THE PRESENCE OF FOLLICULAR FLUID IN THE PORCINE OVIDUCT AND ITS CONTRIBUTION TO THE ACROSOME REACTION

by

© Christiane Hansen

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science

Department of Animal Science
Macdonald College of McGill University
Montreal, Quebec, Canada

March, 1989

Suggested Short Title: Follicular Fluid in the Porcine Oviduct.

THE PRESENCE OF FOLLICULAR FLUID IN THE PORCINE OVIDUCT AND ITS CONTRIBUTION TO THE ACROSOME REACTION

ABSTRACT

Christiane Hansen M.Sc. Animal Science

Two experiments were carried out to determine how much follicular fluid enters the porcine oviduct during the process of ovulation and to establish the influence that this fluid has on the sperm acrosome reaction *in vivo*. The distribution of sperm in the porcine reproductive tract was also examined. PMSG/hCG-treated prepubertal gilts were used in both experiments.

In experiment I, 64 gilts were randomly assigned to one of four treatment groups: 1) preovulatory (n=16, surgery 38h post-hCG), 2) ovulatory (n=16, surgery 42h post-hCG), 3) post-ovulatory (n=16, surgery 46h post-hCG) and 4) ovulation blocked (n=16, surgery 46h post-hCG but treated with indomethacin (10mg/kg) 24h after the hCG injection). Plasma, peritoneal fluid, follicular fluid, and tubal fluid samples were collected from all animals when possible. All of these fluid samples were assayed for progesterone content. It was found that less than 0.5% of the available follicular fluid was present in the oviduct when examined during the process of ovulation and that this value had decreased to 0.04% 4h later. In experiment II, the gilts were divided into two groups: 1) surgery and sperm recovery 46h post hCG (n=5, post-ovulatory - follicular fluid released) and 2) surgery and sperm recovery 46h post hCG but treated with indomethacin (10mg/kg) 24h after the hCG injection (n=4,

ovulation blocked - no follicular fluid released). All animals were inseminated 12h prior to surgery. Sperm were recovered from the top 3cm of the uterine horn, from the isthmus and from the ampulla of the excised oviducts and all sperm were stained with an acrosome-specific stain. The mean sperm concentration found in the uterine horn was significantly higher than that found in the other two regions ($p<0.05$). No significant difference in mean sperm concentration was found between the indomethacin treated and control groups. The mean number of acrosome reacted sperm was found to be higher in the uterine horn segments than in the isthmus and ampulla ($p<0.05$) while the percent acrosome reacted sperm was highest in the ampulla ($p<0.05$). The ampulla of ovulating pigs was found to contain a higher percentage of acrosome reacted sperm than the ampulla of pigs where ovulation was blocked ($p<0.05$).

The results of these experiments indicate that little follicular fluid enters the oviduct of the pig during ovulation. These results also suggest that follicular fluid has a stimulatory effect but is not essential for the acrosome reaction.

LA PRESENCE DE LIQUIDE FOLLICULAIRE DANS L'OVIDUCTE ET SA CONTRIBUTION A LA REACTION ACROSOMAIRE CHEZ LES PORCINS

RESUME

Chrisitane Hansen M.Sc. Sciences Animales

Deux expériences ont été conduites pour 1- déterminer la quantité de liquide folliculaire que pénètre l'oviducte au cours de l'ovilation et 2- établir l'influence de ce liquide sur la réaction acrosomaire du sperme in vivo. La distribution du sperme dans le système reproducteur femelle fût aussi examinée. Des truies prébubères ayant reçues des injections de gonadotrophine de juments gestantes (PMSG) et de gonadotrophine chorionique humaine (hCG) ont été utilisées pour les deux expériences.

Au cours de la première expérience soixante-quatre (64) truies ont été devisées au hayard en quatre (4) groupes: 1- pre-ovulation (n=16, chirurgie 38h après injection de hCG); 2- a l'ovulation (n=16, chirurgie 42h après injection de hCG); 3- post-ovulation (n=16, chirurgie 46h après injection de hCG); 4- ovulation inhibée (n=16, injection d'indomethacin (10mg/kg) 24h après injection de hCG, et, chirurgie 46h apres hCG). Des échantillons de plasma, de liquide péritonéal, de liquide folliculaire et de liquide des ovuiducts ont été obtenus de tous les animaux lorsque possible. La concentration de progestérone a été détérminée dans tous ces échantillons. Il a été démontre que seulement 0.5% du liquide folliculaire disponsible était présent dans les oviductes lorsqu' examinés au cours de l'ovulation; 4 heures plus tard seulement 0.04% de ce liquide

était présent dans l'oviducte.

Au cours de la deuxième expérience les truies ont éte divisées en deux groupes: 1- chirurgie et obtention du sperme 46h après injection de hCG (n=5, post-ovulation, liquide folliculaire relâché) et 2- chirurgie et obtention du sperme 46h après injection de hCG mais injection d'indomethacin (10mg/kg) 24h apres l'injection de hCG (n=4, ovulation inhibée, pas de liquide folliculaire relâché). Tous les animaux ont été inéminés 12h avant la chirurgie. Le sperme a été recueilli 1- des première 3cm des cornes utérines, 2- de l'isthme et 3- de l'ampoule des oviductes excis. Tout le sperme a été teinté avec un indicateur ne colorant que l'acrosome. La concentration moyenne de sperme était plus elevée dans les cornes utétines que dans les 2 autres regions ($p,0.05$). Le traitement avec l'indomethacin n'a eu aucun effet sur la concentration de sperme. Le nombre moyen de spermatozoides ayant subis la réaction acrosomaire etait plus élevé dans l'ampoule ($p<0.05$). L'ampoule des truies à l'ovulation contenait un plus haut pourcentage de spematozoides ayant subi la réaction acrosomaire que l'ampoule des truies dont l'ovuluation fût inhibée ($p<0.05$).

Les resultats de ces deux expériences ont donc indiqué que peu de liquide folliculaire pénètre l'oviducte des truies au cours de l'ovuluation. Les résultats suggèrent également que le liquide folliculaire stimule, mais n'est pas essentiel à la réaction acrosomaire.

ACKNOWLEDGEMENTS

I would like to acknowledge the following people for the considerable contribution they made toward the completion of these experiments:

My supervisory committee which included Dr. B.R. Downey, Dr. A. Srikandakumar and Dr. D. Bousquet for their advice, enormous help, and support.

Jan Pika, Denyse Laurin and Jim Algire for their tremendous assistance during surgery.

Denis Hatcher and Chris Wilson for their cooperation in selecting gilts for me each week and for collecting semen even at odd hours.

All of my friends in the Department of Animal Science who made my time here so enjoyable.

Finally I would like to express my gratitude to my parents, Karl-Friedrich and Elke, who have always supported me in whatever I chose to do.

TABLE OF CONTENTS

	PAGE
ABSTRACT	i
RESUME	iii
ACKNOWLEDGEMENTS	v
LIST OF TABLES	vii
LIST OF FIGURES	ix
LIST OF APPENDIX TABLES	x
GENERAL INTRODUCTION	1
LITERATURE REVIEW	4
EXPERIMENT I:	
OBJECTIVES	39
MATERIALS AND METHODS	39
RESULTS	44
DISCUSSION	55
EXPERIMENT II:	
OBJECTIVES	63
MATERIALS AND METHODS	63
RESULTS	68
DISCUSSION	74
GENERAL CONCLUSIONS	80
LITERATURE CITED	82
APPENDIX TABLES	91

LIST OF TABLES

Table		PAGE
1)	Mean Number of Follicles (±SEM) for PMSG/hCG Stimulated Gilts- by Treatment and Side	45
2)	Mean Number of Ovulations (±SEM) for PMSG/hCG Stimulated Gilts- by Treatment and Side	45
3)	Mean Plasma Progesterone Concentration (ng/ml)(±SEM) for PMSG/hCG Stimulated Gilts- by Treatment	48
4)	Mean Progesterone Concentration of Follicular Fluid (ng/ml)(±SEM) for PMSG/hCG Stimulated Gilts- by Treatment and Side	48
5)	Mean Progesterone Content per Follicle (ng) (±SEM) for PMSG/hCG Stimulated Gilts- by Treatment and Side	50
6)	Mean Total Progesterone Content of Tubal Fluid (ng) (±SEM) for PMSG/hCG Stimulated Gilts- by Treatment and Side	50
7)	Mean Progesterone Concentration of Tubal Fluid (ng/ml) (±SEM) for PMSG/hCG Stimulated Gilts- by Treatment and Side	52
8)	Mean Difference (±SEM) Between PCTF and PS for PMSG/hCG Stimulated Gilts- By Treatment and Side	52
9)	Percentage (±SEM) of Follicular Fluid Entering the Oviduct of PMSG/hCG Stimulated Gilts	54
10)	Mean Sperm Concentration (sperm/ml) (±SEM) for PMSG/hCG Stimulated Gilts- by Treatment	69
11)	Mean Number of Acrosome Reacted Sperm (±SEM) for PMSG/hCG Stimulated Gilts- by Treatment and Location	69
12)	Mean Percentage of Acrosome Reacted Sperm (±SEM) for PMSG/hCG Stimulated Gilts- by Treatment and Location	72

Table		Page
13)	Mean Number of Follicles (±SEM) Per Treatment	72

LIST OF FIGURES

FIGURE		PAGE
1)	Number of Follicles and Number of Ovulations For PMSG/hCG Treated Gilts	46
2)	Progesterone Concentration and Content of Porcine Follicular Fluid	51
3)	The Sperm Concentration and Number of Acrosome Reacted Sperm in the Horn, Isthmus and Ampulla of the Porcine Oviduct	70
4)	The Percent Acrosome Reacted Sperm found in the Porcine Reproductive Tract	73

LIST OF APPENDIX TABLES

Table		Page
1)	Statistical Program for Experiment I.....	91
2)	Data for Experiment I.....	92
3)	Abdominal Fluid Progesterone Concentration	95
4)	Composition of Semen Extender	96
5)	Composition of Acrosome Staining Solutions	97
6)	Statistical Program for Experiment II	98
7)	Data for Experiment II	100

GENERAL INTRODUCTION

The study of the many events comprising mammalian fertilization *in vivo* is not an easy feat. It is made difficult not only by the fact that the site of fertilization, the ampullar-isthmus junction, is internal and therefore difficult to observe, but also because the size of the oviduct is so small in the most commonly used laboratory animals (Moore and Bedford, 1978). *In vitro* fertilization has thus become an important tool for many researchers studying the different aspects of fertilization. In addition, the pressures of modern agriculture have led researchers to look for ways to improve upon the results the industry is now able to obtain. Many have looked toward *in vitro* fertilization in an effort to do this. Unfortunately, however, there are still a number of problems associated with *in vitro* fertilization systems. These include the low number of capacitated sperm, the extended period of time required for capacitation, the reduced survival of *in vitro* capacitated sperm, and the lack of an objective assay for capacitation (Singh *et al*, 1980). *In vitro* fertilization in farm animals has thus, to date, met with only limited success, although success rates for humans and laboratory animals have been much more encouraging.

The majority of the problems associated with *in vitro* fertilization can be explained by the fact that the systems

that have been developed still do not mimic the *in vivo* situation well enough to ensure consistent, high success rates. Many different media and conditions have been experimented with and different combinations of materials are continually being tried in an effort to remedy this situation. Among the products often included in these media are the different products of ovulation, particularly follicular fluid. Follicular fluid has been shown, *in vitro*, to possess the ability to promote sperm capacitation and the acrosome reaction in a number of species (eg. Yanagimachi, 1969b (hamster); Iwamatsu and Chang, 1969 (mouse)). It has also been shown to contain compounds such as proteoglycans which possess acrosome reaction inducing ability (Lenz *et al*, 1982). Furthermore, follicular fluid has been shown to reduce the number of pig sperm attaching to and penetrating zona free hamster eggs *in vitro* and the suggestion has been made that certain follicular fluid components may be involved in the prevention of polyspermy (Ramsoondar, 1986).

It seems remarkable that with all of the work that has been carried out using follicular fluid in the various *in vitro* situations described above, that no one has ever actually measured the amount of follicular fluid entering the oviduct *in vivo* after ovulation. This was the focus of Experiment I.

The factor or factors in the female reproductive tract that cause capacitation and the acrosome reaction are still unknown, as is their source (Bedford, 1983). It has been

suggested that the endocrine status of the female may control the ability of the tract to cause capacitation of sperm. This possibility is supported by experiments such as those of Chang (1958) and Viriyapanich and Bedford (1981) which showed that the uteri of progesterone-dominated rabbits and the oviducts of progesterone-dominated hamsters will not cause capacitation. On the other hand, the different regions of the female reproductive tract may also differ in their ability to induce capacitation (Hunter and Hall, 1974). In addition, as has already been mentioned, follicular fluid and other products of ovulation may be important substances involved in capacitation and acrosome reaction induction.

Unfortunately, because of the inherent difficulties associated with studying capacitation and the acrosome reaction *in vivo*, the majority of experiments done to date have been *in vitro* studies. Very few have examined the potential role of follicular fluid or the other products of ovulation in an *in vivo* setting. An experiment done by Herz *et al* (1985) examining the acrosome reaction of bovine sperm did, however, suggest that follicular fluid or some other ovulatory product may be involved in capacitation and acrosome reaction induction *in vivo*. Unfortunately the results of this study were not conclusive in this regard. Experiment II of the present study was thus designed to attempt to clarify this situation at least in the pig.

LITERATURE REVIEW

FOLLICULAR DEVELOPMENT

During embryonic life, the mammalian ovary contains a fixed number of primordial follicles lying mainly near its periphery. Each oocyte in a primordial follicle is surrounded by a single layer of flattened granulosa cells and each follicle is separated from its neighbours by layers of stromal and interstitial tissue.

The development of the follicular epithelial cells into a single layer of cuboidal cells surrounding the oocyte marks the formation of a primary follicle from a primordial follicle. At puberty and during each subsequent cycle, a number of primary follicles are recruited through endocrine stimulation and develop into secondary follicles. This developmental process involves not only an increase in the number of granulosa cell layers surrounding each oocyte, but also an increase in the size and maturity of the oocytes. In addition, this process involves the formation of the zona pellucida, a membrane-like structure, around the oocyte.

It should be noted that although several follicles may begin the maturation process, only a small, species characteristic number will ever reach the point of ovulation. The others become atretic and disappear. The granulosa cells of the few follicles that are destined to develop further, however, continue to proliferate. In addition, the stromal tissue adjacent to these follicles

becomes arranged concentrically around the follicle forming the theca layer (Hadley, 1984). Finally, as the follicle continues to enlarge, an antrum begins to form and eventually a tertiary or mature Graafian follicle is formed.

THE GRAAFIAN FOLLICLE

The Graafian follicle is a mature tertiary follicle which appears as a fluid filled structure on the surface of the ovary (Bearden and Fuquay, 1984). The size of the fully grown Graafian follicle varies significantly among species. It can range from a few millimetres in mice to 5 cm in horses (Edwards, 1974).

Several different cell layers, some of which have been alluded to earlier, have been identified in the Graafian follicle. All of these are of importance in follicular function. The outer most layer of such a follicle is known as the theca externa layer. It is more fibrous than the next layer, the theca interna. Both of these layers are well supplied with blood vessels. The theca interna is separated from the inner most cell layer, the granulosa layer, by a basement membrane. This basement membrane prevents the entry of the capillary network into the granulosa layer. Communication between the theca layers and the granulosa layer is thus through the diffusion of material across the basement membrane only. The granulosa cells of the Graafian follicle surround the antrum which is filled with a viscous,

component rich fluid called follicular fluid. In addition, the cumulus oophorus, a mound of granulosa cells upon which the ovum rests, is located on one side of the antrum.

Both the theca interna and the granulosa cell layers are actively involved in the production of the various hormones that are so important in the reproductive process. According to the two cell theory, first proposed by Dorrington et al (1978) for testicular estrogen production and later adapted to the ovary by Fortune and Armstrong (1978), the theca interna cells produce increasing amounts of androgen in response to LH stimulation.These androgens then diffuse the basement membrane for conversion to estrogen by the granulosa cells in response to FSH. The porcine system, it should be noted, differs slightly in this regard. Porcine granulosa cells can produce estradiol in the absence of added androgen due to an abundance of androgen stores of thecal origin in the granulosa cells themselves. In addition, both porcine granulosa and theca cells can aromatize androgens to estradiol. The granulosa and theca cells are, furthermore, responsible for the production of progesterone in the corpus luteum and are also believed to secrete other compounds that have been identified in follicular fluid and which are believed to help regulate ovarian function (Bearden and Fuquay, 1984).

FORMATION OF FOLLICULAR FLUID

Follicular fluid is believed to originate from two sources: plasma and the cells of the follicle (McNatty, 1978). It appears as a liquid accumulating between the layers of granulosa cells in growing follicles long before the formation of the antrum. According to McNatty (1978), the secretion of mucopolysaccharides or proteoglycans by the granulosa cell layer helps even this early follicular fluid retain its jellylike matrix.

Follicle-stimulating hormone (FSH) and estrogen are responsible for ensuring that the granulosa cells of a growing follicle proliferate in an orderly arrangement, that is, spatially arranged so that a large antrum is formed. Only if this occurs can larger volumes of follicular fluid than those present in the intracellular spaces accumulate.

The antrum of a growing follicle, in addition to being filled by the products produced by the granulosa cells, is probably also filled by the influx of interstitial fluids (McNatty, 1978). As was mentioned earlier, it is important to note that the basement membrane and granulosa layer do not prevent the diffusion of interstitial fluid into the antrum. What is influenced by this membrane is the rate of transfer of different molecular weight substances and thus the composition of follicular fluid may be altered with respect to that of other body fluids such as blood.

PHYSICAL PROPERTIES OF FOLLICULAR FLUID

Follicular fluid is a slightly viscous solution that may vary in colour from a deep yellow to almost colourless. This colour variation may be due to degenerative changes occurring in follicles from ovaries that are excised and not immediately aspirated. This is postulated because fluid aspirated from follicles *in vivo* is normally only yellow in colour.

Most researchers have reported that the pH of follicular fluid is similar to that of plasma or slightly lower. Shalgi *et al* (1972), for example, reported that the pH of human follicular fluid is approximately 7.3 with the partial pressure of carbon dioxide (pCO_2) being the major factor regulating follicular pH. Some non-volatile fatty acids in follicular fluid may, however, also influence pH. In Shalgi's experiment, pCO_2 ranged from 17 to 54 mm Hg whereas that of blood ranged from 39 to 54 mm Hg. The partial pressure of O_2 tends to be highly variable in follicular fluid and, according to McNatty (1978), may be important for oocyte maturation during the final stages of follicular growth.

HORMONAL COMPONENTS OF FOLLICULAR FLUID

As was stated earlier, follicular growth and antrum formation are dependent upon the presence of various hormones. In fact, the exposure of the granulosa cells and

the oocyte of each follicle to a slightly different hormonal milieu than its neighbour allows one to explain why some follicles mature and ovulate whereas others become atretic (McNatty, 1978; Lacker, 1987). While several of these hormones come to the ovary via the circulation, many more are produced by the follicular cells themselves and are of great importance for normal reproductive functions. Follicular fluid is thus a very rich source of steroids and a good source of many other hormones. Some of these will be discussed in the following paragraphs.

The Gonadotrophins

The gonadotrophins, particularly FSH and luteinizing hormone (LH), are very important hormones involved in the regulation of ovarian function. Both of these hormones are produced by the anterior pituitary gland and are secreted into the circulation through which they reach the ovary.

In the ovary, FSH acts primarily on the granulosa cells to stimulate estrogen production and follicular development. The preovulatory surge of LH, on the other hand, is important in follicular rupture and luteinization.

FSH and LH have been found in the fluid of some, but not all, human antral follicles. In general, the highest levels of these hormones are found in follicular fluid when plasma concentrations are also elevated. However, LH is only found in follicular fluid if FSH is also present (McNatty, 1978). In addition, Bjersing et al (1972) found that the

concentration of LH in sheep follicular fluid was greater than 75 ng/ml, much higher than in plasma.

The number of gonadotrophin binding sites are known to change in both quantity and quality as the follicle grows (Edwards, 1974). According to Channing (1972), a 15 fold increase in LH receptor quantity, as judged by the binding of ^{125}I-hCG, can be seen as the porcine follicle grows.

It should be mentioned that prolactin (PRL), the third gonadotrophin, is also detectable in follicular fluid. Generally, however, PRL concentrations in follicular fluid are highly variable ranging from values much lower than those found in plasma to values much higher. In humans, though, follicular fluid PRL levels have been shown to vary with the developmental stage of the follicle despite this high fluctuation (McNatty, 1978).

The Steroids

The ovarian follicle is the source of three major types of steroid hormones: progestins, androgens, and estrogens (Hadley, 1984). It has been known for many years that the concentration of these steroids varies during follicular development in all species (YoungLai, 1972; Edwards, 1974; Hunter et al, 1976). The highest concentrations of androgens and estrogens are found in the follicular fluid of enlarging follicles, that is, during the follicular phase of the cycle. As ovulation approaches, however, the concentration of these hormones abruptly declines in many species. This is

believed to be due to a distinct alteration in follicular steroidogenesis (Hilliard and Eaton, 1971). Just prior to ovulation, follicles have been shown to contain very high concentrations of progestins (McNatty et al, 1975; Hunter et al, 1976).

Progesterone, testosterone, and estradiol 17B are each representative of one of the three types of steroid hormones mentioned above. Each will be discussed in greater detail below.

Progesterone concentrations in follicular fluid have been shown to increase coincident with the fall in the mitotic rate and increasing hypertrophy of the granulosa cells and with the appearance of LH in human follicular fluid (McNatty, 1978). In addition, studies in the hamster and sow have suggested that granulosa cells more actively synthesize progesterone prior to ovulation (Norman and Greenwald, 1972; Hunter et al, 1976). In fact, Hunter et al (1976) showed that progesterone concentrations in porcine follicular fluid exceeded 1000 ng/ml just prior to ovulation. Such an increase in progesterone may, however, not be characteristic of all species (eg. sheep) (Wheeler et al, 1975; Moor et al, 1975; McNatty, 1978).

Testosterone concentrations in human follicular fluid have been found to be between 10 and 3000 times higher than those in peripheral plasma (McNatty, 1978). The testosterone found in follicular fluid is believed to be produced by the theca interna cells of the follicle (McNatty et al, 1976)

and serves as a precursor for estrogen production along with other C 19 androgens such as androstenedione.

Estradiol 17B is believed to be secreted by the granulosa cells of the follicle. According to McNatty (1978), estradiol concentrations are 40 to 40000 times higher in human follicular fluid than in the peripheral plasma and comparable levels have been found in the follicular fluids of rhesus monkeys, cows, mares, and sows (Short, 1962a,b; Channing and Coudert, 1976; Hunter et al, 1976). As was mentioned earlier, follicular estradiol levels decline just prior to ovulation.

NON HORMONAL COMPONENTS OF FOLLICULAR FLUID

Follicular fluid contains many non-hormonal components in addition to the hormonal ones already mentioned. Among these are electrolytes, carbohydrates, proteins and proteoglycans.

The electrolytes sodium and potassium have been measured in the follicular fluid of many species including humans and cattle (McNatty, 1978). In general, sodium concentrations were found to be slightly higher than those in serum whereas potassium concentrations consistently exceeded those in serum. The importance of sodium and potassium is believed to lie in their regulation of the osmotic pressure of follicular fluid. The concentrations of other electrolytes (eg. Mg^{2+}, Cl^{-}, Ca^{2+} etc.) have also been measured in the follicular fluid of a number of species. The concentrations of these ions were found to be similar to those in serum

(Shalgi, 1972; McNatty, 1978). Some authors have suggested that potassium is secreted by the granulosa cells during follicular expansion (Edwards, 1974).

Several different carbohydrates have been measured in follicular fluid. In general, glucose is the major carbohydrate identified, comprising 80% of the total carbohydrate content of follicular fluid (McNatty, 1978). Fructose, ribulose, and lactic acid have been identified in trace amounts (Edwards, 1974). It has been noted, however, that the concentrations of glucose and other compounds decrease rapidly in the follicular fluid of excised ovaries so that the low levels of some of the carbohydrates reported may not be physiologically correct.

The protein composition of the follicular fluid of many species has been thoroughly studied. Most serum proteins have thus far been identified in follicular fluid. A study done by McGaughey (1975), for example, examined the protein content and composition of porcine follicular fluids. It was found that the follicular fluids all contained more albumin and alpha globulins, but less gamma globulins than serum. In addition, the fluids from follicles at different physiological stages exhibited differences in their protein patterns. This difference may be related to oocyte maturation.

Over the years, histochemical analysis has made it possibe to identify the presence of proteoglycans in follicular fluid. Proteoglycans are polysaccharide- protein

complexes. They are made up of glycosaminoglycans (GAGs) which are polysaccharides composed of repeated disaccharide units. These units are generally covalently bound to a protein core to form the proteoglycan. Chondroitin sulfate and heparan sulfate are the two GAGs generally found in follicular fluid (Bellin and Ax, 1984). However, chondroitin sulfate seems to be the predominant GAG of the proteoglycans in rat, pig and cow follicular fluid (Yanagishita and Hascal, 1979; Ax and Ryan, 1979; Grimek and Ax, 1982).

Proteoglycans tend to accumulate in ovarian follicular fluid during follicular growth. In addition, the GAG composition and degree of sulfation of proteoglycans has been shown to vary during the ovulatory cycle. Ax and Ryan (1979) showed that the concentration of chondroitin-like material decreased eight fold with follicular maturation, while concentrations of heparan sulfate decreased two fold. The degree of sulfation of the chondroitins was shown to increase as the follicle matured.

The roles proteoglycans play in reproductive processes are still not completely clear. It has been suggested that GAGs may play a role in the control of oocyte maturation <u>in vivo</u> by preventing cumulus expansion (Eppig, 1981) and specific GAGs may also be indicators of atresia. For example, Bellin and Ax (1984) reported that healthy bovine follicles had elevated estrogen concentrations and relatively low concentrations of progesterone and chondroitin sulfate. Atretic follicles, on the other hand,

generally contained low amounts of estrogen with elevated progesterone and chondroitin sulfate levels. The suggestion has also been made that the decrease in GAG concentration that occurs as the follicle matures contributes to the conversion of the granulosa cells to LH dependence and that proteoglycans may enhance the acrosome reaction in sperm (Lenz et al, 1982). In fact, Lenz et al (1982) showed that the addition of bovine follicular proteoglycan or chondroitin sulfates ABC significantly increased the incidence of the acrosome reaction in bovine sperm incubated in vitro.

FOLLICULAR FLUID AND THE OVIDUCT

As ovulation approaches, the attachment of the oocyte and its adjacent cell mass to the follicle wall becomes weaker. Eventually, the oocyte and surrounding cumulus cells completely detach and lie free in the follicular fluid (McNatty, 1978). When ovulation occurs, one of the major functions of follicular fluid thus appears to be to help the oocyte escape from the follicle. Without the presence of follicular fluid to facilitate the movement of the relatively small oocyte through the relatively large antrum, it would be very difficult for it to be extruded.

A second important role of follicular fluid appears to be to ensure the safe transport of the oocyte to the oviduct. It is important to realize that follicular fluid is not released explosively from the follicle at the time of

ovulation (Edwards, 1974). Instead it slowly oozes out from the ruptured stigma and in species such as the guinea pig, rabbit and human, for example, its high viscosity helps ensure that the oocyte remains on the surface of the ovary. Movement of the fimbria over the ovary then allows the cilia to sweep the oocyte from the surface of the ovary into the ampulla. In rodents, the role of follicular fluid is slightly different. In these animals the oocyte does not adhere to the surface of the ovary but is, instead, shed into the periovarial sac that surrounds the ovary. Follicular fluid is believed to contribute to the medium around the ovary in these species (Edwards, 1974).

Short (1964) suggested that follicular fluid may also act to enhance tubal transport of the oocyte due to its high steroid content. It is now believed, however, that this is not likely since steroidogenesis declines so dramatically prior to ovulation in many species (Edwards, 1974). Prostaglandins found in follicular fluid may, however, be involved.

SPERM MORPHOLOGY

Spermatozoa are, as Bellve and O'Brien put it (1983), "intricate and highly polarized cells which have evolved structurally and functionally to ensure efficient transmission of the paternal genome to the oocyte at fertilization." Spermatozoa are composed of a head, which

consists almost entirely of nucleus, and a tail. The sperm nuclei of mammalian sperm are generally more elongated and condensed than those of somatic cells. In general, sperm nuclei take on a shape characteristic for each species of mammal (Bellve and O'Brien, 1983).

Surrounding the dense chromatin of mammalian sperm is the nuclear envelope. This envelope is made up of two membranes lying 7-10 nm apart (Fawcett, 1975). Both the nuclear membrane and the plasma membrane of the sperm have been shown to fuse at the caudal end of the sperm head (Friend and Fawcett, 1974) forming the posterior ring. Below this ring the two membranes again separate and extend into the neck region (Fawcett, 1975). Nuclear pores are found in this "redundant envelope" (Friend and Fawcett, 1974). The attachment site of the tail, kncwn as the implantation fossa, lies at the base of the nucleus. The nuclear and plasma membranes are again in close proximity of each other at this point.

Above the posterior ring, the perinuclear material generally covers the nucleus. In addition, the post acrosomal lamina forms an envelope around the caudal portion of the nucleus immediately underneath the plasma membrane.

The tip of the nucleus is covered by the acrosome. The size and shape of the acrosome varies significantly among species. In most mammals, however, the acrosome is a continuous organelle (Bellve and O'Brien, 1983). The plasma membrane of the sperm overlies the acrosomal membranes. The

acrosome has been shown to contain a variety of hydrolytic enzymes including acid phosphatase, arylsulfatase, B N acetylglucosaminidase, phospholipase and hyaluronidase (Srivastava et al, 1965; Stambaugh and Buckley, 1970). It is believed that many of these enzymes play an important role in fertilization.

The tail of mammalian sperm can be divided into four distinct regions: 1) the neck region located, as mentioned earlier, at the base of the nucleus; 2) the midpiece- a region characterized by helically arranged mitochondria, an axoneme and outer dense fibers; 3) the principal piece containing the axoneme and outer dense fibers responsible for sperm motility and surrounded by a fibrous sheath; and finally 4) the end piece also containing the axoneme, but with an altered arrangement of microtubules surrounded by a less dense fibrous sheath (Bellve and O'Brien, 1983). Together the different constituents of the sperm tail are capable of producing the whip-like movement that propels the sperm.

CAPACITATION

Epididymal maturation is, according to Bedford (1979), required by the spermatozoa of all mammalian species. This process involves numerous metabolic, physiological, as well as morphological changes in the sperm. Before a spermatazoon leaving the male reproductive tract is capable of fertilizing an egg, however, it must also undergo a

maturation process in the female reproductive tract known as capacitation. The need for this process was first recognized more than 35 years ago by Austin (1951) and Chang (1951) who presented evidence to show that ejaculated rat and rabbit sperm had to be exposed to the female reproductive tract environment for several hours before acquiring the ability to fertilize an egg. In fact, the need for capacitation has now been extended to include all mammalian spermatozoa that have been studied to date (Moore and Bedford, 1983).

The term capacitation , coined in 1952 by Austin, has become a functional term that encompasses all changes a spermatozoon must undergo in the female tract or in in vitro incubations in order to successfully interact with and fertilize an egg. The term, however, generally does not include the acrosome reaction, as this is believed to be an independent event (Bedford, 1970; Meizel, 1984) and can, in fact, now be temporally separated from capacitation as such (Yanagimachi and Usui, 1974). It should be noted, however, that many scientists still disagree about which events are strictly part of capacitation and which are strictly part of the acrosome reaction, since a clear distinction between the two is extremely difficult to make (Clegg, 1983). Capacitation and the acrosome reaction are thus often discussed together. It is generally agreed, however, that the events occurring during capacitation directly prepare the spermatozoa for the acrosome reaction.

The time required to achieve functional capacitation in

vivo varies from 1.5 to 6 hours depending on the species in question (Hunter, 1982). In addition, the different regions of the female reproductive tract differ in their potential to capacitate spermatozoa (Moore and Bedford, 1983). For some species, such as the pig, the uterotubal junction and the oviduct are probably of primary importance in capacitation (Chang and Hunter, 1975). However, as was shown by Hunter and Hall (1974), the sequential exposure of the spermatozoa to the uterus and then the oviduct accelerates the process. For other species, namely the vaginal depositors (eg. ruminants), the uterus seems to play a more prominent role in capacitation.

The endocrine status of the female has also been shown to alter the ability of the reproductive tract to capacitate sperm (Moore and Bedford, 1983). While the capacitating potential of the oviduct is quite difficult to regulate, high plasma estrogen levels tend to promote capacitation in the uterus while high levels of progesterone will suppress capacitation. According to Moore and Bedford (1983), the results obtained from endocrine studies seem to suggest that capacitation is thus mediated through specific female reproductive tract factors whose identity remains a mystery. One thing that is certain, however, is that the factors are not species specific (Saling and Bedford, 1981).

It has been suggested that a number of factors normally present at ovulation may facilitate capacitation or even trigger the acrosome reaction. For example, Yanagimachi

showed as early as 1969 that follicular fluid appeared to stimulate spermatozoa to become fully capacitated and undergo the acrosome reaction. Similarly, Gwatkin et al (1972) showed that cumulus oophorus also appeared to stimulate the acrosome reaction. Moore and Bedford (1978), however, have clearly shown that fertilization of washed ova can occur in vivo and in vitro in the absence of follicular products. It also seems that the factors responsible for initiating the sequence of events leading to and including the acrosome reaction do not come from the egg.

THE ACROSOME REACTION

Austin and Bishop (1958) were the first to recognize that the acrosome reaction was an important step in mammalian fertilization. They observed that the acrosomal caps of hamster and guinea pig sperm were first modified and then lost before the sperm began to penetrate the zonae pellucidae of their respective oocytes.

While the precise biological significance of the acrosome reaction is not completely clear, its function appears to be at least two-fold. Its first function is thought to be the release or exposure of acrosomal enzymes that are believed to enable the sperm to penetrate the egg investments, whereas the second function is probably to trigger a physiological change in the sperm plasma membrane which renders it capable of fusing with the egg plasma membrane.

Microscopic observations and ultrastructural studies have shown that the acrosome reaction involves membrane vesiculations between the plasma membrane and the outer acrosomal membrane beneath it at the anterior end of the acrosome (Piko and Tyler, 1964; Barros et al, 1967). This results in the formation of gaps through which the contents of the acrosome can diffuse. These gaps or pores are quite numerous but their arrangement enables much of the plasma membrane overlying the acrosome and its proposed receptors for binding to the zona pellucida to be retained while at the same time allowing the acrosomal contents to be released (Moore and Bedford, 1983). It should be noted that fertilizing spermatozoa are not believed to undergo extensive vesiculation in the equatorial region under normal conditions (Bedford and Cooper, 1978).

Species differences are thought to exist in the speed at which the acrosome reaction occurs. In vitro experiments have shown that in guinea pig sperm preincubated in a calcium (Ca^{2+}) free medium, the acrosome reaction occurs within one minute of exposure to Ca^{2+} (Yanagimachi and Usui, 1974). Hamster sperm, on the other hand, require approximately 20 minutes to undergo the acrosome reaction in vitro (Talbot and Franklin, 1976). The amount of time required by spermatozoa in vivo to completely acrosome react is unknown.

HYPERACTIVATION OF SPERM

The term hyperactivation refers to a change in the pattern of beat of the sperm tail that occurs in the sperm of some mammals as a result of capacitation. This swimming behaviour was first described by Yanagimachi (1969a,b) and is characterized by "vigorous whiplash like beating of the flagellum, with the sperm head tracing erratic figure eights" (Yanagimachi, 1981). It is believed to be a physiological phenomenon since hamster sperm with this motility pattern have been observed through the wall of the ampulla around the time of fertilization (Yanagimachi, 1970). Even though hyperactivation appears to occur at the same time as the acrosome reaction, it is separable from this event (Barros and Berrios, 1977) and should be treated as a separate phenomenon.

The physiological significance of hyperactivated motility for fertilization remains uncertain. It may help promote sperm transport from the isthmus to the ampulla of the oviduct (Cummins, 1982) and it has been postulated that hyperactivation gives the sperm the thrust it requires for penetration of the cumulus oophorus and then the egg.

The initiation of hyperactivation is influenced by many factors. The absence of Ca^{2+} from the incubation medium, for example, will prevent initiation of hyperactivation. In addition, the presence of zinc in an incubation medium and cold temperatures will prevent hamster and mouse sperm from becoming hyperactivated (Aonuma et al, 1980). The presence

of caffeine and glucose, on the other hand, tends to promote hyperactivation of mouse sperm (Fraser, 1979; Fraser and Quinn, 1980).

REQUIREMENTS FOR CAPACITATION AND/OR ACROSOME REACTION INITIATION

While many aspects of capacitation and the acrosome reaction remain unclear, a number of steps or requirements are now recognized as being essential. The first of these appears to be the availability of calcium. A number of researchers (Yanagimachi and Usui, 1974; Talbot et al, 1976; Green, 1978) have shown that the addition of extracellular Ca^{2+} will initiate a normal acrosome reaction in sperm kept in capacitating media depleted of Ca^{2+} or containing ionophore A23187. In addition, as Clegg (1983) suggested, increased levels of intracellular calcium may also be important in such things as the activation of acrosin, the membrane modifications occurring before vesiculation, and in the control of adenylate cyclase activity.

A second important step in the capacitation process appears to be the modification of membrane components. Early studies using standard transmission electron microscope techniques failed to show any such alterations. However, procedures involving plant lectins or antisperm antibodies have since shown that proteins or glycoproteins are altered during capacitation. For example, Nicolson and Yanagimachi

(1972) showed that plant lectins readily bind and agglutinate rabbit and hamster sperm. In addition, Gordon et al (1974) showed that concanavalin A binds uniformly over the head of freshly ejaculated sperm. Capacitated sperm, however, were found to bind concanavalin A in the post acrosomal region only. Changes in membrane lipid composition may also be important for the acrosome reaction. For example, a decrease in the cholesterol content of the rat sperm plasma membrane during capacitation appears to be essential for the occurrence of the acrosome reaction (Davis, 1976,1978,1980).

A third important event that must occur during capacitation is the removal of sperm surface components. It is well known that freshly ejaculated sperm are normally coated with a variety of macromolecular substances that are important in protecting the sperm. If these substances are not removed during capacitation or if high concentrations of them are present in the capacitating medium, the acrosome reaction will not occur (Yanagimachi, 1981).

A number of intracellular components are also thought to be involved in capacitation and the acrosome reaction. ATP, ADP, cAMP, cGMP, adenylate cyclase, phosphodiesterase, and protein kinase are known to be intricately involved in such things as sperm motility, metabolism and survival (Yanagimachi, 1981). The precise roles these nucleotides and related enzymes play in the capacitation-acrosome reaction sequence has, however, still to be established.

MOLECULAR MECHANISM INVOLVED IN CAPACITATION AND THE ACROSOME REACTION

Due to the complexity of the series of events that make up capacitation and the acrosome reaction, researchers still do not agree on the molecular mechanism involved in these processes. A number of different hypotheses have been put forward, however. Several of these are briefly outlined below. The outline is based on one published by Yanagimachi in 1981.

The Meizel Hypothesis (Meizel, 1978- Hamster sperm)

Meizel hypothesizes that some unknown mechanism initiates the flow of Ca^{2+} into the space between the outer acrosomal and plasma membranes. This, in turn, causes stimulation of a calcium dependent ATPase and thus increases Ca^{2+} uptake by the acrosome. The conversion of proacrosin to biologically active acrosin is stimulated by the increased amount of Ca^{2+} taken up by the acrosome. Acrosin is then thought to activate a phospholipase in the acrosome. This phospholipase breaks membrane phospholipids into free fatty acids and lysophospholipids. The presence of lysophospholipids is thought to facilitate the fusion of the outer acrosomal membrane and the plasma membrane by increasing the perturbance of the lipids found in the outer acrosomal membrane.

The Green Hypothesis (Green,1978)

Green hypothesizes that the sperm plasma membrane

contains an acetylcholine-like receptor that is normally occupied by an antagonist. This antagonist must be removed during the capacitation process. Once the antagonist is removed, the receptor can be stimulated by a specific factor (possibly follicular cells or the zona pellucida) to allow an influx of Ca^{2+}. This causes an efflux of Mg^{2+} and protons. One of these events (influx or efflux) triggers membrane fusion. A calcium dependent proteinase or some similar factor then converts proacrosin to acrosin. The acrosin subsequently solubilizes the acrosomal matrix causing the acrosome to swell.

The Davis Hypothesis (Davis,1978)

Davis hypothesizes that a lipid exchange that occurs between the sperm membrane and the cholesterol rich lipoprotein vesicles in seminal plasma stabilizes the membrane. During capacitation, cholesterol is transferred from the membrane to albumin resulting in destabilization. Aggregatiom of membrane phospholipids then occurs, as Ca^{2+} binds to the polar membrane phospholipid heads of both the outer acrosomal membrane and the plasma membrane. This leads to membrane fusion.

The Gordon Hypothesis (Gordon, 1973; Gordon et al, 1978)

Gordon hypothesizes that the removal of a seminal plasma factor activates a calcium independent ATPase in the sperm plasma membrane. This ATPase then transports extracellular Ca^{2+} into the space between the outer acrosomal and plasma

membranes. This Ca^{2+} is transported into the acrosome after it activates a calcium dependent ATPase on the outer acrosomal membrane. The Ca^{2+} then converts proacrosin to acrosin and by binding to membrane phospholipids causes adhesions of the plasma and outer acrosomal membranes. Fusion of the two membranes occurs. The fused membranes are, in turn, partially digested by the acrosin thus causing vesiculation and enzyme release.

The Yanagimachi Hypothesis (Yanagimachi, 1981)

Yanagimachi hypothesizes that a Ca^{2+} carrier protein in the sperm plasma membrane adsorbs material during its transit down the epididymis and vas deferens and during exposure to seminal plasma. These coating materials are removed or modified during capacitation by simple elution, enzymatic action or by nonspecific substances such as albumin. In some species specific substances may also be involved. The removal or alteration of the coating material or the specific substances themselves then activate the Ca^{2+} carrier protein and Ca^{2+} enters into the space between the outer acrosomal and plasma membranes. This intracellular calcium allows the two membranes to come closer together by neutralizing the negative charges on both membranes and induces a phase transition and a lateral phase separation of the membrane phospholipids. A phospholipase in the plasma and outer acrosomal membranes is also activated by the Ca^{2+}. Membrane phospholipids are thus cleaved into

lysophospholipids and free fatty acids. The lysophospholipids, in turn, induce increased membrane perturbance, thus facilitating membrane fusion.

FOLLICULAR FLUID, CAPACITATION AND THE ACROSOME REACTION

The role of follicular fluid in sperm capacitation and the acrosome reaction has long been debated. As early as 1964, Yanagimachi and Chang observed that hamster eggs could be fertilized *in vitro* in the absence of uterine and oviductal secretions. This led to the hypothesis that follicular fluid was playing an important role. During the years that followed, capacitation of hamster sperm was achieved using bovine follicular fluid (Gwatkin and Andersen, 1969; Yanagimachi, 1969 a,b) and mouse eggs were fertilized by Iwamatsu and Chang (1969) in the presence of this fluid. In addition, the follicular fluid of several species, including humans and pigs, has been shown to possess acrosome reaction inducing ability (Mukerjee and Lippes, 1972; Oliphant *et al*, 1977). Unfortunately, however, the results of some experiments have not always been as clearly in favour of a role for follicular fluid in capacitation and the acrosome reaction as those just mentioned. For example, evidence has been presented that shows that the eggs of mice, rabbits and hamsters can be fertilized *in vitro* after the cumulus cells have been removed and the eggs repeatedly washed (Fraser *et al*, 1971;

Miyamoto and Chang, 1972). In addition, as was mentioned earlier, Moore and Bedford (1978) have reported that fertilization of washed ova can occur *in vivo* and *in vitro* in the absence of follicular products. In view of this, it becomes obvious that a lot of work remains to be done before a definite conclusion about the role of follicular fluid can be drawn.

SPERM TRANSPORT IN THE FEMALE REPRODUCTIVE TRACT

Species differences are known to exist in the site of semen deposition. In cattle and sheep, for example, a rather small amount of semen is ejaculated into the vagina close to the cervix. In horses and pigs, on the other hand, a large volume of semen is deposited through the cervix directly into the uterus. Interestingly enough, however, even though the site of semen deposition varies enormously among the different species, the actual number of sperm reaching the site of fertilization is remarkably similar. In both cases, this number represents only a small fraction of those ejaculated.

Stages of Sperm Transport

Sperm transport in the female reproductive tract can be divided into three stages: rapid transport; colonization of reservoirs; and slow release.

Rapid Transport

This phase of sperm transport is most evident during the

interval immediately following insemination. It is characterized by almost instantaneous passage of sperm along the entire length of the reproductive tract and has been observed in every mammalian species examined. It is presumed to result from a passive transfer of sperm due to female visceral contractions (Overstreet, 1983). It is not known whether these first spermatozoa entering the oviduct participate in fertilization. It has been proposed that fertilization occurs only when a critical number of sperm reach the site of fertilization (Hafez, 1987). In addition, it has been found that nearly all of the sperm recovered from the rabbit oviduct after rapid transport are immobile (Overstreet and Cooper, 1979). Furthermore, these researchers reported that 98% of these sperm had visibly disrupted acrosomes and 15% had separated into head and tail. It is possible, therefore, that, as Overstreet (1983) suggested, the biological importance of rapid sperm transport relates not to sperm passage through the oviduct but to events occurring in the lower tract.

Colonization of Sperm Reservoirs

In mammals where sperm are deposited into the vagina, large numbers of sperm are trapped in the complex mucosal folds of the cervix. In fact, micelles of the cervical mucus aid in directing the sperm to the cervical crypts where the reservoir is formed (Hafez, 1987).

In animals where sperm are ejaculated into the uterus, sperm reservoirs are found at the uterotubal junction (eg.

pig) or in the endometrial glands (eg. dog) (Hafez, 1987).

It has been observed that the sperm concentration gradients found in the different parts of the female reproductive tract are important for fertility. According to Hafez (1987), the more sperm there are that enter the cervical reservoir, for example, the more that will reach the oviduct and the longer a critical concentration of sperm will be maintained in the oviduct.

Slow Release

Slow release refers to the sequential release of sperm from sperm reservoirs. It not only involves the contraction of the myometrium and mesosalpinx but also requires the innate motility of the sperm. Slow release ensures that sperm will be continuously available to enter the oviduct over a period of time and thus increases the chances of fertilization.

REGULATION OF SPERM TRANSPORT AND MIGRATION

Active sperm migration through the female reproductive tract depends on the flagellar activity of the sperm cells as well as on their interaction with the various secretions and structures in the tract (Overstreet, 1983). Each portion of the reproductive tract is, therefore, involved in a different aspect of migration and transport regulation.

The Cervix

The cervix is a canal with very thick walls made up

almost entirely of connective tissue. Its mucosa consists of an intricate system of grooves, clefts and crypts and it is richly endowed with mucus secreting glands. The cervical mucus is a hydrogel which consists of two main elements: cervical mucin and soluble components. Mucin is a carbohydrate-rich glycoprotein with a fibrillar system of long molecules linked to form a three dimensional network. The soluble components of mucus include organic compounds such as maltose, glucose, amino acids, peptides, proteins and lipids and various inorganic salts.

The blood levels of the ovarian hormones greatly affect the consistency of the cervical mucus and thus these levels are important for the maintenance of a cervical population of sperm. Under estrogen dominance, for example, the mucus becomes extremely thin and watery and the mucus fibrils orient themselves in parallel chains (Harper, 1982). This permits sperm to swim up through the mucus. Under progesterone dominance, on the other hand, the mucus tends to be very viscous and rubbery. It has no apparent micellar structure at this time and, thus, does not allow sperm penetration. An imbalance of estrogen and progesterone can thus significantly affect the transport of sperm by altering the characteristics of the cervical mucus (Hafez, 1987).

The Uterus

The transport of sperm through the uterus is believed to be fairly rapid. Data from numerous experiments have shown that sperm can reach the oviduct in a matter of minutes

(Harper, 1982). It is generally thought that the contraction of the vagina and the myometrium plays a major role in this rapid transit since sperm would be unable to swim the required distance in the time observed. This assumption is further supported by the fact that inanimate objects such as carbon particles or dead sperm and radiolabelled solutions are moved quickly from one end of the uterus to the other. This uterine motility may by stimulated by agents, such as perhaps prostaglandins, found in seminal plasma.

It is believed that the presence of sperm in the uterus induces a leukocytic response in the endometrium (Hafez, 1987). This response is of particular importance since the migration of leukocytes into the uterine lumen and their phagocytosis of sperm appears to be the major mechanism involved in the removal of these cells from the female tract (Hafez, 1987). It should be noted that the ingested sperm do not necessarily have to be dead or damaged ones. Many healthy, live sperm are also removed in this manner.

The Uterotubal Junction

The uterotubal junction is, as the name implies, the point where the uterus and the oviduct join. The uterotubal junction varies from being anatomically quite simple in some species to being extremely complex in others. Its precise physiological function is not yet clear. In species such as the hamster or rat, it seems to be a very efficient sperm barrier. The junction may also limit the passage of dead

sperm into the oviduct. In addition, it may act as a valve under hormonal control since it is quite difficult to force liquids or gases through the junction, except at estrus or when the eggs are passing into the uterus (Harper, 1982).

The Oviduct

The oviduct is a relatively narrow tube extending from the uterus to the ovary. The lumen of the oviduct tends to be quite narrow, as large, numerous mucosal folds project into the tube. Many secretory cells line the oviduct, especially in the isthmic region, and secretions from these cells fill the lumen of the tube. Cilia also line parts of the oviduct.

As was mentioned earlier, the uterotubal junction tends to be closed except at estrus or when the eggs are passing into the uterus. Tubal secretions thus normally flow passively into the peritoneal cavity. The cilia lining the oviduct, however, habitually beat towards the uterus. The fluid flow created by the ciliary action is believed to be confined to a shallow layer though and the direction of flow is likely to be reversed by constrictions such as the ampullar-isthmus junction. Even this fluid will thus eventually flow through the fimbriated end of the oviduct and into the peritoneal cavity (Harper, 1982).

Sperm transport through the oviduct is believed to be controlled by several mechanisms. Peristalsis and antiperistalsis of the oviductal musculature, contractions of the mucosal folds and the mesosalpinx, fluid currents and

counter-currents created by the action of cilia lining parts of the oviduct and the opening and closing of the intramural portion may all be involved in the transport of sperm to some extent (Hafez, 1987).

It is interesting to note that sperm transport in the oviduct is not a continuous process. Spermatozoa have been found to remain in the lower isthmus for extended periods of time and it is generally agreed that their steady transit to the ampullar-isthmus junction only occurs at about the time of ovulation (Harper, 1982). Why this occurs remains a mystery, although it may have something to do with conserving energy to improve the likelihood of fertilization once eggs are available.

FERTILIZATION

Fertilization leading to the formation of a zygote is the ultimate objective behind all reproductive phenomena. It is generally considered to be a dual process in that it has both embryologic and genetic aspects (McLaren, 1974). Embryologically fertilization involves activation of the ovum by the sperm whereas genetically it involves the introduction of hereditary material from the male into the ovum. Fertilization is said to start with the collision between an oocyte and a sperm and end after their pronuclei have emerged and fused (Bearden and Fuquay, 1984).

The first step in mammalian fertilization involves

penetration of the sperm through the cumulus oophorus, if this is still present, and binding of the sperm to the zona pellucida. It is interesting to note that cumulus dispersion was once thought to be essential for successful sperm penetration of the egg. This is, however, not true since the sperm of many species (eg. rabbit, mouse) have been shown to penetrate the egg long before the cumulus cells are lost (Yanagimachi, 1981). As was mentioned earlier, enzymes associated with the sperm head are involved in its penetration through the cumulus complex.

Once sperm have bound to the zona pellucida, a process which does not appear to require capacitation, at least in vitro (Russel et al, 1980), the next obstacle a sperm must overcome before it can enter the egg is the zona pellucida itself. It is probable that enzymes associated with the sperm acrosome facilitate its passage through the zona. Capacitation and the acrosome reaction are thus essential prerequisites for zona penetration (Hunter, 1982; Moore and Bedford, 1983). In addition, the hyperactivated motility that has been shown to occur as a result of capacitation gives the sperm the extra thrust they require to penetrate the zona (Bedford, 1983).

After penetration of the zona by the sperm, fusion with the egg usually follows rapidly. According to Johnson et al (1975), sperm-egg fusion may occur anywhere on the surface of the egg except over a microvilli-free area overlying the metaphase spindle. It is believed that the segment of the

plasma membrane remaining over the equatorial region of the sperm fuses first with the oolemma (Bedford and Cooper, 1978; Talbot and Chacon, 1982). Fusion then proceeds caudally as far as the post acrosomal region until the post acrosomal plasma membrane becomes continuous with the oolemma and until finally the tail plasma membrane merges with the oolemma (Yanagimachi, 1978).

Sperm-egg fusion has been shown to initiate a number of important changes within the egg. One of the most striking results of egg activation in some species (eg. pig, cow) is the shrinking of the vitellus. At the same time, the sperm head in the vitellus normally swells and becomes gel-like in consistency (McLaren, 1974). Numerous nucleoli appear within the sperm nucleus at this time and a nuclear membrane develops. The resulting structure is referred to as the male pronucleus.

Formation of the female pronucleus begins soon after sperm entry into the egg and extrusion of the second polar body. The male and female pronuclei then develop synchronously until at some point during their maximum development they come into contact. The pronuclei then begin to shrink and the nucleoli and nuclear membranes disappear. Shortly before the first cleavage, two groups of chromosomes then become visible. They subsequently come together to form one group which represents the prophase of the first cleavage mitosis (McLaren, 1974). Fertilization is then complete.

EXPERIMENT I: Follicular Fluid Entering the Porcine Oviduct

OBJECTIVES

The purpose of this experiment was to determine the quantity of follicular fluid entering the porcine oviduct during the process of ovulation in an effort to establish whether or not this follicular fluid could be involved in sperm-egg interaction *in vivo*. Progesterone was used as a marker to indicate the presence of follicular fluid in the oviduct as its concentration has been shown to increase so dramatically in porcine follicular fluid just prior to ovulation (Hunter *et al*, 1976). It was thus proposed that an increase in the progesterone concentration of tubal fluid would be indicative of follicular fluid entering the oviducts.

MATERIALS AND METHODS

Animals

Sixty four prepubertal Landrace gilts (Macdonald College Herd) were used in this experiment. These gilts weighed between 52 and 88 kg and were housed at the Macdonald College Swine Facilities. They were fed a commercial 14% crude protein pelleted ration and provided with water *ad libitum*.

Follicular growth and development was stimulated in the gilts using 750 I.U. of pregnant mare's serum gonadotropin

(PMSG, Equinex, Ayerst Laboratories, Montreal) followed 72 hours later by 500 I.U. of human chorionic gonadotropin (hCG, A.P.L., Ayerst Laboratories, Montreal). In this model, ovulation will occur 42±2 hours later.

Four different treatment groups were used in this experiment. In Group I, the preovulatory group, surgery was performed 38 hours after the hCG injection (n=16). In Group II, surgery was carried out 42 hours post hCG. All pigs in this group (n=16) were thus in the process of ovulating. Surgery was performed on Group III animals (post ovulatory, n=16) 46 hours after the hCG injection. Group IV animals (ovulation blocked, n=16) were injected with indomethacin (10 mg/kg,Merck Frosst, Kirkland, Quebec) suspended in propylene glycol (Fisher Scientific Company, Fair Lawn, N.J.) 24 hours following the hCG injection. Surgery was performed on these animals 22 hours later (46 hours post hCG).

Fluid Collection

Prior to surgery, all gilts were injected with 0.045 mg/kg Atropine sulphate (Vetcom Inc., St. Liboire, Que.) and 2ml of Innovar-vet (M.T.C. Pharmaceuticals, Mississauga, Ont.). Anaesthesia was induced using halothane (M.T.C. Pharmaceuticals, Mississauga, Ont.) and maintained using a halothane closed circuit system. The reproductive tract was exposed by midventral laparotomy and a peritoneal fluid sample was collected (groups II, III, and IV). A small glass

tube with a polypropylene extension was then inserted into the fimbrial end of one oviduct. A blunt 20 gauge needle on a 3 ml syringe was finally inserted through the uterotubal junction and the oviductal contents were flushed out through the glass/polypropylene tubing into a 13 X 100 mm test tube using 1.5 ml of warm saline. The ovary was then clamped off at the base and an ovariectomy was performed. The ovary was kept in saline until the follicular fluid was aspirated. The same procedure was repeated on the other side of the reproductive tract. The tubal fluid collected was centrifuged for 10 minutes at 1000 x g to remove all cellular debris, the supernatant fluid was aspirated, its volume recorded and then frozen at -20C in scintillation vials until the time of the progesterone assay.

Follicular fluid was aspirated from all follicles (Groups I, II, and IV) larger than 7 mm in diameter with a 23 gauge needle on a 1 ml syringe within 10 minutes of ovariectomy. The aspirated follicular fluid was centrifuged for 10 minutes at 1000 x g to remove cells and debris. The supernatant fluid was aspirated from individual test tubes following the centrifugation, again using a 23 gauge needle on a 1 ml syringe, and the volume collected was recorded. All fluid was then placed into individual 10 ml scintillation vials, capped tightly and frozen at -20C until the time of the assay for progesterone. In all groups, the follicular size, number of follicles and, when appropriate, number of ovulation points were recorded.

Blood samples were taken from all gilts during the surgery. This was done by inserting a 20 gauge Vacutainer needle into an ear vein and then collecting the blood in a heparinized Vacutainer (Becton Dickinson, Mississauga, Ont.). Tubes containing the blood samples were centrifuged immediately after collection for 15 minutes at 1000 x g. The plasma was then aspirated and placed into scintillation vials. All samples were capped tightly and frozen at -20C until it was time to assay them for progesterone.

Oviductal Volume Determination

For purposes of subsequent calculation, it was necessary to determine the volume of the oviduct. This was done by first inserting a small glass tube into the fimbrial end of the oviduct. A blunt 20 gauge needle on a 1 ml syringe filled with erythrosin B stain was then inserted through the uterotubal junction. The stain was slowly injected into the oviduct and at its first appearance in the glass tube, the volume injected was recorded. The average oviductal volume was found to be 200 ul of fluid.

Progesterone Assay

All peritoneal fluid, follicular fluid, tubal fluid, and plasma samples collected were assayed for progesterone concentration using the Coat a Count assay kit (Diagnostic Products Corporation, Los Angeles, California). Briefly, duplicates of 100 ul of the different standards provided by

the kit or 100 ul of sample were pipetted into individual progesterone antibody coated tubes. One millilitre of buffered (I^{125}) progesterone was then added to every tube as well as to two "total count tubes". Tubes were vortexed and then incubated for 3 hours at room temperature. Finally, the contents of each tube, except the total count tubes, were decanted and the tubes were counted for 1 minute in a gamma counter. It is important to note that all follicular fluid samples were subjected to a 1:1500 dilution to bring the progesterone concentration into the assay range. The minimum detectable level of this progesterone assay was 0.004 ng/ml.

Statistical Analysis

Data obtained in this experiment were analysed using the General Linear Models (GLM) procedures of the Statistical Analysis System (SAS, 1982). Differences between the means of the various variables examined were determined using Duncan's New Multiple Range Test. A complete outline of the statistical program used is given in Appendix Table 1.

RESULTS

Data collected for experiment I are summarized in Appendix Table 2.

Table 1 summarizes the mean number of follicles (NOF) observed in the different treatments and for each side of the reproductive tract. The mean number of follicles clearly varied slightly between treatments. The lowest number of follicles were seen in those pigs examined 38 hours after the injection of hCG whereas pigs examined 46 hours post hCG had the largest number of follicles. The number of follicles at 38 hours post hCG was significantly lower than the number of follicles at 46 hours post hCG ($p<0.05$). The mean number of follicles on the left ovary was found to be significantly higher than the mean number of follicles on the right ovary ($p<0.05$).

The mean number of ovulations (NOOV) that occurred in the treatment groups and on the two sides of the reproductive tract are given in Table 2. The number of ovulations observed followed the pattern one would expect to obtain at the times used. No ovulations were seen in the group of pigs examined 38 hours after the hCG injection nor in the group treated with indomethacin and examined 46 hours post hCG. At 42 hours post hCG, 53% of all follicles had ovulated while at 46 hours post hCG 100% ovulation was observed (Figure 1). There was no significant difference between sides in the mean number of ovulations observed.

Table 1. Mean Number of Follicles (±SEM) For PMSG/hCG Stimulated Gilts- by Treatment and Side.

	Treatment				
Side	38h	42h	46h	46h(Indo.)	Mean±SEM
Left	7.1 ±1.2	7.9 ±1.2	9.7 ±0.9	8.7 ±1.0	8.3[a] ±0.5
Right	5.5 ±0.8	7.7 ±1.2	7.8 ±0.7	6.1 ±1.0	6.8[b] ±0.5
Mean ±SEM	6.3[a] ±0.7	7.8[ab] ±0.8	8.7[b] ±0.6	7.3[ab] ±0.7	

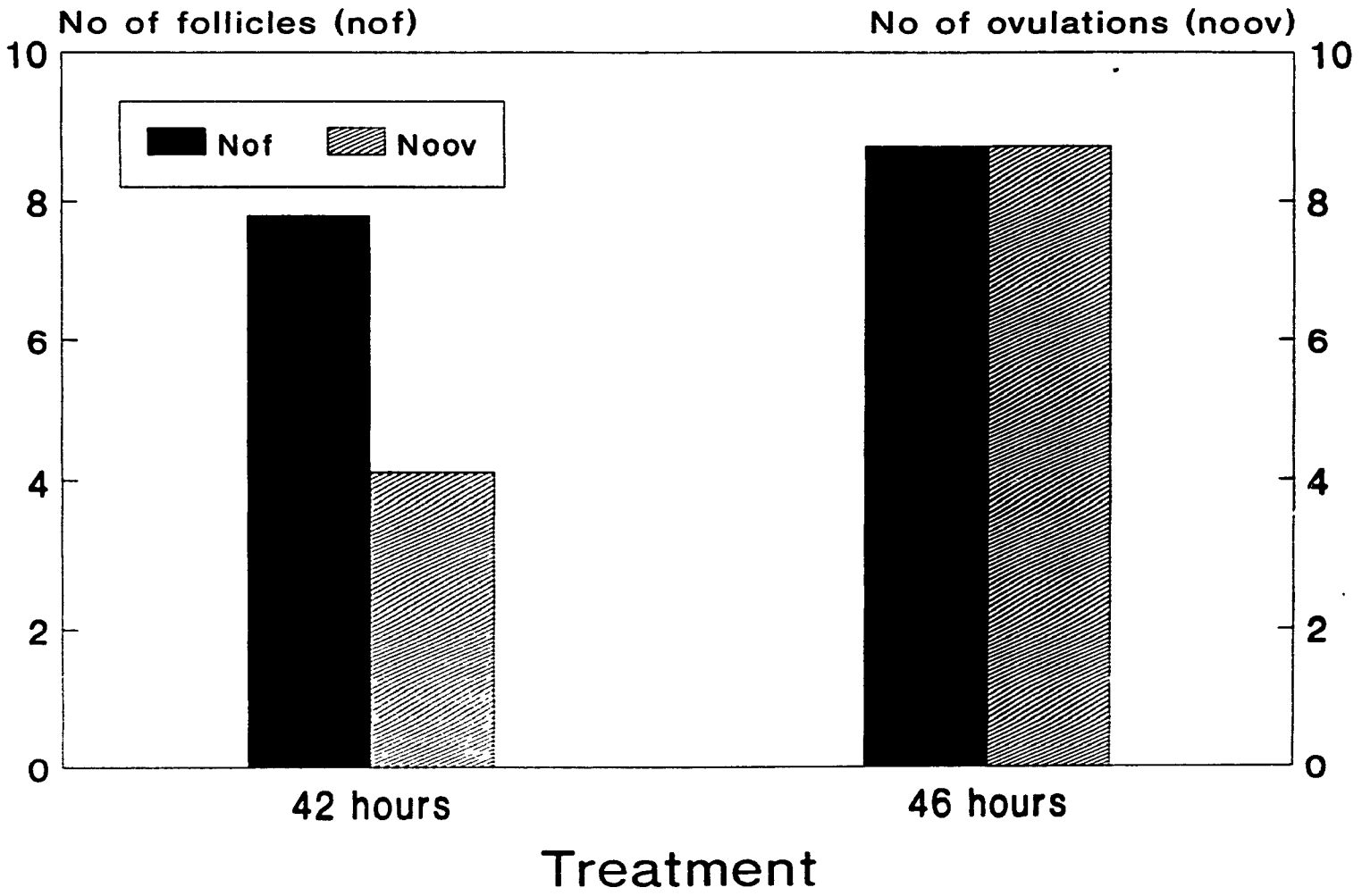
Means (±SEM) within rows or columns bearing different superscripts are different ($p<0.05$).

Table 2. Mean Number of Ovulations (±SEM) For PMSG/hCG Stimulated Gilts- By Treatment and Side.

	Treatment				
Side	38h	42h	46h	46h(Indo.)	Mean±SEM
Left	0	3.7 ±0.6	9.7 ±0.9	0	6.7[a] ±0.6
Right	0	4.5 ±0.5	7.8 ±0.7	0	6.2[a] ±0.5
Mean ±SEM	0	4.1 ±0.4	8.7 ±0.6	0	

Means (±SEM) within rows or columns bearing different superscripts are different ($p<0.05$)

Figure 1. # of Follicles and # of Ovulations For PMSG/hCG Treated Gilts.



The mean plasma progesterone concentrations (PS) for the four treatment groups are given in Table 3. The mean plasma progesterone concentration was found to be lowest in those pigs examined at 38 hours post hCG and highest in pigs examined 46 hours post hCG ($p<0.05$). The mean plasma progesterone level at 38 hours post hCG was shown to be significantly lower than at 42 hours and at 46 hours post hCG respectively ($p<0.05$), but not different from the level found in the indomethacin treated group examined at 46 hours. Mean plasma progesterone in the indomethacin treated group also did not differ significantly from levels at 42 hours. At 46 hours post hCG, plasma progesterone concentrations were significantly higher than at all other times ($p<0.05$).

Table 4 summarizes the mean progesterone concentration in follicular fluid (PFF) at the different times and for the two sides of the reproductive tract. At 38 hours, the mean progesterone concentration in follicular fluid was found to be significantly lower than that found in the group of pigs examined at 42 hours and that found in the indomethacin treated group examined at 46 hours ($p<0.05$). Mean follicular fluid progesterone concentrations did not differ significantly between 42 and 46 hour indomethacin treated groups nor was the mean follicular fluid progesterone concentration different between sides.

In Table 5 the mean progesterone content per follicle (PPF) is reported for the four treatment groups and the two

Table 3. Mean Plasma Progesterone Concentration (ng/ml) (±SEM) For PMSG/hCG Stimulated Gilts- By Treatment.

	Treatment			
	38h	42h	46h	46h(Indo.)
Mean ±SEM	0.85[a] ±0.14	1.36[b] ±0.14	1.85[c] ±0.16	1.27[ab] ±0.20

Means (±SEM) bearing different superscripts are different ($p<0.05$).

Table 4. Mean Progesterone Concentration of Follicular Fluid (ng/ml) (±SEM) For PMSG/hCG Stimulated Gilts- By Treatment and Side.

	Treatment				
Side	38h	42h	46h	46h(Indo.)	Mean±SEM
Left	2249 ±321	6994 ±2063	-	6761 ±1624	4944[a] ±842
Right	1828 ±254	4079 ±463	-	6039 ±1142	3957[a] ±582
Mean ±SEM	2031[a] ±204	5536[b] ±1088	-	6387[b] ±966	

Means (±SEM) within rows or columns bearing different superscripts are different ($p<0.05$).

sides of the reproductive tract. The mean follicular progesterone content did not differ significantly between 38 hour and 42 hour pigs. This, as can be seen in Figure 2, is in sharp contrast to the results presented above for the mean follicular fluid progesterone concentration. A possible explanation for this and its implication will be discussed later. The mean progesterone content per follicle was significantly increased in those pigs treated with indomethacin and examined at 46 hours post hCG ($p<0.05$). Mean progesterone content per follicle did not differ significantly between sides.

The mean total progesterone content of tubal fluid (TPTF) and the mean progesterone concentration of tubal fluid (PCTF) for the four treatment groups and the different sides of the reproductive tract are presented in Tables 6 and 7, respectively. As is evident from these tables, both the mean total progesterone content of tubal fluid and the mean progesterone concentration of tubal fluid were highest for those pigs examined 42 hours post hCG. The 42 hour group did, however, not differ significantly from the 46 hour group with respect to these variables. The mean total progesterone content and mean progesterone concentration of tubal fluid were not statistically different in pigs examined at 38 hours post hCG and pigs examined at 46 hours post hCG but treated with indomethancin. Values in these two groups were, however, significantly lower than those found in 42 and 46 hour pigs ($p<0.05$). Mean total progesterone

Table 5. Mean Progesterone Content Per Follicle (ng) (±SEM) For PMSG/hCG Stimulated Gilts- By Treatment and Side.

	Treatment				
Side	38h	42h	46h	46h(Indo.)	Mean±SEM
Left	233 ±33	380 ±94	-	1087 ±326	610[a] ±150
Right	202 ±34	289 ±103	-	1444 ±599	736[a] ±266
Mean ±SEM	217[a] ±23	339[a] ±68	-	1272[b] ±343	

Means (±SEM) within rows or columns bearing different superscripts are different ($p<0.05$).

Table 6. Mean Total Progesterone Content of Tubal Fluid (ng) (±SEM) For PMSG/hCG Stimulated Gilts- By Treatment and Side.

	Treatment				
Side	38h	42h	46h	46h(Indo.)	Mean±SEM
Left	0.30 ±0.11	1.46 ±0.57	1.44 ±0.49	0.13 ±0.02	0.82[a] ±0.20
Right	0.28 ±0.12	1.58 ±0.54	1.05 ±0.34	0.21 ±0.06	0.77[a] ±0.17
Mean ±SEM	0.29[a] ±0.08	1.52[b] ±0.39	1.23[b] ±0.30	0.17[a] ±0.03	

Means (±SEM) within rows or columns bearing different superscripts are different ($p<0.05$).

Figure 2. P4 Concentration and Content of FF for PMSG/hCG Treated Gilts.

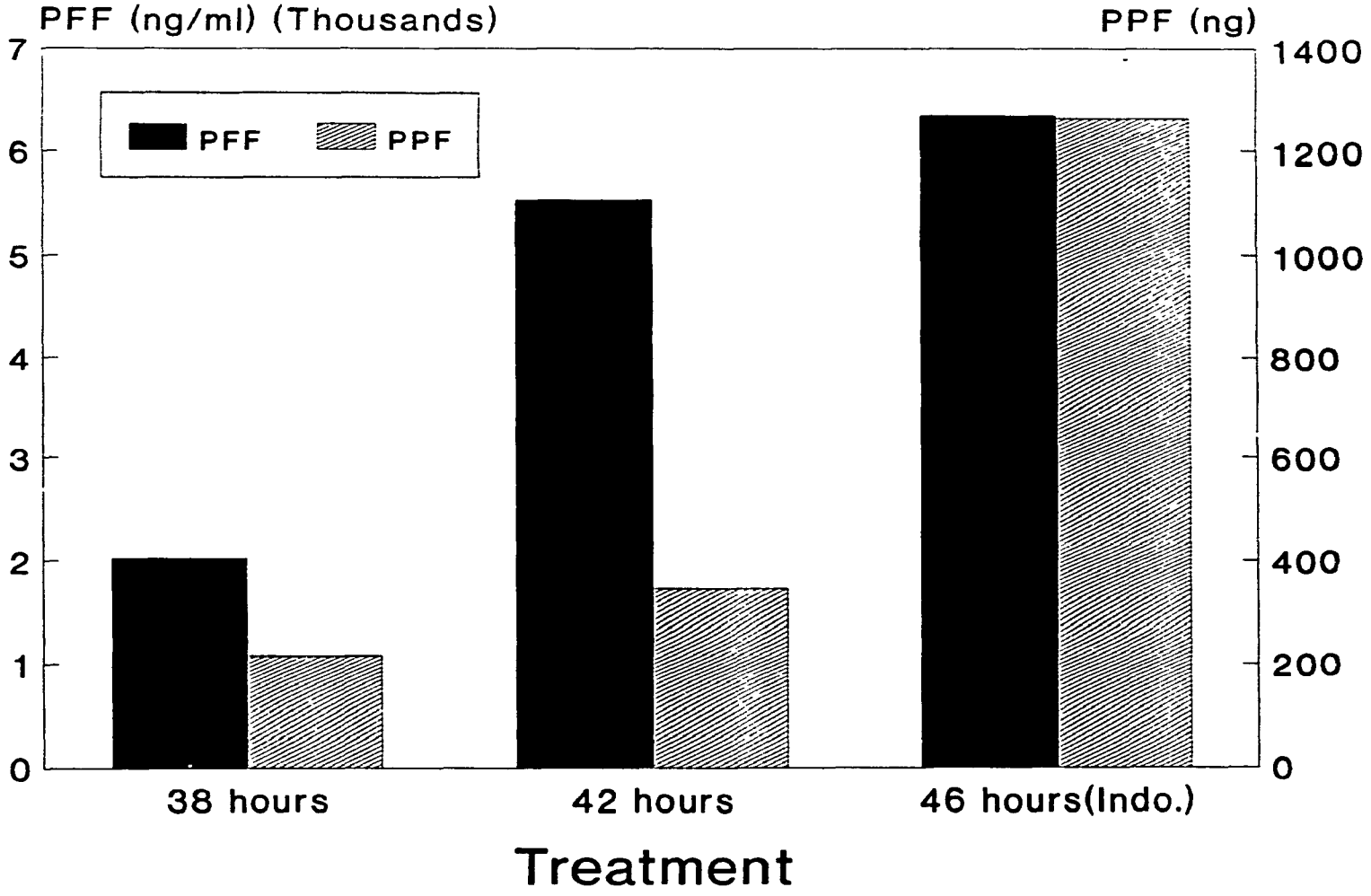


Table 7. Mean Progesterone Concentration in Tubal Fluid (ng/ml) (±SEM) For PMSG/hCG Stimulated Gilts- By Treatment and Side.

	Treatment				
Side	38h	42h	46h	46h(Indo.)	Mean±SEM
Left	1.51 ±0.55	7.32 ±2.85	7.18 ±2.43	0.64 ±0.10	4.11[a] ±0.99
Right	1.38 ±0.58	7.90 ±2.72	5.25 ±1.72	1.05 ±0.29	3.85[a] ±0.86
Mean ±SEM	1.44[a] ±0.39	7.62[b] ±1.93	6.15[b] ±1.45	0.86[a] ±0.17	

Means (±SEM) within rows or columns bearing different superscripts are different ($p<0.05$).

Table 8. Mean Difference (±SEM) between PCTF and PS For PMSG/hCG Stimulated Gilts- By Treatment and Side.

	Treatment				
Side	38h	42h	46h	46h(Indo.)	Mean±SEM
Left	0.80 ±0.56	6.04 ±2.85	5.31 ±2.46	-0.63 ±0.28	2.85[a] ±0.99
Right	0.68 ±0.59	6.58 ±2.72	3.41 ±1.70	-0.34 ±0.22	2.58[a] ±0.86
Mean ±SEM	0.74[a] ±0.40	6.32[b] ±1.93	4.30[b] ±1.45	-0.48[a] ±0.17	

Means (±SEM) within rows or columns bearing different superscripts are different ($p<0.05$).

content and mean progesterone concentration of tubal fluid did not differ significantly between sides.

Table 8 contains the mean values obtained for the difference (DIFF) between the progesterone concentration of tubal fluid and the plasma progesterone concentration for the four treatments and the two sides of the reproductive tract. The difference was found to be highest for those pigs examined 42 hours after the injection of hCG. This value did not differ significantly from the value obtained at 46 hours. The difference in the progesterone concentration of tubal fluid and the progesterone concentration of plasma was not statistically different at 38 hours and at 46 hours (indomethacin). The value obtained for the 46 hour indomethacin treated pigs was the only one to be negative, however. The 38 hour and 46 hour (indomethacin) values were significantly lower than those at 42 and 46 hours ($p<0.05$). The side of the reproductive tract had no significant effect on the mean difference between the progesterone concentration of tubal fluid and the plasma progesterone concentration.

Table 9 gives the mean percentage of the follicular fluid available from the follicles that was estimated to have entered and was thus present in the oviduct (HM). The manner in which this value was calculated is shown in Appendix Table 1. The percentage obtained at 42 hours post hCG was found to be significantly higher than that obtained at 46 hours post hCG ($p<0.05$).

Table 9. Percentage (±SEM) of Follicular Fluid Entering the Oviduct of PMSG/hCG Stimulated Gilts.

	Treatment	
	42h	46h
Mean	0.509[a]	0.039[b]
±SEM	±0.10	±0.06

Means (±SEM) bearing different superscripts are different ($p<0.05$).

DISCUSSION

Yanagimachi stated in 1969(b) that "at ovulation a large volume of follicular fluid is released from mature ovarian follicles. At least a part of this fluid must be transported into the oviduct together with the cumulus oophorus containing the eggs". Since then very little research has been carried out to advance our knowledge on this subject much beyond this simple statement and others like it. The results of the present experiment demonstrate, however, that Yanagimachi's statement is at least partially true. Approximately 0.5% of the available follicular fluid was found to be present in the oviduct of gilts examined 42 hours after the injection of hCG. By 46 hours, this value had decreased to 0.04%. Therefore although, as Yanagimachi suggested, some of the follicular fluid that is released at ovulation does get into the oviduct, the amount appears to be rather minute.

The fact that the proportion of the available follicular fluid present in the oviduct decreased between 42 and 46 hours raises an interesting point. During that four hour period a significant quantity of follicular fluid (progesterone) must have escaped from the oviduct in some manner. Although the possiblity exists that some of this progesterone was lost via diffusion into the blood vessels or lymphatic vessels that feed the walls of the oviduct, another explanation appears to be that the fluid was lost

through backwash into the peritoneal cavity. Mean progesterone concentrations in peritoneal fluid are given in Appendix Table 3. As can be clearly seen from these values, the progesterone concentration of peritoneal fluid was much higher for 42 and 46 hour pigs than for indomethacin treated pigs examined at 46 hours. These two concentrations also tend to be higher than plasma progesterone concentrations at these same time periods. Clearly follicular fluid is escaping into the peritoneal fluid. Although most of this follicular fluid is probably fluid that never made it into the oviduct in the first place, the fact that the progesterone concentration of the peritoneal fluid at 46 hours tends to be slightly higher than that at 42 hours suggests that some of the follicular fluid that enters the oviduct may be expelled again over time.

The fact that the amount of follicular fluid actually entering the oviduct is extremely small is further emphasized when one considers a point that was alluded to earlier. The mean progesterone concentration of follicular fluid at 42 hours post hCG was found to be 5536 ± 1088 ng/ml. This concentration was significantly higher than the one measured at 38 hours (2031 ± 204 ng/ml) but not different from that found in the follicular fluid of indomethacin treated pigs at 46 hours (6387 ± 966 ng/ml). It also compares favourably with the range of progesterone concentrations reported by Hunter *et al* (1976) for porcine follicular fluid (3400 to 6400 ng/ml). The mean progesterone content per

follicle at 42 hours in this experiment meanwhile was found to be 339±68 ng. Unlike what was seen for the progesterone concentration, however, this value was not different from the figure of 217±23 ng that was found at 38 hours post hCG. It was also significantly lower than the 1272±343 ng that were found in 46 hour (indomethacin) follicles. It is difficult to rationalize how the progesterone content of follicles at 42 hours can be the same as that at 38 hours while at the same time the progesterone concentration is much greater than at 38 hours.

The only possible explanation for the discrepancy mentioned above relates back to the manner in which the progesterone content value was obtained. Unlike the progesterone concentration figure, this value was not measured directly but rather was calculated using the progesterone concentration figure and a number of other measurable variables. Among these the one most likely to be directly responsible for causing the discrepancy is the volume of follicular fluid obtained from each ovary. This volume was measured during the course of the experiment using a syringe and needle. This manner of measurement was, as it turns out, probably too inaccurate for the purposes of the calculation. Not only could some follicular fluid get lost in the needle but also only 0.01 ml increments could be measured. Fortunately, this failure in the experimental procedure does not detract from the overall conclusion that can be drawn from the results. As becomes clear when one

examines the formula used to calculate the amount of follicular fluid entering the oviduct, an underestimation of the progesterone content value, as must have occurred, would lead to an overestimation of the amount of follicular fluid entering the oviduct. It can be safely assumed, therefore, that the values given for the amount of follicular fluid in the oviduct are actually larger than they really are.

One of the most striking concerns that emerges as one examines the results of this experiment is the possibility of progesterone entering the tubal fluid in a manner other than via follicular fluid. This does not appear to be the case, however. An examination of Table 8 shows that at both 42 and 46 hours post hCG, the progesterone concentration of tubal fluid was much higher than that of plasma. If progesterone was getting into the tubal fluid via the blood stream one would expect to find the progesterone concentration of plasma to be higher than that of tubal fluid. Since the progesterone concentration of tubal fluid is much greater than that of plasma at both 42 and 46 hours, the only way progesterone could get into the tubal fluid from the blood vessels would be through an active transport mechanism and that seems highly unlikely.

Having thus established that the amount of follicular fluid entering the oviduct is extremely small, one must consider the significance of the fluid that does get in. After all, the progesterone concentration of tubal fluid is raised significantly, from a statistical point of view,

after ovulation and this leads one to assume that the concentration of other follicular fluid factors is also increased significantly. The question that must be asked, however, is whether or not such an increase is biologically significant.

In vitro experiments such as those of Yanagimachi (1969), Mukherjee and Lippes (1972) and Oliphant et al (1977) examining the capacitating and acrosome reaction inducing effects of follicular fluid all employed concentrations much larger than those that have now been shown to exist in vivo. Yanagimachi, for example, used whole follicular fluid to capacitate hamster sperm while Oliphant et al used concentrations ranging from 20 to 80%. It seems quite likely, in retrospect, that at such unphysiological concentrations many biological fluids may have some sort of effect on sperm. In fact, Yanagimachi (1970) showed that hamster sperm could be capacitated by both homologous and heterologous heat treated sera.

The fact that the sera used by Yanagimachi (1970) required inactivation raises an interesting point. Non heat treated sera were shown in his experiment to be highly toxic to sperm. Similar results were obtained by Mukherjee and Lippes (1972) for follicular fluid. In their experiment, follicular fluid without inactivation was not effective in inducing the acrosome reaction in rat and human sperm. Although Yanagimachi (1969b) did not report any detrimental effect of using pure follicular fluid to capacitate sperm,

the fact that such an effect may exist would support the finding that very little fluid enters the oviduct. It would also diminish the possibility of follicular fluid playing some important role in capacitation and acrosome reaction induction in vivo.

Another point that bears consideration is that follicular fluid contains a high concentration of progesterone at the time of ovulation. This was, as has been mentioned, in fact one of the reasons why progesterone was chosen as a marker to indicate the presence of follicular fluid in the oviduct. Progesterone has, however, been shown by a number of investigators to be detrimental to normal sperm transport and fertilization. Day and Polge (1968), for example, reported that the percentage of eggs fertilized was reduced and the incidence of polyspermy was greatly increased in animals injected with progesterone 24 to 36 hours before ovulation. In addition, injection of progesterone before ovulation caused an accelerated rate of egg transport. Similarly, Hunter (1972) showed that polyspermic fertilization was significantly higher in eggs recovered from oviducts where microdroplets of progesterone had been injected beneath the serosa of the tubal isthmus. Findings such as these, therefore, again support the findings of this experiment in principle as it would appear that large amounts of follicular fluid with high progesterone content would be detrimental rather than beneficial in the oviduct.

As was mentioned in the results section of this

experiment, the number of follicles differed statistically between the two sides of the reproductive tract. Further examination of the data, however, indicates that this difference is not biologically significant. When one compares the number of follicles obtained with either the total progesterone content of tubal fluid or the progesterone concentration of tubal fluid on the two sides of the reproductive tract, one finds that, although the left side had a significantly greater number of follicles than the right side (8.3 vs 6.8), this difference was not reflected in the progesterone content or concentration of the tubal fluid on these sides. Tubal fluid progesterone content/concentration did not differ statistically between sides.

A final comment that can be made about the results of this experiment relates to the timing of ovulation in PMSG/hCG treated gilts. Dziuk and Baker's 1962 paper entitled "Induction and Control of Ovulation in Swine' details an experiment done to develop the PMSG/hCG model for gilts. In this experiment, it was found that 94% of animals examined had ovulated 40 hours after the hCG injection. Refinement of the method eventually led to the conclusion that ovulation in gilts stimulated with PMSG and hCG occurs 42$\pm$2 hours after the injection of hCG. The results of the present experiment confirm this time frame for ovulation very nicely. At 38 hours, none of the pigs examined had even a single ovulation point. By 42 hours, on the other hand,

53% of all follicles had ovulated (Fig. 1). Finally at 46 hours, 100% ovulation had occurred.

EXPERIMENT II: Porcine Follicular Fluid and the Sperm Acrosome Reaction *in vivo*.

OBJECTIVES

The purpose of this experiment was to determine whether or not the presence of follicular fluid and the other products of ovulation in the female reproductive tract have any effect on the proportion of spermatozoa undergoing the acrosome reaction in the uterine horn, isthmus and ampulla of the tract of PMSG/hCG stimulated gilts and to examine the sperm distribution in the reproductive tract of these gilts. In the experiment, all ovulatory products were effectively eliminated from the oviducts of half of the experimental animals by blocking ovulation with indomethacin. The remainder of the animals were allowed to ovulate normally.

MATERIALS AND METHODS

Animals

Prepubertal Landrace gilts (Macdonald College Farm) weighing between 55 and 73 kg were used in this experiment. All gilts were housed in the Swine Facilities at Macdonald College and were fed a commercial (14% crude protein) pelleted ration and water ad libitum. Follicular growth and development was stimulated in these gilts by the intramuscular injection of 750 I.U. of pregnant mare's serum gonadotropin (PMSG, Equinex, Ayerst Laboratories, Montreal) followed 72 hours later by 500 I.U. of human chorionic gonadotropin (hCG, A.P.L., Ayerst Laboratories, Montreal).

The animals were randomly assigned to one of two treatment groups. In Group I, surgery was performed 46 hours after the injection of hCG (post ovulatory group, n=5). In Group II, surgery was also performed 46 hours post hCG but these animals received an additional injection of 10 mg of indomethacin (Merck Frosst, Kirkland, Quebec)/ kg of body weight suspended in propylene glycol (Fisher Scientific Company, Fair Lawn, N.J.) 24 hours after the hCG injection (ovulation blocked, n=4). Group I animals received a sham injection of propylene glycol at 24 hours post hCG. All animals were inseminated 12 hours prior to surgery (approximately 10 billion sperm/insemination).

Semen Collection and Processing

Semen was collected by hand from a single mature Landrace boar of proven fertility on the Macdonald College Farm about 3-4 hours prior to insemination. The ejaculate was filtered to remove all of the bulbourethral gel fraction and immediately transported to the laboratory. There the semen was mixed with an extender of proven quality (Composition, Appendix Table 4) in a ratio of 1:3. The extended semen was then stored at room temperature in a dark drawer until the inseminations were carried out. A slide was prepared for purposes of live-dead staining immediately after the semen was extended as well as just prior to insemination by placing a drop of extended semen on a warm microscope slide and adding a drop of Eosin-Fast Green

stain (Sorensen, 1979). The two were mixed thoroughly and a thin smear was made. The slide was then immediately dried on a hot plate and examined under a light microscope. Dead sperm had pink stained heads using this procedure while live sperm remained unstained. Sperm concentrations in the extended semen were determined by diluting a semen sample in a Unopette reservoir (Becton-Dickinson, Rutherford, N.J.) designed for white blood cell counts. A hemocytometer was then charged with a drop of the diluted semen and sperm were counted under 100X magnification. Sperm motility was routinely observed after semen collection and prior to insemination.

Spermatozoa Recovery

Prior to surgery, all gilts were given an intramuscular injection of 0.045 mg/kg Atropine sulphate (Vetcom Inc., St. Liboire, Que.)and 2 ml of Innovar-vet (M.T.C. Pharmaceuticals, Mississauga, Ont.). Anaesthesia was induced by halothane (M.T.C. Pharmaceuticals, Mississauga, Ont.) and maintained using a halothane closed circuit system. The reproductive tract was then exposed by midventral laparotomy and the top portion of each uterine horn and both oviducts and ovaries were excised and placed into warm saline (37 C). Almost immediately after removal, the portions of the reproductive tract were divided into three sections: the top 3 cm of the uterine horn, the isthmus and the ampulla. The contents of each section were

then flushed into individual testtubes with 3 ml of warm saline. All samples were centrifuged at 1000 x g for 10 minutes after which the majority of the supernatant was carefully aspirated. The pellet remaining in each tube was then resuspended in the remaining saline and the volume of the suspension in each tube was measured. The number of follicles and ovulation points on each ovary were also recorded.

Acrosome Staining Procedure

Slides were made by placing a 25 ul drop of sperm suspension on a glass slide and allowing it to dry at room temperature for 2 hours. Slides were then stained according to the method of Bryan and Akruk (1977). The composition of the staining solutions is given in Appendix Table 5. Briefly, all slides were stained for 30 minutes at room temperature with solution I. The slides were then blotted between layers of Kimwipe (Kimberly-Clark, Toronto) and rinsed briefly in 1.0% aqueous acetic acid (Mallinckrodt Inc., Paris, Kentucky). Next, all slides were drained and stained with a mixture of equal parts of solutions II and III for 7 minutes. The slides were then rinsed thoroughly with distilled water adjusted to a pH of 4.6-5.0 with acetic acid, blotted and allowed to air dry. A drop of Permount (Fisher Scientific Company, Fair Lawn, N.J.) was finally applied to the slide and a cover slip mounted.

Slide Examination

All slides were examined under a light microscope and the proportion of acrosome reacted sperm on each slide was determined. The acrosome on acrosome intact sperm appeared as a cherry red cap on the sperm head. Acrosome reacted sperm had white, capless heads.

Determination of Sperm Concentrations

The sperm concentration in each segment of the reproductive tract excised was estimated for each gilt. For the uterine horn and isthmus segments, sperm concentrations were determined by diluting a sample of fluid in a WBC Unopette as was described earlier and then counting the sperm in a hemocytometer. For the ampulla segments, sperm concentrations were determined by counting the number of sperm found in a 25 ul drop of fluid.

Statistical Analysis

Data obtained in this experiment were analysed using the General Linear Models (GLM) procedures of the Statistical Analysis System (SAS, 1982). Differences between the means of the various variables examined were determined using Duncan's New Multiple Range Test. Where appropriate, least square means analysis was used to explain the interactive effects. A comlete outline of the statistical program used is given in Appendix Table 6.

RESULTS

Data collected for experiment II are summarized in Appendix Table 7.

Mean sperm concentrations (CS) found in the top three centimeters of the uterine horn, the isthmus and the ampulla of the gilts examined in this experiment are given in Table 10. The uterine horn segment was shown to contain a significantly higher concentration of sperm than the other two segments ($p<0.05$). The sperm concentrations found in the isthmus and ampulla did not differ significantly from each other, although there was a definite tendency for the isthmus to contain a greater concentration of sperm. The progressive decrease in sperm concentration from region to region that was observed is illustrated in Figure 3. No significant difference was found between the mean sperm concentration for the two treatment groups.

The mean number of acrosome-reacted sperm (ARS) found in the different regions of the reproductive tract are summarized in Table 11. The uterine horn segment contained a significantly greater number of acrosome-reacted sperm than the isthmus or ampulla ($p<0.05$). No statistical difference was shown between the number of acrosome-reacted sperm in the isthmus and ampulla of the oviduct. The isthmus did, however, show a strong tendency to contain more acrosome reacted sperm. As can be seen from Figure 3, the same pattern of marked

Table 10. Mean Sperm Concentrations (Sperm/ml) (±SEM) For PMSG/hCG Stimulated Gilts- By Treatment and Location.

	Location			
Trt	Horn	Isthmus	Ampulla	Mean±SEM
Control	305500 ±83240	63668 ±24315	1004 ±246	123391[a] ±37045
Indo.	353916 ±131600	65220 ±28210	5523 ±2487	141553[a] ±53334
Mean ±SEM	327019[a] ±72398	64358[b] ±17876	3012[b] ±1203	

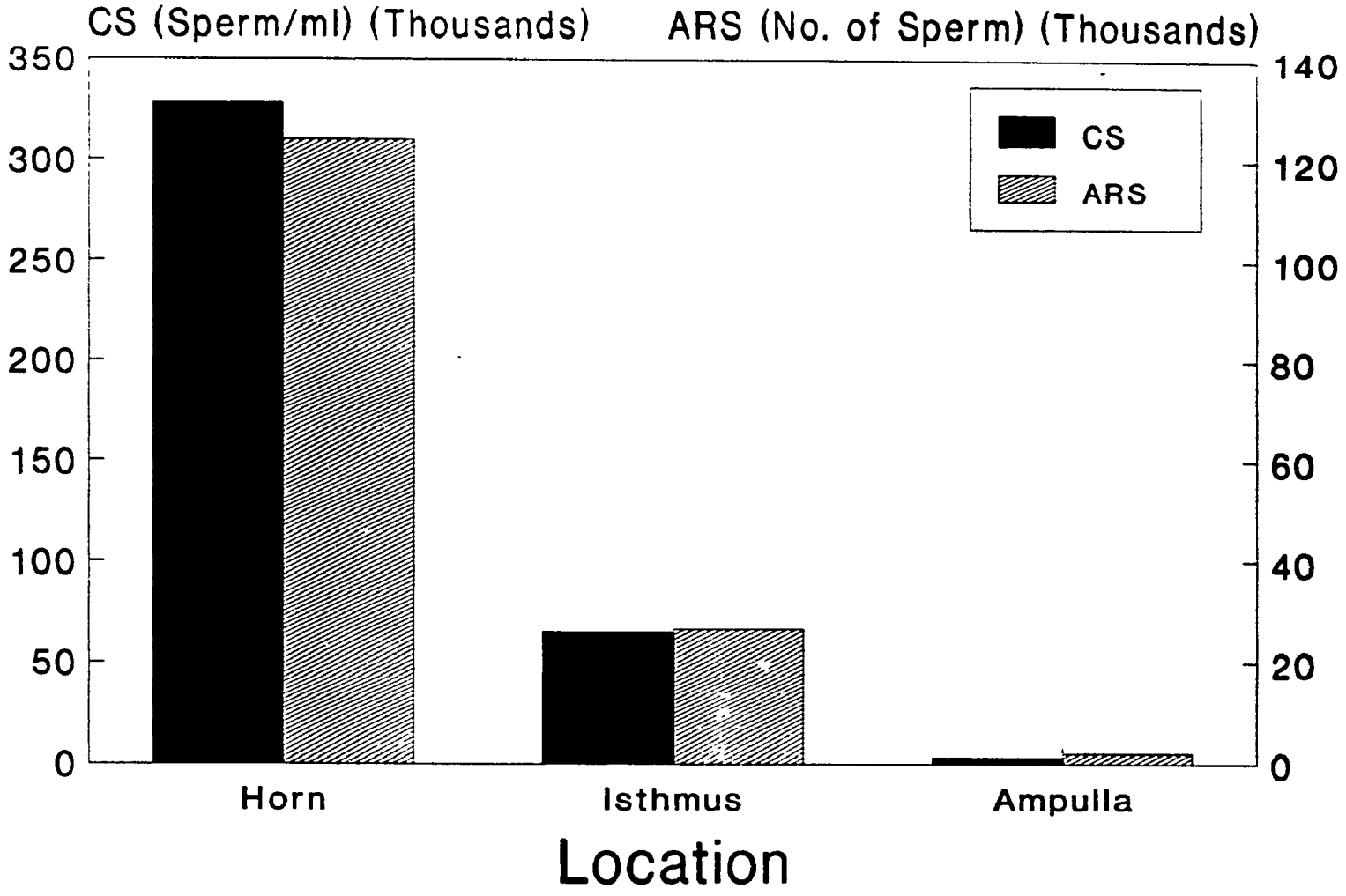
Means (±SEM) within rows or columns bearing different superscripts are different ($p<0.05$).

Table 11. Mean Number of Acrosome Reacted Sperm (±SEM) For PMSG/hCG Stimulated Gilts- By Treatment and Location.

	Location			
Trt	Horn	Isthmus	Ampulla	Mean±SEM
Control	115636 ±32831	25791 ±10639	741 ±184	47389[a] ±14392
Indo.	133797 ±49823	26980 ±12680	2522 ±1397	54683[a] ±20212
Mean ±SEM	123707[a] ±27858	26319[b] ±7921	1866[b] ±735	

Means (±SEM) within rows or columns bearing different superscripts are different ($p<0.05$).

Figure 3. Sperm Concentration and # of Acrosome Reacted Sperm



decline was seen in the number of acrosome reacted sperm in the different segments as was seen in the sperm concentration. Treatment was found to have no effect on the mean number of acrosome reacted sperm.

Table 12 summarizes the mean percentage of acrosome reacted sperm (%A) found in the different segments of the reproductive tract that were examined. As can be seen from Figure 4, the %A did not differ significantly between the horn and the isthmus segments. The percentage of acrosome reacted sperm found in the ampulla was significantly higher than in the other two regions, ($p<0.05$). No significant difference in the mean percentage of acrosome reacted sperm was found overall between the control and indomethacin treated groups. Even though the interaction effect was not significant ($p=0.22$), least squares analysis was done to determine the effect of follicular fluid on the percentage of acrosome reacted sperm in the ampulla. The percentage (73.1%) in the ampulla of the control pigs was found to be significantly higher than the percentage (64.1%) observed in the ampulla of indomethacin-treated pigs ($p<0.05$).

In Table 13, the mean number of follicles (NOF) per treatment are given. No significant difference was found between the mean number of follicles counted on the ovaries of pigs in each treatment group.

Table 12. Mean Percentage of Acrosome Reacted Sperm (±SEM) For PMSG/hCG Stimulated Gilts- By Treatment and Location.

	Location			
Trt	Horn	Isthmus	Ampulla	Mean±SEM
Control	37.4 ±1.5	37.8 ±2.0	73.1 ±3.2	49.4[a] ±3.4
Indo.	36.1 ±1.8	36.8 ±2.5	64.1 ±3.9	45.7[a] ±3.2
Mean ±SEM	36.8[a] ±1.1	37.3[a] ±1.5	69.1[b] ±2.7	

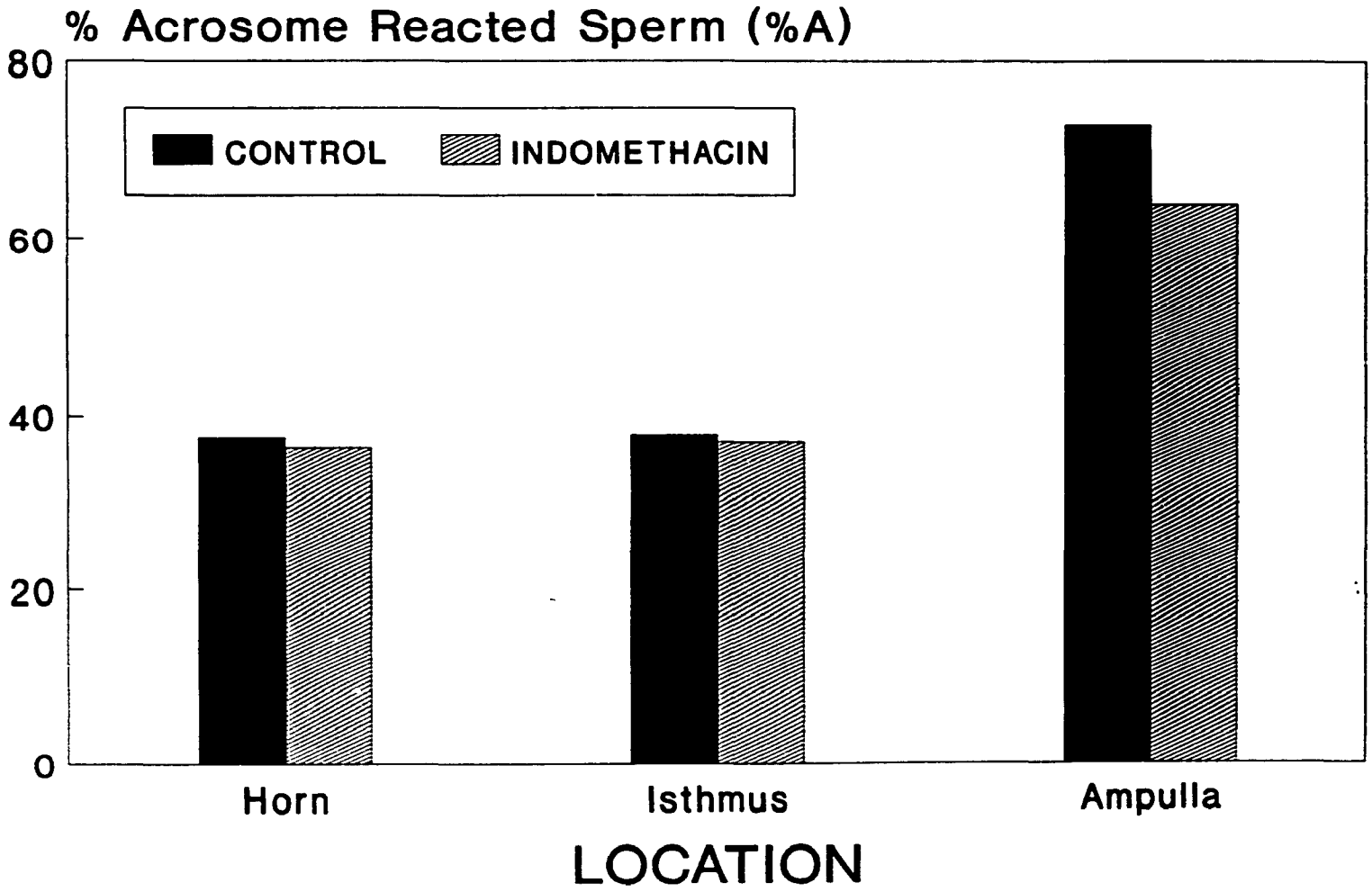
Means (±SEM) within rows or columns bearing different superscripts are different ($p<0.05$).

Table 13. Mean Number of Follicles (±SEM) Per Treatment.

Trt	NOF
Control	5.2± 0.8[a]
Indo.	6.3± 0.7[a]

Means (±SEM) bearing different superscripts are different ($p<0.05$).

Figure 4. % Acrosome Reacted Sperm Found In The Porcine Reproductive Tract.



DISCUSSION

Herz and colleagues (1985) reported that the largest frequency of acrosome-reacted sperm in the bovine was found in the ampulla of the oviduct ipsilateral to a dominant follicle or recent ovulation. These researchers speculated that this high level of acrosome reaction found in the ampulla of the oviduct on the ovulatory side was due to some product of ovulation. While their hypothesis was supported by the observation that all except two cows with a very high frequency of acrosome-reacted sperm in the ampulla had ovulated prior to the time the oviductal contents were recovered, it could not be conclusively proven by the data gathered in the experiment. Further research was thus needed.

The administration of indomethacin has been shown to be an extremely effective way to block ovulation in the pig (Ainsworth et al, 1979). Luteal function in indomethacin-treated pigs has, however, been shown to be unimpaired and treated and untreated animals have been reported to exhibit similar plasma progesterone profiles (Ainsworth et al, 1979). Only prostaglandin levels were shown to be decreased by the treatment. The use of indomethacin thus allows one to exclude follicular fluid from the oviduct with 100% efficiency while not altering the endocrine status of the animal. This treatment method therefore provided an excellent vehicle with which to further the work of Herz and colleagues, at least in the pig, and at the same time provide data to help interpret

the significance of the results of experiment I of this study.

When one considers the results obtained in experiment II of this study, one will observe that the data confirm, in many respects, the speculations of Herz and colleagues. The data obtained in this experiment indicate that there is no difference in the mean number of acrosome-reacted sperm between the two treatments nor a difference in the overall mean percent acrosome reacted sperm for these treatments. The minute amount of follicular fluid that was shown to be present in the porcine oviduct at both 42 and 46 hours post hCG thus clearly does not have an effect on the overall numbers obtained for the two treatments. Interestingly enough, however, when one examines the mean percentage of acrosome reacted sperm in the ampulla only, one will see that the ampulla of control animals contained a significantly higher percentage of acrosome-reacted sperm than the ampulla of indomethacin-treated gilts. It remains questionable, however, if this difference has any biological significance. After all, only one fully capacitated and acrosome reacted sperm is theoretically required for successful fertilization. A small difference in the percentage of acrosome reacted sperm about an egg, as is seen here, may thus not affect the chance of a successful encounter. On the other hand, it might and this possibility should be examined in the future.

Another interesting point that emerges from the data collected in this experiment is the fact that the percentage of acrosome-reacted sperm in the ampulla is significantly

higher than that in both the isthmus and the uterine horn. This finding is again in agreement with the results reported by Herz et al. Since this increase in percent acrosome-reacted sperm in the ampulla occurs whether follicular fluid is present or not, follicular fluid must thus not be the prime factor responsible for inducing the acrosome reaction. However, as mentioned above, since the percentage of acrosome-reacted sperm is clearly higher in the ampulla in the presence of follicular fluid, it may contain a factor or factors that stimulate the acrosome reaction. This conclusion is also supported by the findings of Moore and Bedford (1983). It is very likely, therefore, that the factor or factors that are responsible for acrosome reaction induction are present in the oviduct or perhaps the ampulla of the porcine oviduct.

First and Parrish (1988) reported that heparin, a glycosaminoglycan constituent of oviductal fluid, will capacitate sperm in vitro. These researchers showed that heparin will always capacitate ejaculated bovine sperm in both a dose and time dependent manner and that its capacitating activity is dependent on its sulfation. In addition, Susko-Parrish et al (1985) showed that glucose will inhibit the capacitation of sperm by heparin. It has been suggested that heparin may be the in vivo capacitating factor and its presence in oviductal fluid may thus influence where most sperm undergo the acrosome reaction. Its presence in follicular fluid would also explain why follicular fluid has been shown to possess capacitating and acrosome reaction

inducing activities but why, in this experiment, the presence or absence of follicular fluid did not significantly affect the overall number or percentage of acrosome reacted sperm. The addition of such small amounts of heparin to oviductal fluid via follicular fluid entering the oviduct would probably make little difference in terms of total acrosome reaction inducing potential when one considers the quantities already there. On the other hand, it may give a little boost to the acrosome reaction inducing potential of the ampulla specifically.

Didion and Graves (1986) reported that fewer sperm underwent a true acrosome reaction in diestrous cows than in estrous cows. This observation relates to the fact that the endocrine status of the female can influence the capacitating and acrosome-reaction inducing potential of the female reproductive tract. It is interesting to note that as there was no significant difference in the overall number or percent acrosome reacted sperm between the indomethacin and control groups, the endocrine status of these animals must not have been significantly different. This thus confirms the findings of people such as Ainsworth et al (1979) who, as mentioned previously, reported the luteal function and plasma progesterone profiles of indomethacin-treated gilts to be normal.

When one examines the data obtained for the sperm concentrations in the different segments of the reproductive tract, one will notice that there is a considerable decline

as one moves from the horn (327019±72398 sperm/ml) to the ampulla (3012±1203 sperm/ml) of the pig. This observation supports the concept that very few sperm actually reach the site of fertilization in mammals and also emphasizes the fact that sperm reservoirs exist at the utero-tubal junction (Rigby, 1966) and in the lower isthmus of the porcine oviduct (Hunter, 1981, 1984). The decline in sperm numbers and the existence of reservoirs are, as has been mentioned, very important for proper fertilization.

An examination of the sperm concentration found in each segment of the reproductive tract reveals that there is a tendency for the concentration to be higher in each segment of the indomethacin-treated group than in the same segment of the control group. Although none of the sperm concentrations within a specific segment are statistically different for the two treatment groups, the fact that this trend exists may indicate that the decrease in prostaglandins that results from indomethacin treatment (Ainsworth et al, 1979) has an effect on sperm transport.

Finally, it should be mentioned that although the percentage acrosome-reacted sperm found in the horn and isthmus may appear to be a little bit high (approx. 37%), the general trend observed compares well with that reported by Herz et al (1985) for the bovine. In both cases the horn and isthmus segments did not differ from each other in terms of the percentage of sperm that had undergone the acrosome reaction, while the ampulla contained a significantly greater

proportion of acrosome-reacted sperm. In addition, the percent acrosome-reacted sperm found in the semen used for insemination in this experiment was relatively high, ranging from 12 to 20%. The increase in acrosome-reacted sperm in the horn and isthmus was thus, in reality, not that great.

GENERAL CONCLUSIONS

At ovulation only a minute amount (0.5%) of follicular fluid actually enters the porcine oviduct. It is likely that this follicular fluid is attached to the cumulus oophorus cells surrounding the egg at this time and that almost all of the remaining follicular fluid that is released simply escapes into the peritoneal cavity. The experiment also showed that the percentage of available follicular fluid found in the oviduct decreases with time. This may result from a backwash of fluid out of the oviduct into the peritoneal cavity.

Although the small amount of follicular fluid that does enter the oviduct significantly alters the environment that the sperm encounter, as indicated by the progesterone concentration, this change is not responsible for inducing the acrosome reaction in these sperm. The follicular fluid entering the oviduct does, however, stimulate the acrosome reaction in the ampulla as is indicated by the fact that the percentage of acrosome-reacted sperm found in the ampulla of control pigs was significantly greater than that found in the ampulla of indomethacin-treated pigs.

While there was no difference between indomethacin-treated and control animals in terms of the overall number or percentage of sperm undergoing the acrosome reaction, the ampulla of animals in both groups contained a significantly higher number and percentage of acrosome-reacted sperm. The

ampulla of the oviduct must, therefore, contain a significant quantity of a specific acrosome reaction inducing factor. What this factor is remains to be determined but it appears that it is not a specific follicular fluid component nor some other product of ovulation.

The possibility that the production of the acrosome reaction inducing factor in the ampulla is influenced by the endocrine status of the animal cannot be ruled out based on the results of this study. An investigation of this possibility in the near future would seem appropriate. The fact that indomethacin treatment does not significantly alter the endocrine status of pigs was partially supported by the experiments included in this study. Although luteinization appears to have been slightly delayed by the indomethacin treatment as indicated by the plasma progesterone concentration found in treated and untreated animals at 46 hours post hCG, the ability of the respective oviducts of these pigs overall to induce the acrosome reaction was not affected. Any endocrine stimulation that may be necessary for production of the acrosome reaction inducing factor must thus have been sufficient. The indomethacin-treated PMSG/hCG gilt model thus appears to have a useful application in experiments where the exclusion of ovulatory material from the oviducts and the timing of ovulatory events is desirable just as was postulated.

LITERATURE CITED

Ainsworth, L., Tsang, B.K., Downey, B.R., Baker, R.D., Marcus, G.J., and Armstrong, D.T., 1979. Effect of indomethacin on ovulation and luteal function in gilts. Biol.Reprod. 21:401-411.

Aonuma, S., Okabe, M., Kawaguchi, M., and Kishi, Y., 1980. Studies on sperm capacitation. IX. Movement characteristics of spermatozoa in relation to capacitation. Chem.Pharm.Bull.Jpn. 28:1497-1502.

Austin, C.R., 1951. Observations on the penetration of the sperm into the mammalian egg. Aust.J.Sci.Res. B4:581-596.

Austin, C.R., 1952. The capacitation of mammalian sperm. Nature (London) 170:326.

Austin, C.R., and Bishop, M.W., 1958. Role of the rodent acrosome and perforatorium in fertilization. Proc.R.Soc.London Ser. B 149:241-248.

Ax, R.L., and Ryan, R.J., 1979. The porcine ovarian follicle. IV. Mucopolysaccharides at different stages of development. Biol.Reprod. 20:1123-1132.

Barros, C.,and Berrios, M., 1977. Is the activated spermatozoon really capacitated?. J.Exp.Zool. 201:65-72.

Barros, C., Bedford, J.M., Franklin, L.E., and Austin, C.R., 1967. Membrane vesiculation as a feature of the mammalian acrosome reaction. J.Cell Biol. 34:C1-C5.

Bearden, H.J., and Fuquay, J., 1984. The female reproductive system. In: Applied Animal Reproduction. pp. 9-23. Reston Publishing Company, Virginia.

Bedford, J.M., 1970. Sperm capacitation and fertilization in mammals. Biol.Reprod. Suppl. 2:128-158.

Bedford, J.M., 1983. Significance of the need for sperm capacitation before fertilization in eutherian mammals. Biol.Reprod. 28:108-120.

Bedford, J.M., and Cooper, G.W.,1978. Membrane fusion events in the fertilization of vertebrate eggs. In: Cell Surface Reviews, Vol. 5. (G. Poste and G.L. Nicholson, eds.). pp. 65-125. North Holland, Amsterdam.

Bellin, M.E., and Ax, R.L., 1984. Chondroitin sulfate: An indicator of atresia in bovine follicles. Endocrinology 114:428-434.

Bellve, A.R., and O'Brien, D.A., 1983. The mammalian spermatozoon: Structure and temporal assembly. In: Mechanism and Control of Animal Fertilization. (J.F. Hartmann, ed.). pp. 55-137. Academic Press, New York.

Bjersing, L., Hay, M.F., Kann, G., Moor, R.M., Naftolin, F., Scaramuzzi, R.J., Short, R.V., and YoungLai, E.V., 1972. Changes in gonadotrophins, ovarian steroids and follicular morphology in sheep at oestrus. J.Endocr. 52:465-479.

Bryan, J.H.D., and Akruk, J.R., 1977. A naphthol yellow S and erythrosin B staining procedure for use in studies of the acrosome reaction of rabbit spermatozoa. Stain Technol. 52:47-51.

Chang, M.C., 1951. Fertilizing capacity of spermatozoa deposited in fallopian tubes. Nature (London) 168:697-698.

Chang, M.C., 1958. Capacitation of rabbit spermatozoa in the uterus with special reference to the reproductive phases of the female. Endocrinology 63:619-628.

Chang, M.C., and Hunter, R.H.F., 1975. Capacitation of mammalian sperm: biological and experimental aspects. In: Handbook of Physiology. Vol. 5, Section 7. (D.W. Hamilton and R.O. Green, eds.) pp. 339-351. American Physiological Society, Washington, D.C.

Channing, C.P., 1972. Comparison of number of gonadotropin receptors in granulosa cells harvested from small, medium and large pig follicles. (Abstract) Biol.Reprod. 7:127-128.

Channing, C.P., and Coudert, S.P., 1976. Contribution of granulosa cells and follicular fluid to ovarian estrogen secretion in the rhesus monkey in vivo. Endocrinology 98:590-597.

Clegg, E.D., 1983. Mechanisms of mammalian sperm capacitation. In: Mechanism and Control of Animal Fertilization. (J.F. Hartmann, ed.). pp. 178-212. Academic Press, New York.

Cummins, J.M., 1982. Hyperactivated motility patterns of ram spermatozoa recovered from oviducts of mated ewes. Gamete Res. 6:53-63.

Davis, B.K., 1976. Inhibitory effect of synthetic phospholipid vesicles containing cholesterol on the fertilizing capacity of rabbit spermatozoa. Proc.Soc. Biol.Med. 152:257-261.

Davis, B.K., 1978. Inhibition of fertilizing capacity in mammalian spermatozoa by natural and synthetic vesicles. In: Symposium on the Pharmacological Effect of Lipids. AOCS Monogr. No. 5. pp. 145-158.

Davis, B.K.,1980. Interaction of lipids with the plasma membrane of sperm cells I. The antifertilization action of cholesterol. Arch.Androl. 5:249-254.

Day, B.N., and Polge, C., 1968. Effects of progesterone on fertilization and egg transport in the pig. J.Reprod.Fertil. 17:227-230.

Didion, B.A., and Graves, C.N., 1986. In vivo capacitation and acrosome reaction of bovine sperm in estrous and diestrous cows. J.Anim.Sci. 62:1029-1033.

Dziuk, P.J., and Baker, R.D., 1962. Induction and control of ovulation in swine. J.Anim.Sci. 21:697-699.

Edwards, R.G., 1974. Follicular Fluid. J.Rerod.Fertil. 37:189-219.

Eppig, J.J., 1981. Regulation by sulfated glycosaminoglycans of the expansion of cumuli oophori isolated from mice. Biol.Reprod. 25:599-608.

Fawcett, D.W., 1975. The mammalian spermatozoon. Dev.Biol. 44:394-436.

First, N.L., and Parrish, J.J., 1988. Sperm maturation and in vitro fertilization. Congress Proceedings, 11th International Congress on Animal Reproduction and Artificial Insemination. pp.169-168.

Friend, D.S., and Fawcett, D.W.,1974. Membrane differentiations in freeze-fractured mammalian sperm. J.Cell Biol. 63:641-664.

Fraser, L.E., 1979. Accelerated mouse sperm penetration in vitro in the presence of caffeine. J.Reprod.Fertil. 57: 377-384.

Fraser, L.E., and Quinn, P.J., 1981. A glycolytic product is obligatory for initiation of the sperm acrosome reaction and whiplash motility required for fertilization in the mouse. J.Reprod.Fertil. 61:25-35.

Fraser, L.R., Dandekar, P.V., and Vaidya, K.A., 1971. In

vitro fertilization of tubal rabbit ova partially or totally denuded of follicular cells. Biol.Reprod. 4:229-233.

Gordon, M., Dandekar, P.V., and Bartoszwicz, W., 1974. Ultrastructural localization of surface receptors for concanavalin A on rabbit spermatozoa. J.Reprod.Fertil. 36:211-214.

Green, D.P.L.,1978. The induction of the acrosome reaction in guinea pig spermatozoa by the divalent metal cation ionophore A23187. J.Cell Sci. 32:137-151.

Grimek, H.J., and Ax, R.L.,1982. Chromatographic comparisons of chondroitin containing proteoglycan from small and large bovine ovarian follicles. Biochem.Biophys.Res. Commun. 104:1401-1406.

Gwatkin, R.B.L., and Andersen, O.F., 1969. Capacitation of hamster ova by bovine follicular fluid. Nature (London) 224:1111-1112.

Gwatkin, R.B.L., Anderson, O.F., and Hutchinson, C.F., 1972. Capacitation of hamster spermatozoa in vitro: the role of cumulus components. J.Reprod.Fertil. 30:389-394.

Hadley, M.E., 1984. Hormones and female reproductive physiology. In: Endorinology. pp.426-429. Prentice Hall Inc., New Jersey.

Hafez, E.S.E., 1987. Transport and survival of gametes. In: Reproduction in Farm Animals. (E.S.E. Hafez, ed.). pp. 168-188. Lea and Febiger, Philadelphia.

Harper, M.J.K., 1982. Sperm and egg transport. In: Germ Cells and Fertilization. (C.R. Austin and R.V. Short, eds.). pp. 102-127. University Press, Cambridge.

Herz, Z., Northey, D., Lawyer, M., and First, N.L., 1985. Acrosome reaction of bovine spermatozoa in vivo: Sites and effects of stages of the estrous cycle. Biol.Reprod. 32:1163-1168.

Hilliard, J., and Eaton, L.W., 1971. Estradiol-17 B, progesterone and 20 hydroxypregn-4-ene-3-one in rabbit ovarian venous plasma. 2. From mating through implantation. Endocrinology 89:522-527.

Hunter, R.H.F., 1972. Local action of progesterone leading to polyspermic fertilization in pigs. J.Reprod.Fertil. 31:433-444.

Hunter, R.H.F., 1981. Sperm transport and reservoirs in the pig oviduct in relation to the time of ovulation.

J.Reprod.Fertil. 63:109-117.

Hunter, R.H.F.,1982. Interrelationships between spermatozoa, the female reproductive tract, and the egg investments. In: Control of Pig Reproduction. (D.J.A. Cole and G.R. Foxcroft, eds.). pp. 49-63. Butterworths, London.

Hunter, R.H.F., 1984. Pre-ovulatory arrest and peri-ovulatory redistribution of competent spermatozoa in the isthmus of the pig oviduct. J.Reprod.Fertil. 72: 203-211.

Hunter, R.H.F., and Hall, J.P., 1974. Capacitation of boar spermatozoa: synergism between uterine and tubal environments. J.Exp.Zool. 188:203-214.

Hunter, R.H.F., Cook, B., and Baker, T.G., 1976. Dissociation of response to injected gonadotropin between the Graafian follicle and oocyte in pigs. Nature (London) 260:156-158.

Iwamatsu, T., and Chang, M.C., 1969. In vitro fertilization of mouse eggs in the presence of bovine follicular fluid. Nature (London) 224:919-920.

Johnson, M.H., Eager, D., and Muggleton-Harris, A., 1975. Mosaicism in organization of concanavalin A receptors on surface membrane of mouse egg. Nature (London) 257: 321-322.

Lacker, H.M., Beers, W.H., Meuli, L.E., and Akin, E., 1987. A theory of follicle selection: 1. Hypotheses and Examples. Biol.Reprod. 37:570-580.

Lenz, R.W., Ax, R.L., Grimek, H.J., and First, N.L., 1982. Proteoglycan from bovine follicular fluid enhances an acrosome reaction in bovine spermatozoa. Biochem. Biophys.Res.Commun. 106:1092-1098.

McGaughey, R.W., 1975. A comparison of the fluids from small and large ovarian follicles of the pig. Biol.Reprod. 13:147-153.

McLaren, A., 1974. Fertilization, cleavage and implantation. In: Reproduction in Farm Animals. (E.S.E Hafez, ed.) pp.143-165. Lea and Febiger, Philadelphia.

McNatty, K.P., 1978. Follicular fluid. In: The Vertebrate Ovary. (R.E. Jones, ed.). pp. 215-259. Plenum Press, New York.

McNatty, K.P., Hunter, W.M., McNeilly, A.S., and Sawers, R.S., 1975. Changes in the concentration of pituitary and steroid hormones in the follicular fluid of human

Graafian follicles throughout the menstrual cycle. J. Endocrinol. 64:555-571.

McNatty, K.P., Baird, D.T., Bolton, A., Chambers, P., Corker, C.S., and McLean, H., 1976. Concentration of oestrogens and androgens in human ovarian venous plasma and follicular fluid throughout the menstrual cycle. J. Endocrinol. 71:77-85.

Meizel, S., 1978. The mammalian sperm acrosome reaction: a biochemical approach. In: Development in Mammals, Vol. 3. (M.H. Johnson, ed.). pp. 1-62. North Holland, Amsterdam.

Meizel, S., 1984. The importance of hydrolytic enzymes to an exocytotic event, the mammalian sperm acrosome reaction. Biol.Rev. 59:125-157.

Miyamoto, H., and Chang, M.C., 1972. Fertilization in vitro of mouse and hamster eggs after removal of follicular cells. J.Reprod.Fertil. 30:309-312.

Moor, R.M., Hay, M.F., and Seamark, R.F., 1975. The sheep ovary: Regulation of steroidogenic, haemodynamic and structural changes in the largest follicle and adjacent tissue before ovulation. J.Reprod.Fertil. 45:595-604.

Moore, H.D., and Bedford, J.M., 1978. An in vivo analysis of factors influencing the fertilization of hamster eggs. Biol.Reprod. 19:879-885.

Moore, H.D., and Bedford, J.M., 1983. The interaction of mammalian gametes in the female. In: Mechanism and Control of Animal Fertilization. (J.F. Hartmann, ed.). pp. 453-497. Academic Press, New York.

Mukherjee, A.B., and Lippes, J., 1972. Effect of human follicular and tubal fluids on human, mouse, and rat spermatozoa in vitro. Can.J.Genet.Cytol. 14:167-174.

Nicolson, G.L., and Yanagimachi, R., 1972. Terminal saccharides on sperm plasma membranes: identification of specific agglutinins. Science 177:276-279.

Norman, R.L., and Greenwald, G.S., 1972. Follicular histology and physiological correlates in the preovulatory hamster. Anat.Rec. 173:95-108.

Oliphant, G., Cabot, C.L., and Singhas, C.A., 1977. Nature of the rabbit acrosome reaction inducing activity of follicular fluid. J.Reprod.Fertil. 50:245-250.

Overstreet, J.W., 1983. Transport of gametes in the reproductive tract of the female mammal. In: Mechanism

and Control of Animal Fertilization. (J.F. Hartmann, ed.). pp. 499-543. Academic Press, New York.

Overstreet, J.W., and Cooper, G.W., 1979. The time and location of the acrosome reaction during sperm transport in the female rabbit. J.Exp.Zool. 209: 97-104.

Pavlok, A., and McLaren, A., 1972. The role of cumulus cells and the zona pellucida in fertilization of mouse eggs in vitro. J.Reprod.Fertil. 29:91-97.

Piko, L., and Tyler, A., 1964. Fine structural studies of sperm penetration in the rat. Proc. Vth Int. Congr. Anim. Reprod. A.I. Trento 2:372-377.

Ramsoondar, J.J., 1986. The regulation of sperm-egg interaction in vitro by a porcine follicular fluid protein. M.Sc. Thesis, McGill University, Montreal.

Rigby, J.P., 1966. The persistence of spermatozoa at the uterotubal junction of the sow. J.Reprod.Fert. 11: 153-155.

Russel, L.D., Peterson, R.N., Blumershine, R., and Freund, M., 1980. Morphological observations on the binding of boar sperm to porcine zona pellucida. Scanning Electron Microscopy III:407-412.

Saling, P.M., and Bedford, J.M., 1981. Absence of species specificity for mammalian sperm capacitation in vivo. J.Reprod.Fertil. 31:433-444.

SAS User's Guide. 1982. Statistical Analysis System Institute, Inc., Cary, NC.

Shalgi, R., Kraicer, P.F., and Soferman, N., 1972. Gases and electrolytes of human follicular fluid. J.Reprod.Fertil. 28:335-340.

Short, R.V., 1962a. Steroids in the follicular fluid and the corpus luteum of the mare: A "two cell type" theory of ovarian steroid synthesis. J.Endocrinol. 24:59-63.

Short, R.V., 1962b. Steroids present in the follicular fluid of the cow. J.Endocrinol. 23:401-411.

Short, R.V., 1964. Ovarian steroid synthesis and secretion in vivo. Recent Progr.Horm.Res. 20:303-340.

Singh, J.P., Babcock, D.F., and Lardy, H.A., 1980. Induction of accelerated acrosome reaction in guinea pig sperm. Biol.Reprod. 22:566-570.

Sorensen, A.M.(Jr.)., 1979. Animal Reproduction: Principles and Practices. McGraw-Hill, Inc., USA.

Stambaugh, R., and Buckley, J., 1970. Comparative studies of the acrosomal enzymes of rabbit, rhesus monkey and human spermatozoa. Biol. Reprod. 3:275-282.

Srivastava, P.N., Adams, C.E., and Hartree, E.F., 1965. Enzymatic action of acrosomal preparations on the rabbit ovum in vitro. J.Reprod.Fertil. 10:61-67.

Susko-Parrish, J.L., Parrish, J.J., and Handrow, R.R., 1985. Glucose inhibits the action of heparin by an indirect mechanism. Biol.Reprod. 32 (Suppl. 1):80.

Talbot, P., and Franklin, L.E., 1976. Morphology and kinetics of the hamster sperm acrosome reaction. J.Exp.Zool. 198:163-176.

Talbot, P., Summers, R.G., Hylander, B.L., Keough, E.M., and Franklin, L.E., 1976. The role of calcium in the acrosome reaction: analysis using ionophore A23187. J.Exp.Zool. 198:383-392.

Talbot, P., and Chacon, R.S., 1982. Ultrastructural observations on binding and membrane fusion between human sperm and zona pellucida-free hamster oocytes. Fertil.Steril. 37:240-248.

Viriyapanich, P., and Bedford, J.M., 1981. Sperm capacitation in the fallopian tube of the hamster and its suppression by endocrine factors. J.Exp.Zool. 217: 403-407.

Wheeler, A.G., Baird, D.T., Land, R.B., and Scaramuzzi, R.J., 1975. Increased secretion of progesterone from the ovary of the ewe during the preovulatory period. J.Reprod.Fertil. 45:519-522.

Yanagimachi, R., 1969a. In vitro acrosome reaction and capacitation of golden hamster spermatozoa by bovine follicular fluid and its fractions. J.Exp.Zool. 170: 269-280.

Yanagimachi, R., 1969b. In vitro capacitation of hamster spermatozoa by follicular fluid. J.Reprod.Fertil. 18: 275-286.

Yanagimachi, R., 1970a. In vitro capacitation of golden hamster spermatozoa by homologous and heterologous blood sera. Biol.Reprod. 3:147-153.

Yanagimachi, R., 1970. The movement of golden hamster spermatozoa before and after capacitation.

J.Reprod.Fertil. 23:193-196.

Yanagimachi, R., 1978. Sperm-egg association in mammals. In: Current topics in developmental biology, vol.12. (A.A. Moscana, and A. Monroy, eds.), pp. 83-105, Academic Press, New York.

Yanagimachi, R., 1981. Mechanisms of fertilization in mammals. In: Fertilization and Embryo Development in vitro. (L. Mastroianni and J.D. Biggers, eds.). pp. 81-182. Plenum Press, New York.

Yanagimachi, R., and Chang, M.C., 1964. in vitro fertilization of golden hamster ova. J.Exp.Zool. 156:361-376.

Yanagimachi, R., and Usui, N., 1974. Calcium dependence of the acrosome reaction and activation of guinea pig spermatozoa. Exp.Cell Res. 89:161-174.

Yanagishita, M., and Hascal, V.C., 1979. Biosynthesis of proteoglycans by rat granulosa cells cultured in vitro. J.Biol.Chem. 254:12355-12364.

YoungLai, E.V., 1972. Effect of mating on follicular fluid steroids in the rabbit. J.Reprod.Fertil. 30:157-159.

Appendix Table 1. Statistical Program for Experiment I

```
data one;infile 'a:kumar1';
input trt $ 1 pig $ 2-3 ps 4-6 .2 side $ 7 nof 8-9 vtf 10-12 .2
      vff 13-15 .2 ptf 16-20 .2 pff 21-26 .1 noov 27-28;
* trt: a=38h b=46h c=46i d=42h,ps:serum progesterone,
  nof:number of follicles, vtf:volume tubal fluid,
  vff:volume follicular fluid, ptf:progesterone tubal fluid,
  pff:progesterone folliclar fluid, noov:number of ovulations;

data two;set one;
tptf=ptf*vtf;ppf=(vff/nof)*pff;

data three;set two;
pctf=tptf*5;pof=ppf*noov;

data four;set three;
diff=pctf-ps;hm=(tptf/pof)*100;

data five;set four;

proc sort;by trt;proc means;by trt;var ps pff ptf tptf ppf
diff pctf nof noov;run;

proc sort;by side;proc means;by side;var ps pff ptf tptf ppf
diff pctf nof noov;run;

proc sort;by trt side;proc means;by trt side;var ps pff ptf
tptf ppf diff pctf nof noov;run;

proc glm;class trt;
model ps=trt;
means trt/duncan;run;

proc glm;class trt side;
model nof noov ppf tptf pctf diff hm=trt side trt*side;
lsmeans trt side trt*side/pdiff stderr;
means trt side/duncan;run;
```

Appendix Table 2. Data for Experiment I

OBS	TRT	PIG	PS	SIDE	NOF	VTF	VFF	PTF	PFF	NOOV
1	a	01	0.75	r	8	1.15	0.85	0.05	1917.0	0
2	a	01	0.75	l	13	1.15	1.20	0.86	2328.0	0
3	a	02	0.78	r	9	1.25	0.90	1.41	1213.5	0
4	a	02	0.78	l	7	1.25	0.60	0.43	1539.0	0
5	a	03	1.18	r	6	1.00	0.75	0.58	2208.0	0
6	a	03	1.18	l	18	1.35	1.70	0.29	3498.0	0
7	a	04	0.70	r	3	1.10	0.30	0.52	4455.0	0
8	a	04	0.70	l	8	1.30	0.80	1.17	4008.0	0
9	a	05	0.50	r	5	1.30	0.58	0.07	1969.5	0
10	a	05	0.50	l	1	1.30	.	0.01	.	0
11	a	06	2.34	r	5	1.20	0.15	0.01	1174.5	0
12	a	06	2.34	l	1	1.30	.	0.01	.	0
13	a	07	0.95	r	6	1.20	0.80	0.16	2437.5	0
14	a	07	0.95	l	3	1.00	0.30	0.21	1938.0	0
15	a	08	3.13	r	10	1.35	0.45	.	2491.5	0
16	a	08	3.13	l	14	1.30	0.90	.	3580.5	0
17	a	09	0.44	r	4	1.20	0.30	0.19	1642.5	0
18	a	09	0.44	l	8	1.40	0.70	0.16	2146.5	0
19	a	10	0.67	r	10	1.00	1.20	0.14	2419.5	0
20	a	10	0.67	l	8	1.10	0.80	0.12	2532.0	0
21	a	11	0.53	r	1	1.32	.	0.01	.	0
22	a	11	0.53	l	6	1.50	0.65	0.05	3984.0	0
23	a	12	0.31	r	3	1.40	0.46	0.01	732.0	0
24	a	12	0.31	l	4	1.25	0.57	0.01	711.0	0
25	a	13	0.45	r	3	1.20	0.60	0.14	2272.5	0
26	a	13	0.45	l	9	1.40	1.50	0.09	2635.5	0
27	a	14	0.29	r	10	1.25	1.35	0.08	709.5	0
28	a	14	0.29	l	4	1.20	0.55	0.08	550.5	0
29	a	15	0.30	r	3	1.30	0.35	0.13	1038.0	0
30	a	15	0.30	l	3	1.15	0.35	0.08	1357.5	0
31	a	16	0.32	r	2	0.45	0.23	0.10	732.0	0
32	a	16	0.32	l	7	1.05	1.20	0.08	676.5	0
33	b	01	2.53	r	9	1.10	.	5.43	.	9
34	b	02	2.19	r	7	1.10	.	0.25	.	7
35	b	02	2.19	l	13	1.30	.	2.09	.	13
36	b	03	1.06	r	11	1.10	.	0.36	.	11
37	b	03	1.06	l	6	1.15	.	0.10	.	6
38	b	04	3.24	r	7	1.37	.	0.11	.	7
39	b	04	3.24	l	8	0.38	.	5.24	.	8
40	b	05	3.31	r	9	1.33	.	0.96	.	9
41	b	05	3.31	l	9	1.30	.	0.70	.	9
42	b	06	1.71	r	6	1.35	.	0.41	.	6
43	b	06	1.71	l	9	1.45	.	0.26	.	9
44	b	07	1.81	r	8	1.35	.	0.93	.	8
45	b	07	1.81	l	11	1.05	.	2.64	.	11
46	b	08	1.91	r	7	0.90	.	0.34	.	7
47	b	08	1.91	l	10	1.20	.	0.71	.	10
48	b	09	2.94	r	8	1.32	.	0.83	.	8
49	b	09	2.94	l	15	1.20	.	1.06	.	15

OBS	TRT	PIG	PS	SIDE	NOF	VTF	VFF	PTF	PFF	NOOV
50	b	10	2.00	r	15	0.96	.	0.88	.	15
51	b	10	2.00	l	10	1.15	.	0.16	.	10
52	b	11	1.00	r	9	0.35	.	4.00	.	9
53	b	11	1.00	l	14	1.35	.	5.15	.	14
54	b	12	0.55	r	7	1.35	.	0.77	.	7
55	b	12	0.55	l	6	0.92	.	0.86	.	6
56	b	13	0.71	r	7	1.30	.	0.29	.	7
57	b	13	0.71	l	3	1.18	.	0.07	.	3
58	b	14	2.52	r	3	1.00	.	0.18	.	3
59	b	14	2.52	l	13	1.25	.	0.39	.	13
60	b	15	1.24	r	7	0.90	.	0.66	.	7
61	b	15	1.24	l	9	1.07	.	0.55	.	9
62	b	16	0.73	r	5	1.30	.	0.84	.	5
63	c	01	1.22	r	3	0.95	0.40	0.08	5703.0	0
64	c	01	1.22	l	5	1.10	1.20	0.12	5460.0	0
65	c	02	1.26	r	12	1.20	1.60	0.15	4995.0	0
66	c	02	1.26	l	14	0.95	2.00	0.10	6540.0	0
67	c	03	0.60	r	3	1.20	0.32	0.10	3270.0	0
68	c	03	0.60	l	7	1.20	0.75	0.10	4621.5	0
69	c	04	2.68	r	8	1.30	2.00	0.18	12495.0	0
70	c	04	2.68	l	11	1.30	2.10	0.19	12945.0	0
71	c	05	1.03	r	4	0.42	0.40	0.11	3700.5	0
72	c	05	1.03	l	6	1.05	0.50	0.11	3081.0	0
73	c	06	1.96	r	10	1.25	1.00	0.09	4773.0	0
74	c	06	1.96	l	10	0.45	0.85	0.16	3300.0	0
75	c	07	4.49	r	12	1.85	1.75	0.51	19755.0	0
76	c	07	4.49	l	15	0.90	2.75	0.30	25725.0	0
77	c	08	1.57	r	8	0.95	1.20	0.09	4215.0	0
78	c	08	1.57	l	8	1.25	1.40	0.09	3855.0	0
79	c	09	0.74	r	4	1.35	0.57	.	3058.5	0
80	c	09	0.74	l	12	1.20	1.40	.	4438.5	0
81	c	10	0.47	r	5	1.45	0.50	0.08	2595.0	0
82	c	10	0.47	l	6	1.25	0.55	0.06	1720.5	0
83	c	11	0.37	r	5	1.25	0.75	0.09	5356.5	0
84	c	11	0.37	l	.	0.15	.	0.09	.	0
85	c	12	.	r	2	1.15	3.40	0.41	5415.0	0
86	c	13	1.70	r	2	1.05	0.15	0.08	5275.5	0
87	c	13	1.70	l	4	1.30	0.55	.	4623.0	0
88	c	14	0.36	r	7	1.15	0.85	0.18	5295.0	0
89	c	14	0.36	l	9	1.20	1.90	0.14	7890.0	0
90	c	15	0.39	r	1	1.20	0.10	0.13	.	0
91	c	15	0.39	l	3	1.15	0.25	0.10	4845.0	0
92	c	16	0.14	r	12	1.68	1.70	0.13	4680.0	0
93	c	16	0.14	l	12	0.90	1.48	0.15	5610.0	0
94	d	01	1 20	r	9	1.30	0.90	5.04	4767.0	3
95	d	01	1.20	l	10	1.30	0.90	0.48	4660.5	3
96	d	02	1.07	r	12	1.20	0.18	4.55	4116.0	7
97	d	02	1.07	l	15	1.00	0.35	7.48	2652.0	6
98	d	03	1.34	r	19	0.40	1.55	0.58	4665.0	5
99	d	03	1.34	l	15	0.50	1.75	0.19	4545.0	1
100	d	04	0.73	r	5	1.25	0.01	0.54	.	4
101	d	04	0.73	l	6	1.00	0.00	0.72	.	6

102	d	06	0.70	r	12	1.05	1.60	0.65	5074.5	2

OBS	TRT	PIG	PS	SIDE	NOF	VTF	VFF	PTF	PFF	NOOV
103	d	06	0.70	l	14	1.20	1.75	0.12	4515	0
104	d	07	0.38	r	5	1.35	0.01	0.62	.	3
105	d	07	0.38	l	4	1.00	0.02	0.42	.	2
106	d	08	0.61	r	8	1.50	0.02	0.23	.	7
107	d	08	0.61	l	2	1.15	0.00	0.34	.	1
108	d	09	1.47	r	3	1.15	0.10	1.21	1935	2
109	d	09	1.47	l	11	1.25	0.40	1.37	16005	8
110	d	10	1.82	r	8	1.10	.	.	.	8
111	d	10	1.82	l	8	1.25	0.10	3.02	9585	6
112	d	11	1.26	r	.	1.20	.	0.47	.	4
113	d	12	3.00	r	3	1.25	0.10	0.19	3915	2
114	d	12	3.00	l	5	0.75	.	0.43	.	5
115	d	13	0.97	r	5	0.90	.	0.38	.	4
116	d	13	0.97	l	4	1.30	.	1.20	.	3
117	d	14	2.44	r	7	1.40	.	2.32	.	5
118	d	14	2.44	l	3	1.10	.	0.92	.	2
119	d	15	2.49	r	6	1.35	.	0.46	.	6
120	d	15	2.49	l	8	.	.	.	.	5
121	d	16	0.82	r	6	1.2	.	0.78	.	6
122	d	16	0.82	l	6	1.0	.	0.77	.	4

Appendix Table 3. Abdominal Fluid Progesterone Concentration (ng/ml)

Time	Progesterone Concentration
42 hours	2.07±0.49
46 hours	2.19±0.45
46 hours (Indo.)	0.55±0.21

Appendix Table 4. Composition of the Semen Extender

Compound	g/l
Dextrose (anhydrous)	29
Sodium Citrate	10
Sodium Bicarbonate	2
Potassium Chloride	0.3
Streptomycin	0.7

Appendix Table 5. Composition of Acrosome Staining Solutions

Solution 1:	0.1% Naphthol Yellow S in 1.0% Aqueous Acetic Acid
Solution 2:	0.2% Aqueous Naphthol Yellow S
Solution 3:	0.2% Aqueous Erythrosin B

Appendix Table 6. Statistical Program for Experiment II

```
data one;
input trt $ 1 pig $ 2-3 side $ 4 nof 5-6 loc $ 7
a 8-9 cs 10-15 ars 16-21;
cards;
a07r05h33152000050160
a07r05i25000480000120
a07r05a52000580000302
a07l03h37144000053280
a07l03i35000920000322
a07l03a71000180000128
a09r03h40894000357600
a09r03i38108000041040
a09r03a83001040000863
a09l05h31592000183520
a09l05i45032000014400
a09l05a77001920001478
a11r02h40060000024000
a11r02i36002480000893
a11r02a67000640000429
a11l03h37082000030340
a11l03i42010800004536
a11l03a77000720000554
a13r07h38360000136800
a13r07i38082000031160
a13r07a82001960001607
a13l08h48385000184800
a13l08i45248000111600
a13l08a65002320001508
a15r10h33174000057420
a15r10i42052000021840
a15r10a86000360000310
a15l06h37212000078440
a15l06i32100000032000
a15l06a71000320000227
b08r09h33136000044880
b08r09i40001400000560
b08r09a60000100000060
b08l06h29142000041180
b08l06i30004000001200
b08l06a75000160000120
b10r08h37870000321900
b10r08i34066000022440
b10r08a57001960001117
b10l06h40230000092000
b10l06i31104000032240
b10l06a42012560005275
b12r04h37008000002960
b12r04i30000560000168
```

```
b12r04a75000200000150
b12l03h31042000013020
b12l03i36001800000648
b12l03a71000320000227
b16r06h37963333356433
b16r06i48126000060480
b16r06a66012040007946
b16l08h45440000198000
b16l08i45218000098100
b16l08a67016840011283

data two;set one;

proc sort;by trt;proc means;by trt;var cs ars a;run;
proc sort;by loc;proc means;by loc;var cs ars a;run;
proc sort;by trt loc;proc means;by trt loc;var cs ars a;run;

proc glm;class trt loc;
model cs ars a=trt loc trt*loc;
lsmeans trt*loc/stderr pdiff;
means trt loc/duncan;run;
```

Appendix Table 7. Data for Experiment II

OBS	TRT	PIG	SIDE	NOF	LOC	A	CS	ARS
1	a	07	r	5	h	33	152000	50160
2	a	07	r	5	i	25	480	120
3	a	07	r	5	a	52	580	302
4	a	07	l	3	h	37	144000	53280
5	a	07	l	3	i	35	920	322
6	a	07	l	3	a	71	180	128
7	a	09	r	3	h	40	894000	357600
8	a	09	r	3	i	38	108000	41040
9	a	09	r	3	a	83	1040	863
10	a	09	l	5	h	31	592000	183520
11	a	09	l	5	i	45	32000	14400
12	a	09	l	5	a	77	1920	1478
13	a	11	r	2	h	40	60000	24000
14	a	11	r	2	i	36	2480	893
15	a	11	r	2	a	67	640	429
16	a	11	l	3	h	37	82000	30340
17	a	11	l	3	i	42	10800	4536
18	a	11	l	3	a	77	720	554
19	a	13	r	7	h	38	360000	136800
20	a	13	r	7	i	38	82000	31160
21	a	13	r	7	a	82	1960	1607
22	a	13	l	8	h	48	385000	184800
23	a	13	l	8	i	45	248000	111600
24	a	13	l	8	a	65	2320	1508
25	a	15	r	10	h	33	174000	57420
26	a	15	r	10	i	42	52000	21840
27	a	15	r	10	a	86	360	310
28	a	15	l	6	h	37	212000	78440
29	a	15	l	6	i	32	100000	32000
30	a	15	l	6	a	71	320	227
31	b	08	r	9	h	33	136000	44880
32	b	08	r	9	i	40	1400	560
33	b	08	r	9	a	60	100	60
34	b	08	l	6	h	29	142000	41180
35	b	08	l	6	i	30	4000	1200
36	b	08	l	6	a	75	160	120
37	b	10	r	8	h	37	870000	321900
38	b	10	r	8	i	34	66000	22440
39	b	10	r	8	a	57	1960	1117
40	b	10	l	6	h	40	230000	92000
41	b	10	l	6	i	31	104000	32240
42	b	10	l	6	a	42	12560	5275
43	b	12	r	4	h	37	8000	2960
44	b	12	r	4	i	30	560	168
45	b	12	r	4	a	75	200	150
46	b	12	l	3	h	31	42000	13020
47	b	12	l	3	i	36	1800	648
48	b	12	l	3	a	71	320	227
49	b	16	r	6	h	37	963333	356433

OBS	TRT	PIG	SIDE	NOF	LOC	A	CS	ARS
50	b	16	r	6	i	48	126000	60480
51	b	16	r	6	a	66	12040	7946
52	b	16	l	8	h	45	440000	198000
53	b	16	l	8	i	45	218000	98100
54	b	16	l	8	a	67	16840	11283