BOAR SPERMATOZOA DEVELOP ABILITY TO BIND TO OVIDUCT EPITHELIUM DURING PASSAGE THROUGH THE EPIDIDYMIS

Thesis submitted by
Santiago Torda Peña, Jr. DVM (*LSU*)
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James Cook University

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DECLARATION OF ETHICS

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *Joint NHMRC/AVCC Statement and Guidelines on Research Practice* (1997), the *James Cook University Policy on Experimentation Ethics, Standard Practices and Guidelines* (2001), and the *James Cook University Statement and Guidelines on Research Practice* (2001). The research methodology received clearance from the James Cook University Experimentation Ethics Review Committee (Animal Ethics approval number A1007).

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STATEMENT ON THE CONTRIBUTION OF OTHERS

Professor Phillip Summers supervised the research reported in this thesis, carried out the surgical castrations of the boars, provided advice and assistance with the preparation of the thesis and was a co-author on all papers resulting from this thesis.

A stipend was provided by the Australian Agency for International Development (AusAID) for the duration of the research candidature. Project costs were met from IRA and Reproduction Service accounts held by Professor Summers.

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Santiago T. Peña, Jr. Date

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The LORD bless thee, and keep thee: The LORD make his face shine upon thee, and be gracious unto thee: The LORD lift up his countenance upon thee, and give thee peace. Numbers 6: 24-26 (Holy Bible, KJV)

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To all of you, I tender this humble piece of work.

ABSTRACT

The aim of this study was to investigate the relationship of maturation of spermatozoa in the epididymis and the ability to bind to oviduct epithelium. It was hypothesized that testicular spermatozoa need to pass through the regions of the epididymis in order to acquire the ability to bind to the oviduct.

Spermatozoa were collected from the rete testis and the caput, corpus and cauda epididymides from 10-14 month-old Large White or Large White x Landrace boars. Boars were first unilaterally castrated and then slaughtered four to five weeks later to obtain the second testicle, epididymis and seminal vesicles. Oviducts were obtained from slaughtered pre-pubertal gilts and explants from the isthmus and ampulla prepared. Spermatozoa were suspended in modified Androhep medium, added to oviduct explants and incubated at 39^0 C in a humidified atmosphere containing 5% CO_2 in air for 15 minutes. The number of spermatozoa attached to 1.25 mm^2 of explant was counted after fixation and staining of explants.

The possibility of using oviducts from slaughtered cows rather than porcine oviducts was examined using ejaculated spermatozoa. Significantly more ejaculated spermatozoa bound to the isthmus of gilts than cows hence, porcine oviducts were used in the succeeding experiments. There was a sequential increase in the number of spermatozoa that bound to the oviductal epithelium from the rete testis to the cauda epididymidis (2 ±0.30, 4.36 ±0.53, 9.3 ±1.60 and 15±1.22 for rete testis, caput corpus and caudal spermatozoa on isthmic explants, respectively). Significantly more ($P \leq 0.05$) spermatozoa, either ejaculated or epididymal, bound to isthmus than ampulla explants (26.33±2.27 and 13.55±1.42 ejaculated spermatozoa on isthmic and ampullary explants, respectively).

Incubation in medium containing albumin and asialofetuin which are known to contain mannose and lactose respectively inhibited the binding of epididymal spermatozoa to oviduct explants (3.42±0.56 caudal spermatozoa on isthmic explants in medium with albumin and 14.75±2.02 caudal spermatozoa on isthmic explants in modified Androhep medium).

The number of spermatozoa from the caput and corpus that bound to oviduct explants significantly ($P \leq 0.05$) increased after incubation in caudal fluid for 30 minutes (7.52±1.10 and 12.78±1.64 corpus spermatozoa on isthmic explants for modified Androhep and caudal fluid, respectively). This result suggests that caudal fluid has distinct features that directly or indirectly influence the attachment of spermatozoa to oviduct epithelium. The motility of spermatozoa also decreased after incubation in caudal fluid for 30 minutes. This result is not surprising because *in vivo*, spermatozoa remain in a quiescent state during storage in the cauda epididymidis.

Exposure of epididymal spermatozoa to seminal plasma for 30 minutes significantly reduced the number of spermatozoa that bound to oviduct explants while exposure for one minute significantly increased the number of bound caput spermatozoa. Incubation in seminal plasma also caused capacitation of spermatozoa and this is the likely reason for the reduction in the number of bound spermatozoa. There is also the possibility that some components in the seminal plasma may form a coating over the plasma membrane of spermatozoa sufficient to inhibit the expression of pre-existing binding molecules. On the other hand, the seminal plasma may also be a source of binding molecules for immature caput spermatozoa. There was an increase in the motility of spermatozoa after exposure to seminal plasma.

In conclusion, this study found that as spermatozoa pass down the epididymis, there is an increase in the number of spermatozoa that bind to oviduct explants. This result was interpreted to mean that the maturation of spermatozoa in the epididymis involves the acquisition of the ability to bind to oviduct epithelium.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
ATP	adenosine triphosphate
BSP	bovine seminal plasma protein
cAMP	cyclic Adenosine Monophosphate
cAMP-PKA	cAMP-protein kinase A
CTC	chlortetracycline
CRISP	cysteine-rich secretory protein
CTP	cholesterol transferase protein
DNA	deoxyribonucleic acid
EAP	oestrous-associated glycoprotein
E-RABP	epididymal retinoic acid-binding protein
FITC-sZP	fluorescein-conjugated solubilized zona pellucida
GPX	glutathione peroxidase
g	g-force/g-load/gravitational acceleration
HBPs	heparin-binding proteins
HE	human epididymal protein
HPLC	high performance liquid chromatography
HTM-IVOS	Hamilton-Thorne Machine-integrated visual optical system
kDa	kilodalton
K^+/Na^+ ratio	potassium/sodium ratio
MAN2B	135 kDa alpha-D-mannosidase
MMPs	matrix metalloproteinases
mRNA	messenger ribonucleic acid
OECM	oviduct epithelial cell monolayer
OGP	oestrogen-dependent protein
PAGE	polyacrylamide gel electrophoresis
PGDS	prostaglandin D2 synthase
pI	isoelectric point
pOGP	porcine oestrogen-dependent protein
SDS	sodium dodecyl sulfate
TALP	Tyrode's albumin lactate phosphate

CHAPTER 1

INTRODUCTION

1.1 Introduction

Food animals are very important resources of proteins and other nutrients in the human diet. They provide a number of essential amino acids and vitamins not usually found in plants. Of the many animal resources available throughout the world, pigs as well as poultry are the major and more stable meat sources. The consumption of meat per animal species worldwide is shared between pork (43%), poultry (27%), beef/veal (26%) and 4% from others (Du, 2001). The worldwide demand for meat has been predicted to increase by more than four percent above the consumption of the past decade (FAS, 2004). This is largely due to the increasing demand and growing economies of developing nations in the Asian region in which by the year 2020, developing nations will utilise 60% of the world's meat and 52% of the world's milk production (ILRI, 2004).

The demand for food has been projected to increase significantly between 3000 kcal/person/day in 2015 and above in 2030 (FAO, 2003). Comparative data of the dietary intake per capita of major meats in USA, Australia and The Philippines is 23%, 20.38% and 11.23 % for pork; 23%, 30.56% and 7.44% for chicken; and 29%, 35.16% and 2.81% for beef (LDC, 2004). These data indicate that pig meat plays a very important role in a country like The Philippines where pig farming is the largest livestock industry. In addition to being a major source of food, pig farming also provides business and employment opportunities for people. In 2002, the total production of pork reached as much as 1.67 M tonnes valued at about 86.72B pesos of which smallholder farmers contributed 76.8% of the total production (SwiIN, 2004).

Fertility is an important aspect in animal production. Unfortunately often the fertility of males doesn't receive as much attention as it should in the management of the herd. In pigs, boar fertility is usually related to the prolificacy of the sow as measured by the farrowing rate, litter size or the number of returns to service (Hughes and

Varley, 1980; Colenbrander *et al.*, 1993), but this system doesn't really guarantee the highest culling standards for boars especially if the animal under consideration has an outstanding genetic merit.

The quest for understanding the complex attributes of fertility in male is of paramount concern. Whether it is by natural or artificial insemination of spermatozoa into the female reproductive tract, the highest possible fertility of spermatozoa is always a must as it constitutes 50% in the actual success attainable in every conception. While much attention has been directed to understanding the complex cellular events in the production of spermatozoa and their effect on fertility, there has been a limited focus on aberrations in the function of the epididymis and their role in infertility of domestic animals. While much attention has been directed to understanding the complex cellular events in the production of spermatozoa and their effect on fertility, there has been a limited focus on aberrations in the function of the epididymis and their role in infertility of domestic animals.

One of the organs whose anatomical and physiological attributes are almost unknown to the secular world is the epididymis. This is probably due to its relatively small size, not so conspicuous location and thought to be just an integral part of the testicle. The epididymis is an organ structure comprised of a strongly convoluted duct system with a length of about 60 metres in the boar, 40 in the bull and 20 in man (Mann and Lutwak-Mann, 1981). Upon leaving the testes and passing through the efferent ducts, spermatozoa enter the epididymis. The word epididymis was coined in 1830 by Sir Astley Cooper when he first described this organ (Mann and Lutwak-Mann, 1981):

"Of the epididymis: This body may be considered as an appendix to the testis, and its name is derived from its being placed upon this organ, as the testes were anciently called didymi. It is of a crescenti form; its upper edge is rounded, its lower edge is thin. Its anterior and upper extremity is called its caput, the middle part its body and the lower part its cauda."

The epididymis is divided into three major compartments: the head (caput), the body (corpus) and the tail (cauda) (Mann and Lutwak-Mann, 1981; Briz *et al.*, 1993). The epididymis has a significant role in the maturation of testicular spermatozoa (Hafez, 1993). Testicular spermatozoa are both immotile and infertile (Brooks, 1983) and

despite the fact that the post-testicular mammalian spermatozoon is equipped with a modified nucleus of unique DNA content and a highly organized cytoplasm, it does not have all the capabilities which are essential both for survival in the female reproductive tract or the capacity to fertilise oocytes (Amann *et al.*, 1993). The epididymis serves as an area where the full development and maturation of spermatozoa can continue leading to the acquisition of progressive motility and fertilising capacity.

A comprehensive understanding of the biological and physiological functions of the epididymis is still limited. Although the epididymis has been part of biological investigations since early in the 19th century, it was only in the 1970's when the anatomical features, biochemistry and functionality of the epididymis has been deeply considered. A complex array of proteins and secretory products in the epididymis has been identified (Syntin *et al.*, 1996), but a detailed understanding of the cellular changes that occur to spermatozoa at different sites of the epididymis is still largely unknown.

While the epididymis performs a crucial role in the fertility of the male, the oviducts also have a number of roles that are essential for successful reproduction. The mammalian oviduct is not just a passive conduit between the uterus and the ovaries but is the primary organ involved in sperm capacitation, fertilisation and early development of the embryo (Hunter, 1998; Rodriguez-Martinez, 2001). As with the epididymis, the functional significance of the oviduct was not immediately appreciated.

Those spermatozoa that reach the oviduct in the pig are mostly retained at the utero-tubal junction and attached to the caudal portion of the isthmus. These regions constitute the sperm reservoir which in pigs is established one to two hours after insemination (Hunter, 1981). The sperm reservoir is known to function in three major ways (Topfer-Petersen *et al.*, 2002). Firstly, it prolongs sperm viability with respect to the onset of ovulation regardless of the time of insemination. Spermatozoa stored in the caudal isthmus retain their viability for 36 h or longer (Hunter, 1984). Secondly, it controls capacitation to coincide with the onset of ovulation. With the limited life of spermatozoa, capacitation must be controlled for it to happen at the

right time. The oviduct regulates this complex sequence of cellular and membrane changes (Petrunkina *et al.*, 2003). Thirdly, the sperm reservoir prevents polyspermy (Hunter, 1993).

The mechanisms by which spermatozoa bind to the epithelium of the oviduct are still largely unclear. However, the physical environment of the oviduct and the involvement of a carbohydrate-mediated interaction between spermatozoa and oviductal epithelium appear to be the basis for this complex sperm-to-oviduct interaction (Pollard *et al.*, 1991; Green *et al.*, 2001). At oestrus, the lumen of the oviduct becomes smaller due to oedema of the lamina propria in response to high concentrations of circulating oestrogens. In the pig, the caudal isthmus of the oviduct secretes a characteristic visco-elastic and tenacious mucus just before ovulation (Hunter, 1998). After ovulation, the quantity and the viscosity of the luminal secretions decrease.

On the other hand, a carbohydrate-mediated interaction has been widely observed in many animal species and with other cellular interactions such as between spermatozoa and Sertoli cells (Raychoudhury and Millette, 1997) and between spermatozoa and the zona pellucida (Oehninger *et al.*, 1991). Oligosaccharides and lectins present in the plasma membrane and mucosal surface of spermatozoa and the oviduct, respectively, function as ligand and receptors which actively participate in sperm-oviduct interaction. Different sugar monomers, oligosaccharides or glycoproteins inhibit the binding of spermatozoa to oviductal epithelial cells or explants in a species specific manner (Suarez, 2001). However, it is not yet known how far these identified receptor-ligand systems influence this interaction between spermatozoa and the oviduct epithelium (Hunter, 2003). It has been found recently that proteins secreted by the seminal vesicles of the bull adhere to the plasma membrane of spermatozoa and functions as primary oviduct-binding proteins (Gwathney *et al.,* 2003, 2006).

The relationship that exists between the epididymis and the oviducts is the fact that while spermatozoa progress through different regions of the epididymis, it would be expected that a number of maturational changes occur to spermatozoa which are

necessary for the development of ligands or receptors essential for binding to the oviductal mucosa. Spermatozoa undergo maturation processes principally within the caput and corpus regions of the epididymis and are stored in the cauda. Further to this is the possible influence of certain components of the seminal plasma on the interaction of spermatozoa to the oviductal epithelium.

1.2 Working Hypothesis

Testicular spermatozoa of the boar need to pass through the caput, corpus and cauda epididymidis to develop the full capacity for attachment to the epithelium of the oviduct.

1.3 Objectives

- Examine and compare the capacity of spermatozoa from the rete testes, caput, corpus and cauda epididymidis of the boar to attach to the isthmic and ampullary epithelium with the use of an *in vitro* binding assay.
- Determine whether the binding is species-specific by examining the attachment of porcine epididymal spermatozoa to the bovine oviduct.
- Investigate the influence of secretions of the seminal vesicle on the capacity of porcine epididymal spermatozoa to bind to the oviductal epithelium.
- Determine if incubation of spermatozoa from the caput and corpus with fluid from the cauda epididymidis will influence the binding capacity of caput and corpus spermatozoa.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Introduction

This literature review is divided into three major areas. Firstly, the developmental changes and maturational processes that occur to spermatozoa in the epididymis with special reference to the boar are discussed. These include the morphological and ultrastructural features of the epididymal epithelium and its characteristic secretory products which play significant roles in maturation of boar spermatozoa. Secondly, the structural anatomy and histology of the mammalian oviduct will be considered. The oviductal fluid content and composition is also discussed particularly with respect to oviductal regions and the stage of the oestrous cycle. Thirdly, a discussion will focus on the interaction of spermatozoa with the oviduct both *in vitro* and *in situ*. This includes the morphological features of the binding of spermatozoa to oviduct epithelium, its mechanism, significance and other factors which might be involved in the formation of the sperm reservoir.

2.2 A General Perspective of the Role of the Epididymis

While research on testicular and ovarian physiology has been undertaken for many years, a detailed study of the epididymis and its crucial role in the maturation of spermatozoa has only occurred in the past few decades. The epididymis is important in the final development of the motility and fertilising capacity spermatozoa (Brooks, 1983; Amann *et al.*, 1993; Hafez, 1993). It also enables the production of a population of spermatozoa with different potential capacities to fertilise oocytes (Amann *et al.*, 1993) which is important to increase the likelihood of fertilized oocytes in a given time. This heterogeneity in the sperm population is essential with respect to the time of ovulation, especially in a species like the pig. Pigs ovulate about 40-42 hours from the beginning of oestrus and thus spermatozoa which are able to maintain full potential and remain viable for an extended period of time will be the ones most likely to fertilise oocytes.

The maturation processes principally occurs within the caput and corpus epididymidis, while the prime purpose of the cauda is to store spermatozoa. Interestingly, complete maturation of spermatozoa and the development of the full capacity to fertilise oocytes have never been obtained *in vitro* with testicular spermatozoa or those retrieved from the proximal epididymis (Moore and Akhondi, 1996; Dacheux *et al.*, 2003). However, when epididymal spermatozoa were incubated with epididymal cell cultures, an increase in the motility had been observed and in some cases, the capacity to fertilise oocytes also improved although the binding to the zona pellucida did not improve (Moore and Akhondi, 1996). Early studies have reported that the electrophoretic profiles of epididymal fluid from different regions were different to other body fluids including blood plasma. The presence of hundreds of proteins present across all mammalian species (Dacheux *et al.*, 2003) with some uniquely found and specific to the epididymal secretions suggests that such proteins were the molecular agents responsible for the many significant changes that occur to spermatozoa as they traverse the epididymal duct (Holland and Nixon, 1998).

The epididymis also performs a lot of other functions, which are directly or indirectly essential to the successful penetration and fertilisation of the oocyte. These include the following: secretion of epididymal plasma; modification and development of the plasma membrane of spermatozoa; eradication of old and dead spermatozoa; concentration of spermatozoa; maintenance of the viability of spermatozoa during storage in the cauda epididymidis and sperm transport (Mann and Lutwak-Mann, 1981; Amann *et al.*, 1993). Transport of spermatozoa along the epididymal duct is achieved by the periodic contractions of the smooth muscle fibers lining the epididymal duct of the caput and corpus and the low-frequency contractions in sections around the smooth muscle of the cauda (Robaire and Hermo, 1988; Turner, 1991). The cauda also produces short but forceful contractions mediated by its resident nerve cells (Jones, 1999).

2.3 The Fertilising Ability of Epididymal Spermatozoa

Ejaculated spermatozoa and those directly taken from the cauda are comparable in terms of maturity status and fertility potential (Yanagimachi, 1994). There are some

reports that state that that caudal spermatozoa of the rat (Shalgi *et al.*, 1981) and pigs (Nagai *et al.*, 1984) fertilise more oocytes *in vitro* than ejaculated spermatozoa. Spermatozoa from the cauda epididymidis of the boar penetrated more zona-intact oocytes than ejaculated spermatozoa that were subjected to similar pre-treatment conditions (Nagai *et al.*, 1984). The same authors added that spermatozoa were incapable of penetrating oocytes when they were exposed to the seminal plasma.

The fertilising ability of epididymal spermatozoa of the boar had been studied by Holtz and Smidt (1976). It was found that essential changes occur to spermatozoa as they move down the epididymis and these changes specifically involve the acquisition of fertilising capacity. The fertilisation rate of spermatozoa improved considerably from the caput to the cauda, although complete maturation was not achieved until spermatozoa reached the cauda. This is consistent with the general observation that maturation of spermatozoa is finalised only when spermatozoa have arrived in the proximal cauda epididymidis (Turner, 1995).

Furthermore, while spermatozoa from the upper corpus epididymidis of the boar demonstrate penetration and activation of some oocytes from ipsilateral oviduct, spermatozoa from the cauda epididymidis did not from oocytes recovered from the contralateral oviduct (Hunter *et al.*, 1976). It was suggested that cauda spermatozoa were possibly invested with inhibitory compounds which stabilized the plasma membrane during the epididymal transit (Hunter *et al.*, 1976). Exposure to those factors could delay expression of capacitation unlike those spermatozoa liberated from the proximal portion of the epididymal duct. Substances known to inhibit capacitation are present between the proximal and distal portions of the epididymal duct (Hunter *et al.*, 1976). High concentrations of enzyme inhibitors (i.e. trypsin inhibitor) are present in boar rete testis fluid and epididymal plasma (Suominen and Setchell, 1972). Because of this, the ability of spermatozoa to penetrate oocytes would be likely to be blocked since the expression of the necessary enzymes is inhibited. Further studies on biochemical and physiological aspects relating to these limitations in understanding are needed. In the hamster and mouse, acquisition of the ability to penetrate oocytes is normally achieved when spermatozoa have reached the cauda (Mann and Lutwak-Mann, 1981). While penetration of oocytes is possible by rabbit spermatozoa isolated from the corpus, the fertilisation rate was still minimal.

2.4 Comparative Morphology of Spermatozoa from the Caput, Corpus and Cauda Epididymides

The quality of spermatozoa irrespective of the animal species is greatly influenced by a number of factors which can be either environmental (i.e., photoperiod, relative humidity, temperature and handling) and/or within the animal itself including but not limited to the breed, age, viral or bacterial infection (Swierstra, 1973; Colenbrander *et al.*, 1993). In intensive swine production where artificial insemination is used, the frequency of semen collection can substantially affect the concentration as well as motility characteristics and morphology of spermatozoa (Bonet *et al.*, 1991). A number of important factors which determine the maximum frequency of semen collection without severely affecting the performance of the boar include the rate of sperm production by the animal, the concentration of spermatozoa per ejaculate and the duration of the passage of spermatozoa in the epididymal tract. By using auto-radiographic methods, Swierstra (1968) determined that the time required by boar spermatozoa to travel through the epididymis was three days for the caput, two days for the corpus, and four to nine days for the cauda. The duration of epididymal transit also varies between species and can be lowered by as much as 10-20 % when the frequency of collection/ejaculation is increased (Setchell, 1991).

The comparative morphology of boar spermatozoa collected from different regions of the epididymis had been extensively discussed (Briz *et al.*, 1993; 1995; 1996). The proportion of mature or immature spermatozoa with a proximal or distal cytoplasmic droplet differed significantly as spermatozoa pass down the epididymal tract. In the caput, 99% of spermatozoa have a proximal cytoplasmic droplet while only 1% of spermatozoa in the corpus have a proximal cytoplasmic droplet. On the other hand, the percentage of immature spermatozoa with a distal cytoplasmic droplet is 0.5% in the caput, 81% in the corpus and 18% in the cauda. Malformations in epididymal spermatozoa were also apparent and can be divided into three main types. These are a folded tail that begins at the Jensen's ring, which develops in the cauda due to the failure of the expulsion of the distal cytoplasmic droplet; the coiling of the tail which originates in the corpus; and the fusion of tails which also develops in the corpus (Briz *et al.*, 1996). Other malformations include bicephalic spermatozoa, anomalous cephalic form, macrocephalic spermatozoa and microcephalic spermatozoa.

Caput spermatozoa are also characterised by a mitochondrial sheath that is composed of larger mitochondria of unequal sizes and low electron-dense matrices. The opposite is true for those spermatozoa from the cauda. Caput spermatozoa are also characterised by the high level of acrosomal protuberance and the mid-piece being highly flexible. Spermatozoa with detached heads as well as the tendency to agglutinate also increased in the cauda.

2.5 Histological Features of the Boar Epididymis

The interaction of spermatozoa with the epididymal milieu is an essential event in the life of spermatozoa as this leads to the over-all structural changes to spermatozoa which subsequently affect sperm functions. Thus the histological make-up of the epididymal epithelium is one important factor to consider in the characterisation of the physiological mechanisms within the epididymal duct. Moreover, a good understanding of epididymal histology would help direct a focus in research specific to regions where the epididymal epithelium brings specific changes to the spermatozoon and in this way facilitate an understanding of the molecular aspects of sperm maturation (Amann *et al.*, 1993).

Briz *et al*. (1993) and Stoffel and Friess (1994) have undertaken an intensive morphological study on the ductus epididymidis of the boar (*Sus domesticus*) hence, the following discussion is largely based on their work. As with other animal species, the epididymis of the boar is divided into three main regions: the head (caput); a narrower body (corpus or isthmus); and the tail (cauda). It can also be further subdivided into regions based on morphological characteristics: the caput comprising of the medial limb (initial segment); the bend (proximal caput) and lateral limb (distal caput); the corpus; and the proximal and distal cauda (Stoffel and Friess, 1994).

In general, the whole epididymal duct of the boar appears to be histologically similar being lined with a pseudostratified epithelium with stereocilia and covered with a muscular connective sheath, although variations in the duct diameter, epithelial height, and length of stereocilia occur among the regions (Briz *et al.*, 1993). A similar description was given by Bassols *et al*., (2004) for epididymal epithelial cells

cultured *in vitro*. Cultured epididymal epithelial cells first appeared as a columnar or cuboidal shape but gradually changed to a flattened, polygonal shape (Bassols *et al.*, 2004).

The epithelium in the caput region of the epididymis has the greatest height and becomes progressively smaller in the corpus and cauda. The total membrane surface of the stereocilia is also greater in the caput as well as in the corpus while stereocilia in the caudal region become less abundant and are rather short. The cell height within the initial segment ranges from 40 μm and 60 μm, which gives the lumen a characteristic stellate appearance. The lumen of the proximal caput through to the distal cauda is spherical, and the epithelial height decreases from approximately 85 μm in the proximal caput and about 35 μm in the distal cauda. The distance from one basement membrane to another varies in between regions. The initial segment is between 450 μm and 550 μm, the proximal caput is from 425-460 μm and the distal caput is about 350 μm increasing rapidly from 500 μm in the distal corpus to 700 μm and over 1mm in the proximal and distal cauda, respectively (Stoffel and Friess, 1994).

The blood to the caput and corpus epididymidis is supplied by two branches arising from the testicular artery while the majority of the cauda receives blood from the deferential artery (Stoffel and Friess, 1994). After encircling the testis, the testicular artery joins the epididymal branch commencing at the caput epididymidis. The close association of the epididymal branches with the pampiniform plexus is also worth noting here. Anastomoses can be observed at the level of the vascular cone and drainage is accomplished by the epididymal veins (Stoffel and Friess, 1994).
The peritubular smooth muscle is comprised of seven to ten concentric layers, which extend to the distal corpus. In the proximal cauda, a considerable gradual increase (two to three fold) in the thickness of the periductal muscle can be observed (Jones, 1999). An abrupt increase by the same magnitude is also found in the distal cauda. The boar epididymal tubule is also surrounded and supported by a larger amount of connective tissue than the efferent ductules (Bassols *et al.*, 2004). This is an important point to consider when obtaining epididymal epithelium for *in vitro* culture.

Vascularization is more intense in the caput and corpus while the cauda contains a much thicker muscular-connective tissue sheath. The cavity of the cauda is also noted for the more frequent appearance of body cells, described as spherical in shape with a single nucleus, measuring about 6 μm in diameter and with scarce, granular cytoplasm (Briz *et al.*, 1993).

2.6 Ultrastructural Characteristics of the Boar Epididymis

There are five major types of epithelial cells that are found throughout the three regions of the epididymal duct: principal, basal, clear, narrow, and basophilic cells (Briz *et al.*, 1993). The boar epididymis also has another group called mitochondria-rich cells (Stoffel and Friess, 1994). Macrophages and wandering leucocytes are also present within the connective tissue that surrounds the epididymal duct (Setchell *et al.*, 1994).

The principal cells comprise the majority of the epithelial cells and occupy about 70-90% of the epididymal epithelium in most species (Amann *et al.*, 1993). They are columnar with a slightly basophilic cytoplasm, stereocilia, and a pale oval nucleus containing one or two prominent nucleoli. They extend from the basal membrane where they adhere by means of hemidesmosomes up to the free surface of the epithelium. The principal cells also contain abundant secretory granules as well as mitochondria and cisternae of endoplasmic reticulum lying in the supranuclear cytoplasm.

The pyramidal basal cells with a diameter of about 6.5 μm and a maximum height of 5.5 μm are distributed along the length of the epididymal duct. They are attached by hemidesmosomes to the basal membrane. The nucleus is sub-spherical in shape with deep infoldings, and the cytoplasm contains scattered mitochondria and cisternae of rough endoplasmic reticulum.

The clear cells are slightly vacuolated cells distributed amongst the principal cells, bearing basal outfoldings that contact the basal membrane. Their nucleus is medially located and it bears a deep infolding, which progressively define asymmetrical nuclear lobes. The perinuclear cytoplasm is highly vacuolated and contains abundant

spherical mitochondria and some secretion granules which have a well defined electron-dense matrix.

A small number of narrow cells are also present arranged along with the other epithelial cells. The nucleus is bilobed and elongated, enclosing a dense granular euchromatin and a cytoplasm, which is highly vacuolated. The spheroidal basophilic cells, which are located at different levels between principal cells, are characteristically unique exhibiting high nucleoplasmic difference in staining. They have a rounded nucleus and a cytoplasm containing sparse numbers of mitochondria, some cisternae of rough endoplasmic reticulum, and also some lysosomes of the primary and secondary type.

The mitochondria-rich cells are sporadically located between principal cells in the initial segment and the proximal caput (Stoffel and Friess, 1994). They have a broad apex, which expands over the neighbouring cells and a basally projecting slender stem. As the name implies, the most peculiar characteristic of these cells is the presence of numerous mitochondria.

The epididymal duct sheath is composed of muscular-connective tissue, consisting of two layers of smooth muscle tissue: the thick internal or longitudinal layer and a thinner external or circular layer. As the region of the epididymal canal becomes more distal, the intrinsic connective tissue sheath becomes more fibrous. In addition, the cauda region contains a much thicker muscular connective tissue sheath. Tight junctions are also commonly observed between the smooth muscle cells. The cauda is also characterised by bundles of collagen fibres intruding between myoblasts and thus indentations in the contour of the cells can be observed (Briz *et al.*, 1993).

In culture, the three regions of the epididymis show similar ultrastructural characteristics when examined by electron microscopy (Bassols *et al.*, 2004). Moreover, cultured boar epididymal epithelial cells have a tight adherence to each other through interdigitations, desmosomes, and tight junctions located on their lateral and basal membranes, with some apparent intercellular spaces present. The nuclei are oval with prominent nucleoli. The cytoplasm contains numerous mitochondria, extensive rough endoplasmic reticulum and a well-developed Golgi

apparatus. Moreover, multivesicular bodies, residual bodies, lipid droplets, dense granules and bundles of filaments (10 nm) are also present.

The features of cultured boar epididymal epithelium are similar to the epithelium *in situ* (Bassols *et al.*, 2004). These include the cell polarity, epithelial integrity, and absorptive and secretory activity. The numerous stereocilia frequently found on the apical surface of the cells is evidence of this characteristic polarity. The epithelial integrity is achieved and maintained by interdigitations and junctional complexes in the lateral and basal membranes. The absorptive and secretory activities of boar epididymal cells *in vitro* was also demonstrated by the presence of numerous pinocytic vesicles in the apical cytoplasm, multivesicular bodies, residual bodies, an extensive rough endoplasmic reticulum, a well-developed Golgi apparatus, abundant ribosomes and numerous other vesicles in perinuclear regions.

2.7 Physiological Correlations

Much has yet to be understood about the specific functions of the cells comprising the epididymal epithelium. Briz *et al*. (1993) suggested that the basal, principal, clear, and narrow cells could be the four developmental stages of the same cell type. The proximal principal cells are known to specialize in fluid-phase and receptor-mediated endocytosis and secretory activity (Amann *et al.*, 1993). Renewal of principal cells is the basic function of basal cells. As basal cells mature to columnar cells with apical secretory granules, they become clear cells capable of apocrine function and finally undergo regression to become narrow cells. Basophilic cells have been suggested to be leukocytes which have migrated from the underlying connective tissue to the epididymal duct cavity (Briz *et al.*, 1993).

It should be noted that the absorptive activity is particularly prominent in the proximal and distal caput as determined by ultrastructural examination. This is largely due to the presence of extensive cisternae of rough endoplasmic reticulum and the well-developed Golgi apparatus both in the distal caput and corpus regions which participate in active protein synthesis and secretion (Stoffel and Friess, 1994). The abundant vascularization of the caput and the corpus is correlated with their

greater synthetic and secretory activity and possibly the transport of testicular androgens (Briz *et al.*, 1993).

The abundant muscular-connective tissue sheath of the cauda is related to the basic function of this region as a storage area and the intense contraction needed during ejaculation. The physiological mechanism correlated to this contraction is the presence of abundant collagen fibres and muscular components within the muscular sheath itself. More importantly, tight junctions present in large numbers between smooth muscle cells are responsible for facilitating the overall contraction of the caudal region of the epididymis (Briz *et al.*, 1993) and thus providing a forceful expulsion of spermatozoa during ejaculation.

A detailed study of the lymphatic drainage system of the epididymis has been done mainly in the rat with lymphatic sinusoids observed in intertubular spaces of the epididymal duct (reviewed by Setchell *et al.*, 1994). Most of the vessels come out from the caput with only one or two from the corpus and one from the cauda. These vessels join to the main testicular lymphatic trunk and together with the spermatic artery end up in the para-aortic group of lymph nodes.

2.8 The Epididymal Plasma

The epididymal plasma is composed of fluid coming from different sources. This includes the testes from the secretions of the Sertoli cells; the epididymal spermatozoa from the metabolism of phospholipids producing small amounts of epididymal glycerylphosphorylcholine; and the remainder as a direct result of the biosynthetic activity of the epididymal epithelium (Mann and Lutwak-Mann, 1981; Dacheux *et al.*, 2003). The epididymal plasma has a particularly high K^+/Na^+ ratio and has unique biochemical features due to the presence of a wide range of organic constituents including glycerylphosphorylcholine and carnitine, sialomucoproteins, lipoproteins, and various enzymes (Mann and Lutwak-Mann, 1981).

2.8.1 Epididymal secretory proteins

The epididymal duct provides suitable and appropriate secretory elements necessary for the maturation of the spermatozoa. Among the most important of these elements

are the epididymal secretory proteins (Dacheux *et al.*, 2003). They are secreted into the lumen apically, and make contact with, or are adsorbed onto the surface of spermatozoa (Kirchhoff, 1998). Other proteins including those secreted by Sertoli cells are reabsorbed in the caput. Epididymal proteins that are not secreted by the epididymal epithelium may come from the epididymal blood supply that transcytose to the epididymal lumen (Dacheux *et al.*, 2003).

Complete characterisation and identification of all the proteins secreted by the mammalian epididymal epithelium has not been fully accomplished. At present, only a small number have been named and identified and the number varies with species and the author (Dacheux *et al.*, 2003). To date, fewer than 20 proteins have been named in 15 species and more than one species secretes several (about 15) of these proteins.

Proteins are secreted in specific regions of the epididymis with the proximal region being the most active. In all the species studied, most of the epididymal proteins that have been identified include clusterin, glutathione peroxidase (GPX), lactoferrin, epididymal retinoic-acid-binding protein (E-RABP), osidases (hexosaminidase, mannosidase, and galactosidases), cholesterol transferase protein (CTP)/human epididymal proteins (HE1), and prostaglandin D2 synthase (PGDS) (Dacheux *et al.*, 1998a).

A similarity exists among these proteins with regard to changes in their luminal concentration. This includes a balance between secretion, reabsorption and degradation rates. It appears also that the similar components found in equivalent epididymal regions follow a pattern of secretion, which is similar in most animals (Dacheux *et al.*, 1998b). Secretory proteins of the epididymis are characteristically polymorphic both in molecular mass and isoelectric point (pI) (Dacheux *et al.*, 2003).

2.8.2 Epididymal secretory proteins of the boar and related compounds

Proteins present in the epididymal fluid can be grouped into unregionalized and regionalized proteins (Syntin *et al.*, 1996). Most of the unregionalized proteins are not secreted in the epididymal lumen but are probably secreted by the surrounding tubular tissues. There were no major differences observed in the extratubular

medium. On the other hand, electrophoretic studies revealed that those proteins present within the epididymal lumen were highly regionalized. Only a small number of proteins are secreted by all regions of the epididymis (Syntin *et al.*, 1996). The proximal part of the epididymis is the primary source of proteins. In the caput alone, at least a hundred protein compounds are secreted. In the boar, the secretory activity in the proximal region is about six to eight times more than the distal region (Dacheux *et al.*, 2003). This high synthetic activity is largely related to the secretion of the two compounds, "train" A (25-41 kDa, pI 5-6.6) and "train" F/G (40 kDa) which represent 34.5% and 31.4%, respectively of the total proteins secreted by the epididymis (Syntin *et al.*, 1996). The corpus secretes about 13 proteins, among which two trains have been identified, while the cauda secretes only two minor proteins comprising less than 0.25% of the total regionalized proteins (Syntin *et al.*, 1996). All together, 83%, 16%, and 1% of the overall protein secretion is secreted in the caput, corpus and cauda, respectively. A total of 146 epididymal proteins have been identified as secreted by the boar epididymis (Syntin *et al.*, 1996). There are at least five regions physiologically distinguishable throughout the epididymal duct with reference to the origin and intensity of protein secretion. These are the proximal caput, the middle caput, the proximal corpus, the distal corpus, and the cauda (Syntin *et al.*, 1996).

The regional differentiation in the secretory activity of the epididymal organ is progressively established as the animal matures (Syntin *et al.*, 1999). A number of mechanisms are associated with the maintenance of this regionalization in the adult boar. Of the 48% of all proteins secreted, 33.6% are dependent upon androgen stimulation while 14.4% are dependent upon androgen repression. Forty-seven percent are controlled by some other factor and about 5% were unregulated. Moreover, the secretion of at least half of the specific proteins in the proximal caput alone is influenced by the factors that are coming from the testes (Syntin *et al.*, 1999).

Using N-terminal amino microsequencing, clusterin, glutathione peroxidase, retinol-binding protein, lactoferrin, beta-N-acetyl-hexosaminidase, alpha-mannosidase, and procathepsin L have been identified as major proteins secreted by the porcine epididymis (Syntin *et al.*, 1999). About 70% of the secretory activity of the

epididymis of the boar is particularly related to the production of clusterin, lactoferrin, glutathione peroxidase, and prostaglandin D2 synthase (Dacheux *et al.*, 2003).

Clusterin, a cystine- rich secretory protein (CRISP) is considered to be one of the most intensively secreted proteins consisting of about 25-30 % of the total secretion in several species (Dacheux *et al.*, 2003). This is referred to as train F/G in the epididymis and T2 in the testis (Syntin *et al.*, 1996). This protein is only found in the proximal epididymis of the boar, close to the secretion site. The proportions of other proteins seem to be species dependent.

Glutathione peroxidase, which represents train B, is secreted in the caput region. It comprises about 1.5% of the total production of epididymal proteins and it is partially reabsorbed as it passes through the epididymis accounting for about 2.8% of the total proteins in the cauda (Syntin *et al.*, 1996). However, the concentration of glutathione peroxidase in the porcine epididymis is considerably lower compared to the optimal concentration of the purified protein (Okamura *et al.*, 1997). Thus, glutathione peroxidase is considered to be enzymatically quiescent in the boar epididymis in terms of its functional activity. Glutathione peroxidase protects spermatozoa from untimely acrosome reaction and helps to maintain the fertilising ability of spermatozoa in the epididymis (Okamura *et al.*, 1997).

Retinoic acid-binding protein (train N) is secreted by the corpus and throughout the posterior epididymis. The secreted isoforms however tend to become more basic in the cauda (Syntin *et al.*, 1996).

A porcine homolog of the major protein human epididymal protein (HE1) has been purified from cauda epididymal fluid, but the HE1 homolog mRNA has only been detected in the caput and corpus epididymidis (Okamura *et al.*, 1999). The HE1 homolog is secreted into the epididymal fluid as a 19-kDa glycoprotein. Subsequently its sugar moiety is gradually processed to form a 16-kDa protein as it passes through the epididymis. The HE1 homolog is capable of binding cholesterol, suggesting a major involvement in the lipid composition of the sperm membrane during maturation in the epididymis (Okamura *et al.*, 1999). Furthermore, this

porcine homolog was related to the other human epididymal proteins HE3, HE4, HE5, and HE12 (Schafer *et al.*, 2003).

The occurrence of a 40kDa protein in the fluid of the distal caput epididymidis of the boar has been known for quite a long time. Okamura *et al*. (1995) identified this protein as procathepsin L. Northern blot analysis has demonstrated the abundance of this protein in the distal caput. Procathepsin activity was demonstrated only in the distal caput and it was absent in proximal and mid-caput. However, in the mid-caput preceding the site of procathepsin L secretion, the mRNA for prostaglandin D2 synthase was found to be very high. The mRNA of prostaglandin D2 synthase enhances cathepsin L expression in cultured cells thus suggesting a possible regulatory mechanism by prostaglandin D2 synthase in the locally restricted expression and secretion of cathepsin L in the epididymis (Okamura *et al.*, 1995).

Lactoferrin is secreted by the distal portion of the epididymis in various animal species. In boars, lactoferrin is synthesized from the distal caput to the caudal region of the epididymis and is first secreted as a 75 kDa protein which becomes a 70 kDa glycoprotein upon reaching the cauda (Jin *et al.*, 1997). One of its primary actions is to remove free iron in fluids and inflamed areas, thus preventing free radical-mediated damage as well as transporting metals from invading microbial and neoplastic cells (reviewed by Dacheux *et al.*, 2003). It is also known as an important component of the non-specific immune system by participating in various anti-bacterial, anti-mycotic, anti-viral, anti-neoplastic and anti-inflammatory activities. In addition, lactoferrin might be also involved in preventing tail-to-tail agglutination of mature spermatozoa (Jin *et al.*, 1997).

A number of glycoconjugates have been discovered in the boar epididymal fluid using lectins that specifically recognised sugar residues. Stereocilia, the cellular apex and Golgi areas of the principal cells were the main cellular structures stained by lectins. Also, *N*-and *O*-glycoproteins were closely associated with the principal cells and the cytoplasm of principal cells in the three regions of the epididymal duct contained a granular distribution with *Galanthus nivalis*, a lectin that binds alpha 1, 3-mannose. A subpopulation of *Maackia amurensis*-positive epithelial cells whose morphology is similar to the principal cells is present. These are well distributed

throughout the whole epididymal duct and their function is thought to be related to the production of glycoproteins that contain the specific sequence neuraminic acid α2, 3 galactose ß1, 4-*N*-acetylglucosamine ß1 (Calvo *et al.*, 2000).

Furthermore, lectin staining was not only confined to the regions of the duct epithelium but also to spermatozoa along the epididymal duct (Calvo *et al.*, 2000). In the boar, spermatozoa from the caput epididymidis were weakly stained by *Helix pomatia* and *Glycine max* (both with affinity for terminal alpha-acetylgalactosamine) whereas an increase in staining was found in spermatozoa from the corpus and cauda. Moreover, staining with *Arachis hypogea* (binds to galactose beta 1, 3-*N*-acetylgalactosamine alpha 1) was observed in spermatozoa from the caput epididymidis but not on spermatozoa from the corpus and cauda. These findings all suggest that while glycan molecules are present along the epididymis, the specificity of spermatozoa to bind certain carbohydrate molecules depends upon their location in the epididymis.

Anti-agglutinin is one of the specific proteins secreted in the boar epididymis and inhibits sperm head-to-head agglutination. By using the biotin-streptavidin method, this protein was localized in the large Golgi apparatus of all the principal cells of the caput and corpus epididymidis and was closely associated with the luminal surfaces, particularly around and between stereocilia (Dacheux and Dacheux, 1988). The anti-agglutinin protein has been purified from the cauda epididymal plasma using precipitation with ammonium sulfate, anion-exchange chromatography, and reverse-phase HPLC (Harayama *et al.*, 1996). Characterization by electrophoretic and membrane blotting techniques have also confirmed that porcine anti-agglutinin contains sialic acid residues involved in the immunoreactivity and molecular heterogeneity of the protein (Harayama *et al.*, 1996).

2.8.3 Secretory enzymes in the epididymal fluid of the boar

The epididymal fluid contains several secretory enzymes that are involved in glycoprotein metabolism or protein activation. Two groups of enzymes which are secreted abundantly in different regions of the epididymis in many species are glycosidases and glycosyltransferases (Dacheux *et al.*, 2003). The glycosyltransferases include galactosyltransferase, glucosaminyltransferase,

fucosyltransferase, and sialyltransferase (Tulsiani *et al.*, 1998). These enzymes operate *in vivo* at their optimum pH level (6.6-7) although competition may occur with other enzymes such as pyrophosphatase and phosphatases, which may result in the reduction of their function with the epididymal luminal fluid (Tang, 1998). The most frequently occurring glycosidases in different species are mannosidases, beta hexosaminidase, and beta gluroronidase. In boars, at least two kinds of alpha-D-mannosidase (lysosomal type enzyme and 135 kDa alpha-D-mannosidase (MAN2B) have been identified (Jin *et al.*, 1999). The lysosomal type consists of 63 and 51 kDa subunits at equimolar amounts. It can cleave alpha1-2 linked mannosyl residues and less likely, 1-3 and alpha 1-6 linked mannosyl residues. The activity of the lysosomal type is greater than the MAN2B2 suggesting that the lysosomal type alpha-D-mannosidase is the predominant active enzyme in the porcine epididymis (Jin *et al.*, 1999).

Caput principal cells of the boar epididymis contain tubulovesicular structures characterised by sparsely granulated endoplasmic reticulum. These are poorly developed in the proximal caput but exist in large numbers in the apical cytoplasm of the distal caput principal cells. They have glucose-6-phosphatase activity but their main function has yet to be determined (Geissbuhler *et al.*, 1998). An enzyme, beta-D-galactosidase has been reported in the rat and is capable of digesting a glycoprotein *in vitro* (Tulsiani *et al.*, 1998). This suggests that there are enzymes in the epididymal duct which could be involved in modifications of the sperm surface glycoproteins during epididymal maturation.

Proteases and protease inhibitors are also present in the epididymal fluid and are involved in various physiological processes. In the boar, several proteases have been identified that are actively secreted by the epididymal epithelium, such as cathepsins (D, S and procathepsin L) and matrix metalloproteinases (MMP2 -3-9) (Dacheux *et al.*, 2003). By using a monodimensional and bidimensional zymography, Metayer *et al.*, (2002) demonstrated that the epididymal fluid of the boar contains gelatinases. Moreover, all gelatinases with molecular weights of greater than 54 kDa were considered to be metalloproteases. The cauda epididymidis of the boar specifically contains metalloprotease proteins of around 60 kDa (Metayer *et al.*, 2002). However, the exact actions of these enzymes have not yet been completely elucidated. They

might also be inhibited by a number of protease inhibitors which originate from the testes such as cystin C, alpha$_2$ – macroglobulin (Peloille *et al.*, 1997) and other tissue inhibitors (Metayer *et al.*, 2002).

2.9 Maturation of Boar Spermatozoa in the Epididymis

As spermatids differentiate to become mature spermatozoa, their capacity for biosynthetic activity decreases (reviewed by Amann *et al.*, 1993). Spermatozoa are therefore largely dependent upon exogenous molecules and ions and modify or degrade preformed substances such as glycoproteins, carbohydrates and lipids to provide energy (Bedford and Hoskins, 1990).

As spermatozoa are transported along the epididymis, induced changes occur in them, subject to the uniqueness of the luminal fluid, whose composition is controlled regionally by the secretions of the epididymal epithelium as well as the selective removal of water and some undesirable substances (Amann, 1987; Dacheux *et al.*, 1991; Turner, 1995). The many changes which occur to the spermatozoa during the epididymal transit include: modifications of sperm dimensions; increase in their susceptibility to cold shock; increased surface negative charge; reduced whole-cell isoelectric point; increase in disulfide cross linking, changes in lipid, proteins and antigenic composition; modifications in enzyme activity and alterations in lectin binding to the cell surface (reviewed by Brooks, 1983). A number of structural changes also occur in spermatozoa during this maturation process, including the alteration and migration of the cytoplasmic droplet, the reduction in the amount of the cytoplasm, alterations in the size, shape, and contents of organelles such as the acrosome and sperm membranes (reviewed by Mann and Lutwak-Mann, 1981). The over-all maturation changes that occur to spermatozoa during their maturation in the epididymis can be divided largely into two areas. These are modification of the DNA-protein complex and structural changes in the plasma membrane.

2.9.1 Modifications of the DNA-protein complex

Caput spermatozoa are incapable of pronuclear formation. A specific factor is activated or added to spermatozoa in the caput epididymidis to prevent this from occurring. Moreover, the nucleus of the spermatozoon becomes more resistant to

dissolution by sodium dodecyl sulfate (SDS) or SDS plus dithiothreitol, as spermatozoa are transported along the epididymal tract (Bedford and Hoskins, 1990). This increase in resistance may be the result of increased disulfide binding which leads to the stabilization of the chromatin within the nucleus. Interestingly, the cytoplasm of the oocyte has the only biological medium necessary to break down this DNA-nucleoprotein complex by inducing chromatin decondensation at fertilisation (Amann *et al.*, 1993).

2.9.2 Modifications of the plasma membrane

The plasma membrane of the spermatozoon is uniquely subdivided into well-defined regional domains that differ in composition, organization, and the corresponding functions. The diversity in the nature of the surface of the spermatozoan has been revealed in studies of surface charge, lectin binding to specific sugar moieties, freeze-fracture patterns, intramembranous particle distribution, membrane fluidity, lipid composition, protein composition, and antibody labeling (reviewed by Eddy and O'Brien, 1994). This variability in the composition and organization of the sperm plasma membrane suggests that the sperm surface is a mosaic of restricted surface domains which perform specialized functions.

Most sperm surface domains are likely to be established during spermiogenesis However, during maturation in the epididymis, spermatozoa undergo additional shape and surface changes acquiring the final form and composition of some surface domains. Domains on the middle piece and the acrosomal region are the ones mostly affected or influenced by epididymal maturation (Eddy and O'Brien, 1994). Remodelling of the plasma membrane is achieved by modification or realignment of the pre-existing membrane components, addition of new components to the membrane and removal or loss of membrane components (Jones, 1998).

Lipids of the cell membrane of spermatozoa are characterized by the following 1) the occurrence of large amounts (30-40%) of plasmalogens; 2) a high content of polyunsaturated fatty acids; mostly 20:4, 2:5; and 3) a relatively low cholesterol/ phospholipid ratio of about 0.26 to 0.45, depending upon the species (Jones, 1998). In porcine spermatozoa, phospholipids make up 70% of the total plasma membrane lipid followed by steroids with a cholesterol/phospholipid molar ratio of 0.12

(Nikolopoulo *et al.*, 1985). Free fatty acids constitute a comparatively small amount of the lipid, though diacylglycerol is present in about the same quantity as glycolipid (Nikolopoulo *et al.*, 1985). The phospholipid/protein ratio of the porcine sperm plasma membrane is estimated to be 0.68 on a weight basis. This amount however may vary in different regions/domains.

It has been well demonstrated in several studies with different species that during the transit of spermatozoa along the epididymis, there is a decrease in the total cellular content of phospholipids at a rate of between 25% and 48% (reviewed by Jones, 1998). Several studies also support the fact that the loss of specific classes of phospholipids from the maturing spermatozoa caused significant apparent changes to spermatozoa. The plasma membrane of caudal epididymal spermatozoa contains a different composition of lipid components to testicular spermatozoa (reviewed by Jones, 1998). In the boar, bull, ram, and rat spermatozoon, the whole sperm lipid is reduced during transit within the epididymal duct (reviewed by Eddy and O'Brien, 1994). While the amount of cholesterol is reduced in the boar spermatozoon, there are no significant changes in the cholesterol/phospholipid ratio (Evans *et al.*, 1980). There are also decreases in the amounts of phosphatidylethanolamine and phosphatidylinositol, an increase in dermosterol, cholesterol sulfate, phosphotidylcholine, phosphoinositides, fatty acids, and diacylglycerol although the degree of saturation of fatty acids remains the same (Evans *et al.*, 1980). Changes that occur to the plasma membrane of the spermatozoon as it undergoes maturation along the epididymis were also postulated to explain the greater sensitivity of ejaculated spermatozoa to the cold shock (reviewed by Eddy and O'Brien, 1994). Boar spermatozoa are very sensitive to the effects of cold shock (Watson, 1995).

In all species studied, specific proteins present on testicular spermatozoa are removed or modified during the transit along the epididymal duct (Dacheux *et al.*, 1998b). The uptake of glycoproteins from the epididymal secretion is an important pathway for membrane remodelling in the epididymis. As stated in section 2.8.2, a number of glycoproteins are synthesized and secreted in the specific regions of the epididymis and many bind to spermatozoa (Jones, 1998). Changes in glycoprotein and saccharide composition and to a lesser degree glycolipids, are considered to be the

most important factors for most modifications in sperm-surface charge and lectin-binding properties of spermatozoa during the epididymal maturation (Eddy and O'Brien, 1994).

Most changes to boar spermatozoa occur in the proximal region of the epididymis. For example, a 95 kDa protein is no longer detected when spermatozoa enter the proximal caput. Similarly, a 105-115 kDa testicular sperm protein was not detected until spermatozoa reach the distal caput. This illustrates certain processes existing specifically in the caput as well as the sensitivity of the sperm membrane protein to be processed by specific epididymal enzymes (Dacheux *et al.*, 1998a). Some proteins are strongly attached or interposed into the plasma membrane of spermatozoa (Dacheux *et al.*, 2003). An example of such proteins which are found in the head of rat and tail of the monkey spermatozoon belong to the CRISP family. This protein is strongly bound to the post-acrosomal and equatorial planes and may possibly be involved in binding to oocytes and in prolonging the viability of spermatozoa (Sivashnamugam *et al.*, 1999).

2.9.2.1 Immunity related modifications

The passage of spermatozoa along the epididymis also allows the development of surface characteristics, which enables prolonged survival of spermatozoa in the female reproductive tract (Amann *et al.*, 1993; Jones, 1999). The epididymis may also secrete some immunosuppressive proteins which the spermatozoon may carry into the female reproductive tract that could act to prevent or reduce its immunological recognition (reviewed by Amann *et al.*, 1993).

Modification or unmasking several surface proteins from spermatozoa during epididymal transit has also lead to the identification of several new antigens on the spermatozoal surface (Eddy and O'Brien, 1994; Dacheux *et al.*, 1998b). Consequently, the profile and distribution of protein antigens on the plasma membrane of spermatozoa changes continuously. Also, pre-existing surface components may be redistributed from multiple domains to a single domain or from one domain into another in the course of epididymal maturation (Eddy and O'Brien, 1994). These antigens may also form by adsorbing glycoproteins directly from the luminal secretion, processing by endoproteolysis of acquired or pre-existing

glycoproteins and the migration of proteins to new membrane domains (Dacheux *et al.*, 1998b). Along with this process is the spatial realignment of plasma membrane structures in order to gain the right position where they become functionally active (Jones, 1999). However, it is not yet clear whether these molecules have a significant participation in the fertilisation process or have other functions related to sperm survival in the cauda epididymidis, the acquisition of motility or capacitation in the female reproductive tract (Jones, 1998).

2.9.2.2 Modifications in the mitochondria, microtubular elements and the acrosome

The mitochondria and microtubular elements of the tail of the spermatozoon are also modified in the epididymis (Amann *et al.*, 1993). This is to generate energy for progressive motility once spermatozoa are ejaculated.

The plasma and mitochondrial membranes of an intact spermatozoon are modified allowing changes of the mechanisms of the flux of ions, substrates and metabolites relative to ATP production, and eventual energy transduction into contraction of dynein arms and sliding axonemal fibres. The plasma membrane that covers the tail is modified in the corpus epididymidis. This change leads to a unique resistance to hypo-osmotic stress and a transitory increase in permeability to small particles. This helps in the transmembrane movements of molecules (i.e. substrates) and facilitates adjustment of the internal environment (reviewed by Amann *et al.*, 1993). Membrane remodelling also allows greater potential in the utilisation of glycosylable substrates, the response to dibutyryl cAMP for caffeine and the subsequent production potential for ATP (Bedford and Hoskins, 1990). Caffeine as well as bicarbonate are not potent stimulators of Ca^{2+} uptake in boar spermatozoa (Okamura *et al.*, 1992). Rather, the uptake of calcium largely depends upon the presence of substrates for respiration particularly glucose and pyruvate. This means that high energy levels and mitochondrial functions greatly affect the uptake of calcium ions by porcine spermatozoa. Caffeine and bicarbonate activate many different sperm functions by increasing the concentration of cAMP. An increase in the net activity of Ca^{2+} as it accumulates into the spermatozoon has also been observed during maturation in the epididymis (Okamura *et al.*, 1992).

By using freeze-fracture techniques, porcine spermatozoa from the distal cauda demonstrated geometric arrays of intra-membranous particles in the anterior border of the acrosome (Eddy and O'Brien, 1994). A distinct hexagonal array has been observed to develop as spermatozoa reach the cauda epididymidis. It begins at the margin of the acrosome and then subsequently extends to the post-acrosomal region (Suzuki, 1981). Filipin incorporation in the plasma membrane increases in the principal and equatorial segments as boar spermatozoa mature within the epididymis (Seki *et al.*, 1992).

2.9.2.3 Zona-binding proteins

Proteins that bind to the zona pellucida are present in the acrosomes of porcine spermatozoa from all regions the epididymis. However, zona binding sites are most common on the surface of spermatozoa that come from the corpus and cauda epididymidis, and only a small percentage from caput spermatozoa (Burkin and Miller, 2000). Consistently, testicular and caput spermatozoa are not capable of binding to oocytes (Jones, 1989). Zona binding proteins were concentrated over the acrosomal ridge of acrosome-intact boar spermatozoa.

2.9.3 Concentration of spermatozoa during the epididymal transit

Sperm concentration is also a significant event in the life of spermatozoa during epididymal passage (Mann and Lutwak-Mann, 1981). The density of spermatozoa can be increased to as high as 10^{10}/ml (Dacheux *et al.*, 2003) when excessive fluids have been absorbed in the caput.

2.10 Protection of spermatozoa during storage in the cauda epididymidis

The metabolic rate of spermatozoa is regulated during their storage in cauda. This is important to minimize the production of reactive oxygen species that are potentially damaging to spermatozoa (Jones, 1999). This is where the role of the cauda epididymidis in suppressing the metabolic rate of spermatozoa is greatly appreciated. This condition can be reversed by as much as 3-5 times when spermatozoa are activated by dilution (Jones and Murdoch, 1996). Furthermore, the cauda has a wide lumen that also provides a considerable capacity for sperm storage and slows the transit of spermatozoa along the epididymis (Jones, 1999), thus minimizing energy expenditure.

2.11 The Oviduct

While the epididymis performs a crucial role in male fertility, the oviducts also perform functions that are essential for successful reproduction. The mammalian oviduct is not just a passive conduit between the uterus and the ovaries but is the primary organ involved in sperm capacitation, fertilisation and early development of the embryo (Hunter, 1998; Rodriguez-Martinez *et al.*, 2001). For many years, the function of the oviduct has been neglected but it was only by about the mid-1980's when the beneficial effect of oviductal cells was demonstrated in the *in vitro* development of sheep embryos (Gandolfi and Moor, 1987) and with a similar report in humans (Bongso *et al.*, 1992) that the physiological significance of the oviduct was appreciated and that more research studies about this organ begun.

2.11.1 Structural anatomy and histology of the oviduct

The mammalian oviducts or uterine tubes are two bilateral, tortuous structures which serve anatomically as conduits between the ovaries and the uterine horns (Priedkalns, 1981). Like the epididymis, the oviduct can be divided into three segments (Banks, 1981). They are the infundibulum, a large funnel-shaped region; the thin-walled ampulla which is the middle segment and the isthmus, a narrow muscular portion which joins the uterine horns. Fertilisation occurs in between the isthmus and ampulla. Functionally, the infundibulum with its a finger-like fimbriae serves to secure the released ova during ovulation and together with contractions in the myosalpinx, aid in the movement of gametes and embryos (Hunter, 1998). The cranial part of the oviduct is densely packed with kinocilia that aid the migration of ova by beating toward the uterus thereby directing the current of luminal fluid (Banks, 1981; Priedkalns, 1981). Depending upon the species, the embryo requires about 2.5 days to traverse the isthmus with the aid of peristaltic muscular contractions and the beating of the cilia. The isthmus serves to trap and prepare spermatozoa necessary for different sperm functions such as the capacitation and hyperactivation which are important for the successful fertilisation.

The mucosa of most mammalian oviducts is composed of a simple columnar epithelium with motile cilia in most of the cells (Priedkalns, 1981). Pseudostratified columnar epithelium however do appear in ruminants and pigs (Dellmann, 1971). Two types of cells are apparent in the mucosa. They are the ciliated cells which serve

to transport ova and the secretory cells which provide nutritive elements for the embryo (Dellmann, 1971).

The oviductal mucosa is continuous with the submucosa as there is no lamina muscularis (Dellmann, 1971; Priedkalns, 1981). The ampullary region in the cow is characterized by numerous longitudinal folds each with secondary and tertiary folds. The height of the folds gradually decrease towards the end of the tube and only a few primary folds can be seen with no secondary and tertiary folds in the utero-tubal junction (Dellmann, 1971; Priedkalns, 1981). The tunica muscularis of the infundibulum and ampulla is thin and is comprised primarily of an inner circular smooth muscle layer and with only a few longitudinal muscles. The isthmus on the other hand has the best developed tunica muscularis consisting of an inner longitudinal, middle circular and outer longitudinal muscle layers (Banks, 1981) which blends with the circular muscle layer of the uterus (Priedkalns, 1981).

Physiological changes in the activity of ciliated and secretory cells occur during the ovarian cycle. These changes are thought to be influenced by steroids such as oestrogen and progesterone. Oestrogen treatment causes hyperplasia, maturation and increase in the height of non-ciliated secretory cells. In the cow, ciliated cells become prominent during oestrus while the opposite is true during the luteal phase (Priedkalns, 1981). The percentage of ciliated epithelial cells in the porcine isthmus does not change significantly throughout the oestrous cycle (Walter and Bavdek, 1997). While ciliated cells are prominent in all regions during the follicular phase, they are less in the ampulla and a significant decrease occurs in the fimbriae during the luteal phase. Many cytoplasmic protrusions are present in the non-ciliated cells of ampullary and fimbrial epithelium during the luteal phase but not in the isthmus.

The pattern of pseudo-stratification of the oviduct epithelium of the sow is similar in the ampulla and the infundibulum being high columnar with two-three nuclei levels during oestrus. The opposite occurs during dioestrus and anoestrus (one-nucleus level). The epithelial cells in the isthmus are mostly low columnar with a more consistent pattern in all stages and only a small degree of increasing epithelial height during pro-oestrus. During the follicular phase, ciliated cells are more prominent and dense while the opposite is true during the luteal phase, in which bulbous processes

of the secretory cells tended to hide the appearance of cilia. The mitotic activity is greatest in the infundibulum during pro-oestrus. Moreover, while the appearance of secretory granules tends to be different among regions, the isthmus has the least degree of change as compared to the ampulla. Secretory granules mostly appeared during oestrus (Abe and Oikawa, 1992; Jiwakanon *et al.*, 2005).

2.11.2 Blood supply to the oviduct

The blood supply to the oviducts is derived from the utero-ovarian artery (Dellmann, 1971). Capillary networks of lymph vessels can be seen in the mucosal and serosal layers and drain into the lumbar lymph nodes (Priedkalns, 1981). The lymphatic vessels in the utero-tubal junction reabsorb part of the seminal plasma especially in species where semen is deposited in the uterus (Hunter, 1998). Both myelinated and non-myelinated nerve fibers derived from the sympathetic system are present along with the blood vessels (Dellmann, 1971; Priedkalns, 1981). Many arterioles, venules and small veins are present in the ampulla and infundibulum (Jiwakanon *et al.*, 2005). Moreover, the lower portion of the isthmus is supplied with blood rich in ovarian hormones including androstenedione, oestradiol, progesterone and prostaglandin F_2 (Hunter *et al.*, 1983). This is attributed to the countercurrent exchange between the venous drainage of the ovary and the arterial supply to the oviduct. Because of this, the isthmus is very responsive to changes in concentrations of ovarian hormones.

2.12 The Oviduct Luminal Fluid

The oviductal fluid consists both of a transudate from the blood plasma and the different compounds that are synthesised by the oviductal epithelium which largely originates from the ampulla (Nancarrow and Hill, 1994). It is composed mainly of electrolytes, energy substrates, vitamins, lipids, growth factors, amino acids and proteins. Fluid secretion by the oviductal epithelium is influenced by stage of the oestrous cycle. Most of the secretory activities occur when gametes and/or embryos are present within the oviduct or just after the animal has ovulated (Hunter, 1998). In the sheep, maximum secretion occurs just before ovulation, about 48 hours after an oestrogen surge (Sutton *et al.*, 1984). Regional differences in the chemical composition of the oviductal fluid are apparent (David *et al.*, 1969; Roblero *et al.*,

1976). Studies in pigs have demonstrated the regulatory role of the different segments of the oviduct especially the infundibulum and the ampulla and the stage of the oestrous cycle on the synthesis and secretion of oviductal proteins (Buhi *et al.*, 1992; Buhi *et al.*, 1997). The presence and amount of hormones, ions and other secretions in the oviductal fluid are greatly influenced by stages of the oestrous cycle (Nichol *et al.*, 1992). In the oestrous rabbit, oviductal fluid forms at the rate of 16.8 ul/h which is equivalent to about 400 ul/day (Leese and Gray, 1985).

Concentrations of phospholipids, cholesterol and protein in the oviduct fluid of the cow have been described for both the non-luteal (serum progesterone ≤ 1.5 ng/ml) and the luteal (serum progesterone > 1.5 ng/ml) phases of the cycle (Killian *et al.*, 1989). The volume and osmolality of the fluid present within the lumen tends to be higher during the non-luteal than the luteal phase. Daily concentration of protein, cholesterol and phospholipid are also higher during the non-luteal phase, although phospholipids comprised a much higher percentage. Phosphatidycholine and lysophosphatidylcholine comprised 40% of the phospholipids while phosphatidylinositol and lysophophatidylinositol were 20%. The ratios of cholesterol and phospholipid and of cholesterol and protein increase and decrease respectively at the non-luteal phase. There is also a rise in the amount of high-density lipoprotein in the oviductal fluid of the cow during the follicular stage which can serve as acceptor for sperm cholesterol (Ehrenwald *et al.*, 1990). The changing values of these molecules with respect to the stage of the oestrous cycle suggests significant implications for the function of spermatozoa lodged in the oviduct for a time in preparation for fertilisation. In fact, the cholesterol concentration of spermatozoa must decrease as a requirement for capacitation of spermatozoa in the oviduct (Davis, 1981).

The luminal fluid from different segments of the oviduct at different stages of the cycle influences sperm-egg binding and fertilisation *in vitro*. Way *et al*. (1997) demonstrated an improved fertilising potential of gametes co-incubated with secretions from the isthmus and the ampulla. The motility of spermatozoa was also affected when co-incubated with the luminal fluid of the isthmus irrespective of the stage of the oestrous cycle (Grippo *et al.*, 1992). A decrease in motility from about 71.7% to 34.0% occurred after incubation. Moreover, there was also an increase in

both the viability and the ability to fertilise bovine oocytes when spermatozoa were incubated with non-luteal ampullary fluid (Grippo *et al.*, 1992).

Secretory cells lining the oviductal lumen synthesise and secrete high molecular weight glycoproteins in response to oestrogen. Early experiments by Oliphant *et al*. (1984) found an oestrogen-dependent protein (OGP) synthesized *de novo* by non-ciliated cells. This protein belongs to a chitinase-like family of proteins but lacks the chitinase enzyme activity. Three major isoforms of OGP [(p)OGP-E1, E2 and E3] have been identified in the porcine oviduct. Oestrogen-dependent proteins bind to the zona pellucida, peri-vitteline space and the plasma membrane of ovulated oocytes and oviductal embryos (Suzuki *et al.*, 1995). These findings imply a significant role of the oestrogen-dependent glycoprotein in the fertilisation and development of the embryo.

The bovine ampulla synthesises significantly more OGP than the other regions of the oviduct with the greatest amount being secreted at oestrus (Wagner and Killian, 1992). The protein is 95 kDa and associates with bovine oocytes along with other fluid proteins with apparent molecular masses of 80, 74, 60, 45 and 30 kDa (Staros and Killian, 1998). In pigs, OGP is able to increase the percentage of normal embryos by reducing the rate of polyspermy. This is because it binds to the zona pellucida and hinders further binding by other spermatozoa as well as modifying binding sites on the zona pellucida (McCauley *et al.*, 2003).

Oviductal fluid proteins of apparent molecular weights of 97, 75, 55, 48, 34, 28, and 24 kDa are closely associated with the plasma membrane of bovine spermatozoa (Rodriguez and Killian, 1998). It was thought that fluid proteins specific to the isthmus may differ functionally than proteins in the ampulla. Isthmic fluid proteins are likely to be involved in the regulation of sperm capacitation and sperm motility while ampullary fluid proteins may influence the final preparatory steps of fertilisation and the acrosome reaction (Rodriguez and Killian, 1998).

The concentration of calcium is significantly greater in the isthmus than in the ampulla especially during oestrus (Grippo *et al.*, 1992). The concentration of magnesium also differs between stages of the oestrous cycle and is lower than the

plasma concentration. The potassium concentration is much higher in the oviductal fluid than in serum. This indicates that the non-luteal oviductal fluid possesses considerable characteristics in terms of sustaining the viability and the capacity of spermatozoa to penetrate oocytes.

2.13 Binding of Spermatozoa to the Oviduct

Placental mammals deposit semen either into the vagina, cervix or uterus (Yanagimachi, 1994). The stallion deposits directly into the uterus, the boar in the cervix and uterus, and in other species such as ruminants, cats, dogs, lagomorphs and humans, deposit their semen into the vagina. Semen ejaculated by males of different species differs significantly in terms of volume and concentration of spermatozoa. Millions of spermatozoa are deposited into the female reproductive tract upon ejaculation but only a few thousand are able to pass through the utero-tubal junction and reach the oviductal isthmus (Suarez *et al.*, 1997).

Upon reaching the oviduct, spermatozoa do not ascend directly to the site of fertilisation. In many species, most spermatozoa are trapped in the caudal isthmus being bound to ciliated epithelial cells forming what is called the sperm reservoir (Hunter, 1981; Suarez *et al.*, 1991; Yanagimachi, 1994; Topfer-Petersen, 1999; Hunter, 2002; Suarez, 2002b). The sperm reservoir has been studied in sheep (Hunter and Nichol, 1993); rat (Cortes *et al.*, 2004); hamsters (Smith and Yanagimachi, 1991); cattle (Lefebvre *et al.*, 1995) and in pigs (Hunter, 1981).

The sperm reservoir is believed to have three major functions. Firstly, it prolongs the viability of spermatozoa in relation to the onset of ovulation. Secondly, it controls the timing of the capacitation of spermatozoa. Thirdly, it regulates the transport of spermatozoa to the ampulla while preventing polyspermic fertilisation (Topfer-Petersen *et al.*, 2002).

2.13.1 The role of the sperm reservoir

2.13.1.1 Sperm motility and viability

A critical need for the maintenance of sperm viability becomes paramount especially if the female is inseminated several hours before ovulation. Hamster spermatozoa are

able to maintain their viability as determined by flagellar movement for a prolonged period when they are bound to the mucosal surface and epithelial crypts of the isthmus (Smith and Yanagimachi, 1990). Similar results were also found in cattle (Pollard *et al.*, 1991) and the rabbit (Smith and Nothnick, 1997). Bovine spermatozoa bound to the apical surface of the oviductal epithelial cells *in vitro* maintained their motility for 48 hours and fertilising capacity for up to 30 hours. Rabbit spermatozoa co-incubated with apical membrane vesicles from peri-ovulatory oviductal tissue were also able to maintain their viability for 48 hours whereas the motility of spermatozoa co-incubated with apical membrane vesicles from postovulatory and anovulatory oviduct or kidney tissues declined after 12 hours.

Hormonal regulation of the viability of spermatozoa bound to oviduct cells was observed by Lapointe *et al*. (1995) and granulosa cells and oviductal cells in culture could possibly impart 'nondialyzable, heat-labile factors' that are capable of promoting or maintaining the motility of spermatozoa (Ijaz *et al.*, 1994). Furthermore, the motility of bovine spermatozoa was maintained longer when incubated with oviduct epithelial cell monolayers (OECM) from the isthmus than with OECM from the ampulla (Chian and Sirard, 1995). Of 13 major proteins in the oviduct apical membrane vesicles, three were most abundant just before ovulation. While the exact functions of these proteins have not been identified, they might possibly assist in the transport of molecules in membrane vesicles and promote sperm viability.

Similar studies have been done in the pig by Suarez *et al*., (1991), Fazeli *et al*., (2003), Petrunkina *et al*., (2004) and Romar *et al*., (2005). Suarez and colleagues demonstrated the capacity of intact porcine oviduct epithelium to maintain the motility of spermatozoa for at least 24 hours of incubation. The same result was obtained by Fazeli *et al*., (2003) with spermatozoa incubated with apical plasma membranes prepared from isthmic and ampullary tissues regardless of the stage of the oestrous cycle. A significantly lower percentage of spermatozoa remained viable after incubation for 24 hours in the Tyrode's medium than after incubation with apical plasma membrane preparations from the follicular isthmus (31%± 9 and 60%±11, respectively) (Petrunkina *et al.*, 2004).

2.13.1.2 Fertilising capacity of spermatozoa that bind to oviductal tissues

A thorough investigation on the effect of oviductal epithelial cells on the fertilisation of pig oocytes was made by Kano *et al*. (1994). When spermatozoa were incubated at a concentration of 1 x 10^5 cells/ml, the penetration rates were significantly higher in the presence of oviductal epithelial cells. Similar results were found by Romar *et al*., (2005). Furthermore, bovine spermatozoa incubated with OECM conditioned medium had a 93% penetration rate compared to 67% with fresh modified-TALP medium (Chian and Sirard, 1995). Similar results were also found with frozen-thawed bovine spermatozoa incubated with polarized endosalpingeal epithelial cells (Pollard *et al.*, 1991) and in human spermatozoa against zona-free hamster oocytes incubated with bovine oviductal epithelial cells (OEC) (90% penetration rate), macaque OEC (85%) and human OEC (73%) (Ellington *et al.*, 1998). Inclusion of an oviduct-specific, oestrus-associated glycoprotein (EAP) (85-95 kDa) glycoprotein secreted by the oviductal epithelium during co-culture also improved the fertilising capacity of bovine spermatozoa (King *et al.*, 1994).

2.13.1.3 Control of sperm capacitation

Sperm capacitation is the final maturational step that spermatozoa undergo whereby many different biochemical changes occur to the plasma membrane subsequently allowing the acrosome reaction to occur upon binding to the zona pellucida. The regulation of capacitation is important in order for it to happen at the right time relative to the viability of spermatozoa. If spermatozoa capacitate earlier than ovulation, their plasma membrane would soon become delicate and their energy reserves become depleted. The regulation of this complex sequence of cellular and membrane changes *in vivo* is one of the primary roles of the isthmus (Petrunkina *et al.*, 2001a; Petrunkina *et al.*, 2003).

Among the preliminary events during the capacitation process is the increase of the intracellular calcium, bicarbonate, and hydrogen peroxide. These collectively activate adenyl cyclase to produce cyclic AMP, which in turn activates protein kinase A to phosphorylate certain proteins (Breitbart and Naor, 1999). In the pig, sperm capacitation is associated with tyrosine phosphorylation and tyrosine-like activity (Tardif *et al.*, 2001). Phosphorylation of canine spermatozoa was significantly lower when they are bound to either canine or porcine oviductal cells (Petrunkina *et al.*,

2003). In addition, certain functional changes such as calcium concentration were also inhibited in spermatozoa when they were bound to the oviduct epithelium. Motile equine spermatozoa bound to oviductal epithelial cells from the isthmic epithelium had a significantly lower calcium concentration than unbound spermatozoa (Dobrinski *et al.*, 1997) suggesting the ability of sperm-oviduct interaction to maintain a lower calcium concentration and delay the occurrence of capacitation.

The capacitation of spermatozoa can also be influenced by the region of the oviduct. Conditioned medium prepared from the oestrual isthmus capacitated significantly more spermatozoa than from the oestrual ampulla (Anderson and Killian, 1994). This may be due to the higher content of glycosaminoglycans in the oestrual isthmus than in the ampulla.

As previously mentioned, the stability of the plasma membrane is important prior to ovulation. Capacitated spermatozoa are unable to bind to oviduct explants from cattle (Lefebvre and Suarez, 1996) and horses (Dobrinski *et al.*, 1997). Tienthai *et al*., (2004) demonstrated the capacity of the oviduct to preserve the stability of the plasma membrane of spermatozoa about 8-10 hours before ovulation. These results suggest that the oviducts are able to select functionally competent spermatozoa and prolong their life span by controlling the onset of capacitation.

Interesting results have been reported by Fazeli *et al*. (1999). Boar spermatozoa incubated with oviduct explants for an hour become capacitated. It is not known how this temporal change of events occurred but it was suggested that the oviduct may promote capacitation after a certain period of time and this degree of control might be beneficial especially when ovulation becomes eminent.

Controversy exists about whether capacitation is completed in the sperm reservoir or further along the oviduct near the site of fertilisation. As demonstrated in many *in vitro* studies, only capacitated spermatozoa are released from the sperm reservoir probably in association with the subsequent hyperactivation (Ho and Suarez, 2001). In this regard, Hunter (1998) argued that the distance (6-8 cm) and the distinct fluid composition (Nichol *et al.*, 1992) on the way towards the site of fertilisation were

also significant factors that could influence capacitation. In addition, only a small percentage of spermatozoa are able to capacitate completely at any given point of time (Lynham and Harrison, 1998). These reports confirm the importance of capacitation occurring just before ovulation to maximize the fertilising potential of spermatozoa by preserving their energy reserves.

2.14 Mechanisms Involved in the Formation of the Sperm Reservoir

During mating in the pig, the boar deposits on average at least 30 x 10^9 spermatozoa. However, 70 to 99% percent of spermatozoa are quickly eliminated from the uterus (Rodriguez-Martinez *et al.*, 2005). Within 30 minutes (Hunter, 1981) sufficient numbers of spermatozoa are already present within the oviducts to achieve fertilisation and a 100% fertilisation rate can be obtained in most animals one to two hours after semen deposition.

There are two subpopulations of spermatozoa in the sperm reservoir of pigs (reviewed by Rodriguez-Martinez *et al.*, 2005). The first one which comprises the majority is present inside the deep crypts of the utero-tubal junction and the deep lateral recesses formed by isthmic plicae. Spermatozoa in these sites are in groups with their heads directed towards the epithelium and their tails in straight or gently curved formation. The other subpopulation is localized in the central lumen of the sperm reservoir where they are stacked within the mucoid material of the oviductal lumen. During ovulation, hundreds to thousands of spermatozoa are released restrictedly but gradually to the site of fertilisation to ensure a continuous supply of fertilising spermatozoa.

The mechanisms by which spermatozoa bind to the epithelium of the oviduct is still largely unclear. However, this interaction can be brought upon by a number of different interrelated situations and cannot be related to only one single factor. The physical environment of the oviduct and the involvement of a carbohydrate-mediated interaction between spermatozoa and oviductal epithelium provide some understanding of the binding mechanism (Suarez *et al.*, 1991; Green *et al.*, 2001). The association between spermatozoa and the oviduct is quite strong because

ordinary flushing of the oviduct with physiological medium does not dislodge many spermatozoa (Suarez *et al.*, 1991).

Other factors that might be involved in interaction between spermatozoa and oviduct epithelium include the decreased motility of spermatozoa in the isthmus, a low temperature of the sperm reservoir, a characteristic ionic milieu with low pH and bicarbonate levels and the inhibited entry of calcium into spermatozoa when bound to oviductal epithelium (reviewed by Rodriguez-Martinez *et al.*, 2005).

Temperature is a physiological specialisation which has the ability to influence the physiology of the binding process (Hunter, 1998). A temperature gradient is said to be apparent in the oviduct especially during the follicular stage. The temperature of the caudal isthmus is much cooler than the ampulla prior to ovulation (Hunter and Nichol, 1986). This temperature gradient disappears after ovulation which suggests that it might be involved in sperm storage in the sperm reservoir or in peri-ovulatory events of sperm activation.

2.14.1 Physical aspects

In oestrous females, the lumen of the oviduct becomes extremely small due to the oedematous reaction of the tisue in response to high concentrations of circulating oestrogen. In fact, the lumen is practically concealed by the oedema specifically the lamina propria. Cross sections of the lumen of the bovine oviduct have clear branching patterns within the lumen as exhibited by the presence of many longitudinal folds in the mucosa (Suarez *et al.*, 1997). The size of the lumen of these longitudinal branches is very narrow from only few microns (10 μm) and only some measuring 100 μm across. These narrow passages are also filled with mucopolysaccharides. In the pig just before ovulation, the caudal isthmus secretes a characteristic mucus-like glycoprotein which is whitish in colour, visco-elastic and is very tenacious (Hunter, 1998). The mucus in the isthmus serves as a plug that further impedes spermatozoal passage (Suarez *et al.*, 1991; Suarez *et al.*, 1997). It also prevents the entry of uterine fluids and leukocytes into the oviduct while gametes and embryos are present (Hunter, 1998). The quantity and the viscosity of the luminal secretions decrease after ovulation (Hunter, 2002). Furthermore, the muscular layer of the oviduct especially at the isthmus also contains many alpha-adrenergic

receptors which upon activation during peak oestrogen concentration causes strong contractions (Hunter, 1984). These contractions also may further decrease the lumen of the oviduct.

Thus, the oedematous nature, the luminal size, the presence of mucus and oviductal contractions become significant factors that serve as barriers and severely limit spermatozoal migration. Because of this, many spermatozoa become concentrated at the utero-isthmic junction and the first two centimetres of the adjacent isthmus forming the sperm reservoir (Hunter, 1981; Hunter, 1984; Suarez *et al.*, 1991; Suarez *et al.*, 1997; Rodriguez-Martinez *et al.*, 2005).

2.14.2 Carbohydrate recognition

In depth knowledge regarding the molecular basis of the binding of spermatozoa to the oviduct epithelium is still limited. However, a carbohydrate-mediated interaction has been demonstrated in many animal species. The glycocalyx of the male gamete and the oviductal epithelium contains several different sets of glycan structures and also lectin-like molecules (Topfer-Petersen *et al.*, 2002). Oligosaccharides and lectins function as ligand and receptors, respectively in the sperm-oviduct association. However, it is not known how far these identified receptor-ligand systems influence this interaction between the spermatozoon and the oviduct epithelium (Hunter, 2003).

Different sugar monomers, oligosaccharides and glycoproteins inhibit the binding of spermatozoa to oviductal epithelial cells or explants in a species specific manner (Waberski *et al.*, 2005). The involvement of carbohydrate molecules in the binding of spermatozoa to the oviduct was first reported in hamsters where sialic acid and fetuin competitively restrict the binding of spermatozoa (Demott *et al.*, 1995). The effect was significantly higher in the isthmus than in the ampulla. In the horse, neutral terminal galactose also has a similar effect (Dobrinski *et al.*, 1996) and in cattle, fucose is involved (Lefebvre *et al.*, 1997). In pigs, mannose inhibits the binding of spermatozoa while galactose, fucose and glucose does not (Green *et al.*, 2001).

A glycoprotein known as SBG has been identified in the isthmus of gilts that binds to the periacrosomal membranes of spermatozoa (Marini and Cabada, 2003). This glycoprotein is involved in exposing the terminal, neutral galactose as well as *O*-

linked Galbeta1-3GalNAc. These molecules are recognized by spermadhesins, a family of proteins in the seminal plasma of pigs (Dostalova *et al.*, 1995a; Calvete *et al.*, 1996b).

By using a competitive inhibition assay, Wagner *et al.* (2002) identified a complementary receptor system that recognized the involvement of a specific oligosaccharide in the binding of boar spermatozoa to the oviduct. Ovalbumin and asialufetuin inhibited the attachment of spermatozoa to the oviductal cells. Sugars which are located in the N-and-O-linked carbohydrate side chains are sialic acid, galactose, N-acetylglucosamine and mannose. The results indicated that a high affinity binding site involving oligomannose moieties and a low affinity binding site recognizing galactose residues are highly involved in the attachment of boar spermatozoa to the oviduct.

The inhibitory capacity of a number of glycoproteins as well as fucoidan, a polymer of fucose have been examined in cattle (Lefebvre *et al.*, 1997). Only fucoidan inhibited and reduced the binding of spermatozoa to oviductal explants. Increasing the concentrations of fucose increased the inhibitory activity. There was no significant difference observed in the binding of spermatozoa between the isthmic and ampullary explants. Moreover, while capacitated bovine spermatozoa lost their capacity to bind to fucose, they gain the ability to bind to mannose (Revah *et al.*, 2000) which also happens to be one component of the bovine zona glycoproteins (Katsumata, 1996). This observation is very important to consider in understanding the changing patterns in the composition of the sperm plasma membrane glycoproteins.

2.14.2.1 Lectin binding patterns in the pig oviduct

Lectins have been used to detect various carbohydrates in the porcine oviduct (Walter and Bavdek, 1997). The apical glycocalyx of both ciliated and nonciliated cells in all the regions of the oviduct were labeled by wheat germ lectin with the strongest reaction in the isthmus. *Helix pomatia* lectin labeled kinocilia of ciliated and Golgi regions of the non-ciliated cells in the ampulla and infundibulum at oestrus. *Lotus tetragonolobus agglutinin* reacted with alpha-L-fucose residues and these were present in the isthmus but not in the ampulla and infundibulum. The sugars detected

by the lectins used included alpha-D-mannose, alpha-D-glucose, D-N-acetylgalactosamine, D-N-acetyglucosamine, beta-D-galactose, and alpha-L-fucose. These results suggest regional and cyclic differences in the occurrence of glycoconjugates in the oviductal epithelium of the pig which could affect the binding patterns of spermatozoa to the oviduct.

2.15 Features of the Binding of Spermatozoa to Oviductal Explants

In the various studies that have been undertaken in different species, spermatozoa that bound to the oviduct epithelial cells share common characteristics. These include motility, a normal morphology, an intact acrosome (Thomas *et al.*, 1994a), the lack of the intracytoplasmic droplet (Petrunkina *et al.*, 2001b), a lower intracellular calcium content, sperm head proteins that have not undergone tyrosine phosphorylation (Petrunkina *et al.*, 2001a), a normal chromatin structure (Ellington *et al.*, 1999) and a uncapacitated state (Lefebvre and Suarez, 1996; Fazeli *et al.*, 1999).

Boar spermatozoa attach to the oviductal epithelial cells by the rostral part of their heads (Petrunkina *et al.*, 2001b) and it takes 15 minutes for a significant number to bind (Green *et al.*, 2001). Bovine spermatozoa bind to the apical surfaces of the mucosal epithelium in furrows between neighboring ciliated cells or between ciliated and non-ciliated cells (Pollard *et al.*, 1991) and to oviductal explants from both the isthmus and the ampulla of pre-ovulatory and postovulatory heifers within 15 minutes of incubation (Lefebvre *et al.*, 1995). The motility of spermatozoa that bound was very high. Contact between spermatozoa and the oviductal epithelium was made between the apical region of the oviduct cells and the acrosome of spermatozoa. In the rabbit, no binding could be found between the oviductal plasma membrane vesicles and the mid-piece or tails of the spermatozoa (Smith and Nothnick, 1997).

Spermatozoa bind to both ciliated and non-ciliated oviductal cells in the horse (Thomas *et al.*, 1994b). This is different to the general observation among other species in which spermatozoa tend to only bind to ciliated epithelial cells. Moreover, the motility of spermatozoa bound to explants from cyclic mares was significantly

higher than those spermatozoa bound to explants from a mare in post-ovulatory or di-oestrus stage.

2.16 Sperm-to-Oviduct Binding in Relation to the Region of the Oviduct, the Stage of the Oestrous Cycle and the Reproductive Status of the Animal

Differing results have been reported on the effect of the region of the oviduct, hormonal influence and the parity of the animal on the capacity of spermatozoa to bind to the oviductal epithelium. On the one hand, the binding of boar spermatozoa between the isthmic and the ampullary explants did not differ significantly. A similar result has been observed between the follicular and the luteal stages. This is consistent with the earlier observations by Suarez *et al*. (1991), however, the addition of exogenous oestradiol enhanced the binding process to both the isthmus and the ampulla. Moreover, Petrunkina *et al*. (2001b) found no significant difference in the binding of spermatozoa to oviductal explants from the gilt or the sow.

On the other hand, Raychoudhury and Suarez (1991) found a preferential binding of boar spermatozoa to the isthmus than the ampulla. A similar result has been found in the horse (Thomas *et al.*, 1994a). Furthermore, the presence of oestrus but not di-oestrus concentrations of steroids in the medium increased the percentage of spermatozoa attaching the oviductal epithelium of both the isthmus and the ampulla. These results imply that increased levels of steroids during oestrus have a significant effect on the binding of spermatozoa particularly to the isthmus.

2.17 Sperm-to-Oviduct Binding between Epididymal and Ejaculated Spermatozoa and between Capacitated and Uncapacitated Spermatozoa

The binding capacity of epididymal and ejaculated spermatozoa has been studied by Petrunkina *et al*. (2001b). The fact that ejaculated spermatozoa had been previously in contact with the seminal plasma is important as the binding index of boar epididymal spermatozoa (15.1 ±6.1 per 0.01mm^2) was significantly less than ejaculated spermatozoa (28.4 ±11.4 per 0.01 mm^2). More uncapacitated boar spermatozoa bind to the oviduct than capacitated spermatozoa (Fazeli *et al.*, 1999). Similar results were found in cattle (Lefebvre and Suarez, 1996) and horses

(Dobrinski *et al.*, 1997). As discussed earlier, capacitation destabilizes the plasma membrane of spermatozoa. The presence of the sperm reservoir is therefore important in preserving the integrity of the plasma membrane while waiting for ovulation.

2.18 The Release of Spermatozoa from the Sperm Reservoir

The release of spermatozoa from the epithelium has been attributed to changes in the ultrastructural or biochemical structure of the plasma membrane of spermatozoa. Incubation of bovine spermatozoa with endosalpingeal cells promoted hyperactivation of spermatozoa after 12 hours (Pollard *et al.*, 1991). Moreover, preincubation of bull spermatozoa with capacitating medium for four hours significantly reduced the number of spermatozoa that bound to oviductal epithelium (Lefebvre and Suarez, 1996). Similar results were found in hamsters (Smith and Yanagimachi, 1991) and humans (Pacey *et al.*, 1995). All these results support the idea that specific changes occur to the plasma membrane of spermatozoa during capacitation that leads to the loss of the affinity of spermatozoa to their equivalent carbohydrate ligands on the oviductal epithelial cells (Topfer-Petersen *et al.*, 2000).

Furthermore, some evidence suggests that oviductal cells may secrete certain compounds that can initiate and promote the capacitation of spermatozoa and the subsequent hyperactivation. The rising concentrations of hormones which can be transferred locally to the oviduct (Hunter *et al.*, 1983) before ovulation may influence the release of these substances (Chian *et al.*, 1995). Mahmoud and Parrish (1995) have demonstrated the capacitation-enhancing effect on bovine spermatozoa of soluble substances prepared from oviducts. Oviductal contractions increase near the time of ovulation which can hamper the binding process (Suarez *et al.*, 1991). The muscle layer as well as the mucosa of the oviduct contain catecholamines (Hunter, 2002), a potent neurotransmitter agent. In the isthmus of rabbits, more of the non-drenergic terminals are present in circular muscles and catecholamine receptors have been observed in hamster spermatozoa (Bavister *et al.*, 1979).

Strong flagellar movements by spermatozoa after capacitation and the subsequent hyperactivation are also factors that force spermatozoa to loosen their attachment

from the oviduct epithelium Activated spermatozoa are released consistently in small numbers during ovulation *in vivo*. During this process, capacitation is completed on their way to the ampullary-isthmic junction for the fertilisation. The beneficial effect of the controlled release of spermatozoa from the sperm reservoir is the fact that the ratio between the fertilising spermatozoa and the number of ovulated oocytes becomes almost equal (Hunter, 2002).

2.19 Sperm-Oviduct Binding and the Role of the Seminal Plasma

Upon ejaculation, spermatozoa are suspended in a mixture of secretory products from the accessory glands as well as the cauda, collectively called the seminal plasma. Unlike other species, the boar ejaculates a large volume of semen normally between 250 and 500 ml. The seminal plasma has unique biochemical characteristics as defined by many different substances secreted by the accessory glands. It would therefore be likely that components of the seminal plasma could influence the interaction of spermatozoa with the oviductal epithelium. As mentioned earlier, fewer epididymal spermatozoa bound to oviduct explants than ejaculated spermatozoa.

A protein with an apparent molecular weight of 16.5 kDa and an estimated pI of 5.8 has been isolated from the seminal plasma of the bull (Ignotz *et al.*, 2001). It was found that only a small amount of this protein could be extracted from capacitated compared to uncapacitated spermatozoa and when it is incorporated back to the culture medium, it is able to associate again with capacitated spermatozoa and restore the fucose binding properties. This partly explains why capacitated spermatozoa are unable to bind to oviduct epithelium.

On the other hand, a molecule with an apparent fucose-binding property is present in the plasma membrane of bovine spermatozoa (Revah *et al.*, 2000). The fucose-binding molecule was blocked by certain components of the seminal plasma which could include free sugars, glycopeptides, or glycolipids. Specific components therefore in the seminal plasma might also hinder the binding capacity of spermatozoa by forming a coat over the binding molecule or act as a competitive inhibitor to the corresponding ligand.

Another group of molecules of physiological importance to oviduct physiology are the heparin-binding proteins (HBPs). Heparin-binding proteins are present in the seminal plasma, coat the surface of spermatozoa upon ejaculation and are known to influence the action of heparin (Miller, 1990). Heparin-binding proteins are group of strongly acidic glucosaminoglycans and heparin sulphate glycosaminoglycans produced by most animal cells (Kjellen and Lindahl, 1991) that can influence various biological processes by binding to proteins associated or secreted onto cellular surfaces. They are also capable of changing or influencing the activity of proteins which they bind. The epithelial cells of the female reproductive tract release heparin-like glycosaminoglycans in larger amounts during the follicular stage that initiate capacitation in some animal species (Therien, 1995).

The names of major HBPs are different among animals studied so far. The HBPs in the boar belong to a protein family called spermadhesins and constitute the bulk of the proteins present in seminal plasma (Sanz *et al.*, 1993). Members of this protein group are structurally unrelated to their bovine and equine HBP counterparts. Boar spermadhesins (AQN-1, AQN-3, PSP-I and AWN) are a group of 14-16 kDa seminal plasma and sperm-associated lectins (Calvete *et al.*, 1996a; Calvete *et al.*, 1996b) that could influence sperm capacitation and gamete interaction. The HBPs in the seminal plasma of the bull and stallion are called PDC-109, BSP-A3, and BSP-30K, and HSP-1 and HSP-2, respectively. They are polypeptides with 109-121 amino-acid residues composed of one or more 13-15 repeating residues of O-glycosylated N-terminals followed by two domains arranged in tandem each sharing the consensus sequence of fibronectin type-II modules (Calvete *et al.*, 1996b).

The species-specific difference of heparin-binding proteins among animals determines how they influence sperm physiology. It depends also on how they interact with phospholipids on surfaces of spermatozoa. In cattle for example, HBPs have particular affinity to phosphorylcholine which reflects the way they bind to the sperm surface (Desnoyers and Manjunath, 1992). Heparin-binding proteins present in the seminal plasma of the bull bind to phosphorylcholine-containing lipids of spermatozoa from the cauda during ejaculation and capacitation. It is worth noting

that the heparin binding domain for AWN is present in the same site where the carbohydrate-binding surface has been also located (Calvete *et al.*, 1996b).

2.20 Conclusion

The mammalian epididymis is a complex organ structure which performs significant functions in the maturation of spermatozoa. It is divided into different regions with a distinct histological anatomy and secretes many different products especially glycoproteins that are directly or indirectly involved in the development of various sperm functions. The caput and corpus specialise in the secretion of proteins and other substances while the cauda stores spermatozoa before ejaculation.

The oviducts are primarily involved in the formation of the sperm reservoir and fertilisation of oocytes. After semen deposition, spermatozoa bind to epithelial cells of the caudal isthmus while waiting for ovulation. The binding of spermatozoa to oviduct epithelium enables them to maintain viability, delay capacitation and prevent polyspermy. The mechanisms involved in sperm-to-oviduct binding are still unclear although the physical structure of the cauda isthmus especially during oestrus and a carbohydrate recognition process are likely to be the main mechanisms. While caudal spermatozoa are able to bind to oviductal epithelium, the literature contains no information of where epididymal spermatozoa develop this ability and what might be the molecular mechanisms involved.

CHAPTER 3

MATERIALS AND METHODS

3.1 Boars

Large White or Large White x Landrace boars either purchased from a commercial piggery at 16 weeks of age or born at the School of Veterinary and Biomedical Sciences, James Cook University, Townsville, were reared until 10-14 months of age in the animal facilities of the School. Ethics approval to conduct experimental work with animals was given by the James Cook University Animal Ethics Committee (Approval number A1007).

3.2 Preparation of oviductal explants for the binding Assay

The procedures were modified from Suarez *et al*. (1991), Petrunkina *et al*. (2001b) and Wagner *et al*. (2002). Oviducts were obtained from gilts slaughtered at the Charters Towers abattoir, about 130 km from James Cook University, Townsville. The gilts were about 20 weeks of age and not cycling as determined by the absence of corpora lutea. Both oviducts from a gilt were removed and placed in a 30 ml container with 25 ml of normal phosphate buffered saline solution (PBS). The phosphate buffered saline solution contained 150 mM NaCl, 11.7 mM NaH_2PO_4 and 2.5 mM KH_2PO_4 and was adjusted to pH 7.4 and 280 mOsm/kg. The samples were placed in a polystyrene box with an ice pack and transported to the laboratory in an air-conditioned car.

In the laboratory, the mesentery of the oviduct was cut and separated to straighten the oviducts to aid in the identification of the isthmus and ampulla. One end of the oviduct was then pinned with a 19G needle (1.1 mm x 38 mm) on a sterile platform using the lid of the 96 well flat bottom culture tray (NUNCLON ^{TM}, InterMed) used for incubation of oviduct explants. The other end was held with a pair of fine forceps and the oviduct opened longitudinally with small fine scissors passing through the length of the oviduct. Small pieces (2-3 mm^2) of the oviductal mucosa including the underlying stroma were cut from the isthmus and the ampulla with a scalpel blade.

The pieces of oviduct explants were placed in a modified Tyrode's solution (TALP) (Table 3.1) at pH 7.4 and 300 mOsm/kg. In cases where the oviducts were not used immediately, they were stored for up to two hours at 4^0 C until used.

Table 3.1 Composition of Modified Tyrode's Solution

Chemical Ingredients	Amount
Sodium chloride	96.0 mM
Potassium chloride	3.1 mM
Magnesium sulfate	0.4 mM
Calcium chloride	2.0 mM
Glucose	5.0 mM
Sodium dihydrophosphate	0.3 mM
Sodium bicarbonate	15.0 mM
Sodium lactate	21.6 mM
Sodium pyruvate	2.2 mg/ml
HEPES	20.0 mM
BSA (Sigma A4378)	6.0 mg/ml
Nano-Pure deionised water	1000.0 ml

The same procedure was used in the preparation of tracheal explants except that only the mucosa was used. Explants were equilibrated first for 20 minutes in modified Tyrode's solution at 39^0 C in a humidified atmosphere containing 5% CO_2 in air before adding spermatozoa. The viability of explants was checked before use by examination for ciliary movements of the epithelium.

In preliminary work, oviducts were also collected from non-pregnant cows slaughtered at the Australian Meat Holdings abattoir, Townsville. The procedures were the same as for pigs, except oviducts were collected from cows that contained a mature corpus luteum.

3.3 Preparation of spermatozoa and determination of motility characteristics

In a preliminary experiment, fresh chilled boar semen was used. It was purchased from Premier Pig Genetics, Wacol Pig Artificial Breeding Centre, Grindle Road, Wacol, Australia. The semen used was from the same boar (Large White PPG 114) and usually arrived a day before the experiment was undertaken. Before use, the semen was examined for sperm concentration and motility characteristics using a computer-aided semen analyser (CASA) (Hamilton Thorne Research. IVOS version 10, Beverly, MA, USA) and diluted to 5 x 10^6 spermatozoa per ml with modified Androhep solution (Table 3.2) at pH 7.4 and 290 mOsm/kg (Waberski *et al.*, 1994; Petrunkina *et al.*, 2001b). For the rest of the experiments, sperm samples were prepared from the epididymis of boars. The left epididymis was obtained by unilateral castration and the right when the boar was slaughtered four to five weeks later. Within a minute of collection from the rete testis, caput, corpus and cauda, the sperm samples were analysed for motility characteristics as described for ejaculated spermatozoa. Collection of spermatozoa from the rete testis and epididymis took about 15 minutes. Sperm samples were collected from the middle caput (E-2-E3), middle corpus (E-6) and cauda (E-8) (Syntin *et al.*, 1996; Syntin *et al.*, 1999). Caudal and testicular semen was aspirated using a tuberculin syringe (1ml) while spermatozoa from the middle caput and the middle corpus were collected by a gentle scraping action of the blunt end of a scalpel blade after a small incision was made. The average time interval between the collection of oviducts and addition of spermatozoa to explants was about six hours.

3.3.1 CASA settings and definitions

In this study, the main software setting of the CASA were set as follows: 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 the illumination intensity, 2381. The temperature of the slide chamber was set at 39^0 C.

The following are the definitions used for describing the various motility parameters of spermatozoa (Mortimer, 1997; Mortimer, 2000).

- Motile is the population of spermatozoa that were moving at or above a minimum speed as determined by values defined under the set-up of the CASA whereas the motility is the ratio of motile to non-motile spermatozoa.
- Progressive motility is the number of spermatozoa moving with both an average path velocity (VAP) > medium VAP cut off MVV) and straightness (STR) > the threshold straightness (So).
- Rapid spermatozoa is the percentage of motile spermatozoa with VAP > MVW.
- Average-path velocity (VAP) indicates the length of the general trajectory of spermatozoa in μm/sec and is calculated by taking the length of the average path over a certain period of time.
- Straight-line velocity (VSL) is the value (always the lowest) that corresponds to the straight-line distance (μm/sec) between the first and the last points of the trajectory and correcting for time which then gives the net space gain within a time period.
- Curvilinear velocity (VCL) is the distance (μm/sec) traveled by spermatozoa along its curvilinear path.
- Amplitude of lateral head displacement (ALH) is the approximation of the flagellar beat envelope and is the width of the lateral movement of the sperm head in μm.
- Beat cross frequency (BCF) indicates the flagellar beat frequency (in Hertz), that is, the number of times the sperm head crosses the direction of movement.
- Straightness (STR) compares the straight-line and the average-paths (VSL/VAP X 100) and indicates the relationship between the net space gain and the spermatozoon's general trajectory, expressed in percent.
- Linearity (LIN) compares the straight-line and curvilinear paths (VSL/VCL X 100) and is the expression of the relationship of the distance between the first and the last points of the trajectory.

The overall population of spermatozoa was subdivided into four motility categories: rapid where VAP >MVW; medium where LVV (low VAP cut-off) < VAP < MVW:

slow where VAP < LVV or VSL < low VSL cut-off (LVS); and static, the fraction of all spermatozoa that were not moving during the analysis (Yeung *et al*., 1997).

Table 3.2 Composition of the Modified Androhep Solution

Chemical Ingredients	Amount
Glucose	144.0 mM
Tri-sodium citrate-2-hydrate	27.2 mM
Sodium bicarbonate	14.3 mM
HEPES	37.0 mM
Nano-Pure deionised water	1000.0 ml

3.4 Co-incubation of spermatozoa and explants

Each explant from the isthmus and ampulla was equilibrated first in a 60 µl droplet of modified TALP medium in a 96 flat bottom well culture tray for 20 min at 39^0 C in a humidified atmosphere containing 5% CO_2 in air. The sperm suspension in modified Androhep medium was also equilibrated in the same conditions for at least five min and then 20 µl of the sperm suspension were added to the explant and incubated for 15 min at 39^0 C in a humidified atmosphere containing 5% CO_2 in air. After incubation, the explants were immediately washed twice in modified Tyrode's solution in a small dish with the aid of a fine strainer to free loosely attached spermatozoa. The explants were fixed in 2% formaldehyde in 0.1 M sodium phosphate buffer plus 0.01% $CaCl_2$ at pH 7.3.

3.5 Fixation and counting bound spermatozoa

The explants were fixed overnight at 4^0 C. The next day, the explants were rinsed in three changes of 10 mM phosphate-buffered saline solution at pH 7.3, stained with Gill's Hematoxylin (Table 3.3) for fifteen seconds and were rinsed again five times. The explants were then mounted on a glass slide flooded with enough glycerol to delay the drying of tissues while they are examined under the microscope. The explants were covered with coverslips immobilized by petroleum jelly (Vaseline) as a support, and examined for bound spermatozoa with a light microscope at 400 x

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 thus giving a counted area of 1.25 mm^2 per explant. Only spermatozoa that bound to the epithelium and not to the stroma were seen and counted.

Table 3.3 Composition of Gill's Haematoxylin stain

Chemical Ingredients	Amount
Distilled water	730.0 ml
Ethylene glycol	250.0 ml
Haematoxylin (Cl 75290)	2.0 g
Sodium iodate	0.2 g
Aluminum sulphate	17.6 g
Glacial acetic acid	20.0 ml

The reagents were combined in the order given and mixed for one hour at room temperature.

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

For this experiment, 36 oviducts (left and right) were collected from 18 cows in the mid-luteal stage of the oestrous cycle and 18 non-cycling gilts. Each week, a set up was composed of four to six oviducts from two to three animals. From every oviduct, three explants were taken from both the isthmus and the ampulla giving a total of 108 oviductal explants from each region of the oviduct for all 36 oviduct samples. Six bovine and six porcine tracheal explants were also prepared as controls. Ejaculated boar spermatozoa were used in this experiment. The semen samples were purchased from Premier Pig Genetics at Wacol Pig Artificial Breeding Centre, Wacol, Australia. The semen was contained in an AI bottle and was shipped to the School's laboratory in a polystyrene box with an ice pack (see also section 3.3).

3.7 The binding of boar epididymal spermatozoa to porcine oviducts

Each set-up consisted of epididymal spermatozoa from one boar and four oviducts from two gilts. Unilateral castration of the left testicle was performed to coincide

with the delivery of oviducts to the laboratory. The boars were premedicated with atropine sulphate at 5mg/kg estimated body weight (atrophine 0.6 mg/ml; Apex Laboratories Pty Ltd, Somersby, NSW) followed five minutes later by intramuscular injections of ketamine hydrochloride 6mg/kg (Ketamine 100mg/ml; Parnell Laboratories Pty Ltd, Alexandria, NSW) and xylazine hydrochloride 1mg/kg (Ilium Xylazet 100 mg/ml; Troy Laboratories Pty Ltd, Smithfield, NSW). When the boar was anaesthetised, the scrotum was prepared aseptically and a single incision made to extrude the left testicle. Large haemostats were applied to the spermatic cord and three sutures (3, 5 metric chromic catgut) applied to the spermatic cord before removal of the testicle. Interrupted sutures (3, 5 metric chromic catgut) were used to suture the parietal vaginal tunic and the scrotal skin was closed with mattress sutures (Vicryl, 3.0 metric; Ethicon). The boar was given 1200 mg oxytetracycline intramuscularly (Engemycin; Intervet Australia Pty Ltd, Bendigo, Victoria). The castrated testicle was put to a polystyrene box with an ice pack and brought to the laboratory.

When the oviductal explants had been prepared, the testicle with the epididymis attached was dissected and separated from the tunica albuginea. Epididymal spermatozoa were taken from a small incision made in the middle caput, middle corpus and cauda epididymides (Syntin *et al.*, 1999) and also from the rete testis by longitudinally cutting the testicle to expose the mediastinum. The incised epididymis was gently squeezed with the thumb and index finger and the contents aspired using a sterile tuberculin syringe. Collection of spermatozoa from the rete testis and the epididymis took about 10 minutes. Each sample was placed in an Eppendorf tube containing one ml of modified Androhep medium and the concentration and motility of spermatozoa determined as described previously.

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

In conjunction with the binding of epididymal boar spermatozoa to oviducts from gilts as described previously, a separate experiment was conducted whereby a comparison in the binding capacity of epididymal boar spermatozoa was made between oviducts from sows and gilts. In this experiment, 10 to 12 oviduct explants for each epididymal sperm sample (i.e., rete testis, caput, corpus and cauda) and each

animal treatment was examined. The representative explants were pooled separately from two sows and two gilts that were slaughtered at the same time. One of the sows was raised in the School of Veterinary and Biomedical Sciences and the other was a culled sow from a commercial piggery. Upon examination of their ovaries, the sows were considered to be in the follicular stage. The gilts were the same as described in previous experiments.

3.8 The binding of epididymal spermatozoa after dilution with either albumin, asialufetuin and modified Androhep solutions

The process of preparing oviductal explants and the collection of epididymal spermatozoa were similar as described in previous experiments. Oviductal explants were replicated six times for each sperm sample (i.e. cauda, corpus, caput, rete testis); diluting solution (i.e. modified Androhep, albumin, asialofetuin), and oviductal regions (i.e. isthmus, ampulla). The testicle and epididymis from six boars were used in this experiment. Only one boar was used on a particular day. After the explants had been prepared, the epididymal spermatozoa were collected and diluted in modified Androhep solution for determination of sperm concentration and motility characteristics. After determining the concentration, spermatozoa were diluted again to a concentration of 5 $x10^6$/ml. At this stage, each sperm sample from different epididymal regions was diluted with one ml of albumin, asialofetuin and modified Androhep solution. The modified Androhep solution served as the control. The concentration of albumin (from chicken egg white, Grade V, product A5503, Sigma-Aldrich, Castle Hill, NSW) or asialufetuin (from fetal calf serum, Type I, product A4781, Sigma-Aldrich, Castle Hill, NSW) was 5 mg/ml in modified Androhep solution. Chicken albumin was used because it closely resembles the composition of ovalbumin which contains mannose, the sugar mostly responsible in the inhibition of the binding of porcine spermatozoa to oviduct epithelium. The sperm samples were equilibrated for at least five minutes in a humidified atmosphere containing 5% CO_2 in air at 39^0 C before adding to the oviductal explants which had been incubated beforehand in the same conditions for at least 15 minutes. The explants and spermatozoa were incubated for 15 minutes at 39^0 C with 5% CO_2 in air. The process of fixing the explants, mounting to slides and microscopic examination was the same as described in section 3.5.

3.9 The binding of epididymal spermatozoa after incubation in caudal fluid

Oviductal explants and testicles were collected as described previously. The explants were replicated six times for each epididymal sperm sample (i.e. caput in modified Androhep; caput in caudal plasma; corpus in modified Androhep; corpus in caudal fluid; and cauda in modified Androhep) and between the isthmus and the ampulla. The epididymides of seven boars were used in this experiment. The boars were either unilaterally castrated at the School of Veterinary and Biomedical Sciences or slaughtered at the abattoir in Charters Towers. After the arrival of oviducts and testicles, the contents of the cauda epididymidis was first collected into small centrifugation vials and centrifuged for 30 minutes at 1200 ***g***. The centrifugation was repeated to fully extract the caudal fluid. While centrifugation was underway, the oviductal explants were prepared as described earlier. The caudal fluid was collected into Eppendorf tubes and divided accordingly between the corpus and caput spermatozoa as a diluting medium. Sperm samples were then collected from the caput, corpus and cauda and diluted in either modified Androhep or the caudal fluid. Cauda spermatozoa were only diluted with the modified Androhep. The sperm samples were incubated in a humidified atmosphere containing 5% CO_2 in air at 39^0 C for 30 minutes and then were analyzed for sperm concentration and motility characteristics. While computing for the desired sperm concentration of 5 $x10^6$/ml, the sperm samples were centrifuged for 10 minutes at 600 ***g***. The supernatant was rejected and replaced with the modified Androhep solution to the desired concentration. The sperm samples were equilibrated for at least five minutes in a humidified atmosphere containing 5% CO_2 in air at 39^0 C before adding to the oviduct explants which were also pre-equilibrated in the same conditions for at least 15 minutes. The explants and spermatozoa were incubated for 15 minutes at 39^0 C with 5% CO_2 in air. The explants were fixed, mounted on slides and examined as described previously.

3.10 The binding of epididymal spermatozoa to oviductal epithelium after incubation with seminal plasma

The boars were slaughtered at the Charters Towers abattoir four to five weeks after the unilateral castration. The right testicle and seminal vesicles of a boar and the oviducts from two gilts were collected into separate plastic bags, placed in a

polystyrene box with an ice pack and transported to the laboratory in an air-conditioned car. Upon arrival, a small incision was made into the seminal vesicles and the seminal plasma was poured into an Erlenmeyer flask, covered and refrigerated at 4^0 C while the oviducts and epididymal sperm samples were prepared, as previously described. Of the five boars that were used in this experiment, the seminal plasma from each boar was used except for one case where no seminal plasma was able to be collected from the seminal vesicles. Instead, the seminal plasma of a boar killed earlier and stored at -20^0 C was used.

Two sets of assays were carried out. The first was a duplicate of section 3.7. The second assay used epididymal spermatozoa that were first incubated with seminal plasma. In this assay, 50 µl of the sperm sample from each epididymal region was added to a small vial containing one ml of seminal plasma that has been previously equilibrated in a humidified atmosphere containing 5% CO_2 in air at 39^0 C for 30 minutes. After the addition of spermatozoa, the samples were incubated at 39^0 C for 30 minutes and then centrifuged at 600 ***g*** for 10 minutes. The supernatant was carefully removed and replaced with one ml of modified Androhep medium. The concentration and motility was determined and the sample adjusted to 5×10^6 spermatozoa per ml with modified Androhep medium. The spermatozoa were incubated with oviduct explants as previously described.

In another similar experiment, spermatozoa were mixed with the seminal plasma for only a minute prior to incubation. After sperm samples were collected from each epididymal region and the rete testis, they were mixed gently with seminal plasma in an Eppendorf tube using a pipette at room temperature and immediately centrifuged. The rest of the procedure including incubation of spermatozoa with explants, fixation, mounting with slides and counting the number of spermatozoa that bound to explants were the same as described in sections 3.4 and 3.5. Motility characteristics of spermatozoa before and after centrifugation were determined following the procedures in section 3.3 as well as the capacitation status and the percentage of live and dead spermatozoa (see sections 3.9.1 and 3.9.2) prior to incubation with oviduct explants.

3.10.1 Determination of the percentage of live and dead spermatozoa

The eosin –nigrosin staining assay was used to assess the percentage of live and dead spermatozoa from the rete restis and the epididymis. The stain solution was composed of 0.67 g eosin and 10 g nigrosin in 100 ml distilled water. This was brought to boil and cooled in room temperature. The solution was stored in a vessel protected from light. Fifty microliters of each sperm sample were mixed with an equal amount of the stain solution. After 30 seconds of gentle mixing, 10 µl of the sperm suspension were placed onto a glass side and a smear was made. Two smears were prepared for each sperm sample. Two hundred spermatozoa from each sample were assessed as dead or alive. Those spermatozoa which appear completely free from any stain were considered live while those spermatozoa which were stained from pinkish to purplish were considered dead.

3.10.2 Capacitation status of spermatozoa

The procedure used for the chlortetracycline (CTC) assay was as described by Wang *et al*. (1995) and Green and Watson (2001). Forty-five microliters from each sample were mixed with an equal amount of CTC solution. The CTC solution was composed of 750 uM/l CTC (product C4881, Sigma-Aldrich, MO, USA) and 5 mM/l cysteine (product C-7352, Sigma-Aldrich, MO, USA) in a buffer containing 130 mM/l NaCl and 20 mM/l Tris (hydroxymethyl) aminomethane (product T1503, Sigma-Aldrich, MO, USA) at pH 7.4. The CTC solution was prepared on the day it was used. Spermatozoa were then fixed by adding 10 µl of filtered glutaraldehyde (EM Grade; 1% v/v in 1M Tris; (12.11g to 100ml H_20) at pH 7.8. Ten µl of fixed sperm suspension was placed onto a glass slide and mixed with a drop of 1, 4-diazabicyclo [2.2.2] octane (DABCO, 0.22 M; product D-2522, Sigma-Aldrich, MO, USA) dissolved in glycerol: phosphate-buffered saline (9:1; stored below 0^0 C) to prevent fading of fluorescence. Two slides were prepared from each epididymal region. The slides were examined with fluorescent microscopy with a blue-violet illumination (excitation at 355-425 mm) (Leitz, Wetzlar).

Two hundred spermatozoa from each segment were examined from the two slides. Staining patterns were identified as F-full fluorescence of the entire head (uncapacitated); B-a fluorescence-free band in the post-acrosomal domain (capacitated); and AR-low fluorescence over the entire head with a band of bright

fluorescence across the entire equatorial segment (acrosome-reacted cells) (Wang *et al*., 1995). Spermatozoa were then grouped into uncapacitated and capacitated; the capacitated ones include those whose acrosome had reacted.

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3.11 Data Presentation and Analyses

Data analyses were done using the Statistical Package for Social Sciences software version 11 (SPSS vs.11). Graphical presentation of the data was made using the Excel program of the Microsoft Office Professional Edition 2003. Statistical differences involving two variables such as comparing the binding capacity of spermatozoa between porcine and bovine oviducts and between the isthmus and the ampulla were calculated using the Student's T-test. Analysis of variance (ANOVA) was used to compare the binding capacity of spermatozoa among the three regions of the epididymis and also among boars. Tukey test (Post hoc test of significance) for multiple comparison of means was used to determine homogeneous subsets in variables tested by ANOVA. In the event that data have not passed the assumptions of parametric tests, analyses were performed after each data point was transformed accordingly using Log_{10} otherwise, non-parametric tests i.e. Mann-Witney U test, Kruskal-Wallis H tests were used. In case of Mann-Whitney U test, a Bonferroni adjustment was applied to the normal alpha (level of significance) when more than two variables were tested (Zar, 1999). The level of significant difference was set at $P \leq 0.05$.

CHAPTER 4
BINDING OF BOAR EPIDIDYMAL SPERMATOZOA TO OVIDUCTAL EPITHELIUM

4.1 Introduction

The volume of semen and concentration of spermatozoa ejaculated by males of various species differs significantly. While millions of spermatozoa are deposited into the female reproductive tract upon coitus or during artificial insemination, only a few thousand pass through the utero-tubal junction and reach the caudal isthmus (Suarez *et al*., 1997). Those spermatozoa which are morphologically abnormal are phagocytosed before they gain access to the oviducts (Yanagimachi, 1994).
While a number of spermatozoa reach the site of fertilisation within minutes after semen deposition (Hafez and Hafez, 2000) they do not necessarily fertilise oocytes (Hunter and Wilmut, 1982). Instead, a second population reaches the oviduct several hours after insemination and most are trapped in the isthmus and are held until the ovulation becomes eminent. During this time, spermatozoa bind to ciliated epithelial cells of the isthmus forming what is called the sperm reservoir (Hunter, 1981; Suarez *et al.*, 1991; Yanagimachi, 1994; Suarez, 2002a; Topfer-Petersen *et al.*, 2002). In the pig, at least 4000-5000 spermatozoa are present in the isthmus before ovulation occurs (Hunter, 1997). The sperm reservoir provides the right number of fertilising spermatozoa at the proper physiological time to ensure a successful fertilisation and development of the newly formed embryo. It has been observed that by controlling the number of spermatozoa that are released from the sperm reservoir, it is possible to equalise the number of fertilising spermatozoa and ovulated oocytes during the process of fertilisation and thus polyspermy can be prevented (Hunter, 1993).

While there are publications on the binding of ejaculated and epididymal spermatozoa to oviduct epithelium, there has been no systematic study to determine where and when do spermatozoa develop the ability to bind to oviductal epithelium. That is, have spermatozoa developed this capacity when they are formed in the testis or do they develop binding capacity during their 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 shaping the biochemical changes to the plasma membrane of spermatozoa (Jones, 1989; Dacheux *et al.*, 1998b; Dacheux *et al.*, 2005). It is possible that the changes could include the formation of molecules responsible for binding of spermatozoa to epithelium of the isthmus.

The aim of the work described in this Chapter was to determine the ability of spermatozoa from the rete testis and various sites in the epididymis of the boar to bind to oviductal epithelium using an oviduct explant assay. There was also a partial assessment of the possible carbohydrate-binding molecules present on boar epididymal spermatozoa.

MATERIALS AND METHODS

In a preliminary study, the binding capacity of ejaculated boar spermatozoa was compared between oviducts from cows and gilts (see section 3.6). Fresh chilled semen was purchased from Premier Pig Genetics, Wacol Pig Artificial Breeding Centre, Grindle Road, Wacol, Australia. The semen used was from the same boar (Large White PPG 114) whose ejaculate was purchased on eight occasions for this study. Before use, the semen was examined for motility and concentration of spermatozoa using a computer-aided semen analyser (CASA) (Hamilton Thorne Research. IVOS version 10, Beverly, MA, USA) (see section 3.3). Oviducts were collected from cyclic cows at the mid-luteal phase that were slaughtered at the Australian Meat Holdings abattoir, Townsville while oviducts from gilts where collected at the Charters Towers abattoir. The oviduct explants were prepared as described in section 3.2.

Epididymal spermatozoa were obtained from boars raised in the animal facilities of the School of Veterinary and Biomedical Sciences, James Cook University, Townsville. The boars were either castrated unilaterally in the School (see section 3.7) or killed at the Charters Towers abattoir. Sperm samples were collected from the rete testis, middle caput, middle corpus and the cauda epididymides (see section 3.3). Each set-up consisted of epididymal spermatozoa from one boar and four oviducts

from two gilts. The oviducts came from about 20-week old, non-cyclic gilts slaughtered at the Charters Towers abattoir. Preparation of oviduct explants and determination of the motility characteristics of spermatozoa were done as described in sections 3.2 and 3.3, respectively. The binding capacity of boar epididymal spermatozoa was also compared between oviducts from sows and gilts (see section 3.7.1). Ten to 12 oviduct explants were used for each sperm sample and each animal treatment. Sows were considered to be in the follicular stage while oviducts were from non-cyclic gilts.

The procedures for this experiment on oviduct binding molecules were outlined in section 3.10. Oviductal explants were replicated six times for each epididymal sperm sample, diluting solution and oviductal regions. The testicles and epididymides from six boars were used in this experiment. The collection and preparation of oviduct explants and epididymal spermatozoa were similar as described in sections 3.2 and 3.3. Epididymal spermatozoa were incubated in 5mg/ml albumin or asialufetuin in Androhep as well as modified Androhep as a control for at least five minutes in a humidified atmosphere containing 5% CO_2 in air at 39^0 C before adding to the oviductal explants. The explants and spermatozoa were incubated for 15 minutes at 39^0 C with 5% CO_2 in air and fixed, mounted and examined as described in section 3.5.

RESULTS

4.2 Binding of ejaculated boar spermatozoa to porcine and bovine oviductal explants

In preliminary studies, a comparison was made of the binding of ejaculated boar spermatozoa to oviductal epithelium from cows and gilts to determine if oviduct explants from cows could be used in place of explants from gilts. Significantly more ejaculated boar spermatozoa attached to isthmic explants than ampullary explants whereas no significant difference was found between bovine isthmic and ampullary explants. Only small numbers of spermatozoa were bound to the tracheal explants (controls) of both species (Fig.4.1).

There was a significant difference in the number of spermatozoa that bound to the isthmus of the pig when compared to the isthmus of the cow. The mean (± SEM) number of spermatozoa that bound to the porcine isthmus was 26.33 ± 2.26 per 1.25 mm^2 while 18.85 ± 1.93 spermatozoa per 1.25 mm^2 were bound to the bovine isthmus. The number of spermatozoa that bound to porcine ampullae was not significantly different to bovine ampullae.

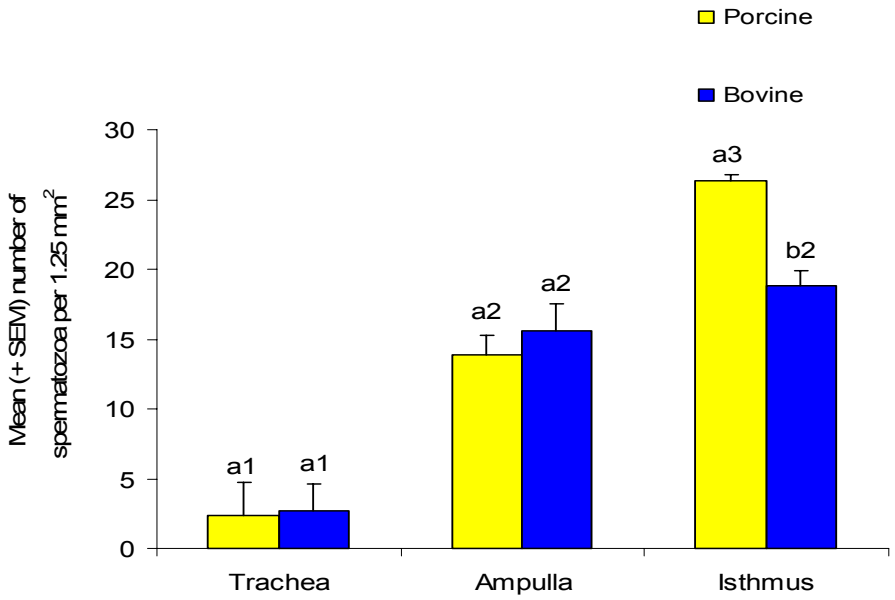

Figure 4.1 Binding of ejaculated porcine spermatozoa to porcine and bovine oviductal and tracheal explants. Different numbers indicate significant difference between types of explants. Different letters indicate significant difference between animal species ($P < 0.05$). N = 121 oviduct explants from 18 gilts and 18 cows for each oviduct region for each animal species; 35 tracheal explants for each animal species; 8 ejaculates from a Large White boar PPG 114.

4.3 Motility of epididymal spermatozoa

The mean percentage of motile spermatozoa from the three regions of the epididymis and the rete testis was determined within a minute of collection (Figure 4.2). The percentage of motile spermatozoa was significantly greater in samples from the cauda and the lowest in samples from the rete testis. While the motility characteristics of spermatozoa were much higher and in the same range in all regions

of the epididymis than in the rete testis, they were not significantly different from each other (Table 4.1).

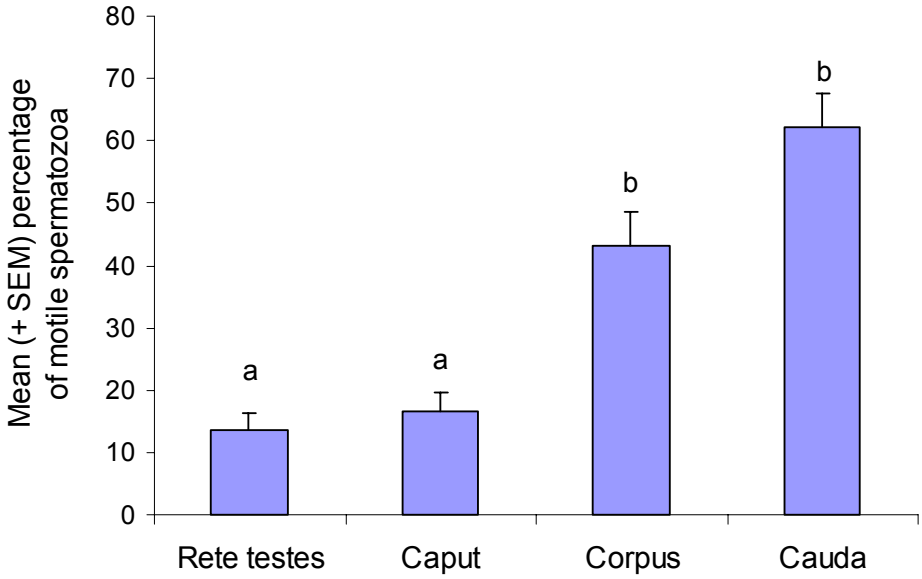

Figure 4.2 The mean (+ SEM) percentage of motile spermatozoa from the rete testis and epididymis. Different letters indicate a significant difference ($P < 0.05$). N = 14 boars.

Table 4.1 Motility characteristics of epididymal spermatozoa immediately after collection.

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

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. No significant differences in the motility parameters between epididymal regions ($P < 0.05$). N = six testicles

4.4 Binding of boar epididymal spermatozoa to the oviductal epithelium of sows and gilts

There were no significant differences in the binding of boar epididymal spermatozoa to oviducts from sows and gilts except for binding of caudal spermatozoa to the isthmus and corpus spermatozoa to the ampulla. While more spermatozoa from the cauda bound to the isthmic explants from sows, more spermatozoa from the corpus bound to the ampullary explants from gilts (Fig. 4.3 and 4.4).

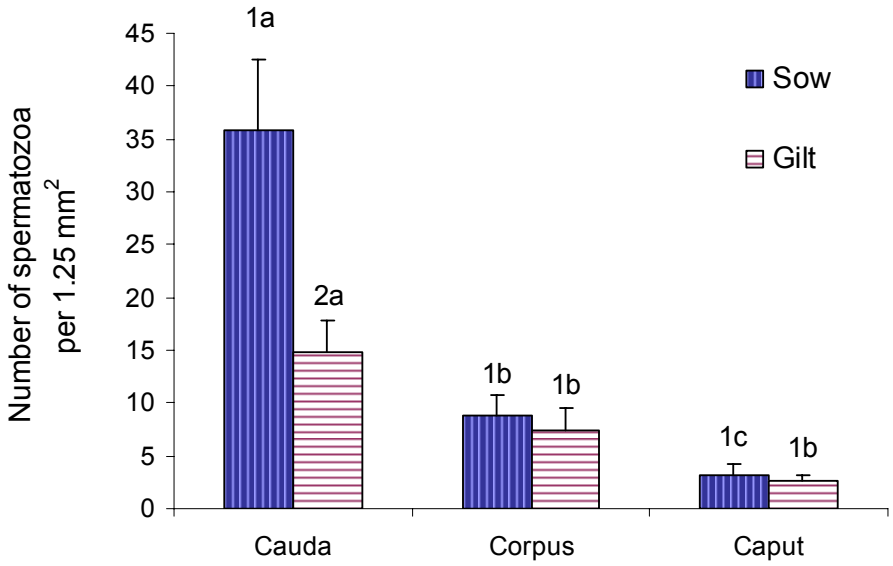

Figure 4.3 Binding of epididymal spermatozoa to the isthmus. Different numbers indicate significant differences between sows and gilts within each epididymal region while different letters indicate significant differences between regions of the epididymis for each type of explants (i.e., sow or gilt). N = 10-12 explants each from two sows and two gilts for each epididymal sperm sample.

4.5 Developmental influence on the binding ability of boar spermatozoa from the rete testis and the caput, corpus and cauda epididymidis to porcine oviducts

The number of spermatozoa that bound to the oviductal epithelium increased progressively from the rete testis to the cauda (Figure 4.5). Significantly more spermatozoa were bound to isthmic than ampullary explants. The number of spermatozoa from the rete testis that bound to isthmic explants was 2 ± 0.30 per 1.25

mm^2. The number of bound spermatozoa increased significantly along the regions of the epididymis with the maximum number of 15 ± 1.22 per 1.25 mm^2 for the cauda. The same was true with the ampullary explants except that the number of bound spermatozoa from the corpus was not significantly different to caudal spermatozoa. There was no significant difference in the number of spermatozoa from the caput that bound to the isthmus when compared to spermatozoa from the cauda epididymidis that bound to the ampulla.

Significantly more ejaculated spermatozoa attached to the isthmus than caudal spermatozoa but there was no significant difference in the binding capacity to the ampulla (Fig.4.6).

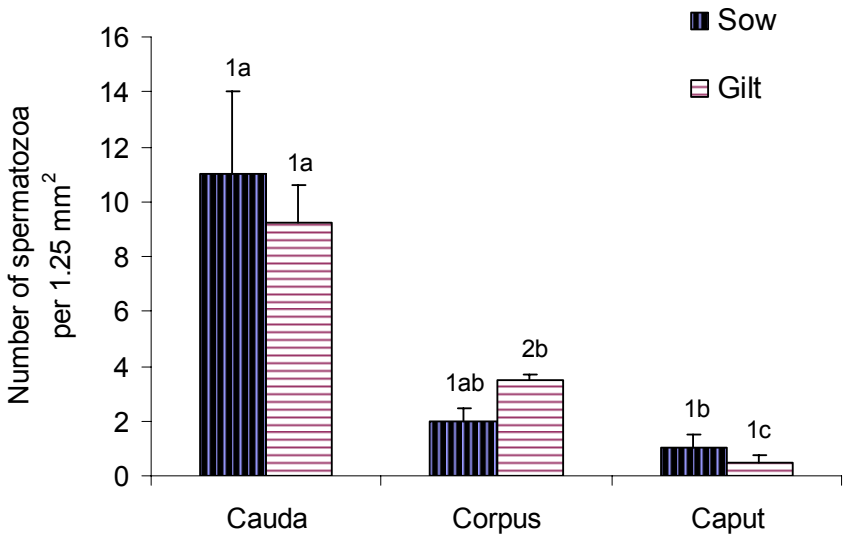

Figure 4.4 Binding of epididymal spermatozoa to the ampulla. Different numbers indicate significant differences between sows and gilts within each epididymal region while different letters indicate significant differences between regions of the epididymis for each type of explants (i.e., sow or gilt). N = 10-12 explants each from two sows and two gilts for each epididymal sperm sample.

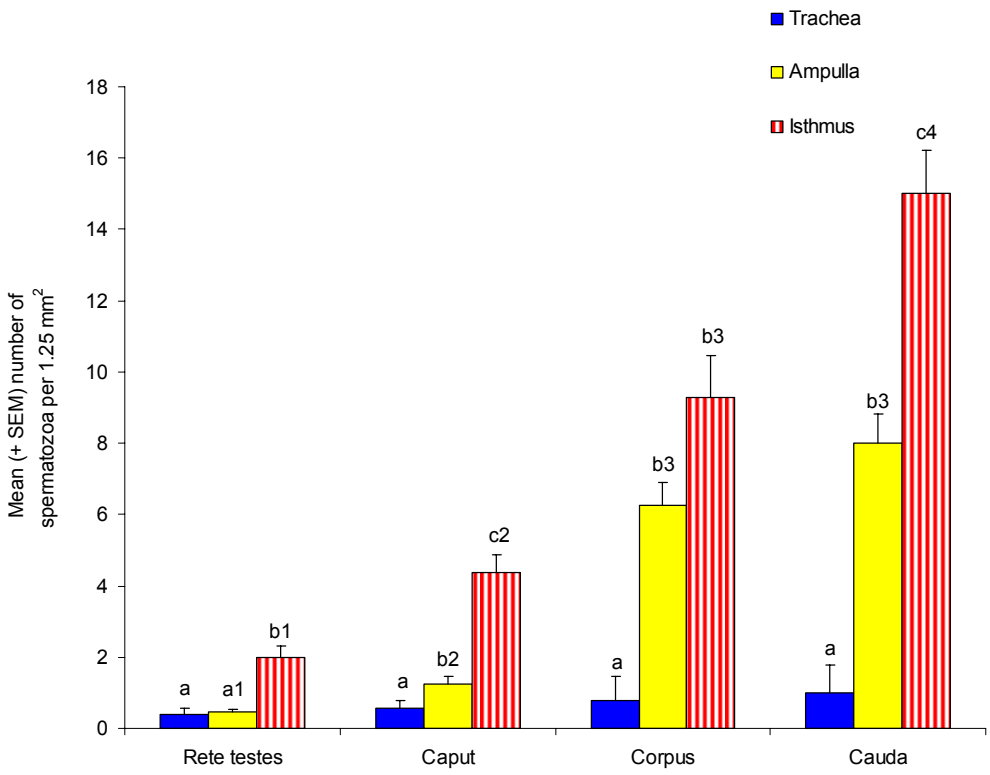

Figure 4.5 Binding of boar spermatozoa from the rete testis and epididymis to the isthmus and ampulla. Histograms with different letters indicate significant differences ($P < 0.05$). N = 84 oviduct explants for each epididymal sperm sample for each oviduct region; 21 tracheal explants for each epididymal region; seven testicles.

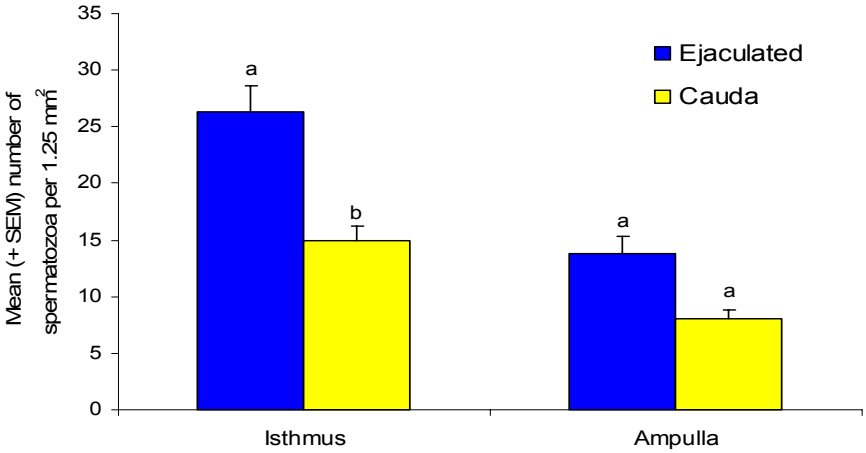

Figure 4.6 Comparison of the binding of ejaculated and caudal spermatozoa to isthmic and ampullary explants. Different letters indicate a significant difference between sperm samples ($P < 0.05$). N = 121 and 84 explants for ejaculated and caudal spermatozoa, respectively; eight ejaculates from a Large White boar PPG 114 for ejaculated spermatozoa and seven testicles for caudal spermatozoa.

4.6 Comparison of the binding capacity of epididymal spermatozoa between boars

While the number of spermatozoa that bound to the isthmus was not significantly different between the seven boars for spermatozoa taken from the rete testes, a significant difference was found between spermatozoa from the caput of boars S1 and S5. Significantly more spermatozoa from the corpus of boars S2 and S3 bound to the isthmus than of boar S5. Significant differences were also apparent for cauda spermatozoa between boars S3 and S5. Overall, epididymal spermatozoa from boar S3 had the highest binding capacity while spermatozoa from boar S5 showed the lowest binding capacity (Fig. 4.7).

The number of spermatozoa from the rete testis and the caput that bound to the ampulla were similar between the boars while marked differences were found in corpus and caudal spermatozoa (Fig. 4.8). The binding of spermatozoa from the corpus was significantly different between boar S2 and boar S5, and between boar S5 and boar S3. In the cauda, significant differences were apparent between boars S2 and S5, boars S5 and S7, and between boars S7 and S9. Similar to the isthmus, bound spermatozoa to the ampulla was the lowest in boar S5 while the highest was in boar S7.

There was a general consistency in the number of spermatozoa between the left and the right testicle of each boars that bound to isthmic and ampullary explants except for boar S5 and boar S7 (Figures 4.9 and 4.10). In boar S5, significantly more spermatozoa from the caput and cauda of the left testicle bound to the isthmus as well as spermatozoa from the corpus and cauda that bound to the ampulla than spermatozoa from the same epididymal regions of the right testicle. Significantly more caudal spermatozoa of the right testicle of boar S7 bound to the ampulla than caudal spermatozoa from the left testicle.

4.7 Carbohydrate-binding molecules recognized by epididymal spermatozoa

There was a significant inhibition of binding of epididymal spermatozoa by medium containing ovalbumin or asialufetuin compared with the control modified Androhep medium (Figures 4.11 and 4.12). The inhibitory effect was similar for explants from

the isthmus and ampulla. There was no significant effect of incubation on binding of spermatozoa from the rete testis by ovalbumin or asialofetuin compared to the control.

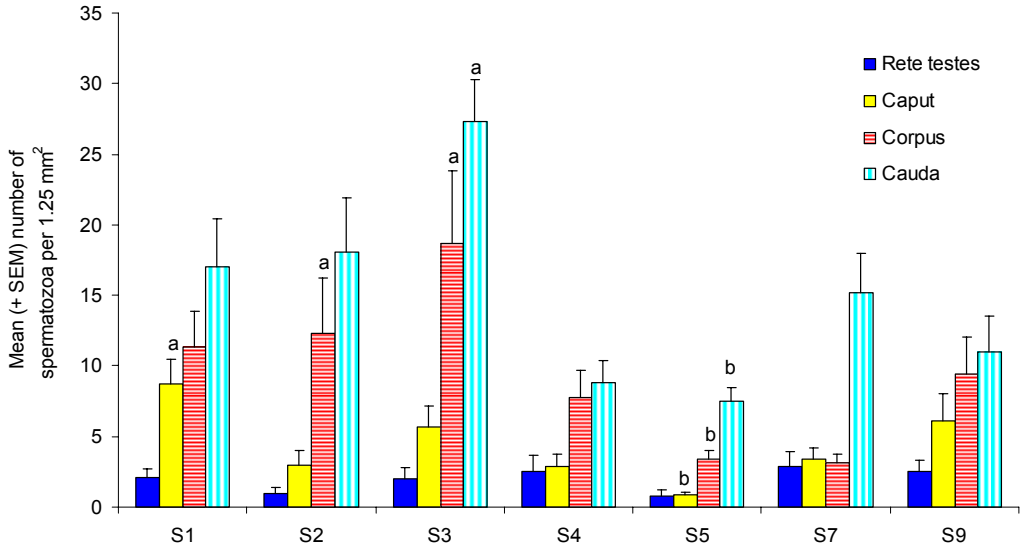

Figure 4.7 Comparison between boars in the binding of spermatozoa from the rete testis and epididymis to isthmic explants. Different letters indicate significant differences between boars within a particular sperm sample ($P < 0.05$). N = 12 explants for each boar; seven boars.

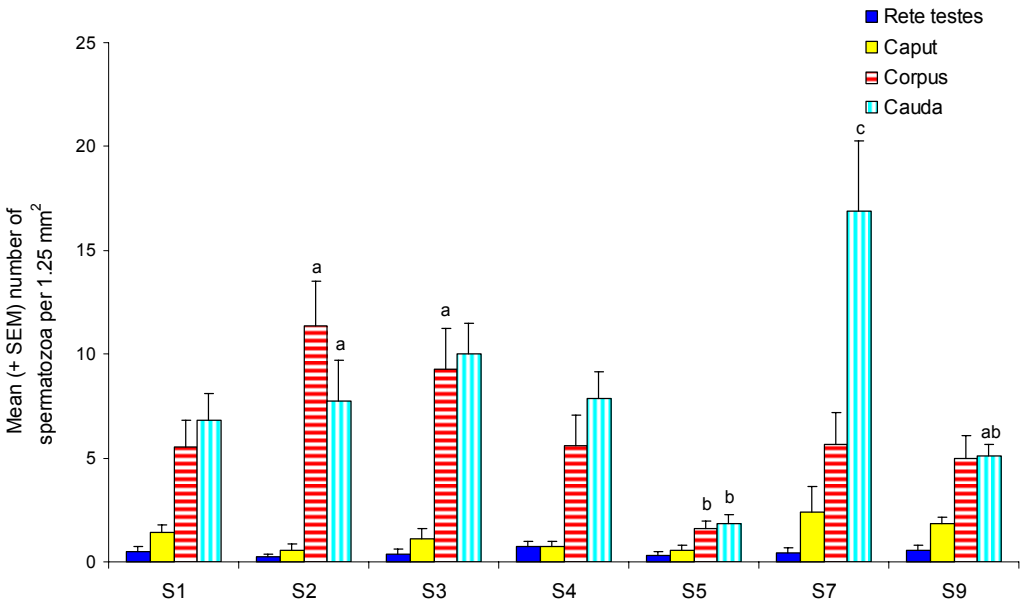

Figure 4.8 Comparison between boars in the binding of spermatozoa from the rete testis and epididymis to ampullary explants. Different letters indicate significant differences between boars within a particular sperm sample ($P < 0.05$). N = 12 explants for each boar; seven boars.

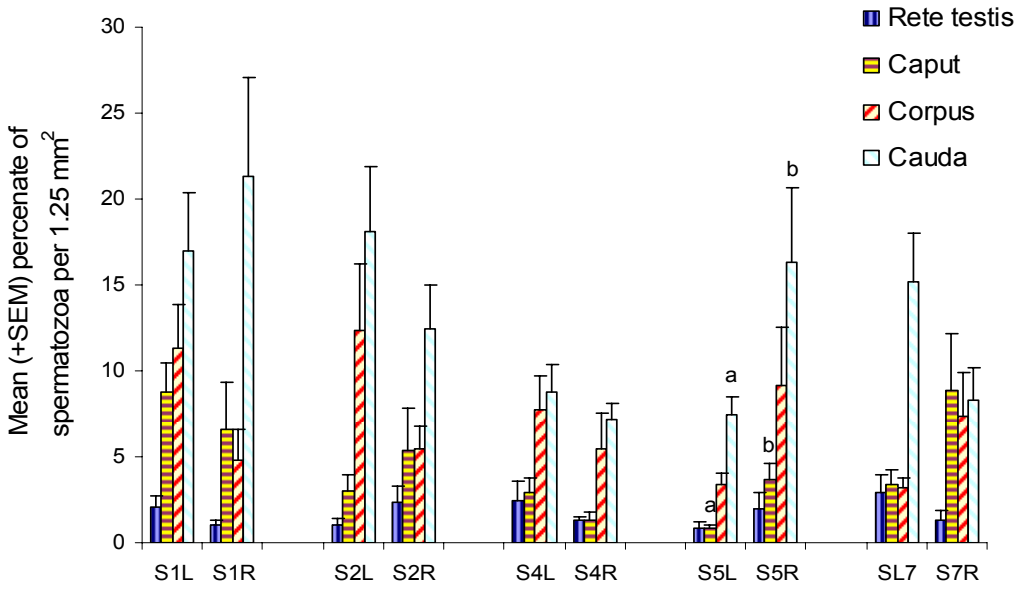

Figure 4.9 Comparison between the left and the right testicles of boars in the binding capacity of spermatozoa to isthmic explants. N = 12 and six explants for each sperm sample and for the left and the right testicle, respectively. L = left; R = right. N= five boars. Different letters indicate significant differences ($P < 0.05$).

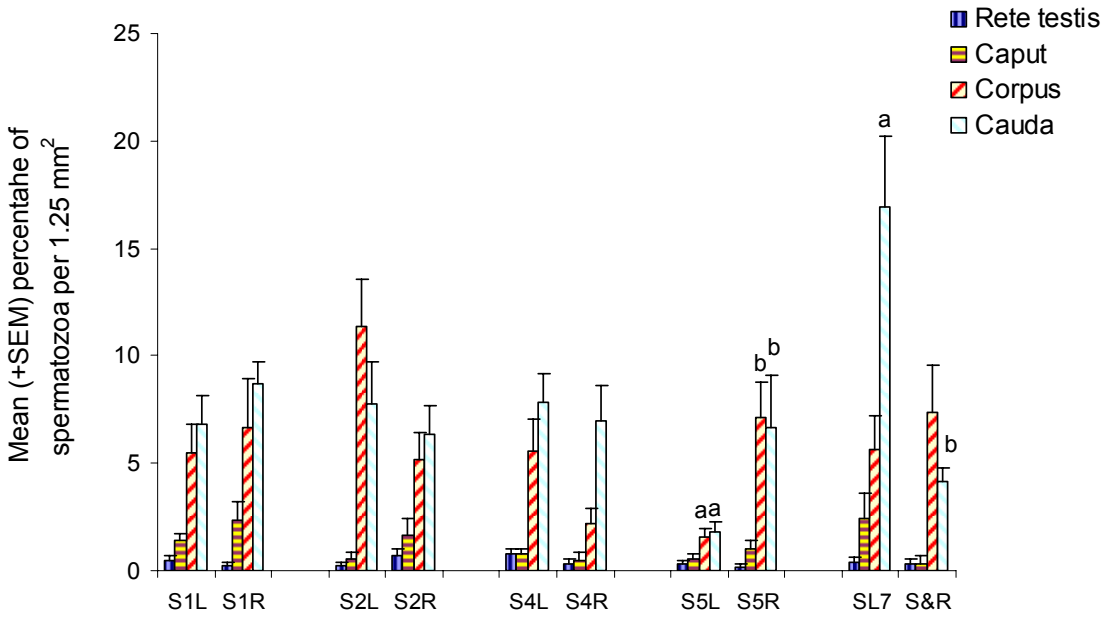

Figure 4.10 Comparison between the left and the right testicles of boars in the binding capacity of spermatozoa to ampullary explants. N = 12 and six explants for each sperm sample and for the left and right testicle of each boar respectively. L = left; R = right. N = five boars. Different letters indicate significant differences ($P < 0.05$).

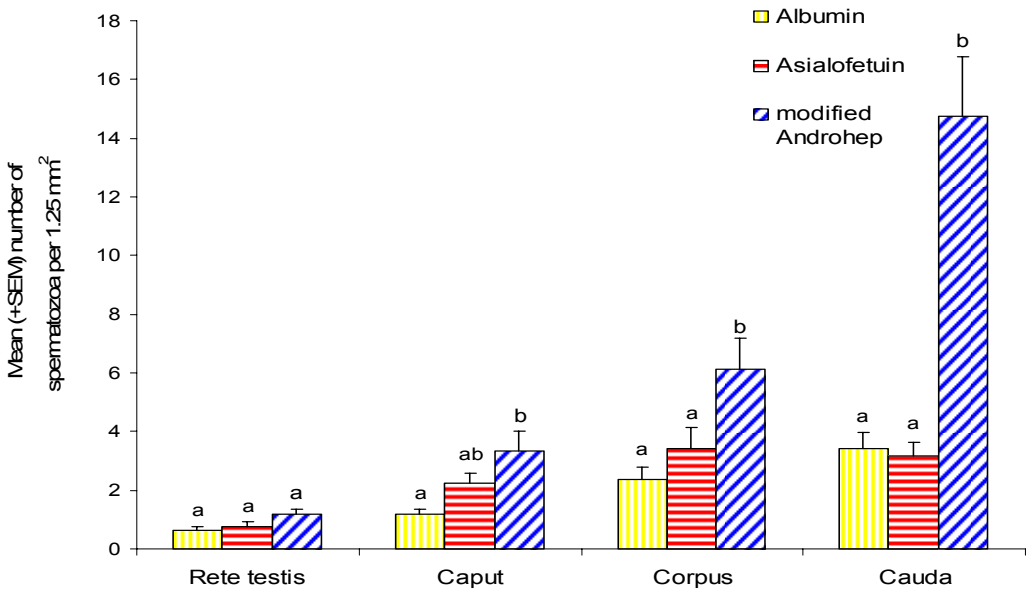

Figure 4.11 The binding of epididymal spermatozoa to isthmic explants after incubation of spermatozoa in medium containing either albumin, asialofetuin or modified Androhep medium. Different letters indicate significant differences between the three media at each epididymal region ($P < 0.05$). N = 31 explants for each sample of spermatozoa and each oviductal region. Six testicles.

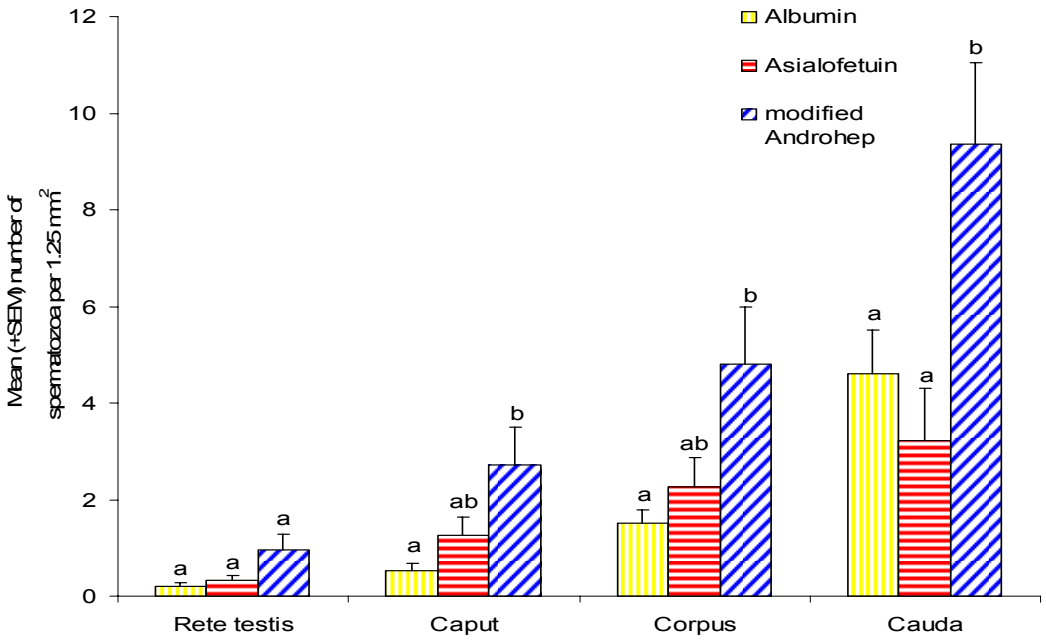

Figure 4.12 The binding of epididymal spermatozoa to ampullary explants after incubation of spermatozoa in medium containing either albumin, asialofetuin or modified Androhep medium. Different letters indicate significant differences between the three media at each region ($P < 0.05$). N = 31 explants for each sample of spermatozoa and for each oviductal region. Six testicles.

DISCUSSION

There was a sequential increase in the number of spermatozoa that bound to the oviductal epithelium from the rete testis to the cauda epididymidis. The sequential increase in binding occurred to explants from both the isthmus and ampulla with significantly more spermatozoa from all epididymal sites binding to the isthmus. These results imply that there are developmental changes to spermatozoa as they pass through the epididymis which will increase the number of spermatozoa that are capable of binding to oviductal epithelium.

There are limited reports in the literature on the binding of epididymal spermatozoa to oviductal epithelium and no reports have been found that have examined the binding of spermatozoa from different sites of the epididymis. It is known that in the pig the number of caudal spermatozoa that bind to oviductal explants is about half that of ejaculated spermatozoa (Petrunkina *et al.*, 2001b). The results obtained in this current study are consistent with the work of Petrunkina *et al*. (2001b).

Given that the evidence in the literature indicates that carbohydrate-recognition mechanisms are involved in sperm-oviduct binding (Topfer-Petersen, 1999; Green *et al.*, 2001; Suarez, 2001), then there are several possible ways by which spermatozoa could develop these mechanisms. One possibility is that the molecules are secreted by epididymal epithelium and attach to the head of spermatozoa. In the pig, one such carbohydrate-binding molecule is AWN which is found on epididymal spermatozoa and seems to accumulate on spermatozoa as they travel along the epididymis (Dostalova *et al.*, 1994; Ekhlasi-Hundrieser *et al.*, 2002). Alternatively, secretions of the epididymal epithelium structurally modify pre-existing molecules of the apical region of spermatozoa so that they acquire the ability to bind to carbohydrates on the surface of oviductal epithelium (Gatti *et al*., 2004).

The use of a heterologous system for studies on the binding of spermatozoa to oviductal epithelium has been examined in several research protocols including binding of human spermatozoa to oviducts of cattle and macaques (Ellington *et al.*, 1998), canine spermatozoa to porcine oviducts (Petrunkina *et al.*, 2004) and cryopreserved stallion spermatozoa to oviductal cells of cattle (Ellington *et al.*,

1999). Heterologous systems have been used mostly for logistical reasons. This is particularly true in studies with human spermatozoa where an adequate supply of disease-free oviduct tissues from women is not possible (Ellington *et al.*, 1998). Therefore when variation between species is minimal, much of the effort, time and cost of the study can be reduced.

There have been no reports on the ability of porcine spermatozoa to bind to oviductal epithelium of cows. The possible use of bovine oviducts for the work described in this study was investigated because of their ready availability from a large cattle abattoir a short distance from the laboratory whereas the nearest source of porcine oviducts was from an abattoir 130 km from the laboratory. While the number of canine spermatozoa that bound to canine and porcine oviducts was similar (Petrunkina *et al.*, 2004), the number of ejaculated boar spermatozoa that bound to the isthmus of cows was significantly less than those bound to the isthmus of gilts although there was no significant difference in the number that bound to the ampulla. This result implies a strong species specificity in the binding property of boar spermatozoa relative to carbohydrate-binding lectins and glycoconjugates present in the plasma membrane of spermatozoa and oviductal epithelium. Carbohydrate-binding lectins and glycoconjugates present on the plasma membrane of the head of spermatozoa and the surface of oviductal epithelium may vary considerably among species (Demott *et al.*, 1995; Lefebvre *et al.*, 1997; Suarez, 2001). Based on this result, it was concluded that it was necessary to use porcine oviducts for studies on the binding capacity of spermatozoa from the epididymis despite the increased cost and logistical difficulties of using porcine oviducts.

It was necessary to use oviducts from pre-pubertal gilts in these studies. There were two reasons for this. The literature reports that there is no difference in the number of ejaculated boar spermatozoa that bind to oviducts from gilts compared to cyclic sows (Petrunkina *et al.*, 2001b) although the authors didn't specify the age of the gilts and whether they were pre-or post-pubertal. The second reason was that the abattoir mostly only killed pigs up to about 20 weeks of age. Sows culled for old age or infertility were only occasionally killed and thus a consistent supply of sow oviducts could not be assured. Nevertheless, there was an opportunity to obtain oviducts from two sows of known history to compare the binding of spermatozoa with that of gilts.

Unlike the results of Petrunkina *et al*.(2001b), there was preferential binding of caudal spermatozoa to the isthmus from sows but not to the ampulla. However, oviducts from more sows need to be examined to confirm this result. A different mechanism might be involved in the physiology of sow oviducts to secure more spermatozoa as a reservoir in the isthmus thus ensuring a higher fertility potential. Nevertheless, it is interesting to find that spermatozoa are able to bind to immature oviducts signifying the unique microanatomy and biochemistry of the oviductal epithelium. It could be assumed that as the animal matures and the oviduct is exposed to ovarian hormones, significant changes occur in the oviduct both histologically and biochemically favoring increased spermatozoal attachment.

Studies that have used binding assays have employed different strategies in the utilization of oviductal epithelium. Hormone treatments and the use of oviductal epithelial monolayers cultured *in vitro* have been successfully demonstrated in various animals (Pollard *et al.*, 1991; Green *et al.*, 2001; Petrunkina *et al.*, 2003). The *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 considerable differences are highly likely between *in vivo* and *in vitro*. Epithelial cultures also have problems of overgrowth by non-epithelial cells (Raychoudhury and Suarez, 1991) as well as loss of binding capacity on repeated culture (Green *et al.*, 2001). For these reasons and to mimic as much as possible the *in vivo* conditions, oviductal explants were used to preserve the integrity of the oviduct mucosa.

More spermatozoa, either ejaculated spermatozoa or those obtained from the epididymis, bound to the isthmus than the ampulla. The reason for this is not entirely very clear but may be attributed to differences in the epithelial structure, regional secretions and biochemical features between the isthmus and the ampulla. Other workers have found no differences in the binding capacity of spermatozoa to the isthmus and ampulla of pigs (Suarez *et al.*, 1991; Petrunkina *et al.*, 2001b) or cattle (Lefebvre *et al.*, 1995), although Raychoudhury and Suarez (1991) 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 oviductal explants. However, the difference between the isthmus and

ampulla could be due to secondary effects of long-term culture conditions or due to the influence of sticky secretions of the isthmus coating the ampullary epithelium that is typical *in vivo* but not *in vitro* after two weeks of culture (Raychoudhury and Suarez, 1991). More spermatozoa from the horse and human were also reported to bind to the isthmus than to the ampulla (Thomas *et al.*, 1994a; Baillie *et al.*, 1997). Regional differences in the expression of glycoconjugates are apparent between segments of the porcine oviduct and the stage of the oestrous cycle as binding sites for *Lotus tetragonolobus*, a lectin which specifically binds to L-α-L-fucose, can only be seen in isthmic epithelial cells (Walter and Bavdek, 1997). On the other hand, binding sites for *Triticum vulgaris*, a lectin which binds to D-*N*-acetyl-glucosamine were predominant on the infundibulum and ampullary ciliated epithelial cells and only during oestrus.

The current study found significant differences between boars in the capacity of spermatozoa to bind to oviductal epithelium. These differences imply that individual differences in the level of fertility between boars could be attributed to the number of spermatozoa that form the sperm reservoir. A similar observation has been made by other workers in pigs (Petrunkina *et al.*, 2001b) and in the horse (Thomas *et al.*, 1994a). It was interesting to find that there was a general consistency in the binding of epididymal spermatozoa from the right and left testicle of the same boar. This would be expected as the distribution of androgens to the left and the right epididymis should be the same and therefore the secretory functions of the epididymis, and as a consequence the maturation events of spermatozoa, should be the same in the left and right epididymis.

The carbohydrate-binding molecules of epididymal spermatozoa were examined by allowing spermatozoa to bind to carbohydrate parts of albumin and asialufetuin molecules. As the molecular sites on spermatozoa that bind to carbohydrates on oviduct cells are already bound with albumin or asialufetuin molecules, they are unable to bind to oviduct epithelium. This experiment aimed to demonstrate indirectly what carbohydrate molecules on oviduct epithelial cells the epididymal sperm binding molecules will recognize. The results were consistent with the work of Wagner *et al.* (2002). Both albumin and asialofetuin significantly reduced the binding of spermatozoa to oviduct epithelium. The ovalbumin that was used in

current study contains 10 mannose residues per mole (Sigma, 2002). Ovalbumin has one N-glycosylation site and contains two high mannose type glycans [(Man)(5)(GlcNAc)(2) and (Man)(6)(GlcNAc)(2)] and a minor hybrid type glycan [(Hex)(4)(GlcNAc)(5)] while bovine asialufetuin contains three N-glycosylation sites (with Galb1, 4GlcNAc antennae) and three O-glysosylation sites (containing Galb1,3GalNAc with sialic acid in the a2,3 and a2,6 positions in fetuin) (Harvey *et al.*, 2000; Wagner *et al.*, 2002). In carbohydrate-binding studies in pigs, mannose as well as maltose and lactose inhibited the binding of porcine spermatozoa to oviductal epithelium (Green *et al.*, 2001; Wagner *et al.*, 2002). The reduction in the number of epididymal spermatozoa that bound to oviduct explants may therefore be attributed to mannose from ovalbumin and lactose from asialufetuin. While the literature does not provide any specific information on the carbohydrate binding characteristics of epididymal spermatozoa, the results suggest that ejaculated as well as epididymal spermatozoa have similar carbohydrate binding properties. This further implies the importance of epididymal maturation of spermatozoa for the acquisition of binding molecules that recognize mannose residues.

One important contributing factor in the fertilising capacity of spermatozoa is motility (Hafez and Hafez, 2000). The development of motility potential by spermatozoa during their maturation in the epididymis has been fully established (Yanagimachi, 1994). The current study found a progressive increase in the motility of spermatozoa from the caput to the cauda epididymidis. This is consistent with the fact that a number of maturational aspects that occur to spermatozoa during their maturation in the epididymis relate to the development of their motility potential (reviewed by Setchell *et al.*, 1993; Jones and Murdoch, 1996) These include the increase in cAMP concentrations and the Ca^{2+}-ATPase activity during the epididymal transit (Hoskins *et al*., 1975) decrease in the intracellular pH (Gatti *et al*., 1993), decrease in the concentration of free calcium ions and the development of glucose transport into spermatozoa (Hiipakka and Hammerstedt, 1978) and the lowered exchange rate of calcium ions into the mitochondria (Vijayaraghavan and Hoskins, 1990). It was reported that the cAMP content of ram spermatozoa from the caput and corpus were significantly lower than spermatozoa from the distal cauda (Amann *et al.*, 1982). Also, when cAMP was added to immature spermatozoa from the caput of the bull, there was an observed improvement in the motility potential of spermatozoa

which was further increased by the addition of forward motility protein (Acott and Hoskins, 1981). The forward motility protein was found to bind to spermatozoa in the caput and become concentrated in spermatozoa at the cauda epididymidis (Acott and Hoskins, 1981). This shows how important epididymal maturation is for the acquisition of motility potential of spermatozoa, a key feature which determines the fertility of male.

In conclusion, this study demonstrated that boar spermatozoa from the rete testis and the epididymis are able to bind to the isthmus and the ampulla of gilts and sows. Significantly more spermatozoa from the cauda epididymidis bound to oviduct epithelium than the remainder of the epididymal regions and more bound to the isthmus than the ampulla.

CHAPTER 5

EXPOSURE TO CAUDAL EPIDIDYMAL FLUID INCREASES THE BINDING ABILITY OF SPERMATOZOA FROM THE CORPUS AND THE CAPUT EPIDIDYMIDIS

5.1 Introduction

As the cauda is the storage site of spermatozoa prior to ejaculation, it is logical to assume that this would be the part of the epididymis where most of the ability to bind to oviductal epithelium would develop. The data presented in Chapter 4 indicate that the number of caudal spermatozoa that bound to oviduct explants was substantially greater than spermatozoa from the corpus and caput. These data could be interpreted to mean that the increase in the binding of caudal spermatozoa was induced by secretory products of the caudal epithelium or through an interaction of secretory products of the corpus and caput with those of the cauda.

There are two important events that occur as spermatozoa pass along the epididymal tract. These are the loss and addition of certain proteins from and onto the sperm surface (Brooks and Tiver, 1984). Lost proteins from the sperm surface are accumulated in the epididymal plasma while those proteins that are added onto the sperm surface come from epididymal secretions (Brooks and Tiver, 1984). Being the last segment of the epididymis, it follows that many secretory products from the caput and corpus will accumulate in the cauda. For this reason, it is likely that the caudal fluid would be substantially different from other regions of the epididymis. Spermatozoa are stored in the cauda epididymidis for several days or weeks (Setchell *et al.*, 1993; Jones and Murdoch, 1996) so there is an ample opportunity for secretory products of the cauda to influence spermatozoa.

There are many products in caudal fluid some of which are secreted under the influence of androgens (De Pauw *et al.*, 2003). Among the important secretory products that are found to be highly expressed in the cauda is the cysteine-rich secretory protein (CRISP) (Udby *et al.*, 2005), a family of proteins that are involved in spermiogenesis, capacitation and binding of the spermatozoon to the oocyte.

Factors have been also identified in the cauda epididymal fluid that appear to sustain motility of bovine spermatozoa *in vitro* (Reyes-Moreno *et al.*, 2002) as well as proteins that are associated with fertility in dairy bulls (Moura *et al.*, 2006). The cauda as well as the corpus of the hamster also secretes HEP64, a 64 kDA glycoprotein that is able to bind specifically to dead and/or dying spermatozoa (NagDas *et al.*, 2000).

The aim of this experiment was to determine whether the incubation of caput and corpus spermatozoa with caudal fluid would increase the number of spermatozoa that bound to oviductal explants.

MATERIALS AND METHODS

The oviduct explants were replicated six times for each epididymal sperm sample or treatment (i.e., caput spermatozoa in modified Androhep; caput spermatozoa in caudal plasma; corpus spermatozoa in modified Androhep; corpus spermatozoa in caudal fluid; and cauda spermatozoa in modified Androhep) and between the isthmus and the ampulla. Seven testicles and epididymides from boars either castrated unilaterally at the School of Veterinary and Biomedical Sciences or killed at the Charters Towers abattoir were used.

After the arrival of oviducts and testicles at the laboratory, some of the contents of the cauda epididymidis was first collected into an Eppendorf tube and centrifuged for 30 minutes at 1200 ***g.*** Collection of the caudal fluid was done by making an incision in the cauda with a scalpel blade and the caudal semen was either aspirated with a tuberculin syringe or allowed to flow directly into an Eppendorf tube. The centrifugation was repeated to fully extract the caudal fluid. While centrifugation was underway, the oviductal explants were prepared as described in section 3.2. After centrifugation, the supernatant (the caudal fluid) was aspirated and placed into two separate Eppendorf tubes, one to be used for caput spermatozoa and the other for corpus spermatozoa. Sperm samples were then collected from the caput, corpus and cauda and diluted in either modified Androhep or the caudal fluid. Cauda spermatozoa were only diluted with the modified Androhep. The sperm samples

were incubated in a humidified atmosphere containing 5% CO_2 in air at 39^0 C for 30 minutes and then were analyzed for sperm concentration and motility characteristics (see section 3.3). Before adding to oviduct explants, spermatozoa were prepared to a concentration of 5 x10^6/ml as described in section 3.8. The remainder of the procedures including co-incubation and fixation of explants and the counting of bound spermatozoa were undertaken as in previous experiments (see also sections 3.5 and 3.5).

RESULTS

5.2 Motility of epididymal spermatozoa after incubation in caudal fluid

The percentage of motile epididymal spermatozoa as well as the motility characteristics was assessed immediately after incubation with either modified Androhep or caudal fluid (Figure 5.1; Table 5.1). Caudal spermatozoa had the highest percentage of motile spermatozoa followed by spermatozoa from the corpus and the caput. However, when spermatozoa from the corpus but not in caput were incubated with caudal fluid, there was a significant reduction in their motility when compared to spermatozoa incubated with modified Androhep medium.

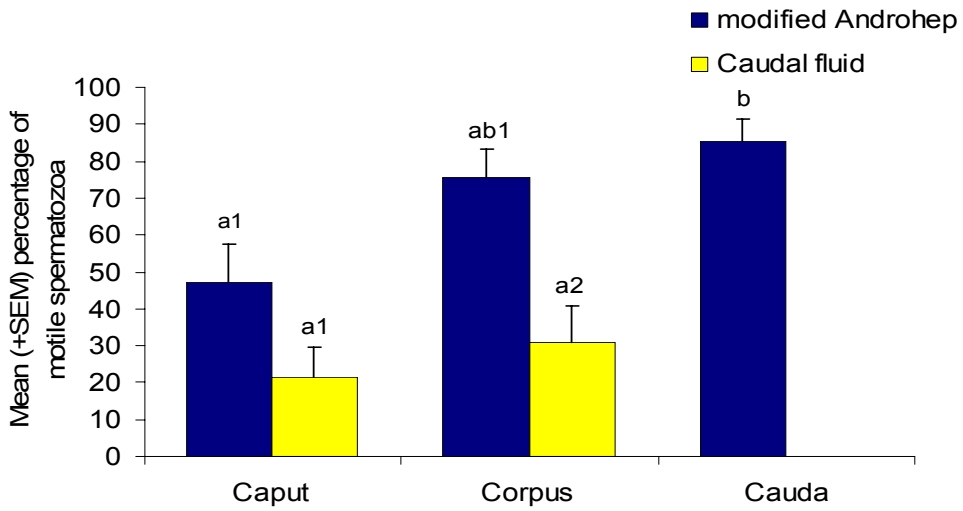

Figure 5.1 The reduction in the motility of spermatozoa from the caput and the corpus after incubation in caudal fluid. Different letters indicate a significant difference between sperm samples while different numbers indicate a significant difference between the caudal fluid and modified Androhep medium ($P < 0.05$). N= five and six epididymides for caput and corpus samples, respectively.

The average path velocity, straight-line velocity and curvilinear velocity of spermatozoa from the caput were significantly higher in modified Androhep medium than in caudal fluid. In the corpus, only the curvilinear velocity and the beat cross frequency were significantly higher in modified Androhep medium than in caudal fluid.

Table 5.1 Motility characteristics (mean ± SEM) of epididymal spermatozoa after incubation in either modified Androhep medium or caudal fluid.

Motility Parameters	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	15.6±4.25	21.48±8.05	19.35±1.64[a]	9.95±2.34[b]	20.50±0.89
STR	72.8±2.84	65.20±16.18	70.50±1.65	62.50±12.57	69.67±1.12
LIN	46.2±3.76	45.60±11.52	41.17±2.75	41.17±8.57	39.33±1.05

mAndro = modified Androhep medium; CF = caudal fluid; 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 significant differences between caput and corpus spermatozoa ($P < 0.05$); N = five and six epididymides for caput and corpus, respectively.

5.3 Binding of epididymal spermatozoa after incubation in caudal fluid

As previously described in Chapter 4, the binding of caudal spermatozoa to either the isthmus or the ampulla was significantly greater than spermatozoa from the rest of the epididymal duct. However, when spermatozoa from either the caput or corpus were incubated with caudal fluid, there was a significant increase in their binding capacity to both isthmic and ampullary explants (Figure 5.2 and Figure 5.3). In all cases, the binding of epididymal spermatozoa to the isthmus was significantly higher than with ampulla.

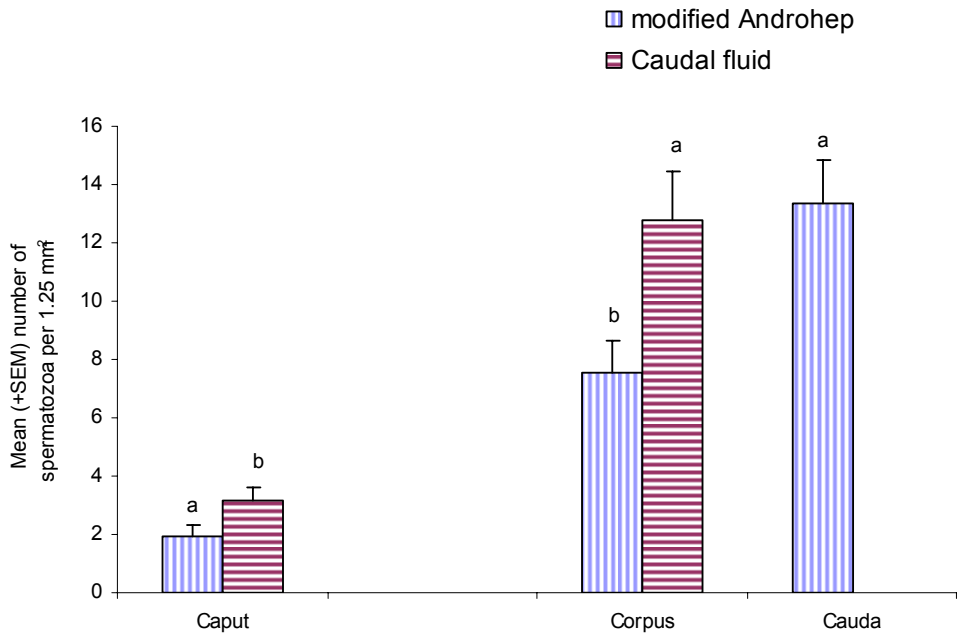

Figure 5.2 The influence of incubation of epididymal spermatozoa in caudal fluid on the binding of spermatozoa to isthmic explants. Different letters signify significant differences between the caudal fluid and modified Androhep medium ($P < 0.05$). N =36 explants for each epididymal sperm sample and each oviductal region; six and seven epididymides for caput and corpus samples, respectively.

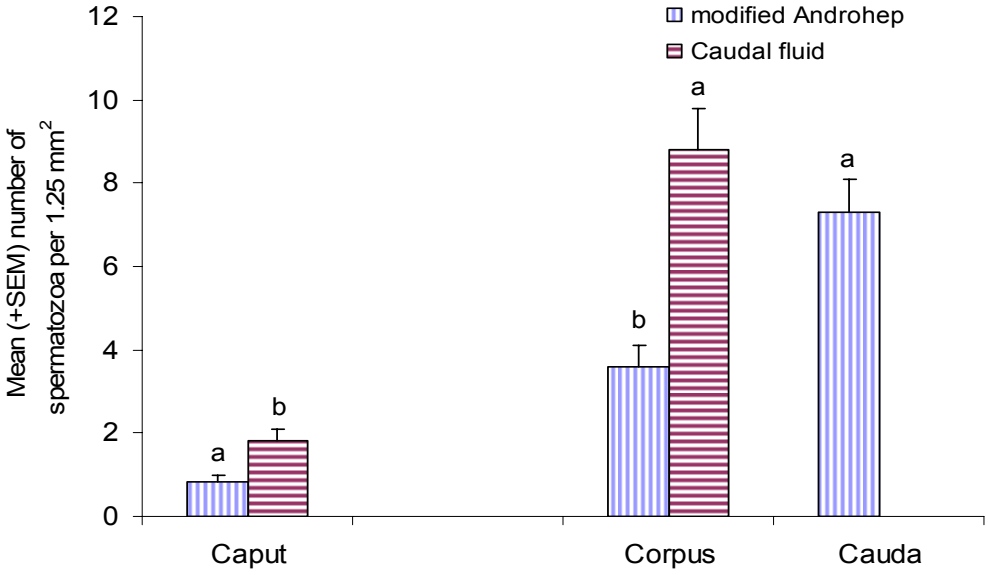

Figure 5.3 The influence of incubation of epididymal spermatozoa in caudal fluid on the binding of spermatozoa to ampullary explants. Different letters signify significant differences between the caudal fluid and modified Androhep medium ($P < 0.05$). N =36 explants for each epididymal sperm sample and each oviductal region; six and seven epididymides for caput and corpus samples, respectively.

DISCUSSION

Incubation of spermatozoa from the corpus with caudal fluid for 30 minutes is able to increase the number of spermatozoa that bound to the isthmus and ampulla to a level comparable with spermatozoa from the cauda. The same but lesser effect occurred with spermatozoa from the caput. This indicates that caudal fluid possesses some distinct features that directly or indirectly influence the interaction between spermatozoa and oviduct epithelium. It would be interesting to know if the same effect could be pronounced by only a short exposure (eg. one minute) of spermatozoa to caudal fluid.

Spermatozoa need binding receptors which could be in the form of glycoproteins to bind to carbohydrate ligands on the oviduct epithelium. The increase in the binding capacity of caput and corpus spermatozoa can be largely attributed to these lectin-like molecules. Caudal fluid contains a number of glycoconjugates that can be detected in lectin-binding studies (Arenas *et al.*, 1996) and it is highly likely that spermatozoa from the caput and corpus acquired these binding molecules during incubation with caudal fluid.

While it can be argued that not all the glycoconjugates are directly produced in the cauda, it is likely that during spermatozoal passage, some glycoconjugates from the proximal epididymis are transported in epididymal plasma down to the cauda and are made even more available for spermatozoa during storage. Acquisition of glycoconjugates by spermatozoa has been readily demonstrated in lectin-studies in mice (Rankin *et al.*, 1989).

The incubation of caput and corpus spermatozoa in caudal fluid may also led to the acquisition of fertility-related glycoproteins (Schroter *et al.*, 1999). In human spermatozoa, this glycoprotein is called “major maturation antigen” (Moore *et al.*, 1989) or sperm membrane glycoprotein (Eccleston *et al.*, 1994) because it was only detected in caudal spermatozoa and is not found in testicular (Brown *et al.*, 1983) or caput spermatozoa (Jones, 1989). A fertility factor has been also described in spermatozoa of dairy bulls (Moura *et al.*, 2006). The specific region from where this protein originates is still unclear but it would have been transferred from the

epididymal epithelium onto the sperm surface during epididymal transit (Kirchhoff and Hale, 1996). Since this protein is only found in caudal spermatozoa, it is logical to think that spermatozoa have acquired it during their long stay in the cauda. While it is not yet known to which specific ligands this protein has the highest affinity (oviduct or zona pellucida ligands), it is likely that this protein also promotes binding of spermatozoa to oviduct epithelium in a manner that is not yet known and increases the number of spermatozoa attached to the isthmus thus improving fertility. In the same manner, caput and corpus spermatozoa may also acquired this protein during incubation in caudal fluid which would improve their ability to bind to oviduct explants.

Spermatozoa stored within the cauda epididymidis need to be held in an immotile state to avoid exhaustion of energy reserves. The way epididymal fluid acts upon this is complex and not fully understood (Setchell *et al.*, 1993). It is therefore not surprising that the percentage of motile spermatozoa and the motility characteristics of spermatozoa incubated in caudal fluid should be considerably less than those spermatozoa incubated in modified Androhep medium.

Another feature of the caudal fluid which may have reduced the motility of caput and corpus spermatozoa after incubation is the pH and the bicarbonate concentration. The pH of the caudal fluid of the boar (pH 6.5) is lower than in more proximal regions (pH 7.2) and the concentration of bicarbonate (3-4 mM) is considerably less than the rete testis fluid (30 mM) (Rodriguez-Martinez *et al.*, 1990). These factors are known to have a role in ensuring spermatozoa in the cauda are quiescent (Acott and Carr, 1984; Rodriguez-Martinez *et al.*, 1990; reviewed by Setchell *et al.*, 1993).

In conclusion, this study demonstrated the fluid of the cauda epididymidis of the boar is able to modify the glycocalyx of caput and corpus spermatozoa to increase the number of spermatozoa that bound to oviduct explants. Whether this is because of structural modification of the glycocalyx or addition of glycoproteins or oligosaccharides to spermatozoa requires further investigation.

CHAPTER 6

INFLUENCE OF SEMINAL PLASMA ON THE BINDING ABILITY OF EPIDIDYMAL SPERMATOZOA

6.1 Introduction

Spermatozoa mix with the secretions from the cauda epididymidis and the accessory sex glands long before and upon ejaculation, respectively. These secretions are collectively called the seminal plasma. This leads to a direct contact of the plasma membrane of the spermatozoon with different components of the seminal plasma to form a coating over the acrosomal region (reviewed by Topfer-Petersen, 1999; Haase *et al.*, 2005). The fact that the seminal plasma contains variable amounts of different molecules and biological substances means that seminal plasma could influence the capacity of spermatozoa to bind to oviductal epithelium.

In *in vitro* studies, about twice as many ejaculated boar spermatozoa bound to oviduct explants than spermatozoa from the cauda (Petrunkina *et al.*, 2001b). Nevertheless, the ability of cauda spermatozoa and those from the more proximal regions of the epididymis to bind to oviduct epithelium suggests that some or all of the caudal epididymal spermatozoa have an inherent capacity for attachment to oviduct epithelium and are not solely dependent upon the seminal plasma. The maturation processes that occur to spermatozoa during their passage in the epididymal duct would be expected to have a key role in the acquisition of this binding capacity. Carbohydrate binding molecules may be acquired by spermatozoa from secretions of the epididymis or pre-existing molecules on spermatozoa are modified to expose carbohydrate binding sites during the epididymal maturation. Many studies have reported that seminal plasma contains lectin-like molecules belonging to the spermadhesin group of proteins. These proteins coat the plasma membrane of the sperm head during ejaculation and act as receptors to carbohydrate ligands present on the oviduct epithelium (Sanz *et al.*, 1993; Dostalova *et al.*, 1994; Dostalova *et al.*, 1995b; Ekhlasi-Hundrieser *et al.*, 2005).

The aim of this study is to determine whether incubation of epididymal

spermatozoa with seminal plasma would increase the number of spermatozoa that bound to oviduct explants.

MATERIALS AND METHODS

About four to five weeks after the unilateral castration for studies in Chapter four and five, the boars were slaughtered at the Charters Towers abattoir. The right testicle, seminal vesicles and oviducts from two pre-pubertal gilts were collected into separate plastic bags, placed in a polystyrene box with an ice pack and transported to the laboratory in an air-conditioned car. Upon arrival, a small incision was made into the seminal vesicles and the seminal plasma was collected into an Erlenmeyer flask, covered and refrigerated at 4^0 C while the oviducts and epididymal sperm samples were prepared (see section 3.2). Five testicles from five boars were used in the binding assay, of which the seminal plasma from each boar was used except for one case where no seminal plasma was able to be collected. In this case, the seminal plasma of a boar killed earlier and stored at -20^0 C was used.

Two sets of assays were carried out. The first was a duplicate of section 3.7. The second assay used epididymal spermatozoa that were first incubated with seminal plasma (see section 3.9). In this assay, prior to incubation with oviduct explants, sperm samples from each epididymal region were incubated with seminal plasma at 39^0 C for 30 minutes and then centrifuged at 600 ***g*** for 10 minutes. The supernatant was carefully removed using a pipette and the sperm pellet was resuspended with modified Androhep medium. The concentration and motility was determined and the sample adjusted to 5×10^6 spermatozoa per ml with modified Androhep medium (section 3.3). Spermatozoa were incubated with oviduct explants and fixed as previously described (see sections 3.4 and 3.5). Motility characteristics, capacitation status and percentage of live and dead spermatozoa were also determined for epididymal spermatozoa from four testicles (see sections 3.3, 3.9.1 and 3.9.2).

In another experiment, the binding of spermatozoa from three testicles to oviductal explants was tested after incubation in seminal plasma for one minute. The spermatozoa were incubated with oviduct explants, fixed and mounted onto a glass

slide as described in sections 3.4 and 3.5. Motility characteristics, capacitation status and the viability of spermatozoa from three testicles before and after incubation for one minute in either modified Androhep medium or seminal plasma were also assessed (see sections 3.3, 3.9.1 and 3.9.2).

RESULTS

6.2 The percentage of motile and motility characteristics of epididymal spermatozoa before and after incubation with seminal plasma

The percentage of motile epididymal spermatozoa from nine testicles were assessed immediately after collection and after incubation for 30 minutes in either modified Androhep or seminal plasma (Figures 6.1 and 6.2). Immediately after collection, the percentage of motile spermatozoa in seminal plasma was significantly greater in samples from the caput and corpus than spermatozoa in modified Androhep medium but the percentage of motile caudal spermatozoa in seminal plasma was not significantly different to modified Androhep medium. Similar results were observed after incubation for 30 minutes. There was a slight increase in the motility of caudal spermatozoa after incubation for 30 minutes in either modified Androhep or seminal plasma but this was not significant. The opposite was true for caput spermatozoa except that the decrease in the motility of spermatozoa after incubation in the seminal plasma was significant. In the corpus, the motility of spermatozoa increased after incubation with modified Androhep but decreased after incubation with seminal plasma, but these changes were not statistically significant.

The motility characteristics of spermatozoa from four testicles were assessed before and after incubation for 30 minutes in modified Androhep and seminal plasma. The results were relatively similar in all regions of the epididymis and the rete testis except for the average path velocity of corpus spermatozoa and the linearity of caput spermatozoa which were significantly different before and after incubation (Table 6.1). Incubation of caudal spermatozoa for 30 minutes in seminal plasma significantly increased all the motility parameters except straight-line velocity and

amplitude of lateral head displacement. However, there were no significant changes in the motility characteristics of spermatozoa from the corpus, caput and rete testis.

Before incubation, the amplitude of lateral displacement and beat cross frequency were significantly different between caudal spermatozoa in modified Androhep medium and caudal spermatozoa in seminal plasma. The average path velocity, curvilinear velocity and straight-line velocity of corpus spermatozoa differed significantly between modified Androhep medium and seminal plasma before incubation. While there were no significant differences in the motility characteristics of spermatozoa from the caput and rete testis between modified Androhep medium and seminal plasma before incubation, significant differences were found in the amplitude of lateral displacement and curvilinear velocity of spermatozoa from the caput and rete testis, respectively after incubation.

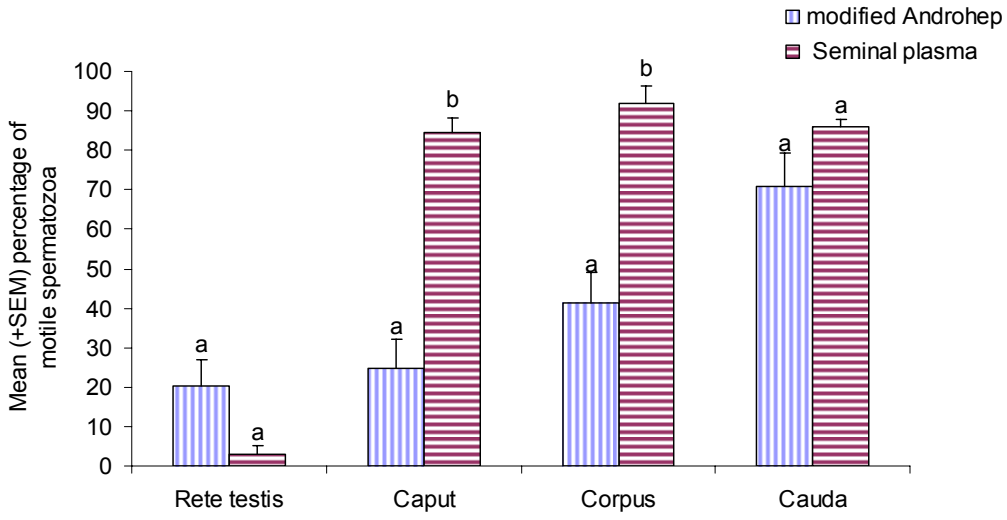

Figure 6.1 The mean (+ SEM) percentage of motile spermatozoa from the rete testis and the epididymis of boars immediately after collection into either modified Androhep medium or seminal plasma. Different letters indicate a significant difference between modified Androhep and seminal plasma ($P < 0.05$). N = nine testicles.

While incubation of epididymal spermatozoa in modified Androhep medium for one minute did not change significantly the motility of spermatozoa, significant changes were observed in spermatozoa from the rete testis and caput but not in the cauda and

corpus after incubation for one minute in seminal plasma (Fig. 6.3). There were no significant differences in the motility characteristics of rete testis and epididymal spermatozoa between the modified Androhep medium and seminal plasma before incubation. After incubation, the motility of spermatozoa from the corpus and caput but not in cauda and rete testis were significantly higher in seminal plasma than in modified Androhep medium.

The motility characteristics of spermatozoa before and after incubation for one minute in modified Androhep medium didn't change significantly between the regions of the epididymis except for curvilinear velocity and the amplitude of lateral head displacement of spermatozoa from the corpus. On the other hand, significant changes were found in the motility characteristics of spermatozoa after incubation for one minute in seminal plasma. These include the amplitude of lateral head displacement and beat cross frequency of caudal spermatozoa and straightness and linearity of both corpus and caput spermatozoa. The motility characteristics of epididymal spermatozoa did not differ significantly between modified Androhep medium and seminal plasma except for the straight-line velocity of corpus spermatozoa before incubation and the average path velocity and curvilinear velocity of caput spermatozoa after incubation.

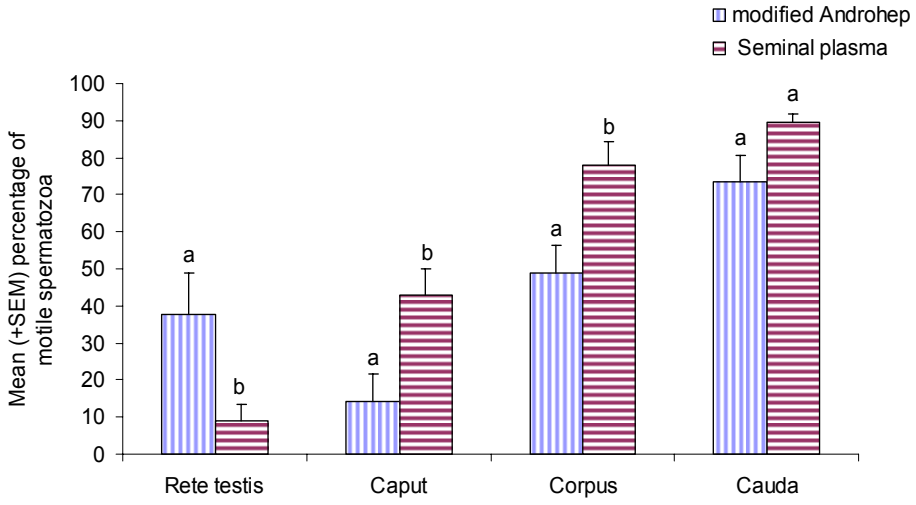

Figure 6.2 The mean (+ SEM) percentage of motile spermatozoa from the rete testis and the epididymis of boars after incubation for 30 minutes in either modified Androhep medium or seminal plasma. Different letters indicate a significant difference between modified Androhep and seminal plasma ($P < 0.05$). N = nine testicles.

Table 6.1 Motility characteristics (mean ± SEM) of spermatozoa before and after incubation for 30 minutes in either modified Androhep medium or seminal plasma.

Motility Parameters		Rete testis		Caput		Corpus		Cauda	
		mAnd	SP	mAnd	SP	mAnd	SP	mAnd	SP
VAP	B	8.7±6.6	3.6±3.3	37.5±7.3	35.2±1.5	50.9±39.7[a1]	38.2±1.1[2]	41.9±3.6	35.3±1.7[a]
	A	16.5±6.4	1.9±1.9	24.2±10.0	33.7±1.3	38.9±3.8[b]	37.0±0.9	46.5±2.7	46.0±3.6[b]
VSL	B	6.2±4.4	1.9±1.9	27.9±5.5	25.5±1.1	35.6±2.8[1]	26.8±1.0[2]	29.9±1.9	26.3±1.5
	A	11.1±4.1	1.6±1.6	14.7±6.4	26.0±0.9	29.0±2.1	26.4±1.1	35.2±2.3	32.1±2.3
VCL	B	12.0±9.5	5.8±5.8	62.5±11.7	62.8±2.6	97.0±10.2[1]	70.4±1.1[2]	82.1±8.5	60.4±3.2[a]
	A	26.6±9.7[1]	3.5±3.5[2]	42.9±18.0	55.3±3.0	75.7±8.3	66.0±2.2	81.9±7.9	85.7±7.3[b]
ALH	B	0.3±0.2	0.0±0.0	2.8±0.7	3.6±0.1	3.58±0.73	4.1±0.2	5.2±0.51	3.8±0.2[2]
	A	0.7±0.4	0.0±0.0	1.2±0.6[1]	3.3±0.4[2]	3.91±0.4	4.1±0.1	4.4±0.3	4.4±0.3
BCF	B	2.6±1.8	3.8±3.8	10.8±3.8	14.3±0.9	18.2±2.5	15.5±0.8	20.6±1.9[1]	12.9±0.6[a2]
	A	10.8±4.1	2.5±2.5	5.5±2.8	11.8±1.4	18.0±3.1	14.7±0.6	16.7±2.0	18.7±1.3[b]
STR	B	16.5±11.2	7.3±37.3	54.3±11.6	71.7±1.0	70.8±4.3	70.4±1.2	73.2±2.6	74.1±1.4[a]
	A	37.4±13.5	11.0±11.0	24.2±10.6	70.9±7.2	77.2±2.6	71.2±1.5	74.9±2.3	69.9±1.4[b]
LIN	B	13.9±9.5	4.6±4.6	39.8±8.4[a]	42.4±1.3	42.9±6.7	39.7±0.7	40.7±3.5	45.0±2.2[a]
	A	24.6±9.6	5.1±5.1	14.1±6.3[b]	49.5±2.3	43.6±2.2	41.5±2.1	46.1±3.7	39.0±1.0[b]

mAnd = modified Androhep; SP = seminal plasma; B = before and A = after incubation; 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 significant differences before and after incubation while different numbers indicate significant differences between modified Androhep medium and seminal plasma ($P < 0.05$). N = four testicles

6.3 Capacitation status of spermatozoa before and after incubation in modified Androhep medium or seminal plasma

While there was no significant difference in the capacitation status of spermatozoa from each epididymal region for each treatment, there was a decline in the percentage of non-capacitated spermatozoa after incubation for 30 minutes in modified Androhep medium and seminal plasma (Fig. 6.4). Correspondingly, the percentage of capacitated and acrosome-reacted spermatozoa also increased. In the cauda, the percentage of non-capacitated spermatozoa after incubation in seminal plasma was significantly lower compared to spermatozoa before incubation and those spermatozoa incubated in modified Androhep medium. Similar results were found

for spermatozoa from the corpus, caput and the rete testis. For corpus and caudal spermatozoa, there was no significant difference in the percentage of acrosome-reacted spermatozoa before and after incubation in modified Androhep medium but was significantly higher for spermatozoa incubated in seminal plasma. A similar result was found for spermatozoa from the caput but this was not significantly different between modified Androhep medium and seminal plasma after incubation.

The capacitation status of spermatozoa was relatively similar between modified Androhep medium and seminal plasma after incubation for one minute (Fig. 6.5). However, the percentage of capacitated spermatozoa from the caput was significantly higher in modified Androhep than in seminal plasma and significantly more non-capacitated spermatozoa from the cauda were found in seminal plasma than in modified Androhep.

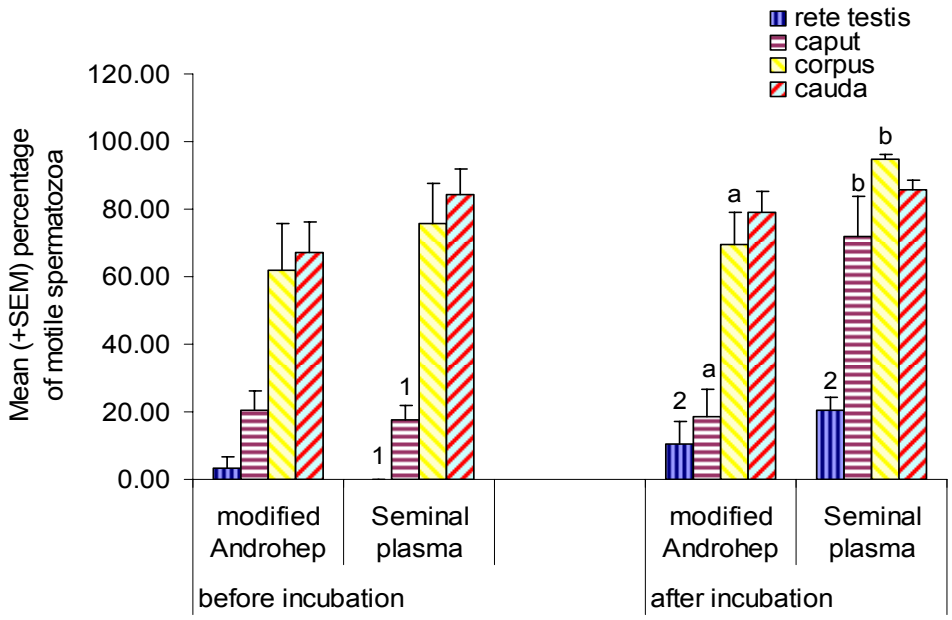

Figure 6.3 The mean (+ SEM) percentage of motile spermatozoa from the rete testis and the epididymis of boars before and after incubation for one minute in either modified Androhep medium or the seminal plasma. Different letters indicate a significant difference between modified Androhep and seminal plasma either before or after incubation within an epididymal region. Different numbers indicate a significant difference before and after incubation in either modified Androhep medium or seminal plasma ($P < 0.05$). N = three testicles

Table 6.2 Motility characteristics (mean ± SEM) of spermatozoa before and after incubation for one minute in either modified Androhep medium or seminal plasma.

Motility		Rete testis		Caput		Corpus		Cauda	
Parameters		mAnd	SP	mAnd	SP	mAnd	SP	mAnd	SP
VAP	B	0	0	22.1±9.	14.6±7.1^{a2}	45.2±2.6	37.9±2.3^{a}	41.3±4.2	40.7±3.2
	A	0	0	24.8±5.7	47.2±4.9^{b}	51.3±2.9	49.4±2.5^{b}	49.1±3.9	44.2±3.6
VSL	B	0	0	14.4±6.4	11.9±5.6^{a}	33.4±2.4^{1}	26.7±1.2^{a2}	30.6±2	28.6±2.4
	A	0	0	19.7±4.1	32.0±3.2^{b}	36.4±2.5	33.6±1.5^{b}	35.3±2.7	30.8±2.0
VCL	B	0	0	43.4±19.4^{1}	19.2±10.0^{a2}	78.3±4.3^{a}	64.7±6.2^{a}	72.1±10.4	69.8±4.8
	A	0	0	40.9±10.2	84.6±11.3^{b}	94.3±5.2^{b}	94.7±46.4^{b}	88.3±8.9	87.7±6.7
ALH	B	0	0	2.3±1.1	1.2±0.8^{a}	4.2±0.2^{a}	3.4±0.4^{a}	4.1±0.2	3.7±0.3^{a}
	A	0	0	3.0±0.8	4.1±0.6^{b}	4.8±0.2^{b}	4.7±0.2^{b}	4.8±0.3	4.8±0.4^{b}
BCF	B	0	0	7.8±3.0	4.0±3.2^{a}	17.4±1.0	15.7±0.9^{a}	19.4±2.2	14.9±1.2^{a}
	A	0	0	21.6±5.4	17.6±1.4^{b}	20.2±1.5	22.9±1.3^{b}	20.5±1.0	23.8±1.6^{b}
STR	B	0	0	32.7±14.6	42.2±19.4	73.5±3.1	71.3±2.9	78.8±2.6	69.5±0.9
	A	0	0	67.3±14.0	69.2±2.1	71.2±1.7	65.8±2.1	72.8±1.3	70.8±1.7
LIN	B	0	0	18.2±8.1	35.3±17.2	45.3±3.5	45.0±3.6	47.3±3.5	42.0±1.7
	A	0	0	45.0±10.2	42.4±3.1	41.6±2.9	37.5±1.1	43.2±2.4	37.8±1.3

mAnd = modified Androhep; SP = seminal plasma; B = before and A = after incubation; 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 before and after incubation while different numbers indicate a significant difference between modified Androhep and seminal plasma ($P < 0.05$). N = three testicles

6.4 The viability of spermatozoa before and after incubation in modified Androhep medium or seminal plasma

The viability of spermatozoa did not vary significantly between before incubation in modified Androhep or after incubation for 30 minutes in either modified Androhep or seminal plasma (Fig 6.6). The mean percentage of live spermatozoa in all treatment conditions was about 90%. The same was true when spermatozoa were incubated for one minute in either modified Androhep medium or seminal plasma (Fig 6.7).

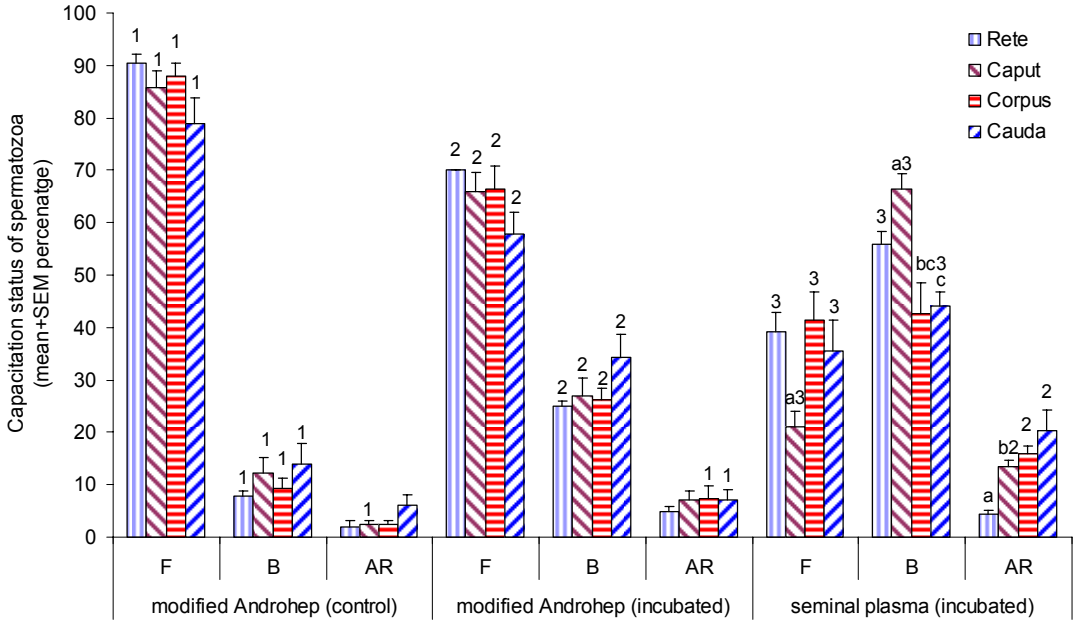

Figure 6.4 The capacitation status of spermatozoa before incubation in modified Androhep medium (control) or after incubation for 30 minutes in either modified Androhep medium or seminal plasma. Different letters indicate a significant difference between epididymal regions for a particular capacitation pattern within each treatment. Different numbers indicate a significant difference between treatments for a particular capacitation pattern within the same epididymal region ($P < 0.05$); F = non-capacitated, B = capacitated, AR = acrosome-reacted; N = four testicles

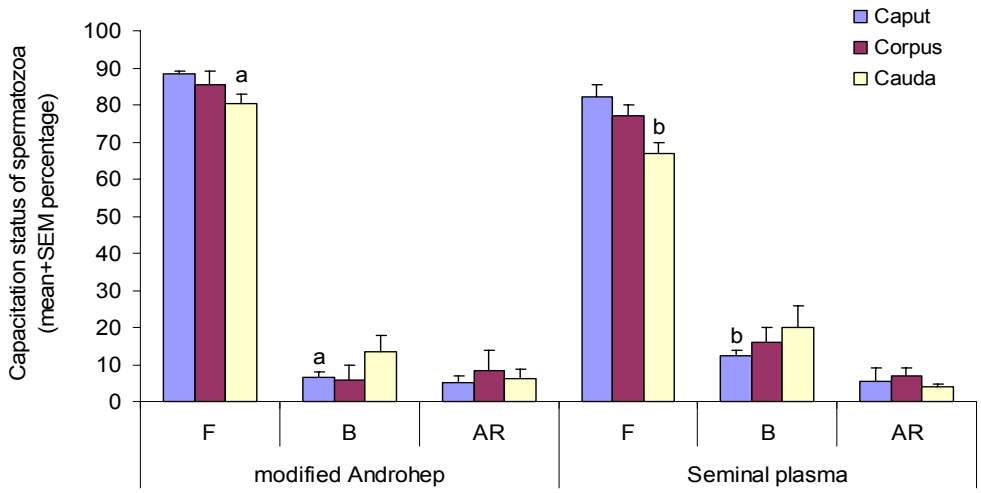

Figure 6.5 The capacitation status of spermatozoa after incubation for one minute in either modified Androhep medium (control) or seminal plasma. Different letters indicate a significant difference between modified Androhep and seminal plasma ($P < 0.05$); F = non-capacitated, B = capacitated, AR = acrosome-reacted; N = three epididymides

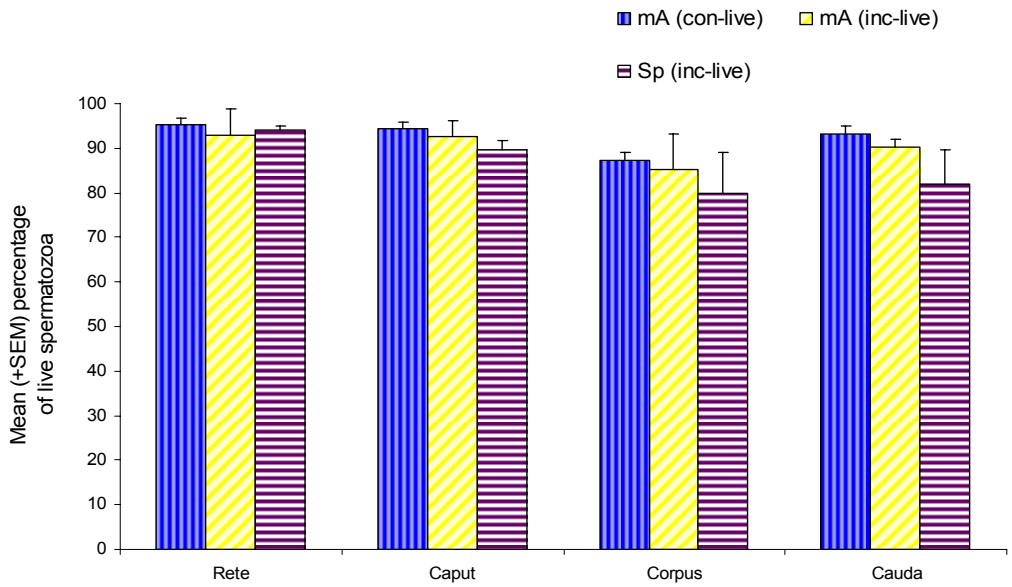

Figure 6.6 The mean (+SEM) percentage of live spermatozoa before incubation in modified Androhep medium or after incubation for 30 minutes in either modified Androhep medium or seminal plasma. There was no significant difference between treatments ($P < 0.05$). mA = modified Androhep; SP = seminal plasma; con = control (before incubation); inc = incubated. N = four testicles.

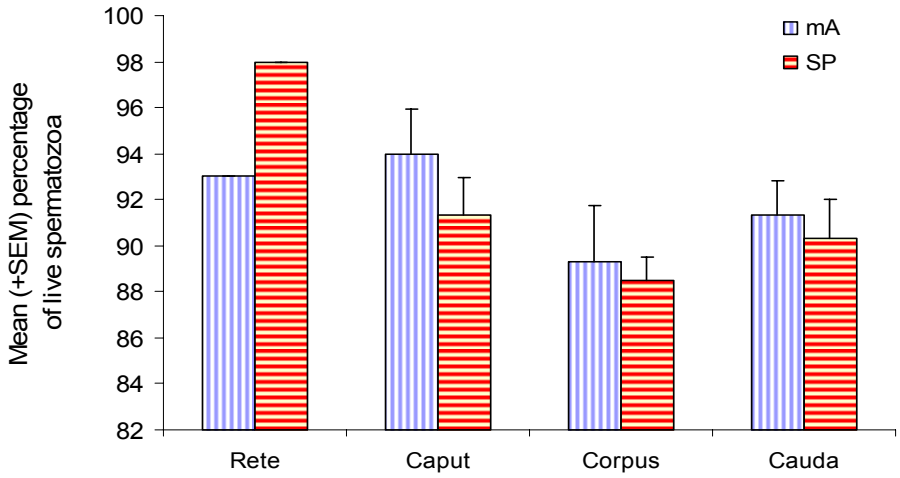

Figure 6.7 The mean (+SEM) percentage of live spermatozoa after incubation for one minute in either modified Androhep medium or seminal plasma. There was no significant difference between treatments ($P < 0.05$). mA = modified Androhep; SP = seminal plasma. N = three testicles. Spermatozoa from the rete testis could only be obtained from one testicle.

6.5 The influence of seminal plasma on the binding capacity of epididymal spermatozoa to oviductal epithelium

There was no significant difference in the binding of spermatozoa from the rete testis and the caput to isthmic explants between spermatozoa diluted in modified Androhep medium and those incubated with seminal plasma. However, significantly more spermatozoa from the corpus and cauda diluted in modified Androhep medium bound to isthmic explants than spermatozoa incubated in the seminal plasma. Similar results were also found for the ampulla. The number of cauda spermatozoa incubated in modified Androhep medium that bound to the isthmus and ampullary explants was 13.13 ± 1.74 spermatozoa per 1.25 mm^2 and 6.65 ± 0.68 spermatozoa per 1.25 mm^2 respectively, while only 6.23 ± 0.87 spermatozoa per 1.25 mm^2 and 4 ± 0.47 spermatozoa per 1.25 mm^2, respectively for spermatozoa incubated with seminal plasma (Fig. 6.8 and 6.9).

Incubation of spermatozoa for one minute in either modified Androhep medium or seminal plasma had no significant effect on the number of spermatozoa that bound to isthmic explants (Fig 6.10). The only significant effect was that more spermatozoa from the caput incubated in seminal plasma bound to ampullary explants (Fig 6.11)

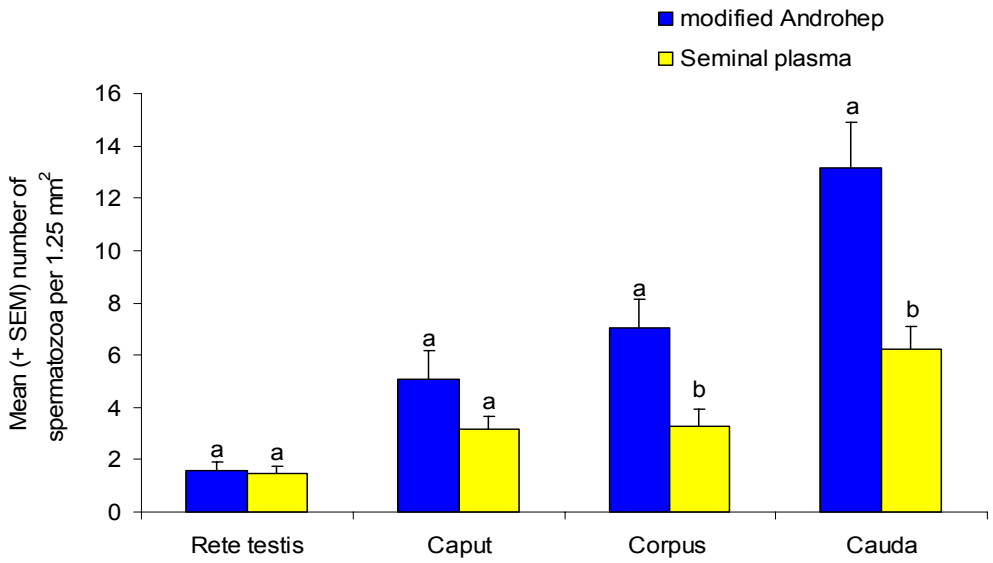

Figure 6.8 Effect of incubation for 30 minutes in either modified Androhep medium or seminal plasma on the binding of spermatozoa from the rete testis and epididymis to isthmic explants. Different letters indicate a significant difference between modified Androhep and seminal plasma ($P < 0.05$). N = 30 oviduct explants for each epididymal region and for each medium; N = five testicles

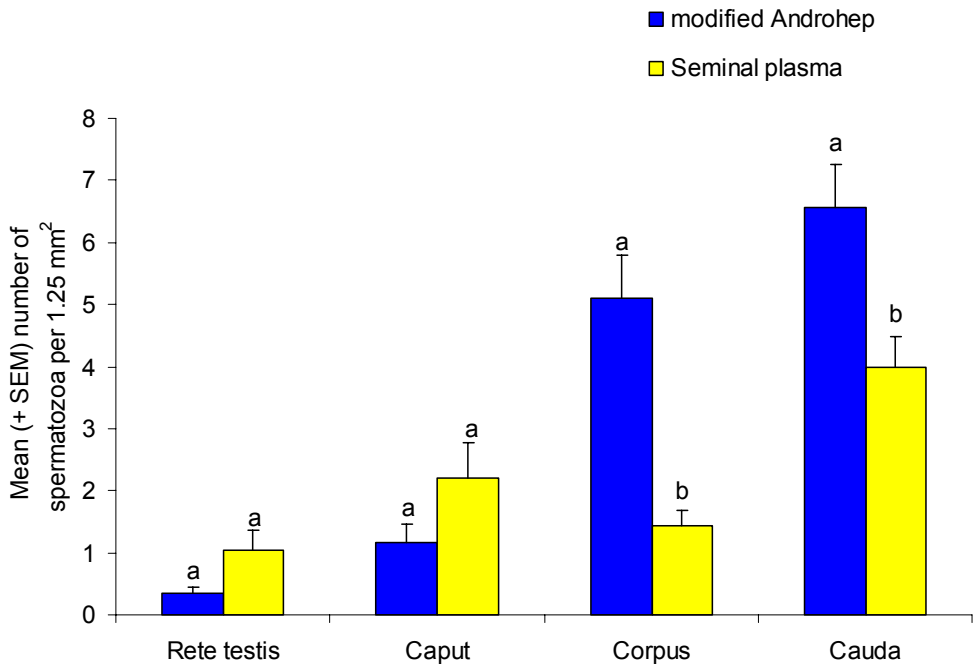

Figure 6.9 Effect of incubation for 30 minutes in either modified Androhep medium or seminal plasma on the binding of spermatozoa from the rete testis and epididymis to ampullary explants. Different letters indicate a significant difference between modified Androhep and seminal plasma ($P < 0.05$). N = 30 oviduct explants for each epididymal region and for each medium; N = five testicles

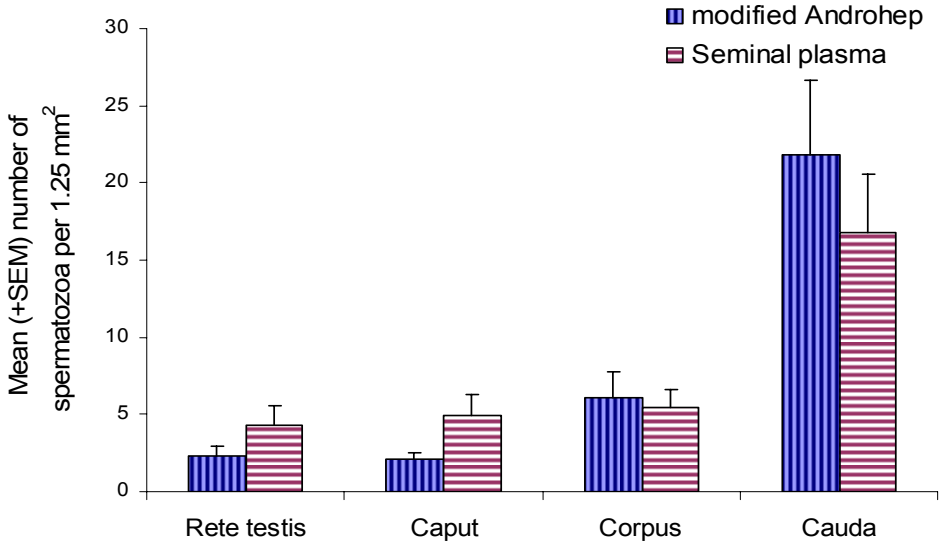

Figure 6.10 Effect of incubation for one minute in either modified Androhep medium or seminal plasma on the binding of spermatozoa from the rete testis and epididymis to isthmic explants. There was no significant difference between modified Androhep and seminal plasma ($P < 0.05$). N = 18 oviduct explants for each epididymal region and for each medium. N = three testicles

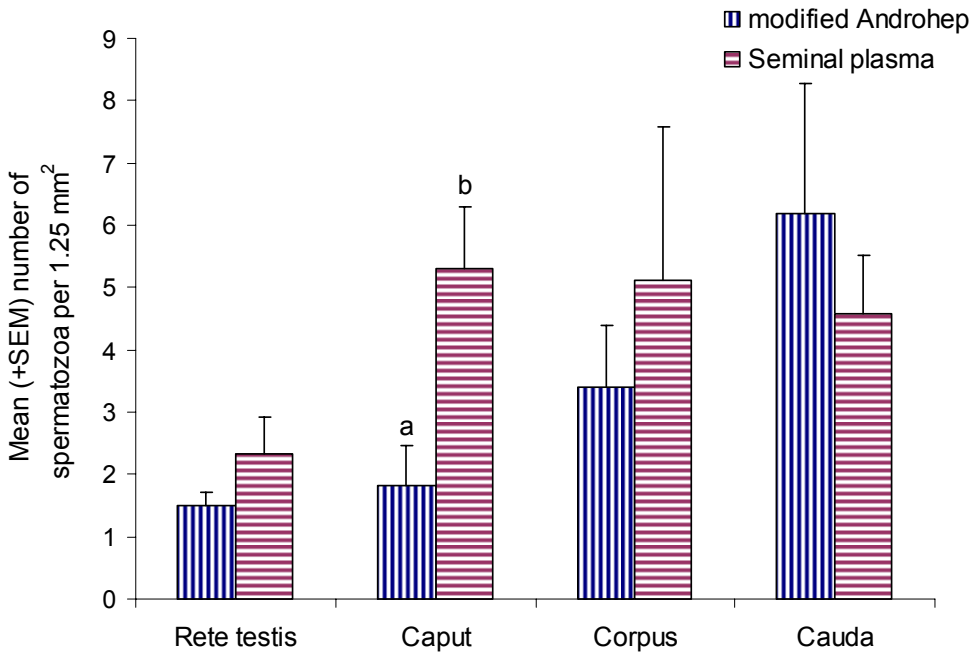

Figure 6.11 Effect of incubation for one minute in either modified Androhep or seminal plasma on the binding of spermatozoa from the rete testis and epididymis to ampullary explants. Different letters indicate a significant difference between modified Androhep and seminal plasma ($P < 0.05$). N = 18 oviduct explants for each epididymal region and for each medium. N = three testicles

DISCUSSION

The current study found a negative effect of incubation of epididymal spermatozoa in seminal plasma for 30 minutes on the binding capacity of spermatozoa to oviduct epithelium. This result is contrary to the work of others as much of the literature supports a positive effect of seminal plasma on the binding of spermatozoa to oviduct epithelium particularly in cattle (Gwathmey *et al.*, 2003, 2006). The molecular studies on the spermadhesin family of proteins led to the conclusion that seminal plasma plays a major role in sperm-to-oviduct interaction in that, upon ejaculation, spermatozoa acquire binding molecules from seminal plasma that assist them to bind to oviduct epithelium (Dostalova *et al.*, 1995b; Calvete *et al.*, 1996a; Topfer-Petersen, 1999). However, it could also be argued that while spermatozoa gain these binding molecules from the seminal plasma, the plasma membrane of spermatozoa can also be coated with other substances present in the seminal plasma which may directly or indirectly inhibit the binding process (Revah *et al.*, 2000). In fact some

studies in humans and domestic livestock have found reduced fertility rates after exposing spermatozoa to seminal plasma (reviewed by Maxwell and Johnson, 1999). Needless to say, components of the seminal plasma do not reach the site of fertilisation suggesting its dispensability during the fertilisation process.

It should be remembered that in this study spermatozoa were incubated with secretions of the seminal vesicle alone. *In vivo*, boar spermatozoa mix with all the components of the seminal plasma including those from the prostate gland and possibly the ampullae. There could be other substances in the seminal plasma which can coat the sperm head making the binding receptors inaccessible for carbohydrate ligands on the oviduct (Revah *et al.*, 2000). While seminal plasma plays a major role in the decapacitation of spermatozoa upon ejaculation, it could also serve as a significant coating over the plasma membrane of spermatozoa and thus preventing exposure of glycoconjugates. In fact, as discussed in section 2.3, porcine spermatozoa were unable to penetrate oocytes after exposure to seminal plasma *in vitro*. This coating by seminal plasma could prevent unnecessary binding of spermatozoa to pre-oviduct epithelium. As spermatozoa travel along the female reproductive tract, the coating could be lost through the interaction of spermatozoa with secretions of the female reproductive tract fluid thus making the binding receptors on spermatozoa available to bind to specific ligands present on oviduct epithelium.

The reduction in the number of spermatozoa that bound to both the isthmus and ampulla after incubation for 30 minutes in seminal plasma led to an investigation of the capacitation status of spermatozoa. As mentioned previously (see section 3.13.1.3), capacitated spermatozoa have reduced binding capacity than uncapacitated spermatozoa. While the percentage of live spermatozoa did not change significantly, it was found that the percentage of capacitated spermatozoa from all regions of the epididymis increased after incubation for 30 minutes in seminal plasma. It was concluded that it was highly likely that the capacitation effect of incubating spermatozoa in seminal plasma for 30 minutes reduced the number of spermatozoa that bound to oviduct explants. The possibility of induction of capacitation of spermatozoa by seminal plasma directly or indirectly is controversial. While bovine seminal proteins are involved in the efflux of sperm cholesterol and phospholipid

which occur during capacitation (Therien *et al.*, 1995; Therien *et al.*, 1999; Jonakova *et al.*, 2000; Manjunath and Therien, 2002), incubation of human spermatozoa in seminal plasma led to inhibition of protein tyrosine phosphorylation (Tomes *et al.*, 1998), a trigger of sperm capacitation. In the current study, the percentage of spermatozoa with an intact acrosome also decreased. This is consistent with the results of Way *et al*. (2000) with caudal bull spermatozoa where the number of acrosome-intact live spermatozoa decreased after incubation with the accessory gland secretions.

While there was no significant difference on the number of epididymal spermatozoa that bound to the isthmus after incubation in seminal plasma for one minute, a significant increase was found in the number of spermatozoa from the caput that bound to ampulla. This could imply that the seminal plasma has the potential of imparting binding molecules at least to immature spermatozoa from the caput.

The current study also found an increased in the motility of spermatozoa after dilution in seminal plasma before and after incubation than with modified Androhep medium. Indeed, the seminal plasma has been appreciated in its classic role as the medium for the optimum motility of spermatozoa upon ejaculation. While seminal plasma provides glycosylable nutrients that favour survival of spermatozoa, it also provides a physical environment that promotes mobility of spermatozoa.

Another possible reason for the increase in the motility of spermatozoa after exposure to seminal plasma is the presence of a low molecular weight factor in porcine seminal plasma that stimulates adenylate cyclase activity (Okamura and Sugita, 1983). This protein factor increases the cAMP concentrations of both ejaculated and epididymal spermatozoa (from the corpus and cauda). The increased level of cAMP therefore leads to further activation of adenylate cyclase and in turn increases the motility of spermatozoa. In cattle, seminal plasma plus theophylline increases the motility of spermatozoa (Hoskins *et al.,* 1975).

In conclusion, this study found that incubation of epididymal spermatozoa with seminal plasma reduced the number of spermatozoa from the corpus and cauda that bound to oviduct epithelium while not affecting spermatozoa from the rete testis and

caput. Further investigation is required to elucidate the molecular components of seminal plasma that may inhibit or promote sperm-oviduct interaction.

CHAPTER 7

GENERAL DISCUSSION

7.1 Scope of research project

While the literature provides ample information on how spermatozoa are produced in the testis and their effect on fertility, it has only been in the past few decades that considerable attention has been directed to understanding the complex events that occur in the maturation of spermatozoa in the epididymis and the possible relationships of the maturation processes to the fertility of domestic animals. In the early studies on male reproductive biology, the epididymis had been considered merely as a structure that provided a passageway for spermatozoa from the testis to the external male genitalia as well as storage site for spermatozoa. However, it soon was realized that the epididymis had an indispensable role in the maturation of spermatozoa not only in the acquisition of motility potential but also the capacity to fertilise oocytes. In addition to understanding the biology of the epididymis, another motivation for research work was the possibility of interfering with epididymal function to provide alternative means to fertility control of humans.

While the capacity of spermatozoa to bind to the zona pellucida is important to achieve successful fertilisation, the same is true with the ability of spermatozoa to bind to oviduct epithelium. The formation of a sperm reservoir in the caudal isthmus is important in prolonging the viability of spermatozoa as well as preventing undue capacitation and polyspermic fertilisation. The question is where do spermatozoa develop the ability to bind to oviduct epithelium? The hypothesis tested in this thesis was that testicular spermatozoa need to pass down the epididymis in order to acquire the ability to bind to oviduct epithelium. The results reported in this thesis using the pig as a model support this hypothesis. There was a progressive increase in the number of spermatozoa that bound to the oviductal epithelium from the rete testis to the cauda epididymidis and spermatozoa preferentially bound to the isthmus than the ampulla (see Chapter 4). This result was interpreted to mean that there are indeed sequential developmental changes to spermatozoa during their maturation in the epididymis that is connected with their ability to bind to oviduct epithelium. While

the current study found significantly fewer epididymal spermatozoa bound to oviduct explants than ejaculated spermatozoa, this is consistent with a previous report (Petrunkina *et al.*, 2001b).

The literature provides evidence that carbohydrate-recognition mechanisms are involved in sperm-oviduct binding in that carbohydrate molecules on the surface of the oviduct are recognised by binding molecules on the plasma membrane of spermatozoa (Topfer-Petersen, 1999; Green *et al.*, 2001; Suarez, 2001). While specific carbohydrate residues on oviduct cells have been identified that are recognised by spermatozoa of various species, what is not completely understood are the specific binding molecules present on spermatozoa. Nevertheless, it is logical to think that it is highly likely that the molecules are secreted by epididymal epithelium and attach to the head of spermatozoa during epididymal transit. In the pig, once such carbohydrate-binding molecule is AWN which has been found on epididymal spermatozoa and seems to accumulate on spermatozoa as they travel along the epididymis (Dostalova *et al.*, 1994; Ekhlasi-Hundrieser *et al.*, 2002). Alternatively, secretions of the epididymal epithelium may modify the structure of pre-existing molecules of the apical region of spermatozoa to enable to bind to carbohydrates on the surface of oviductal epithelium. In cattle, significant amounts of proteins that belong to bovine seminal plasma family of proteins (BSPs): PDC-109 (BSP-A1/-A2), BSP-A3, and BSP-30-kDa perform a crucial role in the formation of the oviductal sperm reservoir (Gwathmey *et al*., 2003, 2006)

While spermatozoa from one species can bind to oviduct epithelium of another species i.e. human spermatozoa to oviducts of cattle and macaques (Ellington *et al.*, 1998), canine spermatozoa to porcine oviducts (Petrunkina *et al.*, 2004) and cryopreserved stallion spermatozoa to oviductal cells of cattle (Ellington *et al.*, 1999), there has been no reports on the binding of porcine spermatozoa to oviducts of cattle. This was examined in this thesis. The use of heterologous systems has been mostly due to logistical reasons. In studies with human spermatozoa, a ready supply of disease-free oviduct tissues from women is not possible (Ellington *et al.*, 1998), thus when species variation is not significant, much of the effort, time and cost of the study can be reduced. In this study, the use of oviducts from cattle was investigated

because of an adequate supply from a cattle abattoir a short distance from the laboratory whereas the nearest source of porcine oviducts was from an abattoir 130 km from the laboratory. It was found that significantly fewer ejaculated porcine spermatozoa bound to oviducts of cattle than oviducts of pigs. This result was contrary to the binding of canine spermatozoa to porcine oviducts (Petrunkina *et al.*, 2004) and human spermatozoa to bovine oviducts (Ellington *et al.*, 1998). The result therefore suggests strong differences between cattle and pigs in carbohydrate-binding property of porcine spermatozoa and the glycoconjugates present on the surface of oviducts.

There was a significant difference in the number of caudal spermatozoa that bound to the isthmus of cyclic sows compared to pre-pubertal gilts. The literature reports no difference in the number of ejaculated boar spermatozoa that bound to oviduct epithelium from sows and gilts (Petrunkina *et al.*, 2001b). While more oviducts from sows and gilts are needed to confirm this result, it was interesting to find that epididymal spermatozoa do bind to immature oviducts and implies that more glycoconjugates are present on the oviduct epithelium under the influence of ovarian hormones. The fact that the literature reported there was no difference between sows and gilts in the number of spermatozoa that bound to isthmus explants was the reason that in the absence of a consistent supply of sow oviducts, oviducts from pre-pubertal gilts were used in this study.

More spermatozoa, either ejaculated spermatozoa or those obtained from the epididymis, bound to the isthmus than the ampulla. While there are differences in the literature on the binding of spermatozoa between the isthmus and ampulla, the apparent differences in the expression of glycoconjugates in different regions of the oviducts (Walter and Bavdek, 1997) could play major role on the preferential binding of spermatozoa to the isthmus. Apart from the fact that the physical features of the caudal isthmus promotes the formation of the sperm reservoir, it is reasonable to think that the epithelium of the caudal isthmus has developed mechanisms that would favor increased affinity for spermatozoa to enable the other changes to occur including capacitation and hyperactivation in preparation for fertilisation at the junction between the isthmus and the ampulla.

The significant differences found in the capacity of spermatozoa of some boars to bind to oviduct epithelium is worth noting as these differences could imply differences in the fertility of boars. None of the boars had mated sows so it was not possible relate the fertility of the boars to the results of binding of spermatozoa to oviduct explants. In fact, sperm-to-oviduct binding assays has been used in the prognosis of fertility status of boar spermatozoa (Waberski *et al.*, 2005). Other workers using pigs (Petrunkina *et al.*, 2001b) and horses (Thomas *et al.*, 1994a) have found similar results. The general consistency in the number of spermatozoa either from the right and the left testicle of a boar that bound to oviduct epithelium indicates the validity of the binding assay in terms of repeatability.

The carbohydrate-binding property of epididymal spermatozoa was examined by allowing spermatozoa to bind to carbohydrate parts of albumin and asialufetuin molecules. There was a significant inhibition by albumin and asialofetuin on the number of spermatozoa that bound to oviduct explants. Earlier reports have found that the binding of ejaculated spermatozoa to oviduct epithelium is mediated by the ability of spermatozoa to recognise mannose, maltose and lactose residues and that much stronger inhibition was induced by mannose (Green *et al.*, 2001; Wagner *et al.*, 2002). Since albumin and asialufetuin contain mannose and lactose respectively, it suggests similarity between ejaculated and epididymal spermatozoa in recognizing carbohydrate molecules responsible for the attachment of spermatozoa to oviduct epithelium.

The development of motility potential by spermatozoa during their maturation in the epididymis has been fully established (reviewed by Setchell *et al.*, 1993; Jones and Murdoch, 1996). The current study found a sequential increase in the percentage of motile spermatozoa from the rete testis to the cauda epididymidis. The increase in the motility of spermatozoa is brought about by a concerted action of several factors including the increase in cAMP concentrations and the Ca^{2+}-ATPase activity (reviewed by Jones and Murdoch, 1996), a decrease in the intracellular pH (Gatti *et al*., 1993), a decrease in the concentration of free calcium ions and the capacity of glucose transport into spermatozoa (Hiipakka and Hammerstedt, 1978) and the lowered exchange rate of calcium ions into the mitochondria (Vijayaraghavan and Hoskins, 1990).

It was demonstrated in Chapter 5 that when caput and corpus spermatozoa were incubated in caudal fluid for 30 minutes, their ability to bind to oviduct epithelium increased. This indicates the caudal fluid has special features that directly or indirectly influence the attachment of spermatozoa to oviduct epithelium. While the cauda only secretes a small percentage of the proteins that have been identified in the epididymis, it is likely that some of the secretions from the proximal regions of the epididymis accumulate in the cauda in association with the transport of spermatozoa to the cauda. As spermatozoa need receptors to bind to carbohydrate ligands on the oviduct epithelium, it is highly likely that most of these binding molecules are finalised in the plasma membrane of spermatozoa during their long stay in cauda. However, the period of time that spermatozoa need to be in cauda to obtain binding ability may not be long as incubation of caput spermatozoa in caudal fluid for 30 minutes was sufficient to significantly increase the number of spermatozoa that bound to oviduct explants.

The motility of spermatozoa also decreased after incubation in caudal fluid for 30 minutes. This result is not surprising because *in vivo*, spermatozoa remain in a quiescent state during storage in cauda. While the manner by which epididymal fluid acts upon spermatozoa to induce immotility during this period is complex and not fully understood, it has been demonstrated that the lower pH (pH 6.5) and bicarbonate concentration (3-4 mM) in the caudal fluid are responsible for the quiescent state of spermatozoa (Acott and Carr, 1984; Rodriguez-Martinez *et al.*, 1990; Setchell *et al.*, 1993).

Exposure of epididymal spermatozoa to seminal plasma for 30 minutes significantly reduced the number of spermatozoa that bound to oviduct explants (see Chapter 6). These results however, are contrary to reports that used ejaculated spermatozoa (Dostalova *et al.*, 1995b; Calvete *et al.*, 1996a; reviewed by Topfer-Petersen, 1999). Moreover, a number of proteins have been identified from bovine seminal plasma which enable bovine spermatozoa to bind to oviduct epithelium (Gwathmey *et al*., 2003, 2006). Nevertheless, it is also possible that other components of the seminal plasma could act as a coating over the plasma membrane of spermatozoa and inhibit the expression of binding molecules (Revah *et al.*, 2000). In fact, porcine

spermatozoa were unable to penetrate oocytes after exposure to seminal plasma *in vitro* (Nagai *et al.*, 1984). Upon ejaculation, this coating over the plasma membrane of spermatozoa serves as decapacitation factor and prevents unnecessary binding of spermatozoa to pre-oviduct epithelium. As spermatozoa travel along the female reproductive tract, the coating could be lost through the interaction of spermatozoa with the female reproductive tract fluid thus making the binding receptors on spermatozoa available to bind to specific ligands present on oviduct epithelium.

Incubation of epididymal spermatozoa in seminal plasma for 30 minutes caused capacitation but had no significant effect on viability. This therefore led to the conclusion that the increased capacitation was the reason why incubation in seminal plasma for 30 minutes reduced the number of spermatozoa that bound to oviduct explants. While there are inconsistencies in the literature about the possibility of capacitation of spermatozoa in seminal plasma, some workers have found that bovine seminal proteins are involved in the efflux of sperm cholesterol and phospholipid which occur during capacitation (Therien *et al.*, 1995; Therien *et al.*, 1999; Jonakova *et al.*, 2000; Manjunath and Therien, 2002) and some have demonstrated in human spermatozoa that seminal plasma inhibits protein tyrosine phosphorylation (Tomes *et al.*, 1998), a trigger of sperm capacitation. While there were no significant changes in the capacitation status and viability of epididymal spermatozoa after exposure to seminal plasma for one minute, the number of spermatozoa that bound to the isthmus also did not change, although significantly more caput spermatozoa bound to ampulla.

The seminal plasma has been the classic medium being used in the dilution of spermatozoa for optimum performance and as expected this study found an increase in the motility of spermatozoa after exposure to seminal plasma. There are a number of possible reasons for this including the presence of glycosylable nutrients being made available to spermatozoa, a low molecular factor being present in porcine seminal plasma that stimulates adenylate cyclase activity (Okamura and Sugita, 1983) and the physical environment that seminal plasma provides which is conducive to spermatozoal mobility.

7.2 Future research directions

This study has highlighted a number of areas of research work which would lead to a better understanding of how boar spermatozoa develop the ability to bind to oviduct epithelium. The following are topics that need investigation:

- There is a need to determine the structure and composition of the binding molecules on spermatozoa. This knowledge could then be used to determine where in the epididymis the binding molecules are produced, if the molecules are produced in all regions of the epididymis or if progressively more are produced in the distal epididymis than the proximal epididymis.
- A study needs to be conducted to determine how long do spermatozoa from the corpus need to be exposed to caudal fluid to achieve binding equivalent to cauda spermatozoa and what are the molecular changes that are induced by caudal fluid. Would incubation for more than 30 minutes increase the number of spermatozoa that bind or would incubation for less than 30 minutes give the same result?
- The number of binding sites on epididymal spermatozoa needs to be quantified to help provide answers as to why binding to oviduct epithelium is not an all or none phenomenon. For example, why do some spermatozoa from the caput bind to the isthmus? Does this mean that some caput spermatozoa have the required number of binding molecules and if so, why do some spermatozoa have the required number of binding sites and others don't.
- The role of seminal plasma in the binding of epididymal spermatozoa to oviduct explants needs further clarification. The finding that fluid from seminal vesicles reduced the number of spermatozoa that bound is opposite to what was expected. Experiments need to be done to determine if complete seminal plasma that contains secretions from all the accessory glands will increase the number of bound spermatozoa and whether the number of spermatozoa that bind is influenced by how long spermatozoa are exposed to seminal plasma. For example, to particularly mimic the *in vivo* situation, would incubation in seminal plasma for six hours increase the number of bound spermatozoa?

7.3 Conclusion

In conclusion, this study demonstrated that boar spermatozoa from the rete testis and the epididymis are able to bind to the isthmus and the ampulla of gilts and sows. Significantly more spermatozoa from the cauda epididymidis bound to oviduct epithelium than the remainder of the epididymal regions and significantly more bound to the isthmus than the ampulla. Exposure of spermatozoa from the corpus to caudal fluid increased the number of spermatozoa that bound to oviduct epithelium. Whether this is because of structural modification of the glycocalyx or addition of glycoproteins or oligosaccharides to spermatozoa requires further investigation. Lastly, seminal plasma reduced the number of spermatozoa from the corpus and cauda that bound while not affecting spermatozoa from the rete testis and caput.

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