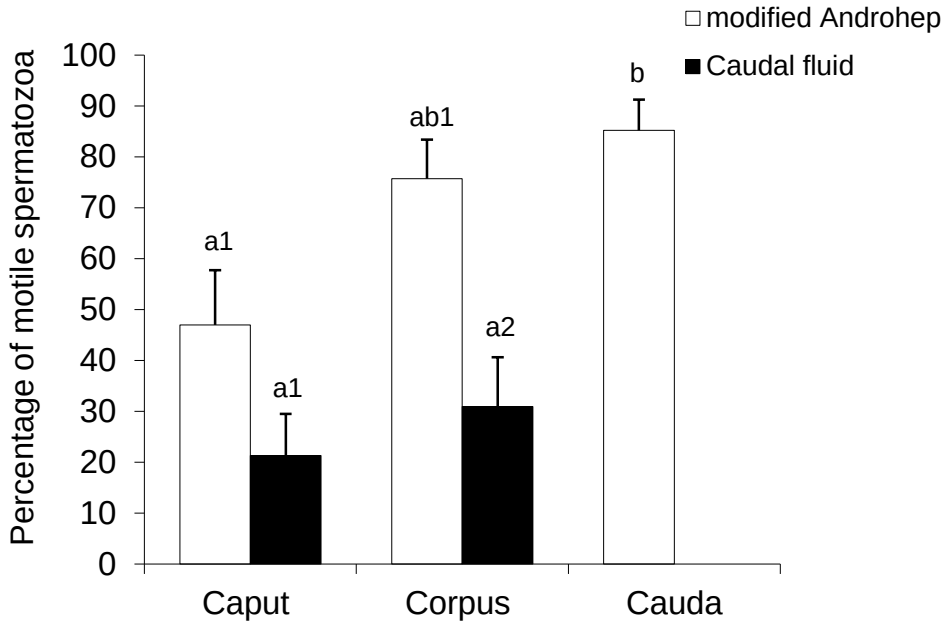


Figure 7. The mean (+ SEM) percentage of motile spermatozoa from different regions of the epididymis after incubation in modified Androhep medium *versus* caudal fluid. Different letters indicate a significant difference between epididymal regions per treatment, while different numbers indicate a significant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

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

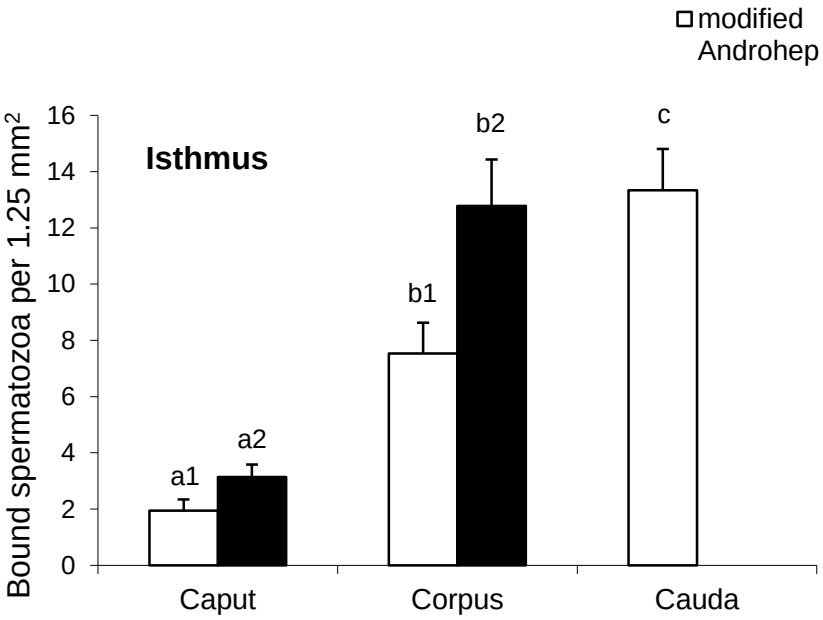
The percentage of motile epididymal spermatozoa as well as their motility characteristics were assessed immediately after incubation with either modified Androhep or caudal fluid (Fig. 7 and Table 2). Caudal spermatozoa had the highest mean percentage of motile spermatozoa in Androhep (higher than caput; $P \leq 0.05$) followed by spermatozoa from the corpus, then caput. However, when spermatozoa from the caput and the corpus were incubated with caudal fluid, there was a reduction in their motility (significant for corpus; $P \leq 0.05$) when compared to spermatozoa incubated with modified Androhep medium (Fig. 7). The average path velocity, straight-line velocity and curvilinear velocity of spermatozoa from the caput were higher ($P \leq 0.05$) in modified Androhep medium than in caudal fluid. In the corpus, only the curvilinear velocity and the beat cross frequency were higher ($P \leq 0.05$) in modified Androhep medium than in caudal fluid (Table 2).

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

Discussion

There are limited reports in the literature on the binding of epididymal spermatozoa to oviductal epithelium and, to our knowledge, this is the first study to compare oviduct binding of spermatozoa from different regions of the epididymis. We report here that the capacity of testicular spermatozoa to bind to the oviduct and form the sperm reservoir appears to develop progressively during maturation in the epididymis. In addition, we demonstrate for the first time that caudal fluid can enhance oviduct binding of immature epididymal spermatozoa to levels equivalent to that of mature caudal spermatozoa. The isthmus of the oviduct was found to bind

a)



b)

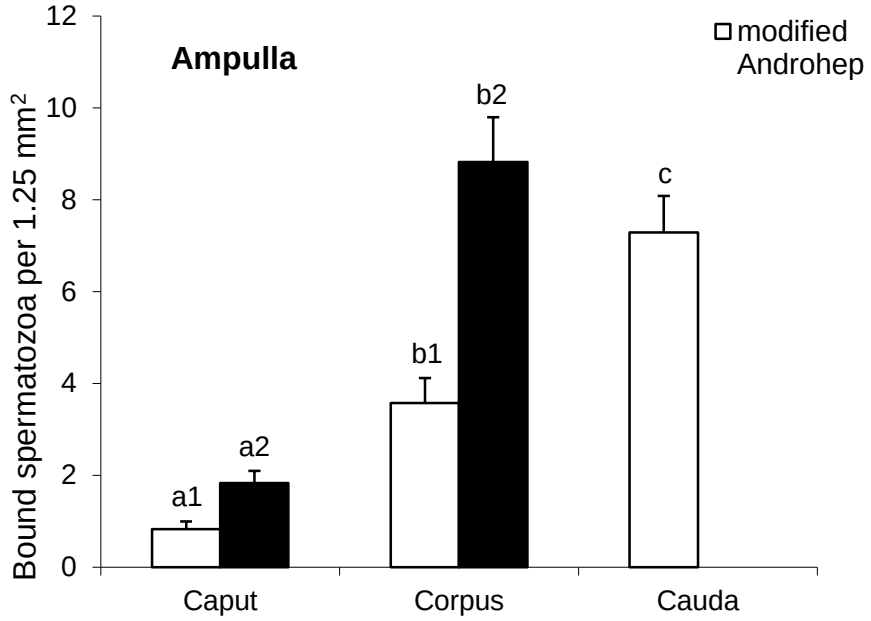


Figure 8. The influence of pre-incubation in modified Androhep medium *versus* caudal fluid on binding of epididymal spermatozoa to a) isthmic explants and b) ampullary explants (mean + SEM). Different letters indicate a significant difference between epididymal regions per treatment, while different 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.

spermatozoa most effectively and this appears to be most prominent during the follicular phase in sexually mature animals. Moreover, binding to the isthmus occurs preferentially in a species-specific manner. Lastly, considerable variability among males exists in the oviduct binding capacity of their spermatozoa.

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

There are many products in caudal fluid, some of which are secreted under the influence of androgens [32]. Among the important secretory products found to be highly expressed in the cauda, is the cysteine-rich secretory protein (CRISP) family of proteins that are involved in spermiogenesis, capacitation and binding of the spermatozoon to the oocyte [33]. Factors have been also identified in caudal fluid that are associated with fertility in dairy bulls [34], and appear to sustain motility of bovine spermatozoa *in vitro* [35]. In our study, 30 minutes pre-incubation of caput and corpus spermatozoa with caudal fluid significantly increased the binding of spermatozoa both to the isthmus and ampulla when compared to caput and corpus spermatozoa maintained in modified Androhep. This potentially indicates that caudal fluid contains distinct factors that could directly or indirectly enhance the interaction between spermatozoa and oviduct epithelium. Importantly, it demonstrates how caudal fluid can accelerate binding capacity (and potential fertility) of immature sperm, which could have important implications in animal production systems. It

would be interesting to determine the optimum duration required for immature spermatozoa to be exposed to caudal fluid in order to acquire maximum binding capacity. Caudal sperm in modified Androhep served as positive control and no preparations were made where caudal sperm was pre-incubated with caudal fluid. This is because the spermatozoa was extracted from the cauda where essentially it had already been in extensive contact with the caudal constituents. Interestingly, comparable rates of binding can be observed between corpus spermatozoa incubated in caudal fluid and caudal spermatozoa (Fig. 8). While this comparison is not ideal, it demonstrates that even 30 min pre-incubation in caudal fluid is sufficient to confer oviductal binding capacity equivalent to that of mature spermatozoa. Whether longer pre-incubation will further improve binding capacity in caput spermatozoa remains to be determined. .

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

putative explanation for the increased binding of immature boar spermatozoa after incubation in caudal fluid.

In addition, specific proteins rich in sphingomyelin (and with a high cholesterol/phospholipid ratio) are known to be secreted by the epididymal epithelium, and are capable of regulating both sperm motility and fertilising ability [42]. These are associated with epididymosomes. Examples include enzymes involved in the polyol pathway and a cytokine (MIF; macrophage migration inhibitory factor) believed to be selectively transferred to spermatozoa during epididymal transit. Similar to epididymosomes are prostasomes (prostate-derived small membrane vesicles) that are found along the male reproductive tract and particularly in ejaculated semen [43]. Epididymosomes and prostasomes can greatly influence the environment through which spermatozoa pass by allowing the transfer of new biologically active proteins, as well as contributing to lipid and cholesterol content. These in turn allow spermatozoa to gain new adhesion molecules that could facilitate inter-cellular communication between the sperm surface and the oviduct epithelium and in return promote binding [44]. Specifically, prostasomes secreted in a timely manner under hormonal control are believed to be involved with post-testicular sperm maturation due to their immunosuppressive activity, improvement in sperm motility, and their modulation of capacitation [reviewed in 43, 45].

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

The use of a heterologous system for studying sperm binding to oviductal epithelium has been examined in several species including the binding of human spermatozoa to the oviducts of cows and macaques [47], canine spermatozoa to porcine oviducts [48] and stallion spermatozoa to bovine oviductal cells [49].

Heterologous systems are mainly used for logistical reasons, particularly in humans where an adequate supply of disease-free oviduct tissues is not always available [47]. When variation between species is minimal; much of the effort, time and cost of the study can be reduced. This study is the first to examine the ability of porcine spermatozoa to bind to the oviductal epithelium of cows. Bovine oviducts were considered for use in this study because they were readily availability from a large cattle abattoir in close proximity to the laboratory. By contrast, the nearest source of porcine oviducts was from an abattoir 130 km away. While the number of canine spermatozoa that bound to canine and porcine oviducts was similar [48], the number of ejaculated boar spermatozoa in our study that bound to the isthmus (but not ampulla) was significantly less in cows than gilts. This result suggests that binding is preferentially species-specific because carbohydrate-binding lectins and glycoconjugates present on the plasma membrane of the sperm head and surface of oviductal epithelium may vary considerably among species [29, 50]. Thus we concluded that it was necessary to use porcine oviducts for remaining experiments despite the increased cost and logistical difficulties associated with such an experimental set-up.

However, differences in oviduct receptivity caused by the reproductive cycle (luteal phase cows *vs.* non-cycling gilts) cannot be excluded. Different results have been reported on the effect of (i) the region of the oviduct, (ii) steroid hormones, and (iii) the reproductive status of the animal on the capacity of spermatozoa to bind to the oviductal epithelium. While no significant difference in the binding of spermatozoa to oviductal explants from either follicular or luteal phase pig oviducts has been observed, the addition of exogenous oestradiol was found to enhance sperm binding to both the isthmus and ampulla [51]. In our study, it was necessary to use oviducts from pre-pubertal gilts because a consistent supply of sow oviducts could not be assured from the abattoir, which primarily slaughtered pigs up to about 20 weeks of age. Moreover, previous literature reported no difference in the number of ejaculated boar spermatozoa that bound to the oviducts of gilts compared to cycling sows [11], although the authors didn't specify the age nor pre/post-pubertal status of gilts used. Nevertheless, there was an opportunity to compare the binding capacity of

spermatozoa to the oviducts from two sows of known history with that of gilts. In contrast to previous results in pigs [11] and cows [52], we found preferential binding of caudal spermatozoa to the isthmus but not ampulla of follicular-phase sows compared to non-cycling gilts. This is consistent with studies in the horse [53] in which the presence of oestrus (but not diestrus) concentrations of steroids in the medium increased the percentage of spermatozoa attaching to both the isthmus and ampulla of the oviduct. These results imply the significant involvement of increased levels of oestrogen in the binding of spermatozoa to oviducts of sexually mature sows compared to pre-pubertal gilts. However due to the small samples size, oviducts from more sows need to be examined to confirm this result.

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

More ejaculated or epididymal spermatozoa bound to the isthmus than the ampulla. The reason for this may be attributed to differences in the epithelial structure, regional secretions and biochemical features that exist between the isthmus and ampulla. Studies report no differences in the binding capacity of spermatozoa to the isthmus and ampulla of pigs [11, 51] or cattle [52], although Raychoudhury and Suarez [55] found more porcine spermatozoa bound to the isthmus (10.8 ± 0.4 spermatozoa per 0.3 mm^2) than to the ampulla (5.6 ± 0.4 spermatozoa per 0.3 mm^2). They suggested that the presence of a high concentration of oestrogen during oestrus favoured the binding of spermatozoa to isthmic explants. Moreover, spermatozoa from the horse and human have also been reported to bind in greater numbers to the isthmus than to the ampulla [56, 57]. It is important to realise that regional differences in the expression of glycoconjugates are apparent between segments of the porcine

oviduct, and across the different stages of the oestrous cycle, thereby affecting the available binding sites [58]. It seems logical that the difference observed with binding is consistent with the normal physiological functions of the oviduct in these two regions: i.e. sperm storage and reservoir formation in the utero-tubal junction and isthmus, *versus* sperm-oocyte binding, acrosome reaction and fertilization in the ampulla. Thus one would expect binding sites to be reduced in the ampulla because this is where sperm must locate and fertilise oocytes without binding to false targets such as the epithelium.

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

The acquisition of motility by spermatozoa during their maturation in the epididymis is well established [4]. The current study found a significant increase in the motility of spermatozoa from the corpus and caudal epididymis when compared to spermatozoa from the rete testis and caput. This indicates that motility of boar spermatozoa predominantly develops from the corpus onwards. This is consistent with several other maturational changes that occur during epididymal transit that facilitate sperm motility [15]. These include changes in cAMP concentrations between epididymal regions [60]; decrease in intracellular pH [61]; decrease in free calcium ion concentration and glucose transport into spermatozoa [62]; and a decrease in the exchange of calcium ions into mitochondria [63]. Acott and Hoskins [64] demonstrated that when cAMP was added to immature bovine spermatozoa from the caput, sperm motility increased and was further enhanced by the addition of forward motility protein. Moreover, forward motility protein binds to spermatozoa in the caput

and becomes concentrated on spermatozoa in the caudal epididymis. Thus, in addition to the capacity for binding to the oviductal epithelium, the acquisition of sperm motility during epididymal maturation is critical to successfully establish the functional sperm reservoir prior to fertilization.

Spermatozoa from the caudal epididymis need to be stored in an immotile state to avoid exhaustion of energy reserves. It is not yet fully understood how this is mediated by caudal fluid [14], however pH and bicarbonate concentration are known to play a role [65]. In the boar, the pH of the caudal fluid (pH 6.5) is lower than in more proximal regions (pH 7.2), while the concentration of bicarbonate (3-4 mM) is considerably less than in rete testis fluid (30 mM) [65]. It is therefore not surprising in the present study that the proportion of motile spermatozoa and their motility characteristics after incubation in caudal fluid were significantly less than those incubated in modified Androhep medium. It is important to note that in our study motility parameters predominantly associated with velocity (i.e. VAP, VSL and VCL; see Table 2) were affected. This is consistent with a reduction in progressive motility/sperm metabolic rate possibly by factors in caudal fluid that may act to prolong sperm storage in the cauda, rather than as a consequence of sperm death *per se*.

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

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